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Optimisation of Poly(γ-Glutamic Acid) Production by *Bacillus velezensis* NRRL B – 23189 in Liquid Fermentation with Molasses as the Carbon Source without Addition of Glutamic Acid

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Abstract – *Poly (γ-glutamic acid), also known as γ-PGA, is an extracellular polymer produced by microbial fermentation. It is water-soluble, edible, biodegradable, non-toxic towards humans and the environment, and it has many available sites for drug conjugation and a powerful ability to solubilise hydrophobic molecules. This work reports the application of molasses, citric acid and ammonium sulphate in the fermentation by Bacillus velezensis NRRL-23189 to produce γ-PGA and the detection of molasses consumption without the use of glutamic acid as a nutrient. Different concentrations of molasses, citric acid and ammonium sulphate were studied. The fermentation was agitated at 200 rpm at 27ºC for 72 h, with an initial pH of 6.5 (NaOH 2N and HCl 2N). Spectrophotometric analyses were used to measure concentrations of γ-PGA and the residual sugar from molasses degradation. The maximum production of γ-PGA was 4.82 g/l, in a medium with molasses (200g/l), citric acid (12.5g/l) and ammonium sulphate (8g/l) in a fermentation that also resulted in the maximum sugar consumption.* ***Copyright © 2012 Praise Worthy Prize S.r.l. - All rights reserved.***

Keywords*: Bacillus, Molasses, Poly (γ-Glutamic Acid), Fermentation, Biosynthesis, Nutritional Requirements*

I. Introduction

Poly(γ-glutamic acid), known as γ -PGA, is a biodegradable anionic polymer that is water-soluble [1]-[5] and non-toxic to humans and the environment [2], [6], [7]. γ-PGA is polymerised via γ -amide linkages between α-amino and γ-carboxylic acid groups linking subunits of D- and L- glutamic acid [3]-[6], [8]-[10]. Ivánovics and co-workers discovered the compound in 1937 as a capsule polymer of *Bacillus anthracis* [1], [12]. Later, γ-PGA was identified in other *Bacillus spp.* as a fermentation product, for example in *Bacillus subtilis* IFO 3335 [1], *Bacillus licheniformes*, *Bacillus megaterium and Bacillus amyloliquefaciens* [12]-[13][14]. After reference [12] showed that γ-PGA was produced as a fermentation product in a culture broth by *Bacillus subtillis*, numerous studies were stimulated [10]. γ-PGA is secreted by the cell wall and forms the capsule during growth [15] and is also excreted into culture media [15], [16]. γ-PGA biosynthesis in bacteria is carried out in two steps. The first step is the synthesis of L- and D- glutamic acid, and in the second step, D- and L- glutamic acid units are joined together [17]. γ-PGA is mainly produced from citric acid and ammonium sulphate (Fig. 1).

It is presumed that L-glutamic acid is produced from citric acid through isocitric acid and α-ketoglutaric acid in the tricarboxylic acid cycle (TCA), and γ-PGA is polymerised from this glutamic acid.

A large amount of γ-PGA is thus produced from citric acid and ammonium sulphate [10].

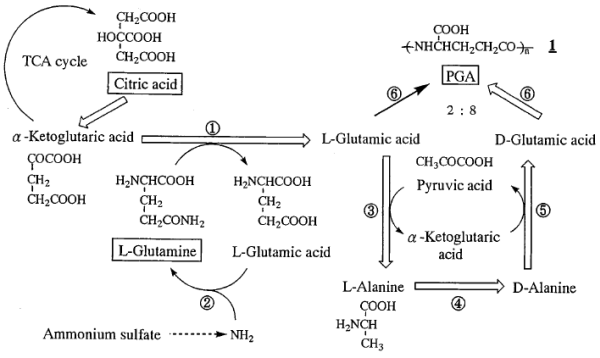

Fig. 1. γ-poly(glutamic acid) (PGA) synthesis in *Bacillus subtilis* IFO3335: 1, glutamine: 2-oxoglutarate aminotransferase, 2, glutamine synthetase; 3, L-glutamic acid: pyruvic acid aminotransferase; 4, alanine racemase; 5, D-glutamic acid: pyruvic acid aminotransferase; 6, PGA polymerase; TCA, tricarboxylic acid [10]

γ-PGA's characteristics enable its wide application as a biodegradable thickener, humectant, sustained release material and drug carrier in the food, cosmetic medical [1], [10], [18] and pharmaceutical industries [3]. γ-PGA and its derivatives can potentially be used as substitutes for petroleum-based hydrogels and thermoplastic polymers because γ -PGA is highly water-absorbent and biodegradable [19].

γ -PGA production by bacteria has diverse nutritional requirements [1], [10]. Some microorganisms require different carbon sources, others have unknown nitrogen sources and many of these do not require glutamic acid. Other bacteria grow only in the presence of glutamic acid [10] and some require biotin to produce γ -PGA [1], [10]. Reference [1] found that glutamic acid was not assimilated when added to the medium. The glutamic acid subunit in γ -PGA is produced from citric acid and ammonium sulphate. γ -PGA production is significantly induced by L-glutamate and the presence of L-glutamate results in the synthesis of highly elongated chains [20].

This paper reports the effect of molasses, citric acid and ammonium sulphate concentrations on γ-PGA production by *Bacillus velezensis* NRRL B – 23189 without the addition of glutamic acid to the fermentation medium. Molasses sugar consumption was also studied.

II. Materials and Methods

II.1. Bacterial Strain and Medium

Bacillus velezensis NRRL B – 23189 from the ARS Culture Collection (NRRL) was used in the study. BHI medium (brain heart infusion) with 20 g/l of bacteriological agar, both from Oxoid, was used for culture maintenance. Bacterial cells were incubated in agar slants at 32°C for 48 h and stored at 4 °C.

A medium reported by Bajaj and co-authors (2009) was modified for use in γ -PGA production as follows (g/l): K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.2; $MnSO_4.7H_2O$, 0.05. The molasses, citric acid and ammonium sulphate concentrations used are shown in Table I. The initial pH was adjusted to 6.5 by using 6 N NaOH, 2 N NaOH and/or 2 N HCl. The medium was sterilised in an autoclave for 15 min at 121 °C.

II.2. Inoculum and Fermentation

The inoculum was produced as described below. A loopful of cells from a slant was collected and transferred to 50 ml of BHI medium in a 250-ml conical flask and incubated at 32 °C and 200 rpm for 12 h in a shaking incubator (TECNAL, mod. TE 421).

Fermentation was carried out in 250-ml Erlenmeyer flasks, each containing 45 ml of the sterile production medium. The medium was inoculated with 10 % (v/v) of a 12-h-old *Bacillus velezensis* NRRL 23189 culture containing approximately 2×10^{-3} g cells/ml BHI medium. The flasks were incubated for 72 h on a rotary shaker at 27 ± 2 °C and 200 rpm. Each fermentation assay was repeated twice. The samples were collected at the end of 72 h of fermentation for γ -PGA and residual sugar quantifications.

II.3. Surface-Response Methodology

The surface-response methodology (SRM) was used to obtain a model for γ-PGA production and sugar consumption.

A Central Composite Design (CCD) was used to formulate a medium that provides optimal support for γ -PGA production by *Bacillus velezensis*.

The factorial planning had 4 central points and yielded a total of 18 treatments. The factors and levels studied are described in Table I.

TABLE I
FACTORS AND LEVELS STUDIED IN THE CCD WITH 18 OBSERVATIONS

Factors	Factor level*[2]		
	-1	0	+1
Molasses - 54 % TRS*[1] (x1)	140.5	200	259.5
Citric Acid (x2)	5.06	12.5	19.94
Ammonium Sulfate (x3)	4	8	12

*[1]TRS: Total reducing sugars.
*[2] Components (g/l).

The results were analysed using Statistic 5.0 software.

When several factors are involved, the model can be expressed as follows:

$$Y = bo + \sum_{i=1}^{4} bi\ xi + \sum_{i=1}^{4} bii\ xi 2 \sum_{i \neq j=1} \sum bij\ xi\ xi \qquad (1)$$

where *bo*, *bi*, *bii* and *bij* are the intercept term, linear, quadratic coefficient and interactive coefficient, respectively, and *xi* and *xj* are coded independent variables.

II.3.1. Determination of γ -PGA and Residual Sugar From Molasses

Cells were separated from the broth by centrifugation for 20 min at 2300 g (Excelsa Baby II, FANEM, model 206-R). The supernatant was collected for determination of the γ-PGA and residual sugar concentrations.

The γ-PGA concentrations were determined according to the method reported by [21].

The residual sugar concentration was monitored turbidometrically at 540 nm according to the method reported by [22].

III. Results and Discussion

Table II shows γ-PGA production and sugar consumption.

Reference [2] and reference [1] showed that when the glycerol concentration or other carbon source was increased in fermentation broth, a large amount of γ -PGA production occurred. When molasses was used as the carbon source, increased concentrations did not increase γ-PGA biosynthesis. When the concentration of molasses was 200 g/l or more, a decrease in γ-PGA production occurred.

An important observation is that sugar consumption was high when γ-PGA production was high.

TABLE II
Γ-PGA PRODUCTION AND SUGAR CONSUMPTION

Treatments	Factors			γ -PGA ($g.l^{-1}$)	TRS* ($g.l^{-1}$)
	x1	x2	x3		
1	-1(140.5)	-1(5.06)	-1(4)	2.284	35.205
2	+1(259.5)	-1(5.06)	-1(4)	2.036	86.804
3	-1(140.5)	+1(19.94)	-1(4)	4.444	27.094
4	+1(259.5)	+1(19.94)	-1(4)	3.700	74.034
5	-1(140.5)	-1(5.06)	+1(12)	2.319	30.200
6	+1(259.5)	-1(5.06)	+1(12)	2.674	84.215
7	-1(140.5)	+1(19.94)	+1(12)	4.550	36.413
8	+1(259.5)	+1(19.94)	+1(12)	2.355	109.929
9	-1.68(100)	0(12.5)	0(8)	3.736	14.496
10	+1.68(300)	0(12.5)	0(8)	3.665	102.853
11	0(200)	-1.68(0)	0(8)	3.063	47.630
12	0(200)	+1.68(25)	0(8)	3.559	35.205
13	0(200)	0(12.5)	-1.68(1.28)	4.338	29.683
14	0(200)	0(12.5)	+1.68(14.72)	3.700	39.347
15	0(200)	0(12.5)	0(8)	4.904	27.612
16	0(200)	0(12.5)	0(8)	4.621	22.780
17	0(200)	0(12.5)	0(8)	5.117	26.231
18	0(200)	0(12.5)	0(8)	4.630	24.160

*TRS: Total reducing sugars

TABLE III
REGRESSION COEFFICIENTS OF CCD

Factor	Regression Coefficients	Pure error	t	p	95 % Confidence interval	
					Lower bound	Upper bound
Interceptation/Mean	4,845	0,316	15,353	0,000	4.117	5.573
Molasses (L*)	-0,216	0,171	-1,263	0,242	-0.611	0.178
Molasses (Q*)	-0,517	0,178	-2,908	0,020	-0.927	-0.107
Citric Acid (L)	0,481	0,171	2,813	0,023	0.087	0.876
Citric Acid (Q)	-0,655	0,178	-3,683	0,006	-1.064	-0.243
Ammonium Sulfate (L)	-0,120	0,171	-0,702	0,503	-0.514	0.274
Ammonium Sulfate (Q)	-0,404	0,1780	-2,275	0,053	-0.814	0.006
Molasses X Citric Acid	-0,381	0,223	-1,704	0,127	-0.896	0.135
Molasses X Ammonium Sulfate	-0,106	0,223	-0,474	0,648	-0.621	0.409
Citric Acid X Ammonium Sulfate	-0,239	0,223	-1,069	0,316	-0.754	0.276

*L = linear, Q = quadratic

The maximum γ-PGA production occurred in treatments 15, 16, 17 and 18. These treatments are central points at level 0, and correspond to 200 g/l molasses, 12.5 g/l citric acid and 8 g/l ammonium sulphate. Assays 3, 7 and 13 showed similar γ-PGA production but with different media compositions.

The minimum γ-PGA production occurred in trials with low molasses, citric acid and/or ammonium sulphate concentrations or in which all the concentrations were maximum (Figs. 2, 4, 6). An equation for γ -PGA production was developed based on a regression analysis of the experimental data:

$$Y = 4.845 - 0.216x1 - 0.517x1^2 + 0.481x2 + \\ -0.655x2^2 - 0.120x3 - 0.404x3^2 - 0.381x1 \cdot x2 + \\ -0.106x1 \cdot x3 - 0.239x2 \cdot x3 \quad (2)$$

Eqn. (2) produced results that were highly significant ($P < 0.05$), as shown in Table III, and the value of the coefficient of determination (R^2=0.810) was satisfactory.The factor effect that more influences the γ-PGA production was citric acid ($p < 0.05$). In general, all effects present little influenced on the γ-PGA production.

An equation for sugar consumption was developed based on a regression analysis of the experimental data:

$$Y = 24.497 + 27.434x1 + 14.960x1^2 + \\ +8.858x2^2 + 6.418x3^2 \quad (3)$$

Eq. (3) produced results that were highly significant ($P < 0.10$) (Table IV), and the value of the coefficient of determination ($R^2 = 0.897$) was satisfactory.

TABLE IV
REGRESSION COEFFICIENTS OF THE CCD

Factor	Regression Coefficients	Pure error	t	p	90 % Confidence interval	
					Lower bound	Upper bound
Interceptation/ Mean	24.498	5.471	4.478	0.0006	14.809	34.186
Molasses (L*)	27.434	2.965	9.252	0.0000	22.183	32.686
Molasses (Q*)	14.960	3.081	4.855	0.0003	9.503	20.416
Citric Acid (Q)	8.858	3.081	2.875	0.0130	3.402	14.315
Ammonium Sulfate (Q)	6.418	3.081	2.083	0.0576	0.962	11.874

*L = linear, Q = quadratic.

All factors shown in Table IV influenced sugar consumption ($p < 0.10$). The effects of molasses, citric acid and ammonium sulphate on γ-PGA production and residual sugar concentrations are shown in Figures 2-7, respectively. The non-explicit variables were fixed at the central point (level 0) for the surface construction.

Maximum γ-PGA productivity was obtained using 4 different formulations of the initial concentrations of molasses, citric acid and ammonium sulphate. The figures show that maximum γ-PGA production occurred when the initial concentrations of molasses, citric acid and ammonium sulphate were between 140 and 200 g/l, 12.5 and 19.94 g/l, 4 and 12 g/L, respectively.

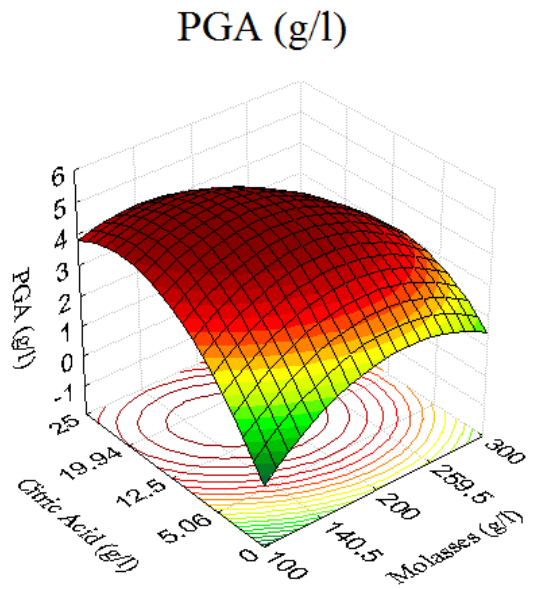

Fig. 2. Surface-response plot for the effect of molasses (x1) and citric acid (x2) on γ-PGA production

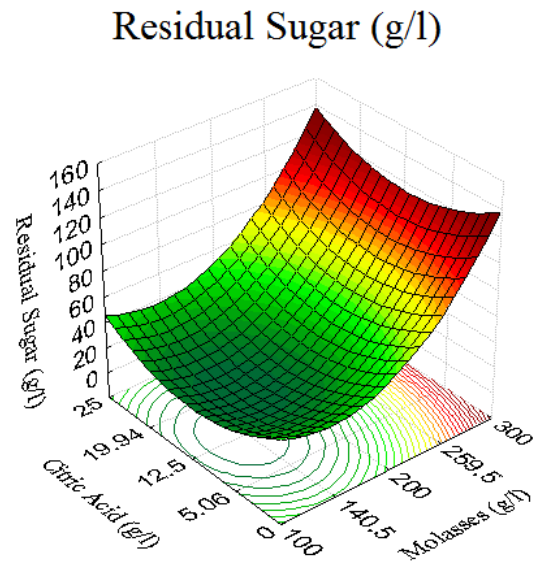

Fig. 3. Surface-response plot for the effect of molasses (x1) and citric acid (x2) on sugar consumption. The molasses contained 54% total reducing sugar

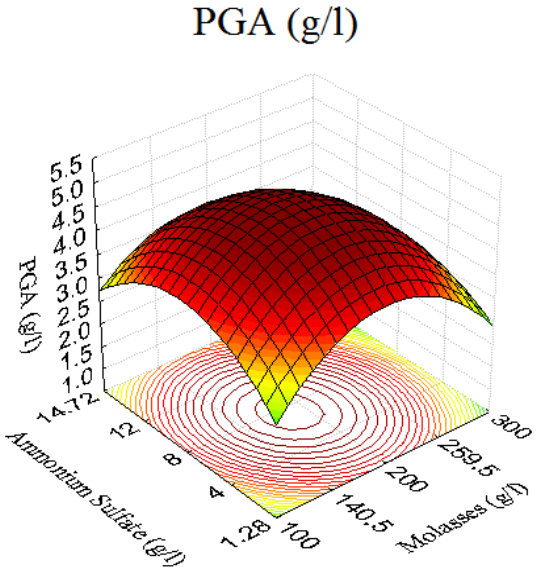

Fig. 4. Surface-response plot for the effect of molasses (x1) and ammonium sulfate (x3) on γ -PGA production

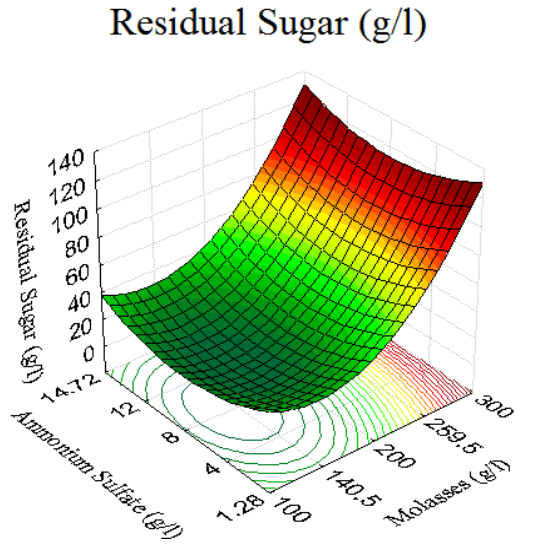

Fig. 5. Surface-response plot for the effect of molasses (x1) and ammonium sulfate (x3) on sugar consumption. The molasses contained 54% total reducing sugar

The maximum γ-PGA production was obtained when sugar consumption was at his maximum.

To choose the best result, assays 3, 7, 13 and 15 were duplicated. The results for γ-PGA production were analysed using the Tukey test 5% with Statistic 5.0 software. The results are shown in Table V.

TABLE V
Γ-PGA PRODUCTION BY *BACILLUS VELEZENSIS* NRRL B - 23189, AT 27 °C FOR 72 H, INITIAL PH 6.5 AND 200 RPM

Treatments	γ -PGA ($g.L^{-1}$)
3	4,249 ± 0,351[a]
7	4,391 ± 0,050[a]
13	4,338 ± 0,351[a]
15	4,798 ± 0,200[a]

Matching letters indicate samples that did not differ statistically, and different letters indicate samples that differed at the 95 % confidence level

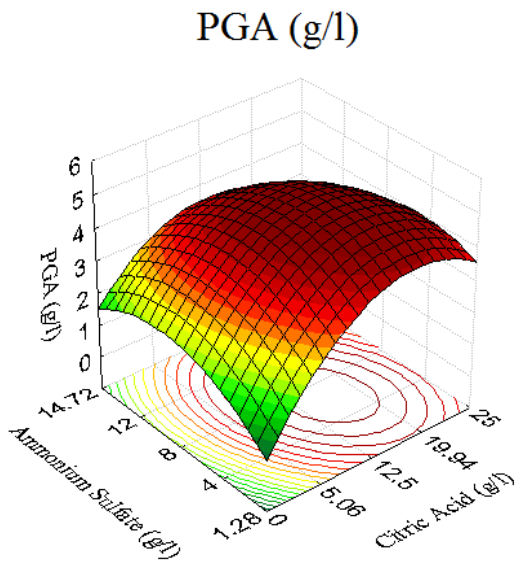

Fig. 6. Surface-response plot for the effect of citric acid (x2) and ammonium sulfate (x3) on γ -PGA production

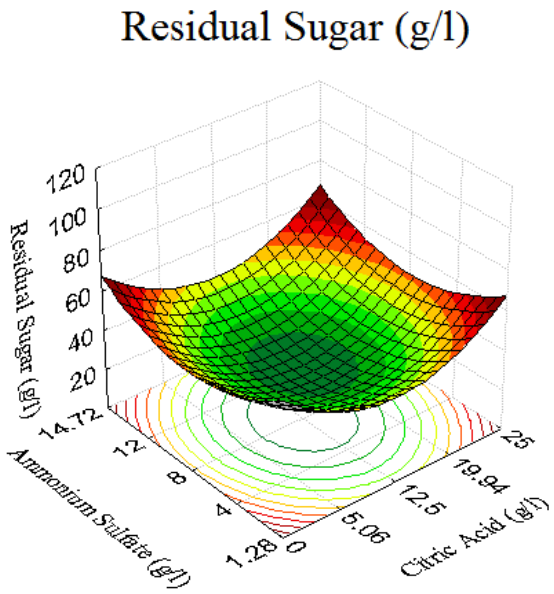

Fig. 7. Surface-response plot for the effect of citric acid (x2) and ammonium sulfate (x3) on sugar consumption. The molasses contained 54 % total reducing sugar

These experiments show that the amounts of molasses, citric acid and ammonium sulphate in the culture medium are important determinants for γ-PGA productivity by *Bacillus velezensis* NRRL 23189.

Increased concentrations of the carbon source increased the γ-PGA production only at the beginning of the fermentation, contrary to the observations of some references [2] and [16]. At concentrations of molasses, citric acid and ammonium sulphate above 200, 19.94 and 12 g/l, respectively, there was no increase in γ-PGA production. Several bacterial strains have been recently studied for γ-PGA production. Reference [21] reported a maximum productivity of 10.4 g/l using *Bacillus subtilis* BL53 cultivated at 37°C for 96 h. Reference [11] reported a maximum productivity of 46.4 g/l using *Bacillus subtilis* ZJU-7 cultivated at 37 °C for 48 h. In other reported work [23], γ-PGA was produced using *Bacillus licheniformis* NCIM 2324 cultivated at 37°C for 96 h with maximum productivity of 26.12 g/l. *Bacillus velezensis* NRRL 23189 produces γ-PGA in media without glutamic acid, which is an advantage when compared with other *Bacillus* strains. This fact is important for industrial cost reduction because glutamic acid is a fermentation product, and the cost of molasses is cheaper than that of the purified sugars.

IV. Conclusion

Molasses, citric acid and ammonium sulphate in a fermentation medium without glutamic acid yielded γ -PGA production in batch fermentations using *Bacillus velezensis* NRRL 23189. A maximum γ -PGA concentration of approximately 4.8 g/l was achieved with 4 different formulations of the fermentation medium that also showed maximum sugar consumption. The production of γ-PGA without glutamic acid in the culture medium is advantageous.

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