African Journal of Biotechnology Vol. 9(33), pp. 5399-5406, 16 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Combined amino acids modulation with H_2O_2 stress for glutathione overproduction in *Candida utilis*

Bin Wang[1], Guobin Liang[1], Quanfa Zhou[1], Jianghui Xie[2] and Yiwei Mo[2]*

[1]School of Chemistry and Environmental Engineering, Jiangsu Teachers University of Technology, Changzhou 213001, China.
[2]South Subtropical Crop Research Institute, Chinese Academy of Tropical Agricultural Science, Zhanjiang 524091, China.

Accepted 22 July, 2010

Strategies of amino acids addition coupled with H_2O_2 stresses were developed for glutathione (GSH) overproduction in high cell density (HCD) cultivation of *Candida utilis*. Based on the fact that glycine shows two functions of promoting cells growth as well as GSH production, precursor amino acids modulations of feeding glycine at 4 mmol/l/h at exponential phase and adding precursor amino acids (glutamic acid 42 mmol/l, glycine 40 mmol/l, and cysteine 36 mmol/) at stationary phase were conducted. As a result, cell density reached 114.8 g/l at 45 h and glutathione yield of 2136 mg/l was achieved at 60 h, which was 12.5 and 90.2% higher than the control, respectively. Furthermore, the novel strategies of amino acids modulation combined with H_2O_2 additions (24 mmol/l at 21 h, 26 mmol/l at 29 h, 28 mmol/l at 37 h and 30 mmol/l at 45 h) were adopted to maximize glutathione production. Final glutathione yield reached 2448 mg/l after 60 h cultivation, suggesting the strategies developed as being feasible for GSH overproduction.

Key words: Amino acids, glutathione (GSH), high cell density (HCD) cultivation, *Candida utilis*, H_2O_2 stresses.

INTRODUCTION

Glutathione (GSH), as the most abundant non-protein thiol compound, is widely distributed in organisms (Meister and Anderson, 1983). The major functions of GSH can be summarized into three ways of serving as antioxidant, immunity booster, and detoxifier (Pastore et al., 2003). As a powerful and versatile defense molecule (Wu et al., 2004), GSH has been widely used in pharmaceuticals and also has potentials to be used in food additives and cosmetics (Sies, 1999), provided that its price can be further reduced by optimizing production methods. It was impractical to extract GSH from plant or animal active tissues as a result of limited raw materials. GSH synthesized by chemical method was also unrealistic because separation of L-form from D-isomer is needed (Douglas, 1989). Accordingly, biotechnological production of GSH has been extensively exploited. Enzymatic GSH production was not commercialized because of relatively higher production cost. Alternatively, GSH production by yeast fermentation was efficient and practical.

As an intracellular product in yeast, a combination of high cell density and high intracellular GSH content can result in maximal GSH production. Biomass can be enhanced by high cell density (HCD) cultivation techniques (Tong et al., 2003; Liang et al., 2008a). However, an increase in cell biomass will inevitably lead to decreased intracellular GSH content. Therefore, addition of precursor amino acids was considered as a practical and effective alternative.

It was demonstrated that cysteine, glycine and glutamic acid are most important precursor amino acids for increasing GSH production by *S. cerevisiae* or *C. utilis* (Wen et al., 2004; Yin et al., 2008). Cysteine can enhance intracellular GSH content significantly, but it inhibits cells growth at the same time. As a result, optimal time for

*Corresponding author. E-mail: moyiwei08@yahoo.cn. Tel: +86-759-2859272. Fax: +86-759-2858168.

Abbreviations: PEP, Phosphoenolpyruvate; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione; **DCW,** dry cell weight; **HCD,** high cell density cultivation.

cysteine addition is at stationary phase as cells stop propagating, and single-shot addition of cysteine is better than other addition ways in enhancing GSH production (Alfafala et al., 1992a,b). Meanwhile, glycine also showed vital role on increasing GSH production besides cysteine (Wen et al., 2006). However, modes of glycine addition for GSH production were scarcely known. In previous study, we observed that glycine showed two functions of promoting cells growth and increasing GSH production compared to glutamic acid and cysteine in batch culture of *C. utilis* (Liang et al., 2008b).

As it is known, ways and times of HCD cultivation distinctively differed from batch culture for fermentative GSH production. Accordingly, how to apply amino acids modulation developed in batch culture to HCD cultivation is very important for maximizing GSH production. However, relative knowledge in this field has never been elucidated systematically. Moreover, under physiological condition, GSH exists in both reduced (GSH) and oxidized (GSSG) forms, which was accounting for less than 1% of total GSH (Dale and Henry, 2002). Oxidative stress induced by H_2O_2 can result in increased GSSG level which is detrimental to cell normal metabolism. To maintain a relatively stable redox state in cells, oxidized GSSG must be converted to reduced GSH, a reaction catalyzed by GSH reductase at the cost of NADPH (Carmel-Harel and Storz, 2000). As a result, a higher total GSH yield can be achieved. It was demonstrated that H_2O_2 stress can induce GSH accumulation in *C. utilis* (Liao et al., 2008). Furthermore, in our previous study, strategies of oxidative stresses by H_2O_2 addition were developed to enhance GSH production in batch fermentation of *C. utilis* (Liang et al., 2008c). So, can the strategies developed in batch culture be further applied in HCD cultivation? Moreover, would oxidative stresses by H_2O_2 combined with amino acids addition lead to GSH overproduction? However, to date, there was no report on enhancing GSH production by combining amino acids addition with oxidative stress.

Accordingly, effects of amino acids addition coupled with H_2O_2 stresses on GSH production in HCD cultivation of *C. utilis* were investigated in present study. Firstly, influences of ways or methods of addition of glycine on GSH production at cells growth phase were investigated. Then amino acids modulations of feeding glycine at constant rate during exponential phase and adding three precursor amino acids at stationary phase were adopted. Finally, the novel strategies of amino acids modulation coupled with H_2O_2-induced oxidative stresses were developed for GSH overproduction in HCD cultivation of *C. utilis*.

MATERIALS AND METHODS

Microorganism and culture media

Candida utilis WSH 02-08 was used in this study. The seed culture, containing (g/l) glucose 20, peptone 20, and yeast extract 10 at pH 6.0, was prepared in a 250 ml flask on a reciprocal shaker. Culture conditions of temperature, agitation rate, and growth period were fixed at 30°C, 200 rpm, and 20 h, respectively.

High cell density cultivation (HCD) in 7 L fermentor

HCD cultivation of *C. utilis* was done as described in previous method (Liang et al., 2008a). After 45 h cultivation with three-stage operation modes (batch culture, exponential glucose feeding and constant glucose feeding), cell concentration reached the maximal value of 102 g/l and corresponding GSH yield was 981 mg/l. The whole cultivation process was divided into cells growth phase (0 - 45 h) including exponential period (9 - 21 h) and stationary phase (45 - 60 h).

Modes of glycine addition at cells growth phase

Whole HCD cultivation period for GSH production lasted 45 h in 7 L fermentor. Different modes of glycine addition were shown as follows:

1. Single glycine addition: glycine with different concentrations (12, 18, 24, and 30 mmol/l) were added separately to the medium at various cultivation times (9, 15, 21, 27, 33 and 39 h) in a 7 l fermentor.
2. Multiple glycine additions: Based on single glycine addition, two, three and four points additions of glycine with three concentrations (12, 18 and 24 mmol/l) were carried out at different times (9, 15, 21 and 27 h), respectively.
3. Continuous glycine addition: glycine was fed continuously at a feeding rate of 2, 4, and 6 and 8 mmol/l/h, respectively, to the medium in a 7 l fermentor. Feeding of glycine began at 9 h and stopped at 21 h.

Addition of precursor amino acids at stationary phase

The ratios between precursor amino acids were kept the same and their addition concentrations (glutamic acid 37mmol/l, glycine 35mmol/l, and cysteine 32 mmol/l) achieved in previous HCD cultivation (Liang et al., 2008b), were amplified based on biomass obtained in this cultivation. As a result, optimal concentrations of three amino acids (glutamic acid 42 mmol/l, glycine 40 mmol/l, and cysteine 36 mmol/l) were ascertained in this study and added at 45 h as cells stopped propagating.

Optimization of H_2O_2 addition during HCD cultivation

For H_2O_2 -induced oxidative stress, 200 mmol/l H_2O_2 solution was used in HCD cultivation. To achieve an efficient GSH production and minimize inhibition of cells growth, H_2O_2 were added during the periods of 21 to 45 h. According to biomass obtained in HCD cultivation, H_2O_2 addition concentrations were determined by amplifying its amounts added in previous batch culture (Liang et al., 2008c). That is H_2O_2 concentrations at 24, 26, 28, and 30mmol/l added separately at 21, 29, 37, and 45 h.

Analytical procedures

Culture broth of 25 mL was centrifuged at 3500×*g* for 15 min 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, 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 a constant weight.

Table 1. Effect of single glycine addition on cell growth and GSH production.

Concentration of glycine (mmol/l)	Addition time (h)	DCW (g/l)	GSH yield (mg/l)	GSH content (%)
Control		102.1±0.2	981±0.3	0.96±0. 03
12				
	9	107.1±0.06	1075±0.7	1.02±0. 04
	15	105.4±0.06	1063±0.6	1.01±0. 03
	21	104.6±0.04	1047±0.4	1.01±0. 02
	27	104.4±0.02	1043±0.4	0.99±0. 01
	33	104.2±0.04	1030±0.3	0.99±0. 03
	39	104.1±0.02	1021±0.2	0.98±0. 02
18				
	9	108.4±0.06	1102±0.6	1.02±0. 04
	15	106.8±0.06	1086±0.6	1.01±0. 03
	21	105.2±0.04	1071±0.5	1.02±0. 02
	27	104.8±0.02	1058±0.3	1.01±0. 02
	33	104.6±0.04	1043±0.2	0.99±0. 01
	39	104.3±0.04	1027±0.3	0.98±0. 04
24				
	9	109.6±0.08	1146±0.8	1.05±0. 03
	15	108.5±0.08	1130±0.7	1.04±0. 02
	21	105.6±0.06	1094±0.6	1.04±0. 04
	27	105.3±0.04	1081±0.5	1.02±0. 01
	33	104.8±0.02	1053±0.3	1.01±0. 03
	39	104.5±0.02	1032±0.3	0.99±0. 03
30				
	9	109.3±0.08	1142±0.7	1.04±0. 02
	15	108.2±0.06	1126±0.6	1.04±0. 02
	21	105.3±0.06	1091±0.5	1.03±0. 01
	27	105.1±0.04	1078±0.4	1.03±0. 04
	33	104.5±0.04	1052±0.3	1.01±0. 02
	39	104.1±0.02	1032±0.2	0.99±0. 01

All experiments were repeated at least twice and average of results was used for analysis.

RESULTS

Effects of glycine addition ways on cells growth and GSH production at cells growth phase

Among precursor amino acids, both cysteine and glycine significantly influenced GSH production, but glutamic acid did not show an effective effect (Wen et al., 2004). Moreover, compared with cysteine that increased intracellular GSH but inhibited cells growth simultaneously, glycine showed two functions of promoting cells growth and GSH production especially during cells rapid growth stage (Liang et al., 2008b). As the status quo of HCD cultivation differs from that in batch culture, it is necessary to investigate the effects of glycine addition ways on cells growth and GSH production during this period.

Effects of single glycine addition on cells growth and GSH production

Firstly, to investigate the effects of single addition of glycine on GSH production during cells growth phase, glycine at 12, 18 24 and 30 mmol/l were added separately at every time point of 9, 15, 21, 27, 33 and 39 h in HCD cultivation of *C. utilis*. DCW and GSH yield were determined at 45 h as cells propagation stopped.

As shown in Table 1, addition times and amounts of glycine markedly affected cells growth and GSH production. More interestingly, DCW and GSH yield were promoted significantly by glycine addition during cells exponential growth period. For instance, with 24 mmol/l glycine added

Table 2. Effects of multiple glycine addition on cells growth and GSH production.

Number	Concentration of glycine (mmol/l)				Experimental results		
	9 h*	15 h	21 h	27 h	DCW (g/l)	GSH yield (mg/l)	GSH content (%)
1	12	18	0	0	109.4±0.06	1219±0.6	1.11±0. 01
2	12	0	24	0	110.3±0.04	1251±0.5	1.14±0. 02
3	12	0	0	24	107.5±0.05	1242±0.4	1.15±0. 02
4	0	18	24	0	111.4±0.04	1256±0.8	1.13±0. 03
5	0	18	0	24	108.2±0.06	1257±0.6	1.16±0. 02
6	0	0	24	24	110.1±0.03	1246±0.5	1.13±0. 01
7	12	18	24	0	112.1±0.04	1283±0.7	1.15±0. 02
8	12	18	0	24	110.8±0.06	1261±0.5	1.14±0. 03
9	12	0	24	24	111.2±0.05	1253±0.6	1.13±0. 02
10	0	18	24	24	110.5±0.06	1267±0.4	1.14±0. 01
11	12	18	24	24	112.4±0.04	1294±0.7	1.15±0. 02

DCW = Dry cell weight; GSH = reduced glutathione.
*Addition time.

at 9 h, DCW and GSH yield reached the highest values of 109.6 g/l and 1146 mg/l, which were 7.3 and 16.8% higher than the control. So it is rational to ponder whether or not glycine added with several spots (lower at the beginning followed by higher levels addition) at exponential phase of 9 to 21 h can achieve higher GSH production compared to single addition. Accordingly, glycine addition with multi-points at exponential growth phase were investigated in the following works.

Effects of multiple spots of glycine additions on cells growth and GSH production

Based on the above results that glycine is greatly needed during periods of 9 to 21 h and 24 mmol/l glycine can meet cells growth and GSH production, three (12, 18 and 24 mmol/l) levels of glycine were used to study the effects of multi-point additions (two points, three points and four points) on GSH production.

As indicated in Table 2, after 45 h cultivation, three points glycine addition (12 mmol/l at 9 h, 18mmol/L at 15 h and 24mmol/L at 21 h) achieved a second highest GSH yield of 1283 mg/l, which was 30.7% higher than the control. Meanwhile, it is very interesting to observe that GSH yield reached 1294 mg/l by four points glycine addition, which was just a slightly higher than three spots addition, indicating frequent induction by glycine after exponential phase can not result in an optimal GSH yield. The reason that glycine added after exponential growth phase did not lead to efficient GSH production perhaps can be attributed to two points: First, slowing down of cells growth after exponential phase results in glycine synthesized by cells themselves can meet cell metabolism; Secondly, exogenous supply of glycine can be directly fed into glycolytic pathway and leads to accumulation of pyruvate and acetate, inhibiting cells growth. As a result, glycine added after 21 h showed insignificant effects on cells growth and GSH production. Concisely, glycine addition with three points during exponential phase can be feasible for increasing GSH production.

Effects of glycine feeding at constant rates on cells growth and GSH production

As is known from above results, glycine added with several points at exponential phase effectively promoted cells growth and GSH production. Would keeping glycine at fixed concentration in medium result in a more efficient effect than multiple-point addition? To compare this effect, glycine feeding at constant rates (2, 4, 6 and 8mmol/l/h) during the periods of 9 to 21 h were carried out. As indicated in Table 3, cells growth and GSH production were greatly affected by feeding rates, indicating glycine effects were dose-dependent. After 12 h glycine feeding, DCW and GSH yield under feeding rate of 4 mmol/l/h reached the highest values of 114.8g/l and 1324 mg/l, increased by 2.1 and 3.2% respectively, compared to the three points glycine addition mentioned above. More fascinatingly, the total glycine exhausted with 4 mmol/l/h feeding rate was 11% lower than three-point addition, which indicated that comparatively higher GSH yield can be achieved but with less production cost.

Enhanced GSH production by two-step amino acids modulation

Evidently, glycine fed at constant rate showed a better effect on cells growth and GSH yield than other addition ways at the exponential phase. Moreover, owing to the characteristics that cysteine increases intracellular GSH but inhibits cell growth at the same time, therefore, cysteine

Table 3. Effect of continuous glycine-feeding on cells growth and GSH production.

Glycine-feeding rate (mmol/l/h)	DCW (g/l)	GSH yield (mg/l)	GSH content (%)
0 (control)	104.2±0.03	1012±0.5	0.97±0.01
2	111.5±0.04	1292±0.6	1.16±0.02
4	114.8±0.02	1324±0.6	1.15±0.01
6	114.1±0.04	1318±0.7	1.16±0.03
8	113.2±0.03	1308±0.6	1.16±0.02

DCW = Dry cell weight; GSH = reduced glutathione.

accompanied by glutamic acid and glycine addition at stationary phase was considered as a practical alternative to enhance GSH production. In our previous research, with three amino acids (glutamic acid 37 mmol/l, glycine 35 mmol/l, cysteine 32 mmol/l) added at stationary phase of 45 h, final GSH yield reached 1841 mg/l at 60 h (Liang et al., 2008d). It is reasonably expected that glycine fed at exponential phase followed by adding three precursor amino acids at stationary phase can bring a better effect on GSH yield but release growth inhibition by cysteine in the meanwhile. Accordingly, a two-step amino acids modulation was adopted as follows: glycine feeding at 4 mmol/l/h from 9 to 21h and adding a mixture of three precursor amino acids (glutamic acid 42 mmol/l, glycine 40 mmol/l, cysteine 36 mmol/l) at 45 h. Furthermore, to test the effects of proposed strategies, GSH yield of two-step amino acids addition were compared with those of no amino acids addition, single glycine feeding at exponential phase and single addition of mixed amino acids at stationary phase.

Without amino acids addition, maximal DCW reached 102.1 g/l at 45 h and GSH yield was 1123 mg/l after 60 h cultivation [Figure 1 (a - d)]. In contrast, with 4 mmol//h glycine feeding during 9 to 21 h, DCW of 114.8 g/l was attained at 45 h and GSH yield reached 1503 mg/l at 60 h. Meanwhile, with only addition of three amino acids (glutamic acid 37mmol/l, glycine 35mmol/l, and cysteine 32 mmol/l) at 45 h, final GSH yield of 1841 mg/l was achieved at 60 h. While by feeding glycine at 4mmol/l/h from 9 to 21 h and adding three precursor amino acids at 45 h, final GSH yield reached 2136 mg/l at 60 h. In brief, by applying the proposed amino acids modulation, final GSH yield increased by 90.2% compared with the control.

Maximizing GSH production by coupling amino acids modulation with H_2O_2 stresses

As it is known, the ratios of reduced glutathione (GSH) to oxidized glutathione (GSSG) in yeast cell were kept stable under physiological condition. Oxidative stress induced by H_2O_2 can cause increased GSSG level being detrimental to cell metabolism. To maintain stable redox, oxidized GSSG must be transferred to reduced GSH. We previously applied H_2O_2-induced oxidative stresses to stimulating GSH production in batch culture of *C. utilis* (Liang et al., 2008c). So, it is reasonably to believe that combining amino acids modulation with H_2O_2 stress would bring a better effect on GSH production.

To minimize inhibition of cells growth and achieve an efficient GSH production, H_2O_2 concentrations of 12 16, 20, 24 mmol/l were added separately at times of 21, 29, 37 and 45 h. DCW and GSH yield shown in Figure 2 were determined at 45 and 60 h, respectively. It was evident that, without H_2O_2 stresses, DCW reached 114.8 g/l at 45 h and final GSH yield of 2136 mg/l was achieved at 60 h. In comparison, by combining H_2O_2 stresses with amino acids addition, cell concentration was slightly decreased from114.8 to 112.3 g/l at 45 h. However, final GSH concentration reached 2448 mg/l after 60 h cultivation, which was 14.5% higher than that with amino acids modulation only. It was indicated that the novel strategies of combining amino acids modulation with H_2O_2 -induced oxidative stresses are being feasible for GSH overproduction on larger scales.

DISCCUSSION

Being an intracellular product, maximal GSH production by yeast fermentation can be achieved by increasing cell density and improving intracellular GSH content. External factors, such as temperature and pH, affected cells growth and GSH synthesis distinctly. As a result, the strategies of two-stage control system were developed to favor cells growth and GSH production simultaneously (Wei et al., 2003; Liang et al., 2009). Besides external factors, intracellular precursor amino acids synthesized by cells themselves are not enough for GSH production (Wada and Takagi, 2006; Penninckx., 2002). Moreover, increasing biomass by HCD cultivation inevitably resulted in lowered GSH content. Accordingly, enhancing intracellular GSH content was accomplished through taking up precursor amino acids by cells from external surrounding.

Among precursor amino acids, both cysteine and glycine showed a better effect on GSH production in contrast to

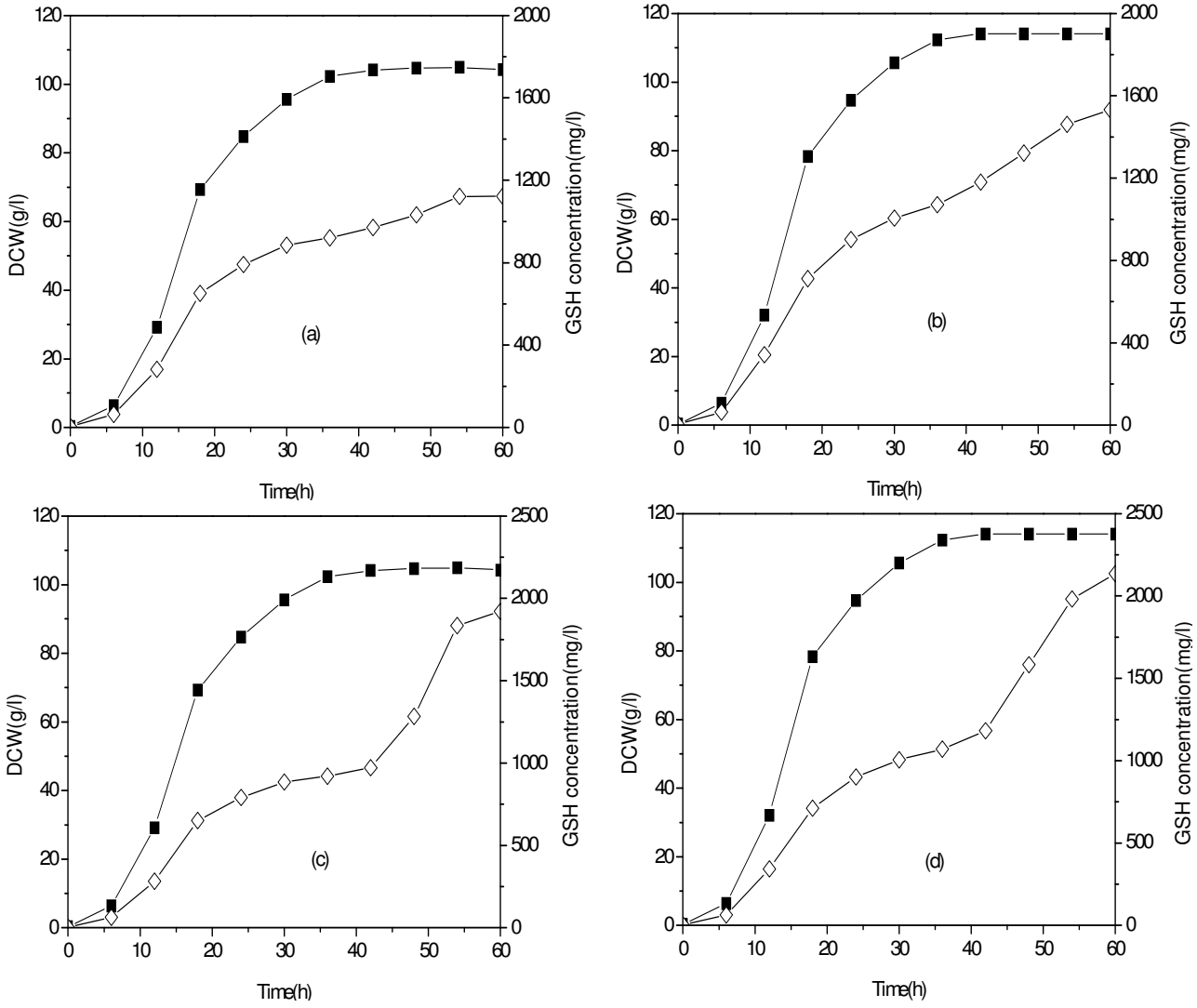

Figure 1. Effects of amino acids modulation on GSH production. (a) Without amino acid addition; (b) With glycine feeding at exponential phase; (c) Only with three precursor amino acids added at stationary phase; (d) With glycine feeding at exponential phase and mixed amino acids addition at the stationary phase. ■, Dry cell weight; □, Intracellular GSH concentration.

glutamic acid (Wen et al., 2004). As a key amino acid for enhancing GSH production, modes and times of cysteine addition have been extensively investigated (Alfafala et al., 1992a,b). Comparatively, few data were available on applying glycine to GSH production in *C. utilis* or *S. cerevisiae*.

We previously demonstrated that, at cell rapid growth phase, glycine showed two functions of promoting cells growth and GSH production in batch culture of *C. utilis* as compared to glutamic acid and cysteine (Liang et al., 2008b). In this study, whole HCD cultivation was divided into two phases of cells growth and GSH production. Firstly, we concentrated on investigating effects of glycine addition modes on GSH production at cells growth phase. Results indicated that DCW and GSH yield reached the highest of 114.8 g/l and 1324 mg/l at 45 h by feeding glycine at 4mmol/l/h during exponential periods. More interestingly, the total glycine consumed was decreased from 54 to 48mmol/l but with higher DCW and GSH yield compared to multi-points addition.

It is known that glucose was initially degraded to phosphoenolpyruvate (PEP) which was converted to pyruvate. And there was no return route for pyruvate to PEP provided that glucose was in sufficient supply. Thus, when three carbon precursor from PEP-pool is produced, serine, glycine and even one-carbon unit can be in full supply to satisfy cells growth and certain products (such as glycine) synthesis. However, at exponential phase, PEP has to be converted to other substances besides glycine for cells growth, which leads to decreased glycine level. Furthermore, glycine needed for cells growth and GSH synthesis during exponential stage increased

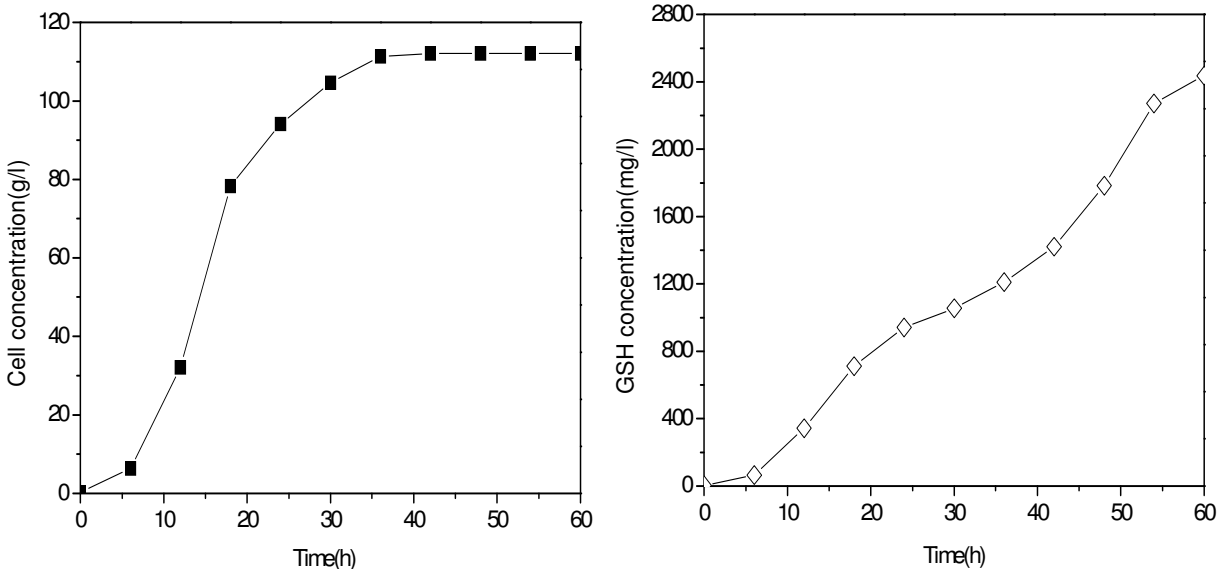

Figure 2. Effects of amino acids modulation coupled with H_2O_2 stress on GSH production. ■, Dry cell weight; □, Intracellular GSH concentration.

comparatively. As a result, the supply of glycine is vital during this period.

Based on aforementioned results that an optimal biomass and GSH yield can be achieved at 45h by feeding glycine at cell exponential phase. GSH production was further enhanced by adding three amino acids at stationary phase. And final results documented the effectiveness of proposed amino acids modulation.

In brief, by adopting amino acids modulation of feeding glycine at 4mmol/l/h during 9 to 21 h and adding amino acids (glutamic acid 42mmol/l, glycine 40 mmol/l, and cysteine 35mmol/1) at 45 h, a final total GSH yield of 2136mg/l was achieved after 60 h cultivation.

Moreover, based on the phenomenon that H_2O_2-induced oxidative stress can stimulate GSH accumulation, we combined aforementioned amino acids modulation with H_2O_2 stress to maximize GSH production. However, owing to the characteristics that H_2O_2 inhibited cells growth simultaneously, H_2O_2 was added after exponential phase for GSH overproduction while minimizing its negative effect on cells growth. As expected, by adopting the strategies of multiple H_2O_2 stresses (24 mmol/l at 21 h, 26 mmol/l at 29 h , 28 mmol/l at 37 h, and 30 mmol/l at 45 h) coupled with amino acids modulation, final GSH yield reached 2448 mg/l at 60 h and intracellular GSH content was 2.18%. It was suggested that combing amino acids modulation with H_2O_2 stress shows a more significant effect on GSH production than those only with amino acids modulation (Yin et al., 2008; Liang et al., 2008d) or H_2O_2-induced oxidative stress(Liao et al., 2008; Liang et al., 2008c).

In summary, in this study, the whole HCD cultivation process was divided into two phases of cells growth and GSH production. Based on the fact that glycine is greatly needed during exponential phase and H_2O_2-induced oxidative stress can stimulate GSH accumulation, glycine was fed constantly to promote cells growth as well as GSH production at exponential phase. In addition, three precursor amino acids were added at stationary phase to increased intracellular GSH content. Further amino acids modulation coupled with H_2O_2 stress showed maximal GSH yield, suggesting the feasibility of proposed strategies for GSH overproduction in HCD cultivation of *C. utilis*.

ACKNOWLEDGEMENTS

The study acknowledged the support in grants from Key Program of ministry of Science and Technology in China (2008BAC46B04) and Jiangsu Teachers University of Technology (KYY09003).

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.

Carmel-Harel O, Storz G (2000). Roles of the glutathione-and hioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu Rev Microbiol. 54: 439-461.

Dale AD, Henry JF (2002). Cellular glutathione and thiols metabolism. Biochem. Pharmacol. 64: 1019-1026.

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

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). Optimization of amino acids addition for efficient production of glutathione in *Candida utilis*. Biochem. Eng. J. 41: 234-240.
Liang GB, Liao XY, Du GC, Chen J (2008c). A new strategy to enhance glutathione production by multiple H_2O_2-induced oxidative stresses in *Candida utilis*. Bioresour. Technol. 100: 350-355.
Liang GB, Liao XY, Du GC, Chen J (2008d). Elevated glutathione production by adding precursor amino acids coupled with ATP in high cell density cultivation of *Candida utilis*. J. Appl. Microbiol.105: 1432-1440.
Liang GB, Wang B, Xie JH, Mo YW (2009). Novel pH control strategy for glutathione overproduction in batch cultivation of *Candida utilis*. Afr. J. Biotechnol. 8: 6337-6345.
Liao XY, Zhang WY, Zhu Z, Chen J, Du GC (2008). Effect of H_2O_2 stress on glutathione production by *Candida utilis*. Chin. J. Bio-Eng. 24(6): 1046-1050.
Meister A, Anderson ME (1983). Glutathione. Annu. Rev. Biochem. 52: 711-760.
Pastore A, Federici G, Bertini E, Piemonte F (2003). Analysis of glutathione: implication in redox and detoxification. Clin. Chim. Acta. 333: 19-39.
Penninckx MJ (2002). An overview on glutathione in *Saccharomyces* versus nonconventional yeasts. FEMS Yeast Res. 2: 295-305.
Sies H (1999). Glutathione and its role in cellular functions. Free Radic. Biol. Med. 27: 916-921.
Tietze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. Anal. Biochem.. 27: 502-522.
Tong Q, Wang G, Du GC, Chen J (2003). Effect of feeding method on production of glutathione by *Saccharomyces cerevisiae*. Ind. Microbiol. 33: 19-22.
Wada M, Takagi H (2006). Metabolic pathways and biotechnological production of L-cysteine. Appl. Microbiol. Biotechnol. 73: 48-54.
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.
Wen SH, Zhang T, Tan TW (2006). Maximizing production of glutathione by amino acid modulation and high-cell-density fed-batch culture of *Saccharomyces cerevisiae*. Process Biochem. 42: 108-111.
Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004). Glutathione metabolism and its implications for health. J. Nutr. 134: 489-492.
Yin LH, Wu XY, Liao XY, Du GC, Chen J (2008). Cultivation of *Candida utilis* WSH02-08 for production of glutathione by amino acids addition strategy. Chin. J. Process Eng. 8(2): 333-338.