Appl Biochem Biotechnol (2010) 162:280–294
DOI 10.1007/s12010-009-8801-y

Production and Characterization of a Novel Laccase with Cold Adaptation and High Thermal Stability from an Isolated Fungus

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Received: 25 May 2009 / Accepted: 28 September 2009 /
Published online: 20 October 2009

Abstract A new white-rot fungus SYBC-L1, which could produce an extracellular laccase, was isolated from a decayed *Elaeocarpus sylvestris*. The strain was identified as *Pycnoporus* sp. SYBC-L1 according to the morphological characteristics and ribosomal ITS1-5.8S-ITS2 RNA genomic sequence analysis. The highest laccase activity of 24.1 $U ml^{-1}$, which was approximately 40-fold than that in basal medium, was achieved in optimal culture medium in submerged fermentation. The laccase produced by *Pycnoporus* sp. SYBC-L1 was not only a cold adaptation enzyme with a relative catalytic activity of 30.2% at 0°C but also a high thermostable enzyme. The half-lives at 60, 70 and 80°C were 85.5, 37.2, and 2.6 h, respectively. The laccase could effectively decolorize weak acid blue AS and diamond black PV up to 88% and 74.7%, respectively, within 2 h in the absence of any redox mediators. The results suggested *Pycnoporus* sp. SYBC-L1 was a potential candidate for laccase production and industrial application.

Keywords Laccase · *Pycnoporus* sp. SYBC-L1 · Submerged fermentation · Cold adaptation · Thermal stability · Decolorization

Introduction

Laccase (benzenediol–oxygen oxidoreductase, EC1.10.3.2) is a blue-multicopper oxidase that oxidizes a wide variety of organic and inorganic compounds, including mono-, di-, and polyphenols, aromatic amines, carboxylic acids, and non-phenolic substrates. Although it is applied variously in industry [1, 2], the catalytic diversity or capacity of laccase still

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depends upon their properties. To screen novel laccase-producing strain is convenient to obtain laccase with suitable characteristics. It has been demonstrated that laccase distributes in numerous fungi, a variety of plant species [3], and some bacteria and insects [4], but the efficient producers are white-rot fungi, such as *Pleurotus ostreatus*, *Trametes pubescens*, *Coriolus hisutus*, *Neurospora crassa*, *Pycnoporus cinnabarinus*, etc. [5, 6]. The genus *Pycnoporus* seems to be one of the most efficient laccase producer and has been described as a model organism for laccase production [7].

Laccase production is greatly influenced by a myriad of factors, such as medial components, metal ions, aromatic or phenolic compounds, etc. Carbon and nitrogen sources are the main factors that impact laccase synthesis. Previous papers showed that the formations of laccase by most white-rot fungi required a higher C/N ratio in cultures [8, 9]. Pointing et al. [8] found that the maximal laccase activity of *Pycnoporus sanguineus* was achieved in high carbon and low nitrogen medium, while Wang et al. [10] reported N-rich and C limitation could stimulate a higher laccase production by *Monotospora* sp. Recently, some low-cost agro-residues, e.g., mandarin peelings, grape seeds, and banana skins, were exploited as the carbon and nitrogen source for laccase production [11–13]. This strategy is becoming an increasing interest for laccase production.

Laccase is known as an environmentally friendly "green" enzyme, and it can be exploited for a number of industrial and environmental applications, such as paper pulping and bleaching, textile dye bleaching, bioremediation, and detoxification [1, 2]. Because of its high-rate capability of decolorizing a wide range of dyes, many studies have focus on its applications in textile dyes biodegradation [14]. Osma et al. [5] showed that *T. pubescens* laccase had high potential for decolorization two structurally different dyes, such as the anthraquinonic dye (Remazol Brilliant Blue R, RBBR) and the triphenylmethane dye (Methyl Green, MG). Recently, several species of the genus *Pycnoporus* and their laccase have been successfully applied in the decolorization of natural pigments or synthetic dyes. The laccase purified from *P. sanguineus* efficiently decolorized Remazol Brilliant Blue R in the absence of added redox mediators [15]. It can be seen that laccase has great potential in the synthetic dyes degradation. Therefore, searching for potential laccase to apply in dye industry is becoming an important task.

During the investigation of screening ligninolytic fungi, we obtained a unique strain SYBC-L1, which could secrete laccase with some novel properties. However, its taxonomy needed further investigation and the factors that influence the laccase production remained unclear. The purpose of this paper was to identify the strain SYBC-L1, to evaluate the effects of carbon and nitrogen sources on the laccase production, to detect the influences of temperature and pH value in the laccase activity and stability, and to test the capacities of the laccase in decolorization of wool dyes.

Materials and Methods

Screening of Laccase-Producing Strains

The microorganisms were mainly isolated from the decayed wood or soil samples collected from Jiangsu, Hebei, Shandong and Inner mongolia Provinces in China.

The soil samples were added, respectively, into sterile water in Erlenmeyer flask, and a serial dilution (10^{-1} to 10^{-9}) was prepared, then 0.1 ml of dilution water was distributed onto potato dextrose agar (PDA) screening medium. The fresh fruiting body of each fungus was split by a knife; a small part of basidiocarp was then picked up and put onto PDA Petri

dish. The PDA screening medium contained potato 20%, glucose 2.0%, agar 1.5%, and α-naphthol 0.5 mM. The laccase activity was recorded by the appearance of purple-red color around the margin of colonies, and the preliminary experiments selected eight promising strains.

These laccase-producing potential strains were then tested through flask culture. Five mycelial plugs with 0.3-cm diameter were cut out from each actively growing mycelial edge and inoculated into 50 ml of basal medium reported by Tien and Kirk [16] in 250-ml flask then incubated at 30°C on a rotary shaker at 200 rpm for 12 days. The strain SYBC-L1 with the highest extracellular laccase production was selected as the study strain in this test.

Identification of SYBC-L1 by Morphological and Molecular Methods

The morphological characters of SYBC-L1 were identified by incubating the strain on the PDA Petri dish. Its mycelia and conidia were observed with optical microscope (OM, LEICA, Germany) and scanning electron microscope (SEM, QUANTA-200, FEI, Netherlands). Its colony morphology was recorded by Nikon digital camera (Nikon Corporation, Japan).

Genomic DNA template was prepared by standard phenol-chloroform extraction. Sequences of ITS-5.8S rDNA were amplified by PCR using the universal primer pairs of ITS5 (5′-GGAAG TAAAA GTCGT AACAA GG-3′)/ITS4 (5′-TCCTC CGCTT ATTGA TATGC-3′) [17]. The PCR was started at 94°C for 2 min, followed by 34 cycles of denaturation (1 min at 94°C), annealing (45 s at 51°C), and extension (1.5 min at 72°C). After 34 cycles of reaction, a 10-min extension time was used. The amplification products were analyzed on a 1% agarose gel, purified by used DNA extraction kit and cloned into the pMD 18-T vector (TaKaRa, China). The sequence of 5.8S rDNA of SYBC-L1 was compared with those submitted in GenBank using the GenBank BLAST search [18].

Optimization of Culture Medium and Conditions for Laccase Production

To investigate the effects of carbon and nitrogen sources on laccase production, glucose was substituted with different carbon source and ammonium tartrate was replaced by the inorganic or organic nitrogen source (summarized in Table 1). The L_{16} (4^5) orthogonal array was designed for laccase production by *Pycnoporus* sp. SYBC-L1, and the data were analyzed using MINITAB 15.1.1 software. Table 2 documents the L_{16} (4^5) orthogonal array of four variables at four different concentrations. To study the effect of copper, gradient concentrations of Cu^{2+} (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mM, final concentration) was added to the optimal medium; meanwhile, 1.5 mM Cu^{2+} was applied in the culture at different time of the shake cultivation (days 1, 3, 5, 7, 9, and 11). In addition, the two important fermentation conditions of incubation temperature (25, 28, 30, 33, 35, and 37°C) and initial pH (2.0 to 8.0) for laccase production were also investigated.

Laccase production was performed at 30°C on a rotary shaker at 200 rpm, in 250-ml flask containing 50 ml of synthetic medium. After 12 days of cultivation, the broths were centrifuged at 12,000×*g*, then the supernatants were used for laccase assay, and the highest activity was taken as 100%.

Laccase Assay and Characterizations

Laccase activity was determined spectrophotometrically at 470 nm by following the oxidation of 2,6-dimethoxyphenol (DMP, Aldrich, $\varepsilon_{470\ nm}$=49.6 $mM^{-1}cm^{-1}$) at pH 3.0 in

Table 1 Effects of carbon and nitrogen sources on laccase production by *Pycnoporus* sp. SYBC-L1.

Carbon source (20 gl^{-1})	Relative activity (%)	Nitrogen source (10 gl^{-1})	Relative activity (%)
Glucose	38.9	Ammonium tartrate	1.7
Sucrose	21.6	Beef extract	56.3
Maltose	22.4	Peptone	43.8
Xylan	26.1	Yeast extract	73.1
Cellubiose	25.9	Glutamine	23.3
Carboxymethyl-cellulose sodium	100.0	Asparagines	20.5
Starch	37.7	Soybean meal powder	100.0
β-dextrin	23.7	NH_4Cl	14.2
Barley bran	96.9	NH_4NO_3	15.6
Filter paper	80.3	$NH_4H_2PO_4$	19.9
Rice straw	27.1	$(NH_4)_2SO_4$	15.8

Data are mean values from triplicate experiments; SD was less than 10%

0.1 M citrate/Na_2HPO_4 buffer [19]. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of 2,6-DMP per minute.

The effects of pH and temperature on laccase activities were determined over a pH range of 3.0–10.0 and temperature gradient of 0–100°C with DMP as the substrate, and the highest laccase activity was taken as 100%. The pH stability of laccase was examined by incubating the fermentation broth at 30°C in the citrate/Na_2HPO_4 buffer (pH 2.0–10.0), while the thermal stability was carried out by incubating enzymes at 0–100°C, then the activities of laccase were measured periodically with DMP as described above. Taken the

Table 2 The L_{16} (4^5) orthogonal array design for laccase production by *Pycnoporus* sp. SYBC-L1.

Run	(A) Barley bran	(B) Glucose	(C) Soybean meal powder	(D) $NH_4H_2PO_4$	Laccase activity (U ml^{-1})
1	1 (70 gl^{-1})	1 (50 gl^{-1})	1 (40 gl^{-1})	1 (0.50 gl^{-1})	1.4±0.1
2	1	2 (70 gl^{-1})	2 (20 gl^{-1})	2 (1.25 gl^{-1})	6.2±0.3
3	1	3 (60 gl^{-1})	3 (10 gl^{-1})	3 (0.75 gl^{-1})	8.1±0.3
4	1	4 (80 gl^{-1})	4 (30 gl^{-1})	4 (1.00 gl^{-1})	4.8±0.2
5	2 (80 gl^{-1})	1	2	3	1.1±0.1
6	2	2	1	4	4.1±0.1
7	2	3	4	1	9.0±0.2
8	2	4	3	2	2.0±0.1
9	3 (50 gl^{-1})	1	3	4	11.1±0.1
10	3	2	4	3	1.6±0.0
11	3	3	1	2	3.1±0.1
12	3	4	2	1	6.6±0.1
13	4 (60 gl^{-1})	1	4	2	5.3±0.2
14	4	2	3	1	16.7±0.2
15	4	3	2	4	12.4±0.2
16	4	4	1	3	10.0±0.1

Data are mean values from triplicate experiments; SD was less than 10%

laccase activity without buffer or heating treatment as 100%, the relative activities were calculated by it.

Decolorization of Wool Dyes by Laccase

The characteristics of the two commercial wool dyes (azo dye diamond black PV and anthraquinone dye weak acid blue AS) were summarized in Table 4. The effects of most important physicochemical conditions of reaction temperature and pH on the decolorization by the crude laccase were studied in details. The reaction mixture (4.0 ml) contained of dye's stock solution (100 mg l^{-1}, final concentration) and extracellular crude laccase (2.0 Uml^{-1}, final concentration) in 0.1 M citrate/Na_2HPO_4 buffer (pH 2.0–6.0). The reactions were incubated at 20–70°C for 2 h in complete darkness without shaking. The test containing the same amount of compounds and heat-denaturated laccase was performed as the control.

Dye decolorization was determined spectrophotometrically by monitoring the decrease in absorbance at the wavelength of maximum absorbance for each dye and expressed in terms of percentage following the equation: $(A_0-A_t)\times 100/A_0$, where A_0 is the initial absorbance, and A_t is the absorbance after incubation for a certain time.

Determination of Reducing Sugar and Biomass

Reducing sugar was measured by the dinitrosalicylic acid method using glucose as a standard, according to Ghose [20].

Biomass was determined in terms of glucosamine content in course of fermentation following the method of Desgranges et al. [21].

Data Analysis

All data were collected from two separate experiments both with at least triplicates and analyzed by SPSS 11.5. The means was compared by independent *t* test.

Results

Identification of Strain SYBC-L1

The basidiocarp of SYBC-L1, represented in Fig. 1a, was collected from a decayed stem of *Elaeocarpus sylvestris* on Long mountain, Wuxi, Jiangsu. The basidiocarp was resupinate without stem, and it was soft when fresh and hard when dry. The surface color was orange to crimson. The size of cap was 3.0 to 7.0 cm wide and up to 0.5 cm thick. The front of cap had pubescence and ring zone, the back had tube. Figure 1b shows the shape of tube, it was slight round but smaller with only 0.10–0.17/mm. When touched with 10% KOH, basidiocarp tissue of SYBC-L1 turned to be dark gray, and this character was consistent with that of the genus *Trametes* and *Pycnoporus*.

The strain SYBC-L1 grew well on PDA plate with a diameter of 70–80 mm after 5 days (Fig. 1c). These fast-growing aerial hyphae were white and downy. They were obviously presented concentric zones and radial morphology on the margin of colonies (Fig. 1c). At this stage, generative hyphae observed by SEM were hyaline with thin walls to thick walls (Fig. 1d). Figure 1e shows the generative hyphae were with clamp connections. However, the clamp connections were hardly seen by SEM because the samples needed treatments

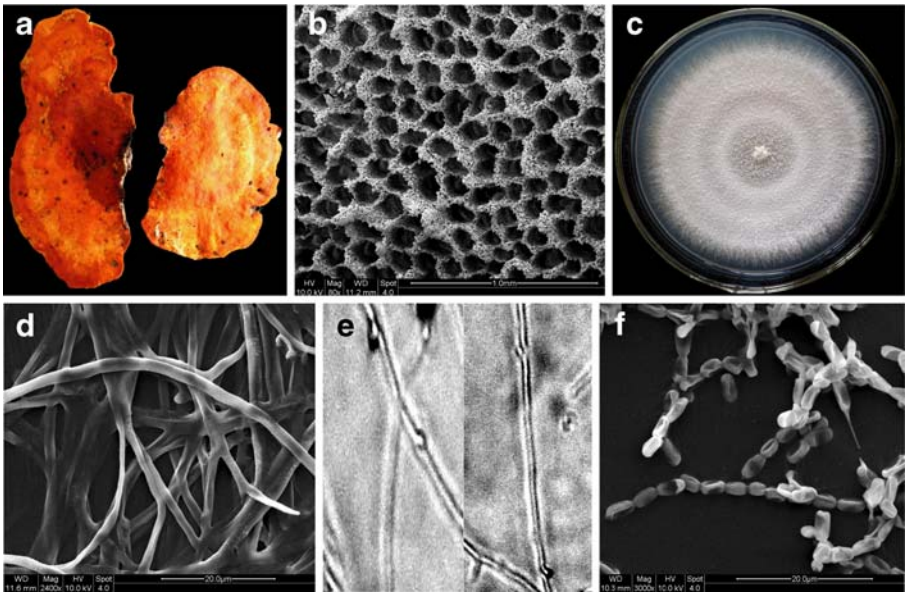


Fig. 1 The morphological structure of SYBC-L1. **a** The fruiting body. **b** Tubes of cross section by SEM (80×). **c** *Top view* of SYBC-L1 with white mycelium on PDA plate (30°C, 5 days). **d** Generative hyphae by SEM (2,400×). **e** Clamp connection structure by OM (1,000×); **f** Arthrospore by SEM (3,000×)

before scanning and this process made the hyphae too dry to observe. With the time of development, some white powders, which were observed to be arthrospores resulting from the fragmentation of white aerial hyphae, were formed on the surface of fungus. The arthrospore could be observed clearly, it was single cell, ellipse, and bunchiness with the size of 1.4×3.0–2.0×4.0 μm (Fig. 1f). The morphological characteristics of this strain revealed that SYBC-L1 was quite similar to genus *Trametes* and *Pycnoporus*.

The GenBank accession number of the ITS1-5.8S-ITS2 DNA sequence of strain SYBC-L1 was EU888831. Through BLAST search and homology analysis with other ITS sequences in the GenBank database, the sequence fragment was closed to that of the genus *Pycnoporus* and had 100% maximal identity with the top two *P. sanguineus* (GenBank accession code: EU661890 and AF363770).

As a result, the isolated strain SYBC-L1 was identified as a *Pycnoporus*, located in phylum *Basidiomycota*, order *Polyporales*, family *Polyporaceae*, genus *Pycnoporus*, and named as *Pycnoporus* sp. SYBC-L1.

Effect of Carbon and Nitrogen Sources on Laccase Production

Table 1 demonstrates that the laccase formation by *Pycnoporus* sp. SYBC-L1 was affected obviously by carbon and nitrogen sources. Compared with macromolecular carbon sources, these small molecular sugars gave much low laccase productions, although their mycelia grew well in the medium. The highest level of laccase production was found with carboxymethyl-cellulose sodium salt as carbon source, the second one was with barley bran. From an economic point of view, barley bran was selected as the suitable macromolecular carbon source. Moreover, the experiment found that when a compounded carbon (the ratio of barley bran and glucose was 1) was used, laccase production by

Pycnoporus sp. SYBC-L1 was approximately 40% higher ($P<0.05$) than that with barley bran individually (data not shown). It was observed that soybean meal powder was the best one for laccase production in all tested nitrogen sources (Table 1). Organic nitrogen source gave higher yields of laccase activity, while lower laccase activity was obtained with inorganic nitrogen sources. The results showed that organic nitrogen (soybean meal powder) combined with inorganic nitrogen ($NH_4H_2PO_4$) facilitated to obtain a higher laccase production (data not shown). Thus, in this paper, the best choice for laccase production was with the compounded carbon and nitrogen sources.

The orthogonal matrix methods have been successfully used to optimize the fermentation medium for metabolite production in fermentation process. In this paper, the L_{16} (4^5) orthogonal array was used to evaluate the carbon and nitrogen sources and optimize their concentrations for laccase production. Delta values and rank analysis helped to evaluate the greatest effective factor on the production of tested objectives [22]. According to the result displayed in Table 3, the order of effective factors on laccase production was barley bran > soybean meal powder > $NH_4H_2PO_4$ > glucose. It indicated that macromolecular carbon sources had significant impact on laccase production. Moreover, MINITAB software revealed that the optimal concentrations of these nutritional components were barley bran 60 gl^{-1}, glucose 60 gl^{-1}, soybean meal powder 10 gl^{-1}, and $NH_4H_2PO_4$ 0.5 gl^{-1}. The result was in agreement with the published data that high C- and N-limiting levels helped to enhance laccase formation in fungi [8, 9]. Under the optimal medium established, the maximum laccase activity of 17.6 Uml^{-1} was obtained, which was approximately 30-fold ($P<0.05$) than that in the basal medium.

Effect of Copper Ion on Laccase Production

The laccase formation of *Pycnoporus* sp. SYBC-L1 was increased significantly with supplementing copper ion in the culture medium (Fig. 2a). It could be seen from Fig. 2a that increasing the concentration of Cu^{2+} from 0.5 to 2.0 mM resulted in considerable increase in laccase activity; moreover, when the concentration was up to 3.5 mM, the laccase production was still higher than that without Cu^{2+}. The highest value of 22.9 Uml^{-1} was observed at 2.0 mM copper ion (data not shown), which was 1.8-fold ($P<0.05$) than that without copper ion (Fig. 2a).

Figure 2b shows the addition time of Cu^{2+} influenced the laccase formation. When 2.0 mM Cu^{2+} was supplemented to the optimized medium after 1 day of growth, the highest laccase activity was found. With the adding time of Cu^{2+}, the laccase formation decreased gradually. The result revealed that high concentration of Cu^{2+} added at the beginning time of incubation had no growth inhibition on *Pycnoporus* sp. SYBC-L1, but the mycelia became dark. Galhaup and Haltrich [23] explained the phenomenon to be caused by the

Table 3 Response table for means.

Level	(A) Barley bran	(B) Glucose	(C) Soybean meal powder	(D) $NH_4H_2PO_4$
1	5.1	4.7	4.7	8.4
2	4.1	7.2	6.6	4.2
3	5.6	8.2	9.5	5.2
4	11.1	5.9	5.2	8.1
Delta	7.1	3.4	4.8	4.3

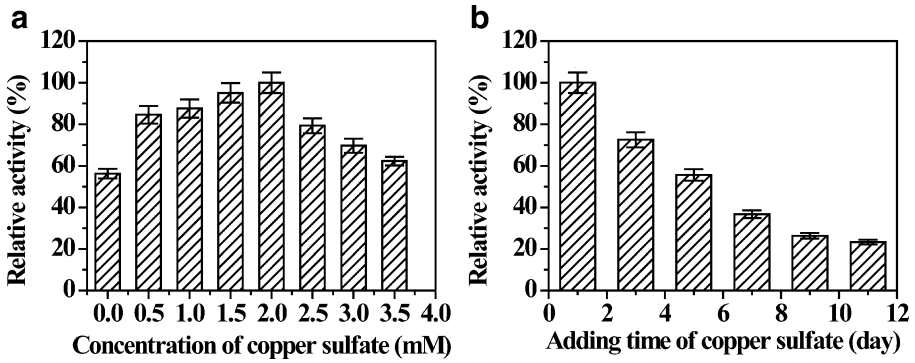


Fig. 2 Effect of various concentrations of $CuSO_4$ (**a**) and varing times of its addition (**b**) on laccase production by *Pycnoporus* sp. SYBC-L1. Values given are the means of at least triplicate experiments, and *error bars* represent the SD

hyphal melanin which could lead mycelia to become much darker with the concentration of copper ion in the growth medium.

Effect of Culture Conditions on Laccase Production

Since enzyme production is also affected by the physical culture conditions, the effects of incubation temperature and initial medium pH on laccase formations were then assessed. In Fig. 3a, the optimum incubation temperature of *Pycnoporus* sp. SYBC-L1 was determined to be 35°C, and this culture temperature was much higher than that of most fungi [24] but was similar to that of bacteria [4]. Figure 3a displays higher laccase yield appearing at the temperature range from 30 to 35°C; however, when culture temperature was lower than 28°C or higher than 35°C, the laccase production was reduced markedly.

As shown in Fig. 3b, the optimal initial pH was 3.5. When initial pH value of the medium was ranged from 3.0 to 7.0, *Pycnoporus* sp. SYBC-L1 was found to produce higher laccase; nevertheless, when initial pH was below 2.0 or above 8.0, growth inhibition was observed, and this led to a lower laccase production. Previous papers suggested that the expression of laccase by most white-rot fungi required an acidic initial pH [25]; however, there was an interesting phenomenon that a much higher laccase yield appeared at pH 7.0 which suggested that neutral conditions also favored for accase production. Figure 3c exhibits laccase production was not only delayed but also retarded in buffered medium. If the medium was buffered against with 0.1 M citrate/Na_2HPO_4 buffer, there was no shift of pH and no laccase production at the early stage of culture, which implied that the release of alkali compounds might switch the laccase production.

Growth and Laccase Production in Submerged Fermentation

Figure 4 reveals the relationship between the biomass, reducing sugars consumption, pH value, and laccase production. In early stage of growth, mycelia were at a slow growth; correspondingly, the reducing sugars were also consumed slightly. With the growth of cell, the reducing sugars decreased rapidly, and it reduced to one sixth of the initial content at the 10th day. At the same time, the biomass of *Pycnoporus* sp. SYBC-L1 reached to the maximum. During the fermentation, the pH value of the broth decreased in the initial

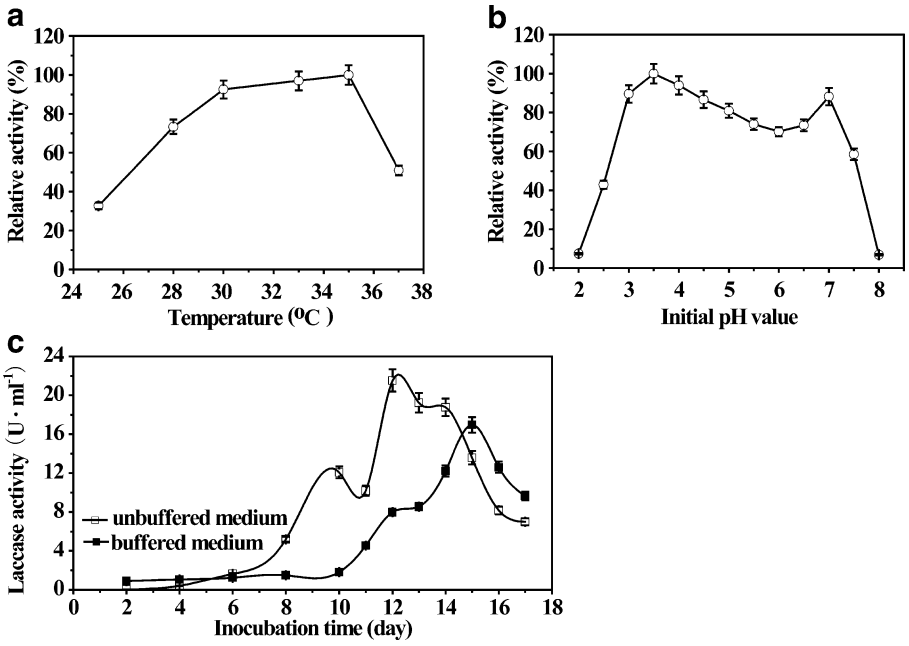


Fig. 3 Effect of incubation temperature (**a**), initial pH (**b**), and unbuffered or buffer (0.1 mM citrate/Na_2HPO_4 buffer) medium (**c**) on laccase production by *Pycnoporus* sp. SYBC-L1. Values given are the means of at least triplicate experiments, and *error bars* represent the SD

period, following the increase of the consumption of reducing sugars. When the pH exceeded 4.0, the fungus started to produce laccase.

A lag phase for fungus growth with almost no extracellular laccase activity was detected in Fig. 4. Laccase production increased greatly from the 8th day of incubation and reached

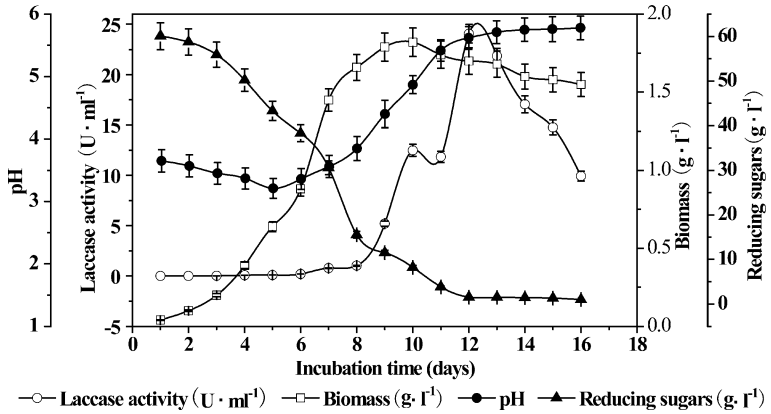


Fig. 4 Time course of *Pycnoporus* sp. SYBC-L1 under the optimized condition in flask cultures on a rotary shaker at 200 rpm. Values given are the means of at least triplicate experiments, and *error bars* represent the SD

the highest activity of 24.1 Uml^{-1} at the 12th day; thereafter, the laccase yield dropped rapidly. The production maxima was attained earlier as compared with some white-rot fungi such as *Phlebia floridensis* (20th day) [26] and *P. cinnabarinus* ss3 (15th day) [27].

Characterizations of Laccase

In Fig. 5a, the pH stability analysis revealed that the laccase had a broad stable pH range (from 4.0 to 10.0). After 72 h incubation at 30°C, the enzyme showed a different stability pattern: the laccase activity decreased rapidly at pH 2.0; there remained only 43% activity at pH 4.0 and 8.0; the residual activity was above 70% at pH 10.0 which with a half-life of more than 130 h. The result indicated that *Pycnoporus* sp. SYBC-L1 laccase was more stable in an alkaline condition.

The optimal temperature of laccase from *Pycnoporus* sp. SYBC-L1 was determined to be 70°C, which was similar to most basidiomycete laccase (50–70°C). The reaction temperature range where the enzyme was active was remarkably wide. Notably, *Pycnoporus* sp. SYBC-L1 laccase behaved high catalytic activities at low temperature (Fig. 5b). This paper detected that it showed about 50–70% of relative activity at 10–30°C; in addition, it remained to exhibit 30.2% of the maximal activity at 0°C. The results displayed *Pycnoporus* sp. SYBC-L1 laccase was adapted to a cold environment. According to the description that cold adaptation enzyme is capable of catalyzing the biochemical reactions at low temperature [28–30], *Pycnoporus* sp. SYBC-L1 laccase was regarded as a cold adaptation enzyme.

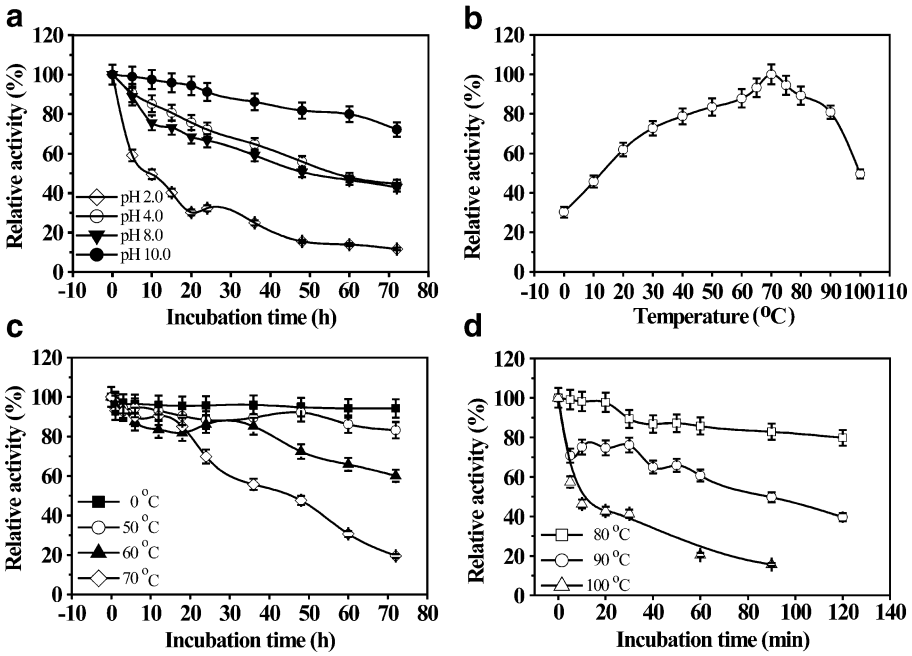


Fig. 5 Effect of pH value and temperature on the activity of *Pycnoporus* sp. SYBC-L1 laccase. **a** The pH stability (incubation at 30°C). **b** The optimal temperature. **c**, **d** The thermal stability. Values given are the means of at least triplicate experiments, and *error bars* represent the SD

Pycnoporus sp. SYBC-L1 laccase not only had a higher catalytic activity at low temperature but also was quite stable at a broad temperature range. The enzyme activity remained almost unaltered after 72 h of incubation at 0°C and lost approximately 10% activity when it was incubated at 50°C. The half-lives of laccase at 60 and 70°C were 85.5 and 37.2 h, respectively (Fig. 5c). Figure 5d shows, after 2 h of incubation at 80°C, the residual activity was 39.6% and its half-life decreased to 2.6 h. When the temperature increased to 100°C, the half-life was only 8.7 min. The results indicated that laccase obtained from *Pycnoporus* sp. SYBC-L1 could be identified as a high thermostable enzyme.

Dye Decolorization by Laccase

Two diverse structures of commercial wool dyes, azo dye (diamond black PV) and anthraquinone dye (weak acid blue AS), could be decolorized efficiently by crude laccase of *Pycnoporus* sp. SYBC-L1 without additional redox mediators. The maximal decolorization rates of the tested wool dyes were both observed at 40°C (Fig. 6a). About the optimal pH value of decolorization, as shown in Fig. 6b, the former was 4.5, while the latter was detected at pH 4.0. Figure 6c shows at the time of early 30 min, the decolorization rates were at a rapidly ascending stage, but from 60 min forward, it increased slowly. The decolorization percentage for weak acid blue AS was more than 88% after incubation at 40°C for 2 h at pH 4.0, whereas, it was lower for diamond black PV with only a decolorization percentage of 74.7% at 40°C and pH 4.5. The difference in the decolorization efficiencies might arise from the different structures which resulted in the different redox potentials (Table 4).

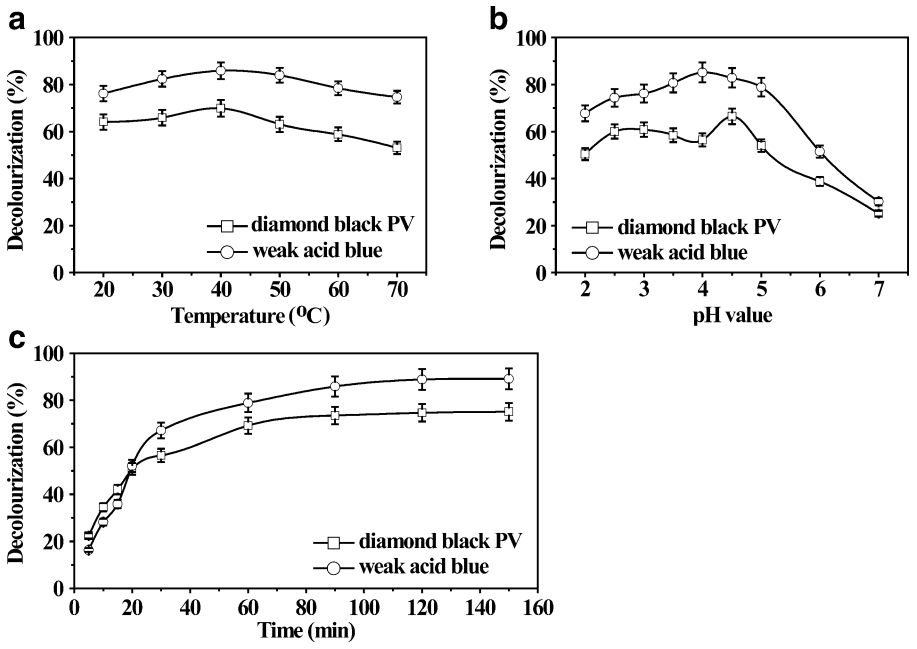
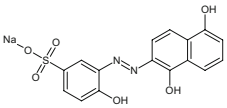
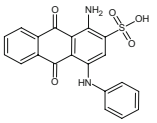


Fig. 6 Effect of temperature (**a**), pH value (**b**), and incubation time (**c**) on decolorization of wool dyes by *Pycnoporus* sp. SYBC-L1 laccase. Values given are the means of at least triplicate experiments, and *error bars* represent the SD

Table 4 Characteristics of the wool dyes.

Dye	Class	λ_{max} (nm)	CI name	Structure
Diamond black PV	Azo dye	535	Mordant black 9	
Weak acid blue AS	Anthraquinone dye	625	Acid anthraquinone blue 25	

CI color index

Discussion

In this paper, a new laccase-producing strain SYBC-L1 was isolated from a decayed *E. sylvestris* in the south of China. The characteristics of morphology and the 5.8S rDNA analysis of this isolated strain proved it was in fact a strain of *Pycnoporus*. Because of its unique bright orange-red color, *Pycnoporus* is well known as a cinnabar-red polypore [7]. It is a type of bracket fungi that specializes in rotting trees or logs. Among the white-rot fungus, *Pycnoporus* is a promising candidate since it is not only a high-laccase producer but also a versatile producer—its fruitbodies possess antibacterial and antitumor properties [31]. It distributes mainly in America, Asia, and Europe. In China, there are two species—*Pycnoporus coccineus* and *P. sanguineus*—they are similar in appearance, but the latter appears rarely. In this study, many morphological characteristics of *Pycnoporus* sp. SYBC-L1 were similar to those of Chinese *Pycnoporus* strains: (1) basidiocarp was orange to reddish-orange, fading with age; (2) the front of cap had pubescence and the back had tube; (3) generative hyphae was with clamp connection which is the typical structure of polypore. However, some special characteristics were found on the strain: (1) Cap and tube were thinner and smaller than the common strains; (2) the pore was smaller (the diameter was only 0.10–0.17 mm). Molecular character is another method in strains identification. Lomascolo et al. [18] reported *P. sanguineus* strains from China constituted a homogeneous subgroup and the 5.8S rDNA analysis supported the proposal that *Pycnoporus* sp. SYBC-L1 also belonged to this group. The special morphological characteristics mentioned in this paper suggested that *Pycnoporus* sp. SYBC-L1 was not completely consistent with other Chinese *Pycnoporus* strains, but a new strain.

Recently, there is a promising trend towards the reutilization of some cheap organic wastes to produce value-added products. Meza et al. [27] reported *P. cinnabarinus* ss3-produced laccase with sugarcane bagasse. Osma et al. utilized mandarin peelings [13] and banana skin [5] as the best carbon source and support-substrate, respectively, to produce laccase by *T. pubescens*. In addition, cassava, tomato pomace, grape seed, grape stalk, coconut, and wheat/rice straw were also used in the laccase production in solid-state fermentation (SSF) [12, 13, 32]. However, compared with liquid culture, SSF existed several problems, for example, it was difficult to control the fermentation process and apply on large scale. In this paper, a high laccase yield was demonstrated to be obtained in submerged fermentation by using the agro-residues (barley bran and soybean meal powder as the best carbon and nitrogen sources). This strategy, used for laccase production in this

test, was with higher activities but lower cost; thus, it was meaningful from the economical point of view.

Pycnoporus sp. SYBC-L1 had a simple ligninolytic system, neither lignin peroxidase nor manganese peroxidase activity had been detected, but laccase was produced. A maximum laccase activity of 24,050 UI^{-1} (laccase productivity of 2,004 $UI^{-1}day^{-1}$) was obtained in the medium with higher C/N ratio. The result was in agreement to those reported by Pointing et al. [8], Nyanhongo et al. [9], and Arockiasamy et al. [33], who found that a high carbon and low nitrogen medium was suitable for laccase production. The best *Pycnoporus* strain for laccase production was *P. cinnabarinus* ss3, which was a monokaryotic strain obtained from the dikaryotic *P. cinnabarinus* I-937. *P. cinnabarinus* ss3 produced laccase with an activity of 266,600 UI^{-1} induced by ethanol and ferulic acid, while the laccase production by *P. cinnabarinus* I-937 was at a low level of 9,500 UI^{-1} [7, 34]. Many literatures showed laccase productions by most *P. sanguineus* or *P. coccineus* strains did not exceed 5,000 UI^{-1} [8, 35]. Lomascolo et al. [18] reported Chinese *P. sanguineus* strains showed laccase activities ranging from 11,000–17,000 UI^{-1}. However, in our study, the laccase yield of *Pycnoporus* sp. SYBC-L1 was substantially not only higher than most reported *Pycnoporus* strains but also had a great rising space by adding some suitable inducers. Therefore, it was an attractive candidate for the laccase production.

Not only enzyme yield but also the character is another important factor for application. Many studies have shown the reaction temperatures of most fungal laccase were high (above 50°C), but few have studied the catalytic abilities at low temperature. Kim et al. [36] found the optimal temperature of *Phlebia tremellosa* laccase was 20°C, but there was no studies about the activities at even lower temperature. Jordaan and Leukes [37] exhibited *Trametes versicolor* and *UD4* laccase retained about 60% of its activity at a low temperature value (30–40°C), while this paper found *Pycnoporus* sp. SYBC-L1 laccase showed much higher activity with above 70% of relative activity at the same temperature value. However, many previous researches had no reports about the laccase activities at 0°C. This study displayed *Pycnoporus* sp. SYBC-L1 laccase had much higher catalytic activity with 30.2% of the maximal activity at 0°C; moreover, the activity was only about three times lower when the reaction temperature was shifted from 0 to 70°C, which implied that it possessed the typical characteristic of cold adaptation enzyme that it was resistant to a cold environment. Therefore, the *Pycnoporus* sp. SYBC-L1 laccase was a kind of cold adaptation enzyme. To the best of our knowledge, this was the first report about the activity of *Pycnoporus* laccase at 0°C. Generally, most cold adaptation enzymes are originated from the microorganisms that grow in "extreme" cold environment, such as the south or north poles or seawaters. However, in this paper, this cold adaptation laccase was secreted from a mesophilic fungus. In some bio-catalytic operations, the biological reagents need to play their roles in low temperature conditions, so it is important to find some new enzymes. The beneficial property of the *Pycnoporus* sp. SYBC-L1 laccase could meet the demands with a significant activity.

In some harsh industrial conditions, enzymes with favorable properties, such as resisting to protein denature at higher temperature or pH, are important for commercial applications. This investigation used extracellular laccase supernatants from *Pycnoporus* sp. SYBC-L1 to assess the thermal and pH stabilities. Many thermostable laccases have been reported in *P. sanguineus*, *Physisporinus rivulosus*, *Peniophora* spp., and *Fomes sclerodermeus* [25, 38, 39]. In our test, *Pycnoporus* sp. SYBC-L1 laccase was quite stable below 50°C and the half-lives at 60, 70, and 80°C were 85.5, 37.2, and 2.6 h, respectively; in addition, it kept half activity after incubation for 8 min at 100°C. The results showed it was more stable than

those crude laccases reported previously. For example, Jordaan and Leukes [37] described *Carpophore* crude laccase remained almost fully activity after 9 h at 60°C, while the *Peniophora* spp. laccase showed a half-life of only 5 h at the same temperature [38]. In another paper, Papinutti et al. [40] reported that after 6 h incubation at 50°C, the residual ability of *F. sclerodermeus* crude Lc2 was blow 15%, while Lc1 and Lc3 were almost completely inactivated. Furthermore, *Pycnoporus* sp. SYBC-L1 laccase was even more stable than most purified *Pycnoporus* laccase. For example, Laccase of *P. sanguineus* showed a rapid loss of activity beyond 35°C [8] or 50°C [15]. Dantán-González et al. [39] found the half-life of *P. sanguineus* enzyme was only 0.21 h at 70°C. Recently, Garcia et al. [41] reported a thermostable *P. sanguineus* CCT-4518 laccase, which the half-life was 20 min at 80°C; however, our *Pycnoporus* sp. SYBC-L1 laccase was much better than it. Many investigations were focused on the thermal stability of laccase, but few were referred to laccase with high stability under an alkaline condition. Generally, alkaline culture conditions seem in favor of an alkali-stable enzyme production, but the *Pycnoporus* sp. SYBC-L1 laccase obtained from an acid medium (pH 3.5) was stable in an alkaline condition. In addition, it was noteworthy that the laccase stability was enhanced with pH increasing.

Nowadays, compared with many other methods, enzyme-based decolorization is becoming efficiently due to its potential to remove pollutants without creating some harsh side effects. The abilities of white-rot fungi laccases with crude or purified form to decolorize azo and anthraquinone dyes have been studied widely, particularly in *P. tremellosa*, *Pleurotus sajor-caju, P. ostreatus*, and *T. versicolor*; however, most required the help of redox mediators, such as HBT, ABTS, purpurat, etc. [42]. In this investigation, crude laccase obtained from *Pycnoporus* sp. SYBC-L1 was able to efficiently decolorize the azo dye diamond black PV and anthraquinone dye weak acid blue AS in the absence of any redox mediators. The test showed that the variations of temperature (from 20 to 70°C) had little effect on decolorization. From an industrial application of view, this catalytic property had a great value, since the decolorization could be implemented at room temperature with a high decolorization rate. In conclusion, this study confirmed the ability of *Pycnoporus* sp. SYBC-L1 laccase in decolorizing two different structures of wool dyes, which implied that it could be extended to decolorize the industrial effluents.

In summary, this paper had screened a new laccase producing strain of *Pycnoporus* sp. SYBC-L1. A high laccase production was obtained with a low cost by using some cheap raw materials. It was worthy of note that *Pycnoporus* sp. SYBC-L1 laccase was proven to be not only a cold adaptation enzyme but also fairly thermostable one. These attractive characteristics suggested it to be an excellent candidate in some harsh industrial applications. In addition, the laccase showed the potentiality in the decolorization of wool dyes.

References

1. Riva, S. (2006). *TRENDS in Biotechnology, 24*, 219–226.
2. Couto, S. R., & Herrera, J. L. T. (2006). *Biotechnology Advances, 24*, 500–513.
3. Ahmadi, N., Mibus, H., & Serek, M. (2008). *Journal of Plant Growth Regulation, 27*, 320–330.
4. Alexandre, G., & Zhulin, I. B. (2000). *Trends Biotechnology, 18*, 41–42.
5. Osma, J. F., Herrera, J. L. T., & Couto, S. R. (2007). *Dyes and Pigments, 75*, 32–37.
6. Bohlin, C., Jönsson, L. J., Roth, R., & van Zyl, W. H. (2006). *Applied Biochemistry and Biotechnology, 129*(1–3), 195–214.
7. Herpoe, I., Moukha, S., Lesage-Meessen, L., Sigoillot, J. C., & Asther, M. (2000). *FEMS Microbiology Letters, 183*, 301–306.

8. Pointing, S. B., Jones, E. B. G., & Vrijmoed, L. L. P. (2000). *Mycologia, 92*, 139–144.
9. Nyanhongo, G. S., Gomes, J., Gubitz, G., Zvauya, R., Read, J. S., & Steiner, W. (2002). *Bioresource Technology, 84*, 259–263.
10. Wang, J. W., Wu, J. H., Huang, W. Y., & Tan, R. X. (2006). *Bioresource Technology, 97*, 786–789.
11. Rosales, E., Couto, S. R., & Sanromán, M. A. (2007). *Enzyme and Microbial Technology, 40*, 1286–1290.
12. Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T., et al. (2008). *Bioresource Technology, 99*, 457–462.
13. Osma, J. F., Saravia, V., Herrera, J. L. T., & Couto, S. R. (2007). *Chemosphere, 67*, 1677–1680.
14. Russo, M. E., Giardina, P., Marzocchella, A., Salatino, P., & Sannia, G. (2008). *Enzyme and Microbial Technology, 42*, 521–530.
15. Lu, L., Zhao, M., Zhang, B. B., Yu, S. Y., Bian, X. J., Wang, W., et al. (2007). *Applied Microbiology and Biotechnology, 74*, 1232–1239.
16. Tien, M., & Kirk, T. K. (1988). *Methods in Enzymology, 161*, 238–249.
17. White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). In M. A. Innis, D. H. Gelfland, J. J. Sninsky & T. J. White (Eds.), *PCR protocols: a guide to methods and applications* (pp. 315–322). New York: Academic.
18. Lomascolo, A., Cayol, J.-L., Roche, M., Guo, L., Robert, J.-L., Record, E., et al. (2002). *Mycological Research, 106*, 1193–1203.
19. Jolivalt, C., Madzak, C., Brault, A., Caminade, E., Malosse, C., & Mougin, C. (2005). *Applied Microbiology and Biotechnology, 66*, 450–456.
20. Ghose, T. K. (1987). *Pure and Applied Chemistry, 59*, 257–268.
21. Desgranges, C., Vergoignan, C., Georges, M., & Durand, A. (1991). *Applied Microbiology and Biotechnology, 35*, 200–205.
22. Revankar, M. S., & Lele, S. S. (2006). *Process Biochemistry, 41*, 581–588.
23. Galhaup, C., & Haltrich, D. (2001). *Applied Microbiology and Biotechnology, 56*, 225–232.
24. Revankar, M. S., Desai, K. M., & Lele, S. S. (2007). *Applied Biochemistry and Biotechnology, 143*(1), 16–26.
25. Hildén, K., Hakala, T. K., Maijala, P., Lundell, T. K., & Hatakka, A. (2007). *Applied Microbiology and Biotechnology, 77*, 301–309.
26. Arora, D. S., & Gill, P. K. (2000). *Bioresource Technol, 73*, 283–285.
27. Meza, J. C., Auria, R., Lomascolo, A., Sigoillot, J. C., & Casalot, L. (2007). *Enzyme and Microbial Technology, 41*, 162–168.
28. Georlette, D., Bentahir, M., Claverie, P., Collins, T., D'Amico, S., Delille, D. et al. (2001) In M. De Cuyper & J. W. M. Bulte (Eds.), *Physics and chemistry basis of biotechnology* (pp. 177–196). The Netherlands: Kluwer Academic Publishers.
29. Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.-P., Claverie, P., Collins, T., et al. (2000). *Trends in Biotechnology, 18*(3), 103–107.
30. Marshall, C. J. (1997). *Trends in Biotechnology, 15*(9), 359–364.
31. Eggert, C. (1997). *Microbiological Research, 152*(3), 315–318.
32. Lorenzo, M., Moldes, D., Couto, S. R., & Sanroman, A. (2002). *Bioresource Technology, 82*, 109–113.
33. Arockiasamy, S., Krishnan, I. P. G., Anandakrishnan, N., Seenivasan, S., Sambath, A., & Venkatasubramani, J. P. (2008). *Applied Biochemistry and Biotechnology, 151*(2–3), 371–379.
34. Lomascolo, A., Record, E., Herpoël-Gimbert, I., Delattre, M., Robert, J. L., Georis, J., et al. (2003). *Journal of Applied Microbiology, 94*, 618–624.
35. Litthauer, D., van Vuuren, M. J., van Tonder, A., & Wolfaardt, F. W. (2007). *Enzyme and Microbial Technology, 40*, 563–568.
36. Kim, Y., Yeo, S., Kim, M. K., & Choi, H. T. (2008). *FEMS Microbiology Letters, 284*, 172–175.
37. Jordaan, J., & Leukes, W. D. (2003). *Enzyme and Microbial Technology, 33*, 212–219.
38. Niku-Paavola, M. L., Fagerström, R., Kruus, K., & Viikari, L. (2004). *Enzyme and Microbial Technology, 35*, 100–102.
39. Dantán-González, E., Vite-Vallejo, O., Martínez-Anaya, C., Méndez-Sánchez, M., González, M. C., Palomares, L. A., et al. (2008). *Internation microbiology, 11*, 163–169.
40. Papinutti, L., Dimitriu, P., & Forchiassin, F. (2008). *Bioresource Technology, 99*, 419–424.
41. Garcia, T. A., Santiago, M. F., & Ulhoa, C. J. (2007). *Applied Microbiology and Biotechnology, 75*, 311–318.
42. Chhabra, M., Mishra, S., & Sreekrishnan, T. R. (2008). *Applied Biochemistry and Biotechnology, 151*(2–3), 587–598.