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A Simple Substrate Feeding Strategy using a pH Control Trigger in Fed-Batch Fermentation

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Abstract A simple automated glucose feeding strategy based on pH control was developed to produce high-cell-density fed-batch fermentation. In this strategy, the pH control scheme utilized an acidified concentrated glucose solution to lower the pH. The frequency of glucose addition to the fermentor is determined by the culture's growth kinetics. To demonstrate the effectiveness of the coupled pH and glucose control strategy in biomass and/or secondary metabolite production, several fed-batch fermentations of indigenous *Escherichia coli* and recombinant *E. coli* were carried out. Both strains produced biomass with optical density of greater than 40 at 600 nm. We also tested the glucose control strategy using two types of pH controller: a less sophisticated portable pH controller and a more sophisticated online proportional-integral-derivative (PID) controller. Our control strategy was successfully applied with both controllers, although better control was observed using the PID controller. We have successfully demonstrated that a glucose feeding strategy based on a simple pH control scheme to indirectly control the glucose concentration can be easily achieved and adapted to conventional bioreactors in the absence of online glucose measurement and control.

Keywords *E. coli* · Fed-batch · Glucose regulation · pH control

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Introduction

Control of glucose concentration in fed-batch fermentation is important and has received much attention [1–3]. Production performance and control of biosynthetic products are greatly affected by the glucose level used in the fermentation processes [4]. The build-up of unwanted metabolites through overflow metabolism, such as ethanol and acetate, occurs when abundant glucose is available [5–8]. To avoid overflow metabolism, fermentation should be carried out using low-glucose concentration where the glucose will be a limiting factor [3, 9]. Overflow metabolism is well documented in fed-batch fermentation of *Escherichia coli* [1, 6, 10]. Many control schemes have been developed to regulate glucose concentration in fermentation processes [11–13]. However, problems still arise in these glucose concentration control schemes associated with dead time in the process control feedback loop. Dead time results when the glucose concentration measurement is far longer than the system's dynamic response time. That is, the glucose consumption rate is high enough that a phase lag results even for sophisticated on-line control [9]. Thus, the controller is responding to a glucose concentration that no longer exists in the fermentor; larger phase lags result in worse control. These lags will be even longer for off-line glucose measurement.

We report on a control scheme based on pH designed to keep glucose concentration near the optimal level for biomass production. Culture pH has been used as a trigger to feed substrate in fed-batch fermentation of *E. coli* [14]. By coupling the glucose feeding strategy with pH control, a rapid control response to glucose depletion can be achieved by avoiding unnecessary delays associated with glucose measurement. Unlike the strategy by Wang and Lee [14], which employed manual feeding based on pH, this scheme utilizes the acid side of the pH control to automatically introduce the glucose into the fed-batch fermentation. A pH change during cell growth and lysis is the basis for this approach. During active fermentation, cells produce organic acids lowering pH; ammonium hydroxide is added to maintain optimal pH for cell growth (pH 6.6–6.8 for *E. coli*). Carbon source depletion is known to cause cell lysis [15]; associated with this is an increase in pH, which is used to trigger acid/glucose feed. Fed-batch fermentation of indigenous *E. coli* and recombinant *E. coli* were used to evaluate the performance of this control scheme in terms of biomass production and recombinant protein expression.

Materials and Methods

Indigenous *E. coli*, aseptically collected and isolated from a natural spring in the Savoy Experimental Watershed (Savoy, AR), was cultured in the laboratory for this study. A seed culture for fed-batch fermentation was prepared by culturing a loop-full of the *E. coli* transferred from a healthy colony into presterilized Luria–Bertani (LB) broth overnight with continuous shaking at 37°C. The composition of the startup solution for the reactor was glucose (20 g L^{-1}), KH_2PO_4 (13.5 g L^{-1}), $(NH_4)_2HPO_4$ (4 g L^{-1}), $MgSO_4 \cdot H_2O$ (1.4 g L^{-1}), citric acid (1.7 g L^{-1}), and trace metals solution (1.0 mL L^{-1}). This was made up in deionized water, and the startup solution was steam sterilized. The composition of trace metals solution was 2.8 mg $FeSO_4 \cdot 7H_2O$, 0.13 mg $ZnCl_2$, 0.20 mg $CoCl_2 \cdot 6H_2O$, 0.20 mg $Na_2MoO_4 \cdot 2H_2O$, 1.4 mg $CaCl_2 \cdot 2H_2O$, 0.6 mg $MnSO_4 \cdot H_2O$, 0.05 mg H_3BO_3, 10.0 mg $MgSO_4 \cdot H_2O$, and 0.1 mg $CuCl_2 \cdot 2H_2O$ per liter of 6.0-N hydrochloric acid. The glucose and the metal salts solution were sterilized separately and added later into the reactor after cooling to 50°C. The acid/glucose feed solution and other conditions for the fermentations

are given in Table 1. The startup and the concentrated glucose feed solutions were both steam sterilized at 121°C for 30 min. A 0.5-M ammonium hydroxide solution was prepared for pH control and used as a nitrogen source.

The chemically competent recombinant *E. coli* DH5α, purchased from Gibco Life Technologies (Rockville, MD, USA), was transformed with plasmid p6xHisGFPuv using heat-shock treatment [16]. The seed culture for recombinant *E. coli* fed-batch fermentation was prepared by incubating the recombinant cells in LB broth using the same conditions as described for the indigenous species. The initial startup solution for the recombinant cell fermentation process consisted of glucose (5 g L^{-1}) as carbon source, 11.28 g L^{-1} M9 mineral salts [NaH_2PO_4 (6 g L^{-1}), KH_2PO_4 (3 g L^{-1}), NH_4Cl (1 g L^{-1}), and NaCl (2 g L^{-1})] as macronutrients/micronutrients. Thiamine (10 mg L^{-1}) and ampicillin (100 mg L^{-1}) were used for selection purposes. For the recombinant *E. coli*, the glucose level in the feed stock was lowered from 300 to 175 g L^{-1}. The composition of minerals in the glucose feed stock was the same as that of the glucose feed stock for indigenous *E. coli*. All solutions were heat sterilized, except for thiamine and ampicillin, which were filter sterilized. The same concentration ammonium hydroxide solution was used in the fermentation for both the recombinant and the indigenous *E. coli*.

Fed-Batch Process

Initially, 350 mL of the startup solution was aseptically poured into a 1-L bioreactor (BIOFLO-C30, New Brunswick Scientific, Edison, NJ, USA, or Aplikon ADI series 1000), preheated to 37°C, and its pH adjusted to 6.7 before inoculation. A gel-based pH probe (Orion, Beverly, MA, USA) was connected to a pH-triggered pump controller (model PHCD 212, PRESTO-TEK, Santa Ana, CA, USA). The controller independently activated the acid/glucose feed and ammonium hydroxide pumps. The pH of the fed-batch fermentation processes for both species of *E. coli* was kept between 6.6 and 6.8 which were the lower and the upper set points for the pump controller. Pure oxygen gas was supplied to the reactor (Table 1), and the agitation rate was fixed at 400 rpm to ensure excess dissolved oxygen for the aerobic fermentation (Fig. 1). To start the fermentation processes, an inoculum was poured into the reactor until the cell suspension's optical density ($OD_{600\ nm}$) measured 0.1 absorbance unit (AU) (Beckman model DU640 spectrophotometer). A bolus of 30 mg of thiamine powder dissolved in deionized water

Table 1 Experimental conditions.

	Glucose feed concentration (g L^{-1})[a]	$MgSO_4$ feed concentration (g L^{-1})	O_2 sparging rate (mL s^{-1})	*E. coli* strain	Target glucose concentration (g L^{-1})	Controller
Run 1	175	20	2	Indigenous	20	Presto-Tek
Run 2	175	20	2	Indigenous	20	PID
Run 3	175	20	2	GFP	5	Presto-Tek
Run 4	175	20	2	GFP-His[b]	5	Presto-Tek
Run 5	300	10	2	Indigenous	20	PID
Run 6	300	10	2	Indigenous	20	Presto-Tek
Run 7	700	20	4	Indigenous	20	Presto-Tek

[a] The glucose feed solution was acidified with 1 mL L^{-1} of 6 M HNO_3

[b] The GFP protein in this run was modified to have a histadine tail to improve post fermentation recovery

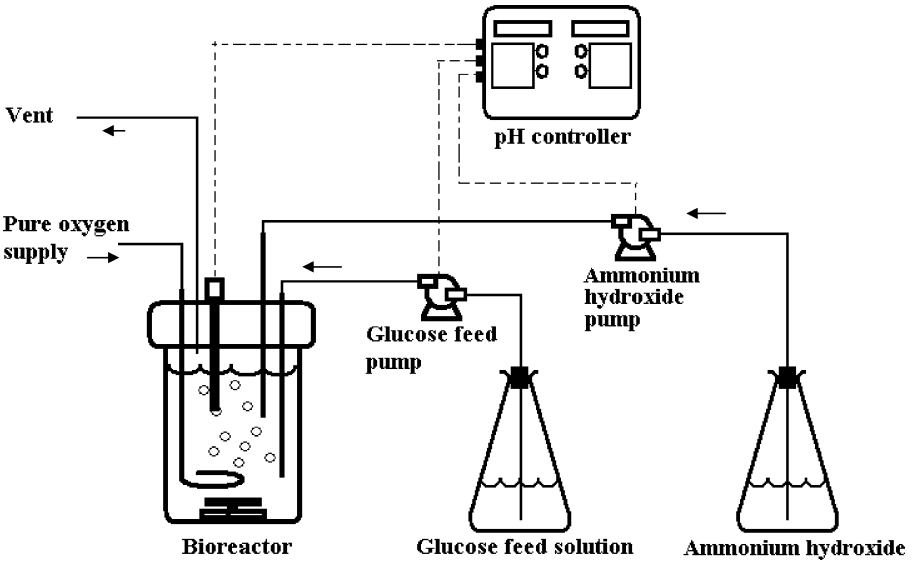

Fig. 1 Schematic representation of experimental setup. *Dashed* and *solid lines* represent electrical signal and tubing, respectively

was regularly pulsed into the reactor during fermentations together with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) and ampicillin. However, only thiamine solution was injected in the fed-batch fermentation of indigenous *E. coli*. The quantity of the ampicillin pulsed was calculated based on the volume of the cell suspension at the time of pulsing to maintain a final ampicillin concentration of 100 mM. The cumulative weight of acid/glucose and the ammonium hydroxide vessels were each measured regularly to track the total quantity of the acid/glucose and base solutions pumped into the bioreactor. The fluorescence intensity of the recombinant *E. coli* suspension was measured off-line to determine the quantity of green fluorescence protein (GFP) produced.

Process Control Model

The production of organic acids, such as acetate, succinate, fumerate, etc., is typical for aerobic fermentation of glucose by *E. coli* [14, 16]. In addition, it is well known that cell starvation and lysis leads to increase the pH of the fermentation broth [14, 17, 18]. This behavior serves as the basis for the simple control scheme presented in Fig. 2. Optimal growth conditions for *E. coli* occur in the pH range 6.6 to 6.8. Using these as lower and upper set points with the controller yields the following strategy, which exploits the controller dead band. When the pH falls below 6.6 to 6.57 during active growth as the result of organic acid byproducts, the ammonium hydroxide pump is activated until the pH rises to 6.67 when the controller stops pumping. As the cells continue to consume glucose, eventually glucose depletion induced cell lysis begins and pH rises. When pH reaches 6.79, the acid/glucose solution is fed until pH is reduced to 6.67. The precise pH values in this control scheme can be manipulated by adjusting the size of the dead band around each set point. Because the cell concentration in the fed-batch fermentation was high, small changes in cell metabolism are adequate to cause a detectable change in the reactor pH.

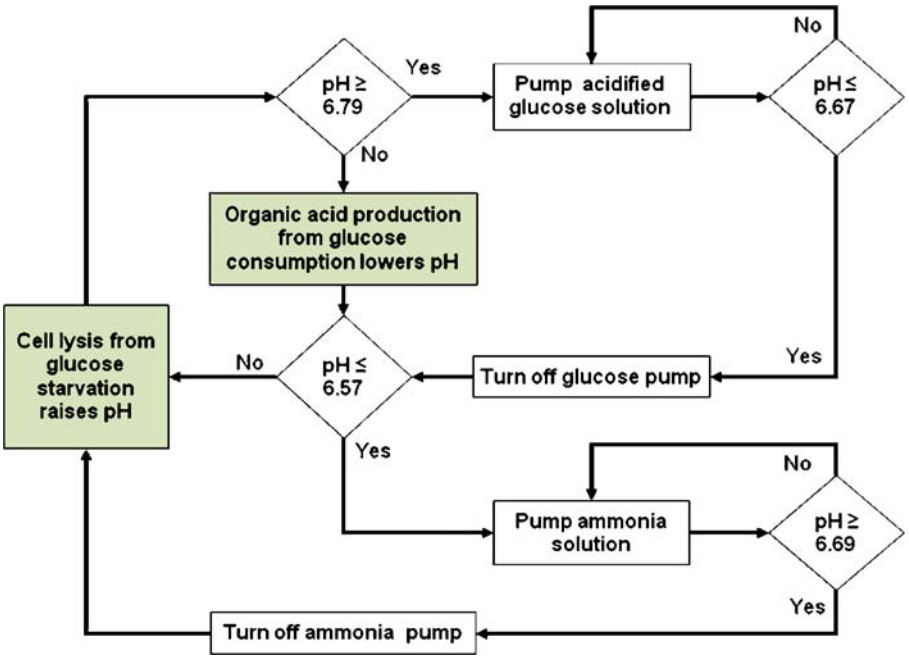

Fig. 2 Flow chart showing coupled pH and glucose feeding logic. The *highlighted boxes* are not part of the control system but represent fermentation processes that cause pH changes that allow for the control algorithm to be implemented. For an upper set point of 6.8, the PRESTO-TEK Model PHCD 212 controller's internal dead band is between 6.79 (glucose pump on) and 6.67 (glucose pump off), respectively. Similarly, for a lower set point of 6.6, the controller's internal dead band for ammonium hydroxide pump on/off signal was 6.57 and 6.69, respectively. The size of the dead band is determined by the controller's hardware; it can be increased, but these are the minimum settings

The PRESTO-TEK dual-channel controller was used in analog mode, without computer control, using the Aplikon fermentor for data acquisition. The Aplikon unit is equipped with a computer controlled proportional-integral-derivative (PID) feedback control system (Bioconsole ADI 1025 control loop). For runs using the control scheme outlined above with the PID controller, the pH setpoint was 6.5 and the PID controller was used in strictly proportional mode with the instrument default setting of P=100 with a dead zone of 0.02 pH units. With this setting the pH was controlled with a range of only 0.05 pH units around the setpoint; therefore, additional tuning of the control settings was not performed.

Analytical Methods

The biomass, metabolite production, and glucose concentration were monitored offline throughout the fermentation processes: biomass was monitored by optical density at 600 nm and the fluorescence intensity of the cell broth was measured using a fixed wavelength (395 nm excitation and 509 nm emission) fluorometer (Shimadzu RF Mini 150 Spectrofluorometer). The glucose concentration in the reactor was determined using the Trinder glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Results and Discussion

Coupled pH and Glucose Control Scheme

Although the glucose concentration in the bioreactor using our control scheme is determined by the pH of the cell suspension, the glucose regulation and the pH control can be viewed as separate processes. As shown in Fig. 3a, when there is sufficient glucose in the reactor to support cell activity, the pH fluctuates as a result of acid/base neutralization between the organic acids produced from the cell's activity and the addition of ammonium hydroxide (up to 10 h). When the glucose level is too low to maintain cell growth (Fig. 3c), cell lysis increased the overall pH of the cell suspension (arrows on insets 1 and 2), triggering the addition of acid/glucose. The pH set point for triggering acid feed is not shown on the plot because the frequency of data collection was not high enough to catch the short spike in pH. The acid/glucose solution was added as a rapid pulse, and the frequency of each pulse was closely tied to cell growth through the pH feedback mechanism described above; the pulses became increasingly frequent as the cell biomass increased as the fermentation enters the exponential growth phase – this is apparent in Fig. 3c, although there are only three cycles shown – 10 h is required to deplete the glucose in the first cycle, 6 h in the second, and only 3 h in the third cycle. The increase in the frequency of the pH fluctuations, shown in Fig. 3a, following the addition of glucose to the fermentor at both 10 and 15.75 h demonstrates the system response that leads to the utility of this control approach. These fluctuations were mediated by cell growth, which was of course dependent on the substrate availability and the ammonium hydroxide pulses (each upward spike in pH).

Depending on the goal of the fermentation, the level of cell starvation can be adjusted by increasing (for lower glucose concentration before the next pulse) or decreasing the upper pH set point or by adjusting the acid/glucose ratio. It is also feasible to manipulate this through the acid-to-glucose ratio in the feed solution. An important point is that oxygen can become limiting in these systems, as shown in Fig. 3e, where the saturation level approaches zero despite continuous sparging with pure oxygen.

For tests performed with digital PID control, the fluctuations in pH (data not presented) were on the order of 0.04 pH units stabilizing to 0.02 pH units; approximately a factor of 5 smaller fluctuations using the digital control compared to the analog control.

Biomass and Secondary Bioproduct Production

The optical density for the two *E. coli* strain cultures clearly showed that pH-triggered glucose-feeding scheme coupled with pH control could be applied to either the indigenous strain or genetically modified *E. coli* (see Fig. 4). For indigenous *E. coli*, the final optical density for the bacterial suspension ranged from 18 to 50 with a lag phase of about 5 h. In contrast, the lag phase for the recombinant *E. coli* was about 20 h. This difference in lag phase was likely due to the difference in the glucose concentration at startup. Because the production of GFP is favored at low glucose concentration [16], the initial glucose concentration for the recombinant *E. coli* and the concentration throughout the fermentation must be kept below 4.5 g L^{-1}. In contrast, for a process focused on biomass production, the glucose level can be elevated as in the fermentation of indigenous strain *E. coli* (30.0 g L^{-1}). This was easily manipulated by changing the ratio of nitric acid/glucose in the glucose feed solution, as shown in Fig. 4, where the lag was shortened and biomass production elevated for a high glucose feed concentration (run 7). If the glucose concentration was

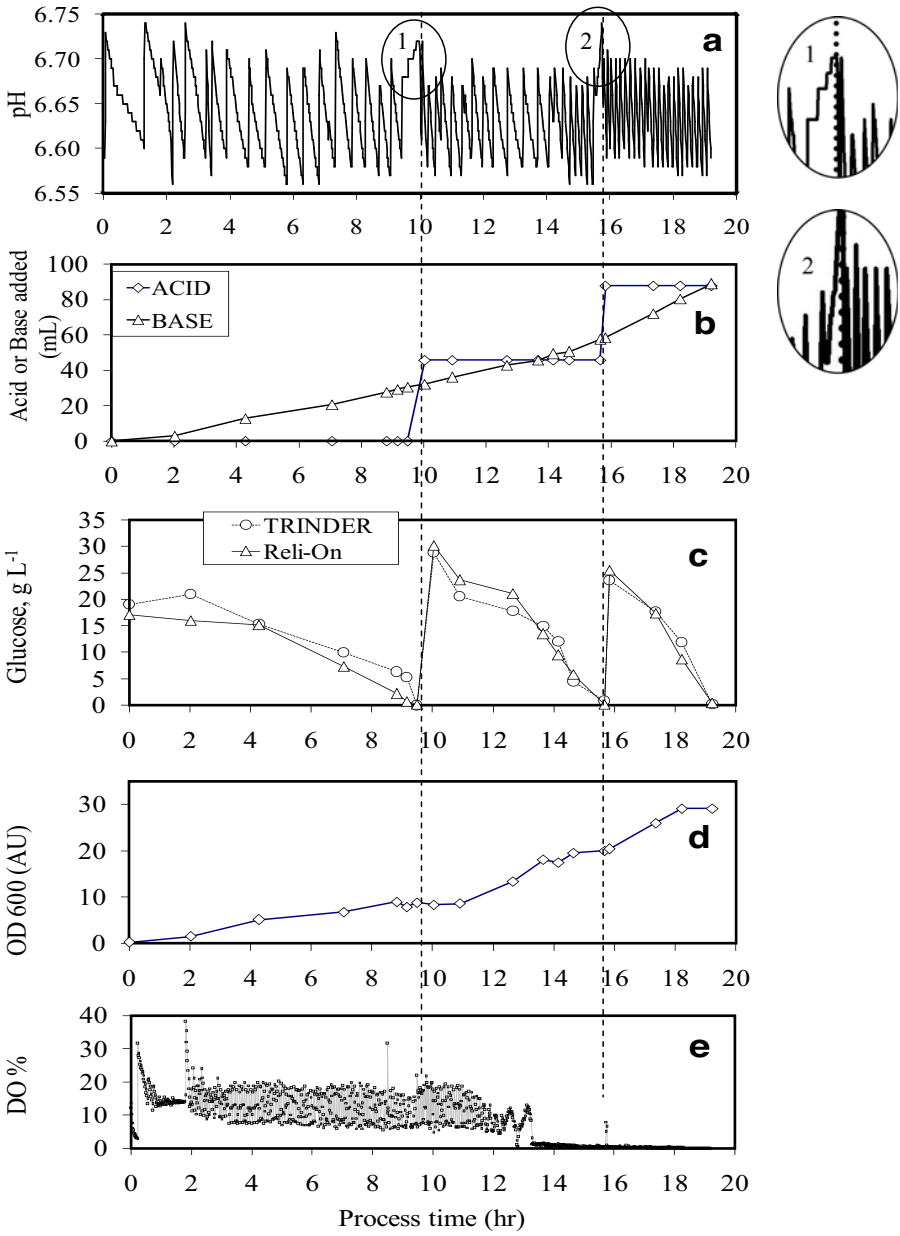

Fig. 3 Operating parameters for run 6. *Vertical lines* mark the times when the acid/glucose solution was pulsed to the reactor. *Insets* show the increase of pH resulting from starvation lysis as the glucose is depleted. **a** pH, **b** acid or base added, **c** glucose, **d** OD 600, **e** DO%

increased while keeping the volume of nitric acid added the same, as demonstrated in the fermentation of indigenous *E. coli*, the glucose concentration reached in the reactor after each glucose feed pulse is between 25 and 35 g L^{-1}. In Fig. 4, the sudden drop in the optical density, at 28 h, for the recombinant *E. coli* (run 4) was attributed to overdose of

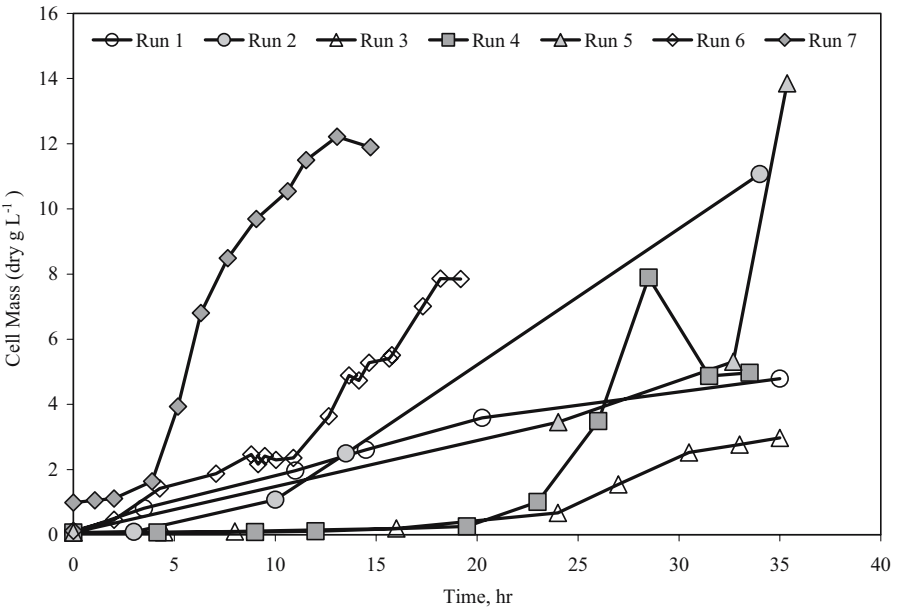

Fig. 4 Effect of reactor conditions and glucose feed concentration on biomass production for the control scheme

acid/glucose feed and subsequent corrective addition of ammonium hydroxide. Instability in the pH measurement resulting from poor selection of the pH probe for the controller was the probable cause for the overdose of acid/glucose. We repeatedly observed large spike/overdosing of the feed solutions using a liquid electrolyte-filled pH probe, which was

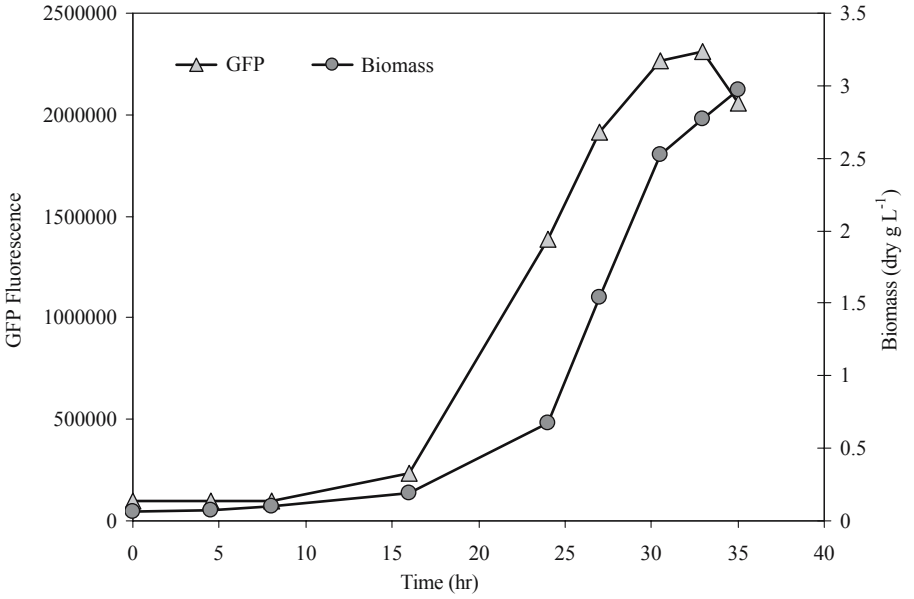

Fig. 5 GFP production from run 3

subject to drift effects. Using a more stable gel-based Orion pH probe eliminated these problems.

Figure 5 presents the results of the production of GFP by the recombinant *E. coli* in terms of fluorescence intensity demonstrating that the control scheme can be used for both biomass production and for expression of recombinant proteins.

Conclusion

Our proposed glucose feeding scheme coupled with pH control was successfully applied to the fermentation of indigenous and recombinant *E. coli*. Through careful manipulation of pH of the glucose feed solution, the quantity of glucose added to the reactor can be controlled and its concentration in the reactor can be regulated. These results clearly show the potential for this simple strategy but do not represent optimal conditions for this fed-batch control scheme. This control system is simple and can be easily adapted for processes that require the addition of substrate automatically in a series of pulses using a conventional bioreactor. If digital control is available, the scheme can achieve very tight pH control.

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