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Synthesis of Poly-γ-Glutamate in Solid-State Fermentation and Its Application in Biocontrol

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ABSTRACT

Poly- gamma- glutamic acid (γ-PGA) is a natural polymer with diverse applications across multiple industries. However, its use in agriculture is limited due to high production costs. This study aimed to optimize the cost-effective production of γ-PGA through Solid-State Fermentation (SSF) using *Bacillus velezensis* UTB96, evaluate the concentration and molecular weight of γ-PGA suitable for agricultural applications, particularly in strawberry cultivation, and explore the impact of γ-PGA on extending the shelf-life of strawberry fruits during cold storage. Initially, the production of γ-PGA using SSF with *B. velezensis* UTB96 was investigated, along with an evaluation of the influence of physicochemical factors on the molecular weight of γ-PGA. Based on the results, three different molecular weights of γ-PGA were identified: 1156.43 kDa, 734.38 kDa, and 296.55 kDa. These were selected for greenhouse trials to assess their effectiveness in controlling gray mold on strawberry plants. The results showed that by utilizing agricultural wastes, including sesame flour, wheat straw, and banana peel in SSF methodology, γ-PGA could be produced at a rate of 70 g/kg of dry weight of the culture medium. Analyzing the impact of γ-PGA on reducing gray mold revealed that this compound could enhance the plant's defense. A significant increase in the activity of ascorbate peroxidase and phenylalanine ammonia-lyase (PAL) enzymes was observed, along with the production of polyphenolic compounds such as ellagic acid. Consequently, these mechanisms improved the plant's flexibility and tolerance to the fungus, helping to maintain the quality of the fruits during cold storage.

Keywords: *Botrytis cinerea*, Molecular weight, γ-PGA, Solid-state fermentation.

INTRODUCTION

There is increasing interest in finding effective and sustainable alternatives to chemical

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pesticides. One promising option is biological control. Various microbial Biological Control Agents (BCAs) have been developed in recent decades to tackle fungal and bacterial diseases. Many studies have focused on microorganisms such as *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. (Bonaterra *et al.*, 2022). *Bacillus velezensis* is a well-known strain recognized for its beneficial effects on plant growth and its role in biocontrol as a gram-positive rhizobacterium (Fan *et al*., 2018). Recent research has investigated the microencapsulation of this bacterium with natural polymers and nanoparticles to control diseases like the *Rhizoctonia solani* fungus in beans (Moradi Pour *et al.*, 2021) and pistachio gum (Moradi Pour *et al*., 2022). Numerous studies have highlighted the potential of bacteria, particularly *Bacillus* species, in direct antibiosis, competition, and the secretion of various secondary metabolites in the rhizosphere (Fan *et al*., 2018). A significant secondary metabolite produced by *B. velezensis* is poly-gamma-glutamic acid (γ-PGA). This biopolymer is biodegradable, non-toxic, environmentally safe, and hypoallergenic, making it highly sought after in various industries. Research on this biological compound has shown that γ-PGA can significantly enhance plant performance and morphological characteristics. It also improves Soil Microbial Biomass Carbon (SMBC) and Nitrogen (SMBN), boosts soil enzyme activity, and increases plant resistance to both biotic and abiotic stresses (Song *et al*., 2019).

Given the widespread application of γ-PGA across various industries, its industrial production through Submerged Fermentation (SmF) using *Bacillus* bacteria is common. However, this method is expensive, which restricts its use in agriculture. In contrast, Solid-State Fermentation (SSF) presents a cost-effective alternative by utilizing inexpensive and readily accessible raw materials. Furthermore, SSF provides various economic and engineering benefits, such as lower energy consumption and simplified equipment and infrastructure requirements (Chen *et al.*, 2005).

Therefore, this study investigates innovative approaches for the cost-effective production of γ-PGA through solid-state fermentation, utilizing affordable waste materials for agricultural purposes. Additionally, it examines the effects of environmental factors on the molecular weight of γ-PGA. Another critical aspect addressed in this article is the mechanisms by which γ-PGA enhances the resistance of strawberry plants to necrotrophic fungi, particularly *Botrytis cinerea*, the most prevalent and destructive fungal pathogen affecting strawberries. Additionally, the study investigates the effect of pre-harvest application of γ-PGA on the shelf life of strawberry fruits during cold storage.

MATERIALS AND METHODS

1: γ-PGA Production in Solid-State Fermentation (SSF) and Its Identification

Preparation of *Bacillus velezensis* UTB96 Bacteria:

In this study, the *Bacillus velezensis* strain UTB96 was obtained from the microbial collection at Tehran University. The bacteria were cultured in a nutrient broth medium (NB) at 37°C and 200 rpm for 16 hours to serve as the inoculum. This medium contained approximately 3×10^7 cells per mL.

Substrate for Solid-State Fermentation

To achieve optimal γ-PGA production, a balanced combination of protein, sugar, and carbon sources is essential. For the economical production of γ-PGA, the recommended medium consists of a blend of sesame flour (as the protein source), wheat straw (as the carbon source), banana peel (providing sugar and minerals), and cow manure (as a mineral source). Various experiments were conducted with different ratios of these components to determine the most effective combination for production.

Extraction and Purification of γ-PGA

The extraction and purification of γ-PGA were performed according to the method described by Goto and Kunioka (Goto and Kunioka, 1999).

Identification and Characterization of γ-PGA

The produced γ-PGA was identified and characterized using SDS-PAGE (Yu *et al*., 2016), FT-IR (Khalil *et al*., 2018), and spectrophotometric methods (Zeng *et al*., 2012).

Determining the Molecular Weight and Investigating the Effect of Environmental Physicochemical Factors on the Molecular Weight of γ-PGA

The effects of the following physicochemical factors on the molecular weight of γ-PGA were studied by varying one factor at a time:

a) Incubation temperature (27 to 42°C)
b) Initial moisture level of the fermentation medium (50 to 75 %)
c) Initial pH level of the fermentation medium (5 to 8)
d) Fermentation time (12 to 96 hours)

The average molecular weights of the γ-PGA product obtained under these specified environmental conditions were determined using Gel Permeation Chromatography (GPC). The

mobile phase consisted of 50 mM phosphate buffer at pH 6.8, with a flow rate of 1.0 mL/min. Molecular weights were calculated relative to polystyrene standards.

2: Studying the Impact of γ-PGA on Strawberry Resistance to *B. cinerea* Fungus and Plant Biochemical Mechanisms

The effect of γ-PGA on the resistance of strawberry plants to *B. cinerea* was evaluated under greenhouse conditions. For this research, all fermented culture media containing γ-PGA in three different molecular weights (high: 1156.43 kDa, medium: 734.38 kDa, and low: 296.55 kDa) were selected for experimentation. These media were dried in an oven at 70°C for 3 days and then crushed using a mill. The resulting powders were mixed with a combination of perlite and cocopeat in a 50:50 ratio to serve as the substrate for strawberry cultivation.

The concentrations of γ-PGA investigated were 10, 20, 50, and 100 mg/kg of the cultivation bed. Each treatment involved five 1.5-liter pots, with two strawberry cv. 'Camarosa' seedlings planted in each pot, which were irrigated daily with 100 cc of drinking water. The light-dark photoperiod was set to 8 hours of light and 16 hours of darkness. At the end of the flowering stage, the plants were inoculated with a *B. cinerea* fungus suspension containing 10^6 spores per mL. The control treatment (Sh0) was inoculated with water.

Treatments

The treatments consisted of two control groups and γ- PGA groups:

a) Control treatment group:

- Sh0: Control treatment without fermentation substrate, with or without γ-PGA, and without contamination by *B. cinerea* fungus.
- Sh-: Control treatment without fermentation substrate, with or without γ-PGA, and with contamination by *B. cinerea* fungus.
- Sh 10,20, 50, and 100 mg: In this investigation, the entire solid-state fermentation substrate was added to the pots. To assess the impact of compounds produced by bacteria other than γ-PGA, the fermentation substrate without the γ-PGA compound was added to the culture medium at concentrations of 10, 20, 50, and 100 mg/kg of soil. This group of control treatments was inoculated with the *B. cinerea* fungus.

b) γ-PGA treatment group: The treatments consist of fermentation substrate containing γ-PGA with molecular weights of 296.55, 734.38, and 1156.43 kDa. Each of these molecular weights was investigated at four concentrations: 10, 20, 50, and 100 mg/kg of soil. The γ-PGA group treatments were inoculated with the fungus *B. cinerea.*

Ascorbate Peroxidase (APX) Enzyme Activity Assay

Sampling of strawberry leaves for the investigation of biochemical characteristics was conducted immediately before inoculation and on days 3, 7, 10, 20, and 30 post-inoculations, coinciding with the first fruit harvest. The samples were promptly transferred to a -80 freezer for storage until examination. The activity of the APX enzyme was determined using the method described by Braga *et al.* (Braga *et al.*, 2009).

Phenylalanine Ammonia Lyase (PAL) Enzyme Activity Assay

The activity of phenylalanine ammonia-lyase was assessed by converting L-phenylalanine into trans-cinnamic acid following the protocol outlined by Tovar *et al.* (Tovar *et al.*, 2002). PAL enzymatic activity was reported as units per gram of fresh leaf weight.

Assay of Ellagic Acid (EA)

The concentration of ellagic acid was measured using the spectrophotometric method established by Wilson and Hagerman (Wilson and Hagerman, 1990).

3: Measurement of Physicochemical Characteristics of Fruits in Storage Conditions

To determine the effectiveness of γ-PGA on the shelf life and quality of fruits in storage, physicochemical tests were conducted. These tests included assessing weight loss, firmness, Total Soluble Solids (TSS), and Titratable Acidity (TA) using established methods (Farida *et al.*, 2023). Forty fruits were harvested from each treatment and stored in a cold room at a temperature of 4°C and a humidity of 90% for 10 days. The samples were analyzed on days 0, 3, 6, and 10.

Apparent Decay of Fruits

To assess decay, 20 fruits from each treatment were randomly selected, and the average decay for each treatment was calculated using the numerical scale defined by Babalare *et al.* (2007). Fungal decay was evaluated through visual examination and microscopic observation of fungal mycelium growth. The numerical scale ranged from 5 (indicating no decay) to 1 (indicating more than 16% decay), with intermediate values representing different levels of decay: 4 (less than 5%), 3 (6-10%), and 2 (11-15%) (Babalar *et al.*, 2007).

4. Statistical Analysis

This study utilized a completely randomized design. The data were analyzed using SAS 9.1.3 statistical software (2001). Duncan's multiple range test was employed at the 5%

significance level to compare the means. The experiments were conducted in triplicate.

RESULTS AND DISCUSSION

1: γ-PGA Production by SSF and Its Identification

Substrate of SSF

Studies have shown that nitrogen and carbon sources, particularly sugars, play a crucial role in γ-PGA production (Sung *et al.*, 2005). Therefore, in this study, the optimal ratio between sesame flour, which serves as a nitrogen source, and banana peel, which acts as a sugar source, was determined (Table 1, Table 2). In all the examined samples, the amounts of wheat straw was 150 g and manure in the substrate - 50 g and remained constant. Subsequently, based on this ratio, the proportion of cow manure to wheat straw was calculated. To determine the optimal ratio, various combinations were examined (Table 3). The most effective composition of the fermentation substrate comprised (g/kg dry weight of substrate): 600g sesame flour, 200g banana peels, 160g wheat straw, and 40g cow manure, resulting in a γ-PGA production rate of 70 g/kg dry weight (DW) of the substrate.

Table 1. Main composition in selected substances for solid-state fermentation.

The main composition	Minerals	Carbohydrate	Carbon source (sugar)	Protein
Substances	Cow manure	Wheat straw	Banana peel	Sesame flour
Amount	34 %	42.56 g/100 g	39.8 g/100g	45.8 g/100g

Table 2. The optimal ratio of sesame flour to banana peel (g).

Sample	Sesame flour	Banana peel	Kg of the	The average production of γ-PGA per dry weight of the substrate
1	200	600		55/75[d]
2	300	500		61/5[c]
3	400	400		65[b]
4	500	300		68/33[a]
5	600	200		69/16[a]
6	700	100		60/2[c]

Table 3. The optimal ratio of wheat straw to manure and between sesame flour and banana peel (g).

Sample	Sesame flour	Banana peel	Wheat straw	Manure	The average production of γ-PGA per Kg of the dry weight of the substrate
1	600	200	180	20	$62/5^{c}$
2	600	200	170	30	$62/5^{c}$
3	600	200	160	40	70^{a}
4	600	200	150	50	69^{b}
5	600	200	140	60	$52/5^{e}$
6	600	200	100	100	$33/25^{g}$
7	600	200	160	40	$67/5^{b}$
8	400	200	380	20	54^{e}
9	400	200	360	40	$57/5^{d}$
10	400	200	340	60	$56/75^{d}$
11	400	200	320	80	$42/5^{f}$

Different letters indicate significant differences between production values of γ-PGA ($P<0.05$).

Identification and Characterization of γ-PGA

Various analytical tools were employed to identify and characterize the γ-PGA produced by *B. velezensis* UTB96 through SSF. As shown in Figure 1- a, the Fourier-Transform Infrared (FT-IR) spectrum of γ-PGA displayed amide bands at 1648.6 cm^{-1} and carbonyl group C=O at 1402.43 cm^{-1}. Additionally, it exhibited C-N stretching vibrations at 1075.76 cm^{-1} and an O-H stretching band at 3447.26 cm^{-1}.

The chemical structure of the γ-PGA obtained in this study was consistent with the structure elucidated by Ho *et al*. (2006) (Khalil *et al*., 2018). Notably, based on previous studies (Rajan *et al*., 2014) and the absorption bands of amide groups, the secondary structure of γ-PGA was identified as an α-helix motif.

Figure 1- b displays the UV absorption spectrum of γ-PGA in deionized water, covering the range of 190 – 340 nm. According to the orbital law, peptide excitation absorption peak, resulting from the presence of carbonyl and amide groups, typically occurs around 200 nm (Braga *et al*., 2009). This aligns with our study's findings that the γ-PGA biopolymer exhibited maximum absorption at 200 nm (216 nm).

Studies have demonstrated the effectiveness of methylene blue staining as a qualitative method for measuring γ-PGA production (Yu *et al.*, 2016). In this investigation, the production of γ-PGA by *B. velezensis* UTB96 was successfully confirmed using methylene blue staining, as shown in Figure 1- c.

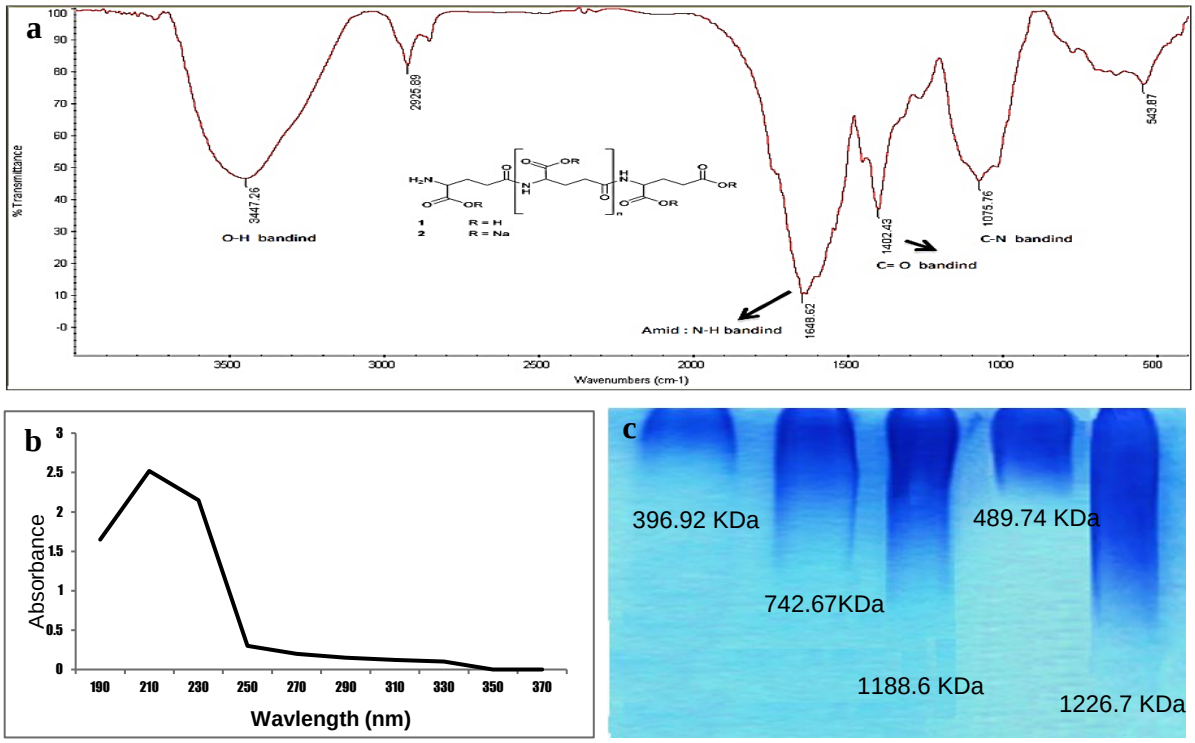


Figure 1. γ-PGA Identification, a: FT-IR absorption peaks (cm^{-1}), b: UV spectral analysis, c: SDS-PAGE for methylene blue stained samples containing purified γ-PGA.

The mechanism by which the cationic dye methylene blue is absorbed by γ-PGA is linked to the active sites (COO^-) present on the surface of the polymer. The absorption of methylene blue occurs spontaneously (Ogata *et al*., 2017). The increases of the concentration or molecular weight of γ-PGA is indicating on a longer peptide chain and increasing the number of active sites (COO-). This increase in active sites enhances dye absorption, as demonstrated by the results.

Poly-γ- Glutamic Acid Molecular Weight

Microbial production of γ-PGA can result in molecular weights ranging from 100 to over 2,000 kDa. Several factors influence the efficiency and molecular weight of γ-PGA (Sung *et al*., 2005). To investigate the effect of temperature on the molecular weight of γ-PGA, five experiments were conducted at varying temperatures. As shown in Figure 2- a, the maximum γ-PGA molecular weight (1117.53 kDa) was observed at 37°C, with similar values around 1112.9 kDa at 32°C. A decrease in the molecular weight was noted at 42°C, although this change was not significant compared to the 37°C. This reduction may be attributed to rapid cell growth at 42°C during the initial stages, which depletes nutrients in the medium and leads to the utilization of γ-PGA as a nitrogen and carbon source for bacterial cells in the later stages of

fermentation. Additionally, temperature can influence the activity of enzymes involved in synthesizing the γ-PGA amino acid chain, potentially affecting the molecular weight (Ajayeoba *et al*., 2019).

The effect of moisture on the molecular weight of γ-PGA is illustrated in Figure 2- b. There was a direct relationship between humidity and the molecular weight of γ-PGA; as humidity increased to 65%, the molecular weight of γ-PGA also rose. However, a subsequent decrease in molecular weight was observed beyond this point. Moisture plays a vital role in oxygen and mass transfer during solid-state fermentation (SSF). Reports indicate that the activity of the pgsBCA enzyme complex is ATP-dependent, with ATP levels being influenced by oxygen availability. Therefore, when media humidity is at its optimal level, both enzyme complex activity and the molecular weight of γ-PGA are expected to increase (Sung *et al*., 2005).

In Figure 2- c, it is shown that as the pH increased from 5 to 8, the molecular weight of γ-PGA rose from 630 kDa to 1121.4 kDa. There was no significant difference in molecular weight between pH values from 6.5 to 8. Under acidic conditions, the carboxylic acid groups do not ionize, causing the γ-PGA structure to adopt an α-helical conformation, which results in decreased stability and decomposition of the compound (Seo *et al.*, 2008), leading to a reduction in molecular weight. In contrast, at higher pH levels, the ionic hydration of γ-PGA induces a conformational shift from α-helix to random coil (Seo *et al*., 2008), thereby increasing the compound's molecular weight.

At 96 hours of fermentation, the molecular weight of γ-PGA increased significantly between 12 and 36 hours. However, after 48 hours, a downward trend in molecular weight was observed. By the end of the 96-hour fermentation period, the molecular weight of γ-PGA showed a 46% decline compared to the 36-hour fermentation period (Figure 2- d). When γ-PGA consists of longer monomeric chains, its molecular weight is higher. Therefore, extending the fermentation time up to 36 hours provides the polymerase enzyme systems with more time to polymerize monomers, increasing the molecular weight of γ-PGA. Prolonging fermentation beyond 36 hours can create adverse conditions such as nutrient scarcity, oxygen depletion, and low humidity levels, which subsequently reduce the activity of the γ-PGA polymerase enzyme. In response, bacteria may hydrolyze γ-PGA as a source of carbon and nitrogen to ensure their survival (Cao *et al*., 2018), leading to a decrease in the molecular weight of γ-PGA.

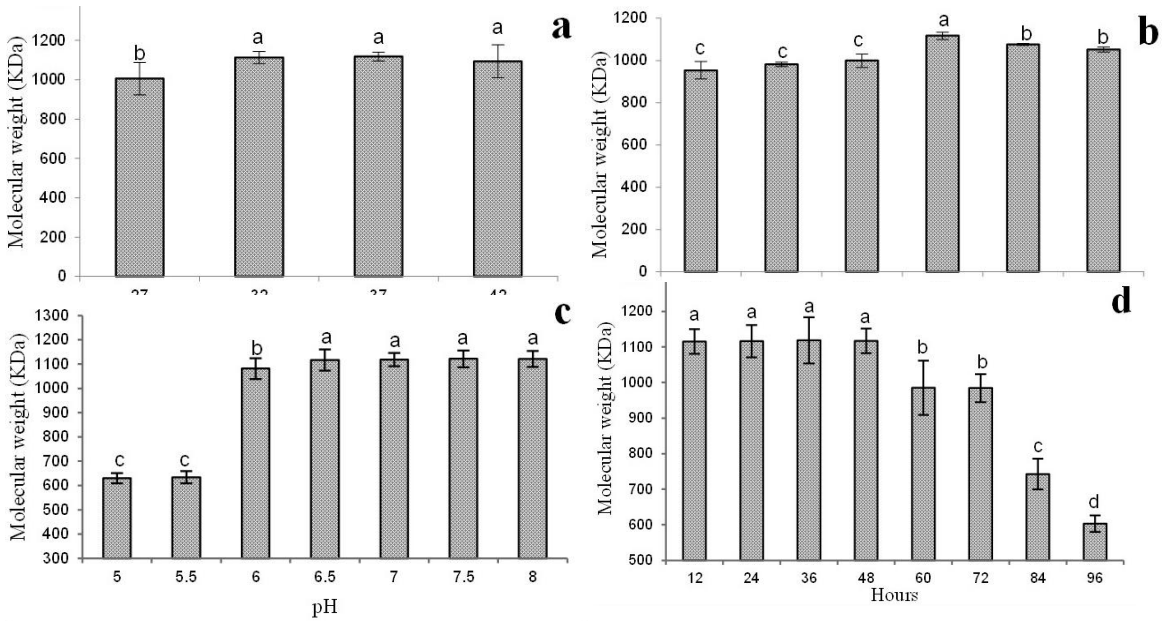


Fig 2. Effect of different factors on γ-PGA molecular weight, a: temperature, b: initial moisture, c: Initial pH, d: incubation time. Different letters in each figure indicate significant differences between the states of the investigated factor (P‹0.05).

2: γ-PGA Effect on Strawberry Plant Resistance to Gray Mold Disease

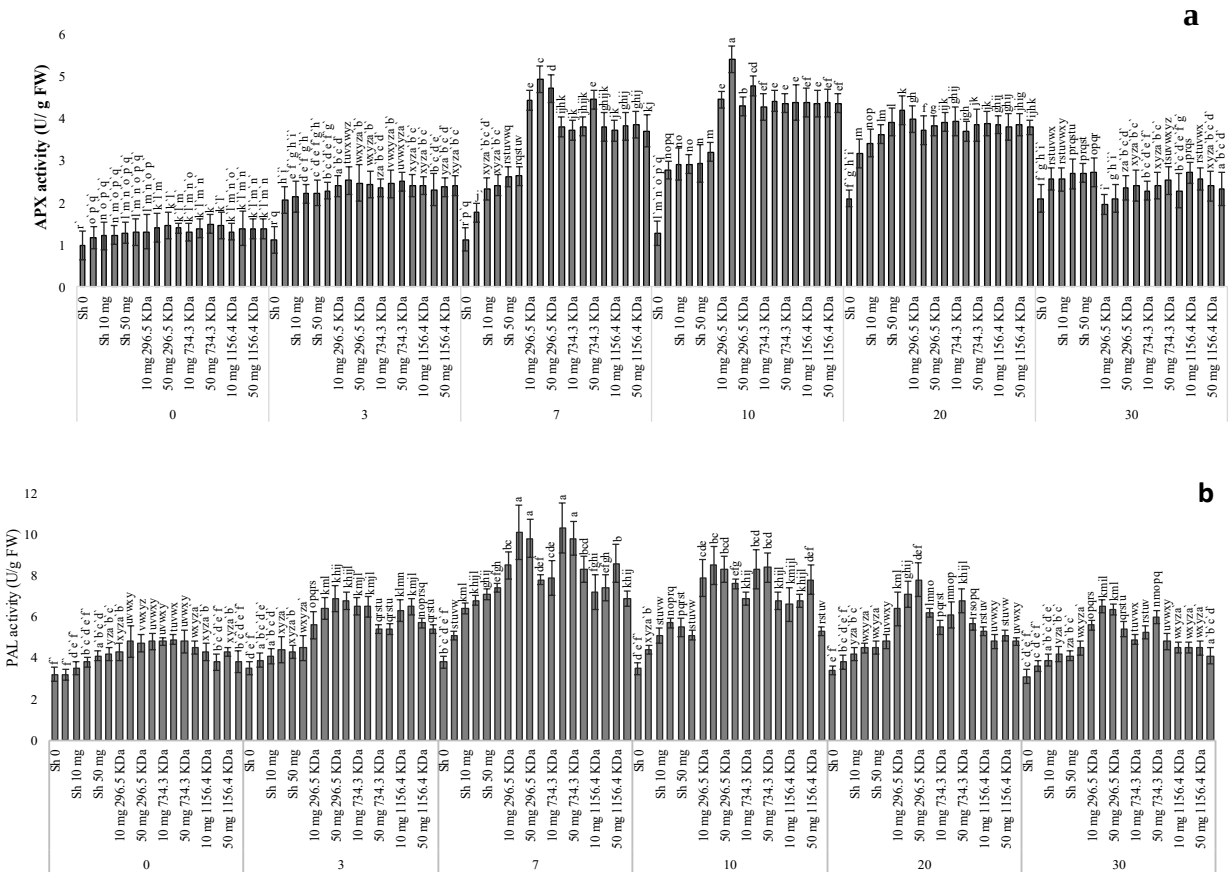
Ascorbate Peroxidase (APX) Enzyme Assay

The activity of the APX enzyme in the leaves of plants treated with γ-PGA showed an increasing trend until the tenth day. Notably, the increase in enzyme activity was significantly higher in the γ-PGA treatments compared to the infected control (Sh-). On the tenth day, the highest enzyme activity was recorded in the treatment of 20 mg 296.55 kDa, which was 50 times higher than that of the negative control (Fig. 3- a). After the tenth day, the activity of the ascorbate peroxidase enzyme began to decline. In contrast, the control treatments continued to show an increase in enzyme activity until the twentieth day (Fig. 3- a). This continued increase in the control could be attributed to the emergence of new infections or the spread of the fungal pathogen *B. cinerea* within the plant tissues.

APX is a key enzyme that converts ascorbate to dehydroascorbate, effectively removing peroxides, particularly H_2O_2, from plant cells (Navari-Izzo *et al*., 1997). Based on the results obtained, it can be concluded that γ-PGA positively influences the plant's antioxidant system, facilitating the metabolism of reactive oxygen species (ROS) and preventing the penetration and spread of the necrotrophic fungus *B. cinerea* in strawberry plants.

Phenylalanine Ammonia Lyase (PAL) Enzyme Assay

The application of γ-PGA significantly increased the activity of the PAL enzyme from the third to the seventh day following the inoculation of *B. cinerea* in the γ-PGA treatments compared to the control groups. The highest increases in PAL activity were observed in the treatments with 20 and 50 mg of 296.55 kDa, as well as 50 and 20 mg of 734.38 kDa. In contrast, the uninfected (Sh0) and negative (Sh-) control groups exhibited the lowest levels of phenylalanine ammonia-lyase activity (Fig. 3- b). PAL plays a crucial role in the biosynthesis of polyphenolic compounds, including flavonoids, phenylpropanoids, and lignin in plants. Research suggests that γ-PGA can enhance the activity of the PAL enzyme by activating the ROS signaling pathway, leading to increased enzyme activity (Lei *et al.*, 2015). Furthermore, studies have indicated that stimulating the phenylpropanoid pathway to produce polyphenolic and flavonoid compounds can help neutralize and prevent excessive ROS production caused by *B. cinerea* infection (Kumar *et al.*, 2020; Perkowski and Warpeha., 2019).



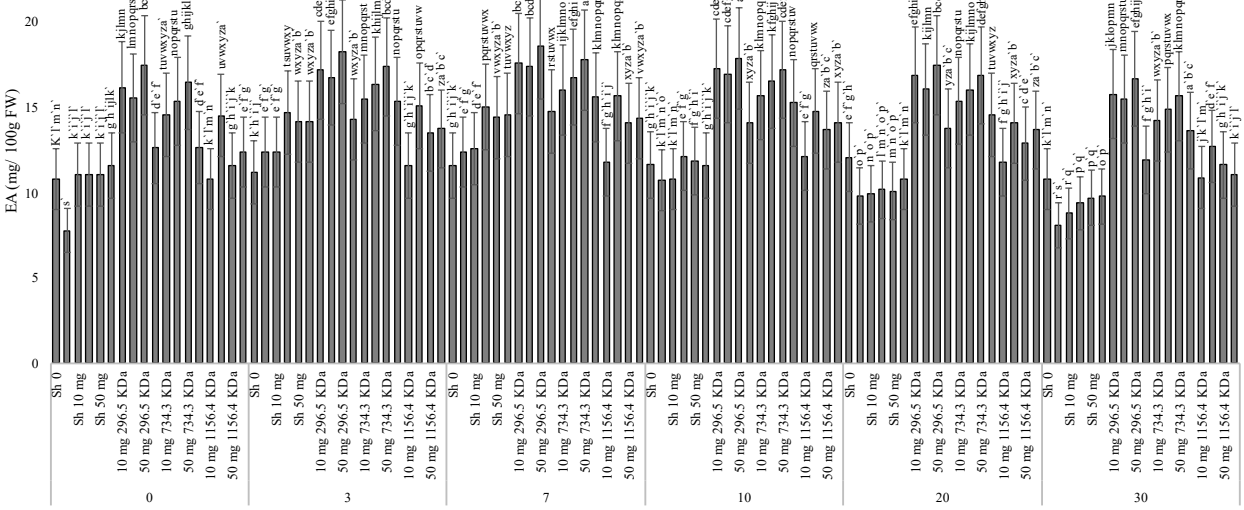
*The order of the columns in the days examined is: Sh0, Sh-, Sh10, Sh20, Sh50, Sh100, 10 mg 296.5 kDa, 20 mg 296.5 kDa, 50 mg 296.5 kDa, 100 mg 296.5 kDa, 10 mg 734.3 kDa, 20 mg 734.3 kDa, 50 mg 734.3 kDa, 100 mg 734.3 kDa, 10 mg 1156.4 kDa, 20 mg 1156.4 KDa, 50 mg 1156.4 KDa and 100 mg 1156.4 kDa.

Figure 3. The effect of γ-PGA on the activity of enzymes involved in strawberry plant defense, a: Ascorbate peroxidase (APX), b: Phenylalanine ammonia- lyase (PAL).

Impact of γ-PGA on the Ellagic Acid (EA) Content in Leaves

The level of ellagic acid in strawberry leaves continued to rise until the seventh day, with the greatest increase observed in the treatment with 50 mg of 296.55 kDa (18.6 mg/100 g FW). Subsequently, a significant decline in ellagic acid was noted in the control treatments, while in the γ-PGA treatments, this decline exhibited a more gradual trend (Fig. 4).

Within plant cells, ellagic acid exists in both free and covalently-bound forms, such as EA glycosides and ellagitannins. Although the level of free ellagic acid in cells is initially low, it tends to increase during biotic and abiotic stresses. Ellagic acid has demonstrated efficiency in absorbing free radicals (Williams *et al*., 2014), Therefore, the rise in ellagic acid levels during the compatible interaction between *B. cinerea* and strawberries may indicate its direct involvement in protecting plant cells through the modulation of the redox balance. Additionally, this compound contributes to defense against fungal pathogens by damaging ergosterol (Lei *et al*., 2015). As a result, ellagic acid leads to pathogen death by disrupting ergosterol and compromising the integrity of the fungal membrane.



*The order of the columns in the days examined is: Sh0, Sh-, Sh10, Sh20, Sh50, Sh100, 10 mg 296.5 kDa, 20 mg 296.5 kDa,50 mg 296.5 kDa, 100 mg 296.5 kDa, 10 mg 734.3 kDa, 20 mg 734.3 kDa, 50 mg 734.3 kDa,100 mg 734.3 kDa,10 mg 1156.4 kDa, 20 mg 1156.4 KDa, 50 mg 1156.4 KDa and 100 mg 1156.4 kDa.

Figure 4. γ-PGA effect on the activity of Ellagic acid (EA) in strawberry leaves inoculated with *B. cinere.*

3: Measurement of Physicochemical Characteristics of Fruits in Storage Conditions

As storage time increased, fruits exhibited a decrease in weight, firmness, and titratable acidity. Notably, the control treatments showed the most significant reduction compared to the

γ-PGA treatments (Table 4). The lowest reduction was observed in the treatments with 50 mg and 20 mg of 296.55 kDa, as well as 50 mg of 734.38 kDa.

In terms of Total Soluble Solids (TSS) analysis, a gradual increase was noted across all treatments throughout the storage period. The smallest increase was recorded in the treatment with 50 mg of 296.55 kDa, which was 27.4% lower than the Sh- treatment.

Physicochemical changes during fruit storage are primarily caused by respiration, water loss, the consumption and breakdown of stored compounds and metabolites during metabolic activities, and the activity of degrading enzymes (Fawole *et al*., 2020). Research has shown that γ-PGA operates through various mechanisms, including reducing the activity of cell wall degrading enzymes (Wang *et al*., 2020), inhibiting abscisic acid signal transmission (Shan *et al*., 2023), and enhancing the antioxidant capacity in fruits. These actions collectively contribute to maintaining cell integrity, preventing water loss, reducing decay, and delaying aging in fruits. As a result, γ-PGA plays a crucial role in preserving the quality of fruits during storage.

Apparent Decay of Fruits

At the end of ten days, no symptoms of fungal infection were observed in the γ-PGA treatments, while the fruits in the control treatments exhibited moderate to severe rotting (Table 4). As storage time increases, the texture of strawberries softens due to changes in the cell wall structure (Brummell *et al*., 1999). The antioxidant activity of γ-PGA and its ability to maintain the integrity of the cell wall, helps prevent rapid deterioration of the fruit tissue and maintains its hardness. This, in turn, inhibits the penetration and spread of fungi into the fruit tissue.

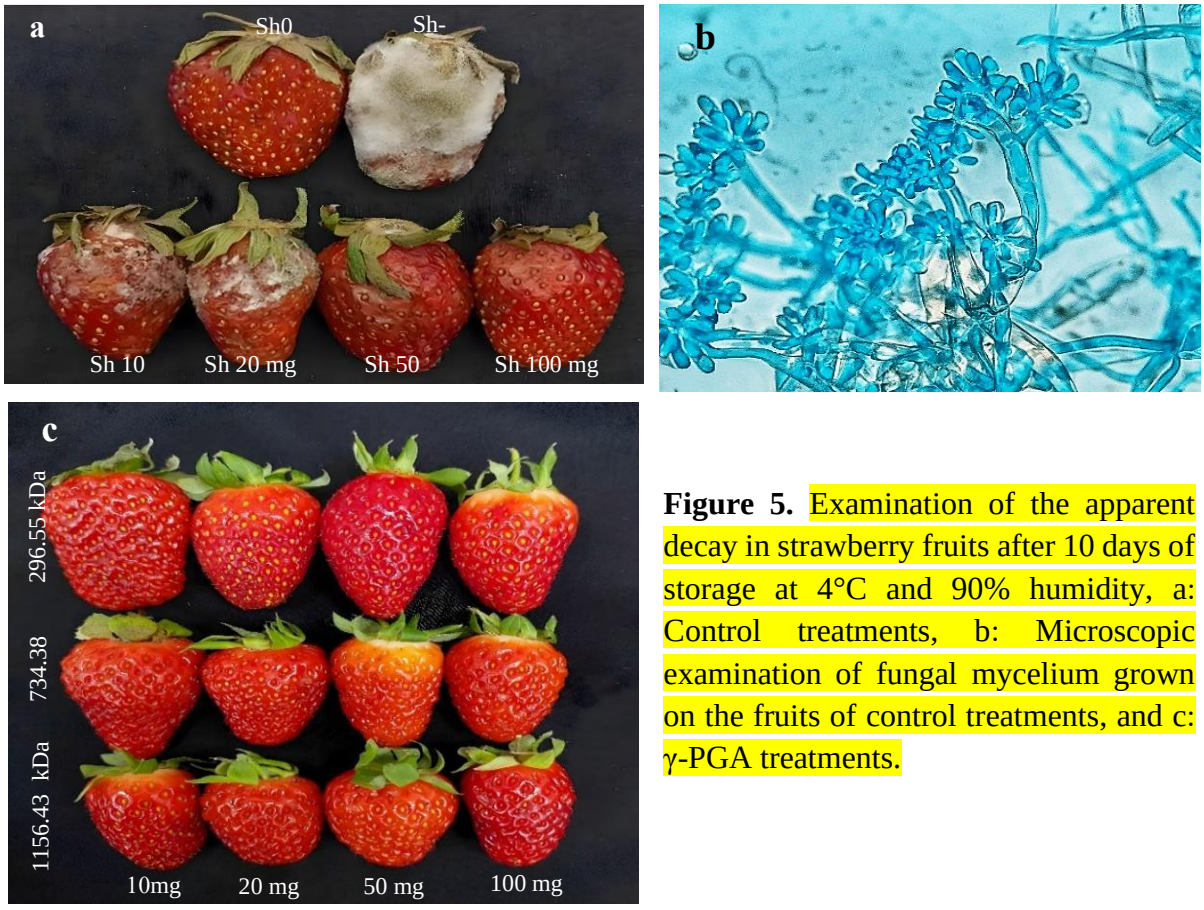


Figure 5. Examination of the apparent decay in strawberry fruits after 10 days of storage at 4°C and 90% humidity, a: Control treatments, b: Microscopic examination of fungal mycelium grown on the fruits of control treatments, and c: γ-PGA treatments.

Table 4. The impact of different treatments on the physicochemical properties of strawberry fruits after 10 days of cold storage.

Indicator Treatment	Weight loss (%)	TA (%)	TSS (%)	Firmness (kg/m^2)	Apparent decay
Sh 0	5.3^{b}	0.62^{cdefg}	8.3^{a}	0.64^{g}	3.11^{b}
Sh-	5.9^{a}	0.47^{g}	8.4^{a}	0.6^{g}	1.04^{e}
Sh 10 mg	4.5^{c}	0.5^{fg}	8^{ab}	0.62^{g}	1.95^{d}
Sh 20 mg	4.28^{cd}	0.53^{efg}	7.9^{ab}	0.73^{fg}	1.95^{d}
Sh 50 mg	4.2^{cd}	0.58^{defg}	7.6^{bc}	0.81^{ef}	2.83^{c}
Sh 100 mg	4.12^{d}	0.6^{defg}	7.3^{cd}	0.84^{def}	2.52^{c}
10 mg 296.5 kDa	2.9^{hi}	0.72^{bcd}	6.7^{ef}	0.95^{cde}	4.79^{a}
20 mg 296.5 kDa	1.6^{kl}	1.15^{a}	6.5^{fg}	1.14^{ab}	4.79^{a}
50 mg 296.5 kDa	1.5^{l}	0.8^{b}	6.1^{g}	0.9^{de}	4.79^{a}
100 mg 296.5 kDa	2.3^{j}	0.72^{bcd}	6.7^{ef}	0.98^{cd}	4.79^{a}
10 mg 734.3 kDa	2.9^{hi}	0.69^{cde}	7^{def}	0.88^{de}	4.79^{a}
20 mg 734.3 kDa	3.06^{gh}	0.74^{bcd}	6.5^{fg}	0.83^{ef}	4.79^{a}
50 mg 734.3 kDa	1.9^{k}	0.7^{dc}	6.5^{fg}	1.2^{a}	4.79^{a}
100 mg 734.3 kDa	3.23^{fg}	0.64^{cdef}	6.8^{def}	0.91^{cde}	4.79^{a}
10 mg 1156.4 kDa	2.7^{i}	0.77^{bc}	6.6^{ef}	1.05^{bc}	4.79^{a}
20 mg 1156.4kDa	2.8^{hi}	0.68^{cde}	7.1^{cde}	0.9^{de}	4.79^{a}
50 mg 1156.4 kDa	3.6^{e}	0.66^{cdef}	6.9^{def}	0.92^{cde}	4.79^{a}
100 mg 1156.4 kDa	3.5^{ef}	0.64^{cdef}	7.2^{cde}	0.98^{cd}	4.79^{a}

* Different letters in columns show significant differences (P‹0.001).

CONCLUSIONS

The findings of this research demonstrate that through solid-state fermentation and

manipulation of fermentation conditions, it is possible to produce γ-PGA compounds in significant quantities with diverse molecular weights. The results indicate that γ-PGA, particularly at low to medium molecular weights and concentrations, could enhance plant defense against necrotrophic pathogens, such as *B. cinerea*, by activating antioxidant mechanisms and boosting the generation of defense compounds. This enhancement could also prolong the storage life of strawberry fruit. The outcomes of this study present a promising avenue for researchers and experts in agriculture to further investigate the potential applications of γ-PGA in various fields, including biological control.

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تولید پلی-γ-گلوتامات به روش فرمانتاسیون بستر جامد و کاربرد آن در کنترل زیستی

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چکیده

پلی- گاما- گلوتامیک اسید (γ-PGA) یک پلیمر طبیعی با کاربردهای متنوع در صنایع مختلف است. با این حال، استفاده از آن در کشاورزی به دلیل هزینه‌های بالای تولید محدود است. اهداف این مطالعه: تولید بهینه و مقرون به صرفه γ-PGA از طریق تخمیر حالت جامد (SSF) با استفاده از باکتری*Bacillus velezensis* UTB96، تعیین غلظت و وزن مولکولی مناسب γ-PGA برای کاربردهای کشاورزی، به‌ویژه در کشت توت فرنگی، و در نهایت بررسی تأثیر γ-PGA تولید شده بر افزایش عمر مفید میوه‌های توت‌فرنگی در طول انبارمانی است. ابتدا تولید γ-PGA با استفاده از SSF و باکتری *Bacillus velezensis* UTB96 مورد بررسی قرار گرفت و تأثیر عوامل فیزیکوشیمیایی بر وزن مولکولی γ-PGA ارزیابی شد. بر اساس نتایج، سه وزن مولکولی مختلف γ-PGA شناسایی شد: 1156.43، 734.38 و 296.55 کیلودالتون. این وزن‌ها برای آزمایش‌های گلخانه‌ای به منظور ارزیابی اثربخشی آن‌ها در کنترل کپک خاکستری روی گیاه توت فرنگی انتخاب شدند. نتایج نشان داد که با استفاده از ضایعات کشاورزی، از جمله کنجاله کنجد، کاه گندم و پوست موز در روش SSF، می‌توان γ-PGA را با نرخ ۷۰ گرم در کیلوگرم وزن خشک محیط کشت تولید کرد. تحلیل تأثیر γ-PGA بر کاهش بیماری کپک خاکستری نشان داد که این ترکیب می‌تواند مقاومت گیاه را بهبود بخشد. افزایش قابل توجهی در فعالیت آنزیم‌های آسکوربات پراکسیداز و فنیل‌آلانین آمونیالیاز (PAL) همراه با تولید ترکیبات پلی‌فنولی مانند اسید الاژیک مشاهده شد. در نتیجه، این مکانیسم‌ها انعطاف‌پذیری و تحمل گیاه را در برابر قارچ بهبود بخشیدند و منجر به حفظ کیفیت میوه‌ها در طول نگهداری در سردخانه شدند.