PARTIAL PURIFICATION AND CHARACTERIZATION OF CHITIN DEACETYLASE PRODUCED BY *Bacillus thermoleovorans* LW-4-11

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ABSTRACT

Purifikasi Parsial dan Karakterisasi Enzim Kitin Deasetilase yang Dihasilkan *Bacillus thermoleovorans* LW-4-11. Kitin deasetilase yang dihasilkan dari bakteri *Bacillus thermoleovorans* LW-4-11 telah dimurnikan menggunakan amonium sulfat 70% diikuti dengan perlakuan panas (suhu 70^{o}C selama 1 jam). Kemurnian enzim yang dipisahkan dari medium fermentasi meningkat 4,28 kali dengan aktivitas spesifik sekitar 4,37 mU/mg. Enzim memiliki suhu dan pH optimum masing-masing 80^{o}C dan 6,0 dalam substrat *O-hydroxyethylated chitin* (glycol chitin). Kitin deasetilase ini relatif tahan panas dengan waktu-paruh sekitar 30 menit pada suhu 80 ^{o}C. Enzim dihambat oleh ion Li^+, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^+ pada konsentrasi 1 mM, tetapi diaktifkan oleh EDTA 1 mM.

Kata kunci: Purifikasi parsial, karakterisasi, kitin deasetilase, *B. thermoleovorans* LW-4-11

INTRODUCTION

Chitin, a homopolymer of β-(1-4)-linked *N*-acetyl-D-glucosamine, is one of the most abundant, easily obtained, and renewable natural polymers, second only to cellulose. It is commonly found in fungi, marine vertebrates, and insects (Sandford 1989; Patil *et al*. 2000). Chitin is an insoluble material and its industrial use is still limited. Chitosan, a partially deacetylated form of chitin, is water soluble and has a large number and a wide variety of important applications (Muzzarelli 1996; Somashekar and Joseph 1996; Shahidi *et al*. 1999; Tsigos *et al*. 2000).

Chitosan is produced by the thermochemical deacetylation of chitin which leads to heterogeneous end-product owing to the severity of the treatment (Chang *et al*. 1997; Kolodziejska *et al*. 2000). The enzymatic conversion of chitin to chitosan using chitin deacetylase (CDA) provides an attractive alternative to presently employed chemical process. CDA catalyses the conversion of chitin to chitosan by the deacetylation of *N*-acetyl-D-glucosamine residues in chitin oligosaccharide (Tsigos *et al*. 2000). CDA's have been identified and characterized from several extracts of fungi (Gao *et al*. 1995; Deising and Siegrist 1995; Alfonso *et al* 1995; Tokuyasu *et al*. 1996; Tsigos and Bouriotis 1995; Christodoulidou *et al*. 1999). However, CDA's from bacteria have been rarely reported. This paper describes the isolation, characterization, and partial purification of thermostable CDA from *B. thermoleovorans* LW-4-11.

MATERIALS AND METHODS

Materials

Chitin, glycol chitosan and glucosamine were purchased from Sigma Chemicals. Glycol chitin was prepared from glycol chitosan with reacetylation using acetic anhydride (Trudel and Asselin 1990). Reagents for protein determination and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were purchased from Bio-Rad. All other chemicals were the highest grade available.

Microorganims and Cultivation

B. thermoleovorans LW-4-11, a chitinase producing bacterium, was used for the production of CDA. The strain was isolated from Langoan hot spring water in North Sulawesi by the method of Srinivasan (2000) on minimal medium containing of (g l^{-1}): Bacto yeast extract, 10; $(NH_4)_2SO_4$, 4: KH_2PO_4, 0,15 g; and 100 mg chitin (100 mesh). For enzyme production, bacterium was cultured on Thermus medium (Takayanagi *et al*, 1991) containing $(NH_4)_2$ SO_4, 0,7%; K_2HPO_4, 0,1%; NaCl, 0,1%; $MgSO_4$ $7H_2O$, 0,01%, Bacto yeast extract, 0,2%, Bacto trypton, 0,1% and colloidal chitin, 1% and incubated at 70 ^oC for 3 days.

Preparation of Enzyme

The bacterial cells were separated from culture broth by centrifugation at 10 000 x g for 15 minutes. The supernatant was brought to 70% saturation with ammonium sulphate at 4 ^oC and allowed to settle down overnight. The precipitate was recovered by centrifugation (10 000 x g, 15 minutes) and the pellet formed was solubilized in 20 mM Tris-HCl buffer, pH 7,0. The solution was dialyzed overnight against the same buffer at 4 ^oC and then incubated at 70 ^oC for 1 h to remove other heat-unstable proteins.

Chitin Deacetylase Assay

Chitin deacetylase activity was estimated using glycol chitin as a substrate (Tokuyasu *et al*. 1996). The assay mixture contained 0,15% glycol chitin dissolved in 20 mM tetraborate/HCl buffer (pH 8,5). Reaction was initiated by the addition of 200 µl (crude extract) or 50 µl (ammonium sulphate precipitated) enzyme solution to 123 µl of reaction mixtures, incubated at 70 ^{o}C for 30 min. The reaction was terminated by the addition of 200 µl of 33% (v/v) acetic acid. The control was prepared by adding the enzyme solution after inactivation. Upon termination of the reaction, the concentration of glucosamine residues produced by the deacetylation reaction was estimated by oxidation using $NaNO_2$, followed by a spectrophotometric method using indole/HCl (Dische and Borenfreund 1950). Protein content was determined using the dye-binding method with bovine serum albumin fraction V as a standard (Bradford 1976).

Effect of pH, temperature and additives

The pH and temperature optima of the enzyme as well as the effect of metal ion concentration on the activity were measured after incubation for 30 min at various pHs, temperatures (60-100 ^{o}C) and metal ion concentration, respectively. The pH was adjusted by using the following buffers: glycine-HCl (pH 3,0-5,0), NaH_2PO_4 – Na_2HPO_4 (pH 6,0-8,0), borate (pH 8,0-9,0), and glycine-NaOH (pH 10-12). Metal ions analysed included LiCl, $MnCl_2$, $ZnCl_2$, $CoCl_2$, $MgCl_2$, and $NiCl_2$,. The effect of EDTA upon the enzyme activity was also examined at a concentration of 0,1 mM.

The thermostability of enzyme was measured by the residual CDA activities after incubation of enzyme solution at optimum temperature with the corresponding buffers. Samples were withdrawn every 15 minutes and the activities were measured.

RESULTS

Isolation and Identification of Microorganism

Microorganisms isolated from hot water collected at different locations in Langoan, North Sulawesi were screened on agar plates containing 1% of colloidal chitin at 70 ^{o}C for 4 days. Colonies that grew well under such conditions were isolated and retained for subsequent screening. Eighty strain of bacteria were obtained from the first screening and then were subcultured in liquid media containing 0,5% colloidal chitin. After incubation, the supernatants were collected for measurement of CDA activity. One strain showed the highest activity and used throughout the study. It produced CDA extracellularly at the maximum level after 4th days cultivation (Fig 1). According to 16S rRNA sequence, the strain was identified as *B. thermoleovorans* (Suhartono 2002).

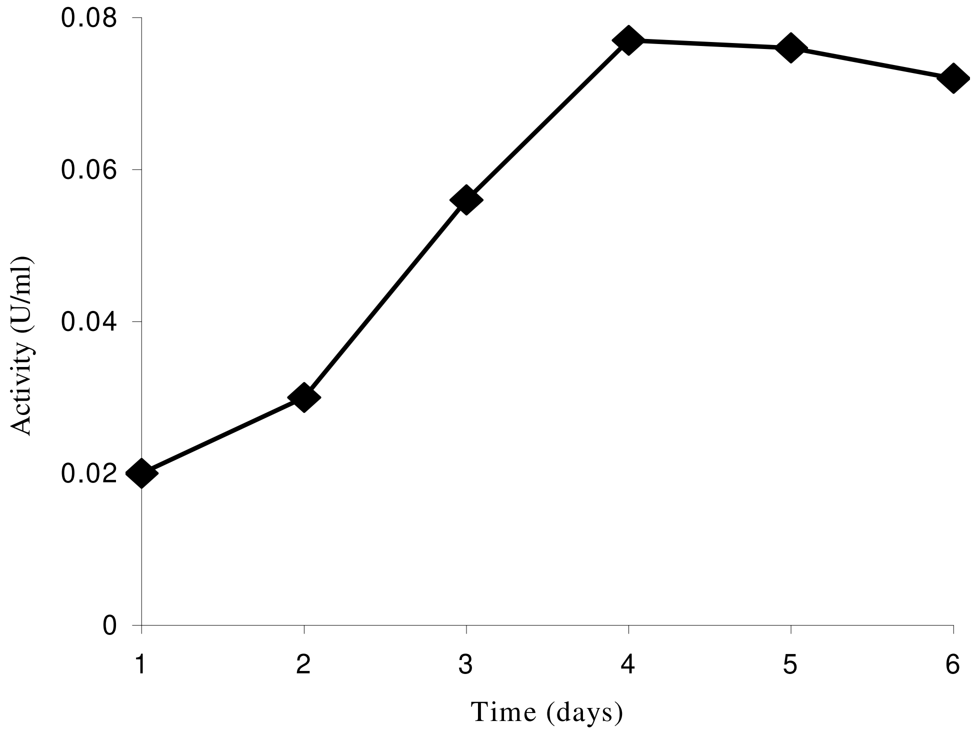

Fig 1. Time course of CDA production by *B. thermoleovorans* LW-4-11

Enzyme Preparation

The result of partial purification procedure are summarized in Table 1. The enzyme from 4 days cell-free medium of LW-4-11 was precipitated by the addition of ammonium sulphate up to 80% saturation. This step resulted in 1.50-fold increase in specific activity. The heat treatment of enzyme increased the purity of enzyme to be 4,28 fold.

Table 1. Partial Purification Steps of CDA Produced by *B. thermoleovorans* LW-4-11

Steps	Total Activity (mU)	Protein (mg)	Specific Activity (mU/mg)	Purification (Fold)	Recovery (%)
Free-Cell Medium	21,4	2,10	10,2	1,00	100,0
Ammonium Sulfate ppt (70%)	18,5	1,21	15,3	1,50	86,4
Heat Treatment (70 ^oC, 1 h)	7,9	0,18	43,7	4,28	36,7

Characterization of Enzyme

The effect of pH, temperature and addition of additives on CDA activity was studied by using glycol chitin as a substrate under the standard assay conditions. The dialyzed CDA exhibited maximum activity at pH 6,0 (Fig 2). The optimum temperature of enzyme was found to be 80 ^oC (Fig 3). To examine the thermostability of the enzyme, CDA solution in 50 mM sodium tetraborate buffer (pH 7.0) was allowed to stand during 5 h at various temperatures, and the residual activity was measured. The enzyme was stable up to 80 ^oC with a loss of more than 80% (data not shown). However about 50% of the original CDA activity was lost after incubation at 90 ^oC for 40 minutes and at 100 ^oC after 5 minutes, respectively (Fig. 3). The CDA was activated by EDTA. It was inhibited by Li^+, Mn^+, Zn^+, Co^+ and Ni^+ tested as chlorides at 1 mM concentration (Fig. 4).

DISCUSSION

Chitosan is usually produced from chitin by thermochemical deacetylation. This process results in a polymer product having a broad distribution of molecular mass and a heterogeneous extent of deacetylation. However, for many potentially important applications, uniform material with specific physical and chemical properties is required (Martinou *et al*. 1993). An alternative procedure that would exploit the enzymatic deacetylation of chitin was using chitin deacetylase. The method did not cause the degradation of sugar chain and produces no alkaline wastes (Tokuyasu *et al*. 1996). Chitin deacetylases (EC 3.5.1.41) which represent a class of hydrolytic enzymes were mainly found in fungi. It was first demonstrated in extracts of the fungus *Mucor rouxii* (Davis and Garcia 1984). Chitin deacetylase activity also had been found in other *Zygomycetes* (Trudel and Asselin 1990; Alfonso *et al*. 1995), in *Colletotrichum lindemuthianum* (Tsigos *et al*. 2000), in *Uromyces viciae-fabae* (Deising and Siegrist 1995), in *Saccharomyces cerevisiae* (Mishra *et al.* 1997; Christodoulidou *et al*. 1999) and in cucumber leaves infected with *Colletotrichum lagenarium* (Siegrist and Kauss 1990).

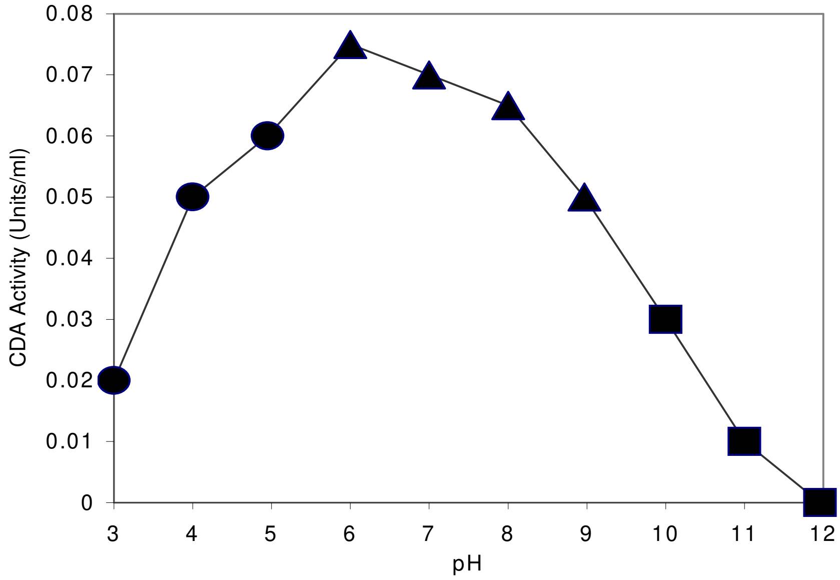

Figure 2. The optimal pH of chitin deacetylase from *B. thermoleovorans* LW-4-11; ●, 0,2 M citrate buffer; ▲, 0,2 M phosphate buffer; and ■, 0,2 M glycine-NaOH buffer

CDA from other microorganisms mainly bacteria was rarely reported. We have isolated and characterized CDA from acidophillic *Bacillus* sp. isolated from Kamojang Crater West Java (Natsir 2000; Rahayu 2000) and *B. stearothermophilus* from Langoan, North Sulawesi (Toharisman *et al*. 2001). In this study, we tried to isolate another thermophile from hot spring water and sediment from North Sulawesi having different characteristics. *Bacillus thermoleovorans* LW-4-11 was able to grow at 70 ^{o}C and had the high CDA activity. It produced CDA extracellularly having optimum temperature of 80 ^oC. It might be one of the highest optimum temperatures for CDA as all optimum temperature of CDAs reported so far was 50 ^oC (Gao *et al*. 1995; Deising and Siegrist 1995; Alfonso *et al*. 1995; Tokuyasu *et al*. 1996; Tsigos and Bouriotis 1995; Christodoulidou *et al*. 1999).

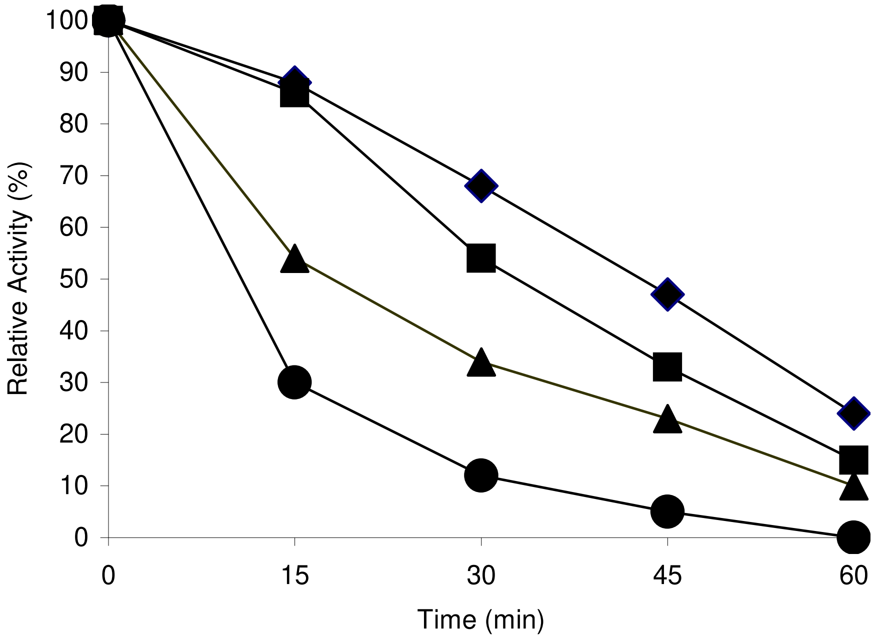

Figure 3. Enzyme stability at; ◆, 70 ^oC; ■, 80 ^oC; ▲, 90 ^oC; and ●, 100 ^oC

The activity of the enzyme was retained by over 50% when it was kept for 45 minutes at 70 ^oC. The half-lifes of the activity (T ½) at 80 and 90 ^oC were about 30 min and 20 min, respectively. When the enzyme was incubated at 70 ^oC for 1 h, heat-labile proteins were denatured. This strategy was applied in partial purification processes described in this paper and could purify enzyme of 4,28 fold with overall yield of 36,7%. Similar procedures were conducted for CDA purification from *M. rouxii* at 50 ^oC for 10 minutes (Martinou *et al*, 1993) and from *Bacillus sp*. at 50 ^oC for 24 h (Rahayu 2000).

Other characteristics of the enzyme were also performed. The optimum pH of the enzyme was 6,0, similar to CDA from *M. rouxii* (Kafetzopoulos *et al*. 1993) Most of the optimum pH of CDAs reported so far was between 4,5 and 8,5 (Tsigos *et al*. 2000). The effect of cations were those similar to those of CDA of *C. lindemuthianum* (Tsigos and Bouriotis 1995; Tokuyasu *et al.* 1996) and *Aspergillus nidulans* (Alfonso *et al*. 1995). The enzyme was activated by EDTA, whereas and inhibited by metal ions such as Zn^{2+}, Mn^{2+} and Cu^{2+} (1 mM).

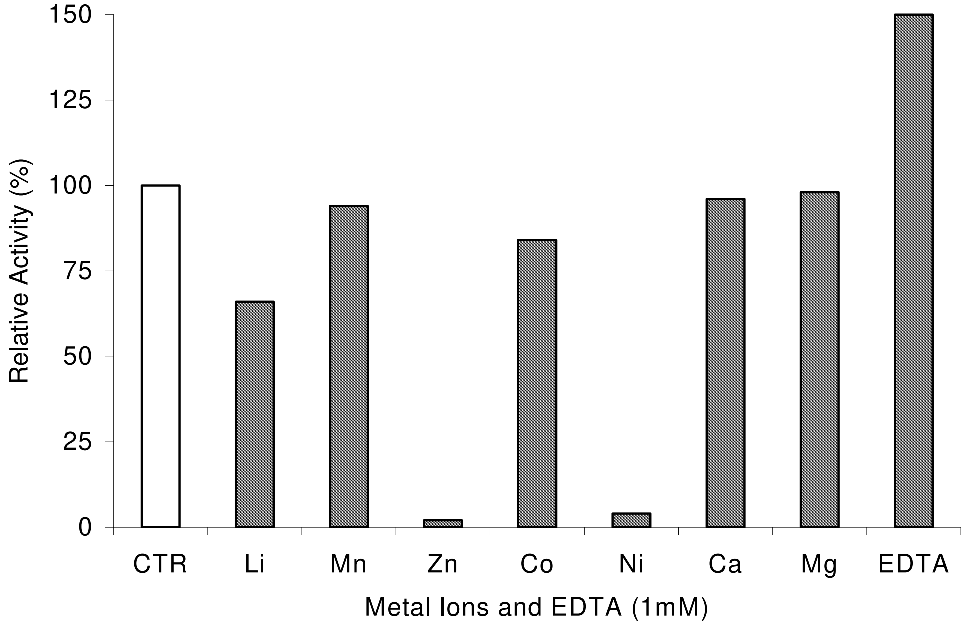

Figure 4. Effect of various cations and EDTA (1 mM) on CDA activity

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