

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

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Received 19 August 2005; received in revised form 25 November 2005; accepted 29 November 2005

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).

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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 bonding. 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 bonds 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 extracellular 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 $12,700 \times g$ 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 $12,700 \times g$ (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 Lab-conco 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 -galactosidase (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 μ mol 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 $t \rightarrow \infty$), *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].

2.8. Statistical analysis

A completely randomized design was carried out in quadruplicate for the screening of the chitinolytic fungi. The programme SPSS version 8.0 (SPSS Inc., USA 1997) computed the analysis of variance with yield of chitinases per gram of initial dry substrate as response variable. The means were compared with Tukey multiple range test ($P \leq 0.05$).

3. Results and discussions

3.1. Modifications of the substrate

The IR spectra of α -chitin samples for several deacetylation treatment times with alkali are shown in Fig. 1. It is observed the elimination of the characteristic -NH peaks of chitin (Fig. 1, spectrum A) at 3269 cm^{-1} ; this absorption band is attenuated or eliminated at higher deacetylation degrees. The intensity of the IR peaks at 1663 and 1626 cm^{-1} assigned to the primary amide decrease due to the elimination of the carboxyl group. The peak at 1561 cm^{-1} corresponding to the secondary amide remains in all the samples, with an increase in its concentration after 3 and 6 days (Fig. 1, spectra B and C), as it was considered chitosan after 6 days.

The solubility of deacetylated α -chitin was measured at different days of treatment (Fig. 2). It is observed a progressive increase throughout the deacetylation process, however, there is an observed reduction in solubility at the 5th day of reaction (71% solubility) due to the formation of a gel, although, this increase in solubility was newly gained later on. Sannan et al. [20] reported a similar behavior in heterogeneous processes, which was explained as a consequence of those residual acetamide groups present in the crystalline region of chitin.

M_w measurements were obtained after 4 days of treatment as the samples of previous days were insoluble in the acetic acid/sodium acetate solution (Fig. 2). The determination of

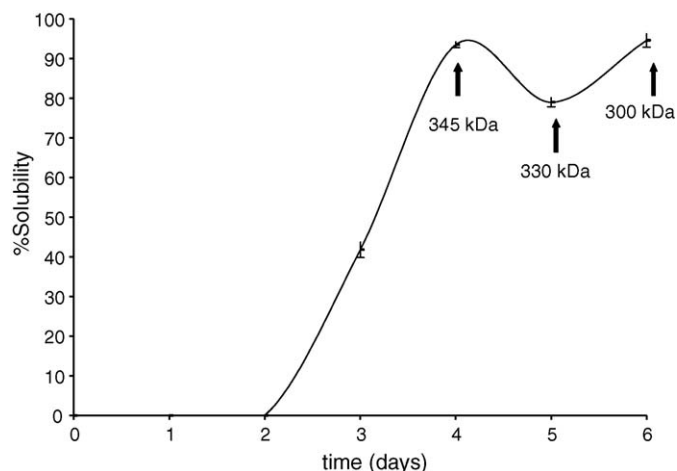


Fig. 2. Solubility percentage in 0.1 M acetic acid and molecular weights of α -chitins deacetylated with 65% (w/v) alkali in homogeneous conditions.

M_w of chitin and chitosan involves constraints such as the limited type of solvents that can be used for chitin and the presence of microgels during chitosan dissolution in acid.

Kurita et al. [21] reported that the chitin with suitable characteristics for enzymatic hydrolysis possesses a DD of 40–60%, solubility higher than 60% and a M_w of 300 kDa. The characteristics of the α -chitins employed in the present study in the enzymatic hydrolysis were 90% of solubility, 55% DD and with a M_w of 343.5 kDa.

3.2. Strain selection and crude enzyme obtaining from submerged fermentation

L. fungicola and *L. lecanii* have been reported as mycoparasitic and entomopathogenic fungi, for instance

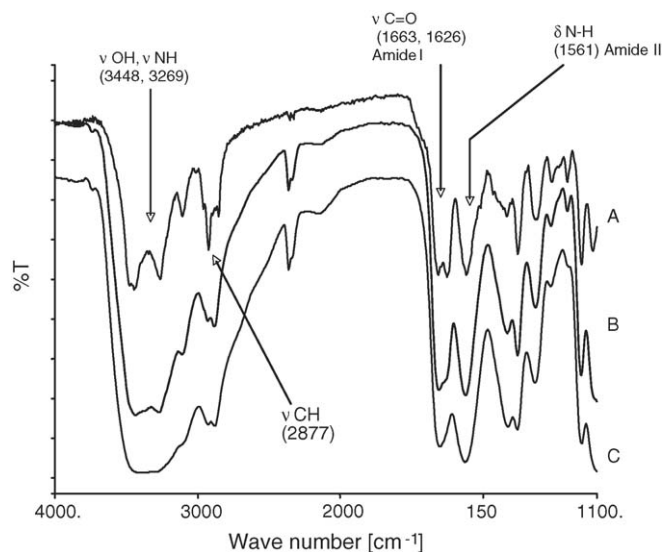


Fig. 1. IR of α -chitins deacetylated with alkali (65%, w/v) at several time of reaction: (A) 1 day, (B) 3 days and (C) 6 days in KBr tablets.

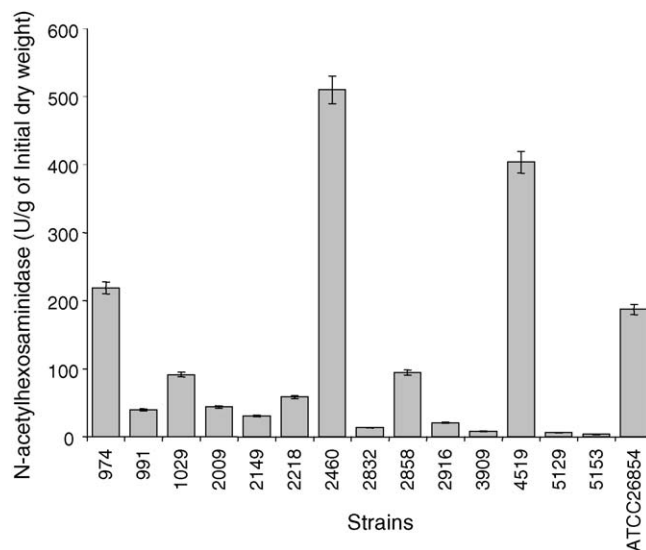


Fig. 3. N-Acetylhexosaminidase activities of 15 strains of *Lecanicillium* determined after 5 days of incubation at 25°C in submerged fermentations with added chitin at pH 5. Each bar is the mean of four replicates.

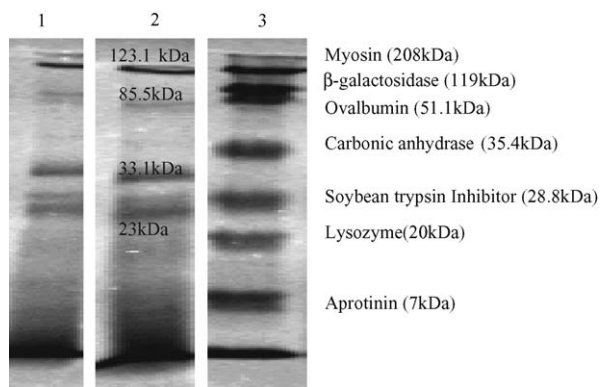


Fig. 4. SDS-PAGE of proteins of ultrafiltrated and freeze-dried enzymatic extract of *L. fungicola* obtained by submerged fermentation. Lanes 1 and 2: enzymatic extract UFL (6 μ g of protein in lane 1 and 8 μ g in lane 2). Lane 3: standard proteins with known molecular weight (Bio-Rad, Richmond): myosin (208 kDa), β -galactosidase (119 kDa), bovine serum albumin (94 kDa), ovalbumin (51.1 kDa), carbonic anhydrase (35.4 kDa), soybean trypsin inhibitor (28.8 kDa), lysozyme (20 kDa) and aprotinin (7 kDa).

L. fungicola is reported as responsible of the dry bubble disease in *Agaricus bisporus*. The attack of the fungus to the host is the result of combining mechanical pressure and secretion of enzymes such as chitinases and proteases [22]. During the experiments carried out for the strain selection *L. fungicola* (USDA 4519), strains of *L. lecanii* USDA 2460, USDA 974 and ATCC 26854 displayed the highest NHase activities ($P \leq 0.05$) in submerged fermentation (Fig. 3).

The SDS-PAGE of the freeze-dried crude enzyme, shown in Fig. 4, presents four protein bands assigned to molecular weights of 123.1, 85.5, 33.1 and 23 kDa. The detected M_w of *L. fungicola* proteins did not coincide with the earlier reported work, for instance, chitobiase and chitosanase of *Verticillium alboatrum* were reported with M_w of 64 and 58 kDa, while enzymes of *V. lecanii* presented up to 45 kDa [23,24].

The highest chitinolytic activity was determined at pH 6, however, partially deacetylated chitins displayed increased solubility at pH 5; therefore, the latter was set in the chitin hydrolysis experiments. The optimal pH and temperature values for Endo and NHase as well as the activities at pH 5.0 of UFL extract are shown in Table 1. The optimum pH determined for UFL differed from those reported in the literature for *V. lecanii* and *V. alboatrum* that are in the acidic range of 3.7 and 4.0, respectively. The temperature value was in agreement with previous reports [23,24].

Table 1
Chitinolytic activities of the enzymatic extracts of *L. fungicola*

pH	T ($^{\circ}$ C)	Specific activities (U/mg of protein)	
		<i>N</i> -Acetylhexosaminidase	Endochitinase
6 ^a	40 ^a	410	747
5.0 ^b	40 ^b	355	619

^a Optimal pH and T .

^b pH and temperature selected for the chitin hydrolysis media.

Table 2

Residual chitinolytic activities of enzymatic extract of *L. fungicola* determined after 168 h of chitin hydrolysis

Substrate	Residual activity (%)	
	<i>N</i> -Acetylhexosaminidase	Endochitinase
α -Chitin	5	73
Deacetylated α -chitin	60	47
Deacetylated β -chitin	20	16

3.3. Chitin hydrolysis using UFL for oligosaccharide production

The chitinolytic activities during hydrolysis were evaluated during the time course of reaction, and the percentages of residual activity were measured considering the enzymatic units obtained at the 7th day respect to the initial ones (Table 2). The NHase activity remained ca. 60% when modified α -chitin was used, while the endochitinase activity was only detected at 47%. These losses in the NHase and Endo activities can be explained by the adsorption on the substrate since chitin is known to be a suitable support for enzyme immobilization [9,25].

The maximum product concentration (P_{\max} 2.8 mmol/l) was up to 16-fold higher for deacetylated α -chitin than for the crystalline one (Table 3), which is above the reported value for commercial pectinase [26].

In the production of chitin oligosaccharides from crystalline α -chitin and deacetylated chitins, the slightly acidified reaction media favored the hydrolysis due to protonation of the amino groups. As a result of the increased on electrostatic repulsion among these amino groups, the β -1,4-glycosidic linkage of chitin was more exposed thereby more susceptible for the recognition of the enzyme.

The chitin oligosaccharides production with deacetylated chitins, shown in Fig. 5, pointed out a higher production with the β form than for the α one. However, it is remarkable that the velocity is significantly higher with the α -chitin compared to β form (Table 3). Indeed, the maximum chitin oligosaccharides production with this deacetylated α form is reached at 48 h and it remains constant after that, whereas deacetylated β -chitin does not display its P_{\max} until 144 h. According to the maximum chitin oligosaccharides production rate (V_{\max}), which was experimentally obtained and it is shown in Table 3, the V_{\max} is higher when α -deacetylated chitin is used, notwithstanding the maximum production is achieved

Table 3

Kinetic constants for the chitin oligosaccharides production by the use of enzymatic extract from *L. fungicola* estimated with Gompertz model

	P_{\max} (mmol/l)	b	k (h ⁻¹)	Correlation coefficient (R)	V_{\max} (mmol/l h)
α -Chitin	0.17	5.47	0.021	0.901	0.0014
Deacetylated α -chitin	2.77	4.49	0.082	0.970	0.0836
Deacetylated β -chitin	4.44	7.41	0.022	0.971	0.0363

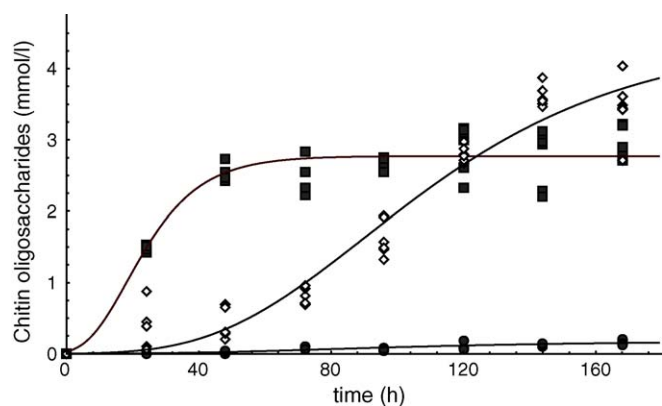


Fig. 5. Production of chitin oligosaccharides employing enzymatic extract of *L. fungicola* with (●) α -chitin crystalline, (■) α -deacetylated chitin and (◇) β -deacetylated chitin as substrates.

with the β form. When both acetylated and deacetylated α -chitin are compared in the chitin oligosaccharides production, the highest V_{\max} of the deacetylated form is explained due to the absence of pending acetyl groups in the biopolymer backbone and the exposition of the amorphous regions of this substrate to the enzymes, thus improving the catalytic activities (Table 3). The crude enzyme might also contain chitosanases that could produce oligosaccharides by endohydrolysis of β -1,4-linkages between D-glucosamine residues in partly acetylated regions of the biopolymer, i.e. recognition of GlcN–GlcNAc links or GlcNAc–GlcN links.

4. Conclusions

The use of partially deacetylated α -chitin in a slightly acidified reaction media as substrate of *L. fungicola* chitinases yielded up to 16-fold chitin oligosaccharides concentration compared with the obtained with crystalline α -chitin; as well, the maximum production rate was significantly higher for α -chitin than for β -chitin. It is concluded that the speed of production of oligosaccharides is favored when the α -form is partially deacetylated, which can be a crucial factor in the designing of this enzymatic process for a particular application.

Acknowledgements

The authors would like to thank to SEP-CONACYT (2004-C01-46173 Government of Mexico) for research funding and granting to Miss Ramírez-Coutiño, to Prof. Humber Curator of USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) for facilitating some of strains used in this work and to Dr. Miquel Gimeno for reviewing the manuscript.

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