Part I Biosynthesis of L-glutamate-rich γ-PGA

Materials and methods

1.1 Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. γ-PGA natural producers *B. subtilis* Ia1a and *B. licheniformis* ATCC 9945a were used as sources of the PGA biosynthetic genes. L-glutamic acid producers *C. glutamicum* ATCC13032 and *C. glutamicum* F343 (industrial strain) were used as chassis organisms, and *E. coli* DH5α was used for plasmid construction. The plasmid pZM1 was used for gene cloning and plasmid construction.

Table 1 Bacterial strains and plasmids used in this study.

Plasmids/Strains	Description	Sources
pZ8-1	Used for point mutation	Lab collection
pZM1-Ptac	Inducible expression plasmid, promoter Ptac	Lab collection
B. subtilis Ia1a	Produce γ-PGA with high yield naturally	(Ibrahim et al., 2016)
B. licheniformis ATCC 9945a	Produce γ-PGA with high yield naturally	(Birrer et al., 1994)
C. glutamicum ATCC13032	Can accumulate L-glutamic acid	Lab collection
C. glutamicum F343	Industrial strain which can accumulate L-glutamic acid with high titer	(Zheng et al., 2012)
PGA01	*C. glutamicum* ATCC13032 harbors the genes *pgsBCA* in inducible expression plasmid	This study
PGA02	*C. glutamicum* ATCC13032 harbors the genes	This study

	*capB*CA* in inducible expression plasmid	
PGA03	*C. glutamicum* F343 harbors the genes *pgsBCA* in inducible expression plasmid	This study
PGA04	*C. glutamicum* F343 harbors the genes *capB*CA* in inducible expression plasmid	This study

1.2 Construction of plasmids and recombinant strains

The genes *pgsB*, *pgsC*, *pgsA* from *B. subtilis* strain Ia1a and the genes *capB*, *capC* and *capA* from *B. licheniformis* ATCC 9945a were amplified using corresponding genomic DNA as template. All the primers used in this study are listed in Table 2. The genes were then cloned into plasmid pZM1 and assembled in a monocistronic form using a previously published method (Xu et al., 2012). The transformation method of plasmids into *C. glutamicum* was carried out following a previously published protocol (van der Rest et al., 1999).

Table 2 Primers used in Part I.

Primers	**Sequence (5' to 3')**	**Notes**
pgsB-Nde-F	TATACATATGTGGTTACTCATTATAGCCTGTGC	Clone the gene *pgsB*,
pgsB-BamH-R	CGCGGATCCCTAGCTTACGAGCTGCTTAACCTTG	mutate the NdeI site in
pgsB-R1	CCATGACATCCATGTGGTCTTCTAAAAC	*pgsB* and construction of
pgsB-F2	GTTTTAGAAGACCACATGGATGTCATGG	the recombinant plasmids
pgsC-Nde-F	TATACATATGTTCGGATCAGATTTATACATCG	Clone the gene *pgsC* and
pgsC-BamH-R	CGCGGATCCttaaattaagtagtaaacaaacatgatagc	construction of the
		recombinant plasmids
pgsA-Nde-F	TATACATATGAAAAAAGAACTGAGCTTTCAT	Clone the gene *pgsA* and

pgsA-BamH-R	CGCGGATCCTTATTTAGATTTTAGTTTGTCGCTATG	construction of the recombinant plasmids
CapB-Nde-F	AAAACTGCAGCATATGTGGGTAATGCTATTAGCCTG	Clone the gene *capB*, mutate the *Nde*I site in *capB* and construction of the recombinant plasmids
CapB-BamH-R	CGCGGATCCCTAGCTAACGAGCTGCTTAATCTTG	
mutation-1-F	CGACTTTAAACATTTGGAAGC	
mutation-1-R	GCTTCCAAATGTTTAAAGTCG	
mutation-2-F	CGTCAAAGCGTATGAAGCAG	
mutation-2-R	CTGCTTCATACGCTTTGACG	
CapC-Nde-F	TATACATATGTTTGGATCAGATTTATATATCGC	Clone the gene *capC* and construction of the recombinant plasmids
CapC-BamH-R	CGCGGATCCttagattagatagtaagcatacataatgacg	
CapA-Nde-F	TATACATATGAAAAAACAACTGAACTTTCAGG	Clone the gene *capA* and construction of the recombinant plasmids
CapA-BamH-R	CGCGGATCCTCATTTGTTCACCACTCCGT	

1.3 Medium and cultivation methods

γ-PGA natural producers *B. subtilis* Ia1a and *B. licheniformis* ATCC 9945a were grown overnight in a modified medium E (Leonard et al., 1958) at 37 °C and 220 rpm. The modified medium E contains (per liter): 12.0 g, citric acid; 7.0 g, NH_4Cl; 0.5 g, $MgSO_4 \cdot 7H_2O$; 0.5 g, K_2HPO_4; 0.15 g, $CaCl_2 \cdot 2H_2O$; 0.104 g $MnSO_4 \cdot H_2O$; and 0.04 g, $FeCl_3 \cdot 6H_2O$ (pH 7.4). *E. coli* used for plasmid propagation was grown in Luria-Bertani (LB) medium containing 5 g/L Yeast extract, 10 g/L tryptone, and 10 g/L NaCl. The medium for preparation of recombinant *C. glutamicum* seeds (seed medium)

contained 25 g/L glucose, 1.5 g/L K_2HPO_4, 0.6 g/L $MgSO_4$, 0.005 g/L $MnSO_4$, 0.005 g/L $FeSO_4$, 25 g/L corn steep liquor, and 2.5 g/L urea. Fermentation medium for *C. glutamicum* ATCC13032 is composed of 100 g/L glucose, 30 g/L $(NH_4)_2SO_4$, 3.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.02 g/L $FeSO_4$, 0.02 g/L $MnSO_4$, 5.0×10^{-5} g/L biotin, 4.5×10^{-4} g/L thiamine-HCl, 3.0×10^{-5} g/L protocatechuic acid and 60 g/L $CaCO_3$. The medium for *C. glutamicum* F343 is composed of 40 g/L glucose, 1.0 g/L K_2HPO_4, 0.6 g/L $MgSO_4$, 0.002 g/L $MnSO_4$, 0.002 g/L $FeSO_4$, 5.0×10^{-5} g/L thiamine, 15 g/L corn steep liquor, and 3.0 g/L urea. For *C. glutamicum* and its recombinant strains, colonies were inoculated into seed medium with kanamycin (25 mg/L) and cultured for 12 h at 30 ^{o}C (for *C. glutamicum* ATCC13032) or 38 ^{o}C (for *C. glutamicum* F343) in 220 rpm shaker. Then, the preculture (10%, v/v) was inoculated into 25 mL fermentation medium with kanamycin (25 mg/L) in 250-mL Erlenmeyer flasks and grown for 36 h at 30 ^{o}C (for *C. glutamicum* ATCC13032) or at 38 ^{o}C (for *C. glutamicum* F343) in 220 rpm shaker. IPTG (1 mM) was added 3 h after inoculation to induce for gene expression.

1.4 Analytical methods

Sampling for the measurement of cell growth, glucose, glutamic acid and γ-PGA was performed every 4 h during fermentation. Cell growth was determined by measuring the optical density at 600 nm (OD_{600}) on a Biotek Synergy plate reader. Before the determination of OD_{600}, the samples were diluted by distilled water to the OD_{600} range of 0.2-0.8, and then the OD_{600} values in the diluted samples were measured. The concentration of glucose was analyzed on an Agilent 1200 Series

HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with a ZORBAX column (4.6×250 mm) and refractive index detector (RID). The mobile phase consisted of acetonitrile (75%, v/v) and the flow rate was set at 2 mL/min. For L-glutamic acid analysis, glutamic acid was derivatized by phenylisothiocyanate (PITC). The level of L-glutamic acid was analyzed by HPLC according to the modified method in our lab (Xu et al., 2015). The mobile phase A was prepared by adding 15.2 g sodium acetate into 1850 mL H_2O followed by adjusting pH to 6.5 with acetic acid, and addition of 140 mL acetonitrile. Mobile phase B consisted of acetonitrile (80%, v/v). The flow rate was set at 1 mL/min. Gel permeation chromatography (GPC) was performed to both determine the molecular weight of purified γ-PGA samples and to determine the concentration of γ-PGA in cultures. The HPLC system used was equipped with Shodex KB00 series columns (two KB80 M, one KB802.5) and a refractive index detector (RI detector). The mobile phase consisted of 0.3 M Na_2SO_4 and the flow rate was set at 0.6 mL/min. One-dimensional (1-D) proton (^1H) NMR spectra were recorded on a Bruker Spectrometer (600 MHz) in deuterated dimethyl sulfoxide (DMSO) (Birrer et al., 1994).

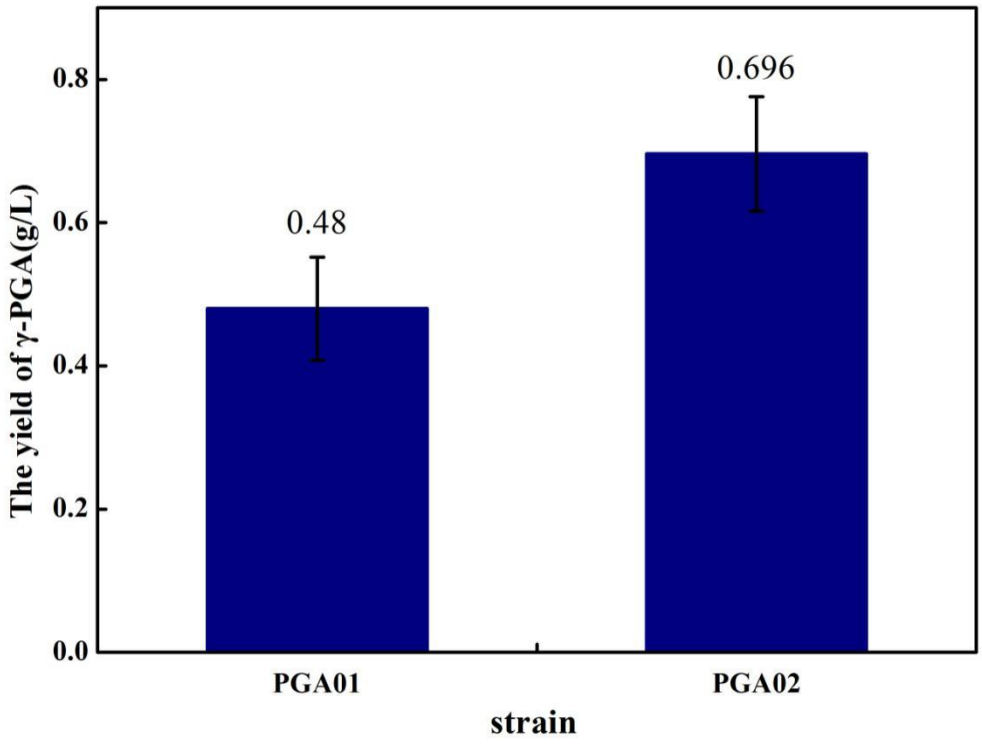
Results

1.5 Biosynthesis of γ-PGA in C. glutamicum ATCC13032

The pathway consisting of *pgsB*, *pgsC* and *pgsA* was first introduced in *C. glutamicum* ATCC13032 (PGA01) and the γ-PGA production accomplished using this recombinant strain was 0.48 g/L (Fig.1A). Similarly, the inducible expression of *capB*, *capC* and *capA* in *C. glutamicum* ATCC13032 (PGA02) led to γ-PGA production of 0.696 g/L (Fig.1A). The production of L-glutamic acid by PGA01 reached 0.48 g/L while

L-glutamic acid achieved by PGA02 was 0.33 g/L (Data not shown). In both cases, we verified the chemical structure of the produced γ-PGA by using ^{1}H NMR (Fig. 1B) and confirmed the presence of γ-PGA in the fermentation samples by GPC.

(A)



(B)

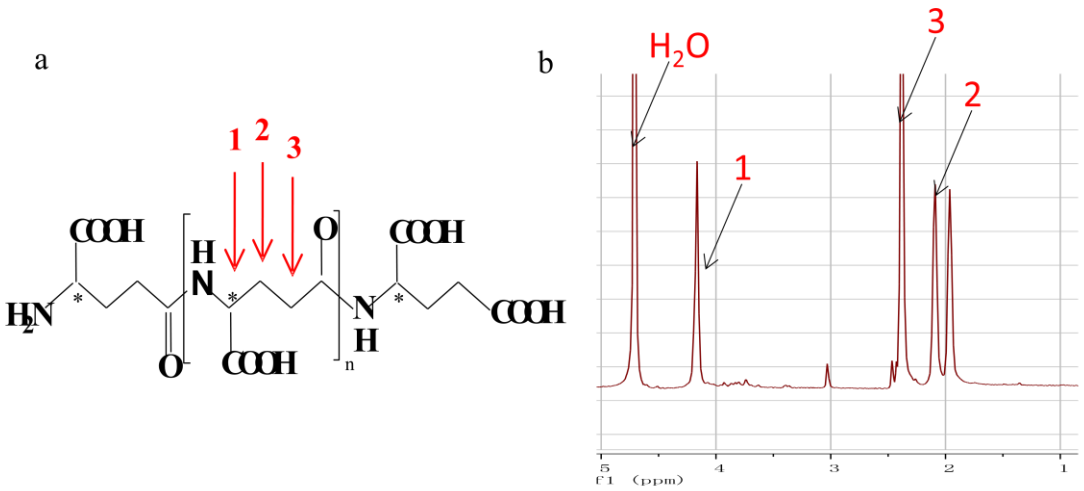


Fig.1. γ-PGA production of strains carrying the *pgsBCA* or *capB*CA* genes under the P_{tac} promoter and characterization of produced γ-PGA by ^{1}H NMR

(A) γ-PGA production by strain PGA01 and PGA02; (B) Structural formula of γ-PGA and ^{1}H NMR

of the samples

1.2 Biosynthesis of γ-PGA in industrial strain C. glutamicum F343 with high yield

The limited supply of L-glutamic acid in *C. glutamicum* ATCC 13032 may represent a bottleneck in the production of γ-PGA. Thus, the industrial strain *C. glutamicum* F343 with high L-glutamic acid production capability, which can grow well even under high temperature (38 °C), was evaluated as a host for γ-PGA production(Zheng et al., 2012). Subsequently, inducible expression of *pgsBCA* or *capB*CA* in *C. glutamicum* F343 (PGA03 and PGA04) was investigated. As a result, the host strain *C. glutamicum* F343 expressing *pgsBCA* under the control of the P_{tac} promoter produced 1.60 g/L γ-PGA, while a slightly higher γ-PGA titer was achieved from the strain carrying the *capB*CA* genes (1.78 g/L) (Fig.2).

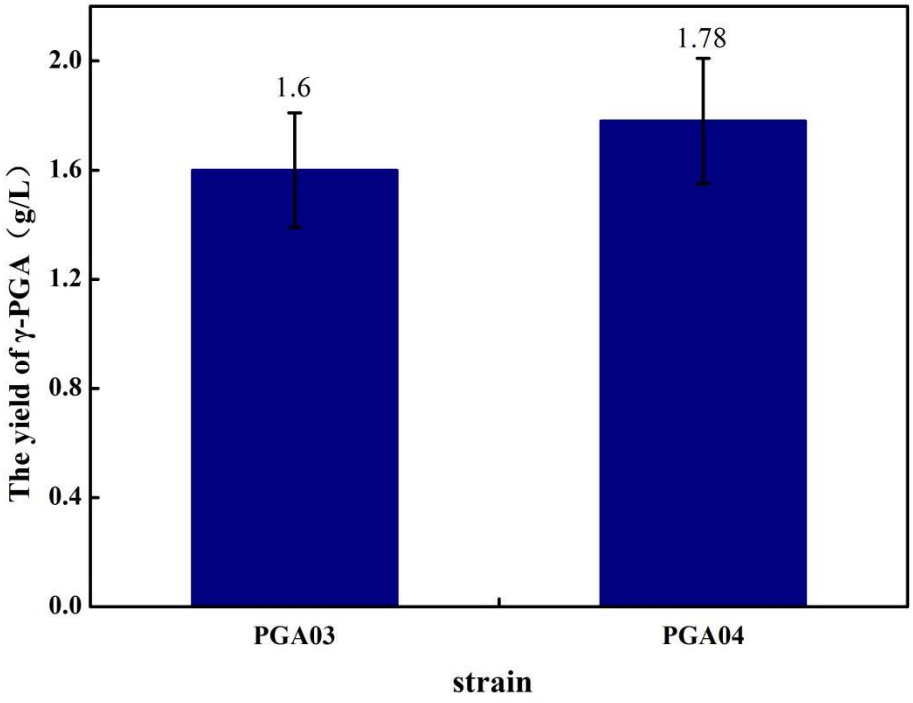


Fig.2 Production of γ-PGA by strain PGA03 and PGA04

The effect of induction time (addition of inducer at 0, 2, 3, 4 and 5 h after starting the shake flask culture) on the γ-PGA production was investigated. As shown in Figure.3A, γ-PGA titer was very sensitive to the induction time. At the same time, an induction time of 2 h gave the highest γ-PGA titer (14.067 g/L). As shown in Figure.3B, after optimizing the fermentation, PGA04(optimized) is about 20 times

higher than PGA02, and about 8 times higher than PGA04. At this point, we can say that we have achieved high yield of γ-PGA.

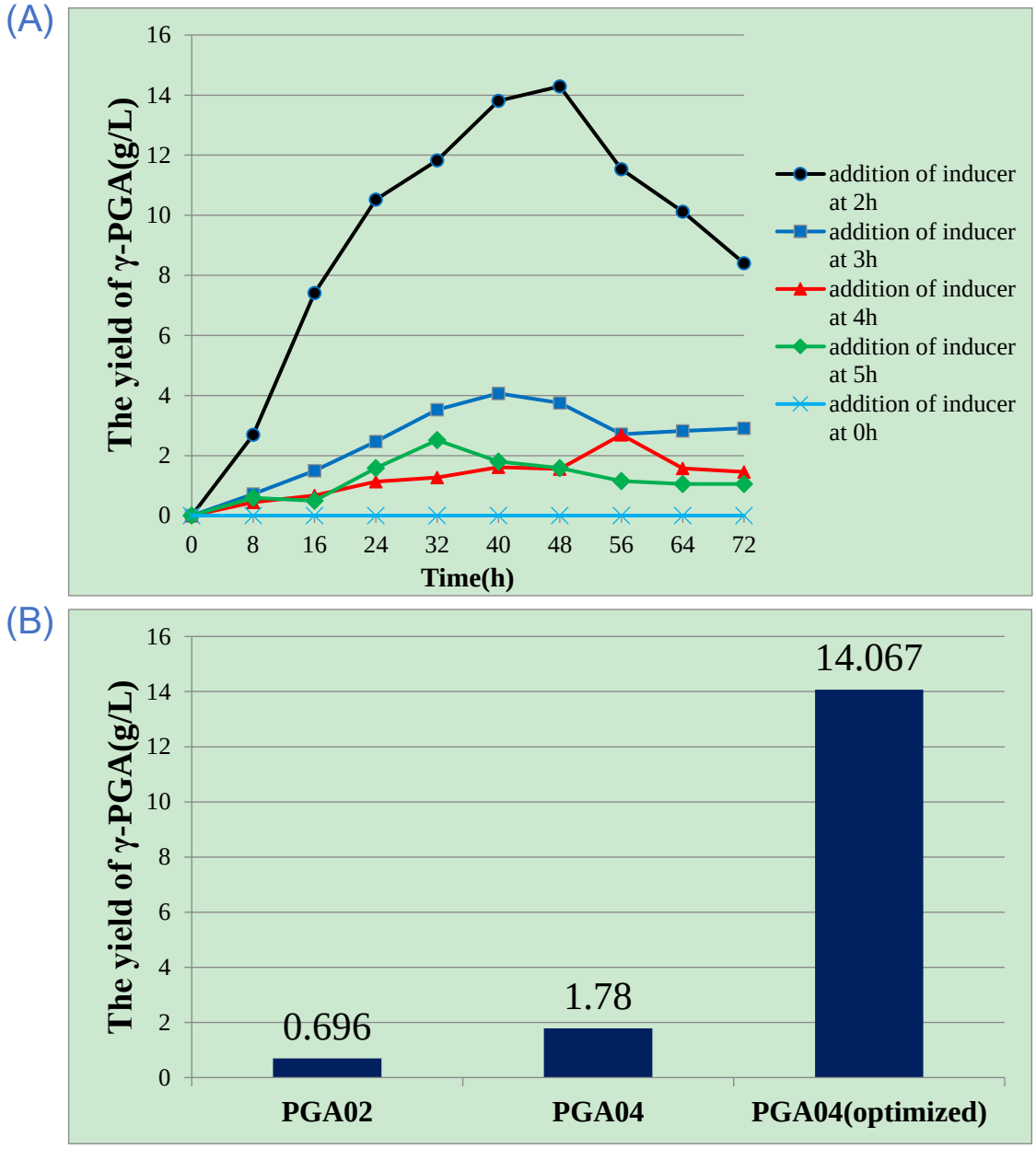


Fig.3

(A). Optimization of induction time of recombinant strain PGA04.

(B). Comparison of fermentation data of PGA02, PGA04, and PGA04(optimized)

PGA02 represents the yield of γ-PGA obtained by introducing *capB*CA* into *C. glutamicum* ATCC13032;

PGA04 represents the yield of γ-PGA obtained by introducing *capB*CA* into *C. glutamicum* F343;

PGA04(optimized) represents the yield of γ-PGA obtained by fermentation optimization in *C. glutamicum* F343-*capB*CA*.