

Isolation of a Microbe to Produce N-acetylchitooligosaccharides and Characterization of Its Chitinase

江佩軒、吳淑姿 ; 瑞澤

E-mail: 9708285@mail.dyu.edu.tw

ABSTRACT

In this study, a microbe, named DYU-Too13, was isolated from soil of Huatan Township in Changhua County to degrade chitin into N-acetylchitooligo- saccharides. The microbe was first cultivated in various chitin sources of the chitin broth (CB) media. When -chitin was the sole carbon source, the highest yield of N-acetylchitotriose was 1.02 g/L at 120 h. To study the effect of nitrogen source on the production of N-acetylchitooligosaccharides, various nitrogen sources were considered. The highest yield of N-acetylchitotriose was 1.62 g/L at 96 h when DYU-Too13 was cultivated in a peptone medium. The microbe was also cultivated in media containing various -chitin contents (1% - 6%) to study the effect of the content on the production of N-acetylchitooligosaccharides. The yield of N-acetylchitotriose reached a maximum (2.72 g/L) at 96 h in a medium containing 6% -chitin. When DYU-Too13 was cultivated in a medium with various peptone contents, the highest yield of N-acetylchitotriose was 1.902 g/L at 96 h in a medium containing 0.09 g peptone. When the microbe was cultivated in media with various NH₄Cl contents, the highest yield of N-acetylchitopentose was 0.246 g/L at 96 h in a medium containing 0.06 g NH₄Cl. Effect of cultivating temperature (30, 35 and 40) was also examined on the varieties and contents of N-acetylchitooligosaccharides. When DYU-Too13 was cultivated at 30 and 35 , the highest yields of N-acetylchitotriose at 96 h were 1.158 and 1.359 g/L, respectively. At 40 , major products at 96 h included N-acetylglucosamine (2.07 g/L), N-acetylchitotriose (0.327 g/L), and N-acetylchitotetraose (0.744 g/L). The optimum reacting temperature for the crude chitinases was 50 , and the optimum reacting pH was 6.0. The crude chitinases was quite stable at pH 8 and in the temperature range of 10~30 .

Keywords : DYU-Too13 ; chitinases ; N-acetylchitotriose

Table of Contents

封面內頁	簽名頁	授權書	iii	中文摘要	iv	英文摘要	v	誌謝	vi	目錄	vii	圖目錄	xii	表目錄	xvi	1. 緒論	1	2. 文獻回顧	2	2.1 幾丁質	2	2.1.1 幾丁質之分子結構與特性	2	2.1.2 幾丁質的功能與應用	4	2.1.3 幾丁質的製備	4	2.2 幾丁質分解	7	2.2.1 幾丁質分解之分類	9	2.2.2 幾丁質作用形機制	10	2.2.3 幾丁質分解之應用	13	2.2.4 幾丁質分解之活性分析	14	2.3 N-乙醯幾丁寡醣	15	2.3.1 N-乙醯幾丁寡醣之製備	15	2.3.2 N-乙醯幾丁寡醣之應用	16	3. 材料與方法	19	3.1 實驗架構	19	3.2 實驗儀器	19	3.3 實驗藥品	21	3.4 培養基與試劑	22	3.4.1 培養基組成	22	3.4.2 膠態幾丁質之製備	22	3.4.3 sMcIlvaine buffer之配製	24	3.4.4 還原醣呈色劑之配製	24	3.5 實驗方法	24	3.5.1 菌株篩選、保存及鑑定	24	3.5.2 菌株生長曲線測定	26	3.5.3 還原醣測定	26	3.5.4 幾丁質分解活性測定	26	3.5.5 蛋白質濃度測定	27	3.5.6 幾丁質水解產物分析	27	3.5.7 酵素之特性分析	27	4. 結果與討論	29	4.1 幾丁質分解菌之篩選	29	4.1.1 幾丁質活性與還原醣量之分析	29	4.1.2 幾丁質之水解產物之分析	31	4.1.3 菌株DYU-Too13之初步鑑定	31	4.2 以不同碳源培養DYU-Too13	36	4.2.1 不同碳源培養DYU-Too13之還原醣量影響	36	4.2.2 不同碳源培養DYU-Too13之幾丁質分解活性的影響	38	4.2.3 不同碳源培養DYU-Too13之蛋白質含量的影響	38	4.2.4 不同碳源培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	40	4.3 以不同氮源培養DYU-Too13 (-幾丁質粉末為碳源)	45	4.3.1 不同氮源培養DYU-Too13之還原醣量影響	45	4.3.2 不同氮源培養DYU-Too13之幾丁質分解活性的影響	47	4.3.3 不同氮源培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	49	4.4 以不同氮源培養DYU-Too13 (-幾丁質粉末為碳源)	50	4.4.1 不同氮源培養DYU-Too13之還原醣量影響	53	4.4.2 不同氮源培養DYU-Too13之幾丁質分解活性的影響	55	4.4.3 不同氮源培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	57	4.5 以不同濃度之 -幾丁質粉末培養DYU-Too13	63	4.5.1 不同濃度 -幾丁質粉末之培養DYU-Too13之還原醣量的影響	63	4.5.2 不同濃度 -幾丁質粉末培養DYU-Too13之幾丁質分解活性的影響	65	4.5.3 不同濃度 -幾丁質粉末培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	68	4.6 以不同濃度之蛋白培養DYU-Too13	71	4.6.1 不同濃度之蛋白培養DYU-Too13之還原醣量的影響	72	4.6.2 不同濃度之蛋白培養DYU-Too13之幾丁質分解活性的影響	74	4.6.3 不同濃度之蛋白培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	76	4.7 不同濃度之氯化銨培養DYU-Too13	79	4.7.1 不同濃度之氯化銨培養DYU-Too13之還原醣量的影響	79	4.7.2 不同濃度之氯化銨培養DYU-Too13之幾丁質分解活性的影響	81	4.7.3 不同濃度之氯化銨培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	83	4.8 不同溫度培養DYU-Too13	86	4.8.1 不同溫度培養DYU-Too13之還原醣量影響	87	4.8.2 不同溫度培養DYU-Too13之幾丁質分解活性的影響	87	4.8.3 不同溫度培養DYU-Too13之N-乙醯幾丁寡醣種類與生成量的影響	90	4.9 粗酵素之特性分析	93	4.9.1 最適反應溫度之測定	93	4.9.2 最適反應pH值之測定	95	4.9.3 溫度穩定性之測定	95	4.9.4 spH值穩定性之測定	95	5. 結論	100	參考文獻	102	圖目錄	圖2.1 幾丁質之製
------	-----	-----	-----	------	----	------	---	----	----	----	-----	-----	-----	-----	-----	-------	---	---------	---	---------	---	-------------------	---	-----------------	---	--------------	---	-----------	---	----------------	---	----------------	----	----------------	----	------------------	----	--------------	----	-------------------	----	-------------------	----	----------	----	----------	----	----------	----	----------	----	------------	----	-------------	----	----------------	----	----------------------------	----	-----------------	----	----------	----	------------------	----	----------------	----	-------------	----	-----------------	----	---------------	----	-----------------	----	---------------	----	----------	----	---------------	----	---------------------	----	-------------------	----	------------------------	----	----------------------	----	------------------------------	----	----------------------------------	----	--------------------------------	----	---	----	----------------------------------	----	------------------------------	----	----------------------------------	----	---	----	----------------------------------	----	------------------------------	----	----------------------------------	----	---	----	------------------------------	----	---------------------------------------	----	---	----	--	----	-------------------------	----	----------------------------------	----	-------------------------------------	----	--	----	-------------------------	----	-----------------------------------	----	--------------------------------------	----	---	----	---------------------	----	------------------------------	----	----------------------------------	----	---	----	--------------	----	-----------------	----	------------------	----	----------------	----	------------------	----	-------	-----	------	-----	-----	------------

備8 圖2.2 幾丁質分解酵素之作用機制11 圖2.3 醣類水解酵素之水解機制可分為保留與反轉兩類12 圖3.1 實驗架構流程圖20 圖4.1 以CB培養基培養菌株P1~P4之還原醣之變化32 圖4.2 以CB培養基培養菌株P1~P4之幾丁質?活性變化33 圖4.3 菌株DYU-Too13於膠態幾丁質培養基產生透明環之型態34 圖4.4 菌株DYU-Too13之位相差顯微照相圖 (1000X)35 圖4.5 不同碳源對菌株DYU-Too13之還原醣量影響37 圖4.6 不同碳源對菌株DYU-Too13之幾丁質?活性影響39 圖4.7 不同碳源對菌株DYU-Too13之蛋白質含量的影響41 圖4.8 以 -幾丁質粉末對菌株DYU-Too13之生合成N-乙醯幾丁寡醣的種類與生成量之影響42 圖4.9 以 -幾丁質粉末對菌株DYU-Too13之生合成N-乙醯幾丁寡醣的種類與生成量之影響43 圖4.10 以膠態幾丁質對菌株DYU-Too13之生合成N-乙醯幾丁寡醣的種類與生成量之影響44 圖4.11 不同氮源對菌株DYU-Too13之還原醣的影響(以 -幾丁質粉末為碳源)46 圖4.12 不同氮源對菌株DYU-Too13之幾丁質?活性的影響(以 -幾丁質粉末為碳源)48 圖4.13 不同氮源培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量(以 -幾丁質粉末為碳源)51 圖4.14 不同氮源培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量(以 -幾丁質粉末為碳源)52 圖4.15 不同氮源對菌株DYU-Too13之還原醣的影響(以 -幾丁質粉末為碳源)54 圖4.16 不同氮源對菌株DYU-Too13之幾丁質?活性的影響(以 -幾丁質粉末為碳源)56 圖4.17 不同氮源培養菌株DYU-Too13於48 h之N-乙醯幾丁寡醣的種類與含量(以 -幾丁質粉末為碳源)58 圖4.18 不同氮源培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量(以 -幾丁質粉末為碳源)59 圖4.19 不同氮源培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量(以 -幾丁質粉末為碳源)60 圖4.20 以1%~6%之 -幾丁質粉末對菌株DYU-Too13還原醣量之影響64 圖4.21 以1%~6%之 -幾丁質粉末對菌株DYU-Too13幾丁質分解?活性的影響66 圖4.22 以1%~6%之 -幾丁質粉末培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量69 圖4.23 以1%~6%之 -幾丁質粉末培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量70 圖4.24 以不同含量之蛋白?培養對菌株DYU-Too13還原醣量之影響73 圖4.25 以不同含量之蛋白?培養對菌株DYU-Too13幾丁質分解?之影響75 圖4.26 不同含量之蛋白?培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量77 圖4.27 不同含量之蛋白?培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量78 圖4.28 以不同含量氯化銨培養對菌株DYU-Too13還原醣量之影響80 圖4.29 以不同含量之氯化銨培養對菌株DYU-Too13幾丁質分解?之影響83 圖4.30 不同含量之氯化銨培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量84 圖4.31 不同含量之氯化銨培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量85 圖4.32 以不同溫度培養對菌株DYU-Too13還原醣量之影響88 圖4.33 以不同溫度培養對菌株DYU-Too13幾丁質分解?之影響89 圖4.34 不同溫度培養菌株DYU-Too13於72 h之N-乙醯幾丁寡醣的種類與含量91 圖4.35 不同溫度培養菌株DYU-Too13於96 h之N-乙醯幾丁寡醣的種類與含量93 圖4.36 溫度對菌株DYU-Too13之粗酵素液反應活性之影響94 圖4.37 pH值對菌株DYU-Too13之粗酵素液反應活性之影響96 圖4.38 溫度對菌株DYU-Too13之幾丁質分解?之穩定性影響97 圖4.39 pH值對菌株DYU-Too13之粗酵素液穩定性影響98 表目錄 表2.1 幾丁質與幾丁聚醣之應用4 表3.1 培養基之組成23 表3.2 McIlvaine 緩衝溶液25 表4.1 幾丁質分解菌株之來源與代號30 表4.2 以CB培養基培養菌株P1~P4之N-乙醯幾丁寡醣之種類與含量30

REFERENCES

- 1.江晃榮。1998。生體高分子(幾丁質、膠原蛋白)在食品工業上的應用。食品資訊, 150: 19-25。
- 2.吳豐智、曾如玲。1997。神奇物質-幾丁質和幾丁聚醣。化工技術, 5 (7): 196-201。
- 3.李宜玲。2004。利用Aeromonas caviae DYU-BT4之幾丁質水解酵素水解幾丁質生產N-乙醯幾丁寡醣。大葉大學生物產業學系研究所論文, 彰化。
- 4.林玲慧。2005。生產N-乙醯幾丁寡醣菌株之篩選與幾丁質?之分離純化。大葉大學生物產業學系研究所論文, 彰化。
- 5.袁國芳。1999。幾丁質與幾丁聚醣在食品工業上之應用。食品工業, 31(10): 19-23。
- 6.梁舜欣。1990。N-乙醯幾丁寡醣製備。台灣大學農業化學研究所碩士論文, 台北。
- 7.連德昇。2007。幾丁質分解酵素之生產與其基因選殖。大葉大學生物產業科技學系博士論文, 彰化。
- 8.陳坤上、黃佩芬、陳聰松、陳幸臣。1996。幾丁寡醣製備條件之探討。食品科學, 23(6): 874-883。
- 9.陳榮輝。2001。幾丁質、幾丁聚醣的生產製造、檢測與應用。第777-787頁。科學發展月刊。
- 10.龜山猶一。1981。化學分析試藥配製法。正文書局, 台北。
- 11.鍾竺均、陳偉。2003。生物技術概論。新文京開發出版, 台北。
- 12.Aiba, S. 1994. Preparation of N-acetylchitooligosaccharides from lysozymic hydrolysates of partially N-acetylated chitoasns. Carbohydr. Res., 261: 297-306.
- 13.Allan, C. R. and Hadwiger, L. A. 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition, Experimental Mycology., 3: 285-287.
- 14.Boller, T., Gehri, A., Mauch, F. and Vogeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties and possible function. Planta., 157: 22-31.
- 15.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.
- 16.Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
- 17.Brameld, K. A. and Goddard III, W. A. 1998. The role of enzyme distortion in the single displacement mechanism of family 19 chitinase. Proc. Natl. Acad. Sci., 95:4276-4281.
- 18.Brine, C. J. and Austin, P. R. 1981. Chitin variability with species and method of preparation. Comp. Biochem. Physiol., 69 B: 283-286.
- 19.Davis, G. and Herrissant, B. 1995. Structure and mechanisms of glycosyl hydrolase. Struc., 3: 853-859.
- 20.Frankowski, J., Lorito, M., Scala, F., Schmid, R., Berg, G. and Bahl, H. 2001. Purification and properties of two chitinolytic enzymes of Serratia plymuthica HRO-C48. Arch. Microbiol., 176: 421-426.
- 21.Gomez Ramirez, M., Rojans 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 chitinase. J. Microbiol. Meth., 56(2): 213-219.
- 22.Hicks, K. B. 1988. Isolation of oligomeric fragments by preparative high-performance liquid chromatography. Method Enzymol., 161: 410-416.
- 23.Imoto, T. and Yagishita, K. 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem., 35(7): 1154-1156.
- 24.Jeon, J. Y.

and Kim, K. S. 2000. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. *Carbohydrate Polymers*, 41: 133-141.

25. Jeuniaux, C. 1966. In *Method in Enzymology*. Academic Press, New York, 8: 644-654.

26. Kendera, D. F. and Hadwiger, L. A. 1984. Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. *Experimental Mycology*, 8: 276.

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

28. Kobayashi, M., Watanabe, T., Suzuki, S. and Suzuki, M. 1990. Effect of N-acetyl-chitohexaose against *Candida albicans* infection of tumor-bearing mice. *Microbiol. Immunol.*, 34: 413-426.

29. Koga, D., Tuskamoto, T., Sueshige, N., Usumi, T. and Ide, A. 1989. Kinetics of chitinase from yam, *Diocorea oppositifolia*. *Agric. Biol. Chem.*, 3(12): 3121-3126.

30. Kumar, R. and Majeti, N. V. 2000. A review of chitin and chitosan applications. *Reactive and Functional Polymer*, 46: 1-27.

31. Kurita, K. 2001. Controlled functionalization of the polysaccharide chitin. *J. Mol. Biol.*, 120: 167-181.

32. Mayo, S. L., Olafson, B. D. and Goddard III, W. A. 1990. Dreiding: a generic force field for molecular simulations. *J. Phys. Chem.*, 94: 8897-8909.

33. Minke, R. and Blackwell, J. 1978. The structure of β -chitin. *J. Mol. Biol.*, 120: 167-181.

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

35. Nampoothiri, M. K., Baiju, T. V., Sandhya, C. and Sabu, A. 2004. Process optimization for antifungal chitinase production by *Trichoderma harzianum*. *Process Biochem.*, 39: 1583-1590.

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

37. 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.

38. Patil, R. S., Ghormade, V. and Deshpande, M. V. 2000. Chitinolytic enzymes: an exploration. *Enzyme. Microb. Technol.*, 26(7): 473-483.

39. Robbins, P. W., Albright, C. and Benfield, B. 1988. Cloning and expression of a streptomyces plicatus chitinase (chitinase-63) in *Escherichia coli*. *J. Biol. Chem.*, 263(1): 443-447.

40. Roberts, G. A. F. 1982. *Chitin Chemistry*. The MacMillan Press, London.

41. Suzuki, K., Midami, T., Okawa, Y., Tokoro, A., Suzuki, S. and Suzuki, M. 1986. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydr. Res.*, 151: 403-408.

42. Suzuki, K., Okawa, Y., Hashimoto, K., Suzuki, S. and Suzuki, M. 1984. Protective effect of chitin and chitosan on experimentally induced murine candidiasis. *Microbiol. Immunol.*, 28: 903-912.

43. Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S. and Suzuki, M. 1986. Effect on N-acetylchitooligosaccharides on activation of phagocytes. *Microbiol. Immunol.*, 30: 777-787.

44. Suzuki, K., Tokoro, A., Suzuki, S. and Suzuki, M. 1985. Enhancing effects of N-acetylchitooligosaccharides on the active oxygen-generating and microbicidal activities of peritoneal exudate cells in mice. *Chem. Pharm. Bull.*, 33: 886-888.

45. Takayanagi, T., Ajisaka, K., Takiguchi, Y. and Shimahara, K. 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. *Biochim Biophys Acta*, 1078(3): 404-410.

46. Tokoro, A., Kobayashi, M., Tatewaki, N., Suzuki, K., Okawa, Y., Mikami, T., Suzuki, S. and Suzuki, M. 1989. Protective effect of N-acetyl-chitohexaose on *Listeria monocytogenes* infection in mice. *Microbiol. Immunol.*, 33: 357-367.

47. Usui, T., Hayashi, Y., Nanjo, F., Sakai, K. and Ishido, Y. 1987. Trans-glycosylation reaction of a chitinase purified from *Nocardia orientalis*. *Biochim Biophys Acta*, 923(2): 302-309.

48. Yamada, A., Shibuya, N., Kodama, O., and Akatsuka, T. 1993. Induction of phytoalexin formation in suspension-cultured rice cells by N-acetylchitooligosaccharides. *Biosci. Biotech. Biochem.*, 57: 405-409.