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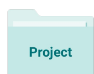
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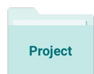
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Short Communication

Ultrasonication and steam-explosion as chitin pretreatments for chitin oligosaccharide production by chitinases of *Lecanicillium lecanii*

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HIGHLIGHTS

- Sonication and steam explosion are proposed for chitin pretreatment and their further use in enzymatic hydrolysis.
- Chitosans were also evaluated as substrates for chitinases.
- Steam explosion reduces crystallinity of chitin and enhanced enzymatic hydrolysis.

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ABSTRACT

In this study, chitin oligosaccharides have been successfully produced using chitinases from submerged fermentation of *Lecanicillium lecanii*. The highest Hex, Chit and Prot production was 0.14, 0.26 and 2.05 U/mg of protein, respectively, which were attained varying pH from 5 to 8 after 96 h. Culture conditions conducted at constant pH of 6 resulted in significantly lower enzyme production. The crude enzyme was partially purified by salting out with $(NH_4)_2SO_4$ followed by size exclusion chromatography to isolate the chitinase mixture for further chitin hydrolysis assays. In this regard, chitin substrates were pretreated with sonication and steam explosion *prior to* enzymatic reaction. Structural changes were observed with steam explosion with 11.28% reduction of the crystallinity index attained with the lowest chitin/water ratio (0.1 g/mL). Pretreated chitins reached the highest production of reducing sugars (0.37 mg/mL) and GlcNAc (0.59 mg/mL) in 23.6% yield.

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1. Introduction

Chitin is a naturally abundant polysaccharide composed of β-1–4 linkages of mainly *N*-acetyl-D-glucosamine (GlcNAc) repeat units, and in lesser extent (<80%) of D-glucosamine units (GlcN). Chitin and chitosan presented several commercial uses and are industrially obtained from exoskeletons of crustaceans (Jang et al., 2004). There is an increasing biomedical interest on chitin oligosaccharides $(GlcNAc)_{2-7}$ owing to the biological activities such as antibacterial, antitumor and immune enhancing effects (Kuroiwa et al., 2009). The chemical methods for chitin hydrolysis involve the use of acids at high concentrations, where the formed oligomers might be deacetylated in a process with poor reaction control. Alternatively, enzymatic methods offer selective hydrolysis in mild and environmentally friendly conditions for chitin oligosaccharide production. The enzymatic hydrolysis of chitin is carried out by a complex chitinolytic system that include endochitinases (EC 3.2.1.1.4), exochitinases (EC 3.2.1.14), chitobiase (EC 3.2.1.30) and β-*N*-acetyl hexosaminidases (EC 3.2.1.52) (Chavan and Deshpande, 2013). *Lecanicillium lecanii* has attracted much interest owing to its application in biological control and chitinases production in submerged and solid substrate cultures (Bing-Lan et al., 2003; Marín-Cervantes et al., 2008; Fenice et al., 2012). Noteworthy, *L. lecanii* has been reported to produce several proteases during cultivation in submerged culture using a medium supplemented with grasshopper cuticle (Bidochka and Khachatourians, 1994). The presence of proteases in the crude enzyme (CE) might reduce the chitinolytic activity in the hydrolysis and therefore, partial purification of chitinases is pursued.

However, in addition to these direct effects on enzyme production, further chitinase-mediated hydrolyses of chitin are generally restricted by the strong dipole–dipole interactions among biopolymer chains that define highly crystalline domains. To circumvent this drawback, we have investigated the effect of sonication and steam explosion (SE) as chemical-free methods. Generally, sonication in chemical processes includes faster reaction rates, higher product yields and reduction on energy consumption. Recently,

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Ajavakom et al. (2012) examined the ultrasonic wave and microwave assisted acidic hydrolysis of chitin toward the monosaccharide GlcNAc production, however, to this date reports on ultrasonication-assisted enzymatic hydrolysis of chitin or chitosan are absent in the literature. Additionally, SE is pointed as an useful method to increase cellulose accessibility for enzyme in several biomass sources regarding critical factors as the opening of crystalline domains and increased pore size volumes (Li et al., 2007), and alike to the low wave radiation methods, the application of SE as pretreatment for enzymatic chitin hydrolysis have not been reported hitherto. This study describes the advantages of the chitin oligosaccharide production from sonicated and SE chitinous substrates by partially purified chitinases of *L. lecanii*.

2. Methods

2.1. Microorganism and culture conditions

L. lecanii ATCC 26854 was cultured on potato dextrose agar at 25 °C for 7 days and maintained at 4 °C *prior to* use. Spore suspension was prepared with sporulated cultures of strains by shaking with sterile Tween 80 solution (0.01% v/v) up to a concentration of 10^7 spores/mL. Culture was conducted in a 3L reactor (Applikon BV, Holland) at 102 rpm, 1 vvm, 25 °C for 96 h, using Czapeck medium with the following composition in g/L: $NaNO_3$ (3.73), Na_2HPO_4 (3.0), $MgSO_4$ (0.5), $FeSO_4$ (0.096), KCl (0.5) pH was set at 5 for 72 h and shifted to 6 for 24 h.

2.2. Determination of enzymatic activities and protein contents

Samples were centrifuged at 12,700*g* and 4 °C for 25 min, the supernatants were considered as the CE and analyzed for *N*-acetylhexosaminidase activity (Hex) using as substrate *p*-Nitrophenyl-β-*N*-acetylglucosamine where one unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute. The determination of endochitinase (Chit) activity was carried out using colloidal chitin and the unit of enzyme activity was defined as the amount of enzyme required to reduce the turbidity of a colloidal chitin suspension in 0.05 absorbance units. Protease activity (Prot) of the crude enzyme was determined using casein substrate 1% (w/v) and the enzyme activity was defined as the amount of enzyme needed to produce an increase in absorbance of 0.001/min at 280 nm. Protein determination was carried out by the method of Bradford (BioRad, USA).

2.3. Purification methods

CE was precipitated by adding ammonium sulfate, leading to 60% of saturation then centrifuged at 18,566*g* at 4 °C for 40 min. The precipitates (concentrated CE) were solubilized in 0.05 M Tris–HCl buffer containing 0.15 M NaCl at pH 7.8 and injected in a Sephacryl™ S-100 High Resolution 26/60 column (GE Healthcare, USA). Elution was carried out using an isocratic flow of 1.3 mL/min of solution 0.05 M Tris–HCl buffer containing 0.15 M NaCl at pH 7.8.

2.4. Electrophoresis and zymograms

SDS–PAGE was carried out using wide range molecular mass markers (Bio-Rad), the electrophoresis gel was stained by silver nitrate (Bio-Rad, USA) and analyzed with the ImageJ 1.41o software (National Institutes of Health, USA). The presence of enzyme activities was observed in the zymograms with the substrates 4-methylumbelliferyl-*N*-*N*-*N*-acetyl-β-D-glucosamine for Chit activity and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamine for Hex activity (Trudel and Asselin, 1989).

2.5. Pretreatment of chitin by FPT deacetylation, sonication and SE

Chitin was obtained and purified from lactic acid fermentation. Heterogeneous deacetylation of chitins was carried out according to the freezing–pumping–thawing (FPT) method (Pacheco et al., 2011). Chitin was pulverized and sieved to a particle size of 177 μm before treatment. A chitin sample was treated by sonication for 20 min in citrate phosphate buffer 50 mM at pH 6 (Barreto-Cardoso et al., 2001). Another sample of chitin was subjected to SE in a 100 mL stainless steel high-pressure reactor equipped with an external ceramic body heating jacket, manometer, two high-pressure valves (Swagelok, USA) and safety rupture disk (Swagelok, USA). Chitin ratios of 0.1, 0.2, 0.4 and 2 g/mL of deionized water were prepared and SE process was carried out at a temperature of 180 °C and 1 MPa (Li et al., 2007) varying residence time of 1, 3, 5 and 8 min.

2.6. Chitinous substrate characterization: degree of acetylation (DA) and crystallinity index (I_{CR})

Chitin was ground (size 106 μm) and dried at 80 °C. ATR-FTIR spectra of the samples were acquired at 24 °C and 30% RH by coupling the ATR accessory (Perkin Elmer, USA) to FTIR equipment (Perkin Elmer 100, UK) The spectra were collected by averaging 60 scans at 4000–550 cm^{-1} spectral resolution. For DA determination, samples of chitin were treated according to the procedure described by Percot et al. (2003) using DCl/D_2O and for the case of chitosan samples were dissolved in HCl/D_2O, both samples were analyzed by proton nuclear magnetic resonance (^1H NMR) spectroscopy in a Bruker (Advance III 500, Germany) at 200 MHz with 3-(trimethylsilyl)propionic acid as internal reference. DA of chitins and chitosans were calculated from integration of assigned signals on the NMR spectra as reported by Pacheco et al. (2011). I_{CR} of chitinous substrates were determined by X-ray diffraction measurements in a diffractometer (Bruker D8 Advance) with an incident radiation CuKα and wave length of $\lambda = 1.5418$ Å in the range of $2\theta = 4.5–50$ °C with steps of 0.02°. I_{CR} of the samples was determined according to the method reported by Focher et al. (1990) using the intensities of the (1 1 0) peaks at around 2θ 20° (corresponding to the maximal intensity) and at 2θ 16° (corresponding mainly to the amorphous halo contribution).

2.7. Chitinase-mediated hydrolysis of chitin

Hydrolysis was conducted in a citrate phosphate buffer 50 mM pH 5, the substrate concentrations for both sonicated and SE treated chitins was 2.5 mg/mL, an enzyme concentration of 0.1 U/mL and stirring speed of 200 rpm at 40 °C (Ramirez-Coutiño et al., 2010). Reaction medium was centrifuged for 10 min at 12,700*g* and 4 °C, collecting the supernatant and filtered through a 0.45 μm membrane.

2.8. Determination of reducing sugars and GlcNAc by HPLC

The amount of reducing sugars was determined in the filtrates from the enzymatic reaction media, which were lyophilized and dissolved in mobile phase of acetonitrile/water (75:25 (v/v) *prior to* injection in an Agilent 1260 Infinity HPLC equipped with a Shodex Asahipak NH_2P-50 4E column (4.6 × 250 mm). Column was eluted at 1.4 mL/min flow and a refractive index (Agilent 1260 Infinity) was used as detector. GlcNAc (Sigma, USA) was used as standard for both techniques. Yield of GLcNAc ($Y_{GLcNAc/chitin}$) was

calculated by the ratio of GLcNAc produced from chitin substrate after enzymatic hydrolysis.

2.9. Determination of degree of polymerization (DP) of chitin oligosaccharides by MALDI-TOF spectrometry

Filtrates were ultrafiltrated through a cut-off membrane of 5 kDa (Millipore, USA), the permeates were freeze-dried and the residue was dissolved in methanol/water 1:1. Then, 0.5 μL was mixed on the target with 2 μL of the matrix (25 g/L of 2,5-dihydroxybenzoic acid in acetonitrile, containing 0.1% (v/v) of trifluoroacetic acid). Samples were dried at room temperature under a stream of air. Mass spectra were recorded using a MALDI-TOF/TOF 4800 Plus Analyzer (Applied Biosystems) equipped with a YAG laser (Y3Al5O12) of 355 nm, analyses were performed using 6500 mv in reflection mode.

3. Results and discussion

3.1. Partial purification of chitinolytic enzymes

The specific activity of CE after salting out varied from 0.14 to 0.45 U/mg of protein for Hex, from 0.26 to 0.67 U/mg for Chit and from 2.05 U/mg to 3.14 U/mg for Prot. Concentrated CE was purified by SEC where fractions from 20 to 27 displayed the highest activities with peaks in fractions 21 and 22 of 0.19 U/mL and 0.72 U/mL (13.84 and 3.75 U/mg of protein) for Hex and Chit, respectively (see Supplementary data 1 for Hex and Chit activities in SEC fractions). Noteworthy, the fractions of SEC with chitinolytic activities did not present Prot. The SDS–PAGE of fractions collected from SEC are shown in Fig. 1a, where only two bands with 38 kDa and 50 kDa remained after the chromatography. The presence of both activities was corroborated with the zymograms for both Chit and Hex (Fig. 1b and c). In agreement with our findings, a previous work by Bing-Lang et al., (2003) show protein bands with chitinolytic activity of 35 and 50 kDa produced in submerged culture of *L. lecanii*, and another work by Rocha-Pino et al. (2011) found bands with exochitinase activity of 32 and 45 kDa.

3.2. Characterization of chitins treated with FPT deacetylation, sonication and SE

The measured I_{CR} and DA for initial native chitin were 88.13% and 98.14%, respectively, which are similar to that reported by Pacheco et al. (2011) in their chitin obtained from lactic acid fermentation (8 I_{CR} 6.4% and DA 94%) (see Supplementary data 2 in the online version of this article for the I_{CR} and DA data of chitin). In the present study, the I_{CR} of chitin samples decreased after the SE for all treatments and remained constant after 2 min of exposure (see Supplementary data 3 for a graphical representation of I_{CR} variations versus exposure time for different treatments). Similar behavior was observed in DA, with maximum decrease after the SE (8 min residence time) treatments of 4.52% (0.2 g/mL) (see Supplementary data 4 in the online version of this article for the DA data of SE chitins). This is consistent with the chitin deacetylation studies using SE conducted by Focher et al. (1990) where the initial order of the chitin molecules was not irreversibly modified and could be recovered during the regeneration or heating steps. The highest decrease in I_{CR} (11.28%) was found at 0.1 g of chitin per mL of water, which presented 3.7% decrease of DA. In agreement with earlier report by Focher et al. (1990), the sample of chitosan presented higher decrease in I_{CR} values than those measured after both SE and sonicated chitins (see Supplementary data 2 in the online version of this article for the I_{CR} and DA data of sonicated chitin), indeed, the highest and the lowest decrease in I_{CR} were 19.73% and 2.63% for chitosan (DA 4.86) and sonicated chitin, respectively (see Supplementary data 2 and 4).

IR spectra for native chitin and those after treatments display the expected two strong absorption bands at 1652 and 1620 cm^{-1} assigned to carbonyl stretching, however, an additional shoulder is observed at 1633 cm^{-1} in the spectra of treated samples (see Supplementary data 5 for examples of FT-IR spectra of native and treated chitins). In this regard, Jang et al. (2004) reported that the amide I band is split in the highly crystalline α-chitin spectrum while generally this region in the less crystalline β-chitin is resolved in a single peak owing to lowest dipole–dipole interactions. The native chitin band at 1650 cm^{-1} in our spectra decrease considerably and the shoulder at 1633 cm^{-1} was not observed after the SE treatments which might be in agreement with a reduction of amino-based hydrogen bonding. It can be observed a broad band in the C–O stretching (1021 cm^{-1}) after the SE treatment (see Supplementary data 5), which might also be ascribed to the reduction of hydrogen bonding. The IR spectrum of sonicated chitins was identical to native chitin (see Supplementary data 5). However, depolymerization was observed in low extension after sonication (Fig. 2) which might be ascribed to the vibrational motion of the molecular structure thus generating cavitation bubbles and production of energy (Barreto-Cardoso et al. 2001). On the contrary, reducing sugars were not detected at $t = 0$ for SE treated chitins, pointing out SE as a less chemically aggressive method than sonication. On the other hand, DA slightly decreased from 98.14% in native chitin to 92.70% and 93.37% after the SE and sonication process, respectively (see Supplementary data 2 and 4). The experimental results evidenced that the structural modifications owing to the SE aimed to facilitate the enzymatic hydrolysis without drastic changes in the overall degree of acetylation and without depolymerization.

3.3. Chitinases-mediated hydrolysis of sonicated, SE chitins and chitosans

The reducing sugars were monitored from 24 to 144 h for all substrates as well as the GlcNAc production by HPLC as the final product of exo and endo-acting enzyme. As observed in Fig. 2, the sonicated chitin sample had its maximum production at 72 h with 0.12 mg/mL of reducing sugars and 0.25 mg/mL of GlcNAc (10.14% $Y_{GLcNAc/chitin}$). The behavior until 72 h could be attributed to the depolymerization produced during sonication which provided substrate for Hex and the plausible explanation for the further decrease might be ascribed to transglycosylation activity, as reported elsewhere (Chavan and Deshpande, 2013). SE treated chitin/water ratio of 0.1 g/mL for 8 min presented its maximum at 144 h with reducing sugars and GlcNAc of 0.37 mg/mL and 0.59 mg/mL, respectively (23.6% $Y_{GLcNAc/Chitin}$). For SE treated chitin sample at 0.4 g/mL for 5 min, the production also reached the highest values at 144 h but relatively low reducing sugars (0.18 mg/mL) and GlcNAc (0.38 mg/mL, 15.1% $Y_{GLcNAc/Chitin}$). Our results are similar to those reported by Fukamizu and Kramer (1985) with 0.44 mg/mL of reducing sugars and maximum production at 7 h. These authors employed a mixture of chitinases purified from insect moulting fluid and reported that this mixture enhanced the production of oligosaccharides compared to Hex. The reducing sugar and GlcNAc production from enzymatic hydrolysis of chitosans was lower than those obtained from sonicated and SE chitins reaching maximum values after 96 and 120 h of reaction. Noteworthy, the hydrolysis by family 18 chitinases presented a substrate-assisted catalysis, thus the enzyme requires an acetylated unit. It has been shown that chitinases form *Serratia marcescens* can degrade chitosans with an acetylated fraction (F_A) as low as 0.13 (Sorbotten et al., 2005). In our study, the accessibility of chitosans as

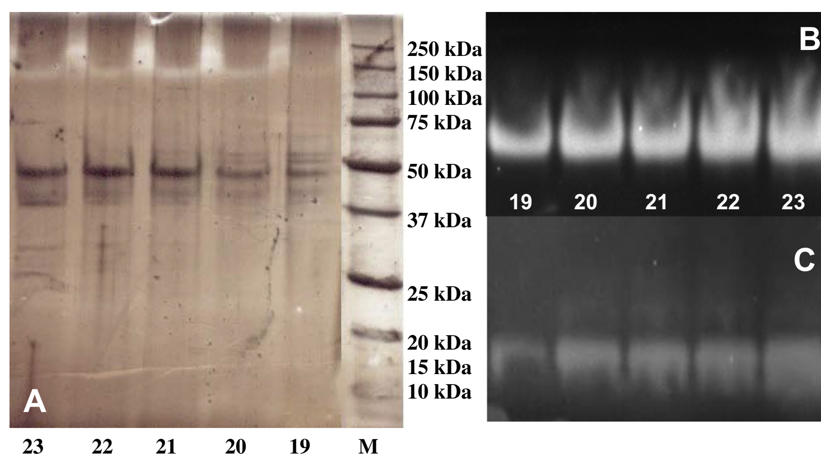

Fig. 1. SDS–PAGE of fractions obtained from SEC. (A) Zymograms of chitinolytic activity (fluorescent zones): Hex (B) and Chit (C).

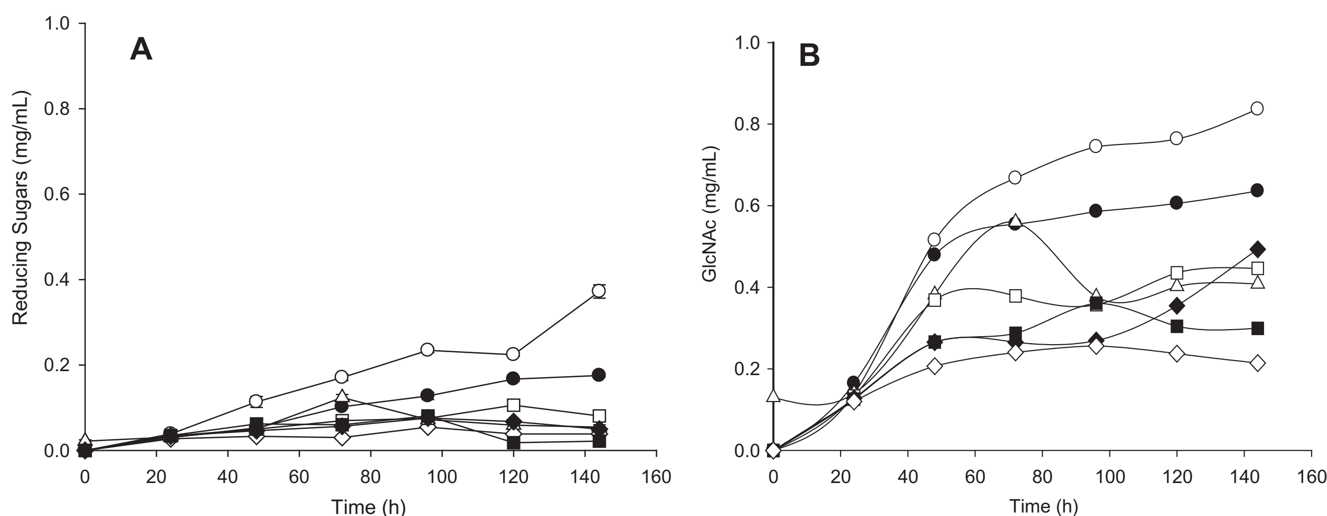

Fig. 2. Time course of enzymatic hydrolysis of chitins and chitosans by *L. lecanii* partially purified chitinases: (A) reducing sugars; (B) GlcNAc determined by HPLC. Substrates: native chitin purified from lactic acid fermentation (empty diamond), sonicated (triangle), SE 0.1 g/mL for 8 min at 180 °C (empty circle), SE 0.4 g/mL for 5 min at 180 °C (filled circle), chitosan DA 52 prepared by FPT (filled diamond), chitosan DA 23 prepared by FPT (empty square) and chitosan DA 4.86 prepared by FPT (filled square).

substrates improved the hydrolysis, reducing sugars and GlcNAc were progressively produced during 72 h of reaction with chitosans (DA of 4.86, 23 and 52) with higher amounts than that for chitin. However, after this time a steep decline in reducing sugars was observed for chitosan with low DA (4.86), this behavior might be due to the low availability of acetylated units (Fig. 2). Nevertheless, the highest amounts of reducing sugars (0.11 mg/mL) and GlcNAc (0.068 mg/mL, 2.72% $Y_{GLcNAc/Chitin}$) were determined with chitosan DA 23 (Fig. 2).

3.4. Determination of DP of chitin-oligomers by MALDI-TOF

The hydrolysis of SE and sonicated chitins gave oligosaccharides with DP of 2, 3, 4 and 5 and similar F_A in samples. However, the number of homologs (oligomers with same DP but different F_A) and intensities with SE were higher than that with sonicated substrates. The MALDI-TOF spectra of samples from the enzymatic hydrolysis of SE chitins corresponding to the highest concentration (144 h) and that for commercial sample of chito oligosaccharides are available for comparison in the Supplementary data 6 and 7 in the online version of this article. The spectrometric studies pointed out the achievement of higher acetylated chitin oligosaccharides (F_A 0.28) than commercial chitin oligosaccharides (F_A 0), which might be of interest in further biological activities.

4. Conclusions

The enzymatic hydrolysis of our biological chitin was remarkably enhanced by a pretreatment with SE which decreased the biopolymer crystallinity with low DA and the absence of depolymerization. Treatment with lowest ratio of chitin to water and longest residence times presented the highest reduction in DA and I_{CR} thus improving the enzymatic hydrolysis. The mixture of partially purified chitinases of *L. lecanii*, protease-free, probed successful for oligomerization of pretreated chitins to achieve chitin oligosaccharides with DP up to 5.

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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.biortech.2013.08.003.

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