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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 102×10^3 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 450×10^3 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 1×10^6 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 β-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

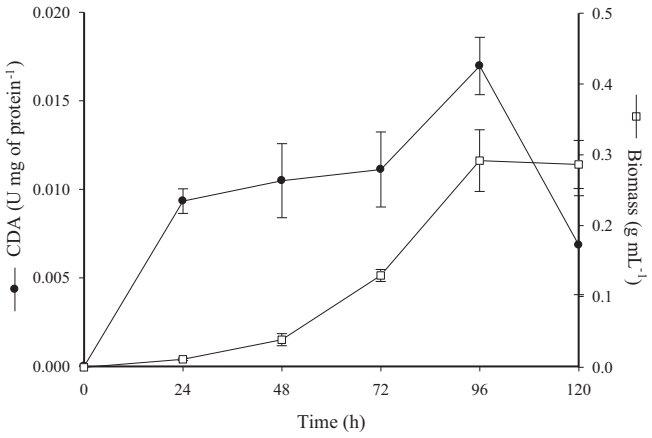

Fig. 1. Time course of chitin deacetylases activity (●) and biomass production (□) of *C. gloeosporioides*, during 120 h of submerged culture at 28 °C and pH 6.

acid (0.1 M) for 24 h at 25° C. The solutions obtained were filtered through filter paper (Whatman 40, USA) and dried overnight at 100 °C. Solubility was determined by gravimetry and reported as percentage of soluble matter. Average M_W of chitins was determined using an automatic capillary viscometer (Viscologic TI 1 SEMATech, USA) in *N,N*-dimethylacetamide containing LiCl (5 wt%). Mark–Houwink–Kuhn–Sakurada parameters were $\alpha = 0.69$ and $K = 2.4 \times 10^{-4}$ L g^{-1} (Pacheco et al., 2011). DA determination on structurally modified chitins was carried out by ^{1}H NMR in DCl/D_2O 20% wt wt^{-1} with 3-(trimethylsilyl) propionic acid as internal reference (Pacheco et al., 2011). Spectra were recorded in a Bruker AC 200 spectrometer (USA) at 200 MHz at 25 °C. DA was calculated by ^{1}H NMR according to the procedure described by Hirai, Odani, and Nakajima (1991). X-ray diffraction measurements were carried out with a Bruker D8 (USA) Advance diffractometer with CuKα incident radiation (wavelength λ = 1.5418 Å) in the 2θ range from 4.5 °C to 50 °C with steps of 0.02°. I_{CR} of chitin samples was determined according to the method reported by Focher, Beltrame, Naggi, and Torri (1990) from normalized diffractograms using the intensities of the (1 1 0) peaks at around $2\theta \cong 20°$ (corresponding to the maximum intensity) and at $2\theta \cong 16°$ (corresponding to the amorphous halo contribution) according to Eq. (2).

$$I_{CR} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (2)$$

The enzymatic reactions with these substrates (7.5 mg mL^{-1}) were carried out at an initial CDA activity of 0.15 U mL^{-1} within concentrated crude enzyme and a reaction time of 12 h.

2.6. Statistical analyses

The data were subjected to analyses of variance and multiple comparison of means by Tukey Kramer test in order to determine significant differences of CDA activities among the chitinous substrates ($p < 0.05$) using the statistical program NCSS (PASS and GESS, USA).

3. Results and discussion

3.1. Chitin deacetylases production

The time course of the maximum CDA production by *C. gloeosporioides* in submerged culture was evaluated during 120 h of fermentation. The highest extracellular specific activity was 0.018 (U mg of protein^{-1}) after 96 h of cultivation followed by a stationary phase (Fig. 1), thereupon, the CDA production decrease to 66%.

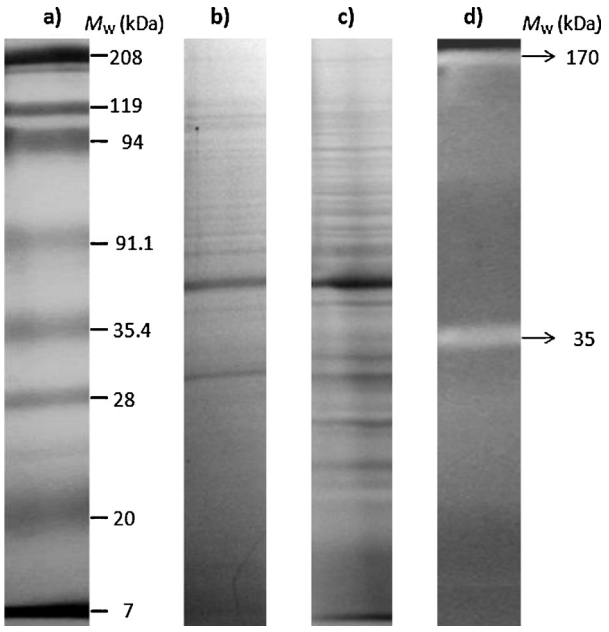

Fig. 2. SDS-PAGE stained with coomassie blue: (a) molecular weight standards; (b) crude enzyme of *C. gloeosporioides*; (c) concentrated crude enzyme and (d) zymogram of concentrated crude enzyme under seminative conditions with ethylene glycol chitin stained with Calcofluor white.

The intracellular CDA activity was determined after cell disruption obtaining ca. 0.001 U mg of protein^{-1}, which was 18-fold less than that excreted to the culture media, and therefore intracellular enzymes were disregarded in this study. Regarding the literature, extracellular CDA secreted to media was related to mycelial growth in *Absidia glauca* and *M. anisopliae* (Tokuyasu et al., 1996; Zhao et al., 2010) and also to the end of fungal growth during autolysis in *C. lindemuthianum* (Tokuyasu et al., 1996). Enzyme produced by *C. lagenarium* has also been detected during plant pathogenesis of cucumber (Siegrist & Kauss, 1990). In comparison, our maximum specific activity from the culture filtrate was higher than that reported by Tsigos and Bouriotis (1995) after 72 h of cultivation of *C. lindemuthianum* (0.002 U mg of protein^{-1}) and similar to that reported by Tokuyasu et al. (1996) (0.019 U mg of protein^{-1}) with a concentrated crude enzyme obtained by precipitation with ammonium sulfate, however, that authors report up to 432 h culture.

The protein profile of the produced extracellular enzymes after 96 h of culture is shown in Fig. 2. M_W of proteins with CDA activity, as detected in zymograms under seminative conditions, showed two bands assigned to 35 kDa and 170 kDa (Fig. 2d), which were in the M_W range of previously reported CDAs (Zhao et al., 2010).

3.2. CDA activity with reacetylated chitosan

The CDAs activity from *C. gloeosporioides* present in the concentrated crude enzyme was evaluated using reacetylated chitosan (DA 52%) with ca. 70% of soluble matter in the acidic medium, and M_W of 460×10^3 g mol^{-1} as substrate (Table 1). Acetate was rapidly produced during the initial 2 h of reaction for all the substrate concentrations, and the steady state was reached after 3 h. The acetate production values obtained during the deacetylation upon the substrate feed were adjusted to the Gompertz model ($R^2 > 0.98$). A highest production of 12.8 μmol mL^{-1} was attained using 7.5 mg mL^{-1} concentration but without significant differences ($p < 0.05$) compared to that at 5 mg mL^{-1} (12.5 μmol mL^{-1}), as shown in Fig. 3. The highest k obtained was 0.027 h^{-1} corresponding to 10 mg mL^{-1} (Fig. 3). The increase of substrate concentration could increase the rate of acetate production owing to the increased

Table 1
Soluble matter, crystallinity index (I_{CR}), molecular weight (M_W) and degree of acetylation (DA) of chitinous samples before and after enzymatic deacetylation.

Samples	Initial				After enzymatic deacetylation	
	Soluble matter in acetic acid 0.1 M (%)	I_{CR}	M_W (1×10^3 g mol^{-1})	DA (%)	M_W (1×10^3 g mol^{-1})	DA (%)
Reacetylated chitosan	69 ± 1.8	0	458 ± 32	52 ± 1	328 ± 56.2	37 ± 2
I[a]	0	86.4	1200 ± 73	95.5 ± 1	1198 ± 92.6	95.3 ± 1
II[b]	0	78.5	851 ± 96	94.5 ± 2	839 ± 82.3	94.41 ± 2
β-chitin	2 ± 0.4	84	831 ± 25	89.2 ± 1	799 ± 22.0	83.7 ± 1
D-I[c]	25 ± 1.1	72.7	783 ± 24	72.8 ± 1	713 ± 23.6	60.8 ± 1
MiWe-I[d]	1.6 ± 0.8	82.7	413 ± 12	91 ± 2	371 ± 5.8	88.38 ± 1
I-Spg[e]	10.4 ± 0.7	79.2	334 ± 26	92.4 ± 1	329 ± 8.3	86.29 ± 2
Precipitated I-Spg[f]	2.6 ± 1.3	74.7	267 ± 7	89.4 ± 2	275 ± 2.3	81.55 ± 1.6
H-I[g]	19.8 ± 1.6	59.4	102 ± 9	79.9 ± 2.9	86 ± 7.4	55.3 ± 3

[a] Biological chitin.
[b] Chemical chitin.
[c] Biological chitin chemically deacetylated.
[d] Microwave biological chitin.
[e] Biological chitin sponge.
[f] Precipitated from biological chitin sponge.
[g] Hydrolyzed biological chitin.

amount of available *N*-acetyl groups. Nevertheless, low acetate production was determined with substrate concentrations above 10 mg mL^{-1}, which might be ascribed to enzyme inhibition by the acetate.

3.3. Characterization of chitinous substrates

The solubility of D-I and H-I were 19.8% and 25%, respectively, while others exhibit solubilities lower than 10% (Table 1). I_{CR} were higher than 80% for I, and β-chitin, whereas that of II was lower (78.5%), which might be attributed to the previous chemical extraction process. Similar value was observed for I-spg. On the other hand, MiWe-I showed only 4% I_{CR} reduction compared to I. H-I presented the lowest I_{CR} value (59.4%) and D-I and precipitated I-Spg had I_{CR} values ca. 75% (Table 1). Regarding the molecular weights, chitin and the less modified chitins as D-I, presented the highest M_W (>750 × 10^3 g mol^{-1}), whereas, MiWe-I displayed 413 × 10^3 g mol^{-1} and H-I and precipitated I-Spg exhibited M_W of 102 × 10^3 g mol^{-1} and 267 × 10^3 g mol^{-1}, respectively (Table 1). All X-ray diffraction patterns for I, II, MiWe-I, I-Spg and precipitated I-Spg displayed the five crystalline reflections assigned to α-chitin (Pacheco et al., 2011), which are indexed as 020, 110, 120, 101 and 130 or given in angle form with strong reflection at 2θ of 9–10°, 2θ of 19–20° and minor reflection at 2θ of 26° (Fig. 4). For the sponge samples, a reflection located at 2θ of 30° was assigned to the calcium carbonate used in the sponge preparation. β-chitin X-ray diffraction profile was similar to the profile reported by Lavall., Assis, and Campana-Filho (2007) with less intense bands at 2θ of 10° and a broad and more diffuse at 2θ of 26.4° than that in I and II (Fig. 4). D-I displayed only 3 reflections, a strong peak at 2θ of 19° and two diffuse bands and 2θ of 9° and 26°. The hydrolyzed chitin showed a significant decrease in the crystalline reflection and only 2θ of 19° was conserved, which is the major peak in the crystalline structure of chitin (Kumirska et al., 2010). The reacetylated chitosan did not show crystal reflections, which indicate an amorphous structure (Fig. 4).

3.4. Evaluation of CDA activity on chitinous substrates

The CDA activity of concentrated crude enzyme was determined at the best reaction conditions obtained previously for reacetylated chitosan substrate, which achieved 15% of deacetylation. Crude enzyme of CDAs (0.15 U ml^{-1}) was able to produce up to 25% of deacetylation in the relatively highly crystalline (I_{CR} = 60%) H-I (80% DA) substrate (Table 1). In addition, the M_W was minimally

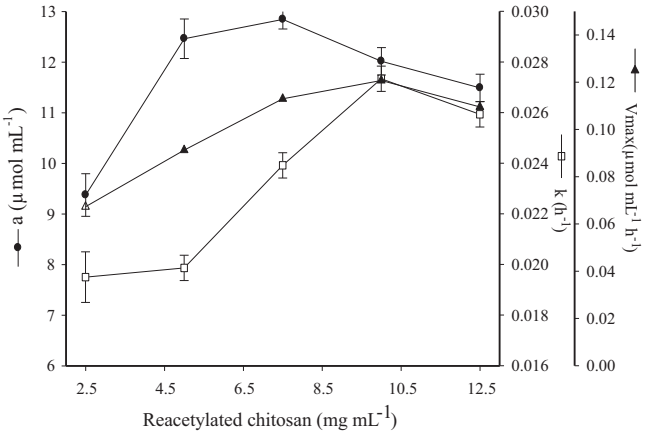

Fig. 3. Kinetic parameters of acetate production from reacetylated chitosan estimated by Gompertz model (Eq. (1)) obtained after 12 h of enzymatic deacetylation with concentrated crude enzyme of *C. gloeosporioides*.

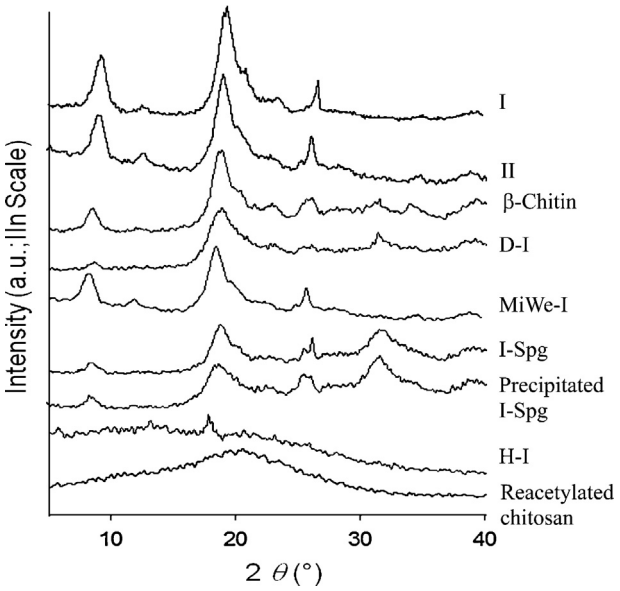

Fig. 4. X-ray diffraction patterns of I, II, β-chitin, D-I, MiWe-I, I-Spg, precipitated I-Spg, H-I and reacetylated chitosan.

reduced from ca. 102 kDa to ca. 86 kDa which accounts for this enzymatic deacetylation route. Contrarily however, low deacetylation was obtained in insoluble chitinous substrates, I and II with only 0.2% and 0.35%, respectively, which presumably underwent only in the amorphous regions (Table 1). This is in agreement with previous reports of Martinou, Kafetzopoulos, and Bouriotis (1995) and Stevens et al. (1997) on CDAs activity from *M. rouxii* and *A. coerulea*, respectively. Noteworthy, MiWe-I had remarkably lower M_W than I and II but similar I_{CR} and solubility values. Then, the enzyme addition to these substrates gave only a slight deacetylation of less than 3% for MiWe-I and significantly none for I and II, neither the M_W values for the latter two presented significant differences ($p < 0.05$). Regarding this, it was reported that the crystallinity (high I_{CR}) in chitins restricted the action of the enzyme (Beaney et al., 2007; Win & Stevens, 2001) and according to that crystallinity is a more significant factor than average biopolymer chain lengths on enzyme-mediated deacetylations. As an exception to this, however, our β-chitin, which presented remarkably high I_{CR} (84%), gave 5.5% decrease of acetylation degree. The latter might be explained for higher enzymatic assimilation than α-chitin due to the weaker intermolecular forces among chains present in the β form. In addition, β-chitin crystalline structure might enable deacetylation at the surface of crystallites, as reported by Lamarque, Viton, and Domard (2004) in their chemical deacetylation studies on β-chitin. On the other hand, an enzymatic deacetylation up to 12% was achieved for D-I, which had higher solubility and lower I_{CR} and M_W values than chitin. This clearly points out to the increased enzyme accessibility to the pendant *N*-acetyl groups in this substrate. In concordance also with this, D-I initial DA (72.8%) might favor deacetylation as it has been reported that partially deacetylated chitin (with DA of 60%), thereby less crystalline biopolymer, was a better substrate for CDA from *C. lindemuthianum* (Tsigos and Bouriotis, 1995). Regarding I-Spg and Precipitated I-Spg substrates, also relatively low deacetylation (6%) was attained that may also be attributed to their relatively high crystalline structure in addition to low solubility. Then, according to the experimental results, it is suggested that crystallinity and solubility have a higher impact in our CDA-mediated reaction than M_W. In this regard, the Pearson's analyses between changes in DA ($\Delta DA = DA_{initial} - DA_{final}$) and solubility was correlated as significantly positive, whereas ΔDA and I_{CR} or M_W were correlated significantly negative ($p < 0.05$) (see supplementary data 1 in the online version of this article for the Pearson's correlation coefficients). The results evidenced that reduction of I_{CR} is a key factor to improve this enzymatic reaction i.e. I_{CR} for H-I (31% lower than that in I), whereas the reacetylated chitosan was amorphous (Table 1), noteworthy, Pearson correlation analyses showed that crystallinity was less significant than solubility and M_W.

4. Conclusion

CDAs from *C. gloeosporioides* were successfully produced. The crude enzymatic extract attained up to 25% deacetylation on a chitin substrate of moderate molecular weight (ca. 100 kDa) and crystallinity from hydrolyzed biologically produced chitin. The present study on the produced CDA from *C. gloeosporioides*-mediated deacetylations of several chitinous substrates, pointed out to the improved solubility in acidic reaction media as the most important factor followed closely by decrease in the crystallinity index and in a lesser extent, the M_W.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.03.051.

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