

本土菌株Aeromonas hydrophila DYU-Too18之幾丁質分解探討

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摘要

本研究的目的為，篩選可生產幾丁質分解之菌株，實驗菌株Aeromonas hydrophila DYU-Too18以CB培養基培養，探討不同碳、氮源對其還原醣生成量、幾丁質分解活性、pH值及N-乙醯幾丁寡醣產量之影響，並進行幾丁質分解純化與特性分析。以 β -chitin為碳源比以 β -chitin為碳源有較高的還原醣生成量，且可生成N-乙醯幾丁三醣；以5% β -chitin培養時，還原醣量達到最高，可生成1.42 g/L；以酵母萃取物與蛋白質混合物為氮源可生成較高的N-乙醯幾丁三醣，其中以0.4 g/L為氮源，所生成之N-乙醯幾丁三醣為最高，可達1.17 g/L。以含5% β -chitin與0.4 g/L酵母萃取物與蛋白質混合物培養此菌株96 h，離心取得粗酵素液，經硫酸銨沉澱、透析、DEAE-Sepharose CL-6B及Sephadry S-100等純化步驟後，酵素之比活性為3.66 U/mg protein，純化倍率為1.21。純化酵素之最適反應溫度為40°C，最適反應pH值為5.0，而Fe²⁺、Hg²⁺、Zn²⁺對幾丁質分解活性抑制較大，尤以Hg²⁺幾乎完全抑制酵素活性。

關鍵詞：N-乙醯幾丁三醣、純化、幾丁質分解？

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參考文獻

- 沈明來。1995。試驗設計學。九州圖書文物有限公司。台北。
- 林玠伯。2009。以Aeromonas sp. DYU-Too13生產N-乙醯幾丁寡醣及其幾丁質?之特性分析。大葉大學生物產業科技學系。彰化。
- 林逸群。2003。連續生產PHBV-丙酸戊酸對菌體生長及PHBV生產之影響。大葉大學食品工程研究所碩士論文。彰化。
- 高宇。2006。生物化學。鼎茂圖書出版股份有限公司。台北。
- 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。
- 陳錦坤、許清輝、李錦榆、林忠亮、方炳勳、黃冬梨、吳奇生。2001。在電泳片上直接分析chitinase活性的新方法。90學年度技術與教學研討會論文專輯:21-23。明志技術學院。台北。
- 楊懿淑。2006。統計學。滄海書局。台中。
- 龜山猶一。1981。化學分析試藥配製法。正文書局。台北。
- Akaboshi M., K. Kawai and A. Waki, 1972, Preparation of 2-Acetamido-2-deoxy- β -D-glucose oligosaccharides from acid hydrolyzates of chitin by electrolytic desalting and exclusion Chromatography. Analytical Biochemistry. 46:687-690.
- T. Boller, A. Gehri, F. Mauch and U. Vogeli, 1983, Chitinase in bean leaves: introduction by ethylene, purifi, properties and possible function. Planta. 157:22-31.
- M. Bradford, 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.
- I. Bravo-Osuna, G. Millotti, C. Vauthier and G. Ponchel, 2007, In vitro evaluation of calcium binding capacity of chitosan and thiolated chitosan poly (isobutyl cyano- acrylate) core-shell nanoparticles. International Journal of Pharmaceutics. 338(1-2):284-290.
- H. G. Byun, Y. T. Kim, P. J. Park, X. Lin and S. K. Kim, 2005, Chitoooligosaccharides as a novel β -secretase inhibitor. Carbohydrate Polymers. 61:198-202.
- K. L. Chang, J. Lee and W. R. Fu, 2000, HPLC analysis of N-acetyl-chito-oligosaccharides during the acid hydrolysis of chitin. Journal of Food and Drug Analysis. 8:75-83.
- S. W. Chen and H. C. Chen, 1999, Effect of oral administration of Cellulomonas flavigena NTOU 1-degraded chitin hydrolysate on physiological change in rates. Food Sci. Agric. Chem. 1:186-189.
- M. G. Davila, J. M. S. Casiano and F. J.

Millero, 1989, The adsorption of Cd(II) and Pb(II) to chitin in seawater. *Journal of colloid and Interface science.* 137(1):102-110. 17.Davila M. G.and F. J. Millero, 1989, The adsorption of copper to chitin in seawater. *Geochimica et Cosmochimica Acta.* 54:761-768. 18.Donzelli, B. G. G., G. Ostroff and G. E. Harman, 2003, Enhanced enzymatic hydrolysis of langostino shell chitin with mixture of enzymes from bacterial and fungal sources. *Carbohydrate Research.* 338:1823-1833. 19.Fernandes, J. C., F. K. Tavaaria, J. Soares, O. S. Ramos, M. J. Monteiro, M. E. Pintado and F. X. Malcata, 2008, Antimicrobial effects of chitosans and chito- oligosaccharides, upon *Staphylococcus aureus* and *Escherichia coli*, in food model systems. *Food Microbiology.* 25:922-928. 20.Hirano, S. and Nagao, N., 1989, Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agricultural and Biological Chemistry.* 53:3065 – 3066. 21.Huang, R., N. Rajapakse and S. K. Kim, 2006, Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives. *Carbohydrate Polymers.* 63:122-129. 22.Ikeda, M., K. Miyauchi, A. Mochizuki and M. Matsumiya, 2009, Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*. *Protein Expression and Purification.* 65:214-222. 23.Imoto, T. and K. Yagishita, 1971, A simple activity measurement of lysozyme. *Agric. Biol. Chem..* 35(7):1154-1156. 24.Je J. Y., P. J. Park and S. K. Kim, 2004, Free radical scavenging properties of hetero -chitooligosaccharidesusing an ESR spectroscopy. *Food and Chemical Toxicology.* 42:381-387. 25.Jeon, Y. J. and S. K. Kim, 2001, Potential immuno-stimulating effect of antitumoral fraction of chitosan oligosaccharides. *Journal of Chitin and Chitosan.* 6:163 – 167. 26.Jeuniaux, C., 1966, Chitinases, *Methods Enzymol.* 8:644-654. 27.Kumar, M. N. V. R., 2000, A review of chitin and chitosan applications. *Reactive & Functional polymers.* 46:1-27. 28.Lavall, R. L., O. B. G. Assis and S. P. Campana-Filho, 2007, -Chitin from the pens of *Loligo* sp.: extraction and characterizaion. *Bioresource technology.* 98:2465-2472. 29.Liang, T. W., Y. J. Chen, Y. H. Yen and S. L. Wang, 2007, The antitumor activity of the hydrolysates of chitious materials hydrolyzed by crude enzyme from *Bacillus amyloliquefaciens* V656. *Process Biochemistry.* 42:527-534. 30.Lin, C. W., L. J. Chen, P. L. Lee, C. I. Lee, J. C. Lin and J. J. Chiu, 2007, The inhibition of TNF- -induced E-selectin expression in endothelial cells via the JNK/NF- B pathways by highly N-acetylated chitooligosaccharides. *Biomaterials.* 28:1355-1366. 31.Molano, J., A. Duran and E. Cabib, 1977, A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* 83(2):648-656. 32.Nil, 1996, Market of chitin and chitosan. *Bio.Industry.* 13:52-61. 33.Ning, W., F. Chen, B. Mao, Q. Li, Z. Liu, Z. Guo Z. He, 2004, N-Acetylchito- oligosaccharides elicit rice defence responses including hypersensitive response-like cell death, oxidative burst and defence gene expression. *Physiological and Molecular Plant Pathology.* 64:263-271. 34.Otakara, A., M. Mitsutomi and Y. Uchida, 1979, Purification and some properties of chitinase from *Vibrio* sp.. *J. Ferment. Technolo.* 57(3):169-177. 35.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. 36.Prashanth, K. V. H. and R. N. Tharanathan, 2005, Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. *Biochimica et Biophysica Acta.* 1722:22-29. 37.Ramirez, M. G., L. I. R. Avelizapa, N. G. R. Avelizapa and R. C. Camarillo, 2004, Colloidal chitin stained with Remazol Brilliant Blue R,a useful substrate to select chitinolytic microorganisms and to evaluate chitinase. *Journal of Microbiological Methods.* 56(2):213-219. 38.Righetti, P. G., B. D. Harnes, E. Gianazza, C. Gelfi, and M. Chiari, 1990, Gel Electrophoresis of Protein. IRL Oxford, 149-216. 39.Rinaudo, M., 2006, Chitin and chitosan: Properties and applications. *Progress in Polymer Science.* 31:603-632. 40.Stanley, W. L., G. G. Watters, B. Chan and J. M. Mercer, 1975, Lactase and enzyme bound to chitin with glutaraldehyde. *Biotech. Bioeng.* 17:315-326. 41.Tsai, G. J., W. H. Su, H. C. Chen and C. L. Pan, 2002, Antimicrobial activicity of shrimp chitin and chitosan from different treatments and aoolications of fish preservation. *Fisheries. Sci.* 68:170-177. 42.Wang, S. L., T. Y. Lin, Y. H. Yen, H. F. Liao and Y. J. Chen, 2006, Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. *Carbohydrate Research.* 341:2507-2515. 43.Wang, Z., L. Zheng, S. Yang, R. Niu, E. Chu and X. Lin, 2007, N-Acetylchito- oligosaccharides is a potent angiogenic inhibitor both in vivo and in vitro. *Biochemical and Biophysical Research Communication.* 357:26-31. 44.Wu H., Z. Yao , X. Bai , Y. Du and B. Lin, 2008, Anti-angiogenic activities of chitooligosaccharides. *Carbohydrate Polymers.* 73:105-110. 45.Wu, G. J. and G. J. Tsai, 2007, Chitooligosaccharides in combination with interferon- increase nitric oxide production via nuclear factor- B activation in murine RAW264.7 macrophages. *Food and Chemical Toxicology.* 45:250-258. 46.Yang, Y. M., R. G. Shu, J. Shao, G. F. Xu and X. X. Gu, 2006, Radical scavenging activity of chitooligosaccharide with different molecular weights. *European Food Research and Technology.* 222:36-40. 47.Yuli, P. E., M. T. Suhartono, Y. Rukayadi, J. K. Hwang and Y. R. Pyun, 2004, Characteristics of thermostable chitinase enzymes from the indonesian *Bacillus* sp. 13.26. *Enzyme and Microbial Technology.* 35:147-153. 48.Zhou, D., L. Zhang, J. Zhou and S. Guo, 2004, Cellulose/chitin beads for adsorption of heavy metals in aqueous solution. *Water Research.* 38:2643-2650.