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EVALUATION OF WHITE-ROT FUNGI FOR TREATMENT OF ORGANIC WASTES WITHOUT ENVIROMENTAL IMPACT

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Summary

Industrialized economies extract huge amounts of natural resources from the environment and convert them to produce various commodities, thus enabling us to enjoy a comfortable and convenient life. On the other hand, pollutants associated with the production and consumption of commodities as well as post-consuming commodities go back into the environment as residues of these economics. The size of this material cycle between human activities and the natural environmental is enormous, and far beyond the reproduction speed of natural resources and the environmental carrying capacity. To establish a habitat system for sustainable development, the architecture of returning system of huge amounts of wastes to ecosystem without environmental impact should be developed.

White-rot fungi are naturally-occuring organisms that cause the decay of fallen trees by degrading lignin, a complex, three-dimensional, heterogenous polymer that provides structural support for plants. The degradation of a variety of environmentally persistent pollutants by this organism has been reported. In the present study, the evaluation of white-rot fungi for treatment of organic wastes without environmental impact was reported.

1. Screening and evaluation of lignin-degrading fungi

Fungi and bacteria are the principal degraders of organic matter. White-rot fungi belong to the group of basidiomycetes that colonize wood in nature and preferentially decompose lignin, complex three-dimentional non-repeating polymer in woody plants which is resistant to attack by most microorganisms, to cause white rotting of wood. They have several potential advantages over other degrading microorganisms in the degradation of persistent organic compounds. During recent decades, research on ligninolytic fungi has greatly intensified because of their potential applications in a variety of biotechnological fields. The applications include biotransformation of lignocellulosic biomass, biopulping and biobleaching, decolorizing various dye pollutants, and degrading dioxins and chlorinated phenols.

White-rot fungi can degrade lignin using an extracellular, rather nonspecific, free-radical based biodegradation system. White-rot fungi variously secrete one or more of three extracellular enzymes that are essential for lignin degradation, and which combine with other processes to effect lignin degradation. The three enzymes comprise: two glycosylated heme-containing peroxidases, lignin peroxidase (LiP, E.C. 1.11.1.14) and manganese peroxidase (MnP, E.C. 1.11.1.13), and a copper-containing phenoloxidase, laccase (E.C. 1.10.3.2).

A number of decayed wood samples (1212 samples) were collected from natural resources. Among them, 387 samples showed ligninolytic activity, and from them we obtained 122 strains as lignin-degrading fungi. At first, we compared weight losses of beech wood meals between new isolates and typical lignin-degrading fungi, *Phanerochaete chrysosporium* and *Trametes versicolor*, during fungal treatment for 30 days, as indexes of ligninolytic activities. Many strains showed much lower in weight losses than *P.*

chrysosporium and *T. versicolor*, 12.2% and 12.7%, respectively. The wood meals-sample (13 samples) which had more than 10% weight losses by decaying with new isolates were determined lignin contents, and selection factors were calculated. We obtained 11 lignin-degrading fungi which showed higher ligninolytic activities and selectivities than *P. chrysosporium* and *T. versicolor*. Eight of the 11 strains showed higher bleaching abilities of hardwood unbleached kraft pulp than did the 2 known fungi. The ligninolytic enzymes were characterized. MnP exhibited great activity compared with those of LiP and phenol oxidase in bleaching cultures of 7 strains which could bleach kraft pulp. The higher brightness increase was observed in fungal treated pulp, the higher MnP activities were detected in the cultures. These fungal strains should be good candidates for treatment of organic wastes without environmental impact.

2. **Screening and evaluation of lignin-degrading fungi in hypersaline conditions**

Marine fungi are often found on decaying lignocellulosic substrates such as proproots, pneumatophores, branches, leaves and driftwood in the intertidal region of mangrove stands. Researchers have paid attention to the underestimated but important role of fungi in the degradation of organic materials in marine and hypersaline ecosystems. There has been considerable research on the classification and growth of marine fungi, but most studies have not focused on their lignin-degrading ability, tolerance to hypersaline ecosystems and enzyme characterization. The primary purpose of this research is to screen the marine fungi that have high lignin-degrading ability in hypersaline environments. The screened salt-tolerant white rot fungi are expected to be effective in bioremediation of the polluted marine environment. In the present research, several marine fungal strains were selected based on their delignification ability, from 28 mushrooms and driftwood collected from the mangrove stands in Okinawa, Japan.

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, and there were 28 samples in which a red zone was observed in Bavendamm reaction out of 53 samples. The 28 strains that had potential lignin-degrading ability were isolated and used in the following experiments. Of the 28 strains, 25 samples, not including *P. chrysosporium* and one of the 28 strains, MG-60, could grow at 10% sea salt concentration, while, all of these 28 strains and *P. chrysosporium* could grow well at 0% sea salt concentration. However, the growth speed of the 25 samples was much slower at 10% sea salt concentration than that at 0% sea salt concentration.

We examined the decolorization ability of the 28 isolated strains under hypersaline conditions. Since 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. In light of this, the 28 isolated fungi were inoculated in the 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. While 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. In order to screen the hypersaline-tolerant strains, these 28 isolated strains were inoculated on the medium with 10% sea salts. Seven strains, MG-03, MG-04, MG-05, MG-24, MG-27, MG-32 and MG-53, were selected for their superior decolorization ability. MG-60 did not grow at 10% sea salt concentration. It was suggested that these 7 strains had stronger decolorization ability and higher tolerance to hypersaline conditions than other strains. Due to 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 was focused on these 8 strains. First, we determined the decolorization ability of the 8 selected strains at different sea salt concentrations. 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 strains were incubated with wood meals at different sea salt concentrations for 30 days. After incubation, the weight loss and the residual lignin content of wood meals were analyzed to evaluate the delignification ability of the strains. The results demonstrate that MG-60 had higher delignification efficiency and better lignin-degrading selection factors than did the other strains at 0%, 3% and 5% sea salt concentrations.

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 white rot type has been reported from three marine basidiomycetes, *Digitatispora marina*, *Halocyphina villosa* and *Nia vibrissa*. The fungus MG-60 has been classified as a kind of basidiomycete, of the *Phlebia* family, based on microscopic observation of its mycelium and determination of its 18SrDNA sequence.

A marine white-rot isolate, *Phlebia* sp. MG-60 secreted LiP, MnP and laccase under different sea salt incubation conditions. Its MnP production was strongly enhanced by adding 3% sea salts. The crude enzyme secreted at 3% sea salt concentration by *Phlebia* sp. MG-60, in which the main component was MnP, was then used to bleach unbleached hardwood kraft pulp *in vitro*. The pulp was brightened 11 points by 4 U of MnP, and the kappa number was decreased 6 points when only 0.5 mM H_2O_2 was added continuously. When 0.5 mM H_2O_2 was added at the initial bleaching, the pulp brightness increased 6 points with the dosage of 4 U of MnP. This screened salt-tolerant fungal strain is expected to be effective in bioremediation of the polluted marine environment.

3. Screening and evaluation of dioxin-degrading fungi

Polychlorinated dibenzo-*p*-dioxins (polyCDDs) and dibenzofurans (polyCDFs) are well known for their strong toxicity and mutagenicity. Contamination by these undesirable compounds has been a serious environmental problem. In the process of producing chlorine-containing herbicides, in the bleaching of paper pulp by using chlorine compounds, and during combustion of domestic and industrial waste, unintentionally formed polyCDDs and polyCDFs have been released into the environment as recalcitrant contaminants and have been found in many environment matrices. In Japan storage of soil, incinerated ash, and chlorine herbicides that have been highly contaminated by dioxins has become a serious problem. Effective bioremediation process for this problem has not yet been developed, although many trials have been carried out.

The biodegradation of dibenzo-*p*-dioxins (DDs) and dibenzofurans (DFs) by bacterial strains have been studied extensively, and degradation of DDs and DFs by filamentous fungi and yeast has also been reported. Although there are many reports of the biodegradation of dioxins, most reports describe the degradation of the non-chlorinated dioxins. Existing studies on the bacterial degradation of chlorinated DDs and DFs have been shown that when the chlorine displacement number increases, the degradation rate decreases. In addition, the degradation rate tends to greatly lower when DDs and DFs have been displaced in two aromatic rings with chlorine. The purpose of this study was to select some fungi that have high dioxin degrading and/or mineralizing ability than the reported strains, and we selected some candidates for bioremediation.

The strains used for screening were 74 strains belonging to 66 species of 19 genera and 62 unidentified strains which have been kept in our laboratory. All identified strains belong to the aphylloporales family. The selected genera were *Aleurodiscus* (three species, three strains), *Bjerkandera* (one species, one strain), *Ceriporia* (five species, six strains), *Ceriporiopsis* (four species, four strains), *Coriolus* (seven species, 10 strains), *Fomitopsis* (one species, two strains), *Gloephyllum* (one species,one strain), *Hericium* (two species, two strains), *Merulius* (one species, one strain), *Microporus* (one species, one strain), *Nigroporus* (one species, one strain), *Oligoporus* (two species, two strains), *Phanerochaete* (13 species, 13 strains), *Phlebia* (16 species, 19 strains), *Punctularia* (one species, one strain), Punus (one species, one strain), *Pycnoporus* (one species, one strain), *Stereum* (four species, four strains) and *Tyromyces* (one species, one strain). All fungi were incubated on a potato dextrose or malt extract agar plate at 25 or 30˚C, and 6-mm diameter disks were punched from the edge of the mycelium. Five disks were placed into each 100-ml Erlenmeyer flask containing 10 ml of the medium. The culture was incubated statically at 30˚C under ambient atmosphere. After incubation for 5 days, 40 µl of 25 mM dibenzo-*p*-dioxin (DD) solution in N,N-dimethylformamide (DMF) was added to each inoculated flask, after which the headspaces were flushed with oxygen and each flask was sealed with a glass stopper and sealing tape. The cultures were incubated for another 5 days. At the end of the incubation, an internal standard (dibenzofuran) was added

to the culture, mycelia were crushed in liquid nitrogen with culture medium. Acetone (10 ml) was added and then the mixture was homogenized. Biomass was removed by centrifugation at 3000 x g for 15 min. The resulting supernatant was analyzed by HPLC in order to determine DD quantitatively after the filtration by membrane filter. [U-^{14}C] 2,7-DiCDD was chosen as a representative model dioxins for *in situ* biodegradation studies. The cultivation was carried out as described above. After incubation for 5 days 50 µl of a 5mM [U-^{14}C] 2,7-diCDD (DMF solution) was added to each inoculated flask (315 kdpm flask^{-1}). Flasks were flushed every 3 days with oxygene (99%) and $^{14}CO_2$ was trapped in 10 ml of an ethanolamine-containing scintillation fluid. The trapped $^{14}CO_2$ was measured according to the ^{14}C radioactivity on a LSC.

In the present experimental condition, 27 strains among 136 tested in this study in all trial strains did not grow. However, it was observed that more than 20 % of added DD was consumed by eight strains belonging to four genera and four unidentified strains, and these strains were selected. These selected fungi, *Aleurodiscus disciformis* strain IFO-6280, *Ceriporia* sp. strain MZ-340, *Phanerochaete subceracea* strain TMIC 32054, *Phlebia lindtneri* strain GB-1027, *Phlebia subserialis* strain HHB-9678-Sp, *Phlebia subochrecea* strain HHB-8494-Sp, *Phlebia tremellosus* strain TMIC 30511 *Phlebia* sp. strain MG-60, unidentified strains HS-010, KD-070, MZ-227 and OK-190, were used in the next experiment. The fungal strains MZ-227, *Phlebia* sp. MG-60 and *P. lindtneri* showed higher cumulative CO_2 production rates compared to other nine fungi. MZ-227, *Phlebia* sp. MG-60, and *P. lindtneri* converted 250 nmol of 2,7-diCDD to 196, 155 and 149 nmol of $^{14}CO_2$, respectively, during a 30-day incubation period. In the uninoculated control, O_2 flash led to negligibly volatile radioactivity after the initial addition of [U-^{14}C] 2,7-diCDD.

Since it was shown that the white-rot fungus *Phlebia lindtneri* could mineralize 2,7-dichlorodibenzo-*p*-dioxin (2,7-diCDD) extensively, *P. lindtneri* was used to degrade both-ring chlorinated dioxins, 2,7-diCDD and 2,8-dichlorodibenzofuran (2,8-diCDF). After preincubation for 5days, 5mM substrate (2,7-diCDD or 2,8-diCDF) in *N,N*-dimethylformamide was added to each inoculated flask (0.25 µmol per flask), after which the headspace was flushed with oxygen and each flask was sealed with a glass stopper and sealing tape. Flasks were flushed with O_2 every 5days. After additional incubation, the whole culture was homogenized, washed by water and a small amount of acetone, and the washes were pooled. The residual biomass was air-dried and was Soxhlet-extracted with ethyl acetate (6 h). All pooled water layers were acidified to pH 2.0 with 0.1 N HCl and extracted with ethyl acetate (three times). Ethyl acetate extracts were mixed and dried over anhydrous sodium sulfate and evaporated to dryness at 40 ˚C. Recovery of substrates was determined by gas chromatography-mass spectrometry (GC-MS). The concentrate was cleaned up on a 12 × 80-mm silica gel column cartridge. The silica gel column was pre-eluted with *n*-hexane (6 ml), and eluted with ethyl acetate/ *n*-hexane (25:75, 6 ml). The latter fraction was analyzed by GC-MS after evaporation and derivatization using diazomethane in ether. As sterile controls, cultures of *P. lindtneri* that were killed by adding sodium azide (5-10 mg/ flask) after preincubation were used.

2,7-diCDD and 2,8-diCDF, were metabolized by the white-rot fungus *P. lindtneri*. 2,7-diCDD disappeared linearly in the culture of *P. lindtneri*; over a 20-day incubation period, with only 45% remaining in the culture. One of the metabolites produced by *P. lindtneri* from a 5-day incubated culture with 2,7-diCDD or 2,8-diCDF was identified by GC-MS. *P. lindtneri* was shown to metabolize 2,7-diCDD and 2,8-diCDF to hydroxy-diCDD and hydroxy-diCDF, respectively. The metabolites identified in this study were not in accordance with the LiP catalytic reaction proposed for *P. chrysosporium*, but indicated hydroxylated compounds common to mammals as well as fungi. It was assumed that the hydroxylated and/or further metabolic products were rapidly metabolized. Although an experimental confirmation is necessary, hydroxylation would result in lowering the ionization potential of recalcitrant chlorinated aromatic compounds, and would form suitable structures for the further metabolism by ligninolytic enzymes such as MnP and laccase.