**Regulation of poly- γ -glutamic acid production in *Bacillus subtilis* GXA-28 by
potassium**

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ABSTRACT

It is a simple and effective method that controlling the production of fermented products by metal ions. In this study, effect of KCl on γ -PGA production by *B. subtilis* GXA-28 was investigated. K^+ was proved to be the true effector, and concentration of 201.2 mM was optimized for γ -PGA production. Addition of KCl to medium effectively reduced broth viscosity from 4289.5 mPa·s to 748.0 mPa·s by 82.6% and increased γ -PGA yield from 18.36 g/L to 25.62 g/L by 39.5%. Although high concentration KCl (>67.1 mM) suppressed cell growth, the maximum biomass with 201.2 mM had been brought forward to 16 h and had a marked effect in prolonging cell viability. Metabolic flux and key enzyme activities analysis indicated that enhanced GDH, GOGAT and GLR are important for γ -PGA production. Specifically, the expression of *pgsB* was improved 9.6-fold by KCl added, which might be another reason for enhanced γ -PGA production. In addition, that modulation of the D/L-glutamate ratio in γ -PGA and molecular weight could also be accomplished by variation of KCl concentrations. This work provided a simple control strategy for γ -PGA production, and it will be applied to γ -PGA production on a large scale in the future.

Keywords: Poly- γ -glutamic acid; Potassium; *Bacillus subtilis*; Yield; Viscosity

1. Introduction

Poly- γ -glutamic acid, abbreviated as γ -PGA, is a promising biopolymer that polymerized via the α -amino and γ -carboxylic groups of D- and L-glutamic acid residues. It has great value for development based on its excellent water-solubility and plasticity [1]. However, the diversity of yield, stereochemical composition, molecular weight and broth viscosity, which affected by variety of *Bacillus* species, environmental and nutritional conditions, have constrained its production and industrial application.

Currently, the γ -PGA yield is between 0 and 50 g/L by batch fermentation in different strains, which is the most important factor to affect its industrial application [2]. Microbial produced γ -PGA can have molecular weights ranging from 100 kDa to 10,000 kDa and the ratio of L-isomer in stereochemical composition ranging from 0 to 100% [1, 3], depending on strains and culture conditions. However, γ -PGA with different molecular weight and stereochemical composition has different applications. Besides, the viscosity of culture media increased with γ -PGA production, which may be the major factor limiting the increase of γ -PGA yield even using a high-producing strain. Therefore, how to control γ -PGA yield, stereochemical composition, molecular weight and broth viscosity is very important for the γ -PGA production. With the exception of screening specific strains, regulating medium component is an effective method.

Metallic ions as a component required of culture media, have been proved to play an important role in the cell growth, γ -PGA yield, stereochemical composition

and molecular weight of γ -PGA producing strain. For example, γ -PGA yield was increased in the presence of Ca^{2+} in the *B. subtilis* CGMCC 2108 [4]. Mn^{2+} has an effect on prolonging the viability of cells which lead to the γ -PGA yield increased, and increasing the proportions of D-isomers of γ -PGA in *B. licheniformis* 9945A [5]. It was found that high concentration Na^+ could reduce the γ -PGA molecular weight in *B. licheniformis* WX-02 [6] and *B. subtilis* (*chungkookjang*) [7]. Besides, no γ -PGA was produced in the absence of K^+ or Mg^{2+} in the *Bacillus* sp. RKY3 [8]. The results of our previous studies showed that the K^+ phenomenon was also present in *B. subtilis* GXA-28. However, the relationships between K^+ and cell growth, γ -PGA yield, stereochemical composition, molecular weight and broth viscosity have not been investigated in γ -PGA producing strain.

In this study, we examined the effect of K^+ on γ -PGA production in detail, and evaluated this in scale-up fermentation by batch fermentation. Furthermore, the possible mechanisms of the K^+ regulation at the metabolic and transcriptional level were explored by metabolic flux analysis and RT-PCR method, respectively.

2. Methods

2.1. Microorganism and medium

B. subtilis GXA-28 (CCTCC M 2012347) was used as γ -PGA producer [9]. The seed medium contained glucose $10.0 \text{ g}\cdot\text{L}^{-1}$, yeast extract $5.0 \text{ g}\cdot\text{L}^{-1}$, L-glutamate $5.0 \text{ g}\cdot\text{L}^{-1}$, KH_2PO_4 $0.5 \text{ g}\cdot\text{L}^{-1}$, and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ $0.1 \text{ g}\cdot\text{L}^{-1}$. In fermentation medium, glucose, yeast extract and L-glutamate were adjusted to 20.0, 2.5 and $20.0 \text{ g}\cdot\text{L}^{-1}$. Besides, KCl with concentration from 0 to 335 mM (0 to $25.0 \text{ g}\cdot\text{L}^{-1}$) was added to the

1 fermentation medium. Specially, adding 3.67 mM ($0.5 \text{ g} \cdot \text{L}^{-1}$) KH_2PO_4 to fermentation
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3 culture to ensure that γ -PGA was produced normally, and using it as the control group
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5 of KCl adding experiment. Initial pH was adjusted to 7.2 ± 0.1 .
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8 9 2.2. Fermentation in shake flask and fermentor

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11 Fermentation in 250 mL flask and 3.6 L fermentor (INFORS HT, Switzerland)
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13 were performed as our previous study [10]. In brief, the GXA-28 cells were
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15 inoculated into 30 mL of seed medium in 250 mL flask and aerobically cultured at 45
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17 °C for 12 h with shaking at 200 rpm. A 2% (v/v) inoculums of seed culture were
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19 transferred to a 250 mL flask containing 50 mL fermentation medium, and cultured at
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21 45 °C with 200 rpm. The batch fermentation was performed in a 3.6 L fermentor
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23 containing 2 L fermentation medium and 50 μL antifoam (Sigma, USA) was added.
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25 The seed culture (100 mL) was inoculated to the medium to start the cultivation at 45
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27 °C. The pH was automatically controlled at 7.2 ± 0.1 by adding 2N NaOH and/or 2N
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29 HCl. The aeration rate was maintained at 1.2 vvm and the agitation speed was
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31 gradually increased from 200 rpm to 400 rpm to increase dissolved oxygen.
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34 35 2.3. Metabolic flux and enzyme activity analysis

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37 The metabolic model for γ -PGA biosynthesis and stoichiometric representations
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39 in *B. subtilis* GXA-28 were established in our previous study [10]. The extracellular
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41 metabolites of glucose, glutamate, biomass, γ -PGA, O_2 , and CO_2 were measured to
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43 calculate metabolic flux using Matlab 7.1. All the flux distributions were normalized
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45 by the glucose uptake rate on a basis of $100 \text{ mmol} \cdot \text{g DCW}^{-1} \cdot \text{h}^{-1}$.
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49 Cells grown in batch fermentation for 20 h were harvested by centrifugation, and
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prepared the cell extracts for isocitrate dehydrogenase (ICDH), glutamate dehydrogenase (GDH), glutamate α -oxoglutarate aminotransferase (GOGAT), α -oxoglutarate dehydrogenase complex (ODHC), and glutamate racemase (GLR) assays [10-12]. Of which, enzyme activities of ICDH, GDH, GOGAT and ODHC were determined by appearance or disappearance of NADH or NADPH ($\epsilon=6.22/(\text{mmol/L})/\text{cm}$) at 340 nm. One unit of activity was defined as the amount of enzyme catalyzing 1 μmol of NADH or NADPH per min. GLR was analyzed by HPLC for D-glutamate, and one unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-glutamate per min. Protein concentration was determined by Bradford method with bovine serum albumin as the standard [13].

2.4. Real-time PCR

RNA extraction and reverse transcriptase reaction were performed using the EZ-10 DNAaway RNA Mini Preps Kit (BBI, BS88133) and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1621), respectively. The relative quantitative real-time polymerase chain reaction (RT-PCR) was performed using 2xSG Fast qPCR Master Mix (SYBR Green) kit (BBI, B639271) on Roche Light Cycle 480. The *pgsB* (GeneBank: KP178960), one of the key gene of the γ -PGA synthase, is the target of analysis. 16s rDNA (GeneBank: JN815234) was selected as the endogenous reference gene. Primer pairs of these two genes were designed by the Primer Premier 5.0 software: F_{pgsB} (5'-GAGCGAGCCTGGGCACT T-3') and R_{pgsB} (5'-CGGTCATCGGTCGG GTAAC-3'), $F_{16s\text{ rDNA}}$ (5'-TGCCGCAATGGACGAAA G-3') and $R_{16s\text{ rDNA}}$ (5'-TGTAAGGT GCCGCCCTATTC-3'). Comparative analysis

of $2^{-\Delta\Delta CT}$ was used to calculate the fold change in gene expression relative to the initial control, which based on the amplification efficiency of the reference gene was consistent with the target gene[14].

2.5. Analytical methods

Dry cell weight (DCW) was determined by the method of Zeng et al. [10]. The viable cell was determined by the number of cfu per milliliter fermentation liquor (1 cfu = 1 colony forming unit) on the broth agar plate according to ten-fold dilution method. The γ -PGA yield was measured by UV or HPLC method [15]. The weight-average molecular weight (Mw) and number-average molecular weight (Mn) were determined by gel permeation chromatography (GPC) [9]. Viscosity of broth was measured by a viscometer (NDJ-8S, Jingtian, Shanghai, China). The ratio of D/L-glutamate in γ -PGA was determined by OPTA-derivatization method [11]. The concentration of glucose and L-glutamate were measured by a biosensor equipped with glucose and L-glutamic acid oxidase electrode (SBA-40D, Shandong Academy of Sciences, China).

2.6. Statistical analyses

Each experiment was carried out in triplicates, and three parallel experiments were performed for each experiment. One-way analysis of variance and the least significant difference (LSD) were used to interpret the difference in means at the 95% confidence level.

3. Results and discussion

3.1. Effects of KCl on cell viability and γ -PGA yield

Figure 1 showed that the time course of cell viability and γ -PGA yield in medium containing various KCl concentrations. Generally, the yield that improved by change of environmental conditions, to a large extent, had relation with higher cell viability. However, it is interesting that γ -PGA yield increased with KCl concentrations, but the cell viability decreased from 9.65×10^8 cfu·mL⁻¹ without KCl to 4.16×10^8 cfu·mL⁻¹ with 335.3 mM (25 g·L⁻¹) KCl. To the best of our knowledge, low concentration of K⁺ had a protective effect enabling cells to survive during osmotic stress [16], but K⁺ had an analogous osmotic stress toxic as Na⁺ when it present excessively [17]. In addition, the maximum yield of γ -PGA was obtained at 22 h regardless of KCl concentrations. The yield was 22.57 ± 0.86 g·L⁻¹ with productivity 1.03 g·L⁻¹·h⁻¹ and apparent conversion rate 113% as 201.2 mM KCl added, which was 1.36-fold higher than that without KCl added. Although high concentration KCl (>67.1 mM) suppressed cell growth, the maximum viable count with 201.2 mM had been brought forward to 16 h and had a marked effect in prolonging cell viability. This may be one of the reasons for improving γ -PGA yield. A similar phenomenon also appeared in *B. licheniformis* ATCC 9945A, Mn²⁺ has effective in prolonging the viability of cells which lead to the γ -PGA yield increased [5].

K⁺ and Cl⁻ coexist in culture medium when KCl was added, and which of these two ions is truly responsible for γ -PGA production. To investigate this, KH₂PO₄ and NaCl were selected to replace KCl at 201.2 mM. There are similar effects on γ -PGA production by KH₂PO₄, but no obvious regulating effects on γ -PGA production by

NaCl (data not shown). Based on above results, K^+ was proved to be the true effector, and has a certain regulation effect on γ -PGA yield.

3.2. Glucose and glutamate utilization

B. subtilis GXA-28 can be produced γ -PGA under relatively simple nutritional components, of which glucose was chosen as a preferred carbon source and L-glutamate was required as substrate. Then, the residual glucose and glutamate as a function of both KCl concentration and culture time were measured (Fig. 2). The glucose utilization increased as the culture medium containing low concentration K^+ (≤ 67.1 mM), which was consistent with the observation of high cell viability. It was interesting that the residual glutamate all remained about 7 g/L at the end of fermentation, which may be considered as “background level of substrate” (Table 1). However, the total amount of glutamate including glutamate unit in γ -PGA and residual glutamate in broth was much more than the added glutamate, and increased with the KCl concentration. It was deduced that the substrate of γ -PGA came from two parts: one is from extracellular glutamate which exists in the medium by artificial added, and others derived from intracellular glutamate which obtained via glycolysis and the tricarboxylic acid cycle by glucose. Based on above results, it was speculated that glucose was mainly used to cell grow and little was used to participate in γ -PGA synthesis when the culture containing low concentration KCl, but more glucose was involved in γ -PGA synthesis when the concentration of KCl increased. Therefore, addition of KCl can be used as a control strategy to improve γ -PGA yield by enhancing the conversion rate of carbon source.

As shown in Table 1, one-way analysis of variance by F-test was used to interpret the difference in results of five group for γ -PGA production. Results showed that all parameter obtained from γ -PGA production were significant, including cell viability, γ -PGA yield, residual glucose, residual L-glutamate, total L-glutamate, productivity, and apparent conversion rate. The maximum yield and apparent conversion rate were obtained at 201.2 mM KCl, whether or not it was significant compared with other experimental group. Thus, comparative analysis of the difference between the 201.2 mM KCl group and other concentrations KCl group by the least significant difference method (LSD) was carried out (Table 2). Compared with 0, 13.4 and 67.1 mM KCl group, all parameter obtained from 201.2 mM KCl group were significant, except total L-glutamate with 67.1 mM KCl group. However, compared with 335.3 mM KCl group, many parameter were not significant, including γ -PGA yield, residual L-glutamate, total L-glutamate, productivity, and apparent conversion rate. These results indicated that it was not obvious promoting effect on γ -PGA production by addition of KCl unceasingly as more than 201.2 mM.

3.3. Molecular weight and stereochemical composition of γ -PGA regulated by KCl

It was known that γ -PGA with different molecular weight and stereochemical composition have different applications. For example, γ -PGA with low enough molecular weight and high content of D-glutamate was preferred used for medical applications because it provide the proper sustained release profile during biodegradation [18]. But, γ -PGA with high molecular weight γ -PGA (over 2.0×10^6 g $\cdot$ mol $^{-1}$) and high content of L-glutamate was generally used as a viscosity-adding

agent or an immunological enhancement effect on mammals due to its excellent bio-compatibility [19]. Therefore, looking for some simple strategies for controlling the γ -PGA molecular weight and stereochemical composition is valuable to meet the needs of different applications.

As shown in Table 3, the weight-average molecular weight (M_w) and number-average molecular weight (M_n) increased respectively from 3.05×10^6 g·mol⁻¹ to 4.95×10^6 g·mol⁻¹ by 62.3% and 1.52×10^6 g·mol⁻¹ to 2.95×10^6 g·mol⁻¹ by 94.1%, and the polydispersity still remained relatively stable, when KCl added from 0 to 335.3 mM. The result differs from other metal ions. For example, there were no readily interpretable correlations between Mn^{2+} and γ -PGA molecular weight in *B. licheniformis* ATCC9945A [5], while high concentration of Na^+ could reduce γ -PGA molecular weight in *B. licheniformis* WX-02 [6] and Ca^{2+} have no effect on γ -PGA molecular weight in *B. subtilis* CGMCC2108 [4]. These diversities may be due to the differences of strain properties and γ -PGA synthetase system, which need to be further investigated.

That modulation of the D/L-glutamate ratio in γ -PGA can also be accomplished by variation of KCl concentration (Table 2). The percentage L-glutamate content decreased by approximately 12% with KCl added to 335.3 mM, which showed a similar effect with Mn^{2+} in *B. licheniformis* ATCC9945A [5]. Above results showed that KCl has a significant regulating effect on the molecular weight and stereochemical composition of γ -PGA, so it is very likely to develop into a simple control strategy for industrial production.

3.4. Control of fermentation broth viscosity

The culture medium for γ -PGA production becomes highly viscous as the γ -PGA concentration increased and exhibits non-Newtonian rheology, which limits dissolved oxygen and mass transfer, then inhibits cell growth and γ -PGA production. Thus, the viscosity of the fermentation broth is one of the most important influencing factors in γ -PGA production. As shown in Fig. 3, it's significantly that the viscosity of broth decreased sharply with the increase of KCl concentrations. The maximum viscosity in 335.3 mM KCl was 521.1 ± 28.5 mPa·s, which reduced by 85.4% than that of without KCl added. Moreover, the viscosity of culture medium in 201.2 and 335.3 mM KCl were at a relatively low level during the whole fermentation process. To the best of our knowledge, there was no report about that the viscosity of γ -PGA broth was significantly decreased by KCl. Why does KCl affect the viscosity of γ -PGA solution so much? It presumed that this phenomenon was due to the change of γ -PGA conformation caused by KCl. Previous studies from He et al. showed that the γ -PGA was in a helical conformation at low ionic strength, while changed to a mixture of helix and β -sheet at 0.1 M, and adopted predominantly β -sheet at 0.5 M [20]. These results indicated that KCl can be used to control the viscosity of γ -PGA fermentation broth, thereby improving γ -PGA production.

3.5. Characterization of γ -PGA production with KCl addition in fermentor by batch fermentation

Shake flask experiment results showed that high concentration KCl had obvious effects on γ -PGA production. To evaluate this in scale-up fermentation, the time

courses of γ -PGA yield, cell growth, broth viscosity, glucose and glutamate consumption in 3.6-L fermentor without (Fig. 4A) or with (Fig. 4B) KCl were investigated. Results showed that γ -PGA production in fermentor was consistent with in shake flask. The maximum yield of γ -PGA was obtained at 22 h regardless of A or B, but the yield in B was $25.62 \pm 0.95 \text{ g}\cdot\text{L}^{-1}$ with productivity $1.16 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, which was 1.4-fold higher than that in A. Although the viable count decreased after adding KCl, the maximum growth in B had been brought forward to 16 h and had a marked effect in prolonging cell viability. However, it's interesting that the glucose in A was consuming much faster than in B, but the residual glutamate all remained about $6 \text{ g}\cdot\text{L}^{-1}$ at the end of fermentation. The viscosity of broth decreased sharply from $4289.5 \pm 198.0 \text{ mPa}\cdot\text{s}$ in A to $748.0 \pm 32.0 \text{ mPa}\cdot\text{s}$ in B by 82.6%. These results indicated that KCl concentration was one key influencing factor for γ -PGA production in *B. subtilis*. Thus, adding KCl can be used as an effective strategy to control γ -PGA fermentation.

3.6. Analysis of KCl regulation by metabolic analysis and RT-PCR

In order to investigate the possible mechanisms of KCl regulation at the metabolic and transcriptional level, the metabolic flux and enzyme activities of key metabolic branch without (Fig. 5A) or with (Fig. 5B) KCl were measured. Furthermore, the gene of *pgsB* that responsible for catalyzing the γ -PGA synthesis in γ -PGA synthetase complex was also assayed.

As shown in Fig. 5, the carbon flux from iso-citrate to α -oxoglutarate increased and from α -oxoglutarate to succinyl-CoA decreased with KCl added. Thus, the carbon flux from α -oxoglutarate to glutamate reached $19.2 \text{ mmol}\cdot\text{g DCW}^{-1}\cdot\text{h}^{-1}$ when KCl

1 added, which was 3.84-fold higher than that of without KCl. The changes of enzyme
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3 activity of ICDH, ODHC, GDH and GOGAT were consistent with the flux
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5 distribution. Generally, GDH and GOGAT are responsible for the shunt from
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7 α -oxoglutarate to glutamate in many bacteria [12]. In this case, the activity of
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9 GOGAT was $4.9 \times 10^{-2} \text{ U} \cdot \text{mg}^{-1}$ when KCl added, which was 2.88-fold higher than
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11 that of without KCl. However, the activity of GDH was improved 2.13-fold by KCl
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13 added. The result showed that the contribution of GOGAT to the conversion of
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15 α -oxoglutarate to glutamate was slightly more than that of the GDH. Furthermore, the
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17 extracellular glutamate uptake rate between A and B are not significantly different.
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19 Therefore, the higher yield of γ -PGA reached $25.62 \text{ g} \cdot \text{L}^{-1}$ with KCl added, which
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21 mainly resulted from the increase of intracellular glutamate. In addition,
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23 transcriptional analysis showed that the expression of *pgsB* was improved 9.6-fold by
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25 KCl added, which might be another reason for the improved γ -PGA production.
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29 Previous reported that the conversion of L-glutamate to D-isomer in *B. subtilis*
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31 was mainly mediated by glutamate racemase [21]. Thus, in order to investigate the
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33 possible explanation for the observed stereochemical modulation by KCl in *B. subtilis*
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35 GXA-28, the metabolic flux and enzyme activities of glutamate node were analyzed.
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37 Results showed that the flux from L-glutamate to D-isomer increased from 22.9 to
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39 $37.6 \text{ mmol} \cdot \text{g DCW}^{-1} \cdot \text{h}^{-1}$ and the activities of GLR increased from 0.42 to $0.53 \text{ U} \cdot \text{mg}^{-1}$
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41 when KCl added. Thereby, the carbon flux from D-glutamate to γ -PGA reached 35.2
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43 $\text{mmol} \cdot \text{g DCW}^{-1} \cdot \text{h}^{-1}$, which was increased 78.7% that that of without KCl. These
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45 results indicated that GLR plays an important role during the process of γ -PGA
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1 stereochemical regulation by KCl.

2 3 4 **4. Conclusions**

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6 The present study investigated the effect of KCl on γ -PGA production in detail
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8 by *B. subtilis* GXA-28. Results showed that addition of KCl could significantly
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10 increase the yield and molecular weight and reduce the broth viscosity. The γ -PGA
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12 stereochemical composition also can be regulated by KCl. Furthermore, the effect of
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14 KCl regulation was verified in the fermentor. The yield improvement and
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16 stereochemical modulation might be due to the changes of metabolic flux and enzyme
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18 activities around key metabolic branch. This work provided a simple control strategy
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20 for γ -PGA production, and it will be applied to γ -PGA production on a large scale in
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22 the future.
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45 Engineering.
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Figure legends

Figure 1 Time course of cell viability (A) and γ -PGA yield (B) in medium containing various KCl concentrations by *B. subtilis* GXA-28. KCl: 0 mM ($\square$); 13.4 mM ($\blacktriangle$); 67.1 mM ($\diamond$); 201.2 mM ($\bullet$); 335.3 mM ($\star$).

Figure 2 Time course of the utilization of glucose (A) and L-glutamate (B) in medium containing various KCl concentrations by *B. subtilis* GXA-28. KCl: 0 mM ($\square$); 13.4 mM ($\blacktriangle$); 67.1 mM ($\diamond$); 201.2 mM ($\bullet$); 335.3 mM ($\star$).

Figure 3 Time course of fermentation broth viscosity with various KCl concentrations by *B. subtilis* GXA-28. KCl: 0 mM ($\square$); 13.4 mM ($\blacktriangle$); 67.1 mM ($\diamond$); 201.2 mM ($\bullet$); 335.3 mM ($\star$).

Figure 4 Time courses of γ -PGA production in 3.6-L fermentor by batch fermentation. A: control; B: addition of 201.2 mM KCl. Error bars are given showing standard deviations for n=3.

Figure 5 Carbon flux distribution and enzymes activities at α -oxoglutarate, glutamate racemization and γ -PGA synthesis branch point in *B. subtilis* GXA-28 without (A) or with (B) KCl. The metabolic fluxes were given in units of $\text{mmol} \cdot \text{g DCW}^{-1} \cdot \text{h}^{-1}$. The unit of enzyme activity was $\text{U} \cdot \text{mg}^{-1}$ protein. Abbreviations: ICDH, isocitrate dehydrogenase; ODHC, α -ketoglutarate dehydrogenase; GDH, glutamate dehydrogenase; GOGAT, glutamate α -oxoglutarate aminotransferase; GLR, glutamate racemase; iso-CIT, isocitric acid; α KG, α -ketoglutarate; SUC-CoA, succinyl coenzyme A; in-[L]-GLU, intracellular L-glutamic acid; ex-[L]-GLU, extracellular L-glutamic acid; in-[D]-GLU, intracellular D-glutamic acid.

Table 1 Effect of KCl on the γ -PGA production and one-way analysis of variance.

KCl ^a	Cell viability	γ -PGA	Residual glucose	Residual L-glutamate	Total L-glutamate	Productivity	App. conversion
(mM)	(cfu·mL ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹)	(g·L ⁻¹) ^b	(g·L ⁻¹ ·h ⁻¹)	rate (%) ^c
0	9.65×10^8	16.54 ± 0.55	0 ^d	7.87 ± 0.25	26.72 ± 0.87	0.75 ± 0.03	82.7 ± 2.77
13.4	9.04×10^8	17.55 ± 0.65	0	7.55 ± 0.36	27.55 ± 0.99	0.80 ± 0.03	87.7 ± 3.27
67.1	8.91×10^8	19.87 ± 0.77	0	7.75 ± 0.28	30.39 ± 1.15	0.90 ± 0.04	99.3 ± 3.80
201.2	6.43×10^8	22.57 ± 0.86	2.45 ± 0.08	6.67 ± 0.27	32.38 ± 1.13	1.03 ± 0.04	113 ± 4.33
335.3	4.16×10^8	22.23 ± 0.88	4.22 ± 0.23	6.76 ± 0.30	32.09 ± 1.30	1.01 ± 0.04	111 ± 4.40
F ^e	73.69 (*) ^f	38.84 (*)	939.59 (*)	11.12 (*)	16.59 (*)	38.23 (*)	38.67 (*)
F _{0.01(4, 10)} ^g	5.99						

^a Fermentation was carried out in 250 mL flask at 45 °C and 200 rpm with various KCl concentrations. The other components of the culture were the same, including glucose 20.0 g/L, yeast extract 2.5 g/L, L-glutamate 20.0 g/L, KH₂PO₄ 0.5 g/L, and MgSO₄·7H₂O 0.1 g/L.

^b Total amount of L-glutamate was amount of γ -PGA $\times$ 147/129 plus residual L-glutamate in the culture broth, 147/129 was coefficient [22].

^c Apparent conversion rate was defined as the ratio of γ -PGA to the amount of L-glutamate added to the medium.

^d Can not be detected by SBA-40D biosensor.

^e One-way analysis of variance by F-test.

^f The difference in results of five group was significant if the numerical value was greater than $F_{0.01(4, 10)}$, and marked with an asterisk.

^g Obtained by F-value table.

Table 2 Comparative analysis of the difference between the 201.2 mM KCl group and other concentrations KCl group by the least significant difference method (LSD).

KCl (mM)	Cell viability (cfu·mL ⁻¹)	γ-PGA (g·L ⁻¹)	Residual glucose (g·L ⁻¹)	Residual L-glutamate (g·L ⁻¹)	Total L-glutamate (g·L ⁻¹)	Productivity (g·L ⁻¹ ·h ⁻¹)	App. conversion rate (%)
0	0.76 (*) ^a	6.03 (*)	2.45 (*)	1.2 (*)	5.66 (*)	0.28 (*)	30.17 (*)
13.4	0.62 (*)	5.02 (*)	2.45 (*)	0.89 (*)	4.83 (*)	0.23 (*)	25.13 (*)
67.1	0.56 (*)	2.7 (*)	2.45 (*)	1.08 (*)	1.99	0.13 (*)	13.53 (*)
335.3	0.53 (*)	0.34 (NS) ^b	1.78 (*)	0.09 (NS)	0.29 (NS)	0.02 (NS)	1.73 (NS)
LSD _{0.01} ^b	0.28	1.95	0.28	0.76	2.85	0.09	9.75

^a The numerical value represents that the value difference between the 201.2 mM KCl group and other concentrations KCl group. The difference was significant if the numerical value was greater than LSD_{0.01}, and marked with an asterisk.

^b NS represent not significant.

Table 3 Effect of KCl on the molecular weight and stereochemical composition of γ -PGA.

KCl (mM)	Mw ($\times 10^6$ g $\cdot$ mol $^{-1}$)	Mn ($\times 10^6$ g $\cdot$ mol $^{-1}$)	Polydispersity	L-Isomer (%)
0	3.05 $\pm$ 0.29	1.52 $\pm$ 0.15	2.01	30.52 $\pm$ 1.18
13.4	3.72 $\pm$ 0.35	1.78 $\pm$ 0.19	2.09	27 $\pm$ 1.12
67.1	3.95 $\pm$ 0.38	1.96 $\pm$ 0.22	2.02	24.31 $\pm$ 0.95
201.2	4.92 $\pm$ 0.37	2.57 $\pm$ 0.23	1.91	19.63 $\pm$ 0.89
335.3	4.95 $\pm$ 0.43	2.95 $\pm$ 0.26	1.68	18.79 $\pm$ 0.81
F ^a	14.91 (*) ^b	22.87 (*)	1.10 (NS) ^c	73.78 (*)
F _{0.01(4, 10)} ^d	5.99			

^a One-way analysis of variance by F-test.

^b The difference in results of five group was significant if the numerical value was greater than F_{0.01(4, 10)}, and marked with an asterisk.

^c NS represent not significant.

^d Obtained by F-value table.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5