APPENDIXES

Appendix A: Media

1. Potato dextrose broth

potato	200 g
dextrose	20 g
distilled water	1000 ml

2. Potato dextrose agar

potato	200 g
dextrose	20 g
agar	15 g
distilled water	1000 ml

3. Dulbecco's modified Egale's medium (DEME)

L-arginine.HCl	0.084 g
L-cystine.2HCl	0.0626 g
L-glutamine	0.584 g
Glycine	0.03 g
L-histidine.HCl.H_2O	0.042 g
L-isoleucine	0.105 g
L-leucine	0.105 g
L-lysine.HCl	0.146 g
L-phenylalanine	0.066 g
L-serine	0.042 g

L-threonine	0.095	g
L-tryptophan	0.016	g
L-tyrosine.2Na.2H_2O	0.10379	g
L-valine	0.094	g
Choline chloride	0.004	g
Folic acid	0.004	g
myo-inositol	0.0072	g
Niacinamide	0.004	g
D-pantothenic acid	0.004	g
Pyridoxal.HCl	0.004	g
Riboflavin	0.0004	g
Thiamine.HCl	0.004	g
Calcium chloride	0.2	g
Ferric nitrate.9H_2O	0.0001	g
Magnesium sulfate	0.09767	g
Potassium chloride	0.4	g
Sodium chloride	6.4	g
Glucose	4.5	g
Sodium phosphate	0.109	g
Phenol red.Na	0.0159	g
Distilled water	1000	ml

Supplemented with: 10% fetal bovine serum, 0.1% gentamycin and 1% non-essential amino acid

Appendix B: Calculation of enzyme activity

The enzyme activities of ligninolytic enzyme are calculated based on Beer-Lambert's law. Beer-Lambert's law is the relationships between the absorbance of the solution each directly increase with the concentration of the absorbing substrate and the path length passed through by the beam. Therefore, Beer's law relates these quantities as follow (Douglas, 1965)

$$A = \varepsilon bc$$

Where A is absorbance of the sample at specific wavelength, ε is a constant number called molar extinction coefficient, b is path length of radiation in centimeter (cm) and c is concentration of an adsorbing solution in mole per liter (mole/l). The molar extinction coefficient value of substrates showed in Table 6.1

Enzymatic activity calculation

The activity of enzyme is the oxidation of substrate to oxidize the product that could be observed under different wavelength, which depends on substrate. According to the definition of enzyme activity that is "one unit was defines as the amount of enzyme producing a one unit change of absorbance per minute". Two chemicals, DMP and veratryl alcohol were used as standard substrates. DMP was used for the assay of manganese peroxidase, manganese independent peroxidase laccase, respectively. While the oxidation of veratryl alcohol to be veratraldehyde will be used for the assay of lignin peroxidase.

Table 6.1 Extinction coefficient values of substrates. (Saparrat *et al*., 2002)

Substrate	**Maximum absorbance (nm)**	**ε ($mM^{-1}cm^{-1}$)**
ABTS	420	36.0
DMP	470	49.6
Syringaldazine	310	65.6
Guaiacol	480	0.57
Veratryl alcohol	310	0.93

According to Beer's law and enzyme activity in IU lead to modify the equations for enzyme activity calculation as follow:

$$C = [(A_1 - A_0)*d]/[\varepsilon*b*t]$$

In the above equation

C is enzyme activity in µmole/min ml or unit/ml

A is the different of absorbance from to 0 to 1

ε is molar extinction coefficient in $mM^{-1}cm^{-1}$

b is path length of radiation in cm

t is time in minute

d is dilution fold.

When the suitable of enzyme solution was used 100 µl in 1000 µl total volume of reaction mixture and ε470 of DMP oxidation is 49.6 $mM^{-1}cm^{-1}$

According to equation

$C = [(A_1 - A_0)*d]/[\varepsilon*b*t]$

$= [(1.573 - 0.487)*10]/[49.6\ mM^{-1}cm^{-1} * 1\ cm * 1\ min]$

$= 0.219\ mM/min$

$= 0.219 * 10^{-3} * 10^{6}\ [\mu mole/(10^{-3})/min]$

$= 0.219 * 10^{-3} * 10^{6} * 10^{-3}\ (\mu mole/ml\ min)$

$= 0.219\ \mu mole/ml\ min$

$= 0.219\ unit/ml$

Therefore, the activity of laccase is 0.219 unit/ml

Appendix C: Total sugar determination by phenol sulfuric method (Dobois *et al*., 1956)

Reagents

Sulfuric acid 95.5%

Phenol 5%

Method

1. Add 1 ml of phenol solution mixed in 1 ml of sample solution

2. Add sulfuric acid 5 ml, wait 10 min, mixed and wait again about 30 min

3. Measuring an absorbance at 490 nm, compare with standard curve of glucose in concentration 20-200 µg/ml (distilled water as a blank)

Table 6.2 Optical density of glucose solution by varying concentration at 490 nm

Glucose (µg/ml)	OD at 490 nm
0	0.000
20	0.186
40	0.375
60	0.555
80	0.751
100	0.92
120	1.092
140	1.261
160	1.378
180	1.658
200	1.803

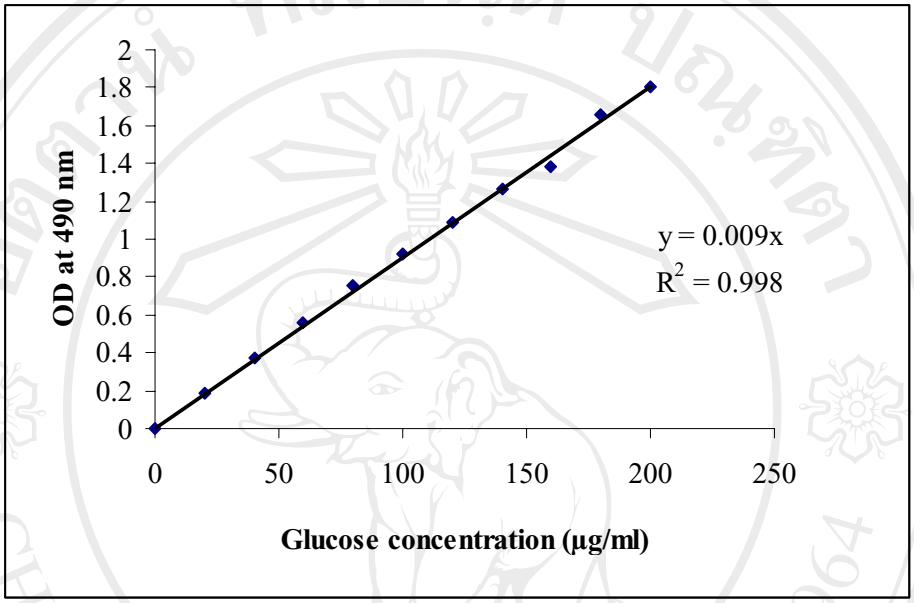

Figure 6.1 Standard curve of total sugar by phenol sulfuric method

Appendix D: Total plate count

Total plate count in the spread plate method was showed in Figure 6.2

Number of colony forming unit (CFU) = (1/dilution factor) x number of colonies

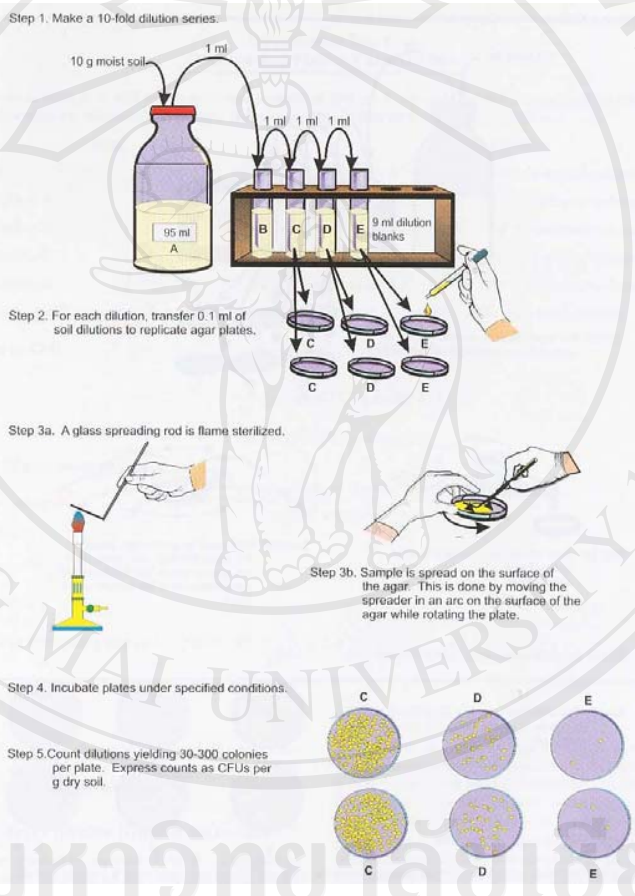

Figure 6.2 Dilution and spread plate

Source: Maire *et al*., 1999

Appendix E: Chemical oxygen demand by closed reflux, titrimetric method (APHA, 1998)

Reagents

1. Standard potassium dichromate digestion solution 0.01667 M

Add to about 500 ml distilled water 4.903 g $K_2Cr_2O_7$, primary standard grade previously dried at 150 ^{O}C for 2 h, 167 ml conc H_2SO_4, and 33.3 g Hg SO_4. Dissolve, cool to room temperature, and dilute to 1000 ml.

2. Sulfuric acid reagent

Add Ag_2SO_4, reagent or technical grade, crystals or powder, to conc H_2SO_4 at the rate of 5.5 g Ag_2SO_4/kg h2SO4. Let stand 1 to 2 days to dissolve. Mix.

3. Ferroin indicator solution

Dissolve 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4.7H_2O$ in distilled water and dilute to 100 ml.

4. Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10M

Dissolve 39.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$ in distilled water. Add 20 ml conc H_2SO_4, cool, and dilute to 1000 ml. Standardize solution daily against standard $K_2Cr_2O_7$ digestion solution.

Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination Refer to table 6.3 for proper sample and reagent volumes. Place sample in culture tube and add digestion solution. Tightly cap tubes, and invert each several times to mix completely.

Place tubes in block digester preheated to 150 ^{O}C and reflux or 2 h behind a protective shield. Cool to room temperature and place vessels in test tube rack. Add 0.05 to 0.10 ml (1 to 2 drops) ferroin indicator and stir rapidly while titrating with standardized 0.10M FAS. The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear with in minutes. In the sane manner

reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

COD as mg O_2/l = ((A-B) x M x 800) / ml sample

Where:

A = ml FAS used for blank,

B = ml FAS used for sample,

M = molarity of FAS, and

8000 = milliequivalent weight of oxygen x 1000 ml/l

Table 6.3 Sample and reagent quantities for various digestion vessels

Digestion Vessel	Sample (ml)	Digestion solution (ml)	Sulfuric acid reagent (ml)	Total final volume (ml)
16 x 100 mm	2.50	1.50	3.5	7.5
20 x 150 mm	5.00	3.00	7.0	15.0
25 x 150 mm	10.00	6.00	14.0	30.0
ampules	2.50	1.50	3.5	7.5

Appendix F: Toxicity test by *Daphnia magma* in short-term tests (APHA,1998)

Preparation of test materials and medium

Make up test solutions and control in 100 ml quantities in 125 ml wide-mouth-glass bottles or equivalent vessels.

Performing tests

After preparing test solutions, segregate neonates that have been released from the mother's brood chambers during the preceding 24 h at 20^{O}C or 25^{O}C and collect in one vessel (use neonates cultured at the test temperature). Introduce the same number of neonates (at least 10) into each test vessel and control. Use a plastic, disposable pipet with a 5-mm bore for collecting and transferring neonates. Alternatively, use a glass bulb pipet.

Introduce neonates to test solutions by releasing them below the surface of the solution. Observe animals regularly, ideally after 1 h and 4 h and daily thereafter. A 48-h exposure is generally accepted for a *Daphnia* acute toxicity test. Record number of motile animals in each test vessel. Consider an animal nonmotile if it shows no independent movement even after gentle squirting with test solution from a pipet (nonmotile animals are not necessarily dead). At threshold concentrations of such substances as ethanol, acetone, and chlorobutanol, animals may show no movement and the hart may have ceased to beat but no transfer to dilution water they will recover. However, such animals maintained in the test medium will die. In addition to immobilization, note behaviors and features such as the number of *Daphnia* that are on bottom, lethargic, swimming, caught on the bottom or on debris, floating on surface, swimming erratically, or have a flared carapace.

Record conditions of the medium such as whether it is cloudy or if any particulate matter, precipitate, undissloved material, or film is present. Continue an observation for a

minimum of 48 h or as long as there is no more than 10% control mortality. Run tests in replicates of at least three.

Criteria for test acceptability

An acceptable test will have no more than 10% mortality in the negative control.

Appendix G: *Ex vivo* assay for cytotoxic activity

Cell lines preparation (Freshney, 2004)

Cancer and normal cell lines used were the COR-L23 large cell lung carcinoma and the 3T3 Albino Swiss mouse embryo fibroblast, respectively. According to their growth profiles, the optimal plating densities of the cell line were determined (5×10^3 and 7×10^3 cells/well for COR-L23 and 3T3, respectively) to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay.

Cytotoxicity assay

For the assay, cells were washed with phosphate buffer saline (PBS) (Oxoid Ltd., UK) free of magnesium and calcium. The PBS was decanted and cells detached with 0.025% trypsin-EDTA (Sigma) and medium was added to a volume of 15 ml. The cell pellet, obtained by centrifugation (1, 500×g, 5 min) was resuspended in 10 ml of medium to make a single cell suspension, viable cells density being counted by trypan blue exclusion in a haemocytometer and the diluted with medium to give the previously-determined optimal plating densities for COR-L23 and 3T3, respectively. 100 µl/well of these cell suspensions were seeded in 96-well microtiter plate and incubated at 37 ^{o}C to allow for cell attachment. After 24h the cells were treated with the synthetic and real wastewater in before and after decolorization, respectively. Each extract was initially dissolved in either DMSO. Vinblastine (Sigma, Lot No. 093K1612) was used as positive controls.

Sulphorhodamine B (SRB) assay

The sulphorhodamine B cytotoxicity assay determines total cell number by measuring cellular protein. The protein binds to the dye, sulphorhodamine B, and it is then extracted from the cells in a Tris base solution. This assay was conducted according to the originally described by Skehan *et al*. (1990). In briefly, cells were fixed by layering 100µl of ice-cold 40% trichloroacetic acid (TCA, Aldrich Chemical) on top of the growth medium. Cells were incubated at 4 ^{o}C for 1h, after which plates were washed five times with cold water, the excess water drained off and the plates left to dry in air. SRB stain (50 µl; 0.4 in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min, after which they were washed with 50 ml 1% acetic acid, rinsed four times until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM tris base pH 10.5 (Sigma) were added to each well to solubilize the dye. The plates were shaken gently for 20 min on a gyratory shaker and the absorbance (OD) of each well was read on a microplate reader (Metrohm) at 492 nm. Cell survival was measured as the percentage absorbance compared to control (non-treated cells). The IC_{50} values were calculated by probit analasis software.

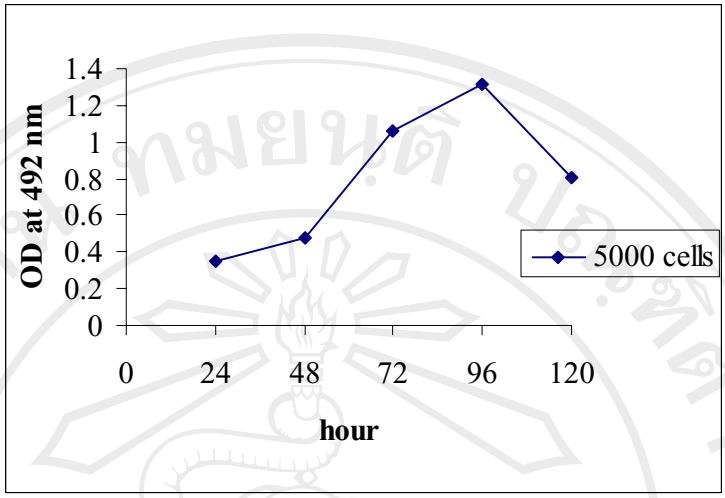

Figure 6.3 Growth profile of COR-L23 cell line with inoculum of 5000 cells/well

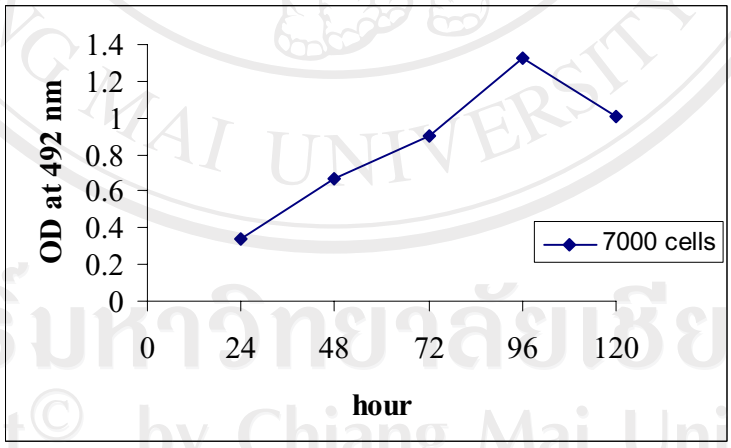

Figure 6.4 Growth profile of 3T3 cell line with inoculum of 7000 cells/well

Appendix H: Figures of the dye and cell before and after decolorization

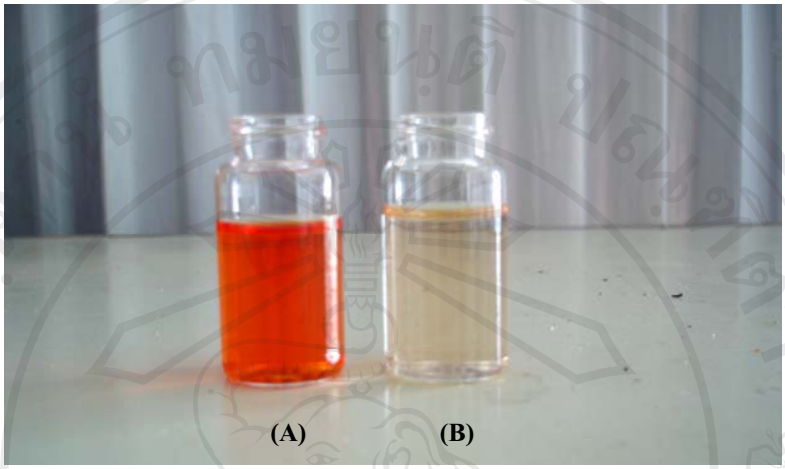

Figure 6.5 The color removal of synthetic wastewater

(A) Before decolorization

(B) After decolorization 24 h

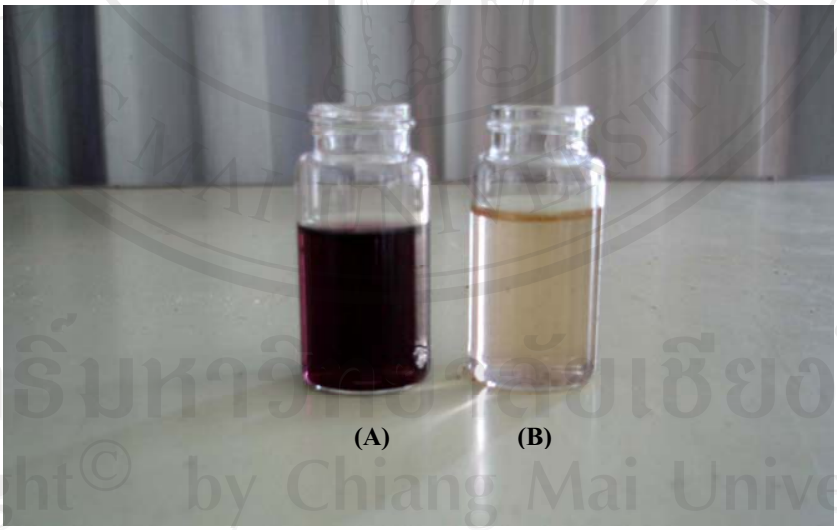

Figure 6.6 Color removal of real wastewater

(A) Before decolorization

(B) After decolorization in 48 h

Appendix I: Industrial Effluent Standards

Table 6.4 Industrial Effluent Standards

Parameters	Standard Values	Method for Examination
1. pH value	5.5-9.0	pH Meter
2. Total Dissolved Solids (TDS)	- not more than 3,000 mg/l depending on receiving water or type of industry under consideration of PCC but not exceed 5,000 mg/l - not more than 5,000 mg/l exceed TDS of receiving water having salinity of more than 2,000 mg/l or TDS of sea if discharge to sea	Dry Evaporation 103-105 °C, 1 hour
3. Suspended solids (SS)	not more than 50 mg/l depending on receiving water or type of industry or wastewater treatment system under consideration of PCC but not exceed 150 mg/l	Glass Fiber Filter Disc
4. Temperature	not more than 40°C	Thermometer during the sampling
5. Color and Odor	not objectionable	Not specified
6. Sulphide as H_2S	not more than 1.0 mg/l	Titrate
7. Cyanide as HCN	not more than 0.2 mg/l	Distillation and Pyridine Barbituric Acid Method

Table 6.4 (Continued)

Parameters	Standard Values	Method for Examination
8. Fat, Oil & Grease (FOG)	not more than 5.0 mg/l depending of receiving water or type of industry under consideration of PCC but not exceed 15.0 mg/l	Sovent Extraction by Weight
9. Formaldehyde	not more than 1.0 mg/l	Spectrophotometer
10.Phenols	not more than 1.0 mg/l	not more than 1.0 mg/l
11.Free Chlorine	not more than 1.0 mg/l	not more than 1.0 mg/l
12.Pesticides	not detectable	not detectable
13.Biochemical Oxygen Demand (BOD)	not more than 20 mg/l depending on receiving water or type of industry under consideration of PCC but not exceed 60 mg/l	-Azide Modification at 20 °C , 5 days
14.Total Kjedahl Nitrogen (TKN)	not more than 100 mg/l depending on receiving water or type of industry under consideration of PCC but not exceed 200 mg/l	Kjeldahl
15.Chemical Oxygen Demand (COD)	not more than 120 mg/l depending on receiving water of type of industry under consideration of PCC but not exceed 400 mg/l	Potassium Dichromate Digestion

Table 6.4 (Continued)

Parameters	Standard Values	Method for Examination
16.Heavy metals		Atomic Absorption Spectro Photometry; Direct Aspiration or Plasma Emission Spectroscopy ; Inductively Coupled Plama : ICP
1. Zinc (Zn)	not more than 5.0 mg/l	
2. Chromium (Hexavalent)	not more than 0.25 mg/l	
3. Chromium (Trivalent)	not more than 0.75 mg/l	
4. Copper (Cu)	not more than 2.0 mg/l	
5. Cadmium (Cd)	not more than 0.03 mg/l	
6. Barium (Ba)	not more than 1.0 mg/l	
7. Lead (Pb)	not more than 0.2 mg/l	
8. Nickel (Ni)	not more than 1.0 mg/l	
9. Manganese (Mn)	not more than 5.0 mg/l	
10. Arsenic (As)	not more than 0.25 mg/l	Atomic Absorption Spectrophotometry; Hydride Generation, or Plasma Emission Spectroscopy; Inductively Coupled Plasma : ICP
11. Selenium (Se)	not more than 0.02 mg/l	
12. Mercury (Hg)	not more than 0.005 mg/l	Atomic Absorption Cold Vapor Technique

Note: 1) PCC Pollution Control Committee

2) The standards were summarized from the Notification of the Ministry of Science, Technology and Environment, No. 3, B.E. 2539 (1996) and it specifies that pollution sources that the above standards are to be applied are factories group II and III issues under the Factory Act B.E.2535 (1992) and every kind of industrial estates.

3) Notification of the Pollution Control Committee, No. 3, B.E. 2539 (1996) dated August 20, B.E. 2539 (1996) has issued types of factories (category of factories issued under the Factory Act B.E.2535 (1992) that are allowed to discharge effluent having different standards from the Ministerial Notification No. 3 above as follows :

1. BOD up to 60 mg/l
- animal furnishing factories (category 4 (1))
- starch factories (category 9 (2))
- food from starch factories (category 10)
- textile factories (category 15)
- tanning factories (category 22)
- pulp and paper factories (category 29)
- chemical factories (category 42)
- pharmaceutical factories(category 46)
- frozen food factories (category 92)

2. COD up to 400 mg/l
- food furnishing factories (category 13 (2))
- animal food factories (category 15 (1))
- textile factories (category 22)
- pulp and paper factories (category 38)

3. TKN
- 100 mg/l - effective after 1 year from the date published in the Royal Government Gazette of the Ministerial Notification No. 4
- 200 mg/l - effective after 2 year from the date published in the Royal Government Gazette of the Ministerial Notification No. 4 for the following factories:
    1. food furnishing factories (category 13 (2))
    2. animal food factories (category 15 (1))

Source: Notification the Ministry of Science, 1996

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Marine and Freshwater Mycology Symposium at Chiang Mai University during November 14th-19th, 2004

Oral presentation in the topic "Decolorization of textile wastewater by immobilized *Coriolus versicolor* RC3 in repeated batch system with the effect of sugar addition " In 1st Chiang Mai University Conference at Chiang Mai University during December 8th -10th, 2005