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Biochemical Characteristics of Chitinase Enzyme from *Bacillus* sp. of Kamojang Crater, Indonesia

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Chitinase and chitindeacetylase and enzymes capable of degrading chitin into chitooligomers and chitosan. The chitinases characterized and purified in this study were extracted from the acidophillic *Bacillus* sp. isolated from Kamojang Crater West Java Indonesia.

When grown in liquid media containing colloidal chitin, the optimum chitinase activity of the acidophilic isolate was reached after 4–5 days of incubation. The optimum temperature and pH of the chitinase and chitin deacetylase were found at 37°C and pH 5. When incubated at pH 5, the activity of chitin deacetylase was increased; after 3 h, the activity was 1.5 times of the control. The enzyme was stable at pH 4, after 2 h incubation, the activity was still 80% of the control. The chitinase and chitin deacetylase activities were not influenced by Mg^{++} nor Ca^{++}. Ni^{++} and Cu^{++} inhibited the chitinase activity, while chitin deacetylase activity was not affected by Cu^{++} addition. When 1 mM of EDTA was added, the enzyme activity was reduced from 40 to 50%.

Keywords: Chitinase; Chitin deacetylase; Enzyme characteristics; Incubation

INTRODUCTION

Chitin, a polymer of β1,4-*N*-acetylglucosamine units is the second most abundant polymer in nature, after cellulose. Chitin is present in insects, crustacea and in most fungi. Commercial chitin can be produced from crabs and shrimp shells. Chitosan is the deacetylase form of chitin and is made through deacetylation process. Chitin and chitooligomers produced by enzymatic hydrolysis of chitin can be used in human health care. Due to the charge present in the molecule, chitosan find wider usages, examples of chitosan applications are: as flocculant, as food preservatives and stabilizer, in enzyme and cell immobilization, protein separation, cell recovery and in reverse osmosis technology [3–5,7,10]. For some applications, uniformly deacetylated chitosan is required. It is recognized that the thermodeacetylation process currently used to produce commercial chitosan is non specific and the product shows variability in the degree of deacetylation. The commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive, reliable sources of active and stable chitinase and chitin deacetylase preparations. Production of inexpensive chitinolytic enzymes is also important in the utilization of shellfish waste in solving environmental problems as well as promoting the economic value of marine products.

Chitinase (EC 3.2.1.14) and *N*-acetylglucosamidase (GlcNAcase) (EC 3.2.1.30) are enzymes capable of hydrolyzing insoluble chitin to oligo and monomeric components. Chitinase can be classified in two major categories, endochitinase (EC 3.2.1.14) which cleaves chitin randomly at internal sites, generating soluble low molecular mass multimers of *N*-acetylglucosamine, and exochitinase (EC 3.2.1.29) which catalyze the progressive release of monomers or dimers from the non reducing end of the chitin microfibril. The enzymes found in numerous bacteria, fungi, insects, plants, and animals are involved in natural protection mechanisms; therefore, soil borne microorganisms that produce chitinese are considered as potential biocontrol agents against fungi [3,7,10]. Chitinolytic bacteria are typically detected by the production of clearing zones on agar containing chitin.

Current limitation on the use of industrial enzymes are due to the cost of isolation of the enzymes from natural resources, their instability, and their activity within narrow

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temperature and pH ranges. Scientists have focused their attention on microorganisms, which have developed the capability during the course of evolution of living under extreme environmental conditions. As an example, the acidophilles as factories for production of novel enzymes capable of standing particularly aciduous environments become valuable for specific uses.

This research is a preliminary study on the exploration of chitinase producing microorganisms, isolated from West Java Indonesia, in particular, those of an acid nature. In this preliminary exploration, we present our result on the biochemical characteristics of the potential chitinase and chitin deacetylase enzymes.

MATERIALS AND METHODS

Enzyme Production

Chitinolytic isolates were screened from samples collected in Kamojang West Java Indonesia using a selective media containing $(NH_4)_2\ SO_4$ 0.7%, K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4{\cdot}7H_2O$ 0.01%, Yeast Extract 0.2%, Tripton 0.1%. Incubation temperature and pH were 37–50°C and pH 3–5. The microorganisms which produced the largest clearing zone were selected, transferred and purified for morphological and biochemical analysis.

The selected chitinolytic bacteria (Isolate K-22) were grown in similar liquid media, at pH 5 and 37°C. At specific times of incubation, samples were taken and assayed for protein [1] and enzyme activity. The enzymes were isolated at optimum fermentation time, and fractionated using 40–50% ammonium sulphate. The resulting fractions were dialyzed against 20 mM Tris buffer (pH 7) using a specific membrane (Sigma, 12,500 molecular cut off) and chromatographed onto DEAE Sephadex A 50. The enzymes were eluted with a 0–1 M NaCl gradient in 20 mM Tris buffer (pH 7).

Preparation of Colloidal Chitin and Assay for Enzyme Activity

Commercial chitin (20 g) was mixed with concentrated HCl (400 ml), stored at 4°C for 24 h, filtered through glass wool, and centrifuged to obtain the precipitated substance. The colloidal chitin was recovered after adjusting the pH to 7 with NaOH, followed by centrifugation at 600*g* for 15 min.

The method used for analyzing chitinase activity was that reported by Ueda and Arai [12]. A reaction mixture containing 1 ml of 0.3% of colloidal chitin, 2 ml of 0.1 M acetate/phosphate buffer (pH 4 or 7) and 1 ml of enzyme solution was incubated for 1 h at 37°C. The remaining chitin in the reaction mixture was measured by solution turbidimetricaly at 660 nm. One unit of activity was defined as the amount of enzyme causing a 0.001 decrease in the absorbance at 660 mm/min.

The method used for analyzing chitin deacetylase activity was that reported by Tokuyasu *et al.* [11] using a water soluble glycol chitin as the substrate. The enzyme was assayed in 20 mM sodium tetraborate/HCl buffer (pH 5.5) using 0.15% glycol chitin as a substrate. The reaction was initiated by addition of 40 µl of enzyme solution to 160 µl of a reaction mixture. 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 following oxidation with sodium nitrite, followed by addition of indole HCl as described by Dische and Borenfreund [2].

RESULTS

The acido-chitinolytic isolates from Kamojang, were screened based on the typical clearing zone surrounding the colonies observed in the selective media. The chitinolytic index was measured as the ratio of the diameter of the clearing zone upon the diameter of the colonies. The selected isolate (K-22) were capable of growth at pH 3.5–5, but the clearing zone were more intensive when they were incubated at pH 5. The isolate was characterized as spore forming, gram positive rod shaped bacteria of the *Bacillus* type.

When grown in the chitin containing liquid media, the optimum fermentation time was 4–5 days (Fig. 1). This is considered longer compared to chitinase production from the thermophiles isolated from the same area, which showed optimum enzyme activity after 24–30 h of incubation (research still in progress). Our study and others, find that the chitinases were optimally produced in the stationary phase, indicating the utilization of simpler carbon sources during the first step of growth (Fig. 1).

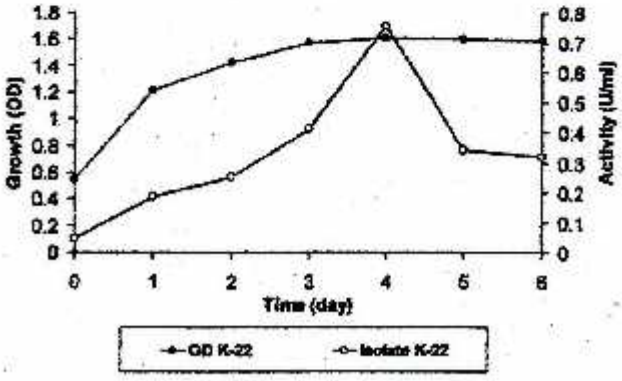

FIGURE 1 Growth of the chitinolytic *Bacillus* K-22 in chitin containing media. The growth was measured at 660 mM. For the chitinase assay, colloidal chitin was used as the substrate as mentioned under "Materials and Methods" section. The enzyme activity was reported as U/ml filtrate. •: Growth (OD = Optical Density K-22), ○: Chitinase activity.

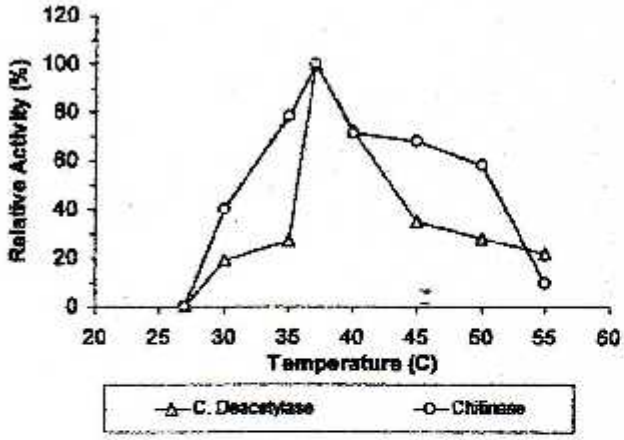

FIGURE 2 The effect of temperature on the chitinase and chitin deacetylase activity of *Bacillus* K-22. The substrate used for the chitinase assay was colloidal chitin as described by Ueda and Arai [12], while for the chitin deacetylase assay, glycol chitin was used as described by Tokuyasu *et al.* [11]. The activity of optimum temperature was regarded as 100% (1.2 IU/mg for chitinase and 1.3 U/mg for chitin deacetylase). O: Chitinase, Δ: Chitin deacetylase.

Among the chitinase isolates, the highest enzyme activity was shown by the K-22 isolate (0.755 U/mg). This isolate was used for further characterization.

The optimum temperature for the chitinase and chitin deacetylase activity was 37°C, while the optimum pH was 5 (Figs. 2–4). Figure 5 shows that the chitin deacetylase produced by the Kamojang *Bacillus* K-22 was more stable compared with the chitinase at low pH (Fig. 4). When incubated at pH 4, the chitin deacetylase activity was still at 60% of the control, while that of the chitinase was almost completely inactivated after 1 h incubation. At pH 5, the chitin deacetylase activity was in fact increased up to 1.5 times of the control, during the 2.5 h incubation, while that of the chitinase was reduced to 70% of the control following 2 h incubation.

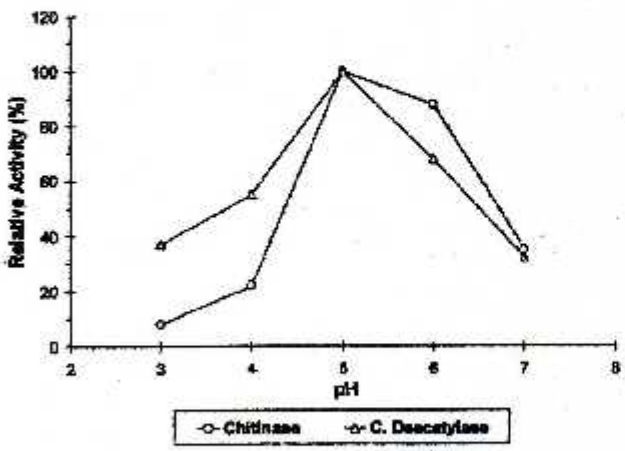

FIGURE 3 The effect of pH on the chitinase and chitin deacetylase activity of *Bacillus* K-22. The substrate used for the chitinase assay was colloidal as described by Ueda and Arai [12], while for the chitin deacetylase assay, glycol chitin was used as described by Tokuyasu *et al.* [11]. The activity at optimum temperature was set at 100% (1.2 IU/mg for chitinase and 1.3 U/mg for chitin deacetylase) O: Chitinase, Δ: Chitin deacetylase.

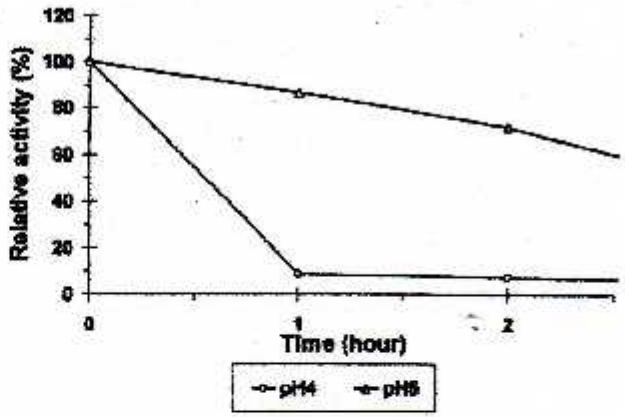

FIGURE 4 Stability of chitinase at pH 4 and 5. The enzyme was incubated for various times in citrate phosphate buffer 0.1 M (pH 4 and 5) at 37°C. The enzyme activity set as 100% before incubation (time: zero) was 1.1 U/mg. O: pH 4, Δ: pH 5.

The effect of divalent cations on chitinase activity is varied, depending on the enzyme sources. Chitinase excreted from *Colletotrichum lindemuthianum* is activated by Co^{++} [11], while that of *Bacillus* is activated by Ca^{++} and Mn^{++} [8]. Chitinase from *Pseudomonas* K-187 was activated by Cu^{++}, while that of *Aeromonas* was activated by Ni^{++} [12,13]. We found that divalent cations Mg^{++}, Mn^{++} and Ca^{++} do not affect significantly the enzyme activity, while Co^{++}, Zn^{++}, Cu^{++} and particularly Ni^{++} inhibited the chitinase (Table I). The chitin deacetylase activity was not affected by Mg^{++}, Ca^{++}, and Cu^{++}, while Mn^{++}, Ni^{++} and Co^{++} inhibit the enzyme. Nevertheless, the strong inhibition of the enzyme by 1 mM EDTA implies a requirement for metal ion for optimum enzyme activity.

DISCUSSION

During our preliminary exploration of the indigenous chitin degrading enzymes, we found that Kamojang, West Java is particularly rich in thermophillic as well as

TABLE I The effects of divalent cations (1 mM) and EDTA (1 mM) upon chitinase and chitin deacetylae activities

Treatment	Relative activity (%)	
	Chitinase	Chitin deacetylase
Control	100	100
EDTA	55	68
$MgCl_2$	116	105
$CaCl_2$	113	100
$MnCl_2$	113	82
$CoCl_2$	88	85
$NiCl_2$	67	88
$ZnCl_2$	86	93
$CuCl_2$	70	103

Experiments were conducted as described in the "Materials and Methods" section. Enzyme activity of the control is regarded as 100% (0.8 IU/mg for chitinase and 1.4 U/mg for chitin deacetylase). Activities of the enzymes treated with divalent cations were calculated as percentage of the control.

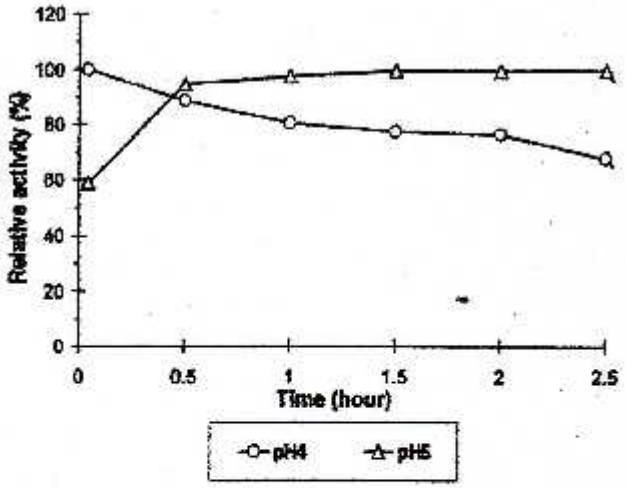

FIGURE 5 Stability of chitin deacetylase at pH 4 and pH 5. The enzyme was incubated for various times in citrate phosphate buffer 0.1 M (pH 4 and 5) at 37°C. The activity at 100% was 1.5 U/mg. O: pH 4, Δ: pH 5.

acidophilic microorganisms producing these enzyme (research on the thermostable chitinase is still in progress). Since chitin is a common structural component of the arthropod exoskeleton and fungal cell walls, it is possible that the plants and insects indigenous to this area may represent a worthwhile population to study the diversity of enzymatic reaction in a variety of species. In addition, chitinolytic enzymes together with other extracellular hydrolases are the enzymes most frequently considered critical in facilitating invasion of insects. The soil borne microorganisms that produce chitinolytic enzymes found in this area can be considered potential biocontrol agents against fungi and nematode causing disease in agricultural crops.

The incubation time for enzyme production in our study is considered longer compared to our study on chitinase production by the thermophillies isolated from the same area. However, Okazaki *et al.* [6], reported that the optimum incubation for chitinase production from *Streptomycetes* sp. J-13-3 was as long as 4 days, at 28°C, while that for *Pseudomonas aeruginosa* K-187 was 3 days, when incubated at 45°C [13].

Reports on the biological function and biochemical characteristics of chitin deacetylases from microorganisms have not been extensive as those of the chitinase enzymes. The enzyme is regarded important as part of the natural defence mechanism of the organism. Chitin deacetylase catalyse the conversion of chitin to chitosan by deacetylation of *N*-acetylglucosamine residues. The use of chitin deacetylase for chitosan production in contrast to the presently used chemical procedures offers the possibility of a controlled, non degradable and environmentally friendly process resulting in the production of novel, well defined chitosan oligomers and polymers. Thus far, the enzyme has been isolated from *Saccharomyces cerevisiae* and several fungi, including *Colletotrichum lindermuthianum* [7,10,11]. Presently, screening of microorganisms capable of excreting chitin deacetylase is important for future biotechnological application, as the enzyme is considered to be a versatile tool in biotechnology [10]. The acid stable chitin deacetylase from the Indonesian *Bacillus* K-22 is quite unique, and will be important, not only for enzymatic production of chitosan from the abundant chitin substances and waste marine products, but also for molecular studies of its stable protein conformation under acid conditions. As reported in this study, the enzyme could retain 70% of its activity after incubation at pH 4 for 2.5 h.

Acknowledgements

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