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Influences of Cultural Medium Component on the Production of Poly(γ-glutamic acid) by *Bacillus* sp. RKY3

Duk-Yeon Jung[1], Sunok Jung[2], Jong-Sun Yun[3], Jin-Nam Kim[1], Young-Jung Wee[1], Hong-Gi Jang[4], and Hwa-Won Ryu[5]*

[1] Department of Material Chemical and Biochemical Engineering, Chonnam National University, Gwangju 500-757, Korea
[2] BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea
[3] BioHelix, Naju 520-811, Korea
[4] Korean Institute of Natural Science Inc., Naju 520-330, Korea
[5] School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea

Abstract In this study, the cultural medium used for the efficient production of γ-PGA with a newly isolated *Bacillus* sp. RKY3 was optimized. It was necessary to supplement the culture medium with L-glutamic acid and an additional carbon source in order to induce the effective production of γ-PGA. The amount of γ-PGA increased with the addition of L-glutamic acid to the medium. The addition of 90 g/L L-glutamic acid to the medium resulted in the maximal yield of γ-PGA (83.2 g/L). The optimum nitrogen source was determined to be peptone, but corn steep liquor, a cheap nutrient, was also found to be effective for γ-PGA production. Both the γ-PGA production and cell growth increased rapidly with the addition of small amounts of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$. *Bacillus* sp. RKY3 appears to require Mg^{2+}, rather than Mn^{2+}, for γ-PGA production, which is distinct from the production protocols associated with other, previously reported bacteria. *Bacillus* sp. RKY3 may also have contributed some minor γ-PGA depolymerase activity, resulting in the reduction of the molecular weight of the produced γ-PGA at the end of fermentation.

Keywords: *Bacillus*, biodegradable polymer, culture medium, glutamic acid, poly(γ-glutamic acid)

INTRODUCTION

Currently, a great deal of interest has focused on several biodegradable polymers, including poly(lactic acid), poly(3-hydroxybutyrate), poly(γ-glutamic acid), and bacterial cellulose [1-3]. Among these, poly(γ-glutamic acid) (γ-PGA), discovered in the capsule of *Bacillus anthracis*, is an unusual anionic polypeptide, consisting of glutamic acid units that are polymerized *via* amide linkages between the α-amino and γ-carboxylic acid functional groups [4,5]. γ-PGA, which has been considered a promising biodegradable polymer, is a naturally occurring, water-soluble, and edible compound, which is also nontoxic in both the human body and the general environment [5-7]. There has been a great deal of interest in γ-PGA and its derivatives in a wide range of industries, including food, cosmetics, medicine, and water-treatment, as they may prove useful in a broad range of applications, including their uses as thickeners, humectants, drug carriers, water absorbants, bioflocculants, and biological adhesives [5,7].

Several *Bacillus* strains can synthesize γ-PGA, and these are generally divided into two groups, based on their requirement for glutamic acid [5,6]. In other words, one group is glutamic acid-dependent, and the other glutamic acid-independent. The former group includes *B. licheniformis* ATCC 9945 [8], *B. subtilis* IFO 3335 [6], and *B. subtilis* F-2-01 [10], and the latter *B. subtilis* TAM-4 [11] and *B. licheniformis* A35 [12]. *Bacillus* strains have been intensively studied with regard to their capacity to produce γ-PGA, with research focusing on the optimal culture conditions, metabolic pathways, and enzymes related to γ-PGA synthesis [4,7,13,14]. Several research groups have reported that the nutritional requirements of γ-PGA-producing microorganisms, in terms of carbon, nitrogen, and mineral sources, varied with the strain employed [5].

In this study, we attempted to optimize the cultural medium, with regard to carbon, nitrogen, and mineral sources, for the efficient γ-PGA production *via* batch cultures of the newly isolated *Bacillus* sp. RKY3, which was found to be able to produce γ-PGA from L-glutamic acid in high concentrations.

***Corresponding author**
Tel: +82-62-530-1842 Fax: +82-62-530-1869
e-mail: hwryu@chonnam.ac.kr

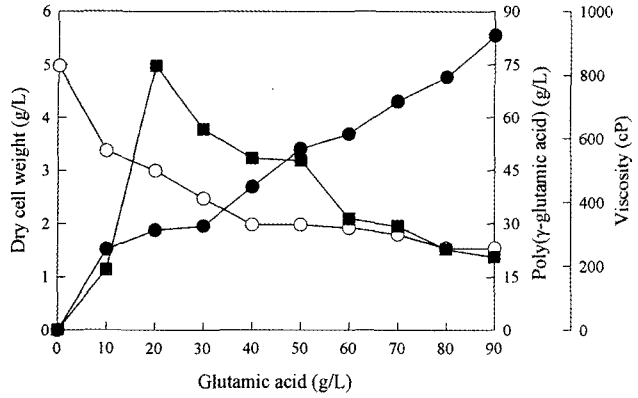

Fig. 1. The effects of the L-glutamic acid concentration on poly(γ-glutamic acid) production, cell growth and viscosity. Symbols: -●-, poly(γ-glutamic acid); -○-, dry cell weight; -■-, viscosity.

MATERIALS AND METHODS

Strain and Fermentation

The bacterial strain used in this study was *Bacillus* sp. RKY3 KCTC 10412BP, which was isolated from soil [15]. It was grown in a medium composed of L-glutamic acid (20 g/L), citric acid (12 g/L), glycerol (20 g/L), NH_4Cl (5 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), peptone (2 g/L), $CaCl_2 \cdot 2H_2O$ (0.15 g/L), $MnSO_4 \cdot H_2O$ (0.104 g/L), and $FeCl_3 \cdot 6H_2O$ (0.04 g/L). The cells were inoculated into 100 mL of medium in a 250-mL flask, and incubated at 38°C with shaking at 200 rpm for 24 h, which was then used as the inoculum in all experiments. The flask culture for the optimization of the culture medium was incubated at 38°C in a rotary shaker for 24 h. To investigate the viscosity and molecular weight profiles during γ-PGA production, fermentation was performed in a KF-7L fermenter (Kobiotech, Incheon, Korea) with 3 L of the optimized medium at 38°C and 300 rpm. Samples were periodically withdrawn for further analyses.

Isolation of γ-PGA

After the fermentation steps, cells were separated from the culture broth by centrifugation for 30 min at 32,000 × *g*, from which the γ-PGA was isolated by ethanol precipitation. The culture supernatant was then poured into four volumes of ethanol and gently stirred. The resulting precipitate was dried under a vacuum at 60°C until its weight was constant, and then its dry weight was determined [16].

Analytical Methods

Cell growth was determined by measuring the absorbance at 660 nm using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The viscosity of the cell-free culture broth was then measured with a DV-II+Pro Digital Rheometer (Brookfield, MA, USA) equipped with a spindle SPE-41, at different shear rates. Silicon oil (44.8 cP and 496 cP at 25°C) was used as a standard for the viscosity measurement. The molecular weight of the isolated γ-PGA was measured with a gel permeation chromatography (GPC) system, equipped with an Ultrahydrogel Linear column (300 × 7.8 mm; Waters, MA, USA) and refractive index detector (RID-10A; Shimadzu). The column was eluted with a mobile phase of 0.1 M $NaNO_3$, at a flow rate of 0.8 mL/min, with the temperature controlled at 40°C during the measurements. Pullulan was used as a standard for the molecular weight analysis. By-products of the fermentation, such as polysaccharide and proteins, were measured with phenol-sulfuric acid method and thin layer chromatography (TLC) [17,18].

RESULTS AND DISCUSSION

Effects of L-Glutamic Acid on γ-PGA Production

The effects of different L-glutamic acid concentrations on the γ-PGA production and cell growth in *Bacillus* sp. RKY3 were assessed within the range 0~90 g/L. As shown in Fig. 1, no γ-PGA was produced without glutamic acid supplementation of the medium, but the amount of γ-PGA increased with increasing glutamic acid addition to the medium, while the cell growth decreased gradually. These results may be due to the substrate inhibition. The highest yield (92.4%) of γ-PGA (83.2 g/L) was achieved with the addition of 90 g/L L-glutamic acid. There are some previous reports with the γ-PGA yield of more than 65% [8-10], but our result (83.2 g/L) is thought to be highest and most economical. The viscosity of the cell-free culture broth increased with increasing amounts of glutamic acid added, up to 20 g/L, but thereafter, the viscosity decreased gradually. Troy [8] and Birrer *et al.* [19] previously observed a decrease in the medium viscosity with the cultivation of *B. licheniformis*, thereby suggesting the existence of γ-PGA depolymerase, which could be responsible for the breakdown of γ-PGA. It can be deduced that *Bacillus* sp. RKY3 also exhibits some degree of γ-PGA depolymerase activity. Two types of γ-PGA producing bacteria exist: glutamic acid-dependent and glutamic acid-independent microorganisms [5,6]. Our results indicated that *Bacillus* sp. RKY3 is clearly of the glutamic acid-dependent variety, as the production of γ-PGA using this strain was dependent upon the amount of glutamic acid added to the medium.

Effects of Carbon Sources on γ-PGA Production

The effects of different carbon sources on the production of γ-PGA by *Bacillus* sp. RKY3 were investigated. Twenty grams per liter of various carbon sources was added to the medium. As shown in Table 1, *Bacillus* sp. RKY3 produced no γ-PGA without supplementation with an additional carbon source. Among the carbon sources tested, maltose, fructose, glucose, sucrose and glycerol were all favorably utilized by the RKY3 strain. The RKY3 strain grew well in media supplemented with lactose, ga-

Table 1. Effect of carbon sources on the poly(γ-glutamic acid) production, cell growth and by-product content

Carbon sources (20 g/L)	Poly(γ-glutamic acid) (g/L)	Dry cell weight (g/L)	By-product content (%)
None	ND	1.51	–
Lactose	ND	1.57	–
Galactose	2.1	2.39	–
Xylose	ND	2.41	–
Starch	1.7	2.07	+
Maltose	25.6	2.23	–
Fructose	29.4	2.35	–
Glucose	28.4	2.19	–
Sucrose	25.8	2.18	–
Glycerol	32.1	1.84	–

ND: not detected
–: less than 1%
+: more than 1%

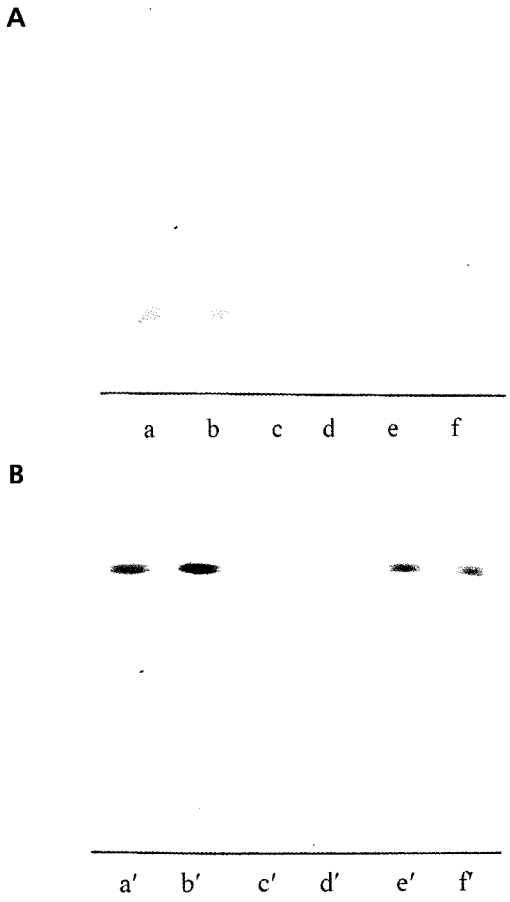

Fig. 2. Thin layer chromatograms of poly(γ-glutamic acid) hydrolyzates. (A) butanol:acetic acid:water (3:1:1, v/v), (B) butanol:acetic acid:water (3:1:1, v/v) + 96% ethanol:water (63:37, v/v); glutamic acid 1 g/L (a, a', b, b'), glutamic acid 0.1 g/L (c, c', d, d'), poly(γ-glutamic acid) hydrolyzate 0.5 g/L (e, e', f, f').

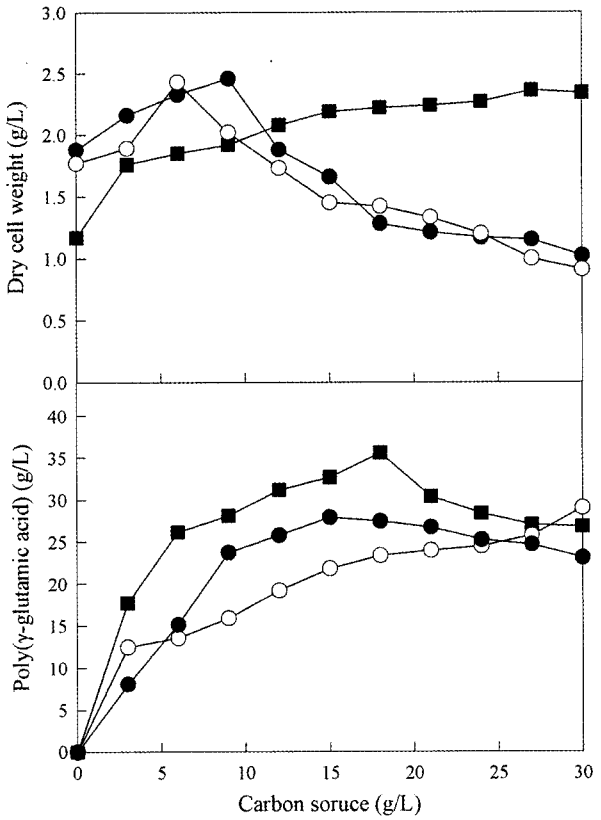

Fig. 3. The effects of various concentrations of glucose, fructose, or glycerol, as the carbon source, on poly(γ-glutamic acid) production and cell growth. Symbols: -●-, glucose; -○-, fructose; -■-, glycerol.

lactose, xylose, or starch as a carbon source, but those carbon sources had little effect on the γ-PGA production. Despite the relatively low degree of cell growth, the largest amount of γ-PGA (32.1 g/L) was obtained in media supplemented with glycerol as a carbon source.

Table 1 shows that the RKY3 strain did not produce polysaccharide as a by-product in the media except for starch. In addition, as shown in Fig. 2, thin-layer chromatography of the hydrolyzed γ-PGA was performed on a silica gel TLC plate and visualized with 0.2% ninhydrin indicated a single spot with an R_f value identical to that of authentic glutamic acid. Furthermore, the ninhydrin and biuret reactions for the isolated γ-PGA were negative, which indicated the peptide bond of γ-PGA is γ-bond. From these above results, the produced biopolymer was found to be γ-PGA consisted of only glutamic acid.

Therefore, we further assessed the effects of various concentrations of glycerol, glucose, and fructose, all of which appeared to be good carbon sources, with regard to cell growth and γ-PGA production. As shown in Fig. 3, when glucose was utilized as the carbon source, the cell growth peaked at 9 g/L of glucose (2.46 g-dry cell weight/L), but the maximum γ-PGA production was obtained with at 15 g/L of glucose (27.9 g-γ-PGA/L). In the case of fructose, the cell growth profiles were similar to those seen

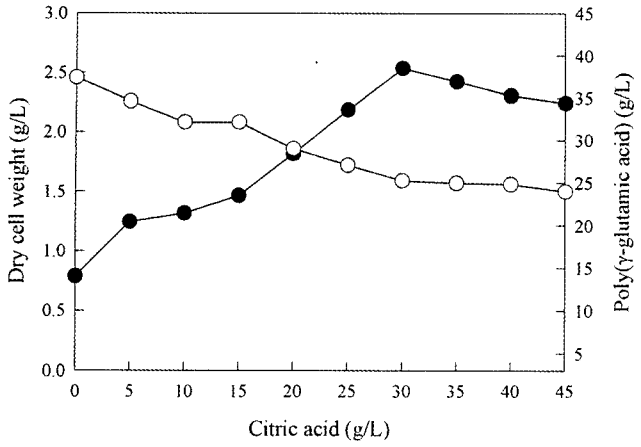

Fig. 4. The effects of the citric acid concentration on poly(γ-glutamic acid) production and cell growth. Symbols: -●-, poly(γ-glutamic acid); -○-, dry cell weight.

Table 2. Effect of nitrogen sources on the poly(γ-glutamic acid) production and cell growth

Nitrogen sources (2 g/L)	Poly(γ-glutamic acid) (g/L)	Dry cell weight (g/L)
None	ND	0.15
Ammonium sulfate	ND	0.15
Urea	ND	0.29
Corn steep liquor	32.3	3.72
Polypeptone	ND	0.23
Peptone	44.1	2.12
Yeast extract	39.8	2.31
Beef extract	40.1	2.34
Malt extract	11.5	1.04

ND: not detected

with glucose, but the amount of γ-PGA increased gradually with increasing fructose concentration. The highest yield of γ-PGA, 29 g/L, was obtained from the medium supplemented with fructose as the carbon source. Although glycerol exhibited minor stimulatory effects on cell growth, the amount of γ-PGA produced with glycerol was clearly larger than those with glucose or fructose as the carbon source. The most abundant γ-PGA (35 g-γ-PGA/L) was produced with the addition of 18 g/L of glycerol to the medium.

Effect of Citric Acid on γ-PGA Production

We attempted to determine the effects of the citric acid concentration on γ-PGA production, as it is an important precursor for γ-PGA biosynthesis [6]. As shown in Fig. 4, the most abundant yield of γ-PGA, 38 g/L, was obtained with the addition of 30 g/L citric acid to the medium. The yield of γ-PGA increased with increasing amounts of citric acid addition to the medium, but decreased at citric

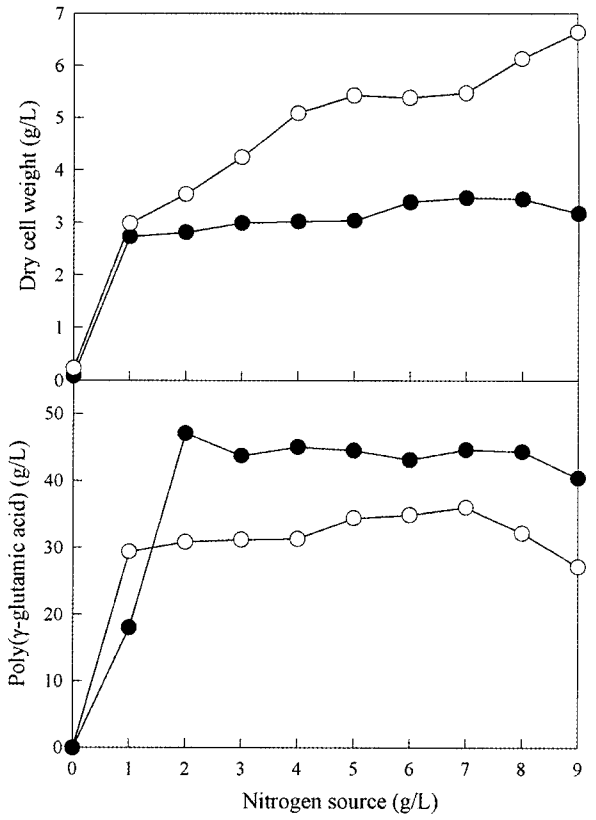

Fig. 5. The effects of various concentrations of peptone or corn steep liquor, as a nitrogen source, on poly(γ-glutamic acid) production and cell growth. Symbols: -●-, peptone; -○-, corn steep liquor.

acid levels above 30 g/L. The cell growth decreased proportionally to the amount of citric acid addition to the medium, which might be caused by the substrate inhibition with high concentration of citric acid.

Effects of Nitrogen Sources on the Production of γ-PGA

In order to investigate the effects of nitrogen sources on the γ-PGA production and cell growth, we tested several organic and inorganic nitrogen sources. Two grams per liter of various nitrogen sources was added to the medium. As shown in Table 2, *Bacillus* sp. RKY3 produced no γ-PGA without the addition of an extraneous nitrogen source. When inorganic nitrogen sources, such as ammonium sulfate and urea, were added to the medium, no γ-PGA was produced, which may be attributable to the lack of cell growth stimulation. However, the maximum γ-PGA yield, 44 g/L, was obtained in medium supplemented with peptone as the nitrogen source. The addition of corn steep liquor to the medium resulted in stimulation of both the γ-PGA production and cell growth, and culminated in the production of 32.3 g/L γ-PGA. This result indicates that, because corn steep liquor is an agricultural waste, it can be used to economically produce γ-PGA instead of using an expensive nitrogen

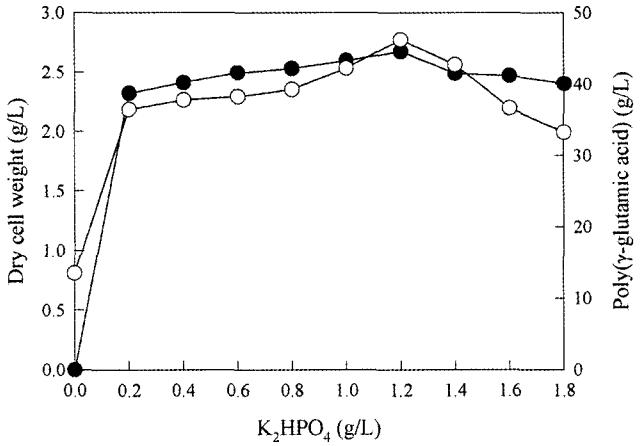

Fig. 6. The effect of K_2HPO_4 concentration on poly(γ-glutamic acid) production and cell growth. Symbols: -●-, poly(γ-glutamic acid); -○-, dry cell weight.

source such as yeast extract and peptone for industrial purposes. It is necessary, however, that process of ethanol precipitation for γ-PGA purification should be improved for more economical production of γ-PGA.

We further attempted to evaluate the effects of peptone and corn steep liquor concentrations on the γ-PGA production. As shown in Fig. 5, in the case of corn steep liquor, although the cell growth was higher, the γ-PGA production was lower than with the use of peptone. The amount of γ-PGA produced reached 47 g/L and 36 g/L with 2 g/L peptone or 8 g/L corn steep liquor as the nitrogen source, respectively.

Effects of K_2HPO_4 on γ-PGA Production

The effects of various concentrations of K_2HPO_4 on the γ-PGA production and cell growth were investigated. As shown in Fig. 6, no γ-PGA was produced without the addition of K_2HPO_4, due to the low cell growth. However, the cell growth increased abruptly with the addition of a small amount of K_2HPO_4. The optimal K_2HPO_4 concentration for the production of γ-PGA was determined to be 1.2 g/L, which yielded the largest amount of γ-PGA, 44.6 g/L.

Effects of Mg^{2+} and Mn^{2+} on γ-PGA Production

We attempted to characterize the effects of Mg^{2+} and Mn^{2+} on the γ-PGA production and cell growth, as these have been reported to significantly stimulate γ-PGA production on their addition to the medium [20,21]. As shown in Fig. 7, no γ-PGA was produced without the additional Mg^{2+}, but the addition of a small amount of Mg^{2+} resulted in stimulation of both the γ-PGA production and cell growth. The maximum yield of γ-PGA, 49.3 g/L, was obtained with the addition of 3.0 g/L of $MgSO_4 \cdot 7H_2O$ to the medium. However, the amount of γ-PGA obtained decreased proportionally to the amount of Mn^{2+} added to the medium. Troy [8] previously reported that the enzyme system of *B. licheniformis*, which is involved in γ-PGA production, required Mg^{2+}, and could not be replaced by another divalent cation. Leonard *et al.* [22] found that the γ-PGA synthetase system of the same organism uses Mn^{2+} as a cofactor, which also could not be place by Mg^{2+} or any other divalent cation. According to our results, *Bacillus* sp. RKY3 appears to require Mg^{2+} rather than Mn^{2+} for its production of γ-PGA, which is quite similar observation to Troy's study [8].

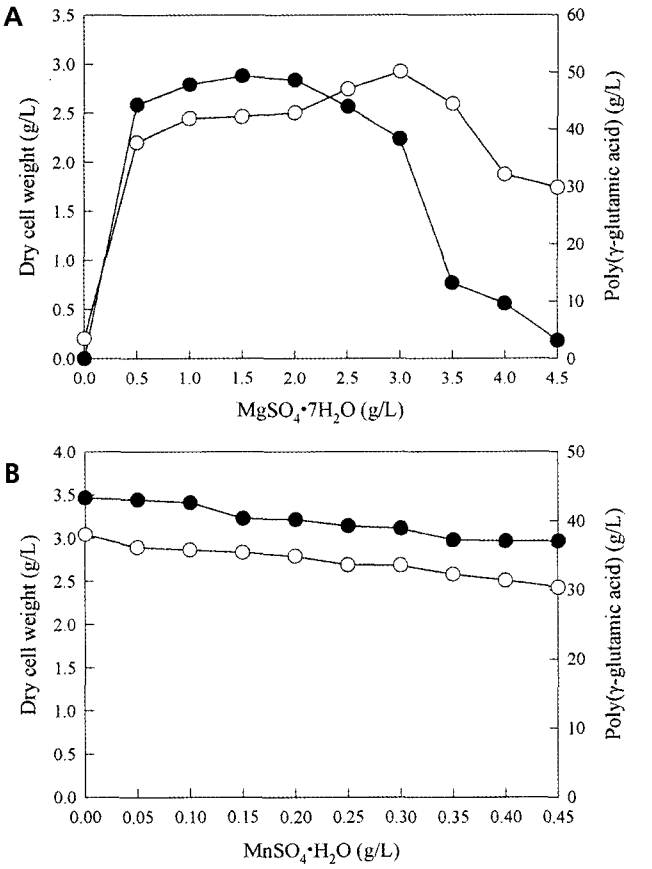

Fig. 7. The effects of various concentrations of $MgSO_4 \cdot 7H_2O$ (A) and $MnSO_4 \cdot H_2O$ (B) on poly(γ-glutamic acid) production and cell growth. Symbols: -●-, poly(γ-glutamic acid); -○-, dry cell weight.

Production of γ-PGA in a 7-L Fermenter

The production of γ-PGA was carried out in a 7-L fermenter, containing 3 L of the medium that had been optimized *via* the batch culture of *Bacillus* sp. RKY3. Fig. 8 shows the time courses of γ-PGA production, cell growth, viscosity change, and molecular weight distribution during the fermentation. More than 28 g/L of γ-PGA was produced after 48 h of fermentation, and the viscosity of the broth increased gradually, eventually reaching 650 cP, with a γ-PGA productivity of 0.6 $gL^{-1}h^{-1}$. The molecular weight of the γ-PGA produced increased up to 49.3 kDa at 36 h of fermentation, but decreased to

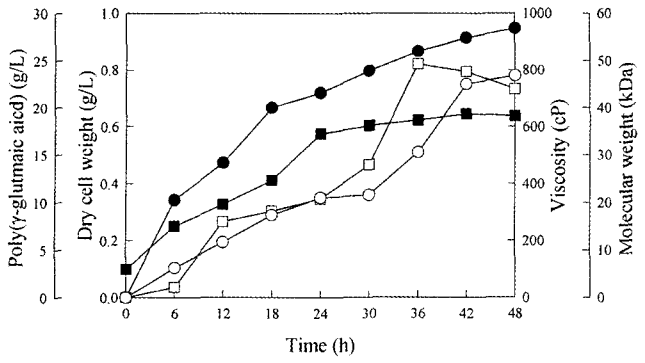

Fig. 8. The profiles of poly(γ-glutamic acid) production, cell growth, viscosity and molecular weight during batch fermentation in a 7-L jar fermenter with 3 L of optimized medium. Symbols: -●-, poly(γ-glutamic acid); -○-, dry cell weight; -■-, viscosity; -□-, molecular weight.

44.0 kDa by the end of fermentation, which may be attributed to the contribution of γ-PGA depolymerase. Although a culture medium for the efficient production of γ-PGA was formulated in this study, it will be necessary to further optimize the culture conditions, including the aeration rate, agitation speed, culture pH, and culture method (fed-batch or continuous), using a laboratory-scale fermenter for enhancing the production and molecular weight of γ-PGA by *Bacillus* sp. RKY3.

CONCLUSION

γ-PGA has recently been considered a promising biodegradable polymer, which can be produced independently of petrochemical resources. γ-PGA could be utilized in a broad range of applications across several industries. In order to efficiently produce γ-PGA, we investigated the culture medium by batch culture of a novel γ-PGA-producing bacterium, *Bacillus* sp. RKY3. The RKY3 strain was found to be able to produce high yields of γ-PGA from media containing various carbohydrate and nitrogen sources, when supplemented with L-glutamic acid and citric acid. *Bacillus* sp. RKY3 appears to require Mg^{2+} rather than Mn^{2+} for its efficient γ-PGA production, which may also be a critical factor. In the case of our laboratory-scale fermenter experiment, *Bacillus* sp. RKY3 was determined to produce as much as 28.4 g/L γ-PGA, after 48 h of fermentation, from a medium supplemented with 30 g/L L-glutamic acid. However, it must be necessary to optimize the culture conditions such as the aeration rate, agitation speed, culture pH, and culture method (fed-batch or continuous). Therefore, we now focus our further studies on the optimization of the culture conditions to enhance the molecular weight of γ-PGA.

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