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Purification and Characterization of *Streptomyces* sp. IK Chitinase

Sebastian Margino[1*], Agustinus Joko Nugroho[2] and Widya Asmara[3]

[1]Department of Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Master Program for Biotechnology, Graduate School, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3] Research Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Streptomyces sp. IK isolated from compost inoculants, could produce extra cellular chitinase in a medium containing 0.2% (w/v) colloidal chitin, fermented for 96 hours at 30°C. The enzyme was purified by a combination of ammonium sulphate precipitation and DEAE-Cellulose anion-exchange chromatography. On SDS-polyacrylamide gel electrophoresis analysis, the purified enzyme showed a mass of 71 kDa. Chitinase was optimally active at pH of 6.7 and at 37°C. Km value and Vmax of the protein for colloidal chitin were 2.92 mg/ml and 4.26 ìg/h, respectively.

Key words : chitinase, *Streptomyces*, purification, characterization

Introduction

Chitin, an insoluble linear α-1,4-linked polymer of *N*-acetyl-*D*-glucosamine (GlcNAc), is the second most abundant polymer in nature. This polysaccharide is found in the cell walls of fungi and exoskeletons of insects and crustaceans (Tsujibo *et al*., 2003). Chitin and its derivatives are interesting because of their vary biological functions, e.g., as immunoadjuvants, as flocculants of wastewater sludge, and as agrochemicals. The addition of chitin to soil reduces populations of fungal plant pathogens and plant-parasitic nematodes. Such biological activities of chitin oligomers are dependent on chain and solubility.

A wider range of organisms has the ability of producing chitinases (EC.3.2.14), including those non-chitin-bearers such as bacteria, plants, and vertebrates (Cohen-Kupiec and Chet, 1998). In bacteria, chitinases are used mainly for their nutrition and parasitism purpose (Wang and Chang, 1997). To completely degrade chitin into free *N*-acetylglucosamine (GlcNAc), synergistic and consecutive action of different types of chitinases and other enzymes are needed. According to the characteristics of hydrolyzing chitin, the chitinases are classified into two types, exochitinase and endochitinase. Endochitinases cleaved randomly inside the chains of chitin and cut them into shorter fragments. Exochitinases (exo-*N*,*N*-α-diacetylchitobiohydrolase) or chitobiosidase, hydrolyze chitin from the terminal end and release chitobiose. Another enzyme named *N*-acetylglucosaminidases (EC 3.2.1.30) hydrolyzes short oligomers, typically chitobiose dimer units, and releases *N*-acetylglucosamine (Nielsen and Sarensen, 1999).

Recently chitin and chitinases are receiving more and more attention from biologists. *N*, *N*-α-diacetylchitobiose has been widely used as starting material for synthesis of biological active compounds

***Corresponding author** :
Sebastian Margino,
Department of Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora Bulaksumur, Yogyakarta, Indonesia 55281

(Terayama *et al.*, 1993; Kobayashi *et al.*, 1997). Chitinases promise to be safer pesticides (than chemical ones) and microbial control agents due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development (Herrera-Estrella and Chet, 1999). Chitinase activity in human serum has recently been detected, and it may play an important role in defending the invasion of fungal pathogens.

Bacteria, fungi, plants and insects are four major objects of chitinase research. In bacteria, *Bacillus* and *Streptomyces* are intensively studied for their high productivity of chitinases (Christodoulou *et al*., 2001). *Streptomyces* sp. IK is a newly isolated strain from inoculant compost (Srumbung, Magelang, Central Java, Indonesia) that have a high chitinase activity in the culture medium. The aim of this research was to purify the crude of chitinase from *Streptomyces* sp. IK, and to characterize the enzyme.

Materials and Methods

Bacterial strain and culture condition

Streptomyces sp. IK was isolated from compost inoculant (Srumbung, Magelang, Central Java, Indonesia). Cultures were maintained on Nutrient agar slants and incubated at 30^0C for 7 days. The conidia were then inoculated into a 250 ml Erlenmeyer flask containing 50 ml liquid medium, cultured at 30^0C for 48 – 72 h on a shaker until most conidia broke off.

The liquid medium for bacterial growth contained 0.7 g/l K_2HPO_4; 0.3 g/l KH_2PO_4; 0.5 g/l $MgSO_4.5H_2O$; 0.01 g/l $FeSO_4.7H_2O$; 0.001 g/l $ZnSO_4$; and 0.001 g/l $MnCl_2$. The optimal condition of *Streptomyces* sp. IK, consist of pH medium was 7, saturation of chitin 0.2% (w/v), with inoculums saturation consist of 10% (v/v), and incubation time for 4 days.

Preparation of colloidal chitin

Colloidal chitin was prepared from purified chitin according to the method of Vessey and Pegg (1991).

Purification of chitinase

After cultivation, the cells were removed by centrifugation at 4,000 rpm and 4^0C for 20 min. Proteins in the cell-free culture broth (1,000 ml) were fractionated with 40-90% (w/v) saturation $(NH_4)_2SO_4$ and then were collected by centrifugation at 4,000 rpm for 40 min. The protein precipitate was dissolved in 50 mM Potassium Buffer Phosphate Solution (PBS), pH 6.7, and the insoluble materials were removed by centrifugation at 15,000 rpm for 5 min. The derived supernatant (with the highest enzyme activity) was applied onto a DEAE Cellulose column (1 by 20 cm, Pharmacia Biotech) pre-equilibrated with 50 mom PBS pH 6.7. The column was washed with 1-2 bed volumes of 0.2 M Noah, 2-3 bed volumes of 50 mom PBS pH 6.7, and then eluted with distilled water. The flow rate was maintained at 0.3 ml/min. The fractions with chitinase activity were pooled, concentrated and kept at – 20^0C, for the next analysis.

Protein estimation

Protein concentrations were measured according to Bradford (1976) method using bovine serum albumin as a standard.

Enzymatic activity assay

Chitinase activity was claimed as turbidity relatively reduction percentage to the colloidal chitin without enzyme (control) (Harman *et al.*, 1993). The five hundred μl of enzyme solution was added to 500 μl of 1% (w/v) colloidal chitin, 50 mM PBS (pH 6.7) and incubated at 28^oC for 24 h. After incubation, in to reaction tube was added 3 ml dH_2O . The reduction of turbidity was measured by vis spectrophotometer at 510 nm, and distilled dH_2O was used as a control. One unit of chitinase activity was defined as the amount of enzyme needed to reduce 5% turbidity of colloidal chitin

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass of purified chitinase was estimated by sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel according to Laemmli (1970) method. The gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250.

Effect of optimal pH and temperature on the activity

The optimal pH on the purified chitinase was determined at different pH values (3-11) under standard assay conditions. The buffer systems used were as follows: 0.1 M citrate-phosphate buffer, pH 3.0-6.5; 0.1 M phosphate buffer, pH 6.5-8.5; 0.1 M glycine-NaOH buffer, pH 8.5-11.0. The optimal temperature for the chitinase activity was determined in the range of 20°C to 65°C under standard assay conditions.

Results and Discussion

Enzyme fractionation using ammonium sulphate

Streptomyces sp. IK was originally isolated from compost inoculants (Srumbung, Magelang, Central Java, Indonesia) and had high chitinase activity in the culture broth medium. Proteins present in culture filtrate were extracted by ammonium sulphate 40%, 50%, 60%, 70%, 80% and 90% (w/v). The result indicated that at saturation 50% was able to produce a maximum chitinase activity that was 111.99 U/mg, and followed 111.63 U/mg for saturation 60% (w/v) (Figure 1).

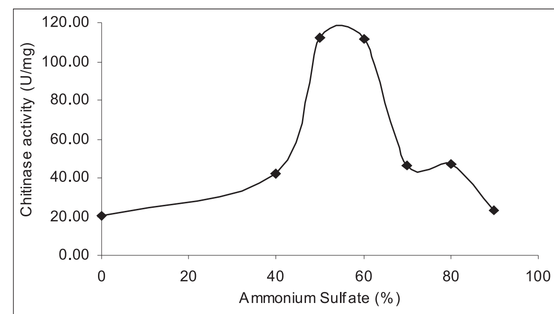

Figure 1. Difference effect of ammonium sulphate saturation on purifying chitinase protein of *Streptomyces* sp. IK

Furthermore, ammonium sulphate in this saturation level 50% (w/v) was used for precipitating protein in crude enzyme. The added salt (ammonium sulphate), will pressed out water molecule from protein and causing the hydrophobic condition of protein compounds (Harris and Angal, 1990).

Enzyme dialisate fractionation using ion exchange chromatography

The result of ammonium sulphate precipitation was dialyzed by 0.05 M PBS pH 6.7, then dialyzed protein precipitate was

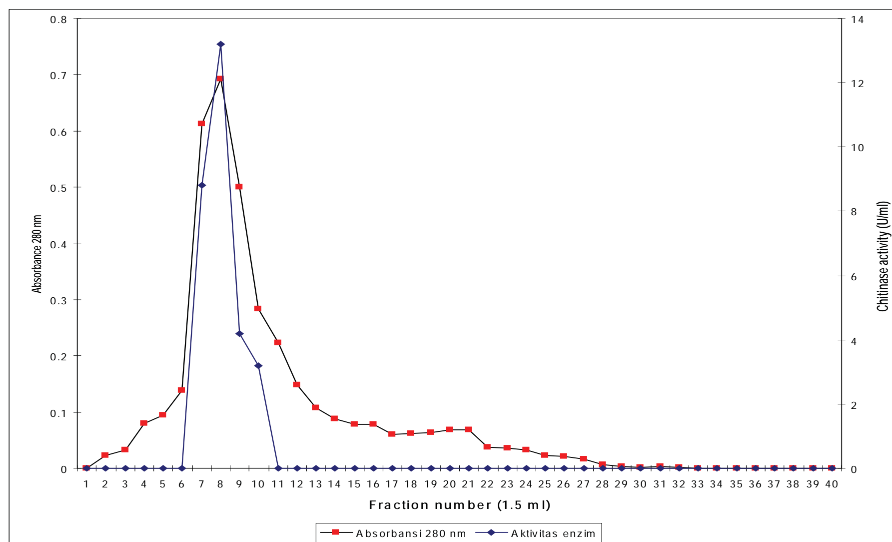

Figure 2. Elution profile of chitinase on DEAE Cellulose anion-exchange chromatography

Table 1. Purification of *Streptomyces* sp. IK chitinase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Recovery (%)
Crude enzyme	166.50	8571.50	51.48	1.00	100.00
50% ammonium sulphate precipitation	15.56	1740.00	111.79	2.17	20.30
DEAE Cellulose	2.90	1470.00	506.55	9.84	17.15

subjected to gel filtration for purification. Partial purification by single-step procedure was done using *DEAE Cellulose* filtration, result presented at Figure 2.

This experiment has measured 100 fractions to find out protein contents of each fraction (based on absorption value at 280 nm). In the gel filtration, one protein peak was observed, which formed by fractions number 6 to 12 (Figure 2) and high chitinase activity were detected on the fractions number 7 to 10. Furthermore, the samples were collected into one tube, then be freeze dried for analyzing the protein pattern using electrophoresis.

The enzyme purification level using *DEAE Cellulose* may increase enzyme purity as much as 9.84 fold (Table 1).

Table 1 showed that precipitation using ammonium sulphate and dialysis resulted in the enzyme purity level as much as 2.17-fold compared with crude enzyme, with over all yields of 20.30 %. In addition, this step was able to reduce the number of protein from 166.50 mg into 15.56 mg (more than 10 fold). DEAE- Cellulose was able to procure the pure chitinase up to 9.8-fold with recovery level as much as 17.15% (Table 1). Purification of crude chitinase from *Streptomyces* sp. M-20 was increased to 6-fold using DEAE-Cellulose and Sephadex G100 filtration (Kim *et al*., 2003).

Characterization of the chitinase
Molecular weight of chitinase

The character of chitinase protein produced by *Streptomyces* sp. IK could be performed on 10% SDS-PAGE (Figure 3). The molecular weight of chitinase single protein

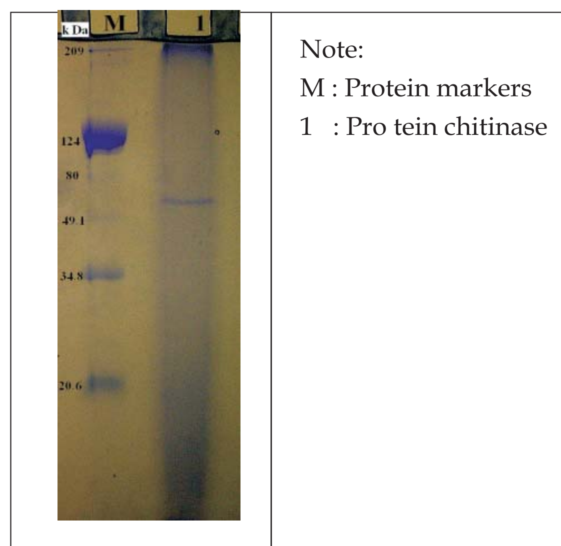

Figure 3. SDS-PAGE analysis of *Streptomyces* sp. IK chitinase

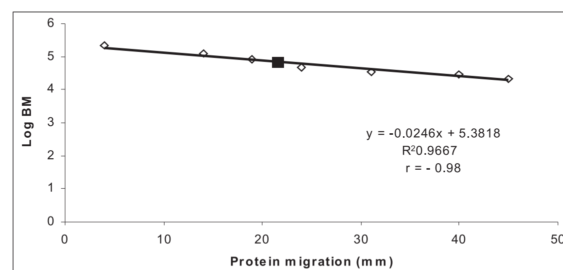

Figure 4. Determination of molecular weight of chitinase at optimum pH

that result from *DEAE Cellulose* isolation was determined using relative mobility calibration curve of standard polypeptide (Figure 4). The molecular weight estimation was determined using regression equation Y = 5.3818 – 0.0246 X (with r = 0.98).

The chitinase molecular weight of *Streptomyces* sp. IK was about 71 kDa. It was supposed that this band is chitinase,

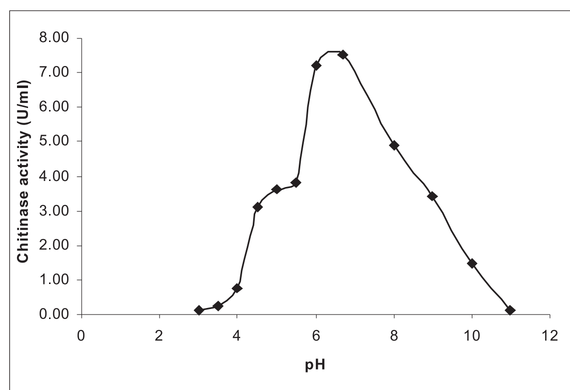

Figure 5. The effect of pH on enzymatic activity of the purified chitinase

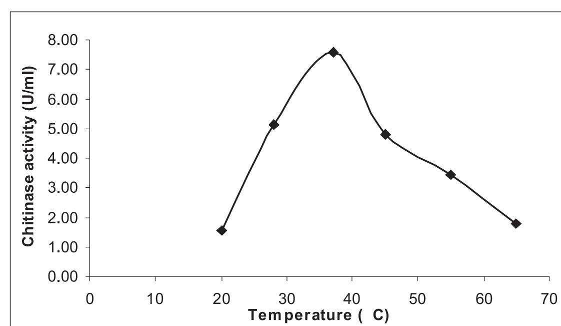

Figure 6. The effect of temperature on enzymatic activity of the purified chitinase

this approved by the increasing of chitinase activity and its enzyme purity. Sakai *et al.,* (1998) reported that chitinases had the molecular weight L, M and S for *Bacillus* sp. MH-1 were 71, 62 and 53 kDa, respectively. Another research conducted by Mukherjee and Sen (2006), showed that chitinase of *Streptomyces venezuelae* P10 on SDS-PAGE producing one band with molecular weight 66 kDa. *Bacillus brevis* showed its chitinase molecular weight was 85 kDa (Sheng *et al.*, 2002).

The result showed that the most optimum pH value was 6.7 with chitinase activity up to 7.52 U/ml. The difference effect of pH buffer to enzyme activity was presented at Figure 5.

Figure 5 showed that optimum pH for chitinase activity was 6 – 7. Wilson and Walker (2005) stated that enzyme activity was determined by the presence of proton donor and acceptor at the expected ionization level so that procured an optimum pH. Dixon and Webb (1979), stated that the best enzyme activity at pH condition was about 5.5 -7.5, furthermore, when in more acid condition (pH 4 – 5) or alkali (pH 8 -10), then the enzyme was not active or the activity decreased. The pH optimum of enzyme was not must equal to the pH of environmental culture medium, but could be lower or higher than environment pH. Scopes (1994) stated that the presences of a little pH change may cause the changing of load, enzyme and group conformation at the active side so that disturbed the reaction of enzyme - substrate. The pH for enzyme activity has usually narrow turn. Tsujibo *et al.,* (1992) stated that chitinase of actinomycetes and fungus activity was in the acid condition. Jeaniaux (1966) stated that chitinase of *Streptomyces* had an optimum pH at value 6.3 and will be obstructed its activity at pH 4.5.

The optimum temperature of chitinase

A maximum chitinase activity was achieved at temperature 37^{0}C with activity up to 7.56 U/ml (Figure 6). The chitinase activity was decreased at temperature above 40^{0}C. Commonly, the chitinase activity has range between 25^0 – 45^{0}C with optimum temperature 37^{0}C. The optimum temperature of chitinase activity S. *antibioticus* (Jeaniaux, 1966) and *Streptomyces* CU 36 (Kamel *et al.*, 1993) was at temperature 40^{0}C and 45^{0}C, respectively.

At temperature 20^{0}C to 40^{0}C, an increasing enzyme activity was occurred, this event indicated the speed of reaction increased with the increasing of temperature. The high temperature at the certain limitations will increase a chemistry reaction, similarly with reaction which catalyzed by the enzyme, while the temperature above 40^{0}C the chitinase activity decreased. This event occurred because the temperature treated was higher than its optimum temperature, so that enzyme has

Table 2. Computation data of determining Km value and Vmax chitinase

No.	[S]	1/[S]	Vi	1/Vi	Regression Equation
1	0.25	4.00	0.000401	2496.000	Y = 685.66 X – 234.97
2	0.50	2.00	0.000850	1176.000	
3	0.75	1.33	0.001509	662.857	Km = 2.92 mg/ml Vmax = 4.26 µg/h
4	1.00	1.00	0.002289	436.800	

loosed its catalytic activity. One cause the loss of catalytic activity was the occurrence of denaturizing process by high temperature.

Denaturation will caused the occurrence of change or the damaged of three dimension structure from the enzyme protein, so the bounds within it (vanderwal, hydrophobic, hydrogen and electrostatic bounds) have changed. Furthermore, the changing of enzyme protein structure will cause a part or the whole of protein function becomes not active (Harris and Angal, 1990)

Determination of Vmax and Km value

Determination of Vmax and Km value were based on the pH condition and optimum temperature that have been procured. In this event, the increase of substrate saturation will increasing enzyme activity to achieve certain limitations at the certain substrate saturation as well, so that with the increasing of substrate after optimum limitations will not increase the enzyme activity.

Michaelis Menten's (Km) constant value analysis and maximum speed (Vmax) can be seen at Table 2. According to above computation, there was quantitative relation between the speed (Vi) with substrate saturation [S]. Michaelis Menten's (Km) constant was procured about 2.92 mg/ml and maximum speed (Vmax) as much as 4.26 mg/h with regression equation Y = 685.66 X – 234.97 and have correlation value about r = 0.99.

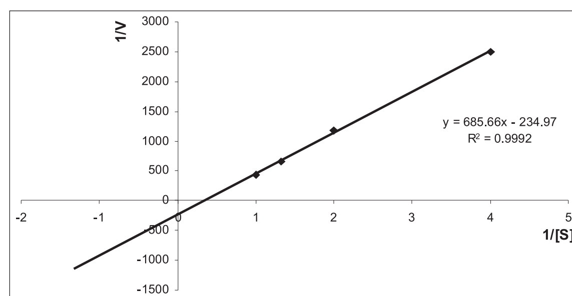

Figure 7. Lineweaver – Burk plot

Joo (2005) reported that Km and Vmax values (to the colloidal chitin) of *Streptomyces halstedii* AJ-7 in sequence was 3.2 mg/ml and 118 mmol/h. Km *Streptomyces* sp. IK chitinase value was smaller (Km = 2.92 mg/ml) compared to the Km S. *halstedii* AJ-7 value (Km = 3.2 mg/ml). Wilson and Walker (2005) stated that the lower Km value of an enzyme showed the higher enzyme activity. The result of qualitative relationship between the speeds of reaction (V) with substrate saturation [S], was drawn in linear form through Lineweaver – Burk plot (Figure 7). as the conclusion, purification of *Streptomyces* sp.IK chitinase by ion exchange chromatography (*DEAE Cellulose*) was able to increase the enzyme specific activity, from 51.48 U/mg to 506.55 U/mg. Isolated chitinase had molecular weight 71 kDa, Vmax 4.26 mg/h, Km value 2.92 mg/ml; at pH 6.7 and temperature 37°C. chitinase had optimum activity.

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References

Bradford, M.M. 1976. A Rapid and sensitive Method for Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-dye binding. *Anal. Biochem*. **72**,248 – 254.

Christodoulou, E., Duffner, F. and Vorgias, C.E. 2001. Overexpression, purification, and characterization of a thermostable chitinase (Chi 40) from *Streptomyces thermoviolaceus* OPC-520. *Protein Expr. Purif*. **23**,97-105.

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

Dixon, M. and Webb, E. 1979. *Enzymes*. Logman Group Ltd. London.

Harman, G.E., Hayes, C.K., Lorito,M., Broadway, R.M., Di Pietro,A.,Peterbauer, C., and Tronsmo, A.1993. Chitinolytic Enzymes of *Trichoderma harzianum:* Purification of Chitobiosidase and Endochitinase. *Phytopathol.* **83**, 313-318.

Harris, E.L.V. and Angal, S. 1990. *Protein Purification Method*. Oxford University Press. New York.

Herrera-Estrella, A., and Chet, I. 1999. Chitinases in biological control. *EXS*. **87**,171-184.

Jeaniaux, C. 1966. Chitinases. In: E.F. Neufeld and V. Ginburg (Eds.), *Complex Carbohydrate*s, *Methods in Enzymology*. Vol. VII, pp. 644 – 650. Academic Press. New York.

Joo, G.J. 2005. Purification and Characterization of an Extracellular Chitinase from The Antifungal Biocontrol Agent *Streptomyces halstedii. Biotechnol. Lett.* **27**(19),1483 - 1476.

Kamel, Z., Heikel, N., and Fahmy, F. 1993. Extracellular Chitinase from *Streptomyces s*p. and its antifungal activity. *Acta Pharm. Turcica* **35**, 135-143.

Kim, KJ., Yang, Y.J., and Kim, J.G. 2003. Purification and characterization of chitinase from *Streptomyces* sp. M-20. *J. Biochem. Mol. Biol*., **36**,185-189

Kobayashi S., Kiyosada,T., and Shoda, S. 1997. A novel method for synthesis of chitobiose via enzymatic glycosylation using a sugar oxazoline as glycosyl donor. *Tetrahedron Lett*. **38**, 2111-2112.

Laemmli, U.K. 1970. Cleavage of Structural Proteins During The Assembly of The Head of Bacteriophage T4. *Nature*, **227,** 680 – 685.

Mukherjee, G. and Sen, S.K. 2006. Purification, Characterization, and Antifungal Activity of Chitinase from *Streptomyces venezuelae* P10. *Curr. Microbiol*. **53**(4), 265-269.

Nielsen, M.N., and Sarensen, J. 1999. Chitinolytic activity of *Pseudomonas fluorescens* isolates from barley and sugar beet rhizosphere. *FEMS Microbiol. Ecol.* **30**, 217-227.

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**, 3397 - 3402.

Scopes, R.K. 1994. *Protein Purificatio*n, *Principles and Practic*e. Third edition. Springer-Verlag. New York.

Sheng, L., Zhi-An, Z., Zhen-Rong, G., Chen, B., and Wei-Da, H.. 2002. Purification and Characterization of a Novel Chitinase from *Bacillus brevis. Acta Biochem.Biophys.Sinica*. **34** (6), 690 – 696.

Terayama, H., Takahashi, S ., and Kuzuhara, H. 1993. Large-scale preparation of *N,N*-â-diacetylchitobiose by enzymic degradation of chitin and its chemical modifications. *J. Carbohydr. Chem.* **12**, 81-93.

Tsujibo, H., Kubota,T.,Yamamoto,M., Miyamoto, K., and Inammori, Y. 2003. Characterization of chitinase genes

from an alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131. *Appl. Environ. Microbiol*. **69**, 894-900.

Tsujibo, H., Yoshida, Y., Miyamoto, K., Hasegawa, T., and Inammori, Y.1992. Purification and Properties of Two Types of Chitinases Produced by an Alkalophilic Actinomycete.*Biosci. Biotechnol. Biochem*. **56**, 1304 – 1305.

Vessey, J.C. and Pegg, G.F. 1991. Autolysis and Chitinase Production in Cultures of Verticillium albo-atrum. *Trans. Br. Mycol. Soc*. **60**, 133 – 143.

Wang, S.L., and Chang, W.T. 1997. Purification and characterization of two bifunctional chitinases/ lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Appl. Environ. Microbiol*. **63**, 380-386.

Wilson, K. and Walker, J. 2005. *Practical Biochemistry: Principles* and Techniques. Fifth edition. Cambridge University Press. Cambridge.