APPENDIXES

Appendix A: Media

1. Malt extract agar (1 litre)

Malt extract	20	g
Peptone casein	2	g
Glucose	10	g
Agar	15	g
pH 6.0		

2. Modified Kirk's medium (1 litre; used from the topic 3.2.3.1 through out the research)

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
Glucose	1	g
Yeast extract	0.05	g
Orange II	20	mg
pH 5.5 – 5.6 (no adjustment)		

Trace element solution (1 litre) prepared as

$CuSO_4 \cdot 5H_2O$	0.10	g
$FeSO_4 \cdot 7H_2O$	0.10	g
$MnSO_4 \cdot H_2O$	0.10	g
$ZnSO_4 \cdot 7H_2O$	0.10	g

3. Difference in medium composition used in each experiment is explained in Table 6.1.

Table 6.1 Difference of medium composition used in each experiments

Part	Batch decolorization						Immobi-lization		Continuous decolorization			
Topic	3.2.1.1	3.2.1.2	3.2.1.3	3.2.1.4	3.2.1.5[5]	3.2.1.6[6]	3.2.2.1	3.2.2.2	3.2.3.1[7]	3.2.3.2[8]	3.2.3.3[9]	3.2.3.4
NH_4^+ / N-source (g/l)	0.04[1]	0.04[2]	varied[3]	-[4]	-[4]	-[4]	Malt extract broth was used	No medium used	-[4]	-[4]	-[4]	-[4]
KH_2PO_4 (g/l)	0.2	0.2	0.2	0.2	0.2	0.2			0.2	0.2	0.2	0.2
$MgSO_4 \cdot 7H_2O$ (g/l)	0.05	0.05	0.05	0.05	0.05	0.05			0.05	0.05	0.05	0.05
$CaCl_2 \cdot 2H_2O$ (g/l)	0.01	0.01	0.01	0.01	0.01	0.01			0.01	0.01	0.01	0.01
Trace element (ml/l)	10	10	10	10	10	10			10	10	10	10
Glucose (g/l)	10	varied	1	1	1	1			1	1	1	1
Yeast extract (g/l)	0.05	0.05	0.05	0.05	0.05	0.05			0.05	0.05	0.05	0.05
Orange II (mg/l)	20	20	20	20	20	20			20	20	20	varied
pH	6.0	6.0	6.0	varied	6.5	6.5			5.5[10]	5.5[10]	5.5[10]	5.5[10]
Note	[1]varying in NH_4^+ source	[2]NH_4^+ oxalate was used	[3]NH_4^+ oxalate was varied	[4]N-limited condition was used	[5]varying in temperature	[6]ligninolytic enzymes study			[7]warming-up time study	[8]varying in HRT	[9]varying in PUF size	[10]no adjustment

Appendix B: Ligninolytic enzyme assay

1. Acetate buffer preparation (Gomori, 1990)

Stock solution

A: 0.2 M solution of acetic acid (11.55 ml in 1,000 ml)

B: 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na•3H_2O$ in 1,000 ml)

Proportion between A and B

x ml of A + y ml of B, diluted to a total of 100 ml

x	y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.3	30.0	4.8
14.8	**35.2**	**5.0**
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

2. Calculation of enzyme activity

Example 1

In the case of laccase, MIP and MnP, if the initial and final absorbance were 0.000 and 0.016, respectively, molar extinction coefficient (measured by DMP) is 49.6 $mM^{-1}cm^{-1}$, dilution ratio was 1/10, cuvet width was 1 cm and time of measurement was 30 sec. Take each parameters into the formula from 3.2.1.6 and calculate as the following.

Ligninolytic activity = (A1 – A0) / εdbt

when A0 = 0.000

A1 = 0.016

ε = 49.6 $mM^{-1} cm^{-1}$

d = 1/10 or 0.1

b = 1 cm

t = 30 sec or 0.5 min

therefore Ligninolytic activity = (0.016 – 0.000) / (49.6)(0.1)(1)(0.5)

$(mM^{-1} cm^{-1} cm\ min)^{-1}$

= 0.016 / 2.48 mM / min

= 0.00645 (mmol/l) / min

= 0.00645 $(10^3\ \mu mol) / (10^3\ ml\ min)$

= 0.00645 μmol / (min ml) or U/ml

= 6.45 mU/ml

Example 2

In the case of LiP, if the conditions in Example 1 were used and molar extinction coefficient of VA is 9.3 $mM^{-1} cm^{-1}$, the activity can be calculated as:

LiP activity = (0.016 – 0.000) / (9.3)(0.1)(1)(0.5)

$(mM^{-1} cm^{-1} cm\ min)^{-1}$

= 0.016 / 0.465 mM / min

= 0.03441 (mmol/l) / min

= 0.03441 $(10^3\ \mu mol) / (10^3\ ml\ min)$

= 0.03441 μmol / (min ml) or U/ml

= 34.41 mU/ml

3. Transformation of DMP and VA

DMP (2,6-Dimethoxyphenol) was used as a substrate to detect activity level of MnP and laccase. When DMP was oxidized by catalytic activities of those enzymes, DMP would transform to DMQ (2,6-Dimethoxyquinone) as Figure 6.1.

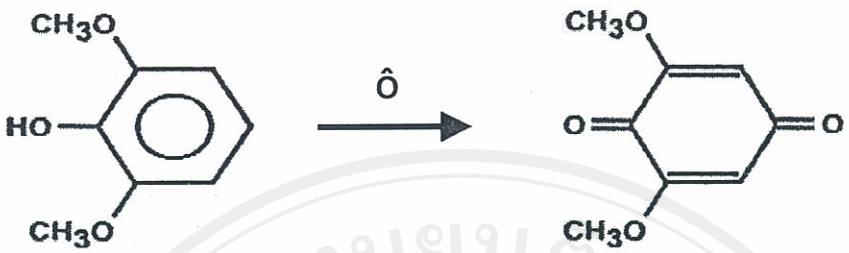


Figure 6.1 Oxidative transformation of DMP

DMQ have a maximum absorbance at 468-470 nm. When it was detected in time scan mode of spectrophotometry after adding of the enzymes, It can indicate the enzyme activities. While VA (Veratryl alcohol or 3,4-Dimethoxybenzyl alcohol) was used as a substrate to detect activity level of LiP. When VA was oxidized by catalytic activities of LiP, VA would transform to veraltraldehyde or 3,4-dimethoxybenzaldehyde as Figure 6.2.

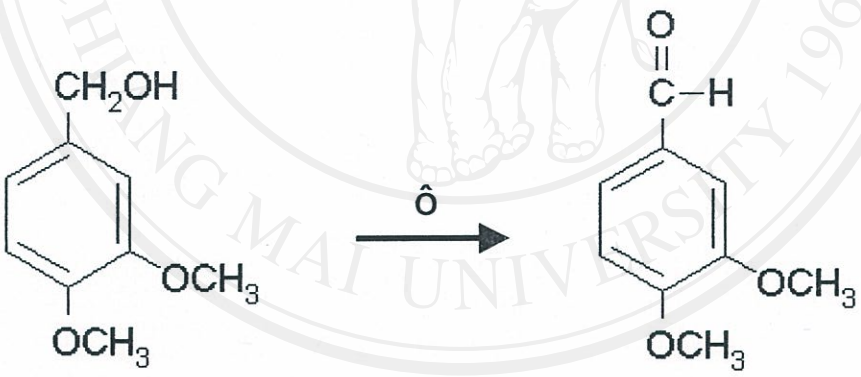


Figure 6.2 Oxidative transformation of veratryl alcohol

In the same way, veratraldehyde have a maximum absorbance at 310 nm. When it was detected in time scan mode of spectrophotometry after adding of the enzymes, It can indicate the enzyme activities.

Appendix C: Standard curve of Orange II concentration

Orange II (Acid orange 7, Acid orange A, or 4-[2-Hydroxy-1-naphthylazo] benzenesulfonic acid sodium salt; $C_{16}H_{11}N_2NaO_4S$) have a maximum adsorbance at 483 nm. When concentration of the dye was varied, we obtain a standard curve as a Table 6.2.

Table 6.2 Absorbances at 483 nm in various Orange II concentration

Orange II concentration (ppm)	Absorbances (483 nm)
0	0
1	0.058
5	0.296
10	0.578
20	1.14
30	1.663
40	2.093
50	2.377

When the above data were plotted, the standard curve of Orange II concentration was obtained as Figure 6.3a. However, Linear relationship between concentration and absorbances was occurred at about only 0-20 ppm of dye concentration (R^2=0.9999; Figure 6.3b) meaning that if Orange II concentrate more than 20 ppm, absorbances from spectrophotometer should be rejected because of decreasing of R^2. If the dye sample give an absorbance more than about 1.2, the sample has to be diluted until an absorbance lower than 1.2 for correcting the data.

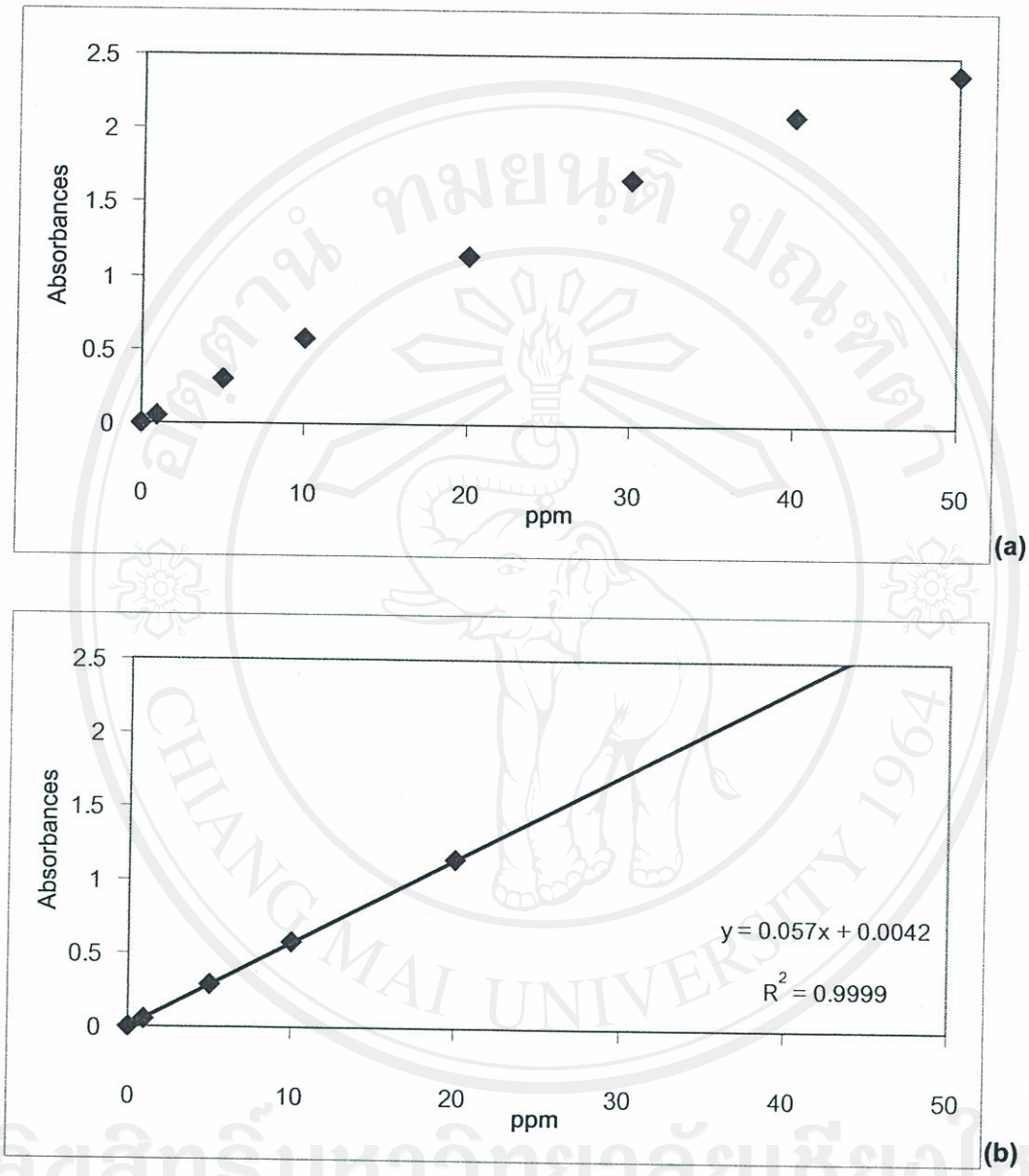


Figure 6.3 Standard curve of Orange II concentration

(a) Original plot

(b) Specified plot at 0-20 ppm

Appendix D: Morphology of *C. versicolor*

C. versicolor look like a tail of turkey thus their common name is turkey tail mushroom as Figure 6.4. While *C. versicolor* RC3 used in this research was collected and isolated from Rukkachat park, Chiang Mai province. Fruiting body and mycelia of the RC3 strain are shown in Figure 6.5.

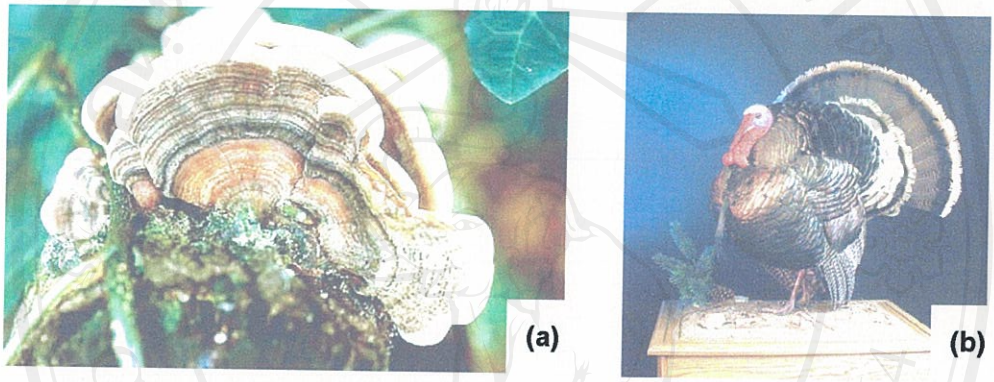


Figure 6.4 Morphology of *Coriolus versicolor*

(a) Fruiting bodies of *C. versicolor*

(b) Comparison with tail of turkey

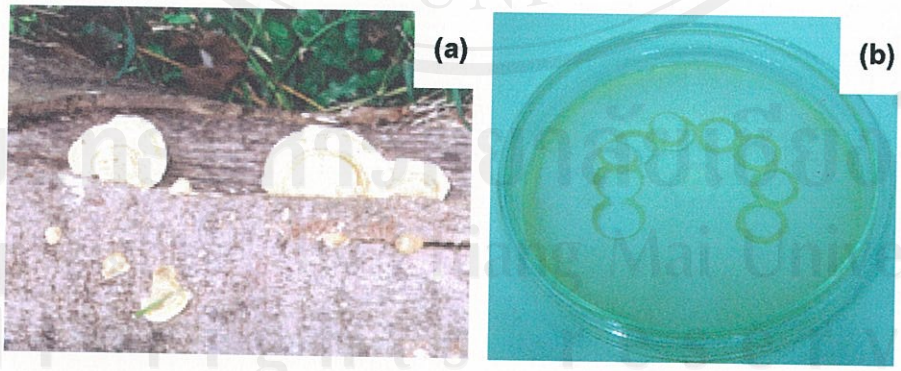


Figure 6.5 Morphology of *Coriolus versicolor* RC3

(a) Fruiting body

(b) Mycelium grown on agar plate

Appendix E: Some scene review of the experiments

Figure 6.6 Batch decolorization study in effect of nitrogen concentration, each flasks showed a different decolorization rate

Figure 6.7 Preliminary study of continuous decolorization using packed bed bioreactor, a good decolorization efficiency could be achieved in room temperature

Figure 6.8 Color comparison between influent, 98% decolorization effluent and pure water from left to right

Figure 6.9 Foam in foam trapping column generated large amount in initial period of cultivation from protein rich broth remaining in immobilized polyurethane foam, the foam would be decreased and stabilized when continuous running time was increased

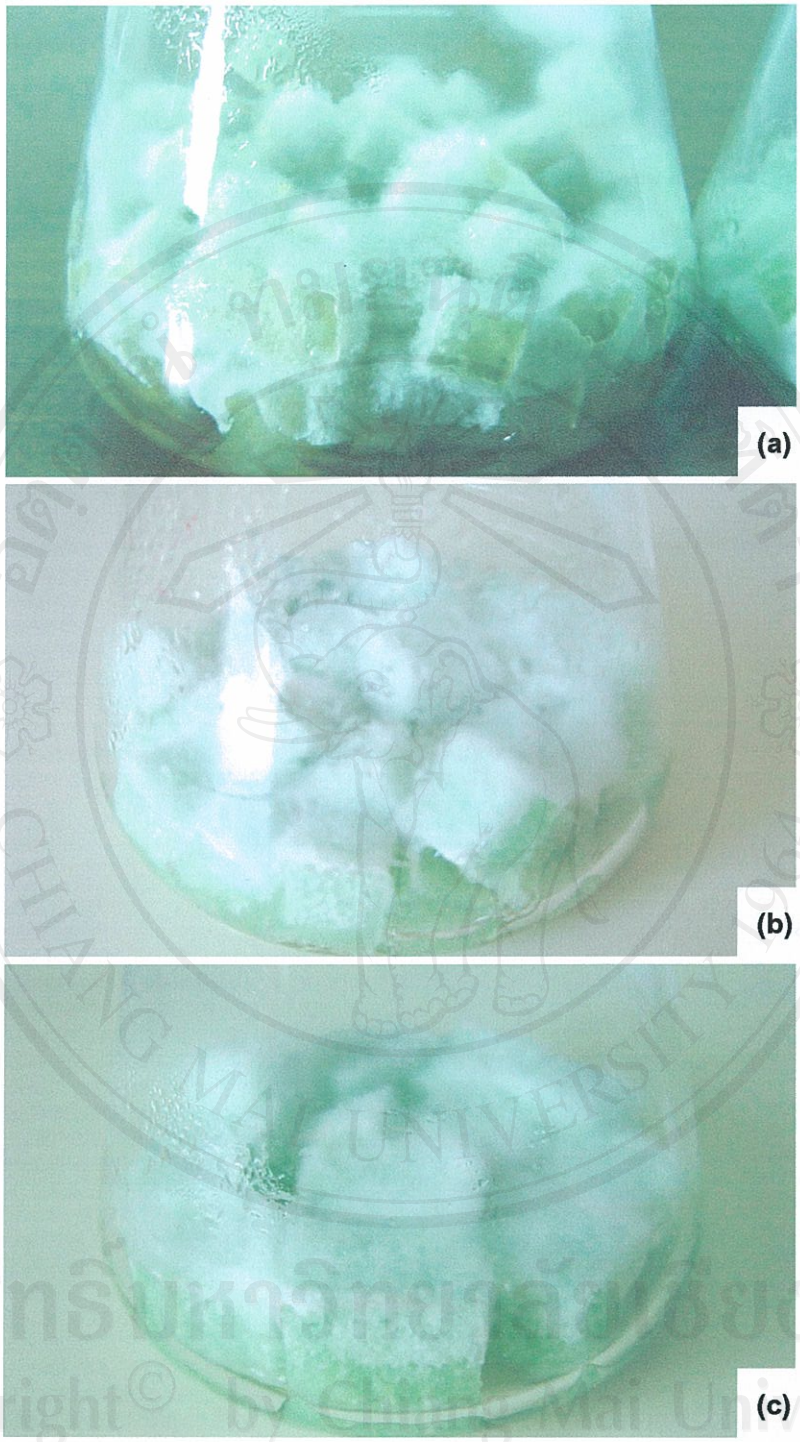


Figure 6.10 Immobilized RC3 strain on various size of PUF

(a) 1 cm^3 PUF

(b) 1.5 cm^3 PUF

(c) 2 cm^3 PUF

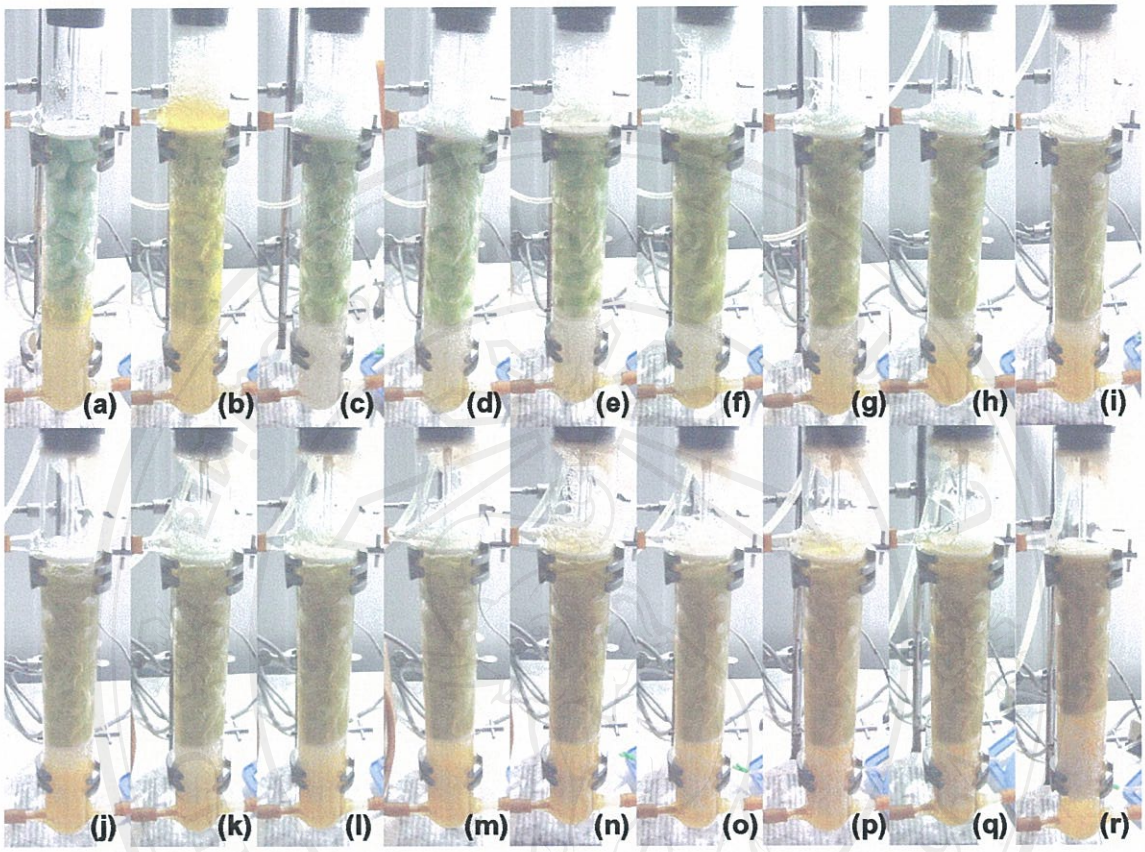


Figure 6.11 Development in the reactor packed with 1.5 cm^3 PUF

(a) Medium feeding after the column was packed by immobilized PUF

(b) The column was filled by the medium and incubated for 5 hours without feeding

(c) After 5 hours of incubation

(d) Beginning of continuous medium feeding

(e) Day 2 of incubation **(f)** Day 3 of incubation

(g) Day 4 of incubation **(h)** Day 5 of incubation

(i) Day 6 of incubation **(j)** Day 7 of incubation

(k) Day 8 of incubation **(l)** Day 9 of incubation

(m) Day 10 of incubation **(n)** Day 11 of incubation

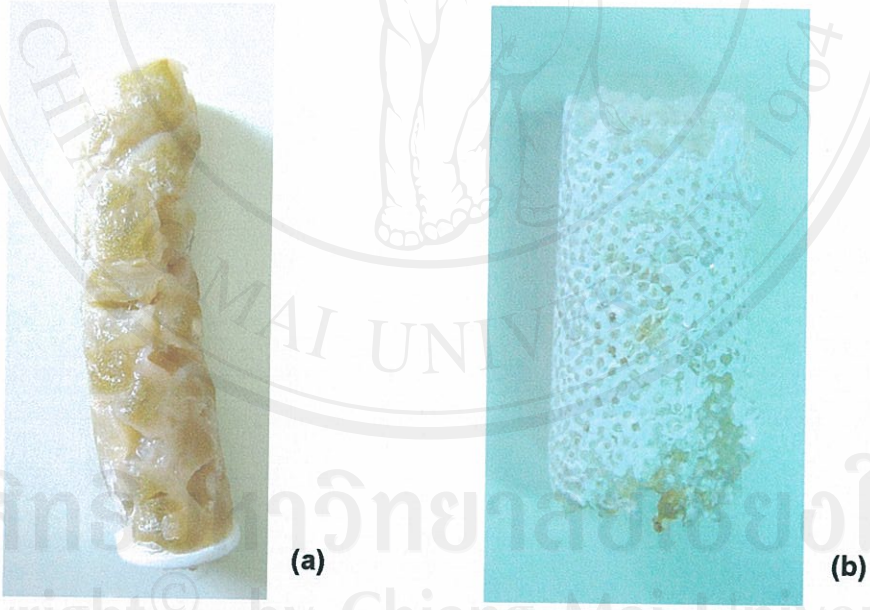
(o) Day 12 of incubation **(p)** Day 13 of incubation

(q) Day 14 of incubation **(r)** The reactor clogged in Day 15

Figure 6.12 Clogging pattern of 1 cm^3 PUF, packed PUF layer was lifted by applied air

Figure 6.13 Clogging pattern of 1.5 cm^3 PUF, glass bead layer was lifted by applied air

Figure 6.14 Clogging pattern of 2 cm^3 PUF, effluence port of the reactor was clogged by the mycelium as in red circle



(a) **(b)**

Figure 6.15 High cell density obtained almost 2 weeks of cultivation, they could be put out from the reactor in stable shape

(a) high cell density in PUF

(b) high cell density in glass beads

Figure 6.16 Comparison of color intensity of Orange II between 0, 20, 50 and 100 ppm from left to right

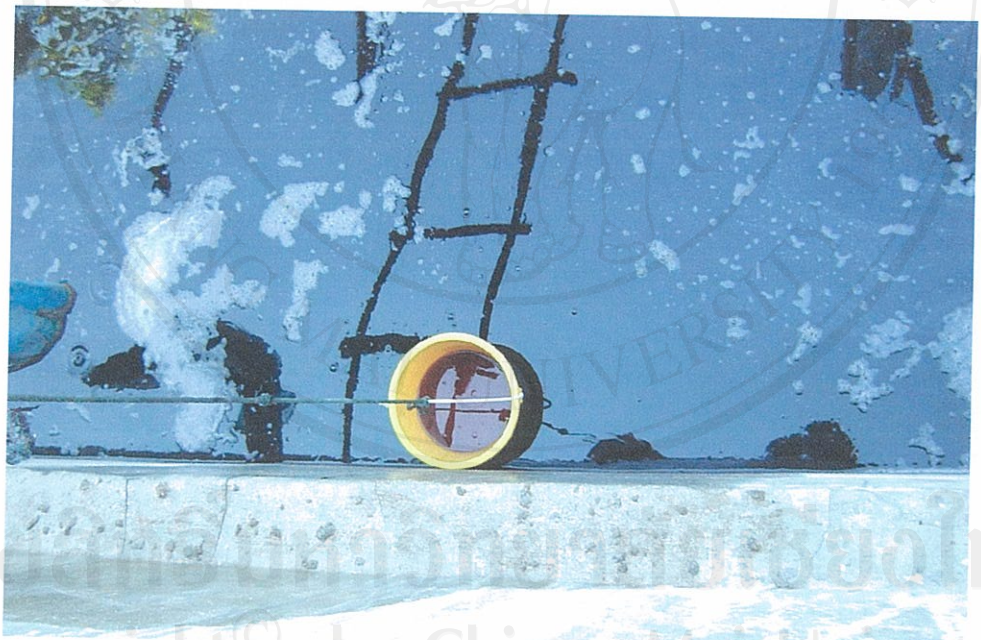


Figure 6.17 Textile wastewater from Batik factory in stabilization pond

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