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Isolation, Identification, Optimal Culture Conditions, and Enzyme Production of a Wood-decay Fungus *Flavodon* sp. GSM-11

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A wood-decay fungus *Flavodon* sp. GSM-11 was isolated from imported wood *Gluta tavoyana* of Burma. It was identified as *F. flavus* based on ITS gene amplification and phylogenetic analysis. Culture characteristics of mycelia showed that it demonstrated favorite carbon source of glucose, favorite nitrogen sources of soybean meal and silkworm pupa meal, favorite C/N ratio of 10/1, optimal temperature of 36 °C, and optimal pH of 7.0, respectively. Undergoing an 8 day liquid fermentation with PD medium, *F. flavus* manifested considerably high extracellular laccase activity both in mycelia and in broth. Copper ions can stimulate laccase production at a concentration range of 1.0-5.0 mmol/L. The highest laccase production (485.0 ± 19.2 U/mL) was obtained with a Cu^{2+} adjunction concentration of 5.0 mM at 6th day of the fermentation. The strain also produced phytase, protease, and RNase with low levels, but was devoid of lectin.

Key words: Culture condition, Enzyme production, *Flavodon flavus*, ITS identification, Laccase.

Wood decay is a common biological process and is essential for the global carbon cycle. During wood decay process, lignin and cellulose, the two most abundant organic compounds on Earth, are degraded to carbon dioxide and water with a release of energy to maintain forest processes[1, 2]. Depending on types of wood decay they cause, fungi are classified as white-rots, brown-rots, and soft-rots. Among the three groups, white-rot fungi are considered as the most efficient ecosystem engineers, because they can break down all major wood components including cellulose, hemicelluloses and lignin[3]. The wood-decay ability of white-rot fungi is due to their possession of lignin degrading enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac), and etc [3, 4]. Recently, extensive studies of fungal laccases have been carried out owing to their potential applications in a variety of biotechnological processes: lignocellulosic biotransformation, environmental decolorizing and detoxification, bioremediation, chemical synthesis, and also food industries[5,6].

Flavodon flavus, a wood-decay fungus, belongs to order Polyporales and family Meruliaceae. To date, only a few literatures focused on the species which manifests effective lignin degrading enzymes and potential biotechnological applications. *F. flavus* PSU-MA201, isolated from leaves of mangrove (*Rhizophora apiculata*) from Thailand coasts, demonstrated a new rare difuranylmethane natural product flavodonfuran[7]. *F. flavus* NIOCC strain 312, which was isolated from sea grass of India coast, manifested decolorizing

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activity in low-nitrogen, nutrient-rich, and sugarcane bagasse media[8]. The strain 312 also produced the three lignin degrading enzymes LiP, MnP, and laccase[3]. In the present study, another strain of *F. flavus* was isolated from a kind of rosewood (*G. tavoyana*) from Burma, not from any sea habitats. Initial studies showed that the strain manifested considerably high laccase activity in PD medium. The goal of the present work was to clarify culture characteristics of mycelial growth and enzyme production in liquid fermentation.

MATERIALS AND METHODS

Strain

Fruiting bodies of *Flavodon* sp. were collected from imported wood *G. tavoyana* produced in Burma. The strain, numbered as GSM-11, was isolated from the fruiting bodies and collected in College of Biological Sciences and Engineering, Beijing University of Agriculture.

Culture media

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

Strain isolation

Pure culture mycelia were obtained using the tissue isolation technique. In brief, the fruiting bodies were firstly undergone a surface disinfection with 20% ethanol. Inner tissue was subsequently collected and planted on PDA pieces, followed by incubation at 25 °C for about a week under dark. Pure culture colonies were collected for further identification.

ITS gene amplification and sequence analysis

Total genomic DNA was extracted from fungal mycelia grown on PDA plates using the CTAB method[9]. The internal transcribed spacer (ITS) regions of 5.8S rDNA gene was amplified and sequenced using fungal universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3')[10]. Polymerase chain reaction (PCR) was performed in a volume of 50 µL under standard conditions. The PCR production was sequenced by Invitrogen Corp. (Shanghai, China). Sequence homolog analysis was performed by Basic Local Alignment Search Tool (BLAST) in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Fungi classification was based on 5.8S rDNA gene sequence similarity to BLAST hits. Subsequently, a neighbour-joining phylogenetic tree was generated with 5.8S rDNA related sequences using the MEGA 4.0 software[11].

Effect of carbon sources on mycelial growth

To determine the effect of carbon sources, mycelia of strain GSM-11 were grown in various carbon sources media based on the basic medium. Various carbon sources instead of glucose (20 g/L) with the same carbon content were evaluated. The assayed carbon sources included glucose, lactose, maltose, mannitol, sorbitol, starch, and sucrose. The mycelia were previously 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 placed at the center of new petri plates with the various assay media, and incubated at 25 °C for 5-14 d until mycelial growth of any treatments firstly reached the edges of plates. Finally, growth ratio [colony radius (mm) / incubation time (d)] and growth vitality (mycelial density) 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

In this assay, various nitrogen source media were improved based on the basic medium. The assayed nitrogen sources included beef extract, glycine, soybean meal, silkworm pupa meal, urea, NH_4NO_3, and $(NH_4)_2SO_4$. Their assay amounts were just the same nitrogen content of soybean peptone (2 g) in the basic medium. Growth ratio and vitality were subsequently determined after incubation at 25 °C for about 5-14 d. Basic medium without any soybean peptone was used as a negative control. All determinations were performed in triplicate.

Effect of C/N ratio on mycelial growth

To determine the effect of C/N ratio (ratio of carbon to nitrogen) on mycelial growth of strain GSM-11, its favorite 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 ratio of 10/1, 20/1, 30/1, 40/1, 50/1, and 60/1, respectively. All the petri plates were incubated at 25 °C for 5-14 d. Growth ratio and vitality of mycelia were finally evaluated. All determinations were performed in triplicate.

Effect of temperature on mycelial growth

In this assay, a modified enrichment medium was used. In the assayed medium, glucose and soybean peptone were replaced by the optimal carbon and nitrogen sources, respectively. The C/N ratio reached to the optimal one, while the pH value maintained to 7.0-7.2. The assayed petri plates were incubated at various temperatures of 20, 24, 28, 32, 36, and 40 °C for 5-14 d with final evaluation of growth ratio and vitality. All determinations were performed in triplicate.

Effect of pH values on mycelial growth

To determine the effect of pH values on mycelial growth, the above-mentioned medium was used with improved pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. All the petri plates were incubated at 25 °C for 5-14 d. Growth ratio and d vitality of mycelia were finally evaluated. All determinations were performed in triplicate.

Assay for enzyme production in liquid fermentation

In this assay, various enzyme activities were determined in both broth and mycelia. The assayed enzymes included laccase, phytase, protease, ribonuclease, and lectin. Mycelia were pre-incubated in PD medium and in an orbital shaking incubator at 180 rpm and 25 °C for 5 days. Subsequently, the broth was collected and used as the liquid seed with an inoculation of 5% (v/v) in the liquid fermentation using PD medium. After 8 days of cultivation at 180 rpm and 25 °C, the fermented broth was centrifuged at 12000 rpm for 15 min at 4 °C. Both the supernatant (broth) and the deposition (mycelia) were collected for further evaluation.

Laccase activity was determined using ABTS (2, 2'-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt) as the substrate. The assay was performed at pH 5.2 with a standard protocol previously described by Sun *et al* [5]. Enzyme unit (U) 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. Phytase activity was evaluated at pH 2.5 and 5.2 respectively using sodium phytate as the substrate and a modified ferrous sulfate–molybdenum blue method [12]. One unit of phytase activity was defined as the amount of enzyme that released l ¼mol phosphate per minute under the assay conditions. Protease activity was assayed at pH 7.2 using casein as the substrate. Enzyme unit was represented as a 0.001 absorbance increase per minute per mL of the reaction mixture under the assay conditions [13]. Ribonuclease (RNase) activity was determined at pH 5.2 and 7.2 respectively using yeast tRNA as the substrate [14]. 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. Lectin (hemagglutination) activity was determined towards 2% rabbit red blood cell suspension following Li's description[15]. One hemagglutination unit was the reciprocal of the highest dilution of the sample inducing hemagglutination. All determinations were performed in triplicate.

Effect of Cu^{2+} on laccase production

In this assay, $CuSO_4$ solution (1.0 mol/L) was added into PD medium with final Cu^{2+} concentrations of 0, 1.0, 2.0, 5.0, and 10 mmol/L, respectively. Mycelia of strain GSM-11 were cultivated in an orbital shaking incubator at 180 rpm and 25 °C. The broth was continuously collected at 0, 48, 96, 144, and 192 h, and laccase activity in broth was subsequently assayed with the mentioned ABTS assay.

RESULTS AND DISCUSSION

Phylogenetic analysis based on ITS sequences

An expected 5.8S rDNA gene sequence of 637 bp was obtained by PCR amplification. The sequence was deposited in the GenBank database with an accession number of JQ638521.1. It demonstrated the highest similaritiy (over 99%) with sequences generated from *F. flavus* based on

BLAST analysis. A 5.8S rDNA region phylogenetic tree of strain GSM-11 was obtained based on ITS sequences of fungi from genus *Flavodon* and other Polypores species using the neighbor-joining method (Fig. 1).

In previous researches, two *F. flavus* strains PSU-MA201 and 312 were isolated from coasts of Thailand and India, respectively[3, 7]. All the three strains manifested lignin degrading activities. To date, not much information is available in the literature regarding the culture and fermentation characteristics of *F. flavus*.

Effect of culture conditions on mycelial growth

The favorite carbon source on mycelial growth was determined to glucose with the fastest growth ratio of 6.3 ± 0.0 mm/d and the best growth vitality (Table 1). Mycelia can also grow very well on other carbon sources as sucrose and mannitol, whereas mycelia were weak and thin on the lactose medium. The favorite nitrogen source of strain GSM-01 was determined to soybean meal (6.4 ± 0.1 mm/d) and silkworm pupa meal (6.3 ± 0.1 mm/d) with the fastest growth ratio and best growth vitality (Table 2). Based on the nitrogen test, growth of strain GSM-11 showed significant differences towards the assayed nitrogen sources. In the urea group, mycelial growth ratio were just about 20% of that in the favorite groups. It can not survive without nitrogen sources. Its favorite C/N ratio for mycelial growth was 10/1 with the mycelial growth ratio of 6.4 ± 0.1 mm/d (Fig. 2). The strain preferred a low C/N ratio rather than higher ones. The best growth temperature of strain GSM-11 was observed at 36 °C with a mycelial growth ratio of 8.9 ± 0.1 mm/d, which was about 2-fold higher than that at 20 °C (Fig. 3). The favorite pH value of strain GSM-01 was determined to 7.0 with the fast mycelial growth ratio of 6.3 ± 0.1 mm/d and the best growth vitality (Fig. 4).

Mycelia of GSM-11 grow well using normal carbon sources as glucose, sucrose, mannitol, maltose, etc. Glucose is its favorite carbon source, whereas lactose is hard to be used. Just like the present study, a cultural mushroom *Coprinus atramentarius* and a wild mushroom *Tricholoma matsutake* regard glucose as their favorite carbon source for mycelial growth[16, 17]. A mycorrhizal fungus *Suillus granulatus* also manifests a optimal carbon source of glucose with the fastest growth ratio and best growth vitality[18]. On the other hand, *Lepista nuda*, which is a famous delicious wild mushroom, prefers sucrose rather than glucose as its favorite carbon source [19]. *Paecilomyces sinclairii*, which was reported to produce exopolysaccharides, also demonstrates a

Table 1. Effect of carbon sources on mycelial growth of *F. flavus* GSM-11

Carbon sources	Mycelial growth rate (mm/d)	Mycelial growth vitality
Glucose	6.3 ± 0.0 a	+ + +
Sucrose	6.1 ± 0.4 ab	+ +
Mannitol	6.0 ± 0.2 bc	+ + +
Maltose	5.8 ± 0.5 bc	+ +
Sorbitol	5.6 ± 0.1 cd	+ +
Starch	5.4 ± 0.1 de	+ +
CK	5.1 ± 0.1 e	+
Lactose	4.4 ± 0.1 f	+

Note: Different small letters mean significant differences (<0.05). + means weak, + + means ordinary, + + + means vigorous.

Table 2. Effect of nitrogen sources on mycelial growth of *F. flavus* GSM-11

Nitrogen sources	Mycelial growth rate (mm/d)	Mycelial growth vitality
Soybean meal	6.4 ± 0.1 a	+ + +
Silkworm pupa meal	6.3 ± 0.1 a	+ + +
Beef extract	4.3 ± 0.1 b	+ +
NH4NO3	3.5 ± 0.1 c	+ +
Glycine	3.0 ± 0.1 d	+ +
(NH4)2SO4	2.3 ± 0.0 e	+
Urea	1.5 ± 0.1 f	+
CK	0 g	-

Note: Different small letters mean significant differences (<0.05). + means weak, + + means ordinary, + + + means vigorous.

Table 3. Enzyme activities of in broth and mycelia of *F. flavus* GSM-11

Enzymes		In Broth (U/mL)	In mycelia (U/g)
Laccase		302.4 ± 6.6	1354.8 ± 47.0
Protease		0.2 ± 0.0	2.6 ± 0.4
Phytase	pH 2.5	2.0 ± 0.8	4.6 ± 1.3
	pH 5.2	3.8 ± 0.7	7.4 ± 1.1
RNase	pH 5.2	3.7 ± 0.3	19.9 ± 2.0
	pH 7.2	3.5 ± 0.4	15.0 ± 1.7
Lectin		0	0

favorite carbon source of sucrose for its mycelial growth and exopolysaccharides (EPS) production[20]. Anothe edible mushroom *Morchella esculent* was reported that mushrooms from different origins manifest different optimal carbon sources. *M. esculent* from Beijing (China) prefers sucrose as its favorite carbon source, whereas *M. esculent* from Wuhan (China) likes starch better[21].

Both soybean meal and silkworm pupa meal can be used as optimal nitrogen sources for mycelial growth of GSM-11. Both *C. atramentarius* and *M. esculent* from Wuhan demonstrate their

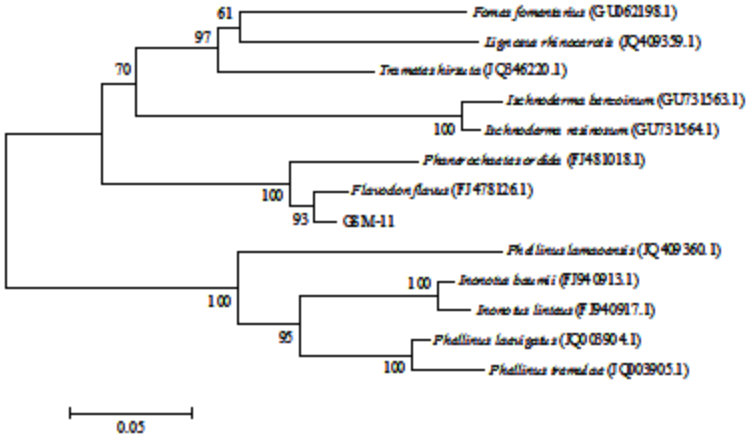

Fig. 1. 5.8S rDNA/ITS region phylogenetic tree of *F. flavus* GSM-11 obtained by the neighbor-joining method

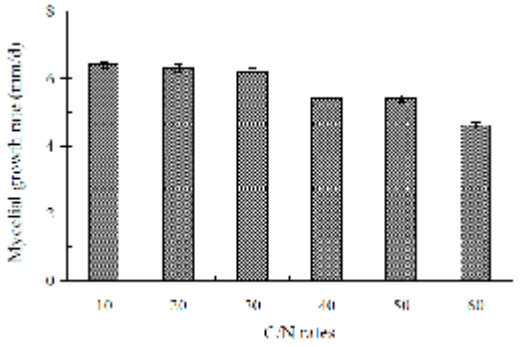

Fig. 2. Effect of C/N rates on mycelial growth of *F. flavus* GSM-11

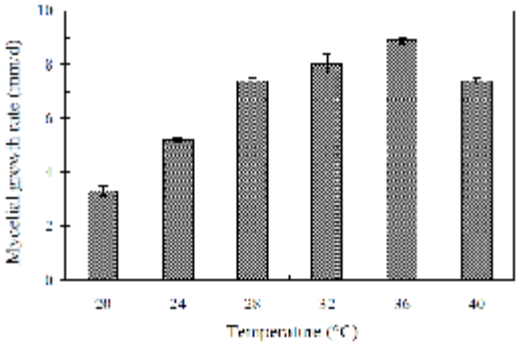

Fig. 3. Effect of temperature on mycelial growth of *F. flavus* GSM-11

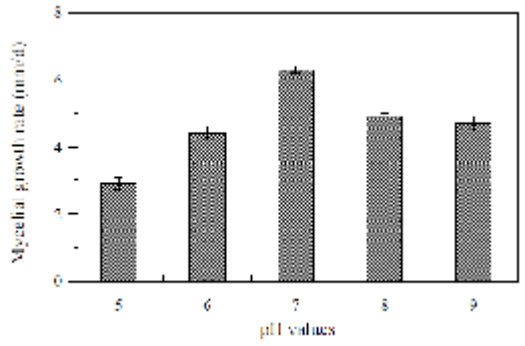

Fig. 4. Effect of pH values on mycelial growth of *F. flavus* GSM-11

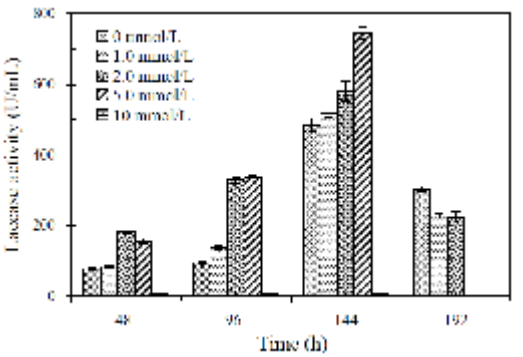

Fig. 5. Effect of Cu^{2+} on laccase production in liquid fermentation

favorite nitrogen source of peptone, whereas *M. esculent* from Beijing prefers beef extract and yeast extract as its favorite[16, 21]. *L. nuda* finds favor in yeast extract as its optimal nitrogen source [19]. *P. sinclairii* demonstrates its favorite nitrogen source of corn steep powder for its mycelial growth and exopolysaccharides production[20]. Just like *C. atramentarius* and *M. esculent*, *F. flavus* prefers organic nitrogen sources better than inorganic ones as NH_4NO_3 and $(NH_4)_2SO_4$ [16, 21]. On the other hand, *S. granulatus* prefers inorganic sources as NH_4Cl and KNO_3 better than organic ones[18].

The ratio of carbon to nitrogen balance is very important to both mycelial growth and reproductive growth of fungi[22]. Most of fungi prefer a C/N ratio range of 10/1-20/1 for their best mycelial growth, and a C/N ratio range of 30/1-40/1 for their best reproductive growth. In the present study, *F. flavus* prefers its favorite C/N ratio of 10/1 for mycelial growth, whereas the optimal C/N ratio for mycelial growth of *L. nuda* and *Pleurotus eryngii* were reported to be 20/1 and 30/1, respectively[19, 23]. An optimum C/N ratio for mycelial growth of *Lignosus rhinocerus* is approximately 10/1 using 2% glucose as a carbon source [24]. On the other hand, *P. sinclairii* manifests its optimal C/N ratio of 20/1 and 12/1 for its mycelial growth and EPS production, respectively[20]. While an optimum C/N ratio of 40/30 results in the highest mycelial growth of *T. matsutake*, 40/50 ratio results in the highest EPS production[17]. C/N ratio range of 1/3-1/5 supports the best mycelial growth of the mushroom *Lentinus tuberregium*[22].

F. flavus demonstrates an optimal temperature of 36 °C and an optimal pH value of 7.0 for its best mycelial growth. The edible mushroom *L. nuda* need a temperature of 24 °C and a pH range of 6.5-7.0 for its best mycelial growth[19], whereas the optimal conditions for mycelial growth of *L. rhinocerus* are 30 °C at pH 6 and 7[24]. A medicinal mushroom *Cordyceps nutans* manifests an optimal temperature of 25 °C and grows relatively well in neutral to weak alkaline pH levels (pH 7.0-9.0) compared with acidic pH levels[25]. Mycelial growth of two plant pathogenic fungi *Mycogone perniciosa* and *Verticillium fungicola* is the fastest at the temperature of 25 °C, and greatly decreases at 30 °C[26]. Most of other fungi prefer an optimal temperature range of 25-28 °C and a neutral acidic condition for their best mycelial growth. The present strain manifests considerably high optimal temperature of 36 °C. The fungus was isolated from Burma which is a tropical country. That is why it adapts high temperature as 36 °C.

Enzyme production and Effect of Cu^{2+} on laccase production in liquid fermentation

After 8 days of liquid fermentation, laccase, protease, ribonuclease, phytase, and lectin activities were determined. The results were shown in Table 3. GMS-11 manifested extremely high laccase activities with 302.4 ± 6.6 U/mL and 1354.8 ± 47.0 U/g in broth and mycelia, respectively. Low protease, phytase, and RNase activities were also obtained. It was devoid of lectin activity in broth and mycelia. Copper ions demonstrated high increasing activity on laccase production in PD medium as shown in Fig. 5. The highest extracellular laccase activity of 745.28 ± 18.7 U/mL was obtained at 6th day of cultivation with a Cu^{2+} adjunction concentration of 5.0 mM. In the CK group (copper ions free), extracellular laccase also reached its highest activity at 6th day with the amount of 485.0 ± 19.2 U/mL, which was about 65% of 5.0 mM copper ions treatment group. Extracellular lassase activity underwent a sharp decrease after 6 day fermentation. Laccase activity of 5.0 mM copper ions treatment group almost vanished at 8th day, whereas the CK group remained only about 60% of its highest level.

As a wood-decay fungus, *F. flavus* strain GSM-11 manifests considerably high extracellular laccase activity both in mycelia and in broth. *Cerrena unicolor*, which is an excellent laccase producer, demonstrates a 15 U/mL of laccase activity towards ABTS on the 8th day of cultivation with a Cu^{2+} adjunction concentration of 0.1 mM[27]. In the present study, the highest extracellular laccase activity was determined to be 745.28 ± 18.7 U/mL which is about 50-fold of that of *C. unicolor*. Another wood-decay mushroom *Trametes pubescens* reaches its highest laccase production of 333 U/mL with Cu^{2+} adjunction concentration of 2 mM [28]. It suggests that the present strain processes great potential application in extracellular laccase production and further utilization.

Just like many other fungi, laccase production of the strain GSM-11 can be promoted by Cu^{2+} adjunction during the cultivation [27, 29-31]. The highest laccase production of the present

strain was obtained with a Cu^{2+} adjunction concentration of 5.0 mM at 6th day of the fermentation, whereas the optimal copper ions adjunction concentration of *C. unicolor* and *Proteus hauseri* were reported to be 0.1 mM[27] and 3.0 mM[31], respectively. Although copper ions with high concentration are harm to mycelial growth, the possible activation mechanism of copper ions with high concentration is might because they can enhance the laccase genetic transcription level during the laccase synthesis[31].

CONCLUSIONS

In conclusion, a wild strain of *F. flavus* was isolated from Burmese rosewood (*G. tavoyana*). Its taxonomic analysis was determined by ITS gene amplification and phylogenetic analysis. Culture characteristics of mycelial growth and enzyme production in liquid fermentation were intensively studied. The strain manifests extremely high extracellular laccase activity, which makes it great useful in lignin degradation and other biotechnological processes.

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