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Effects of nitrogen source and carbon/nitrogen ratio on batch fermentation of glutathione by *Candida utilis*

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Abstract−Several inorganic/organic N-containing substances were tested as nitrogen source for efficient glutathione production by *C. utilis* WSH 02-08. Although the strain could assimilate all the inorganic/organic nitrogen, urea and ammonium sulfate were found more favorable to cell growth and glutathione biosynthesis in a flask, respectively, and an optimal C/N ratio existed for each as 5.6 mol/mol and 8.3 mol/mol. A mixed nitrogen source of urea and ammonium under diverse C/N ratios could not boost glutathione fermentation despite the many mixed strategies that were introduced. Batch glutathione production in a stirred fermentor, using the sole or mixed nitrogen sources of urea and ammonium sulfate under their optimal C/N ratios, were conducted; urea was further proved to be the best nitrogen source for glutathione production. The reason was then quantitatively described by kinetic model, together with the distribution of flux for metabolites in metabolic network of glutathione biosynthesis by *C. utilis* WSH 02-08.

Key words: Glutathione, Nitrogen Source, C/N Ratio, Kinetic Analysis, Metabolic Flux Analysis

INTRODUCTION

Glutathione (γ-glutamyl-L-cysteinylglycine, reducing form of GSH) is one of the most abundant tripeptide compounds widely distributed in living organisms [1]. Glutathione has been found to fulfill many physiological roles in higher eukaryotic organisms, *i.e.*, serving as antioxidant, immunity booster and detoxifier [2,3]. Specially, glutathione plays an important role in maintaining the normal cellular redox environment [4], which helps to protect DNA, proteins and other biomolecules against oxidative damage generated by reactive oxygen species [5]. Though generally used as a medicine, glutathione also has the potential to be used as a food additive [6] and in the cosmetic industry [7], the demand for this major non-protein thiol compound is now expanding.

The fermentative production of glutathione using sugar materials is regarded as one of the most promising methods, and yeasts such as *S. cerevisiae* and *C. utilis* are the commonly used microorganisms [8]. Belonging to the Crabtree negative [9] and Kluyver positive yeast [10], *C. utilis* possesses very weak catabolic repression effect when sugars are used as substrate. In addition, *C. utilis* also has the ability to utilize hexose and pentose degraded from lignocellulosic biomass as carbon source [11]. These characteristics make *C. utilis* an ideal cell plant for glutathione production, especially on an industrial scale.

Besides producing strain screening, nutrients in the culture medium are surely very important for the fermentative production of glutathione; the concentration of nutrients as well as the ratio of carbon source to nitrogen source (C/N ratio) should also be optimized to obtain high production, high yield and high productivity of glutathione [8]. Many researchers have focused on the process optimization to achieve high glutathione concentration by increasing the intracellular glutathione content and cell density; these attempts include adding constituent amino acids to the medium, using fed-batch culture, and other suitable cultivation strategies [8,12,13]. Since glutathione is a compound consisting of nitrogen element at a high level (13.68%), it is necessary to select an appropriate nitrogen source and its proper concentration during glutathione biosynthesis. Khan et al. recommended that ammonium sulfate was the best nitrogen source for glutathione production by *S. cerevisiae* [14], but ethanol was easily produced followed by the consumption of ammonium ions in this genus of yeast. So, organic nitrogen sources, *i.e.*, peptone, beef extract and yeast extract, were commonly used in the fermentative production of glutathione in most cases [15]. To date, few studies have been involved in the effect of nitrogen source and C/N ratio on glutathione production by *C. utilis*. In this paper, we have studied the batch culture of *C. utilis* with several nitrogen sources under different C/N ratios for glutathione production in flasks and fermentors, especially concentrated on the process kinetics and metabolic flux of intermediates in batch fermentation of glutathione.

MATERIALS AND METHODS

1. Chemicals

5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), NADPH, dinitrosalicylic acid (DNS), glutathione reductase, and standard glutathione were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals used were all of standard analytical grades.

2. Microorganism and Medium

C. utilis WSH 02-08 was used throughout this study. The strain was maintained by being monthly subcultivated on slants consisting of seed medium with 2% (w/v) agar. Seed medium contained glu-

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cose (20 g/L), peptone (20 g/L) and yeast extract (10 g/L) with an initial pH of 6.0. The fermentation medium for glutathione production in flasks or fermentors consisted of glucose (30 g/L), KH_2PO_4 (3 g/L), and $MgSO_4$ (0.25 g/L); the nitrogen sources and their concentrations were specified in each experiment according to C/N ratios. The medium was steam-sterilized except for urea, which was microfiltered by a Sartorious® membrane with the pore size of 0.20 μm. Glucose was sterilized separately and added before inoculation.

3. Culture Conditions

The culture from a fresh slant was inoculated into 500 mL baffled shake flasks containing 50 mL seed medium, incubated at 30 °C for 20 h on a reciprocal shaker with a rotation speed of 200 rpm to prepare precultures. The seed was then inoculated into the fermentation medium with an inoculum size of 10% (v/v). Glutathione fermentation in flasks was conducted in triplicate at 30 °C and 200 rpm for 30 h with an initial pH of 6.0.

Batch glutathione fermentation was carried out in a 5 L stirred fermentor (5BG-2002, BAOXING BIO-ENGINEERING EQUIPMENT CO., LTD) with a working volume of 3 L, using an aeration rate of 1.0 vvm and an initial agitation rate of 300 rpm. The cultivation in the fermentor was carried out at 30 °C for 30 h. The agitation rate was altered between 300 rpm and 600 rpm to ensure a dissolved oxygen level not lower than 35% of air saturation. The pH was controlled automatically at 5.5 throughout the fermentation by adding 3 mol/L H_2SO_4 or 3 mol/L NaOH solution.

4. Analytical Methods

Some 25 mL culture broth was centrifuged at 3,000 rpm for 10 min and washed twice with distilled water to collect biomass. Dry cell weight (DCW) was determined by heating the wet biomass at 70 °C to a constant weight. In addition, wet cells were extracted with 40% (v/v) ethanol at 30 °C for 2 h and then centrifuged at 10,000 ×g for 20 min; the supernatant was used for glutathione assay. Glutathione concentration was determined according to the method described by Tietze [16]. Glucose and ammonium sulfate concentration were both measured by colorimetric methods [17].

Glutathione was bio-synthesized intracellularly in *C. utilis* and was not usually excreted into the medium, so the intracellular glutathione content was defined as follows [13]:

$$\text{Intracellular glutathione content/\%} = \frac{\text{Glutathione concentration/(mg/L)}}{\text{DCW/(g/L)}\times 10} \neq 100\%$$

The results shown from the flasks represent the average of three parallel experiments. The data from fermentor cultures represent the average of two parallel samples.

5. Kinetic Analysis on Batch Glutathione Fermentation

Kinetic parameters as specific glucose consumption rate (q_s), specific growth rate (μ) and specific glutathione production rate (q_p) were defined and calculated by using formula as follows:

$$q_s = \frac{1}{X}\frac{dS}{dt} = \frac{1}{X}\lim_{\Delta t\to 0}\frac{\Delta S}{\Delta t} \quad (1)$$

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{1}{X}\lim_{\Delta t\to 0}\frac{\Delta X}{\Delta t} \quad (2)$$

$$q_p = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\lim_{\Delta t\to 0}\frac{\Delta P}{\Delta t} \quad (3)$$

The software used to determine parameters is Statistica 6.0 (StatSoft, OK, USA) together with the quasi-Newton method.

6. Metabolic Flux Analysis on Batch Glutathione Fermentation

Metabolic networks of glutathione biosynthesis that were constructed in the former study [18] consisted of 67 metabolites and 65 reactions *in vivo*. Carbon flux of metabolites was calculated by the software of Matlab 6.5 based on the assumption of pseudo-steady state for the intermediates according to the method described by Nielsen [19].

RESULTS AND DISCUSSION

1. Effect of Nitrogen Sources on Cell Growth and Glutathione Production

The chemical composition of the culture medium, particularly

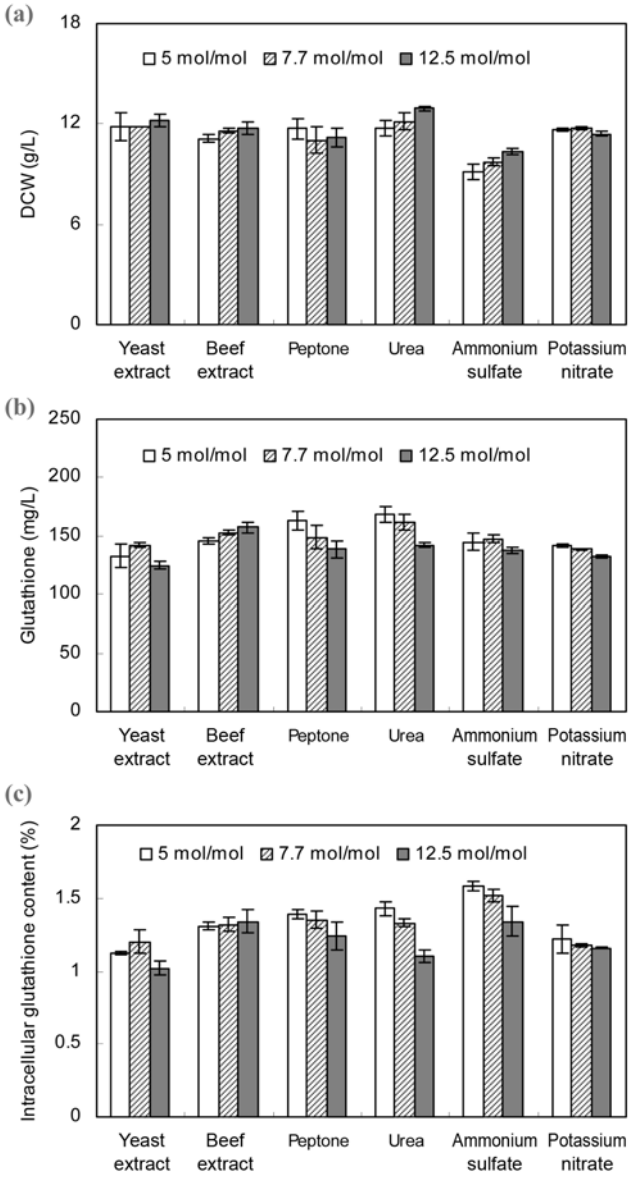

Fig. 1. Effect of different nitrogen sources on cell growth and glutathione production by *C. utilis* WSH 02-08 under diverse C/N ratios.

the concentration of nitrogen, is the mean of physiological control and regulation of microorganism metabolism [20,21]. *C. utilis* is a species of yeast having a wide nutritional spectrum, which can utilize hexose and pentose as carbon source, together with organic and inorganic substances as nitrogen source [11]. The cultivation of *C. utilis* WSH 02-08 in flasks using different N-containing substances like yeast extract, beef extract, peptone, urea, ammonium sulfate and potassium nitrate was performed to investigate the effect of nitrogen sources on glutathione fermentation. Fig. 1 shows the cell growth and glutathione production using different nitrogen sources under diverse C/N ratios of 5.0, 7.7 and 12.5 mol/mol. Firstly, all the N-containing substances we chose can be utilized as nitrogen source in this study, but the tendency on cell growth and glutathione production for each substance was different. Organic nitrogen sources seem more suitable to cell growth; about 20% of extra DCW was achieved and more glutathione was concomitantly accumulated, compared to that of ammonium sulfate. However, as to the intracellular glutathione content, organic nitrogen sources cannot boost the ability of glutathione bio-synthesis by *C. utilis* WSH 02-08, and ammonium sulfate was confirmed to be the best substance to maintain intracellular glutathione content to a higher level of 1.5%. Secondly, C/N ratio showed less effect on cell growth except for urea and ammonium sulfate, while glutathione production and intracellular glutathione content varied greatly with C/N ratio. Since glutathione contained high percentage of N element (13.68%), more N-containing substance will be needed within glutathione bio-synthesis and accumulation by *C. utilis* WSH 02-08, and the strain preferred to lower C/N ratio like 5.0 mol/mol for most nitrogen sources in this study.

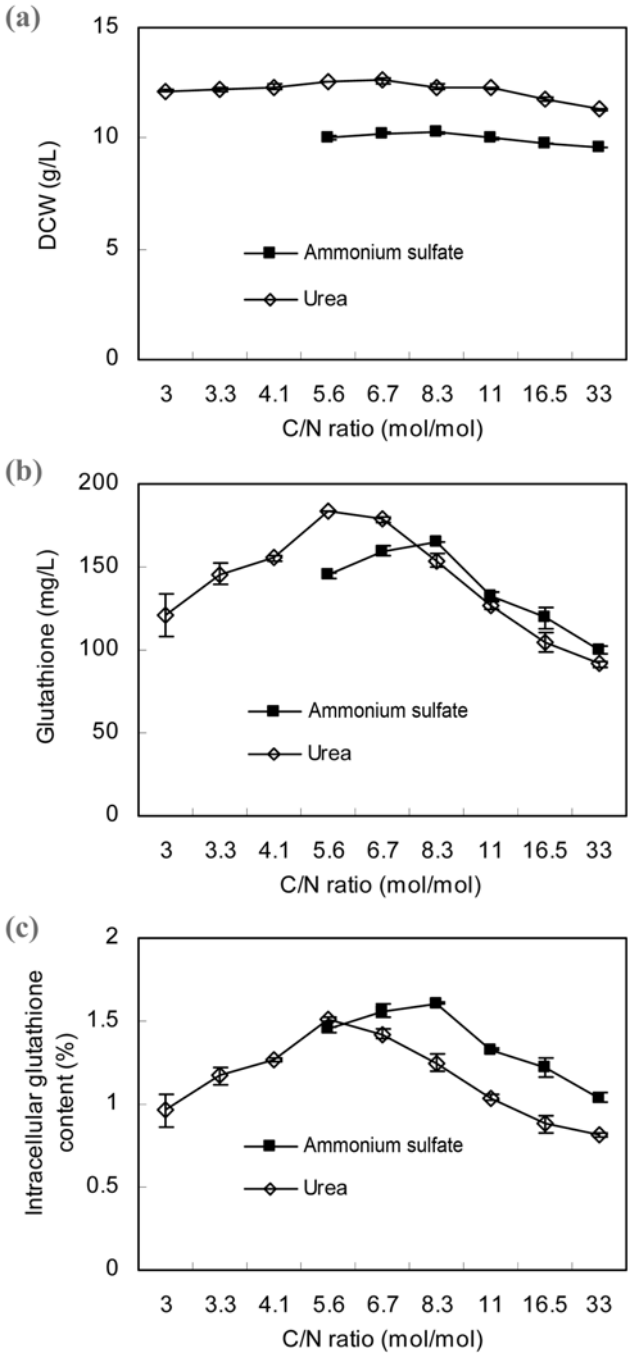

Fig. 2. Comparison of cell growth and glutathione production by *C. utilis* WSH 02-08 under diverse C/N ratios when using ammonium sulfate or urea as the sole nitrogen source.

2. Effect of Urea and Ammonium Sulfate on Cell Growth and Glutathione Production under Diverse C/N Ratios

Based on the results above, we chose urea and ammonium sulfate, two kinds of cheap and commonly used substances, as nitrogen source for further study. Fig. 2 shows the effect of C/N ratio on cell growth and glutathione production when using urea and ammonium sulfate, respectively, as the sole nitrogen source. It was indicated that cell growth scarcely altered with the concentration of nitrogen sources. However, there has an obvious difference in DCW between two substances; urea is more favorable to cell growth of *C. utilis* WSH 02-08 than ammonium sulfate. Different from cell growth, glutathione bio-synthesis is more sensitive to C/N ratio, and there exists an optimal C/N ratio for glutathione production by both nitrogen sources. Under the optimal C/N ratio of 5.6 mol/mol and 8.3 mol/mol, not only were the maximum glutathione concentrations of 184.1 mg/L and 165.3 mg/L achieved, but the highest levels of intracellular glutathione content of 1.51% and 1.61% were also obtained by urea and ammonium sulfate, respectively. Although urea can bring more DCW (18.7%) and glutathione (10.2%) compared to ammonium sulfate at their respective optimal C/N ratios, the ability of glutathione bio-synthesis by *C. utilis* WSH 02-08 decreased when using urea as the sole nitrogen source, and the maximum intracellular glutathione content lowered by 6.2%. Accordingly, urea is a benefit to cell growth and ammonium sulfate is favorable to the ability of glutathione bio-synthesis. It is necessary to make it clear whether DCW and glutathione can be improved when using mixed nitrogen source by combining urea and ammonium sulfate together.

To test this idea, mixing urea and ammonium as nitrogen source was applied in flasks under diverse C/N ratios of 5.6, 6.7 and 8.3 mol/mol. Table 1 illustrates the effect of mixed nitrogen source on cell growth and glutathione production; the trend of DCW, glutathione and intracellular glutathione content using mixed nitrogen source under different C/N ratios is most similar to those of the sole nitrogen source. However, if the C/N ratio is fixed at one level (*e.g.*, 5.6 mol/mol), we can find that no matter which kind of mixed strategy is used, DCW is still lower than that of urea, glutathione and

Table 1. Effects of mixed nitrogen sources of ammonium sulfate and urea on cell growth and glutathione production under diverse C/N ratios

C/N (mol/mol)	Total N (mmol/L)	Mixed strategy		DCW (g/L)	Glutathione (mg/L)	Intracellular glutathione content (%)
		NH_4^+-N (mmol/L)	Urea-N (mmol/L)			
8.3	120	0	120	12.32±0.12	153.9±3.8	1.25±0.05
		30	90	12.20±0.15	139.6±2.8	1.16±0.03
		60	60	12.13±0.08	150.3±2.3	1.24±0.02
		90	30	11.64±0.16	168.0±1.9	1.44±0.01
		120	0	10.26±0.06	165.3±0.3	1.61±0.01
6.7	150	0	150	11.92±0.12	159.6±2.7	1.34±0.04
		30	120	11.57±0.17	166.5±2.3	1.44±0.03
		60	90	11.33±0.11	147.5±2.0	1.30±0.02
		90	60	11.32±0.04	143.6±3.8	1.27±0.03
		120	30	11.00±0.04	133.8±3.7	1.22±0.02
		150	0	10.22±0.06	140.8±1.6	1.38±0.01
5.6	180	0	180	12.22±0.02	183.8±1.6	1.50±0.02
		30	150	12.20±0.04	175.8±2.8	1.44±0.03
		60	120	12.15±0.09	163.8±2.3	1.35±0.01
		90	90	11.99±0.05	156.3±2.5	1.30±0.01
		120	60	11.88±0.04	151.5±2.3	1.28±0.02
		150	30	11.31±0.05	148.0±1.9	1.31±0.02
		180	0	11.13±0.07	148.0±4.3	1.33±0.03

intracellular glutathione content can scarcely exceed those derived from ammonium sulfate. From these results, a mixed nitrogen source cannot boost DCW and glutathione production even if the mixed strategies are introduced under different C/N ratios.

3. Batch Glutathione Fermentation under Different Nitrogen Sources

The effect of the sole and mixed nitrogen sources on cell growth and glutathione production by *C. utilis* WSH 02-08 in a 5 L stirred

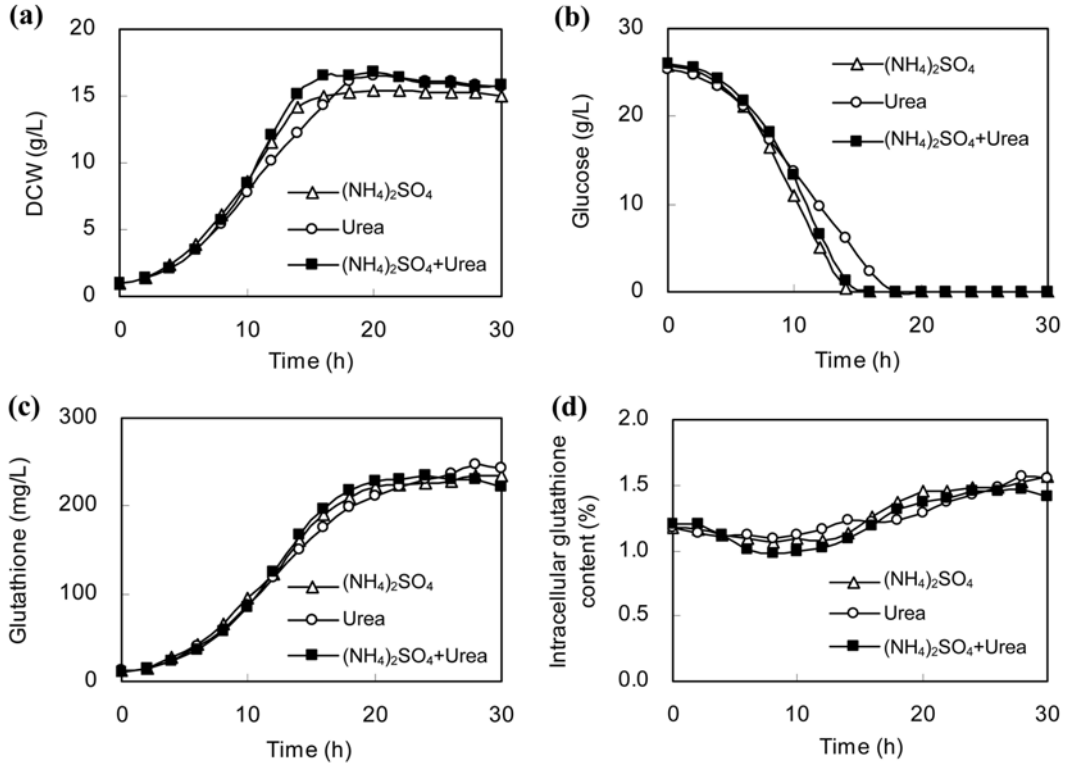

Fig. 3. Time-courses of batch culture of *C. utilis* WSH 02-08 for glutathione production with ammonium sulfate at C/N ratio of 8.3 mol/mol, urea at C/N ratio of 5.6 mol/mol and mixed nitrogen source at C/N ratio of 6.7 mol/mol.

Table 2.Comparison of parameters within batch glutathione production using different nitrogen sources

Parameters	Nitrogen source		
	$(NH_4)_2SO_4$	Urea	$(NH_4)_2SO_4$+Urea
Initial glucose concentration/(g/L)	25.8	25.3	26.0
Initial N concentration/(mmol/L)	120	180	150 (90+60)
C/N ratio/(mol/mol)	8.3	5.6	6.7
N comsuption/(mmol/L)	84.0	96.4	89.7
Maximum DCW/(g/L)	15.40	16.48	16.76
Maximum glutathione production/(mg/L)	234.6	246.4	233.5
Maximum intracellular GSH content/%	1.57	1.56	1.46
Glucose consumption rate/(g/L·h^{-1})	1.84	1.41	1.63
Average specific glucose consumption rate/h^{-1}	0.296	0.256	0.291
Average specific growth rate/h^{-1}	0.135	0.138	0.143
Average specific glutathione production rate/(mg/g·h^{-1})	1.20	1.24	1.24
Biomass yield on glucose/(g/g)	0.60	0.65	0.64
Glutathione yield on glucose/(mg/g)	9.09	9.74	8.98
Biomass productivity/(g/L·h^{-1})	0.70	0.82	0.84
Glutathione productivity/(mg/L·h^{-1})	7.82	8.80	8.34

Table 3. Kinetic parameters of cell growth and glutathione production within different culture phases of batch culture of *C. utilis* WSH 02-08

Nitrogen source	Average specific growth rate (h^{-1})		Average specific glutathione production rate (mg/g·h^{-1})	
	During glucose consumption	After glucose consumption	During cell growth	After cell growth
$(NH_4)_2SO_4$	0.187	0.013	1.49	0.12
Urea	0.153	0.034	1.63	0.26
$(NH_4)_2SO_4$+Urea	0.178	0.016	1.61	0.07

fermentor is illustrated in Fig. 3, and the parameters within batch glutathione fermentation are summarized in Table 2. Glucose consumption was much slower when the nitrogen source contained urea; cell growth retardation also occurred, but more DCW and glutathione were achieved compared to that of ammonium sulfate (Table 2). Furthermore, much higher DCW (about 16 g/L) appeared as long as glucose was exhausted than that from flasks, which would be due to a better oxygen supply in the fermentor [22]. Glutathione can still be produced by the yeast even if the cultivation reached the stationary phase (Fig. 3), indicating that glutathione production by *C. utilis* WSH 02-08 is a partial growth-associated process. It is also obvious in Table 2 that more N element was consumed when using urea as the sole nitrogen source, followed with the parameters associated with glutathione all being larger. The reason for this phenomenon should rely on the kinetics analysis and metabolic flux analysis on batch glutathione fermentation process.

4. Kinetics Analysis on Batch Glutathione Fermentation

Most kinetic parameters of batch glutathione fermentation can reflect the ability of glucose consumption, cell growth and glutathione formation by *C. utilis* WSH 02-08, which were expressed as specific glucose consumption rate (q_s), specific growth rate (μ) and specific glutathione production rate (q_p). The amount of q_s, μ and q_p, and the relationship among them can help us to quantify and analyze the process of batch glutathione fermentation.

The processes of batch glutathione fermentation under the sole or mixed nitrogen sources were divided into two stages by glucose consumption and cell growth, respectively. Based on this division, we can investigate the cell growth before/after glucose consumption, and glutathione production before/after cell growth. Table 3 lists the kinetic parameters of μ and q_p using three kind of nitrogen sources. It was obvious that urea could boost cell growth more than two-fold after glucose consumption, although about 20% less growth ability during glucose consumption. Besides, the average specific glutathione production rate was maintained at high levels not only during cell growth but also after cell growth, from which we can conclude that *C. utilis* WSH 02-08 has stronger ability of glutathione production using urea all along the cultivation.

As is known, the relationship between q_p and μ was described by Gaden [23] as follows:

$$q_p=\alpha\mu+\beta \quad (4)$$

Where, α and β can represent the ability of product formation during and after cell growth, respectively. In this study, non-linear estimation was introduced to calculate α and β with the data from

Table 4. Kinetic parameters of glutathione production estimated with different nitrogen sources

Nitrogen source	α (mg·g^{-1})	β (mg·g^{-1}·h^{-1})	R^2 value
$(NH_4)_2SO_4$	8.11	0.55	0.972
Urea	9.52	0.62	0.964
$(NH_4)_2SO_4$+Urea	7.17	0.58	0.946

Fig. 3, and the results are summarized in Table 4. When urea was used as the nitrogen source for the cultivation of *C. utilis* WSH 02-08, higher α and β could be simultaneously achieved in the Gaden equation, compared to those with ammonium sulfate. Therefore, it is not strange that urea can bring more glutathione production in a batch process.

5. Metabolic Flux Analysis on Batch Glutathione Fermentation

To make clear the flux distribution of metabolites involved in glutathione bio-synthesis by *C. utilis* WSH 02-08, a metabolic flux analysis was applied by dividing the batch process into cell growth phase (before 14 h) and glutathione further producing phase (after 14 h). Table 5 illustrates the metabolic flux of metabolites in the main nodes of metabolic network for glutathione formation. It was shown that r_{17} with urea was larger than r_{17} with ammonium sulfate and mixed nitrogen source, which indicated that more carbon flux flowed directly to HMP pathway to synthesize ribose and nucleic acids for biomass preparation, instead of flowing along the EMP pathway and TCA cycle to generate energy (r_2, r_3 and r_{10} were all low). To metabolize in this way, more NADPH formed in HMP pathway could also offer a necessary reductive environment *in vivo* for glutathione biosynthesis and accumulation by using urea as nitrogen source. Furthermore, if we concentrated on r_{24}, r_{25}, r_{26}, r_{35}, r_{62} and r_{63}, more carbon flux flowed to the metabolites concerned with glutathione biosynthesis including glutamic acid, cysteine and glycine; hence more glutathione was achieved with urea than other nitrogen sources. When we turned to biomass formation by comparing the fluxes flowing to the compositions of cells like amino acids, polysaccharides, glycerol, and fats (r_4, r_{31}, r_{40}, r_{55}, r_{56} and r_{57}), it is not difficult to realize why more biomass (r_{61}) could also be obtained with urea in batch glutathione fermentation.

Table 5. Metabolic flux distribution for typical metabolites within glutathione biosynthesis by *C. utilis* WSH 02-08

Metabolite	Vectors	Metabolic flux					
		A-I	B-I	A-II	B-II	A-III	B-III
G6P	r_1	100	100	100	100	100	100
	r_2	74.3	31.7	52.8	22.8	55.6	28.4
	r_{17}	21.4	62.7	42.2	71.3	39.4	65.9
	r_{55}	4.3	5.6	5.0	5.9	5.0	5.7
GA3P	r_3	80.7	62.8	71.6	59.2	72.8	61.4
	r_4	1.0	1.2	1.1	1.3	1.1	1.2
	r_5	80.0	67.0	73.4	64.5	74.2	66.1
	r_{21}	2.7	18.7	10.7	22.1	9.6	19.9
PG	r_5	80.0	67.0	73.4	64.5	74.2	66.1
	r_6	74.6	60.0	67.2	57.0	68.2	58.9
	r_{24}	12.3	16.0	14.0	17.2	13.7	16.5
PYR	r_7	71.6	56.1	63.8	53.0	64.8	55.0
	r_8	52.5	31.8	42.1	27.5	43.4	30.2
	r_9	22.3	28.4	25.4	29.6	25.0	28.9
	r_{31}	4.2	5.4	4.9	5.6	4.8	5.5
	r_{32}	1.8	2.3	2.1	2.4	2.1	2.4
AcCoA	r_8	52.5	31.8	42.1	27.5	43.4	30.2
	r_{10}	84.1	36.7	60.2	26.9	63.3	33.1
	r_{56}	1.2	1.5	1.3	1.5	1.3	1.5
	r_{57}	1.2	1.5	1.3	1.5	1.3	1.5
KG	r_{11}	83.3	35.7	59.3	25.9	62.4	32.1
	r_{12}	59.1	16.3	37.6	7.2	40.4	12.9
	r_{35}	41.3	52.9	47.1	55.4	46.3	53.8
OAA	r_9	22.3	28.4	25.4	29.6	25.0	28.9
	r_{10}	84.1	36.7	60.2	26.9	63.3	33.1
	r_{16}	45.9	11.3	28.5	3.9	30.8	8.6
	r_{40}	25.4	31.9	28.9	32.8	28.5	32.2
GSH	r_{25}	1.6	1.8	1.8	1.9	1.8	1.9
	r_{26}	6.5	8.5	7.5	9.0	7.3	8.7
	r_{62}	2.0	3.1	2.3	3.8	2.2	3.3
	r_{63}	1.1	2.1	1.3	2.9	1.2	2.4
BIOM	r_{61}	60.2	77.9	69.4	80.8	68.3	79.0

Note: A, culture phase before 14 h; B, culture phase after 14 h; I, ammonium sulfate as the sole nitrogen source; II, urea as the sole nitrogen source; III, mixed nitrogen source with ammonium sulfate and urea

CONCLUSIONS

Glutathione, a kind of bioactive thiol compound, usually serves as an important pharmaceutical. Although glutathione is successfully produced on an industrial scale using biotechnological methods, many researchers still focused on the production of this tripeptide for higher concentration, higher yield and higher productivity [8]. In this report, several nitrogen sources were tested for the production of glutathione by *C. utilis* WSH 02-08. Although *C. utilis* WSH 02-08 could assimilate all the inorganic and organic nitrogen, urea and ammonium sulfate were still found to be the most favorable nitrogen sources for cell growth and glutathione bio-synthesis in flasks, respectively. A high or low C/N ratio inhibits glutathione production, which demonstrates that maintaining an optimal C/N ratio (5.6 mol/mol for urea and 8.3 mol/mol for ammonium sulfate, respectively) is important for efficient glutathione production. Mixed nitrogen source of urea and ammonium under diverse C/N ratios indicated that DCW and glutathione production cannot be further boosted even if several mixed strategies are introduced. Batch glutathione fermentation using the sole or mixed nitrogen sources of urea and ammonium sulfate under their optimal C/N ratios was carried out; urea was further proved to be the best nitrogen source for cell growth and glutathione production. The reason for the improvement of glutathione by urea was then quantitatively described in detail by the comparison of parameters obtained from fermentation kinetic model, together with the distribution of flux for metabolites in metabolic network of glutathione production by *C. utilis* WSH 02-08. If we use the favorable nitrogen source of urea and its optimal C/N ratio of 5.6 mol/mol, the maximum DCW and glutathione could reach 16.48 g/L and 246.4 mg/L, respectively, after 30 h of cultivation of *C. utilis* WSH 02-08 in batch glutathione fermentation.

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APPENDIX

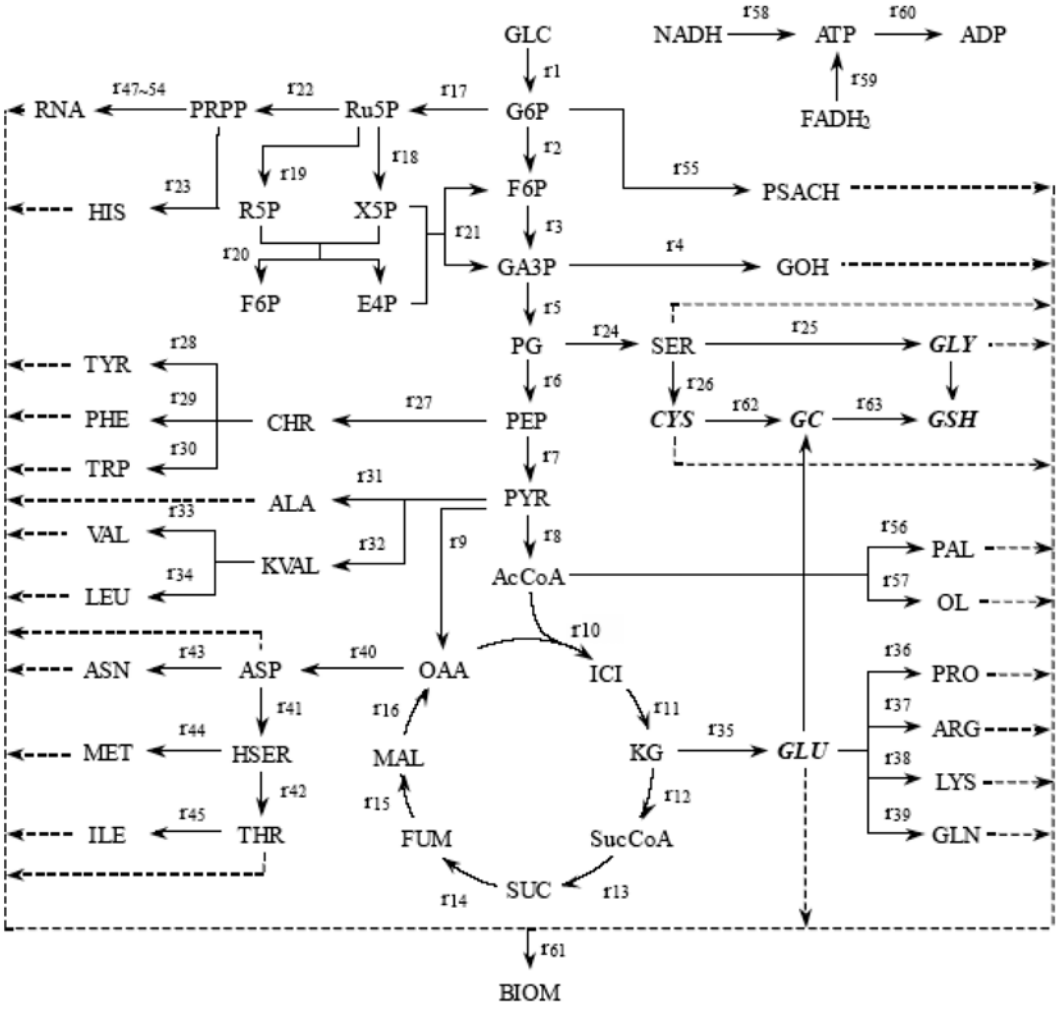

Fig. 1. Metabolic network for glutathione biosynthesis with *C. utilis* WSH 02-08 under batch cultivation.

Table 1. Intracellular reactions for cell growth and glutathione formation by *C. utilis* WSH 02-08

Glycolysis pathway	
r_1	GLC+ATP→G6P+ADP
r_2	G6P→F6P
r_3	F6P+ATP→2 GA3P+ADP
r_4	GA3P+NADH→GOH+NAD+Pi
r_5	GA3P+NAD+Pi+ADP→PG+NADH+ATP
r_6	PG→PEP
r_7	PEP+ADP→PYR+ATP
Tricarboxylic acid cycle	
r_8	PYR+CoA+NAD→AcCoA+NADH+CO_2
r_9	PYR+ATP+CO_2→OAA+ADP+Pi
r_{10}	OAA+AcCoA→ICI+CoA
r_{11}	ICI+NADP→α-KG+NADPH+CO_2
r_{12}	α-KG+CoA+NAD→SucCoA+NADH+CO_2
r_{13}	SucCoA+ADP+Pi→SUC+ATP+ CoA
r_{14}	SUC+FAD→FUM+$FADH_2$
r_{15}	FUM→MAL
r_{16}	MAL+NAD→OAA+NADH
P1entose phosphate pathway	
r_{17}	G6P+2 NADP→Ru5P+2 NADPH+CO_2
r_{18}	Ru5P→X5P
r_{19}	Ru5P→R5P
r_{20}	X5P+R5P→F6P+E4P
r_{21}	X5P+E4P→F6P+GA3P
Amino acids synthesis	
r_{22}	Ru5P+2 ATP→PRPP+2 ADP
r_{23}	PRPP+GLN+NH_3+2 NAD+NADPH+3 ATP+CO_2→HIS+α-KG+2 NADH+NADP+3 ADP+6 Pi
r_{24}	PG+GLU+NAD→SER+α-KG+NADH+Pi
r_{25}	SER+THF→GLY+MET
r_{26}	SER+AcCoA+4 NADPH+ATP→CYS+AC+CoA+4 NADP+ADP+Pi
r_{27}	2 PEP+E4P+ATP+NADPH→CHR+ADP+4 Pi+NADP
r_{28}	CHR+GLU+NAD→TYR+α-KG+NADH+CO_2
r_{29}	CHR+GLU→PHE+α-KG+CO_2
r_{30}	CHR+GLN+PRPP+SER→TRP+GLU+2 Pi+GA3P+PYR+CO_2
r_{31}	PYR+GLU→ALA+α-KG
r_{32}	2 PYR+NADPH→KVAL+NADP+CO_2
r_{33}	KVAL+GLU→VAL+α-KG
r_{34}	KVAL+AcCoA+GLU+NAD+ATP→LEU+α-KG+CoA+CO_2+NADH+ADP+Pi
r_{35}	α-KG+NH_3+NADPH→GLU+NADP
r_{36}	GLU+ATP+2 NADPH→PRO+ADP+Pi+2 NADP
r_{37}	2 GLU+AcCoA+5 ATP+NADPH+ASP+NH_3+CO_2→ARG+CoA+α-KG+AC+5 ADP+5 Pi+FUM+NADP
r_{38}	2 GLU+AcCoA+3 ATP+2 NADPH+2 NAD→LYS+CoA+α-KG+CO_2+3 ADP+3 Pi+2 NADP+2 NADH
r_{39}	GLU+NH_3+ATP→GLN+ADP+Pi
r_{40}	OAA+GLU→ASP+α-KG
r_{41}	ASP+ATP+2 NADPH→HSER+2 NADP+ADP+Pi
r_{42}	HSER+ATP→THR+ADP+Pi
r_{43}	ASP+NH_3+2 ATP→ASN+2 ADP+2 Pi
r_{44}	HSER+SucCoA+CYS+MTHF+ATP→MET+CoA+SUC+PYR+NH_3+ADP+Pi+THF
r_{45}	THR+PYR+NADPH+GLU→ILE+NH_3+NADP+CO_2+α-KG
Amino acid polymerization	
r_{46}	0.0820 GLU+0.0285 GLN+0.0448 PRO+0.0437 ARG+0.0776 LYS+0.0502 SER+0.0787 GLY + 0.0019 CYS+0.0806 ASP +0.0277 ASN+0.0518 THR+0.0138 MET+0.0524 ILE+0.1246 ALA+0.0719 VAL+0.0803 LEU+0.0364 PHE+0.0277 TYR +0.0076 TRP+0.0179 HIS+4 ATP→4.8248 PROT+4 ADP+4 Pi

Table 1. Continued

Nucleotide synthesis	
r_{47}	PRPP+2 GLN+GLY+4 ATP+ASP+2 FTHF+CO_2→IMP+4 ADP+6 Pi+2 GLU+ 2 THF+FUM
r_{48}	IMP+ASP+ATP→AMP+ADP+Pi+FUM
r_{49}	IMP+NAD+2 ATP+GLN→GMP+2 ADP+2 Pi+GLU+NADH
r_{50}	GLN+PRPP+2 ATP+ASP+NAD→UMP+2 ADP+4 Pi+GLU+NADH
r_{51}	UMP+2 ATP→UTP+2 ADP
r_{52}	UTP+GLN+ATP→CTP+ADP+Pi+GLU
r_{53}	CTP+2 ADP→CMP+2 ATP
RNA synthesis	
r_{54}	0.2330 AMP+0.2330 GMP+0.3060 UMP+0.2280 CMP+3.2279 ATP→9.446 RNA+3.22791 ADP+3.22791 Pi
Synthesis of glycogen and polysaccharides	
r_{55}	0.16667 G6P+0.16667 ATP→PSACH+0.16667 ADP+0.33333 Pi
Synthesis of fatty acids	
r_{56}	8 AcCoA+15 ATP+15 NADPH+O_2→PAL+8 CoA+15 ADP+15 NADP+15 Pi
r_{57}	9 AcCoA+17 ATP+17 NADPH+O_2→OL+9 CoA+17 ADP+17 NADP+17 Pi
Oxidative phosphorylation	
r_{58}	NADH+0.5 O_2+1.53 ADP+1.53 Pi→NAD+1.53 ATP
r_{59}	$FADH_2$+0.5 O_2+0.5 (P/O) ADP+0.5 (P/O) Pi →0.5 (P/O) ATP+FAD
ATP consumption for maintenance	
r_{60}	ATP→ADP+Pi
Biomass formation	
r_{61}	0.47003 PROT+0.35376 PSACH+0.05234 RNA+0.00344 PAL+0.00344 OL+0.00266 GOH→BIOM
Glutathione formation	
r_{62}	GLU+CYS+ATP→GC+ADP+Pi
r_{63}	GC+GLY+ATP→GSH+ADP+Pi
Transfer of 1-carbon compounds	
r_{64}	THF+ATP+NADH+CO_2→FTHF+ADP+Pi+NAD
r_{65}	THF+CO_2+3 NADH→MTHF+3 NAD