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Production of Poly-γ-glutamic acid by *Bacillus licheniformis*: Synthesis and Characterization

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Abstract

Poly-γ-glutamic acid (γ-PGA), one type of amino acid polymers, consists of D-and L-glutamic acids linked with amide bonding between α-amino and γ-carboxylic acids. Naturally, γ-PGA could be biodegradable polymer and water-solubleapplied in various industries such as food, cosmetics, and pharmaceutical industries. Variation of initial L-glutamic acid concentration affecting on the production of γ-PGA by fermentation of *Bacillus licheniformis* was studied. The batch fermentation process consists of preparing the inoculum and expanding the culture of *B. licheniformis* into E medium (pH 6.5) for γ-PGA production. γ-PGA was recovered by using methanol precipitation together with lyophilization. For characterization, the molecular size of γ-PGA products was analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and that for characterization of γ-PGA with Fourier Transform Infrared Spectroscopy (FT-IR). The results showed that initial concentrations of L-glutamic acid(0, 15 30, 45, 60, 75 g/L) affected on the production of γ-PGA significantly. In addition, the L-glutamic acid of 75 g/L could contribute the best production of 12.64 g/L γ-PGA, which higher than those concentrations of 45 and 60 g/L L-glutamic acid. Therefore, an increase of the L-glutamic acid concentration correlated well with an increased concentration of γ-PGA polymer. The observations gave an alternative for possible process development of the production of γ-PGA. This is directly applicable in industries, as well as enhancing academic progress in bio-polymer field.

Key words: Poly-γ-glutamic acid, L-glutamic acid, *Bacillus licheniformis*, Bio-polymer

Introduction

Poly-γ-glutamic acid (γ-PGA) is a natural polymer, consists of D- and L-glutamic acids linked with amide bonding between α-amino and γ-carboxylic acids. γ-PGA can be synthesized by *Bacillus* strains via fermentation process. Naturally, some *Bacillus* spp. can produce γ-PGA not only intracellularly, but also extracellularly. γ-PGA is interestingly applicable as a raw material used in various industries, such as the substance improving a dough surface of making bread, the thickening agent in foods, the material applied for drug delivery, the application in fiber formation and the biodegradable thermoplastic film, as well as, the absorbent for removal of water and heavy metals. There are several researches reported that *Bacillus* spp. produced γ-PGA depending on the suitable conditions of fermentation. *B. licheniformi*s, one of *Bacillus* spp. strains was reported that it could produce extracellular polymer (γ-PGA) having high molecular weight. The fermentation broth of *B. licheniformis* formed highly viscosity due to its production of γ-PGA. An increased viscosity is likely to decrease the

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volumetric oxygen transfer, and leads to oxygen limitation and causes decreasing cell growth. This problem becomes more significant due to cells need to be grown to a higher density. A phenomenon has been reported the L-glutamic acid units are polymerized in a ribosome-independent manner via amide linkages between the α-amino and γ-carboxylic acid functional groups[1], which affected on the production of γ-PGA significantly. Moreover, there was a report about optimum pH for growth of *B. licheniformis* ATCC9945 is 6.5[2], which was the optimal pH for cell growth and γ-PGA production as well. However, there are several factors which affect on fermentation process and still are limited to study such as an addition of L-glutamic acid.

In this study, the variation of concentrations of L-glutamic acid, i.e. 0, 15, 30, 45, 60, 75 g/L was compared in culture medium E (pH 6.5) for the production of γ-PGA.The effect of L-glutamic acid concentration on amount and molecular size of γ-PGA products was studied and the characterization of γ-PGA were determined by FT-IR spectroscopy and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Experimental Procedure

Strain Information

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

Production of γ-PGA in Flask Culture

B. licheniformis ATCC® 9945™, the γ-PGA producer, was culturedon NA medium and then inoculated into 25 ml of E medium (pH 6.5) which contained L-glutamic acid 20 g, citric acid 12 g, glycerol 80 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeCl_3.6H_2O$ 0.04 g, $CaCl_2.2H_2O$ 0.15 g and $MnSO_4.H_2O$ 0.104 gper liter[2]. The culture was performed at 30°C under shaking condition at 250 rpm for 48 hours. The cell suspension was transferred to 225 ml of E medium (pH 6.5) and incubated under above similar conditions for another 72 hours. The initial L-glutamic acid concentrations in the range of 0, 15, 30, 45, 60, 75 g/L were investigated for γ-PGA production.

Purification of γ-PGA

The cell cultures were separated from the culture broth by centrifugation for 20 min at 12,000 rpm to remove cells. The supernatant was poured into 4 volumes of methanol with gentle stirring for 24 hours to precipitate the γ-PGA. The resulting precipitate containingcrude γ-PGA was collected by refrigerated centrifuge (12,000 rpm, 10ºC, for 40 min). The crude γ-PGA was then dissolved in distilled water at a concentration of 10 g/L, and any insoluble contaminants were removed by centrifugation (14,000 rpm, 10°C, for 20 min). The γ-PGA was further purified by dialysis (membrane with the molecular weight cut off of 12,400) against 1 L of distilled water three times for 12 hours to remove salts from aqueous γ-PGA solution. Finally, the γ-PGA slurry was lyophilized to prepare as γ-PGA powder[3]. An amount of γ-PGA was determined using ninhydrin with glutamic acid as a standard [6],[7].

Measurement of FT-IR Spectroscopy

A characterization of γ-PGA structure was analyzed by Fourier Transform Infrared Spectroscopy (FT-IR) (a Bruker Tensor 27 instrument). The KBr mode was carried out to determine the PGA produced from the L-glutamic acid added at 0, 15, 30, 45, 60, 75 g/L and recorded the transmission spectra in the range of 4000–400 cm^{-1}.

Molecular Size Estimation of γ-PGA

The molecular size of γ-PGA was measured by the mobility on the gel. It was estimated by using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 12% acrylamide and SeeBlue®Plus2 Pre-Stained standard protein makers which consists of myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin&Bchain (4 kDa). The γ-PGA was then visualized by Pierce Silver Stain Kit.

Statistic Analysis

The confidence interval statistical test of cell growth was estimated by using the statistical analysis program from the Trendline/Regression Type and Display R-squared value options of Microsoft Excel.

Results and Discussion

Effect of L-glutamic Acid Concentrations on Cell Growth

The effect of L-glutamic acid concentrationson the cell growth of *B. licheniformis* ATCC® 9945™ was studied. The results showed that the addition ofinitial L-glutamic acid concentrations at 0, 15, 30, 45, 60, 75 g/L contributed the cell growth. In addition, cell densities of all experiments increased gradually through the cultivation and found to be the highest at 30-36 hr of culture time. Afterwards, the bacterial growth was found to decrease around 48-72 hours of the cultivations as shown in Figure 1. Besides, the growths of *B. licheniformis* ATCC® 9945™ at various initial L-glutamic acid concentrations (0-75 g/L) were estimated using a confidence interval statistical test. The results showed that at 45, 60, 75 g/L L-glutamic acid, with a 95% confidence interval statistics testing, their R^2 values for the bacterial growth were 0.971, 0.969 and 0.956, respectively. This indicated that the growths of *B. licheniformis* ATCC® 9945™ at these L-glutamic acid concentrations (45-75 g/L) were potential to produce higher quality of γ-PGA, as shown in Figure 2. The increasing of initial concentration of L-glutamic acid affected on an increased amount of γ-PGA products[8]. The highest γ-PGA concentration of 12.64 g/L was obtained from using 75 g/L L-glutamic acid. Besides, the amount of γ-PGA reached 10.49 g/L and 10.52 g/L in the presence of 45 g/L and 60 g/L L-glutamic acid, respectively. During the bacterial growth, L-glutamic acid, citric acid and glycerol are main substrates for the γ-PGA synthesis. In addition, γ-PGA was not produced after the depletion of citric acid and glutamic acid in the culture medium of fermentation process. For *B. licheniformis* ATCC® 9945™, glycerol was slightly used for cell growth. Also, L-glutamic acid was previously reported to be able to activate relevant enzymes synthesizing γ-PGA, such as glutamate synthase, glutamate polymerase and glutamate dehydrogenase. Moreover, bacterial cells were able to synthesize γ-PGA when both citric acid and L-glutamic acid were consisted in the culture medium during fermentation[2],[3].

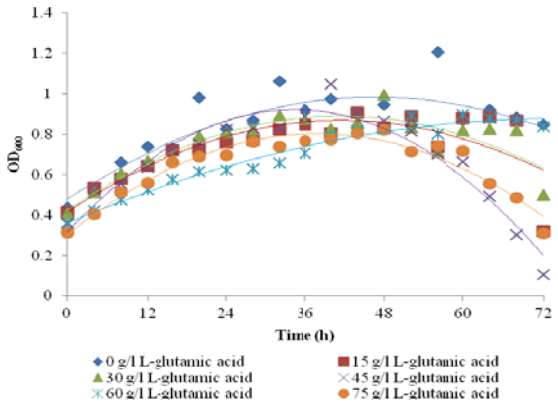

Figure 1. The growth of B. licheniformis ATCC® 9945™ in shake flask culture at various initial concentrations of L-glutamic acid between 0-75 g/L.

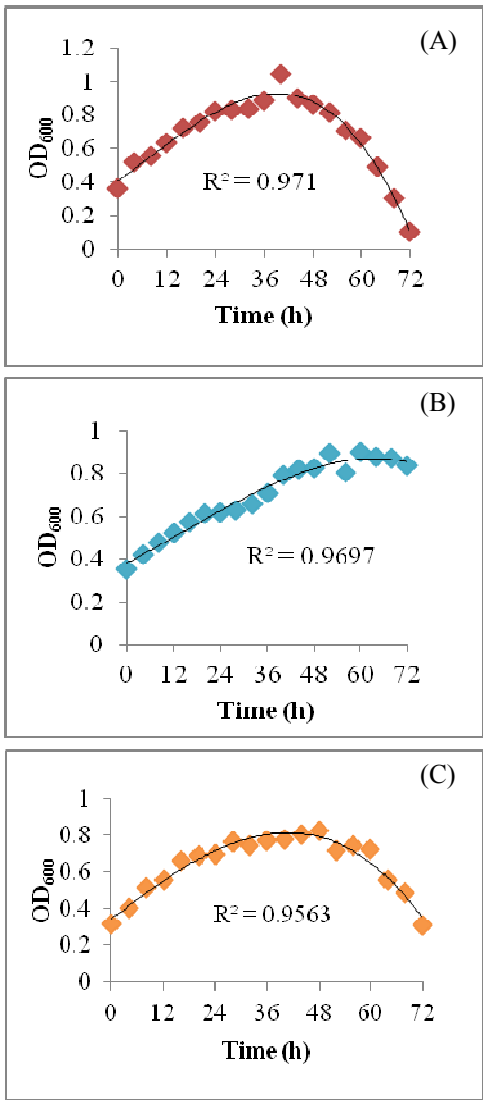

Figure 2. The growth of B. licheniformis ATCC® 9945TM in shake flask culture at various initial concentrations of L-glutamic acid, (A) 45, (B) 60, and (C) 75 g/L.

FT-IR spectroscopy Analysis

The results of γ-PGA analysis by Fourier Transform Infrared Spectroscopy (FT-IR) were given in Figure 3. The different profiles of γ-PGA at various initial L-glutamic acid concentrations (45, 60, 75 g/L) were observed. The results showed that at 75g/L L-glutamic acid presented clearly the characteristic peaks of pure γ-PGA produced from *B. licheniformis* ATCC® 9945™ . The position of peaks representing C=O stretch of free carboxylic acids at 1649 cm^{-1}, asymmetric COO^- stretch at 1582 cm^{-1} with a broad band due to peak-overlap of N–H/C–N deformation and symmetric COO^- at 1464 cm^{-1}. Additionally, the appearance of the peak position is quite clear, as compared with those three typical peaks of pure γ-PGA. These are representing C=O stretch of free carboxylic acids at 1653.3 cm^{-1}, asymmetric COO^- stretch at 1582.9 cm^{-1} with a broad band due to peak-overlap of N–H/C–N deformation and symmetric COO^- at 1403.9 cm^{-1}[4]. Therefore, the initial concentration of L-glutamic acid at 75 g/L found suitable for the γ-PGA production in this study, due to clear appearance of the peaks similarly to pure γ-PGA[4]. Moreover, the result indicated that the increased concentration of L-glutamic acid (45-75 g/L) could increase the production of γ-PGA.

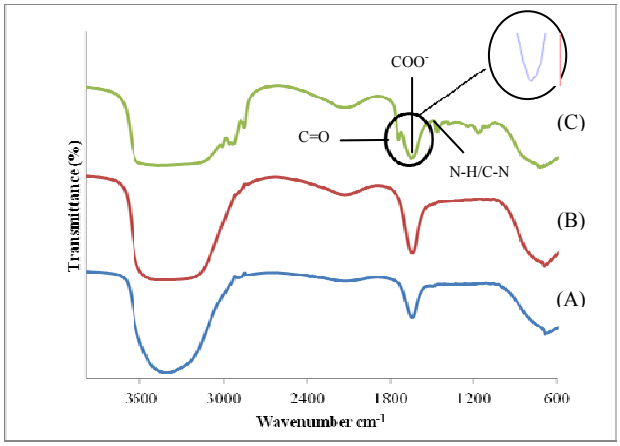

Figure 3. Overlaid Fourier Transform Infrared Spectra (FT-IR) in KBr of γ-PGA production by *B.licheniformis* ATCC® 9945™ at the various initial L-glutamic acid concentrations, 45g/L (line A), 60 g/L (line B) and 75 g/L (line C).

Effect of L-glutamic Acid Concentrations on γ-PGA Molecular size

The molecular size of γ-PGA which produced via *B. licheniformis* ATCC® 9945™ fermentation was estimated by SDS-PAGE method. The bands of γ-PGA at the various initial L-glutamic acid concentrations (0-75 g/L) from 72 hour of culture time were shown in Figure 4. The results showed that no bands of γ-PGA was obtained from no adding L-glutamic acid, whereas adding L-glutamic acid at 15, 30, 45, 60 and 75 g/L gave the γ-PGA bands at the size over 98 kDa. This indicated that an increase of L-glutamic acid concentrations in the medium could induce clearly the production of γ-PGA from the fermentation of *B. licheniformis* ATCC® 9945™. In contrast, an increase of the L-glutamic acid concentrations did not affect on the molecular size of γ-PGA products, bsut contributed in the production of higher concentration of γ-PGA products. Moreover, the concentrations of L-glutamic acid at 60, 75 g/L showed apparently longer bands, which resulted from depolymerization to several sizes of polymers. As the result, *B. licheniformis* ATCC® 9945™ might abundantly produce an extracellular enzyme such as PGA depolymerase during γ-PGA production, which directly affects on the quality of γ-PGA products. Therefore, the enzyme activities involved in γ-PGA synthesis needed to optimize for the higher production of γ-PGA from fermentation process[1],[5].

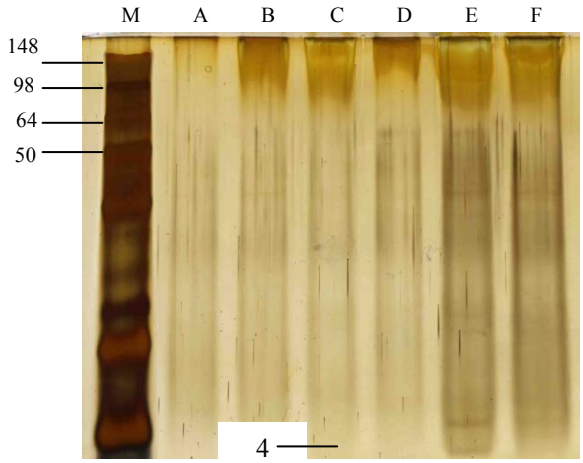

Figure 4. SDS-PAGE (12% acrylamide) of γ-PGA production by B. licheniformis ATCC® 9945™ at the various initial L-glutamic acid concentrations; 0 g/L (land A), 15 g/L (land B), 30 g/L (land C), 45 g/L (land D), 60 g/L (land E) and 75g/L (land F) for 72 hours and SeeBlue®Plus2 Pre-Stained standard protein makers (land M) which consists of myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin & B chain (4 kDa).

Conclusions

The γ-PGA polymer could be obtained through the fermentation of *B. licheniformis* ATCC® 9945TM with an addition of L-glutamic acid. The increasing of initial concentration of L-glutamic acid contributed on the growth of *B. licheniformis* ATCC® 9945TM and enhanced the higher concentration of γ-PGA production. However, the increasing of initial concentration of L-glutamic acid in the culture medium did not affect on changing the γ-PGA size.

Acknowledgements

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