

Production of N-acetylglucosamine by *Chitinibacter tainanensis*

曾士維、?瑞澤 吳淑姿

E-mail: 9510864@mail.dyu.edu.tw

ABSTRACT

N-acetylglucosamine has been used as a treatment for osteoarthritis, inflammatory bowel diseases and as an enhancer of skin moisture. N-acetylglucosamine has also become a popular research topic in the area of food processing and medical applications. Current methods for producing N-acetylglucosamine have advantages and drawbacks. In this study, N-acetyl-glucosamine was produced in a two-stage culture of *Chitinibacter tainanensis*. In the first stage, the medium glucose concentration was increased to create a high cell density. In the second stage, the medium pH was controlled to increase the productivity of N-acetylglucosamine. To investigate the effect of glucose concentration on the biomass, *Chitinibacter tainanensis* was cultivated in media with various glucose concentrations. The highest biomass production was obtained when *Chitinibacter tainanensis* was cultivated in a 0.3% glucose medium. In this case, the microbe was first cultivated in the 0.3% glucose medium, and 8 h later was transferred to a Bushnell-Hass broth containing 2% chitin, the reducing sugars (mainly N-acetylglucosamine) had a maximum productivity of 14.7 g/L after 96 h of incubation. The highest chitinase activity was 725.3 U/L after 72 h of incubation; the N-acetyl-glucosamine production was the highest (15.8 g/L) at 144 h, and the conversion rate from chitin into N-acetyl-glucosamine was 79.0%. To investigate the effect of glucose concentration on the biomass, *Chitinibacter tainanensis* was cultivated in media containing various glucose concentrations. The result showed that the medium containing 2.5% glucose yielded the highest biomass, and therefore, the first stage culture contained 2.5% glucose as a carbon source. When *Chitinibacter tainanensis* was cultivated in a two-stage process in a fermenter, minor modifications were made, such as a set pH of 7 and use of a modified BH-1 medium containing 2.5% glucose. With this process, the maximum biomass (2.42 g/L) was attained at 24 h. In the second stage, the culture was transferred to a modified medium containing 2% chitin (designated as Medium BH-2). At 96 h, the biomass, reducing sugars and chitinase activity reached their maximums of 1.99 g/L, 16.1 g/L, and 800.3 U/L, respectively. During cultivation of *Chitinibacter tainanensis*, chitin was hydrolyzed into N-acetylglucosamine with a maximum production of 15.6 g/L at 72 h, and a chitin conversion rate of 77.8%. When *Chitinibacter tainanensis* was cultivated in a two-stage culture, the first stage culture was transferred into the second stage with a modified BH-2 medium after 24 h. The pH in the culture was not regulated at this stage and fell rapidly from 7.0 to 5.31 by the 48 h. Maximum chitinase activity (710 U/L) was reached at 72 h, and the reducing sugars reached maximum concentration (16.7 g/L) at 96 h. The production of N-acetylglucosamine in the second stage was highest (16.1 g/L) at 120 h, and the chitin conversion rate was 80.1%. When the chitin concentration was raised to 4%, *Chitinibacter tainanensis* was cultivated using a two-stage fermentation with no pH control in the second stage. The maximum concentrations of reducing sugars (33.7 g/L) and biomass (2.47 g/L) were both achieved at 120 h. The chitinase activity reached the highest (650.0 U/L) at 72 h. N-acetyl-glucosamine production was highest (31.2 g/L) at 96 h, when the chitin conversion rate was 78.0%.

Keywords : N-acetylglucosamine ; *Chitinibacter tainanensis* ; two-stage culture

Table of Contents

授權書iii	中文摘要iv	英文摘要vi	致謝viii	目錄ix	圖目錄xii	表目錄xiv	第一章 緒言1	第二章 文獻回顧3	2.1 幾丁質3	2.1.1 幾丁質類之分子結構與性質3	2.1.2 幾丁質之功能與應用4	2.1.3 幾丁質之製備7	2.2 幾丁質?7	2.2.1 幾丁質水解酵素之分類7	2.2.2 幾丁質? 漣@用形機制8	2.2.3 幾丁質? 漱掬M分佈11	2.2.4 幾丁質? 瑰野?3	2.2.5 幾丁質? 吨艦R14	2.3 N-乙醯葡萄糖胺15	2.3.1 N-乙醯葡萄糖胺的功能與應用16	2.4 N-乙醯葡萄糖胺之製造方法17	2.4.1 化學法17	2.4.2 酵素水解法17	第三章 材料與方法20	3.1 實驗架構20	3.2 實驗儀器20	3.3 實驗藥品22	3.4 培養基23	3.4.1 基礎培養基23	3.4.2 發酵培養基23	3.5 實驗菌株25	3.5.1 <i>Chitinibacter tainanensis</i> 25	3.5.2 菌種保存25	3.6 菌體顯微鏡觀察25	3.6.1 光學顯微鏡之觀察25	3.6.2 位相差顯微鏡之觀察25	3.7 膠態幾丁質之製備27	3.8 批次發酵槽培養27	3.8.1 批次發酵培養條件27	3.8.2 操作步驟29	3.9 分析方法30	3.9.1 生長曲線30	3.9.2 N-乙醯葡萄糖胺? 30	3.9.3 還原醣31	3.9.4 生質量31	3.9.5 殘餘葡萄糖31	3.9.6 水解產物32	第四章 結果與討論33	4.1 菌株基本特性33	4.2 菌株 <i>Chitinibacter tainanensis</i> 之生長型態33	4.3 菌株 <i>Chitinibacter tainanensis</i> 之生長曲線33	4.4 葡萄糖濃度的效應(搖瓶培養)37	4.5 葡萄糖濃度的效應(發酵槽培養)44	4.6 <i>Chitinibacter tainanensis</i> 之兩階段培養44	4.6.1 pH調控47	4.6.2 第二階段培養pH不調控49	4.6.3 以4%幾丁質於發酵槽之培養51	第五章 結論55	參考文獻57	附錄64	圖目錄	圖2.1 纖維素、幾丁質及幾丁聚糖之結構5	圖2.2 幾丁質之製備9	圖2.3 幾丁質酵素的水解路徑10	圖2.4 以化學法製造N-乙醯葡萄糖胺18	圖3.1 實驗架構流程圖21	圖4.1 於位相差顯微鏡下(1,000x)觀察
--------	--------	--------	--------	------	--------	--------	---------	-----------	----------	---------------------	------------------	---------------	-----------	-------------------	--------------------	--------------------	-----------------	------------------	----------------	------------------------	---------------------	-------------	---------------	-------------	------------	------------	------------	-----------	---------------	---------------	------------	---	--------------	---------------	------------------	-------------------	----------------	---------------	------------------	--------------	------------	--------------	--------------------	-------------	-------------	---------------	--------------	-------------	--------------	---	---	----------------------	-----------------------	---	--------------	---------------------	-----------------------	----------	--------	------	-----	-----------------------	--------------	-------------------	-----------------------	----------------	-------------------------

之Chitinibacter tainanensis 34 圖4.2 Chitinibacter tainanensis培養於膠態幾丁質平板培養基 35 圖4.3 Chitinibacter tainanensis 於BH培養基中之生長曲線 36 圖4.4不同葡萄糖濃度培養Chitinibacter tainanensis之生質量的變化 38 圖4.5不同葡萄糖濃度培養Chitinibacter tainanensis之pH的變化 39 圖4.6以2%幾丁質粉末之BH培養基培養Chitinibacter tainanensis之N-乙醯葡萄糖胺? “吡B還原糖及pH的變化 40 圖4.7 Chitinibacter tainanensis生產之N-乙醯葡萄糖胺的高效能液相層析圖 42 圖4.8以2%幾丁質粉末BH培養基培養Chitinibacter tainanensis 生產N-acetylglucosamine 43 圖4.9不同葡萄糖濃度培養於Chitinibacter tainanensis 之生質量變化 45 圖4.10不同葡萄糖濃度於Chitinibacter tainanensis培養過程之葡萄糖的變化 46 圖4.11 Chitinibacter tainanensis於發酵培養基中N-乙醯葡萄糖胺之變化 48 圖4.12 Chitinibacter tainanensis於2.5%葡萄糖生長培養基中生質量、葡萄糖及pH值之變化 50 圖4.13 Chitinibacter tainanensis於發酵培養基中幾丁質? “吡B還原糖及生質量之變化 52 表目錄 表3.1 基礎培養基 24 表3.2 發酵培養基 24 表3.3微量金屬溶液組成 26 表3.4 Chitinibacter tainanensis 之型態特性分析 28 表3.5 Chitinibacter tainanensis 之生理特性分析 28 表 4.1以不同培養條件培養菌株Chitinibacter tainanensis之活性、還原醣、生質量、N-乙醯葡萄糖胺產量及轉化率之變化 54

REFERENCES

- 中文部份 1. 李宜玲、吳淑姿、余世宗、?瑞澤。2005。具分解幾丁質能力菌株之篩選。台灣農業化學與食品科學43(6): 410-418。 2. 阮勝威。1996。由靈芝子實體經萃取後之廢渣所製成之薄膜對於天竺鼠傷口及組織纖維母細胞之影響。第12-14頁。臺北醫學院醫學研究所碩士論文。台北。 3. 徐世昌。2001。生物性高分子 幾丁質與幾丁聚醣之介紹與應用。化工資訊15(2): 36-45。 4. 陳俊任。1993。Aeromonas sp. No.16.所生產幾丁質分解酵素之研究。第61-69頁。國立台灣大學農業化學研究所碩士論文。台北。 5. 陳錦坤。2005。以生物轉化法生產N-乙醯葡萄糖胺之研究。台灣幾丁質幾丁聚醣研討會:第98-99頁。5月28日。南台科技大學化學工程與材料工程系、台灣幾丁質幾丁聚醣學會。台南。 6. 陳錦坤、方炳勳、林忠亮、蔡丞佳、許清輝、廖權能、魏國銘、黃冬梨、吳奇生。2005。以共培養方式探討Chitinibacter tainanensis 生產N-乙醯葡萄糖胺的作用機轉。台灣幾丁質幾丁聚醣研討會:第173-177頁。5月28日。南台科技大學化學工程與材料工程系、台灣幾丁質幾丁聚醣學會。台南。 7. 諸葛健。2005。現代發酵微生物實驗技術。第45-47頁。化學工業出版社。北京，中國。 8. 蘇南維、李敏雄。1998。Listonella damsela NTU-FC-6幾丁質酵素之生產與基本性質之探討。中國農業化學會誌。36(1):65-76。 英文部份 1. Aiba, S. 1992. Studies on chitosan: 4. Lysozymic hydrolysis of partially N-acetylated chitosans. Int. J. Biol. Macromol. 14:225-228. 2. Aiba, S. 1993. Studies on chitosan: 6. Relationship between N-acetyl group distribution pattern and chitinase digestibility of partially N-acetylated chitosans. Int. J. Biol. Macromol. 15: 241-245. 3. Bhushan, B. 2000. Production and characterization of a thermostable chitinase from a new alkalophilic Bacillus sp. BG-11. J. Appl. Microbiol. 88: 800-808. 4. Boller, T., Gehri, A., Mauch, F. and Vogeli, U. 1990. Chitinase in bean leaves: induction by ethylene, purification, properties and possible function. Planta. 157: 22-31. 5. Busam, G., Kassemeyer, H. H. and Matern, U. 1997. Differential expression of chitinase in Vitis vinifera L responding to systemic acquired resistance activators or fungal challenge. Plant Physiol. 115: 1029-1038. 6. Chandy, T., and Sharma, C. P. 1992. Chitosan beads and granules for oral sustained delivery of nifedipine: in vitro studies. Biomaterials. 13: 949-952. 7. Chandy, T. and Sharma, C. P. 1993. Chitosan matrix for oral sustained delivery of ampicillin. Biomaterials. 14: 939-944. 8. Chern, L. L., Stackbrandt, E., Lee, S. F., Lee, F. L., Chen, J. K and Fu, H. M. 2004. Chitinibacter tainanensis gen. nov., sp. nov., a chitin-degrading aerobe from soil in Taiwan. Int. J. Syst. Evol. Microbiol. 54: 1387-1391. 9. Chung, L.Y., Schmidt, R. J. and Hamlyn, P. F. 1994. Biocompatibility of potential wound management products: fungal mycelia as a source of chitin/chitosan and their effect on the proliferation of human F1000 fibroblast in culture. J. Biomed. Mat. Res. 28: 463-469. 10. Clendennen, S. P., Jacobs, A. K. and Dry, I. B. 1997. Differential gene expression in ripening banana fruit. Plant Physiology. 115: 463-469. 11. Deshpande, M.V. 1986. Enzymatic degradation of chitin and its biological application. J. Sci. Ind. Res. 45: 273-275. 12. Drovanti, A., Bignamini, A. A. and Rovati, A. L. 1980. Therapeutic activity of oral glucosamine sulfate in osteoarthritis: A placebo-controlled double-blind investigation. Clinical Therapeutics, 3: 260-272. 13. Elad, Y. I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by Trichoderma harzianum. Can. J. Microbiol.. 28: 719-725. 14. Escott, G. M., Adam, D. J. 1995. Chitinase activity in human serum and leukocyte. Infect. Immun. 63: 4770-4773. 15. Evans, E. E. 1962. The use of basic polysaccharides in histochemistry and cytochemistry: IV. Precipitation and agglutination of biological materials by Aspergillus polysaccharides and deacetylated chitin. J. Histochem. Cytochem. 10: 24-28. 16. Frandberg, E. and Schnurer, J. 1994. Evaluation of a chromogenic chito- oligosaccharide analogue, p-nitrophenyl-N, N' -diacetylchitobiose, for the measurement of the chitinolytic activity of bacteria. J. Appl. Bacteriol. 76: 259-263. 17. Fukada, Y., Kimura, K. and Ayaki, Y. 1991. Effect of chitosan feeding on intestinal bile acid metabolism in rats. Lipids. 26: 395-399. 18. Gomez Ramirez, M., Rojas Avelizapa, L. I., Rojas Avelizapa, N. G. and Cruz Camarillo, R. 2004. Colloidal chitin stained with Remazol Brilliant blue R, a useful substrate to select chitinolytic microorganisms and to evaluate chitinases. J. Microbiol. Meth. 56(2): 213-219. 19. Haran, S., Schickler, H. and Chet, I. 1996. Molecular mechanisms of Chitinolytic enzymes involved in the biocotrol activity of Trichoderma harzianum. Canadian Journal of Microbiology. 142: 2321-2331. 20. Herrmann, J. B. and Woodward, S. C. 1972. An experimental study of wound healing accelerators. Am. Surg. p 26-34. 21. Hiraga, K., Oda, K. and Aiba, S. I. 2002. Production of N-acetyl-D-glucosamine from -chitin by crude enzymes from Aeromonas hydrophila H-2330. Carbohydr. Res. 337: 761-763. 22. Imoto, T and K. Yagishita. 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem. 35: 1154-1156. 23. Jeuniaux, C. 1966. In methods in Enzymology. p.644-654. Academic Press. New York, USA. 24. Kamel, M. and Alnahdi, M. 1992. Inhibition of superoxide anion release from human polymorphonuclear leukocytes by N-acetyl-galactosamine and N-acetyl- glucosamine. Clinical Rheumatology. 11(2): 254-260. 25. Kajimoto, O., Sakamoto, K., Takamori, Y.,

Kajitani, N., Imanishi, T., Matsuo, R. and Kajitani, Y. 1998. Therapeutic activity of oral glucosamine hydrochloride in osteoarthritis of the knee: A placebo-controlled, double-blind, cross-over study. *Nippon Rinsho Eiyo Gakkaishi*. 20: 41-47.

26. Knorr, D. 1984. Use of chitinous polymer in food. *Food Technol.* 1: 85-88.

27. Kuk, J. H., Jung, W. J., Jo, G. H., Kim, Y. C., Kim, K. Y. and Park, R. D. 2005. Production of N-acetyl-D-glucosamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. *Appl. Microbiol. Biotechnol.*68(3): 384-389.

28. Kumar, R. and Majeti, N. V. 2000. A review of chitin and chitosan applications. *Reactive and functional polymers*. 46: 1-27.

29. Leah, R., Tommeruo, H., Svendsen, I. and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* 266(3) : 1564-73.

30. Lorito, M. 1996. Mycoparasitic interaction relieves binding of the Crel carbon catabolite repressor protein to promoter sequences of the ech42 (endochitinase-encoding) gene in the *Trichoderma harzianum*. *Proceeding of the National Academy of Sciences of the United States of America*. 93: 14868-14872.

31. Mori, T., Okumura, M., Matsura, M., Ueno, K., Tokura, S., Okamoto, Y., Minami, S. and Fujinaga, T. 1997. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. *Biomaterials*. 18: 947-951.

32. Molano, J., Duran, A. and Cabib, E. 1977. A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* 83(2): 648-656.

33. Muzzarelli, R. A. A. 1985. In the polysaccharides, Aspinall, G. O.(Ed.), p.427-451.

34. Muzzarelli, R. A., Mattioli-Belmonte, M. and Tietz, C. 1994. Stimulatory effect on bone formation exerted by modified chitosan. *Biomaterials*. 15: 1075-1081.

35. Nakajima, M., Atsumi, K., Kifune, K. 1986. Chitin is an effective material for sutures. *Japan. J. Surg.* 16: 418-424.

36. Nishimura, K., Nishimura, S., Seo, H., Nishi, N., Tokura, S. and Azuma, I. 1987. Effect of multiporous microspheres derived from chitin on the activation of mouse peritoneal macrophages. *Vaccine*. 5: 136-140.

37. Nishimura, S., Nishi N., Tokura, S. 1986. Bioactive chitin derivatives. Activation of mouse-peritoneal macrophages by O-carboxymethyl chitins. *Carbohydr. Res.*146: 251-258.

38. Otakara, A., Mitsutomi, M. and Uchida, Y. 1979. Purification and some properties of chitinase from *Vibrio* sp. *J. Ferment. Technol.* 57(3): 169-177.

39. Overdijk, B. and Steijn, G. J. V. 1994. Human serum contains a chitinase: identification of an enzyme, formerly described as 4-methylumbelliferyl-tetra- N-acetylchitotetraoside hydrolase (MU-TACT hydrolase), *Glycobiology*. 4(6): 797-803.

40. Peluso, G., Petillo, O. and Ranieri M. 1994. Chitosan-mediated stimulation of macrophage function. *Biomaterials*. 15: 1215-1220.

41. Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinit, M. and Aiba, S. I. 2002. Quantitative production of 2-acetamido-2-deoxy- D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337: 557-559.

42. Revah-Moiseev, S. and Carroad, A. 1981. Conversion of the enzymatic hydrolysate of shellfish waste chitin to single-cell protein. *Biotechnol. Bioeng.* 23: 1067-1078.

43. Roberts, W. K. and Selitrennikoff, C. P. 1988. Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol.* 134: 169-176.

44. Salvatore, S., Heuschkel, R., Tomlin, S., Davies, S. E., Walker-Smith, J. A., French, I. and Murch, S. H. *Aliment. Pharmacol. Ther.* 2000. 14:1567-1579.

45. Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Sukwattanasinit, M., Pichyangkura, R. and Aiba, S. I. 2003. Enzymatic production of N-acetyl-D-glucosamine from chitin. Degradation study of N-acetyl- chitooligosaccharide and the effect of mixing of crude enzymes. *Polym.* 51: 391-395.

46. Shyu, S. S., Mi, F. L., Wu, Y. B., Lee, S. T., Shyong, J. Y. and Huang, R. N. 2001. Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials*. 22: 165-173.

47. Sirica, A. E. 1971. Selective aggregation of L1210 leukemia cells by the polycation chitosan. *J. Nat. Cancer Inst.* 47: 377-388.

48. Suzuki, S., Okawa, Y., Okura, Y., Hashimoto, K. and Suzuki, M. 1982. Proceedings of the second international conference on chitin and chitosan. Sapporp, Japan., p. 210-212

49. Suzuki, S., Watanabe, T., Mikami, T., Matsumoto, T. and Suzuki, M. 1992. Immuno-enhancing effects of N-acetyl-chitohexanose. In advance in chitin and chitosan, p 96-105.

50. Usami, Y., Okamoto Y. and Minami, S. 1994. Chitin and chitosan induce migration of bovine polymorphonuclear cells. *J. Veter. Med. Sci.* 56: 761-762.