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The Autolysis of Industrial Filamentous Fungi

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ABSTRACT: Fungal autolysis is the natural process of self-digestion of aged hyphal cultures, occurring as a result of hydrolase activity, causing vacuolation and disruption of organelle and cell wall structure. Previously, authors have considered individual aspects of fungal lysis, in terms of either an enzyme, a process or an organism. This review considers both the physiology and morphology of fungal autolysis, with an emphasis on correlations between enzymological profiles and the morphological changes occurring during culture degeneration. The involvement of the main groups of autolytic hydrolases is examined (i.e., proteases, glucanases, and chitinases), in addition to the effects of autolysis on the morphology and products of industrial bioprocesses. We call for a concerted approach to the study of autolysis, as this will be fundamental for research to progress in this field. Increased understanding will allow for greater control of the prevention, or induction of fungal autolysis. Such advances will be applicable in the development of antifungal medicines and enable increased productivity and yields in industrial bioprocesses. Using paradigms in existing model systems, including mammalian cell death and aging in yeast, areas for future study are suggested in order to advance the study of fungal cell death.

KEY WORDS: fungal autolysis, enzymology, morphology, cell death, bioprocessing.

I. INTRODUCTION

Filamentous fungi are ubiquitous. Not only can they be serious pathogens, they are also of tremendous environmental and economic importance to man. These organisms recycle nutrients in the biosphere and produce a valuable range of products, including antibiotics, organic acids, enzymes, heterologous proteins, and food products such as Quorn®. Improved understanding of the mechanisms and factors causing fungal autolysis (self-digestion) has the potential to bring considerable improvements in bioprocesses.

In the case of chronic fungal infections in immunocompromised patients, and the problem of fungal pests in agriculture, new targets for drug discovery are required (due to the decreasing efficacy of existing antifungals) in order to bring about the death of the fungus.[1] Fungal autolysis is a multistage process involving partial permeabilization of the cellular envelope and leakage of intracellular material in its latter stages. These characteristics of fungal cell death have been likened to the activity of the antifungal agent amphotericin B, which increases acidification and pore formation allowing cations and Ca^{2+} to diffuse out of the organism, causing osmotic lysis.[2]

In the bioprocessing industries, manufacturers usually want to prevent autolysis, either to continue antibiotic production, or to prevent degradation of heterologous protein products by autolytic proteases.[3] Autolysis during the production of penicillins[4,5] and cephalosporin C,[6] has been correlated with increased extracel-

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lular acylase activity. Although this enzyme degrades both antibiotics, its activity might be economically advantageous, as the respective products, 6-aminopencillanic acid and 7-aminocephalosporanic acid, are the precursors for semisynthetic antibiotic production. Autolysis may also be desirable because it might promote intracellular product recovery, for example, the release of lipid granules containing the antibiotic fusidic acid.[7] Although such lysis may assist downstream processing, McNeil et al. (1998) noted that greatly increased filtration times during cell removal, led to complete filter blockage.[8] Such problems could increase processing times, which is a serious concern for the recovery of susceptible products from fermentation fluids.

In reviewing recent research on fungal autolysis, we found that most falls into two main categories, physiology and morphology. The former is concerned with enzymological profiles,[9,10,11] while the latter generally focuses on fungal morphology in liquid culture in stirred tank bioreactors.[8,12,13,14] In this review, we critically evaluate this research, summarizing current understanding of fungal autolysis and propose possible directions for future research. With no recent summation of autolysis in the filamentous fungi,[15,16,17] it seems timely to evaluate findings pertaining to this process. Although there may be conserved features of autolysis relevant to an array of fungal bioprocesses, many research groups have focused on one particular area of autolysis, such as one class of enzyme, organism, or process. This review seeks to take a more holistic approach to the phenomenon.

II. THE PHYSIOLOGY OF FUNGAL AUTOLYSIS

A. Autolysis in Liquid Culture, Nutrient Recycling, and Cryptic Growth

In submerged cultivation of filamentous fungi on an industrial scale, widespread cellular breakdown occurs toward the end of the stationary phase of growth. At this time the physiological process of autolysis is readily observed[8,12] and the activity of key classes of hydrolytic enzymes increase, resulting in a weakened cell structure. Increased hyphal fragmentation can then result from mechanical stress. The activity of these hydrolases leads to the measurable consequences of autolysis, such as biomass decline, ammonia release, and hyphal degradation.[8,12] Autolysis can also occur within fungal pellets, which can make up a significant proportion of the culture, due to mass transfer limitations.[18,19] In this review, we use the term autolysis to describe the dynamic process of cell death in filamentous fungi.[8,12]

Autolysis is a natural part of filamentous fungal bioprocesses, and its onset can be advanced or retarded by both intrinsic and extrinsic factors (Figure 1). In many instances, autolysis is also a means of survival, with portions of the culture existing by recycling lytic products, freed by hydrolases, which can be utilized by actively growing areas, that is, extending hyphal tips. This type of growth is termed *cryptic growth*,[20] and can occur as a result of nutrient starvation.[21,22] Recently, it has been observed in *Penicillium chrysogenum*, where cryptic growth maintained the growth of two-celled fragments after extensive fragmentation in liquid, shake flask cultures,[22] and prevented further reduction in dry cell weight, with carbon and nitrogen requirements met by amino acid degradation (Figure 2).[23] Cryptic growth has also been observed in chemostat cultures of both *P.chrysogenum*[91] and *Aspergillus niger*,[24] where protein production was maintained, without an increase in biomass.[24] Although the activation and regulation of autolytic hydrolases is complex, the major groups may be classified on the basis of the specific substrate degraded, that is, proteases, glucanases and chitinases.

B. Proteases

Fungal proteases degrade aberrant proteins, assist in protein folding, activate enzymes, and

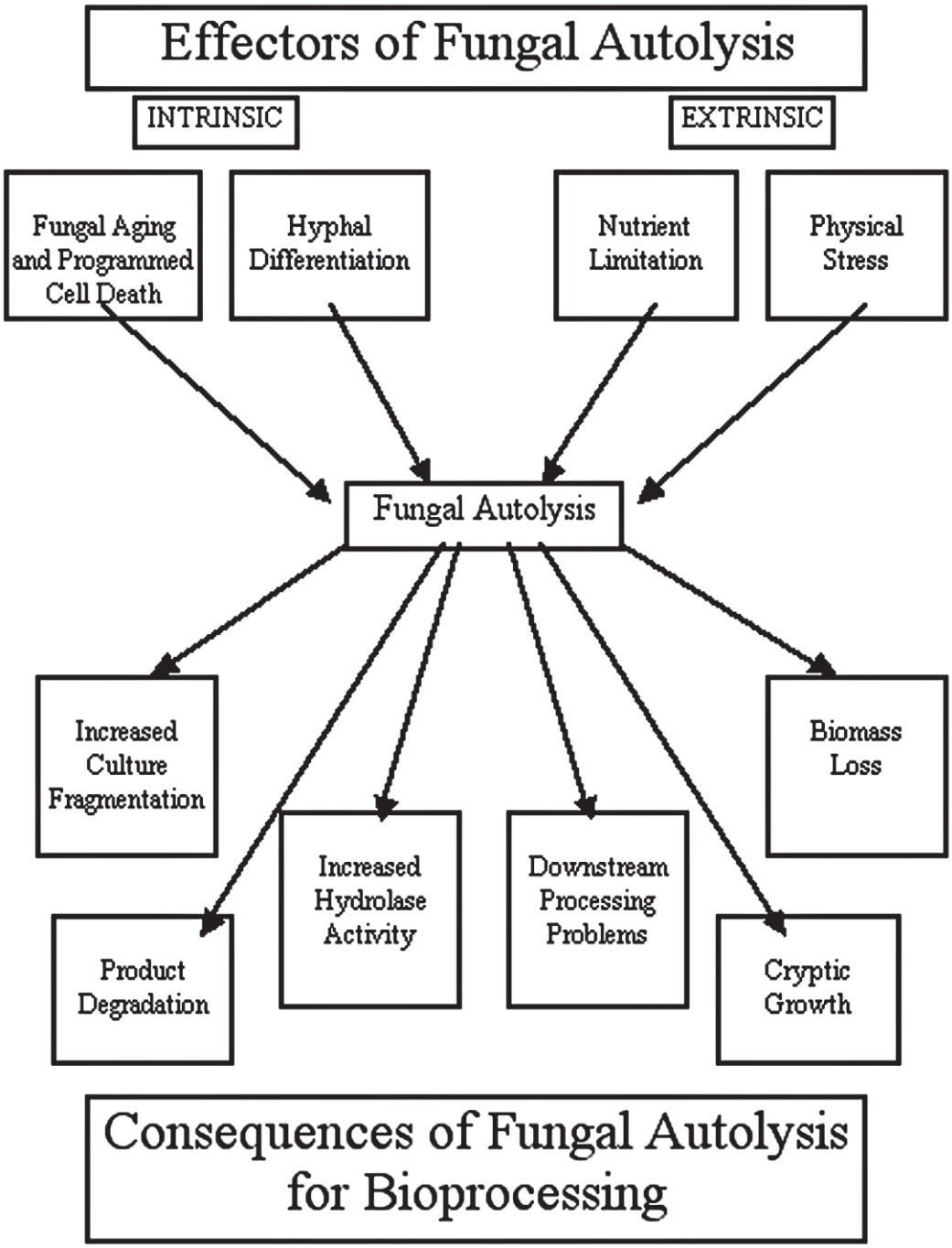

FIGURE 1. Overview of fungal autolysis.

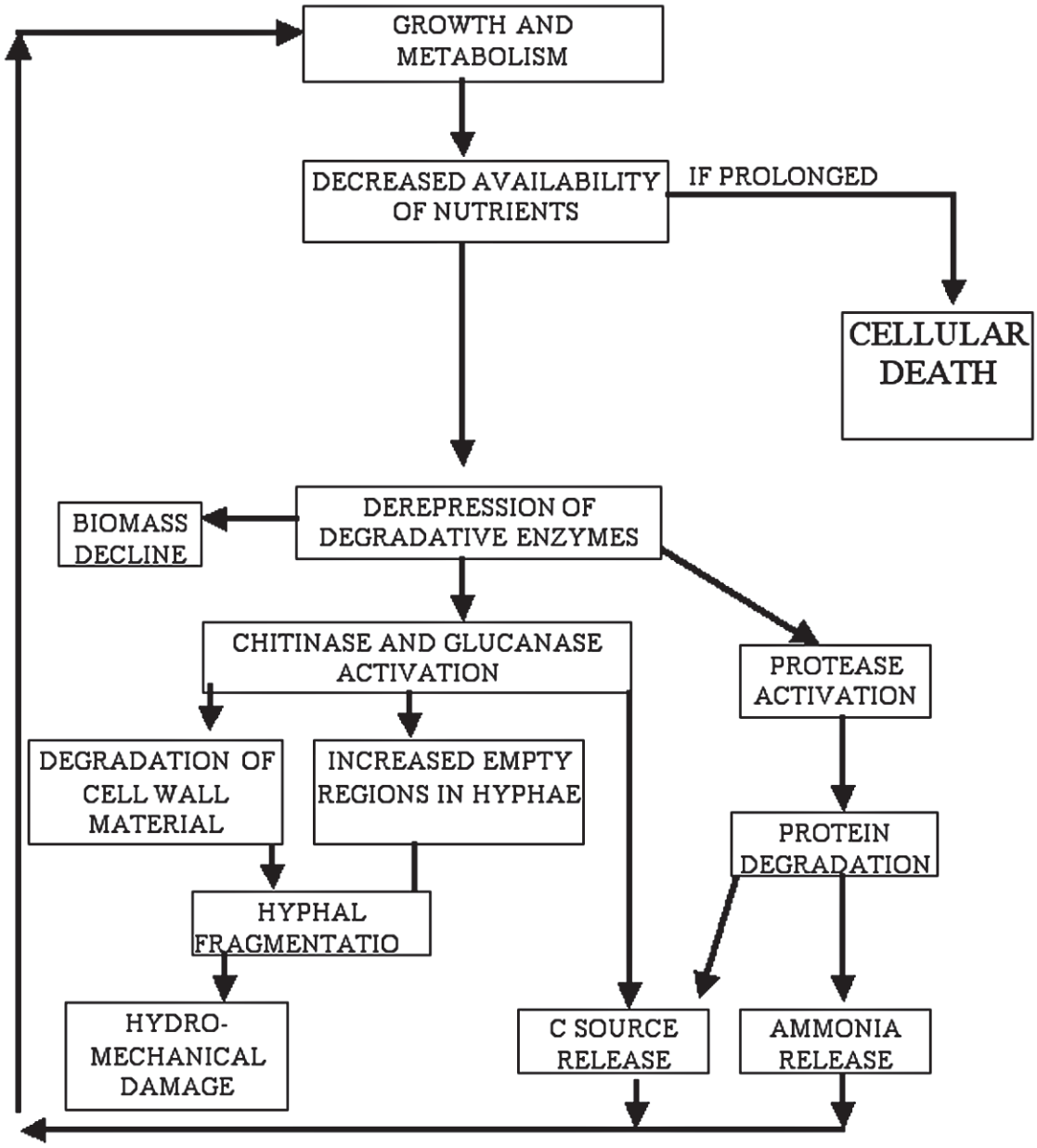

FIGURE 2. Events associated with nutrient starvation-induced autolysis.

during autolysis allow cryptic growth from liberated amino acids.[8,11,22] In liquid cultures, Santamaria et al. (1988)[11] found 50% of the fungi studied increased extracellular proteolytic activity during autolysis. Protease activity in *Aspergillus nidulans* is generally repressed in the presence of low-molecular-weight forms of C, N, S, and P.[25,26] These products of proteolytic degradation can feedback, reducing protease biosynthesis, demonstrating the cyclical nature of nutrient provision during autolysis, where protease activity is reduced when sufficient protein has been degraded for survival. This is also true in younger cultures, as there is little degradation of intracellular protein under conditions of nutrient excess. Protein turnover also occurs in static cultures. For example, nitrogen-limited cultures of *Schizophyllum commune*[27] contain a vacuolar protease, ScPrB,[28] which mediates proteolysis, with resulting amino acids translocated to the growing tips by cytoplasmic streaming.[29] Proteolysis was observed in the mid-sections of the colony with concomitant physical lysis noted at the colony center,[30] that is, physiological changes precede physical lysis. Thus, specific proteolytic activities can serve as markers for autolysis.[31] In *P. chrysogenum*, derepression of intracellular proteolysis occurs under carbon starvation,[14] enabling cryptic growth,[21,23] which precedes gross culture degradation.[8] Indeed, carbon sources like glucose[21,22] and lactose can both inhibit proteolysis in *P. chrysogenum*.[32] Although carbon limitation may induce autolysis, some carbon is necessary to drive proteolysis. This has been demonstrated in chemostat cultures of *A. nidulans* where carbon limitation caused proteolytic release of ammonia, while chemostat cultures fed with only the maintenance ration of glucose released no ammonia.[33] Although similar levels of intracellular proteolytic activity were detected during exponential growth and autolysis in *P. chrysogenum*,[8] the actual proteases involved may differ, that is, serine[21] and aspartyl proteases[34] were involved in culture degradation and the early stages of autolysis,[34] while metalloprotease activity maintained cryptic growth during carbon starvation in *P. chrysogenum*.[21,22]

C. Glucanases

Glucanase activity occurs in all stages of the fungal life cycle, including autolysis.[8,9,35] In hyphal tip extension, β-glucanases and β-glucan synthases act in concert, inserting glucan oligomers into the cell wall, conferring some resistance to digestion.[36,37,38] This would suggests that nascent β-glucan is more susceptible to degradation. However, the hyphal tip's resistance to lysis has been attributed to a balance of synthesis and hydrolysis, and the variety of β-linkages.[39] Glucanases occur in the cell wall and cytoplasm, or can be secreted, playing a role in carbon source procurement and cell wall lysis.[8,39,40,41] β-glucanase activity can be both exo- and endo-acting.[42] This allows for the efficacious degradation of complex β-glucans, releasing individual glucose molecules for recycle via cryptic growth.[41] Increased β(1,3)-glucanase activity accompanies autolysis of *P. oxalicum* in liquid culture,[37,43] thereby enabling the utilization of cellular polysaccharides, decreasing the biomass of the culture.[9] As with proteases, glucanase activity increases during carbon starvation, immediately prior to autolysis in cultures of *Neurospora* and *Botrytis* spp.[35] Catabolite repression also controlled glucanase activity in *P. oxalicum*,[40] *Penicillium italicum*,[44] and *Acremonium* spp.,[41] that is, low concentrations of carbon increased enzyme activity at the end of the growth period, prior to autolysis. In one case, a tenfold increase in glucanase activity was noted, compared with that during exponential growth.[43] Also, carbon source derepression of β-glucanase activity during autolysis[8,37,41,43] generated glucan oligomers from the autolytic turnover of the cell wall.[43] Despite their short half-life, these glucan oligomers also increased glucanase activity.[45] Glucan monomers, the ultimate products of carbohydrase activity, have been proposed as indicators of autolysis.[9,11] However, their short half-life

would seem to preclude their use. Importantly, *de novo* protein synthesis is responsible for increased glucanase activities during autolysis, not simply the release of intracellular enzymes by lysis. [41,43] Thus demonstrating a coordinated response to starvation during autolysis.

D. Chitinases

Chitin exists in fungal cell walls as fibrillar polymers of β(1,4)-*N*-acetyl-D-glucosamine,[36] and during normal growth chitinases degrade nascent chitin at the apical tip, with concomitant insertion of chitin oligomers by chitin synthetase.[39,46] Chitinolytic activity is defined as the consecutive hydrolysis of chitin and chitodextrins by chitinase (endo-acting) and *N*-acetyl-D-glucosaminidase (exo-acting).[10] The importance of chitinase activity in autolysis has been demonstrated, and several studies have examined this activity as a means to control fungal plant pests.[47,48,49] As with glucanases, chitinase biosynthesis in *Penicillium* and *Aspergillus* spp. is considered to be an inducible phenomenon,[50] controlled by catabolite repression,[51] triggered by chitin oligomers arising from autolytic degradation.[47] The control of chitinolytic activity affects morphology, where catabolite repression of subapical chitinases (relieved by carbon starvation) increased subapical hyphal branching.[51] In *P.chrysogenum*, the chitinase *N*-acetyl-β-D-hexosaminidase is a cytosolic enzyme, accumulating intracellularly in stationary phase, and degrades polysaccharide reserves during starvation. This enzyme has no demonstrable role in cell wall synthesis,[52] suggesting a unique role in the recycle of chitin oligomers, generated by endo-chitinases. Chitin deacetylase activity also increased during the autolysis of *A. nidulans*.[53] This enzyme converts chitin to chitosan. Activity increased in the presence of endo-chitinases, which perhaps enabled the deacetylase to access the polymer. Recently, a trisaccharide, allosamidin, was found to inhibit chitinase activity and to decrease mycelial fragmentation in *A. chrysogenum*.[54] Although growth did not increase, such a simple solution to fungal autolysis, if generally applicable, might be of some use in the bioprocessing industries.

Continued characterization of autolytic enzymes is essential, because species-specific control mechanisms of the three groups of hydrolases during autolysis are known.[32,55,56] The study of conserved fungal components, for example, the regulatory CREA protein might prove useful, because this protein mediates carbon catabolism.[41] With consensus recognition motifs of the global regulator proteins CREA and AREA (nitrogen regulation) found in promoter regions of pepAB and pepF protease genes in *Aspergillus niger*,[3] it may be hypothesized that the activation of the autolytic state may depend on the levels of several nutrients. Hydrolases may also act on other lytic enzymes; for example, chitinase activity might decrease as a result of proteolytic action, or could activate chitinases during autolysis.[30] Thus, the interactions occurring during autolysis (Figure 2) are both complex, and at some times contradictory.

III. THE MORPHOLOGY OF FUNGAL AUTOLYSIS

A. Hyphal Fragmentation, Differentiation, and Autolysis

Typically, when the growth phase ends in fungal cultures, gross culture degradation becomes apparent, as autolysis and fragmentation are no longer balanced by growth.[57,58] Hyphal fragmentation in bioreactors has been described as a function of shear stress, that is, when the forces of agitation and turbulent flow exceed the tensile strength of the hyphal cell wall.[59] The breaking point of each hypha will be determined by its individual characteristics, for example, age, physiological condition, and length.[42] As a hypha differentiates, tensile strength varies along its length, allowing frag-

mentation to occur.[58,60] Growth and differentiation occur as a result of cytoplasmic translocation toward the hyphal tip, generating a physiological age gradient along the length of a hypha. Vacuole size in each compartment increases with age and vacuolation generally precedes autolysis in distal regions.[33,61,62] This vacuolation occurs as the organism attempts to maintain turgor pressure for cytoplasmic streaming toward the growing apex.[42,63] However, extensive vacuolation can reduce growth rate, tip extension rates, and branching frequency due to the reduced availability of cytoplasm for subapical branching.[64] Fragmentation can then occur in aged, vacuolated hyphae,[18,20] as they have a reduced compartmental turgor pressure and tensile strength.[42, 65]

In *P. chrysogenum,* a relationship between physiology and fragmentation was first suggested by Righelato et al. (1968).[20] More recently, studies have correlated morphology with impeller geometry, power input, and the kinetics of mycelial fragmentation. Such studies are useful in attempting to understand the relationship between physics and physiology in fungal bioprocesses.[66,67,68,72] Unfortunately, some confusion still surrounds hyphal fragmentation, which is said to be definable by first-order kinetics.[69,70] Hyphal breakage appeared to be spontaneous and unaffected by agitation intensity in chemostat cultures of *P. chrysogenum*.[60] However, some authors claim that hyphal cell wall strength is constant,[71] while others state that the composition of hyphal cell walls vary along their length.[58] It is logical to assume that the effects of mechanical damage are complicated by vacuolation, age, and size of the hyphal compartment, and the accumulation of toxic metabolites.[63] Intracellular events affecting hyphal integrity and morphology need to be considered in conjunction with the effects of physical stresses on hyphal elements in filamentous fungal cultures.[12,66] Clearly, the study of hyphal fragmentation and its relationship to autolysis in bioreactors is incomplete. However, proteolysis and autolysis recently have been related to fragmentation in stirred bioreactors[8] and shake-flask cultures[22] of *P.chrysogenum*. These results provide further evidence that both physical and physiological characteristics significantly impact autolysis.

B. Measurement of Autolysis Using Image Analysis

Submerged filamentous fungal cultures exhibit a heterogeneous morphology of pellets, clumps and freely dispersed hyphae.[18] Computerized image analysis can quantify many aspects of these cultures, including autolysis, providing meaningful morphological information,[59] applicable to many industrial bioprocesses.[72] Image analysis also allows researchers to determine the physiological nature of individual hyphal elements (Figure 3),[61,73] and the quantification of proportions of hyphae that contain cytoplasm are vacuolated or degenerated.[42,68,74] Unfortunately, these studies, although extremely detailed, were not specifically directed toward detection of autolyzed hyphae. However, Vanhoutte et al. (1995)[13] were able to distinguish between vacuolated and dead hyphal compartments of *P. chrysogenum*, using a combination of staining techniques, phase contrast microscopy and semiautomated image analysis. Degenerated segments were found to comprise 50% of the total freely dispersed hyphal elements measured. Recently, manual image analysis measurements of autolyzing cultures have yielded similar results[8,12] and emphasized the importance of considering process-specific parameters when studying a particular system.[5,72,75] Image analysis also allows morphological modelling of fungal processes, allowing the prediction and assessment of specific technical problems. Although models for the growth of filamentous organisms in liquid culture exist, cell death has been neglected in even the most recent studies on filamentous organisms.[59,76] Fortunately, autolysis has been considered in terms of physiology (i.e., fragmentation of vacuolated regions was attributed to enzymatic activity and shear forces).[68] However, fragmentation and autolysis in

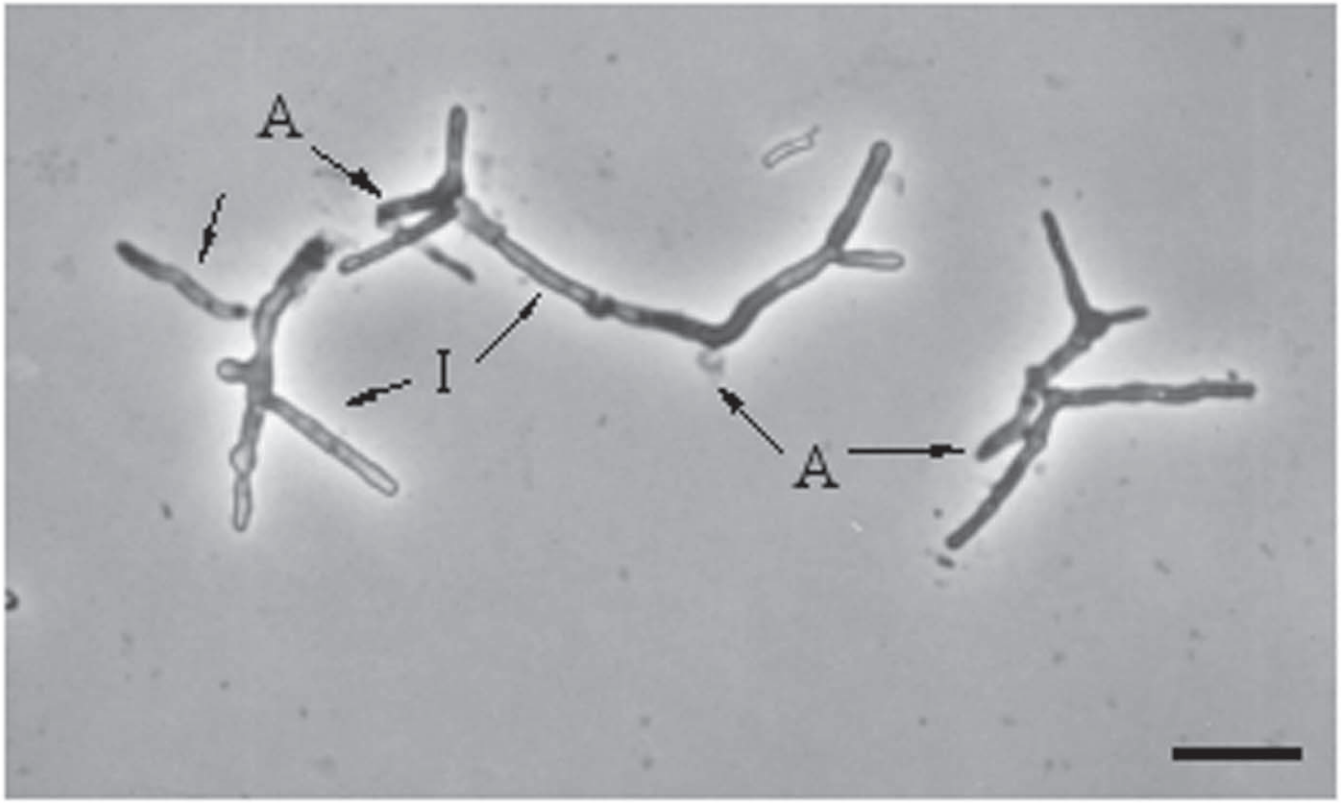

FIGURE 3. Phase contrast image of freely dispersed hyphal elements of *P. chrysogenum* from the biomass decline phase (180 h) of a stirred tank reactor batch culture. Autolyzed regions (A) and intact hyphae (I) are shown (Bar = 20 μm).

P. chrysogenum were grouped together into "biomass loss". Although this study by Paul et al. (1994b)[68] obtained a good correlations between predicted and actual values, the proportion of hyphae damaged by autolysis vs. physical fragmentation during the course of a bioprocess remains to be fully elucidated.

Biologically active stains have also been employed to clarify the status of aging hyphal regions. For example, Agger et al. (1998)[64] applied a morphologically structured model for α-amylase production in *Aspergillus oryzae*, using image analysis and the fluorescent stains calcufluor white (chitin/β-glucans) and $DiOC_6$ (staining organelles on the basis of their membrane potential). The ratio between these areas was deemed the proportion of active cells, while inactive areas (vacuolated and devoid of organelles) were deemed the "hyphal compartment", even though nucleic acid synthesis has been observed in such areas.[69] Nevertheless, these data were then used to simulate steady state and transient conditions in chemostat and batch processes.[64] This approach shows how physiology, image analysis, staining,[13,61] and a simple structured model can be used to more accurately characterize degenerated hyphal regions,[18,61] and incorporate aging and autolysis into process models. For example, Marek et al. (1999)[77] used staining methods to identify characteristic apoptotic morphologies,[78] such as septal plugging, cytoplasmic shrinkage, DNA fragmentation, and cytoplasmic bodies. In addition, Marek et al. (1999)[77] observed evacuated cell walls, similar to the degraded hyphae measured by McNeil et al. (1998)[8] and Harvey et al., (1998).[12] Also, dying cells failed to exclude the impermeant dye propidium iodide, while fragmented nuclei, within shrunken cytoplasm, were revealed by staining with DAPI.[77]

IV. FUTURE PARADIGMS IN FUNGAL AUTOLYSIS

With a great deal of data from the study of cell death in mammalian systems, it may be possible to apply such theories and protocols to the study of autolysis in the filamentous fungi, that is, cell aging (senescence) is distinct from apoptosis (programmed cell death) and necrosis[78,79] in mammalian cells. While necrosis is uncontrolled cell death, apoptosis occurs as a result of a lesser stress and has been cited as the mode of autolytic cell death in fungal systems.[77] Mammalian apoptosis is characterized by nucleolus enlargement, cytoplasmic condensation, and nuclear break up, leading to the formation of apoptotic bodies containing fragmented chromatin, which are then exocytozed.[71] These characteristics are similar to those in fungi. Indeed, a common apoptotic marker, DNA fragmentation, occurs in *Phytopthora infestans, Neurospora crassa,* and *Rhizoctonia solani*.[77] DNase and RNase activities have been related to carbon source depletion in *A.nidulans*,[80] linking environmental stress with apoptotic physiological events.

Existing hypotheses suggest aging increases vulnerability to programmed cell death by conferring loss of function, or that aging results from accumulated damage, decreasing the organisms ability to produce antioxidants and thereby maintain homeostasis.[81] Oxidative stress, specifically the generation of reactive oxygen species, can damage protein, DNA, and lipids.[82] In *P. chrysogenum*, increased copies of the superoxide dismutase gene prolonged growth,[83] while the use of lactose promoted production of the antioxidant glutathione.[84] Little is known about the effects of reactive oxygen species in the filamentous fungi. However, investigations aimed at understanding the relationship between oxidative stress and fungal autolysis could be relevant to submerged fungal bioprocesses. This significance is due to the maintenance of high dissolved oxygen concentrations for most fungal cultures at the industrial scale. Under these conditions, these organisms will be exposed to high levels of continued oxidative stress.

From this review it is clear that the area most lacking in the study of fungal cell death is at the molecular level. However, other eukary-

otes may supply appropriate models to follow, as conservation of aging mechanisms[85] has been demonstrated. That is, a DNA helicase implicated in premature aging in humans has a yeast homologue.[86] Other discoveries have included the cloning of yeast genes that postpone senescence and suppress autolysis.[87] Although telomere shortening has been implicated in aging,[88] telomeres of the *Podospora anserina* had no significant role in the aging process.[89] Other studies with *P.anserina* discovered that the accumulation of an autoreplicative mitochondrial plasmid (mtDNA)[90] both correlated with aging,[91] and decreased protein synthesis,[77,92] suggesting a link between cell death and respiratory potential.

V. CONCLUSIONS

A better understanding of fungal autolysis offers the potential to control fungal growth in situations where unwanted or uncontrolled fungal growth can have a negative impact on human health, industrial productivity or the environment. These advances will come from a greater knowledge of the mechanisms controlling hydrolase activity and through the study of resulting hyphal morphologies. However, these indicators are late events in autolysis. To characterize the sequence of events leading to fungal autolysis, it will be necessary to look to apoptosis pathways in other eukaryotic systems. In simple terms, we should seek to control autolysis in the filamentous fungi, so that induction (for disease treatment) and prevention (to increase production in bioprocesses) can occur as required. Success in this will require the co-operation of molecular biologists, enzymologists, and fungal physiologists.[78,93]

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