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A novel strategy on the high-cell-density cultivation of *Candida utilis* for the enhanced production of glutathione

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Abstract−Efficient glutathione production by high-cell-density cultivation of *Candida utilis* was investigated. A series of batch glutathione fermentations were carried out and the optimal initial glucose concentration was found to be about 26 g/L. Then, fed-batch fermentation under diverse feeding strategies was used to enhance glutathione production with a total glucose concentration of 150 g/L. Constant glucose feeding strategy cannot meet the requirement of cells at the late period of feeding, while exponential glucose feeding strategy cannot satisfy the needs of cells at the beginning of feeding. Based on the results above, a polynomial glucose feeding strategy was developed to provide enough glucose for cells along with the cultivation, under which both the cell and glutathione productivity were satisfactorily improved. Furthermore, fed-batch fermentation under this strategy with a total glucose concentration of 200 g/L was successfully performed, the dry cell weight and glutathione concentration reached 91.2 g/L and 825 mg/L, respectively.

Key words: Glutathione, *Candida utilis*, Fed-batch Fermentation, High-cell-density Cultivation

INTRODUCTION

The tripeptide γ-L-glutamyl-L-cystinylglycine (glutathione) is one of the major antioxidant molecules of cells and is thought to play a vital role in buffering the cell against reactive oxygen species and toxic electrophiles [1]. In addition to its pharmaceutical applications, glutathione is also being used in food additives and cosmetics [2]. Glutathione can be extracted from some active tissues; it can also be produced by chemical method, enzymatic reaction, and microbial fermentation. Among these methods, microbial fermentation is considered as the most promising approach in industry [3]. As glutathione is an intracellular product in yeast, a combination of high intracellular glutathione content and high cell density leads to high glutathione concentration.

High-cell-density cultivation of microorganisms can improve microbial biomass and product formation substantially to obtain high production, high yield and high productivity [4]. This technique can also provide additional advantages such as a decreased culture volume, decreased waste-water, decreased investment in the equipment, and enhanced downstream processing [5,6]. Nonetheless, limiting factors still need to be taken into account, including substrate inhibition, oxygen and heat-transfer limitations, accumulation of cellular by-products and inhibition of cellular respiration by a high concentration of dissolved CO_2 [7]. Fed-batch fermentation is usually used in high-cell-density cultivation to prevent or reduce substrate-associated growth inhibition by controlling nutrient supply, and has been widely employed for the production of various bio-products including primary and secondary metabolites, proteins, and other biopolymers [8].

Many studies on the production of glutathione by high-cell-density cultivation have been performed in *S. cerevisiae*. Sakato and Tanaka [9] developed a feed forward/feedback control system to control the sugar feeding rate in order to maximize glutathione yield. Alfafara et al. [10] developed a fuzzy logic controller for the control of ethanol concentration and utilized it to realize the maximum production of glutathione in yeast fed-batch culture. Wang et al. [11] achieved high glutathione yield and dry cell weight after 52 h in high-cell-density cultivation of *S. cerevisiae* for glutathione production. The results were obtained by controlling glucose feeding rate, which was followed with the changes of ethanol concentration and respiratory quotient (RQ). *Candida utilis*, a kind of Crabtree-negative [12] and Kluyver positive [13] yeast, which can use both hexose and pentose, is another commonly used microorganism on an industrial scale for glutathione production [3]. Liang et al. [14] improved glutathione production by adding precursor amino acids in fed-batch fermentation of *Candida utilis* WSH02-08. Even though, few papers had focused on which method was more suitable for the high-cell-density cultivation of *C. utilis* for glutathione production. In this study, we have successfully developed a novel glucose feeding strategy of fed-batch culture followed with a polynomial equation for efficient glutathione production, which was based on the strategies from constant rate and exponential rate of glucose feeding.

MATERIALS AND METHODS

1. Microorganism and Seed Media

C. utilis SZU 07-01, which was screened and collected in our lab of industrial microbiology, was used in this study. The strain was maintained at 4 °C on the slant contained seed media (20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone, pH 6.0) and 20 g/L agar, and sub-cultured once a month.

2. Seed Preparation and Inoculation

The seed culture was grown in 500 mL baffled shake flasks con-

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taining 50 mL seed media on a rotating shaker at 200 rpm and 30 °C for 20 h. Then the culture was used to inoculate into the fermentation broth, and the ratio of seed culture to fermentation media was set at 10% (v/v).

3. Batch and Fed-batch Fermentation

Batch and fed-batch fermentation were carried out in a 5 L stirred fermentor (Minifors 5 L, Swiss) with 3 L fermentation media contained (g/L): glucose 30, $(NH_4)_2SO_4$ 8, KH_2PO_4 3 and $MgSO_4$ 0.25, $FeSO_4$ 0.01, $MnSO_4$ 0.01, $ZnSO_4$ 0.003, $CuSO_4$ 0.001. The fermentor was equipped with sensors for the control of temperature, pH, and dissolved O_2 concentration. The bioreactor was operated at 30 °C and pH 5.5, the pH was automatically held by adding 3 mol/L H_2SO_4 and 3 mol/L NaOH solutions. Dissolved O_2 concentration was kept over 35% [15] relative to saturation by controlling manually the agitation rate (300 rpm-900 rpm); the aeration rate and the initial agitation rate were kept at 1.67 vvm and 300 rpm, respectively. Fed-batch fermentation was initiated like batch fermentation, except that the concentrated medium was fed beginning at 12 h. The feeding medium contained glucose concentration of 600 g/L.

4. Analytical Methods

A culture broth of 10 mL was centrifuged at 6,000×g for 10 min, and the wet cells were extracted with 40% (v/v) ethanol at 30 °C for 2 h, then centrifuged at 8,000×g for 10 min, the supernatant was used for glutathione assay.

The wet cells from 25 mL culture broth were centrifuged at 6,000 ×g for 10 min, and the dry cell weight (DCW) was determined after drying the wet cells at 70 °C to a constant weight. Glucose was determined by the DNS method [16]. Glutathione concentration was determined according to the method described by Tietze [17]. The intracellular glutathione content (IGC) was described as follows:

$$\text{Intracellular glutathione content/(\%)} = \frac{\text{Glutathione concentration/(mg/L)}}{\text{DCW/(g/L)}\times 100}\times 100\%$$

RESULTS

1. Effect of Initial Glucose Concentrations on Cell Growth and Glutathione Production

Batch culture of *C. utilis* SZU 07-01 was carried out under diverse initial glucose concentrations ranging from 16 g/L to 80 g/L; cell growth and glutathione production are illustrated in Fig. 1. It was shown that the yeast grew well on glucose as the sole carbon source, and the DCW and glutathione concentration increased along with the initial glucose concentration. However, the intracellular glutathione content decreased when the glucose concentration was higher than 40 g/L.

The parameters within batch glutathione production under different glucose concentrations are summarized in Table 1. It was indicated that the yeast can assimilate and consume glucose as much as possible in a given culture time, which resulted in a higher bio-

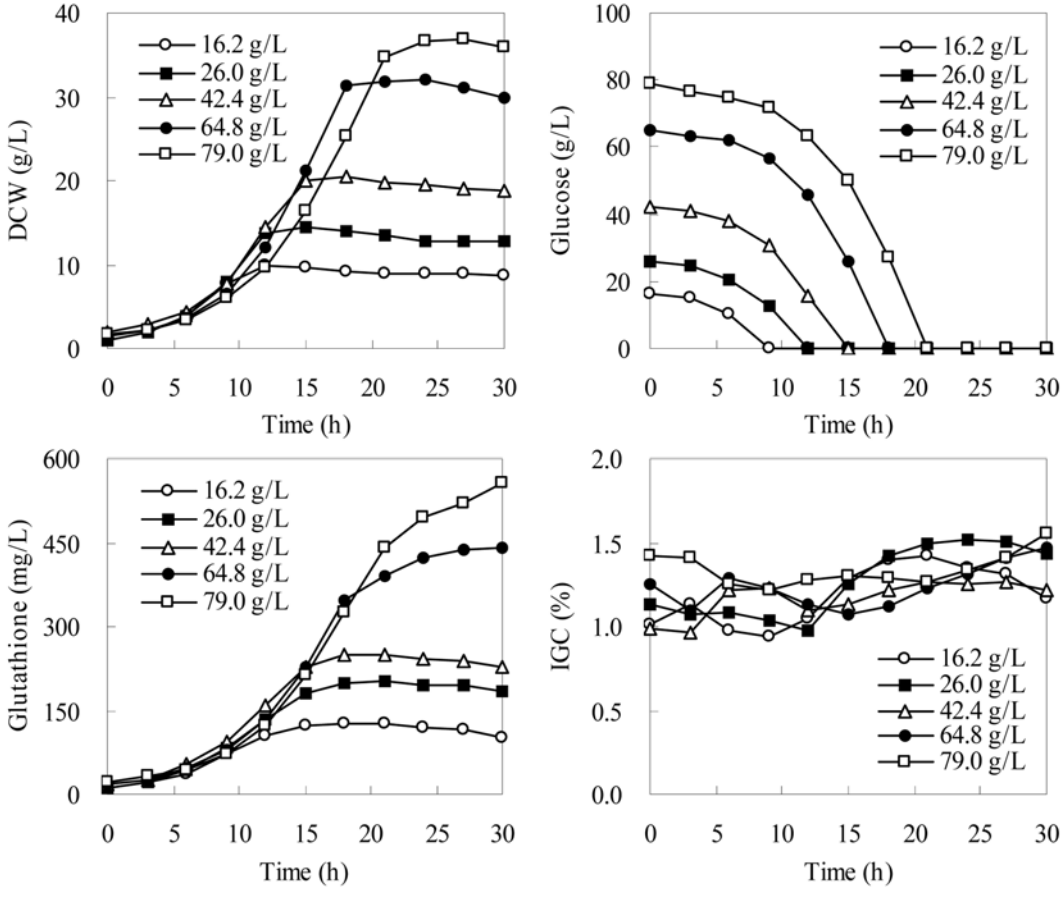

Fig. 1. Effect of initial glucose concentrations on batch glutathione fermentation.

Table 1. Comparison of parameters in batch glutathione production under diverse initial glucose concentrations

Parameters	Results				
Initial glucose concentration/(g/L)	16.2	26.0	42.4	64.8	79.0
Maximum dry cell weight/(g/L)	9.94	14.38	20.49	32.08	36.92
Maximum glutathione production/(mg/L)	128.1	202.4	248.5	439.7	557.1
Maximum intracellular glutathione content/%	1.43	1.49	1.26	1.47	1.55
Glucose consumption rate/(g/(L·h))	1.80	2.16	2.83	3.60	3.76
Average specific growth rate/h^{-1}	0.18	0.17	0.16	0.14	0.13
Biomass yield on glucose/(g/g)	0.61	0.55	0.48	0.50	0.47
Glutathione yield on glucose/(mg/g)	7.91	7.80	5.85	6.79	7.05
Biomass productivity/(g/(L·h))	0.83	0.96	1.14	1.34	1.37
Glutathione productivity/(mg/(L·h))	6.10	9.64	11.83	14.66	18.57

mass and glutathione productivity at a higher glucose concentration. However, the yield of biomass and glutathione on glucose decreased when the glucose concentration increased. So, batch fermentation cannot bring high production, high yield and high productivity of biomass and glutathione simultaneously; we should turn to fed-batch culture for efficient glutathione production. In addition, from the results in Table 1, the initial glucose concentration of about 26 g/L was more suitable for batch glutathione fermentation under comprehensive consideration.

2. Fed-batch Glutathione Production under Constant Glucose Feeding Rates

Fed-batch culture under constant substrate feeding rate is a simple way to improve microbial biomass and product formation [18]. First, a high-cell-density culture of *C. utilis* SZU 07-01 was conducted by glucose feeding after 12 h under different constant feeding rate of 4.0, 5.0, 6.7 and 8.0 g/(L·h) until the total glucose concentration of 150 g/L to find if there existed a suitable glucose feeding rate (Fig. 2). Because high dissolved oxygen concentration (over

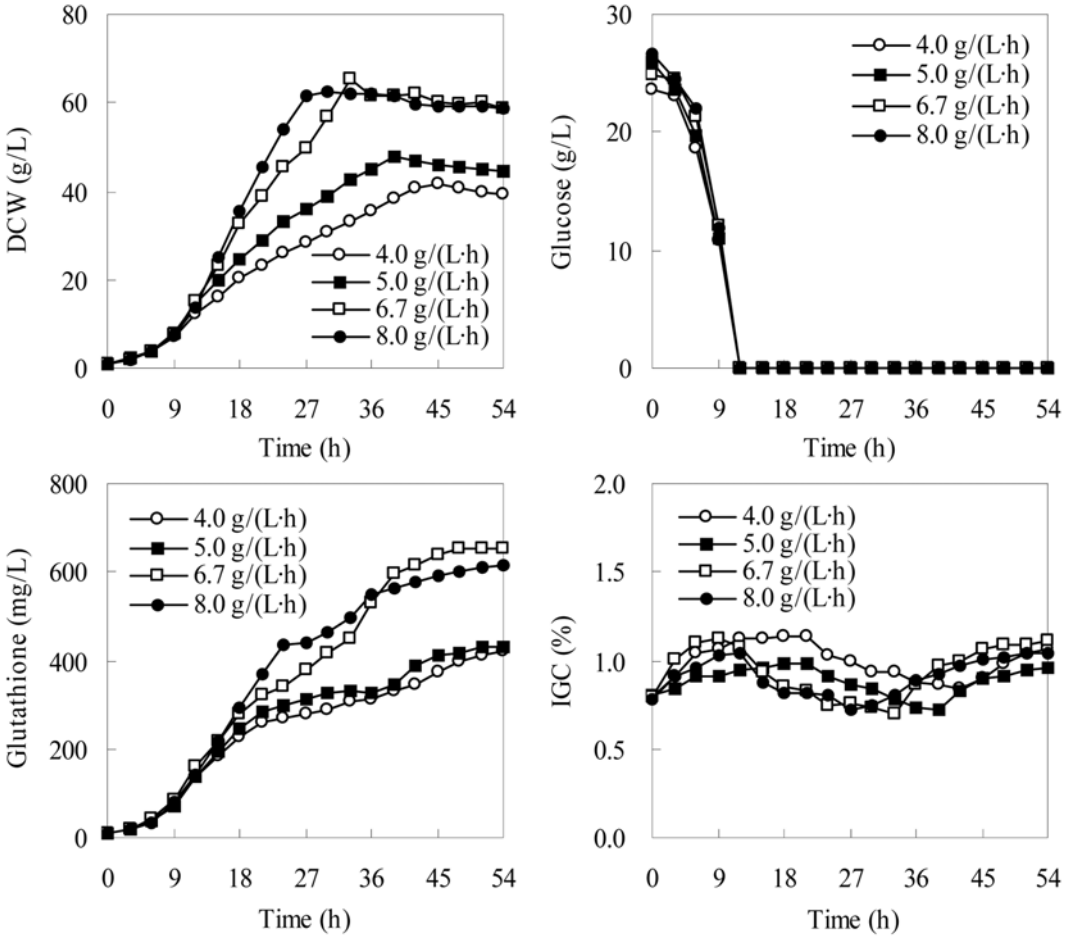

Fig. 2. Time-courses of fed-batch glutathione fermentation under glucose feeding at different constant rates.

Table 2. Comparison of parameters for glutathione production under diverse fed-batch feeding strategies with a total glucose concentration of 150 g/L

Parameters	Constant feeding				Exponential feeding			Polynomial feeding
	I	II	III	IV	I	II	III	
Total glucose/g	431.1	437.7	434.7	439.8	437.4	442.5	430.8	434.1
Culture time/h	54	54	54	54	54	48	48	42
Maximum dry cell weight/(g/L)	41.50	47.74	65.09	62.35	68.03	70.00	65.52	68.92
Maximum glutathione production/(mg/L)	419.9	430.4	654.3	613.7	653.0	672.6	642.8	691.3
Maximum intracellularglutathione content/%	1.13	0.99	1.11	1.04	1.00	1.12	1.08	1.13
Biomass yield on glucose/(g/g)	0.29	0.33	0.45	0.43	0.47	0.47	0.46	0.48
Glutathione yield on glucose/(mg/g)	2.92	2.95	4.52	4.19	4.48	4.56	4.48	4.8
Biomass productivity/(g/(L·h))	0.92	1.22	2.03	2.08	2.06	2.33	2.43	2.55
Glutathione productivity/(mg/(L·h))	7.78	7.97	12.12	11.36	12.09	14.01	13.39	16.46

Note: Constant feeding I, II, III and IV were representative for glucose feeding at constant rates of 4.0, 5.0, 6.7 and 8.0. g/(L·h), respectively. Exponential feeding I, II and III were representative for the cultivation mode of glucose feeding with the specific growth rate of 0.08 h^{-1}, 0.10 h^{-1} and 0.14 h^{-1}, respectively

35% of saturation) was manually maintained by modulating the agitation rate in the bioreactor throughout the cultivation, the yeast could consume glucose quickly, and there was no glucose accumulation even when the glucose feeding rate reached 8.0 g/(L·h). DCW increased all along with glucose consumption, and DCW reached the maximum level when glucose was exhausted. The parameters within the constant glucose feeding processes are also listed in Table 2. Higher glucose feeding rate is more favorable for biomass, and it can bring more biomass production, biomass yield and biomass productivity, simultaneously. However, higher glutathione production, glutathione yield and glutathione productivity can only be obtained at the constant glucose feeding rate of 6.7 g/(L·h); the maximum glutathione concentration increased by 56%, 52% and 7% compared to that with the constant glucose feeding rate of 4.0,

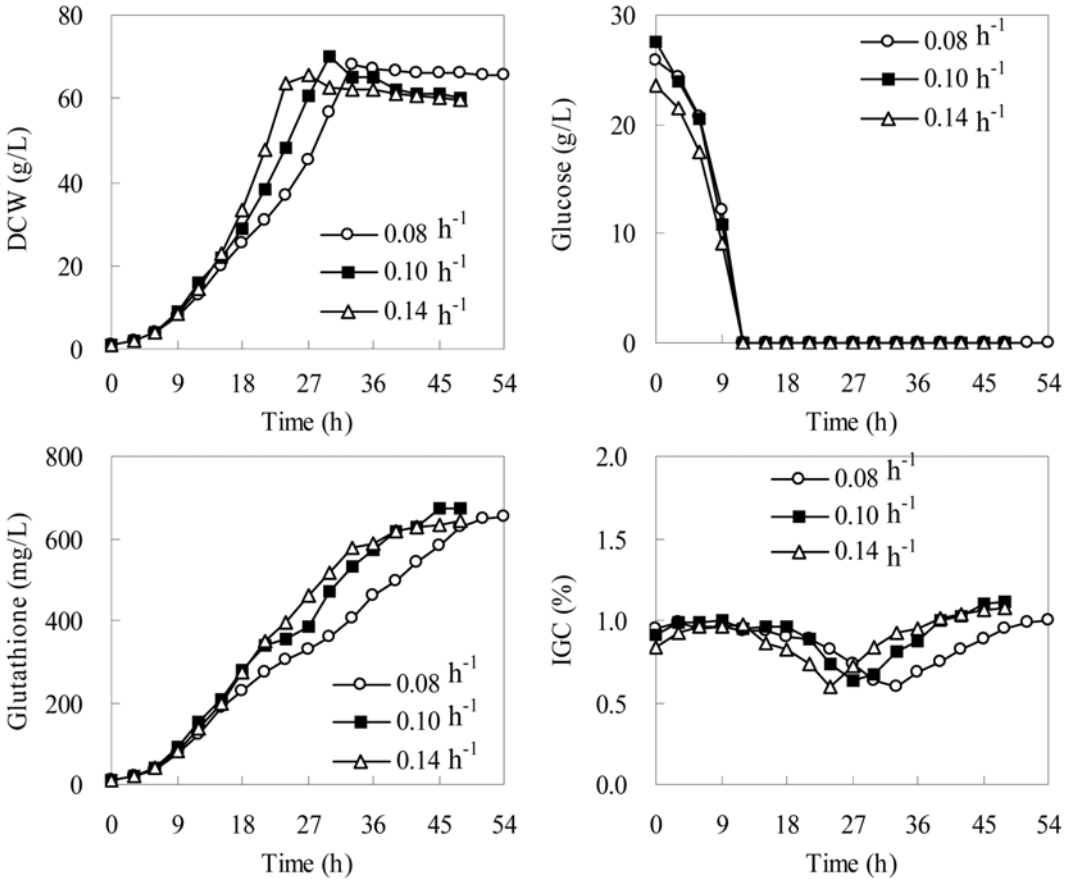

Fig. 3. Time-courses of fed-batch glutathione fermentation under different specific growth rates at an exponential rate of glucose feeding.

5.0, and 8.0 g/(L·h), respectively. Therefore, it can be concluded that the most appropriate constant glucose feeding rate for efficient glutathione production in fed-batch cultivation is 6.7 g/(L·h).

3. Fed-batch Glutathione Production under Exponential Glucose Feeding Rates

Exponential feeding is another simple but powerful method that allows cells to grow at a constant specific growth rate [19]. The nutrient feeding rate can be determined by Eq. (1), which is derived from a mass balance with the assumption of a constant cell yield on substrate and constant maintenance coefficient throughout the fermentation [19].

$$F = \frac{\mu(VX)_0}{Y_{X/S}(S_F - S)}\exp(\mu t) \quad (1)$$

F is the feeding rate (mL/h), μ is the specific growth rate (h^{-1}), t is the time after feeding (h). V_0, X_0 and S are the initial volume of medium (mL), concentration of biomass (g/L) and residual glucose concentration (g/L) at feeding time of 0 h, respectively. Y_{XS} is the biomass yield on glucose (g/g); S_F is the glucose feeding concentration (g/L).

Based on the results of batch fermentation with the initial glucose concentration of 26 g/L, the initial levels of parameters within Eq. (1) were set as follows: V_0=3 L, Y_{XS}=0.55, X_0=13.8 g/L, S=0, and S_F=600 g/L. If the data of μ is given, the glucose feeding rate of F can be calculated quickly from Eq. (1).

Fed-batch cultivation of *C. utilis* SZU 07-01 was performed with exponential glucose feeding strategy after 12 h of batch fermentation. Fig. 3 illustrates the time-course of cell growth and glutathione production under three specific growth rates of 0.08 h^{-1}, 0.10 h^{-1} and 0.14 h^{-1}. Glucose was consumed completely by the yeast under the three specific growth rates and cells grew almost exponentially with glucose consumption. Glutathione concentration increased while intracellular glutathione content decreased accompanied with the improvement of biomass. Among the three specific growth rates, better cell growth and glutathione production were both achieved at 0.10 h^{-1}, and the maximum DCW and glutathione concentration reached 70.0 g/L and 672.6 mg/L, which were both higher than those at 0.08 h^{-1} and 0.14 h^{-1}. Table 2 also indicates that the biomass and glutathione productivity at 0.1 h^{-1} were all higher than that at 0.08 h^{-1} and 0.14 h^{-1}. Moreover, the specific growth rate of 0.1 h^{-1} can also bring a higher biomass and glutathione yield on glucose. So, 0.1 h^{-1} is a suitable choice for efficient glutathione production under glucose feeding at an exponential rate by *C. utilis* SZU 07-01.

4. High-cell-density Cultivation of *C. utilis* for the Enhanced Production of Glutathione Using the Polynomial Glucose Feeding Strategy

Within fed-batch culture of microorganisms, a suitable substrate feeding rate is very important for efficient product formation. Now, let's analyze the processes of fed-batch cultivation of *C. utilis* SZU 07-01 for glutathione production. First, when we apply a constant glucose feeding rate strategy, glucose can usually meet the requirement of the yeast at the beginning of feeding, but the increasing of yeast cells will result in the shortage of glucose for each cell. Second, if the exponential glucose feeding strategy is adopted, the limitation of glucose supply will occur just from the beginning of feeding. Therefore, the potential of the yeast on glucose consumption cannot be stimulated to the maximum all along with the fed-batch cultivation, whether glucose feeding at a constant rate or an exponential rate. To solve this problem, we developed a useful operation mode of glucose feeding followed with a polynomial equation based on the results above: the constant flow rate of 6.7 g/(L·h) was the optimal for glucose supply between 12 h and 18 h while a flow rate under μ=0.10 h^{-1} was the optimal after 18 h. The volume of the glucose feeding solution (F, mL/h) can be calculated by the following Eq. (2) within which the coefficients were estimated by the SPSS software.

$$F = 34.566 - 4.994*t + 1.235*t^2 - 0.041*t^3 \quad (2)$$

As shown in Table 2, after cultivation of *C. utilis* SZU 07-01 under this strategy, the maximum DCW and the maximum glutathione concentration reached 68.92 g/L and 691.3 mg/L, respectively, which were the highest among all the strategies used in this study. In the end, the intracellular glutathione content was obtained at 1.13% after 42 h cultivation. Also, according to the results in Table 2, higher biomass and glutathione productivity were both obtained because of short culture time. It was also indicated that the polynomial glucose feeding strategy worked well under the total glucose concentration of 150 g/L.

To confirm the polynomial glucose feeding strategy, fed-batch fermentation of glutathione with a total glucose concentration of

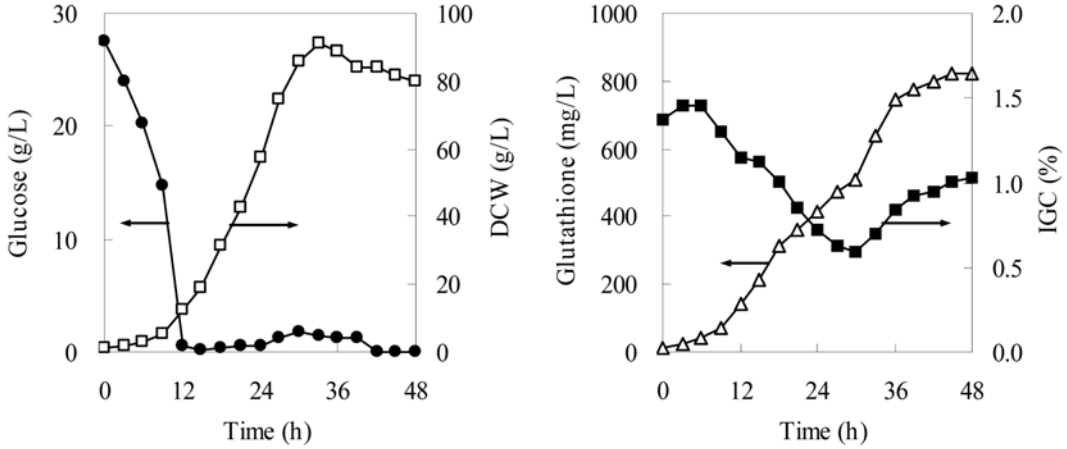

Fig. 4. Fed-batch glutathione fermentation using the polynomial glucose feeding strategy for the enhanced production of glutathione.

200 g/L was further carried out (Fig. 4). Using this model, a maximum DCW of 91.2 g/L and a total glutathione concentration of 825 mg/L were obtained after 48 h cultivation, together with a final intracellular glutathione content of 1.03%. The biomass and the glutathione productivity achieved quite a high value of 2.76 g/(L·h) and 17.19 mg/(L·h), respectively.

DISCUSSION

A major objective of fermentation in research and industry is to obtain the maximum volumetric productivity, which means obtaining the highest possible amount of product in a given volume within a certain time [4]. Although many microorganisms can be grown to high cell densities, the maximum biomass concentration does not always correlate with the maximum productivity. In this study, high biomass productivity and high glutathione productivity were both obtained.

Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product [20]. Various strategies have been developed to control the nutrient concentration within the optimal range, and have been applied to the high-cell-density culture of several microorganisms [8]. Oxygen and temperature control using substrate feeding have been used by Vanags and co-workers to simplify the system (by cutting costs) for aeration/mixing and for heat removal, retaining a pO2 and temperature that are optimal for the culture [21].

In this study, we developed a polynomial strategy since glucose feeding at constant rate cannot satisfy cells for growing in the prophase, and exponential rate may serve excess glucose during the anaphase of feeding. The strategy worked well as there was no glucose left, and both the biomass yield and the glutathione yield reached high levels. Enhanced biomass productivity and glutathione productivity were also achieved, which is much more important. When the strategy was used for the cultivation of *Candida utilis* SZU 07-01 under a higher glucose concentration of 200 g/L, the cell density and glutathione concentration reached 91.2 g/L and 825 mg/L together with the biomass and the glutathione productivity of 2.76 g/(L·h) and 17.19 mg/(L·h), respectively. Amino acids addition was confirmed as a successful way to obtain a high amount of glutathione concentration in the high-cell-density cultivation of *S. cerevisiae* [11,22] and *C. utilis* [14]. The addition of amino acids will be used in our following work.

In conclusion, based on the batch fermentation and fed-batch fermentations of glutathione production with a total glucose concentration of 150 g/L by *C. utilis* SZU 07-01 under glucose feeding at constant and exponential rate, we developed a polynomial glucose feeding strategy. The strategy worked well with the total glucose concentration of both 150 g/L and 200 g/L, which gives us a new way for the enhanced production of glutathione by high-cell-density cultivation of *C. utilis*.

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