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INVESTIGATION OF OPTIMAL CONDITIONS FOR THE PRODUCTION OF POLY GAMMA GLUTAMIC ACID (Γ -PGA) FROM A LOCAL ISOLATE OF THE BACILLUS MEGATERIUM

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Abstract

This study involved the production of poly-γ-glutamic acid (γ-PGA) using a local isolate of the *Bacillus megaterium* bacteria. Two natural carbon sources were used for qualitative detection and quantitative estimation using thin layer chromatography [TLC]. The study also evaluated its ability to grow in a production medium and improve productivity by manipulating its components and environmental conditions using molasses (sugarcane), corn syrup, glucose, fructose, sucrose, and some sources of nitrogen. The best sources and optimal concentrations of carbon and nitrogen were selected. The optimal conditions for production of γ-PGA were by using a semi-synthetic medium by preparing the molasses as a carbohydrate source at 10% concentration, while ammonium nitrate provided the best source of nitrogen at 2%. This study showed that the optimum temperature and initial pH for achieving better γ-PGA productivity from the studied bacteria was at 37° C and 6.5, respectively. Higher productivity was achieved by agitation and aeration at a ratio of 5:1 of the fermentation media size to flask volume in the rotary shaker incubator at 180 rpm/min, using an inoculum size of 2.8×10^6 cfu/mL in the production medium till the total γ-PGA concentration reached 7.22 mg/ml. The γ-PGA was then extracted from a productive

medium after precipitation and obtaining pure crystals, and its purity determined using Fourier-Transform Infrared Spectroscopy (FTIR).

Keywords: (γ-PGA), B. megaterium, Molasses, corn syrup, Fermentation, FTIR.

دراسة الظروف المثلى لإنتاج متعدد حامض كاما كلوتاميك (γ–PGA) من عزلة محلية لبكتريا Bacillus megaterium

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الخلاصة

شملت هذه الدراسة إنتاج متعدد حامض الكلوتاميك من نوع كاما (γ–PGA) باستخدام العزلة المحلية لبكتريا *Bacillus megaterium* ومن ثم إجراء الكشف النوعي والتقدير الكمي بواسطة كروماتوغرافيا الطبقة الرقيقة (TLC)، ودراسة قدرة هذه العزلة على النمو باستخدام مصدرين طبيعيين للكربون هما المولاس وشراب الذرة، كما تم استخدام سكريات نقية من الكلوكوز والفركتوز والسكروز مع بعض مصادر النيتروجين لتحسين إنتاجيته (γ–PGA) من خلال التلاعب بمكونات وسط الإنتاج والظروف البيئية. وتم اختيار أفضل مصدر للكربون والنيتروجين بتركيزات مثالية من خلال دراسة الظروف المثلى لإنتاج (γ–PGA) باستخدام وسط شبه صناعي وذلك بتحضير المولاس كمصدر للكربوهيدرات مما أعطى أفضل إنتاجية لـ (γ–PGA) عند تركيز 10% ونترات الأمونيوم كأفضل مصدر نتروجين عند تركيز 2%، أظهرت الدراسة أن بكتريا *B. megaterium* اعطت أفضل انتاج من (γ–PGA) عند درجة الحرارة المثلى والأس الهيدروجيني الأولي الأمثل 37 °م و6.5 على التوالي، كذلك طريق التحريك والتهوية باستعمال نسبة حجم وسط التخمير إلى حجم الدورق 5:1 في حاضنة هزازة عند 180 دورة في الدقيقة، وباستخدام حجم اللقاح 2.8 × 10^6 وحدة تكوين المستعمرة/ مل في وسط الإنتاج حيث وصل التركيز الكلي لـ (γ–PGA) إلى 7.22 ملغم/مل. تم استخلاص (γ–PGA) من الوسط الإنتاجي بعد الترسيب بالإيثانول المبرد والحصول على بلورات نقية تم الكشف عن نقاوة (γ–PGA) من خلال استخدام مطيافية الاشعة تحت الحمراء.

كلمات مفتاحية: متعدد حامض الكلوتاميك، B. megaterium، المولاس، شراب الذرة، التخمرات، التحليل الطيفي FTIR.

Introduction

Poly gamma-glutamic acid (γ-PGA) is a kind of biopolymer natural material with good biocompatibility and degradability widely used in food products, medicine, environmental protection, cosmetics, and agricultural applications. It acts as a flavor enhancer in beef burgers and texture enhancer in mayonnaise and vegetable soup. In 1942, Bovarnick conducted a study that demonstrated the unrestricted secretion of PGA into the medium after the fermentation of *B. subtilis* (1). γ-PGA is a high-molecular-weight amino acid homopolymer formed by the D or L type glutamic acid connected by γ-amide bonds. The relative molecular weight of γ-PGA is generally between 100000 and 1 million daltons. It is water-soluble, anionic, and non-toxic (16) which explains its important role in our daily lives, as it is biodegradable, safe to eat, and non-polluting (4).

Production of γ-PGA in microorganisms was by using l-glutamic acid, citric acid, and ammonium sulfate as carbon and nitrogen sources to activate its synthesis enzyme system (18). PGA is typically divided into two isoforms, that is alpha-poly glutamic acid (α-PGA) and γ-PGA. The latter is the form in which peptide bonds are created between the amino group of glutamic acids and the carboxyl group on the glutamic acid side (10). Several bacillus species possess the capability to excrete γ-PGA as a extracellular byproduct during the fermentation process a growth medium (8). Microbial synthesis of γ-PGA is carried out in four successive steps: racemization, polymerization, regulation, and degradation. These steps have a first-rate position within the γ-PGA manufacturing process with specific enzymes (3). Bacillus is the most common genus for producing PGA and some species of this genus include *B. licheniformis* and *B. subtilis*. γ-PGA has widespread applications such as a thickener, adding bitterness, relieving agent, cryoprotectant, sustained release material, drug carrier, curable biological adhesive, biodegradable fibers, highly water-absorbable hydrogels, biopolymer flocculants, and heavy metal absorber (7).

Biopolymers are polymers made of organisms like plants, animals, and microorganisms (12 and 20). γ-PGA was first discovered by Ivanovics and Bruckner as a component of capsules of *Bacillus anthracis*. They observed that it was released into the medium on autoclaving or aging and autolysis of the cells (6). The *B. megaterium* bacterium has been rarely studied in producing γ-PGA from some food factory waste to prepare a semi-natural production medium for its production. This study used molasses and corn syrup as a carbon source to produce γ-PGA by microbial fermentation, and optimal conditions were determined to attain its highest productivity.

Materials and Methods

This study was conducted at the biotechnology field, Department of Food Sciences, College of Agricultural Engineering Sciences, University of Baghdad using a local isolate of the *B. megaterium* bacteria and the Vitek 2 system obtained from the college.

Preparation of the seed medium: The seed medium was prepared according to (13) and comprised 40 glucose 0.1 $MnSO_4.H_2O$, 2.5 NaCl, 1 KH_2PO_4, 1 K_2HPO_4, 0.5 $MgSO_4.7H_2O$, and 10 yeast extract (g/L).

Basal production medium (BPM): The media was prepared according to (10) as the main production medium, KH_2PO 1 g, urea 2 g, $(NH_4)_2SO$ 2 g, and modified for this study with Biotin 3 µg, NaCl 0.5, $FeSO_4.7H_2O$ 0.05, $MgSO_4.7H_2O$ 0.5 (g/ L).

Carbon sources: Pure sugars (glucose, sucrose, and fructose) and food factory waste extracts (corn syrup and sugarcane molasses).

Qualitative detection of γ-PGA produced from locally isolated *B. megaterium*: The qualitative detection of γ-PGA was based on the method described by (17) using thin layer chromatography (TLC). Quantification of γ-PGA to estimate the amounts produced by *B. megaterium* was by using the separation solution of butanol, acetic acid, and distilled water at a 20:20:80 ratio. The color of the separated stains was developed using a ninhydrin detector (0.2% ethyl alcohol), the plates were dried, and the stains scraped and placed in test tubes containing 5 ml ethanol (75%). The absorbance was read at the 570 nm wavelength by returning to the standard curve (Figure 1).

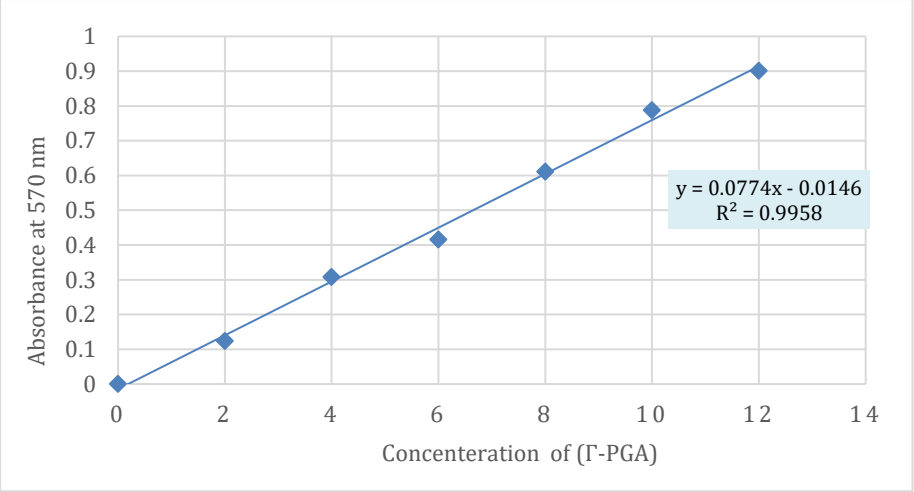

Fig. 1: Standard curve for estimating the γ-PGA concentrations.

Bacterial growth measurement: Measured using the absorbance spectrophotometric method according to (14) by taking a specific volume of the bacterial trap into the production medium for recording using the spectrophotometer at the 600-nm absorption wavelength. Total plate count was used to estimate the number of live bacteria at each density measurement (number of live cells/ml) in order to construct the growth curve shown in Figure 2.

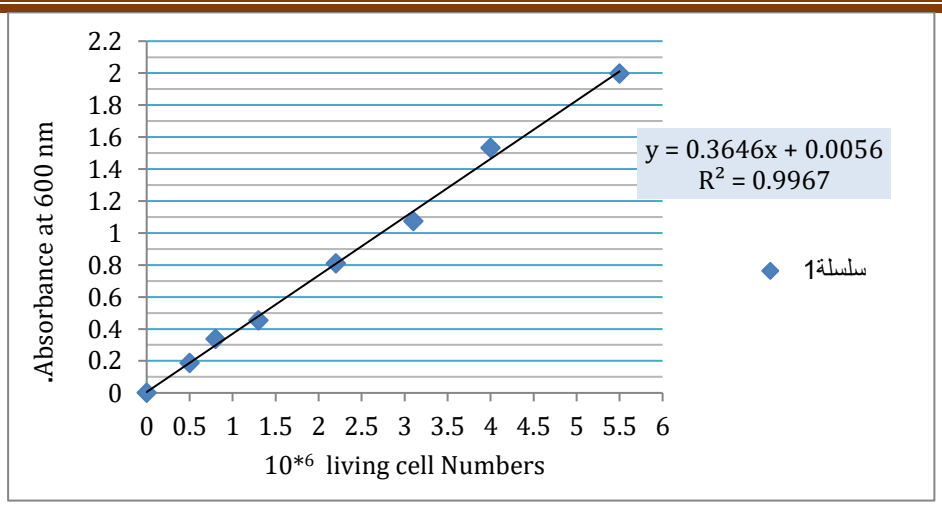

Fig. 2: Standard curve for the live cells count.

Determining optimal conditions for producing γ-PGA:

Carbon source: The effect of the carbon source on the production of γ-PGA was tested using synthetic sugars, including glucose, fructose, and sucrose. Two natural carbon sources obtained from local markets were tested, including molasses and corn syrup. They were added in a constant 4% concentration to the production medium, incubated at 37° C at an initial pH of 7 in a shaker incubator at 120 rpm using a vaccine from *B. megaterium* in a primary concentration of 2.8×10^6 for 48 hrs. The period was used to test the optimum carbon source for producing γ-PGA.

Sugarcane molasses concentration: Different concentrations of 2, 4, 6, 8, 10, and 12% of the natural extracts were used to achieve the best productivity.

Nitrogen source: Potassium nitrate, ammonium phosphate, ammonium chloride, and ammonium nitrate were added. The ammonium sulfate, peptone, and urea were at 1% concentration in the production medium, taking into account the optimal concentrations of the carbon source.

Nitrogen concentration: Different concentrations of ammonium nitrate at 0.5, 1, 2, and 3% were tested to determine the best nitrogen source for γ-PGA production.

Temperature: Six different temperatures 25, 28, 31, 34, 37, and 40° C were applied taking into consideration the optimum conditions achieved in previous experiences.

pH: Using the production medium with primary pH 5.5, a difference of half a pH between treatments 5.5, 6, 6.5, 7, 7.5, 8 was applied taking into consideration the optimum conditions achieved in previous experiences.

Moving speed and ventilation: The effect of the rotating incubator's speed was studied at 40, 60, 80, 120, 160, 180, and 220 rpm while ventilation effects were examined using a ratio based on flask volume and production medium (30). The experiment was carried out with different sized flasks containing a fixed production medium at ratios of 3:1, 5:1, 6:1, 10:1, 15:1, and 20:1.

Purifying γ-PGA produced by the *B. megaterium*: Following the method of (23), the culture broth was collected and centrifugation carried out at 6,000 xg for 20 min to remove the cells. The supernatant was collected and titrated to pH 3 by 1 M (H_2SO_4) and stored at 4° C for 12 hrs until crystals of glutamic acid formed and precipitated in the beaker. The crystals were collected by filtration with a Buchner

funnel and washed with distilled water twice to remove impurities. The drying process used an electric-air oven to transform it into dry material (28).

Results and Discussion

Production of γ-PGA) by *B. megaterium*: The results from separating using thin layer chromatography (TLC) showed one spot for both the γ-PGA sample produced by the bacteria and the standard sample (Sigma-Aldrich American Company), as seen in Figure 3. The Rf value for the γ-PGA model under study was equal to the standard model at 0.35.

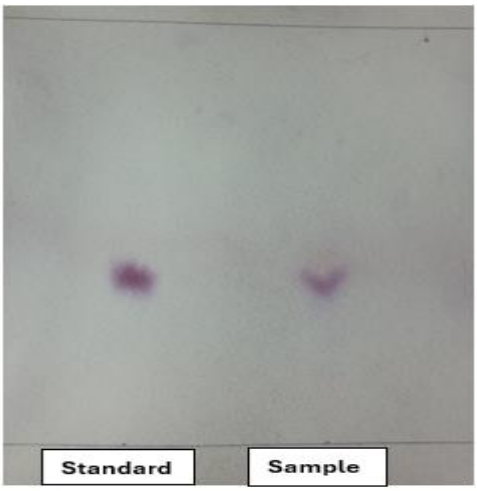

Fig. 3: Separation and quantitative detection of γ-PGA produced from B. megaterium and the standard sample.

Determining optimal conditions for producing γ-PGA:

Effect of the carbon source: Five carbon sources were used in producing the γ-PGA: two natural ones, namely molasses and corn syrup, and three pure synthetic sugars comprising glucose (Glu), sucrose (Suc), and fructose (Fru) after adding them to a 10% concentration of the primary productive medium. An inoculum of the *B. megaterium* at 2.8×10^6 cfu/ml. was used. Figure 4 shows that glucose gave the highest productivity for γ-PGA reaching 1.76 mg/ml, almost similar to the 1.73 mg/ml for the molasses followed by corn syrup at 1.44 mg/ml and close to that for fructose at 1.49 mg / mL. This offers opportunities to exploit food factory wastes for their intercalations in fermentation production and obtaining fermentation products, thus reducing production costs environmental pollution (29). This finding is close to (5) in using agricultural waste, with cassava peels, corn residue, and pineapple residue as the only carbon sources for produce γ-PGA.

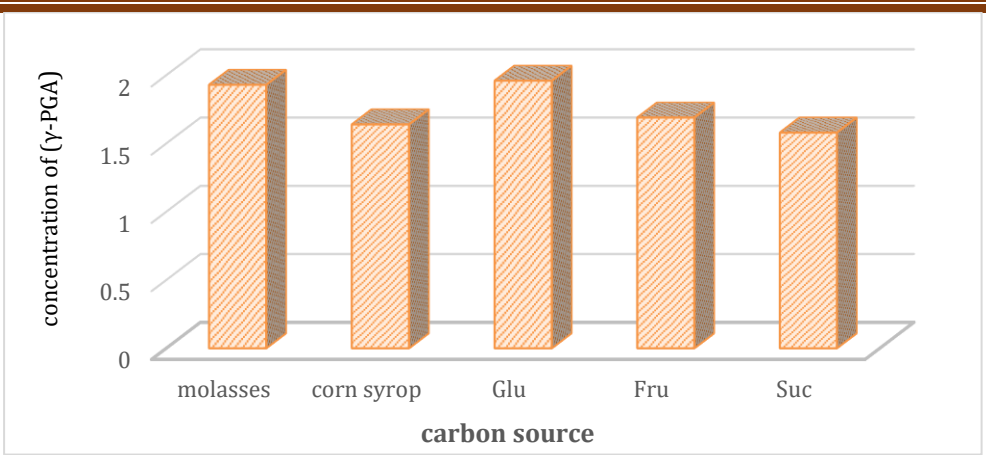

Fig. 4: Production of γ-PGA by B. megaterium using different carbon sources at 4% concentration.

The effect of ideal concentrations of molasses in the production medium on γ-PGA yield: As seen in Figure 5 the productivity of γ-PGA increased at higher concentrations of molasses using different medium concentrations in the production medium with the highest yield of 2.74 mg/ml obtained at the 10% concentration. This could be due to the sugarcane molasses added with $FeSO_4 \cdot 7H_2O$ at production medium which increased the volume of intracellular metabolites, including amino acids, organic acids, as well as the up regulation of the glycolysis pathway and TCA cycle (15). At the same time, γ-PGA production declined at higher glucose concentrations as glucose tends to inhibit the basic materials in some enzyme activity or increase carbon-nitrogen ratios in the media (19).

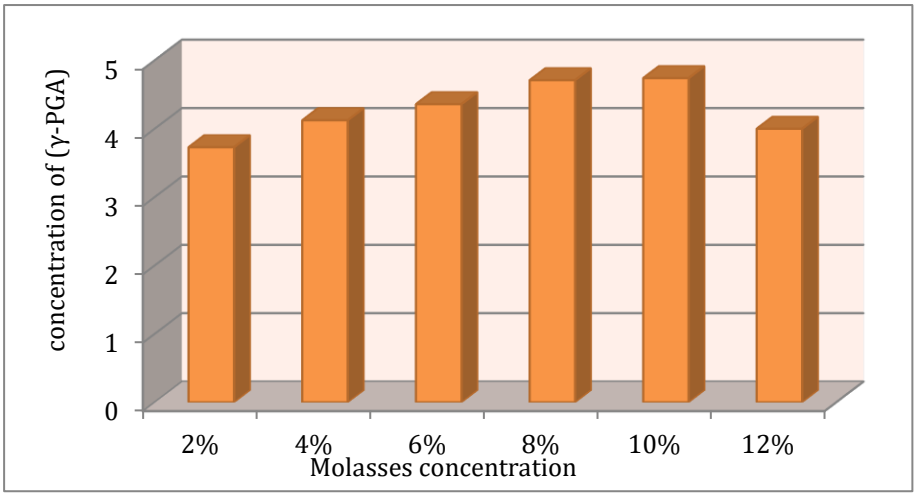

Fig. 5: Effect of different concentrations of molasses on production of γ-PGA from B. megaterium.

Nitrogen sources: Seven nitrogen sources were tested of which two were organic (peptone and urea) and five were inorganic (potassium nitrate, ammonium phosphate, ammonium chloride, ammonium nitrate, and ammonium sulfate). They were studied separately at a uniform concentration of 1% after fixing the carbon source type and concentration (molasses at 10% concentration) at 37° C and pH 7. Figure 6 shows the effect of these nitrogen sources on γ-PGA production with ammonium nitrate outperforming the others at 4.585 mg/ml, followed by ammonium sulfate, ammonium

chloride, and ammonium phosphate at 4.17, 3.615, and 3.355 mg/ml, respectively. At the same time, urea excelled as an organic nitrogen source for γ-PGA production at 3.06 mg/ml while the use of peptone generated the lowest production level. In comparing sources for γ-PGA production, the inorganic ammonium nitrate was found to be superior to the organic urea. This could be due to the free matter required for the production of γ-PGA being more readily available in inorganic nitrogen sources such as the NH^{+4} ions (13). Bacteria producing γ-PGA prefer ammonium salts to other nitrogen sources; the ammonium ion NH^{+4} in these salts is responsible for converting the glutamate dehydrogenase (GDH) enzyme from glutarate into glutamate (22).

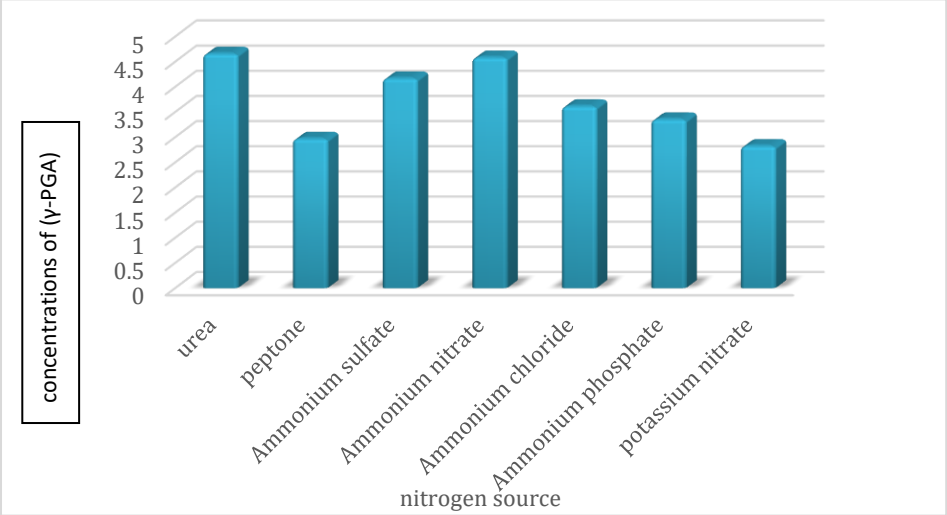

Fig. 6: Production of γ-PGA by B. megaterium using different nitrogen sources at 1% concentration.

Different concentrations of ammonium nitrate at 0.5, 1, 2, and 3% were tested to determine the optimum levels for γ-PGA production. As seen in Figure 7, the highest γ-PGA production was at 3.72 mg/ml at 2% ammonium nitrate concentration. Production decreased at lower concentrations to 3.28 mg/mL and 3.5 mg/mL at 0.5% and 1% ammonium nitrate, respectively, and at concentrations above 2% to 3.58 mg/ml (3% concentration). This result is relative to the (21) when using 2% ammonium sulfate as a nitrogen source to obtain the highest concentration of γ-PGA using the *Brevibacterium* strain.

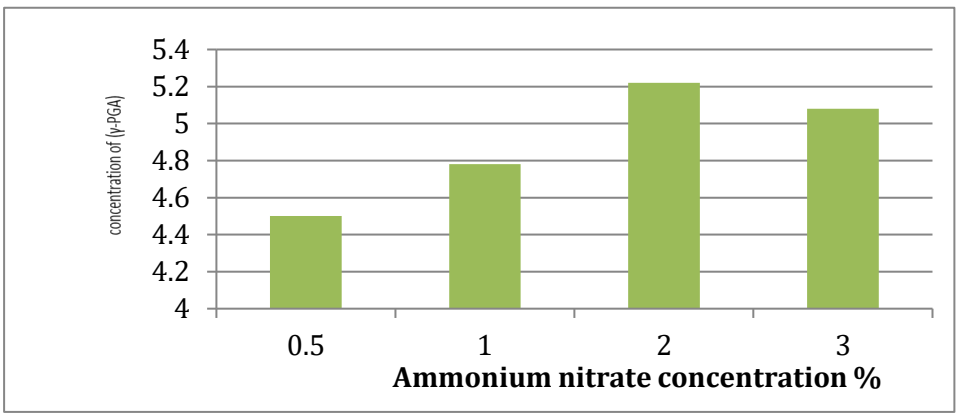

Fig. 7: Optimal concentrations of ammonium nitrate in producing γ-PGA from B. megaterium.

The effect of temperature on γ-PGA production: Six temperature levels 25, 28, 31, 34, 37, and 40° C were examined for γ-PGA production in a medium whose components were determined in previous experiments and at a pH of 7. Figure 8 shows that 37° C was the optimal temperature as production reached 6.33 mg/ml, while 25 and 28° C temperatures reduced it to 3.52 and 4.2 mg/ml, respectively. Also, γ-PGA production decreased to 5.54 and 2.1 mg/ml at higher incubation temperatures of 34 and 40°C, respectively. The bacterial productivity of amino acids and polymers decreases in temperature between 30 - 35°C due to lower glucose metabolism efficiency (11).

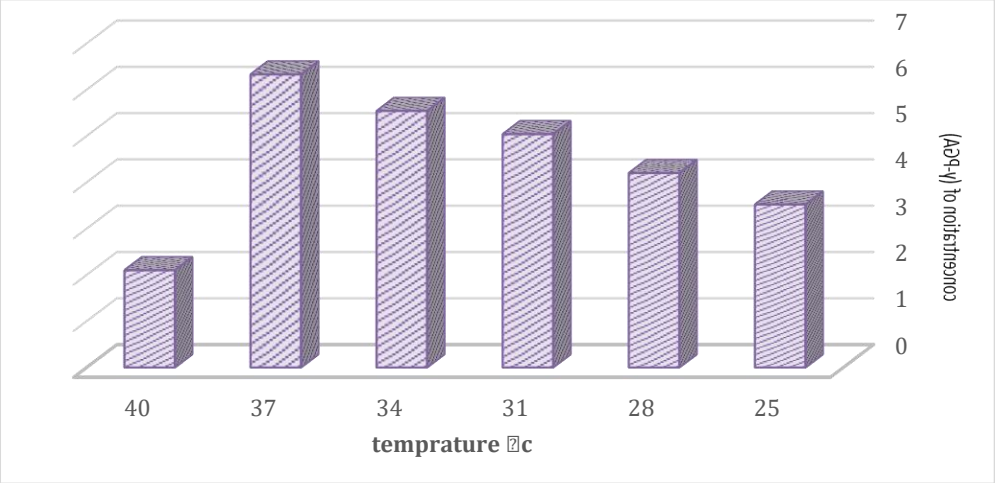

Fig. 8: Determining the optimum temperatures for γ-PGA production by B. megaterium.

pH value: The primary pH of a production medium is among the most important factors affecting the production of γ-PGA from the *B. megaterium* bacteria. pH levels of .5, 6, 6.5, 7, 7.5, and 8 in medium containing 10% molasses and 2% ammonium nitrate and at 37° C were used for that purpose in this study. Figure 9 shows that the highest γ-PGA yield at 7.22 mg/ml was achieved at the 6.5 pH level and the lowest at pH 5.5 (3.12 mg/ml). It also decreased (to 3.65 mg/ml) when pH was raised to 8.

The pH factor has apparent effects on the physiology of microorganisms, such as through nutrient solubility and absorption, enzyme activity, cell membrane morphology, by-products, and oxidation and reduction reactions (24). Following completion of the production stage, bacterial cell growth intensity (OD 600 nm) was measured with γ-PGA quantitation at the tested pH. The density of cell growth increased from 0.5 at the initial pH 5.5 to 1.108 at pH 6 and 2.44 at pH 7, but declined to 2.35 at pH 7.5. The increased growth intensity may be due to the pH of the culture medium being near parity to the nature of the physiological system of the microscopic organism that affects nutrient solubility and absorption and enzymatic activity. It could also be due to the effect on the morphological nature of the cell membrane and the formation of metabolic products (1).

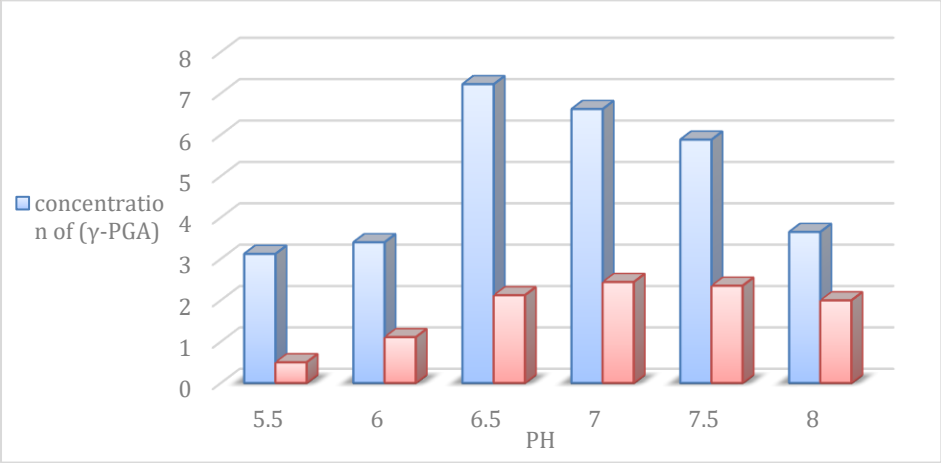

Fig. 9: Effect of initial pH on γ-PGA production from B. megaterium.

Ventilation and stirring effects: The effects of ventilation and stirring were studied using a rotary shaker incubator to determine the ratio between the volume of the productivity medium and flask size. It took into consideration the optimal conditions to produce γ-PGA identified in previous experiments on culture medium components and environmental conditions. Differences in medium sizes in the flask determine the intensity of movement and oxygen processing changes (2). This effect may be due to differences in the volume of the production medium inside the flask leading to changes in speed and the availability of oxygen (27).

Figure 10 shows that the 5:1 ratio and rotational speed of 180 rpm gave the highest γ-PGA productivity at 8.56 mg/ mL at the optimum temperature of 37° C There was a decrease in γ-PGA production in both cases of abundant ventilation represented by the sixth and fifth treatments (20:1) and (12:1). For the limited ventilation represented by the first treatment (3:1) and in the sixth and fifth treatments, γ-PGA production increased with rotational speed to 1.92 mg/mL and 2.7 mg/mL, respectively at 160 rpm, then decreased at 180 and 220 rpm. At the same time, cell growth increased (based on optical density) to reach a maximum of 4.6 at the 15:1 ratio and 180 rpm rotational speed.

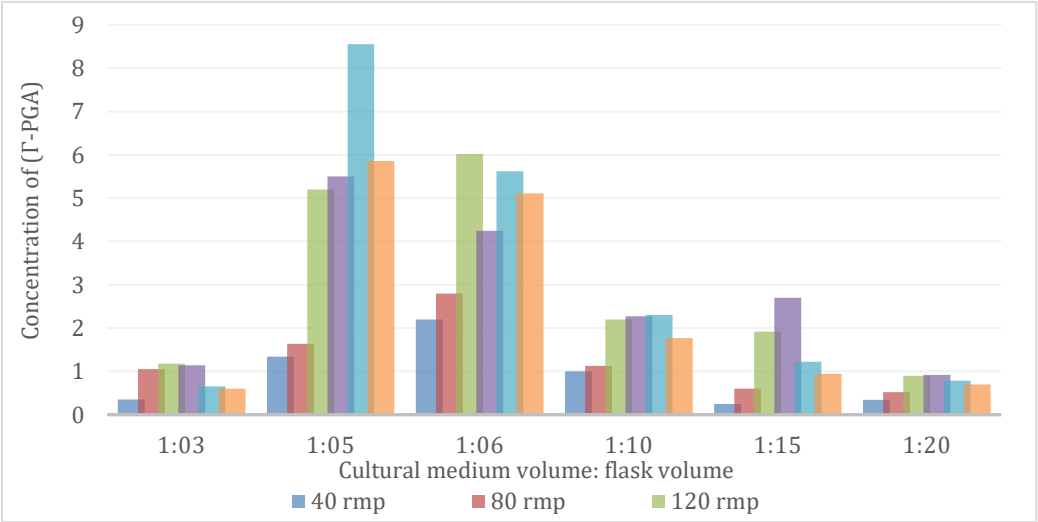

Fig. 10: Ventilation and stirring effects on γ-PGA production by *B. megaterium*. Diagnosis using FTIR infrared spectroscopy

In addition to the standard sample, a sample of γ-PGA produced by *B. megaterium* was subjected to diagnosis using infrared spectroscopy to detect influential groups for each of the tested samples. Table 1 shows the locations of the most essential peaks and bands of the active functional groups (25).

Table 1: FTIR absorption beams of γ-PGA for the standard and this study's samples.

Elastic frequency (cm^{-1}) of γ-PGA sample in this study	Elastic frequency (cm^{-1}) of γ-PGA standard sample	Functional groups
3452.58	3433.29	O-H (Carboxylic acid)
3104.53	3059.81	N-H (Amines)
1643.35	1666.5	C=O (Carboxylic acid)
2962.66	2966.52	C-H

Figures 11 and 12 show each tested sample's FTIR infrared spectroscopy results. The two samples show locations of the important peaks and bands of the practical, functional groups. There is a comprehensive bundle at the elastic frequency at wave number 3452.58 cm^{-1} for the sample produced and 3433.29 cm^{-1} for the standard sample that is back to the O-H group. There is also a peak at 3104.53 cm^{-1} and 3059.81 cm^{-1} for each of the study and standard samples, respectively, that is back to the N-H amino group. Two bundles are also apparent for both samples at wave numbers 1643.35 cm^{-1} and 1666.50 cm^{-1} of the C=O carboxyl group. A bundle appears in each of the spectra of the γ-PGA under study and the standard polymer at elastic frequencies of 2962.66 cm^{-1} and 2966.52 cm^{-1}, respectively.

The FTIR findings in this study are similar to that of (26). They obtained peaks representing numerical values for all active groups at the amplitude-frequency 3061.49 cm^{-1} belonging to the N-H group, while the stretch frequency of 1613.75 cm^{-1} returned to the C=O group, reaching a peak at wave number 2742.03 cm^{-1} of the C-H group.

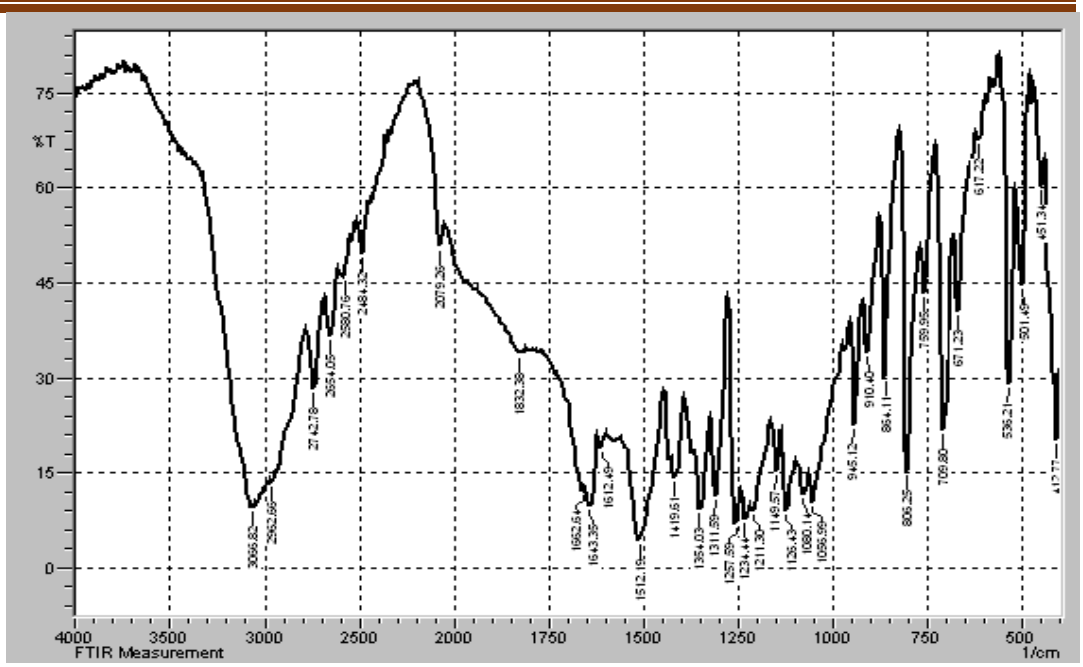

Fig. 11: FTIR absorption spectrum of γ-PGA produced by *B. megaterium.*

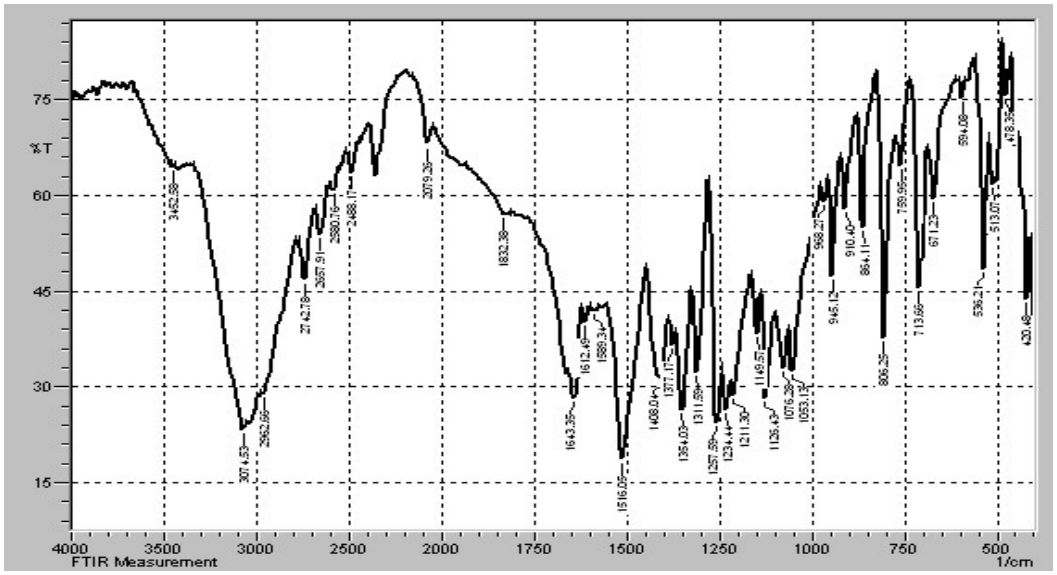

Fig. 12: FTIR absorption spectrum of standard γ-PGA sample.

Conclusions

This study examined the use the B. megaterium bacteria isolated locally to produce γ-PGA. The highest γ-PGA productivity (7.22 mg/ml) was attained from bacteria fermented under optimal conditions using molasses and ammonium nitrate as the main sources of carbon and nitrogen at 10% and 2% concentrations, respectively, and a temperature of 37° C and initial pH of 6.5. γ-PGA crystal deposits were also found in the production medium by acidification up to the isoelectric point. It was possible to isolate and recrystallize them and study their purity using FTIR.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Author 1: methodology, writing and original draft preparation; Eman J. Al-Attar, Amer H. H. Alzobaay and Suzan K. Hasan writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement:

No Informed Consent Statement.

Data Availability Statement:

No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

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