J. Microbiol. Biotechnol. (2004), 14(4), 647–652

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Study of Thermostable Chitinase Enzymes from Indonesian *Bacillus* K29-14

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Received: September 30, 2002
Accepted: April 21, 2004

Abstract Thermophilic microorganisms capable of producing chitinase enzymes were screened from samples collected from several crater and geothermal areas. The chitinolytic microorganisms were grown in a selective medium containing colloidal chitin. The *Bacillus* K29-14 isolate was found to exhibit the highest chitinase and chitin deacetylase activities. When grown in a chitin-containing medium, the isolate produced extracellular chitinase after 24 h of incubation. The optimum temperature and pH for the chitinase were 55°C and pH 7, respectively, while those for the chitin deacetylase were 55°C and pH 8, respectively. The thermostable chitinase and chitin deacetylase also retained 80- 90% of their activity after incubation for 5 h at 70°C. The divalent cations $CoCl_2$ and $NiCl_2$ increased the chitinase activity, while $ZnCl_2$ inhibited the enzyme. The chitin deacetylase was also activated by the presence of $MgCl_2$ and inhibited by $MnCl_2$, $NiCl_2$, and $CaCl_2$. A zymogram analysis revealed several forms of chitinase, with a 67 kDa form being the major enzyme.

Key words: Chitinase, chitin deacetylase, microorganism, stability

Chitin, a polymer of β-1,4-N-acetylglucosamine units, is the second most abundant polymer in nature, after cellulose. Chitin is present in insects, crustacea, and most fungi. Chitinase (EC 3.2.1.14) and N-acetylglucosamidase (GlcNAcase) (EC 3.2.1.30) are enzymes capable of hydrolyzing insoluble chitin into its oligo and monomeric components. The enzymes found in numerous bacteria, fungi, insects, plants, and animals are involved in natural protection mechanisms. Most notable among the chitin degrading prokaryotes are gliding bacteria, pseudomonads, vibrios, enteri bacteria, actinomycetes, bacilli, and clostridia. Chitinolytic bacteria are typically detected and screened through the production of clearing zones on a chitin-containing agar used as a selective medium [9, 15].

Chitin, chitooligomers (produced by enzymatic hydrolysis of chitin), and chitosan, which is the deacetylation product, have found numerous applications in industries, environmentally friendly techniques, and medicine. Chitin deacetylase catalyzes the conversion of chitin to chitosan [8, 9, 15]. The commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive and reliable sources of active and stable chitinase preparation. The current limitation on the use of industrial enzymes is due to their instability after extraction and separation from the cell producers.

Scientists have focused their attention on microorganisms that are capable of living in unnatural environments. As such, thermophiles, as factories for the production of heat-stable enzymes, have become very valuable for industry, health care, and research on protein stability at elevated temperatures. Accordingly, the current study presents several characteristics of chitinase enzymes from thermophiles isolated from a geothermal area in West Java, Indonesia.

Materials and Methods

Screening of Chitinase-Producing Thermophilles

Soil and liquid samples were collected from several geothermal areas in West Java, Indonesia. A selective solid medium containing $(NH_4)_2SO_4$ 0.7%, K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01%, Yeast Extract 0.2%, Trypton 0.1%, 1% of colloidal chitin, and 1.5% agar was used to screen the chitinase-producing microorganisms. The incubation

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temperatures were 50°C or higher. The microorganisms which produced the typical clearing zone were selected, further isolated, and purified. The chitinolytic index, i.e., the ratio of the diameter of the clearing zone to the diameter of the colony, was analyzed. The microorganisms that produced a high chitinolytic index was selected for further experiments.

Characterization of K29-14 Isolate

The morphology and physiological characteristics of K29-14 were determined according to the method of Slepecky and Hemphill [13]. The isolation of the genomic DNA and PCR amplification and sequencing of the PCR product for an analysis of the 16S-rRNA were conducted as suggested by Sambrook *et al.* [12], and Marchesi *et al.* [7], respectively, while a further analysis and comparison of the sequence data was performed according to Van de Peer and De Wachter [18].

Enzyme Production and Purification

The chitinolytic bacteria were grown in a similar liquid medium (without an agar) at 55°C. At specific times during the incubation, samples were taken and assayed for enzyme activity. The production of the enzymes was conducted using the optimum fermentation time. At the end of the incubation, the filtrate was separated from the cells by centrifugation at 3,000 ×*g* for 15 min. The protein was then precipitated using 40–50% ammonium sulfate and dissolved in 0.02 M Tris buffer, pH 7. After incubation at 55°C for 24 h and centrifugation to eliminate any denatured (non-thermostable) proteins, the enzymes were dialyzed in an appropriate buffer using a membrane (Sigma, 12,500 molecular cutoff), and loaded onto a DEAE Sephadex A 50 column. The proteins were eluted with a linear gradient of 0–1 M NaCl in 20 mM of Tris buffer. The proteins were analyzed according to Bradford [1].

Assay for Enzyme Activity

The method used for analyzing the chitinase activity was according to Ueda and Arai [17]. A reaction mixture containing 1 ml of 0.3% colloidal chitin, 2 ml of 0.1 M phosphate buffer (pH 7.0), and 1 ml of the enzyme solution was incubated for 1 h at 55°C. The remaining chitin in the reaction mixture was measured turbidimetrically at 660 nm. One unit of activity was defined as the amount of enzyme causing a 0.001 decrease in the absorbance at 660 nm per minute.

The chitin deacetylase activity was analyzed according to the method of Tokuyasu *et al.* [16] using a water-soluble glycol chitin (Sigma) as a substrate. The enzyme was assayed in 20 mM of Sodium tetraborate/HCl buffer using 0.15% glycol chitin as a substrate. The reaction was initiated by the addition of 40 µl of the enzyme solution into 160 µl of the reaction mixture. The incubation time was 20 min at 30°C, and the reaction was terminated by the addition of 200 µl of 33% (w/v) acetic acid. Upon termination of the reaction, the concentration of glucosamine residues produced by the deacetylation reaction was spectrophotometrically estimated after oxidation with sodium nitrite, followed by the additon of indole HCl according to the method of Dische and Borenfreund [4].

Characterization of Chitinase Enzymes

The optimum temperature for the enzymes was analyzed using a chitinase and chitin deacetylase assay at different temperatures. The activity assay was performed at pH 7. The temperature stability was determined by measuring the residual activity after various periods of preincubation in phosphate buffer at 55°C and 70°C (pH 7).

The optimum pH for the enzymes was measured at 55°C using Universal (borate) buffer solutions with different pHs.

To determine the effect of metal ions on the enzyme activity, the enzyme solution was preincubated with 1 mM of metal ions such as $CaCl_2$, $COCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$. The effect of EDTA on the enzyme activity was also analyzed.

The molecular weight was determined by SDS PAGE [6]. The electrophoresis was run at 100 mA, 100 Volts for

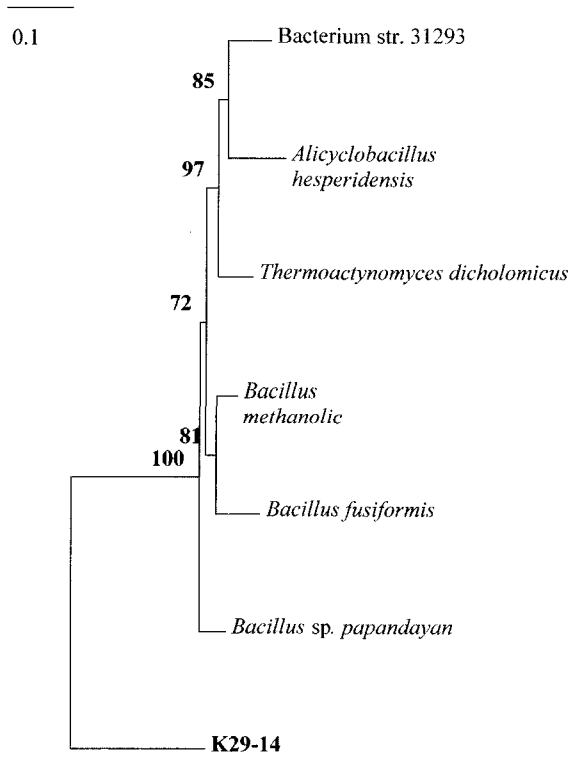
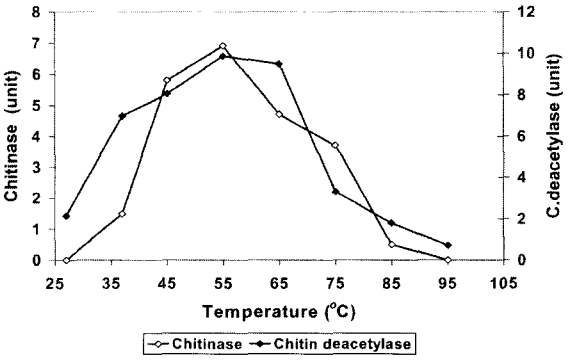


Fig. 1. Phylogenetic tree based on sequence analysis of 16S-rRNA gene. The numbers in the tree are the percentages of the bootstrap replicate.

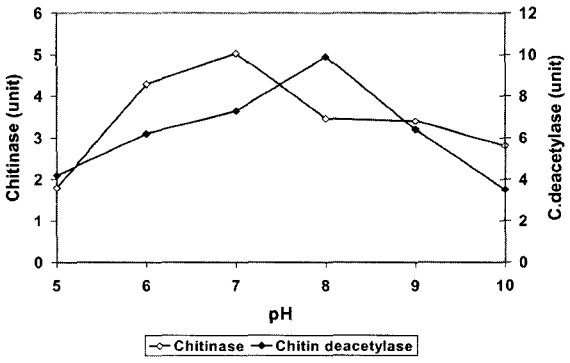
90- 120 min and the protein bands were stained with Coomasie Brilliant Blue R 250. Zymogram analysis was conducted by following the method of Cottrell *et al.* [3]. A 0.01% of glycol chitin was mixed with acrylamide to form a gel. After electrophoresis at 50 mA, 30 Volts, and 4°C for 5 h, the gel was incubated at 55°C for 2 h in 100 mM of sodium acetate solution (pH 6) containing 1% of Triton X 100. The analysis of the hydrolyzed chitin was conducted by incubation in 0.01% calcofluor white M2R (Sigma) - 0.5 M Tris solution (pH 9) for 5- 10 min, followed by overnight washing in aquadest before detection under UV light.

RESULTS

A number of colonies were found to be capable of growing at temperatures from 55°C to 70°C. Among the chitinolytic isolates from the K29 sampling site, K29-14 was identified as the best chitinase producer, showing the highest chitinolytic index. This isolate was then further characterized. The

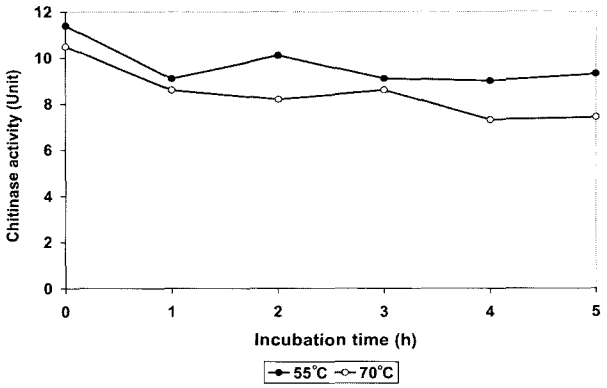


(a) Optimum temperature for chitinase and chitin deacetylase from K29-14 isolate

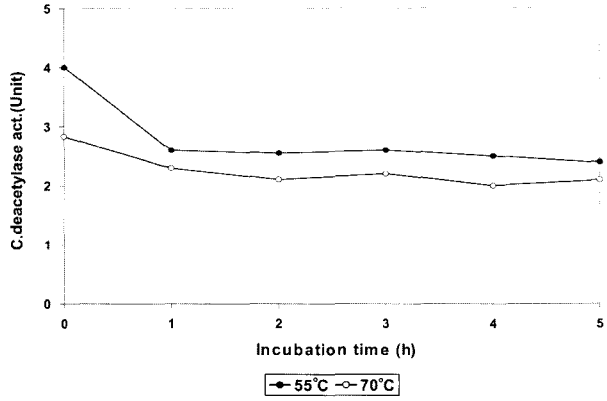


(b) Optimum pH for chitinase and chitin deacetylase from K29-14 isolate

Fig. 2. Optimum temperature (a) and optimum pH (b) for chitinase and chitin deacetylase of *Bacillus* K29-14 isolate.
The enzyme solution was incubated at different temperatures (a) or different pHs (b) as indicated. The enzyme activities were measured as described in the Materials and Methods. (-○-) Chitinase activity; (-●-) chitin deacetylase activity.



(a) Heat stability of chitinase from K29-14 isolate



(b) Heat stability of chitin deacetylase from K29-14 isolate

Fig. 3. Heat stability of chitinase (a) and chitin deacetylase (b) from *Bacillus* K29-14 isolate.
The enzyme solution was kept at 55°C (-●-) or 70°C (-○-) for 5 h. Samples were taken and analyzed every hour.

morphological and physiological characteristics of the isolate indicated a bacilli type, and the analysis of the 16S-rRNA gene revealed that the isolate was closest to *Bacillus papandayan* (Fig. 1).

Although capable of growing at 70°C, the Gram-positive, spore former *Bacillus* K29-14 grew and produced chitinase enzymes best at 55°C. The chitinase and chitin deacetylase of the K19-14 isolate exhibited their maximum activities after 24 h of incubation (2.5 unit/mg protein for chitinase and 7.5 unit/mg protein for chitin deacetylase). At this time, the microorganisms were in the stationary phase. The optimum pH for the chitinase of K29-14 was 7, and the optimum temperature was 55°C, while those for the chitin deacetylase were pH 8 and 55°C, respectively (Figs. 2a and 2b). When K29-14 chitinase and chitin deacetylase were incubated at 70°C, 80- 90% of the activity still remained after 5 h (Fig. 3). Therefore, they were also stable at elevated temperatures.

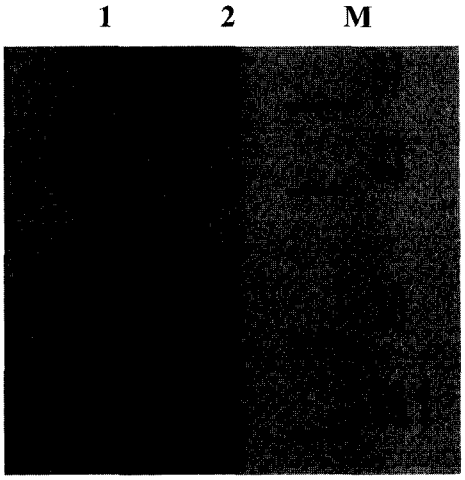
Table 1 indicates that 1 mM of $CoCl_2$ and $NiCl_2$ activated K29-14 chitinase. Yet, a different result was produced as

Table 1. Effect of 1 mM of divalent cations on chitinase activities.

Divalent cations	Enzyme activity (%)	
	Chitinase	Chitin deacetylase
Control	100	100
$CaCl_2$	75	50
$CoCl_2$	137	87
$MgCl_2$	96	144
$MnCl_2$	103	20
$NiCl_2$	131	50
$ZnCl_2$	20	106
EDTA	103	105

regards to the effect of divalent cations on the activity of K29-14 chitin deacetylase, i.e., Mg^{2+} was found to be an activator, while Mn^{2+}, Ni^{2+}, and Ca^{2+} reduced the enzyme activity.

According to zymogram analysis of the chitinases, the purified major enzyme showed molecular weight of 67 kD (Fig. 4) after DEAE chromatography. The result of ion-exchange chromatography is presented in Table 2. The enzyme fractionated by ammonium sulfate exhibited enzyme (chitinase) activity at 20, 43, 67, and 94 kDa, however, the 20, 43, and 94 kDa chitinase activites were not detected after further purification (chromatography), indicating that the amounts of these molecules were much less compared with the 67 kDa protein.



1. : Purified enzyme
2. : Crude enzyme
M : Molecular weight marker (67, 43, 30, and 20.1 kd)

Fig. 4. Results of zymogram analysis of chitinase from *Bacillus* K 29-14 isolate.
For zymogram analysis, experiments were conducted by following the method of Cottrell *et al.* [3]. A 0.01% of glycol chitin was mixed with acrylamide to form a gel. After electrophoresis, the gel was incubated at 55°C for 2 h in 100 mM of sodium acetate solution (pH 6) containing 1% of Triton X-100. Analysis of the hydrolyzed chitin was conducted by incubation in 0.01% calcofluor white M2R (Sigma) - 0.5 M Tris solution, pH 9, for 5- 10 min, followed by overnight washing in aquadest before detection under UV light.

DISCUSSION

The locations selected for screening the thermophilic microorganisms in the current study were several known geothermal areas and craters in West Java, Indonesia. It has been found that the Kamojang crater and geothermal area in West Java are good sources for microorganisms producing chitin-degrading enzymes. Since chitin is a common structural component of arthropod exoskeleton and fungal cell walls, it is possible that the plants surrounding this geothermal area could be good sources for studying the diversity of these insects and fungi. In addition, chitinolytic enzymes together with other extracellular hydrolases are most frequently considered as critical in facilitating insect invasions. As such, the soilborne microorganisms that produce chitinolytic enzymes found in the Kamojang area have potential to be further developed as biocontrol agents against fungi and nematodes causing diseases in agricultural crops.

The existance of thermophilic microorganisms has been known for several decades. Many of these microorganisms are bacteria, and some belong to the archea family. In earlier studies by the current authors, a large number of hydrolases-producing thermophiles were identified including proteolytic, amylolytic, cellulolytic, and xylanase-producing thermophiles. However, among these isolates, thermo-chitinolytic isolates were the smallest percentage. The chitinolytic thermophile isolates identified in the current study were capable of growing at 70°C, yet the best condition for producing stable chitinase is generally at 55°C.

Most of the chtinase-producing microorganisms isolated in the current study were found to be Gram-positive,

Table 2. Chitinase activity during purification.

Step	Protein (mg)	Activity (unit)	Specific activity (unit/mg protein)	Purification fold
Crude extract	115	554	4.8	
Amonium sulfate precipitation	19.5	107	5.5	1.2
Heat treatment at 55°C	7.7	87	11.3	2.4
Dialysis	5.1	77	15.1	3.2
DEAE	2.9	70	24.1	5.0

motile, spore-forming, and aerobic Bacilli type. The *Bacillus* K29-14 was determined by a sequence analysis of its 16S-rRNA gene as being closest to *Bacillus papandayan*.

The current exploration of selected areas in West Java identified thermophilic chitinase and chitin deacetylase-producing enzymes that may be potentially useful for food, medical, agricultural, and other biotechnological applications, as well as for the development of biocontrol agents. The heat-stable chitinases produced by the thermophilic microorganisms in the current study generally exhibited optimal enzymatic activity at neutral pH.

Wang and Chang [19] reported the presence of two extracellular chitinases from *Pseudomonas aeruginosa* with an optimum pH and temperature at pH 7-8 and 40°C-50°C, respectively. The enzymes were also reported as stable at 50°C-60°C. Other chitinases isolated from thermophiles have shown an optimum activity at 45°C-75°C, while the extremophile *Pyrococcus* excretes chitinase with an optimum temperature of 85°C [14]. Meanwhile, the chitinases from the Indonesian *Bacillus* K29 showed an optimum temperature at 55°C, and were quite stable at 70°C.

The effect of divalent cations on chitinase activity differs among different sources of chitinases. The chitinase excreted by *Colletotrichum lindemuthianum* is activated by Co^{2+} [16], while the chitinase from *Bacillus* MH-1 is activated by Ca^{2+} and Mn^{2+} [11]. The Ag^{2+} ion, which is an activator of the *Aeromonas* chitinase, is also an inhibitor of the *Bacillus* chitinase [11, 17]. Ni^{2+} and Mn^{2+} have been identified as inhibitors of the chitinase from *Colletotrichum lindemuthianum* [16], while Cu^{2+} activates the chitinase from *Pseudomonas aeruginosa* [19]. In this current study, the thermostable chitinase and chitin deacetylase from the Indonesian *Bacillus* K29-14 appeared to be rather specific, since they were only activated by Co^{2+} and Ni^{2+} cations. As such, K29-14 chitinase and chitin deacetylase may not strictly require a metal ion cofactor for their optimum activities, as reflected by the inability of 1 mM EDTA to inhibit the enzyme activity.

Reports on the biological functions and biochemical characteristics of the chitin deacetylases from microorganisms have not been as numerous as those on chitinase. Chitin deacetylase is regarded as an important part of the natural defence mechanism of an organism [5, 15]. Chitin deacetylase catalyzes the conversion of chitin into chitosan by the deacetylation of N-acetylglucosamine residues. The use of chitin deacetylase for chitosan production as opposed to the presently used chemical procedures offers the possibility of a controlled, and nondegradable process, resulting in the production of novel and well-defined chitosan oligomers and polymers. Until now, the enzyme has only been isolated from *Saccharomyces cerevisiae* [2] and several fungi, such as *Colletotrichum lindemuthianum* [16]. The 30 kDa chitin deacetylase from *Colletotrichum lindemuthianum* fungi shows an optimum temperature at 60°C and is not inhibited by sodium acetate [16]. However, the heat-stable chitin deacetylase from the Indonesian *Bacillus* K29-14 in the current study was found to be sodium acetate insensitive.

The practical aspect of the purification of a heat-stable enzyme is that a thermostable enzyme can retain its active conformation while other heat-labile proteins denature at elevated temperature. This strategy was applied to the purification of K29-14 chitinase and chitin deacetylase, and the result turned out to be quite satisfactory. The occurrence of two types of chitinase has been reported in *Streptomyces* [10]. Three chitinases with different molecular weights are excreted by *Bacillus* sp. as reported by Sakai *et al.* [11]. Ueda and Arai [17] reported the presence of multiple forms (8) of chitinases from *Aeromonas* sp. with molecular weights ranging from 89 to 120 kDa. Bacteria produce different forms of chitinase enzymes to meet nutritional needs, probably to hydrolyze the diversity of chitin molecules found in nature. The chitinases identified in the current study were molecules of 20, 43, 67, and 94 kDa, although the 67 kDa molecule was found to be the major enzyme.

Acknowledgment

This research was supported by a Research Grant for the "Exploration of chitinase enzymes from the Indonesian thermophiles" from the Bioproduct Research Center Yonsei University Korea 1999-2000.

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