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Screening and Production of Ligninolytic Enzyme by a Marine-Derived Fungal *Pestalotiopsis* sp. J63

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Abstract Marine-derived fungi are prone to produce structurally unique secondary metabolites, a considerable number of which display the promising biological properties and/or industrial applications. Among those, ligninolytic enzymes have attracted great interest in recent years. In this work, about 20 strains were isolated from sea mud samples collected in the East China Sea and then screened for their capacity to produce lignin-degrading enzymes. The results showed that a strain, named J63, had a great potential to secrete a considerable amount of laccase. Using molecular method, it was identified as an endophytic fungus, *Pestalotiopsis* sp. which was rarely reported as ligninolytic enzyme producer in the literature. The production of laccase by *Pestalotiopsis* sp. J63 was investigated under submerged fermentation (SF) and solid state fermentation (SSF) with various lignocellulosic by-products as substrates. The SSF of rice straw powder accumulated the highest level of laccase activity (10,700 IU/g substrate), whereas the SF of untreated sugarcane bagasse provided the maximum amount of laccase activity (2,000 IU/ml). The value was far higher than those reported by other reports. In addition, it produced 0.11 U/ml cellulase when alkaline-pretreated sugarcane bagasse was used as growth substrate under SF. Meanwhile, the growth of fungi and laccase production under different salinity conditions were also studied. It appeared to be a moderately halo-tolerant organism.

Keywords Marine-derived fungi · Laccase · Salinity · Submerged fermentation · Solid state fermentation

Introduction

In recent years, lignin-degrading enzymes have been extensively studied because of their potential biotechnological applications in various industrial sectors. These applications include: (1) biotransformation of lignocellulosic biomass to feeds, fuels and chemicals; (2) biopulping; (3) biobleaching of paper pulps; (4) decolorizing and detoxifying Kraft bleach effluents; (5) degradation of highly toxic environmental chemicals; (6) biosensor; (7) cosmetics; (8) food and others [1–5]. Ligninolytic enzyme is a big family of various isoforms of extracellular enzyme, of which lignin peroxidase (EC 1.11.1.14), manganese-dependent

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peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2) are the three major classes. These enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates, but also in the degradation of various xenobiotic compounds such as polycyclicaromatic hydrocarbons (PAHs), which are highly recalcitrant carcinogenic environmental pollutants, and various industrial dyes or effluents which could also pose a potential health risk because of their detrimental biological effects [6, 7]. Saparrat et al. [8] reported that white rot basidiomycete *Coriolopsis rigida* degraded wheat straw lignin and both the aliphatic and aromatic fractions of crude oil from contaminated soil. Arun's team [9] investigated PAHs' degradation potential by five basidiomycetes fungi through in vivo and in silico approach positively correlated with their capacity to express ligninolytic enzymes.

Different groups of fungi were reported as producers of ligninolytic enzymes, including *Phanerochaete chrysosporium*, and *Trametes versicolor*, *Pleurotus pulmonarius*, *Pycnoporus sanguienus*, *Neurospora crassa*, and *Coriolus versicolor*. Among them, white rot fungi are regarded as the most efficient producers which secrete ligninolytic enzymes in nature. However, a few attempts have been conducted to explore some novel fungi with ligninolytic capacity from marine environment in the literature [2, 10–13]. Theoretically, marine-derived fungi should have the great potential to produce biologically active secondary metabolites which are different from those produced by their terrestrial counterparts because they are adapted to marine harsh environment (high pressure, low temperature, oligotrophic nutrient, high salinity, etc.) [14, 15]. In addition, sea grasses and mangrove plants are the major contributors of lignocellulosic substrates in highly productive marine ecosystems, and fungi are believed to play a critical role in degrading lignocellulose in these ecosystems [2, 16]. In other words, they are the ideal habitats for these microorganisms. Only recently, this group of microorganisms has been studied to exploit their potential to generate new natural products and biomass degradation. Taking these factors into account, our aim was to screen for some microbes with outstanding ligninolytic enzyme activity from the ocean.

Agro-wastes from crop cultivation and food processing constitute vast available renewable resources for microbial conversion into different value-added products [17]. This has attracted great attention because agro-based raw materials such as wheat bran, sugarcane bagasse, corn cob, and rice straw have the advantages of being used as a sole source of energy and C-pool and are, most importantly, environment friendly [18, 19]. The selection of appropriate plant residues suitable for fungal growth and desired enzymes syntheses is a crucial step to develop an efficient technology of enzyme production [20, 21]. Also, a variety of plant raw materials have been successfully utilized as growth substrate to produce ligninolytic enzyme under different cultivation methods, submerged or solid state fermentation (SSF) [22–25]. Submerged fermentation (SF) involves the growth of microbes in a liquid medium abundant in nutrients and with a high oxygen concentration. The industrial production of enzymes is mainly performed by SF [26]. Meanwhile, SSF is generally defined as the growth of microorganism on a solid material in the absence or near absence of free water by Pandey [27]. Compared with the SF method, it is regarded as the most appropriate process for filamentous fungi growth and lignocellulosic enzymes production, because it provides growth conditions that are similar to the natural habitats of these fungi. And yet, for all its strengths, SSF has had its own disadvantages including the mass- and heat-transfer limitation and the lack of kinetic and design data on various fermentation processes. Most notably, its culture time is too long [28]. Even so, SSF continues to attract increasing interest from researchers because of the broad availability of raw materials, its superior product yields and simplified downstream processing. This study utilizes different agro-wastes and plant residues as growth substrates to investigate the production of ligninolytic enzymes through SSF and SF, respectively.

The objectives of this study were (1) to isolate fungi from sea grasses and sediments samples collected in the East China Sea, (2) to screen these fungi strains for ligninolytic activities and (3) to assess the potential of selected agro-wastes and plant residues as growth substrates for enhancing enzyme activity under SF or SSF.

Materials and Methods

Fungal Isolation

Sea mud, sea grass, mangroves and sediment samples were collected from Nanji Island off the east coast of China in the Pacific Ocean. Fungi were isolated by placing pieces of samples on basic medium (BM) plate containing antibiotic solution to prevent bacterial growth. As soon as fungal mycelia were observed to colonize, minimal amounts of mycelia were picked up by a sterile toothpick and subcultured on the modified potato dextrose agar (PDA) plate. It was also used for maintenance of the cultures. The modified PDA was prepared with artificial seawater instead of distilled water.

The BM medium contained 10 g/l yeast extract, 20 g/l glucose and 20 g/l agar.

The ingredients of artificial seawater were 29.8 g/l NaCl, 0.73 g/l KCl, 10.7 g/l $MgCl_2 \cdot 6H_2O$, 5.4 g/l $MgSO_4 \cdot 7H_2O$, 1.1 g/l $CaCl_2 \cdot 2H_2O$.

Qualitative Analysis for Ligninolytic Enzyme

The isolates were screened for peroxidase enzyme activity by growing them on plates of the modified medium containing 10 g/l glucose, 2 g/l ammonium tartrate, 2.6 g/l peptone, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l KH_2PO_4, 0.2 g/l K_2HPO_4, 0.1 g/l Azure B and 20 g/l agar, 1 l artificial seawater. The pH was adjusted to 5 prior to autoclaving at 121°C for 20 min.

The ability of the fungal strains to produce laccase was demonstrated on plates as mentioned above, except that Azure B was substituted by 4 mM guaiacol.

The plates were incubated at 30°C for 10–30 days. Any colony that decolorized dye Azure B or produced an intense brown halo under and around it was considered as a positive reaction for ligninolytic activity.

Taxonomic Characterization of Marine-Derived Fungi and Phylogenetic Tree

The fungus J63 was screened for having the great potential to secrete laccase enzyme, and then deposited at the China Center for Type Culture Collection (CCTCC). The fungus was identified by molecular methods, the D1/D2 region of 25–28S ribosomal DNA analysis. For ITS-5.8S-ITS2, regions were amplified with the following primers: ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [29]. The PCR reaction products were purified and sequenced by TAKARA (TAKARA Co. Ltd. Japan).

The sequence was compared with ITS-rDNA sequence data at the public database Genbank by using the BLASTn sequence match database. The sequence was aligned by using the CLUSTAL X program, and phylogenetic and molecular evolutionary analyses were conducted using MEGA 4. The Kimura two-parameter model was used to estimate evolutionary distance. The phylogenetic tree was constructed using the neighbour-joining algorithm with bootstrap values calculated from 1,000 replicates [13].

Lignocellulosic Substrates and Chemicals

Rice straw powder, wheat bran, bean-pod powder, sugarcane bagasse, corn cob powder and the peel of pomelo were obtained from the local market. Water hyacinth, one of the acknowledged ten hazardous grasses in the world, was collected from a local pool. All residues were dried at 60°C and milled for SF and SSF. Rice straw, sugarcane bagasse and corn cob powder were also chosen as SF substrates and treated with alkaline or not prior to inoculation. For alkaline pretreatment, the substrates were immersed in 10% NaOH solution at the room temperature for 1 day and then washed to neutral with water and then dried to constant weight.

All chemicals were analytical grade unless stated otherwise. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic) (ABTS) was purchased from Sigma (USA).

Effect of Salinity on Growth and Enzyme Production

The effect of salinity on fungal growth and enzyme activity of the marine-derived fungus were investigated by preparing the basal medium plus different amounts of sodium chloride, yielding to final concentrations of 0, 5, 10, 20, 30, 40 and 50 g/l salinity. For biomass, the culture samples were filtered through filter papers in vacuum; the mycelia were washed by the distilled water and then dried to a constant weight. Biomass was calculated by subtracting the initial weight from the final weight.

The basal medium was composed of 10 g/l glucose, 2 g/l ammonium tartrate, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$ and 2 g/l yeast extract and 10 ml/l mineral element solution.

Mineral element solution contained 0.5 g EDTA, 0.2 g $FeSO_4$, 0.01 g $ZnSO_4{\cdot}7H_2O$, 0.003 g $MnCl_2{\cdot}4H_2O$, 0.03 g H_3BO_4, 0.001 g $CuCl_2{\cdot}2H_2O$, 0.02 g $CoCl_2{\cdot}6H_2O$, dissolved with 1 l distilled water.

Effect of pH on Enzyme Production

To investigate the effect of pH on laccase production, the modified basal medium was adjusted to pH 3.5, 4, 4.5, 5, 5.5, 6 and 6.5 prior to autoclaving, respectively. The modified basal medium was identical to the basal medium described above except for preparing with artificial seawater instead of distilled water.

Effect of Copper on Enzyme Production

To estimate the effect of copper on laccase yield, different volumes of a sterilized stock copper sulphate solution (200 mM) were added to the modified basal medium, giving the final concentration of 0, 100 μM, 500 μM, 1 mM, 1.5 mM, 2 mM copper, respectively.

The time of addition of copper to induce laccase secretion was also studied. The following experiments were carried out by adding copper (final concentration 100 μM) to the modified basal medium at various culture times: day 0, day 1, day 2, day 3 and day 4, respectively.

Fermentation Condition

The SF of selected substrates were carried out on a rotary shaker at 28°C and 160 rev/min in 100-ml flasks containing 50 ml of the basal medium plus with artificial seawater which were replaced glucose by plant residues at concentration of 20 g/l and supplemented with 100 μM $CuSO_4{\cdot}5H_2O$. The initial pH was adjusted to 5 prior to sterilization. A 5% (v/v)

inoculum was used for growing culture. Samples (1 ml) were taken from each flask at a certain time of interval during the culture period and centrifuged at 10,000 rev/min for 10 min, 4°C. The supernatants were used for enzyme assay.

The SSF of selected substrates were performed in 100-ml flasks containing 5 g of plant residues moistened with 15 ml of the mineral element solution without glucose and supplemented with 2 g/l ammonium tartrate, 2 g/l yeast extraction and 100 μM $CuSO_4 \cdot 5H_2O$. The initial pH was adjusted to 5 prior to autoclaving. A 10% (v/v) inoculum was used for growing culture. After 3, 5, 7, 9, 11, and 18 days of growth, a certain amount of samples were extracted with NaAc–HAc buffer (pH 4.5) at the ratio of 1:5 (w/v) at 28°C for 3 h in a static condition, then centrifuged at 10,000 rev/min for 10 min at 4°C. The supernatant was used for enzyme assay.

Enzyme Assays

Laccase activity was determined by using of 2,2-azino-bisethylbenthiazoline-6-sulphonate (ABTS) as substrate according to Soares et al. [27]. The mixture contained 2.5 ml sodium acetate buffer (0.1 M, pH 4.5), 0.4 ml ABTS solution (0.5 M) and 0.1 ml appropriate diluted crude enzyme solution. The ABTS oxidation was measured by monitoring the increase in absorbance at 420 nm. One enzyme unit was defined as the amount of enzyme that oxidized 1 μmol ABTS per min. In SSF, the activity was expressed in IU per gram of fermented substrate (IU/g).

The total cellulase activity (filter paper activity [FPA]) was assayed according to IUPAC recommendations by using filter paper as the substrate (Ghose 1987). A reaction mixture containing a string of filter paper (Whatman No. 1), 0.5 ml of a 50 mM citrate buffer (pH 5.0) and 0.5 ml appropriately diluted supernatant was incubated at the 50°C for 60 min.

In all experiments described in this work, duplicates were set up for each tested parameter. The means of duplicate values for all data in the experiments were tested using one-way analysis of variance.

Results and discussion

Construction of Phylogenetic Tree

About 20 fungi were isolated from sea mud, sea grass and mangrove samples. However, most of them had no or weak capacity to decolorize Azure B (data not presented). Among those fungi, one isolate (designated as J63) used in the present work showed positive reaction for laccase activity when grown in the presence of guaiacol medium. This strain was deposited in the China Center for Type Culture Collection. Based on internal transcribed spacer ITS sequence aligned with GenBank database, fungal J63 was shown to have 100% similarity to ascomycete *Pestalotiopsis microspora*. The sequence of ITS was deposited in the GenBank under the accession number HQ339955. The phylogenetic tree is shown as Fig. 1.

Effect of Salinity on Laccase Production and Biomass Yield

Due to *Pestalotiopsis* sp. J63, an endophytic fungus, isolated from marine environment, has adapted to sodium chloride or other inorganic haloid solution. Thus, it is reasonable to suppose that its biologically active secondary metabolites will differ from those of their terrestrial counterparts [30]. So the growth of fungus and production of laccase enzyme by

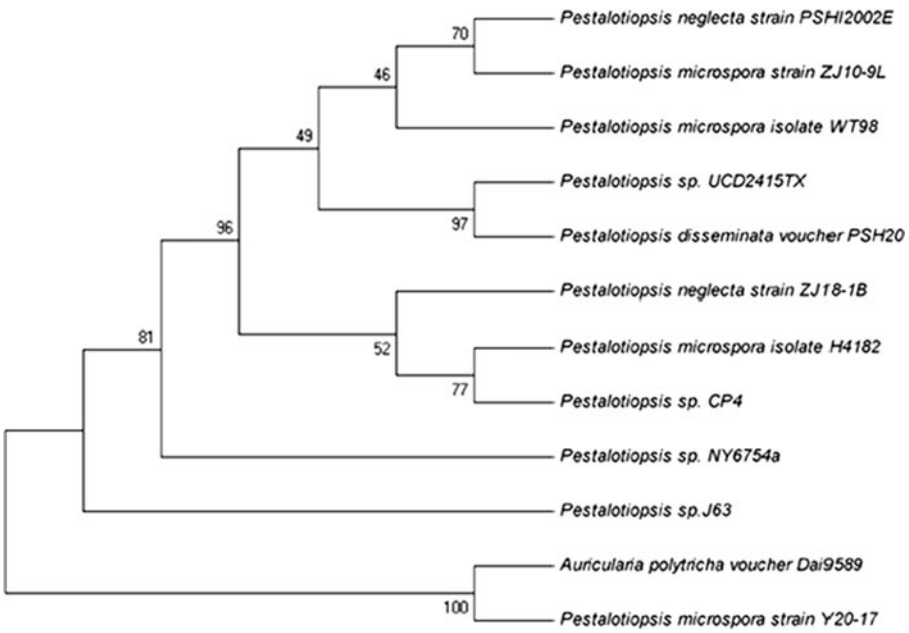

Fig. 1 Phylogenetic tree based on ITS analysis (Kimura two-parameter model; neighbour-joining algorithm and 1000 replicate bootstrap)

J63 were firstly investigated under different salinity conditions, especially for the use of this enzyme under the condition of high salt concentrations in the future. The results are revealed in Figs. 2 and 3.

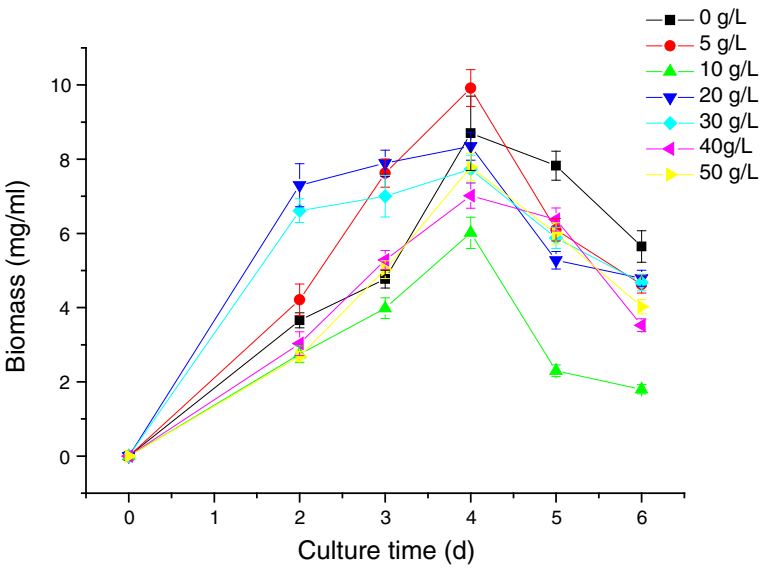

Fig. 2 Fungal biomass (dry weight) cultured at the different concentrations of salt. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

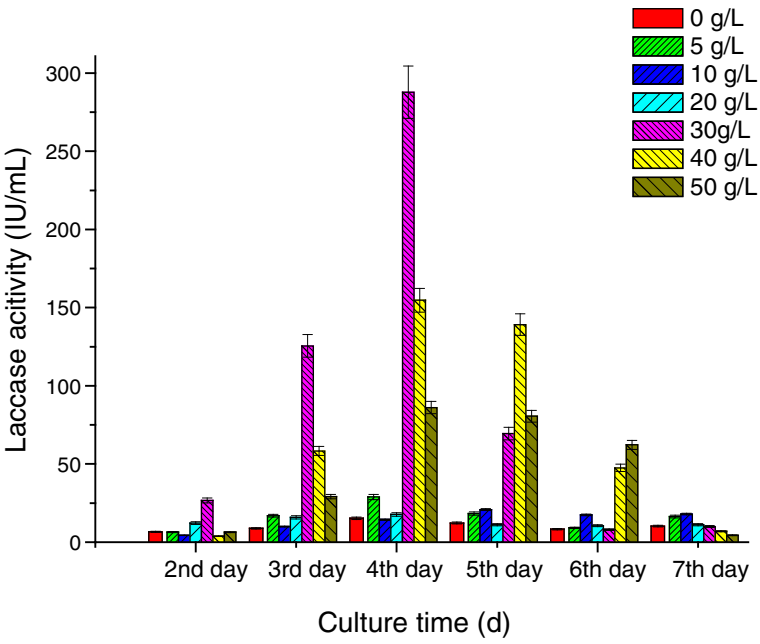

Fig. 3 Effect of different salinity on laccase production by *Pesatlotiopsis* sp. J63 when grown in the basal medium. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

The maximum biomass was reached at 5 g/l salinity about 10 mg/ml, whereas the highest level of laccase activity was observed when the marine-derived fungus J63 was cultured in 30 g/l of salinity, approximately to 300 IU/ml. The results were similar to those obtained by D'Souza et al. [3], in which the maximum laccase was produced in 25 ppt (25 g/l) of salinity. Consequently, this fungus appeared to be a marine-adapted strain of its terrestrial counterpart *Pestalotiopsis* sp. as evidenced by its growth and laccase production in medium containing salt. According to the data obtained in this work, *Pestalotiopsis* sp. J63 can be defined as a moderately salt-tolerant microorganism.

Effect of pH on Laccase Production

As shown in Fig. 4, the optimal pH for *Pestalotiopsis* sp. J63 to stimulate laccase production is about 5.0. From pH 4.5 to pH 5.0, it is beneficial to maintain high level of enzyme activity and its stability. Obviously, strain J63 cannot tolerate an alkaline environment. It can only adapt to a mild acid condition.

Effect of Copper on Laccase Production

Laccase is a copper-containing polyphenol oxidase protein. Therefore, copper ion in the medium as micronutrient plays a crucial role in forming a metal active site. It is essential during the gene transcription process to express laccase protein.

Figure 5 shows the impact of different concentrations of Cu^{2+} on laccase production. When copper is absent in the medium, *Pestalotiopsis* sp. J63 produced a minimal level of laccase, approximately 31 IU/ml. Meanwhile, with increase of Cu^{2+} concentration, ranging

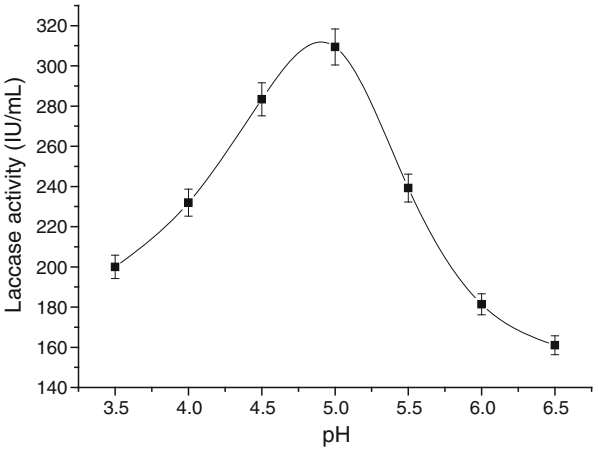

Fig. 4 Effect of pH on laccase production by *Pestalotiopsis* sp J63. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

from 0 to 0.5 mM, enzyme activity soared dramatically and peaked at 0.5 mM Cu^{2+} (411 IU/ml). Furthermore, laccase activity remarkably declined when copper concentration exceeded 0.5 mM. We noticed that strain J63 was almost completely suppressed in the medium with a final concentration of Cu^{2+} at 1.5 and 2 mM, respectively. It was reported that a high level of Cu^{2+} in the cultivation medium has a toxic effect on fungus growth [31]. Our results further showed that *Pestalotiopsis* sp. J63 is not similar to a number of previously studied fungi. Hao et al. [32] previously reported that some strains of *Pestalotiopsis* sp., which were isolated from the organic layers of the forest floor, produced high levels of laccase even when the culture medium was supplemented with 2 mM Cu^{2+}. Perhaps this is one of the differences between the marine microorganism and its terrestrial counterpart.

Due to the previous results, we chose 100 μM Cu^{2+} as the optimal concentration to perform subsequent experiments. Cu^{2+} was added to the culture on the initial stage of fermentation, and the first, second, third and fourth days of fermentation, respectively. It was observed (see Fig. 6) that addition of Cu^{2+} on the first and second days of incubation enhanced laccase activity remarkably. In particular, it reached the highest level, 419.44 IU/ml, on the first day

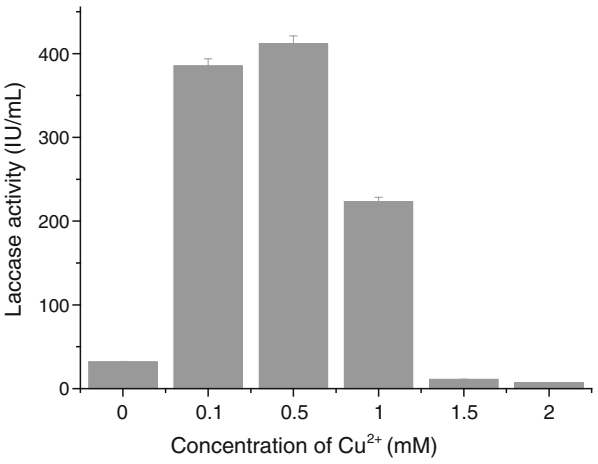

Fig. 5 Effect of Cu^{2+} concentration on laccase production by *Pestalotiopsis* sp. J63. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

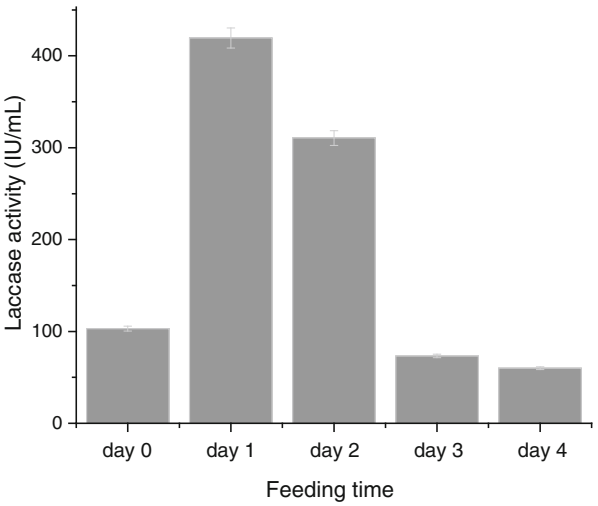

Fig. 6 Effect of different Cu^{2+} addition time on laccase formation by *Pestalotiopsis* sp. J63. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

of cultivation. The addition of Cu^{2+} at the time of inoculation led to poor growth and thus delayed laccase production. There was probably an impact of toxicity on fungal growth. As a result, addition of $CuSO_4$ after sufficient growth had occurred would be ideal. This would overcome the toxic effect of copper on biomass and yet retain its positive effect on laccase secretion [33].

Screening of Agro-wastes to Produce Laccase Enzyme Under SSF

There is a trend to utilize agro-waste raw materials to produce diverse value-added products through the bioconversion method, especially in contemporary times. In conclusion, there are five advantages in reutilization those agro-wastes: (1) they are abundant in carbon source; (2) they are easily available; (3) they are very inexpensive; (4) they are renewable, in contrast to petroleum-based resources; (5) most importantly, they are environmental friendly [34].

Furthermore, natural lignocellulosic agricultural residues contain comparable amounts of lignin and/or cellulose and hemicellulose, which act as inducers of laccase. Moreover, most of them are rich in sugars, which — because of their organic nature — are easily metabolized by microorganisms. This makes the whole process much more economical [26]. Therefore, seven plant residues were chosen as growth substrates to produce laccase. The first signs of growth were seen 2–3 days after inoculation, while complete colonization of the substrates were seen within 5–8 days except for the media supplemented with sugarcane bagasse and corncob powder. The profile of laccase productivity during degradation of various agro-wastes by *Pestalotiopsis* sp. J63 is shown in Fig. 7.

As shown in Fig. 7, the tested growth substrates exhibited marked difference in laccase secretion during cultivation. Among these, the rice straw powder appeared to be an excellent substrate for laccase production. Laccase activity soared dramatically and reached the maximum value of 10,700 IU/g substrate on the seventh day. Rice straw, containing cellulose (35–40% w/w) and hemicellulose (25–30% w/w) in close association with lignin (10–15% w/w) [35], is one of the most abundant lignocellulosic crop residues throughout the world with a production of 800 million dry tons/year. Thus, use of rice straw to produce value-added co-products appears to be a promising way to meet the dual goal of utilizing agricultural wastes and obtaining environment-friendly products [35–37].

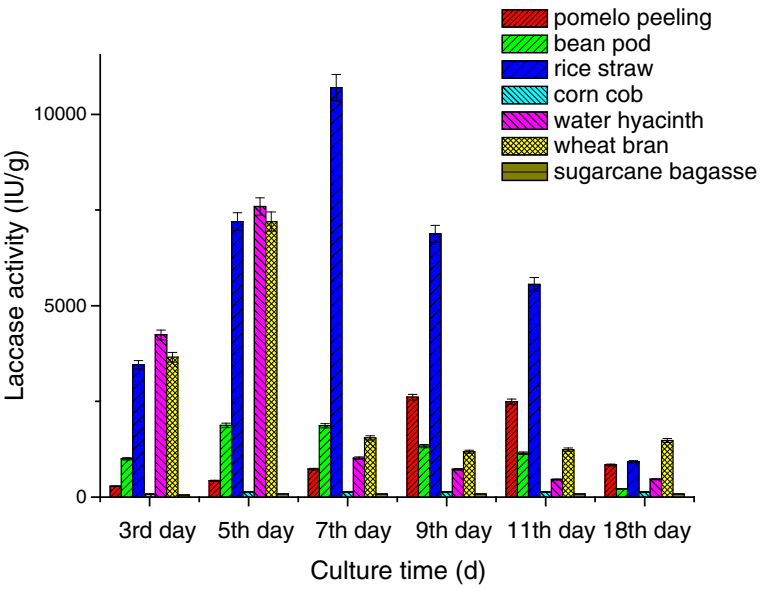

Fig. 7 Time course of production of laccase by *Pestalotiopsis* sp. J63 when grown on different plant residues via solid state fermentation. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

Water hyacinth (*Eicchornia Crassipes*) is one of the most prominent fresh aquatic plants found throughout the tropical and sub-tropical areas [38]. Due to its quick colonization on vast areas of water surface, it can cause a great deal of problems. Therefore, it could be a nuisance. Recently, attention is being devoted to the utilization of water hyacinth since efforts to control plant growth by chemical, biological and mechanical tools have met with little success. The real challenge is not how to get rid of it but how to benefit from it [39]. To the best of our knowledge, there is no report in the literature on the production of ligninolytic enzymes by *Pestalotiopsis* sp. isolated from marine environment using water hyacinth. Tentatively, we would exploit its potential in this work. In addition to cellulose (18% w/w), hemicellulose (49% w/w) and lignin (3.5% w/w), water hyacinth is rich source of protein and fiber [40], which have a high potential to induce laccase secretion. Figure 7 indicates that the medium supplemented with water hyacinth can also attain a high level of laccase productivity, 7,593.3 IU/g substrate, on the fifth day. Its productivity was even higher than that produced by rice straw powder as growth substrate at the same fermentation time. Subsequently, however, it declined dramatically to a very low degree onward. In a word, water hyacinth is an appropriate substrate for laccase production. In addition, a similar result can be seen from the medium supplemented with wheat bran.

In contrast, laccase activity was hardly detected in the medium composed of sugarcane bagasse or corncob powder. Theoretically, sugarcane bagasse has a great potential to produce a high level of laccase, because it has a large amount of soluble and insoluble sugar. But the results were contrary to the previous work done by other researchers [41–43]. It is worth noting that the different physical characteristics (such as roughness, porosity and particle size) of growth substrates can notably affect laccase production [44]. The sugarcane bagasse used in this study was of very bulky physical feature like a sponge, so it can absorb a substantial amount of free-flowing liquid. Therefore, the medium containing sugarcane

bagasse did not look as moist as the other media used as substrates. The possible explanation for its poor laccase secretion, according to our scheduled experimental method, is that the fungus cannot propagate enough under such a hydropenic environment; as a result, it cannot enter into a secondary metabolism stage (it should be noted that ligninolytic enzymes including laccase are formed in secondary metabolism). Consequently, there was little amount of laccase secretion in the medium.

Effect of Alkaline-Pretreated Substrates on the Production of Laccase Under SF

The close physical and chemical association between lignin and plant cell-wall polysaccharides is a major limitation to the efficient utilization of lignocellulosic materials for bioconversion. A variety of pretreatment technologies have been applied to agricultural residues in an effort to increase the materials digestibility for the subsequent enzymatic hydrolysis. Alkaline pretreatment is a typical chemical pretreatment method. Alkaline pretreatment of lignocellulosic feedstock causes swelling, leading to decreased a degree of polymerization and crystallinity, increased internal surface area, disruption of lignin structure, and separation of structural linkages between lignin and carbohydrates [45]. Knill et al. reported that lignocelluloses pretreated with alkaline had a much more porosity structure, and were thus more suitable for filamentous fungi growth.

Subsequently, three raw materials (rice straw powder, sugarcane bagasse and corncob powder) were chosen as substrates in SF for production of laccase based on the results (discussed in Section 3.5). Altogether, there are two sets of these three plant residues, one set was pretreated with alkaline, and the other set without any physical or chemical treatment for SF by strain J63. Next, the effect of two sets of raw materials as growth substrates on producing laccase was investigated under SF. In addition, the production of total cellulase (filter paper activity) was also investigated. The results of laccase activity and FPase activity by *Pestalotiopsis* sp. J63 are shown in Figs. 8 and 9, respectively.

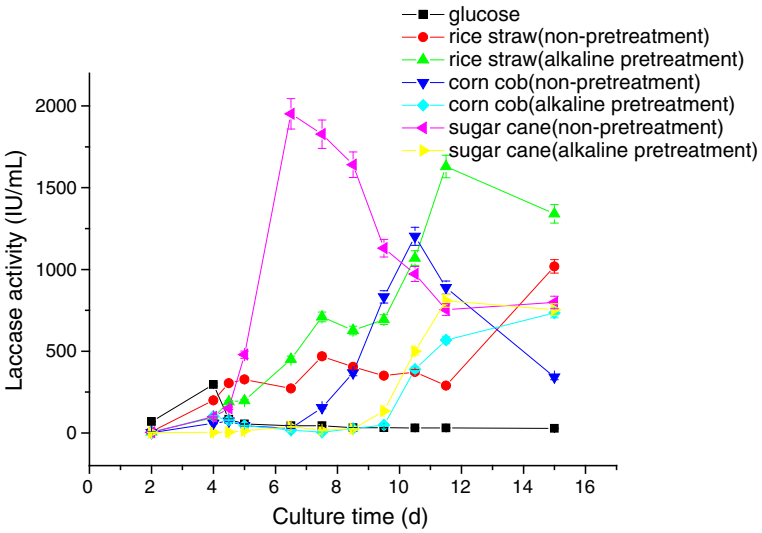

Fig. 8 Time course of production of laccase by *Pestalotiopsis* sp. J63 when grown on a series of pretreated or untreated substrates via submerged fermentation. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

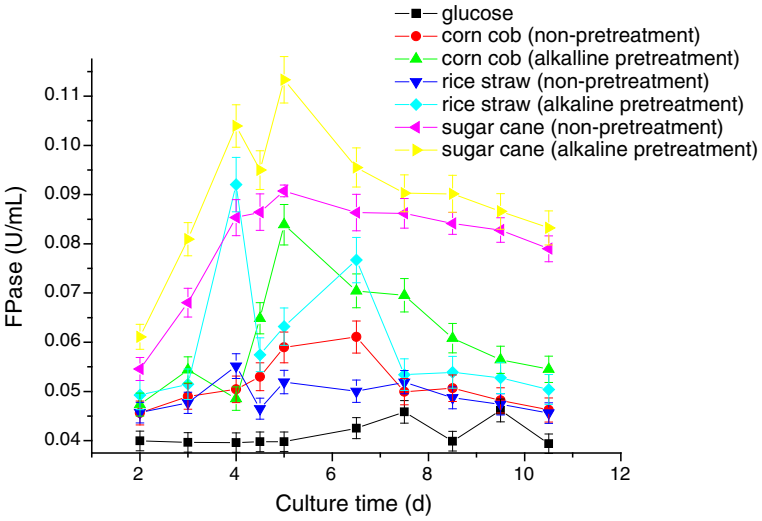

Fig. 9 Time course of production of FPase by *Pestalotiopsis* sp. J63 when grown on a series of pretreated or untreated substrates via submerged fermentation. Values shown are the mean of duplicate cultivation experiments and error bars represent SD

Interestingly, the data in this study demonstrated that utilization with untreated sugarcane bagasse as substrate can accumulate much more laccase than the other selected residues (approaching 2,000 IU/ml), and it was about 6.5-fold higher than that produced by glucose (control substrate). In contrast to SSF of sugarcane bagasse, the result was encouraging because this time sugarcane bagasse lived up to its terrific potential as a substrate in producing laccase. High laccase activity was revealed in SF of sugarcane bagasse without pretreatment at 168 h, whereas only a trace of laccase activity was detected in SF of sugarcane bagasse with alkaline pretreatment. In general, the results obtained from SF using sugarcane bagasse without pretreatment is much better than that from sugarcane bagasse with pretreatment. Similarly, the results showed that corn cob also can stimulate laccase production only if the appropriate pretreatment method and appropriate cultivation process are used. Compared to the SSF of corn cob, laccase activity could be detected in the media both using non-pretreatment corn cob and using alkaline pretreated corn cob as growth substrate under SF. Moreover, corn cob without pretreatment produced better results for laccase activity, reaching its peak value 1,200 IU/ml on the 252th hour of incubation period, while the maximum level of laccase yield obtained from the medium supplemented with alkaline pretreated corn cob was only 733 IU/ml.

As is well known, lignocellulosic materials are abundant in cellulose, hemicellulose and lignin. The component analysis of sugarcane bagasse, corn cob and rice straw before and after alkaline pretreatment by other research teams revealed (see Table 1) that the content of lignin and hemicellulose decreased largely, while cellulose increased after alkaline treatment. Lignin is mainly comprised of phenolic acids linked through ester, ether, or acetal bonds to other components of the plant cell wall. The major phenolic acid compounds are cinnamic acids such as ferulic acid (FA) and *p*-coumaric acid (*p*-CA). Using alkaline treatment, lignin will be dissolved by cleavage of ester linkages in lignin–polysaccharides complexes, leading to release phenolic acid. Torre et al. [46] found that the concentrations of PA and *p*-CA in the alkali-treated hydrolysate of corn cob increased

Table 1 The analysis of composition of corresponding dry solid residues, either before (I) or after (II) alkaline pretreatment

	Cellulose (% w/w)	Hemicellulose (% w/w)	Lignin (% w/w)
Sugarcane bagasse[a]			
I	34.1	29.6	19.4
II	68.0	12.2	9.3
Corn cob[b]			
I	40.7	31.1	11.7
II	49.3	26.5	2.70
Rice straw[c]			
I	38.3	28	14.9
II	59.3	10.9	9.5

[a] Quoted from Maeda et al. [48]

[b] Quoted from Chen et al. [49]

[c] Quoted from Zhang and Cai [50]

significantly. This meant that the content of phenolic acid in the solid residue decreased after hydrolysis. This was of no advantage to secrete laccase. Although extracellular laccases are constitutively produced in small amounts, their production can be remarkably stimulated by the presence of a wide variety of inducers, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5-xylidine, *p*-anisidine, or veratryl alcohol [47]. On the other hand, the compositions of these lignocellulosic wastes are usually rich in sugar, either soluble or insoluble, ensuring sufficient growth as well as water-soluble aromatic compounds capable of inducing or stimulating biosyntheses of laccase enzyme. So, if pretreated with alkali and then washed to neutral, they lost a large amount of carbohydrate and aromatic compounds. In this way, it was not beneficial to fungi growth.

Figure 9 reveals that cellulase was barely detected by strain J63 (only 0.05 U/ml) when grown in the medium prepared with glucose as sole carbon source. Nevertheless, strain J63 can accumulate a certain amount of FPase when cultivated in the media supplemented with various lignocellulosic residues as growth substrates. It seemed that lignocellulose can stimulate cellulase production. The maximum amount of FPase was produced by sugarcane bagasse with pretreatment, reaching 0.11 U/ml. It must be correlated with the increase in the cellulose content in the alkali pretreated lignocellulosic residues.

In conclusion, we are more likely to synthesize a large amount of laccase when there is a high content of lignin or lignin derivatives, such as natural lignocelluloses without any pretreatment, whereas cellulase is prone to be produced in the hydrolyzed material.

Conclusions

In this study, an endophytic fungus *Pestalotiopsis* sp. J63, which has a great capacity to produce laccase, was isolated from the marine environment of East China Sea and characterized taxonomically by molecular method. Through this investigation, *Pestalotiopsis* sp. J63 appeared to be a moderate halo-tolerant marine-derived fungus. Subsequently, the high yields of laccase were achieved in SF and SSF with cheap plant wastes by *Pestalotiopsis*

sp. J63. The data presented in this study demonstrate that sugarcane bagasse performs well with SF, whereas rice straw powder favours SSF. There are a few reports indicating that the lignocellulose fermentation method may have a considerable impact on enzyme production by white-rot fungi. Therefore, the study underlines the need to explore more lignocellulosic substrates with different compositions to fully exploit the potential of laccase-producing fungi. However, further studies are required to elucidate the mechanism by which some complex substrates stimulate enzyme production.

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