Optimization of Some Nutritional Conditions and α-Ketoglutaric Acid Concentration as PGA Precursor for Maximizing PGA Production from *Bacillus* sp. 42 and *Bacillus sonorensis* 44

Rania F. Ahmed*

1Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo 11241, Egypt.

**Author’s contribution**

The sole author designed, analysed, interpreted and prepared the manuscript.

**Article Information**

DOI: 10.9734/MRJI2020/v30i230196

Editor(s):

(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.

Reviewers:

(1) Igiebor Francis Aibuedefe, Wellspring University, Nigeria.

(2) Kambire Otto, Peleforo Gon Coulibaly University, Côte d'Ivoire.

Complete Peer review History: [http://www.sdiarticle4.com/review-history/56107](http://www.sdiarticle4.com/review-history/56107)

Received 28 January 2020
Accepted 04 April 2020
Published 07 April 2020

**ABSTRACT**

Poly gamma glutamic acid is a biodegradable, water soluble and non-toxic edible biopolymer, PGA has nylon back bone similar structure and expressed as bio-nylon. Various bacterial strains produced PGA on of them *Bacillus* sp. such as *B. subtilis*, *B. lichenformans* and *B. sonorensis*. Polymer yield was affected with medium composition as nitrogen and carbon sources. The current experimental was carried out using shake flask technique for PGA production during 72 of fermentation. The highest biomass was achieved at glycerol media and glucose media for PGA yield and productivity being 2.31, 9.65 $\text{g} \cdot \text{L}^{-1}$ and 0.134 $\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, respectively of *B. sonorensis* 44. Of nitrogen source, organic source (yeast extract) was higher PGA yield and productivity than inorganic sources ($\text{NH}_4\text{NO}_3$) which reduced PGA yield about 28.7 and 36.02% of *Bacillus* sp. 42 and *B. sonorensis* 44, respectively. Production media supplemented with 0.5 and 0.75 $\text{g} \cdot \text{L}^{-1}$ α-keto-glutaric acid increased PGA yield about 1.24fold for both *Bacillus* strains. Osmotic pressure of 2.55 MPa (3% NaCl) enhanced PGA yield about 1.18 and 1.24fold of *Bacillus* sp. 42 and *B. sonorensis* 44, respectively. Furthermore, the
highest PGA was received using medium containing glucose and yeast extract (as C and N sources). α-keto-glutaric acid and osmotic potential has an induction effect for polymer accumulation.

Keywords: γ-PGA; carbon sources; nitrogen sources; α-Keto-glutaric acid; NaCl osmotic stress; Bacillus sp.42 and B. sonorensis 44.

1. INTRODUCTION

Poly gamma glutamic acid is a water soluble exopolymer which consist of glutamic acid polymerization. γ-PGA polymer has two stereochemical structure D-glutamic acid, L-glutamic acid, and copolymer of both D- and L-glutamic acid [1]. The polymerization of PGA catalyzed γ-PGA synthase complex in a ribosome independent manner [2]. The PGA polymer produced from different kinds of bacterial strains such as Bacillus genera like Staphylococcus, Natrialba, Lysinibacillus, and Fusobacterium [3,4,5,6]. PGA produced protected bacterial cell against from destructive condition, phagocytosis, antibacterial agent, phage infection and nutritional depletion during stationary phase [7,8,9]. PGA polymer known as eco-friendly polymer of its biodegradable, non-toxic and edible properties [10].

Poly-γ-glutamic acid has many different varieties of application scales and including food processing, improving calcium solubility and enhanced food texture, antifreeze agent, stabilizer for ice cream and reduced uptake of oil during deep-fat frying [11,12,9]. PGA was used in waste water treatment as heavy metal biosorption, basic day removable and high flocculating activity of PGA [13,14]. Medical scale, effectively reducing the tumour size, drug carrier, field of tissue engineering and coating wound dressing [15,16].

γ-PGA production carried out through chemical, biological methods, biotransformation, peptide synthesis, chemical synthesis and microbial fermentation [17]. Poly-γ-PGA production using microbial fermentation has many economic and environmental advantages, as inexpensive raw materials, minimal environmental pollution, high natural product purity, and mild reaction conditions as compared with other methods [18]. In this view, the study was carried to evaluated the best nutritional condition such as, carbon, nitrogen and minerals content of production medium. Also, evaluation the NaCl osmotic pressure and PGA concentration on PGA yield.

2. MATERIALS AND METHODS

2.1 Effect of Carbon Sources on PGA Production

Both Bacillus strains were cultivated on medium M (7.5% glucose, 1.8% NH₄Cl, 0.15% K₂HPO₄, 0.035% MgSO₄ & 0.005% MnSO₄) supplemented with different carbon sources (fructose, galactose, sucrose, lactose, glycerol, citrate and glucose). Elementary flasks (250 ml) contained production med. M (100 ml) were inoculated with 1% standard inoculum and incubated at 30°C and 150 rpm for 72 h. At the end of incubation period, sample of 10ml was collected and centrifugation at 10000 rpm for 10 min at 4°C, pellet was washed twice with distilled water and re-centrifugated, the pellets were dried at 80°C till constant weight. Culture supernatant was used to determine residual sugar and polymer dry weight.

2.2 Combination the Most Efficient of Carbon Sources on PGA Production

Production med. M was supplemented with the most efficient carbon sources. In combination ratio of 1:1:1, 1:2:1 & 1:1:2 (glucose: glycerol: citrate) and 1:1, 1:2 & 2:1 (glycerol: citrate). At the end of fermentation process, Bacillus sp. biomass and PGA dry weight were determined.

2.3 Effect of Nitrogen Sources on PGA Production

Six organic nitrogen (casine, peptone, tryptone, proteose peptone and yeast extract) and 3 inorganic nitrogen (NH₄SO₄, NH₄NO₃ and KNO₃) were study for the most efficient source of PGA yield using med. M. After fermentation, Bacillus sp. biomass and PGA dry weight as well as residual sugar were determined.

2.4 Effect of Mineral Solution Addition on PGA Production

Mineral solution with the following composition (1 mM trace solution of: CaCl₂, FeSO₄, ZnCl₂ & MnSO₄) were added to the production media in a
trail ranged from 0.25 to 1.75 ml of mineral solution/L production medium. At the end of fermentation, *Bacillus* sp. biomass, PGA dry weight and residual sugar were determined.

2.5 Impact of Using α-keto glutaric Acid as PGA Precursor

A trail of α-keto glutaric acid ranged from 0.25 to 1.25 gl⁻¹ were added to the production med. M at the end of fermentation period, *Bacillus* sp. biomass, PGA dry weight and residual sugar were determined.

2.6 Effect of NaCl Osmotic Pressure on PGA Production

The PGA production media (med. M) were supplemented with different NaCl concentration to give osmotic pressure ranged from 0.85 to 5.95 MPa (1 to 6% NaCl). Then production media were inoculated with tested *Bacillus* strains, after fermentation process, biomass PGA dry weight and residual sugar were determined.

2.7 Standard Inoculum

TB media were inoculated with loopful of tested *Bacillus* strains (These strains were previously isolated from soil and identified according to their pheno and genotypes characteristics [19]) and incubated 30°C for 24 h using rotary shaker at 150 rpm, each 1 ml of culture contain 0.95 gl⁻¹ dry weight was used as standard inoculum in this study.

2.8 PGA Recovery

About 10 ml of culture was centrifugation at 10000rpm for 10 min at 4°C, then pellets were dried at 80°C till constant weight after washing twice with D.W (growth dry weight), to recover PGA, supernatant was precipitated using ice-cold ethanol (1:2 volume ratio), then kept at 4°C overnight. Precipitate PGA was collected by recentrifugation at 7000 rpm for 10 min at 4°C, for further purification, the precipitation step was repeated. Finally, pellets were dried at 80°C till constant weight [20].

2.9 Glucose Determination

Residual sugar in the culture supernatant was determined using potassium freecyanide as described by Park and Johnson [21].

2.10 PGA Parameters

**PGA productivity** = gm PGA/ fermentation time (h) gl⁻¹h⁻¹, according to Lee [22].

**PGA yield to biomass** (Y/\(\text{m} \)) = gm PGA/ gm biomass dry weight gl⁻¹ according to Grothe et al. [23].

**PGA conversion coefficient (%)** = gm PGA X 100/gram utilized sugar, according to Ramadan et al. [24].

2.11 Statistical Analysis

The standard error was analyzed with Microsoft Office Excel 2013.

3. RESULTS AND DISCUSSION

3.1 Effect of Carbon Sources

Glycerol achieved the highest biomass dry weight of *B. sonorensis* 44 and *Bacillus* sp. 42 followed by citrate. While the highest figures of PGA dry weight, productivity and yield were recorded of glucose followed by glycerol and citrate, The values being 9.65gl⁻¹, 0.134 gl⁻¹h⁻¹ & 4.6 gg⁻¹, respectively of *Bacillus sonorensis* 44 and 8.64 gl⁻¹, 0.12 gl⁻¹h⁻¹ & 4.02 gg⁻¹, respectively of *Bacillus* sp. 42, (Fig. 1a). The high accumulation of PGA of glucose media related to the ability of *Bacillus* sp. to utilized glucose through TCA. Which also used for PGA generation [25]. As more, applied high concentration of glucose (120 gl⁻¹) in PGA production media maximized the PGA yield to 46.4 gl⁻¹ [26].

3.2 Combination the Most Efficient Carbon Sources on PGA Production

Fig. 1b. shows that the highest biomass, PGA & productivity were estimated under combination of glucose: citrate at ratio of 1:1:1 for *B. sonorensis* 44 (2.28, 11.32 gl⁻¹ & 0.157 gl⁻¹h⁻¹). While *Bacillus* sp. 42 has the same trend of 1:2:1 ratio (2.23, 9.59 gl⁻¹ & 0.133 gl⁻¹h⁻¹). Moreover, the elimination of glucose from production media decreased biomass and polymer dry weight. The reduction in biomass and polymer dry weight were recorded at glycerol: citrate (1:2) of *B. sonorensis* 44 being 1.46 and 5.12 gl⁻¹ and *Bacillus* sp. 42 being 1.97 and 7.97l gl⁻¹, respectively. This depletion may relate to the fast
utilization of glucose than glycose furthermore substitution of glucose in media formulation in stated of glycerol supported metabolism of citrate and glutamate. Which known as PGA precursors and led to high yield of PGA [27,28]. On contrast, application of citric acid with other carbon sources, polysaccharide formed either had little or no γ-PGA accumulation [29].

3.3 Impact of Nitrogen Sources

The effect of nitrogen sources (organic and inorganic) were study during 72 h of incubation. The highest biomass dry weight (2.22 gl⁻¹) was recorded on yeast extract and casine for B. sonorensis 44 and (2.43 gl⁻¹) on casine for Bacillus sp. 42, as in Fig. 2. Yeast extract and peptone achieved the highest PGA dry weight, productivity and yield being 10.98gl⁻¹, 0.135 gl⁻¹h⁻¹ & 5.64 gg⁻¹ of Bacillus 42 and B. sonorensis 44, respectively. Inorganic carbon source NH₄NO₃ was unfavorable nitrogen source for growth and PGA production. Biomass and PGA dry weight reduced about 40.6 and 28.7% for Bacillus sp. 42 and 39.67 & 36.02%, respectively. These data were in line with other investigation, who found that, addition of yeast extract as nitrogen sourced increased PGA yield of Bacillus subtilis 168 [30,25]. Furthermore, organic nitrogen sources were favorable for PGA production than inorganic sources which have non-significant effect on PGA production. On the other hand, addition of sufficient ammonium ions was found to be necessary for efficient conversion of citric acid to glutamic acid [28]. With regard to glucose consumption, there was no direct effect on PGA generation. As seen the highest consumed glucose figures were 1.63 and 1.95 gl⁻¹ of tryptone and casine, respectively and low polymer production (8.32 and 8.01 gl⁻¹ PGA).

![Fig. 1. Effect of carbon sources (a) and mixture of glucose: glycerol: citrate of different ratio (b) on PGA yield](image-url)
Fig. 2. Effect of organic and inorganic nitrogen sources on biomass and PGA yield

3.4 Effect of Mineral Solution Concentration

Serial trails of mineral solution ranged from 0.25 to 1.75 ml/L were added to production medium M. Biomass, PGA and productivity were increased gradually with mineral concentration then declined. The highest figures were achieved by both Bacillus sp. 42 (2.05, 9.81 gl⁻¹ & 0.136 gl⁻¹h⁻¹, respectively) and B. sonorensis 44 (2.16, 10.55 gl⁻¹ & 0.147 gl⁻¹h⁻¹, respectively) at 1.5 ml/L (Fig. 3). The addition of mineral solution of FeCl₃MnSO₄-7H₂O, and NaCl at low concentration (range of 0.07 to 0.1% w/w) to cooked soybean stimulated the PGA accumulation of Bacillus subtilis 168 in a significant way. These inorganic salts such as FeCl₃ may act as components of the coenzyme participating in the metabolism of bacteria, thus positively affecting the enzymatic reaction of c-PGA synthesis [30,31].

3.5 Effect of α-keto-glutaric Acid Concentrations

In this study, different concentration of α-keto-glutaric acid were added to production media as PGA precursor. The biomass, polymer dry weight and productivity increased gradually with concentrations as seen in Fig. 4, and reach the peak at 0.5 and 0.75 gl⁻¹ α-keto-glutaric acid then begin to decline. PGA dry weight increased about 1.24fold for both Bacillus strain. α-ketoglutaric acid was serve as a direct precursor of PGA synthesis through TCA cycle. α-ketoglutaric acid was converted to L-glutamic acid which catalyzed by glutamic dehydrogenase. Then glutamic acid polymerized into PAG by the action of the enzyme glutamine synthase [32,33].

3.6 Effect of NaCl Osmotic Pressure on PGA Yield

Data in Fig. 5. shown that the additionally osmotic pressure which conducted of NaCl concentration enhanced growth, PGA dry weight, consumed sugar (g/L) as well as polymer productivity. Osmotic pressure of 2.55 MPa (3%NaCl) introduced the highest values being 2.2, 10.34, 2.31 gl⁻¹ and 0.144 gl⁻¹h⁻¹ for Bacillus sp. 42 and 2.41, 11.7, 2.38 gl⁻¹ and 0.163 gl⁻¹h⁻¹, respectively of B. sonorensis 44. These incremental in PGA in recorded by other investigators [34,7] who found that addition of
NaCl (2%w/v) to production media affected PGA yield, molecular weight and stereochemistry production significantly of *B. licheniformis* CCRC 12826. As well as increasing molecular weight of PGA produced under osmotic pressure show very promising industrial applications [9]. While increasing osmotic pressure led to slow down the growth of *Bacillus* sp. 42 and complete inhibition of PGA generation of *Bacillus* sp.42 and *B. sonorensis* 44 growth.

**Fig. 3.** Effect of mineral solution (ml/L) of PGA yield
4. CONCLUSION
Poly gamma glutamic acid on of the polymer which known as bio-nylon. With back bone similar to nylon structure. PGA produced from different bacterial species among them Bacillus sp. Biomass dry weight of Bacillus sp. 42 and B. sonorensis 44 was received of glycerol media. PGA highest figures were recorded with glucose. the highest biomass and PGA were estimated under combination of glucose: glycerol: citrate at ratio of 1:1:1 for B. sonorensis 44. Organic nitrogen sources were more favorable for PGA accumulation specially yeast extract and
peptone. Also, addition of α-keto-glutaric acid (0.5 and 0.75 gl-1) and NaCl osmotic pressure (2.55 MPa) enhanced PGA production of Bacillus sp. 42 and B. sonorensis 44.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Shih L, Van YT The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresource Technology. 2001;79:207-225. DOI: 10.1016/S0960-8524(01)00074-8
2. Yang X. Preparation and characterization of γ-poly glutamic acid copolymer with glycol-diglycidyl ether. Procedia Environmental Sciences. 2011;8:11-15. DOI: 10.1016/j.proenv.2011.10.004
3. Kambourova M, Tangney M, Priest FG. Regulation of polyglutamic acid synthesis by glutamate in Bacillus licheniformis and Bacillus subtilis. Applied Environmental Microbiology. 2001;67:1004–1007.
4. Meerak J, lida H, Watanabe Y, Miyashita M, Sato H, Nakagawa Y, Tahara Y. Phylogeny of γ-polyglutamic acid-producing Bacillus strains isolated from fermented soybean foods manufactured in Asian countries. J. Gen. Appl. Microbiol. 2007;53:315-323. DOI: https://doi.org/10.2323/jgam.54.159
5. Candela T, Moya M, Haustant M, Fouet A. Fusobacterium nucleatum, the first Gram-negative bacterium demonstrated to produce polyglutamate. Canadian Journal Microbiology. 2009;55:627–632.
6. Cao M, Geng W, Song C, Xie H, Guo W, Jin Y, Wang S. Glutamic acid independent production of poly-C-glutamic acid by Bacillus amyloliquefaciens LL3 and cloning of pgsBCA genes. Bioresource Technology. 2011;102:4251–4257.
7. Shimizu K, Nakamura H, Ashiuchi M. Salt-Inducible Bionylon Polymer from Bacillus megaterium. Appl. Environ. Microbiol. 2007;73:2378–2379. DOI:10.1128/AEM.02686-06
8. Tamang JP. Naturally fermented ethnic soybean foods of India. J. Ethnic Foods. 2015;2:8–17. DOI:10.1016/j.jef.2015.02.003
9. Ogunleye A, Bhat A, Irorere VU Hill, D, Williams C, Radecka I. Poly-C-glutamic acid: Production, properties and applications. Microbiol. 2015;161:1–17. DOI: 10.1099/mic.0.081448-02015
10. Shih L, Wu J. Microbial production of biopolymers and polymer precursors. Applications and perspectives. Caister Academic Press, Poole, UK; 2009.
11. Mitsuki M, Mizuno A, Tanimoto H, Motoki M. Relationship between the antifreeze activities and the chemical structures of oligo-and poly glutamic acids. J. Agric. Food Chem. 1998;46:891-895. DOI: 10.1021/jf970797m
12. Shyu YS, Sung WC Improving the emulsion stability of sponge cake by the addition of γ-polyglutamic acid. J.Mar. Sci. Technol. 2010;18:895-900. DOI: 10.6119/JMST
13. Ho GH, Ho TI, Hsieh KH, Su YC, Lin PY, Yang J, Yang KH, Yang SC. γ-polyglutamic acid produced by Bacillus Subtilis (Natto): Structural characteristics, chemical properties and biological functionalities. J. Chin. Chem. Soc. 2006; 53:1363-1384. DOI: 10.1002/jccs.200600182
14. Abdel-Fattah Y, Soliman N, Berekaa M. Application of Box-Behnken design for optimization of poly-γ-glutamic acid production by Bacillus Licheniformis SAB-26. Res. J. Microbiol. 2007;2: 664-670. DOI: 10.3923/jm.2007.664.670
15. Matsuo K, Koizumi H, Akashi M, Nakagawa S, Fujita T, Yamamoto A, et al. Intranasal immunization with poly (γ-glutamic acid) nanoparticles entrapping antigenic proteins can induce potent tumor immunity. J. Control. Release. 2011;152: 310-316.
16. Candela T, Fouet A. Poly-gamma-glutamate in bacteria. Mol. Microbiol. 2006; 60:1091-1098.
17. Ashiuchi M. Occurrence and biosynthetic mechanism of poly-gamma-glutamic acid, in Hamano Y (ed.), Amino-Acid Homopolymers Occurring in Nature, Springer, New York, N.Y; 2010.
18. Ahmed RF, Khalil HB. Isolation, identification and evaluation of Egyptian Bacillus sp. isolates for producing poly gamma glutamic acid. JABM. 2020;19:1-13.
9945a. Biotechnol. Bioengineer. 1998;57: 430- 437.
28. Cromwick AM, Gross RA. Investigation by NMR of metabolic routes to bacterial g- poly (glutamic acid) using 13C labeled citrate and glutamate as media carbon sources. Can. J. Microbiol. 1995;41:902–909.
29. Bhunia B, Mukhopadhy D, Goswami S, Mandal T, Dey A. Improved production, characterization and flocculation properties of poly (γ)-glutamic acid produced from Bacillus subtilis. J. Biochem. Technol. 2012;3:389-394.
30. Jian X, Shouwen C, Ziniu Y. Optimization of process parameters for poly c-glutamate production under solid state fermentation from Bacillus subtilis CCTCC202048. Process Biochem. 2005; 40:3075–3081. Ratha PD, Jhon Y. Factors increasing poly-c-glutamic acid content of cheonggukjang fermented by Bacillus subtilis 168. Food Sci Biotechnol. 2019; 28:103–110. DOI: org/10.1007/s10068-018-0424-z
31. Buescher JM, Margaritis A. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Crit. Rev. Biotechnol. 2007; 27:1-19. DOI: 10.1080/07388550601166458
32. Kongkrom N, Shi ZP, Chisti Y, Sirisansaneeyakul S. Enhanced production of poly-γ-glutamic acid by Bacillus licheniformis TISTR 1010 with environmental controls. Appl Biochem Biotechnol. 2017;182:990–999. Morris G, Charalampopoulos MR, D. Radecka I. Bacillus subtilis natto: A nontoxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. Amb Express. 2013;3:36. DOI: 10.1186/2191-0855-3-36

© 2020 Ahmed; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/56107