

Purification and Characterization of a Solvent Stable Protease from *Bacillus* sp.TKU004

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ABSTRACT

The protease-producing bacterium, *Bacillus* sp.TKU004, was isolated from soil in the central Taiwan. The optimized culture was composed of 2% squid pen powder(SPP), 0.1%K₂HPO₄, 0.05%MgSO₄ · 7H₂O at pH7. The bacterium was incubated in 250mL Erlenmeyer flask containing 100mL of above liquid medium and kept shaking at 30 for four days. The protease of *Bacillus* sp.TKU004 was produced under the optimized culture condition. The supernatant was first precipitated by ammonium sulfate. The further purification and separation procedures of protease were processed by DEAE-Sepharose, CM-Sepharose ionic exchange chromatography, and Sephacryl S-200 gel chromatography. The overall activity yield of the purified protease were 14%, with specific protease activities of 0.062 U/mg. The optimum temperature and pH of the enzyme were 60 and 7 respectively, and the enzyme was stable at

Keywords : *Bacillus* sp.TKU004, protease, squid pen, deproteinization

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REFERENCES

- 1.王三郎。1999。海洋未利用生物資源之回收再利用。生物資源 生物技術。1 (1):1-8。
- 2.王三郎 編著。1996。水產資源利用學:第23~25頁。高立圖書出版社。台北,台灣。
- 3.王三郎 編著。1997。應用微生物學:第56~57頁。高立圖書出版社。台北,台灣。
- 4.江晃榮。1996。新生技產品:幾丁質、幾丁聚醣(甲殼質)產業現況與展望。經濟部IT IS叢書。
- 5.吳許得。2003。不同處理方法及來源所製得幾丁質的性質及其所誘導之幾丁質?活性比較。國立海洋大學食品科學研究所博士論文。基隆。
- 6.邱少華。1997。利用綠膿桿菌K-187發酵蝦蟹殼廢棄物生產幾丁質?之應用及量產條件之研究:第33~34頁。私立大葉工學院食品工程研究所碩士論文。彰化。
- 7.張文智。2003。利用*Bacillus cereus*發酵蝦蟹殼廢棄物所生產抗真菌酵素之分離純化及其應用:第10~11頁。國立中興大學食品科學研究所博士論文。台中。
- 8.張虎,杜昱光,虞星炬。1999。幾丁寡糖與殼寡糖的製備和功能。中國生化藥物雜誌:99。
- 9.楊政國。1999。利用枯草菌進行蝦蟹殼去蛋白之研究:第99頁。私立大葉工學院食品工程研究所碩士論文。彰化。
- 10.楊孟琳。2002。以微生物水解蝦殼蛋白質分離幾丁質後的廢液作為植物液肥之可行性:第88頁國立海洋大學食品科學研究所碩士論文。基隆。
- 11.蘇遠志,黃世佑。2002。微生物化學工程學。第372~374頁。華香園出版社。台北,台灣。
- 12.蕭瑞昌。1997。利用水溶性幾丁聚醣以薄膜超過濾法去除微量之金屬離子:第77頁。私立元智工學院碩士論文。桃園。
- 13.Alamgir, K., Keith, W., Mark, P. M. and Helena, N. 2003. Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. *Protein Expr. Purific.* 32:210-220.
- 14.Alam, S. I., Dube, S., Reddy, G. S. N., Bhattacharya, B. K., Shivaji, S. and Singh, L. 2004. Purification and characterisation of extracellular protease produced by *Clostridium* sp. from Schirmacher oasis, Antarctica. *Enzyme Microb. Technol.* 36:824-831.
- 15.Adinarayana, K., Ellaiah, P. and Prasad, D. S. 2003. Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis* PE-11. *AAPS PharmSciTech.* 4:56.
- 16.Azeredo, L. A. I. De., Freire, D. M. G. Soares, R. M. A., Leite, S. G. F. and Coelho, R. R. R. 2004. Production and partial characterization of the thermophilic proteases from *Streptomyces* sp. isolated from Brazilian cerrado soil. *Enzyme Microb. Technol.* 34:354 – 358.
- 17.Akuzawa, R., Tottori, A., Tsukahara, K. and Okitani, A. 1997. Purification and characterization of a cysteine proteinase from *Lactococcus lactis* ssp. *lactis* IAM 1198. *Intl. Dairy J.* 7:429-434.
- 18.Beg, Q. K. and Gupta, R. 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb. Technol.* 32:294 – 304.
- 19.Basma, G., Alya, S. K. and Moncef, N. 2003. Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb. Technol.* 32:513 – 518.
- 20.Caldas, C., Cherqui, A., Pereira, A. and Simoes, N. 2002. Purification and Characterization of an Extracellular Protease from *Xenorhabdus nematophila* Involved in Insect Immunosuppression. *Appl. Environl. Microbiol.* 1297-1304.
- 21.Einosuki, M. Fumiko, Y. and Hiroyuki, K. 1993. Preparation and crystallization of D-glucosamine oligosaccharides with dp 6-8. *Carbohydr. Res.* 239:227-237.
- 22.Fredy, J. R., Luis, A. G., Jose, A. S., Mario, D. and Luis, M. Q. 2001. Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. *Process Biochem.* 36:507 – 515.
- 22.Francoise, B., Pierre, S., Anne, T., Mohamed, M., Pereillo, J. M., Joel, C., Guillemot, J. C., Maffrand, J. P. and Herbert, J. M. 1996. Purification and characterization of a novel protease from culture filtrates of a *Streptomyces* sp. *FEMS Microbiol. Lett.* 141:213-220.
- 23.Gupta, M. N. 1992. Enzyme function in organic solvents. *Eur. J. Biochem.* 203:25 – 32.
- 24.Gupta, A., Roy, I., Khare, S. K. and Gupta, M. N. 2005. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA. *J. Chromatogr. A.* 1069:155 – 161.
- 25.Hittu, M. and Vasu, P. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *Intl. J. Food Microbiol.* 42:139-145.
- 26.Imoto., T. and Yagishita, K. 1971. A simple activity measurement by lysozyme. *Agric. Biol. Chem.* 35:1154 – 6.
- 27.Jose, I. D. V., Dolores, D. A., Pilar, D. V., Joaquin, S. L. and Arturo, P. E. 1996. Purification and Characterization of an Extracellular Aspartate Protease from *Phycomyces blakesleeana*. *Fungal Genetics Biol.* 20:115 – 124.
- 28.Jadwiga, K. S. 1998. Purification and partial characterization of a neutral protease from a virulent stain of *Bacillus cereus*. *Int. J. of Biochem. Cell Biol.* 30:579-595.
- 29.Jiang, Y. and Dalton, H. 1994. Chemical modification of the hydroxylase of soluble methane monooxygenase gives one form of the protein with significantly increased thermostability and another that functions well in organic solvents. *Biochim. Biophys. Acta.* 1201:76-84.
- 30.Kumar, C. G. 2002. Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Let. Appl. Microbiol.* 34:13-7.
- 31.Kobayashi, T., Hakamada, Y., Adachi, S., Hitomi, J., Yoshimatsu, T., Koike, K. Kawai, S. and Ito, S. 1995. Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. (*Bacillus clausii*). *Appl. Microbiol. Biotechnol.* 43:473-81.
- 32.Karadzic, I., Masui, A. and Fujiwara, N. 2004. Purification and Characterization of a Protease from *Pseudomonas aeruginosa* Grown in Cutting Oil. *J. Biosci. Bioeng.* 3:145-152.
- 33.Kenji, A., Kukizo, M., Shuichiro, M. and Ryu, S. 1995. Anaerobic synthesis of extracellular proteases by the soil bacterium *Bacillus* sp. AM-23: Purification and characterization of the enzymes. *Soil Biol. Biochem.* 27:1377-1382.
- 34.Kim, S. S., Kim, Y. J. and Rhee, I. K. 2001. Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. *Arch. Microbiol.* 75:458 – 461.
- 35.Kumar, S., Sharma, N. S., Saharan, M. R. and Singh, R. 2005. Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. *Process Biochem.* 40:1701 – 1705.
- 36.Knorr, D. 1984. Use of chitinous polymer in food. *Food Technol.* 1:85-89.
- 37.Maria, J. B., Mar, R., Felix, N., Miguel, A. A., Maria, E. and Juan J. C. 2002. Purification and Characterization of an Extracellular Protease from *Penicillium chrysogenum* Pg222 Active against Meat Proteins. *Appl. Environ. Microbiol.* 3532-3536.
- 38.Martinez, P. and Arnold, F. H. 1991. Surface-charge substitutions increase the stability of alpha-lyticprotease IN organic-solvents. *J. Am. Chem. Soc.* 113:6336 – 6337.
- 39.Matta, H. and Punj, V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *Int. J. Food Microbiol.* 42:139-45.
- 40.Moreira, K. A., Porto, T. S., Teixeira, M. F. S., Porto, A. L. F. and Filho, J. L. L. 2003. New alkaline protease from *Nocardiopsis* sp: partial purification and characterization. *Process Biochem.* 39:67-72.
- 41.Muzzarelli, R. 1977. Enzymatic synthesis of chitin and chitosan. In *Chitin*.

42. Mink, R. and Blackwell, J. 1978. The structure of α -chitin. *J. Mol. Biol.* 120:167-170. 43. Nagano, H. and To, K. 2000. Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. *Biosci. Biotechnol. Biochem.* 64:181-3. 44. Nongporn, H. T., Anongnat, P. and Prasery, S. 1999. Purification and characterization of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS719. *J. Biosci. Bioeng.* 87:581-587. 45. Noboru, N., Claudia, T., Tianyan, S. and Masaaki, I. 2004. Purification and characterization of a novel metalloprotease isolated from *Aeromonas caviae*. *FEMS Microbiol. Lett.* 237:127 – 132. 46. Outtrup, H. and Jorgensen, S. T. 2002. The importance of *Bacillus* species in the production of industrial enzymes. In applications and systems of *Bacillus* and relatives. Edited by R. Berkley. Blackwell Science Inc., Malden, Mass. 206 – 218. 47. Ogino, H., Yasui, K., Shiotani, T., Ishihara, T. and Ishikawa, T. 1995. Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. *Appl Environ Microbiol.* 61:4258-62. 48. Ogino, H., Watanabe, F., Yamada, M., Nakagawa, S., Hirose, T. and Nogushi, A. 1999. Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PST-01. *J. Biosci. Bioeng.* 87:61 – 8. 49. Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D. and Mohan, R. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem.* 31:135 – 152. 50. Park, O. J., Jeon, G. J. and Yang, J. W. 1999. Protease-catalyzed synthesis of disaccharide amino acid esters in organic media. *Enzyme Microb. Technol.* 25:455 – 462. 51. Persson, M., Wehtje, E., and Adlercreutz, P. 2002. Factors governing the activity of lyophilised and immobilised lipase preparations in organic solvents. *Chem. Bio. Chem.* 3:566-571. 52. Qasim, K. B. and Rani, G. 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microbial. Technol.* 32:294 – 304. 53. Rashbehari, T., Binita, S. and Rintu, B. 2003. Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. *Process Biochem.* 38:1553-1558. 54. Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62: 597 – 635. 55. Rawlings, N. D. and Barrett, A. J. 1993. Evolutionary families of peptidases. *Biochem. J.* 290:205 – 218. 56. Ramachandran, N. and Madharam, P. 1982. Metal binding property of chitosan from different sources. Plenum Press, Sapporo, Japan. 187-190. 57. Sookkheo, B., Sinchaikul, S., Phutrakul, S. and Chen, S. Y. 2000. Purification and Characterization of the Highly Thermostable Proteases from *Bacillus stearothermophilus* TLS33. *Protein Expr. Purif.* 20:142 – 151. 58. Sergeeva, M. V., Paradkar, V. M. and Dordick, J. S. 1997. Peptide synthesis using proteases dissolved in organic solvents. *Enzyme Microb. Technol.* 20:623-628. 59. Schallmeyer, M., Singh, A. and Ward, O. P. 2004. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* 50. 60. Sareen, R., Uwe, T., Bornscheuer, and Mishra, P. 2004. Synthesis of kyotorphin precursor by an organic solvent-stable protease from *Bacillus licheniformis* RSP-09-37. *J. Mol. Catal. B: Enzymatic.* 32:1 – 5. 61. Sushil, K., Neeru, S., Sharma, M. R., Saharan, and Randhir, S. 2005. Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. *Process Biochemistry.* 40:1701 – 1705. 62. Schallmeyer, M., Singh, A. and Ward, O. P. 2005. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* 50:1 – 17. 63. Thangam, E. B. and Rajkumar, G. S. 2002. Purification and characterization of alkaline protease from *Alcaligenes faecalis*. *Biotechnol. Appl. Biochem.* 35:149 – 154. 64. Vulfson, E. N., Halling, P. J. and Holl, H. L. 2001. Enzymes in nonaqueous solvents, Methods and Protocols, Humana Press, Totowa, N. J. 65. VanBoven, A., Tan, P. S. T. and Koning, W. N. 1988. Purification and characterization of dipeptidase from *Streptococcus cermoris* Wg2. *Appl. Environ. Microbiol.* 54: 43-49. 66. Wang, S. L., Chen, Y. H., Wang, C. L., Yen, Y. H. and Chern, M. K. 2005. Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. *Enzyme Microbial. Technol.* 36:660-665. 67. Wang, S. L., Chang, W. T. and Lu, M. C. 1995. Production of chitinase by *Pseudomonas aeruginosa* K-187 in a shrimp and crab powder as a carbon source. *Proc. of National Sci. (B)* 19:105-112. 68. Wang, S. L. and Chio, S. H. 1998. Deproteinization of shrimp and crab shell with the protease of *Pseudomonas aeruginosa* K-187. *Enzyme Microb. Technol.* 22:629-633. 69. Wang, S. L., Yieh, T. C. and Shih, I. L. 1999. Production of antifungal compound by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. *Enzyme Microb. Technol.* 25:142- 148. 70. Wang, S. L., Yieh, T. C. and Shih, I. L. 1999. Purification and characterization of a new antifungal compound produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Enzyme Microb. Technol.* 25:439-446. 71. Wang, H. T. and Hsu, J. T. 2005. Optimal protease production condition for *Prevotella ruminicola* 23 and characterization of its extracellular crude protease. *Ecol. Environ. Microbiol.* 11:155-162. 72. Ward, O. P. 1991. Bioprocessing. Open University Press, Buckingham, U. K. 73. Yasuda, M., Aoyama, M., Sakaguchi, M., Nakachi, N. and Kobamoto, N. 1999. Purification and characterization of a soybean-milk-coagulating enzyme from *Bacillus pumilus* TYO-67. *Appl. Microb. Biotechnol.* 51:474 ± 479. 74. Yang, J. K., Shih, I. L., Tzeng, Y. M. and Wang, S. L. 2000. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microb. Technol.* 26:406 – 413. 75. Yoo, J. I., Lee, Y. S., Song, C. Y. and Kim, B. S. 2004. Purification and characterization of a 43-Kilodalton Extracellular serine proteinase from *Cryptococcus neoformans*. *J. Clin. Microb.* 722 – 726. 76. Zukowski, M. 1989. Production of commercially valuable products. In *Biotechnology Handbook No. 2. Bacillus*. Edited by C. R. Harwood. Plenum Publishing Corporation, New York. 311 – 337.