Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(6):608-611

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Co-expression of quinic acid biosynthesis key gene in *Escherichia coli*

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ABSTRACT

Quinic acid is one of small important molecular compound which belong to aromaticity, and it is widely used in the field of foodstuff and chemical， as well as medicine. Genic project bacteria which product quinic acid is constructed Using the metabolism project technique. new metabolic quinic acid is synthesized through colishikimic acid way of coliform. Using the metabolism project tradition research strategy, the content of enzyme is enhanced through increasing copy of essential enzyme gene, as well as relieving the feedback inhibition which the essential enzyme receives to maintain activity of enzyme, then the distribution of microorganism's metabolism flows is adjusted. The goal is flowing the carbon metabolism to the greatest degree of producting of quinic acid.

Key words: quinic acid, biosynthesis, co-expression

INTRODUCTION

Quinic acid (Quinic acid, QA) was first discovered in 1790, also known as cinchona acid, commonly found in cinchona bark, tobacco leaves, carrot leaves, apples, pears, coffee and other plants [1], and rarely appears in the bacterium. The medicinal value of quinic acid monomer is not large, but as a raw material or pharmaceutical intermediates, are increasingly been found and applied [2]. With the development of the pharmaceutical, food, chemical industry, As an important fine chemical products and pharmaceutical intermediates, quinic acid and its derivatives expanded the scope, and the features gradually being discovered, such as anti-inflammatory, anti-aging et al. [3]. The demand in the international market also expanding. This experiment confirmed that the *aro*E gene from *E.coli* genome encoding shikimate dehydrogenase has Quinate dehydrogenase activity. And *aro*F^{m} and *aro*E were cloned into expression plasmid pBV220 individually or in series. In consideration of the order of the gene and the type of the promoter will influence the amount of expression of the two genes in series, I designed several combinations: change the order of the two genes; two genes co-expression with one promoter and the two genes were under their own independent promoter. Finally obtained successful expression and have good biological activity. Provide a new idea for quinic acid biosynthesis and thus biological synthesis of aromatic compounds.

EXPERIMENTAL SECTION

Strains and plasmids (Table 1)

Table 1 Strains and plasmids

Strains and plasmids	Characters	Source and reference
Strains		
*Escherichia coli*31884	Donor of genome	ATCC
Escherichia coli DH5α	Recipient strain	Tigen company
Plasmids		
pBV220	AP^r expressing vector	Reference
PEF-Ⅰ	AP^r *aro*E *aro*F^mcis-promoter	This work
PEF-Ⅱ	AP^r *aro*E *aro*F^m trans-promoter	This work
PFE-Ⅰ	AP^r *aro*F^m *aro*E cis-promoter	This work
PFE-Ⅱ	AP^r *aro*F^m *aro*E trans-promoter	This work

Enzymes and reagents: dNTP, TaqDNA polymerase and reaction buffer, restriction enzymes *Eco*RI, *Bam*HI, *Bgl*Ⅱ etc purchased from TaKaRa, TPP (Merck), PEP, E4P (Sigma), Protein Marker (Beijing DingGuo companies), other chemical reagents were analytical grade.

***aro*E and *aro*F^m gene cloning, recombinant protein expression**

gene manipulation techniques and methods, see Ref. [4] [5]. **sequence analysis** was measured by Shanghai Boya Biotechnology Co., Ltd.. The recombinant plasmids pBV220-*aro*E、pBV220-*aro*F^m、PEF-Ⅰ、PEF-Ⅱ、PFE-Ⅰand PFE-Ⅱ were transformed into the strain DH5α, picked single colony and Extracted plasmids,analyzed result by restriction enzyme analysis and PCR, and then pick a single colony inoculated into LB liquid medium containing ampicillin, shaken overnight at 37 ℃, Expand the culture to 20mL by 2%, The culture was incubated at 30℃ in a rotary set at 150 rpm until the optical density (OD600) reached 0.4~0.6. At this time, the cells were cultured at 42℃ for about 4 h for induction of the proteins.

The cells were harvested by centrifugation at 8000rpm for about 2min, mixed with loading buffer, boiled 10min, centrifugated at 12000rpm for 10min, SDS-PAGE assay with 10% separating gel.

Enzyme activity assays of the recombinant strains

Cell cultured and induced, cells were collected by centrifugation at 4 ℃, 5000g for 20min, then cell were washed twice by 100mmol/L Tris-HCl (pH7. 5) and resuspended in the respective buffer, sonicated in an ice water bath; supernatant was collected by centrifugation at 4 ℃, 10000g for 20min. Enzyme activity assays of *aro*E and *aro*F^m according to the literature [6] and [7].

RESULTS AND DISCUSSION

Co-expression of *aro*E and *aro*F^m

As shown above, Fragments and plasmids were digested, phosphorylated, ligated, transformed into *E. coli* DH5α, and then picked the colony, extracted plasmids, identified by restriction enzyme. The results are shown in Figure2 and 3.

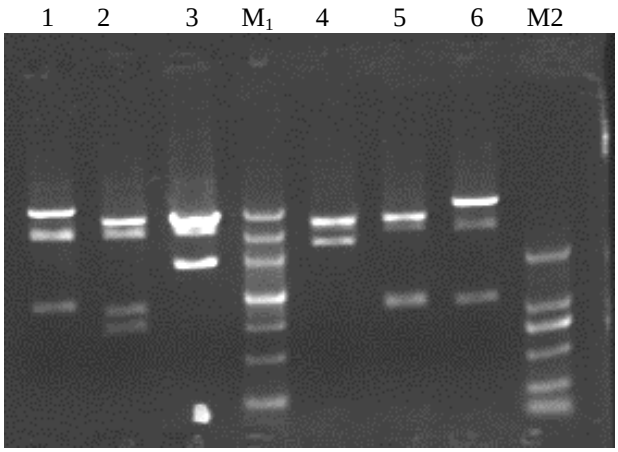

Figure 2 Identification of PEF Ⅰ and PEF Ⅱ with different digestion

M1 : DNA Marker Ⅲ; M2 : DNA Marker DL2000; PEF Ⅰ (4,5,6); PEF Ⅱ (1,2,3); lanes 1,4 : double digestion with BglII + BamHI; lanes 2,5 : double digestion with EcoRI + BamHI; lanes 3,6 : digestion with EcoRI

PEF Ⅰ and PEF Ⅱindicate different in series, resulted in different digestion result

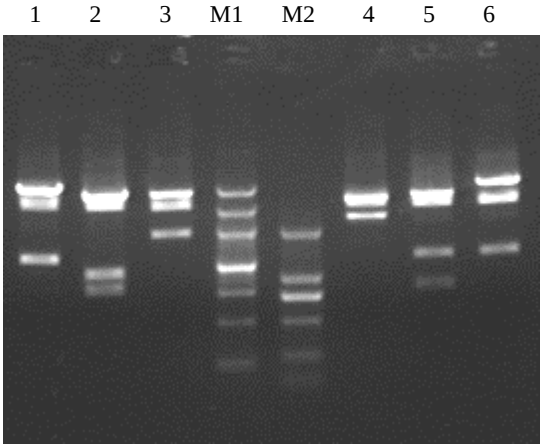

Figure 3 Identification of PFE Ⅰ and PFE Ⅱ with different digestion

M1:DNA Marker Ⅲ; M2 :DNA Marker DL2000; PFE Ⅰ (4,5,6); PFE Ⅱ (1,2,3); lanes 1,4 : double digestion with BglII + BamHI; lanes 2,5 : double digestion with EcoRI + BamHI; lanes 3,6 : digestion with EcoRI

PFE Ⅰ and PFE Ⅱindicate different in series, resulted in different digestion rusult

Expression of *aro*E and *aro*F^{m} in *E. coli*

Cultured and induced strains contain pBV220, pBV220-aroE, pBV220-aroFm, PEF-Ⅰ, PEF-Ⅱ, PFE-Ⅰ, PFE-Ⅱand DH5α host bacteria, SDS-PAGE results were shown in Figure 4. There were 30kD and 39kD protein band appeared in strains contain pBV220-aroE, pBV220-aroF^{m} respectively, and there were 30kD and 39kD protein band appeared meanwhile in strains contain PEF-Ⅰ、PEF-Ⅱ、PFE-Ⅰ、PFE-Ⅱ.

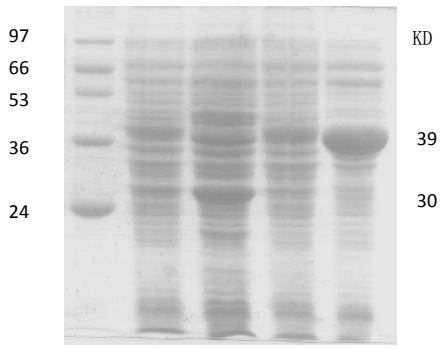

Figure 5 SDS-PAGE analysis of single and tandem expression of *aro*E and *aro*F^{m}

1.DH5α/PFE-Ⅱ 2.DH5α/PFE-Ⅰ 3.DH5α/PEF-Ⅱ;4.DH5α/PEF-Ⅰ; M. Protein marker

Activity assay of *aro*E and *aro*F^{m}

Activity of aroE and aroFm were assayed according to 16 and 17 respectively, Activity of Crude extract from empty host bacteria was 1.0, The results are shown in Table 2.

Table.2 Enzyme activity assays of the recombinant strains

Strains /Plasmids	cloning genes	Relative specific activities of enzymes (increased folds)	
		DS	DHQase
E. coli DH5α/ pBV220	(Control)	1.0	1.0
E. coli DH5α/ PEF- I	*aro*E, *aro*F^{m}	7.0	2.3
E. coli DH5α/ PEF- II	*aro*E, *aro*F^{m}	4.5	2.1
E. coli DH5α/ PFE- I	*aro*F^{m}, *aro*E	7.8	4.0
E. coli DH5α/ PFE- II	*aro*F^{m}, *aro*E	3.0	3.2

DISCUSSION

The synthesis of Quinic acid in large quantities has been a problem, There are four methods from foreign reports about quinic acid preparation: the plant extracts, chemical synthesis, enzymatic and microbial direct fermentation [8,9]. Quinic acid originally derived from plant extracts, this method is low yield and high cost, therefore rarely used. Later quinic acid by chemical synthesis methods, but requires a lot of raw materials, complex process, and produce harmful substances. Therefore, an urgent need a method that is economical and environmentally friendly for synthesis of quinic acid. So enzymatic synthesis emerged, which use shikimate as the substrate to catalyze Synthesis of quinic acid, although this method has the advantages of simple process, short cycle, and high yield, but because of the high price of substrate and enzyme and limited sources, so that its application has been limited. In recent years w ith the development of biotechnology, especially the rise of metabolic engineering, metabolic engineering research continue to apply for the biosynthesis of aromatic compounds[10], it make direct synthesis of quinic acid using microbial fermentation, which was an economical and environmentally friendly method, become possible. In recent years, with the application of genetic engineering techniques in metabolic engineering, many laboratory are trying to construct engineering strains using genetic engineering. In *E. coli* DAHP synthase encoded by *aro*F gene catalyze erythrose-4-phosphate and phosphoenolpyruvate to generate DAHP, but the reaction was subjected the feedback inhibition of prodct Phe, Tyr and Trp. Shikimate dehydrogenase encoded by *aro*E also has quinate dehydrogenase activity can catalyzes the shikimate pathway intermediate 3-Dehydroquinic acid to generate quinic acid. In this experiment, first Clone and expression of *aro*E gene and *aro*F^{m} gene which has anti-feedback inhibition, on the basis of high expression *aro*E and *aro*F^{m}, achieve co-expression of these two genes , and then improve DAHP synthase and Quinate dehydrogenase activity of the engineering bacteria.So that by increasing the number of gene copies and the removal of feedback inhibition to achieve the increase of enzyme amount and improvement of activity, lay the foundation for industrial production of high-producing strain of quinic acid.

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