Pulsed Addition of Limiting-Carbon During *Aspergillus oryzae* Fermentation Leads to Improved Productivity of a Recombinant Enzyme

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Abstract: Fungal morphology in many filamentous fungal fermentations leads to high broth viscosity which limits oxygen mass transfer, and often results in reduced productivity. The objective in this study was to determine if a simple, fed-batch, process strategy—pulsed addition of limiting-carbon source—could be used to reduce fungal broth viscosity, and increase productivity of an industrially relevant recombinant enzyme (glucoamylase). As a control, three *Aspergillus oryzae* fed-batch fermentations were carried out with continuous addition of limiting-carbon. To determine the effect of pulse-feeding, three additional fermentations were carried out with limiting-carbon added in 90-second pulses, during repeated five-minute cycles. In both cases, overall carbon feedrate was used to control dissolved oxygen concentration, such that increased oxygen availability led to increased addition of limiting-carbon. Pulse-fed fermentations were found to have smaller fungal mycelia, lower broth viscosity, and improved oxygen mass transfer. As a result, more carbon was added to pulse-fed fermentations that led to increased enzyme productivity by as much as 75%. This finding has significant implications for the bioprocessing industry, as a simple process modification which is likely to cost very little to implement in most production facilities, has the potential to substantially increase productivity. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **82:** 111–117, 2003.
Keywords: fed-batch; morphology; filamentous fungi; fermentation

INTRODUCTION

The world market for industrial enzymes has been estimated to be worth over $1.6 billion, with almost half of these enzymes produced in filamentous fungal fermentations (Müller 2001). This is because many strains of filamentous fungi have been classified GRAS (generally regarded as safe), can grow on relatively inexpensive substrates, and can both produce and secrete tremendous amounts of recombinant protein (van den Hondel et al., 1992; Ward et al., 1992). Unfortunately, the fungal morphology in many of these fermentations leads to high viscosity that can impede mixing and mass transfer, and ultimately leads to reduced productivity. Despite ongoing study, there has been relatively little success in reducing broth viscosity in industrial-scale systems (Olsvik and Kristiansen, 1994). Early approaches involved addition of water or media to dilute the broth (Buckland et al., 1988b; Olsvik and Kristiansen, 1992a; Taguchi and Miyamoto, 1966) or increased agitation to fragment the mycelia (Olsvik and Kristiansen 1992b; Smith et al. 1990; van Suijdam and Metz, 1981); they have not proven to be consistently effective (Olsvik and Kristiansen, 1994). More recent attempts to use metabolic engineering to modify morphology (McIntyre et al. 2001; Müller, 2001) illustrate a clever approach and appear quite promising , but will apparently involve significant genetic manipulation and thus may be difficult to implement.

The filamentous fungus *Aspergillus oryzae* has been used for more than 2000 years for the production of food products in Asia (Cook and Campbell-Platt, 1994), and today is widely used in the bioprocessing industry for the production of a variety of both native and recombinant enzymes (Christensen, 1994). In a previous study using *A. oryzae*, we showed a relatively simple process modification during fed-batch fermentation could be used to reduce fungal broth viscosity (Bhargava et al., 2002). An *A. oryzae* wild-type strain was grown in pilot-scale fermentors, with either continuous or pulsed addition of glucose. In both pulse-fed and continuously fed batches, the same total amount of glucose was added. We found pulsing had no effect on growth or extracellular (native) protein production, but made a substantial difference in fungal morphology. Fungal elements grown in pulse-fed fermentations were significantly

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smaller, and as a result viscosity was up to 50% lower during pulse-fed batches.

In the present study, we examine whether pulsed addition of limiting carbon during fed-batch fungal fermentation can be used to increase the productivity of a recombinant protein. In the study described above (Bhargava et al., 2002), an *A. oryzae* wild-type strain was grown on glucose to relatively low biomass concentrations, and thus encountered no oxygen limitation (Table I). In this study, we grew a recombinant *A. oryzae* strain producing a commercially important enzyme (glucoamylase). Maltodextrin was added as limiting-carbon source, in an attempt to reach relatively high biomass concentration and potential oxygen limitation. Instead of adding the same total amount of carbon, dissolved oxygen (DO) concentration was used as the control parameter for overall feed-rate. As a result, increased oxygen availability led to increased addition of limiting-carbon. We find that pulsed addition of limiting-carbon yielded smaller mycelia and led to reduced broth viscosity. This allowed addition of more carbon during pulse-fed batches and resulted in increased enzyme productivity.

MATERIALS AND METHODS

Strain and Growth Conditions

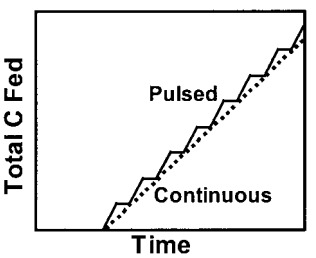
Aspergillus oryzae strain AMG #13 was obtained from J. Lehmbeck (Novo Nordisk A/S, Bagsvaerd, Denmark), is a transformant of IFO4177 (Institute for Fermentation, Osaka, Japan), contains the *A. niger* glucoamylase gene, under control of the *A. oryzae* Taka α-amylase promoter on a pBoel-777-like construct (Christensen et al., 1988), and has been the subject of previous studies (Amanullah et al., 1999; Bocking et al., 1999). For storage, freeze-dried spores were suspended in 0.1% Tween 80 solution and glycerol was added to a final concentration of 30% (v/v). Spore suspension was maintained at − 70°C. For inoculation, frozen spores were germinated on fresh agarose slants as described previously (Bhargava et al., 2002), allowed to sporulate, and used to inoculate 20-L seed fermentors. Seed media had the following composition per liter: Glucose 20.0 g, $(NH_4)SO_4$ 2.5 g, yeast extract 10.0 g, KH_2PO_4 1.5 g, NaCl 1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, and $CaCl_2 \cdot 2H_2O$ 0.10 g. Just prior to inoculation, 1 mL of sterile trace mineral solution (Bhargava et al., 2002) was added. Temperature was maintained at 30°C, air flow was 1.0 VVM, and impeller speed was held constant at 550 rpm. Gaseous NH_3 was used to maintain pH at 3.3, to prevent pellet formation (Carlsen et al., 1996). Seed culture was grown until oxygen uptake rate reached an arbitrary value of 0.3 $mmol \cdot L^{-1} \cdot min^{-1}$, at which time 5% (v/v) seed culture was used to inoculate experimental fermentations.

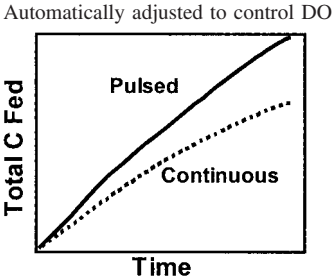
Fermentation Conditions

Fermentations were carried out in 20-L stainless steel fermentors, each with three 6-bladed, Rushton-style impellers. An initial volume of 14 L defined medium was used, with the following composition per liter: $(NH_4)SO_4$ 2.5 g, KH_2PO_4 3.75 g, NaCl 2.5 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, $CaCl_2 \cdot 2H_2O$ 0.25 g. No carbon source was used in the initial medium composition. After sterilization, 4.9 mL filtered sterile trace mineral solution (Bhargava et al., 2002) was added. Culture pH was maintained at 6.0 with NH_3, and total pressure was maintained at 0.4 bar g. Air flow-rate was increased from 0.3 to 1.0 VVM in the first 10 h of fermentation, and then maintained at this value for the remainder of each batch. Similarly, impeller speed was increased from 400 to 750 in the initial 10 h, and then maintained at this value. DO set point was linearly decreased in the first 40 h

Table I. Comparison between this study and previous study (Bhargava et al., 2002), illustrating differences in strains, products, mass-transfer conditions, and feed strategies employed.

Previous study		Current study
Strain	*A. oryzae* – wild-type	*A. oryzae* – recombinant
Product	Native extracellular proteins	Glucoamylase
Growth media	Defined	Defined
Limiting carbon	Glucose	Maltodextrin
Biomass conc.	Low	High
Oxygen mass transfer	No limitation	Potential for limitation
Initial condition	Small amount of glucose added initially	No initial carbon
Carbon feed rate	Preset	Automatically adjusted to control DO





of fermentation from 100 to 20%, where it remained for the duration of each batch. In all fermentations, maltodextrin feed was added as the sole carbon source and was used to control DO according to the set-point. In control fermentations, maltodextrin was added in continuous fashion, while in test fermentations feed was added in pulses, with feed-pump on for 90 s during each repeated 300-s cycle. Samples were withdrawn at regular intervals for measurement of biomass (measured as dry cell weight), viscosity, morphology, and glucoamylase activity as described below.

Morphology

As described previously (Li et al. 2000; 2002a; 2002b), images of fungal elements, which included both freely dispersed mycelia and clumps, were analyzed to quantify morphology. Samples for image analysis were prepared by mixing 1 mL broth with an equal volume of fixative solution (Paul and Thomas, 1998) and stored at 4°C for later analysis. For analysis, fixed samples were diluted to a final concentration of 0.2 $g.L^{-1}$ DCW to prevent artifacts from cell overlap, and diluted sample was placed on slides, covered with cover glass, and used for image capture. Images were captured using a CCD video camera (Sony) mounted on an inverted stage phase contrast microscope (IMT-2, Olympus) and digitized by a frame grabber card (G-3, Scion) installed on a Macintosh computer (Quadra 950). Image analysis was done using NIH Image V1.6 (a public domain image-processing program developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Clumps and freely dispersed mycelia were measured together using average projected area. Since hyphae have approximately constant width, projected area is a close measure of volume and thus can be used to quantify biomass (Justen et al., 1998a; 1998b).

Rheological Analysis

All rheological tests were performed using a rotational viscometer (DVII+, Brookfield) with a "vane and cup" geometry. The vane and cup system, its calibration, and the rheological testing procedure used have been described previously (Marten et al., 1997). The Herschel-Bulkley equation ($\tau = \tau_y + K\gamma^n$; where τ is overall shear stress, $N.m^{-2}$; τ_y is yield stress, $N.m^{-2}$; K is consistency index, Pa/s^n; and n is power law index, dimensionless) was used to describe rheological character of all batches, and apparent viscosity (η, $Pa.s^n$) is calculated as the ratio of average shear stress (τ_{avg}, $N.m^{-2}$) to average shear rate (γ_{avg}, s^{-1}), determined as described previously (Marten et al., 1997).

Glucoamylase Activity Assay

Glucoamylase activity was measured using a modification (Amanullah et al., 1999; Bocking et al., 1999), of a procedure originally described by Holm (Holm, 1986). This assay is based on the spectrophotometric detection of *p*-nitrophenol (pNP) release due to glucoamylase hydrolytic action, on the substrate *p*-nitrophenyl-α-D-glucopyranoside (pNPG). In alkaline pH, pNP absorbs at 400 nm and can thus be used as a measure of glucoamylase enzyme activity. To carry out the assay, broth samples were diluted 10- to 20-fold (depending upon the activity level) and 1 mL diluted sample was mixed with 2 mL colorless pNPG substrate solution (0.1% w /v), with pH maintained at 4.3–4.4 via 1*M* acetate buffer, and incubated at 30°C for 20 minutes. Reaction was terminated by adding 3 ml of 0.1 M sodium carbonate buffer (pH = 11.6). Resulting absorbance at 400 nm was measured immediately and used to determine activity, where one unit is defined as the amount of enzyme that hydrolyzes 1 μmol of maltose per minute at 30°C.

RESULTS AND DISCUSSION

The goal in this study was to determine whether pulsed addition of limiting-carbon during *Aspergillus oryzae* fermentations, could be used to significantly increase recombinant enzyme productivity. As a control, three identical fermentations were carried out with a continuous stream of limiting-carbon (maltodextrin). To determine the effect of pulsing, three additional fermentations were carried out with limiting-carbon fed in pulses (feed pump on for 90 s during repeated 300 s cycles). Note that mixing time for a similar system was approximately 10 s (Marten et al. 1997), significantly shorter than the cycle time used here.

In both cases, the overall feed addition rate (i.e., $g\ h^{-1}$) was automatically controlled to maintain the dissolved oxygen (DO) set-points shown in Figure 1A. During pulse-fed fermentations, this strategy resulted in more carbon being added (Fig. 1B), and more biomass being produced (Fig. 1C). Note that the deviation from DO set-point during pulse-fed fermentations occurred because the carbon addition rate necessary to drive down the DO was greater than the capacity of the feed pumps used. If this constraint had not occurred, the amount of carbon added to pulse-fed batches would have been even higher, and it is likely this would have resulted in even greater biomass concentrations during pulse-fed batches. In spite of the differences in biomass concentration, Figure 1D shows the average biomass yield factor ($Y_{x/s}$) was nearly identical during both pulse-fed and continuously-fed batches. Concentration of total free sugar (maltodextrin) was measured indirectly at regular time intervals by measuring refractive index (RI) values (data not shown). RI values remained constant at a low level throughout each batch, indicating no accumulation of maltodextrin.

Figure 2 shows the recombinant glucoamylase activity profiles for continuously fed and pulse-fed fermentations. Enzyme activity increases similarly in both cases, until approximately 30 h. At this point however, activity in pulse-fed fermentations continues to rise rapidly (until approximately 50 h) while that in continuously fed batches rises more slowly. As a result, activity titer values are between 20 and 75% higher during pulse-fed fermentations.

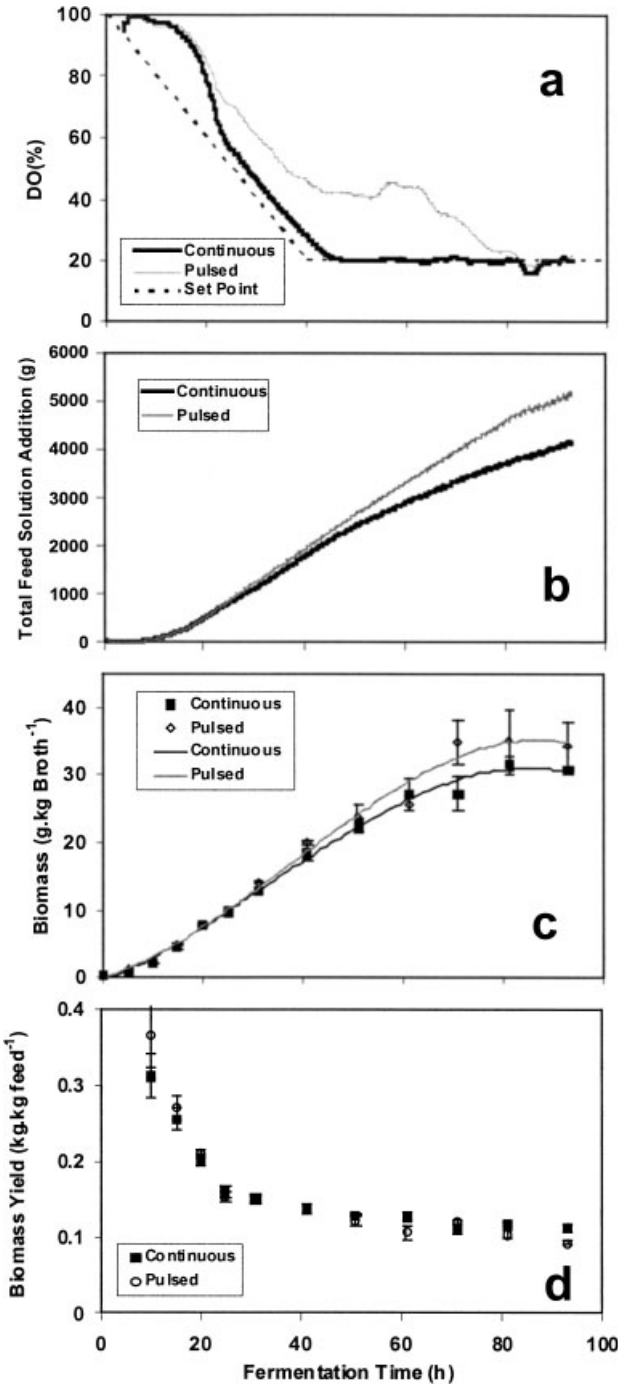


Figure 1. Results from three pulsed and three continuously fed *A. oryzae* fermentations, operated in 20-L fermentors. (a) Dissolved oxygen (DO) set-point and profiles, (b) average biomass concentration, (c) total carbon feed solution added, and (d) biomass yield factor, vs. time. In (b) and (d), error bars represent standard error and lines drawn in to illustrate trends. Each line in (a) and (c) represents the average of three fermentations.

Data in Figure 3 explain the difference in productivity between continuously fed and pulse-fed batches. Figure 3A depicts how fermentation-broth viscosity changed during these experiments. In continuously fed batches, viscosity rose rapidly until approximately 25 h, at which point it began to increase more slowly. This corresponds well with

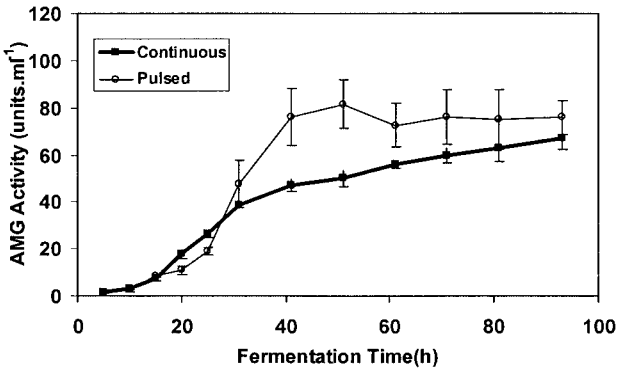


Figure 2. Product (glucoamylase) activity profile for three pulse-fed and three continuously fed fermentations. Error bars represent standard error.

changes at approximately 25 h in oxygen-uptake rate (OUR; Fig. 3B) and oxygen mass-transfer coefficient (k_La; Fig. 3C), both of which were lower in continuously fed fermentations. Taken together, these data imply high viscosity

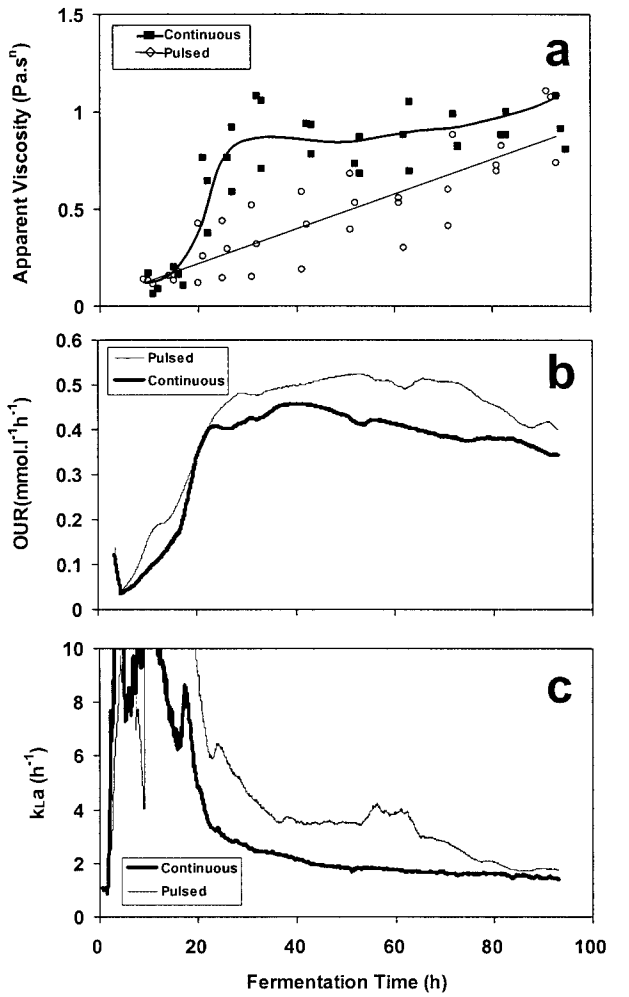


Figure 3. (a) Apparent viscosity (at a shear rate = 20 s^{-1}), (b) oxygen-uptake rate (OUR), and (c) oxygen mass-transfer coefficient (k_La) for the three pulse-fed and three continuously fed fermentations described here. Lines drawn in (a) illustrate trends, those in (b) and (c) represent average values.

broth in continuous fermentations led to a significant reduction in oxygen mass transfer and as a result, less oxygen was available to the culture. Because DO levels were used to control the carbon feed rate, this led to a reduction in the total amount of carbon added, and subsequently in biomass concentration and enzyme productivity. In contrast, Figure 3A shows fermentations fed in pulses had significantly lower viscosity that rose slowly over the entire course of these fermentations. Because viscosity was lower, both OUR and k_La were higher (Fig. 3B & C), and as a result more oxygen was available to the culture. This in turn allowed an increased carbon feed rate, which led to higher biomass and enzyme productivity. Thus, the relatively low viscosity during pulse-fed fermentations led to improved oxygen mass transfer, allowing addition of more carbon, resulting in increased biomass and recombinant enzyme productivity.

To explain the difference in viscosity between pulse-fed and continuous experiments, we point to previous studies which have shown fungal-broth viscosity is typically a function of biomass, and often a strong function of morphology (Metz et al., 1979; Olsvik and Kristiansen, 1994). In fact, we have shown previously that when *A. oryzae* are fed limiting-carbon in pulses (vs. continuously) significantly smaller fungal elements (i.e., combination of freely dispersed mycelia and clumps) result, that lead to a reduction in broth viscosity of up to 50% (Bhargava et al., 2002). The micrographs in Figure 4 show typical fungal elements found in the fermentations described here, and serve to illustrate how average size was determined. Similar digital images were taken of over 100 fungal elements from each fermentation, and image analysis software was used to determine the projected area of each element. The mean projected area was determined for all elements (i.e., > 600) from each type of fermentation, and a comparison is shown in Figure 4. At 25 h there is no statistically significant difference in fungal-element size between continuously fed and pulse-fed fermentations ($P_{value} > 0.5$). However, consistent with our previous finding there is a statistically significant difference in size at 31, 50, and 70 h ($P_{value} < 0.001$) with fungal elements in pulse-fed fermentations found to be smaller than those in continuously fed batches. Thus, even though biomass was higher during pulse-fed fermentations, the reduction in fungal-element size was sufficient to significantly lower broth viscosity.

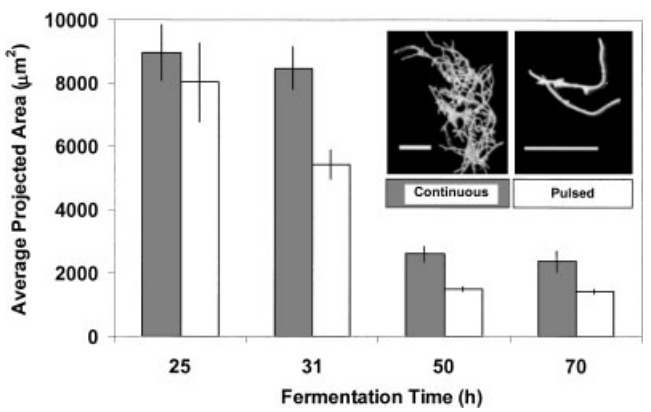


Figure 4. Size difference, reported as mean projected area, between mycelial elements in continuously fed and pulse-fed fermentations. Projected area of over 100 fungal elements from each fermentation (i.e., > 300 total for each bar) used to determine mean. Error bars represent standard error. Average size of fungal elements is significantly smaller at 31, 50, and 70 hours ($P_{value} < 0.001$). Micrographs show typical fungal elements in each type of fermentation. Bar size in each micrograph = 250 μm.

A number of previous fermentation studies have been conducted to determine the effect of cyclic exposure to nutrients (both carbon and oxygen). All of these studies however, were performed to better understand the effects of less-than-ideal mixing in large-scale bioreactors, and involved fermentations with either bacteria (DeLeon et al., 1995; Kataoka et al., 1986; Namdev et al., 1993; Oosterhuis et al., 1985; Pickett et al., 1979; Schweder et al., 1999; Yegneswaran and Gray, 1991) or yeast (Abel et al., 1994; Fowler and Dunlop, 1989; Heinzle and Dunn, 1985; Namdev et al., 1992; Sweere et al., 1988a; 1988b; Welles and Blanch, 1976). We are aware of only two studies carried out with filamentous fungi (Larsson and Enfors, 1985; Vardar and Lilly, 1982) which, in contrast to the present study, were preformed to better understand the effects of cyclic exposure to oxygen, not carbon, on production of a secondary metabolite, not a protein. Despite this, previous studies may shed some light on the behavior described here. In early work with bacteria (Brooks and Meers, 1973), limiting-carbon was fed to chemostat-grown cells in large pulses, of short duration. Immediately after each nutrient pulse, a decreasing dissolved oxygen curve implied a period of carbon availability was accompanied by cellular growth. However, this carbon was consumed prior to the next pulse (i.e., DO curve began to rise between pulses), and cells were considered to be in a period of carbon starvation. Biomass yield was lower in pulse-fed fermentations. When yeast were exposed to a similar pulsing strategy (Heinzle and Dunn, 1985; Heinzle et al., 1981; Sweere et al., 1988a; Welles and Blanch, 1976;), less biomass and more ethanol were produced. In discussing these findings, Welles and Blanch (Welles and Blanch, 1976) suggest cells exposed to cycling periods of carbon availability and starvation strive to synthesize components necessary to survive in both environments. A similar hypothesis was offered by Pickett and Bazin (Pickett et al., 1979) in attempting to describe an increase in protein fraction found in pulse-fed *E. coli* cells. While we did not find biomass yield was reduced during pulsing, we did observe a similar trend in dissolved oxygen curves (data not shown), implying fungi in this study were undergoing repeated cycles of carbon availability followed by starvation. In light of this, we offer the following hypothesis to explain how pulse-feeding might have led to altered fungal morphology. There is some indication that expression of hydrolytic activities implicated in endogenous metabolism (McIntyre et al., 2000; Perez-Leblic et al., 1982; Pitson et al., 1991; Rodriguez et al., 1995; Sahai and Manocha, 1993), may occur in phases (Rober et al. 1986;

McIntyre et al. 2000). These enzymes respond to starvation conditions and are thought to target older portions of the mycelium, allowing a re-proportioning of resources to permit continued growth at the apical tip (Bainbridge et al., 1971; McIntyre et al. 1999; 2000). Pulsed feeding may have elicited expression of some of these activities leading to physiological changes associated with autolysis. This would have led to weakening of older parts of the mycelium, which would then be more susceptible to fragmentation from impeller shear in the fermentor. Consistent with this hypothesis, is the recent finding that in *A. oryzae* protein production (both native and recombinant) does not decrease with increased hyphal fragmentation (Amanullah et al., 1999). Additional research will be required to understand the cellular mechanisms responsible for our results.

CONCLUSION

Problems with mixing and mass transfer in filamentous fungal fermentations are relatively well established, and currently there are few solutions available (Olsvik and Kristiansen, 1994). The results presented here demonstrate for *A. oryzae*, a major host used for industrial enzyme production, pulsed addition of limiting-carbon combined with DO controlled feeding can be used to increase recombinant protein productivity during fed-batch fungal fermentation. This appears to occur because pulsing of limiting-carbon affects fungal morphology, leading to smaller fungal elements. This, in turn leads to reduced broth viscosity which allows increased oxygen mass transfer, which allows more carbon to be added to the culture. This finding has significant implications for the bioprocessing industry, where it would be relatively simple and inexpensive to implement a pulsed-feeding strategy during fed-batch operation of most fungal fermentations. The data reported here imply this could lead to substantial increases in productivity of recombinant protein.

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