CHAPTER 3

ISOLATION AND IDENTIFICATION OF LACCASE PRODUCING WHITE-ROT FUNGI

3.1 Introduction

Lignocellulosic biomass refers to plant biomass that is composed of cellulose, hemicellulose, and lignin. The carbohydrate polymers (cellulose and hemicellulose) are tightly bound to the lignin. The organisms predominantly responsible for lignocelluloses degradation are fungi, and the most rapid degraders in this group are basidiomycetes (Rabinovich *et al*., 2004). White-rot fungi are known to be capable of using lignin as a sole carbon and energy source. Many white-rot fungi have been employed for production of lignin degrading (ligninolytic) enzymes and for delignifying which used as biological method for lignocellulosic materials (Sánchez, 2009). These fungi present more effectively and more specifically degrade lignin than brown- and soft-rot fungi.

Laccase (EC 1.10.3.2) is an important enzyme in fungal ligninolytic systems involved in lignin degradation (Gianfreda *et al*., 1999). This enzyme, belonging to a family of multicopper-containing enzymes, catalyzes the oxidation of a variety of phenolic compounds, polyamines, aryl diamines, and lignin, with concomitant reduction of O_2 to water (Thurston, 1994; Solomon *et al*., 1996). Most laccases were reported and studied from fungi such as *Coriolus versicolor* (Madhavi and Lale, 2006), *Phanerochaete chrysosporium* (Elisashvili *et al*., 2008) and *Coriolopsis*

(*Trametes*) *polyzona* (Jaouani *et al*., 2006). Laccase has attracted considerable interest for application in many fields including the pulp, paper (Bajpai, 1999), and food industry (Durán *et al*., 2002).

In this study, we isolated white-rot fungi from natural forest and studied of their potential of laccase production with purpose to apply white-rot fungus for biological pretreatment of lignocellulosic biomass in bio-ethanol production.

3.2 Materials and Methods

3.2.1 Fungal collection and isolation

White rot fungi were collected from native forest and rotting linocellulosic materials from the north of Thailand; Chiang Rai and Chiang Mai provinces, during raining season (May to August, 2008). Hyphal tip isolation technique (Goh, 1999) was done for isolate pure culture of white rot fungi. The presence of aerial mycelium and spore mass color morphology were recorded after 5 days incubation on PDA medium. The pure strain were stored at 4^{o}C in PDA slants and inoculated once in every 3 months.

3.2.2 Primary screening of laccase producing white-rot fungi

Primary screening of the isolate for laccase production was carried out on lignin modifying basal medium (LBM) containing (L^{-1}), KH_2PO_4, 1 g; $C_4H_{12}N_2O_6$, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.01 g; yeast extract, 0.01 g; $CuSO_4.5H_2O$, 0.001 g; $Fe_2(SO_4)_3$, 0.001 g; $MnSO_4.H_2O$, 0.001 g and the polymeric anthrapyridone chromophore poly R-478 0.2% (w/v) (Pointing, 1999). One mycelium plug of fungi sample was growth on LBM agar and incubated at 37^{o}C under static condition. *Coriolus versicolor* RC3, a thermotolerant white-rot fungus isolated from Chiang Mai

province, Thailand (Khanongnuch *et al*., 2004) was used as reference microorganism for laccase production.

3.2.3 Laccase assay

Laccase production was measured by oxidation of 2,6-dimethoxyphenol (DMP). The reaction mixture containing (ml^{-1}), 50 µl of 4 mM DMP; 500 µl of 50 mM sodium acetate buffer (pH 5.0); 350 µl of distilled water and 100 µl of enzyme sample. DMP oxidation was monitored by determination of an increasing in absorbance at 470 nm (ε_{470}=49.6 $mM^{-1}cm^{-1}$) (Patana *et al*., 2006). One activity unit was defined as the amount of enzymes necessary to oxidize 1 µmol of substrate per minute. Activities will be compared according to the enzyme activity (unit/ml) and specific activity (unit/mg protein).

3.2.4 Molecular identification

3.2.4.1 DNA extraction and DNA sequencing

Total genomic DNA was extracted from fresh mycelium of 5 days white rot fungal colony according to modification of the rapid preparation of DNA from filamentous fungi (Raeder and Broda, 1985). Fungal mycelia were scraped off and transferred to 1.5 ml centrifuge tube. Then, sterile white quartz sand and 300 µl of preheated (65^{o}C) 2X CTAB buffer were added to 1.5 ml centrifuge tube. Mycelium suspension was ground with sterile chopstick for 10 minutes and incubated at 60 ^{o}C for 30 to 60 minutes and gently inverted centrifuge tube every 15 minutes. One volume of phenol per chloroform per isoamyl alcohol (25:24:1) was added and mixed before centrifuge each centrifuge tube at 13000 rpm for 30 minutes. The top extracted solution layer was removed and transferred into a new 1.5 ml centrifuge tube. The cold absolute ethanol 1 ml was added and gently inverted and the tube was stored

overnight at -20 ^{o}C for DNA precipitation. The DNA pellet in each centrifuge tube was collected by centrifugation at 11000 rpm for 30 minutes at 4 ^{o}C. The pellet was washed with 70% ethanol twice and dried at room temperature. Then, DNA pellet was dissolved in 50 µl of TE buffer containing 20 µg/ml RNase. The purity of DNA was determined by running DNA samples by electrophoresis in 1% (w/v) agarose. Agarose gel was soaked in ethidium bromide (10 mg/ml) for 10 minutes and checked DNA band under UV light.

Molecular identification of the fungus was carried out by sequence analyses of the internal transcribed spacer (ITS) regions of rRNA gene. PCR amplification of ITS region was performed using the forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al*., 1999). PCR reaction tubes were composed of 10 µl of PCR master mix (50 units/ml of *Taq* DNA polymerase, 400 mM each: dATP, dGTP, dCTP, dTTP and 3 mM $MgCl_2$), 1.0 µl of 10 µM primers (ITS4 and ITS1), 2.5 µl of DNA template and 11.5 µl of deionized water to adjust the volume of PCR reaction as 25 µl. Each PCR reaction tube was carried out under following condition; 2 minutes initial denaturation at 95 ^{o}C, follow by 30 cycles of 30 second denaturation at 95 ^{o}C, 30 second annealing at 50 ^{o}C, 1 minutes extension at 72 ^{o}C, and 10 minutes final extension at 72 ^{o}C. The PCR reaction products were examined by electrophoresis in 1% (w/v) agarose gel and the bands stained with ethidium bromide. PCR products were purified using the PCR clean-up gel extraction (NucleoSpin® Extract II). The purified product was directly sequenced by Macrogen Inc., (Seoul, South Korea).

3.2.4.2 Phylogenetic analysis

The ITS region of rDNA gene sequences were aligned with known nucleotide sequence in database of the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) using the BLAST search option, available public data base Genbank for comparing with rDNA sequencing data. Phylogenetic tree for rDNA sequences was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The sequences were taken together in the calculations of levels of sequence similarity using CLUSTAL W 1.74 (Higgins *et al*., 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Pairwise distances between sequences were calculated by using the maximum composite likelihood and showed in units of the number of vase substitution per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons. The phylogenetic tree was conducted in the MEGA 4 program (Tamura *et al*., 2007). The PHYDIT program version 3.1 was used to establish sequence similarity percentages.

3.2.4 Production of fruiting body of white-rot fungus

To produce fungal fruiting body, orange peel was used as solid substrate and various liquid solution, MYG solution (Tanabe *et al*., 1989), basal media (Mikiashvili *et al*., 2006) or distilled water were prepared in grass tubes 3.5×3.5 cm diameter and 12 cm height. Mycelium plugs of white-rot fungus were inoculated into glass tube contained 60% of humidity. Solid state fermentation condition was static, 12/12 hour light/dark and 37^{o}C for 15 days. After that the glass tube were transferred to 28^{o}C incubator, mycelium and fruiting body morphology were recorded after 40th day.

3.3 Results and discussion

3.3.1 Primary screening of laccase producing white-rot fungi

White-rot fungal isolates were screened for their potential to degrade the Poly-R dye. The solid cultures of the fungal isolates grown on LBM agar contained Poly-R 478 resulted in only 24 positives from total 31 isolates which decolourised the dye, turned from purple to yellow colour around fungal colony after 4 days incubation indicated presence of ligninolytic enzyme (Fig. 3.1a) when compared with negative results (Fig. 3.1b). Poly-R 478 dye was a synthetic dye with a lignin-like structure, the dye is common used as a indicator for laccase production (Jang *et al*., 2009; Korniłłowicz-Kowalska and Rybczyńska, 2012).

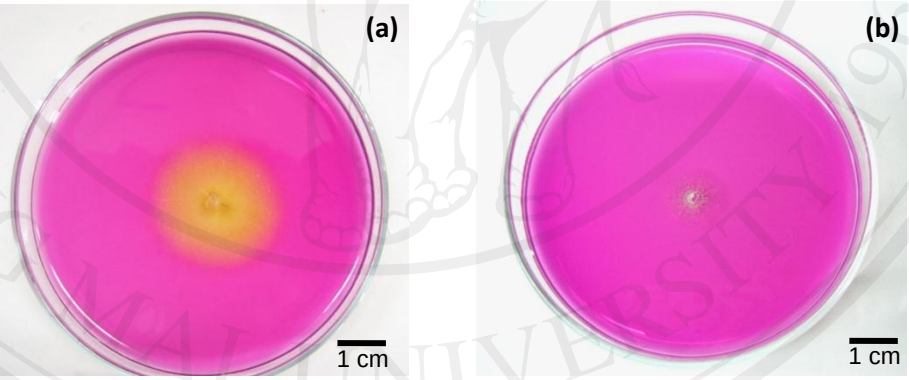


Fig.3.1 Primary screening for ligninolytic enzyme production.

(a) postive result, LBM agar turned from purple to yellow color around colony and **(b)** negative result on LBM agar (no change of color).

After that the positive isolates were determined for enzyme activity following lacccase assay by using 2,6-DMP as substrate, the results showed in Table 3.1. Three isolates which presented high level of laccase activity were selected to culture with 1% of wheat bran, laccase producing substrate (Souza *et al*., 2010) under solid condition and compared with the laccase producer, *C. versicolor* RC3 as positive control (Chawachart *et al*., 2004). As shown in Fig. 3.2, white-rot fungus strain WR710-1 produced high activity of laccase when compared with the other strain samples and the positive control. Thus, fungus strain WR710-1 was selected, identified and used for further experiments.

Table 3.1 Laccase activity level produced from fungal samples

Isolation code No.	Laccase activity level*
WR 29 5-1	+
WR 29 5-2	+
WR 29 5-3	++
WR 29 5-4	n.
WR 29 5-5	n.
WR 29 5-6	++
WR 29 5-7	++
WR 29 5-8	+++
WR 29 5-9	n.
WR 29 5-10	n.
WR 29 5-11/1	+
WR 29 5-11/2	+
WR 29 5-12	+
WR 29 5-13	n.
WR 29 5-14	n.
WR 29 5-15	+++
WR 29 5-16	n.
WR 2 6-1	++
WR 2 6-2	n.
WR 14 8-1	n.
WR 14 8-3	+
WR 7 10-1	+++
WR 7 10-3	n.
WR R9	n.
Coriolus versicolor RC3**	+++

* Laccase activity level ; n. = no activity, + = 0.0001 – 0.001 U/ml, ++ = 0.0011- 0.01 U/ml, and +++ = 0.011-0.1 U/ml. ** Positive control of this experiment

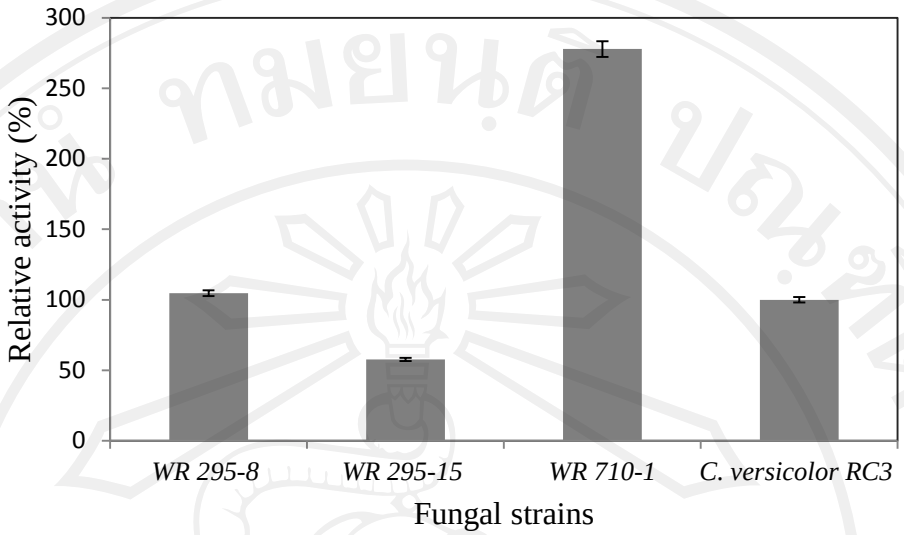


Fig. 3.2 Relative activity of laccase from different fungal strains compared with positive control, *C. versicolor* RC3 (defined relative activity as 100%).

Seven day incubated under solid condition with 1% of wheat bran as substrate. Error bars represent the standard deviation from the mean of three replications.

3.3.2 Molecular identification of selected strain

The ITS region of rDNA sequence can be obtained and aligned with known nucleotide sequences in GenBank databases using BLAST search option to calculate percentage of identity. The percentage of similarities of the rDNA gene sequences between fungal strain WR710-1 and closely related white-rot fungi were showed in Table 3.2. The results of percentage of identity and percentage of average similarity analyzed by PHYDIT program are shown that white-rot fungus isolate WR 710-1 was closely related with *Trametes polyzona*. Analysis of the ITS region sequence indicated that the isolate WR 710-1 could be a member of genus *Trametes* with 99% similarity.

The nucleotide sequence of *T. polyzona* strain WR710-1 was deposited in the GenBank database under accession number JN848329.

Table 3.2 Percentage of similarities of the ITS gene sequences between fungal strain WR710-1 and closely related white-rot fungi

Fungal strain	Size (bp)	Similarity (%)	Accession number
WR710-1	941	-	This study
Trametes polyzona voucher BKW017	644	99	JN 164977.1
T. polyzona voucher BKW01	844	99	JN 164980.1
T. polyzona voucher BKW004	637	99	JN 164978.1

To investigate inter- and intra-relationships of the members in some part of the genus *Trametes*, the obtained sequences were aligned with the sequences of type strains retrieved from GenBank databases. A phylogenetic tree was constructed by NJ method. The sequence of *Grifola frondora* was used as an outgroup to root the tree. From Fig. 3.3, the final Neighbor-joining phylogenetic tree indicated that isolate WR710-1 was positioned between group of *T. polyzona* (syn. *Coriolopsis polyzona*) and *T. versicolor* under bootstrap supported value of 99%.

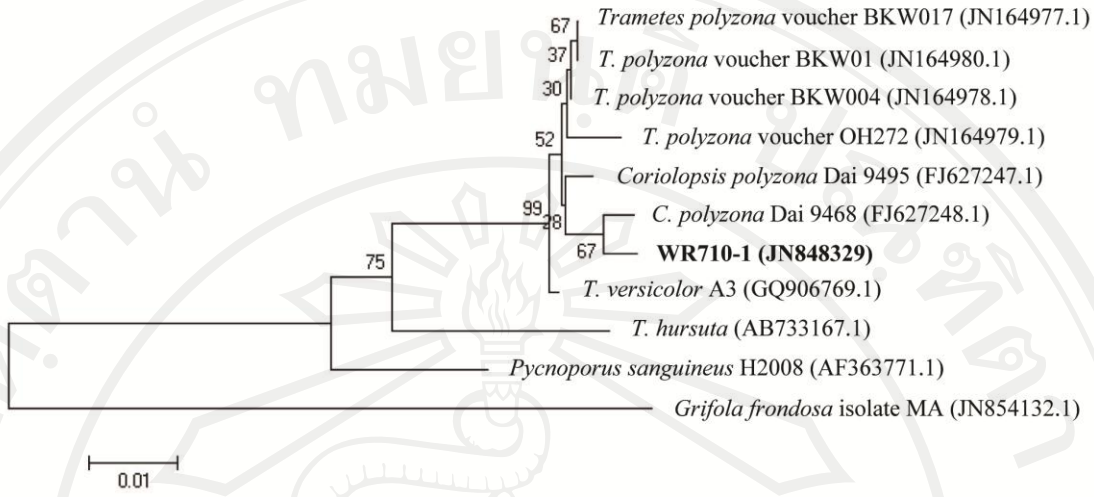


Fig. 3.3 Neighbor-joining phylogenetic tree showing the position of *T. polyzona* WR710-1 based on sequences of ITS region.

The numbers at the branching points are the percentages of occurrence in 1000 bootstrapped tree. Bar indicates 1 nucleotide substitution per 100 nucleotides.

3.3.3 Taxonomic characterization of *Trametes polyzona* WR710-1

White-rot fungus isolate WR710-1 was identified to *T. polyzona* which belonged to the Basidiomycetes, Family *Polyporaceae*, Genus *Trametes*. The phenotype of young fresh sample was flat to globular shape with white color, various sizes 1.5×1.6 to 3×4.2 cm (Fig. 3.4). Mature basidiocarp or fruiting body was up to 15 cm wide, common on dead trunks, opten in exposed situations. The upper surface is light to cinnamon-brown, lower surface creamy (Fig. 3.5).

Fig. 3.4 The sample of *T. polyzona* WR 710-1.

Trametes polyzona produce basidiocarps annual to biennial, sometimes reflexed, commonly broadly attached, less often dimidiate, solitary or imbricate, single pilei flabelliform to reniform, up to 10 cm wide and 15 cm long, 2-7 mm thick at the base, coriaceous and flexible to corky; pilear surface yellowish-ochraceous when fresh, soon darker, fulvous, ochraceous-brown or greyish-brown, in old specimens frequently with green tints due to algae, tomentose to slightly hispid in numerous sulcate to flat, concentric zones, tomentum 1-3 mm thick, margin thin, flat to undulating, often lobed and incised; pore surface cream to beige when fresh, darkens to golden-brown or fulvous, pores round to angular, on average 2-3 per mm, on oblique stibstrates somewhat elongated radially and up to 1 mm long, tubes concolorous with the pore surface, in section often lighter than the trama, up to 4 mm long, sometimes stratified; context ochraceous to golden- brown, darker towards the base, duplex, lower part fibrous and semiglossy in section, upper part loose and more faded, in old specimens it may become greyish-brown to dark brown, the two parts usually easy to distinguish in sections and sometimes with a separating thin black line, lower part up to 3 mm thick. Basidiocarps may persist from one rainy season to the nest one. Distribution in Asia; China, Japan, Taiwan, northern Thailand and Vietnam (Núñez and Ryvarden, 2001).

Fig. 3.5 Mature basidiocarp of *T. polyzona* (Copyright ©2010 Danny Newman)**.**

Mycelial colonies of *T. polyzona* on PDA medium were off-white, showing high density, velvety texture, and abundant aerial hyphae (Fig. 3.6a). The growth rate of *T. polyzona* WR 710-1 completely colonized on Petri dish in 5 days at 37°C. The light micrograph under compound microscrope was shown in Fig. 3.6b, the fungus were generative hyphae with clamp connections, thin-walled and 1.5-2.5 µm wide.

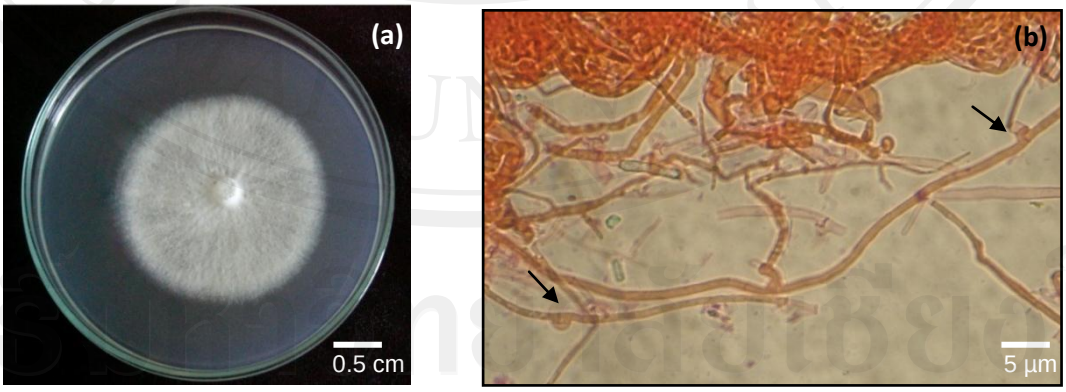
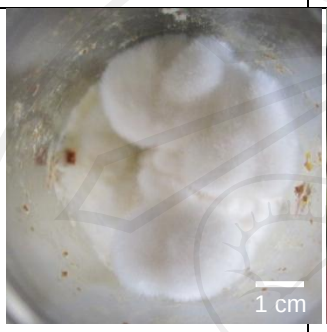
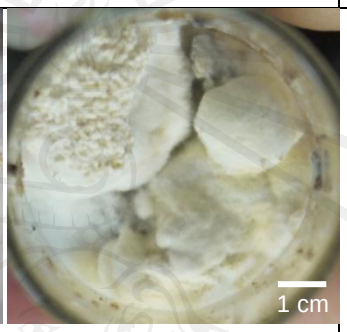
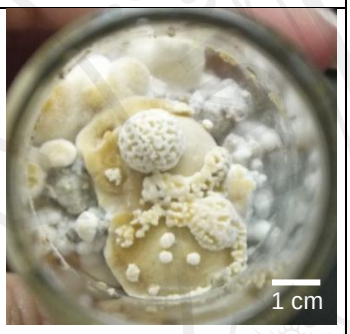


Fig. 3.6 *T. polyzona* WR710-1, (**a**) colony morphology and (**b**) light micrograph with clamps connection (arrow point) grown on PDA for 3 days at 37°C.

3.3.4 Production of fruiting body of white-rot fungus

Orange peel was used as solid substrate and distilled water, basal and MYG solution were used as liquid solution for white-rot fruiting body production. After 40 days at 28^{o}C, 12/12 hr light/dark and static condition, white-rot fungus *T. polyzona* form white global mycelium obviously presented in MYG solution (Table 3.3). White-rot fungi produced white fluffy cotton wool-like growth, usually found under humid conditions (Coleman, 2005). About 60th day incubation, *T. polyzona* grew on orange peel with basal and MYG solution form structure like pore on the top of white rot mycelium. After approximately 85 days, white rot turned to brown colored and then it wilted, died and decay. White-rot fungus was difficult generated to macro structure or fruiting body. Fruiting body formation of white-rot fungi was not so successful because physiological and development process were unclear, in addition, humidity and aeration also affected fruiting body formation (Lin *et al*., 2006).

Table 3.3 Fruiting body of *T. polyzona* WR710-1 produced under solid state fermentation with different liquid solution

Time (day)	Liquid solution in solid state fermentation (60% v/w)		
	Distilled water	Basal solution	MYG
40			
85