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Screening and Evaluating of Wood-Rotting Fungi for Lignin Degradation and Ligninolytic Enzyme Production (Ⅱ)*1

- Laccase Production by Lignin-Degrading Fungi -

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리그닌分解와 리그닌分解酵素 生産을 위한 木材腐朽菌의 選拔과 評價 (Ⅱ)*1

- 리그닌分解菌에 의한 laccase 生産 -

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요 약

리그닌分解能이 높은 菌株로 選拔된 *Coriolus versicolor*-13 (CV-13), LKY-7 및 LKY-12 세 菌株에 대하여 菌體外 laccase生産을 檢討하였다. Glucose-peptone broth에서 菌體外 laccase活性은 CV-13의 경우 3일 以上 培養後에 나타났고 LKY-7과 LKY-12菌株의 laccase 活性은 培養 2일째에 檢出되었다. 炭素源으로서는 maltose가 glucose와 비슷한 laccase 生産效果를 나타냈고 窒素源으로서는 有機態窒素가 無機態 窒素보다 效果的이었다. Laccase 誘導物質로서는 2,5-Xylidine이 가장 우수하였으며 1mM 以下의 濃度에서는 誘導效果가 크게 나타났으나 1.5mM 以上의 濃度에서는 laccase生産이 抑制되었고, 菌絲生長 初期에 添加하는 것이 效果的으로 나타났다. SDS-PAGE 후, CV-13菌株의 菌體外 蛋白質에서는 약 69, 66, 25, 23, 19kDa 크기의 laccase band가 5개 나타났고 LKY-7 菌株에서는 27kDa과 19kDa 크기의 2개 band가, LKY-12 菌株에서는 22, 20, 17kDa 크기의 laccase band가 3개 나타났다.

Keywords : Laccase, carbon source, nitrogen source, 2,5-xylidine, laccase band

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1. INTRODUCTION

The importance of phenoloxidases in lignin degradation has been discussed for a long time. These enzymes catalyze the removal of one electron from phenolic hyroxyl groups and from amino groups of a wide range of phenols and aromatic amines(Mayer & Harel, 1979). Laccase, one of the phenoloxidases, is an extracellular enzyme that is produced abundantly by white-rot fungi. In many wood-rotting fungi, the production of laccase corresponds with the presence of ligninolytic activity, thus suggesting a role for this enzyme in lignin biodegradation. However, the role of laccase in biodegradation of lignin has not been well established. Although that is not fully understood, generally a positive correlation between laccase production and lignin degradation was reported(Arora & San du, 1985 · 1987). In our laboratory, three isolates of *Coriolus versicolor*-13, LKY-7, and LKY-12 which were high active in lignin degradation and laccase activity were screened. These isolates were considered to be able to use in biological pulping and bleaching and ligninolytic enzyme production(Jung *et al.*, 1995). Therefore, the present study was undertaken to evaluate and to establish the laccase production potential of these three isolates under different nutritional conditions and various laccase inducers addition for lignin degradation and ligninolytic enzyme production.

2. MATERIALS & METHODS

2.1 Strains and culture condition

The lignin degrading fungi, Coriolus versicolor-13, LKY-7, and LKY-12 which were screened for lignin degradation and ligninolytic enzyme production in our laboratory, were used throughout this study. Standard liquid cultures in 250㎖ Erlenmeyer flasks contained 35㎖ of glucose-peptone broth, which consisted of the following (per liter) : 30g of glucose, 10g of peptone, 1.4g of KH_2PO_4, 0.5g of $MgSO_4$, 2㎎ of Thiamin-HCl, and 20㎎ of $CuSO_4$. The pH was adjusted to 5 before autoclaving. The screened fungi were incubated on this stationary culture at 29℃ for 12 days. Mycelium weight and extracellular laccase activity were measured periodically at 2 days interval.

The effects of different sources of carbon and nitrogen on mycelial growth and laccase production were investigated. The carbon sources were monosaccharides(glucose, fructose), disaccharides (lactose, maltose), polysaccharides(starch, cellulose), and phenolic compound(lignosulfonic acid, sodium salt). The amount of the carbon sources added were calculated so that the same amount of glucose in glucose-peptone broth was added. And the nitrogen sources were two inorganic compounds(ammonium chloride, ammonium nitrate) and four organic compounds(arginine, asparagine, glutamic acid, yeast extract). The nitrogen sources were added at a final concentration of 0.026% N, whereas yeast extract at 1% level in the medium.

To induce the laccase production, cycloheximide(0.003mM), ferulic acid(1mM), gallic acid (1mM), guaiacol(1mM), vanillic acid(1mM), and 2,5-xylidine(1mM), were added to the glucose-peptone broth, respectively. The effective inducer and concentration and addition time on laccase production were invesitigated.

Stationary incubation was done at 29℃ for 7 days. In all experiments, the dry weight of mycelium was determined by filtering sample through a preweighed Whatman NO. 4 filter paper. The residual mycelium was washed with distilled water, dried overnight at 80℃ and then weighed.

2.2 Preparation of extracellular crude enzyme and laccase assay

After incubation, mycelial pellets were seperated with a Buchner funnel equipped with Whatman NO. 4 filter paper under reduced pressure. To the filtrates were added ammoni-

um sulfate (0.9 saturation) which were kept over night at 4℃. The precipitates were collected by centrifuging of 12,000rpm for 20 minutes, and were extracted with phosphate buffer(pH 5.0). The filtrates were used as a crude-enzyme solution for laccase assay and the precipitates were used in analysis of the isozyme patterns of laccase.

Laccase activity was measured by monitoring the oxidation of syringaldazine (ε = 62,500) spectrophotometrically at 525nm. The reaction mixture contained 0.18M citric acid, 0.36M phosphate buffer(pH 6.0), 0.075mM syringaldazine, and suitable amount of crude-enzyme solution in total volume of 3*ml*. Enzyme boiled for 5 min. was used in the control.

2.3 Polyacrylamide gel electrophoresis

Isozyme patterns of laccase were displayed by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE). The precipitates were applied to a 4% stacking and 10% running gel, and laccase bands were visualized by soaking the gels in 0.1M sodium acetate buffer(pH 5.0) containing 1mM guaiacol.

3. RESULTS & DISCUSSION

3.1 Effect of incubation time on mycelial growth and laccase production

Three lignin-degrading fungi screened, *Coriolus versicolor*-13(CV-13), LKY-7 and LKY-12, were incubated at 2 days intervals for 12 days in glucose-peptone broth. Results of mycelial growth (mycelium weight) and laccase production were shown in Fig. 1 The three isolates grew rapidly during 8 days after inoculation and reached a maximum growth on 10 days of incubation, after which time it persisted constant growth. The mycelium weight of CV-13 isolate was highest in the three isolates, and LKY-12 isolate was comparatively slow glowing.

Laccase activity of LKY-7 and LKY-12 isolates were first detected in the extracellular fluid on 2 days incubation, whereas laccase activity of CV-13 were detected on 4 days incubation. All the three isolates approximately produced maximum laccase activity up to 8 and 10 days incubation, which indicated maximum laccase activity during active growth of mycelium. Maximum laccase production was observed in LKY-12 isolate with 85.68 nmole/*ml*/min. It was observed that CV-13 isolate was highest myceli-

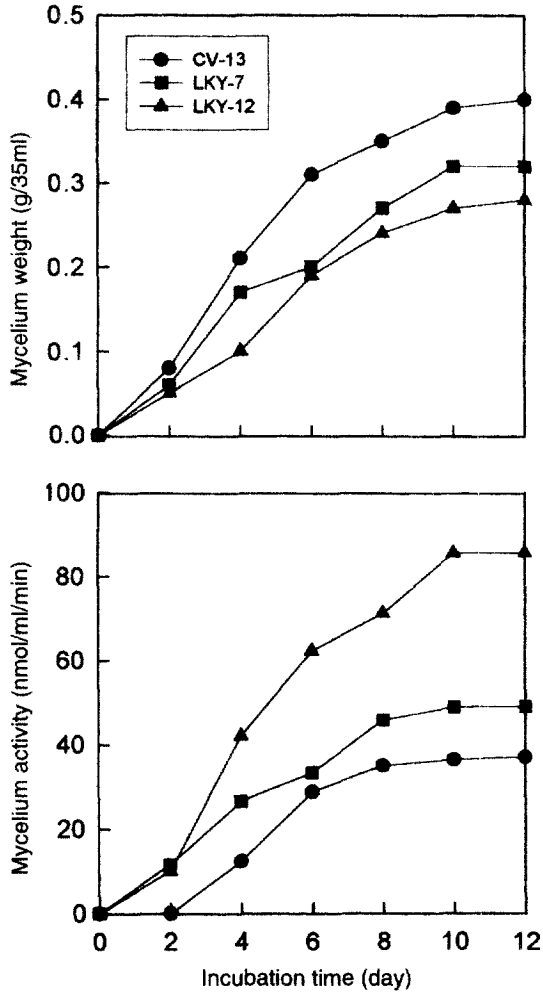

Fig. 1. Mycelium weight and laccase activity of lignin-degrading fungi in glucose-peptone broth.

Table 1. Effect of carbon sources on mycelial growth and laccase production.

Carbon sources	CV-13		LKY-7		LKY-12	
	M.W	L.A	M.W	L.A	M.W	L.A
Control	0.3354	30.59	0.2681	41.55	0.2331	67.33
Xylose	0.2783	20.81	0.1975	26.32	0.2069	35.31
Fructose	0.1524	8.12	0.1029	20.18	0.1274	25.08
Maltose	0.3035	28.76	0.2716	42.13	0.2245	66.27
Lactose	0.2823	27.25	0.2145	39.76	0.2598	59.49
Cellulose	0.0996	3.34	0.0724	4.25	0.0521	9.22
Starch	0.1186	7.54	0.1062	6.15	0.0987	13.37
Lignosulfonic acid	0.0565	-	0.0453	1.56	0.0124	8.34

Notes : *Coriolus versicolor*-13. M.W : mycelium weight (g/35*ml*). L.A : laccase activity (nmol/*ml*/min)

um weight but laccase production was much less than that of other isolates. Therefore, no consistent correlation between laccase production and mycelium build up was observed in this experiment. However, it was clearly showed that laccase production of three isolates is relation to its mycelial growth, being maximum during its active growth. Wood(1977) also made similiar observation that laccase concentration increases during mycelial growth and declines rapidly at the onset of fruiting.

3.2 Effect of carbon and nitrogen sources on mycelial growth and laccase production

The effect of different carbon sources on the mycelial growth and laccase production by the three isolates were summarized in table 1. When the various carbon sources were supplimented instead of glucose, mono and disaccharides showed better effect in mycelial growth and laccase production than polysaccharides and lignosulfonic acid. It was thought to be due to the lack of ability to degrade high molecular weight substances in these three isolates. Supplimentation of maltose and lactose as carbon source was almost the same efficiency in mycelial growth and laccase production as glucose. Especially, the supplimentation of maltose enhanced effectively the laccase production in case of LKY-7 and LKY-12 isolates. Xylose and fructose were also commonly utilized by most fungi (Hervey *et al*, 1978). However, although the effect of xylose on mycelial growth was similar to that of lactose, the effect on laccase production was low. And fructose as same monosaccharides was noticeably less efficient than any other mono and disaccharides on mycelial growth and laccase production.

To evaluate the effects of nitrogen sources on mycelial growth and laccase production, two inorganic and four organic compounds were added as nitrogen source instead of peptone in glucose-peptone broth to give a final concentration of 0.026% N, exception of yeast extract. The results were summarized in table 2. In all cases of the addition of various nitrogen sources, mycelial growth and laccase production by three isolates were shown to be low as compared with peptone(control). Although no apparent increase of mycelial growth and laccase production by various nitrogen sources were observed, organic nitrogen sources seemed generally to be more effective than inorganic nitrogen sources. And of the various nitrogen sources, the addition of yeast extract was found to be more effective on mycelial growth and laccase production than any other organic nitrogen resources. Arginine and asparagine were comparatively efficient in mycelial growth, but were less efficient in laccase production than

Table 2. Effect of nitrogen sources on mycelial growth and laccase production.

Nitrogen sources	CV-13		LKY-7		LKY-12	
	M.W	L.A	M.W	L.A	M.W	L.A
Control	0.3354	30.59	0.2681	41.55	0.2331	67.33
Amm. chloride	0.0635	2.11	0.0343	4.43	0.0787	2.19
Amm. n itrate	0.1554	4.18	0.1244	6.38	0.1098	5.43
Arginine	0.2645	17.25	0.2017	20.84	0.2136	21.46
Asparagine	0.2775	20.12	0.2319	24.31	0.2245	29.78
Glutamic acid	0.2043	12.45	0.1567	16.23	0.1834	20.25
Yeast extract	0.3079	28.12	0.2292	37.52	0.2135	59.78

yeast extract. These results are in agreement with those of Kalisz*et al.* (1986) who stated that the L-isomers of asparagine and argenine normally are good nitrogen sources for fungal growth.

3.3 Effect of laccase inducer on mycelial growth and laccase production

To enhance the laccase production, various phenolic compounds as laccase inducer were added to glucose-peptone broth after 3 days incubation. The results of the mycelial growth and extracellular laccase activity with various phenolic compounds as a laccase inducer were summarized Table 3. The addition of phenolic compounds was observed to be funtioned as not only inducer but also inhibitor in mycelial growth and laccase production. Of the 6 phenolic compounds, 2,5-xylidine was found to be the best inducer for laccase production, followed by ferulic acid. And the addition of guaiacol enhanced the laccase production to some extent in case of LKY-7 isolates.

In contrast, the addition of cycloheximide which has been known to be effect on eukaryote, gallic acid and vanillic acid had practically no effects on laccase production. The addition of these phenolic compounds in glucose-peptone broth were shown to be inhibited the mycelial growth and laccase production. The highest levels of laccase production was observed in LKY-12 isolate with 80.45 nmol/*ml*/min by adding 1 mM of 2,5-xylidine, followed by LKY-7 isolate with 51.68 nmol/*ml*/min.

The extracellular laccase activities of three isolates with various concentrations of 2,5-xylidine were shown in Fig. 2. The laccase activity increase with 2,5-xylidine concentrations was

Table 3. Effect of laccase inducer on mycelial growth and laccase production.

Laccase inducer	CV-13		LKY-7		LKY-12	
	M.W	L.A	M.W	L.A	M.W	L.A
Control	0.3354	30.59	0.2681	41.55	0.2331	67.33
Control	0.3354	30.59	0.2681	41.55	0.2331	67.33
Cycloheximide	0.2019	17.68	0.1314	21.79	0.1585	27.31
Gallic acid	0.2563	20.45	0.1618	30.13	0.1645	38.27
Guaiacol	0.3235	29.85	0.2512	46.82	0.2187	60.25
Ferulic acid	0.3346	38.97	0.2839	47.45	0.2419	78.19
Vanillic acid	0.3268	30.95	0.2624	42.17	0.2395	67.45
2,5-Xylidine	0.3414	36.24	0.2795	49.23	0.2552	80.45

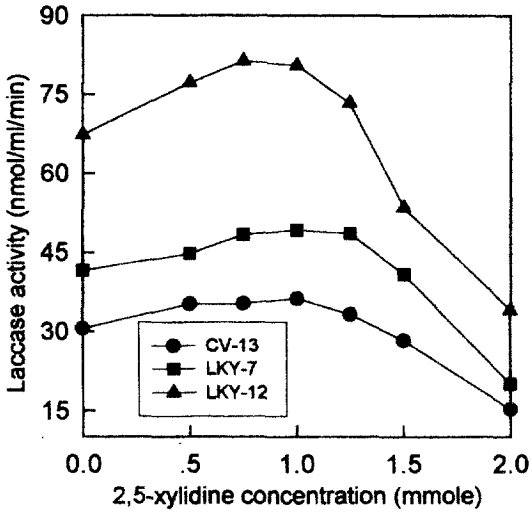

Fig. 2. Effect of 2,5-xylidine concentration on laccase activity.

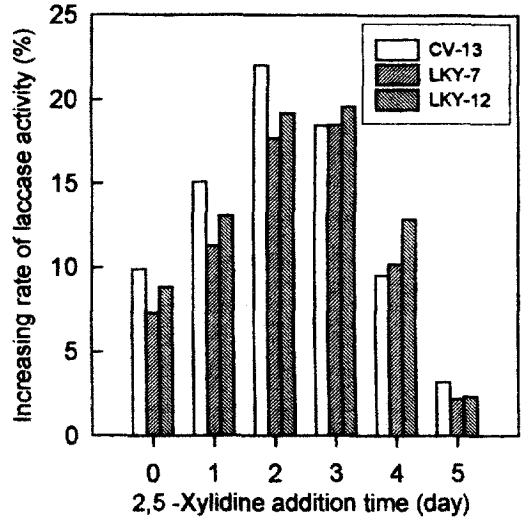

Fig. 3. Effect of 2,5-xylidine addition time on laccase activity.

high in LKY-7 isolate as compared with CV-13 and LKY-7 isolates, and reached a maximum at about 0.75 to 1.0 mM. That is, low concentrations of 2,5-xylidine tended to stimulate laccase production, but as the concentration of 2,5-xylidine was increased beyond 1.5mM, the laccase acctivity was gradually repressed. A higher concentration of 2,5-xylidine appeared to be apparently inhibitory in the laccase production of these isolates.

Fig. 3 showed the increasing rate of laccase activity versus control with the addition time of 2,5-xylidine. The addition time of 2,5-xylidine had quite different effect on laccase production. In general, the effect of 2,5-xylidine was high when it was added in initial growth period. The highest increasing rate of laccase activity were obtained when 2,5-xylidine was added after 2 days incubation in case of CV-13 isolates, and after 3 days incubation in LKY-7 and LKY-12 isolates. Additions after 5 days incubation showed almost no effects on the laccase production.

3.4 Laccase activity after SDS-PAGE

The extracellular protein of CV-13, LKY-7, and LKY-12 isolates were subjected to SDS-PAGE. After electrophoresis, the separation gel was soaked in 0.1M sodium acetate buffer (pH 5) containing 1 mM guaiacol to dectected laccase band. The patterns of laccase band of three isolates were shown in Fig. 4. A total of eight different guaiacol-oxidizing bands were produced by these three isolates, two laccase bands at 23 kDa and 18 kDa being approximately common to CV-13 and LKY-7 isolates. Several laccase isozymes have been reported for Coriolus versicolor (Bourbonnais *et al.*, 1990; Roy-Arcand *et al.*, 1991). CV-13 isolate appeared to be have three major band of laccase with molecular weight of approximately 69, 66, and 25kDa range, and two miner band with 23 and 19kDa range. LKY-7 isolate had two major band estimated molecular weight of 23 and 18kDa range. LKY-12 isolate had three major band with molecular weight of 22, 20, and 17kDa range.

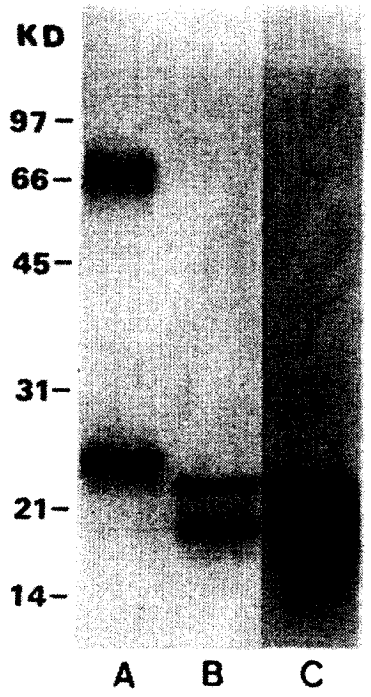

Fig. 4. Laccase activity after SDS-PAGE.
Notes: A : *Coriolus versicolor*-13.
B : LKY-7.
C : LKY-12.

4. CONCLUSIONS

Three wood-rotting fungi of CV-13, LKY-7 and LKY-12 which were screened for lignin degradation and ligninolytic enzyme production were tested for their laccase production. Laccase activity of LKY-7 and LKY-12 isolates were first detected in the extracellular fluid of glucose-peptone broth on 2 days incubation, whereas laccase activity of CV-13 isolate were detected on 4 days incubation. And it was observed that laccase production of three isolates is relation to its mycelial growth. Supplimentation of maltose and lactose as carbon source was almost the same efficiency in mycelial growth and laccase production as glucose. Of the various nitrogen sources, inorganic nitrogen sources seemed generally not to be utilized as efficiently as organic nitrogen sources. 2,5-xylidine was shown to be the best inducer for laccase production of these three isolates, followed by ferulic acid. Low concentrations(<1.0mM) of 2,5-xylidine tended to stimulate laccase production, but as the concentration of 2,5-xylidine was increased beyond 1.5mM, the mycelial growth and the laccase activity was gradually repressed. In general, the effect of laccase induce was high when 2,5-xylidine was added in initial growth period. Isozymes pattern of laccase by three isolates appeared to be have eight different bands, two laccase bands at 23kDa and 18kDa being approximately common to CV-13 and LKY-7 isolates. CV-13 isolate appeared to be have three major bands and two miner bands. LKY-7 isolate have two major bands, and LKY-12 isolate have three major bands. Estimated molecular weight of laccase bands of CV-13, LKY-7, and LKY-12 isolates were approximately between 69 and 19kDa, between 23 and 18kDA, and between 22 and 17kDa, respectively.

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