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Influences of Culture Medium Components on the Production Poly (γ-Glutamic Acid) by *Bacillus subtilis* GS-2 Isolated *Chungkookjang*

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청국장에서 분리한 *Bacillus subtilis* GS-2에 의한 Poly(γ-Glutamic Acid) 생산의 최적 배양조건

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국문요약

Poly-γ-glutamic Acid(γ-PGA)를 다량 생산하는 균주를 우리나라의 전통발효식품인 청국장으로부터 *Bacillus subtilis* GS-2를 분리하였다. 이 균은 glutamic acid 의존형 균으로, 이 균에 의한 γ-PGA 생산 최적 조건을 검토한 바, 단순배지(L-glutamic acid 2.0%, glucose 1.0%, NH_4Cl 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, pH 7.0)로 진탕배양(220 rpm) 하였을 때, 배양시간 48시간, 최적온도 33℃, 그리고 초기 pH 6.5로 나타났다. 영양원으로 glutamic acid 3%, sucrose 3%, NH_4Cl 0.25%, KH_2PO_4 0.15%, $MgSO_4 \cdot 7H_2O$ 0.015%에서 γ-PGA 최대 생산량이 31.0 g/ℓ이었다.

Key words: *Chungkookjang*, culture medium, poly(γ-glutamic acid), PGA, soybean paste

INTRODUCTION

Soybeans are an important edible plant source of protein and oil as well as of phytochemicals such as genistein, phytic acid, tocopherol and saponin. In particular, soybean curd and sprouts are eaten as processed foods and used for fermented foodstuffs (Oh et al. 2007). In Korea, *Chungkookjang* is a traditional fermented-soybean foods made from boiled soybean with *Bacillus subtilis* from rice straw and is very similar to *natto*. Consumption of *Chungkookjang* is associated with antioxidant, antimicrobial, blood pressure lowering, antidiabetic, and other beneficial effects, which may arise from isoflavons, peptides, phenols, and other flavonoids produced during fermentation (Back et al. 2008).

γ-PGA(poly-γ-glutamic acid) is freely secreted into the growth medium of *Bacillus subtilis* as a product of fermentation and several *Bacillus* species have now been shown to produce extracellular γ-PGA. An unusual anionic polypeptide, γ-PGA is made of D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups (Bovarnick 1942; Back et al. 1954). The ratio of D/L content in γ-PGA changes readily depending on culture conditions and on bacterial strains. The γ-PGA produced by *B. subtilis* strains F-2-01, IFO 3335, and TAM-4 can contain as much 80% D-isomer (Shih and Van 2001).

γ-PGA was first discovered as a component of capsules of *B. anthracis* (Oh et al. 2007). Later, several γ-PGA producing

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strains were isolated from many kinds of organisms. Many *Bacillus* strains produce γ-PGA as a capsular material in the form of an extracellular viscous material (Hara and Ueda 1982; Cheng et al. 1989; Goto and Kunioka 1992).

As a viscosity element in fermented soybean products such as *Chungkookjang* and *Natto*, γ-PGA is biodegradable, edible and non-toxic toward humans and the environment. It can vary in molecular weight from ten thousand to several hundred thousand depending on the kinds of strains used. For this reasons, potential applications of γ-PGA and its derivatives have been of interest for their potential application in a broad range of industrial fields such as food, cosmetics, medicine, water-treatment, etc. (Yamanaka 1991; Borbely et al. 1994; Choi et al. 1995; Hasebe and Inagaki 1999). Therefore, it is very important to identify microorganisms that can produce γ-PGA abundantly in inexpensive media.

Some investigations have been initiated into the enhancement of productivity, simplification of nutrient requirements and improvement of culture conditions for γ-PGA production. The nutrient requirements such as carbon sources, nitrogen sources, amino acid, metal ions, etc. vary according to the strain used and the mechanisms of γ-PGA synthesis are also different (Francis et al. 2003).

In this study, a *B. subtilis* GS-2 isolated from the Korean traditional seasoning food, *Chungkookjang* was found to produce large amounts of γ-PGA with high productivity when supplied with very simple and inexpensive nutrients.

MATERIALS AND METHODS

1. Materials and Reagents

Chungkookjang was purchased at different local markets. All chemicals used were of reagent grade.

2. Screening and Identification of γ-PGA-producing *B. subtilis* GS-2

The γ-PGA producing bacterial strain GS-2 was isolated from the traditional fermented soybean food, *Chungkookjang*, through serial dilution and inoculation on an isolation medium with the following composition: L-glutamic acid 1.0%, glucose 1.0%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, agar 1.5% (pH 7.0). After incubation at 37℃ for 48 hr, any highly mucoid and high growth colonies that appeared on the plates were picked up, and used to inoculated 50 ㎖ of a basal production medium containing L-glutamic acid 2.0%, glucose 1.0%, NH_4Cl 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01% (pH 7.0) in 300 ㎖ Erlenmeyer flasks. The flasks were incubated at 37℃ on a rotary shaker at 220 rpm for 24 hr. Culture broth that contained γ-PGA was noticeably viscous. The relative viscosity of culture broth by various bacteria was measured and compared and the concentration of γ-PGA in highly viscous culture broths was determined. One strain GS-2 was identified as the best γ-PGA-producing organism and it was selected and maintained on a nutrient agar slant medium. A stock culture of this strain was subcultured at regular intervals of 1 month and stored under refrigeration. The isolated GS-2 strain was identified the method suggested by the Bergey' manual and 16S rRNA sequencing was carried out as described in Seo et al. (2008). It was identified as *B. subtilis* GS-2 (Bang et al. 2011).

3. Production of γ-PGA by Flask Culture

A loopful of GS-2 cells was first inoculated into 50 ㎖ of the basal production medium (pH 7.0) and aerobically incubated at 37℃ for 24 hr with a rotary shaker (220 rpm). A 2% inoculum was then transferred to 300 ㎖ Erlenmeyer flasks containing 50 ㎖ of basal production medium. The flask culture was incubated at 37℃ for given time on a rotary shaker at 220 rpm.

4. Determination of Dry Cell Weight and Purification of Poly(γ-glutamic acid) Hydrolysates

After fermentation, the culture broth was poured into two volumes of dH_2O and gently stirred. The diluted culture broth was centrifuged at 20,000×g for 20 min and the resulting precipitate was washed with distilled water 10 times, dried in a drying oven at 80℃ for 2～4 hr, and the dry cell weight (DCW) was determined. The γ-PGA in the culture broth supernatant was purified by the ethanol precipitation method reported by Goto and Kunioka (1992). Briefly, the culture supernatant was then adjusted to pH 3.0 with 6 M HCl and incubated at 4℃ for 12 hr. Four volumes of ethanol were added to the supernatant, and this solution was left to stand for one day, then centrifuged at 25,000×g for 30 min at 4℃. The pellet (crude γ-PGA) was harvested and then dissolved in a minimal volume of distilled water. After dialysis against de-ionized water, the γ-PGA was lyophilized, and its dry weight was determined.

5. Analysis of Polyglutamic Acid and Polysaccharide

The residual protein content in the purified γ-PGA pre-

paration was determined by the Bradford dye binding method (1976). Total sugar content was determined by the modified phenol/sulfuric acid method (Dubois et al. 1956).

6. Identification of Glutamic acid

The hydrolysate of γ-PGA with 6 M HCl was chromatographed by TLC against authentic glutamic acid as using *n*-butanol-acetic acid-water (12:3:5, w/w) as the solvent system. The plates (Silica Gel-60 F254: Merck Co., 20×20 ㎝) were dried and sprayed with acetone containing 0.2% ninhydrin to visualize the amino acid (Kambourova et al. 2001).

RESULTS AND DISCUSSION

1. Effect of Culture Conditions on Production of γ-PGA

Production of γ-PGA by GS-2, was investigated with respect to optimal culture conditions such as the time courses, temperature, and initial pH. The bacterial, GS-2 strain was cultivated in the basal production medium described above. This basal medium is a medium of choice for many researchers who have investigated production of γ-PGA by *Bacillus* sp. (Shih et al. 2003). All experiments were carried out in triplicate and the data were expressed as mean±SD of triple determinations. The concentration of γ-PGA and dry cells weight were measured at each sampling point during the cultivation process.

The time course study in Table 1 shows that concentration of γ-PGA and dry cells weight were maximal at 9.2 g/ℓ and 2.44 g/ℓ, respectively, at 48 hr. With further culture, γ-PGA production decreased slightly with time, whereas DCW did not decreased only within 72 hr. These decrease may be due to partial enzymatic hydrolysis of the γ-PGA during late stage fermentation. This hypothesis was supported by previous studies on *natto* mucilage, which was hydrolyzed by a depolymerase produced by *Bacillus* sp., resulting in decreased viscosity (Suzuki and Tahara 2003).

The culture temperature for optimum γ-PGA production (9.12 g/ℓ) was 33℃ and for optimum DCW (2.55 g/ℓ) was 37℃. The γ-PGA production in GS-2 was quite similar to the results reported by Ashiuchi et al. (2001) and Kubota et al. (1993). Similar data have also been reported for many kinds of *Bacillus* sp. that shows temperature optima ranging from 30 to 37℃.

In general, the pH of culture broth is one of the most critical environmental factors that affects cell growth and end product formation. The GS-2 strain was cultivated under conditions of different initial pH (5.0～9.0) on a rotary shaker (220 rpm) at 33℃ for 48 hr. The highest levels of γ-PGA production (10.2 g/ℓ) was observed at pH 6.5 while the greatest DCW (2.40 g/ℓ) was found at pH 7.0 (Table 1). A variety of *Bacillus* sp. have shown pH optima of 6.5～7.5 for γ-PGA production and cell growth.

Table 1. Optimum cultural conditions for γ-PGA production

Conditions		γ-PGA (g/㎖)	Cell growth (DCW, g/㎖)
Cultural time (hr)	24	6.8	1.88
	36	8.7	2.44
	48	9.2	2.44
	60	8.3	2.50
	72	7.4	2.40
	84	7.0	2.35
	96	5.1	2.30
Cultural temperature (℃)	20	1.50	0.70
	25	2.20	1.15
	30	5.70	2.20
	33	9.12	2.25
	37	7.80	2.55
	40	4.20	1.15
	45	1.40	0.38
Initial pH	5.5	4.4	1.15
	6.0	7.2	2.15
	6.5	10.2	2.38
	7.0	9.0	2.40
	7.5	8.2	2.22
	8.0	7.3	2.20
	8.5	5.2	1.20

2. Identification of Poly(γ-Glutamic Acid) Hydrolysates

As shown Fig. 1, thin-layer chromatography of the hydrolyzed γ-PGA resulted in a single spot with an Rf value identical to that of authentic glutamic acid. Ninhydrin and biuret reactions for the isolated, unhydrolyzed γ-PGA were negative, which indicated the peptide bond of the γ-PGA was a γ-bond. Based on these results, the isolated biopolymer was concluded to be γ-PGA consisting only of glutamic acid.

3. Effect of Carbon Source

Fig. 1. The thin layer chromatography of hydrolysate of purified γ-PGA. A: authentic glutamic acid, B: hydrolysate of purified γ-PGA.

A 2% inoculum was cultivated on rotary shaking (220 rpm) in the basal production medium (pH 6.5) containing various carbon sources at a final concentration of 1% for 48 hr. Among 13 carbon sources examined, sucrose, starch, glucose, and citrate were highly effective for γ-PGA production (12.13, 11.75, 10.21, and 9.83 g/ℓ, respectively). But polysaccharide was also produced from starch as by-product in the medium. Citrate, sucrose, glucose and starch were also relatively favorable for cell growth (2.70, 2.67, 2.58, and 2.48 g/ℓ, respectively, data not shown). For subsequent study, sucrose was selected as a suitable carbon source. The effect of varying the concentration of sucrose on the γ-PGA production are shown in Fig. 2. The most abundant yield of γ-PGA, 16.75 g/ℓ, was obtained with the addition of 30 g/ℓ sucrose to the basal production medium. The most abundant yield of DCW, 2.80 g/ℓ, was obtained with the addition of 50 g/ℓ sucrose. The amount of γ-PGA decreased gradually at sucrose levels above 30 g/ℓ, and cell growth decreased at sucrose levels above 50 g/ℓ. Goto and Kunioka (1992) also noted that citric acid was the best supplemental carbon source for the production of γ-PGA by *B. subtilis* IFO 3335. Cheng et al. (1989) found that glucose was the best carbon source for the production of γ-PGA by *B. licheniformis* A35. Ogawa et al. (1997) indicated that maltose was the best carbon source for the production of γ-PGA by *B. subtilis* (*natto*) MR-141. Our results were similar to those reported by Ashiuchi et al. (2001), who determined that sucrose was the best carbon source for the production of γ-PGA by *B. subtilis Chungkookjang*.

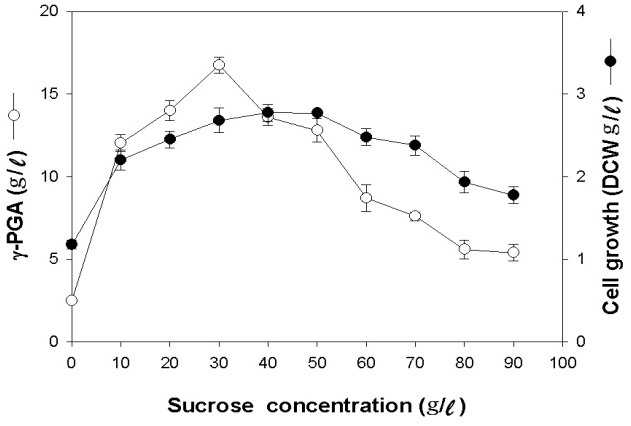

Fig. 2. The effects of sucrose concentration on γ-PGA production and cell growth. Sucrose concentration is 0 to 90 g/ℓ in basal production medium containing L-glutamic acid 2.0%, NH_4Cl 0.5%, 0.5%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.01% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ in a rotary shaker (220 rpm) for 48 hr.

4. Effect of Nitrogen Source

Several inorganic nitrogen sources including NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)NO_3$, $NaNO_3$ and KNO_3, and organic nitrogen sources including yeast extract, peptone, beef extract, malt extract, soytone and casamino acid, at concentration of 5 g/ℓ were tested for their effects on γ-PGA production. Of these, NH_4Cl and $(NH_4)_2SO_4$

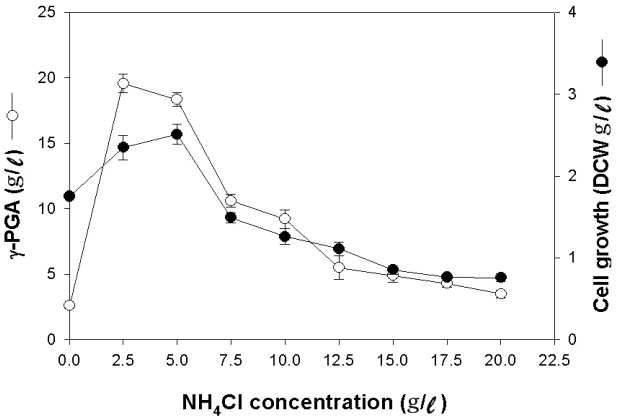

Fig. 3. The effects of NH_4Cl concentration on γ-PGA production and cell growth. NH_4Cl concentration is 0 to 20 g/ℓ in the basal production medium containing sucrose 3.0%, L-glutamic acid 2.0%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ on a rotary shaker (220 rpm) for 48 hr.

were the best suitable for prodution of γ-PGA and for cell growth. Addition of NH_4Cl to the medium resulted in stimulation of both γ-PGA production and cell growth, and resulted in production of 18.22 g/ℓ γ-PGA. When yeast extract was used, although γ-PGA production and cells growth was higher, that for other nitrogen sources, polysaccharide also appeared as a by-product in the medium (data not shown). Further evaluation of NH_4Cl concentration, as shown in Fig. 3, indicated that 2.5 g/ℓ NH_4Cl resulted in production of 19.57 g/ℓ γ-PGA while 5.0 g/ℓ resulted in DCW of 2.65 g/ℓ. Thus, using 2.5 g/ℓ NH_4Cl as a nitrogen source, resulted in a higher production γ-PGA than the 7 g/ℓ result achieved by Yoon et al. (2000), the 18 g/ℓ result of Ito et al. (1996), or the 4 g/ℓ result of Kambourova et al. (2001).

5. Effect of Amino Acids

The effect of amino acids on γ-PGA production by GS-2 was investigated by adding various amino acids at a final concentration of 2% to the basal production medium containing sucrose 3%, NH_4Cl 0.25%, $MgSO_4 \cdot 7H_2O$ 0.01%, KH_2PO_4 0.05% (pH 6.5) in 300 mℓ Erlenmeyer flasks. L-glutamic acid was the most suitable for production of γ-PGA and for cell growth (data not shown). The optimum concentration of L-glutamic acid for γ-PGA production was 30 g/ℓ, which gave the highest yield of γ-PGA, at 22.11 g/ℓ. However, the amount of γ-PGA and cell growth decreased gradually at L-glutamic acid levels above 30 g/ℓ and 40 g/ℓ (Fig. 4). The γ-PGA-producing bacteria can be divided into two types: glutamic acid-dependent and glutamic acid-independent producers. For the former, γ-PGA production depends on a supply of glutamic acid in the medium, whereas the later, can produce considerable amounts of γ-PGA, even in the absence of glutamic acid because of the operation of the *de novo* pathway of glutamic acid synthesis within the cells. The most notable glutamic acid-dependent bacteria are *B. subtilis* IFO 3335 (Goto and Kunioka 1992), *B. subtilis* MR-141 (Ogawa et al. 1997), *B. subtilis Chungkookjang* (Ashiuchi et al. 2001) and *B. subtilis* F-2-01 (Kubota et al. 1993). The most notable glutamic acid-independent strains are *B. subtilis* TAM-4 (Ito et al. 1996), *B. subtilis* (*natto*) 5E (Muraso 1969) and *Bacillus licheniformis* S-173 (Kambourova et al. 1997). The role of glutamic acid differs in these strains. In the case of *B. subtilis* MR-141, glutamic acid is converted to γ-PGA directly (Ogawa et al. 1997), whereas in *B. subtilis* TAM-4, glutamic acid activates enzymes in the pathway of γ-PGA synthesis as a regulator and is not assimilated itself (Goto and Kunioka 1992). As shown in Fig. 4, in the absence of glutamic acid, no γ-PGA was produced in GS-2. Therefore, our strain GS-2 was shown to be at glutamic acid-dependent bacterium.

6. Effects of Phosphate Salts

The effect of several phosphate salts was tested on γ-PGA production. The positive significant effect of KH_2PO_4 and K_2HPO_4

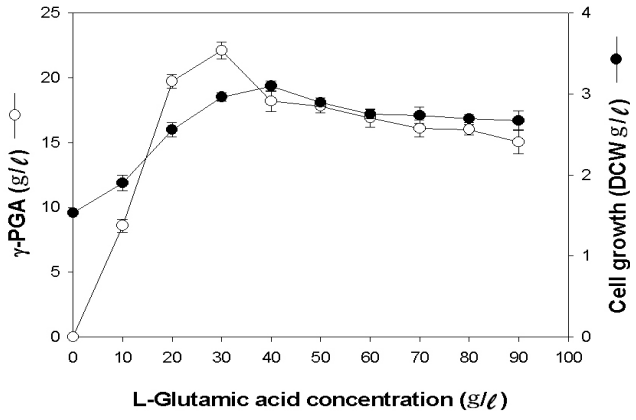

Fig. 4. The effects of L-glutamic acid concentration on γ-PGA production and cell growth. L-glutamic acid concentration is 0 to 90 g/ℓ in the basal production medium containing sucrose 3.0%, NH_4Cl 0.25%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ on a rotary shaker (220 rpm) for 48 hr.

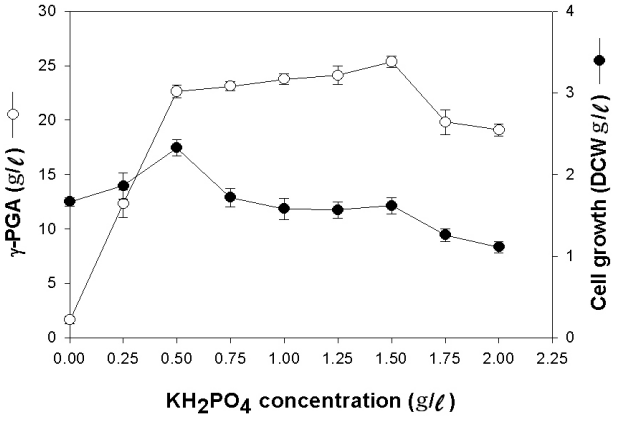

Fig. 5. The effects of KH_2PO_4 concentrationon γ-PGA production and cell growth. KH_2PO_4 concentration is 0 to 2 g/ℓ in the basal production medium oontaining sucrose 3.0%. NH_4Cl 0.25%, L-glutamic acid 3.0%, $MgSO_4 \cdot 7H_2O$ 0.01% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ on a rotary shaker (220 rpm) for 48 hr.

can be attributed to the mechanism of γ-PGA synthetase in *B. licheniformis*, as proposed by Troy (1973). In this mechanism, the γ-PGA synthetase activation is accompanied by the cleavage ATP to AMP, resulting in greater production of polyglutamate polymer. Phosphate is an essential factor for GS-2 cell growth and γ-PGA production. Among many phosphate salts, KH_2PO_4 was the most suitable for these processes (data not shown). The optimum concentration of KH_2PO_4 for γ-PGA production was 1.5 g/ℓ of KH_2PO_4 and gave the highest yield of γ-PGA, at 25.36 g/ℓ. However, the production of γ-PGA and cell growth decreased gradually at KH_2PO_4 levels above 1.5 g/ℓ and 0.5 g/ℓ, respectively (Fig. 5).

7. Effects of Metallic Ions

We endeavored to characterize the effects of metal ions on γ-PGA production and cell growth, as significant stimulation of γ-PGA production has previously been repotred following their addition to culture media (Leonard et al. 1958). Among the metal ions tested at 0.1%, $MgSO_4 \cdot 7H_2O$ had a stimulatory effect, with the highest yield of γ-PGA, at 25.71 g/ℓ (data not shown). The maximum yield of γ-PGA, at 30.20 g/ℓ, was obtained following the addition of 0.15 g/ℓ of $MgSO_4 \cdot 7H_2O$ to the medium. However, further increases in $MgSO_4 \cdot 7H_2O$ concentration began to be inhibitory to γ-PGA production (Fig. 6).

There are many reports of increased γ-PGA production following addition of sodium chloride to culture media. Based on our results, the addition of sodium chloride to the medium, decreased γ-PGA production and inhibited cell growth as NaCl concentration increased (data not shown). Thus, our results were contrast to those reported by Ogawa et al. (1997) which showed NaCl to be significant factor for γ-PGA production.

8. Time Course of γ-PGA Production

The time courses of γ-PGA production and cell growth in the optimal culture medium containing sucrose 3%, NH_4Cl 0.25%, L-glutamic acid 3%, KH_2PO_4 0.15%, $MgSO_4 \cdot 7H_2O$ 0.015% (pH 6.5) in 300 mℓ Erlenmeyer flasks at 33℃ by rotary shaking (220 rpm) were investigated (Fig. 7). After 6 hr of cultivation, the cells started to growth and after 12 hr of cultivation, γ-PGA production started to produce. The concentration of γ-PGA and the cells in the culture broth was measured at each sampling point during the cultivation process. The concentration of extracellular γ-PGA increased and reached its highest value of 31.0 g/ℓ at 48 hr. After then, γ-PGA production decreased slightly (Fig. 7). This may be due to the partial enzymatic hydrolysis of the γ-PGA in late stage of fermentation. Such a hypothesis was supported for *natto* mucilage, which was hydrolyzed by depolymerase produced by *Bacillus* sp., resulting in decreased viscosity (Suzuki and Tahara 2003). Compared to previous reports about the γ-PGA yield by *B. licheniformis* A35 (8～12 g/ℓ) (Cheng et al. 1989), *B. licheniformis* ATCC 9945 (17～23 g/ℓ) (Troy 1973), *B. subtilis*

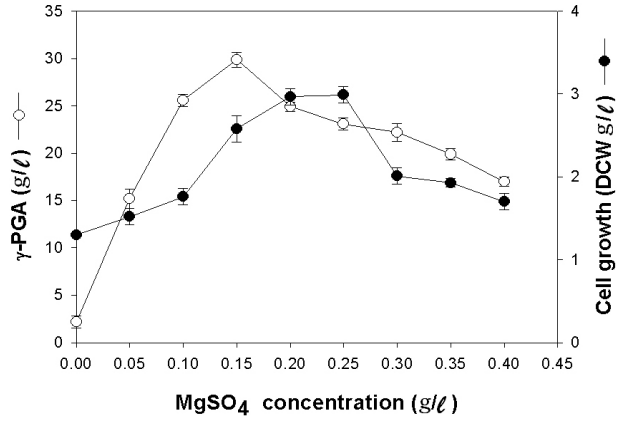

Fig. 6. The effects of $MgSO_4$ concentration on in γ-PGA production and cell growth. $MgSO_4$ concentration is 0 to 0.4 g/ℓ in the basal production medium containing sucrose 3.0%. NH_4Cl 0.25%, L-glutamic acid 3.0% and KH_2PO_4 0.15% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ on a rotary shaker (220 rpm) for 48 hr.

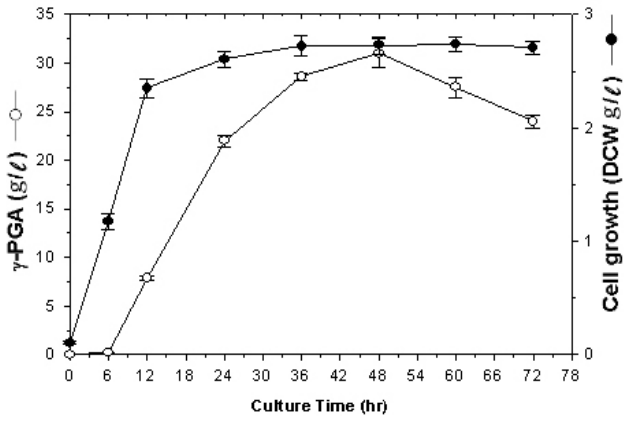

Fig. 7. Time course of γ-PGA production associated with cell growth. The cultivation was done in the medium containing sucrose 3.0%, NH_4Cl 0.25%, L-glutamic acid 3.0%, KH_2PO_4 0.15% and $MgSO_4 \cdot 7H_2O$ 0.015% (pH 6.5) in 300 mℓ Erlenmeyer flask. The culture was incubated at 33℃ on a rotary shaker (220 rpm) for each given time.

IFO 3335 (10～20 g/ℓ) (Kunioka and Goto 1994), *B. subtilis* TAM-4 (20 g/ℓ) (Ito et al. 1996), a novel *B. subtilis* HA (22.5 g/ℓ) (Seo et al. 2008) and *Bacillus* sp. YN-1 (27 g/ℓ) (You et al. 2005), and considering that the utilization of glutamic acid in defined medium, A *B. subtilis* GS-2 could produce 31.0 g/ℓ of γ-PGA from 30 g/ℓ glutamic acid. Therefore, the conversion of glutamic acid in optimized culture medium by *B. subtilis* GS-2 is considered to be efficient.

ABSTRACT

A bacterium strain GS-2 isolated from the Korean traditional seasoning food, *Chungkookjang* and was determined to produce large amounts of γ-PGA with high productivity when provided with simple nutrients (L-glutamic acid 2.0%, glucose 1.0%, NH_4Cl 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, pH 7.0). In this study, the culture medium for this strain was optimized for the production of γ-PGA. The *Bacillus subtilis* GS-2 required supplementation with L-glutamic acid and other nutrients for maximal production of γ-PGA. The optimal culture conditions for γ-PGA production were a 48 hr culture time, a temperature of 33℃ and initial pH of 6.5 by rotary shaking (220 rpm). A maximum γ-PGA production of 31.0 g/ℓ was obtained with L-glutamic acid (30 g/ℓ), sucrose (the main carbon source, 30 g/ℓ), NH_4Cl (the main nitrogen source, 2.5 g/ℓ), KH_2PO_4 (1.5 g/ℓ) and $MgSO_4 \cdot 7H_2O$ (0.15 g/ℓ) in the culture medium.

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