Morphological Alterations of *Penicillium chrysogenum* Caused by Carbon Dioxide

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Scanning electron microscopic studies showed that the morphology of *Penicillium chrysogenum* Thom in fermentation cultures changed in response to changes in CO₂ concentration. At influent CO₂ partial pressures between 0.03% (air) and 8%, the predominant morphological form was filamentous, whereas at 15% CO₂, swollen and stunted hyphae predominated, and a significant quantity of spherical or yeast-like cells was observed.

INTRODUCTION

The morphology of submerged mycelial organisms can be affected by a variety of environmental variables. Pirt & Callow (1959) showed that pellet formation and growth of the normal filamentous forms of *Penicillium chrysogenum* in a continuous submerged fermentation were determined by the pH of the medium. Average hyphal branch length was 200 µm for mycelia grown at pH 6.0 compared with 20 µm at pH 7.4. At pH 7.4 large numbers of swollen yeast-like cells were observed, indicating the beginning of pellet formation. These morphological alterations attributable to pH were reversible. When *P. chrysogenum* was grown in glucose-limited chemostat cultures at 25 °C and a dilution rate of 0.09 h⁻¹, hyphal branch length varied with pH, attaining a maximum value of 110 µm at pH 6.0 compared with that of 45 µm at pH 8.0 (Miles & Trinci, 1983). Variation of the diameters of hyphae grown at different pH values was not significant. The authors suggested that pH 6.0 is optimal for hyphal extension of *P. chrysogenum*.

Cell morphology is affected by the nitrogen source (Pirt & Callow, 1959). Replacement of ammonium sulphate by corn steep liquor increased hyphal length, reduced the frequency of hyphal branching and prevented formation of swollen cells at pH 7.4. Nutrient formulation is also important. Morton (1961) observed that conidiation of *Penicillium griseofulvum* Dierckx was rapidly induced in a medium containing a high concentration of glucose but lacking assimilable nitrogen. However, *P. chrysogenum* remained vegetative upon nitrogen exhaustion. Specific growth rate also affects cell morphology of *P. chrysogenum*. Righelato *et al.* (1968) found that vegetative growth occurred at a specific growth rate of 0.023–0.075 h⁻¹. As the growth rate was increased within this range, the occurrence of pellets and swollen cells increased. Conidiation occurred at specific growth rates below 0.014 h⁻¹.

Ho & Smith (1986) demonstrated that exposure of *P. chrysogenum* cultures to a range of moderate to high dissolved CO₂ concentrations retarded antibiotic production, decreased growth rate and caused marked morphological changes. This paper describes the effect of CO₂ concentration on the mycelial morphology of *P. chrysogenum*.

Abbreviation: SEM, scanning electron microscopy.

METHODS

The sporulation culture. *Penicillium chrysogenum* Thom, strain Wisconsin 54-1255 (ATCC 28089), was used. This strain is capable of producing up to 1 g penicillin per litre of fermentation medium. Activated spores were grown on agar slants (Ho & Smith, 1986) for 7–10 d. These mature surface cultures were stored at 4 °C and remained stable for up to three months at this temperature.

Inoculum development. An inoculum for the fermentation culture was prepared by removing spores from mycelia by mild agitation with sterile glass beads in 300 ml distilled water. Spore suspensions contained approximately 10^6 spores ml⁻¹ as determined with a haemocytometer (Fisher Scientific).

Bench-scale process. A Biostat S laboratory fermentation system (12.5 l capacity) was used (Ho & Smith, 1986). Growth medium (Ho & Smith, 1986) was added to the vessel aseptically. The pH was adjusted to 6.5 by addition of concentrated NH₄OH or a solution of 1 M-H₂SO₄ and 1.5 M-(NH₄)₂SO₄; glucose was sterilized separately. pH was measured with an Ingold steam-sterilizable pH electrode and controlled with a home-made pH controller. Dissolved CO₂ was monitored with a steam-sterilizable CO₂ electrode (Ingold Electrodes). Effluent CO₂ concentration was measured with an Esterline Angus infrared gas analyser. Dissolved O₂ was measured with an Ingold dissolved O₂ electrode.

Unless otherwise mentioned, the following conditions were held constant: temperature, 25 °C; rotational speed, 950 r.p.m.; air flow rate, 8 l min⁻¹ (1 vol. per culture vol. per min); glucose concentration, 40 g l⁻¹; and pH, 6.5. The influent gas was air with various proportions of N₂ replaced by equivalent amounts of CO₂ to give influent CO₂ partial pressures of 0.03% (air), 8.2%, 15% and 20%. The influent gas stream was fed to the fermentation vessel at constant temperature, pressure and flow rate for each fermentation run.

Specimen preparation for scanning electron microscopy (SEM). The following procedure was developed to fix and dehydrate the mycelial specimens for SEM studies. The morphological state of the cells in these specimens compared well with that of cells observed by light microscopy.

A 5 ml sample from the culture vessel was added to 25 ml 2% (v/v) glutaraldehyde solution at the same osmolarity as the culture medium. The glutaraldehyde solution was decanted from settled cells after incubation at 4 °C for 2 h. Cells were then resuspended in a buffer solution of 30.5 g KH₂PO₄ in 100 ml H₂O, and stored at 4 °C.

Fixed cells (5 ml) were injected through a Nucleopore filter (pore size 0.8 µm). A pre-filter was used to remove any particulate materials that might be present in the reagents and to protect the cells. All subsequent treatments were made through this pre-filter by injection. Portions (25 ml) of ethanol, 10%, 25%, 50%, 75% and 100% (v/v) in H₂O, were injected through the filter in successive washings, each of about 5 min duration. Freon 113 (50%, v/v) in ethanol (40 ml) was then injected through the specimen for 10 min, followed by 40 ml 100% Freon 113 for a further 10 min. The specimen was dried with injected air and stored in a desiccator.

The specimen was frozen very quickly in Freon cooled in liquid N₂, and either stored in liquid N₂ or moved to a cold stage in a vacuum coating unit. The specimen was introduced into a freeze-drying apparatus consisting of a vacuum system with a cold surface close to the specimen.

RESULTS AND DISCUSSION

Lyophilized spores of *P. chrysogenum* are shown in Fig. 1. Characteristically, these are the asexual conidiospores that are produced on agar slant cultures. The diameter of the spores was 5.5 µm. Hyphae of *P. chrysogenum* grown for 48 h under control conditions (no CO₂ added to the influent air) are shown in Fig. 2. The mycelium was composed of a network of long, thin, diffuse hyphae, whose average thickness was 2.0 µm. Characteristically, hyphae grew from a central branch, and the hyphal tips were smooth and round, indicating an actively growing site (Righelato *et al.*, 1968; Trinci & Righelato, 1970; Collinge *et al.*, 1978; Miles & Trinci, 1983). It is believed that any deviance from this morphological form would indicate altered metabolism.

Fig. 3 shows a hypha from mycelium grown for 48 h at an influent CO₂ concentration of 8.2%. The predominant morphological form was filamentous. The hyphal branching frequency was greater than that observed in the control experiment; this would presumably result in a high growth rate as the quantity of actively growing sites was increased. The diameter of the hyphae was 2.0–2.5 µm. Other morphological changes started to become apparent at this CO₂ concentration. An altered hyphal tip is shown in Fig. 4. Such swelling of the hyphal tips was accompanied by an increased hyphal branching frequency near the tip.

P. chrysogenum grown for 48 h with an influent CO₂ concentration of 15% had a morphology similar to that of yeast cells and spores (Fig. 5). Some filamentous growth was noted, however; this originated from spores, 6.0–7.0 µm in diameter, which resembled the spherical or yeast-like forms present in the culture. It is possible that the spherical forms were simply stunted spores.

Fig. 1

Fig. 1 SEM micrograph of lyophilized spores. Bar, 5 μ m.

Fig. 2

Fig. 2. SEM micrograph of a hyphal tip from a culture grown for 48 h without additional CO₂. Bar, 5 μ m.

Fig. 3

Fig. 3. SEM micrograph of hypha from a culture grown for 48 h under 8.2% CO₂. Bar, 5 μ m.

Fig. 4

Fig. 4. SEM micrograph of hypha from a culture grown for 48 h under 8.2% CO₂, showing abnormal tip morphology. Bar, 5 μ m.

Fig. 5

Fig. 5. SEM micrograph of swollen spores and hyphae from a culture grown for 48 h under 15% CO₂. Bar, 10 µm.

Fig. 6

Fig. 6. SEM micrograph of mycelium from a culture grown for 56 h under 20% CO₂. Bar, 10 µm.

The hyphae that developed from these spores were 2.5–3.0 µm in diameter, and some swollen and deformed hyphae were observed (Fig. 5). The diameters of the hyphae and the spores were 20% and 12%, respectively, greater than those in the control cultures.

Part of a mycelium grown for 56 h at an influent CO₂ concentration of 20% is shown in Fig. 6. The individual cells within the hyphae can be seen to have become swollen, causing the hyphae to have a 'bumpy' appearance. The diameter of the hyphae was 4.0–7.0 µm. The diameters of the spores could not be measured because of difficulty in identifying them. Almost all the hyphal tips were severely deformed, indicating an adverse effect of high CO₂ concentration on mycelial growth. There appeared to be an increase in the branching frequency near the hyphal tips. Fig. 7 shows hyphae grown for 88 h with an influent CO₂ concentration of 20%. The main difference in comparison to the mycelia harvested at 56 h was in the length of the hyphae, since linear growth had continued and the hyphal cross-sectional diameter remained constant. Hyphae from an 88 h culture are shown at a higher magnification in Fig. 8. The mycelial structure appeared to be of the aberrant form described by Pirt & Callow (1959), Morton (1961) and Bent & Morton (1963). Not only were the hyphae much deformed, but their cell wall was also punctured, an effect not observed in the cultures harvested at 56 h.

The major results of the present study are summarized in Table 1. There are several possible explanations for the effects observed. *P. chrysogenum* cultures exposed to high concentrations of CO₂ consisted of swollen hyphae in which individual cells were identifiable by the bumpy surface of the cell walls (Figs 6–8). This suggests that conidiation occurred in these conditions. In typical submerged mycelial fermentations, conidiation would normally occur under adverse environmental conditions (Morton, 1961; Bent & Morton, 1963; Righelato *et al.*, 1968).

As CO₂ concentration increased, so did the degree of hyphal swelling, suggesting that either high CO₂ concentrations affected the membrane transport properties of the cell or the cells were subject to osmotic swelling. CO₂ may alter cell membranes by affecting the fatty acid core, and

Fig. 7

Fig. 7. SEM micrograph of swollen hyphae from a culture grown for 88 h under 20% CO₂. Bar, 10 µm.

Fig. 8

Fig. 8. SEM micrograph of a deformed and punctured hypha from a culture grown for 88 h under 20% CO₂. Bar, 5 µm.

Table 1. *Effect of CO₂ on morphology of P. chrysogenum*

Samples were taken from batch fermentations as described in Methods. Observations and measurements were made using SEM.

Influent CO ₂ concn (%)	Incubation time (h)	Hyphal diam. (µm)	Spore diam. (µm)	Hyphal morphology
0.03 (air)	48	2.0	5.5	Hyphae long, thin and diffuse (Fig. 2) Tips smooth and rounded
8.2	48	2.0–2.5	NA	Filamentous hyphae (Fig. 3) Branching frequency of hyphae and tips (Fig. 4) increased
15	48	2.5–3.0	6.0–7.0	Some deformed, swollen hyphae (Fig. 5)
20	56	4.0–7.0	ND	All hyphae severely swollen and deformed (Fig. 6) Much increased branching frequency
20	88	4.0–7.0	ND	Much increased branching frequency (Fig. 7) Swollen hyphal walls punctured (Fig. 8)

NA, Not applicable; ND, not determined.

HCO₃⁻ ions may affect the charge of the phospholipid head groups and proteins at the surface of the membrane (Alberts *et al.*, 1983; Houslay & Stanley, 1982). It is thus possible that CO₂ caused the hyphal swelling by altering membrane transport processes.

Many fungi can grow in either a filamentous or a yeast-like form depending on environmental conditions (Stewart & Rogers, 1983). Our results demonstrated that *P. chrysogenum* could grow in a yeast-like form in the presence of high CO₂ concentrations, whereas at low to moderate CO₂

concentrations, growth was predominantly filamentous with some yeast-like cells present, the proportion depending on CO₂ concentration and culture age.

Chitin synthesis and the resulting chitin fibrils determine the shape or direction of mycelial growth (Stewart & Rogers, 1983). Linear growth occurs as the chitin fibrils form a microfibril net around the circumference of the hyphae, whereas spherical growth results from the elimination of rearrangement of the chitin synthesis sites (Stewart & Rogers, 1983). Possibly, as the concentration of CO₂ was increased in the medium, the chitin synthesis sites on *P. chrysogenum* cells were reduced and spherical or yeast-like growth occurred as a result.

The branching frequency of *P. chrysogenum* hyphae increased in the presence of added CO₂ (Table 1). This increase in hyphal branches might be related to a higher transcellular electric current (Jaffe, 1981; Harold *et al.*, 1985) under these conditions. A variety of fungi drive currents of protons which enter the apical zone of growing hyphal tips and leave distally (Jaffe & Nuccitelli, 1974). An inward current of protons often precedes the outgrowth of new tips (Jaffe *et al.*, 1974; Jaffe, 1981; Kropf *et al.*, 1983, 1984; Harold *et al.*, 1985). The CO₂ in the gas mixtures sparged into the fermentation medium would form carbonic acid, which would be readily converted to H⁺ and HCO₃⁻. In consequence, a higher quantity of protons would be present in the medium. The resulting higher endogenous electric currents could induce more growing hyphae, thus resulting in a higher branching frequency as compared to that of the control fermentation.

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