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High yield of poly-γ-glutamic acid from *Bacillus subtilis* by solid-state fermentation using swine manure as the basis of a solid substrate

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Abstract

Solid-state fermentations (SSF), using swine manure as the basis of a solid substrate, were carried out for high yield of poly-γ-glutamic acid (γ-PGA) by *Bacillus subtilis* CCTCC202048. Fermentation medium and process parameters were optimized through three orthogonal array designs. The optimal medium consisted of 62.3% (w/w, dry weight basis) swine manure, 25.0% soybean cake, 5.0% wheat bran, 5.0% glutamic acid, 2.5% citric acid and 0.2% $MnSO_4 \cdot H_2O$. The optimal process parameters were 15.0 g medium with initial moisture content 60% and initial pH 9.0 in 250 ml flask, inoculation at mid-log phase with a 4% inoculum level and cultivation for 48 h at 37 °C. The average-PGA yield (6.0%) in triplicate under optimal conditions was obtained on the laboratory scale while it was 4.5% at compost experiment. These would lay a foundation for lessening the pollution of swine manure, increasing fertilizer efficiency and exploring a late-model organic fertilizer that retains water and nutrients.

Keywords: *Bacillus subtilis*; Poly-γ-glutamic acid; Swine manure; Solid-state fermentation; Compost

1. Introduction

Pig husbandry is one of the most flourishing industries in livestock husbandry at all times. With the rapid development of intensified pig breeding, more and more swine manure has caused serious environmental problems such as eutrophication of ground water and atmosphere pollution, which have raised social serious concerns (Fan et al., 2000; Hsu and Lo, 2001; Liao et al., 1997; Westerman et al., 2000). Polyamino acids are used as environmental-friendly fertilizer synergists to increase nutrient utilization and to enhance the yield and quality of crops (Kinnersley et al., 1994; Koskan et al., 1998). For example, thermal polyaspartate (TPA) is an active ingredient of AmiSorb used as a chemical fertilizer to increase nutrient utilization (Evers, 1999). Poly-γ-glutamic acid (γ-PGA) is synthesized by a number of species of the genus *Bacillus*. It is an unusual anionic homo-polyamide that is made of D- and L-glutamic acid units connected by amide linkages between α-amine and γ-carboxylic acid groups (Kunioka and Choi, 1998; Oppermann et al., 1998; Shih et al., 2001). The construction of γ-PGA is similar to that of TPA. γ-PGA is water-soluble, water-absorptive, biodegradable, metal-binding, edible and non-toxic toward humans and

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environment (Ashiuchi and Misono, 2002; Ashiuchi et al., 2003; Chen et al., 2003; Mclean et al., 1990; Shih et al., 2001). These attractive properties make it of interest for potential application as an environmental-friendly fertilizer synergist like TPA and other polyamino acids (Evers, 1999; Kinnersley et al., 1994; Koskan et al., 1998). Recently, Hoppensack et al. (2003) and Potter et al. (2001) have shown that *Bacillus licheniformis* was able to grow in liquid swine manure in the presence of sodium gluconate or citrate and glycerlol. Their experiments indicated that the ammonia content was severely reduced and resulted in production of 0.16–0.85 g/l γ-PGA.

In this study, swine manure was used as the basis of solid substrate for the high yield of γ-PGA under solid-state fermentation (SSF) by *Bacillus subtilis* CCTCC202048. SSF medium and the process parameters were optimized by three orthogonal array designs.

2. Methods

2.1. Microorganism

Bacillus subtilis CCTCC202048, isolated from local soil samples by our laboratory (Chen et al., 2003), was maintained on agar slants with the following broth medium (g/l): beef extract 10, peptone 5, sodium chloride 10, agar 18 and pH 7.2–7.4.

2.2. Swine manure

Swine manure was obtained from the hogpen of *Huazhong Agricultural University*. It contains 64% moisture. Dry swine manure contains 42% total carbohydrate, 3.6% total nitrogen, 20% ash, 6.5% phosphorus, 1.5% potassium, 5.3% calcium and 1.8% magnesium.

2.3. Fermentation medium

Swine manure, supplemented with different materials purchased from a local market, was used as the solid substrate (Tables 1 and 4). Initial pH and moisture content of the substrates were set at 7.0 and 65%, respectively. Twenty grams of substrate in 250 ml conical flasks were mixed thoroughly to achieve uniformity and then autoclaved at 121 °C for 20 min.

Table 1
Factors and their levels of orthogonal experiment $L_{27}(3^{13})$

Constituent	Symbol	Concentration (%)		
		Level 1	Level 2	Level 3
Soybean cake	A	5.0[a]	10.0	15.0
Wheat bran	B	5.0	10.0	15.0
Glutamic acid	C	1.0	2.0	3.0
Citric acid	D	1.0	2.0	3.0
$MnSO_4 \cdot H_2O$	E	0	0.2	0.6

[a] The data were the percentage of different component of medium (dry weight) to medium (dry weight); the percentage of swine manure (%) = 100(%) – Σ the percentage of the rest component in medium (%).

2.4. Solid-state fermentation

Solid-state fermentation was carried out, unless otherwise stated, by the following paragraphs. A loopful of cells from the slant were transferred into 250 ml conical flasks containing 30 ml broth medium and incubated to mid-log phase (OD_{600} = 1.4) for 4–5 h on a rotary shaker operating at 180 rpm at 37 °C. Twenty grams of substrate in 250 ml conical flasks (prepared as described above) were inoculated with a 5% inoculum level, mixed carefully under strictly aseptic conditions with sterile glass rods, sealed with eight layers of gauze and then incubated in a chamber with relative humidity above 80% at 37 °C for 48 h in a static mode.

2.5. Orthogonal array design

Fermentation media were optimized through two orthogonal array design tables of $L_{27}(3^{13})$ and $L_8(2^7)$ shown in Table 2 and Table 5 (Lee et al., 1997), respectively. Process parameters were optimized by the orthogonal experiment $L_{18}(3^7)$ (Table 6).

2.6. Compost experiment

Based on optimal medium, 17,300 g swine manure, supplemented with 2500 g soybean cake and 500 g wheat bran, 3800 ml acidified nutrient solution containing 500 g glutamic acid, 250 g citric acid and 20 g $MnSO_4 \cdot H_2O$, was mixed thoroughly to achieve uniformity. The unautoclaved substrates were inoculated with a 4% inoculum level, mixed thoroughly, stacked 10 cm deep and then incubated for 72 h in the open air. Fermented matter was turned over at 12 h interval. In the fermentation period, the ranges of atmospheric temperature and humidity were 20–35 °C and 50–85%, respectively.

2.7. Extraction of γ-PGA

When fermentation was terminated, fermented matter was entirely transferred to 500 ml conical flasks, 10 volumes of distilled water was added (w/v, based on initial dry weight of the substrate) and the mixture was mixed at room temperature (20 ± 2 °C) on a rotary shaker (150 rpm) for 1 h. The whole contents were filtered through muslin cloth. After filtering twice using the same approach, the filtrates were pooled and the total volume was recorded. A 50.0 ml of filtrate was sampled for quantitative determination of ammonia. A 10.0 ml

Table 2
Experimental design and results of the orthogonal experiment $L_{27}(3^{13})$

	A[a]	B	A × B		C	A × C		D	A × D		E	e	e	γ-PGA[b] (%, w/w)
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	3.22 ± 0.03[c]
2	1	1	1	1	2	2	2	2	2	2	2	2	2	3.68 ± 0.04
3	1	1	1	1	3	3	3	3	3	3	3	3	3	3.80 ± 0.03
4	1	2	2	2	1	1	1	2	2	2	3	3	3	3.68 ± 0.04
5	1	2	2	2	2	2	2	3	3	3	1	1	1	3.60 ± 0.02
6	1	2	2	2	3	3	3	1	1	1	2	2	2	3.80 ± 0.04
7	1	3	3	3	1	1	1	3	3	3	2	2	2	3.60 ± 0.05
8	1	3	3	3	2	2	2	1	1	1	3	3	3	3.40 ± 0.04
9	1	3	3	3	3	3	3	2	2	2	1	1	1	3.88 ± 0.04
10	2	1	2	3	1	2	3	1	2	3	1	2	3	3.20 ± 0.04
11	2	1	2	3	2	3	1	2	3	1	2	3	1	3.90 ± 0.04
12	2	1	2	3	3	1	2	3	1	2	3	1	2	3.86 ± 0.02
13	2	2	3	1	1	2	3	2	3	1	3	1	2	3.78 ± 0.03
14	2	2	3	1	2	3	1	3	1	2	1	2	3	3.90 ± 0.04
15	2	2	3	1	3	1	2	1	2	3	2	3	1	3.86 ± 0.05
16	2	3	1	2	1	2	3	3	1	2	2	3	1	3.80 ± 0.05
17	2	3	1	2	2	3	1	1	2	3	3	1	2	3.60 ± 0.02
18	2	3	1	2	3	1	2	2	3	1	1	2	3	3.90 ± 0.03
19	3	1	3	2	1	3	2	1	3	2	1	3	2	3.93 ± 0.05
20	3	1	3	2	2	1	3	2	1	3	2	1	3	4.16 ± 0.04
21	3	1	3	2	3	2	1	3	2	1	3	2	1	4.23 ± 0.04
22	3	2	1	3	1	3	2	2	1	3	3	2	1	3.90 ± 0.04
23	3	2	1	3	2	1	3	3	2	1	1	3	2	4.04 ± 0.03
24	3	2	1	3	3	2	1	1	3	2	2	1	3	4.18 ± 0.02
25	3	3	2	1	1	3	2	3	2	1	2	1	3	3.96 ± 0.04
26	3	3	2	1	2	1	3	1	3	2	3	2	1	4.12 ± 0.05
27	3	3	2	1	3	2	1	2	1	3	1	3	2	4.20 ± 0.03
K_1[d]	32.66 ± 0.37	33.98 ± 0.32	34.12 ± 0.30	34.52 ± 0.32	33.07 ± 0.35	34.44 ± 0.37	34.51 ± 0.34	33.31 ± 0.32	34.24 ± 0.33	34.23 ± 0.32	33.87 ± 0.29			
K_2	33.80 ± 0.32	34.74 ± 0.32	34.32 ± 0.33	34.70 ± 0.35	34.40 ± 0.33	34.07 ± 0.29	34.09 ± 0.32	35.08 ± 0.35	34.13 ± 0.39	35.03 ± 0.38	34.94 ± 0.37			
K_3	36.72 ± 0.32	34.46 ± 0.37	34.74 ± 0.38	33.96 ± 0.34	35.71 ± 0.33	34.67 ± 0.35	34.58 ± 0.35	34.79 ± 0.34	34.81 ± 0.29	33.92 ± 0.31	34.37 ± 0.35			

[a] The symbols were the same as those in Table 1; e was the error effect.
[b] The γ-PGA yields (%, w/w) were the percentage of γ-PGA amount to medium amount (dry weight).
[c] Values were mean of three determinations with standard deviation (±).
[d] $K_i^A = \Sigma$ γ-PGA yield at A_i. Values were mean of three determinations with standard deviation (±).

Table 3
The interaction between soybean cake (A) and citric acid (D)

Soybean cake	Citric acid		
	D_1	D_2	D_3
A_1[a]	10.42 ± 0.11[b]	11.24 ± 0.12	11.00 ± 0.10
A_2	10.66 ± 0.11	11.58 ± 0.10	11.56 ± 0.11
A_3	12.23 ± 0.12	12.26 ± 0.11	12.23 ± 0.11

[a] The symbols were the same as those in Table 1.
[b] Value = $\Sigma(A_i, D_j)$ ($i = 1$ and $j = 1$) as shown in Table 2. Values were mean of three determinations with standard deviation (±).

Table 4
Factors and their levels of the orthogonal experiment $L_8(2^7)$

Constituent[a]	Symbol	Concentration (%)	
		Level 1	Level 2
Soybean cake	A[b]	20.0[c]	25.0
Glutamic acid	C	4.0	5.0
Citric acid	D	2.0	2.5
$MnSO_4 \cdot H_2O$	E	0.2	0.4

[a] Wheat bran was an invariable factor and its concentration was kept at 5%.
[b] The symbols were the same as those in Table 1.
[c] The data were the percentage of different component of medium (dry weight) to medium (dry weight); the percentage of swine manure (%) = 100(%) − Σ the percentage of the rest component in medium (%).

of filtrate was centrifuged for 20 min at 12,000 rpm. The supernatant containing γ-PGA was poured into four volumes of cold ethanol to precipitate the γ-PGA. The resultant precipitate containing crude γ-PGA was collected by centrifugation at 12,000 rpm for 20 min and redissolved in deionized water at equimultiple volume. Any insoluble contaminants were removed again by centrifugation for 20 min at 12,000 rpm. The concentration of aqueous γ-PGA solution was determined by gel permeation chromatography.

2.8. Quantitative analysis of γ-PGA

Quantitative analysis of γ-PGA was carried out by high performance liquid chromatography (Waters, the United States) using TSK Gel G6000 PW_{XL} gel permeation chromatogram column (7.8 mm × 300 mm, Tosoh, Tokyo, Japan) equipped with an UV detector (Waters 2487). Samples were eluted with a mixture of 25 mM sodium sulfate solution:acetonitrile (8:1) at a flow rate of 0.5 ml/min and detected at 220 nm. The purified γ-PGA was used as a standard.

2.9. Plate count of bacteria

Fermented matter was adequately mixed with 10 volumes of sterilized water (w/v, based on initial dry weight of the substrate). The mixture was aseptically diluted to suitable concentration by decuple dilution. A 0.1 ml of diluted mixture was spread on broth agar plates in quintuplicate. The average colony was counted after incubating for 18 h at 37 °C.

2.10. Quantitative determination of ammonia

Culture filtrates, prepared as described above, were diluted if necessary to a suitable ammonia concentration. The ammonia content was determined with a pNH_3-1 type gas-sensitive electrode (Shanghai Precision & Scientific Instrument Co. Ltd., China; detection limit: 10^{-6} mol/l) according to the instructions provided by the manufacturer.

3. Results and discussion

3.1. Selection of medium components

Carbon compounds are the sources of carbon skeleton and energy of microorganism cells, while nitrogen sources provide N element with protoplasm and other structure of cells. Dry swine manure tested contains 42% total carbohydrate and 3.6% total nitrogen. The carbon:nitrogen ratio is 11.7:1. Based on the characteristic of the strain and the component of conventional medium E used in the literature, some substrates (e.g., glucose, lactose, sucrose, glycerol, glutamic acid and citric acid) were selected as the supplement of swine manure, respectively. After initial pH of the substrate was set at 7.0, the yield of γ-PGA was still much low and the cell number was small if a single supplement was mixed with swine manure. However, both the γ-PGA yield and the number of viable cells increased if swine manure was combined with a mixture of glutamic acid and citric acid. In addition, seven substrates, viz. wheat bran, rice bran, corn flour, peptone, soybean cake, peanut cake and rapeseed cake were used to prepare seven sets of substrates combined with swine manure, respectively. Among seven different substrates tested, wheat bran and soybean cake were found to be more compatible supplement with swine manure, supporting the strain growth and γ-PGA formation. Accordingly, wheat bran, soybean cake, citric acid and glutamic acid were chosen as the supplemented component of swine manure in further fermentation experiments for high yield of γ-PGA. Both stereochemical compositions and biosynthesis of γ-PGA were significantly affected by Mn^{2+} or Mg^{2+} concentration. Mn^{2+} or Mg^{2+} was considered as the cofactor of γ-PGA synthetase (Ashiuchi et al., 1999; Perez-Camero et al., 1999). Dry swine manure tested contains 1.5% potassium, 5.3% calcium and 1.8% magnesium, so $MnSO_4 \cdot H_2O$ was also selected as the medium component.

3.2. Optimization of SSF medium

3.2.1. Preliminary optimization of SSF medium

An $L_{27}(3^{13})$ orthogonal array design with five factors and three interactions between different factors, each with three different levels, was used to optimize the medium composition (Table 1). The yield of γ-PGA, determined for each experimental design, was shown in Table 2. According to the orthogonal method, the analysis of variance for the experimental designs was calculated, and the significant levels of each medium variable and their interactions were determined by Fisher's *F*-test. The *F*-test's table value of $F_{(2,4)}$ is 18.00 (at 1% level), 6.94 (at 5% level) and 4.32 (at 10% level); and the *F*-test's table value of $F_{(4,4)}$ for interaction is 16.00 (at 1% level), 6.39 (at 5% level) and 4.11 (at 10% level). Among five variable factors, soybean cake (A), glutamic acid (C) and citric acid (D) were significant with a probability of 99% as the calculated value (121.8 for A, 48.40 for C and 25.03 for D) exceeded the table value of $F_{(2,4)}$ at 1% level; $MnSO_4 \cdot H_2O$ (E) was also significant with a probability of 95% as the calculated value 7.98 exceeded the table value of $F_{(2,4)}$ at 5% level; wheat bran (B), however, had no significant influence on γ-PGA yield as the calculated value 4.10 was less than the table value of $F_{(2,4)}$ at 10% level. The interaction between soybean cake (A) and citric acid (D) had a significant effect on γ-PGA yield as the calculated value 6.40 exceeded the table value of $F_{(4,4)}$ at 5% level, but soybean cake (A) and wheat bran (B), soybean cake (A) and glutamic acid (C) had no significant interactions as the calculated values (3.45 for AB and 2.25 for AC) were less than the table value of $F_{(4,4)}$ at 10% level. The analysis of interaction between soybean cake (A) and citric acid (D) suggested that combination of A_3D_2 was the best as shown in Table 3. Soybean cake is an excellent nitrogen source, while citric acid plays an important role in biosynthesis of γ-PGA (Cromwick and Gross, 1995). The tiny differences among A_3D_1, A_3D_2 and A_3D_3 also suggested that the suitable carbon-to-nitrogen ratio was beneficial to high yield of γ-PGA by *Bacillus subtilis* CCTCC202048.

3.2.2. Further optimization of SSF medium

Based on the magnitude of K (Table 2), among four significant factors above, soybean cake (A) and glutamic acid (C) had a positive effect on yield of γ-PGA and their initial concentrations were increased above high levels in further optimization experiments. Citric acid (D) and $MnSO_4 \cdot H_2O$ (E) had slightly positive effect and their concentrations were modified between middle levels and high levels. In contrast, wheat bran (B) had no significance on γ-PGA yield, and its concentration was kept at low level as an invariable factor. Therefore, these four factors were selected as supplemented component of swine manure for further optimization and their levels were modified through orthogonal experiment design $L_8(2^7)$ (Table 4). The final experiment results were shown in Table 5. The data were also analyzed for statistical significance as described above. The calculated values of $F_{(1,1)}$ about soybean cake (A), glutamic acid (C), Citric acid (D), $MnSO_4 \cdot H_2O$ (E), the interaction DA and DC were 20.25, 56.25, 30.25, 2.25, 25.00 and 16.00, respectively; while the *F*-test's table value of $F_{(1,1)}$ is 161.0 (at 5% level) and 39.9 (at 10% level). Therefore, only glutamic acid (C) was significant on the yield of γ-PGA to some extent. All the other variables and interactions were insignificant. Based on the magnitude of K (Table 5), the optimized combination was $A_2C_2D_2E_1$, viz. 62.3% (w/w, dry weight basis) swine manure, 25% soybean cake, 5% wheat bran (as an invariable factor in this orthogonal experiment), 5%

Table 5
Experimental design and results of the orthogonal experiment $L_8(2^7)$

Run	D	A[a]	D × A	C	D × C	e	E	γ-PGA[b] (%, w/w)
	1	2	3	4	5	6	7	
1	1	1	1	1	1	1	1	4.74 ± 0.10[c]
2	1	1	1	2	2	2	2	4.96 ± 0.08
3	1	2	2	1	1	2	2	4.92 ± 0.08
4	1	2	2	2	2	1	1	5.16 ± 0.12
5	2	1	2	1	2	1	2	5.00 ± 0.10
6	2	1	2	2	1	2	1	5.12 ± 0.10
7	2	2	1	1	2	2	1	5.04 ± 0.08
8	2	2	1	2	1	1	2	5.06 ± 0.06
K_1[d]	19.78 ± 0.38	19.82 ± 0.38	19.80 ± 0.32	19.70 ± 0.36	19.84 ± 0.34	19.96 ± 0.38	20.06 ± 0.40	
K_2	20.22 ± 0.34	20.18 ± 0.34	20.20 ± 0.40	20.30 ± 0.36	20.16 ± 0.38	20.04 ± 0.34	19.94 ± 0.32	
Optimal level	2	2		2			1	

[a] The symbols were the same as those in Table 1.
[b] The γ-PGA yields (%, w/w) were the percentage of γ-PGA amount to medium amount (dry weight).
[c] Values were mean of three determinations with standard deviation (±).
[d] $K_i^A = \Sigma$ γ-PGA yield at A_i. Values were mean of three determinations with standard deviation (±).

Table 6
Experimental design and results of the orthogonal experiment $L_{18}(3^7)$

Run	A Amount of substrate (g)	B Moisture content (%)	C Inoculation time (h)	D Inoculum level (%)	E pH	F Cultivation temperature (°C)	G Cultivation time (h)	γ-PGA[a] (%, w/w)
1	15.0	60	3.0	4	7.0	30	36	5.40 ± 0.10[b]
2	15.0	65	4.5	6	8.0	34	48	4.80 ± 0.08
3	15.0	70	6.0	8	9.0	37	60	3.86 ± 0.06
4	25.0	60	3.0	6	8.0	37	60	4.58 ± 0.02
5	25.0	65	4.5	8	9.0	30	36	4.48 ± 0.06
6	25.0	70	6.0	4	7.0	34	48	4.20 ± 0.04
7	35.0	60	4.5	4	9.0	34	60	5.10 ± 0.08
8	35.0	65	6.0	6	7.0	37	36	4.10 ± 0.10
9	35.0	70	3.0	8	8.0	30	48	4.18 ± 0.04
10	15.0	60	6.0	8	8.0	34	36	5.79 ± 0.06
11	15.0	65	3.0	4	9.0	37	48	5.70 ± 0.08
12	15.0	70	4.5	6	7.0	30	60	4.60 ± 0.05
13	25.0	60	4.5	8	7.0	37	48	5.78 ± 0.06
14	25.0	65	6.0	4	8.0	30	60	4.26 ± 0.03
15	25.0	70	3.0	6	9.0	34	36	4.86 ± 0.08
16	35.0	60	6.0	6	9.0	30	48	5.42 ± 0.10
17	35.0	65	3.0	8	7.0	34	60	4.26 ± 0.04
18	35.0	70	4.5	4	8.0	37	36	5.04 ± 0.05
K_1[c]	30.15 ± 0.43	32.07 ± 0.42	28.98 ± 0.36	29.70 ± 0.38	28.34 ± 0.39	28.34 ± 0.38	29.67 ± 0.45	
K_2	28.16 ± 0.29	27.60 ± 0.39	29.80 ± 0.38	28.36 ± 0.43	28.65 ± 0.28	29.01 ± 0.38	30.08 ± 0.40	
K_3	28.10 ± 0.41	26.74 ± 0.32	27.63 ± 0.39	28.35 ± 0.32	29.42 ± 0.46	29.06 ± 0.37	26.66 ± 0.28	
R[d]	2.05 ± 0.84	5.33 ± 0.74	2.17 ± 0.77	1.35 ± 0.80	1.08 ± 0.85	0.72 ± 0.75	3.42 ± 0.68	
Optimal level	1	1	2	1	3	3	2	

[a] The γ-PGA yields (%, w/w) were the percentage of γ-PGA amount to medium amount (dry weight).
[b] Values were mean of three determinations with standard deviation (±).
[c] $K_i^A = \Sigma$ γ-PGA yield at A_i. Values were mean of three determinations with standard deviation (±).
[d] $R^A = \max\{K_i^A\} - \min\{K_i^A\}$. Values were mean of three determinations with standard deviation (±).

glutamic acid, 2.5% citric acid and 0.2% $MnSO_4 \cdot H_2O$. The mean yield of three parallel experiments under optimal conditions was 5.20%.

3.3. Optimization of process parameters

There were many factors affecting γ-PGA yield by one-factor-at-a-time method, such as amount of substrate, initial moisture content, inoculation time, initial pH, cultivation temperature, inoculum level and cultivation time. According to orthogonal experiment design $L_{18}(3^7)$, a total of seven factors above, each with three different levels (Table 6), were selected in the study. The final experiment results and the effects of those factors on γ-PGA production were also shown in Table 6. Based on the magnitude order of R (Table 6), the effect of the factors on the yield of γ-PGA decreased in the following order: initial moisture content (B), cultivation time (G), inoculation time (C), amount of substrate (A), inoculum level (D), initial pH (E) and cultivation temperature (F). Based on the magnitude order of K (Table 6), the optimal combination was $A_1B_1C_2D_1E_3F_3G_2$, namely 15 g medium in 250 ml flask, initial moisture content of solid substrate 60%, inoculation time of 4.5 h (mid-log phase), initial pH of the medium 9.0, cultivation temperature at 37 °C, inoculum level of 4.0% and cultivation time of 48 h. The mean yield of three parallel experiments under optimum fermentation medium and optimum process parameters was 6.0%. The γ-PGA yield increased about 15% against process parameters before optimization.

3.4. Time course of fermentation

The time courses of γ-PGA production, the cell growth and the changes of ammonia content under optimal medium and process parameters were monitored for 72 h as shown in Fig. 1. The γ-PGA production and the number of viable cells were increased rapidly after 6 h. The number of viable cells reached the maximum of 10.8×10^{10} colony-forming units (cfu)/g at 42 h and decreased slowly thereafter. The production of γ-PGA reached the maximum (6.0%, w/w) at 48 h and then decreased gradually. The results suggested that γ-PGA produced by *Bacillus subtilis* CCTCC202048 was associated partially with cell growth. As shown in Fig. 1, the ammonia content declined slowly at the lag growth phase and decreased sharply at the exponential growth

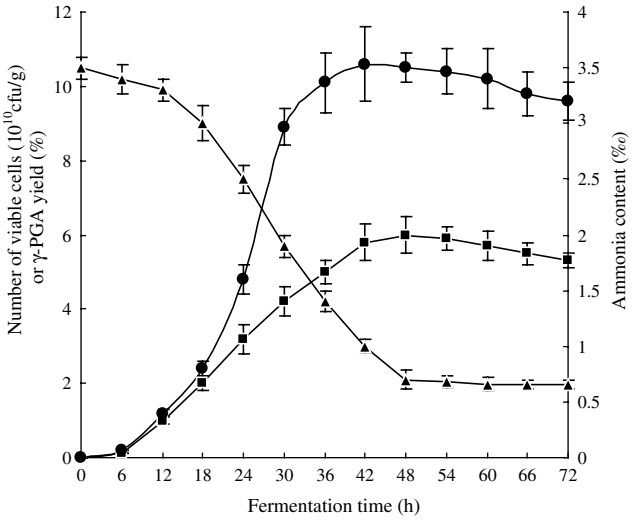

Fig. 1. Typical time courses of γ-PGA yield (■), ammonia content (▲) and number of viable cells (●) by *Bacillus subtilis* CCTCC202048 in optical condition. Error bars were given showing standard deviations for $n = 3$.

phase from 3.3‰ to 0.7‰, and then remained approximately constant until the fermentation was terminated. It seemed probable that ammonia was converted into γ-PGA and biomass.

3.5. Compost experiment

Compost experiment on the scale of 24 kg was conducted applying the knowledge gained from the above optimal experiments. The yield of γ-PGA reached 4.5% after 72 h compost fermentation. It indicated that *Bacillus subtilis* CCTCC202048 had viability and high productivity of γ-PGA in the open air. The strain, isolated from soil samples, belongs to *Bacillus subtilis*, so it is harmless to environment. γ-PGA is normally produced by submerged fermentation (SmF). In the course of SmF, γ-PGA fermentation broth has a highly viscous and exhibits non-Newtonian rheology (Richard and Margaritis, 2003). The rheological behavior of the fermentation broth causes serious problems of mixing, heat transfer and oxygen supply, thus limiting the maximum γ-PGA concentration achievable and the product quality as well as increasing manufacturing costs. By utilizing swine manure and agro-industrial materials as media, SSF requires lower manufacturing costs, while achieves higher product concentrations with simpler process and less pre-processing energy than SmF. These make this technique promising for decontamination of livestock and fowls feces, improvement fertilizer efficiency and amelioration the ability of organic fertilizer to retain water and nutrients.

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