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Fermentative Production of L-Theanine in *Escherichia coli* via the Construction of an Adenosine Triphosphate Regeneration System

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Abstract: Theanine is a non-protein amino acid that is highly represented in tea plants and is one of the delicious ingredients in tea. In recent years, the global market demand for theanine has continued to rise, and the industry has developed rapidly. Here, we designed and constructed a promising pathway in *Escherichia coli* to produce L-theanine. This biosynthesis pathway employs four enzymes to achieve the production of L-theanine. This route involves the co-expression of four functional enzymes: γ-glutamylmethylamide synthetase (GMAS) from *Methyloversatilis* universalis, polyphosphate kinase (PPK) from *E. coli*, alanine transaminase from *Bacillus subtilis* (BsAld), and alanine decarboxylase from *Camellia sinensis* (CsAlaDC). Polyphosphate kinase from *Escherichia coli* was overexpressed in *E. coli* FD02, constructing an ATP regeneration system that increased the titer of L-theanine by 13.4% compared to *E. coli* FD01. A titer of 334 mg/L of L-theanine was produced via engineering strain FD03 in shake flasks. Moreover, glutamine permease from *Saccharomyces cereviside* (GNP1) was overexpressed in *E. coli* FD04, and the L-theanine titer increased by 14.7%. Finally, 2.9 g/L of L-theanine was obtained via FD04 in a 1 L bioreactor. In addition, the molecular docking results indicated that L-glutamate could bind to the hydrophobic cavity of GMAS due to the formation of hydrogen bonds and hydrophobic interactions with the surrounding amino acid residues.

Keywords: L-theanine; γ-glutamylmethylamine synthase; alanine decarboxylase; ATP regeneration system; transporter

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1. Introduction

The recent developments in bioinformatics and synthetic biology have enabled a further understanding of enzymatic reactions and the establishment of effective industrial biotransformation systems. Recently, many important high-value chemicals have been produced in microorganisms through synthetic biology and bioinformatics, such as ferulic acid [1], quercetin [2], carminic acid [3], 5-aminovalerate [4], 3-hydroxycadaverine [5], vanillin [6], 2-hydroxyglutarate [7], and 5-hydroxyvaleric acid [8].

Theanine is a unique flavor substance in tea leaves. As a representative non-protein amino acid in tea leaves, it is one of the umami components of tea leaves [9]. The chemical molecular formula of L-theanine was first isolated from green tea in 1949 [10]. Theanine found naturally in tea is predominantly in the L-theanine, whereas synthetic theanine is a mixture of both the D- and the L-theanine [11]. Theanine is widely used in food, medicine, healthcare, daily cosmetics, and other industries [12–14] (Figure 1). L-theanine is extensively used as a food additive to enhance the taste and flavor of products [15]. As a health supplement, it has various functions, including promoting relaxation, preventing

and treating Alzheimer's disease [16], and improving sleep quality [17]. Theanine possesses the characteristics of umami, sweetness, and taste, and is suitable for enhancing the palatability of various foods [18]. Theanine has a fresh sweetness and caramel flavor similar to monosodium glutamate, which can relieve bitterness and enhance the freshness of tea [19]. Theanine is most commonly used in tea drinks, baked snacks, and desserts [20].

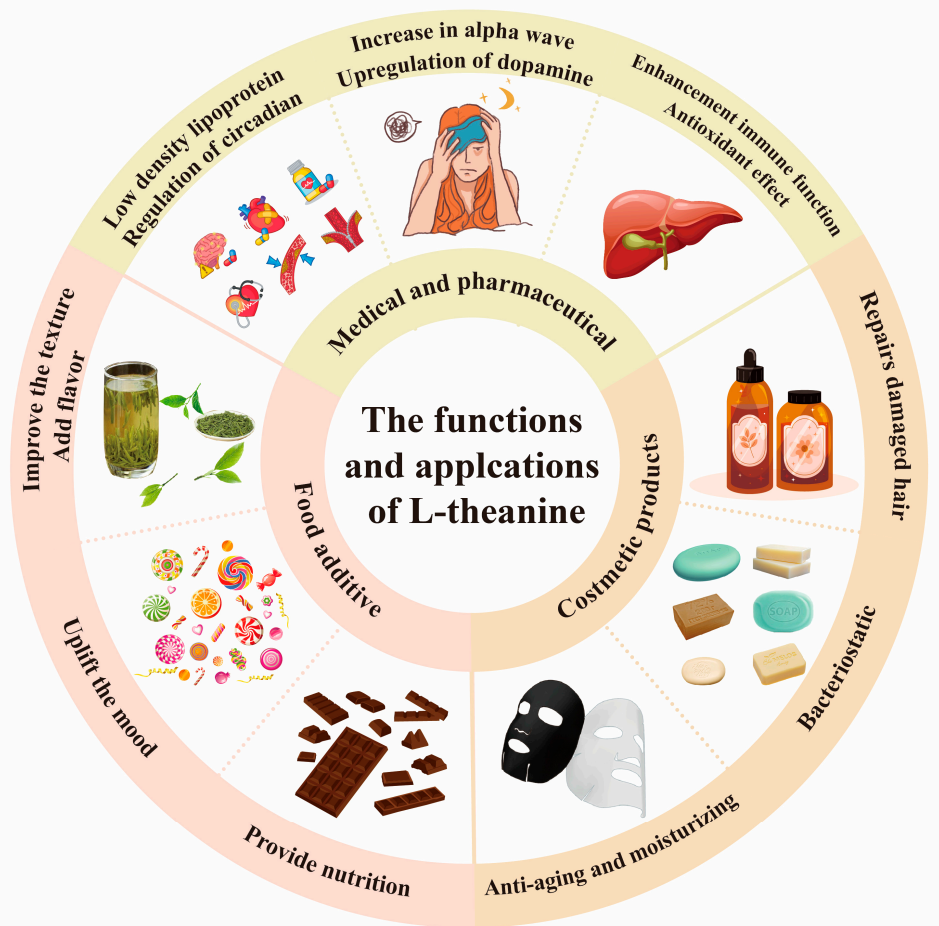

Figure 1. The functions and applications of L-theanine.

In 2020, the market output value of L-theanine surpassed USD 50 million [21]. The theanine market is expected to be worth more than USD 60 million by 2025 [22]. At present, due to the limited sources of theanine, the many extraction steps, the low extraction rate, and the high cost, the application range of theanine is greatly limited [19]. Early studies mainly focused on using chemical methods for synthesis or purification, resulting in products containing effective L-theanine decomposing into the toxic substance D-theanine. This not only resulted in a difficult separation and complicated procedures but also incurred high costs and unsatisfactory economic benefits [23–25]. In contrast, the microbial synthesis of L-theanine has the advantages of sustainability and environmental protection, while significantly adding economic benefits. Therefore, it is a promising strategy for the production of L-theanine [26].

The microbial synthesis pathways of L-theanine can be mainly divided into two routes: the glutamate-mediated pathway and the glutamine-mediated pathway [27,28]. In the glutamate-mediated pathway, glutamate and ethylamine are used as precursors, and ATP is needed to provide energy simultaneously [29]. Yang et al. [30] screened a γ-glutamylmethylamide synthetase (GMAS) from *Methylovorus mays*. Through the optimization of the protein expression and reaction conditions, 34.49 g/L of L-theanine was successfully produced. Fan et al. [31] achieved a high L-theanine production of 70.6 g/L by heterologously introducing GMAS from *Paracoccus aminovorans* in a 5 L bioreactor through

fed-batch fermentation. Yao et al. [32] utilized an engineered γ-glutamylcysteine synthetase (γ-GCS) to synthesize L-theanine. Through multiple rounds of directed evolution, the best-performing mutant enzyme, 13B6, exhibited a 14.6-fold increase in L-theanine production and a 17.0-fold increase in catalytic efficiency for ethylamine.

The glutamine-mediated pathway utilizes γ-glutamyl transfer reactions with glutamine and ethylamine as precursors [33]. In the glutamine-mediated synthesis pathway of L-theanine, glutamine synthetase (GLS) and γ-glutamyltransferase (GGT) play a primary catalytic role by catalyzing the formation of L-theanine from glutamine and ethylamine. Unlike the glutamate-mediated synthesis pathway, this pathway does not require ATP. Zhang et al. [15] cloned and expressed the γ-glutamyltranspeptidase from *Bacillus amyloliquefaciens* in *B. subtilis*, conducting a biosynthetic reaction using live cells. Finally, 190 mM of L-theanine was obtained through a combination of mutagenesis and promoter screening. On the other hand, Li et al. [34] employed a cell-free reaction with purified enzymes, using salt-tolerant mutants of the γ-glutamyltranspeptidase variant (V319A/S437G) to enhance the enzyme catalytic activity, which led to a significant increase in the yield of L-theanine from 58% to 83%. Yang et al. [35] conducted a cell-free reaction combined with carrier immobilization utilizing purified enzymes, which produced over 70 g/L of L-theanine within 18 h through a fed-batch conversion process. Sun et al. [36] isolated endophytic bacteria from tea plants and successfully produced L-theanine. Although the titer of L-theanine was only 31.875 μg/L, this indirectly suggests the existence of a microbial synthesis pathway for the production of L-theanine that does not require the addition of ethylamine. Table 1 presents the strain types, engineered strategies, substrate types, and L-theanine production titers and yields.

Table 1. The strain types, engineered strategies, substrates, and L-theanine production titers and yields.

Host	Engineered Strategy	L-Theanine Titer (g/L)	L-Theanine Yield (g/g)	Substrate	Reference
E. coli	Enzyme-catalyzed reaction	33.0	0.95	Glutamine	[15]
E. coli	Whole-cell catalytic reaction	16.5	0.66	Glutamate	[30]
E. coli	Engineering of a one-step fermentation pathway from sugar and ethylamine	70.6	0.42	Glucose	[31]
E. coli	Optimization of PPK to reduce ATP consumption	30.4	0.871	Sodium glutamate	[32]
E. coli	Point mutation, pure enzyme catalysis	26.1	0.83	Glutamine	[34]
B. subtlis	Random mutagenesis and high-throughput screening	70.6	0.67	Glutamine	[35]
E. coli	Enzyme-catalyzed reaction	78.3	0.94	Glutamine	[37]
C. glutamicum	Batch make-up fermentation strategy	42.0	0.196	Glucose	[38]
E. coli	Expression of GS	31.35	0.6	Sodium glutamate	[39]
B. altitudinis	Enzyme-catalyzed reaction	2.2	0.6–0.65	Glutamine	[40]
P. nitroreducens	Enzyme-catalyzed reaction	47.0	0.458	Glutamine	[28]

In this study, an ATP regeneration system was established based on polyphosphate kinase (PPK) to regenerate ATP, using polyphosphate as a substrate. In addition, alanine transaminase and alanine decarboxylase (AlaDC) are overexpressed in *E. coli*, transforming the pyruvate into ethylamine and, thus, establishing a biotransformation system for L-theanine without the further addition of ethylamine. Moreover, a glutamine permease (GNP1) from *Saccharomyces cereviside* was overexpressed in *E. coli* FD04 to increase the rate of L-theanine transport out of cells, thereby increasing L-theanine production.

2. Materials and Methods

2.1. Strains and Plasmids

The strains and plasmids involved in this work are listed in Table 2. Plasmid pETDuet-1 and *E. coli* BL21(DE3) were preserved in our laboratory and used as cloning and expression hosts. The nucleotide sequences of the genes *gmas* from Methyloversatilis universalis, ppk from *E. coli*, *Ald* from *Bacillus subtilis* (BsAld), *AlaDC* from *Camellia sinensis* (CsAlaDC), and *gnp1* from *Saccharomyces cerevisiae* are available in the GenBank database, with the accession numbers of WP_008064112, EGI12137, WP_003220655, and QIH45886.1, respectively. Genes encoding PPK, alanine dehydrogenase (Ald), and glutamine permease (GNP1) were PCR amplified from *E. coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae genomic* DNA using Green DNA polymerase (TsingKe Biotech, Beijing, China), respectively. The genes encoding GMAS and alanine decarboxylase (AlaDC) from *Camellia sinensis* were synthesized by TsingKe Biotech (TsingKe Biotech, Beijing, China). In order to establish an ATP regeneration system, the *gmas* and *ppk* genes were inserted into pETDuet-1, and then the plasmid pETDuet-1-gmas-ppk was generated. In addition, in order to build a de novo synthetic pathway of L-theanine from glucose, the *bsAld* and *csAlaDC* genes were inserted into pETDuet-1-gmas-ppk, and then the plasmid pETDuet-1-gmas-ppk-bsAld-csAlaDC was generated. Furthermore, the glutamine permease gene *gnp1* from *Saccharomyces cerevisiae* was inserted into the plasmid pETDuet-1-gmas-ppk-BsAld-CsAlaDC to generate the plasmid pETDuet-1-gmas-ppk-BsAld-CsAlaDC-gnp1. Amplicons were inserted into the pETDuet-1 vector using the In-Fusion cloning system (TaKaRa Bio), and the resulting plasmids were introduced into *E. coli* BL21(DE3) via electroporation.

Table 2. The strains and plasmids used in this study.

Strains or Plasmids	Description	Sources
Strains		
BL21(DE3)	Wild type	Novagen
FD01	*E. coli* BL21(DE3) harboring plasmid pETDuet-1-gmas	This study
FD02	*E. coli* BL21(DE3) harboring plasmid pETDuet-1-gmas-ppk	This study
FD03	*E. coli* BL21(DE3) harboring plasmid pETDuet-1-gmas-ppk-bsAld-csAlaDC	This study
FD04	*E. coli* BL21(DE3) harboring plasmid pETDuet-1-gmas-ppk-bsAld-csAlaDC-gnp1	This study
Plasmids		
pETDuet-1	Empty plasmid, Amp^R	[41]
pETDuet-1-gmas	pETDuet-1 carries a γ-glutamylmethylamide synthetase (GMAS) gene from *Methyloversatilis universalis*, Amp^R	This study
pETDuet-1-gmas-ppk	pETDuet-1 carries a γ-glutamylmethylamide synthetase (GMAS) gene from *Methyloversatilis universalis* and a polyphosphate kinase (PPK) gene from *E. coli*, Amp^R	This study
pETDuet-1-gmas-ppk-bsAld-csAlaDC	pETDuet-1 carries a γ-glutamylmethylamide synthetase (GMAS) gene from *Methyloversatilis universalis*, a polyphosphate kinase (PPK) gene from *E. coli*, an alanine transaminase gene from *Bacillus subtilis* (bsAld), and an alanine decarboxylase gene from *Camellia sinensis* (csAlaDC), Amp^R	This study
pETDuet-1-gmas-ppk-bsAld -csAlaDC-gnp1	pETDuet-1 carries a γ-glutamylmethylamide synthetase (GMAS) gene from *Methyloversatilis universalis*, a polyphosphate kinase (PPK) gene from *E. coli*, an alanine transaminase gene from *Bacillus subtilis* (bsAld), an alanine decarboxylase gene (AlaDC) from *Camellia sinensis*, and a glutamine permease gene from *Saccharomyces cereviside* (gnp1), Amp^R	This study

2.2. Cultivation Conditions

Recombinant *E. coli* BL21(DE3) cells carrying the corresponding plasmids were cultured in 2 mL of LB medium in a test tube (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl) with 100 mg/L of ampicillin for 12 h at 37 °C and 220 rpm. Then, 20 μL

of the culture was transferred into a 100 mL flask containing 20 mL of medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 mmol/L $MgCl_2 \cdot 6H_2O$, 150 mmol/L $(NaPO_3)_6$ and 100 μg/mL of Amp) at 37 °C and 220 rpm. After the OD_{600} reached 0.6, 0.5 mM IPTG was added; at the same time, L-glutamate and ethylamine were added, and culturing was continued at 30 °C for 12 h. For the whole-cell biotransformation of *E. coli* FD01 and FD02, *E. coli* wet cells were then harvested via centrifugation at 10,000 rpm (15 min, 4 °C) and washed a minimum of 3 times and stored at −20 °C. Then, the cells were added to 3 mL reaction mixture (OD_{600} = 20) containing 200 mM L-glutamate, 200 mM ethylamine, 10 mmol/L $MgCl_2 \cdot 6H_2O$, and 150 mmol/L $(NaPO_3)_6$. In both *E. coli* FD03 and FD04, the production of L-theanine does not require the addition of exogenous ethylamine and glutamate. Ethylamine is generated through the conversion of pyruvate by BsAld and CsAlaDC, while glutamate is obtained from glucose though the TCA cycle.

The production of L-theanine in recombinant *E. coli* FD04 was conducted in a 1 L bioreactor. The composition of the medium includes 40 g/L glucose, 7.5 g/L $K_2HPO_4 \cdot 3H_2O$, 1.6 g/L, $(NH_4)_2SO_4$, 1.6 g/L $MgSO_4 \cdot 7H_2O$, 0.00756 g/L $FeSO_4 \cdot 7H_2O$, 2 g/L citric acid, 0.02 g/L Na_2SO_4, 0.0064 g/L $ZnSO_4$, 0.0006 g/L $Cu_2SO_4 \cdot 5H_2O$, 0.004 g/L $CoCl_2 \cdot 6H_2O$, 150 mmol/L $(NaPO_3)_6$, and 100 μg/mL ampicillin. The pH was maintained at 6.8 with $NH_3 \cdot H_2O$. The rotational speed and temperature were 220 rpm and 30 °C, respectively.

2.3. Homology Modeling and Molecular Docking

The theoretical structure of native GMAS was generated via the SWISS-MODEL online server. The modeled GMAS structure was hydrotreated for subsequent molecular docking. The initial structure was processed with AutoDockTools 1.5.6, which preserved the original charge of the protein and generated a PDBQT file for docking. The ligand L-glutamate was docked into the pocket of the GMAS using the AutoDock 4.2.6 package, where the lowest energy conformation in the largest cluster was considered to be the approximately natural complex model. The energy optimization used the Amber14 force field.

2.4. Analytical Methods

L-Theanine, L-glutamate, and glucose were detected via HPLC (Agilent Technologies 1200 series, Hewlett-Packard). The sample was derived with phenyl isothiocyanate (PITC) for the detection of L-theanine and L-glutamate [42]. The L-Theanine and L-glutamate contents were analyzed via HPLC with a C18 column (4.6 × 250 mm). For the analysis of L-theanine and D-theanine, the Chirex®3126 (D)-penicillamine LC column (4.6 × 250 mm, Phenomenex, Torrance, CA, USA) was used in the HPLC system [43]. Samples were centrifuged and filtered using a 0.22 μm membrane. The column temperature was maintained at 35 °C, and the flow rate was 1.0 mL/min.

3. Results and Discussion

3.1. Construction of a PPK-Based ATP Regeneration System for Enhancing the Production of L-Theanine in E. coli

To improve the fermentative production of the target product, L-theanine, we first planned to establish a genetically engineered *E. coli* strain by designing an ATP regeneration system utilizing PPK (Figure 2). In this pathway, GMAS from *Methyloversatilis universalis* and PPK from *E. coli* were inserted into pETDuet-1, to generate the plasmid pETDuet-1-gmas-ppk. The plasmid pETDuet-1-gmas-ppk was transferred into *E. coli* BL21(DE3) to generate the engineering strain. Meanwhile, PPK utilized polyphosphates as substrates to convert ADP into ATP, achieving ATP regeneration.

Whole-cell biotransformation systems typically exhibit robustness against harsh reaction conditions and have advantages such as a higher stability and storage capability compared to purified enzymes [44]. Pan et al. [45] successfully established an ATP regeneration system utilizing PPK and employed cost-effective substrates, including glutamine and ethylamine, for the whole-cell biocatalytic synthesis of L-theanine. A conversion rate

of 66.34% for glutamine was achieved through the optimization of the whole-cell catalytic reaction conditions [45].

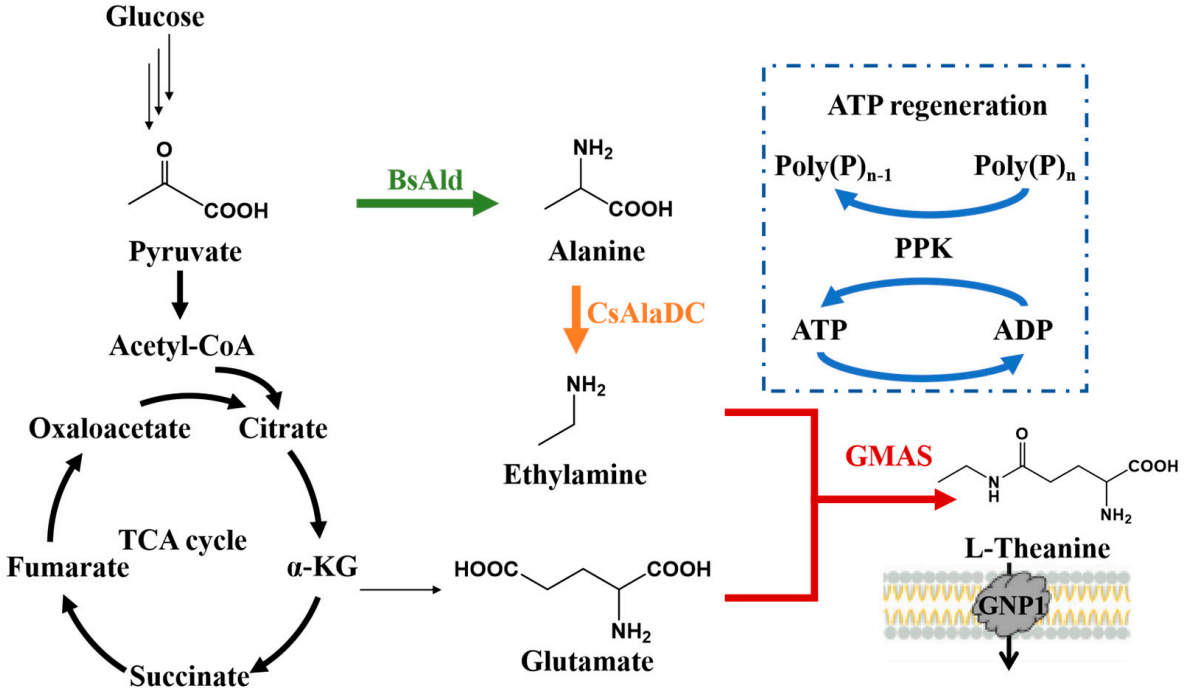

Figure 2. A synthetic pathway of L-theanine coupled with an ATP regeneration system and an L-theanine transport system in *E. coli*.

3.2. Molecular Docking Studies

To gain a better insight into the interaction between GMAS and L-glutamate at the molecular level, a molecular docking study was performed. Binding mode analysis was performed to specifically clarify the binding mechanism. The binding pattern diagram of the L-glutamate molecule with the GMAS protein is shown in Figure 3. As shown in Figure 3c,d, the docking results for GMAS and L-glutamate show that L-glutamate formed seven hydrogen bonds with the side chain Trp189, Tyr191, Ser239, Arg317, and Arg312. In addition, the binding of GMAS with L-glutamate could be attributed to a hydrophobic interaction, because strong hydrophobic interactions of L-glutamate with His237 and Arg323 were formed. The molecular docking results showed that L-glutamate could bind to the hydrophobic cavity of GMAS due to the formation of hydrogen bonds and hydrophobic interactions with the surrounding amino acid residues. Due to the impact of the GMAS catalytic efficiency, there is still room for further improvement in the production of L-theanine. Through molecular docking, some key sites that affect the catalytic activity of GMAS were identified. For the next step, semi-rational design can be used to perform saturation mutations on key sites to enhance the catalytic activity of GMAS and, thereby, increase L-theanine production.

3.3. Whole-Cell Catalytic Synthesis of L-Theanine in Engineered E. coli FD01 and FD02

To optimize the ATP regeneration system based on PPK, the effects of the temperature, IPTG concentration, and substrate concentration on the production of L-theanine were investigated. By adjusting the temperature, pH, and substrate concentration, we determined the optimal conditions for the production of L-theanine in engineered *E. coli* FD01 and *E. coli* FD02 (Figure 4).

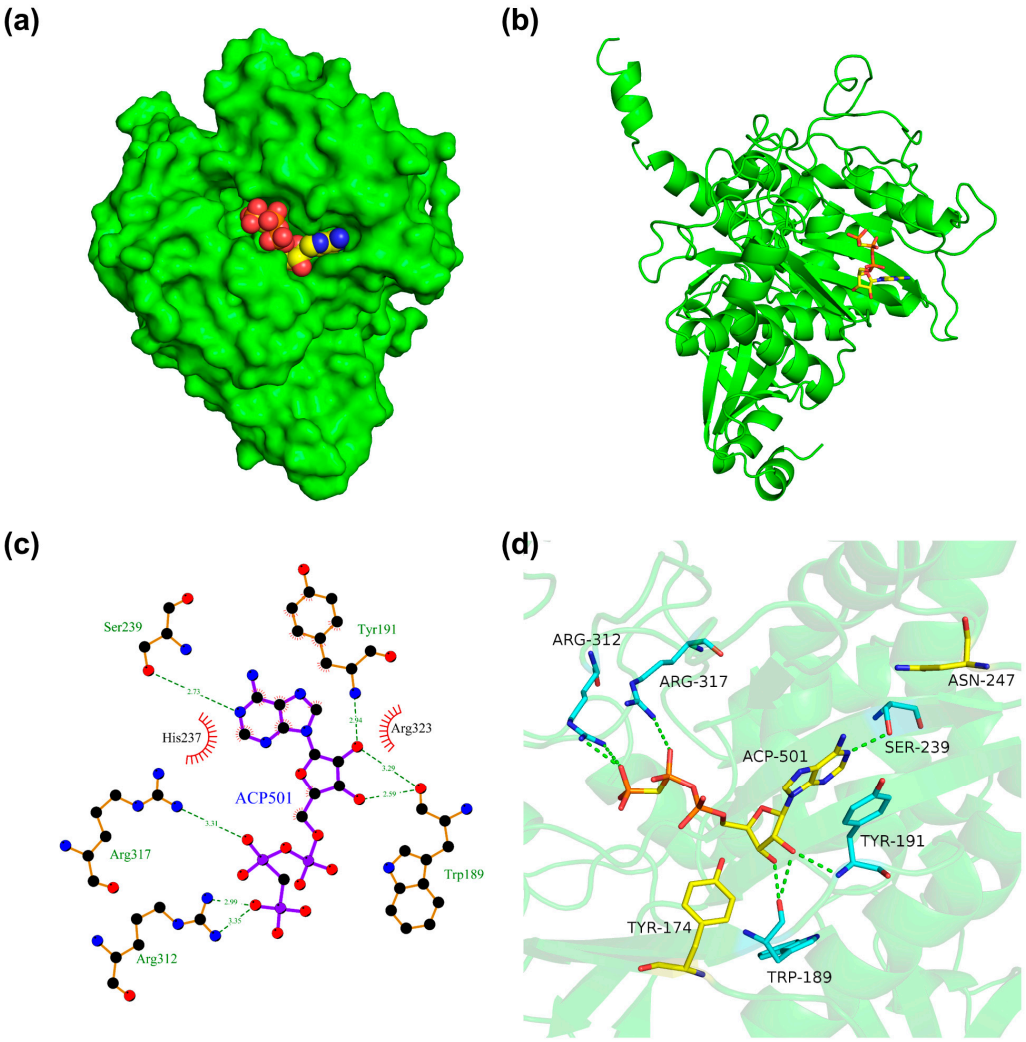

Figure 3. The binding pattern diagram of the L-glutamate molecule with the GMAS protein. (**a**) The distribution of the L-glutamate molecule on the GMAS protein surface; (**b**) the spatial position of the L-glutamate molecule in the GMAS protein; (**c**) the 2D interaction diagram of the L-glutamate molecule with the GMAS protein, with the green dashed line denoting hydrogen bonding; (**d**) the 3D interaction diagram of the L-glutamate molecule with the GMAS protein.

The temperature is one of the key factors influencing the efficiency of enzymatic reactions. To improve the biocatalytic efficiency and ensure the effectiveness of whole-cell catalysis, the optimal reaction temperature was discussed. Figure 3a,b show the production of L-theanine via the engineered strains *E. coli* FD01 and *E. coli* FD02 at different temperature gradients (ranging from 30 °C to 50 °C). The results in Figure 4A demonstrate that the engineered strain *E. coli* FD01 exhibited the highest L-theanine production at 30 °C, and the titer of L-theanine decreased with the increase in temperature when the temperature exceeded 30 °C. Therefore, the optimal temperature for whole-cell catalysis in *E. coli* FD01 is 30 °C. Figure 4B shows that the highest L-theanine production in the engineered strain *E. coli* FD02 was achieved at 37 °C, and that the titer of L-theanine decreased at higher temperatures. Hence, the optimal temperature for whole-cell catalysis in *E. coli* FD02 was 37 °C.

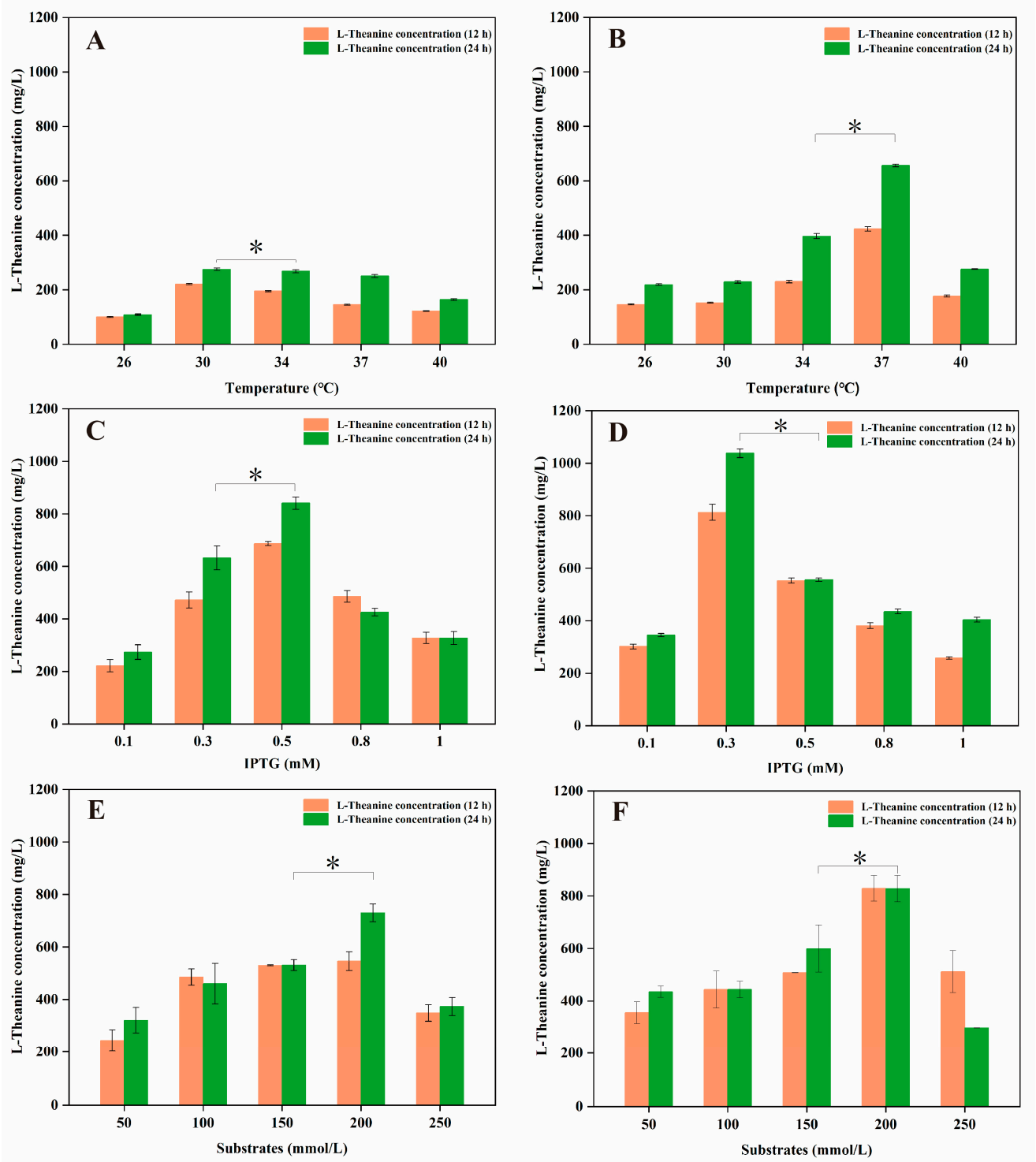

Figure 4. The optimization of whole-cell transformation conditions for the production of L-theanine via engineered *E. coli* FD01 (**A**,**C**,**E**) and FD02 (**B**,**D**,**F**). Statistics were performed by two-tailed Student's *t*-test. * $p < 0.05$. Each experiment was done at least in triplicate. Error bars indicate standard errors of the means.

The concentration of IPTG during protein induction can have an impact on protein expression. On the one hand, IPTG competitively binds to inhibitory proteins to initiate the transcription of target proteins and, if the concentration of IPTG is too low, it does not reach the saturation concentration for binding to lac-inhibitory proteins, which is unfavorable for inducing protein expression. On the other hand, a high concentration of IPTG will promote

the formation of inclusion bodies, which will affect the quality of the enzyme protein. In this study, Figure 4C,D show the effect of different IPTG concentrations on the production of L-theanine. Figure 4C,D show that the engineered strain *E. coli* FD01 achieves the highest L-theanine production at an IPTG concentration of 0.5 mM, while the engineered strain *E. coli* FD02 exhibits the highest L-theanine production at an IPTG concentration of 0.3 mM. Thus, the IPTG concentrations for L-theanine production in *E. coli* FD01 and *E. coli* FD02 were set to 0.5 mM and 0.3 mM, respectively.

In the whole-cell catalytic production of L-theanine, the titer of L-theanine increases with the addition of L-glutamate under conditions of excess enzyme involvement. However, the substrate ethylamine has a high toxicity to *E. coli* and can disrupt the cells involved in the reaction. Therefore, this study explored the optimal substrate addition amount. The ratio of L-glutamate to ethylamine was set at 1:1, and the concentrations of L-glutamate were set to 50 mmol/L, 100 mmol/L, 150 mmol/L, 200 mmol/L, and 250 mmol/L. Figure 4E,F show that the highest L-theanine production in the engineered strains *E. coli* FD01 and *E. coli* FD02 is achieved when the L-glutamate concentration is 200 mmol/L (1 L-glutamate: 1 ethylamine). Both too-low and too-high substrate concentrations have an impact on L-theanine production, so subsequent experiments were conducted at a substrate concentration of 200 mmol/L (1 L-glutamate: 1 ethylamine).

3.4. De Novo Biosynthesis of L-Theanine via Engineered E. coli FD03

Ethylamine is an essential starting material in the synthesis of L-theanine. However, its high toxicity could have significant negative impacts on human health and the natural environment [46]. As a result, researchers have been led to explore efficient intracellular pathways for ethylamine synthesis to produce L-theanine [37]. Ethylamine in tea plants is mainly produced through the decarboxylation reaction of alanine, but the microbial synthesis pathway for ethylamine has not been reported yet [47]. Hagihara et al. [23] constructed two de novo synthetic pathways from glucose to theanine. One pathway is the AlaDC pathway, which involves the simultaneous expression of GMAS from *Pseudomonas syringae*, CsAlaDC, and BsAld from *Bacillus subtilis* 168 in *E. coli*. The other pathway, referred to as the transaminase-mediated pathway, involves the amination reaction of acetaldehyde. Benninghaus et al. identified and screened a transaminase, PpTA8, derived from *Pseudomonas putida* KT2440, which effectively promotes the amination of acetaldehyde [48]. Finally, this pathway utilizes the simultaneous expression of GMAS, BsAld, PpTA8, and the endogenous acetaldehyde dehydrogenase EutE in *E. coli*, thereby establishing a novel de novo synthetic pathway for L-theanine.

In the one-step fermentation process of L-theanine production from glucose, temperature plays a crucial role as a significant factor affecting the fermentation efficiency. This study explores the optimal temperature to enhance L-theanine production. While keeping the inducer IPTG concentration, substrate concentration, reaction system size, and pH unchanged, a temperature gradient was set in order to investigate the best reaction temperature. As shown in Figure 5A, the engineered strain *E. coli* FD03 achieved the highest L-theanine yield at 30 °C, and there was little difference in L-theanine production between 34 °C and 37 °C, possibly due to the gradual impact of temperature on enzyme activity within this temperature range, followed by a gradual decrease in the L-theanine yield as the temperature increased.

Next, the optimal IPTG concentration was explored while the other reaction conditions were kept constant. The gradient of IPTG concentration was set. As depicted in Figure 5B, the highest L-theanine production for the engineered strain *E. coli* FD03 was obtained at an IPTG concentration of 0.8 mM. When the IPTG concentration was 0.5 mM and 1.0 mM, the L-theanine yield was almost the same, indicating that excessively high IPTG concentrations could inhibit L-theanine synthesis in the engineered strain *E. coli* FD03. Thus, the optimal fermentation conditions for the engineered strain *E. coli* FD03 comprise a temperature of 30 °C, an IPTG concentration of 0.8 mM, and a pH of 7.0.

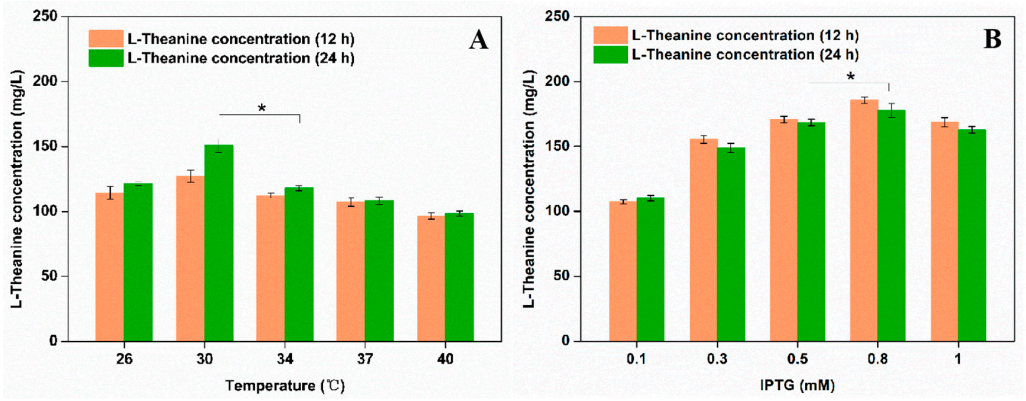

Figure 5. The production of L-theanine from glucose via recombinant *E. coli* FD03. The optimization of temperature (**A**) and IPTG (**B**) for the production of L-theanine in engineered *E. coli* FD03. Statistics were performed by two-tailed Student's *t*-test. * $p < 0.05$. Each experiment was done at least in triplicate. Error bars indicate standard errors of the means.

3.5. The Enhancement of L-Theanine Production via the Overexpression of a Glutamine Permease

Currently, the transport mechanism of theanine in tea plants remains unclear and is the subject of continuous research and analysis. Some researchers have confirmed the use of yeast libraries for genetic screening and, through functional analysis, they have identified and confirmed glutamine permease GNP1 as the specific transport protein for theanine in yeast, making it one of the candidate genes for encoding theanine transporters [49]. Furthermore, from an economic and environmental perspective in industrial production, glucose is one of the more economically viable and environmentally friendly carbon sources [31,50]. Therefore, glucose is highly suitable for use as the source material.

Firstly, the plasmid pETDuet-1-gmas-ppk-bsAld-csAlaDC-gnp1 was constructed and transformed into *E. coli* BL21(DE3), resulting in the formation of *E. coli* FD04 (Table 1). Figure 6 shows that the optimal reaction conditions for *E. coli* FD04 were 30 °C, pH 7.0, and an IPTG concentration of 0.3 mmol/L. Under the optimal conditions, the maximum titer of L-theanine produced via the engineered *E. coli* strain FD03 was approximately 334 mg/L, while *E. coli* FD04 achieved a maximum titer of 383 mg/L. This indicates that *E. coli* FD03 has a lower capacity for L-theanine production compared to *E. coli* FD04, with a 14.7% decrease in the production rate of L-theanine in *E. coli* FD03 compared to *E. coli* FD04. These results indicated that the overexpression of the theanine transport protein enhanced the synthesis of L-theanine in *E. coli* FD04. All of the theanine was L-theanine. The optical purity of the L-theanine was 100%.

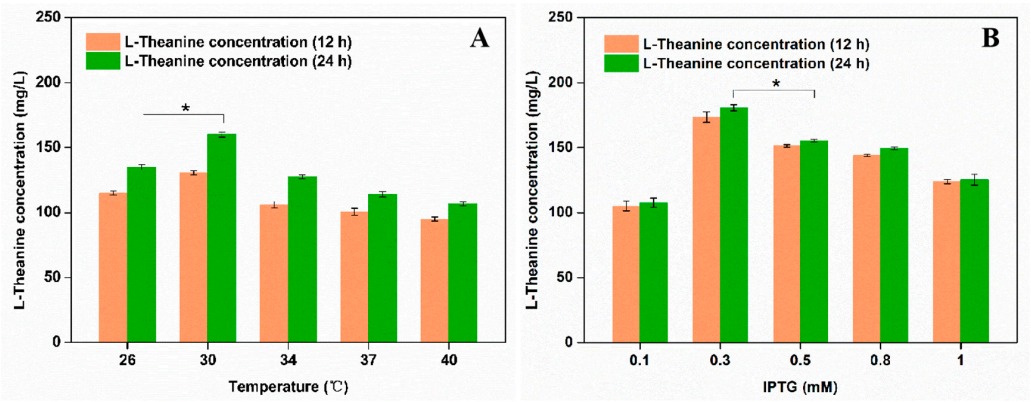

Figure 6. The production of L-theanine from glucose via recombinant *E. coli* FD04. The optimization of temperature (**A**) and IPTG (**B**) for the production of L-theanine in engineered *E. coli* FD04. Statistics were performed by two-tailed Student's *t*-test. * $p < 0.05$. Each experiment was done at least in triplicate. Error bars indicate standard errors of the means.

3.6. Production of L-Theanine from Glucose via Recombinant E. coli FD04 in a 1 L Bioreactor

Figure 7 shows the fermentation process curve of L-theanine in the engineered *E. coli* FD04 within a 1 L bioreactor. From 0 to 16 h, the metabolism of the bacteria was highly active, and glutamine permease was rapidly expressed. Glucose, as the carbon source in the reaction, was catalyzed to produce L-theanine. The production of L-theanine steadily increased with the reaction time and, at 20 h of fermentation, the accumulated maximum yield of L-theanine reached 2.9 g/L, successfully achieving the one-step fermentation of L-theanine from glucose.

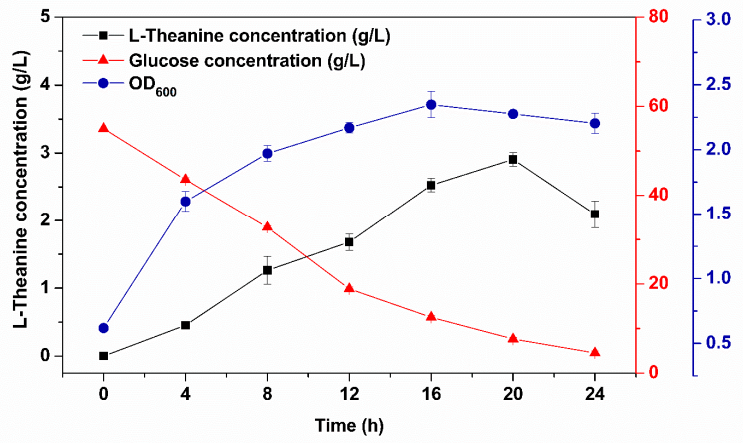

Figure 7. The production of L-theanine via recombinant *E. coli* FD04 in a 1 L bioreactor.

4. Discussion

This study primarily confirms that the γ-glutamylmethylamide synthetase from *Methyloversatilis universalis* and the polyphosphate kinase from *E. coli* can catalyze the production of L-theanine from the substrates L-glutamine and ethanolamine, thereby demonstrating the advantage of constructing an ATP regeneration system to enhance the biosynthesis of L-theanine. Simultaneously, the heterologous expression of the yeast glutamine permease from *Saccharomyces cerevisiae* confirms the feasibility of enhancing L-theanine synthesis through the overexpression of the theanine transport protein, indicating significant prospects for the application of one-step fermentation methods using microorganisms in the production of L-theanine.

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References

1. Zhou, Z.; Zhang, X.; Wu, J.; Li, X.; Li, W.; Sun, X.; Wang, J.; Yan, Y.; Shen, X.; Yuan, Q. Targeting cofactors regeneration in methylation and hydroxylation for high level production of Ferulic acid. *Metab. Eng.* **2022**, *73*, 247–255. [CrossRef] [PubMed]
2. Yu, S.; Shan, X.; Lyv, Y.; Zhou, J. Bioproduction of quercetin using recombinant thermostable glycosidases from *Dictyoglomus thermophilum*. *Bioresour. Bioprocess.* **2022**, *9*, 48–58. [CrossRef]
3. Zhang, Q.; Wang, X.; Zeng, W.; Xu, S.; Li, D.; Yu, S.; Zhou, J. De novo biosynthesis of carminic acid in *Saccharomyces cerevisiae*. *Metab. Eng.* **2023**, *76*, 50–62. [CrossRef] [PubMed]

4. Rohles, C.; Pauli, S.; Giesselmann, G.; Kohlstedt, M.; Becker, J.; Wittmann, C. Systems metabolic engineering of *Corynebacterium glutamicum* eliminates all by-products for selective and high-yield production of the platform chemical 5-aminovalerate. *Metab. Eng.* **2022**, *73*, 168–181. [CrossRef] [PubMed]
5. Prell, C.; Vonderbank, S.-A.; Meyer, F.; Pérez-García, F.; Wendisch, V.F. Metabolic engineering of *Corynebacterium glutamicum* for de novo production of 3-hydroxycadaverine. *Curr. Res. Biotechnol.* **2022**, *4*, 32–46. [CrossRef]
6. Chen, Q.; Jiang, Y.; Kang, Z.; Cheng, J.; Xiong, X.; Hu, C.Y.; Meng, Y. Engineering a Feruloyl-Coenzyme A Synthase for Bioconversion of Phenylpropanoid Acids into High-Value Aromatic Aldehydes. *J. Agric. Food Chem.* **2022**, *70*, 9948–9960. [CrossRef] [PubMed]
7. Xiao, D.; Zhang, W.; Guo, X.; Liu, Y.; Hu, C.; Guo, S.; Kang, Z.; Xu, X.; Ma, C.; Gao, C.; et al. A D-2-hydroxyglutarate biosensor based on specific transcriptional regulator DhdR. *Nat. Commun.* **2021**, *12*, 7108. [CrossRef] [PubMed]
8. Sohn, Y.J.; Kang, M.; Baritugo, K.-A.; Son, J.; Kang, K.H.; Ryu, M.-H.; Lee, S.; Sohn, M.; Jung, Y.J.; Park, K.; et al. Fermentative High-Level Production of 5-Hydroxyvaleric Acid by Metabolically Engineered *Corynebacterium glutamicum*. *ACS Sustain. Chem. Eng.* **2021**, *9*, 2523–2533. [CrossRef]
9. Liu, S.-H.; Li, J.; Huang, J.-A.; Liu, Z.-H.; Xiong, L.-G. New advances in genetic engineering for l-theanine biosynthesis. *Trends Food Sci. Technol.* **2021**, *114*, 540–551. [CrossRef]
10. Saeed, M.; Naveed, M.; Arif, M.; Kakar, M.U.; Manzoor, R.; Abd El-Hack, M.E.; Alagawany, M.; Tiwari, R.; Khandia, R.; Munjal, A.; et al. Green tea (*Camellia sinensis*) and l-theanine: Medicinal values and beneficial applications in humans-A comprehensive review. *Biomed. Pharm.* **2017**, *95*, 1260–1275. [CrossRef]
11. Chen, S.; Kang, J.; Zhu, H.; Wang, K.; Han, Z.; Wang, L.; Liu, J.; Wu, Y.; He, P.; Tu, Y.; et al. L-Theanine and Immunity: A Review. *Molecules* **2023**, *28*, 3846. [CrossRef] [PubMed]
12. Chen, Z.; Wang, Z.; Yuan, H.; He, N. From Tea Leaves to Factories: A Review of Research Progress in l-Theanine Biosynthesis and Production. *J. Agric. Food Chem.* **2021**, *69*, 1187–1196. [CrossRef]
13. Jang, H.S.; Jung, J.Y.; Jang, I.S.; Jang, K.H.; Kim, S.H.; Ha, J.H.; Suk, K.; Lee, M.G. L-theanine partially counteracts caffeine-induced sleep disturbances in rats. *Pharm. Biochem. Behav.* **2012**, *101*, 217–221. [CrossRef]
14. Vuong, Q.V.; Golding, J.B.; Nguyen, M.H.; Roach, P.D. Preparation of decaffeinated and high caffeine powders from green tea. *Powder Technol.* **2013**, *233*, 169–175. [CrossRef]
15. Zhang, R.; Zheng, L.; Zhou, L.; Xiang, L.; Jiang, B.; Zhang, T.; Chen, J. Characterization of alkaline *Bacillus amyloliquefaciens* γ-glutamyltranspeptidase expressed in *Bacillus subtilis* and its application in enzymatic synthesis of L-Theanine. *Process Biochem.* **2023**, *131*, 125–132. [CrossRef]
16. Wang, X.; Liang, H.; Mao, B.; Hu, C.; Tang, D. The Current Situation and Prospect of the Utilization of Theanine. *Chinatea* **2021**, *43*, 6–10.
17. Shuai, Y.; Shen, Y.; Huang, H.; Li, J. Applications and regulation of functional ingredient L-Theanine. *China Food Addit.* **2013**, *S1*, 181–185.
18. Jagim, A.R.; Harty, P.S.; Tinsley, G.M.; Kerksick, C.M.; Gonzalez, A.M.; Kreider, R.B.; Arent, S.M.; Jager, R.; Smith-Ryan, A.E.; Stout, J.R.; et al. International society of sports nutrition position stand: Energy drinks and energy shots. *J. Int. Soc. Sport. Nutr.* **2023**, *20*, 2171314. [CrossRef]
19. Mu, W.; Zhang, T.; Jiang, B. An overview of biological production of L-theanine. *Biotechnol. Adv.* **2015**, *33*, 335–342. [CrossRef]
20. Zhang, J.; Rong, S.-F.; Gong, G.-M.; Qiu, Z.-C. Comparison of Determination of Theanine in Different Tea Species. *Food Sci.* **2008**, *29*, 335–337.
21. Xiao, S.; Qian, R.; Hu, S.; Fu, Z.; Bai, T.; Wang, W.; Cheng, J.; Zhang, J. Advances in the Production of Theanine by Plants and Microorganisms. *Fermentation* **2023**, *9*, 543. [CrossRef]
22. Zhang, Z.; Long, M.; Zheng, N.; Deng, Y.; Wang, Q.; Osire, T.; Xia, X. Redesign of gamma-glutamyl transpeptidase from Bacillus subtilis for high-level production of L-theanine by cavity topology engineering. *Appl. Microbiol. Biotechnol.* **2023**, *107*, 3551–3564. [CrossRef] [PubMed]
23. Hagihara, R.; Ohno, S.; Hayashi, M.; Tabata, K.; Endo, H. Production of L-Theanine by *Escherichia coli* in the Absence of Supplemental Ethylamine. *Appl. Environ. Microbiol.* **2021**, *87*, e00031-21. [CrossRef] [PubMed]
24. Zhang, Y.; Chen, B.; Huang, Z.; Shi, Z. Preparative Isolation and Purification of L-Theanine by HPLC. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 875–884. [CrossRef]
25. Gu, H.; Jiang, Y.; Wang, J. A Practical Synthesis of Ethyl L-Glutamine (L-Theanine). *Org. Prep. Proced. Int.* **2004**, *36*, 182–185. [CrossRef]
26. Feng, J.C.; Yang, C.; Zhao, Z.H.; Xu, J.J.; Li, J.; Li, P. Application of Cell-Free Protein Synthesis System for the Biosynthesis of L-Theanine. *ACS Synth. Biol.* **2021**, *10*, 620–631. [CrossRef] [PubMed]
27. Wen-Xian, Z.; Xing-Hui, L.I.; Li-Yuan, W.; Wan-Ping, F.; Hao, C. Construction of *E. coli* Recombinant Engineered Strain for Theanine Biosynthesis with GS Gene Embedded. *J. Tea Sci.* **2008**, *28*, 242–248.
28. Tachiki, T.; Yamada, T.; Mizuno, K.; Ueda, M.; Shiode, J.-I.; Fukami, H. γ-Glutamyl Transfer Reactions by Glutaminase from *Pseudomonas nitroreducens* IFO 12694 and Their Application for the Syntheses of Theanine and γ-Glutamylmethylamide. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1279–1283. [CrossRef]
29. Miyake, K.; Kakita, S. A novel catalytic ability of gamma-glutamylcysteine synthetase of *Escherichia coli* and its application in theanine production. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 2677–2683. [CrossRef]

30. Yang, S.Y.; Han, Y.H.; Park, Y.L.; Park, J.Y.; No, S.Y.; Jeong, D.; Park, S.; Park, H.Y.; Kim, W.; Seo, S.O.; et al. Production of L-Theanine Using Escherichia coli Whole-Cell Overexpressing gamma-Glutamylmethylamide Synthetase with Bakers Yeast. *J. Microbiol. Biotechnol.* **2020**, *30*, 785–792. [CrossRef]
31. Fan, X.G.; Zhang, T.; Ji, Y.Q.; Li, J.; Long, K.Y.; Yuan, Y.; Li, Y.J.; Xu, Q.Y.; Chen, N.; Xie, X.X. Pathway engineering of *Escherichia coli* for one-step fermentative production of L-theanine from sugars and ethylamine. *Metab. Eng. Commun.* **2020**, *11*, e00151. [CrossRef]
32. Yao, J.; Li, J.; Xiong, D.; Qiu, Y.; Shi, G.; Jin, J.M.; Tao, Y.; Tang, S.Y. Development of a highly efficient and specific L-theanine synthase. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 3417–3431. [CrossRef] [PubMed]
33. Pan, X.; Yu, J.; Du, Q.; Zeng, S.; Liu, J.; Jiao, Q.; Zhang, H. Efficient synthesis of γ-glutamyl compounds by co-expression of γ-glutamylmethylamide synthetase and polyphosphate kinase in engineered *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 573–583. [CrossRef]
34. Li, Z.; Zhu, R.; Liu, Y.; Li, J.; Gao, H.; Hu, N. Gamma-Glutamyltranspeptidase from Bacillus amyloliquefaciens: Transpeptidation activity enhancement and L-theanine production. *Enzym. Microb. Technol.* **2020**, *140*, 109644. [CrossRef]
35. Yang, T.; Liu, S.; Liu, H.; Long, M.; Chen, P.; Zhang, X.; Xu, M.; Rao, Z. Semi-quantitative activity assays for high-throughput screening of higher activity gamma glutamyl transferase and enzyme immobilization to efficiently synthesize L-theanine. *J. Biotechnol.* **2021**, *330*, 9–16. [CrossRef] [PubMed]
36. Sun, J.; Chang, M.; Li, H.; Zhang, Z.; Chen, Q.; Chen, Y.; Yao, Y.; Pan, A.; Shi, C.; Wang, C.; et al. Endophytic Bacteria as Contributors to Theanine Production in *Camellia sinensis*. *J. Agric. Food Chem.* **2019**, *67*, 10685–10693. [CrossRef] [PubMed]
37. Chi, M.C.; Lin, M.G.; Huang, Y.F.; Chen, Y.Y.; Wang, T.F.; Lin, L.L. Enzymatic synthesis of L-theanine from L-glutamine and ethylamine by *Bacillus licheniformis* gamma-glutamyltranspeptidase and its mutants specialized in transpeptidase activity. *Biocatal. Agric. Biotechnol.* **2019**, *22*, 101393. [CrossRef]
38. Ma, H.K.; Fan, X.G.; Cai, N.Y.; Zhang, D.Z.; Zhao, G.H.; Wang, T.; Su, R.; Yuan, M.; Ma, Q.; Zhang, C.L.; et al. Efficient fermentative production of l-theanine by *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 119–130. [CrossRef]
39. Yamamoto, S.; Wakayama, M.; Tachiki, T. Cloning and expression of Pseudomonas taetrolens Y-30 gene encoding glutamine synthetase: An enzyme available for theanine production by coupled fermentation with energy transfer. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 500–507. [CrossRef]
40. Sharma, E.; Lal, M.K.; Gulati, A.; Gulati, A. Biochemical Characterization of gamma-Glutamyl Transpeptidase from *Bacillus altitudinis* IHB B1644 and Its Application in the Synthesis of l-Theanine. *J. Agric. Food Chem.* **2023**, *71*, 5592–5599. [CrossRef]
41. Hu, B.; Yu, H.; Zhou, J.; Li, J.; Chen, J.; Du, G.; Lee, S.Y.; Zhao, X. Whole-Cell P450 Biocatalysis Using Engineered *Escherichia coli* with Fine-Tuned Heme Biosynthesis. *Adv. Sci.* **2023**, *10*, 2205580. [CrossRef] [PubMed]
42. Cheng, J.; Tu, W.; Luo, Z.; Gou, X.; Li, Q.; Wang, D.; Zhou, J. A High-Efficiency Artificial Synthetic Pathway for 5-Aminovalerate Production From Biobased L-Lysine in *Escherichia coli*. *Front Bioeng. Biotechnol.* **2021**, *9*, 633028. [CrossRef] [PubMed]
43. Cheng, J.; Huang, Y.; Mi, L.; Chen, W.; Wang, D.; Wang, Q. An economically and environmentally acceptable synthesis of chiral drug intermediate l-pipecolic acid from biomass-derived lysine via artificially engineered microbes. *J. Ind. Microbiol. Biotechnol.* **2018**, *45*, 405–415. [CrossRef] [PubMed]
44. Griffiths, M.W.; Muir, D.D. Properties of a thermostable β-galactosidase from a thermophilic *Bacillus*: Comparison of the enzyme activity of whole cells, purified enzyme and immobilised whole cells. *J. Sci. Food Agric.* **1978**, *29*, 753–761. [CrossRef]
45. Pan, X.R.; Liu, J.Z.; Zhang, H.J.; Jiao, Q.C. Synthesis of L-Theanine by Whole Cell Catalyst Co-expressing PPK and GMAS. *Fine Chem.* **2019**, *36*, 1827–1832. [CrossRef]
46. Fu, X.M.; Liao, Y.Y.; Cheng, S.H.; Deng, R.F.; Yang, Z.Y. Stable Isotope-Labeled Precursor Tracing Reveals that L-Alanine is Converted to L-Theanine via L-Glutamate not Ethylamine in Tea Plants In Vivo. *J. Agric. Food Chem.* **2021**, *69*, 15354–15361. [CrossRef] [PubMed]
47. Ji, Y.; Zhou, Y.; Zhang, T.; Xie, H.; Dong, D.; Chen, N.; Fan, X. Production technology of L—Theanine and its application prospect. *Food Ferment. Ind.* **2022**, *48*, 303–311. [CrossRef]
48. Benninghaus, L.; Walter, T.; Mindt, M.; Risse, J.M.; Wendisch, V.F. Metabolic Engineering of Pseudomonas putida for Fermentative Production of L-Theanine. *J. Agric. Food Chem.* **2021**, *69*, 9849–9858. [CrossRef]
49. Dong, C.X.; Li, F.; Yang, T.Y.; Feng, L.; Zhang, S.P.; Li, F.D.; Li, W.H.; Xu, G.H.; Bao, S.L.; Wan, X.C.; et al. Theanine transporters identified in tea plants (*Camellia sinensis* L.). *Plant J.* **2020**, *101*, 57–70. [CrossRef]
50. Liu, K.; Peng, Y.; Lin, L.; Gong, Z.; Xiao, W.; Li, Y. L-Theanine Regulates the Abundance of Amino Acid Transporters in Mice Duodenum and Jejunum via the mTOR Signaling Pathway. *Nutrients* **2023**, *15*, 142. [CrossRef]