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Isolation, screening, and production of poly-γ-glutamic acid by *Bacillus* sp. KMUTT06 using glucose syrup from cassava starch and monosodium glutamate

Kanokorn Changto[1], Somporn Moonmangmee[2] and Duangtip Moonmangmee[1*]

[1]Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, THAILAND
[2]Biodiversity Research Center, Thailand Institute of Scientific and Technological Research, Pathum Thani 10120, THAILAND
[*]Corresponding author: duangtip.moo@kmutt.ac.th

ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is a versatile biopolymer characterized by its non-toxic, water soluble, biodegradable making it suitable for a wide range of application. However, the high cost of production remains a major challenge. This study aimed to isolate *Bacillus* sp. strains from fermented soybean products for γ-PGA production, evaluate production using glucose syrup and monosodium glutamate (MSG), and assess antioxidant activity. Seven isolates of *Bacillus* sp. were obtained from fermented soybean samples. The isolate KMUTT06 showed that 99.93% similarity to *B. tequilensis* KCTC13622^T, *B. inaquosorum* KCTC13429^T, *B. cabrialesii* TE3^T and *B. cabrialesii* TSO23^T and exhibited the highest γ-PGA production at 1.116 mg/ml, with highest relative viscosity. Using 2.5% (w/v) glucose syrup concentration (w/v) showed that highest of γ-PGA (16.546 ± 2.988 mg/mL) compared to 2.0% (w/v) dextrose and 2% (w/v) MSG produced the highest of γ-PGA (2.589 ± 0.328 mg/mL) compared to L-glutamic acid. Antioxidant activity of γ-PGA produced by *Bacillus* sp. KMUTT06 had IC_{50} values of 0.650 ± 0.022 mg/mL (DPPH), 3.050 ± 0.148 mg/mL (ABTS), and a FRAP value of 8.806 ± 0.358 μmol Fe^{2+}/g. In conclusion, *Bacillus* sp. KMUTT06 may be regarded as a candidate strain for γ-PGA production, demonstrating that glucose syrup and MSG can be used instead of dextrose and L-glutamic acid respectively, for producing γ-PGA with discernible antioxidant activity, further study needed suggesting its potential for various functional industrial applications.

Keywords: Antioxidant, Biopolymer, Fermented soybean, Glucose syrup, Poly-γ-glutamic acid

INTRODUCTION

Poly-γ-glutamic acid (γ-PGA) is known as a biodegradable biopolymer that can be produced by several microorganisms, especially *Bacillus* sp. This group of bacteria is widely used because they are efficient in producing γ-PGA and it's safe in general industrial use [1]. γ-PGA consists of D-glutamic acid, L-glutamic acid, or a mixture of their stereoisomers (enantiomers). Structurally, it contains amide bonds linking the amino and carboxyl groups. γ–PGA is a homopolyamide and has several useful properties, including being edible, negatively charged, antibacterial, non-toxic, biodegradable, water-soluble, and naturally occurring [2]. This polymer is widely used in many fields such as medicine, wastewater treatment, the food industry, cosmetics, and agriculture [3]. γ–PGA is naturally found in the soybean fermented products by *Bacillus* sp., like natto, soybean paste, miso, cheonggukjang and other fermented soybean products. Many studies have isolated strains capable of producing γ–PGA from these products [4]. The fermentation process led to bacteria producing a slimy substance that is beneficial to health as a good prebiotic for the gut, promoting probiotic growth [5]. However, the high costs of production are primary economic problem due to the expense of laboratory-grade raw material, particularly carbon sources like dextrose and substrate like L-glutamic acid [6]. While other research several used other carbon source including cane molasses [7] and raw straw hydrolysate [8], there remains a limitation about purity of carbon source.

To address this, Thailand is an agricultural country with an important economic crop of cassava, especially grown across 48 provinces (excluding the south region), covering a total farmland area of approximately 7.9 million rai and yielding over 30.23 million tons in 2013 [9]. Hence, many products are made from cassava at low cost of raw material and in an environment friendly by using all parts of cassava. Specifically, glucose syrup is made from the processing of cassava starch hydrolysis, with an enzyme or acid to break down starch molecules into smaller units [10].

Furthermore, Monosodium glutamate (MSG) is also derived from cassava through the fermentation of plant-based materials, such as sugarcane, beetroot,

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cassava, or corn. Therefore, using glucose syrup from casava starch and MSG can reduce production costs by replacing expensive glucose and L-glutamic acid typically used in conventional media. Additionally, another study has demonstrated that γ–PGA has significant antioxidant activity, scavenging free radicals like 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radicals, and superoxide radicals [11]. These activities contributed to reducing oxidative stress or the increase in reactive oxygen species (ROS), such as superoxide, hydroxyl radicals, and hydrogen peroxide, which are linked to damage in carbohydrates, nucleic acids, proteins, and fats, leading to conditions such as inflammation, lung injury, cancer, aging, and vascular issues [11]. This study aimed to isolate and select *Bacillus* sp. strains from fermented soybean products available in local markets in Thailand, for γ–PGA production using low-cost carbon sources, namely glucose syrup from cassava starch and monosodium glutamate (MSG). Furthermore, crude γ–PGA was tested for its antioxidant activities.

MATERIALS AND METHODS

1. Sample collection screening and isolation of Bacillus sp. for producing γ-PGA

Weigh one gram of sample from fermented soybeans from Chiang Mai and natto, then dissolved it in 9 mL of sterile distilled water and vortexed. After that, it was subjected to heat shock at 80°C for 10 minutes. Then, the serial dilution technique was used, and the samples were spread plate onto 2xTY agar (1.6% Typtone (w/v), 1.0% Yeast extract (w/v), 5% NaCl (w/v) and 1.5% agar (w/v)). The plates were incubated at 37°C for 24-48 hours. Colonies exhibiting slimy or irregular shapes were selected and re-streaked onto 2xTY agar plates, then incubated at 37°C for 24-48 hours. The purified isolates were subsequently stored in 20% glycerol at -20°C for further analysis.

2. Screening of γ-PGA producing bacterial strains for producing γ-PGA

2.1 Cell culture

The cell culture concentration was adjusted to a turbidity of 5.5 McFarland, and a 5% (v/v) inoculum was used. It was then inoculated into 100 mL of culture medium, which consisted of 2% dextrose (w/v), 0.25% yeast extract (w/v), 3% L-glutamic acid (w/v), 0.05% KH_2PO_4 (w/v), 0.05% K_2HPO_4 (w/v), and 0.01% $MgSO_4{\cdot}7H_2O$ (w/v) [12]. The culture was incubated at 200 rpm and 37°C for 48 hours.

2.2 Viscosity measurement of γ-PGA

5 mL of culture supernatant was collected for analysis at ambient temperature (25 ± 2°C) to determine the relative viscosity using an Ostwald capillary viscometer (No.3, Climbing Co.,Ltd, Japan). The flow time was recorded using a digital stopwatch, starting when liquid meniscus passed the upper mark and stopping when it reached the lower mark. All measurements were performed in triplicate (n=3). The relative viscosity was calculated using the following equation [13].

Relative viscosity:

$$\rho = \frac{mass}{volume}\left(\frac{kg}{m^3}\right) \quad (1)$$

$$\mu r = \frac{\rho_{sample} \times t_s}{\rho_{water} \times t^{\circ}} \times \rho_{water} \quad (2)$$

Where: ρ refers to density, $\rho_{(sample)}$ refers to the density of the sample, $\rho_{(water)}$ refers to the density of distilled water, t_s is the falling time of the sample (seconds), t° is the falling time of distilled water (seconds), and μr refers to the relative viscosity.

2.3 Detection of γ-PGA by agarose gel electrophoresis

Agarose gel electrophoresis was performed using a modified method from [14]. A 3% agarose gel (w/v) was prepared in 1x TAE buffer and cast into a gel tray (13 x 12.2 cm) with 13 wells (9 mm spacing). For sample preparation, 10μl of culture supernatant was mixed with 2μl of 6x loading dye (1:5 ratio). Then the mixture was loaded into the gel wells. Agarose gel ran at 50 volts for 60 minutes. After the electrophoresis, gel was stained in 0.5% methylene blue (w/v) for 15 minutes and gently washed with distilled water until the bands were directly visible on gel.

2.4 γ-PGA Content measurement using safranin O

The standard γ-PGA (FUJIFILM Wako Pure Chemical corporation) was prepared at 0.000, 0.025, 0.050, 0.075, 0.100, and up to 0.3 mg/mL. to make a calibration curve. One mL of the γ-PGA solution (standard and sample) was mixed with 1 mL of 0.85% NaCl (w/v), followed by the addition of 1 mL of 0.1% safranin O (w/v). After incubating the mixture for 30 minutes, 1 mL of 0.06 M citrate buffer (pH 6.0) was added, and the solution was centrifuged at 3,000 rpm for 10 minutes. The resulting supernatant was then diluted 25-fold with 0.85% sodium chloride (w/v) solution, and its absorbance was measured at 520 nm using a spectrophotometer. The γ-PGA content was calculated by comparing the absorbance with a standard calibration curve (R^2=0.99) [15].

3. Identification of Bacillus sp. using 16S rRNA sequencing

Bacillus sp. strains capable of producing γ-PGA were grown on 2xTY an agar plate at 37°C for 16-24 hours. Then resuspended in saline-EDTA buffer, lysed with lysozyme, incubated at 37°C, and treated with 10% SDS (w/v) at 55–60°C. DNA was extracted using phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) [16] precipitated with sodium acetate and ethanol, and analyzed by agarose gel electrophoresis. 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R, and the purified PCR products

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were used to carry out sequencing. After sequencing, the homology of the 16S rRNA gene was examined using NCBI BLASTn. The sequences were then aligned in BioEdit (version 7.7.1), and phylogenetic relationships were constructed in MEGA12 [17] using the Neighbor-Joining method with 1,000 bootstrap replicates.

4. Comparison of monosodium glutamate and L-glutamic acid in γ-PGA production

The highest γ-PGA content was achieved using *Bacillus* sp. KMUTT06; consequently, this strain was chosen for further production and experimental studies, into the culture media with MSG and L-glutamic acid concentrations varying at 2%, 3%, and 4% (w/v), based on the modified medium [12] consisting of 3% dextrose (w/v), 0.25% yeast extract (w/v), 0.05% KH_2PO_4 (w/v), 0.05% K_2HPO_4 (w/v), and 0.01% $MgSO_4 \cdot 7H_2O$ (w/v). The cultures were incubated under shaking conditions at 200 rpm and 37°C for 3 days. After incubation, the culture supernatant was used to analyze γ-PGA content following the method in section 2.4.

5. Comparison of glucose syrup and dextrose for γ-PGA production

The γ-PGA production was using *Bacillus* sp. KMUTT06, into the culture media with glucose syrup (DE60) and dextrose concentrations varying at 2.5%, 3.5%, and 4.5% (v/v) was based on the modified medium m [18] consist of 0.042% $CaCl_2$ (w/v), 0.4% peptone (w/v), 5% MSG (w/v), 1.2% NaCl (w/v), 0.125% $MgSO_4 \cdot 7H_2O$ (w/v), 0.4% KH_2PO_4 (w/v), and 0.008% $MnSO_4$ (w/v). The cultures were incubated under shaking conditions at 200 rpm and 37°C for 3 days. After incubation, the culture supernatant was used to analyze γ-PGA content following the method in section 2.4

6. Characterization of γ -PGA using HPLC

The γ-PGA was hydrolyzed with 6 M HCl at 110°C for 24 hours. The pH was neutralized by mixing the sample with sodium hydroxide and distilled water on ice, followed by adding 0.2 M sodium citrate buffer (pH 2.2). The mixture was centrifuged at 15,000 x g for 5 minutes. The hydrolyzed sample was collected and analyzed using HPLC with a Waters e2695 system. The column used was a SunFire C18 (100Å, 3.5 µm), with an eluent consisting of 10mM K_2HPO_4 in 5% methanol (v/v). The flow rate was set to 0.5 mL/min, and the analysis was conducted using a Photodiode Array (PDA) detector. The column temperature was maintained at 25°C [19].

7. Antioxidant properties analysis using DPPH, ABTS, and FRAP methods

7.1 DPPH Radical Scavenging Assay

A 0.1 mM DPPH solution (100 µL) in methanol was tested with 100 µL of γ-PGA extract at varying concentrations (0.5 - 5.0 mg/mL) and incubated at room temperature for 30 minutes in the dark. The control was prepared by mixing 100 µL of DPPH solution with 100 µL of distilled water. Absorbance was measured at 517 nm using a microplate reader. Ascorbic acid was used as the standard solution. Three measurements were taken, and the percentage of radical scavenging activity was calculated using the following equation [20].

$$I\% = \frac{(A_o - A_s)}{A_o} \times 100 \quad (3)$$

Where: A_o is the abs. of the sample mixed with DPPH, A_s is the abs. of the DPPH solution , I% is percentage of radical scavenging activity.

7.2 The 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) (ABTS) assay

A 7 mM $ABTS^{\bullet+}$ solution was mixed with 2.45 mM potassium persulfate in equal volumes and incubated in the dark at room temperature for 12-16 hours. The solution was then diluted in methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. The $ABTS^{\bullet+}$ solution (180 µL) was mixed with γ-PGA extract (20 µL), incubated at room temperature for 10 minutes in the dark. The control sample was prepared by mixing 20 µL of $ABTS^{\bullet+}$ solution with 180 µL of distilled water. Absorbance was measured at 734 nm using a microplate reader. Ascorbic acid was used as the standard solution. Three measurements were taken, and the percentage of radical scavenging activity was calculated [20].

$$I\% = \frac{(A_o - A_s)}{A_o} \times 100 \quad (4)$$

Where: A_o is the abs. of the sample mixed with ABTS, and A_s is the abs. of the ABTS solution I% is percentage of radical scavenging activity.

7.3 Ferric-reducing antioxidant power (FRAP) assay

A 180 µL FRAP solution was mixed with 20 µL of the sample. The mixture was incubated at 37°C for 30 minutes in the dark, and absorbance was measured at 593 nm using a spectrophotometer, compared with ascorbic acid as a standard solution. The concentration of the ferrous ion complex in the solution was calculated by substituting values from a ferrous ion standard curve [20].

8. Statistical analysis

The data were analyzed using SPSS. All experiments were performed in 3 replicates, and the results are presented as the mean ± standard deviation (SD). Differences between treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by pairwise comparisons with Tukey's post-hoc test. Statistical significance was considered at $p < 0.05$, and all analyses were carried out using SPSS.

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RESULTS AND DISCUSSION

1. Isolation of Bacillus sp. capable of producing γ-PGA

The isolation of *Bacillus* sp. from fermented soybean products is shown in Table 1. A total of seven bacterial isolates were obtained. Six isolates, namely KMUTT01–KMUTT06, were collected from fermented soybean products in Chiang Mai and one isolate, namely KMUTT07, was obtained from a commercial natto product.

Bacillus sp. is generally capable of producing γ-PGA due to its safety and is commonly used in various industries. The strains such as *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* are frequently used in the food and pharmaceutical industries, along with in the production of enzymes for food processing and fermentation [21]. These strains are highly considered for their safety, reliability, and efficiency in industrial processes, making them suitable for commercial use [22]. Their extensive application in various fields is caused by their ability to produce γ–PGA effectively and their suitability for large-scale production, as the bacteria can grow rapidly and express the γ-PGA effectively [21]. Based on these reasons, *Bacillus* sp. are regarded as excellent candidates for commercialization in both the food and pharmaceutical industries [23]. In addition, Bacillus sp. is a spore-forming microorganism capable of surviving under unfavorable conditions. During sporulation, vegetative cells differentiate into spores, which can subsequently germinate and resume vegetative growth when conditions become favorable. Therefore, a heat shock treatment was employed to selectively isolate spore-forming bacteria by eliminating non-spore-forming microorganisms from the samples [24, 25]. As follow the heat shock treatment was employed to selectively isolate these spore-forming bacteria while eliminating on spore forming microorganisms from the samples.

Table 1 Bacterial strains and isolation sources.

Isolation Number	Source
KMUTT01	
KMUTT02	
KMUTT03	Fermented soybean
KMUTT04	products, Chiang Mai
KMUTT05	
KMUTT06	
KMUTT07	Commercially natto products

2. Viscosity measurement of γ-PGA

The viscosity of culture supernatants is a parameter for selecting bacterial strains capable of efficiently producing γ-PGA. As shown in Figure 1, the supernatant derived from isolate KMUTT06 exhibited the highest relative viscosity, measuring 188.376 ± 5.263, followed by KMUTT05 with a viscosity of 183.004 ± 5.196, and KMUTT02, which displayed a relatively lower viscosity of 58.607 ± 2.017.

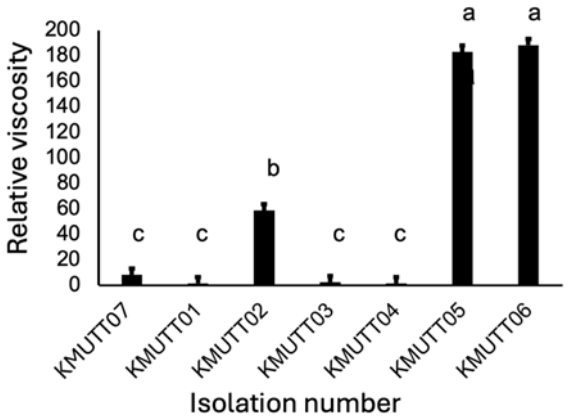

Figure 1 Viscosity of the culture supernatant produced by the isolated *Bacillus* sp.

These results agree with previous studies suggesting that polymers with higher molecular weights typically high ~~of~~ flow resistance, leading to an increase in viscosity. The relationship between molecular weight and viscosity is associated with the entanglement and interaction of polymer chains that slow down fluid movement, thereby increasing resistance to flow [26]. Larger polymer molecules are more likely to entangle, further increasing the resistance to flow [27]. The correlation between higher molecular weight and increased viscosity is important for selecting bacterial strains capable of producing γ-PGA. A high molecular weight is typically used for industrial applications where viscosity is a crucial factor. In this study, it is shown that KMUTT6 has the highest viscosity, suggesting more efficient γ-PGA production. It could be an advantage in commercial applications requiring high-viscosity formulations, such as the precipitation of heavy metals, skincare moisturizers, and drug carriers. In the study of [28] shown that the γ-PGA chain structure in the α-helix form significantly enhances viscosity at low pH, which causes molecular entanglement and increased resistance to flow. On the other hand, γ-PGA high pH exhibits a structure like a random coil and leads to changes in viscosity, as the molecules unfold and electrostatic forces cause the polymer chains to separate further. Therefore, isolate KMUTT06 was selected for further investigation.

3. Detection of γ-PGA by agarose gel electrophoresis

The agarose gel in Figure 2 shows the results of γ–PGA production by various bacterial isolates. The first lane labeled as M contains the γ–PGA standard used as a reference for comparison with isolated sample production. The lanes number 2 to 8 showed that a band from isolates KMUTT01 to KMUTT07 that stained with methylene blue, a band that shows differences in thickness and colour intensity. With

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isolates KMUTT07, KMUTT02, KMUTT05, and KMUTT06 showing darker, thicker bands, indicating higher concentrations and larger molecular sizes of γ–PGA compared to the other isolates.

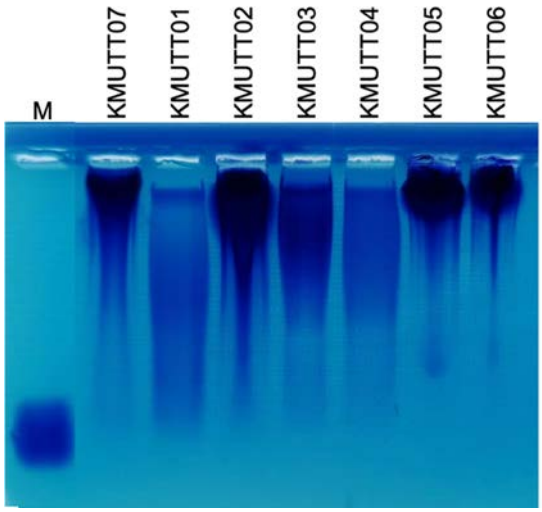

Figure 2 Agarose gel electrophoresis of standard γ-PGA (lane M) and culture supernatants (crude PGA) from isolates KMUTT01-07 stained with methylene blue.

All of the isolated γ–PGA show bands move more slowly than the standard γ–PGA (400 kDa). Thus, the isolated γ–PGA has larger molecular weights than 400 kDa because the band travel is directly correlated to the size of the γ–PGA molecules. Generally, a shorter migration distance indicates a higher molecular weight, while increased band thickness and intensity reflect a higher γ-PGA concentration. These findings correspond with the principle that macromolecules with higher molecular weights migrate more slowly through the gel matrix [29]. The increase in both molecular weight and concentration is the primary cause of enhanced viscosity in the fermentation broth [30]. Thereby, longer polymer chains and higher concentrations increase the physical resistance to flow and promote the molecular entanglement effect in γ-PGA solution that shows high viscosity in the culture supernatant. Additionally, the molecular weight of γ–PGA can affect its functionality and properties. Larger γ–PGA molecules tend to form more viscous solutions, which can be important for applications in industries like food and pharmaceuticals, where viscosity plays a critical role in product performance [31].

In addition, methylene blue is a basic dye that is used to stain the gel because of its ability to bind to the negatively charged carboxyl groups (COO-) of γ–PGA. As a result, the cationic dye effectively detects the anionic COO^- groups in γ–PGA, resulting in visible bands on the gel. Therefore, the methylene blue staining method, combined with the electrophoresis technique, is a rapid screening technique for identifying strains capable of producing γ–PGA. The ability of methylene blue to bind to the anionic COO- groups on γ–PGA molecules is crucial for visualizing the polymer during electrophoresis [19]. This property makes methylene blue an appropriate dye stain for screening γ–PGA production in bacterial cultures.

4. *γ-PGA Content*

The capacity for PGA production was evaluated between seven isolated strains as follows KMUTT01 to KMUTT07. As presented in Table 2, all isolates show the ability to synthesize γ-PGA, with production levels varying between 41.774 and 111.649 g/L.

Table 2 γ-PGA content of *Bacillus* sp. isolates from fermented soybean products.

Isolation Number	γ-PGA content (g/L)
KMUTT01	58.0160
KMUTT02	102.456
KMUTT03	84.3880
KMUTT04	41.7740
KMUTT05	110.541
KMUTT06	111.649
KMUTT07	95.1510

Among the screened isolates, KMUTT06 exhibited the highest biosynthetic potential, achieving a γ-PGA yield of 111.649 g/L. High production levels were also observed in KMUTT05 and KMUTT02, which yielded 110.541 g/L and 102.456 g/L, respectively. Conversely, KMUTT04 showed the lowest production capacity at 41.774 g/L. Based on these results, isolate KMUTT06 was selected as the candidate strain for subsequent optimization studies using cost-effective substrates. Interestingly, the γ–PGA yield of 111.600 g/L is higher than the previous reported including [32], which achieved 30.200 g/L, 19.800 g/L [33].

5. Identification of Bacillus sp. using 16S rRNA sequencing

The result of bacterial isolate identification using 16S rRNA showed high genetic relationships with the *Bacillus subtilis* group. Phylogenetic analysis indicates that strains KMUTT01–KMUTT07 are related as follows in table 3. KMUTT01 exhibited 99.79% and 99.78% sequence similarity to *B. siamensis* KCTC13613^T and *B. velezensis* CR-502^T, respectively. The difference of three bases suggests that it may represent a strain closely related to these species. KMUTT02 showed 99.72% similarity to *B. tequilensis* KCTC13622^T, *B. inaquosorum* KCTC13429^T, *B. cabrialesii* TE3^T and *B. cabrialesii* TSO23^T, with a four-base-pair difference, displaying KMUTT02 closely within this cluster. The KMUTT03 showed 99.57% similarity to *B. velezensis* CR-502T and 99.36% to *B. siamensis* KCTC13613^T, whereas KMUTT04 exhibited 99.85% sequence similarity with both *Bacillus siamensis* KCTC13613 and *B. velezensis* CR-502^{T}with different

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with three base pairs different, and showed 99.64% similarity with five base pair differences to *B. nematocidal* B-16[t]. KMUTT05 and KMUTT06 exhibited the highest similarity values at 99.93% to *B. tequilensis* KCTC13622[T], *B. inaquosorum* KCTC13429[T], *B. cabrialesii* TE3[T] and *B. cabrialesii* TSO23[T] and showed a single base pair difference, indicating a near relationship.

In contrast, KMUTT07 showed 99.85% similarity to *B. subtilis* with a difference of five base pairs, which suggests a closer relationship with *B. subtilis* than with other related species. Collectively, this result indicated that the KMUTT isolates are genetically related to the *B. subtilis* species complex, especially with *B. siamensis*, *B. velezensis*, and *B. tequilensis*, and may include potential novel or variant strains within this phylogenetic group. In summary, the sequence similarity analysis reveals that all KMUTT isolates belong to the *Bacillus subtilis* species complex, exhibiting specific close genetic relationships with *B. siamensis*, *B. velezensis*, and *B. tequilensis*. Isolates KMUTT01, KMUTT03, and KMUTT04 clustered with the *B. velezensis B. siamensis* group, while KMUTT02, KMUTT05, and KMUTT06 were associated with the *B. tequilensis* clade, and KMUTT07 was more closely related to *B. subtilis*. The phylogenetic tree shown in Figure 3 supports a close genetic relationship between *B. tequilensis*, *B. subtilis*, and *B. velezensis*. This indicated that *B. velezensis*, *B. amyloliquefaciens*, and *B. vallismortis* are genetically closely related and belong to the *B. amyloliquefaciens* operational group within the *B. subtilis* species complex.

Despite underway taxonomic debates regarding the classification of *B. velezensis* and *B. amyloliquefaciens*, recent phylogenomic analyses provide interesting evidence suggesting that these species should be classified separately based on genetic differences [34]. Additionally, isolates KMUTT01, KMUTT03, and KMUTT04 are likely to share several biological traits with these species, such as amylase production, plant growth promotion, and antimicrobial activity, traits commonly associated with *B. velezensis* and B. amyloliquefaciens [35]. However, relationships with bootstrap values below 70% indicate variability; further investigation is needed to increase the consistency of the analysis. This limitation arises from the use of a single gene, which may be deficient for notable species with high genetic similarity, such as the *B. subtilis* complex that shares highly similar 16S rRNA sequences. The KMUTT isolates were identified as being closely related to *B. velezensis*, *B. siamensis*, and *B. subtilis*, species frequently utilized for industrial γ-PGA production [11, 36, 37]. Our study confirms that the KMUTT isolates show similarity with the *Bacillus* species complex, which is commonly used it. Identification is necessary because different strains indicate unique advantages regarding their biosynthetic efficiency. Furthermore, the structural attributes of the synthesized γ-PGA specifically its D/L-glutamate ratio and molecular weight, are intrinsically linked to the specific species and their respective metabolic pathways [3].

Finally, future studies should utilize multilocus sequence typing (MLST) techniques to improve both the accuracy and resolution of species identification. It would also be useful to include genes beyond the commonly used 16S rRNA, such as *gyrB*, *rpoB*, *atpD*, and *recA*. Moreover, integrating biochemical and morphological data will be essential to confirm species classification more clearly [34, 38].

Table 3 16S rRNA gene sequence identity of isolate KMUTT06 compared with reference strains.

Isolation number	Accession number	Reference type strain	Identity (%)
KMUTT01	PX755919	*B. siamensis* KCTC13613 [T]	99.79%
		B. velezensis CR-502[T]	99.78%
KMUTT02	PX755920	*B. tequilensis* KCTC13622[T]	99.72%
		B. inaquosorum KCTC13429[T]	99.72%
		B. cabrialesii TE3[T]	99.72%
		B. cabrialesii TSO23[T]	99.72%
KMUTT03	PX755921	*B. velezensis* CR-502[T]	99.57%
		B.siamensis KCTC13613[T],	99.36%
KMUTT04	PX755922	*B. siamensis* KCTC13613[T]	99.85%
		B. velezensis CR-502[T]	99.85%
		B. nematocida B-16[t]	99.64%
KMUTT05 and KMUTT06	PX755923	*B. tequilensis* KCTC13622[T]	99.93%
		B. inaquosorum KCTC13429[T]	99.93%
		B. cabrialesii TE3[T]	99.93%
	PX755924	*B. cabrialesii* TSO23[T]	99.93%
KMUTT07	PX755925	*B. subtilis* NCIB3610[T]	99.85%

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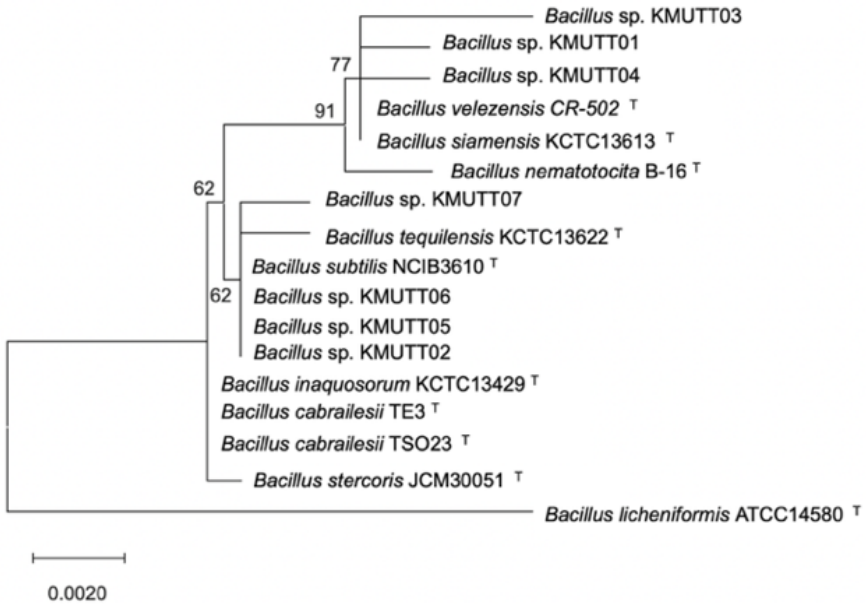

Figure 3 Phylogenetic relationships of the isolated *Bacillus* sp. based on 16S rRNA gene sequences constructed using the neighbor-joining method. Bootstrap values, expressed as percentages from 1,000 bootstrap replicate.

6. γ-PGA Characterization

The analysis of the amino acid composition of γ-PGA was determined by HPLC on the γ-PGA hydrolysate sample. The result shows in Figure 4A and 4B the retention times similar to standards mixed of D and L-glutamic acid (3.885 minute) with retention times of 3.580 minutes. This close agreement in retention times confirms that the biopolymer is composed of glutamic acid subunits, thereby verifying the identity of the product as γ-PGA.

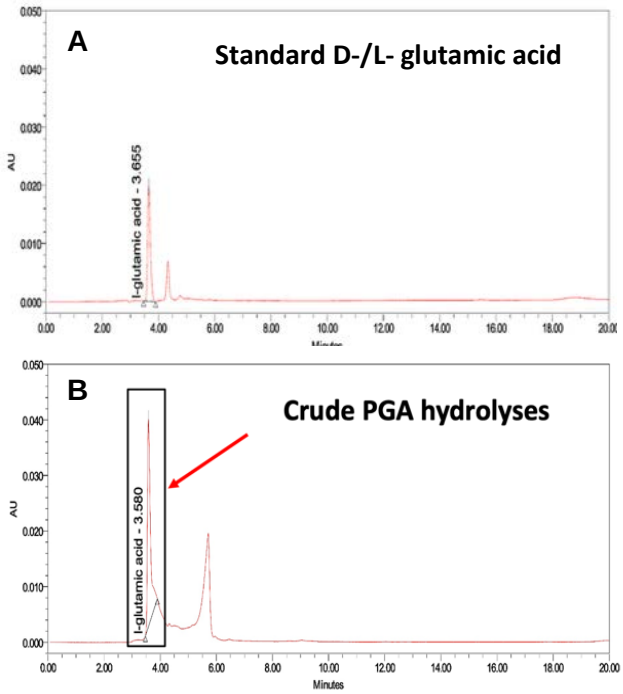

Figure 4 The γ-PGA analysis of amino acid composition using HPLC, (A) Mixed standard of L- and D- glutamic acid (B) Crude γ-PGA hydrolyses.

7. Comparison of MSG and L-glutamic acid for γ-PGA production

This study showed the production of γ -PGA by *Bacillus* sp. KMUTT06 using monosodium glutamate (MSG) compared efficacy with L-glutamic acid at various concentrations of 2.0%, 3.0%, and 4.0% (w/v). As shown in Figure 5 at a concentration of 2.0% (w/v) MSG shows higher γ-PGA production at 2.589 ± 0.328 mg/mL than L-glutamic acid (1.582 ± 0.120 mg/mL).

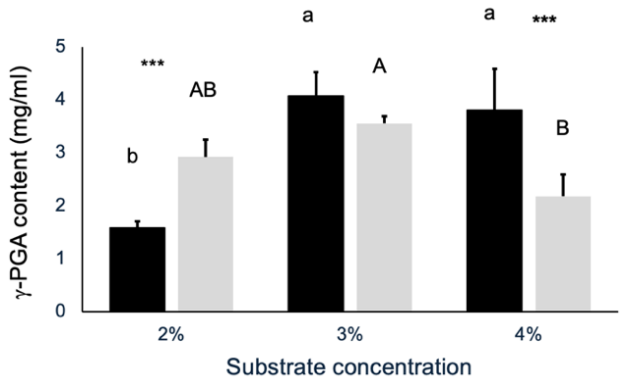

Figure 5 γ-PGA Production by *Bacillus* sp. KMUTT06 when cultured in media containing L-glutamic acid, black bars: L-glutamic acid and grey bars as MSG at concentrations of 2.0%, 3.0%, and 4.0% (w/v). The symbol *** indicates a statistically significant difference between the L-glutamic acid and monosodium glutamate groups.

However, a higher concentration at 4.0% (w/v) shows that L-glutamic acid produces significantly more γ-PGA than MSG. Interestingly, at 3.0% (w/v) there is no difference in γ-PGA production observed

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between of two substrates. Thus, the concentration of the substrate it's a key to the efficiency of γ-PGA production. As shown in Figure 5, substrate concentration had an impact on production efficiency, with remarkable variations in γ-PGA content at different substrate concentrations. At 2% (w/v) L-glutamic acid shows the lowest γ-PGA production significant differences when compared to the other concentrations. Concentrations of 3% (w/v) and 4% (w/v) of L-glutamic acid demonstrated significant increase in γ-PGA production was demonstrated, with the highest γ-PGA content achieved at 3% (w/v), which reached 4.080 ± 0.450 mg/mL. This result indicates that the 3% (w/v) concentration of L-glutamic acid was optimal for γ-PGA production. For MSG, significant differences in γ-PGA production were observed at concentrations of 3% (w/v) and 4%, (w/v) while 2% (w/v) MSG showed no difference with 3% (w/v) and 4% (w/v). The highest γ-PGA content when using MSG as substrate shows at 3% (w/v) MSG, which is a production value of 3.554 ± 0.140 mg/mL.

These results suggested that substrate concentration effected to γ-PGA production, with both MSG and L-glutamic acid exhibiting different levels of efficiency depending on their concentration. The production of γ-PGA by *Bacillus* sp. KMUTT06 is likely affected by the solubility of the substrate because higher solubility ensures better substrate availability and faster uptake by the cells, whereas excessive concentrations may lead to increased viscosity or osmotic stress that inhibits bacterial growth.

Generally, amino acids are soluble in water but insoluble in nonpolar organic solvents. However, glutamic acid has a very low solubility of approximately 0.88 g/100mL [39] because of its stable crystalline structure, which exists as a zwitterion having both negative and positive charges, leading to intermolecular ionic attractions [40]. Additionally, the carboxyl group in the structure promotes an extensive network of hydrogen bonding. These factors combined result in high lattice energy, requiring a lot of energy to break the crystal structure and hydrate the individual molecules [39]. On the other hand, MSG is much easier to dissolve, reaching 74 g/100 mL, which higher 84 times higher than the acid form [41]. Based on its structure, sodium ion (Na^+) are surrounded by water molecules, leading to a pre-existing hydration shell effect easier and faster for water to pull the molecules apart compared to the very stiff and tightly packed zwitterionic structure of L-glutamic acid [42]. MSG has a structure that has the ability to solubilize compared to L-glutamic acid, which may facilitate easier absorption by the bacterial cells. [43].

However, at higher concentrations, the presence of sodium ions in MSG may affect the osmotic balance within the cells, potentially impacting the efficiency of the γ-PGA production process. This could lead to stress in cellular function that may either inhibit the production of γ-PGA, depending on the concentration and the physiological response of the bacterial cells [44]. In addition, *Bacillus* sp. KMUTT06 showed the highest production in medium that has a substrate (MSG or L-glutamic acid) maybe this strain it's glutamate dependence but additional investigate is prefer.

8. Comparison of glucose syrup and dextrose for γ-PGA production

This study compared the effects of glucose syrup and dextrose on the production of γ-PGA by *Bacillus* sp. KMUTT06 using concentrations of carbon source at 2.5%, 3.5%, and 4.5% (w/v), as shown in Figure 6, the glucose syrup significantly produced γ-PGA content more than dextrose at the same concentrations of 2.5% and 3.5% (w/v). At a 2.5% (w/v) concentration, glucose syrup resulted in 16.546 ± 2.988 mg/mL of γ-PGA content, whereas dextrose produced only 5.704 ± 0.523 mg/mL. This significant difference in γ-PGA content at 2.5% (w/v) demonstrates that glucose syrup may be more efficiently utilized by the bacterial cells at this concentration.

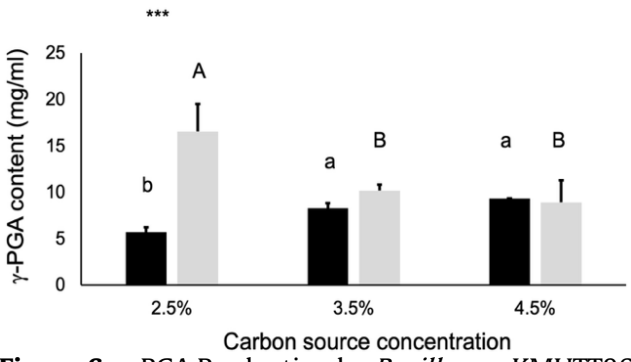

Figure 6 γ-PGA Production by *Bacillus* sp. KMUTT06 when cultured in media containing glucose syrup, grey bars and black bars as dextrose at concentrations of 2.5%, 3.5%, and 4.5% (w/v). The symbol *** indicates a statistically significant difference between the dextrose and glucose syrup groups.

However, at higher concentrations of 3.5% and 4.5% (w/v) there is no difference in γ-PGA production was observed between glucose syrup and dextrose, implying that the efficiency of both carbon sources is similar at these concentrations. While the type of carbon source significantly affected γ-PGA production at 2.5% and 3.5% (w/v) thus substrate concentration also played a key role on γ-PGA production. At 2.5% (w/v) glucose syrup showed the highest γ-PGA content at 16.546 ± 2.988 mg/mL, which was significantly higher than the production levels at both 3.5% and 4.5% (w/v). This indicates that glucose syrup is more effective at lower concentrations, potentially due to its higher solubility and more efficient absorption by the

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bacterial cells. On the other hand, for dextrose, the highest γ-PGA content was observed at 4.5% (w/v) concentration, it is producing 9.349 ± 0.017 mg/mL, which was significantly higher than the yields at 2.5% (w/v) and 3.5% (w/v).

This study indicated that dextrose may require higher concentrations to reach optimal γ-PGA production because its simplest sugar is less readily utilized at lower concentrations compared to glucose syrup.

Whereas glucose syrup shows higher performance for γ-PGA production by *Bacillus* sp. KMUTT06. This is attributed to its diverse sugar composition; the glucose syrup used in this study (DE60) typically consists of approximately 34% dextrose, 33% maltose, 10% maltotriose, and 23% higher sugars (w/v) [45]. This specific distribution of saccharides likely promotes better fermentation and utilization by the bacteria, resulting in a higher yield of γ-PGA at lower concentrations.

While increasing dextrose concentration provided more carbon sources for product synthesis, higher concentrations of glucose syrup led to a decline in γ-PGA yield. Furthermore, excessive sugar concentrations may induce osmotic stress, impair microbial metabolism and limit production efficiency at 4.5% (w/v) concentration. Although maltose results in slower growth than glucose, it supports higher biomass and enzyme activity, potentially enhancing γ-PGA production. However, increasing the initial carbon concentration can diminish specific enzyme efficiency and trigger osmotic stress, which impairs microbial metabolism and limits overall production yields [46]. Therefore, while glucose syrup proves to be a superior carbon source at lower concentrations, optimization of substrate concentration is critical to minimize osmotic stress and maximize γ-PGA yield [44].

The production of γ-PGA is largely influenced by the cost of carbon sources. Therefore, selecting a low-cost carbon source that can support high product yields is a critical factor for improving the overall economic feasibility of the production process. In recent years, many studies have explored the use of by-products of industrial and agricultural processes as carbon sources including molasses from the sugar industry [47], which has been reported to yield high levels of γ-PGA, glycerol waste from the biodiesel industry [48], and hydrolyzed rice straw or starch wastewater derived from agricultural residues [49]. Utilizing such low-cost carbon sources not only enhances γ-PGA production without compromising product quality but also promotes waste valorization and contributes to environmental sustainability. In this study, glucose syrup is a low-cost substrate obtained from the cassava starch-processing industry, showed that it significantly produces a γ-PGA higher yield than dextrose despite being used at low concentration. This result suggests that carbon sources with a diverse sugar composition, such as glucose syrup, may contribute to promoting efficient microbial metabolism and γ-PGA synthesis compared to pure sugars.

Therefore, glucose syrup has a potentially and economically viable carbon source for industrial-scale γ-PGA production, demonstrating both of enhanced fermentation efficiency and reduced production costs.

9. Antioxidant properties of γ-PGA from Bacillus sp. *KMUTT06*

The study of the antioxidant activity of γ-PGA of *Bacillus* sp. KMUTT06 using the DPPH method shows in Figure 7A that γ-PGA had an IC_{50} value of 0.650 ± 0.022 mg/mL, indicating it has antioxidant potential. However, its antioxidant activity was significantly lower than that of ascorbic acid, which had a much lower IC_{50} value of 0.0038 ± 0.004 mg/mL. This result is similar to previous research [50], which demonstrated that γ-PGA could scavenge DPPH radicals at concentrations ranging from 0.625 to 5.0 mg/mL, with radical scavenging activity levels of approximately 40-47%. These results confirm that γ-PGA exhibits antioxidant properties less efficient than ascorbic acid because it's a naturally occurring and well-known antioxidant with an enediol structure that rapidly electron and hydrogen atom to neutralize free radicals [51] more effectively than the larger γ-PGA polymer.

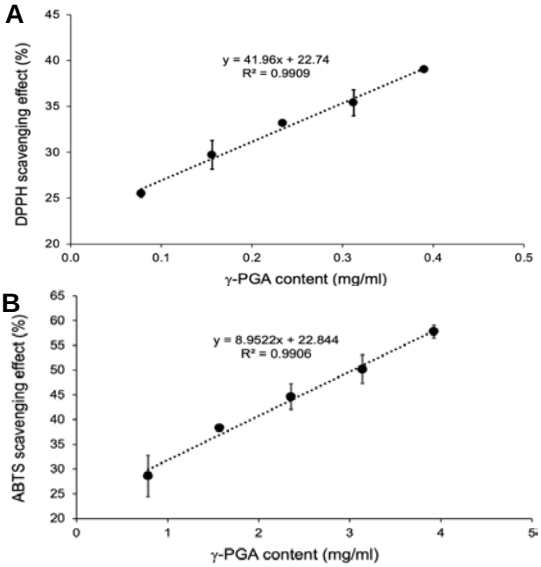

Figure 7 A linear relationship between DPPH (A) and ABTS (B) scavenging effect (%) and γ-PGA content (mg/ml) produced by *Bacillus* sp. KMUTT06.

Regarding the purity of the sample, the γ-PGA used in this study was a partially purified extract by ethanol. The presence of residual peptides or other impurity metabolites during the recovery

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process might have influenced the antioxidant performance, suggesting that higher levels of purification could potentially refine its specific activity.

The antioxidant activity of γ-PGA is likely attributed to its chemical structure, which is a polypeptide composed of D- and/or L-glutamic acid units linked by amide bonds between the γ-amino and γ -carboxylic groups [52]. These structural properties provide γ-PGA with the ability to donate electrons or hydrogen atoms from its carboxyl (-COOH) and amide (-CO-NH-) groups to DPPH radicals, thereby reducing their reactivity and scavenging the radicals. This potential mechanism effectively neutralizes oxidative stress, despite its lower efficiency compared to ascorbic acid [11].

γ-PGA still demonstrates notable antioxidant potential, which could make it a valuable compound for applications in many areas, such as food preservation or pharmaceutical formulations, where antioxidants are used to neutralize oxidative stress. Furthermore, this study investigated with ABTS assay to further monitor the antioxidant activity as showed in Figure 7B of γ-PGA produced from *Bacillus* sp. KMUTT06. The results from the ABTS assay indicated that γ-PGA had an IC_{50} value of 3.050 ± 0.148 mg/mL, which is still higher than ascorbic acid, which exhibited an IC_{50} value of 0.0021 ± 0.0002 mg/mL. Further analysis shows ascorbic acid had significantly higher antioxidant activity compared to γ-PGA. However, an interesting observation from this experiment is that when compared to the previous report showing that γ-PGA at a concentration of 10 mg/mL could reduce ABTS radical activity by only 42.1% [50].

Furthermore, the Ferric Reducing Antioxidant Power (FRAP) assay demonstrates the ability of γ-PGA to reduce ferric ions. These results showed that γ-PGA had a FRAP value of 8.806 ± 0.358 μmol Fe^{2+} per gram, which is lower than the FRAP values of ascorbic acid had a FRAP value of 1928.549 ± 55.634 μmol Fe^{2+} per gram and gallic acid had a FRAP value of 7388.949 ± 20.418 μmol Fe^{2+} per gram. Consequently, γ-PGA is significantly less efficient in reducing iron ions compared to both ascorbic acid and gallic acid, by a difference of approximately 219 and 839 times, respectively.

The lower FRAP value observed for γ–PGA can be attributed to its chemical structure, which differs from that of ascorbic acid and gallic acid. Both ascorbic acid and gallic acid contain multiple hydroxyl groups that are highly effective for donating electrons to reduce ions [51]. In contrast, γ–PGA lacks hydroxyl-rich functional groups. However, the antioxidation mechanism for γ–PGA in the FRAP assay may dependence more on its metal-chelating ability through its side-chain carboxylic groups rather than direct reduction, which explains its relatively lower efficiency compared to standard phenolic antioxidants.

The high efficiency of gallic acid is attributed to its multiple hydroxyl groups [53], which function as potent electron donors. Similarly, ascorbic acid exhibits strong reducing capacity through the single electron transfer (SET) mechanism [54], allowing for the rapid reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Finally, the observed antioxidant potential of γ-PGA suggests that it could still be useful in applications where moderate antioxidant activity is required, particularly in combination with other antioxidants or in formulations where its effect can be enhanced. Thus, further studies of its antioxidant efficiency or exploring its potential synergistic effects with other compounds could enhance its applicability in various industries.

CONCLUSIONS

This study isolated seven *Bacillus* sp. from fermented soybean products and commercial natto. Among the isolates, *Bacillus* sp. KMUTT06 showed the highest γ-PGA production, as proved by its high viscosity and band electrophoresis profile, showing thicker than another isolates, and quantitative chemical analysis of the γ-PGA at 1.116 mg/mL. The identity of the produced polymer was confirmed as γ-PGA through acid hydrolysis followed by HPLC analysis. The results showed that the hydrolysis of γ-PGA led to its to breakdown into glutamic acid. The *Bacillus* sp. KMUTT06 strain has a similarity of 99.93% to KCTC13622^T, *B. inaquosorum* KCTC13429^T, *B. cabrialesii* TE3^T and *B. cabrialesii* TSO23^T that prove KMUTT06 isolated similarly with these strains. Additionally, the result comparison of glutamate sources shows that MSG was more effective in promoting γ-PGA production than pure L-glutamic acid, particularly at concentrations of 2.0% (w/v). Glucose syrup proved to be a more efficient carbon source for γ-PGA production than dextrose, especially at lower concentrations, because of its broader sugar composition that enhances microbial growth and metabolism. Antioxidant activity estimates by using DPPH, ABTS, and FRAP assays shows antioxidant properties of γ-PGA were less potent compared with ascorbic acid and gallic acid.

Nevertheless, the antioxidant activity of γ-PGA is of interest to increase potential for use in various applications within the food and pharmaceutical industries. In conclusion, further studies on its antioxidant efficiency or exploring potential synergistic effects with other compounds could enhance its applicability. Furthermore, thorough characterization, purification, and proving whether the strain is glutamic acid-dependent or independent are essential for its application in various fields.

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DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors used Grammarly and Gemini to improve the language and translation of this manuscript. The authors have reviewed and edited the final text and take full responsibility for its content.

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