

# Continuous Production of Poly(hydroxybutyrate-co-hydroxyvalerate)-Effect of Propionate and Valerate on the Microbial Gro

林逸群、?瑞澤

E-mail: 9300181@mail.dyu.edu.tw

## ABSTRACT

PHA is a kind of polyesters produced by microorganisms. *Ralstonia eutropha* can produce PHA when the carbon source is still sufficient, but one of necessary nutrient sources (e.g., nitrogen) is lacking. *R. eutropha* can produce PHBV (poly-hydroxybutyrate-co-hydroxyvalerate) if organic acidic substrates such as sodium propionate and valerate are fed during the second stage of microbial growth. However, excessive sodium propionate and valerate may be toxic to *R. eutropha* and hinder microbial growth and PHBV accumulation. Therefore, the feeding rate of acidic substrate must be properly controlled. When acid substrates are fed, bacteria can take them into the pathway of synthesizing PHBV. A continuous stirred tank bioreactor is used to enhance the effect of limiting nutrients on the PHBV accumulation. The production rates of PHBV in bacteria intracellular for different acidic substrates and C/N ratios are compared. In this study, *R. eutropha* was cultivated in a continuous stirred tank bioreactor with various dilution rates, C/N ratio (20, 30, and 40) and acid substrates (sodium propionate or sodium valerate) in order to explore the microbial growth, the PHBV accumulation, the consumption of glucose, nitrogen, and sodium propionate or valerate during fermentation. Results show that PHBV was about 37% of the dry biomass and PHBV production rate 0.094 g/L · h, the highest peak occurring at the dilution rate of 0.060 h<sup>-1</sup>. In the case of feeding sodium propionate, the biomass contains about 14%(w/w) PHBV at a dilution rate of 0.060 h<sup>-1</sup>, the average molar ratio of HB/HV approximately maintained at 90:10, and the yield of HV is 0.040 g per gram of propionate. In the case of feeding sodium valerate, the biomass contains about 37%(w/w) PHBV at the dilution rate of 0.060 h<sup>-1</sup>, the average molar ratio of HB/HV approximately maintained at 60:40, and the yield of HV is 0.131 g per gram of valerate. It has been shown that feeding sodium valerate can promote the accumulation of HV better than sodium propionate. In this study, the dilution rate, C/N ratio, and acid substrate were selected as manipulating factors. Main effects as well as their interactions were taken into consideration. The results were analyzed through statistical software called Statistica. The effect of manipulating factors on the microbial growth, the PHBV accumulation, the consumption of glucose, nitrogen, sodium propionate, and sodium valerate during fermentation could be obtained through the ANOVA (analysis of variances). Experimental results show that the higher the dilution rate is during the high rate, the higher the HB productivity will be. Feeding sodium valerate seems better to the HV production than feeding sodium propionate. Experimental results also show that the production of HB decreases, if the C/N ratio increases. The effect of manipulating factors on the production of PHBV is not that substantial.

Keywords : PHBV, continuous stirred tank reactor, dilution rate, *Ralstonia eutropha*, C/N ratio, analysis of variance

## Table of Contents

第一章 緒論 .....	1	第二章 文獻回顧 .....	3	2.1 前言 .....	3
.....	3	2.2 生物分解性塑膠的檢測方式與標準規範 .....	4	2.3 分解性塑膠的種類 .....	6
.....	6	2.3.1 微生物合成聚合物 .....	6	2.3.2 化學合成聚合物 .....	9
.....	9	2.3.3 天然聚合物 .....	10	2.3.4 光分解性塑膠 .....	10
.....	10	2.4 PHB與PHBV簡介 .....	12	2.4.1 合成 .....	12
.....	12	2.4.2 物理與化學性質 .....	16	2.4.3 PHB(V)的代謝過程 .....	20
.....	20	2.5 微生物的代謝作用 .....	27	2.5.1 初級代謝的生合成 .....	27
.....	27	2.5.2 次級代謝的生合成 .....	27	2.5.3 微生物生長與產物形成的關係 .....	30
.....	30	2.6 發酵工程概述 .....	31	2.6.1 批次發酵 .....	32
饋料發酵 .....	34	2.6.2 連續發酵 .....	36	2.6.3.1 連續式發酵的特點 .....	36
.....	36	2.6.3.2 連續發酵的控制 .....	38	2.7 生物分解性塑膠的應用 .....	39
.....	39	2.8 生物分解性塑膠的市場 .....	41	2.8.1 國外市場 .....	41
.....	41	2.8.2 中國大陸市場 .....	43	2.8.3 國內市場 .....	43
.....	43	2.9 PHBV測定 .....	46	2.9.1 定性與定量 .....	46
.....	46	2.9.2 結構測定 .....	47	2.10 實驗設計 .....	47
.....	48	2.10.1 實驗設計的目的與意義 .....	48	2.10.2 假設檢定 .....	48

.....48	2.10.3 變異數分析	.....49	第三章 材料與方法
.....53	3.1 實驗材料	.....53	3.1.1 菌株
.....53	3.1.2 藥品	.....53	3.1.3 培養基
.....55	3.3 實驗方法與流程	.....56	3.2 儀器
.....56	3.3.2 預培養	.....56	3.3.1 活化
.....56	3.3.4 發酵槽培養	.....57	3.3.3 連續式發酵培養
.....59	3.4.1 菌體	.....59	3.4 分析方法
.....59	3.4.2.1 葡萄糖	.....59	3.4.2 碳源
.....59	3.4.3 氮源	.....60	3.4.2.2 丙酸鈉與戊酸鈉
.....61	3.4.4.1 GC條件設定	.....62	3.4.4 PHB與PHBV
.....62	3.5 實驗設計	.....63	3.4.4.2 樣品中PHB與PHBV定量
.....66	4.1 前言	.....66	第四章 結果與討論
.....66	4.2 添加丙酸鈉於連續式發酵生產PHBV	.....66	4.2.1 於C/N 20的連續發酵
.....66	4.2.1.1 菌體生長與PHBV生產	.....67	4.2.1.2 葡萄糖、丙酸鈉與氮源之消耗情形
.....69	4.2.2 於C/N 30的連續發酵	.....74	4.2.2.1 菌體生長與PHBV生產
.....74	4.2.2.2 葡萄糖、丙酸鈉與氮源之消耗情形	.....79	4.2.3 於C/N 40的連續發酵
.....81	4.2.3.1 菌體生長與PHBV生產	.....81	4.2.3.2 葡萄糖、丙酸鈉與氮源之消耗情形
.....86	4.3 添加戊酸鈉於連續式發酵生產PHBV	.....88	4.3.1 於C/N 20的連續發酵
.....88	4.3.1.1 菌體生長與PHBV生產	.....89	4.3.1.2 葡萄糖、戊酸鈉與氮源之消耗情形
.....93	4.3.2 於C/N 30的連續發酵	.....95	4.3.2.1 菌體生長與PHBV生產
.....95	4.3.2.2 葡萄糖、戊酸鈉與氮源之消耗情形	.....100	4.3.3 於C/N 40的連續發酵
.....102	4.3.3.1 菌體生長與PHBV生產	.....102	4.3.3.2 葡萄糖、戊酸鈉與氮源之消耗情形
.....107	4.4 實驗設計-變異數分析	.....109	4.4.1 HB產率
.....109	4.4.2 HV產率	.....109	4.4.2 HV產量
.....115	4.4.4 HV產量	.....115	4.4.3 HB產量
.....120	4.5 結論	.....122	4.4.5 PHBV產率
.....122	4.5.1 比較不同稀釋率的生長情況	.....122	4.5.1 比較不同稀釋率的生長情況
.....122	4.5.2 比較不同C/N比的生長情況	.....122	4.5.2 比較不同C/N比的生長情況
.....122	4.5.3 比較不同酸類基質的生長情況	.....128	4.5.3 比較不同酸類基質的生長情況
.....129	第五章 結論與未來展望	.....128	5.1 結論
.....129	5.2 未來展望	.....129	5.2 未來展望
.....132	參考文獻	.....132	附錄
.....139		.....139	

## REFERENCES

- 王韻婷 (2000) 高分子生物塑膠生產菌之分離、特性分析及其高分子合成基因之選殖, 台灣大學農業化學研究所碩士論文, 台北。
- 王奕隆 (1998) 由Alcaligenes eutrophus生產生物可分解塑膠的能量模式, 大葉大學食品工程學系碩士論文, 彰化。
- 王鼎 (2001) 統計學, 鼎茂圖書出版有限公司, 台北。
- 王建龍和文湘華 (2001) 現代環境生物技術, 清華大學出版社, 北京。
- 沈曉復 (2000) 生物分解性塑膠之發展及認識, 塑膠資訊, 49: 20-32。
- 沈明來 (2000) 生物檢定統計法, 九州圖書文物有限公司, 台北。
- 向明 (1998) 生物技術的發展與應用(田蔚城編彙), 九州圖書, 台北, 151-164。
- 何志煌 (1998) 生物技術的發展與應用(田蔚城編彙), 九州圖書, 台北, 207-212。
- 吳易凡 (2001) 生物分解性塑膠之發展動向, 塑膠資訊, 50: 41-49。
- 林碧洲 (1996) 分解性塑膠的回顧與展望, 產業透析, 34-39。
- 林家慶 (2002) 連續式發酵生產PHBV之研究, 大葉大學食品工程學系碩士論文, 彰化。
- 洪哲穎和陳國誠 (1992) 回應曲面實驗設計法在微生物酵素生產上之應用, 化工, 39(2): 3-18。
- 洪世淇 (2001) 生物分解性塑膠的技術與市場展望, 化工資訊, 61-65。
- 柯志強 (1997) 生物分解性塑膠之發展及認識, 塑膠資訊, 12: 59-71。
- 姜燮堂 (2001) 分解性塑膠, 產業調查與技術, 173: 28-40。
- 黃建銘 (2001) 生物可分解塑膠對環境的益處與未來發展趨勢, 環保月刊, 2(1): 176-181。
- 黃泰銘 (1997) 纖維材料之環保新意識-生物分解性高分子, 紡織速報, 9(5): 18-23。
- 徐惠美 (2000) 生物分解性塑膠, 化工資訊, 81-84。
- 梅樂和 (1999) 生化生產工藝學, 科學出版社, 北京。
- 楊建俊 (1996) 生物分解性高分子之發展及應用, 生物產業, 7(2): 123-127。
- 劉世忠 (2000) 生物可分解性塑膠業, 華銀月刊, 595: 34-38。
- 鄭瑞洲 (1999) 可分解性塑膠, 科技博物, 5(3): 96-100。
- Anderson, A. J. and E. A. Dawes (1990) Occurrence, metabolism, metabolic role and industrial use of bacterial polyhydroxyalkonates, Microbiol., 54 : 450-472.
- Barham, P. J. and A. Selwood (1982) Extraction of poly(beta-hydroxybutyric acid), Eur. Pat. Appl. 58480.
- Berger, E., J. A. Ramsay, B. A. Ramsay, C. Chavarie and G. Braunegg (1989) PHB recovery by hypochlorite digestion of non-PHB biomass, Biotech. Tech., 3 : 227-232.
- Brandl, H., R. A. Gross, R. W. Lenz and R. C. Fuller (1990) Plastics from bacteria and for bacteria, Biochem. Eng., 41 : 77-93.
- Braunegg G., B. Sonnleitner and R. M. Lafferty (1978) Extraction of poly(beta-hydroxybutyric acid), Eur. J. Appl. Microbiol. Biotechnol., 629.
- Bolembergen, S., D. A. Holden, G. K. Hamer and T. L. Bluhm (1986) Studies of composition and crystallinity of Bacterial poly( -hydroxybutyrate-co- -hydroxyvalerate), Macromolecules., 19 : 2865-2871.
- Byrom, D. (1987) Polymer synthesis by microorganism, technology and economics, Biotech., 5 : 246-250.
- Chen, Y., G. Yang; Q. Chen (2002) Solid-state NMR study on the structure and mobility of the noncrystalline region of poly(3-hydroxybutyrate) and

poly(3-hydroxybutyrate-co-3-hydroxyvalerate), *Polymer*, 43 : 2095-2099. 31. Chung, D. M., M. H. Choi, J. J. Song, S. C. Yoon, I. K. Kang and N. E. Huh (2001) Intracellular degradation of two structurally different polyhydroxyalkanoic acids accumulated in *Pseudomonas putida* and *Pseudomonas citronellolis* from mixtures of octanoic acid and 5-phenylvaleric acid, *Macromolecules.*, 29 : 243-250. 32. Cornibert, J. and R. H. Marchessault (1972) Physical properties of Poly- $\beta$ -hydroxybutyrate, IV. Conformational analysis and crystalline structure, *J. Mol. Biol.*, 71 : 735-756. 33. Doi, Y., M. Kunioka, Y. Nakamura and K. Soga (1998) Nuclear magnetic resonance studies on unusual bacterial copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate, *Macromolecules.*, 21 : 2722-2727. 34. Du, G., J. Chen, J. Yu and S. Lun (2001) Continuous production of poly-3-hydroxybutyrate by *Ralstonia eutropha* in a two-stage culture system, *Biotech.*, 88 : 59-65. 35. Du, G., J. Chen, J. Yu and S. Lun (2001) Feeding strategy of propionic acid for production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with *Ralstonia eutropha*, *Biochem. Eng.*, 8 : 103-110. 36. Gao, H. J., Q. Wu and G. Q. Chen (2002) Enhanced production of D-(-)-3-hydroxybutyric acid by recombinant *Escherichia coli*, *Microbiology Letters*, 213 : 59-65. 37. Holmes, P. A. (1985) Applications of PHB-A microbially produced biodegradable thermoplastic, *Phys. Technol.*, 16 : 32-36. 38. Haywood G.W., A. J. Anderson, L. Chu and E.A. Dawes (1988) Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxy-alkanoate synthesizing organism *Alcaligenes eutrophus*, *FEMS Microbiol. Lett.*, 52 : 91-96. 39. Houmiel, K. L., S. Slater, D. Broyles, L. Casagrande, S. Colburn, K. Gonzalez, T. A. Mitsky, S. E. Reiser, D. Shah, N. B. Taylor, M. Tran, H. E. Valentin and K. J. Gruys (1999) Poly( $\beta$ -hydroxybutyrate) production in oilseed leucoplasts of *Brassica napus*, *Planta*, 209 : 547-550. 40. Jan, S., C. Roblot, J. Courtois, B. Courtois, J. N. Barbotin and J. P. Seguin (1996) <sup>1</sup>H NMR spectroscopic determination of poly 3-hydroxybutyrate extracted from microbial biomass, *Enzyme and Microbial Technology*, 18 : 195-201. 41. Juettner R. R., R. M. Lafferty and H. J. Knackmuss (1975) Beta-hydroxybutyrate polymer, *Eur. J. Appl. Microbiol. Biotechnol.*, 1 : 233-236. 42. Kunioka, M., Y. Kawaguchi and Y. Doi (1989) Production of biodegradable co-polyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*, *Appl. Microbiol. Biotechnol.*, 30 : 569-573. 43. Lafferty, R. M. and E. Heinze (1978) Microbiological methods, U.S. Pat. 4,138,291. 44. Lafferty, R. M. and W. Korsatko (1988) Microbial production of poly- $\beta$ -hydroxybutyric acid, *Biotechnol.*, 6b : 135-176. 45. Liu, F. W., L. D. Ridgway, T. Gu and Z. Shen (1998) Production of poly- $\beta$ -hydroxybutyrate on molasses by recombinant *E. coli*, *Biotechnology letters.*, 20 : 345-348. 46. Park, C. H. and V. K. Damodaran (1994) Biosynthesis of poly(3-hydroxybutyrate-co-hydroxyvalerate) from ethanol and pentanol by *Alcaligenes eutrophus*, *Biotechnol. Prog.*, 10: 615-620. 47. Peoples, O. P. and A. J. Sinskey (1989a) Poly- $\beta$ -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16: identification and characterization of the PHB polymerase gene (phbC), *J. Biol. Chem.*, 264 : 15293-15297. 48. Ramsay, B. A., G. M. Znoj and G. C. David (1986) Formal kinetics of poly- $\beta$ -hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H16 and *Mycoplanarubra* R14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures, *Appl. Environ. Microbiol.*, July : 152-156. 49. Schmidt, J., B. Biederman and H. Schmiechen (1985) Ger.(East) Pat. DD 223,428. 50. Slater S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgett and K. J. Gruys (1998) Multiple  $\beta$ -ketothiolases mediate poly( $\beta$ -hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*, *J. Bacteriol.*, 180 : 1979-1987. 51. Vanlautern, N. and J. Gilain (1982) Process for separating poly-beta-hydroxybutyrate from a biomass, U.S. Pat. 4,310,684. 52. Wendlandt, K. D., M. Jechorek, J. Helm and U. Stottmeister (2001) Producing poly-3-hydroxybutyrate with a high molecular mass from methane, *Biotech.*, 86 : 127-133. 53. Williams, J. P. (1989) Production of poly- $\beta$ -hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources, *Appl. Microbiol. Biotechnol.*, 31 : 329-333. 54. Yamane, T. (1993) Yield of poly-D-(3)-hydroxybutyrate from various carbon sources: a theoretical study, *Biotechnol. Bioeng.*, 41 : 165-170. 55. Yan, Y. B., Q. Wu and R. Q. Zhang (2000) Dynamic accumulation and degradation of poly(3-hydroxyalkanoate)s in living cells of *Azotobacter vinelandii* UWD characterized by <sup>13</sup>C NMR, *Microbiology Letters*, 193 : 269-273. 56. Yim, K. S., S. Y. Lee and H. N. Chang (1996) Synthesis of poly-(3-hydroxybutyrate-co-hydroxybutyrate) by recombinant *Escherichia coli*, *Biotechnol. and Bioeng.*, 49 : 495-503. 57. Yu, G. E., R. H. Marchessault (2000) Characterization of low molecular weight poly( $\beta$ -hydroxybutyrate)s from alkaline and acid hydrolysis, *Polymer*, 41 : 1087-1098.