Biotechnology and Bioprocess Engineering 15: 635-640 (2010)
DOI 10.1007/s12257-009-0059-2

RESEARCH PAPER

Effect of Aeration and Agitation on Synthesis of Poly (γ-glutamic acid) in Batch Cultures of *Bacillus licheniformis* NCIM 2324

Ishwar B. Bajaj and Rekha S. Singhal

Received: 19 March 2009 / Revised: 30 May 2009 / Accepted: 2 June 2009
© The Korean Society for Biotechnology and Bioengineering and Springer 2010

Abstract The effects of aeration and agitation on the production and molecular weight of poly (γ-glutamic acid) (PGA) were systematically investigated in batch fermentor cultures of *Bacillus licheniformis* NCIM 2324. A high aeration rate and agitation speed enhanced the growth of *B. licheniformis* NCIM 2324, but did not always lead to high PGA production. Additionally, PGA production actually decreased at very high aeration rates and agitation speeds. The maximum PGA concentration was obtained at 750 rpm and 1 vvm. Rheological studies revealed that fermentation broth during production of PGA exhibited pseudoplastic behavior. The effects of aeration and agitation on the molecular weight of PGA were also studied, and the rate and extent of the decrease in the molecular weight of PGA as a function of time were found to be much greater at high aeration than low aeration. The PGA production of 46.34 g/L with a specific productivity of 0.17 g-PGA/g-biomass/h and a PGA yield of 0.48 with respect to total substrate observed in the present study are much higher than the values reported in previously conducted studies.

Keywords: Poly (γ-glutamic acid), biopolymer, agitation, aeration, rheology, molecular weight

1. Introduction

Poly (γ-glutamic acid) (PGA) is a water-soluble, biodegradable biopolymer produced by microbial fermentation that consists of D- and L-glutamic acid monomers connected by amide linkages between α-amino and γ-carboxyl groups. Potential applications of PGA and its derivatives have been of interest in past few years in a broad range of industrial fields including the food, pharmaceutical, and medicinal industries [1].

Agitation and aeration are important for all aerobic processes, and have a significant effect on the yield of most biopolymers, including pullulan [2], xanthan [3], gellan [4], curdlan [5], and hyaluronic acid [6]. As with other biopolymers, PGA synthesis and molecular weight characteristics are likely to be influenced by aeration and agitation, since the high viscosity of the fermentation broth decreases the efficiency of nutrient and oxygen transfer [7]. Both agitation and aeration are involved in overall mass and oxygen transfer in the process fluid to different extents. Agitation controls nutrient transfer and the distribution of air and oxygen, while aeration determines the oxygenation of the culture and also contributes to bulk mixing of the fermentation fluid, especially where mechanical agitation speeds are low [8].

The effects of medium components on PGA production by *B. licheniformis* are well documented [9-12], but there is very little information available regarding the effects of aeration and agitation on PGA production. Richard and Margaritis [13] studied the key oxygen mass transfer coefficient and broth rheology parameter during fermentative production of PGA by *B. subtilis* IFO 3335 at a constant airflow rate of 2 vvm. Their results showed that the fermentation broth exhibited pseudoplastic behavior during the late exponential growth phase and early stationary phase. Cromwick *et al.* [7] studied the simultaneous effect of pH and aeration on the production of PGA by *B. licheniformis* and found that an increase in aeration rate from 0.5 to 1.0 vvm increased the PGA yields. Because the effect of pH and aeration were determined simultaneously,

Ishwar B. Bajaj* and Rekha S. Singhal
Food Engineering and Technology Department, Institute of Chemical Technology, University of Mumbai, Mumbai 400-019, India
Tel: +91-22-2414-5616; Fax: +91-22-2414-5614
E-mail: ishbajaj1@yahoo.co.in

the effect of aeration on PGA synthesis was not clearly established. The effect of agitation and aeration on the molecular weight of PGA and viscosity of fermentation broth during PGA production has also not been clearly established.

We previously investigated the effect of medium components and precursors on the production of PGA by *B. licheniformis* NCIM 2324 [11,12]. In the present study, the effect of agitation and aeration on the growth of *B. licheniformis* NCIM 2324, PGA production, substrate consumption, rheology of the fermentation broth, and the molecular weight of the PGA were systematically investigated in batch fermentor cultures of *B. licheniformis* NCIM 2324.

2. Materials and Methods

2.1. Materials

All chemicals used in present study were of AR grade and were purchased from Hi-Media Limited, Mumbai, India. HPLC grade sodium chloride was purchased from S.D. Fine Chemicals Limited, Mumbai, India. PGA was generously provided by Vedan Enterprise Corporation, Taiwan.

2.2. Bacterial strain and medium

A bacterial strain of *B. licheniformis* NCIM 2324 was used in the present study. The medium used for the growth and maintenance contained (g/L) peptone (5); yeast extract (1.5); beef extract (1.5); sodium chloride (5); and agar (20) at pH 7 ± 0.2. Bacterial cells in agar slants were incubated at 37°C for 48 h and stored at 4°C.

For the production of PGA, a medium reported by Bajaj and Singhal [12] was used. Specifically, the medium contained (g/L): glycerol (62.4); citric acid (15.2); L-glutamic acid (20); ammonium sulphate (8.0); K_2HPO_4 (1); $MgSO_4 \cdot 7H_2O$ (0.5); $CaCl_2 \cdot 2H_2O$ (0.2); $MnSO_4 \cdot 7H_2O$ (0.05); L-glutamine (0.5 mM); and α-ketoglutaric acid (10 mM). The initial pH of the medium was adjusted to 6.5 using 2 N NaOH and/or 2 N HCl. The medium was sterilized in an autoclave for 15 min at 121°C.

2.3. Inoculum and fermentation

A loopful of cells from a slant was transferred to 20 mL of the seed medium in a 100 mL conical flask and incubated at 37°C and 200 rpm for 16 h. This was then used as the inoculum. Batch cultivations were conducted in a Biostat B5 (B. Braun Biotech-Sartorius) fermenter with a 2.5 L working volume. The bioreactor was a stirrer driven vessel type Univesssel ® 5 L that was jacketed, had a height to diameter ratio of 2:1 and an internal concave bottom that enabled optimum mixing even at low stirring speeds. The reactor had four baffles with two six-flat-blade disc impellers measuring 64 mm in diameter (Di), spaced 11.5 cm from each other. The bioreactor was also supplied with two membrane filters for sterile aeration and exhaust. A stainless steel ring gas sparger located at the base of the impeller was used to sparge sterile air/oxygen during the fermentation.

The fermenter was inoculated with 1% (v/v) 16 h old *B. licheniformis* NCIM 2324 culture containing approximately 3×10^7 cells/mL into 2.5 L of sterile fermentation medium. All fermentation runs were conducted in batch mode under controlled temperature conditions of 37°C. The pH was automatically controlled using a sterile pH electrode (Hamilton) at 6.5 ± 0.05 by adding 2 M HCl or 2 N NaOH. The dissolved oxygen (DO) concentration was measured using a sterilizable polarographic electrode (Mettler-Toledo InPro6000 Series). The effect of aeration and agitation on PGA production was evaluated by changing the aeration rate in the range of 1.0 ~ 3.0 vvm and the agitation speed in the range of 250 ~ 1,000 rpm.

2.4. Analytical determinations

The PGA concentration was determined using the method described by Chen *et al*. [14]. A Jasko HPLC system fitted with a PL-aquagel-OH gel permeation chromatogram column (7.8 × 300 mm, Polymer Laboratories Ltd., UK) and UV detector was used for PGA analysis. Samples were eluted with 0.1 mM sodium chloride at a flow rate of 1 mL/min and detected at 220 nm. The purified PGA was used as a standard.

A total of 20 mL of properly diluted culture broth was centrifuged for 20 min at 10,000 rpm and 4°C to separate the cell mass. The detained cell pellet was then dried in a hot air oven at 80°C to a constant weight to determine the dry cell weight.

Glycerol in the fermentation broth was estimated by a colorimetric procedure described by Bok and Demain [15]. The concentration of citric acid was determined by the method described by Marrier and Boulet [16]. The concentration of glutamic acid was determined by HPLC under conditions identical to that for quantification of PGA [11].

Rheological measurements were conducted using a Haake Rotoviscometer (VT550) with a sensor system NV (cup) and NV (rotor). Viscosity was measured at shear rates ranging from 27.05 to 2705/sec (27.05, 44.90, 75.19, 125.5, 245.0, 349.4, 583.1, 971.6, 1610.0, and 2705/sec). For comparison of the viscosity of different fermentation fluids, a constant shear rate of 245.0/sec was selected.

The molecular weight of PGA was measured using a Jasko HPLC system fitted with PL-aquagel-OH gel permeation chromatogram columns (2 × PL aquagel-OH mixed 8 μm, 300 × 7.5 mm, Polymer Laboratories Ltd., UK) and a refractive index (RI) detector. Poly ethylene oxide stan-

dards were used to construct a standard curve. The eluant was 0.2 M $NaNO_3$ (pH 7) and the flow rate was set at 1 mL/min.

3. Results and Discussion

3.1. Cell growth

Fig. 1 describes the effect of aeration and agitation on biomass formation of *B. licheniformis* NCIM 2324. The results revealed that the growth of *B. licheniformis* NCIM 2324 was closely linked to the aeration rate and agitation speed. Biomass accumulation increased as the agitation speed increased, and the maximum dry cell weight (DCW) (4.21 g/L) was obtained at 1,000 rpm and 1 vvm (Fig. 1). This was likely due to the improved oxygen and nutrient supply to the cells at higher agitation speed [8]. An increase in the aeration rate from 1 to 3 vvm also improved the cell growth (Fig. 1). *B. licheniformis* NCIM 2324 is an aerobic bacteria, and increased aeration might have provided sufficient oxygen to the bacteria even in the highly viscous fermentation broth, resulting in proper growth of bacteria. These results are in accordance with the results of a study conducted by Cromwick *et al*. [7], who found that approximately 2 g/L of DCW of *B. licheniformis* ATCC 9945A were produced with an agitation speed of 250 rpm at 1.0 vvm aeration and that approximately 4 g/L of DCW were produced with an agitation speed of 800 rpm at 2.0 vvm aeration.

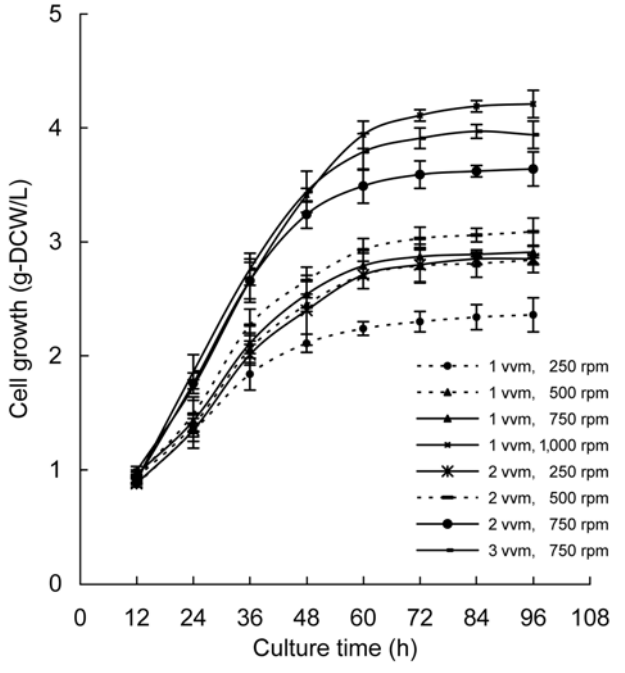

Fig. 1. Biomass formation in batch cultures of *B. licheniformis* NCIM 2324 under different agitation and aeration conditions.

3.2. PGA production and nutrient utilization

The PGA production increased as the rate of aeration and agitation increased up to the optimum level, above which PGA production decreased (Fig. 2). The maximum PGA production of 46.34 ± 1.12 g/L was obtained at an agitation speed of 750 rpm and an aeration rate of 1 vvm. At 1,000 rpm and 1 vvm, and 750 rpm and 3 vvm, the PGA concentration was 22.28 ± 1.04 and 25.93 ± 1.18 g/L, respectively, despite the improved cell growth. These findings suggest that at higher aeration and agitation speeds, cell growth was stimulated at the expense of PGA production. The increase in PGA production by increasing aeration was previously reported by Cromwick *et al*. [7], who observed an increase in PGA production from 12 to 24 g/L in response to an increase in the aeration rate from 1 to 2 vvm. The enhancement of biopolymer production by agitation and aeration was also observed in experiments conducted to evaluate polymers such as pullulan [17] and xanthan [3]. However, in the present study, there appeared to be a limit to the positive effect of agitation and aeration on PGA production, with the PGA concentration falling at very high agitation speeds and aeration rates. A decrease in the production of biopolymers such as gellan gum under very high aeration and agitation conditions was reported by Giavasis *et al*. [4], but no previous studies have reported a decrease in PGA production under very high aeration and agitation conditions.

The effects of aeration and agitation on utilization of medium components were also studied. The results revealed that utilization of citric acid, glycerol and glutamic acid improved as the aeration rate and agitation speed increased (Figs. 3A, 3B, and 3C). Under higher aeration and agitation conditions (1 vvm, 1,000 rpm; and 3 vvm, 750 rpm), mass transfer was far better, and hence the rate of utilization of nutrients was greater [8]. However, the result of biomass

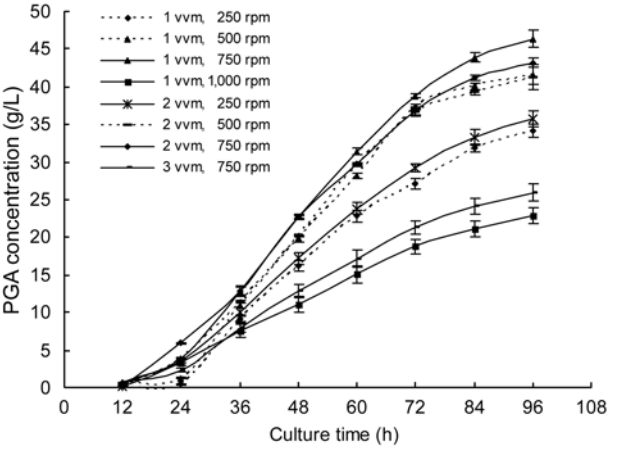

Fig. 2. PGA production in batch cultures of *B. licheniformis* NCIM 2324 under different agitation and aeration conditions.

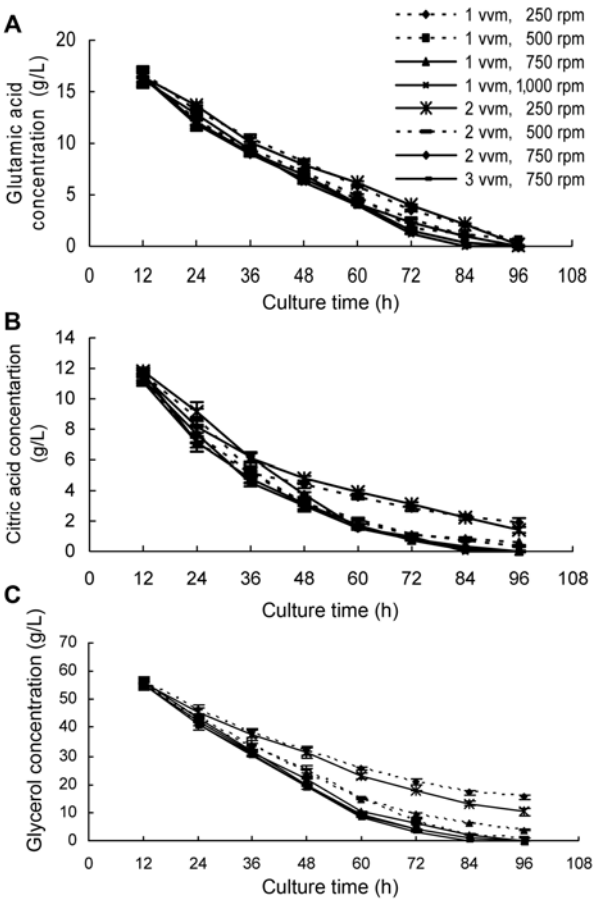

Fig. 3. Consumption of (A) glutamic acid; (B) citric acid; and (C) glycerol in batch cultures of *B. licheniformis* NCIM 2324 under different agitation and aeration conditions.

and PGA production suggest that, at high aeration and agitation, nutrients were utilized by *B. licheniformis* NCIM 2324 for growth and not for PGA production. Under lower aeration and agitation conditions (1 vvm, 250 rpm; 1 vvm, 500 rpm; and 2 vvm, 250 rpm), the rate of utilization of the substrates was much lower and the nutrients were not completely utilized, even at the end of fermentation, which resulted in less PGA and biomass production. At 1 vvm and 750 rpm, where the highest PGA production was observed, the utilization rate was slightly lower when compared to the higher aeration conditions; however, glycerol, glutamic acid, and citric acid were completely utilized at the end of fermentation. The increase in the consumption of glycerol, glutamic acid, and citric acid with an increase in aeration during production of PGA by *B. licheniformis* ATCC 9945A was also reported by Cromwick *et al.* [7].

In various studies conducted to evaluate fermentative production of PGA, glycerol or L-glutamic acid remained unutilized at the end of fermentation, and may have acted as impurities leading to the need for increased steps in downstream processing [18,19]. Contrary to those results, in the present study, both glycerol and L-glutamic acid were completely utilized at the end of fermentation.

The maximum PGA (46.34 g/L) produced in the present study was far greater than the L-glutamic acid (20 g/L) added to the production medium, suggesting that glutamic acid is produced in *B. licheniformis* NCIM 2324 by some other mechanism. In our previous studies, we reported that a large amount of endogenous L-glutamic acid was produced in *B. licheniformis* from citric acid through isocitric acid and α-ketoglutaric acid in the tricarboxylic acid cycle *via* the glutamate dehydrogenase (GD) pathway, as well as the pathway involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) [12]. D-glutamic acid is presumably produced from L-glutamic acid through an indirect conversion mechanism. It is believed that PGA is polymerized from externally provided L-glutamic acid and endogenously formed D and L-glutamic acid, and hence the amount of PGA produced was greater than the amount of L-glutamic acid supplied in the fermentation medium.

Fig. 4 shows the DO concentration (as percentage of air saturation) in the broth during fermentative production of PGA by *B. licheniformis* NCIM 2324 under different agitation and aeration conditions. In all of the fermentation conditions, oxygen uptake by *B. licheniformis* NCIM 2324 was higher for up to 24 h of fermentation, after which much less oxygen was required. Based on this data, it can be concluded that the peak oxygen demand, both for biomass and PGA production, occurs during the early phase, while less oxygen was required when PGA was synthesized by *B. licheniformis* NCIM 2324 since the maximum PGA formation was observed after 24 h of fermentation. When the concentration of PGA and broth viscosity reached their maximum values, very little or no oxygen was

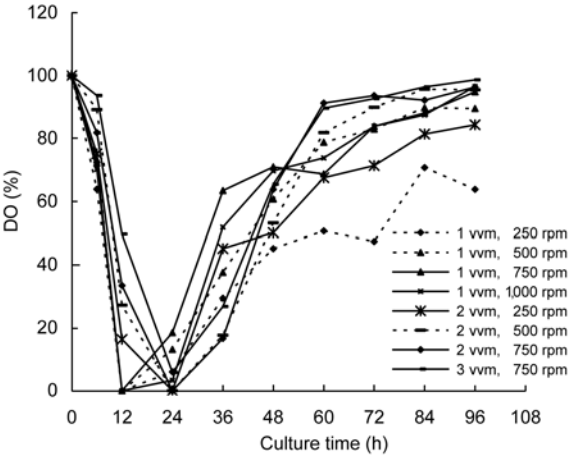

Fig. 4. Dissolved oxygen (DO) concentration during fermentative production of PGA by *B. licheniformis* NCIM 2324 under different agitation and aeration conditions.

utilized. These results are in accordance with the results reported by Richard and Margaritis [13], who also found that the maximum specific oxygen uptake rate occurred at the initial phase of fermentation.

3.3. Rheology of fermentation broth

Figs. 5A and 5B demonstrate the apparent viscosity of the fermentation broth during batch cultures of *B. licheniformis* NCIM 2324 under different agitation and aeration conditions. The viscosity of the fermentation broth decreased as the shear rate increased, suggesting that the fermentation broth during production of PGA exhibits a marked degree of pseudoplasticity (Fig. 5A). Richard and Margaritis [13] also reported pseudoplastic behavior of broth during fermentative production of PGA. The maximum viscosity of 340.8 mPa.S was achieved at 750 rpm and 1 vvm (Fig. 5B), where the maximum PGA concentration was also observed. In all fermentation runs, the time profile of apparent viscosity of fermentation broth was closely correlated with PGA concentration until 24 to 60 h of fermentation, after which the relatively high PGA concentration was accompanied by a lower viscosity than expected for this concentration of PGA. In other words, there was no simple correlation between the concentration of biopolymer formed (Fig. 2) and the apparent viscosity of the fermentation broth (Fig. 5B). Because the rheological characteristics of fermentation broth are dominated by the polymer, the most probable explanation for the decrease in viscosity is changes in the molecular weight of the PGA.

3.4. Molecular weight of PGA

At the end of the fermentation, the molecular weight of PGA was higher in the fermentation runs with low aeration and agitation than in fermentation runs with higher aeration and agitation (Table 1). The molecular weight of PGA was higher in the initial phase of fermentation and decreased thereafter. The rate and extent of the decrease of molecular weight as a function of time was much greater at high aeration and agitation speed than at low aeration and agitation. Cromwick *et al*. [7] reported similar effects of aeration on the molecular weight of PGA. The significant effects of aeration on other biopolymers have also been reported in other studies. For example, Quagliano and Miyaazaki [20] found that low aeration rates (1,100 kDa at 0.5 vvm) produced high molecular weight poly-β-hydroxybutyrate, while high aeration rates (111 kDa at 2.5 vvm) decreased the molecular weight of poly-β-hydroxybutyrate. Giavasis *et al*. [4] found that all processes conducted under moderate aeration resulted in the production of gellan with a higher molecular weight when compared to the processes in which higher aeration was applied.

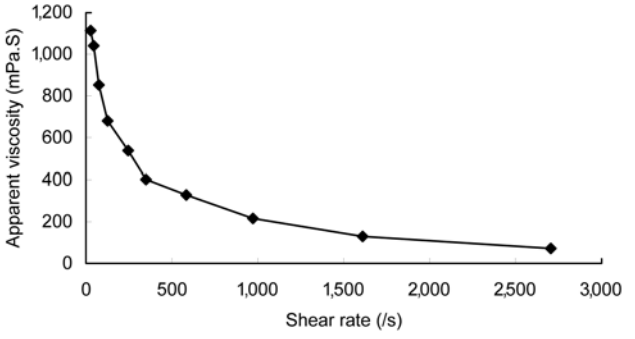

Fig. 5. (A) Pseudoplastic behavior of fermentation broth during fermentative production of PGA. (B) Apparent viscosity of the fermentation broth during batch cultures of *B. licheniformis* NCIM 2324 under different agitation and aeration conditions.

The induction of PGA depolymerase activity by depletion of available carbon sources could be caused by the lower molecular weight PGA produced during fermentation [21]. However, based on the present data, the action of PGA depolymerase alone at high aeration rates did not satisfactorily explain these observations because a reduction in the molecular weight of PGA in processes with high aeration was consistently observed throughout the fermentation (Table 1). PGA depolymerase has generally been shown to be induced only in the late stages of the fermentation, when the culture is in the stationary or the death phase, and exogenous carbon sources are at very low or limiting levels [21]. Because molecular weight is consistently lower under higher aeration conditions than less aerated conditions, factor acting continuously throughout the process would appear to be the probable cause of the reduced polymer molecular weight. It can be concluded that shear forces resulting from increasing the stirring rate from 250 to 750 or 1,000 rpm are responsible for reduction of the molecular weight of PGA.

The specific productivity of PGA (g PGA/g-biomass/h) for the fermentation cultures conducted under different aeration and agitation conditions ranged from 0.05 to 0.17 g-PGA/g-biomass/h (Table 2). For high aeration cultures, the specific productivity was much lower because these conditions supported biomass production rather than PGA

Table 1. Influence of fermentation conditions on the molecular weight of PGA

Fermentation conditions	Molecular weight under various culture times during fermentation (kDa)			
	24 h	48 h	72 h	96 h
1 vvm, 250 rpm	4.1×10^5	5.9×10^5	6.2×10^5	6.9×10^5
1 vvm, 500 rpm	6.3×10^5	1.2×10^6	8.3×10^5	6.8×10^5
1 vvm, 750 rpm	2.1×10^6	1.1×10^6	5.8×10^5	3.3×10^5
1 vvm, 1,000 rpm	1.8×10^6	7.4×10^5	4.1×10^5	8.9×10^4
2 vvm, 750 rpm	2.4×10^6	2.6×10^6	7.1×10^5	3.2×10^5
3 vvm, 750 rpm	1.3×10^6	6.1×10^5	3.5×10^5	1.1×10^5

Table 2. Specific productivity of PGA and PGA yield with respect to total substrate under different agitation and aeration conditions

Fermentation conditions	PGA (g/L)	Specific productivity (g-PGA/ g-biomass/h)	PGA yield (g PGA/g) total substrate*
1 vvm, 250 rpm	34.13 ± 1.54	0.15	0.35
1 vvm, 500 rpm	41.63 ± 1.39	0.15	0.43
1 vvm, 750 rpm	46.34 ± 1.12	0.17	0.48
1 vvm,1,000 rpm	22.28 ± 1.04	0.05	0.23
2 vvm, 250 rpm	35.78 ± 1.05	0.13	0.36
2 vvm, 500 rpm	41.13 ± 0.62	0.13	0.42
2 vvm, 750 rpm	43.16 ± 0.81	0.12	0.44
3 vvm, 750 rpm	25.93 ± 1.18	0.06	0.26

*Total substrate = glycerol + glutamic acid + citric acid.

production. The maximum specific productivity (0.17 g-PGA/g-biomass/h) was obtained at 1 vvm aeration and 750 rpm agitation, and the maximum PGA yield with respect to total substrate utilized was also observed under these conditions (Table 2).

4. Conclusion

Both aeration rate and agitation speed are key parameters in the fermentative production of PGA. High aeration and high agitation favor cell growth, but are not necessary for PGA production. High agitation and aeration stimulates cell growth at the expense of PGA synthesis. The highest PGA production of 46.34 g/L with a specific productivity of 0.17 g-PGA/g-biomass/h and PGA yield with respect to total substrate of 0.48 was obtained at 1 vvm and 750 rpm. Fermentation broth during production of PGA exhibited a pseudoplastic behavior. The time profile of the apparent viscosity of fermentation broth was closely correlated with PGA concentration until 48 to 60 h of fermentation, after which there was no simple correlation between the concentration of biopolymer and the apparent viscosity. The molecular weight of PGA was higher in the initial phase of fermentation and decreased thereafter. The rate and extent of the decrease of molecular weight as a function of time was much greater at high aeration rate and agitation speed than at low aeration and agitation. PGA depolymerase and shear forces resulting from increasing aeration and agitation were responsible for reduction of the molecular weight of PGA during fermentation.

References

1. Shih, I. L. and Y. T. Van (2001) The production of poly(γ-glutamic acid) from microorganism and its various applications. *Bioresour. Technol.* 79: 207-225.
2. Wecker, A. and U. Onken (1991) Influence of dissolved oxygen concentration and shear rate on the production of pullulan by *Aureobasidium pullulans*. *Biotechnol. Lett.* 13: 155-160.
3. Peters, H. U., H. Herbst, P. Hesselink, H. Lunsdorf, A. Schumpe, and W. D. Deckwer (1989) The influence of agitation rate on xanthan production by *Xanthomonas campestris*. *Biotechnol. Bioeng.* 34: 1393-1397.
4. Giavasis, I., L. M. Harvey, and B. McNeil (2006) The effect of agitation and aeration on the synthesis and molecular weight of gellan in batch cultures of *Sphingomonas paucimobilis*. *Enz. Microbial Technol.* 38: 101-108.
5. Lawford, H. and J. Rousseau (1989) Effect of oxygen on the rate of β-1,3-glucan microbial exopolysaccharide production. *Biotechnol. Lett.* 11: 125-130.
6. Huang, W. C., S. J. Chen, and T. L. Chen (2006) The role of dissolved oxygen and function of agitation in hyaluronic acid fermentation. *Biochem. Eng. J.* 32: 239-243.
7. Cromwick, M., G. A. Birrer, and R. A. Gross (1996) Effects of pH and aeration on γ-poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. *Biotechnol. Bioeng.* 50: 222-227.
8. McNeil, B. and L. Harvey (1993) Viscous fermentation products. *Crit. Rev. Biotechnol.* 13: 275-304.
9. Shih, L., Y. T. Van, and Y. N. Chang (2002) Application of statistical experimental methods to optimize production of poly(γ-glutamic acid) by *Bacillus licheniformis* CCRC 12826. *Enz. Microbial. Technol.* 31: 213-220.
10. Suo, C., L. H. Mei, J. Huang, and Q. Sheng (2007) Selection of γ-poly glutamic acid high yield strain by 60Co γ-irradiation and the optimization of its culture medium. *J. Chem. Eng. Chin. Univ.* 21: 820-825.
11. Bajaj, I. B., S. S. Lele, and R. S. Singhal (2009) A statistical approach to optimization of fermentative production of poly(γ-glutamic acid) from *Bacillus licheniformis* NCIM2324. *Bioresour. Technol.* 100: 826-832.
12. Bajaj, I. B. and R. S. Singhal (2009) Enhanced production of poly-(γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324 by using metabolic precursors. *Appl. Biochem. Biotechnol.* 59: 133-141.
13. Richard, A. and A. Margaritis (2003) Rheology, oxygen transfer, and molecular weight characteristics of poly(glutamic acid) fermentation by *Bacillus subtilis*. *Biotechnol. Bioeng.* 82: 299-305.
14. Chen, X., S. Chen, M. Sun, and Z. Yu (2005) Medium optimization by response surface methodology for poly-γ-glutamic acid production using dairy manure as the basis of a solid substrate. *Appl. Microbiol. Biotechnol.* 69: 390-396.
15. Bok, S. H. and A. L. Demain (1977) An improved colorimetric assay for polyols. *Anal. Biochem.* 81: 18-20.
16. Marier, J. R. and M. Boulet (1958) Direct determination of citric acid in milk with an improved, pyridine acetic anhydride method. *J. Dairy Sci.* 41: 1683.
17. McNeil, B. and B. Kristiansen (1987) Influence of impeller speed upon the pullulan fermentation. *Biotechnol. Lett.* 9: 101-104.
18. Xu, H., M. Jiang, H. Li, D. Lu, and P. Ouyang (2005) Efficient production of poly(γ-glutamic acid) by newly isolated *Bacillus subtilis* NX-2. *Proc. Biochem.* 40: 519-523.
19. Yoon, S. H., J. H. Do, S. Y. Lee, and H. N. Chang (2000) Production of poly-γ-glutamic acid by fed-batch culture of *Bacillus licheniformis*. *Biotechnol. Lett.* 22: 585-588.
20. Quagliano, J. C. and S. S. Miyaazaki (1997) Effect of aeration and carbon/nitrogen ratio on the molecular mass of the biodegradable polymer poly-β-hydoxybutyrate obtained from *Azotobacter chroococcum* 6B. *Appl. Microbiol. Biotechnol.* 48: 662-664.
21. Birrer, G. A., A. M. Cromwick, and R. A. Gross (1994) γ-Poly-(glutamic acid) formation by *Bacillus licheniformis* 9945A: Physiological and biochemical studies. *Int. J. Biol. Macromol.* 16: 265-275.