The Effects of Aeration on Glucose Catabolism in *Penicillium expansum*

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SUMMARY

Polyacrylamide-disc gel electrophoresis and quantitative enzyme assays showed that the pathways of glucose catabolism and secondary metabolism in *Penicillium expansum* were dependent on the degree of aeration of the cultures. The isoenzyme patterns and specific activities of aldolase and succinate dehydrogenase indicated that glycolysis and the tricarboxylic acid cycle operated under conditions of both limited and efficient aeration (i.e. in cultures grown statically or on an orbital shaker). At high levels of aeration the growth rate was faster and synthesis of extracellular pectolytic enzymes was enhanced, whilst the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed that the pentose-phosphate shunt was important in glucose catabolism during the trophophase of growth. In contrast, under conditions of low aeration this latter pathway was virtually undetectable, growth was slower, pectolytic enzyme production low and large concentrations of secondary metabolites (6-methylsalicylic acid, patulin and citrinin) accumulated.

INTRODUCTION

It is well known that culture conditions of fungi can markedly affect the general growth characteristics and the production of secondary metabolites. An illustration of this is the considerable decrease in the amount of patulin produced when mycelial mats of *Penicillium patulum* growing on liquid media are disturbed (Tanenbaum & Bassett, 1958).

Bu'Lock and co-workers (Bu'Lock *et al.* 1965; Bu'Lock, 1965) have investigated the relationship between the formation of secondary metabolites, including patulin, and general metabolic development in fungi, particularly in *Penicillium urticae*. Patulin is a common metabolite of many species of *Penicillium* and *Aspergillus*, and is of interest because of its toxicity and the frequency with which it has been detected in mouldy foodstuffs (Brian, Elson & Lowe, 1956; Walker, 1969; Tauchmann, Toth & Leistner, 1971; Reiss, 1972). The fungus also secretes pectolytic enzymes causing a soft rot in infected fruit (Cole & Wood, 1961) and potentially dangerous concentrations of patulin have been detected in commercially prepared apple juice contaminated with infected fruit (Scott *et al.* 1972).

In view of local interests in fruit processing and the implications of patulin as a potential health hazard we have begun an investigation of the effect of aeration on glucose catabolism by *Penicillium expansum* and its relationship to the formation of secondary metabolites and extracellular pectic enzymes. We report the variations in activity and multiple forms of some key enzymes of primary metabolism in relation to secondary biosynthesis by *P. expansum* under culture conditions of high and low aeration.

METHODS

Organism and growth conditions. The original culture of *Penicillium expansum* was isolated from infected apples (Walker, 1969) and maintained on slopes of potato-glucose agar at 4 °C.

Malt extract medium (2 %, v/v) fortified with bacteriological peptone (0.3 %, w/v) was autoclaved (100 ml in a 500 ml conical flask) at 121 °C for 15 min. Flasks were inoculated with 2×10^7 spores (in 5 ml sterile water) and grown at 27 °C, either under static conditions or on an orbital shaker at 250 rev./min and 3 inch throw.

Dry weight measurements were made after filtering the mycelium through Miracloth, washing twice with distilled water and drying for 48 h at 70 °C.

Determination of secondary metabolites. Samples of the culture medium (20 ml) were acidified with dilute HCl and extracted with ethyl acetate (2 $\times$ 25 ml). The extracts were concentrated under reduced pressure and taken up in ethanol (0.1 ml) for chromatography on Whatman No. 1 paper using benzene-acetic acid-water (136:72:3, v/v) or 2 % (v/v) acetic acid. Chromatograms were viewed under short- or long-wavelength u.v. light, before and after exposure to NH₃ fumes, and then sprayed with diazotized *p*-nitroaniline to visualize the phenolics. Patulin was detected with phenylhydrazine by the method of Yamamoto (1956). 6-Methylsalicylic acid (6-MSA), patulin and citrinin were identified by co-chromatography with authentic specimens and by comparison of their u.v. spectra in 95 % ethanol recorded on a Pye-Unicam SP 1800 spectrophotometer.

For time-course experiments the metabolites were assayed at 24 h intervals over a 7-day period by reading the extinction of each extract at 276 nm (patulin), 305 nm (6-MSA) and 330 nm (citrinin).

Extraction and assay of extracellular enzymes. Lyophilized culture filtrates of *P. expansum* were dissolved in 0.2 M-phosphate/citrate buffer (pH 5.0) and assayed for pectolytic enzyme activity.

Polygalacturonase was detected by the 'cup-plate' method of Dingle, Reid & Solomons (1953) and assayed by the viscometric method of Bell, Etchells & Jones (1955). Pectinesterase was measured by the titrimetric method of Cole & Wood (1961).

Pectate transeliminase activity was assayed as the increase in E_{235} of sodium polypectate (0.5 %, w/v) in 0.05 M-phosphate/citrate buffer (pH 5.0) plus enzyme, against a blank containing boiled enzyme, using the second sample position of the Pye-Unicam SP 1800 spectrophotometer. The assay was carried out at 20 °C.

Extraction of mycelial enzymes. *Penicillium expansum* was separated from the culture fluid by filtration through Miracloth, washed thoroughly with distilled water and excess moisture removed by blotting. Soluble proteins were extracted by macerating weighed amounts of mycelium with acid-washed quartz sand in 0.05 M-tris-HCl buffer pH 8.0 at 4 °C in a pestle and mortar. The macerates were centrifuged at 0 °C (30 000 *g* for 90 min) and the supernatant used as the crude protein extract.

Protein concentration was measured by the method of Lowry *et al.* (1951) after precipitation with 3 M-trichloroacetic acid using bovine serum albumin as a standard.

Enzyme assays. Glucose 6-phosphate dehydrogenase (G6-PD) (D-glucose 6-phosphate: NADP oxidoreductase, EC. 1.1.1.49) was assayed spectrophotometrically by the method of Kornberg & Horecker (1955). One unit of activity was the amount of enzyme that caused an increase in extinction of 1.0 extinction units/min at room temperature.

6-Phosphogluconate dehydrogenase (6-PGD) (6-phospho-D-gluconate: NADP oxidoreductase, EC. 1.1.1.44) was measured by the same method as for G6-PD except that 6-phosphogluconate was used as substrate.

Aldolase (D-fructose-diphosphate aldolase, EC. 7.1.2.7) was determined by coupling glyceraldehyde 3-phosphate production to the reduction of NAD by glyceraldehyde 3-phosphate dehydrogenase (Brubaker, 1968). One unit of aldolase activity caused an increase in extinction of 0.1 extinction units/min at room temperature.

Succinate dehydrogenase [succinate: (acceptor) oxidoreductase, EC. 1.3.99.1] activity was measured by following the decrease in E_{600} as the dye 2,6-dichlorophenolindophenol was reduced in an assay system containing: 2.5 mM 0.2 M-sodium succinate (1.0 ml), 2,6-dichlorophenolindophenol (0.1 ml), 0.05 M-phosphate buffer pH 7.0 (1.8 ml) and enzyme extract (0.1 ml). One unit of activity was that amount of enzyme that caused a decrease in E_{600} of 0.1 extinction units/min at room temperature.

Gel electrophoresis. Proteins were separated by disc electrophoresis (2.5 mA/gel tube for 1 h at 0 °C) on 7.5 % (w/v) polyacrylamide gels made up in 0.025 M-tris-HCl buffer pH 8.9. The bridge buffer was 0.005 M-tris-glycine, pH 8.3. Samples containing up to 300 mg protein were applied to the gels, with bromophenol blue as tracker dye. After separation the gels were rinsed in ice-cold buffer and stained for proteins with Coomassie brilliant blue (0.25 % in 25 % v/v, methanol containing 7 % v/v, acetic acid) for 10 min and destained overnight in 25 % (v/v) methanol containing 7 % (v/v) acetic acid.

Dehydrogenases were located by selective staining using the NADPH-coupled reduction of phenazine methosulphate (PMS) and *p*-nitroblue tetrazolium (NBT) (Brewer & Sing, 1970). Gels were destained overnight in 25 % (v/v) methanol containing 7 % (v/v) acetic acid and photographed on Ilford Pan F film. Isoenzyme patterns are represented here as line diagrams drawn from the negatives.

Reagents. Biochemicals and enzymes were purchased from Sigma. Cyanogum for polyacrylamide gels, PMS and laboratory chemicals (Analar grade) were from BDH, and *p*-NBT was purchased from Calbiochem.

RESULTS

General effects of agitation on secondary metabolism and extracellular enzyme production by P. expansum

The growth and general metabolic rate of *P. expansum* was greatly increased by shaking (Fig. 1) which increased the degree of aeration of the culture medium (Freedman, 1970). The major products of secondary metabolism in *P. expansum* are the acetate-derived polyketides patulin and citrinin, large quantities of which were detected in the static cultures (Fig. 1*a*) whereas much lower concentrations were found in shake cultures (Fig. 1*b*). A similar trend was observed for 6-MSA (Fig. 1), the precursor of patulin (Bu'Lock & Ryan, 1958).

Penicillium expansum has been reported to secrete a number of pectolytic enzymes (Cole & Wood, 1961; Spalding, Wells & Allison, 1973), but only polygalacturonase was present in our culture filtrates, whilst a very low level of pectinesterase was detected in shake cultures only. Qualitative 'cup-plate' assays for polygalacturonase showed that this enzyme was much more active in shake cultures, and this was corroborated by quantitative viscometric assays (Fig. 2). In both shake and static cultures a fall in activity was observed after about 96 h of growth, the magnitude of the fall in shake cultures being approximately double that observed in static culture.

Intracellular enzymes from P. expansum

Soluble proteins from static and shake cultures were extracted at 24 h intervals throughout the growth period and separated by electrophoresis on polyacrylamide gels. After staining

Fig. 1. Growth curve and secondary metabolite production by *P. expansum* under (a) static conditions and (b) in shake cultures. ●, Dry weight; ○, patulin (E_{276}); ▲, 6-MSA (E_{305}); △, citrinin (E_{330}). Commencement of sporulation is shown by the arrow.

Fig. 2. Time-course of extracellular enzyme production, showing dry weight in static (●) and shake (○) cultures, and polygalacturonase activity in static (▲) and shake (△) cultures.

with Coomassie brilliant blue distinct differences were observed in the protein patterns of the two sets of extracts (Fig. 3a). Consequently, we decided to attempt to elucidate the observed effects of aeration on *P. expansum* metabolism by investigating four enzyme systems, representative of the two main pathways of glucose catabolism, using quantitative assays supplemented by selective enzyme staining after separation by gel electrophoresis.

The enzyme systems considered were two representatives of the pentose-phosphate (PP)

Fig. 3. Diagrammatic interpretation of the electrophoretic patterns of proteins and enzymes from shake and static cultures at different stages of development. (a) Total soluble protein; (b), G6-PD; (c), 6-PGD; (d) aldolase; (e) SDH. The intensities of the bands are indicated by the degrees of shading.

Fig. 4. Time-course of enzyme activities in static (●) and shake (○) cultures. (a), G6-PD; (b), 6-PGD; (c) aldolase; (d) SDH.

Fig. 5. Diagrammatic interpretation of zymograms showing the effects of sequential changes in aeration on enzyme activity. (a) G6-PD; (b) 6-PGD; (c) SDH. Culture conditions: A, static 42 h; B, static 42 h → shake 6 h; C, static 42 h → shake 6 h → static 6 h; D, shake 42 h; E, shake 42 h → static 6 h; F, shake 42 h → static 6 h → shake 6 h. The intensities of the bands are indicated by the degrees of shading.

shunt (G6-PD and 6-PGD), aldolase as a measure of glycolysis, and a tricarboxylic acid (TCA) cycle enzyme, succinate dehydrogenase (SDH).

The electrophoretic patterns of the enzymes studied are shown in Fig. 3*b, c, d, e*. Although it was possible to estimate the relative levels of the enzymes from the number and widths of the bands and from the intensity of staining on zymograms, interpretation of electrophoretic data was reinforced by quantitative assays of the enzymes (Fig. 4).

Fig. 6. The effects of sequential changes in aeration on the production of (●) patulin (E_{276}) and (○) citrinin (E_{330}) by *P. expansum*. Culture conditions: (a) static → shake 6 h → static; (b) shake → static 6 h → shake. The change-over points are marked by arrows.

The enzyme activities and zymogram patterns suggested that utilization of glucose by *P. expansum* took place via different pathways dependent on the culture conditions. Efficient aeration appeared to favour the PP shunt in addition to glycolysis and the TCA cycle, whereas under static conditions the PP shunt was not involved. Secondary metabolism was also apparently affected by these culture conditions. To confirm these observations and minimize the effect of nutrient depletion, cultures were maintained under both static and shake conditions for an initial period of 42 h and then conditions were interchanged for a period of 6 h. Zymograms of enzyme preparations extracted before and after the change in conditions are shown in Fig. 5. It was also observed that, in these experiments, cessation of shaking caused an increase in secondary metabolite production whereas the commencement of shaking slowed the production of secondary metabolites (Fig. 6).

DISCUSSION

In all experiments the results of quantitative assays correlated well with the trends indicated in the zymograms, i.e. the mechanism of glucose catabolism depended on the degree of aeration of the cultures.

In shake culture, where p_{O_2} was high, the first two enzymes of the PP shunt (G6-PD and 6-PGD) showed high activity in the initial stages of growth, which corresponds to the 'trophophase' of Bu'Lock *et al.* (1965) where 'balanced growth' (Borrow *et al.* 1961) takes place. Thereafter the activity of these enzymes declined, as the 'idiophase', where 'species-peculiar' metabolic activities occur, became established. The zymograms of these enzymes (Fig. 3*b, c*) showed several bands in the early stages that were absent later. This was most marked in the case of 6-PGD where only a single band existed in the idiophase.

When less oxygen was available to the growing cultures, as under static conditions, the activities of the PP pathway enzymes were minimal during the trophophase (Fig. 4*a, b*). Initially a single band was observed for 6-PGD, with a second appearing after 96 h. A similar increase in the number of bands was observed for G6-PD, which suggested that the PP pathway was not used by static cultures until the idiophase.

The Embden-Meyerhof glycolysis pathway was of equal importance in both static and shake cultures (Figs. 3*d* and 4*c*). After an initial peak at 48 h there was a fall in aldolase activity between 72 and 96 h which corresponded approximately to the change from trophophase to idiophase, and then the activity increased again. The TCA cycle operated in both static and shake cultures. Maximum activity of the representative enzyme, SDH, was attained during the trophophase of aerated cultures where several isozymes appeared, but when the fungus had reached its idiophase only one form of the enzyme remained. However, in static cultures this particular isoenzyme appeared first, and it was not until the beginning of the idiophase that other isoenzymes became apparent.

Thus the pathways of glucose catabolism and secondary metabolism in shake culture for *P. expansum* are in agreement with the situation found in *P. urticae* (Bu'Lock *et al.* 1965). However, the degree of aeration affected both the primary and secondary metabolism of *P. expansum*. Figure 1 shows that 6-MSA was produced first, under both conditions, and that patulin, which is derived from 6-MSA via a series of gentisyl derivatives (Tanenbaum & Bassett, 1958), built up later. The yield of patulin in static cultures was, however, approximately three times that in shake culture. Likewise citrinin, another acetate-derived secondary metabolite, was formed in large quantities only in static culture. In contrast, the production of extracellular pectic enzymes was considerably stimulated in shake cultures (Fig. 2). One effect of higher levels of aeration, therefore, seems to be to enhance primary metabolic activities such as enzyme synthesis and to lessen the production of secondary metabolites which would normally occur when *P. expansum* is grown in static culture. These ideas are reinforced by the results shown in Figs. 5 and 6, which show that as soon as static cultures were exposed to an increase in aeration the PP pathway was activated. Shaking stopped the activity of G6-PD, and secondary metabolites began to accumulate whilst 6-PGD diminished and further patulin and citrinin production ceased.

Any explanation of these observations must, at this stage, be largely a matter of conjecture. The major difference initially between static and shake cultures is the amount of oxygen available, but substrate exhaustion could become a limiting factor in later stages and this could possibly bring about some of the effects described. However, the sequential 'change-over' experiments shown in Figs. 5 and 6 would seem to militate against this possibility. Therefore, in shake cultures there was a generally higher metabolic rate, and sufficient oxygen

for the oxidative enzymes of the PP shunt to operate during the trophophase in addition to glycolysis, which occurred at the same rate in both static and shake cultures. However, static cultures, growing at a slower rate, could obtain sufficient energy and reducing potential (as NADPH) from glycolysis and the TCA pathway. Metabolism of hexoses by the PP pathway generates NADPH, which is necessary for many essential biosynthetic reactions such as fatty acid biosynthesis, whereas the pathways of secondary metabolism from acetyl CoA to aromatic polyketides have little need for NADPH (Bu'Lock, 1965). Therefore, both processes requiring NADPH, and general 'growth' are stimulated in shake culture, whilst polyketides (patulin, citrinin) accumulate in static culture. A possible reason for their accumulation may be to prevent a build-up of C_2 -units and/or 6-MSA. Bu'Lock (1967) has stated that concentrations of about 2×10^{-4} to 6×10^{-4} M-6-MSA caused a general inhibition of metabolic processes in *P. urticae*. If this is the case and the production of secondary metabolites by *P. expansum* in static culture is essentially an excretory or detoxication mechanism, then the question arises as to the fate of the 6-MSA produced in shake culture. Here the amount of patulin produced is insufficient to account for all the 6-MSA initially formed (see Fig. 1*b*), and it yet remains to establish the fate of this intermediate. It is also of interest to note that although 6-MSA is usually considered to be a secondary metabolite, its initial production here seems to occur when replicatory growth is still occurring. These observations suggest that 6-MSA may have some other, non-secondary, role in fungal metabolism.

Glycolysis and the TCA cycle operated in *P. expansum* under conditions of both low and high aeration. The activity of SDH reached a maximum in shake culture during the trophophase (Fig. 4*d*), but was attained during the later idiophase in static cultures. Moreover, gel electrophoresis showed that several isoenzymes of SDH that were present during shaken growth were absent from static-grown mycelium. Bu'Lock (1967) proposed that changes related to the TCA cycle affect secondary metabolite production as the pathway for the oxidation of acetyl CoA, and has shown that during the trophophase a high proportion of cycle intermediates are withdrawn, whilst at the same time a large pool of α -amino acids accumulates in the mycelium. The relationship between TCA cycle intermediates and transaminating α -amino acids is important in protein synthesis, and is also responsible for utilization of a larger proportion of reduced coenzymes (e.g. NADPH) from the PP shunt. In view of Bu'Lock's work, and as our results show that increased O_2 availability seems to divert *P. expansum* from secondary metabolite production to increased extracellular pectolytic enzyme synthesis, a closer look at the effects of aeration on the operation of the TCA cycle seems desirable.

Another point of interest for future studies is the relationship between sporulation and the processes of secondary metabolism with respect to the effects of aeration. Spore formation in *P. expansum* occurred after about 72 h (Fig. 1*a*). This was approximately the time of transition from trophophase to idiophase, and is the point where catabolic pathways change and where secondary metabolism really begins. High aeration has been reported to inhibit spore germination in *Aspergillus nidulans* (Rowley & Bull, 1973), and Bu'Lock (1961) observed some correlations between spore formation and primary and secondary metabolism in fungi. Furthermore, our replacement culture experiments were usually unsuccessful once the mycelial mat had begun to sporulate. Thus, our results suggest that catabolism, secondary metabolism and spore formation are interrelated and affected commensurately by the level of aeration of the cultures.

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