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재수화된 유청분말내 *Bacillus subtilis* ATCC 663에 의해 다른 탄소원들로부터 생산된 폴리글루타믹산의 특징

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Properties of Polyglutamic Acid Produced by *Bacillus subtilis* ATCC 6633 in Rehydrated Whey Powder Supplemented with Different Carbon Sources

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Abstract: In this study, the use of rehydrated whey powder (RWP) solutions containing different carbon sources (citric acid, ammonium sulfate, or glutamic acid) for γ-poly(glutamic acid) (γ-PGA) by *Bacillus subtilis* ATCC 6633 was explored. After 72 h of fermentation at 30 °C, cell growth, γ-PGA production, molecular weight by SDS-PAGE, rheological properties and NMR analysis of γ-PGA were determined. The growth of *B. subtilis* was significantly different during 72 h of fermentation in RWP, medium E, and RWP containing citric acid or glutamic acid. These results showed the dynamic viscosity of the 1% polymer solution was 2.5 Pa·s at 10 1/sec shear rate. The maximum γ-PGA concentration was 1.57 g/100 mL in RWP containing glutamic acid, citric acid and ammonium sulfate. Therefore, RWP as a waste product with being efficient and more economic fermentation medium to produce γ-PGA can be ideal for the industrial production.

Keywords: polyglutamic acid, *Bacillus subtilis*, whey powder, carbon sources, NMR.

Introduction

Extracellular polymers produced by microorganisms are usually beneficial and more profitable for industry practice. For example, microbial polysaccharides such as xanthan gum (*Xanthomonas campestris)*, dextran (*Leuconostoc mesenteroides*), curdlan (*Agrobacterium tumefaciens* and *Alcaligenes faecalis*), pullulan (*Aureobasidium pullulans*), gellan (*Pseudomonas elodea*), and alginate (*Azotobacter vinelandii)* are common food industry ingredients.[1] *Bacillus subtillis* is also known to produce extracellular polymers including mostly γ-poly (glutamic acid) (γ-PGA).[2-4] This edible polymer has received considerable attention due its water solubility. Its structure is unusual because it is a homopolymer of glutamic acid that has amide linkages between glutamate γ-carboxyl and α-amino groups.[5] These and other features make it of interest for applications in medicine, foods, plastics, and oil recovery.

Production of γ-PGA by *Bacillus* species has been extensively studied under different conditions.[2,5-12] Medium E, consisting of L-glutamic acid, citric acid, glycerol, NH_4Cl, K_2HPO_4, $MgSO_4$-$7H_2O$, $FeCl_3$-$6H_2O$, $CaCl_2$-$2H_2O$, $MnSO_4$-H_2O was formulated for PGA production by Leonard *et al*..[7] In general, the concentration of L-glutamic acid, citric acid and ammonium sulfate strongly affects the amount of γ-PGA concentration in the final medium.[10-12] However, the addition of glucose as a carbon source decreases γ-PGA production and increases by-product polysaccharide by *B. subtilis*.[10,12]

Whey, a by-product of cheese processing in the dairy industry, also has attracted considerable attention due to its high nutritional content and functional properties.[13] Whey powder contains lactose (78%, w/v), protein (5.5-5.6%, w/v), lipid (0.6%), trace amounts of vitamins (A, B1, B2, B6, B12, vita-

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min C, folic acid), minerals (calcium (0.9%), magnesium (0.1%), sodium (0.8%), potassium (2.2%), phosphorous (0.8%), iron (10 ppm), copper (4 ppm), zinc (48 ppm), manganese (24 ppm) and organic acids.[14] Whey can be used as a medium for various microorganisms to manufacture polysaccharides, surfactants, enzymes, vitamins, or antibiotics.[15-17] Whey permeate can be used as a fermentation substrate for *Pseudomanas spp.* or *Klebsiella oxytoca* to produce polysaccharides or rhamnolipid biosurfactants.[18-21] Moreover, we also reported that rehydrated whey powder solutions can be used as fermentation media for *B. subtilis* to produce polysaccharides or surfactins.[22] However, the effect of supplementation of whey with different carbon sources on the production of polysaccharide was not included in our previous study.

In this study, we investigated the effect of L-glutamic acid, citric acid and/or ammonium sulfate as a carbon or nitrogen source in rehydrated whey powder for production of γ-PGA and polysaccharides by *B. subtilis* ATCC 6633. The polymers formed were analyzed by SDS Page and NMR to determine whether the medium formulation resulted in γ-PGAs of variable molecular weight.

Experimental

Culture Preparation. *Bacillus subtilis* ATCC 6633 was obtained from the Health Department of Refik Saydam Hifzisiha in Ankara, Turkey. The culture was stored in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, Mich., U.S.A.) containing 6% yeast extract and glycerol at -60 ºC until use. Two successive transfers (30 ºC for 24 h) were prepared in 10 mL of TSB (Difco) using a 1% inoculum, after which the culture was pelleted by centrifugation (12000×g/30 min/4 ºC) and resuspended in sterile water for subsequent inoculation.

Preparation of the Media. Rehydrated whey powder (RWP) (20% w/v) was prepared using whey powder composed of 83-85% lactose, 5.5-6.5% protein, 5.5% mineral, 2% moisture and 0% fat obtained from a dairy plant (Palmalat, Port Elizabeth, South Africa). After adjustment of pH to 7.00, RWP was autoclaved at 121 ºC for 15 min and cooled to room temperature. L-glutamic acid (0, 2, or 3% (wt/v)), citric acid (0, 2, or 3% (wt/v)) and/or ammonium sulfate (0, 0.5, or 0.75% (wt/v)) were added at three different concentrations to RWP medium and Medium E (2% L-glutamic acid, 2% citric acid, 8% glycerol, 7% NH_4Cl, 0.5% K_2HPO_4, 0.5% $MgSO_4$-$7H_2O$, 0.04% $FeCl_3$-$6H_2O$, 0.15% $CaCl_2$-$2H_2O$, 0.104% $MnSO_4$-H_2O). One loop of the *B. subtilis* suspension was added to 100 mL of media in 250 mL flasks which were incubated at 30 ºC for 72 h with shaking (150 rpm).

Bacterial Enumeration. Vegetative cells of *B. subtilis* were quantified in all media during 72 h of fermentation at 30 ºC. Serial dilutions prepared 0.1% (w/v) peptone water were surface-plated on tryptic soy agar (Merck) with the plates counted after 25-30 h of incubation at 30 ºC.

Purification of Polyglutamic Acid. *B. subtilis* was cultivated aerobically in the media tested at 30 ºC and 150 rpm. The cells were harvested by centrifugation (5000×g, 30 min, 4 ºC) after which the supernatant solution was centrifuged (12000×g, 30 min, 4 ºC) to remove residual cells. γ-PGA was precipitated from the supernatant by adding acetone at a volumetric ratio of 3:1 and stored at 4 ºC for 24 h.[23] The supernatant was concentrated by ultrafiltration using a 30000 molecular weight cut off membrane (Model 8010, Millipore Corp., Bedford, MA, U.S.A.). The retentate was washed twice with deionized water, and freeze-dried. The PGA concentration was determined by gel permeation chromatography (GPC) (TSK gel GMPWXL and GMPW column, Tosoh, Tokyo, Japan; Asahipak GS-620HQ, Showa Denko, Tokyo, Japan). Pullulan (Sigma-Aldrich, U.S.A.) was used as a standard. Twenty μg of a polymer sample in 20 μL of developing buffer was injected into the GPC and developed with 50 mM phosphate buffer (pH 7.0) at a flow rate of 0.8 mL/min. Total carbohydrate levels were estimated using the phenol sulfuric method.[24] A purified form of Gellan® CM (Sigma-Aldrich, U.S.A.) was used as the standard.

NMR Analysis. Polymer composition was confirmed using nuclear magnetic resonance (NMR). Analysis of ^{1}H and ^{13}C NMR was conducted with a NMR spectrometer (Varian Unityionva 500 NMR Spectrometer, MO) using DMSO-d_6 as an internal reference.

Viscosity and Shear Testing. Viscosity and shear rate of the 1% PGA solution was measured at 21, 30, 35, 40, 50 and 60 ºC at pH 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 using a viscometer (Rheolab QC, Anton Paar, Ahland, VA, U.S.A.).

Molecular Weight of γ-PGA by SDS-PAGE. The degree of PGA polymerization was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel. The protein standards (ovalbumin, 45 kDa; serum albumin, 66.2 kDa; phosphorylase b, 97 kDa; β-galactosidase, 116 kDa; acid phosphatase, 130 kDa) were stained with Coomassie brilliant blue and, after destaining in 7% acetic acid-10% methanol, the gel was stained for PGA with 0.5% methylene blue in 3% acetic acid and destained in water.

Statistical Analysis. All experiments were replicated three times. Two-way analysis of variance was performed using the SAS Statistical Analysis System.[25] Means were compared using the Duncan Grouping test at p=0.05.

Results and Discussion

Bacterial Growth. Growth and polyglutamic acid production ability of *B. subtilis* was determined by using RWP as a medium with supplementation of different carbon and nitrogen contents such as ammonium sulfate, glutamic acid, citric acid. The results showed that *B. subtilis* ATCC 6633 grew in both RWP and RWP media containing L-glutamic acid, citric acid, and/or ammonium sulfate (Table 1). Numbers of *B. subtilis* significantly increased in RWP medium from 4.3 to 10.92 $\log_{10}$ CFU/ml after 72 h at 30 ºC (p<0.05). *B. subtilis* reached populations of 11.09, 10.94 or 10.84 $\log_{10}$ CFU/mL in RWP media containing either citric acid or L-glutamic acid alone or citric acid and L-glutamic acid (2:2) together after 72 h of fermentation, respectively. Addition of L-glutamic acid did not significantly decrease cell growth (p>0.05). In the present study, cell growth was similar for each media used except that containing ammonium sulfate (p<0.05). In fact, the addition of ammonium sulfate to RWP solution significantly decreased the growth (p<0.05).

In our previous study, RWP was shown as an ideal media for

Table 1. Growth of *B. subtilis* During 72 h of Fermentation at 30 ºC

	Fermentation time (h)			
	Cell growth $\log_{10}$ CFU/mL			
Media	0	24	48	72
Medium E	4.5±0.09^{aA}**	9.87±0.23^{bA}	10.67±0.54^{cA}	11.23±0.53^{cA}
20: 0:0:0*	4.3±0.22^{aA}	9.47±0.32^{bA}	10.53±0.45^{cA}	10.92±0.44^{cA}
20:2:0:0	4.2±0.16^{aA}	9.56±0.29^{bA}	10.48±0.23^{cA}	11.09±0.12^{cA}
20:3:0:0	4.6±0.21^{aA}	9.76±0.34^{bA}	10.63±0.33^{cA}	10.88±0.56^{cA}
20:0:2:0	4.1±0.22^{aA}	9.47±0.45^{bA}	9.98±0.67^{cAB}	10.94±0.18^{cA}
20:0:3:0	4.3±0.18^{aA}	9.69±0.34^{bA}	10.63±0.25^{cA}	10.88±0.43^{cA}
20:0:0:0.5	4.6±0.1^{aA}	8.97±0.43^{bAB}	9.54±0.45^{cA}	10.12±0.49^{cAB}
20:0:0:0.75	4.4±0.25^{aA}	8.45±0.37^{bAB}	9.33±0.35^{cA}	9.97±0.97^{cB}
20:2:2:0	4.7±0.15^{aA}	9.67±0.76^{bA}	10.57±0.89^{cA}	10.84±0.59^{cA}
20:2:3:0	4.5±0.19^{aA}	9.87±0.58^{bA}	10.77±0.13^{cA}	11.15±0.65^{cA}
20:3:2:0	4.3±0.09^{aA}	9.75±0.45^{bA}	10.45±0.27^{cA}	10.96±0.92^{cA}
20:3:3:0	4.4±0.08^{aA}	9.39±0.23^{bA}	10.65±0.77^{cA}	11.13±0.86^{cA}
20:2:0:0.5	4.5±0.21^{aA}	8.99±0.78^{bAB}	9.67±0.48^{cA}	10.56±0.24^{cAB}
20:2:0:0.75	4.1±0.12^{aA}	9.12±0.82^{bA}	9.76±0.53^{cA}	10.49±0.54^{cAB}
20:3:0:0.5	4.3±0.07^{aA}	8.78±0.44^{bAB}	9.45±0.35^{cA}	10.36±0.43^{cAB}
20:3:0:0.75	4.6±0.09^{aA}	9.09±0.49^{bA}	10.12±0.29^{cA}	10.73±0.33^{cA}
20:0:2:0.5	4.3±0.19^{aA}	8.95±0.75^{bAB}	9.89±0.60^{cA}	10.95±0.96^{cA}
20:0:2:0.75	4.2±0.27^{aA}	8.96±0.59^{bAB}	10.18±0.22^{cA}	10.78±0.67^{cA}
20:0:3:0.5	4.7±0.12^{aA}	8.76±0.67^{bAB}	9.87±0.19^{cA}	10.89±0.29^{dA}
20:0:3:0.75	4.6±0.05^{aA}	8.89±0.99^{bAB}	9.95±0.92^{cA}	11.09±0.75^{dA}
20:2:2:0.5	4.5±0.08^{aA}	9.44±0.92^{bA}	10.54±0.57^{cA}	11.10±0.34^{cA}
20:2:3:0.5	4.3±0.06^{aA}	9.34±1.03^{bA}	10.65±0.49^{cA}	11.13±0.19^{cA}
20:3:2:0.5	4.4±0.07^{aA}	9.23±0.23^{bA}	10.45±0.68^{cA}	10.98±0.44^{cA}

*Whey powder: citric acid: glutamic acid: ammonium sulfate (%, wt/v). Mean±standard deviation (n=3).

**Means in the same column with different uppercase alphabets or in the same row with different lowercase alphabets are significantly different (p<0.05).

the growth of *B. subtilis* ATCC 6633.[22] Generally, in the present study, supplementation of RWP with glutamic acid and/or citric acid significantly made a difference in the growth of *B. subtilis* compared to the control (RWP) (p<0.05). In agreement with our findings, Kunioko and Gotto[26] and Gotto and Kunioko[11] also reported that the addition of glutamic acid or high levels of ammonium sulfate depressed the growth of *B. subtilis* in media containing citric acid.

Production of γ-Polyglutamic Acid. Table 2 shows the amount of γ-PGA produced by *B. subtilis* in the media tested during 72 h of fermentation. Only 0.23 and 0.57 g/100 mL of γ-PGA were produced in RWP media and Medium E, respectively. The addition of 2 or 3% of citric acid or glutamic acid alone in RWP did not significantly change γ-PGA production; however, production of γ-PGA increased 4-5 times using citric acid and glutamic acid together in RWP media (P>0.05). Moreover, the addition of 0.5% ammonium sulfate with 2 or 3% citric acid also significantly increased the production of γ-PGA from 0.23 to 0.49 or 0.50 g/100 mL, respectively (P<0.05). However, the presence of 0.5 or 0.75% ammonium sulfate alone or with glutamic acid in RWP medium slightly decreased the yield of γ-PGA. According to the results, the maximum amount of γ-PGA (1.57 g/100 mL) was synthesized by *B. subtilis* in RWP media when ammonium sulfate was used with both glutamic acid and citric acid.

Table 2. Levels of γ-Polyglutamic Acid and Polysaccharides Produced by *B. subtilis* in RWP Media with Different Carbon Sources during 72 h of Fermentation

Media	γ-PGA (g/100 mL)	Polysaccharides (g/100 mL)
Medium E	0.54±0.02^{b}**	0.03±0.01^{a}
20: 0:0:0*	0.23±0.08^{a}	0.54±0.06^{c}
20:2:0:0	0.24±0.05^{a}	0.22±0.01^{b}
20:3:0:0	0.28±0.06^{a}	0.20±0.01^{b}
20:0:2:0	0.45±0.07^{a}	0.12±0.02^{a}
20:0:3:0	0.34±0.03^{a}	0.11±0.02^{a}
20:0:0:0.5	0.14±0.08^{a}	0.05±0.01^{a}
20:0:0:0.75	0.12±0.06^{a}	0.04±0.01^{a}
20:2:2:0	0.99±0.07^{c}	0.15±0.02^{a}
20:2:3:0	1.13±0.04^{c}	0.16±0.03^{b}
20:3:2:0	0.87±0.01^{c}	0.17±0.01^{b}
20:3:3:0	1.23±0.03^{c}	0.13±0.02^{a}
20:2:0:0.5	0.49±0.03^{b}	0.12±0.05^{a}
20:2:0:0.75	0.32±0.02^{a}	0.10±0.01^{a}
20:3:0:0.5	0.50±0.02^{b}	0.13±0.02^{a}
20:3:0:0.75	0.34±0.03^{a}	0.17±0.04^{b}
20:0:2:0.5	0.21±0.02^{a}	0.12±0.03^{b}
20:0:2:0.75	0.19±0.05^{a}	0.11±0.01^{b}
20:0:3:0.5	0.25±0.03^{a}	0.09±0.02^{b}
20:0:3:0.75	0.24±0.04^{a}	0.14±0.05^{b}
20:2:2:0.5	1.34±0.06^{cd}	0.09±0.07^{a}
20:2:3:0.5	1.45±0.04^{d}	0.05±0.01^{a}
20:3:2:0.5	1.54±0.07^{d}	0.08±0.03^{a}
20:3:3:0.5	1.57±0.06^{d}	0.07±0.02^{a}

*Whey powder: citric acid: glutamic acid: ammonium sulfate (%, wt/v).
**Mean±standard deviation (n=3). Means in same column with different alphabets are significantly different (p<0.05).

PGA-producing bacteria were divided into two groups: glutamate-dependent and glutamate-independent producers.[26,27] In the former, PGA yield increased upon addition of glutamate to the medium, however considerable γ-PGA can be produced even in the absence of glutamate using the de novo pathway for L-glutamate synthesis.[26] As shown in Table 2, γ-PGA production was low when glutamic acid was not used as a nitrogen source; thus *B. subtilis* ATCC 6633 could be subsequently classified as a glutamate-dependent PGA producer. Most literature reports suggest using 2-3% of L-glutamic acid for the production of γ-PGA.[4,28] γ-PGA is an extracellular polymer produced from the intracellular glutamic acid via a membranous synthesis mechanism. Therefore, the amount of intracellular glutamic acid is important for the biosynthesis of PGA, and an increase in its level would result in more effective production of the polymer.[29] Similarly, Zhang *et al.*[30] demonstrated that addition of 2 g/L ammonium sulfate and 20 g/L glutamic acid into monosodium glutamate waste liquid used as a fermentation medium could provide economical production of γ-PGA by *B. subtilus* NX-2 in this study. Citric acid is also one of the main components for γ-PGA production. Kunioka and Goto[26] and Cronwick and Gross[3] proved that citrate was indeed a precursor substrate for polymer production, presumably via the tricarboxylic (TCA) cycle. Supplemented citric acid could be shifted to α-ketoglutaric by the TCA cycle and the α-ketoglutaric was then changed to glutamate through the glutamate synthetic pathway. According to the studies, the glutamate units of PGA come from both additional glutamate and the glutamate synthesized by glutamate synthetic pathway in this strain.[12,31]

Besides carbon sources, nitrogen sources such as ammonium sulfate or ammonium chloride are known to support production of PGA.[32,33] A free amino group necessary for bio-

synthetic pathway of PGA production can be readily derived from ammonium sulfate or ammonium chloride. Supportively, in the present study, ammonium sulfate addition with citric acid and glutamic acid made a significant difference in the amount of PGA in the medium. Moreover, the effects of ammonium sulfate on γ-PGA synthesis by *B. subtilis* were also examined by Gotto and Kunioka.[11] When no ammonium sulfate was added to the L-glutamic/citric acid medium, they reported that a small amount of γ-PGA was produced. However, when 2.5 g/L ammonium sulfate was added, PGA yield greatly increased. Similar to the present study, Kuniko and Gotto [26] reported that 0.96 g/100 mL γ-PGA was produced by *B. subtilis* IFO 3335 in a medium containing 3% L-glutamic acid, 2% citric acid and 0.5% ammonium sulfate.

Inorganic salts like $CaCl_2$ and $MnSO_4$ also have a significant effect on yield as well as stereochemical composition of γ-PGA.[34] Huang *et al.*[27] observed that the addition of $CaCl_2$ effectively reduced viscosity of culture broth and increased consumption of extracellular glutamic acid by 11.4%, leading to a higher γ-PGA yield compared to the control. In the current study, no minerals were added since RWP medium had a very rich mineral content (calcium (0.9-1.0%), magnesium (0.1-0.2%), sodium (0.8-0.9%), potassium (2.2-2.4%), phosphorous (0.8-1.0%), iron (10-12 ppm), copper (4-6 ppm), zinc (48-52 ppm), and manganese (24-26 ppm)).[14,27]

Polysaccharide Production. Some polysaccharide by-product formation (0.54 g/100 mL) was also observed during *B. subtilis* fermentation in this study in RWP (Table 2). In the current study, the presence of glutamic acid or ammonium sulfate in the media significantly reduced polysaccharide production ($p<0.05$). However, the amount of polysaccharide in the medium was comparably higher in the presence of citric acid. Minimum polysaccharide production (0.03 g/100 mL) occurred when glutamic acid, citric acid and ammonium sulfate were added into RWP media.

Similar to our previous study in which 51 to 76 g/100 mL polysaccharide was produced by *B. subtilis* in RWP during 72 h of fermentation.[22] RWP did not have any glucose but the lactose content was high. *B. subtilis*, which possesses β-galactosidase (lactose-hydrolyzing enzyme), can use lactose as a carbon source by hydrolyzing it to glucose and galactose to produce PGA and polysaccharides.[28] Nevertheless, Xu *et al.* [29] reported that *B. subtilis* NX-2 could not produce any γ-PGA or polysaccharide when lactose was used as a carbon source. According to these studies, glucose and excess citric acid in the media lead to polysaccharide production through TCA cycle and glycogenesis.[11,35]

NMR. ^{13}C NMR analysis of γ-polyglutamate showed the following chemical shifts: 56.43 ppm for β-CH_2 group, 31.61-34.01 ppm for γ-CH_2 group, 182.21 ppm for CO group, and 182.69 ppm for COO- group (Figure 1). ^1H NMR for γ-polyglutamate in D_2O produced the following chemical shifts: 3.98 ppm for -CH proton; 1.98 and 1.80 ppm for β-CH_2 proton; and 2.19 ppm for γ-CH_2 proton (Figure 2). This NMR spectrum is quite similar to that of γ-PGA reported by Wu and Ye[36] and Perez-Camero *et al.*.[37] Hence, there is a good possibility that the polymer produced by *B. subtilis* contained the structure of PGA. There are also -CH_2O, -CH_2OH, -CH-OH groups observed in the ^1H NMR spectrum and CHOH-CHOH bonds in the ^{13}C NMR spectrum (Figures 1, 2). These chemical shifts indicate that the polymer might contain several polysaccharides as well as γ-PGA.

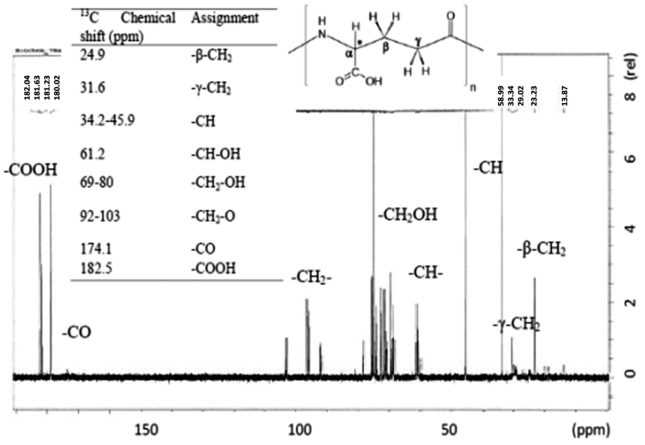

Figure 1. ^{13}C NMR spectrum of polymer produced by *B. subtilis* in RWP media with different carbon sources.

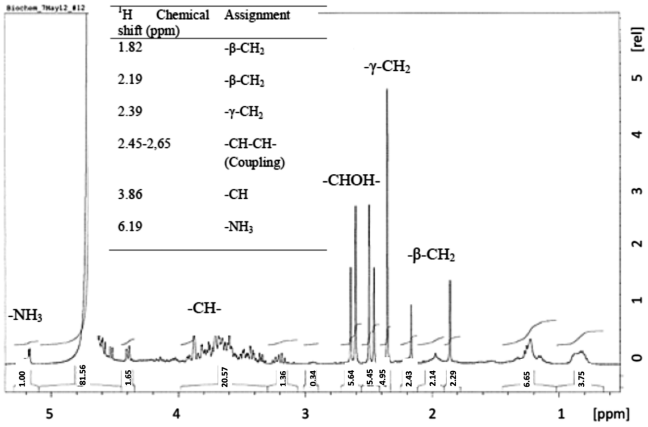

Figure 2. ^1H NMR spectrum of polymer produced by *B. subtilis* in RWP media with different carbon sources.

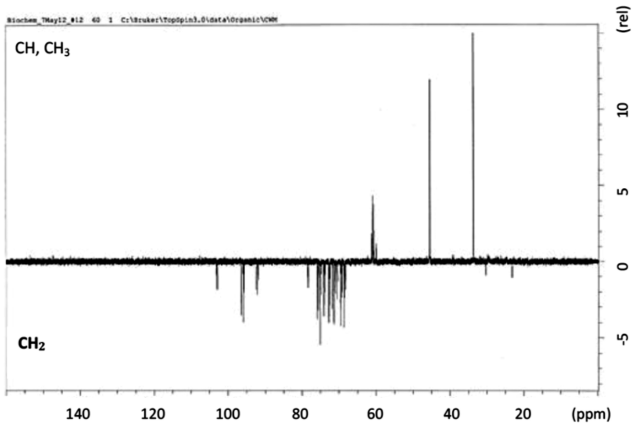

Figure 3. ^{13}C NMR DEPT 135 spectrum of polymer produced by *B. subtilis* in RWP media with different carbon sources.

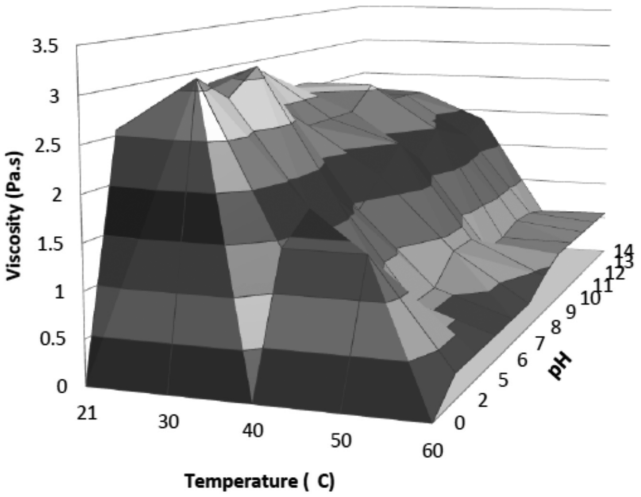

Figure 4. Dynamic viscosity of 1.0% PGA solution at different temperatures and pH values.

^{1}H and ^{13}C NMR techniques have been widely used to estimate the structural parameters of polymers. However, these two methods have limitations in resolving the resonance of tertiary and quaternary carbons of -CH, -CH_2 and -CH_3 in the aliphatic region. With the emergence of various multi-pulse one- and two-dimensional NMR techniques, interpretation of the ^{13}C NMR spectra has been greatly facilitated. Recently, distortion less enhancement by polarization transfer (DEPT) has been developed to simplify and assign the resonances in the overlap regions of p3 C NMR spectra. In this study, the DEPT 135 technique was used to confirm C atoms in our structure. The DEPT 135 spectrum does not show carbon atoms without any proton bonds (Figure 3) which is why the chemical shift at 182.21 for CO group and 182.69 for COO- group did not appear. Moreover, in this kind of spectrum, the peaks on the positive side of the graph represent -CH or -CH_3 and the peaks on the lower side represent -CH_2. Thus, the DEPT 135 spectrum confirmed that the chemical shifts were 56.43 ppm for -CH_2 group, 31.61 ppm for -CH_2 group, 34.01 ppm for -CH_2 group, 34.2-45.9 ppm for -CH group, 61.2 ppm for CH-OH group, 182.21 ppm for -CO group, and 182.69 ppm for COO- group.

Viscosity and Shear Rate. The temperature and pH dependent viscosity of a 1% γ-PGA solution were measured at a constant shear rate (10.21/s) (Figure 4). Viscosity of the γ-PGA solution generally decreased with increasing temperature and decreasing pH. Maximum dynamic viscosity of the 1% γ-PGA solution was observed at 30 ºC, pH 6 as 3.13 Pa.s a the minimum viscosity of 0.28 Pa.s seen at 60 ºC, pH 2. Increased viscosity at higher pH values may be attributed to the dissociation of carboxyl groups and to the conformational change of PGA from the helical to the randomly coiled form.[38] The change of dissociation of the ionizable groups caused by the temperature shift has a major impact on dynamic rigidity although the temperature dependency of this dissociation is usually small. Similarly, Ho *et al*.[39] found that increasing the pH and decreasing the temperature promoted viscosity of a γ-PGA solution measured as 100-400 cps. These results also indicate that the polymer solution was non-Newtonian and pseudoplastic. Increasing the speed rate decreased polymer shear stress (data not shown). Non-Newtonian flow behavior is often observed for microbial biopolymers.[35,40]

Molecular Weight of γ-PGA. The molecular weight of the precipitated peptide was about 1.3×10^5 Da for each media used in this study by SDS-PAGE and GPC (Figure 5). According to these results, using different media did not change the molecular weight of γ-PGA produced by *B. subtilis*. Moreover, PGA hydrolysis was minimal in the some of the media including RWP with glutamic acid, citric acid, RWP with glutamic acid and RWP with citric acid after fermentation as reported by others.[34,41]

The γ-PGA produced by *Bacillus* sp. generally has a high molecular weight in the range of 10^5-10^6 Da.[34] Some studies suggested that final molecular weight can decrease as fermentation time increases, owing to an enzyme that catalyzes the hydrolytic breakdown of γ-PGA.[26,42] However, in the current study, hydrolysis was not observed RWP or RWP containing ammonium sulfate. Similarly, these studies showed that the addition of ammonium sulfate to the medium for γ-PGA production depressed cell growth and lead to the production of γ-PGA with a high molecular weight by reducing the hydrolysis.[41,34] Kunioko and Gotto[26] also showed that addition of

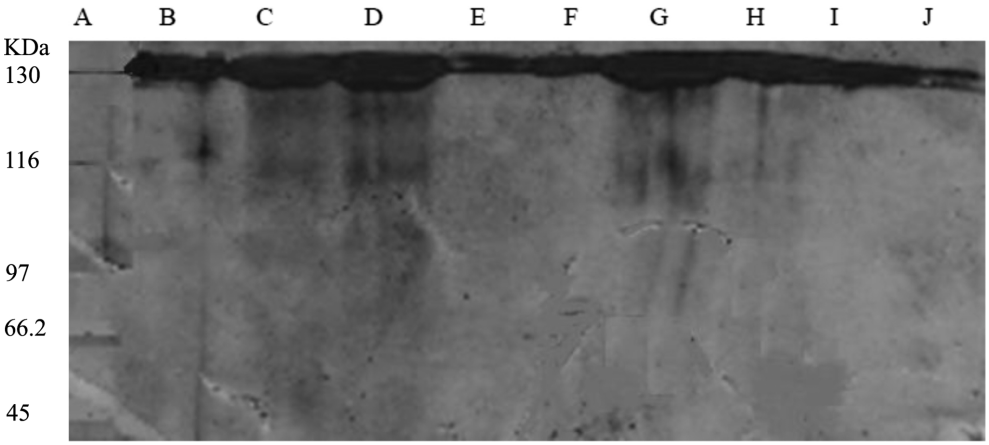

Figure 5. Molecular weight of PGA produced in different media including A: Standards, B: Medium E, C: Whey powder/glutamic acid/citric acid/ammonium sulfate, D: Whey powder/glutamic acid, E: Whey powder/glutamic acid/citric acid, F: Whey powder/glutamic acid/ammonium sulfate, G: Whey powder/citric acid, H: Whey powder/citric acid/ammonium sulfate, I: Whey powder/ammonium sulfate, J: Whey powder.

higher than 0.25% of ammonium sulfate reduced the hydrolysis of γ-PGA during stationary phase.

Conclusions

As a conclusion, the results showed that RWP medium can be used as a fermentation substrate to produce γ-PGA by using *B. subtilis* ATCC 6633 and also indicated that the presence of glutamic acid, citric acid and ammonium sulfate in RWP medium improved production of γ-PGA. For a broader application, the cost of bioproducts is one of the main economic determinants of the process. Reducing the cost of biopolymer production by optimizing the fermentation medium is a prerequisite for industrial application. Using RWP as a low-cost and high nutrient content fermentation medium for production of γ-PGA will meet this industry objective.

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