0273-2289/1559-0291 (Online)/06/133/0271/$30.00

Efficient Production of Poly-γ-glutamic Acid by *Bacillus subtilis* ZJU-7

Feng Shi, Zhinan Xu,* and Peilin Cen

Institute of Bioengineering, Department of Chemical Engineering and Bioengineering, Zhejiang University, Hangzhou 310027, China, E-mail: znxu@zju.edu.cn

Received September 15, 2005; Revised November 23, 2005; Accepted November 23, 2005

Abstract

A strain with high poly-γ-glutamic acid (γ-PGA) production was isolated from fermented bean curd, a traditional Chinese food. The strain was named *Bacillus subtilis* ZJU-7 according to 16s rDNA sequencing and its taxonomic characters. The culture conditions for γ-PGA production were evaluated. The most suitable carbon and nitrogen sources were sucrose and tryptone, respectively. Exogenous L-glutamic acid was necessary for γ-PGA production, and the production of γ-PGA increased on the addition of L-glutamic acid to the medium. In the medium containing 60 g/L of sucrose, 60 g/L of tryptone, 80 g/L of L-glutamic acid, and 10 g/L of NaCl, the yield of γ-PGA reached 54.4 g/L after cultivation at 37°C for 24 h, which was the highest γ-PGA production compared with values reported in the literature. The average molecular mass of γ-PGA produced was about 1.24×10^6 Daltons. *B. subtilis* ZJU-7 is genetically stable and can synthesize levan instead of γ-PGA without the addition of L-glutamic acid to the medium.

Index Entries: Poly-γ-glutamic acid; 16s rDNA; L-glutamic acid; sucrose; *Bacillus subtilis*.

Introduction

Poly-γ-glutamic acid (γ-PGA) is a type of water-soluble and biodegradable polymer that is made of D- and L-glutamic acid units linked by amide linkages between γ-amino and γ-carboxylic acid groups. Therefore, multifarious applications have been developed in the food and cosmetic as well as pharmaceutical industries. γ-PGA may be important as a therapeutic tool in the treatment of osteoporosis because it can increase Ca^{2+} solubility in vivo and in vitro, thereby enhancing intestinal Ca^{2+} absorption *(1)*.

*Author to whom all correspondence and reprint requests should be addressed.

High water-absorbent and biodegradable γ-PGA derivatives have potential as substitutes for hydrogels and thermoplastic polymers synthesized from petroleum *(2,3)*. Furthermore, γ-PGA may function as an adaptation agent in various environments, such as heavy metal ion absorbent *(4)*.

Ivanovics et al. *(5)* first discovered γ-PGA as the capsule of *Bacillus antharacis*. It is also well known that the mucilage of "natto" is a mixture of γ-PGA and fructan produced by *B. natto (6)*. Furthermore, it has been reported that many species of *B. subtilis* have γ-PGA-producing ability, since Bovarnick and colleagues showed that γ-PGA was freely secreted to the culture medium *(7–9)*. A number of factors, such as carbon source, nitrogen source, metal ions, and temperature, have a significant effect on γ-PGA production. *B. subtilis* (*natto*) has been studied intensively, and many studies have been conducted on the optimization of culture medium and conditions to improve γ-PGA production.

In the present study, a strain named *B. subtilis* ZJU-7 with high γ-PGA production was isolated and identified. The composition of the medium and culture conditions were evaluated to enhance the accumulation of γ-PGA.

Materials and Methods

Isolation of PGA-Producing Strains

Various fermented bean curd samples were purchased from market in China. The samples were suspended in distilled water and boiled for 5 min before they were spread on the isolation medium. The components of the isolation medium were as follow: 5 g/L of glucose, 5 g/L of tryptone, 10 g/L of L-glutamic acid, 2.5 g/L of NaCl, and 15 g/L of agar. The final pH of the medium was adjusted to 7.0. After 48 h of incubation at 37°C, some sticky clones appeared on the plates. Then these clones were transferred to 10 mL of basal medium in a 100-mL flask and incubated in a rotary shaker at 200 rpm and 37°C for 24 h. The components of the basal medium were as follow: 20 g/L of glucose, 20 g/L of tryptone, 40 g/L of L-glutamic acid, and 10 g/L of NaCl. Because of the high viscosity of the γ-PGA solution, the relative viscosity of each broth was measured and compared. The concentration of γ-PGA was determined by the method described later. Among various strains evaluated, the strain ZJU-7 was selected as the best PGA-producing microorganism and was maintained in agar slant medium.

Identification of Strain ZJU-7

The taxonomic characters of the strain ZJU-7 were studied according to *Berg's Manual of Systematic Bacteriology (10)*. The 16s rDNA sequence of the strain was also determined. Primers used for amplification of the 16s rDNA gene were as follows: fd1 (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-TAC GGH TAC CTT GTT ACGACT T-3'). Polymerase chain reaction (PCR) and restriction fragment analyses were performed as

described in Hansen and Hendriksen *(11)*. The sequences determined in the present study were aligned with the 16s rDNA sequences retrieved from the GeneBank database.

Optimization of Culture Conditions

B. subtilis ZJU-7 cultivation conditions were optimized in flask scale. The strains of *B. subtilis* ZJU-7 were transferred from the agar slant medium to 100-mL flasks containing 10 mL of the basal culture medium, and the cells were incubated in a rotary shaker at 37°C and 200 rpm for 12 h. One milliliter of culture medium of the *B. subtilis* ZJU-7 was first inoculated into 50 mL of seed medium containing basal medium plus 10 g/L of glucose and 10 g/L of L-glutamic acid in a 500-mL flask and aerobically cultured at 37°C for 24 h with shaking at 200 rpm as a seed. Then, 1 mL of seed was transferred to a 250 mL flask containing 30 mL of basal medium plus 20 g/L of carbon source, 20 g/L of nitrogen source, and 40 g/L of L-glutamic acid. Flask culture was carried out at 37°C in a rotary shaker at 200 rpm for 24 h. Six different carbon sources (glucose, sucrose, maltose, lactose, starch, citrate acid and glycerol) were used, respectively, to examine the effect of carbon sources on the production of γ-PGA. The concentration of each carbon source was 40 g/L, and 40 g/L of L-glutamic acid with 40 g/L of tryptone was added as precursor of γ-PGA synthesis. To evaluate the effect of nitrogen sources on the production of γ-PGA, seven nitrogen sources (peptone, tryptone, yeast extract, ammonium sulfate, soybean, maize flour, fish protein concentrate) were examined at 40 g/L with 40 g/L of optimized carbon source and 40 g/L of L-glutamic acid added. Finally, the effect of L-glutamic acid on the production of γ-PGA was studied in the range of 0–120 g/L. After optimization of the composition of the medium, the effects of other factors, such as incubation time, culture temperature, and rotation speed, on γ-PGA production were also tested.

Separation, Purification, and Identification of γ-PGA

γ-PGA was purified by the methanol precipitation method proposed by Goto and Kunioka *(8)*. Cells were separated from the broth by centrifuging for 20 min at 12,000 rpm and 4°C. Then, 4 vol of methanol was poured into the supernatant and left overnight with gentle stirring. The precipitate was collected by centrifuging for 10 min at 10,000*g* and 4°C and then dissolved in distilled water to a concentration of 10 g/L, and the insoluble contaminants were removed by filtration. The aqueous solution was desalted by dialysis (mol wt cutoff of 10,000) against 11 vol of distilled water for 12 h with three water exchanges. The absence of free L-glutamic acid and polysaccharides in the solution was confirmed by L-glutamate dehydrogenase-coupled assay *(12)* and by the phenol–sulfuric acid method *(13)*, respectively. The solution was finally lyophilized and the dry matter was determined as γ-PGA.

Analysis

Cell density was determined by measuring the optical density at 660 nm (OD_{660}). Dry cell weight (g/L) was determined by centrifuging 4–8 mL of cell suspension in preweighed tubes, washing with distilled water, and drying at 95°C to a constant weight. The average molecular size of γ-PGA was estimated by the gel permeation chromatography (GPC) method according to Kunioka *(14)*.

According to a method described previously *(12)*, γ-PGA was hydrolyzed by 6 *M* HCl under vacuum at 110°C for 24 h, and the glutamate monomers generated were analyzed by high-performance liquid chromatography (HPLC) with an Agilent C18 column (Agilent, Palo Alto, CA). The concentration of sucrose in the culture medium was measured according to the method suggested by Lee and Lin *(15)*. The concentrations of L-glutamic acid and γ-PGA in the broth were determined by a CE instrument (Agilent) with a 60-cm fused-silica capillary pillar (the length to the charge-coupled device system is 46.0 cm; Supelco, Bellefonte, PA). The instrument was equipped with a diode array detector set at 195 nm (direct detection), a temperature control device maintained at 25°C, and a data acquisition and treatment software package supplied by the manufacturer (HP ChemStation, rev A.06.01). The electrophoresis system was operated under reversed polarity and constant voltage conditions of 25 kV. A 30 mmol/L borate solution was used as an electrolyte solution. The capillary was flushed sequentially with NaOH (1 min), water (1 min), and electrolyte solution (4 min) as a precondition. The running time of the electrophoresis is 15 min. The glutamic acid and γ-PGA standards were purchased from Sigma (St. Louis, MO).

Results

Isolation and Identification of Strain ZJU-7

About 500 vicious colonies have been selected from the isolation medium (described in Materials and Methods) and 47 strains have been proved to have γ-PGA-producing ability. The strain ZJU-7 was found to be the best γ-PGA producer, and Table 1 summarizes the results of the taxonomic characteristics of this strain.

Applying genes encoding 16s rDNA as molecular markers has become a routine technique in the phenotypic classification system. Using general primers and PCR, the 16s rDNA sequence of the strain ZJU-7 was obtained and submitted to GeneBank with accession no. DQ086154. The sequence was aligned with the 16s rDNA sequences retrieved from the GeneBank database. The BLAST result shows that 16s rDNA isolated from the strain ZJU-7 has a similarity of 99.9, 99.8, and 99.6% to that of *B. subtilis* MO 07, *B. subtilis* WL 07, and *B. subtilis* KL 077, respectively. Accordingly, the strain ZJU-7 was classified as a *B. subtilis* species and named *B. subtilis* ZJU-7. The strain has been maintained in the China General Microbiogical Culture Collection Center (CGMCC) with the accession no. CGMCC 1250.

Table 1
Taxonomical Characteristics
of Newly Isolated γ-PGA Producer *B. subtilis* ZJU-7[a]

Gram stain	+
Shape	Rod 0.5–0.8 × 2.0–3.0 μm
Motility	–
Nitrite reduction	+
Starch and casein hydrolysis	+
Gelatin liquefaction	+
Voges-Proskauer test	+
Citrate utilization	+
Oxidase and catalase formation	+
Denitrification	–
Methyl-red test	–
Urease and indole formation	–
Ammonium salt utilization	–

[a](+), positive; (–), negative.

Different from the γ-PGA-producing strains reported before, the colony shape of *B. subtilis* ZJU-7 will change with different culture media. The colony was moist, circular with a smooth surface and regular edge when sufficient L-glutamic acid was supplied, and the colony size was 20 mm or larger in diameter after 24 h of incubation. However, the colony size of *B. subtilis* ZJU-7 was less than 5 mm with insufficient L-glutamic acid supply (0–5 g/L) and the colony shape was rough, folding with the hollow center with an irregular edge in dry medium.

Separation, Purification, and Identification of γ-PGA

The precipitate derived from the broth was hydrolyzed using 6 *M* HCl under vacuum at 110°C for 24 h. The HPLC results showed that glutamine was almost the only product (>97.8%). The results of both the L-glutamate dehydrogenase-coupled assay and the phenol–sulfuric acid reaction also confirmed the absence of free L-glutamate and polysaccharides in the precipitate *(12)*.

Effect of Culture Medium on γ-PGA Production

Effect of Carbon Source on γ-PGA Production

The effect of carbon sources on γ-PGA production of *B. subtilis* ZJU-7 was examined; the results are presented in Table 2. It is obvious that when sucrose was used as the carbon source for *B. subtilis* ZJU-7, γ-PGA production reached 20.3 g/L, which is the highest among carbon sources evaluated. Glucose was the best carbon source for cell growth, but the γ-PGA production was only 13.5 g/L.

The effect of sucrose concentration on γ-PGA production was further explored; the results are shown in Fig. 1. Without sucrose supply in the

Table 2
Production of γ-PGA From Medium
With Various Carbon Sources Using *B. subtilis* ZJU-7

Carbon source	OD_{660}	γ-PGA (g/L)
None	0	0
Glucose	16.312	13.5
Sucrose	10.705	20.3
Maltose	9.150	7.1
Lactose	9.753	11.3
Starch	6.330	9.5
Citric acid	4.277	8.8
Glycerol	8.137	10.6

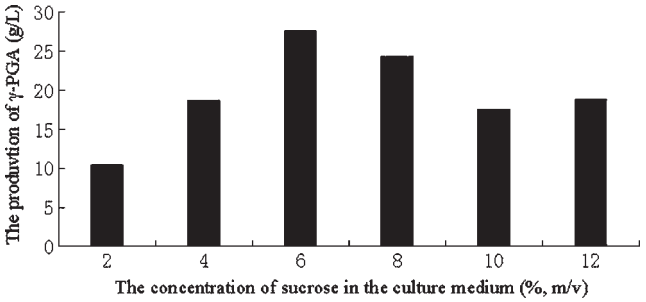

Fig. 1. Effect of sucrose concentration on γ-PGA production by *B. subtilis* ZJU-7.

culture medium, the cells grew rather slowly, and almost no γ-PGA was detected in the broth (not shown in Fig. 1), whereas the production of γ-PGA increased with an increase in sucrose concentration. When sucrose concentration was 60 g/L, the highest γ-PGA production of 27.6 g/L was obtained. A further increase in sucrose concentration led to a decrease in γ-PGA production because of substrate inhibition.

Influence of Nitrogen Source on γ-PGA Production

The effect of nitrogen sources on γ-PGA production was studied; the results are given in Table 3. In each experiment, the medium contained 60 g/L of sucrose, 40 g/L of each kind of nitrogen source, 40 g/L of L-glutamic acid, and 10 g/L of NaCl. The results indicated that inorganic nitrogen source could not support cell growth and product synthesis, whereas tryptone was the most suitable nitrogen source among them. Both cell density and γ-PGA production were the highest when tryptone was added as a nitrogen source.

The effect of tryptone concentration on γ-PGA production was also examined; the experimental data are shown in Fig. 2. When tryptone concentration was in the range of 8–10%, γ-PGA production was higher than 40 g/L. Because the addition of L-glutamic acid, the precursor of γ-PGA

Table 3
Effect of Nitrogen Source on γ-PGA Production

Nitrogen source	OD_{660}	γ-PGA (g/L)
Peptone	11.88	11.3
Tryptone	13.02	19.6
Yeast extract	10.71	10.7
Ammonium sulfate	0	0
Soybean	10.04	7.6
Maize flour	7.755	8.6
Fish protein concentrate	9.217	13.8

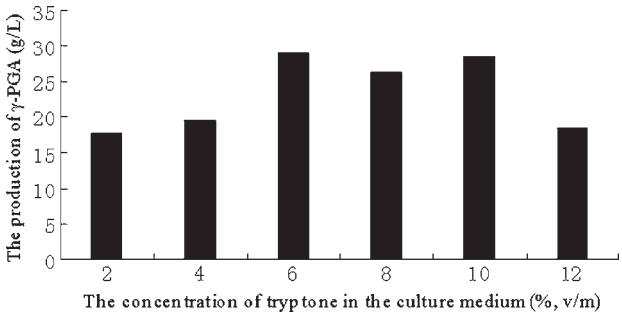

Fig. 2. Effect of tryptone concentration on γ-PGA production by *B. subtilis* ZJU-7.

synthesis, was only 40 g/L, the excess γ-PGA production was probably caused by the L-glutamic acid hydrolyzed from the tryptone.

Effect of L-Glutamic Acid on γ-PGA Production

According to the nutrient requirements, γ-PGA-producing bacteria are divided into two groups: L-glutamic acid-dependent and -independent bacteria *(16)*. The preliminary experimental data showed that without the addition of L-glutamic acid, *B. subtilis* ZJU-7 could not accumulate γ-PGA, which indicated that the strain is an L-glutamic acid-dependent one. Then, the effect of L-glutamic acid concentration on γ-PGA production was studied; the results are shown in Fig. 3. It was obvious that a high initial L-glutamic acid concentration was favorable for γ-PGA accumulation; however, when the initial L-glutamic acid concentration was higher than 80 g/L, γ-PGA production was almost unchanged. The initial L-glutamic acid concentration of 60–80 g/L was appropriate. When the initial L-glutamic acid was 80 g/L and cultured for 36 h, the yield of γ-PGA reached a maximum of 51.6 g/L.

In the medium without L-glutamic acid, it was observed that the viscosity of the fermentation broth of *B. subtilis* ZJU-7 still increased, which indicated that some polymers other than γ-PGA were synthesized, which was confirmed as levan according to the method reported by Shih and Yu *(17)*.

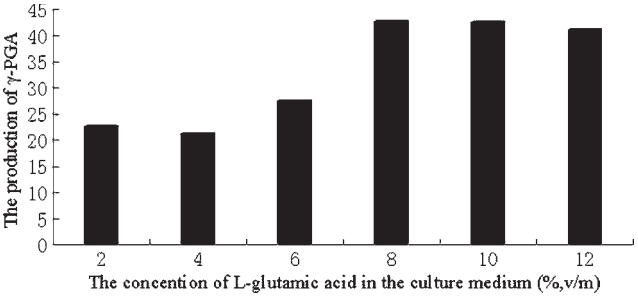

Fig. 3. Effect of L-glutamic acid concentration on γ-PGA production by *B. subtilis* ZJU-7.

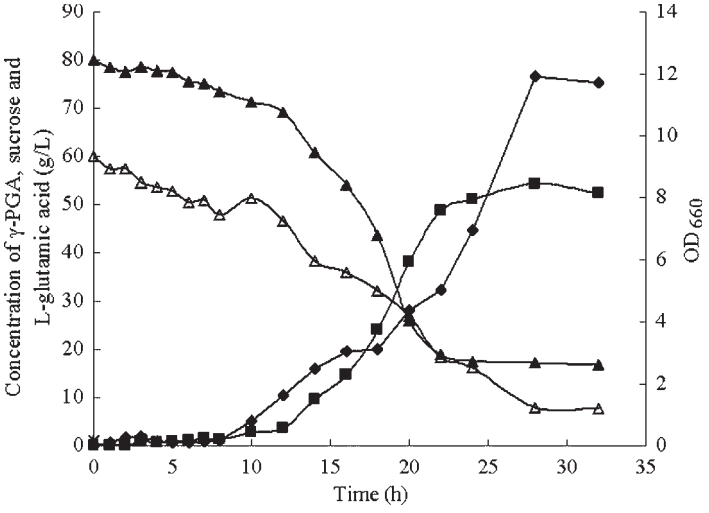

Fig. 4. Time courses of bacterial growth and γ-PGA, sucrose, and L-glutamic acid concentration in medium during cultivation: (◆) cell OD_{660}; (■) γ-PGA concentration; (▲) L-glutamic acid concentration; (△) sucrose concentration.

Time Courses of *B. subtilis* ZJU-7 Fermentation for γ-PGA Production

Figure 4 shows the time courses of bacterium growth, γ-PGA accumulation, as well as sucrose and L-glutamic acid consumptions at the initial concentrations of sucrose, L-glutamic acid, and tryptone of 60, 80, and 60 g/L, respectively, in batch fermentation. The lag phase required about 10 h. In the exponential growth phase, the cell density and γ-PGA accumulation increased simultaneously. After 24 h of cultivation, cell growth entered the stationary phase, and in the meantime, the accumulation of γ-PGA ceased to increase, demonstrating that this accumulation was cell growth dependent. The highest γ-PGA concentration of 54.4 g/L was observed after fermentation for 28 h. The residual concentrations of sucrose and L-glutamic acid were 5.0 and 16.7 g/L, respectively, which meant that the conversion ratio from L-glutamic acid to γ-PGA was about 73%. After separation and

Table 4
Comparison of *B. subtilis* ZJU-4 With Other Previously Reported Strains

Strain/reference	Main nutrients	Culture time (h)	γ-PGA (g/L)	Molecular mass (Daltons)
B. licheniformis ATCC 9945a *(9)*	20 g/L Glutamine, 80 g/L glycerol, 12 g/L citric acid	96	17–23	$1.4–9.8 \times 10^5$
B. subtilis IFO3335 *(14)*	30 g/L Glutamine, 20 g/L citric acid	48	10–20	$1.0 \times 10^5–2.0 \times 10^6$
B. subtilis TAM-4 *(16)*	75 g/L Fructose, 18 g/L NH_4Cl	96	22	$6.0 \times 10^5–1.6 \times 10^6$
B. subtilis F-2-01 *(19)*	70 g/L Glutamine, 1 g/L glucose	96	48	1.2×10^6
B. subtilis ZJU-7	60 g/L Sucrose, 80 g/L glutamine	24	54.4	1.23×10^6

purification, the average molecular weight of γ-PGA was measured by the GPC method, and the result showed that the average molecular weight of γ-PGA produced by *B. subtilis* ZJU-7 was as high as 1.24×10^6 Daltons. Table 4 summarizes the γ-PGA production of *B. subtilis* ZJU-7 compared with other γ-PGA-producing strains.

Discussion

B. subtilis ZJU-7 is an L-glutamic acid-dependent strain for γ-PGA production and its notable characteristics are fast growth rate and high γ-PGA production with a high molecular weight. The batch fermentation period and γ-PGA production for *B. subtilis* ZJU-7 reported in the literature were in the range of 48–96 h and 10–50 g/L, respectively, whereas for our study they were only 24 h and 54.4 g/L. The high molecular weight of γ-PGA (1.24×10^6 Daltons) produced by *B. subtilis* ZJU-7 explains that the activity of depolymerase secreted by the bacteria is rather low.

Goto and Kunioka *(8)* concluded that citric acid was the best carbon source for cell growth and γ-PGA production by *B. subtilis* IFO3335. Furthermore, Birr et al. *(9)* found citric acid to be the best carbon source for *Bacillus licheniformis* ATCC 9945a. In the present work, the results showed that sucrose was the best carbon source for cell growth and γ-PGA accumulation, which suggests that the metabolic pathways in *B. subtilis* ZJU-7 are different from those of the other L-glutamic acid-dependent strains for γ-PGA production.

Shih and Yu *(17)* found that *B. natto* was able to produce γ-PGA and levan simultaneously when sucrose and L-glutamic acid were added to the medium at a different ratio. However, for *B. subtilis* ZJU-7, it has been found that it can produce levan only without the addition of L-glutamic acid to the culture medium. When the concentration of L-glutamic acid in the medium is higher than 2%, it is observed that γ-PGA is the only product (>98%). When sucrose is used as the carbon source, it can be hydrolyzed into glucose and fructose. The accumulation of levan during *B. subtilis* ZJU-7 cultivation without the addition of L-glutamic acid demonstrates that the strain is capable of producing fructose polymerase. The disappearance of levan at high L-glutamic acid concentration indicates that L-glutamic acid is a strong inhibitor of or repressor for fructose polymerase synthesis.

Several studies indicated that the reproducibility of *B. subtilis* for γ-PGA production was low and that γ-PGA production dropped quickly after several rounds of subcultivation. This phenomenon is owing to the interruption of an insert gene, *comP*, by IS4*Bsn (18)*. It is suggested that the IS4 element is important for the physiological characteristics of *B. subtilis*. However, as for *B. subtilis* ZJU-7, we have found that PGA production was rather stable even after repeated subcultivation and/or storage in Luria-Bertani medium for almost half a year at –20°C.

Conclusion

B. subtilis ZJU-7 is a suitable strain for γ-PGA production with various advantages: fast cell growth rate, high γ-PGA production with a high molecular weight, and stable genetic characteristics. It is worthy of further study at genetic and enzymatic levels. Furthermore, it is important to optimize its cultivation conditions to enhance γ-PGA production.

Acknowledgment

This work is the prophase special project of key basic research supported by the Ministry of Science and Technology, China (project no. 2004CCA05500).

References

1. Kadowaki, M. and Noguchi, T. (2001), *Biosci. Biotechnol. Biochem.* **65,** 516–521.
2. Choi, H. J. and Kunioka, M. (1995), *Radiat. Phys. Chem.* **46,** 175–179.
3. Ashiuchi, M., Tani, K., Soda, K., and Misono, H. (1998), *J. Biochem.* **123,** 1156–1163.
4. Richie, S. M. C., Bachas, L. G., and Olin, T. (1999), *Langmuir* **15,** 6346–6357.
5. Ivanovics, G. and Bruckner, V. (1937), *Z. Immunitatsforsch.* **90,** 304–318.
6. Fugii, H. (2001), *Nippon Nogeikagaku Kaishi* **37,** 407–411.
7. Bovarnick, M. (1942), *J. Biol. Chem.* **145,** 415–424.
8. Goto, A. and Kunioka, M. (1992), *Biosci. Biotechnol. Biochem.* **63(1),** 105–110.
9. Birr, G. A., Cromwick, A. M., and Gross, R. A. (1994), *Int. J. Biol. Macromol.* **16(5),** 265–275.
10. Sneath, P. H. A., Krieg, N. R., and Holt, J. G. (1986), *Berg's Manual of Systematic Bacteriology [M],* vol. 12, Willams & Wilkins, Baltimore.
11. Hansen, B. M. and Hendriksen, N. B. (2001), *Appl. Environ. Microbiol.* **67,** 185–189.
12. Ashiuchi, M., Tani, K., Soda, K., and Misono, H. (1998), *J. Biochem.* **123,** 1156–1163.
13. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–356.
14. Kunioka, M. (1994), *Appl. Microbiol. Biotechnol.* **40,** 867–872.
15. Lee, Y. H. and Lin, T. I. (1996), *J. Chromatogr. B* **681,** 87–97.
16. Ito, Y. Tanaka, T. Ohmachi, T., and Asada, Y. (1996), *Biosci. Biotechnol. Biochem.* **60,** 1239–1242.
17. Shih, I. L. and Yu, Y. T. (2005), *Biotechnol. Lett.* **27,** 103–106.
18. Nagai, T., Phan Tran, L. S., Inatsu, Y., and Itoh, Y. (2000), *J. Bacteriol.* **182,** 2387–2392.
19. Kubota, H. (1993), *Biosci. Biotechnol. Biochem.* **57(7),** 1212–1213.