CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and chemical reagents

3.1.1 Media and chemical reagents

List of chemical reagents	Production companies
Acetic acid, glacial	SCHARLAU
Agar	HELICOPTER
Ammonium chloride	CARLO ERBA
di-Ammonium hydrogen citrate ($C_6H_{14}N_2O_7$)	UNILAB
Ammonium nitrate	MERCK
Ammonium oxalate monohydrate ($C_2H_8N_2O_4 \cdot H_2O$)	BAKER
Ammonium phosphate monobasic	CARLO ERBA
Ammonium sulfate	SCHARLAU
Ammonium tartrate ($C_4H_{12}N_2O_6$)	SIGMA
Calcium chloride dihydrate	CARLO ERBA
Copper (II) sulfate	CARLO ERBA
2,6-Dimethoxyphynol	ALDRICH
Ferrous sulfate heptahydrate	MERCK
Glucose	FLUKA
Hydrochloric acid	MERCK
Hydrogen peroxide	CARLO ERBA
Malt extract	MERCK
Magnesium sulfate	UNIVA
Manganese (II) sulfate monohydrate	SCHARLAU
Orange II	SIGMA
Peptone casein	SCHARLAU

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List of chemical reagents	Production companies
Polyurethane foam	Charoenpan Intergroup, Ltd.
Potassium dihydrogen phosphate	MERCK
Sodium acetate trihydrate	MERCK
Sodium hydroxide	MERCK
Veratryl alcohol	SIGMA
Yeast extract	SCHARLAU
Zinc sulfate heptahydrate	MERCK

3.1.2 Equipment

List of equipment	Production companies
Analytical balance	PRECISA
Aquarium air pump	AQUASYSTEM
Autoclave	IWAKI ACV-3167
Autopipette	BIOHIT
Centrifuge	HARMONIC SERIES
Hot air oven	MEMMERT
Hot plate and stirrer	LABINCO Model L334
Incubator shaker	KUHNER
Laminar air flow cabinet	LABCONCO
Microcentrifuge	EPPENDROFF
Microwave	SHARP Model R-242
Peristaltic pump	EYELA MP-1000-H
pH meter	CONSORT C 830
Spectrophotometer	SPECTRONIC 20 GENESYS
UV/VIS Spectrophotometer	JASCO V-530
Vortex-2 Genie	BOHEMIA
Water bath	SHELAB

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3.2 Methods

3.2.1 Batch decolorization

3.2.1.1 Effect of ammonium sources on dye decolorization

From the previous preliminary experiment, ammonium salt was concluded as the most effective nitrogen source for growth and decolorization of *C. versicolor* RC3. Various kind of ammonium salts including ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium phosphate ($NH_4H_2PO_4$), ammonium sulfate ($(NH_4)_2SO_4$), ammonium oxalate ($C_2H_8N_2O_4 \cdot H_2O$), ammonium tartrate ($C_4H_{12}N_2O_6$), ammonium citrate ($C_6H_{14}N_2O_7$), and no addition of ammonium salt were separately included in the modified Kirk's medium (Robinson *et al.*, 2001b), which the medium composition (per litre) are as the following.

NH_4^+	0.04	g
KH_2PO_4	0.20	g
$MgSO_4 \cdot 7H_2O$	0.05	g
$CaCl_2 \cdot 2H_2O$	0.01	g
Trace element solution	10	ml (see Appendix A)
Glucose	10	g
Yeast extract	0.05	g
Orange II	20	mg
pH 6.0		

The experimental conditions for batch experiment are as follow:

- Microorganism: white-rot fungus *Coriolus versicolor* RC3 grown on Malt extract agar (MA; see Appendix A)
- Experimental volume: 80 ml of each experiments in 250 ml Erlenmeyer flask
- Sterilization: autoclaving in 121°C for 15 min
- Inoculation: a plug (1 cm diameter) of the RC3 strain (grown on MA for 3 day) per flask
- Incubation: 120 rpm of rotary shaker and 37°C ambient temperature
- Sampling: 3 ml by aseptic technique at 0, 24, 48, 60, 72 and 84 hours

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For sample analysis, all samples were measured and analyzed for pH measured by pH meter. Decolorization was measured by spectrophotometry at 483 nm (centrifugation at 6,000 rpm for 10 min was necessary for removing of mycelium). Final biomass was measured by dry cell weight determination (filtrated the culture broth through Whatman no.1 filter paper in vacuum condition and dried the filter cake at 105°C for 2 hours before weight measurement). Statistical analysis of F-test and LSD test were performed by the Statistix version *7.0* software and significant different was analyzed at $p < 0.05$.

3.2.1.2 Effect of glucose concentration on dye decolorization

Medium composition used in this experiment was the same as described in 3.2.1.1, but glucose concentration was varied in concentration of 0, 0.5, 1, 5, 10 and 20 g/l. Ammonium salt was supplemented 0.04 g/l from the selected ammonium oxalate and no addition of ammonium salts was used as control experiment. Other procedures were performed with the condition described previously (see 3.2.1.1).

3.2.1.3 Effect of nitrogen concentration on dye decolorization

The medium used was described as 3.2.1.1, except selected glucose concentration was 1 g/l and the selected ammonium oxalate salt was varied with concentration of 0, 0.1, 0.2, 0.5, 1.0 and 2.0 g/l. Other procedures were performed as 3.2.1.1.

3.2.1.4 Effect of initial pH on dye decolorization

The medium compositions were consisted of the same ingredients as 3.2.1.1, but the condition as no addition of ammonium salts with 1 g/l glucose was used in this experiment. The initial pH of the medium was varied at 3.5, 4.5, 5,0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.5, respectively. Other procedures were also performed as 3.2.1.1.

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3.2.1.5 Effect of ambient temperature on dye decolorization

The medium used was a same composition as 3.2.1.4, the initial pH was adjusted to 6.5 and the ambient temperature was varied in 25, 30, 35, 37, 40 and 45°C. Other procedures were also performed as 3.2.1.1.

3.2.1.6 Monitoring of ligninolytic enzymes production during dye decolorization

The medium compositions were consisted of ingredients as described 3.2.1.5 and other procedures were followed to 3.2.1.1.

Ligninolytic activities were assayed by oxidation of 2,6-dimethoxyphynol (DMP) and veratryl alcohol (VA). The reaction mixtures are shown in Table 3.1.

Table 3.1 Reaction mixtures of ligninolytic enzymes assay (µl)

Reagents	Laccase	MIP	MnP	LiP
2 mM DMP	100	100	100	-
2 mM VA	-	-	-	100
50 mM acetate buffer pH 5	500	500	500	500
1 mM H_2O_2	-	100	100	100
1 mM $MnSO_4$	-	-	100	-
Water	300	200	100	200
Enzyme sample	100	100	100	100

Oxidation of DMP and VA were measured at 470 and 310 nm, respectively. One unit of enzyme activity was defined as amount of enzyme that liberated an oxidative products 1 µmole per minute. The calculation was demonstrated as the formula below, molar extinction coefficient of DMP and VA are 49.6 and 9.3 $mM^{-1}cm^{-1}$, respectively (see also in Appendix B).

Ligninolytic activity = (A1 – A0) / εdbt

When A0 = initial absorbance

A1 = final absorbance

ε = molar extinction coefficient ($mM^{-1}cm^{-1}$)

d = dilution ratio

b = light path or cuvet width (cm)

t = time (min)

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3.2.2 Cell immobilization

3.2.2.1 Effect of polyurethane foam volume and incubation time on cell immobilization

1. Medium preparation: 50 ml Malt extract broth (MB; according to Appendix A with out agar) in 250 ml Erlenmeyer flasks were used.
2. Polyurethane foam (PUF) preparation: PUF used (13.75 kg/m^3 density) was cut to about 1 cm^3 (Figure 3.1), then washed in boiled water for 10 min to remove some impurities and dried in 50^{o}C hot air oven over night.
3. PUF were varied in 0, 0.5, 1, 1.5 and 2 g (Figure 3.2) while incubation times were varied in 24, 48, 72, 96, 120, 144 and 168 hours.
4. Sterilization, inoculation and incubation were the same as described in 3.2.1.1.
5. Sampling: colonized PUF were taken and washed by tap water for removing of mobilized cell from PUF.
6. Biomass was measured by dry cell weight determination.

3.2.2.2 Packed bed bioreactor design and configuration

Packed bed bioreactor design was modified from Feijoo *et al.* (1995) as showed in Figure 3.3. The main component is a glass column with 30 cm height and 4 cm of internal diameter. The base of the glass column was filled with 180 g of glass beads (3 mm diameter) and covered by porous Teflon lid. Working volume was 201 ml from 16 cm height of working area and covered again by porous Teflon lid connected to rubber cork with glass rod. The glass column consist of 4 opening end; feed inlet, air inlet, feed outlet and air outlet on the rubber cork. The entire reactor was shown in Figure 3.3. Influent from medium tank and effluent from over flow were controlled by peristaltic pump. Aeration was introduced by air pump. Foam was controlled by foam trapping column. The terminal of aeration line was dipped in 1 N NaOH for contamination control.

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Figure 3.1 Polyurethane foam preparation

Figure 3.2 Variation of polyurethane foam in 50 ml Malt extract broth

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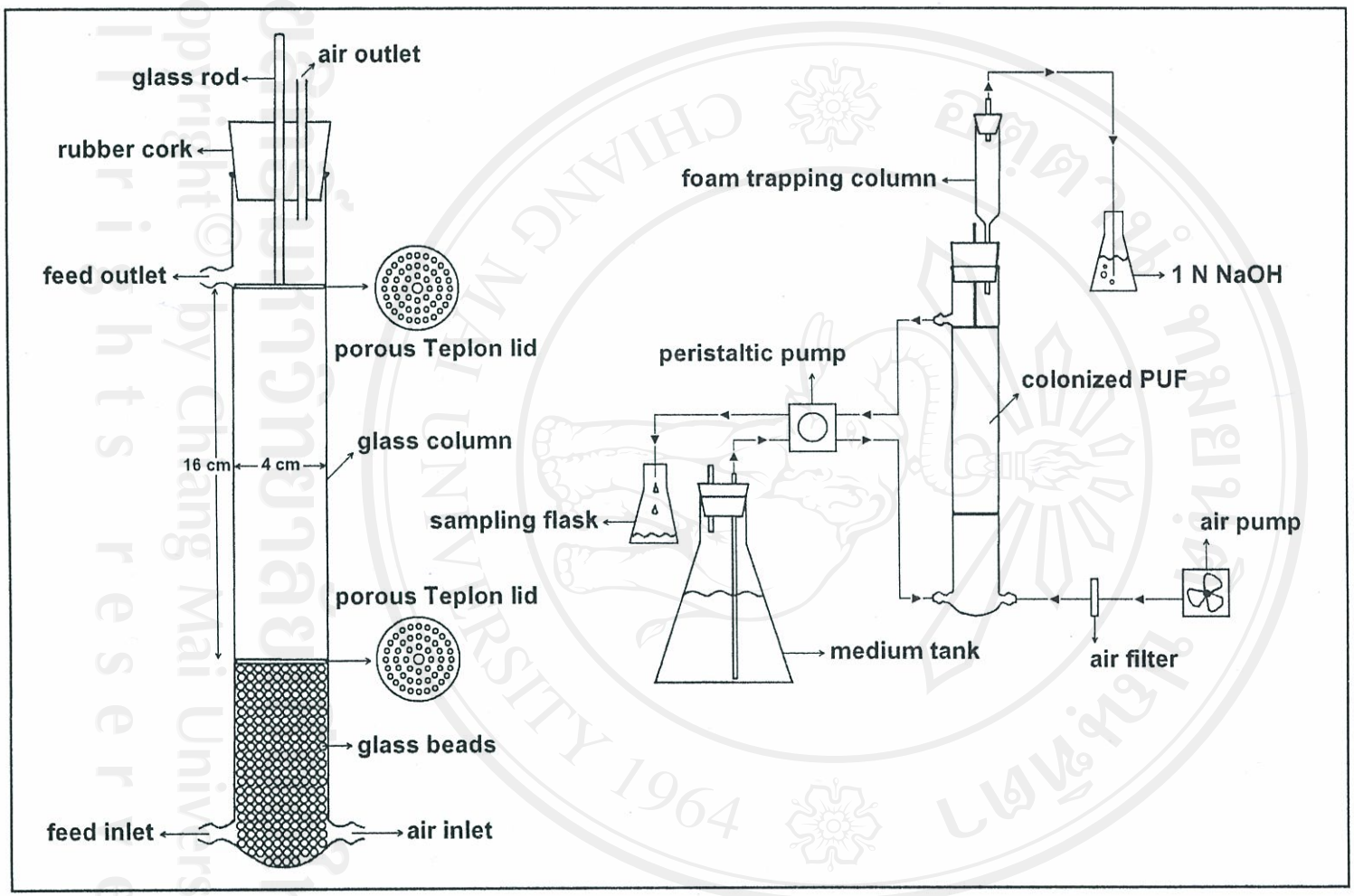

Figure 3.3 Packed bed bioreactor design and configuration

3.2.3 Continuous decolorization

3.2.3.1 Warming-up time of bioreactor

a. A medium composition was modified Kirk's medium prepared as Appendix A.
b. Bioreactor was set as explained in 3.2.2.2 without medium feeding part.
c. Packed the column (201 ml working volume) with fungal colonized PUF (2 g PUF per 100 ml MB incubated with the RC3 strain for 4 day) by aseptic technique.
d. Filled the column with the medium.
e. Incubated in 37°C and 5 vvm of aeration measured by replacing specific volume of water by air in specific time and controlled by manual valve.
f. Samples were take in 3 ml with 1 hour interval until the 90% of decolorization was obtained.
g. Analysis method was following as 3.2.1.1.

3.2.3.2 Effect of hydraulic retention times on dye decolorization

a. Medium composition: follow as described in 3.2.3.1.
b. Bioreactor setting: follow as described in 3.2.2.2.
c. Column packing and incubation condition: follow as described in 3.2.3.1.
d. Warming-up was 5 hours before continuous feeding.
e. Hydraulic retention times (HRT) were varied as Table 3.2.
f. Sampling: over flow sampling.
g. Analysis: follow as described in 3.2.1.1.

3.2.3.3 Effect of polyurethane foam sizes on dye decolorization

This experiment used the same procedure as in 3.2.3.2, but size and amount of PUF used in the reactor were varied as show in Table 3.3. Preparation of PUF in this study is shown in Figure 3.4. The HRT used was 8 hours.

In cell immobilization on 1.5 and 2 cm^3 PUF, a suitable proportion 1:50 (w/v) of PUF and MB was used as described in 3.2.3.1.

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Table 3.2 Flow parameters used in packed bed bioreactor (201 ml working volume)

HRT (hr)	Dilution rate (hr^{-1})	Medium flow rate (ml/hr)	Dye loading rate (g/l/d)
5	0.20	40	0.096
6	0.16	33	0.08
8	0.12	25	0.06
12	0.08	16.5	0.04

Table 3.3 Size and amount of polyurethane foam used in this experiment

Size (cm^3)	Amount (g)
1	2.0
1.5	1.75
2	1.5

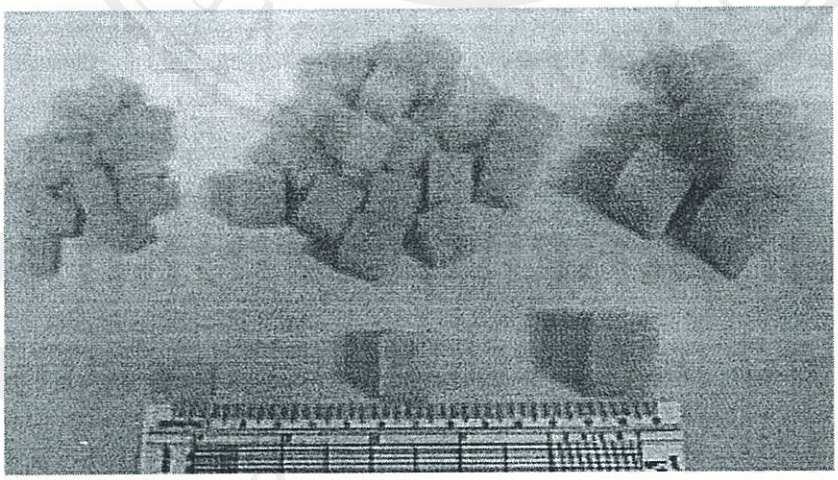

Figure 3.4 Polyurethane foam preparation in different sizes

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3.2.3.4 Effect of dye concentration on dye decolorization

The procedure in the topic 3.2.3.2 was used in this experiment, but Orange II concentration in the medium was varied up to 50, and 100 ppm. The selected condition of 1.75 g of 1.5 cm^3 PUF and 8 hours of HRT was performed. Absolute dye removal measured by using a standard curve (see Appendix C).

Relation of dye concentration under a value of 8 hour HRT and dye loading rate were demonstrated in Table 3.4.

Table 3.4 Dye loading rate in various dye concentration

Dye concentration (ppm)	Dye loading rate (g/l/d)
20	0.06
50	0.15
100	0.3

3.2.4 Decolorization of Batik wastewater

Textile wastewater used in this decolorization study was collected from Batik factory in Lamphun province (provided by Asst. Prof. Dr. Tapana Cheunbarn, Maejo University). The physicochemical properties of wastewater were reddening color with a maximum absorbance at 405 nm and pH was 7.75. Before starting this experiment, filtration of this wastewater through Whatman No. 1 filter papar in vacuum condition was necessary for removing any large solid impurities. After that, separated into two parts, 100% wastewater and 50% diluted wastewater, and varied in several conditions as Table 3.5. Other experimental steps were performed the same as synthetic wastewater as described in 3.2.1.1.

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Table 3.5 Treatments used in Batik wastewater decolorization study

No.	Treatments	Wastewater	Inoculation	Medium addition
1	100C	100%	No	No
2	100I	100%	Yes	No
3	100G	100%	Yes	Glucose 1 g/l
4	100M	100%	Yes	Medium[a]
5	50C	50%	No	No
6	50I	50%	Yes	No
7	50G	50%	Yes	Glucose 1 g/l
8	50M	50%	Yes	Medium[a]

Note: [a] Medium composition (per litre): KH_2PO_4 0.20 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, Trace element solution 10 ml (Appendix A), Glucose 1 g, Yeast extract 0.05 g

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