A Fermentation Strategy for Anti-MUC1 C595 Diabody Expression in Recombinant *Escherichia coli*

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Abstract The development of fermentation conditions for the production of C595 diabody fragment (dbFv) in *E. coli* HB2151 clone has been explored. Investigations were carried out to study the effect of carbon supplements over the expression period, the comparison of C595 dbfv production in synthetic and complex media, the influence of acetic acid upon antibody production, and comparison of one-stage and two-stage processes operated at batch or fed-batch modes in bioreactor. Yeast extract supplied during expression yielded more antibody fragment than any other carbon supply. The synthetic medium presented higher specific productivity (0.066 mg dbFv g^{-1} dry cell weight) when compared to the complex medium (0.044 mg dbFv g^{-1} DCW). The comparison of fermentation strategies demonstrated that (1) one-stage fed-batch fermentation performed higher C595 dbFv production than that operated in batch mode which was significantly affected by acetate concentration; (2) a two-stage batch operation could enhance C595 dbFv production. It was found that a concentration of 12.3 mg L^{-1} broth of C595 dbFv and a cell concentration of 10.8 g L^{-1} broth were achieved at the end of two-stage operation in 5-L fermentation.

Keywords: diabody fragment, recombinant *E. coli* fermentation, C595 dbFv, cell concentration, specific productivity

INTRODUCTION

MUC1 mucins are high molecular weight glycoproteins that are normally expressed on the apical surfaces of specialized glandular epithelia [1,2]. Their major physiological functions are as protective agents and biological lubricants. MUC1 mucins have been the subject of medical studies, are frequently elevated and/or altered in cancer and thus have potential uses as tumour markers. For example, in breast carcinomas, their expression is frequently up-regulated and they may be secreted into the blood circulation [3]. Determination of the levels of MUC1 antigen in the blood has been exploited as a measure of tumour burden and changing levels reflect the response to therapy [4,5]. The C595 monoclonal antibody has been proved to be a reagent of clinical utility and employed in immunoassays for the measurement of circulating mucin in breast cancer patients [6-8] and it also has been used *in vivo* diagnostic tests for the identification of malignant ovarian tumours by immunoscintigraphy [9].

Despite the success of C595 in cancer diagnosis, potential problems exist in its use as a therapeutic agent. These include immunogenicity due to its murine origin and poor tumour penetration characteristics because of its large molecular size (150 kDa) [3]. The former is of particular hindrance in therapies where multiple administrations may be required. These problems may be solved by engineering small antibody fragments. Small antibody fragments have high renal clearance and relatively weak antigen binding capacity because of their small size and monovalency [3]. This has led to the development of smaller C595 diabody molecules (molecular weight: 60 kDa), which are essentially scFv dimers [10]. The C595 diabody constructs possess an improved biological half-life and greater binding affinities [11]. The C595 diabody shows similar binding characteristics to the parental C595 monoclonal antibody and it can be expressed in the *E. coli* culture exploiting recombinant technology [3].

As compared to other expression system such as mammalian cell, the recombinant *E. coli* production required the simpler fermentation media used and shorter

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fermentation times and may present lower cost. The fermentation tactics are often necessarily generic as well, including the maintenance high growth rates and the achievement of high cell density whilst assuring high levels of product accumulation [12]. In the present study, investigations were made to explore the effect of carbon supplements over the expression period, the comparison of C595 dbfv production in synthetic and complex culture, the influence of acetic acid on anti-MUC1 C595 dbFv production in synthetic media, and comparison of batch and fed-batch strategies in one-stage and two-stage processes operated in 5-L scale bioreactor.

MATERIALS AND METHODS

Microorganism

E. coli HB2151 clone 8.3 containing plasmid pCANTAB 5E was kindly provided from Dr. M. Price (CRC laboratories, University of Nottingham).

Growth Medium

Synthetic medium (SM) (g L^{-1}): glucose, 15; Na_2SO_4, 2; $(NH_4)_2SO_4$, 2.7; NH_4Cl, 0.5; K_2HPO_4, 14.6; NaH_2PO_4, 3.5; $(NH_4)_2$-H-citrate, 1.0; thiamine, 0.01; 1 M $MgSO_4$, 2 mL L^{-1}; trace element solution, 3 mL L^{-1}. The trace element solution was composed of (g L^{-1}): $CaCl_2 \cdot H_2O$, 0.74; $ZnSO_4 \cdot 7H_2O$, 0.18; $MnSO4 \cdot H_2O$, 0.1; Na_2 EDTA, 20.1; $FeCl_3$, 9.7; $CuSO_4 \cdot 5H_2O$, 0.1; $CoSO_4 \cdot 7H_2O$, 0.21. The sucrose, glucose, and 1 M $MgSO_4$ were autoclaved separated. Thiamine and 100 mg L^{-1} concentration of ampicillin were filtered sterilised into the fermenter by using 0.2 μm sterilised filter (Whatman, UK).

The complex medium, Superbroth (SB) (g L^{-1}): glucose, 15; bacto-tryptone (Difco, UK), 35; yeast extract (Difco), 20; NaCl, 5. Glucose solution was sterilised separately. Sterile filtered of ampicillin was added to a final concentration of 100 mg L^{-1}.

The Comparison of dbFv Production in Synthetic and Complex Mediums

The comparison of dbFv production in synthetic and complex mediums was routinely operated as two-stage productions. The *E. coli* HB2151 clone 8.3 was cultivated in the shake flask culture (500 mL) with super broth (SB) and SM feedstocks containing 5% (w/v) glucose at 37°C agitated at 200 rpm. The cells were subsequently centrifuged and the supernatants were discarded. The cell paste was re-suspended with glucose-free fresh medium with 2% of yeast extract (supplement for SM culture only). The inducer, IPTG (100 mg L^{-1}), was added into shake flask cultures. The cultures (100 mL) were routinely maintained at 25°C and 200 rpm. The C595 dbFv was then released using an enzymatic method and analysed by ELISA.

The Influence of Acetic Acid Accumulation upon C595 dbFv Production in Synthetic Medium

The recombinant *E. coli* cells were culitivated overnight in the SM culture (100 mL) with 10% (w/v) glucose at 37°C and 200 rpm. The *E. coli* cultures were centrifuged while presumably the stationary phase was achieved and re-suspended in the sterile glucose-free medium. The isopropyl-*D*-thiogalactopyranoside (IPTG) at concentration of 100 mg L^{-1} was subsequently introduced. Stock solutions of acetic acid were added to cell cultures to make up the final working concentrations of 0.5, 1, 5, and 10 g L^{-1} of acetate, respectively, and these were used to contrast with cultures containing no acetic acid solution. The *E. coli* cells were continually cultivated at 25°C and 200 rpm for 12 h. The methods for releasing and detection of C595 dbFv are described below.

The Effect of Carbon Source during Induction Stage on dbFv Production

The recombinant *E. coli* was grown overnight in several shake flasks containing 100 mL of synthetic medium (SM) culture with 5% (w/v) glucose as the initial carbon source. The cultures were centrifuged and re-suspended with fresh glucose-free medium while the glucose concentration was measured below the assay limitation (*i.e.* $<<0.01$ g L^{-1}) whereupon the glucose was exhausted. The 2 or 5% (w/v) of yeast extract, sucrose, and glycerol were introduced as the carbon supplement during induction stage. The IPTG inducer, with a concentration of 100 mg L^{-1}, was injected to initiate expression. The cultures were controlled at the temperatures of 37 and 25°C in growth and induction stages, respectively. The C595 dbFv was then released using an enzymatic method and analysed using immunoreactivity by enzyme-linked immunosorbant assay (ELISA).

The Comparison of Fermentation Strategies in Various Operation Modes (scale 4 litres)

The 5-L fermenter (Electrolab series 300, UK) having 4-L working volume was inoculated with 10% (v/v) *E. coli* inoculum in the exponential phase from the shake flask cultures. All the fermentations were incubated at 37°C and pH 7.0, by controlled addition of 2 M H_2SO_4 and 35% NH_3 solution and then maintained at 25°C during the induction phase. The minimum dissolved oxygen tension (DOT) was maintained above 20% by adjusting the agitation rate (200~300 rpm) and airflow rate (L h^{-1}). Polypropylene glycol (2%, w/v) was used as the antifoam solution. For one-stage entation strategies in various operation modesn batch fermentation when cell reached stationary phase, the C595 dbFv production was initiated following the addition of IPTG (100 mg L^{-1}) into the fermentation broth. The procedure was continued at 25°C for at least 8 h. The whole fermentation broth was harvested and treated by addition of 0.05% sodium azide and stored at 4°C. In the one-stage fermentation using fed-batch strategies, the glucose solution was

pumped at a constant feeding rate of 3 g h^{-1}. After 14 h cultivation (cell density reached approximately 10 g L^{-1}), the glucose feeding was suspended for an hour. The IPTG inducer was subsequently introduced to begin the protein expression. For the two-stage batch operation, the whole cell broth was harvested by centrifugation [JS-21 centrifuge (Beckman, USA) for 15 min at 3,440 g] when cell growth had stopped. Cell paste was re-suspended in an equivalent volume of sterile culture (glucose-free). The inducer, isopropyl β-D-thiogalactopyranoside (IPTG), wasemployed into the bioreactor to initiate C595 dbFv production. The whole cell broth (after 8 h of post-production) was treated with 0.05% sodium azide and stored at 4°C. Carbon-sources comprising of 2% (w/v) yeast extract were applied during the induction stage for all operations.

Enzymatic Method to Release Intracellular dbFv from *E. coli*

The dbFv mainly accumulated inside of the *E. coli* cell as an intracellular, periplasmic product. An enzymatic method exploiting lysozyme to release dbFv was adopted [13].

Biomass and Carbon Source Estimation

Dry cell weight (DCW) was estimated as the mean of triplicate measurements. One mL cell broth sample was pelleted in pre-dried and pre-weighed 1.5 mL Eppendorf tubes using a microcentrifuge at 7,200 g for 5 min. After re-suspension in 1 mL of deionised water and further centrifugation, pellets were dried in open Eppendorf tubes overnight in an oven at 96°C. Biomass was estimated after re-weighing the tubes. Optical density measurements of the original samples were estimated using a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) at a wavelength of 500 nm. A plot of DCW against OD500 of cell broth was established as a standard curve for determining DCW from fermentation samples. Glucose concentration was determined by using BM-Test 1-44 kit (Boehringer & Mannheim, UK). The sample (1 mL) obtained from fermentation was micro-centrifuged at 7,200 g for 5 min. The supernatant (20 μL) containing approximately glucose concentration (*i.e.* in the range of 0.01 to 7.92 g L^{-1}) was applied to a commercial test strip for first 60 sec reaction. Subsequently, test strip was wiped firmly with clean cotton wool and then was inserted into the glucose reader to determine glucose concentration. The values of glucose concentrations are yielded after second 60 sec estimation.

Immunoreactivity by Enzyme-Linked Immunosorbant Assay (ELISA)

A 96-well microtitre plates (Falcon 3912, Becton Diclinson, Oxford, UK) coated with antigen A-G (amino acid sequence: APDTRPAPG) conjugated to BSA (M. R. Price, CRC Laboratories, University of Nottingham, UK) at a working concentration of 2 μg mL^{-1}. The wells were washed four times with phosphate buffered saline PBS/Tween and the remaining non-specific binding sites were blocked in PBS for 1 h. After four washes in PBS/Tween, semi-logarithmic serial dilutions of the determined samples were added at 50 μL/well along with appropriate positive and negative controls and incubated for 1 h. Plates were again washed four times and anti-E-tag/HRP (Pharmacia) at 1/1,000 in PBS was added at 50 μL/well and incubated for 1 h. The plates were washed four times with PBS/Tween. ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) substrate was added to the wells at 100 μL/well. Analysis of colour development was performed over a 10-min period using a Milenia kinetic analyser (Diagnostic Products Corporation, Llanberis, UK).

Acetic Acid Assay

The concentration of acetic acid was detected using an HP1050 series system connected with an Aminex HPX-87H column, 300 × 7.8 mm (BioRad, UK) and RI detector. The mobile phase was 0.008 M degassed H_2SO_4 and the operating flow rate was 0.5 mL min^{-1}.

Sodium Dodecyl Shlphate-Polyacrlamide Gel Electrophoresis (SDS-PAGE)

Gels (4~12%) were run under reducing conditions using a vertical slab electrophoresis instrument (Protean IIxi Cell, BioRad, CA, USA). Low molecular weight markers (Pharmacia) were employed to estimate the molecular weight (MW) of fractionated proteins. The low molecular weigh marker consists of: phosphorylase b, MW 94 kDa; albumin, MW 67 kDa; ovalbumin, MW 43 kDa; carbonic anhydrase, MW 30 kDa; trpsin inhibitor, 20.1 kDa; β-lactabumin, MW 14.4 kDa.

RESULTS AND DISCUSSION

The key economic drivers for antibody manufacture in upstream processes are identified including fermentation titre and high productivity which are critical determinants of economic success [14]. Therefore, the strategies for studying optimization of C595 dbFv fermentation are depicted in Fig. 1 and results are discussed as following.

The Comparison of C595 dbFv Production in Synthetic and Complex Mediums

The dry cell weight (DCW) and concentration of C595 dbFv against induction time were plotted and as shown in Fig. 2. It indicated that the complex medium (SB) yielded higher cell density and product concentration compared to the synthetic medium in the same time course. The cell dry weight and C595 dbFv concentration expressed relatively to the volume of original broth were 7.24 g L^{-1} and 3.22 mg L^{-1} for SB and for that experiment with SM were 3.10 g L^{-1} and 2.06 mg L^{-1}, respectively. However, the specific productivity (defined as mg product per g

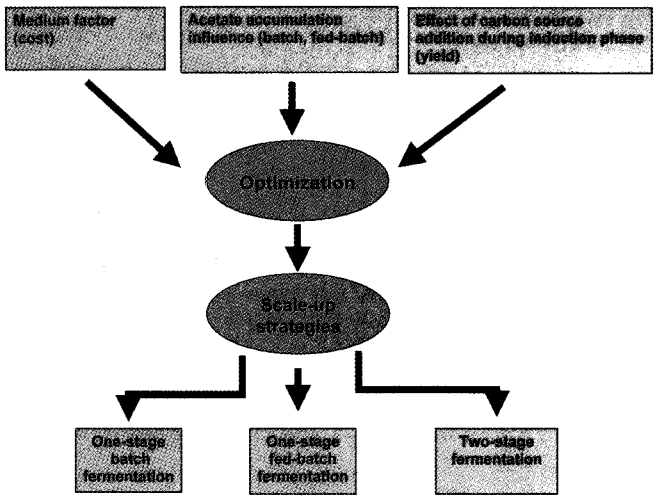

Fig. 1. Study scheme of fermentation strategies for C595 dbFv production in recombinant *E. coli*.

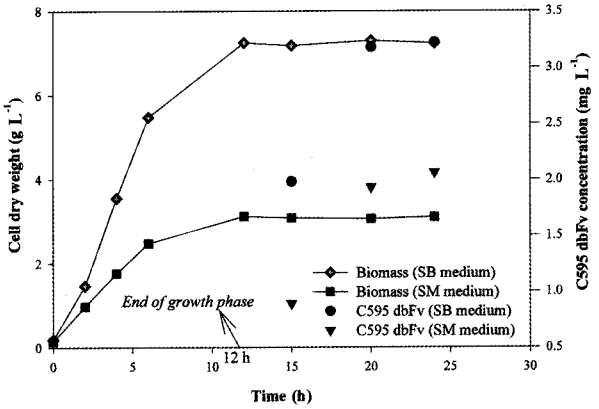

Fig. 2. Comparison of C595 dbFv expression with complex and synthetic mediums in shake flask scale.

DCW per hour [15]) was reduced within the complex medium (0.044 mg dbFv g-DCW^{-1} h^{-1}) when compared to the synthetic medium (0.066 mg dbFv g-DCW^{-1} h^{-1}). This may be attributed to the fact that the plasmid copy number of recombinant *E. coli* was decreased with increasing growth rate in the shake flask culture. A similar phenomenon was reported by Seo and Bailey [16]. It was found that medium was known to essentially affect the plasmid stability in *E. coli* expression system [17]. Nevertheless, since the growth rates in these batch experiments were varied by employing different growth media, it is not clear that the difference in plasmid stability was due only to the change in growth rate. The changes observed could have also been the result of nutrient effects. Acetate quickly formed and accumulated when grow recombinant *E. coli* in complex culture had a dramatically impact upon protein expression but no significant influence on cell growth [15]. The amount of antibody produced decreased spectacularly in the presence of acetic acid (>0.5 g L^{-1}) in the SB culture [18]. This may be one of reasons resulted in low specific productivity of C595 dbFv performed in the complex medium. Although the SB culture indicated the attractiveness for C595 dbFv production in Fig. 2, but defined medium (SM) was employed for subsequent investigations due to it yielded higher specific productivity and have advantages on offering lower material costs.

The Effect of Acetic Acid Accumulation on C595 dbFv Production in Synthetic Medium (SM)

Acetic acid was commonly generated during *E. coli* fermentations because of the easily utilisable glucose and fast cell growth or limitation of dissolved oxygen. Acetate have been reviewed to be inhibitory to the growth of *E. coli* [19], varying concentrations of acetic acid were added to the production flasks to determine its influence on the growth of the recombinant *E. coli* HB2151 strain and the biosynthesis of C595 dbFv. As shown in Table 1, it indicated that the cell growth was not inhibited in the

Table 1. Summary of acetate effect upon C595 dbFv expression

Acetic acid concentration (g L^{-1})	Dry cell weight (g L^{-1})	C595 diabody (mg L^{-1})	Specific productivity (mg g-DCW^{-1} h^{-1})
0.0	3.58	2.08	0.05
0.5	3.95	1.93	0.04
1.0	3.20	0.40	0.01
5.0	4.06	0.03	0.00
10.0	3.71	0.00	0.00

presence of acetic acid. However, the produced C595 dbFv and specific productivity significantly decreased with increasing acetic acid concentration to 1 g L^{-1}. The result showed the same phenomenon observed in the antibody production using complex culture [18]. This demonstrates that it is important to minimize the generation of extra-cellular acetic acid in culture medium in order to achieve maximum yield of C595 dbFv.

The Effect of Carbon Source during Induction Stage upon dbFv Production

The importance of the added carbon source upon the metabolism of recombinant *E. coli* during expression was explored in this study. The results are presented in Fig. 3 and demonstrate that the production of C595 dbFv was apparently increased within the addition of carbon supplements. The C595 dbFv concentration with no added carbon source after 12 h induction time was 0.36 mg L^{-1}. The *E. coli* cultures with 2 and 5% of yeast extract additive yielded 1.51 and 1.54 mg L^{-1} of C595 dbFv, respectively, which were approximately 5-fold higher. The values of specific productivity for the experiments without and with 2 and 5% of yeast extract addition were 0.014, 0.058, and 0.057 mg dbFv g-DCW^{-1} h^{-1}, respectively. Fig. 3 indicated that the yeast extract provided not only the carbon source but also other unknown requirements for *E. coli* cells during the induction phase which have high- est concentration of C595 dbFv expressed mg per litre of original broth. With higher concentrations of car-

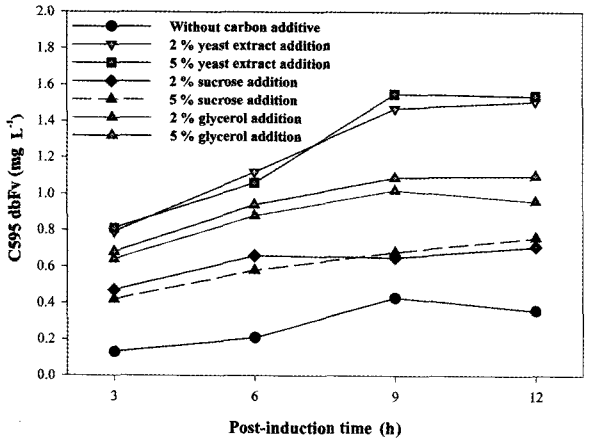

Fig. 3. Comparison of different addition of carbon supplements during expression stage.

bon supplements, the antibody fragment concentration did not show an increasing trend. From the experiments, it was observed that the cell density was very constant with addition of yeast extract, sucrose or glycerol in the induction stage.

Comparison of Fermentation Strategies

The commercial production of recombinant proteins for industrial and medical use has increased significantly in recent years due to of low manufacturing costs; processes using *E. coli* remain the production systems of choice [19]. As discussed earlier, the key factor to scale up C595 dbFv production is to diminish the formation of acetate during fermentation process. A straightforward approach to prevent acetate formation is to force glucose limited cells to grow by employing fed-batch fermentation [19,20]. The comparison of batch and fed-batch fermentations in one-stage operations were undertaken at the 5-L scale bioreactor and the results were presented in Figs. 4A and 4B. The characteristics comparison of different fermentation strategies was summarised in Table 2. Acetic acid concentrations were estimated in the end of growth and induction phase, respectively. To compare the concentrations of acetate in end of growth and induction stage in each operation mode showed similar results. The formation of acetic acid was terminated under glucose limitation condition during induction phase in both operations. However, it revealed that concentration of acetic acid was 10% lower in the end of growth phase while using constant feeding of glucose (see Table 2). Therefore, a lower biomass and C595 dbFv concentration (*i.e.* 6.90 DCW g L^{-1} and 1.30 C595 dbFv mg L^{-1}, respectively) was obtained at the end of the one-stage batch fermentation. The C595 dbFv yield was improved to 5.2 mg L^{-1} as well as economic effect was retrenched from 1.10 to 0.28 £ medium mg^{-1} C595 dbFv by introducing fed-batch strategy.

The two-stage batch fermentation including a centrifugation step was designed in order to reduce influences of present acetate upon production C595 dbFv and consequently achieve high yield. The dry cell weight (DCW), concentration of C595 dbFv and economic effect were 10.78 g L^{-1}, 12.28 mg L^{-1}, and 0.22 £ mg L^{-1}, respectively. The acetic acid recorded at the end of growth stage was 1.20 g L^{-1}, respectively. However, the concentration of was reduced to 0.41 g L^{-1} after a centrifugation step. As summarized in Table 2, similar DCW were obtained in one-stage fed-batch and two-stage batch fermentations but yield of C595 dbFv increased 2-fold. The better performance for two-stage fermentation was due to the removal of acetic acid presence in the broth and the cell paste was resuspended in glucose free medium. It is well known that overfeeding of glucose for the aerobic *E. coli* fermentation results in acetic acid production [20] which promotes growth inhibition and the reduction of productivity [21-23]. As a result, the strategies to achieve high productivity of recombinant C595 dbFv have primarily focused on minimising organic acid production under minor variations (*i.e.* includes a centrifugation step and the induction phase is initiates in glucose free medium) of the expression system. Fig. 6 shows the SDS-PAGE analysis of C595 dbFv produced from one-stage fed-batch and two-stage batch fermentations. The stained protein bands of produced C595 dbFv from fermentation appeared at the location of 30 kDa (compare Lanes 1, 2, and 3 in Fig. 5) confirmed that the C595 dbFv has been produced.

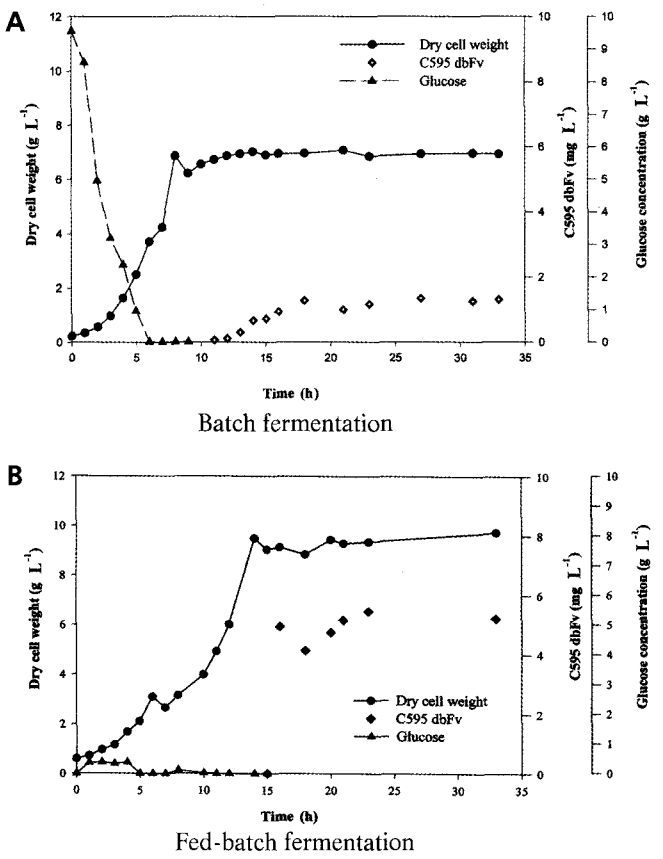

Fig. 4. Comparison of batch and fed-batch fermentations in one-stage operation for the production of C595 dbFv from *E. coli* HB2151 in 5-L bioreactor.

Table 2. Summary of fermentation characteristics of C595 dbFv production in recombinant *E. coli* HB2151

Fermentation modes	One-stage batch	One-stage fed-batch	Two-stages batch
Working volume in growth phase (L)	4.00	4.00	4.00
Working volume in induction phase (L)	4.00	4.00	4.00
Total culture volume required (L)	4.00	4.00	8.00
Dry cell weight (g L^{-1})	6.90	9.70	10.78
C595 dbFv yield (mg L^{-1})	1.30	5.20	12.28
Total medium cost (£)	5.80	5.80	11.02
Acetic acid concentration in the end of growth phase (g L^{-1})	1.26	1.14	1.20
Acetic acid concentration in the end of induction phase (g L^{-1})	1.20	1.12	0.41
Economic effect (£ medium mg-dbFv^{-1})	1.10	0.28	0.22

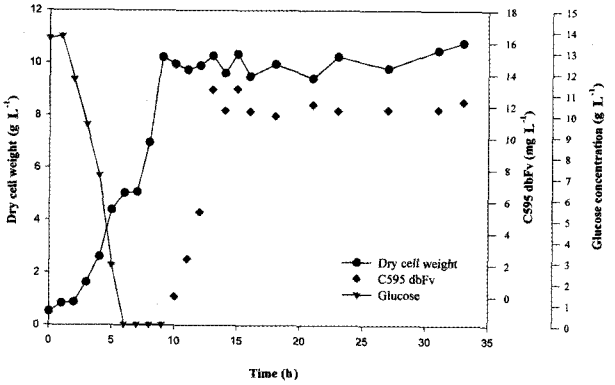

Fig. 5. Profiel of two-stage batch fermentation of *E. coli* HB2151 in 5-L bioreactor.

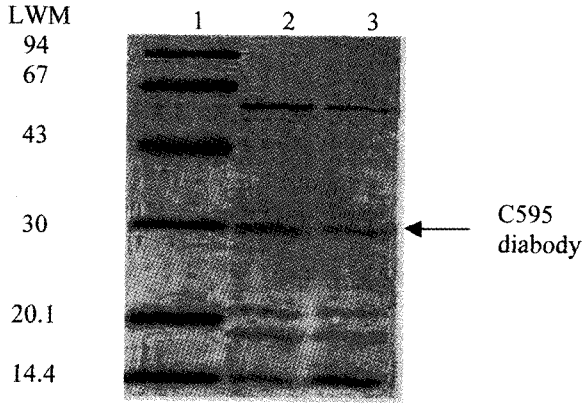

Fig. 6. SDS-PAGE analysis of the released C595 dbFv from the different fermentations. Lane 1 contains the standard low molecular weight (LMW) markers obtained from Amersham Pharmacia Biotech. Lanes 2 and 3 carried C595 dbFv obtained from two-stages and one-stage batch fermentations, respectively.

CONCLUSIONS

The results demonstrated that the carbon-source additives in the induction stage can increase C595 dbFv yield and yeast extract supplied during expression yielded more antibody fragment than any other carbon supply. However, the C595 dbFv expressed in recombinant *E. coli* in SM culture was significantly affected by presence of acetic acid (>0.5 g L^{-1}). The comparison of fermentation processes demonstrated that fed-batch technique could improve C595 dbFv production in one-stage fermentation but a two-stage operation remarkably increased C595 dbFv yield even through it involved more unit operations. This consequence may benefit the downstream processing of C595 dbFv.

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