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Full Length Research Paper

Novel pH control strategy for glutathione overproduction in batch cultivation of *Candida utilis*

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The effects of pH values on cell growth and glutathione (GSH) production were studied in batch cultivation of *Candida utilis*. According to the fact that lower pH value favors cells growth but retards GSH production and higher pH value promotes GSH production while inhibits cells growth, a pH-shift strategy, optimized via simulating Gauss function, was developed. By applying two-stage pH-shift strategy of controlling pH at 5.0 for first 7.5 h and switching to 6.0 afterwards, final GSH yield and productivity reached 279 and 12.7 mg/l/h after 22 h cultivation, increased by 30 and 42%, respectively, compared to constant pH 5.5 operation. Moreover, by feeding glucose instead of sulphuric acid (H_2SO_4) solution to control pH, maximal GSH yield of 315 mg/l was achieved, suggesting application of pH-shift strategy for GSH overproduction as being feasible.

Key words: Batch fermentation, *Candida utilis*, glutathione (GSH), gauss function, pH-shift strategy.

INTRODUCTION

Glutathione (GSH), as the most abundant non-protein thiol compound, is widely distributed in living organisms (Meister and Anderson, 1983). GSH is an important antioxidant for protecting DNA, proteins and other biomolecules against oxidative damage caused by reactive oxygen species (Pastore et al., 2003; Meister, 1994; Sies, 1999; Mehdi and Penninckx, 2000). As GSH was increasingly interesting in medical treatment, health care, functional foods and cosmetics, the commercial demand has been expanding (Wu et al., 2004).

With discovery in many living organisms, solvent extraction of GSH from animal or plant active tissues was widely exploited. However, limited raw materials available and relatively low intracellular GSH content made end-product expensive, hampering its practical application. Subsequently, it was demonstrated that GSH could be synthesized by chemical method (Harington and Mead, 1935). However, chemically synthesized GSH was an optically inactive mixture. As only L-form is physiologically active, an optical resolution is required to separate L-form from its D-isomer (Douglas, 1989). Following observation of biosynthesis of GSH in isolated liver and characterization of GSH biosynthetic pathway (Bloch, 1949), explorations on the enzymatic and fermentative production of GSH were extensively pursued. To date, enzymatic production of GSH has not been comercialized because of relatively high production cost. Alternatively, GSH production by yeast fermentation is efficient and practical.

Saccharomyces cerevisiae and *Candida utilis* are currently used microorganisms for GSH production on industrial scales. In recent years, studies on enhancing GSH production were mainly concentrated on increasing cell biomass and intracellular GSH content via fed-batch culture and amino acid additions (Wen et al., 2004; Wang et al., 2007; Liang et al., 2008a). However, the other methods or strategies developed for GSH production are still little known.

It was documented that GSH plays a crucial role in maintaining cell physiological function under environmental stress (Mehdi and Penninckx, 2000; Izawa et al., 1995; Smirnova et al., 2001). Also, intracellular GSH was accumulated greatly to protect cells from injuries, such as,

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by oxidative and osmotic stresses (Pompella et al., 2003; Ruiz and Blumwald, 2002). Accordingly, in the previous study, we applied H_2O_2-induced oxidative stresses to stimulating GSH production in *C. utilis* (Liang et al., 2008b).

Additionally, for batch GSH production, the optimal conditions for cell growth and GSH formation may be quite different (Li et al., 2004). GSH formation rate, yield and productivity varied with medium composition and environmental parameters, including pH and temperature. To obtain high GSH yield and productivity, it is vital to optimize conditions for cells growth and GSH formation during batch fermentation. For example, based on the fact that the optimal temperature for cells growth and GSH formation differed, a temperature-shift strategy was developed to enhance GSH production in batch culture of *C. utilis* (Wei et al., 2003).

Being an intracellular product, GSH fermentation was related to biomass and intracellular GSH content, which were dramatically affected by broth pH value. Tragically, there was no report about the influence of broth pH on GSH formation in yeasts GSH batch fermentation.

Accordingly, effects of broth pH values on cells growth and GSH formation in batch culture of *C. utilis* were investigated in this paper. Based on the results that the optimal pH values for cells growth and GSH formation differed, a novel two-stage pH-shift strategy, optimized by simulating Gauss function, was developed to enhance GSH production. Furthermore, by feeding glucose replacing H_2SO_4 for controlling pH, a maximal GSH production was achieved.

MATERIALS AND METHODS

Microorganism and seed culture

A high-GSH yeast strain of *C. utilis* WSH 02-08 was used in this study. Seed medium contained (g/l) glucose 20, peptone 20 and yeast extract 10 at pH 6.0. Seed culture was grown in a 250 ml flask containing 50 ml medium on a shaker at 200 rpm and 30°C for 20 h.

Batch cultivation in 7 l fermentor

Batch cultivation of GSH in 7 l fermentor was accorded to the methods described by (Liang et al., 2008b). Medium for batch culture contained (g/l): glucose 30, yeast extract 5, $(NH_4)_2SO_4$ 10, KH_2PO_4 3 and anhydrous $MgSO_4$ 0.25. 10% (v/v) of seed culture was inoculated into a 7 l fermentor with a working volume of 5 l. The pH was controlled automatically by adding 3 mol/l H_2SO_4 or 3 mol/l NaOH solutions. Agitation speed and temperature were controlled at 300 rpm and 30°C, respectively.

Feeding glucose to control pH

Based on the two-stage pH-shift strategy, glucose solution of 500 g/l was used to replace 3 mol/l H_2SO_4 solution for automatically controlling pH at 5.0 for first 7.5 h and 6.0 afterwards.

Calculation of specific rates of cells growth, glucose consumption and GSH production

Specific cell growth rate (μ_{cell}) was determined from the slope of semilogarithmic plot of cell density versus fermentation time.

$$\mu_{cell} = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta x}{\Delta t}$$

Specific glucose consumption rate (q_s) was determined from the slope of semilogarithmic plot of residue glucose concentration versus fermentation time.

$$q_s = \frac{1}{s}\frac{ds}{dt} = \frac{1}{s}\lim_{\Delta t \to 0}\frac{\Delta s}{\Delta t}$$

Specific GSH production rate (q_p) was determined from the reciprocal of cell density and GSH yield versus fermentation time.

$$q_p = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta p}{\Delta t}$$

Analytical methods

Culture broth of 25 ml was centrifuged at 3500 g for 15 min at 4°C and cells were washed twice with ice-cold saline (0.85% NaCl, w/v). Wet cells were extracted with 40% (v/v) ethanol at 30°C for 2 h and centrifuged at 5000 g for 20 min at 4°C and the supernatant was used for GSH assay. GSH concentration was determined according to the method described by (Tietze, 1969). Dry cell weight (DCW) was determined after drying cells at 105°C to constant weight. Glucose concentration was measured according to colorimetric method (Miller, 1959). Intracellular ATP was determined by high performance liquid chromatography (HPLC) according to the method described by Veciana-Nogues (Veciana-Nogues et al., 1997). Three precursor amino acids were detected according to the method (Wang et al., 2006).

All experiments were repeated twice and average results were used for analysis.

RESULTS

Time courses of GSH fermentation at natural pH values

To ascertain the suitable pH scope for cells growth and GSH formation, GSH batch culture with 30 g/l glucose without pH control was carried out in 7 l fermentor. Status quo of GSH production at the natural pH (initial pH in the broth was adjusted to 6.5 and not controlled afterwards) is shown in Figure 1. Apparently, pH in the broth decreased gradually and reached 2 or so at 16 h. At this time, glucose was nearly exhausted and cells stopped growing at 18 h. It was noted that cells growth became slowly as pH decreased to about 4 after 8 h cultivation, suggesting inhibition of cells growth occurs. With cultivation further extending, GSH yield was increased correspondingly and a maximal yield of 168 mg/l was achieved at 24 h. How-

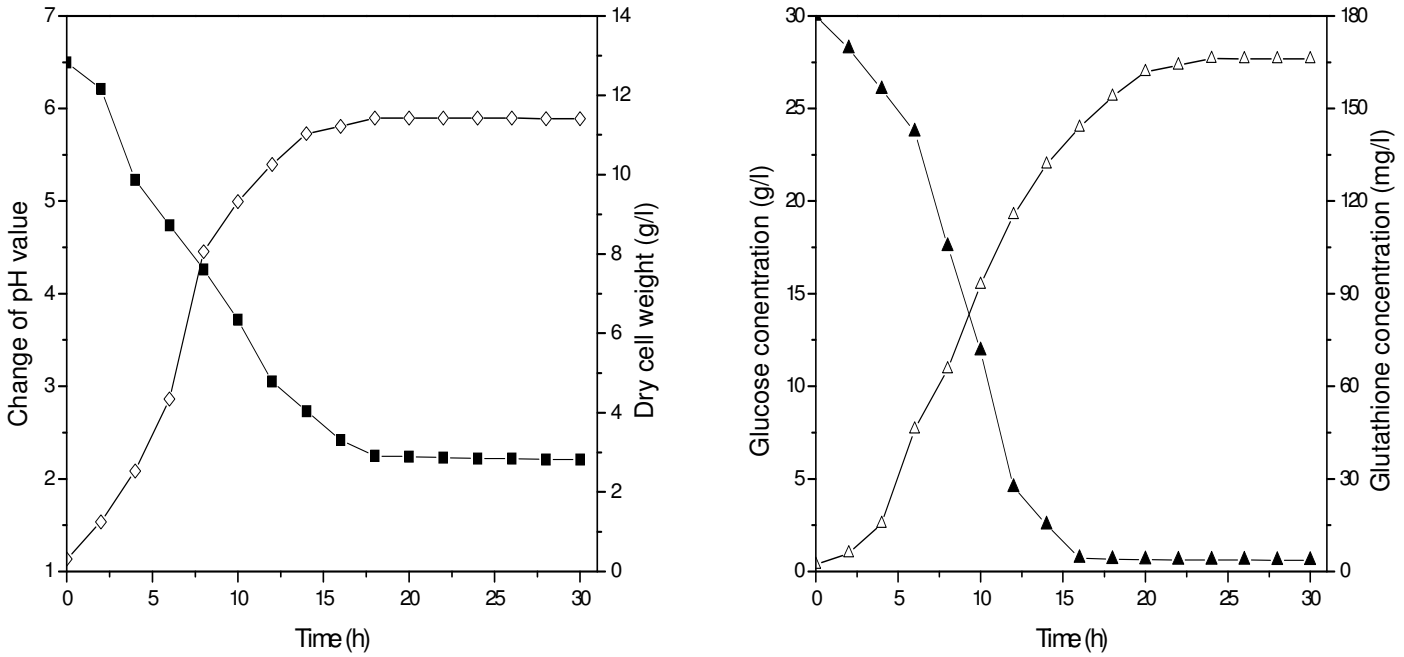

Figure 1. Time course of cells growth, glucose consumption and GSH production at natural pH. ■, changes of pH; ◇, dry cell weight; ▲, glucose concentration; △, glutathione concentration.

ever, we noticed that only 28 mg/l GSH was synthesized during 18 to 24 h, which indicated that GSH production was severely inhibited and the reason can be attributed to the lowered pH value. Accordingly, it is necessary to investigate role of pH at different values on cells growth and GSH production.

Effects of different pH values on cells growth and GSH production

Based on above results that little cells growth occurred as pH was lower than 4.0, the effects of pH values at the range of 4.0 - 6.5 on GSH production were studied and results were shown in Figure 2. Cells growth was greatly affected by pH values. For example, at pH 5.0, cells grew very quickly and final biomass reached 17.22 g/l at 12 h, which was higher than that at other pH values. It was suggested that the optimal pH for cells growth was 5.0. Meanwhile, profiles of μ_{cell} at different pH values showed that μ_{cell} at pH 5.0 was higher than at other pH values before 8 h, documenting the highest biomass being achieved with pH 5.0. Moreover, at pH 5.0, glucose was almost consumed at 10 h and q_s was the highest at first 6 h, also demonstrating the maximal DCW achieved above.

In addition, maximal GSH yield (mg/l) reached 192, 208, 212, 215, 210 and 202 with pH at 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5, respectively. It was indicated that the impact of broth pH values on total GSH yield was insignificant. Profiles of q_p at different pH values had similar tendencies. With extending of cultivation, q_p was found to increase gradually exhibiting a climacteric peak after cells growth stopped and then decrease to a lower level. However, the maximal q_p, duration of reaching peak and decreasing rate varied at different pH values. We observed that at first 10 h and after 16 h, q_p at pH 6.0 was the highest. At pH 5.0 and 5.5, glucose was consumed at 10 and 12 h, respectively and a rapid increase in q_p was observed. As a result, lowered q_p at pH 6.0 during 10 to 16 h was observed as compared to at pH 5.5 and 5.0. Accordingly, by comprehensively analyzing q_p at various pH values, we can reasonably believe that the optimal pH for GSH formation should be 6.0.

Concisely, as the optimal pH for cells growth (pH at 5.0) and GSH formation (pH at 6.0) varied, at early cultivation phase, it is appropriate to control pH at lower value to favor cells growth and at mid-and later-fermentation phase, higher pH was suitable to maintain high q_p for GSH overproduction.

Enhancement of GSH production with pH-shift strategy

Based on above results, we reasonably speculate that pH can be controlled at 5.0 to favor cells growth and 6.0 benefit GSH formation. However, as it is known, the ultimate aim for batch GSH fermentation was to maximize GSH yield and minimize cultivation times simultaneously. So, how to determine the shift time for pH value changing from 5.0 to 6.0 is central to achieving maximal GSH yield and productivity. In general, shift time for pH value

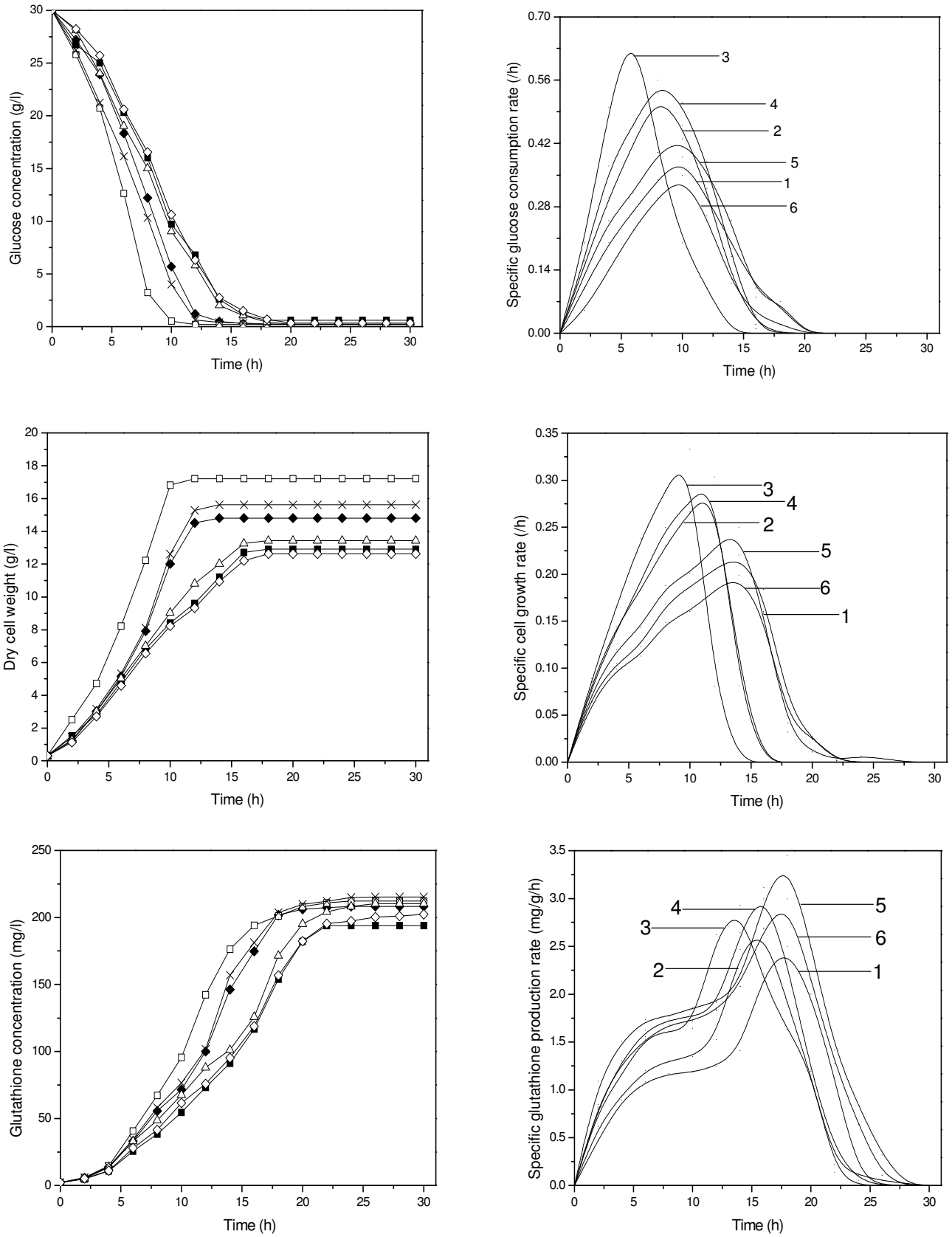

Figure 2. Time course of cells growth, glucose consumption and GSH production at different pH. ■ and 1, pH 4.0; ◆and 2, pH 4.5; □ and 3, pH 5.0; × and 4, pH 5.5; △ and 5, pH 6.0; ◇ and 6, pH 6.5;

Table 1. Effect of different pH shift time on GSH production.

Shift time (h)	0	2	4	6	8	10	12	14	16
GSH concentration (mg/l)	212	231	256	270	274	262	238	228	218

The shift time means the point at which pH value changes from 5 to 6.

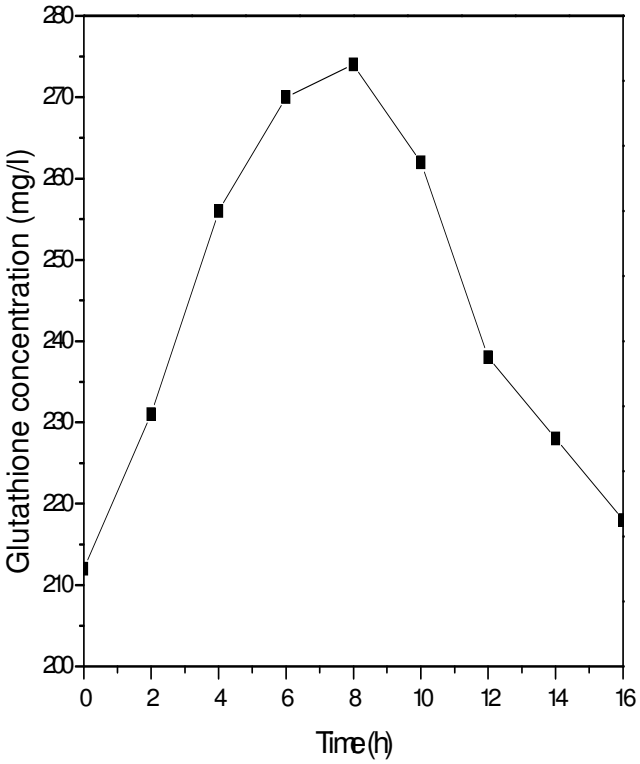

Figure 3. GSH production with different pH shift time. ■, glutathione concentration;

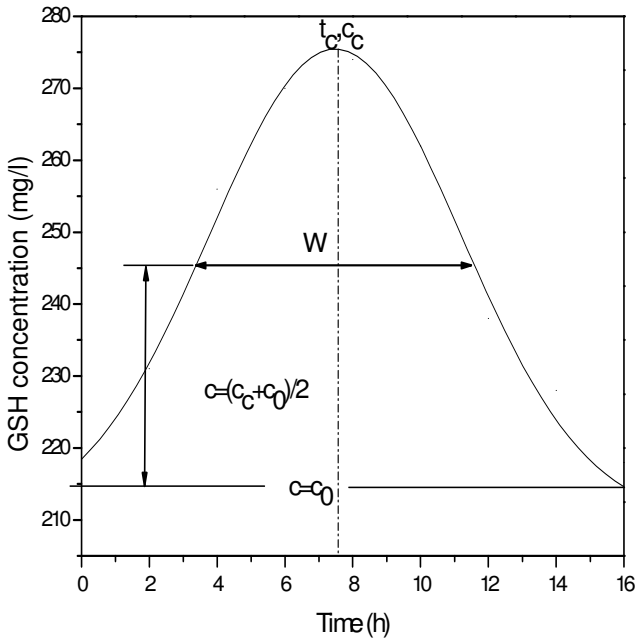

Figure 4. Optimization of GSH production by simulating Gauss function.

changing was empirically determined. For example, in batch microbial production of transglutaminase, a pH-shift strategy was developed by comparing specific rates of cells growth and transglutaminase formation (Zheng et al., 2002). Apparently, although the results achieved by this pH-shift strategy were better than single-step constant pH value, it was still not the optimal one.

Accordingly, to achieve the highest GSH yield, a peak function of Gauss was adopted in this study. Initially, GSH yields with different shift time for pH values changing from 5.0 to 6.0 were determined and shown in Table 1. And then based on the experiments data, GSH yield versus shift time was plotted. As shown in Figure 3, GSH yield increased correspondingly with an increase in shift time and reached the highest point at 8 h followed by a gradual decrease. As profile tendency of Figure 3 was very similar to that of Gauss function, it was further simulated by Gauss function using software Origin 7.5 and the simulated one was indicated in Figure 4. The formula for Gauss (t, c_0, t_c, W, A) function is shown in equation (1):

$$c = c_0 + \frac{A}{W\sqrt{\pi/2}} e^{-2\frac{(t-t_c)^2}{W^2}} \quad (1)$$

Where c_0 is the baseline offset; t_c is the center of the peak; W equals 0.849 the width of the peak at half height; A is the area under the peak.

Regression coefficients for GSH yield were indicated in Table 2 and GSH yield can be achieved according to these coefficients. Correlation coefficient of regression models of GSH yields was 97.67%, which means a high fitting accuracy.

In the aforementioned equation, c represents GSH yield; maximal GSH yield of 276 mg/l was calculated according to the parameters in Table 2. To test the accuracy of maximal GSH yield achieved by simulated Gauss function, the pH-shift experiment, in which pH was controlled at 5.0 for first 7.5 h and switched to 6.0 afterwards, was further operated in 7 l fermentor. As shown in Figure 5, after 22 h cultivation, final GSH yield reached 279 mg/l, which was about 1% higher than the simulated one, indicating the effectiveness of optimized strategy. More importantly, by adopting this strategy, GSH produc-

Table 2. Regression coefficients for GSH yield by Gauss function.

Parameter	Value	Error
c0	209.9663	6.43137
tc	7.4862	0.19537
W	7.40513	0.94255
A	607.60679	121.75065
2	0.97692	

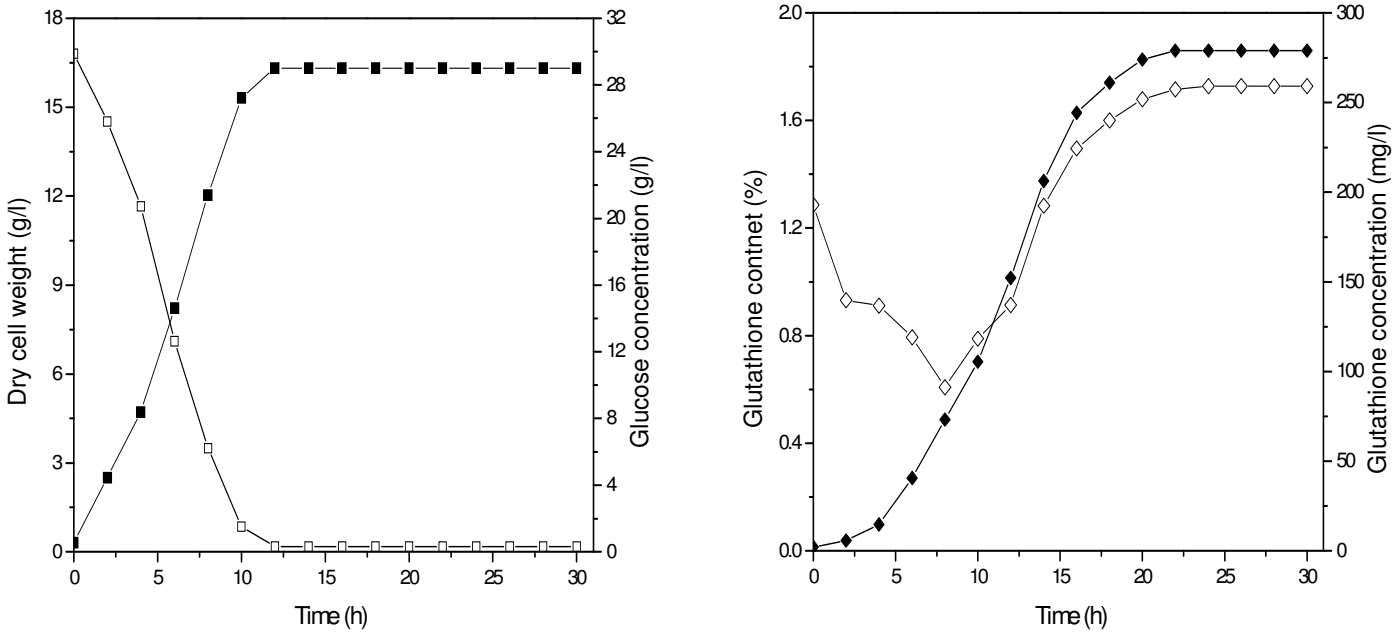

Figure 5. Enhanced GSH production with two-stage pH-shift strategy. □, glucose concentration; ■, dry cell weight; ◇, glutathione content; ◆, glutathione concentration.

tivity was also significantly improved.

In short, by controlling pH at 5.0 for first 7.5 h and switching to 6.0 afterwards, final GSH yield and productivity reached 279 and 12.7 mg/l/h, increased by 30 and 42%, respectively, as compared to constant pH 5.5 operation.

Dynamic changes of intracellular precursor amino acids and ATP during whole culture process

Glucose, as commonly used carbon source for GSH fermentation, was split and converted to intermediates and ATP for GSH production. To investigate whether or not precursor amino acids and ATP originated from glucose degradation can meet cells growth and GSH production, dynamic changes of three intracellular amino acids and ATP levels were assayed. As indicated in Figure 6, precursor amino acids levels increased gradually and reached the highest values as cells stopped proliferation. In contrast, their correspondingly intracellular contents were kept in relatively lowed levels at the initial phase and began to rapidly decrease after the cessation of cells growth. More interestingly, compared to cysteine and glycine, intracellular glutamic acid content was slightly higher, which was consistent with our previous study (Liang et al., 2008c).

Profile of intracellular ATP level also showed a similar trend as precursor amino acids. For the fermentative GSH production, ATP was mainly generated from glycollysis and citric acid cycle, suggesting ATP availability is glucose-dependent. After glucose exhaustion, ATP generation will be ceased. While we noticed that GSH production increased continuously after glucose consumption. Under normal conditions, energy produced by cells was stored in several forms. As cellular ATP was exhausted, energy stored in other forms can be converted to ATP. Accordingly, we convincingly believe that energy in other forms was converted to ATP continuing GSH production after glucose was exhausted. In short, with glucose exhaustion and cultivation extending, a rapid decrease in precursor amino acids and ATP concentration will inevitably limit GSH further production.

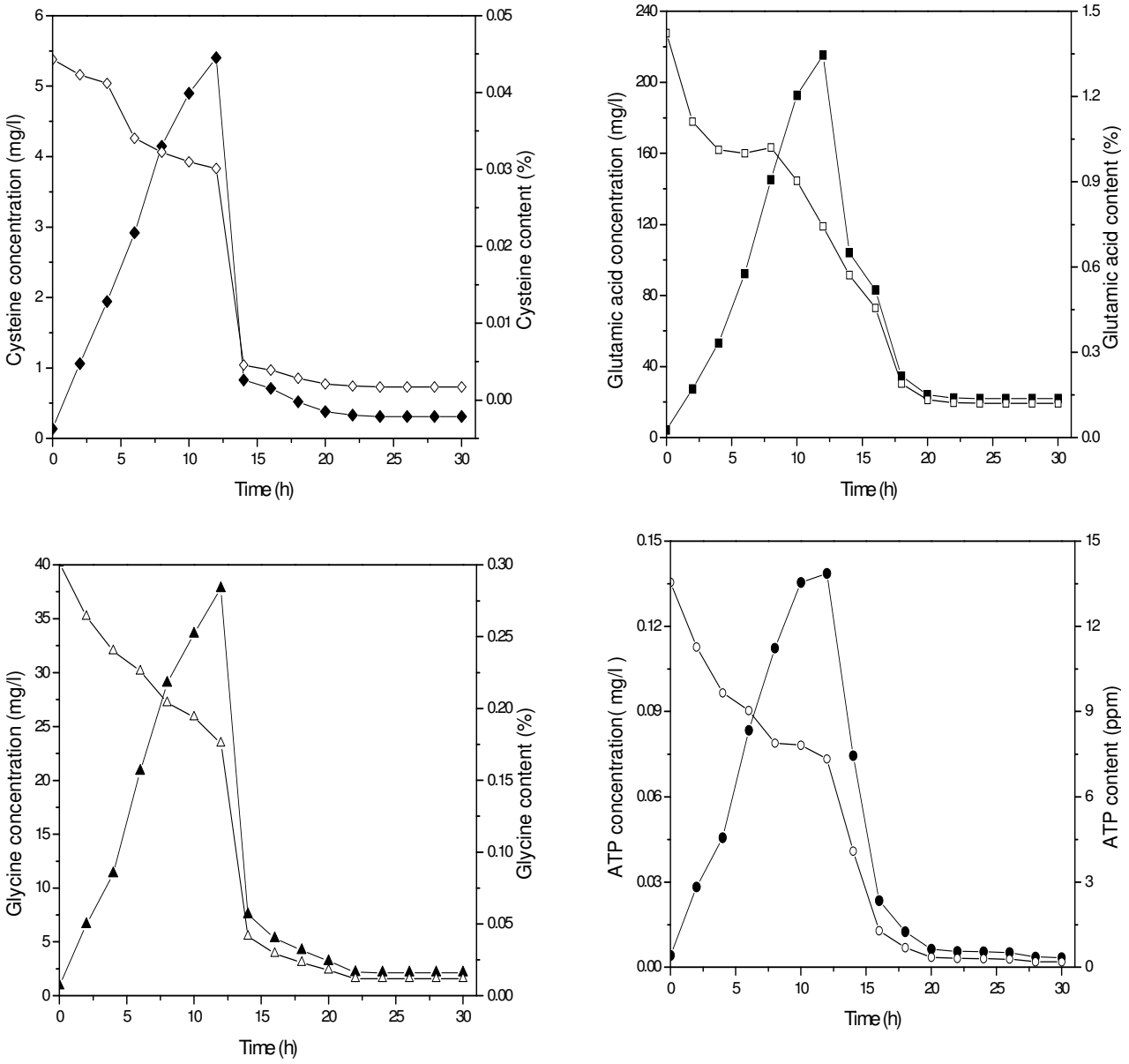

Figure 6. Dynamic changes of intracellular precursor amino acids and ATP levels. ◆ and ◇, cysteine concentration and content; ■ and □, glutamic acid concentration and content; ▲and △, glycine concentration and content; ● and ○, ATP concentration and content.

Feeding glucose to adjust pH as well as generate precursor amino acids and ATP for GSH overproduction

In batch GSH fermentation, at cells growth phase, glucose was converted to pyruvate followed by citric acid cycle to carbon dioxide (CO_2). As a result, only NaOH solution was needed to adjust broth pH at. In contrast, at the stationary phase, as broth pH value increased immediately after glucose consumption, H_2SO_4 solution must be added. So 500g/l glucose solution was used to replace H_2SO_4 for adjusting medium pH. Fascinatingly, as indicated in Figure 7, the final GSH yield reached 315 mg/l after 22 h cultivation, which was 13% higher than using H_2SO_4 for adjusting medium pH. It was indicated that glucose feeding shows two functions of promoting GSH production and adjusting pH.

DISCCUSSION

Micro-organisms, such as *S. cerevisiae* and *C. utilis*, have been applied in GSH fermentative production on industrial scales. The ultimate aim for GSH fermentation

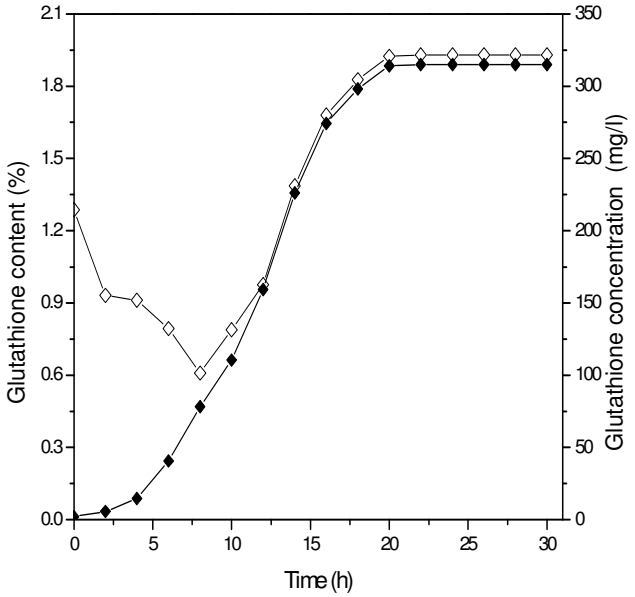

Figure 7. GSH overproduction by feeding glucose in pH-shift strategy. ◇, glutathione content; ◆, glutathione concentration.

was to achieve a high GSH yield which can be obtained by increasing cell density and intracellular GSH content. However, an increased biomass can inevitably result in lowered intracellular GSH content. Alternatively, addition of precursor amino acids was an easy approach (Alfafala et al., 1992a, 1992b).

As it is known, the major functions of plasma membrane were to regulate what comes in and goes out of cells. Fermentation pH can determine complex physiological parameters such as membrane permeability and cell morphology. So a change in broth pH will affect membrane osmosis to certain ions and thus substance absorption. Many authors have examined effects of broth pH on growth kinetics of microorganisms and concluded that pH was the important environmental factors affecting cells growth and products formation (Tang et al., 1989; Amanullah et al., 2001). For instance, actions of enzymes that catalyze all metabolic reactions were governed by broth pH (Elibol, 2002). Unfortunately, effects of broth pH values on GSH production in batch GSH fermentation were scarcely known.

As an intracellular product, GSH yield was intimately related to cell density and intracellular GSH content. Broth pH, as a vital environmental factor, significantly affects cells growth and GSH formation. Accordingly, how to maximize GSH yield by optimizing broth pH is the point we tried to explore in this research. Based on the results that the optimal for cells growth and GSH formation differed, a pH-shift strategy, optimized via simulating gauss function, was developed. As a result, GSH yield was significantly enhanced by this pH-shift strategy.

Moreover, as it is known, GSH biosynthesis occurs in two ATP-dependent steps catalyzed by γ-glutamylcysteine and GSH synthetase in the presence of cysteine, glutamic acid and glycine (Meister, 1988). It was indicated that GSH production is closely related to the availability of ATP and three precursor amino acids.

For batch GSH fermentation, the whole process was divided into two phases of cell growth and GSH synthesis. At cell growth phase, three precursor amino acids and ATP can meet GSH production. In contrast, after glucose exhaustion, generation of precursor amino acids and ATP will be ceased. To adjust broth pH as well as generate ATP and three precursor amino acids for GSH production, glucose was fed instead of sulphuric acid (H_2SO_4) solution to maximize GSH production and the results verified the effectiveness of this proposed strategy.

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REFERENCES

Alfafala CG, Kanda A, Shioi T, Shimizu H, Shioya S, Suga K (1992a). Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 36: 538-540.

Alfafala CG, Miura K, Shimizu H, Shioya S, Suga K (1992b). Cysteine addition strategy for maximum glutathione production in fed-batch culture of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 37: 141-146.

Amanullah A, McFarlane CM, Emery AN, Nienow AW (2001). Scale-down model to simulate spatial pH variations in large-scale bioreactors. Biotechnol. Bioeng.73: 390-399.

Bloch K (1949). The synthesis of glutathione in isolated liver. J. Biol. Chem. 179: 1245-1254.

Douglas KT (1989). Chemical synthesis of glutathione and analogs. Coenzymes Cofactors, 3: 243-279.

Elibol M (2002). Product shifting by controlling medium pH in immobilized *Streptomyces coelicolor* A3(2) culture. Process Biochem. 37: 1381-1386.

Harington CR, Mead TH (1935). Synthesis of glutathione. Biochem. J. 29: 1602-1611.

Izawa S, Inoue Y, Kimura A (1995). Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. FEBS Lett. 368: 73-76.

Li Y, Wei GY, Chen J (2004). Glutathione: a review on biotechnological production. Appl. Microbiol. Biotechnol. 66: 233-242.

Liang GB, Du GC, Chen J (2008a). A novel strategy of enhanced glutathione production in high cell density cultivation of *Candida utilis*-cysteine addition combined with dissolved oxygen controlling. Enzyme Microb. Technol. 42: 284-289.

Liang GB, Liao XY, Du GC, Chen J (2008b). A new strategy to enhance glutathione production by multipleH_2O_2-induced oxidative stresses in *Candida utilis*. Bioresour. Technol. 100: 350-355.

Liang GB, Liao XY, Du GC, Chen J (2008c). Optimization of amino acids addition for efficient production of glutathione in *Candida utilis*. Biochem. Eng. J. 41: 234-240.

Mehdi K, Penninckx M (2000). A short review on the role of glutathione in the response of yeast to nutritional, environmental, and oxidative stresses. Enzyme Microbiol. Technol. 26: 737-742.

Meister A, Anderson ME (1983). Glutathione. Ann. Rev. Biochem. 52: 711-760.
Meister A (1988). Glutathione metabolism and its selective modification. J. Biol. Chem. 17: 205-208.
Meister A (1994). Antioxidant functions of glutathione. Life Chem. Rep.12: 23-27.
Miller GL (1959). Use of 3, 5-dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426-428.
Pastore A, Federici G, Bertini E, Piemonte F (2003). Analysis of glutathione: implication in redox and detoxification. Clin. Chin. Acta. 333: 19-39.
Pompella A, Visikis A, Paolicchi A, DeTata V, Casini AF (2003). The changing faces of glutathione, a cellular protagonist. Biochem. Pharmacol. 66: 1499-1503.
Ruiz JM, Blumwald E (2002). Salinity-induced glutathione synthesis in *Brassica napus*.Planta. 214: 965-969.
Sies H (1999). Glutathione and its role in cellular functions. Free Radic. Biol. Med. 27: 916-921.
Smirnova GV, Krasnykh TA, Oktyabrsky ON (2001). Role of glutathione in the response of *Escherichia coli* to osmotic stress. Biochemistry (Moscow), 66: 973-978.
Tang IC, Okos MR, Yang ST (1989). Effects of pH and acetic acid on homoacetic fermentation of lactate by *Clostridium formicoaceticum*. Biotechnol. Bioeng. 34: 1063-1074.
Tietze F (1969). Enzymatic method for quantitative determination of monogram amounts of total and oxidized glutathione: application to mammalian blood and other tissue. Anal. Biochem. 29: 502-522.
Veciana-Nogues MT, Izquierdo-Pulido M, Vidal-Carou MC (1997). Determination of ATP related compounds in fresh and canned tuna fish by HPLC. Food Chem. 59: 467-472.
Wang YH, Feng JL, Pan ZQ, Fang XX, Li, BR (2006). The analysis of 18 free amino acids by liquid chromatography/mass spectrometry. Health Lab. Technol. 16: 161-163(in Chinese).
Wang Z, Tan TW, Song J (2007). Effect of amino acids addition and feedback control strategies on the high-cell-density cultivation of *Saccharomyces cerevisiae* for glutathione production. Process. Biochem. 42: 108-0111.
Wei GY, Li Y, Du GC, Chen J (2003). Application of a two-stage temperature control strategy for enhanced glutathione production in the batch fermentation by *Candida utilis*. Biotechnol. Lett. 25: 887-890.
Wen SH, Zhang T, Tan TW (2004). Utilization of amino acids to enhance glutathione production in *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 35: 501-507.
Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004). Glutathione metabolism and its implications for health. J. Nutr. 134: 489-492.
Zheng MY, Du GC, Chen J (2002). pH control strategy of batch microbial transglutaminase production with *Streptoverticillium mobaraense.* Enzyme Microb. Technol. 31: 477-481.