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Cloning, characterization and expression of an exo-1,3-beta-glucanase gene from the antagonistic yeast, *Pichia guilliermondii* strain M8 against grey mold on apples

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Abstract

The strain M8 of *Pichia guillermondii* isolated from the carposphere of apples (cv. Golden Delicious) showed a high efficacy in controlling grey mold, caused by *Botrytis cinerea*, on apples under semi-commercial conditions. Moreover, *P. guilliermondii* M8 produced high amounts of active exo-1,3-beta-glucanase in Lilly-Barnett minimal salt medium with different carbon sources, which greatly inhibited *B. cinerea in vitro* and *in vivo* tests. Therefore, an exo-1,3-beta-glucanase gene, named as *PgExg1* (GenBank accession number HQ113463) was cloned from the genomic DNA of the strain M8 by genome walking. The sequencing and the nucleotide BLAST analysis indicates that no introns are present inside the gene, which was confirmed by amplifying the full gene from complementary DNA (cDNA) of the yeast. An open reading frame of 1,224 bp encoding a 408-amino acid (aa) protein with a calculated molecular weight (M_r) of 46.9 kDa and an isoelectric point (pI) of 4.5 was characterized. Protein BLAST and phylogenetic tree analysis of the deduced amino acid sequences from the *PgExg1* gene suggested that the glucanase produced by *PgExg1* gene belongs to the Glycoside Hydrolase Family 5. Expression of *PgExg1* in *Escherichia coli* BL21 (DE3), followed by identification with Western-blotting, purification with Ni-NTA and analysis with enzyme assay, yielded homogeneous recombinant PgExg1. At its optimal pH of 5.0 and its optimal temperature of 40°C, the recombinant enzyme protein showed the highest activity towards laminarin, while the highest stability was obtained when the enzyme was stored at pH of 7.0 and temperature of 4°C.

Keywords: Biological control; Enzyme characterization; Molecular cloning; Postharvest pathogens; SDS-PAGE.

1. Introduction

Due to the occurrence of fungicide-resistant pathogen strains (Holmes and Eckert, 1999), the increasing concerns for public safety and the subsequent cancellation of some of the most effective fungicides (Spadaro and Gullino, 2004), new strategies are required to control postharvest pathogens (Janisiewicz and Korsten, 2002). One of the most promising alternatives to reduce pesticide use is biological control using microbial antagonists. This approach has been attracting increasing attention from the fruit growers and the researchers engaged in the control of postharvest diseases (Wisniewski et al., 1995; Ippolito et al., 2000; Spadaro et al., 2002). Among the microbial antagonists, yeasts received particular attention from the researchers, searching for new biocontrol agents against the pathogens on fruits and vegetables, because generally they have minimal negative toxicological or environmental impact (Spadaro et al., 2008; Zhang et al., 2010a).

Understanding the mechanism of biocontrol is essential for developing appropriate commercial formulations, application methods, and to maximize the effectiveness of biological control agents (BCAs). For antagonistic yeasts, few modes of action have been proposed such as competition for limiting nutrients and site exclusion (Bencheqroun et al., 2007; Saravanakumar et al., 2008), induction of host resistance (Ippolito et al., 2000), and mycoparasitism (Chan and Tian, 2005). However, recently more attention has been paid to study the production of lytic enzymes by BCAs due to their roles in breaking down the pathogen cell wall and inhibiting the spore germination (Smits et al., 2001; Masih and Paul, 2002).

Most phytopathogenic fungi have cell walls composed of complex polymers of β-1,3- and β-1,6-glucans, mannoproteins, as well as some chitin which play an important role in maintaining the cell integrity and protecting against biotic and abiotic stresses (Smits et al., 2001; Cheng et al., 2009). In the fungal cell wall, chitin acts as a structural backbone and is arranged in regularly ordered layers, and

β-1,3-glucan is a filling material arranged in an amorphous manner and the most abundant component of the fungal cell walls (Marcello et al., 2010). Thus, breakdown of the fungal cell wall requires the participation of different enzymes, especially β-1,3-glucanases and chitinase (Simmons, 1994). β-1,3-glucanases can hydrolyze the substrate by two possible mechanisms: (1) exo-β-1,3-glucanase (EC3.2.1.58) hydrolyzes β-glucans by sequentially cleaving glucose residues from the nonreducing end, and (2) endo-β-1,3-glucanase (EC 3.2.1.39) cleaves β-linkages at random sites along the polysaccharide chain, releasing shorter oligosaccharides and glucose (Monteiro and Ulhoa, 2006).

Lorito et al. (1994) found that β-1,3-glucanases were directly involved in the mycoparasitism interaction between *Trichoderma* species and their hosts. More recently, it was proven that β-1,3-glucanases participate directly or indirectly to the mechanism of biocontrol of some yeasts antagonistic against plant pathogens (Masih and Paul, 2002; Chan and Tian, 2005).

Among the potential yeast antagonists, the yeast *Pichia guilliermondii* was effective in controlling different postharvest pathogens on a number of fruits and vegetables, such as *Penicillium digitatum* on grapes (Droby et al., 1997), *Penicillium expansum* on apples (Tian et al., 2002; Scherm et al., 2003), *Rhizopus nigricans* on tomatoes (Zhao et al., 2008) as well as anthracnose on chilli (Chanchaichaovivat et al., 2008). *B. cinerea* is the causal agent of grey mold on pome and stone fruits, and could cause large economic losses worldwide. However, information about the application of *P. guilliermondii* for controlling grey mold on apples is still limited and there is no report on cloning and characterization of the β-1,3-glucanase gene from the antagonistic yeast, *P. guilliermondii*.

Therefore, the objectives of this research were i) to clone and characterize exo-β-1,3-glucanase gene from *P. guilliermondii* strain M8 to reveal its phylogenetic relationship with β-1,3-glucanases from other fungi, and ii) to express the exo-β-1,3-glucanase gene and demonstrate the activity of exo-β-1,3-glucanase

in controlling pathogens to prove its involvement in the biocontrol activity of the yeast.

2. Materials and methods

2.1 Microorganisms, fruit, vectors and molecular kit

The antagonistic strain M8 of *Pichia guilliermondii* was obtained from the rhizosphere of maize cultivated in northern Italy (Zhang et al., 2011) and maintained on nutrient yeast dextrose agar (NYDA) slants (nutrient broth 8 g l^{-1}, yeast extract 5 g l^{-1}, glucose 10 g l^{-1} and agar 20 g l^{-1}). The yeast was grown in YPD (20 g l^{-1} dextro-glucose, 20 g l^{-1} peptone casein, 10 g l^{-1} yeast extract) at 25°C for 48 h on a rotary shaker (200 rpm). Five strains of *B. cinerea* were provided by AGROINNOVA. They were maintained on PDA (potato dextrose agar) slants at 4°C and the conidia were harvested from pathogen mycelia grown on PDA in Petri dishes at 25°C for 7 days. The required concentrations of the conidia were determined using a Bürker chamber (Knittel, Germany). Apples (cv. Golden Delicious) for the biocontrol experiments were bought from supermarkets at a maturity suitable for marketing.

The oligonucleotides and the vector pGEM-T and pET-23a(+) used in this study were obtained from Promega (Madison, WI, USA). *Eschericha coli* strain DH5α and strain BL21 were obtained from Invitrogen (Eugene, OR, USA). The kits for DNA and RNA extraction (RNeasy and DNeasy), QIAquik PCR purification, Reverse transcript PCR, Plasmid extraction, and QIAquik Gel extraction as well as the materials for PCR were purchased from Qiagen (Hilden, Germany). The kit “Gene Walking Made Easy”, the monoclonal anti-polyhistidine as first antibody and the A1293-AlkPhos APA Mouse Fab ads HIgG as second antibody for Western-blotting were purchased from Sigma (St Louis, MO, USA), and the materials for enzyme assays, protein purification and SDS-PAGE were purchased from Merck company (Darmstadt, Germany).

2.2 Preparation of cell wall and β-1, 3-glucanase activity assay

Cell wall preparations (CWP) of the pathogen were prepared as described by Saligkarias et al. (2002) with small modifications. Briefly, the pathogen mycelium was harvested from cultures grown in potato dextrose broth (PDB) medium for 72 h. the mycelium was filtered through four-layer cotton gauzes, washed twice with deionized water through Whatman No.1 filter paper and centrifuged (Centrifuger: 6K15, Sigma, Germany) at 500g for 2 min. After removing the supernatant, the mycelial mats were sonicated with a probe type sonicator (USC6000, Malaysia) for 20 min and centrifuged at 500×g for 5 min. The supernatant was removed and the pellet was resuspended in deionized water. Then the crushed mycelium was resuspended into an equal volume of Tris/HCl buffer (50 mM and pH 7.2), centrifuged at 1900×g for 15 min, and the supernatant was discarded. The pellet was subjected to three successive cycles of centrifugation and resuspension. The final pellet was frozen with liquid N_2, lyophilized and stored at -20°C for further studies.

The yeast strain M8 was grown in modified Lilly-Barnett minimal salt medium (Lilly and Barnett, 1951) containing 2 mg ml^{-1} CWP as sole carbon source in 30 ml of culture media incubated at 25°C on a rotary shaker at 150 rpm for 0, 24, 72, 96 and 120 h. Culture filtrates from each individual flask were collected by centrifuging at 7,000g for 8 min, and the supernatants were used for the β-1, 3-glucanase (EC3.2.1.58) activity assay. The assay was carried out by measuring the amount of reducing sugars released from laminarin (L9634, Sigma), using glucose as standard. A reaction mixture was prepared by adding 250 µl of 50 mM potassium acetate buffer (pH 5.0) containing 2.5 mg of laminarin per ml into 250 µl of culture filtrate (Chan and Tian, 2005). The enzyme-substrate mixture was incubated at 40°C in a water bath (D-3508 Melsungen, Germany) for 2 h. Then 0.5 ml of dinitrosalicylic acid reagent was added,

147 the sample was boiled at 100°C for 5 min and after cooling, 2 ml deionized water was added and the
148 absorbance was determined spectrophotometrically at 595 nm. Background levels of reducing sugars were
149 determined with a supernatant substrate at time zero just prior to boiling at 100°C for 5 min. The protein
150 concentration of the enzyme solution was determined according to Bradford (1976) by using bovine serum
151 albumin (A1933, Sigma) as a standard. The specific activity was expressed as micromoles of glucose per
152 milligram protein per hour (Fan et al., 2002). Each treatment had three replications and the experiment was
153 repeated twice.
154
155 **2.3 Purification of exo-β-1,3-glucanase and its activity against *B. cinerea***
156 Purification of β-1,3-glucanase protein was carried out according to Tseng et al. (2008). The strain M8
157 was grown in modified Lilly-Barnett minimal salt medium as described in section 2.2 and incubated at
158 25°C on a rotary shaker (150 rpm) for 72 h. The strain M8 culture supernatant were collected by
159 centrifuging at 7,000 g for 8 min. Ammonium sulfate (Merck) was added to 1000 ml of the culture
160 supernatant and stirred at 4°C overnight to give up to 75% saturation. Following centrifugation at 18,000 g
161 for 30 min at 4°C, the precipitated protein pellets were resuspended in 3 ml deionized water. A 0.1%
162 protease inhibitor cocktail (Sigma, USA) was added, and samples were dialyzed (dialysis membrane
163 molecular weight cut off = 12,000 kDa) against a 100-fold volume of deionized water at 4°C for 24 h and
164 then stored at -20°C for further studies.
165 To assess the activity of the purified crude enzyme against the pathogen, *B. cinerea* was grown on
166 PDA in Petri dishes at 25°C for 4 days, and then two small wells were made close to the pathogen mycelia
167 by removing the medium with a cork borer. One hundred µl of purified enzyme solution was added into
168 one well and 100 µl of uncultured medium was added into the other, serving as a control (CK). After

incubation for two days, the inhibition of the pathogen mycelia was investigated.

To determine the activity of the purified crude enzyme against the pathogen *in vivo*, the enzymes were tested directly on wounded apples according to Jijakli and Lepoivre (1998), with small modifications. Aliquots of 100 µl of the purified crude enzymes were pipetted into each wound site. After 2 hours incubation at room temperature, the wounds were inoculated with 30 µl of *B. cinerea* suspension at the concentration of 10^5 spores ml^{-1}. Inoculated control fruits were rinsed with 100 µl deionized water, before pathogen inoculation. A blank control, treated with 100 µl of deionized water, was included. When dry, apples from different treatments were randomly packed in commercial plastic trays and stored at 20°C for 3 days, when the diameters of the rotten lesions were measured.

2.4 Cloning of the exo-β-1,3-glucanase gene

To clone the β-1,3-glucanase gene from the genomic DNA of *P. guilliermondii* strain M8, degenerate primers were designed according to the conserved protein sequences (VRIPIGYW and DHHHYQVF) obtained by using alignments of glucanase genes already published: DPf 5'-GTNCGNATHCCNATHGGNTAYTGG-3' and DPr 5'-AANACYTGRTARTGRTGRTGRTGRTC-3'. The amplification was performed by Grads-PCR with 0.5°C as gradient for annealing temperature from 45 to 60ºC. The reaction mixture (20 µl) contained 1 µl DNA template (50 ng), 200 mM each deoxynucleotide triphosphate, 2 µl 10 X buffer (Taq DNA Polymerase, Qiagen), 0.7 mM each primer, and 1.0 U Taq DNA Polymerase (Qiagen). The temperatures of Grads-PCR program were: 95°C, 3 min; 32 cycles: 94°C, 15 s; 45-60°C (0.5°C as gradient), 45 s; 72°C, 30 s; with final extension 72°C, 10 min; 4°C. The PCR products were purified by agarose (2.0%, w/v) gel electrophoresis in TEB buffer, stained with sybr-safe, excised from the gel and purified with a QIAquick gel extraction kit (Qiagen) according to the supplier's

191 instructions. The purified fragments were ligated into pGEM-T (Promega) cloning vector before
192 transformation into chemically competent cells of *E. coli* strain DH5α (Invitrogen). Positive transformants
193 were selected at 37°C on Luria Broth (LB) agar added with ampicillin (100 µg ml^{-1} of LB agar), IPTG (50
194 mM) and X-gal (80 µg ml^{-1}) for blue / white screening of recombinant colonies. Transformed *E. coli*
195 strains were confirmed for the presence of insert using universal primers M13F
196 (5'-CACGACGTTGTAAAACGAC-3') and M13R (5'-GGATAACAATTTCACACAGG-3').

197 To amplify and identify 5' and 3' flanking regions of β-1,3-glucanase gene from genomic DNA of *P.*
198 *guilliermondii* strain M8, specific primers and restriction enzymes were designed according to the obtained
199 sequences (Table 1) and the chemical kit "Gene Walking Made Easy" (UVS1, Sigma) was used according
200 to the supplier's instructions. The fragments from Vectorette *Cla* I and *EcoR* I library of *P. guilliermondii*
201 strain M8 were purified, ligated into pGEM-T cloning vector and sequenced as described above. The
202 obtained sequences were then subjected to BLAST and ORF Finder programs to assemble the whole
203 sequence of the targeted gene.

204 To amplify the β-1,3-glucanase gene from the cDNA of *P. guilliermondii* strain M8, specific primers
205 were designed according to the *PgExg1* gene sequence obtained from the genomic DNA of *P.*
206 *guilliermondii* M8: forward primer, 5'-ATGCTTCCATACTTCTTTATGATG-3' and reverse primer,
207 5'-CTAGAATTTACATTGGTTGGGATA-3'. Total RNA was extracted from *P. guilliermondii* M8 using
208 RNeasy, a RNA extraction kit, according to the manufacture's protocol (Qiagen). The concentration and
209 quality of the total RNA were evaluated by measuring the absorbance of 280 nm and ratio of 260/280 nm
210 with a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). The
211 first-strand cDNA of the RNA was obtained using QuantiTect® Reverse Transcription Kit (Cat. 205313,
212 Qiagen). The PCR reaction mixtures were prepared with the first-strand cDNA of *P. guilliermondii* M8 as

template and the PCR programs followed: 95°C, 3 min; 34 cycles: 94°C, 30 s; 58°C, 45 s; 72°C, 1min 30 s; 72°C, 10 min; 4°C. The PCR products were purified and sequenced as described above.

2.5 DNA sequence and computer analysis

BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for the nucleotide sequence assembling analysis, deduction of the amino acid sequence and database searches. Multiple sequence alignments of DNA and amino acid sequence were performed using the programs of DNA-MAN 6.0 and Clustal W (http://www.ebi.ac.uk/Tools/es/Cgi-bin/clustalw2). The phylogenetic tree of the exo-β-1,3-glucanases was generated using ClustalX 2.09 and MEGA 4.1 by neighbour-joining method.

2.6 Expression of the *PgExg1* gene in *E. coli* BL21

To further characterize the exo-β-1,3-glucanases encoded by the *PgExg1* gene, the gene expression was performed by transforming the gene into *E. coli* BL21 (DE3) and inducing expression with IPTG. To amplify the *PgExg1* fragment without signal sequence or terminator from the cDNA of *P. gulliermondii* strain M8, the primers forward *Eco*RI 5'-G/AATTCGAATTTACATTGGTTGGGATA-3' (bases underlined encode *Eco*RI restriction site) and reverse *Hin*dIII 5'-A/AGCTTATAACTCGCCGAGGC-3' (bases underlined encode *Hin*dIII restriction site) were used. The amplicon was ligated into pET-23a(+) with restriction sites of *Eco*RI and *Hin*dIII to produce the construct of pET-23a(+)-*PgExg1*. The expression construct was then transformed into *E. coli* BL21 (DE3) for the expression of the *PgExg1* gene. The transformants with the plasmid pET-23a(+) served as control. The transformants were selected after growth in LB broth containing 100 μg ml^{-1}ampicillin and incubated on a rotary shaker (150 rpm) at 37°C until

OD_{600nm} reached 0.4-0.8, followed by induction with IPTG (final concentration 1.0 mM). The bacterial strains were then grown at 37°C for 5-6 h.

2.7 SDS-PAGE and Western-blotting

To characterize the recombinant proteins produced by the transformants of pET-23a(+)-*PgExg1*, SDS-PAGE was performed. The bacterial strains were grown and induced with IPTG as described above. The crude recombinant exo-β-1,3-glucanase in the supernatant was obtained by ultrasonication and removal of cell debris of the induced cells of BL21 containing the plasmids pET-23a(+)-*PgExg1*, and then subjected to SDS-PAGE. The protein extracts from the BL21 containing the empty vector pET-23a(+) were used as controls. After SDS-PAGE analysis, the recombinant proteins (6×His-tagged fusion proteins) were then purified by affinity chromatography with the kit of Ni-NTA for purification of 6×His-tagged proteins (No. 102, Qiagen) according to the supplier's instructions. For SDS-PAGE analysis, the concentration of stacking gel was 7% and the concentration of separating gel was 12%.

To further confirm the specific protein expressed by the *PgExg1* gene, western-blotting with monoclonal anti-polyhistidine as primary antibody and A1293-AlkPhos APA Mouse Fab ads HIgG as secondary antibody was performed.

2.8 Effect of pH and temperature on the activity and stability of the recombinant β-1,3-glucanase *PgExg1*

The effect of pH on the activity of the recombinant β-1,3-glucanase of *PgExg1* was determined by incubating the recombinant enzyme between pH 3.0-11.0 using the standard assay conditions described in section **2.2**. In a similar way, the effect of temperatures on the recombinant β-1,3-glucanase was

investigated by incubating the recombinant enzyme at different temperatures (4-60°C) using the standard assay conditions described before. The relative activity at different pH values and at different temperatures was referenced to activity at pH 5.0 and 40°C (relative activity = 100%).

The effect of pH value on the enzyme stability was tested by 2 h pre-incubation of the recombinant enzyme in 0.05 M potassium acetate (pH 3.0-11.0). The remaining activities of the recombinant enzyme were measured immediately after this treatment with the standard method described above. The relative activity was referenced to the recombinant enzyme activity at pH 7.0 prior to incubation (relativity activity = 100%). The effect of temperature on the enzyme stability was examined by pre-incubating the enzyme at different temperatures (4-60°C) for 2 h. The residual activity was immediately measured as described above. The sample pre-incubated at 4°C was used as a reference (relative activity = 100%). Three replicates for each treatment were prepared.

2.9 Antifungal activity of the recombinant PgExg1 against *B. cinerea in vitro*

The antifungal activity of the recombinant enzyme PgExg1 against *B. cinerea* was assessed in PDB (Potato Dextrose Broth, Merck) under sterile conditions as reported by Lorito et al. (1993), with small modifications. The recombinant proteins were obtained and purified as described before. The protein concentration of the purified enzyme solution was determined according to Bradford (1976) by using bovine serum albumin (A1933, Sigma) as a standard. The conidia of *B. cinerea* were harvested from 7 day-old culture grown on PDA at 25°C by centrifugation and resuspended in sterile Ringer solution. The required concentrations of pathogen conidia were adjusted with the help of a Bürker chamber (Knittel, Germany). Aliquots (300 µl) of *B. cinerea* spore suspension (1×10^6 spores/mL) in Ringer solution were transferred to tubes containing 2.4 ml PDB and 300 µl of the purified recombinant enzyme solution (80 µg

279 ml^{-1}) was added to the tubes and reached a final concentration of 8 µg ml^{-1} in PDB, serving as the enzyme
280 treatment (pathogen + enzyme). Aliquots (300 µl) of thermally inactivated enzymes, by boiling at 100°C
281 for 10 min, or sterile distilled water were added to the tubes instead of the enzyme solution as controls
282 (pathogen+ inactivated enzyme, and pathogen+ water). The tubes were incubated at 25 °C on a rotary
283 shaker (200 rpm) for 18 h, and 100 spores/replicate were observed microscopically and their germination
284 rate and germ tube length were measured. Three replications of three tubes were prepared for each
285 treatment and the experiment was repeated twice.
286
287 **2.10 Statistical analysis**
288 All the experiments were performed at least twice. Data from all the experiments were analyzed using
289 analysis of variance (ANOVA) and the SPSS version 12.0 (SPSS, 1989-2003). The treatment means were
290 separated at the 5% significance level using Duncan's Multiple Range Test (DMRT).
291
292 **3. Results**
293 **3.1 Production and activity of β-1, 3-glucanase**
294 The antagonistic yeast *P. guilliermondii* strain M8 produced extracellular β-1,3-glucanase in culture
295 media in the presence of purified fungal cell walls, sucrose or glucose used as a sole carbon source. it
296 produced more extracellular β-1,3-glucanase after 24 h than 48 h incubation in Lilly-Barnett minimal salt
297 media supplemented with *B. cinerea* cell walls, sucrose or glucose used as sole carbon sources. While after
298 72 h incubation period, the yeast increased the extracellular β-1,3-glucanase activities, it reached the
299 maximum after 96 h in minimal salt media with *B. cinerea* cell walls (133 U µmol glucose released/mg
300 protein/h), sucrose (125 U) and glucose (126 U) as sole carbon sources. At 120 h, the β-1,3-glucanase

activities started to decrease (Fig. 1).

An obvious inhibition of the pathogen mycelia by the purified enzyme was observed as compared to the control after incubation on PDA at 25°C for two days (Fig. 2a). Moreover, in presence of purified crude enzyme, the decayed lesions (15 mm in diameter) caused by *B. cinerea* on apples were markedly reduced when compared to the control inoculated with the pathogen alone (35 mm in diameter) (Fig. 2b).

3.2 Cloning exo-β-1,3-glucanase gene

To clone the partial gene sequence encoding an extracellular exo-β-1,3-glucanase in *P. guilliermondii* M8, degenerate primers were designed according to the conserved amino acid sequences of extracellular exo-β-1,3-glucanases from different yeast species (Fig. 3). With the degenerate primers, Grads-PCR produced a reliable fragment of around 500 bp. Analysis of the sequence by BLAST program suggested that a fragment of the putative exo-β-1,3-glucanase gene was isolated and it contained the consensus motif (IGIEALNEPL) of the signature pattern of Family 5 hydrolases that is highly conserved among the exo-β-1,3-glucanases. New primers were designed from the sequence of the putative exo-β-1,3-glucanase fragment in combination with the kit Gene Walking Made Easy (Table 1) using the 5' and 3' flanking regions of the fragment. The new amplicons were sequenced, assembled and subjected to BLAST and ORF Finder analysis. Finally, the whole gene (GenBank accession number HQ113463), designated as *PgExg1*, encoding the exo-1,3-β-glucanase, was cloned from the genomic DNA of the antagonistic yeast strain M8 (Fig. 4).

To amplify the ORF encoding exo-1,3-β-glucanase from the cDNA of *P. guilliermondii* M8, new specific primers were designed according to the sequence of the whole gene. PCR on the first-strand cDNA of *P. guilliermondii* M8 as template with the new primers produced one specific fragment of the same size

(1,224 bp) of the genomic DNA. After sequencing analysis of the specific fragment obtained from the cDNA, it was found that the ORF from the cDNA shared the same nucleotide sequence with that of the genomic DNA, confirming that no intron exists inside the exo-1,3-β-glucanase gene (*PgExg1*) sequence.

3.3 Analysis of *P. guilliermondii* M8 *PgExg1* gene

As shown in Fig. 4, *P. guilliermondii* M8 *PgExg1* gene has an open reading frame of 1,224 bp encoding a 408-amino acid (aa) protein with a calculated molecular weight (M_r) of 46.9 kDa. Signal peptide analysis of the protein deduced from the *PgExg1* gene indicated that the signal peptide has **15** amino acids. The peptide bond between the 15th and 16th amino acid would be cleaved by a signal peptidase (Fig. 4). Prediction of isoelectric point of the deduced protein sequence revealed that the deduced protein from the *PgExg1* gene had a pI of 4.54.

Using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/es/Cgi-bin/clustalw2), alignment of the deduced amino acid sequence of *PgExg1* with those of other exo-β-1,3-glucanases retrieved from NCBI database indicated that the deduced amino acids shared a consensus motif (IGIEALNEPL) which is the signature pattern of GH Family 5 hydrolases, suggesting that PgExg1 belongs to the GH 5 Family (Fig. 4). Moreover, a BLAST search performed using the *PgExg1* ORF as query sequence indicated that the enzyme PgExg1 has high sequence identity and similarity to exo-β-1,3-glucanases (EC 3.2.1.58) from other yeasts (Table 2). The latter enzymes are members of the Glycoside Hydrolase (GH) Family 5 (formerly known as cellulose family A). In addition, the PgExg1 with a pI of 4.54 is an acidic protein, which are similar to other exo-β-1,3-glucanases previously obtained from many yeasts and has the signature pattern of GH Family 5 hydrolases which indicated that PgExg1 belongs to GH Family 5.The topology of the phylogenetic tree indicated that the amino acids deduced from *PgExg1* were also closely related to that of

other exo-glucanases of other *Candida* or *Pichia* species (Fig. 5). The tree clustered the glucanases into three subgroups. *P. anomala* EXG1 (CAA05243) and *S. cerevisiae* EXG2 (CAA92719) clustered into one subgroup. *T. stipitatus* [ATCC10500] Exg1 (XP_002483905.1), *S. cerevisiae* Exg2p (CAA92719.1) and *L. edodes* EXG (BAD97445.1) belonged to another subgroup. PgExg1 and the remaining glucanases formed another subgroup. Among the glucanases in the phylogenetic tree, PgExg1 is the closest to the glucanase CoEXG1 (Genbank accession number-AAM 21469) of *C. oleophila*.

3.4 Expression of the *PgExg1* gene in *E. coli*

SDS-PAGE on the cell free extracts from the induced cells of *E. coli* BL21 (DE3) containing the plasmids pET-23a(+)-*PgExg1* presented one specific band with a molecular mass of about 47 kDa. This was a fusion hybrid protein and was similar to the size of 46.9 kDa as calculated from the deduced amino acid sequence of *PgExg1* gene. This conclusion was confirmed by the result of a Western blotting assay which directly demonstrated that the specific band with a molecular mass of about 47.0 kDa in SDS-PAGE was actually the His-tagged fusion protein of the recombinant enzyme (Fig. 6).

3.5 Effect of pH and temperature on the activity and stability of the recombinant enzyme

The activity of the recombinant exo-β-1,3-glucanase was higher at pH 5.0 (Fig. 7a). At pH 4.0 and 6.0, the recombinant enzyme still had 47% and 40% relative activity, respectively. However, at pH higher than 8.0, the enzyme activity was very low. At the optimum pH of 5.0, the recombinant enzyme had the highest activity at 40°C (Fig. 7b).

The residual activity of the recombinant exo-β-1,3-glucanase pre-incubated in buffers at various pH was higher at pH 7.0. Moreover, when pre-incubated at pH values between 4.0 and 10.0, the recombinant

enzyme still had high residual activities (Fig. 7c), indicating that the recombinant enzyme had a good stability in a wide range of pH values. When the recombinant enzyme was pre-incubated at temperatures between 4 and 30°C for 2 h, its residual activity was still very high (Fig. 7d). However, it greatly decreased above 37°C, suggesting that the stability of the recombinant enzyme was easily affected by temperature.

3.6 Antifungal activity of the recombinant PgExg1 in controlling the growth of *B. cinerea in vitro*

No significant differences in germination percentage were observed among the treatments, however, the germ tube length (97 µm) of the pathogen spores in presence of the recombinant enzyme PgExg1 was significantly lower compared to the controls (217 and 223 µm) (Table 3).

4. Discussion

Though much work has been done to develop biocontrol agents against postharvest pathogens of fruits, most of the biocontrol efficacy evaluation with antagonistic yeasts was carried out only under lab conditions. Moreover, so far few postharvest biocontrol products such as Biosave® (*Pseudomonas syringae* Van Hall) and Shemer® (*Metschnikowia fructicola* Kurtzman & Droby) active against *B. cinerea* on apples are commercially available (Droby et al., 2009). Previous research (Zhang et al., 2011) showed that the yeast *P. guilliermondii* strain M8 had a good efficacy in controlling grey mold on apples under semi-commercial conditions. Moreover, even though *P. guilliermondii* has been successfully used to control postharvest diseases on a number of fruits and vegetables (Tian et al., 2002; Scherm et al., 2003), very little research has been done to study its efficacy in controlling grey mold, caused by *B. cinerea*, on apples.

The knowledge of the mechanism of biocontrol is an important factor in enhancing the biocontrol

activity and for establishing screening criteria to select new antagonists (Qin et al., 2003). However, the mechanisms of BCAs against postharvest pathogens are still poorly understood, mainly due to the fact that appropriate methods and technologies to study the complex interactions of antagonists and pathogens in fruit wounds are limited, although some mechanisms have been explained. Currently, various mechanisms have been described, including antibiosis, production of lytic enzymes, parasitism, induced resistance and competition for limiting nutrients and space (Janisiewicz and Korsten, 2002). Generally, more than one mechanism is involved in the interactions of one antagonist against the pathogens.

The production of hydrolytic enzymes able to degrade fungal cell walls has been reported as an important mechanism of suppression of pathogens on fruits by BCAs. Howell (2003) found that a β-1,3-glucanase produced by *Trichoderma* spp. was directly involved in inhibiting some fungal pathogens. Masih and Paul (2002) showed that β-1,3-glucanase secreted by *Pichia membranaefaciens* had good biocontrol activity against *B. cinerea* causing grey mold of grapevine. *P. membranaefaciens* grown with cell wall of the pathogen as sole carbon source produced higher activity of glucanase but less activity in the presence of sucrose as sole carbon source (Masih and Paul (2002). However, our research showed that *P. guilliermondii* strain M8 produced high activities of the enzyme when grown in the presence of sucrose or the pathogen cell wall as sole carbon source, suggesting that the strain M8 has a stronger capacity to produce the extracellular β-1,3-glucanase than *P. membranaefaciens*. Moreover, in this research the biocontrol activity of exo-β-1,3-glucanase secreted by *P. guilliermondii* was examined using the native enzyme and the recombinant enzyme to inhibit the pathogens. The result indicated that although the recombinant enzyme PgExg1 did not decrease the germination rate of the pathogen conidia, it significantly reduced the germ tube elongation. The results from the tests *in vivo* and *vitro* of enzyme activities against pathogens suggested that the secretion of exo-1,3-β-glucanase is involved in the biocontrol activity of *P.*

411 *guilliermondii* strain M8 against pathogens. To further determine its specific role, such as determinant or
412 additional role, in the biocontrol mechanisms, exo-1,3-β-glucanase deficient mutants will be generated.

413 Due to the potential role of exo-β-1,3-glucanase in the biocontrol (Oelofse et al., 2009), glucanase
414 genes have been cloned and characterized from some microbial antagonists. Monteiro and Ulhoa (2006)
415 biochemically characterized a β-1,3-glucanase from *Trichoderma koningii* induced by the cell wall of
416 *Rhizoctonia solani*. Marcello et al. (2010) successfully cloned and characterized an exo-β-1,3-glucanase
417 gene from the mycoparasitic fungus *Trichoderma asperellum*. Although production of β-1,3-glucanase
418 proved to be an important mechanism of biocontrol for some antagonistic yeast against pathogens, little
419 work was done in cloning and characterizing β-1,3-glucanase genes from antagonistic yeasts. Grevesse et
420 al. (2003) isolated a gene, *PaEXG2* (CAA11018), from the antagonistic yeast *Pichia anomala* strain K. Xu
421 et al. (2006) cloned and characterized an exo-β-1,3-glucanase from the yeast *Pichia pastoris*. As far as we
422 know, there is no published report on cloning and characterizing β-1,3-glucanase genes from the
423 antagonistic yeast *P. guilliermondii.*

424 In this research, the gene *PgExg1* encoding exo-β-1,3-glucanase was successfully cloned from the
425 antagonistic yeast *P. guilliermondii* strain M8 by using " genome walking" and PgExg1 was confirmed to
426 be one member of GH 5 Family. Moreover, PgExg1 was comprehensively compared with the
427 exo-β-1,3-glucanases of other yeasts in similarities, identities as well as molecular weights and pIs. In
428 comparison, the PgExg1 had extremely high identity and similarity to the exo-β-1,3-glucanase
429 (XP_001385760.2) isolated from the yeast *Pichia stipitis* CBS 6054 (Jeffries et al., 2007). Moreover, the
430 phylogenetic tree indicated that PgExg1 has very high homology to the glucanase CoEXG1 which has
431 been characterized as an exo-β-1,3-glucanase of the yeast *C. oleophila* (Segal et al., 2002). These results
432 further confirmed that PgExg1 is one member of the GH 5 family. The ORF size of exo-β-1,3-glucanases

433 of diverse yeasts ranged from 408 to 562 amino acids, and the molecular weights ranged from 45.7 to 63.5
434 kDa. Interestingly, the pIs of all exo-β-1,3-glucanases are below 7.0, suggesting that although the
435 exo-β-1,3-glucanase genes from various yeast species are different in length, the enzymes they code are
436 acidic proteins.

437 *PgExg1* gene was expressed in *E. coli* BL21 (DE3) and yielded homogeneous recombinant PgExg1.
438 This confirmed the possibility of producing a large quantity of PgExg1 through gene expression in *E. coli*.
439 Activity and stability are the most important characteristics of one enzyme. In this research, optimal pH
440 and temperature for the activity and stability of the purified recombinant exo-β-1,3-glucanase obtained
441 from *E. coli* BL21 were investigated. As to activity, it has been reported that the optimum pH range of
442 β-1,3-glucanases from fungi is generally between pH 3.0 and pH 6.0 (El-Katatny 2008), exceptions are
443 enzymes from *Phytophthora infestans* and *Polyporus* species, which are more active at pH 7.0 (Pitson et
444 al., 1993). Our result from PgExg1 is consistent with this report. El-Katatny (2008) showed that the
445 temperature optimum of β-1,3-glucanase preparations from *Trichoderma harzianum* (free or immobilized)
446 was in the range from 50 to 60°C. However, in this study, the optimal temperature of the recombinant
447 β-1,3-glucanase PgExg1 was lower than that of *T. harzianum* β-1,3-glucanase.

448 The pH and thermal stability is vital to enzyme activity. Like other β-1,3-glucanase, the recombinant
449 PgExg1 showed the highest stability at neutral situations and a high stability in a wide range of pH values.
450 When stored at 4°C, the recombinant enzyme exhibited the highest activity under the same conditions as
451 compared to other storage temperatures, suggesting that low temperatures are compatible with the enzyme
452 activity on fruits during storage. Stored at lower temperature, the enzyme exhibited higher stability, which
453 could be attributed to prevention of thermal denaturation (Hayashi and Ikada 1990).

454 In conclusion, the yeast *P. guilliermondii* strain M8 produced high activity of exo-1,3-β-glucanase

when grown in different carbon sources. Moreover, an exo-1,3-β-glucanase gene, *PgExg1*, of *P. guilliermondii* strain M8 was successfully cloned and characterized, and the recombinant PgExg1 expressed in *E. coli* BL21 had a strong activity in controlling the growth of *B. cinerea*.

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Tables

Table 1

Primers and vectorette units used for amplification and identification flanking regions of *PgExg1* by genome walking with the kit “Gene Walking Made Easy”

Flanking regions of the gene	Primers	Restriction digestion enzymes	Vectorette library
5’ prime	5’-TAGGATCATTGTCCAAAAG-3’	*Mae* II	*Cla* I vectorette units
3’ prime	5’-CCAATGCTTGGAATGTGG-3’	*Mfe* I	*EcoR* l vectorette units

599 **Table 2**. Amino acid sequence identities and similarities between *P. guilliermondii* PgExg1 and other fungal exo-beta-1,3-gluancase and some characteristics of the

600 glucanases

Source*	Accession No.	Identity (%)	Similarity (%)	Size (AA)	Predicted molecular weight (kDa)	pI
Pichia guilliermondii PgExg1	HQ113463	-	-	408	46.9	4.54
Pichia angusta EXG	CAA86948.1	52	69	435	49.3	4.59
Pichia stipitis CBS 6054 EXG	XP_001385760.2	66	77	438	50.0	4.71
Pichia anomala EXG2	CAA11018	58	72	427	45.7	4.45
Pichia anomala EXG1	CAA05243	46	62	498	58.1	4.79
Saccharomyces cerevisiae EXG1	AAA34599.1	54	69	448	51.3	4.57
Saccharomyces cerevisiae Exg2p	CAA92719.1	45	60	422	47.1	6.14
Saccharomyces cerevisiae YJM789 EXG	EDN63719.1	52	66	445	51.9	5.58
Saccharomyces cerevisiae EXG2	CAA92719	41	56	562	63.5	5.18
Candida tropicalis MYA-3404 EXG	XP_002550037.1	62	76	435	49.6	4.52
Pichia pastoris EXG	AAY28969.1	57	72	414	47.8	4.53
Candida oleophila EXG1	AAM21469	62	75	425	48.8	4.48
Candida dubliniensis CD36 EXG	XP_002416951.1	64	76	438	50.0	5.06
Candida albicans SC5314 EXG	XP_721488.1	64	76	438	50.0	5.36
Wickerhamomyces anomalus EXG	ABK40520.1	58	72	427	49.2	4.66
Williopsis saturnus EXG	ACP74152.2	60	74	417	47.7	4.72
Yarrowia lipolytica EXG	CAA86952.1	50	67	421	48.2	4.98
Talaromyces stipitatus [ATCC10500] Exg1	XP_002483905.1	45	59	424	46.8	4.78
Lentinula edodes EXG	BAD97445.1	42	57	421	46.1	4.58

601 *The β-1,3-glucanases are retrieved from NCBI database. Predicted molecular weight and pI were calculated at the website: http://expasy.org/cgi-bin/pi_tool.

602 Identity and similarity were performed by alignments at the website: http://blast.ncbi.nlm.nih.gov.

603 **Table 3**

604 Antifungal activity of the recombinant PgExg1 in controlling the growth of *B. cinerea in vitro*. The
605 pathogen spores were co-cultured with the recombinant enzyme PgExg1 in PDB, serving as the enzyme
606 treatment (Pathogen+ Enzyme), and inactivated enzymes or sterile distilled water instead of the enzyme
607 solution served as controls (Pathogen+ Inactivated enzyme, and Pathogen+ Water).

608

Treatments	Antifungal activity of the recombinant enzyme PgExg1 in controlling the growth of *B .cinerea in vitro*	
	Germ tube length (%) **	Germination rate (%) **
Pathogen+enzyme	97 ± 6.6 a	97 ± 0.6 b
Pathogen+inactivated enzyme	217 ± 12.4 b	99 ± 1.0 a
Pathogen+water (Control)	223 ± 8.3 b	99 ± 0.6 a

609

610 ** The results are the mean of two independent experiments. "±" stands for standard error of the means.
611 Values of each column followed by different letters show significant difference ($P<0.05$) according to
612 analysis by Duncan's Multiple Test (SPSS 13.0).

613

Figures and Captions

Fig. 1. β-1,3-glucanase (EC3.2.1.58) activity of *P. guilliermondii* strain M8 grown in Lilly-Barnett minimal salt medium supplemented with 2 mg ml^{-1} CWP (cell wall preparation of *B. cinerea*) (▒), glucose (▒) or sucrose (▒) as sole carbon source at 25°C for 120 h. The specific activity was expressed as micromoles of glucose per milligram protein per hour (µmol glucose released/mg protein). Bars represented standard deviations of the means.

Fig. 2. Inhibition *in vitro* and *in vivo* of *B. cinerea* growth by purified crude enzymes produced by *P. guilliermondii* strain M8. For the test *in vitro*, a) purified enzyme solution was added to two wells made near the edge of the 4 day-old *B. cinerea* colony, and after two days, inhibition of the pathogen mycelia growth was measured. For the test *in vivo*, b) aliquots of 100 µl of the purified crude enzymes were pipetted into each wound site of apples. Incubated for 2 h at room temperature, inoculated with 30 µl of *B. cinerea* spore suspension at 10^5 spores ml^{-1} and stored at 20°C for 3 days, when the diameters of the rotten lesions were observed.

Fig. 3. Alignment of the deduced amino acid sequence of eight exo-β-1,3-glucanase genes retrieved from NCBI database: Pa-EXG, *P. angusta* EXG (CAA86948.1); Pp-EXG, *P. pastoris* EXG (AAY28969.1); Pa-EXG2, *P. anomala* EXG2 (CAA11018); Cd-EXG, *C. dubliniensis* CD36 EXG (XP_002416951.1); Ca-EXG, *C. albicans* SC5314 EXG (XP_721488.1); Ps-EXG, *P. stipitis* CBS 6054 EXG (XP_001385760.2); Co-EXG1, *C. oleophila* EXG1 (AAM21469); Sc-EXG, *S. cerevisiae* YJM789 EXG (EDN63719.1). Multiple sequence alignment of proteins was performed by using the DNAMAN 6.0.

Identical residues are highlighted by black boxes.

Fig. 4. The nucleotide sequence and deduced amino acid sequence of exo-1,3-β-glucanase gene *PgExg1* from *P. guilliermondii* M8. One GC box, two TATA boxes, and one CAAT box were highlighted in bold, respectively. The signal peptide cleavage site is indicated by an arrowhead. Numbers on the left represent nucleotide positions and numbers in bold on the left represent amino acid positions. The signature pattern of Family 5 hydrolases was underlined. Asterisk (*) represents the terminator of *PgExg1*. The underlined (AATAATA) is the potential polyadenylation signal. CA and GT-rich sequences were highlighted within the black box.

Fig. 5. Phylogenetic analysis of PgExg1 and closely related β-1,3-glucanases. The β-1,3-glucanases are retrieved from NCBI database as seen in Table 2. Phylogenetic analysis was performed using the program MEGA 4.1. The numbers at node indicate the bootstrap percentages of 1000 resamples. The DDBJ/Genbank/EMBL accession numbers are indicated in parentheses.

Fig. 6. Confirmation of the recombinant exo-β-1,3-glucanase encoded by the *PgExg1* gene in *E. coli* BL21 by Western-blotting with monoclonal anti-polyhistidine as first antibody and A1293-AlkPhos APA Mouse Fab ads HIgG as second antibody. Lane PgExg1 represents the protein extracts from the cells of recombinants containing pET23a(+)-*PgExg1*; Lane CK, the protein extracts from the cells of recombinants containing the empty vector pET23a(+).

Fig. 7. Effects of pH (a) and temperature (b) on the activity of recombinant exo-β-1,3-glucanase encoded

by the *PgExg1* gene in *E. coli* BL21 and pH (c) and temperature (d) on stability of the enzyme. The relative activity at different pH's and temperature was calculated based on the activity of recombinant PgEXg1 obtained by the reaction at pH 5.0 and 40°C (relative activity = 100%). For the effect of pH value on the stability, the relative activity was based the recombinant enzyme activity at pH 7.0 (relativity activity = 100%). For the effect of temperature on the stability, the sample pre-incubated at 4°C was used as a reference (relative activity = 100%). The details were seen in the section (2. 9) of Materials and Methods. There were three replicates of each treatment. Bars indicate ± SD of the means.