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ORIGINAL ARTICLE

Chitin-degrading enzymes from an actinomycete ectosymbiont of *Acromyrmex subterraneus brunneus* (Hymenoptera: Formicidae)

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Abstract Microbes have many mechanisms to exert their inhibitory activity against target pests. One such mechanism involves the production and secretion of hydrolytic enzymes, such as chitinases, which are produced naturally by plants in response to attack by insect herbivores and phytopathogens and have been sought as an additional factor to enhance pest management. Thus, our main aim was to screen the diverse actinomycete community associated with the integument of *Acromyrmex subterraneus brunneus* for a chitinase-producing strain and to characterize its chitinases. We identified isolate ENT-21—a *Streptomyces* sp.—as a chitinase-producer and our data indicate that this isolate produces a chitinolytic complex that contains a chitinase and a high-molecular-weight β-*N*-acetylglucosaminidase (>100 kDa) when cultured in Chitin-Czapek broth. The presence of chitinases in the genome of this isolate was checked by diagnostic PCR, and two chitinase genes belonging to family 18 group A and family 19 were verified. The chitinolytic activity of the crude extract was observed at pH values ranging from 3.8 to 11.0, with the highest chitinase activities recorded at pH 9.0 and 9.5, whereas optimum β-*N*-acetylglucosaminidase activity was observed over a narrow pH range, between pH 4.7 and 5.1. We describe some biochemical and molecular properties of the chitinase and β-*N*-acetylglucosaminidase produced by ENT-21, and discuss the potential for exploitation of these enzymes for pest control.

Keywords *Streptomyces* · Chitinases · β-*N*-acetylglucosaminidase · Biotechnology

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Introduction

Chitin is the second most abundant polysaccharide in nature and is a major constituent of the integument of arthropods and of the cellular wall of many phytopathogenic fungi (Tharanathan and Kittur 2003; Dahiya et al. 2006). Chitin is also a component of the membrane that covers and protects the gut epithelial cells from abrasion and microorganism attack in most insects, which also indicates a functional role in the compartmentalization of the gut lumen (Lehane and Billingsley 1996; Lehane 1997; Terra and Ferreira 2005). Because of the important role of this membrane in the digestive physiology of insects and in the cell wall structure of fungi, chitinases (EC 3.2.1.14)—enzymes that are able to randomly hydrolyze β-1,4 links between *N*-acetylglucosamine (NAG) residues of chitin—have been advocated and exploited as an alternative strategy to target chitin for insect and plant pathogen control (Oppenheim and Chet 1992; Kramer and Muthukrishnan 1998; Herrera-Estrella and Chet 1999; Arakane and Muthukrishnan 2010; Chandrasekaran et al. 2012). Because of the abundance of chitin, chitinases for industrial application in food (Shahidi and Kamil 2001) and drug processing (Dahiya et al. 2006) have also been sought, as well as for the decomposition of chitinous residues (Das et al. 2012), among other applications. Chitinases are grouped according to their amino acid composition in families 18 and 19

of glycosyl hydrolases. Family 18 chitinases are commonly distributed among all organisms, while family 19 chitinases are most commonly reported in plants and microorganisms (Williamson et al. 2000).

β-*N*-acetylglucosaminidases (EC 3.2.1.52) participate in the processing of chitin by hydrolyzing chitooligosaccharides produced by chitinases, releasing NAG units. β-*N*-acetylglucosaminidases also have interesting biotechnological applications, such as control of fungi (Horsch et al. 1997) or synthesis of chitooligossacharides (Usui et al. 1990).

Microorganisms have been considered a useful reservoir for chitinolytic enzymes (Bhattacharya et al. 2007) and as *Streptomyces* stands out in the production of these enzymes (Williamson et al. 2000; Chater et al. 2010), it has been intensively surveyed for the identification of species that can produce chitinases or investigated for the optimization of enzyme production (Nawani and Kapadnis 2005). Ants have been demonstrated to carry a large diversity of actinobacterium associated with their sternum, which has been demonstrated to be a source of molecules active against the parasitic fungus that infects the fungus garden these ants cultivate (Currie et al. 2006). We have recently shown that *Acromyrmex subterraneous bruneus* (Hymenoptera: Formicidae) carry a diversity of culturable actinobacteria associated with its integument (Zucchi et al. 2011a), and have also demonstrated the potential some of these actinobacteria have to produce active molecules against plant pathogens (Zucchi et al. 2010, 2014) and enzymes for biotechnological applications (Zucchi et al. 2011b). As a large number of isolates associated with *A. subterraneus bruneus* were identified as *Streptomyces* spp. (Zucchi et al. 2011a), and this actinobacterium is known to have the potential to secrete chitinases (Williamson et al. 2000; Chater et al. 2010), we aimed to select for and characterize a chitin-degrading actinomycete strain among the ectosymbionts associated with this leaf-cutting ant and to describe its chitinolytic activity.

Materials and methods

Selection of a chitinolytic isolate

A total of 19 actinobacteria strains previously isolated from the integument of the leaf-cutting ant *Acromyrmex subterraneus brunneus* (Zucchi et al. 2011a) were surveyed for their chitinolytic activity. The isolates were inoculated on Chitin-Czapek agar (CCz) to stimulate chitinase production. The CCz agar was composed of (w/v) 1.0 % colloidal chitin, 0.2 % $NaNO_3$, 0.1 % K_2HPO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4{\cdot}7H_2O$, 1.6 % agar and pH was adjusted to 7. The inoculated plates were incubated at 28 °C for 10 days. Chitin hydrolysis was detected by the visualization of a degradation halo surrounding the colonies. Only the most active strain was selected for further studies for molecular typing and characterization of the chitinolytic activity.

Molecular characterization of the selected strain

The selected strain of actinomycete (ENT-21) has been partially characterized by the partial sequencing of the 16S rRNA gene, and phylogenetic analysis has placed ENT-21 in a clade represented by *Streptomyces* (Zucchi et al. 2011a). A better molecular characterization of this strain was sought by extending the sequence of the 16S rRNA gene to an almost complete sequence (1,427 nucleotides), and by multilocus sequence typing (MLST) analysis.

Genomic DNA was extracted from the selected isolate (ENT-21) and PCR amplification and 16S rRNA gene sequencing achieved following Zucchi et al. (2011a, b). The almost complete 16S rRNA gene sequences were aligned manually using MEGA version 5 software (Tamura et al. 2011) against corresponding sequences of the type strains of *Streptomyces* species retrieved from the GenBank database using the EzTaxon-e server (Kim et al. 2012).

Isolate ENT-21 was also submitted to MLST to enhance the characterization of its taxonomic position. Thus, in addition to 16S rRNA gene, four housekeeping genes (*atp*D, *gyr*B, *rec*A and *rpo*B) were analyzed following Guo et al. (2008). Prior to phylogenetic analysis, these genes were aligned individually using MEGA 5 software against corresponding sequences of close relative strains of *Streptomyces* species retrieve from the MLST Database for *Streptomyces* (http://pubmlst.org/streptomyces). A 2,385 nucleotide concatenated sequence (16S rRNA_*atp*D_*gyr*B_*rec*A_*rpo*B) was assembled and used for phylogenetic analysis. The 16S rRNA and MLST genes sequences were deposited in the GenBank database under the accession numbers KF704237–KF704241.

Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein 1981), maximum-parsimony (Fitch 1971) and neighbor-joining (NJ, Saitou and Nei 1987) tree-making algorithms drawn from the MEGA 5 and PHYML (Guindon and Gascuel 2003; Tamura et al. 2011) packages, and an evolutionary distance matrix for the NJ algorithm was generated using the Jukes and Cantor (1969) model. The topologies of the evolutionary trees were evaluated by bootstrap analysis (Felsenstein 1985) of the NJ method based on a 1,000 replicates using MEGA 5.0.5 software. The root position of the 16S rRNA NJ tree was inferred by using a sequence derived from *Streptomyces albus* subsp. *albus* DSM 40313^T (GenBank accession no. AJ621602) as an outgroup.

Growth conditions and enzyme sampling

A pre-inoculum was prepared using two plugs (0.5 cm) of an ENT-21 stock culture in conical flasks containing 50 mL ISP-

2 broth (Shirling and Gottlieb 1966) incubated at 28 °C for 5 days. After this period, 1 mL aliquots were inoculated into four conical flasks containing 50 mL CCz broth, and incubated at 28 °C under constant agitation (180 rpm). Each flask was considered as one replicate. The enzyme produced was sampled every 24 h by taking a 2 mL aliquot from each culture flask. The supernatant containing the chitinase was separated from the cellular fraction by centrifugation (15,000 *g*× 15 min×4 °C), and later assayed for chitinase and β-*N*-acetyl-glucosaminidase activity and protein quantification.

Enzyme assays and protein quantification

Chitinase activity was measured using colloidal chitin-Azure (Sigma) as substrate following Ramírez et al. (2004). The substrate was prepared by incubating 1 g chitin-Azure in 25 mL 85 % phosphoric acid at room temperature (approximately 20 °C) for 24 h under constant agitation (150 rpm). The colloidal chitin-Azure was collected by centrifugation (10,000 *g*, 25 °C, 10 min) and washed with distilled water until pH neutralization. Each enzymatic reaction contained 400 μL 0.3 % colloidal chitin-Azure (w/v) prepared in glycine-NaOH buffer (50 mM, pH 9.0; Dawson et al. 1989) incubated with 200 μL of the crude enzyme extract. The reaction was interrupted by the addition of 200 μL 2 *N* HCl. Samples were then centrifuged (10,000 *g*, 10 min) and the supernatant collected prior to quantification by spectrophotometry at 550 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to increase the value of absorbance of the supernatant by 0.001 unit of the spectrophotometer read per minute.

β-*N*-Acetylglucosaminidase was measured by the detection of *p*-nitrophenol released from *p*-nitrophenyl-*N*-acetyl-β-glucosaminide, following Terra et al. (1979) after modifications. Briefly, the crude enzyme extracts (50 μL) were incubated at 30 °C with 50 μL *p*-nitrophenyl-*N*-acetyl-β-glucosaminide (10 mM) prepared in citrate buffer (50 mM, pH 4.5; Dawson et al. 1989). Reactions were interrupted by adding 500 μL 50 mM NaOH prior to the quantification of the released *p*-nitrophenol by spectrophotometry at 405 nm. A standard curve of *p*-nitrophenol (8–80 nmol) prepared under the same conditions as the enzymatic assay was used as a reference. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the release of 1 nmol *p*-nitrophenol per minute.

The optimum pH of both enzymes was determined in 50 mM citrate-phosphate (pH 2.6–7.0), 50 mM Tris-maleate (pH 7.0–8.6) and 50 mM glycine-NaOH (pH 8.6–10.5) buffer systems (Dawson et al. 1989), using the evaluated substrates under the same conditions as described previously. Blank of enzyme (no substrate added to the reaction) and blank of substrate (no enzyme added to the reaction) were conducted to avoid spontaneous color formation by the enzyme extract or color formation as a result of substrate hydrolysis by the conditions of the assays, respectively.

The sample protein concentrations were determined following Bradford (1976), using the commercial reagent Coomassie Plus™ Protein Assay (Pierce), and bovine serum albumin as the standard, according to the manufacturer's instructions.

Amicon filtration

The crude enzyme extract was filtered using Amicon 100 kDa molecular weight cutoff (MWCO) filter units (Millipore, Bedford, MA), according to the manufacturer's instructions. The obtained fractions, the retentate (molecules larger than the MWCO) and the filtrate (molecules smaller than the MWCO), were analyzed to determine their chitinase and β-*N*-acetylglucosaminidase activities using colloidal chitin-Azure (pH 5.0 and pH 10.0) and *p*-nitrophenyl-*N*-acetyl-β-glucosaminide (pH 5.0 and pH 10.0), respectively, as previously described.

Molecular and bioinformatics studies of the chitinase genes

Degenerate PCR primers targeted to gene fragments from families 18 (groups A and B) and 19 of streptomycete chitinases and from streptomycete chitin-binding genes were used to evaluate the presence of chitinase sequences in the genomic DNA of isolate ENT-21. The primers sets (SC1F/SC2R, C31F/C41R, SCBF/SCBR and F19F2/F19R) and PCR gene amplifications followed Williamson et al. (2000). Amplified fragments were subjected to DNA sequencing for a better characterization of the detected genes. PCR products were purified by ethanol precipitation (Sambrook and Russell 2001) and subjected to bidirectional sequencing using the gene-specific primers of the original amplifications (SC1F and SC2R for family18 group A chitinase gene, and F19F2 and F19R for family 19 chitinase gene) using the ABI DYEnamic ET (GE®) reagent in an ABI3100 DNA Analyser (Applied Biosystems, Foster City, CA), following the manufacturer's guidelines. Chromatograms were visualized with FinchTV v.1.4.0 (Geospiza, http://geospiza.com/), sequence quality evaluated by considering a Phred value threshold ≥20 and both sequenced strands assembled in a consensus sequence using the tools available in MEGA v. 5.0.5 software (Tamura et al. 2011). The deduced amino acid sequence was obtained by translation on Expasy translate tool (http://web.expasy.org/translate/) and searched for homology by BLASTp in NCBI non-redundant protein database (http://blast.ncbi.nlm.nih.gov/). Sequences were aligned on ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A search for

conserved domains was performed using the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html) (Marchler-Bauer et al. 2011). The partial gene sequences of Family 18 and Family 19 chitinases from *Streptomyces* sp. ENT-21 (ENT-21 chi18 and ENT-21 chi19, respectively) were deposited in the GenBank database under the accession numbers (KF710044 and KF710043).

Results

Selection of an isolate with chitinolytic activity

Out of the 19 isolates tested, 7 displayed a potential to hydrolyze chitin. All chitinase-positive isolates were previously putatively identified as *Streptomyces* (Zucchi et al. 2011a), and isolates ENT-21, ENT-25 and ENT-34 demonstrated the

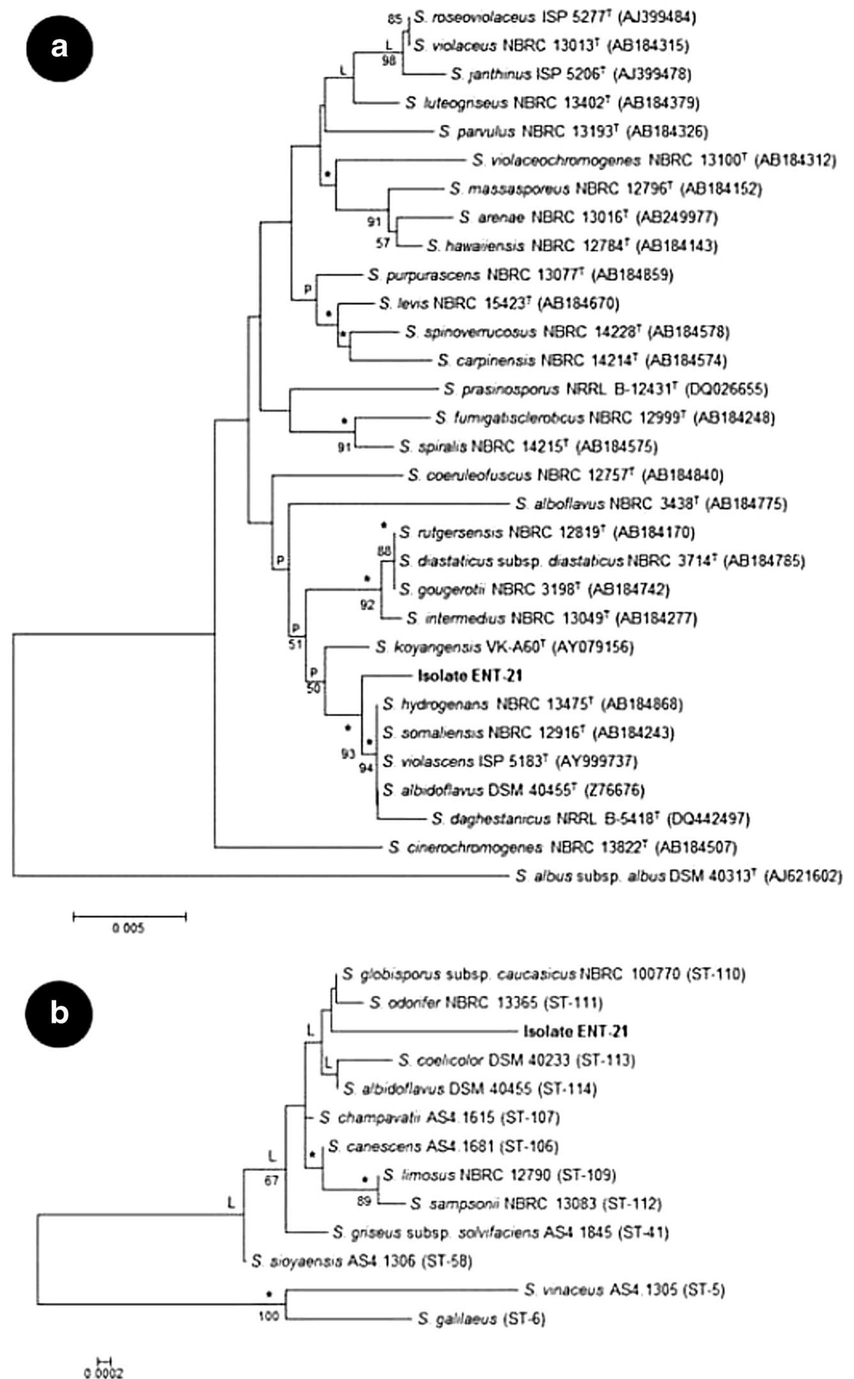

Fig. 1 Neighbour-joining (NJ)-based trees showing relationships between isolate ENT-21 and the type strains of the phylogenetically close *Streptomyces* species. **a** 16S rRNA gene sequences (~1,400 bp). **b** Multi locus analysis based on concatenated 16S rRNA_*atp*D_*gyr*B_*rec*A_*rpo*B genes. *Asterisks* Branches of the tree that were also recovered with the maximum-likelihood (ML) and maximum-parsimony (MP) tree-making algorithms; *L*, *P* branches recovered by the ML or MP tree-making algorithms, respectively. Numbers at the nodes are percentage bootstrap values based on a NJ analysis of 1,000 resampled datasets; only values above 50 % are given. *Bar* 0.0002–0.005 substitutions per nucleotide position

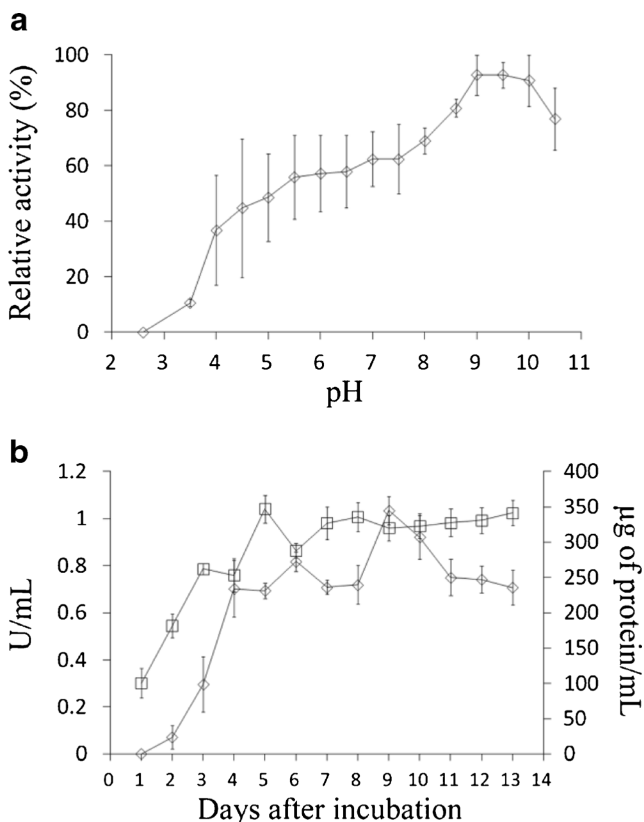

Fig. 2 **a** Optimum chitinase activity secreted by *Streptomyces* sp. ENT-21 cultivated on chitin medium, and **b** chitinolytic activity at pH=9.0 (*diamonds*) and total protein content (*squares*) of the supernatant of *Streptomyces* sp. ENT-21 cultivated on chitin medium. Colloidal chitin-Azure was used to detect the activity of chitinase and total protein content was determined with Coomassie Blue G-based test using BSA as reference. *Bars* Standard error from three replications

strongest response. Isolate ENT-21 was selected for further chitinase characterization.

Isolate characterization and phylogeny

The classification of isolate ENT-21 as a *Streptomyces* was confirmed by its assignment to the periphery of the *Streptomyces albidoflavus* 16S rRNA gene subclade [clade 112; *sensu* Labeda et al. (2012)]—a taxon that was supported by all of the tree-making algorithms and by a 93 % bootstrap value (Fig. 1a). Isolate ENT-21 was related most closely to the type strain of *Streptomyces hydrogenans* NRBC 13475^{T}; the two organisms shared a 16S rRNA gene similarity of 99.6 %, which corresponds to 5 nucleotide (nt) differences at 1,408 sites. The organism also shared a relatively high 16S rRNA gene similarity (99.2 % similarity, 11 nt differences at 1,419–1,421 sites) with the type strains of the *Streptomyces somaliensis*, *Streptomyces albidoflavus* and *Streptomyces violascens*.

Phylogenetic analysis using the MLST alleles resolved the isolate *Streptomyces* sp. ENT-21 in a distinct phyletic line between the *S. albidoflavus* and *S. odorifer* MLST subclades (Fig. 1b). Although this taxonomic position was not supported by a high bootstrap value, it also appeared in the maximum-likelihood (ML) tree-making algorithm analysis.

Partial characterization of the chitinolytic activity in the crude extract of *Streptomyces* sp. ENT-21

The chitinolytic activity of the extract secreted by *Streptomyces* sp. ENT-21 cultivated in CCz broth was observed in a pH range from 3.8 to 11.0. The highest values of chitinase activity were obtained at pH 9.0 and 9.5 (Fig. 2a).

Fig. 3 Multiple alignment of the peptide coded by the family 19 chitinase gene from *Streptomyces* sp. ENT-21 (*1* ENT-21 chi19) with other family 19 chitinases (*2* AFI99893.1; *3* ACJ22968.1; *4* ACJ22969.1; *5* EKC94082.1; *6* ACJ22966.1; *7* YP_003679267.1; *8* YP_004803472.1; *9* ZP_04710133.1; *10* ZP_04710133.1; *11* 1DXJ_A). Highlighted *E* are conserved residues involved in chitin hydrolysis

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 1)ENT-21 chi19      AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 2)S. sampsonii      AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 3)S. sp VC-YC6652   AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 4)S. sp VC-YC6653   AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 5)S. sp SM8        AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 6)S. sp VC-YC6650   AFLANVSHETGG-------------LVHIKEVNEANYPHYCDRNQPYGCPAG 39
 7)N. dassonvillei   AFLANVSHETGG-------------LVHIRETNEANYPHYCDGNQPFGCPAG 39
 8)S. sp SirexAA-E   AFLANVSHETGG-------------LVHIVEQNTANYPHYCDTSQSYGCPAG 39
 9)S. sp W007        AFLANVSHETGG-------------LFYIKEVNEANYPHYCDATQSYGCPAG 39
10)S. roseosporus    AFLANVSHETGG-------------LVYIKEVNEANYPHYCDASQPYGCPAG 39
11)Jack_Bean         AFLAQTSHETTGGAAGSPDGPYAWGYCFVTERDKSN--KYCDPGTP--CPAG 48
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 1)ENT-21 chi19   QAAYYGRGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 2)S. sampsonii   QAAYYGRGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 3)S. sp VC-YC6652 QAAYYGRGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 4)S. sp VC-YC6653 QAAYYGRGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 5)S. sp SM8      QAAYYGRGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 6)S. sp VC-YC6650 QAAYYGRGPIQLSWNFNYRAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 7)N. dassonvillei QAAYYGRGPIQLSWNYNYKAAGDALGIDLLNNPYLVEQNASVAWRTGLWYWNTQ-  93
 8)S. sp SirexAA-E QAAYYGRGPIQLSWNFNYKAAGDALGIDLLGNPWQVEQNASVAWKTGLWYWNTQ-  93
 9)S. sp W007     QAAYYGRGPIQLSWNFNYKAAGDALGINLLANPYLVEQDAAVAWKTGLWYWNTQ-  93
10)S. roseosporus QSAYYGKGPIQLSWNFNYKAAGDALGIDLLNNPYLVEQNAAIAWKTGLWYWNTQ-  93
11)Jack_Bean      KS-YYGRGPIQLTHNYNYAQAGRALGVDLINNPDLVARDAVISFKTAIWFWMTPQ 102
                  :: ***:*****: *:**  ** ***::*: **  * ::* ::::*.:*:* *
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The crude extract of isolate *Streptomyces* sp. ENT-21 demonstrated an increased chitinolytic activity from the 2nd to the 4th day of incubation in CCz broth (Fig. 2b). After this period, one peak of activity was observed at day 9 of incubation. Total protein content of the crude extract reached its maximum at the 5th day of incubation, remaining almost unaltered until the end of the observation period (Fig. 2b).

Molecular detection of chitinase genes and bioinformatics

The molecular screening of chitinases demonstrated two gene groups encoding chitinases in the genome of *Streptomyces* sp. ENT-21, but no genes for chitin-binding proteins or chitinases belonging to family18 group B were detected. The genes encoding chitinases belong to the family 18 group A and family 19.

Alignment of the partial deduced amino acid sequence of family 19 chitinase from *Streptomyces* sp. ENT-21 (ENT-21 Chi19) showed that ENT-21 Chi19 is highly homologous to other sequences of family 19 chitinases from *Streptomyces* and *Nocardiopsis*, showing 100 % identity with the chitinases from *Streptomyces* sp. VC-YC6652 and *Streptomyces* sp. VC-YC6653 (ACJ22968.1; ACJ22969.1) (Fig. 3). The alignment of ENT-21 Chi19 (Fig. 3), as well as the conserved domain search on CDD showed the presence of the two residues of glutamic acid (E) correctly positioned for chitinolytic activity (Hahn et al. 2000).

Partial deduced aminoacid sequence of family 18 class A from *Streptomyces* sp. ENT-21 (ENT-21 Chi18) was also highly homologous to other chitinases found in the NCBI non-redundant protein database. ENT-21 Chi18 has 82 % identity with the family 18 chitinase from *Streptomyces* sp. SM8 (EKC94133.1) (Fig. 4). CDD analysis indicated that the

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1)ENT-21 chi18            YTRRVSRR--VATLDQPLRGNFNQLRKFKAKYPHIKVLWSFGGWTWSGGF  48
2)S. pristinaespiralis    YTADQSVDGKADTWDQPLRGNFNQLRKLKAQYPHIKVLWSFGGWTWSGGF  50
3)S. roseosporus          YTADQSVDGVADTWDQPLRGNFNQLRKLKAKFPHIKVLWSFGGWTWSGGF  50
4)S. sp SM8               YTAAESVDGVADTWDQPLRGNFNQLRKLKAKYPHIKVLWSFGGWTWSGGF  50
5)S. sp S4                YTAAESVDGVADTWDQPLRGNFNQLRKLKAKYPHIKVLWSFGGWTWSGGF  50
6)S. albus                YTAAESVDGVADTWDQPLRGNFNQLRKLKAKYPHIKVLWSFGGWTWSGGF  50
7)S. peucetius            YTADQSVDGVADTWDQPLRGNFNQLRKLKAKYPHIKVLWSFGGWTWSGGF  50
8)S. halstedii            YTADQSVDGVADTWDQPLRGNFNQLRKLKAKYPHIKILWSFGGWTWSGGF  50
9)S.griseoflavus          YTADQSVSGTADTWDQPLRGNFNQLRQLKAKYPHIKVLYSFGGWTYSGGF  50
                          **   *    . * ************::**::****:*:******:****

1)ENT-21 chi18            TDAVKNPAAFAKSCHDLVEDPRWADVFDGIDLDWEYPNACGLSCDSSGPA  98
2)S. pristinaespiralis    GQAVQNPAAFAQSCYDLVEDPRWADVFDGIDLDWEYPNACGLTCDTSGPA 100
3)S. roseosporus          GQAVQNPAAFAQSCYDLVEDPRWADVFDGIDLDWEYPNACGLSCDTSGPA 100
4)S. sp SM8               TDAVKNPAAFAKSCHDLVEDPRWADVFDGIDLDWEYPNACGLSCDSSGPA 100
5)S. sp S4                TDAVKNPAAFAKSCHDLVEDPRWADVFDGIDLDWEYPNACGLSCDSSGPA 100
6)S. albus                TDAVKNPAAFAKSCHDLVEDPRWADVFDGIDLDWEYPNACGLSCDSSGPA 100
7)S. peucetius            GQAVQNPAAFAQSCYDLVEDPRWADVFDGIDLDWEYPNACGLSCDTSGPA 100
8)S. halstedii            GDAAKNPAAFAESCYGLVEDPRWADVFDGIDIDWEYPNACGLTCDTSGPD 100
9)S.griseoflavus          GQAAQNPAAFAKSCKQVVEDPRWADVFDGIDIDWEYPNACGLTCDTSGPA 100
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1)ENT-21 chi18            ALKNMVQAMRAQFGTD-LVTAAITADASSGGKLDAADYAGAAQYFDWYNV 147
2)S. pristinaespiralis    AFKNMMQAMRTKFGANNLVTAAVTADASSGGKIDAADYGGAAQYLDWYNV 150
3)S. roseosporus          AIKTMADAMRAKFGANYLVTAAITADASDGGKIDAADYAGAAKSFDWYNV 150
4)S. sp SM8               ALKNMVQAMRAQFGTD-LVTAAITADASSGGKLDAADYAGAAQYFDWYNV 149
5)S. sp S4                ALKNMVQAMRAQFGTD-LVTAAITADASSGGKLDAADYAGAAQYFDWYNV 149
6)S. albus                ALTNMVQAMRAQFGTD-LVTAAITADASSGGKLDAADYAGAAQYFDWYNV 149
7)S. peucetius            AFKNMMQAMRAKFGTNNLVTAAVTADASSGGKIDAADYGGAAQYIDWYNV 150
8)S. halstedii            ALKGLTSALRAKFGKDYLVTAAITADGSEGGKIDAADYAGAAQSLDWYNV 150
9)S.griseoflavus          AFKNLSQALRAEFGNDYLITAAITADGSAGGKIDAADYGGAAQYLDWYNV 150
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1)ENT-21 chi18            MTYDFFGAWDKTGPTAPHSALNSYSGIPKADFHSAAAIAKLKAKGVPASK 197
2)S. pristinaespiralis    MTYDFFGAWAAKGPTAPHSPLTSYSGIPQAGFNSAEAIAKFKAKGVPAKK 200
3)S. roseosporus          MTYDFFGAWAKQGPTAPHSPLTSYAGIPQAGFNSADAIAKLKAKGVPAKK 200
4)S. sp SM8               MTYDFFGAWDKTGPTAPHSALNSYSGIPKADFHSAAAIAKLKAKGVPASK 199
5)S. sp S4                MTYDFFGAWDKTGPTAPHSALNSYSGIPKADFHSAAAIAKLKAKGVPASK 199
6)S. albus                MTYDFFGAWDKTGPTAPHSALNSYSGIPKAEFHSAAAIAKLKAKGVPASK 199
7)S. peucetius            MTYDFFGAWAKNGPTAPHSPLTSYPGIPQAGFNSAEAIAKFKAKGVPAAK 200
8)S. halstedii            MTYDFFGAWDAKGPTAPHSPLTSYEGIPKAGFSSADAISKLKAKGVPASK 200
9)S.griseoflavus          MTYDYFGAWDKAGPTAPHSPLNSYNGIPKEGFNSAAAISKLRAKGVPASK 200
                          ****:****   *******.*.** ***:  * ** **:*::****** *

1)ENT-21 chi18            LLLGIGFYGRGWTGVTQDAPG 218
2)S. pristinaespiralis    LLLGIGFYGRGWTGVTQSAPG 221
3)S. roseosporus          LLLGIGFYGRGWTGVTQAAPG 221
4)S. sp SM8               LLLGIGFYGRGWTGVTQDAPG 220
5)S. sp S4                LLLGIGFYGRGWTGVTRDAPG 220
6)S. albus                LLLGIGFYGRGWTGVTQDAPG 220
7)S. peucetius            LLLGIGFYGRGWTGVTQSAPG 221
8)S. halstedii            LLLGIGFYGRGWTGVTQDAPG 221
9)S.griseoflavus          LLLGIGFYGRGWTGVSQAAPG 221
                          ***************:: ***
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Fig. 4 Multiple alignment of the peptide coded by the family 18 chitinase gene from *Streptomyces* sp. ENT-21 (ENT-21 chi18) with another family 18 chitinases (*1* ENT-21 chi18-; *2* ZP_06909435.1; *3* ZP_04711422.1; *4* EKC94133.1; *5* ZP_09182055.1; *6* ZP_06593565.1; *7* AAF43629.1; *8* BAF49409.1; *9* ZP_07310707.1). Residues involved in chitin hydrolysis are *highlighted*

residues of the catalytic site of family 18 chitinases (DXDXE) of ENT-21 Chi18 are positioned correctly (Fig. 4).

Partial characterization of β-*N*-acetylglucosaminidase activity in the crude extract of *Streptomyces* sp. ENT-21

β-*N*-Acetylglucosaminidase maximum activity occurred in acid conditions, with an optimum at 4.5–5.0 (Fig. 5a). The activity of β-*N*-acetylglucosaminidase increased at a constant rate until the 10th day, remaining unaltered until the end of the assay (Fig. 5b). The total protein content indicated that the crude extract obtained after the fermentation of CCz broth by *Streptomyces* sp. ENT-21 reached its maximum protein content at the 5th day of incubation, which remained unaltered until the end of the observation period (Fig. 5b).

Amicon filtration analysis

Analysis of the fractions obtained by filtration in 100 kDa MWCO Amicon indicated that the proteins in the retentate (>100 kDa) hydrolyzed *p*-nitrophenyl-*N*-acetyl-β-glucosaminide at pH 5.0, but not colloidal chitin-Azure at either pH 5.0 or 10.0. On the other hand, the filtrate presented chitinolytic activity against colloidal Chitin-Azure at both pHs.

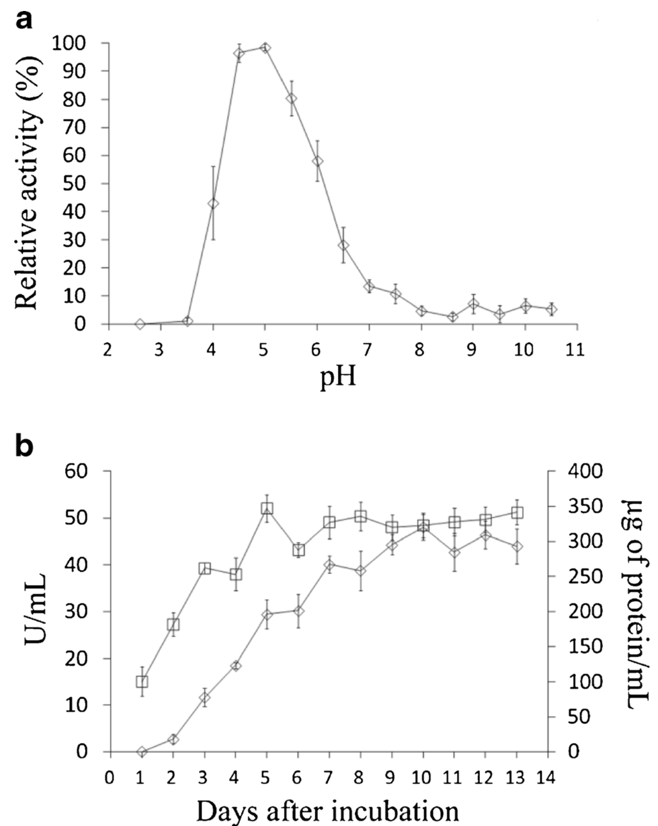

Fig. 5 **a** Optimum pH of the β-*N*-acetylglucosaminidase secreted by *Streptomyces* sp. ENT-21 cultivated on chitin medium, and **b** β-*N*-acetylglucosaminidase activity at pH =4.5 (*diamonds*) and total protein content (*squares*) of the supernatant of *Streptomyces* sp. ENT-21 cultivated on chitin medium. *p*-Nitrophenyl-*N*-acetyl-β-glucosaminide was used to detect the activity of β-*N*-acetylglucosaminidase and total protein content was determined with Coomassie Blue G-based test using BSA as reference. *Bars* Standard error from three replications

Discussion

The results of the present study confirm and extend those reported by Zucchi et al. (2011a) in showing that the isolate ENT-21 has morphological and molecular genetic properties in line with its classification in the genus *Streptomyces*. Although the closest related sequences differed in both analyses, the data indicate that isolate ENT-21 may form a novel taxon. The use of MLST has been proved as a powerful tool in unraveling the taxonomic status of strains that share high 16S rRNA similarities values (Rong et al. 2009; Rong and Huang 2010). Consequently, MLST has been proposed as a potential candidate to substitute DNA:DNA relatedness studies (Rong and Huang 2012)—the most robust method for the circumscription of bacterial species (Rosselló-Mora and Amann 2001). Nevertheless, a polyphasic approach is still needed to confirm the correct taxonomic status of ENT-21.

Utilization of chitin as sole carbon source involves the hydrolysis of this polysaccharide, which is unavailable for absorption and metabolic processing, into chitobiosides or monomeric unities of NAG, which can be assimilated and metabolized by microorganisms (Wang et al. 2002). In the present study, we demonstrated that the isolate *Streptomyces* sp. ENT-21 has the required enzymes to break down chitin into monomers of NAG, as we detected chitinase activity (3.2.1.14), corresponding to primary chitin hydrolysis, and β-*N*-acetylglucosaminidase (3.2.1.52) activity, corresponding to secondary hydrolysis of the products from the primary chitin hydrolysis.

Actinomycetes, particularly *Streptomyces*, have been described as reservoirs of chitinases (Kawase et al. 2004; Chater et al. 2010), and is not uncommon to find reports of such bacteria encoding up to 13 chitinases/chitosanases (Bentley et al. 2002; Wu et al. 2011). Although there are indications from our data that the chitinolytic activity of *Streptomyces* sp. ENT-21 may involve more than one chitinase, it is not possible at present to be sure if the chitinolitic activity detected was due to a single secreted enzyme or to a mixture of chitinases secreted by *Streptomyces* sp. ENT-21. Our data suggests the existence of at least two chitinases. First, because we detected two chitinase gene families in the genome of *Streptomyces* sp. ENT-21, and secondly, because we observed two distinct peaks of activity at different pH values (one peak close to pH 6.0 and another to pH 9.0). This hypothesis is supported by the identification of two distinct chitinases from

Isoptericola jiangsuensis with optimum activity at pH values similar to those we report (Wu et al. 2011). However, purified family 18 (subfamily A) chitinases (Chi18aC and Chi18aD) of *Streptomyces coelicolor* A3(2) were shown to have their best activity at quite different pH values (extremely low and high pH values) (Kawase et al. 2006)—a behavior unlike that observed for the crude extract of *Streptomyces* sp. ENT-21 reported herein.

Nevertheless, the chitinase activity observed and the possibility to have different chitinases produced by *Streptomyces* sp. ENT-21 as indicated by the diversity observed in its genome shows the potential for biotechnological exploitation of this actinomycete and/or its enzymes. Family 19 chitinases, for example, are highly common in plants and are reported to be involved in plant responses against pathogenic fungi. The antifungal activity of these chitinases has already been demonstrated and the potential for their exploitation for plant disease control is extremely high (Dahiya et al. 2006). Partial sequence comparisons of the family 19 chitinase gene of *Streptomyces* sp. ENT-21 demonstrated a gap of 13 amino acids between the glutamic acid (E) residues of the active site when compared to plant chitinases of family 19 glycoside hydrolases of Class I and II (Hahn et al. 2000). The 13 amino acid gap detected refers to the loop II structure of family 19 chitinases, but the absence of this loop is conserved in chitinases from *Streptomyces* and from Class IV plant chitinases (Watanabe et al. 1999). Interestingly, the loop II structure is not related with the enzymes' chitinolytic activity and its deletion may result in an increased hydrolytic activity (Mizuno et al. 2008). Therefore, the family 19 chitinase detected in the genome of *Streptomyces* sp. ENT-21 has the potential to be functional. Moreover, the data obtained by using amicon filters suggest that proteins larger than 100 kDa were related to a β-*N*-acetylglucosaminidase but not to an exochitinase, since no chitinolytic activity was recorded in the fraction containing these large proteins (>100 kDa).

Chitinases have been used effectively to control insect pests, especially lepidopteran and coleopteran species, by targeting the midgut peritrophic membrane—an environment that is alkaline to most lepidopterans (Kramer and Muthukrishnan 1998; Terra and Ferreira 2005; Arakane and Muthukrishnan 2010; Martínez et al. 2012; Nagpure and Gupta 2013) and suitable for chitin hydrolysis by the secreted chitinase from the isolate *Streptomyces* sp. ENT-21. Furthermore, the use of the chitinase(s) secreted by *Streptomyces* sp. ENT-21 could be associated with its β-*N*-acetylglucosaminidase to enhance the effectiveness of peritrophic membrane disruption. This hypothesis is supported by data showing that synthesis and degradation of the insect peritrophic membrane are mediated by a balance between the activity of chitin synthases, chitinases and β-*N*-acetylglucosaminidases (Filho et al. 2002). Thus, the introduction of foreign chitinolytic enzymes could interfere with the delicate balance and interplay between chitin-degrading enzymes and chitin synthases in insects, and have a potential deleterious impact on their growth and development. Similarly, many reports have highlighted the effects of chitinase and β-*N*-acetylglucosaminidase on fungi development, also showing the potential use of these enzymes for fungal control (Marco et al. 2004).

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References

Arakane Y, Muthukrishnan S (2010) Insect chitinase and chitinase-like proteins. Cell Mol Life Sci 67:201–216

Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141–147

Bhattacharya D, Nagpure A, Gupta RK (2007) Bacterial chitinases: Properties and potential. Crit Rev Biotechnol 27:21–28

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254

Chandrasekaran R, Revathi K, Nisha S, Kirubakaran SA, Sathish-Narayanan S, Senthil-Nathan S (2012) Physiological effect of chitinase purified from *Bacillus subtilis* against the tobacco cutworm *Spodoptera litura* Fab. Pestic Biochem Physiol 104:65–71

Chater KF, Biró S, Lee KJ, Palmer T, Schrempf H (2010) The complex extracellular biology of *Streptomyces*. FEMS Microbiol Rev 34: 171–198

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 6:81–83

Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782

Das SN, Neeraja C, Sarma PVSRN, Prakash JM, Purushotham P, Kaur M, Dutta S, Podile AR (2012) Microbial chitinases for chitin waste management. In: Satyanarayana T, Johri BN, Prakash A (eds) Microorganisms in environmental management. Springer, Dordrecht, pp 135–150

Dawson RMC, Elliott DC, Elliott WH, Jones KM (eds) (1989) Data for biochemical research. Oxford University Press, NewYork

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Filho BPD, Lemos FJA, Secundino NFC, Pascoa V, Pereira ST, Pimenta PFP (2002) Presence of chitinase and β-*N*-acetylglucosaminidase in the *Aedes aegypti*: a chitinolytic system involving peritrophic matrix formation and degradation. Insect Biochem Mol Biol 32:1723–1729

Fitch WM (1971) Toward defining the course of evolution: minimum change for specific tree topology. Syst Biol 20:406–416

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704

Guo YP, Zheng W, Rong XY, Huang Y (2008) A multilocus phylogeny of the *Streptomyces* griseus 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. Int J Syst Evol Microbiol 58:149–159

Hahn M, Hennig M, Schlesier B, Höhne W (2000) Structure of jack bean chitinase. Acta Crystallogr D 56:1096–1099

Herrera-Estrella A, Chet I (1999) Chitinases in biological control. Chitin Chitinases 87:171–184

Horsch M, Mayer C, Sennhauser U, Rast DM (1997) β-*N*-acetylhexosaminidase: a target for the design of antifungal agents. Pharmacol Ther 76:187–218

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism, vol 3. Academic, New York, pp 21–123

Kawase T, Saito A, Sato T, Kanai R, Fujii T, Nikaidou N, Miyashita N, Watanabe T (2004) Distribution and phylogenetic analysis of famly 19 chitinases in Actinobacteria. Appl Environ Microbiol 70(2): 1135–1144

Kawase T, Yokokawa S, Saito A, Fujii T, Nikaidou N, Miyashita K, Watanabe T (2006) Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *S. coelicolor* A3(2). Biosci Biotechnol Biochem 70(4):988–998

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Kramer KJ, Muthukrishnan S (1998) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27:887–900

Labeda DP, Goodfellow M, Brown R, Ward AC, Lanoot B, Vanncanneyt M, Swings J, Kim SB, Liu Z, Chun J, Tamura T, Oguchi A, Kikuchi T, Kikuchi H, Nishii T, Tsuji K, Yamaguchi Y, Tase A, Takahashi M, Sakane T, Suzuki KI, Hatano K (2012) Phylogenetic study of the species within the family *Streptomycetaceae*. Antonie Van Leeuwenhoek 101:73–104

Lehane MJ (1997) Peritrophic matrix structure and function. Annu Rev Entomol 42:525–550

Lehane MJ, Billingsley PF (1996) Biology of the insect midgut. Chapman & Hall, London

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39(D):225–229

Marco JL, Valadares-Inglis MC, Felix CR (2004) Purification and characterization of an *N*-acetylglucosaminidase produced by a *Trichoderma harzianum* strain which controls *Crinipellis perniciosa*. Appl Microbiol Biotechnol 64:70–75

Martínez CP, Echeverri C, Florez JC, Gaitan AL, Góngora CE (2012) *In vitro* production of two chitinolytic proteins with an inhibiting effect on the insect coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) and the fungus *Hemileia vastatrix* the most limiting pests of coffee crops. AMB Express 2:22 [http://www.amb-express.com/content/2/1/22]

Mizuno R, Fukamizo T, Sugiyama S, Nishizawa Y, Kezuka Y, Nonaka T, Suzuki K, Watanabe T (2008) Role of the loop structure of the catalytic domain in rice class I chitinase. J Biochem 143:487–495

Nagpure A, Gupta RK (2013) Purification and characterization of an extracellular chitinase from antagonistic *Streptomyces violaceusniger*. J Basic Microbiol 63:871–877

Nawani NN, Kapadnis BP (2005) Optimization of chitinase production using statistics based experimental designs. Process Biochem 40: 651–660

Oppenheim AB, Chet I (1992) Cloned chitinases in fungal plant-pathogen control strategies. Trends Biotechnol 10:392–394

Ramı́rez MG, Avelizapa LIR, Avelizapa NGR, Camarillo RC (2004) Colloidal chitin stained with Remazol Brilliant Blue R®, a useful substrate to select chitinolytic microorganisms and to evaluate chitinases. J Microbiol Methods 56(2):213–219

Rong XY, Huang Y (2010) Taxonomic evaluation of the *Streptomyces griseus* clade using multilocus sequence analysis and DNA–DNA hybridization, with proposal to combine 29 species and three subspecies as 11 genomic species. Int J Syst Evol Microbiol 60:696–703

Rong XY, Huang Y (2012) Taxonomic evaluation of the *Streptomyces hygroscopicus* clade using multilocus sequence analysis and DNA–DNA hybridization, validating the MLSA scheme for systematics of the whole genus. Syst Appl Microbiol 35(1):7–18

Rong XY, Guo YP, Huang Y (2009) Proposal to reclassify the *Streptomyces albidoflavus* clade on the basis of multilocus sequence analysis and DNA-DNA hybridization, and taxonomic elucidation of *Streptomyces griseus* subsp. solvifaciens. Syst Appl Microbiol 32:314–322

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25(1):39–67

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:404–425

Sambrook J, Russell DW (2001) Molecular cloning—a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shahidi F, Kamil YVAJ (2001) Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Technol 12:435–464

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Evol Microbiol 16:313–340

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Terra WR, Ferreira C (2005) Biochemistry of digestion. In: Gilbert LI, Iatrov K, Gill S (eds) Comprehensive Molecular Insect Science 4: 171–224. Elsevier, Oxford

Terra WR, Ferreira C, Bianchi AGD (1979) Distribution of digestive enzymes among the endoperitrophic and ectoperitrophic spaces and midgut cells of *Rhynchosciara* and its physiological significance. J Insect Physiol 25:487–494

Tharanathan RN, Kittur FS (2003) Chitin—the undisputed biomolecule of great potential. Crit Rev Food Sci 43:61–87

Usui T, Matsui H, Isobe K (1990) Enzymic-synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium-sulfate. Carbohydr Res 203:65–77

Wang F, Xiao X, Saito A, Schrempf H (2002) *Streptomyces olivaceoviridis* possesses a phosphotransferase system that mediates specific, phosphoenolpyruvate-dependent uptake of *N*-acetylglucosamine. Mol Genet Genomics 268:344–351

Watanabe T, Kanai R, Kawase T, Tanabe T, Mitsutomi M, Sakuda S, Miyashita K (1999) Family 19 chitinases of *Streptomyces* species: characterization and distribution. Microbiology 145: 3353–3363

Williamson N, Brian P, Wellington EMH (2000) Molecular detection of bacterial and streptomycete chitinases in the environment. Antonie Van Leeuwenhoek 78:315–321

Wu Y, Liu F, Liu Y, Zhang Z, Zhou T, Liu X, Shen Q, Shen B (2011) Identification of chitinases Is-chiA and Is-chiB from *Isoptericola jiangsuensis* CLG and their characterization. Appl Microbiol Biotechnol 89:705–713

Zucchi TD, Almeida LG, Dossi FCA, Cônsoli FL (2010) Secondary metabolites produced by *Propionicimonas* sp. (ENT-18) induce histological abnormalities in the sclerotia of *Sclerotinia sclerotiorum*. BioControl 55:811–819

Zucchi TD, Guidolin AS, Consoli FL (2011a) Isolation and characterization of actinobacteria ectosymbionts from *Acromyrmex subterraneus brunneus* (Hymenoptera, Formicidae). Microbiol Res 166:68–76

Zucchi TD, Rossi GD, Cônsoli FL (2011b) Characterization of a β-amylase from *Propionicimonas* sp. ENT-18 ectosymbiont of *Acromyrmex subterraneus brunneus*. Ann Microbiol 61: 985–990

Zucchi TD, Almeida LG, Moraes LAB, Cônsoli FL (2014) Albocycline, the main bioactive compound from *Propionicimonas* sp. ENT-18 against *Sclerotinia sclerotiorum*. Ind Crops Prod 52C:264–268