Production of Fuculose-1-Phosphate Aldolase Using Operator–Repressor Titration for Plasmid Maintenance in High Cell Density *Escherichia coli* Fermentations

Olga Durany,[1,2] Philip Bassett,[2] Amanda M.E. Weiss,[2] Rocky M. Cranenburgh,[2] Pau Ferrer,[1] Josep López-Santín,[1] Carles de Mas,[1] Julian A.J. Hanak[2]

[1]Departament d'Enginyeria Química, Escola Tècnica Superior d'Enginyeria, Universitat Autònoma de Barcelona, Spain; telephone: ++34-935812141; fax: ++34-935812013; e-mail: pau.ferrer@uab.es
[2]Cobra Biomanufacturing plc, The Science Park, Keele, Staffordshire, ST5 5SP, United Kingdom; telephone: ++44(0)-1782-714181; fax: ++44(0)-1782-714167/8; e-mail: phillip.bassett@cobrabio.com.

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Abstract: We report a novel application for the operator–repressor titration (ORT) plasmid maintenance system. The ability of ORT to maintain a plasmid during production of DNA has been demonstrated previously. In this study, we have used the ORT system to maintain a plasmid during high cell density cultivation and expression of a recombinant protein. No evidence of plasmid loss was seen during protein expression at high cell densities. In addition, the quantity of protein produced using this system was similar to traditional plasmid maintenance systems. © 2005 Wiley Periodicals, Inc.

Keywords: operator–repressor titration; *Escherichia coli*; fuculose-1-phosphate aldolase; heterologous protein production; fed-batch cultivation

INTRODUCTION

Escherichia coli remains one of the most attractive systems for high yield recombinant protein production at industrial scale, providing that post-translation modifications are not required. With exponential fed-batch strategies, the accumulation of metabolites leading to growth inhibition, principally acetic acid, can be minimised and high cell concentrations (over 100 g dry cell weight/L) are easily obtained using inexpensive media (Yee and Blanch, 1992). Combining this with elevated levels of expression of the desired product enables high process productivities for recombinant proteins at industrial scale in *E. coli*.

Once the fermentation conditions and expression systems have been optimised for a given recombinant protein, the two key factors that must be considered for maximising yield are (i) plasmid maintenance and (ii) the metabolic burden due to other expressed sequences on the host cell. Often the plasmid-associated metabolic burden is an important element in plasmid maintenance, and even if the expression of a transgene does not contribute directly to plasmid loss, there is often a reduction in recombinant protein yield or the rate of cell growth (Bentley et al., 1990).

There are a number of systems for plasmid selection and maintenance, and all depend on the expression of a plasmid-borne selectable marker gene (for a review, see Hanak and Cranenburgh, 2001). The traditional approach has been to use an antibiotic in the culture and antibiotic resistance genes on the plasmid. This has a number of drawbacks in recombinant protein production, including degradation during cell culture resulting in a reduced selection pressure, and the risk of product contamination (Baneyx, 1999). Certain antibiotics, including the β-lactams, are already prohibited by regulatory authorities for use in the production of biotherapeutics.

To prevent recombinant plasmid loss in long term high cell density cultivations avoiding use of antibiotic resistances, alternative strategies have been developed. One common approach is to exploit the post-segregational killing mechanisms (PSK) found in many naturally-occurring plasmids. These consist of a toxin and an associated antitoxin, with the toxin having a longer half-life than the antitoxin. The loss of the plasmid and subsequent rapid degradation of the antitoxin results in killing of the host cell by the remaining toxin molecules. However, Cooper and Heinemann (2000) have shown that PSK mechanisms do not increase plasmid stability, as cultures containing similar plasmids with and without a PSK system were grown for approximately 200 generations and equivalent numbers of plasmid-containing cells were present in both. PSK systems have instead evolved to give the host plasmid an advantage in plasmid–plasmid competition in bacterial populations.

Olga Durany's present address is Oryzon Genomics, Parc Científic de Barcelona, Josep Samitier, 1-5, 08028 Barcelona, Spain.

Correspondence to: Philip Bassett; Pau Ferrer

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Another common maintenance strategy is the utilisation of a plasmid-borne gene that complements a host auxotrophy. This strategy avoids problems associated with antibiotic supplementation but it is still necessary to employ plasmids carrying unnecessary genes for recombinant protein production causing extra metabolic burden to the host strain due to their constitutive transcription and translation. Depending on the gene used, there may be limitations on the composition of the growth medium.

The operator–repressor titration system (ORT) utilises engineered *E. coli* strains possessing essential genes for host survival under the control of the *lac* operator/promoter system (Williams et al., 1998). This system allows the growth of the modified host strain only when the *lac* promoter is induced (by addition of β-galactose or IPTG), or when the cell is transformed with a multicopy plasmid containing the short *lac* operator sequence. Therefore, no expressed genes are required for plasmid stability, so avoiding a significant factor contributing to metabolic burden. The ORT strain DH1*lacdapD* enables antibiotic-free selection and stable maintenance of recombinant plasmids in complex media, with *dapD* as the essential chromosomal gene controlled by *lacO/*P (Cranenburgh et al., 2001).

ORT has previously been used to successfully maintain plasmids during the manufacture of DNA for use in DNA vaccine clinical trials (Hanke and McMichael, 2000). Here, we present the first results employing the ORT system for the production of a recombinant protein in high cell density cultures. The metabolic burden associated with expression of the recombinant protein and growth during high cell density cultivation will provide a novel test for the ORT plasmid maintenance system. The *E. coli* enzyme fuculose-1-phosphate aldolase (Fuc-1-PA), involved in carbohydrate biosynthesis, has been chosen as model protein for this study.

One of the main limitations for the industrial development of processes employing biocatalysts for organic chiral synthesis is the commercial availability of the DHAP dependent aldolases. Previous works with XL1-Blue MRF′(pTrc*fuc*), a recombinant *E. coli* strain for the production of Fuc-1-PA under control of the IPTG inducible promoter *trc* and ampicillin resistance as a selection pressure, has allowed optimisation of an exponential fed-batch strategy to achieve high cell density cultures and optimise specific Fuc-1-PA production simultaneously (Durany et al., 2005). Nevertheless, this system is not industrially scalable because of antibiotic dependence. In the present article, we test the ORT system for improved Fuc-1-PA production during high cell density cultivation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

To construct the ORT plasmid pORT1aLfuc, with *fucA* expression controlled by the λ promoters, the *trc* expression plasmid pTrcfuc was used (Garcia-Junceda et al., 1995). A *Pst*I-*Eco*RI fragment, containing the λ cI857 repressor and λ Pl and Pr promoters, was inserted into *Pst*I-*Eco*RI-cut pORT1 (Cranenburgh et al., 2001) to create pORT1L. Then *fucA* was removed from pTrcfuc with an *Nco*I-*Bst*BI digest, the *Nco*I site was blunt-ended and the fragment ligated to pORT1L that had been cut with *Eco*RV and *Bsp*DI to create pORT1Lfuc. Finally, the *bla* gene was excised from pORT1Lfuc using *Fse*I to create pORT1aL*fuc*, selected in ORT strain DH1*lacdapD*.

All strains were preserved at −80°C in glycerol stocks prepared from aliquots of exponential phase cultures grown in Luria–Bertani media (LB).

Media Composition

LB medium with a composition of 10 g/L soy peptone, 5 g/L yeast extract and 5 g/L NaCl, was used during strain evaluation and inoculum cultivation. A defined mineral medium (MD) (Durany et al., 2004), utilising glucose as the sole carbon source, was also used in shake flask experiments for new strain characterisation and for all bioreactor cultivation experiments. Batch-MD medium for shake flask cultures and the batch phase of bioreactor cultivations was composed of 25 g/L glucose, 13.23 g/L K_2HPO_4, 2.65 g/L KH_2PO_4, 2.04 g/L NaCl, 4.10 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.026 g/L $FeCl_3$, 0.01 g/L thiamine and 2.86 mL/L trace elements solution. The trace elements solution consisted of 1.44 g/L $CaCl_2 \cdot 2H_2O$, 0.04 g/L $Cl_3Al \cdot 6H_2O$, 0.87 g/L $ZnSO_4 \cdot 7H_2O$, 0.16 g/L $CoCl_2 \cdot 6H_2O$, 1.55 g/L $CuSO_4 \cdot H_2O$, 0.01 g/L H_3BO_3, 1.42 g/L $MnCl_2 \cdot 4H_2O$, 0.01 g/L $NiCl_2 \cdot 6H_2O$ and 0.22 g/L Na_2MoO_4. The glucose concentration was reduced to 10 g/L for growth in shake flasks.

The feed-MD medium for high cell density fermentations consisted of 582 g/L glucose, 10.7 g/L $(NH_4)_2SO_4$, 11.6 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $FeCl_3$, 0.35 g/L thiamine and 76.6 mL/L trace elements solution. Phosphates were not included in the feeding solution in order to avoid co-precipitation with magnesium salts. Instead, a concentrated phosphate solution containing 500 g/L K_2HPO_4 and 100 g/L KH_2PO_4 was employed during the fed-batch phase to avoid their depletion (Durany et al., 2005).

As DH1*lacdapD* is auxotrophic for L-proline, the amino acid was also included in MD media composition. The amount of L-proline required was found to be proportional to the amount of glucose consumed. Batch phase MD medium was supplemented with 0.13 g L-proline/g glucose. The feed solution contained 0.02 g L-proline/g glucose.

Cultivation Conditions

The inocula for all fermentation experiments were grown at 30°C. Starter cultures were generated by first inoculating 50 mL of LB medium with 50 μL of a glycerol stock. This culture was incubated overnight and 10 mL were used to inoculate 200 mL of MD medium in a 2 L shake flask. Once the optical density (600 nm) reached 3.0 U, 350 mL were used to inoculate 3.5 L of MD medium, starting the batch phase of the fermentation.

Fermentation cultivation was conducted in 5/7 L glass bioreactors (Z61103CT07, FT Applikon Ltd., Pershore, Worcestershire, UK). During the fermentation, the pH was controlled at 7.00 ± 0.05 with the addition of 25% (v/v) NH_4OH. The temperature was maintained at 30°C before induction. The dissolved oxygen was controlled at 20% of saturation by manipulation of the stirrer speed. The end of the batch phase was identified by a reduction in the oxygen consumption rate and an increase in pH to the upper limit of the dead-band. The fed-batch phase began with the addition of the MD-feed solution. The rate at which the feed solution was introduced to the culture was calculated using Equation 1. A material balance of glucose and biomass in a fed-batch bioreactor can be combined and integrated between time $t_0 = 0$ and time $t = t$ to obtain a continuous feeding equation (Lee, 1996; Wilms et al., 2001):

$$F = \frac{\mu_{set} \cdot X_0 \cdot V_0 \cdot e^{(\mu_{set} \cdot \Delta t)}}{S_f \cdot Y_{X|S}}, \quad (1)$$

where F is the feed rate (L/h), μ_{set} desired specific growth rate (per h), X_0 biomass concentration (g/L) at time t_0, V_0 liquid volume in the fermenter (L) at time t_0, Δt difference in time (h), S_f glucose concentration in the feed solution (g glucose/L) and $Y_{X/S}$ apparent yield coefficient biomass/glucose (g DCW/g glucose).

The expression of Fuc-1-PA from DH1*lacdapD*(pORT1aL*fuc*) was regulated by the λ promoter. Expression of Fuc-1-PA by DH1*lacdapD*(pORT1aL*fuc*) was therefore induced by a temperature shift from 30 to 42°C.

Analytical Methods

The methods used to measure biomass concentration, Fuc-1A-P activity and percentage of intracellular Fuc-1A-P are referenced in Durany et al. (2005). Concentrations of glucose and ammonia in the fermentation medium were analysed using a Vitros® DT60 II System (Axis Shield, Kimbolton, Cambridgeshire, UK) with the appropriate slides. To quantify the concentration of acetic acid in the fermentation medium an enzymatic kit was used (Roche Diagnostics, Mannheim, Germany, E0148261). If the concentration of glucose, ammonia or acetic acid exceeded beyond the range of any of the methods then the sample was diluted with distilled water. Replica plating was carried out in order to assess plasmid stability. Diluted culture samples were spread on to LB agar plates with and without 1 mM IPTG. The plates were incubated for 24 h at 37°C and the numbers of colonies were compared to determine if there were any signs of plasmid instability.

RESULTS

Adaptation of a High-Cell Density Cultivation Strategy for Growth of ORT Strains

To achieve high cell density cultivations of DH1*lacdapD* (pORT1aL*fuc*), a simple exponential fed-batch strategy was used (Yee and Blanch, 1992). A batch phase where the culture would deplete the available glucose was followed by a fed-batch phase where glucose was added to the culture. This system minimised the production of inhibitory products, such as acetate, by maintaining low growth rates. The specific growth rate of the cultivation was controlled by modifying the glucose feed rate, calculated using Equation 1. Previous studies using XL1-Blue MRF′(pTrc*fuc*) (Durany et al., 2005) had shown that this simple strategy achieved high cell densities when the growth rate was maintained at 0.1/h and so the experiments using DH1*lacdapD*(pORT1aL*fuc*) used the same rationale. As the new plasmid contained the λ promoter, non-induced growth was conducted at 30°C and induced growth at 42°C.

A defined medium using glucose as the sole carbon source was used in both growth phases. The glucose concentration at the start of the batch phase was set at 25 g/L, all other components were added in light excess to support growth until glucose depletion. At this point, a feed solution containing a high concentration of glucose supplemented with magnesium and microelements was fed into the fermenter according to Equation 1.

DH1*lacdapD* is auxotrophic for L-proline and thiamine. The medium was supplemented with these compounds. It has been reported that the relative consumption of auxotrophic amino acids can change depending on media composition, glucose concentrations, cultivation strategy and specific growth rate. Excess amino acids can reduce maximal biomass concentrations and growth rate (Favre-Bulle et al., 1993; Mak et al., 1995; Rothen et al., 1998). In addition to this, supplementation of amino acids can be very expensive compared to the costs of the other components. Therefore, after the proline auxotrophy had been identified, the concentration of proline was optimised with respect to glucose. A series of fed-batch fermentations, under non-induced conditions, were carried out with excess proline (0.5 g/L) in the growth medium in order to estimate the amount of proline consumed per gram of glucose ($Y_{L\text{-proline/glucose}}$). The ratio between proline and glucose consumed in a given period of time (i.e. $Y_{L\text{-proline/glucose}}$) was calculated to be 0.13 g L-proline/g glucose for the batch phase and 0.2 g L-proline/g glucose for the fed-batch phase at a specific growth rate of 0.1/h.

Based on previous results (Durany et al., 2004), using XL1-Blue MRF′(pTrcFuc), the yield of glucose to biomass was initially set at 0.4 g DCW/g glucose. The yield was then determined experimentally for DH1*lacdapD*, by maintaining the growth rate at 0.1/h and measuring the amount of glucose consumed compared to biomass generated. The yield coefficient obtained was 0.41 g DCW/g glucose, which is very similar to that of the previous strain.

High-Cell Density Fermentation Using an ORT Strain, DH1*lacdapD*(pORT1aL*fuc*), for Recombinant Fuc-1-PA Expression

Following the adaptation of the cultivation strategy to the requirements of DH1*lacdapD*(pORT1aL*fuc*), it was possible

to conduct further experiments to assess the new plasmid. Expression of Fuc-1-PA was induced during high cell density cultivation of DH1*lacdapD*(pORT1aL*fuc*) in order to assess the plasmid maintenance using ORT and the protein expression system under these conditions. Two identical fermentations were carried out: the first was induced at a biomass concentration of 25 g DCW/L; the second was not induced. Following the induction of the first fermentation, by a temperature shift from 30 to 42°C, the oxygen consumption rate increased beyond the oxygen transfer rate of the vessel. To prevent oxygen limitation and to allow the fermentation to continue, the air supply was exchanged for a 50% oxygen inlet air concentration. The fermentation continued until it reached a final biomass concentration of 46.7 g DCW/L. The non-induced culture reached a concentration of 40 g DCW/L before requiring oxygen supplementation and reached a final concentration of 51.4 g DCW/L (Fig. 1, Table I).

The results from the non-induced culture show the efficiency of this exponential fed-batch strategy using ORT strains. High cell densities were achieved, the growth rate was maintained at the set point of 0.1/h and there was no accumulation of glucose or acetate in the culture (Fig. 1). This indicates that the $Y_{\text{biomass/glucose}}$ coefficient had been accurately estimated. As this strategy allows the growth rate to be controlled at a low level it suggests that supplementation with pure oxygen would achieve cell densities compar-

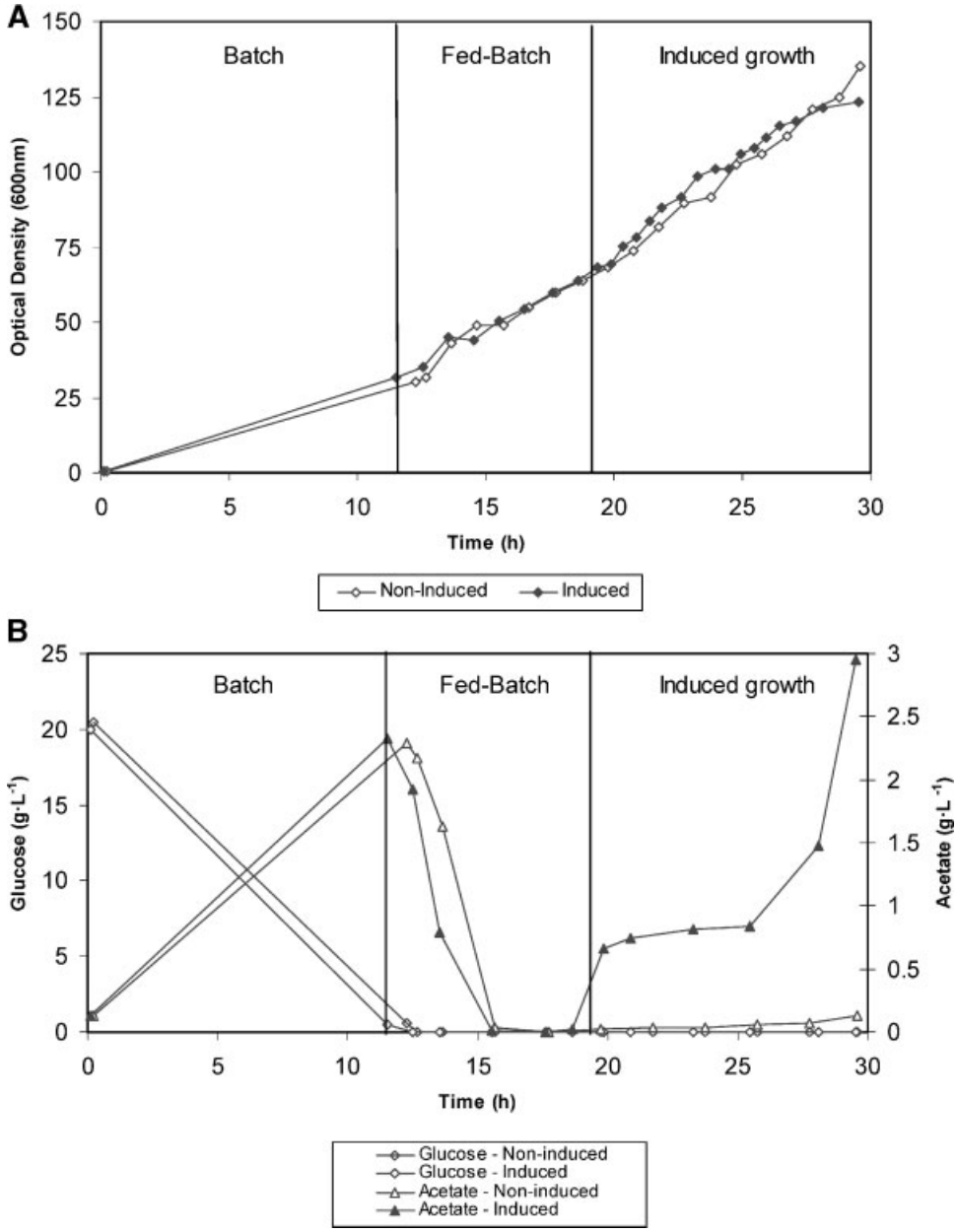

Figure 1. Fed-batch growth profiles of *E. coli* DH1*lacdapD*(pORT1aL*fuc*). **A**: Optical density (600 nm) measurements of induced (FB-7L-I) and non-induced (FB-7L-NI) fed-batch fermentation. **B**: Profile of glucose (g/L) and acetate (g/L) concentrations from induced and non-induced fed-batch fermentation. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table I. Description of the growth phases during the fed-batch cultivations of DH1*lacdapD*(pORT1aL*fuc*), FB-7L-NI and FB-7L-I.

Cultivation	Duration (h)	Induction (h)	Final volume (L)	Final biomass concentration (g DCW/L)	Culture phase	Period (h)	μ (per h)	$Y_{X/S}$ (g DCW/g glucose)
FB-7l-NI	29.5	N/A	4.78	51.4				
					Batch	0–12	—	—
					Fed-batch	12–29.5	0.095	0.386
FB-7l-I	29.5	19.0	4.71	46.7				
					Batch	0–12	—	—
					Fed-batch			
					Before ind.	12–19	0.107	0.479
					After ind.	19–25	0.099	0.393
						25–28	0.069	0.285
						28–29.5	0.036	0.169

able to those reported for similar feeding strategies (Horn et al., 1996; Korz et al., 1995), around 100 g DCW/L.

The percentage of Fuc-1-PA as intracellular protein and the specific activity of Fuc-1-PA (AU/g DCW) from the induced fermentation are summarised, respectively, in Figures 2 and 3B. The results show that DH1*lacdapD*(pORTaL*fuc*) allows well regulated expression of Fuc-1-PA. A constant percentage of intracellular Fuc-1-PA (around 7% of total intracellular protein) was maintained under non-induced conditions. Conversely, over expression was soon detected after temperature induction and final Fuc-1-PA accumulation approached 28% of total intracellular protein, corresponding to a specific concentration of 845 AU/g DCW.

The effects of metabolic burden associated with Fuc-1-PA over expression and growth at 42°C on the host were not observed until 6 h after induction. During these initial 6 h the growth rate was maintained at 0.1/h. There was no excessive acetate accumulation despite the temperature increase, which tends to increase host metabolic activity, growth rate (Rinas, 1996) and activate the well-described heat shock response (Bahl et al., 1987; Dong et al., 1995; Ito et al., 1986; Parsell and Sauer, 1989). After the initial 6 h post-induction, growth progressively slowed and acetic acid began to accumulate (Fig. 1), but did not reach inhibitory concentrations, over 5 g/L (Riesenberg et al., 1991). Growth stopped completely approximately 9 h after induction. A final biomass concentration of 46 g DCW/L was achieved. The growth profile and accumulation of acetate during the final 3 h of the induced culture was significantly different from the non-induced culture despite maintaining optimal dissolved oxygen conditions until the end of the growth of both cultures. It is, therefore, probable that the cessation of growth of the induced culture was a consequence of host metabolic burden associated with both Fuc-1-PA over expression and heat shock response following the temperature increase.

The production of active Fuc-1-PA in relation to growth is shown in Figure 3. The amount of active Fuc-1-PA produced (total AU Fuc-1-PA) increased in proportion to the biomass (total g DCW). When growth began to slow down and eventually stop (28–30 h), production of active enzyme also ceased. As the production of active Fuc-1-PA appears so closely linked to cellular viability, it is likely that production of Fuc-I-PA stopped due to a decrease in viability rather than plasmid instability. The maximum specific concentration of Fuc-1-PA activity, 845 AU/g DCW, was achieved 6 h post-induction.

To estimate plasmid stability, replica plating was carried out at regular intervals during the fermentation. Dilutions of 10^{-7} and 10^{-8} were made on culture samples and then plated onto LB agar with and without 1 mM IPTG. The plates were incubated for 24 h at 37°C. The number of colonies were counted to calculate any segregational plasmid loss. Two examples of the viable cell counts from replica plating, before and after induction, are shown in Figure 4. No segregational plasmid loss was detected throughout the entire

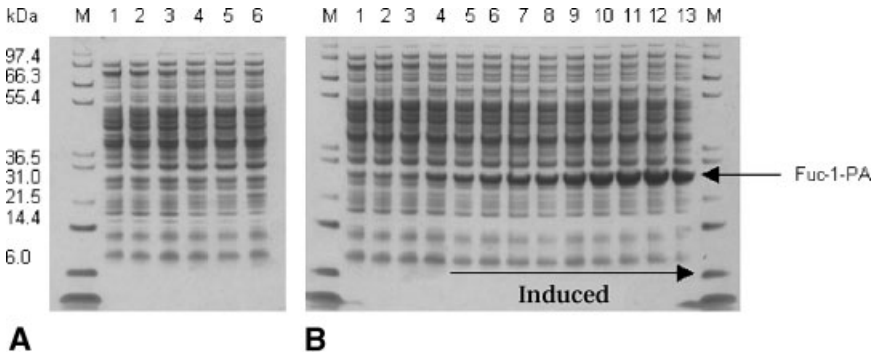

Figure 2. SDS–PAGE analysis of whole cell lysates (preparations of similar biomass content) from samples taken during fed-batch cultivations **(A)** FB-7L-NI (not induced control cultivation). **B**: FB-7L-I (induced cultivation). M, molecular weight standards. A: **1**-14.67 h, **2**-16.67 h, **3**-19.75 h, **4**-22.75 h, **5**-25.75 h, **6**-28.45 h, (B) **1**-16.53 h, **2**-18.62 h, **3**-19.37 h, **4**-19.87 h, **5**-20.37 h, **6**-20.87 h, **7**-21.37 h, **8**-21.87 h, **9**-22.37 h, **10**-23.95 h, **11**-25.90 h, **12**-26.45 h, **13**-28.12 h.

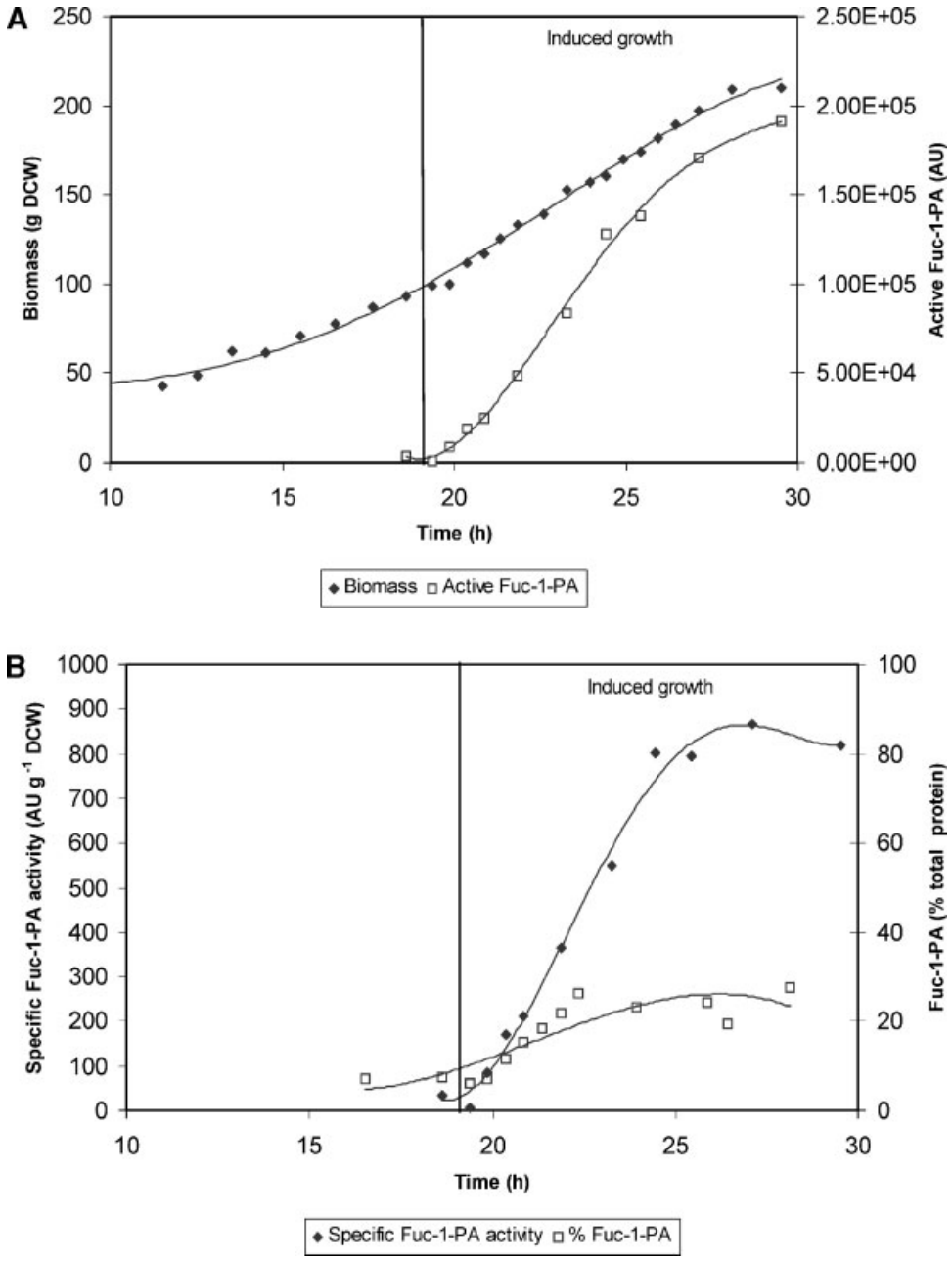

Figure 3. Fuc-1-PA production during fed-batch fermentation of DH1*lacdapD*(pORT1aL*fuc*), FB-7l-I. **A**: Profile of total biomass (g DCW) and total Fuc-1-PA activity (AU). **B**: Profile of specific Fuc-1-PA concentration (AU/g DCW) and Fuc-1-PA as percentage total intracellular protein. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

fermentation. The post-induced replica plate cell numbers in Figure 4 highlight that the plasmid was maintained throughout 4.7 h of the increased metabolic burden related to the higher growth temperature and expression of the protein.

DISCUSSION

One of the most important factors influencing the productivity of recombinant protein expression is that of plasmid stability. Plasmid-free cells can quickly out grow plasmid-containing cells and reduce volumetric protein yields. It is, therefore, important to maintain plasmid stability during the cultivation of recombinant *E. coli*. The majority of plasmid maintenance systems rely on the expression of plasmid-borne selectable marker genes. These types of selection systems have several disadvantages. Expression of a selectable marker from a plasmid can increase the plasmid-associated metabolic burden. Often the selectable marker confers antibiotic resistance; maintenance then takes place by culturing in the presence of the antibiotic. The use of antibiotics has become undesirable in several areas of biotechnology due to economic, environmental and regulatory reasons. This is particularly true when the product is destined for clinical use where the antibiotic will have to be removed

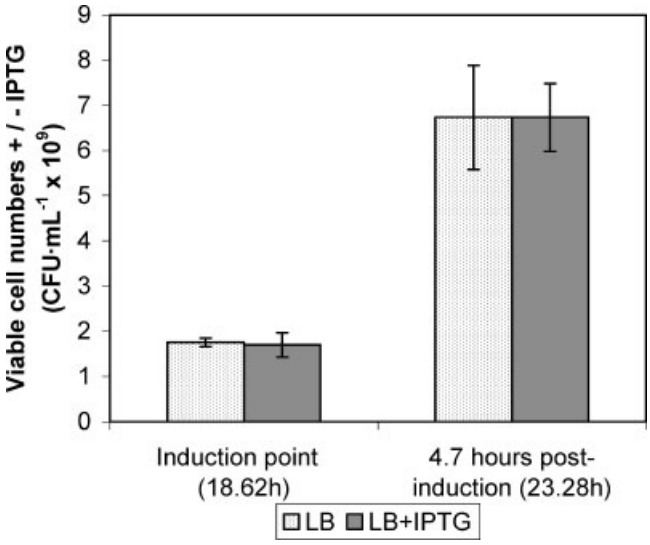

Figure 4. Viable cell numbers on LB agar with and without 1 mM IPTG to estimate plasmid maintenance from the high-cell density fermentation of DH1*lacdapD*(pORT1aL*fuc*). LB plate counts represent only plasmid-containing cells, as the cells require repressor titration by the operator on the plasmid to prevent cell lysis. LB+IPTG plate counts also represent plasmid-free cells, as IPTG induces expression of DAP in the absence of operator–repressor titration.

during purification. In addition the final product will require testing for residual antibiotic.

During the development of fermentation strategies it is often necessary to increase the yield of a recombinant protein. High cell density cultivation is a well-reported method for increasing the volumetric yield of recombinant proteins (Yee and Blanch, 1992). Generally the culture is grown in a defined medium and a fed-batch strategy is used to maintain the growth rate at a low level; decreasing the growth rate helps to ensure the oxygen consumption rate of the culture is minimal. In this way, it is possible to achieve very high cell densities (Horn et al., 1996; Korz et al., 1995). The stress placed upon the cells during high cell density cultivation (Yoon et al., 2003) and metabolic burden imposed by expression of a recombinant protein (Bhattacharya and Dubey, 1995) can have a significant effect on the growth and productivity of the fermentation. It is possible that these stresses placed on to the culture may affect plasmid maintenance.

The quantity of Fuc-1-PA produced using the ORT system was very similar to that of a traditional plasmid maintenance system (ampicillin selection) used in previous work (Durany et al., 2005). We have also established that the process can be carried out at 50 L scale without segregational plasmid loss, despite the increase in generation number. We have previously shown that even the low copy number plasmids commonly used for recombinant protein expression can be selected by ORT (Cranenburgh et al., 2004), but in these experiments we have chosen a high copy number expression plasmid to further increase the metabolic burden. With the temperature increase necessary for λ expression, even plasmids that possess the low copy number (pBR322-type) pMB1 origin of replication will undergo a significant increase in copy number (Lin-Chao et al., 1992), thus increasing the likelihood of plasmid loss in traditional plasmid maintenance systems.

During these experiments, it was evident that the λ promoter enabled well-regulated control of Fuc-1-PA expression. Minimal amounts of Fuc-1-PA expression were detected before induction and following a temperature shift from 30 to 42°C Fuc-1-PA accumulated to around 30% of total intracellular protein. Despite allowing such well-regulated expression of recombinant proteins, the λ system has several disadvantages associated with the temperature shift to 42°C. Increasing the temperature of the culture decreases the solubility of oxygen in the medium and increases the specific oxygen consumption rate. The demand for oxygen following induction can be significant and may limit the biomass concentration at which the culture can be induced due to the transfer capacity of the vessel. Growth of *E. coli* at 42°C will undoubtedly activate the heat shock response. The stress caused during growth at an elevated temperature was apparent during this study. Following induction, growth progressively slowed and acetate began to accumulate. Despite these potential problems results have shown that a well designed growth strategy, where low growth rates are maintained, can mitigate the undesirable effects of λ expression.

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