Effects of media components and agricultural by-products on γ -polyglutamic acid production by *Bacillus toyonensis* As8

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Abstract

Background. Poly- γ -glutamic acid (γ -PGA) provides an environmentally friendly alternative to plastic materials which have widely polluted the environment.

Objectives. The microbial production of γ -PGA, an amino acid biopolymer with glutamic acid subunits, was investigated using renewable agricultural residues in an attempt to find cheaper substitutes for conventional synthetic media components.

Material and methods. Bacteria which produce γ -PGA were isolated through depolymerizing *Coix lacryma-jobi*, a cellulosic grass, and the effects of various carbon and nitrogen sources, temperature, inoculant load, incubation period, and pH on γ -PGA yield were determined after submerged fermentation. Bacterial growth was measured turbidimetrically at 550 nm. The γ -PGA produced was characterized using Fourier transform infrared (FT-IR) spectroscopy and the polymer shape was determined using scanning electron microscopy (SEM).

Results. The best γ -PGA producer was molecularly identified as *Bacillus toyonensis* As8. The conditions which produced the highest γ -PGA yield were glucose, ammonium sulfate, 25°C, a pH of 5.5, and an incubation period of 48 h. This bacterium yielded the most γ -PGA (26.45 g/L) on cassava peels, while other agro-wastes (corn cob, sorghum leaves, *Coix noir* leaves, and rice bran) also supported bacterial growth with lower γ -PGA yields than conventional carbon sources. The wrinkled γ -PGA had absorbance peaks of hydroxyl, amide, carbonyl, and amine groups comparable with the ranges of those found in commercial γ -PGA.

Conclusions. The use of agricultural by-products as fermentation substrates increased γ -PGA yield and may therefore be used as substitute components in γ -PGA production.

Key words: agricultural wastes, biopolymer synthesis, poly- γ -glutamic acid characteristics, *Bacillus toyonensis*

Introduction

Poly- γ -glutamic acid (γ -PGA) is a non-toxic, anionic, water-soluble, and biodegradable homopolyamide consisting of D- and L-glutamic acid units polymerized by γ -amide linkages and found between α -amino and γ -carboxylic acid groups^{1–3} (with the molecular formula $(C_5H_7NO_3)_n$). In light of the many undesirable properties associated with the use of chemically manufactured products, γ -PGA – along with many other biopolymers – has enjoyed a growing interest due to its biodegradability and non-toxicity. When compared with other production methods – such as chemical synthesis, peptide synthesis and biotransformation – microbial fermentation is considered the most cost-effective, having numerous advantages: minimal environmental pollution, potential production using inexpensive raw materials, high natural product purity, and mild reaction conditions.^{3–5} This important biopolymer has been found in species from all domains of life, including archaea, bacteria and eukaryotes.^{6,7} The traditional Japanese food natto – *Bacillus subtilis*-fermented soybeans – contains a naturally occurring mucilaginous mixture of γ -PGA and fructan. Apart from *Bacillus*, many other species have been reported to produce γ -PGA, such as *Planococcus*, *Sporosarcina*, *Staphylococcus*, *Fusobacterium*, *Natrialba*, and *Hydra*.^{3–7} At present, microbial fermentation of biomass is still the most preferred means of commercial γ -PGA production.⁵

The desirable properties of γ -PGA as a safe, biodegradable, edible, eco-friendly, and water-soluble biopolymer make it and its derivatives important as food thickeners, bitterness-relieving agents,⁸ humectants, cryoprotectants,⁹ sustained-release materials, drug delivery agents, biological adhesives, heavy metal absorbers, bioflocculants, dye-removing agents, fertilizer synergists, and biodegradable plastics. Other potential applications may include its use as a contrast agent or vaccine adjuvant, or in the areas of immobilization, microencapsulation, gene delivery, and tissue engineering.^{1,8–10}

Although the process leading to the microbial fermentative biosynthesis of γ -PGA is well-known, some challenges remain, such as the cost and suitability of substrate media for optimal yields, a fact which limits economically viable commercial applications. In addition to the important efforts currently directed at finding a lasting solution to various problems associated with γ -PGA production for commercial applications, there is a need to continue the search for potential γ -PGA producers with unique properties. In this study, the effects of media components and the suitability of agricultural byproducts as substrates for γ -PGA production in flask fermentation by various bacteria were investigated.

Material and methods

Sample collection, microorganisms and screening for γ -PGA production on a solid medium

The *Bacillus toyonensis* (*B. toyonensis*) As8 used in this experiment was isolated from samples of decomposing *Coix lacryma-jobi* collected from a fallow agricultural farm of the University of Ibadan, Nigeria. The bacteria were isolated through serial dilutions of the samples¹¹ inoculated using the pour plate method on nutrient agar and incubated at 30°C for 24 h. Bacterial colonies observed on the surface of the nutrient agar were picked at random, based on differences in colonial morphology, and were streaked onto the surface of nutrient agar to obtain distinct colonies representative of a single pure isolate.

Screening for γ -PGA production

Pure cultures of each bacterial isolate were cultivated on a solid medium composed of 1 g/L glucose, 0.5 g/L yeast extract, 1 g/L L-glutamic acid, 0.05 g/L KH_2PO_4 , 0.01 g/L $MgSO_4$, and 15 g/L agar (pH 7.0) at 37°C for 24 h.¹² Sticky, highly viscous colonies forming on the agar – examined for stickiness with gentle touches using a sterile inoculating needle – were considered γ -PGA-producing bacterial isolates. The isolates (10% v/v 24-hour-old broth culture, equivalent to 10⁸ CFU/mL) selected from the solid screening medium as described previously were grown at 37°C on a shaker incubator (ZHWHY211E, New Brunswick Scientific Company, New Jersey, US) at 150 rpm for 72 h in a conical flask containing 100 mL of screening broth (10 g/L L-glutamic acid, 10 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $MnSO_4$, and 0.05 g/L $FeCl_3 \cdot 7H_2O$). After 72 h of incubation, the broth culture was centrifuged at 12,000 rpm for 20 min at 4°C to obtain cell-free supernatants, after which an equal volume of cold ethanol was added to the supernatant to yield a fibrous precipitate regarded as the crude γ -PGA. This precipitate was concentrated with centrifugation and was subsequently oven-dried at 55°C to a constant weight; the weight was then measured.^{12,13} The best γ -PGA-producing bacteria were identified with morphological, biochemical and 16S rRNA gene sequencing using universal primers.^{11,14,15}

Recovery and quantification of the produced γ -PGA

The cells were separated from the fermentation broth with 20 min of centrifugation at 12,000 rpm to obtain a cell-free supernatant. The γ -PGA was precipitated from the supernatant through the addition of 4 volumes of 95% ethanol with gentle stirring. The mixture was then stored

in a refrigerator (Haier THERMOCOOL BD-124E, HPZ Nigeria) at 4°C for 12 h. The resulting precipitate containing crude γ -PGA was collected using a high-speed refrigerated centrifuge (Hitachi Himae CR21GII, Hitachi Group, Tokyo, Japan) at 12,000 rpm for 20 min at 10°C. The crude γ -PGA was oven-dried at 55°C to a constant weight, which was then measured.⁶

Effects of medium components and environmental conditions on γ -PGA production

The effects of environmental conditions and the components of the medium on γ -PGA production using the selected bacterial isolates were investigated using the one-factor-at-a-time method with a PGA basal medium containing 5 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/L MnSO_4 , and 0.05 g/L $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$.¹³ Variables such as the effects of different carbon sources (glucose, fructose, maltose, lactose, sucrose, citric acid, and starch) and nitrogen sources (peptone, urea, yeast extract, L-glutamic acid, ammonium sulfate, ammonium chloride, and sodium nitrate), pH (4.5 to 9.0 in 0.1M phosphate buffers), incubation temperature (25°C, 30°C, 35°C, 40°C, and 45°C), different inoculant loads (1% and 10%), and incubation period (24–96 h) on the production medium were investigated in order to determine the ones which are most conducive to γ -PGA production. To measure the effects of the different carbon sources, the basal medium contained 20 g/L L-glutamic acid, 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/L MnSO_4 , and 0.05 g/L $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$. The L-glutamic acid was then substituted for each of the sugars listed above. Likewise, to test the effects of nitrogen sources, the basal medium contained 20 g/L glucose, 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/L MnSO_4 , and 0.05 g/L $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$; $(\text{NH}_4)_2\text{SO}_4$ was then substituted for the other nitrogen sources.

To measure the effects of pH, incubation temperature and incubation period, the basal medium consisted of 20 g/L L-glutamic acid, 20 g/L glucose, 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L K_2HPO_4 , 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/L MnSO_4 , and 0.05 g/L $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$.¹³ A loopful of 18–24-hour-old nutrient agar culture of the γ -PGA-producing bacterium was transferred into the PGA broth and incubated at 35°C for 24 h. A 10% v/v dilution of this preparation was used as an inoculant for the experiments. The fermentation flasks were incubated with agitation at 150 rpm over 4 days. Bacterial growth was determined with optical density using a Jenway 6405 UV-VIS spectrophotometer (Cole-Parmer, UK) at 550 nm.^{6,16}

The effects of different agricultural wastes (corn cob, sorghum leaves, *Coix noir* leaves, cassava peel, and rice bran) as carbon sources for γ -PGA production by the selected γ -PGA producers were investigated. Freshly collected agricultural

wastes were oven-dried at 45°C, pulverized and sieved to obtain powder-sized particles. Each of these substrates (20 g/L) was added into the γ -PGA production medium as the major source of carbon and autoclaved at 121°C for 15 min, after which they were allowed to cool to room temperature and were inoculated as described previously. They were then incubated at 35°C on a shaker incubator (ZHWY211E, New Brunswick Scientific Company, New Jersey, US) at 150 rpm for 72 h. At the end of the incubation, the fermentation broth was diluted with an equal volume of sterile distilled water and centrifuged using the refrigerated centrifuge at 12,000 rpm for 20 min to recover the γ -PGA; the yield was then measured.^{6,16} The best environmental variables and agricultural wastes for the highest γ -PGA yield in these experiments were used to produce γ -PGA.

In all cases, the means of triplicate experimental readings were used.

Characteristics of γ -PGA

The peaks of the spectra of key functional groups in the γ -PGA produced in the experiments were identified using Fourier transform infrared (FT-IR) spectroscopy. Their absorption spectra, with peaks corresponding to specific bonds in the γ -PGA product, were compared with standards characteristic of amine (C–N), carbonyl (C=O), amide (N–H), and hydroxyl (OH) groups in the ranges of 1085–1165 cm^{-1} , 1394–1454 cm^{-1} , 1620–1655 cm^{-1} , and 3400–3450 cm^{-1} , respectively.^{17,18} Scanning electron microscopy (SEM) was also used to determine the surface morphology of the polymer produced. An FEI Inspect S50 scanning electron microscope (FEI Company, Japan) with an acceleration voltage of 10 kV was used. The polymer samples were placed on a metallic stub and sputtered with gold film under vacuum; images were taken at different levels of magnification.

Results and discussion

Screening of isolates for γ -PGA production

Approximately 14% of the 36 isolates obtained from the decomposing *Coix lacryma-jobi* (As8, Is6, Is7, Is13, and Is14) produced viscous colonies when screened. The highest yield from the submerged fermentation, 16.53 g/L of γ -PGA, was recovered from the As8 fermentation culture. This was followed by isolates Is14, Is6, Is7, and Is13 with γ -PGA yields of 14.82 g/L, 14.07 g/L, 12.58 g/L, and 9.42 g/L, respectively. The 5 γ -PGA-positive isolates were Gram-positive, facultative, anaerobic spore formers and were presumptively identified as *Bacillus* species (Table 1). Phenotypic and biochemical techniques have been successfully used in the past to identify different microorganisms in microbiology.¹⁶ Isolate As8, the most prolific γ -PGA producer, was capable of metabolizing different

Table 1. Morphological and biochemical characteristics of poly- γ -glutamic-acid (γ -PGA)-producing bacteria

γ -PGA-producing isolates	As8	Is6	Is7	Is13	Is14
Gram's reaction	+	+	+	+	+
Morphology	short rods	rods	rods	rods	rods
Endospore staining	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Proteolysis	+	+	+	+	+
Hemolysis	+	–	–	–	–
Lecithinase test	+	–	+	–	+
Catalase test	+	+	+	+	+
Citrate utilization	+	+	–	+	+
Methyl red test	–	–	–	–	–
Voges–Proskauer test	+	+	+	+	+
Glucose	+	–	+	–	+
Fructose	+	+/-	+	–	+
Lactose	–	–	–	+	+
Maltose	+	+/-	–	+/-	+
Sucrose	+	–	–	+	+
Galactose	–	–	+/-	–	+
Raffinose	–	+	–	–	+/-
Probable identity of the organism	<i>Bacillus</i> sp. As8	<i>Bacillus</i> sp. Is6	<i>Bacillus</i> sp. Is7	<i>Bacillus</i> sp. Is13	<i>Bacillus</i> sp. Is14

(+) – positive; (–) – negative; (+/–) – variable.

sugars (such as glucose, fructose and maltose) and tested positive for utilizing catalase, protease, lecithinase, and citrate, as well as for starch hydrolysis and gelatin hydrolysis. The isolate As8, however, did not metabolize lactose, galactose or raffinose. Genotypically, *Bacillus* sp. As8 was 96% percent similar to the *B. toyonensis* strain BCT-7112 and was therefore referred to as *B. toyonensis* As8.

Growth and γ -PGA production responses of *Bacillus toyonensis* As8 to physicochemical modifications of medium components

Bacillus toyonensis As8 (the representative colony depicted in Fig. 1A) had the highest γ -PGA yield (16.53 g/L) with glucose and fructose as carbon sources, while its yield in a starch-based medium was the lowest (4.06 g/L) – even though that substrate was highly conducive to bacterial growth (Fig. 1B). All of the sugars used as carbon sources supported bacterial growth. These findings may be attributed to the fact that simple sugars are more desirable for bacterial metabolism since less energy is required to incorporate them into the metabolism of a cell. However, Ju et al.¹⁹ obtained a higher γ -PGA yield with 30 g/L of starch in the fermentation medium and *B. subtilis* MJ80, yielding 48.3 g/L of γ -PGA. The findings that citric acid and starch supported bacterial growth may be attributed to the ease with which these compounds convert into glutamic acids, through the tricarboxylic acid cycle and finally into poly- γ -glutamate.²⁰

Fig. 1. (A) Colonial view of *Bacillus toyonensis* As8. (B) Effect of different carbon sources on the growth and poly- γ -glutamic-acid (γ -PGA) yield of *Bacillus toyonensis* As8

Ammonium sulfate supported both bacterial growth (optical density: 1.854) and γ -PGA production (19.95 g/L) by *B. toyonensis* As8 (Fig. 2). Organic peptone and malt extract did support γ -PGA production, though less so than inorganic ammonium sulfate. Because γ -PGA was produced in the medium containing ammonium sulfate in the absence of L-glutamic acid, we may deduce that *B. toyonensis* As8 can be classified as an L-glutamic acid-independent γ -PGA-producing strain.³

There was a progressive reduction in γ -PGA yield as the incubation temperature increased (Fig. 3), indicating that the γ -PGA yield from *B. toyonensis* As8 was temperature-dependent.¹⁶ Production was highest at 25°C, with a dry weight of 18.25 g/L. The lowest yield of 6.10 g/L was observed at 45°C, at which temperature the γ -PGA producer exhibited the highest turbidimetric reading. In a related study, the optimal growth temperature for *Bacillus licheniformis* NRC20 was reported to be 30°C, while the highest γ -PGA yield was obtained at 35°C.^{16,21}

From Fig. 4, while the lowest γ -PGA production was recorded at an acidic pH (4.5), the highest was at pH 5.5 (26 g/L). At neutral pH and above, the γ -PGA yield was reduced to about 64% of the highest yield though the bacterial growth was highest. The highest fermentative production of γ -PGA was reported at a pH of 6.5.²² The level of pH significantly affects bacterial nutrient solubility and uptake, enzyme activity, and cell membrane morphology, thus impacting the formation of by-products (γ -PGA release).¹⁶ The fact that the highest γ -PGA yield was recorded at pH 5.5 may indicate that this pH influenced microbial metabolism to favor a higher γ -PGA release.

Fig. 2. Effect of different nitrogen sources on the growth and poly- γ -glutamic-acid (γ -PGA) yield of *Bacillus toyonensis* As8

Fig. 3. Effect of incubation temperature on the growth and poly- γ -glutamic-acid (γ -PGA) yield of *Bacillus toyonensis* As8

Fig. 4. Effect of pH on the growth and poly- γ -glutamic-acid (γ -PGA) production of *Bacillus toyonensis* As8

Effects of inoculant concentration and incubation period on γ -PGA production

Over the 96 h, bacterial growth increased in the 2 flask experiments. However, the highest γ -PGA yield (23.65 g/L) was observed after 48 h of incubation in the production medium inoculated with 10% of *B. toyonensis* As8. After this, there was a progressive reduction in yield from both media (Fig. 5A,B). Ju et al.¹⁹ reported the highest γ -PGA yield by a *B. subtilis* strain at the end of a 5-day incubation before a fall in yield and concluded that – for that strain – the longer the incubation time, the higher the production of γ -PGA. There was a progressive increase in optical density proportional to increasing incubation time. It was reported that the γ -PGA product could serve as a source of glutamate for the producing

Fig. 5. Effect of (A) 1% and (B) 10% inoculation with different incubation periods on the growth and poly- γ -glutamic-acid (γ -PGA) production of *Bacillus toyonensis* As8

strain during its late stationary phase of life to sustain cell metabolism while nutrients and energy become limited,²³ which might be the reason for the reduction in the γ -PGA recovered. This also agrees with another report that the glutamic acid product of γ -PGA degradation catalyzed by γ -glutamyl hydrolase was utilized by the bacteria as a source of carbon and nitrogen.²⁴

γ -PGA production using agricultural residues

Many carbon- and nitrogen-based agro-industrial wastes (such as rice straw, wheat bran, corn bran, corn cob, sugarcane bagasse, cotton stalk, sorghum stover, and soybean cake) have been used as substrates in microbial fermentation because they can be biologically converted to 6-carbon and 5-carbon compounds which are funneled into the main carbon metabolism via glycolysis and the pentose phosphate pathway.^{20,25} Although all of the agro-substrates supported γ -PGA production, the highest yield was obtained with cassava peel (22.26 g/L) as the sole source of carbon (Table 2), an indication that the carbohydrate it contained could easily be metabolized by *B. toyonensis* As8 into simpler forms for subsequent conversion into α -ketoglutaric acid (a precursor metabolite of L-glutamine) in the citric acid cycle (TCA). There was an increase in γ -PGA production when 10% of the γ -PGA producer was cultivated under optimal culture conditions in a medium that now contained either cassava peel, fructose or Coix leaves with ammonium sulfate, at 25°C and a pH of 5.5, over 48 h to yield 26.45 g/L, 23.23 g/L and 16.87 g/L of γ -PGA, respectively.

Table 2. Effects of various agricultural wastes as carbon sources on poly- γ -glutamic-acid (γ -PGA) production by *Bacillus toyonensis* As8

Agro-waste – carbon source	γ -PGA [g/L]
Corn cob	6.03
Sorghum leaves	10.03
Coix noir leaves	8.37
Cassava peels	22.26
Rice bran	8.35

Characterization of the γ -PGA produced

Fourier transform infrared spectroscopy of the γ -PGA produced by *B. toyonensis* As8 revealed the key characteristic functional groups of γ -PGA (Fig. 6). The absorbance spectrum had peaks ranging from 441.26 cm^{-1} to 3771.78 cm^{-1} , where functional groups including hydroxyl, amide, carbonyl, and amine groups – common features of γ -PGA – were represented. This is similar to the results obtained by Kedia et al.,¹⁸ who measured over 100 scans and wavelength ranges of 400–4000 cm^{-1} using the same technique. The results of the present study for the γ -PGA produced by *B. toyonensis* As8 – amide absorption

at 1639.38 cm^{-1} , carbonyl absorption at 1439.00 cm^{-1} and hydroxyl absorption at 3417.00 cm^{-1} – are in agreement with those of other authors^{17,19,26} who independently reported a strong amide (N–H) absorption at ~ 1620 – 1655 cm^{-1} , a weaker carbonyl (C=O) absorption at ~ 1394 – 1454 cm^{-1} , a strong hydroxyl (OH) absorption at ~ 3400 – 3450 cm^{-1} , and a characteristically strong amine (C–N) absorption in the range of 1085–1165 cm^{-1} .

Fig. 6. FTIR Spectra of the poly- γ -glutamic-acid (γ -PGA) produced by *Bacillus toyonensis* As8

Figure 7 shows the scanning electron micrograph of the γ -PGA produced by *B. toyonensis* As8. The polymer particles were clumped together as agglomerates and the surfaces of the agglomerates were rough, wrinkled and non-uniform, which indicates the polymeric material might be non-free-flowing, loosely packed and porous.

Fig. 7. Scanning electron micrograph of the poly- γ -glutamic-acid (γ -PGA) produced by *Bacillus toyonensis* As8

Conclusions

In this study, the effects of media components and various agricultural wastes as substrates for the microbial production of γ -PGA were investigated. Cassava peels as the sole source of carbon supported the highest γ -PGA yield for the non-glutamic-acid-dependent *Bacillus toyonensis* As8 (26.45 g/L) at a pH of 5.5 and at 25°C.

Also, the key groups/peaks present in the product were characteristic of the γ -PGA. Although they have been used in the past as substrates due to their abundance in our environment, cassava peels and many other agricultural by-products could be harnessed as substrates for large-scale γ -PGA production in a bid to reduce the high costs associated with commercial γ -PGA production.

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