REFERENCES

Abeles, F. B., Bosshart, R. P., Fuence, L. E., and Habig, W. H. 1970. Preparation and purification of glucanase and chitinase from bean leaves. *Plant Phyiol.* 47: 129-134.

Aiba, S.1992. A convenient assay for chitinase that uses partially N-acetylated chitosan as substrate. *Carbohydr. Res.* 230: 373-376.

Alam, M. M., Mizutani, T., Isono, M., Nidaidou, N., and Watanabe, T. 1996. Three chitinase genes (*ChiA*, *ChiC* and *ChiD*) comprise three chitinase system of *Bacillus circulans* WL-12. *J. Ferment. Bioeng.* 82: 28-36.

Altschul, S.F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410

Armand, S., Tomita, H., Heyraud, A., Gey, C., Watanabe, T., and Henrissat, B. 1994. Stereochemical course of the hydrolysis reaction catalyzed by chitinase A1 and D from *Bacillus circulans* WL-12. *FEBS Lett.* 343: 177-180.

Bassler, B. L., Yu, C., Lee, Y. C., and Roseman, S. 1991. Chitin utilization by marine bacteria degradation and catabolism of chitin oligosaccharides by *Vibrio furnisii*. *J. Biol. Chem.* 266: 24276-24286.

Begiun, P. 1990. Molecular biology of cellulose degradation. *Annu. Rev. Microbiol.* 44: 219-248.

Blaak, H, Scheleman, J. Herissat, B, and Schrempf, H. 1993. Characteristics of an exochitinase from *Streptomyces olovaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. *Eur J. Biochem.* 214: 659-669.

Blackwell, J. 1988. Physical methods for the determination of chitin structure and conformation. *Method Enzymol* 161: 435-442.

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.

Brameld, K. A., and Goddard III, W. A. 1998. *Proc. Natl. Acad. Sci. U.S.A.* 95: 4276-4281.

Brurberg, M. B., Nes, I. F., and Eijsink, V. G. 1996. Comparative studies of chitinases A and B from *Serratia marcescens*. *Microbiology* 142: 1581-1589.

Chen, A. C., Mayer, R. T., and Deoach, J. R. 1982. Purification and characterization of chitinase from the stable fly, *Stomoxysi calcitrans*. *Arch Biochem. Biophys.* 216: 314-321.

Chernin, L. S., Fuente, L. D. L., Sobulev, V., Haran, S., Vorgias, L. E., Oppenheim, A. B., and Chet, I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* 63:834-839.

Clarke, P. H. 1956. The occurrence of chitinase in some bacteria. *J. Gen. Microbiol.* 14: 188-196.

Claus, D. and Berkeley, R. E. W. 1986. Genus *Bacillus* Cohn 1872, pp. 1105-1139.

Cody, R. M. 1989. Distribution of chitinase and chitobiase in *Bacillus*. *Curr. Microbiol.* 32: 71-93.

Cohen-Kupiec, R., and Chet, I. 1998. The molecular biology of chitin digestion. *Curr. Opin. Biotechnol.* 9: 270-277.

Collinge, D. B., Krage, K. M., Mikkelse, J. D., Rasmussen, U., and Vad, K. 1993. Plant chitinases. *Plant J.* 3: 31-40.

Deane, E. E., Whipps, J. M., Lynch, J. M., and Peberdy, J. F. 1998. The purification of a *Trichoderma harzinum* exochitinase. *Biochim Biochys Acta.* 1383: 101-110.

Edberg, S. C. 1992. US EPA human health assessment: *Bacillus licheniformis*. Unpublished, U.S. Environmental Protection Agency, Washington, D.C.

Edwards, U., Rogall, T., Blocher, H., Emade, M., and Bottger, E. C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. 17(19): 7843-7853.

Estrella, A. H., and Chet, I. 1999. Chitinases in biological control. Chitin and chitinases. Jolles, P. and Muzzarelli, R. A. A. editors. pp 171-180.

Farrar, W. E. 1963. Serious infections due to "nonpathogenic" organisms of the genus *Bacillus*. *Am. J. Med.* 34: 134.

Flach, J., Pilet, P. E., and Jolles, P. 1992. What's new in chitinase research? *Experientia* 48: 701-716.

Fuchs, R. L., McPherson, S. A., and Drahos, D. J. 1986. Cloning of a *Serratia marcescens* gene encoding chitinase. *Appl. Environ. Microbiol.* 19: 677-286.

Fujii, T., and Miyashita, K. 1993 Multiple domain structure in a chitinase gene (*chi C*) of *Streptomyces lividans*. *J. Gen. Microbiol.* 139: 677-686.

Gish, W., and States, D.J. 1993. Identification of protein coding regions by database similarity search. *Nature Genet.* 3:266-272.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. 1991. Domains in microbial β 1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55: 303-315.

Gooday, G. W. 1999. Aggressive and defensive roles for chitinases. Chitin and chitinases. Jolles, P. and Muzzarelli, R. A. A. editors. pp 157-166.

Gooday, G. W., Zhu, W. Y., and O'Donnell, R. W. 1992. What are the roles of chitinases in the growing fungus? *FEMS Microbiol. Lett* 100: 387-392.

Gooday, G. W. 1990. The ecology of chitinase decomposition. *Adv. Microb. Ecol.* 11: 387-430.

Gomes, C. S., Semedo, L. T. A. S., Soares, R. M. A., Linhares, L. F., Ulhoa, C. J., Alviano, R. C., and Coelho, R. R. R. 2001. Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *J. Appl. Microbiol.* 90: 653- 661.

Gordon, R. E., Haynes, W. C., and Pang, CH-N. 1973. The genus Bacillus. U.S. Department of Agriculture Agricultural Handbook no. 427. U.S. Department of Agriculture, Washington DC,

Guex, N. and Peitsch, M. C. 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Eletrophoresis.* 18: 2714-2723.

Hara, S., Yamamura, Y., Fujii, Y., Mega, T., and Ikenake, T. 1989. Purification and characterization of chitnase produced by *Streptomyces erythraeus*. *J. Biochem.* 105: 484-489.

Harpster, M. H., and Dunsmuir, P. 1989. Nucleotide sequence of the chitinase B gene of a *Serratia marcescens* QMB1466. *Nucleic Acids Res.* 17: 5395.

Henrissat, B., and Bairoch, A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293: 781-788.

Henrissat, B. 1991. A classification of glycosyl hydrolyses bases on amino acid sequence similarities. *Biochem. J.* 280: 309-316.

Herwig, R. P., Pellerin, N. B., and Irgens, R. L. 1988. Chitinolytic bacteria and chitin minerlization in the marine waters and sediments along the Antarctic peninsula. *FEMS Microbiol. Ecol.* 53: 101-112.

Hiraga, K., Shou, L., Kitazawa, M., Takahashi, S., Shimada, M., Sato, R., and Oda, K. 1997. Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330. *Biosci. Biotech. Biochem.* 61: 174-176.

Imoto, T., and Yagishita, K. 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35(7): 1154-1156.

Inbar, J., and Chet, I. 1981. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. *Microbiology* 141, 2823-2829.

Jeuniaux, C. 1966. Chitinases. *Method Enzymol* 8: 644-649.

Jones, J. D. C., Grady, K. L., Suslow, T. V., and Bedbrook, J. R. 1986. Isolation and characterization of genes encoding two enzymes from a *Serratia marcescens*. *EMBO J.* 5: 467-473.

Joshi, S., Kozlovski, M., Richens, S., and Comberbach, D. M. 1989. Chitinase and chitobiase production during fermentation of genetically improved a *Serratia liquefaciens*. *Enzyme Microbiol. Technol.* 11: 289-296.

Joshi, S., Kozlowski, M., Selvrj, G., Iyer, V. N., and Davies, R. W. 1988. Cloning of genes of the chitin utilization regulon of *Serratia liquefaciens*. *J. of Bateriol.* 170: 2984-2988.

Kamei, K., Yamamura, Y., Hara, S., and Ikenaka, T. 1989. Amino acid sequence of chitinase from *Streptomyces erythraeus*. *J. Biochem.* 105: 979-985.

Kaschnitz. R., Peterlik, M., and Weiss, H. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* 30: 148-452.

Koga, D., Isogai, A., Sakuda, S., Matsumoto, S., Suzuki, A., Kimura, S., and Ide, A. 1987. *Agri. Biol. Chem.* 51: 471-476.

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., and Nagamatsu, Y. 1997. Purification and characterization of *Bombyx mori* chitinases. *Insect Biochem. Mol. Biol.* 27: 759-767.

Kono, M., Matsui, T., Shimizu, C., and Koga, D. 1990. Purification and some properties of chitinase from the stomach of Japanese eel, *Anguilla japonica*. *Agric. Biol. Chem.* 54: 937-987.

Kramer, K., and Koga, D. 1986. Insect chitin. *Insect Biochem.* 16: 851-877.

Kuranda, M. J., and Robbins, R. W. 1991. Chitinase is required for cell separation during growth of *Saccharomyces cervisiae*. *J. Biol. Chem.* 266: 19785-19767.

Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lindsay, G. J. H., and Gooday, G. W. 1985. Action of chitinase in spines of the diatom *Thalassiosira fluviatilis*. *Carbohydr. Polymers* 5: 131-140.

Lowe, G., Sheppard, G., Sinnot, M. L., and Williams, A. 1967. *Biochem. J.* 104: 893-899.

Madden, T.L., Tatusov, R.L. & Zhang, J. 1996. Applications of network BLAST server. *Meth. Enzymol.* 266:131-141.

Martin, M. 1991. The latex of *Hevea brasiliensis* contains high levels of both chitinase and chitinase/lysozymes. *Plant Physiol.* 95: 469-476.

Melechers, L. S., Groot, M. A., Knaap, J. A., Postein, A. S., Buurlage, M. B. S., Bol, J. F., 'Cornelissen, B. J. C., Elzen, P. J. M., and Linthorst, J. M. 1994. A new class of tobacco chitinases homologous to bacterial exo-chitinase displays antifungal activity. *Plant J.* 5: 469-480.

Minke, R., and Blackwell, J. 1978. The structure of α-chitin. *J. Molec. Biol.* 120: 167-181.

Miyashita, K., and Fujii, T. 1993. Nucleotide sequence and analysis of a genes (chiA) for a chitinase from *Streptomyces lividans* 66. *Biosci. Biotechnol. Biochem.* 57: 1961-1968.

Miyashita, K., Fujii, T., and Sawada, Y. 1991. Molecular cloning and characterization of chitnase gene from *Streptomyces lividans* 66. *J. Gen. Microbiol.* 137: 2065-2072.

Monreal, J., and Rees, E. T. 1968. The chitinase of a *Serratia marcescens*. *Can. J. Microbiol.* 137: 689-696.

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

Morimoto, K., Kanta, S., Kimura, T., Sakka, K., and Ohmiya, K. 1997. Cloning, sequencing and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel Cadherin-link domains and a chitin-binding domain. *J. Bacteriol.* 179: 7306-7314.

Nawani, N. N., and Kapadnis, B. P. 2001. One-step purification of chitinase from *Serratia marcescens* NK1, a soil isolate. *J. Appl. Microbiol.* 90: 803-808.

Ohtakara, A. 1986. Viscoimetric assay for chitinase. *Method Enzymol* 161: 426-430.

Ohtakara, A., Yoshida, M., Murakmi, M., and Izumi, T. 1981. Purification and characterization of β-N-acetylhexosaminidase from *Pynoporus cinnabariunus*. *Agric. Biol. Chem.* 45: 239-247.

Okazaki, K., Kato, F., Watanabe, N., Tasuda, S., Masui, Y., and Hayakawa, S. 1995. Purification and properties of two chitinases from *Streptomyces* sp. J-13. *Biosci. Biotechnol. Biochem.* 59: 1586-1587.

Peitsch, M. C. 1996. ProMod and Swiss-Model: Internet-based tools for automated comparative protein modeling. *Biochem Soc Trans.* 24:274-279.

Peitsch, M. C. 1995. Protein modeling by E-mail Bio/Technology. 13,658-660.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S., and Vorgias, C. E. 1994. Crystal structure of bacterial chitinase at 2.3 A° resolution. *Stucture* 2: 1169-1180.

Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwathanasininitt, M., and Aiba, S. 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337: 557-559.

Piszkiewiez, D. and Briuce, T. C. 1968. *J. Am. Chem. Soc.* 90: 2156-2163.

Punja, Z. K., and Zhang, Y. Y. 1993. Plant chitinases and their roles in resistance to fungal diseases. *J. Nematol.* 25: 526-540.

Reissig, J. L., Strominger, J. l., and leloir, L. F. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* 217: 956-966.

Robbins, P.W., Overbye, K., Albright, C., Benfiedk, B., and Pero, J. 1992. Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene.* 111: 69-76.

Roberts, R. L., and Cabib, E. 1982. *Serratia marcescens* chitinase: One step purification and use for the determination of chitin. *Anal. Biochem.* 127: 402- 412.

Robertson, E. F., Dannelly, K. H., Malloy, P. J., and Reeves, H. C. 1987. Rapid isoelectric focusing in a vertical polyacrylamide minigel system. *Anal. Biochem.* 167: 290-294.

Rudall, R. M. 1963. The chitin/protein complexes of insect cuticles. In: Beament, J. W. L, Treherne, J. E., and Wigglesworth, V. B., editors. Advances in insect physiology. London, New York: Academic Press, pp 257-313.

Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M. 1998. Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Appl. Environ. Microbiol.* 64(9): 3397-3402.

Sakai, K., Narihar, M., Kasama, Y., Wakayama, M., and Moriguchi, M. 1994. Purification and characterization of thermostable β-N-acetylhexosaminidase of *Bacillus stearothermophilus* CH-4 isolated from chitin-containing compost. *Appl. Environ. Microbiol.* 60(8): 2911-2915.

Shaikh, S. A., and Deshpande, M.V. 1993. Chitinolytic enzymes: their contribution to basic and applied research. *World J. Microbiol. Biotech.* 9: 468-475.

Shinshi, H., neuhaus, J. M., Ryals, J., and Meins, F. Jr. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can rise by transposition of sequences encoding a cysteine-rich domian. *Plant Molecular Biol.* 14: 357-368.

Sirit, Y., Vorgias, C., Chet, I., and Oppenheim, A. B. 1995. Cloning and Primary Structure of the *chiA* Gene from *Aeromonas caviae*. *J. Bacteriol.* 177: 4187-4189.

Tabbara, K. F., and Tarabay, N. 1979. Bacillus licheniformis corneal ulcer. *Am. J. Ophthalmol* 87(5): 717-9.

Takaguchi, Y., and Shimahara, K. 1989. Isolation and identification of a thermophilic bacterium producing N, N'-diacetylchitobiose from chitin. *Agric. Biol. Chem.* 53 (6): 1537-1541.

Takayanagi, T., Ajidaks, K., Takiguchi, Y., and Shimahara, K. 1991. Isolation and characterization of thermostable chitinase from *Bacillus licheniformis* X 7u. *Biochim. Biophys. Acta.* 1078: 404-410.

Tanaka, T., Fujingo, S., Mishiaki, S, Fukni, T., Takagi, M., and Imanaka, T. 1999. A unique chitinase with dual actives sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. *Appl. Environ. Microbiol.* 65: 5838-53443

Tantimavanich, S., Pantuwatana, S., Bhumiratana, A., and Panbangred, W. 1998. Multiple chitinases enzymes from a single gene of *Bacillus licheniformis* TP-1. *J. Ferment Bioeng.* 85(3): 259-265.

Tracey, M.V. 1957. Chitin. *Rev. Pure Appl. Chem.* 7: 1-14.

Trachuk, L. A., Revina, L. P., Shemyakina, T. M. Chestukhina, G. G. and Stepanov, V.M. 1996. Chitinases of *Bacillus licheniformis* B-6839: isolation an properties. *Can. J. Microbiol.* 42: 307-315.

Trudel, J., and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* 178: 362-366.

Tsukamoto, T., Koga, D., Ide, A., Idhibahi, T., Horino-Motsuchige, M., Yagashita, K. and Imoto, T. 1984. Purification and some properties of chitinases from yam, *Dioscorea opposita* THUNB. *Agric. Biol. Chem.* 48: 931-939.

Tsujibo, H., Hatano, N., Endo, H., Miyamoto, K., and Inamori, Y. 2000. Purification and characterization of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520 and cloning of the encoding gene. *Biosci. Biotechnol. Biochem.* 64: 96-102.

Tsujibo, H., Minoura,K., Miyamoto, K., Endo, H., Moriwaki, M., and Inamori, H. 1993. Purification and properties of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Appl. Environ. Microbiol.* 59: 620-622.

Tsujibo, H., Yoshida, Y. and Miyamoto, K. 1992. Purification properties and partial amino acid sequence of chitinase from a marine *Alteromaonas* sp. strain O-7. *Can. J. Microbiol.* 38: 391-397.

Ueda, M., Fujiwara, A., Kawaguchi, T., and Arai, M. 1995. Purification and some properties of six chitinases from *Aeromonas* sp. 10S-4. *Biosci. Biotechnol.Biochem.* 59: 2162-2164.

Ueda, M., Kawaguchi, T., and Arai, M. 1994. Molecular cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas* sp. No. 10S-24. *J. Ferment Bioeng.* 78: 205-211.

Ueno, H., Miyashita, K., Sawada, Y., and Obe, Y. 1990. Purification and some properties of extracellular chitinases form *Streptomyces* species S-84. *J. Gen. Appl. Microbiol.* 36: 377-392.

Usui, T., Hayashi, Y., Nanjo, F., Sakai, K., and Ishido, Y. 1987. Transglycosylation reaction of a chitinase purified from *Norcardia orientalis*. *Biochim. Biophys. Acta* 923: 302-309.

van Aaltem, D. M. F., Synsted, B., Brudberg, M. B., Haugh, E., Riise, B. E., Eijsink, V. G. H., and Weierenga, R. W. 1999. *Proc. Natl. Acad. Sci.* U.S.A. 97: 5842-5847.

van Scheltbga, A. C. T., Armand, S., Kalk, K. H., Isogai, A., henrissat, B., and Dijkstra, B. W. 1995. Stereochemistry of chitin hydrolysis by plant chitinase/lysozyme and X-ray structure with complex with allosamidin: Evidence for substrate assisted catalysis. *Biochemistry* 34: 15619-15623.

Vyas, P., and Deshpandee, M. V. 1989. Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. *J. Gen. Appl. Microbiol.* 35: 343-350.

Wang, S. L., Chang, W. T., and Lu, M.C. 1995. Production of chitinase by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as carbon source. *Proc. Natl. Sci.* Counc. ROC B 19: 105-112.

Watanabe, T., Uchida, M., Kobori, K., and Tanaka, H. 1994. *Biosci. Biotech. Biochem.* 58: 2283-2285.

Watanabe, T., Ito, Y., Yamada, T., Hashinmoto, M., Sekine, S., and Tanaka, H. 1994b. The roles of the C-terminal domain and type-III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J. Baceriol.* 176: 4465-4472.

Watanabe, T., Kobori, K., Miyahsita, K., Fujii, T., Sakai, M., Uchida, M. and Tanaka, H. 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 268: 18567-18572.

Watanabe, T., Oyanagi, M., Suzuki, K., Ohnishi, K., and Tanaka, H. 1992. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryote chitinases and class III plant chitinases. *J. Bacteriol.* 174: 408-414.

Watanabe, T., Oyanagi, W., Suzuki, K., and Tanaka, H. 1990a. Chitinase A_1 in chitin degradation. *J. Bacteriol.* 172: 4017-4022.

Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K., and Tanaka, H. 1990b. Gene cloning of chitinase A_1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. *J. Biol. Chem.* 265: 15659-15665.

Wiegel, J., and Ljungdahl, L. G. 1986. The importance of thermophilic bacteria in biotechnology. *Crit. Rev. Biotechnol.* 3:39.

Wilson, D. B., Spezio, M., Irwin, D., Karplus, A., and Taylor, J. 1995. Comparison of enzymes catalyzing the hydrolysis of inducible polysaccharides. *Enzyme Degradation.* 618: 1-12.

Woytowich, A. E., Selvaraj, G., and Khachatourians, G. G. 2000. Analysis of the *chiB* gene of *Serratia liquefaciens*. *J. Biotechnol.* 80: 277-283.

Yabuki, M., Mizushina, K., Amatotsu, T., Ando, A., Fujii, T., Shimada, M., and Yamashita, M. 1986. Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophila* subsp. *Anaerogenes* A52. *J. Gen. Appl. Microbiol.* 32: 25-38.

Yabuki, M., Kasai, Y., Ando, A., and Fujii,T. 1984. Rapid method of converting fungal cells into protoplast with a high regeneration frequency. *Exp. Mycol.* 8: 386-390.

Yamaoka, H., Hayashi, H., Karita, S., Kimura, T., Sakka, K., and Ohmiya, K. 1999. Purification and some properties of a chitinase from *Xanthomonas* sp. strain AK. *J. Biosci. Bioeng.* 88: 328-330.

Yannis, p., Gali, P., Giorgos, T., Constantionus, E. V., Amos, B. O., and Kyriacos, P. 2001. High solution structure analyses of mutant chitinase a complexes with substrates provide new insight into the mechanism of catalysis. *Biochemistry* 40: 11338-11343.

Yu, C., Lee, A. M. Bassler, B. L., and Roseman, S. 1990. Chitin utilization by marine bacteria. *J. Biol. Chem.* 266: 24260-24267.

Zhang, J., and Madden, T.L. 1997. PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation. *Genome Res.* 7:649-656.

Zhu, B. R. C., Lo, J. Y., Li, Y. T., Li, Sc., Li, J. M., Gildenmeister, O. S., Laine, R. A., and Ou, C. Y. 1992. Thermostable, salt, tolerant, wide pH range novel chitobiase from *Vibrio parahemolyticus*: Isolation, characterization, molecular cloning and expression. *J. Biochem.* 112: 163-167.

APPENDICES

APPENDIX A

PURIFICATION OF THERMOSTABLE CHITINASE PRODUCED BY *Bacillus licheniformis* SK-1

Sanya Kudan and Rath Pichyangkura*
Department of Biochemistry, Faculty of Science, Chulalongkorn university, Bangkok, Thailand 10330

Key words: chitinase, *Bacillus licheniformis*, glycoprotein

ABSTRACT

Chitinase was partially purified from the culture medium of *Bacillus licheniformis* SK-1 by colloidal chitin affinity adsorption followed by DEAE-cellulose column chromatography. The partial purified enzyme showed a single protein band on native polyacrylamide gel electrophoresis. The isoelectric point of the major component in the partial purified chitinase was 4.62. The partial purified chitinase showed a major band with MW 72 kDa and 2 minor bands with MW 58, 70 kDa on SDS-PAGE, respectively. In addition, the partial purified chitinase showed a glycoprotein nature on native polyacrylamide gel when stained with Schiff's reagent. The partial purified chitinase revealed two activity optima at pH is 6 and 8 when colloidal chitin was used as substrate. The enzyme exhibited a broad activity temperatures ranging between 40 to 70 °C, with optimum at 55 °C. The K_m and V_{max} of the partial chitinase was 0.23 mg colloidal chitin ml^{-1} and 0.45 U ml^{-1}.

*Corresponding author.

INTRODUCTION

Chitinases, a group of enzymes capable of degrading chitin to low-molecular-weight products, have been shown to be produced by a number of microorganisms. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shellfish wastes that not only solves environmental problems but also promotes the economic value of the marine products [1]. Thus, chitinolytic enzymes have been purified from many microorganism, and their enzymatic properties have been investigated. *Bacillus* spp. is regarded as a group of bacteria particularly efficient in the breakdown of chitin. [2]. Previously, thermostable endochitinases, which are useful for the preparation of chitobiose [3], have been isolated from *B. licheniformis* X-7u [4], and *Streptomyces thermoviolaceus* OPC-520 [5], while thermostable exochitinase from *B. staerothermophilus* CH-4 [6] was characterized. *B. licheniformis* SK-1 produces thermostable chitinase when cultured in a medium containing colloidal chitin as a carbon source. We were able to use crude chitinase from this microorganism to prepare GlcNAc from crystalline chitin [7]. This paper describes the purification and some properties of chitinases from the culture medium of *B. licheniformis* SK-1.

EXPERIMENTAL PROCEDURE

Microorganism and culture. SK-1 was isolated from soil in Angthong Province, Thailand. SK-1 was cultured at 50 °C on 0.02% colloidal chitin minimum medium (CCMM) agar plate, 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4$, 0.6% KH_2SO_4 and 1.0% K_2HPO_4, pH 7.5 and 2% agar. For liquid culture, the medium containing 0.1% CCMM. The microorganism was incubated with shaking at 50 °C in CCMM for 4-5 days.

Purification of chitinase. SK-1 was cultured with shaking at 50 °C in 0.1% CCMM (2L) for 4-5 days. The cells were removed by centrifugation (8,000 X g ; 20 min) to obtain culture fluid. The culture fluid was stirred gently with fresh colloidal chitin (10 mg/mg of protein) overnight at 0 °C for affinity adsorption [8]. The colloidal chitin was then washed three times with 10 mM potassium phosphate buffer (KPB, pH 6.0) and collected by centrifugation. The precipitated colloidal chitin was resuspended in 20 ml of KPB and incubated at 50 °C overnight to digest the colloidal chitin. The digested solution was dialyzed against 25 mM Tris-HCl buffer, pH 7.5. The dialyzed enzyme was applied to a DEAE-cellulose column, previously equilibrated with 25 mM Tris-HCl buffer, pH 7.5, and eluted with 0-1.0 gradient of NaCl.

Enzyme assay. Chitinase activity was assayed in 1.5 ml of a reaction mixture containing 0.1% colloidal chitin in 0.1 M phosphate buffer pH 6.0 and 0.1 ml of enzyme solution. After incubation at 50 °C for 10 min, the reaction was stopped by boiling, then centrifuged. Reducing sugar produced in the supernatant was measured by the modified Schales method [9]. One unit of chitinase activity was defined as the amount of enzyme that liberate reducing sugar corresponding to one μmole of *N*-acetyl-D-glucosamine per minute.

Protein measurement. Protein measurement was performed by the method of Bradford et al.[10] using bovine serum albumin as the standard. For chromatographic profile, the protein concentration was estimated by measuring the absorbance at 280 nm.

Determination of the isoelectric point (pI) of the enzyme. Isoelectric focusing was performed according to Robertson et al. [11] with ampholine carrier ampholytes pH 3-10 at final concentration of 1%. After electrofocusing, the gel was stained with Coomassie brilliant blue R-250.

Glycoprotein in native protein and activity stain on PAGE. The enzyme preparations were analyzed by nondenaturing PAGE using 7.5% gel, according to Laemmli [12]. Three sets of gel with identical samples were electrophoresis. After electrophoresis, one gel was stained for protein with Coomassie blue R-250, the second gel was stained for activity, using glycol chitin as substrate [14]. The glycoprotein nature of the enzymes was detected in the third gel (7-15% gradient) using Schiff's reagent as a staining agent according to the methods described by Kaschnitz et al.[13].

Determination of molecular weight and activity staining. The molecular weights of the enzyme was determined by 10% SDS-PAGE. In electrophoresis, standard proteins were used. The activity of chitinase in SDS-PAGE was detected according to the method of Trudel and Asselin [14].

Optimal pH of chitinase. The chitinase activity was measured at various pHs by a colorimetric method, using colloidal chitin as a substrate. The enzyme was preincubated in 0.1 M citrate buffer at pH 3-6, 0.1 M phosphate buffer at pH 6-8 and 0.1 M Tris-HCl buffer at pH 8-10, at 50 °C for 30 min. After which, the enzyme activity was assay.

Optimal temperature of chitinase. The enzyme was preincubated in 0.1 M phosphate buffer pH 6.0 at different temperatures from 40 to 70 °C for 30 min and assayed for chitinase activity.

RESULTS AND DISCUSSION

The partial purified enzyme gave a single protein band on native polyacrylamide gel electrophoresis and isoelectric focusing gel (Fig.1). The isoelectric point was 4.62 by isoelectric focusing. The partial purified enzyme gave a three bands on SDS-PAGE (Fig.2). The molecular weight was 58, 70 and 72 kDa, as judged by SDS-PAGE.

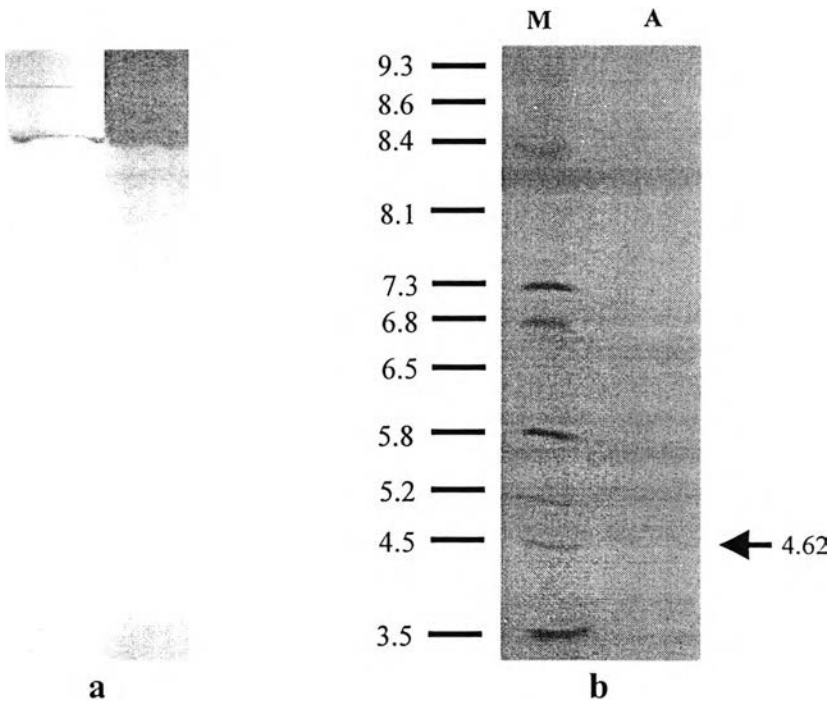


Figure.1 Native gel of the partial purified chitinase from *B. licheniformis* SK-1(a); lane A: partial purified chitinase, lane B: chitinase activity). IEF pattern of chitinase (b); lane M: IEF standard marker, lane A: partial purified chitinase.

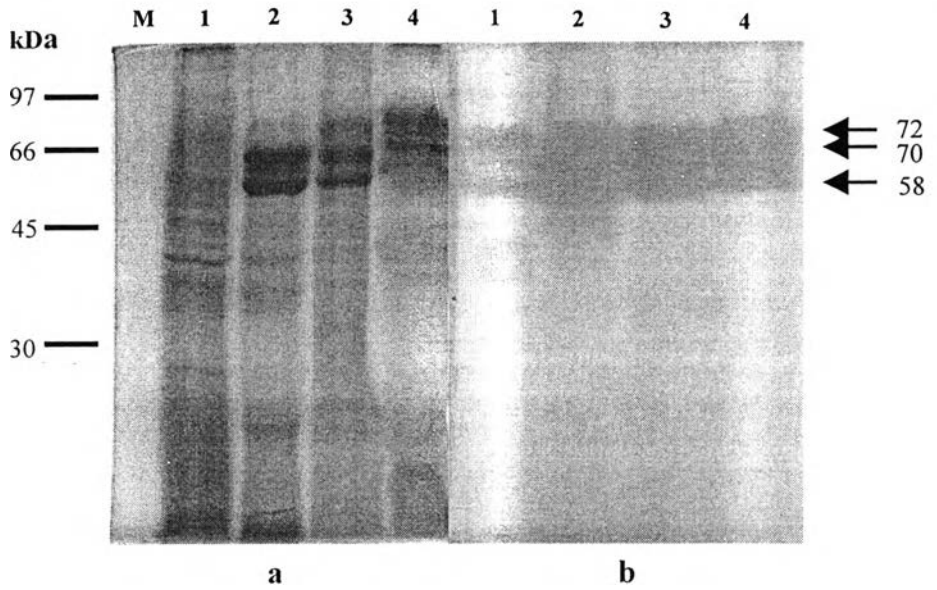


Figure 2. SDS-PAGE of the partial purified chitinases, protein stain (a) and activity stain (b). Lane M: standard marker, lane 1: crude chitinases, lane 2 : affinity adsorption, lane 3: bound column peak 1, lane 4: bound column peak 2.

It has been shown earlier that the thermophilic strain of *B. licheniformis* X-7u produced four forms of chitinase [4]. Two of the chitinases had the molecular masses of 89 and 76 kDa with different N-terminal sequence, whereas the other two have formed
presumably owing to limited proteolysis of the chitinase 76 C-terminal region Hence, it appears that strain used in our work produced a different array of chitinase.

Multiplicity of chitinase produced by bacterial cells might be considered as a rather common phenomenon. *Serratia marcescens* [16, 17, 18, 19], *S. liquefaciens* [20], *Streptomyces Olivaceoviridis* [21, 22], *Streptomyces pliccatus* [23], *B. circulans* [24, 25, 26], and *B. licheniformis* X 7-u [4] have been shown to produce several forms of chitinase. These forms reflect the multiplicity of structural genes coding for chitinases or a result of post-translational modifications, among which limited proteolysis plays a prominent role.

Hence, *B. licheniformis* appears to produce an array of chitinase that might be useful for adaptation of these bacteria to utilize the sources of chitin encountered in their habitat [15]

Polyacrylamide gel electrophoresis of the purified enzyme sample stained positive with Schiff's reagent, suggesting the glycoprotein nature of the enzymes (Fig. 3).

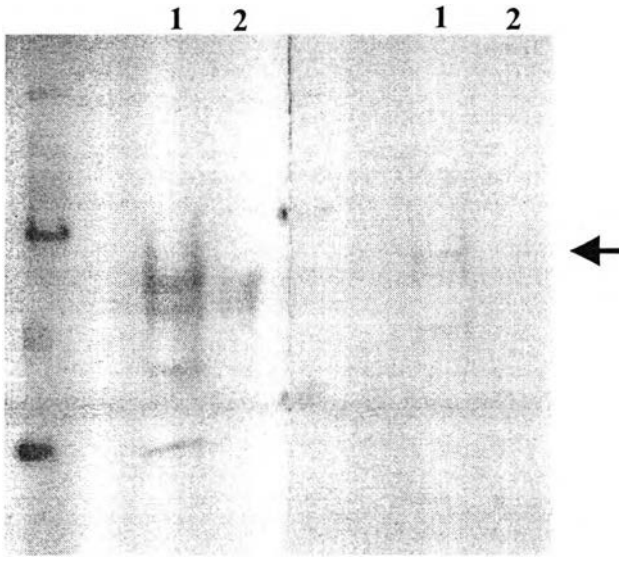


Figure 3. Glycoprotein staining. Lane M: positive control (transferrin, BSA), lane1 : crude chitinases, lane 2. partial chitinases.

The enzyme showed optimal activity at 2 pHs: 6 and 8 (Fig.4). *B. licheniformis* was also shown to possess two pH optima of activity against glycol and colloidal chitin [4, 15], i.e., in both acidic and alkaline solutions, whereas only one optimum activity was observed at pH 5 towards low molecular mass substrates. This unusual property, peculiar to some bacterial chitinases, seems to reflect a difference in pH influence on the activity of an enzyme and the structure of the high molecular mass substrate [15].

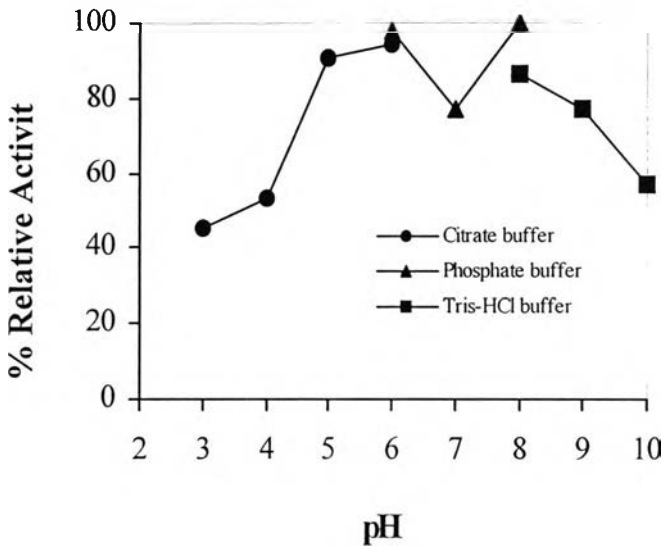


Figure 4. Effect of pH of *B. licheniformis* SK-1 chitinase activity.

The chitinase had a high optimum temperature at 55 °C (Fig. 5) compared with the enzymes from other mesophile bacteria [27] and fungi [28]; with optimum temperature around 45 °C.

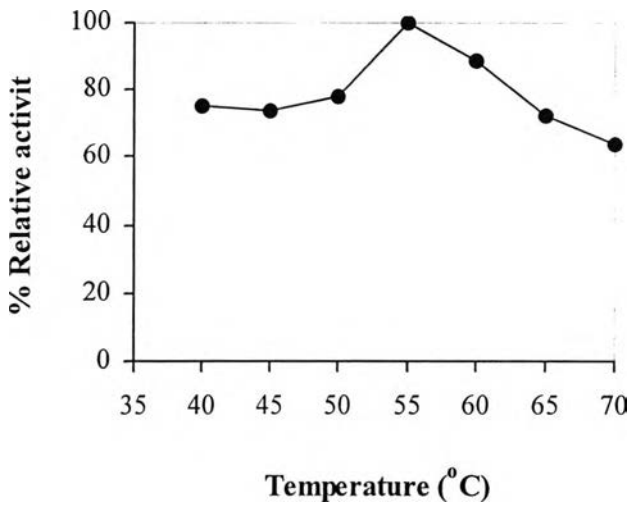


Figure 5. Effect of temperature on *B. licheniformis* SK-1 chitinase activity.

When the activity of the partial purified chitinase was followed at various substrate concentrations using colloidal chitin as the substrate, the profile followed Michaelis-Menten kinetics. The K_m value calculated from Lineweaver-Burk plots (Fig. 6) was 0.23 mg/ml for colloidal chitin and the V_{max} was 0.45 U ml^{-1}.

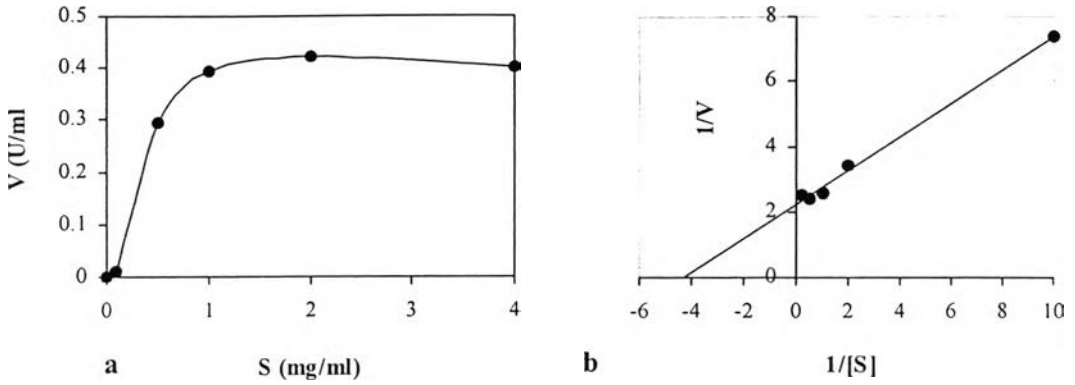


Figure 6. Dependence of the reaction rate of chitinase on the substrate concentration.
a) Saturation curve
b) Lineweaver-Burk plot

CONCLUSIONS

Chitinase was partially purified from *B. licheniformis* SK-1 with molecular weight of 58, 70 and 72 kDa on SDS-PAGE. It stained positive for glycoprotein and its isoelectric was 4.62. The optimum pH and temperature of the chitinase from *B. licheniformis* SK-1 was at pH 6 and 8, and 55 °C. The K_m of the chitinase was 0.23 mg colloidal chitin ml^{-1} and the V_{max} was 0.45 U ml^{-1}.

ACKNOWLEDGMENT

The research work was supported by the graduate school foundation, Chulalongkorn university, Thailand. We would like to thank Dr. Tipaporn Limpaseni for critical reading of this manuscript.

REFERENCE

1. Wang, S. L., Chang, W. T., and Lu, M.C. 1995. Production of chitinase by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as carbon source. *Proc. Natl. Sci.* Counc. ROC B 19: 105-112.
2. Gooday, G. W. 1990. The ecology of chitinase decomposition. *Adv. Microb. Ecol.* 11: 387-430.
3. Takaguchi, Y., and Shimahara, K. 1989. Isolation and identification of a thermophilic bacterium produing N, N'-diacetylchitobiose from chitin. *Agric. Biol. Chem.* 53(6): 1537-1541.
4. Takayanagi, T., Ajidaks, K., Takiguchi, Y., and Shimahara, K. 1991. Isolation and characterization of thermostable chitinase from *Bacillus licheniformis* X 7u. *Biochim. Biophys. Acta.* 1078: 404-410.
5. Tsujibo, H., Minoura,K., Miyamoto, K., Endo, H., Moriwaki, M., and Inamori, H. 1993. Purification and properties of a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Appl. Environ. Microbiol.* 59: 620-622.

6. Sakai, K., Narihar, M., Kasama, Y., Wakayama, M., and Moriguchi, M. 1994. Purification and characterization of thermostable β-N-acetylhexosaminidase of *Bacillus stearothermophilus* CH-4 isolated from chitin-containing compost. *Appl. Environ. Microbiol.* 60(8): 2911-2915.
7. Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwathanasininitt, M., and Aiba, S. 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337: 557-559.
8. Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M. 1998. Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Appl. Environ. Microbiol.* 64(9): 3397-3402.
9. Imoto, T., and Yagishita, K. 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35(7): 1154-1156.
10. 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.
11. Robertson, E. F., Dannelly, K. H., Malloy, P. J., and Reeves, H. C. 1987. Rapid isoelectric focusing in a vertical polyacrylamide mnigel system. *Anal. Biochem.* 167: 290-294.
12. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
13. Kaschnitz. R., Peterlik, M., and Weiss, H. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* 30: 148-452.
14. Trudel, J., and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* 178: 362-366.
15. Trachuk, L. A., Revina, L. P., Shemyakina, T. M. Chestukhina, G. G. and Stepanov, V. M. 1996. Chitinases of *Bacillus licheniformis* B-6839: isolation an properties. *Can. J. Microbiol.* 42: 307-315.
16. Fuchs, R. L., McPherson, S. A., and Drahos, D. J. 1986. Cloning of a *Serratia marcescens* gene encoding chitinase. *Appl. Environ. Microbiol.* 19: 677-286.
17. Jones, J. D. C., Grady, K. L., Suslow, T. V., and Bedbrook, J. R. 1986. Isolation and characterization of genes encoding two enzymes from a *Serratia marcescens*. *EMBO J.* 5: 467-473.
18. Harpster, M. H., and Dunsmuir, P. 1989. Nucleotide sequence of the chitinase B gene of a *Serratia marcescens* QMB1466. *Nucleic Acids Res.* 17: 5395.
19. Monreal, J., and Rees, E. T. 1968. The chitinase of a *Serratia marcescens*. *Can. J. Microbiol.* 137: 689-696.
20. Joshi, S., Kozlovski, M., Richens, S., and Comberbach, D.M. 1989. Chitinase and chitobiase production during fermentation of genetically improved a *Serratia liquefaciens*. *Enzyme Microbiol. Technol.* 11: 289-296.
21. Miyashita, K., Fujii, T., and Sawada, Y. 1991. Molecular cloning and characterization of chitnase gene from *Streptomyces lividans* 66. *J. Gen. Microbiol.* 137: 2065-2072.
22. Miyashita, K., and Fujii, T. 1993. Nucleotide sequence and analysis of a gens (chiA) for a chitinase from *Streptomyces lividans* 66. *Biosci. Biotechnol. Biochem.* 57: 1961-1968.
23. Robbins, P.W., Overbye, K., Albright, C., Benfiedk, B., and Pero, J. 1992. Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene.* 111: 69-76.

24. Watanabe, T., Oyanagi, W., Suzuki, K., and Tanaka, H. 1990a. Chitinase A_1 in chitin degradation. *J. Bacteriol.* 172: 4017-4022.
25. Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K., and Tanaka, H. 1990b. Gene cloning of chitinase A_1 from *Bacillus circulans* WL-12 revealed its evolutionnary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. *J. Biol. Chem.* 265: 15659-15665.
26. Watanabe, T., Oyanagi, M., Suzuki, K., Ohnishi, K., and Tanaka, H. 1992. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryote chitinases and class III plant chitinases. *J. Bacteriol.* 174: 408-414.
27. Yabuki, M., Mizushina, K., Amatotsu, T., Ando, A., Fujii, T., Shimada, M., and Yamashita, M. 1986. Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophila* subsp. *Anaerogenes* A52. *J. Gen. Appl. Microbiol.* 32: 25-38.
28. Ohtakara, A., Yoshida, M., Murakmi, M., and Izumi, T. 1981. Purification and characterization of B-N-acetylhexosaminidase from *Pynoporus cinnabariunus*. *Agric. Biol. Chem.* 45: 239-247.

Quantitative production of 2-Acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase

Rath Pichyangkura[a*], Sanya Kudan[a], Kamontip Kuttiyawong[a], Mongkol Sukwattanasinitt[b], Sei-ichi Aiba[c]

[a] *Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand,*

[b] *Center for bioactive Compounds, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.*

[c] *The Special Division for Human Life Technology, National Institute of Advanced Industrial Science and Technology, Osaka 563-8577, Japan.*

Abstract

Fine powdered α– and β-chitin can be completely hydrolyzed with chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) for the production of 2-acetamido-2-deoxy-D-glucose (GlcNAc). Crude chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 were used to digest α– and β–chitin powder. Chitinase from *Burkholderia cepacia* TU09 produced GlcNAc over 85% yield from β-chitin and α-chitin within 1 day and 7 days, respectively. *Bacillus licheniformis* SK-1 chitinase completely hydrolyzed β-chitin within 6 days, giving the final GlcNAc yield of 75% along with 20% of chitobiose. However, only a 41% yield of GlcNAc was achieved from digesting α-chitin with *Bacillus licheniformis* SK-1 chitinase.

Key words: Chitin, Chitinase, *N*-Acetyl-D-glucosamine, 2-Acetamido-2-deoxy-D-glucose, *Bacillus licheniformis*, *Burkholderia cepacia*

2-Acetamido-2-deoxy-D-glucose (*N*-Acetyl-D-glucosamine, GlcNAc) and 2-amino-2-deoxy-D-glucose (D-glucosamine, GlcN) have recently been promoted for treatment or as nutriceutical agents for patients with osteoarthritis and inflammatory bowel disease.[1,2] In contrast to GlcN hydrochloride or sulfate, both of which have a bitter taste, GlcNAc has sweet taste which can be conveniently used in daily consumption. However, GlcNAc has not been widely commercialized mainly due to the lack of an economical process for production of this compound that is acceptable for food and medicine. The current acid hydrolysis of chitin using concentrated HCl is inefficient, and poses environmental and technical concerns.[3] On the other hand, hydrolysis of chitin with enzymes can produce GlcNAc under mild and environmentally friendly conditions. An approach whereby commercially available crude enzymes were used to hydrolyze amorphous chitin substrate was carried out.[4]

* Corresponding author. *Fax: +66-2-2185418; E-mail:rath.p@chula.ac.th.*

Unfortunately, this method added an additional substrate preparation step into the production of GlcNAc. The work on commercially available crude enzymes was also extended to a production of GlcNAc by direct hydrolysis of β-chitin powder.[5, 6] These reports have shown that enzymatic hydrolysis of chitin can produce GlcNAc in relatively higher yields than the acid hydrolysis. Nevertheless, the remaining major impediment of an enzymatic hydrolysis process is the extremely low hydrolytic susceptibility of the natural chitin substrate, due to its high crystallinity. We would thus like to show herein for the first time that crystalline chitin in both α- and β-forms could be cleanly hydrolyzed, producing GlcNAc in virtually quantitative yield.

Powdered α-chitin (14 μm in size) from crab shells and β-chitin (3 μm in size) from squid pens were used as substrates for digestion by crude bacterial chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1. A typical reaction contains100 mU/mL (1 unit = the amount of enzyme that produces 1 μmole of GlcNAc per minute from colloidal chitin) of the enzyme and 10-40 mg/mL of the substrate, unless indicated otherwise. Digestion reactions were carried out in 3-5 mL of 0.1 M citrate-phosphate buffer, pH 6.0, in 10 mL glass vials. The reactions were incubated in a shaking water bath, with moderate shaking, at 37°C and 50°C when the enzyme from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 was used, respectively. At each time point, a portion of the reaction mixture was withdrew, diluted with H_2O then mixed with CH_3CN (at the ratio 31/69), filtered, and analyzed by HPLC (column: Shodex Asahipak NH2P-50; flow rate: 1 mL/min; mobile phase: 31/69 H_2O-CH_3CN; detection: UV at 210 nm). The amount of GlcNAc in the reaction mixture was determined from a calibration curve of GlcNAc standard.

The percent yield of GlcNAc production increased with the reduction of substrate/enzyme ratio. Although, at the substrate/enzyme ratio of 100 mg/U, chitinase from *Bacillus licheniformis* SK-1 completely hydrolyzed β-chitin, it gave a mixture of GlcNAc and *N,N'*-diacetylchitobiose [$(GlcNAc)_2$] (**Table 1**). The gradual increase of the GlcNAc/$(GlcNAc)_2$ product ratio with incubation time implied the presence of low β-*N*-acetylhexosaminidase (EC 3.2.1.52) activity in the crude enzyme from *Bacillus licheniformis* SK-1 under the reaction conditions. On the other hand, hydrolysis of β-chitin with chitinase from *Burkholderia cepacia* TU09 gave mostly GlcNAc with a trace amount of chitotriose. At the substrate/enzyme ratio of 100 mg/U, a 90% yield of GlcNAc was obtained within one day, and a quantitative yield was realized upon prolonged incubation (**Table 2**).

The tightly packed chitin strands of α-chitin are known to have low susceptibility to enzymatic hydrolysis. We found that when chitinase from *Bacillus licheniformis* SK-1 was used, it was unable to completely hydrolyze α-chitin. Only 41% of α-chitin was hydrolyzed in 6 days, even when the concentration of enzyme used in the reaction was 10-fold of the amount that was used to completely hydrolyze β-chitin (**Table 3**). We speculate that the crystalline domains in α-chitin were completely resistant to digestion by chitinase from *Bacillus licheniformis* SK-1. The GlcNAc produced was probably liberated from amorphous regions of the substrate.
Chitinase from *Burkholderia cepacia* TU09 showed superior characteristic in hydrolyzing α-chitin as 85% yield of GlcNAc was achieved after 7 days of incubation (**Table 4**). It is worth noting that the hydrolysis of α-chitin with chitinase *from Burkholderia cepacia* TU09 consists of two steps. First, a rapid hydrolysis step in the first 24 hours, where we believe that the amorphous portion (~40%) of the chitin

particle is hydrolyzed. The second step is a slower step, where the remaining tightly packed chitin is slowly hydrolyzed. Because of this slower degradation rate, 300 mU/mL of enzyme was used to ensure sufficient amount of active enzyme present throughout the hydrolysis.The isolation and characterization of the enzymes used here will be published elsewhere.

Table 1. Production of GlcNAc from β-chitin by chitinase from *Bacillus licheniformis* SK-1.

β-chitin/enzyme[a] (mg/U)	Digestion time (day)	% Yield[b]		
		GlcNAc	$(GlcNAc)_2$	Total
400	1	9	22	31
	3	18	27	45
	6	25	22	47
200	1	16	18	34
	3	34	38	72
	6	46	29	75
100	1	28	50	78
	3	53	40	93
	6	75	20	95

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.
[b] HPLC yield.

Table 2. Production of GlcNAc from β-chitin by chitinase from *Burkholderia cepacia* TU09.

β-chitin/enzyme[a] (mg/U)	Digestion time (day)	% Yield[b] GlcNAc
400	1	31
	3	57
	6	65
200	1	62
	3	81
	6	84
100	1	90
	3	96
	6	100

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.
[b] HPLC yield.

Table 3. Production of GlcNAc from α-chitin by chitinase from *Bacillus licheniformis* SK-1.

α - Chitin/Enzyme (mg/U)	Digestion time (day)	% Yield GlcNAc[a]
	1	32
10	3	40
	6	41

[a] [E] = 1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.
[b] HPLC yield.

Table 4. Production of GlcNAc from α-chitin by chitinase from *Burkholderia cepacia* TU09.

α - Chitin/Enzyme[a] (mg/U)	Digestion time (day)	% Yield[b] GlcNAc
100	1	37
	3	54
	7	57
33	1	41
	3	57
	7	85

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.
[b] HPLC yield.

We have demonstrated here for the first time that chitinase from certain bacteria can completely hydrolyze both powdered α– and β-chitin to give GlcNAc in very high to quantitative yield. The cleanliness of the reaction, mild conditions, ease of substrate preparation, and high production yield undeniably render the approach of using enzyme more attractive than the current acid hydrolysis process for the production of GlcNAc. Despite all these beneficial factors in using bacterial chitinase, care must be taken in further development to ensure food safety and enhance cost efficiency for industrial production of GlcNAc.

Acknowledgements

This work was sponsored by Thailand-Japan Technology Transfer Project (TJTTP) under the Oversea Economy Cooperation Fund (OECF), Agency of Industrial Science and Technology (AIST) under the Institute for Transfer of Industrial Technology (ITIT) Fellowship Program, and Japan International Science and Technology Exchange Center (JISTEC) under the Science and Technology Agency (STA) Fellowship. We would also like to thank Sunfive Company for providing α- and β-chitin powder.

References

1. Talent, J. M.; Gracy, R. W. *Clin. Ther.*, 1996, *18*, 1184-1190.

2. Salvatore, S.; Heuschkel, R.; Tomlin, S.; Davies, S. E.; Walker-Smith, J. A.; French, I; Murch, S. H. *Aliment. Pharmacol. Ther.*, 2000, *14*, 1567-1579.
3. Sakai, K. *Chitin Chitosan Handbook*, Japan Society of Chitin and Chitosan, Gihodo, Tokyo, 1995, pp 209-210.
4. Zhu, H.; Sukwattanasinitt, M.; Pichyangkura, R.; Miyaoka, S.; Yunoue, M.; Mruaki, E.; Aiba, S. *Chitin and Chitosan -Chitin and Chitosan in Life-* Ed. Uragami, T.; Kurita, K.; Fukamiso, T., Kodansha Scientific Ltd., Tokyo, 2001, pp330-331.
5. Sashiwa, H.; Fujishima, S.; Yamano, N.; Kawasaki, N.; Nakayama, A.; Muraki, E.; Aiba, S. *Chem. Lett.*, 2001, pp 308-309.
6. Sukwattanasinitt, M.; Zhu, H.; Sashiwa, H.; Aiba, S. *Carbohydr. Res.* in press.

APPENDIX B

Table B. Biochemical characteristics of the bacteria strain SK-1.

Characteristics	Reaction	Characteristics	Reaction
Gram reaction	+ve	Fermentative production of acid from	
Fermentative production of acid from		Salicine	+
Glycerol	+	Cellobiose	+
Erythritol	-	Maltose	+
D-arabinose	-	Lactose	+
L-arabinose	+	Melibiose	-
Ribose	+	Sucrose	+
D-xylose	+	Trehalose	+
L-xylose	-	Inuline	+
Adonitol	-	Melezitose	-
□□β-methyl –D-xylose	-	D-raffinose	-
Galactose	+	Starch	+
D-glucose	+	Glycogen	+
D-fructose	+	Xylitol	+
D-mannose	+	□β-gentiobiose	+
L-sorbose	-	D-turanose	+
Rhamnose	-	D-lyxose	-
Dulcitol	-	D-tagatose	+
Inoitol	-	D-fucose	-
Manntal	+	L-fucose	-
Sorbitol	+	D-arabitol	-
α-methyl-D-mannoside	-	L-arabitol	-
α-methyl-D-glucoside	+	Gluconate	+
N-acetyl-glucosamine	+	2-keto-gluconate	-
Amygdaline	+	5-keto-gluconate	-
Arbutine	+	Esculine	+

Remark: +ve = Gram positive bacteria; + = Positive reaction; - = Negative reaction

APPENDIX C

Preparation for non-denaturing polyacrylamide gel electrophoresis

Stock reagents

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

acrylamide	29.2 g
N, N'-dimethylene-bis-acrylamide	0.8 g

Adjust volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydoxymethyl)-aminomethane	18.17 g

Adjust pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

2 M Tris-HCl pH 8.8

Tris (hydoxymethyl)-aminomethane	24.0 g

Adjust pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCl pH 6.8

Tris (hydoxymethyl)-aminomethane	6.06 g

Adjust pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl pH 8.8

Tris (hydoxymethyl)-aminomethane	12.0 g

Adjust pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

Solution B (1.5 M Tris-HCl pH 8.8)

2 M Tris-HCl pH 8.8	75 ml
Distilled water	25ml

Solution B-SDS (1.5 M Tris-HCl pH 8.8)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8)

1 M Tris-HCl pH 8.8	50 ml
Distilled water	50 ml

Solution B-SDS (1.5 M Tris-HCl pH 6.8)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

5x Sample buffer

1 M Tris-HCl pH 6.8	3.1 ml
Glycerol	5 ml
1% Bromophenol blue	0.5 ml
Distilled water	1.4 ml

Non-denaturing electrophoresis buffer, 1 liter (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g

Dissolved in distilled water to 1 liter without pH adjustment (final pH should be 8.3).

SDS electrophoresis buffer, 1 liter (25 mM Tris, 192 glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g
SDS	1 g

Dissolved in distilled water to 1 liter without pH adjustment (final pH should be 8.3).

Preparation of non-denaturing PAGE

10% Separating gel

30% Acrylamide solution	3.3 ml
Solution B	2.5 ml
Distilled water 4.2 ml	
10% $(NH_4)_2S_2O_8$	50 ml
TEMED	10 ml

3% Separating gel

30% Acrylamide solution	0.4 ml
Solution C	1.0 ml

Distilled water	2.6 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µ

Preparation of SDS-PAGE

10% Separating gel

Prepare as described for non-denaturing gel but using with solution B containing SDS instead solution B.

3% Separating gel

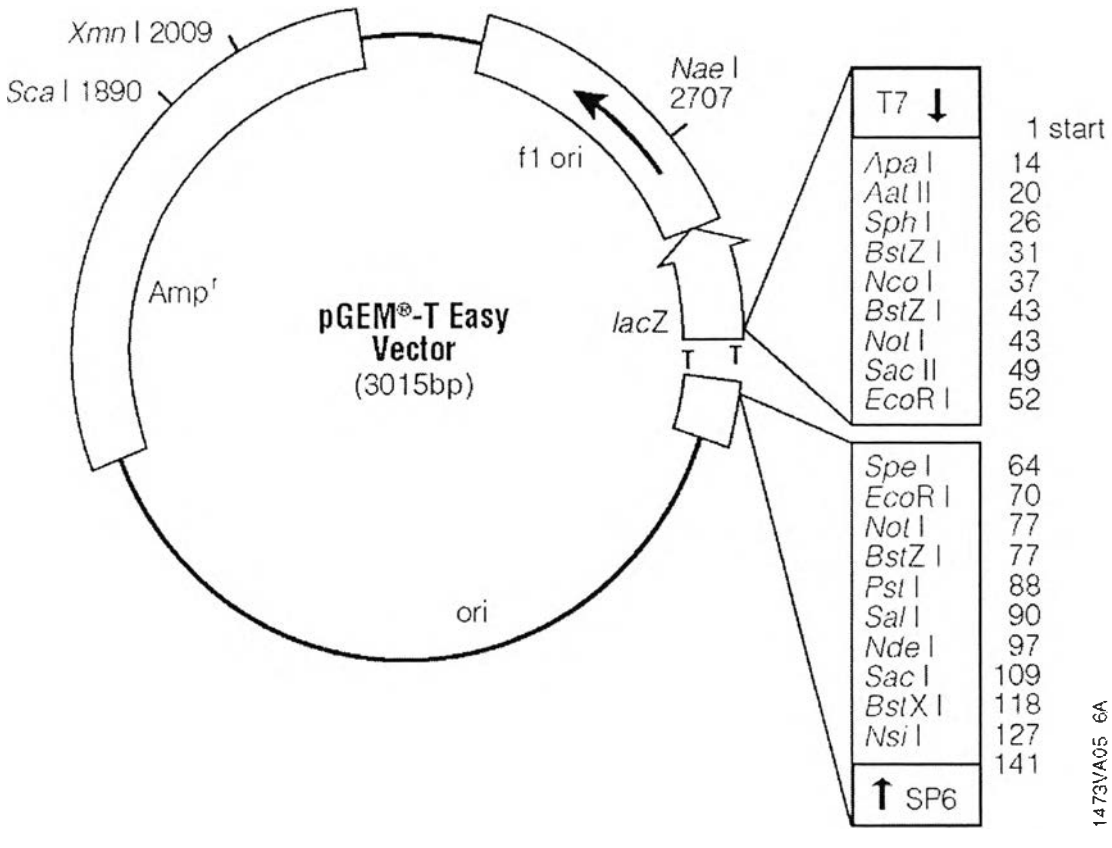
Prepare as described for non-denaturing gel but using with solution C containing SDS instead solution C.

Preparation of gel system IEF

30% Acrylamide solution	3.3 ml
Distilled water	4.2 ml
Ampholyte solution pH 3-10	288 µl
Mixed well and degassed before added:	
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	20 µl

APPENDIX D

Restriction map of pGEM-T easy



APPENDIX E

Standard curve of N-acetyl-D-glucosamine for chitinolytic enzyme assay by colorimetric method.

Standard curve for N-acetyl-D-glucosamine (GlcNAc) was made by determining the absorbance value at 420 nm of standard N-acetyl-D-glucosamine according to the method of Schale.

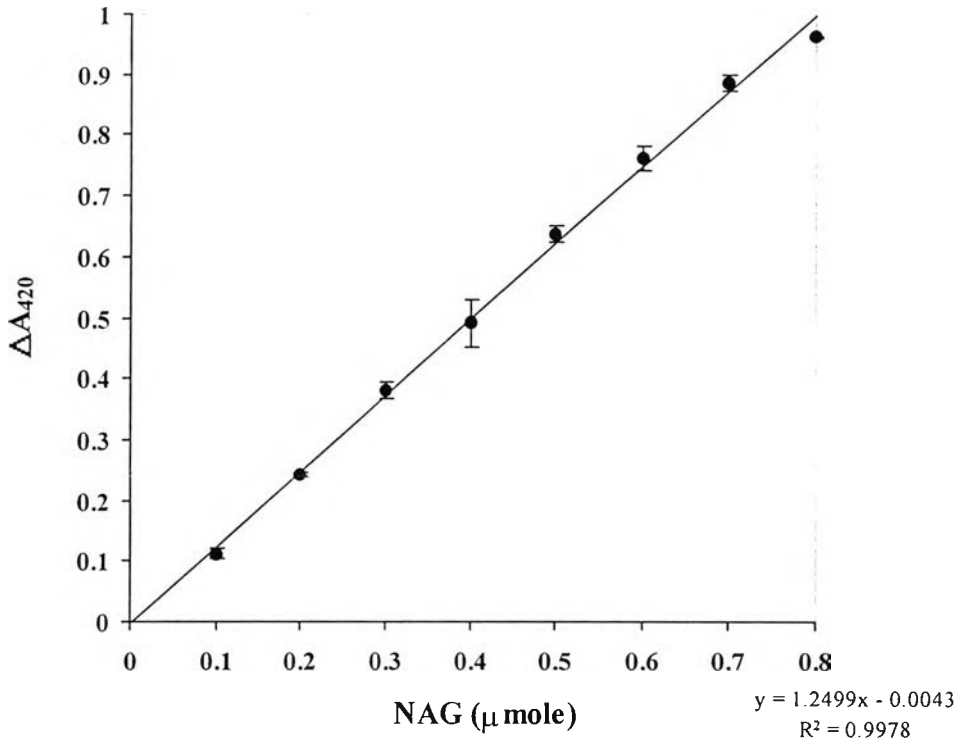


Figure E1. Correlation between final concentration of standard N-acetyl-D-glucosamine and optical density (absorbance) at 420 nm.

Standard curve of p-nitrophenol for chitinolytic enzyme assay by colorimetric method.

Standard curve for p-nitrophenol was made by determining the absorbance value at 420 nm of standard p-nitrophenol according to the colorimetric method.

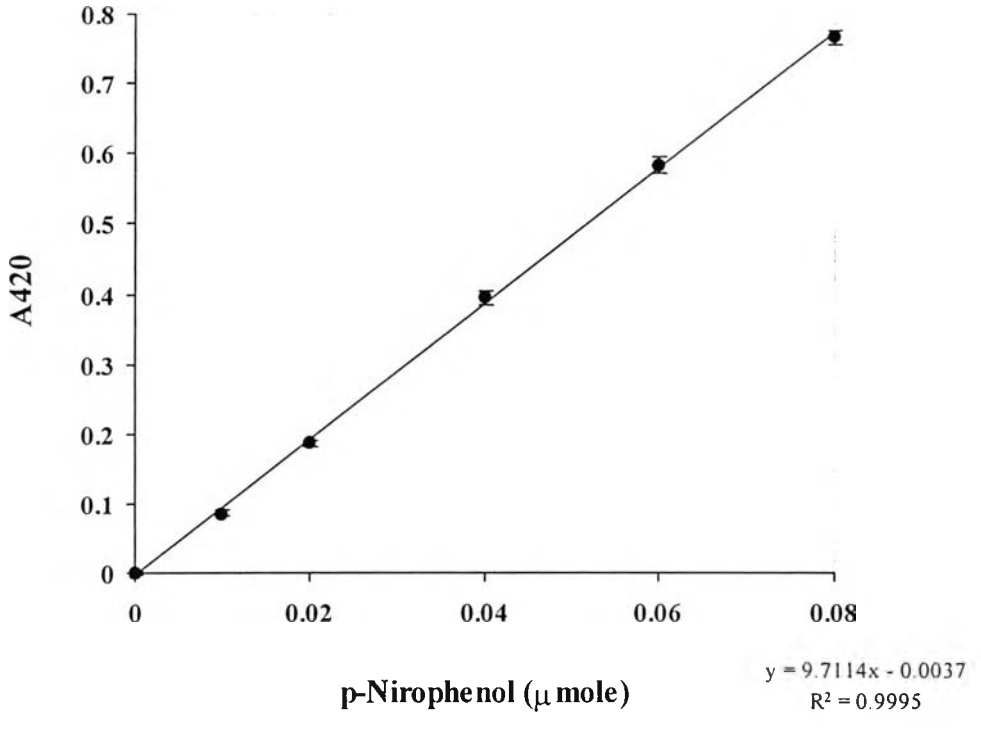


Figure E2. Correlation between final concentration of standard p-nitrophenol and optical density (absorbance) at 420 nm.

APPENDIX F

Standard curve of protein concentration by Bradford's colorimetric method.

Standard curve for bovine serum albumin (BSA) was made by determining the absorbance value at 595 nm of BSA according to the method of Bradford.

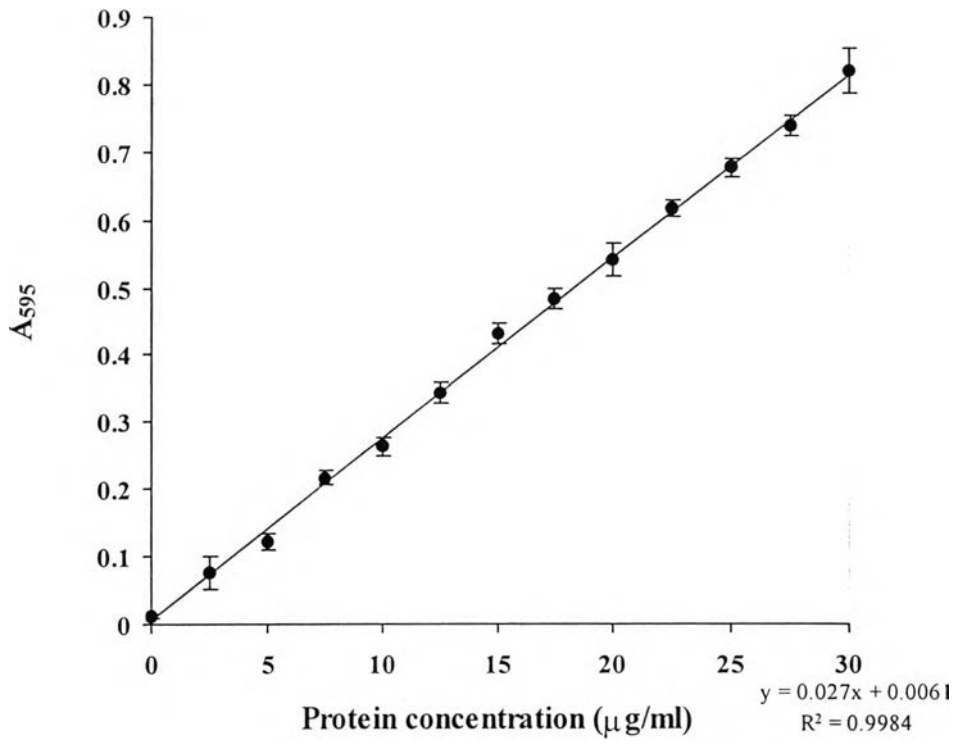


Figure F Relationship between standard protein (BSA) concentration and optical density (absorbance) at 595 nm.

APPENDIX G

Calibration curve of standard pI markers

The migration distance from cathode of pI standard markers w measured and plotted against pI's.

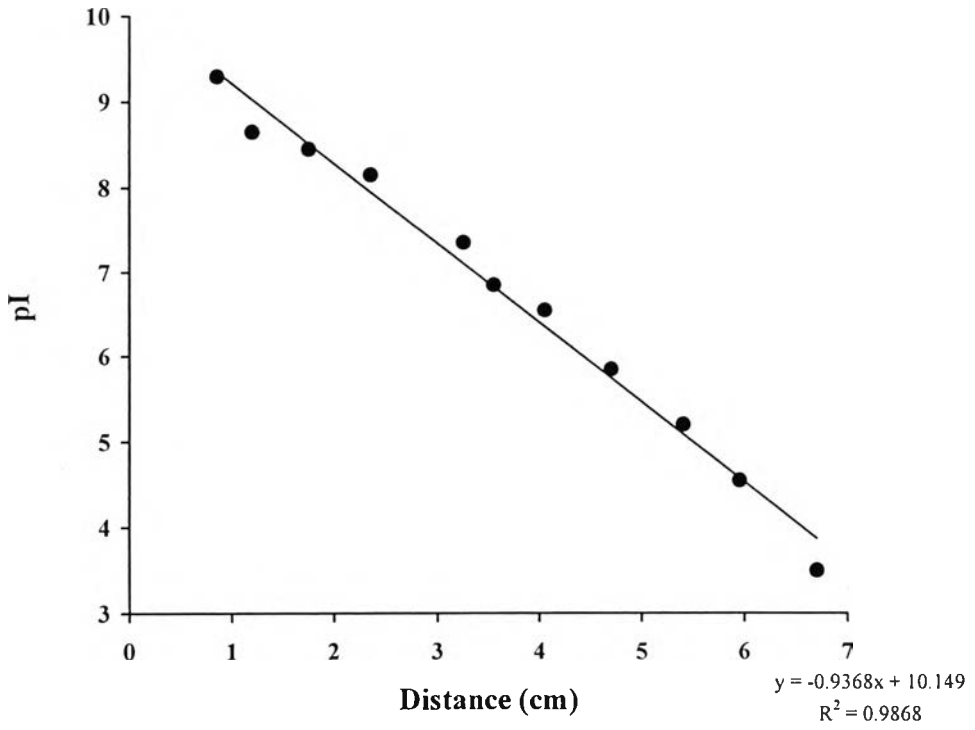


Figure G. Calibration curve for standard pI markers.

APPENDIX H

LIST OF PUBLICATIONS

Abstract

Kudan S., Achakulwisut, E., Weeratien, K., Tertdee, A., Chisawatana, O. and Pichyangkura, R. 2000. Properties and Characterization of Chitinase from *Bacillus licheniformis* Strains SK-1 and RN001. Poster presented at the 2nd Joint Seminar on Development of Thermotolerant Microbial Resources and their Applications, 21-25 November, Yamagushi University, Yamaguchi, Japan. Abstracts book. p.133(P3-15).

Kudan, S. and Pichyangkura, R. 2001. Identification and characterization of thermophilic bacterium which produce chitinase isolate SK-1. Poster presented at the 27th Congress on Science and Technology of Thailand, 16-18 October, Lee Gardens Plaza Hotel, Hat Yai, Songkla, Thailand. Abstracts book. p.547 (17-16P-47).

Proceeding

Kudan, S., and Pichyangkura, R. Purification of thermostable chitinase produced by *Bacillus licheniformis* SK-1. Proceeding of the Fifth Asia Paciffic Chitin-Chitosan Symposium and Exhibition, Bangkok, Thailand, March 13-15, 2002. in press.

Paper

Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwathanasininitt, M., and Aiba, S. 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337: 557-559.

Nucleotide sequence

GenBank accession no., AF411341

BIOGRAPHY

Mr. Sanya Kudan was born on June 4th 1974 in Sukhothai. After He finished Mattayom VI in 1991 from Mattayom Wat Nongkhaem School, he was enrolled in the Biotechnology, Ramkhamhaeng University and graduated with a B.S. in 1997. After which, he worked in Associate Professor Dr.Jerapan Krungkai's laboratory for 2 years. He entered the graduate program for M.Sc. in Biochemistry at Chulalongkorn University in 1999.