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A novel strategy of enhanced glutathione production in high cell density cultivation of *Candida utilis*—Cysteine addition combined with dissolved oxygen controlling

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Abstract

Effects of dissolved oxygen (DO) concentration on glutathione (GSH) production and cysteine oxidation were investigated in high cell density cultivation of *Candida utilis*. Lower DO concentration favors cysteine absorption but retards GSH production. Higher DO promotes GSH production but accelerates cysteine oxidation in the broth. A two-step DO control strategy was developed and compared for the potential in enhancing GSH production and cysteine absorption. By using the two-step DO control strategy, a 40% decrease in cysteine addition and a 13% increase in GSH production are observed as compared with that at constant DO of 40%.

Keywords: *Candida utilis*; Glutathione (GSH); High cell density cultivation; Cysteine addition; Dissolved oxygen (DO)

1. Introduction

Glutathione (GSH), a low molecular-mass thiol, functions in many cellular processes including protection of cells against oxidation and is of increasing interest in medical treatment, foods and cosmetics industry [1].

GSH production by yeast fermentation is efficient and practical. As an intracellular product in yeast, the ultimate goal of the fermentative production of GSH is to achieve a high total GSH yield by increasing cell density and intracellular GSH content.

Enhancement of cell concentration can be achieved by process optimization. For instance, Sakato and Tanaka [2] developed a feeding control system to achieve a high cell density for maximizing GSH yield. Meanwhile, addition of precursor amino acids required for GSH is another easy approach to increase specific GSH production rate (P_G) by enhancing intracellular GSH content. Alfafala et al. [3] investigated the effects of cysteine and related compounds on GSH production. Their results showed that cysteine is a key amino acid for increasing the specific GSH production rate.

In *Saccharomyces cerevisiae* cysteine for GSH synthesis can be supplied by cells to synthesize and to take up from the medium.

Recent advancement in biochemical studies has enabled researchers to use various approaches to obtain cysteine in fermentation, but high-level microbial production of cysteine has not yet been achieved [4]. For fermentative production of GSH, cysteine synthesized by cells is not enough as a precursor for synthesis of GSH.

Therefore, a suitable cysteine addition strategy is needed to enhance GSH production. It was demonstrated that single-shot addition of cysteine is better than continuous or several additions [5]. And the increase in specific GSH production rate (P_G) by single shot method can be achieved without growth inhibition if cysteine dose is maintained at 0.7 mmol/g cell or less.

However, cysteine in the medium was oxidized rapidly after its addition. How to enhance the transporting degree of cys-

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teine from the medium into cells in reduced form is central to promoting cysteine utilization rate for GSH production.

S. cerevisiae or *Candida utilis* employed in fermentative production of GSH are aerobic. Dissolved oxygen (DO) concentration, a very important factor, affects cell growth and GSH production markedly. Autoxidation of cysteine to cystine by dissolved oxygen in phosphate buffer with pH 7.4 was reported and the result indicated that cysteine is completely oxidized after incubation at 37 °C for 2 h [6]. However, in the literature there are no details on oxidation degree of cysteine addition in *S. cerevisiae* or *C. utilis* cultivation for GSH production.

In this paper, a detailed research about effects of DO concentrations on cysteine oxidation and GSH biosynthesis were carried out. The results showed that lower DO benefits cysteine uptake but retards GSH synthesis and vice versa. Two-step DO control strategy is applied to enhance cysteine uptake degree for GSH production and can efficiently enhance GSH yield in high cell density cultivation of *C. utilis*.

2. Materials and methods

2.1. Microorganism and culture media

A high-GSH yeast strain, *C. utilis* WSH 02-08, was used in this study. The seed medium contained (g/l): glucose 20, peptone 20, and yeast extract 10 at pH 6.0. The seed culture was prepared in a flask on a reciprocal shaker at 200 rpm and 30 °C for 20 h. The medium for batch fermentation contained (g/l): glucose 15, ammonium sulfate 8, KH_2PO_4 3 and $MgSO_4$ 0.25.

2.2. Batch and fed-batch cultivation

Initial glucose was 15 g/l for batch culture in a 7 l fermentor and 500 g/l for the feed medium, including (per litre) 1 g $MgSO_4$, 30 g $(NH_4)_2SO_4$ and 6 g KH_2PO_4. Ten percent (v/v) of seed culture was inoculated into a 7 l fermentor with a working volume of 5 l. A total amount of 450 g glucose was fed with an exponential feeding after 8 h of batch fermentation. The exponential feeding was stopped at 20 h cultivation, followed by feeding at a constant rate of 5.5 g/(l h) to 45 h.

2.3. Control of DO and pH

During the cell growth phase, the aeration was maintained at 1 vvm and the agitation was operated at 400 rpm. In GSH synthesis after cysteine addition, DO was controlled at 5% in former 3 h, and 20% in later 12 h by maintaining aeration at 1 vvm and adjusting agitation in the range of 50–200 rpm. The pH was controlled automatically at 5.5 by adding 3 mol/l H_2SO_4 or 3 mol/l NaOH solutions.

Under the same condition, each experiment was carried out at least three times.

2.4. Analytical methods

A culture broth of 25 ml was centrifuged at 3500 × *g* for 15 min and the cells were washed twice with ice-cold saline (0.85% NaCl, w/v). The 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 and intracellular total thiols determination. Glutathione concentration was determined according to the method described by Tietze [7]. Total thiols concentration was determined based on the method described by Ellman [8]. Dry cell weight (DCW) was determined after drying the cells at 105 °C to a constant weight.

3. Results and discussion

3.1. High cell density cultivation of C. utilis

The whole process of GSH fermentation is composed of three phases. It began with a short batch phase. After the carbon source was exhausted, a fed-batch culture with glucose feeding was followed to obtain high cell density. Then, cysteine, a precursor of GSH, was added into the broth in the transformation phase to enhance intracellular GSH content.

Riesenberg [9] reported that *E. coli* growth was inhibited when initial glucose concentration exceeded 50 g/l. Our previous study showed that the suitable initial glucose for batch culture of *C. utilis* was 15 g/l and after 8 h cultivation the glucose was consumed. It is, therefore, important to develop a fed-batch culture to achieve high cell density.

In recent years, direct substrate feedback control and indirect feedback control schemes [10–12] have been applied in high cell density cultivation of *S. cerevisiae*. However, the mentioned feeding strategies are sophisticated and difficult to scale up because accurate and expensive instruments are needed. Li et al. [13] compared the effects of different glucose feeding methods on GSH fermentation by *E. coli* and found that exponential feeding can greatly improve cell concentration, cell productivity and total GSH concentration.

In our study, exponential feeding strategy was applied after 8 h batch fermentation. Derived from a mass balance with an assumption of constant cell yield on substrate, the following equation of feeding rate was derived from and has been applied to the production of baker's yeast [14].

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

where F is the feeding rate (ml/h); μ is the specific growth rate (h^{-1}); 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 0 h, respectively; $Y_{X/S}$ is the yield coefficient of biomass on glucose (g/g); and S_F is the glucose concentration in the feeding solution (g/l).

Based on batch fermentation analysis, the values of V_0 (3.5), $Y_{X/S}$ (0.7), and X_0 (10.5) were measured experimentally, S_F was set at 500 g/l, if the value of specific growth rate μ was set, the feeding rate can be determined.

By setting μ (h^{-1}) at 0.15, 0.2 and 0.25, a set of flow rate values can be calculated from Eq. (1). Since continuously changing

Table 1
Effects of different specific growth rates (μ) on cell growth and GSH production

Parameters	Results		
Nominal specific growth rate (h^{-1})	0.15	0.2	0.25
Maximum Dry cell weight (g/l)	55.4	81.2	64.7
Maximum GSH production (mg/l)	509.7	763.2	580.3
Maximum Intracellular GSH content (%)	0.92	0.94	0.90
Actual average specific growth rate (h^{-1})	0.132	0.198	0.143
Biomass yield on glucose (g/g)	0.56	0.63	0.17
GSH yield on glucose (mg/g)	6.45	6.74	1.84