

Study on Production of Poly(glutamic acid) Using Bacillus subtilis DYU1 and Its Flocculation Property

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ABSTRACT

In this study, we use *B. subtilis* DYU1 as γ -Polyglutamic acid (γ -PGA) producer to investigate the effect of medium compositions and incubated conditions on γ -PGA production in flask. The maximum γ -PGA production (32.1 g/L) was obtained when it was grown in the medium containing 30 g/L L-glutamic acid. The applicable temperatures for producing γ -PGA were between 35 – 38 °C. The addition of amino acid solution to the medium could not enhance the γ -PGA production. On the other hand, we also investigate the effect of agitation speed and aeration rate on the γ -PGA production in a 5-L fermentor. Furthermore, in order to reduce the production costs, the molasses was used as basic production medium, at the same time the waste could be reused. The results suggest that the molasses has high potential for producing γ -PGA. The rheological properties of γ -PGA fermentation broth were studied using a rotational viscometer at several pHs and temperatures. The mathematical models were developed for determining the apparent viscosity of γ -PGA fermentation broth as affected by temperature and γ -PGA concentration. Potential applications of γ -PGA as humectant and flocculant was also study in this research and, the results show that γ -PGA has high moisture-absorption and moisture-retention capacities. Furthermore, the γ -PGA solution also has high moisture-retention capacity. Three kinds of γ -PGA (as flocculant) and the polyaluminum chloride (PAC) (as coagulant) were use to perform the flocculation test of *E. coli*. The γ -PGA produced from *B. subtilis* DYU1 was found to have excellent flocculating ability for *E. coli* suspension. Moreover, mechanisms describing the flocculation process with γ -PGA were proposed based on the experimental observations.

Keywords : *Bacillus subtilis* DYU1, γ -PGA, fermentor, molasses, rheological properties, flocculation

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REFERENCES

1. 劉榮元, 2005, 篩選聚穀胺酸生產菌株及其絮凝性質之研究。大葉大學生物產業科技研究所碩士論文, 彰化。2. 呂媚君, 2007, 利用篩選之菌株 *Bacillus subtilis* DYU1 生產具穀胺酸之研究。大葉大學生物產業科技研究所碩士論文, 彰化。3. 楊思廉, 1993, 工業化學概論

, 五洲出版社, 台北, pp. 431-443. 4. Afendra, A. S., Yiannaki, E. E., Palaiomyliou, M. A., Kyriakidis, D. A., Drinas, C. 2002. Co-production of ice nuclei and xanthan gum by transformed *Xanthomonas campestris* grown in sugar beet molasses. *Biotechnology Letters* 24: 579 – 583. 5. Akagi, T., Higashi, M., Kaneko, T., Kida, T. and Akashi, M. 2005a. In vitro enzymatic degradation of nanoparticles prepared from hydrophobically-modified poly(γ -glutamic acid). *Macromolecular Bioscience* 5: 598 – 602. 6. Akagi, T., Kaneko, T., Kida, T. and Akashi, M. 2005b. Preparation and characterization of biodegradable nanoparticles based on poly(γ -glutamic acid) with L-phenylalanine as a protein carrier. *Journal of Control Release*. 108: 226 – 236. 7. Allen, D. G. and Robinson, C. W. 1989. Hydrodynamics and mass transfer in *Aspergillus niger* fermentations in bubble column and loop bioreactors. *Biotechnology and Bioengineering*. 34: 731- 740. 8. Allen, D. G. and Robinson, C. W. 1990. Measurement of rheological properties of filamentous fermentation broths. *Chemical Engineering Science*. 45: 37- 48. 9. Arrhenius, S. 1889. On the reaction velocity of the inversion of cane sugar by acids. *Zeitschrift fur Physikalische Chemie*. 4: 226- 232. 10. Ashiuchi, M. and Misono, H. 2002a. Poly- γ -glutamic acid. In *Biopolymers Vol. 7*, S.R. Fahnestock, and A. Steinb?chel, eds.(Weinheim, Germany: Wiley-VCH) pp. 123 – 174. 11. Ashiuchi, M., and Misono, H. 2002b. Biochemistry and molecular genetics of poly- γ -glutamate synthesis. *Applied Microbiology and Biotechnology* 59:9 – 14. 12. Ashiuchi, M., Kamei, T., and Misono, H. 2003a. Poly- γ -glutamate synthetase of *Bacillus subtilis*. *Journal of molecular catalysis. B, Enzymatic* 23:101 – 106. 13. Ashiuchi, M., Kamei, T., Baek, D. H., Shin, S. Y., Sung, M. H., Soda, K., Yagi, T., and Misono, H. 2001a. Isolation of *Bacillus subtilis*(chungkookjang), a poly- γ -glutamate producer with high genetic competence. *Applied Microbiology and Biotechnology* 57:764 – 769. 14. Ashiuchi, M., Nawa, C., Kamei, T., Song, J.J., Hong, S.P., Sung, M.H., Soda, K., and Misono, H. 2001b. Physiological and biochemical characteristics of poly- γ -glutamate synthetase complex of *Bacillus subtilis*. *European Journal of Biochemistry* 268:5321-5328. 15. Ashiuchi, M., Shimanouchi, K., Nakamura, H., Kamei, T., Soda, K., Park, C., Sung, M. H., and Misono, H. 2004. Enzymatic synthesis of high-molecular-mass poly- γ -glutamate and regulation of its stereochemistry. *Applied and Environmental Microbiology* 70:4249 – 4255. 16. Ashiuchi, M., Soda, K., and Misono, H. 1999a. Characterization of *yrcC* gene product of *Bacillus subtilis* IFO 3336 as glutamate racemase Isozyme. *Bioscience, biotechnology, and biochemistry* 63:792-798. 17. Ashiuchi, M., Soda, K., and Misono, H. 1999b. A poly- γ -glutamate synthetic system of *Bacillus subtilis* IFO 3336: Gene cloning and biochemical analysis of poly- γ -glutamate produced by *Escherichia coli* clone cells. *Biochemical and Biophysical Research Communications* 263:6 – 12. 18. Ashiuchi, M., Tani, K., Soda, K., and Misono, H. 1998. Properties of glutamate racemase from *Bacillus subtilis* IFO 3336 producing poly- γ -glutamate. *The Journal of Biochemistry* 123:1156-1163. 19. Atkins, P. 2001. *The elements of physical chemistry*. (3rd ed.). Oxford University Press Inc., New York. 20. Atkinson, B., Matvituna, F. 1983. *Biochemical engineering and biotechnology handbook*. ISBN: 0333332741; 978-033333274-021. Avichezer, D., Schechter, B., Arnon, R. 1998. Functional polymers in drug delivery: carrier-supported CDDP (cis-platin) complexes of polycarboxylates-effect on human ovarian carcinoma. *Reactive and Functional Polymers* 36: 59-69. 22. Bajaj I. B., Lele, S. S., and Singhal, R. S. 2009. A statistical approach to optimization of fermentative production of poly(γ -glutamic acid) from *Bacillus licheniformis* NCIM 2324. *Bioresource Technology* 100: 826-832. 23. Bai, F., Zeng, C., Yang, S., Zhang, Y., He, Y. and Jin, J. 2008. The formation of a novel supramolecular structure by amyloid of poly-L-glutamic acid. *Biochemical and Biophysical Research Communications* 369: 830-834. 24. Baranska, H., Labudzinska, A., and Terpinski, J., “ *Laser Raman Spectroscopy-Analytical Applications*, ” Ellis Horwood, Chichester, UK (1987). 25. Beaulieu, M., Beaulieu, Y., Me?linard, J., Sithian, P., Goulet, J. 1995. Influence of Ammonium Salts and Cane Molasses on Growth of *Alcaligenes eutrophus* and Production of Polyhydroxybutyrate. *Applied and Environmental Microbiology* 61(1): 165-169. 26. Bender, H., Rodriguez-Eatun, S., Ekanemesang, U. and Phillips, P. 1994. Characterization of metal-binding biofloculans produced by the cyanobacterial component of mixed microbial mats. *Applied and Environmental Microbiology* 60: 2311-2315. 27. Bhattacharya, S., Bhat, K. K. and Raghuvver, K. G. 1992. Rheology of bengal gram cicer arictinum flour suspensions. *Journal of Food Engineering* 17: 83-96. 28. Birrer, G. A., Cromwick, A. M. and Gross, R. A. 1994. γ -Poly(glutamic acid) formation by *Bacillus licheniformis* 9945A. physiological and biochemical studies. *International Journal of Biological Macromolecules* 16: 265-275. 29. Borb?ly, M., Nagasaki, Y., Borb?ly, J., Fan, K., Bhogle, A., and Sevoian, M. 1994. Biosynthesis and chemical modification of poly(γ -glutamic acid). *Polymer Bulletin* 32: 127-132. 30. Borst A. H., Haverich, G., Walterbush, G., Maatz, W., Messmer, M. 1982. Fribin adhesive: an important hemostatic adjunct in cardiovascular operation. *Journal of Thoracic and cardiovascular surgery* 84: 548-553. 31. Bovarnick, M. 1942. The formation of extracellular D(-)glutamic acid polypeptide by *Bacillus subtilis*. *The Journal of Biological Chemistry* 145:415-424. 32. Bratby, J. 1980. *Coagulation and flocculation*, Ch. 8, Upland Press, Croydon, UK. 33. Buescher, J. M. and Margaritis, A. 2007. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries *Criterion Review Biotechnology* 27: 1-19. 34. Candela, T. and Fouet, A. 2006. Poly- γ -glutamate in bacteria. *Molecular Microbiology* 60: 1091-1098. 35. Cejka, A. 1985. Preparation of media. In: Rehm, H.-J., Reed, G., Brauer, H.(eds.). *Biotechnology*, Vol. 2. Weinheim(Germany): VCH, 629 – 698. 36. Cell Therapeutics Inc. 2000. Company press release. www.cticseattle.com. 37. Cheng, C., Asada, Y., and Aaida T. 1989. Production of γ -polyglutamic acid by *Bacillus subtilis* A35 under denitrifying conditions. *Agricultural and Biological Chemistry* 53:2369-2375. 38. Chen, G. Q., and Page, W. J. 1997. Production of poly- γ -hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. *Biotechnology Techniques* 11: 347 – 350. 39. Choi, H. J., Kunioka, M. 1995a. Preparation conditions and swelling equilibria of hydrogel prepared by γ -irradiation from microbial poly(γ -glutamic acid). *Radiation Physics and Chemistry* 46: 175-179. 40. Choi, H. J., Yang, R., Kunioka, M. 1995b. Synthesis and characterization of pH-sensitive and biodegradable hydrogels prepared by γ -irradiation using microbial poly(γ -glutamic acid) and poly(ϵ -lysine). *Journal of Applied Polymer Science* 58: 807-814. 41. Choma C., Clavel T., Dominguez H., Razafindramboa N., Soumille H., Nyugen C., Schimtt P. 2000. Effect of temperature on growth characteristics of *Bacillus cereus* TZ415, *Int. J. Food Microbiol* 55: 73 – 77. 42. Cromwick, A. M., Birrer, G. A., and Gross,

R. A. 1996. Effects of pH and aeration on γ -poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. *Biotechnology and bioengineering* 50: 222-227.

43. Cumming, R. H., Robinson, P. M., and Martin, G. F. 1996. Flocculation of *E. coli* with cationic polymers. *Bioseparation* 6: 17-23.

44. Daninippon Pharmaceutical Co, Ltd. 1972. Ice cream stabilizer, JP Patent 19735/72.

45. Dearfield, K. L., Abernathy, C. O., Ottley, M. S., Brantner J. H. and Hayes, P. F. 1988. Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity and carcinogenicity. *Mutation Research* 195: 45-77.

46. Deng, S. B., Bai, R. B., Hu, X. M., and Luo, Q. 2003. Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. *Agricultural and Biological Chemistry* 60:588-593.

47. Do, J. H., Chang, H. and Lee, S. 2001. Efficient recovery of poly(glutamic acid) from highly viscous culture broth. *Biotechnology and Bioengineering*. 76: 219-223.

48. Du, G., Yang, G., Qu, Y., Chen, J. and Lun, S. 2005. Effects of glycerol on the production of poly(γ -glutamic acid) by *Bacillus licheniformis*. *Process Biochemistry* 40: 2143-2147.

49. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28: 350-356.

50. Editorial. 2006. China launches huge water quality study, *Chemical & Engineering News* 84: 8.

51. Esser K., Kues U. 1983. Flocculation and its implication for biotechnology. *Process Biochemistry* 18:21 – 3.

52. Fabián, S. G., Amelia M. A., and Juan M. D. 2004. Activated carbon fibers from Nomex by chemical activation with phosphoric acid. *Carbon* 42:1419 – 1426.

53. Ferreira, I. M. P. L. V. O., Gomes, A. M. P. and Ferreira, M. A. 1998. Determination of sugars, and some other compounds in infant formulae, follow-up milks and human milk by HPLC-UV/RI. *Carbohydrate Polymers* 37: 225-229.

54. Friedman, B. A. and Dugan, P. R. 1968. Concentration and accumulation of metallic ions by the bacterium *Zoogloea*. *Developments in Industrial Microbiology* 9: 381-388.

55. Fujii, H. 1963. On the formation of mucilage by *Bacillus natto*. Part III. Chemical constitutions of mucilage in natto (1). *Nippon Nogeikagaku Kaishi*: 37, 407-411.

56. Fujita, M., Ike, M., Tachibana, S., Kitada, G., Kim, S. M., and Inoue, Z. 2000. Characteristics of a bioflocculant produced by *Citrobacter* sp. YKF04 from acetic and propionic acids. *Journal of Bioscience and Bioengineering* 89: 40 – 46.

57. Gao, H., Jiang, T., Han, B., Wang, Y., Du, J., Liu, Z. and Zhang, J., 2004. Aqueous/ionic liquid interfacial polymerization for preparing polyaniline nanoparticles. *Polymer* 45:3017-3019.

58. Gardner, J. M. and Troy, F.A. 1979. Chemistry and biosynthesis of the poly(γ -D-glutamyl)capsule in *Bacillus licheniformis*. Activation, racemization, and polymerization of glutamic acid by a membranous polyglutamyl synthetase complex. *The Journal of Biological Chemistry* 254:6262 – 6269.

59. Goldman J. C., Carpenter E. J. 1974. A kinetic approach to effect of temperature on algal growth. *Limnology and Oceanography* 19: 756 – 766.

60. Goto, A. and Kunioka, M. 1992. Biosynthesis and hydrolysis of poly(γ -glutamic acid) from *Bacillus subtilis* IFO 3335. *Bioscience, Biotechnology, and Biochemistry* 56: 1031-1035.

61. Gouda, M. K., Swellam, A. E., Omar, S. H. 2001. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiological Reviews* 156: 201 – 207.

62. Gough, S., Flynn, O., Hack, C. J., Marchant, R. 1996. Fermentation of molasses using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3: simplex optimization of media supplements. *Applied Microbiology and Biotechnology* 46:187 – 190.

63. Gross, A. 1998. Bacterial γ -poly(glutamic acid). In *Biopolymers from renewable resources*, D.L. Kaplan, ed. (New York: Springer Berlin Heidelberg) 195 – 219.

64. Gross, R. A., McCarthy, S. P., Shah, D. T. 1995. Gamma-poly(glutamic acid) esters. US Patent 5: 378-807.

65. Hantula J., and Bamford D. H. 1991b. Bacterial phage resistance and flocculation deficiency of *Flavobacterium* sp. Are phenotypically interrelated. *Applied Microbiology and Biotechnology* 36:105 – 108.

66. Hara, T. 2002. Deser greening. Greening by utilization of microbial macromolecules. *Kobunshi* 49: 367-370.