Research Article

Lisheng Xu*, Furu Wu, Tingting Li, Xingtao Zhang, Qiong Chen, Bianling Jiang, and Qiuxia Xia

Ultrasound-assisted L-cysteine whole-cell bioconversion by recombinant *Escherichia coli* with tryptophan synthase

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Abstract: L-Cysteine is widely used in food, medicine, and cosmetics. In this study, a recombinant *Escherichia coli* whole-cell system with tryptophan synthase was used to complete the biological transformation of L-serine to L-cysteine, and bioconversion of L-cysteine was investigated by tryptophan synthase. The biotransformation of L-cysteine was optimized by response surface methodology. The optimal conditions obtained are 0.13 mol·L^{-1} L-serine, 75 min, 130 W ultrasound operation, where the V_{max} of tryptophan synthase is 25.27 ± 0.16 (mmol·h^{-1}·(g-cells)$^{-1}$). The V_{max} of tryptophan synthase for the biosynthesis without ultrasound is 12.91 ± 0.34 (mmol·h^{-1}·(g-cells)$^{-1}$). Kinetic analysis of the recombinant *Escherichia coli* whole-cell system with tryptophan synthase also showed that under the ultrasound treatment, the K_m values of L-cysteine biosynthesis increase from 1.342 ± 0.11 mM for the shaking biotransformation to 2.555 ± 0.13 mM for ultrasound operation. The yield of L-cysteine reached 91% after 75 min of treatment after 130 W ultrasound, which is 1.9-fold higher than no ultrasound.

Keywords: L-cysteine, ultrasound, tryptophan synthase, L-serine

1 Introduction

L-Cysteine is an amino acid that contains sulphydryl groups, and it is used in medicine, cosmetics, and food [1]. It is mainly derived from hydrolysed keratin in human and animal hair by hydrolysis and extraction. However, this preparation method leads to the unpleasant odour, and waste treatment of hydrolysed keratin is required [2]. Biocatalytic synthesis is environmentally friendly with little or no byproducts [3,4]. L-Cysteine is formed from L-serine in many microorganisms. *O*-Acetyl-L-serine is synthesized from acetyl-CoA and L-serine by L-serine *O*-acetyltransferase [5]. Enzymatic methods for producing L-cysteine include enzymatic synthesis [6]. Nakatani et al. reported that NrdH and Grx1 reduce SSC to L-cysteine. Expression of CysI and NrdH enhances L-cysteine yield [7]. Duan et al. reported the coinstantaneous cloning and expression of atcA and atcB for L-cysteine synthesis [8]. Joo et al. reported the synthesis of L-cysteine by *Corynebacterium glutamicum* with sulphur supplementation. These authors investigated the effect of combined expression of the transcriptional regulator CysR and CysE [9,10]. Enhancement by GlpE overexpression in *E. coli* for L-cysteine overproduction was successful [11]. The biosynthesis process of L-cysteine was investigated, and the microbial reactions were studied [12]. Wei et al. reported the engineering of a microorganism, *Corynebacterium glutamicum*, for the biosynthesis of L-cysteine [13]. Some microorganisms were used to synthesize L-cysteine, such as *Lactococcus lactis* [14], *Pseudomonas putida* [15], and *Mycobacterium tuberculosis* [16]. The productivity of L-cysteine synthesis by *C. glutamicum* was 290 mg^{-1} [17]. The results of a previous study show that ultrasound treatment can change the membrane permeability of microorganisms to improve the substrate reaction [18]. Zheng et al. reported that the biotransformation rate of water-soluble yeast β-glucan reached 36.2% under ultrasound [19]. Sharma et al. reported that sugar was synthesized by *Aspergillus assiutensis* VS34 under ultrasound [20]. Yao et al. observed greatly increased production of fumigaclavine C by *Aspergillus fumigatus* CY018 under ultrasound [21]. The biotransformation efficiency of astaxanthin was increased by *Phaffia rhodozyma* MTCC 7536 under ultrasonic treatment [22]. Tryptophan synthetase (EC 4.2.1.20) is a heterotetramer with an aaββ subunit structure in *Escherichia coli*. The enzyme can synthesize L-tryptophan with

* **Corresponding author: Lisheng Xu,** Department of Life and Food Science, Suzhou University, Suzhou, 234000, China, e-mail: xulisheng111@163.com
Furu Wu, Tingting Li, Xingtao Zhang, Qiong Chen, Bianling Jiang, Qiuxia Xia: Department of Life and Food Science, Suzhou University, Suzhou, 234000, China

indole and L-serine as substrates [23]. The trp B and trp A genes (or trp BA genes) coexist in the tryptophan operon of the *E. coli* genome. The activities of both subunits increase upon complex formation and are further regulated by an intricate and well-studied allosteric mechanism. The function of a subunit is to decompose indole-3-glycerol phosphate into indole and glyceraldehyde-3-phosphate, while the function of the β subunit is to synthesize L-tryptophan. A direct evolution strategy was applied to engineer tryptophan synthase from *E. coli* to improve the efficiency of L-5-hydroxytryptophan synthesis [24]. The rate of L-cysteine formation from L-serine and sodium hydrosulphide with tryptophan synthase was 47%. The yield of L-cysteine synthesized by tryptophan synthase was low. The synthesis of L-cysteine by ultrasound-assisted tryptophan synthase was rare. Our innovations gave a high yield of L-cysteine with ultrasound-assisted tryptophan synthase. In the present study, tryptophan synthase (*trp*BA-*trp*A) was expressed in *E. coli* BL21. L-Cysteine was synthesized from L-serine and sodium bisulphide using tryptophan synthase by response surface methodology (RSM) under ultrasound treatment.

2 Materials and methods

2.1 Chemicals

Yeast powder, beef extract, and peptone were purchased from Alighting Biochemical Technology Co. Ltd. (Shanghai, China). L-Serine, L-cysteine, and sodium bisulphide were purchased from Sinopharma Co. Ltd. (Wuhan, China). Agarose, IPTG, and ampicillin were purchased from Nanjing Jitian Biotechnology Co. Ltd. (Nanjing, China). Plasmid extraction kits and gel recovery kits were purchased from Aisjin Biotechnology Co. Ltd. (Hangzhou, China). Chemicals were analytical reagents.

2.2 Cloning and expression of tryptophan synthase

The tryptophan synthase gene from *E. coli* K-12 was obtained from the NCBI. *trp* BA, *trp* A were amplified using the primers P1(CCCCATATGACAACATTACTTAAC)/P2(CCCGAATTCTTAACTGCGCGTTT), P1(CCGCATATGATGGAACGCTAC)/P2(ATTCTCGAGTTAACTGCGCGT). The genes of tryptophan synthase were inserted into the pETDuet-1 plasmid to generate the pETDuet-*trp* BA-*trp* A plasmid (Figure 1a). *trp* BA and *trp* A genes were amplified by PCR and constructed with relevant plasmids. The amplified fragments were identified as *trp* BA and *trp* A genes and sequence determination. The constructed plasmids were identified by PCR to prove the correctness of the constructed genes. Then, the recombinant plasmid with the tryptophan synthase gene (*trp* BA-*trp* A) was transformed into *E. coli* BL21.

2.3 Bioconversion of L-serine to L-cysteine

To ensure that the recombinant *Escherichia coli* whole-cell system with tryptophan synthase has the ability to

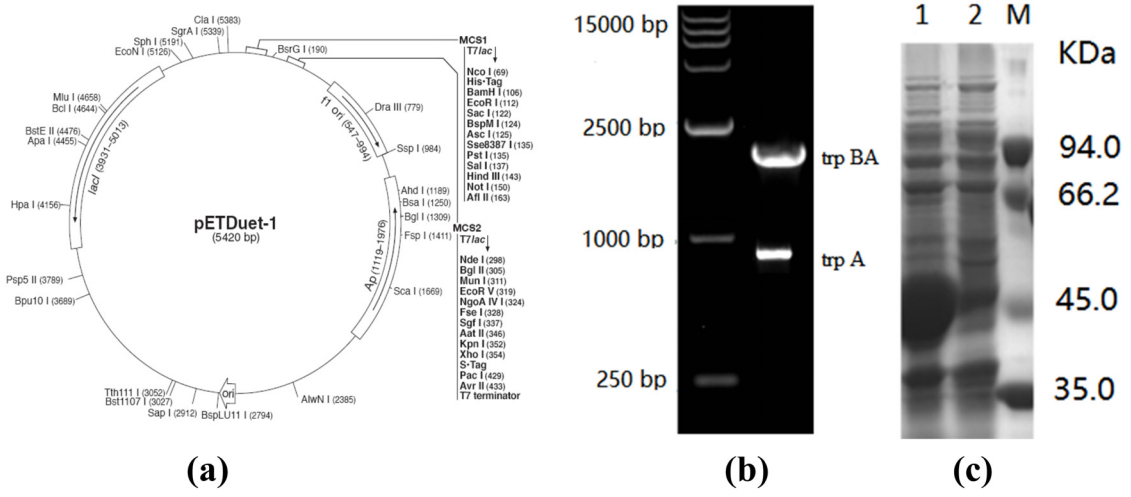

Figure 1: (a) Physical map of pETDuet-1. (b) Agarose gel electrophoresis analysis of the *trp*BA and *trp* A genes. (c) SDS-PAGE analysis of tryptophan synthase (*trp*BA-*trp* A).

biosynthesize L-cysteine from L-serine, the whole-cell system with tryptophan synthase was centrifuged at 15,000×*g* for 15 min. The reaction mixture containing 0.1 mol·L^{-1} L-serine, 0.1 mol·L^{-1} NaHS, and 10 mg·mL^{-1} of the whole-cell system with tryptophan synthase was diluted using 0.1 M PBS buffer and incubated at 37°C, pH 8.0, under ultrasound treatment. All experiments with ultrasound and the control experiment without ultrasound treatment were performed with three replicates. After the reaction of the recombinant *Escherichia coli* whole-cell system with tryptophan synthase, the supernatant of the reaction mixture was obtained for analysis using an amino acid analyser (L-8900, Japan).

2.4 Analysis of L-serine and L-cysteine

The product of L-cysteine in the reaction mixture was analysed using an amino acid analyser (L-8900, Japan) with a separation column (4.6 mm × 60 mm, 50°C, sulphonate-type cationic resin). The injection volume was 20 µL.

2.5 Ultrasound operation of bioconversion

The effect of ultrasound on L-cysteine biosynthesis was investigated. The reaction of the recombinant *E. coli* whole-cell system with tryptophan synthase was executed in an ultrasonic tank with a power series ranging from 60 to 200 W (SB-120D, China). The recombinant *Escherichia coli* whole-cell system with tryptophan synthase was added to 10 mL of the substrate containing 0.1 mol·L^{-1} L-serine, 0.1 mol·L^{-1} NaHS, and 10 mg·mL^{-1} whole cells at 37°C with ultrasound for different times.

2.6 Kinetic studies on ultrasound effects

To study the effects of L-cysteine from L-serine under ultrasound, the kinetic data of L-cysteine biosynthesis were obtained. The tryptophan synthase activity was determined under different concentrations (0.05–0.2 mol·L^{-1}) of L-serine. The kinetic constants K and V of tryptophan synthase were calculated under different concentrations.

2.7 Optimization of L-cysteine biosynthesis

The bioconversion of L-cysteine was studied by response surface methodology. Factors such as L-serine, time, and ultrasound power were selected for analysis. The levels of the variables under ultrasonic power are shown in Table 1.

Table 1: Variables and levels defined in the Box–Behnken design

Factor	Variables	Low level (−1)	High level (+1)
X_1	L-Serine	0.05	0.2
X_2	Time	30	120
X_3	Ultrasound power	60	200

The data of the recombinant *Escherichia coli* whole-cell system with tryptophan synthase were analysed via response surface methodology. All the experiments were done in triplicate. The results were presented as the mean ± SD (SPSS 22.0).

3 Results and discussion

3.1 Expression of tryptophan synthase

The complete coding region of the *trp* BA and *trp* A genes from *E. coli* was obtained (Figure 1b). The reaction was carried out in a 50 µL volume, and the mixture containing 37.4 µL of water (nuclease-free), 5 µL of 10× *Taq* buffer (Mg^{2+} free), 3 µL of $MgCl_2$ (25 mM), 1 µL of dNTP mixture (10 mM), 1 µL genomic DNA, 1 µL of each 3′ and 5′ primer, and 0.6 µL of *Taq* DNA polymerase (5 U·mL^{-1}). *E. coli* [pETDuet-*trp*BA-*trp*A/BL21(DE3)] were tested for tryptophan synthase overproduction with ampicillin (100 µg·mL^{-1}). At 6 h after IPTG induction, cells were harvested and lysed, and intracellular proteins were analysed by SDS-PAGE. Recombinant tryptophan synthase (*trp* BA-*trp* A) appeared as two intense protein bands (45 and 30 kDa) (Figure 1c). Tryptophan synthase has found applications in many fields of synthetic chemistry, in particular, for the production of amino acids. An allosteric heterodimeric enzyme in the form of an αββα complex that catalyzes the biosynthesis of L-cysteine.

3.2 Ultrasound effect on L-cysteine bioconversion

The rate of biotransformation is limited by the mass transfer of substrates or products, which is related to the properties of the reactants or products. Therefore, ultrasonic treatment can improve the efficiency of the ability of recombinant *Escherichia coli* whole-cell system with tryptophan synthase to biotransform L-serine to L-cysteine. As shown in Figure 2a, the yield of L-cysteine

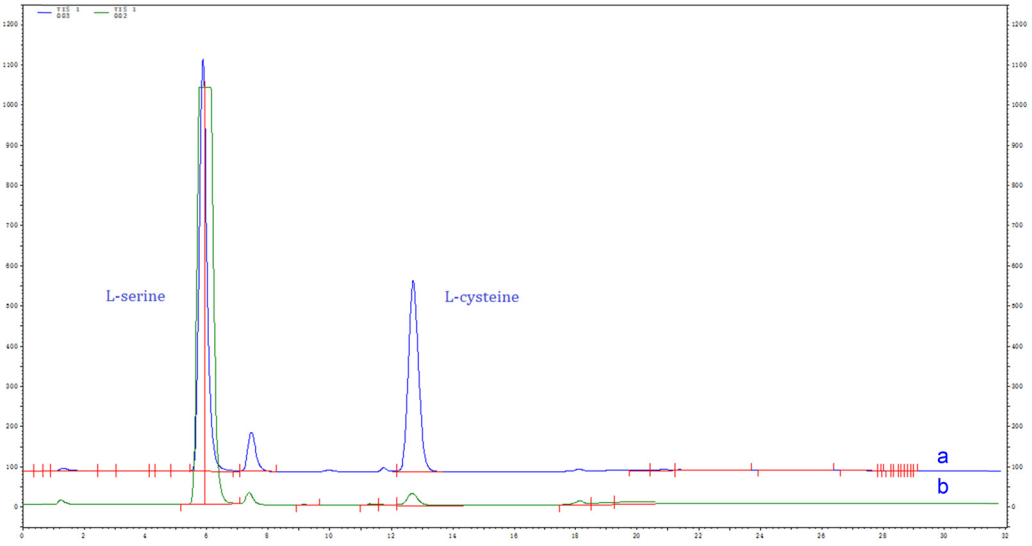

Figure 2: Analysis of the bioconversion of L-serine to L-cysteine by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase. The products were analysed with an amino acid analyser (L-8900, Japan). (a) Ultrasound treatment; (b) no ultrasound treatment (100 W, 60 min, 0.10 mol·L^{-1} L-serine).

reached 57.63% after 60 min of ultrasound treatment (100 W). However, the low yield of L-Cysteine from L-serine occurred within 60 min without ultrasonic treatment, and the yield of L-cysteine was only 13.46% after 60 min (Figure 2b). It is further suggested that ultrasonic treatment can improve the efficiency of the recombinant *Escherichia coli* whole-cell system with tryptophan synthase. Although bioconversion is the most feasible and specific method for production, the efficiency of the product conversion has some limitations, the mass transfer of the substrate through the cell membrane is among the main barrier for high bioconversion efficiency. Recently, ultrasound treatment has been widely used to enhance the efficiency of biocatalysis. It was interesting to find that a positive effect for bioconversion was observed when ultrasound operation was adopted. The acoustic energy with low-frequency ultrasound is beneficial for cell growth [25–27] and metabolite production [28–30]. Singh et al. reported that ultrasound has been used to enhance β-carotene production [31].

3.3 Catalytic kinetics of tryptophan synthase

Ultrasound with 130 W was employed in the bioconversion of L-cysteine with different concentrations of L-serine, and the reaction rates of tryptophan synthase are shown in Figure 3. When the concentration of L-serine was lower than 0.13 mol·L^{-1}, the reaction rate of tryptophan synthase increased sharply. The reaction rate of tryptophan synthase decreased when the concentration of L-serine was higher than 0.13 mol·L^{-1}. The V_{max} of tryptophan synthase was approximately 25.27 ± 0.16 (mmol·h^{-1}·(g-cells)$^{-1}$), whereas that of L-serine was approximately 0.13 mol·L^{-1}. The effect curve shows that a high level of L-serine inhibits biotransformation. To research the ultrasound effect on tryptophan synthase, experiments under different ultrasound power values were conducted. Compared with previous research results, our experimental results significantly improved the yield of L-cysteine. Ultrasound enhanced the transport of L-serine and nutrients across the cell membrane with

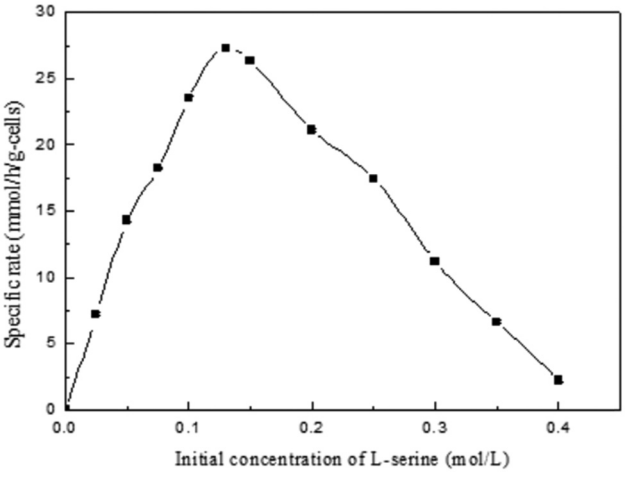

Figure 3: Screening of L-cysteine bioconversion by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase and ultrasound treatment.

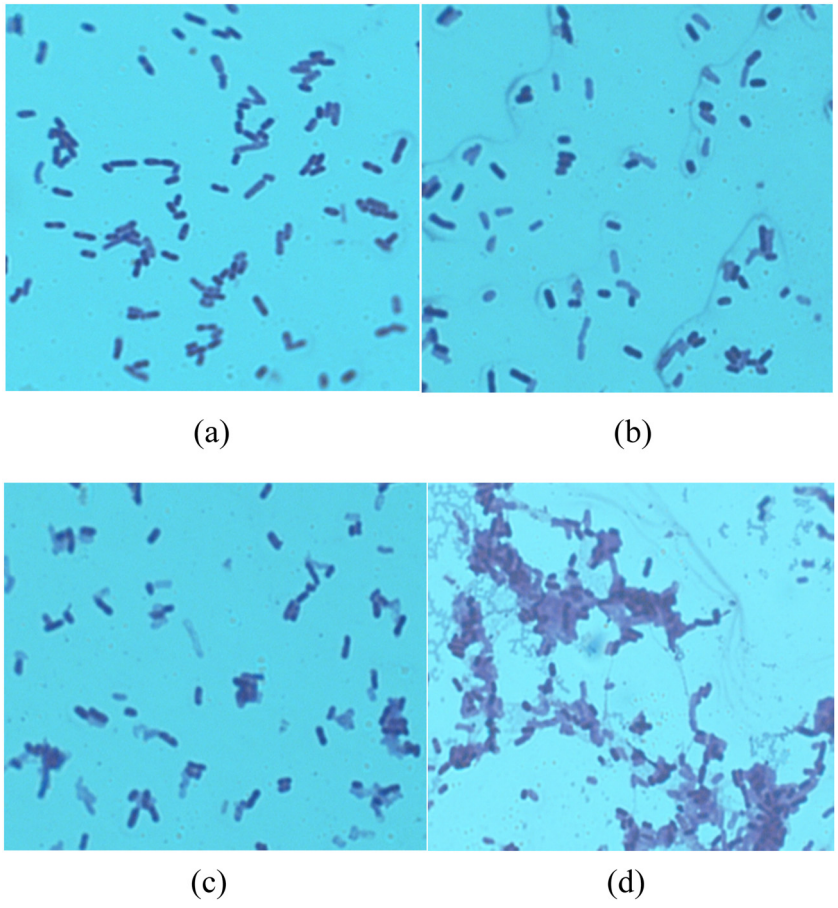

Figure 4: Microbe morphology under different ultrasonic power: (a) 0 W, (b) 100 W, (c) 200 W, and (d) 300 W.

Table 2: Box–Behnken design for L-cysteine by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase

No.	L-Serine (mol·L^{-1})	Time (min)	Ultrasound power (W)	Yield
1	0.13	75.00	60.00	0.34
2	0.13	120.68	130.00	0.81
3	0.20	120.00	200.00	0.77
4	0.13	75.00	130.00	0.89
5	0.20	120.00	60.00	0.65
6	0.13	75.00	247.73	0.57
7	0.13	75.00	130.00	0.91
8	0.13	75.00	130.00	0.89
9	0.20	30.00	60.00	0.53
10	0.13	30.00	130.00	0.34
11	0.13	75.00	130.00	0.9
12	0.05	120.00	60.00	0.23
13	0.20	30.00	200.00	0.34
14	0.05	30.00	60.00	0.44
15	0.05	120.00	200.00	0.34
16	0.25	75.00	130.00	0.65
17	0.13	75.00	130.00	0.91
18	0.13	75.00	130.00	0.91
19	0.13	75.00	130.00	0.9
20	0.05	30.00	200.00	0.23

Table 3: Analysis of variance for the selected quadratic model

Source	Sum of squares	df	Mean square	*F* value	*P*-value Prob >*F*
Model	1.12	9	0.12	8.00	0.0016
A L-serine	0.15	1	0.15	9.76	0.0108
B Time	0.14	1	0.14	8.89	0.0138
C Ultrasound power	3.44 $\times 10^{-3}$	1	3.442 $\times 10^{-3}$	0.22	0.0010
AB	0.053	1	0.053	3.40	0.0151
AC	1.125 $\times 10^{-4}$	1	1.125 $\times 10^{-4}$	7.238 $\times 10^{-3}$	0.0039
BC	0.050	1	0.050	3.19	0.0143
A^2	0.20	1	0.20	13.05	0.0048
B^2	0.14	1	0.14	8.78	0.0142
C^2	0.31	1	0.31	20.27	0.0011
Residual	0.16	10	0.016		
Lack of fit	0.16	5	0.031	467.27	<0.0001
Pure error	3.333 $\times 10^{-4}$	5	6.667 $\times 10^{-5}$		
Cor total	1.27	19			

R-Square = 0.9913; *R*-square adj = 0.9553; root mean square error = 1.4031.

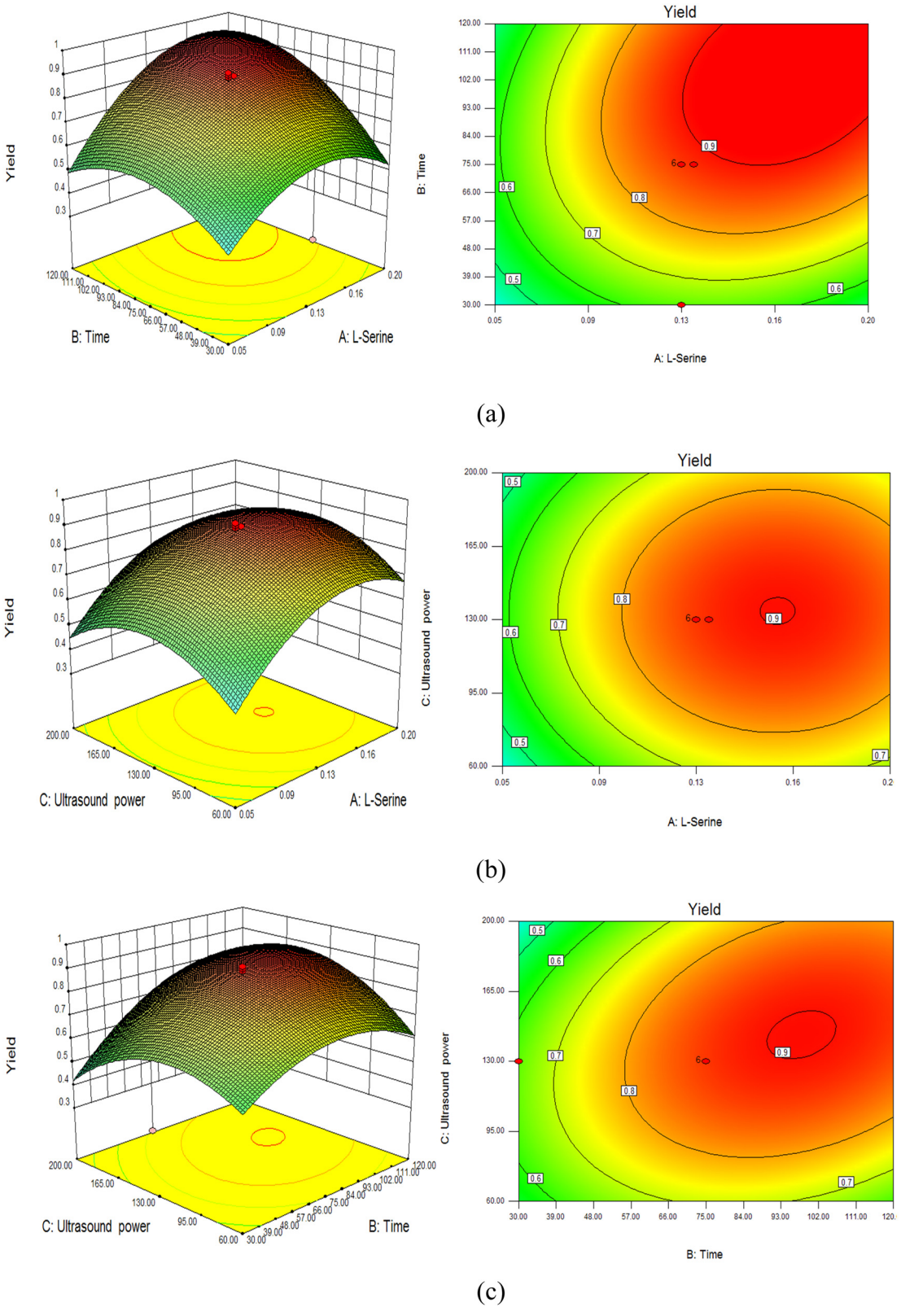

Figure 5: Response surface curves of yield for L-Cysteine by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase. (a) L-Serine and time, (b) L-serine and ultrasound power, and (c) time and ultrasound power.

Table 4: Kinetics data for L-Cysteine bioconversion by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase

Operations	V_{max} (mmol·h^{-1}·(g-cells)$^{-1}$	K_m (mM)
Shaking	12.91 ± 0.34	1.342 ± 0.11
Ultrasound (100 W)	17.81 ± 0.24	1.843 ± 0.13
Ultrasound (110 W)	19.95 ± 0.16	2.041 ± 0.11
Ultrasound (120 W)	21.18 ± 0.31	2.202 ± 0.12
Ultrasound (130 W)	25.27 ± 0.16	2.555 ± 0.13
Ultrasound (140 W)	23.41 ± 0.12	2.435 ± 0.11
Ultrasound (150 W)	18.27 ± 0.18	1.855 ± 0.13
Ultrasound (160 W)	16.11 ± 0.16	1.655 ± 0.13
Ultrasound (170 W)	15.15 ± 0.11	1.584 ± 0.15
Ultrasound (200 W)	13.24 ± 0.51	1.334 ± 0.12

tryptophan synthase [32,33]. The cells of the recombinant *E. coli* whole-cell system with tryptophan synthase after the optimal ultrasound treatment were observed under a microscope. Microbe morphology remains intact at low ultrasonic power (0, 100, and 200 W; Figure 4a–c, respectively). However, the microbe morphology was destroyed under high ultrasonic power (300 W, Figure 4d).

3.4 Experimental design

Parameters for the optimization study were concentration of L-serine, time and ultrasound power. The concentration of L-serine, time and ultrasound power affected the activity of tryptophan synthase. L-Serine (0.05–0.2 mol·L^{-1}), time (30–120 min), and ultrasound power (60–200 W) were selected as the process variables (Table 1). All the experiments were done in triplicate. The Box–Behnken design for L-cysteine by the recombinant *Escherichia coli* whole-cell system with tryptophan synthase is listed in Table 2. A second-order polynomial equation of tryptophan synthase is as follows:

$$Y = 0.92 + 0.21X_1 - 0.18X_2 + 0.34X_3 - 0.011X_1X_2 - 0.22X_2X_3 - 0.011X_1X_3 - 0.061X_1^2 - 0.34X_2^2 - 0.15X_3^2 \quad (1)$$

where Y is the yield of L-cysteine and X_1, X_2, and X_3 are L-serine (0.05–0.2 mol·L^{-1}), time (30–120 min), and ultrasound power (60–200 W). The results showed that all factors (L-serine, time, and ultrasound power) had effects on the yield of L-cysteine (Table 2). To improve the biosynthesis of L-cysteine, the effects of different reaction conditions on L-cysteine production were investigated under different ultrasound power values. Among all the variables, L-serine concentration, time, and ultrasound power had effects on the biosynthesis of L-cysteine. Analysis of variance for the selected quadratic model is

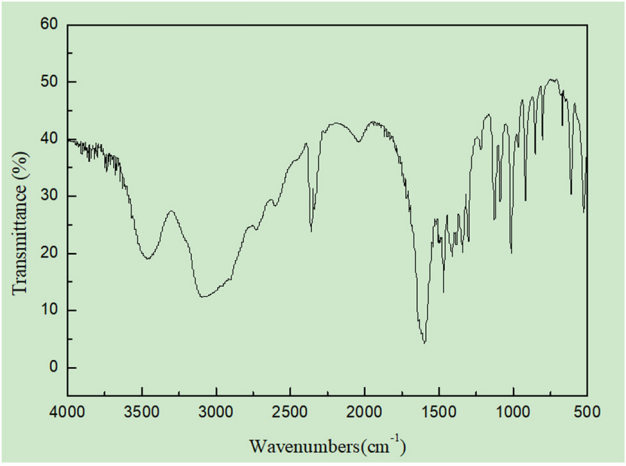

Figure 6: FTIR spectra of (a) L-serine and (b) L-cysteine.

shown in Table 3. L-Cysteine biosynthesis was found to be affected by the ultrasound power, L-serine level, and the length of time (Figure 5). The effects of substrate concentration, time, and ultrasonic on product concentration were very significant and had similar significant effects. The product concentration increased first and then decreased with the increase of substrate concentration, time, and ultrasonic. The optimal bioconversion conditions for L-cysteine by tryptophan synthase were obtained. The optimal conditions obtained are 0.13 mol·L^{-1} L-serine, 75 min, and 130 W, where V_{max} of tryptophan synthase is 25.27 ± 0.16 (mmol·h^{-1} per g-cells). The V_{max} of tryptophan synthase for the biosynthesis without ultrasound is 12.91 ± 0.34 (mmol·h^{-1}·(g-cells)$^{-1}$) (Table 4). The predicted yield of L-cysteine reached 91.7% after 75 min of treatment after 130 W ultrasound. The real yield of L-cysteine reached 91% after 75 min of treatment after 130 W ultrasound, which is 1.9-fold higher than no ultrasound.

3.5 Preparation of L-cysteine

The reaction mixture (1 L) was centrifuged at 6,000 rpm for 20 min to remove the bacterial cells. The supernatant was adjusted to pH 1.0 with 6 mol·L^{-1} hydrochloric acid. H_2S was removed by heating. The reaction mixture was decolorized by activated carbon, and the filtrate was adjusted to pH 5.0 by NaOH (5 mol·L^{-1}). L-Cysteine was oxidized through air in the filtrate and was washed with pure water. Then, the crude L-cystine was dried and decolorized by acid solution, crystallized, and washed. L-Cysteine was prepared by electrolytic reduction of L-cystine and dried to yield 14.26 g L-cysteine. The infrared spectrum of L-cysteine included the spectra of sulphydryl groups showing vibrational absorption (2,500–2,600 cm^{-1}) (Figure 6b). The infrared spectrum of L-serine and L-cysteine included the spectra of amide groups showing vibrational absorption (1,500–1,800 cm^{-1}) (Figure 6a and b).

4 Conclusion

The effects of ultrasound treatment on the biosynthesis of L-cystine by a recombinant *E. coli* whole-cell system with tryptophan synthase were investigated for the first time. The optimal conditions obtained are 0.13 mol·L^{-1} L-serine, 75 min, and 130 W, where the V_{max} of tryptophan synthase is 25.27 ± 0.16 (mmol·h^{-1}·(g-cells)$^{-1}$). The V_{max} of tryptophan synthase for the biosynthesis without ultrasound is 12.91 ± 0.34 (mmol·h^{-1}·(g-cells)$^{-1}$). These results indicated that ultrasound treatment of the recombinant *E. coli* system with tryptophan synthase was useful for industrial L-cysteine biosynthesis.

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