Production and characterization of microbial poly-γ-glutamic acid from renewable resources

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Poly-γ-glutamic acid (PGA) is an anionic, naturally occurring, biodegradable, edible and water soluble polyamide that has widespread applications viz., thickener, cryoprotectant, drug carrier, biological adhesive, biopolymer flocculant, heavy metal absorber, bitterness relieving agent, etc. Here, we evaluated effectiveness of renewable resources for production of γ-PGA using an isolated Bacillus sp. Lignocellulosic biomass such as rice straw, sugarcane trash, sugarcane bagasse, cotton stalk and sorghum stover were evaluated for production of γ-PGA. Pretreatment was carried out using 1% (w/w) H2SO4 for rice straw and 2.5% (w/w) H2SO4 for rest of the biomass, followed by enzymatic hydrolysis using cellulase enzyme. Comparative evaluation of sugar yield from various biomass were done and highest percentage of reducing sugar was obtained from rice straw hydrolysate. The hydrolysate was concentrated to obtain 1.01 g/mL of reducing sugar and was added to the production media for the fermentative production of γ-PGA. The maximum PGA obtained was 82.97 g/L by supplementing rice straw hydrolysate (reducing sugar concentration: 4%) by Bacillus amyloliquefaciens. The γ-PGA was characterized by NMR, FTIR, gel permeation chromatography and amino acid analyzer. Present work shows the feasibility of using lignocellulosic biomass as a cheap and economically efficient carbon source for the fermentative production of microbial γ-PGA.

Keywords: Bacillus sp., Hydrolysis, Lignocellulosic biomass, PGA, Poly amino acids, Submerged fermentation

Poly-γ-glutamic acid (γ-PGA) is an anionic, naturally occurring, water soluble polyamide in which D- and L-glutamate units are formed via γ-amide linkages1,2. It is biodegradable, edible and nontoxic and non-polluting3,4. Poly-γ-glutamic acid, poly-ε-lysine (ε-PL), and cyanophycin are naturally occurring poly amino acids5. Poly amino acids differ from proteins in various aspects. Proteins are composed of a variety of amino acids; the poly amino acid consists of only one type of amino acid (at least in the backbone). A different pathway biosynthesizes poly amino acids in microorganisms. It is produced by ribosome independent manner, D- and L-glutamates are copolymerized in a single filament (g-DL-PGA) by the membrane γ-PGA synthetase6,8,9. It is free from protease attack as it is made up of D-and L-glutamic acid units linked by α-amino and γ-carboxylic acid units9. Usually, proteins and other peptides are made up of α-amino and α-carboxylic acid units which are susceptible to protease attack. It is an optically active polymer with the chiral center in every glutamate residues. It consists mainly of three types including a homopolymer of D-glutamic acid units, a homopolymer of L-glutamic acid units and a copolymer of DL-glutamic acid units.

PGA was first discovered by Ivanovics and Bruckner as a component of capsules of Bacillus anthracis. They observed that PGA was released into the medium on autoclaving, or on aging and autolysis of the cells. It was also present in the mucilage of “natto” (fermented soybeans, a traditional food in Japan) and later found that γ-PGA was freely secreted into the growth medium of Bacillus subtilis as a product of fermentation and several Bacillus species have now been shown to produce γ-PGA outside the cells10,12. It has widespread applications such as a thickener, bitterness relieving agent, cryoprotectant, sustained release material, the drug carrier, curable biological adhesive, biodegradable fibres, highly water-absorbable hydrogels, biopolymer flocculants and heavy metal absorber13,15.
Its extensive applications invite intensive research to improve upon production, recovery, and purification from the fermentation broth. In submerged fermentation, the nutritional requirements for cell growth and culture conditions are the main areas of investigation for enhanced production. Production of α-PGA by submerged fermentation, glucose, glutamic acid, citric acid and glycerol was usually used as carbon sources. Citrate and glutamate are precursor substrates for polymer production. The large scale production of α-PGA is not feasible because of raw material cost. The renewable lignocellulosic biomass is a promising choice as a cost attractive, environmentally friendly and sustainable resource for α-PGA production. The lignocellulosic material, mainly made up of cellulose (40-50%), hemicelluloses (25-50%) and lignin (10-40%) is considered as the suitable substrate for value added products.

In this study, lignocellulosic biomass such as rice straw, sugarcane trash, sugarcane bagasse, cotton stalk and sorghum stover have been pretreated using mild acid followed by enzymatic hydrolysis and screened based on the sugar content. To study the feasibility of hydrolysate as carbon source for PGA production, lignocellulosic biomass was pretreated and hydrolyzed to get reducing sugars, as the sugars were utilized by Bacillus to convert into products.

Materials and Methods
Culture maintenance, media components, and culture conditions
A strain of Bacillus licheniformis was used in this study. The growth and maintenance medium contained (g/L) peptone 5, yeast extract 1.5, beef extract 1.5, sodium chloride 5, and agar 20. Bacterial culture in agar plates were incubated at 37°C for 24 h, stored at 4°C and subcultured at regular intervals.

Mild acid pretreatment of lignocellulosic biomass
Rice straw, sugarcane trash, sugarcane bagasse, cotton stalk and sorghum stover were purchased locally and milled and pretreated using mild acid (H2SO4) under the following conditions. Sugarcane bagasse (2.5% w/w acid loading and 20% solid loading), rice straw (1% w/w acid loading and 15% solid loading), cotton stalk, sorghum stover, sugarcane trash (2.5% w/w acid loading and 25% solid loading), and pretreated at 120°C for 60 min in an autoclave. After pretreatment, biomass was neutralized with 10N NaOH and washed with water and dried. The dry material was used for hydrolysis reaction.

Enzymatic hydrolysis of pretreated ligno-cellulosic biomass
Each pretreated biomass was hydrolyzed using commercial cellulase from Zytex India, Pvt, Ltd., Mumbai, India. The pretreated biomass loading (30% w/w), enzyme (FPU: 20/g), tween 80 (0.05%w/v) and 0.05M citrate buffer (pH-4.8) were added and incubated at 50°C in a shaking water bath at 200 rpm for 48 h. After the reaction, the total hydrolysate was centrifuged at 8000 rpm for 10 min to recover supernatant for analysis of total reducing sugar. Sugar concentrations in lignocellulosic biomass hydrolysate were measured by HPLC (Shimadzu Prominence UFLC) fitted with Rezex RPM Monosaccharide Pb+2 column (Phenomenex, India & RI detector). The column temperature was maintained at 80°C, and the flow rate was maintained at 0.6 mL/min. De-ionized water was used as the mobile phase.

PGA production by Submerged fermentation
A loopful of culture from a plate was transferred to 50 mL of the seed medium (peptone 5g/L, yeast extract 1.5, beef extract 1.5 and sodium chloride 5 g/L and pH 7) in a 250 mL conical flask, incubated at 37°C and 200 rpm for 16 h to reach the cell concentration of 7×10^8 CFU/mL. The cell suspension was inoculated into a modified E medium (1%v/v) pH-6.5 in which glycerol (30 g/L)/ citric acid (4 g/L) were replaced with rice straw hydrolyzate, glutamic acid (30 g/L), NH4Cl (6 g/L), K2HPO4 (1 g/L), MgSO4 (0.05 g/L), CaCl2 (0.2 g/L), FeCl3 (0.03 g/L), MnSO4 (0.05 g/L) and carbon source by varying concentration was added: (a) hydrolysate (3%) + citric acid (4%); (b) glycerol + hydrolysate (3%); (c) glycerol:hydrolysate (1:1) + citric acid; and (d) glycerol + hydrolysate:citric acid (1:1). Then incubated aerobically at 37°C, 200 rpm for 96 h. Further experiments were done for optimization of the hydrolysate concentration. Instead of glycerol, hydrolysate was added by varying concentrations and production of PGA was evaluated.

Downstream processing and Purification of α-PGA
The culture supernatant was collected by broth centrifugation for 8000 rpm for 15 min, and the purification was done by the ethanol precipitation method. To the supernatant added four volumes of ice cold ethanol and kept at 4°C for 12 h. The precipitate
obtained was collected by centrifugation for 8000 rpm for 20 min at 4°C. Dissolve the pellet in distilled water and treated with activated charcoal and centrifuged and collected the supernatant. The polymer was recovered by ethanol precipitation and collected the pellet after centrifugation for 8000 rpm for 20 min at 4°C and dried in an oven.

Characterization of poly gamma glutamic acid

Molecular weight and the molecular weight distribution of γ-PGA was determined by gel permeation chromatography using Shodex OH-PAK SB 804(300×8 mm) column with de-ionized water as mobile phase at a flow rate of 1 mL/min. Dextran standards were used to construct a calibration curve. 1H NMR spectroscopy was done with Bruker, 500 MHz spectrophotometer and samples for NMR were dissolved in D2O solution. The analysis of γ-PGA structure was done by Fourier Transform Infrared Spectroscopy (FTIR). The KBr mode was carried out to determine the PGA produced and recorded the transmission spectra in the range of 400-4000 cm⁻¹. Thermogravimetric analysis (TGA) was carried out for powder samples using a TA Instruments from 25°C to 700°C under N₂ flow at a heating rate of 10°C/min.

Amino acid analysis was done to for further confirmation. The purified material was hydrolyzed with 6N HCl at 110°C for 24 h in a sealed, and evacuated tube and the amino acid compositions were determined by Shimadzu HPLC system equipped with Agilent Zorbax Eclipse AAA. O-Phthaldialdehyde was used as the pre-column derivatization agent, and the derivatization reaction was carried out at a buffering pH of 10.2. The mobile phases were 40 mM Na₂HPO₄ and Acetonitrile:Mehtanol:Water (45:45:10, v/v/v), with gradient elution and run time of 32 min. TLC of PGA hydrolysate was done on silica gel 60 F₂₅₄ and eluted with n-butanol:acetic acid:water 3:1:1 by volume) mixture and visualized with 0.2% ninhydrin.

Results

Sugar concentration from lignocellulosic hydrolysate was measured by HPLC, and highest percentage of sugar yield was obtained from rice straw hydrolysate as shown in Table 1. Compared to other biomass such as sugarcane trash, sugarcane bagasse, cotton stalk and sorghum stover, sugar yield was high in rice straw hydrolysate. Hence, rice straw hydrolysate was chosen as the carbon source for γ-PGA production, and the yield of γ-PGA from rice straw hydrolysate was monitored by the following experiments. Rice straw hydrolysate was concentrated to 1.01 g/mL of initial glucose concentration by lyophilization and was supplemented to PGA production media and fermentation carried out for 96 h. The amount of γ-PGA obtained from the experiment was shown in Fig. 1. Further, optimization of rice straw hydrolysate concentration was done, and the maximum yield was 82.97 g/L from 4% rice straw hydrolysate was shown in Fig 2. Rice straw hydrolysate could replace commercial glycerol/glucose in case of γ-PGA production. The conversion of sugar into poly gamma glutamic acid has taken place so that rice straw hydrolysate could be used as the potent substrate for γ-PGA production. The cost-effective production and the utilization of renewable biomass are the main advantages of using lignocellulosic biomass as the carbon source.

<table>
<thead>
<tr>
<th>Biomass loading (% w/w)</th>
<th>Acid loading (% w/w)</th>
<th>Sugar yield (mg/mL)</th>
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</thead>
<tbody>
<tr>
<td>Rice straw (15)</td>
<td>1.0</td>
<td>25.18</td>
</tr>
<tr>
<td>Sugar cane trash (25)</td>
<td>2.5</td>
<td>8.7</td>
</tr>
<tr>
<td>Sugar cane bagasse (20)</td>
<td>2.5</td>
<td>17.3</td>
</tr>
<tr>
<td>Sorghum (25)</td>
<td>2.5</td>
<td>13.3</td>
</tr>
<tr>
<td>Cotton stalk (25)</td>
<td>2.5</td>
<td>11.75</td>
</tr>
</tbody>
</table>
Downstream processing and Purification of γ-PGA

Recovery of PGA was done by ethanol precipitation and activated charcoal treatment. Activated charcoal treatment was done to remove impurities, and other colored substances from the ethanol precipitated sample to get purified material for further applications. Here, the purification of PGA by activated charcoal treatment was found to be cost-effective as compared to other.

Characterization of PGA

The PGA produced by *Bacillus* sp. generally has high molecular weight. The molecular weight of PGA is dependent on the bacterial strains, medium components, and culture conditions. The molecular weight of PGA produced in this study was approximately 1.3 KDa. Poly gamma glutamic acid with high molecular weight has applications such as flocculating agent, cryoprotectant, thickener in food, etc. HNMR spectrum was shown in Fig. 3. The chemical shift in ppm 1.95-2.07 ppm (β, 2H), 2.26-2.28 ppm (γ, 2H), 3.66-3.69 (α, 1H) corresponds to the peak positions of standard γ-PGA. The FTIR spectrum of PGA was given in Fig. 4. The characteristic peaks of γ-PGA such as asymmetric COO− stretch observed at 1582 cm⁻¹ with a broadband due to peak overlap of N-H/C-H...
deformation and a symmetric COO⁻ stretch at 169 cm⁻¹ were observed in the FTIR spectrum which indicates the purity of the compound. From the thermo gravimetric analysis the initial weight loss at a temperature below 100°C caused by the removal of adsorbed water or surface hydroxyl groups depicted in Fig. 5. Further, weight loss till 700°C was occurred mainly because of the degradation of the polymer material. Characterization of PGA was done by amino acid analysis and thin layer chromatography (TLC). The 6N HCl hydrolysate of PGA was composed solely of glutamic acid units by amino acid analysis. The retention time obtained by amino acid analysis was comparable to that of a glutamic acid standard. From TLC of 6N hydrolysate of PGA obtained a single spot with Rf value identical to that of standard glutamic acid which was performed on a silica gel and visualized with 0.2% ninhydrin.

**Discussion**

Poly gamma glutamic acid has broad applications, and its improved production and purification are the current focus. Due to wide applications of PGA, the researchers focus on the production of PGA from low-cost renewable feedstock, lignocellulosic biomass. PGA was found to be produced from various carbon sources such as sucrose, glucose, fructose and citrate by various Bacillus strains¹⁷. The raw nutrient cost brings a barrier for the fermentative production of γ-PGA. The conversion of agro-industrial residues for poly gamma glutamic acid production could be achieved using lignocellulosic biomass hydrolysate in submerged fermentation and also by solid state fermentation. Soybean cake powder, soybean meal, wheat bran, dairy manure compost, swine manure, sweet potato, monosodium glutamate production residues and corn flour, etc. alone or in combination have been used as the substrate for γ-PGA production by solid state fermentation¹⁸,²⁶. In submerged fermentation, γ-PGA was produced from rice straw hydrolysate, untreated cane molasses, sugarcane juice, waste water containing monosodium glutamate and corncob fiber hydrolysate are some of the cost effective raw materials for γ-PGA production²¹,²². The low-cost corncob fiber hydrolysate was chosen as the alternative complex carbon source to produce γ-PGA by Bacillus subtilis HB-1, and the yield was 24.92g/L²³. γ-PGA was produced from cane molasses by Bacillus subtilis NX-2 immobilized on chemically modified sugarcane bagasse. From this study, by fed-batch fermentation 84 g/L of PGA was produced²⁴.

The production of PGA from untreated cane molasses in a bioreactor and from monosodium glutamate waste liquor was reported. 33.6 g/L of PGA was produced from untreated cane molasses in batch fermentation and to minimize the substrate inhibition fed-batch fermentation was carried out, and the yield was 50.2 and 51.1 g/L from untreated cane molasses and hydrolysed molasses, respectively. Further studies on PGA production was carried out using monosodium waste liquor as carbon source, and resulting PGA concentration of 52.1 g/L was obtained²⁵. Among lignocellulosic biomass, rice straw hydrolysate was the highest report to date; 73 g/L was reported from rice straw hydrolysate by continuous feeding strategy by Bacillus subtilis NX-2²⁶.

In this study, pretreatment and hydrolysis were done for the efficient utilization of sugar from the lignocellulosic biomass for the economical and green production of γ-PGA. Among the biomass used, sugar yield was highest in case of rice straw, and rice straw is one of the widespread lignocellulosic biomass and its annual production proximately 731 million tonnes across the world²⁷. Rice straw utilization in fermentation could be a promising approach to manage this agricultural waste and to produce environmentally friendly products²⁸. γ-PGA generated by fermentation was characterized by NMR, FTIR, GPC and amino acid analyses. The production of γ-PGA from low cost, non-food lignocellulosic material could be achieved by this study.

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