OPTIMIZE ENGINEERED E. COLI FOR ARBUTIN PRODUCTION TO REDUCE COSTS

by

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(Under the Direction of YAJUN YAN)

ABSTRACT

Arbutin is a hydroquinone glucoside compound found in a variety of plants. Due to its well-known skin lightening, antioxidant, antimicrobial, and anti-inflammatory activities, it is widely used in the pharmaceutical and cosmetic industries. In previous studies, a method for biosynthesis of arbutin from simple carbon sources was implemented in E. coli and the titer of arbutin was increased to 4.19 g / L. In this work, the constitutive promoter replaced the inducible promoter of the original artificial pathway, and the participation of the inducer was no longer needed during the fermentation process, which reduced the fermentation cost. In the appropriate host bacteria, the expression intensity of the artificial pathway was adjusted by a series of stepped strength promoters, which reduced the cost of inducers and the titer reached 4.97 *g/L*, and material cost is reduced by 89.79%.

INDEX WORDS: Arbutin, E.coli., Metabolic Engineering, Synthetic Biology, Lower Cost

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CHAPTER 1

Introduction

Metabolic engineering refers to the subject area that uses multi-gene recombination technology to purposefully modify and transform cell metabolic pathways and change cell characteristics. It can be combined with cellular gene regulation, metabolic regulation, and biochemical engineering to construct new metabolic pathways for specific products. It is the application of genetic engineering in industrial production.

In this research, arbutin is the target product. Arbutin is a glycosylated hydroquinone (HQ) mainly extracted from the leaves of bearberry, and it can also be found in some fruits and other plants [2]. It brightens the skin, can quickly penetrate into the skin without affecting skin cells and combines with the tyrosine that causes melanin production, effectively blocking the activity of tyrosinase and the production of melanin and accelerating the decomposition and elimination of myranin; but its inhibition is reversible, unlike hydroquinone which can kill melanocytes, and it is therefore safer than hydroquinone [3][4]. In addition, arbutin also protects the skin from free radicals and is highly hydrophilic, so it is often added to commercially available whitening products. A new type of burn and scald medicine with arbutin as the main component is characterized by rapid pain relief, strong anti-inflammatory power, rapid elimination of redness and swelling, fast healing, and no scarring. As an intestinal anti-inflammatory drug, arbutin has good bactericidal and anti-inflammatory effects, with no toxic or side effects [5][6][7][8].

Initially, arbutin is usually produced by plant extraction or enzymatic methods, which has the disadvantages of low yield and high processing costs [9]. In the previous work, a 4-hydroxybenzoate 1-hydroxylase from Candida parapsilosis CBS604 and a glucosyltransferase from Rauvolfiaserpentina were introduced into E. coli, and an artificial pathway was established to hyphenate arbutin from a simple carbon source. Further enhancement of shikimate pathway genes and the optimization of the glucose concentration in culture medium can achieve 4.19 g / L titer of arbutin [10].

In this study, a group of constitutive promoter lpps (plpps) with different expression strengths was used to replace the original inducible promoter plac-O1. Promoter lpps are selected from a series of promoters obtained by mutating the -10 and -35 regions of the original promoter lpp (plpp), and their strengths vary. For example, the promoter plpp1.0 is the original plpp, and the expression intensity of plpp0.5 is 0.5 times that of the original plpp. The reason for using the plpps is that the original plpp (plpp 1.0) has the similar expression intensity as the plac-O1, which was used in previous research. This can provide a reference for our regulation of gene expression. On the one hand, the constitutive promoter can express genes on its own without adding an inducer, thereby saving production costs. On the other hand, the promoter group can be used to find a better expression intensity combination for related genes, which may further improve the production of arbutin.

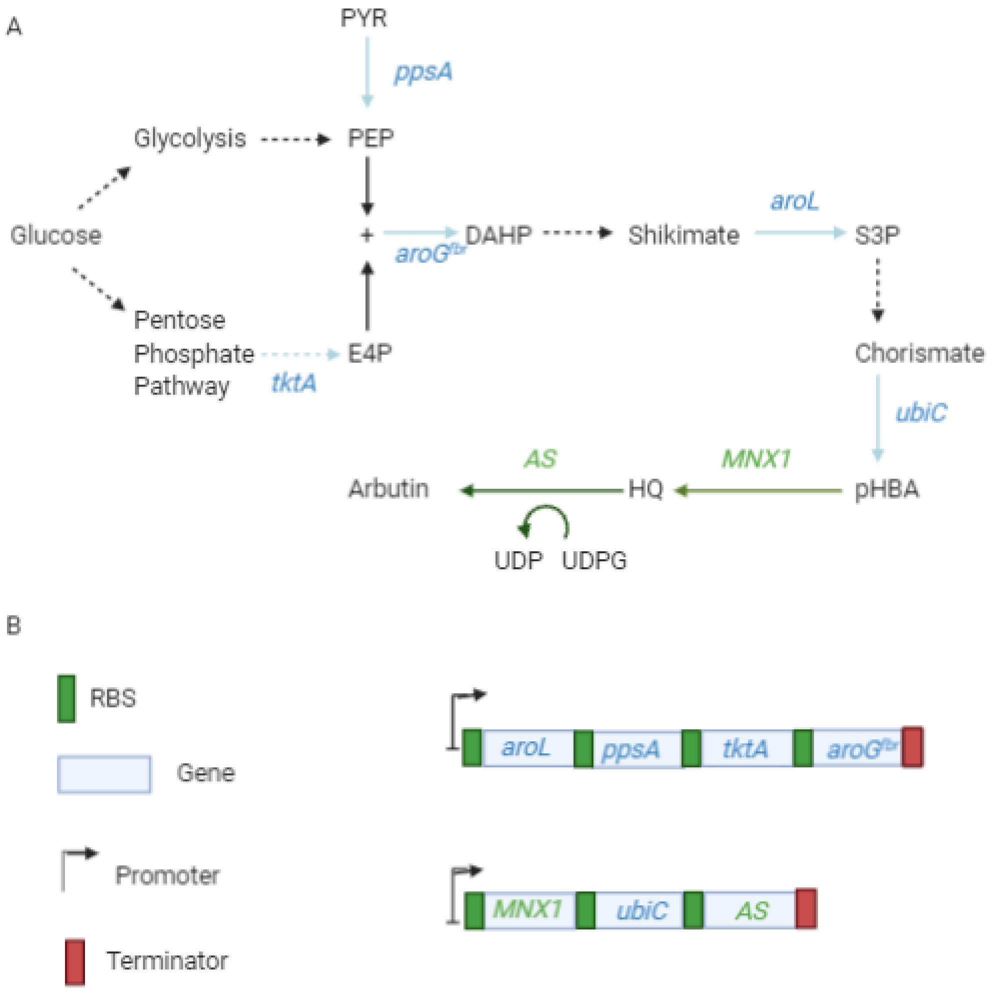

Figure. 1. A. The artificial pathway related to de novo biosynthesis of arbutin. Black arrows indicate native metabolic pathways in E. coli; blue arrows and genes indicate over-expressed native genes of E. coli; green arrows and genes describe the introduced artificial pathway; gray arrows indicate the branch routes of chorismate. PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; PYR, pyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; S3P, shikimate 3-phosphate; pHBA, p-hydroxybenzoic acid; HQ, hydroquinone. *tktA* encodes transketolase; *ppsA* encodes phosphoenolpyruvate synthetase; $aroG^{fbr}$ encodes the feedback inhibition resistant mutant of 2-dehydro-3-deoxyphosphoheptonate aldolase; *aroL* encodes shikimate kinase II; *ubiC* encodes chorismate lyase; *MNX1* encodes 4-hydroxybenzoate 1-hydroxylase; *AS* encodes arbutin synthase. B. Cluster APTA includes $aroG^{fbr}$, *ppsA*, *tktA*, and *aroL*. Cluster MUA contains *ubiC*, *MNX1*, and *AS*. There is a ribose binding site (RBS) in front of each gene and a promoter in front of each cluster.

For the convenience of use, we separate the endogenous genes that need to be over-expressed and the introduced foreign genes in the metabolic pathway of arbutin (**Figure 1, A**) synthesis into two gene clusters. Each cluster is a set of genes guided by one promoter. Because the length of efficient expression after one promoter is limited, we cannot put too many genes behind the same promoter. In addition, the capacity of the plasmid is limited, so we cannot assign separate promoters to each gene. We will use the first letter of all genes in a cluster to form the name of this cluster. Cluster APTA contains 4 over-expressed endogenous genes: *aroG*^{fbr} (the feedback inhibition-resistant mutant of 2-dehydro-3-deoxyphosphoheptonate aldolase), *ppsA* (phosphoenolpyruvate synthetase), *tktA* (transketolase), and *aroL* (shikimate kinase II). The other one is MUA which contains the downstream over-expressed endogenous gene, *ubiC* (chorismate lyase), and is close to arbutin in the metabolic pathway and two introduced foreign genes, *MNX1* (4-hydroxybenzoate 1-hydroxylase) and, *AS* (arbutin synthase). Besides, there is a ribose binding site (RBS) in front of each gene. (**Figure 1, B**)

CHAPTER 2

Materials and methods

2.1 Media

A Luria-Bertani (LB) medium containing 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl was employed for cell inoculation, protein expression and plasmid propagation. An M9Y medium contains 5 g/L yeast extract, 1 g/L NH4Cl, 6 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 246.5 mg/L MgSO4•7H2O and 14.7 mg/L CaCl2•2H2O, A M9Y media supplemented with 30 g/L glycerol was used for de novo production of arbutin. The antibiotics ampicillin (100 µg/mL) and kanamycin (50 µg/mL) were added into the medium if needed. A final concentration of 0.5 mM IPTG was supplemented into the media if needed.

2.2 Strains and plasmids

E. coli strain XL-1Blue was used for plasmids construction and propagation in this research. E. coli QH4 [11] and E. coli QH4-pykAF were used as the host to express proteins for de novo production. Plasmid pZE12-luc and pCS27 were used as the high-copy number and medium-copy number plasmids. **Table 1** lists all the strains and plasmids.

Table 1 List of strains and plasmids used in this study

Strain	Genotype	Source
E. coli XL-1 Blue	recA1endA1gyrA96thi−1hsdR17supE44relA1lac	Stratagene
E. coli QH4	E. coli ATCC31884 ΔpheLA ΔtyrA	Wang et al. (2019) [11]
E. coli QH4-pykAF	E. coli ATCC31884 ΔpheLA ΔtyrA ΔpykAF	Wang et al. (2019)
Plasmid	Description	Source
pZE12- luc	pLlacO1; luc; ColE1 ori; AmpR	(Lutz and Bujard, 1997) [12]
pCS27	pLlacO1; p15A ori; KanR	(Shen and Liao, 2008) [13]
pCS-APTA	pCS27 containing aroL, ppsA, tktA, aroGfbr from E. coli	(Lin et al., 2013) [10]
pZE-MUA (pSXL101)	pZE12-luc containing MNX1 from Candida parapsilosis CBS604, ubiC from E. coli and AS from Rauvolfia serpentina	(Lin et al., 2013)
pZE-plpp0.2-MUA	pZE12-luc containing pLpp0.2, MNX1, ubiC, and AS	This study
pZE-plpp0.5-MUA	pZE12-luc containing pLpp0.5, MNX1, ubiC, and AS	This study
pZE-plpp0.8-MUA	pZE12-luc containing pLpp0.8, MNX1, ubiC, and AS	This study
pZE-plpp1.0-MUA	pZE12-luc containing pLpp1.0, MNX1, ubiC, and AS	This study
pZE-plpp1.2-MUA	pZE12-luc containing pLpp1.2, MNX1, ubiC, and AS	This study
pZE-plpp1.4-MUA	pZE12-luc containing pLpp1.4, MNX1, ubiC, and AS	This study
pZE-plpp1.6-MUA	pZE12-luc containing pLpp1.6, MNX1, ubiC, and AS	This study
pZE-plpp1.8-MUA	pZE12-luc containing pLpp1.8, MNX1, ubiC, and AS	This study
pZE-plpp2.0-MUA	pZE12-luc containing pLpp2.0, MNX1, ubiC, and AS	This study

pZE-plpp1.0-MUA-plpp0.5-APTA	pZE12-luc containing pLpp1.0, MNX1, ubiC, AS; and pLpp0.5, aroL, ppsA, tktA, aroGfbr	This study
pZE-plpp1.0-MUA-plpp0.8-APTA	pZE12-luc containing pLpp1.0, MNX1, ubiC, AS; and pLpp0.8, aroL, ppsA, tktA, aroGfbr	This study
pZE-plpp1.0-MUA-plpp1.0-APTA	pZE12-luc containing pLpp1.0, MNX1, ubiC, AS; and pLpp1.0, aroL, ppsA, tktA, aroGfbr	This study
pZE-plpp1.0-MUA-plpp1.2-APTA	pZE12-luc containing pLpp1.0, MNX1, ubiC, AS; and pLpp1.2, aroL, ppsA, tktA, aroGfbr	This study
pZE-plpp1.0-MUA-plpp1.4-APTA	pZE12-luc containing pLpp1.0, MNX1, ubiC, AS; and pLpp1.4, aroL, ppsA, tktA, aroGfbr	This study

2.3 Arbutin production

The E. coli strain QH4 harboring pCS-APTA and pZE-MUA was first inoculated in 3 mL LB medium at 37 °C for 8h. Then, the culture (200 μL) was cultivated in 20 mL M9Y liquid medium with 30 g/L glucose shakers at 30 °C and induced with 0.5 mM IPTG. Samples were taken after 48h and analyzed by HPLC.

CHAPTER 3

Result

3.1 Feasibility test for producing arbutin in QH4

At the beginning, we need to verify whether the arbutin production strategy is feasible in the selected host strain. We chose E. coli QH4, a derivative of L-phenylalanine (L-Phe) overproducer E.coli ATCC31884 with disrupted L-Phe and L-tyrosine (L-Tyr) branches, as the host strain (**Figure 2, A**). Put the production gene cluster MUA on the high-copy plasmid pZE-luc, and replace the original inducible promoter lac-O1 with the constitutive promoter group plpps. From this we obtained the plasmid group pZE-plpps-MUA expressed by different strengths.

The pZE-plpps-MUA was expressed in E. coli QH4, and after 48 hours of fermentation production, the change trend of yield was obtained when the production gene cluster MUA expression intensity was different (**Figure 2, B**). The results showed that with the increase of the expression intensity of the production gene cluster MUA, the yield of arbutin increased first and then decreased, and reached a maximum of 1.38 g/L at plpp1.0. The yield from plpp0.5 to plpp1.4 was similar, all at about 1.3 g/L. From this, we determined that pZE-plpp1.0-MUA is the best production plasmid in this group of plasmids, but we are not sure whether this result will be changed after the metabolic pathway is rebalanced.

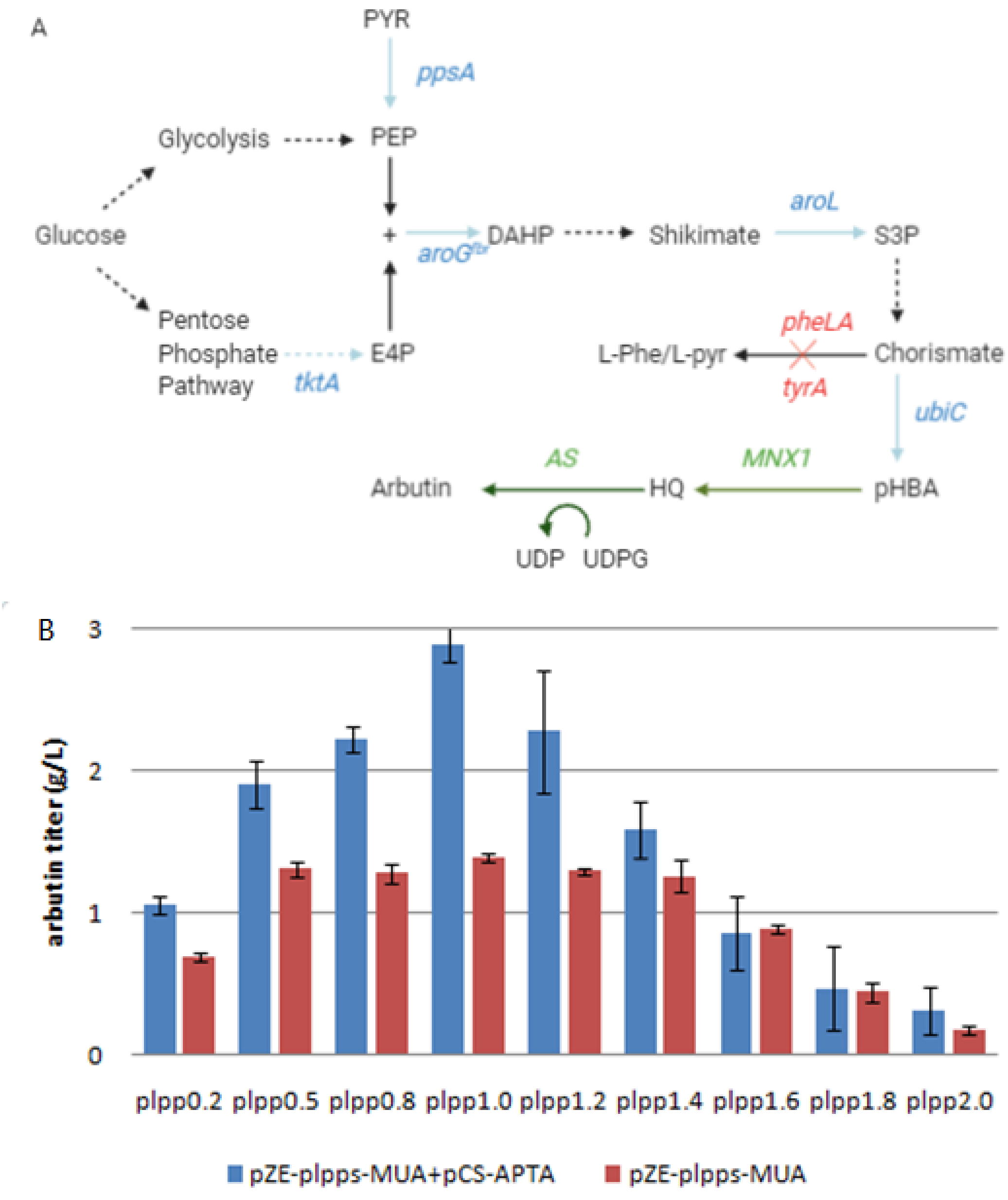

Figure 2. A. Metabolic pathways in E. coli QH4. Black arrows indicate native metabolic pathways in E. coli; blue arrows and genes indicate over-expressed native genes of E. coli; green arrows and genes describe the introduced artificial pathway; gray arrows indicate the branch routes of chorismate; deleted genes are indicated in red. PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; PYR, pyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; S3P, shikimate 3-phosphate; pHBA, p-hydroxybenzoic acid; HQ, hydroquinone. *tktA* encodes transketolase; *ppsA* encodes phosphoenolpyruvate synthetase; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; *aroG*^{fbr} encodes the feedback inhibition resistant mutant of 2-dehydro-3-deoxyphosphoheptonate aldolase; *aroL* encodes shikimate kinase II; *ubiC* encodes chorismate lyase; *MNX1* encodes 4-hydroxybenzoate 1-hydroxylase; *AS* encodes arbutin synthase; *pheL* leader peptide of chorismate mutase-P-prephenate dehydratase; *pheA* encodes prephenate dehydratase; tryA encodes prephenate dehydrogenase. B. De novo biosynthesis of arbutin from glucose. Strain QH4 harboring empty plasmids pCS-27 and pZE12-luc was used as a negative control. In red bar chart, strain QH4 expressing pZE-plpps-MUA was used as arbutin producer. In blue bar chart, strain QH4 expressing pZE-plpps-MUA and pCS-APTA was used as arbutin producer. Production time is 48h. The time point of

induction if needed was used as 0 h. The x-axis shows the specific promoter used in the plpps promoter group. All data points are reported as mean±s.d. from three independent experiments.

3.2 Effects of upstream metabolic pathway enhancement on arbutin production

In the previous section, we determined that pZE-plpp1.0-MUA is the best production plasmid among a group of plasmids. Here we need to verify whether this result will be changed after the metabolic pathway is rebalanced. On the basis of the previous part of the experiment, we continue to introduce the enhancement plasmid pCS-APTA to enhance the expression of upstream metabolic pathways.

The plasmids pZE-plpps-MUA and pCS-APTA were expressed in E. coli QH4. After 48 hours of fermentation, the production trend was obtained when the expression intensity of the production gene cluster MUA was different and the expression of the upstream metabolic pathway was enhanced (**Figure 2, B**). The results showed that after the upstream metabolic pathway was enhanced, the trend of "arbutin production vs the change of the production gene cluster MUA expression intensity" did not change much. With the increase of MUA expression intensity, the yield of arbutin still increased first and then decreased, and reached a maximum value of 3.89 g / L at plpp1.0. Unlike before, the output from plpp0.5 to plpp1.4 shows a difference. Based on this, we determined that pZE-plpp1.0-MUA is the best production plasmid in this group of plasmids, and the results will not change after the metabolic pathway is rebalanced.

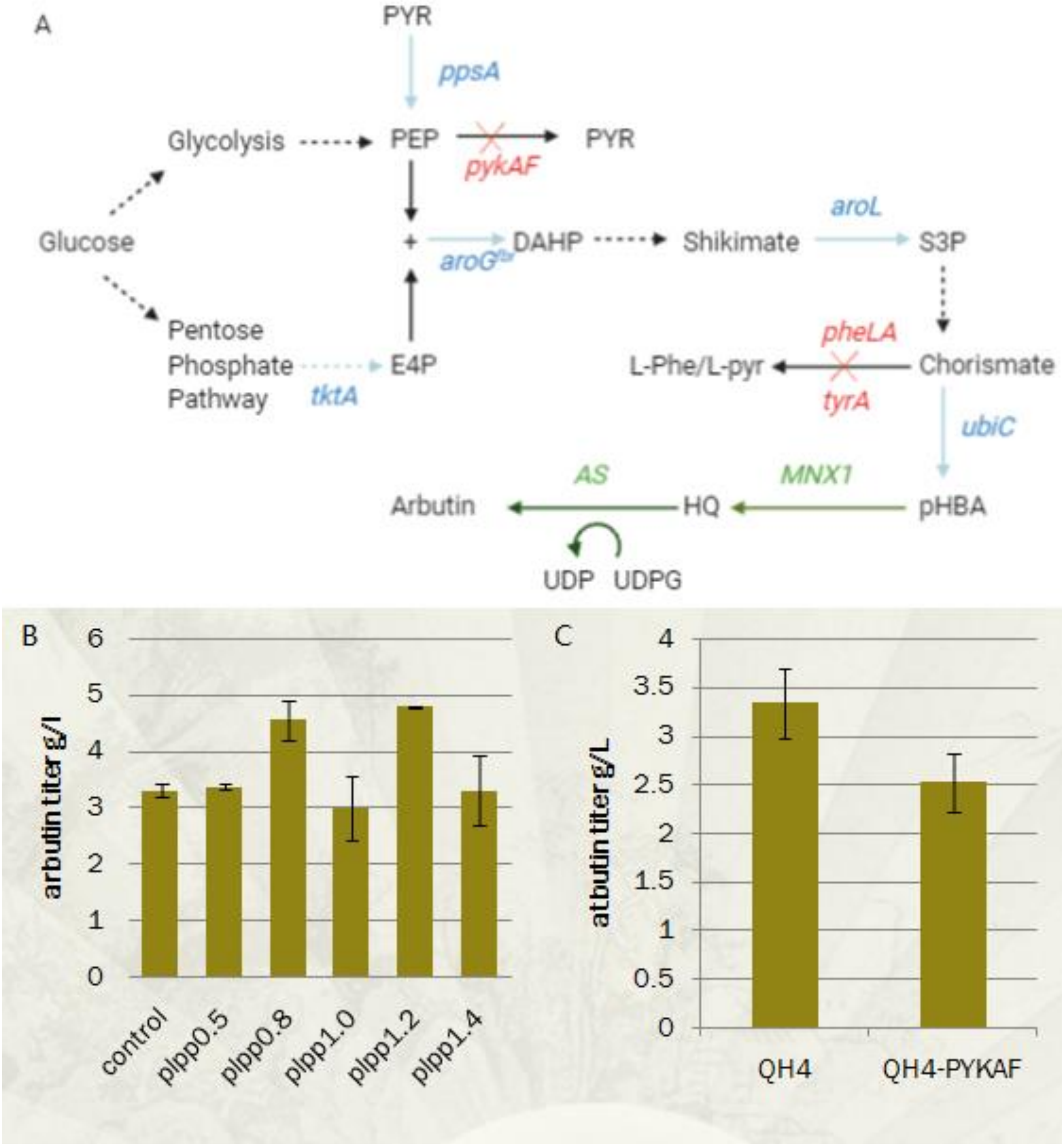

Figure 2. A. Metabolic pathways in E. coli QH4-pykAF. Black arrows indicate native metabolic pathways in E. coli; blue arrows and genes indicate over-expressed native genes of E. coli; green arrows and genes describe the introduced artificial pathway; gray arrows indicate the branch routes of chorismate; deleted genes are indicated in red. PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; PYR, pyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; S3P, shikimate 3-phosphate; pHBA, p-hydroxybenzoic acid; HQ, hydroquinone; PYR, pyruvate. *tktA* encodes transketolase; *ppsA* encodes phosphoenolpyruvate synthetase; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; *aroG*^{fbr} encodes the feedback inhibition resistant mutant of 2-dehydro-3-deoxyphosphoheptonate aldolase; *aroL* encodes shikimate kinase II; *ubiC* encodes chorismate lyase; *MNX1* encodes 4-hydroxybenzoate 1-hydroxylase; *AS* encodes arbutin synthase; *pheL* leader peptide of chorismate mutase-P-prephenate dehydratase; *pheA* encodes prephenate dehydratase; tryA encodes prephenate dehydrogenase; pykA encodes pyruvate kinase II; pykF encodes pyruvate kinase I. B. De novo biosynthesis of arbutin from glucose. Strain QH4 harboring plasmids pCS-APTA and pZE-plpp1.0-MUA was used as a positive control. Strain QH4 expressing pZE-plpp1.0-MUA-plpps-APTA was used as arbutin producer. Production time is 48h. The time point of induction if needed was used as 0 h. The x-axis shows the specific promoter used in the plpps promoter

group. All data points are reported as mean±s.d. from three independent experiments. C. De novo biosynthesis of arbutin from glucose. Strain QH4 harboring empty plasmids pCS-27 and pZE12-luc was used as a negative control. Strain QH4 and QH4-pykAF harboring plasmids pZE-plpp1.0-MUA-plpp0.8-APTA used as arbutin producer. Production time is 48h. The x-axis shows the host strain. All data points are reported as mean±s.d. from three independent experiments.

3.3 Find the optimal expression intensity of related genes to achieve the highest stable yield

Based on the results of the previous part, we determined the optimal expression intensity of the gene cluster MUA, and the enhanced expression of upstream metabolic pathways does not change this result. Therefore, in this part, we can explore the optimal expression intensity of the gene cluster APTA.

The enhanced gene cluster APTA was placed on the plasmid pZE-plpp1.0-MUA, and the original inducible promoter lac-O1 was replaced with a constitutive promoter group plpps. From this we obtained the plasmid group pZE-plpp1.0-MUA-plpps-APTA expressed in different intensities. The copy number of the high-copy plasmid pZE12-luc is twice that of the medium-copy plasmid pCS-27, and the expression intensity of pCS-APTA is still within the control range of pZE-plpp1.0-MUA-plpps-APTA. In addition, all the regulatory genes putting on the same plasmid can simplify the preservation process of the plasmid, and can save a resistance cost in production.

Plasmid pZE-plpp1.0-MUA-plpps-APTA was expressed in E. coli QH4. After 48 hours of fermentation, when the expression intensity of the enhanced gene cluster APTA was different, a production trend was obtained (**Figure 3, B**). The results show that when the expression intensity of the enhanced gene cluster APTA is different, the production efficiency does not reflect an obvious trend. Among them, plpp0.8 and plpp1.2 have the highest yields, both of which reach more than 4.5 g/L. But compared with plpp0.8, plpp1.2 appeared more outliers in the experiment, reflecting greater instability. Therefore, we regard the plasmid pZE-plpp1.0-MUA-plpp0.8-APTA as the best production plasmid.

3.4 Guide more flux to arbutin

In the previous section, we determined the optimal expression intensity of gene cluster MUA and enhanced gene cluster APTA, and obtained the optimal production plasmid pZE-plpp1.0-MUA-plpp0.8-APTA. Here, we want to explore the possibility of further transformation of the host bacteria.

We deleted the pyruvate kinase genes pykA and pykF in E. coli QH4, yielding strain QH4-pykAF (**Figure 3, A**). Plasmid pZE-plpp1.0-MUA-plpp0.8-APTA was expressed in E. coli QH4-pykAF. After 48 hours of fermentation, the difference between the production of the two host bacteria was obtained (**Figure 3, C**). The results show that the arbutin produced by QH4-pykAF is less efficient.

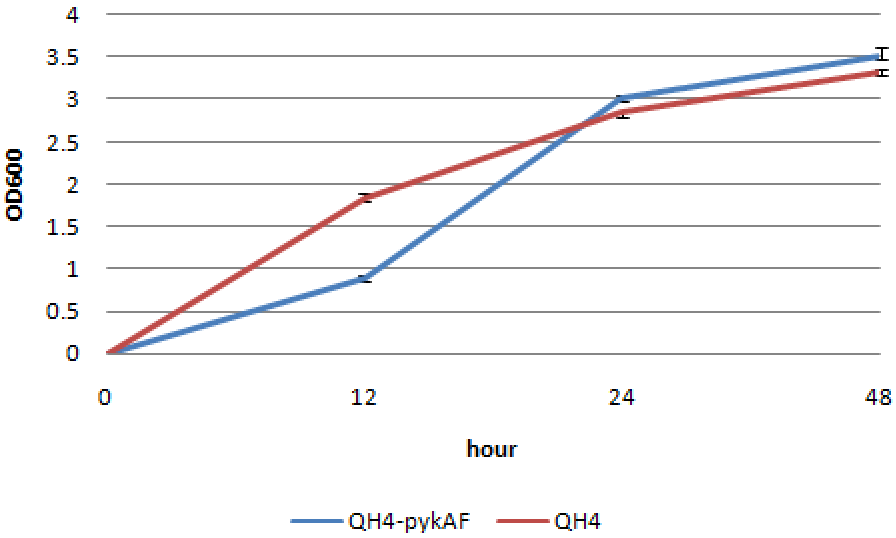

Figure 4 The growth of QH4 and QH4, the x-axis shows the growth time (hours), and the y-axis shows OD600.

According to our analysis, the reason why QH4-pykAF production efficiency is lower is that the knock-out of pykAF affects the growth of the strain, prolonging the growth phase of the strain and shortening the production phase, and the final OD600 of QH4-pykAF is higher than QH4, which makes more carbon sources used for growth rather than production. (**Figure 4**)

3.5 Cost accounting

In cost accounting, we only calculate the use of materials above a certain scale (**Table 2**). The cost of the instrument, power, and the material use too little are not included in the cost for the convenience of calculation, because the service life of the instrument is difficult to calculate accurately, and the power and little used material do not affect the final conclusion. The calculation results show that the material cost of arbutin in the previous study is 11.75 dollar/g, and the material cost of arbutin in this study is 1.2 dollar/g. Compared with the previous study, the material cost of this study is reduced by 89.79%.

Table 2 Shake flasks production material costs when the total amount is 1L

Material	Unit price(dollar)	amount	Price(dollar)	In previous studies	In this study
M9	200(1kg)	17.63(g)	3.526	✓	✓
Yeast Extract	58.30(250g)	5(g)	1.116	✓	✓
Glucose	147(10kg)	30(g)	0.441	✓	✓
IPTG	128(100mM*15mL)	5(ml)	42.67	✓	
Ampicillin	224(25g)	0.1(g)	0.896	✓	✓
Kanamycin	296(25g)	0.05(g)	0.592	✓	
Total				49.241(dollar)	5.979(dollar)
Arbutin production				4.19(g)	4.97(g)
Material costs				11.75(dollar/g)	1.2(dollar/g)

CHAPTER 4

Discussion

In this study, we found the optimal expression intensity of the production gene cluster MUA and enhanced gene cluster APTA in the host bacteria QH4, and constructed a production plasmid pZE-plpp1.0-MUA-plpp0.8-APTA. Compared with previous studies, we replaced the inducible promoter with a constitutive promoter, saving the cost of the inducer in production. And we constructed a large plasmid to replace the two plasmids that were needed before, further saving the use of a resistance in production. These two changes have reduced the cost of production. And one plasmid simplifies the preservation of the plasmid. In addition, the highest titer of this study is 4.97 g/L, which is a slight increase of 18.6% compared with the previous highest titer. In the end, we reduced the material cost of arbutin by 89.79%.

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