Asian Journal of Chemistry; Vol. 23, No. 8 (2011), 3549-3552

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Characterization of Chitinase from *Pseudomonas* sp TNH54 Isolated from Mud Fields

NUNIEK HERDYASTUTI[1,*], SARI EDI CAHYANINGRUM[1] and TRI JOKO RAHARJO[2]

[1]Department of Chemistry, Surabaya State University, Jl. Ketintang Surabaya, Indonesia
[2]Department of Chemistry, Gadjah Mada University, Sekip Utara Yogyakarta, Indonesia

*Corresponding author: E-mail: nherdyastuti@yahoo.com

(*Received*: 19 October 2010; *Accepted*: 25 April 2011) AJC-9844

Chitinase enzyme is produced by the bacterial *Pseudomonas* sp which has been isolated from mud fields. The enzyme was precipitated with ammonium sulphate 0-50 % demonstrated by increasing up to 1.3 fold in purity compared to before fractionation. The molecular mass of the purified chitinase was 26.1 and 29 kDa, estimated by a sodium dodecyl sulfate polyacrylamide gel electrophoresis and was confirmed by activity staining with Calcofluor white M2R. Chitinase was optimally active at pH of 5.0 at 35 ºC. The enzyme showed stability of activity at pH range 3-5 and temperature of 40 ºC, an apparent K_M value of 1.51 mg/mL and V_{maks} 0.35 µmL/mL hour. Among the metals, ions, the Cu^{2+} and Fe^{2+} completely inhibited the activity enzyme but activated in presence of is Mn^{2+} in 10 mM concentration.

Key Words: Chitinase, Characterization, *Pseudommonas* sp, Mud fields.

INTRODUCTION

Chitin, a linear β-1,4-N-acetyl-glucosamine (GlcNAc) polysaccharide[1], is the most abundant renewable natural resource after cellulose and is component of crustacean exoskeleton, fungal cell walls and squid pens. Chitin is a versatile and promising biopolymer with numerous industrial, medical and commercial uses[2]. The biodegradation of chitin requires the synergestic action of several hydrolytic enzymes for efficient and complete breakdown. The catabolism of chitin typically occurs in two steps involving the initial cleavage of the chitin polymer by polysaccharides (chitinase) into chitin oligosaccharides and then further cleavages to N-acetyl-glucosamine monomers by chitobioses[3].

Chitinases (EC.3.2.1.14) a group of enzyme capable of degrading chitin directly to low molecular weight products, have been isolated from different microorganisms, such as fungi and bacteria[4,5]. During the last decades, chitinases have received remarkable attention due their wide range of application, especially in the production of chitooligosaccharides and N-acetyl D-glucosamine[6,7], biocontrol of pathogenic fungi[8,9] and bioconversion of chitin waste to single cell protein[10].

In previous paper, we have TNH54 isolated producing a large amount of extracellular chitinase and showing a high activity. In this study, an extracellular chitinase of the strain was partial purification and characterized.

EXPERIMENTAL

Organism and cultural condition: *Pseudomonas* was isolated from field mud in Ketintang area Surabaya by using a minimal medium containing colloidal chitin. The cells were cultured on LB medium and incubated at room temperature for 20 h in rotary shaker at 150 rpm. Ten per cent of bacterial broth was used as an inoculum and added on to screening medium with the following composition: 0.4 % colloidal chitin, 0.7 % K_2HPO_4, 0.3 % KH_2PO_4, 0.5 % $MgSO_4{\cdot}5H_2O$, 0.01 % $FeSO4{\cdot}7H_2O$, 0.001 % $MnCl_2$ and 0.5 % peptone, incubated at room temperature for 45 h on a rotary shaker (150 rpm). The supernatants were collected for measurements of chitinase activity.

Preparation of enzyme: After incubation, the culture cells were centrifuged (10000 × g, 4 ºC for 20 min). The supernatant was brought to 0-50 % saturation with ammonium sulphate (4 ºC, for 0.5 h) stirring by magnetic stirer. The precipitate was recovered by centrifugation (4 ºC 6000 × g for 0.5 h) and pellet formed was solubilized in 0.1M phosphate buffer pH 7.0. The solution was dialyzed overnight against the same buffer at 4 ºC.

Chitinase assay: Chitinase activity was measured by colorimetric method based on released N-acetyl-glucosamine[11]. The colloidal chitin solution 2.0 mL of 1.25 % (w/v) dissolved in 200 mM potassium phosphate buffer was added to 0.5 mL enzyme solution and incubated for 2 h at room temperature.

After 2 h, place the vials in to a boiling water for 5 min and cool to room temperature by placing the vials in a cold water bath. The suspension was centrifugated (4 ºC, 6000 × g, for 10 min). Place the vials 1.0 mL supernatant was added 2.0 mL deionized water and 1.5 mL colour reagent solution containing 5.3M sodium potassium tartrate and 96 mM 3,5-dinitrosalicyclic acid. Place the mixed solution in to a boiling water for 5 min and allow the containers to cool at room temperature. Transfer the solution to suitable cuvettes and record the absorbance at 540 nm. One unit activity will liberate 1.0 mg N-acetyl D-glucosamine from chitin per hour.

Protein determination: Protein was determined by the Bradford method at 595 nm using Coomassie Briliant Blue G-250 with bovine serum albumin as a standard.

Effect of pH and temperature on chitinase activity and stability: The pH effect was determined by incubated the chitinase solution 0.5 mL at different range pH (2-9) under standard assay condition using colloidal chitin as the substrate. Glycine-HCl buffer 0.1M was used for pH 2, citrate-phosphate 0.1M for pH 3-6, phosphate buffer 0.1M for pH 7-8 and *tris*-HCl buffer 0.1M for pH 9. The enzyme stability was determined after preincubation at various pHs without the substrate for 1 h at 4 ºC. The optimum temperature for the chitinase activity was determined by performing of the standard assay in the range of 25-55 ºC. Thermal stability was determined by assaying the residual chitinase activity for 0.5 h at the previous temperature without the substrate.

Effect of metal ion on chitinase activity: The enzyme was preincubated with a 10 mM concentration of different metals. After 0.5 h, the remaining chitinase activity was measured using the standard assay. The relative inhibition of the calculated enzyme was based on the release of N-acetylglucosamine.

Effect substrate on activity of chitinase: Chitinase enzyme was incubated separately with different substrate in the range 0.2-1.4 g/L using standard assay methods. Following incubation the release of N-acetylglucosamine was measured. The kinetic value K_M and V_{max} determined by Lineweaver-Burk equation.

Electrophoresis and zymogram: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970), using 12 % gel. After electrophoresis, gels were stained either with Coomassie brilliant blue R-250. The chitinolytic activities were detected on gels by zymogram using 0.1 % glycol chitin from acetylation of glycol chitosan[12]. After electrophoresis, the gels were incubated for 2 h at 37 ºC in 100 mM sodium acetate buffer, pH 5.0 containing 1 % (v/v) Triton X-100. The gels were then stained with 0.01 % Calcofluor white M2R in 0.5M *tris*-HCl, pH 8.9, for 7 min. The lytic zones were photographed under the UV-transilluminator.

RESULTS AND DISCUSSION

Many species of bacteria synthesize chitinase for the utilization of chitin as a source of carbon and nitrogen. Microorganism isolated from mud fields in Surabaya were screened in minimal media containing 0.4 % colloidal chitin at room temperature. Six isolate were obtained from the first screening and then they were tested in crude extract. TNH54 isolate was showed the highest activity after 2nd days of cultivation (Fig. 1) and used throughout the experiment. TNH54 isolate was gram negative, rod-cocoid cell, was grew in the temperature 42 ºC and not spore-forming. The bacteria could generate acid from mannitol, sucrose, sorbitol, innositol able to oxidize the glucose and shows the genetic similarity 98 % to *Pseudomonas* sp based on nucleotide sequences of 16S-rRNA.

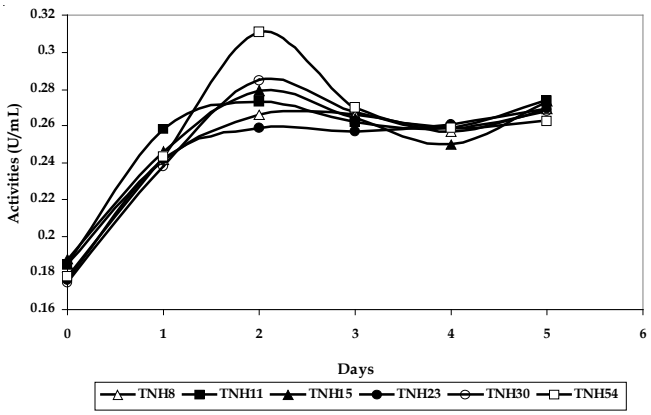

Fig. 1. Chitinase activity of six isolated for five days incubated. TNH54 isolate was showed the higest activity after 2nd days cultivated with 0.331 U/mL activity

Specificity of the purified chitinase to various chitins was investigated and shown as the percentage of the activity to that on colloidal chitin (Table-1). The enzyme showed 77.5 % of relative activity on glycol chitin and 64.1 % on chitosan compared to that on colloidal chitin. However, it showed low chitinolytic activity on chitin and glycol chitosan. An extracellular chitinase produced by *Pseudomonas* sp TNH54 settles on fractionation with 0-50 % ammonium sulphate, demonstrated by increasing up to 1.3 fold in purity compared to before fractionation (Table-2).

TABLE-1
SUBSTRATE SPECIFITY OF THE EXTRACELLULAR CHITINASE FROM *Pseudomonas* sp TNH54

Substrate	Relative activity (%)
Chitin	44.6
Colloidal chitin	100
Glycol chitin	77.5
Chitosan	64.1
Glycol chitosan	39.5

TABLE-2
PARTIAL PURIFICATION STEP OF CHITINASE PRODUCED BY *Pseudomonas* sp

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	9.184	53.46	5.74	1.00	100
$(NH_4)_2SO_4$ (50 %)	2.185	16.46	7.53	1.31	30.79

The effect of pH on chitinase activity determined by varying the pH of the reaction mixture using different buffers. The effect of pH on chitinase were investigated by incubating the enzyme solutions for 1 h at various pH. The enzyme was most active between 2.0 and 5.0 pH and optimum at pH 5.0 (Fig. 2). It was relatively stable at pHs between 2.0 and 6.0 when kept at 4 ºC for 1 h with relative activity to kept 60 %. However, beyond these pH ranges, it rapidly lost its activity. Many research about chitinase, including the present one, showed a pH optimum in the acidic range. There are *Bacillus* sp[7], *Streptomyces* sp. M-209, *Trichoderma viride*[13]. At the same pH, the optimum temperature for chitinase was found to be 35 ºC (Fig. 3) and the chitinase maintained stability in temperature range from 25-40 ºC. The chitinase retained more than 75 % activity at 40 ºC and nearly 50 % activity at 45 ºC for 0.5 h in citrate phosphate buffer (pH 5.0). The enzyme was completely inactivated at 55 ºC.

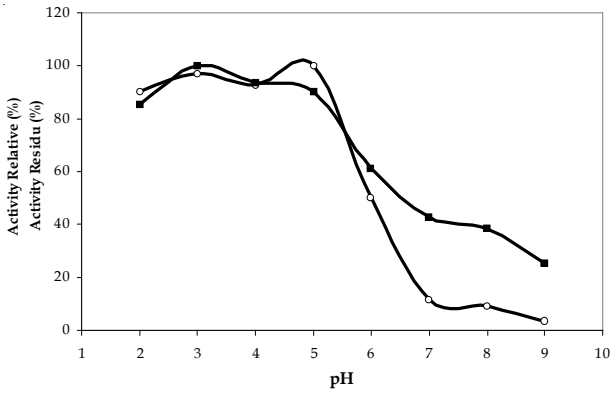

Fig. 2. Optimal pH (■) and stability pH (o) of chitinase enzyme from *Pseudomonas* sp TNH54

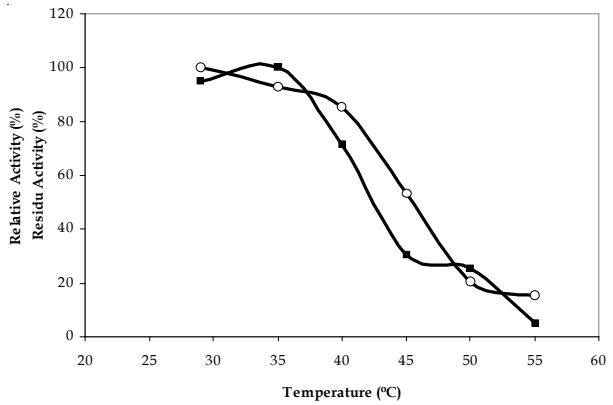

Fig. 3. Optimal temperature (■) and stability of temperature (o) of chitinase enzyme from *Pseudomonas* sp TNH54

Michaelis-Menten constant were determined using the optimal reaction condition in experiments designed to calculate reaction velocities at each substrate concentration. The K_M and V_{max} of chitinase toward colloidal chitin were determined. The apparent K_M and V_{max} of the chitinase for colloidal chitin were 1.51 mg/mL and 21.06 µmol/mL min, respectively (Fig. 4). The K_M values of chitinase from different organisms were 1.43 mg mL^{-1} from *Enterobacter* sp. NRG42; 2.88 mg mL^{-1} for *Enterobacter aerogenes*[14]; 1.4 mg mL^{-1} for chitinase from *Vibrio alginolyticus* and 12 mg mL^{-1} for *Bacillus* sp. BG-11 chitinase[15].

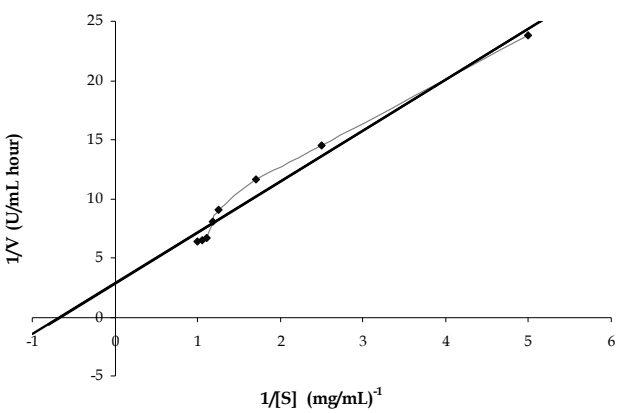

Fig. 4. K_M and V_{max} of chitinase enzyme from *Pseudomonas* sp TNH54 for colloidal chitin

The inhibitory effects of some metal ions on chitinase activity were investigated in an assay system where 10 mM of each ion was present. As shown in Table-3, only addition of Mn^{2+} slightly increases chitinase activity, while Fe^{2+} and Cu^{2+} strongly inhibited the chitinase activity. Similar inhibition by Fe^{2+} and Cu^{2+} but activated by Mn^{2+} for *Bacterium* C4 was reported by Yong[16].

TABLE-3
EFFECT OF METALS IONS ON CHITINASE ACTIVITY

Compounds	Concentration (mmol/L)	Relative activity (%)
None	–	100.0
KCl	10	63.0
$CoCl_2$	10	91.5
$CuCl_2$	10	36.4
$CaCl_2$	10	73.8
$Fe(SO_4)_2$	10	42.6
$MgSO_4$	10	95.6
$MnCl_2$	10	118.6
$ZnSO_4$	10	68.0
$BaCl_2$	10	71.7

The molecular mass of TNH54 isolate was determined by SDS-PAGE and zymogram (Fig. 5). Chitinase was able to hydrolyze glycol-chitin as a substrate in denaturing conditions, expressed the different isoforms as a broad band and two bands at 29 and 26.1 kDa, respectively. Different molecular masses that ranged from 20-69 kDa have been reported for other chitinases[5,17-19]. The molecular mass of *Pseudomonas* sp TNH54 chitinase was obviously different from other *Pseudomonas chitinases*, such as those of *Pseudomonas* sp TKU015 (68 kDa)[20], *Pseudomonas* sp. YHS-A2 (67 kDa)[21], *P. aeruginosa* 385 (58 kDa)[22], *P. aeruginosa* K-187 (30 kDa, 32 kDa)[9] and *Pseudomonas* sp TKU008 (40 kDa)[23].

Conclusion

Chitinase enzyme fom *Pseudomonas* sp TNH54 was precipitated up to ammonium sulphate resulted in 1.31 fold increase specific activity. The molecular mass of the chitinase was estimated to be 26.1 and 29.0 kDa by SDS-PAGE and zymogram. The chitinase has optimal activity at temperature 35 ºC and pH of 5. The chitinase exhibited K_M and V_{max} values of 1.51 mg/mL and 21.06 µmol/mL min for colloidal chitin. Cu^{2+} and Fe^{2+} inhibited chitinase activity at 10 mM concentration.

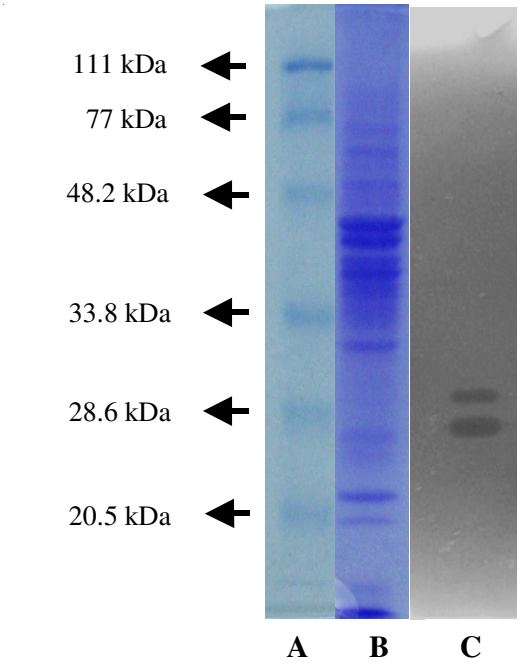

Fig. 5. SDS-PAGE chitinase of *Pseudomonas* sp stained with Comassie blue R-250; A: marker, B: crude extract enzyme, C: zymogram analysis with 0.1 % glycol chitin

ACKNOWLEDGEMENTS

Financial support from DP2M-DIKTI, National Education Department of Indonesia, for this research (Research Grant XVI, 2009)

REFERENCES

1. E. Cabib, *Adv. Enzymol. Relat. Areas Mol. Biol.*, **59**, 59 (1987).
2. N. Dahiya, Tewari and Hoondai, *Electron. J. Biotechnol.*, **8**, 2 (2005).
3. E. Suginta, P.A. Robertson, B. Austin, S.C. Fry and L.A. Fotherfill-Gilmore, *J. Appl. Microbiol.*, **89**, 76 (2000).
4. R.S. Patil, V. Ghormade and M.V. Deshpande, *Microbiol. Technol.*, **26**, 473 (2000).
5. C.J. Huang and C.Y. Chen, *Ann. Microbiol.*, **54**, 289 (2004).
6. R. Pichyangkura, S. Kudan, K. Kultiyawong and M. Sukwattanasinitt, *Carbohydr. Res.*, **337**, 557 (2002).
7. C.D. Woo and H.D. Park, *Biotechnol. Lett.*, **25**, 409 (2003).
8. L.S. Chernin, L. De La Fuente, V. Sobolev, S. Haran, C.E. Vorgias, A.B. Oppenheim and I. Chet, *Appl. Environ. Microbiol.*, **63**, 834 (1997).
9. K.J. Kim, Y.J. Yang and J.G. Kim, *J. Biochem. Mol. Biol.*, **36**, 185 (2003).
10. P.R. Vyas and M.V. Deshpande, *J. Gen. Appl. Microbiol.*, **37**, 267 (1991).
11. J. Monreal and E.T. Reese, *Can. J. Microbiol.*, **15**, 689 (1969).
12. J. Trudel and A. Asselin, *Anal. Biochem.*, **189**, 249 (1990).
13. C.A. Omumashaba, N. Yoshida and K. Ogawa, *J. Gen. Appl. Microbiol.*, **47**, 53 (2001).
14. Y. Tang, J. Zhao, S. Ding, S. Liu and Z. Yang, *Acta Microbiol. Sinica*, **41**, 82 (2001).
15. B. Bushan, *J. Appl. Microbiol.*, **8**, 800 (2000).
16. T. Yong, J. Hong, L. Zhangfu, Z. Li, D. Xiuqiong, T. Ke, G. Shaorong and L. Shigui, *Ann. Microbiol.*, **55**, 213 (2005).
17. S.-L. Wang and W.-T. Chang, *Appl. Environ. Microbiol.*, 380 (1997).
18. Y.-S. Lee, I.-H. Park, J.-S. Yoo, S.-Y. Chung, Y.-C. Lee, Y.-S. Cho, S.-C. Ahn, C.-M. Kim, Y.-L. Choi , *Bioresour. Technol.*, **98**, 2734 (2007).
19. R. Nandakumar, S. Babu, T. Raguchander and R. Samiyappan, *J. Agric. Sci. Technol.*, **90**, 803 (2007).
20. S.L. Wang, S.J. Chen and C.L. Wang, *Carbohydr. Res.*, **343**, 1171 (2008).
21. H.S. Lee, D.S. Han, S.J. Choi, S.W. Choi, D.S. Kim, D.H. Bai and J.H. Yu, *Appl. Microbiol. Biotechnol.*. **54**, 397 (2000).
22. S.E. Thompson, M. Smith, M.C. Wilkinson and K. Peek, *Appl. Environ. Microbiol.*, **67**, 4001 (2001).
23. S.-L. Wang, T.-W. Liang, B.-S. Lin, C.-L. Wang, P.-C. Wu and J.-R. Liu , *J. Microbiol. Biotechnol.*, **20**, 1001 (2010).