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Efficient Production of a Thermophilic 2-Deoxyribose-5-Phosphate Aldolase in Glucose-Limited Fed-Batch Cultivations of *Escherichia coli* by Continuous Lactose Induction Strategy

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Abstract The production of a thermophilic 2-deoxyribose-5-phosphate aldolases (DERA) in *Escherichia coli* BL21 under continuous lactose induction strategy was investigated. The process was combined with the exponential feeding method, controlling the feeding rate to maintain the specific growth rate at 0.15 h^{-1}. The results indicate that the lactose concentration in the feed medium affected directly the expression of the target protein. The use of 50 g/L in the feed medium resulted in the biomass concentration of 39.3 g DCW/L, and an expression level of above 30%, and the maximum final DERA concentration of 16,200 U/L. Furthermore, the acetate concentration remained at a low level in the fed-batch phase, less than 0.5 g/L. In conclusion, combining glucose feeding with lactose induction is a more powerful way to achieve high cell density cultures and to efficiently produce the thermophilic DERA. The results also indicate the potential industrial utility in the scale production of other recombinant proteins.

Keywords 2-Deoxyribose-5-phosphate aldolase · Lactose induction · Continuous · Glucose limited · Fed-batch cultivation

Introduction

Enzymes are now widely exploited as catalysts in asymmetric organic synthesis, due to their exquisite chemo-, region-, and stereospecificity [1, 2]. Aldolases catalyze enantioselective C–C bond formation and generate up to two chiral centers as powerful tools for the industrial synthesis of chiral molecules [3]. 2-Deoxyribose-5-phosphate aldolases (DERAs, EC 4.1.2.4) belong to the class I aldolase and reversibly catalyze the aldol reaction between acetaldehyde

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and D-glyceraldehyde-3-phosphate to form 2-deoxyribose-5-phosphate via a Schiff base intermediate between the active-site lysine and acetaldehyde [4]. The main difference compared to other aldolases is the fact that both substrates and the product are aldehydes. Therefore, DERAs can perform a sequential aldol reaction, starting from three achiral aldehydes and forming 2,4,6,-thideoxy-D-erythro-hexapyranoside [5]. The hemiacetal is employed in the synthesis of the antitumor drugs epothilone A and C, iminocyclitols, and statins.

Many DERAs from different microbial origins have been studied and characterized, including eukaryotes, bacteria, and archaea [6, 7]. Gijsen and Wong were the first to observe *Escherichia coli* DERA catalyzing a double aldol condensation of three acetaldehyde molecules [8]. Recently, much attention has been paid to the isolation and characterization of enzymes from hyperthermophiles due to their higher thermal stability and higher resistance to most chemical denaturants [9]. $DERA_{Pae}$ and $DERA_{Tma}$ from two hyperthermophiles showed much greater catalytic efficiency of sequential aldol condensation using three acetaldehydes as substrates than mesophilic *E. coli* enzyme [10].

E. coli is the most commonly used prokaryotic host because it has been best characterized in terms of its molecular genetics, physiology, and expression systems, and aerobic high cell density cultures of this host are most frequently used to obtain high biomass yields and high recombinant protein concentrations [11]. The *lac* promoter and its derivatives (*tac*, *pac*, *rac*) belong to the strongest bacterial promoters which are frequently used for the induced overexpression of foreign genes in *E. coli*, and they are generally induced by isopropyl-β-D-thiogalactoside (IPTG). IPTG induction is effective at a small dose, and the process is not affected by the presence of glucose. However, IPTG is not only costly, and it also is toxic to humans so that its presence as a contamination of the final purified protein product is problematic. As an alternative, lactose, the natural inducer of the *lac* operon, has been studied to induce recombinant proteins expression as effective as IPTG, and can also enhance the solubility of the overexpressed protein in *E. coli* [12, 13]. Nevertheless, only a limited number of laboratory studies have examined continuous lactose induction strategy to achieve high cell density culture of recombinant *E. coli*, especially in glucose-limited fed-batch cultivations.

In previous studies, the DERA from *Hyperthermus butylicus* was subcloned and overexpression in *E. coli* BL21. The enzyme exhibited higher thermal stability and remarkable resistance to acetaldehyde [14]. In addition, the effects of cell growth rate in fed-batch cultures on the production of the enzyme was also investigated [15]. The current work was undertaken to investigate a continuous lactose induction strategy in glucose-limited fed-batch cultivations of *E. coli* for the production of the thermophilic DERA. Cell growth and the target protein production were discussed. The study was expected to decrease the enzyme production cost, and to establish the base for industrialization.

Materials and Methods

Bacterial Strain and Plasmid

E. coli BL21 (DE3) was used as the host for recombinant thermophilic DERA expression. The DERA expression vector, pET303-DERA008 was introduced into *E. coli* cells, which expressed the target recombinant protein under the control of the IPTG-inducible T7 promoter and employed ampicillin as selection pressure.

Media

The Luria Broth (LB) medium supplemented with antibiotic was used for the preinoculum preparation and shake flask cultures, and LB-agar medium was complemented with 15 g/L agar before sterilization. A semi-synthetic medium (SM-B) with glucose as the sole carbon source was employed in all fermenter cultures [15]. For the fed-batch growth phase, the feeding solution contained (per liter): 600 g glucose, 15.2 g $MgSO_4 \cdot 7H_2O$, 1 mmol ampicillin, and 60 mL trace element solution (TES). For the induction phase, the induction solution contained (per liter): 600 g glucose 15.2 g $MgSO_4 \cdot 7H_2O$, 1 mmol ampicillin, 60 mL TES, and different concentrations of lactose. The TES composition (×40, per liter) was 1.3 g $CoC_{l2} \cdot 6H_2O$, 6.2 g $MnC_{l2} \cdot 4H_2O$, 0.6 g $CuC_{l2} \cdot 2H_2O$, 1.2 g H_3BO_3, 1.1 g $Na_2MoO_4 \cdot 2H_2O$, 5.2 g Zn (II) acetate$\cdot 2H_2O$, and 40.2 g Fe (III) citrate.

Cultivation Conditions

A single colony from selective LB-agar plate containing 1 mM ampicillin 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 OD600 was over 1.5, the culture was used to inoculate all fermentations.

For shake flask experiments, inoculums were inoculated into 500-ml shake flasks containing 100 mL LB medium at an inoculation volume of 3%. The cultures were cultured in duplicate at 37 °C with 180-rpm agitation. When the optical density (OD_{600}) reached 0.8, induction was done by addition of 0.5 mM IPTG or the different concentration of lactose under the same conditions.

Subsequent fed-batch studies were conducted in controlled fermentations of initial 5.0 L SM-B medium in 13 L fermenter (BIOENGINEERING L1523, Bioengineering AG, Switzerland) [15]. All fed-batch fermentations were performed using an exponential feeding profile to keep a constant specific growth rate, 0.15 h^{-1}. Exponential feeding was accorded as the typical equation (Eq. 1):

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

Where $F(t)$ is the continuous feed rate (liters per hour), X_0 and V_0 are the cell concentration (grams DCW per liter) and the culture volume (liter) at the beginning of the fed-batch phase, respectively, S_{f} is glucose concentration in feed solution (grams per liter), and specific growth rate (μ_{set}, per hour). $Y_{\text{X/S}}$ is the biomass growth yield (gram DCW per gram of glucose). The preliminary cultivations revealed that $Y_{\text{X/S}}$ equals to 0.55 g DCW per gram of glucose.

Determination of Recombinant DERA

DERA expression levels in *E. coli* cells were analyzed by SDS-PAGE (15%) of soluble whole cell proteins [15]. DERA activity was monitored by determining D-2-deoxyribose-5-phosphate (DRP)-decomposing activity at 25 °C. The DPR cleavage activity was determined by measuring the oxidation of NADH in coupled assay using triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase [10]. The assay mixture contained 100 mM imidazole HCl buffer (pH 6.5), 0.4 mM DRP, 0.1 mM NADH, 5.5 units of triose-phosphate isomerase

(Rabbit muscle, Sigma), 2 units of glycerol-3-phosphate dehydrogenase (Rabbit muscle, Sigma), and the enzyme preparation in a total volume of 0.5 ml. The change in absorbance of NADH was followed at 340 nm (ε=6.22 mM^{-1} cm^{-1}). One enzyme unit is defined as the amount catalyzing the cleavage of 1 μmol of DRP per minute.

Analytical Methods

Samples periodically taken from the culture broth were immediately centrifuged at 8,000×*g* for 5 min, and the supernatants were stored at 4 °C prior to analysis of glucose, lactose, and acetate. The residual glucose in the broth was determined by biochemical analyzer (BioProfile 300B, NOVA, USA). The total reducing sugar concentration was detected by dinitrosalicylic acid method [16], and the lactose concentration was determined by the total reducing sugar concentration minus residual glucose.

Acetate concentration was analyzed by gas chromatograph (GC-2010, Shimadzu, Japan) equipped with an FID detector and a glass column (PEG-20 M, 30 m×0.25 mm), and the cell density was determined by measuring the culture optical density at 600 nm with a UV-2550 spectrophotometer (Shimadzu, Japan) [15].

Results and Discussion

Preliminary Experiments on Lactose Induction

To test the potential of lactose to induce the thermophilic DERA synthesis in *E. coli*, cells were grown in LB medium and induced by adding lactose to a final concentration of 4.0 gL^{-1}. As shown in Fig. 1a, the biomass growth of the lactose-induced cultures increased, while it of cultures induced with IPTG decreased significantly compared with the non-induced cultures. The cultures halted after 10 h of cultivation, and the cell density (OD_{600}) reached 5.9 with lactose as inducer, while it was only 3.9 under IPTG induction. This is in agreement with the results of previous researches by the batch addition of lactose. The metabolic load of DERA expression reduced cell growth with IPTG inducing, but the added carbon source in the medium when inducing with lactose provided the energy required for continued cell growth [17].

Furthermore, lactose can induce DERA formation efficiently as IPTG (Fig. 1b). The expression level of target protein increased with the induction process progressed and reached a plateau with an approximately equal maximum value after approximately 2 h of induction, above 40%. It was generally accepted that there was inevitably a delay in recombinant protein expression using lactose as inducer [18]. However, no significant difference was observed from the expression level of DERA with the two different inducers. It is explained possibly that the length of lag phase may be a reflection of conversion from lactose to allolactose, and is also related with different strains and recombinant proteins, from several quarters to a few hours reportedly.

The Effect of the Lactose Content on the Production of Active DERA

To examine the effect of the lactose content on cell growth and the expression of the thermophilic DERA, the following cultures with different concentration of lactose were employed (grams per liter): 0.1, 0.4, 1.0, 2.0, 4.0, 8.0, and 14.0, and the results are shown in Fig. 2. The cell growth increased with the initial concentration of lactose ranging from

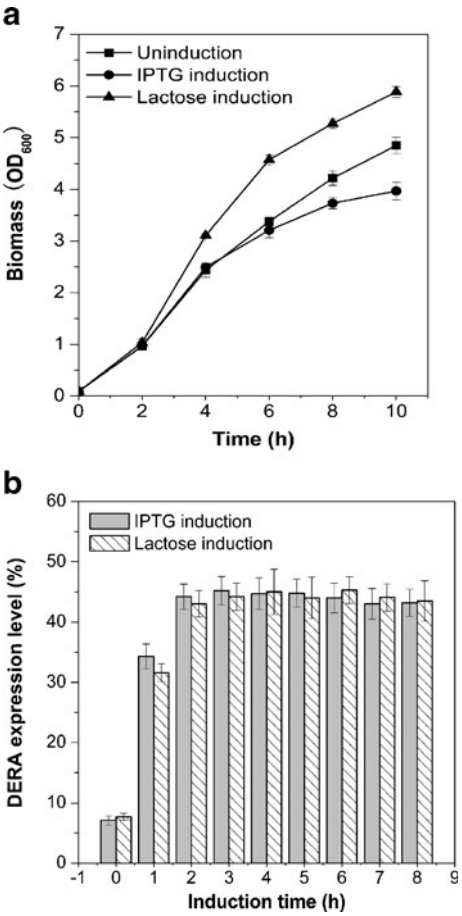

Fig. 1 The growth of *E. coli* BL21 (pET303-DERA008) (**a**) and expression levels of recombinant DERA (**b**) using lactose and IPTG in LB medium

0.1 to 2.0 g/L, but there were no significant differences with the concentration exceeded 2.0 g/L (Fig. 2a).

The production of active DERA is shown in Fig. 2b. The enzyme concentration increased with the lactose content and reached a plateau at a concentration of 2.0 g/L. Based on the results presented by Gombert and Kilikian, the lactose concentration plays an important role in the heterogenous proteins expression [19]. It is clear that the critical amounts of lactose for inducing maximum DERA expression in LB medium was 2.0 g/L, namely, 1.04 g lactose/g DCW. It is worthwhile to note that the cell growth was inhibited slightly at the lactose concentration of 0.1~0.4 g/L. It is possible due to the ways of lactose induction in LB medium (Fig. 2a). Lactose is transported into the cell and first converted into allolactose to induce the expression of recombinant protein. When lactose was sufficient in medium, a part of it would be as carbon source to improve cell growth.

Glucose-Limited Fed-Batch Cultivations Using Lactose as Inducer

To maximize recombinant protein, production means both achieving maximal final culture densities and maximal specific activity leading to maximal volumetric active product

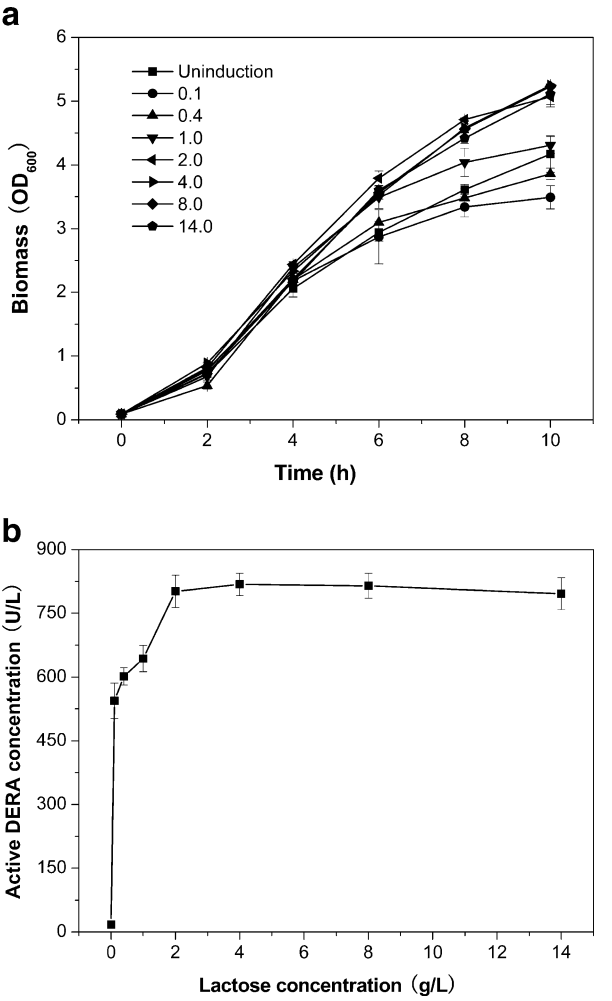

Fig. 2 Effect of lactose concentration on the growth of *E. coli* BL21 (pET303-DERA008) (**a**) and expression levels of recombinant DERA (**b**) in LB medium

concentration. In this work, two exponential fed-batch cultivations induced by lactose were carried out, and μ_{set} of 0.15 h^{-1} was chosen to avoid acetate accumulation. 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 target enzyme production.

Data from a fed-batch cultivation is shown in Fig. 3. The fed-batch culture was performed 4 h, and the biomass yield was 17.1 g/L. Meanwhile, the induction solution containing 210 g/L lactose was added into the culture broth immediately, which was determined based on a maximum expected biomass expected of 40 g/L and the criteria of 1.04 g lactose per gram of DCW. 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 0.5 g/L. However, the lactose concentration continually increased, the maximum concentration at 28.6 g/L, and the residual lactose can also improve cell growth as carbon source after the end of feeding. After approximately 24 h of growth, the culture halted at a final biomass density of 45 g/L (corresponding to a

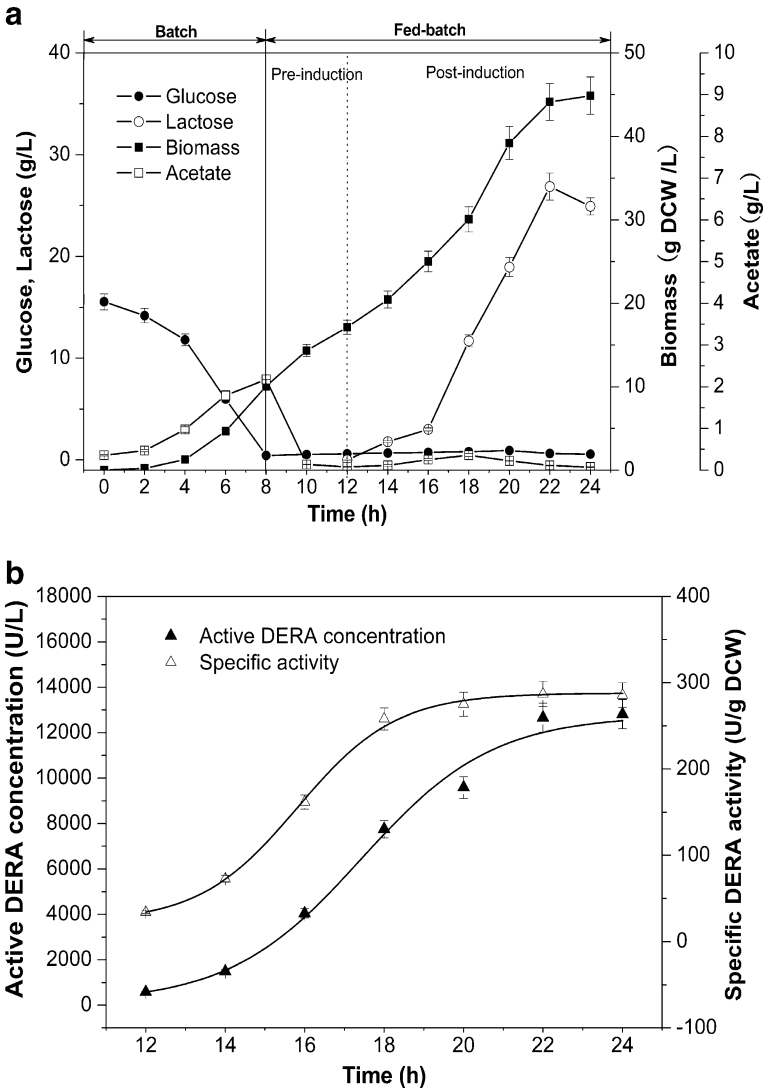

Fig. 3 Time course of biomass and DERA production in a glucose-limited fed-batch culture at 210 g/L lactose

final OD≈115; Fig. 2b). The production of target protein is shown in Fig. 3b, where the active enzyme produced followed parallel to biomass growth and peaked at the end of the culture at 12,800 U/L. After approximately 6 h of induction, the DERA expression levels reached a plateau at above 30%, and the specific activity reached a plateau at 260 U/g DCW.

In order to avoid the high residual concentration of lactose in the medium, a new fed-batch experiment was performed using a feed medium containing 50 g/L (Fig 4). The biomass growth presented a similar tendency with above culture, the final concentration above 39.3 g DCW/L (equivalent to an OD of 105). At the feeding phase, glucose and lactose did not accumulate and remained under 0.5 and 0.3 g/L, respectively,

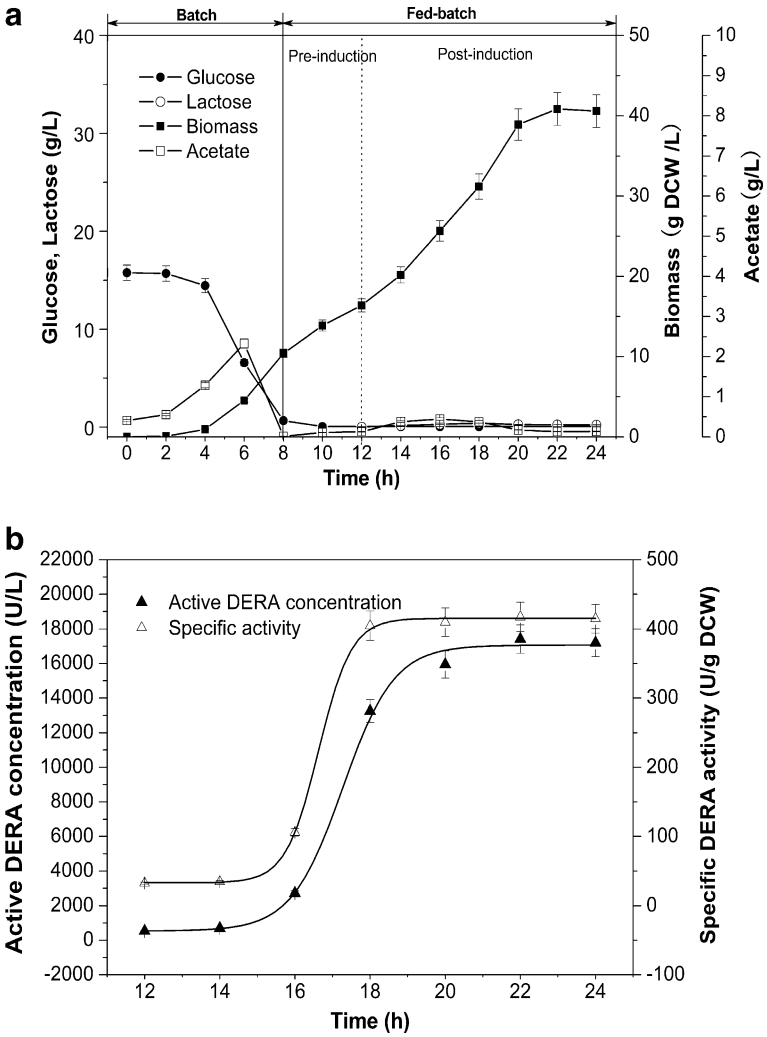

Fig. 4 Time course of biomass and DERA production in a glucose-limited fed-batch culture at 50 g/L lactose

and the acetate concentration was less than 0.5 g/L. The production of active DERA is shown in Fig. 4b. The amount of active enzyme produced in proportion to the biomass, and the expression reached above 30% of total protein after approximately 6 h of induction, and the maximum specific activity was also achieved, about 410 U/g DCW. However, the target protein was nearly expressed in the first 2 h of induction phase. It is possibly explained that the concentration of lactose was very low in the initial stage. The final DERA concentration of 16,200 U/L was obtained. When growth began to slow down and eventually stop, the production of active enzyme also ceased. Therefore, the production of active DERA appears so closely linked to cellular viability, and this final value could be increased if the induction operations proceed to a higher biomass concentration.

Lactose as an inducer for *lac* promoter or its derivative-controlled genes expression is attractive for industrial scale cultivation mainly for cost reasons and the lack of

toxicity [20]. However, lactose cannot be used in processes with glucose as carbon and energy source because of glucose repression and inducer exclusion. Therefore, glycerol was used instead of glucose in former studies [17, 21], or the culture process was completely divided into two phases, namely the biomass growth to a higher density and the recombinant protein expression by lactose [22]. From the view of economy and practice, it would be more interesting to adopt a continuous lactose induction strategy in glucose-limited fed-batch cultivations. Hoffman et al. described the method for high express the *Ricinus communis* stearoyl-acyl carrier protein Δ^9 desaturase in *E. coli* BL21 (DE3), and the glucose concentration in the fermentation medium was determined using an in-line sampler with a glucose flow injection analyzer and was maintained between 0.05 and 0.45 g/L. Under these conditions, the biomass density was 12 g DCW/L, and a maximum of 36% of the total cellular protein was obtained [23]. However, it is possible that the accumulation of acetate due to a higher growth rate inhibited a further increase in biomass and the target protein production. In this study, lactose induction was combined with glucose-limited cultivations controlled by an exponential feeding strategy, and the results are favorable for its application in industrial production.

It is important to note that the optimal lactose content in shaken flasks was not suitable for fed-batch cultures in fermenter. It is possible explained by the role of lactose in the two cultivations. Lactose served both as an inducer as well as a carbon source in LB medium, but it only acted as an inducer. Meanwhile, comparison of the two fed-batch cultures, the expression of target protein was obviously inhibited by the high residual lactose in broth (Figs. 3b and 4b). This is in agreement with the results of Gombert and Kilikian, the protein production decreased probably due to some kind of inhibition, either by a high residual concentration of lactose or by the accumulation of galactose in the medium, and galactose was not consumed by *E. coli* BL21 (DE3) [19]. In addition, the high concentration of lactose seemed to partially repress the T7 promoter due to the glucose liberated by hydrolysis of lactose [24]. The results reflect the fact that optimizing conditions using lactose as the inducer may be a more complex task than IPTG, due to the physiological response of the cells and the fact that it is metabolize by the cells, especially in high cell density fed-batch cultivations. In the glucose-limited fed-batch cultivations, the cells would be in an alternative lifestyle throughout, to consume glucose or to adapt to lactose. At molecular level, the phosphoenolpyruvate: carbohydrate phosphotransferase systems (PTS) is the center of the regulatory network, and represent important uptake systems for a number of carbohydrates. $EIIA^{Crr}$ as the EIIA domain for the glucose-specific PTS is a key regulation molecule for inducer exclusion and catabolite repression in *E. coli* [25].

Conclusions

Inexpensive lactose was used to produce the recombinant thermophilic DERA in *E. coli* BL21 (DE3) instead of IPTG. The enzyme was efficiently induced for overexpression in glucose-limited fed-batch cultivations controlled by combining lactose induction with glucose feeding. The lactose concentration in the feed medium affected directly the expression of the target protein. The use of 50 g/L in the feed medium resulted in the biomass concentration of 39.3 g DCW/L, and an expression level of above 30%, and the final DERA concentration of 16,200 U/L. The process exhibited potential in large-scale fermentations, and also guided the production of other recombinant enzymes.

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