

Article

An Efficient Prephenate Dehydrogenase Gene for the Biosynthesis of L-tyrosine: Gene Mining, Sequence Analysis, and Expression Optimization

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Abstract: L-tyrosine is a key precursor for synthesis of various functional substances, but the microbial production of L-tyrosine faces huge challenges. The development of new microbial chassis cell and gene resource is especially important for the biosynthesis of L-tyrosine. In this study, the optimal host strain *Bacillus amyloliquefaciens* HZ-12 was firstly selected by detecting the production capacity of L-tyrosine. Subsequently, the recombinant expression of 15 prephenate dehydrogenase genes led to the discovery of the best gene, *Bao-tyrA* from *B. amyloliquefaciens* HZ-12. After the overexpression of *Bao-tyrA*, the L-tyrosine yield of the recombinant strain HZ/P43-*Bao-tyrA* reach 411 mg/L, increased by 42% compared with the control strain (HZ/pHY300PLK). Moreover, the nucleic acid sequence and deduced amino acid sequence of the gene *Bao-tyrA* were analyzed, and their conservative sites and catalytic mechanisms were proposed. Finally, the expression of *Bao-tyrA* was regulated through a promoter and 5'-UTR sequence to obtain the optimal expression elements. Thereby, the maximum L-tyrosine yield of 475 mg/L was obtained from HZ/P43-UTR3-*Bao-tyrA*. *B. amyloliquefaciens* was applied for the first time to produce L-tyrosine, and the optimal prephenate dehydrogenase gene *Bao-tyrA* and corresponding expression elements were obtained. This study provides new microbial host and gene resource for the construction of efficient L-tyrosine chassis cells, and also lays a solid foundation for the production of various functional tyrosine derivatives.

Keywords: *Bacillus amyloliquefaciens*; L-tyrosine; prephenate dehydrogenase; recombinant expression; sequence analysis; element regulation



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1. Introduction

The aromatic amino acid L-tyrosine is a nutritionally essential amino acid for humans, and it has been widely used in food additives, dietary supplements, pharmaceuticals, and chemicals [1–3]. Moreover, L-tyrosine is a common precursor for the synthesis of various high-value-added natural active substances such as salvianic acid A, resveratrol, caffeic acid, hydroxytyrosol, salidroside, curcumins, benzylisoquinoline alkaloids (BIAs), and so on [4–9]. These derivatives are endowed with various functions, such as antioxidant, anti-coagulation and anti-inflammatory activities [10,11]. Therefore, L-tyrosine has prospects for broad application as a platform compound, and the demand for nutritional chemicals derived from L-tyrosine is constantly increasing [12–15]. For example, the market-sharing of the common L-tyrosine derivatives of flavonoids will reach 1.26 billion USD by 2026 according to market research reports [16].

Most nutritional chemicals are extracted from plants, and low extraction efficiency cannot adequately meet the growing demand for these naturally active substances [17,18].

Chemical synthesis methods are also applied for nutritional chemicals. However, chemical synthesis processes usually cause problems of environmental pollution, high energy consumption, the generation of different toxic intermediates, and the formation of unstable intermediates, which can lead to a noticeable decrease in the desired chemical productivity and economic yield [16]. Fortunately, green and sustainable biosynthesis methods can provide an alternative strategy for producing valuable chemicals in an environmentally friendly manner [19,20]. These sustainable biosynthesis methods also present the advantages of shorter process cycles, higher efficiency, and simpler extraction processes [21]. At present, the synthesis of various high-value functional nutritional chemicals, such as salvianic acid A and (2S)-naringenin have been achieved [22,23]. An alternative way to produce nutrient chemicals is to develop chassis cells with high yield of L-tyrosine and then introduce different synthesis modules, which results in the synthesis of natural active substances [5,8]. Therefore, the construction of efficient L-tyrosine chassis cells is important for the production of nutritional chemicals derived from L-tyrosine.

Although L-tyrosine has broad applications in the market, the excessive production of L-tyrosine by microorganisms has been a huge challenge to overcome due to the complex gene regulation [24,25]. At present, the L-tyrosine synthesis pathway has been obtained for most microorganisms [16,21,26]. The formation of 4-hydroxyphenylpyruvate from prephenate through oxidative decarboxylation is an important reaction for the synthesis of L-tyrosine [27–29]. The dehydrogenases specially used for L-tyrosine biosynthesis are the TyrA protein family, including prephenate dehydrogenase, cyclohexadienyl dehydrogenase, and aromatic acid dehydrogenase [30–32]. At present, the prephenate dehydrogenase is used in the production of L-tyrosine in several microorganisms by heterologous expression, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Bacillus licheniformis*, etc. [3,24,33]. Xu et al. overexpressed the gene *tyrA* from *B. licheniformis*, indicating that the expression level of *tyrA* plays an important role in improving the production of L-tyrosine [34].

The main microorganism currently used to produce L-tyrosine is *E. coli*, and the existence of endotoxins leads to it being difficult to apply in the food industry [35]. Compared to other microorganisms, *Bacillus* has become an alternative strain [36]. *Bacillus* is a class of Gram-positive bacteria widely existing in nature, which can produce spores in harsh environments. In addition, *Bacillus* species have the advantages of easy cultivation, clear inherited backgrounds, convenient gene modification, short fermentation cycle, and robustness in industrial fermentation [37]. These characteristics have caused *Bacillus* species to be the preferred organisms for the industrial production of various products, such as platform chemicals. Thereby, *Bacillus subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* have been designated Generally Recognized As Safe (GRAS) strains by the US Food and Drug Administration (FDA) [37], and they have been widely used in the production of nutritional chemicals, such as riboflavin [38], spermidine [39], S-adenosylmethionine [40], N-acetylglucosamine, N-acetylneuraminic acid, chondroitin, poly-gamma-glutamic acid, and so on [41]. Additionally, *Bacillus* species can produce a wide range of products after genetic modification using novel synthetic biology strategies and efficient genetic engineering tools [42]. Therefore, it is believed that the production of L-tyrosine by *Bacillus* species is feasible and of great significance.

In this study, a series of food-safe *Bacillus* strains were evaluated to select the optimal host strain for L-tyrosine production, and the recombinant expression of 15 prephenate dehydrogenase-related genes was carried out. The optimal prephenate dehydrogenase gene and corresponding expression element suitable for the host strain were obtained, and its catalytic mechanism was further clarified. L-tyrosine is a key precursor for the synthesis of various functional substances. This study can provide new *Bacillus* host and gene resources for the construction of efficient L-tyrosine chassis cells, which can laid a solid foundation for production of various bioactive derivatives from tyrosine.

2. Materials and Methods

2.1. Strains and Plasmids

All the constructed strains and plasmids in the present study are shown in Table 1. The *B. amyloliquefaciens*-engineered strains were constructed based on the native strain of *B. amyloliquefaciens* HZ-12. All expression vectors derived from pHY300PLK were constructed and prepared using *E. coli* DH5 α as the host strain. The corresponding primers used in the present study are shown in Table S1 in the Supplementary Materials section.

Table 1. Strains and plasmids used in this study.

Strains or Plasmids	Characteristics	Source
<i>Bacillus amyloliquefaciens</i> HZ-12	Wild type	stored in lab
<i>Bacillus subtilis</i> 168	<i>trpC2</i>	stored in lab
<i>Bacillus subtilis</i> SCK6	Erm ^R , 1A751 derivative, <i>lacA::P_{xyIA}-comK</i>	stored in lab
<i>Bacillus subtilis</i> WB800	<i>nprE aprE epr bpr npr::ble nprB::bsr Δvpr wprA::hyg</i>	stored in lab
<i>Bacillus licheniformis</i> JY1	Wild type	stored in lab
<i>Bacillus licheniformis</i> JY3	Wild type	stored in lab
<i>Bacillus licheniformis</i> DW2	Wild type	stored in lab
<i>Bacillus licheniformis</i> WX-02	Wild type	stored in lab
<i>B. licheniformis</i> ATCC14580	Wild type	stored in lab
<i>B. amyloliquefaciens</i> JY6	Wild type	stored in lab
<i>B. amyloliquefaciens</i> AQ	Wild type	stored in lab
HZ/pHY300PLK	HZ-12 with pHY300PLK	this study
HZ/P43- <i>Eco-tyrA</i>	HZ-12 with pHY-P43- <i>Eco-tyrA</i>	this study
HZ/P43- <i>Bao-tyrA</i>	HZ-12 with pHY-P43- <i>Bao-tyrA</i>	this study
HZ/P43- <i>Bld-tyrA</i>	HZ-12 with pHY-P43- <i>Bld-tyrA</i>	this study
HZ/P43- <i>Bsu-tyrA</i>	HZ-12 with pHY-P43- <i>Bsu-tyrA</i>	this study
HZ/P43- <i>Btb-tyrA</i>	HZ-12 with pHY-P43- <i>Btb-tyrA</i>	this study
HZ/P43- <i>Bpum-tyrA</i>	HZ-12 with pHY-P43- <i>Bpum-tyrA</i>	this study
HZ/P43- <i>Cgb-tyrA</i>	HZ-12 with pHY-P43- <i>Cgb-tyrA</i>	this study
HZ/P43- <i>Sce-tyrA</i>	HZ-12 with pHY-P43- <i>Sce-tyrA</i>	this study
HZ/P43- <i>Lpt-tyrA</i>	HZ-12 with pHY-P43- <i>Lpt-tyrA</i>	this study
HZ/P43- <i>Bcoa-tyrA</i>	HZ-12 with pHY-P43- <i>Bcoa-tyrA</i>	this study
HZ/P43- <i>Bce-tyrA</i>	HZ-12 with pHY-P43- <i>Bce-tyrA</i>	this study
HZ/P43- <i>Bmh1-tyrA</i>	HZ-12 with pHY-P43- <i>Bmh1-tyrA</i>	this study
HZ/P43- <i>Bmh2-tyrA</i>	HZ-12 with pHY-P43- <i>Bmh2-tyrA</i>	this study
HZ/P43- <i>Zmc-tyrA</i>	HZ-12 with pHY-P43- <i>Zmc-tyrA</i>	this study
HZ/P43- <i>Pap-tyrA</i>	HZ-12 with pHY-P43- <i>Pap-tyrA</i>	this study
HZ/P43- <i>Psrfa-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>Psrfa-Bao-tyrA</i>	this study
HZ/P43- <i>PytzE-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PytzE-Bao-tyrA</i>	this study
HZ/P43- <i>PylB-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PylB-Bao-tyrA</i>	this study
HZ/P43- <i>Pbay-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>Pbay-Bao-tyrA</i>	this study
HZ/P43- <i>PykzA-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PykzA-Bao-tyrA</i>	this study
HZ/P43- <i>PRBS6-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PRBS6-Bao-tyrA</i>	this study
HZ/P43- <i>PmmgA-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PmmgA-Bao-tyrA</i>	this study
HZ/P43- <i>PabrB-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PabrB-Bao-tyrA</i>	this study
HZ/P43- <i>PbacA-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PbacA-Bao-tyrA</i>	this study
HZ/P43- <i>P12-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>P12-Bao-tyrA</i>	this study
HZ/P43- <i>PylB-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>yIB-Bao-tyrA</i>	this study
HZ/P43- <i>PR5-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PR5-Bao-tyrA</i>	this study
HZ/P43- <i>PRBS6-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>PRBS6-Bao-tyrA</i>	this study
HZ/P43- <i>UTR1-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR1-Bao-tyrA</i>	this study
HZ/P43- <i>UTR2-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR2-Bao-tyrA</i>	this study
HZ/P43- <i>UTR3-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR3-Bao-tyrA</i>	this study
HZ/P43- <i>UTR4-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR4-Bao-tyrA</i>	this study
HZ/P43- <i>UTR5-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR5-Bao-tyrA</i>	this study
HZ/P43- <i>UTR6-Bao-tyrA</i>	HZ-12 with pHY-P43- <i>UTR6-Bao-tyrA</i>	this study
pHY300PLK	<i>E. coli</i> - <i>Bacillus</i> shuttle vector for gene expression, Ap ^r , Tet ^r	stored in lab

Table 1. Cont.

Strains or Plasmids	Characteristics	Source
pHY-P43- <i>Eco-tyrA</i>	pHY300PLK + P43 + <i>Eco-tyrA</i> + TamyL	this study
pHY-P43- <i>Bao-tyrA</i>	pHY300PLK + P43 + <i>Bao-tyrA</i> + TamyL	this study
pHY-P43- <i>Bld-tyrA</i>	pHY300PLK + P43 + <i>Bld-tyrA</i> + TamyL	this study
pHY-P43- <i>Bsu-tyrA</i>	pHY300PLK + P43 + <i>Bsu-tyrA</i> + TamyL	this study
pHY-P43- <i>Btb-tyrA</i>	pHY300PLK + P43 + <i>Btb-tyrA</i> + TamyL	this study
pHY-P43- <i>Bpum-tyrA</i>	pHY300PLK + P43 + <i>Bpum-tyrA</i> + TamyL	this study
pHY-P43- <i>Cgb-tyrA</i>	pHY300PLK + P43 + <i>Cgb-tyrA</i> + TamyL	this study
pHY-P43- <i>Sce-tyrA</i>	pHY300PLK + P43 + <i>Sce-tyrA</i> + TamyL	this study
pHY-P43- <i>Lpt-tyrA</i>	pHY300PLK + P43 + <i>Lpt-tyrA</i> + TamyL	this study
pHY-P43- <i>Bcoa-tyrA</i>	pHY300PLK + P43 + <i>Bcoa-tyrA</i> + TamyL	this study
pHY-P43- <i>Bce-tyrA</i>	pHY300PLK + P43 + <i>Bce-tyrA</i> + TamyL	this study
pHY-P43- <i>Bmh1-tyrA</i>	pHY300PLK + P43 + <i>Bmh1-tyrA</i> + TamyL	this study
pHY-P43- <i>Bmh2-tyrA</i>	pHY300PLK + P43 + <i>Bmh2-tyrA</i> + TamyL	this study
pHY-P43- <i>Zmc-tyrA</i>	pHY300PLK + P43 + <i>Zmc-tyrA</i> + TamyL	this study
pHY-P43- <i>Pap-tyrA</i>	pHY300PLK + P43 + <i>Pap-tyrA</i> + TamyL	this study
pHY- <i>PsrfA-Bao-tyrA</i>	pHY300PLK + <i>PsrfA</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PytzE-Bao-tyrA</i>	pHY300PLK + <i>PytzE</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PylB-Bao-tyrA</i>	pHY300PLK + <i>PylB</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>Pbay-Bao-tyrA</i>	pHY300PLK + <i>Pbay</i> + <i>Bao-tyrA</i> + TamyL	this study
HZ/ <i>PykzA-Bao-tyrA</i>	pHY300PLK + <i>PykzA</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PykzA-RBS6-Bao-tyrA</i>	pHY300PLK + <i>PykzA-PRBS6</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PmmgA-Bao-tyrA</i>	pHY300PLK + <i>PmmgA</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PabrB-Bao-tyrA</i>	pHY300PLK + <i>PabrB</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY- <i>PbacA-Bao-tyrA</i>	pHY300PLK + <i>PbacA</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY-P12- <i>Bao-tyrA</i>	pHY300PLK + P12 + <i>Bao-tyrA</i> + TamyL	this study
pHY-P43- <i>ylB-Bao-tyrA</i>	pHY300PLK + P43- <i>PylB</i> + <i>Bao-tyrA</i> + TamyL	this study
pHY-PR5- <i>Bao-tyrA</i>	pHY300PLK + PR5 + <i>Bao-tyrA</i> + TamyL	this study
pHY-PRBS6- <i>Bao-tyrA</i>	pHY300PLK + PRBS6 + <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR1- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR1- <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR2- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR2- <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR3- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR3- <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR4- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR4- <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR5- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR5- <i>Bao-tyrA</i> + TamyL	this study
pHY-P43-UTR6- <i>Bao-tyrA</i>	pHY300PLK + P43 + UTR6- <i>Bao-tyrA</i> + TamyL	this study

2.2. Recombinant Expression of the Prephenate Dehydrogenase Gene *tyrA*

The cloned prephenate dehydrogenase gene was identified using heterologous expression in *B. amyloliquefaciens* HZ-12 following the procedure reported in our previous study. Thereby, taking the gene *Bao-tyrA* expression strain as an example, the gene fragment of *Bao-tyrA* was amplified with a pair of primers, *Bao-tyrA*-F and *Bao-tyrA*-R, using the *B. amyloliquefaciens* HZ-12 DNA template, and then the gene *Bao-tyrA* was fused with the P43 promoter amplified from *B. subtilis* 168 and the TamyL terminator obtained from *B. licheniformis* WX-02 using overlap extension PCR (SOE-PCR). After digestion of fusion fragment and pHY300PLK plasmid by restriction enzymes of *Bam*HI and *Xba*I, they were ligated to obtain the expression plasmid pHY-P43-*Bao-tyrA*. Finally, this expression plasmid was electro-transformed into the competent cells of *B. amyloliquefaciens* HZ-12, generating the recombinant strain HZ/P43-*Bao-tyrA*. Other recombinant strains were obtained using the same procedure in this study. Moreover, synthetic 5'-UTRs were designed using UTR Library Designer (http://sbi.postech.ac.kr/utr_library, accessed on 6 July 2022) to fine-tune gene expression levels [43].

2.3. Chemicals

In this study, the TransStartFastPfu DNA polymerase and TransStartR easyTaq DNA polymerase were purchased from TransGen Biotech Co., Ltd. (Beijing, China). DNA restriction enzymes, T4 ligase, dNTPs, RNase, and DL5000 Marker were provided by

Takara Biotechnology Co., Ltd. (Dalian, China). The DNA recovery kit and plasmid extraction kit were bought from Omega Bio-Tek, Guangzhou, China. Other chemicals were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.4. Determination of L-tyrosine

To measure L-tyrosine, 1 mL of fermentation broth was vortexed with 0.6 mL of 1 M HCl for 1 h, and centrifuged at $10,000\times g$ for 3 min. The supernatant was then filtered using a 0.22 μm membrane. The HPLC analysis conditions are listed as follows: Agilent 1100 HPLC chromatograph, ZORBAX Eclipse XDB-C18 (4.6 mm \times 250 mm, 5 μm) column, mobile phase of 10% methanol, 90% sodium acetate (100 mM, pH 4.0), flow rate 0.6 mL/min, injection volume 10 μL , column temperature 30 $^{\circ}\text{C}$, and UV detector with a detection wavelength of 280 nm. The L-tyrosine standard was used to calculate the concentration.

2.5. L-tyrosine Fermentation

The bacteria cells were transferred into 50 mL LB liquid medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. After culture at 37 $^{\circ}\text{C}$ and 180 rpm for 12 h, the seed cultures were obtained. Then, 3% (*v/v*) seed cultures were inoculated into the 50 mL L-tyrosine fermentation medium containing 22 g/L glucose, 3 g/L $(\text{NH}_4)_2\text{SO}_4$, 6.75 g/L K_2HPO_4 , 1.25 g/L KH_2PO_4 , 1.5 g/L $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5 g/L sodium citrate, 3 g/L peptone, 6 g/L yeast extract, 4 g/L $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 4 g/L CaCl_2 , 1 g/L $\text{MnSO}_4\cdot 5\text{H}_2\text{O}$, 0.4 g/L $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 0.2 g/L $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 0.1 g/L $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$, 0.1 g/L $\text{CuCl}_2\cdot \text{H}_2\text{O}$, and 0.05 g/L H_3BO_4 , cultured at 37 $^{\circ}\text{C}$ and 180 rpm for 36 h. When necessary, tetracycline was added at the final concentrations of 20 $\mu\text{g}/\text{mL}$.

2.6. Statistical Analysis

Each fermentation experiment was carried out in three independent replicates. SPSS 20.0 (IBM, Armonk, NY, USA) was used to calculate the means and standard deviations, and observe the significance. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was applied to deal with the data and plot the graphs.

3. Results and Discussion

3.1. Screening the Optimal Host Strain for the Synthesis of the Platform Compound L-tyrosine

At present, the host strains for the production of platform compound L-tyrosine by metabolic engineering are concentrated in *E. coli* [26,44]. Juminaga et al. constructed a modular biosynthesis pathway in *E. coli* MG1655, and each module was optimized to achieve the optimal combination. Finally, the L-tyrosine yield reached 2.6 g/L, 79% of the maximum theoretical yield under corresponding fermentation conditions [26]. By using global transcription machinery engineering and high-throughput screening strategies, the *rpoA* mutant *E. coli* strains encoding RNA polymerase subunits were obtained, which could produce 13.8 g/L L-tyrosine in a 2 L fermenter [44]. However, the existence of endotoxins in *E. coli* hinders its applicability in the food industry [35]. In contrast, *Bacillus* species have become a promising alternative due to their advantages of their Generally Recognized As Safe (GRAS) status, good growth on cheap carbon sources, distinct endogenous metabolism, and robustness in industrial fermentations [34]. Hence, a series of *Bacillus* strains (shown in Table 1) stored in this laboratory were verified with L-tyrosine as the monitoring target for obtaining the optimal host strain. As shown in Figure 1, the L-tyrosine yield of the strain *B. amyloliquefaciens* HZ-12 reached 297 mg/L after fermentation for 36 h, much higher than other strains. *B. amyloliquefaciens* HZ-12 has a high initial L-tyrosine yield, and it has also been broadly applied in the biosynthesis of functional substances due to its genetic transformation potential, being able to transform into organic compounds such as spermidine and S-adenosylmethionine [39,40]. Therefore, the *B. amyloliquefaciens* HZ-12 was selected for engineering modification in the subsequent experimental operations.

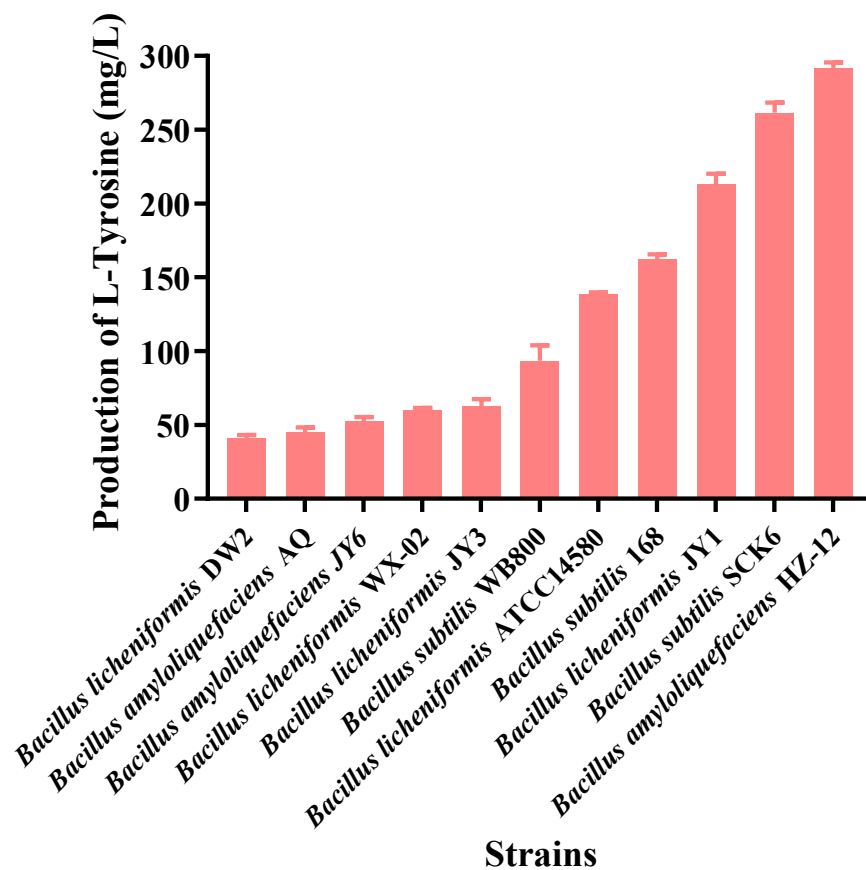


Figure 1. Evaluation of L-tyrosine production by serial host strains.

3.2. Effects of Different Prephenate Dehydrogenase Genes on Biosynthesis of L-tyrosine

At present, the L-tyrosine biosynthesis pathways in *E.coli*, *Bacillus*, and other microorganisms have been analyzed, which has laid a certain theoretical foundation for the subsequent optimization of L-tyrosine production [16,26,33]. Prephenate dehydrogenase is a key pathway enzyme for the synthesis of L-tyrosine, and its activity affects the synthesis of metabolic end products [33,34]. Kim et al. overexpressed the mutant gene *tyrA* from *E.coli*, indicating that the expression level of *tyrA* plays an important role in improving the production of L-tyrosine [24]. Therefore, in order to obtain the optimal prephenate dehydrogenase gene, the gene of bifunctional chorismate mutase/prephenate dehydrogenase was amplified from *E. coli* MG1655, and other prephenate dehydrogenase genes were cloned from *Lactobacillus plantarum*, *S. cerevisiae*, *Corynebacterium glutamate*, *B. subtilis*, *B. licheniformis*, *Bacillus coagulans*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Bacillus cereus*, and *B. amyloliquefaciens*. Moreover, we also cloned the cyclohexadiene dehydrogenase genes from *Zymomonas mobilis* and *Pseudomonas aeruginosa*, another type of catalytic enzyme of TyrA protein family [30]. The 15 obtained gene fragments were separately inserted into plasmid pHY300PLK to generate corresponding expression plasmids. Then, these expression plasmids were electrically transformed into *B. amyloliquefaciens* HZ-12 to obtain recombinant strains, including HZ/P43-Bao-*tyrA*, HZ/P43-Eco-*tyrA*, HZ/P43-Lpt-*tyrA*, HZ/P43-Sce-*tyrA*, HZ/P43-Cgb-*tyrA*, HZ/P43-Bsu-*tyrA*, HZ/P43-Bld-*tyrA*, HZ/P43-Bcoa-*tyrA*, HZ/P43-Bpum-*tyrA*, HZ/P43-Bmh1-*tyrA*, HZ/P43-Bmh2-*tyrA*, HZ/P43-Btb-*tyrA*, HZ/P43-Bce-*tyrA*, HZ/P43-Zmc-*tyrC*, and HZ/P43-Pap-*tyrA*.

The L-tyrosine yields of all the 15 engineered strains were measured after shake-flask cultivation for 36 h under the same experimental conditions. As shown in Figure 2, the highest yield (411 mg/L) was obtained after the enhanced expression of the gene *tyrA* from *B. amyloliquefaciens* HZ-12, which was 42% higher than that of the control strain HZ/pHY300PLK. In addition, the expression of the gene from *E. coli*, *B. subtilis*, *B. licheni-*

formis, *B. pumilus*, *Bacillus megaterium*, *B. thuringiensis*, and *Bacillus cereus* also has a significant improvement effect on the synthesis of L-tyrosine. The data indicate that the efficient expression of the prephenate dehydrogenase gene is crucial for the synthesis of L-tyrosine, and various prephenate dehydrogenase gene resources suitable for L-tyrosine synthesis were obtained, especially the gene *Bao-tyrA* from *B. amyloliquefaciens* HZ-12.

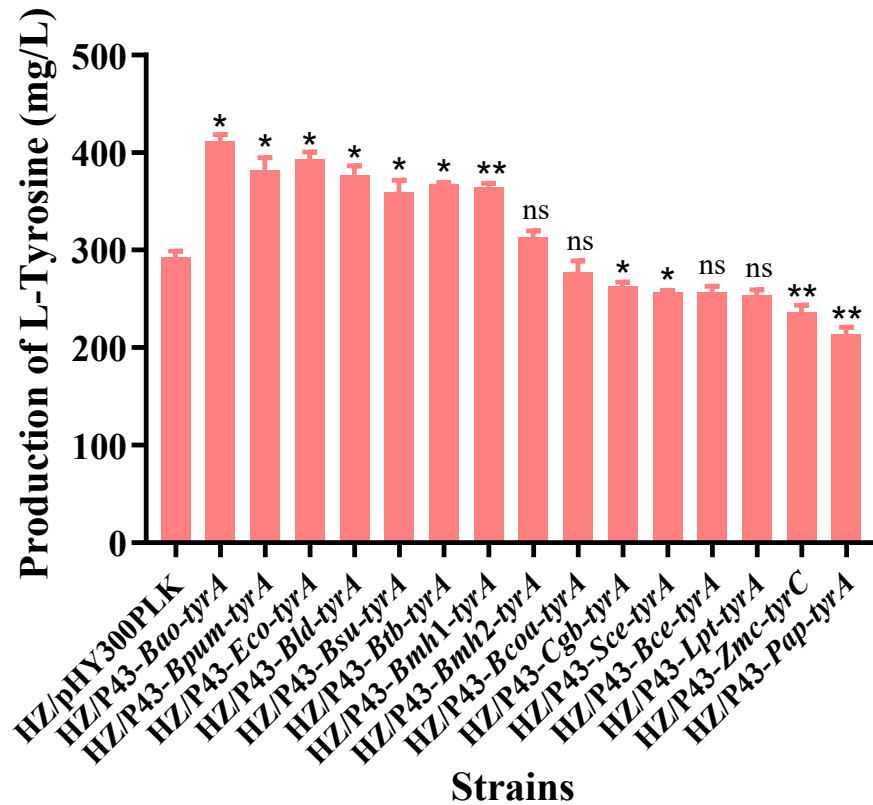


Figure 2. Effects of enhanced expression of the key enzyme genes *tyrA* from different species on L-tyrosine production. Note: * means significant difference ($p < 0.05$), ** means very significant difference ($p < 0.01$), and ns means no significant difference.

3.3. Amino Acid Sequence Analysis and Possible Catalytic Mechanism

Prephenate dehydrogenase catalyzes the synthesis of p-hydroxyphenylpyruvate using prephenate and NAD^+ as substrates [45]. To explain the function of the prephenate dehydrogenase gene *Bao-tyrA* from *B. amyloliquefaciens* HZ-12, the gene sequence (1107 bp) and corresponding amino acids were further analyzed. The gene sequence of *Bao-tyrA* was translated into a sequence containing 368 amino acids by using BioEdit v7.0.9.0 software (Figure 3). Then, the sequence was aligned with the previously reported amino acid sequences (Figure 4), and the similarities with *E. coli* (NC_000913), *C. glutamate* (CAF18797), and *S. cerevisiae* (NC_001134) were 20.89%, 25.86%, and no similarity, respectively. In addition, the TyrA protein from *E. coli* is a bi-functional enzyme that can display the activities of chorismate mutase/prephenate dehydrogenase, while the TyrA protein from *B. amyloliquefaciens* HZ-12 only shows the activity of prephenate dehydrogenase [34,46]. Sequence alignment revealed that these amino acid sequences contained the same conserved sites. Therefore, His131 might assist in the transfer of hydride from prephenate to NAD^+ in the dehydrogenase reaction [45]. Arg294 might interact specifically with the cyclic carboxylate at C-1 of prephenate [47]. This further illustrates the catalytic mechanism of the prephenate dehydrogenase coded by the gene *Bao-tyrA* in *B. amyloliquefaciens* HZ-12.

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1   ATG AAG GAT ACT ATA TTG CTT GCG GGG ATC GGG CTG ATC GGC GGA TCG ATC GCA TTG GCC ATC AAA AAG GAG CAT CCG CAT AAA CGG ATT   90
Met Lys Asp Thr Ile Leu Leu Ala Gly Ile Gly Leu Ile Gly Gly Ser Ile Ala Leu Ala Ile Lys Lys Gly Lys Gly Gly Gly Gly Gly Gly   30

91  ATC GGT GTG GAT ATT TCG GAA GAA CAG CTC ACT GCC GCG CTG AAG CTC GGA ATT ATT GAC GAG GCG GCG TCA TTG AGC GAA GGG GCG   180
Ile Gly Val Asp Ile Ser Glu Glu Gln Leu Thr Ala Ala Leu Lys Leu Gly Ile Ile Asp Glu Arg Ala Gly Ser Leu Thr Glu Gly Ala   60

181 CAG CAA GCG TCT GTT GTC ATT ATC GCC ACG CCT GTC GCC CAA ACG CTG AAA ATG CTC GAT ATG CTG GCC GCA TCA GAC ATC ACC CAT GAA   270
Gln Gln Ala Ser Val Val Ile Ile Ala Thr Pro Val Ala Gln Thr Leu Lys Met Leu Asp Met Leu Ala Ala Ser Asp Ile Thr His Glu   90

271 CTC CTG ATT ACG GAT GTG GGC AGC ACG AAA CAG AAA GTC GTC AGC TAC GCC GAC AAA GTG CTT CCG GCT CAT TAT CAA TTT GTC GGC GGC   360
Leu Leu Ile Thr Asp Val Gly Ser Thr Lys Gln Lys Val Val Ser Tyr Ala Asp Lys Val Leu Pro Ala His Tyr Gln Phe Val Gly Gly   120

361 CAT CCG ATG GCG GGG TCT CAT AAA TCC GGG GCC GCT GCC GCC AAA GAA TTT TTA TTC GAA AAT GCG TTT TAC ATT TTA ACA CCC GGA AAA   450
His Pro Met Ala Gly Ser His Lys Ser Gly Ala Ala Ala Ala Lys Glu Phe Leu Phe Glu Asn Ala Phe Tyr Ile Leu Thr Pro Gly Lys   150

451 TCA ACT TCT AAA GAG TCA GTA ACG CAT CTG AAG GAG CTT TTG AAA GCG GCA AAC GCC CAC TTT GTG GAG ATG ACT CCC GAG GAG CAT GAC   540
Ser Thr Ser Lys Glu Ser Val Thr His Leu Lys Glu Leu Leu Lys Ala Ala Asn Ala His Phe Val Glu Met Thr Pro Glu Glu His Asp   180

541 GGC GTA ACG AGC GTC ATC AGC CAT TTC CCT CAT ATC GTT GCT GCA AGC CTC GTC CAT CAG GCT CAT CAT TCT GAA GAA AAT TAT CCG TTT   630
Gly Val Thr Ser Val Ile Ser His Phe Pro His Ile Val Ala Ala Ser Leu Val His Gln Ala His His Ser Glu Glu Asn Tyr Pro Phe   210

181 631 TTG AAG CGT TTC GCA GCC GGC GGT TTT CGG GAC ATC ACC AGA ATC GCC TCA AGC AGT CCG GCG ATG TGG AGA GAC ATA CTT CTT CAT AAC   720
Leu Lys Arg Phe Ala Ala Gly Gly Phe Arg Asp Ile Thr Arg Ile Leu Arg Pro Leu Ala Ser Ser Ser Pro Ala Met Trp Arg Phe Tyr Asp Leu Tyr Val   240

721 AAA GAT AAA CTG CTT GAC CGC TTC AAA GAA TGG AAA CGG GAG ATT GAC ACG ATC GAA TCT TAT GTA GAA AAT GAA GAT GCG GAC AGC CTG   810
Lys Asp Lys Leu Leu Asp Arg Phe Lys Glu Trp Lys Arg Glu Ile Asp Thr Ile Glu Ser Tyr Val Glu Asn Glu Asp Ala Asp Ser Leu   270

811 TTC GGA TAT TTT AAA AAA GCG AAA GAT TAT CGC GAC GGC CTG CCG CTC CGT CAA AAA GGA GCG ATC CCG TCC TTT TAT GAC CTT TAT GTC   900
Phe Gly Tyr Phe Lys Lys Ala Lys Asp Tyr Arg Asp Gly Leu Pro Leu Arg Gln Lys Gly Ala Ile Phe Pro Ser Asp Thr Tyr Asp Leu Tyr Val   300

901 GAT GTT CCC GAT CAT CCG GGG GTC ATT TCG GAA ATT ACG GCG ATA CTG GCA GAA GAA AAA ATC AGT ATC ACC AAC ATC CGC ATT ATC GAA   990
Asp Val Pro Asp His Pro Gly Val Ile Ser Glu Ile Thr Ala Ile Leu Ala Glu Glu Lys Ile Ser Ile Thr Asn Ile Arg Ile Ile Glu   330

301 991 ACA AGA GAA GAT ATT AAC GGA ATT CTC AGA ATC AGT TTC CAG ACT GAT GAT GAC CGG AAG GCG GCG GAA ACC TGC ATT CAA TCC CGC GCC   1080
Thr Arg Glu Asp Ile Asn Gly Ile Leu Arg Ile Ser Phe Gln Thr Asp Asp Arg Lys Arg Ala Glu Thr Cys Ile Gln Ser Arg Ala   360

1081 AAA TAT GAC ACA TTT TAC GCT GAC TGA   1107
361 Lys Tyr Asp Thr Phe Tyr Ala Asp End   368
    
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Figure 3. Gene sequence (upper part) and deduced amino acids sequence (lower part) of *Bao-tyrA*.

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Bao-tyrA      ... ..ANDTI LLAGI CLIGCSIALAI KKEHPHKMI I CVDI SEEQI TAAI KLGI I DERAGS I TEGRCCAS VVI I TPVAQTLKALDLNLAASDI TH   89
Eco-tyrA      ... ..NV AELTALRDCI DEVDKAL ENLAKRLELVAEVEVKSRFGLPI VVPEREASMLASRRAEAEALCPPELLI EDVRRVARSYSSENCKGFKTLCPSLR   99
Cgb-tyrA      ... ..NCHTCQVITTKMI SRPVC I LGLCLI GSSLRLRLHAANHS VFGYNRSPKCAKS VVEEGFDVSADELEATLQRAAEDALI VLAVPMTAI ESLLBAVHTHAPNN 100
Sce-tyrA      ... ..VSEDKI EQVKATKVI GI I GLGDAGLLYANXFTDAGVGI CCLREYYEYI KKEYIAS AKFELVKNGHLSVRSGLD I IYSMEASNSKI VATYGPSKSVG   99
Consensus
Bao-tyrA      ELL I TDVGS I KCKVYS YADK VLF AHYCFVGC E PNA GSHKSGAAAKE FLENFNI LTP. . . . . GKS ISKES VTH KEL LKAA N AHFVENT P. . . EEH 180
Eco-tyrA      PVVI VGGCGCMGRLEFKALTLISGYCVRI LCE CVDRADI VADAGVIVS VPI HVTECV. . . . . I GKL PPLPKDCI LVDLAVKNGPL CALVAHDGPVL 194
Cgb-tyrA      C. . . FTDVSVKTA VYI AVKARNCHR YVGS E PNA GTANGVSA NDGLE KRAVWVTFECL FGDGDI NS IWI SJ WCKVVCML AVGAEAVPS RVGPHD 196
Sce-tyrA      T. I I NGGCTS CKLPEI EAFEK YLPKDCD I I T VESLHCPKVNTEGQPLVI I NERS CPSEFEFVNSVNAACLKS KCVYI TYEEHEKI TACTCAVTHAFLSN 198
Consensus
Bao-tyrA      CVTSVI SHFPHI VAASL VQAHHSEEN. . . . . YPFLKRF AAGCFRDI TRI ASSS PANVRDI LLFNKDKL LERFKVREI ETI ESYVENETA 267
Eco-tyrA      CLHPNF CPDSSGLACVWVWCGRK. . . . . PEAYQVLECI CVWGARLHRI SAVEHCNNAFI CALRHFATFATGLHLAENVCL 274
Cgb-tyrA      AAARVSHLTHI LAETLAI VGLNGC. . . . . ALSLSL AAGSYRIS TRVAGTDPL VRANCESNAGPLVKALDEALALH. . . . . 269
Sce-tyrA      SAWAKI KI YWTLGVNKKVYGL ENLVKVIN SLRI YS NKWHVYGLAI TNPS AHQCL I QYATATEL FSLNI DNKEQLI TRRLKAKC VFKKHTGLLLLLD 298
Consensus
Bao-tyrA      ES I FCFKKAKDYR DGLPI R I K G. . . . . AI PSEYEL VVDVDPDFGVI S E I TAI LAEEXI S I T NI RI I E I TREDI NGI LRI SF CDDEDNRRAETCI QS RAKYD 363
Eco-tyrA      EQLLAL SSP I YRLELANVRI FAGE. . . . . PCLYADI I ASSERNALI KRYKRFCEAI E LLECGDQAFI DSFRKNEHWFGYACRFQSES RNLIRQAND 370
Cgb-tyrA      . . . . . EAR EGLTAEI. . . . . PNIQLADNRYRSRI RYEA RS GQPRAKESVS. . . . . P T I TS SRP V LHP GP NWEKQI I HAETI GARI E 344
Sce-tyrA      TI I EKYSLS K S I GNSNNK PVPNSHLSL LAI VES WFQLEGI DPEYHNI CS I P L FRI FLGVSE YLFLKPGCLLEQTI DAAI HEKFSI KCDLEFVLS AREVSS 398
Consensus
Bao-tyrA      T E YAD. . . . . 368
Eco-tyrA      NRQ. . . . . 373
Cgb-tyrA      VE. . . . . 346
Sce-tyrA      VVS FANFETI YKKGFCVSKRF FEPALPEANLI GNEM KTI LS HSS DRS AAEKRN 451
Consensus
    
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Figure 4. Comparison of amino acid sequences of the prephenate dehydrogenase gene *tyrA* from *B. amyloliquefaciens* and other species. The conserved active sites (H131 and R294) were marked in red box.

3.4. Effects of Different Promoters of *Bao-tyrA* on Biosynthesis of L-tyrosine

The promoter is one of the key factors that can affect the gene expression level, and it constitutes an important genetic regulatory element in the complex framework of transcriptional control [48,49]. An efficient promoter, SPL-21, was screened from *Streptomyces* to control the expression of *toyF*, which significantly increased the production of *toyocamycin* [50]. A high-strength promoter, P_{tnrQ} , was mined from *B. subtilis* based on transcriptome data, and it could double the amylase expression [51]. Based on genome-wide microarray analyses, a toolbox of novel promoters was obtained from *B. megaterium* to offer versatile promoter strength, and the final progesterone yields of 3.6 mM were found to be increased compared with the control promoter [52]. This indicates that efficient promoters can further improve the production of metabolites. Therefore, in order to further optimize the expression level of the gene *Bao-tyrA*, promoters with different strengths were used, and corresponding recombinant strains were obtained, including HZ/*Psrfa-Bao-tyrA*, HZ/*PytzE-Bao-tyrA*, HZ/*Pylb-Bao-tyrA*, HZ/*Pbay-Bao-tyrA*, HZ/*PykzA-Bao-tyrA*, HZ/*PykzA-PRBS6-Bao-tyrA*, HZ/*PmmgA-Bao-tyrA*, HZ/*PabrB-Bao-tyrA*, HZ/*PbacA-Bao-tyrA*, HZ/*PUTR12-Bao-tyrA*, HZ/*P43-Pylb-Bao-tyrA*, HZ/*PR5-Bao-tyrA*, and HZ/*PRBS6-*

Bao-tyrA. The L-tyrosine yields of all the 13 engineered strains were measured (Figure 5); it was found that different promoters had different effects on the expression of prephenate dehydrogenase. In comparison, the P43 promoter could drive the maximum L-tyrosine production in *B. amyloliquefaciens* HZ-12, while other promoters did not further increase L-tyrosine production. The data indicate that the constitutive promoter P43 has great advantages in the sustained and efficient expression of its regulated genes.

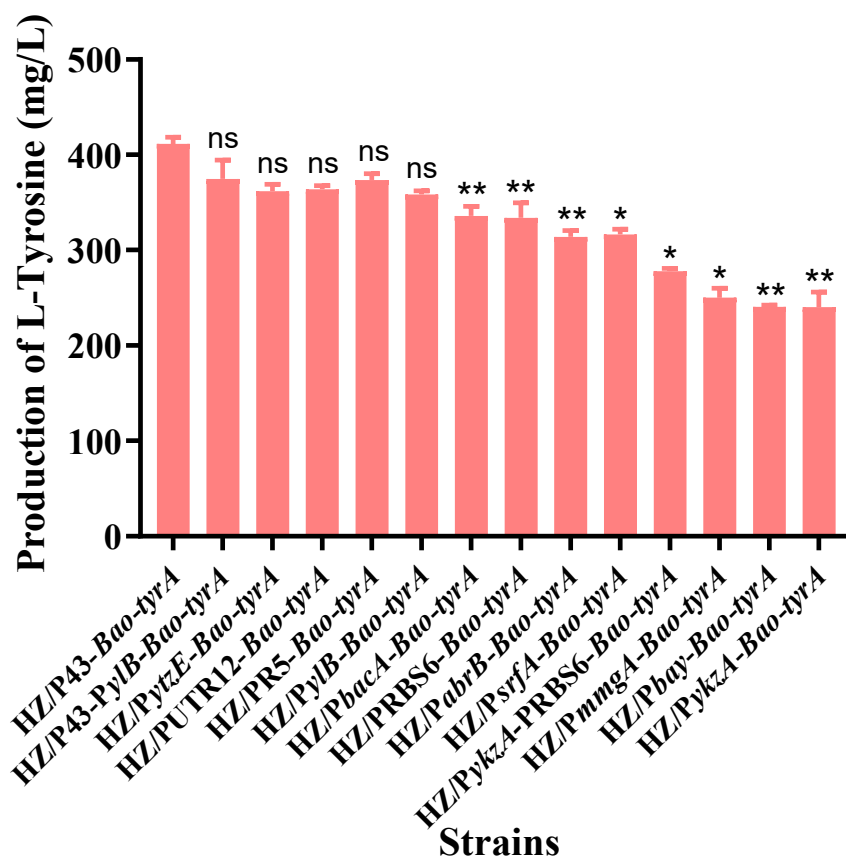


Figure 5. Effect of replacement of promoter of the gene *Bao-tyrA* on L-tyrosine production. Note: * means significant difference ($p < 0.05$), ** means very significant difference ($p < 0.01$), and ns means no significant difference.

3.5. Redesign of the 5'-UTR of the Gene *Bao-tyrA*

In addition to the promoter, 5'-UTR is also a factor affecting gene expression, and can play a role in post transcriptional regulation of genes [53,54]. Kim et al. introduced a constitutive promoter and a synthetic 5'-UTR into each gene of the L-tyrosine synthesis pathway, and then used UTR designer to control the expression level of PEP synthetase. After optimization, the L-tyrosine yield of *E. coli* was increased to 3.0 g/L [2]. Noh et al. introduced a strong inducible tac promoter with synthetic 5'-UTRs designed for high expression to the genes *hemA* and *hemL*, and the yield of 5-aminolevulinic acid was increased to 0.74 g/L after optimization [55]. This demonstrates that the 5'-UTR redesign is an effective strategy for optimizing the biosynthetic pathway. Therefore, to further optimize the expression of gene *Bao-tyrA* at the translation level, the five-terminal UTR sequence of gene *Bao-tyrA* was redesigned to precisely regulate gene expression. The corresponding gene-expression plasmids pHY-P43-UTR1-*Bao-tyrA*, pHY-P43-UTR2-*Bao-tyrA*, pHY-P43-UTR3-*Bao-tyrA*, pHY-P43-UTR4-*Bao-tyrA*, pHY-P43-UTR5-*Bao-tyrA*, and pHY-P43-UTR6-*Bao-tyrA* were constructed. These expression plasmids were then converted into *B. amyloliquefaciens* HZ-12, and corresponding recombinant strains were constructed, including HZ/P43-UTR1-*Bao-tyrA*, HZ/P43-UTR2-*Bao-tyrA*, HZ/P43-UTR3-*Bao-tyrA*, HZ/P43-UTR4-*Bao-tyrA*, HZ/P43-UTR5-*Bao-tyrA*, and

HZ/P43-UTR6-*Bao-tyrA*. The L-tyrosine yields of all these six engineered strains after shake-flask cultivation for 36 h are shown in Figure 6. The redesigned UTR3 could significantly increase the L-tyrosine production to 475 mg/L, which was 16% higher than the control strain HZ/P43-*Bao-tyrA*. The data indicated that the redesign of the five-terminal UTR was a feasible strategy in the metabolic engineering of *Bacillus*.

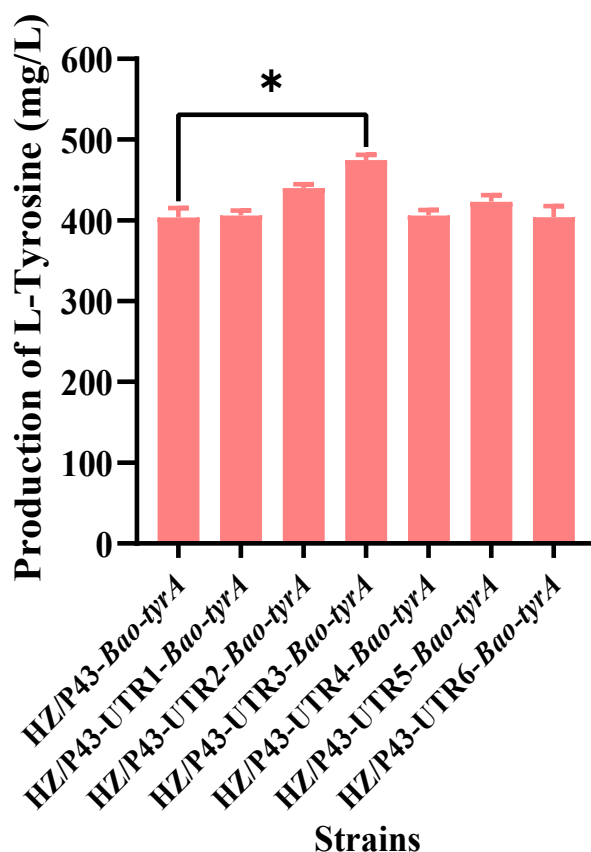


Figure 6. Effects of replacing 5'-UTR sequences of *Bao-tyrA* on L-tyrosine production. Note: * in the figure means significant difference ($p < 0.05$).

The 5'-UTR redesign can be used to fine-tune the level of target gene expression within cells, and has been widely used in *E. coli* and *C. glutamate*. Lee et al. constructed a plug-in inhibitor expression library based on 5'-UTR redesign, and verified the expression range of the library. Subsequently, the library was applied to accurately control the throughput of key metabolic nodes in the synthesis pathway of target substances. Finally, the yield of 3-HP and lycopene reached 2.59 g/L and 11.66 mg/L, which, respectively, increased 16.5 times and 2.82 times compared with the parental strains [56]. Jiang et al. developed two plug-in repressor expression libraries by diversifying the translation levels of *phlF* and *mcbR* based on the 5'-UTR variants to accurately control the metabolic flux, which can reduce the production of L-lysine as a by-product and balance extracellular protein synthesis and cell growth. The two libraries resulted in a 28% and 12% increase in the production of Ectoine compared with the control strain, respectively [57]. This indicates that the redesign of 5'-UTR is important for the precise control of intracellular carbon flux, and can provide some guidance for the subsequent optimization of biosynthesis. However, few studies have been conducted to fine-tune the expression level of target genes in *B. amyloliquefaciens* by redesigning 5'-UTR sequences. This study fills in this gap and verifies that this strategy of redesigning 5'-UTR sequences can fine-tune the intracellular gene expression level in *B. amyloliquefaciens*, which can be further applied to the production of more target compounds.

4. Conclusions

This study obtained a food-safe strain, *B. amyloliquefaciens* HZ-12, with a high initial yield of L-tyrosine. A total of 15 prephenate dehydrogenase genes were successfully expressed to screen the optimal gene suitable for the host strain, and the gene *Bao-tyrA* from *B. amyloliquefaciens* HZ-12 was confirmed to be an efficient gene resource. In addition, promoter replacement and five-terminal UTR redesign were also carried out, and it was found that the use of a 5'-UTR-3 sequence driven by a P43 promoter could enhance the expression of prephenate dehydrogenase in *B. amyloliquefaciens*. This study provides new microbial and genetic resources for construction of a L-tyrosine chassis cell, which will be beneficial for metabolic engineering.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods12163084/s1>, Table S1: Primers used in this study.

Author Contributions: X.W. and A.M. designed this study, and contributed reagents and materials; A.J. and P.B. conducted the experimental work; A.J. analyzed the data; A.J., X.W. and A.M. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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