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RESEARCH PAPER

# On-line Specific Growth Rate Control for Improving Reduced Glutathione Production in *Saccharomyces cerevisiae*

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**Abstract** Reduced glutathione (GSH), the abundant bioactive tripeptide in most living cells, is widely used in pharmaceutical, food, and cosmetic industries. Specific growth rate ($\mu$) is a key physiological parameter for GSH high-cell-density cultivation using microbial cell factories. Here, based on a biomass probe, an on-line $\mu$ feedback control was developed to regulate glucose feeding rate during the fed-batch phase for overproducing GSH in *Saccharomyces cerevisiae*. Compared with real-time $\mu$ controlled at 0.15/h, $\mu$ controlled at 0.2/h achieved yeast dry weight (120 g/L), GSH concentration (1.5 g/L), and intracellular GSH content (1.25%), which improved by 9, 150, and 129.1%, respectively. To our knowledge, this is the first report about on-line $\mu$ feedback control for GSH production. On-line $\mu$ control led to 59.38 mg/L/h of GSH productivity and 3.52 mg/g of GSH yield on glucose, which improved by 107.6 and 7.2%, respectively, in comparison with those of traditional ethanol feedback control (maintaining ethanol concentration at 1%). Taken together, the on-line $\mu$ feedback control is a promising method as an efficient alternative to conventional feed control techniques presently practiced in the GSH industry, and has the potential for the production of other valuable chemicals.

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**Keywords:** feedback control, fed-batch phase, reduced glutathione, *Saccharomyces cerevisiae*, specific growth rate

## 1. Introduction

Reduced glutathione (GSH, γ-glutamyl-L-cysteinylglycine) is one of the most abundant tripeptide in living cells [1]. Two soluble enzymes in the cytosol, γ-glutamylcysteine synthetase (also known as glutamate-cysteine ligase, EC 6.3.2.2, Gsh1p) and GSH synthetase (EC 6.3.2.3, Gsh2p), catalyze the ATP-dependent synthesis of GSH [2]. The first reaction of GSH biosynthesis catalyzed by Gsh1p is the rate-limiting step due to the feedback inhibition at both the transcriptional and post-translational levels by GSH [3]. Thus far, GSH is widely used in pharmaceutical, cosmetic, and food industries [2] (*e.g.*, in the treatment of human diseases liver cirrhosis, diabetes, neurodegenerative diseases, and aging [4]). The commercial demand and application areas of GSH will be expanded if the production cost can be further decreased [2].

Improved productivity is an efficient strategy to reduce production cost [2,5]. Currently, GSH is mainly produced *via* fermentation on an industrial scale. Fed-batch cultivation is the most common fermentation mode for achieving high cell density (HCD) and high GSH concentration [2]. Control methods such as dissolved oxygen (DO)-/pH-stat forward control and ethanol/respiratory quotient (RQ) feedback control have been employed to regulate feeding rate for GSH fed-batch cultivation [6,7]. Despite fed-batch culture is the most efficient method to improve GSH productivity, to avoid the decrease of intracellular GSH content, the specific growth rate ($\mu$) should be carefully controlled during fed-batch process [2,8,9]. However, $\mu$ is

typically detected by off-line estimation, which is often time-consuming, inaccurate and influenced by operator judgment and provides only a limited number of measurements during normal shift hours. $\mu$ is one of the most important cellular physiological parameters during the fermentation [10,11], real-time estimation and control of $\mu$ is thus necessary for industrial bioprocess control [10].

To data, on-line $\mu$ can be estimated indirectly through other measurable variables such as biomass, substrate, or products [11]. In our previous work, an *in situ* biomass probe was employed to achieve on-line monitoring of cell growth and estimation of $\mu$ during GSH fermentation [12]. Although GSH production has been well studied [13,14], to our knowledge, there are no reports on real-time $\mu$ control methods for GSH production. Here, we implemented on-line $\mu$ feedback control to regulate glucose feeding rate during the fed-batch phase to achieve simultaneous increases in cell density, GSH concentration, and intracellular GSH content. A classical proportional-integral-derivative (PID) controller was employed to maintain $\mu$ at a desired value. Our work indicates simultaneous improvements in GSH concentration and intracellular GSH content with an on-line $\mu$ control strategy.

## 2. Materials and Methods

### 2.1. Strain, medium, and culture conditions

*S. cerevisiae* T65 was maintained on yeast extract peptone dextrose medium (YPD) agar in our laboratory at 4°C. The seed medium, fermentation medium, and feed medium were described elsewhere [15]. A colony of *S. cerevisiae* was picked into a 500 mL flask containing 100 mL of the seed medium and incubated at 30°C and 220 rpm for 10 h. A 50 L fermentor (FUS-50L(A); NCBio, Shanghai, China) containing 20 L fermentation medium was sterilized at 121°C for 20 min and inoculated with 5% (v/v) yeast seed cultures. Fermentation temperature was controlled at 30°C with air flow rate of 1 vvm, and pH was kept at 5.2 by the automatic addition of 20% ammonia. The agitation speed was controlled at 600 rpm. DO was measured with an autoclavable $O_2$ sensor (Mettler Toledo, Greifensee, Switzerland). Capacitance (*Cap*) was on-line monitored with a Biomass Monitor 220 probe (Aber Instruments, Aberystwyth, UK). During the culture, measurements were taken every 20 sec and averaged over the routine interval of 2 min. The principle of biomass ($X$) estimation by this probe was reported elsewhere [12]. A linear correlation was established between $X$ and dual-frequency *Cap* values at 500 kHz and 10 MHz. On-line $X$ and $\mu$ were calculated by *Cap* measurement using the following equations [12]:

$$X = \alpha Cap$$

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{1}{Cap}\frac{dCap}{dt} \quad (1)$$

Ethanol concentration was real-time determined with an ethanol probe (FC-2002, ECUST, Shanghai, China). All the data including ethanol and *Cap* were monitored, and $X$ and $\mu$ were automatically calculated on-line by bioradar software package (ver. 2.0; NCBio, Shanghai, China). All acquired data (*e.g.*, pH, DO, agitation speed, temperature, total glucose feeding amount, glucose, ethanol, GSH, and *Cap*) were automatically collected by Labveiw (National Instruments, Austin, USA) and inputted into bioradar system for monitoring and analysis of fermentation processes and regulation of these cultivation parameters [6,12].

### 2.2. Cultivation procedure

A typical GSH production can be separated into three stages: batch culture, fed-batch culture, and bioconversion phase [12]. During the batch phase, glucose (60 g/L) was exhausted at ~10 h when ethanol concentration reached the maximum value. After the batch culture, to achieve HCD cultivation, the feeding medium containing 600 g/L of glucose was added to the bioreactor by implementing two on-line control methods: $\mu$ or ethanol feedback control that kept either $\mu$ or ethanol at respective set-point based on the measured values of $\mu$ or ethanol (Fig. 1A). The bioradar program calculated the values of $\mu$ or ethanol every 2 min. A classical PID controller maintained $\mu$ or ethanol at the respective set values by manipulating feeding rate ($F_t$). Fig. 1B is a diagram depicting the feedback control methods in this work. If the measured $\mu$/ethanol value was outside the set-point range, the controller performed the PID mathematical function on the error (the difference between the set-points and the measured value) and output a corrective action that changed the feeding rate for adjusting $\mu$/ethanol to minimize the error (Fig. 1B). Subsequently, at ~26 h by $\mu$ control or at ~36 h by ethanol control, with the addition of 5 mmol/L GSH precursors (cysteine, glutamate, and glycine), the bioconversion phase was initiated to enhance GSH biosynthesis. Antifoam agent was added as required during the fermentation.

### 2.3. Analytical methods

Glucose in the fermentation broth was detected with a glucose kit (Shanghai Institute of Biological Products, Shanghai, China). GSH was extracted from the cells by 40% ethanol and detected with the alloxan method [16]. Intracellular GSH content (%) was calculated by GSH concentration/DCW × 100%. All of the assays were performed in triplicate.

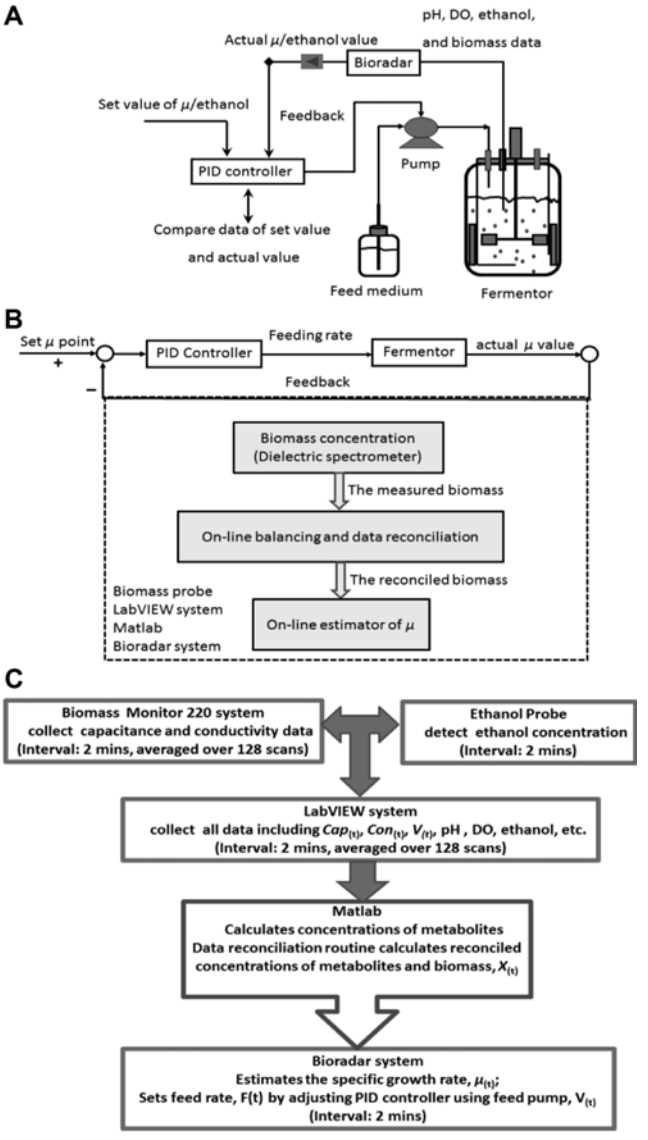


**Fig. 1.** Feedback control based on on-line $\mu$ or ethanol. (A) schematic diagram, (B) block diagram, and (C) implementation flowchart. The variables included in the arrow boxes represent those that are passed from one program to another *via* a dynamic interface.

## 3. Results and Discussion

### 3.1. GSH fed-batch fermentation by on-line $\mu$ feedback control

From the bioprocess point of view, $\mu$ is an essential process variable that characterizes cell physiological state and relates to the biosynthesis of many valuable products [10,11]. For example, Gao *et al.* used an improved artificial neural network pattern recognition model-based glycerol feeding strategy to maintain $\mu > 0.11$/h during recombinant *Pichia pastoris* fermentation. With this feeding strategy, the porcine interferon-α concentration reached 1.43 g/L, which is more than 1.5-fold higher than that of the previously adopted feeding strategy [17]. In GSH production, $\mu$ should be carefully controlled to avoid the decrease of GSH titer and intracellular GSH content [2,9]. Using exponential feeding mode, $\mu$ can significantly affect GSH accumulation in recombinant *E. coli* [18]. Compared with the constant-rate feeding/artificial feedback feeding strategy, exponential feeding (manual controlling $\mu$ at 0.2/h) was greatly improved both cell concentration (80 g/L), cell productivity (3.2 mg/L/h), and GSH concentration (0.88 g/L) [18]. Such a phenomenon also exists in yeast. Exponential feeding (maintaining $\mu$ at 0.13/h with manual adjustment of glucose feeding rate) significantly enhanced both cell concentration (105 g/L) and GSH concentration (0.81 g/L) in *S. cerevisiae* compared with the constant feeding strategy [19]. However, $\mu$ was off-line calculated and controlled in all reports, which cannot promptly reflect cell physiological change for GSH production. Additionally, $F_t$ of exponential feeding could be directly calculated according to the following equation:

$$F_t = \frac{\mu V_0 X_0}{Y_{x/s}(S_F - S_0)} \exp(\mu t) \tag{2}$$

Where $V_0$ is the initial bioreactor volume (L); $X_0$ is the initial biomass concentration (g/L); $S_0$ is the initial glucose concentration in the bioreactor (g/L); $Y_{X/S}$ is the biomass yield on glucose (g/g); and $S_F$ is glucose concentration in the feeding medium.

Owing to a limited number of biomass and glucose measurements during the fermentation, the change of $F_t$ cannot quickly and accurately regulate $\mu$ to the desired value by manual control.

Compared with the above off-line manual control, on-line automatic control of $\mu$ could be more accurate and convenient for bioprocess control of GSH production. We previously enabled on-line calculation of $\mu$, which offers an opportunity to develop an applicable strategy for bioprocess optimization during GSH fermentation [12]. In this work, $\mu$ was applied in the feedback control of *S. cerevisiae* fed-batch culture for GSH production. The overall implementation flowchart is shown in Fig. 1C. All programs in Labview, matlab, and bioradar as well as the dynamic interfaces between the different programs were coded in-house and tested rigorously before this study. After the batch phase, fed-batch phase was carried out based on on-line $\mu$ feedback control. To maintain $\mu$ at the set value, a feedback control was deployed to adjust $F_t$ *via* the PID controller. $F_t$ was changed continuously based on the measured $\mu$ value. Thus, $\mu$ can keep constant with the minimum error. In our previous work, this type of PID controller was also achieved good performance for the feedback control of RQ by regulating

**Table 1.** Effect of different $\mu$ on cell growth and GSH production during the fed-batch phase (without the addition of GSH precursors glycine, cysteine, and glutamate)

| Parameters | Results | | |
|---|---|---|---|
| $\mu$ (/h) | 0.15 | 0.20 | 0.25 |
| Dry cell weight (g/L) | 110 ± 1.0 | 120 ± 2.0 | 135 ± 1.0 |
| GSH concentration(g/L) | 0.60 ± 0.03 | 1.50 ± 0.08 | 1.10 ± 0.05 |
| Intracellular GSH content (%) | 0.55 ± 0.01 | 1.25 ± 0.01 | 0.81 ± 0.01 |
| Cell productivity (g/L/h) | 4.23 ± 0.04 | 4.62 ± 0.08 | 5.19 ± 0.04 |
| GSH productivity (mg/L/h) | 23.08 ± 1.15 | 57.69 ± 3.07 | 42.31 ± 1.92 |
| Cell yield on glucose (g/g) | 0.24 ± 0.01 | 0.22 ± 0.01 | 0.21 ± 0.01 |
| GSH yield on glucose (mg/g) | 1.33 ± 0.07 | 2.78 ± 0.15 | 1.69 ± 0.08 |

All date are the average of three replicate experiments.

glucose feeding rate in *S. cerevisiae* [6].

Here we proposed that if we control $\mu$ at a suitable level, cells might maintain TCA metabolic flux at a moderate level, glucose overflow might be relieved, and more carbon flux might be steadily directed toward GSH biosynthesis. $\mu$ control might achieve our goal of improving both GSH concentration and intracellular GSH content. The effect of $\mu$ at 0.15, 0.20, and 0.25/h on the cell density and GSH production is summarized in Table 1 and Fig. 2. The final GSH concentration was not the highest at $\mu$ = 0.25/h among the three $\mu$ settings studied. The reason might be that, owing to overflow metabolism resulted by speedy glucose uptake rate [20], byproducts such as glycerol and ethanol (data not shown) in the broth quickly accumulated and resulted in the decrease of the carbon flux toward GSH biosynthesis. To limit the accumulation of byproducts in the broth, $\mu$ value was set at 0.20/h, and glucose feed rate was changed by PID controller when $\mu$ either exceed 0.21/h or dipped below 0.19/h. At $\mu$ = 0.20/h, GSH concentration improved by 36.3% over that at $\mu$ = 0.25/h. However, further decreasing $\mu$ to 0.15/h caused a lower biomass concentration (110 g/L) and a very lower GSH concentration (0.6 g/L). It may be that different $\mu$ affect the activity of key enzymes for GSH biosynthesis such as Gsh1p and Gsh2p, and result in different GSH levels in cells. A similar phenomenon was found by manual $\mu$ control for GSH and S-adenosyl-L-methionine co-production [19]. Table 1 showed that different levels of $\mu$ have a significant effect on intracellular GSH content, GSH productivity, and GSH yield on glucose. To our knowledge, this is the first report about on-line $\mu$ feedback control method for GSH production. In addition, in comparison with GSH production in the literatures (Table 2), $\mu$ controlled at 0.20 resulted in the higher GSH concentration and productivity as well as concomitant improvements in cell density and intracellular GSH content.

### 3.2. Comparison of on-line $\mu$ feedback control with ethanol feedback control in the GSH industry

The industrial GSH production has been successful to practice fed-batch fermentation where $F_t$ is manipulated by ethanol feedback control [6,15]. Hence, we compared on-line $\mu$ feedback control (controlling $\mu$ at 0.20/h) and ethanol feedback control (maintaining ethanol concentration

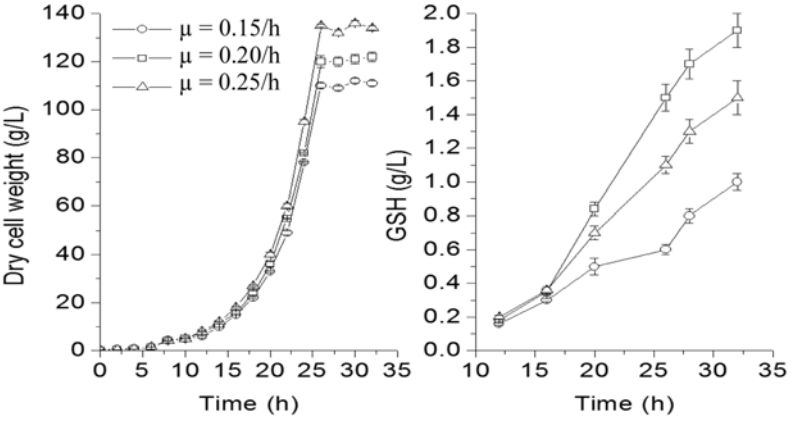


**Fig. 2.** Effect of different $\mu$ on cell growth and GSH production.

**Table 2.** Summary of GSH production with different control strategies during the fed-batch phase (without the addition of GSH precursors)

| Strain | Control mode | Biomass (g/L) | GSH (g/L) | Intracellular GSH content (%) | GSH productivity (mg/L/h) | Reference |
|---|---|---|---|---|---|---|
| *S. cerevisiae* KY6186 | Model-based feeding | 63.8 | 2.36 | ~5.0 | ~39.3 | [7] |
| *S. cerevisiae* KY5711 | Ethanol feedback[a] | ~1.0 | ~0.05 | ~5.0 | ~12.5 | [8] |
| *S. cerevisiae* KY5711 | Model-based feeding | ~11 | ~0.09 | ~0.8 | ~9 | [8] |
| *S. cerevisiae* GE-2 | Ethanol feedback[b] | ~90 | ~0.90 | ~1.0 | ~21.4 | [15] |
| *S. cerevisiae* GE-2 | Ethanol feedback[c] | ~90 | ~0.90 | ~1.0 | ~37.5 | [15] |
| *S. cerevisiae* LYCC7048 | Ethanol feedback[d] | ~95 | ~0.53 | ~0.56 | ~37.9 | [21] |
| *S. Cerevisiae* ZJUS1 | Exponential feeding | 105 | 0.81 | 0.77 | 13.5 | [22] |
| *S. cerevisiae* T65 | RQ feedback[e] | 112 | 1.50 | 1.34 | 46.9 | [6] |
| *S. cerevisiae* T65 | $\mu$ feedback[f] | 120 | 1.50 | 1.25 | 57.6 | In this study |
| *P. pastoris* D18 | Constant-rate feeding | ~90 | 0.92 | ~1.0 | 32.8 | [23] |
| *C. utilis* WSH 02-08 | Exponential feeding[g] | 81.2 | 0.76 | 0.94 | 38 | [24] |
| *C. utilis* SZU 07-01 | Constant-rate feeding | 65 | 0.65 | 1.11 | 12.1 | [25] |
| *C. utilis* SZU 07-01 | Exponential feeding[h] | 70 | 0.67 | 1.12 | 14 | [25] |
| *C. utilis* SZU 07-01 | Polynomial feeding | 68.9 | 0.69 | 1.13 | 16.5 | [25] |
| *H. polymorpha* DL-1 | DO-stat[i] | 72 | 0.91 | 1.26 | ~6.5 | [26] |
| *H. polymorpha* MOXp-GSH2 | DO-stat[i] | 72 | 2.26 | 3.15 | ~16.1 | [26] |
| *E. coli* WSH-KE1 | Constant-rate feeding | 77.5 | 0.78 | 1.0 | 18.8 | [18] |
| *E. coli* WSH-KE1 | Artificial feeding | 64 | 0.45 | 0.7 | 7.0 | [18] |
| *E. coli* WSH-KE1 | Exponential feeding | 80 | 0.88 | 1.1 | 35.2 | [18] |

[a]Maintaining a constant ethanol concentration by fuzzy logic control.
[b]Keeping ethanol concentration at ~1%.
[c]Controlling ethanol concentration descending rate between 0.1 and 0.15%/h.
[d]Keeping ethanol concentration at ~ 0.2 g/L.
[e]Keeping RQ concentration at 0.65.
[f]Keeping $\mu$ at 0.20/h.
[g]Controlling $\mu$ at ~0.20/h.
[h]Controlling $\mu$ at ~0.10/h.
[i]Keeping DO at 30%.

**Table 3.** Comparison of two different feedback control schemes

| Feedback control mode | $\mu$[a] | Ethanol[b] |
|---|---|---|
| Time (h) | 32 | 42 |
| Dry cell weight (g/L) | 122 ± 2.0 | 113 ± 5.7 |
| GSH yield (g/L) | 1.90 ± 0.1 | 1.20 ± 0.1 |
| GSH content (%) | 1.67 ± 0.05 | 1.06 ± 0.02 |
| Cell productivity (g/L/h) | 3.81 ± 0.07 | 2.69 ± 0.14 |
| GSH productivity (mg/L/h) | 59.38 ± 3.2 | 28.60 ± 2.4 |
| Cell yield on glucose (g/g) | 0.23 ± 0.01 | 0.31 ± 0.21 |
| GSH yield on glucose (mg/g) | 3.52 ± 0.19 | 3.28 ± 0.28 |

[a]Keeping $\mu$ at 0.20/h.
[b]Keeping ethanol concentration at 1%.

at ~1%) for GSH production (Fig. 3 and Table 3). GSH concentration (1.9 g/L, Fig. 3A), yeast dry weight (122 g/L, Fig. 3B), GSH content (1.56%) and GSH productivity (59.38 mg/L/h) achieved with $\mu$ control were significantly higher than those obtained with ethanol control (Table 3). Importantly, $\mu$ control increased GSH yield on glucose (3.52 mg/g) by 7.2% over that obtained with ethanol control (3.28 mg/g). Despite $\mu$ control required more expensive equipment (biomass probe) than ethanol control did (ethanol probe), it may still result in a lower total production cost because of a shorter cultivation period, a lower byproduct concentration (*e.g.*, ethanol, Fig. 3C), a higher GSH content, and a higher GSH concentration with $\mu$ feedback control over ethanol feedback control. Hence, on-line $\mu$-based control method could be an efficient alternative to ethanol-based control method in the GSH industry. In addition, Min *et al*. found that a two-step control of $\mu$ could significantly enhance the production of a novel disintegrin, saxatilin, in recombinant *P. pastoris* [27]. Our future experiments could also explore to optimize the on-line $\mu$ control method (*e.g.* two-stage $\mu$ feedback control strategy) to further improve GSH production. The biosynthesis of many products is closely dependent on controlling $\mu$ [10], our feeding strategy based on $\mu$ control is potentially suitable for the production of other metabolites accumulated in the cells.

Transformation phase is an important step for GSH production [6], which can significantly stimulate GSH biosynthesis by the addition of GSH precursors. GSH titer and productivity could also gradually improve with the

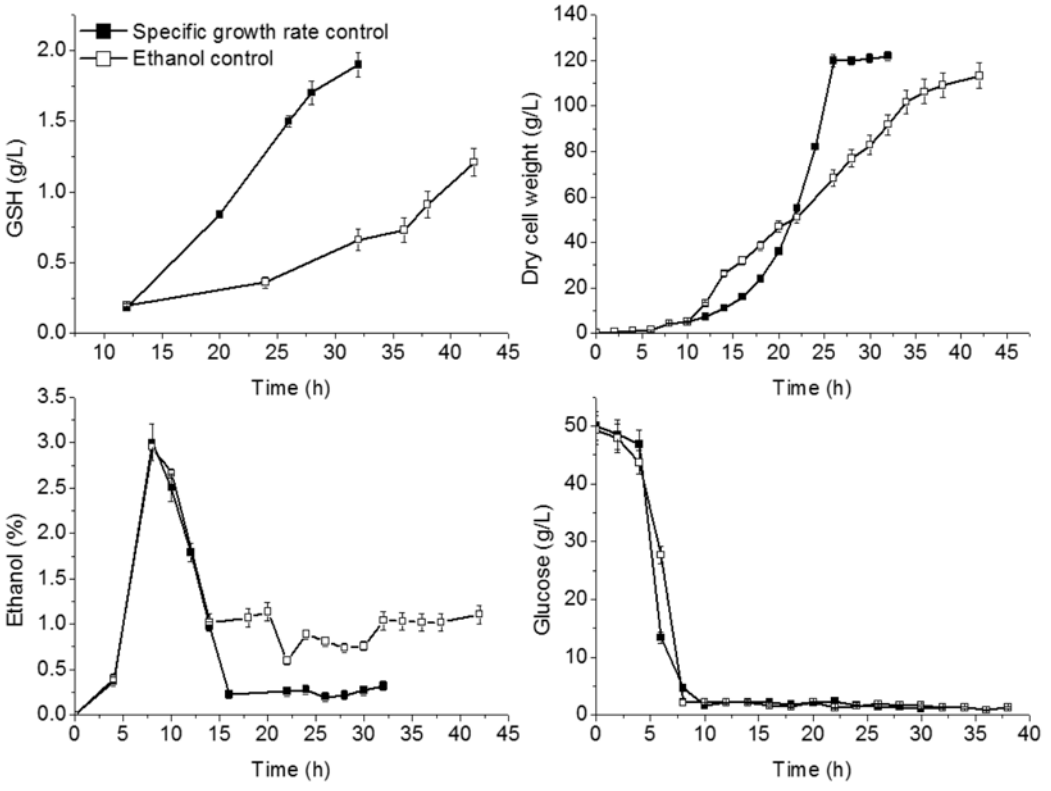


**Fig. 3.** Comparison of on-line $\mu$ (controlling $\mu$ at 0.20/h) and ethanol (maintaining ethanol concentration at 1%) feedback control schemes. (A) GSH titer, (B) dry cell weight, (C) ethanol concentration, and (D) glucose concentration.

increasing concentration of precursors [23,28]. For example, GSH concentration significantly enhanced from 2.03 to 4.15 g/L when the precursors concentration increased from 5 to 15 mmol/L [23]. Only 5 mmol/L GSH precursors were added in our experiment, which led to 20% improvement of GSH production (Fig. 3A). Hence, a strategy of combining on-line $\mu$ control and increased precursor addition may improve GSH productivity. Moreover, metabolic engineering of the high-yield strains is important to GSH production. Engineered targets such as Gsh1p, Gsh2p, GshF (a bacterial bifunctional enzyme possessing both GSH1p and Gsh2p activities), MET14 (adenylylsulfate kinase), MET16 (3'-phosphoadenylsulfate reductase), CYS4 (cystathionine beta-synthase), and DEF1 (RNAPII degradation factor) have been demonstrated to improve GSH biosynthesis [14,29,30]. Therefore, the production cost of GSH could be further decreased by an engineered strain deploying with on-line $\mu$ control strategy.

## 4. Conclusion

Based on a biomass probe, an on-line $\mu$ feedback control strategy was developed to control glucose feeding rate during the fed-batch phase for GSH production. The present GSH production study indicates that glucose feed with $\mu$ set at 0.20 is an easily implemented control strategy that enhanced cell growth and increased both GSH yield and intracellular GSH content. Compared with the conventional ethanol feedback control, $\mu$ feedback control represents an effective alternative. Moreover, this feeding strategy based on $\mu$ feedback control has the potential for the cultivation of other organisms or production of other valuable chemicals.

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## References

1. Penninckx, M. (2000) A short review on the role of glutathione in

the response of yeasts to nutritional, environmental, and oxidative stresses. *Enz. Microb. Technol*. 26: 737-742.
2. Li, Y., G. Y. Wei, and J. Chen (2004) Glutathione: A review on biotechnological production. *Appl. Microbiol. Biotechnol*. 66: 233-242.
3. Korz, D. J., U. Rinas, K. Hellmuth, E. A. Sanders, and W. D. Deckwer (1995) Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J. Biotechnol*. 39: 59-65.
4. Navarro, J., E. Obrador, J. Carretero, I. Petschen, J. Avino, P. Perez, and J. M. Estrela (1999) Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth *in vivo*. *Free Radic. Biol. Med*. 26: 410-418.
5. Cha, J. Y., J. C. Park, B. S. Jeon, Y. C. Lee, and Y. S. Cho (2004) Optimal fermentation conditions for enhanced glutathione production by *Saccharomyces cerevisiae* FF-8. *J. Microbiol*. 42: 51-55.
6. Xiong, Z. Q., M. J. Guo, Y. X. Guo, J. Chu, Y. P. Zhuang, N. S. Wang, and S. L. Zhang (2010) RQ feedback control for simultaneous improvement of GSH yield and GSH content in *Saccharomyces cerevisiae* T65. *Enz. Microb. Technol*. 46: 598-602.
7. Sakato, K. and H. Tanaka (1992) Advanced control of glutathione fermentation process. *Biotechnol. Bioeng*. 40: 904-912.
8. Alfafara, C. G., K. Miura, H. Shimizu, S. Shioya, K. I. Suga, and K. Suzuki (1993) Fuzzy control of ethanol concentration its application to maximum glutathione production in yeast fed-batch culture. *Biotechnol. Bioeng*. 41: 493-501.
9. Shimizu, H., K. Araki, S. Shioya, and K. I. Suga (1991) Optimal production of glutathione by controlling the specific growth rate of yeast in fed-batch culture. *Biotechnol. Bioeng*. 38: 196-205.
10. Dabros, M., M. M. Schuler, and I. W. Marison (2010) Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced online measurements of biomass. *Bioproc. Biosyst. Eng*. 33: 1109-1118.
11. Schuler, M. M. and I. W. Marison (2012) Real-time monitoring and control of microbial bioprocesses with focus on the specific growth rate: current state and perspectives. *Appl. Microbiol. Biotechnol*. 94: 1469-1482.
12. Xiong, Z. Q., M. J. Guo, Y. X. Guo, J. Chu, Y. P. Zhuang, and S. L. Zhang (2008) Real-time viable-cell mass monitoring in high-cell-density fed-batch glutathione fermentation by *Saccharomyces cerevisiae* T65 in industrial complex medium. *J. Biosci. Bioeng*. 105: 409-413.
13. Wang, Y., D. Wang, G. Wei, and C. Wang (2013) Improved co-production of S-adenosylmethionine and glutathione using citrate as an auxiliary energy substrate. *Bioresour. Technol*. 131: 28-32.
14. Ge, S., T. Zhu, and Y. Li (2012) Expression of bacterial GshF in *Pichia pastoris* for glutathione production. *Appl. Environ. Microbiol*. 78: 5435-5439.
15. Shang, F., Z. Wang, and T. Tan (2008) High-cell-density cultivation for co-production of ergosterol and reduced glutathione by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol*. 77: 1233-1240.
16. Wen, S. H., T. Zhang, and T. W. Tan (2005) Optimization of the amino acid composition in glutathione fermentation. *Proc. Biochem*. 40: 3474-3479.
17. Gao, M. J., Z. Y. Zheng, J. R. Wu, S. J. Dong, Z. Li, H. Jin, X. B. Zhan, and C. C. Lin (2012) Improvement of specific growth rate of *Pichia pastoris* for effective porcine interferon-alpha production with an on-line model-based glycerol feeding strategy. *Appl. Microbiol. Biotechnol*. 93: 1437-1445.
18. Li, Y., J. Chen, Y. Y. Mao, S. Y. Lun, and Y. M. Koo (1998) Effect of additives and fed-batch culture strategies on the production of glutathione by recombinant *Escherichia coli*. *Proc. Biochem*. 33: 709-714.
19. Lin, H. P., J. Tian, J. F. You, Z. H. Jin, Z. N. Xu, and P. L. Cen (2004) An effective strategy for the co-production of S-adenosyl-L-methionine and glutathione by fed-batch fermentation. *Biochem. Eng. J*. 21: 19-25.
20. Vemuri, G. N., M. A. Eiteman, J. E. McEwen, L. Olsson, and J. Nielsen (2007) Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A*. 104: 2402-2407.
21. Nisamedtinov, I., K. Kevvai, K. Orumets, J. J. Rautio, and T. Paalme (2010) Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding. *Appl. Microbiol. Biotechnol*. 87: 175-183.
22. Liu, H., J. P. Lin, P. L. Cen, and Y. J. Pan (2004) Co-production of S-adenosyl--methionine and glutathione from spent brewer's yeast cells. *Proc. Biochem*. 39: 1993-1997.
23. Fei, L., Y. Wang, and S. Chen (2009) Improved glutathione production by gene expression in *Pichia pastoris*. *Bioproc. Biosyst. Eng*. 32: 729-735.
24. Liang, G., G. Du, and J. Chen (2008) A novel strategy of enhanced glutathione production in high cell density cultivation of *Candida utilis*-Cysteine addition combined with dissolved oxyen controlling. *Enz. Microb. Technol*. 42: 284-289.
25. Nie, M., G. Y. Wei, N. Shao, and X. G. Ge (2010) A novel strategy on the high-cell-density cultivation of *Candida utilis* for the enhanced production of glutathione. *Kor. J. Chem. Eng.* 27: 1246-1251.
26. Ubiyvovk, V. M., V. M. Ananin, A. Y. Malyshev, H. A. Kang, and A. A. Sibirny (2011) Optimization of glutathione production in batch and fed-batch cultures by the wild-type and recombinant strains of the methylotrophic yeast *Hansenula polymorpha* DL-1. *BMC Biotechnol*. 11: 8.
27. Min, C. K., J. W. Lee, K. H. Chung, and H. W. Park (2010) Control of specific growth rate to enhance the production of a novel disintegrin, saxatilin, in recombinant *Pichia pastoris*. *J. Biosci. Bioeng*. 110: 314-319.
28. Wang, Y., D. Wang, G. Wei, and N. Shao (2012) Enhanced co-production of S-adenosylmethionine and glutathione by an ATP-oriented amino acid addition strategy. *Bioresour. Technol*. 107: 19-24.
29. Suzuki, T., A. Yokoyama, T. Tsuji, E. Ikeshima, K. Nakashima, S. Ikushima, C. Kobayashi, and S. Yoshida (2011) Identification and characterization of genes involved in glutathione production in yeast. *J. Biosci. Bioeng*. 112: 107-113.
30. Hara, K. Y., K. Kiriyama, A. Inagaki, H. Nakayama, and A. Kondo (2012) Improvement of glutathione production by metabolic engineering the sulfate assimilation pathway of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol*. 94: 1313-1319.