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The Fed-Batch Production of a Thermophilic 2-Deoxyribose-5-Phosphate Aldolase (DERA) in *Escherichia coli* by Exponential Feeding Strategy Control

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Abstract 2-Deoxyribose-5-phosphate aldolase (DERA) catalyzes a sequential aldol reaction useful in synthetic chemistry. In this work, the effect of a feeding strategy on the production of a thermophilic DERA was investigated in fed-batch cultures of recombinant *Escherichia coli* BL21 (pET303-DERA008). The predetermined specific growth rate (μ_{set}) was evaluated at 0.20, 0.15, and 0.10 h^{-1}, respectively. The DERA concentration and volumetric productivity were associated with μ_{set}. The cells synthesized the enzyme most efficiently at μ_{set}=0.15 h^{-1}. The maximum enzyme concentration (5.12 g/L) and total volumetric productivity (0.256 $gL^{-1}h^{-1}$) obtained were over 10 and five times higher than that from traditional batch cultures. Furthermore, the acetate concentration remained at a relatively low level, less than 0.4 g/L, under this condition which would not inhibit cell growth and target protein expression. Thus, a specific growth rate control strategy has been successfully applied to induce fed-batch cultures for the maximal production of the thermophilic 2-deoxyribose-5-phosphate aldolase.

Keywords 2-Deoxyribose-5-phosphate aldolase · Fed-batch culture · Feeding strategy · Enzyme production · Acetic acid

Introduction

The asymmetric aldol condensation reaction is important in synthetic organic chemistry [1]. The ability to form C–C bonds and generate up two new stereocenters allows the organic chemist to fashion a broad range of both natural and novel poly-

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hydroxylated compounds [2]. Although considerable progress based on non-biological methods has been made, these methods generally require low temperature and organic solvent to achieve high asymmetric induction. However, enzyme-catalyzed aldol condensations can occur at neutral pH and room temperature and display the great potential in the coming future [3]. The class I aldolase, 2-deoxyribose-5-phosphate aldolase (DERA, EC 4.1.2.4), reversibly catalyzes the aldol reaction of acetaldehyde with D-glyceraldehyde-3-phosphate to form 2-deoxyribose-5-phosphate via a Schiff base intermediate between the active-site lysine and acetaldehyde [4, 5]. Unlike other aldolases, both substrates and the product of DERA are aldehydes; therefore, DERA can perform sequential aldol reactions [5-7]. This type of reaction series can be used to synthesize chiral 2,4,6-trideoxy-D-erythro-hexapyranoside, as the key intermediates in the production of important groups of pharmaceutical products, like atorvastatin, which inhibits HMG-CoA reductase and lowers blood cholesterol.

The use of enzyme-catalyzed technology on an industrial scale is limited by the lack of commercially available enzymes. Thus, economically feasible and reliable enzyme production processes are needed. *Escherichia coli* is the most commonly used host strain for expression of heterologous proteins and biocatalysts [8, 9]. These recombinant proteins are usually synthesized intracellularly in either the cytoplasm or the periplasmic space [10], and overall productivity is a function of both the cell density and the specific yield. A number of methods have been developed to grow *E. coli* to high density for the production of recombinant proteins. One of the most popular is fed-batch culture with controlled nutrient feeding [11-13]; this type of method includes exponential feeding strategies and the traditional on-line feedback methods, such as pH stat, dissolved oxygen stat (DO-stat), and substance stat [14, 15]. Due to the inherent characteristics of *E. coli* cells growth, the exponential feeding strategy is usually adopted because this method makes it easy to achieve high cell density with optical density at 600 nm (OD_{600}) above 200 [16, 17]. The strategy has also been developed to allow cells to grow at constant specific growth rates [18, 19], and with the advantage that acetate production can be minimized. Acetate inhibits cell growth and recombinant protein expression. Therefore, the exponential feeding strategy aims to control the specific growth rate under the critical value of acetate formation. The method is empirical and requires sophisticated control techniques. Improper feeding strategies will lead to the starvation or the accumulation of the restrictive nutrient substance, which cause cell disruption or acetic acid accumulation. Therefore, the appropriate directed fed-batch control strategy must be determined for different recombinant protein expression system [20].

Since aldolases production is growth-associated in *E. coli*, the over-expression of these enzymes exerts an extremely high metabolic burden on host cells, especially when grown under glucose limiting conditions [21]. The preparation of recombinant DERA from *E. coli* strain DH5α had been primarily studied by Wong et al. [5]. However thermophilic DERAs showed much greater catalysis of sequential aldol condensation and higher resistance to high concentration of acetaldehyde [7]. The production of other recombinant aldolases has been studied in fed-batch cultures, as fuculose-1-phosphate aldolase and rhamnulose 1-phosphate aldolase [22, 23]. In this study, the fed-batch control strategy for production of a thermophilic DERA was investigated thoroughly. The gene was from *Hyperthermus butylicus* and was cloned and expressed in *E. coli* BL21. An exponential feeding strategy at different predetermined special growth rates (μ_{set}) was employed to determine the method for maximal DERA productivity in a laboratory bioreactor. These strategies can be used to guide process development for production of other growth-associated proteins.

Materials and Methods

Strain

E. coli BL21 (DE3) (pET303-DERA008) was used as a recombinant strain for 2-deoxyribose-5-phosphate aldolase expression in both batch and fed-batch cultures. This strain contains the gene for DERA from *H. butylicus* under the control of the IPTG-inducible T7 promoter and employs ampicillin as a selection marker. The strain was stored at −80 °C in glycerol stocks.

Media

Luria–Bertani (LB) medium plus antibiotic was used for the preinoculum preparation. The composition was 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl. LB–agar medium was complemented with 15 g/L agar before sterilization.

An optimum semi-synthetic medium (SM-B) with glucose as the sole carbon source was employed in all shake-flask and fermentor cultures. The SM-B medium contained 20 g/L glucose, 32.3 g/L yeast extract (special for microbial medium, Xiwang, China), 15.3 g/L K_2HPO_4, 9.7 g/L KH_2PO_4, 2.63 g/L $MgSO_4{\cdot}7H_2O$, 0.018 g/L EDTA-Na_2, 1 mM ampicillin, and 2.44 mL trace element solution (TES). The glucose and $MgSO_4$ solutions were sterilized separately.

The TES composition (40×) was 1.3 g/L $CoC_{12}{\cdot}6H_2O$, 6.2 g/L $MnC_{12}{\cdot}4H_2O$, 0.6 g/L $CuC_{12}{\cdot}2H_2O$, 1.2 g/L H_3BO_3, 1.1 g/L $Na_2MoO_4{\cdot}2H_2O$, 5.2 g/L Zn (II) acetate·$2H_2O$, and 40.2 g/L Fe (III) citrate.

Feeding medium in fed-batch experiments consisted of 600 g/L glucose, 15.2 g/L $MgSO_4{\cdot}7H_2O$, 1 mM ampicillin, and 76.6 mL L^{-1} TES.

Cultivation Conditions

Inoculum

A unit of glycerinated storage was drawn in lines on a selective LB-agar plate (1 mM ampicillin) and incubated overnight at 37 °C statically. A single clone was inoculated overnight in 15 mL LB medium in a 50-mL test tube at 37 °C on a rotary shaker at 180 rpm. This initial preinoculum culture was transferred to a 500-mL Erlenmeyer flask containing 250 mL SM-B liquid medium and was shaken at 180 rpm at 37 °C. When OD_{600} was over 1.5, the culture was used to inoculate the batch and fed-batch fermentations.

Batch and Fed-Batch Fermentations

Batch and fed-batch fermentations were carried out using a BIOENGINEERING L1523 bioreactor (Bioengineering AG, Switzerland) equipped with a 13-L fermentation vessel. The inoculum culture was used to inoculate 5 L of SM-B medium in the fermentation vessel. The inoculation volume was 5%, which resulted in an initial OD_{600} of approximately 0.1. The temperature was kept at 37 °C throughout the fermentation. Ammonium was supplemented as a concentrated base solution (25%, *w*/*v*, $NH_3{\cdot}2H_2O$) to control pH at 7.00±0.1 and to control the ammonium concentration in the fermentation. To maintain the DO levels above 30%, the agitation speed was between 300 and 1,000 rpm, the reactor pressure was enhanced (≤0.8 atm), and pure oxygen was also used when required. The end of the batch phase was identified by a reduction in the oxygen

consumption rate and an increase in pH. Induction was performed by adding IPTG in a single pulse. The amount of IPTG was 61.7 μmol IPTG per each gram of dry cell weight (DCW) expected at the end of the culture. All cultures were performed at least in duplicate.

Feeding Control Programs

Fed-batch fermentations were performed using an exponential feeding profile to keep a constant specific growth rate. Glucose was the limiting substrate. Exponential feeding was based on the equation [24]:

$$F(t) = \frac{\mu_{\text{set}} X_0 V_0 \exp(\mu_{\text{set}} t)}{S_{\text{f}} Y_{\text{X/S}}} \tag{1}$$

where $F(t)$ is the continuous feed rate (L/h); X_0 and V_0 are the cell concentration (g DCW/L) and the culture volume (L) at the beginning of the fed-batch phase, respectively; S_{f} is the glucose concentration in feed solution (g/L); and μ_{set} is the predetermined specific growth rate (h^{-1}). $Y_{\text{X/S}}$ is the growth yield biomass/glucose (g DCW g^{-1} glucose). In our studies, the feed rate (F) was altered every hour to simulate the exponential feeding process.

Analytical Procedures

The cell density was determined by measuring the culture OD_{600} with a UV-2550 spectrophotometer (Shimadzu, Japan). The cell pellets were washed twice and diluted with a 0.9% NaCl solution to obtain an OD_{600} between 0.2 and 0.8. A standard curve was used to convert the OD_{600} to DCW. In our studies, one optical density unit was equal to 0.38 gDCW/L.

The glucose concentrations were determined by the dinitrosalicylic acid method [25]. Acetate concentration was analyzed using a gas chromatograph with an FID detector and a glass column packed with Thermon 300 (Shimadzu, Japan) [18]. The injector and column temperatures were 180 and 100 °C, respectively. Nitrogen was used as the carrier gas at flow rate of 1 mL/min. The FID detector temperature was 200 °C. An acetate peak appeared 6.7 min after sample injection. A calibration curve constructed from acetic acid standards was used to obtain the acetate concentration.

DERA production in *E. coli* cell was analyzed by SDS-PAGE (15%) of soluble whole cell proteins. The expression level of the protein was determined using a gel imaging system (GE, USA) and expressed as a percentage of the total protein. The culture samples were centrifuged at 8,000 rpm for 5 min, and the harvested cells were re-suspended in phosphate buffer solution (0.1 M, pH7.4) to a final OD_{600} about 8. Cell suspensions were placed in ice and disrupted by sonication (Sonicator 4000, MISONIX, USA). The crude cell extract was then centrifuged at 12,000 rpm for 30 min and the precipitate was discarded. The total soluble protein concentrations were determined by the Coomassie brilliant blue dye-binding method with bovine serum albumin as the standard. Concentration of the target protein was determined as the total soluble protein concentration multiplied by the expression level.

Results and Discussion

Batch Cultures

Initial Erlenmeyer flask experiments at different induction starting times were performed. Results allowed us to determine that the optimal induction opportunity was the prophase of

logarithmic growth. Batch fermentation was carried out in a bioreactor, and the results were shown in Fig. 1. After culture for 4 h at OD_{600} of about 3.0, 2 mmol IPTG was added to induce the expression of DERA. The growth was stopped after 11 h when glucose was depleted. The biomass of 10.0 g DCW/L was obtained (equivalent to an OD_{600} of 26.3) (Fig. 1a). $Y_{X/S}$ was calculated from the start of the exponential growth phase, 0.55 gcell g^{-1} glucose. The final enzyme concentration and the total volumetric productivity were only 0.47 g/L and 0.043 $gL^{-1}h^{-1}$, respectively. During the culture, acetate was produced, and the final concentration was 2.07 g/L (Fig. 1b). When the acetate concentration was above 1.0 g/L, the protein expression levels began to decrease from the maximal value of 34.1% to 21.3% finally (Fig. 1c).

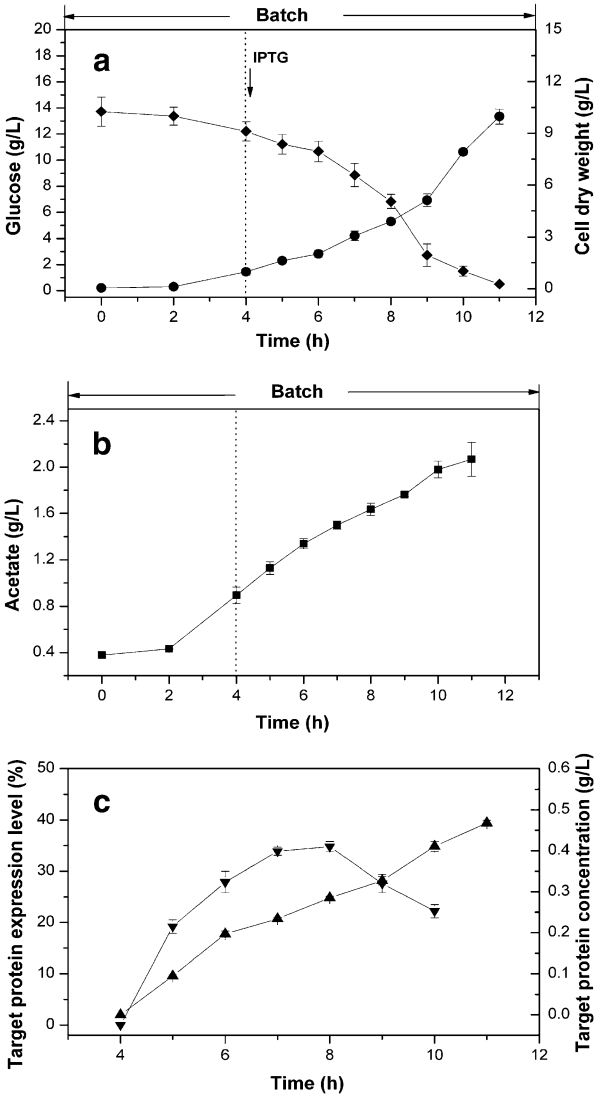

Fig. 1 **a**–**c** Batch culture course of *E. coli* BL21 (pET303-DERA008) in a bioreactor. IPTG was added at 4 h. *Circles* biomass (g DCW L^{-1}), *diamonds* glucose (g/L), *squares* acetate (g/L), *triangles* target protein concentration (DERA, g/L), *inverted triangles* target protein expression level (%)

In batch cultures, the accumulation of acetate was one of the main problems encountered in high-density cultivation of recombinant *E. coli* [12, 16]. Acetate metabolism and its effect on *E. coli* growth have been studied extensively during the past few decades. The general view is that the acetate accumulation is due to rapid growth on glucose, which leads to saturation of the TCA cycle and saturation of the electron transport process [26, 27]. It is a general consensus that fed-batch strategies control the cell growth rate and inhibit acetate production to improve recombinant protein synthesis.

Fed-Batch Culture with μ_{set}=0.20 h^{-1}

To maximize recombinant protein production, it is critical that maximal final biomass density (g DCW/L) and maximal volumetric concentrations be achieved at the end of the growth period. As a result, feeding strategies must be optimized for each recombinant protein expression system. In all the fed-batch fermentations of this study, V_0=5 L, S_f= 570 g/L, $Y_{X/S}$=0.55 gcell g^{-1} glucose, X_0 was the actual biomass concentration in each culture, and μ_{set} was 0.20, 0.15, and 0.10 h^{-1}.

Data from a fed-batch cultivation with μ_{set}=0.20 h^{-1} are shown in Fig. 2. The fed-batch phase was divided into two parts. The first part was used to allow the biomass density to reach an appropriate scale, and the second one was the induction for the recombinant enzyme production.

The fed-batch culture was performed for 4 h, and the biomass yield was 21.0 g/L; 19.4 mmol of IPTG was injected into the fermentor, which was determined based on a maximum expected biomass expected of 45 g/L and the criteria of 61.7 μmol IPTG per gram of DCW. After the induction, the biomass specific growth rate decreased suddenly, from 0.19 to 0.10 h^{-1}, which was less than the μ_{set} of 0.20 h^{-1}. Synchronously, the acetate and glucose began to accumulate (Fig. 2a, b). This indicates that the biomass growth and the consumption rate of glucose were inhibited. Consequently, the glucose accumulation was accelerated and was no longer limiting. In this case, acetate accumulated, even at low growth rates, because the rate of glucose consumption (q_s) was at its maximum value as glucose was in excess [28, 29]. Konstantinov et al. also reported that glucose enters the fermentative pathway, and acetic acid is formed when the carbon flow exceeds the capacity of the oxidative pathway [26].

After approximately 22 h of growth, the culture halted at a final biomass density of 50.3 g/L and acetate concentration of 4.2 g/L (Fig. 2a, b). The production of DERA is shown in Fig. 2c. As the induction proceeded, the target protein expression level first increased and then decreased, and DERA concentration improved and reached a plateau after 6 h of induction. The results were mainly due to the accumulation of acetate. The previous researches have also identified the adverse effects of acetate in growth medium [30, 31]. During the early stages of fed phase, acetate can be utilized as the carbon source. When the concentration of acetate was over 1.0 g/L, DERA expression level decreased. In this process, the maximal enzyme expression level and concentration were 42.1% and 3.88 g/L, but with the accumulation of acetate after induction, the final expression level was decreased to 28.7%. The total volumetric productivity of DERA was 0.173 $gL^{-1}h^{-1}$. Therefore, the fed-batch method with μ_{set}=0.20 h^{-1} was improper for the production of recombinant DERA in *E. coli* BL21 that the accumulation of acetate inhibited the cell growth rate and decreased the expression of recombinant protein.

Fed-Batch Culture with μ_{set}=0.15 h^{-1}

Another fed-batch experiment was performed with the μ_{set} of 0.15 h^{-1} (Fig. 3). The culture process was the same as described above. After 4 h of culture, 19.4 mmol of IPTG was

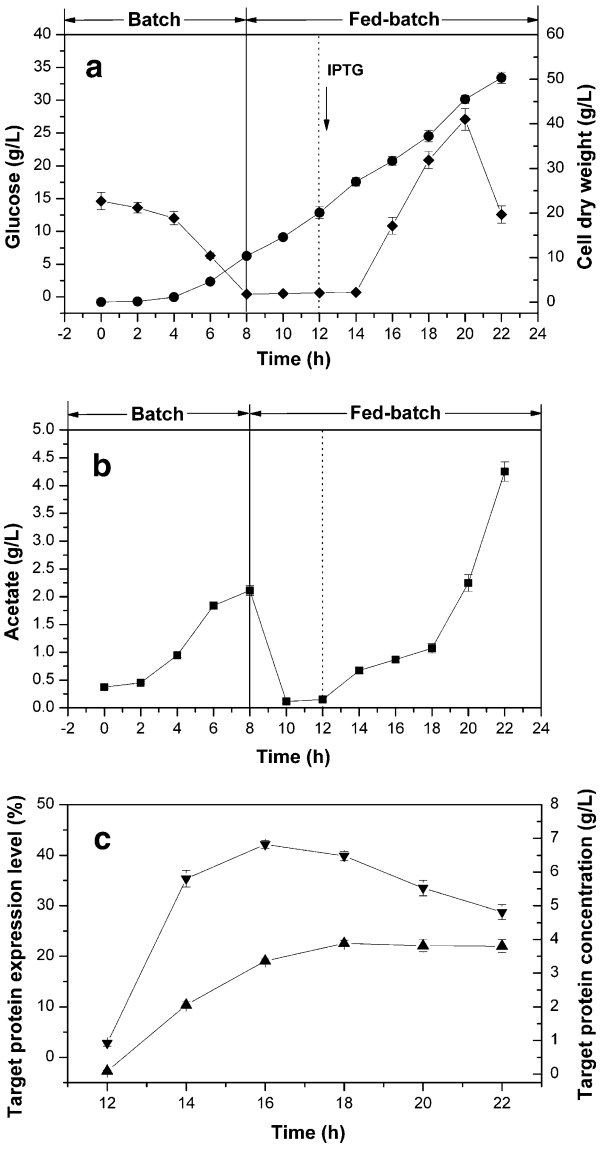

Fig. 2 a–c Fed-batch culture course of *E. coli* BL21 (pET303-DERA008) with the μ_{set}= 0.20 h^{-1}. IPTG was added after cultivated for 12 h. *Circles* biomass (g DCW L^{-1}), *diamonds* glucose (g/L), *squares* acetate (g/L), *triangles* target protein concentration (DERA, g/L), *inverted triangles* target protein expression level (%)

added for induction of DERA expression. At this time, the biomass concentration in bioreactor was 19.0 g/L. In the fed-batch culture process, acetate and glucose did not accumulate obviously, and the glucose concentration was maintained below 1.0 g/L, and the acetate concentration was less than 0.4 g/L. At the end of the culture phase, the biomass concentration was above 43.8 g/L (Fig. 3a, b). During the pre-induced phase of fed-batch culture, it was calculated from the experiment data that the experimental value of μ_{exp} was 0.15 h^{-1}, whereas the value decreased to 0.11 h^{-1} after induction, and it was similar to the results of fed-batch cultivation with μ_{set}=0.20 h^{-1}.

The DERA production is shown in Fig. 3c. After 4 h of induction, the target protein expression was maintained at a stable level of about 40% through to the end of culture. In the induction process, the DERA concentration increased with the biomass growth and

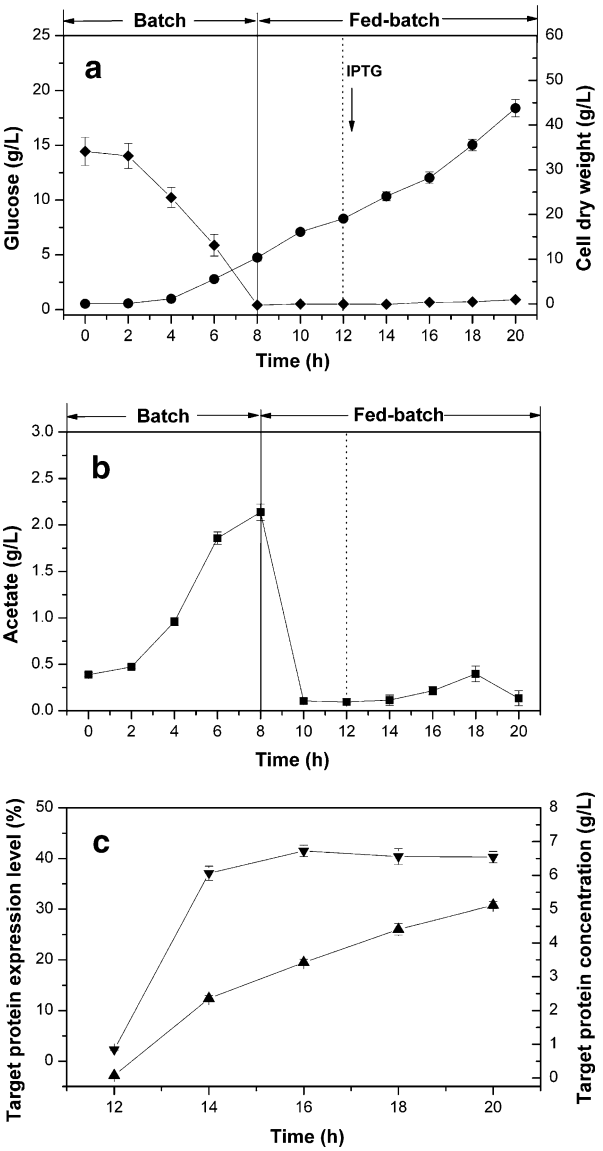

Fig. 3 a–c Fed-batch culture course of *E. coli* BL21 (pET303-DERA008) with the μ_{set}= 0.15 h^{-1}. IPTG was added at 12 h. *Circles* biomass (g DCW L^{-1}), *diamonds* glucose (g L^{-1}), *squares* acetate (g L^{-1}), *triangles* target protein concentration (DERA, g L^{-1}), *inverted triangles* target protein expression level (%)

peaked at the end of the culture period at 5.12 g/L with total volumetric productivity of 0.256 $gL^{-1}h^{-1}$, which were over 10 and 5-fold higher than conventional batch culture, respectively. The DERA concentration in the whole cell determined by SDS-PAGE at different fermentation times is shown in Fig. 4. In this fed-batch culture, the highest biomass concentration gave the highest enzyme concentration.

Fed-Batch Culture with μ_{set}=0.10 h^{-1}

Another fermentation profile is shown in Fig. 5. The fed-batch phase started at 8 h with μ_{set}=0.10 h^{-1}. In this experiment, glucose and acetate did not accumulate and remained

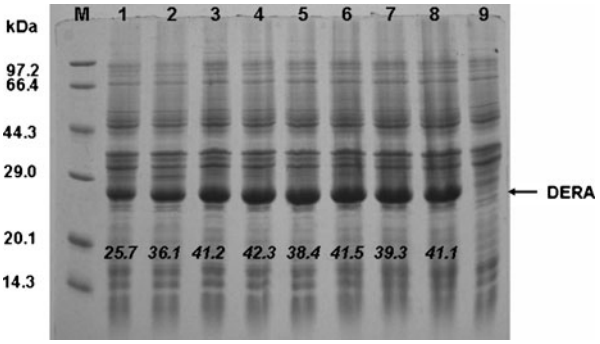

Fig. 4 SDS-PAGE of samples of the fed-batch fermentation of *E. coli* BL21 (pET303-DERA008) with the μ_{set}=0.15 h^{-1}. *M* indicates molecular mass standard. Lane labels indicate induction time: (*1*) 1 h, (*2*) 2 h, (*3*) 3 h, (*4*) 4 h, (*5*) 5 h, (*6*) 6 h, (*7*) 7 h, (*8*) 8 h, and (*9*) 0 h (induction start). *Arrow* marks the target protein, DERA. DERA as a percentage of total protein is shown in *italics* under the DERA protein band

under 1.0 and 0.15 g/L, respectively (Fig. 5a, b). After 4 h of fed-batch culture, the biomass concentration reached 15.8 g/L. At this time, a certain amount of IPTG was added. After approximately 6 h of induction, the target protein expression reached a plateau at above 40% (Fig. 5c), and the induction phase lasted 12 h. At the end of culture, the final biomass concentration was 37.8 g/L. The DERA concentration increased with the biomass, and the final value was 4.31 g/L with total volumetric productivity of 0.179 $gL^{-1}h^{-1}$.

When μ_{set} was set to the value of 0.10 h^{-1}, the acetate did not accumulate with the biomass growth and protein expression, and the acetate concentration was maintained under 0.10 g/L. However, the lower feeding rate directly led to decrease the final DERA concentration and total volumetric productivity. Chen et al. have investigated the effect of specific growth rate (μ) on the production of a recombinant nuclease in fed-batch cultures and found that the specific nuclease production rate increased with increasing μ [18]. The results were similar to the previous researches and further confirmed that feeding rates are important for the production of recombinant protein in fed-batch cultures.

In the above fed-batch cultures, it was not ignorable that the actual experiment special growth rates (μ_{exp}) after induction were less than the values of μ_{set}, even when the acetate concentrations were maintained a lower level. These results were principally related with the metabolic burden associated with recombinant protein expression in the cells. The transition on growth performance showed that cells shifted the usage of the available substrate from anabolic to catabolic pathways to increase their ability to produce energy for plasmid-encoded protein synthesis, stress protein synthesis, and maintenance requirements. Özkan et al. observed that the expression of fusion protein GI-malE in recombinant *E. coli* XL1 induced by IPTG caused a severe retardation in cell growth rate from μ=0.066 to 0.006 h^{-1} and increased acetic acid production [32]. The results were basically due to the pathway fluxes, where induced cells at very low growth rates allocated more substrate to the EMP pathway in order to meet the higher demand for ATP. Hoffman et al. estimated that heat shock proteins accounted for 25% of the total proteins synthesized during high-temperature-induced expression of hFGF-2, and the additional synthesis of the heat shock proteins required an additional 20% of the cell's biosynthetic energy [33]. Therefore, the fed-batch strategies according to Eq. 1 need to be further modified by considering the metabolic burden associated with recombinant proteins expression, which will be a study emphasis in our further research.

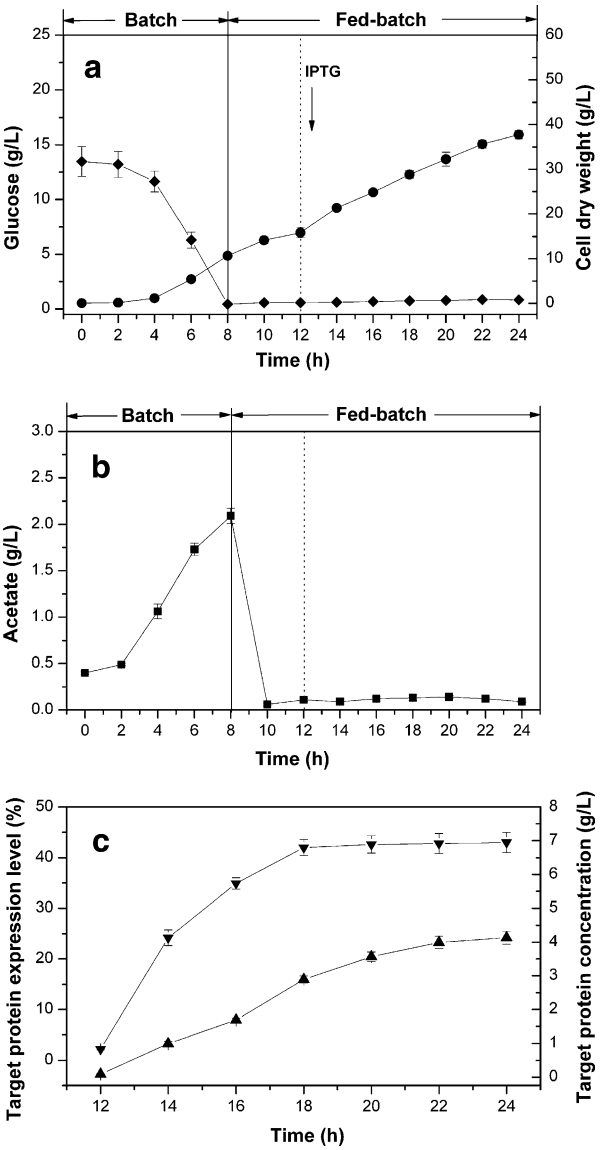

Fig. 5 Fed-batch culture course of *E. coli* BL21 (pET303-DERA008) with the μ_{set}= 0.10 h^{-1}. IPTG was added at 12 h. *Circles* biomass (g DCW L^{-1}), *diamonds* glucose (g L^{-1}), *squares* acetate (g L^{-1}), *triangles* target protein concentration (DERA, g L^{-1}), *inverted triangles* target protein expression level (%)

Conclusions

DERA catalyzes a sequential aldol reaction that is useful to synthesize a key fragment of atorvastatin. In this study, the production of a thermophilic DERA produced in *E. coli* BL21 (pET303-DERA008) with different feeding methods with different μ_{set} have been evaluated. The host could synthesize the enzyme most efficiently at μ_{set}=0.15 h^{-1}. The maximum enzyme concentration and total volumetric productivity, 5.12 g/L and 0.256 g $L^{-1}h^{-1}$, respectively, were over 10- and 5-fold higher than conventional batch culture. In this case, the acetate concentration remained at a relatively low level and did not inhibit cell

growth and target protein expression. This efficient process for the production of thermophilic DERA was designed from the point of view of recombinant enzyme concentration and productivity. This process is economically and technically viable and can be used as a guide for production of other synthetically useful enzymes.

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References

1. Kobayashi, S., Uchiro, H., Shiina, I., & Mukaiyama, T. (1993). *Tetrahedron, 49*, 1761–1772.
2. Dean, S. M., Greenberg, W. A., & Wong, C. H. (2007). *Advanced Synthesis & Catalysis, 349*, 1308–1320.
3. Chen, L., Dumas, D. P., & Wong, C. H. (1992). *Journal of the American Chemical Society, 114*, 741–748.
4. Samland, A. K., & Sprenger, G. A. (2006). *Applied Microbiology and Biotechnology, 71*, 253–264.
5. Wong, C. H., Garcia-Junceda, E., Chen, L., Blanco, O., Gijsen, H. J. M., & Steensma, D. H. (1995). *Journal of the American Chemical Society, 117*, 3333–3339.
6. Liu, J. J., Hsu, C. C., & Wong, C. H. (2004). *Tetrahedron Letters, 45*, 2439–2441.
7. Sakuraba, H., Yoneda, K., Yoshihara, K., Satoh, K., Kawakami, R., Uto, Y., et al. (2007). *Applied and Environmental Microbiology, 73*, 7427–7434.
8. Riesenberg, D., & Guthke, R. (1999). *Applied Microbiology and Biotechnology, 51*, 422–430.
9. Matsui, T., Sato, H., Yamamuro, H., Misawa, S., Shinzato, N., Matsuda, H., et al. (2008). *Journal of Biotechnology, 134*, 88–92.
10. Choi, J. H., Keum, K. C., & Lee, S. Y. (2006). *Chemical Engineering Science, 61*, 876–885.
11. Korz, D. J., Rinas, U., Hellmuth, K., Sanders, E. A., & Deckwer, W. D. (1995). *Journal of Biotechnology, 39*, 59–65.
12. Lee, J., Lee, S. Y., Park, S., & Middelberg, A. P. J. (1999). *Biotechnology Advances, 17*, 29–48.
13. Lau, J., Tran, C., Licari, P., & Galzzzo, J. (2004). *Journal of Biotechnology, 110*, 95–103.
14. Kim, B. S., Lee, S. C., Lee, S. Y., Chang, Y. K., & Chang, H. N. (2004). *Bioprocess and Biosystems Engineering, 26*, 147–150.
15. Yano, T., Kurokawa, M., & Nishizawa, Y. (1991). *Journal of Fermentation and Bioengineering, 71*, 345–349.
16. Shiloach, J., & Fass, R. (2005). *Biotechnology Advances, 23*, 345–357.
17. de Durany, O., Mas, C., & Josep, L. S. (2005). *Process Biochemistry, 40*, 707–716.
18. Cheng, L. C., Hor, L. I., Wu, J. Y., & Chen, T. L. (2003). *Biochemical Engineering Journal, 14*, 101–107.
19. de Jaume, P., Mas, C., & Josep, L. S. (2006). *Biochemical Engineering Journal, 29*, 235–242.
20. Yee, L., & Blanch, H. W. (1992). *Nature Biotechnology, 10*, 1550–1556.
21. de Jaume, P., Mas, C., & Josep, L. S. (2008). *Biochemical Engineering Journal, 41*, 181–187.
22. Durany, O., Bassett, P., Weiss, A. M. E., Cranenburgh, R. M., Ferrer, P., Josep, L. S., et al. (2005). *Biotechnology and Bioengineering, 91*, 460–467.
23. Vidal, L., Ferrer, P., Alvaro, G., Benaiges, M. D., & Caminal, G. (2005). *Journal of Biotechnology, 118*, 75–87.
24. Lee, S. Y. (1996). *Trends Biotechnology, 16*, 98–105.
25. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428.
26. Konstantinov, K., Kishimoto, M., Seki, T., & Yoshida, T. (1990). *Biotechnology and Bioengineering, 36*, 750–758.
27. Han, K., Lim, H. C., & Hong, J. (1992). *Biotechnology and Bioengineering, 39*, 663–671.
28. Hua, Q., Yang, C., Oshima, T., Mori, H., & Shimizu, K. (2004). *Applied and Environmental Microbiology, 70*, 2354–2366.
29. Emmerling, M., Dauner, M., Ponti, A., Fiaux, J., Hochuli, M., Szyperski, T., et al. (2002). *Journal of Biotechnology, 184*, 152–164.

30. Johnston, W., Cord-Ruwisch, R., & Cooney, M. J. (2002). *Bioprocess and Biosystems Engineering, 25*, 111–120.
31. Bauer, K. A., Ben Bassat, A., Dawson, M., De la Puente, V. T., & Neway, J. O. (1990). *Applied and Environmental Microbiology, 56*, 1256–1302.
32. Ozkan, P., Sariyar, B., Utkur, F. O., Akman, U., & Hortacsu, A. (2005). *Biochemical Engineering Journal, 22*, 167–195.
33. Hoffman, F., & Rinas, U. (2001). *Biotechnology and Bioengineering, 76*, 333–340.