論文の内容の要旨

応用生命工学専攻
平成 20 年度博士課程入学
氏名　　羅　鋒
指導教員名 五十嵐 泰夫

論文題目

The promotion of laccase expression induced by interspecies interaction and its application to biomass pretreatment

（微生物間相互作用により誘導されるラッカーゼ発現促進とそのバイオマス前処理への応用）

Laccases have been paid much attention both from researchers and from industries in recent decades due to their possible involvement in the transformation of a wide phenolic and non-phenolic compounds and lignin, as well as bioremediation of highly recalcitrant aromatic environmental pollulants.

In nature, we can observe various occurrences of symbioses, synergies or competitions between or among diverse microorganisms, and interestingly, the secretion of fungal laccase is also involved in the biocontrol phenomenon. It is found that some fungi can promote the production of laccase during interactions with other organisms. This leads us to a possible way to upgrade laccase production to solve environmental problems, and to make or improve pretreatment processes of lignocellulosics by interspecies interactions.

Therefore, the aim of this study is to screen out the active lignolytic fungi and laccase-promoting interspecies interactions among white-rot fungi and its application to the pretreatment process of lignocellulosic biomass.

Chapter I. Laccase expression induced by co-culture and inductive materials

Firstly, this section was performed to screen out the most highly efficient cellulolytic fungi and the most active lignolytic fungi from these 10 species of fungi shown in Table 1:

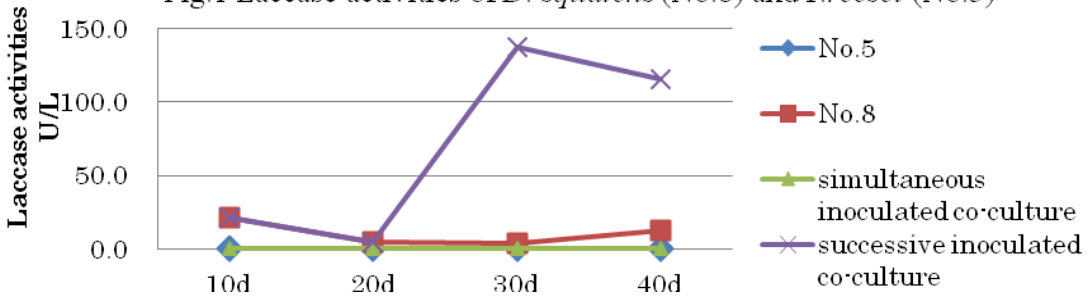
It was found that *T.reesei* (No.5) and *A.nidulans* (No.6) were the most efficient to degrade avicel and xylan (as culture media), while *D. squalens* (No.8) and *A. mellea* (No.10) exhibited the most predominant ability to delignify rice straw by the indicators of laccase and Manganese peroxidase (MnP) activity.

Table1 White-rot basidiomycete strains used in this study.

No.	Fungus Code	Species of fungus	No.	Fungus Code	Species of fungus
1	NBRC 30776	*Pleurotus ostreatus*	6	NBRC 4340	*Aspergillus nidulans*
2	NBRC 30388	*Trametes versicolor*	7	DSMZ 1016	*Panus tigrinus*
3	NBRC 9076	*Cyathus stercoreus*	8	DSMZ 9615	*Dichomitus squalens*
4	ATCC 90467	*Ceriporiopsis subermispora*	9	DSMZ 6909	*Phanerochaete chrysoporium*
5	NBRC 31326	*Trichoderma reesei*	10	NBRC 7037	*Armillaria mellea*

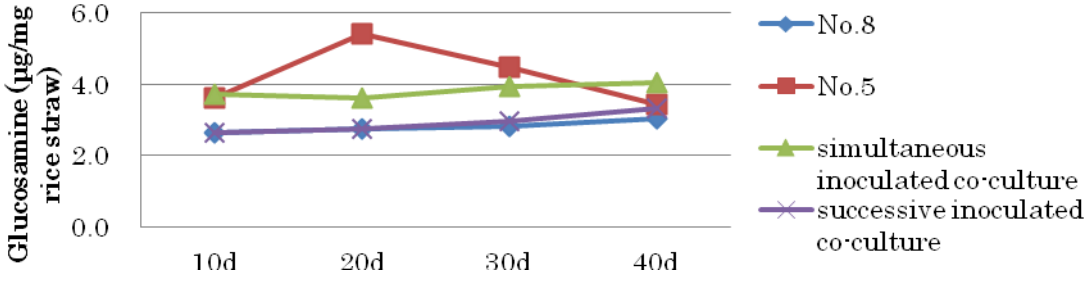
It is postulated that the two cellulolytic fungi and two lignolytic fungi would cooperate each other due to the synergy of cellulases and lignolytic enzymes in degradation of lignocellulosics. To construct the synergic combination between fungi, four sets of two-species groups, *D.squalens* and *T. reesei*, *D.squalens* and *A.nidulans*, *A.mellea* and *T. reesei* or *A.mellea* and *A.nidulans* have been incubated for 40d in rice straw by simultaneous inoculation or successive inoculation (the latter was inoculated after the former had been cultivated for 20d). Unexpectedly, hardly laccase activites were detected in all simultaneous inoculated co-cultures during the whole incubation, but in successive inoculated co-cultures, the laccase activities in pure culture phase (for 20 days) can be amplified by inoculating the latter fungus in three two-species groups, *D.squalens* and *T.reesei*, *D. squalens* and *A.nidulans*, or *A. mellea* and *T. reesei*, significantly, laccase activity was upgraded 20 folds in *D.squalens* and *T.reesei*, and 7 folds in *D. squalens* and *A.nidulans*.

Fig.1 Laccase activities of *D. squalens* (No.8) and *T.reesei* (No.5)



This study took the group of *D.squalens* (Ds) and *T.reesei* (Tr) as an example to verify the phenomenon of laccase increase. Obviously, it was shown that laccase from *D.squalens* was induced by inoculation of *T.reesei*. It was not because cell growth by some synergies between them brought laccase increase as *T.reesei* showed little growth in successive inoculated co-culture after vaccination in 20d and since 20d, *D.squalens* has exhibited no obvious changes in its quantities, too (Fig.2), and moreover, no laccase activity was detected in *T.reesei*'s pure culture (Fig.1).

Fig.2 The microbial quantities during incubation



What are the inductive materials? No laccase induction phenomenon appeared in non-lignin media(PDB) by the same way of successive inoculated co-culture, but the addition of cell-free supernatant from pure culture *T.reesei*'s 5-day cultivation in rice straw (Supernatant Tr) to PDB media cultivating *D.squalens* could strongly induce laccase, and the autoclaved supernatant can also bring laccase induction. So the inducers proved to be heat-stable, and may relate to lignin or its derivatives. Then three basic lignin component Units, p-hydroxyphenyl (H) acid, Guaiacyl (G) acid and syringyl (S) acid were added to *D.squalens* non-lignin PDB media, respectively and S unit was found to be able to induce laccase at the same extent as rice straw.

Additionally, all the ultrafiltrate fractions from Supernatant Tr by 10K-cut-off or 5K-cut-off ultrafilter can actively induce laccase as Supernatant Tr, which shows that not only a sole component but a series of materials with continuous molecular sizes from 0 to over 10KD containing S unit can be the inductive materials, therefore it is concluded that the partially degraded lignin with 0 to over 10KD molecular mass degraded by *T.reesei* have induced laccase expression from *D. squalans*.

To verify this postulation, soluble lignin in Supernatant Tr was completely depolymerized to monomolecular lignin component units by alkaline nitrobenzene oxidation and about a 3 folds of S component unit concentration was detected than in *D.squalens* culture in rice straw. Besides it, few laccases were found in *D. squalens* culture cultivated in submerged powder media of red pine, a softwood species, in which lignin contains no S unit. Undoubtedly, the conclusion above has been proved.

Chapter II. Purification and characterization of pure culture's laccase and induced laccase

In this chapter, the laccases from *D.squalens* pure culture and induced by co-culture were purified and characterized. Two kinds of laccases, Lac1 and Lac 2 have been purified, and Lac 1 was the minor laccase while Lac 2 represented the major laccase in *D.squalens* laccase systems. And the peak of Lac 2 was apparent to be amplified by induction, but it is unclear whether Lac 1 was induced or not.

Table 2. Comparison on the characteristics between laccase from pure culture or co-culture

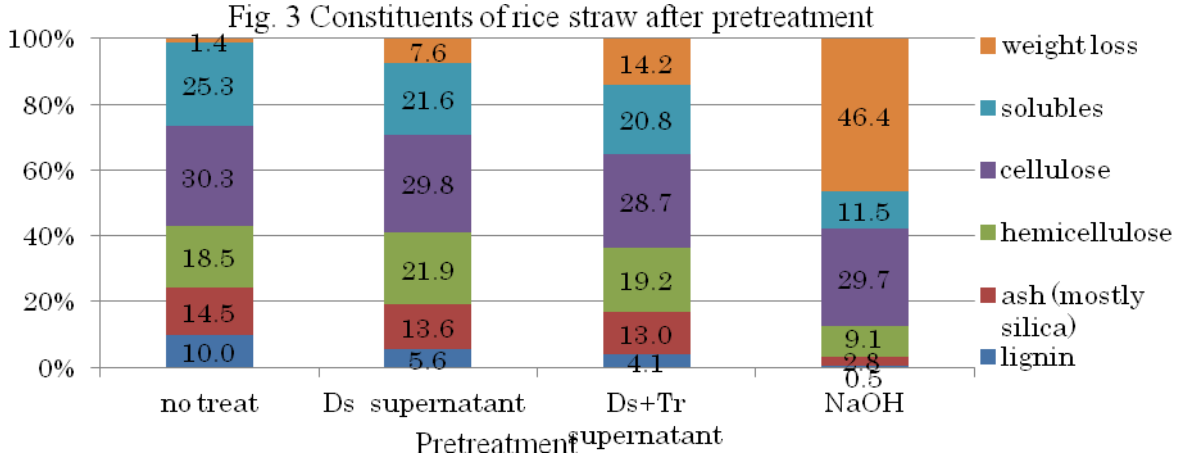
Parameters	**Lac 2 from pure culture**	**Lac 2 induced by co-culture**
Molecular mass	60 (denatured); 51.5 (native)	60 (denatured); 50.0 (native)
Isoelectric focusing	3.8	3.7
Temp. of optimal activitiy	65 °C	65°C
pH of optimal activity	2.5	2.5
N-terminal amino acid sequence	GIGPVTDLTITNADIAPDDF	GIGPVTDLTITNADIAPDDF

Based on the characteristics between two Lac 2 (from pure culture and co-culture) shown in Table 2 and elution profiles, it was concluded that no novel laccase was induced and the two laccases are of the same laccase.

Chapter III. Lignolytic pretreatment of rice straw by crude induced laccase

As yet there are no reports that lignocellusic biomass was pretreated efficiently by laccase. This work has had a trial to delignify rice straw by crude laccase as pretreatment process using the alkaline pretreatment (NaOH) and no treatment as control experiments and assessed its effect from various angles.

Fig. 3 Constituents of rice straw after pretreatment



From Fig.3, crude laccases from Ds pure culture and co-culture can depolymerize lignin of rice straws by 44% and 59% in 5d, respectively. Moreover, by further analysis of dioxane extraction from pretreated solid residues, it can be known that 18.6% and 24.7% rice straw have been partially depolymerized by laccase from Ds pure culture and co-culture, respectively, and what is more favorable is that, in the laccase-pretreated liquid residues, concentrations of aromatic compound were even lower than no-treated samples. This showed that the depolymerized lignin in liquids had completely oxidized. Although alkaline pretreatment (NaOH) delignified 95% of lignin (Fig.3) and the 5% lignin residue can also be extracted by dioxane as partially degraded lignin, the residual aromatic compounds in pretreated liquids showed a very high concentration (104.4 g/L).

Moreover, results of saccharifying the pretreated rice straw showed that there was no change in detected glucose conc. compared to not-treated straw. By further analysis of adsorption technique using two dyes having high affinity to cellulose, results showed that the increase of exposed cellulose was little, and from Fig.3, negligible silica (ash) removal was observed, showing that cellulose was still encrusted by silica.

Conclusions

D.squalens is a more efficient lignin-degrading fungus in rice straw media than many famous wood-rotting fungi and its laccase production can be further induced by co-culture with *T.reesei*. The lignin partially degraded by *T.reesei* strongly induced the *D.squalens* laccase, Lac 2. The crude induced laccase delignified 59% of rice straw lignin, and further partially depolymerized 24.7% of lignin and moreover, thoroughly decomposed these fall-off lignin to non-aromatic materials.

Though laccase cannot remove silica affecting saccharification from rice straw, still its use is promising for pretreatment of herbaceous plants with low silica content as well as woody materials.