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Production and optimization of poly-γ-glutamic acid by *Bacillus subtilis* BL53 isolated from the Amazonian environment

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Abstract The aims of this research were to screen and characterize a new microbial source of γ-PGA, to optimize aspects of culture conditions and medium composition using central composite design and response surface methodologies. The influence of bioreactor stirring rates on the production of γ-PGA was also investigated and the oxygen volumetric mass transfer coefficients ($k_L a$) were established. The most productive strain was identified by 16S rDNA analysis as *Bacillus subtilis*, and its γ-PGA production in rotatory shaker was threefold increased under optimized conditions (37 °C, pH 6.9, and 1.22 mM Zn^{2+}), compared to conventional medium. In bioreactor, the γ-PGA production was further increased, reaching 17 g l^{-1}, 70 % higher than shaker cultures. γ-PGA production showed high dependency on oxygen transfer. At $k_L a$ of 210 h^{-1}, the cultivation time could be reduced to 48 h, about 50 % of the time required for operations at $k_L a$ 55 h^{-1}.

Keywords Poly-gamma-glutamic acid · *Bacillus subtilis* · Biopolymer · Stirring rate · Oxygen volumetric transfer rate

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Introduction

Poly-gamma-glutamic acid (γ-PGA) is a homo-polyamide of D- and L-glutamic acid units [1]. This polymer presents γ-amide linkages between α-amino and γ-carboxylic groups as opposed to α-amino linkages in proteins. Consequently, this polyamide is resistant to proteases [2]. γ-PGA is water soluble, biodegradable, edible, biocompatible, non-toxic, and non-immunogenic. Applications of this biopolymer and its derivatives have been widely reported for food, chemical, medical, and environmental-related industries, as flocculants in water and wastewater treatment, thickener, cryoprotectant and functional ingredient in food processing, and as an anticancer drug-release agent in medicine [1, 3–8], but these applications are still very limited due to the high cost of γ-PGA.

γ-PGA was first identified 70 years ago as a component of the *Bacillus anthracis* capsule [1, 9]. Further studies have also shown its presence in extracellular viscous material of other non-pathogenic *Bacillus* species such as *B. subtilis*, *B. licheniformis*, *B. megaterium*, and *B. halodurans* [1, 9–11]. A few Gram (+) bacteria, archeobacteria, and some eukaryotes can also synthesize γ-PGA [2, 3]. γ-PGA biosynthesis is a membrane-associated process, which requires ATP and glutamic acid as a substrate and cations as cofactors. The enzymatic complex PgsBCA (polyglutamate synthetase) is responsible for the polymerization and transport of γ-PGA through the cellular membrane [2, 3]. The role of PgsBCA complex was described in detail by Ashiuchi and co-workers [11–13]. Initially, glutamic acid binds to the ATP molecule, which is converted to ADP providing energy for the polymerization. PgsBCA acts as an amide-ligase, the subunits PgsB and PgsC forming a catalytic site, while the subunit PgsA removes the elongated chain from the active site allowing for the addition of

a new monomer. According to Ashiuchi et al. [13], Mg^{2+} is essential, and Zn^{2+} acts as a booster to PgsBCA activity in vitro.

In recent years, the demand for environmentally safe materials, especially those obtained from renewable resources, have increased the interest for γ-PGA biosynthesis and its applications [4, 8, 14]. *Bacillus* γ-PGA producers have been isolated from soil and traditional foods, mainly in Asian countries [12, 15–20]. Some researchers have investigated the influence of nutritional and process parameters on γ-PGA production in order to seek strategies to increase yield of production to allow cost-effective industrial production of this polymer.

The media formulation for the production of γ-PGA is still one of the major industrial problems, mainly due to cost. The glutamic acid monomers incorporated in γ-PGA can be obtained from culture medium or can be synthesized from precursors derived from tricarboxylic acid (TCA) cycle by microorganisms [1, 3]. Medium "E", developed by Leonard and co-workers [21], is frequently used to grow *Bacillus* for γ-PGA production in submerged cultivation [22–25]. Glycerol, citric acid and L-glutamic acid as medium components are reported as necessary for biomass and γ-PGA production [22–28]. Citric and L-glutamic acids act as precursors of polymer [1, 3, 26], while glycerol acts as a co-substrate [27].

The analysis of the influence of oxygen transfer mechanism is of fundamental importance in aerobic bioprocesses, especially those involving the production of biopolymers that change the rheological properties of culture broth over time, and is regarded as the key factor for understanding and optimizing these processes. However, relatively few studies have investigated the influence of the oxygen mass transfer on the production of γ-PGA [26, 29, 30], and the optimal oxygen volumetric transfer rates for γ-PGA production have never been reported.

In this context, the aims of this research were to identify new microbial sources of γ-PGA, characterize them, and to apply the central composite design and response surface methodology in order to optimize culture conditions in terms of pH, temperature, and Zn^{2+} concentration to enhance γ-PGA production. Furthermore, we scaled up the γ-PGA cultivation in bioreactors, evaluated the influence of stirring rate over the oxygen uptake rate, oxygen volumetric mass transfer coefficient, cell viability and production and productivity of γ-PGA.

Materials and methods

Microorganisms

A total of 87 different bacterial strains of our own collection composed of isolates from the aquatic and dumped soils of the Amazonian environment in Brazil were pretested for biopolymer production. Six of them, named as BL15, BL16, BL32, BL53, BL62, and BL74, showed highly viscous culture broths and were chosen for γ-PGA production screening. These strains were kept as lyophilized reference stocks in the Culture Collection of the Microbiology Department of The Federal University of Rio Grande do Sul State, Brazil. For working purposes, isolates were kept in 20 % glycerol at −20 °C. Bacteria isolates were reactivated twice in Luria–Bertani (LB) broth, streaked onto LB agar plates and subcultured onto LB slants. Strain slants were kept under refrigeration until usage.

Cultivation medium

Microorganisms were cultivated in medium E (ME), which was composed of (in g l^{-1}) L-glutamic acid, 20; citric acid, 12; glycerol, 80; NH_4Cl, 7; $MgSO_4·7H_2O$, 0.5; $FeCl_3·6H_2O$, 0.04; K_2HPO_4, 0.5; $CaCl_2·2H_2O$, 0.15, and $MnSO_4·H_2O$, 0.04 [21].

Inoculum preparation

The inocula preparation for cultivations were prepared by transferring a loopful of cells from LB slants into 50 ml LB broth in 250 ml Erlenmeyer flasks and incubated at 37 °C in a rotatory shaker at 180 rpm. The inocula were standardized to 1.0 ± 0.1 OD at 600 nm and added to cultivation medium at 4 % (volume fraction).

Screening γ-PGA-producing strains

Screening cultivations were carried out on medium E, with the final pH adjusted to 6.5 using either 1 M HCl or NaOH. Each strain was inoculated into 25 ml by cultivation on medium E in 125-ml Erlenmeyer flasks and incubated at 37 °C for 96 h in a rotatory shaker at 180 rpm. Concentration of γ-PGA in culture medium was measured and the results were subjected to analyses of variance (ANOVA) followed by Fisher LSD ($p < 0.01$). The ANOVA and LSD were performed with Statistica 7.0 (Statsoft Inc., Tulsa, USA). All shake experiments were run in triplicates.

Identification and characterization of γ-PGA producer strain

The best producer of γ-PGA was characterized and identified by 16S rDNA analysis and by morphological and physiological characteristics. DNA from a bacterial suspension equivalent to a McFarland 0.5 (200 μl) was extracted using the QIAamp DNA minikit (Quiagen®) following the manufacturer instructions. PCR reaction was

performed using the Platinun Taq Supermix (Invitrogen) and 10 μl of the extracted DNA in a final volume of 50 μl. The 16S rDNA gene was amplified using primers 285 and 261 under cycling conditions previously described [31]. PCR products were separated by agarose gel electrophoresis and the ethidium bromide-stained bands were visualized under UV light. After confirmation, the remaining PCR mix was purified using the AccuPrep(R) PCR Purification Kit (Bioneer) and submitted to direct sequencing using the BigDye Terminator kit (Applied Biosystems), as directed by the manufacturer instructions. Following sequencing, the nucleotide sequence was subjected to BLAST sequence analysis using the NCBI (National Center for Biotechnology Information) website, and the nucleotide sequence was deposited in GenBank database.

Morphological and biochemical characterization was performed according to the Manual of Bergey [32] and MacFadin [33], and also included additional references.

Optimization of culture parameters for γ-PGA production by response surface methodology (RSM)

The influence of temperature, initial culture pH, and concentration of Zn^{2+} supplemented to medium E were evaluated for the best producer of γ-PGA strain in experiments conducted with 25 ml of medium in 125-ml Erlenmeyer flasks in rotary shaker at 180 rpm. A 2^3 central composite design ($k = 3$, $\alpha = 1.68$), including 6 axial points and 3 replicates at the central point was designed, resulting in 17 runs, and the three culture parameters were optimized for γ-PGA production by response surface methodology (RSM). A second-order polynomial regression model was obtained from experimental design according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \qquad (1)$$

where Y is the response variable (γ-PGA production); β_0 is the constant; β_i, β_{ii}, and β_{ij} are the coefficients for the linear, quadratic, and interaction effects, respectively; X_i and X_j are the coded independent variables or factors.

Analysis of variance (ANOVA) was employed in order to evaluate the significance of the regression model. Significance of regression coefficients was determined by Student's t test and the variance explained by the model is given by the multiple determination coefficient, R^2. Experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft Inc., Tulsa, USA).

The time course of γ-PGA production was evaluated for 96 h using the optimal parameters obtained. Submerged cultures were conducted with 25 ml of medium in 125-ml Erlenmeyer flasks, incubated in rotatory shaker at 180 rpm, and samples were taken for quantification of γ-PGA, and count of viable cells.

Submerged cultivations in bioreactors

Submerged batch cultures were performed in a fully instrumented 5-l stirred tank bioreactor (Biostat B bioreactor, B. Braun Biotech International, Germany), under the optimized conditions using an aeration rate of 2.0 vvm (2 l of air/liters of medium/min) and stirring agitation of 500, 750, and 1,000 rpm. The cultivations were carried out without pH control. Assays were carried out with 2.5 l of culture medium including 4 % of inoculum in LB broth standardized to 1.0 ± 0.1 OD at 600 nm. A small amount of 0.2 % (volume fraction) of Antifoam 204 (organic non-silicone Emulsion, Sigma, USA) was added for foaming control. During the time course of cultivation, samples were taken for quantification of γ-PGA, dry biomass, and for count of viable cells. All bioreactor experiments were run in duplicates.

Determination of growth kinetic parameters

Curves of viable cell growth were fitted to the Logistic Model shown in Eq. 2 [34] by using the OriginPro 8.0 software (OriginLab, USA).

$$X = \frac{X_{\max}}{1 + e^{-\mu_{\max}(t-t_c)}} \qquad (2)$$

where, X is the total count of viable cells (CFU ml^{-1}) and t is time (h). The parameters $X_{\max}$, $\mu_{\max}$ and t_c are obtained from the experimental data fit. $X_{\max}$ represents the maximum amplitude of X in the stationary phase plateau, $\mu_{\max}$ is the maximum specific growth rate (exponential phase) and t_c is the time in which $X = X_{\max}/2$.

Determination of OUR, Q_{O2} and k_La

Dissolved oxygen concentration (DOC) in the culture broth was measured using a polarographic electrode (Mettler-Toledo, Germany), and was expressed as percentage of oxygen saturation. An acquisition board connected to a computer running MATLAB (Mathworks, USA) was used to acquire data of dissolved oxygen. Oxygen uptake rate (OUR), specific rate of oxygen consumption (Q_{O2}), and volumetric oxygen transfer coefficient (k_La) were determined following the dynamic gassing-out method [35]. The parameters OUR, Q_{O2}, and k_La were calculated within the exponential growth phase (at 3, 6 and 8 h), except for 500 rpm, which was calculated only at 3 h of run because dissolved oxygen was very low after this time.

Analytical methods

The amount of γ-PGA was determined by spectrophotometric method based on the complexation reaction of γ-PGA with cetylmethylammonium bromide (CTAB) [36]. In this method CTAB binds very specifically to γ-PGA, and form a water-insoluble, highly dispersed micelle-like complex, resulting in an increase in turbidity, which is measured at 400 nm [36, 37]. γ-PGA (MM of 70–100 kDa, Sigma-Aldrich, USA) was used as spectrophotometric standard. Procedure for polymer extraction from culture medium was as follows. Trichloroacetic acid solution was added to samples until pH was reduced to 3.0 in order to separate cells and proteins [22, 36]. The mixture was kept at 50 °C for 30 min, and centrifuged at $15,000g$, 15 °C for 30 min. The supernatant was collected, the pH adjusted to 7.0 ± 0.1 and five volumes of ethanol were added to samples. A second centrifugation was conducted at $15,000g$, 4 °C and 30 min. The pellet was dissolved in buffer solution pH 7.0 ± 0.1 and the complexation reaction was carried out.

Biomass was determined as dry cell weight by centrifugation of the culture medium at $3,000g$ for 20 min at 4 °C; cell pellets were twice washed and dried at 75 °C until constant weight. The viable cells were determined by direct plate count procedure using the spread plate technique. After successive decimal dilutions of the cultivation broth, the adequate dilutions were spread on to Agar Plate Count Medium and incubated at 37 °C for 18 h.

Results and discussion

Screening and identification of γ-PGA-producing strains

Table 1 shows the γ-PGA production by BL15, BL16, BL32, BL53, BL62, and BL74 strains in ME after 24, 72 and 96 h. It can be observed that all strains had the ability to produce γ-PGA, except for BL32. The highest productions were obtained for BL53 and BL74 strains. BL53 produced approximately 4 g l^{-1} γ-PGA in 72 h of cultivation. As a comparison, for a well-studied strain B. licheniformis CCRC 12826, Shih and co-workers [24] obtained 5.27 g l^{-1} of γ-PGA, but only after 96 h of cultivation in medium E under similar cultivation conditions. After 96 h all strains showed a decline on γ-PGA production, probably due to PGA depolymerase enzymes, which could decrease the yield of γ-PGA during stationary phase [3]. Results also showed that BL53 produced significantly more γ-PGA at early growth stages when compared to BL74 strain. The strain identification showed that BL74 could be morphologically and physiologically

identified as B. anthracis. Consequentially, BL74 was discharged and only BL53 strain was selected for further experiments.

The 16S rDNA sequence obtained from BL53 strain was deposited in NCBI GenBank database under the accession number JQ359757. This nucleotide sequence was compared to available sequences at NCBI GenBank database, and revealed 100 % of similarity with several B. subtilis and Bacillus amyloliquefaciens strains, and also to some Bacillus polyfermenticus and Bacillus siamensis. Taxonomic studies have indicated that Bacillus species are very similar to each other with respect to 16S rDNA, and B. amyloliquefaciens is indeed closely related to B. subtilis on the basis of 16S–23S ITS nucleotide sequences [38–40]. Nevertheless, some physiological characteristics allowed distinguishing among these species [32, 40–44]. The morphological and physiological characterization of BL53 strain showed that it is aerobic, rod-shaped, spore-forming, catalase-positive, Gram(+) bacterium, capable of producing acid from glucose, as a typical Bacillus sp. [32]. The positive citrate reaction, negative oxidase test, growth at 50 °C, no acid formation from lactose and sorbitol, and growth in NaCl 7 % but not in NaCl 10 % allowed for differentiating from other Bacillus species and for the identification of BL53 as B. subtilis. All morphological and physiological characteristics for BL 53 strain are shown in Table 2.

Optimization of cultivation using the CCD and RSM

Although previously described for the production of γ-PGA, ME has not been statistically optimized for cultivation conditions such as temperature, pH, and the influence of Zn^{2+} concentration. The Zn^{2+} cation was pointed out by Ashiuchi and co-workers [13] as an important factor in the enzymatic in vitro synthesis of γ-PGA, however, this cation is not present in the composition of the ME. Moreover,

Table 1 Screening of γ-PGA producers in medium E, pH 6.5 at 37 °C, in shaker at 180 rpm for 24, 72 and 96 h

Strains	γ-PGA production (g l^{-1})		
	24 h	72 h	96 h
BL15	0.11 ± 0.01^d	2.09 ± 0.06^c	0.02 ± 0.02^d
BL16	0.68 ± 0.00^c	1.46 ± 0.17^d	0.04 ± 0.06^d
BL32	0.13 ± 0.10^d	0.05 ± 0.00^f	0.07 ± 0.01^d
BL53	1.63 ± 0.06^a	3.67 ± 0.18^a	2.63 ± 0.13^b
BL62	0.12 ± 0.01^d	0.79 ± 0.41^e	1.19 ± 0.01^c
BL74	1.11 ± 0.05^b	3.18 ± 0.59^b	3.11 ± 0.05^a

Results are mean $\pm$ SD of three runs. Means with different letter in the same column are significantly different from each other ($p < 0.01$)

Table 2 Physiological and morphological characterization of *Bacillus subtilis* BL53

Characteristics	
Shape	Rod
Gram stain	+[a]
Sporulation	+
Nitrate reduction	+
Hydrolysis of	
Starch	+
Gelatin	+
Indole formation	−[b]
Voges-Proskauer test	+
Citrate utilization	+
Catalase test	+
Oxidase test	−
Phenylalanine desaminase	−
Acid from	
Glucose	+
Lactose	−
Xylose	−
Raffinose	−
Mannitol	+
Sorbitol	−
Growth at 50 °C	+
Growth in 7 % NaCl+	+
Growth in 10 % NaCl+	−

[a] Positive

[b] Negative

since *B. subtilis* BL53 is a new strain for bioprocess, it is important to assess its physiological characteristics to define optimal operating conditions. Therefore, the influence of temperature, initial pH of cultivation, and Zn^{2+} concentration on γ-PGA production by *B. subtilis* BL53 was investigated using a 2^3 central composite design (CCD). Table 3 shows the coded and actual values of 3 factors and the observed γ-PGA production for each run at 72 h of cultivation, time of the highest production. The effects estimation showed that the negative quadratic effect of initial pH was the most significant ($p = 0.067$) on γ-PGA production, followed by the positive linear effect ($p = 0.115$) and negative quadratic effect ($p = 0.118$) of Zn^{2+} concentration. The strong influence of pH was reported for some other *Bacillus* strains. Richards and Margaritis [45] reported the highest production of γ-PGA for pH between 6.5 and 7.5, for *B. subtilis* IFO 3335 strain, growing at 37 °C. Cromwick and co-workers [26] observed the strong influence of pH on the production of γ-PGA by *B. licheniformis* ATCC 9945A, correlating it to cell viability and to the preferential use of citrate via TCA cycle. According to these authors, the consumption of glycerol

and glutamic acid remained approximately constant under any pH range, but the consumption of citrate was increased under pH 6.5. Wu and co-workers [46] showed that the glutamic acid uptake by cells was also dependent on the medium pH, and reported the best production of γPGA at pH 6.5–7.0, suggesting that the influence of pH might be due to the participation of H^+ in the transport driving force of glutamate throughout membrane.

In order to determine the optimal values for the 3 studied variables, a second-order model was obtained from regression of CCD data. All coefficients were considered in the fitted model presented in Eq. (1), except for X_1X_2 (Zn^{2+} concentration–initial pH interaction), and X_2X_3 (temperature–initial pH interaction), which were the least significant effects. Equation 1 can now be rewritten as:

$$Y = 12.0 - 1.01\,X_1 + 2.10\,X_2 - 0.884\,X_3 - 1.62\,X_1^2 - 2.27X_2^2 - 3.13\,X_3^2 - 2.44\,X_1X_2 \tag{3}$$

where Y is the response variable (γ-PGA production, g l^{-1}), X_1, X_2 and X_3 are the coded independent variables temperature, Zn^{2+} concentration and initial pH, respectively. The analysis of variance demonstrated that the model is highly significant, as evidenced by Fisher's F test with very low p value ($p = 0.0024$), and presenting insignificant lack of fit ($p = 0.84$). The determination coefficient R^2 was 0.87, indicating that 87 % of variation was explained by the model. These results suggest that the proposed model satisfactorily represents the phenomenon under analysis. The fitted response for the regression model is plotted in Fig. 1, showing the contour plots for each two variables. The maximal predicted γ-PGA production was determined by the fitted model as 12.3 g l^{-1} when the temperature, initial pH, and Zn^{2+} concentration were 37 °C, 6.9, and 1.22 mM, respectively. The experimental validation of the model in shaker flasks produced 10.4 g l^{-1} of γ-PGA.

Ashiuchi et al. [12] demonstrated that the optimal pH for the in vitro synthesis of γ-PGA by PgsBCA complex is around 7.0, using enzyme-associated membrane from *B. subtilis* subspecies *chungkookjang*. Richards and Margaritis [29] showed that optimal pH for γ-PGA production by *B. subtilis* IFO 3335 was also 7.0. Finally, for *B. licheniformis* ATCC 9945, optimum pH was defined as 6.5 [26]. These studies performed using the traditional 'one-factor-at-a-time' technique without verifying the interaction among factors, which is only possible in factorial designs. Shih, Van, and Chang [24] used CCD to evaluate medium composition and initial medium pH. Nevertheless, the chosen range of pH was very narrow (6.0–7.0), resulting in the exclusion of pH from the design due to its low significance.

Table 3 Central composite design and observed γ-PGA yield at 72 h by *Bacillus subtilis* BL53

Run	Coded factors			Actual factors			γ-*PGA* (g l^{-1})
	X_1	X_2	X_3	Temperature (°C)	Zn^{2+} (mM)	Initial pH	
1	−1	−1	−1	32	0.70	6.0	8.86
2	−1	−1	+1	32	0.70	8.0	9.60
3	−1	+1	−1	32	1.40	6.0	10.0
4	−1	+1	+1	32	1.40	8.0	8.91
5	+1	−1	−1	42	0.70	6.0	5.10
6	+1	−1	+1	42	0.70	8.0	5.24
7	+1	+1	−1	42	1.40	6.0	11.0
8	+1	+1	+1	42	1.40	8.0	9.53
9	−1.68	0	0	29	1.05	7.0	9.75
10	+1.68	0	0	45	1.05	7.0	9.52
11	0	−1.68	0	37	0.46	7.0	7.60
12	0	+1.68	0	37	1.64	7.0	9.80
13	0	0	−1.68	37	1.05	5.3	8.79
14	0	0	+1.68	37	1.05	8.7	6.20
15 (C)	0	0	0	37	1.05	7.0	10.4
16 (C)	0	0	0	37	1.05	7.0	13.2
17 (C)	0	0	0	37	1.05	7.0	12.4

γ-PGA mean ± SD at central point: 12.0 ± 1.44 g l^{-1}, X_1 temperature, X_2 Zn^{2+} concentration, X_3 initial pH, (C), Central point of CCD

Although initial pH played an important role in the γ-PGA production by *B. subtilis* BL53 and other *Bacillus* strains, the experiments in this study indicated that strict control of pH was not necessary, since pH tends to buffer around 7.0–7.5 (data not shown), which are optimal values for γ-PGA production after 24 h of cultivation. Therefore, *B. subtilis* BL53 cultures contrasted with that of other strains, as reported for *B. licheniformis* CCRC 12826 [23], where the initial pH of 6.5 dropped to 5.6 after 36 h of cultivation at 37 °C. Cromwick and co-workers [26] reported that in the cultivation of *B. licheniformis* ATCC 9945, the pH was acidified from 7.4 to 5.6 between 48 and 96 h, at 37 °C.

As shown on the analysis of the effects (Table 3), Zn^{2+} concentration was a relevant factor in the production of γ-PGA by *B. subtilis* BL53. Ashiuchi et al. [13] observed that the Zn^{2+} was able to boost the action of Mg^{2+} on activation of complex PgsBCA responsible for γ-PGA enzymatic synthesis. Soliman and co-workers [17] using Plackett–Burman analysis to investigate γ-PGA production by a new strain of *B. licheniformis,* also identified Zn^{2+} concentration to have a positive effect on γ-PGA production, but it was negative for biomass formation. Negative effects Zn^{2+} concentration on biomass was not evidenced for *B. subtilis* BL 53 (data not shown).

Kinetics of *B. subtilis* BL53 cultivation

In order to evaluate the kinetics of *B. subtilis* BL53 cultivation and the effects of small scaling-up of the process,

cultivations were performed in Erlenmeyer flasks (125 ml) and 5 l stirred tank bioreactors, with results shown in Fig. 2. For the bioreactor, aeration rate of 2 vvm and stirred agitation of 500 rpm were used to improve aeration of the system and prevent the oxygen depletion, while in the rotatory shaker, 180 rpm was adopted. The number of viable cells rapidly increased after 6 h in bioreactors. At this time, γ-PGA began to be excreted to the medium broth. Kinetics of both shaker and bioreactor suggests that γ-PGA produced by *B. subtilis* BL53 was partially associated with cell growth, similar to what is reported for other *Bacillus* strains [18, 47, 48].

Shaker cultivations produced 10.4 g l^{-1} of γ-PGA in 72 h, while in the bioreactor a 70 % higher production (17 g l^{-1}) was obtained, strongly suggesting that aerobic metabolism is essential for this biopolymer production. Since γ-PGA is an extracellular polymer, the culture broth becomes viscous as cultivation time progresses, which may lead to oxygen limitation.

Effects of dissolved oxygen concentration, stirring rate, OUR, Q_{O2}, and k_La on cultivation for γ-PGA production

Dissolved oxygen concentration of medium was monitored during cultivation of *B. subtilis* BL53 for production of γ-PGA and results are shown in Fig. 3. The DOC declined sharply before 12 h of cultivation indicating high oxygen demand by cells during the exponential phase, exceeding
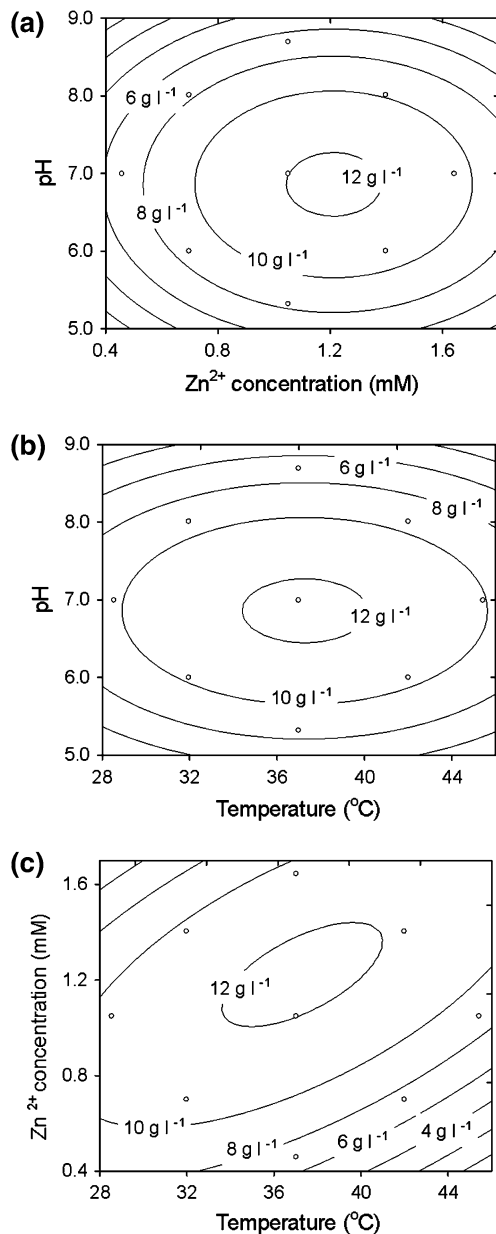
Fig. 1 Influence of independent variables on production γ-PGA at 72 h. **a** Initial pH and Zn^{2+} concentration, **b** initial pH and temperature and **c** Zn^{2+} concentration and temperature. Variables that are not plotted are fixed at their optimal value

Fig. 2 γ-PGA production by *Bacillus subtilis* BL53 under optimized CCD conditions in rotatory shaker at 180 rpm (*wingdings*), and in bioreactor at 500 rpm and 2 vvm (*filled circle*). Viable cells in rotatory shaker (*square*), and in bioreactor (*filled square*). Results are the mean of triplicates for shaker experiments and duplicates for bioreactor experiments

oxygen transfer capacity in most conditions, except for 1,000 rpm, when DOC was around 10 %. The results indicate the difficulty in cultivations with high γ-PGA concentrations, which directly affects oxygen mass transfer capacity of the system. Data in Table 4 show that there was an increase in oxygen demand (OUR) from 3 to 6 h in cultures performed at 750 and 1,000 rpm, with a subsequent reduction at 8 h. Similar results were demonstrated by Richards and Margaritis [29] in the first hours of cultivation of *B. subtilis* IFO 3335 in a bioreactor operating at
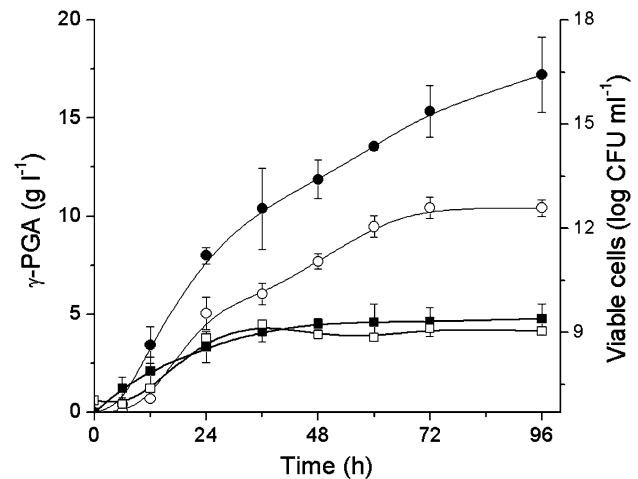
2 vvm and 700 rpm, however, the maximal OUR was 20 mmol O_2 l^{-1} h^{-1} and occurred later, after 10 h of cultivation. Normally, OUR of bacteria increases during the exponential growth phase because, at this stage, there is a high substrate consumption, decreasing thereafter due to reduced metabolic activity of cells as it approaches stationary phase [50]. The highest value of Q_{O2} for *B. subtilis* BL53 occurred at the beginning of exponential phase around 11–12 mmol O_2 g^{-1} h^{-1} for all conditions (Table 4). These values are within the range of Q_{O2} values for other biopolymers produced by microorganisms as reviewed by Garcia-Ochoa et al. [50], which are in the range between 0.2 and 31.2 mmol O_2 g^{-1} h^{-1}. In the production of xanthan gum by *Xanthomonas campestris* NRRL 1775, for example, Q_{O2} values ranged from 2 to 15 mmol O_2 g^{-1} h^{-1}, and the maximal value was observed after 5 h of cultivation [51]. Richard and Margaritis [29] also showed a peak of Q_{O2} at the beginning of cultivation of *B. subtilis* IFO 3335 during production of γ-PGA. Considering the cultivation time of 3 h, which corresponded to early-exponential growth phase, the increase in stirring rate from 500 to 1,000 rpm caused an increase of approximately four times on the value of k_La (Table 4). Bandaiphet and Prasertsan [52] observed the same effect in the production of an exopolysaccharide by *Enterobacter cloacae*, when the increase from 500 to 1,200 rpm caused an increase of approximately nine times in the value of k_La, while the increase in the aeration rate was far less effective.

Figure 4 shows the growth kinetics for *B. subtilis* BL53 at 37 °C in the bioreactor operating using aeration rate of 2 vvm at different stirring rates. For the three conditions, a

concentration of viable cells around 10^9 CFU ml^{-1} was reached after 36 h, with no loss of cell viability until the end of cultivation (96 h), even at high stirring rates. Similar results were reported by Cromwick and co-workers [26] for *B. licheniformis* ATCC 9945A cultures under milder aeration conditions (0.5 vvm and 250 rpm). In many cases, the oxygen transfer controls microbial growth and may affect the biological process [50]. As data in Table 5 show, the increase in the stirring rate produced a small increase in the specific growth rate of the logarithmic phase (μ_{max}), but cell viability was not affected, probably due to the shear protective effect of γ-PGA, also reported for mammalian cells [53]. The correlation between oxygen availability and microbial growth in the exponential phase can be perceived from data on Fig. 3 and the kinetic parameters in Table 5. As more abrupt was the decline in DOC in the first hours of cultivation, the lower was the specific growth rate, extending the exponential phase.

Figure 5 depicts the production of γ-PGA as a function of stirring rate. Results showed a positive effect of increased stirring rates on the productivity of the biopolymer, but did not affect its final concentration, which was kept around 17 g l^{-1} until 96 h. Cromwick and co-workers [26] were the first to investigate the influence of different conditions of aeration/agitation on the production of γ-PGA. These authors tested two different conditions (0.5 vvm/250 rpm and 1 vvm/800 rpm) for the cultivation of *B. licheniformis* ATCC 9945A and showed that the production of the polymer was twice as high at the higher aeration. Bajaj and Singhal [49] evaluated the effects of aeration and agitation on cultivations of *B. licheniformis* NCIM 2324, reporting higher concentrations of γ-PGA with increased agitation and, to a lesser extent, by
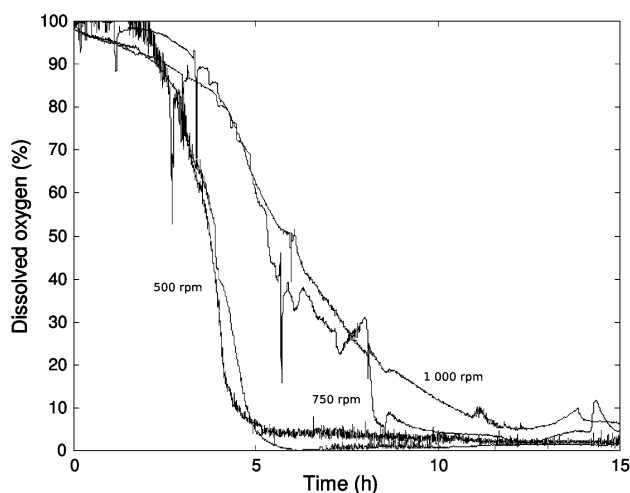
Table 4 OUR, Q_{O2} and k_La for *Bacillus subtilis* BL53 cultures carried out at 2 vvm, and 500, 750 or 1,000 rpm

Stirring rate (rpm)	Time (h)	Q_{O2}	OUR	K_La
500	3	12	1.5	55
750	3	12	0.97	93
750	6	12	3.4	72
750	8	0.35	0.49	38
1,000	3	11	0.80	210
1,000	6	3.4	6.8	190
1,000	8	0.22	1.9	170

OUR oxygen uptake rate (mmol O$_2$ g^{-1} h^{-1}), CV = 2.6 %

Q_{O2}, specific oxygen uptake rate (mmol O$_2$ g biomass^{-1} h^{-1}), CV = 1.8 %

k_La, volumetric oxygen transfer coefficient (h^{-1}), CV = 11 %

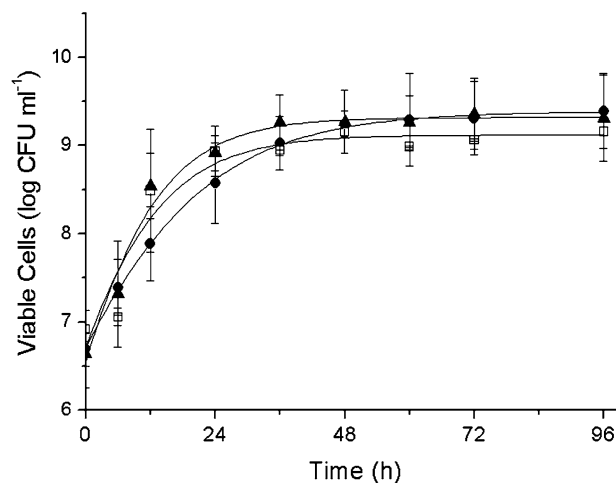CV, is the coefficient of variation calculated using data of experiments at 500 rpm

Fig. 4 Viable cells during γ-PGA production by *Bacillus subtilis* BL53, cultivation conducted at 2 vvm and stirring rates of 500 rpm (*filled circle*), 750 rpm (*square*), and 1,000 rpm (*filled triangle*). Results are the mean of duplicates and *curves* are obtained by fit to the Logistic Model in Eq. (2)

increased aeration. However, k_La was not calculated in those studies, so it is not possible to make further considerations regarding the transfer of oxygen. Comparing the γ-PGA production by *B. subtilis* BL53 (Fig. 5) to their respective k_La values for each stirring rate (Table 4), it can be observed that a higher k_La during exponential phase leads to a higher γ-PGA production. The k_La decreases along time cultivation, as a direct influence of γ-PGA concentration, an effect already observed for other biopolymers systems, such as xanthan gum [54].

The maximal productivity of γ-PGA, which was obtained at 1,000 rpm ($k_La = 170$ h^{-1}, middle exponential phase), was about 0.8 g l^{-1} h^{-1} (Fig. 6), exceeding the highest productivity obtained by Cromwick and co-workers [26] for *B. licheniformis* ATCC 9945A, and Bajaj and

Fig. 3 Dissolved oxygen concentration (expressed as percentage of medium saturation) during cultivation for production of γ-PGA by *Bacillus subtilis* BL53 under 2 vvm and different stirring rates: 500, 750, and 1,000 rpm

Table 5 Kinetic parameters ± SD from the Logistic Model fit for the data of viable cells

Stirring rate (rpm)	X_{max} (log CFU ml^{-1})	μ_{max} (h−1)	T_c (h)	R^2
500	9.39 ± 0.02	0.062 ± 0.002	14.7 ± 0.5	0.999
750	9.12 ± 0.14	0.095 ± 0.026	10.8 ± 3.8	0.904
1,000	9.32 ± 0.08	0.102 ± 0.077	8.4 ± 1.6	0.974

X_{max} viable cells, μ_{max} specific growth rate, t_c time at $X = X_{max}/2$, R^2 multiple determination coefficient

Singhal [49] for *B. licheniformis* NCIM 2324; it was similar to that reported by Richards and Margaritis [29] for *B. subtilis* IFO 3335. However, the highest productivity of γ-PGA from *B. subtilis* BL53 occurred before 24 h of cultivation, earlier than reported by the other authors. Using the strategy of increasing oxygen transfer through stirring rate, it was possible to reduce the time of process to <48 h at $k_L a$ of 210 h^{-1}, about 50 % of the time required to produce de same amount of γ-PGA operating at $k_L a$ of 55 h^{-1}. This result is especially relevant for future industrial applications because the enhancement of productivity contributes to reducing process costs.

The difficulty in producing γ-PGA along the time course of cultivation is related to the increase in the medium viscosity that negatively affects oxygen transfer. The sharp decline in the γ-PGA concentration shown in Fig. 6 for 1,000 rpm may be due the high shear rate, which could breakdown the biopolymer molecule. This effect has already been reported for the production of other biopolymers such as xanthan gum [55], gellan gum [56], and
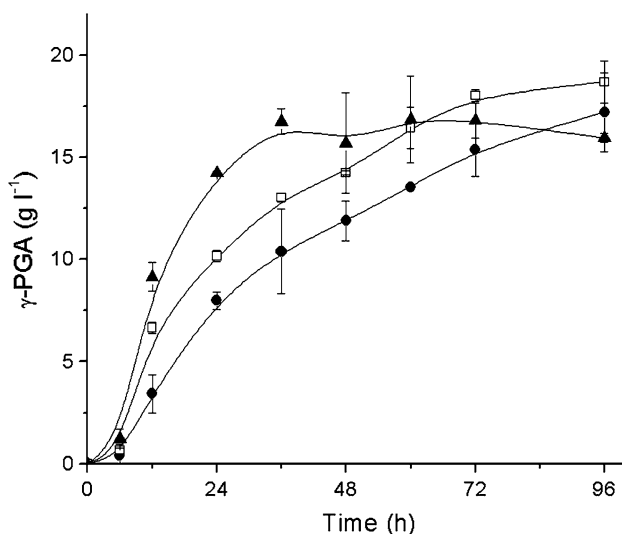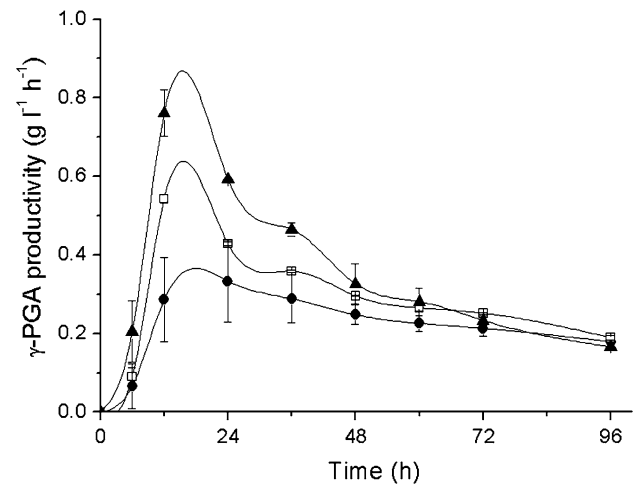

Fig. 6 Productivity γ-PGA by *Bacillus subtilis* BL53 for cultures conducted at 2 vvm and stirring rates of 500 rpm (*filled circle*), 750 rpm (*square*), and 1,000 rpm (*filled triangle*). Results are the mean of duplicates and *curves* are obtained by fit to the Logistic Model in Eq. (2)

an exopolysaccharide produced by *E. cloacae* [52]. However, the influence of depolymerase enzymes, in the specific case of γ-PGA, cannot be dismissed, since their activities are induced under conditions of carbon source limitations or increased concentration of γ-PGA [26].

Conclusions

Exploring biodiversity is a key step in the development of bioprocess for new or alternative sources of industrially important products. In this study, five strains isolated from Amazonian environment showed the ability to produce γ-PGA when cultured in medium E. The best producer strain was characterized and identified as *B subtilis*. γ-PGA production could be optimized concerning temperature, pH, and Zn^{2+}, which is an important inducer of the biopolymer. The scaling-up of γ-PGA production in bioreactors, where agitation strategies were evaluated and the best $k_L a$ determined, increased its production in more than 70 % compared to shaker cultivations. The production of γ-PGA by *B. subtilis* BL53 showed to be highly dependent on oxygen transfer, and productivity could be increased, while cultivation time could be reduced to half the time compared to conditions where $k_L a$ was not optimal, thereby contributing to reducing process costs.

Fig. 5 Production of γ-PGA by *Bacillus subtilis* BL53 for cultivation conducted at 2 vvm and stirring rates of 500 rpm (*filled circle*), 750 rpm (*square*), and 1,000 rpm (*filled triangle*). Results are the mean of duplicates and *curves* are obtained by fit to the Logistic Model in Eq. (2)

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