

Screening of *Candida utilis* and medium optimization for co-production of S-adenosylmethionine and glutathione

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Abstract—An effective S-adenosylmethionine and glutathione enriching yeast mutant of *Candida utilis* CCTCC M 209298 was first screened from plates containing 0.5 g/L of DL-ethionine by complex mutagenesis with UV and γ -ray in this study. Medium components optimization for enhanced co-production of S-adenosylmethionine and glutathione by *C. utilis* CCTCC M 209298 was further carried out using response surface methodology. The significant factors influencing S-adenosylmethionine and glutathione co-production were selected by Plackett-Burman design as sucrose, KH_2PO_4 and L-methionine, and Box-Behnken design was applied for further optimization studies. Based on these approaches, the optimized concentrations on medium components for higher co-production of S-adenosylmethionine and glutathione were sucrose 35.4 g/L, $(\text{NH}_4)_2\text{SO}_4$ 10 g/L, KH_2PO_4 12.3 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L, CaCl_2 0.05 g/L and L-methionine 4.6 g/L. The medium optimization by response surface methodology led to a total production of 589.3 mg/L on S-adenosylmethionine and glutathione, which was 2.4-fold increased compared with the medium without optimization.

Key words: S-adenosylmethionine, Glutathione, Co-production, *Candida utilis*, Medium Optimization

INTRODUCTION

S-adenosylmethionine (SAM) and reduced glutathione (GSH) are both significant active molecules *in vivo* that have key roles in cell growth and metabolism. SAM is biosynthesized by L-methionine and ATP under the catalysis of SAM synthetase [1]. As the most important methyl donor, SAM is involved in the methyl transferring processes catalyzed by kinds of methyltransferases, and modulates the metabolism of DNA, RNA and proteins [2]. At present, SAM is extensively utilized for the treatment of liver diseases, arthritis and depressive disorder [3]. GSH is a tripeptide produced intracellularly in the presence of ATP and its three precursor amino acids (L-glutamate, L-cysteine and glycine); it can be found in most mammalian and many prokaryotic cells and is the most abundant intracellular thiol [4]. GSH fulfills many physiological functions in higher eukaryotic organisms, serving as antioxidant, immunity booster and detoxifier [5]. Especially, GSH plays an important role in maintaining the normal cellular redox environment [6], which results in its wide utilization in medicine, food and the cosmetic industry. Therefore, the commercial demand for GSH keeps rapidly expanding [7].

In recent years, the production of both SAM and GSH has attracted wide attention because of their application in pharmacy. Besides being extracted from some active tissues, SAM and GSH both can be produced by chemical method, enzymatic reaction and microbial fermentation. Among these methods, biotechnological synthesis has been widely exploited for its high O/I ratio while compared

with extraction and chemical method. Furthermore, yeast fermentation was characterized as the most efficient and practical method for SAM and GSH production [7,8]. Due to the tightly interrelated metabolic pathways, SAM and GSH both participate in the thiol metabolism of cells, which will lead to a contradiction in the co-production of the two compounds [3]. Researchers had already used *S. cerevisiae* for the efficient co-production of SAM and GSH [8,9], and the production of SAM and GSH can reach 900 mg/L and 390 mg/L when cultured in a 15 L stirred-tank fermentor [8]. Even so, no report was focused on another commonly used industrial yeast, *Candida utilis*, for the co-production of SAM and GSH until now.

Response surface methodology (RSM) is the collection of statistical techniques for experiment design, model development, evaluation factors, and optimum conditions search [10]. Now it is extensively applied in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation, and food manufacturing processes [11,12]. RSM design was used for further study of the influences of major factors and interaction between them on the response value, which is based on the results of sole-experiment and Plackett-Burman (PB) design. PB design is a method of choice for initial screening of medium components. Following that, a Box-Behnken (BB) experiment is usually used for further optimization by determining the influence of key factors with a minimum number of experiments [10].

In this work, we have made great efforts on the screening of a mutant of *C. utilis* CCTCC M 209298 for SAM and GSH co-production, which was obtained by complex mutagenesis on *C. utilis* SZU07-01 using UV and γ -ray, and the optimal medium components for the mutant were further studied. We attempt to formulate a suitable medium in hope of improving the co-production of SAM

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and GSH using statistical optimization methods like PB and BB.

MATERIALS AND METHODS

1. Yeast Strains

Parental strain of *C. utilis* SZU07-01, which can only accumulate GSH intracellularly, was used for UV and γ -ray complex mutagenesis. *C. utilis* CCTCC M 209298, an effective SAM and GSH-enriching yeast strain, screened from the plates contained 0.5 g/L of DL-ethionine after complex mutagenesis of the parental strain by sequential treatment with UV and γ -ray, was now preserved in the China Center for Type Culture Collection. Yeast strains were maintained in 20% glycerol under -70°C , or on nutrient agar slants for a short period of time.

2. Medium and Culture Conditions

The seed medium contained (g/L) glucose 20, peptone 20 and yeast extract 10 at pH 6.0. The inoculum was prepared by transferring colonies from a fresh agar slant into a 50-mL seed medium in a 500-mL Erlenmeyer flask, and incubated at 30°C for 24 h on a rotary shaker at 200 rpm. The seed culture was then transferred to a 500-mL flask containing 50-mL fermentation medium with an inoculum size of 10% (v/v). The starting medium for flask fermentation contained (g/L) glucose 30, ammonium sulfate 8, KH_2PO_4 3, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 and L-Met 1 at pH 5.5. Flask fermentation was performed at 30°C for 30 h on the shaker at 200 rpm. All experiments were repeated in triplicate.

3. Mutagenesis

UV mutagenesis was performed on freshly cultured yeast cells of *C. utilis* SZU07-01 diluted in 0.9% (w/v) NaCl to a final concentration of 1×10^6 cells/mL. After being irradiated with UV for 60 s, cells with a survival rate of about 25% were plated on seed medium containing DL-ethionine and incubated at 30°C for 2 days. Colonies were selected for further cultivation, and the strain with highest production of SAM and GSH was screened for γ -ray mutagenesis.

γ -ray mutagenesis was done by irradiating the strain with γ -ray (Co-60) under an irradiation dosage rate of 80 Gy/min for 7 min. The surviving cells were also plated on seed medium that contained DL-ethionine. After the complex mutagenesis with UV and γ -ray on *C. utilis* SZU07-01, the mutant of *C. utilis* CCTCC M 209298 was screened for the co-production of SAM and GSH.

4. Analytical Methods

A fermentation broth of 25 mL was centrifuged at $8,000 \times g$ for 10 min, and after being washed twice with distilled water, the wet cells were dried at 70°C to a constant weight for dry cell weight (DCW) determination. GSH was extracted from the wet cells by 40% (v/v) ethanol at 30°C for 2 h, and centrifuged at $8,000 \times g$ for 15 min. The supernatant was used for GSH assay. GSH concentration was determined according to the method described by Tietze [13]. SAM was extracted by 0.35 mol/L H_2SO_4 at 4°C for 2 h, and centrifuged at $8,000 \times g$ for 15 min. The supernatant was used for SAM assay. The concentration of SAM was determined by high performance liquid chromatography (HPLC) according to the method described previously [14]. HPLC was equipped with a SunFire TM ODS column (4.6 \times 200 mm) packed with 5 μm particle size C18 packing material and an UV detector (254 nm). 0.5 mol/L ammonium formate at pH 4.0 adjusted with formic acid was used as the mobile phase with the flow rate of 0.8 mL/min. The concentration of residual carbohydrate was determined by sulfuric acid-phenol method [15].

5. Plackett-Burman Design

PB design is one special type of a two-level fractional factorial design based on the incomplete equilibrium piece principle. It can pick up the main factors from a list of candidate factors with the least number of experiments [16,17]. The possible factors affecting SAM and GSH co-production in this study included sucrose, ammonium sulfate, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCl_2 and L-methionine. The 12-run PB design was used to study these factors and three dummy variables were laid out to estimate the standard error during analysis of data. Each variable was prepared in two levels: -1 for low level while $+1$ for high level, as shown in Tables 1 and 2.

6. Box-Behnken Design

In general, response surface methodology contains Box-Behnken (BB) design and central composite design (CCD). CCD is a five-level fractional factorial design which is tensely dependent on the accuracy of the central point. Therefore, in this work, a three-level fractional factorial design named BB design was applied. Based on the results of the PB design, the BB design was conducted to gain the optimal levels of the main factors picked out by PB experiment. We can get a second-order empirical model from the experimental data about relationship between the response value (total production

Table 1. The factors, levels, and the regression analysis of Plackett-Burman design

Factors (g/L)	Levels		Effect	t (X _i)	P value	Confidence level (%)	
	-1	1					
X ₁	Sucrose	25	35	36.25	7.723	0.0164	98.36
X ₂	(NH ₄) ₂ SO ₄	5	10	-0.06	-0.46	0.6907	30.93
X ₃	KH ₂ PO ₄	5	10	1.302	9.71	0.0104	98.96
X ₄	MgSO ₄ · 7H ₂ O	0.01	0.05	-0.13	-0.93	0.4495	55.05
X ₅	CaCl ₂	0.05	0.1	-0.08	-0.56	0.6321	36.79
X ₆	L-Methionine	4	6	-20.9	-4.45	0.047	95.30
X ₇	Dummy			0.115	0.858	0.4813	51.87
X ₈	Dummy			0.062	0.46	0.6907	30.93
X ₉	Dummy			0.225	1.679	0.2353	76.47

X₁-X₆ represent different assigned variables; X₇-X₉ were three dummy variables used for experimental error estimation; t is the value of variables determined by student's t-test

Table 2. The experiments and results of the Plackett-Burman design

Run	Factor-coded levels									Total production of SAM and GSH (mg/L)
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	
1	1	-1	1	-1	-1	-1	1	1	1	468.0±21.3
2	1	1	-1	1	-1	-1	-1	1	1	463.0±2.6
3	-1	1	1	-1	1	-1	-1	-1	1	332.4±7.3
4	1	-1	1	1	-1	1	-1	-1	-1	462.3±0.0
5	1	1	-1	1	1	-1	1	-1	-1	514.5±5.6
6	1	1	1	-1	1	1	-1	1	-1	483.5±19.2
7	-1	1	1	1	-1	1	1	-1	1	374.6±4.2
8	-1	-1	1	1	1	-1	1	1	-1	433.4±0.0
9	-1	-1	-1	1	1	1	-1	1	1	315.1±2.6
10	1	-1	-1	-1	1	1	1	-1	1	474.2±14.9
11	-1	1	-1	-1	-1	1	1	1	-1	357.5±0.0
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	327.2±0.0

X₁-X₆ represent different assigned factors and X₆-X₈ are the dummy factors; '-1' is for low level of factors and '+1' is for high level of factors

Table 3. Design of Box-Behnken experimental variables

Variables	Symbols	Coding (g/L)		
		-1	0	1
Sucrose	X ₁	30	35	40
KH ₂ PO ₄	X ₂	5	10	15
L-Methionine	X ₃	2	4	6

Table 4. Design and results of Box-Behnken experiment

Run	X ₁	X ₂	X ₃	Total production of SAM and GSH (mg/L)	
				-1	0
1	-1	-1	0	478.0±0.6	
2	-1	1	0	514.5±10.0	
3	1	-1	0	477.4±0.6	
4	1	1	0	550.1±0.6	
5	0	-1	-1	465.3±6.9	
6	0	-1	1	484.3±3.0	
7	0	1	-1	490.0±3.2	
8	0	1	1	545.3±0.6	
9	-1	0	-1	477.4±1.1	
10	1	0	-1	452.9±0.0	
11	-1	0	1	499.9±6.1	
12	1	0	1	523.8±3.9	
13	0	0	0	580.0±2.0	
14	0	0	0	579.7±2.0	
15	0	0	0	580.0±2.0	

of SAM and GSH) and the variables through polynomial regression analysis. The BB design was prepared according to Tables 3 and 4.

7. Software for Experimental Design and Statistical Analysis

Statistical Analysis System (SAS) Version 9.0 was used for the experimental design and statistical analysis of the experimental data. The quality of fit for the regression model equation was expressed by the coefficient of determination R² and its statistical significance

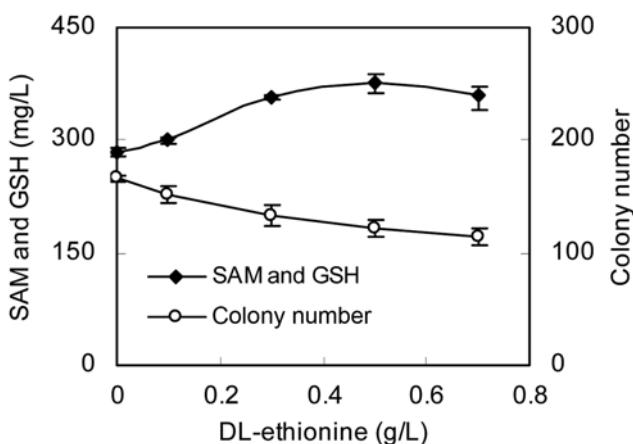


Fig. 1. Effect of DL-ethionine on the cell growth and the capacity of co-production of SAM and GSH by mutants of *C. utilis* screened from plates (Φ 9 cm) after complex mutagenesis using UV and γ-ray. The data were the averages of the results from 20 mutants.

was determined by an F test. The significance of the regression coefficients was checked by a t-test. All of the experiments were performed in triplicate, and the average value was used for SAS analysis.

RESULTS

1. Effect of DL-ethionine on Cell Growth and Co-production of SAM and GSH

DL-ethionine is a structural analog of SAM and GSH, so the strain which can resist high concentrations of DL-ethionine will possibly accumulate high concentrations of SAM and GSH intracellularly. Based on this consideration, the mutants after UV and γ-ray complex mutagenesis were all screened from the plates containing DL-ethionine. The effects of DL-ethionine on cell growth and the co-production of SAM and GSH are illustrated in Fig. 1. The number of colony on plates decreased following with the addition of DL-ethionine; however, strains which had the ability to grow on plates

containing DL-ethionine were proven to accumulate higher levels of SAM and GSH, especially under a DL-ethionine concentration of 0.5 g/L. Mutants screened from the plates containing 0.5 g/L of DL-ethionine were also found to be more favorable to the utilization of L-methionine; hence the co-production of SAM and GSH was accordingly increased. Among these mutants, an effective SAM and GSH-enriching yeast strain of *C. utilis* CCTCC M 209298 was selected. The production of SAM and GSH of the mutant was increased by 131.4% and 26.0%, respectively, compared to the parental strain of *C. utilis* SZU07-01. Moreover, the conversion rate of L-methionine to SAM can still reach 60% under L-methionine concentration of 10 g/L [18].

2. The “Single-Factor” Experiments on Medium Composition

Optimal nutrients such as carbon source, nitrogen source, inorganic salts and kinds of growth factors are very crucial to cell growth and metabolism of microorganisms. In this section, kinds of carbon sources and nitrogen sources were investigated in order to choose the most efficient nutrients for the co-production of SAM and GSH by *C. utilis* CCTCC M 209298, and results are illustrated in Fig. 2. It was shown that sucrose and $(\text{NH}_4)_2\text{SO}_4$ were the best carbon source and nitrogen source, respectively. Furthermore, suitable concentrations of carbon source and nitrogen source were further studied (Fig. 3), and the total production of SAM and GSH reached the highest when the concentration of sucrose was 30 g/L and $(\text{NH}_4)_2\text{SO}_4$ was

10 g/L, respectively.

The effects of inorganic salts like KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and CaCl_2 on the co-production of SAM and GSH were investigated (Fig. 4). It was indicated that suitable concentrations of inorganic salts were also necessary, and the optimal concentrations were as follows: KH_2PO_4 10 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g/L and CaCl_2 0.05 g/L. L-Methionine is the restricted precursor for SAM formation, a suitable addition of L-methionine can significantly increase the production of SAM, but high concentration of L-methionine would result in a relatively low conversion of L-methionine to SAM. As shown in Fig. 4, the co-production of SAM and GSH with a 5 g/L L-methionine addition was increased by 130% compared with the control (233.5 mg/L) without the addition of L-methionine.

3. Main Factors Picked up by Plackett-Burman Experiment for the Co-production of SAM and GSH

The levels of the variables for the PB design were selected (Table 1) according to the previous single-factor experiments. Based on the selection, a 12-run PB experiment was chosen to pick up the main factors in the fermentative process for the co-production of SAM and GSH (Table 2). The main factors were picked up at the confidence level of 95% based on their effects. According to the *t*-test results in Table 1, sucrose, KH_2PO_4 and L-methionine were considered as the three major factors affecting the co-production of SAM and GSH by *C. utilis* CCTCC M 209298. The rest of the factors

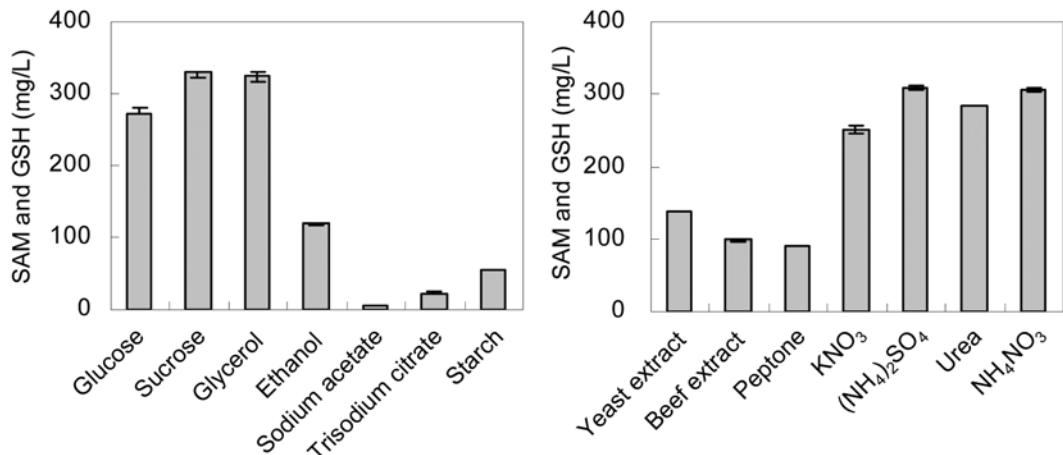


Fig. 2. Effects of carbon sources and nitrogen sources on the co-production of SAM and GSH.

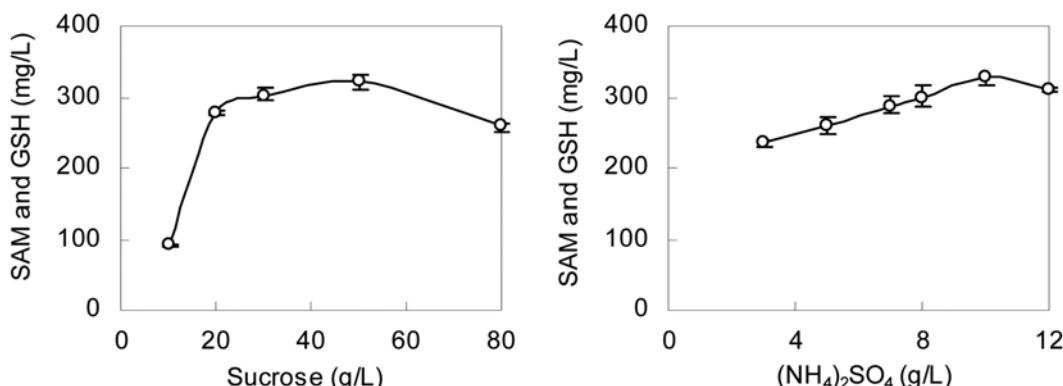


Fig. 3. Effects of sucrose and $(\text{NH}_4)_2\text{SO}_4$ concentrations on the co-production of SAM and GSH.

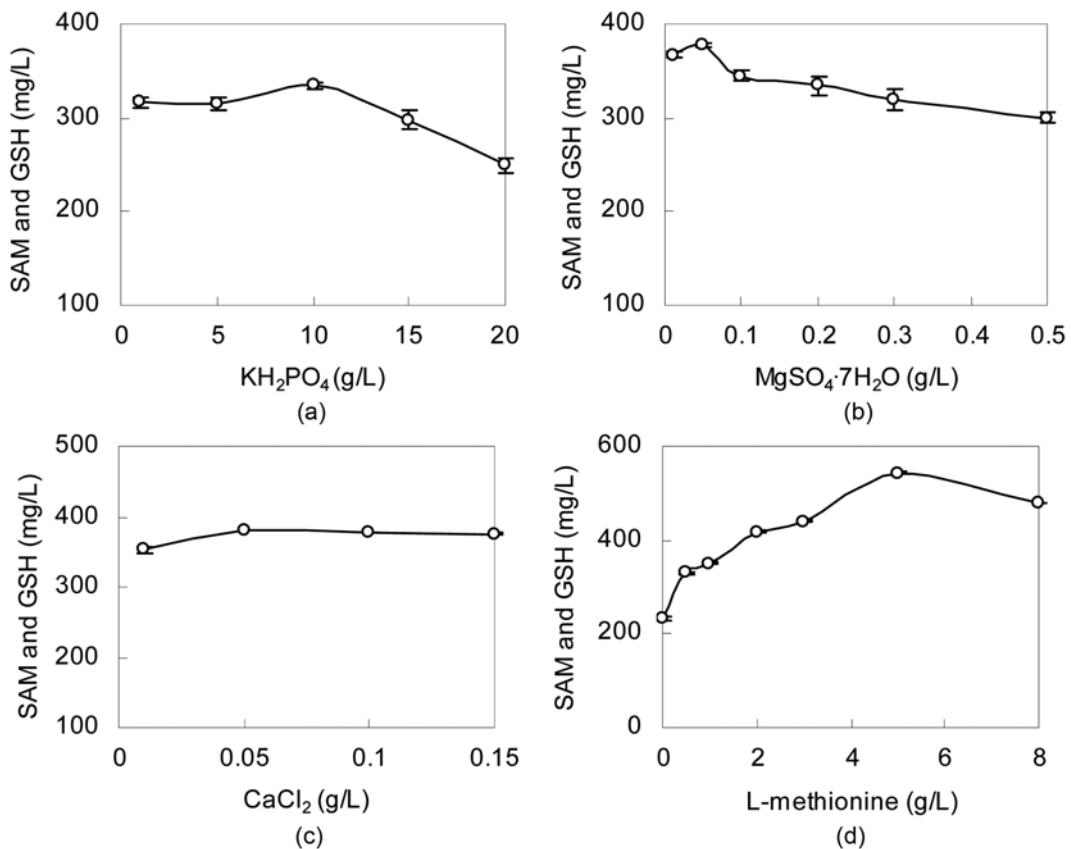


Fig. 4. Effects of inorganic salts and L-methionine on the co-production of SAM and GSH.

level below 90%, so they were considered insignificant.

4. Optimization of Screened Medium Components for the Co-production of SAM and GSH

The major factors including sucrose, KH_2PO_4 and L-methionine were selected for further optimization by using BB. Based on the results of PB experiment, the BB experiment was designed and conducted, as is shown in Tables 3 and 4. Each of the three major factors (sucrose, KH_2PO_4 and L-methionine) was designed in three levels (Table 3). The BB experiment results were analyzed by SAS 9.0 and illustrated in Table 5, which indicated that the experimental

results of BB could be fitted into a second-order regression model as follows:

$$\begin{aligned} Y = & 579.9 + 4.3X_1 + 24.362X_2 + 20.962X_3 \\ & - 41.313X_1^2 + 9.05X_1X_2 - 33.587X_2^2 \\ & + 12.1X_1X_3 + 9.075X_2X_3 - 50.088X_3^2 \end{aligned} \quad (1)$$

where, Y is the response variable (total production of SAM and GSH) and X_1 , X_2 and X_3 are the concentrations of sucrose, KH_2PO_4 and

Table 5. Coefficient estimates by the regression model

Term	Coefficient	SE Coef.	t	Pr> t
Constant	579.9	4.267	135.9	<0.0001
X_1	4.3	2.613	1.65	0.161
X_2	24.362	2.613	9.32	0.0002**
X_3	20.962	2.613	8.02	0.0005**
X_1X_1	-41.313	3.846	-10.74	0.0001**
X_2X_2	-33.587	3.846	-8.73	0.0003**
X_3X_3	-50.088	3.846	-13.02	<0.0001**
X_1X_2	9.05	3.695	2.45	0.0580
X_1X_3	12.1	3.695	3.27	0.0221*
X_2X_3	9.075	3.695	2.46	0.0575

*Statistically significant at 95% of confidence level

**Statistically significant at 99% of confidence level

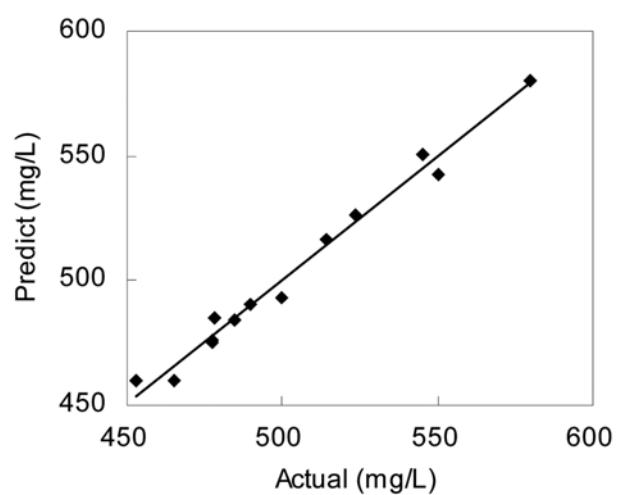


Fig. 5. Plot of predicted vs actual SAM and GSH co-production by *C. utilis* CCTCC M 209298.

L-methionine, respectively.

Fig. 5 illustrates the actual values for SAM and GSH co-production and the predicted values calculated by the model Eq. (1). Significance of coefficients has been reported to be directly proportional to t test and inversely to P value [19]. The smaller the P values, the bigger the significance of the corresponding coefficient. It was further indicated from Table 5 that KH_2PO_4 , L-methionine and second-order sucrose, KH_2PO_4 and L-Met were highly significant over 99% confidence level. An analysis of variance for the model

Table 6. Analysis of variance

Variance sources	DF	SS	MS	F	P
Regression	9	26862.8	2984.8	54.64	<0.0001
Residual error	5	273.1	54.6		
Total	14	27135.9			
R^2		97.2%			

F-test was used for the analysis of variance, a bigger F value and smaller P value represented a higher confidence level of the regression model
DF, degree of freedom; SS, sum of square; MS, mean square

was also conducted and summarized in Table 6; the value of R^2 was 97.2%, which indicated that the model congressed well with the practice and can be used to analyze and estimate the co-production of SAM and GSH by *C. utilis* CCTCC M 209298.

Compared to the second-order equation, the response surface plots shown in Fig. 6 can display a clear view of the influences of three major factors and interactions between them on the co-production of SAM and GSH. The shapes of contour curves showed that there were great effects on each other. From the response surface analysis, the optimal concentrations of three major factors can be achieved as follows: sucrose 35.4 g/L, KH_2PO_4 12.3 g/L, L-methionine 4.6 g/L, and the extreme production of SAM and GSH was forecasted as 588.7 mg/L.

5. Validation of the Model

According to the results derived from the “single-factor” experiments and the following PB and BB experiments, the optimal medium compositions for the co-production of SAM and GSH by *C. utilis* CCTCC M 209298 can be summarized as follows (g/L): sucrose 35.4, $(\text{NH}_4)_2\text{SO}_4$ 10, KH_2PO_4 12.3, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, CaCl_2 0.05 and L-Met 4.6. To validate the prediction of the model, addi-

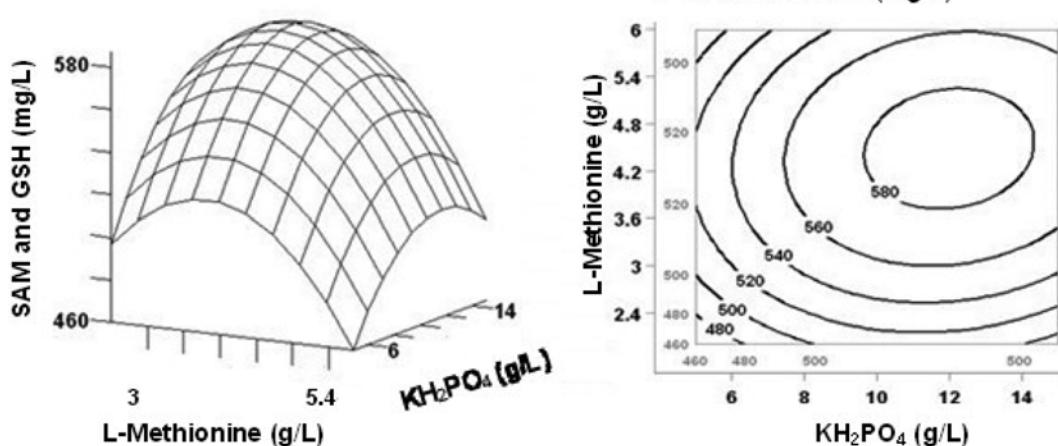


Fig. 6. The surface and contour plots of response surface methodology showing the effect of L-methionine and KH_2PO_4 on the co-production of SAM and GSH, under a fixed sucrose concentration of 35 g/L.

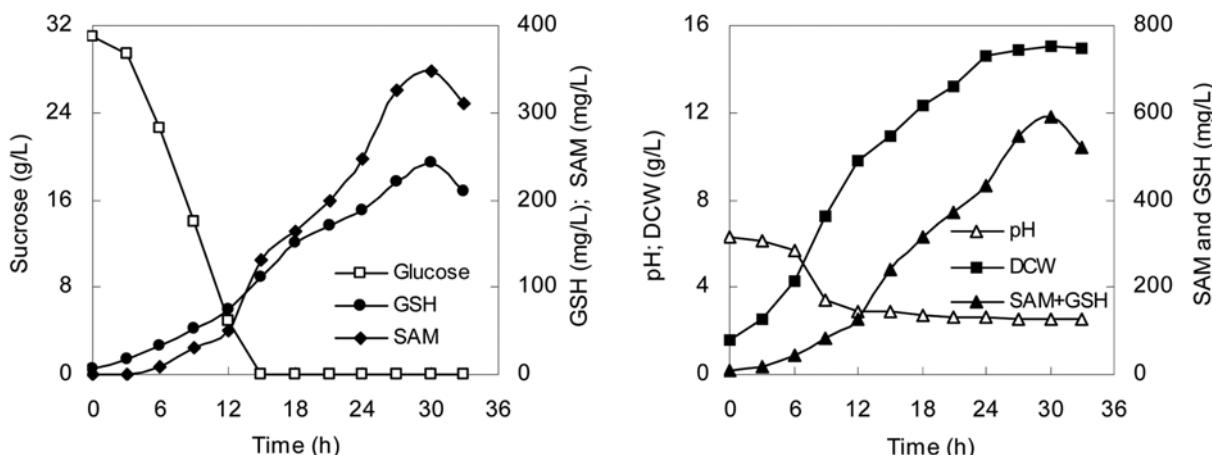


Fig. 7. Time-courses of SAM and GSH co-production by *C. utilis* CCTCC M 209298.

tional experiments in shake flasks were carried out using this combination of nutrients (Fig. 7). Under this circumstance, the maximum total production of SAM and GSH reached 589.3 mg/L, which agreed well with the forecasted value (588.7 mg/L). That is, the model was accurate and reliable for describing the process of SAM and GSH co-production by *C. utilis* CCTCC M 209298. Moreover, the total production of SAM and GSH under the optimal medium was 2.4 times higher than the starting medium.

DISCUSSION

In recent years, the microbial production of both SAM and GSH has attracted wide attention because of their expanding application in medicine. Many efforts had been made by researchers on the efficient production of either SAM or GSH, among which SAM or GSH was the sole target product. In this work, we obtained a high positive strain of *C. utilis* CCTCC M 209298 by UV and γ -ray complex mutagenesis followed with screening from plates containing 0.5 g/L of DL-ethionine, which has the capacity for the co-production of SAM and GSH. The present work is the first report on the screening of *C. utilis* and medium optimization for efficient co-production of SAM and GSH.

The carbon and nitrogen sources are the most important factors affecting cell growth together with SAM and GSH production. According to the single-factor experiments, the strain can utilize both organic and inorganic carbon sources and nitrogen sources efficiently, which indicates that *C. utilis* CCTCC M 209298 has a broad spectrum of nutrition. Sucrose and ammonium sulfate were found to be the optimal carbon and nitrogen source, respectively (Fig. 2), under the consideration of the yields of SAM and GSH production to nutrients. Furthermore, the effect of ion salts as K^+ , Mg^{2+} and Ca^{2+} on the co-production of SAM and GSH was also studied, and high concentrations of K^+ together with low concentrations of Mg^{2+} and Ca^{2+} were found to be beneficial to the co-production of SAM and GSH (Fig. 4).

Sucrose, KH_2PO_4 and L-methionine were picked out by PB design as the major factors that influenced the co-production of SAM and GSH. Among the three major factors, sucrose served as cell growth motor while L-methionine was the major precursor of the two target products. Moreover, KH_2PO_4 was also picked as a significant factor for SAM and GSH co-production at 99% confidence level (Table 1). The possible explanation could derive from two aspects: (1) high concentration of K^+ can promote Na^+/K^+ pump on the membrane running more efficiently for aerobic microorganism like *C. utilis* [20]; (2) K^+ is critical to the activity of SAM synthetase, for there was K^+ -binding site on it [21]; hence an adequate supply of K^+ can increase the biosynthesis of SAM. The results from BB design showed that there were great interactions among the three major factors of sucrose, KH_2PO_4 , and L-methionine. According to the second-order equation, the optimal medium components for the co-production of SAM and GSH by *C. utilis* CCTCC M 209298 were obtained (g/L): sucrose 35.4, $(NH_4)_2SO_4$ 10, KH_2PO_4 12.3, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCl_2$ 0.05 and L-methionine 4.6. The validation of the model showed that the co-production of SAM and GSH with optimized medium was 589.3 mg/L, which increased by 140.0% compared to the original level 245.3 mg/L without optimization.

In addition, medium components for the maximum production

of SAM or GSH by *C. utilis* CCTCC M 209298 were also worked out by using the same response surface methodology approach. It was shown that the optimal concentrations of three major factors for SAM production were (g/L) sucrose 35, KH_2PO_4 10.9 and L-methionine 4.7, while for GSH were (g/L): sucrose 40, KH_2PO_4 14.34 and L-methionine 5.8. According to the data, we can find that the optimal concentration values of the three major factors for SAM and GSH co-production were between them for SAM or GSH single-production, indicating that critical paradox existed in the co-production of SAM and GSH, which needed to be further studied.

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