J Wood Sci (2002) 48:147–152

ORIGINAL ARTICLE

Xin Li · Ryuichiro Kondo · Kokki Sakai

Studies on hypersaline-tolerant white-rot fungi I: screening of lignin-degrading fungi in hypersaline conditions

Received: November 10, 2000 / Accepted: April 16, 2001

Abstract To search for marine fungi that have significant lignin-degrading ability in a hypersaline environment, eight strains of marine fungi were selected from 28 strains isolated from mushrooms and driftwood sampled from mangrove stands in Okinawa, Japan. We evaluated the decolorization ability, delignification ability, and biobleaching properties of the strains; then strain MG-60 was screened as a hypersaline-tolerant lignin-degrading fungus. We have summarized its growth, decolorization ability, and biobleaching properties at various sea salt concentrations. The strain has been estimated to belong to the *Phlebia* family.

Key words White-rot fungi · Decolorization · Delignification · Biobleaching · Tolerance to sea salts

Introduction

During recent decades research on ligninolytic fungi has greatly intensified because of their potential applications in a variety of biotechnology fields. The applications include biotransformation of lignocellulosic biomass, biopulping and biobleaching,[1,2] decolorizing various dye pollutants,[3–6] and degrading dioxins[7] and chlorinated phenols.[8]

Because of the environmental and economic concerns, pulp and paper mills have adopted a white water recycling system to reduce the freshwater usage during the pulping, bleaching, and papermaking operations. The dissolved inorganic materials are almost completely recycled and accumulate in the white water or process streams, although the suspended particles in the white water are cleaned up. These inorganic materials that are not pulping or bleaching chemicals are referred to as nonprocess elements (NPEs).[9] The main NEPs dissolved in the white water were reported to be $CaSO_4$, Na_2SO_4, and NaCl.[10] Therefore, screening of salt-tolerant white rot fungi is a fundamental and primary study for effective biopulping and biobleaching with recycled white water.

Marine fungi are often found on decaying lignocellulosic substrates such as proproots, pneumatophores, branches, leaves, and driftwood in the intertidal region of mangrove stands.[11] Since Cribb and Cribb first reported marine fungi from mangroves in Australia in 1955,[12] researchers have paid attention to the underestimated but important role of fungi in the degradation of organic materials in marine and hypersaline ecosystems.[13] There has been considerable research on the classification and growth of marine fungi,[14–18] but most studies have not focused on their lignin-degrading ability, tolerance to hypersaline ecosystems, and enzyme characterization.

The primary purpose of this research was to screen marine fungi that have high lignin-degrading ability in hypersaline environments. The screened salt-tolerant white-rot fungi are expected to be effective for biobleaching with recycled white water and for employment as alternative microorganisms for bioremediation of the polluted marine environment.

For the present research several marine fungal strains were selected based on their delignification ability and biobleaching properties from 28 mushrooms and driftwoods collected from mangrove stands in Okinawa, Japan. One of them, MG-60, was found to have outstanding decolorization ability, delignification ability, and biobleaching properties.

Materials and methods

Fungal strains

Phanerochaete chrysosporium ME-466 was provided by Dr. H. Wariishi, Faculty of Agriculture, Kyushu University and

X. Li · R. Kondo (✉) · K. Sakai
Laboratory of Forest Chemistry and Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
Tel. +81-92-642-2989; Fax +81-92-642-2989
e-mail: kondo@agr.kyushu-u.ac.jp

Part of this paper was presented at the 44th Lignin Symposium, Gifu, Japan, October 1999

has been maintained in our laboratory. Mushrooms (MG-01–MG-13) and driftwood (MG-20–MG-47 and MG-50–MG-63) were collected from mangrove stands in Okinawa, Japan. Discs cut from the mushrooms and driftwood were sterilized with 70% alcohol, washed with sterilized water, and then inoculated on 20 ml of beech wood meal medium. The medium was composed of 2% beech (*Fagus* sp.) wood meal (100 mesh pass), 0.02% guaiacol, 1 ppm benomyl, and 1.6% agar. The medium was adjusted by HCl to pH 5.5 before sterilization.[19] The strains, which showed red zones in the Bavendamm reaction on the wood meal medium, were isolated. The selected fungi were inoculated on potato dextrose agar (PDA) (Difco Laboratories, USA) plates and incubated at 30°C for the following experiments.

Evaluation of fungal growth at different conditions

The fungi were inoculated on PDA media containing various sea salt concentrations and incubated at 30°C to evaluate their speed of growth. To determine the effect of temperature on fungal growth, the fungus was inoculated on PDA medium and incubated at 25°C, 30°C, and 37°C. The diameter of the growth zone was measured every 24 h and used to evaluate the fungal speed of growth.

Decolorization of synthetic dye

The synthetic dye Poly R-478 (Sigma, USA) at 0.02% strength,[3] 2% agar, and sea salts (Sigma) were added to 20-ml Kirk high-carbon and low-nitrogen (HCLN) medium.[20] This mixture is called PAK medium here. The sea salt concentration was adjusted to 0%, 3%, 5%, 7%, and 10%. The fungal mycelium disc, 5 mm in diameter, from the PDA plate was inoculated in a 9-cm Petri dish containing 20 ml of PAK medium and cultivated at 30°C. For determination of decolorization ability, the culture was autoclaved for 1 min at 121°C in accordance with a previously described method,[3] and the decolorization efficiency was evaluated by examining the ratio of absorbance at 513 and 362 nm.[3]

Delignification ability

A 3-g portion of extractive-free beech wood meal (60–80 mesh) and 7.5 ml HCLN medium at various sea salt concentrations were mixed in a 100-ml Erlenmeyer flask and then sterilized (121°C for 20 min). Five fungal disks (d = 6 mm) punched from the edge of the PDA culture were inoculated on the wood meal medium and incubated at 30°C for 30 days. After incubation, the sample was washed three times with water and then dried at 105°C to a constant weight. The weight loss of wood meals was determined.

The Klason lignin content and acid-soluble lignin content were determined before and after incubation. The lignin-degrading selection factor (SF) for each strain was calculated as follows.

SF = lignin loss/holocellulose loss

in which holocellulose loss was calculated as follows.

Holocellulose loss = weight loss − lignin loss

The weight loss, residual lignin content, and SF are employed to evaluate the delignification ability of the strain.

Biobleaching under hypersaline conditions

Five fungal disks punched from the edge of the PDA plate were added to 50 ml of potato dextrose broth (PDB) medium (Difco) and incubated at 30°C for 7 days. The mycelia separated from the PDB media were washed with sterilized water and then homogenized with a sea salt solution (0%, 3%, 5%, 7%, 10%) for 20 s (low speed) in a sterile Waring Blender 7010. A 4-g portion of unbleached hardwood kraft pulp (UKP) was mixed with 8 ml of sea salt solution and then sterilized at 121°C for 20 min. After 8 ml of mycelia suspension (mycelium dry weight 15 mg/ml) was added to the prepared UKP, the mixture was cultivated in a stationary position at 30°C. Pulp brightness was determined with a colorimeter (CR-200, Minolta, Japan), and the result was multiplied by a coefficient to adjust to ISO brightness.

Results and discussion

Isolation of hypersaline-tolerant lignin-degrading fungi

Mushrooms (MG-01–MG-13) and decayed driftwood (MG-20–MG-47, MG-50–MG-63) were collected and inoculated on beech wood meal medium. A red zone was observed in 28 of 53 samples with the Bavendamm reaction. The 28 strains that had potential lignin-degrading ability were isolated and used in the following experiments.

Of the 28 strains, 25 not including *P. chrysosporium* and one of the 28 strains (MG-60) grew at 10% sea salt concentration, and all of the 28 strains and *P. chrysosporium* grew well at 0% sea salt concentration. However, the growth speed of the 25 samples was much slower at 10% sea salts than at 0% sea salts (data not shown).

We examined the decolorization ability of the 28 isolated strains under hypersaline conditions. Because ligninolytic fungi can degrade many dye pollutants, decolorization of the synthetic dye Poly R-478 is often employed to evaluate the delignification ability of fungi.[3,21,22] Thus the 28 isolated fungi were inoculated in PAK medium to isolate the hypersaline-tolerant lignin-degrading fungi. We detected the decolorization ability of the 28 stains and *P. chrysosporium* incubated with 0% sea salts for 7 days; the results are shown in Fig. 1. Although the other isolated fungi showed no obvious decolorization efficiency, the highest decolorization ability of one of the 28 strains (MG-60) to Poly R-478 was observed at 0% sea salt concentration. Although *P. chrysosporium* was reported to decolorize polymeric dyes,[3] its decolorization ability was lower than that of MG-60 in these culture conditions. To screen for hypersaline-tolerant strains, these 28 isolated strains were inoculated on PAK medium with 10% sea salts. Seven strains (MG-03, MG-04,

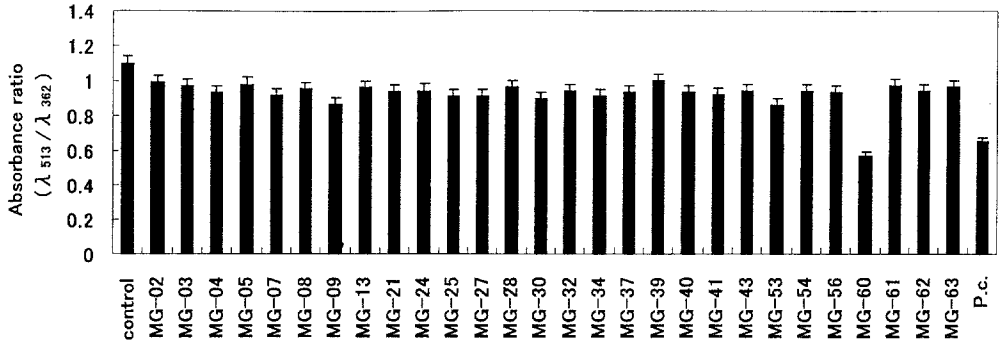

Fig. 1. Decolorization of Poly R-478 by marine fungi at 0% sea salt concentration (incubation time 7 days)

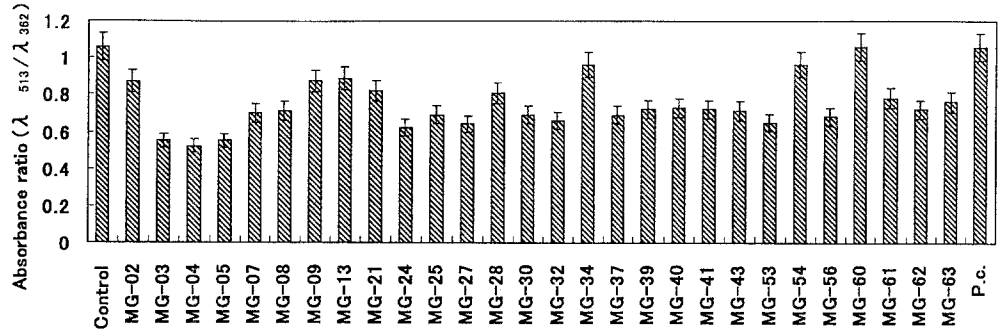

Fig. 2. Decolorization of Poly R-478 by marine fungi at 10% sea salt concentration (incubation time 20 days)

MG-05, MG-24, MG-27, MG-32, MG-53) were selected for their superior decolorization ability (Fig. 2). Unfortunately, MG-60 did not grow at 10% sea salt concentration. It was suggested that these seven strains had stronger decolorization ability and higher tolerance to hypersaline conditions than other strains. Because of the outstanding decolorization ability of MG-60 at 0% sea salt concentration and the excellent hypersaline tolerance of the seven strains with superior decolorization ability, the following research focused on these eight strains.

Lignin-degrading properties of the eight selected strains

First, we determined the decolorization ability of the eight selected strains at various sea salt concentrations. Figure 3 indicates that the decolorization ability of MG-60 was much higher than that of the other seven strains when the sea salt concentration was lower than 5%.

The eight strains were incubated with wood meals at various sea salt concentrations for 30 days. After incubation the weight loss and residual lignin content of wood meals were analyzed to evaluate the delignification ability of the strains. The results are illustrated in Figs. 4 and 5. The results demonstrate that MG-60 had higher delignification efficiency and better lignin-degrading SFs than did the other strains at 0%, 3%, and 5% sea salt concentrations, although it did not grow at higher concentrations. This means that the strain can degrade lignin efficiently, but that holocellulose was also slightly damaged. This property is valuable when MG-60 is employed for biobleaching and biopulping.

As an alternative evaluation method, we analyzed the degradation ability of the eight strains to residual lignin in UKP at various sea salt concentrations. All eight strains

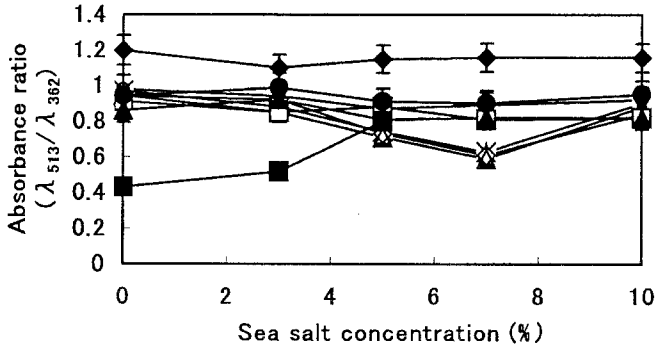

Fig. 3. Decolorization of Poly R-478 by the eight selected strains at various sea salt conditions (incubation time 14 days). *Filled diamonds*, control; *asterisk*, MG-03; *open diamonds*, MG-04; *open circles*, MG-05; *open triangles*, MG-24; *open squares*, MG-27; *filled circles*, MG-32; *filled triangles*, MG-53; *filled squares*, MG-60

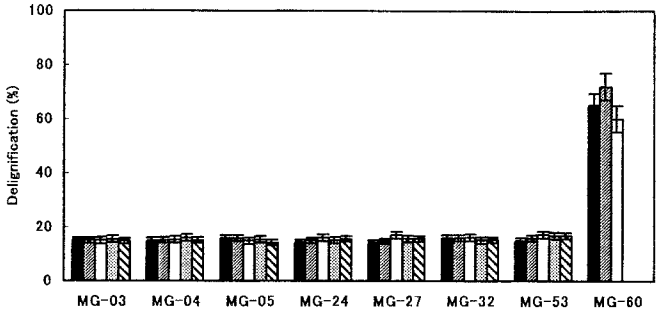

Fig. 4. Delignification ability of the eight selected strains at 0% (*black bars*), 3% (*gray bars*), 5% (*open bars*), 7% (*grayish-white bar*), and 10% (*hatched bars*) sea salt concentrations (incubation time 30 days)

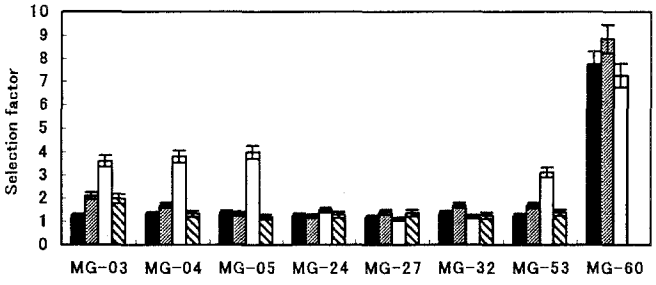

Fig. 5. Lignin-degrading selection factor of the eight selected strains at 0% (*black bars*), 3% (*gray bars*), 5% (*open bars*), and 10% (*hatched bars*) sea salt concentrations (incubation time 30 days)

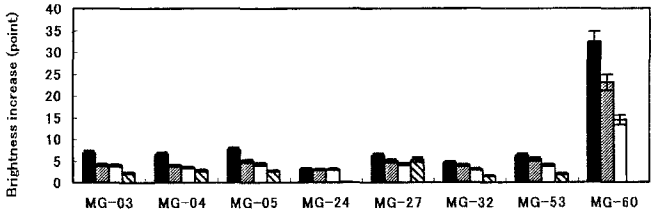

Fig. 6. Brightness increase of unbleached kraft pulp (UKP) bleached with the eight selected strains at 0% (*black bars*), 3% (*gray bars*), 5% (*open bars*), and 10% (*hatched bars*) sea salt concentrations (cultivation time 8 days)

could brighten the pulp to some degree when they grew at various sea salt concentrations (Fig. 6). The pulp brightness bleached by MG-60 was much higher than the bleaching by the other seven strains at 0%, 3%, and 5% sea salt concentrations. We concluded that MG-60 had better biobleaching ability than the other seven strains even at a certain degree of hypersalinity.

We observed that the fungus MG-60 has a good lignin-degrading ability at sea salt concentrations ≤5%. Therefore, we selected it as a hypersaline-tolerant lignin-degrading fungus and employed it in the following research.

Characterization of the fungus MG-60

We summarized the characteristics of the screened fungus MG-60 with regard to its growth at various temperatures, decolorization ability, and biobleaching properties at 0%, 3%, and 5% sea salt concentrations. First, we compared the speed of growth of MG-60 with that of the well-known white-rot fungus *P. chrysosporium* at various sea salt concentrations (Fig. 7). The higher the sea salt concentration, the slower the growth of MG-60 and *P. chrysosporium*. Even at 5% sea salt concentration, growth of the two strains was observed, with MG-60 growing faster than *P. chrysosporium* at the same concentration. We deduced that MG-60 had better tolerance to a hypersaline environment than did *P. chrysosporium*.

We determined the effect of temperature on the growth of MG-60 at 0% sea salt concentration, the results of which are shown in Fig. 8. The fungus grew faster at 30°C than it did at 25°C or 37°C, so we regarded 30°C as the proper incubation temperature for MG-60.

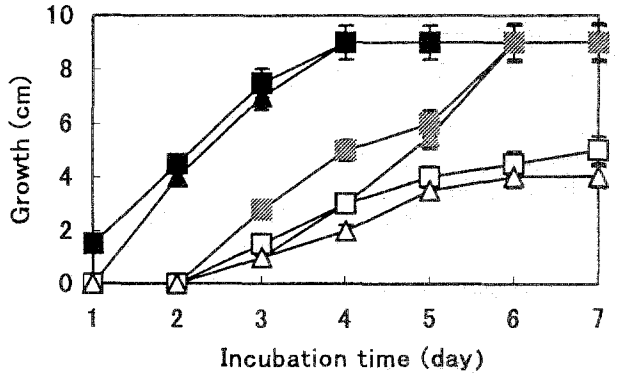

Fig. 7. Growth of the fungus MG-60 (quadrilateral mark) and *Phanerochaete chrysosporium* (*triangle marks*) at 0% (*black marks*), 3% (*gray marks*), and 5% (*open marks*) sea salt concentrations

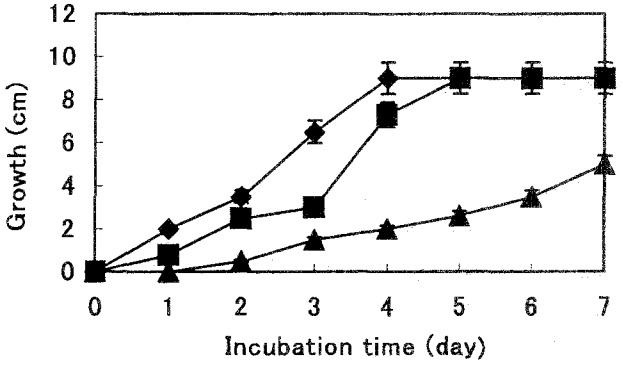

Fig. 8. Growth of the fungus MG-60 under 25°C (*squares*), 30°C (*diamonds*), and 37°C (*triangles*) incubation conditions

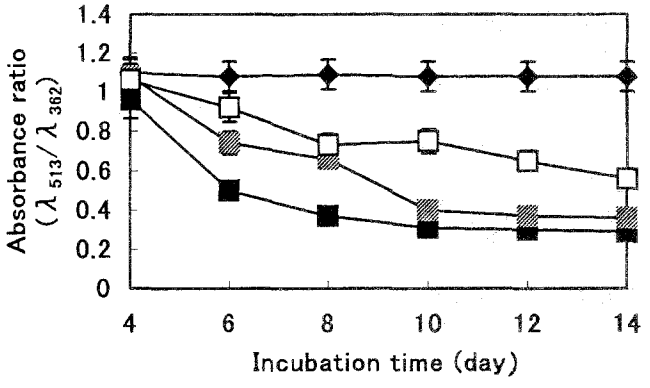

Fig. 9. Decolorization of Poly R-478 by the fungus MG-60 at 0% (*filled squares*), 3% (*gray squares*) and, 5% (*open squares*) sea salt concentrations

As shown in Fig. 9, increasing sea salt concentration decreased the decolorization ability, although decolorization was detected even at the 5% sea salt concentration. The maximum decolorization efficiency occurred at 10 days of incubation at both 0% and 3% sea salt concentrations, and the decolorization process lasted for 14 days of incubation at 5% sea salt concentration.

The UKP was bleached with *P. chrysosporium* and MG-60 at three sea salt concentrations (0%, 3%, 5%). The

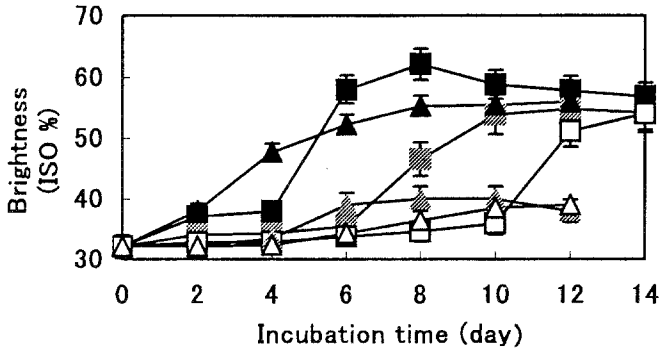

Fig. 10 Brightness increase of UKP during biobleaching with the fungus MG-60 (*squares*) and *P. chrysosporium* (*triangles*) at 0% (*filled marks*), 3% (*gray marks*), and 5% (*blank marks*) sea salt concentrations

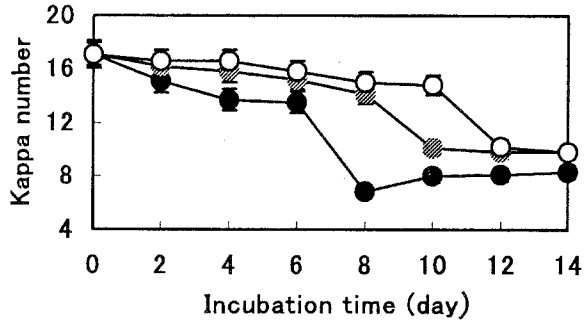

Fig. 11 Decrease in the kappa number of UKP bleached with the fungus MG-60 at 0% (*filled circles*), 3% (*gray circles*), and 5% (*open circles*) sea salt concentrations

bleached pulp properties are shown in Figs. 10 and 11. Increasing the sea salt concentration decreased the pulp brightness gain at the same incubation time that the pulp was bleached by both MG-60 and *P. chrysosporium*. The pulp was brightened by MG-60 at various speeds at the three sea salt concentrations, with the brightness increase at 0% sea salt concentration being sharper than that at 3% or 5% concentrations. The maximum brightness increases were observed after days 4, 6, and 10 of incubation with MG-60 at 0%, 3%, and 5% sea salt concentrations, respectively. It appears that MG-60 may secrete different enzymes or have different enzyme activities at different sea salt concentrations. Although the pulp brightness was improved by *P. chrysosporium* at 0% sea salt concentration, it was lower than the brightness produced by MG-60. Furthermore, the pulp was only slightly brightened by *P. chrysosporium* at 3% and 5% sea salt concentrations, whereas MG-60 improved the pulp brightness by about 20 points even when 5% sea salt was added. These results indicate that MG-60 had a stronger lignin-degrading ability than *P. chrysosporium*, especially in a hypersaline environment. The pulp kappa number was decreased by MG-60 along with the increase in brightness (Fig. 11). It is clear that despite its hypersaline-tolerant property, the less hypersaline the conditions, the stronger the lignin-degrading ability of MG-60.

Marine fungi grow in marine ecosystems and are often found on decayed lignocellulosic substrates. Most of them probably belong to the soft-rot fungi, but the white-rot type has been reported from three marine basidiomycetes: *Digitatispora marina, Halocyphina villosa*, and *Nia vibrissa*.[23-25] The fungus MG-60 has been classified as a type of basidiomycete, of the *Phlebia* family, based on microscopic observations of its mycelium and determination of its 18Sr DNA sequence.[26]

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