The production of poly-γ-glutamic acid from monosodium glutamate waste (ami-ami) by *Bacillus licheniformis* ATCC 9945

Methawee Chintrakool[1], Nuttawut Kongklom[1,2] and Sarote Sirisansaneeyakul[1,2,*]

[1]*Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, 50 Ngam Wong Wan Road, Ladyaow, Chatuchak, Bangkok 10900, Thailand*
[2]*Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Kasetsart University, Chatuchak, Bangkok 10900, Thailand (CASTNAR, NRU-KU, Thailand)*
**e-mail: sarote.s@ku.ac.th*

Abstract

Poly-γ-glutamic acid (γ-PGA) is one type of amino acid polymers. γ-PGA has biodegradable potential, ability to consume and dissolve in water without toxicity which may affect the environment. It has multifarious potential applications in foods, pharmaceuticals, healthcare, water treatment and other fields. γ-PGA has been synthesized by bacterial fermentation process. However, high-cost medium is bottleneck for γ-PGA production. Therefore, this study focused on solving the high-cost medium with using ami-ami (monosodium glutamate/ MSG waste, ami-ami) instead of glucose or commercial carbon sources. The ami-ami was used as the low-cost carbon and nitrogen sources for γ-PGA production by *B. licheniformis* ATCC 9945. The results showed that an initial glutamate concentration of 40 g L^{-1} was optimal for γ-PGA production, and the γ-PGA concentration of 1.84 ± 0.10 g L^{-1} with the γ-PGA productivity of 0.041 ± 0.11 g L^{-1} h^{-1} were obtained. In addition, an initial glutamate concentration at 60 g L^{-1} showed the highest substrate inhibition (the lowest K_i) vigorously affected on cell growth and γ-PGA production. These results suggested that the low-cost ami-ami can be used for the environmental-friendly and economical production of γ-PGA by *B. licheniformis* ATCC 9945.

Keywords: poly-γ-glutamic acid, γ-PGA, glutamate, monosodium glutamate waste, ami-ami, *Bacillus licheniformis* ATCC 9945

Introduction

Poly-γ-glutamic acid or γ-PGA is an unusual anionic, naturally occurring homo-polyamide that is made of D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups (Shih and Van 2001). It has properties of water-soluble, biodegradable, edible and nontoxic toward humans and the environment. Therefore, γ-PGA is interesting in a broad range of industrial fields such as food, cosmetics, medicine and water treatment (Shih et al. 2005). γ-PGA can be synthesized by *Bacillus* strains via fermentation process. γ-PGA production by *Bacillus* strain depends greatly on the composition of the medium which can be divided into two groups; glutamic acid dependent and independent bacteria. From the previous reports, the glutamic acid dependent bacteria have potential for γ-PGA production higher than those without glutamic acid (Kunioka 1997; Feng et al. 2007; Buescher and Margaritis 2007; Bajaj and Singhal 2011). For glutamic acid dependent bacteria, L-glutamic acid plays an important role in the γ-PGA producing strains. Whereas, it shared up to 50% of raw material costs and is not economical for commercial scale production (Zhang et al. 2012a). Ami-ami (monosodium glutamate waste) is an alternative raw material as low-cost medium for γ-PGA producing strains. Moreover, the reducing sugar, ammonium salt and glutamate from ami-ami are also expected to be suitable low-cost material for γ-PGA production. In this study, the

economical production of γ-PGA by *B. licheniformis* ATCC 9945 with ami-ami was investigated to reduce the medium cost from the γ-PGA fermentation process. This finding is useful for an industrial scale production of γ-PGA.

Methodology

Microorganism

Bacillus licheniformis ATCC 9945 ordered from ATCC, United States of America (University Boulevard; Manassas, Virginia) was cultured and stored at Thailand Institute of Scientific and Technological Research (TISTR).

Medium and cultivation conditions

Ami-ami (pH 2.5), the monosodium glutamate waste was obtained from Ajinomoto Co. Ltd., Samutprakarn, Thailand. It consisted of 0.27 kg L^{-1} reducing sugar, 0.135 kg L^{-1} ammonium salt and 1.19 kg L^{-1} glutamate. Glutamate from the ami-ami was desired as the main carbon source for γ-PGA production. Ami-ami was filtered by filter paper and sterilized at 121°C for 15 min. The cooled solution was used as the culture medium.

B. licheniformis ATCC 9945, the γ-PGA producer, was cultured on NA slant and then inoculated into 250-mL flask which contained 10 mL of ami-ami (pH 6.5). The cultures were performed at 37 °C in shaking condition at 250 rpm for 24 h. The cell suspension (10 mL) was transferred to 90 mL of ami-ami (pH 6.5) in a 500-mL flask, then incubated at the similar temperature and shaking condition for 48 h. The ami-ami solution was diluted with distilled water to obtain the initial glutamate concentration of 20, 40 and 60 g L^{-1}. The final concentration of reducing sugar and ammonium salt were also adjusted to 14 g L^{-1} and 5 g L^{-1} by using glucose and ammonium chloride, respectively, based on those concentrations found with 60 g L^{-1} glutamate.

Analytical methods

Two milliliters of fermentation broth was accurately taken at an interval sampling time. The samples were then centrifuged (12,000 ×*g*, 30 min). The supernatants were collected and properly stored for the measurements of reducing sugar, NH_4Cl, glutamate and γ-PGA. The cell pellets were washed twice with distilled water, and dried at 105°C to a constant weight to determine the dry cell weight (DCW). Reducing sugar was measured by the dinitrosalicylic acid method (Miller 1959). Glutamate was measured by spectrophotometer (Paraskevas et al. 2002; Park et al. 2005). The ammonium salt concentration was measured by phenol-hypochlorite method (Weatherburn 1967). The γ-PGA concentration was measured by using cetylmethylammonium bromide (CTAB) (Zhang et al. 2012b). In this method, CTAB binds very specifically to γ-PGA, and form a water-insoluble, highly dispersed micelle-like complex, resulting in an increase of turbidity (Ashiuchi 2011). The γ-PGA was precipitated from the supernatants with 3-fold volumes of cold anhydrous ethanol at 4°C overnight. The precipitated γ-PGA was collected by centrifuged at 12,000 ×*g* and 4°C for 20 min and dissolved in distill water. The γ-PGA solution (2 mL) was mixed with CTAB (2 mL) and incubated at room temperature for 3 min. The γ-PGA concentration was measured with the turbidity by spectrophotometer at 400 nm. Data were expressed as mean values ± standard deviation (SD). Duncan's one-way ANOVA was carried out using the IBM SPSS Statistics 21.0 software for Windows (IBM corp., USA). Differences were considered statistically significant when the *P* value was less than 0.05. The γ-PGA production was investigated with various initial concentrations of glutamate (20, 40 and 60 g L^{-1}). The kinetic study was mathematically described by the proposed model constructed upon the following assumptions (Shuler and Kargi 1992);

(i) The substrate inhibition resulted from glutamate on the bacterial growth is given as Equation (1), modified from Monod equation.

$$\frac{dX}{dt} = \left[\frac{\mu_{max} \cdot S}{K_S + S + \left(\frac{S^2}{K_i} \right)} \right] X \quad (1)$$

(ii) The uptake of glutamate for growth, cell maintenance and the production of γ-PGA is given as Equation (2).

$$\frac{dS}{dt} = -\left[\left(\frac{1}{Y_{X/S}} \right) \left(\frac{dX}{dt} \right) + m_S X + \left(\frac{1}{Y_{P/S}} \right) \left(\frac{dP}{dt} \right) \right] \quad (2)$$

(iii) Since γ-PGA is generally described as a mixed growth associated product, thus, α and β were represented as the growth-associated and non-growth associated constants, respectively. Therefore, the production kinetics of γ-PGA is given as Equation (3).

$$\frac{dP}{dt} = (\alpha\mu + \beta)X \quad (3)$$

(iv) The consumption ratio of carbon and nitrogen sources for growth and γ-PGA production is given as Equation (4). Where *C* is carbon source concentration (defined as reducing sugar + glutamate), and *N* is nitrogen source concentration (defined as ammonium salt).

$$C/N = \left(\frac{C_2 - C_1}{N_2 - N_1} \right) \quad (4)$$

Estimation of model parameters

To estimate the parameters, the model profiles were fitted to the experimental data using the Berkeley Madonna™ (www.berkeleymadonna.com) software. The set of parameter values that resulted in the best fit between the model and the data were taken as the final values. The value of coefficient of determination (R^2) was used to test the difference between the model-predictions and the observations.

Results

Glutamate concentration affecting on γ-PGA production from ami-ami medium

The feasibility of using ami-ami as a low cost substrate in γ-PGA fermentation by *B. licheniformis* ATCC 9945 with various glutamate concentrations were investigated in shake flasks for 48 h (Figure 1). The results showed that *B. licheniformis* ATCC 9945 could utilize the reducing sugar, ammonium salt and glutamate from ami-ami for growing and producing γ-PGA. The highest biomass ($C_{X,max}$) obtained from the initial 20, 40 and 60 g L^{-1} glutamate was found no significant difference (p>0.05) (data not shown) (Table 1). The highest specific growth rate (μ) of 0.050 ± 0.024 h^{-1} was derived from the initial 20 g L^{-1} glutamate (Table 1). As compared to the initial 60 g L^{-1} glutamate (1.890 ± 0.190 g L^{-1} γ-PGA), the maximal γ-PGA, γ-$(PGA)_m$ obtained from the initial 40 g L^{-1} glutamate (1.840 ± 0.100 g L^{-1} γ-PGA) was not significantly different (p>0.05) (data not shown) (Table 1). Importantly, the highest γ-PGA productivity (Q_P) and the specific rate of γ-PGA production (q_P) derived from the initial glutamate concentration at 40 g L^{-1} were 0.041 ± 0.011 g L^{-1} h^{-1} and 0.030 ± 0.010 g g^{-1} h^{-1}, respectively (Table 1). The results indicated clearly the consumption of carbon sources (reducing sugar and glutamate) and nitrogen source (ammonium salt) by *B. licheniformis* ATCC 9945. With the initial glutamate concentration at 20, 40 and 60 g L^{-1}, the bacteria consumed the carbon sources at 61%, 35% and 32%, respectively, and the nitrogen source at 28%, 28% and 31%, respectively (Table 1). The consumption ratio of carbon and nitrogen sources (*C/N* ratio) demonstrated the performance of the strain consuming substrates,

for example, the higher nitrogen uptaken for enhancing the cell mass defined at the lower *C/N* ratio, especially found at the initial 60 g L^{-1} glutamate (Table 1). When the concentrations of reducing sugar and ammonium salt were markedly lowered during 40-48 h culture time, the production of γ-PGA was retarded (Figure 1). Clearly, both critical concentrations were found at ~3 g L^{-1}, as the decreased γ-PGA was observed (Table 1).

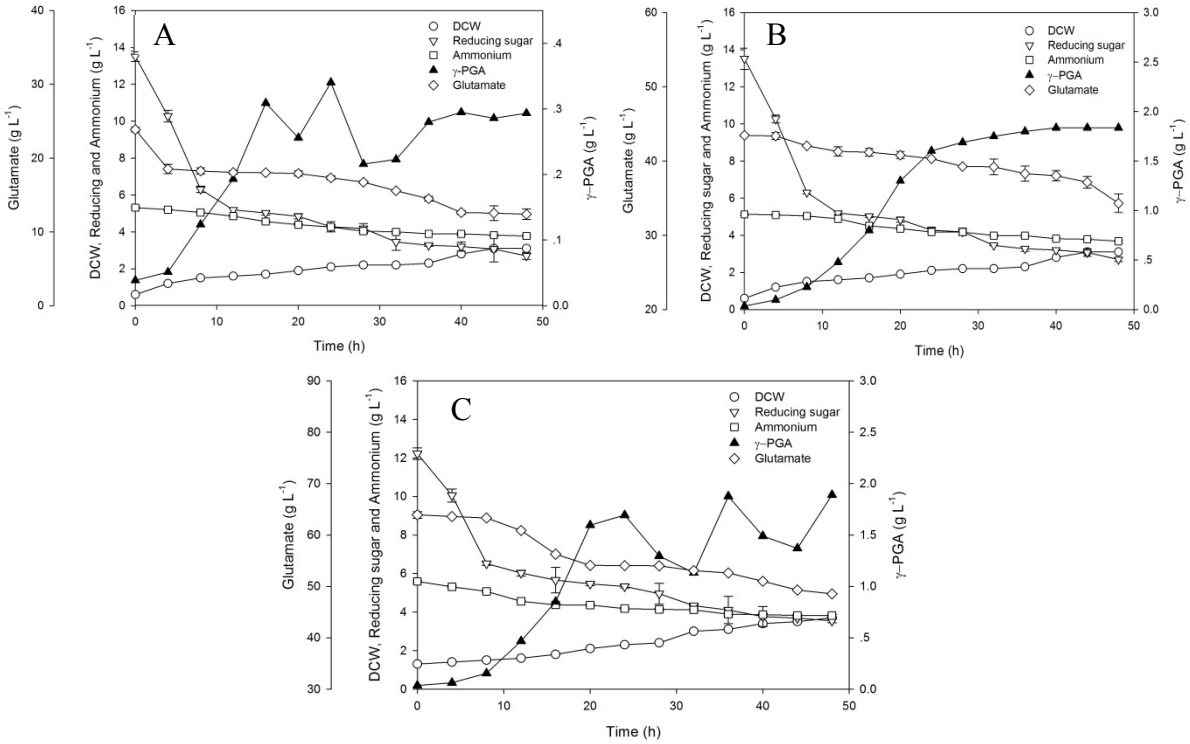

Figure 1 The batch production of γ-PGA in ami-ami medium varying initial glutamate concentrations in shake flasks, (A) 20 g L^{-1}, (B) 40 g L^{-1} and (C) 60 g L^{-1}.

Table 1 The kinetic parameters of γ-PGA production affected by glutamate concentration.

Parameter	Glutamate concentration (g L^{-1})		
	20	40	60
C_{Xmax} (g L^{-1})	3.100 ± 0.150	3.100 ± 0.230	3.500 ± 0.250
μ (h^{-1})	0.050 ± 0.024	0.030 ± 0.016	0.024 ± 0.015
γ-$(PGA)_m$ (g L^{-1})	0.340 ± 0.170	1.840 ± 0.100	1.890 ± 0.190
γ-$(PGA)_f$ (g L^{-1})	0.293 ± 0.100	1.830 ± 0.120	1.870 ± 0.110
Q_P (g L^{-1} h^{-1})	0.013 ± 0.003	0.041 ± 0.011	0.039 ± 0.015
q_P (g g^{-1} h^{-1})	0.010 ± 0.001	0.030 ± 0.010	0.019 ± 0.013
C/N (g g^{-1})	14.743 ± 0.150	13.743 ± 0.120	13.598 ± 0.140
% Consumption			
Carbon	61	35	32
(reducing sugar + glutamate)	(8.7 + 52.3)	(2.6 + 32.4)	(2.2 + 29.8)
Nitrogen	28	28	31

Note: The fermentation kinetic parameters were calculated according to Sirisansaneeyakul et al. (2012). All data are presented as mean ± SD. C_{Xmax}, the maximal biomass; γ-$(PGA)_m$, the maximal γ-PGA; γ-$(PGA)_f$, the final γ-PGA. Carbon sources were reducing sugar and glutamate, nitrogen sources were ammonium chloride and/or ami-ami ammonium salt

Kinetic modeling of the γ-PGA production in shake flask

The γ-PGA production model described with the substrate inhibition from initial 20, 40 and 60 g L^{-1} glutamate were summarized in Table 2. The proposed model showed a good agreement with the observations in the present study, whose determination coefficients (R^2) were found highly acceptable ($\geqslant$ 0.8) (Table 2). Among various initial glutamate concentrations, the substrate inhibition constant (K_i) derived from an initial 20 g L^{-1} glutamate was the highest, which demonstrated the least substrate inhibition to bacterial growth. Whereas the substrate inhibition constant (K_i) derived from an initial 60 g L^{-1} glutamate was the lowest to represent the highest substrate inhibition. Subsequently, its biomass yield from substrate ($Y_{X/S}$) found to be the lowest. The yields of biomass from substrate (reducing sugar plus glutamate) and γ-PGA from glutamate ($Y_{P/S}$) were found highest with an initial glutamate concentration of 20 and 40 g L^{-1}, respectively. The model described fairy good the γ-PGA production (Figure 2), especially that with the initial 40 g L^{-1} glutamate, whereas those with the initial 20 and 60 g L^{-1} glutamate predicted slightly higher than the observations. As a result, the bacterial growth was optimized at 20 g L^{-1} glutamate, while that of γ-PGA production was found reasonably at 40 g L^{-1} glutamate. Furthermore, the production of γ-PGA was expressed definitely as bacterial growth association, which observed remarkably only from the growth-associated constant (α) (Table 2).

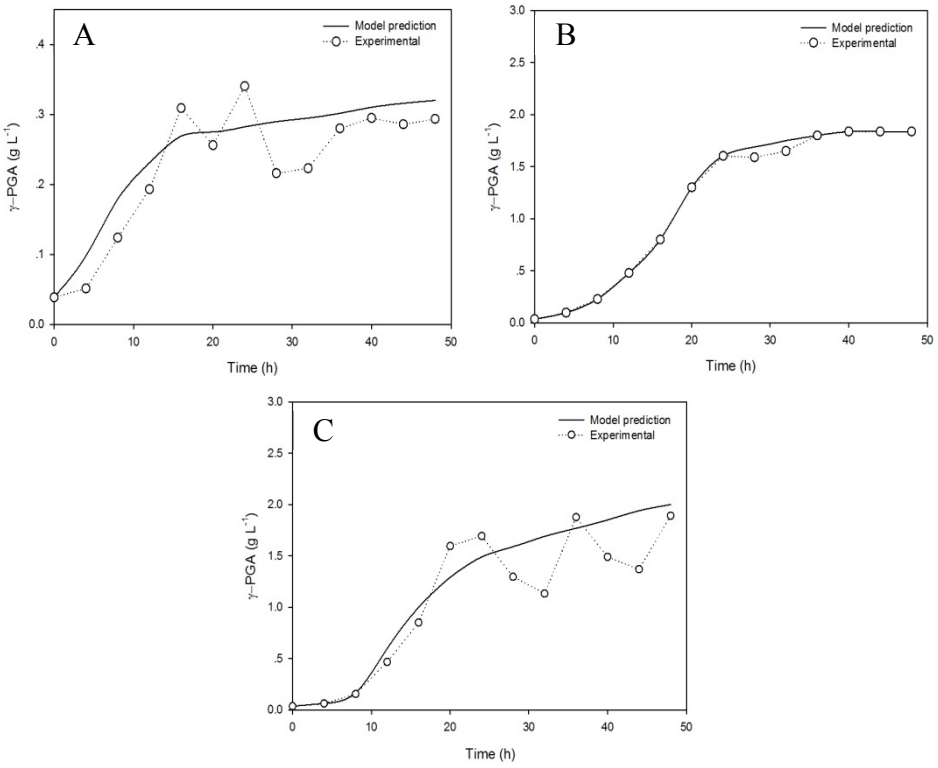

Figure 2 Comparison between observation and prediction for the production of γ-PGA from ami-ami medium varying initial glutamate concentrations in shake flask, (A) 20, (B) 40, and (C) 60 g L^{-1}.

Table 2 Kinetic parameters estimated by fitting the models to experimental data obtained from γ-PGA production with various glutamate concentrations in shake flask culture.

Kinetic model		Parameter	Glutamate concentration (g L^{-1})		
			20	40	60
Cell growth	$\frac{dX}{dt} = \left[\frac{\mu_{max} \cdot S}{K_S + S + \left(\frac{S^2}{K_i}\right)}\right] X$	μ_{max} (h^{-1})	0.074	0.074	0.074
		K_S (g L^{-1})	18.411	18.411	18.411
		K_i (g L^{-1})	1,112	94.164	55.173
		R^2	0.805	0.824	0.919
Glutamate consumption	$\frac{dS}{dt} = -\left[\left(\frac{1}{Y_{X/S}}\right)\left(\frac{dX}{dt}\right) + m_S X + \left(\frac{1}{Y_{P/S}}\right)\left(\frac{dP}{dt}\right)\right]$	m_S (g g^{-1} h^{-1})	0.004	0.004	0.004
		$Y_{X/S}$ (g g^{-1})	0.797	0.513	0.320
		$Y_{P/S}$ (g g^{-1})	0.064	0.942	0.210
		R^2	0.817	0.938	0.813
γ-PGA production	$\frac{dP}{dt} = (\alpha\mu + \beta)X$	α (g g^{-1})	0.863	0.863	0.863
		β (g g^{-1} h^{-1})	0	0	0
		R^2	0.801	0.998	0.868

Discussion

The production of γ-PGA from MSG waste (ami-ami) by *B. licheniformis* ATCC 9945 was potential to be elucidated with the substrate inhibition for the bacterial growth and γ-PGA production. In the present work, an initial glutamate concentration of 40 g L^{-1} was optimal for the γ-PGA production, regarding the highest specific and volumetric rates of γ-PGA production. An initial 60 g L^{-1} glutamate did not improve the γ-PGA production due to its severe substrate inhibition (the lowest K_i, 55.173 g L^{-1}). Moreover, the substrate consumption was lowest found at an initial 60 g L^{-1} glutamate (Table 1). This demonstrated clearly with their lowest kinetic parameters, i.e. μ, $Y_{X/S}$ and $Y_{P/S}$ (Table 2). As compared to an initial 20 g L^{-1} glutamate, an initial 40 g L^{-1} glutamate enhanced the production of γ-PGA (the higher $Y_{P/S}$) favorably rather than the biomass (the lower $Y_{X/S}$) (Table 2). As well as, both the mass and volumetric specific rate of γ-PGA production are superior.

Conclusion

In this study, the feasibility of ami-ami used for the γ-PGA production by *B. licheniformis* ATCC 9945 was demonstrated in a low-cost defined medium. These results suggested that the γ-PGA production from ami-ami medium is obviously potential. The finding is promising to develop a low-cost industrial medium for γ-PGA production from MSG waste, and results in an environmental friendly γ-PGA for various applications.

Acknowledgements

This research was supported by Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, and Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Kasetsart University, Thailand.

References

Ashiuchi M. (2011) Analytical approaches to poly-γ-glutamate: Quantification, molecular size determination, and stereochemistry investigation. J Chromatogr B 879:3096–3101.

Bajaj I., Singhal R. (2011) Poly (glutamic acid)–An emerging biopolymer of commercial interest. Bioresour Technol 102:5551–5561.

Buescher J.M., Margaritis A. (2007) Microbial biosynthesis of poly glutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Crit Rev Biotechnol 27:1–5.

Feng S., Zhinan X., Peilin C. (2007) Microbial production of natural poly amino acid. Sci China Ser B-Chem 50:291–303.

Kunioka M. (1997) Biosynthesis and chemical reactions of poly(amino acid)s from microorganisms. Appl Microbiol Biotechnol 47:469–475.

Miller G.L. (1959) Use of dinitrisalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426 –429.

Paraskevas G., Atta-Politou J., Koupparis M. (2002) Spectrophotometric determination of lisinopril in tablets using 1-fluoro-2,4-dinitrobenzene reagent. J Pharmaceut Biomed 29:865–872.

Park C., Choi J.C., Choi Y.H., Nakamura H., Shimanouchi K., Horiuchi T., Misono H., Sewaki T., Soda K., Ashiuchi M., Sung M.H. (2005) Synthesis of super-high-molecular-weight poly-γ-glutamic acid by *Bacillus subtilis* subsp. *chungkookjang*. J Mol Catal B-Enzym 35:128–133.

Shih I.L., Van Y.T. (2001) The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresour Technol 79:207–225.

Shih I.L., Wu P.J., Shieh C.J. (2005) Microbial production of a poly(γ-glutamic acid) derivative by *Bacillus subtilis*. Process Biochem 40:2827–2832.

Shuler M.L., Kargi F. (1992) Bioprocess engineering: basic concept. New Jersey. Prentice-Hall, Inc.

Sirisansaneeyakul S., Wannawilai S., Chisti Y. (2013) Repeated fed-batch production of xylitol by *Candida magnoliae* TISTR 5663. J Chem Technol Biotechnol 88:1121–1129.

Weatherburn M.W. (1967) Phenol-hypochlorite reaction for determination of ammonia. Anal Chem 39:971–974.

Zhang D., Feng X., Zhou Z., Zhang Y., Xu H. (2012a) Economical production of poly(γ-glutamic acid) using untreated cane molasses and monosodium glutamate waste liquor by *Bacillus subtilis* NX-2. Bioresour Technol 114:583–588.

Zhang Y., Chen G., Zeng W., Wu K., Liang Z. (2012b) Studies on the UV spectrum of poly(γ-glutamic acid) based on development of a simple quantitative method. Int J Biol Macromol 51:83 –90.