Journal of Molecular Catalysis B: Enzymatic 126 (2016) 10–17

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enhancement of alpha-ketoglutaric acid production from L-glutamic acid by high-cell-density cultivation

Xiangchen Fan[a,b,c], Ruidong Chen[a,b,c], Lele Chen[a,b,c], Liming Liu[a,b,c,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China
[b] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China
[c] Laboratory of Food Microbial-Manufacturing Engineering, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

ARTICLE INFO

Article history:
Received 15 August 2015
Received in revised form 13 January 2016
Accepted 13 January 2016
Available online 16 January 2016

Keywords:
l-Glutamate oxidase
α-KG
Lactose
High-cell-density culture
Whole-cell transformation

ABSTRACT

In this study, an efficient strategy of high-cell-density cultivation was exploited to obtain recombinant L-glutamate oxidase (LGOX), which was used to produce alpha-ketoglutaric acid (α-KG) from L-glutamic acid. First, lactose was used to replace isopropyl β-D-1-thiogalactopyranoside as a cheaper and more effective inducer. Second, a novel, two-stage feeding strategy was proposed, in which the exponential feeding mode was first performed in a 5-L fermenter until the maximum dissolved oxygen (DO) concentration was reached, which was followed by a DO-stat feeding mode. Based on the two-stage feeding strategy and by optimizing the induction strategies, the maximal cell density and LGOX activity reached 48.4 g/L and 156.1 U/mL, respectively, after 20 h of cultivation. When a whole-cell biocatalyst was used, the titer of α-KG was up to 127.2 g/L from 132 g/L L-glutamic acid in 24 h.

1. Introduction

Alpha-ketoglutaric acid (α-KG) is an important dicarboxylic acid in the tricarboxylic acid cycle (TCA), and it plays a central role in carbon and nitrogen utilization [1]. It is widely used in the food industry, as well as in medicine, agriculture, and other fields [2–5]. Especially, it is currently being investigated as a cancer therapy in clinical trials [6–8], and it also be used as a co-substrate for the synthesis of hydroxyl amino acids, such as 4-hydroxyproline [9], 4-hydroxyisoleucine [10], etc. Currently, there are three approaches to produce α-KG: chemical synthesis, microbiological production, and enzymatic transformation. Chemical synthesis from diethyl succinate and diethyl oxalate has successfully been used in the large-scale industrial production of α-KG, but it results in a low yield and produces toxic chemicals and solvents that generate environmental hazards [4,5]. Microbiological production of α-KG has been studied for several decades and it had gotten great progress (α-KG yield was up to 186 g/L) [11], although different by-products are produced [12] and a long fermentation period is required [2,13]. Enzymatic transformation, which is environmentally friendly, requires less energy, has a high selectivity, produces fewer by-products, and is non-toxic, has received increasing attention [14–16].

To produce more α-KG via a biocatalyst, a highly efficient enzyme source must be selected. Until now, two enzymes have been reported to produce α-KG from L-glutamic acid: including L-amino acid oxidase (LAAO) [17] and L-glutamate oxidase (LGOX) [14]. LAAO has a low substrate specificity, and it is highly subject to competitive inhibition and by a high α-KG concentration [15,17], which results in a decreased conversion ratio and low productivity. Using a *Bacillus subtilis* whole-cell biocatalyst, which was generated by inserting an L-amino acid deaminase and deleting the α-KG utilization pathway, the α-KG titer increased from 4.65 g/L to 12.21 g/L [18]. LGOX has a high specificity and a low K_m value with respect to L-glutamic acid, and this enzyme resulted in a maximum α-KG yield of 104.7 g/L from 110 g/L L-glutamic acid in 24 h [14]. Thus, LGOX has great potential in the industrial production of α-KG, as it meets the following criteria: 100 g/L of substrate loading, a 98% conversion ratio, a reaction time that is less than 24 h, and 5 g/L of biocatalyst loading [14,19].

In our previous study, a recombinant *Escherichia coli* strain expressing LGOX was constructed for the production of α-KG from L-glutamic acid. A strategy of increasing the expression of LGOX in shake flasks was developed, and LGOX activity reached 0.59 U/mL after 7 h of cultivation. However, 1.5 U/mL of LGOX were needed to produce the highest α-KG titer (104.7 g/L) from 110 g/L L-glutamic acid in 24 h [14]. Thus, large amounts of cells and enzymes are required for large-scale α-KG production. In general, the major limiting factor for the industrial production of α-KG is the low biomass of the recombinant strain and the low LGOX activity. The

* Corresponding author. Fax: +86 0510 85197875.
E-mail address: mingll@jiangnan.edu.cn (L. Liu).

http://dx.doi.org/10.1016/j.molcatb.2016.01.009
1381-1177/

most effective and broadly applied method to increase biomass is high-cell-density cultivation (HCDC) [20]. To increase enzymatic activity, different culture methods and induction strategies can be used to increase the concentration of biomass, as well as the specific cellular productivity [21].

In this study, three steps were performed to achieve high-level expression of LGOX. First, lactose replaced isopropyl β-D-L-thiogalactopyranoside (IPTG) as the inducer, which decreased the production cost. Second, a novel, two-stage feeding strategy was developed to increase cell density. Third, different feeding strategies were performed to enhance the expression of recombinant LGOX. Finally, the cost-effectiveness of the whole-cell transformation of L-glutamic acid to α-KG using HCDC cells was investigated.

2. Materials and methods

2.1. Bacterial strains and culture medium

A recombinant *E. coli* strain expressing LGOX was prepared by inserting plasmid pET28a expressing LGOX into *E. coli* BL21 (DE3) [14]. This strain was constructed successfully in our laboratory, and stored frozen at −70 °C in stock solutions of LB medium containing 15% (v/v) glycerol. LB medium (5 g/L yeast extract, 10 g/L peptone, and 5 g/L NaCl) was used for the preparation of the seed culture. Terrific broth (TB) medium (4 g/L glycerol, 24 g/L yeast extract, 12 g/L peptone, 2.31 g/L KH_2PO_4, and 12.54 g/L K_2HPO_4) was used in the shake flask experiments and batch/fed-batch cultivations. Kanamycin (0.1 g/L) was added to the media to maintain the plasmid. The feeding solution was composed of 500 g/L glycerol and 30 g/L $MgSO_4{\cdot}7H_2O$. The induction medium contained 200 g/L lactose.

2.2. Shake flask and batch experiments

A seed culture was started by inoculating 100 μL of frozen glycerol stock into 25 ml of LB medium in a 250-ml shake flask and culturing for 8–10 h at 37 °C with shaking 200 rpm. The seed culture was than inoculated into TB medium (4% v/v) for the shake flask fermentations. The biomass increase was checked by measuring the absorbance at 600 nm with a spectrophotometer (UV-mini 1240, Shimadzu, Kyoto, Japan) and by determining the cell dry weight of 5-mL broth samples after centrifugation at 10,800 × *g* for 10 min (5804R, Eppendorf, Enfield, CT, USA), followed by two washes with deionized water and oven drying at 70 °C to a constant weight. Cultures were induced when the optical density at 600 nm (OD_{600}) was around 0.6–0.65, at which time the growth temperature was reduced to 30 °C. Preliminary experiments to compare growth and enzyme production were performed in TB medium, with induction by 1, 3, 5, or 7 g/L lactose. Control experiments were performed in parallel in the same media using the optimal IPTG induction conditions [14].

In the fermentation tank, we used soya peptone and Angel yeast (Angel Yeast Co., Ltd., Hubei, China) instead of peptone and yeast extract in the TB medium. Batch fermentations were performed in 5-L bioreactors (Baoxing, Shanghai, China) in a 2-L working volume. The temperature, agitation rate, and aeration rate were initially adjusted to 37 °C, 400 rpm, and 1.0 vvm, respectively, after inoculation with 80 mL of the seed culture. When the OD_{600} reached 2.0, lactose was added to a final concentration of 5 g/L after reducing the temperature to 30 °C, and the induction continued for 5 h.

2.3. Feeding strategies to increase cell density

A 5-L bioreactor equipped with pH and pO_2 probes (Mettler Toledo, Greifensee, Switzerland) and four peristaltic pumps for the addition of antifoaming agent, acid, base, and feeding solutions was used for the fed-batch experiments. The initial bioreactor parameters and inoculum concentration were the same as in the batch experiments. In the batch phase, dissolved oxygen was kept at 30–40% saturation by controlling both the inlet air and the agitation rate. After depletion of the initial glycerol in the batch medium, the DO level began to rise and the feeding solution was initiated. Then, different feeding methods were performed. In the DO-stat feeding strategy, the feeding solution was added via a peristaltic pump when the DO level was above 30% saturation. The second experiment employed an exponential feeding strategy with a specific growth rate (μ) of 0.25 h^{-1}. The third experiment combined exponential feeding (μ = 0.25 h^{-1}) and the DO-stat strategy. In the exponential feeding stage, the DO level was maintained at 30–40% until the agitation rate and aeration exceeded the maximum set values (900 rpm and 3.0 vvm), at which time there was a shift to the DO-stat feeding method. During all the fermentations, the pH was maintained at 7.0 by the addition of 25% (v/v) NH_4OH. Foam was controlled by the addition of a silicone-based anti-foaming reagent. In all fermentations, cells were induced with 5 g/L lactose when the OD_{600} reached its maximum value, after which the OD_{600} barely changed, or even declined slightly, over the last 2 h. Subsequently, the growth temperature was reduced to 30 °C.

Exponential feeding was performed using Eq. (1), which was obtained from the mass balance of the cells and substrate, and by assuming a quasi-steady state and a constant fermentation volume [22,23]:

$$M_s(t) = F(t)S_0 = \left[\mu(t)/Y_{x/s} + m\right] X_{t0}V_{t0}\exp\left[\int_0^t \mu(t)\,dt\right] \quad (1)$$

where $M_s(t)$ is the mass feeding rate of glycerol (g/h), V_{t0} is the medium volume in the bioreactor (liters), X_{t0} is the mass concentration at the start of the feeding step (g/L), t is the time (h), $\mu(t)$ is μ as a function of the process time (h^{-1}), S_0 is the glycerol concentration (g/L) in the feeding solution, $F(t)$ is the feeding rate (L/h), $Y_{x/s}$ is the yield of biomass/substrate (g/g), t_0 is the time at the start of feeding (h), and m is the specific maintenance coefficient (g/h/g). The yield coefficient ($Y_{x/s}$) and the maintenance coefficient (m) were set to 0.5 and 0.025 g/L/g, respectively, for all experiments.

2.4. Strategy to enhance LGOX activity in fed-batch experiments

The optimum feeding strategy was determined to be a combination of exponential feeding and DO-stat feeding. Then, different methods were used to enhance the expression of the recombination protein. In the first experiment, different μ values, such as 0.25, 0.4, and 0.55 h^{-1}, were adopted during the pre-induction in the exponential phase. In this phase, carbon source feeding was achieved through a feeding pump that was controlled by the Eq. (1) until the agitation rate and aeration exceeded the maximum set values. Then, it shifted to the DO-stat feeding method. Additionally, the time of induction was optimized, starting from cell densiy values of 9, 18, or 27 g/L. Furthermore, induction was performed after setting the temperature to 30 °C and by using 5 or 10 g/L lactose at cell density values of 18 g/L or 5 g/L lactose + 5 g/L lactose at cell density values of approximately 18 and 27 g/L. In all cases, the final biomass was harvested after 18–20 h of growth by centrifuging the broth at 4 °C and 8000 × *g* for 5 min. The centrifuged biomass was frozen at −70 °C.

2.5. Production of α-KG from L-glutamic acid via LGOX

The transformation reaction was optimized under the following conditions: 50 mL of 0.1 M phosphate buffer (pH 6.5) were added to a 500-mL flask on a rotary shaker (200 rpm) at 30 °C, which included

a whole-cell biocatalyst, L-glutamic acid, catalase, and 4–5% triton X-100. Different concentration of whole-cell biocatalyst, L-glutamic acid, and Catalase were investigated to optimize α-KG production. Three different levels (L-glutamic acid, catalase and whole-cell biocatalyst) was designed as a L9-orthogonal array. All measurements were taken in triplicate and experiments were repeated three times to evaluate the standard deviation.

2.6. Measurement of LGOX activity

LGOX activity was measured by using a 4-aminoantipyrine system [24] (per assay: 1.0 ml of 121.5 μg/mL 4-aminoantipyrin, 1.4 ml of 0.26 μL/mL *N*,*N*-dimethyl aniline, 0.1 ml of 60 U/mL horseradish peroxidase, and 0.5 ml of 11 mg/mL L-glutamate in a total volume of 3.0 mL). The reaction was started by the addition of LGOX and then the absorbance at 550 nm was measured after 10 min. The value of A_{550} should range from 0.2 to 0.5; otherwise, the results were not usable. One unit of LGOX activity was defined as the amount of enzyme that liberated 1 μmol H_2O_2 per min:

$$\text{LGOXactivity(U/mL)} = \frac{A_{550}}{14.3 \times 1} \times \frac{3.1}{0.1} \times \frac{1}{10} \times C \quad (2)$$

where A_{550} is the absorbance at 550 nm, 14.3 is the extinction value of every millimole of the quinine imine compounds, 3.1 is the total reaction liquid volume (mL), 0.1 is the enzyme volume (mL), 10 is the reaction time (min), and *C* is the dilution multiples.

2.7. HPLC analysis

The glycerol and acetate concentrations in the medium during the fermentation were measured by HPLC (Agilent 1260, Agilent, USA) [25], by using an ion-exchange column (Supelcogel C-610-H, 30 cm × 7.8 mm, Supelco, USA) at 30 °C and with a 0.1% (v/v) H_3PO_4 buffer at the flow rate of 0.5 mL/min. Glycerol was detected by RI detector, while acetate was detected by UV detector at 210 nm. The α-KG concentration was measured by HPLC [26], by using ZORBAX SB-Aq reversed-phase column at 35 °C with mobile phase (19.7 g/L Na_2HPO_4 + 1.2% H_3PO_4 + 1% acetonitrile, pH 2.0) at the flow rate of 1.0 mL/min and UV detector at 210 nm.

3. Results

3.1. Lactose as induction agent for LGOX production

The effect of lactose concentrations on LGOX activities was investigated at an OD_{600} of 0.6, and the results are illustrated in Fig. 1A. LGOX activity increased with lactose concentration, from 0 to 5 g/L, and the maximum LGOX activity (4.53 U/mL) was observed at 5 g/L. Moreover, the optimal induction time was 5 h, at which time the maximum recombinant LGOX activity achieved was 4.78 U/mL (Fig. 1B), an increase of 53.2% compared with an IPTG-induced control experiment (3.12 U/mL). Furthermore, lactose was used in the 5-L batch-fermentation to improve the LGOX yield. Fig. 1C shows that LGOX activity reached its maximum value (9.43 U/mL) after 5.5 h of growth, at which time cell density reached 4.40 g/L (OD_{600} = 10.2), representing 97.3% and 54.5% improvements compared with the lactose-induced shake flask.

3.2. Feeding strategies to increase cell growth

Two different feeding methods, including DO-stat and exponential feeding, were applied to increase cell density and LGOX activity. When glycerol was completely exhausted or the DO level increased sharply (after about 4 h of growth), the DO-stat feeding strategy was initiated by feeding glycerol via a peristaltic pump to maintain the DO value below 30%. Fig. 2A shows that the final

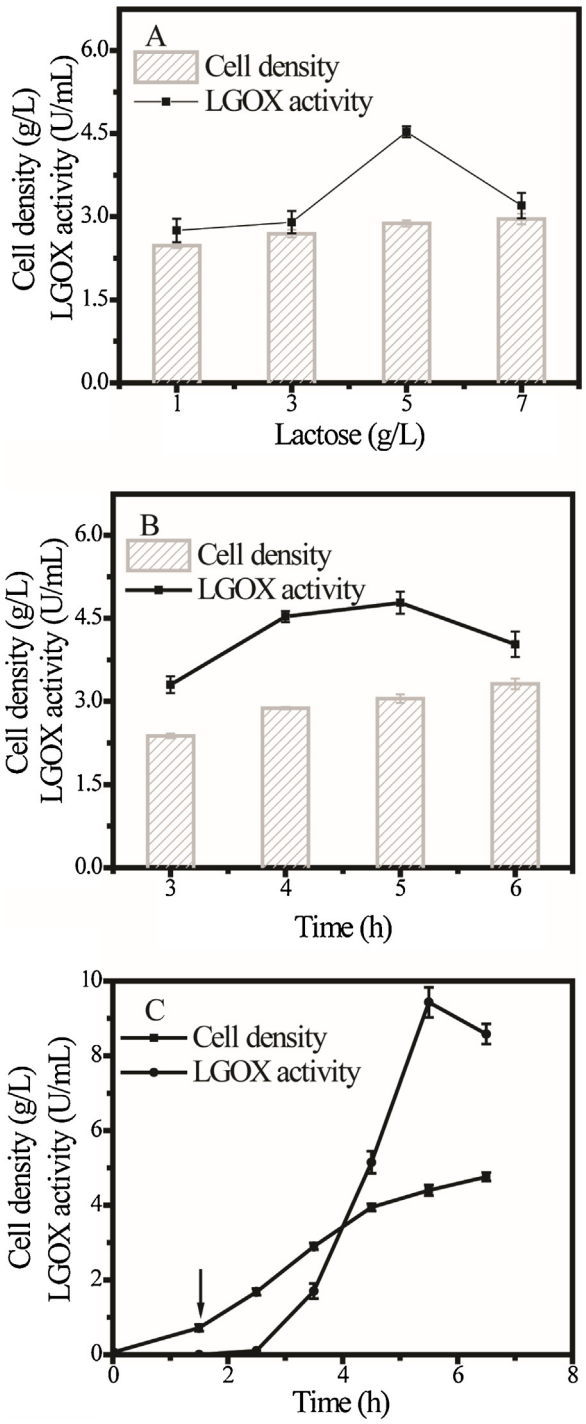

Fig. 1. Expression condition optimized on recombinant LGOX using lactose inducer, and batch fermentation. (Arrows show the points of induction).

cell density and LGOX activity of the DO-stat method were 21.4 g/L (OD_{600} = 47.7) and 35.6 U/mL, respectively, after 16 h of growth and 5 h of induction. Throughout the fermentation period, the acetate concentration was below 0.68 g/L, which benefitted the expression of the recombinant protein, and the glycerol concentration was below 4 g/L. However, the cell density was not sufficient to meet industrial requirements, as it was only 7.9-fold greater than that of the shake flask experiment. Thus, an exponential feeding strategy (μ = 0.25 h^{-1}) was performed, and the final cell density was 33.7 g/L (OD_{600} = 74.6), which was 57.5% higher than that of the DO-stat feeding strategy, and the LGOX activity was 33.9 U/mL after 16 h of

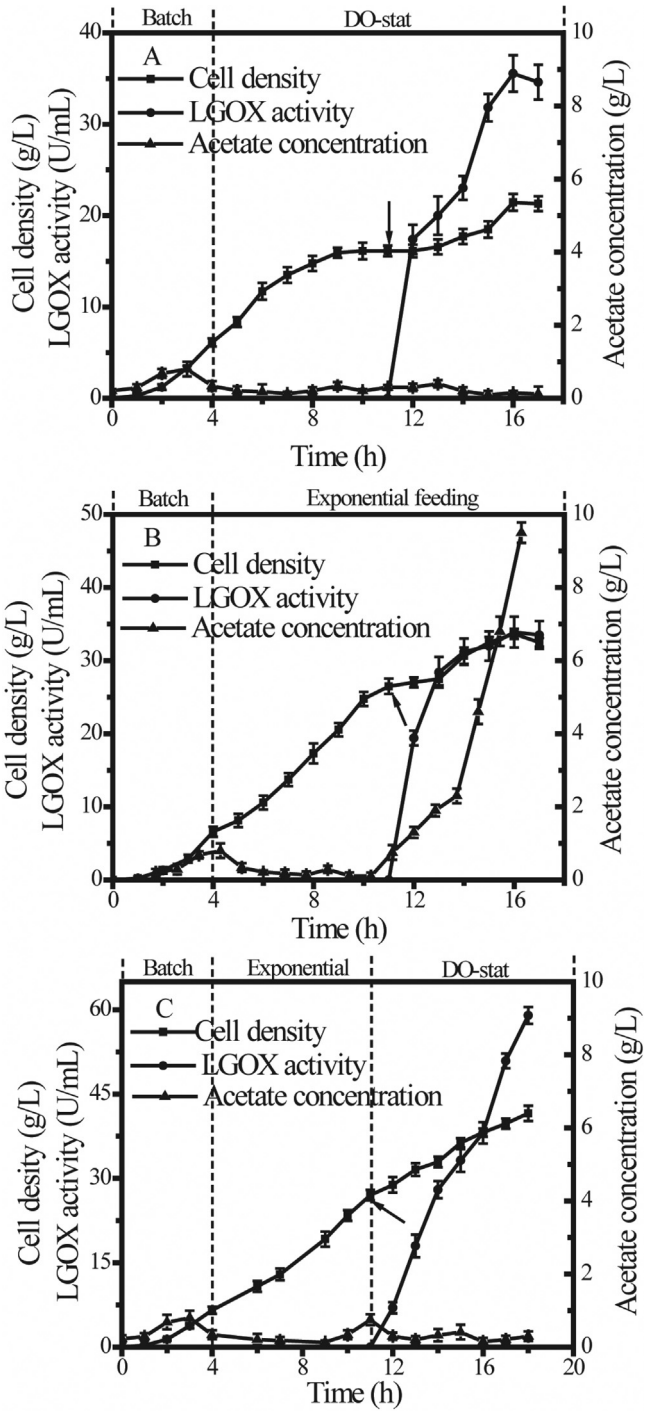

Fig. 2. Time courses of HCDC under DO-stat feeding, exponential feeding and the two-stage feeding strategy (Arrows show the points of induction).

growth (Fig. 2B). After 12 h, acetic acid, a major by-product, began to accumulate, reaching 2 g/L at 14 h, which further inhibited cell growth and LGOX expression [27].

The above results (Fig. 2 and Table 1) demonstrated that during the pre-induction of the single exponential feeding strategy, the biomass productivity was 36% higher than that of the single DO-stat feeding strategy. However, the LGOX specific activity in the single DO-stat strategy increased by 66% compared with that of the single exponential feeding. Thus, an exponential feeding at the mid-fermentation stage was advantageous to cell growth, while a DO-stat feeding strategy at a later stage enhanced LGOX production and further increased the cell density by decreasing acetate accumulation. To increase the cell density and LGOX production, a two-stage feeding strategy, as opposed to a single feeding strategy, should be used: after the initial amount of glycerol was exhausted after 4 h of growth, the fermentation shifted to exponential feeding. In the exponential feeding stage, the DO level was maintained at 30–40% of saturation until the agitation rate reached its maximum set value of 900 rpm. Then, the feeding strategy shifted to DO-stat strategy to avoid the acetate accumulation that began after approximately 11 h of growth.

The time course of the feeding shift strategy is shown in Fig. 2C, and the data are summarized in Table 1. With the two-stage feeding strategy, the maximum cell density (41.6 g/L), LGOX activity (59 U/mL), and biomass production (2.31 g/L/h) were achieved at 18 h, representing 93.9, 66, and 72.3% increases compared with those of the single DO-stat feeding strategy, and 23.4, 74, and 10% increases compared with those of the single exponential feeding strategy, respectively. Furthermore, after induction, the cell density of the two-stage strategy increased from 27.0 to 41.6 g/L, higher than that of the single exponential feeding strategy, in which the cell density increased from 26.5 to 33.7 g/L. These results demonstrated that the proposed two-stage feeding strategy improved the cell density and LGOX activity.

3.3. Induction strategies to enhance LGOX production

On the basis of the two-stage feeding strategy, some methods to increase LGOX activity were performed. First, a different μ value during exponential feeding can significantly influence recombinant protein synthesis [28,29]. The effect of μ (0.25 h^{-1}, 0.4 h^{-1} and 0.5 h^{-1}) on the OD_{600}, LGOX activity, and acetate production were investigated, and the results are illustrated in Fig. 3. After the exponential feeding stage, μ values of 0.25 h^{-1}, 0.4 h^{-1}, and 0.55 h^{-1} were shifted to DO-stat feeding at 11, 8, and 7 h, respectively. The cell density during pre-induction increased more quickly from 0.25 h^{-1} to 0.55 h^{-1}, whereas the final cell densities were similar, about 41.6, 42.7, and 43.2 g/L, respectively, at 20 h (Fig. 3A). However, the LGOX activity achieved its maximum value, 75.6 U/mL when μ was 0.4 h^{-1}, 28.1 and 8% higher than at μ values of 0.25 h^{-1} and 0.55 h^{-1}, respectively (Fig. 3B). In all the fermentations using the two-stage feeding strategy, the acetate concentration was always under the inhibiting level of 2 g/L and the residual glycerol concentration was always below 1 g/L after 4 h of growth. Among them, a μ value of 0.4 h^{-1} was confirmed as the basic condition of the exponential feeding stage because of its higher LGOX production.

Second, the effects of induction at different cell densities (9, 18, or 27 g/L) on the cell density and LGOX activity, based on a μ value of 0.4 h^{-1} during the exponential feeding stage, were examined. The final cell densities of the low and intermediate cell density inductions were similar, about 48 g/L, which were 12.4% higher than that of the highest cell density induction (Fig. 4A). The maximum LGOX activity (101.3 U/mL) was achieved during the intermediate cell density induction after 20 h of growth, while the LGOX activities following the low and high cell density inductions were 83 U/mL and 80 U/mL, representing increases of 22 and 26.6%, respectively (Fig. 4B). Therefore, the optimal induction density was 18 g/L.

On the basis of the above results, the effects of lactose concentration on cell density and LGOX activity were investigated. The final cell densities in all the fermentations reached equivalent values of about 48 g/L (Fig. 5A), whereas the LGOX activities differed greatly. The LGOX activity reached 156.1 U/mL during the 10 g/L lactose induction, 54.1% higher than that of the 5 g/L lactose induction. Furthermore, it was 45.7% higher than the two-step induction method (107.1 U/mL).

Table 1
Comparison of parameters of HCDC with different feeding strategies.

Parameters	DO-stat	Exponential feeding	Two-stage feeding strategy
Fermentation time (h)	16	16	18
The final cell density (g/L)	21.4 ± 0.91	33.7 ± 0.51	41.6 ± 1.36
Productivity of biomass (g/L/h)	1.34	2.1	2.31
LGOX activity (U/mL)	35.6 ± 2.0	33.9 ± 2.1	59 ± 2.9
Specific activity (U/mg)	1.66	1.0	1.42
The time of induction (h)	11	11	11
Cell density at induction (g/L)	15.9 ± 0.45	26.5 ± 0.60	27.0 ± 0.72
Biomass productivity at pre-induction (g/L/h)	1.45	2.41	2.46
The maximum acetate concentration (g/L)	0.68 ± 0.12	9.5 ± 0.2	0.75 ± 0.15

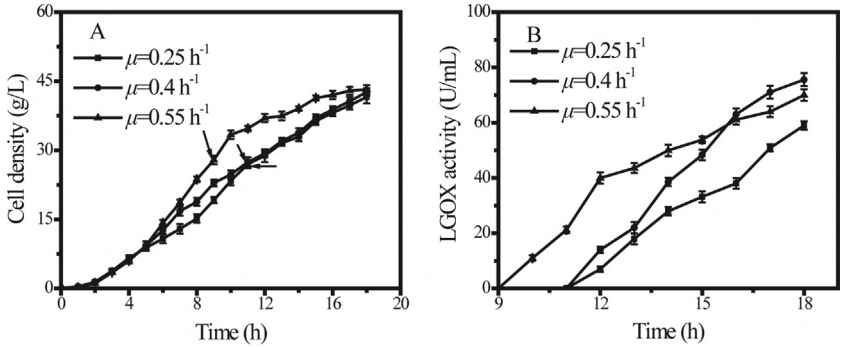

Fig. 3. The effect of μ value on cell density, LGOX activity in HCDC (Arrows show the points of induction).

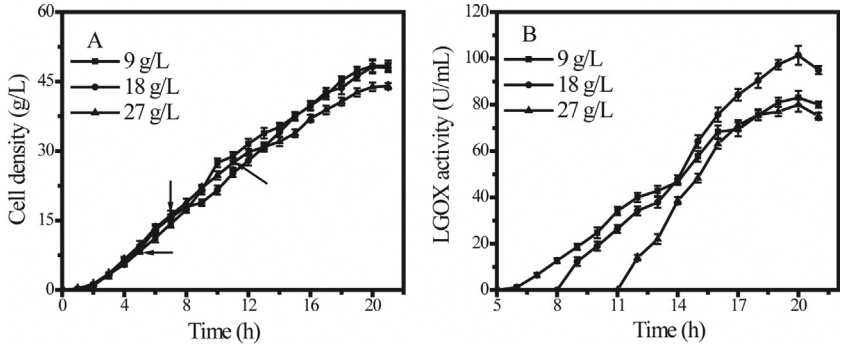

Fig. 4. The effect of induction phase on the activity of recombinant LGOX in fed-batch experiments (Arrows show the point of induction).

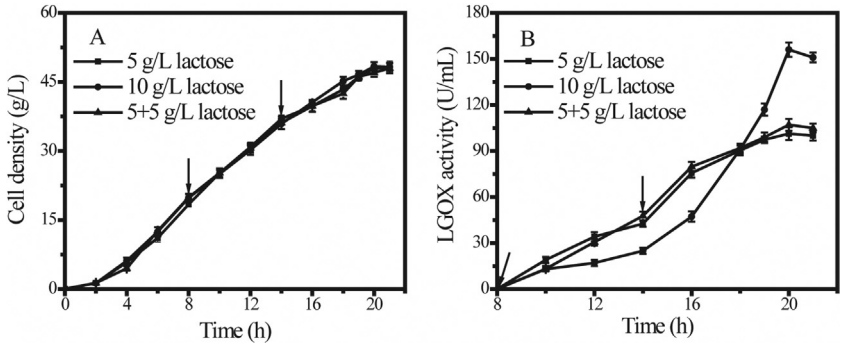

Fig. 5. Comparison of three induction condition by lactose (Arrows show the point of induction; 5 g/L, 10 g/L on behalf of the final inducer concentration at 8 h, 5 + 5 g/L on behalf of two-step inducted at 8 h and 14 h).

3.4. Enzymatic transformation of L-glutamic acid into α-KG in HCDC cells

From the above experiments, we can intuitively understand the effect of every stage of HCDC to produce LGOX for industrial-scale applications (Table 2). In the first step, lactose was used instead of IPTG for induction, and the LGOX activity increased by 53.2%. In the second step, the two-stage feeding strategy was used to increase the cell density from 2.76 to 41.6 g/L. In the last step, different induction strategies were performed on the basis of the two-stage feeding strategy, and the LGOX activity increased from 59 to 156.1 U/mL. Through all three steps, the final cell

Table 2
Comparison of different stages in HCDC to product recombinant LGOX.

Parameter	IPTG-induced in shake flask/A	Lactose-induced in shake flask/B	The two-stage feeding strategy/C	The optimal induction strategy/D
Fermentation time (h)	7	7	18	20
The final cell density (g/L)	2.49 ± 0.23	2.76 ± 0.23	41.6 ± 1.36	48.4 ± 0.91
Productivity of biomass (g/L/h)	0.36	0.39	2.31	2.42
LGOX activity (U/mL)	3.12 ± 0.2	4.78 ± 0.3	59 ± 2.9	156.1 ± 5.4
Specific activity (U/mg)	1.25	1.64	1.42	3.23
Productivity of LGOX (U/L/h)	445.7	647.1	3277.8	7804
The maximum acetate concentration (g/L)	–	–	0.75 ± 0.15	1.32 ± 0.19
Glycerol consumption (g/L)	–	–	150 ± 5.0	155 ± 4.5

Table 3
Orthogonal array design to improve α-KG production.

Run	Factors A	B	C	A Glumatic acid (g/L)	B Catalase (U/mL)	C Whole-cell biocatalyst (U/mL)	α-KG titer (g/L)
1	1	2	3	110	900	6	109.8 ± 1.2
2	1	3	2	110	1200	4.5	110.4 ± 1.2
3	1	1	1	110	600	3	94.7 ± 3.1
4	2	3	3	132	1200	6	116.2 ± 0.8
5	2	2	1	132	900	3	102.2 ± 4.9
6	2	1	2	132	600	4.5	103.8 ± 1.0
7	3	3	1	154	1200	3	104.5 ± 2.9
8	3	1	3	154	600	6	104.3 ± 3.3
9	3	2	2	154	900	4.5	108.5 ± 6.0
K_1				104.967	100.933	100.467	
K_2				107.400	106.833	107.567	
K_3				105.767	110.367	110.100	
R				2.433	9.434	9.633	
Q				A_2	B_3	C_3	

density, LGOX activity, LGOX specific activity, biomass productivity, and LGOX productivity reached 48.4 g/L, 156.1 U/mL, 3.23 U/mg, 2.42 g/L/h, and 7,804 U/L/h, representing 19.4-, 50-, 2.6-, 6.7-, and 17.5-fold increases, respectively, compared with those in the shake flask experiment with IPTG induction.

The highly efficient expression of recombinant LGOX in *E. coli* has great potential for the large-scale production of α-KG. A newly generated whole-cell biocatalyst was used to increase α-KG titer, and the optimum proportions of L-glutamic acid, catalase, and whole-cell biocatalyst were examined by using an orthogonal array design (Table 3). The order of influence was whole-cell biocatalyst > catalase > L-glutamic acid, and whole-cell biocatalyst and catalase contributed the almost equivalent effect larger than L-glutamic acid. Optimal conditions for α-KG production were 132 g/L L-glutamic acid, 1200 U/mL catalase and 6 U/mL whole-cell biocatalyst ($A_2B_3C_3$), and the highest α-KG titer was observed to be 116.2 g/L.

The influence of Mn^{2+} addition on the production of α-KG is checked (Fig. 6A). The optimal concentration of Mn^{2+} was 1 mmol/L. Under the optimal conditions of 30 °C and pH 6.5, the highest α-KG titer reached 127.2 g/L from 132 g/L L-glutamic acid, with conversion ratio 97.0%, increased by 9.5% as compared with no Mn^{2+} addition. From the conversion time profiles (Fig. 6B), it can be seen that the maximum titer of α-KG was reached at 24 h. The initial rate of production was about 10.2 g/L/h, and the average rate of production was about 5.3 g/L/h.

4. Discussion

To increase the production of α-KG, the cell density and LGOX activity were improved in this study. High cell density is required to ensure that there are a sufficient number of cells to produce the large amounts of enzyme required for industrial processes. Thus, the two-stage feeding strategy with lactose induction, which consisted of an exponential feeding strategy combined with a DO-stat feeding strategy, was demonstrated to be the optimal feeding method, as it increased the cell density from 2.49 to 41.6 g/L. On the basis of the two-stage feeding strategy, different strategies were used to increase LGOX expression, and the maximum cell density and LGOX activity reached 48.4 g/L and 156.1 U/mL, respectively, in 20 h (Table 2). As a result, 127.2 g/L α-KG was obtained from 132 g/L L-glutamic acid in 24 h by optimizing HCDC whole-cell transformation.

Regarding the choice of inducer, IPTG is routinely used to induce recombinant protein in the laboratory because of its stability, which results from the fact that it is not metabolized. However, its toxicity and high price increase production costs during industrial-scale fermentations. Lactose is a less expensive and nontoxic alternative [30]. Additionally, it can be used a carbon source, which benefits cell growth [31]. Therefore, IPTG is usually replaced by lactose in industrial-scale fermentations. For example, the use of lactose, as opposed to IPTG, increased the production of recombinant human interleukin-24 (rhIL-24) in *E. coli* [31]. Moreover, recombinant *E. coli* expressing human keratinocyte growth factor-2 were induced by lactose in a 30-L fermentation, which increased the cell mass by 84% compared with an IPTG induction, while maintaining a comparable expression level [32]. Furthermore, this study demonstrates that lactose is a perfect substitute for IPTG as an inducer, and it increased the cell density and LGOX activity by 10.8 and 53.2%, respectively (Table 2).

A common feeding strategy to increase cell growth is to use indirect feedback methods (such as pH-stat [33] or DO-stat [34]), predetermined feeding methods (such as exponential feeding [29,33]), feeding according to glucose uptake or demand [35], and other methods. The DO-stat method is based on the finding that the DO level increases sharply when the substrate is depleted; therefore, the substrate concentration can be maintained within a desired range, which avoids the accumulation of by-products below the oxygen transfer threshold [34,36], although it does not result in a higher specific growth rate or a greater final cell

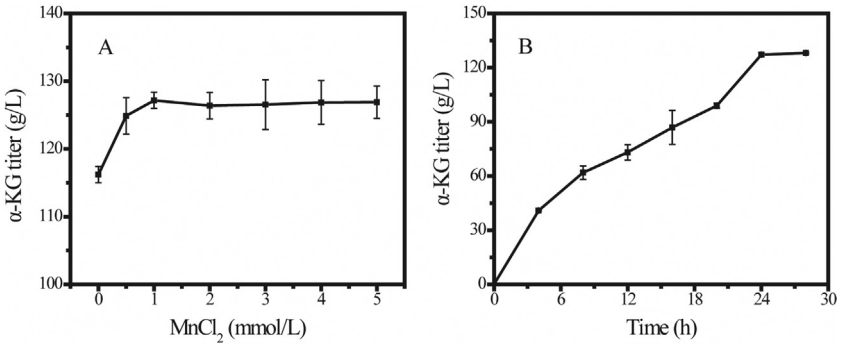

Fig. 6. The effect of Mn^{2+} on α-KG production and time course of α-KG production.

density. Exponential feeding is a simple but efficient method that has been successfully used for the HCDC of several non-recombinant and recombinant *E. coli* strains, and it can quickly produce a large amount of biomass under adequate DO conditions. However, it results in the accumulation of acetate during longer periods of exponential feeding [36]. In general, a high cell density culture needs to maintain an adequate DO level by adjusting the agitation speed or aeration rate, sometimes using pure oxygen, to avoid the accumulation of acetic acid [25,36–38]. However, pure oxygen is expensive and increases production costs when used in large quantities. Therefore, a new feeding strategy was developed for industrial-scale applications: the two-stage feeding strategy in which exponential feeding was combined with DO-stat feeding was used, and it resulted in a cell density of 41.6 g/L at 18 h, which was 93.9% higher than that of a single DO-stat strategy and 23.4% higher than that of a single exponential feeding strategy. In earlier experiments, a two-stage feeding strategy, which employed an exponential feeding method during the pre-induction phase and gradient-decreasing feeding method, increased α-cyclodextrin glycosyltransferase (α-CG) production in recombinant *E. coli* BL21(DE3) from 48 to 275.3 U/mL [39]. Alkaline polygalacturonate lyase (PGL) reached 371.86 U/mL, which is the highest PGL production by a recombinant *E. coli* expression system, using an exponential feeding strategy combined with a constant feeding approach [40]. An exponential feeding combined with a pH-stat strategy yielded 101 g/L of recombinant *E. coli*, which was higher than those of a single exponential and pH-stat feeding methods [33]. However, a two-stage feeding strategy that combined exponential feeding and DO-stat strategies has not been reported for high cell density cultures of recombinant *E. coli*.

Induction conditions must be optimized to maximize the expression of heterologous proteins in recombinant *E. coli* [41]. Additionally, different μ values during pre-induction and post-induction can have a significantly influence on recombinant protein synthesis [28]. By optimizing the μ values during the exponential feeding stage, as well as the induction time and inducer concentration, the LGOX activity increased from 59 U/mL to 156.1 U/mL, a 165% increase. Moreover, even though the fermentation time was only 20 h in this study, the production of LGOX increased by 50-fold and the cell density increased by 19.4-fold. Hence, the production cost of LGOX declined, which increased α-KG production by a whole-cell biocatalyst. Lastly, the titer of α-KG was up to 127.2 g/L by whole-cell transformation, with conversion ratio 97.0%. And, 1 mmol/L Mn^{2+} was optimum for α-KG production in whole-cell transformation system which was much lower than the previous studies (3 mmol/L [14] and 5 mmol/L [42]), because LGOX activity in whole-cell was declined slower than crude enzyme in the process of transformation and need not too much activator Manganese ion. This study is the first to report the use of recombinant LGOX in a high-cell-density culture.

Acknowledgements

This work was supported by the Key Technologies R&D Program of Jiangsu Province (BE2013612 & BE2014652), the Fundamental Research Funds for the Central Universities (JUSRP51303A), and the National Natural Science Foundation of China(NO. 21422602).

References

[1] C.D. Doucette, D.J. Schwab, N.S. Wingreen, J.D. Rabinowitz, Nat. Chem. Biol. 7 (2011) 894–901.
[2] I.G. Morgunov, S.V. Kamzolova, V.A. Samoilenko, Appl. Microbiol. Biotechnol. 97 (2013) 8711–8718.
[3] T.V. Finogenova, I.G. Morgunov, S.V. Kamzolova, O.G. Chernyavskaya, Appl. Biochem. Microbiol. 41 (2005) 418–425.
[4] U. Stottmeister, A. Aurich, H. Wilde, J. Andersch, S. Schmidt, D. Sicker, J. Ind. Microbiol. Biotechnol. 32 (2005) 651–664.
[5] C. Otto, V. Yovkova, G. Barth, Appl. Microbiol. Biotechnol. 92 (2011) 689–695.
[6] F. Donnarumma, R. Wintersteiger, M. Schober, J. Greilberger, V. Matzi, A. Maier, M. Schwarz, A. Ortner, Anal. Sci. 29 (2013) 1177–1182.
[7] B.M. Wagner, F. Donnarumma, R. Wintersteiger, W. Windischhofer, H.J. Leis, Anal. Bioanal. Chem. 396 (2010) 2629–2637.
[8] K. Michail, H. Juan, A. Maier, V. Matzi, J. Greilberger, R. Wintersteiger, Anal. Chim. Acta 581 (2007) 287–297.
[9] F. Falcioni, L.M. Blank, O. Frick, A. Karau, B. Buhler, A. Schmid, Appl. Environ. Microbiol. 79 (2013) 3091–3100.
[10] J. Ogawa, H. Yamanaka, J. Mano, Y. Doi, N. Horinouchi, T. Kodera, N. Nio, S.V. Smirnov, N.N. Samsonova, Y.I. Kozlov, S. Shimizu, Biosci. Biotechnol. Biochem. 71 (2007) 1607–1615.
[11] V. Yovkova, C. Otto, A. Aurich, S. Mauersberger, G. Barth, Appl. Microbiol. Biotechnol. 98 (2014) 2003–2013.
[12] L.M. Liu, Y. Li, Y. Zhu, G.C. Du, J. Chen, Metab. Eng. 9 (2007) 21–29.
[13] Z.Z. Yu, G.G. Du, J.W. Zhou, J. Chen, Bioresour. Technol. 114 (2012) 597–602.
[14] P.Q. Niu, X.X. Dong, Y.C. Wang, L.M. Liu, J. Biotechnol. 179 (2014) 56–62.
[15] L. Liu, G.S. Hossain, H.D. Shin, J.H. Li, G.C. Du, J. Chen, J. Biotechnol. 164 (2013) 97–104.
[16] U.T. Bornscheuer, G.W. Huisman, R.J. Kazlauskas, S. Lutz, J.C. Moore, K. Robins, Nature 485 (2012) 185–194.
[17] G.S. Hossain, J.H. Li, H.D. Shin, G.C. Du, L. Liu, J. Chen, Appl. Microbiol. Biotechnol. 98 (2014) 1507–1515.
[18] G.S. Hossain, J.H. Li, H.D. Shin, L. Liu, M. Wang, G.C. Du, J. Chen, J. Biotechnol. 187 (2014) 71–77.
[19] S. Luetz, L. Giver, J. Lalonde, Biotechnol. Bioeng. 101 (2008) 647–653.
[20] D. Riesenberg, R. Guthke, Appl. Microbiol. Biotechnol. 51 (1999) 422–430.
[21] A.K. Gombert, B.V. Kilikian, J. Biotechnol. 60 (1998) 47–54.
[22] R. Khalilzadeh, S.A. Shojaosadati, N. Maghsoudi, J. Mohammadian-Mosaabadi, M.R. Mohammadi, A. Bahrami, N. Maleksabet, M.A. Nassiri-Khalilli, M. Ebrahimi, H. Naderimanesh, J. Ind. Microbiol. Biotechnol. 31 (2004) 63–69.
[23] D.J. Korz, U. Rinas, K. Hellmuth, E.A. Sanders, W.D. Deckwer, J. Biotechnol. 39 (1995) 59–65.
[24] C.C. Allain, L.S. Poon, C.S. Chan, W. Richmond, P.C. Fu, Clin. Chem. 20 (1974) 470–475.
[25] O.F. Restaino, U. Bhaskar, P. Paul, L. Li, M. De Rosa, J.S. Dordick, R.J. Linhardt, Appl. Microbiol. Biotechnol. 97 (2013) 3893–3900.
[26] D.D. Zhang, N. Liang, Z.P. Shi, L.M. Liu, J. Chen, G.C. Du, Biotechnol. Bioproc. E 14 (2009) 134–139.
[27] J. Shiloach, R. Fass, Biotechnol. Adv. 23 (2005) 345–357.
[28] J.H. Choi, K.C. Keum, S.Y. Lee, Chem. Eng. Sci. 61 (2006) 876–885.
[29] V. Babaeipour, S.A. Shojaosadati, R. Khalilzadeh, N. Maghsoudi, F. Tabandeh, Biotechnol. Appl. Biochem. 49 (2008) 141–147.
[30] S. Jana, J.K. Deb, Appl. Microbiol. Biotechnol. 67 (2005) 289–298.
[31] M.I. Amirzada, M.L. Yu, X.H. Gong, Y. Chen, R.Y. Zhu, J.Y. Lei, J. Jin, J. Ind. Microbiol. Biotechnol. 41 (2014) 135–142.

[32] H.S. Tian, L. Tang, Y. Wang, X.J. Wang, L.L. Guan, J. Zhang, X.P. Wu, X.K. Li, Int. J. Pept. Res. Ther. 17 (2011) 123–129.
[33] B.S. Kim, S.C. Lee, S.Y. Lee, Y.K. Chang, H.N. Chang, Bioproc. Biosyst. Eng. 26 (2004) 147–150.
[34] J. Ding, M. Gao, G. Hou, K. Liang, R. Yu, Z. Li, Z. Shi, J. Chem. Technol. Biotechnol. 89 (2014) 1948–1953.
[35] B.S. Kim, S.C. Lee, S.Y. Lee, H.N. Chang, Y.K. Chang, S.I. Woo, Enzyme Microb. Technol. 16 (1994) 556–561.
[36] S.Y. Lee, Trends Biotechnol. 14 (1996) 98–105.
[37] S.J. Park, S.-J. Lee, K.-H. Kim, S.-K. Kim, Biotechnol. Bioproc. E 16 (2011) 733–738.
[38] M. De Mey, S. De Maeseneire, W. Soetaert, E. Vandamme, J. Ind. Microbiol. Biotechnol. 34 (2007) 689–700.
[39] J. Cheng, D. Wu, S. Chen, J. Chen, J. Wu, J. Agric. Food Chem. 59 (2011) 3797–3802.
[40] S.Y. Fang, J.H. Li, L. Liu, G.C. Du, J. Chen, Bioresour. Technol. 102 (2011) 10671–10678.
[41] S.J. Chen, M.C. Chang, C.Y. Cheng, J. Ferment. Bioeng. 84 (1997) 610–613.
[42] P.Q. Niu, Z.Y. Zhang, L.M. Liu, Chin. J. Biotechnol. 30 (2014) 1318–1322.