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Response of *Penicillium chrysogenum* to oxygen starvation in glucose- and nitrogen-limited chemostat cultures

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Abstract

The response of glucose- or nitrogen-limited chemostat cultures of an industrial strain of *Penicillium chrysogenum* to a period of oxygen starvation was assessed. Indicators of autolysis and morphological changes were monitored quantitatively using a computerised image analysis system. Despite previous reports that O_2 starvation was a likely contributory factor bringing about the onset of autolysis, levels of the indicators of autolysis were modest in this study during and following the anoxic period. Indeed, the cultures remained active at a much reduced level following anoxia, for a length of time dependent on the nature of the nutrient limitation previously imposed. The recovery of cultures from variable periods of anoxia was examined by inoculating aliquots of the bioreactor culture into shake flasks with fresh, complete medium; no recovery or autolysis was observed after 3 or more h of O_2 starvation when the culture (from either the glucose or nitrogen limited chemostat) was inoculated into shake flasks. However, culture survival, as assessed by regrowth after a period of anoxia, was dependent on the nature of the limiting nutrient before anoxia, with N-limited cultures showing improved survival relative to C (energy limited). This study clearly identifies the processes of cell death (cessation of metabolic activity) and cellular autolysis as distinct phenomena.

Keywords: P. chrysogenum; Oxygen starvation; Autolysis

1. Introduction

The processes of growth and hyphal elongation in filamentous fungi are the subject of numerous studies and the mechanisms and control are well understood [1,2]. Less well understood, however, are the processes of cell death and autolysis (where cells cease growing and begin to break down by action of their own enzymes [3]). In filamentous fungi, the development of autolysis in hyphal sections may be looked on as a loss of productive capacity, with potentially significant consequences [4,5]. It has been shown that production of some secondary metabolites, such as antibiotics from filamentous fungi, may be related to the physiological state of the organism and that degenerated cells may not contribute to secondary metabolite production [6]. Furthermore, it has been shown that many of the enzymes released or activated during autolysis may degrade the product [7,8]. The rate at which autolysis occurs in a fungal culture and the factors influencing the process are, therefore, of interest, particularly in bioprocesses using economically important filamentous fungi.

In large scale batch and fed batch cultivation of *Penicillium chrysogenum*, the onset of autolysis coincides with substrate limitation towards the end of the penicillin production phase [9,10]. At this time, an increasing proportion of empty, degenerated cells are observed in the culture [5] with a progressive loss of cytoplasmic ultra-structure [11], and, for some hyphal sections, only empty hyphal walls remain [12]. In industrial production of penicillin, there is a noticeable loss of the product through degradation reactions [8], and an increase in the activities of penicillin G and penicillin V amidohydrolase activities also has been demonstrated during fungal autolysis [7]. Fungal autolysis could, thus, have economic implications.

It has been previously reported that the degree of autolysis within a culture was impossible to judge microscopically [11] due to the lack of synchrony, which is a feature of the morphology of filamentous fungi in submerged cultivation [5,13]. However, several studies of hyphal differentiation in *P. chrysogenum* have highlighted the importance of

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the recent advances in image analysis technology used in studies of the morphology of filamentous fungi [6,9,10,14]. From such studies, it appears that autolysis is most likely to occur in the highly vacuolated or degenerated regions of hyphae [10,14]. In these regions, it is thought that proteins [15,16], cell wall polysaccharides and cytoplasmic material [7,11,17] may be degraded. Increased protease activity has been observed in *P. chrysogenum* when extensive vacuolation and fragmentation were taking place, as well as a switch to the use of amino acids as an energy source[18]. It is generally assumed that components excreted from degenerating cells are immediately scavenged by healthy cells for maintenance or growth [19,20]. In some respects, there are similarities between fungal autolysis and the process of apoptosis in higher eucaryotes [21]. What remains to be elucidated is the sequence of events in fungal autolysis from limitation or starvation in the culture to breakdown of macromolecules and degradation of the hyphal structure.

With many organisms, it has been reported that the synthesis and secretion of proteases occurs when the medium is deficient in one or more nutrients [22]. In *Aspergillus nidulans*, this occurs when the medium is deficient in one or more of carbon, nitrogen, or sulphur [23,24]. A number of proteolytic enzymes in *Schizophyllum commune* have their activities increased during nitrogen limited growth of the mycelium [25]; under these conditions sustained mycelial growth is thought to involve mobilization of nitrogen-rich molecules from older portions of the mycelium and the subsequent redistribution of these molecules to growing apices.

In addition to the importance of nutrient deficiency, examination of the effect of O_2 starvation or limitation is particularly relevant because, at industrial scale, cultures may be exposed to repeated or prolonged periods of O_2 starvation [26], which, in addition to inhibiting growth, may lead to increased autolysis [4]. If this were the case, it might be a contributory factor to the generally observed decrease in the productivity of fungal cultures with increasing operational scale [27]. Indeed, penicillin production is widely reported as being severely affected by decreases in dissolved oxygen tension (DOT) [28,29]. An additional reason for investigating the impact of oxygen starvation/limitation on fungal autolysis is that, in the absence of oxygen, the normal energy generation metabolism will be disrupted (as in carbon starvation); thus, one might, a priori, expect anoxia to have a major impact on autolysis. Previous reports indicate that oxygen starvation is one of the main contributory factors to the onset of autolysis [30,31], although the impact of oxygen starvation on morphology and degeneration of mycelial cultures has not yet been assessed quantitatively.

For the above reasons, the present study concentrates on the effects of oxygen starvation in chemostat cultures of *P. chrysogenum*, where changes made to the inlet gas composition may be studied in a steady-state environment. Using continuous culture processes provides a steady reference point from which to monitor the effect of changes made to the environment of the culture [8,22]. In this study, the response of glucose (C and energy)-limited and ammonium (N source)-limited steady-state cultures to oxygen starvation was monitored. Also of interest was the level of recovery possible in cultures that previously had been exposed to periods of oxygen starvation to determine whether the process of autolysis can be slowed or halted once started in a culture. The key factor being investigated is recovery from the onset of autolysis, or commitment to the process of autolysis. Computerized image analysis was used to quantify morphological changes in the cultures.

2. Materials and methods

2.1. Submerged liquid processes

2.1.1. Micro-organism

An industrial strain of *P. chrysogenum* (supplied by SmithKline Beecham Pharmaceuticals, Irvine, UK) was used throughout. Spores for inoculum purposes were prepared from a stock spore suspension inoculated into sealed flasks containing sterile, cooked rice. These were incubated at 25°C for 7 days. Spores then were harvested in sterile distilled water, with gentle shaking, and filtered through sterile glass wool. The resulting spore suspensions were used as inocula for shake flasks at a level of 10^6/ml in the flasks.

The medium composition for inoculum development and the batch phase of the continuous processes contained (per m^3) (modified from Nielsen et al. [33]) 10 kg of sucrose, 70 kg of lactose, 1.6 kg of KH_2PO_4, 7.0 kg of $(NH_4)_2SO_4$, 0.04 kg of $FeSO_4.7H_2O$, 0.1 kg of $MgSO_4.7H_2O$, 0.5 kg of KCl, 0.05 kg of $CaCl_2.7H_2O$, 5 ml/l of a trace element solution containing (per m^3) 1 kg of $CuSO_4.5H_2O$, 4 kg of $ZnSO_4.7H_2O$, 4 kg of $MnSO_4.H_2O$. The pH was adjusted to 5.9 by addition of 2 M NaOH. The bioreactor inoculum was 0.8 dm^3 of a 72-h-old shake flask culture grown at 25°C and 200 rev./min.

Continuous fermentations were commenced at 50 h into the batch phase. Processes were carried out with either glucose or ammonium as the limiting nutrient. The feed medium composition for the continuous phase of bioreactor experiments for glucose limitation was the same as the medium described above (modified from Henriksen et al. [34]) except that it contained (per m^3) 15 kg of glucose (replacing the sucrose and lactose above) and 6.5 kg of phenoxyacetic acid (neutralized with 2 M NaOH and added to the medium). For nitrogen limitation, the medium was as the continuous medium described above except that it contained (per m^3) 20 kg of glucose and 3.5 kg of $(NH_4)_2SO_4$. Sterile continuous medium was pumped into the fermenter by using a Watson Marlow pump (Type 501) at a dilution rate of 0.08 h^{-1}.

2.1.2. Bioreactor experiments

The bioreactor used in this study was a Biostat ED ES10 (B. Braun Biotech International, Melsungen, Germany) with a working volume of 8 dm^3. Fermentations were performed at 25°C, and stirrer speed was 800 rev./min. The pH was controlled throughout at 5.9 by automatic addition of either 2 M NaOH or 10% (v/v) H_2SO_4. The culture was aerated at a rate of 1 vvm. Gas flow rates and pressures were monitored and independently controlled by the use of a gas mixing unit (Model 881611, B. Braun Biotech International) and a thermal mass flow meter (B. Braun Biotech International).

In addition to the standard glucose-limited or nitrogen-limited continuous processes (where air was the input gas), these processes also were subject to gassing with oxygen-free nitrogen (OFN) (at 1 vvm) for 5 h, after which time the air supply was reconnected. After the anoxic period, the feed medium was switched off and the process was batched. The dynamic response of the cultures to step changes in the influent oxygen level was monitored closely during these periods.

2.1.3. Shake flask studies

Fifteen 500-ml shake flasks containing 200 ml of the continuous culture medium as above (depending on which nutrient was limiting in the fermentation) each were inoculated with culture broth from the fermenter at 0, 1, 2, 3, and 4 h after the start of gassing with OFN to give a final concentration of biomass in the flasks of 0.1 g/l. The flasks were incubated at 25°C and 200 rev./min for 5 days with three flasks from each set being removed at 24-h intervals for analysis.

2.1.4. Analyses

Dry weight was estimated by filtering 0.01 dm^3 of the culture broth through Whatman GF/C filter paper (diameter, 4.25 cm). Filter cakes were washed with 2 vol of sterile distilled water, dried for 20 min in a microwave oven (650 W) on low power, and cooled before weighing. The amount of total carbohydrate in culture filtrates was determined by the method of Dubois et al [35]. The concentration of glucose in culture filtrates was determined by using a kit (Cat. No. 716251) by Boehringer Lewes Ltd. The concentration of NH_4^+ was determined by the method of Burton and Watson–Craik [36]. The ethanol concentration in culture filtrates was determined enzymatically by using a kit (Cat. No. 176290) provided by Boehringher Ltd. Elemental composition analysis (relative percentages of C, H, and N) of washed, dried, and powdered biomass samples was determined by using a Carlo ERBA 1106 analyzer. All analyses were carried out in triplicate.

The concentration of carbon dioxide in the exit gas was monitored with a model ADC 7000 infrared gas analyzer (The Analytical Development Co., Cambridge, UK), and the concentration of oxygen in the fermenter off-gas was monitored with a series 500 Servomex Paramagnetic oxygen analyzer (Sybron Taylor, Sussex, UK).

2.1.5. Morphological analysis

Morphological characteristics of the culture were measured using a Seescan (Cambridge, UK) dedicated image analysis system that produced digitized images from a Nikon Optiphot-2 microscope, on which was mounted a Sony CCD video camera (Model XC-77CE). Measurements were made manually on frozen images by using a mouse to skeletonize individual hyphal trees ("organisms") and, thus, to measure hyphal dimensions. The total magnification used was ×200.

For individual hyphal elements, the mean hyphal length, the mean branch length, the mean total hyphal length, and the mean hyphal growth unit were found for each sample. The mean values were estimated from measurements of 50 mycelial particles for each fermentation time-course sample. The degree of degradation (as an indication of autolysis) also was found for each sample by calculating the percentage of empty compartments within the hyphae.

3. Results

3.1. Glucose-limited chemostat

Biomass, total carbohydrate, ammonia and penicillin concentrations, carbon dioxide evolution rate, oxygen uptake rate, percentage of empty regions in the hyphae, and percentage of "organisms" with empty regions over the time course of the process are shown in Fig. 1. The time course for these variables during the 5-h period with oxygen-free nitrogen as the sparge gas (i.e. complete oxygen starvation or anoxia) is given in Fig. 2. The trends in the morphological indices over this period are shown in Fig. 3. The concentration of glucose was estimated in the culture during the previous steady-state (with air as the sparge gas) and at 0 h in Figs. 1 and 2 and was not found to be present; thus cultures were designated glucose-limited despite the presence of carbohydrates in the filtrates (these may have been products of low level autolysis occurring throughout the process).

Whole broth samples were removed from the fermenter at 0, 1, 2, 3, and 4 h after commencement of sparging with OFN and inoculated into shake flasks, and the cultures were grown for 5 days. The biomass concentration and percentage of empty regions (extent of autolysis) after 5 days of cultivation for each of the samples are shown in Fig. 4.

3.2. Nitrogen-limited chemostat

Biomass, total carbohydrate, ammonia and penicillin concentrations, carbon dioxide evolution rate, oxygen uptake rate, percentage of empty regions in the hyphae, and the percentage of "organisms" with empty regions over the time course of the process are shown in Fig. 5. The time course for these parameters during the 5-h period with oxygen-free nitrogen as the sparge gas is given in Fig. 6,

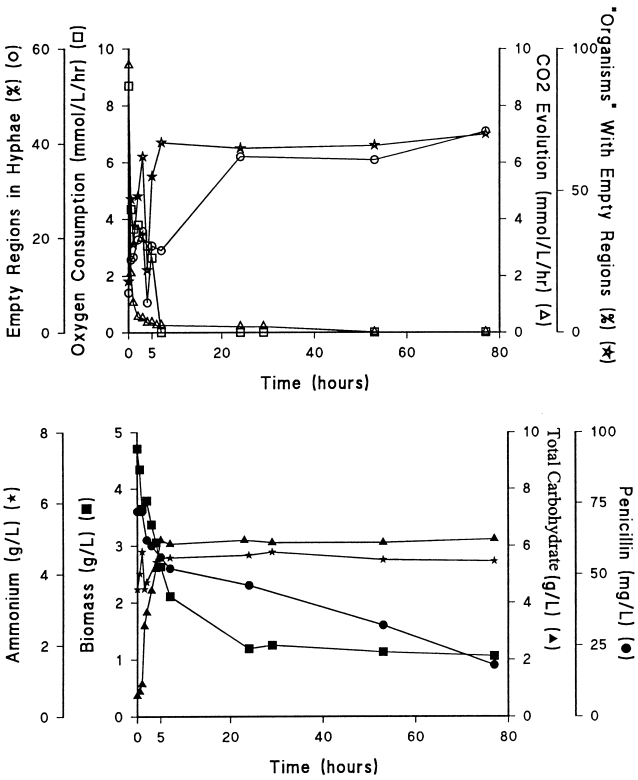

Fig. 1. Biomass (g/l), total carbohydrate (g/l), ammonia (g/l) penicillin (mg/l) concentrations, oxygen uptake rate (mmol l/h), carbon dioxide evolution rate (mmol l/h), percentage of empty regions in hyphae, and percentage of "organisms" with empty regions over the time course of the glucose-limited continuous fermentation. Zero time points represent previous steady-state values. Air was replaced by OFN as the sparge gas between 0 and 5 h. Dilution rate during the continuous phase was 0.08 h^{-1}. The process was batched up after 5 h.

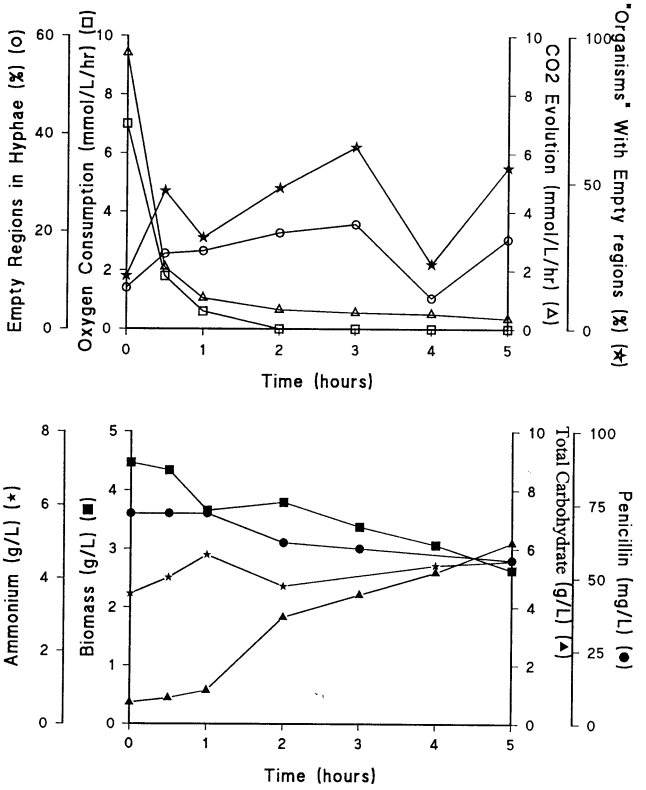

Fig. 2. Biomass (g/l), total carbohydrate (g/l), ammonia (g/l) penicillin (mg/l) concentrations, oxygen uptake rate (mmol l/h), carbon dioxide evolution rate (mmol l/h), percentage of empty regions in hyphae, and percentage of "organisms" with empty regions over the time course of the glucose-limited continuous fermentation when air was replaced by OFN as the sparge gas. Zero time points represent previous steady-state values. Dilution rate was 0.08 h^{-1}.

and the morphological parameters over this period are shown in Fig. 7.

As above, whole broth samples were removed from the fermenter at hourly intervals during sparging with OFN and inoculated into shake flasks, and the cultures were grown for 5 days. The biomass concentration and percentage of empty regions at the end of the cultivation period for each of the samples are shown in Fig. 8. Ethanol concentrations for both glucose- and nitrogen-limited processes at various time points are given in Table 1. Elemental composition of carbon- and nitrogen-limited steady-state cultures before and after the period of oxygen starvation is given in Table 2. The 0 time sample values in Figs. 1–3 and 5–7 represent the steady-state values for the cultures.

4. Discussion

4.1. Glucose-limited chemostat

From Fig. 1, it can be seen that after the period of oxygen starvation (0 to 5 h), uptake of nitrogen or carbon sources from the medium ceases. The biomass levels decline between 0 and 24 h whereafter the values remain constant. This indicates that autolysis and cellular breakdown of the organism is occurring within the 24-h period following introduction of OFN as the sparge gas. Culture degradation with release of carbon may be suggested by the fact that the

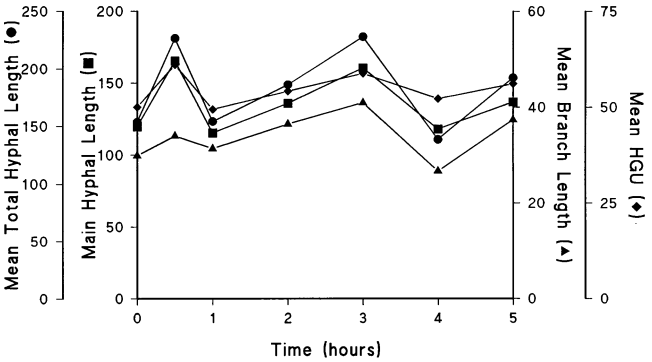

Fig. 3. Mean main hyphal length, mean branch length, mean total hyphal length, and mean hyphal growth unit (all um) over the time course of the glucose-limited process when air was replaced by OFN as the sparge gas. Zero time points represent previous steady-state values.

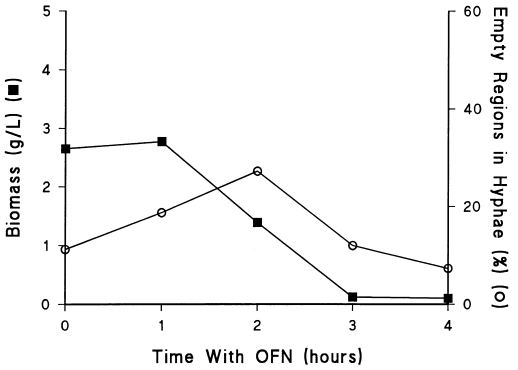

Fig. 4. Biomass concentration (g/l) and percentage of empty regions in the hyphae after 5 days of cultivation in shake flasks for samples removed from the glucose-limited chemostat 0, 1, 2, and 3 h after commencement of OFN sparging.

total carbohydrate concentration in the filtrate increases during and following oxygen starvation (Figs. 1 and 2). Further evidence for autolysis is given by the increase in the percentage of empty regions within the hyphae (i.e., regions without cytoplasm) during this period. Progressive loss of cytoplasmic ultra structure, with only empty hyphal walls remaining, has been designated previously an indicator of autolysis [6,13]. However, from 24 h onward, the culture appears metabolically inactive with no CO_2 evolution or O_2 consumption observed. Apart from the penicillin level (which shows a decline consistent with previously reported

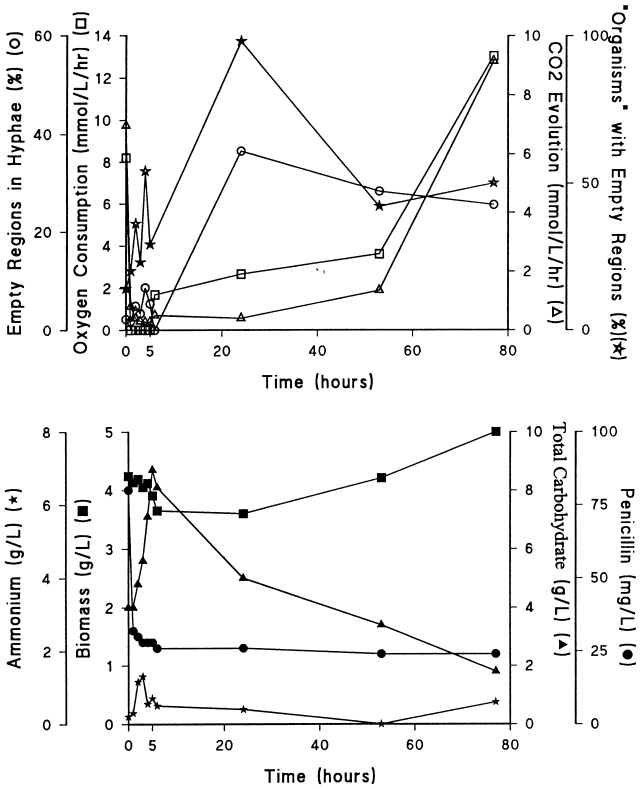

Fig. 5. Biomass (g/l), total carbohydrate (g/l), ammonia (g/l) penicillin (mg/l) concentrations, oxygen uptake rate (mmol l/h), carbon dioxide evolution rate (mmol l/h), percentage of empty regions in hyphae, and percentage of "organisms" with empty regions over the time course of the nitrogen-limited continuous fermentation. Zero time points represent previous steady-state values. Air was replaced by OFN as the sparge gas between 0 and 5 h. Dilution rate during the continuous phase was 0.08 h^{-1}. The process was batched up after 5 h.

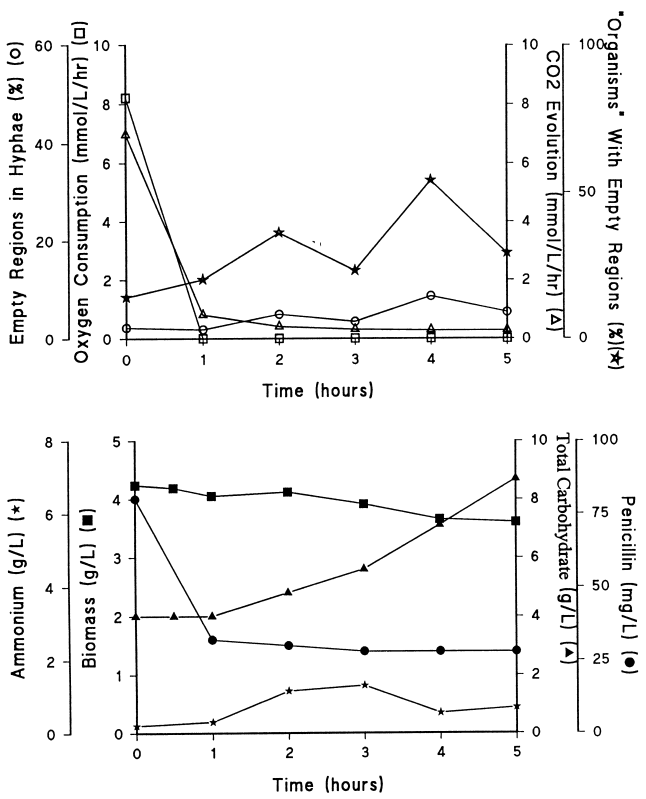

Fig. 6. Biomass (g/l), total carbohydrate (g/l), ammonia (g/l), penicillin (mg/l) concentrations, oxygen uptake rate (mmol l/h), carbon dioxide evolution rate (mmol 1/h), percentage of empty regions in hyphae, and percentage of "organisms" with empty regions over the time course of the nitrogen-limited continuous fermentation when air was replaced by OFN as the sparge gas. Zero time points represent previous steady-state values. Dilution rate was 0.08 h^{-1}.

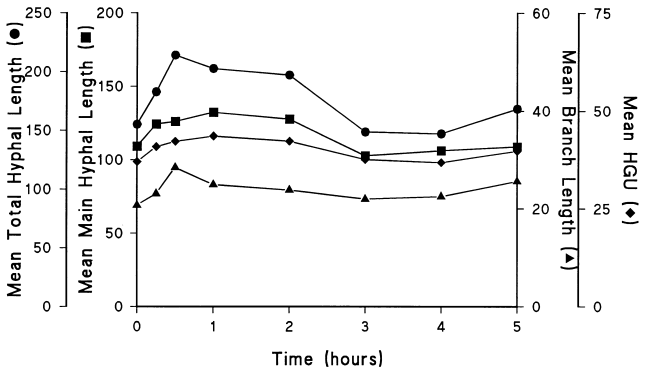

Fig. 7. Mean main hyphal length, mean branch length, mean total hyphal length, and mean hyphal growth unit (all in micrometers) over the time course of the nitrogen-limited process when air was replaced by OFN as the sparge gas. Zero time points represent previous steady-state values.

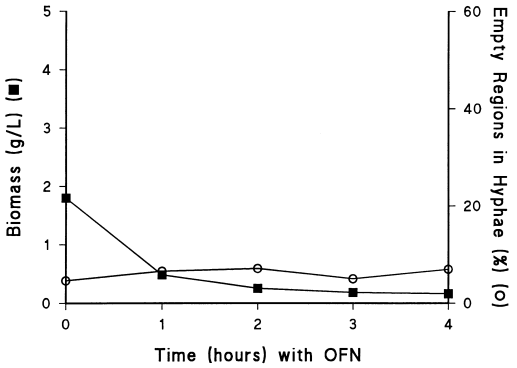

Fig. 8. Biomass concentration (g/l) and percentage of empty regions in the hyphae after 5 days of cultivation in shake flasks for samples removed from the nitrogen-limited chemostat 0, 1, 2, and 3 h after commencement of OFN sparging.

degradation rates [10]), all parameters (Fig. 1) remain relatively constant from 24 h to the end of the experimental period.

On a shorter time scale, changes were apparent in the process within 30 min of the onset of OFN sparging (Fig. 2) with a decline in biomass observed and an increase in both the residual glucose and nitrogen levels. An increase in the percentage of empty regions was observed as the period of oxygen starvation proceeded up to 3 h, followed by a decline at 4 h. These observations can be explained by consideration of Fig. 3. At the 4-h point, there was a decrease in the values for all key morphological parameters. This decline was probably a result of fragmentation of the hyphae [16,37], which is most likely to have occurred at the weakened empty regions [11,12,14,16,20,38]. The resultant fall in the percentage of empty regions at this point suggests that the empty hyphal sections were fragmented and distributed into the medium. An important conclusion that may be drawn from these results is that, once started, the process of autolysis is not an inevitable fate for the entire hyphal element; rather, the process can be contained or restricted to within certain segments of the organism, and following the fragmentation of these weakened, empty regions, typically, a decrease in the overall level of empty regions is observed.

Between 4 and 5 h after the onset of gassing with OFN (Fig. 2) an increase was observed in the percentage of empty regions and in the value of the morphological indices (Fig. 3). It has been reported for *Aspergillus nidulans* that regeneration of a fragmented and previously starved culture can occur, as each fragment would consist of a number of hyphal compartments, of which only one would need to be viable for subsequent growth [19]. During the final hour of oxygen starvation in the current study, the hyphae were elongating (demonstrated by an increase in total hyphal length in Fig. 3), but there was, however, a decline in the biomass concentration (Fig. 2) over this period. The simultaneous increase in both the percentage of empty regions and the percentage of organisms with empty regions provides evidence that recycling of components within individual organisms, as proposed in a previous study [22], was occurring during this time.

No recovery in the culture was apparent after the period of oxygen starvation and no regrowth of cultures occurred in shake flasks after 3 or more h with OFN (Fig. 4). The percentage of empty regions in the hyphae did show an increase initially; however, after 5 days of cultivation, the levels in the samples taken at 3 and 4 h into the OFN sparging were similar to those in the samples that were removed from the fermenter. This was also true for the biomass levels in these samples, indicating that, in these cases, there was no recovery or regrowth in the cultures.

Table 1
Ethanol concentration in filtrate samples from the glucose- and nitrogen-limited processes at steady-state, the end of the oxygen starvation period, and during the batch up phase

Process time point (h)	Ethanol concentration (mg/l)	
	Glucose-limited process	Nitrogen-limited process
Steady-state	11	16
5 (end of OFN period)	134	131
24 after anoxic period	10	8
48 after anoxic period	4	4
72 after anoxic period	6	4

Table 2
Elemental composition (percentage C, N, and H) of carbon and nitrogen-limited steady-state cultures before (0 h OFN), during (5 OFN), and after (72 h) the period of oxygen starvation (where the sparge gas was OFN)

Time (h)	C	N	H
Carbon-limited culture			
0 OFN	47.8 (1.5)	4.8 (0.1)	6.8 (0.1)
5 OFN	46.9 (1.3)	4.8 (0.2)	6.6 (0)
72	45.9 (1.4)	4.0 (0.2)	6.7 (0.1)
Nitrogen limited culture			
0 OFN	46.8 (1.5)	8.9 (0.4)	6.2 (0.2)
5 OFN	46.7 (0.5)	7.1 (0.1)	6.5 (0.4)
72	46.7 (1.5)	6.8 (0.5)	6.4 (0.1)

4.2. Nitrogen-limited chemostat

After an initial decline during the period of oxygen starvation (Fig. 5), the biomass concentration increased from 20 h onwards; this was coupled with utilization of carbohydrate. This, and the resumption of O_2 consumption and CO_2 evolution, indicated recovery of the organism in the fermenter following the period of oxygen starvation. The nitrogen source was exhausted at 48 h, with ammonium appearing in the medium at 77 h. Over this period, it is likely that recycling of nitrogen sources within organisms was occurring. There was a relatively high level of empty regions in the hyphae at this stage in the process, which may indicate that turnover within hyphal elements was providing

the nitrogen requirement and that the increase in ammonium concentration at the end of the process was a result of protein degradation [6].

During the 5-h period in which OFN was the sparge gas (Fig. 6) there was a decrease in the biomass concentration and a fall in the level of carbohydrate utilization. In these cultures, however, there was an increase in the percentage of empty regions within the hyphae (Fig. 7), again indicating breakdown and recycling of components within individual organisms. Apart from the appearance of autolysis within the culture, the organisms appear to be metabolically inactive after ≈2 h with OFN. When the results of the regrowth experiment results are considered (Fig. 8), it can be seen that there is no regrowth in the cultures removed from the fermenter after 3 or 4 h sparging with OFN. However, there is clear regrowth in the fermenter after the period of oxygen starvation.

From the above it is clear that the responses of *P. chrysogenum*, in terms of both morphology and growth, to periods of anoxia are quite distinct, dependent on the nature of the nutrient limitation previously imposed on the culture. One possible theory may be advanced for the regrowth of the culture in the nitrogen-limited chemostat but not in the glucose-limited process. It has been widely reported for other filamentous fungi [17,27,39] that proteolytic enzymes have their activities elevated during periods of nitrogen limitation, resulting in N-stress-induced autolysis. This may explain the survival of the culture under N-limited conditions, with a high rate of recycling of cellular components through proteolysis, which was not induced in the glucose-limited process, providing the necessary nitrogen for continued survival. The excess carbon in the medium at the start of the oxygen starvation period (Fig. 5) may have provided the energy required to drive the autolytic process, which was not possible in the case where glucose was the limiting nutrient (Fig. 1) [14].

Examination of Table 2 reveals evidence of nitrogen storage by the nitrogen-limited culture with a higher nitrogen content in the cells in these cultures than in those from the glucose-limited process. Carbon and hydrogen levels in the biomass are similar for both processes and are in agreement with previously reported figures for the elemental composition of this organism [40]. In the carbon-limited process, the level of nitrogen in the biomass declines slightly over the period of oxygen starvation and for the period following this. This may point to the reason why this culture did not recover as well as the nitrogen-limited culture from the period of anoxia. In this (nitrogen-limited process) the level of nitrogen in the biomass decreases during oxygen starvation, suggesting, in this case, utilization of internal nitrogen reserves.

It has been shown that the process of autolysis occurs in distinct stages, and, in the early stages, is an energy-requiring (active) process; thus, the C source excess in the N-limited cultures may have permitted more extensive nutrient recycling and survival of the period of anoxia. Many proteases have been shown to be energy-dependent [41], especially those induced during periods of nutritional stress. It is thus possible that in the absence of a sufficient external energy source, the proteolytic activation, which is considered an important feature of autolysis [20,27] and nutrient recycling, cannot occur in the C-limited culture, and key systems required for recovery are inactivated, preventing culture regrowth after the anoxic period.

It has been previously reported that O_2 starvation promotes autolysis in cultures of filamentous fungi [6,30,31], with autolysis being defined as a step in the path towards cell death and cessation of metabolic activity. However, this was not observed in this study in which the development of autolyzed or degraded regions within the hyphae appeared to be dependent on the availability of energy to recycle nutrients, with no increase in the percentage of empty regions once cellular metabolism had ceased. Indeed, there appeared to be no significant increase in autolysis as a result of oxygen starvation. Surprisingly, as it has been reported that energy (O_2 and carbon) is required for the maintenance of functional cell envelopes, the level of empty regions within the hyphae remained low during the 5-h periods of oxygen starvation (Figs. 2 and 6). Results of this study demonstrate, therefore, that the process of autolysis (breakdown of cellular material from within) may be induced by oxygen starvation but only where there is excess carbon as an energy source for the catabolism.

In both processes, some CO_2 evolution was noted during the period of oxygen starvation; this was accompanied by a low level of ethanol production (Table 1), indicating fermentation by *P. chrysogenum* under anaerobic conditions. Such behavior in other filamentous fungi has been reported previously; the alcoholic fermentation pathway is required in *Aspergillus nidulans* for anaerobic survival [42,43]. There is, however, no recovery in the glucose-limited process after this 5-h period with OFN, and CO_2 evolution ceases. In the nitrogen-limited process, CO_2 evolution continues with the recovery and continued respiration of the organism for the time period following oxygen starvation. It is apparent that *P. chrysogenum*, in this study also may be using the alcoholic fermentation pathway for survival during the periods of oxygen starvation. This study demonstrates a clear distinction between the active, energy-consuming process of autolysis, which may contribute to the survival of culture elements in times of nutritional stress, and the process of cell death (the complete suspension of metabolic activity).

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