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Original Research Article

Enhancement of L-Serine Production by *Corynebacterium glutamicum* SYPS-062 Directly from Sucrose

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Abstract

Purpose: *To improve the production of L-serine from sucrose directly by wild type Corynebacterium glutamicum SYPS-062.*
Methods: *The culture medium for the production of L-serine was optimized using a statistical experimental design. Sucrose, ammonium sulfate ($(NH_4)_2SO_4$) and biotin were the key factors, based on previous studies. A three-level Box–Behnken factorial design combined with response surface methodology were used to optimize the medium for the production of L-serine. Validation experiments were also carried out to establish the adequacy and accuracy of the model obtained.*
Results: *Under optimal conditions (sucrose 81 g/l, $(NH_4)_2SO_4$ 30 g/l and biotin 27 µg/l), L-serine concentration increased to 14.90 g/l which is about 2.5-fold increase over that of the original medium (6 g/l). L-serine concentration in a 5 L fermenter reached 16.4 g/L.*
Conclusion: *The results show that statistical medium design method is effective in improving L-serine production. The optimization medium is considered fundamental and useful for the development of C. glutamicum SYPS-062 cultivation process for efficient production of L-serine on a large scale.*

Keywords: *L-Serine, Sugar, Medium optimization, Corynebacterium glutamicum Box–Behnken factorial design, Response surface methodology*

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INTRODUCTION

L-serine is wildly applied in the pharmaceutical and the cosmetic industries [1]. The current production still relies mainly on the extraction of L-serine from protein hydrolysates or from molasses, as well as on the enzymological conversion of glycine plus a C1 compound to L-serine [2]. However, the dependence on expensive substrates and low productivity make them less attractive. However, fermentative production of L-serine from sugar has received more attention.

Compared with other amino acids, L-serine is a central intermediate for a number of cellular reactions, which makes its fermentation production from sugar difficult [3,4]. *Corynebacterium glutamicum* has been successfully applied to produce amino acids, including L-glutamate and L-lysine [5-7]. However, there is limited information on the direct fermentation production of L-serine from sugar using *C. glutamicum*.

Genetically-engineered strains of *Corynebacterium glutamicum* have been constructed based on the studies of the key enzymes of L-serine metabolic pathway, which

include 3-phosphoglycerate dehydrogenase (PGDH, serA), phosphhydroxypyruvate phosphoserine aminotransferase (PSAT; serC), and phosphoserine phosphatase (PSP; serB) were identified and characterized in *C. glutamicum*. Furthermore, two enzymes were found to be involved in L-serine degradation, namely, serine hydroxymethyl transferase (SHMT; glyA) and L-serine dehydratase (L-SerDH; sdaA) [8-10].

The strategies for constructing recombinant *C. glutamicum* to produce L-serine have mainly focused on removing the feedback inhibition of PGDH by L-serine and overexpressing serAΔ197 together with serC and serB, which yielded only traces of L-serine. However, when these strategies were employed together with reduction in the expression of glyA, a transient L-serine accumulation of up to 16 mM (1.68 g/L) was achieved. In addition, when sdaA was deleted simultaneously, the resulting strain accumulated up to 86 mM (9.03 g/L) L-serine [11]. This strain was further improved by reducing folate supply [12], which might be the first report on direct fermentative production of L-serine from sugar.

Recently, recombinant *Escherichia coli* producing L-serine from glucose was constructed by Yu [13], and 0.13 mmol/g of L-serine was obtained. In order to enhance L-serine concentration, the glyA gene expression was increased in *Methylobacterium* sp. MB200 that produced 11.4 g/l of L-serine [14]. However, the recombinant strains are often less stable than the wild-type strain. Obtaining a wild strain capable of accumulating L-serine from sugar may be commercially attractive.

In our previous study, a wild-type strain SYPS-062, was isolated from soil samples, and identified as *C. glutamicum* that could accumulate 6 g/l of L-serine from sucrose [15,16]. L-serine production was relatively low, but it might be the first report on direct fermentative production of L-serine from sucrose by wild-type *C. glutamicum*. In the present study, to further improve L-serine production, a three-level Box–Behnken factorial design combined with response surface methodology (RSM) were used to optimize the fermentation medium, and validation experiments were also investigated.

EXPERIMENTAL

Bacterium

A wild-type *C. glutamicum* SYPS-062 was stored at our laboratory (obtained from mud culture collection). It was deposited in the China General Microbiological Culture Collection Center with the accession number of 1843 [16].

Medium and cultivation conditions

The composition of the medium for slant or plate culture is: 10 g/l peptone, 10 g/l beef extract, 5 g/l yeast powder, 3 g/l NaCl and 20 g/l agar. The seed medium contained: 30 g/l glucose, 20 g/l $(NH_4)_2SO_4$, 20 g/l corn steep liquor, 1 g/l K_2HPO_4, 1.5 g/l urea and 0.5 g/l $MgSO_4•7H_2O$.

All the basic fermentation cultures were carried out in (250 mL) Erlenmeyer flasks containing 20 ml fermentation medium based on the experimental design (Table1), where base medium composition was: 80 g/l sucrose, 30 g/l $(NH_4)_2SO_4$, 3 g/l K_2HPO_4, 0.5 g/l $MgSO_4•7H_2O$, 0.02 g/l $FeSO_4•7H_2O$, 0.02 g/l $MnSO_4•H_2O$, 30 g/l $CaCO_3$, 32.5 µg/l biotin and 450 µg/l vitamin B1. To inoculate the fermentation medium, 5 % (v/v) of the seed culture was added, and the initial pH of the medium was adjusted to 7.0. The fermentations were carried out in a rotary shaker incubator at 115 rpm, 96 h and 30 ^{o}C under aerobic conditions. Various medium compositions were selected based on Table 2. All experiments were carried out in duplicate.

Batch fermentation in 5 L fermenter

The batch fermentation was carried out in 5L fermenter (Korea Co. Ltd, KF-5l) equipped with impeller, oxygen and pH electrodes, under the following conditions: medium volume 3 L, inoculation volume 5 % (v/v), at 30 ^{o}C, 96 h, initial pH 6.8, aeration rate 0.8 vvm, and agitation speed 180 rpm.

Analytical methods

The cell concentration was determined from the absorbance at 562 nm, while the dry cell weight (DCW) was measured gravimetrically after the cells were centrifuged at 2000 g for 15 min. Subsequently, the cell pellets were washed with 2 volumes of distilled water and then dried at 105 °C to a constant weight. The experimental result was 1 x absorbance value = 0.1925 g/l DCW.

The concentration of L-serine was measured by an Agilent 1100 HPLC with the following parameters: column, Hypersil ODS-C 18 4 mm × 125 mm; temperature, 40 °C; flow rate, 1.0 ml/min; detection, fluorescence detector; Em, 450 nm; Eluent A, 20 mmol/l Na-acetate; and Eluent B, 20 mmol/l Na-acetate : methanol : acetonitrile at a ratio of 1:2:2 (v/v). Sucrose concentration was measured by using resorcin spectrophotometric method.

Experimental design and evaluation

According to the results of our earlier experiment (data not shown), the initial concentrations of sucrose, $(NH_4)_2SO_4$ and biotin significantly influenced the yield of L-serine by SYPS-062. A Box-Behnken factorial design with three factors of three levels, including three replicate points, was used for fitting a second-order response surface. Table 1 gives the factors, their values, and the experimental design. A mathematical model was subsequently developed to describe the relationships between the process indicates (L-serine production) and the medium component contents in the second-order equation. The yield of L-serine was regressed with respect to the medium component contents by the least squares method as follows:

$$Y = A_0 + \sum A_iX_i + \sum A_{ii}X_i + \sum A_{ij}X_iX_j \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where Y is the predicted response variable; A_0, A_i, A_{ii}, A_{ij} are constant regression coefficients of the model, and X_i, X_j (i = 1, 3; j = 1, 3, i ≠ j) represent the independent variables (medium composition) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2.

Statistical analysis

Data from the Box–Behnken factorial design shown in Table 1 were used for determining the regression coefficients of the second-order multiple regression models. The analysis of regression and variance (ANOVA) was carried out using the RSREG procedure of the SAS statistical package procedure (version 8.1, SAS Institute, USA). Canonical analysis, which is used to predict the shape of the curve generated by the model, was also carried out. The contour plot analysis was made by keeping one independent variable at constant level, changing the other two independent variables, and then calculating the response variables using RSREG analysis.

RESULTS

Optimization of L-serine production

Table 1 shows considerable variation in L-serine productions with different fermentation medium composition. The concentration of L-serine ranged from 11.52 g/l to 14.79 g/l, and the run # 8 and #15 had the minimum and maximum concentrations, respectively. The centre point in the design was repeated three times to estimate the error. The analysis of variance (t-test) for this experiment is shown in Table 2. The coefficient of determination (R^2) is 99.87, this indicated that, the accuracy and general ability of the polynomial model was good, and analysis of the response trends using the model was considered to be reasonable.

Table 1: Box-Behnken experimental design matrix with experimental values of L-serine productivity

Trial number	Sources (g/l) X_1	$(NH_4)_2SO_4$ (g/l) X_2	Biotin (µg/l) X_3	L-serine (g/l) Y
1	70	25	32.5	12.98
2	70	30	15	13.16
3	70	30	50	14.16
4	70	35	32.5	12.98
5	80	25	15	13.07
6	80	25	50	13.25
7	80	35	15	13.79
8	80	35	50	11.52
9	90	25	32.5	13.34
10	90	30	15	14.14
11	90	30	50	12.52
12	90	35	32.5	12.64
13	80	30	32.5	14.67
14	80	30	32.5	14.70
15	80	30	32.5	14.79

Table 2: The least-square fit and parameters (significant of regression coefficient)

Parameter	Master Model	Predictive Model
Mean	13.447333	13.447333
Root MSE	0.055182	0.055182
R-square	99.87%	99.87%
CV	0.4104	0.4104

MSE = mean square error and CV = coefficient variation

The regression coefficients, along with the corresponding P-values, for the model of L-serine production by SYPS-062, are presented in Table 3. The P-values are used to check the significance of each coefficient, which also indicate the interaction strengths among each independent variable. Table 3 shows that, the regression coefficients of the linear term and quadratic coefficients of X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, X_1X_3 and X_2X_3 was significant at 5 % level, the polynomial model for predicting L-serine production Y was regressed by only considering the significant terms as shown below:

$$Y = 14.72 + 0.24X_1 - 0.25X_2 - 0.55X_3 - 0.57X_1^2 - 0.83X_2^2 - 0.98X_3^2 - 0.34X_1X_2 - 0.17X_1X_3 - 0.61X_2X_3$$

The shape of the contour plots indicates whether the mutual interactions between the independent variables were significant. An elliptical nature of the contour plots indicates that the interactions between the independent variables are significant. From the contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variables' pairs be easily understood. The fitted contour plots for the L-serine production by the above model were shown in Figs 1 - 3, respectively. The contour plots based on independent variable sucrose (X_1) and $(NH_4)_2SO_4$ (X_2) were shown in Fig 1, while the third independent variable, biotin (X_3) was kept constant. The interaction between the two independent variables (sucrose, $(NH_4)_2SO_4$) and the response variable (L-serine production) can be easily understood by examining the contour plots. It was obvious that the yield of L-serine was sensitive even when sucrose and $(NH_4)_2SO_4$ concentration was subject to small alterations. An increase of the L-serine yield could be significantly achieved with the increases of sucrose and $(NH_4)_2SO_4$ concentrations. Under certain conditions (81.1 g/l sucrose, coded as 0.11; 29.9 g/l $(NH_4)_2SO_4$, coded as -0.02), a maximal contour (Y = 14.77) could be predicted (Fig 1), indicating that further increases in sucrose and $(NH_4)_2SO_4$ concentrations would not increase the production levels anymore.

The effect of sucrose and biotin on L-serine production is shown in Fig 2. The contour plot showed the effect of substrate concentration and biotin on the L-serine production, while $(NH_4)_2SO_4$ was fixed at its middle level. When the biotin concentration in medium was at a fixed level, the production of L-serine production increased gradually with increasing sucrose substrate concentration.

The effects of $(NH_4)_2SO_4$ and biotin on L-serine production are shown in Fig 3. The contour plot showed the effect of $(NH_4)_2SO_4$ and biotin on the L-serine production, while sucrose was fixed at its middle level. These 3D plots and their respective contour plots provide a visual interpretation of the interaction between two factors and facilitated the identification of the optimum experimental conditions.

Table 3: Analysis of variance (ANOVA) for the response of serine production ($p < 0.05$)

Parameter	DF	Estimate	Standard error	t-value	Pr > |t|
Intercept	1	14.720000	0.031859	462.03	<.0001
X_1	1	0.240000	0.019510	12.30	<.0001
X_2	1	-0.251250	0.019510	-12.88	<.0001
X_3	1	-0.551250	0.019510	-28.26	<.0001
X_1^2	1	-0.573750	0.028717	-19.98	<.0001
X_1*X_2	1	-0.340000	0.027591	-12.32	<.0001
X_2^2	1	-0.826250	0.028717	-28.77	<.0001
X_1*X_3	1	-0.170000	0.027591	-6.16	0.0016
X_2*X_3	1	-0.612500	0.027591	-22.20	<.0001
X_3^2	1	-0.986250	0.028717	-34.34	<.0001

The canonical analysis revealed a maximum production of L-serine was 14.77 g/l when the concentrations of sucrose, $(NH_4)_2SO_4$ and biotin were set at 81.1 g/l, 29.9 g/l and 27.1 µg/l, respectively.

Validation of the models

To confirm the model adequacy for predicting the maximum L-serine production, three additional experiments in shake flasks under this optimum medium composition were performed. The mean value of L-serine concentration was 14.90 g/l, which was in excellent agreement with the predicted value (14.77 g/l).

Batch fermentation in 5 L fermenter

Fermentation was scaled up from flask to 5-liters fermenter (effective volume of 3 l). The optimal values of sucrose, $(NH_4)_2SO_4$ and biotin were 81 g/l, 30 g/l and 27 µg/l, respectively.

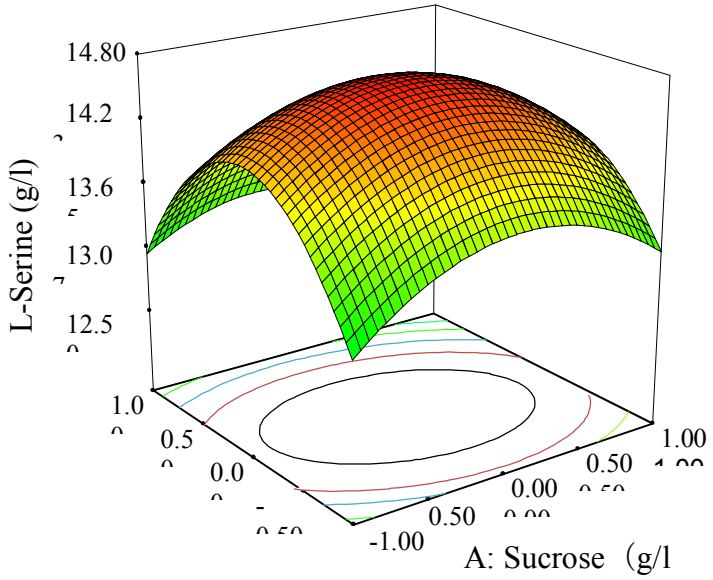

Fig 1: Response surface curve for L-serine production by *C. glutamicum* SYPS-062 showing the interaction between sucrose and $(NH_4)_2SO_4$

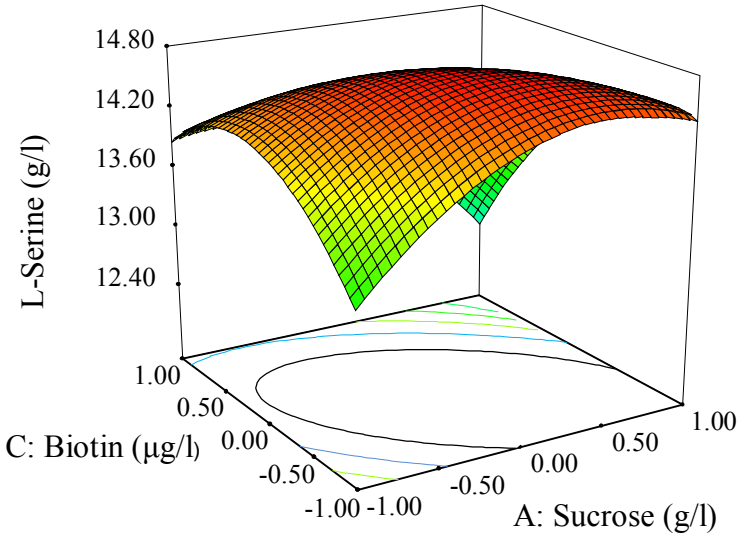

Fig 2: Response surface curve for L-serine production by *C. glutamicum* SYPS-062 showing the interaction between sucrose and biotin

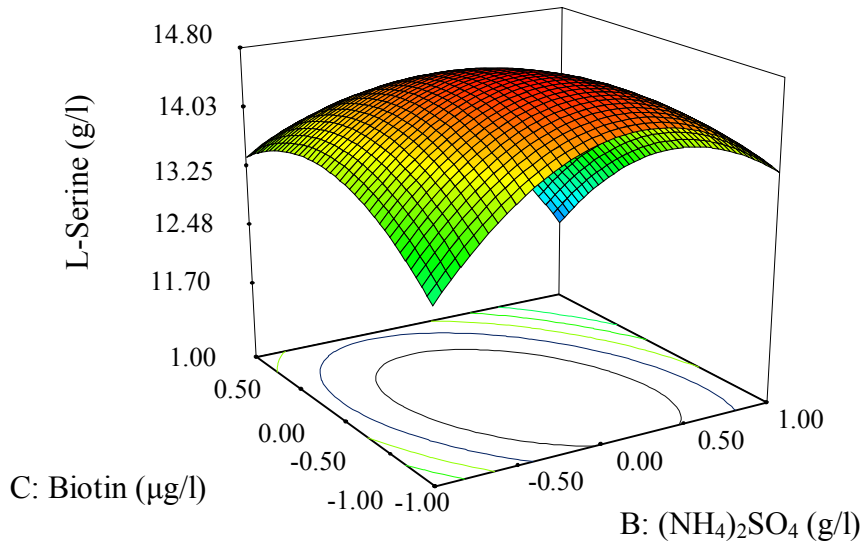

Fig 3: Response surface curve for L-serine production by *C. glutamicum* SYPS-062 showing the interaction between $(NH_4)_2SO_4$ and biotin

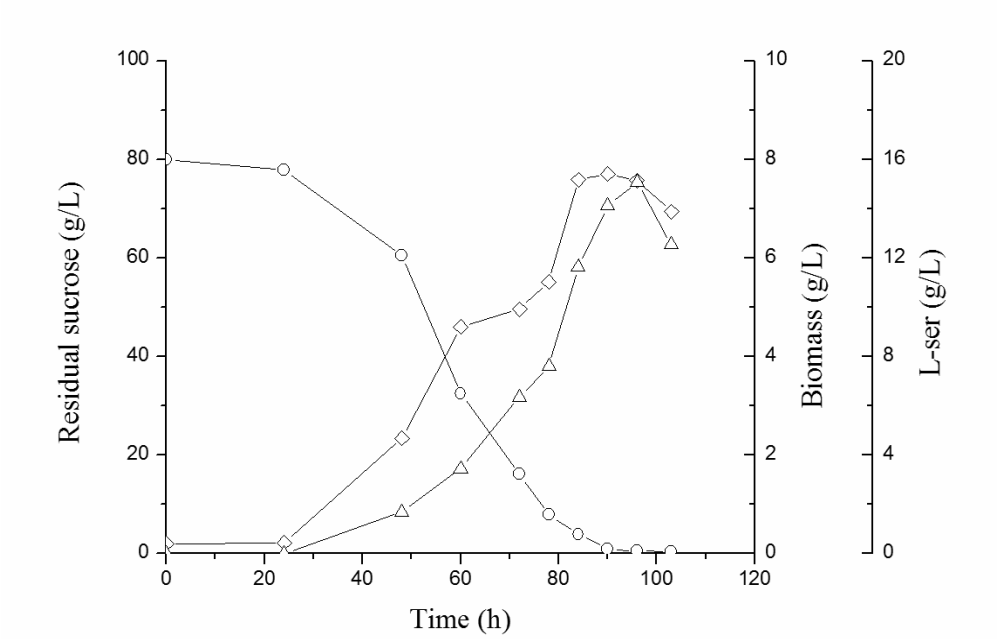

Fig 4: Fermentation kinetics of *C. glutamicum* SYPS-062 in a 5 L fermenter. (○) residual sucrose, (◇) biomass, (△) L-serine

Fig. 4 showed the profile of L-serine production using the optimized medium in the 5-liters fermenter. Under optimum conditions, the production of L-serine was 16.4 g/l.

DISCUSSION

Among the many methods for L-serine production, microbial production is considered one of the most effective methods and has been widely studied. In this study we were interested in the amino acid-synthesizing capabilities of *C. glutamicum*, which is traditionally used for the large-scale production of L-glutamate and L-lysine [5-7]. However, information on the direct fermentative production of L-serine from sugar with wild *C. glutamicum* is scanty [17,18].

Due to the key position in the precursor supply, L-serine should be regarded as an intermediate of central metabolism. It has been reported that about 15 % of glycolytic flux is directed into the L-serine biosynthetic pathway. About 7.5 % of the total carbon flux toward L-serine is utilized for cellular demand in *C. glutamicum* [19,20]. So it is difficult to accumulate L-serine in *C. glutamicum*.

L-serine accumulation can be achieved by reducing further degradation of L-serine in *C. glutamicum* [11,12]; however, the decrease in L-serine degradation leads to the decline in biomass. This suggests that L-serine biosynthesis occurred mainly at the consumption of growth, and it would be optimal to decrease the specific growth rate for L-serine accumulation.

Compared with the model strain *C. glutamicum* ATCC13032, which accumulates little L-serine, *C. glutamicum* SYPS-062 grows slowly, and the degradation of L-serine to glycine is weaker, which might be the critical reason for L-serine accumulation [21,22]. The whole genome sequencing, along with transcriptomic and proteomic studies, may provide further information in this regard. To improve L-serine accumulation, future studies should focus on the ways to coordinate the relationship between biomass and L-serine production in *C. glutamicum* SYPS-062.

CONCLUSION

Statistical optimization method for fermentation process is a powerful tool for the optimization of L-serine production by SYPS-062. Under optimal conditions, the predicted value of L-serine production can reach 14.77 g/l by canonical analysis. The results show that the predicted values agreed well with the experimental values. The results of batch fermentation also showed that the production of L-serine (16.4 g/l) can enhanced by SYPS-062 in optimized culture medium. The results of this study provide useful information and reference for L-serine industrial production.

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