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Enzymatic hydrolysis of chitin in the production of oligosaccharides using *Lecanicillium fungicola* chitinases

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Abstract

Lecanicillium fungicola was selected among 15 strains as chitinase producer and it was used to obtain the crude enzyme (UFL) in submerged fermentation (SF) with added chitin. The UFL displayed at pH 6 and 40 °C the highest endochitinase (Endo) and *N*-acetylhexosaminidase (NHase) activities, 747 and 410 U/mg, respectively. Four bands of proteins with molecular weights of 123.1, 85.5, 33.1 and 23 kDa were detected in UFL by SDS-PAGE. In order to increase solubilities of the substrates, α and β-chitins were treated with alkali; degrees of deacetylation (DD) were determined 55 and 50%, respectively. Thereafter, chitin hydrolysis with UFL was carried out at 40 °C and pH 5, Endo and NHase at these conditions were 619 and 355 U/mg of protein, respectively. The partial deacetylation as well as the use of acidified reaction media improved significantly the enzyme efficiency in terms of yields of chitin oligosaccharides produced and process time. The maximum chitin oligosaccharides concentration (P_{max}) obtained from α- and β-deacetylated chitins were 2.77 and 4.44 mmol/l, respectively; whereas for α-chitin it was determined a very small amount of product (0.17 mmol/l). Despite of these results, the maximum production rate (V_{max} 0.0836 mmol/l h) for α-deacetylated chitin was significantly higher than β-deacetylated chitin (0.0363 mmol/l h).

Keywords: Chitin oligosaccharides; *N*-Acetylglucosamine; *Lecanicillium fungicola*; Chitinases; Enzymatic hydrolysis; Chitin

1. Introduction

Chitin is a crystalline polysaccharide widely spread in nature with three structures: α-, β- and γ-chitins. α-Chitin is the most abundant isomorphous form, it is tightly compacted due to its crystalline structure where the chains are in antiparallel fashion favoring strong hydrogen bounding. β-Chitin has an arrangement in parallel with weaker intermolecular forces that leads to a less stable molecule than α-chitin. The third polymorphic form is γ-chitin which is a mixture of both α- and β-chitins. α-Chitin is not soluble and does not swell in common solvents, whereas β-chitin can be swollen in water as well as dissolved in formic acid [1].

The solubility of chitin is enhanced by partial deacetylation under mild conditions that do not degrade the polymer, thus increasing the polarity and electrostatic repulsion of the amino groups. Besides, the loss of the crystalline structure is a consequence of the reduction of the hydrogen bounds caused by the elimination of acetyl groups. It has been reported that chitins with a degree of acetylation (DA) of 0.45–0.55 display good solubility in aqueous media [1,2].

On the other hand, there is a growing interest in the derivatives obtained from the chitin hydrolysis, chitoligomers, *N*-acetylglucosamine and glucosamine [3]. These monomers have been obtained by chemical hydrolysis; however, the enzymatic methods have been studied as an alternative to the conventional processes [4–6], where the enzymes involved are divided in endochitinases and exochitinases [3]. However, it has been reported that chitin and chitosan can be hydrolyzed with other enzymes, such as cellulases, pectinases and lysozymes [7–9], where the degree of deacetylation (DD) of chitin is an important factor on the activity of these enzymes, for instance chitins with >60% DD were reported as better substrate for chitosanases of *Aspergillus* [10].

Generally β-Chitin is preferred due to its higher solubility and swelling compared with α-chitin. Nevertheless, the interest

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in using α-chitin relays in its abundance, i.e. crustacean wastes, and its relatively low cost.

In despite of the fact that fungal chitinases are produced constitutively during apical growth and also as extracelullar enzymes induced by addition of chitin [11,12], there is not enough information on its application for chitoligomers production. The aim of this work was to determine the hydrolytical activity of the crude enzyme from *Lecanicillium fungicola* on crystalline and deacetylated α-chitins and its comparison with β-deacetylated chitin.

2. Materials and methods

2.1. Materials

α-Chitin was obtained from shrimp wastes by acid lactic fermentation and purified until a concentration of 2 and 1% (dry weight basis) of residual proteins and minerals, respectively [13].

N-Acetylglucosamine was purchased from Sigma (St. Louis, MO, USA) and used without further purification. All other chemicals used were analytical grade and they were used as supplied.

2.2. Substrate modification

α-Chitins with a particle size of 2 mm were modified by homogeneous deacetylation using 65% (w/v) of alkali solution. DD, solubility and molecular weights (M_W) were determined at several times of reaction (the samples were taken every 24 h during 6 days). β-Chitin was purified from squid and deacetylated with 20% alkali at 100 °C with DD of 50% following the methodology of Kurita et al. [14].

2.3. Analysis of chitins

The DD of the substrates, crystalline and partially deacetylated α- and β-chitins, were determined by elemental analysis (CHN) (Perkin-Elmer Series II, Connecticut, USA), infrared spectra (IR) were obtained with KBr tablets (Perkin-Elmer Spectrum GX FT-IR System). The solubility was determined by dissolving 0.5% (w/v) of chitins in acetic acid (0.1 M) solutions at room temperature, followed by filtering off, rinsing and drying any undissolved material that was weighed directly to determine percentage of chitin dissolved. M_W were obtained by intrinsic viscosity with 2% acetic acid/sodium acetate 0.2 M at 25 °C and using the values of $K = 13.8 \times 10^{-5}$ and $a = 0.85$ for the specific viscosity constants of the solvent following the equation of Mark–Houwink–Sakurada [15].

2.4. Microorganisms and cultivation conditions

The microorganisms used were from the collections of entomopathogenic fungal cultures ARSEF (USDA-ARSEF) and American type culture collection (ATCC): *Lecanicillium lecanii* (USDA-ARSEF 974; 991; 1029; 2009; 2149; 2460; 2832; 2858; 2916; 3909; 5129; 5153 and ATCC 26854), *Lecanicillium chlamydosporium* (USDA-ARSEF 2218) and *L. fungicola* (USDA-ARSEF 4519). The fungal strains were maintained on potato dextrose agar slants at 4 °C until needed. The spore suspension was obtained by agitation with a solution of 0.1% (v/v) of Tween 80 at concentration of 10^7 spores/ml.

2.5. Screening of chitinolytic strains of Lecanicillium in submerged fermentations using chitin

The screening on enzyme producers was carried out in flasks with Czapeck medium supplemented with 10 g/l of chitin or glucose (control) and adjusted to pH 5. Spore suspensions (10^7 spores/ml) from each strain were inoculated into Czapeck media with added chitin or glucose. The inoculated media were incubated on a rotary shaker at 180 rpm at 25 °C. Samples were taken at the 6th day, after centrifugation at 12,700 × *g* and 4 °C during 25 min; the supernatants were used for the enzyme assays and protein determination. The results are shown as yield of *N*-acetylglucosaminidase based on initial dry weight substrate (U/g of initial dry substrate) considering chitin as substrate [12].

2.6. Submerged fermentations (SF)

L. fungicola SF were carried out in a 3-l instrumented bioreactor (Applikon B.V, Holland) using Czapeck medium supplemented with 10 g/l of chitin at pH 5, and submerged fermentations with added glucose (10 g/l) were used as a control.

2.6.1. Crude enzyme (UFL)

The submerged culture was harvested by centrifugation at 4 °C and 12,700 × *g* (Beckman J2-MI, USA). The culture supernatant (1550 ml) was reduced in volume to approximately (150 ml) by ultrafiltration with a molecular weight cut off membrane of 10 kDa (Millipore Pellicon XL equipment, Bedford, Massachusetts). Then the retentate was freeze-dried (Lyph-Lock 6 Labconco 195, Kansas City, Missouri) and it was used for further characterization (SDS-PAGE, optimal pH and temperature profile) and chitin hydrolysis experiments as the crude enzyme.

2.6.2. Electrophoresis analysis of the crude enzyme

The M_W of UFL was determined by electrophoresis using the technique of Laemmli [16]. The protein bands were analyzed by densitometry (Gel-Doc 100, Bio-Rad and the software Image J version 2.1 for Windows), using a known molecular weight standard proteins (Bio-Rad, Richmond): myosin (208 kDa), β-galaçtosidase (119 kDa), bovine serum albumin (94 kDa), egg albumin (51.1 kDa), carbonic anhydrase (35.4 kDa), soybean trypsin inhibitor (28.8 kDa), lysozyme (20 kDa) and aprotinin (7 kDa).

2.6.3. Enzymatic activities

The endochitinase (Endo) and *N*-acetylhexosaminidase (NHase) activities were determined by the techniques described by Tronsmo and Harman [17] using a spectrophotometer (JENWAY 6305 Essex, UK). The NHase activity unit was defined as "the amount of enzyme required to release 1 μmol of *p*-nitrophenol per milliliter of crude enzyme per minute". The unit of Endo activity was defined as "the amount of enzyme required to reduce 5% the turbidity of a 1% (w/v) colloidal chitin solution, under the established conditions of pH, time and temperature". The range of temperatures tested varied from 5 to 90 °C, and pH from 1 to 3 (glycine/HCl buffer), from 4 to 7 (citrate–phosphate buffer) and from 8 to 10 (phosphate buffer). The protein content of crude enzyme was determined by the dye binding procedure [18].

2.7. Enzymatic hydrolysis

The hydrolysis experiments were carried out by fixing the substrate concentration (5 mg/ml), and adding 0.02% NaN_3 as antimicrobial agent and 5 mg/ml of UFL. The pH of the reaction media was set at pH 5 in 50 mM of citrate–phosphate buffer. The quantification of chitin oligosaccharides was carried out by means of spectrometric measurements of reducing sugars (Spectrophotometer JENWAY 6305 Essex, UK), using NAG as standard for the calibration curve [19]. The concentration of chitin oligosaccharides in the liquid was determined after filtration of the reaction medium.

2.7.1. Production data treatment

An integrated Gompertz model was used to analyze kinetic chitin oligosaccharides production data. In this logistic-like model, the product P (oligosaccharides) is a function of time t according to the following equation:

$$P = P_{max}\exp(-b\exp(-kt))$$

where P_{max} is the maximum product concentration (at $t \to \infty$), b a constant related to the initial conditions (when $t = 0$, then $P = P_0 = P_{max}\exp(-b)$) and k is the production rate constant. The constants P_{max}, b and k were evaluated from the production data using a non-linear estimation programme STATISTICA (StatSoft Inc.). The maximum chitin oligosaccharides production rate V_{max} was calculated from the parameters of the Gompertz model as $V_{max} = 0.368kP_{max}$ [13].