JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 861-868

Isolation, Classification, Culture Conditions, and Enzyme Production of a White Rot Fungus *Cerrena* sp. GSM-01

Shan-Shan Wang[1,2], Qing-Jun Chen[1], Kai Cui[2], Zi-Jiang Wang[2], Shou-Nan Wang[2], Hai-Wen Hu[2], and Guo-Qing Zhang[2]*

[1] Beijing Key Laboratory for Agricultural Application and New Technique, College of Plant Science and Technology, Beijing University of Agriculture, Beijing 102206, China.
[2] Key Laboratory of Urban Agriculture (North) of Ministry of Agriculture, College of Biological Sciences and Engineering, Beijing University of Agriculture, Beijing 102206, China.

(Received: 23 February 2015; accepted: 10 April 2015)

As white rot fungi, *Cerrena* species manifest efficient lignin degrading enzymes for bioremediation applications. A fungal strain from the genus *Cerrena* was isolated from its fruiting bodies using the tissue isolation method and numbered as GSM-01. It was identified as *Cerrena unicolor* using ITS identification methods. Optimal culture conditions of mycelia were: favorite carbon source of maltose, favorite nitrogen source of yeast extract, favorite C/N rate of 10/1, favorite growth factor of vitamin B_1, optimal temperature of 32 °C, and optimal pH of 6.5. It demonstrated extremely high laccase activity in liquid fermentation. The highest extracellular laccase activity of 2855.0 ± 41.6 U/mL was obtained in the broth with a Cu^{2+} adjunction concentration of 1.0 mM after a 8th day of cultivation. It is about 190-fold of that of reported *C. unicolor* strains and indicated its further applications. It also manifested considerably high RNase and phytase activities, but was devoid of protease and lectin activities.

Key words: *Cerrena unicolor*, ITS identification, Laccase, Liquid fermentation, Mycelia.

Lignin, one of the most abundant organic polymers on Earth, is a complex chemical compound most commonly derived from wood. In nature, white rot fungi are believed to the most efficient microorganisms in lignin degradation which is very important for the global carbon cycle[1, 2]. They produce enzymes, such as laccases, which can break down lignin, cellulose, and hemicelluloses. Recently, applications of white rot fungi have been extensively attracted because it can reduce the energy requirement and environmental impacts[3]. Extensive studies of white rot fungi have revealed that they have three different types of potentially ligninolytic enzymes: laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) [3, 4]. Laccase (benzenediol : oxygen oxidoreductase, EC 1.10.3.2) catalyzes the oxidation of various phenolic and inorganic compounds via a one-electron transfer mechanism using molecular oxygen as the electron acceptor[5, 6].

Cerrena unicolor, commonly known as the mossy maze polypore, is a very aggressive decay fungi species which even can attack living trees and causes extensive white-rot[7]. It was previously reported to be an excellent producer of laccase[8-10]. In the present study, a strain of *C. unicolor* was isolates from the living tree of *Ligustrum sinense*. Initial studies showed that the strain manifested extremely high laccase activity in PD medium. The goal of the work presented here was to clarify culture conditions for mycelial growth and enzyme production in liquid fermentation.

* To whom all correspondence should be addressed.
Tel & Fax: 86-10-80797308;
E-mail: zhanggqbua@163.com

MATERIALS AND METHODS

Strain

Mushroom fruiting bodies from genus *Cerrena* were collected from the tree truck of *L. sinense* located in the campus of Beijing University of Agriculture (Beijing, China). Pure culture strain was collected in Beijing University of Agriculture (numbered as GSM-01) and also in China General Microbiological Culture Collection Center (CGMCC No.7713).

Culture media

Potato dextrose agar (PDA) medium contained (g/L): potato 200 g, dextrose 20 g, agar 20 g, pH 7.0–7.2, distilled water 1000 mL. It was used for isolation and culture collection. Potato dextrose (PD) medium contained (g/L): potato 200 g, dextrose 20 g, pH 7.0–7.2, distilled water 1000 mL. It was used for liquid culture. Basic medium (BM) contained (g/L): glucose 20 g, soybean peptone 2 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, vitamin B_1 10mg, agar 20 g, pH 7.0–7.2, distilled water 1000 mL. Enrichment medium (EM) contained (g/L): glucose 20 g, soybean peptone 2 g, potato 200 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, vitamin B_1 10 mg, agar 20 g, pH 7.0–7.2, distilled water 1000 mL. BM and EM were used in optimal culture condition tests.

Strain isolation

Pure culture mycelia were obtained using the tissue isolation method[11]. In brief, the fruiting bodies were firstly undergone a surface disinfection with 70% ethanol. Subsequently, inner tissue was collected and planted on PDA media, and incubated at 25 °C for about a week under dark. Pure culture colonies were collected and for further studies.

ITS gene amplification and sequence analysis

Total genomic DNA was extracted using the CTAB method[12]. The entire Internal transcribed spacer (ITS) region (ITS-1, 5.8S, and ITS-2) was amplified by PCR, using universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). PCR reactions were performed in a volume of 50 µL under standard conditions[13]. The PCR production was sequenced by Invitrogen Corp. (Shanghai, China), and compared with ITS sequences in GenBank using the Blast tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, a neighbour-joining phylogenetic tree based on ITS sequences was mapped using the MEGA 4.0 software[14].

Effect of carbon sources on mycelial growth

In this assay, mycelial cultures of strain GSM-01 were grown in various carbon sources media based on the basic medium. The assayed carbon sources included glucose, lactose, maltose, mannitol, sodium carboxymethyl cellulose, sorbitol, starch, and sucrose. Same carbon content of different carbon sources instead of glucose (20 g/L) were used in the basic medium. Strain GSM-01 was cultured in petri plates (100 mm × 15 mm) using PDA medium at 25 °C for 7 d. Subsequently, mycelial colonies (5 mm in diameter) were collected and placed at the center of new petri plates with the various assay media. Incubation of the petri plates were carried out at 25 °C for 5–14 d until mycelial growth of any treatments firstly reached the edges of plates. Colony diameter and growth vigor were measured daily. Growth rate [colony radius (mm) / incubation time (d)] and dry weight of mycelia were evaluated. Basic medium without any glucose was used as a negative control. All determinations were performed in triplicate.

Effect of nitrogen sources on mycelial growth

To determine the effect of nitrogen sources, mycelial cultures were grown in modified basic media in which various nitrogen sources replaced same nitrogen content of soybean peptone (2 g). The assayed nitrogen sources included beef extract, glycine, soybean meal, soybean peptone, urea, yeast extract, NH_4NO_3, and $(NH_4)_2SO_4$. All the petri plates were carried out at 25 °C for 5–14 d. Growth rate and dry weight of mycelia were finally evaluated. Basic medium without any soybean peptone was used as a negative control. All determinations were performed in triplicate.

Effect of C/N rates on mycelial growth

In this assay, optimal carbon and nitrogen sources instead of glucose and soybean peptone were used in the improved basic media. In the assayed media, carbon sources were equal to glucose (20 g), while nitrogen sources were changed until reaching C/N rates of 10/1, 20/1, 30/1, 40/1, 50/1, and 60/1, respectively. All the petri plates were carried out at 25 °C for 5–14 d. Growth rate and dry weight of mycelia were

finally evaluated. All determinations were performed in triplicate.

Effect of growth factors on mycelial growth

In this assay, equal amount of various growth factors including vitamin B_2, vitamin B_6, vitamin C, corn syrup, and inositol were replaced vitamin B_1 in the basic medium. All the petri plates were carried out at 25 °C for 5–14 d. Growth rate and dry weight of mycelia were finally evaluated. Basic medium without vitamin B_1 was used as a negative control. All determinations were performed in triplicate.

Effect of temperature on mycelial growth

To determine the effect of temperature on mycelial growth, a modified enrichment medium was used. In the assayed medium, glucose, soybean peptone, and vitamin B_1 were replaced by the optimal carbon source, nitrogen source, and growth factor, respectively. The C/N rate reached to the optimum, while the pH value maintained to 7.0–7.2.The assayed petri plates were carried out at various temperature of 16, 20, 24, 28, 32, and 36 °C for 5–14 d with final evaluation of growth rate and mycelia dry weight. All determinations were performed in triplicate.

Effect of pH values on mycelial growth

In this assay, the medium in the temperature assay was used with improved pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. All the petri plates were carried out at 25 °C for 5–14 d. Growth rate and dry weight of mycelia were finally evaluated. All determinations were performed in triplicate.

Assay for enzyme production in liquid fermentation

In this assay, laccase, protease, ribonuclease, phytase, and lectin activities were determined. Mycelial colonies were pre-incubated in PD medium and in an orbital shaking incubator at 180 rpm and 25 °C for 5 days. The broth was used as the liquid seed with an inoculation of 5% (v/v) for the liquid fermentation using PD medium. After 8 days of cultivation at 180 rpm and 25 °C, the fermented broth was centrifuged a 12000 rpm for 15 min at 4 °C. Both the supernatant (broth) and the deposition (mycelia) were subsequently collected. Enzyme activities in both broth and mycelia were determined. All determinations were performed in triplicate.

Laccase activity was determined at pH 5.2 following Sun's assay protocol and using ABTS (2, 2'-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt) as the substrate[15]. One unit (U) of laccase activity was defined as the amount of enzyme required to produce one absorbance increase at 405 nm per minute per mL of the reaction mixture under the assay conditions. Protease activity was assayed following the protocol as described by Wang et al and using casein as substrate at pH 7.2[16]. Enzyme unit was represented as a 0.001 absorbance increase per minute in the supernatant per mL of the reaction mixture under assay conditions. Ribonuclease (RNase) activity was determined at pH 5.2 and 7.2 respectively using yeast tRNA as the substrate[17]. One unit of ribonuclease activity was defined as the amount of enzyme that produced one absorbance increase at 260 nm per min in per mL of reaction mixture under the assay conditions. Phytase activity was determined using sodium phytate as the substrate and following a modified ferrous sulfate–molybdenum blue method at pH 2.5 and 5.2 respectively as described by Zhang et al[18]. One unit of phytase activity was defined as the amount of enzyme that released l μmol phosphate per minute under the assay conditions. Lectin activity was determined towards 2% rabbit red blood cell suspension following Chan's description[19]. One hemagglutination unit was the reciprocal of the highest dilution of the sample inducing hemagglutination.

Effect of Cu^{2+} on laccase production

In this assay, $CuSO_4$ solution (1 mol/L) was added into PD medium for liquid fermentation with final Cu^{2+} concentrations of 0, 1.0, 2.0, 5.0, and 10 mmol/L, respectively. The broth was collected at 0, 48, 96, 144, and 192 h, and laccase activity in broth was subsequently assayed.

RESULTS AND DISCUSSION

Phylogenetic analysis based on ITS sequences

An expected 631 bp DNA fragment of ITS gene sequence was obtained by PCR amplification. The sequence was deposited in the GenBank database with an accession number of JQ798288.1. BLAST analysis indicated that, it

manifested the highest similaritiy (over 99%) with sequences generated from *C. unicolor*. A phylogenetic tree for its phylogenetic position was constructed based on ITS sequences of Polypores fungi (Fig. 1). The result indicated that strain GSM-01 and *C. unicolor* formed one cluster with the similaritiy of 100%. In addition, morphological characteristics of strain GSM-01 also falls well within the descriptions of *C. unicolor* in the taxonomy handbooks[20].

Effect of carbon sources on mycelial growth

The mycelia can grow using all the tested carbon sources (Table 1). The favorite carbon source was determined to maltose based on both mycelial dry weight and growth rate. The ranking of its mycelial dry weight towards the carbon sources was: maltose > starch > glucose > sucrose > mannitol > sorbitol > sodium carboxymethyl cellulose > lactose > CK, with a highest amount in maltose of 57.6 ± 6.0 mg. On the other hand, the fastest mycelial growth rate (10.7 ± 0.5 mm/d) was obtained in glucose test group. In the lacose and CK groups, mycelial growth rates were 3.8 ± 0.6 mm/d and 4.3 ± 0.9 mm/d respectively, which was just about 40% of that of the glucose group.

Effect of nitrogen sources on mycelial growth

The favorite nitrogen source of strain GSM-01 was determined to yeast extract, followed by soy peptone, based on both mycelial dry weight and growth rate (Table 2). It can use all the tested organic or inorganic nitrogen sources with a dry weight ranking of 'yeast extract > soy peptone > beef extract > soybean meal > urea > NH_4NO_3 > $(NH_4)_2SO_4$ > glycine > CK'. The strain likes organic nitrogen sources better than inorganic ones.

Effect of C/N rates on mycelial growth

The strain prefers a low C/N rate rather than higher ones. Its favorite C/N rate for mycelial growth was 10/1 with the mycelial dry weight of 38.3 ± 1.1 mg and the growth rate of 3.9 ± 0.1 mm/d, respectively (Table 3). The ranking of its mycelial dry weight towards various C/N rates was: 10/1 > 20/1 > 40/1 > 60/1 > 50/1 > 30/1. In this assay, the growth rates of all the test groups were very close (about 3.8–4.0 mm/d), whereas their mycelial mass exhibited high variability. In 10/1 group the mycelia was much whiter and thicker than that in 30/1 group.

Effect of growth factors on mycelial growth

The favorite growth factor of strain GSM-01 was determined to vitamin B_1 based on both mycelial dry weight and growth rate (Fig. 2A, B). The ranking of its mycelial dry weight towards various growth factors was: vitamin B_1 > vitamin C > vitamin B_2 > inositol > corn syrup > vitamin B_6.

Effect of temperature on mycelial growth

In this assay, maltose, yeast extract, C/N rate of 10/1, and vitamin B_1 were used as the carbon source, nitrogen source, C/N rate, and

Table 1. Effect of carbon sources on mycelial growth of *Cerrena unicolor* GSM-01

Carbon sources	Dry weight of mycelia (mg)	Mycelial growth rate (mm/d)
Maltose	57.6 ± 6.0 a	9.2 ± 1.3 de
Starch	55.1 ± 5.5 a	9.2 ± 0.4 cd
Glucose	48.7 ± 6.4 ab	10.7 ± 0.5 a
Sucrose	41.0 ± 4.4 bc	10.4 ± 0.7 ab
Mannitol	33.4 ± 6.2 cd	10.0 ± 0.9 bc
Sorbitol	27.1 ± 5.6 de	10.5 ± 2.0 a
Sodium carboxymethyl cellulose	25.6 ± 7.5 de	8.2 ± 0.6 de
Lactose	20.5 ± 6.1 e	3.8 ± 0.6 e
Negative control	16.7 ± 2.3 e	4.3 ± 0.9 e

Note: Different small letters mean significant differences (P<0.05).

Table 2. Effect of nitrogen sources on mycelial growth of *Cerrena unicolor* GSM-01

Nitrogen sources	Dry weight of mycelia (mg)	Mycelial growth rate (mm/d)
Yeast extract	30.5 ± 1.6 a	8.6 ± 0.1 a
Soy peptone	28.5 ± 3.4 a	8.8 ± 0.3 a
Beef extract	22.4 ± 1.2 b	8.3 ± 0.2 b
Soybean meal	20.6 ± 2.8 b	6.6 ± 0.1 d
Urea	19.3 ± 1.2 bc	6.6 ± 0.2 d
NH_4NO_3	15.5 ± 4.0 cd	7.9 ± 0.1 c
$(NH_4)_2SO_4$	14.1 ± 3.3 d	7.9 ± 0.1 c
Glycine	14.0 ± 0.7 d	4.0 ± 0.2 f
Negative control	11.8 ± 1.0 d	4.9 ± 0.3 e

Note: Different small letters mean significant differences (P<0.05).

growth factor, respectively. The best growth condition was observed at 32 °C with a mycelial dry weight of 48.2 ± 6.0 mg and a growth rate of 13.2 ± 0.2 mm/d, respectively (Fig. 3A, B). The mycelia can grow among the assayed temperature range of 16–36 °C, with a mycelial dry weight ranking of '32 °C > 36 °C > 28 °C > 24 °C > 20 °C > 16 °C'. As a white rotting fungus, fruiting bodies of *Cerrena unicolor* often emerge in hot seasons. That is why strain GSM-01 manifests a much higher favorite growth temperature.

Effect of pH values on mycelial growth

The favorite pH value of strain GSM-01 was determined to 6.5, with a mycelial dry weight of 35.5 ± 6.6 mg and a growth rate of 8.9 ± 0.1 mm/d, respectively (Fig. 4A, B). The ranking of its mycelial dry weight at various pH values was: 6.5 > 5.0 > 5.5 > 7.5 > 9.0 > 7.0 > 8.5 > 8.0. *The strain* GSM-01, just like most of fungi species, requires acidic conditions for its optimal growth.

Enzyme production in liquid fermentation

After liquid fermentation, laccase, protease, ribonuclease, phytase, and lectin activities were determined in both broth and mycelia. The results were shown in Table 4. GMS-01 manifested extremely high laccase activities of 673.6 ± 18.5 U/mL and 2822.7 ± 312.3 U/g in both broth and mycelia, respectively. Considerably high RNase and phytase were obtained, whereas protease and lectin activity were devoid.

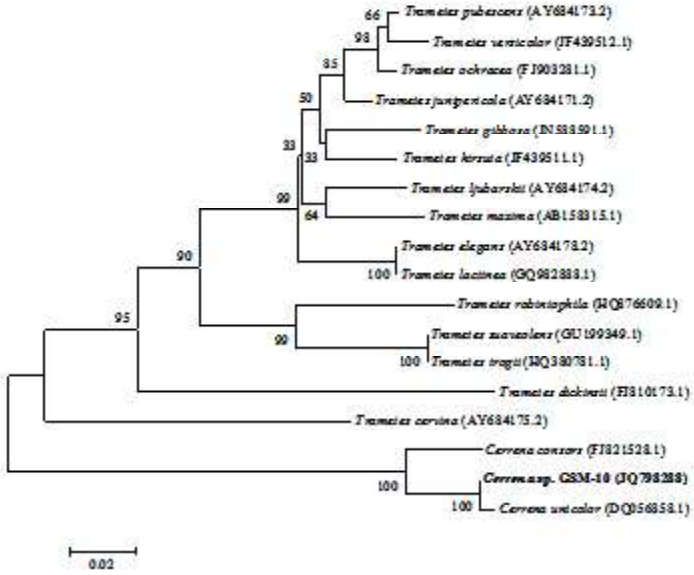

Fig. 1. Phylogenetic analysis *Cerrena unicolor* GSM-01 and other Polypores species based on their ITS sequences

Table 3. Effect of C/N rates on mycelial growth of *Cerrena unicolor* GSM-01

C/N rates	Dry weight of mycelia (mg)	Mycelial growth rate (mm/d)
10/1	38.3 ± 1.1 a	3.9 ± 0.1 ab
20/1	30.5 ± 1.6 b	4.0 ± 0.0 a
40/1	22.4 ± 1.2 c	4.0 ± 0.1 a
60/1	20.4 ± 0.8 c	3.8 ± 0.0 b
50/1	15.5 ± 4.0 d	3.9 ± 0.1 ab
30/1	14.1 ± 3.3 d	4.0 ± 0.0 a

Note: Different small letters mean significant differences (*P*"ÿ0.05).

Table 4. Enzyme activities of in broth and mycelia of *Cerrena unicolor* GSM-01

Enzymes		In Broth (U/mL)	In mycelia (U/g)
Laccase		673.6 ± 18.5	2822.7 ± 312.3
Protease		0	0
RNase	pH 5.2	4.3 ± 1.8	64.9 ± 16.7
	pH 7.2	11.4 ± 0.6	84.4 ± 23.0
Phytase	pH 2.5	10.4 ± 0.5	31.0 ± 4.4
	pH 5.2	9.1 ± 2.6	20.8 ± 2.8
Lectin		0	0

Laccase activity of *Cerrena unicolor* was reported in previous studies[7-9, 21]. A laccase from *C. unicolor* C-139 was fermented in the Lindenberg and Holm medium with Cu^{2+} adjunction. The obtained laccase level in culture liquid was about 65,000 nkat/L (3.9 IU/mL) towards syringaldazine[8]. *C. unicolor* VKM F-3196 manifested a 15 U/mL of laccase activity towards ABTS on the 8th day of cultivation with its optimal Cu^{2+} adjunction concentration of 0.1 mM[9]. In the present, laccase

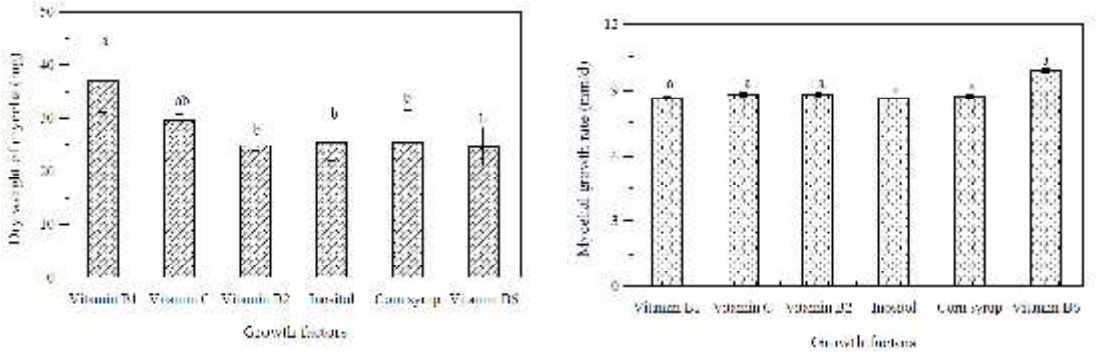

Fig. 2. Effect of growth factors on mycelial growth of *Cerrena unicolor* GSM-01: a) Dry weight of mycelia, b) Mycelial growth rate (mm/d). Different small letters mean significant differences (P<0.05).

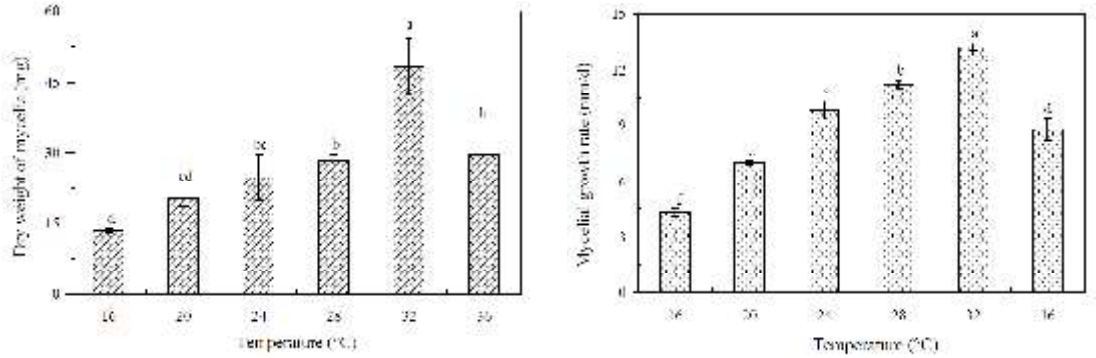

Fig. 3. Effect of temperature on mycelial growth of *Cerrena unicolor* GSM-01: a) Dry weight of mycelia, b) Mycelial growth rate (mm/d). Different small letters mean significant differences (P<0.05).

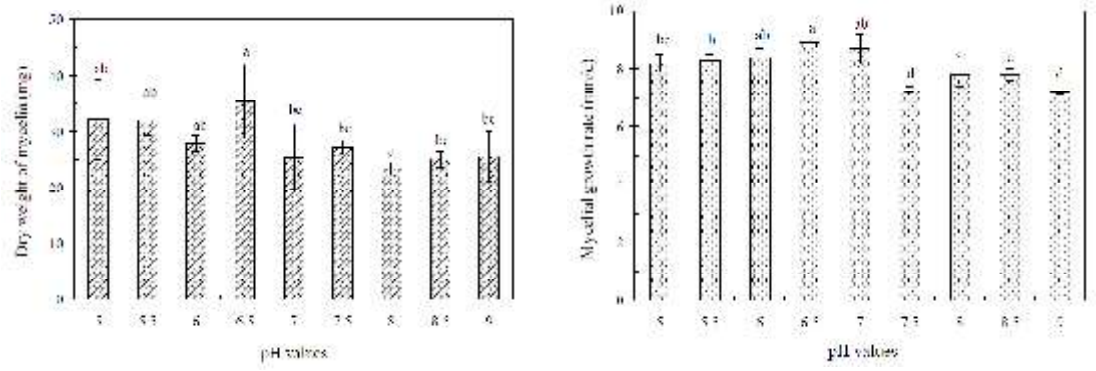

Fig. 4. Effect of pH values on mycelial growth of *Cerrena unicolor* GSM-01: a) Dry weight of mycelia, b) Mycelial growth rate (mm/d). Different small letters mean significant differences (P<0.05).

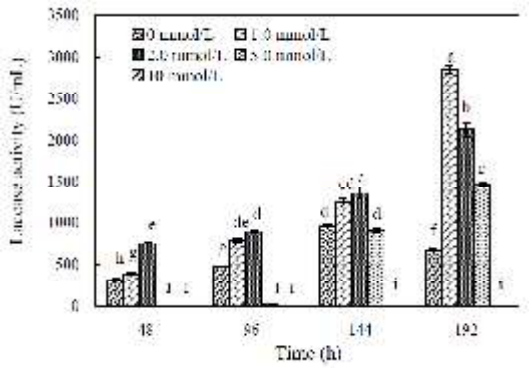

Fig. 5. Effect of Cu^{2+} on laccase production in liquid fermentation

activity in the broth was as high as 673.6 ± 18.5 U/mL without any Cu^{2+} adjunction, which was about 45-fold of that of *C. unicolor* VKM F-3196 with its optimal Cu^{2+} adjunction.

Effect of Cu^{2+} on laccase production

Activation of Cu^{2+} on laccase production was shown in Fig. 5. The highest extracellular laccase activity of 2855.0 ± 41.6 U/mL was obtained in the broth with a Cu^{2+} adjunction concentration of 1.0 mM after a 8th day of cultivation. It is a 4.2-fold of that of Cu^{2+} free (CK) group and about 190-fold of that of *C. unicolor* VKM F-3196[9]. Since copper is a heavy metal, mycelial growth of GSM-01 was intensely inhibited when the Cu^{2+} adjunction concentration was higher than 5.0 mM. The mycelia can not survive with a Cu^{2+} concentration of 10 mM.

Laccase is usually called multicopper oxidases with copper atoms in the catalytic centre[22, 23]. Cu^{2+} adjunction during the cultivation can promote laccase production in many fungi species including *C. unicolor*[8, 9], *Phlebia radiate*[24], and *Proteus hauseri*[25], etc. The optimal Cu^{2+} adjunction concentration of GSM-01 is 1.0 mM, which is different from that of *C. unicolor* VKM F-3196 (0.1 mM)[9] and *P. hauseri* ZMd44 (3.0 mM) [25]. Zheng et al speculated that the possible activation mechanism of copper ions with high concentration was they can enhance the laccase genetic transcription level during the laccase synthesis[25]. Based on the present study, laccase activity of 5.0 mM Cu^{2+} group emerged at 6th day, and was considerably higher than the CK group at 8th day. It indicates that copper ions might transform into laccase and low the copper ions level in media. That is why the lacase activity suddenly increased since 6th day.

CONCLUSION

In the present study, a white-rotting fungus with extremely high extracellular laccase activity was isolated from the tree trunk of *L. sinense*. It was determined as *C. unicolor* by ITS gene amplification and phylogenetic analysis. Culture condition studies showed that it manifested favorite carbon source of maltose, favorite nitrogen source of yeast extract, favorite C/N rate of 10/1, favorite growth factor of vitamin B_1, optimal temperature of 32 °C, and optimal pH of 6.5. It demonstrated extremely high laccase activity in liquid fermentation with extracellular laccase activity of 2855.0 ± 41.6 U/mL in the broth after an 8th day of cultivation.

ACKNOWLEDGMENTS

This work was financially supported by National Grants of China (31200070, and 2012BAD14B09), Beijing NOVA Program, Beijing Higher Education Young Elite Teacher Project (YETP1714), and Beijing Grants of Undergraduate Research Program.

REFERENCES

1. Eggert, C., Temp, U., and Eriksson, K. E. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl Environ Microbiol*, 1996; **62**(4): 1151-8.
2. Ko, E. M., Leem, Y. E., and Choi, H. T. Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. *Appl Microbiol Biotechnol*, 2001; **57**(1-2): 98-102.
3. Wesenberg, D., Kyriakides, I., and Agathos, S. N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol Adv*, 2003; **22**(1-2): 161-87.
4. Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N. S., Hofrichter, M., and Rogalski, J. Biodegradation of lignin by white rot fungi. *Fungal Genet Biol*, 1999; **27**(2-3): 175-85.
5. Morozova, O. V., Shumakovich, G. P., Gorbacheva, M. A., Shleev, S. V., and Yaropolov,

A. I. "Blue" laccases. *Biochemistry (Mosc)*, 2007; **72**(10): 1136-50.
6. Zhang, G. Q., Wang, Y. F., Zhang, X. Q., T.B., N., and Wang, H. X. Purification and characterization of a novel laccase from the edible mushroom *Clitocybe maxima*. *Process Biochemistry*, 2010; **45**(5): 627-33.
7. Michniewicz, A., Ullrich, R., Ledakowicz, S., and Hofrichter, M. The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. *Appl Microbiol Biotechnol*, 2006; **69**(6): 682-8.
8. Rogalski, J. and Janusz, G. Purification of extracellular laccase from *Cerrena unicolor*. *Prep Biochem Biotechnol*, 2010; **40**(4): 242-55.
9. Lisova, Z. A., Lisov, A. V., and Leontievsky, A. A. Two laccase isoforms of the basidiomycete *Cerrena unicolor* VKMF-3196. Induction, isolation and properties. *J Basic Microbiol*, 2010; **50**(1): 72-82.
10. Elisashvili, V., Kachlishvili, E., and Bakradze, M. Dependence of activities of polysaccharide hydrolases and oxidases from *Cerrena unicolor* on the source of carbon and aromatic acids in culture media. *Prikl Biokhim Mikrobiol*, 2002; **38**(3): 243-7.
11. Liu, Y. P., Wang, Z. J., Cui, K., and Zhang, G. Q. Isolation, Identification, Culture Conditions, and Enzyme Production of a Wild Mushroom *Lepista* sp. *Chin J Appl Environ Biol*, 2012; **18**(804-9).
12. Doyle, J. and Doyle, J. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull*, 1987; **19**(11-5).
13. Wang, P., Liu, Y., Yin, Y., Jin, H., Wang, S., Xu, F., Zhao, S., and Geng, X. Diversity of microorganisms isolated from the soil sample surround *Chroogomphus rutilus* in the Beijing region. *Int J Biol Sci*, 2011; **7**(2): 209-20.
14. Tamura, K., Dudley, J., Nei, M., and Kumar, S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol*, 2007; **24**(8): 1596-9.
15. Sun, J., Chen, Q. J., Cao, Q. Q., Wu, Y. Y., Xu, L. J., Zhu, M. J., Ng, T. B., Wang, H. X., and Zhang, G. Q. A laccase with antiproliferative and HIV-I reverse transcriptase inhibitory activities from the mycorrhizal fungus *Agaricus placomyces*. *J Biomed Biotechnol*, 2012; 2012(736472.
16. Wang, S. X., Liu, Y., Zhang, G. Q., Zhao, S., Xu, F., Geng, X. L., and Wang, H. X. Cordysobin, a novel alkaline serine protease with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Cordyceps sobolifera*. *J Biosci Bioeng*, 2012; **113**(1): 42-7.
17. Ngai, P. H. and Ng, T. B. A ribonuclease with antimicrobial, antimitogenic and antiproliferative activities from the edible mushroom *Pleurotus sajor-caju*. *Peptides*, 2004; **25**(1): 11-7.
18. Zhang, G. Q., Dong, X. F., Wang, Z. H., Zhang, Q., Wang, H. X., and Tong, J. M. Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from Aspergillus ficuum NTG-23. *Bioresoure Technol*, 2010; **101**(11): 4125-31.
19. Chan, Y. S., Wong, J. H., Fang, E. F., Pan, W., and Ng, T. B. Isolation of a glucosamine binding leguminous lectin with mitogenic activity towards splenocytes and anti-proliferative activity towards tumor cells. *PLoS One*, 2012; **7**(6): e38961.
20. William, C. R. 2003. Mushrooms of West Virginia and the Central Appalachians. University Press of Kentucky, Lexington.
21. Pegasova, T. V., Zwart, P., Koroleva, O. V., Stepanova, E. V., Rebrikov, D. V., and Lamzin, V. S. Crystallization and preliminary X-ray analysis of a four-copper laccase from *Coriolus hirsutus*. *Acta Crystallogr D Biol Crystallogr*, 2003; 59(Pt 8): 1459-61.
22. Baldrian, P. Fungal laccases - occurrence and properties. *FEMS Microbiol Rev*, 2006; **30**(2): 215-42.
23. Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. Multicopper oxidases and oxygenases. *Chem Rev*, 1996; **96**(7): 2563-606.
24. Makela, M. R., Lundell, T., Hatakka, A., and Hilden, K. Effect of copper, nutrient nitrogen, and wood-supplement on the production of lignin-modifying enzymes by the white-rot fungus *Phlebia radiata*. *Fungal Biol*, 2013; **117**(1): 62-70.
25. Zheng, X., Ng, I. S., Ye, C., Chen, B. Y., and Lu, Y. Copper ion-stimulated McoA-laccase production and enzyme characterization in *Proteus hauseri* ZMd44. *Journal of Bioscience and Bioengineering*, 2013; **115**(4): 388-93.