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Expression of single domain antibody ToxA5.1 in recombinant *Escherichia coli*: Part II - Effects of medium composition and operation conditions

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ABSTRACT

A novel llama single domain antibody (pSJF2H-ToxA5.1) neutralizing *Clostridium difficile* toxin A (TcdA) was expressed in recombinant *Escherichia coli* TG1. The effects of several parameters on the expression of ToxA5.1 were studied in Erlenmeyer flasks and bioreactors. Amongst the parameters studied, yeast extract and temperature proved to have the most effects on the expression of ToxA5.1 both in flasks and in bioreactors. Inducer concentration and induction timing must also be taken in consideration to enhance ToxA5.1 yields. In flasks, under conditions tested, a yield of 182 mg/g DCW was achieved. In bioreactors, *E. coli* TG1-ToxA5.1 was grown to a final biomass concentration of 12.26 g DCW/L in defined media using a dual-point pH-stat fed-batch control strategy. Expression of ToxA5.1 required an increase in the fermentation temperature from 28 ºC to 37 ºC, supplementation of yeast extract of 54 g/L in the feed solution and the addition of 1 mM inducer molecule, isopropyl β-D-1-thiogalactopyranoside (IPTG). Under these conditions, ToxA5.1 was expressed to 127 mg ToxA5.1/L in bioreactors.

KEYWORDS: *Escherichia coli*, recombinant protein expression, Fed-batch fermentation, pH-stat

INTRODUCTION

Clostridium difficile is a Gram-positive sporulating bacterium that generally spreads by fecal-oral route and causes, especially in persons undergoing antibiotic treatment, symptoms from light diarrhea to fatal pseudomembranous colitis [1], which are known as *C. difficile* infections (CDI). The number of CDI cases have constantly increased since 2000 [2] and it is projected that 450 000 to 750 000 [3] annual cases could be diagnosed in the US [3]. Moreover, a hypervirulent strain (BI/NAP1/027) was responsible for a series of outbreaks in Québec, Canada [4]. This hypervirulent strain has been reported worldwide including the US, Asia, UK and some European countries [5, 6]. CDIs are causing multiple problems in hospital settings and, amongst these, increased healthcare costs, which were estimated at over 3.2 billion $/year for the USA alone in 2007 [2], mainly due to longer stay and higher rate of readmission. Emerging therapies against *C. difficile* have been reviewed [7] and toxin neutralization using monoclonal antibodies shows promising results [8]. Some single domain antibodies (sdAb) [9] have also shown *C. difficile* toxin neutralization abilities [8, 10]. One such sdAb is ToxA5.1, which has proven to be effective at recognizing conformational epitopes and neutralizing the cytopathic effects of toxin A on fibroblast cells in an *in vitro* assay [10]. Because of their pH stability [11] and proteolytic degradation resistance [12], sdAbs are good candidates for oral therapy and, therefore, large quantities of sdAbs would be required. As opposed to monoclonal antibodies, sdAb do not require extensive refolding or glycosylation to be fully functional and, therefore,

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can be expressed at high levels in prokaryotic cells such as *E. coli* [13].

When expressing recombinant protein for lab-scale applications such as protein characterization, small volumes of culture are sufficient to satisfy these applications. However, when high volumetric productivity is required as it is the case when expressing recombinant protein for commercial applications, a large volume of high biomass concentration is highly desirable. Commercial protein production relies on high cell density cultures which significantly reduce capital investments as well as operating costs [14, 15]. Protein expression factors affecting cell growth also have a profound effect on recombinant protein expression. Other factors ought to be investigated for optimal protein production and these can be separated into two types: the operating conditions such as pH, temperature, feeding strategy and timing of induction; and the composition of the fermentation medium (complex or defined), carbon source, medium additives, and inducer molecule used.

Recombinant protein expression, in most cases, needs to be induced. At a judicious moment, a change in the environment, commonly performed by adding an inducer, frees the target gene(s) from repression and initiates the protein expression phase.

Since recombinant protein expression can produce an important metabolic stress on cells [16], being able to control the moment of induction is critical. Inducing cells too early would be detrimental to biomass production as induction creates a metabolic burden in which most of the cell's energy is diverted towards protein expression. When recombinant product is intracellular as is the case with ToxA5.1, premature induction might result in a lower volumetric productivity (g protein/L culture h) due to the lower biomass concentration. If recombinant product is growth-associated, a later induction phase is not recommended since cells are not growing as actively, which reduces specific productivity (g protein/DCW h). Timing of the induction is also important as the fraction of plasmid-bearing organisms tends to decrease with fermentation time even if selective pressure, in the form of antibiotics, is applied [17].

Other additives to the fermentation medium can increase specific productivity and, amongst these, yeast extract is one of the most studied. Yeast extract has been reported by multiple sources to enhance specific productivity of recombinant proteins expressed by different *E. coli* strains [18-21].

In this study, we have investigated the effects of temperature on *E. coli* TG1 growth and ToxA5.1 expression, a single domain antibody that neutralizes *Clostridium difficile* enterotoxin A [10], as well as the effects of yeast extract, timing of induction and inducer concentration in order to optimize ToxA5.1 expression in a bioreactor. The results of this study were then integrated with a Dual Point pH-Stat (DPPS) feeding strategy developed in a previous study [22] to achieve high level expression of Tox5.1 in a fed-batch fermentation process.

MATERIALS AND METHODS

Bacterial strain and plasmid

DNA encoding ToxA5.1 gene, a llama single domain antibody with specificity for *C. difficile* toxin A [10], was cloned into the expression vector pSJF2H [23] 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). Recombinant strains were grown in 20 g/L lysogeny broth (LB) medium (Fisher Scientific, Pittsburgh, PA). Colony from an overnight culture on LB+ampicillin (100 μg/mL) plate (37 °C) was sub-cultured in 50 mL of defined medium [24] 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.

Medium preparation

Batch medium and feeding solution used in this study are identical to those reported by Korz *et al.* [24] except that 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, thiamine HCl 4.5 mg/L, 10 mL/L trace

metal solution which consisted of ethylene-diaminetetraacetic 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 ampicillin 100 mg/L. Fed-batch feeding solution consisted of glucose 600 g/L, $MgSO_4$ 20 g/L, 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. For experiments using the modified feeding solution, the feeding solution was supplemented with 54 g/L of yeast extract (Oxoid, Fisher Scientific).

Protein quantification

Cells were sampled as follows: 1 mL of broth was centrifuged at 13 400 rfc for 2 min in a microcentrifuge (Thermo Electron Corp. IEC Micromax) at room temperature. Supernatant was discarded and pellet was frozen at -20 °C. Cell lysis buffer (300 µL of 1X buffer/pellet) was prepared using Promega Corporation (Madison, WI) Fast Break lysis reagent 10X, 1 mM phenylmethylsulphonyl fluoride (PMSF) (Bio Basic Inc. Markham, ON) and 3 U DNAse 1 (Promega Corp.). Cell lysis was carried out on a rocking platform (Boekel Scientific, Feasterville, PA) at room temperature for 30 min. Total and soluble fractions of crude cell lysates were analyzed on 4-12% discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [25]. Bovine serum albumin standards (Thermo Scientific, Rockford, IL) of 10, 30, 100, and 200 µg/mL were loaded on every gel providing quantitative estimation of expressed ToxA5.1. Gels were stained using Fermentas PageBlue Protein Staining Solution (Glen Burnie, MD) fast protocol followed by overnight destaining in distilled water. Quantification of scanned gels was achieved via densitometry using ImageJ software [26].

Effect of temperature and media on cell growth

Primary inoculum was seeded in 25 mL of defined medium containing 25 g/L of glucose and 100 µg/mL ampicillin in 125 mL Erlenmeyer flasks at 30 °C for 18 h in an orbital shaker at 200 rpm, to serve as inoculum for experiments. Enough inoculum from the overnight culture to obtain an OD_{600} of ca. 0.1 was added to 25 mL of defined medium containing 25 g/L of glucose and 100 µg/mL ampicillin in 125 mL Erlenmeyer flasks. Flasks were then incubated at 28 ºC or 37 ºC and growth curves were obtained by measuring OD_{600}. The same procedures except without the addition of glucose were repeated for growth in 20 g/L LB medium (25 mL of 20 g/L LB medium containing 100 µg/mL ampicillin in a 125 mL Erlenmeyer flask).

Effect of temperature on growth and expression

Testing the effect of temperature was done in LB medium and cultures were prepared as described in the previous section. The cultures were grown either at 28 ºC or 37 ºC until biomass reached an OD_{600} of ca. 0.6 at which point the cultures were induced with 2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega, Madison, WI). Some cultures were then placed on an orbital shaker (200 rpm) at 28 ºC while the remaining cultures were placed at 37 ºC on a second orbital shaker (200 rpm) to get the following growth:induction temperature scheme: 28:28, 37:37, and 28:37. For example 28:28 means that cells were grown at 28 °C until OD_{600} reached ca.0.6, at which point the culture was induced and placed at 28 °C for the expression; 28:37 means that cells were grown at 28 °C, induced at OD_{600} ca. 0.6 and then incubated at 37 °C for the expression. All cultures were induced for a period of 18 h after which protein quantification was performed as previously mentioned.

Effect of yeast extract, induction timing and inducer concentration in defined medium

To investigate the effect of yeast extract, induction timing and inducer concentration on ToxA5.1 expression in defined medium, a fractional factorial design was used. The low, intermediate and high values of the fractional factorial design, as summarized in Table 1, were: 0, 5 and 10 g/L for yeast extract; 0.4, 0.8 and 1.2 for the OD_{600} at which the induction was performed, and 0, 1 and 2 mM for the inducer concentration (IPTG).

Table 1. Fractional factorial design values for yeast extract, biomass at the time of induction and IPTG concentration.

Order	Experimental conditions	Parameter setting		
		Yeast extract g/L	Optical density OD_{600}	IPTG mM
1	- - +	0	0.4	2
2	+ - -	10	0.4	0
3	0 0 0	5	0.8	1
4	- + -	0	1.2	0
5	+ + +	10	1.2	2

Cultures were prepared as previously described except that the defined medium contained 10 g/L of glucose. Briefly, the culture was grown to the appropriate induction OD_{600} on an orbital shaker (200 rpm) at 37 ºC, then, induction was performed.

Inoculum preparation for fermentation in bioreactors

Primary inoculum was added to four 500 mL Erlenmeyer flasks, each containing 100 mL of defined medium [24] with 10 g/L of glucose and 100 µg/mL of ampicillin. Cultures were grown at 30 °C for 12 h in an orbital shaker at 200 rpm. Cultures were transferred to two 250 mL centrifuge bottles and centrifuged (2,550 rcf at 20 °C) for 20 min in a tabletop centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). 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 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 ca. 0.15.

Expression of ToxA5.1 in bioreactor adapting the dual-point pH-stat feeding strategy

Fermentations were performed in New-Brunswick Scientific BioFlo110 3-L bioreactors (Edison, NJ) with a working volume of 1.5 L. The batch phase was carried out in 1.5 L of defined medium containing 7 g/L of glucose. Temperature (28 °C) and agitation (with two 6-blade Rushton impellers mounted on a single shaft at 300 rpm) were kept constant via the control module of the bioreactors. During the batch phase, the medium pH was controlled by the addition of a 25% v/v solution of NH_4OH (Fisher Scientific) when the pH dropped below the setpoint of 6.6. When pH was higher than the upper pH setpoint of 6.8, a glucose pulse was injected which caused the medium pH to decrease. Dissolved oxygen (DO) was set at 20% air saturation and a constant airflow of 1 vvm was bubbled into the bioreactors. When the oxygen demand could not be satisfied with the air supply, pure oxygen was mixed in the airflow using the BioFlo110 gas mixer module.

Upon glucose exhaustion in the batch medium, a dual-point pH control strategy [22] was implemented using a LabVIEW (National Instruments, Vaudreuil-Dorion, QC) interface and New-Brunswick Scientific OPC (Object Linking and Embedding (OLE) for Process Control) server. Briefly, a pulse of feeding solution corresponding to 10 g glucose/L of culture (for a total of 15 g of glucose per pulse) was injected every time pH reached the upper pH setpoint of 6.8. Upon injection of the feeding solution, glucose catabolism causes the medium pH to decrease and as mentioned previously NH_4OH solution was used to keep the medium pH above the lower pH setpoint of 6.6. Considering the dynamics of the system, a subroutine was included in the LabVIEW program to prevent the injection of a second glucose pulse for a period of 5 min thus preventing glucose over-feeding, which may lead to the accumulation of acetate. Upon glucose exhaustion in the medium, acidic metabolites get consumed causing the medium pH to increase until the upper pH setpoint is reached. This constitutes a complete feeding cycle. Another pulse of feeding solution is then injected which corresponds to the start of a new feeding cycle.

Induction of the expression of ToxA5.1, which marked the end of the growth phase and the beginning of the protein expression phase, was carried out according to the conditions specified in Table 2. The feeding solution was also replaced by the modified feeding solution, which was the same as the feeding solution except being supplemented with 54 g/L of yeast extract.

Glucose quantification

Concentrations of glucose 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 (H_2SO_4, 5 mM) was run at 0.6 mL/min. Glucose was quantified using a refractive index (RI) detector.

RESULTS

Effects of media and temperature on cell growth

The effect of temperature and medium composition on *E. coli* TG1 growth can be seen in Figure 1. Growth at both of the tested temperatures (28 and 37 ºC) in LB medium was faster than in the defined medium. It is interesting to note that the maximal biomass concentration achieved in LB medium was 1.20 g DCW/L, which was obtained at the lower temperature of 28 °C. Similarly, the maximal biomass level achieved in defined medium was of 1.22 g DCW/L again when cells were grown at the lower temperature of 28 °C. Growth in both media at 37 °C yielded final biomass concentrations of 0.90 and 0.84 g DCW/L in the defined and LB medium, respectively.

Protein expression in flasks

The effect of temperature on the expression of ToxA5.1 can be seen in Figure 2. It is clear that ToxA5.1 cell content was greater when the expression took place at a temperature of 37 °C. When cells were grown and recombinant protein expressed at 28 °C, ToxA5.1 cell content was only 19 mg/g DCW, whereas it was 120 mg/g DCW when cells were grown at 28 °C and recombinant protein expression was performed at 37 °C. The highest protein cell content was achieved when cells were grown and recombinant proteins were expressed at 37 °C. Under these conditions, ToxA5.1 cell content was of 182 ± 32 mg/g DCW.

Effects of yeast extract, biomass concentration at induction time and IPTG concentration were tested using a fractional factorial design. It can be seen from Figure 3 that a culture without yeast extract or IPTG (- + -) did not lead to protein expression while the presence of IPTG alone (- - +) resulted in the expression of 0.3 mg ToxA5.1/L. The single factor having the largest effect on protein expression was yeast extract as 2.7 mg ToxA5.1/L was observed when a concentration of 10 g/L (+ - -) was used. Yeast extract and IPTG concentrations of 5 g/L and 1 mM, respectively, and induction performed at mid-exponential phase (OD_{600} ca. 0.8) (0 0 0) resulted in protein expression of 1.7 mg ToxA5.1/L. The highest ToxA5.1 expression, which was 10 mg/L, was obtained when yeast extract and IPTG were present at concentrations of 10 g/L and 2 mM, respectively, and the induction performed at OD_{600} value of 1.2 (+ + +).

Protein expression in bioreactor

Several expression conditions that were tested during the fed-batch phase in bioreactors are summarized

Table 2. Summary of conditions tested for the expression of ToxA5.1 in bioreactors.

Induction temperature ºC	Inducer concentration mM	Biomass at time of induction g DCW/L	Final biomass g DCW/L	ToxA5.1 concentration mg/L	Incubation time h
28	1	21	22.5	ND	18
28	2	15	20.1	ND	18
37	1	15	19.5	< 1	18
37	1[a]	5	12.3	127	7

ND: not detectable.

[a]feeding solution contained 54 g/L yeast extract.

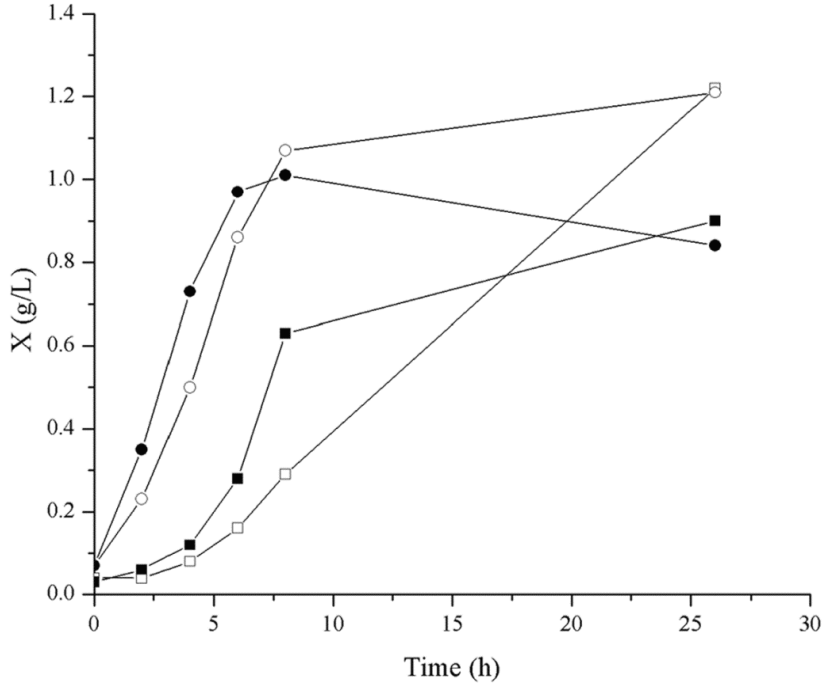

Figure 1. Effect of growth temperature and medium composition on *E. coli* TG1+ToxA5.1 growth without induction. Defined medium containing 25 g/L glucose at 28 ºC (–□–) and 37 ºC (–■–), LB medium at 28 °C (–○–) and 37 °C (–●–). Standard deviation is not shown for clarity (n = 4). The standard deviation average values were of 0.01 g/L for the defined medium and 0.03 g/L for the complex medium.

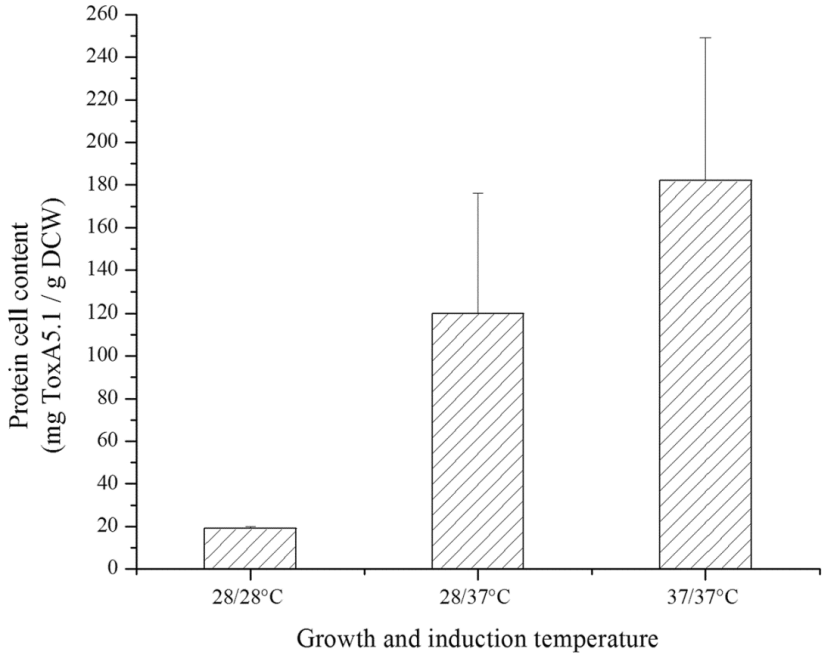

Figure 2. ToxA5.1 recombinant protein content in *E. coli* TG1 induced at OD_{600} = 0.6 with 2 mM IPTG for 18 h in LB medium at different combinations of cell growth/protein expression temperatures. Error bars are showing the highest value of two replicates for each condition.

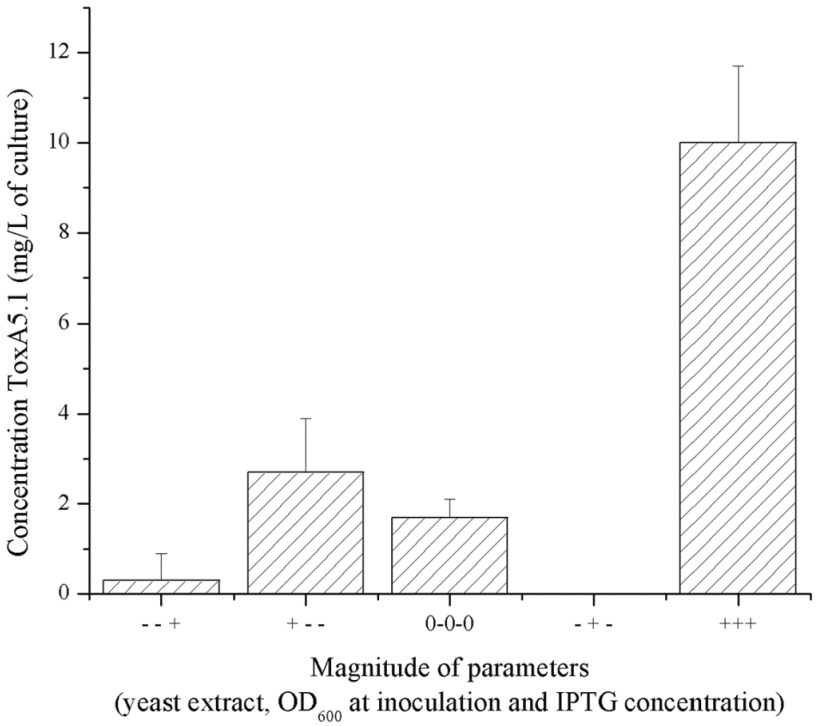

Figure 3. Effect of yeast extract, induction timing and inducer concentration on ToxA5.1 expression in defined medium at 37 °C with initial glucose concentration of 5 g/L. Error bars are 1σ (n = 3).

in Table 2. It can be seen that a temperature of 28 °C with induction at mid or high biomass concentration (15 or 21 g DCW/L, corresponding to cultures in the middle or later exponential phase, respectively) did not yield any detectable level of ToxA5.1 even after 18 h of induction, no matter if the inducer concentration was 1 or 2 mM. Increasing the induction temperature to 37 °C and inducing the culture at a mid-biomass concentration of 15 g DCW/L (i.e., middle-exponential-phase induction) with 1 mM IPTG resulted in very little ToxA5.1 expression (< 1 mg/L) after 18 h. Only when cells were induced with 1 mM IPTG at 37 °C, at a low biomass concentration of 5 g DCW/L (i.e., when the culture was in early exponential phase) and with yeast extract supplemented in the feeding solution did ToxA5.1 expression occurred. After 7 h of induction, 127 mg/L of ToxA5.1 was obtained.

A typical ToxA 5.1 expression experiment using the DPPS feeding strategy is presented in Figure 4. Initially, the batch phase medium contained 7 g/L of glucose, which was being consumed over a period of 8 h as indicated by a decrease in pH value to the lower setpoint of 6.6. Upon reaching this lower setpoint, ammonia was fed to the culture keeping the medium pH at 6.6. After 8 h of fermentation, the acidic by-products produced during the catabolism of glucose and responsible for the decrease of pH started to get consumed, as indicated by the increase of the pH value from 6.6 to 6.67 in approximately 3 h. This is an indicator of glucose depletion from the culture since glucose is a more favored carbon source than the salts of the acidic metabolites of its catabolism, e.g. acetate [27]. Once pH reached 6.67, the batch phase was considered to be completed. During the batch phase, the biomass concentration reached 1.43 g DCW/L and the yield of biomass on glucose ($Y_{X/S}$) was 0.204 g DCW/g glucose (Table 3). The fed-batch phase was initiated manually by injecting a pulse of feeding solution containing 15 g of glucose (for a concentration in the medium of 10 g/L), which caused a rapid pH decrease in the fermentation medium. The amount of glucose that was injected upon fed-batch initiation was consumed in approximately 2 h as indicated by an increase in the medium pH after 14 h.

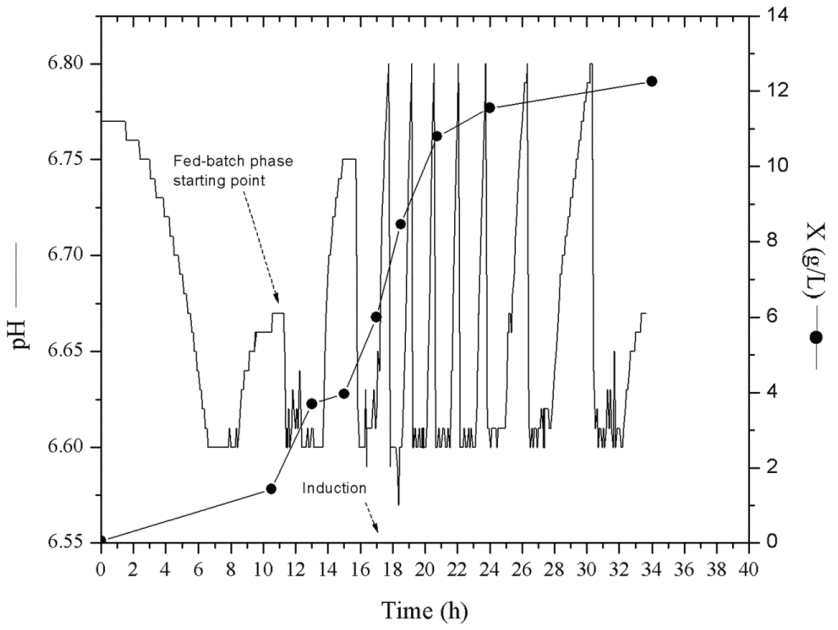

Figure 4. Fermentation of *E. coli* TG1 in defined medium at 28 °C using a dual-point pH-stat control strategy coupled with feeding pulses of 15 g glucose per pulse. Induction was performed at 37 °C with IPTG concentration of 1 mM in the bioreactor and with an induction feeding solution consisting in 600 g/L of glucose (15 g glucose per pulse), 54 g/L of yeast extract (1.34 g yeast extract per pulse) and trace metals.

Table 3. Parameters of batch and fed-batch fermentation phases.

	Batch phase	Fed-batch phase	Overall
Glucose (g/L)	7	137	144
Yeast extract (g/L)	NA	9.45	9.45
C (mg protein/L)	ND	127	127
X (g DCW/L)	1.4	10.8	12.2
$Y_{X/S}$ (g DCW/g Glucose)	0.204	0.079	0.085
$Y_{P/X}$ (mg ToxA5.1/g DCW)	ND	11.0	11.0
$Y_{P/S}$ (mg ToxA5.1/g Glucose)	ND	1.31	1.31
P (mg ToxA5.1/L h)	ND	5.77	3.53

NA: Not applicable; ND: not detectable.

As aforementioned, culture pH increase was caused by the consumption of the acidic metabolite salts resulting from glucose catabolism. Once the medium pH value reached 6.75 (15 h), a second pulse of feeding solution was injected in the culture. The latter glucose pulse (15 g) was consumed in approximately 1 h after which pH increased to a value of 6.8. At that point, the induction was performed by increasing the culture temperature from 28 °C to 37 °C and by injecting IPTG in the fermentation broth to achieve a concentration of 1 mM IPTG. Upon induction, the feeding solution was replaced by the formulation containing 54 g/L of yeast extract described in the materials and methods section. This modified feeding solution was added to the fermentation every time pH reached a value of 6.8 indicating depletion of glucose and consumption of the acidic by-products from the fermentation broth. The fermentation lasted a total of 34 h and a final biomass concentration of 12.26 g DCW/L was obtained. From Table 3, it can be seen that during the fed-batch phase, 137 g of glucose and 9.45 g of yeast extract were fed to the culture resulting in a biomass

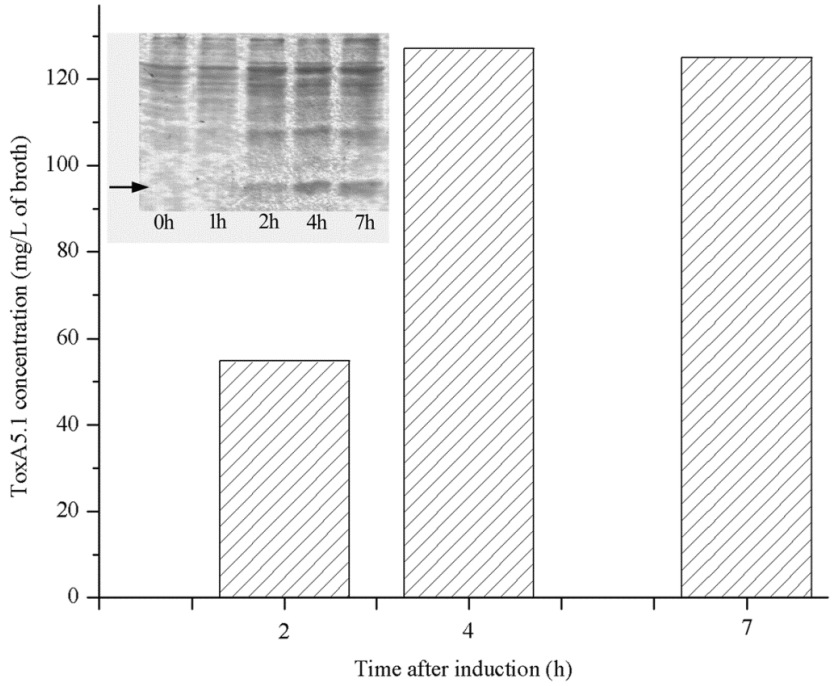

Figure 5. Time course expression of recombinant protein ToxA5.1 single domain antibody against *C. difficile* toxin A in *E. coli* TG1 during fed-batch fermentation. Arrow pointing ToxA5.1.

yield on glucose ($Y_{X/S}$) of 0.079 g DCW/g glucose. As shown in Figure 5, at the time of induction and for 1 hour post-induction, no recombinant protein was detected. ToxA5.1 expression was detected 2 h post-induction. After 7 h of induction, a total of 127 mg ToxA5.1/L was obtained resulting, as can be seen in Table 3, in ToxA5.1 cell content ($Y_{P/X}$) of 11 mg/g DCW. An yield of ToxA5.1 over glucose ($Y_{P/S}$) of 1.31 mg/g glucose and a productivity of 3.53 mg/L h of ToxA5.1 were calculated.

DISCUSSION

Effect of temperature and media on cell growth

Results presented in Figure 1 show that complex medium, such as LB, can support higher growth rate than defined medium. This may be explained by the fact that the complex medium contains growth factors and other trace elements present in the yeast extract that enhance bacterial growth. In complex medium, the temperature had a minor effect on biomass growth shortly after inoculation but after 26 h, a higher biomass concentration was obtained at a lower temperature of 28 °C than at a temperature of 37 °C with final biomass concentrations of 1.20 and 0.90 g DCW/L, respectively. In the defined medium, however, the temperature had an impact early during the growth phase and, after 6 h, the growth rate for the culture at 37 °C was higher than at 28 ºC. After 26 h, similar to the complex medium, biomass concentration was higher at 28 °C (1.22 g DCW/L) than at 37 °C (0.84 g DCW/L). Lower biomass concentration at higher temperature can be explained by the fact that *E. coli* growth follows an Arrhenius type equation with an optimal temperature range between 28 °C and 37 °C outside which growth rate diminishes drastically [28]. It has been well established that an increase in temperature would lead to an increase in the rates of two opposite reactions: the reaction that leads to cell growth and the endogenous metabolic reaction where cell materials are consumed to sustain the viability of cells. When the environmental temperature is lower than the optimal temperature, an increase in temperature will result in an increase in the cell growth reaction that is larger than the increase of endogenous metabolic reaction, leading to the increase in the overall or net specific cell growth rate. On the other hand, when the temperature is

higher than the optimal temperature, a temperature increase will result in an increase in the endogenous metabolism that is larger than the increase of growth metabolism, leading to a decrease in net specific cell growth rate [28].

Effect of temperature on cell growth and ToxA5.1 expression

The hypothesis proposed to explain the effect of temperature on cell growth can also be used to understand why the cell protein content is higher at 37 °C than it is at 28 °C as observed in Figure 2. When *E. coli* TG1 cells were grown and protein expression induced at 28 °C, ToxA5.1 content was almost 10 times less than when cells were grown and protein expression carried out at 37 °C, where a protein concentration of 182 mg ToxA5.1/g DCW was obtained. It is reported in the literature that a lower temperature might have an effect on the expression of certain genes [29, 30]. For instance, it was demonstrated that the expression of the OmpA gene, which is also present in the plasmid used in this study, was less efficient at a temperature lower than 28 °C when compared to a temperature of 37 °C [31]. Similarly, the absence of the recombinant protein K1 capsular antigen was reported by Bortolussi and Ferrieri [32] when *E. coli* cells were grown and gene expression performed at a temperature lower than 30 °C for a short period of time. When examining results presented in Figure 2, it can be seen that increasing the temperature from 28 °C during the growth phase to 37 °C during the induction phase did not impair the ability of the transformed *E. coli* cells to express the recombinant ToxA5.1 gene, as a ToxA5.1 content of 120 mg/g DCW was obtained when the growth temperature was 28 °C and the induction temperature was 37 °C.

Effect of yeast extract, induction time and inducer

The effect of yeast extract supplementation on protein expression during the induction phase can clearly be seen in Figure 3. The presence of yeast extract had the greatest impact on the protein expression of all of the tested parameters. Even in the absence of inducer, expression of ToxA5.1 was 2.7 mg/L in the presence of yeast extract. A synergistic effect of yeast extract and inducer is clearly evidenced and resulted in a protein expression of 10 mg/L. It is interesting to note that a concentration of 10 g/L of yeast extract without inducer resulted in a higher ToxA5.1 expression than a concentration of 5 g/L combined with 1 mM of inducer. As mentioned earlier, yeast extract is comprised of complex nitrogen sources which are building blocks for proteins as well as some growth factors and it is a known fact that protein expression is enhanced when yeast extract is present [20, 33]. It was observed in our studies (data not shown) that the plasmid pSJF2H used in this study, which was derived from a high copy number vector, pUC8 [34-36], did not have a tight expression regulation. These results seem to suggest that, when complex medium was used to grow *E. coli* TG1+pSJF2H+ToxA5.1 cells, the repressor responsible for preventing recombinant expression, may not fully bind to the operator site on the lac operon, allowing a certain level of transcription of the genes downstream of the promoter [35, 37].

Fermentation in bioreactors

A typical fermentation experiment is shown in Figure 4. Using a dual point pH-stat control strategy to feed glucose was successful in achieving a biomass concentration of 21 g/L as can be seen in Table 2. This feeding strategy can produce biomass of up to 27 g DCW/L when no induction was attempted [22]. The dual point pH-stat control strategy relies on the fact that when glucose is consumed, acidic by-products are produced which cause medium pH to decrease. Upon exhaustion of glucose, those acidic by-products are consumed resulting in medium pH increase as seen in Figure 4. Using this strategy ensures that glucose is completely consumed before the next feeding cycle and also that acidic by-products, such as acetate, are consumed. This strategy can therefore prevent accumulation of both glucose and acetate in the fermentation medium which, if not controlled, can be detrimental to cell growth. Unlike exponential feeding schemes, it is not the amount of feed that increases to support bacterial growth but rather the frequency at which feeding takes place as seen in Figure 4 where each peak correspond to a new feeding cycle. Several conditions under which ToxA5.1 was not expressed are reported in Table 2. From this table, the importance of temperature, timing and duration of induction as well as supplementation with yeast extract for expression

of ToxA5.1 in defined medium are evidenced. At low temperature of 28 °C, even after 18 h of induction using 2 mM IPTG in the late mid-exponential phase (15 g DCW/L), no amount of ToxA5.1 was detectable. Increasing the temperature to 37 °C during induction at late mid-exponential phase only resulted in very little expression of ToxA5.1. Successful expression of ToxA5.1 was achieved only when induction was performed at the early mid-exponential phase at a temperature of 37 °C with inducer concentration of 1 mM IPTG and a supplement of yeast extract in the feeding solution. Under these conditions, it was possible to obtain 127 mg/L of ToxA5.1 in the fermentation culture. It is interesting to note that even if the fermentation lasted over a period of 34 h, the final biomass concentration reached only 12.26 g DCW/L which is lower than the 27 g/L obtained in previous studies where no induction occurred [22]. This could be explained by the fact that the induction conditions produce a metabolic burden [16] in which most of the cell's energy was diverted towards protein expression, which is detrimental to biomass production, and this phenomenon is reported to be more significant with complex media (e.g. LB) than with minimal medium (e.g. M9) [16].

CONCLUSION

In conclusion, it was shown that using a dual point pH-stat control strategy, *E. coli* TG1 expressing the single domain antibody against *C. difficile* enterotoxin A could be grown to a final biomass of 12.26 g DCW/L with ToxA5.1 cell content of 11 mg/g DCW. The optimal induction condition was to increase temperature from 28 °C to 37 °C, enrich feeding solution by including 54 g/L yeast extract in it, and induce in the early exponential phase. Under these conditions, ToxA5.1 was expressed at a concentration of 127 mg/L. It is clear from the results that yeast extract supplementation is required for protein expression when *E. coli* is grown in defined medium. Since growth control is not as critical during induction phase, larger concentration of yeast extract, more akin to those used in Terrific Broth (TB) medium should be supplemented at the time of induction. Timing of induction as well as temperature at which induction is done must also be considered as both influences ToxA5.1 titer.

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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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