Production of Chitinase and N-Acetylchitooligosaccharides from Bacillus sp. DYU-Too 20

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ABSTRACT

In this study, Bacillus sp. DYU-Too 20 was isolated from Hsinchu. The aim of this study was to investigate the effects of an optimal condition for production of N-acetylchitotetrasaccharide by Bacillus sp. DYU-Too 20. In addition, the chitinase produced by this strain was purified and characterized. The optimal condition of Bacillus sp. DYU-Too 20 to investigate the effects of carbon source on the production of N-acetyltetrasaccharide. When α-chitin was the sole carbon source, the major product was N-acetyltetrasaccharide. Especially, the highest production of N-acetyltetrasaccharide (0.144 g/L) was obtained in a medium of 4% α-chitin; NH_4Cl seemed to be a better nitrogen source to produce N-acetyl- tetrasaccharide, and the production was 0.256 g/L in a medium containing 0.1 g/L NH_4Cl; The highest yield of N-acetyltetrasaccharide (0.284 g/L) was obtained at 35°C. The crude enzyme was obtained from a culture of Bacillus sp. DYU-Too 20 in medium containing 4% α-chitin and 0.1 g/L NH_4Cl at 35°C. The purification procedures included precipitation by ammonium sulfate, dialysis, and anion exchange chromatograph (DEAE-Sepharose CL-6B). From DEAE-Sepharose gel chromatographic diagram, one peak of Fraction 83-95 possessed chitinase activity. Hence, the above chitinase was used to hydrolyze colloidal chitin solution, the hydrolysates were separated through centrifuge and lyophilization, and its composition was analyzed by HPLC. The hydrolysates contained N-acetyl- glucosamine, N-acetylchitodisaccharide and N-acetyltetrasaccharide. . Through electrophoresis, the molecular weight of the chitinase was 26 kDa.

Keywords : chitinase、Bacillus sp. DYU-Too 20、N-acetyl-glucosamine、N-acetylchitodisaccharide、N-acetyltetrasaccharide 、Optimum condition

Table of Contents

封面內頁 簽名頁 中文摘要 iii 英文摘要 iv 誌謝 v 目錄 vii 圖目錄 xi 表目錄 xiv 1. 緒論 1 2. 文獻回顧 2 2.1 幾丁質 2 2.1.1 幾丁質類之分子結構與性質 2 2.2 幾丁質相關衍生物與應用 4 2.2.1 免疫活性 4 2.2.2 幾丁質之應用 5 2.3 N-乙醯幾丁寡醣與幾丁寡醣 6 2.4 幾丁質分解酵素 6 2.4.1 幾丁質分解酵素之來源 7 2.4.2 幾丁質分解酵素之應用 8 2.4.3近年幾丁質分解?之研究 9 3. 材料與方法 12 3.1 實驗架構 12 3.2 實驗藥品 12 3.3 實驗器材 14 3.4 試劑及培養基配製 15 3.4.1培養基組成 15 3.4.2膠態幾丁質製備 15 3.4.3 McIlvaine buffer之配製 16 3.5實驗方法 16 3.5.1菌株篩選、保存與活化 16 3.5.2 菌株生長曲線之測定 19 3.5.3 酵素法製備N-乙醯幾丁寡醣 19 3.6 分析方法 19 3.6.1幾丁質分解酵素之活性測定 19 3.6.2蛋白質濃度測定 20 3.6.3幾丁質之水解產物的處理與分析 20 3.7幾丁質分解酵素之純化與特性分析 21 3.7.1幾丁質分解酵素之純化 21 3.7.2 酵素分子量 22 3.7.2.1聚丙烯醯胺膠體電泳分析 22 4. 結果與討論 24 4.1 菌株於膠態幾丁質培養生長情形 24 4.2 篩選幾丁質酵素生產菌株 24 4.3 菌株粗發酵液之特性分析 28 4.3.1 菌株Ze1 28 4.3.2 菌株Ze2 28 4.3.3 菌株Ze3 31 4.3.4 菌株Ze4 33 4.3.5 菌株Ze5 33 4.3.6 篩選菌株之水產分析 35 4.4 菌株DYU-Too 20之基本特性分析 38 4.5 不同碳源濃度培養菌株 42 4.5.1 幾丁質?活性分析 42 4.5.2 還原醣與pH值變化 42 4.5.3 水解產物分析 46 4.6 α-幾丁質濃度 48 4.6.1 幾丁質?活性分析 48 4.6.2 還原醣與pH值變化 48 4.6.3 水解產物分析 50 4.7 氮源 54 4.7.1 幾丁質?活性分析 54 4.7.2 還原醣量與pH值變化 54 4.7.3 水解產物分析 57 4.8 NH_4Cl濃度 60 4.8.1 幾丁質?活性分析 60 4.8.2 還原醣與pH值變化 60 4.8.3 水解產物分析 64 4.9 溫度 64 4.9.1 幾丁質?活性分析 66 4.9.2 還原醣量與pH值變化 66 4.9.3 水解產物分析 70 4.10 幾丁質?之分離純化 70 4.10.1 硫酸銨沉澱 70 4.10.2 離子交換層析 72 4.10.3 膠體過濾層析 72 4.10.4 酵素分子量測定 75 5. 結論 79 5.1 結論 79 5.2 展望 80 參考文獻 81 附錄 88

REFERENCES

1.李妍蒨。2009。IL-6和惡性胸水肺腺癌臨床及免疫調控相關性。國立成功大學臨床醫學研究所，台南，台灣。 2.黃安德。1998。利用部分純化之Amycolatopsis orientalis 細胞外N-乙醯葡萄糖胺酵素製備N-乙醯幾丁寡醣。國立海洋大學水產食品科學系碩士學位論文，基隆，台灣。 3.龜山猶一。1981。化學分析試藥配製法。正文書局。台北。 4.Alias, Z. and Tan, I. K. P. 2005. Isolation palm oil-utilising polyhydroxy- alkanote (PHA) producing bacteria by an enrichment technique. Bioresource Technology, 96 : 1229-1234. 5.Anderson, A. J. and Dawes, E. A. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological Reviews. 54:450-472. 6.Ashby, R. D., Solaiman, D. K. Y. and Foglia, T. A. 2002. The synthesis of short and medium-chaiN-length poly (hydroxyalkanoates) mixtures from glucose or alkanoic acid grown Pseudomonas oleovorans. J Ind. Microbiol Biotechnol. 28 : 147-53. 7.Bucci, D. Z., Tavares, L. B. B. and Sell, I. 2005. PHB packaging for the storage of food products. Polymer Testing. 24 : 564-571 8.Chang, K. L., Lee, J. and Fu, W. R. 2000.

HPLC analysis of N-acetyl-chito- oligosaccharides during the Acid Hydrolysis of Chitin. Journal of Food and Drug Analysis. 8(2):75-83 9.Chet, I. and Inbar, J. 1994. Biological control of fungal pathogens. Applied Biochemistry and Biotechnology. 48(1): 37-43. 10.Chre ’ tiennot, D. M. J., Giraud, G. M. M., Vaulot, D., Putaux, J. L. and Chanzy, H. 1997. The chitinous nayure of filament ejected by Phaeocystis (Prymnesiophycae). Journal of Phycology. 33:666-72. 11.Clesceri, L. S., Greenberg, A. E. and Eaton, A. D. 2001. Standard Methods for the Examination of Water and Wastewater, p. 409-421, American Public Health Association, Washington, DC. 12.Collinge, D. B., Kragh, K. M., Mikkslesen, J. D., Nielsen, K. K., Rasmussen, U. and Vad, K. 1993. Plant chitinases. Journal of Phycology. 3: 31-40. 13.Dou, J. L., Tan, C. Y., Du, Y, G., Bai, X. F., Wang, K. Y., and Ma, X. J. 2007. Effects of chitooligosaccharides on rabbit neutrophils in vitro. Carbohydrate Polymers. 69(2):209-213 14.Eggink, G., Wal, V. H., Huijberts, G. N. M. and Waard, D. P. 1993. Oleic acid as a substrate for poly-3-hydroxybutyrate formation in Alcaligenes eutrophus and Pseudomonas putida. Industrial Crops and Products. 1 : 157-63. 15.Fathi, F., Kermani J., Abozari M., Alasvand M. and Ahmadi S. 2008. Assessment of Capability of Fetal and Adult Neural Stem Cells to Differentiate into Endothelial Cells. Scientific Journal of Kurdistan University. 13: 1-12. 16.Feng, J., Zhao, L., and Yu, Q. 2004. Receptor-mediated stimulatory effect of oligochitosan in macrophages. Biochemical and Biophysical Research Communications. 317:414-420. 17.Fidler, S. and Dennis, D. 1992. Polyhydroxyalkanoate production by recombinant Escherichia coli. Federation of European Microbiological Societies Microbiology Letters Microbiology Reviews. 103 : 231-6. 18.Fukui, T., Kichise, T., Yoshida, Y. and Doi, Y. 1997. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyheptanoate) terpol- ymers by recombinant Alcaligenes eutrophus. Biotechnology Letters. 19 (11) : 1093-1097. 19.Hahn, S. K., Chang, Y. K. and Lee, S. Y. 1995. Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus and recombinant Escherichia coli. Applied and Environmental Microbiology. 61 : 34 – 9. 20.Hart, P. J., Pfluger, H. D., Monzingo, A. F., Hollis, T. and Robertus, J. D. 1995. The refined crystal structure of an endochitinase from hordeum vulgare L. seeds at 1.8 A resolution. Journal of Molecular Biology. 248:402-413. 21.Hsu, S. C. and Lockwood, J. L. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Journal of Applied Microbiology. 29: 422-426. 22.Huang, R., Niranjan, R. and Se-Kwon, K. 2006. Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives. Carbohydr. Polymers. 63:122-129. 23.Lee, I. Y., Kim, M. K., Chang, H. N. and Park, Y. H. 1995. Regulation of poly-β-hydroxybutyrate biosynthesis by nicotinamide nucleotide in Alcaligenes eutrophus. Federation of European Microbiological Societies Microbiology Letters. 131:35-39. 24.Lemoigne, M. 1926. Products of dehydration and of polymerization of β-hydr- oxybutyric acid. Bull. Soc. Chem. Biotechnology. 8:770-782. 25.Lemos, P. C., Serafim, L. S. and Reis, M. A. M. 2005. Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. Journal of Biotechnology. 122 (2) : 226-238. 26.Jeuniaux, C. 1966. Chitinases. Methods Enzymol., 8: 644-650. 27.Jolles, P. and Muzzarelli, R. A. A. 1999. Chitin and Chitinases. Birkhauser Verlarg, Basel. 28.Kahar, P., Tsuge, T., Taguchi, K. and Doi, Y. 2004. High yield production of polyhydroxyalkanoates from soybean oil by Ralstonia eutropha and its recombinant strain. Polymer Degradation and Stability. 83 : 79-86. 29.Khanna, S. and Srivastava, A. K. 2005. Statistical media optimization studies for growth and PHB production by Ralstonia eutropha. Process Biochemistry. 40 : 2173-2182. 30.Kim, B. S., Kim, S. C., Lee, S. Y., Chang, H. N., Chan, Y. K. and Woo, S. I. 1994. Production of poly(3-hydroxybutyric acid) by fed batch culture of Alcaligenes eutrophus with glucose concentration control. Biotechnol. Bioeng. 43:892-898. 31.Kim, B. S., Lee, S.Y. and Chang, H. N. 1992. Production of poly-beta-hydrox- ybutyrate by fed-batch culture of recombinant Escherichia coli. Biotechnol Letters. 14 : 811-6. 32.Ko, S., Takeshi, M., Yoshio, O., Akio, T., Shigeo, S. and Masuko, S. 1986. Antitumor effect of hexa-N-acetylchitohexose and chitohexose. Carbohydrate Research. 151:403-408. 33.Koga, K, Iwamoto, Y., Sakamoto, H., Hatano, K., Sano, M. and Kato, I. 1991. Purification and characterization of beta-N-acetylhexosaminidase from Trichoderma harzianum. Agricultural and biological chemistry. 55: 2817-2823. 34.Krajewska, B. 2004. Application of chitiN- and chitosaN-based materials for enzyme immobilizations: a review. Enzyme Microbiol Technol. 35:26-39. 35.Kumar. R. and Majeti, N. V. 2000. A review of chitin and chitosan applications. Reactive and functional polymers. 46:1-27. 36.Kumar, M. S., Mudliar, S. N., Reddy, K. M. K. and Chakrabarti, T. 2004. Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant. Bioresource Technology. 95 : 327-330. 37.Kupiec, C. R. and Chet, I. 1998. The molecular biology of chitin digestion. Current Opinion in Biotechnology. 9: 270-277. 38.Maeda, Y. and Kimura, Y. 2004. Antitumor effects of various low-molecular- weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice. Journal of Nutrition. 134: 945-950. 39.Mass W. A., Mass A. and Tighe B. 1998. A review of biodegradable polymers: uses,current developments in the synthesis and characterization of biodegradable polyesters. Blends of biodegradable polymers and recent advances in biodegradation studies. Journal of Polymer Research. 47:89. 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.Mona, K. G., Swellam, A. E. and 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 Research. 156 : 201-207. 42.Muzzarelli, R. A. 1977. Chitin. p. 262-270. Oxford Pergamon Press, Londom. 43.Omar, S., Rayes, A. and Eqaab, A., Vob, I. and Steinbuchel, A. 2001. Optimization of cell growth and poly(hydroxybutyrate) accumulation on date syrup by a Bacillus megaterium strain. Biotechnology Letter. 23 : 1119-1123. 44.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. 45.Pflug, S., Richter, S. M. and Urlacher, V. B. 2007. Development of a fed-batch process for the production of the cytochrome P450 monooxygenase CYP102A1 from Bacillus megaterium in E. coli. Journal of Biotechnology. 129 : 481-488. 46.Pichyangkura P., Kudan S., Kuttiyawong K., Sukwattanasinitt M., and Aiba S. 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. Carbohydrate Research. 337:

557-559. 47.Preusting, H., Hazenberg, W. and Witholt, B. 1993. Continuous production of poly(3-hydroxyalkanoates) by P. oleovorans in a high cell density, two-liquid- phase chemostat. Enzyme and Microbial Technology. 15 : 311-316. 48.Punja, Z, K. and Zhang, Y. Y. 1993. Plant chitinase and their roles in resistance to fungal diseases. Journal of Nematology. 25(4): 526-540. 49.Qin, C., Du, Y., Xiao, L., z. and Gao, X. 2002. Enzymic preparation of water-soluble chitosan and their antitumor activity. International Journal of Biological Macromolecules. 31:111-317. 50.Revah-Moiseev, S. and Carroad, A. 1981. Conversion of the enzymatic hydro- lysate of shellfish waste chitin to single-cell protein. Biotechnology and Bioengineering. 23: 1067-1078. 51.Righetti, P. G., Gianazza, E., Gelfi, C., Chiari, M. Hames, B. D. and Richwood, D. 1990. In: Gel Electrophoresis of Proteins: A Practical Approach. (Eds.) Oxford University Press, New York. 149-216. 52.Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. Progress in Polymer Science. 31:603-632. 53.Rodriguez, M. and Ransohoff, R. M., 2003. InterleukiN-6 protects anterior horn neurons from lethal virus-induced injury. Journal of Neuroscience. 23:481-492. 54.Sakurada, M., Morgavi, D. P., Komatani, K., Tomita, Y. and Onodera, R. 1997. Purification and characteristics of an autolytic chitinase of Piromyces communis OTS1 from culture medium. Current Opinion in Microbiology. 35: 48-51. 55.Salvatore J., Cooper B., Shah I., and Kummet T. 2000. Primary pancreatic lymphoma: a case report, literature review, and proposal for nomenclature. Medical Oncology. 17:237-47 56.Shang, L., Jiang, M. and Chang, H. N. 2003. Poly(3-hydroxybutyrate) synthesis in fed-batch culture of Ralstonia eutropha with phosphorus limitation under different glucose concentrations. Biotechnology Letter. 25 : 1415-1419. 57.Shang, L., Yim, C. Y., Park, H. G. and Chang, H. N. 2004. Sequential feeding of glucose and valerate in a fed-batch culture of Ralstonia eutropha for production of poly(hydroxybutyrate-co-hydroxyvalerate) with high 3-hydroxyvalerate raction. Biotechnology Progress. 20 : 140-144. 58.Skoog, D. A., West, D. M. and Holler, F. J. 1993. The determination of amine nitrogen by the Kjeldahl method. Analytical Chemistry. 6 : 575-578. 59.Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S. and Suzuki, M. 1986. Antitumor effect of hexa-N-acetylchitohexose and chitohexaose. Carbohydrate Research. 151:403-408. 60.Taguchi, S., Nakamura, H., Kichise, T., Tsuge, T., Yamato, I. and Doi, Y. 2003. Production of polyhydroxyalkanoate (PHA) from renewable carbon sources in recombinant Ralstonia eutropha using mutants of original PHA synthase. Biochemical Engineering Journal. 16 : 107-113. 61.Tang, S. C., Chang M. C. and Cheng, C. Y. 1998. Use of colloid chitin and diatomaceous earth in continuous cake-filtration fermentation to produce creatinase. Proc. Biochem. 33: 519-526. 62.Tokoro, A., Suzuki, K, Mikami, T., Okawa, Y, Suzuki, S. and Suzuki M. 1989. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. Carbohydrate Research. 151(15):403-408 63.Tsukada, K., Matsumoto, T., Aizawa, K., Tokoro, A., Naruse, R. and Suzuki, S. 1990. Antimetastatic and growth-inhibitory effects of N-acetylchitohexose in mice bearing Lewis lung carcinoma. Japanese Journal of Cancer Research. 81:259-265. 64.Wang, W., Sun, J., Hollmann, R., Zeng, A. P. and Deckwer, W. D. 2006. Proteo- mic characterization of transient expression and secretion of a stress-related metalloprotease in high cell density culture of Bacillus megaterium. Journal of Biotechnology. 126 : 313-324. 65.Wiwat, C., Siwayaprahm, P. and Bhumiratana, A. 1999. Purification and characterization of chitinase from Bacillus circulans No. 4.1. Current Microbiology. 39: 134-140. 66.Van, A., Aalten, D. M., Komander, D., Synstad, B., Gaseidnes, S., Peter, M. G. and Eijsink, V. g. 2001. Structural insights into the catalytic mechanism of a family 18 exochitinase. Proceedings of the National Academy of Sciences. 98:8979-898. 67.Volova, T., Shishatskaya, E., Sevastianov, V., Efremov, S. and Mogilnaya, O. 2003. Results of biomedical investigations of PHB and PHB/PHV fibers. Biochemical Engineering Journal. 16:125-133. 68.Vyas, P. R. and Deshpande, M. V. 1989. Chitinase production by Myrothecium verrucaria and its significance for fungal mycelia degradation. The Journal of General and Applied Microbiology. 35: 343-350. 69.Vyas, P. R and Deshpande, M. V. 1991. Enzymatic hydrolysis of chitin by Myrothecium verrucaria chitinase complex and its utilization to produce SCP. The Journal of General and Applied Microbiology. 37: 267-275. 70.Xu, Q., Dou, J., Wei, P., Tan, C., Yun, X., Wu, Y., Bai, X., Ma, X., and Du, Y. 2008. Chitooligosaccharides induce apoptosis of human hepatocellular carcinoma cells via up-regulation of Bax. Carbohydrate Polymers. 71:509-514. 71.Yan, Q., Du, G. and Chen, J. 2003. Biosynthesis of polyhydroxyalkanoates (PHAs) with continuous feeding of mixed organic acids as carbon sources by Ralstonia eutropha. Biochemistry. 39 : 387-391. 72.Yusof, N. L., Wee, A., Lim, L. Y. and Khor E. 2003. Flexible chitin filmsas potential wound-dressing materials: wound model studies. Journal of Biomedical Materials Research Part A. 66A:224-32.