Microbiology Research Journal International

30(2): 34-42, 2020; Article no.MRJI.56107
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

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

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Author's contribution

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

Article Information

DOI: 10.9734/MRJI/2020/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 Ollo, Peleforo Gon Coulibaly University, Côte d'Ivoire.
Complete Peer review History: http://www.sdiarticle4.com/review-history/56107

Original Research Article

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. lichanformans* 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 gl^{-1} and 0.134 $gl^{-1}h^{-1}$, respectively of *B. sonorensis* 44. Of nitrogen source, organic source (yeast extract) was higher PGA yield and productivity than inorganic sources (NH_4NO_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 gl^{-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

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highest PGA was received using medium containing glucose and yeast extract (as C and N_2 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. ɣ-PGA polymer has two stero-chemical 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-ɣ-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% NH_4Cl_2, 0.15% K_2HPO_4, 0.035% $MgSO_4$ & 0.005% $MnSO_4$) 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 tell 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 (NH_4SO_4, NH_4NO_3 and KNO_3) 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: $CaCl_2$, $FeSO_4$, $ZnCl_2$ & $MnSO_4$) 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 re-centrifugation 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 sugarin 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) $gl^{-1}h^{-1}$, according to Lee [22].

PGA yield to biomass($Y_{c/x}$) = gm PGA/ gm biomass dry weight gg^{-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 $gl^{-1}h^{-1}$& 4.6 gg^{-1}, respectively of *Bacillus sonorensis* 44 and 8.64 gl^{-1}, 0.12 $gl^{-1}h^{-1}$ & 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].

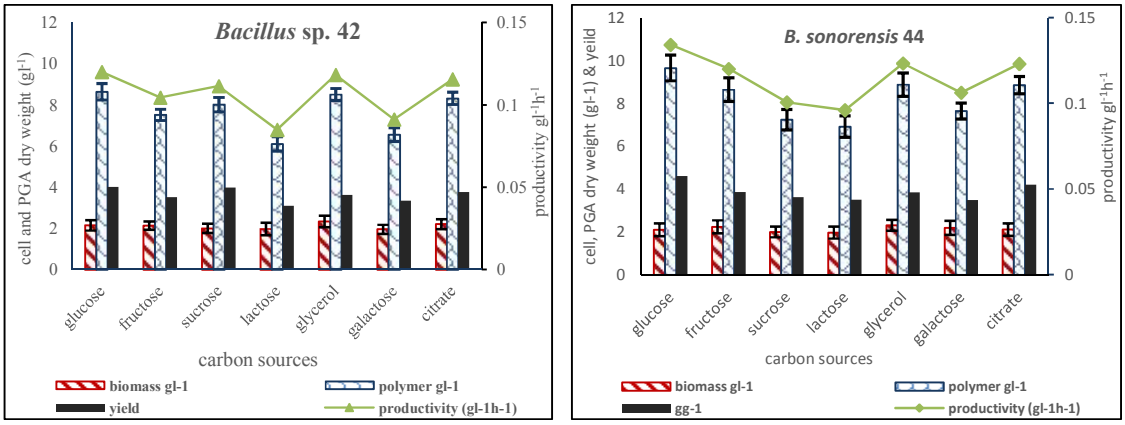
3.2 Combination the Most Efficient of Carbon Sources on PGA Production

Fig. 1b. shows that the highest biomass, PGA & productivity were estimated under combination of glucose: glycerol: citrate at ratio of 1:1:1 for *B. sonorensis* 44 (2.28, 11.32 gl^{-1} & 0.157 $gl^{-1}h^{-1}$). While *Bacillus* sp. 42 has the same trend of 1:2:1 ratio (2.23, 9.59 gl^{-1}& 0.133 $gl^{-1}h^{-1}$). 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

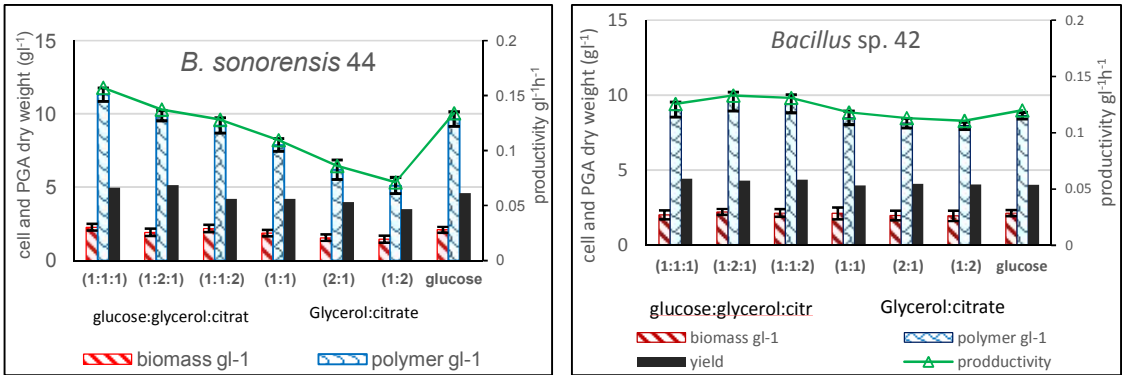
utilization of glucose than glycose furthermore substitution of glucose in media formulation in stated of glycerol supported metabolism of citrate and glutamate. Which known as PGA precursors and led to high yield of PGA [27,28]. On contrast, application of citric acid with other carbon sources, polysaccharide 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 $gl^{-1}h^{-1}$ & 5.2 gg^{-1} and 11.84 gl^{-1}, 0.164 $gl^{-1}h^{-1}$& 5.64 gg^{-1} of *Bacillus* 42 and *B. sonorensis*44, respectively. Inorganic carbon source NH_4NO_3 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).



(A)



(B)

Fig. 1. Effect of carbon sources (a) and mixture of glucose: glycerol: citrate of different ratio (b) on PGA yield

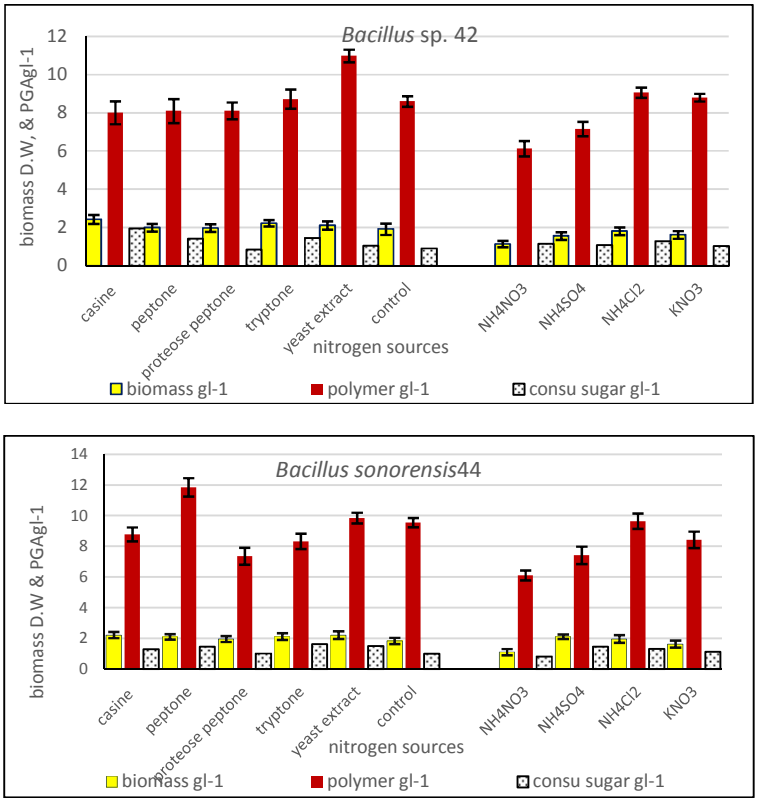


Fig. 2. Effect of organic and inorganic nitrogen sources on biomass and PGA yield

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 $gl^{-1}h^{-1}$, 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 $FeCl_3$Mn SO4-7H_2O, 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 $FeCl_3$ 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 $gl^{-1}h^{-1}$ for *Bacillus* sp. 42 and 2.41, 11.7, 2.38 gl^{-1} and 0.163 $gl^{-1}h^{-1}$,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. lichenifomis* 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.

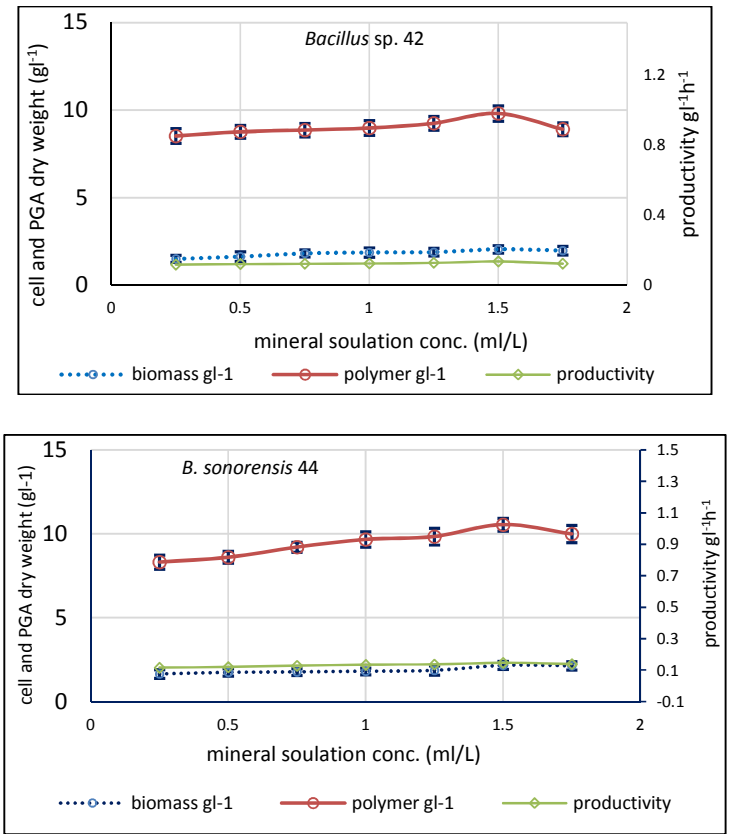
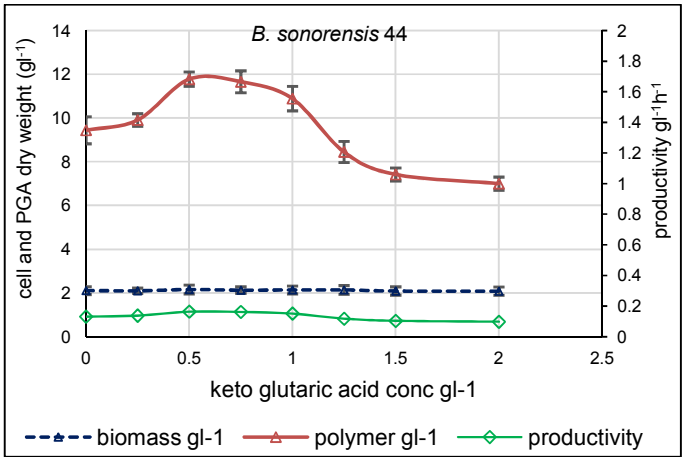


Fig. 3. Effect of mineral solution (ml/L) of PGA yield



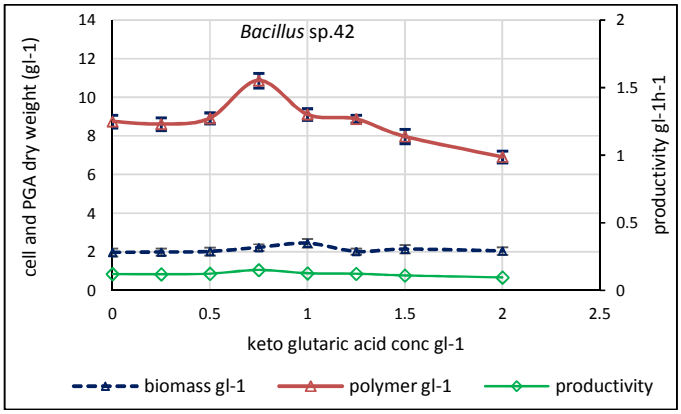
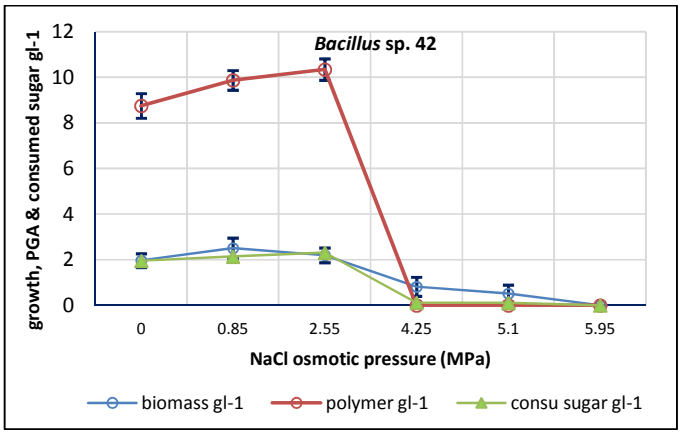


Fig. 4. Effect of serial concentration of α-keto-glutaric acid on PGA yield



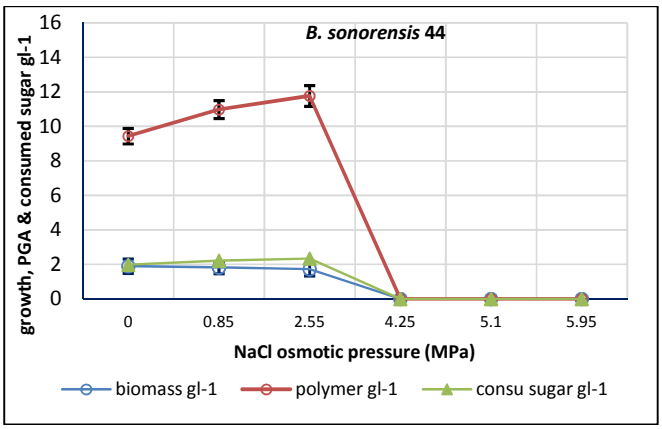


Fig. 5. Effect of NaCl osmotic pressure (MPa) on PGA yield

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.

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Peer-review history:
The peer review history for this paper can be accessed here:
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