Metabolic engineering of *Escherichia coli* for efficient production of L-alanyl-L-glutamine

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Abstract

Background: L-alanyl-L-glutamine (AQ) is a functional dipeptide with high water solubility, good thermal stability and high bioavailability. It is widely used in clinical medicine, post-operative rehabilitation, sports health care and other fields. AQ is mainly produced by chemical synthesis which is complicated, time-consuming, labor-consuming, low yield and accompany with by-products. It is highly desirable to develop an efficient biotechnological process for AQ production.

Results: A metabolic engineered *E. coli* strain for AQ production was developed by over-expressing L-amino acid-ligase (BacD) from *Bacillus subtilis*, peptidases including PepA, PepB, PepD, PepN and dipeptide transport system Dpp were inactivated. In order to use the more readily available substrate, glutamic acid, a glutamine synthetic module from glutamic acid to glutamine was constructed by introducing glutamine synthetase (GlnA), *glsA-glsB* catalyze the first step in glutamine metabolism and *glnE-glnB* involved in the ATP-dependent addition of AMP/UMP to a subunit of glutamine synthetase were blocked which resulted in increased glutamine supply. This glutamine synthetic module combined with AQ synthetic module to develop the engineered strain that using glutamic acid and alanine for AQ production. The expression of BacD and GlnA was further balanced to improve the AQ production. The engineered strain p15/AQ10 was used in the whole-cell biocatalysis and 65.6 mM AQ was produced with productivity of 7.29 mM/h and conversion rate of 65.6%.

Conclusion: Metabolic engineered strains were developed for AQ production. Strategies including inactivation of peptidases, screening of BacD, introducing glutamine synthetic module, and balancing the glutamine and AQ synthetic modules were applied to improve the yield of AQ. This work provides the biosynthetically industrial potential for efficient production of AQ by microbial cell factory.

Background

Glutamine (L-Gln) plays an extremely important role in maintaining intestinal function[1-4], promoting immune function[5, 6], maintaining homeostasis of internal environment[7] and improving the adaptability of organism to stress[8]. Under disease or stress, demand outpaces supply, and glutamine becomes conditionally essential[9]. The supply of exogenous L-glutamine or glutamyl dipeptide is an important nutritional solution to reduce glutamine deficiency in vivo, used to be applied in the clinical treatment. However, some characters of glutamine such as low solubility in water, easy decomposition and poor thermal stability, and production of toxic pyroglutamate during heating sterilization restrict its application in medicine. Otherwise, as a result of the unstable nature, the low yield of glutamine is a great challenge in the purification process[10].

L-alanyl-L-glutamine (abbreviated as AQ) is a dipeptide molecule of glutamine and alanine, which makes it more stable and water-soluble than glutamine by itself. AQ functions like glutamine, which is hydrolyzed to glutamine and alanine in vivo. AQ has higher bioavailability and short half-life[11], and

cannot cause cumulative damage to the body, so it is used as a substitute for glutamine in clinical practice.

The demand of AQ is increasing with the expansion of new applications and the development of new products about AQ. Chemical and biotechnological methods for producing AQ have been developed [12-16]. AQ can be synthesized by chemical condensation of benzyloxycarbonyl-L-alanine and methyl glutamate by activated ester [17] or triphenyl phosphine/hexachloroethane condensation, which usually requires complex steps such as amino acid activation, subunit protection, and removing protectant. Enzymatic process for AQ production had been developed by an α -amino acid ester acyltransferase [18, 19]. Tabata and Hashimoto engineered *Escherichia coli* strain by expressing L-Amino Acid α -Ligase (BacD) which catalyzes the formation of dipeptide AQ in an ATP-dependent manner and produced more than 100 mM AQ in 47h fermentation [20]. Whole-cell biocatalysis can realize enzyme cascade reaction, improve catalytic efficiency, and make the preparation simpler.

In this study, metabolically engineered *E. coli* overexpressing BacD was developed for AQ production, and in order to reduce preparation cost, we introduced a glutamine biosynthesis module in which glutamine product from glutamic acid catalyzed by glutamine synthetase (Fig.1).

A metabolic engineered *E. coli* strain for AQ production was developed by over-expressing L-amino acid-ligase (BacD) from *Bacillus subtilis* and peptidases of *E. coli* were inactivated. In order to use the more readily available substrate, synthetic module of glutamine from glutamic acid by introducing glutamine synthetase (GlnA) was constructed and optimized. The glutamine synthetic module was combined with AQ synthetic module to use glutamic acid and alanine for AQ production. The protein expression of BacD and GlnA was further balanced to improve the AQ production. An whole-cell biocatalytic process for AQ production was developed by the engineered strain. This work provides an environmentally friendly, highly efficient and cost-effective process for industrial synthesis of AQ.

Results

Construction of the AQ Synthesis Module

E. coli strain p01/BW25113 expression BacD from *Bacillus subtilis* (BsBacD) which catalyzes the formation of AQ from alanine and glutamine was constructed for production of AQ, and 2.0 mM AQ was obtained (Fig.2).

In *E. coli*, peptidases encoded by *pepA*, *pepB*, *pepD* and *pepN*, have been reported to degrade a broad spectrum of dipeptides [20, 21], inactivation of them might reduce AQ degradation. It has been reported that a transportation system coded by the *dpp* operon which is responsible for import of dipeptides, and deletion of *dpp* increase AQ accumulation [22]. By knocking out the genes *pepN*, *pepA*, *pepB*, *pepD* and *dpp*, the strain (AQ09), the degradation of AQ was alleviated. In starting host BW25113, 20 mM AQ was completely degraded at 3 hours, while only 1.3 mM in the chassis AQ09 after 6 hours. 3.3 mM AQ was obtained by the strain (p01/AQ09) in a whole-cell catalytic system after 18 h (Fig.2). The result

demonstrate inactivation of peptidases and dipeptide transport system Dpp reduce the degradation of AQ and thus increase AQ production.

Screening of BacD with excellent properties

BacD is a key enzyme involved in AQ synthesis, BacD with efficient catalytic properties is screened. BacD from different species was codon optimized and synthesized by Nanjing Generay (China) and then cloned in strain AQ10 (*BW25113*, $\Delta glnEB$, $\Delta glsAB$, $\Delta lpxM$, $\Delta pepABDN$, Δdpp) separately, including *Bacillus altitudinis*, *Bacillus subtilis*, *Beta vulgaris*, *Bifidobacterium longum subsp. Infantis*, *Perkinsus marinus*, *Pseudomonas fluorescens*, *Bacillus safensis*, *Vibrio campbellii*, *Streptomyces rubrolavendulae*. The strains was used in the whole-cell bioconversion for AQ synthesis. The result showed that strain with BaBacD (from *Bacillus altitudinis*) produced higher amount of AQ (19.2 mM) than strains with other BacDs. While 5.6 mM AQ was obtained by strain with BsBacD (from *Bacillus subtilis*) (Fig.3). Although, BvBacD (from *Beta vulgaris*), VcBacD (from *Vibrio campbellii*), SrBacD (from *Streptomyces rubrolavendulae*) were well-expressed but only 3.0 mM, 1.8 mM, 0.5 mM AQ was obtained separately (Fig.3). BsaBacD (from *Bacillus safensis*), BloBacD (from *Bifidobacterium longum subsp. Infantis*), PmBacD (from *Perkinsus marinus*), PfBacD (from *Pseudomonas fluorescens*) were poor expressed and very low amount of AQ was detected.

Construction of glutamine synthetic module

To use the more readily available substrate glutamic acid, glutamine synthetase from *Corynebacterium glutamicum* (CgGlnA) which convert glutamic acid to glutamine was cloned in *E. coli* resulted in the strain p00/BW25113. 22.4 mM glutamine was obtained in the whole-cell bioconversion by p00/BW25113. During nitrogen-rich growth, glutamine synthetase adenylyltransferase /deadenylase which encoded by *glnE* interacts with PII-1 protein encoded by *glnB*, jointly reduce the activity of glutamine synthetase, the *glnE-glnB* deficiency resulted in an increase glutamine accumulation [23-25]. The *glnE-glnB*-deficient strain ($\Delta glnE\Delta glnB$) expressing *glnA* was constructed (p00/AQ02), and 27.8 mM glutamine was achieved which was 24.1% higher than that of wild-type strain (Fig.4). In *E. coli*, glutamine degrades into glutamic acid by glutaminase GlsA (encoded by *glsA*) and GlsB (encoded by *glsB*) [26], and further decomposed into α -ketoglutarate. *glsA* and *glsB* were deleted in *E. coli* resulted in strain AQ04 ($\Delta glsA\Delta glsB$), which was then transformed with plasmid p00. 33.8 mM was obtained by strain p00/AQ04 exhibited increased glutamine production after 18 hours of bioconversion. Further, *glnE*, *glnB*, *glsA*, *glsB* and *lpxM* which involved in one of the last two acylation reactions needed to synthesize KDO2-lipid A in *E. coli* was knockout, and 46.5 mM glutamine was accumulated with a conversion rate of 93.0 % (Fig.4). p00/AQ06 was used in the thereafter study.

Combination of AQ synthetic module and glutamine synthetic module

To achieve AQ production from glutamic acid and alanine, AQ synthetic module and glutamine synthetic module was combined. The strain AQ10 was obtained constructed by knocking out the genes *pepN*, *pepA*, *pepB*, *pepD*, *dpp*, *glnE*, *glnB*, *glsA*, *glsB* and *lpxM*, the degradation of AQ was alleviated, glutamine

catabolism was effectively weakened as well. And 7.9 mM AQ was obtained by the strain (p01/AQ10), which was four times as much as the original strain p01/BW25113 (Fig.2).

Plasmid co-expression BsBacD and CgGlnA was transformed in host strain AQ10. The engineered strain was used in the whole-cell biocatalysis for AQ production from alanine and cheaper substrate glutamic acid.

And 29.8 mM AQ was obtained (Fig.5) , which was much higher than that of p11/AQ09–p11/AQ06–p11/AQ04–p11/BW25113. The result showed that *glnE-glnB* deficiency and glutaminase *glsA*, *glsB* knock-out enhancing glutamine supply and the inactivation of peptidases PepA, PepB, PepD, PepN alleviate AQ degradation and transporter Dpp knock-out promote the efflux of AQ, all of that contributed to enhanced AQ production.

Regulation of the two module by balancing the protein expression

To regulation of the two module flux for improved AQ synthesis, the protein expression of BacD and GlnA was studied. BaBacD or BsBacD was co-expressed with CgGlnA. Four plasmids p11 (pYB1s-*CgglmA-BsbacD*), p12 (pYB1s-*BsbacD-CgglmA*), p13 (pYB1s-*CgglmA-BabacD*), p14 (pYB1s-*BabacD-CgglmA*) were constructed (Fig.6c), and transformed into the host AQ10 separately. Both of L-Amino Acid α -Ligase and glutamine synthetase were poorly expressed when BaBacD was co-expressed with CgGlnA, and led to a less AQ produced. However, when CgGlnA was co-expressed with BsBacD, both of the protein were well-expressed (Fig.s4), and contributed to higher yield of AQ after 18h of bioconversion, that is 29.8 mM when *CgglmA* was inserted in front of *BsbacD* (p11/AQ10) and 22.3 mM when *CgglmA* was expressed behind *BsbacD* (p12/AQ10), respectively (Fig.6a). 22.8 mM intermediate product glutamine was detected when *CgglmA* was put first, higher than that *BsbacD* was put first, which suggested that the well-expression of CgGlnA could ensure adequate supply of glutamine. The result hinted that increasing the expression of BsBacD might be tried to further improve the synthesis of AQ.

In order to enhance BsBacD expression, RBS of *BsbacD* was replaced to upregulate targeted mRNA's translation initiation rate in recombinant strain. Predicting translation rate and designing new RBS was carried out by RBS Calculator 2.0[27-29]. p15/AQ10 expressed more BsBacD (Fig.s5), AQ production increased 76.1% compared to p11/AQ10 (Fig.6b).

Optimization of the conditions of whole cell biocatalysis

The whole-cell biocatalytic conditions were investigated, including temperature, pH, glucose feeding and ratio of substrates. AQ production hit a high point at 30°C (Fig.7a) and pH 9.0 (Fig.7b). For synthesis a molecules of AQ, two molecules of ATP are needed, because BacD and GlnA are ATP dependent enzymes. ATP can be supplied by oxidation of glucose. It was known that, if there is too much glucose, acetic acid is accumulated. So three strategies of batch flow glucose was tried, 1) 50 mM glucose at once; 2) 10 mM every 3 hours; 3) 20 mM every 3 hours. When low concentration of glucose (10 mM) was fed every three hours (Fig.7c), glucose was fully utilized (Fig.7d), only a small amount of acetic acid

accumulated (Fig.7e), which hint that 10mM glucose fed every three hours matched AQ productivity. 65.6 mM AQ obtained with productivity of 7.29 mM/h and conversion rate of 65.6% after 9 hours of reaction, which is twice as high as before, by p15/AQ10, from 100 mM glutamic acid and 125 mM alanine, fed 10 mM glucose every three hours, at 30 °C and pH9.0 (Fig.7f).

Discussion

In this study, metabolic engineered *E. coli* strains for AQ production were constructed by overexpressing L-amino acid-ligase from *Bacillus subtilis*, knocking out dipeptidases PepA, PepB, PepD , PepN which involved in AQ degradation, and transporter Dpp involved in transporting AQ into cells, and 25.9 mM AQ was obtained. Glutamine can be synthesized by glutamine synthetase from glutamic acid and ammonium in a ATP-dependent manner. To use the more readily available substrate, a synthetic module from glutamic acid to glutamine was constructed by overexpressing glutamine synthetase from *Corynebacterium glutamicum*. Glutaminases GlsA-GlsB catalyzes glutamine to glutamic acid, which is the main pathway of glutamine catabolism, GlnE interacts with GlnB that undergoes the deadenylation/deuridylylation of glutamine synthetase when the intracellular nitrogen concentration is high, thus reduce the activity of glutamine synthetase. GlsA, GlsB, GlnE, GlnB were blocked which resulted in increased glutamine supply with a bioconversion of 93.0 % from glutamic acid. Then AQ synthetic module and glutamine synthetic module was combined, 29.8 mM AQ production was achieved. The two module flux were balanced better by tuning the expressing of BsBacD and CgGlnA. By replacing RBS of *BsbacD*, AQ production increased by 76.1%. Further, the whole-cell biocatalytic conditions were optimized, 65.6 mM AQ was obtained with productivity of 7.29 mM/h after 9 hours of reaction by p15/AQ10. The metabolic engineering strategies in this study can be applied in the synthesis of other high value-added dipeptides and oligopeptides, such as aspartame [30] and alitame [31].

By metabolic engineering of *E. coli* and optimization of process, a conversion rate of 65.6% from glutamic acid to AQ was obtained. BacDs have been reported to have insufficient substrate specificity to form other by-products, such as different dipeptides or longer oligopeptides [32], which undoubtedly greatly increases the cost of downstream separation and purification of target products. In this study, the extracellular concentrations of Ala-Ala reached 12.4 mM after 18 h of reaction, no other dipeptides or longer oligopeptides were detected in the conversion. It was reported that the activity of BacD was inhibited by its product AQ [33]. Further studies are underway to improve the system by screening of BacD with higher enzymatic activity and substrate specificity, and removing of AQ by dipeptide efflux pump.

Conclusions

In this study, metabolic engineering strategy of *E.coli* for the synthesis of AQ by whole-cell biocatalysis from glutamic acid and alanine was developed. The engineered *E.coli* strain, p15/AQ10, produced 65.6mM AQ with a conversion rate of 65.6% from glutamic acid. Deletion of *glnE* and *glnB* combined with

elimination of *glsA* and *glsB* increased glutamine supply. Inactivation of peptidases PepA, PepB, PepD, PepN and dipeptide transport system Dpp contributed to increasing yield of AQ. Increase the expression of BacD resulted in more AQ produced. This study showed the new opportunity of biologically industrial production of AQ. The metabolic engineering strategies in this study can be applied in the synthesis of other high value-added dipeptides and oligopeptides.

Materials And Methods

Construction of plasmids and strains

Escherichia coli K12 (BW25113) was used for protein expression. Bacteria strains and plasmids used in this study are listed in Tables 1. Target genes(*CgglA*, *BsbacD*, *BabacD*, *BlabacD*, *BvbacD*, *VcbacD*, *BlabacD*, *PfbacD*, *BlobacD*, *PmbacD*, *BsabacD*, *SrbacD*) were synthesized and codon-optimized by Generay (Shanghai, China), then ligated into pYB1a at the XhoI and EcoRI sites by the Gibson assembly method[34]. Primers used in this study are listed in Table S1. Inactivation of genes were conducted by using the CRISPR–Cas9 system[35].

Culture condition

Strains were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37 °C and 220 rpm. Antibiotics (ampicillin 100 µg/mL, or streptomycin 40 µg/mL) was added as required. For protein expression, auto-inducing ZYM medium (per liter: tryptone 10 g, yeast extract 5 g, glycerol 5 g, glucose 0.5 g, l-arabinose 2 g, Na₂HPO₄ 25 mM, KH₂PO₄ 25 mM, NH₄Cl 50 mM, Na₂SO₄ 5 mM, MgSO₄ 2 mM and trace elements containing 0.05 mM FeCl₃, 0.02 mM CaCl₂, 0.01 mM MnCl₂, 0.01 mM ZnSO₄, and 0.002 mM each of CoCl₂, NiCl₂, Na₂Mo₄, Na₂SeO₃, and H₃BO₃) was used[36], and the strains induced at 30 °C for 12–16 h.

Whole-cell biocatalysis conditions

Cells after induction were harvested by centrifugation at 4200 rpm for 10 min and washed with 0.85% NaCl solution once and then used in whole-cell biocatalysis for the production of AQ [37]. For AQ synthesis from glutamine and alanine, the conversion system (pH7.0) contained 50 mM MOPS (morpholine propane sulfonic acid) buffer, 50mM glutamine, 50 mM alanine, 10 mM magnesium chloride, 50 mM glucose to form a cell suspension from a starting biomass of OD_{600nm} = 30. The bioconversion reaction was performed at 30°C and 220 rpm in a test tube. Glucose was supplemented frequently 10mM every three hours. For glutamine synthesis, the cells were suspended in 1 mL bioconversion medium (pH7.0) containing 50 mM MOPS buffer, 50mM sodium glutamate, 100 mM ammonium chloride, 10 mM magnesium chloride, 50 mM glucose to form a cell suspension from a starting biomass of OD_{600nm} = 30. For AQ production from glutamic acid and alanine, the conversion system (pH9.0) contained 50 mM MOPS buffer, 100mM sodium glutamate, 100 mM alanine, 100 mM ammonium chloride, 10 mM magnesium chloride, 50 mM glucose.

In the process of optimizing the whole-cell catalytic conditions, the bioconversion reaction was performed at different temperature (range from 20-45°C) and pH (6.0-11.0). When optimizing the strategy of glucose feeding, three different strategies were investigated: 1) 50 mM glucose at one time; 2) 50 mM glucose, fed in five times, 10mM every 3 hours; 3) 100 mM glucose, fed in five times, 20 mM every 3 hours. When the concentration of substrate was studied, different concentration of alanine was added once ranging from 100mM to 200mM, and 100 mM sodium glutamate concentration was added.

Analytical methods

Cell density was estimated by measuring the optical density at 600 nm. Proteins expression was analyzed by SDS-PAGE. The concentrations of glucose and acetate in the supernatant were determined by HPLC equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion column (7.8×300 mm; Hercules, CA, USA), and refractive index detector. Analysis was performed at 65 °C with a mobile phase of 5 mM H₂SO₄ at a flow rate of 0.5 mL/min.

Dipeptides AQ and amino acids including glutamine, glutamic acid, alanine, were derivatized using 9-fluorenylmethoxy carbonyl chloroformate and measured by HPLC as Kazuhiko Tabata described[38]. The method was modified. The mobile phase A and B were adjusted to acetonitrile and 50mM sodium acetate, the gradient program was slightly modified as follows: 0 min, solvent A-solvent B at 10:90; 0 to 20 min, a linear increase in solvent A to A-B at 60:40; 20 to 24 min, a linear increase to A-B at 100:0; 24 to 27 min, held at A-B at 100:0; 27 to 28 min, a linear decrease in solvent B to A-B at 10:90. The column temperature was set at 30°C. 5 µL of sample was injected to the HPLC system for analysis, the flow rate was set at 0.6 mL/min.

Declarations

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Additional file

Additional file : Table S1. Primers used in this study. Table S2. Sequences of three RBS fragments. Figure S1. HPLC chromatogram of AQ production by engineered strain. Figure S2. Expression of strain p00/BW25113. Figure S3. Expression of BacD from different species. Figure S4. Expression of engineered strains co-expressing BaBacD /BsBacD and CgGlnA. Figure S5. Expression of engineered strains with different RBS of BsbacD.

Abbreviations

AQ, L-alanyl-L-glutamine; GlnA, glutamine synthetase; BacD, L-Amino Acid α -Ligase;

SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Authors' contributions

J-M Z carried out the main work, collected and analyzed the data, and drafted the manuscript. WY, B-H W, QL, X-T Z, Q-X G, J-Z L participated in the research. B-XL supervised the work and participated in data analysis, and revised the manuscript. J-Z H and Y T participated in the conception and design of the study and finalized the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional file.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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References

1. Stehle P, Kuhn KS: **Glutamine: an obligatory parenteral nutrition substrate in critical care therapy.** *Biomed Res Int* 2015, 2015:545467.
2. Labow BI, Souba WW: **Glutamine.** *World Journal of Surgery* 2014, 24:1503-1513.
3. Fuentes-Orozco C, Anaya-Prado R, González-Ojeda A, Arenas-Márquez H, Cabrera-Pivaral C, Cervantes-Guevara G, Barrera-Zepeda LM: **L-Alanyl-L-glutamine-supplemented parenteral nutrition**

- improves infectious morbidity in secondary peritonitis.** *Clinical Nutrition* 2004, 23:13-21.
4. Laserra JLF, Carmona TG: **L-Alanyl-L-glutamine Dipeptide-Supplemented Total Parenteral Nutrition.** In *Diet and Nutrition in Critical Care*. Edited by Rajendram R, Preedy VR, Patel VB. New York, NY: Springer New York; 2014: 1-11.
 5. Moore SR, Guedes MM, Costa TB, Vallance J, Maier EA, Betz KJ, Aihara E, Mahe MM, Lima AA, Oria RB, Shroyer NF: **Glutamine and alanyl-glutamine promote crypt expansion and mTOR signaling in murine enteroids.** *Am J Physiol Gastrointest Liver Physiol* 2015, 308:G831-839.
 6. Mundi MS, Shah M, Hurt RT: **When Is It Appropriate to Use Glutamine in Critical Illness?** *Nutr Clin Pract* 2016, 31:445-450.
 7. R. R. W. J. van der Hulst, Meyenfeldt, M. F. V. , & Soeters, P. B. . (0). **Glutamine: a gut essential amino acid.**
 8. Zhou Y, Zhang P, Deng G, Liu X, Lu D: **Improvements of immune status, intestinal integrity and gain performance in the early-weaned calves parenterally supplemented with L-alanyl-L-glutamine dipeptide.** *Vet Immunol Immunopathol* 2012, 145:134-142.
 9. Furst, P. , Pogan, K. , & Stehle, P. . (1997). **Glutamine dipeptides in clinical nutrition.** *Nutrition*, 13(7–8), 731-737.
 10. Li, S. L. , Li, C. , Liu, Y. S. , Wang, X. L. , & Cao, Z. A. . (2003). **Separation of (l)-glutamine from fermentation broth by nanofiltration.** *Journal of Membrane Science*, 222(1-2), 191-201.
 11. Tan B, Liu H, He G, Xiao H, Xiao D, Liu Y, Wu J, Fang J, Yin Y: **Alanyl-glutamine but not glycyl-glutamine improved the proliferation of enterocytes as glutamine substitution in vitro.** *Amino Acids* 2017, 49:2023-2031.
 12. Sano T, Sugaya T, Inoue K, Mizutaki S I , & Kasai M: **Cheminform abstract: process research and development of l-alanyl-l-glutamine, a component of parenteral nutrition.** *ChemInform*, 31(39).
 13. Neuh User-Berthold M , Eufinger H: **Utilization of alanyl-L-glutamine and glycyl-L-glutamine during long-term parenteral nutrition in the growing rat.** *Amino Acids* (Vienna), 1992, 3(3):293-302.
 14. Yokozeki K, Hara S: **A novel and efficient enzymatic method for the production of peptides from unprotected starting materials.** *J Biotechnol* 2005, 115:211-220.
 15. Fields G B, Noble R L: **Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids.** *International Journal of Peptide and Protein Research*, 35(3), 161-214.
 16. Wang M, Qi W, Yu Q, Su R, He Z: **Kinetically controlled enzymatic synthesis of dipeptide precursor of L-alanyl-L-glutamine.** *Biotechnol Appl Biochem* 2011, 58:449-455.
 17. Akabori, S., S. Sakakibara, and Y. Shimonishi. 1961. **Protection of amidinitrogen for peptide synthesis. A novel synthesis of peptide containing Cterminal glutamine.** *Bull. Chem. Soc. Jpn.* 34:739.
 18. Li Y, Yuan W, Gao J, Fan C, Wu W, Bai F: **Production of l-alanyl-l-glutamine by recycling E. coli expressing alpha-amino acid ester acyltransferase.** *Bioresour Technol* 2017, 245:1603-1609.

19. Tanaka T, Takagi K, Saddam HM, Takeda Y, Wakayama M: **Purification and Characterization of Elizabethkingia L-Amino Acid Esterase: an Enzyme Useful for Enzymatic Synthesis of the Dipeptide, Valyl-Glycine.** *Appl Biochem Biotechnol* 2017, 183:362-373.
20. Tabata K, Hashimoto S: **Fermentative production of L-alanyl-L-glutamine by a metabolically engineered Escherichia coli strain expressing L-amino acid alpha-ligase.** *Appl Environ Microbiol* 2007, 73:6378-6385.
21. Lutz W, Jazdzewski B: **Degradation of intracellular proteins.** *Pol Tyg Lek*, 35(33), 1275-1278.
22. Smith, M. W. , Tyreman, D. R. , Payne, G. M. , Marshall, N. J. , & Payne, J. W. . (1999). **Substrate specificity of the periplasmic dipeptide-binding protein from escherichia coli: experimental basis for the design of peptide prodrugs.** *Microbiology*, 145 (Pt 10)(10), 2891.
23. Jiang P , Ninfa A J: **Escherichia coli PII signal transduction protein controlling nitrogen assimilation acts as a sensor of adenylate energy charge in vitro.** *Biochemistry*, 2007, 46(45):12979-96.
24. Jaggi, R: **The two opposing activities of adenylyl transferase reside in distinct homologous domains, with intramolecular signal transduction.** *EMBO (European Molecular Biology Organization) Journal*, 1997, 16(18):5562-5571.
25. Pahel G, Tyler B: **A new glnA-linked regulatory gene for glutamine synthetase in Escherichia coli.** *Proc Natl Acad Sci U S A* 1979, 76:4544-4548.
26. Brown, G. , Singer, A. , Proudfoot, M. , Skarina, T. , Kim, Y. , & Chang, C. , et al. (2008). **Functional and structural characterization of four glutaminases from escherichia coli and bacillus subtilis.** *Biochemistry*, 47(21), 5724-35.
27. Espah Borujeni A, Channarasappa AS, Salis HM: **Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites.** *Nucleic Acids Res* 2014, 42:2646-2659.
28. Espah Borujeni A, Salis HM: **Translation Initiation is Controlled by RNA Folding Kinetics via a Ribosome Drafting Mechanism.** *J Am Chem Soc* 2016, 138:7016-7023.
29. Espah Borujeni A, Cetnar D, Farasat I, Smith A, Lundgren N, Salis HM: **Precise quantification of translation inhibition by mRNA structures that overlap with the ribosomal footprint in N-terminal coding sequences.** *Nucleic Acids Res* 2017, 45:5437-5448.
30. Tordoff, M. G. , & Alleva, A. M. . (1990). **Effect of drinking soda sweetened with aspartame or high-fructose corn syrup on food intake and body weight.** *American Journal of Clinical Nutrition*, 51(6), 963.
31. Glowaky, R. C. (2009). **Development and Uses of Alitame.** *Acs Symposium Series*.
32. Yokozeki, K. , & Hara, S. . (2005). **A novel and efficient enzymatic method for the production of peptides from unprotected starting materials.** *Journal of Biotechnology*, 115(2), 211-220.
33. Hayashi M, Tabata K, Yagasaki M, Yonetani Y: **Effect of multidrug-efflux transporter genes on dipeptide resistance and overproduction in Escherichia coli.** *FEMS Microbiol Lett* 2010, 304:12-19.

34. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases.** *Nat Methods* 2009, 6:343-345.
35. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F: **Genome engineering using the CRISPR-Cas9 system.** *Nat Protoc* 2013, 8:2281-2308.
36. Studier, F. W. . (2005). **Protein production by auto-induction in high-density shaking cultures.** *Protein Expr Purif*, 41(1), 207-234.
37. Lin B, Tao Y. **Whole-cell biocatalysts by design.** *Microb Cell Fact.*2017;16:106.
38. 38. Tabata K, Ikeda H, Hashimoto S: **ywfE in Bacillus subtilis codes for a novel enzyme, L-amino acid ligase.** *J Bacteriol* 2005, 187:5195-5202.

Table

Table.1 Strains and plasmids used in this study

Strains/plasmids	Characteristics	Source
Strains		
<i>E.coli</i> BW25113	<i>IacI^qrrnB_{T14}ΔlacZ_{WJ16}hsdR514ΔaraBAD_{AH33}ΔrhaBAD_{LD78}</i>	Invitrogen
AQ02	<i>E. coli</i> BW25113, Δ <i>glnE</i> , Δ <i>glnB</i>	This study
AQ04	<i>E. coli</i> BW25113, Δ <i>glsA</i> , Δ <i>glsB</i>	This study
AQ06	<i>E. coli</i> BW25113, Δ <i>glnE</i> , Δ <i>glnB</i> , Δ <i>glsA</i> , Δ <i>glsB</i> , Δ <i>lpxM</i>	This study
AQ09	<i>E. coli</i> BW25113, Δ <i>pepA</i> , Δ <i>pepB</i> , Δ <i>pepD</i> , Δ <i>pepN</i> , Δ <i>dpp</i>	This study
AQ10	<i>E. coli</i> BW25113, Δ <i>glnE</i> , Δ <i>glnB</i> , Δ <i>glsA</i> , Δ <i>glsB</i> , Δ <i>lpxM</i> , Δ <i>pepA</i> , Δ <i>pepB</i> , Δ <i>pepD</i> , Δ <i>pepN</i> , Δ <i>dpp</i>	This study
p11/AQ10	AQ10 expressing p11	This study
p12/AQ10	AQ10 expressing p12	This study
p13/AQ10	AQ10 expressing p13	This study
p14/AQ10	AQ10 expressing p14	This study
p15/AQ10	AQ10 expressing p15	This study
p16/AQ10	AQ10 expressing p16	This study
Plasmids		
pYB1a	P15A origin, P <i>bad</i> promoter, Amp ^R	Our lab
pYB1s	P15A origin, P <i>bad</i> promoter, Str ^R	Our lab
p00	<i>glnA</i> from <i>Corynebacterium glutamicum</i> cloned into pYB1a	This study
p01	<i>bacD</i> from <i>Bacillus subtilis</i> cloned into pYB1a	This study
p02	<i>bacD</i> from <i>Bacillus altitudinis</i> cloned into pYB1a	This study
p03	<i>bacD</i> from <i>Beta vulgaris</i> cloned into pYB1a	This study
p04	<i>bacD</i> from <i>Vibrio campbellii</i> cloned into pYB1a	This study
p05	<i>bacD</i> from <i>Streptomyces rubrolavendulae</i> cloned into pYB1a	This study
p06	<i>bacD</i> from <i>Bacillus safensis</i> cloned into pYB1a	This study
p07	<i>bacD</i> from <i>Bifidobacterium longum subsp. Infantis</i> cloned into pYB1a	This study
p08	<i>bacD</i> from <i>Brevibacillus laterosporus</i> cloned into pYB1a	This study
p09	<i>bacD</i> from <i>Perkinsus marinus</i> cloned into pYB1a	This study
p10	<i>bacD</i> from <i>Pseudomonas fluorescens</i> cloned into pYB1a	This study
p11	<i>CgglmA-BsbacD</i> cloned into pYB1s	This study
p12	<i>BsbacD-CgglmA</i> cloned into pYB1s	This study
p13	<i>CgglmA-BabacD</i> cloned into pYB1s	This study
p14	<i>BabacD-CgglmA</i> cloned into pYB1s	This study

Figures

Figure 1

Schematic presentation of AQ metabolic pathway in engineered *E. coli*. Genes of *glnA* and *bacD* are co-expressed. Discontinued arrows represent the enzymatic activities that have been eliminated. GlsA, glutaminase 1; GlsB, glutaminase 2; GlnE, fused glutamine synthetase deadenylase /glutamine synthetase adenylyltransferase; GlnB, nitrogen regulatory protein PII-1; PepA, aminopeptidase A/I; PepB, aminopeptidase B; Pep-D, peptidase D; Pep-N, aminopeptidase N; Dpp, dipeptide ABC transporter DppABCDF.

Figure 2

Production of AQ by engineered strains overexpressing BsBacD. WT, wild type; BW, AQ06, AQ09, AQ10 were transformed with plasmid p01. Engineered host strains were induced and suspended in a reaction mixture containing 50mM glutamine, 50 mM alanine, 10 mM magnesium chloride. The bioconversion reactions were performed at 30 °C and 200 rpm for 18 h. Glucose was supplemented frequently 10mM every three hours.

Figure 3

AQ production by engineered strains over-expressing BacD from different species. Ba, *Bacillus altitudinis*; Bs, *Bacillus subtilis*; Bv, *Beta vulgaris*; Vc, *Vibrio campbellii*; Pm, *Perkinsus marinus*; Sr, *Streptomyces rubrolavendulae*; Bsa, *Bacillus safensis*; Blo, *Bifidobacterium longum* subsp. *Infantis*; Pf, *Pseudomonas fluorescens*.

Figure 4

Production of glutamine by engineered strains overexpressing GlnA. WT, wild type; BW, AQ02, AQ04, AQ06 were transformed with plasmid p00. Engineered host strains were induced and suspended in a reaction mixture containing 50mM sodium glutamate, 10 mM magnesium chloride, 100 mM ammonium chloride. The bioconversion reactions were performed at 30 °C and 200 rpm for 18 h. Glucose was supplemented frequently 10mM every three hours.

Figure 5

Production of AQ and glutamine by engineered strains co-expressing CgGlnA and BsBacD. WT, wild type; BW, AQ02, AQ04, AQ06 were transformed with plasmid p11. Engineered host strains were induced and suspended in a reaction mixture containing 100mM sodium glutamate, 100 mM alanine, 200 mM ammonium chloride, 10 mM magnesium chloride. The bioconversion reactions were performed at 30 °C and 200 rpm for 18 h. Glucose was supplemented frequently 10mM every three hours.

Figure 6

Regulation of the two module by balancing the protein expression. a Production of AQ in the bioconversions by engineered strains with different expression of BacD and CgglnA. p11, pYB1s-CgglnA-BsbacD; p12, pYB1s-BsbacD-CgglnA; p13, pYB1s-CgglnA-BabacD; p14, pYB1s-BabacD-CgglnA. p11, p12, p13, p14 were transformed into AQ10. b AQ production by strains with different RBS of BsbacD. CK, p11/AQ10. c Modular expression of genes CgglnA and bacD in a ara-operon configuration.

Figure 7

Optimization of the conditions of whole cell catalysis. a AQ production by p15/AQ10 in different pH. b AQ production by p15/AQ10 at different temperature. c AQ production under different glucose feeding strategies. Feeding strategy: 1) 50 mM glucose at once; 2) 50 mM glucose, 10 mM every 3 hours; 3) 100 mM glucose, 20 mM every 3 hours. d Residual glucose under different feeding strategies. e Acetate concentration under different feeding strategies. f AQ production with different initial alanine concentration.