Advances in the Biosynthesis of L-Cysteine

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L-Cysteine is a widely used unique sulfur-containing amino acid with wide application in the food, pharmaceutical, and agricultural industries.

Keywords: L-cysteine ; Escherichia coli ; metabolic engineering ; synthetic biology

1. Introduction

L-cysteine is a sulfur-containing amino acid that plays an important role in the folding of proteins, has a high redox activity in cellular metabolism, is a catalytic residue for a variety of enzymes, and is a sulfur donor compound that is required for the synthesis of Fe/S clusters, biotin, coenzyme A, and thiamine [1][2][3]. In addition to its roles in cellular metabolism, L-cysteine plays a variety of roles in metal binding, catalytic activity, and redox and has a vast array of industrial applications in the production of food, cosmetics, pharmaceuticals, and animal feed [4][5][6].

Chemical hydrolysis of proteins, which are typically extracted from the keratin of animal hair such as feathers, pig hair, etc., is the traditional method of industrial production of L-cysteine [7]. However, this method not only consumes a large amount of hydrochloric acid, but it also causes an unpleasant odor and wastewater treatment problems, which have a significant impact on the environment [7]. To avoid the environmental hazards of this method, scientists have explored biotechnological approaches to synthesize L-cysteine as an alternative to chemical hydrolysis. Fermentation and enzymatic biotransformation are the two most prominent biotechnological methods [8][9]. However, due to the presence of generated L-cysteine products that inhibit the activity of the enzymes, the enzyme bioconversion method presents difficulty in solving the problems of low yield and high cost.

Although fermentation offers a number of advantages, the design and construction of efficient microbial cell factories for fermentative production of L-cysteine remains challenging due to the high toxicity of L-cysteine and the complex regulation of its synthetic pathway [10]. The efficient production of L-cysteine on an industrial scale has not yet been achieved, which is a major challenge for the industrialization of L-cysteine [11][12]. Numerous microorganisms, including bacteria such as *E. coli*, *C. glutamicum*, and *Pantoea ananatis*, have been engineered to produce L-cysteine due to the rapid development of systems metabolic engineering and synthetic biology [13][14][15][16][17]. In comparison to other bacteria, *E. coli* has a rapid growth rate and more developed genetic engineering techniques, whereas *C. glutamicum* is a non-pathogenic, industrial microorganism with developed fermentation technology that is extensively employed in food processing and other industries [13][18]. Therefore, *E. coli* and *C. glutamicum* are the two most studied chassis cells that directly produce L-cysteine from glucose [19][20][21].

2. Advances in the Biosynthesis of L-Cysteine

2.1. Enzyme Biotransformation—Asymmetrical Hydrolysis of DL-2-amino-Δ^2-thiazoline-4- Carboxylic Acid

Since L-cysteine is traditionally obtained by hydrolyzing animal hair, the extraction of 1 kg of L-cysteine requires approximately 10 kg of animal hair and 2.7 kg of hydrochloric acid, a process that not only has a low yield but also produces foul odors and wastewater, causing severe environmental damage [22]. The transformation method uses *Pseudomonas* to enzymatically convert DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) to L-cysteine. This method of converting DL-ATC to cysteine involves three enzymes: ATC racemase, L-ATC hydrolase, and S-carbamoyl-L-cysteine hydrolase [23][24]. The complete procedure consists of three stages (**Figure 1**): (i) conversion of D-ATC to L-ATC by ATC racemase, (ii) ring-opening of L-ATC by L-ATC hydrolase to produce N-carbamoyl-L-cysteine (L-NCC), and (iii) final hydrolysis of L-NCC to L-cysteine by S-carbamoyl-L-cysteine hydrolase [25].

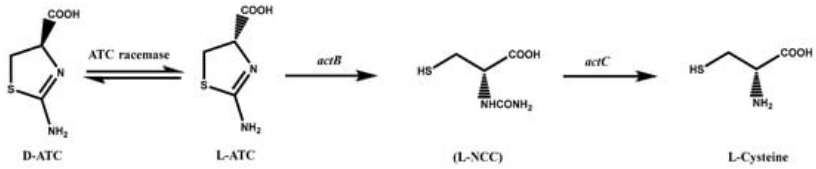

Figure 1. A metabolic pathway of DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) to L-cysteine via *N*-carbamyl-L-cysteine (L-NCC) in *Pseudomonas* species. *atcB* gene encoding L-ATC acid hydrolase; *atcC* gene encoding L-NCC amidohydrolase.

Both L-ATC hydrolase (*atcB*) and S-carbamoyl-L-cysteine hydrolase (*atcC*) genes originated from *Pseudomonas* sp. strain BS. After sequencing by Japanese scientists, the amino acid sequence of the *atcC* gene product was found to be highly homologous to L-*N*-carbamoylase from other bacteria, but the amino acid sequence of the *atcB* gene was novel [8]. *AtcB* was initially identified as a gene encoding an enzyme that catalyzes the thiazoline ring-opening reaction and does not share a high degree of homology with previously described enzymes [26].

Enzymatic bioconversion is to some extent environmentally friendly and has lower energy consumption than hydrolysis of animal hair. However, the high toxicity of L-cysteine inhibits enzyme activity, leading to low efficiency and relatively high cost [8][9].

2.2. Biological Fermentation Methods

2.2.1. L-Cysteine Biosynthesis in *E. coli*

It is well known that in most microorganisms and plants, L-serine is the precursor substance for the synthesis of L-cysteine. The biosynthetic pathway of L-cysteine has been widely reported after many years of research [4][27][28]. In gut bacteria, L-serine is synthesized via a three-step pathway from the glycolytic intermediate 3-phosphoglycerate, and L-cysteine is synthesized via a two-step pathway from L-serine [28]. Firstly, the glycolytic intermediate 3-phosphoglycerate is converted to L-serine by a three-step reaction catalyzed by 3-phosphoglycerate dehydrogenase (PGDH), phosphoserine aminotransferase (PAST), and phosphoserine phosphatase (PSP) (**Figure 2**) [27].

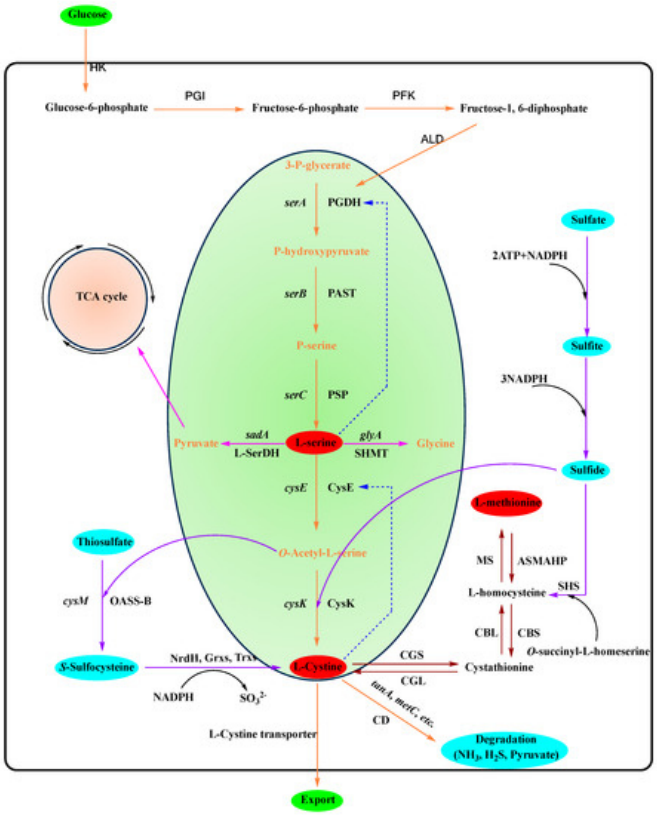

Figure 2. The metabolic pathway of L-cysteine in *E. coli* and *C. glutamicum*. Brown arrows refer to metabolic pathways in *P. aeruginosa*. The solid purple line indicates the conversion of L-serine to other productsDashed lines represent feedback inhibition. The solid orange line indicates the major metabolic pathway of L-cysteine. The solid purple line indicates the source of the sulfide. Italicized fonts on the same arrow line indicate genes encoding corresponding enzymes. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; ALD, aldolase; PGDH, 3-phosphoglycerate dehydrogenase; PAST, phosphoserine aminotransferase; PSP, phosphoserine phosphatase; CysE, serine *O*-

acetyltransferase; CysK, cysteine synthase; L-SerDH, L-serine dehydratase; SHMT, serine hydroxymethyl transferase; CD, L-cysteine desulfhydrases; OASS-B, *O*-acetyl-L-serine sulfhydrylase-A; NrdH and Grxs, glutaredoxins; Trxs, thioredoxins; CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; MS, methionine synthase; SHS, O-succinyl-L-homoserine sulfhydrylase; ASMAHP, S-adenosylmethionine synthase-methyltransferases-S-adenosylhomocysteine hydrolase pathway; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase.

Scientists have conducted studies to address the aforementioned causes of L-cysteine biosynthesis blockage. **Table 1** summarizes the research progress on the fermentation synthesis of L-cysteine by different engineering strains.

Table 1. Progress of fermentation synthesis of L-cysteine by different engineered strains.

Bacterial Strain	Metabolic Strategy	L-Cysteine Production (g/L)	Productivity (g/(L·h))	References
***E. coli* JM240**	**Enhancing biosynthesis**	**0.03**	**/**	[29]
***E. coli* JM39**	**Enhancing biosynthesis**	**0.20**	**0.003**	[30]
***E. coli* W3110**	**Enhancing excretion**	**0.07**	**0.003**	[31]
***E. coli* W3110**	**Enhancing excretion**	**0.15**	**0.007**	[32]
***E. coli* JM39**	**Enhancing biosynthesis and weakening degradation**	**0.60**	**0.013**	[33]
***E. coli* MG1655**	**Enhancing biosynthesis and excretion and weakening degradation**	**1.20**	**0.025**	[34]
***E. coli* BW25113**	**Enhancing biosynthesis and excretion**	**1.23**	**0.026**	[35]
***E. coli* BW25113**	**Enhancing biosynthesis and excretion/weakening degradation**	**1.72**	**0.024**	[20]
***E. coli* JM109**	**Enhancing the sulfur conversion rate**	**7.50**	**0.341**	[14]
***E. coli* BW25113**	**Enhancing biosynthesis and thiosulfate assimilation and weakening degradation**	**8.34**	**0.321**	[10]
***E. coli* W3110**	**Balancing carbon and sulfur module conversion rate**	**11.94**	**0.254**	[36]
***C. glutamicum* IR33**	**Enhancing biosynthesis**	**0.29**	**0.004**	[37]
***C. glutamicum* ATCC13032; *C. glutamicum* ATCC21586**	**Enhanced sulfur metabolism in biosynthesis**	**0.06**	**0.004**	[27]
***C. glutamicum* NBRC12168**	**Enhancing biosynthesis and weakening degradation**	**0.20**	**0.017**	[16]
***C. glutamicum* CYS**	**Enhancing biosynthesis and excretion**	**0.28**	**0.014**	[19]
***C. glutamicum* ATCC13032**	**Enhancing precursor accumulation and weakening degradation**	**0.95**	**0.026**	[15]
***C. glutamicum* Cys -10**	**Enhancing biosynthesis, excretion, and sulfur metabolism and weakening degradation**	**5.92**	**0.082**	[38]
Pantoea ananatis	**Weakening degradation and educing efflux**	**2.20**	**0.079**	[17]

2.2.2. L-Cysteine Biosynthesis in *C. glutamicum*

The metabolic pathway of L-cysteine in *C. glutamicum* is generally the same as that of *E. coli*, and L-serine is also synthesized from the glycolytic intermediate 3-phosphoglyceric acid via a three-step pathway, followed by further conversion to L-cysteine via CysE and CysK [39]. However, wild-type *C. glutamicum* is subjected to a feedback mechanism that produces almost no L-cysteine. In order to allow *C. glutamicum* to produce L-cysteine, the *cysE* gene (the gene is insensitive to feedback inhibition by L-cysteine) encoding an alteration of CysE in the *E. coli* Met256Ile mutant was introduced into *C. glutamicum*, which produces approximately 0.29 g/L of L-cysteine [37]. CysR is a transcriptional regulator that regulates sulfur metabolism in L-cysteine biosynthesis, and overexpression of the *cysR* gene in *C.*

glutamicum resulted in a 2.7-fold higher intracellular sulfide concentration than that of the control strain (empty pMT-tac vector), and overexpression of the *cysE*, *cysK*, and *cysR* genes in *C. glutamicum* resulted in a 3-fold higher L-cysteine production than that of the control [27].

2.2.3. L-Cysteine Biosynthesis in Other Bacteria

It is reported that in addition to *E. coli* and *C. glutamicum*, several other microbial bacteria have been reported to produce L-cysteine, such as *Saccharomyces cerevisiae*, *Lactococcus lactis*, *Mycobacterium tuberculosis*, and *Pseudomonas* species, which can synthesize L-cysteine from L-methionine by the reverse transsulfuration pathway [40][41][42][43][44]. However, it was later demonstrated that L-cysteine synthesis in *Saccharomyces cerevisiae* is exclusively by L-cystathionine β-synthase (CBS; EC 4.2.1.22) and L-cystathionine γ-lyase (CGL; EC 4.4.1.1) via cystathionine [45][46].

3. Metabolic Engineering Strategies for L-Cysteine Biosynthetic Pathway

3.1. Enhanced Accumulation of Precursor L-Serine

The precursor of L-cysteine is L-serine, and enhanced accumulation of L-serine is essential to increase the yield of the target amino acid L-cysteine. Firstly, the metabolic flux from 3-phosphoglycerate to L-serine can be improved, or feedback inhibition caused by high L-serine titers can be reduced, and enzymes in the L-serine biosynthesis pathway have to be regulated to be overexpressed or genetically altered [47]. Secondly, it is important to prevent the degradation of L-serine, which is not only a precursor of L-cysteine but also a precursor of glycine, L-alanine, and L-valine and is even necessary for protein synthesis, phospholipid synthesis, and C1 unit production [48][49]. Therefore, the enzymes of the relevant degradation pathways must be inhibited, or the corresponding genes must be knocked out to ensure that L-serine is converted to L-cysteine as much as possible [50][51][52].

3.2. Increase the Accumulation of Sulfides

Sulfur in L-cysteine, found in plants and bacteria, is primarily derived from sulfate. The sulfur assimilation processes of L-cysteine in *E. coli* are categorized into two main pathways: sulfate assimilation and thiosulfate assimilation pathways. In the sulphate assimilation pathway, *O*-acetyl-L-serine sulfhydrylase A catalyzes the conversion of OAS and sulfide (S^{2-}) into L-cysteine [14]. In the thiosulfate assimilation pathway, the entry of inorganic sulfur into the cell is achieved through the thiosulfate ABC transporter proteins, which are encoded by the genes *cysU*, *cysW*, *cysA*, and *sbp*. Thiosulfate is not only less energy intensive than sulfate but also provides a more efficient source of sulfur, making it the current optimal choice [35][53][54]. As thiosulfate is transported into the cytoplasm, it reacts with OAS via the catalytic action of *O*-acetyl-L-serine sulfhydrylase B, with the consequent formation of *S*-sulfo-L-cysteine (SSC). SSC is ultimately converted to L-cysteine by the enzymes NrdH and GrxA [55]. Thus, optimizing the thiosulfate pathway can enhance sulfide accumulation, thereby enhancing L-cysteine production.

3.3. Decrease the Degradation of L-Cysteine

L-cysteine degradation in *E. coli* is primarily catalyzed by the enzyme CD, which catalyzes L-cysteamine degradation to pyruvate, ammonia, and sulfide [50]. Currently, it has been determined that the degradation activity of the enzyme CD is encoded by the *aceD* gene, and disruption of the *aceD* gene, which encodes the degradation activity of the CD enzyme, has been reported to slow down the degradation of L-cysteine [16][50]. However, the concentration of L-cysteine continues to decrease during the stationary growth phase [16].

3.4. Enhance the Ability of Cells to Output L-Cysteine

Advances in synthetic biology and systems metabolic engineering have provided various tools and techniques for engineering microbial strains for the production of bio-based chemicals [56]. Among these methods, efficient output of target products is a very promising method that can improve the robustness and efficiency of microbial cell factories, providing significant assistance in improving substrate absorption, overcoming metabolic inhibition, protecting cells from toxic compounds, and providing assistance for efficient fermentation production of target products [57][58].

4. Challenges and New Ideas in the Biological Production of L-Cysteine

4.1. In Vitro Metabolic Pathways

In vitro metabolic engineering is an alternative production technology for fermentation that utilizes a cascade reaction composed of purified/semi purified enzymes for chemical production [59][60]. During production, there are no living cells in

in vitro metabolic engineering, so there is no impact of toxic products on cell activity [61]. Recently, it was reported that L-cysteine was produced by in vitro metabolic engineering and that 28.2 mM L-cysteine was produced from 20 mM glucose with a molar yield of 70.5%, surpassing the greatest yield of L-cysteine produced by fermentation at present [62]. In this reaction, the genes for the enzymes needed to synthesize L-cysteine in vitro are put together in a single expression vector and co-expressed in a single strain.

4.2. Explore New Raw Materials

Glycerol is one of the main by-products in biodiesel production, and according to statistics, every 10 kg of biodiesel produces approximately 1 kg of crude glycerol by-products, so utilizing glycerol would be a great asset [63][64]. With continuous research, the conversion of glycerol into high-value-added products has become more competitive [65][66]. The production of L-cysteine from *E. coli* using glycerol instead of glucose as substrate has been recently reported to have been achieved [21]. The metabolic pathway from glycerol to L-cysteine is shorter, and the carbon atom economy is more favorable compared to glucose [67][68].

4.3. Utilization of New Technologies

The desire for efficient microbial cell factories requires constant regulation of metabolism and modulation of gene expression, which is often time-consuming [69]. The emergence of new technologies, such as CRISPR-Cpf1 gene editing and high-throughput screening (HTS), has provided a significant boost to metabolic engineering as a result of the development of synthetic biology [70][71]. In recent years, an efficient CRISPR-Cpf1 genome-editing system has been established in *C. glutamicum*, which holds promise for improving L-cysteine production [72][73][74].

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