APPENDICES

APPENDIX A

CULTURE MEDIA

All media were prepared in 1000 ml of distilled water and sterilized in autoclave at 121^{o}C for 15 min.

Basal media (Mikiashvili *et al.*, 2006)

KH_2PO_4	1	g
$MgSO_4.7H_2O$	0.5	g
NH_4NO_3	0.2	g
Yeast extract	0.1	g
$CaCl_2.2H_2O$	0.01	g
$CuSO_4.5H_2O$	0.001	g
$MnSO_4.H_2O$	0.001	g
$Fe_2(SO_4)_3$	0.001	g
pH	6.0	

Lignin modifying enzyme basal medium (LBM) (Pointing, 1999)

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
Poly R-478 dye	0.02	g
Agar	16	g
pH	6.0	

Malt-Yeast (MY) agar (Yu *et al*, 2009)

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	20	g
Agar	20	g

Malt Yeast Glucose solution (MYG) (Tanabe *et al*., 1989)

Malt extract	10	g
Yeast extract	4	g
Glucose	4	g

Mineral salt solution (Jianmin *et al*., 2008)

$(NH_4)_2.SO_4$	3.5	g
KH_2PO_4	3	g
$MgSO_4.7H_2O$	0.5	g
$CaCl_2.2H_2O$	0.5	g
pH	6.0	

Modified Mineral salt solution (This study)

Peptone	8.9	g
KH_2PO_4	4.6	g
$MgSO_4.7H_2O$	0.9	g
$CaCl_2.2H_2O$	0.7	g
pH	6.0	

Potato dextrose agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g

APPENDIX B

THE CHEMICAL ANALYSIS OF LIGNOCELLULOSIC BIOMASS

Measurement of holocellulose in lignocelluloses substrates (Browing, 1967)

Holocellulose is the total polysaccharide fraction of wood, straw, and so on, that is composed of cellulose and hemicelluloses. It is obtained when the extractives and the lignin are removed from the natural material.

Reagents

- Gracial acetic acid (CH_3COOH)
- Sodium Chlorite ($NaClO_2$)
- Acetone

Procedure

Substrates were ground into small size (0.1 mm) with a hammer mill (Christy and Norris Ltd.). Modified acid chlorite method of Browing (1967) was used to measure holocellulose in the biomass. Dried substrates 1.5 g was add into flask 250 ml that contained 80 ml of distilled water and 0.5 ml of CH_3COOH then 1.5 g of $NaClO_2$ was add to test flasks. The samples were incubated in bath at 70-80^oC for 1 hr, and mixed every 15 min. This method was done three times, total incubation time 4 hr. The test flasks were moved to iced bath at temperature about 10^oC, suspension was transferred to a filtering funnel and the sample was washed by iced distilled water and acetone. Dried the holocellulose samples in an oven at 105^oC, cool in a desiccators and weigh measured.

For each determination, calculated the holocellulose content in the test specimen as follows:

Holocellulose (%) = $A \times 100 / W$

Where, *A* is weight of dried holocellulose (g) and *W* is oven-dry weight of test specimen (g).

Measurement of alpha-cellulose in pulp (T203 om-88, TAPPI 1988*****)

Pulp is extracted consecultively with 17.5 and 9.45% of sodium hydroxide solution at test condition, 25^{o}C. The soluble fraction, consisting of beta- and gamma-cellulose would separated from insoluble alpha-cellulose.

Reagents

- Sodium hydroxide solution, 17.5%(w/v) of NaOH
- Acetic acid 10% (v/v)

Procedure

Place the holocellulose sample from above 0.75 g in flask 250 ml and add 100 ml of sodium hydroxide solution, mixed and incubated in a bath at 25^{o}C. After a period of 30 min from the first addition of the NaOH reagent, add 100 ml of distilled water at 25^{o}C. Leave the flask in the bath for 30 min then suspension was transferred to a filtering funnel and the alpha-cellulose sample was washed by distilled water and 10% (v/v) of acetic acid until pH around 7.0. Dried the alpha cellulose sample in an oven at 105^{o}C, cool in a desiccators and weigh measured.

For each determination, calculated the alpha cellulose content in the holocellulose sample as follows:

Alpha cellulose (%) = $A \times 100 / H$

Where, A is weight of dried alpha cellulose (g) and H is weight of dried holocellulose (g).

Measurement of lignin in lignocelluloses substrates (T222 om-88, TAPPI 1988*)

Lignin represents what is called the "incrusting material" forming a part of the cell wall and middle lamella in wood. In this method of determination, lignin (also known as "Klason lignin") is defined as a wood or pulp constituent insoluble in 72 % sulfuric acid.

Main reagent

- Sulfuric acid 72 % (v/v)

Procedure

The carbohydrates in lignocellulosic biomass are hydrolyzed and solubilized sulfuric acid but the acid-insuluble lignin is filtered off, dried, and weighed. Lignocelluloses substrate 0.5 g was added into flask 250 ml and 72% (v/v) of sulfuric acid, 7.5 ml was added. Stirred and keep the flask in a bath at 2 ^{o}C during dispersion of the material. After the sample is dispersed, cover the flask with glass and keep in a bath at 20^{o}C for 2 hr. Add about 300 to 400 ml of distilled water to dilute the concentration of sulfuric acid to 2% (v/v), total volume of 575 ml. Boil the solution for 4 hr, maintain constant volume either by using a reflux condenser or by frequent addition of hot water. Without stirring up the precipitate, decant or siphon off the supernatant solution through a filtering crucible. Wash the lignin free of acid with hot

water, dried the lignin sample in an oven at 105^{o}C, cool in a desiccators and weigh. For each determination, calculated the lignin content in the test specimen as follows:

Lignin (%) = $A \times 100 / W$

Where, A is weight of lignin (g) and W is oven-dry weight of test specimen (g).

Measurement of ash in lignocelluloses substrates (T211 om-85, TAPPI 1988*)

The ash in wood and pulp is inorganic residue after combustion at a temperature of 575 ±25^{o}C.

Procedure

Heat the empty crucible and cover in muffle furnace at 150^{o}C for 1 hr. Place in a dessicator and cool for 45 min when a porcelain or silica crucible is used. Transfer the lignocelluloses substrate 1 g to the crucible and place it in a muffle furnace at a temperature not higher than about 100^{o}C. Raise the temperature gradually to 575 ^{o}C so that the material becomes carbonized without flaming. Incubated 3 hr to burn away all the carbon, complete ignition is indicated by the absence of black particles. Calculate the ash content as follow:

Ash (%) = $A \times 100 / B$

Where, A is weight of ash (g) and B is weight of test specimen, (g) moisture-free.

* TAPPI; Technical Association of the Pulp and Paper Industry (http://www.tappi.org/s_tappi/index.asp)

APPENDIX C

REDUCING SUGAR ANALYSIS

(Modified from Miller, 1972)

Estimation of reducing sugar by dinitrosalicylic acid (DNS) method

For sugar estimation the DNS method is simple, sensitive and adoptable during of a large number of samples at a time.

Materials

- DNS reagent: dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml of 1% NaOH and store at 4^{o}C. Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.
- 40% Rochelle salt solution (potassium sodium tartrate)

Procedure

- Pipette out 0.5 ml of the sample solution in test tube
- 0.5 ml of DNS reagent was added to the test tube and the contents mixed either by inversion or vortexing
- Place the test tube in boiling water for 5 minutes
- Cool to room temperature.
- The developed color was measured at 540 nm, cuvettes against a reagent blank prepared from 0.5 ml of distilled water and 5 ml of DNS reagent.
- The reducing sugar in sample was determined by using a calibration curve of an glucose or xylose standard

Table C.1 Absorbance at 540 nm of glucose standard at various concentrations

Glucose concentration (mg/ml)	Absorbance at 540 nm
0	0
0.1	0.144
0.2	0.214
0.3	0.310
0.4	0.429
0.5	0.527
0.6	0.625
0.7	0.738
0.8	0.858
0.9	0.929
1	1.089

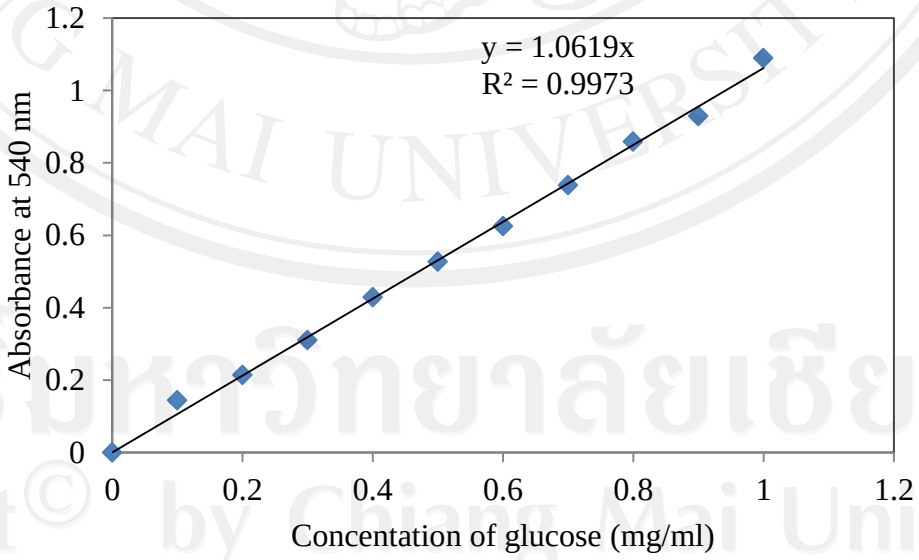

Fig. C.1 Standard curve of glucose concentration against absorbance at 540 nm

Table C.2 Absorbance at 540 nm of xylose standard at various concentrations

Xylose concentration (mg/ml)	Absorbance at 540 nm
0	0
0.1	0.074
0.2	0.224
0.3	0.309
0.4	0.415
0.5	0.483
0.6	0.567
0.7	0.652
0.8	0.738
0.9	0.863
1	0.952

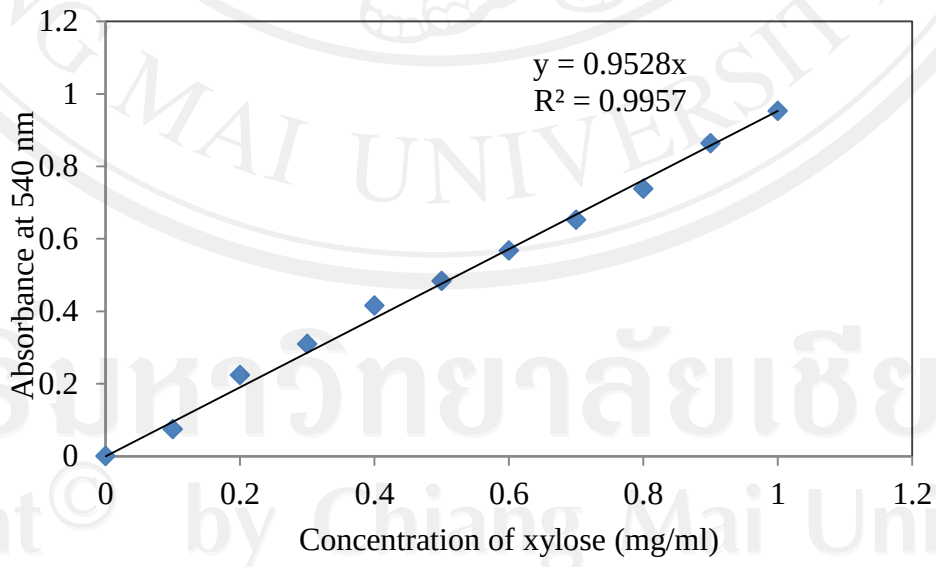

Fig. C.2 Standard curve of xylose concentration against absorbance at 540 nm

APPENDIX D

TOTAL PROTEIN ANALYSIS

(Bradford, 1976)

Estimation of total protein by Bradford method

A rapid determination method which involves the binding of Coomassie Brilliant Blue G-250 to protein. It is the increase in absorption at 595 nm which is monitored.

Reagents

- Protein reagent: Coomassie Brilliant Blue G-250 (100 mg) dissolved in 50 ml of 95% ethanol. Then 100 ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter.
- Standard protein: Bovine serum albumin (BSA) 10 to 100 µg in 0.1 ml of distilled water

Procedure

- Pipette out 0.1 ml of the sample solution in test tube.
- 5 ml of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing
- The developed color was measured at 595 nm after 2 min incubation, cuvettes against a reagent blank prepared from 0.1 ml of buffer and 5 ml of pretein reagent.
- Protein in sample was determined by using a calibration curve of an BSA standard

Table D Absorbance at 595 nm of BSA standard at various concentrations

BSA concentration (µg/ml)	Absorbance at 595 nm
0	0
3	0.038
6	0.169
9	0.238
12	0.305
15	0.380
18	0.437
21	0.559
24	0.659
27	0.717
30	0.789

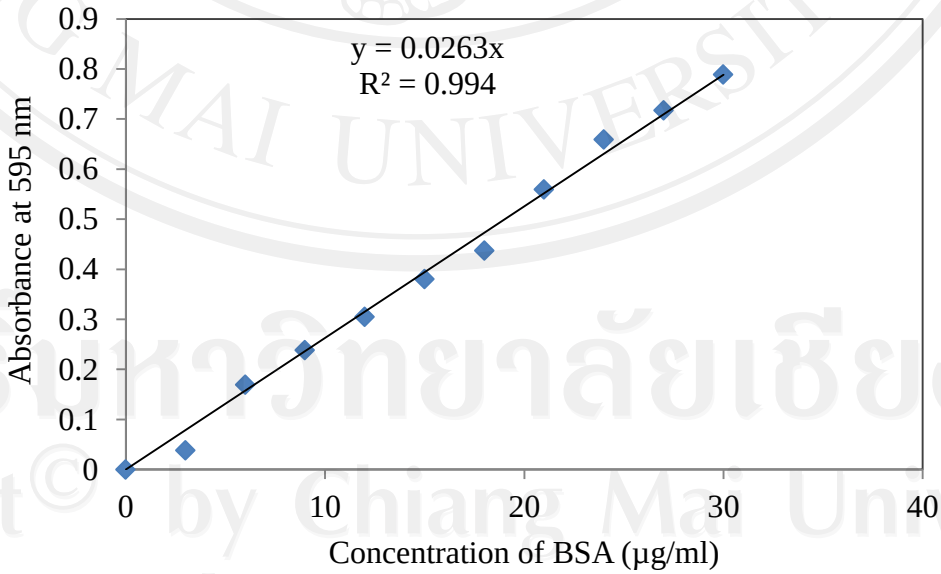

Fig. D Standard curve of BSA concentration against absorbance at 595 nm

APPENDIX E

TOTAL SUGAR ANALYSIS

(Dubois *et al*., 1956)

Estimation of total sugar by phenol-sulfuric acid method

The phenol-sulfuric acid method is a simple and rapid colorimetric method to determine total carbohydrates in a sample. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490 nm.

Reagents

- Phenol 5%: phenol 50 g dissolved in water and distilled to one litre.
- Sulfuric acid 96%
- Standard glucose: stock- 100 mg in 100 ml of water. Working standard- 10 ml of stock diluted to 100 ml with distilled water

Procedure

- Pipette out 0.5 ml of the sample solution in test tube.
- Add 0.5 ml of 5% phenol to test tube
- Add 2.5 ml of 96% sulfuric acid to test tube and shake well.
- After incubated 20 min at 25-30^{o}C, read the color at 490 nm. Calculate the amount of total sugar present in the sample solution using the standard curve.

Absorbance corresponds to 0.5 ml of the test tube = x mg of glucose

100 ml of the sample solution contain = $(x / 0.5) \times 100$ mg of glucose

= % of total sugar present

Table E Absorbance at 490 nm of glucose standard at various concentrations

Glucose concentration (mg/ml)	Absorbance at 490 nm
0	0.000
0.01	0.185
0.02	0.354
0.03	0.525
0.04	0.653
0.05	0.804
0.06	0.983
0.07	1.115
0.08	1.244
0.09	1.402
0.10	1.517

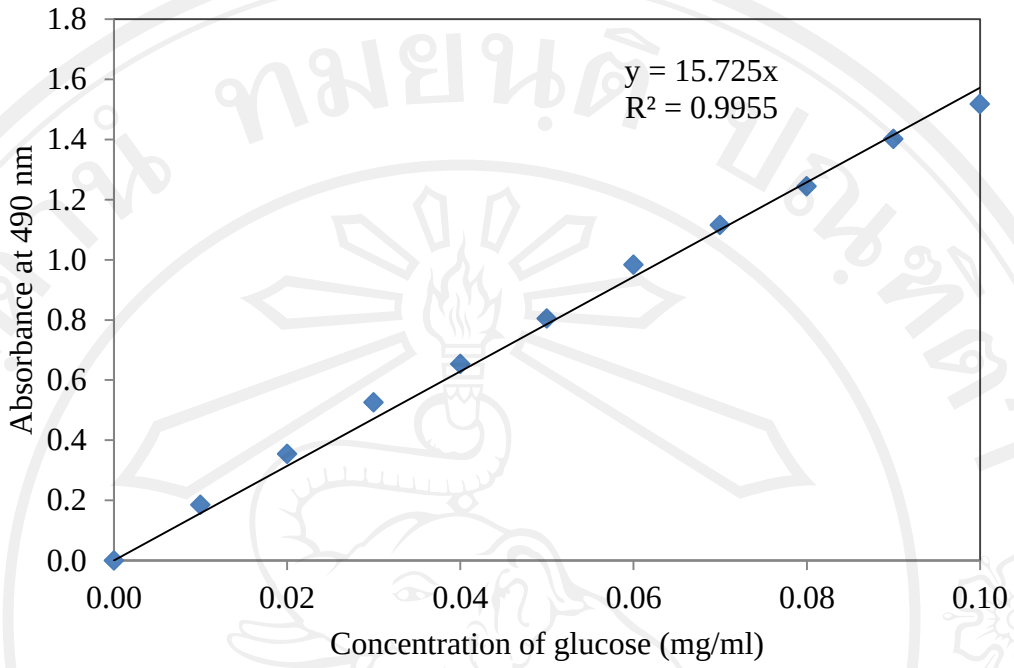

Fig. E Standard curve of glucose concentration against absorbance at 490 nm

APPENDIX F

ETHANOL DETERMINATION

Estimation of ethanol concentration by gas chromatography (GC)

Gas chromatography (Konik HRGC 4000 B) was used in this study.

Reagents

- One percent (v/v) of butanol was used as internal standard
- Ethanol standards: Five ethanol standards were prepared by diluting absolute ethanol with distilled water to cover the range 0.2 to 1% (v/v) ethanol.

Procedure

- Pipette out 10 µl of sample solution in microcentrifuge tube.
- Add 10 µl of 1% (v/v) of butanol to sample tube and mix well.
- Inject mix sample into gas chromatography, read the peak areas of ethanol and butanol.
- The peak area ratio between ethanol in sample and butanol (internal standard) and ethanol concentration (%v/v) were determined
- Ethanol in sample was determined by using a calibration curve of ethanol standard

Table F The peak area ratio between ethanol/butanol and ethanol concentration

Ethanol concentration (%v/v)	the peak area ratio of ethanol/butanol
0	0.000
0.1	0.090
0.2	0.131
0.3	0.181
0.4	0.217
0.5	0.281

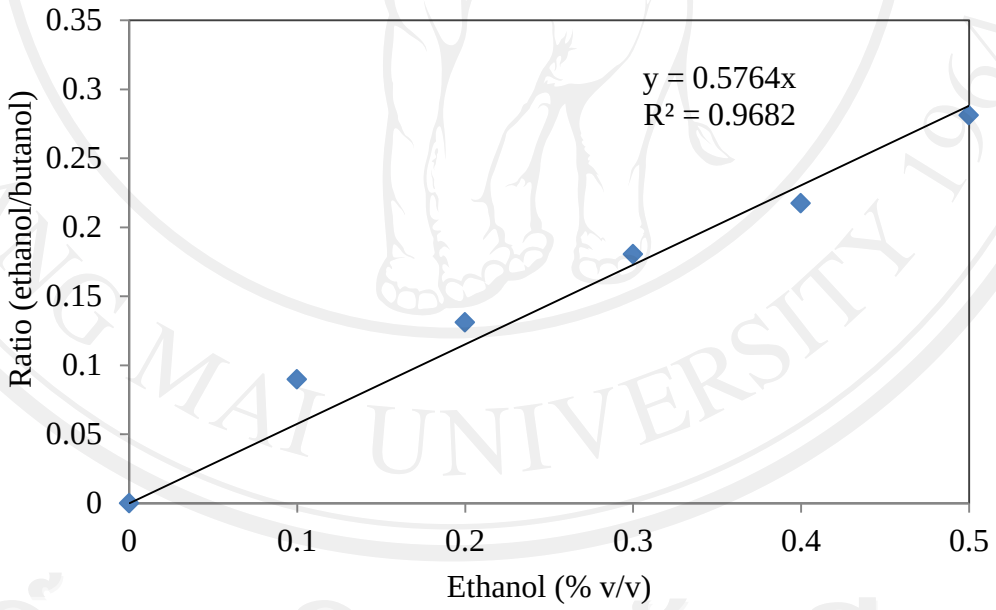

Fig. F Standard curve of ethanol concentration against peak area ratio between ethanol/butanol

APPENDIX G

PHYLOGENITIC TREE ANALYSIS DATA

(Saitou and Nei, 1987)

Phylogenetic tree for sequences of ITS region was constructed using the Neighbor-Joining method. The 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 database of Genbank for comparing with ITS region sequencing data.

Table H Strains examined in this study.

Species names	GenBank accession number	References
Trametes polyzona WR710-1	JN848329.1	This study
Coriolopsis polyzona voucher Dai9495	FJ627247.1	Cui, B.-K.
C. polyzona voucher Dai9468	FJ627248.1	Cui, B.-K.
T. polyzona voucher BKW01	JN164980.1	Justo and Hibbett, 2011
T. polyzona voucher BKW004	JN164978.1	Justo and Hibbett, 2011
T. polyzona voucher BKW017	JN164977.1	Justo and Hibbett, 2011
T. versicolor isolate A3	GQ906769.1	Raval *et al*.
T. polyzona voucher OH272sp	JN164979.1	Justo and Hibbett, 2011
Pycnoporus sanguineus H2008	AF363771.1	Lomascolo *et al*., 2002
Grifola frondosa MAr.Eg	JN854132.1	Mikhail *et al*.

CURRICULUM VITAE

Name Ms. Thanunchanok Chairin

Date of birth March 27th, 1981

Education

1999-2003: B.Sc. (Agriculture) 2nd Class Honor
Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, THAILAND

2003-2005: M.S. (Agriculture)
Department of Agricultural Extension, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, THAILAND

2007-2013: Ph.D. candidate (Biotechnology)
Biotechnology program, Faculty of Graduate School, Chiang Mai University, Chiang Mai 50200, THAILAND

Academic presentations

1. **T. Chairin,** C. Khanongnuch, and S. Lumyong. 2009. Mixture design of agricultural waste substrates for laccase production from white rot fungus. The III International Conference on Environment, Industrial, and Applied Microbiology (BioMicroWorld 2009). December 2-4, University of Lisboua, Lisbon, PORTUGAL

2. **T. Chairin,** C. Khanongnuch, and S. Lumyong. 2012. The combination of agricultural wastes in solid state fermentation, application of mixture design experiment for xylanase production by *Thermoascus aurantiacus* SL16W. The First Asean Plus Three Graduate Research Congress, The First Forum of the Deans of ASEAN Plus Three Graduate Schools (AGRC 2012). March 1-2, The Empress Convention Center, The Empress Hotel, Chiang Mai, THAILAND.

Publications

1. **T. Chairin,** C. Khanongnuch, and S. Lumyong. (2009). Mixture design of agricultural waste substrates for laccase production from white rot fungus. *Proceeding Book of The III International Conference on Environment, Industrial, and Applied Microbiology*. 328-332.

2. **T. Chairin,** C. Khanongnuch, and S. Lumyong. (2012). The combination of agricultural wastes in solid state fermentation, application of mixture design experiment for xylanase production by *Thermoascus aurantiacus* SL16W. *Proceeding Book of The First Asean Plus Three Graduate Research Congress*. 758-763.

3. **T. Chairin**, T. Nitheranont, A. Watanabe, Y. Asada, C. Khanongnuch and S. Lumyong. (2013). Purification and characterization of the extracellular laccase produced from *Trametes polyzona* WR710-1 under solid state fermentation. *Journal of Basic Microbiology*. (In press)

4. **T. Chairin**, T. Nitheranont, A. Watanabe, Y. Asada, C. Khanongnuch and S. Lumyong. (2013). Biodegradation of bisphenol A and decolorization of synthetic dyes by laccase from white-rot fungus, *Trametes polyzon*a. *Applied Biochemical and Biotechnology*. 169: 539-545.