Chitinase and Antifungal Activity of Endophytic Fungi Isolated from *Hedychium coronarium* J. Koenig

Anna MENDROFA
Erman MUNIR (✉)
Yurnaliza YURNALIZA
Anisa LUTFIA
Adrian HARTANTO

Summary

Chitinase (EC 3.2.1.14) is a class of hydrolytic enzyme capable of degrading polymeric chitin, and it has been reported and isolated from many sources. In the present study, we evaluated the ability of endophytic fungi isolated from native *Hedychium coronarium* J. Koenig in producing chitinase following its characterization and the test of its inhibitory activities against *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, a pathogenic fungus causing wilt disease among common corps in North Sumatra. Twelve endophytic fungal isolates were tested for their ability to grow on chitin agar medium, resulting in only two isolates, namely isolate JRE 1A and JRE 4B, capable of producing a visible clear zone indicating the secretion of extracellular chitinase. The two isolates were observed for their morphology and identified on the basis of molecular markers (ITS1–ITS4) within the region of ITS-rDNA, resulting in the species designation of *Trichoderma afroharzianum* JRE 1A and *Aspergillus fumigatus* JRE 4B. The production was detected highest after 4 days of incubation for *A. fumigatus* JRE 4B (4.76 U/mL) and 3 days for *T. afroharzianum* JRE 1A (4.15 U/mL). The crude chitinases showed stable activities within the pH range of 4–7, stimulated by Mn^{2+} and Zn^{2+} and slightly inhibited by K^{+} and Ca^{2+}. Furthermore, the strongest antagonistic activity against *F. oxysporum* was displayed by *T. afroharzianum* (CGI > 70%) followed by *A. fumigatus* (CGI > 30%). The precipitated crude chitinases extracted from the endophytes also inhibited the growth of the tested phytopathogenic fungi.

Key words

biocontrol, chitinase, *Fusarium oxysporum*, ITS-rDNA

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, North Sumatra, Indonesia

✉ Corresponding author: erman@usu.ac.id

Received: September 17, 2020 | Accepted: April 13, 2021

Introduction

Endophytes are ecologically important groups of microorganisms that live inside the healthy tissue of a plant without developing any obvious detrimental effects on their host (Hyde and Soytong, 2008). They are ubiquitously present across plant species, with their prospects upon beneficial properties shared genetically or chemically with their hosts (Naik et al., 2008). Following the progressive trend of endophytic microbial exploration in recent years, endophytic fungi stood out as the most common group of microorganisms elaborated in the laboratory to produce a variety of secondary metabolites such as alkaloids, flavonoids, terpenoids, volatile organic compounds (VOC), phenols and its derivatives, and potential hydrolytic enzymes for many applications (Zhang et al., 2006; Strobel, 2018).

Medicinal plants are promising sources of prominent fungal isolates, especially for their production of extracellular enzymes, with potential use in industrial application or as fungicides to control the growth of phytopathogenic fungi (Meenavalli et al., 2011; Toghueo et al., 2017). Chitinolytic fungi are a diverse functional group of fungi with the ability to secrete extracellular chitinase that decompose chitin, a β-(1,4)-linked polymer of N-acetyl-D-glucosamine, and the cell wall structure of most phytopathogenic fungi along with other versatile bioactive compounds (Hartl et al., 2012).

The information on chitinolytic fungi recovered from the rhizome or root parts of *Hedychium coronarium* is still limited. Recent investigations revealed an assemblage of fungal genera within the leaf parts of Indian cultivar of *H. coronarium* such as *Alternaria, Aspergillus, Bipolaris, Fusarium, Nigrospora, Penicillium*, and *Trichoderma* with the ability to produce amylase, cellulase, pectinase, laccase, and asparaginase (Uzma et al., 2016). In the previous studies, we explored and reported the occurrence of endophytic fungal species residing within the rhizomes of some Zingiberaceous species within the genera of *Amomum, Elettaria, Etlingera, Globba*, and *Hedychium* in North Sumatra (Lutfia et al., 2019a; 2019b; 2019c; 2019d; Munir et al., 2019). Some species were also reported to act as antagonists that inhibit the growth of phytopathogenic fungi (Munir et al., 2020; Lutfia et al., 2020). Meanwhile, the mechanism of inhibition was still unknown but assumed to be the activity of extracellular chitinase produced by the strains. Here, we evaluated two potential strains from our collection to produce chitinase in a batch fermentation study following its enzyme characterization and growth inhibition against *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, a causal agent of wilt disease in some local commodities and *Ganoderma boninense* Pat. (1889), a causal agent of basal stem rot (BSR) in oil palm.

Materials and Methods

Screening of Chitinolytic Fungi

Thirty one endophytic fungal isolates from previous studies on native Zingiberaceous species (*Alpinia* sp., *Amomum centrocephalum*, *Elettaria* sp., *Etlingera elatior*, *Hedychium coronarium*) in the forest of North Sumatra were maintained in potato dextrose agar (PDA, Merck®) slants in the Laboratory of Microbiology, Universitas Sumatera Utara, Medan, Indonesia. Of 31 isolates, only two fungal isolates from *H. coronarium*, namely JRE 1A and JRE 4B which produced visible growth on the chitin agar plate (0.3 g of KH_2PO_4, 0.7 g of K_2HPO_4, 0.5 g of $MgSO_{4.7}H_2O$, 0.01 g of $FeSO_{4.7}H_2O$, 0.001 g of $ZnSO_4$, 0.001 g of $MnCl_2$, 72.7 mL of 1% (w/v) colloidal chitin, 15 g of agar in 1 L of distilled water) as positive indications of the presence of extracellular chitinase.

Molecular Identification of Fungal Isolates Based on ITS-rDNA

Fungal genomic DNA was isolated based on the technical procedure by Wizard® Genomic DNA Purification Kit Protocol (United States). The rDNA-ITS region was amplified using the universal primer for fungi: ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Manter and Vivanco, 2007) with reaction mixture comprised of 12 µL nuclease-free water (NFW), 20 µL GoTaq DNA Polymerase solution, 2 µL ITS-1F primer solution, 2 µL ITS-4 primer solution, and 4 µL DNA template solution with a total volume of 40 µL in Eppendorf tube. The PCR was conditioned as 95°C for 3 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72 °C for 45 sec, and final extension at 72°C for 7 min. The PCR products were visualized on 1% agarose gels and delivered for sequencing (Macrogen, Inc., Singapore). The raw sequences were qualitatively assembled to construct the consensus sequence. The ITS-rDNA sequences were submitted to the NCBI Genbank database to obtain the accession numbers. The phylogenetic tree of rDNA-ITS sequence was constructed based on the maximum likelihood fits of nucleotide substitution models, which produced the lowest Bayesian Information Criterion (BIC) with 1000× bootstrapping using MEGA X (Kumar et al., 2018).

Chitinase Assay

The culture plugs of the two isolates, JRE 1A and JRE 4B, were inoculated in 100 mL of colloidal chitin broth and incubated at 28°C for 7 d at 120 rpm. Cultural filtrates were harvested through centrifugation at 11,000 rpm for 10 min. Chitinase activity was determined as the amount of released *N*-acetyl-D-glucosamine (NAG) under the initial conditions (Monreal and Reese, 1969). A reaction mixture of 2 mL of culture supernatant, 2 mL of phosphate buffer pH 7.0, and 2 mL of 0.3% (w/v) colloidal chitin was incubated at 37°C for 30 min. Then, 1 mL of dinitrosalycilic acid was added to the tube and heated at 100°C for 10 min. The calibration curve with NAG as a standard was used to determine the product concentration in the interval of 0–1000 µg mL^{-1} and the absorbance of the reaction mixture was measured at A540. The whole experiment was conducted in duplicate.

Effect of pH and Metal Ions on Chitinase Activity and Stability

The effect of the pH of the chitinase stability was determined by reacting the enzyme with substrates at different pHs (4–9) by 50 mM acetate, phosphate, and Tris-HCl buffer. The effect of the metal ions was performed by incubating the enzyme in 0.1 mL of 1 mM metal ion solutions such as K^+, Na^+, Ca^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, and Fe^{3+}. The chitinase activity was expressed as a relative activity (%) compared to the initial activity in the previous method.

Antagonistic and Antifungal Test of Chitinolytic Fungi

The antagonistic test against *F. oxysporum* and *G. boninense* was based on a dual culture plate assay in a chitin agar plate. Culture plugs of the phytopathogenic fungi were inoculated at the middle 3 days prior to the inoculation of chitinolytic fungi. The colony growth inhibition (CGI) was measured in percentage (%) after 5 days of incubation (Bivi et al., 2010). Cultural filtrates were concentrated in 70% ammonium sulphate at 10°C and centrifuged at 11,000 rpm for 10 min at 4°C to obtain the pellets. The pellets were resuspended in a phosphate buffer pH 7.0 to achieve the volume of 2 mL crude chitinases. An antifungal test against phytopathogenic fungi was performed in a similar manner to the antagonistic test, with an exception of the inoculation of 10 µL crude chitinase into a blank disk. CGI was measured after 5 days of incubation.

Results and Discussion

Morphology of Fungal Isolates and Growth Performance on Chitin Agar Plate

Two fungal isolates, JRE 1A and JRE 4B, showed good growth on chitin agar plates, which produced a mass of mycelium immediately after incubation at 28°C for 5 d (Fig. 1). A microscopical examination revealed that isolate JRE 1A showed the characteristics of a member of *Trichoderma* by having branched conidiophores clustered into fascicles (Fig. 1A).

Isolate JRE 4B showed the characteristics of a member of *Aspergillus* by having terminate conidiophores in a dome-shaped vesicle with uniseriate phialides on the upper portion of the vesicle (Fig. 1B). A chitin agar plate was used to visualize the production of extracellular chitinase by our isolates. The diameter of clear zone around fungal colonies indicated the hydrolisis of chitin into monomeric products or N-acetyl glucosamine (NAG). A higher degree of hydrolisis was observed from isolate JRE 1A for covering the entire plate with their full colony growth after incubation. The supplementation of colloidal chitin will induce the production of chitinase by chitinolytic microorganisms (Hsu and Lockwood, 1975). The most common source of chitin is crab or shrimp waste pre-treated with strong acids, producing soluble chitin or a colloidal solution. The chitinase may then be induced, producing both the endo- and exochitinase into the screening medium by the strains. Different chitin substrates may also yield a different degree of hydrolisis on the preliminary plate assay, commonly either the α-form, the most abundant in nature, or the β-form, the least common in nature.

Phylogenetic Relationship among ITS-rDNA Database

The maximum likelihood statistical method and the Kimura-2 parameter model were used to construct the phylogenetic trees of the chitinolytic fungal isolates from *H. coronarium*. Here, we constructed two phylogenetic trees to distinguish between *Trichoderma* and *Aspergillus* members (Fig. 2 and 3). Based on the evolutionary distance and bootstrap value (BV) ≥ 60%, each isolate was reliably assigned to the species level of identification. Therefore, the isolate JRE 1A was identified as *Trichoderma afroharzianum* (MT740379), while the isolate JRE 4B was identified as *Aspergillus fumigatus* (MT740380).

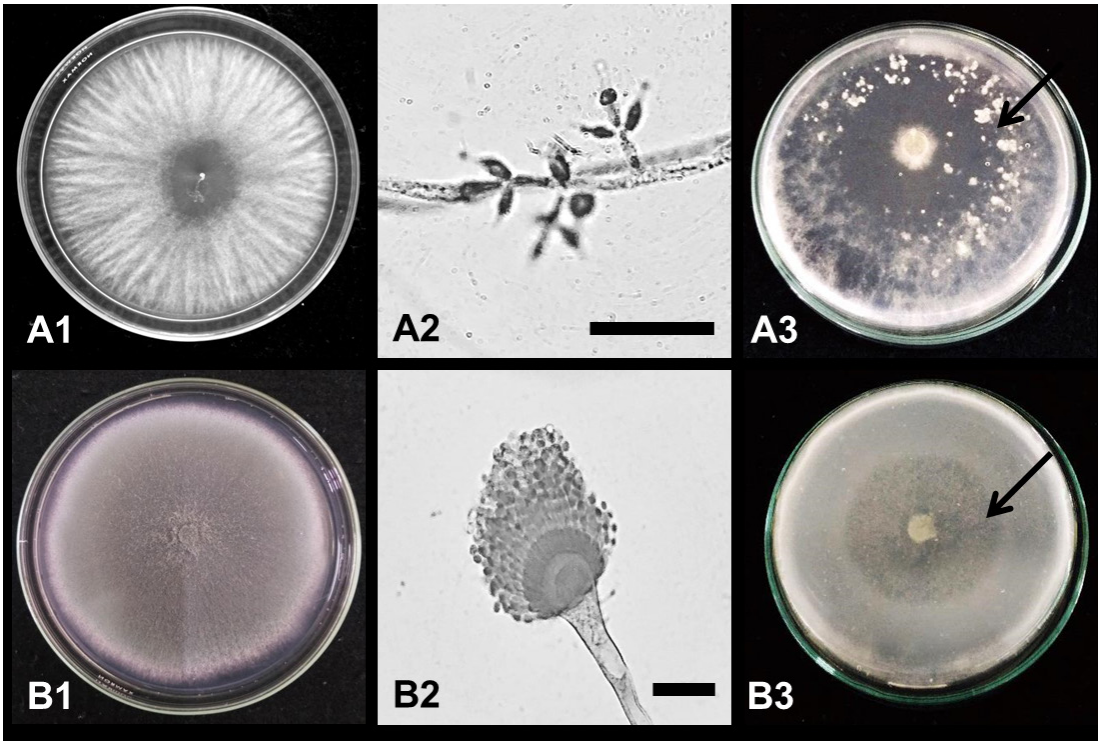

Figure 1. Colony morphology of isolate *Trichoderma afroharzianum* JRE 1A (A1) and *Aspergillus fumigatus* JRE 4B (B1) grown on potato dextrose agar at 25 °C for 3 d. Conidiophore of isolate JRE 1A (A2) and JRE 4B (B2). Extracellular chitinase activity by isolate JRE 1A (A3) and JRE 4B (B3) on chitin agar plate after incubation at 25°C for 7 d. Scale bar = 20 µm

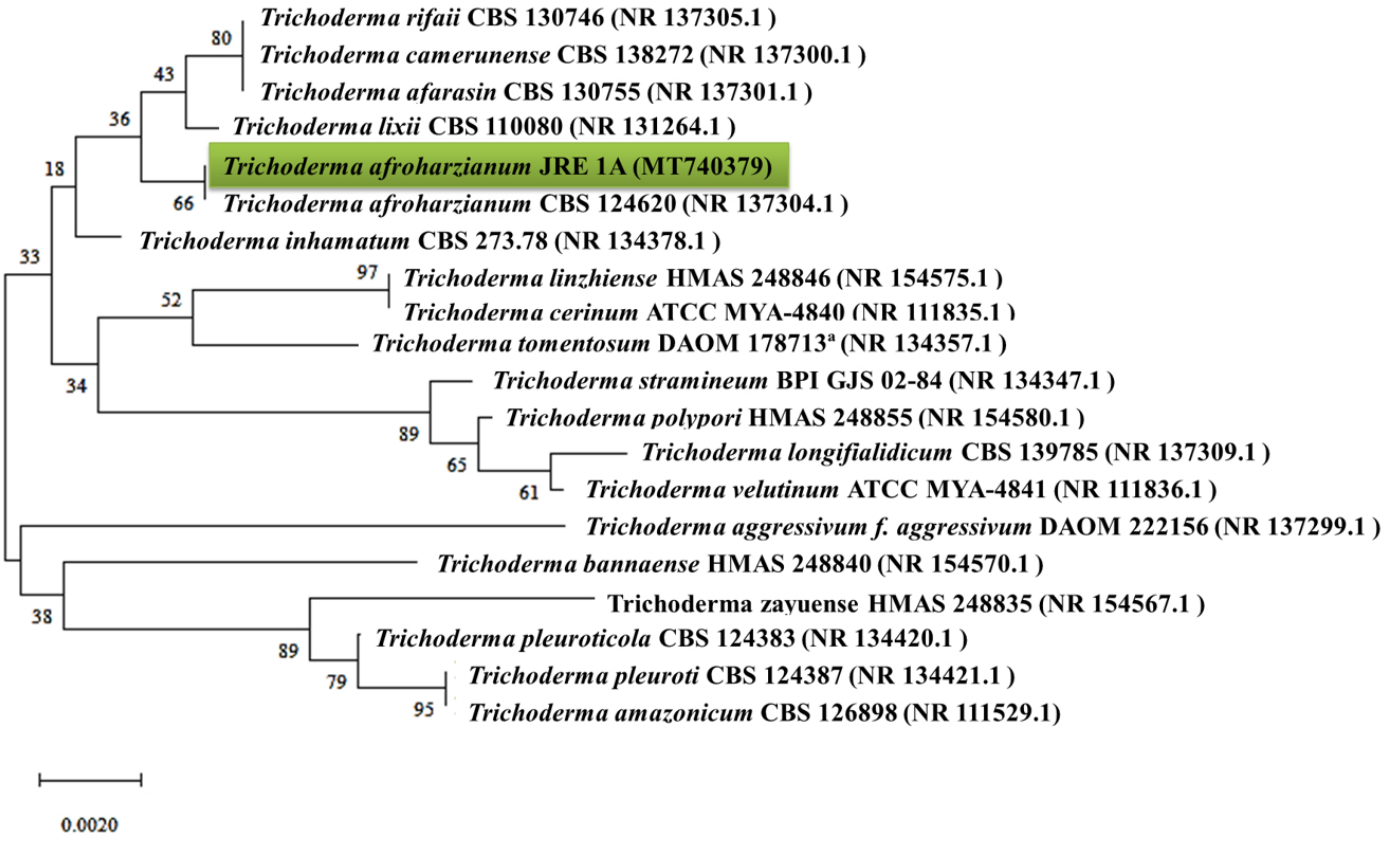

Figure 2. Phylogenetic tree of *Trichoderma* isolates and its members based on rDNA-ITS region

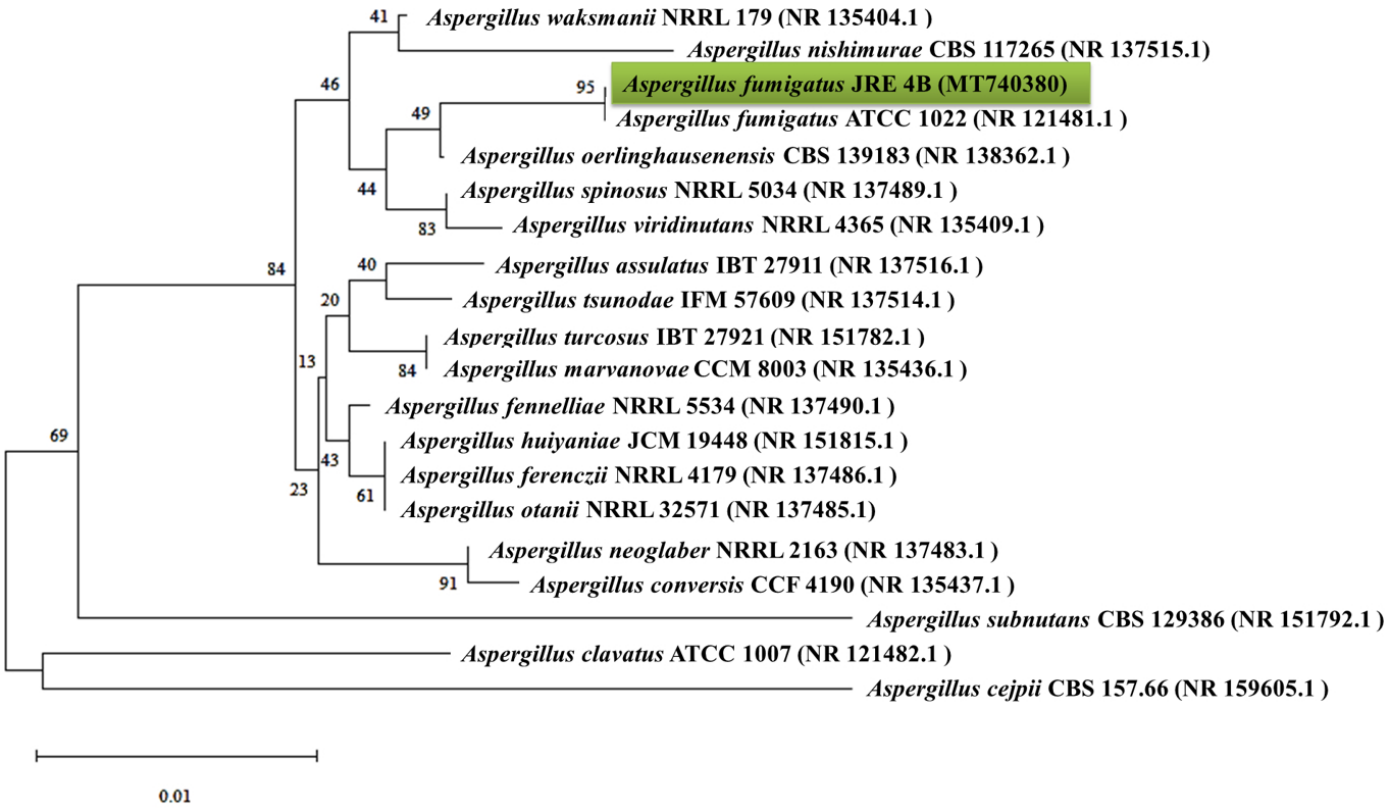

Figure 3. Phylogenetic tree of *Aspergillus* isolates and its members based on rDNA-ITS region

Characteristics of Chitinase Activity by Fungal Isolates

The chitinase activity was determined through daily observation for 7 days in colloidal chitin broth (Fig. 4). The hydrolisis of chitin can be observed visually, as evidenced by the growth of fungal colonies and the clearance of the medium in the absence of available colloidal chitin (Fig. 5). Based on the results, *A. fumigatus* JRE 4B produced the highest chitinase activity (4.76 U/mL) on day 4, followed by *T. afroharzianum* JRE 1A (4.15 U/mL) on day 3. The results showed that each isolate grew at a different rate with the optimum growth, as indicated by the highest chitinase activities produced by the isolates. The higher the number of active-growing cells in a population in a medium, the more the hydrolytic enzyme secreted to optimize the conversion of chitin into their biomasses.

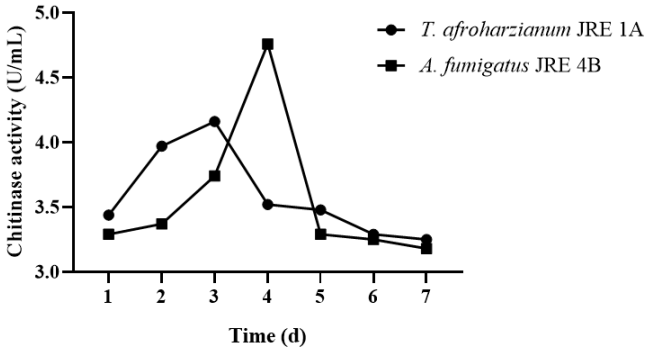

Figure 4. Production and chitinase activity of chitinolytic fungi *Trichoderma afroharzianum* JRE 1A and *Aspergillus fumigatus* JRE 4B in colloidal chitin broth for 7 d of incubation at 28°C

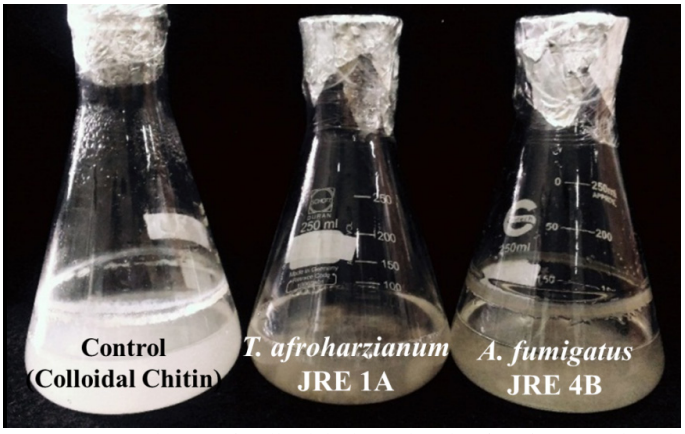

Figure 5. Condition of colloidal chitin broth after fermented with chitinolytic fungi *Trichoderma afroharzianum* JRE 1A and *Aspergillus fumigatus* JRE 4B at 7 d of incubation at 28°C

Members of *Trichoderma* spp. were known as common soil mycoflora that produced a variety of chitinases with a different degree of chitinase activity, such as *Trichoderma harzianum* TUBF 966 (14.7 U/mL) (Sandhya et al., 2004), *T. asperellum* isolates of Pakistan (± 22 U/mL) (Asad et al., 2015), and *T. asperellum* PQ34 (22 U/mL) (Loc et al., 2020), etc. In addition, chitinase activity produced by the members of *Aspergillus* was also reported, though lesser than in the studies using *Trichoderma*, such as *Aspergillus* sp. S1-13 (0.01 U/mg) (Rattanakit et al., 2007), *Aspergillus niger* LOCK 62 (9 U/mg) (Brzezinska and Jankiewicz, 2012), *Aspergillus terreus* isolate of Saudi Arabia (2–65 U/mg) (Farag et al., 2016), etc. It is also noteworthy that different fermentation conditions and chitinolytic strains will yield different maximum chitinase activities in the laboratory.

Based on the enzyme characteristics, our study revealed that chitinase was stable and active in the pH range of 4–7, with optimal activity at pH 7 for *T. afroharzianum* JRE 1A and pH 6 for *A. fumigatus* JRE 4B, which was higher than the initial condition (Fig. 6). A more basic environment affected the chitinase to a significant downfall of activity at pH>7. Moreover, the chitinase of *T. afroharzianum* JRE 1A was more stable than *A. fumigatus* JRE 4B, which retained its activity >80% within the pH range of 4–6, indicating its tolerance toward an acidic environment. Similar results were reported in a study using *T. atroviride* PTCC5220 with optimum chitinase activity at pH 5.0 (Harighi et al., 2006). In general, the chitinases from microbial sources were active within the pH range of 5–8 (Brzezinska et al., 2014).

Depending on the origin of the chitinase produced by various chitinolytic strains, the chitinase activity may be either inhibited or stabilized by the presence of metal ions (Table 1). In our study, most of the tested metal ions showed slight inhibition (>80%), especially toward chitinase activity produced by *A. fumigatus* JRE 4B by the presence of Zn^{2+} and Mn^{2+}, which acted as stimulators. On the contrary, the chitinase activity was widely stimulated for *T. afroharzianum* JRE 1A by the presence of various metal ions, with only documented inhibition by K^{+} and Ca^{2+}. The addition of Mn^{2+} and Zn^{2+} stimulated the chitinase activity of both strains, with the highest being provided by Mn^{2+}. Similar results were reported from a study using purified chitinase produced by *Penicillium* sp. LYG 0704, which was stimulated in the presence of Mn^{2+} (Lee et al., 2009). In other studies using *Trichoderma viride* AUMC 13021, the isolate of mangrove soil was also reported to increase its purified chitinase activity by 40% in the presence of Mn2+, although inhibited by the presence of Hg^{2+}, Zn^{2+}, Cu^{2+}, and Co^{2+} (Abu-Tahon and Isaac, 2020).

Table 1. Effect of metal ions on chitinase activity

Metal ions	Relative activity (%)	
	T. afroharzianum JRE 1A	*A. fumigatus* JRE 4B
Control	100	100
K^{+}	83	80
Ca^{2+}	96	82
Pb^{+}	108	90
Na^{+}	111	91
Cu^{2+}	115	91
Fe^{3+}	118	95
Mg^{2+}	128	94
Zn^{2+}	152	105
Mn^{2+}	156	136

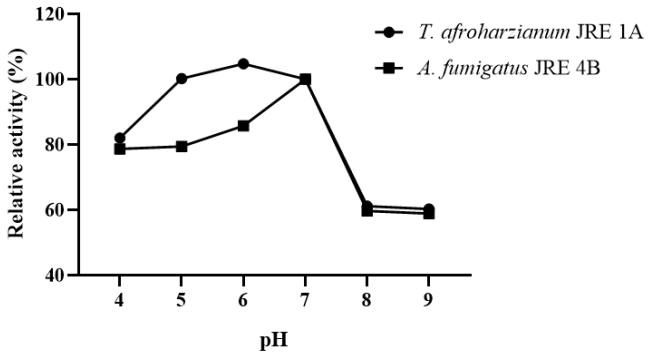

Figure 6. Effect of pH on chitinase activity of *Trichoderma afroharzianum* JRE 1A and *Aspergillus fumigatus* JRE 4B at 37°C. A mixture of acetate, phosphate, and Tris-HCl buffer at a concentration of 50 mM was used

Based on the antagonistic and antifungal tests, it can be seen that *F. oxysporum* was the most sensitive phytopathogenic fungus tested in our study, with the CGI reaching 75% (Fig. 7). Meanwhile, *G. boninense* was inhibited under a low percentage of CGI (<20%) in using both chitinolytic fungi and crude chitinase. When a fungal colony is exposed to chitinase, several changes in the physiological conditions leading to inhibition, such as delayed spore germination and elongation, distortions of hypha, and leakage of cell interior due to digested cell wall by the enzyme, may occur (Elad et al., 1982; Wang et al., 1999; Lima et al., 1999).

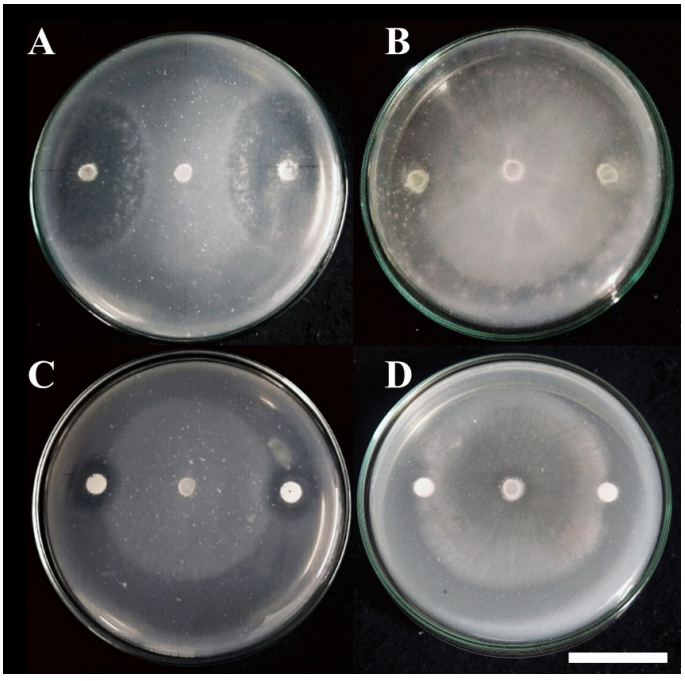

Figure 7. Colony growth inhibition of chitinolytic fungi (*Trichoderma afroharzianum* JRE 1A) against (A) *Fusarium oxysporum*, (B) *Ganoderma boninense*. Antifungal activity of crude chitinase (*Aspergillus fumigatus* JRE 4B) against (C) *Fusarium oxysporum*, (D) *Ganoderma boninense*. Scale bar: 3 cm

Moreover, the chitin structure and composition among fungal species may be intrinsically variable, which explains why, in certain cases, chitinase has not shown any significant antifungal activity against *G. boninense*. Besides the chitinase activity upon the inhibition of hyphal growth, some antagonistic fungi may also secrete other hydrolytic enzymes such as glucanase and protease to effectively digest the cell walls of their competitors or surpass their capacity to metabolize available resources leading to competitive exclusion in a dual culture test. In addition to hydrolytic enzymes, some species of *Trichoderma* were also reported to secrete bioactive metabolites called Trichodermin. The compound belongs to trichothecenes, a large group of terpene-derived secondary metabolite mainly synthesized by *Trichoderma* spp. which may improve their antifungal activity during antagonistic interaction (Wilkins et al., 2003).

Conclusion

Chitinolytic fungi isolated from some Zingiberaceous species in North Sumatra revealed two potential strains, namely *T. afroharzianum* JRE 1A and *A. fumigatus* JRE 4B, from *H. coronarium*. The maximum chitinase activity of these strains reached >4 U/mL within 3–4 d of incubation. The chitinase of *T. afroharzianum* JRE 1A was stable in a wide range of acidic conditions (pH 4–7), but both strains showed a decline in chitinase activity in pH>7. Ion metals such as Mn^{2+} and Zn^{2+} were considered as stimulators for chitinase activity. The use of *T. afroharzianum* JRE 1A and *A. fumigatus* JRE 4B had more potential than the crude chitinase, as shown by the higher CGI (%) against phytopathogenic fungi, *F. oxysporum* and *G. boninense*.

Acknowledgements

This project is fully funded by Universitas Sumatera Utara through scheme of TALENTA Research Grant of 2019.

References

Abu-Tahon, M.A., Isaac, G.S. (2020). Anticancer and Antifungal Efficiencies of Purified Chitinase Produced from *Trichoderma viride* under Submerged Fermentation. J Gen Appl Microbiol 66: 32–40. doi: 10.2323/jgam.2019.04.006

Asad, S.A., Tabassum, A., Hameed, A., Hassan, F.U., Afzal, A., Khan, S.A., Ahmed, R., Shahzad, M. (2015). Determination of Lytic Enzyme Activities of Indigenous *Trichoderma* Isolates from Pakistan. Braz J Microbiol 46: 1053–1064. doi: 10.1590/S1517-838246420140787

Bivi, M.R., Farhana, M.S.N., Khairulmazmi, A., Idris, A. (2010). Control of *Ganoderma boninense:* A Causal Agent of Basal Stem Rot Disease in Oil Palm with Endophytic Bacteria in vitro. Int J Agric Biol 12: 833–839

Brzezinska, M.S., Jankiewicz, U. (2012). Production of Antifungal Chitinase by *Aspergillus niger* LOCK 62 and Its Potential Role in the Biological Control. Curr Microbiol 65: 666–672. doi: 10.1007/s00284-012-0208-2

Brzezinska, M.S., Jankiewicz, U., Burkowska, A., Walczak, M. (2014). Chitinolytic Microorganisms and Their Possible Application in Environmental Protection. Curr Microbiol 68: 71–81. doi: 10.1007/s00284-013-0440-4

Elad, Y., Chet, I., Henis, Y. (1982). Degradation of Plant Pathogenic Fungi by *Trichoderma harzianum*. Can J Microbiol 28: 719 – 725. doi: 10.1139/m82-110

Farag, A.M., Abd-Elnabey, H.M., Ibrahim, H.A.H., El-Shenawy, M. (2016). Purification, Characterization and Antimicrobial Activity of Chitinase from Marine Derived *Aspergillus terreus*. Egypt J Aquat Res 42: 185 – 192. doi: 10.1016/j.ejar.2016.04.004

Harighi, M.J., Motallebi, M., Zamani, M.R. (2006). Purification of Chitinase 42 from *Trichoderma atroviride* PTCC5220. Iran J Biol 19: 203–214

Hartl, L., Zach, S., Seidl-Seiboth, V. (2012). Fungal Chitinases: Diversity, Mechanistic Properties, and Biotechnological Applications. Appl Microbiol Biotechnol 93: 533–543. doi: 10.1007/s00253-011-3723-3

Hsu, S.C., Lockwood, J.L. (1975) Powdered Chitin Agar as A Selective Medium for Enumeration of Actinomycetes in Water and Soil. Appl Microbiol 29: 422–426

Hyde, K.D., Soytong, K. (2008). The Fungal Endophyte Dilemma. Fungal Divers 33: 163–173

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35: 1547–1549. doi: 10.1093/molbev/msy096

Lee, Y.G., Chung, K.C., Wi, S.G., Lee, J.C., Bae, H.J. (2009). Purification and Properties of a Chitinase from *Penicillium* sp. LYG 0704. Protein Expr Purif 65: 244–250. doi: 10.1016/j.pep.2008.12.004

Lima, L.H.C., De Marco, J.L., Felix, C.R. (1999). Synthesis of a *Trichoderma* Chitinase Which Affects the *Sclerotium rolfsii* and *Rhizoctonia solani* Cell Walls. Folia Microbiol 44: 45–49. doi: 10.1007/bf02816220

Loc, N.H., Huy, N.D., Quang, H.T., Lan, T.T., Ha, T.T.H. (2020). Characterisation and Antifungal Activity of Extracellular Chitinase from a Biocontrol Fungus, *Trichoderma asperellum* PQ34. Mycology 11: 38–48. doi: 10.1080/21501203.2019.1703839

Lutfia, A., Munir, E., Yurnaliza, Basyuni, M. (2019a). Screening of Antagonistic Fungi from *Etlingera littoralis* (J. Konig) Giseke Rhizome in Sibolangit Forest, North Sumatra. J Phys Conf Ser 1351: 012025. doi: 10.1088/1742-6596/1351/1/012025

Lutfia, A., Munir, E., Yurnaliza, Y. (2019b). Antagonistic Endophytic Fungi of *Globba pendula* Roxb. from Taman Hutan Raya, North Sumatra against *Staphylococcus aureus* ATCC® 29213™. IOP Conf Ser Earth Env Sci 305: 012003. doi: 10.1088/1755-1315/305/1/012003

Lutfia, A., Munir, E., Yurnaliza, Y. (2019c). Antagonistic Endophytic Fungi of *Hedychium coronarium* J. Koenig from Hutan Sibayak and Taman Hutan Raya, North Sumatra against *Staphylococcus aureus* ATCC® 29213™. IOP Conf Ser Earth Env Sci 305: 012002. Doi: 10.1088/1755-1315/305/1/012002

Lutfia, A., Munir, E., Yurnaliza, Y. (2020). Molecular Identification of Endophytic Fungi from Torch Ginger (*Etlingera elatior*) Antagonist to Phytopathogenic Fungi. Biodiversitas 21: 2681–2689. doi: 10.13057/biodiv/d210641

Lutfia, A., Munir, E., Yurnaliza, Y., Hartanto, A. (2019d). Antagonistic Fungal Endophytes Colonizing Rhizome of *Amomum centrocephalum* A. D. Poulsen from North Sumatera, Indonesia. Int J Adv Sci Eng Inf Tech 9: 1427–1432. doi: 10.18517/ijaseit.9.4.8781

Manter, D.K., Vivanco, J.M. (2007). Use of the ITS Primers, ITS1F and ITS4, to Characterize Fungal Abundance and Diversity in Mixed-Template Samples by qPCR and Length Heterogeneity Analysis. J Microbiol Methods 71: 7–14. doi: 10.1016/j.mimet.2007.06.016

Meenavalli, B., Rajulu, G., Thirunavukkarasu, N., Suryanarayanan, T.S., Ravishankar, J.P., Gueddari, N.E.E., Moerschbacher, B.M. (2011). Chitinolytic Enzymes from Endophytic Fungi. Fungal Divers 47: 43–53. doi: 10.1007/s13225-010-0071-z

Monreal, J., Reese, E.T. (1969). The Chitinase of *Serratia marcescens*. Can J Microbiol 15: 689 – 696. doi: 10.1139/m69-122

Munir, E., Lutfia, A., Yurnaliza, Y. (2019). Records of Culturable Endophytic Fungi Inhabiting Rhizome of *Elettaria* in Hutan Sibayak, North Sumatera. IOP Conf Ser Earth Env Sci 305: 012004. doi: 10.1088/1755-1315/305/1/012004

Munir, E., Lutfia, A., Yurnaliza. (2020). Colonization of *Trichoderma* spp. in Rhizome of Five Zingiberaceous Species from North Sumatra, Indonesia. Int J Agric Biol 24: 831–837. Doi: 10.17957/IJAB/15.1506

Naik, S.B., Shashikala, J., Krishnamurthy, Y. (2008). Fungal Communities in Herbaceous medicinal Plants from the Malnad Region, Southern India. Microbes Environ 23: 24–28. doi: 10.1264/jsme2.23.24

Rattanakit, N., Yano, S.H., Plikomol, A., Wakayama, M., Tachiki, T. (2007). Purification of *Aspergillus* sp. S1–13 Chitinases and Their Role in Saccharification of Chitin in Mash of Solid-State Culture with Shellfish Waste. J Biosci Bioeng 103: 535–541. doi: 10.1263/jbb.103.535

Sandhya, C., Adapa, L.K., Nampoothiri, K.M., Binod, P., Szakacs, G., Pandey, A. (2004). Extracellular Chitinase Production by *Trichoderma harzianum* in Submerged Fermentation. J Basic Microbiol 44: 49 – 58. doi: 10.1002/jobm.200310284

Strobel, G. (2018). The Emergence of Endophytic Microbes and Their Biological Promise. J Fungi 4: 57. doi: 10.3390/jof4020057

Toghueo, R.M.K., Zabalgogeazcoa, I., Vazquez de Aldana, B.R., Boyom, F.F. (2017). Enzymatic Activity of Endophytic Fungi from the Medicinal Plants *Terminalia catappa*, *Terminalia mantaly* and *Cananga odorata*. South Afr J Bot 109: 146–153. doi: 10.1016/j.sajb.2016.12.021

Uzma, F., Konappa, N.M., Chowdappa, S. (2016). Diversity and Extracellular Enzyme Activities of Fungal Endophytes Isolated from Medicinal Plants of Western Ghats, Karnataka. Egyptian J Basic Appl Sci 3: 335 – 342. doi: 10.1016/j.ejbas.2016.08.007

Wang, S.L., Yieh, T.C., Shih, I.L. (1999). Purification and Characterization of a New Antifungal Compound Produced by *Pseudomonas aeruginosa* K-187 in a Shrimp and Crab Shell powder Medium. Enzyme Microb Technol 25: 439–446. doi: 10.1016/S0141-0229(99)00069-1

Wilkins, K., Nielsen, K.F., Din, S.U. (2003). Patterns of Volatile Metabolites and Nonvolatile Trichothecenes Produced by Isolates of *Stachybotrys, Fusarium, Trichoderma, Trichothecium*, and *Memnoniella*. Environ Sci Pollut Res 10: 162–166. doi: 10.1065/espr2002.05.118

Zhang, H.W., Song, Y.C., Tan, R.X. (2006). Biology and Chemistry of Endophytes. Nat Prod Rep 23: 753–771. doi: 10.1039/b609472b

aCS86_15