

## Laccase produced by a thermotolerant strain of *Trametes trogii* LK13

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### Abstract

Thermophilic and thermotolerant micro-organisms strains have served as the natural source of industrially relevant and thermostable enzymes. Although some strains of the *Trametes* genus are thermotolerant, few *Trametes* strains were studied at the temperature above 30 °C until now. In this paper, the laccase activity and the mycelial growth rate for *Trametes trogii* LK13 are superior at 37 °C. Thermostability and organic cosolvent tolerance assays of the laccase produced at 37 °C indicated that the enzyme possessed fair thermostability with 50% of its initial activity at 80 °C for 5 min, and could remain 50% enzyme activity treated with organic cosolvent at the concentration range of 25%-50% (v/v). Furthermore, the test on production of laccase and lignocellulolytic enzymes showed the crude enzymes possessed high laccase level (1000 U g<sup>-1</sup>) along with low cellulose (2 U g<sup>-1</sup>) and xylanase (140 U g<sup>-1</sup>) activity. Thus, *T. trogii* LK13 is a potential strain to be applied in many biotechnological processes.

**Key words:** *Trametes trogii*, thermotolerant fungi, Laccase, thermostability, organic cosolvent tolerance.

### Introduction

To degrade lignocelluloses materials, filamentous fungi produce complex extra- and intracellular enzyme systems which include cellulose, xylan and lignin oxidation enzymes (Abdel-Hamid *et al.*, 2013). Laccase (EC 1.10.3.2, Lac) is one of the most important lignin oxidation enzymes that can non-specifically degrade many phenolic and non-phenolic compounds, such as lignin and many environmental pollutants (including synthetic dyes, toxic substances in industrial effluents, herbicides and pesticides in soil) (Rodríguez Couto and Toca Herrera, 2006). Due to the ability to degrade and detoxify many environmental pollutants, laccases have been widely applied in several biotechnological processes (Rodgers *et al.*, 2009).

Laccases and laccase-like proteins have been described in plants, arthropods, bacteria and bovine rumen microflora, with particular emphasis on the fungi, especially in the white rot basidiomycetes (Beloqui *et al.*, 2006; Claus, 2004). In order to facilitate novel and more efficient bio-catalytic applications at industrial scale, the biochemi-

cal and financial properties of fungi laccase must be improved (Hildén *et al.*, 2009; Piscitelli *et al.*, 2011). Many biochemical properties of laccases closely related to their application, including thermostability, thermotolerance, pH-stability, organic solvent-tolerant (Huang *et al.*, 2011) and high salts-tolerance (Ellouze *et al.*, 2010; Hildén *et al.*, 2009).

Thermophilic and thermotolerant micro-organisms strains have served as the natural source of industrially relevant and thermostable enzymes (Boonlue *et al.*, 2003). Moreover, the thermostable enzymes usually have higher resistance to chemical denaturants, high alkalinity and extreme acidity (Hildén *et al.*, 2009). Currently, thermostable fungi laccases in industrial application are mainly isolated from *Melanocarpus albomyces*, *Myceliophthora thermophila* and *Chaetomium thermophilum*, which all belong to the *Ascomycota* (Hildén *et al.*, 2009).

As a potential white rot basidiomycete source of thermostable laccases, the *Trametes* strains have attracted much attention, such as *T. versicolor*, *T. pubescens* and *T.*

*trogii*. These strains can produce unique extra-cellular oxidative enzymes, including Mn-peroxidase (MnP) and laccase, and the latter is the dominant one. It has been reported that many laccase isozymes from this genus show exceptionally high thermal stability (Hildén *et al.*, 2009). Interestingly, elevating the temperature of the enzyme production or heat shock treatment can improve laccase production in the strains of the *Trametes* genus (Tong *et al.*, 2007; Wang *et al.*, 2012). Thus, the *Trametes* genus is considered to be the potential source of novel and thermostable laccases (Hildén *et al.*, 2009).

It is worth pointing out that *T. trogii* is a thermo-tolerant species preferring sun-exposed habitats, but until now, few related studies of *T. trogii* were conducted over 30 °C, mostly at 28 or 30 °C (Mutlu *et al.*, 2010). In this study, laccase produced by the newly isolated thermo-tolerant *T. trogii* strain LK13 was investigated at 37 °C.

## Materials and Methods

### Isolation and identification of strain

The fungal fruit body growing on the decayed wood was collected from the forest in Lijiang, China. After the elimination of surface contamination, the fragments (approximately 5 mm<sup>2</sup>) of the fruit body were placed on 2% MA (malt extract agar) plate for incubation at 28 °C. The pure cultures were obtained from hyphal tip. Morphological characteristics of the fungal fruit body and mycelia were observed by Micro- and Macro- scope. Molecular identification is performed based on a modified protocol. In brief, total genomic DNA was extracted from the 6-day fresh mycelium using the CTAB method, and the ITS region was amplified using the primer pairs: 5'-GGAAGTAAAGTCGTAACAAGG-3' (forward) and 5'-TCCTCCGTTATTGATATGC-3' (reverse). The amplification was performed as follow: 1 cycle of 94 °C for 1 min; 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C for 26 cycles; followed by a final extension of 10 min at 72 °C. Phylogenetic analysis and construction of an unrooted tree were performed by the software MEGA version 3.0.

### Growth characteristics at different temperatures

To explore the optimal growth temperature of strain, 4-day-old mycelial blocks in the diameter of 5 mm incubated at 28 °C were transferred to the 2% MA plates kept at different temperatures (including 28 °C, 30 °C, 34 °C, 37 °C, 40 °C and 42 °C). The growth rate of the colonies was recorded and the features of clones were also observed.

### Localisation of laccase activity by staining

Localisation of laccase activity was determined according to the method of Hiscox *et al.* (2010). Mycelial block with a diameter of 5 mm from the 2% MA plate was transferred to 9 cm Petri dishes plus 250 mg L<sup>-1</sup> 2, 2'-azino

bis (3-ethylbenzthiazoline-6- sulphonic acid) (ABTS, Sigma, USA), and the green/violet zone was recorded at 2 d, 8 d, 15 d, 1 month and 2 months respectively.

### Effects of temperature and organic solvents on laccase activity

The mycelia from the slant were transferred to the GYP plates (Fan *et al.*, 2011) and incubated at 30 °C for 5 d. Inocula were prepared in 250 mL Erlenmeyer flask containing 50 mL of GYP starting from 4 mycelial plugs (1 cm in diameter). Cultures grow for 5 days at 30 °C were homogenized by beaded glasses (0.3 mm in diameter), and 5% (v/v) aliquots of the mycelia suspension were used as inocula for laccase production. The supernatants of 9-day-old liquid culture of *T. trogii* LK13 were obtained by centrifugation (9 000 rpm, 5 min at 4 °C), and the crude enzyme solutions were used for the further study.

To determine the effect of temperature on laccase activity, the laccase was incubated at a temperature range of 20-85 °C in phosphate citrate buffer (100 mM, pH 4.0) for 5 min and then the residual laccase activity was determined at room temperature. To further determine the half-life at certain temperature, the residual laccase activity was determined after the crude enzyme solutions were incubated at given temperature in phosphate citrate buffer (100 mM, pH 4.0) with different time intervals. To determine the effect of organic solvents on laccase activity, the reaction mixture was added with methanol, isopropanol, acetonitrile, acetone, formalin or DMSO to get the final concentration. The laccase activity of the reaction mixture without organic solvent was recorded as 100%.

### Solid state fermentation (SSF)

Rice straw, bagasse, sawdust, cotton seed coat fragments were obtained in the region of Kunming city. After dried at 80 °C for 4 days, the lignocellulosic materials were milled by a micro-grinder. Through the sieve screen, 1 mm chips were used for the solid state fermentation, which employed 5 g materials in 16 mL culture solution (peptone, 12.5 g L<sup>-1</sup>; malt extract, 30 g L<sup>-1</sup> and 11 mM CuSO<sub>4</sub>) according to the study by Levin *et al.* (2008). The substrates were inoculated with 4 mycelial blocks in a diameter of 1 cm in a 250 mL Erlenmeyer flask, and kept in darkness at 37 °C for 4 weeks. Crude enzyme was obtained by adding 0.05 M sodium acetate buffer (pH 4.8) to the fermented culture at a liquor ratio of 1:10. Subsequently, the mixture was stirred continuously at 200 rpm for 30 min and centrifuged at 10000 rpm for 10 min at 4 °C. The supernatants were used for the enzyme activity assay.

### Enzyme assays

Laccase activity was routinely determined with ABTS ( $\epsilon_{420} = 36 \text{ (mM cm)}^{-1}$ ) as substrate. Mixture of 0.5 mL appropriately diluted crude enzyme and 1.1 mL of 2 mM ABTS in phosphate citrate buffer (100 mM, pH 4.0)

was used to determine the activity. The increase in absorbance was monitored at 420 nm for 3 min (Bourbonnais and Michael, 1990). One unit of the enzyme activity was defined as the amount of the enzyme that oxidized 1  $\mu\text{mol}$  of the ABTS per minute. MnP activity (E.C:1.11.1.13) was measured using phenol red as the substrate in 0.1 M sodium dimethylsuccinate buffer (pH 4.5) at room temperature ( $\epsilon_{610} = 22 \text{ (mM cm)}^{-1}$ ) (Glenn and Gold, 1985). Organic solvent tolerance and the thermal stability assays were performed according to the methods of Farnet *et al.* (2008) and Halaburgi *et al.* (2011), respectively. Exo- $\beta$ -D-1, 4-glucanase and endo- $\beta$ -D-1, 4-xylanase activities were determined according to the method of Levin *et al.* (2008).

## Results

### Microorganism identification

Based on the characteristics of fruity body (Figure 1a) and microscopy observation of hyphae (Figure 1b) combined with the ITS rDNA gene sequence with the 99% identity to that of most strains of *T. trogii* deposited in the Genbank database, the isolated strain LK13 was identified as *T. trogii* (Figure 1c). The sequence was submitted to GenBank with the accession no: JX105361.

### Growth characterization of *T. trogii* LK13 strain at different temperatures

Colonies of *T. trogii* LK13 strain could typically grow at 28–42 °C on 2% MA plate (Figure 2), but the optimal temperature range was 34–37 °C. Mycelium rapidly expanded to the margin of 9 cm Petri dishes within 4 days at 37 °C, while 8 days were needed at 28 °C to cover the same size. Mycelium even could grow at 42 °C (Figure 2), which was the lethal temperature for most filamentous fungi.

### Location of laccase activity by staining

To study the laccase production of *T. trogii* LK13 at different temperatures, in situ staining method of enzyme activity was employed. After the culture for 6 days at 37 °C, a clear green zone with the average diameter of 4 cm was observed around the inoculum block on the plate of MA with 250 mg L<sup>-1</sup> ABTS (Figure 3b), and the next 4 days, the green zone expanded to the margin of the dish and simultaneously was turn into strong violet (Figure 3c). While only light orange was observed on Petri dishes with ABTS at 28 °C even more than 12 days of cultivation (Figure 3e). Based on these observations, *T. trogii* LK13 should produce the laccase at both 28 °C and 37 °C, and the activity was higher at 37 °C.

### Effect of temperature and organic solvents on laccase activity

There was almost no loss of activity at 45 °C and 55 °C (Table 1). With the temperatures increasing to 65 °C, 70 °C and 75 °C, about 11.3%, 15.9% and 22.4% laccase

activities were reduced (Table 1). The enzyme retained about 50% its initial activity at 80 °C, while only 5% activity was remained at 85 °C (Table 1). In addition, the enzyme retained 72.3% and 35.5% of its initial activity at 75 °C for 10 min and 30 min, respectively.

Among all tested organic solvents, 10% (v/v) isopropanol and acetonitrile increased the laccase activity by about 21% and 25% respectively, while the activity slightly declined to 86.9% and 99.2% of the control with the concentration reaching to 20% (v/v) (Table 2). DMSO obviously inhibited the laccase, and 5% (v/v) reagent could lead to the 41.3% loss of activity (Table 2). The inhibiting concentration where 50% of laccase activity remained was about 40% (v/v) for methanol, about 25% (v/v) for acetone and formalin (Table 2).

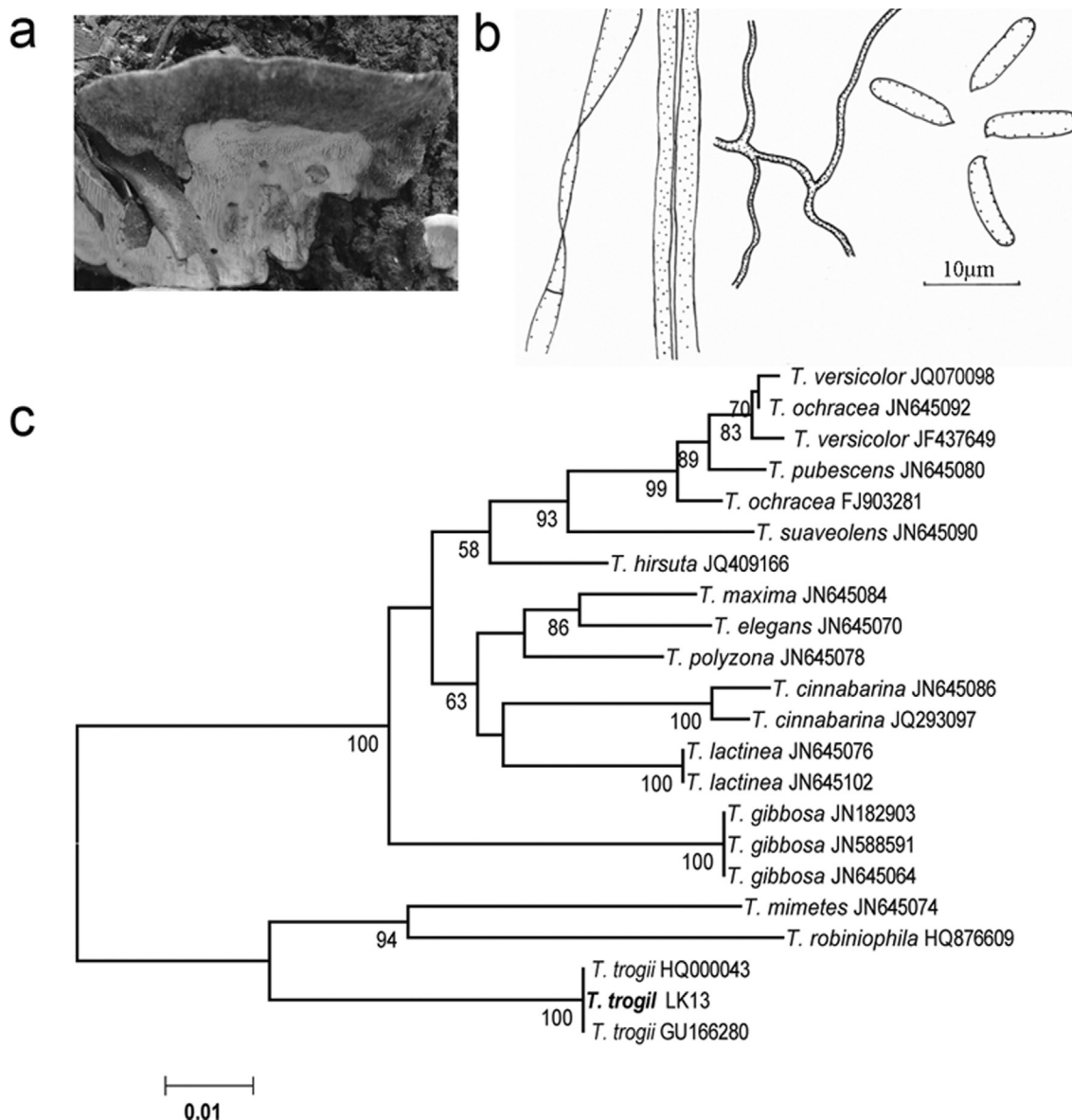
### Production of laccase using solid state fermentation (SSF)

The substrates of rice straw, bagasse, sawdust, cotton seed coat fragments were completely colonized within 7, 9, 4 and 4 days, respectively. Compared with rice straw, all other materials could lead to the fast mycelium-growth and high laccase activity (Table 3). On the culture with cotton seed coat fragments, the activity gradually increased from day 3 and achieved the maximal values of about 1263 U g<sup>-1</sup> at day 7 (Table 3).

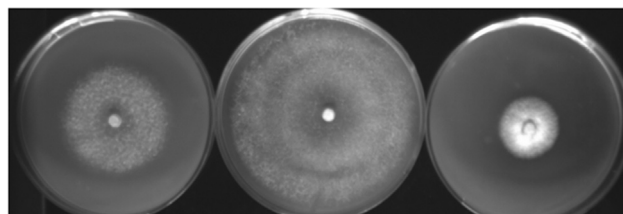
At the same time, the laccase activity reached about 800, 400 and 1000 U g<sup>-1</sup> on rice straw, bagasse and sawdust, respectively (Table 3), and they showed the highest activity at day 14, which were still lower than that in cotton seed coat fragments culture. During the whole fermentation cycle, Exo- $\beta$ -D-1, 4-glucanase activity was always lower than 2.3 U g<sup>-1</sup>, and even on rice straw culture which was cellulose-rich lignocellulosic substrate. The lowest Exo- $\beta$ -D-1, 4-glucanase activity was present in cotton seed coat fragments culture with the values of about 1 U g<sup>-1</sup> on day 7 (Table 3), and extracellular xylanase maintained the activity of 255.6 U g<sup>-1</sup> (Table 3).

## Discussion

Due to harsh industrial process conditions that may include high temperature and/or pressure, high salt concentrations, acidic or alkaline pH, oxidative conditions, high shear forces or short delays, resistant enzymes are required (Hildén *et al.*, 2009). Previous studies have demonstrated that the thermostable enzymes usually are higher resistant to chemical denaturants, high alkalinity and extreme acidity (Hildén *et al.*, 2009). Thermophilic and thermotolerant micro-organisms strains have served as the natural source of industrially relevant and thermostable enzymes (Boonlue *et al.*, 2003). *Phanerochaete chrysosporium* is well studied thermophilic white-rot fungus. However, *P. chrysosporium* strains mainly produce lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP), not



**Figure 1** - Morphological characterization of *T. trogii* LK13 and phylogenetic tree constructed based on ITS sequences. (a) Morphological characteristics of the fruit body of *T. trogii* LK13. (b) Trisomy mycelial systems and spores of *T. trogii* LK13. (c) Neighbour-joining tree based on analysis of ITS sequences. The numbers at the nodes represent bootstrap supported from 1000 replicates, and the values above 50% were shown).

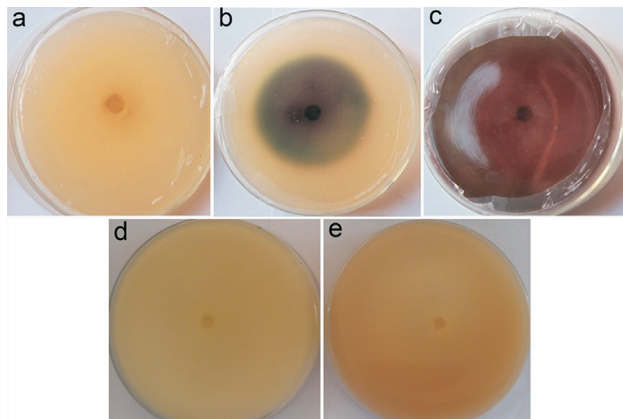


**Figure 2** - Comparison of the mycelial growth rate of *T. trogii* LK13 strain under different temperatures. After the culture for 4 days under 28 °C (Left), 37 °C (Middle) and 42 °C (Right) on 2% MA.

laccase (Abdel-Hamid *et al.*, 2013). Our study certified that the optimal temperature of 37 °C was for both mycelium growth and laccase production in a newly isolated *T. trogii*

LK13 (Figures 2 and 3), which was the first report on the laccase produced by the thermotolerant *T. trogii* strain.

The activities of fungal laccases usually drop suddenly at the temperature above 60 °C (Baldrian, 2006). However, the laccase from *T. trogii* LK13 maintained approximately 88.7% of its initial activity at 65 °C and dropped suddenly at 85 °C (Table 1). The crude laccase from *T. trogii* LK13 retained 84.1% and 77.6% of its initial activity after the incubation at 70 °C and 75 °C for 5 min respectively, which is higher than the laccase from *Cladosporium cladosporioides* and *T. hirsuta* (Halaburgi *et al.*, 2011; Zhang *et al.*, 2009). The  $T_{50}$  of the crude laccase from *T. trogii* LK13 (defined as the temperature at which the enzyme retains 50% of its initial activity) at 70 °C and 75 °C



**Figure 3** - Localisation of the laccase activity of *T. trogii* LK13 strain using in situ staining method. Laccase production under 37 °C at day 6 (b) and day 10 (c), and 28 °C (e) on 2% MA added with 250 mg L<sup>-1</sup> ABTS. (a) and (d) on 2% MA without ABTS as the control for 37 °C and 28 °C, respectively. Laccase with high activity can generate a purple or green zone.

**Table 1** - The stability of the crude laccase from *T. trogii* LK13 under different temperatures.

Temperature (°C)	Remaining activity (%)
0	100
45	100 ± 0.5
55	92.7 ± 3.9
65	88.7 ± 4.6
70	84.1 ± 8.2
75	77.6 ± 2.2
80	53.5 ± 7.6
85	5.7 ± 0.2

was higher than that of laccases from *T. hirsuta*, *Pleurotus cinnabarinus*, *P. ostreatus*, *Coriolopsis gallica* and *T. versicolor* (Bommarius *et al.*, 2006; Maté *et al.*, 2010), while it was lower than that from *Pycnoporus sanguineus* (SCC 108) (Litthauer *et al.*, 2007) and lacIII from *Trametes* sp. HS-03 (Guo *et al.*, 2012). The crude laccase was used for thermostability assay in this study, which may affect the comparability of the purified laccases, but this comparability also indicates that *T. trogii* LK13 is a promising resource of laccase with fair thermostability.

Many substrates of laccases are organic pollutants which contain high concentrations of organic solvents to

**Table 2** - The influence of organic solvents on the crude laccase activity from *T. trogii* LK13.

Organic solvent	Concentration (% v/v)	Remaining activity (%)
Control	/	100
Methanol	40	52.8 ± 18.9
Isopropanol	10	121.6 ± 4.0
	20	86.9 ± 5.2
Acetonitrile	10	125.3 ± 8.3
	20	99.2 ± 17.0
Acetone	25	42.2 ± 0.6
Formalin	25	55.6 ± 6.5
DMSO	5	58.7 ± 3.9

enhance the solubility (Maté *et al.*, 2010). Thus, fungal laccases with organic cosolvent tolerance can make their practical use available (Farnet *et al.*, 2008). In *Marasmius quercophilus*, 50% of the laccase activity remained when ethanol, methanol and acetone were added at the concentration of 60%, 40% and 40% (v/v) respectively (Farnet *et al.*, 2008), which is superior to the crude laccase from *T. trogii* LK13. However, Laccase activity increased by about 21% and 25% respectively when isopropanol and acetonitrile were added at the concentration of 10% (v/v) (Table 2), while laccase of *M. quercophilus* didn't show the increased activity at all tested organic solvents.

Lignocellulose is the most abundant renewable biomass on earth, and it has long been recognized as an alternative source for producing renewable fuels and chemicals (Abdel-Hamid *et al.*, 2013). Pretreatment is a crucial step in the conversion of lignocellulosic biomass to fermentable sugars and biofuels. Using lignin-degrading microorganisms, fungal pretreatment potentially provides an environmentally-friendly and energy-efficient pretreatment technology (Wan and Li, 2012). Fungal pretreatment encounters the challenge of cellulose loss along with delignification because of the tested fungi generally with high cellulose and xylanase activities (Nakagame *et al.*, 2006; Shi *et al.*, 2009). Thus, cellulose-deficient white rot fungi are extremely needed (Nakagame *et al.*, 2006; Shi *et al.*, 2009). In this work, some relevant hydrolytic enzymes were studied, including Exo-β-D-1, 4-glucanase and endo-β-D-1, 4-xylanase. Both of them produced by strain *T.*

**Table 3** - Hydrolytic and oxidative activities of solid-state fermentation materials prepared on various lignocellulose substrates for 7 d.

Substrate	Laccase (U g <sup>-1</sup> )	Exo-β-D-1, 4-glucanase (U g <sup>-1</sup> )	Endo-β-D-1, 4-xylanase (U g <sup>-1</sup> )
Rice straw	800.6 ± 15.3	2.3 ± 0.17	139.3 ± 11.2
Bagasse	400.3 ± 25.6	2.0 ± 0.33	140.2 ± 13.8
Sawdust	1000 ± 50	1.5 ± 0.2	145.6 ± 17.0
Cotton seed coat fragments	1263.8 ± 34	1.1 ± 0.15	158.6 ± 15.8

*trogii* LK13 were detected with much lower activities than that in the strain *T. trogii* MYA 28-11 by Levin *et al.* (2008), and the activities of exo- $\beta$ -D-1,4-glucanase and endo-oxylanase were about 2 U g<sup>-1</sup>, 140 U g<sup>-1</sup> in the strain *T. trogii* LK13 (Table 3) compared with that of 113 U g<sup>-1</sup>, 1176 U g<sup>-1</sup> in *T. trogii* MYA 28-11 respectively.

In this study, the laccase activity and the mycelial growth rate for *T. trogii* LK13 were superior at 37 °C. The laccase produced at 37 °C of this strain possessed good thermostability and organic cosolvent tolerance. Additionally, *T. trogii* LK13 could produce high laccase level (1000 U g<sup>-1</sup>) along with low Exo- $\beta$ -D-1, 4-glucanase and endo- $\beta$ -D-1, 4-xylanase using lignocellulose materials. Thus, *T. trogii* LK13 is a potential strain to be applied in many biotechnological processes.

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