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Glutamic acid independent production of poly-γ-glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of *pgsBCA* genes

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A new glutamic acid independent poly-γ-glutamic acid (γ-PGA) producing strain, which was identified as *Bacillus amyloliquefaciens* LL3 by analysis of 16S rDNA and gyrase subunit A gene (*gyrA*), was isolated from fermented food. The product had a molecular weight of 470, 801 and L-glutamate monomer content of 98.47%. The pre-optimal medium, based on single-factor tests and orthogonal design, contained 50 g/L sucrose, 2 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4$, and provided well-balanced changes in processing parameters and a γ-PGA yield of 4.36 g/L in 200 L system. The γ-PGA synthetase genes *pgsBCA* were cloned from LL3, and successfully expressed by pTrcLpgs vector in *Escherichia coli* JM109, resulting the synthesis of γ-PGA without glutamate. This study demonstrates the designedly improved yield of γ-PGA in 200 L system and the first report of *pgsBCA* from glutamic acid independent strain, which will benefit the metabolized mechanism investigation and the wide-ranging application of γ-PGA.

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1. Introduction

Poly-γ-glutamic acid (γ-PGA) is an unusual macromolecular anionic polypeptide, which consists of D- and L-glutamic acid units polymerized by γ-amide linkages (Ashiuchi and Misono, 2002). Microbial γ-PGA molecules have molecular weight varying from 10 to 1000 k, and their stereochemical structures can be divided into three types: a homopolymer of D-glutamic acid (γ-D-PGA), a homopolymer of L-glutamic acid (γ-L-PGA), and copolymer of random combinations of D-/L-glutamic acid (γ-DL-PGA) (Ashiuchi and Misono, 2003). Many free carboxyls are present in the main chain of γ-PGA, which confer the typical characteristics of polycarboxylic acid, such as super absorbency and moisture retention. These active sites can also be modified by crosslinking, derivative and chelate reactions to yield products used in wide-ranging applications such as hydrogels, flocculants, thickeners, dispersants, drug deliveries, cosmetic, and feed additives (Shih and Van, 2001).

Microorganisms capable of producing γ-PGA mainly belong to the *Bacillus* genus. γ-PGA was first found in *Bacillus anthracis* and serves as a capsule component and important virulence factor, which can help the pathogen to resist phagocytosis and host immune responses (Makino et al., 1989). *Bacillus subtilis* and *Bacillus licheniformis* are the most widely used industrial producers of γ-PGA. The γ-PGA product is secreted into the medium and may protect the organism from the harsh environmental conditions or serve as a carbon, nitrogen, energy source or biofilm formation enhancer (Stanley and Lazazzera, 2005). In addition, Hezayen et al. (2000) identified a new archaeobacteria species *Natrialba aegyptiaca* that can survive in extreme environmental stress (>20% NaCl) due to the strong hydrophilic nature of γ-PGA.

γ-PGA producing strains are divided into two types: one that requires the presence of glutamic acid to produce γ-PGA, while the other does not (Ito et al., 1996). Most of the known γ-PGA producers, such as *B. subtilis* chungkookjang (Ashiuchi and Misono, 2001), *B. licheniformis* NK-03 (Cao et al., 2010) and *B. subtilis* RKY3 (Jeong et al., 2010) belong to the former type, while there are only a few strains of the latter type, including *B. subtilis* C1 (Shih et al., 2005), *B. subtilis* TAM-4 (Ito et al., 1996) and *B. licheniformis* A35 (Cheng et al., 1989). γ-PGA producers with high productivity are mostly L-glutamic acid dependent bacteria, and many genetically engineered strains producing γ-PGA have been constructed (Ashiuchi et al., 1999, 2001; Jiang et al., 2006). However, the bacteria which do not require glutamic acid to produce γ-PGA are of great interest because of the lower cost and simplified process in industrial fermentor production systems. Thus, obtaining effective expression of γ-PGA synthetase (*pgsBCA*) genes in the absence of L-glutamate

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is the key to the success of achieving such γ-PGA producing strains. It was previously reported that the recombinant *Corynebacterium glutamicum* strain harboring a shuttle vector pXMJ19–PGS could express γ-PGA synthetase genes from *B. licheniformis* NK-03 and produce γ-PGA without the addition of L-glutamate (Cao et al., 2010). However, it would be much more valuable if *pgsBCA* genes are cloned and expressed from L-glutamic acid independent γ-PGA producing strain.

In this study, a new glutamic acid independent γ-PGA producing strain from fermented food was isolated and designated as *Bacillus amyloliquefaciens* LL3. The properties of the γ-PGA products were analyzed, and carbon sources, nitrogen sources, and the optimal culture conditions were investigated by single factor and orthogonal design experiments. A 200 L fermentation process was then carried out using the pre-optimized medium to evaluate the scalability of γ-PGA production from the LL3 strain. Furthermore, the *pgsBCA* genes from *B. amyloliquefaciens* LL3 were cloned and expressed in *Escherichia coli*. So far, it is the first report of enhancing the production of γ-PGA in 200 L system and exploring the γ-PGA synthesis genes from glutamic acid independent strains, which will provide a significant step towards the uses in wide-ranging industrial applications.

2. Methods

2.1. Isolation and identification of bacterium

Samples of fermented food (Korea bibimbap paste) bought from a local market were suspended in sterile Luria–Bertani (LB) media (Sambrook and Russell, 2001). After stirring and boiled for about 15 min, the suspensions were serially diluted and spread onto solid isolation medium (basal medium) plates consisting of the following (g/L) (Berekaa et al., 2006): glucose 20, KH_2PO_4 6, K_2HPO_4 14, $MgSO_4$ 0.2, $(NH_4)_2SO_4$ 4, 2 mL of a solution of trace elements (1 mM of $FeSO_4·4H_2O$, $CaCl_2·2H_2O$, $MnSO_4·4H_2O$ and $ZnCl_2$, respectively), and agar 20. The plates were cultivated at 37 °C until mucous colonies were obtained. The pure cultures were stored in 15% glycerol solution at −80 °C.

Cloning of the 16S rRNA gene and detection of other conserved genes by multiplex PCR were employed to identify the highly mucous strain. Amplification of 16S rRNA gene sequence was performed with a Biometra Tpersonal Combi PCR System (Germany), using the 27f and 1492r universal primers (Felske et al., 1999). The sense and antisense primer sequences for 16S rDNA were: AGAGTTTGATCMTGGCTCAG (M = C, A) and CGGYTACCTTGTTACGACTT (Y = T, C). Multiple alignments of 16S rRNA partial gene sequences were performed with BLAST (NCBI), Clustal X program (www.clustal.org), while the construction of a neighbor-joining phylogenetic tree were accomplished with MEGA 4 software (www.megasoftware.net) based on the tests with 1000 bootstrap replicates. Subsequently, a multiplex PCR approach was used to validate the identity of the 16S rRNA gene. The more conserved *B. subtilis* RNA polymerase subunit α (*rpoA*) gene and *B. amyloliquefaciens* gyrase subunit A (*gyrA*) gene were chosen to detect from the strain LL3 by multiplex PCR (Cao et al., 2008), with the model strains *B. subtilis* IFO3335 and *B. amyloliquefaciens* ACCC10225 as controls. The primers sequences were: *rpoA* gene, 5′-CGTAGAGGCCACTTGAGCG-3′ (forward) and 5′-CTGCCGTTACAGTTCCTT-3′ (reverse); *gyrA* gene, 5′-AAATCTGCCCGTATCGTCG-3′ (forward) and 5′-GCGTCACGGCGAATCTCAA-3′ (reverse). The PCR conditions were as follows: 30 cycles of denaturation at 94 °C, 40 s; annealing at 58 °C, 40 s; extension at 72 °C, 40 s. The product sequence was aligned with the referred genes, and the strain designated LL3 was ultimately confirmed with Bergey's Manual of Systematic Bacteriology.

2.2. Characterization of the biopolymer in culture medium

2.2.1. Purification and identification of the biopolymer

γ-PGA was recovered and purified according to the method of Kubota et al. (1993). The fermentation broth was centrifuged at 20,000*g* (20 min, 4 °C), and the cell pellet was dried and weighed. After the supernatant was treated with 4-fold volumes of cold anhydrous ethanol, the precipitate was centrifuged again and dissolved in deionized water. The solution was dialyzed or ultrafiltered to remove low molecular products or insoluble substances and then lyophilized (−50 °C) to obtain pure γ-PGA.

The sample above and a γ-PGA standard were dissolved in deionized water and scanned under 190–800 nm ultraviolet wavelength using a SHIMADZU UV-1800 spectrophotometer (Kyoto, Japan). The sample was hydrolyzed by HCl at 110 °C for 8 h (nitrogen protection), neutralized with NaOH and then analyzed by thin layer chromatography (TLC) on a silica gel plate. The glutamic acid was visualized by the developing agent (*n*-butanol–acetone–water (12:3:5)) and streak reagent (0.2% ninhydrin) (Kambourova et al., 2001). The sample dissolved in heavy water (D_2O) was analyzed with a Nuclear Magnetic Resonance Spectrometer (Varian Infinity plus 400, USA), and the ^1H NMR spectroscopy fingerprints of the sample were compared with that of a standard γ-PGA to confirm that the product was in fact γ-PGA.

2.2.2. Molecular weight determination

The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (PDI) of γ-PGA were measured by gel permeation chromatography (GPC) using an Alltech system controller (Alltech Associates Inc., US) equipped with a Shodex KW804 column (Showa Denko KK, Japan) and Schambeck SFD RI detector (Germany) calibrated by the Shodex Pullulan-82 standard. The eluant containing 0.25 N $NaNO_3$ was brought to natural pH, and the flow rate was set at 0.6 mL/min (Cao et al., 2010). The calibration curve was drawn with the retention time and M_w. Furthermore, the relative viscosity of the cell-free culture, which could represent the approximate yield and molecular weight of γ-PGA (Ito et al., 1996; Shih et al., 2005), was measured with Brookfield Digital Rheometer (model DV-I, USA) equipped with a spindle S00 code at the shear rate of 1 S^{-1}. Distilled water (at 25 °C) was used as a viscosity standard.

2.2.3. Determination of D-/L-glutamic acid composition

The ratio of D-/L-glutamic acid in γ-PGA was determined by reversed phase high performance liquid chromatography (RT-HPLC) with an Alltech GRACE C18 column (Cao et al., 2010). The different chiral forms of γ-PGA in the hydrolysates were separated by FDAA (Marfey's reagent) and subjected to acetonitrile gradient elution, de-modulating at UV 340 nm. The calibration curve was generated by different concentrations and corresponding ratios of the D-glutamic acid peak area.

2.3. Pre-optimization of γ-PGA production by single-factor and orthogonal experimental design tests

2.3.1. Determination of growth curve and fermentation time, pH, carbon source and nitrogen source by single-factor tests

The γ-PGA yield is significantly influenced by the characteristics of a particular strain. Therefore, LB broth was inoculated at 0.5% volume to generate the standard growth curve (optical density at 660 nm, $OD_{660\ nm}$) at 37 °C and evaluate the effects of a range of temperatures (between 25 and 40 °C) and pH levels (between 6.0 and 8.0). Additionally, the optimal fermentation time was assessed by measuring relative viscosity between 0 and 96 h. The optimal carbon sources were selected by testing glucose, fructose, glycerol, sucrose, glutamate, lactose, and promising nitrogen sources were

selected from ammonium sulfate, urea, peptone, beef extract, yeast extract screened by single-factor tests (Bajaj et al., 2009).

2.3.2. Optimization of concentrations of selected medium components by orthogonal experimental design

In this study, an orthogonal experimental design was applied to determine the optimal nutrient concentrations and evaluate their significance on γ-PGA production. Three different variables (factors) and concentrations (levels) of model sources were tested in the experimental design. Among them, sucrose and $(NH_4)_2SO_4$ were selected based previous experience and prior single-factor tests, but $MgSO_4$ was chosen based on published report (Ashiuchi et al., 2004). The ranges of levels tested were also selected on the basis of preliminary experiments (data not shown) using concentrations of (g/L): sucrose, 15, 30, 50; $(NH_4)_2SO_4$, 2, 4, 6 and $MgSO_4$, 0.2, 0.4, 0.6, respectively. For a 3^3 factorial design with three factors at three levels, nine fitting experimental runs were required (Box and Wilson, 1951). Table 1 shows the trial combinations, experimental error list, and γ-PGA yield of two parallel groups. The corresponding ANOVA results are shown in Table 2. Factors with the highest *F*-value and exceeded 95% confidence level ($F_{0.05}$) were considered have efficient γ-PGA production.

2.4. Upscaling cultivation in a 200 L fermentor

2.4.1. Cultivation process of 200 L fermentation

To prepare the seed cells, 6 L of LB medium, inoculated from a 600 mL overnight flask culture of LL3, was cultured in a 10-L jar fermentor (Biotech-3000, Baoxing Bioengineering Equipment Co. Ltd., China) for 20 h when cells were grown to an optical density at 600 nm ($OD_{600\ nm}$) of 1.8. The 6 L seed liquid was then transferred to a scaled-up cultivation system, which was carried out at 37 °C in a 200 L computer controlled fermentor (Biotech-3000, Baoxing Bioengineering Equipment Co., Ltd., China) with 120 L of pre-optimized medium for the orthogonal experimental design (g/L), i.e., sucrose 50, $(NH_4)_2SO_4$ 2, $MgSO_4$ 0.6, KH_2PO_4 6, K_2HPO_4 14, and 2 mL of solution with trace elements (1 mM of $FeSO_4•4H_2O$, $CaCl_2•2H_2O$, $MnSO_4•4H_2O$ and $ZnCl_2$). The pH, agitation speed and sterile air rate were maintained at 7.0, 200 rpm, and 1.5 vvm, respectively. The fermentation proceeded for 48 h at 37 °C and samples were withdrawn at a 4 h interval for further analysis.

Table 2
ANOVA (analysis of variance) table of 3^3 orthogonal experimental design.

Factor	Sum of squares	d.f.	Mean of squares	*F* value	$F_{0.05}$	$F_{0.01}$
Sucrose	0.023	2	0.0115	7.19*	4.10	7.55
$(NH_4)_2SO_4$	0.003	2	0.0015	0.94	4.10	7.55
$MgSO_4$	0.011	2	0.0055	3.44	4.10	7.55
Groups	0.00000089	1	0.00000089	0.00056	4.96	10.01
Pure error	0.003	2	0.0015			
Lack of fit	0.013	8	0.001625	1.08		
Residual	0.016	10	0.0016			
Total	0.0069	17				

* Significant at 5% level.

2.4.2. Determination of processing parameters

The pH and dissolved oxygen (DO) were detected automatically by the fermentor probes. The yield of γ-PGA, viscosity and OD_{600} were measured as described in Section 2.2. The residual concentration of sucrose, following the protein elimination by Carrez agent treatment, was analyzed using a previously reported method (Li et al., 2003).

2.5. Cloning and expression of pgsBCA genes

To amplify the γ-PGA biosynthesis genes (*pgsBCA*), PCR was carried out using chromosomal DNA of *B. amyloliquefaciens* LL3 as a template. Primers used in the study were designed based on the sequences of *B. amyloliquefaciens* FZB42 (Chen et al., 2007) *ywsC–ywtAB genes*, i.e., a sense primer (5′-CGCGGATCCAGAAGGAGATGTCAAAAATCAATG-3′), and an antisense primer (5′-CCCAAGCTTGATTTTCATTTGTTTTTCACTCCGC-3′), which contained restriction sites of *Bam*HI and *Hind*III, respectively (underlined). The amplified DNA fragment was cloned into the pMD19-Simple T Vector (TaKaRa Biotech Co. Ltd., Japan), sequenced and aligned with other known *pgsBCA* sequences obtained from the NCBI database using DNAMAN Software (Lynnon Co., US) and Clustal X, such as *B. subtilis* 168, *B. licheniformis* ATCC14580, *B. pumilus* SAFR-032 and *B. amyloliquefaciens* FZB42.

For the expression of γ-PGA biosynthesis genes, the *pgsBCA* fragment was digested with *Bam*HI and *Hind*III (TaKaRa Biotech Co. Ltd., Japan) from the T vector and sub-cloned into pTrc99A (Gifted from Chinese Academy of Sciences), which contains an inducible *trc* protomer and ampicillin resistance gene (Amp^r). The resultant plasmid pTrcLpgs was transformed into *E. coli* JM109 by

Table 1
Experimental design and results of three-factor-three-level orthogonal design for selecting of optimized γ-PGA production conditions.

Trial number	Factors				γ-PGA yield (g/100 mL)		
	Sucrose (g/L)	$(NH_4)_2SO_4$ (g/L)	$MgSO_4$ (g/L)	Error list	Group 1	Group 2	T_t
1	15	2	0.2	1	0.112	0.097	0.209
2	15	4	0.4	2	0.093	0.077	0.170
3	15	6	0.6	3	0.141	0.196	0.337
4	30	2	0.4	3	0.156	0.158	0.314
5	30	4	0.6	1	0.155	0.180	0.335
6	30	6	0.2	2	0.131	0.198	0.329
7	50	2	0.6	2	0.315	0.210	0.525
8	50	4	0.2	3	0.209	0.145	0.354
9	50	6	0.4	1	0.158	0.213	0.371
T_1	0.716	1.048	0.892	0.915			
T_2	0.978	0.859	0.855	1.024	1.470	1.474	2.944(*T*)
T_3	1.250	1.037	1.197	1.005			
X_1	0.119	0.175	0.149	0.153			
X_2	0.163	0.143	0.143	0.171			
X_3	0.208	0.173	0.200	0.168			

T_i: summation of trial index at the same level, *i* = 1,2,3.
X_i: average value of trial index at the same level, *i* = 1,2,3.

a previously reported method (Sambrook and Russell, 2001). The recombinant *E. coli* strain was inoculated into 5 mL LB medium containing ampicillin (100 μg/mL). After overnight growth at 37 °C with shaking, a 1% (v/v) inoculum was transferred into a 500 mL flask filled with 150 mL LB medium plus 2% L-glutamic acid (or 2% glucose), 0.5% $MgCl_2$ and 100 μg/mL ampicillin. IPTG (1 mM) was added into the culture broth after 24 h (OD_{600} about 1.5) and further cultivation for 36 h. The product was then separated, purified and quantified as described in Section 2.2.

3. Results and discussion

3.1. Isolation, characterization and taxonomic classification of the new strain LL3

From the solid culture plate of the glutamic acid-independent bacteria, three viscous strains were obtained, and the highly viscous isolate LL3 was selected for the following study. Analysis of the morphological and physiological characteristics of LL3 revealed that it was rod-shaped, gram-positive, spore-forming, thermo-tolerant (surviving at 100 °C) and amylase active. Additionally, the colonies were regular shaped, wet and dewy.

The 1438 bp fragment of 16S rRNA partial gene was amplified from the clone, sequenced and registered in GenBank (accession No. EU755383). Comparison of the sequence with other known sequences available from NCBI database revealed that the strain LL3 clearly belonged to the genus *Bacillus*. Fig. 1 shows the phylogenetic tree of LL3 based on multiple alignments of 16S rRNA gene. The strain was almost identical (more than 99% homology) to *B. subtilis* CICC10025 and *B. amyloliquefaciens* CICC22383. To more accurately define the species, *B. subtilis rpoA* and *B. amyloliquefaciens gyrA* genes were amplified by multiplex PCR. The resulting PCR fragment bands were separated by agarose gel electrophoresis and visualized as shown in Fig. 2a. The migration distance of the LL3 band corresponded to that of the model strain of *B. amyloliquefaciens* ACCC10225 and was obviously different from that of *B. subtilis* IFO3335 (about 250 bp). The 691 bp fragment was sequenced and deposited into GenBank (accession No. GQ387663). The result of BLAST analysis revealed that the amplified fragment has the greatest similarity to the *gyrA* sequence of *B. amyloliquefaciens* strain NRRL B-14393 (99%), and the isolated strain was therefore designated as *B. amyloliquefaciens* LL3.

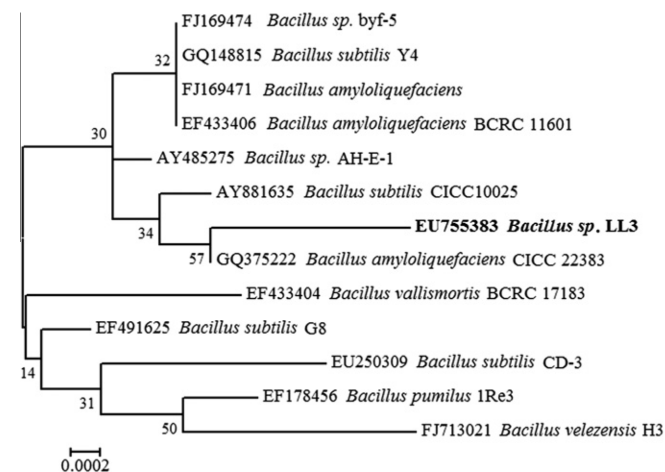

Fig. 1. Phylogenetic relationship of *Bacillus sp.* LL3 (bold fonts) and other *Bacillus* strains based on the neighbor-joining tree analysis of 16S rRNA gene. The bar (0.0002) at the bottom of the tree indicates the substitution per nucleotide position. Numbers at nodes are percentage bootstrap values based on 1000 replications. GenBank accession numbers are given in front of the strains.

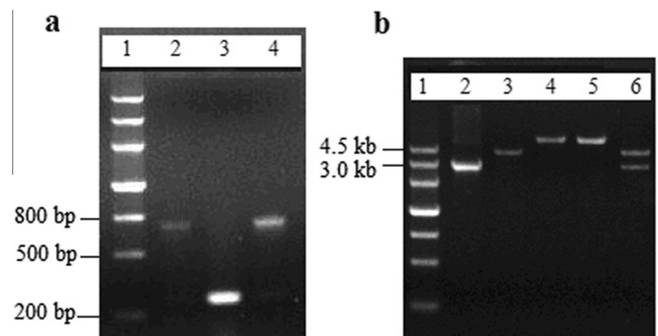

Fig. 2. Electrophoresis analysis of multiplex PCR of LL3 and construction of recombinant plasmid pTrcLpgs. (a) Multiplex PCR of LL3 and two model strains. Lane 1, DNA Marker III TIANGEN; Lane 2, *Bacillus sp.* LL3; Lane 3, *B. subtilis* IFO3335; Lane 4, *B. amyloliquefaciens* ACCC10225. (b) Construction of recombinant plasmid pTrcLpgs from pTrc99A and γ-PGA synthetase *pgsBCA* genes. Lane 1, DNA Marker III TIANGEN; Lane 2, colony PCR product; Lane 3, pTrc99A/*Bam*HI; Lane 4, pTrcLpgs/*Bam*HI; Lane 5, pTrcLpgs/*Hin*dIII; Lane 6, pTrcLpgs/*Hin*dIII + *Bam*HI.

3.2. Characterization of γ-PGA biosynthesized by B. amyloliquefaciens LL3

3.2.1. Identification and qualitative analysis of γ-PGA

The UV scanning spectrum of purified product synthesized by LL3 presented the same absorption peak at 206 nm (γ-amide linkage) as that of the γ-PGA standard. The sample was further confirmed by TLC and ^{1}H NMR spectrum. The 6 N HCl hydrolysate of purified sample was consistent with glutamic acid alone. TLC of the hydrolysate, which was performed on a silica gel plate and visualized with 0.2% ninhydrin, indicated a single spot with an R_f value (R_f = 0.28), almost equal to that of authentic L-glutamic acid (R_f = 0.27). The result of ^{1}H NMR spectrum showed that the chemical shifts of α-H (4.063 ppm), β-H (1.846 ppm and 1.980 ppm), γ-H (2.261 ppm) and N–H (7.732 ppm) from LL3 product nearly overlapped with the peak positions of the Sigma γ-PGA standard as that was reported by Cao et al. (2010) (Fig. S1). However, there were some secondary peaks locating beside the α-H and maybe occupy comparative percent content, which was supposed to be the chemical shifts of hydrogen from impure polysaccharide or protein (hypothesized as Nattokinase from molecular weight analysis, data not shown).

3.2.2. Determination of molecular weight and D-/L-glutamate composition of γ-PGA

The molecular weight of γ-PGA produced by *B. amyloliquefaciens* LL3 was determined by GPC. The retention time was 9.644 min, and the weight-average molecular weight (M_W) was determined to be 470, 801 Da, according to the calibration curve: Log (M_w) = −0.2149*T*r + 7.7554 (*T*r, retention time; R^2 = 0.9964). In addition, the number-average molecular weight (M_n = 456, 681) and the polydispersity index (PI = 1.031) were analyzed by using the Alltech system. It has been suggested that the cultivation conditions and medium components such as rotation speed, medium ionic concentration and fermentation time all affect the molecular weight of the γ-PGA produced (Bajaj et al., 2009; Shih et al., 2002). Whether the above factors affect the molecular weight of γ-PGA produced by *B. amyloliquefaciens* LL3 has yet to be determined. The γ-PGA depolymerase is also known to have an important effect on the molecular weight of γ-PGA. Ashiuchi and Misono (2003); Ashiuchi et al. (2006); Kimura and Itoh (2003); Suzuki and Tahara (2003) and their colleagues had cloned the depolymerase gene *pgdS* (*ywtD*) from *B. subtilis* and purified the expression products from transformed *E. coli* or other host cells. They found that the *pgdS* gene of about 1.2 kb is located down-

stream of *ywsC* and *ywtABC*, a γ-PGA biosynthesis operon. The *pgdS* product, a designated γ-DL-glutamyl hydrolase, shows certain membrane-associated structural features and partial similarity to D/L-endopeptidase. The endopeptidase selectivity assay indicated that PgdS recognizes the D-glutamate unit and favors cleaving the γ-glutamyl bond between two D-glutamate residues in γ-PGA. This hydrolysis reaction is evidently affected by metal ion and time.

D-/L-glutamic acid peak time and area of LL3 γ-PGA hydrolysates were measured by HPLC after pre-column derivation by FDAA. The retention time of the D and L-glutamic acid peaks were at 19.654 min and 17.791 min, respectively, which was consistent with the γ-PGA standard hydrolysates. The area ratio of the D-glutamic acid peaks relative to the total area was 3.121%, and the composition of γ-PGA hydrolysates was determined (D-glutamic acid, 1.53%; L-glutamic acid, 98.47%) according to the calibration curve of weight ratio (m%) against peak area ratio (area%) of D-glutamic acid by the following formula: $Y_{m\%} = 1.1759X_{area\%} + 0.0132$ ($X_{area\%}$: peak area ratio; $R^2 = 0.9973$) (Cao et al., 2010). However, the LL3 γ-PGA composition was greatly different from previous reports of glutamic acid independent γ-PGA production from other strains such as *B. subtilis* TAM4 (D:L = 78:22) (Ito et al., 1996), *B. subtilis* C1 (D:L = 97:3) (Shih et al., 2005), and *B. licheniformis* A35 (D:L = (50–80):(50–20)) (Cheng et al., 1989). γ-PGA with a relatively higher L-glutamic acid ratio can be more easily metabolized in organisms and used in delivery of products. Therefore, production of LL3 could potentially be broadly applied in medicinal applications. There are two different pathways responsible for the conversion of L-glutamic acid to its D-counterpart (Ashiuchi and Misono, 2001; Shih and Van, 2001; Shih et al., 2001): one is through indirect conversion involving pyruvic acid amino-transferase; the other is through direct conversion by glutamate racemase. γ-PGA with diverse compositions can be obtained by controlling the activity of the two enzymes. At present, the glutamate racemase gene (*racE*, GenBank accession No. GQ375412) has been amplified from LL3, the further genetic and apparent research on *racE* will provide better understanding of stereo-chemical regulation and synthesis of γ-PGA.

3.3. Pre-optimization of γ-PGA production of strain LL3

3.3.1. Effect of culture conditions, carbon and nitrogen sources on the growth and γ-PGA yield of LL3 strain

The growth curve of the LL3 strain cultivated in LB broth was measured by $OD_{660\ nm}$ (data not shown) which showed that it grows optimally at 37 °C, pH 7.0, and goes through the logarithmic phase from 2 to 5 h and a steady phase from 12 to 20 h, followed by autolysis. Because γ-PGA yield can be estimated by relative viscosity, the changes were traced in this property and no inconsistency was detected. When scaled to 24 h, maximal viscosity was obtained. Therefore, the time of 24 h was chosen as the optimal time in the fermentation experiment. Since this cycle time is much shorter than that of all previously reported γ-PGA producing strains, LL3 could become one of the most practical industrialized γ-PGA producers. Nevertheless, more research is still needed to confirm the relationship between γ-PGA yield and viscosity.

The effects of carbon and nitrogen sources on biopolymer production by *B. amyloliquefaciens* LL3 were monitored in a shaking flask (140 rpm) inoculated at 1% in basal medium and cultured at 37 °C. As a glutamic acid independent strain with *de novo* L-glutamate synthesis (Shih and Van, 2001), LL3 did not favor glutamate (almost no γ-PGA detected), but absorbed sucrose as the optimal carbon source for the synthesis of γ-PGA (about 1.4 g/L), which provided with a 15% more yield than the second choice of glucose (Fig. S2). The adoption of sucrose was presumed that sucrose could be converted to more easily utilized products glucose and fructose by hydrolase, but whether it was depended on the hydrolysis reaction needs the isotope track of sucrose, glucose and γ-PGA. In basal medium containing sucrose, different nitrogen sources were added to test in selective culture, and the result indicated that $(NH_4)_2SO_4$ was the optimal nitrogen source, i.e., inorganic nitrogen was more favorable for γ-PGA production than organic nitrogen sources (Fig. S2). NH_4^+ (NH_3), served as direct substrate of aminotransferase, which can transform α-ketoglutaric acid and glutamine into glutamic acid for synthesis of γ-PGA.

3.3.2. Orthogonal experimental design

The yield of γ-PGA from LL3 in basal medium was about 0.625 g/L and only reached 1.4 g/L with addition of optimal carbon and nitrogen sources. Therefore, it was necessary to use factorial methods to optimize the medium composition. An orthogonal experimental design is a sequential procedure that allows rapid and efficient determination of optimal conditions and can easily minimize the variance of relative coefficients. Since the factors and their location of the optimum were unknown prior to running orthogonal experiment, it is imaginable that a design with rota that ensures equal precision of estimation in all directions (Shih et al., 2002). Table 1 shows the complete orthogonal or three-level three-factorial design (3^3), and variables with confidence levels exceeding 95% were considered as significant. According to the test statistics and analysis from Table 2, the *F*-value of sucrose was 7.19, falling between $F_{0.05}$ (4.10) and $F_{0.01}$ (7.55), whereas the lack of fit was only 1.08. From the confidence level of variables, it was apparent that sucrose (95–99%) was the most significantly positive variable affecting γ-PGA production, whereas lack of fit of other variables lower than 95% were considered insignificant. This design showed that the γ-PGA produced by *B. amyloliquefaciens* LL3 was increased significantly by 3.2-fold (from 0.625 to 2.63 g/L) when the strain was cultivated in the optimal medium (Trial number 7) composed of sucrose 50 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.6 g/L, as compared with the basal medium. Therefore, the orthogonal experimental design proved to be a useful and feasible tool to enhance γ-PGA production.

It is known that biotin acts as a prosthetic group for many ATP-dependent carboxylases, such as pyruvate carboxylase acetyl-CoA carboxylase, and it can participate in carbon dioxide fixation and carboxylation reactions. Furthermore, the above mentioned carboxylases are significant factors for the formation and secretion of glutamic acid. Biotin has also been reported to have important effects on γ-PGA yield of some glutamic acid-dependent γ-PGA producing strains (Richard and Margaritis, 2003; Ueda, 1989). Consequently, the effect of biotin on cell growth, pH, viscosity and γ-PGA yield of the LL3 strain was investigated. These results indicated that the strain showed an optimal growth in the presence of 1 mg/L biotin, and that the γ-PGA yield improved by about 20% compared to the control in the presence of 0.5 mg/L biotin (data not shown). Huang et al. (2011) reported that Ca^{2+} contributed to the redistribution of the carbon flux and improved the γ-PGA production in *B. subtilis* CGMCC2108 by enhancing the activity of certain key enzymes (ICDH, GDH, and ODHC) around the 2-oxoglutarate branch in γ-PGA synthesis pathway. Therefore, the γ-PGA biosynthesis can be controlled with biotin or metal ions (Ca^{2+}) by promoting glutamic acid synthetic flux.

3.4. The scaled-up system of γ-PGA production in a 200 L fermentor

Based on the pre-optimization culture above, an experiment using a 10 L fermentor with 6 L broth was carried out at 37 °C for about 24 h, and it produced similar steady cell growth and γ-PGA yield as that in a small flask culture (data not shown). Therefore, a second scaled-up system using a 200 L fermentor with 120 L medium was carried out to observe the relative parameters, which

was set at constant pH, agitation speed and sterile air rate. As shown in Fig. 3, during the incubation period, especially before 12 h, the turbidity of the cell suspension, the sucrose utilization and the viscosity remained relatively unchanged, indicating that sucrose was a favorable factor for improving cellular growth. Almost all of the γ-PGA was produced from residual nutrition in the 6 L seed medium. The growth of the cells, the viscosity and consumption of sucrose were increased steadily along the process of fermentation. The drop of the dissolved oxygen, which was appeared steeply at 16 h, was presumed to owe to the exponential growth of the bacterial cells and very high viscosity of the culture. In addition, the γ-PGA depolymerase may begin to exhibit considerable effects at the end of fermentation cycle which would cause a drop in γ-PGA production and the viscosity.

Although the γ-PGA yield reached a maximum of 4.36 g/L at the incubation time of 44 h, which was consistent with the maximum viscosity and about 1.7-fold higher than that obtained in flask culture, this yield was still very low when compared with that obtained with other glutamic acid dependent γ-PGA producing strains. Considering the restrictive factors affecting γ-PGA yield, $(NH_4)_2SO_4$ was determined to be the most significant factor, i.e., 2 g/L $(NH_4)_2SO_4$ could produce a potential yield of 4.455 g/L γ-PGA by total transamination. Therefore, it may be necessary to employ other statistical designs, such as the Plackett–Burman and response surface, as well as genetic modification approaches, including the chromosomal integration of expression control sequence (Yeh et al., 2010) and *Vitreoscilla* hemoglobin gene (Su et al., 2010), to obtain the maximum yield of γ-PGA from the LL3 strain comparable to that reported from other strains (Ito et al., 1996; Shih et al., 2001; Soliman et al., 2005).

3.5. Cloning and expression of pgsBCA genes from the LL3 strain

Much research has indicated that *pgsBCA* genes encode an enzymatic system synthesizing γ-PGA from glutamate in *B. subtilis* (Ashiuchi and Misono, 2002; Ashiuchi et al., 1999, 2001, 2004), namely the γ-PGA synthetase complex. Although the mechanisms of the PgsB, PgsA, PgsC components have been the subject of focus for a long time, there has been little work on the cloning and expression of *pgsBCA* genes from *B. amyloliquefaciens*, especially, from the glutamate independent γ-PGA producing strains.

In this study, a fragment of 3150 bp was amplified and sequenced from *B. amyloliquefaciens* LL3, which consisted of three open reading frames (ORF), *pgsB* (*ywsC*) 1182 bp, *pgsC* (*ywtA*) 450 bp, and *pgsA* (*ywtB*) 1149 bp, and deposited at GenBank (accession No.: *pgsB*, HM034756; *pgsC*, HM034757; *pgsA*, HM034758). In

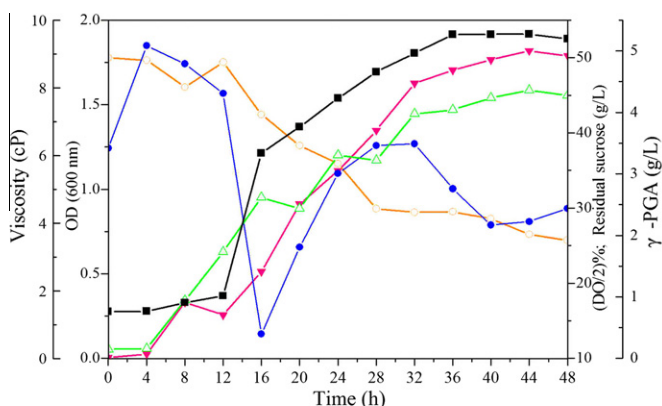

Fig. 3. Time curve of process parameters in 200 L fermentation of glutamate independent *B. amyloliquefaciens* LL3. Cell concentration at 600 nm (OD, –▼–), viscosity of cell free culture (–■–), 1/2 dissolved oxygen ((DO/2)%, –●–), residual sucrose concentration (–○–), the yield of γ-PGA (–△–).

Table 3
The comparison of the *pgsBCA* genes and PgsBCA synthetase complex of LL3 with other strains.

	B. amyloliquefaciens LL3 (%)					
	pgsB	*pgsC*	*pgsA*	*PgsB*	*PgsC*	*PgsA*
B. amyloliquefaciens FZB42 (%)	97.04	97.11	94.87	100	100	98.17
B. subtilis 168 (%)	81.56	83.33	74.06	93.13	93.96	78.53
B. licheniformis ATCC14580 (%)	77.07	80.44	67.95	88.30	85.91	64.52
B. pumilus SAFR-032 (%)	77.50	75.33	63.25	88.80	86.58	59.13
Identity (%)	87.80	88.09	80.19	94.35	94.09	79.85

comparing the ORF and deduced amino acids with those from *B. amyloliquefaciens* FZB42, *B. subtilis* 168, *B. licheniformis* ATCC14580 and *B. pumilus* SAFR-032, it was revealed that PgsB and PgsC had great similarity (about 94.35% and 94.09% identity), while PgsA was the most variably expressed protein in the γ-PGA synthetase complex (79.85% identity) (Table 3). Based on the consensus of PgsB, PgsC, PgsA components and their amide acids structural features from LL3 (Fig. S3), the functions of each synthetase complex (Pgs) component have been validated as that reported (Ashiuchi et al., 1999, 2001, 2003). A commonly structural feature of amide ligases was found in PgsB, and the residues 37–42 (GIRGKS) of the protein displayed the sequence of the ATP-binding motif. PgsC, the most hydrophobic component, has yet to be found from organisms other than γ-PGA producers. The active site of Pgs, which was proposed to constitute from PgsB and PgsC, was capable of assimilating both isomers of glutamic acid as the substrate. Furthermore, as the structure of the complex merged into cell membranes and the transporter of γ-PGA, PgsA showed great diversities in various strains to achieve the elongation and excretion of γ-PGA.

The pTrcLpgs vector harboring *pgsBCA* genes from LL3 was successfully constructed and transformed into *E. coli* JM109 (Fig. 2b). The flask culture of LB plus 2% carbon source and Mg^{2+} showed that the recombinant *E. coli* strain had the capacity of synthesizing γ-PGA by IPTG induction. However, it was surprising that the recombinant strain in glucose medium (0.70 g/L) could provide nearly the same yield of γ-PGA as that of L-glutamic acid (0.75 g/L). So far as concerned, this is the first report of successful biosynthesis of γ-PGA in *E. coli* by the expression of *pgsBCA* genes in the absence of glutamate, and the γ-PGA productivity in *E. coli* was similar to that achieved with the recombinant *Corynebacterium glutamicum* strain harboring a shuttle vector pXMJ19–PGS (Cao et al., 2010). Whether it was a virtue of the *pgsBCA* genes from a glutamate independent strain or an effect of the pTrc99A vector will require more in-depth work. Groups of tests about the production and characteristics of γ-PGA in the two recombinant strains by different additions of L-glutamate would also provide us more reliable gist.

4. Conclusion

Statistical nutrient optimization was implemented to enhance γ-PGA productivity by a newly isolated *B. amyloliquefaciens* LL3. The orthogonal design showed sucrose was the most significantly variable affecting γ-PGA productivity. A 200 L fermentation provided well-balanced changes in processing parameters and a relative high γ-PGA yield of 4.36 g/L. This study described for the first time the cloning and expression of *pgsBCA* genes from glutamic acid independent strain LL3, which produce 0.70 g/L γ-PGA without glutamate. At present, much more statistical approaches and genetically regulatory elements are being examined to maximize the yields and to elucidate the mechanisms of γ-PGA synthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.12.065.

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