

# Optimal Cultivation of Aeromonas veronii DYU-Too19 in Chitin Degradation

盧沛誼、吳淑姿

E-mail: 381992@mail.dyu.edu.tw

## ABSTRACT

Chitin, a polymeric carbohydrates, is widely distributed, and abundant just next to cellulose. When chitin is degraded into N-acetyl-chitooligosaccharides that could have applications in the functional food and environmental protection. N-acetyl-chitooligosaccharides can be obtained by the chitinase method that is more favorable over the alkaline treatment. In this study, a strain, *Aeromonas veronii* DYU-Too 19, was isolated from Dacun Village in Changhua County. The aim of this study was to investigate an optimal condition for the production of reducing sugars by this strain. The effects of carbon and nitrogen sources on the production of reducing sugars, chitinase activity, and pH value were first examined. The one-factor-at-a-time method was first used to investigate the effects of carbon and nitrogen sources on the production of reducing sugars. When  $\alpha$ -chitin was the sole carbon source, higher amounts of reducing sugars were produced. Especially a highest production of reducing sugars (15.4 g/L) was obtained in a medium containing 4%  $\alpha$ -chitin; Peptone seemed to be a better nitrogen source to produce reducing sugars, and 12.5 g/L of reducing sugars were obtained in a medium containing 0.5 g/L peptone. In addition, the response surface methodology was used to search for an optimal condition for culturing *A. veronii* DYU-Too 19. From the analysis of variance, both of the  $\alpha$ -chitin and peptone concentrations were significant for the strain to produce reducing sugars ( $p < 0.05$ ) and the optimal concentrations of  $\alpha$ -chitin and peptone were 4.42% and 0.55 g/L, respectively. The predicted maximal production of reducing sugars was 20.52 g/L. In addition, comparison between the actual observation (20.24 g/L) at the optimal condition and the predicted response showed a close agreement. This indicates that the response surface model could well predict the reducing sugars production of *A. veronii* DYU-Too 19.

Keywords : *Aeromonas veronii* DYU-Too 19、Chitinase, Reducing sugars、Response surface methodology、Optimum condition

## Table of Contents

簽名頁 中文摘要 iii 英文摘要 v 誌謝 viii 目錄 xi 表目錄 xiv 1. 緒論 1 2. 文獻回顧 2 2.1 幾丁質與幾丁聚醣 2 2.2 幾丁質之應用 8 2.3 幾丁質之固態結晶 11 2.4 幾丁質? 12 2.5 N-乙醯幾丁寡醣與幾丁寡醣 13 2.6 反應曲面法 14 3. 材料與方法 16  
3.1 實驗架構 16 3.2 實驗藥品 16 3.3 實驗器材 18 3.4 試劑及培養基之配置 19 3.4.1 培養基組成 19 3.4.2 McIlvaine buffer 21 3.4.3 呈色劑 21 3.4.4 膠態幾丁質之製備 21 3.5 酶素法製備N-乙醯幾丁寡醣 23 3.5.1 菌株篩選 23 3.5.2 菌株保存與活化 23 3.5.3 還原醣含量之測定 23 3.5.4 幾丁質分解?活性分析 24 3.5.5 菌株生長曲線測定 24 3.5.6 幾丁質水解產物之HPLC分析 24 3.6 菌株最適培養之實驗設計 25 4. 結果與討論 28 4.1 幾丁質分解菌株之篩選 28 4.1.1 菌株篩選於培養基之生長情形 28 4.1.2 幾丁質?之性質 28 4.1.3 篩選菌株之水解產物分析 34 4.2 菌株DYU-Too19之基本特性分析 37 4.3 碳源對*Aeromonas veronii* DYU-Too19之影響 37 4.3.1 幾丁質?活性之分析 40 4.3.2 還原醣量與pH值變化 40 4.3.3 碳源濃度 44 4.4 氮源 52 4.4.1 幾丁質?活性之分析 56 4.4.2 還原醣量與pH值變化 56 4.4.3 Peptone濃度 60 4.5 幾丁質分解效率之最適培養 60 4.5.1 實驗設計 64 4.5.2 實驗結果 64  
5. 結論 76 5.1 結論 76 5.2 展望 77 參考文獻 78 附錄 85 圖目錄 圖2.1葡萄糖胺(幾丁聚醣之單體)與葡萄糖(纖維素之單體) 3  
圖2.2 幾丁質經由鹼液去乙醯處理生成幾丁聚醣 5 圖2.3 幾丁質之去乙醯作用：幾丁質經由幾丁質脫乙醯?形成幾丁聚醣及醋酸 6 圖2.4 纖維素、幾丁質及幾丁聚醣之結構 7 圖2.5 -幾丁質之分子排列結構 9 圖2.6 -幾丁質之分子排列結構 10  
圖3.1 實驗流程圖 17 圖4.1 菌株E6、K2及A2於膠態幾丁質培養基產生透明環之型態 30 圖4.2 以CB培養基培養菌株E6、K2及A2之幾丁質分解?活性 31 圖4.3 以CB培養基培養菌株E6、K2及A2之還原醣生成量 32 圖4.4 以CB培養基培養菌株E6、K2及A2之pH值變化 33 圖4.5 *Aeromonas veronii* DYU-Too 19生產之N-乙醯幾丁寡醣高效能液相層析圖 36 圖4.6 *Aeromonas veronii* DYU-Too 19之16S rDNA部份核甘酸序列 38 圖4.7 菌株*Aeromonas veronii* DYU-Too 19於LB培養基中之生長曲線圖 39 圖4.8 不同碳源對*Aeromonas veronii* DYU-Too 19幾丁質分解?活性之影響 41 圖4.9 不同碳源對*Aeromonas veronii* DYU-Too 19還原醣生成量之影響 42 圖4.10 不同碳源培養*Aeromonas veronii* DYU-Too 19之pH值變化 43 圖4.11 不同 -幾丁質濃度對*Aeromonas veronii* DYU-Too 19幾丁質分解?活性之影響 45 圖4.12 不同 -幾丁質濃度對*Aeromonas veronii* DYU-Too 19還原醣生成量之影響 47 圖4.13 不同 -幾丁質濃度培養*Aeromonas veronii* DYU-Too 19之pH值變化 48 圖4.14 不同 -幾丁質濃度對*Aeromonas veronii* DYU-Too 19幾丁質分解?活性之影響 49 圖4.15 不同 -幾丁質濃度對*Aeromonas veronii* DYU-Too 19還原醣生成量之影響 50 圖4.16 不同 -幾丁質濃度培養*Aeromonas veronii* DYU-Too 19之pH值變化 51 圖4.17 不同膠態幾丁質濃度對*Aeromonas veronii* DYU-Too 19幾丁質分解?活性之影響 53 圖4.18 不同膠態幾丁質濃度對*Aeromonas veronii* DYU-Too 19還原醣生成量之影響 54 圖4.19 不同膠態幾丁質濃度培養*Aeromonas veronii* DYU-Too 19

之pH值變化 55 圖4.20 氮源對Aeromonas veronii DYU-Too 19幾丁質分解?活性之影響 57 圖4.21 氮源對Aeromonas veronii DYU-Too 19還原醣生成量之影響 58 圖4.22 氮源培養Aeromonas veronii DYU-Too 19之pH值變化 59 圖4.23 Peptone濃度對Aeromonas veronii DYU-Too 19幾丁質分解?活性之影響 61 圖4.24 Peptone濃度對Aeromonas veronii DYU-Too 19還原醣生成量之影響 62 圖4.25 Peptone濃度培養Aeromonas veronii DYU-Too19之pH值變化 63 圖4.26 -chitin與peptone濃度對還原醣產量影響之反應曲面圖 70 圖4.27 -chitin powder與peptone對還原醣產量之等高線圖 71 圖4.28 菌株Aeromonas veronii DYU Too-19於最適條件培養 73 圖4.29 還原醣產量預測值與觀測值之殘差分析圖 74 表目錄 表2.1 幾丁聚醣及幾丁質來源 4 表3.1 培養基之組成 20 表3.2 McIlvaine緩衝溶液 22 表3.3 中心混成實驗設計表 26 表4.1 幾丁質分解菌株之篩選 29 表4.2 以2% -幾丁質培養菌株E6、A2 及K2之N-乙醯幾丁寡醣種類及含量 35 表4.3 中心混成設計實驗之控制因子 65 表4.4 中心混成試驗之結果 67 表4.5 兩因子中心混成實驗之複迴歸分析表 68 表4.6 反應曲面模式之變異數分析表 69 表4.7 實際實驗數據與回應曲面模式所預估的預測值比較 75

## REFERENCES

1. 林榮耀(總校閱)。2008。Stryer's生物化學第六版。藝軒圖書。台北。
2. 洪哲穎、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。化工專論 39(2):3-18。
3. 洪碧雲。2003。蜡蚧輪枝菌幾丁聚醣?之特性分析及應用研究。朝陽科技大學應用化學所碩士論文。台中。
4. 莊榮輝。2000。酵素化學實驗。國立台灣大學農業化學系生物化學研究室。台北。
5. 連德昇。2002。以本土菌株分解幾丁質生產N-乙醯幾丁寡醣之研究。大葉大學食品工程研究所碩士論文。彰化。
6. 陳嘉芬。2006。細胞生物學。藝軒圖書。台北。
7. 張讚昌、邱南昌、尤封陵、施玲玲、朱敬儀、謝雅如、王瑜琦、李文珍。2003。實用微生物學(含免疫學)。華格那發行。台中。
8. 張玉瓈、徐乃芝、許素菁。2008。生物技術。新文京開發。新北市。
9. 張育祥。2011。菌株 *Bacillus sp.* DYU-Too17 生產N-乙醯幾丁寡醣之最適培養條件。大葉大學生物產業學系研究所論文。彰化。
10. 謝伊金。2007。N-乙醯幾丁寡醣生產菌之篩選與幾丁質分解?之特性分析。大葉大學生物產業學系研究所論文。彰化。
11. 蕭正明。2009。限氮條件下添加有機酸對菌株Yu-3生和成PHBV之影響。大葉大學生物產業學系研究所論文。彰化。
12. Adrangi, S., Mohammad, A. F., Ahmad, R. S, Zargham Sepehrizadeh. 2010. Purification and characterization of two extracellular endochitinases from *Massilia timonae*. Carbohydrate Research., 345:402-407.
13. Bing-Lan Liu., Pao-Min Kao., Yew-Min Tzeng., Kuo-Ching Feng. 2003. Production of chitinase from *Verticillium lecanii* F091 using submerged fermentation. Enzyme and Microbial Technology ., 33:410 – 415.
14. Blanes, M., Pilar, M. D. and Jesus, F. 2007. Inmunosupresion e infección en el paciente transplantado. Enfermedades Infecciosas y Microbiología Clínica., 27(2):143-154.
15. Bhattacharya, D., Nagpure, A., Gupta, R.K. 2007. Bacterial chitinases: properties and potential. Crit. Rev. Biotechnol., 27: 21 – 28.
16. Chre' tiennot-Dinet M-J., Giraud-Guille M-M., Vaulot, D., Putaux, J-L. and Chanzy, H. 1997. The chitinous nature of filament ejected by *Phaeocystis* (Prymnesiophyceae). J Phyco., 33:666 – 672.
17. Chang, K. L., Lee, J. and Fu, W. R. 2000. HPLC analysis of N-acetyl-chito-oligosaccharides during the Acid Hydrolysis of Chitin. J. Food Drug Anal. 8(2):75-83.
18. Choi, J.H., Choi, B.K., Kim, K.Y., Yoo, Y.J., Oh, S.J. 2001. In vitro antimicrobial activity of a chitooligosaccharide mixture. International Journal of Antimicrobial Agents., 18:553 – 557.
19. Chen, C.T., Chien-Jui Huang., Yi-Huei Wang, and Chao-Ying Chen. 2004 .Two-step purification of *Bacillus circulans* chitinase A1 expressed in *Escherichia coli* periplasm. Protein Expression and Purification, 37: 27 – 31.
20. Fenice, M., Selbmann, L., Giambattista, RD., Federici, F. 1998 .Chitinolytic activity at low temperature of an Antarctic strain (A3) of *Verticillium lecanii*. Res Microbiol., 149: 289 – 300.
21. Felse, PA., Panda, T. 1999 .Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Process Biochem., 34: 563 – 566.
22. Fiandra, L., Maro, A. D., Terracciano, I., Sticco, L., Ruocco, M., Corrado, G., Parente, A., Rosa, R. 2010. Journal of Biotechnology., 147:1-6.
23. Frandberg, E., Schnurer, J. Chitinolytic properties of *Bacillus pabuli* K1. 1994 . J Appl Bacteriol ., 76: 361 – 367.
24. Hadwiger, L. A., Kendra, D. F., Fristensky, B. W., and Wagoner, W. 1985. Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. In chitin in Nature and Technology. p. 209-222.
25. Hart, P.J., Pfluger, H.D., Monzingo, A.F., Hollis, T., Robertus, J.D. 1995. The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 Å resolution. J. Mol. Biol., 248: 402 – 413.
26. Hackman, R. H. 1960. Studies on chitin. IV. The nature of - and -chitin. J. Biol. Sci. 18: 935.
27. Hirano, S. and Midorikawa, T. 1998. Novel method for the preparation of N-acylchitosan fiber and N-acylchitosan – cellulose fiber. Biomaterials. 19:293 – 7.
28. Huang, R., Nirajan, R. and Se-Kwon., K. 2006. Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives. Carbohydr. Polymers., 63:122-129.
29. Izabela, S. Santos., Maura Da Cunha, Olga L.T. Machado., Valdirene, M. Gomesa. 2004. A chitinase from *Adenanthera pavonina* L. seeds: purification, characterisation and immunolocalisation. Plant Science.167: 1203 – 1210.
30. Jang, M.-K., Kong, B.-G., Jeong, Y.-I., Lee, C. H., & Nah, J.-W. 2004. Physicochemical characterization of a-chitin, b-chitin and c-chitin separated from natural resources. Journal of Polymer Science: Part A: Polymer Chemistry, 42:3423 – 3432.
31. Jeuniaux, c. 1966. Chitinases. Methods Enzymol., 8: 644-650.
32. Jie, F., Luhang, Z., Qiqi, Y. 2004. Receptor-mediated stimulatory effect of oligochitosan in macrophages. Biochem. biophys. res. commun. 317: 414-420.
33. Kawada, M., Hachiya, Y., Arihiro, A., Mizoguchi, E. 2007. Role of mammalian chitinases in inflammatory conditions. Keio J. Med., 56:21-27.
34. Keyhani, NO., Roseman, S. 1996 .The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Molecular cloning, isolation, and characterization of a periplasmic b-N-acetylglucosaminidase. J Biol Chem. 271:425 – 432.
35. Krajewska, B. 2004. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme Microbiol Technol., 35:126 – 139.
36. Kumar, R., Majeti, N. V. 2000. A review of chitin and chitosan applications. Reactive and functional polymers 46 :1-27.
37. Marguerite, Rinaudo. 2006. Chitin and chitosan:Properties and applications. Prog. Polym. Sci. 31:603-632.
38. Mahadevan, B., Crawford, DL. 1997 .Properties of the

chitinase of the antifungal agent *Streptomyces lydicus* WYEC108. *Enzyme Microb Technol.*, 20:489 – 493. 39. Mathur, NK., Narang, CK. 1990. Chitin and chitosan, versatile polysaccharides from marine animals. *J Chem Educ.*, 67:938 – 942. 40. Matahira, Y., Ito, M. and Sakai, K. 1998. The application of chitin-chitosan to bone filling materials. *Kichin Kitosan Kenkyu: Publ. Nippon Kitin, Kitosan Gakkai.* 4:142-143. 41. Maro, A.D., Terracciano, I., Sticco, L., Fiandra, L., Ruocco, M., Corrado, G., Parente, A., Rosa R 2010. Purification and characterization of a viral chitinase active against plant pathogens and herbivores from transgenic tobacco. *Journal of Biotechnology*147:1-6.42. McKay, G., Blair, H.S. and Gardner, J.R. 2000. Adsorption of dyes on beads and microgranules of chitosan, *Biomaterials* 21 chitin. I. Equilibrium studies, *J. Appl. Polym. Sci.*, 27:1115– 3043. 43. Molinari, L. M., Raissa, Bocchi, Pedroso., Denise, de Oliveira Scoaris , Tania Ueda-Nakamura., Celso, Vataru Nakamura., Benedito, Prado Dias Filho. 2007 .Identification and partial characterisation of a chitinase from Nile tilapia, *Oreochromis niloticus Comparative Biochemistry and Physiology.*, Part B 146:81 – 87. 44. Ohishi, K., Yamagishi, M., Ohta, T., Suzuki, M., Izumida, H., Sano, H. et al. 1996 . Purification and properties of two chitinases from *Vibrio alginolyticus* H-8. *J Ferment Bioeng.*, 82:598 – 600. 45. Patel, A. K., Vijay, Kumar Singh., Ravi, Prakash Yadav., Arthur, J.G. Moir, Medicherla. V. Jagannadham. 2010. Purification and characterization of a new chitinase from latex of *Ipomoea carnea*. *Process Biochemistry.*,45 :675 – 681. 46. Patil, RS., Ghormade, V. 2000. Deshpande MV. Chitinolytic enzymes: an exploration. *Enzyme Microb Technol.*, 26:473 – 483. 47. Pera, LM., Infante, Majoll?, MV., Baigor?, MD. 1997. Purification and characterization of a thermostable and highly specific b-N-acetyl-Dglucosaminidase from *Aspergillus niger* 419. *Biotechnol Appl Biochem.*,26:183 – 187. 48. Pillai, C. K. S., W. Paul and C. P. Sarma, 2009. Chitin and chitosan polymers: Chemistry,solubility and fiber formation. *Progress in Polymer Science.* 34:641-678. 49. Rajapakse, N., Moon, M. K., Eresha, M., Ronghua, H. and Se K. K. 2006. Carboxylated chitooligosaccharides (CCOS) inhibit MMP-9 expression in human fibrosarcoma cells via down-regulation of AP-1. *Biocmica Biophysica et Acta.*, 1760:1780-1788.50. Rinaudo, M. 2006. Chitin and chitosan: Properties and applications. *Prog. Polym. Sci.* 31:603-632. 51. Rogalski, J., Krasowska, B., Glowik, G., Wojcik, W., Targonski, Z. 1997. Purification and some properties of extracellular chitinase produced by *Trichoderma viride* F-19. *Acta Microbiol Pol.*, 46:363 – 375. 52. Tagawa, K., Okazaki, K. 1991. Isolation and some cultural conditions of *Streptomyces* species which produce enzymes lysing *Aspergillus niger* cell wall. *J Ferment Bioeng.*, 71:230 – 236. 53. Tao, Y. Yan, Z-Z., Sun, J-W., Cheng, X-L., Zhang, S-Z. 1990 .Purification and characterization of b-N-acetylhexosaminidase from *Aspergillus tamarii*. *Acta Microbiol Sin.*, 30:259 – 266. 54. Takayanga, T., Ajisaka, K., Takiguchi, Y., Shimahara, K. 1991 .Isolation and characterization of thermostable chitinase from *Bacillus licheniformis* X-7U. *Biochem Biophys Acta.*, 1078:404 – 410. 55. Thamthiakul, S., Suan-Ngay S., Tantimavanich, S., Panbangred, W. 2001 .Chitinase from *Bacillus thuringiensis* subsp. pakistani. *Appl Microbiol Biotechnol.*, 56:395 – 401. 56. Tokoro , A., Suzuki, K., Mikami, T., Okawa, Y., Suzuki, S. and Suzuki, M. 1989. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydr. Res.* 51(15): 403-408. 57. Tsigos, I., Aggeliki, M., Dimitris, K and Vassilis, B. 2000. Chitin deacetylases: new, versatile tools in biotechnology. *Trends Biotechnol.* 18:(7):305-312. 58. Varma, R. S., George, K.J., Balaji, S. and Parthasarathy,V. A. 2009. Differential induction of chitinase in *Piper colubrinum* in response to inoculation with phytophthora capsici, the cause of foot rot in black pepper. *Saudi Journal of Biological Sciences.*, 16:11 – 16. 59. Tweddell, R.J., Jabaji-Hare SH., Charest, PM. 1994 .Production of chitinaseand \_-1,3-glucanase by *Stachybotrys elengans*, a mycoparasite of *Rhizoctonia solani*. *Appl Environ Microbiol.*, 60:489 – 498. 60. Usui, T., Hayashi, Y., Nanjo, F., Ishido, Y. 1984 . Transglycosylation reaction of a chitinase purified from *Nocardia orientail*. *Biochem Biophys Acta.*, 923:302 – 309. 61. Vyas, P., Deshpande, MV. 1989 .Chitinase production by *Myrothecium verucaria* and its significance in fungal mycelia degradation. *J Gen Microbiol.*, 35:343 – 350. 62. Van, Aalten, D.M., Komander, D., Synstad, B., Gaseidnes, S., Peter, M.G., Eijsink, V.G. 2001. Structural insights into the catalytic mechanism of a family 18 exochitinase. *Proc. Natl. Acad. Sci.USA.*, 98:8979-8984. 63. Wipa, S., Chomphunuch, S., Supansa, P., Adeleke, H. A., Robinson, R. C. 2008. Crystal structures of *Vibrio harveyi* chitinase A complexed with chitooligosaccharides: Implications for the catalytic mechanism *J. Struct. Biol.* 62:491 – 499. 64. Yusof, NL., Wee, A., Lim, LY., Khor, E. 2003 .Flexible chitin filmsas potential wound-dressing materials: wound model studies. *J Biomed Mater Res A.*, 66A:224 – 232. 65. Young, ME., Bell, RL., Carroad, PA. 1985 . Kinetics of chitinase production. I. Chitin hydrolysis. *Biotechnol Bioeng.*, 27:769 – 775. 66. Young, ME., Bell, RL., Carroad, PA. 1985 .Kinetics of chitinase production. II. Relation between bacterial growth, chitin hydrolysis, and enzyme synthesis. *Biotechnol Bioeng.*, 27:776 – 780.