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Analysis of Carbon Metabolism and Improvement of γ-Polyglutamic Acid Production from *Bacillus subtilis* NX-2

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Abstract *Bacillus subtilis* NX-2 produces γ-polyglutamic acid (γ-PGA) when using glucose and L-glutamate as carbon sources. The conversion of carbon sources into γ-PGA was analyzed with the ^{13}C-NMR method after enriching the media with ^{13}C-labeled glucose. The results showed that the percentage of γ-PGA monomers derived from glucose was relatively low, approximately 6% and 9%, respectively, with an initial glucose concentration of 30 and 40 gL^{-1}. It was concluded that glucose was utilized mainly as the growth-limiting substrate for cell growth and supplied the required energy during γ-PGA biosynthesis, while L-glutamate was preferred as the main substrate for γ-PGA formation. To achieve an efficient conversion of L-glutamate and enhance the γ-PGA production, a fed-batch culture was proposed by feeding of glucose. By this method, supplied L-glutamate (40 gL^{-1}) was completely depleted, and γ-PGA yield was attained 42 gL^{-1}.

Keywords *Bacillus subtilis* · Batch culture · γ-Polyglutamic acid · Metabolic pathway · Isotope · NMR

Introduction

γ-Polyglutamic acid (γ-PGA) is a homo-polyamide made of D- and L-glutamic acid monomers via γ-amide linkages. It is produced by several *Bacillus* species as an extracellular polymer [1, 2]. γ-PGA is water-soluble, biodegradable, edible, and nontoxic toward human and environment. Its potential applications have earned γ-PGA a growing

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interest since the early 1990s. A substantial amount of research has been devoted to optimizing γ-PGA biosynthesis from either *Bacillus licheniformis* or *Bacillus subtilis*. Medium E was commonly used for cell growth and γ-PGA formation [3–8]. Medium E had a relatively high concentration of carbon sources including glycerol, citrate acid, and L-glutamate. The metabolism of carbon sources occurring during γ-PGA synthesis was varied according to the strain. In the case of *B. subtilis* IFO 3335, exogenous L-glutamate was almost not assimilated in spite of its indispensability in the culture, and citrate acid was considered to be the main substrate responsible for both γ-PGA synthesis and cell growth [3, 5]. In the case of *B. licheniformis* 9945a, citrate acid and L-glutamate were both involved in γ-PGA synthesis [4, 6].

The production of γ-PGA by *B. subtilis* NX-2 has been previously investigated [9, 10]. Compared with medium E, relatively low levels of carbon sources and simple nitrogen source (inorganic ammonium) were used to achieve the efficient production of γ-PGA. Therefore, *B. subtilis* NX-2 was promising to be commercially used in the industrial production of γ-PGA. Glucose and L-glutamate were demonstrated to be the optimum carbon sources with high yield of γ-PGA and no formation of polysaccharide, a side-product reported by Goto and Kunioka [3, 9]. However, the mechanism by which glucose was preferred as the growth-limiting substrate or functioned as the substrate converted into γ-PGA remained unclear. Exogenous L-glutamate was essential for the production of γ-PGA from *B. subtilis* NX-2. The roles of the two carbon sources operative in the γ-PGA synthesis were needed to be further elucidated.

In this paper, the isotope labeling method and NMR analysis were used to analyze and evaluate the metabolism of carbon sources to γ-PGA formation. Based on the knowledge of carbon metabolism, a fed-batch strategy was proposed to achieve an efficient production of γ-PGA from these two carbon sources.

Materials and Methods

Microorganism

B. subtilis NX-2 was isolated from a soil sample [9].

Flask Culture Conditions

B. subtilis NX-2 was first inoculated into 50 ml of seed medium containing glucose 20 gL^{-1}, L-glutamate 10 gL^{-1}, yeast extract 5 gL^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 gL^{-1}, $MgSO_4$ 0.2 g L^{-1} in 500-ml flask and aerobically incubated at 32.5 °C for 16 h with shaking at 220 rpm. Seed culture (0.8 ml) was then transferred to 500-ml flasks containing 80 ml of basal medium comprising NH_4Cl 8 gL^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 gL^{-1}, $MgSO_4$ 0.2 gL^{-1}. Different concentrations of glucose (0–60 gL^{-1}) and L-glutamate (0–60 gL^{-1}) were contained respectively in the cultures. pH was adjusted to 7.5. The flask culture was incubated at 32.5 °C in a rotary shaker at 220 rpm for 56 h.

The above medium formulations were also prepared with ^{13}C-labeled glucose enriched. The uniformly labeled ^{13}C glucose ([U-^{13}C]-D-glucose, with an isotopic purity of 99.0%, Cambridge Isotope Laboratories Inc.) was adopted. [U-^{13}C] glucose was mixed with non-enriched glucose to carry out a series of experiments. The percentages of [U-^{13}C] glucose enriched for cultures forming polymers A, B, C, and D are shown in Table 1. Forty grams per liter of L-glutamate was also used in the experiments.

Table 1 Glucose concentration and percentages of [U-^{13}C] glucose by weight of provided glucose.

Product	Glucose concentration (g L^{-1})	[U-^{13}C] glucose percentage (%)
A	40	0
B	40	5
C	40	10
D	30	10

Batch Culture Conditions in Bioreactor

The reactor runs were performed in a Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA). Initial glucose concentrations of 60 and 30 g L^{-1} were adopted in batch and fed-batch culture, respectively. The medium was autoclaved in the reactor (121 °C for 15 min). Seed culture (80 ml) was then used to inoculate 4 L of the bioreactor culture medium. The aeration rate into the bioreactor was kept at 1.2 vvm, and agitation was kept at 400 rpm. Temperature was controlled at 32.5 °C and the initial medium pH adjusted at 7.5. At predetermined intervals, aliquots of the culture were withdrawn for the determination of biomass, residual substrate, and γ-PGA formation.

The feeding solution for fed-batch culture contained 200 gL^{-1} of glucose. The time point of feeding could be determined by monitoring the glucose concentration in culture broth, and a predetermined amount of feeding solution was added at a constant rate (0.28 ml min^{-1}) before the depletion of glucose in the culture broth.

Preparation of γ-PGA

γ-PGA was isolated and purified by a previously reported method [5].

Analysis

Biomass Determination

Aliquots of samples were withdrawn at predetermined time intervals from the shake flask or fermentor cultures, and cell dry weight (CDW) was determined by centrifuging 1.5 ml aliquots in microfuge tubes, washing the cell with diluted water, and drying under vacuum at 80 °C.

Quantification of Carbon Source in Culture

The concentrations of glucose and L-glutamate remaining in the broth were analyzed by a biosensor equipped with both glucose and L-glutamic acid oxidase electrodes (Institute of Biology, Shandong Academy of Sciences SBA-40C) after separation of the cells by centrifugation [9].

Quantification of γ-PGA

The volumetric yield of γ-PGA in the culture was measured by gel permeation chromatograph (GPC) using an Alltech system controller equipped with Shodex OH pak SB800 Series columns (SB 806M HQ) and a UV detector (Thermo LINEAR UV201) [11].

^{13}C-NMR Measurement

Purified γ-PGA samples were prepared as 5% (*w*/*v*) solutions in d_6-dimethyl sulfoxide (d_6-DMSO). The spectra were recorded at 400.13 Hz on a Bruker Avance 400 SB spectrometer equipped with a HR/MAS probe head (4 mm, 15 kHz). d_6-DMSO was used as a chemical shift and concentration standard. Polymer spectral acquisitions were conducted using the following parameters: 300 K, 10 μs pulse width, 16 K data points, 1.3 s acquisition time, and 2 s delay time. Peak intensities were determined by spectral integration. All NMR data processing was performed using the Bruker XWINNMR.

Results and Discussion

Effects of Glucose and L-glutamate on γ-PGA Production

Shaken flask cultures containing different concentrations of glucose were incubated for 48 h. Forty grams per liter of L-glutamate was used in the cultures. Cell growth and γ-PGA formation were monitored. As shown in Table 2, in the absence of glucose, poor cell growth was observed, and no γ-PGA was produced. The cell growth was enhanced with increased glucose. High level of glucose (above 40 gL^{-1}) resulted in excessive biomass formation but little enhanced γ-PGA production. Glucose was deduced to be a growth-limiting substrate for cell growth. It was noted that, when glucose was provided above 30 gL^{-1}, the total amount of glutamate including the glutamic acid unit of γ-PGA and residual glutamate in the broth was more than the supplied glutamate. As glucose and L-glutamate in the medium were the only possible sources of γ-PGA carbon skeleton, glucose could also serve as a substrate for γ-PGA synthesis.

The effect of L-glutamate on γ-PGA production was investigated with 40 gL^{-1} glucose as an additional carbon source, as shown in Table 3. When no L-glutamate was added to the medium, γ-PGA was scarcely produced. The increased glutamate resulted in higher γ-PGA production (0–40 gL^{-1}). Contrary to the research conducted on *B. subtilis* IFO 3335, which showed little assimilation of L-glutamate during γ-PGA formation, the exogenous L-glutamate was consumed with an increased amount of γ-PGA produced by *B. subtilis* NX-2. It was indicated that L-glutamate was essential for the γ-PGA production and presumed to be an important precursor of γ-PGA. An efficient conversion of L-glutamate

Table 2 Effect of glucose on γ-PGA production.

Glucose added (g L^{-1})	CDW (g L^{-1})	γ-PGA (g L^{-1})	Residual glucose (g L^{-1})	Residual glutamate (g L^{-1})	Total glutamate[a] (g L^{-1})
0	0.984	0.00	0.00	38.96	38.96
20	2.328	17.26	0.00	15.45	35.59
30	2.688	19.37	0.00	19.78	42.37
40	3.181	23.18	0.00	19.32	45.72
50	3.636	23.86	0.00	18.45	45.64
60	4.140	24.13	1.35	17.96	45.46

[a] Total amount of glutamate was calculated as followed: the amount of γ-PGA × 147/129 plus residual glutamate in the culture broth, 147/129 was the coefficient

Table 3 Effect of L-glutamate on γ-PGA production.

Glutamate added (g L^{-1})	CDW (g L^{-1})	γ-PGA (g L^{-1})	Residual glucose (g L^{-1})	Residual glutamate (g L^{-1})	Total glutamate (g L^{-1})
0	1.513	0.00	15.23	0.00	0.00
10	4.176	4.23	8.21	0.72	5.54
20	3.468	9.76	1.54	6.43	17.55
30	3.264	19.35	0.55	13.46	35.41
40	3.181	23.18	0.00	19.32	45.72
50	2.820	23.10	0.00	25.72	52.04
60	2.364	21.32	0.00	36.13	60.42

might contribute to γ-PGA production. It was also observed that despite diverse concentrations of glucose and L-glutamate supplied in the medium, there still remained a considerable amount of residual L-glutamate in the broth.

The above indicated that the repeat units of γ-PGA could come from two origins: One was from glucose through the de novo pathway of glutamic acid synthesis, and the other was derived from the provided exogenous L-glutamate. The metabolism of the two carbon sources operative in the γ-PGA biosynthesis would be further investigated through the ^{13}C labeling method and NMR analysis mentioned below.

Analysis of Carbon Metabolism by ^{13}C Labeling and NMR Spectroscope

Isotope labeling method was a powerful technique for elucidating the key pathways of central carbon metabolism. [U-^{13}C] glucose was used to gain information on the degree by which glucose provided in the medium was converted into polymer repeat units as well as operative metabolic pathway involved in the process. ^{13}C abundance of the provided glucose (P_i) from each culture (see Table 1) could be calculated as followed:

$$P_i = \mathrm{Pl}_i \times 99\% + (1 - \mathrm{Pl}_i)P_0 \quad (1)$$

where Pl_i was the percentage of [U-^{13}C] glucose by provided glucose (i representing B or C or D), P_0 is the ^{13}C natural abundance (1.1%), and 99% represents the isotopic purity. The values of P_B, P_C, and P_D attained by calculation were 6.0%, 10.9%, and 10.9%, respectively, while P_A was equal to P_0.

The purified γ-PGA samples obtained from each culture were analyzed by ^{13}C NMR. Figure 1 shows the representative ^{13}C NMR spectra of products (A, B, C, and D) and their signal intensities for α-C, β-C, and γ-C obtained by spectrometer integration. The conversion of glucose into polymer repeat units resulted in γ-PGA with ^{13}C enrichment at different positions of repeat unit. It is known that NMR measurements usually do not directly quantify isotopomer mole fractions, but they supply linear constraints on the isotopomer distribution. Fractional enrichment measurements could quantify the relative amount of isotopomers labeled at a certain position, relative to the fraction of isotopomers that are not labeled in that particular position [12, 13]. The calculated NMR signal intensity ratios were used to determine the percentage of repeat units formed from glucose (GP_i; Eq 2). Product *A* served as the non-enriched γ-PGA samples for comparison to B, C and D. As glucose and L-glutamate in the medium were the only possible sources of

γ-PGA carbon skeleton, the percentage of repeat units formed from L-glutamate could be expressed as $(1-\mathrm{GP}_i)$.

$$\frac{I_i(X_\mathrm{C})}{I_\mathrm{A}(X_\mathrm{C})} = \frac{\mathrm{GP}_i \times P_i + (1-\mathrm{GP}_i) \times P_0}{P_0} \tag{2}$$

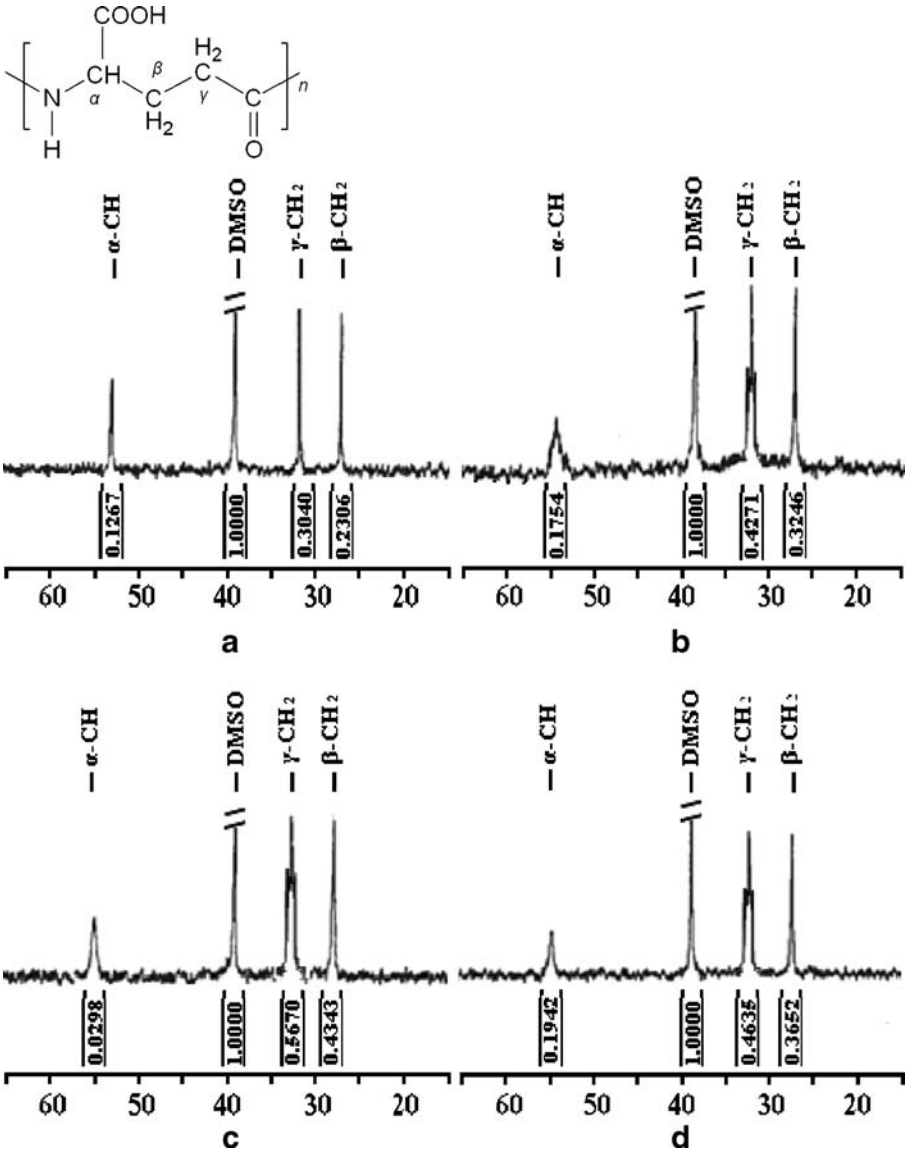

Fig. 1 ^{13}C-NMR spectra of γ-PGA produced with quantitative enriched [U-^{13}C] glucose. *A* Product A (from medium containing 40 gL^{-1} natural abundance [^{13}C] glucose). *B* Product B (from medium containing 40 gL^{-1} glucose enriched with 5% [U-^{13}C] glucose). *C* Product C (from medium containing 40 gL^{-1} glucose enriched with 10% [U-^{13}C] glucose). *D* Product D (from medium containing 30 gL^{-1} glucose enriched with 10% [U-^{13}C] glucose)

Table 4 Values of GP_i calculated from different positions of carbon atoms.

product	GP_i(%)			
	α-C	β-C	γ-C	$\overline{GP_i}$
B	8.63	9.15	9.09	8.96
C	9.14	9.92	9.72	9.59
D	5.99	6.56	5.90	6.15

i represents *B*, *C*, and *D*. $\overline{GP_i}$ represents the average values calculated form from α-C, β-C, and γ-C

where $I_i(X_C)$ was the NMR signal intensity of the products (*i* representing B or C or D, X_C representing α-C or β-C or γ-C), P_i was the ^{13}C abundance of the provided glucose (calculated by Eq 1), and P_0 was the ^{13}C natural abundance (1.1%).

Equation 2 was used to calculate the GP_i values (Table 4). GP_i attained from different positions of carbon atoms (α-C or β-C or γ-C) showed little variation for the enrichment of uniformly ^{13}C-labeled glucose. The average values of $\overline{GP_B}$, $\overline{GP_C}$ and $\overline{GP_D}$ from α-C, β-C, and γ-C were calculated, respectively. The enrichment of [U-^{13}C] glucose was expected to have little effect on the metabolism of glucose to γ-PGA, as shown in Table 4, at the same initial glucose concentration (40 gL^{-1}) enriched with different amount of [U-^{13}C] glucose, and values of $\overline{GP_B}$ and $\overline{GP_C}$ were almost equal (9%). The value of $\overline{GP_D}$ was only 6% with the initial glucose concentration of 30 gL^{-1}.

The conversion of glucose into γ-PGA suggested that γ-PGA formation by *B. subtilis* NX-2 could occur by the glycolysis of glucose to acetyl-CoA and tricarboxylic acid (TCA) cycle intermediates that were then metabolized via the TCA cycle to form α-ketoglutarate, which was a direct glutamate precursor (Fig. 2, route 1 in serif). However, the amount of glucose converted into γ-PGA monomer was relatively low, and only a small fraction of γ-PGA repeat units were derived from provided glucose, which could be interpreted as an indication that most of glucose was utilized as the growth-limiting substrate for cell growth and supplied the required energy during γ-PGA formation, while γ-PGA repeat units were mainly derived from exogenous L-glutamate with retention of the glutamate carbon skeleton (Fig. 2, route 2 in bold). The involvement

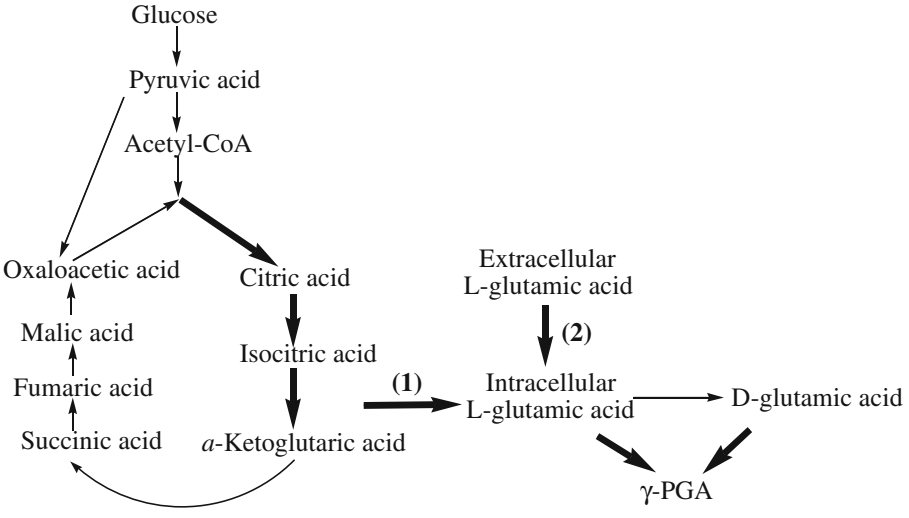

Fig. 2 Proposed pathway of γ-PGA synthesis in *B. subtilis* NX-2 (route 1, derived from glucose through de novo pathway, in serif; route 2, derived from exogenous L-glutamate, in *bold*)

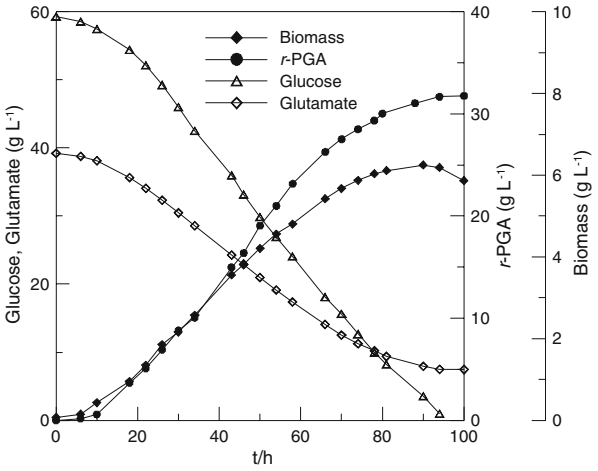

Fig. 3 Profile of batch culture of γ-PGA production from *B. subtilis* NX-2 in fermentor (culture medium: glucose 60 gL^{-1}, L-glutamate 40 gL^{-1}, NH_4Cl 8 gL^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 gL^{-1}, $MgSO_4$ 0.2 gL^{-1})

of glutamate racemase in γ-PGA synthesis had been carried out in our previous work [10].

Improvement of γ-PGA Production by Fed-Batch Culture in Fermentor

The above had proven that γ-PGA repeat units were mainly derived from the supplied L-glutamate. An efficient conversion of L-glutamate to γ-PGA was required for enhancing γ-PGA production. Excessive L-glutamate was helpful to enhance γ-PGA production, but a considerable amount of residual L-glutamate remained in the culture and low conversion rate occurred. Glucose was utilized mainly as the growth-limited substrate for cell growth

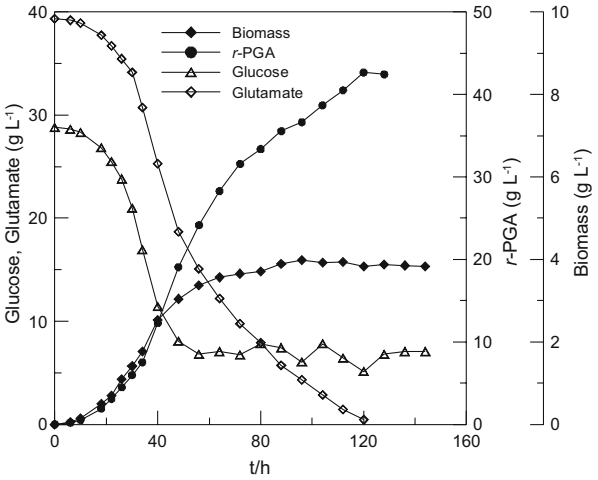

Fig. 4 Profile of fed-batch culture of γ-PGA production from *B. subtilis* NX-2 in fermentor (culture medium: initial glucose 30 gL^{-1}, L-glutamate 40 gL^{-1}, NH_4Cl 8 gL^{-1}, $K_2HPO_4{\cdot}3H_2O$ 2 gL^{-1}, $MgSO_4$ 0.2 gL^{-1})

and supplied the required energy during γ-PGA synthesis. Increased glucose resulted in higher biomass but little enhanced γ-PGA formation. A batch culture was firstly performed to achieve the batch production of γ-PGA. Sixty grams per liter of initial glucose was used in the culture. As shown in Fig. 3, when glucose was exhausted after 94 h of cultivation, a maximum γ-PGA concentration of 31 gL^{-1} was observed, and biomass attained the maximum value of 6.25 gL^{-1}; however, about 10 gL^{-1} of L-glutamate remained unused in the culture. The results indicated that the presence of glucose was indispensable and crucial to the cell growth and conversion of L-glutamate to γ-PGA.

To achieve a more efficient conversion of L-glutamate and less contribution of glucose to excessive cell growth, simultaneously enhancing γ-PGA production, a fed-batch method was proposed: A low level initial glucose (30 gL^{-1}) was contained in the medium to avoid the occurrence of excessive cell growth. When residual glucose was decreased to a low level of about 10 gL^{-1} at 40 h, glucose was fed to the medium and glucose concentration maintained at 5–10 gL^{-1}. As shown in Fig. 4, biomass was kept at a moderate level of about 4 gL^{-1} since the feeding of glucose occurred; however, a more efficient production of γ-PGA was observed. γ-PGA yield reached 35 gL^{-1} at 88 h when glucose was totally utilized 60 gL^{-1}, which was higher than the maximum yield of γ-PGA by none-fed culture mentioned above (31 gL^{-1}, 94 h). After 120 h of cultivation, L-glutamate was completely consumed, and about 80 gL^{-1} glucose was totally utilized; the maximum yield of γ-PGA was achieved 42 gL^{-1}.

Conclusion

In this paper, the metabolism of glucose involved in γ-PGA synthesis was investigated through the ^{13}C labeling method and NMR analysis. The results showed that only a small fraction of γ-PGA repeat units were formed from provided glucose, while most of the supplied glucose was utilized as the growth-limiting substrate for cell growth and supplied the required energy during γ-PGA formation. L-Glutamate was considered as the main substrate responsible for γ-PGA production. High levels of initial glucose concentration resulted in excessive biomass but minimally enhanced the γ-PGA production, and a considerable amount of L-glutamate still remained in the culture. A fed-batch culture offered a feasible-effective method to enhance the conversion of L-glutamate and γ-PGA production. On the basis of this research, a systematic conversion of commercially inexpensive L-glutamate into γ-PGA, the value-added new biomaterials, will be anticipated to be available in our future work.

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