Halo-tolerance of Marine-derived Fungi and their Enzymatic Properties

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Marine environments are unique habitats for microorganisms and represent uninvestigated areas that possess valuable resources. Fungi may also be important because they contribute to marine ecosystems as decomposers. The aims of this study were to investigate the halo-tolerance of marine-derived fungi and their enzymatic properties. Eighteen fungal strains representing 11 different species were used, which included 17 ascomycetes and a zygomycete. The majority were not affected by salinity and showed endo-glucanase (EG) and β-glucosidase (BGL) activities. Interestingly, the cellulolytic enzyme activity derived from *Penicillium chrysogenum* FU42 increased with salinity. To investigate whether this increase was due to an adaptation or an innate ability of the species, *P. chrysogenum* KCTC6933, which originated from a terrestrial environment, was used as a control, and its enzymatic properties were compared. Consequently, *P. chrysogenum* FU42, which was derived from the ocean, showed unique enzymatic properties that might enable the fungus to live in the ocean and contribute to the nutrient cycle in marine ecosystems.

Keywords: Format; Adaptation; Carbohydrate; Decomposition; Enzymatic activity; Halo-tolerance

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INTRODUCTION

Many fungi can withstand severe and extreme environmental conditions. A marine environment is one of the most unique habitats for fungi. Not only does the water have a high salinity, but there is also low water potential, a high sodium ion concentration, low temperatures, and a high hydrostatic pressure, all of which contribute to a harsh environment for fungi (Jones 2000; Raghukumar 2008). The complexity of the marine environment may contribute to physiological changes in fungi to allow adaptation to unique conditions. Recently, a remarkable diversity of fungi was discovered in marine environments, which seems to be important in terms of ecology and biotechnology (Jones 2000). However, there was not sufficient information to determine whether the marine-derived fungi merely survived and endured the harsh conditions or whether they actively played a role and contributed to the marine ecosystem (Sparrow 1937; Kohlmeyer and Kohlmeyer 1979; Vrijmoed 2000). A study by Mouton *et al.* (2012) showed that marine-derived fungi have the potential to survive and grow in the marine environment and can contribute to the carbon and nitrogen cycles. Nevertheless, it is still unsolved how fungi adapt and contribute to marine ecosystems.

Fungi contribute to the decomposition of lignocellulosic materials in nature by producing a range of lignocellulolytic enzymes. Most fungi produce lignocellulolytic

enzymes of various kinds that are released into their environments and cooperate to degrade lignocellulolytic residues. Fungi play important roles in degrading lignocellulosic materials in terrestrial environments and may do so in marine environments. The lignocellulosic materials that come from salt marshes, mangrove swamps, and freshwater wetlands have highly refractory polymeric complexes. Most aquatic animals cannot produce lignocellulolytic enzymes for the direct utilization of lignocellulosic materials (Kristensen 1972). Instead, marine fungi can degrade the complex polymers into digestible and assimilable products for other members in the marine ecosystem. In one of the first studies on this topic (Hyde *et al.* 1998), lignocellulose was decomposed by more than 30 strains of fungi isolated from marine environments. The fungi could withstand reduced oxygen concentrations and degrade organic materials in low-oxygen and anoxic marine sediments by producing lignocellulolytic enzymes.

In this study, we investigated the halo-tolerance of marine-derived fungi and their cellulolytic enzyme profiles to screen for fungi that affect the nutrient cycle in marine ecosystems. In addition, the effect of the salt concentration was measured to distinguish between advantageous fungi and enzymes in living in the marine ecosystem. We also discussed the reason for the increased activity and whether it was a result of adaptation or an innate ability of the species by comparing the marine-derived fungal strain to strains from the same species originating from terrestrial environments.

EXPERIMENTAL

Fungal Isolation and Identification

Agarum cribrosum were collected from sites along the eastern coast of Korea. Sterile latex gloves were worn to transfer samples directly to Zip-lock bags. The samples were transported to the laboratory at 4 °C and processed immediately for fungal isolation. Each sample was gently washed with artificial sea water (ASW) to remove surface debris and soil (Huang *et al.* 2011). Discs of 5-mm in diameter were cut from each sample and placed on potato dextrose agar (PDA; Difco-Becton, Sparks, MD, USA) with ASW. All plates were incubated at 25 °C until the morphology of the cultured fungi could be distinguished (7 to 15 days); then, each strain was transferred to a new PDA plate. The isolated strains were stored in 20% glycerol at -80 °C at the Seoul National University Fungus Collection (SFC). Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Rogers and Bendich 1994). The internal transcribed spacer region (ITS) was amplified using the primer set ITS1F/ ITS4 (White *et al.* 1990). PCR was performed on a C1000 thermocycler (Bio-Rad, USA) using the Maxime PCR PreMix- StarTaq (Intron Biotechnology Inc., Korea) in a final volume of 20 μL containing 10 pmol of each primer and 1 μL of DNA. The PCR products were electrophoresed through a 1% agarose gel stained with STAR (Dyne Bio, Korea) and purified using the ExpinTM PCR SV Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Sequencing was performed in both the forward and reverse directions using the corresponding PCR primers at Macrogen (Seoul, Korea) using an ABI Prism 3700 genetic analyzer (Life Technologies, Gaithersburg, MD). For identification, each sequence was compared with reference sequences available on the GenBank database supplemented with BLAST searches of GenBank. The species with the best BLAST match in the GenBank database was selected for identification (Table 1).

Terrestrial strains of *Penicillium chrysogenum* KCTC6423, KCTC6929, and KCTC6933 from the Korean Collection of Type Cultures (KCTC) were used to compare its enzymatic properties to those of the marine-derived fungi.

Halo-tolerance Assay

Halo-tolerance was determined by cultivating fungi at ASW (29.5 g of Instant Ocean® per liter of distilled water). Instant Ocean® contains all of the important major and minor trace elements to provide a similar elemental composition as that of sea water (Atkinson and Bingman 1997). Spore suspensions were modified to a final concentration of 10^5 spores per mL. Cultivation was performed in a sterile flat-bottom 96-well plates containing 50 µL per well of 2-fold-concentrated nutritional medium. Each well was inoculated with 50 µL of 2-fold-concentrated spore suspension. The microplates were incubated at 25 °C in the dark. Spore germination and fungal growth were determined by measuring the optical density at 750 nm every 12 h for 4 days (Morris and Nicholls 1978; Druzhinina *et al.* 2006). All experiments were carried out in triplicate. Statistical analysis of the data was performed using SAS 9.3 (SAS Institute Inc., Cary, NC). The analysis of variance (ANOVA) was used to estimate the statistical parameters.

Enzyme Production and Assays

For fungal enzyme preparation, 18 fungi were cultivated in Mandels' medium (0.3 g of urea, 1.4 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 0.25 g of yeast extract, 0.75 g of peptone, 5 mg of $FeSO_4 \cdot 7H_2O$, 36 mg of $COCl_2 \cdot 6H_2O$, 1.8 mg of $MnSO_4 \cdot H_2O$, and 2.5 mg of $ZnSO_4 \cdot 7H_2O$ per liter of distilled water), with 1% cellulose as the sole carbon source, at 25 °C with shaking at 150 rpm. After seven days of fermentation, samples of the microbial culture were centrifuged, and the supernatants were collected to determine the activities of the extracellular enzymes and the protein concentration. In the particular assays, filter paper activity (FPA) was assayed according to the method of Ghose (1987). The reducing sugars were measured according to the dinitrosalicylic acid method (Miller 1959). The activities of endo-glucanase (EG) and β-glucosidase (BGL) were determined according to the methods of Lee *et al.* (2011b). The protein content was determined using the Bradford method, with bovine serum albumin as the standard (Bradford 1976).

Effect of Salt Concentration, pH, and Temperature on Cellulase Activity

The EG assay was conducted by varying the incubation conditions, in which salt concentration, pH, and temperature were adjusted, respectively. The effect of salinity was evaluated by incubating crude enzymes with the substrate containing various concentrations of NaCl ranging from 0.1 to 1.0 M at constant pH of 5.5 and temperature of 50 °C. To study thermal stability, a crude enzyme was incubated at different temperatures ranging from 20 to 80 °C at pH 5.5 without NaCl. The effect of pH was measured by varying the pH of the buffer, used in the assay, from 5.0 to 8.5. at 50 °C without NaCl. Subsequently, the remnant EG activity of each treatment was measured by incubating the crude enzyme with 2% (w/v) CMC solution at 50 °C for 30 min using the DNS method. Following the color development, the absorbance was measured at 540 nm. Then, the DNS method, as described in the previous paragraph, was carried out on the incubated samples. This analysis was carried out without further adjusting the salt content of the mixtures. One unit per mL of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose equivalents from CMC per milliliter of culture medium per minute.

RESULTS AND DISCUSSION

Halo-tolerance of Marine-Derived Fungi

Colony growth was examined in media containing ASW to compare the halo-tolerance of marine-derived fungi. As shown in Fig. 1, the majority of marine-derived fungi showed well-adjusted growth in culture containing ASW. The fungi did not show statistically significant differences in growth rate with ASW or a statistically higher growth compared with controls that were cultivated without ASW by analysis of variance (ANOVA) followed by Tukey's test. Among the fungi, *Dendryphiella arenaria* and *D. salina* are of known marine origin. In particular, *D. salina* has been used to investigate the ecophysical and morphophysiological adaptation of marine fungi to salinity as a model species (Clipson and Jennings 1992; Edwards *et al.* 1998). The genera *Arthrinium* and *Fusarium* are regarded as common inhabitants of marine environments (Panno *et al.* 2013). However, *Cladosporium cladosporioides* FU07, *Trichoderma hamatum* FU21, and *Stagonosporopsis heliopsidis* FU41 were inhibited by salt-supplemented media.

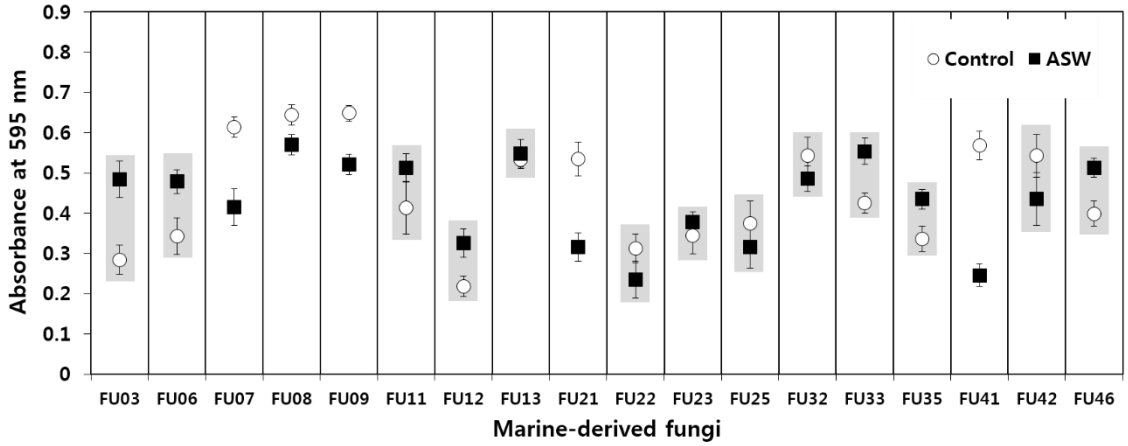

Fig. 1. Halo-tolerance of the marine fungi in ASW (■) and a control media (○). Gray backgrounds indicate that the fungi showed statistically identical growth in ASW or halo-stimulated growth, according to Tukey's test

Adaptations to marine environments require the fungi to endure salinity. Generally, the salinity of sea water is approximately 3.5%, though it can be lower near rivers or in microscopic environments that contain organic materials or floating matter imported from the land. Most of the fungi tested in this study could grow in saline conditions, and some fungi even showed better growth with ASW (Fig. 1). In particular, two species, *Arthrinium phaeospermum* FU03 and *Fusarium equiseti* FU46, exhibited the highest growth in saline conditions, which is analogous to marine environments; these species are considered common inhabitants of marine environments. One explanation is that they have developed abilities to endure saline conditions, such as an ability to accumulate intracellular solutes, which allows the fungi to adapt to salinity (Clipson and Jennings 1992).

Cellulase Activity

To screen for fungal strains that produce potentially valuable halo-stable enzymes, the cellulolytic enzyme activities were investigated. The EG and BGL activities were determined, and the total cellulase activity was measured by determining the FPA. As shown in Table 1, the majority of the samples displayed EG and BGL activities.

Table 1. Protein Concentration and Cellulolytic Enzyme Activities from Marine-Derived Fungi (Means are Given ± SD)

ID	Fungal name	Genbank No.	Protein concentration (mg/mL)	Enzyme activity (U/mL)		
				FPA[a]	EG[b]	BGL[c]
FU03	*Arthrinium phaeospermum*	KP836322	0.074 ± 0.004	ND[d]	0.167 ± 0.025	0.083 ± 0.001
FU06	*Arthrinium phaeospermum*	KP836323	0.049 ± 0.006	ND	0.108 ± 0.011	0.043 ± 0.001
FU07	*Cladosporium cladosporioides*	KP836324	0.016 ± 0.001	ND	ND	0.038 ± 0.001
FU08	*Cladosporium cladosporioides*	KP836325	0.010 ± 0.001	ND	ND	0.038 ± 0.001
FU09	*Cladosporium cladosporioides*	KP836326	0.023 ± 0.007	ND	ND	0.040 ± 0.001
FU11	*Dendryphiella arenaria*	KP836327	0.035 ± 0.012	ND	0.127 ± 0.034	0.070 ± 0.022
FU12	*Dendryphiella arenaria*	KP836328	0.053 ± 0.004	ND	0.113 ± 0.001	0.049 ± 0.001
FU13	*Dendryphiella arenaria*	KP836329	0.030 ± 0.006	ND	ND	0.051 ± 0.007
FU21	*Trichoderma hamatum*	KP836330	0.163 ± 0.019	0.209 ± 0.03*	0.568 ± 0.073*	0.204 ± 0.025*
FU22	*Dendryphiella salina*	KP836331	0.035 ± 0.006	ND	0.118 ± 0.006	0.061 ± 0.005
FU23	*Dendryphiella salina*	KP836332	0.106 ± 0.026	ND	0.222 ± 0.053*	0.123 ± 0.042*
FU25	*Phoma plurivora*	KP836333	0.034 ± 0.002	ND	0.107 ± 0.009	ND
FU32	*Penicillium spinulosum*	KP836334	0.033 ± 0.001	ND	0.156 ± 0.015	0.048 ± 0.001
FU33	*Penicillium spinulosum*	KP836335	0.028 ± 0.001	ND	0.143 ± 0.016	0.049 ± 0.001
FU35	*Mucor circinelloides*	KP836336	0.023 ± 0.001	ND	ND	ND
FU41	*Stagonosporopsis heliopsidis*	KP836337	0.127 ± 0.020	ND	0.419 ± 0.063*	0.038 ± 0.001
FU42	*Penicillium chrysogenum*	KP836338	0.101 ± 0.006	ND	0.307 ± 0.019*	0.293 ± 0.018*
FU46	*Fusarium equiseti*	KP836339	0.129 ± 0.065	ND	0.364 ± 0.067*	0.317 ± 0.034*

*Significantly different (α = 0.05) from control according to Tukey's test
[a] FPA, filter paper activity
[b] EG, endoglucanase
[c] BGL, β-glucosidase
[d] ND, not detected

Trichoderma hamatum FU21, *Dendryphiella salina* FU23, *Stagonosporopsis heliopsidis* FU41, *Penicillium chrysogenum* FU42, and *Fusarium equiseti* FU46 produced significantly high levels of EG. Although these fungi, with the exception of *S. heliopsidis* FU41, also showed significantly higher BGL activities than the other fungi, the activity was lower than those of well-known enzyme producers such as *Aspergillus niger*, *Penicillium oxalicum*, and *Trichoderma harzianum* (Lee *et al.* 2011a). Therefore, the fungi that produced significantly high levels of EG were screened to investigate the halo-stability of their enzymes.

Fungi may be important in the degradation of lignocellulosic materials in marine environments as well as terrestrial environments. In this study, most of the fungi could produce cellulolytic enzymes.

Not only are *T. hamatum* FU21, *P. chrysogenum* FU42, and *F. equiseti* FU46 known cellulase producers, but *D. salina* FU23 and *S. heliopsidis* FU41 also produced significantly high levels of cellulolytic enzymes (Table 1). These fungi could degrade plant material in the marine environment by producing various types of cellulolytic enzymes. Therefore, as decomposers of plant material, marine-derived fungi may play an important role in the elemental cycle by allowing large amounts of lignocellulosic materials to return to the ecosystem food chain.

Halo-Stability of Fungal Enzymes

The halo-stabilities of the fungal enzymes were measured to explore the contribution of marine-derived fungi to the carbon cycle in the ecosystem. The effect of the salt concentration on cellulase activity was analyzed by exposing the crude enzyme to different concentrations of NaCl during the assay, and the results are shown in Fig. 2. For all of the NaCl concentrations, *P. chrysogenum* FU42 had the most halo-tolerant EG activity, followed by *D. salina* FU23 and *F. equiseti* FU46. Compared with Celluclast 1.5 L., *D. salina* FU23 and *P. chrysogenum* FU42 showed an increase in EG activities at the optimal salt concentration (0.5 M and 0.25 M, respectively). In contrast, Celluclast 1.5 L, which is a commercial enzyme, showed decreasing enzyme activity with increasing NaCl concentration.

To assess the aforementioned ability of the fungi, it should be considered that they would grow sufficiently and that their enzymes would retain their own activities in marine environments. *D. salina* FU23, *P. chrysogenum* FU42, and *F. equiseti* FU46 satisfied the requirements and even showed halo-stimulated growth as an effect of salinity for the enzymes with the addition of NaCl.

In particular, the EG activity of *D. salina* FU23 was retained at up to 0.5 M NaCl (99%), and the activity of *P. chrysogenum* FU42 was even increased at 0.5 M NaCl (126%), which resembles the salinity of sea water. Although *T. hamatum* FU21 and *S. heliopsidis* FU41 showed significantly high enzyme activities, their growth was inhibited in saline conditions. It is difficult to expect them to play a role in the degradation of cellulosic materials in marine ecosystems without adequate growth in salinity. Furthermore, their enzyme activities were somewhat decreased in conditions containing similar amounts of NaCl to that in an ocean environment.

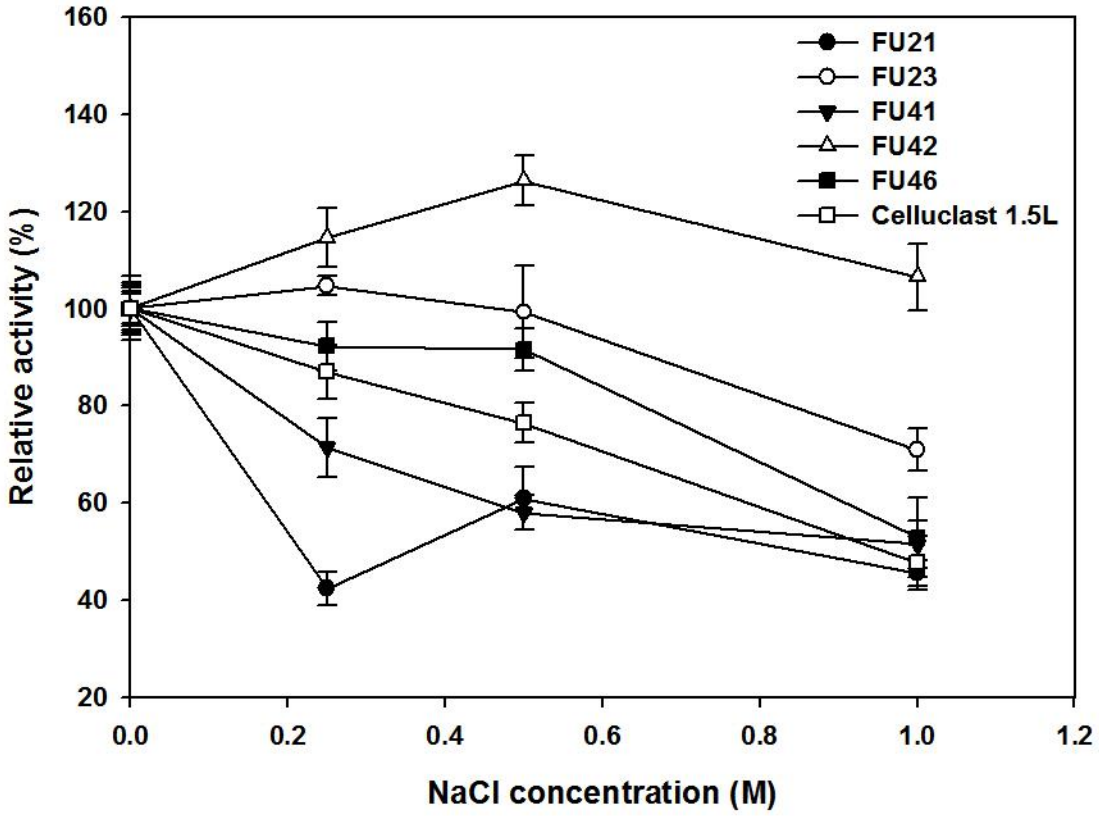

Fig. 2. Effect of salinity on endo-glucanase activity produced by FU21 (●), FU23 (○), FU41 (▼), FU42 (△), FU46 (■), and Celluclast 1.5 L (□)

Comparison between Marine-Derived and Terrestrial Fungi

Additional tests were performed to determine whether the halo-stability of the enzyme from *Penicillium chrysogenum* FU42 resulted from an innate ability of the fungus or was a product of an adaptation to marine environments. In the tests, *Penicillium chrysogenum* KCTC6423, KCTC6929, and KCTC6933, which originated from a terrestrial environment, were used to demonstrate the differences in enzyme production when the fungi were cultivated in ASW. All of the fungi showed a similar or lower protein concentration, FPA, and BGL activity in the marine conditions (Fig. 3). However, *P. chrysogenum* FU42 produced a significantly high yield of EG when the fungus was cultivated in ASW.

To investigate the reason for the increased EG activity by *P. chrysogenum* FU42, the effects of salinity, pH, and temperature were measured on the enzymes obtained from *P. chrysogenum* FU42 and *P. chrysogenum* KCTC6933, which showed statistically higher EG activity than did the other strains. The results showed that the EG from FU42 exhibited the highest activity under the conditions of 0.5 M NaCl, pH 7.5, and 60 °C, whereas KCTC6933, a terrestrial fungus, showed the highest activity at 0.1 M, pH 6.5, and 50 °C (Fig. 4).

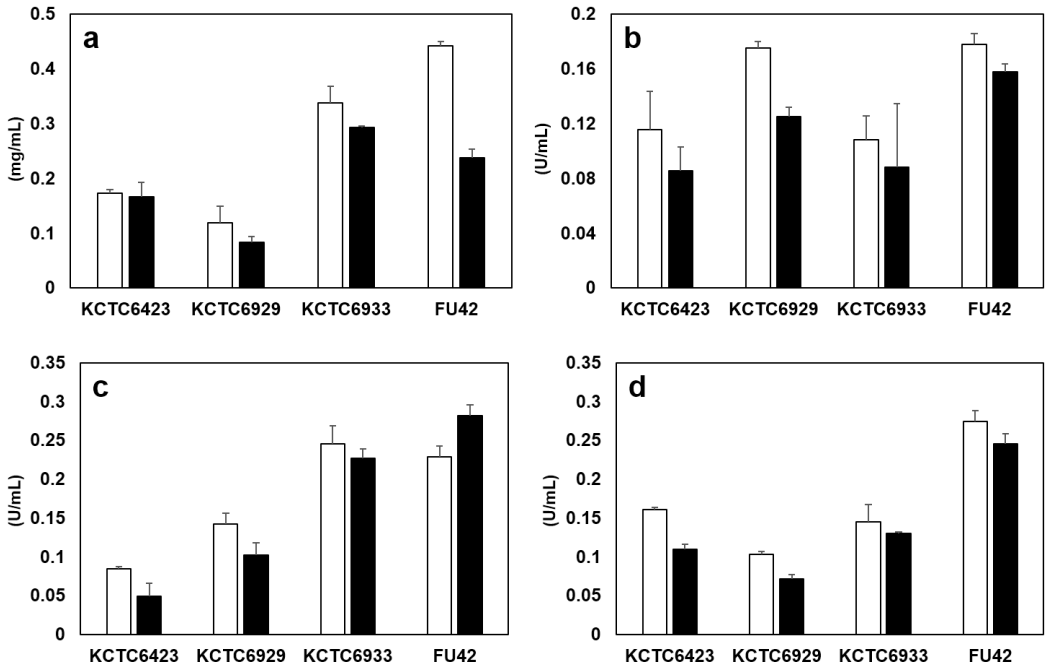

Fig. 3. Comparison of protein concentration and enzyme activities. The error bars represent the standard error of triplicate experiments. (a) Protein concentration; (b) filter paper activity; (c) endo-glucanase; (d) β-glucosidase produced by *Penicillium chrysogenum*, a marine-derived fungus and four terrestrial fungi, cultivated in Mandels' medium (□) and artificial sea water(■).

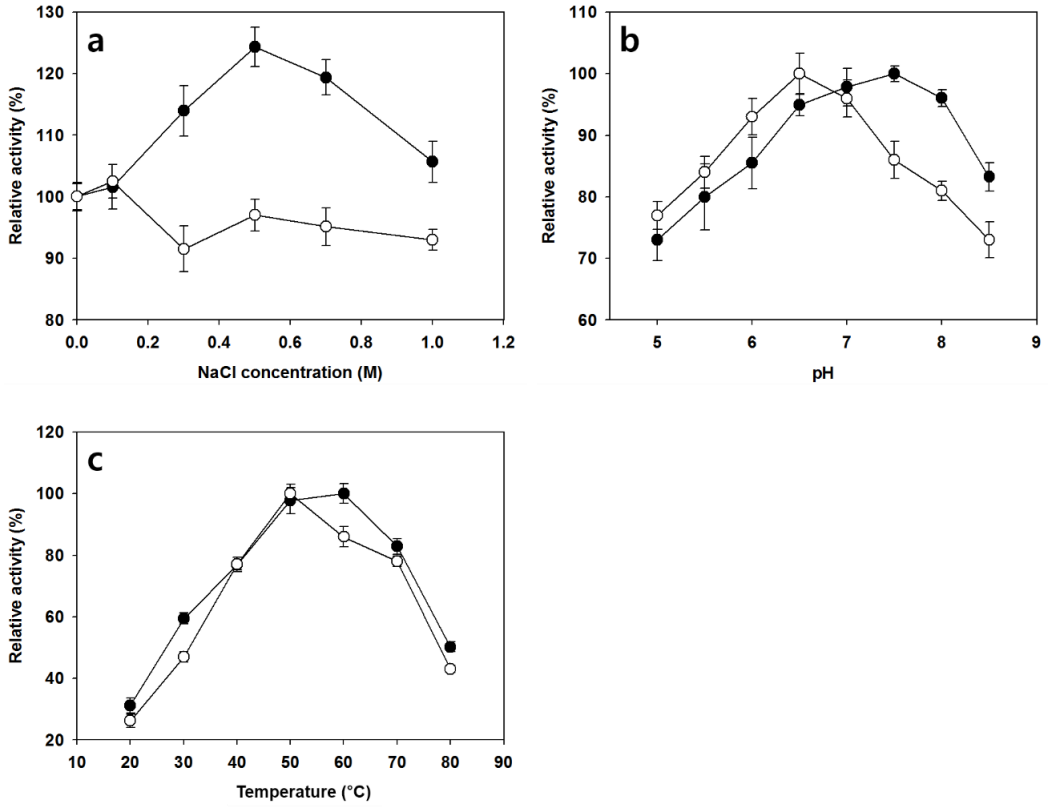

Fig. 4. Comparison of the effects of (a) salinity, (b) pH, and (c) temperature on fungal enzyme activity from *Penicillium chrysogenum* FU42 (●) and KCTC6933 (○)

To investigate whether the increased activity was the result of adaptation or an innate ability of the species, the enzymatic properties of *P. chrysogenum* FU42 were analyzed using identical strains originating from a terrestrial environment as a control. Regarding the influence of salinity on the enzyme activities, the fungal strains were either not affected or showed an increase in their degradative capacities with increasing salt concentration. However, there was an apparent difference: *P. chrysogenum* FU42 showed increased activities at all salt concentrations, with an optimum concentration of 0.5 M NaCl, whereas the activity of the enzyme obtained from KCTC6933 rapidly decreased at 0.3 M NaCl. This unique property of the halophilic enzyme from *P. chrysogenum* FU42 can be exploited for the saccharification of ionic liquid-pretreated lignocellulosic biomass. This enzyme may be able to solve the cost problem for the saccharification of lignocellulose and the development of subsequent products (Skovgaard and Jørgensen 2013). In addition to its halophilic property, the enzyme exhibited the highest activity between pH 7.0 to 8.0, which corresponds to the pH value in the ocean. These results agree with those of previous reports (Jones 2000; Raghukumar 2008), which showed that marine fungi were known to produce functional enzymes at pH 7 to 8. The finding that enzymes of *P. chrysogenum* FU42 are active in extreme conditions is interesting because it indicates that the fungus can trigger and produce much more specific enzymes (Pointing and Hyde 2000; Bonugli-Santos *et al.* 2010). These results indicate that enzymes from marine-derived fungi are considerably different from those produced by their terrestrial counterparts because the enzymes retained their activities in extreme conditions where proteins can be denatured (Raghukumar 2008). It is clear that this marine-derived fungus acquired its abilities in the process of adaptation, which might enable the fungus to live in oceans and contribute to the nutrient cycle of marine ecosystems.

In this study, we investigated the halo-tolerance of marine-derived fungi and their cellulolytic enzyme profiles, and we then compared various enzymatic properties of a marine-derived fungus with an identical species from a terrestrial environment. Interestingly, *P. chrysogenum* FU42 showed unique enzymatic properties that are favorable to living in the ocean. In a future study, functional genomics, such as transcriptomics, proteomics, and epigenomics, would be necessary to demonstrate how the fungi acquire their advantageous properties for marine life and adapt to marine environments.

CONCLUSIONS

1. Marine-derived fungi isolated from *Agarum cribrosum* were investigated to determine their halo-tolerance and cellulolytic enzyme profiles. Among the fungi, *Penicillium chrysogenum* FU42 showed proper abilities to live in the marine environment in both fungal growth and enzymatic properties.
2. *Penicillium chrysogenum* FU42, marine-derived fungus, produced halophilic endo-glucanase, which was differ to the *P. chrysogenum* originated from a terrestrial environment.
3. The presented results provided new information for an enzyme profile of marine-derived fungi, and can contribute to understanding the ecological role of fungi in marine environments, specifically of *P. chrysogenum* FU42. The selection of marine-derived fungi with well-adapted enzymatic properties, even in the presence of high salt

concentrations, would increase the saccharification yields of ionic liquid-pretreated lignocelluloses.

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