CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and chemical reagents

3.1.1 Media and chemical reagents

List of chemical reagents	Production companies
1, 10-phenanthroline monohydrate	MERCK
2, 6-Dimethoxyphynol	ALDRICH
Acetic acid, glacial	SCHARLAU
Agar	MERCK
Commercial textile dye (red)	Mang Korn Thong, THAILAND
Ferrous sulfate heptahydrate	MERCK
Ferrous ammonium sulfate	MERCK
Fetal bovine serum	GIBCO
Glucose	FLUKA
Polyurethane foam	-
Potassium dichromate	MERCK
Potato dextrose agar	-
Potato dextrose broth	-
Silver sulfate	MERCK
Sodium acetate trihydrate	MERCK
Sodium hydroxide	MERCK
Sulfuric acid	MERCK

3.1.2 Materials

List of materials	Production companies
Coconut husk	-
Luffa sponge	-
Nylon sponge	UNI-TOP TRADING
Stainless steel sponge	UNI-TOP TRADING

3.1.3 Equipment

List of equipment	Production companies
Analytical balance	PERCISA
Aquarium air pump	-
Autoclave	IWAKI ACV-3167
Carbondioxide incubator	SHEL LAB
Hot air oven	MEMMERT
Incubator shaker	KUHNER
Laminar air flow cabinet	LABCONCO
Microcentrifuge	EPPENDROFF
Microscope	OLYMPUS
Microwave	SHARP Model R-242
pH meter	CONSORT C830
Spectrophotometer	SPECTRONIC 20 GENESYS
UV/VIS Spectrophotometer	JASCO V-530
Vortex-2 Genie	BOHEMIA

3.2 Methods

3.2.1 Fungal strain and inoculum preparation

The thermotolerant white rot fungus *C. versicolor* RC3, isolated from Chiang Mai, Thailand by Khanongnuch *et al*. (2004), was used through all experiments and maintained on PDA (Appendix A) for 4 days. For free cell experiment, the inoculum was prepared by culturing one agar plug of mycelium, 1 cm diameter, in 100 ml of PDB (Appendix A) and incubated on 120 rpm of rotating shaker at 37^{O}C for 4 days. In case of immobilized cell, one agar plug of mycelium was inoculated on support placed in 100 ml of PDB and incubated at 37^{O}C for 4 days.

3.2.2 Investigation of suitable support

The experimental conditions for investigation of suitable support are as follow:

3.2.2.1 Prepared polyurethane foam, nylon sponge, stainless steel sponge, coconut husk and luffa sponge of 1, 8, 9, 3 and 1.5 g, respectively in 50 ml of PDB.

3.2.2.2 Inoculated with 1 agar plug of *C. versicolor* RC3 mycelium.

3.2.2.3 Incubated on 120 rpm of rotating shaker at 37^{O}C for 4 days.

3.2.2.4 Drained all of medium and added 150 ml of 150 ppm of commercial textile dye

3.2.2.5 Incubated on 120 rpm of rotating shaker at 37^{O}C for 24 hours

3.2.2.6 Sampling and determination for decolorization (%). The samples were centrifuged at 13000 rpm for 2 min and the supernatant was measured by spectrophotometer. Scanning for wavelength spectrum was performed between 200 and 800 nm. Distilled water was used as reference. The maximum peak of absorbance was found at 495 nm for commercial textile dye. Decolorization was determined as the relative decrease of absorbance at their absorbance maxima. Laccase activity

was determined by oxidation of 2,6-Dimethoxyphynol (DMP), which was monitored with an increasing in absorbance at 470 nm (Appendix B). The pH meter was used to measure the pH of the dye.

3.2.2.7 Drained all of dye and added 150 ml of fresh dye and repeated step 3.2.2.5 again until decolorization (%) was observed lower than 90%.

3.2.3 Investigation of dye adsorption on fungal mycelium

Decolorization of dye was determined as relative decrease of absorbance for dye at the absorbance maxima. In an attempt to solubilize any bound dye, the immobilized fungus was homogenized in methanol and the homogenate was centrifuge and absorbance of the supernatant was then determined (Yesilada *et al*., 2003). Mycelium of immobilized fungus cell before and after decolorization was determined by scanning electron microscope.

The effects of heat-killed and cell-washed on dye decolorization were also studied. Heat-killed immobilized fungus was prepared by autoclave 4 days old immobilized fungus at 121^{O}C, 15 minutes and used as inoculum for dye decolorization. While cell-washed was prepared by washed immobilized fungus cell in distilled water 3 times and used as inoculum for dye decolorization.

3.2.4 Investigation of inoculum size and dye concentration

Experiments were performed by using 250 ml shake flask. Flasks were prepared in triplicates and contained 150 ml of commercial textile dye in varied concentration of 150, 500, 750 and 1000 ppm, respectively. Dye solution was autoclave at 121^{o}C, 15 minutes before used. Immobilized *C. versicolor* RC3 on suitable support was used as inoculum in varies inoculum size of 6, 8 and 10%(v/v), respectively. The cultures were incubated on 120 rpm of rotating shaker at 37^{O}C for 24 h sampling and determined decolorization (%), pH and laccase activity every 3 hour.

3.2.5 Decolorization of commercial dye using free cell in 10 litres air bubble bioreactor

Seven and a half litres of dye was filled in 10 liters bioreactor and autoclave at 121^{o}C, 15 minutes. A half litres of fungal cell culture was used as inoculum and supplemented with 0.5 vvm of aeration rate by aquarium air pump with 0.2 µm air filter at room temperature (approximately 28-30^{O}C). Sampling and determination decolorized (%), pH and laccase activity every 2 hours until 24 hours.

3.2.6 Decolorization of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor

The experiment conditions were prepared as same as the topic 3.2.5 except the innoculum. The immobilized cell on suitable support was used as inoculum.

3.2.7 Decolorization of commercial dye using immobilized cell with repeated batch system

The experimental conditions for decolorization of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor with repeated batch system are as follow:

3.2.7.1 Prepared 7.5 litres of dye and 0.5 litres of immobilized fungal culture on suitable support. Supplemented with 0.5 vvm of aeration rate by aquarium pump at room temperature (approximately 28-30^{O}C)

3.2.7.2 Sampling and determination decolorization (%), pH and laccase activity until decolorization was obtained more than 90%

3.2.7.3 Drained 50% of dye volume from the bioreactor and added 50% volume of fresh dye, respectively and repeated the previous step again until decolorization was found lower than 90% in 24 hours.

3.2.7.4 Varied dye removal (%) from 25, 50 and 75%, respectively.

3.2.8 Decolorization of commercial dye using immobilized cell with repeated batch system in non-sterile condition

The experiment conditions were prepared as same as the topic 3.2.6 all material was prepared under non-sterile conditions such as dye solution bioreactor, and aquarium air pump except inoculum.

3.2.9 Effect of sugar addition on decolorization

This experiment was studied as follow:

3.2.9.1 Seven and a half litre of synthetic wastewater and 0.5 liters of inoculum were filled in 10 liters air bubble bioreactor and supplemented with 0.5 vvm of aeration rate by aquarium pump.

3.2.9.2 Sampling and determination decolorization (%), pH, laccase activity, and total sugar (Appendix C) until %decoloriation was more than 90% in 24 h.

3.2.9.3 Drained 50% of dye volume from the reactor and added 50% volume of fresh dye solution. Repeated the previous step again until decolorization was found lower than 90%.

3.2.9.4 If the total sugar was lower than 0.03 g/l the various sugars such as glucose, sucrose and molasses was added in the reactor.

3.2.10 Study on decolorization of real wastewater from Batik factory

3.2.10.1 Characterization of real wastewater

The peak absorbance was measured on spectrophotometer. Scanning was performed between 300-700 nm. Moreover, absorbance, pH, COD (Appendix E), total solid suspended, total plate count (Appendix D) and color of real wastewater were examined before and after decolorization.

3.2.10.2 Decolorization of real wastewater by immobilized *C. versicolor* RC3 on PUF with repeated batch in 10 liters air bubble bioreactor

The experiment conditions were prepared as same as the topic 3.2.10, but the commercial dye was replaced by real wastewater.

3.2.10.3 Decolorization of real wastewater by immobilized *C. versicolor* RC3 on PUF with repeated batch in 50 liters air bubble bioreactor

Prepared 45 liters of real wastewater and 3 liters of inoculum in 50 liters air bubble bioreactor and supplemented with 0.5 vvm of aeration rate by aquarium pump. Sampling and determination decolorization (%), pH, and laccase activity every 6 hour.

3.2.11 Toxicity test of dye before and after decolorization

Dye solution before and after decolorizations were prepared to study the toxicity. *Daphnia magna*, aquatic invertebrate, was used as bioassay as described in Appendix F compare with *Ex vivo* assay for cytotoxic activity (Appendix G). Results were evaluated on the basis of immobilization percentage obtained by dividing the number of immortality animals by total animals. The toxicity of wastewater samples was indicated as toxic when the immortality percent is higher than 50% (Selcuk, 2005).