



## **Isolation, Identification and Evaluation of Egyptian *Bacillus* sp. Isolates for Producing Poly- $\gamma$ -glutamic Acid**

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Author RFA designed the study, performed the experiments, wrote the protocol and shared in writing the first draft of the manuscript. Author HBK managed the analyses of the study, shared in writing the first draft of the manuscript, edited and finalized the manuscript. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

Poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) is a non-toxic, biodegradable, and water-soluble polymer secreted from different microbes. We isolated forty-nine *Bacillus* spp. isolates generating PGA from the soil of different Egyptian regions. A total of 18% of these isolates were found to highly produce PGA in the range of 5- 8 g l<sup>-1</sup>. Two of them, B44 and B42, were identified by the 16SrRNA DNA sequence, and they shared 81% of similarity. The most similar DNA sequences to B44-16SrRNA were *Bacillus* sp. (FJ607057.1) and *Bacillus sonorensis* (KP236346.1) matching 99% and 94% of similarity, respectively. However, B42-16SrRNA was similar 99% to several *Bacillus* species. The tree-building algorithm MEGA-X constructed the phylogenetic tree of 16SrRNA DNA sequences for *Bacillus* 44 and *Bacillus* 42 strains along with other similar *Bacillus* species revealing the distance between them. We also boosted the PGA production of both strains. *Bacillus* 44 strain revealed the highest accumulation level of PGA at 35°C and 72 h of incubation using medium M with inoculum size and agitation speed 3% and 250 rpm, respectively. Both strains secreted the PGA biopolymer with a molecular mass of 55 kDa. This investigation is an attempt of boosting promising Egyptian *Bacillus* sp. isolates for PGA production that may be a seed for industrial production.

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## 1. INTRODUCTION

Recently, the interest in the biopolymer, poly- $\gamma$ -glutamate acid ( $\gamma$ -PGA), has a significant increasing trend. With many applications in the food industry, bioplastic, cosmetic, wastewater treatment, and medicine, this biopolymer is ascendingly desired [1]. PGA polymer is mainly generated by Gram-positive bacteria like the genus *Bacillus*, but other genera like *Staphylococcus*, *Natrialba*, *Lysinibacillus*, and *Fusobacterium* are also found to produce the same biopolymer [2,3,4,5,6]. According to the presence of D-glutamic acid in the production media, the microorganisms secreting PGA were classified into two major groups. Microbes generate glutamic acid-dependent (c-PGA), which usually need glutamic acid in the media for inducing c-PGA synthetase [7,8] and others glutamic acid-independent that produce PGA without D-glutamic acid [9,4]. The glutamic acid-independent bacteria have a lower PGA productivity compared to glutamic acid-dependent bacteria [10]. Therefore, the accumulation of PGA biopolymer in the media usually increases when L-glutamic acid used as a nutrient, so this modification is expensive [11,12].

Unlike most proteins,  $\gamma$ -PGA is synthesized in a ribosome-independent process. The precursor for  $\gamma$ -PGA in several bacteria is a pyruvate molecule that is associated with cell growth. Therefore, medium components supporting cell growth will make enough precursor, pyruvate, for  $\gamma$ -PGA biosynthesis [13]. The biosynthesis of PGA has mainly four stages: (i) racemization for converting glutamate isomerases; (ii) polymerization for polymerizing the  $\gamma$ -PGA at the active site of the synthase complex; (iii) regulation for controlling the production of the polymers; (iv) degradation for cleaving  $\gamma$ -PGA into various fragments [11]. The molecular mass of the native PGA is relatively high ranged from 10 to 2000 KDa [14,15,16,17]. This depends on the type of PGA secreting microbes. Recently, medium composition, hydrolysis, degradation, and degrading enzymes have all been utilized to control the size of PGA [14].

Several *Bacillus* species have been known as  $\gamma$ -PGA producers. The PGA biopolymer was firstly discovered in *Bacillus anthracis* capsules, then found in other *Bacillus* species such as *B. cereus*, *B. subtilis* and *B. natto*, *B. licheniformis*,

*B. thuringensis*, *B. pumilus*, *B. amyloliquefaciens*, *B. mojavensis*, *B. atrophaeus* and *B. megaterium* [18,19,20,21,22]. Till now, new native species of the genus *Bacillus* are discovered to generate a high amount of this biopolymer.

In this study, we screened and identified *Bacillus* spp. isolates from different Egyptian regions for investigating their capabilities of secreting poly- $\gamma$ -glutamate acid. We also optimized the conditions for the best yielding of generated PGA. Also, we identified the molecular size of this bioproduct.

## 2. MATERIALS AND METHODS

### 2.1 Sampling

A total of thirty-five soil samples were collected from different Egyptian governorates including Banysoif, Menoufia, Al-Wady Al-Gadid and Cairo. The samples were picked in pre-sterilized plastic bags, then sorted for further analyses.

### 2.2 Isolation and Characterization of *Bacillus* sp. Isolates

For isolation, an amount of ten grams of each sample was transferred into shaking conical flasks containing 90 ml of PPs solution (0.1% Peptone, and 0.85% NaCl) on a speed of 100 rpm for 15min. Then, the suspensions were heated at 80°C for 15 min to eliminate vegetative cells. Serial dilutions of the suspensions were prepared (up to  $10^{-5}$ ) and 100  $\mu$ l of each diluted sample was spread on TSB medium plate (3% Trypticase soy broth, 0.3%  $K_2NO_3$  and 1% Glucose). The plates were then incubated for 48 h at 30°C till smooth-mucoid colonies appeared. Each colony was picked and purified using the streak plate method. Purified isolates were microscopically examined using Gram-and Schaeffer-Fulton stains to identify *Bacillus* spp. isolates.

### 2.3 Screening *Bacillus* sp. Isolates for PGA Production

The purified isolates were assessed based on the accumulation of PGA. One milliliter of a standard seed culture was inoculated in 100 ml of TSB medium, then incubated for 4 days at 30°C and 150 rpm. After incubation, a sample

(10 ml) from each culture was utilized to measure cell growth and PGA dry weights ( $\text{gl}^{-1}$ ).

## 2.4 Bacterial Genomic DNA Isolation and Quantification

The total genomic DNA of selected isolates that revealed high production of PGA was extracted according to GeneJET genomic DNA purification Kit protocol (Thermo Scientific, Cat No. K0721). Then, the DNA samples were quantified, then stored at 4°C.

## 2.5 Bacterial 16SrRNA PCR Amplification, Purification and Sequencing

A pair of primers (For. 5' AGAGTTTGATCCTGGCTCAG 3'; Rev. 5' GGTTACCTTGTTACGACTT 3') of 16S ribosomal RNA, 16SrRNA, was selected for amplifying an approximate of a 1500 bp of DNA fragment reported in [23]. The PCR reaction was performed according to Maxima Hot Start PCR Master Mix (Thermo Scientific, Cat No. K1051). A volume of 50  $\mu\text{L}$  PCR reaction containing 20  $\mu\text{M}$  of each primer and a 50 ng of genomic DNA sample, 25  $\mu\text{l}$  of 2X Maxima® Hot Start PCR Master Mix and 18  $\mu\text{l}$  of nuclease-free  $\text{H}_2\text{O}$  was prepared. The PCR amplification was performed on a thermal cycler with a cycling profile: denaturation for 10 min at 95°C; 35 cycles of 95°C for 30 sec, 65°C for 60 sec, and 72°C for 90 sec; final extension at 72°C for 10 min. The PCR product was purified using GeneJET™ PCR Purification Kit (Thermo Scientific, Cat No. K0701), then sequenced according to ABI 3730xl DNA manufacturer instructions.

## 2.6 Bioinformatics Resources

Basic Local Alignment Search Tool (BLAST) services provided by the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) were used in this study for comparison of nucleic acid sequences. European Bioinformatics Institute (EBI, <http://www.ebi.ac.uk/>) ClustalW2 was used for multiple sequences alignment and analysis of sequence structure.

## 2.7 Phylogenetic Analysis

For phylogenetic analysis, the tree-building algorithm for maximum likelihood [24] provided by MEGA-X [25] was applied. Initial tree(s) for the heuristic search were obtained automatically

by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (above the branches). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed [26].

## 2.8 Fermentation Time

Elementary flasks (250 ml) provided by 100 ml of TSB medium were used for *Bacillus* 44 and *Bacillus* 42 isolate propagation. Samples (10 ml) of each isolate culture were taken periodically every 12 h under aseptic conditions during 110 h of the incubation period. All collected samples were used to evaluate cell growth and PGA dry weight, as well as sugar consumption.

## 2.9 Media Composition

The cell growth of *Bacillus* sp. isolates and their PGA production were assessed using four different media: TSB (a control medium); Basal m. (2% Glucose, 0.4% Casine hydrolysate, 1.4%  $\text{K}_2\text{HPO}_4$ , 0.6%  $\text{KH}_2\text{PO}_4$ , 0.02%  $\text{MgSO}_4$  and 1 ml of 1 mM trace solution [ $\text{FeSO}_4$ ,  $\text{CaCl}_2$ ,  $\text{MnSO}_4$ , and  $\text{ZnCl}_2$ ]); medium M (1.8%  $\text{NH}_4\text{Cl}_2$ , 7.5% glucose, 0.15%  $\text{K}_2\text{HPO}_4$ , 0.035%  $\text{MgSO}_4$ , 0.005%  $\text{MnSO}_4$ ); medium E (1.2% citric acid, 8% glycerol, 0.7%  $\text{NH}_4\text{Cl}$ , 0.005%  $\text{K}_2\text{HPO}_4$ , 0.05%  $\text{MgSO}_4$ , 0.004%  $\text{FeCl}_3$ , 0.015%  $\text{CaCl}_2$ , 0.0104%  $\text{MnSO}_4$ ).

## 2.10 Optimizing the Environmental Conditions for the Best Yielding of PGA

### 2.10.1 Incubation temperature

Five incubation temperatures (25, 30, 35, 40 and 45°C) were used to find the best temperature for PGA accumulation.

### 2.10.2 Agitation speed

Seven agitation speeds ranged from 100 to 400 rpm were assessed using a rotary shaker at 35°C.

### 2.10.3 Inoculum size

The size of the inoculum ranged from 1 to 6% (v/v) was examined for selecting the standard

amount of tested bacterial isolates for inoculation.

#### 2.10.4 Inoculum physical state

Three different physical states of *Bacillus* sp. isolates were assessed including spores, outgrowth vegetative cells and a mixture of outgrowth vegetative cells and spores.

#### 2.10.5 Standard seed

A loop-full of each *Bacillus* isolate was inoculated in a conical flask containing 100 ml of nutrient broth, then incubated for 24 h at 30°C and 150 rpm. One ml of the culture of each isolate contained about 0.1 g l<sup>-1</sup> was used in this investigation as a standard seed.

#### 2.11 Glucose Determination

Residual sugar in the culture supernatant was determined using potassium free cyanide as described by Park and Johnson [27].

#### 2.12 PGA Recovery

According to a method described by Ashiuchi et al. [28], the PGA from screened bacterial isolates was participated, then collected by a re-centrifugation at a speed of 7000 rpm for 10 min at 4°C. Finally, pellets were dried at 80°C.

#### 2.13 Calculations

##### 2.13.1 Specific growth rate ( $\mu$ ) and doubling time ( $t_d$ )

The specific growth rate ( $\mu$ ) and doubling time ( $t_d$ ) were calculated from the exponential phase according to Painter and Marr [29] using the following equations:

$$\mu = (\ln A - \ln A_0) t^{-1}$$

$$t_d = \ln 2 \cdot \mu^{-1}$$

$\mu$  = Specific growth rate (h<sup>-1</sup>); A = Amount of growth after t time; A<sub>0</sub> = Amount of growth at the beginning of logarithmic Phase; t = Time of the logarithmic phase; t<sub>d</sub> = Doubling time

##### 2.13.2 Number of generations (N)

The number of generations was calculated according to Stanier et al. [30] using the following equation:

$$N = t/t_d$$

N = Number of generations; t = the period of exponential (h); t<sub>d</sub> = doubling time (h)

##### 2.13.3 Multiplication Rate (MR)

The multiplication rate was calculated according to Stanier et al. [30] using the following equation:

$$MR = Nt^{-1}$$

MR = Multiplication rate; N = Number of generations; t = Time of the logarithmic phase

#### 2.14 PGA Parameters

We used the following equations for measuring the accumulation rate of PGA.

##### 2.14.1 PGA conversion coefficient

PGA conversion coefficient (%) = gm PGA X 100/gram utilized sugar, according to Ramadan et al. [31].

##### 2.14.2 PGA yield to biomass

PGA yield to biomass (Y<sub>c/x</sub>) = gm PGA/ gm biomass dry weight gg<sup>-1</sup>, according to Grothe et al. [32].

##### 2.14.3 PGA productivity

PGA productivity = gm PGA / fermentation time (h) g l<sup>-1</sup> h<sup>-1</sup>, according to Lee [33].

#### 2.15 PGA Purification and Determination by SDS-PAGE

PGA was purified according to a protocol described by Ito et al. [33]. SDS-PAGE of proteins was performed using a 10% polyacrylamide gel [34]. The protein was stained by Coomassie brilliant blue, then destained. The standard proteins, bovine serum albumin, was used to determine the molecular size of PGA.

### 3. RESULTS AND DISCUSSION

Soil microbes able to secret polymers such as poly-γ-glutamic acid. This biopolymer commonly produced by the genus *Bacillus*. Of screened bacterial isolates collected from different regions in Egypt, forty-nine were morphemically characterized as *Bacillus* sp. isolates. On the

microscopic level, the isolates were gram-positive, rod-shaped and forming endospores. Several studies reported the importance of *Bacillus* found in the soil, rhizosphere and fermented food that generate exopolysaccharides such as levan, gellan, and curdlan, and Poly- $\gamma$ -glutamic acid [20,35,36,37, 38]. Here, we attempted to isolate and purify *Bacillus* sp. isolates that were assigned by numbers ranged from B1 to B49.

### 3.1 Screening of *Bacillus* spp. Isolates for PGA Production

Based on the dry weight of PGA collected at the end of the incubation period of each grown *Bacillus* sp. isolates (assigned: B1 to B49), nine isolates (18%) revealed a high production of PGA ranged from 5 to 8  $\text{g l}^{-1}$ . Contrary, thirty isolates (61%) found to have a low production of PGA. The residual isolates (21%) with a range of 3-5  $\text{g l}^{-1}$  have a medium production of PGA. Of the highly PGA productive isolates, we selected the best two (B44 and B42) to identify them on the molecular level, to optimize the conditions for yielding PGA, and to identify the molecular mass of generated PGA.

### 3.2 Identification of Highly PGA Productive *Bacillus* spp. Isolates Based on the DNA Sequence of 16SrRNA

From the bacterial genomic DNA of B44 and B42 isolates, a PCR reaction for amplifying the DNA sequence of 16SrRNA was performed. Then, the PCR product was purified and sequenced (methods). The DNA sequence of B44-16SrRNA isolate was identified by BLASTN searching in the GenBank NR database. The most similar DNA sequences to B44-16SrRNA were unidentified species of *Bacillus* (FJ607057.1), and *Bacillus sonorensis* (KP236346.1) matching 99% and 94% DNA sequence similarity, respectively. On the other hand, several *Bacillus* species were found to be similar to B42 isolate, i.e., *B. paramycooides*, *B. cereus*, *B. wiedmannii*, *B. thuringiensis*, *B. albus*, *B. tropicus*, *B. anthracis* and others unidentified at species level (MN566094.1, MN733183.1, MN647026.1, MN428155.1, MN203621.1, MN637798.1, MN456848.1, and MN422007.1) with 99% DNA sequence similarity. The multiple DNA sequence alignment was performed on 16SrRNA of similar species by ClustalW2. The 16SrRNA sequence

analysis of both studied strains, *Bacillus* 44 and *Bacillus* 42, was also performed revealing the genetic distance between them as they were only sharing 81% DNA sequence similarity.

The evolutionary phylogenetic tree was generated using the maximum likelihood method (Fig. 1). For this, the analysis performed on the DNA sequence of *Bacillus* 44- and *Bacillus* 42-16SrRNA and their most similar DNA sequences in the GenBank NR database (KP236346.1, FJ607057.1, JX311358.1, AM110946.1, MN566094.1, MN733183.1, MN647026.1, MN428155.1, MN203621.1, MN637798.1, MN456848.1, and MN422007.1). The constructed tree resulted in two major clusters of *Bacillus* species that mainly belonged to the *Bacillus* 44 strain (Cluster II), and *Bacillus* 42 strain (Cluster 1). This genetic distance indicated that those *Bacillus* sp. strains were from two different species of *Bacillus*. Further genomic studies are essential to be performed to identify these *Bacillus* species strains that may be unprecedentedly designated for PGA production.

### 3.3 Assignment of the Fermentation Time

To further investigate cell growth, PGA production, and sugar consumption of the selected *Bacillus* sp. strains, time-course experiments were performed during batch fermentation using TSB medium. We collected samples every 12 h under aseptic conditions from grown culture through the period of incubation (110 h). Then, the cell growth and PGA dry weights ( $\text{g l}^{-1}$ ), as well as the sugar consumption ( $\text{g l}^{-1}$ ) of each sample for each strain were recorded (Fig. 2). The strains (*Bacillus* 44 and *Bacillus* 42) exhibited by three curves that revealed the rates of cell growth (0.77, 0.69), PGA production (0.86, 0.66) and sugar consumption (0.84, 0.73), respectively. The maximum production of PGA dry weight for both strains was during the stationary phase at 72 h yielding 8.03 and 7.72  $\text{g l}^{-1}$  for *Bacillus* 44 and *Bacillus* 42 strains, respectively (Fig. 2). A similar observation was reported by Thanh et al. [39] who optimized the fermentation time of *Bacillus* sp. at stationary phase, after that time the PGA dry weight was depleted. They interpreted this phenomenon for several reasons: increasing culture viscosity led to decreasing dissolved oxygen; reducing nutrients which affected on the bacterial growth and releasing  $\gamma$ -PGA degrading enzymes [40].

The PGA productivity, yield and conversion coefficient were also calculated (methods) at that time resulted in 0.1115  $\text{gl}^{-1}\text{h}^{-1}$ , 3.2  $\text{gg}^{-1}$  and 590% for B44 strain and 0.107  $\text{gl}^{-1}\text{h}^{-1}$ , 3.64  $\text{gg}^{-1}$ , and 507% for *Bacillus* 42 strain. Also, the comparison between the two strains at the growth parameter level ( $\mu$ ,  $t_d$ , MR, and N) was higher in the *Bacillus* 44 strain (0.149 $\text{h}^{-1}$ , 4.65h, 0.215, and 5.2), than the B42 strain (0.115 $\text{h}^{-1}$ , 6.02h, 0.166 and 4).

### 3.4 Determination of the Best PGA Productive Medium

We screened four media, TSB (as a control), Basal m., medium E, and medium M, for yielding the best production of PGA. At the end of fermentation time (at 72 h), the cell growth and PGA dry weights of *Bacillus* 44 and *Bacillus* 42 strains were recorded. The best medium for yielding PGA was medium M that scored 9.85 and 8.98  $\text{gl}^{-1}$  for *Bacillus* 44 and *Bacillus* 42 strains, respectively (Fig. 3). In parallel, *Bacillus* 44 and *Bacillus* 42 strains grown in the same medium revealed the highest sugar utilization (1.47 and 1.65  $\text{gl}^{-1}$ , respectively). This high production of PGA may be related to the

consumption of glucose and  $\text{NH}_4\text{Cl}_2$  as carbon and nitrogen sources in this media that classified as one of the most efficient C and  $\text{N}_2$  sources [41].

### 3.5 Optimization of the Environmental Conditions for the Best Yielding of PGA

#### 3.5.1 Incubation temperature

Of five incubation temperatures (25, 30, 35, 40 and 45°C) selected for highly PGA accumulation rate, we found that 35°C was the optimum temperature for both *Bacillus* 44 and *Bacillus* 42 strains (Fig. 4A). At the 72 h of the incubation, the cell growth and PGA dry weights were 2.01 and 9.96  $\text{gl}^{-1}$  for B44, and 2.3 and 8.69 for *Bacillus* 42  $\text{gl}^{-1}$ , respectively. The production of PGA was declined after that temperature and completely disappeared at 45°C. These strains could be defined as mesophilic bacteria that have the highest growth rate on the range of 30 to 37°C, and this could have a direct impact on PGA biosynthesis [42,43].

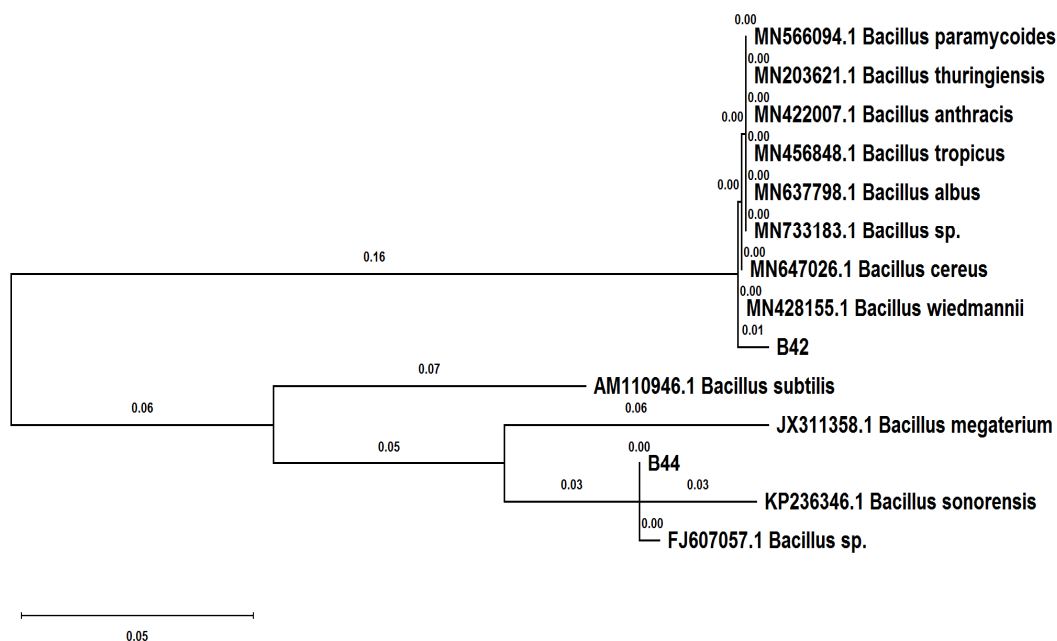


Fig. 1. The phylogenetic tree of *Bacillus* sp. B44 and B42 isolates along with their most similar species constructed by the DNA sequence of 16SrRNA

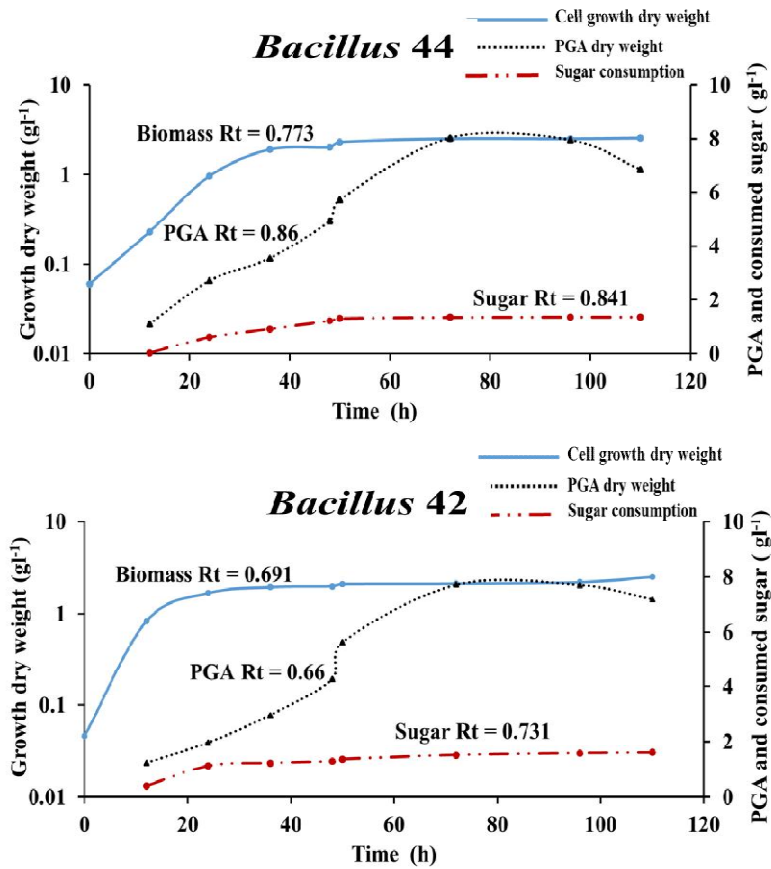


Fig. 2. Time-course of PGA production by *Bacillus 44* and *Bacillus 42* strains during 110 h of incubation period

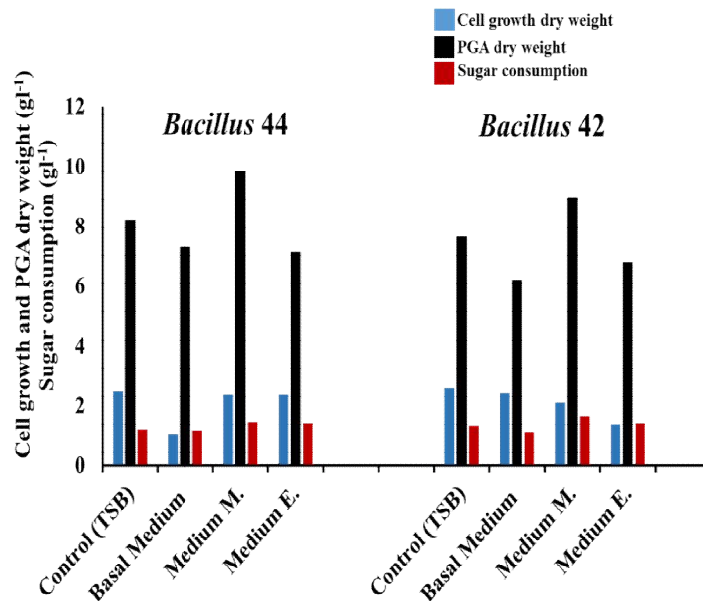


Fig. 3. Evaluating four media types for the best yielding of PGA using *Bacillus 44* and *Bacillus 42* strains

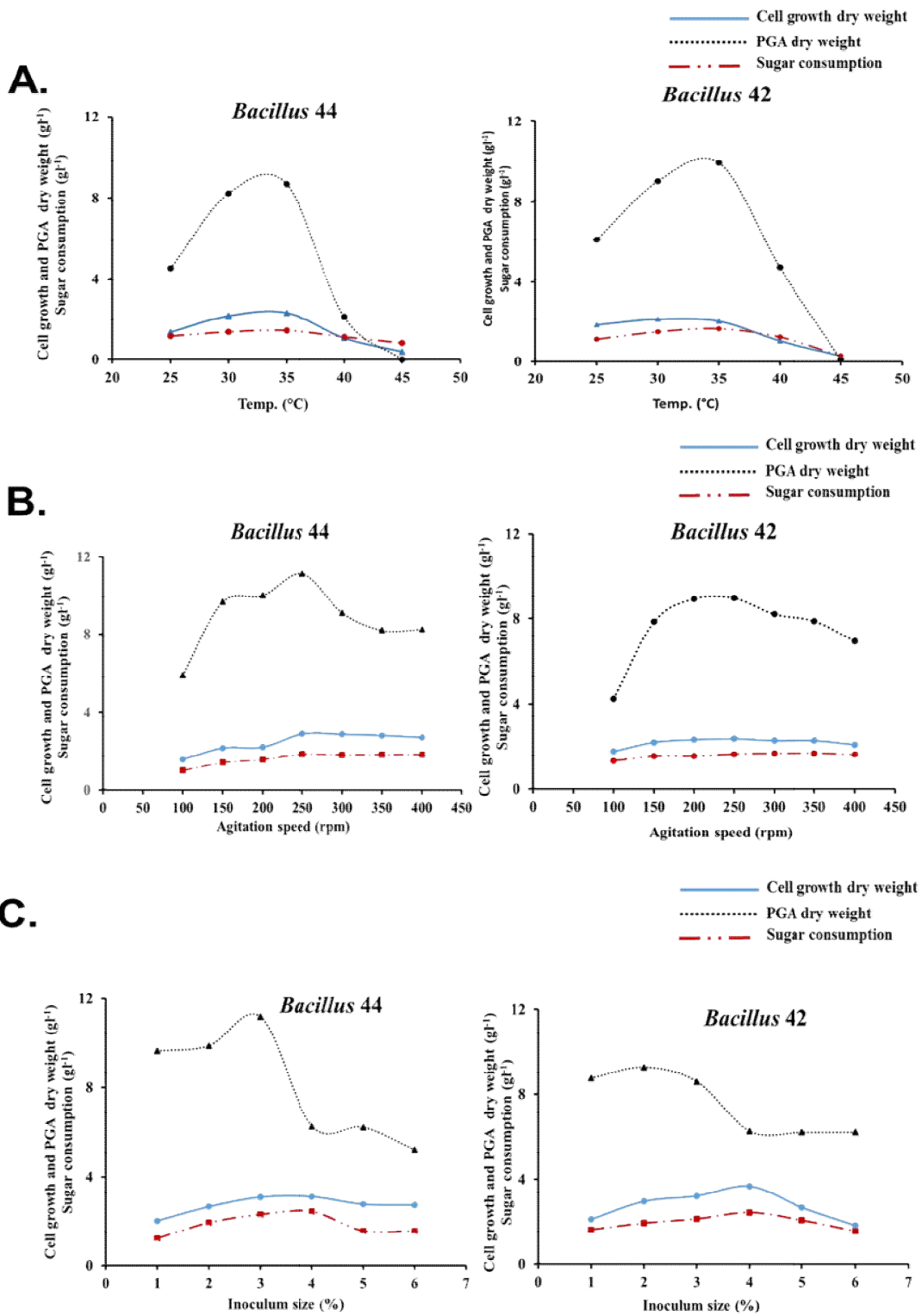


Fig. 4. Optimizing fermentation temperature, agitation speed, and inoculum size for the best yielding of PGA using *Bacillus 44* and *Bacillus 42* strains



### 3.5.2 Agitation speed

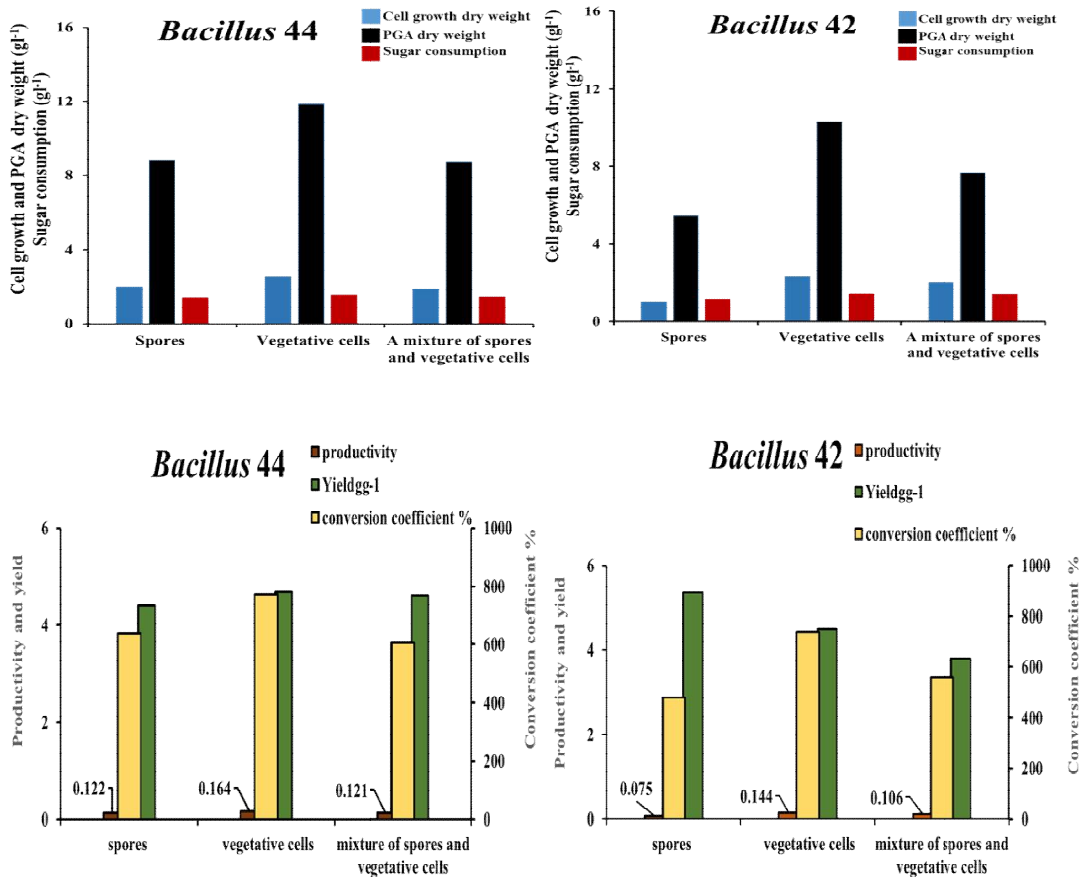
In previous reports, researchers noticed that the genus *Bacillus* is an obligate aerobic, and the levels of O<sub>2</sub> have an important impact on the physiological activity [42]. With these facts, optimizing agitation speed for the best accumulation of PGA was essential. The cell growth and PGA dry weight were incremented with agitation, and the most efficient agitation speed for both strains was at 250 rpm yielding cell growth dry weight (2.88 and 2.35 g<sup>l</sup><sup>-1</sup>), PGA dry weight (11.12 and 8.96 g<sup>l</sup><sup>-1</sup>), and PGA productivity (0.154 and 0.124 g<sup>l</sup><sup>-1</sup>h<sup>-1</sup>) for *Bacillus* 44 and *Bacillus* 42 strains, respectively (Fig. 4B). The importance of aerobic conditions for cell growth and polymer production was related to the oxygen supply for activating the TCA cycle [43]. The reduction of agitation speed could decrease the dissolved O<sub>2</sub> in the medium that resulted in decreasing of the PGA dry weight in our study to record 46.67 and 52.56% for *Bacillus* 44 and *Bacillus* 42, respectively.

### 3.5.3 Inoculum size

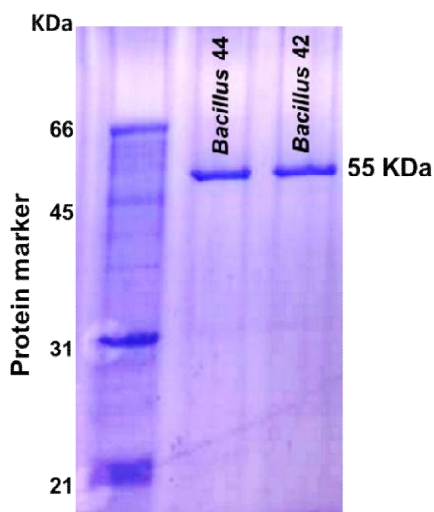
We evaluated six inoculum sizes ranged from 1 to 6%. For *Bacillus* 44 strain, the volume of 3% was the best inoculum for yielding PGA (Fig. 4C). This volume size resulted in 11.18 g<sup>l</sup><sup>-1</sup> PGA dry weight. The PGA productivity, yield, and conversion coefficient of the same strain were 0.155 g<sup>l</sup><sup>-1</sup>h<sup>-1</sup>, 3.62 gg<sup>-1</sup>, and 486%, respectively. On the other hand, the strain *Bacillus* 42 represented the highest production of PGA with the inoculum size 2% (Fig. 4C). Increasing the inoculum over 3% had a negative impact on PGA production. Our results were in the same line with Baxi, [37] investigation who assessed inoculums from 4 to 20% (v/v).

### 3.5.4 Physical state of inoculum

The assessment of the three physical states (spores, outgrowth vegetative cells, and a mixture of spores and vegetative cells) of *Bacillus* sp. strains revealed the importance of



**Fig. 5.** Three inoculum physical states including spores, outgrowth vegetative cells and a mixture of spores and vegetative cells, for the best yielding of PGA using *Bacillus* 44 and *Bacillus* 42 strains



**Fig. 6. The molecular weight of PGA generated from *Bacillus 44* and *Bacillus 42* strains**

young outgrowth vegetative cells in PGA accumulation. This physical state yielded cells with the best growth dry weight (2.52 and 2.3 gl<sup>-1</sup>), PGA dry weight (11.82 and 10.34 gl<sup>-1</sup>) as well as sugar consumption (1.53 and 1.4 gl<sup>-1</sup>) for *Bacillus 44* and *Bacillus 42* strains, respectively (Fig. 5A). Similar results were also reported by [44,45,37] who found that the production of PGA dry weight incremented 2-fold when the media inoculated by young outgrowth vegetative cells at the age of 24 h when compared to another inoculum contained a mixture of outgrowth vegetative cells and spores. In addition, PGA productivity, yield, and conversion coefficient were the most efficient at the same inoculum state (outgrowth vegetative cells) revealing (0.16 gl<sup>-1</sup>h<sup>-1</sup>, 4.69 gg<sup>-1</sup>, 772.5%) for *Bacillus 44* strain, and (0.14 gl<sup>-1</sup>h<sup>-1</sup>, 4.5 gg<sup>-1</sup>, 738.6%) for *Bacillus 42* strain, respectively (Fig. 5B). On the contrary, the inoculation with *Bacillus* spores led to the reduction of cell growth and PGA dry weights (1.01 and 5.42 gl<sup>-1</sup>, respectively) in *Bacillus 42* strain (Fig. 5A). While this did not impact on the PGA production when *Bacillus 44* spores were used for inoculation.

### 3.6 Determination of the Molecular Size of Produced PGA by SDS-PAGE

We analyzed the PGA secreted by both *Bacillus 44* and *Bacillus 42* strains using SDS-PAGE. The PGA biopolymer was participated, then analyzed on 10% SDS-PAGE. The molecular size of generated PGA from *Bacillus 44* and *Bacillus 42*

strains about 55 KDa, (Fig. 6). In general, the molecular mass of PGA was varied based on several environmental factors. The PGA polymer generated by *B. licheniformis* was in the range of 80 to 180 KDa [46]. Many studies reported different molecular weights of ranged from 10 to 2000 KDa that may be impacted by the type of microbes or degradation enzymes [14,15,16,17].

## 4. CONCLUSIONS

Great insights have been gained for manipulating the production of PGA. Because of its biodegradability and non-toxicity, PGA is used commonly in the food, medicine, and wastewater industries. Of Egyptian soil samples, we isolated and identified highly productive PGA *Bacillus* species isolates. On the molecular level, we identified two of them at the genus level. Both were identified and shared 81% of the DNA sequence of 16SrRNA. With other *Bacillus* species comparisons, both species found to belong to separated species of *Bacillus*. We also boosted *Bacillus 44* and *Bacillus 42* strain PGA production. The *Bacillus* species *Bacillus 44* strain revealed the highest accumulation level at 35°C and 72 h of incubation using medium M with inoculum size and agitation speed 3% and 250 rpm, respectively. This strain secreted PGA biopolymer with a molecular mass of 55 KDa. The other strain, *Bacillus 42*, was also optimized for the high secretion of a similar size of PGA. New approaches like molecular biology and genetic engineering can be applied to boost the PGA production for several industrial applications.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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