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Original Communication

Expression of single domain antibody ToxA5.1 in recombinant *Escherichia coli*: Part I - Dual-point pH-stat fed-batch fermentation

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ABSTRACT

A simple yet efficient fed-batch strategy, dual-point pH-stat (DPPS), was developed for the culture of recombinant *Escherichia coli* TG1 expressing ToxA5.1, a single domain antibody neutralizing *Clostridium difficile* toxin A. Cell densities up to 26.6 g DCW/L were achieved with the newly proposed fed-batch strategy. This strategy has shown the potential of enabling high cell density culture of *E. coli* by providing indirect feedback control over feeding of a solution containing glucose and other macronutrients.

KEYWORDS: *E. coli*, fed-batch, fermentation, pH-stat

INTRODUCTION

Drug resistant strains of *Clostridium difficile* are a major health concern in hospital settings [1]. *C. difficile* associated diseases (CDAD) are toxin-mediated, which can be treated by means of lessening the severity of symptoms via toxin inactivation using antibodies [2] to drastically reduce CDAD morbidity. A novel llama single domain antibody (pSJF2H-ToxA5.1) was expressed in recombinant *Escherichia coli* TG1 targeting *C. difficile* enterotoxin A (TcdA) and shown to have toxin-neutralizing activities [3]. Furthermore, a novel purification method was developed in a previous study [4] to selectively recover ToxA5.1. It is therefore of great interest to develop optimized processes for its cost-effective production at large scale, and obtaining high cell density culture (HCDC) is the first step towards this goal.

Developments in biochemical engineering since the early 1990s have led to high cell density culture when cells such as bacteria, yeast or fungi are grown in fed-batch mode. Several researchers reported cell densities for *E. coli* ranging from 17.6 to 134 g/L on a dry cell weight basis (DCW) [5, 6], leading to significant increases in volumetric productivity [5, 7].

Nutrients are required in large quantities if high cell densities are to be achieved but the problem of substrate inhibition and precipitation due to low solubility must be dealt with. It has long been established that substrate inhibition would occur when the concentration of a substrate is above a critical level. For instance, it was reported that for *E. coli*, substrates and nutrients become inhibitory if they are present at the following respective levels: glucose > 50 g/L, NH_4OH > 3 g/L, boron > 44 mg/L, cobalt > 0.5 mg/L, copper > 4.2 mg/L, iron > 1.15 g/L, magnesium > 8.7 g/L, molybdenum > 0.8 g/L, phosphorus > 10 g/L and zinc > 38 mg/L [8]. Possible mechanisms of substrate inhibition include: 1) osmotic pressure increase in the medium when the initial concentrations are too high, thereby leading to detrimental conditions for the cultures [5], 2) some substrates may precipitate at high initial concentration [5] which could possibly make them unavailable for cell growth, and 3) many bacterial strains can

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produce inhibitory products such as acidic metabolic by-products when a specific substrate concentration, such as glucose, is too high [9]. For instance, when *E. coli* is cultivated in high glucose concentration medium, it may overproduce acetic acid as a by-product of glucose catabolism in association with fast cell growth [10]. It is therefore necessary to control the cell growth of these strains below a given specific growth rate, known as the critical growth rate [11-15]. To this end, fed-batch fermentation is commonly used. Fed-batch fermentation is a two-phase process with the first phase being a batch phase where all nutrients required for cell growth are present in non-inhibitory concentrations and a second phase consisting in the feeding of limiting nutrients in a controlled manner to avoid substrate inhibition while supplying sufficient nutrients for HCDC. These limiting nutrients are typically the carbon source (e.g., sugars) and other macronutrients.

Different feeding control strategies have been developed for HCDC in the last few decades. These strategies are implemented either with or without feedback control. Feeding with feedback control can be further classified into two groups: direct or indirect feedback control. Direct feedback control relies on the *in situ* measurement of the concentration of the substrate of primary concern (e.g. in-line glucose analyzer for glucose feeding) while indirect feedback control can be achieved by monitoring one of the more easily measured indicators to infer the key substrate consumption. For instance, when glucose is the key substrate, indirect feedback control indicators such as dissolved oxygen (DO), carbon dioxide evolution, medium pH and cell concentration are commonly used [7]. Feeding without feedback control strategies include intermittent feeding, constant rate feeding, linear feeding, stepwise feeding, and exponential feeding. Some of these strategies such as intermittent feeding, constant rate feeding, linear feeding, and stepwise feeding do not offer good control over the key substrate in the culture and are suitable for a very limited number of processes. On the other hand, more precise strategies, such as exponential feeding, require a thorough knowledge and an accurate model of the kinetics of individual strains under given conditions.

One of the most promising indirect feedback control feeding strategies is the use of a pH probe for the feedback control of glucose feeding, which is called pH-stat feeding. It is advantageous since pH probes are sensitive, reliable, easy to use and readily available in standard fermentation setup [7]. The strategy of pH-stat relies on the pH change of culture, which is closely associated with glucose catabolism, for feeding control. When glucose is exhausted from the medium, the pH of the culture rises because the cells are forced to consume the acidic by-products of glucose catabolism. This change in pH can be utilized to devise a feeding strategy such that whenever the pH rises above a certain preset value, glucose is fed to the fermentation culture [16]. For instance, an on-demand pH-stat glucose feeding strategy was reported to achieve a biomass concentration of *E. coli* of 15 g DCW/L [17].

The potential of fed-batch fermentation is better demonstrated by studies involving more sophisticated strategies. For instance, coupling the pH-stat strategy with fuzzy control, exponential feeding strategy with monitoring of the biomass to compute the proper amount of feeding solution to be added or in-line glucose analyzer to continuously monitor glucose concentration were reported to lead to biomass level of 61 g DCW/L [18], 72 g DCW/L [19], and 18 g/L [20], respectively. Furthermore, it was reported by Korz *et al*. [21] that *E. coli* TG1 wild-type could be cultivated to reach 128 g DCW/L using an exponential feeding strategy. These strategies, however, require either complex control systems or process-specific kinetic models or both to succeed.

In this research, it is demonstrated that a simple dual-point pH-stat feeding strategy has the potential of being developed into a feeding strategy to enable HCDC of recombinant *E. coli* for overexpression of single domain antibody ToxA5.1.

MATERIALS AND METHODS

Bacterial strain and plasmid

DNA encoding ToxA5.1, a llama single domain antibody with specificity for *C. difficile* toxin A [3], was cloned into plasmid pSJF2H [22] via BbsI and BamHI (New England Biolabs, Mississauga, ON) restriction sites. Protein expression was performed in TG1 *E. coli* cells purchased from Stratagene (La Jolla, CA). To test the effect of recombinant gene expression on *E. coli* TG1 growth, three strains were tested: a wild type *E. coli* TG1 (not transformed),

an *E. coli* TG1 transformed with an empty pSJF2H plasmid (i.e., without a DNA insert) and an *E. coli* TG1 transformed with plasmid pSJF2H containing the gene for ToxA5.1. Strains were grown in 20 g/L LB Lennox medium (Fisher Scientific, Pittsburgh, PA) containing 100 μg/ml of ampicillin (Fisher Scientific) and then plated on LB Lennox agar. A colony was then sub-cultured in 50 mL of defined medium [21] containing 10 g/L of glucose and 100 μg/mL of ampicillin in a 250 mL Erlenmeyer flask at 30 °C for 18 h, in an orbital shaker at 200 rpm. The culture, to which sterile glycerol was added to a final concentration of 15%, was then aliquoted as 1.5 mL in 2 mL micro-centrifuge tubes, kept in a freezer (Thermo Electron Corp. Asheville, NC) at -80 °C, and served to prepare inoculum for the bioreactors.

Media and feeding solution

Batch medium and feeding solution used in this study were identical to those reported by Korz *et al.* [21] except that the glucose concentration was 7 g/L or varied as specified in the text. Defined medium consisted of glucose 7 g/L, KH_2PO_4 13.3 g/L, $(NH_4)_2HPO_4$ 4 g/L, $MgSO_4$ 1.2 g/L, citric acid 1.7 g/L, ampicillin 100 mg/L, and 10 mL/L trace metal solution, which consisted of ethylenediaminetetraacetic acid (EDTA) 840 mg/L, $CoCl_2 \bullet 6\ H_2O$ 250 mg/L, $MnCl_2 \bullet 4\ H_2O$ 1500 mg/L, $CuCl_2 \bullet 2\ H_2O$ 150 mg/L, H_3BO_3 300 mg/L, $Na_2MoO_4 \bullet 2\ H_2O$ 250 mg/L, $Zn(CH_3COO)_2 \bullet 2\ H_2O$ 1300 mg/L, Fe (III) citrate 10 g/L, and thiamine HCl 4.5 mg/L. Fed-batch feeding solution contained glucose 600 g/L, $MgSO_4$ 20 g/L, and 10 mL/L trace metal solution, which consisted of EDTA 1300 mg/L, $CoCl_2 \bullet 6\ H_2O$ 400 mg/L, $MnCl_2 \bullet 4\ H_2O$ 2350 mg/L, $CuCl_2 \bullet 2\ H_2O$ 250 mg/L, H_3BO_3 500 mg/L, $Na_2MoO_4 \bullet 2\ H_2O$ 400 mg/L, $Zn(CH_3COO)_2 \bullet 2\ H_2O$ 1600 mg/L, and Fe (III) citrate 4000 mg/L. The ammonium hydroxide solution used for pH control contained 25% v/v of NH_4OH (Fisher Scientific).

Inoculum preparation

Four 500 mL Erlenmeyer flasks each containing 100 mL of defined medium was inoculated with 1 mL of stock culture that was stored in a -80 °C freezer and which was thawed at room temperature before being transferred to the Erlenmeyer flasks. The seeded flasks were incubated at 30 °C for 12 h in an orbital shaker at 200 rpm. The inoculum medium was the same medium as described in the previous section except being supplemented with 10 g/L of glucose and 100 μg/mL of ampicillin. The culture was transferred to two sterile 200 mL centrifuge bottles and centrifuged at 2,550 rcf for 20 min at 20 °C using a Hermle Labortechnik GmbH centrifuge (Wehingen, Germany). The supernatant fluid was discarded and both pellets were re-suspended with 25 mL of defined medium and combined, for a total of 50 mL. The cell density of the inoculum was therefore 8-fold that of the cell density in the original inoculum culture. This concentrated mixture served as inoculum for the bioreactors where a sufficient amount was added to each bioreactor to obtain an initial OD_{600} of approximately 0.15.

Fermentation

Fermentations were performed in New-Brunswick Scientific (Edison, NJ) BioFlo110 3-L bioreactors with a working volume of 1.5 L. Batch phase was carried out in 1.5 L of defined medium containing 100 μg/mL of ampicillin. Temperature (28 °C) and agitation with two 6-blade Rushton impellers mounted on the same shaft (300 rpm) were kept constant via the control module of the bioreactors. During the batch phase, when the pH dropped to a value lower than the setpoint, the culture pH was controlled by the addition of 25% v/v NH_4OH solution (Fisher Scientific). The lower limit of the dissolved oxygen (DO) was set at 20% air saturation and a constant airflow of 1 vvm was sparged into the fermentation medium. When oxygen demand could not be satisfied with the air supply, pure oxygen (Linde Canada, Ottawa, ON) was used to increase the oxygen mole fraction of the inlet gas stream *via* the BioFlo110 gas mix module, while the inlet gas flow rate remained constant at 1 vvm. The bioreactors were inoculated as described in the previous section.

Single-point pH-stat (SPPS) fed-batch feeding strategy

When the single-point pH-stat strategy (SPPS) was employed, the addition of the feeding solution was controlled by the acid pump of the built-in pH control loop of the bioreactor. Instead of feeding an acid solution to lower the pH, feeding solution containing 600 g/L of glucose and other macronutrients was added to the fermentation broth, and organic acids such as acetic acid produced as by-products

of glucose catabolism would result in a decrease of the medium pH. This was done via the bioreactor controller and did not require any particular equipment. When pH decreased below the setpoint of 6.8, NH_4OH (25% v/v) was added to raise the pH.

Dual-point pH-stat (DPPS) fed-batch feeding strategy

This strategy was implemented using the bioreactor pH control loop coupled with a LabVIEW (National Instruments, Vaudreuil-Dorion, QC) interface and New-Brunswick Scientific OPC (Object Linking and Embedding (OLE) for Process Control) server to prevent pH from falling below the lower setpoint or rising above the upper setpoint. When pH decreased below the lower setpoint, which was set to 6.6 in this investigation, NH_4OH (25% v/v) was added to the medium via the pH control loop. When the pH rose above the upper setpoint, i.e., pH 6.8, an injection of 25 mL of feeding solution containing 600 g/L glucose was added, corresponding to the addition of 15 g glucose or elevating the glucose concentration of the culture by 10 g glucose/L. Considering the response time of the system, a LabVIEW subroutine was used to prevent the injection of a second feeding solution pulse within 5 min after an injection pulse, thus avoiding excessive glucose addition.

Analysis of glucose and acetate

The concentrations of glucose and acetate were determined using an Agilent 1200 HPLC unit (Agilent Technologies, Foster City, CA) with a Shodex S-1011 column (Showa Denko K.K., Kawasaki, Japan) and a Shodex SG-1011 guard column. Samples of 50 μL were loaded and the mobile phase (5 mM, H_2SO_4) was run at 0.6 mL/min. Glucose was quantified using a refractive index (RI) detector while acetate was quantified using a UV detector (λ = 210 nm).

RESULTS

Batch fermentation at high initial glucose concentration

It was reported by Korz *et al*. [21] that *E. coli* TG1 wild-type could be grown to reach 128 g DCW/L with an initial glucose concentration of 25 g/L using an exponential feeding strategy. However, our attempt to repeat these experiments failed with cell growth being inhibited in the batch phase, i.e., before the fed-batch phase was started. The glucose, biomass, and acetate profiles of a typical batch phase are shown in Figure 1.

As shown in Figure 1, up to approximately 11.5 h in the batch phase, 11.57 g/L of glucose was consumed to produce 1.78 g/L DCW of biomass for a yield of 0.15 g DCW/g glucose. During that period, the acetate concentration reached 2 g/L. However, further depletion of the remaining 16 g/L of glucose from 11.5 to 24 h in the batch phase only led to a biomass increase of 0.82 g/L DCW with a biomass yield of 0.05 g DCW/g glucose, which is only 1/3 of that observed in the earlier stage (i.e., from time 0 to 11.5 h). In the same period, acetate concentration increased from 2.0 g/L at 11.5 h to 9.16 g/L at 29 h.

At the end of the fermentation, a final biomass concentration of 2.94 g DCW/L was obtained, resulting in an overall yield of biomass on glucose ($Y_{X/S}$) of 0.1 g DCW/g glucose as shown in Table 1. It is worth noting that, after 4 h of fermentation, the acetate level became detectable in the culture and increased quickly thereafter until the end of the batch phase with a final concentration of 9.16 g/L.

Effect of genetic modification on cell growth

The strain of *E. coli* used in our attempts to achieve HCDC using Korz *et al.* [21] approach was a genetically engineered strain bearing the pSJF2H-ToxA5.1 plasmid and the genetic modification could have been responsible for the failure to initiate the fed-batch phase [23]. To test this hypothesis, experiments were performed to compare growth characteristics of *E. coli* TG1 wild-type, *E. coli* TG1 transformed with an empty pSJF2H vector and *E. coli* TG1 recombinant with pSJF2H vector containing the ToxA5.1 gene.

As shown in Figure 2, genetic modification of *E. coli* TG1 did not lead to much difference in the biomass concentration profiles among the wild-type *E. coli* TG1, *E. coli* TG1 transformed with an empty vector and *E. coli* TG1 recombinant with vector containing the ToxA5.1 gene. It was concluded that genetic modification was not causing the low biomass observed during the batch phase.

Single point pH-stat (SPPS)

In single point pH-stat (SPPS), the culture pH was controlled at a single setpoint of 6.8 (Figure 3A) by adding NH_4OH solution (25% v/v) to raise the

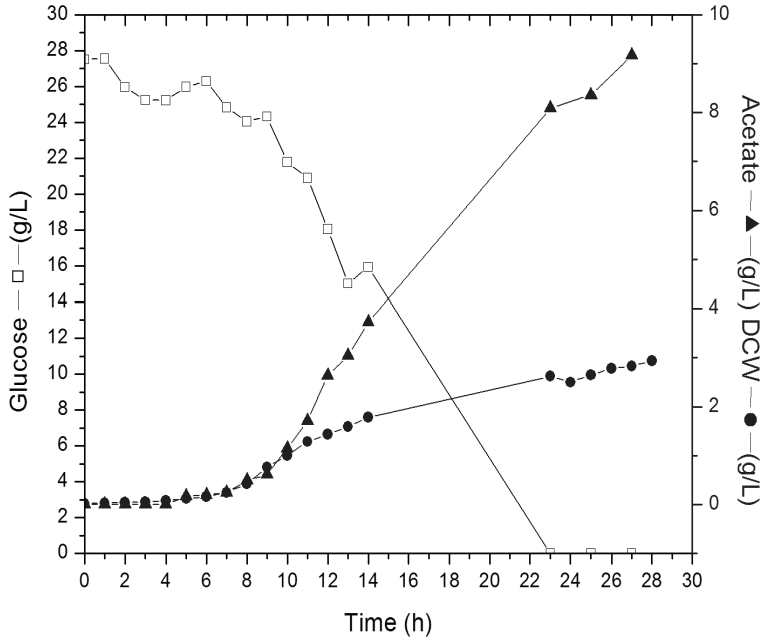

Figure 1. Batch growth of *E. coli* TG1 bearing the plasmid for the expression of ToxA5.1 single domain antibody against *C. difficile*. Cells were grown in defined media containing initially 27 g/L of glucose at 28 °C.

Table 1. Summary of some parameters of different pH-stat experiments.

	Batch				Fed-batch			Overall			
	S_0 g/L	μ_{max} h^{-1}	X g/L	$Y_{X/S}$ g X/g S	S_{fed} g	X g/L	$Y_{X/S}$ g X/g S	S_{total} g	X g/L	$Y_{X/S}$ g X/g S	NH_3 g
Batch	27	2.12	2.94	0.10	---	---	---	---	---	---	---
SPPS	7	0.66	0.48	0.018	---	---	---	---	---	---	---
DPPSL	7	1.41	1.82	0.26	5	0.61	0.12	12	2.43	0.20	1.0
DPPSH	7	2.06	1.97	0.28	318	26.6	0.16[a]	325	26.6	0.17	30.5

[a]$Y_{X/s}$ was calculated using glucose added up to the point where the biomass stopped increasing.

pH and the feeding solution, which is described in Materials and Methods section, to lower the pH. Profiles of key parameters of a typical fed-batch fermentation using SPPS are shown in Figure 3. Figure 3A indicates that the culture pH was well controlled at the setpoint of 6.8 throughout the entire course of the fermentation. Figure 3B shows the outputs of the NH_4OH pump and the feeding solution pump, which were controlled by the built-in pH control loop of the bioreactor. When the output value was positive, it indicates that the NH_4OH pump was activated with NH_4OH solution added. The percentage value represents the actual flow rate in comparison to the total flow rate when the pump is fully operating. A negative output value indicates that the feeding solution pump was operating at the specified percentage of the full capacity of the pump.

As shown in Figure 3B, only NH_4OH solution was added for approximately 18 h into fermentation. Then the feeding solution pump was activated, marking the end of the batch phase and the beginning of the fed-batch phase. During the fed-batch phase, both NH_4OH and feeding solution (containing 600 g/L glucose) were added in an alternating manner to maintain the pH at the setpoint of 6.8 (Figure 3A). The alternating cycles between the

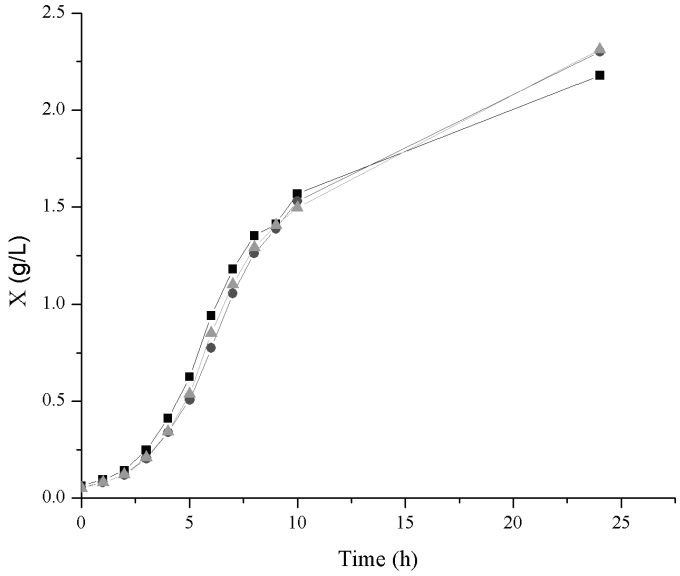

Figure 2. Growth curves of *E. coli* TG1 strains in defined medium (glucose 25 g/L) at 28 °C. *E. coli* TG1 wild type (–■–), *E. coli* TG1 transformed with an empty pSJF2H vector (–▲–) and *E. coli* TG1 recombinant with pSJF2H vector containing the ToxA5.1 gene (–●–). Points represent a triplicate average and standard deviations are not shown for clarity. The average standard deviation observed was 0.024 g DCW/L for wild-type, 0.025 g/L for recombinants containing empty vectors and 0.015 g/L for recombinant *E. coli* TG1.

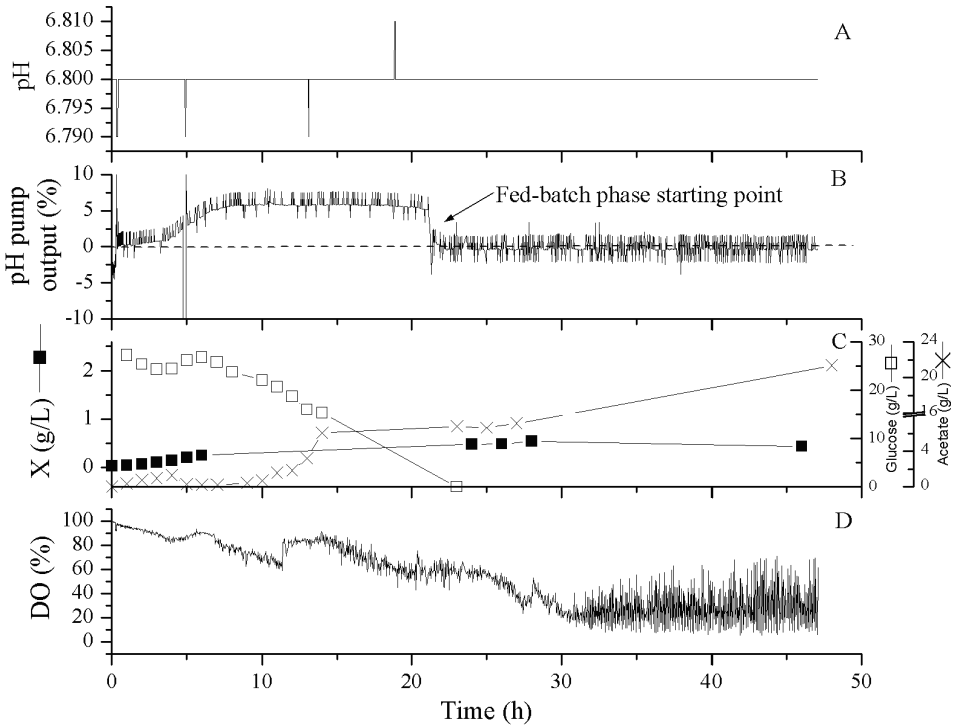

Figure 3. Single-point pH-stat control strategy (SPPS): (A) pH value, (B) output of the pH control module, (C) Biomass profile, and (D) Dissolved oxygen percentage saturation. The axis for acetate is broken into two sections to allow clear depiction of data in the range of 0-5 g/L and 18-22 g/L.

NH_4OH solution and the feeding solution, however, did not correspond to the depletion of neither glucose nor acidic metabolites in the culture as intended. A total of 30.44 g NH_4OH was added during the entire period of fermentation while only 5.0 mL of feeding solution was added during the fed-batch phase, and cell growth stopped shortly after the fed-batch phase started (Figure 3C). It is interesting to observe, as indicated in Figure 3D, that the DO value followed a decreasing trend for approximately 30 h of fermentation, at which point it started oscillating around the set value of 20% air saturation, indicating that the metabolism of *E. coli* was active throughout the entire course of 48 h of fermentation. Figure 3C also shows that glucose was quickly consumed in the batch phase. On the other hand, a quick accumulation of acetate was observed in the period of 10-15 h with concentrations below 15 g/L. However, acetate concentration increased to 21.8 g/L at 48 h of fermentation while no glucose was detected throughout the fed-batch phase, indicating that the glucose added into the culture was mainly directed to the production of acetic acid, which was probably the major cause of the cessation of cell growth in the fed-batch phase. As summarized in Table 1, biomass concentration only reached 0.48 g DCW/L over a period of 48 h whereas during the batch phase, a μ_{max} of 0.66 h^{-1} and a biomass/glucose yield ($Y_{X/S}$) of 0.018 g X/g S were observed.

Dual point pH-stat (DPPS) with low glucose feeding dosage (DPPSL)

The SPPS feeding strategy led to narrow bandwidth cycles, which was not coupled directly to the depletion of either glucose or acidic metabolites as desired and, consequently, failed to prevent acetate from accumulating beyond inhibitive concentration. As a result, HCDC was not achievable. To overcome the narrow bandwidth cycles, a dual-point pH-stat feeding strategy was devised and tested. In this strategy, the pH was controlled in a range between two setpoints: 6.6 to 6.8. When culture pH decreased due to the production of organic acids such as acetic acid during glucose metabolism by *E. coli*, NH_4OH was added to prevent pH from decreasing below the lower setpoint (pH 6.6). On the other hand, when pH increased to the upper setpoint (pH 6.8) due to the consumption of acidic metabolites upon the depletion of glucose in the culture, the feeding solution pump was activated for 3 seconds, adding 0.2 mL of the feeding solution, which corresponded to a glucose dosage of 0.12 g. The pH, biomass, and DO profiles of a typical fermentation employing this strategy are presented in Figure 4.

As shown in Figure 4A, the culture pH started at the upper setpoint of pH 6.8 and decreased gradually to the lower setpoint of pH 6.6 in approximately 7 h after inoculation. The NH_4OH pump was activated at that point to prevent pH from falling below pH 6.6. At approximately 9 h into the fermentation, pH started to increase continuously without the addition of NH_4OH solution, indicating that the glucose had been depleted from the culture and the bacteria were consuming acidic by-products accumulated in the early stage of fermentation. At about 12 h, the injection of the feeding solution started. As shown in Figure 4A, these injections only resulted in slight pH decreases that never reached the lower setpoint of pH 6.6, and therefore were not sufficient to activate the NH_4OH pump. The biomass curve presented in Figure 4B shows that the biomass was rapidly increasing during the batch phase where a μ_{max} of 1.41 h^{-1} (Table 1) and a biomass/glucose yield ($Y_{X/S}$) of 0.26 g X/g S were estimated. Even though more than 40 feeding solution injections were made during the fed-batch phase, this strategy yielded a final biomass of only 2.43 g DCW/L (Figure 4B) and an overall $Y_{X/S}$ of 0.20 g X/g S. The dissolved oxygen profile during the fermentation, as shown in Figure 4C, indicates that the cellular metabolism was active for 13 h, which is evidenced by the decreasing DO or its control to the setpoint of 20%. Then, the metabolic activity slowly decreased, which is signified by a decrease in the oxygen demand as revealed by the gradual increase of DO to finally approach 100%.

Dual point pH-stat (DPPS) with high glucose dosages (DPPSH)

The DPPSL strategy was able to prevent the excess addition of NH_4OH due to the narrow bandwidth cycles, leading to a biomass concentration of 2.43 g DCW/L which is higher than that obtained with SPPS (0.48 g/L). However, the resulting biomass concentration was still far from what is expected for HCDC. It was hypothesized that the glucose

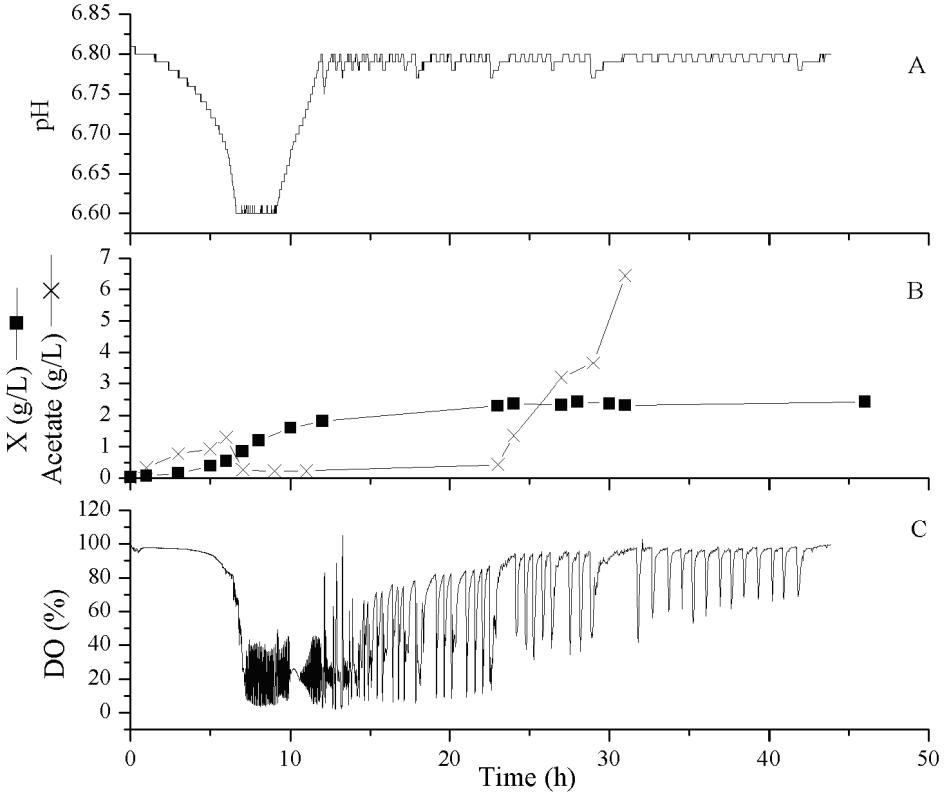

Figure 4. DPPSL control strategy using glucose dosage of 0.12 g per injection: (A) pH value, (B) Biomass profile, and (C) Dissolved oxygen percentage saturation. The initial glucose concentration was 10 g/L.

dosage of each injection was too small to allow accumulation of sufficient amount of acetic acid and other acidic metabolites to reduce culture pH to the lower setpoint, pH 6.6. As a result, no NH_4OH was added to the culture during the entire course of the fermentation and nitrogen starvation probably led to the early cessation of cell growth. To overcome this problem, an increased glucose dosage of 15 g per injection, which corresponded to an addition of 10 g/L of glucose to the culture with every injection, was implemented. The profiles of key parameters of a typical fermentation employing such a strategy are shown in Figure 5.

As shown in Figure 5A, the culture pH decreased gradually from the upper setpoint of pH of 6.8 at the beginning of the fermentation to a pH of 6.6 in approximately 9.5 h and was maintained at this value by the addition of NH_4OH solution until approximately 11 h, indicating that acidic metabolites were accumulating during this period. Then, pH started to increase quickly first and at a lower rate until reaching pH 6.7 at 14 h, indicating that the acidic metabolites accumulated during the earlier stage were consumed during this period of time. The pH was then stabilized at 6.7, a value too low to trigger the addition of glucose. As a result, cell metabolism was inactive during this period, which is confirmed by the stabilization in the pH profile (Figure 5A), biomass profile (Figure 5B), and DO profile (Figure 5C) during this period. At 22 h of fermentation, an injection of 15 g glucose, which corresponds to a concentration of 10 g/L, was added into the culture manually. As shown by the pH, biomass, and DO profiles, cell metabolism was activated immediately after the addition of the feeding medium with the pH decreasing, biomass increasing, and DO dropping to 20% air saturation, which was the setpoint for the DO control.

As shown in Figure 5A, an automatic and well-paced cyclic pattern of pH oscillation between pH 6.6 and pH 6.8 was established with the glucose feeding solution added at pH 6.8 and NH_4OH solution added at pH 6.6. It is worth noting that the frequency of pH oscillation increased with time

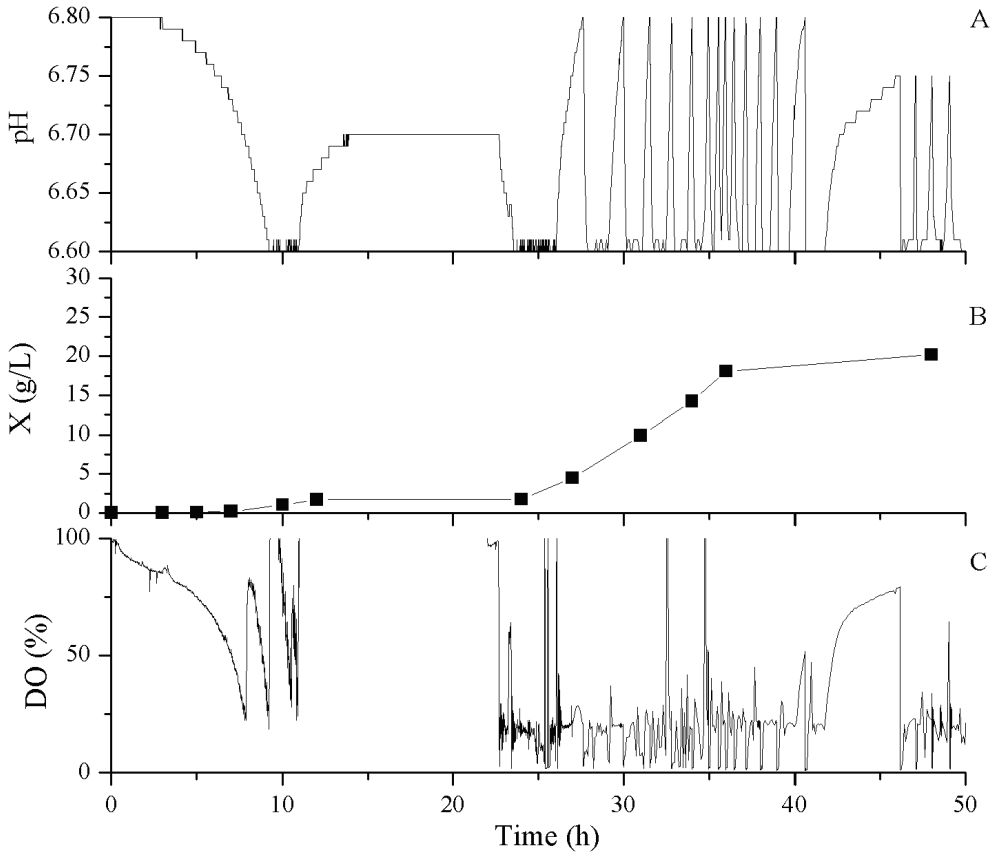

Figure 5. DPPSH control strategy using 7 g/L initial glucose concentration and 15 g per injection (10 g/L glucose) feeding dosage: A, pH; B, biomass; and C, dissolved oxygen.

(Figure 5A), apparently due to the increased rate of consumption of glucose or acidic metabolites owing to the fast-increasing biomass concentration (Figure 5B) during this period of time, which increased from 1.77 g DCW/L after the first manual injection to 20.2 g DCW/L at the end of the fermentation. Upon the initiation of the fed-batch phase, the characteristic cycles were observed and the biomass increased to a final concentration of 20.2 g DCW/L at 32 h at which time growth rate declined (Figure 5B). The decrease in activity in the later stage of the culture, also confirmed by the increasing trend of the DO percentage saturation profile, resulted in a very low biomass increase.

Figure 6 depicts the profiles of pH (Figure 6A), biomass concentration (Figure 6B), and DO (Figure 6C) of another batch of DPPSH fermentation, which was identical to the batch shown in Figure 5 but had an initial glucose concentration of 10 g/L instead of 7 g/L. As shown in Figure 6A, initial culture pH was at the upper setpoint of 6.8 and decreased to the lower setpoint of 6.6 at around 6 h, at which point pH was stabilized by the addition of NH_4OH. Oscillation of pH, which corresponds to the alternating loops of glucose addition and NH_4OH addition, was established and maintained during the period of approximately 22 to 32 h, during which cells were growing quickly (Figure 6B) and DO was maintained at the setpoint of 20% air saturation (Figure 6C), indicating active metabolic activities of cells. Unlike the previous batch, no manual injection of glucose feed was necessary. The highest biomass concentration obtained was 26.6 g/L and the biomass increased to 18.3 g DCW/L in about 9 h after the fed-batch phase started. It is worth mentioning that the pH and DO profiles of the batch phase were less regular compared to that presented in Figure 5, probably due to the high sensitivity of the control system.

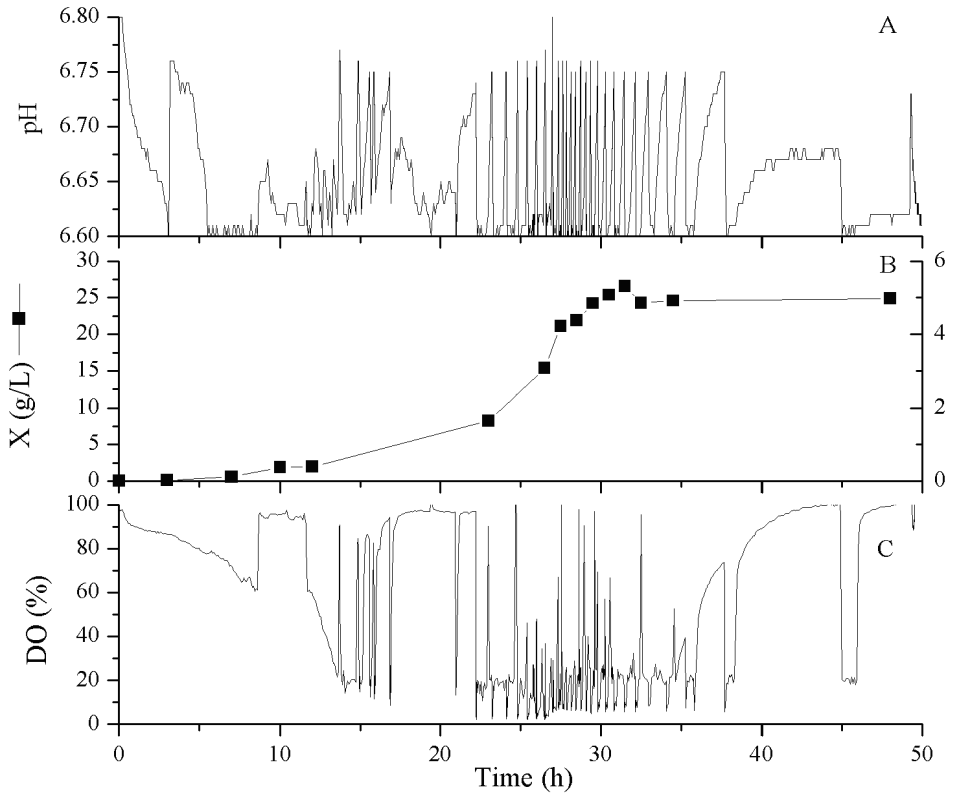

Figure 6. DPPSH control strategy using glucose concentration of 10 g/L and a feeding dosage of 10 g/L glucose: A, pH; B, Biomass; and C, dissolved oxygen (% air saturation).

DISCUSSION

Effect of initial glucose concentration on fed-batch

Our attempt to repeat the results of Korz *et al*. [21] with *E. coli* TG1 wild-type using an exponential feeding strategy with an initial glucose concentration of 25 g/L, which was reported to be able to reach a HCDC of 128 g DCW/L, was not successful. The failure was hypothetically attributed to the quick accumulation of high concentration of acetate in the culture, which reached 9.16 g/L, owing to the high initial glucose concentration. This was compatible with the results of other researchers showing that acetate at that level could lead to complete inhibition of *E. coli* growth [24, 25]. The slow cell growth obtained in these experiments also confirms the observation of other researchers to the effect that acetate at a level of 2.0 g/L or above may inhibit growth of *E. coli* [11-15]. Consequently, the biomass concentration obtained in the SPPS experiments was extremely low.

On the other hand, it was observed that when a pH range of 6.6-6.8 was employed with the DPPS fed-batch, an initial glucose concentration of 7 g/L was not enough to cause the culture pH to increase to the upper setpoint after decreasing to 6.6. It is hypothesised that the initial amount of glucose catabolised did not yield sufficient acidic by-products to push the culture pH to the upper setpoint even after their complete consumption, thus demanding a manual injection of the first feeding solution pulse to trigger the cycles. Furthermore, it was observed that an initial glucose concentration of 10 g/L was able to trigger the injection of the glucose solution automatically. These results highlight the importance of determining the proper initial glucose concentration at a level that is low enough to avoid inhibition of cell growth and, at the same time, high enough to match with the pre-set pH control range.

The narrow bandwidth cycle loop with SPPS

The pH-stat feeding strategy relies on the change of culture pH that is closely related to cellular

metabolism. When glucose is present, metabolic by-products, which are mainly acids such as propionic, acetic and lactic acids [26], are produced and neutralized by the addition of basic solutions such as NH_4OH to maintain the culture pH at the desired setpoint. Then, when glucose is depleted, cells are forced to consume ammonium salts of acidic metabolites, resulting in a pH increase [16]. If this pH increase due to the consumption of accumulated acidic metabolites was large enough to push the pH culture to reach the upper pH setpoint, then it would trigger the addition of glucose to start another feeding cycle.

A key for a successful feeding strategy requires the addition of a feeding solution, which contained glucose and other macronutrients, as well as the NH_4OH solution to be coupled with the depletion of acidic by-products of glucose catabolism and glucose, respectively. Unfortunately, as shown in Figure 3, when the SPPS feeding strategy was used, this was not achieved, thereby, creating narrow bandwidth cycle loops that were independent of the depletion of acidic metabolites or glucose. As shown in Figure 3B, in the fed-batch phase, alternating feeding of glucose and NH_4OH was observed. It is clear, however, that the alternating cycle was not responding to the depletion of glucose or of acidic metabolites but rather to slight pH offsets caused by the addition of either NH_4OH or the feeding solutions. At the end of the batch phase, glucose was depleted and the cells were forced to assimilate acidic metabolites for cell growth and maintenance. The consumption of a small quantity of these acidic metabolites would cause a small increase of pH, push it above the setpoint and, therefore, trigger the addition of glucose. Since glucose is a more favourable carbon source than acidic metabolites such as acetate, cells would assimilate the newly added glucose and produce more acidic metabolites to trigger the addition of a new dose of NH_4OH and, therefore, start another narrow bandwidth cycle feeding loop.

Apparently, the narrow bandwidth cycle feeding loop allows the consumption of very little acidic by-products before triggering the addition of a new pulse of glucose and very little glucose before triggering the addition of a new pulse of NH_4OH. As a result, acidic metabolites such as acetate and added nutrients such as glucose, and pH controlling agent, i.e. NH_4, would all accumulate with time and eventually reach a level that is inhibitive to cell growth. This explanation was confirmed by the accumulation of approximately 21.8 g acetate/L at 48 h of fermentation in the SPPS fed-batch fermentation (Figure 3). The early cessation of cell growth and low biomass concentration were clearly a result of the failure of this strategy to avoid accumulation of high level of acetate.

Effects of glucose feeding dosage in dual-point pH control

The dual-point pH-stat (DPPS) feeding strategy was devised to overcome the narrow bandwidth feeding cycle observed in SPPS. Two different glucose-feeding dosages were tested. Figure 4A shows that when a small glucose feeding dosage of 0.12 g of glucose per injection was used, the pH would oscillate near the upper setpoint of pH 6.8 once it has reached that level due to the consumption of acidic metabolites upon depletion of glucose at the end of batch phase. This is because the glucose dosage per injection was too small to induce the production of acidic metabolites necessary to lower the pH to the lower setpoint of pH 6.6 after each injection. As a result, ammonia was never added to the culture and cells might have experienced nitrogen starvation [27], which caused the cessation of cell growth. To remedy this problem, fermentations with glucose dosages of 15 g of glucose per injection, which would lead to a glucose concentration in the culture of at least 10 g/L, were carried out. As shown in Figure 5 and Figure 6, this dosage was sufficient to maintain well-paced alternating addition of the feeding solution and the NH_4OH solution and obtain cell densities in the range of 22.0-26.6 g/L. Using DPPS feeding strategy does not require calculations for modification of feeding flow rate as is done in exponential feeding [26] or to manually change the amount of glucose fed as required in step-wise feeding strategy [28]. As the time intervals between feeding periods are shortened and the feeding is carried out frequently in a responsive way, DPPS feeding can sustain pseudo-exponential growth. As biomass concentration increases, feeding tends to become more frequent corresponding to increased metabolism of cells. However, the feeding frequency started to decrease

when cell growth slows down, which began at around 35 h in the high glucose dose DPPS batch, as shown in Figure 5A, corresponding to the start of plateau in the biomass concentration profile as shown in Figure 5B and the delayed increase of DO as shown in Figure 5C. This decrease in activity is also corroborated by the dissolved oxygen profile slowly rising over time. The plateau observed in the biomass profile seems to suggest that a critical component is missing from the fermentation medium preventing further growth. It is to be noted that Korz *et al.* [21] did supply phosphorus and ammonia when DCW reached c.a. 40 g/L and 70 g/L, respectively. Also, it is reported in the literature that upon starvation for any of the three nutrients— carbon, phosphorus, or nitrogen— *E. coli* enters stationary phase for survival [27].

CONCLUSION

A simple dual setpoint pH-stat (DPPS) feeding strategy was developed to enhance the growth of a recombinant *E. coli* TG1 strain expressing single domain antibody ToxA5.1. Results indicate that the initial glucose concentration in the medium should be low enough to avoid over-production of inhibitive by-products such as acetic acid and other acidic metabolites. On the other hand, it should be large enough to support substantial cell growth and to accumulate sufficient amount of acidic metabolites, which would be able to create a pH increase that is large enough to reach the upper end of the pre-determined range of pH control (i.e., pH 6.8 in this study), when the acidic metabolites are consumed following glucose depletion to trigger the injection of a dose of glucose. Similarly, the glucose dose added per injection should be small enough to avoid substrate inhibition but sufficiently large to allow the acidic metabolites produced during glucose catabolism to lower the culture pH to the lower setpoint (i.e., pH 6.6 in this study) to trigger the addition of NH_4OH, which serves as an additional nitrogen source for cell growth in the fed-batch phase. Furthermore, using two setpoints for pH control allows cells to fully deplete available glucose as primary carbon source and then acidic metabolic by-products resulting from glucose catabolism as a secondary carbon source. This ensures that neither glucose nor acetate can accumulate to inhibitory concentrations in the culture medium. In this study, 10 g/L of glucose in the initial medium resulted in growth conditions that enabled fed-batch feeding to proceed using the DPPS strategy when a pH range of pH 6.6-6.8 was used. Using injections of 10 g/L of glucose per feeding cycle, cell densities up to 26.6 g DCW/L were achieved with the newly proposed fed-batch strategy. This strategy is simple and effective but further experiments to determine which nutrients are lacking could potentially lead to HCDC.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest for this study.

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