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Optimization of culture on the overproduction of TRAIL in high-cell-density culture by recombinant *Escherichia coli*

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Abstract Different nutrient-feeding cultures were carried out in producing recombinant protein of truncated tumor necrosis factor related apoptosis-inducing ligand (TRAIL) (114–281 amino acids of TRAIL) in *Escherichia coli* strain C600/pBV-TRAIL. The effects of preinduction specific growth rate, postinduction carbon source (glucose and glycerol), and feeding strategies were investigated. The higher preinduction specific growth rate (μ=0.22 h^{-1}) contributed to the increase in the TRAIL production, at which TRAIL was accumulated in bacterial cells as 7.2% of total cellular protein, corresponding to 1.99 g l^{-1} in contrast with 5.1% (1.29 g l^{-1}) at preinduction specific growth rate (μ=0.1 h^{-1}) during high-cell-density culture. Glycerol was superior to glucose as the postinduction carbon source for TRAIL production. Under similar culture conditions, the final concentration of TRAIL was produced 1.59-fold more when glycerol was used as postinduction carbon source than when glucose was used. At the same time, the results showed that it is efficient to adopt the pH-stat feeding strategy at postinduction for the overproduction of TRAIL. The TRAIL production was increased up to 4.51 g l^{-1}, approximately 16.1% of total cellular protein. The mechanisms behind the preinduction specific growth rate effect on the expression level may be ascribed to the leakage secretion of acetate.

Introduction

Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL/Apo2L) is a new member of the TNF superfamily. It is composed of 168 amino acid residues with a total molecular mass of 19 kDa and displays apoptotic activity against various cancer cells with minimal cytotoxicity toward normal tissues both in vitro and in vivo (Wiley et al. 1995; Walczak et al. 1999). Because of its biological activity, TRAIL is attracting considerable interest in the pharmaceutical industry and is currently being developed as a therapeutic agent for various cancer remedies.

High-cell-density culture (HCDC) techniques have been developed to improve the productivity of recombinant proteins in *Escherichia coli* (Babu et al. 2000; Klemom and Strohl 1994; Riesenberg 1991). As is known, secretion of detrimental metabolic by-product acetate is one of the major constraint factors for growth and maximizing the production of heterogeneous proteins in HCDC (Lischke et al. 1993). It has been proven that acetate cannot be detected in the culture broth in which the concentration of glucose was under 0.748 g l^{-1} (Han et al. 1992). In addition, yeast extract is able to prevent the degradation of target protein and improve yield by reducing the acetate secretion (Panda et al. 2000; Mario et al. 2004). It was also found that acetate would not be produced via the Crabtree effect when the growth rate was less than the threshold and acetate could usually be controlled at low concentration level through exponential feeding of the nutrient (Babu et al. 2000). Meanwhile, determination of the specific growth rate is very important in HCDC, obtained through exponential growth feeding model. Many studies have been performed to investigate the effect of preinduction specific growth rates of recombinant *E.coli* on the synthesis of target proteins. Some different results have been published. It was reported that the rate of recombinant protein synthesis in production of human interferon R1 was correlated inversely with the specific growth rate (Riesenberg et al. 1990). Others observed the direct correlation between the specific growth rate and specific recombinant product formation (Siegel and Ryu 1985; Curless et al. 1990, 1994), while some studies considered no correlation between the specific growth rate and specific recombinant product formation (Bech and Carlsen 1990). It was also reported that the nutrient-feeding strategy at postinduction played a key role in the fed-batch cultivation of recombinant *E. coli* (Wong et al. 1998). Because the growth and metabolic activity of the host cells were often influenced by many

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factors, such as toxicity of inducer, upshift of temperature, and expression of foreign proteins after induction, great nutrient to supply the material and energy for the host cell was required. In addition, the final concentration of acetate in the culture also varied depending on the postinduction feeding strategy.

In this report, we described the overproduction of protein TRAIL in the recombinant *E. coli* C600 and investigated various cultural strategies in more details.

Materials and methods

Bacterial strains and plasmids

The *Escherichia coli* strain C600 (supeE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21) harboring the plasmid pBV-TRAIL was used as the host for the expression of recombinant TRAIL with a temperature inducible system (provided by Dr. LH Wang; Wang et al. 2002).

Continuous cultivation: medium, inoculum preparations, bioreactor culture, and control

Stock cultures (−20°C) were activated in Luria–Bertani (LB) broth with ampicillin at a concentration of 100 μg ml^{-1} for 12 h at 30°C. Subsequently, one further preculture (80-ml terrific broth medium in 500-ml shake flask) was inoculated with the first preculture (2% v/v) and incubated on a rotary shaker at 30°C for 12 h. The resultant culture was used to inoculate in 1.6-l medium for continuous cultivation experiments in a 3.7-l bioreactor (KLF2000 3.7 l, Bioengineering AG, Switzerland). The composition of the self-defined medium for batch and continuous cultivation was described in Table 1. Ampicillin (100 μg ml^{-1}) was added in the fed-batch cultures at the beginning. The initial batch culture conditions were as follows: The dissolved oxygen concentration (DOC) was maintained above 30% of air saturation. pH was controlled at 7.0 using 28%(v/v) ammonia water, and the air flow rate was adopted 2 l min^{-1}. When glucose was exhausted, continuous operation was initiated. Throughout the experiment, pH was controlled at 7.2 using 28%(v/v) ammonia water; the dissolved oxygen level was maintained at 30% saturation with agitation cascaded. In each experiment, the culture optical densities (ODs) were maintained at steady state under chemostat operation for at least three residence times prior to adjusting the flow rate to another set value. The samples were drawn from the bioreactor and transferred into shake flask cultures with fresh medium (2× yeast–tryptone (YT) + glucose 10 g l^{-1}) and shifted to 42°C induction when steady state was obtained.

Table 1 Medium composition

Components	Batch and continuous medium (per liter)	Feeding solution (per liter)
Glucose (g)	10.0	700
Yeast extract (g)	10.0	175
Tryptone (g)	10.0	175
KH_2PO_4 (g)	2.0	0
K_2HPO_4 (g)	4.0	0
Na_2HPO_4 (g)	7.0	0
$(NH_4)_2SO_4$ (g)	1.2	0
$MgSO_4$ (g)	1.2	7.2
Trace metal[a] (ml)	3.0	0
Antifoam (ml)	0.5	0

[a]The trace metal solution contains per liter of 5 M HCl: 10 g of $FeSO_4$·7H2O, 2.3 g of $ZnSO_4$·7H2O, 1 g of $CuSO_4$·5H2O, 0.5 g of $MnSO_4$·$5H_2O$, 2.9 g of CaCl·$2H_2O$, 0.12 g of $Na_2B_4O_4$·$10H_2O$, and 0.1 g of $(NH_4)_6Mo_7O_{24}$

HCDC: bioreactor cultivation and controls

The composition of the self-defined medium for HCDC was described in Table 1. The feeding solutions (glucose and $MgSO_4$) were sterilized separately before mixing, and nitrogen sources (YT) were sterilized after mixing. Ampicillin (100 μg ml^{-1}) was added in the fed-batch cultures at the beginning. The DOC was maintained above 30% of air saturation. pH was controlled at 7.0 using 28%(v/v) ammonia water and the air flow rate was adopted 2 l min^{-1} during batch. In fed-batch, the DOC was maintained in 20% of air saturation by manipulating the agitation speed up to 1,000 rpm with agitation cascaded, and pure oxygen was supplied when required. pH was controlled at 7.2 using 28%(v/v) ammonia water. When glucose was exhausted, exponential feeding of feeding solution was carried out, and the flow rate was increased stepwise. The specific growth rate was adjusted by varying the feeding rate, which was predetermined according to the material balance equation (Korz et al. 1995; Lee 1996). Initially, the specific growth rate was set at μ=0.15 h^{-1}. Subsequently, the value was adjusted to 0.22 and 0.10 h^{-1} and maintained 2 h prior to induction. Finally, the value was maintained at 0.10 h^{-1} during induction. Table 3 described the control parameters of different reactor runs. The pH-stat feeding strategy in the study has been described previously (Jeong and Lee 1999). When the measured pH diverged 0.1 U

Table 2 Values from different dilution at steady state

D=μ (h^{-1})	OD	Cell dry weight (gl^{-1})	Glucose (gl^{-1})	Acetate (gl^{-1})	Expression content (%)
0.05	12.42	4.99	0.00	0.00	6.50
0.10	11.70	4.69	0.01	0.00	11.9
0.22	10.88	4.36	0.03	0.15	16.8
0.30	9.77	3.91	0.03	0.35	14.3
0.40	8.58	3.44	0.05	0.54	11.6

higher or lower than the set point, 7.2, the carbon source or the nitrogen source was added via the peristaltic pump. At the stage of the postinduction, carbon source was added according to the pH divergence while the feeding rate of nitrogen source was kept constant. Plasmid stability was determined by plating serially diluted samples onto selective (100 μg ml^{-1} ampicillin) and nonselective LB agar plates and was calculated by taking the ratio of the average number of colonies from three selective plates to the average from three nonselective plates.

Analytical methods

Samples periodically taken from the culture broth were immediately centrifuged at 10,000 rpm for 10 min, and the supernatants were stored at 4°C prior to analysis of glucose and acetate. Residual glucose in the fermentation broth was determined by the glucose kit (Shanghai Institute of Biological Products, China). Acetate were determined by high-pressure liquid chromatography (model 1100, Agilent, USA) equipped with 250×4.6 mm C_{18} column (Agilent) with 0.05 mol l^{-1} $(NH_4)H_2PO_4$ (pH 2.6) as a mobile phase. Dry cell concentrations were estimated using a calibration curve obtained from the relationship between the OD at 600 nm and the dry cell weight (DCW). Higher OD samples were diluted suitably to have an absorbance at the range of 0.1–0.6. The cell pellets which resulted from the centrifugation was washed with physiological saline and dried to constant weight at 80°C. One absorbance unit for the uninduced culture and induced culture was equivalent to 0.40 and 0.44 g l^{-1} of DCW, respectively. Expression level of recombinant TRAIL was determined by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie brilliant blue R250. The product concentration was quantified by measuring the staining intensity of protein bands using a densitometer. The total protein concentration was determined by the lowery assay with bovine serum albumin as a reference protein.

Results and discussion

Effects of different preinduction specific growth rate on TRAIL production

The effects of preinduction specific growth rate on TRAIL production were investigated by continuous cultivation and fed-batch cultivation. In continuous cultivation model, steady-state concentration of cell dry weight, glucose concentration, acetate concentration, and protein content at each dilution rate were showed in Table 2. We observed that acetate acid was excreted at specific growth rate (μ=0.22 h^{-1}) and glucose concentration maintained at low level during the cultivation. Cell mass and substrate concentration exhibited typical patterns over the range of dilution rate. Figure 1a showed the effect of preinduction dilution rate on the expression level of TRAIL after 4 h of induction. The maximal TRAIL content was 16.8% of total cellular protein at dilution rate of 0.22 h^{-1}. According to the curve shown in Fig. 2, the expression level increased with increasing preinduction dilution rate up to 0.22 h^{-1}. When preinduction dilution rate exceeded 0.22 h^{-1}, the expression level of TRAIL was decreased gradually.

In fed-batch cultivation model, the feeding model was performed according to fed-batch-1 (FB-1) and fed-batch-2 (FB-2) (Table 3). The growth curve, acetate concentration, and TRAIL expression level were shown in Fig. 3a and b. The results indicated that the different specific growth rate at preinduction affected acetate formation and foreign protein production. The final cell concentration was obtained at 52.9 g DCW l^{-1}, and acetate concentration was accumulated as high as 3.18 g l^{-1} above the acetate threshold at the end of the culture according to FB-2. Although the preinduction acetate concentration of cell induced at preinduction specific growth rate (μ=0.22 h^{-1}) was higher than that of cell induced at preinduction specific growth rate (μ=0.1 h^{-1}), TRAIL was accumulated in bacterial cells as high as 7.2% of total cellular protein corresponding to 1.99 g l^{-1} compared to 1.29 g l^{-1}, accounting for 5.1% of total cellular protein, in manner of preinduction

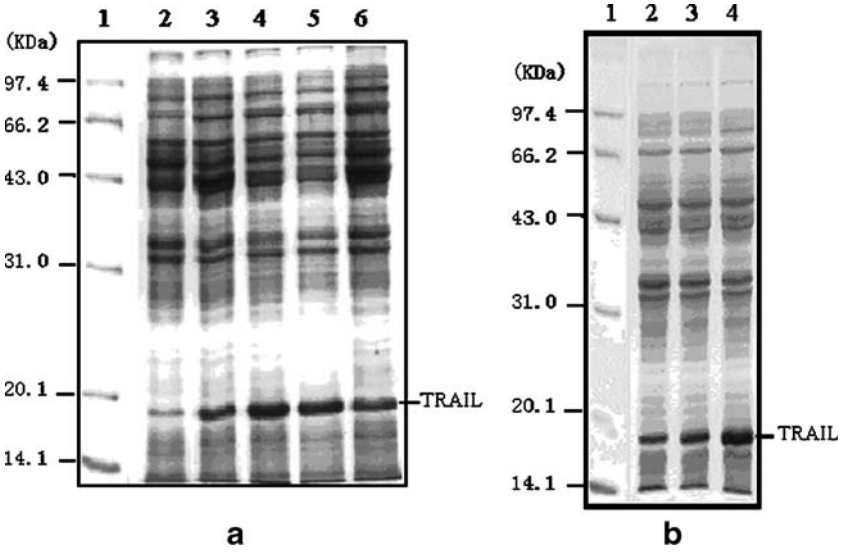

Fig. 1 **a**, **b** SDS-PAGE analysis of TRAIL. **a** The effect of different preinduction special growth rate on the protein content of TRAIL. *Lane 1* molecular mass standard, *Lane 2* 0.05 h^{-1}, *Lane 3* 0.10 h^{-1}, *Lane 4* 0.22 h^{-1}, *Lane 5* 0.30 h^{-1}, *Lane 6* 0.40 h^{-1}. **b** TRAIL expression in HCDC. *Lane 1* molecular mass standard, *Lane 2* protein content produced in FB-1, *Lane 3* protein content produced in FB-2, *Lane 4* protein content produced in FB-4. The *line* indicates the band position of TRAIL protein in the gel. Each fraction corresponds to 15 μl of protein homogenate

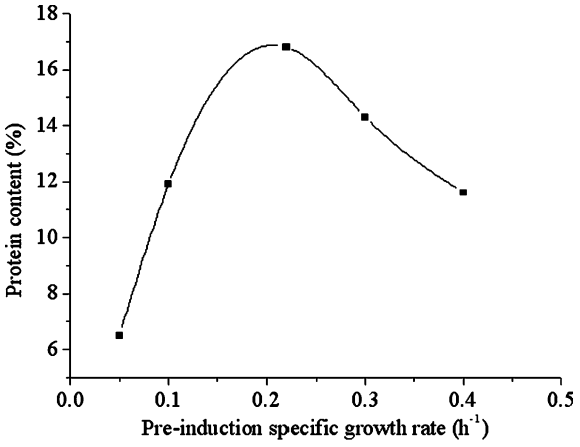

Fig. 2 The relation between different preinduction special growth rate and the expression level of TRAIL

specific growth rate (μ=0.1 h^{-1}) during HCDC. Both plasmids were stable during fed-batch culture (data not shown). The results indicated that the high specific growth rate at preinduction increased the TRAIL production in *E. coli* C600.

Previous studies considered that the mechanism linking specific growth rate with recombinant protein productivity is likely to be related to the change in cellular ribosomal content. We also observed that the acetate increased with the specific growth rate in continuous cultivation model and HCDC model. We found that when the cell from continuous cultivation with μ=0.22 h^{-1} were induced, the expression level of TRAIL was higher than that of the cell induced with lower specific growth rate, even if the acetate acid was produced at the specific growth rate (μ=0.22 h^{-1}). In HCDC model, the similar result that the acetate concentration at preinduction in FB-2 was higher than that of FB-1 was observed, but the expression level was better in FB-2. It could be explained that acetate synthesis, moderate regulation of acetate pathway through certain means, allowed the cells to keep metabolic activity and improved the production of TRAIL. It has been reported that the acetate pathway had important relations between the cellular energy metabolism and foreign protein production through the formation of acetyl-P and adenosine 5c-triphosphate (ATP; Kim and Cha 2003). Note that acetate synthesis could generate the second largest amount of ATP and $NADH_2$ (Han et al. 1992). It was also confirmed that acetate could increase the steady-state expression level of 37 proteins, including periplasmic transporters for amino acids and peptides (ArtI, FliY, OppA, and ProX), metabolic enzymes (YfiD and GatY), the RpoS growth phase regulon, and the autoinducer synthesis protein LuxS (Kirkpatrick et al. 2001). On the other hand, if the capacity of acetate synthesis was eliminated by *E.coli* mutants or engineered strains, the modified strain grew much more slowly than its counterpart (Bauer et al. 1990; Aristidou et al. 1994). The results from our laboratory also found that the TRAIL expression level of the photransacetylase mutant of *E. coli C600* with reduced acetate accumulation was dropped to 50% that of counterpart (date not shown). These results implied that acetate pathway was important for the cell growth and production of heterogeneous protein and the moderate acetate excretion at preinduction could enhance the capability of cell growth and foreign protein synthesis, though high concentration of acetate had harmful effects on host cells.

Effects of different postinduction carbon sources, glucose and glycerol, on TRAIL production

The lower rate of uptake of glycerol than of glucose into the cell appears to cause a reduction in the flux of carbon through glycolysis, greatly decreasing acetate concentration (Lee 1996). The feeding strategies based on the FB-2 and fed-batch-3 (FB-3) in Table 3 were adopted. Two postinduction carbon sources, glucose and glycerol, were compared for TRAIL production. Figure 3b and c compared the fermentation results from different postinduction carbon sources. The results represented that glycerol was a better carbon source than glucose for production of TRAIL in HCDC. For fed-batch cultures using glucose as the postinduction carbon source, the final volumetric concentration of the recombinant protein reached 1.99 g l^{-1}, accounting for 7.2% of total cellular protein, when cells were induced at 40.6 g DCW l^{-1}. For the fed-batch culture using glycerol as postinduction carbon source, the protein

Table 3 Control parameters and results in different reactor runs

Run	Preinduction		Postinduction		Induction cell concentration (g DCW l^{-1})	Maximum cell concentration (g DCW l^{-1})	TRAIL Content (%)	TRAIL Content (g l^{-1})
	Carbon source	Feeding model	Carbon source	Feeding model				
FB-1	Glucose	Exponential μ=0.15[a], 0.10[b]	Glucose	Exponential μ=0.1[c]	33.6	44.8	5.1	1.29
FB-2	Glucose	Exponential μ=0.15[a], 0.22[b]	Glucose	Exponential μ=0.1[c]	40.6	52.9	7.2	1.99
FB-3	Glucose	Exponential μ=0.15[a], 0.22[d]	Glycerol	Exponential μ=0.1[d]	42.2	54.1	11.2	3.16
FB-4	Glucose	Exponential μ=0.15[a], 0.22[d]	Glycerol	pH-stat	41.1	53.4	16.1	4.51

[a]When glucose was exhausted, feeding was initiated according to exponential feeding model (μ_{set}=0.15 h^{-1})
[b]Feeding was switched to exponential to regulate the recombinant cell growth at a desired growth rate (μ_{set}=0.1 h^{-1}), prior to induction (2 h)
[c]Feeding was switched again and regulated the recombinant cell growth (μ_{set}=0.10 h^{-1}) at postinduction
[d]Feeding was switched to exponential to regulate the recombinant cell growth at a desired growth rate (μ_{set}=0.22 h^{-1}), prior to induction (2 h)

expression level could reached up to 11.2% of total cellular protein, and 3.16 g l^{-1} of TRAIL was produced even when induction was manipulated at 42.2 g DCW l^{-1}. We also found that the excretion of acetate was as high as 1.8 g l^{-1} and the acetate concentration was reduced by 70%. It was confirmed that the glycerol as carbon source at postinduction medium decreased the excretion of acetate and contributed to the improvement of TRAIL production.

High concentration acetate usually inhibited the cell growth and the expression of heterogeneous protein. Our previous research showed that high concentration of acetate and residual glucose following the induction were accumulated at the late stage of culture; the highest acetate concentration attained 13 g l^{-1}, and specific cellular TRAIL protein yield was negligible (Shen et al. 2004). Thus, it was critical to control the final acetate concentration in broth, especially at the late stage of culture. The result showed that glycerol as postinduction carbon source was superior to glucose for production of TRAIL in HCDC. It could be explained that the low uptake rate of substrate of *E. coli* grown on glycerol caused the lower acetate excretion (Holms 1986).

Effects of different feeding strategies at postinduction on the foreign protein production

Because glycerol could enhance the TRAIL production, the feeding solution containing glycerol was used at the postinduction in the study. Exponential feeding strategy and pH-stat feeding strategy were performed at postinduction. As shown above, in the exponential feeding strategy, the expression level of TRAIL could reach up to 11.2% of total cellular protein, and 3.16 g l^{-1} of TRAIL was

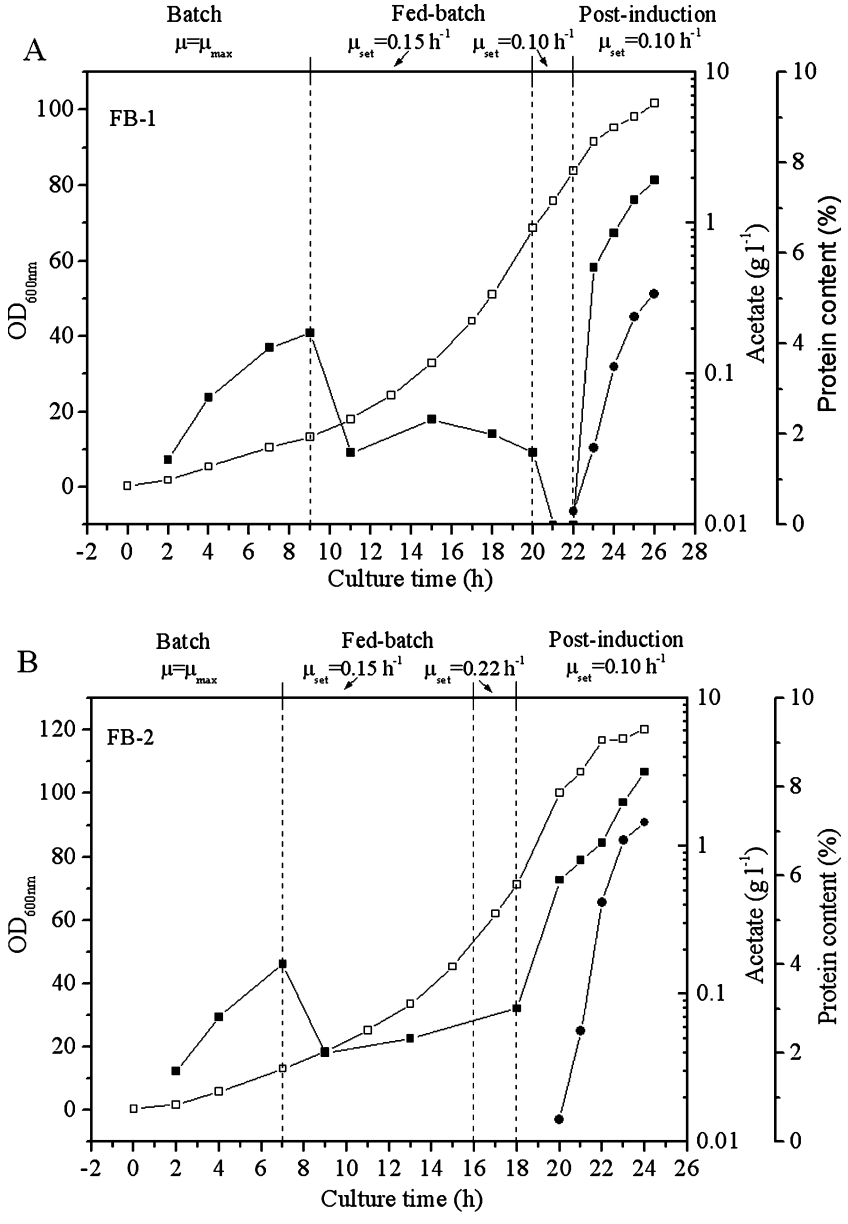

Fig. 3 **a**, **b**, **c**, **d** Time profiles of cell OD (○), TRAIL concentration (•), acetate concentration (▪) in HCDC according different processes. **a** FB-1. **b** FB-2. **c** FB-3. **d** FB-4

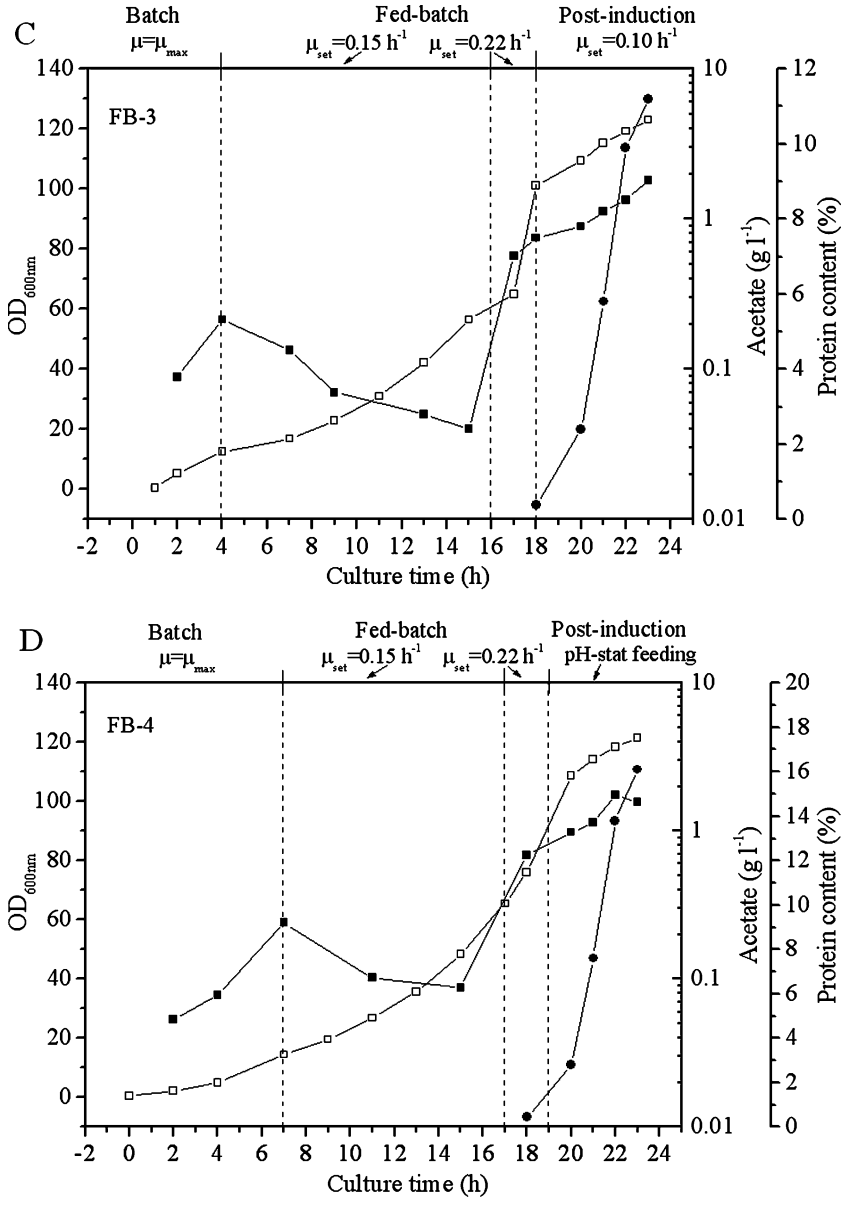

Fig. 3 (continued)

produced. Surprisingly, when pH-stat feeding strategy was applied during the postinduction period, the protein expression level increased rapidly to 16.1% of total cellular protein and the TRAIL protein content in fed-batch-4 (FB-4) was 3.16- and 2.24-fold higher than that obtained in FB-1 and FB-2, respectively (Fig. 1b). The final volumetric concentration of the recombinant protein reached 4.51 g l^{-1}. Moreover, no significant amount of acetate was accumulated in the culture broth. The growth curve, acetate concentration, and TRAIL content were shown in Fig. 3d. Judged from the experiment results, the pH-stat feeding method led to better production of recombinant protein than exponential feeding strategy. We also observed that there was no obvious difference in the final acetate excretion between the two feeding strategies, which showed that the acetate was not the main reason that caused the difference of the protein expression by feeding strategies at postinduction. Maybe, the TRAIL expression level varied depending on the amount and timing of the carbon sources feeding at postinduction to a certain degree. Thus, applying FB-4 feeding strategy was the best choice for the production of TRAIL in recombinant *E.coli*.

Judged from our results, the performance of the expression system during temperature-induced production of the TRAIL protein depended on the overall cell physiology, because it was observed that the final TRAIL expression level was affected by not only preinduction but also postinduction carbon source and feeding strategy. The results displayed that the protein expression by pH-stat feeding strategy during postinduction was better than that of the predetermined exponential feeding, representing an approximate 1.34-fold increase in expression of TRAIL.

Moreover, the recombinant TRAIL expressed in *E. coli* was shown to have strong cytotoxicity to human pancreatic tumor cell line 1990 (Xia et al. 2004). Seemingly, adopting the pH-stat feeding strategy met the cell demand well and promoted the cell capacity for expression of TRAIL. Although we did not understand the details of cell metabolism clearly, the fluctuation of pH during the postinduction reflected the change of cell physiology indirectly. Thus, keeping pH steady state by feeding the carbon and nitrogen source could be advantageous to avoid the accumulation of organic acid and achieve high level expression of target protein. The final acetic acid concentration was only 1.65 g l^{-1}, and the maximum TRAIL concentration was obtained (4.51 g l^{-1}).

Conclusion

To achieve HCDC, the exponential nutrient-feeding strategy has been widely employed to control the specific growth rate at a desired value and minimize the formation of growth-inhibiting by-products such as acetate. However, when they were cultured under severe nutrient limitation, cells could not be well adapted to the changed environment such as high temperature, low dissolve oxygen, expression of heterogeneous protein, and so on. Our results showed that the cells growing at higher growth rate prior to temperature upshift were better adapted to the stressful induction environments and improved the expression of foreign protein. Glycerol was superior to glucose as the postinduction carbon source for TRAIL production. Under similar cultural conditions, 1.59-fold more TRAIL could be produced when glycerol was used as the postinduction carbon source than glucose. Combined feeding strategy was adopted to supply the nutrition and control the final concentration of acetate below threshold (2 g l^{-1}), which increases the heterogeneous protein expression level. We believed that the strategy would be a potential for the production of not only TRAIL but also some other recombinant proteins.

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