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ENVIRONMENTAL BIOTECHNOLOGY: MOLECULAR BREEDING OF BASIDIOMYCETE FUNGAL STRAINS USEFUL FOR RECYCLE/ REUSE OF WASTE PLANT BIOMASS RESOURCE AND EFFICIENT DEGRADATION OF DIOXIN

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1. Introduction : White-rot basidiomycetous fungi are very attractive microorganisms to degrade lignocellulose, composed of cellulose, hemicellulose and lignin, which is the most abundant renewable organic resource on earth. They also degrade aromatic pollutants such as dioxins etc. So far, we have molecular-genetically produced the *Coprinus cinereus* strains useful for an efficient isolation of ethanol-convertible cellulose from rice straw, one of typical waste plant biomass resources and the *Coriolus hirsutus* strains useful for an efficient degradation of dioxin, a typical aromatic pollutant. The details of these our works, that were carried out as an Applied Life Science, are as follows.

2. Production of the *Coprinus cinereus* strains with high lignin- and xylan-degrading activities and their use for an efficient isolation of cellulose from rice straw : Lignocellulosic residues themselves are not high-value materials. However, if we can develop biological methods for the isolation of cellulose, which is convertible into ethanol by fermentation, from waste plant biomass, it is meaningful and remarkable from the point of view of obtaining a source of energy. In order to isolate celllulose from plant biomass, of which major components are cellulose, hemicellulose and lignin, it is required to eliminate lignin and hydrolyze hemicellulose. As described in introduction white-rot basidiomycete fungi degrade lignin. They can also hydrolyze hemicelluloses such as a xylan. However the productions of lignin-degrading enzymes such as manganese (II) peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lac) and xylan-hydrolyzing enzymes such as endo-(beta 1,4)-D-xylanase are all inducible. Therefore the constitutive and high level producution of these enzymes in basidiomycete fungi is important for efficient use of lignocellulosic residues.

2-1. Molecular breeding of the *Coprinus cinereus* strains with high lignin-decolorization and -degradation activities using novel heterologous protein expression vectors : Two chromosome-integrating vectors, pLC1 and pLC2, were used. The former is the pUC19-based vector carrying the *L. edodes ras* gene promoter and *priA* gene terminator, and the latter is the pBR322-based vector carrying the basal promoter and the terminator of the *priA* gene [1]. The MnP cDNA (*mnpc*) derived from *Pleurotus ostreatus* was fused between the promoter and terminator of pLC1 and pLC2, yielding the recombinant plasmids pLC1-mnp and pLC2-mnp. These plasmids were introduced into protoplasts of the monokaryotic *C. cinereus trp1* (Trp^-) strain with the *C. cinereus TRP1*-containing plasmid pCc1001 by co-transformation. We chose *C. cinereus* because of its high growth rate and production of more than ten isozymes of Lac. Two Trp^+ transformants for each plasmid, showing clearly higher lignin-decolorization activities, were obtained through introduction of pLC1-mnp and pLC2-mnp. Southern-blot analysis revealed that the four transformants all possess *mnpc* sequence on their chromosomes. One Trp^+ MnP^+ transformant (named CcTF2-7(Po.MnP)), which was derived from the introduction of pLC2-mnp and carried the highest copies (approx. 10) of *mnpc*, showed remarkably high lignin-decolorization and -degradation activities; at the time of cultivation when only 35% - 40% of the lignin was decolored and degraded by the control Trp^+ transformant obtained by the introduction of pCc1001 alone, almost all of the lignin was decolored and degraded by CcTF2-7(Po.MnP) [1].

2.2. Molecular breeding of the *C. cinereus* strains with high xylan-degradation activities using vectors pLC1 and pLC2 : The *Bacillus subtilis* endo-(beta 1,4)-D-xylanase structural gene (*xyn*) was trimmed away from its signal sequence and then fused after the signal sequence of the basidiomycete *P. ostreatus* MnP cDNA. The resulting modified gene (*xyn'*) was inserted between the promoter and terminator of pLC1 and pLC2. These recombinant plasmids pLC1-xyn' and pLC2-xyn' were introduced into protoplasts of the monokaryotic *C. cinereus trp1* strain with pCc1001. For each plasmid, one Trp^+ Xyn^+ transformant, showing a significantly high xylan-degrading activity was obtained and it was named CcTF1-16(Bs.Xyn) (derived from pLC1-xyn') or CcTF2-11(Bs.Xyn) (derived from pLC2-xyn'). CcTF1-16(Bs.Xyn) and CcTF2-11(Bs.Xyn) were estimated to carry about 6 and 10 copies of the *xyn'* sequence respectively, on their chromosomes. The supernatants of CcTF1-16(Bs.Xyn) and CcTF2-11(Bs.Xyn) obtained from their 18-day cultures contain a xylanase activity seven or nine times as high as that of the control Trp^+ transformant [2].

2-3. Efficient isolation of cellulose from rice straw by using CcTF2-7(Po.MnP) and CcTF2-11(Bs.Xyn) : To examine the usefulness of the molecular-bred C. cineres strains to isolate cellulose from waste plant biomass, we first tested the growth of CcTF2-7(Po.MnP) and CcTF2-11(Bs.Xyn) on solid medium containing rice straw, sawdust of beech wood, or fallen leaves as a waste plant biomass, revealing that rice straw is the most suitable source for culture medium. The two C. cineres strains were mixed-cultured at 27°C in the liquid-medium containing 0.5% (w/v) cut rice straw and 0.025% $MnCl_2$. After 3 weeks, the culture supernatant was extensively treated with crude cellulase, showing the presence in it of 9.3% of the total cellulose of rice straw [3]. When rice straw treated with 0.1 N NaOH (for 2 hrs) or cultured (for 1 week) with Elfvingia applanata were used, the recoveries of the cellulose increased up to 29% [3]. The same experiments were done by using a non-bred control strain, showing the recoveries of the cellulose from the treated or cultured rice straw to be 8%. These results indicate the success in the conversion of approx.

12% of total weight of rice straw into non-precipitable forms of cellulose. This is meaningful and interesting from point of view of obtaining a source of energy from waste material and recycling of natural resources.

3. Efficient degradation of chlorinated dibenzo-*p*-dioxins by using molecular-genetically bred *Coriolus hirsutus* strains : Chlorinated dibenzo-*p*-dioxins (CDDs) have been of public concern for two decades because of their toxicity in animal tests. Extracellular lignin-degrading enzymes LiP and MnP produced by white-rot basidiomycetous fungi have been reported to be involved in degradation of various CDDs [4]. Through various metabolic pathways, the white-rot basidiomycetous fungi convert CDDs to CO_2 and H_2O. Mammalian (Rat etc.) cytochrome P450, CYP1A1 has been reported to play an important role in the metabolism of mono-trichlorodibenzo-*p*-dioxins (M-TriCDDs). The CYP1A1-dependent metabolism includes multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, hydroxylation with elimination of a chloride substituent, and opening of the dioxin ring [4]. All of these reactions appear to be reactions aimed at detoxifying M-TriCDDs. These led us to attempt to produce white-rot mushroom strains producing large amounts of LiP and rat CYP1A1 and examine their degradation/transformation activities of CDDs. We chose *C. hirsutus* that transformation system was already established in this fungus and produces LiP, MnP and Lac as a recipient. The *C. hirsutus* LiP was chosen as a donor.

3-1. Efficient degradation of 2,7-DCDD by using molecular-genetically bred *C. hirsutus* strains producing large amounts of LiP enzyme : The chromosome-integrating vector MIp30 carrying the *C. hirsutus gpd* gene promoter—*L. edodes priA* gene terminator and the selectable marker of *C. hirsutus ARG1* gene was constructed [5]. The *C. hirsutus* LiP gene (*lip*) was fused between the promoter and terminator of MIp30 and the resulting recombinant plasmid MIp30-lip was introduced into protoplasts of monokaryotic *C. hirsutus* Arg^- Leu^- strain (OJ1078), followed by selection of Arg^+ Lip^+ colonies. Southern-blot analysis revealed that two of the Arg^+ Lip^+ transformants, named ChTF6-1(Ch.LiP) and ChTF6-2(Ch.LiP), possess the plural number of copies (approx. 5) of the promoter—*lip*—terminator cassette on their chromosomes [5]. Northern-blot analysis showed that both Arg^+ Lip^+ transformants contained large amounts of *lip* transcripts. The mycelial cells of the transformants were cultivated in BK medium containing 25 g brewer's grains and 100 ml Kirk Basal III medium per liter and grown at 25°C. The LiP activities of the ChTF6-1(Ch.LiP) and ChTF6-2(Ch.LiP) in their culture supernatants were found to be about five times as high as that of the Arg^+ control [5]. The degrading activity of 2,7-DCDD was analyzed as follows. The reaction mixture containing 2,7-DCDD and the culture supernatant were incubated for 20 hr at 25°C and the remaining 2,7-DCDD were extracted by hexane, followed by gas chromatographic analysis. The supernatants of ChTF6-1(Ch.LiP) and ChTF6-2(Ch.LiP) showed remarkably high 2,7-DCDD degradation activities: at the time when only 33.5 % of 2,7-DCDD was degraded by the control Arg^+ transformant, 73.7 % and 63.5 % of 2,7-DCDD were degraded by ChTF6-1(Ch.LiP) and ChTF6-2(Ch.LiP) respectively [5].

3-2. Degradation/transformation of 2,7/2,8-DCDDs by using molecular-genetically bred *C. hirsutus* strains producing rat cytochrome p450, CYP1A1 : To breed the lignin (and M-TriCDDs)-degrading *C. hirsutus* strains producing rat CYP1A1, the expression cassette [*C. hirsutus gpd* promoter-*C. hirsutus gpd* 5'-portion (224-bp sequences of 1st exon—8th base of 4th exon)-rat *cyp1a1* cDNA-*Lentinula edodes priA* terminator] was constructed and then inserted into pUCR1 carrying *C. hirsutus ARG1* gene. The resulting recombinant plasmid, MIp5-(cyp1a1+arg1) was introduced into protoplasts of *C. hirsutus* monokaryotic Arg^- Leu^- strain (OJ1078), obtaining three good Arg^+ transformants. These transformants, namely ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were estimated to carry nine, six, and seven copies of the expression cassette on their chromosomes respectively [6]. Immunoblot analysis revealed that the three transformants produce similar amounts of rat CYP1A1 enzyme. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and recipient strain (OJ1078) were cultivated in 10 ml of MYGC medium containing 10 μg of 2,7/2,8 (at the ratio of 1:1)-DCDDs in an L-shaped tube at 30°C for 5 days with shaking and the remaining amount of intra- and extracellular 2,7/2,8-DCDDs was measured. We chose the 5-day cultivation from the following reason. *C. hirsutus* monokaryotic strain produces only the limited amounts of lignin-degrading enzymes (LiP and MnP), which also degrade 2,7/2,8-DCDDs, and hereby the rat CYP1A1-catalyzed degradation/transformation of 2,7/2,8-DCDDs can be easily assessed. The hexane extracts of the whole cell cultures were subjected to gas chromatography and determined the total amount of the 2,7/2,8-DCDDs remaining both inside the mycelial cells and in the culture medium. In the gas chromatography, 2,7-DCDD and 2,8-DCDD give a single peak. The peaks of the 5 day-cultivations of ChTF5-2(CYP1A1), ChTF5-4(CYP1A1) and ChTF5-6(CYP1A1) showed that the three strains degraded/transformed 2,7/2,8-DCDDs much more efficiently than OJ1078. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), and ChTF5-6(CYP1A1) were indicated to degrade/transform 71.7%, 69.8%, and 69.4% of 2,7/2,8-DCDDs respectively, while recipient OJ1078 was shown to degrade/transform only 11.8% of 2,7/2,8-DCDDs [6]. The results strongly suggest that, at the 5-day cultivation, about 58.5 (70.3-11.8)% of 2,7- and 2,8-DCDD molecules added to culture medium were transported into the mycelial cells and degraded/transformed by rat CYP1A1 enzyme within them. We next examined the level of degradation/transformation of 2,7/2,8-DCDDs in a prolonged cultivation. ChTF5-2(CYP1A1), ChTF5-4(CYP1A1), ChTF5-6(CYP1A1) and OJ1078 were cultivated at 30°C for 16 days, when the production of LiP and MnP reaches to a maximum level. Although a constant recovery (%) of 2,7/2.8-DCDDs from the whole cell culture was not obtained probably owing to much larger mass of mycelial cells, roughly about 85-90% of 2,7/2,8 DCDDs was considered to be degraded by ChTF5-2(CYP1A1), ChTF5-4(CYP1A1) and ChTF5-6(CYP1A1), while about 30-35% was presumably degraded/transformed by OJ1078 [6]. Finally, a much more efficient degradation/transformation of chlorinated dioxins is thought to be achieved by co-cultivation of C. *hirsutus* strains producing rat CYP1A1 and those producing large amounts of LiP.

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