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ORIGINAL PAPER

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A recombinant vaccine against hydatidosis: production of the antigen in *Escherichia coli*

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Abstract A commercial process was developed for producing a recombinant vaccine against hydatidosis in farm animals. The vaccine antigen consisting of a surface protein of the oncospheres of the hydatid worm (*Echinococcus granulosus*), was produced as inclusion bodies in *Escherichia coli*. Fed-batch cultures of *E. coli* using Terrific broth in stirred bioreactors at 37°C, pH 7.0, and a dissolved oxygen level of 30% of air saturation produced the highest volumetric concentrations of the final solubilized antigen. An exponential feeding strategy proved distinctly superior to feeding based on pH-stat and DO-stat methods. The plasmid coding for the antigen was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at 4 h after initiation of the culture. The minimum IPTG concentration for full induction was 0.1 mM.

Keywords *Echinococcus granulosus* · *Escherichia coli* · Hydatid · Hydatidosis · Recombinant vaccine · Veterinary vaccine

Introduction

Escherichia coli is commonly used to produce recombinant proteins because it can be grown to high densities on inexpensive media and its genetics are well understood. Unfortunately, eukaryotic proteins expressed in *E. coli* generally form insoluble inactive aggregates or inclusion bodies (Dempster et al. 1996; Chisti 1998; Makrides 1996; Hoffmann et al. 2001). Recovery of the biologically active protein from the inclusion bodies requires solubilization and refolding of the protein into its native form. The processing involved in recovering the bioactivity from inclusion bodies adds to cost of production of the target molecule (Lilie et al. 1998; Middelberg 2002). Despite this, many products are produced as inclusion bodies because of an absence of other practicable alternatives (Dempster et al. 1996; Hoffmann et al. 2001). Recombinant *E. coli* can be grown to high densities in common media such as Luria broth (LB) (Curless et al. 1990; Li et al. 1999), the synthetic M9 minimal medium (Lim et al. 2000), Terrific broth (TB) (Zanette et al. 1998; Lim et al. 2000) and Super broth (SB) (Madurawe et al. 2000). The optimal growth temperature and pH for wildtype *E. coli* are 37°C pH between 6.4 and 7.2 (Holt et al. 1994). The bacterium is generally grown under aerobic conditions because anaerobic growth provides less energy for metabolic processes such as protein synthesis (Xu et al. 1999). To ensure that oxygen supply does not become limiting, the fed-batch operation is used extensively in cultures (Curless et al. 1990; Oh et al. 1998; Castan and Enfors 2000). This improves biomass and recombinant protein yield relative to batch culture. Fed-batch operation overcomes possible limitations due to a high concentration of substrate and enables growth to be prolonged in comparison with traditional batch fermentations.

Synthesis of recombinant proteins places a substantial metabolic burden on producing cells. To prevent this burden from compromising growth, the biomass growth and rDNA protein production phases are separated by using transcription regulators to switch on protein expression after most of the growth has occurred. Common regulators used in *E. coli* include the *lac*,*trp*, *ara*BAD and *tac* promoters (Makrides 1996). The *lac* promoter used in this work is controlled by the use of isopropyl-β-D-thiogalactopyranoside (IPTG) (Warnes et al. 1991; Hellmuth et al. 1994) or lactose (Kweon et al. 2001). Recombinant protein production is generally induced in the late exponential growth phase.

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Induction in the stationary phase reduces culture viability and can lead to production of proteases that can breakdown the desired recombinant protein (Chisti 1998; Corchero et al. 2001). Induction too early can unnecessarily slow the doubling time of bacterial cells.

This work investigated the production of a vaccine that protects livestock from hydatid disease, or hydatidosis. Hydatidosis is caused by the cysts of the tapeworm *Echinococcus granulosus*. The adult worms inhabit the small intestine of dogs. The eggs from the tapeworms are excreted by dogs and are ingested by herbivore intermediate hosts such as cattle and sheep where they develop into cysts. Humans become accidentally infected. Hydatidosis among humans occurs worldwide. The cysts of *E. granulosus* can grow to contain several liters of fluid and interfere with the normal functioning of organs such as the liver and lungs. The hydatid vaccine *Eg95* is a protein sequence derived from the *E. granulosus* oncospheres and has been shown to provide immunity to natural challenge by *E. granulosus* eggs in sheep and cattle (Heath et al. 2003). The *Eg95* vaccine is produced in recombinant *E. coli*.

Materials and methods

Strain and plasmid

The host strain used was *E. coli* BB4LE392.23 (Strategene, La Jolla, CA, USA), [F' *lacI^q* ZΔM15, proAB, Tn10 (tet^r)]. The recombinant plasmid pGEX used for *Eg95* expression was constructed with the *E. granulosus* DNA fused with a glutathione S-transferase gene of *Schistosoma japonicum* and an ampicillin resistance gene (Lightowlers et al. 1996). Expression was under the control of the *lacI^q* allele where expression was repressed in the absence of IPTG. Production seeds were stored in soybean–peptone–yeast extract broth (SOB) with 20% glycerol at −80°C in the presence of 0.012% ampicillin.

Media and chemicals

Reagents and chemicals were purchased from Sigma (St Louis, MO, USA), BioRad (Hercules, CA, USA), Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). Unless stated otherwise, fermentations used TB that contained per liter: 24 g yeast extract (Merck), 12 g soy-peptone (Merck), 4.8 g potassium di-hydrogen orthophosphate, 2.2 g di-potassium hydrogen orthophosphate and 5 g glycerol. The feed medium of the fed-batch fermentations contained per liter: 315 g glycerol and 315 g yeast extract.

Inoculum development

A frozen culture (1 ml) of recombinant cells was thawed at room temperature and added to 50 ml of TB supplemented with 0.012% ampicillin, in a 250 ml baffled shake flask. The flask was incubated for 16 h on a rotary shaker at 180 rpm, 37°C. For bioreactor culture work, 1 ml of this production seed was added to 150 ml of enhanced TB (supplemented with 0.012% ampicillin) in a 2 l baffled shake flask incubated for 24 h at 180 rpm, 37°C.

Media trials

Media trials were performed to evaluate the *Eg95* recombinant protein production on several media that are commonly used for culturing *E. coli*. Duplicate cultures were carried out in five different media: Luria broth (LB), Terrific broth (TB), Super broth (SB), soybean–peptone–yeast extract broth (SOB), and M9 minimal medium. All the media were formulated as specified in the *Handbook of Microbiological Media* (Atlas 1997). Inoculum (1 ml) was added to 50 ml of each of the five media in separate 250 ml shake flasks incubated at 180 rpm and 37°C. Samples were taken every hour. After 4 h, cultures were induced with 0.1 mM IPTG. Total protein, recombinant protein and final cell density were measured after 8 h.

Inducer concentration trials

Shake flask cultures were used to assess the effect of various concentrations of IPTG on the induction of recombinant protein synthesis. Overnight seed (1 ml) was added to twelve 250 ml shake flasks containing 50 ml of TB and 0.012% ampicillin. Duplicate cultures were induced 4 h after inoculation, with 0.0001, 0.001, 0.01, 0.1, 0.5 and 1 mM concentrations of IPTG. All cultures were incubated at 180 rpm and 37°C. Total protein, recombinant protein and final cell density were measured after 8 h.

Induction time trials

Shake flasks were used to test the effect of induction time on the production of *Eg95*. Overnight seed (1 ml) was added to eight 250 ml shake flasks containing 50 ml of TB and 0.012% ampicillin. Duplicate cultures were induced at 0, 2, 4 and 6 h after inoculation, with 0.1 mM of IPTG. All cultures were incubated at 180 rpm and 37°C. Total protein, recombinant protein and final cell density were measured after 8 h.

Fermenter trials

Bioreactor cultures were performed in 3.3 l roundbottomed stirred fermenter (BioFlo 3000, New Brunswick Scientific, Edison, NJ, USA) of 130 mm vessel diameter. The fermenter was fitted with pH and dissolved oxygen sensors (Mettler Toledo, OH, USA). Temperature was

controlled via a water-filled stainless steel base. Agitation was provided by two centrally mounted six-bladed Rushton turbines spaced 80 mm apart with the lowermost impeller positioned 70 mm above the base of the vessel. Aeration occurred through a perforated pipe sparger ring. Dissolved oxygen (DO) was controlled at 30% of air saturation by using a sequential cascade of agitation between 50 and 800 rpm and aeration between 2 and 10 l/min with pure oxygen blended into the sparged air at high-cell densities. The pH was controlled at 7.0 using 10% phosphoric acid and 10 M sodium hydroxide. Antifoam 289 (Sigma, St Louis, MO, USA) was added automatically to control the foaming. The latter was sensed using a conductivity probe mounted 5 cm above the culture level.

The feed medium contained (per liter) 315 g yeast extract and 315 g glycerol. The medium was adjusted to pH 7.0 using 2 M HCl. Three different feeding strategies were used: a DO-stat, pH stat and exponential feeding. The DO-stat and pH-stat were controlled by the automated BioCommand control software (New Brunswick Scientific). Feed rate was set initially at 15 ml/h, increasing by 0.15 ml/h for every minute after a preset period. For exponential feeding, the rate of feeding was calculated according to following equation (Ejiofor et al. 1996; Lee et al. 1997):

$$F = \frac{\mu VX}{(S_F - S)Y_{x/s}} e^{\mu t}. \quad (1)$$

The desired specific growth rate μ in Eq. 1 was set to 0.15 h^{-1}. In Eq. 1, t is time (h), F is the flow rate of feed at time t, V is the reactor volume (l) at time t, S_F is the substrate concentration in feed (g/l), S is the substrate concentration in the culture broth at time t (g/l), X is the cell concentration at time t (g cell dry weight/l), and $Y_{x/s}$ is the biomass yield coefficient on glucose (g dry cell weight/g).

Unless otherwise stated, the initial volume of the medium in the vessel was 1.4 l. Inoculum (100 ml) consisted of a culture that had been grown for 16 h in a 2 l shake flask (37°C, 180 rpm) to an optical density (595 nm) of approximately 1.5. The incubation temperature was 37°C. Fermentations were induced with 0.1 mM IPTG after 4 h and continued until two sequential reductions in cell density were measured to indicate the onset of the decline phase.

Protein recovery

For shake flask cultures, the intracellular proteins were extracted from the cells with the B-PER™ reagent (Pierce Chemical Company, Rockford, IL, USA). Thus, 40 ml of bacterial broth was centrifuged (3,000*g*, 10 min), the supernatant was decanted off, the pellet was resuspended in 2.5 ml B-PER and agitated by repeatedly drawing through a 1 ml pipette. The sample was then incubated on an orbital shaker for 15 min at room temperature. The sample was then centrifuged (27,000*g*, 15 min) and the supernatant was decanted off. The pelleted inclusion bodies were resuspended in 2.5 ml B-PER with 100 µl of 10 mg/ml lysozyme and incubated for 5 min at room temperature on a rotating tray. 15 ml of 1:20 (vol/vol) solution of B-PER in distilled water was added and the sample was centrifuged at 27,000*g*. The supernatant was decanted off. The pellet was resuspended in 20 ml of 1:20 B-PER and centrifuged at 27,000*g*. The pellet was further washed twice before the extracted inclusion bodies were resuspended in 2 ml of solubilizing buffer. The latter contained (per liter): 484.8 g urea, 11.25 g glycine buffer, 0.37 g EDTA and had been adjusted to pH 9.0 with 10 M NaOH. Dithiothreitol (0.77 g/l) was added just prior to use.

Because of the large volume that had to be processed, recovery of the recombinant proteins from the bioreactor broth followed a slightly different procedure than used for samples from shake flasks. Thus, the broth was centrifuged at 3,500*g* for 30 min. The supernatant was discarded and pellet was suspended in 1 l of wash buffer (per liter: 181.8 g urea, 11.25 g glycine, 0.37 g EDTA, and 0.77 g dithiothreitol). Suspended cells were disrupted in a high-pressure homogeniser (APV-2000 single stage; APV Co., London, UK) operated at 1,000 bar. Disrupted cells were centrifuged at 10,000*g* (30 min), the supernatant was discarded and the pellet was resuspended in 1 l of wash buffer using an ultra-trurrex (Drive T25, IKA) mixer. The centrifugation and resuspension process was further repeated twice. The pelleted inclusion bodies were then suspended in solubilizing buffer (as above) and placed on a shaking tray for 1 h. The slurry was now centrifuged (10,000*g*, 1 h) to remove any insoluble material. The supernatant containing solubilized protein was filtered through a 0.22 µm Durapore tangential flow membrane unit (Millipore, Billerica, MA, USA).

Analytical methods

Culture growth was monitored by measuring optical density at 595 nm using a Genesis 2C spectrophotometer (Thermo Electron Corp, New York, NY, USA). The dry cell weight (DCW, g/l) was estimated from a calibration curve that correlated experimentally measured dry weight to spectrophotometric measurements of optical density.

Total protein measurements used the BioRad protein assay which is based on the Bradford method (Bradford 1976). Protein standards were made using bovine serum albumin (Sigma, St Louis, MO, USA) in distilled water. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was carried out on 0.75 mm thick 15% acrylamide separating gel and 4% slacking gel, using a Mini-Protean II electrophoresis unit (BioRad, Hercules, CA, USA) (Laemmli 1970). Protein bands were visualized with Coomassie brilliant blue stain. Low-molecular weight marker proteins were used as standards (Amersham Biosciences, Uppsala, Sweden).

The quantity of Coomassie stained recombinant protein was evaluated densitometrically from SDS-PAGE gels using an GS-800 imaging densitometer (BioRad, Hercules, CA, USA) and Quantity One image quantification software (BioRad).

For immunological confirmation, the *Eg95* proteins were transferred electrophoretically to a mixed ester nitrocellulose membrane (Hybond-C; Amersham Biosciences, Uppsala, Sweden) in alkaline transfer buffer (2% v/v methanol, 25 mM Tris HCl, 190 mM glycine) using the Mini Trans-blot (BioRad). The filter membrane was then incubated in the blocking solution (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% v/v Tween−20, 5% w/v non-fat milk powder) for 1 h at 37°C. The filters were washed in TBS buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% v/v Tween 20) and then incubated overnight at room temperature in sheep antibody raised against *Eg95* (kindly provided by Dr. D. Heath, Wallaceville Animal Research Center, AgResearch, Upper Hutt, New Zealand) diluted 1/200 in blocking solution. Any unbound primary antibody was removed by washing in TBS buffer. The membrane filter was then incubated for 6 h at room temperature in donkey anti-sheep IgG (Cappel) conjugated to horse radish peroxidase (HRP) diluted 1/1000 in the blocking solution. Unbound secondary antibody was removed by washing in TBS buffer. The blot was developed by incubating in developing solution (5% w/v *o*-phenylenediamine in methanol).

Results and discussion

Media trials

The growth and protein production characteristics of the cells cultured on various media are shown in Table 1. The final cell concentration depended on the medium used. Super broth (SB) achieved the highest final cell density, but TB produced 28% more inclusion body and 11% more *Eg95* on volumetric basis. SOB and SB produced similar levels of inclusion body protein and *Eg95* titres, but because of a much higher final cell density SB had a lower cell-specific *Eg95* titre. Luria broth (LB) produced the lowest final cell density and recombinant protein titre compared to the other complex media tested. The only chemically defined medium used, i.e. the M9 minimal medium, performed poorly achieving a cell density of only 0.371 g DCW/L after 10 h. This is understandable in view of the low level of glucose in M9. No measurable titres of *Eg95* were detected and no inclusion body pellet was recovered. Chemically defined media are generally known to produce slower growth and protein titres than complex media (Zanette et al. 1998). Nonetheless, use of chemically defined media in producing recombinant proteins is a common practice (Lim and Jung 1998; Cserjan-Puschmann et al. 1999; Zhang and Greasham 1999; Kweon et al. 2001) because these media attain more consistent titres, allow easier process control and monitoring, and simplify downstream recovery of the target protein. In the present work, use of complex media is justified, as the final product will be a relatively crude vaccine for nonhuman veterinary applications.

Table 1 Production characteristics of *E. coli* expressing *Eg95* grown on various media

Media[a]	Production characteristics at 8 h			
	Final cell density (DCW g/l)	Inclusion body protein (mg/ml)	Volumetric *Eg95* production (mg/ml)	Specific *Eg95* production (mg/g DCW)
TB	1.123	0.218	0.059	52.6
SB	1.184	0.170	0.053	44.8
SOB	1.078	0.164	0.054	50.1
LB	1.014	0.094	0.031	30.6
M9	0.371	0.007	–[b]	–[b]

[a] Terrific broth (*TB*), Super broth (*SB*), soybean–peptone–yeast extract broth (*SOB*), Luria broth (*LB*), minimal medium (*M9*)
[b] No inclusion body pellet was recovered

The high-cell densities attained (Table 1) with SB and TB are likely explained by these media being rich in yeast extract and phosphate salts compared to the other media used. Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during synthesis of recombinant protein (Lim et al. 2000). Similarly, a high concentration of phosphate is known to be important for attaining high-cell densities of *E. coli*, as phosphate can easily become a limiting nutrient when provided in low doses (Korz et al. 1995). In addition to providing a source of phosphate the phosphate salts in the media provided a buffering capacity to prevent pH fluctuations that can adversely affect normal metabolic activity. The content of *Eg95* in the inclusion body fraction varied little with the medium. All samples contained between 27 and 54% recombinant protein, except for M9 that did not produce inclusion bodies.

As shown in Fig. 1, the pH of the shake flask cultures varied with time and the nature of this trend depended on the medium used. As expected, the unbuffered media such as SOB and LB experienced the most substantial fluctuations in pH. In both these media, the pH drifted outside the optimal recommended pH range for *E. coli* growth. Indeed the two complex media that resisted the pH drift better (i.e. TB and SB; Fig. 1) also yielded higher final cell densities and total inclusion body protein (Table 1). The characteristic initial decline in pH followed by a rise occurred in all complex media (i.e. SB, LB, SOB and TB). This phenomena is known to be caused by the way the cells consume nitrogen sources (Chisti 1999a). During the first 4 to 6 h the pH decreased as cells consumed the free ammonium in the culture media. Once most of the free ammonium had been consumed further nitrogen requirements for growth were met by the decomposition of proteins in the media as indicated by the increase in pH (Chisti 1999a).

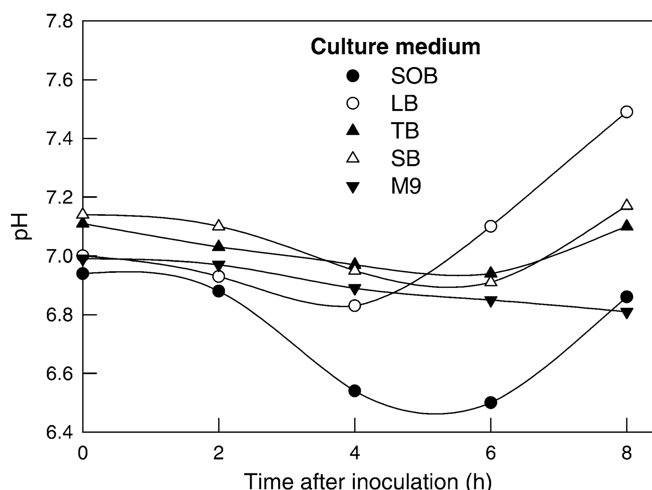

Fig. 1 Broth pH variation in shake flask cultures in various media: minimal medium (*M9*), Luria broth (*LB*), Terrific broth (*TB*), soybean–peptone–yeast extract broth (*SOB*), Super broth (*SB*)

(Decomposition of proteins releases ammonia that takes up a proton to become NH_4^+, causing an increase in pH.) In view of these results, controlled pH cultures at a pH of about 7 are preferable to uncontrolled cultures, for producing high-biomass densities and titres of *Eg95*.

As shown in Table 1, the highest specific titres of *Eg95* were attained with the TB and SOB media; however, the TB medium was superior to SOB because it provided significantly higher volumetric titres of *Eg95* (Table 1). The best growth medium was SB and it achieved a final cell density that was slightly more than attained with the next best TB medium. Unfortunately, the biomass grown in SB had much lower levels of *Eg95* than the TB grown biomass. Apparently, the higher growth rate in SB led to a reduced availability of metabolic resources for synthesis of recombinant protein, even though attempts were made to keep the cell growth and protein production phases separate by induction with IPTG.

Surprisingly, the LB medium that is commonly used in expression of recombinant proteins in *E. coli* (Zanette et al. 1998; Madurawe et al. 2000), produced the lowest final cell density and recombination protein titre. The relative poor performance of LB compared to complex media such as TB and SB has been commented on before for other recombinant proteins (Zanette et al. 1998; Madurawe et al. 2000) and is said to be due to lower amounts of readily accessible carbon and nitrogen in LB (Zanette et al. 1998). Because TB produced the highest specific titre of *Eg95* and simultaneously yielded a high-final biomass density (Table 1), this was the medium used in all subsequent studies.

Inducer concentration trials

The IPTG inducer is expensive. Considering this, the minimum concentration of IPTG required to fully induce the *lac* promoter was identified in shake flask cultures. The inducer concentration trials were conducted in TB as it had been earlier identified as the optimal production medium. Figure 2 shows that the expression of *Eg95* increased with increasing IPTG concentration at induction (i.e. 4 h after inoculation) until a concentration of 0.1 mM. The specific titre of *Eg95* was doubled by raising the inducer concentration from 0.001 mM to 0.1 mM. At a concentration of 0.1 mM the *lac* promoter appeared to be fully induced. In studies with other proteins, IPTG concentrations of between 0.1 mM and 2 mM have been used to induce the *lac* promoter (Yee and Blanch 1993; Bhattacharya and Dubey 1997; Li et al. 1999; Madurawe et al. 2000).

The concentration of IPTG required for complete induction is known to vary widely with clones. This was well demonstrated by Madurawe et al. (2000) who produced 0.01 g/l of decorin-binding lipoprotein in *E. coli* using 0.1 mM of IPTG for induction. In contrast, Robbens et al. (1995) required 2 mM IPTG to produce 0.016 g/l of recombinant murine interleukin-2 also in

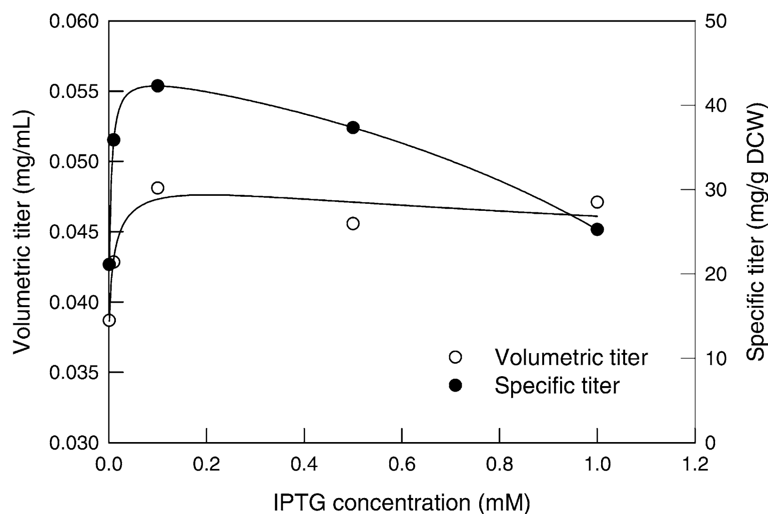

Fig. 2 Specific and volumetric titres at 8 h, of *Eg95* in Terrific broth after induction with various concentrations of IPTG

E. coli. In the present study, the volumetric titre of *Eg95* declined as the concentration of IPTG increased much above 0.1 mM (Fig. 2). This was apparently because the presence of excessive IPTG reduced the final cell density as a consequence of the growth inhibition by the IPTG (data not shown). These results concur with Yee and Blanch (1993) who observed a reduced growth rate in *E. coli* when the IPTG concentration exceeded 1 mM, but associated this to the toxic effect of the expressed recombinant protein.

The *lac* promoter used has been previously reported to be a weak promoter with moderate levels of basal expression occurring under non-induced conditions (Saluta and Bell 1998). Basal expression in this work was found to be 40% of the maximum expression obtained under purposeful induction. This high level of basal expression may be a combined result of partial induction by lactose present in the medium. Terrific broth uses tryptone, a digest of milk that contains lactose. Lactose has been used as a cheap alternative to IPTG to induce the *lac*, but lactose has a much lower induction potential (Kweon et al. 2001) and is consumed whereas IPTG is not consumed. Basal expression is generally undesirable as it reduces the energy available for growth and, if the recombinant protein is toxic to the cells, may lead to growth inhibition or death of the culture. Basal expression can be reduced by replacing the glycerol in the TB with glucose (Guzman et al. 1995). Catabolism of glucose depletes the intracellular level of cAMP that is required by the *lac*B gene to prevent repression (Chang et al. 1998; Lim et al. 2000). Unlike glycerol, glucose is fermented to acetic acid and this can affect culture growth, for example by affecting the pH.

Induction time

Shake flask trials were used to test the effect of induction time on the production of *Eg95*. Cultures were induced with 0.1 mM IPTG. As shown in Fig. 3, induction at seeding severely limited culture growth and subsequent recombinant protein production (Fig. 4) likely as a result of metabolic stress. The expression of foreign genes in *E. coli* places considerable pressure on the cell's metabolic systems and limits the available energy for growth (Jeong and Lee 1999). The specific titre of *Eg95* increased as the uninduced period of growth was increased for up to 4 h (Fig. 4). The volumetric titre of *Eg95* was also greatest when induced 4 h after inoculation (Fig. 4) at which time the culture was at its highest specific growth rate (Fig. 3). This result is consistent with Lim and Jung (1998) who found that recombinant protein production in *E. coli* fermentations was proportional to the specific growth rate at induction as cell metabolic capacity is at its greatest during rapid growth (Lim and Jung 1998).

Induction late in the exponential growth phase (i.e. induction time >4 h) appeared to reduce the specific titre of *Eg95* (Fig. 4). Several studies report that induction late in exponential growth leads to higher volumetric recombinant protein production by increasing the final cell density (Yee and Blanch 1993; Zanette et al. 1998). Yee and Blanch (1993) found that induction late in exponential growth led to 50% more recombinant trypsin being produced; however, the specific cellular titre was decreased. Induction at >4 h when the specific growth rate had begun to decrease (Fig. 3) because of onset of stationary phase, reduced cell specific *Eg95* titres so much so that the volumetric titre was reduced (Fig. 4) despite a higher biomass concentration than at induction at 4 h (Fig. 3). In view of the data in Figs. 3 and 4, the optimal induction time was 4 h. Cellular responses to induction depend on a number of interacting factors including the host/vector system and properties of the expressed protein. Therefore, the timing of induction of new recombinants needs to be empirically determined for each new clone (Cserjan-Puschmann et al. 1999).

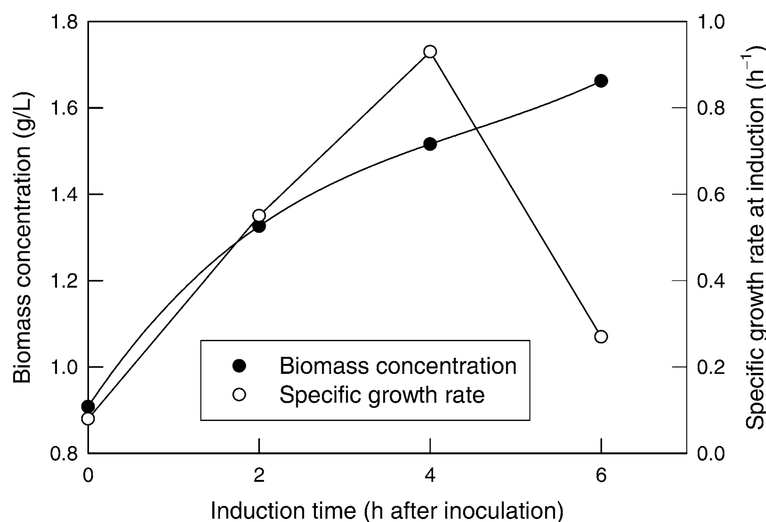

Fig. 3 Effects of induction time on final cell concentration (8 h, Terrific broth) and the specific growth rate at induction. The specific growth rate at 0 h was measured over the hour after inoculation

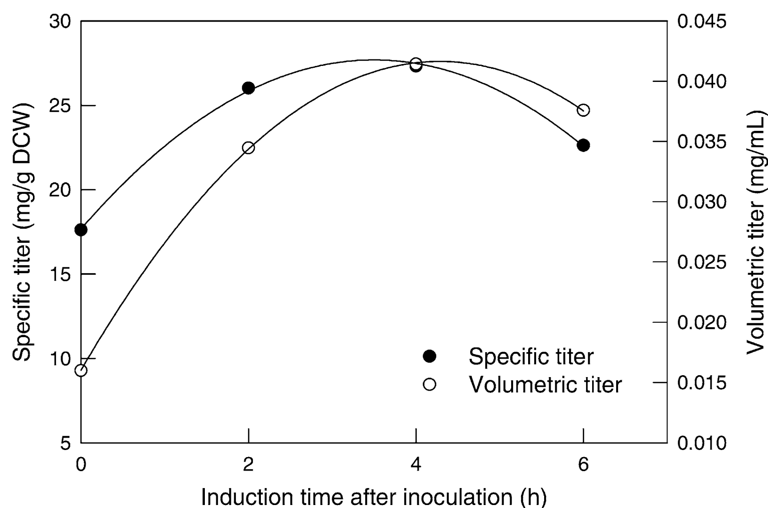

Fig. 4 Effects of induction time on volumetric and specific production of *Eg95* (8 h) in Terrific broth

Dissolved oxygen concentration

Previously *E. coli* fermentations have been controlled at dissolved oxygen (DO) concentrations between 20 and 70% of air saturation (Yee and Blanch 1993; Kleman and Strohl 1994; Korz et al. 1995; Lee et al. 1997; Cserjan-Puschmann et al. 1999). The effect of dissolved oxygen concentration on *Eg95* production in TB was tested in three DO-stat fermentations with DO values controlled at 30, 50 and 70% of air saturation. As shown in Fig. 5, an increase in the DO level increased the growth rate and reduced the final biomass concentration, but the maximum biomass levels were attained earlier in the fermentation. At a DO set-point of 30% the culture grew for 7 h longer before going into stationary growth and produced two-thirds more biomass than when the dissolved oxygen concentration was controlled at 70%. No previous references to increased biomass at lower DO were found; however, the reduction in cell density was likely a result of oxidative damage to cells. Elevated DO levels are known to cause oxidative stress in cells, leading to damage of nucleotides and oxidation of proteins (Konz et al. 1998). The natural environment of *E. coli* contains little dissolved oxygen.

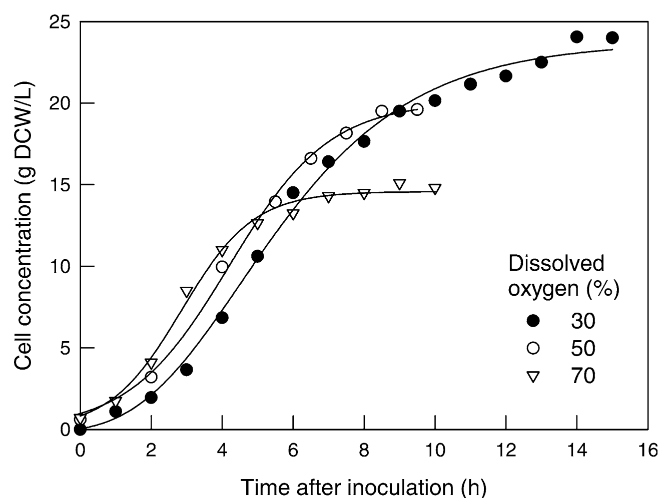

Fig. 5 Effects of dissolved oxygen concentration on biomass growth in Terrific broth batch fermentations

Table 2 shows that the biomass specific titre of *Eg95* increased about threefold when the DO set point was reduced from 70 to 50%, but was not further affected significantly by a reduction in the DO concentration to 30%. The highest concentration of the inclusion body protein and the volumetric titres of *Eg95* were attained at 30% DO level. In view of these results, the best production practice would be to maintain a DO level of between 30 and 50% to ensure that the biomass growth is rapid, the specific productivity is high and the volumetric production is not compromised.

Several studies have reported reduced expression of recombinant proteins in *E. coli* at high levels of dissolved oxygen (Landwall and Holme 1977; Doig et al. 2001). Doig et al. (2001) observed that the high-shear rates required for attaining an elevated level of dissolved oxygen were the real cause of suppressed production of recombinant cyclohexanone monooxygenase (CHMO) in *E. coli*. Maintaining a high-dissolved oxygen level in high-cell density cultures requires intense agitation and aeration that can damage fragile recombinant cells (Chisti and Moo-Young 1996; Chisti 1999b).

Feeding strategy

The use of fed-batch cultures has been shown to significantly increase the cell-density and specific protein production by overcoming inhibitory substrate concentrations encountered in batch culture (Chen et al. 1995; Castan and Enfors 2000). Various strategies exist for controlled feeding of fed-batch cultures. This work investigated the following feeding strategies: DO-stat, pH-stat and exponential feeding.

Table 2 Effect of dissolved oxygen concentration on recombinant *Eg95* protein production

Dissolved oxygen (%)	Inclusion bodytotal protein (mg/ml)	*Eg95* production (mg/ml)	Specific *Eg95* production (mg/g DCW)
30	3.07	0.72	30.0
50	2.70	0.67	34.2
70	0.95	0.16	10.8

A pH-stat feeding system monitors the pH of the fermentation for indications of acetic acid production (Yee and Blanch 1993; Jeong and Lee 1999). The feed rate is increased until the maximum growth rate is reached as indicated by a metabolic overflow causing production of acid and a consequent decrease in pH (Xu et al. 1999; Akesson et al. 2001). The DO-stat operation relies on the fact that specific oxygen uptake reaches a maximum at the maximum growth rate (Yee and Blanch 1993; Hellmuth et al. 1994; Oh et al. 1998; Akesson et al. 1999; Jeong and Lee 1999; Madurawe et al. 2000). Changes in oxygen uptake rate following a pulse of feed are used to determine whether the microorganism is at its maximum growth rate (Oh et al. 1998; Akesson et al. 2001). Exponential feeding makes use of an empirical model of growth, to regulate the feed rate (Yee and Blanch 1993; Ejiofor et al. 1996; Lee et al. 1997).

As shown in Fig. 6, irrespective of the feeding method used all the cultures actively grew for approximately 16 h; however, exponential feeding achieved a high-final cell density of 46.3 g DCW/l. The DO-stat and pH-stat both produced similar final cell densities that were only about half the biomass concentration obtained with exponential feeding. Although, the DO-stat and pH-stat feeding methods are simple and respond rapidly to changes in substrate demand, they rely on online measurements. In addition, DO-stat feeding is liable to control distortions caused by changes in the volumetric oxygen transfer coefficient (Akesson et al. 1999). In contrast to pH-stat and DO-stat methods, exponential feeding does not need any online measured inputs, but relies on substantial a priori knowledge of the culture growth kinetics.

As shown in Table 3, all three feed regimes attained similar specific titres of *Eg95*; however, because of the much greater biomass concentration obtained with exponential feeding, this feeding mode produced >2-fold more total *Eg95* than both the DO-stat and pH-stat modes. This result is consistent with Yee and Blanch (1993) who reviewed a number of feeding regimes and found that exponentially fed cultures achieved superior titres to those that used online feeding involving strategies such as DO-stat and pH-stat. Online feeding systems of course have the advantage that they are capable of adapting to changes in culture growth rate to prevent both starvation and overfeeding. However, online feeding can stress a culture because of continual fluctuations in the culture nutrient levels (Madurawe et al. 2000). Not surprisingly, the pH and DO-stat feedings produced similar final cell densities and titres of *Eg95* as both methods rely on similar environmental indicators of carbon depletion to maintain the culture on the brink of starvation.

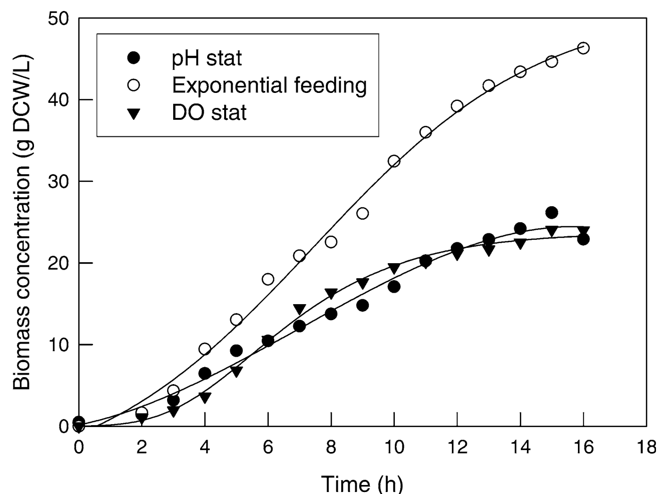

Fig. 6 Biomass growth profiles of fed-batch fermentations (Terrific broth): a comparison of DO-stat, pH-stat and exponential feeding

Using TB as the identified optimal medium in combination with exponential feeding and control of DO level at 30% of air saturation, the final titre of solubilized *Eg95* was increased 29-fold compared to the highest titre attained in shake flask batch cultures (Table 1). Reproducibility was confirmed with two fed-batch fermentations conducted under the above specified optimal conditions. In both cases the final *Eg95* titre was within 0.01 g/l of the result shown in Table 3. The hydatid vaccine is currently being transferred into a commercial production facility.

Table 3 Effect of feeding regimens on biomass, total protein and *Eg95* production

Feeding strategy	Final cell density (g DCW/l)	*Eg95* volumetric titre (mg/ml)	*Eg95* specific titre (mg/g DCW)
DO-stat	24.0	0.72	30.0
pH-stat	22.9	0.74	32.3
Exponential feeding	46.3	1.73	37.4

Concluding remarks

Inclusion body production of *Eg95* in *E. coli* was influenced by the medium, the feeding strategy, the dissolved oxygen concentration and induction time. *E. coli* grown on complex media rich in yeast extract and containing the phosphate buffer system was found to support good growth and produce more recombinant protein than the other media. Terrific broth was the best medium. Fed-batch cultures controlled by exponential feeding gave the highest *Eg95* volumetric titres at 37°C, pH 7.0, and the DO level maintained at 30% of air saturation. IPTG concentration of 0.1 mM was found to be sufficient to fully induce the *lac* promoter. Induction 4 h after inoculation produced the highest titre of *Eg95*.

Feeding strategies involving DO-stat and pH-stat methods are not recommended in view of their low *Eg95* productivities. Antigenically active hydatid protein could be recovered from inclusion bodies produced in recombinant *E. coli*, for use as an effective vaccine for protecting farm animals against hydatid infestations.

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