CHAPTER 4

RESULTS AND DISCUSSION

4.1 Investigation of suitable support

Generally, the fungi favor to grow on solid state media, in this condition they can highly produce biomass and other products. However, fungi can also grow on submerge culture. In the first part of the study, several supports were investigated for using as immobilized material in dye decolorization. This stage of the research focused on the immobilization of *C. versicolor* RC3 by colonization technique on synthetic material such as polyurethane foam, nylon sponge, stainless steel sponge and natural material including coconut husk and luffa sponge. The suitable support obtained from this experiment will be used in air bubble bioreactor, next experiment.

Figure 4.1 show the decolorization by immobilized *C. versicolor* RC3 on several supports. The result showed that the decolorization were more than 90% in all supports except coconut husk that could not be determined the decolorization because this support generated brown color with interfere the measurement.

The colonization method was used for immobilized *C. versicolor* RC3 on the support. When the fungus was immobilized on natural material the coconut husk, the large amount of fungus gave high biomass (6.08 g/l). While the dye decolorization was started, this support generated brown color and interfere the measurement (Table 4.1).

When immobilized this fungus on luffa sponge, the dye decolorization could be repeated for only 3 cycles due to its softness and natural material property. The luffa degradation by the fungus was suggested.

Immobilization on cube of nylon sponge, the fungus grew well on the support. However, the cycle of repeated batch decolorization was found only 5 cycles, which lower than that obtained from polyurethane foam. Nylon sponge is a hard material which produced shear force during cultivation. From this reason, fungal cell might be broken and released to the liquid phase after culturing for a long time. This effect was also presented when immobilized on stainless steel sponge.

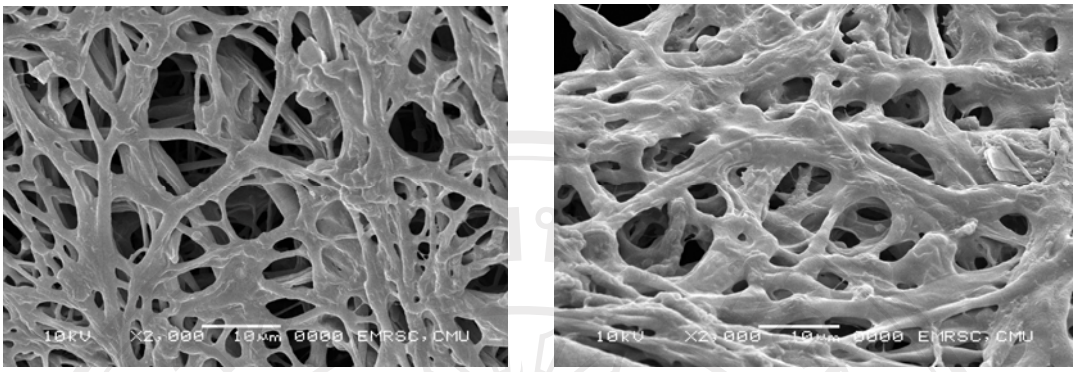
Figure 4.1 Decolorization of commercial dye by immobilized *C. versicolor* RC3 on several supports
(A) Before decolorization
(B) After decolorization for 24 hours

Table 4.1 The investigation of suitable supports for immobilized *C. versicolor* RC3 by colonization technique

Supports	Biomass (g/l)	Cycle of decolorization	Laccase activity (mU/ml)
Polyurethane foam	4.80	6	3.09
Nylon sponge	5.89	5	3.23
Stainless steel sponge	2.85	4	2.90
Coconut husk	6.08	ND	ND
Luffa sponge	3.64	3	4.64

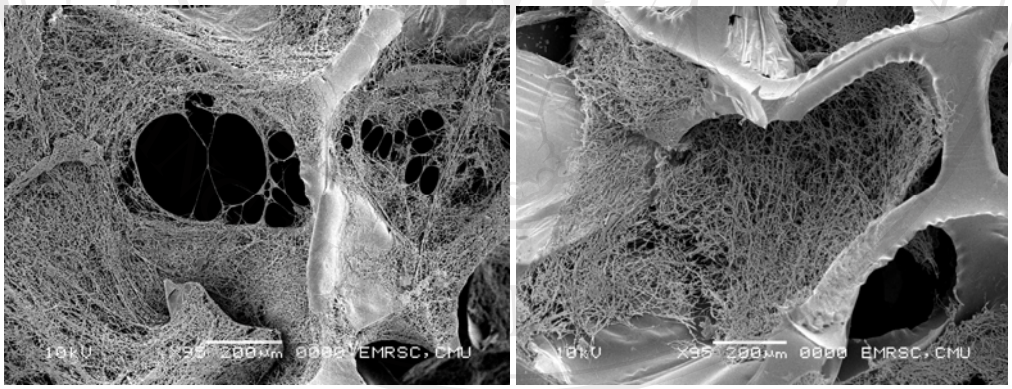
NE: Not Determined

Polyurethane foam immobilization, the fungus grew well attached to the carrier material, the liquid phase remaining clear through whole cultivation. The highest cycle of decolorization 6 cycles was achieved. According to overall aspect of immobilization, polyurethane foam gave very good result and could be selected as the suitable support for decolorization by our strain. Kasinath *et al*., (2003) using polyurethane foam as the solid support for fungal colonization to study the effect of growth conditions on degradation of commercial dyes that support this experiment. Meilgo *et al*., (2001) also used polyurethane foam as immobilized support for degraded azo dye in continuous packed bed bioreactor. The photo of fungal mycelium immobilized on PUF was showed in Figure 4.2 and 4.3.



(A) **(B)**

Figure 4.2 Mycelia of *Coriolus versicolor* RC3 in PUF under scanning electron microscope (SEM)
(A) Before decolorization
(B) After decolorization



(A) **(B)**

Figure 4.3 Immobilized *Coriolus versicolor* RC3 in PUF under scanning electron microscope (SEM)
(A) Before decolorization
(B) After decolorization

4.2 Investigation of dye adsorption on fungal mycelium

4.2.1 Effect of bound dye in immobilized on dye decolorization

Spectrophotometric analysis of methanol extracts of *C. versicolor* RC3 immobilized on polyurethane foam showed that any bound dye in immobilized fungus after 24 hours decolorized in synthetic and real wastewater contained only 0.97 and 0.42%, respectively. These indicated the high percentage of dye decolorization is mainlydue to the microbial metabolism. Similar results were reported by Knapp *et al*., (1997) for Orange II decolorization by the fungus strain F29 and Yesilada *et al*., (2003) for decolorization of textile dyes by *Funalia trogii* pellets.

4.2.2 Effect of heat-killed and cell-washed on dye decolorization

The heat-killed and cell-washed immobilized *C. versicolor* RC3 absorbed the dye and 1.95 and 2.85% of decolorization were obtained, respectively. This observation show that decolorization process by immobilized *C. versicolor* RC3 involves decolorization through the microbial metabolism.

4.3 Investigation of inoculum size and dye concentration for dye decolorization

To determine the maximum dyestuff concentration tolerated by *C. versicolor* RC3 with the suitable inoculun size, experiments with different initial dye concentration and inoculum size were performed. Dye concentration and inoculum size were varied between 150 to 1000 ppm and 6 to 10%, respectively. The concentration of dye substrate can influence the efficiency of dye removal through a combination of some factors including the toxicity of the dye at higher concentrations.

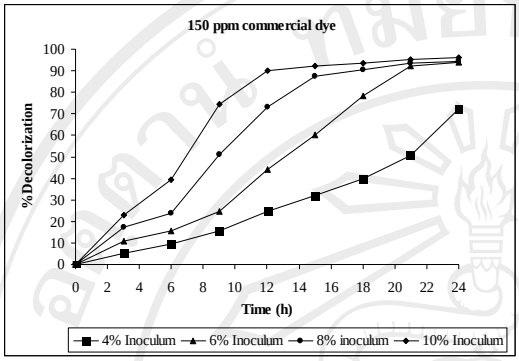
Decolorization at 150 ppm of initial dye concentration is showed in Figure 4.4 A. More than 90% of decolorization was observed in all of inoculum in 24 hours. Inoculum size of 8 and 10% gave the 90% decolorization in 18 hours of incubation

time. Althought at 6% inoculum degraded slowly but 90% decolorization was obtained at 21 hours of incubation time. Similar to decolorization curve of 500 ppm of initial dye concentration (Figure 4.4 B), it was found that inoculum of 8 and 10% gave 90%decolorization in 21 hours of incubation time and 6% of inoculum was observed at the end of incubation time.

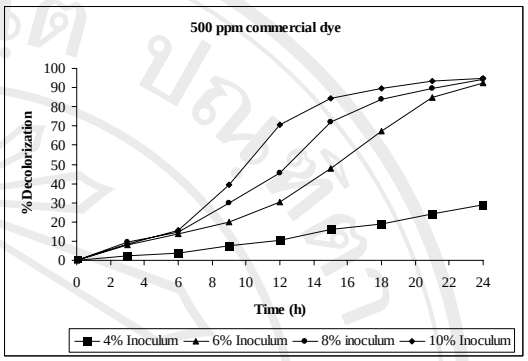
For the initial dye concentration of 750 and 1000 ppm (Figure 4.4 C and 4.4 D), the efficiency of decolorization were lower compare to those of 150 and 500 ppm. This suggests that high concentration of this dye show a toxic that adversely affects the performance. Below 90% decolorization were found in all of inoculum size. The results indicated that more than 90% of color removal was possible at the end of 24 hours of incubation when initial dye concentration below 500 ppm. Inoculum size at 6% was the optimal inoculum for the efficiency of dye decolorization due to the economic reason and that would be benefit when application in large-scale decolorization by immobilized *C. versicolor* RC3 on polyurethane foam. Further experiments will be conducted at 6% inoculum and 150 ppm of dye concentration in large-scale decolorization.

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

The system of commercial dye decolorization using free cell in 10 litres air bubble bioreactor showed in figure 4.5. It was found that all *C. versicolor* RC3 cells settled at the bottom of reactor after 24 h decolorization. The decolorization curve of dye decolorization using *C. versicolor* RC3 is showed in Figure 4.6. The commercial dye decolorization by using free cell in 10 liters air bubble bioreactor was done with 6% of inoculum, 150 ppm of commercial dye, 0.5 vvm of aeration rate at room temperature in 24 hours. It was found that the decolorization started slowly and gave percent decolorization higher than 90% before 20 hours (91.25% in 20 hours). 93.2 % of decolorization and 3.02 mU/ml of laccase activity were obtained in 24 hours (Figure 4.6).



(A)



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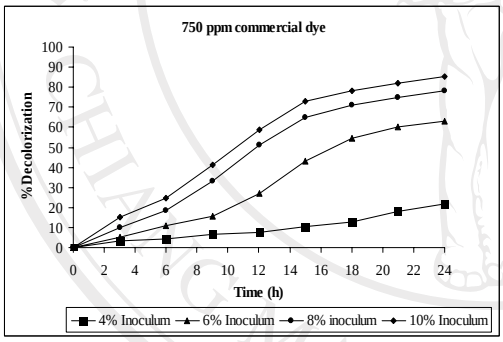

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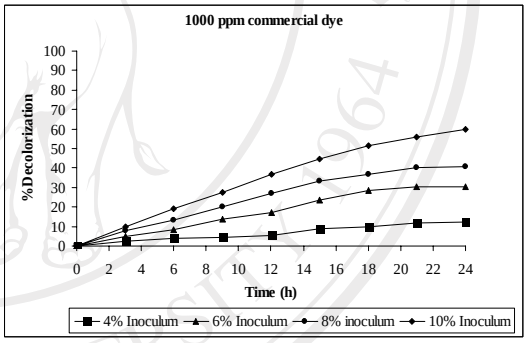

(D)

Figure 4.4 Effect of inoculum size and dye concentration for dye decolorization by immobilized *C. versicolor* RC3
(A) 150 ppm
(B) 500 ppm
(C) 750 ppm
(D) 1000 ppm

Figure 4.5 The system of dye decolorization using free cell of *C. versicolor* RC3

(A) Before decolorization

(B) After decolorization 24 hours

(C) Sample of dye before and after decolorization from 0 to 24 hours

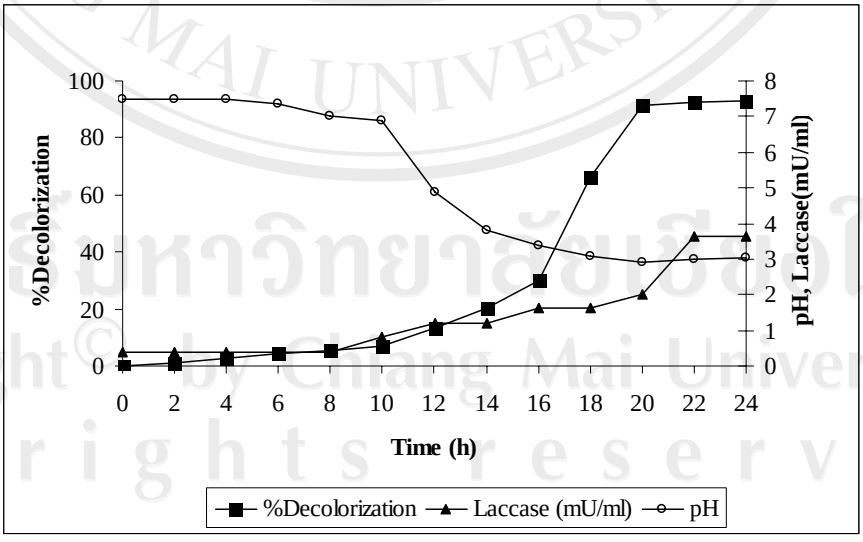

Figure 4.6 Time course of dye decolorization using free cell of *C. versicolor* RC3 in 10 liters air bubble bioreactor

Decolorization increased corresponding with the increasing of laccase quantity. This result showed that a high percentage of decolorization of this commercial dye was mainly caused by microbial degradation, not by adsorption. Similar result was reported by Benito *et al*. (1997), the color adsorption by *Trametes versicolor* mycelium was only 5-10% of the total color removal. In addition, the decreasing of pH was observed, while decolorization (%) and laccase activity increased. This was suggested to involve by the metabolism of this white rot fungus that generally produced acid. This offers many advantages in development of the process for real textile effluent application.

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

The system of commercial dye decolorization using immobilized *C. versicolor* RC3 in 10 liters air bubble bioreactor was showed in Figure 4.7. The figure showed some of *C. versicolor* RC3 mycelium came out of the polyurethane foam and settled at the bottom of the reactor while large amount of cells fixed on polyurethane foam. This was one merit reason for using immobilized cell in repeated batch system. The same resulted as Couto *et al*., (2004) using immobilized white-rot fungus *Trametes hirsute* for decolorisation of textile dyes. Similar result was reported by Zouari *et al*. (2002), the efficiency of immobilized *Phanerochate chrysosporium* on polyurethane foam for degradation of 4-chlorophenol with repeated batch degradation were started quickly and gave decolorization (%) more than 90 % before 20 h. It was found that 95.01 % of decolorization and 3.63 mU/ml of laccase activity were found in 24 hours (Figure 4.8).

C. versicolor RC3 shows a rapid decolorization activity as more than 90% color removal was observed after 18 hours of incubation (92.07% in 18 hours).

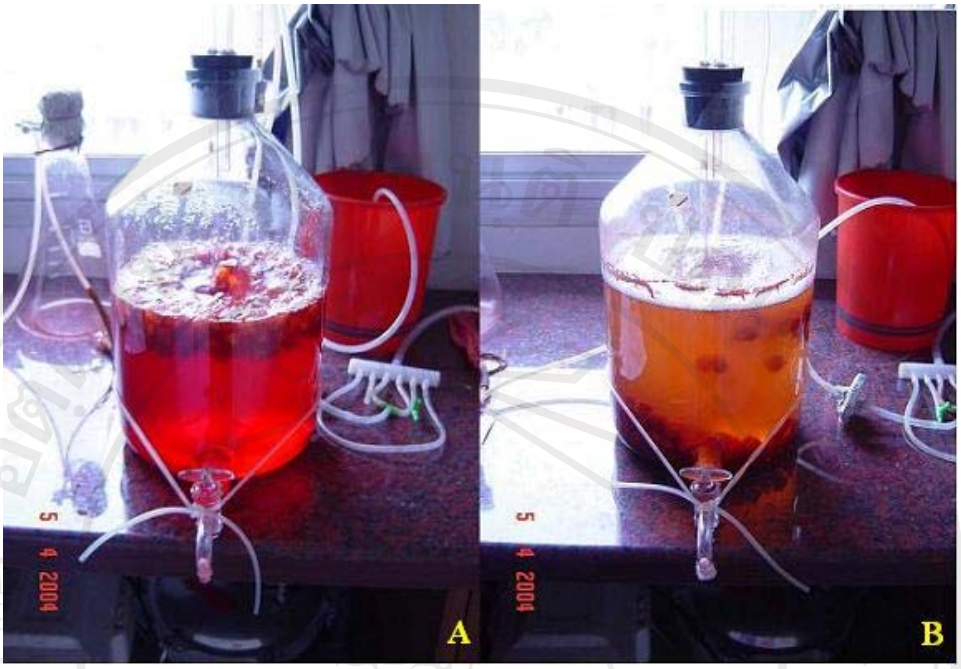

Figure 4.7 The system of dye decolorization using immobilized *C. versicolor* RC3
(A) Before decolorization
(B) After decolorization 24 hours

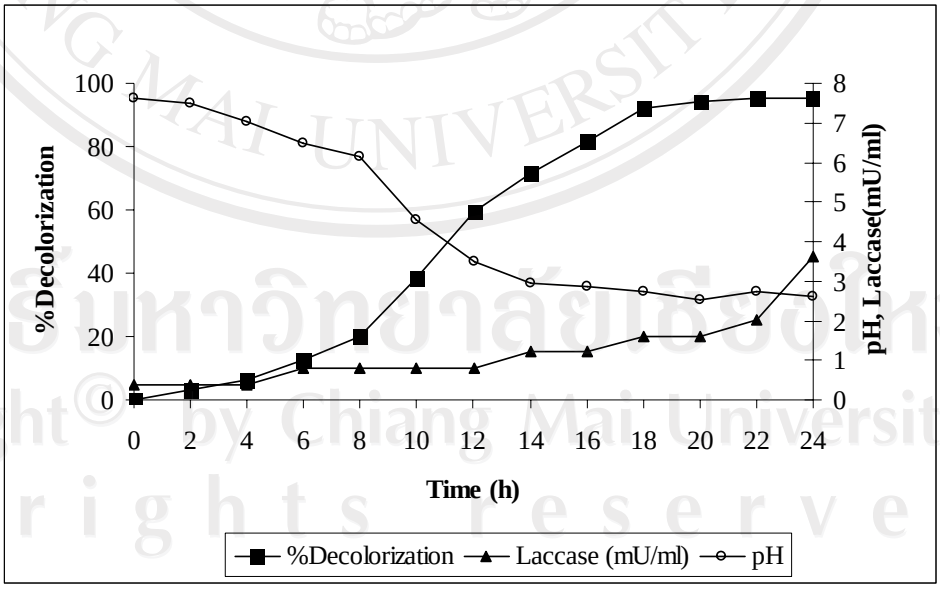

Figure 4.8 Time course of dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 liters air bubble bioreactor

4.6 Decolorization of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor with repeated batch system

Commercial dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 litres air bubble bioreactor with repeated batch system was studied. The repeated batch condition was operated by discharging 50% volume of dye and adding 50% volume of fresh dye for the new cycle. This cycle will be stopped when decolorization (%) was lower than 90% in 24 h. The decolorization profile of repeated batch system is illustrated in Figure 4.9. It is apparent that the repeated batch system affects on the longevity of the decolorization activity. The fungal cell could not degrade dye when repeated for a long time might be the mediator from cell metabolized was discharged.

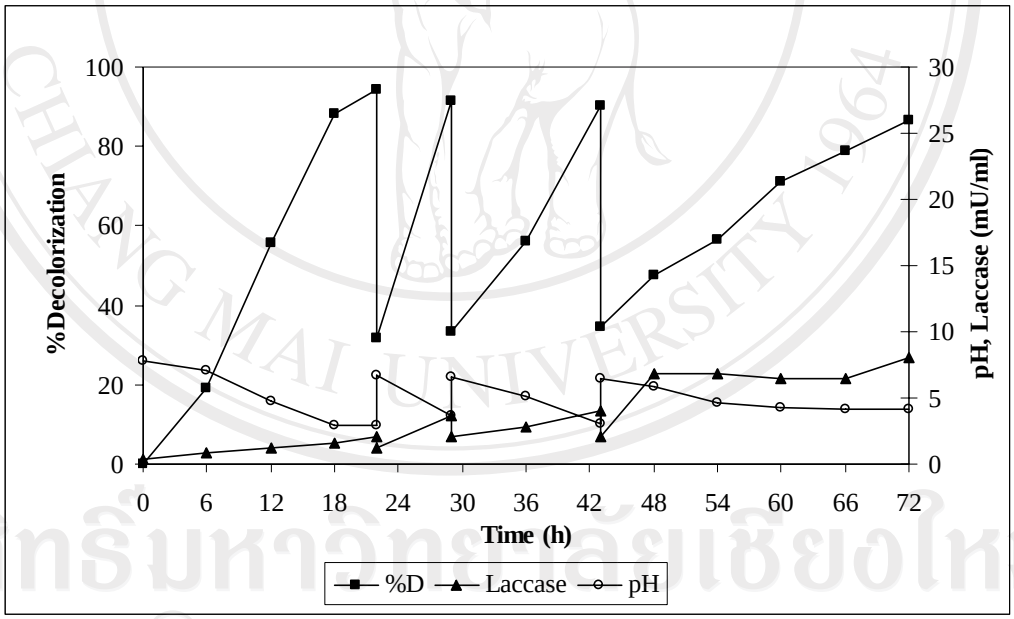

Figure 4.9 Time course of dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 liters air bubble bioreactor with 50% repeated batch system

The repeated batch was started when decolorization (%) more than 90%. In the first cycle, decolorization (%) was 94.15 % in 22 hours, while the second and the third cycle were 91.45 and 90.38 % in 7 and 14 hours, respectively. However, in the fourth cycle decolorization (%) was found more than 90%, but it took 30 h more than 24 hours. These results showed that the decolorization was stabled for 3 cycles of decolorization and gave more than 90 % of decolorization with in 24 hours. The total volume of dye removal was 12 litres in 43 hours. Moreover, the laccase activity was high through out the experiment but the decolorization yield was not stayed for a long time. This was suggested to cause from the exhaust of mediator, an important component for laccase activity.

4.7 Decolorization of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor with variation of dye removal volume in repeated batch system

Commercial dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 litres air bubble bioreactor with varies volume of dye removal repeated batch system was studied. The repeated batch condition was discharged 25 and 75% volume of dye and repeated 25 and 75% volume of fresh dye for the new cycle, respectively. This cycle will be done when decolorization (%) was lower than 90% in 24 hours. The decolorization profile of repeated batch system is illustrated in Figure 4.10 and 4.11. It is apparent that the repeated batch system affects the longevity of the decolorization activity.

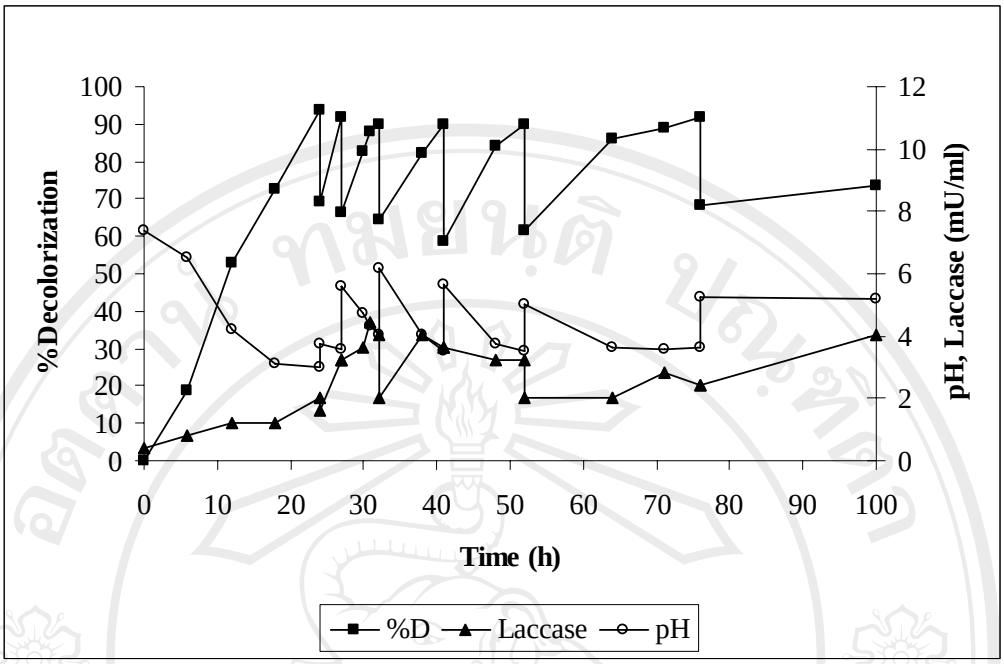

Figure 4.10 Time course of dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 liters air bubble bioreactor with 25% repeated batch system

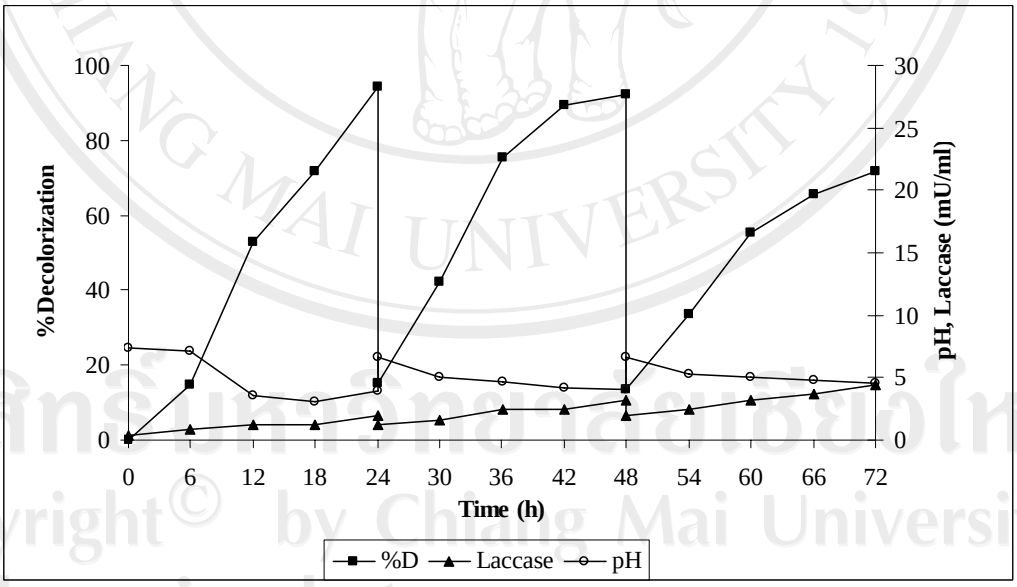

Figure 4.11 Time course of dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 liters air bubble bioreactor with 75% repeated batch system

For 25% of repeated batch, the repeated batch was started when decolorization (%) more than 90%. This system showed 6 cycles of decolorization and gave more than 90% of decolorization in 24 hours. In the first cycle, decolorization (%) was 93.86 % in 24 h, while the others cycle were 92.06, 90.01, 90.01, 90.01, and 91.65 % in 3, 5, 8, 11 and 24 hours, respectively. The total volume of dye removal was 12 litres in 52 hours.

The 75% of repeated batch, the repeated batch was started when decolorization (%) more than 90%. This system gave only 2 cycles of decolorization. Decolorization (%) was 94.4 and 92.09 % for 24 h of each cycle. The total volume of dye removal was 12 litres in 48 hours.

The performance of commercial textile dye decolorization by *C. versicolor* RC3 in various conditions was summarized in Table 4.2. In this experiment, using the repeated batch could remove a large dye volume. Using free and immobilized *C. versicolor* RC3, the volume of dye removal was 8 litres in 20 and 18 hours of decolorization. While repeated batch system was set, the dye removal volume was higher than and gave 12 litres of dye removal in all repeated batch conditions. The number of repeated batch in 25, 50 and 75% repeated batch was 6, 3 and 2 cycles, respectively. The 25% repeated batch gave the highest cycle number, while 75% repeated gave the lowest. However, the volume of dye removal was the same in all repeated batch conditions. In addition with 6% of inoculum, laccase was produced as the same level as in all conditions. That mean dye removal gave same value in all conditions. Time for removal dye in 25, 50 and 75% repeated batch was 100, 43 and 48 h, respectively. It was showed that 50% repeated batch used the shortest time for decolorize 12 litres of dye in 43 h. It was indicated that 50% repeated batch was the suitable condition for commercial textile dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam. However, this system might maintain high number of repeated in long-term condition by supplement of mediator for activity of ligninolytic enzyme. The system will be set by drained 50% of dye volume from the bioreactor and added 50% volume of fresh dye every 24 h for easy to operate, next experiment.

Table 4.2 Concluded performances of commercial textile dye decolorization by immobilized *C. versicolor* RC3 in various conditions

Conditions	Number of repeated batch (cycles)	Volume of dye removal (liters)	Time use in decolorization (h)
Free cell	-	8	20
Immobilized cell	-	8	18
25% repeated batch	6	12	100
50% repeated batch	3	12	43
75% repeated batch	2	12	48

Similar to the results of Zouari *et al*. (2002), the repeated batch decolorization of 4-chlorophenolby immobilized *P. chrysosporium* on polyurethane foam was investigated. Three repeated batches of decolorization were achieved when a regeneration activation medium was added. Moreover, Yesilada *et al*. (2003) also studied the repeated batch decolorization of Astrazone Black FDL, Astrazone Blue FGRL and Astrazone Red FBL dye by fungal pellets and succeeded only 2 cycles of decolorization, with the dye concentration and pellets of 132 mg/l and 370 mg/50ml, respectively.

4.8 Decolorization of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor with repeated batch system in non-sterile condition

Few studies have investigated non-sterile operation with white rot fungus, although the goal of most studies was to evaluate their use to oxidize contaminants wastewater. For the application of white rot fungi in treatment schemes for dye-containing wastewater, the process must be designed either to retain pure culture

conditions or to function under non-sterile conditions. This decolorization experiment of commercial dye using immobilized cell on suitable support in 10 litres air bubble bioreactor with repeated batch system in non-sterile condition was studied. The result was showed in Figure 4.12.

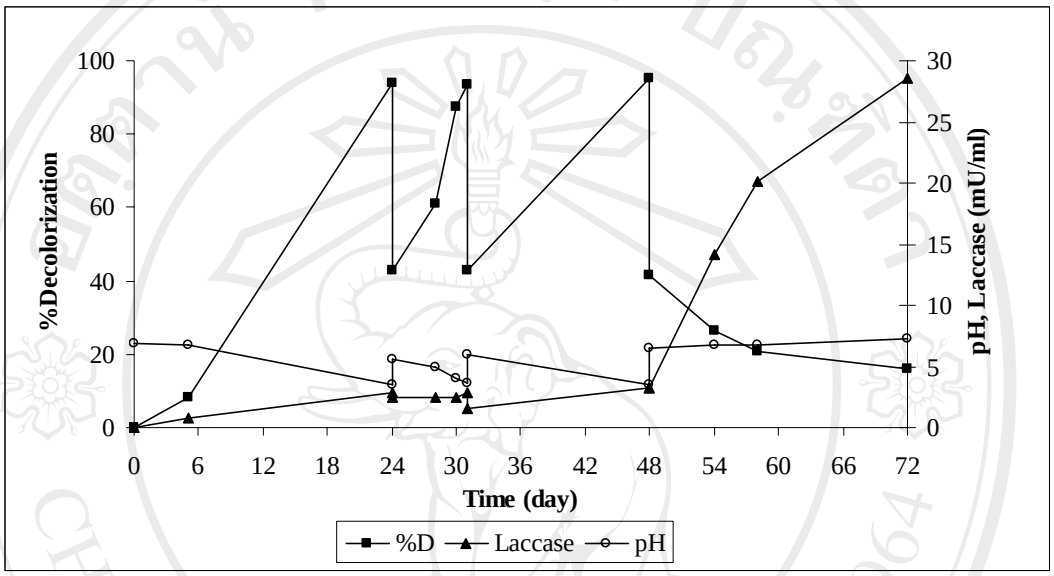

Figure 4.12 Time course of dye decolorization using immobilized *C. versicolor* RC3 on polyurethane foam in 10 liters air bubble bioreactor with repeated batch system in non-sterile condition

In non-sterile decolorization, *C. versicolor* RC3 could grow and produced acid to suppress bacterial growth. Three cycles of repeated batch decolorization was also achieved as the same as sterile condition in the previous study. The maintenance for long-term fungal activity requires either much technical effort to maintain sterile conditions or an effective method to suppress bacterial growth. For next studies, the non-sterile condition will be performed.

4.9 Effect of sugar addition on decolorization

From previous studies, three cycles of 50% repeated batch decolorization by immobilized *C. versicolor* RC3 on polyurethane foam was obtained. Although the laccase activity was found in the end of decolorization time but the fungus could not degrade dye. To improve the efficiency of this system for long-term decolorization, the carbon source was added to the system. Because the fungi consume and grow on readily available carbon sources at the initial stage of growth and then produces secondary metabolites and extracellular enzymes for biodegradation of dyestuffs at low concentrations of carbon or nitrogen. The most readily useable carbon source by white rot fungi is glucose. However, glucose is an expensive carbon source and also is not commonly used in wastewater treatment (Kapdan *et al*., 2000).

To find an inexpensive and more suitable carbon sources, addition of sucrose and molasses were tried in decolorization experiments compared with glucose. Concentration of carbon sources was varied as 20, 5, 3, and 1 g/l, respectively except molasses was varied as 3 and 1 g/l, respectively. Results of decolorization experiments with different carbon sources and concentration were summarized in table 4.3. It was found that more than 10 cycles of decolorization were presented when different carbon sources were added except molasses. Repeated batch decolorization with 1 g/l of molasses was not changed and gave 3 cycles of decolorization as same as no sugar addition. However, 4 cycles of decolorization was obtained when 3 g/l of molasses was added. The high concentration of molasses could not prepare because the brown color interfering to the red color of dyestuff. These results clearly indicated that molasses was not a suitable carbon source for biodegradation of the dyestuff by this fungus, although it is a cheap source of carbon. Glucose addition at 3 g/l gave the highest cycles of decolorization about 16 cycles of decolorization but 3 g/l of sucrose was selected as the suitable supplementation in this experiment because of it is cheaper than glucose, even only 14 cycles of repeated batch decolorization was obtained. The profile of repeated batch decolorization with 3 g/l sucrose addition was illustrated in Figure 4.13.

Table 4.3 Cycles number of decolorization of synthetic wastewater with sugar addition

Sugars	Cycles number of decolorization at various concentrations			
	20 (g/l)	**5 (g/l)**	**3(g/l)**	**1 (g/l)**
Glucose	11	15	16	13
Sucrose	10	14	14	11
Molasses	ND	ND	4	3

ND: Not Determined

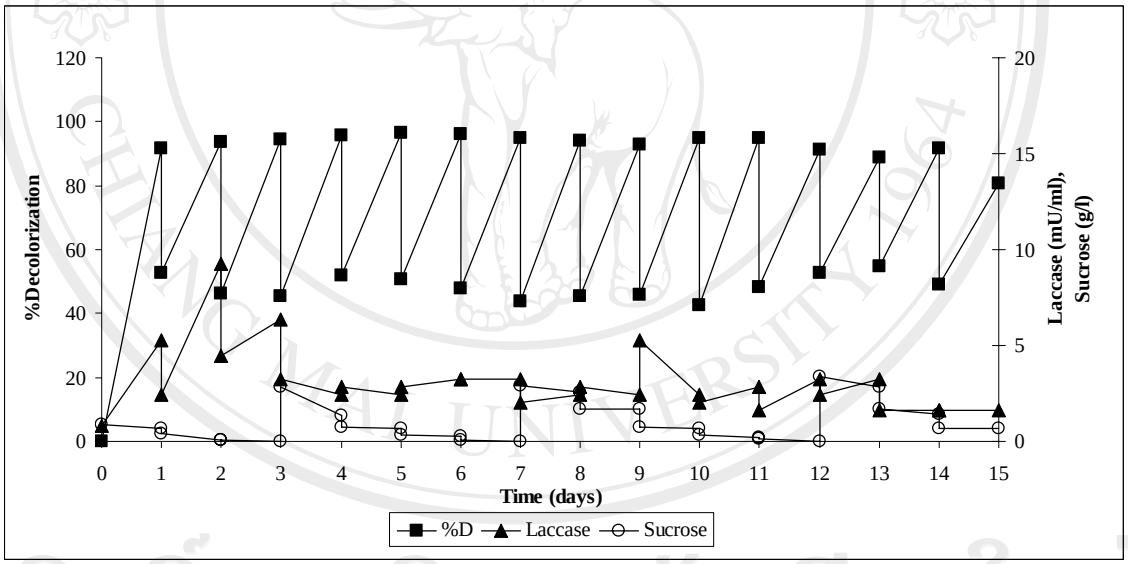

Figure 4.13 Time course of decolorization of commercial dye with repeated batch and added 3 g/l of sucrose

Several researcher studies on the effects of different carbon sources in decolorization and various selected carbon source was reported. Ge *et al*., (2004) used *P. sordida* in decolorization of Basic Blue 22 at the concentration of 200 mg/l

with repeated batch mode and supplement with 5 g/l of sucrose in a rotating biological contactor. Five decolorization phases was obtained and the efficiency of decolorization was 80%. Martins *et al*., (2003) used 5g/l of sucrose to degrade syringol derivatives of azo dye by several fungi and *Tremetes versicolor* showed the best biodegradation performance. Padmavathy *et al*., (2003) investigated the efficiency of azo dye degradation by consortia in presence of various co-substrates, such as glucose, starch, lactose, and sewage and whey water. It was observed that starch was the best source of carbon for decolorization of reactive azo dyes. Assadi *et al*., (2001) decolorized textile wastewater by *P. chrysosporium* and supplemented 0.3 g/l of glucose in textile wastewater 95% decolorization was found. In addition, Kapdan *et al*., (2000) studies the effect of carbon source on decolorization of Everzol Turquoise Blue G by *C. versicolor* MUCL and found that 5 g/l of glucose seem to be the most suitable carbon source.

4.10 Characterization of real wastewater

Textile wastewater generated by the different production step such as sizing of fibers, scouring, desizing, bleaching, washing, mercerization, dyeing and finishing. It also contains high concentrations of organic matter, non-biodegradable matter, toxic substances, detergents and soaps, suspended and dissolved solids and alkalinity. The characterization of real wastewater was needed before decolorization and showed in table 4.4. The color of real wastewater for this experiment was purple but brown, blue, red, green and black were also observed depending on the production process.

Table 4.4 Characteristics of wastewater from Batik factory

Parameters	Values
Absorbance (nm)	500
pH	7.5-8
COD (mg/l)	2000-3600
TSS (mg/l)	50-70
Color	purple

4.11 Decolorization of real wastewater using immobilized cell on suitable support in 10 litres air bubble bioreactor with repeated batch system

The repeated batch system was set when decolorization (%) more than 60% in 24 hours and 4 cycles of repeated batch was found while supplemented with 3 g/l of sucrose (Figure 4.14). It was found that decolorization (%) increased when sucrose was added.

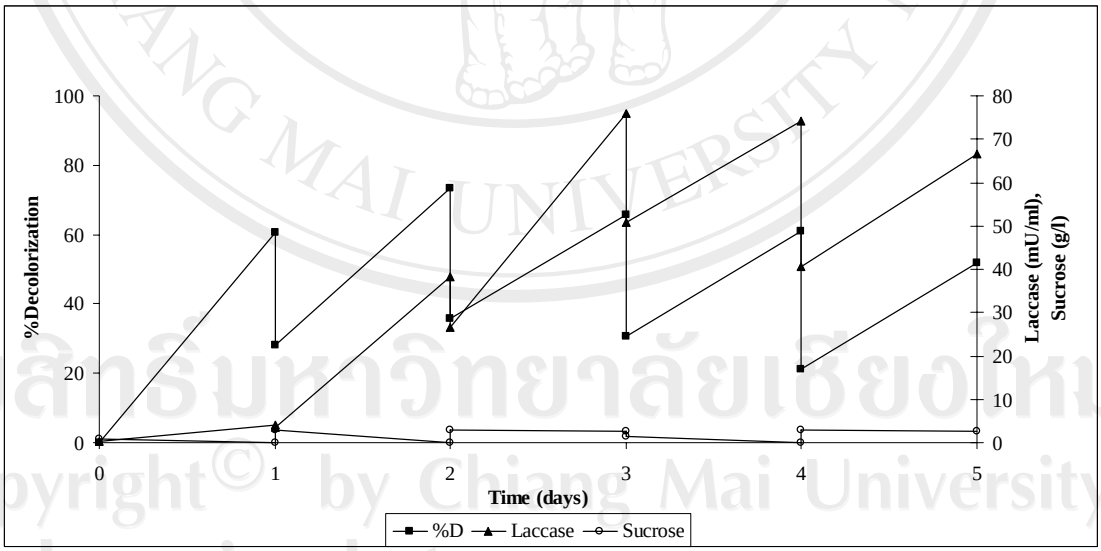

Figure 4.14 Time course of decolorization of real wastewater decolorization with repeated batch and added 3 g/l of sucrose

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

The Figure 4.15 shows the wave length scan of real wastewater before and after decolorization. The peak at 500 nm was found in before decolorized sample. After decolorization this peak was disappeared. This revealed the structural degradation of dye molecules.

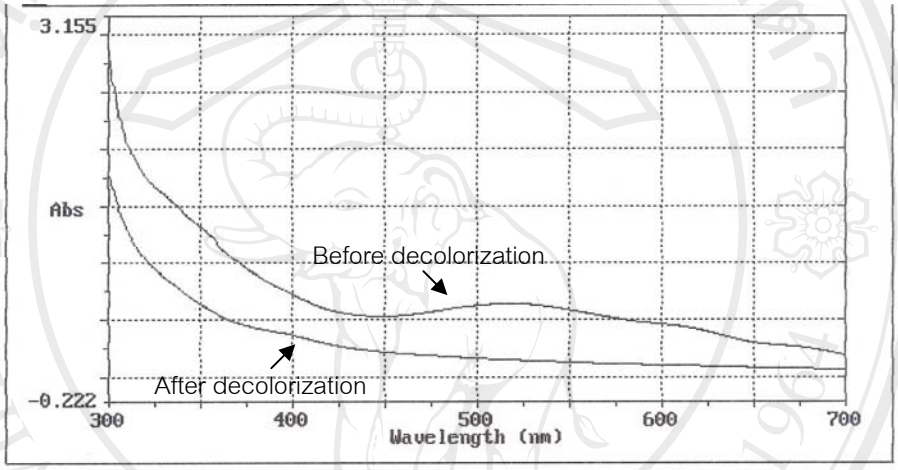

Figure 4.15 Wave length scan of real wastewater from Batik factory

The parameters of real wastewater before and after decolorization in 50 liters air bubble bioreactor was shown in Table 4.5. It was found that *C. versicolor* RC3 could grow in real wastewater and generated an acidic condition. The reduction of the pH is suggested to be the cause of bacterial growth suppression. COD removal was decreased 67% and decolorization was found at 80% in 48 hours. Moreover, 14.1 mU/ml of laccase activity was also found (data not shown). This process removed COD from 3,680 to 1,200 mg/L, however, the COD value is still over water quality standard (120 mg/L) in appendix I. Wastewater treatment methods such as wet land will be required.

Table 4.5 Parameters of real wastewater before and after decolorization in 48 hours

Parameters	Influent	Effluent	%Removal
Peak in WL scan (nm)	500	-	-
pH	8.22	3.91	-
COD (mg/L)	3,680	1,200	67
TSS (g/L)	0.067	0.05	-
Total plate count (cfu)	$2.1*10^5$	$4*10^3$	81
OD_{500}	0.49	0.097	80
Color	purple	colorless	-

4.13 Toxicity test of dye before and after decolorization

Toxicity experiments with *Daphnia* sp. were carried out at different dilutions to evaluate of dye before and after decolorization. The toxicity tests of synthetic wastewater before and after decolorization are shown in Fig. 4.16. The decolorization process significantly reduced toxicity in the synthetic wastewater and the wastewater after decolorization was not toxic up to 80% concentration while untreated wastewater was found to be toxic up to 30% concentration. The result indicated that the raw and decolorized wastewater were toxic at 30 and 80% concentration, respectively.

The toxicity tests of synthetic wastewater with 2 cell lines are shown in Figure 4.17 and 4.18. Raw synthetic wastewater after decolorization was not toxic in fibroblast cell line and also found to be toxic at 90% concentration in cancer cell line while untreated wastewater was toxic at same concentration (70%) in both cell lines. The results were summarized in Table 4.6 and 4.7.

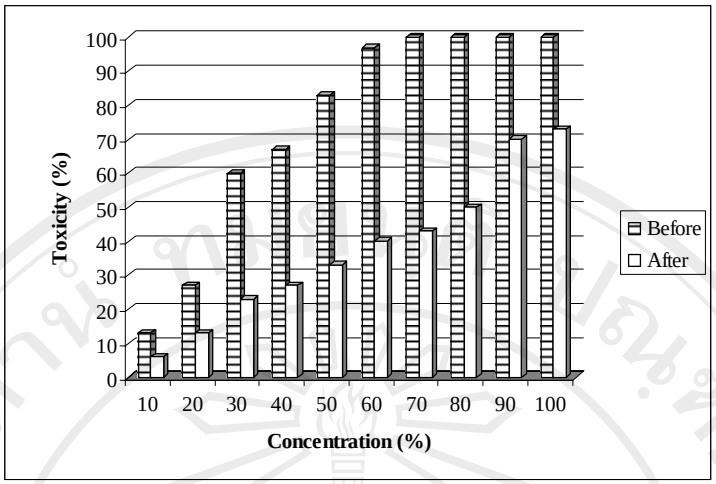

Figure 4.16 Toxicity test of synthetic wastewater with *Daphnia* sp. at different dilutions

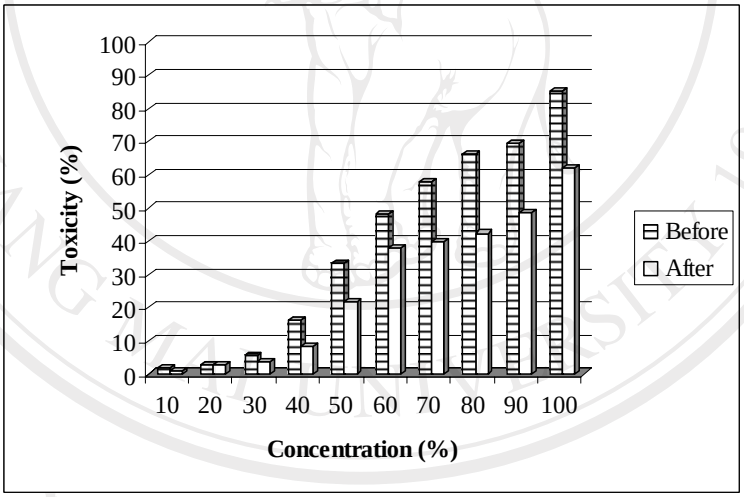

Figure 4.17 Toxicity test of synthetic wastewater with mouse fibroblast cell line (3T3) at different dilutions

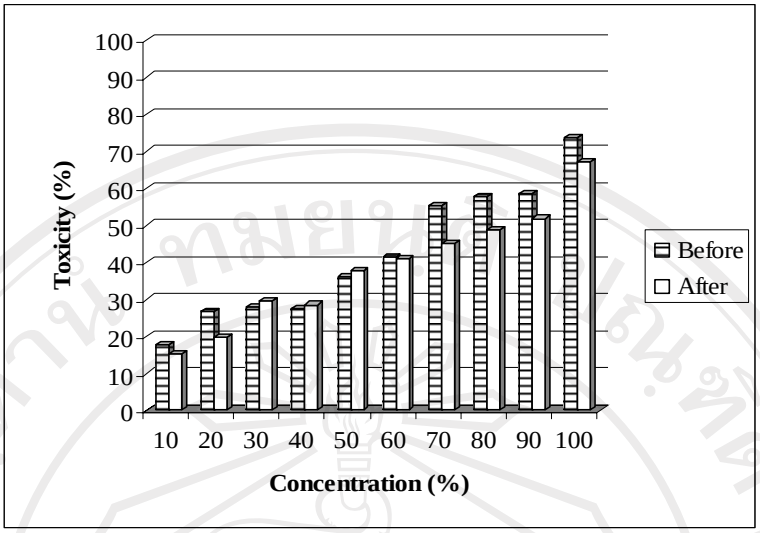

Figure 4.18 Toxicity test of synthetic wastewater with lung cancer cell line COR-L23 at different dilutions

Table 4.6 Toxicity test in *Daphnia* sp. and cell lines before decolorization

Toxicity before decolorization	***Daphnia* sp.**	**3T3**	**COR-L23**
Synthetic wastewater	30% concentration	70% concentration	70% concentration
Real wastewater	40% concentration	70% concentration	No toxic
Orange II	70% concentration	90% concentration	90% concentration

Table 4.7 Toxicity test in *Daphnia* sp. and cell lines after decolorization

Toxicity after decolorization	***Daphnia* sp.**	**3T3**	**COR-L23**
Synthetic wastewater	90% concentration	100% concentration	90% concentration
Real wastewater	60% concentration	No toxic	No toxic
Orange II	No toxic	No toxic	No toxic

For real wastewater, raw wastewater was not toxic to cancer cell line and toxic at 40 and 70% concentration in *Daphnia* sp. and fibroblast cell line. While treated wastewater were not toxic in both cell lines and also found to be toxic at 60% concentration *Daphnia* sp. Decolorization of Orange II dye was also studied to compare with synthetic and real wastewater. It was found that before degradation Orange II was toxic when treated with *Daphnia* sp. and both cell lines while after decolorization the effluent was not toxic with *Daphnia* sp. and both cell lines.