Tropical Journal of Pharmaceutical Research June 2017; 16 (6): 1267-1275
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i6.9

Original Research Article

A new and efficient method for purification of poly-γ-glutamic acid from high-viscosity fermentation broth

Fengqing Wang[1]*, Jinzhong Liang[2], Wei Wang[2], Dawei Fu[2] and Wei Xiao[2]
[1]Department of Biological Engineering, Sichuan University of Science and Engineering, Zigong City, Sichuan 643000, PR China, [2]College of Food Engineering, Harbin University of Commerce, Harbin City, Heilongjiang 150076, PR China

For correspondence: **Email: wangfengqing_100@126.com*

Sent for review: 22 February 2017 *Revised accepted: 24 May 2017*

Abstract

Purpose: *To devise an efficient strategy for the separation and recovery of high-quality γ-PGA by investigation of the physical properties, pigment properties and microfiltration mode of high-viscosity fermentation broth.*
Methods: *The bacterial strain, Bacillus subtilis 115, was used in this study. The viscosity of the fermentation broth was determined by digital viscometer with spindle SP-2 at 25 ^{o}C. The concentrations of glucose and L-glutamate were analyzed with a biosensor equipped with both glucose oxidase and L-glutamate oxidase electrodes. The pigment in the fermentation liquid was scanned with a UV spectrophotometer at wavelength range of 200 - 500 nm and was removed using activated carbon. Measurement of IR spectrum was performed using an IR spectrophotometer with KBr pellet. .*
Results: *The results showed that the γ-PGA yield was 35 g/L. The viscosity of the fermentation broth was 1600 mPa.s at the end of the batch fermentation. After 3-fold dilution, the viscosity was reduced to one-fortieth of the original value at 65 °C for 30 min., which allowed effective removal of Bacillus subtilis 115 from the broth. Maximum UV absorption of the pigment was occurred at 260 nm. The pigment was removed by shaking with 0.6 % activated carbon powder at 50 rpm for 20 min, resulting in 88 % de-colorization. Concentration with hollow-fiber membrane (MWCO 500,000) resulted in complete removal of residual glucose and glutamic acid from the aqueous solution of γ-PGA. The molecular weight of the γ-PGA was 1095 kDa, and its UV scanning spectrum showed an absorption peak at 216 nm. The decomposition temperature (Td) of the γ-PGA was 312.92 oC. Its IR spectrum was consistent with the presence of carboxyl, hydroxyl, carbonyl and amide groups.*
Conclusion: *An efficient method for the extraction and purification of high-quality γ-PGA from high-viscosity fermentation broth.*

Keywords: *Bacillus subtilis 115, γ-Polyglutamic acid, De-pigmentation, Activated carbon, Ultra-filtration, High-viscosity fermentation broth*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

γ - Polyglutamic acid (γ - PGA), a homo - polyamide of D- and L-glutamic acid units, was first discovered as a component of capsules of *Bacillus anthracis* by Ivonovics and Bruckner [1]. It contains linkages between α - amino and γ - carboxylic groups, as opposed to peptide bonds in proteins. Consequently, γ-PGA is resistant to proteases [2]. However, the polymer is environmentally friendly, nontoxic and non - immunogenic, and can therefore be used safely in a variety of rapidly increasing applications. Because of these potential applications, the development of processes for improved production and recovery of γ-PGA from the

fermentation broth is great importance. However, most research on microbial production of γ - PGA are focused on screening of strains, the optimization of medium and culture conditions with the potential to produce high yield, specific enantiomeric composition and desired molecular mass of γ - PGA at reduced cost. There have been few researchs on the extraction and purification of γ - PGA from fermentation broth.

γ - PGA can be synthesized by polymerase on microbial cell membranes, and then excreted into the culture medium. The molecular weight of γ - PGA is high, and varies from 100 to 2000 kDa. Thus the fermentation liquid becomes highly viscous on production of γ - PGA. Cells encapsulated with γ - PGA possess negative charges near neutral pH because of the ionization of carboxyl groups in γ - PGA molecules. The negative surface charges confer high stability on γ - PGA - encapsulated cells in the culture broth. The stability and high viscosity of the broth create difficulties in sedimentation of the cells during the separation process [2]. This is a major concern in the separation of the cells from γ - PGA in the culture broth.

This study was aimed at finding a feasible method of the separation and recovery of high - quality γ - PGA, through investigation of the physical properties, pigment properties and microfiltration mode of the high - viscosity fermentation broth.

EXPERIMENTAL

Materials

Granular and powdered, activated carbon were purchased from Xilong Chemical Limited. The activated carbon granules were sieved through number 30 screen to remove fine carbon powder. The portion of activated carbon granules retained on the screen was washed three times with excess volume of 70 % aqueous ethanol to remove any powdered carbon adhering to the surface of the carbon granules. After evaporation of the ethanol, the washed granules were dried overnight in a hot air oven at 105 ^{o}C.

Preparation of corn saccharification liquid

Saccharification of corn starch was catalyzed by the double-enzyme method. In this process, a 1:3 (w:v) mixture of corn powder and water (in a tank) was liquefied at 85 ^{o}C for 30 min at pH 6.7 - 7.0 using commercial thermo - stable α - amylase (20 KU/g). The temperature of the corn saccharification liquid was rapidly decreased to about 60 ^{o}C, and then the pH was adjusted to 4.5 with hydrochloric acid. Release of monosaccharides from the saccharification liquid was catalyzed by commercial glucoamylase (50 KU/g) for 4 h. The main components of the corn hydrolysate were reducing sugars, cellulose, and protein (70:11:4 on weight basis). After pre-saccharification, the glucose monomer fraction was close to 25 %, and the level of release of glucose and nutrients (including protein) was a function of saccharification time. Complete starch hydrolysis required more than 7 h of pre - saccharification.

Strain and fermentation

The bacterial strain used in this study was *Bacillus subtilis* 115. Batch cultures of *Bacillus subtilis* 115 were carried out in a 30 - L jar fermentor containing 20 L of medium at 37 °C and 150 rpm for 72 h. The medium contained (per liter) 250 mL of corn saccharification liquid (sugar concentration: 200 g/L), $CaCl_2$ (0.42 g), peptone (4 g), monosodium glutamate (50 g), NaCl (12 g), $MgSO_4 \bullet 7H_2O$ (1.25 g), KH_2PO_4 (4 g) and $MnSO_4$ (0.08 g).

Analytical procedures

After separation of the cells by centrifugation, the concentration of glucose and L - glutamate remaining in the medium were determined by a biosensor analyzer with both glucose oxidase and L - glutamate oxidase electrodes (Institute of Biology, Shandong Academy of Sciences SBA - 40D). The viscosity of the culture broth was measured by Digital Viscometer with spindle SP-2 at 25 ^{o}C (DNJ - 5S, China). Infrared spectrometer was used to analyze the absorption characteristics of the samples with KBr pellet (Nicolet - Magna - IR 550, USA).

Removal of pigment in fermentation liquid

Dried activated carbon powder and granules (0.2, 0.4, 0.6, 0.8 and 1.0 g) were separately measured into six 250 mL Erlenmeyer flasks. A flask without activated carbon served as negative control. The cell-free fermentation liquid (100 mL) was added to each flask and the flasks were then sealed with parafilm. The decolorization process was carried out on an Incubator Shaker at 50rpm. The UV absorbance of each liquid fraction was measured at 260 nm.

Statistical analysis

The data are presented as mean ± standard deviation were analyzed Student t-test with Microsoft Excel 2003. $P < 0.05$ was considered statistically significant.

RESULTS

Batch fermentation produced γ-PGA

γ - PGA was produced in a 30 L stirred tank bioreactor under the following conditions: stirring speed of 150 rpm, ventilation rate of 1.6 m^3/h, tank pressure of 0.08 MPa, and culture temperatures between 36.5 and 37.5 ^oC. The yields of γ - PGA as a function of changes in broth nutrient composition with time are shown in Figure 1. Changes in the pH of the fermentation broth were also monitored, and the results are shown in Figure 2. Although pH played an important role in the production of γ - PGA by *Bacillus subtilis* 115, results from the experiments in this study indicated that strict control of pH was not necessary. In the fermentation, monosodium glutamate was a carbon source for **γ -** PGA formation, while the glucose in the corn saccharification liquid served as source of energy for microbial growth and product formation. As shown in Figure 1, the concentration of glucose in the bioreactor rapidly decreased after 10 h. At this time, the release of **γ -** PGA into the broth had commenced. This implies that the production of **γ -** PGA was partially associated with cell growth. The levels of consumption of glutamate and glucose during the fermentation process were about 10 g/L and 60 g/L, respectively, and the yield of γ - PGA was stable, rising to a peak value of 35 g/L.

Effect of temperature on viscosity of fermentation broth at different dilution time

The viscosity of γ - PGA fermentation liquid was too high, it was not conducive to the removal of the cell. Accordingly, the effect of temperature on the viscosity of fermentation broth with different dilution times was evaluated. As shown in Figure 3, the viscosity of the culture broth decreased with increasing temperature and degree of dilution (dilution - fold). When the fermentation liquid was diluted 3-fold, the viscosity reduced from 1600 to 100 mPa.s (6.25 % of initial viscosity) at 35 ^oC, and eventually fell to 20 mPa.s following a 5 - fold dilution at 65 ^oC for 10 min.

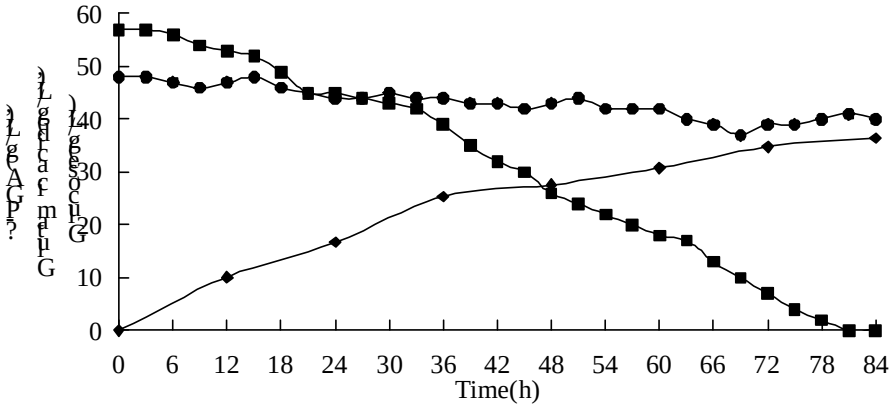

Figure 1: Yield of γ - PGA, concentration of glucose and glutamic acid as function of changes in broth nutrient composition with time. ***Note:*** ■ means the curve of γ - PGA production with time. ● means the curve of glucose consumption with time. ♦ means the curve of glutamic acid consumption with time

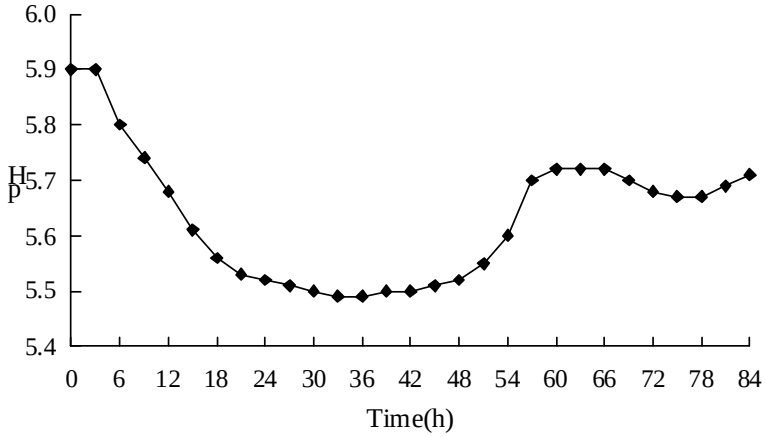

Figure 2: Changes in pH of the fermentation broth with time

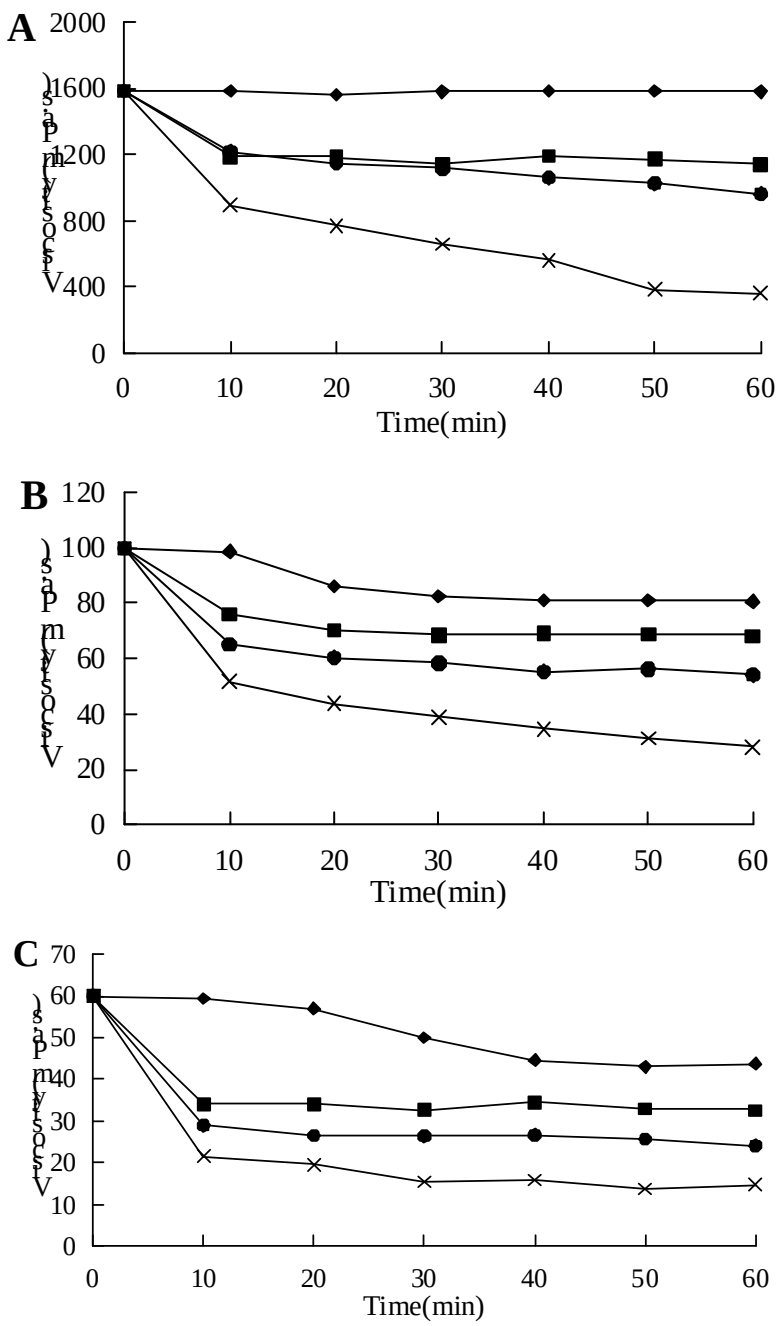

Figure 3: Effect of temperature and dilution on broth viscosity. Culture broth diluted 2-fold (A); culture broth diluted 3-fold (B); culture broth diluted 5-fold (C). ***Note:*** ♦ = fermentation broth was treated at 35 ^{o}C; ■ = fermentation broth was treated at 45 ^{o}C; ● = fermentation broth was treated at 55 ^{o}C. × = fermentation broth was treated at 65 ^{o}C

Effect of pH on the viscosity of fermentation broth at different dilution times

The viscosity of the fermentation broth containing γ - PGA is not only affected by temperature, but also affected by pH. The pH of the culture broth was adjusted to 2.5 - 11.5 with 6 mol/L HCl and 10 mol/L NaOH. The effect of temperature on the viscosity of fermentation broth with different dilution times was evaluated. Figure 4 shows that the viscosity of fermentation broth was affected by pH and dilution fold. At the end of fermentation, the pH of the culture broth was about 5.5. As shown in Figure 4, the viscosity of fermentation broth significantly decreased as the pH decreased from 5.5 to 2.5 and increased from 8.5 to 11.5 with dilution 1 fold.

Characteristics of pigment in fermentation liquid

The fermentation liquor, prior to removal of cells

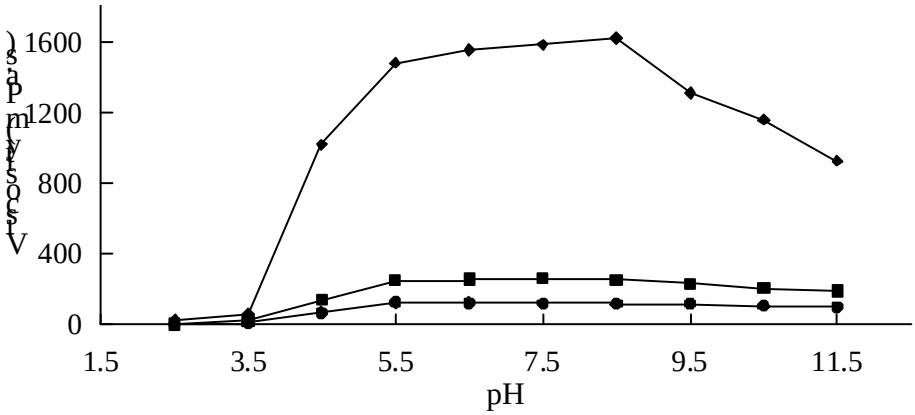

Figure 4: Effect of pH and dilution on broth viscosity. NOTE: ♦ = dilution 1-fold. ■ = dilution 2-fold. ● = dilution 3-fold

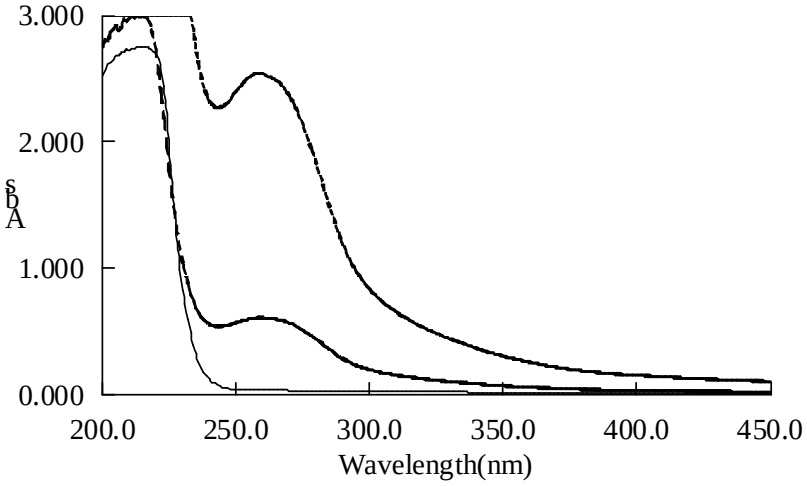

Figure 5: Spectral analysis of the culture broth (treated and untreated with activated carbon), and high purity *γ*-PGA over wavelength range of 200 to 500 nm. ***Note:*** From top to bottom, the curves represent treated and untreated with activated carbon, high purity γ-PGA.

by plate-and-frame filtration, was buff due to pigment. This would affect the quality of γ - PGA products. Thus experiments were carried out to determine the characteristics of the pigment and the most efficient decolorization method to be used. The sample was scanned in a UV spectrophotometer at wavelength range of 200 - 500 nm. As shown in Figure 5, it was found (through comparison with the spectrum of high-purity γ - PGA) that the maximum absorption wavelength of the pigment in the fermentation liquor was 260 nm.

Effect of activated carbon powder and granules on pigment removal

The results indicated that activated carbon was very effective in removal of the pigment. As shown in Figure 6A, the decolorization effect of powdered activated carbon was better than that of granulated activated carbon. The results revealed that under the experimental conditions used (shaking at 50 rpm for 20 min), 0.6 % powdered activated carbon was the most effective concentration for decolorization and removal of impurities from the cell-free culture broth containing *γ*-PGA.

Activated carbon is a non-specific adsorbent which decolorizes by binding to colored components/pigments. The advantage of this method of decolorization is that due to its high molecular weight, γ - PGA does not adsorb readily to activated carbon, unlike low molecular impurities, such as pigments and proteins. The percentage decolorization which is the ratio of the absorbance values at 260 nm before and after decolorization, was up to 88 % as shown in Figure 6 (B). The rate of decolorization remained constant with changes in duration of shaking.

Concentration of γ-PGA aqueous solution by ultra-filtration

The use of concentrated solutions of *γ* - PGA reduces cost of drying. Ultra - filtration is typically

used to concentrate solutions of macromolecules and colloids from solution, the lower limit being solutes with molecular weights of a few thousand Daltons. Because of high molecular weight, γ - PGA aqueous solution could also be concentrated by ultra – filtration. After fermentation the molecular weight of γ - PGA was in the range of 500 - 1200 kDa. Therefore, a membrane having Molecular Weight Cut Off (MWCO) of 100 kDa was chosen as an efficient and economical procedure for concentration of γ - PGA. The γ - PGA solution was concentrated at a pressure of 0.12 MPa using the concentration - dilution - concentration - dilution - concentration method, with a hollow - fiber cartridge having a membrane area of 650 cm^2, as shown in Table 1.

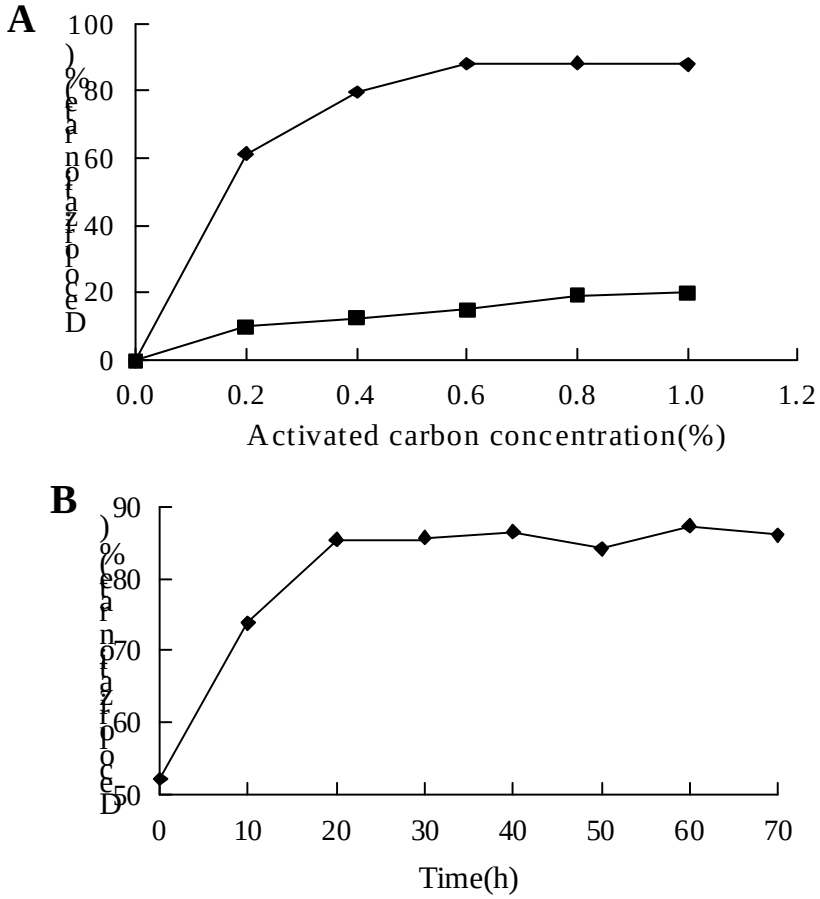

Figure 6: Effect of activated carbon powder and activated carbon granules (A), and effect of shaking time (B) on removal of pigment from fermentation liquid. ***Note:*** ♦ means powdered activated carbon. ■ means granular activated carbon

Table 1: Removal of low molecular weight impurities from γ - PGA solution by hollow-fiber cartridge

Low molecular impurities	Concentrated liquid		Filtration fluid	
	Glutamic acid (g/L)	***Glucose (g/L)***	***Glutamic acid (g/L)***	***Glucose (g/L)***
Before concentration(400mL)	2	12	-	-
Concentrated to 100mL	2	9	2	13
Diluted to 400mL and then concentrated to 100mL	0	3	1	2
Diluted to 400mL and then concentrated to 100mL	0	0	0	1
Diluted to 400mL and then concentrated to 50mL	0	0	0	0

Repeated concentration not only increased the concentration of γ - PGA, but also effectively removed low molecular impurities and improved the purity of the γ - PGA. The final product was pre-frozen at -70 ^oC for 48 h, and then freeze - dried at -55 ^oC for 48 h (Figure 7).

Characteristics of γ-PGA

The molecular weight of γ - PGA was 1095 kDa (as determined using GPC), and the poly - diversity index (Mw/Mn) was 1.060. The spectrum from UV scan of PGA showed

absorption peak at 216 nm. There was no absorption peak in the range of 260 - 280 nm, indicating absence of peptide chain structure in the polymer.

Td (decomposition temperature) was measured with thermal gravimetric analysis (TGA) by heating at a rate of 10 ^{o}C per min. As shown in Figure 8, Td was 312.92 ^{o}C.

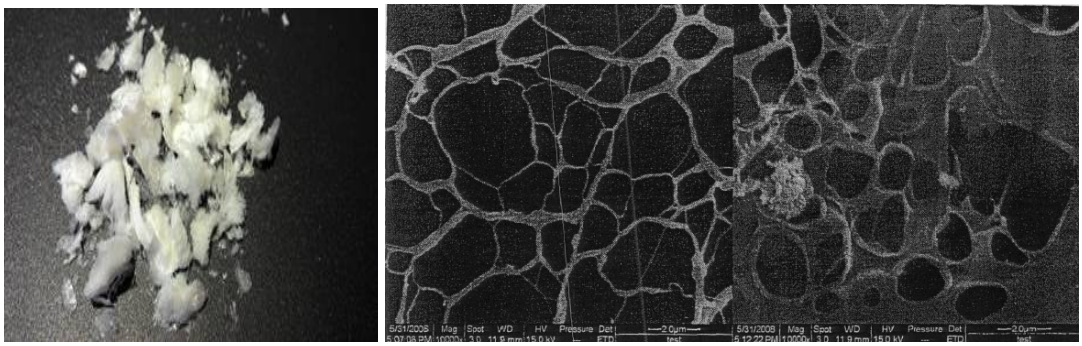

Figure 7: Concentrated solution of γ-PGA pre-frozen at -70 ^{o}C for 24 h, and freeze-dried at -55 ^{o}C for 48 h

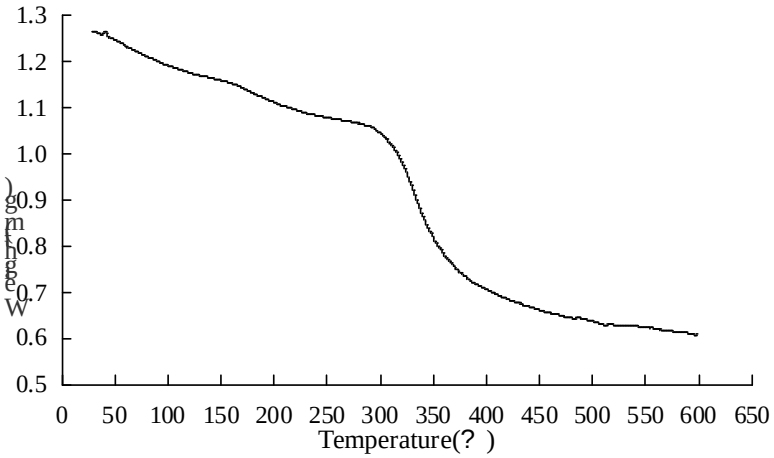

Figure 8: Thermal properties of γ-PGA

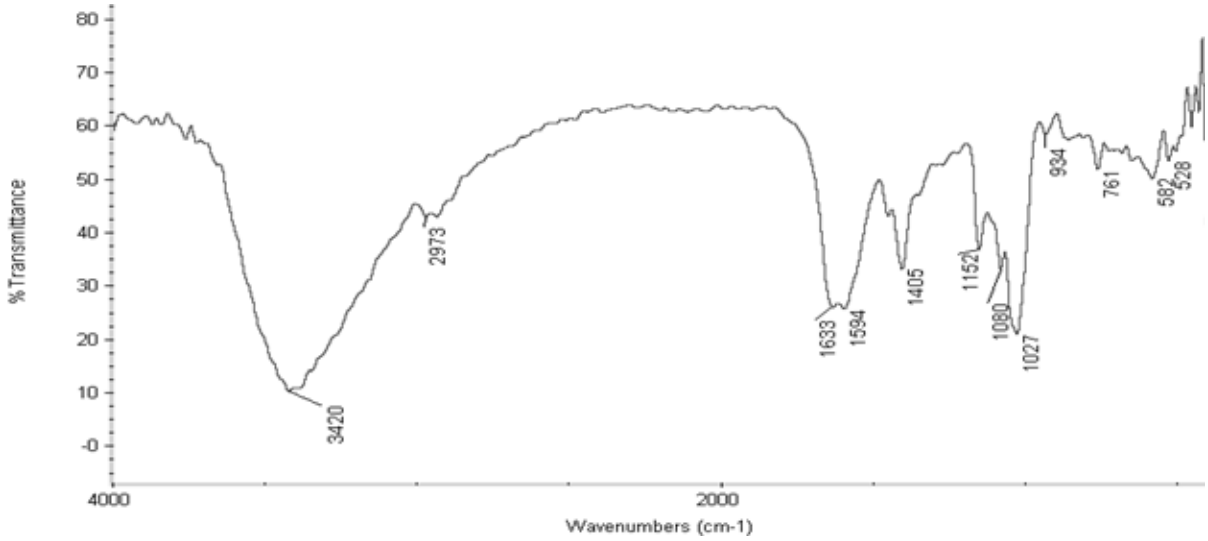

Figure 9: Infrared (FT-IR) absorption spectrum of γ-PGA in KBr pellet

Figure 9 shows the infrared spectrum of *γ* - PGA in KBr pellet: Amide I, N - H bending band at 1633 cm^{-1}; Amide II, stretching band at 1594 cm^{-1}; C = O symmetric stretching band at 1405 cm^{-1}; C - N stretching band at 1152 cm^{-1}; N - H group bending band at 707 cm^{-1}; and O - H stretching band at 3420 cm^{-1}. The absorption peak at 3400 - 3450 cm^{-1} is characteristic of OH stretching

from the bound hydroxyl group and adsorbed water molecules. The absorption peaks around 1600 - 1660 cm^{-1} and 1390 - 1450 cm^{-1} are characteristic of amide groups and C = O groups. The strong absorption peaks observed in the range from 1085 to 1165 cm^{-1} are characteristic of C - N groups. The IR spectrum of the *γ*-PGA conforms to the presence of carboxyl, hydroxyl, carbonyl and amide groups.

DISCUSSION

The production of γ - PGA with *Bacillus subtilis*115 was partially associated with cell growth, which is in agreement with results reported earlier [3-5]. The levels of consumption of glutamate and glucose during the fermentation process were about 10 g/L and 60 g/L, respectively. The change of pH during fermentation was slight, and could be used as a criterion for the normal or not fermentation with the consumption of glucose and glutamic acid.

The first task in the process of refining the γ - PGA was to remove the *B. subtilis* 115 cells from culture broth. However, it was necessary to handle the culture broth first in order to make for efficient removal of the cells due to the high viscosity of the fermentation broth containing γ - PGA. After investigated the effects of dilution ratio, temperature and pH on the viscosity of fermentation broth, it was found that dilution could be used as the most effective pretreatment method, and the cell could be effectively removed by dilution. Because of higher thermal energy of water molecules, the viscosity of γ - PGA solution did not decrease with increasing temperature as expected. Optical Rotatory Dispersion (ORD) studies have shown that strong hydrogen bonds make the γ - PGA assume a tightly compacted a-helix conformation, resulting in a strong hydrophobic character [6]. However, as pH increases, the hydrogen bonds are broken, the a-helical conformation changes into a linear random - coil conformation, and the α-COOH group ionizes to form a -COO- anion. It was reasoned that the viscosity of culture broth containing γ-PGA would decrease at low pH due to the reduction of ionized γ - PGA, which would result in less interaction between water and γ - PGA. It has been reported that the zeta potential of cells was reduced with decreases in pH from 7 to 2 [7]. The cells, which had lost their surface charges at low pH, were aggregated by their interactions. It was also reported in the same studies that separating γ - PGA at low pH was beneficial on a large - scale, because it resulted in significant lowering of energy cost implications [7]. However, this was not a favorable method for isolating γ - PGA because the polymer chains of γ - PGA might be liberated under acidic conditions, which would result in a decrease in molecular weight of γ - PGA.

Although activated carbon can effectively adsorb the pigment in fermentation broth, it was also found that the longer the contact time of activated carbon with the fermentation fluid containing γ - PGA, the harder it became to separate the activated carbon from γ - PGA. It is likely that the activated carbon was trapped by the high molecular weight γ - PGA.

Ultra-filtration not only increased the concentration of γ - PGA, but also effectively removed low molecular impurities and improved the purity of the γ - PGA.

CONCLUSION

This study demonstrates that the problem of extracting and purifying γ-PGA from high viscosity fermentation broth has been resolved using the method developed. The viscosity of the fermentation broth can be greatly reduced by dilution so that cells can be effectively removed. Activated carbon decolorization and ultrafiltration improves the purity of γ-PGA and lower the cost of extraction and purification of γ-PGA, thus making it possible to use γ-PGA more widely.

DECLARATIONS

Acknowledgement

The authors would like to thank all the staff of Key Laboratory of Food Science and Engineering, College of Heilongjiang, as well as all partners for their assistance.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

REFERENCES

1. *Ivanovics G, Bruckner V. The chemical nature of the immuno-specific capsule substance of anthrax bacillus. Naturwissenschaften 1937; 25: 250.*
2. *Shih IL, Van YT. The production of poly-(g-glutamic acid) from microorganisms and its various applications. Bioresour Technol 2001; 79: 207–225.*
3. *Chen X, Chen S, Sun M, Yu Z. High yield of poly([gamma]-glutamic acid) from Bacillus subtilis by solid-state fermentation using swine manure as the basis of a solid substrate. Bioresour Technol 2005; 96: 1872-1879.*
4. *Chen X, Chen S, Sun M, Yu Z. Medium optimization by response surface methodology for poly-γ-glutamic acid production using dairy manure as the basis of a solid substrate. Appl Microbiol Biotechnol 2005; 69: 390-396.*
5. *Xu H, Jing M, Li H, Lu D, Ouyang P. Efficient production of poly([gamma]-glutamic acid) by newly isolated Bacillus subtilis NX-2. Process Biochem 40: 519-523.*
6. *Ho G, Ho T, Hsieh K, Su Y, Lin P, Yang J, Yang K, Yang S. γ-Polyglutamic Acid Produced by Bacillus subtilis (natto): Structural Characteristics, Chemical Properties and Biological Functionalities, J Chinese Chem Soc 2006; 53: 1363-1384*
7. *Jin Hwan Do, Ho Nam Chang, Sang Yup Lee. Efficient Recovery of γ-Poly (Glutamic Acid) From Highly Viscous Culture Broth, Biotechnol Bioeng 2001; 76(3): 219-223.*