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[Article]

Characterization of L-asparaginase-producing *Trichoderma* spp. Isolated from Marine Environments

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L-asparaginase (ASNase) is a therapeutic enzyme used to treat acute lymphoblastic leukemia. Currently, the most widely used ASNases are originated from bacteria. However, owing to the adverse effects of bacterial ASNases, new resources for ASNase production should be explored. Fungal enzymes are considered efficient and compatible resources of natural products for diverse applications. In particular, fungal species belonging to the genus *Trichoderma* are well-known producers of several commercial enzymes including cellulase, chitinase, and xylanase. However, enzyme production by marine-derived *Trichoderma* spp. remains to be elucidated. While screening for extracellular ASNase-producing fungi from marine environments, we found four strains showing extracellular ASNase activity. Based on the morphological and phylogenetic analyses using sequences of translation elongation factor 1-alpha (*tef1α*), the *Trichoderma* isolates were identified as *T. afroharzianum*, *T. asperellem*, *T. citrinoviride*, and *Trichoderma* sp. 1. All four strains showed different ASNase activities depending on the carbon sources. *T. asperellem* MABIK FU00000795 showed the highest ASNase value with lactose as a carbon source. Based on our findings, we propose that marine-derived *Trichoderma* spp. are potential candidates for novel ASNase production.

Keywords: L-asparaginase, Marine fungi, *Trichoderma* spp.

Introduction

The genus *Trichoderma* Persoon belongs to the family Hypocreaceae and contains more than 250 species (Bissett et al., 2015). *Trichoderma* species are ubiquitous and have been isolated from various environments and substrates, such as soil, agricultural fields, and plant debris; as well as in marine resources, including mudflats, sea sand, and seaweeds (Kredics et al., 2014; Kim et al., 2020). Approximately 78 metabolites from *Trichoderma* spp. have been reported (Su et al., 2018), some of which exhibit antimicrobial, antioxidant, and cytotoxic activities (Zhang et al., 2017; Vinale et al., 2016).

Trichoderma is also known to produce various enzymes, such as an array of carbohydrate-active enzymes (CAZymes). Cellulases, hemicellulases, and chitinases, which belongs to glycoside hydrolases (GHs), have been employed in the bioconversion of biomass (di Cologna et al., 2018; Peciulyte et al., 2014). For example, *T. reesei* is a major source of cellulolytic enzymes and is a lignocellulosic biomass degrader (Adav et al., 2012; Bischof et al., 2016). *T. harzianum* has potential biotechnological applications because of its ability to degrade and metabolize lignocellulosic plant materials (Ferreira et al., 2017). In addition, the influence of carbon sources and metals on enzyme production such as cellulase and β-glucosidase in *Trichoderma* spp. have been studied to understand their mechanisms with regard to the induction and regulation of enzymes (Mandels and Reese, 1957; Loewenberg, 1984).

L-asparaginase (ASNase), which converts L-asparagine to aspartic acid, is used to treat acute lymphoblastic leukemia (ALL). ALL is the most common cancer in children aged 2~10 years (Egler et al., 2016). In addition, ASNase is used to remove carcinogenic compound acrylamide found in food (Doriya and Kumar, 2016). ASNase is produced by a variety of plants and microorganisms

Table 1. General information of four marine-derived *Trichoderma* spp. isolated from marine environments

Identity	ID	Substrate	Sampling site	Sampling date	Accession No.
Trichoderma asperellum	MABIK FU00000795	Mudflat	Gilsang-myeon, Ganghwa-gun, Incheon[A]	January 2018	OP515524
T. citrinoviride	MABIK FU00000804	Sea Sand	Hwado-myeon, Ganghwa-gun, Incheon[B]	January 2018	OP535388
T. afroharzianum	MABIK FU00000805	Mudflat	Gilsang-myeon, Ganghwa-gun, Incheon[A]	January 2018	OP535389
Trichoderma sp.	MABIK FU00000822	Sea Sand	Sokcho-si, Gangwon-do[C]	January 2015	OP535390

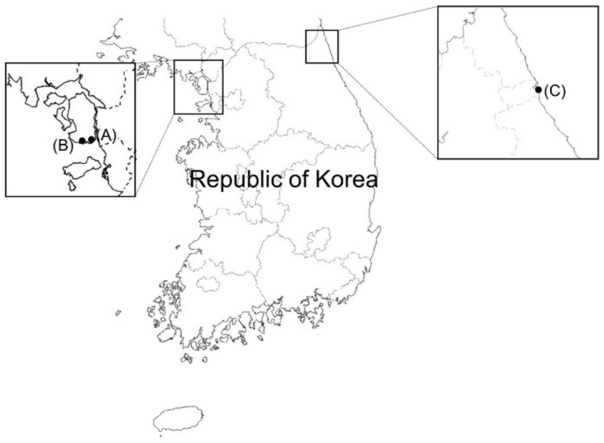

Fig. 1. The map of sampling sites. (A) Hwado-myeon, (B) Gilsang-myeon, Ganghwa-gun, Incheon, and (C) Sokcho Beach in Sokcho-si, Korea. Four strains of *Trichoderma* spp. were isolated and deposited in Marine Microbial BioBank (MMBB). Raw map was obtained from National Geographic Information Institute, Republic of Korea.

including bacteria, fungi, and algae. These microorganisms, especially bacteria, are relatively inexpensive and efficient sources of ASNase. Indeed, ASNase from *Pectobacterium carotovorum* and *Escherichia coli* have been utilized to treat ALL (Ramya et al., 2012). However, due to the side effects induced by bacterial ASNases, such as allergic reactions, fever, and neurological problems, novel ASNases should be explored.

Although the genus *Trichoderma* is well known for producers of valuable metabolites, production of ASNase by *Trichoderma* spp. has rarely been reported. Therefore, in this study, we searched for marine-derived *Trichoderma* spp. with ASNase activity and identified them using morphological and phylogenetic analyses. The effect of carbon sources on the extracellular ASNase activity of *Trichoderma* spp. was investigated. Through this paper, we suggested *Trichoderma* spp. served as ASNase sources potentially.

Materials and Methods

1. Isolation and preparation of *Trichoderma* spp. cultures

Mudflats and sea sand were collected from Incheon and Sokcho-si, Republic of Korea, and diluted with sterile distilled water. The diluted samples were spread on potato dextrose agar (PDA; BD Difco, Sparks, MD, USA) containing 0.01% (w/v) ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% (w/v) streptomycin (Sigma-Aldrich), and incubated at 25℃ for 7~14 days. During that period, individual fungal colonies were picked and transferred to fresh PDA in order to obtain pure cultures. After isolation, fungal strains were cultured on PDA at 28℃ unless otherwise described. Fungal spores and hyphal fractions were suspended in 20% glycerol (v/v) and stored at -80℃. Sample collection and general information for *Trichoderma* isolates are presented in Table 1 and Fig. 1.

2. Morphological observation

To observe the fungal colony morphology, all isolates were pre-cultured on PDA at 28℃ for 2 days. Subsequently, using agar plugs (8 mm diameter) obtained from the pre-cultures on PDA, individual strains were inoculated on PDA, yeast mold agar (YMA; BD Difco), malt extract agar (MEA; BD Difco), and czapek yeast extract agar (CYA; czapek dox broth; BD Difco, 5.0 g/l yeast extract and 15.0 g/l agar), followed by cultivation at 28℃ for 5 days. Colony characteristics such as color and shape were observed by naked eyes.

3. DNA extraction, sequencing, and phylogenetic analysis

To extract genomic DNA, *Trichoderma* spp. were incubated in potato dextrose broth (PDB; BD Difco) at 28℃ for 3 days. Genomic DNA was then extracted from the harvested mycelia previously established protocols (Chung et al., 2019). Polymerase chain reaction (PCR) was conducted using the primers EF1-728F (5'-CAT CGA GAA GTT CGA GAA GG-3') (Carbone and Kohn, 1999) and TEF1LLErev (5'-AAC TTG CAG GCA ATG TGG-3') (Jaklitsch et al., 2005) to amplify the translation elongation factor 1-alpha (*tef1α*) gene. PCR conditions included an initial denaturation of 95℃ for 5 min; followed by 34 cycles of denaturation at 95℃ for 15s, annealing at 58℃ for 30s, and extension at 72℃ for 1 min; and finally a post-polymerization at 72℃ for 15 min. PCR products were purified using a Gel Extraction kit (Qiagen, Hilden, Germany). Subsequently, the purified products were sequenced by Macrogen Inc. (Seoul, South Korea).

The *tef1α* sequences were identified in the GenBank database by BLAST searches. The sequences were aligned and a phylogenetic tree was constructed using MEGA7.0 (Kumar et al., 2016). A phylogenetic analysis was performed using the neighbor-joining method (Kimura two-parameter model), followed by bootstrapping with 1,000 random replicates (Kimura, 1980) in MEGA7.0. The reference *tef1α* sequences were obtained from GenBank database.

The four *Trichoderma* spp. isolates were deposited in Marine Microbial BioBank (MMBB) culture collections of the National Marine Biodiversity Institute of Korea (MABIK) after identification.

4. Screening of L-asparaginase producing *Trichoderma* spp.

To screen ASNase-producing *Trichoderma* spp., Modified Czapek Dox agar (MCDA) (Gulati et al., 1997) was used for the plate assay. The components of 1 ℓ of MCDA were as follows: 2.0 g glucose, 10.0 g L-asparagine, 1.52 g KH_2PO_4, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) $CuNO_3 \cdot 3H_2O$, 0.05% (w/v) $ZnSO_4 \cdot 7H_2O$, 0.05% (w/v) $FeSO_4 \cdot 7H_2O$ and 15.0 g agar, supplemented with 0.005% (w/v) phenol red as indicator of ammonia production. The pH was adjusted to 6.0 using 1 N NaOH. Furthermore, control plates were prepared with $NaNO_3$, instead of L-asparagine, as a nitrogen source. *Aspergillus terreus* MABIK FU00000783 obtained from MMBB was used as the positive control (de-Angeli et al., 1970). Agar plugs (8 mm diameter) from pre-cultured isolates were inoculated on ASNase-containing and control plates, followed by incubation at 28℃ for 5 days. The presence of pink zones around colonies indicated ASNase enzyme activity.

5. Effects of carbon source on extracellular L-asparaginase activities

Agar plugs (diameter 8 mm) of *Trichoderma* isolates were inoculated in 100 ml of Modified Czapek Dox broth (MCDB) and incubated at 28℃ for 5 days with shaking at 120 rpm. The components of the MCDB were the same as those of the MCDA, except for agar and phenol red. To examine the effect of ASNase production depending on different carbon sources, 0.2% (w/v) glucose, fructose, sucrose, lactose, and soluble starch were supplemented to MCDB as the sole carbon source. Supernatants from the four isolates were prepared to investigate extracellular ASNase activity. Briefly, the supernatants were collected using a Miracloth (Merck, New Jersey, USA) and sterilized using syringe filters with 0.2 μm pore size (Corning, NY, USA). ASNase activity was measured using the Asparaginase Activity Assay Kit (ab107922, Abcam, Cambridge, MA, USA), according to manufacturer's instructions (Kim et al., 2015). In the assay, fungal supernatants were used to hydrolyze L-asparagine as a substrate to produce L-aspartate, which is subsequently converted to pyruvate. Pyruvate reacts with the probe to form a stable chromophore, which can be detected at 570 nm. One unit of asparaginase was defined as the amount of asparaginase generating 1.0 μmol of aspartate per minute at 25℃. An enclosed positive control solution, which is an asparaginase enzyme solution, was used to ensure confidence in the results.

For data analysis, two-way ANOVA was used to clarify the effects of species and carbon sources on ASNase activities.

Results and Discussion

1. Colony morphology and growth rate of *Trichoderma* spp. on various media

The morphological characteristics of the four *Trichoderma* strains on PDA, YMA, MEA and CYA were observed (Fig. 2). Strain MABIK FU00000795 formed abundant aerial mycelia and conidia. Its colonies were distributed radically on all types of media. Compared to the white to dark green colonies on PDA, the colonies on YMA, MEA and CYA zonated distinctly with different colors. Strain MABIK FU00000804 had abundant conidia, with broad concentric rings on PDA, YMA and MEA. Its colonies on PDA formed pale olive to pale green conidia, whereas those on YMA formed

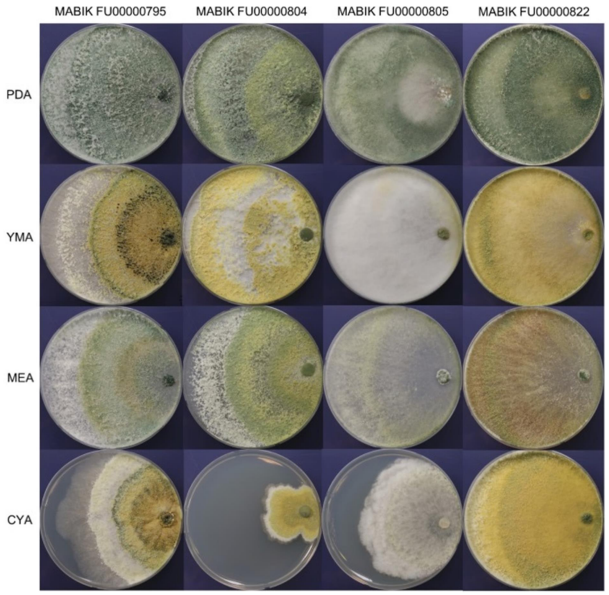

Fig. 2. Colony of marine-derived *Trichoderma* spp. Agar plugs (8 mm diameter) taken from cultured isolates were inoculated at edge positions on PDA, YMA, MEA, and CYA plates. Inoculated plates were incubated at 28℃ for 5 days.

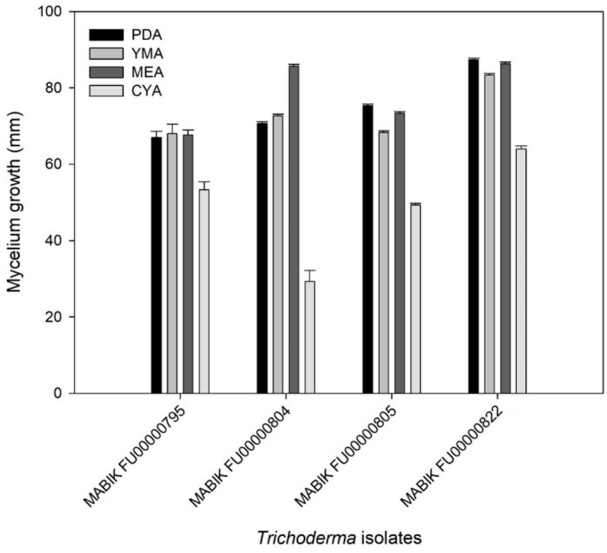

Fig. 3. Mycelial growth of *Trichoderma* spp. After 3 days of culturing at 28℃, mycelial growth of *Trichoderma* isolates on different nutrient media (PDA, YMA, MEA, and CYA).

yellow to grayish yellow pustules. On MEA, colonies zonated distinctly with white and olive-colored conidia in the outermost layer and inner layers, respectively. Furthermore, MABIK FU00000804 had a slow growth on CYA compared on PDA, YMA and MEA, and it had olive-colored conidia around the inoculums. Strain MABIK FU00000805 appeared white and pale green on PDA. Aerial mycelia and cottony conidia were abundant on YMA. On CYA, whitish to pale greenish aerial mycelia appeared hairy to floccose and radical. On all media, strain MABIK FU00000822 formed aerial mycelia that had abundant cottony conidia. Colonies appeared circular, pale green to dark green, and radially distributed on PDA. Meanwhile, yellow-green colonies were observed on YMA, white to olive colonies on MEA, and yellow to dark green colonies on CYA. Diffusing pigments were observed on all media. The pigments were yellow to pale green, orange to brown, yellow to pale green, and brown on PDA, YMA, MEA, and CYA, respectively.

Overall, the four *Trichoderma* spp. showed distinct growths on PDA, YMA, MEA and CYA (Fig. 3). The growth of mycelia on CYA was slower than that on other media. Taken together, the marine-derived *Trichoderma* spp. in this study presented different growth rates and colony morphologies depending on the media.

2. Phylogenetic analysis

Morphological and molecular methods have been used to investigate the taxonomy of *Trichoderma* (Samuels et al., 2002). Distinguishing *Trichoderma* species based only on morphological characteristics is difficult because they are morphologically similar (Kim et al., 2020). Because the internal transcribed spacer rDNA, the universal fungal DNA barcoding marker, is not sufficient to identify certain fungal clades using molecular methods, *tef1α* is recommended as a barcoding marker for the phylogeny of *Trichoderma* (Raja et al., 2017).

In this study, marine-derived *Trichoderma* spp. were identified via phylogenetic analysis of *tef1α* (Fig. 4). For molecular identification, the *tef1α* sequence information were deposited in GenBank (accession numbers were represented in Table 1). The phylogenetic tree contained 36 taxa of *Trichoderma* strains. The four strains (in bold) were grouped into distinct clades: MABIK FU00000795 in the *Hamatum* clade, MABIK FU00000804 in the *Longibrachiatum* clade, and MABIK FU00000805 and MABIK FU00000822 in the *Harzianum* clade. The BLASTN search conducted using *tef1α* sequences showed that MABIK FU00000795 is closely related to *T. asperellum* B1902, C261s, T2007, M01, and CBS 433.97. MABIK FU0000084 was grouped with *T. citrinoviride* DAOM 172792, 18CPHA003, S279 and TRS745. MABIK FU00000795 and MBIK FU00000804 were identified as *T. asperellum* and *T. citrinoviride*,

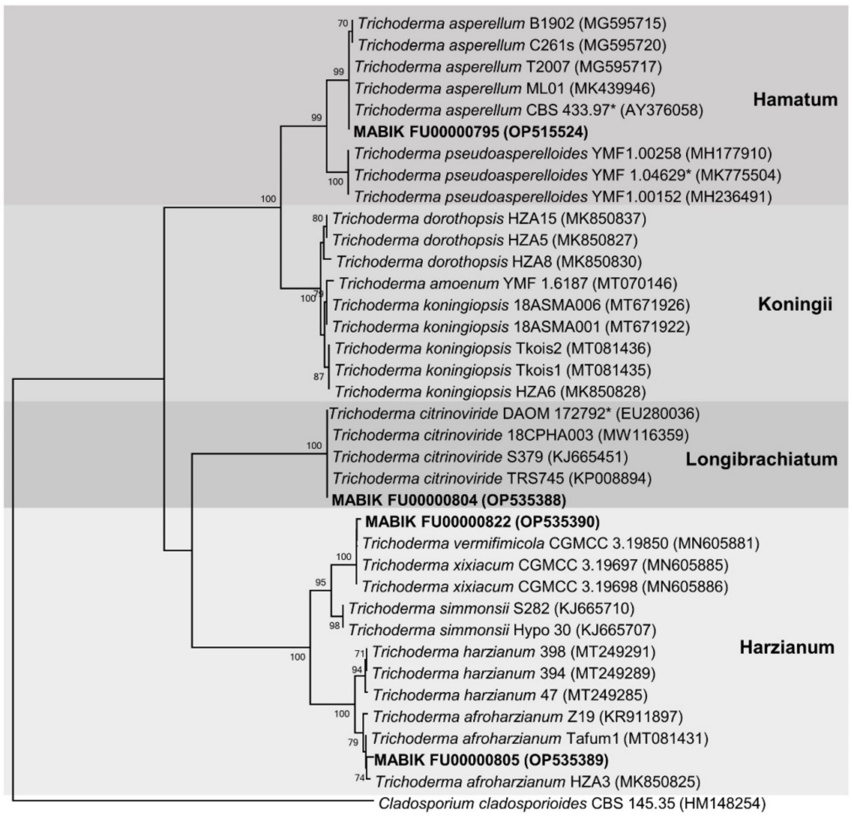

Fig. 4. The neighbor joining tree using sequences of the *tef1α* locus of the genus *Trichoderma*. The numbers at above/below nodes indicate the percentage bootstrap values based on 1,000 replications. Bootstrap values higher than 70% are shown. The scale bar equals the number of nucleotide substitution per site. *Cladosporium cladosporidoides* CBS 145.35 was used as the outgroup. Sequences of type specimens are indicated by asterisk symbols (*) after strain name.

respectively, with high bootstrap value of 99% and 100%, respectively. MABIK FU00000805 showed a high degree of similarity to *T. afroharzianum* Z19, Tafum1, and HZA3 (99.48%, 99.65%, and 99.65% identity), but the bootstrap value was relatively low (74%). As MABIK FU00000822 was grouped with two distinct species, *T. vermifimicola* CGMCC 3.19850 (99.59% identity) and *T. xixiacum* CGMCC 3.19697 (99.68% identity), it was not identified at the species level and thus was designated as *Trichoderma* sp. 1.

3. Screening of L-asparaginase-producing *Trichoderma* spp.

While we screened extracellular ASNase-producing fungi isolated from marine environments, results of the plate assay indicated four *Trichoderma* spp. produced ASNase. *Trichoderma* isolates were cultured on MCDA with L-asparagine as substrate. ASNase activity is accompanied with an increase in the pH due to the production of aspartic acid and ammonia from L-asparagine. Phenol red as the indicator of alkaline pH represented pink zones on the agar plates. The positive control *A. terreus* MABIK FU00000783 exhibited ASNase activity, as determined by the presence of a pink zone around the colony on the MCDA containing asparagine. In contrast, inoculation of *A. terreus* MABIK FU00000783 on the control plate containing $NaNO_3$ as the sole nitrogen source did not produce pink zones. Similarly, all *Trichoderma* isolates showed pink zones around colonies when grown on asparagine-containing MCDA, whereas no pink zones were observed in the control plates containing $NaNO_3$ (Fig. 5). Therefore, we found ASNase-producing fungal strains that showed a pink zone of the colony. Among these *Trichoderma* spp., ASNase production using the plate assay method and the characteristics of purified ASNase of *T. asperellum* have been reported (Uzma et al., 2016; Elsaba et al., 2022). These results indicated that four *Trichoderma* isolates from marine-associated habitats produced extracellular ASNase and showed ASNase activity.

4. Effects of carbon sources on extracellular L-asparaginase activity

Carbon sources stimulate cellular growth, leading to increased enzyme production (El-Naggar et al., 2019). In this study, we evaluated the effects of carbon sources on extracellular ASNase

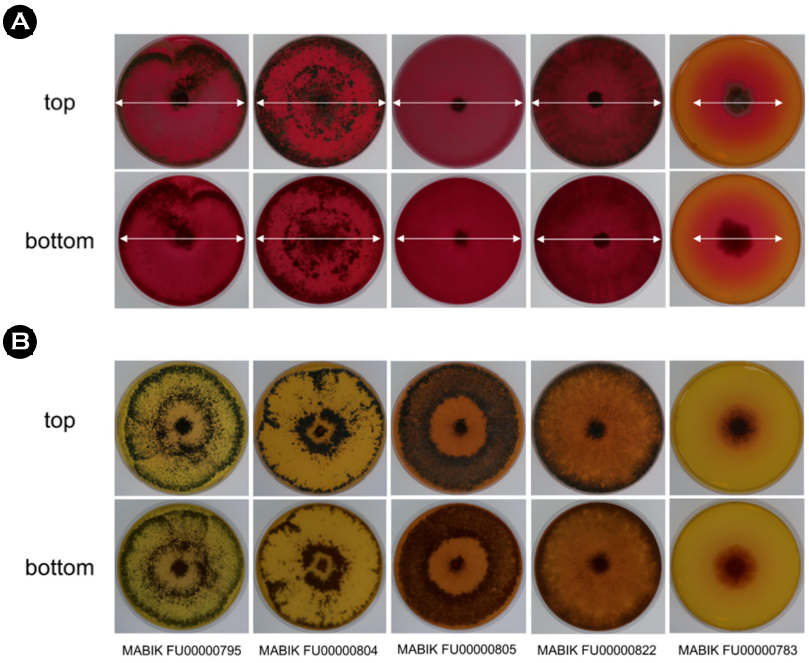

Fig. 5. *Trichoderma* isolates screened for ASNase activity. Four *Trichoderma* isolates were inoculated on MCDA containing (A) 1% L-asparagine and (B) 1% sodium nitrate as a sole nitrogen source. After incubation at 28°C for 5 days, top and bottom view of a colony on two kinds of media. ASNase activity was assessed by the presence of pink zones on the agar plates containing L-asparagine. The white arrow marks indicate where the pink zone is on the agar plates. Plates containing $NaNO_3$ served as control plates. The positive control *Aspergillus terreus* MABIK FU00000783 were inoculated on asparagine-containing and control plates.

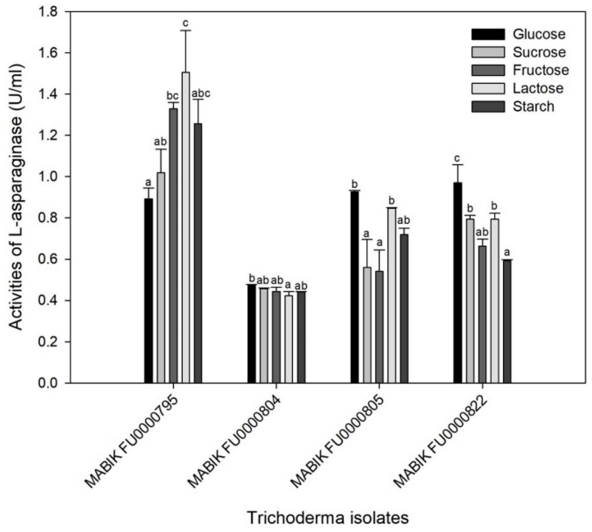

Fig. 6. Quantitative assay for ASNase activity of *Trichoderma* isolates depending on carbons sources. ASNase activity of four *Trichoderma* isolates was tested using Asparaginase Activity Assay Kit (abcam). Five kinds of carbon sources (glucose, fructose, sucrose, lactose and starch) were used. The results showed that all *Trichoderma* spp. exhibited different variations on L-asparaginase activities. The highest value of asparaginase activity in *T. asperellum* MABIK FU00000795 was observed when lactose was utilized as a carbon source. The asparaginase activity unit was defined as the amount of aspartate generated (μmol/ml) per minute at 25°C. Error bars represent standard deviation for n = 3 biological replicates. Significant differences between carbon sources in each strain are indicated by different letters (Two-way ANOVA, Tukey's test, $p < 0.05$).

activity. *Trichoderma* isolates in MCDB with various carbon sources exhibited different ASNase activities. At 0.5% concentration in a growth medium, glucose could repress ASNase production in certain microorganisms, including *Escherichia coli* (Garaev and Golub, 1977; Baskar and Renganathan, 2011). Therefore, to avoid this potential adverse effect of carbon sources, we added 0.2% of carbon sources in this study.

In *T. asperellum* MABIK FU00000795, the highest value of ASNase activity were recorded at 1.51 ± 0.20 U ml^{-1} with lactose as a carbon source (Fig. 6). The values of ASNase activities of MABIK FU00000795 were recorded at 0.89 ± 0.05 U ml^{-1} and 1.02 ± 0.11 U ml^{-1} with glucose and sucrose as the sole carbon source, respectively. In contrast, ASNase activities of *T. afroharzianum* MABIK FU00000805 and *Trichoderma* sp. 1 MABIK FU00000822 were the highest with glucose as a carbon source. The ASNase activity of *T. citrinoviride* MABIK FU00000804 was not significantly different depending on the carbon sources. Additionally, the effects of species and carbon sources on ASNase activities were determined based on two-way ANOVA ($p < 0.001$).

The effects of glucose on ASNase activity vary among the microorganisms. Glucose can lower the enzyme yield as a catabolic repressor (Heinemann and Howard, 1969), and thus generally serves as a poor carbon source for the production of ASNase in some microorganisms (Lincoln et al., 2021; Freitas et al., 2021; Mukherjee et al., 2000). However, glucose serves as a good carbon

source for production of ASNase in some microorganisms such as *Aspergillus*, *Fusarium* and *Streptomyces* strains (Baskar and Renganathan, 2012; Hosamani and Kaliwal, 2011; Deshpande et al., 2014). Therefore, it is important to optimize carbon sources for the production of microbial ASNase.

Conclusion

In this study, we aimed to find novel microbial resources for ASNase production. Four *Trichoderma* isolates, which were identified using *tef1α* sequences, were screened for extracellular ASNase activity. *T. asperellum* MABIK FU0000795 showed the highest ASNase production, with lactose as the sole carbon source. Other carbon sources such as glucose and sucrose served as repressors of the ASNase production of MABIK FU00000795. In contrast, glucose stimulated the enzymatic production of *T. afroharzianum* MABIK FU000805 and *Trichoderma* sp. 1 MABIK FU00000822. This study showed ASNase activity of *Trichoderma* spp. isolated from marine-derived sources. Our results suggest the potential of *Trichoderma* spp. as ASNase producers. The discovery of novel ASNase producers from eukaryotic microorganisms such as *Trichoderma* species may be an alternative source with fewer side effects due to their capacity to mimic the characteristics of human cells.

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