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# Activity of chitin deacetylase from *Colletotrichum gloeosporioides* on chitinous substrates

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ABSTRACT

Production of chitin deacetylases from the phytopathogenic fungus *Colletotrichum gloeosporioides* was successfully achieved by submerged fermentation. The highest specific activity of 0.018 U $mg^{-1}$ of protein was obtained after 96 h of cultivation at pH 6 and 28 °C. Two bands with molecular weights of 35 kDa and 170 kDa determined with SDS-PAGE displayed deacetylase activities as detected in the zymograms. Reacetylated commercial chitosan (52% acetylation degree) was used as substrate for the extracellular crude extract in order to estimate the kinetic parameters of acetate production as undirected deacetylation measurement. The highest acetate production of 12.8 μmol $mL^{-1}$ was obtained using 7.5 mg $mL^{-1}$ of substrate. The produced enzyme from *C. gloeosporioides* achieved up to 25% deacetylation of a chitin substrate (hydrolyzed biological chitin) having 80% degree of acetylation, $M_W$ of $102 \times 10^3$ g $mol^{-1}$ and a crystallinity index of ca. 60%.



## 1. Introduction

Chitin is a β-(1 → 4)-linked polysaccharide composed mostly of *N*-acetyl-D-glucosamine (GlcNAc) repeat units, commonly found in the exoskeleton or cuticles of many invertebrates and in the cell wall of most fungi (Rudrapatnan & Tharanathan Farooqahmed, 2003). Chitosan occurs in the cell wall of certain fungi, such as *Mucor rouxii* (Muzzarelli et al., 2012). The parameter used to distinguish chitin from chitosan is the nitrogen content, which is reflected by the degree of acetylation (DA), so that the polysaccharide is conventionally named chitosan when DA is <0.30 and soluble chitosan salts can be obtained (Muzzarelli, 2011). In order to produce chitosan, high alkali concentrations at high temperatures are used that might cause depolymerization and increased amorphism (Shahidi & Abuzaytoun, 2005). Alternatively, the use of chitin deacetylase (CDA) enzymes is prompted as a useful tool towards biotechnological chitosan production, thus preserving polymer characteristics throughout environmentally friendly processes (Aye, Karuppuswamy, Ahamed, & Stevens, 2006; Beaney, Gan, Magee, Healy, & Lizardi-Mendoza, 2007; Tsigos, Martinou, Kafetzopoulos, & Bouriotis, 2000; Win & Stevens, 2001;). CDAs are glycoproteins with optimum temperature for enzyme activity of 50 °C while optimum pH varies from 4.5 to 8.5 (Aye et al., 2006; Young-Ju, Zhao, Oh, Nguyen, & Park, 2008). Extracellular constitutive CDAs production has been reported from several fungi: *M. rouxii* (Araki & Ito, 1975), *Colletotrichum lindemuthianum* (Kauss & Bauch, 1988; Tsigos & Bouriotis, 1995; Tokuyasu, Ohnishi-Kameyama, & Hayashi, 1996), *Absidia coerulea* (Gao, Katsumoto, & Onodera, 1995), *Aspergillus nidulans* (Alfonso, Nuero, Santamaría, & Reyes, 1995), *Metarhizium anisopliae* (Nahar, Ghormade, & Deshpande, 2004), *Rhizopus nigricans* (Jeraj, Kunic, Lenasi, & Breskvar, 2006), as well as in marine bacteria and insects (Zhao., Park, & Muzzarelli, 2010). The activity of CDAs on chitin oligomers is reported by deacetylation of one unit of sequence of four GlcNAc units (Zhao et al., 2010). However, enzymatic deacetylation in chitin substrates is still challenging mainly due to the insolubility, high molecular weights and crystalline nature of this biopolymer. Low deacetylation degree has been achieved with CDA of *C. lindemuthianum* on several chitinous substrates i.e. shrimp crystalline chitin (0.54%), shrimp chitosan 60% DA (4.8%), crab crystalline chitin (0.5%), crab chitosan with 60% DA (4.0%) (Tsigos & Bouriotis, 1995). Cai and co-workers (2006) reported similar results with CDA from the fungi *Scopulariopsis brevicaulis*, which was evaluated on shrimp crystalline chitin with 3.7% deacetylation despite of high activity of their purified enzyme (12 U $mL^{-1}$), whereas up to 33% and 37% deacetylation were determined but with the amorphous substrates chitosan and amorphous chitin (46% DA) from *Aspergillus niger*, respectively. Several physical and chemical modifications have been proposed to increase the enzymatic deacetylation in chitins, such as heating, sonicating,

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grinding, derivatization, and partial deacetylation (60% DA), as well, the use of chitin of small particle size pretreated with formic acid and the use of low molecular weight chitin, among others (Beaney et al., 2007; Tsigos & Bouriotis, 1995; Win & Stevens, 2001). Then, current research aims at controlled enzymatic deacetylation of less as possible modified chitins in order to preserve some of their properties. In this regard, this work reports the successful production of CDAs from submerged fermentation of the plant pathogenic fungus *Colletotrichum gloeosporioides* and application of the concentrated crude enzyme in deacetylation of chitinous substrates.

## 2. Materials and methods

### 2.1. Materials

Ethylene glycol chitosan was purchased from Sigma (USA) and reacetylated as described by Kauss and Bauch (1988). Commercial chitosan (Kitomer, Marinard Biotech Inc., Canada) with initial degree of acetylation (DA) of 14% and $M_W$ of $450 \times 10^3$ g mol$^{-1}$ was further reacetylated to 52% DA by the method reported by Sorlier, Denuziére, Viton, and Domard (2001). α-Chitin was obtained by biological (I) and chemical (II) methods as reported by Pacheco et al. (2011). β-Chitin was purified chemically as described by Rocha-Pino, Shirai, Arias, and Vazquez-Torres (2008).

### 2.2. Microorganism and submerged fermentation condition

*C. gloeosporioides* strain CF-6 was provided by Culture Collection of Centro de Biotecnologia Genomica of Instituto Politecnico Nacional (Mexico). The microorganism was cultivated on potato dextrose agar (PDA) at 28 °C during 7 days. Spore suspension was obtained by agitation with sterile Tween 80 solution (0.01%). Spores were counted using a Neubauer chamber and the inoculum size was $1 \times 10^6$ spores mL$^{-1}$. Spore suspension was inoculated in a 3 L instrumented bioreactor (Applikon B.V. Holland) containing 2 L of a medium consisting of 15 g of glucose, 6.6 g of glutamic acid, 1 g of $K_2HPO_4$, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1.8 mg of $ZnSO_4{\cdot}7H_2O$, 1 mg of $FeSO_4{\cdot}7H_2O$, 0.3 mg of $MnSO_4{\cdot}H_2O$, 0.4 mg of $CuSO_4{\cdot}5H_2O$, 1 mg of thiamine and 1 mg of nicotinic acid in 1 L of deionized water (Tsigos & Bouriotis, 1995) during 6 days at 28 °C and pH 6. Samples were taken every 24 h and centrifugation at 12,700 × *g* and 4 °C during 25 min. Pellets were recovered and washed out in deionized water, filtered (Whatman 40, USA) and subjected to grinding in a mortar and glass beads with liquid nitrogen in order to determine intracellular CDA activity. Dry weight of mycelia was determined by drying pellets overnight at 100 °C. Supernatants obtained from the fungal culture were used as crude enzyme for further experimentation. Crude enzyme was concentrated by salting out with 80% wt v$^{-1}$ of $(NH_4)_2SO_4$, followed by dialysis of the precipitate against buffer Bis-Tris–HCl pH 5.8. The enzymatic assay and protein determinations were carried out in concentrated and non-concentrated samples. Protein content was determined by Bradford (1976) using serum albumin as standard.

### 2.3. Enzyme activity

CDA activities from culture filtrates, crude enzyme and its concentrate were determined spectrophotometrically as reported by Kauss and Bauch (1988) using ethylene glycol chitin as substrate (Araki & Ito, 1975). A reaction mixture consisted of 100 μL of 50 mM sodium tetraborate/HCl buffer, pH 8.5, 100 μg of substrate in 100 μL of water and 50 μL of enzyme was incubated at 37 °C. The reaction was terminated by the addition of 250 μL of 5% wt v$^{-1}$ of $KHSO_4$. For color formation 250 μL of 5% wt v$^{-1}$ $NaNO_2$ was added. After 15 min, 250 μL of 12.5% wt v$^{-1}$ ammonium sulfamate was added and allowed to stand for another 5 min. Two hundred and fifty microliters of 0.5% wt v$^{-1}$ 3-methyl-2-benzothiazoline hydrazone was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled in tap water and 250 μL of 0.5% wt v$^{-1}$ $FeCl_3$ was added. The developing color was read after 30 min at 650 nm. One unit of activity was defined as the amount of enzyme necessary to release 1 μmol of acetate from glycol chitin per minute. The acetate production as a product of CDA activity was determined by measuring the acetate production from reacetylated chitosan. A constant concentration of crude enzyme with activity of 0.15 U mL$^{-1}$ and 12 h reaction time were used to evaluate the kinetics on several substrate concentrations in 50 mMol of citrate phosphate buffer at pH 5.5 at 45 °C. Acetate concentration was determined by gas chromatography equipped with a FID (Hewlett Packard 5890 series II, USA) and an AT-1000 Alltech (USA) capillary column (0.53 mm × 10 m) (Win & Stevens, 2001). One unit of activity was defined as the amount of enzyme necessary to release 1 μmol of acetate from substrate per minute. Acetate production rates were estimated by the Gompertz model using the non-linear regression program (STATISTICA Stat Soft Inc.), according to Eq. (1).

$$y(t) = a \exp[-b \exp(-kt)] \quad (1)$$

where: $y(t)$ is the acetate produced at time ($t$); $a$ is the maximum product concentration (μmol g$^{-1}$) at $t \to \infty$; $b$ is a constant related to the initial conditions when $t = 0$, then $y(t) = y_0 = a\exp(-b)$; $k$ is the acetate production rate constant (h$^{-1}$). The maximum rate of acetate production ($V_{max}$) was calculated from parameter of the model as $V_{max} = 0.368ak$.

### 2.4. Detection of chitin deacetylases activity by SDS-PAGE

Crude enzyme extracts were subjected to SDS-PAGE (mini Protean II Bio-Rad, USA) under semi native conditions. Samples were prepared with $\beta$-mercaptoethanol without boiling; gels were prepared with addition of glycol chitin (0.1% wt v$^{-1}$) (Trudel & Asselin, 1990). Renaturation of the enzymes after electrophoresis, proceeded by treatment with 0.1 M phosphate buffer (pH 6) and Triton X100 (1% v v$^{-1}$) for 24 h at 37 °C. Further, gels were immersed for 5 min into a freshly prepared solution of Calcofluor white M2R (Sigma–Aldrich, USA) 0.01% (wt v$^{-1}$) in 0.5 M Tris–HCl buffer (pH 9). Gels were then rinsed with deionized water for 1 h. Chitin deacetylase activity was revealed with an UV transilluminator (Gel Doc Bio-Rad, USA) (Trudel & Asselin, 1990). The bands were analyzed with the ImageJ software using a known molecular weight standard proteins (Bio-Rad, USA) for determination of the molecular weight of the enzymes. SDS-PAGE was also conducted following the method of Laemmli (1970). Gels were stained with coomassie brilliant blue R-250 (Bio-Rad, USA) to obtain the protein profile of the extracellular produced enzymes. Broad range of molecular weight ($M_W$) protein standard (Bio-Rad, USA) was used as a reference.

### 2.5. Chitin modifications and characterization

Chemically deacetylated chitins (II) were obtained by the FPT method reported by Lamarque, Viton, and Domard (2005). Microwave deacetylated I (MiWe-I) were obtained by the method reported by Sahu, Goswami, and Bora (2009). Polysaccharides I sponge (I-Spg) and precipitated I (Precipitated I-Spg) were produced by the method described by Flores, Barrera-Rodriguez, Shirai, and Durán-Bazúa (2007). Polysaccharide I was partially deacetylated (D-I) and hydrolyzed (H-I) as described by Ramirez-Coutiño, Marin-Cevantes, Huerta, Revah, and Shirai (2006). Products were further characterized to determine solubility (soluble matter in acetic acid), $M_W$, DA, crystallinity index ($I_{CR}$) and their evaluation as substrates of CDAs. Samples (100 mg) were dissolved in acetic