

Effect of Cycle Time on Fungal Morphology, Broth Rheology, and Recombinant Enzyme Productivity During Pulsed Addition of Limiting Carbon Source

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Abstract: For many years, high broth viscosity has remained a key challenge in large-scale filamentous fungal fermentations. In previous studies, we showed that broth viscosity could be reduced by pulsed addition of limiting carbon during fed-batch fermentation. The objective in this study was to determine how changing the frequency of pulsed substrate addition affects fungal morphology, broth rheology, and recombinant enzyme productivity. To accomplish this, a series of duplicate fed-batch fermentations were performed in 20-L fermentors with a recombinant glucoamylase producing strain of *Aspergillus oryzae*. The total cycle time for substrate pulsing was varied over a wide range (30–2,700 s), with substrate added only during the first 30% of each cycle. As a control, a fermentation was conducted with continuous substrate feeding, and in all fermentations the same total amount of substrate was added. Results show that the total biomass concentration remained relatively unaltered, while a substantial decrease in the mean projected area of fungal elements (i.e., average size) was observed with increasing cycle time. This led to reduced broth viscosity and increased oxygen uptake rate. However, high values of cycle time (i.e., 900–2,700 s) showed a significant increase in fungal conidia formation and significantly reduced recombinant enzyme productivity, suggesting that the fungi channeled substrate to storage compounds rather than to recombinant protein. In addition to explaining the effect of cycle time on fermentation performance, these results may aid in explaining the discrepancies observed on scale-up to larger fermentors. © 2005 Wiley Periodicals, Inc.

Keywords: *Aspergillus*; fed-batch; fermentation; fungi; glucoamylase

INTRODUCTION

Submerged cultivation of filamentous fungi is often characterized by high broth viscosity, which can result in a number of problems (e.g., insufficient oxygen mass transfer, high power requirement, formation of nutrient concentration zones) that have the potential to reduce productivity. While a number of prior attempts to reduce broth viscosity (Buckland et al., 1988; Olsvik and Kristiansen, 1992a, 1992b; Smith et al., 1990; Taguchi and Miyamoto, 1966; van Suijdam and Metz, 1981) have been relatively unsuccessful, we showed a possible solution in a previous study (Bhargava et al., 2003a). We used “pulsed” addition of the limiting carbon source in an *Aspergillus oryzae* fed-batch fermentation to significantly reduce broth viscosity. We demonstrated that this viscosity reduction was achieved without detrimentally affecting cellular metabolic activity or total secreted protein. In addition, the same process strategy led to improved oxygen mass transfer efficiency (Bhargava et al., 2003c) and increased recombinant enzyme productivity (Bhargava et al., 2003b). However, in all our previous studies a “cycle time” (t_c ; defined in Fig. 1) of 5 min was used, and thus the influence of cycle time was not determined. The goal in this study was to determine the effect of cycle time.

In the present study, the same industrially relevant filamentous fungal strain used in previous studies (Bhargava et al., 2003a, 2003b, 2003c) was grown in fed-batch fermentation, with limiting carbon added in pulses of fixed cycle time. A number of duplicate fermentations were carried out to determine the effect of cycle time (30–2,700 s) on fungal morphology, broth viscosity and recombinant enzyme productivity. In all experiments, feed was added only during the first 30% of each cycle, and the feed addition rate was adjusted so that the same amount of carbon was added to all fermentations. We find cycle time has

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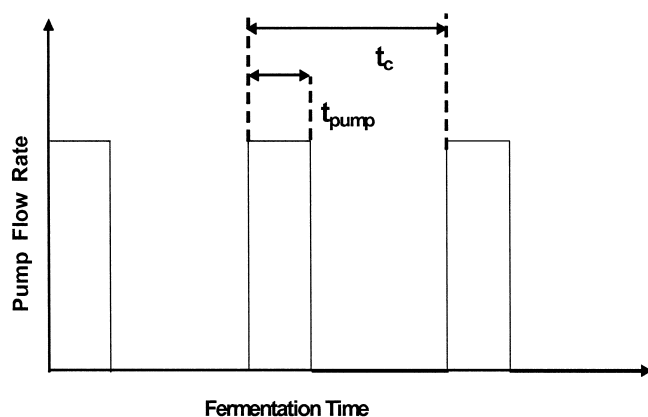


Figure 1. Schematic of feed profile used for experiments. Cycle time (t_c) and pump “on time” (t_{pump}) are defined as shown. In this study, a constant value (0.3) of pulse fraction (PF; defined as t_{pump}/t_c) was used in all fermentations.

relatively little effect on biomass concentration but can have a strong effect on morphology, rheology, and recombinant enzyme productivity.

MATERIALS AND METHODS

Strain and Growth Conditions

A recombinant strain of *A. oryzae* was used to perform all fermentations and contains the *A. niger* glucoamylase gene, under control of the *A. oryzae* Taka α -amylase promoter (Christensen et al., 1988) and has been the focus of several earlier studies (Amanullah et al., 1999; Bhargava et al., 2003a, 2003b, 2003c; Bocking et al., 1999). Spores were extracted and suspended in a solution containing 0.1% Tween 80 and 30% v/v glycerol. Aliquots of 1 mL of solution were stored at -70°C . To prepare inoculum, frozen spores were germinated on fresh agarose slants as described previously (Bhargava et al., 2003a). Once sporulated, they were used to inoculate 20-L seed fermentors with a working volume of 8 L. A seed medium with the following composition (per liter) was used: glucose, 20.0 g; $(\text{NH}_4)\text{SO}_4$, 2.5 g; yeast extract, 10.0 g; KH_2PO_4 , 1.5 g; NaCl, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.10 g, in addition to 1 mL of sterile trace mineral solution (Bhargava et al., 2003a) which was filtered sterile and added prior to inoculation. Temperature was maintained at 30°C , air flow was 1.0 VVM, and impeller speed was controlled at a constant value of 550 rpm. Throughout the run, gaseous NH_3 was used to maintain pH at 3.3, to avoid pellet formation (Carlsen et al., 1996b). The culture (5% v/v) was transferred to experimental fermentations once oxygen uptake rate reached an arbitrary value of $0.3 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$.

Fermentation Conditions

Experimental fermentations were carried out in 20-L, bottom-driven, stainless steel fermentors, configured with

three, 6-bladed, Rushton-type impellers. All fermentations were charged with an initial working volume of 14 L of defined medium with the following composition per liter: $(\text{NH}_4)\text{SO}_4$, 2.5 g; KH_2PO_4 , 3.75 g; NaCl, 2.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 g. To minimize the initial batch phase, no carbon source was added to the initial media. After heat sterilization, the medium was cooled to room temperature and 4.9 mL of filtered sterile trace mineral solution (Bhargava et al., 2003a) was added. Culture pH was maintained at 6.0 with NH_3 , and total pressure was maintained at 0.4 bar g. Impeller speed was increased from 400 to 750 in the initial 10 h and then maintained at this value for the remainder of the course. Similarly, the 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 rest of each batch. Samples were withdrawn regularly and processed immediately for measurement of biomass and viscosity. Simultaneously samples were stored and frozen for morphology and glucoamylase activity measurements as described below.

In all fermentations, maltodextrin feed (approximately 65% carbohydrate) was added as the sole carbon source. The feed rate increased linearly from 0 to 45 g of solution per hour in the initial 15 h and was then maintained at 45 g/h for the remaining period. In all test fermentations, maltodextrin was added in pulses of varying cycle time (Fig. 1) while keeping the feed pump on only during the first 30% of each cycle. As a control, a single fermentation was performed with continuous addition of carbon feed. To be certain that media components other than carbon were not exhausted, 1 L of concentrated media was added after 40 h, composed of all the media components but carbon source.

Morphological, Rheological, and Activity Measurements

To analyze the morphology, samples were prepared by mixing 1 mL of broth with an equal volume of fixative solution (Paul and Thomas, 1998) and stored at 4°C for later analysis. For quantifying morphology, experimental procedure is described elsewhere (Bhargava et al., 2003a; Li et al., 2000, 2002a, 2002b). No pellet formation was observed in all the fermentations performed. For each sample analyzed, a total of 256 images were analyzed in terms of average projected area. In addition, total number of spores was counted for all 256 elements for each sample. Number of spores count for each sample was normalized by total area for 256 elements to generate Figure 3c.

All rheological tests were performed using a similar experimental setup to that described earlier (Marten et al., 1997). The rotational viscometer (DVII+, Brookfield Engineering, Stoughton, MA) and “vane and cup” geometry were calibrated and samples were subjected to rheological measurement based on the procedure described previously (Marten et al., 1997). To describe rheological character of analyzed samples, the Herschel–Bulkley equation $\tau = \tau_y + K\dot{\gamma}^n$ (where τ is the overall shear stress [$\text{N} \cdot \text{m}^{-2}$];

τ_y is the yield stress [$\text{N}\cdot\text{m}^{-2}$]; K is the consistency index [$\text{Pa}\cdot\text{s}^n$]; and n is the power law index [dimensionless] was used, and the apparent viscosity (η , $\text{Pa}\cdot\text{s}$) is calculated as the ratio of average shear stress (τ_{avg} , $\text{N}\cdot\text{m}^{-2}$) to average shear rate ($\dot{\gamma}_{\text{avg}}$, s^{-1}), as described previously (Bhargava et al., 2003a; Marten et al., 1997).

A procedure originally described by Holm (1986) with a slight variation (Amanullah et al., 1999; Bhargava et al., 2003a; Bocking et al., 1999) was used to measure glucoamylase activity.

Statistical Data Analysis

Statistical analyses were performed with SAS System for Windows V9.0 (SAS Institute, Cary, NC). For comparison of variables during different treatments (i.e., cycle times), a general linear hypothesis test was performed, in a linear regression setup, as described in (Myers, 1990). This type of test allows one to determine if there is a statistically significant difference between treatment effects over time. A significance level of 0.05 was chosen, so that a P value < 0.05 is considered significant.

RESULTS AND DISCUSSION

The objective of this study was to determine the effect of pulse-feed frequency on fed-batch *A. oryzae* fermentations. To accomplish this, we carried out a series of duplicate fermentations with cycle times of 30, 60, 300, 900, and 2,700 s. In all experiments, the percentage of pump “on time” was held constant at 30%. As a control, a fermentation was carried out with continuous addition of limiting carbon.

Figure 2a shows that, in all fermentations, the same total amount of carbon (maltodextrin) was added, irrespective of cycle time. Frequent samples were taken and analyzed immediately for sugar content by measuring refractive index. It was observed that there was no accumulation of sugar at any time (data not shown). This suggests that for higher values of cycle time (i.e., 900 and 2,700 s), no substrate was available for extended periods (as long as 1,900 s). Surprisingly, cycle time had relatively little effect on total biomass concentration, which remained essentially unaltered for all of the different fermentations (Fig. 2b) and was essentially a function of the total substrate added. We note, however, that there is a slight but statistically significant increase in biomass concentration (approximately 10%) at the end of fermentations carried out with cycle times of 900 and 2,700 s.

In contrast, viscosity was found to be a strong function of cycle time (Fig. 2c). Viscosity increased during the course

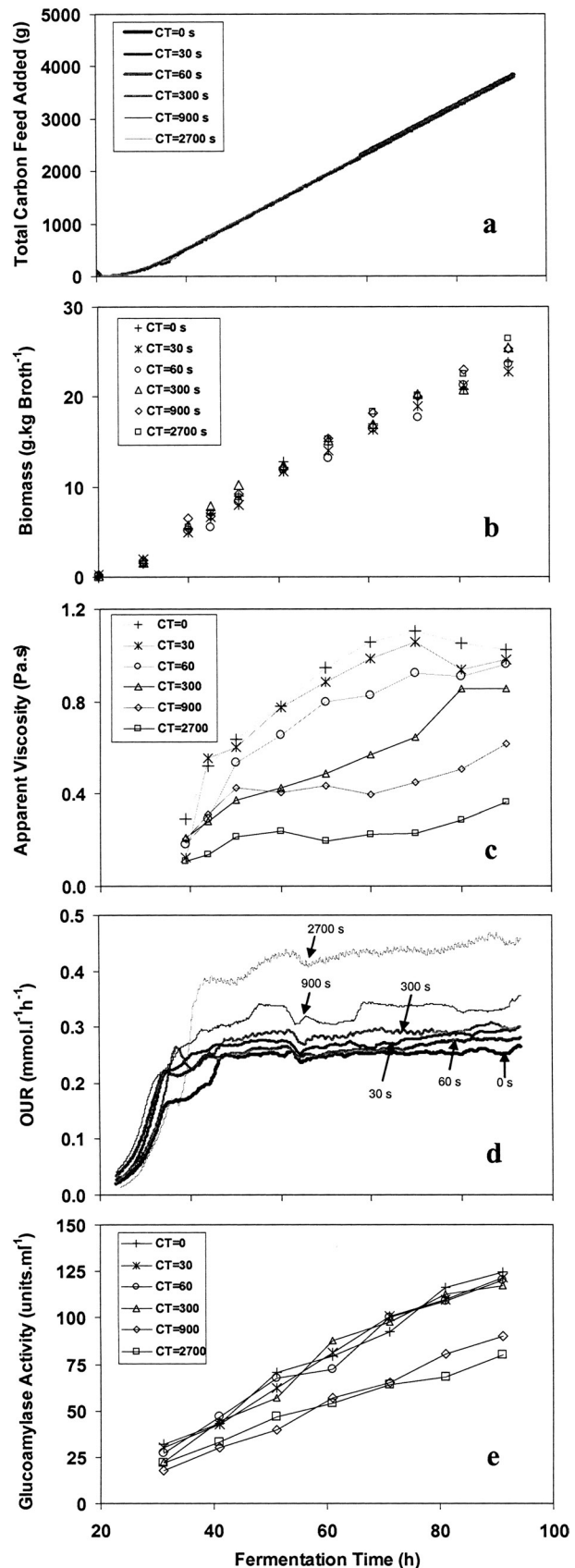


Figure 2. Profiles for (a) total carbon feed added; (b) biomass concentration; (c) apparent viscosity; (d) oxygen uptake rate; and (e) glucoamylase activity for cycle times ranging from 30 to 2,700 s. Duplicate fermentations were conducted at each cycle time, and thus each data point represents the average of two fermentations.

of all fermentations and was highest in the continuously fed fermentation, consistent with findings from our previous studies (Bhargava et al., 2003a, 2003b, 2003c). Viscosity was inversely related to cycle time and the fermentations with the longest cycle time showed an approximate 5-fold reduction in viscosity compared to the continuously fed batch. This result implies that a significant reduction in viscosity can be achieved by manipulating cycle time during pulse feeding.

The reduced viscosity resulted in a higher value of available dissolved oxygen (data not shown), which in turn resulted in increased oxygen uptake values (Fig. 2d), indicating a probable shift in metabolic activity of the cells.

Figure 2e shows glucoamylase activity profiles. It is observed that enzyme activity increased approximately linearly over time for all fermentations irrespective of the cycle time. For the lower values of the cycle time (i.e., 30–300 s) the difference in productivity was found to be trivial, whereas higher values of cycle time (900–2,700 s) resulted in a decrease in glucoamylase activity by as much as 50%. We note that the TAKA promoter used here for recombinant protein expression can experience carbon catabolite repression in the presence of as little as 10 mg/L glucose (Carlsen et al., 1996a). This catabolite repression is mediated through CreA (Agger et al., 2001, 2002) and may act to a limited degree at even lower glucose concentrations (J. Nielsen, personal communication). Thus, while HPLC analysis of the carbon feed in our experiments (maltodextrin) showed <2% glucose (data not shown) and the refractive index showed no measurable sugars, there may have been some degree of catabolite repression in the 900- and 2,700-s fermentations from small accumulations of repressive carbohydrate. This may have contributed to the decrease in glucoamylase activity.

To further investigate the effect of pulsing, samples from all fermentations at 70 h were analyzed to determine morphological properties. A value of 70 h was chosen based on the observation that the greatest differences in viscosity and related parameters (e.g., oxygen uptake rate, dissolved oxygen concentration, and glucoamylase activity) occurred at this time. These data are compared with other relevant parameters in Figure 3. As discussed above, biomass remained relatively unaffected by cycle time (Fig. 3a), whereas viscosity and oxygen uptake rate (Fig. 3b) varied considerably. A reduction in viscosity, an increase in biomass and an increase in available oxygen imply that productivity might also increase with cycle time, but Figure 3b shows this was not the case. Glucoamylase activity expression remained relatively constant for the continuously fed fermentation and for those carried out at shorter cycle times (i.e., 30–300 s) and then decreased by approximately 50% for the higher cycle time values (i.e., 900–2,700 s; see Fig. 3c). Fungal morphology was also a strong function of cycle time. As cycle time increased, the average size of fungal elements (clumps or free mycelia) decreased by approximately 50%. This likely accounted for the large decrease in viscosity with increased cycle time. Note also

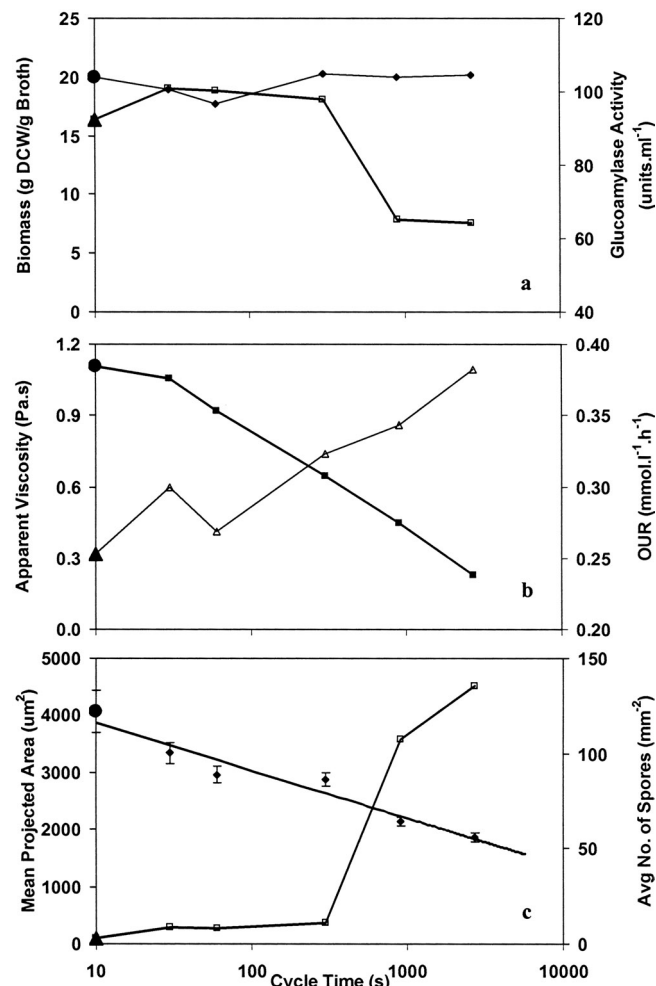


Figure 3. Effect of cycle time on various parameters at 70 h fermentation time. Profiles for (a) biomass concentration (◆) and glucoamylase activity (□); (b) apparent viscosity (■) and oxygen uptake rate, OUR (△); and (c) mean projected area (◆) and spore counts per unit total area (□, 256 fungal elements for each run). Data points represent average for duplicate fermentations or for control fermentation with continuous addition of substrate (●, ▲). Error bars in (c) represent standard error with curve drawn to represent the observed trend based on logarithmic relationship with an r^2 value of 0.94.

that for relatively long cycle times (>900 s) the number of conidiated hyphae increased dramatically. This may be due to the relatively long time period between nutrient additions, where it is likely that fungi entered a preservation mode and channeled available nutrients to conidia instead of recombinant product. This is consistent with the decrease in glucoamylase activity at these same cycle times.

Pulsing of limiting carbon substrate has been used as a tool in a number of previous scale-down studies to simulate nonideal mixing at production scale. It has been well established by these studies that gradients are formed in large-scale bioreactors (Bylund et al., 1998; Larsson et al., 1996; Manfredini et al., 1983; Oosterhuis and Kossen, 1983; Steel and Maxon, 1966), and therefore cells growing inside the bioreactor experience different concentrations of

nutrients for varying lengths of time. The effect of varying cycle time thus can be of high relevance to investigate the effect of gradients on fermentation performance. However, almost all previous studies focused either on bacteria (Kataoka et al., 1986; Oosterhuis et al., 1985; Pickett et al., 1979; Yegneswaran and Gray, 1991) or yeast (Abel et al., 1994b; Heinzle and Dunn, 1985; Welles and Blanch, 1976; Namdev et al., 1992; Sweere et al., 1988a, 1988b). It was reported in these studies that cycle time variation could affect cellular growth, product formation, or cellular metabolism in very distinct ways, depending on the host system and the range of cycle time applied. Besides our own, we are aware of only two previous studies where filamentous fungi have been subjected to pulsing (Larsson and Enfors, 1985; Vardar and Lilly, 1982), and these have focused on secondary metabolite production not protein. The present study, in contrast, focuses on the nutrient pulsing and protein as a product. In spite of many differences in the observations discussed, there seem to be two common points of agreement in almost all the reports including the current study: First, at sufficiently low values of cycle time (of the order of few seconds), system performance remains unaltered (Abel et al., 1994a; Heinzle et al., 1981; Roberts and Slater, 1986), indicating a critical value of cellular response time, below which cells do not differentiate between pulsing and continuous exposure of nutrients. Second, extracellular parameters, such as dissolved oxygen, oscillate with a frequency similar to the frequency of limiting nutrient pulses (Brooks and Meers, 1973; Dawson, 1971; Gilley and Bungay, 1967; Goodwin, 1969; Pickett et al., 1979), emphasizing the fact that in general cellular systems respond very rapidly to changes in local nutrient concentration.

CONCLUSION

In the present study we determined the effect of cycle time during pulsed addition of limiting carbon source in fed-batch *A. oryzae* fermentations. We observed that biomass concentration showed relatively little dependence on cycle time, but fungal morphology, rheology, and productivity changed as a function of cycle time. At high values of cycle time (i.e., 900–2,700 s), pulsing resulted in reduced viscosity and increased oxygen mass transfer rate. However, large values of cycle time also led to increased conidia formation and decreased productivity.

This study leads to a very distinct finding: pulsing of limiting carbon may be used as a tool to enhance filamentous fermentation performance via viscosity reduction until the point when it results in conidia formation. This apparently defines the outer limit of process relevant cycle time. In addition, it shows that since conidia formation in these fungal fermentations occurs at relatively longer starvation conditions, the presence of increased conidiation in larger fermentors may imply the existence of stagnant zones, owing to poor mixing, and thus may

be used to evaluate mixing performance in large-scale fungal fermentations.

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