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Original Article

Isolation and characterization of chitinase from soil fungi, *Paecilomyces* sp.

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ABSTRACT

Chitinolytic fungal strains were isolated from soil in Thailand. They were screened as chitinase producers by testing their shrimp shell digestion ability on potato dextrose agar plates. The chitinase activity was tested with colloidal chitin in culture medium C and basal medium. There was greater activity in culture medium C than in the basal medium. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis from the culture filtrate of medium C showed three protein bands at about 40 kDa, 46 kDa and 56 kDa. The chitinase gene was sequenced from genomic DNA. The obtained sequence consisted of 713 bp upstream, a 1499 bp open reading frame that was interrupted by three introns and 1698 bp downstream sequences. The intron lengths were 63 bp, 57 bp and 110 bp, respectively. The sequence was found to be the most similar to the chitinase gene of *Paecilomyces lilacinus* (EF183511). Pairwise alignment of the 1499 bp and *P. lilacinus* resulted in 72.5% DNA sequence identity, while alignment of the 1269 bp coding sequence and *P. lilacinus* resulted in 78.5% cDNA sequence identity and 83.5% amino acid sequence identity. The protein structure contained two conserved domains of the putative substrate binding site (S-I-G-G) and catalytic domain (D-G-I-D-L-D-W-E), suggesting that this fungal chitinase belonged to the glycosyl hydrolases family 18 chitinase (GH18). Phylogenetic analysis of the chitinase gene from the nematopathogenic fungi suggested that this chitinase sequence was class V chitinase.

Introduction

Some fungi have considerable potential for the biological control of insect pests of plants (Screen et al., 2001; Baratto et al., 2006; He and Xia, 2009). Microorganisms with inhibitory activity against pathogens have also been considered as potential sources of genes for disease resistance (Deng et al., 2007; Pinnamaneni et al., 2010). Many researchers have reported the biological control of pests using insect pathogenic fungi, such as *Paecilomyces* spp. (Khan et al., 2003; Kiewnick and Sikora, 2006), *Metarhizium anisopliae* (Kanzok and Jacobs-Lorena, 2006), *Hirsutella rhossiliensis, H. sinensis* (Sung et al., 2007), *Beauveria bassiana*, *B. brongniartii* (Thomas and Read, 2007) and *Cordyceps militaris* (Zheng et al., 2011). To penetrate the host cuticle, the fungi will produce and secrete hydrolytic enzymes, such as proteases, chitinases and lipases, which have been proposed to be important for the initiation of the infection process (St. Leger et al., 1986). Chitinases are among the group of proteins that insects use to digest the structural polysaccharide chitin in their exoskeletons and gut linings during the molting process (Fukamizo, 2000). Among the biocontrol agents, the chitinolytic ones have been successfully used to control several pathogenic fungi (Nandakumar et al., 2001; Viswanathan and Samiyappan, 2001). Some autolytic enzymes, including chitinases are bound to subapical walls of *Neurospora crassa* and *Aspergillus nidulans* (Rosenberger, 1979). Many fungi produce chitinase enzyme to degrade substrate as a defense mechanism, as well as to gather nutrients for their survival under adverse environmental conditions (St Leger et al., 1986; da Silva et al., 2005). Fungal chitinase enzymes are translated from chitinase genes, which are induced by the presence of chitin (Deng et al., 2007). Additionally, great varieties of chitinase can be found among fungal species, and each species can produce different chitinase isomers, which have

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different catalytic properties. For example, Baratto et al. (2006) reported that the characterization of the *chi2* chitinase gene from *M. anisopliae* var. *anisopliae* is similar to the glycohydrolase family 18. Analysis of secreted chitinases in *M. anisopliae* revealed at least six isoforms (30 kDa, 33 kDa, 43.5 kDa, 45 kDa, 60 kDa and 110 kDa). Three genes coding for chitinases were described in the *Metarhizium*: *chit1* gene and the ortholog *chi1*, coding a 42 kDa endochitinase (Screen et al., 2001); *chi2* (partial sequence, AJ293217) and *chi3*, coding for an endo/exo-acting 30 kDa chitinase (CAC07217) (da Silva et al., 2005). Among filamentous fungi, *Trichoderma* spp. have been widely studied for chitinase production (Matroudi et al., 2008; Saiprasad et al., 2009). In the current study, the chitinase producing ability of fungi isolated from soil in Thailand was estimated, and the DNA and amino acid sequences of the chitinase from fungi were analyzed and compared with those reported chitinase from mycopathogens, entopathogens and nematopathogens. A sequence similarity search was undertaken using GenBank Blast (Altschul et al., 1997) from the US National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/cgi-bin/blast). Furthermore, the phylogenetic relationship of these fungi was constructed using sequences of 18S ribosomal DNA deposited in GenBank.

Materials and methods

Selection of fungi used in the experiment

Three species of fungi (*Fusarium* sp., *Gongronella* sp. and *Paecilomyces* sp.) were isolated from soil and used in this study. The isolated fungi were grown on potato dextrose agar (PDA) at room temperature. For DNA extraction, the mycelia were cultured in 250 mL Erlenmeyer flasks containing 50 mL potato dextrose broth. The incubation conditions were at 28 °C for 7 d.

Screening of antagonistic fungi

The antagonistic fungi were screened using shrimp shells. A 10 mm disk of a pure culture of the fungi (*Fusarium* sp., *Gongronella* sp. or *Paecilomyces* sp.) was placed at the center of a PDA plate and two pieces of shrimp shell were placed close to the culture disk. The plates were further incubated for 7–10 d at room temperature, and the degradation of shrimp shells was evaluated in triplicate. The fungus most effective in digesting the shrimp shell was selected for future studies.

Preparation of culture filtrates

The effects of different media to induce chitinase production were tested using two culture media—basal medium (Vyas and Deshpande, 1989) and medium C (Deng et al., 2007). Each 500 mL flask containing 250 mL medium was inoculated with a culture disk (5 mm diameter) taken from the growing margins of fungi on a PDA plate. The inoculated flasks were then placed on a rotary shaker at 180 rpm and 28 °C. Each treatment was repeated three times. The culture filtrate (250 mL) was used as crude protein extract after incubation for 5 d, 10 d and 20 d. The culture broths from both media were filtered through 0.22 μM bacterial proof filter (Millipore Corporation; Bedford, MA, USA). Culture filtrates were used as sources of crude chitinase and kept throughout the experiment at 4 °C.

Colloidal chitin preparation

Colloidal chitin was prepared from crab shell chitin (Sigma–Aldrich; Saint Louis, MO, USA) following the method of Roberts and Selitrenikoff (1988) with some modification. One hundred grams of chitin flakes were added slowly to 1.75 L concentrated HCl and mixed gently for 3 h on a magnetic stirrer. This solution was then filtered to 20 L of pre-chilled, distilled water with constant mixing and allowed to settle. A dense white precipitate formed that was then centrifuged at 10,000 rpm for 10 min at 4 °C. The precipitate was then washed in cold, distilled water repeatedly until the pH of the wash reached 5.5. The supernatant was discarded and the colloidal chitin was then kept in a refrigerator for future use.

Assay for chitinase activity

Endochitinase activity was measured using the reduction in turbidity (absorbance reading at 510 nm) of a colloidal chitin suspension (Tronsmo and Harman, 1993) after incubation of colloidal chitin and the enzyme solution (1:1 ratio) at 1 h, 2.5 h, 4 h, 5.5 h, 7 h, 8.5 h, 10 h and 12 h at 37 °C. Colloidal chitin suspensions contained 1% (weight per volume) purified colloidal chitin (Vessey and Pegg, 1973) in 100 mM of acetate buffer at pH 5.0 and were sterilized using autoclaving. Chitinase activity was calculated as the percentage reduction in turbidity relative to a similar suspension that contained water rather than enzyme solution. One enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5% (Tronsmo and Harman, 1993). The protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as a standard. Summary statistics were used to obtain the mean ± SE values. ANOVA was used to evaluate the significant differences ($p \leq 0.05$) between means using the SPSS statistical software (version 17; SPSS Inc.; Chicago, IL, USA).

One-dimension protein electrophoresis analysis

Protein analysis was carried out using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 12% gels (Laemmli, 1970). Samples of 100 ng of crude proteins were mixed 1:1 (volume per volume) with 4× sample buffer, then loaded to the gel and electrophoresed for about 90 min at a constant 60 mA (when the two gels were electrophoresed at the same time). The gel was stained with 0.25% Coomassie brilliant blue R-250 and then destained with 10% acetic acid and 40% methanol.

Extraction, amplification and sequencing of 18S rDNA

A mycelium was recovered by centrifugation and filtration through Whatman No. 1 filter paper. The mycelium was then washed twice with sterilized water, added with liquid nitrogen and ground until powdered mycelium was obtained. DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) nucleic acid extraction procedure of Taylor and Powell (1982). The DNA concentration was determined using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific; Waltham, MA, USA) and stored at −20 °C.

The identities of fungi were established using polymerase chain reaction (PCR) amplification and sequencing of 18S rDNA segments. The 18S rDNA gene was amplified with the primer pair NS3/NS7 (Table 1) according to White et al. (1990). The PCR reactions were carried out in 50 μL volumes containing 100 ng of genomic DNA, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM of each dNTP, 5 pmol of each primer and 1U of *Taq* DNA polymerase (Invitrogen; São Paulo, Brazil). The regime in the thermal cycling was one cycle of initial denaturation at 94 °C for 3 min, 35 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and 30 s, extension at 72 °C for 3 min and a final extension at 72 °C for 10 min. PCR products was analyzed using electrophoresis in 1%

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5′ → 3′)
NS3 (White et al., 1990)	GCAAGTCTGGTGCCAGCAGCC
NS7 (White et al., 1990)	GAGGCAATAACAGGTCTGTGATGC
Chi-PF1	AA(C/T)GCTGT(C/T)TACTTCAC(T/C)AAC
Chi-PR1	CC(C/G)TTCTT(C/G)ATGTTGTCGTAC
RAPD primers (PO E-02)	GGTGCGGGAA
PR-IV	ACAGCCATAAACATTCTTGCCC
PF-VI	ACTACCAGGTTCTCCCCAAGTCTGG
RAPD primers (OP AB-13)	CCTACCGTGG

agarose gels, stained by ethidium bromide and visualized under ultraviolet light. The PCR product was purified using a Favor PrepTM GEL/PCR purification kit (FavorgenBiotech Corp.; Ping-Tung, Taiwan). The purified product was ligated into the pGEM-T easy vector (Promega; Madison, WI, USA) and transformed into competent *Escherichia coli* JM 109 cells using the heat-shock method. The cloned products were used for sequencing by First Base (Selangor, Malaysia).

The sequence of 18S rDNA was obtained and compared with the sequences in the NCBI database using a basic local alignment search tool (BLAST) search and aligned with the BioEdit software (Hall, 1999). Construction of a neighbor joining tree and bootstrap analysis of 1000 re-samplings were conducted using the MEGA 6.0 software (Tamura et al., 2013).

Characterization of full-length genomic DNA of chitinase gene

A DNA fragment containing chitinase gene was obtained using PCR with the genomic DNA of *Paecilomyces* sp. as a template and degenerate oligonucleotide primers (Chi-PF1/Chi-PR1) (Table 1), designed based on the endochitinase gene sequences of *Paecilomyces* (EF183511) and other fungi (http://www.ncbi.nlm.nih.gov/). The PCR product was sent for sequencing via DNA cloning and used to design a range of primers. The 5′ region was obtained using PCR with the random primer, OP E-02 (Operon Technologies Inc.; Alameda, CA, USA), and the primer PR-IV, while the 3′ region was obtained using PCR with the primer PF-VI and the random primer, OP AB-13 also from Operon Technologies Inc. PCR was performed using the same reaction as for 18S rDNA with *Taq* polymerase (Takara; Shiga, Japan) and was carried out under the following conditions for: 1) degenerate oligonucleotide primers—initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and final extension was at 72 °C for 3 min; and 2) for 5′ and 3′ regions—initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and final extension at 72 °C for 10 min. The resulting PCR products were analyzed on 1% agarose gel containing ethidium bromide. PCR fragments were purified from gel with the Favor PrepTM GEL/PCR purification kit (Favorgen Biotech Corp.; Ping-Tung, Taiwan) according to the manufacturer's instructions. The purified PCR products were cloned into pGEM-T easy vector (Promega Corporation; Madison, WI, USA) and transformed into JM109 competent cells. Plasmid containing the insert was isolated using the Favor-Prep Plasmid DNA extraction mini kit (Favorgen Biotech Corp.; Ping-Tung, Taiwan) according to the manufacturer's instructions and sent for sequencing. The sequences were assembled using the CAP3 software (Huang and Madan, 1999) and aligned with other chitinase gene sequences using the BioEdit software (Hall, 1999). The distance matrix for all pairwise sequence combinations was analyzed using the neighbor-joining method (Saitou and Nei, 1987) and a phylogenetic tree was constructed with 1000 bootstrap replicates using the MEGA 6.0 software (Tamura et al., 2013). The deduced amino acid sequence from the chitinase gene was used in homology modeling undertaken using the Swiss-Model (Biasini et al., 2014). The protein structure and models were viewed using the software DeepView-Swiss-Pdb Viewer (SPDBV_4.10) (Guex and Peitsch, 1997).

Results

Screening of antagonistic fungi

In the culture method for screening antagonistic fungi, *Paecilomyces* sp. showed higher shrimp shell digestion ability than *Gongronella* sp. while *Fusarium* sp. expressed lower digestion ability than that of the other two species (Fig. 1). Among these, *Paecilomyces* sp. was selected for further studies.

Preparation of culture filtrates

The chitinase activity was evaluated in culture filtrates by adding colloidal chitin and incubating at 37 °C for 1–12 h. The chitinase activity increased steadily throughout the incubation for 12 h for both culture filtrates from *Paecilomyces* sp. in medium C and the basal medium; however, the increase was more substantial in the culture filtrate from medium C (from 20 to 90 Units/mL) than the basal medium (from 10 to 40 Units/mL) as shown in Fig. 2. Additionally, the culture filtrates of medium C for 10 d and 20 d had the highest chitinase activity (at similar levels), while the filtrate of medium C for 5 d was significantly low (Table 2). Furthermore, the protein concentration was higher in the culture filtrate from medium C (0.356 Units/mL) than basal medium over the 20 d of experimentation (Table 3).

One-dimension protein electrophoresis analysis

From the SDS PAGE analysis, no band was observed in all culture filtrates from *Paecilomyces* sp. grown in all treatments on the basal medium and medium C containing colloidal chitin for 5 d, while three strong protein bands with estimated molecular masses of about 40 kDa, 46 kDa and 56 kDa were observed in culture filtrates from fungus grown in medium C for 10 d and 20 d (Fig. 3).

Polymerase chain reaction amplification of 18S rDNA and identification of fungi

Amplification of the 18S rDNA gene resulted in a specific DNA fragment of approximately 800 bp in length (data not shown). The homology of the partial 18S rDNA gene sequence of the selected fungus was compared to other nucleotide sequences in the nucleotide sequence database using the BLAST algorithm in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST searches of the partial 18S rDNA fragment of the selected fungus resulted in sequence similarity to the 18S rDNA fragment of *Purpureocillium* (*Paecilomyces* sp., KM222253), *Paecilomyces* sp. (KC790497), *Paecilomyces carneus* (KC143071), *Purpureocillium* (*Paecilomyces*) *lilacinum* (KC143069), *Paecilomyces lilacinus* (JF824691), *Ascomycete* sp. (EF638694), *Ophiocordyceps heteropoda* (AB084157), *Ephelis japonica* (AB114631) and *Paecilomyces nostocoides* (AB104884) with a 100% identity score. The phylogenetic analysis showed relationships with 73 fungal strains cited from GenBank (Fig. 4).

Sequence analysis

Assembly of the sequence led to the complete gene sequence encoding a chitinase shown in Fig. 5. The obtained sequence consisted of 713 bp upstream, 1499 bp open reading frame that was interrupted by three introns and 1698 bp downstream sequences.

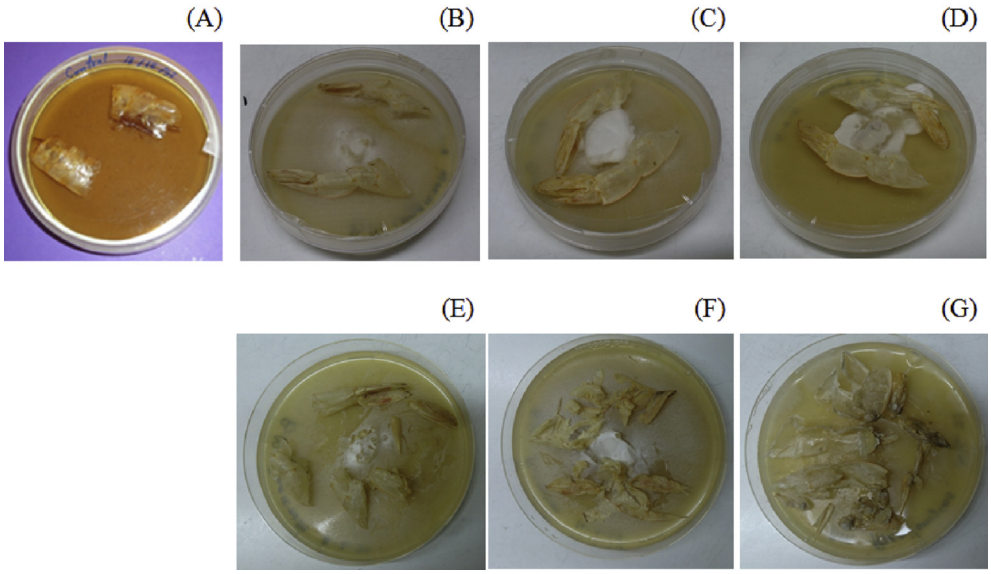

Fig. 1. Degradation of shrimp shells on potato dextrose agar by different fungi: (A) control-no fungal treatment; (B) after treatment of mycelium of *Fusarium* sp. for 5 d; (C) after treatment of mycelium of *Gongronella* sp. for 5 d; (D) after treatment of mycelium of *Paecilomyces* sp. for 5 d; (E) after treatment of mycelium of *Fusarium* sp. for 10 d; (F) after treatment of mycelium of *Gongronella* sp. for 10 d; (G) after treatment of mycelium of *Paecilomyces* sp. for 10 d.

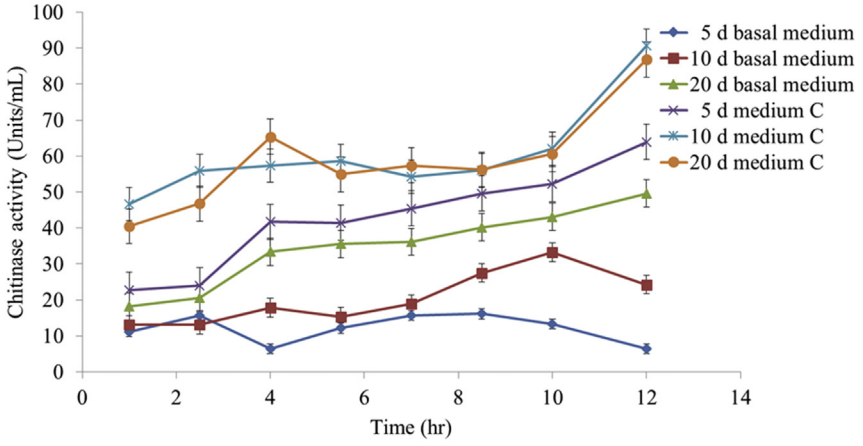

Fig. 2. Influence of medium affecting chitinase production by *Paecilomyces* sp. Each point represents the mean of three independent experiments and error bars indicate ± SE.

Table 2
Chitinase activity (mean ± SE, three independent replicates) of isolated *Paecilomyces* sp. after incubation at 37 °C in medium C and basal medium.

Culture filtrate	Chitinase activity (Units/mL)
Medium C	
5 d	42.5404 ± 2.75567[b*]
10 d	60.1858 ± 3.09047[a]
20 d	58.5546 ± 2.89632[a]
Basal medium	
5 d	12.1012 ± 1.12009[e]
10 d	20.3792 ± 1.77170[d]
20 d	34.5567 ± 2.16877[c]

*Means in the same column with different lowercase superscript letters are significantly different from each other at $p \leq 0.05$.

Table 3
Effect of different media on chitinase production by *Paecilomyces* sp.

Medium	Culture filtrates (Units/mL)		
	5 d	10 d	20 d
Medium C	0.118	0.277	0.356
Basal medium	0.091	0.161	0.286

Intron lengths were 63 bp, 57 bp and 110 bp, respectively. The sequence was most similar to the chitinase gene of *P. lilacinus* (EF183511). Pairwise alignment of the 1499 bp and *P. lilacinus* resulted in 72.5% DNA sequence identity (Fig. 6), while alignment of the 1269 bp coding sequence and *Purpureocillium* (*Paecilomyces*) *lilacinus* resulted in 78.5% cDNA sequence identity (Fig. 7). The phylogenetic trees from fungal chitinase DNA sequences with and without introns are shown in Figs. 6 and 7. The deduced amino acid sequence, encoding a protein of 422 residues, shared 83.5% identity with *Purpureocillium* (*Paecilomyces*) *lilacinus* (ABP37997) as shown in Fig. 8. The amino acid sequence contained the deduced N-terminal (VYFTNWGIYGRN) of fugal chitinase (Fig. 5). It included the consensus motif SIGG (SXGG) corresponding to a substrate-binding site and the catalytic domain consensus motif DGIDLDWE (DXXDXDXE) (Fig. 5), which were highly conserved among chitinases of the glycosyl hydrolase family 18 (Falquet et al., 2002). Moreover, the chitinase cDNA sequence shared 72% similarity with

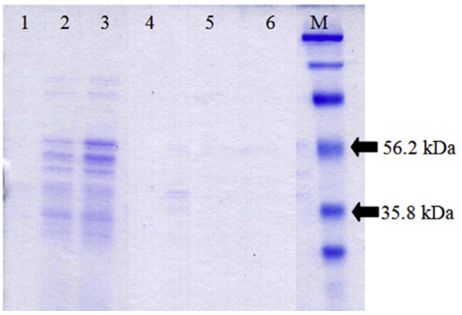

Fig. 3. Secreted proteins from *Paecilomyces* sp. culture filtrate. Lanes 1–3, medium C for 5 d, 10 d and 20 d, respectively; Lanes 4–6, basal medium for 5 d, 10 d and 20 d, respectively. A sample of 30 mg of protein was loaded in each lane. M = protein ladder marker. Arrows indicate band positions of 35.8 kDa and 56.2 kDa.

the chitinase gene, *chi-1*, from the entomopathogenic fungus *Nomuraea rileyi* (ACF10394) as shown in Fig. 7.

Phylogenetic analysis

Family 18 chitinases can be divided into two groups—class III and V fungal chitinases—based on their amino acid sequences (Dong et al., 2007). Fourteen chitinase amino acid sequences, consisting of 13 chitinases in glycosyl hydrolase family 18 and a chitinase in family 19, identified from *Zea diploperennis* (Tiffin and Gaut, 2001) as the outgroup, were aligned using ClustalW (Thompson et al., 1994) and a dendrogram was constructed using the MEGA6.0 software (Fig. 8). Three sequences clustered and formed class III chitinase, while the other 10 sequences, including the 14P 25 from the current study, formed a monophyletic clade of class V chitinase. 14P 25 was comparable to the nematopathogenic *Purpureocillium* (*Paecilomyces*) *lilacinus* (ABP37997).

Modeling of chitinase gene

Homology modeling was studied for three-dimensional structure prediction of chitinase from the *Paecilomyces* sp. in the current study using the X-ray structure of *Lecanicilium psalliotae* (ver112); 3g6m chitinase as a template. The root mean square deviation (RMSD) from the superposition backbone structure and each atom between the chitinase and 3g6m were equal (1.65 Å). The RMSD values revealed that the three-dimensional structure of chitinase from homology modeling was correct and credible. It contained 11 β-sheets and 9 α-helix. Superposition of the structures of 3g6m and the chitinase were in the same structure (Fig. 9).

Discussion

Screening for chitin-degradation of shrimp shells showed that *Paecilomyces* sp. has the greatest degradation capability. Nematophagous fungi, such as *Verticillium chlamydosporium* and *V. suchlasporium* can produce fungal chitinases that might be involved in the breakdown of nematode eggshells (Tikhonov et al., 2002). Chitinases are produced as competition and defense mechanisms against other fungi or to colonize other arthropods including insects (entomopathogenic fungi). They are also proposed to be involved in mycoparasitism by mycoparasitic fungi (Haran et al., 1996).

In this research, a comparison of different culture media showed that the culture filtrate from *Paecilomyces* sp. grown in medium C has significantly greater colloidal chitin degradation than from using the basal medium. Deng et al. (2007) indicated an increased

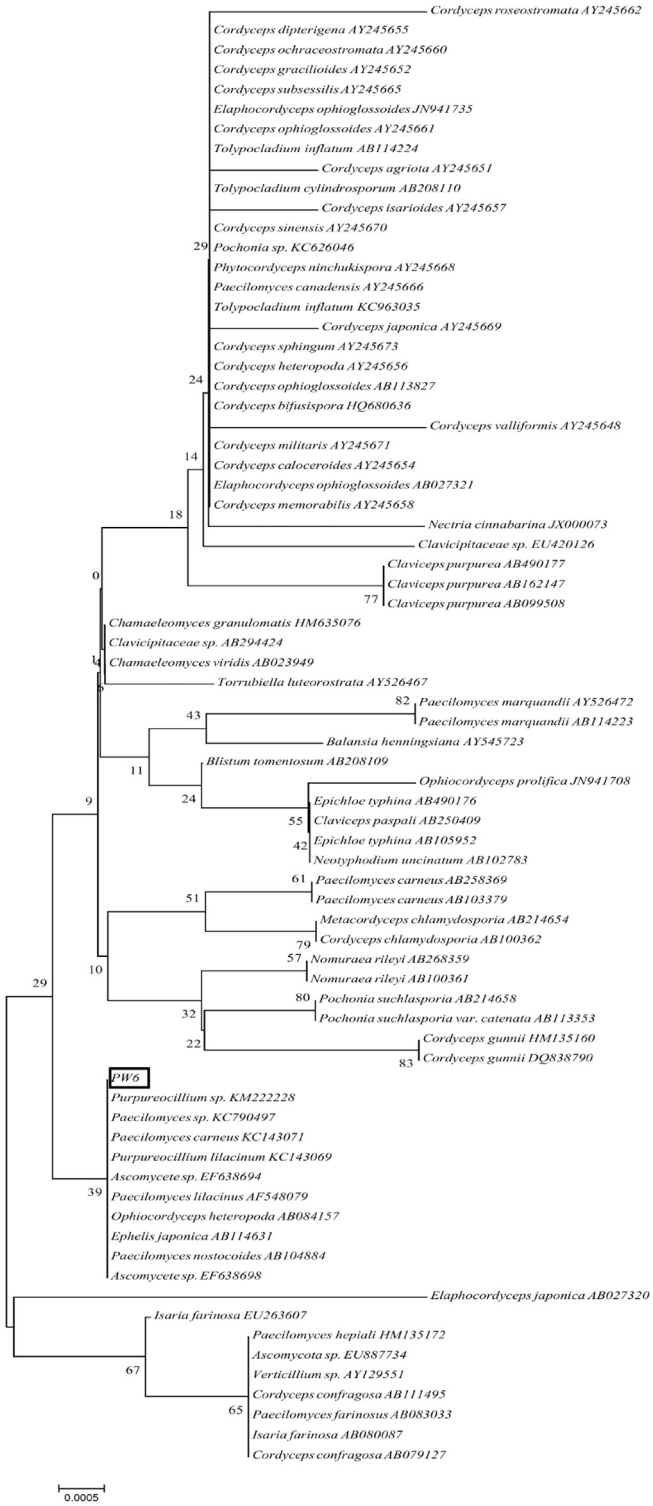

Fig. 4. Dendrogram based on neighbor-joining method of the partial 18S ribosomal DNA sequences of *Paecilomyces* sp. (*PW6*) and the compared fungal strains from GenBank. Numbers at branches indicate bootstrap values (%) for 1000 replications.

production of antifungal enzymes by *Trichoderma atroviride* in medium C containing trace elements that play a crucial role in determining protein and enzyme productions by fungi. The chitinase enzyme had a specific requirement of trace elements (Cu and Mo ions) for chitinase activity (Deng et al., 2007). Furthermore, it was reported that yeast extract and peptone had a repressive effect

Fig. 5. Nucleotide sequence (lowercase letters) of chitinase gene from *Paecilomyces* sp. with 5′ and 3′ flanking regions. Positions of primers used for gene walking are underlined and the primer directions are indicated by arrows. The deduced amino acid sequences (capital letters) are indicated below the corresponding putative coding regions. Amino acid sequences of *Paecilomyces* sp., which are the same as of other fungi, are underlined. The putative substrate binding site and catalytic domain of the fungal chitinase are underlined and in italics. Open boxes are start and stop codons.

on chitinase production by *Aspergillus* sp. (Rattanakit et al., 2002). Similar to these findings, enzyme production in this study was significantly enhanced by the metal ion in medium C. Comparison of the results showed that organic nitrogen sources increase chitinase activity considerably relative to inorganic ones. The results of the SDS-PAGE analysis from the culture filtrate of medium C showed three bands with estimated molecular masses of about 40 kDa, 46 kDa and 56 kDa, which could be chitinase isomers. The molecular mass of chitinase isomers from *Bacillus* MH-1 was 71 kDa, 62 kDa and 53 kDa (Sakai et al., 1998). The sizes of chitinases found in bacteria, fungi, yeasts, plants, actinomycetes, arthropods and humans can range from 20 kDa to about 90 kDa (Bhattachrya et al., 2007).

The phylogenetic analysis using 18S rDNA sequencing indicated high similarity between the *Paecilomyces* sp. and species in five other genera—*Purpureocillium*, *Paecilomyces*, *Ophiocordyceps*,

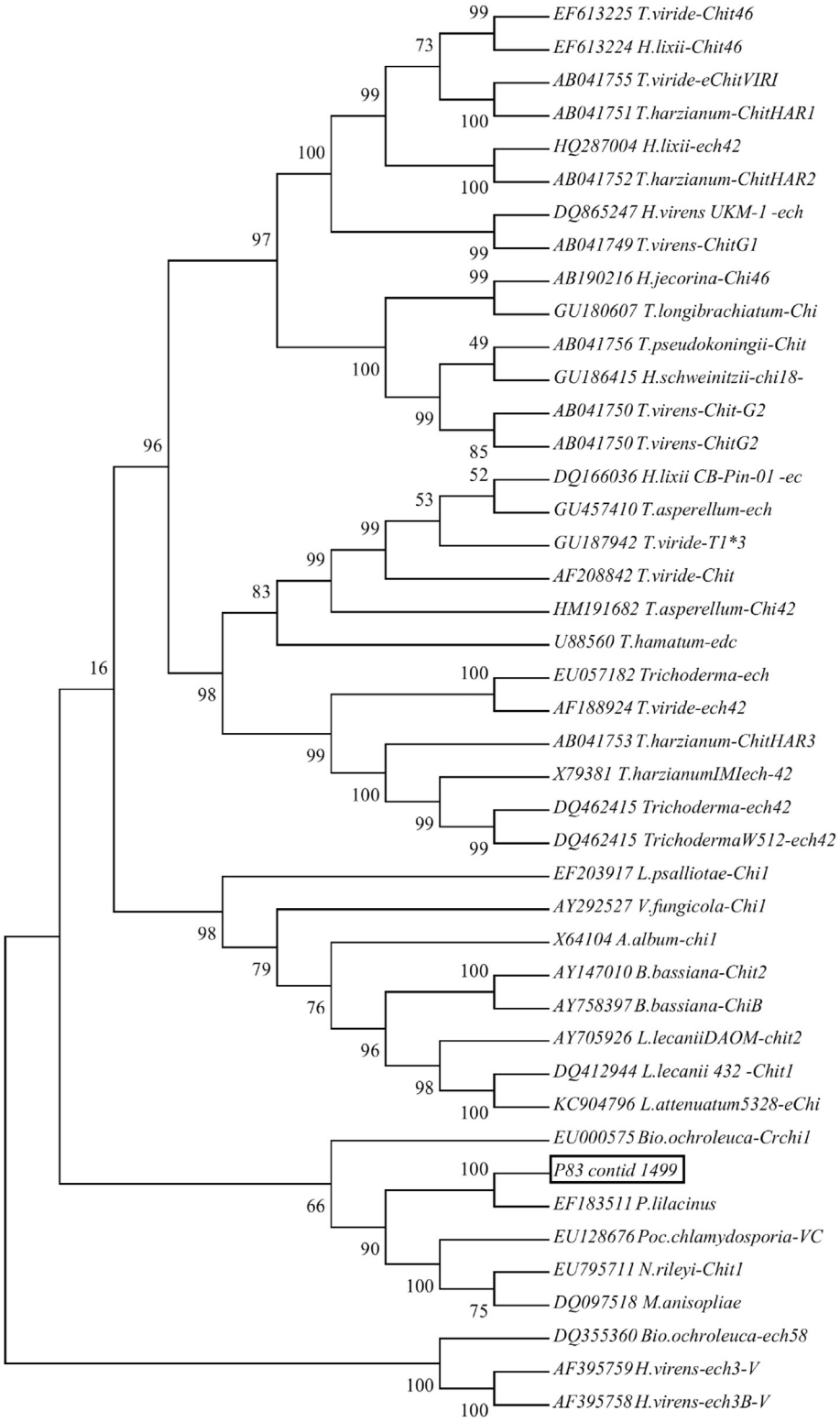

Fig. 6. Dendrogram based on neighbor-joining method of chitinase DNA sequence (1499 bp, from start to stop codons including 3 introns) of *Paecilomyces* sp. (*P83 contid 1499* and boxed in the dendrogram) and the compared fungal strains from GenBank. Numbers at branches indicate bootstrap values (%) for 1000 replications.

Ephelis and *Ascomycete* (Fig. 4). *Purpureocillium* sp., *Ophiocordyceps* sp. and *Ascomycete* sp. are in the family Ophiocordycipitaceae (Sung et al., 2007). Detailed phylogenetic analysis showed that the purple-colored species (*P. nostocoides*, *P. lilacinus*, *Isaria takamizusanensis* and *Nomuraea atypicola*) are closely related (Sung et al., 2007). *Paecilomyces* sp. and *Ephelis* sp. are in the family Clavicipitaceae (Kuldau et al., 1997; Luangsa-ard et al., 2004). *P. lilacinus* and *M. anisopliae* can infect across the insect cuticle (Marti et al., 2006; Fiedler and Sosnowska, 2007) and nematode eggshells (Walters and Barker, 1994; Sun et al., 2006). The phylogenetic

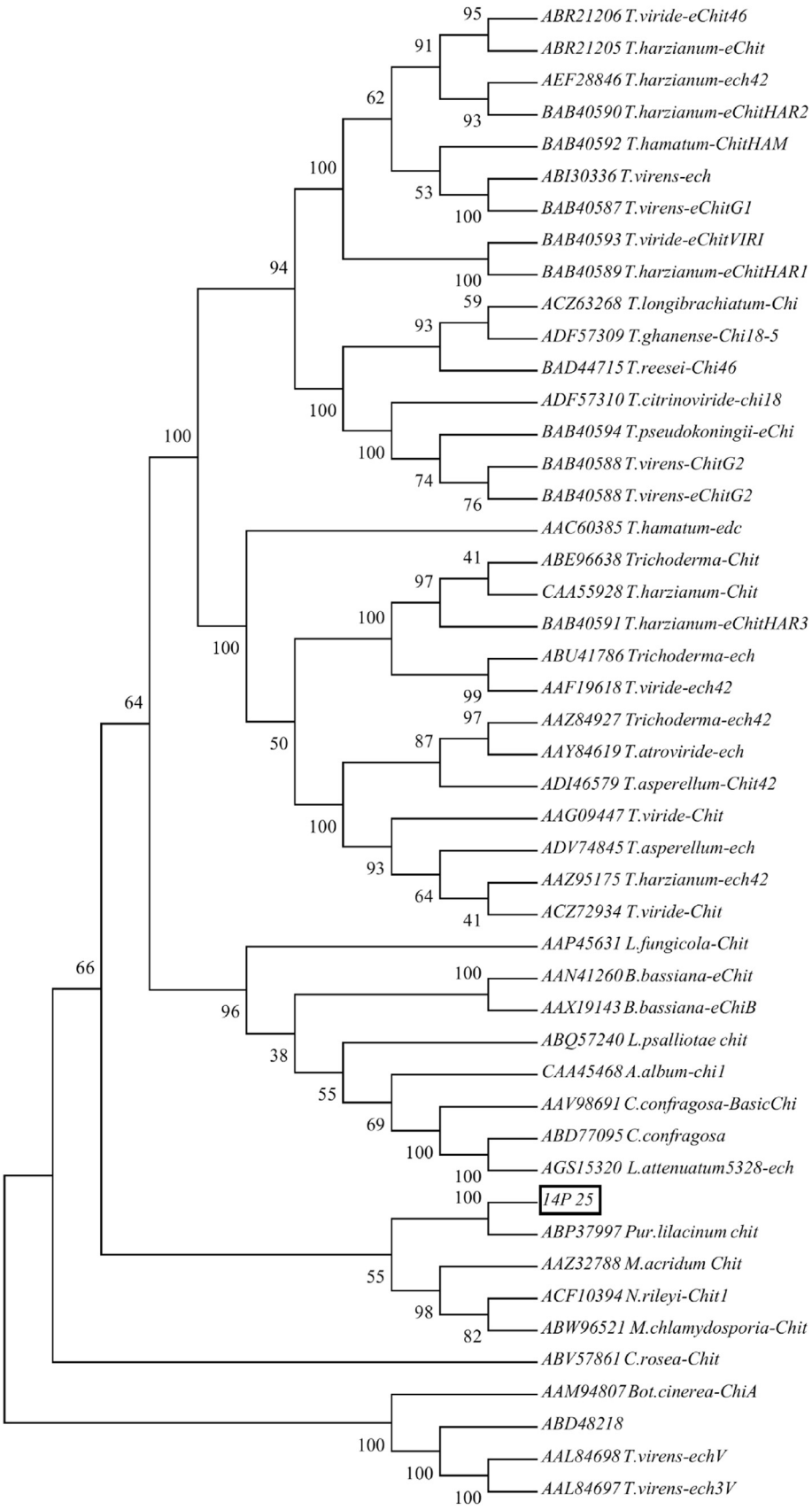

Fig. 7. Dendrogram based on neighbor-joining method of chitinase DNA coding sequence (1269 bp, from start to stop codons) of *Paecilomyces* sp. (*14P 25* and boxed in the dendrogram) and the compared fungal strains from GenBank. Numbers at branches indicate bootstrap values (%) for 1000 replications.

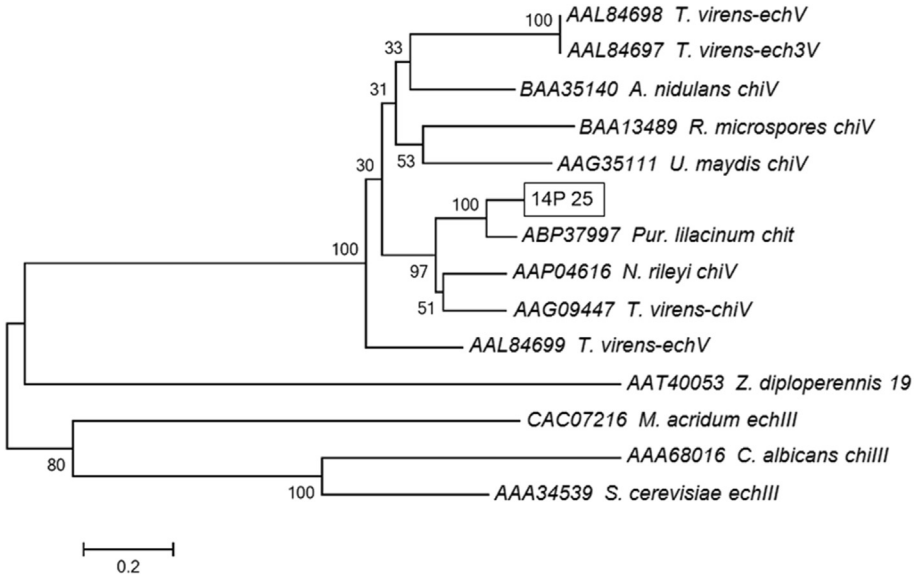

Fig. 8. Dendrogram constructed based on chitinase amino acid sequences using MEGA6.0 software for *Paecilomyces* sp. (14P 25 and boxed in the dendrogram) and the 13 chitinases in class III and class V. Numbers at branches indicate bootstrap values (%) for 1000 replications.

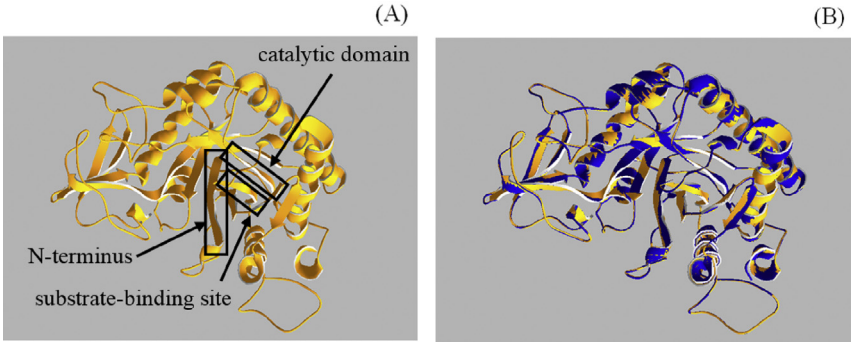

Fig. 9. Three-dimensional model of the chitinase protein constructed by Swiss-PdbView (SPDBV_4.10): (A) homology modeling of the chitinase and its active domains; (B) homology modeling of superposition between the chitinase protein (orange) and 3g6m as template (blue).

analysis of the 18S rDNA gene region in the current study confirmed the information provided by Obornik et al. (2001), indicating the polyphyletic nature of *Paecilomyces*. Others studies, such as the analysis of 5.8S rDNA and internal transcribed spacer sequences in entomogenous *Paecilomyces* species by Mugnier (1998) and Samson (1974), could not effectively identify the species of their samples. Similarly, Luangsa-ard et al. (2004) confirmed the polyphyly of the genus *Paecilomyces* through analysis of the large and small-subunit rRNA gene sequences. A major review of the genus *Paecilomyces* is required, including the analysis of a more conserved gene region to clarify the phylogenetic relationships.

The full-length chitinase gene was obtained using the PCR method. The DNA sequence was found to be most like the chitinase gene of *P. lilacinus*. The deduced amino acid sequence was very similar to chitinase from *Purpureocillium*, which was formerly named *Paecilomyces* (Luangsa-ard et al., 2011).

The deduced amino acid sequence revealed a similarity to the glycosyl hydrolases family 18 [Pfam database;11]. It has the deduced N-terminal of fugal chitinases and it has the consensus motif SXGG corresponding to a substrate-binding site and the catalytic domain consensus motif (DXXDXDXE), highly conserved among fungal chitinases (Perrakis et al., 1994). In previous studies (Yang et al., 2005a, 2005b), a cuticle-degrading protease Ver112 was identified from *L. psalliotae*, and was shown to be capable of degrading nematode cuticle. The crude enzymes isolated from the culture filtrate of *P. lilacinus* 112 showed a higher nematicidal potential than the wild-type strain. Recently, the crystal structures of serine proteases and chitinases from nematophagous fungi were determined (Liang et al., 2010; Yang et al., 2010). Based on the crystal structures of Ver112, the degradation of nematode cuticle/exoskeleton was found to be a key step for the infection by nematophagous fungi. All the proteinous components extracted from *Caenorhabditis elegans* were completely degraded by Ver112 (Liang et al., 2010).

Conclusions

A new chitinolytic fungal strain, *Paecilomyces* sp. was isolated from soil in Thailand. The produced extracellular chitinase activity was assessed spectrophotometrically using colloidal chitin as a substrate in the culture medium. The chitinase activity expressed in medium C was significantly higher than that in the basal medium. Based on the 18S rDNA gene sequence, the *Paecilomyces* sp. was clustered in the same group with *Purpureocillium* sp. *Paecilomyces* sp., *Ophiocordyceps* sp., *Ephelis* sp. and *Ascomycete* sp. The full-length chitinase gene was obtained using the PCR method. Comparison of deduced amino acid sequences elucidated the relationships among chitinases. The amino acid sequence revealed a

similarity to the glycosyl hydrolases family 18 [Pfam database;11]. This *Paecilomyces* sp. was comparable to the nematopathogenic *Purpureocillium* (*Paecilomyces*) *lilacinum* (ABP37997). The analysis showed that the chitinase sequence was class V chitinase. Homology modeling was studied for three-dimensional structure prediction of chitinase and found that it is similar to the X-ray structure of *L. psalliotae*, (3g6m) chitinases.

Conflict of interest

There is no conflict of interest.

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