Chapter 3

Methodology

This chapter deals with batch and continuous experiments to investigate the technical feasibility of using *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 immobilized on Polyurethane Foam (PUF) with the co-substrates addition at varying concentrations (1 to 3 g/l) for the removal of color, BOD and COD of landfill leachate from Nonthaburi solid waste disposal site. To maximize the removal efficiency of removals, batch experiments were carried out to find out the optimum conditions of pertinent factors such as pH, co-substrate dose and contact time, while continuous experiments were performed to evaluate the possibility of using the fungi mentioned previously for leachate treatment application.

3.1 Material and methods

All chemical solutions are certified lab-grade chemicals. All glassware were rinsed with distilled water. Before being used, they were dried in an oven and cooled for further used.

3.1.1 Reagent preparation

Reagents were prepared according to the Standard Methods for the Examination of Water and Wastewater, 20th Edition (1998) for the measurement of the parameters according to Table 3.1.

Table 3.1 Parameters for determining landfill leachate characteristics

Parameter	(Unit)	Analytical Method
pH	-	Standard Method Part 4500 B: Electrometric Method, pH meter (Glass Electrode)
Color	ADMI	ADMI Tristimulus Filter Method (Tristimulus Filter Method Standard Method 2120 E)
COD	mg/l	Standard Method Part 5220 C: Closed Reflux, Titrimetric Method, Dichromate method
BOD_5	mg/l	Standard Method Part 5210 B: 5-day BOD test
Suspended Solids (SS)	mg/l	Standard Method Part 2540 D: Total Suspended Solids Dried at 103-105ºC
NH_3-N	mg/l	Standard Method Part 4500-NH_3 C. Titrimetric Method
Heavy Metal concentrations : Ni, Cr, Cd, Pb, Hg	mg/l	Inductively Coupled Plasma Spectroscopy (ICP Optima 2100DV)

3.1.2 Leachate collection

Leachate was collected from different places and different seasons from Nonthaburi solid waste disposal site as follows:

1. Leachate 1 was collected from the large stabilization pond in May 2006 (1 year old).
2. Leachate 2 was collected from a pipe as discharged from landfill to the stabilization pond in December 2006.

3. Leachate 3 was collected from the garbage truck directly in Nonthaburi district in December 2006.
4. Leachate 4 was collected from a pipe as discharged from landfill to the stabilization pond in March 2008.
5. Leachate 4* was the leachate 4 which is kept in 4ºC room for 1 year.

Samples were collected in 20-L plastic carboys, transported to the laboratory and stored at 4ºC to avoid any decomposition for further experiments.

3.1.3 Fungi collection

T.versicolor BCC 8725 was obtained from BIOTECH Central Research Unit, Pathumthani, Thailand. The fungi were grown in culture tubes containing Potato Dextrose Agar (PDA) at 25ºC for 1 week.

3.1.4 Co-substrate preparation

Three co-substrates as glucose, cassava, and corn starch were used in this study. Glucose and corn starch were obtained commercially. Cassava was obtained in raw and then crushed, grounded and dried. Each co-substrate was kept in the vacuum box in order to protect from moisture that can accelerate the growth of living microorganisms. However, these co-substrates were autoclaved again before used.

3.1.5 Polyurethane Foam (PUF) preparation

Approximately 30,000 pieces of PUF were cut with the size of 1.0cm*1.0cm*1.0cm. The foam pieces were autoclaved before use.

3.2 Experimental Procedure

3.2.1 Sub-culture of Fungi

Potato Dextrose Agar (PDA) was used as a culture media. The boiled agar was poured into culture tubes with caps. The tubes were then kept in autoclave at 121ºC for 15 min and cooled until solidified. The fungi were spread over the PDA in the culture tube. Temperature was controlled at 25ºC for 1 week. Sub-culture was done once a week to obtain active fungi.

3.2.2 Leachate characterization before treatment

The leachate was filtered to remove suspended solids before measurement and was analyzed for pH, COD, BOD_5, SS, NH_3-N according to the Standard Methods for the Examination of Water and Wastewater (1998). The heavy metals: Ni, Cr, Cd, Pb, and Hg, were analyzed using the Inductively Coupled Plasma Mass Spectrometry (ICP/MS Optima 2100DV). The color of the leachate was measured by ADMI Tristimulus Filter Method using Hewlett Packard Spectrophotometer Model 8452A Diode Array.

3.2.3 Mycelial suspension and immobilization of fungi on PUF

Mycelial suspension: Mycelial suspension was prepared by punching four pieces of fungi from the culture tube by using the sterile loop in 100 mL of sterile Potato Dextrose Broth (PDB). Cotton plugs were used to close the flasks which were agitated for 24 hours on a rotary shaker at 150 rpm.

Polyurethane foam (PUF) Sterilization: 80 pieces of PUF were first put into the 1,000 mL PDB in 2,000 mL flasks so that PUF is not too crowded. The flasks were plugged with the cotton and then sterilized in autoclave for 15 minutes prior to use.

Fungi Immobilization on PUF: To 1,000 mL of PDB containing 80 pieces of PUF, 100 mL of mycelial suspension was added in a 2,000 mL flask. The flasks were kept at the ambient temperature (30-33ºC). The PUF were covered with fungal mycelium within 4 days. However, the effects of fungi immobilization on PUF for 4 and 15 days were investigated to see the effect of initial biomass growth on removal efficiency. Ten pieces of PUF with immobilized fungi were used for each varying condition.

Fungi Mobilization: To 1000 ml of PDB, 100 ml of mycelial suspension was added in 2,000 ml flask. Cotton plug was used to close the flask. The flask was incubated for 4 days at the ambient temperature and was agitated for 24 hours at 180 rpm to form fungal balls.

3.2.4 Analytical assays

a) Scanning Electron Microscope (SEM) image

For SEM imaging, the immobilization of *T. versicolor* BCC 8725 on PUF cubes was done with 2% (v/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 2 hours. The specimens were rinsed twice in phosphate buffer for 10 min/each and once in distilled water for 10 min. The specimens were then dehydrated with a graded series of ethanol (10%, 20%, 30%, 50%, 70%, and 95%) for 10 mins each and absolute ethanol for 3 times (10 min/time) by using Critical Point Dryer, Balzers model CPD 020). The dry cubes were mounted and coated with gold using Sputter Coater, Balzers model SCD 040) and observed under a scanning electron microscope (JEOL, model JSM-6400).

b) Enzyme assays

Lignin Peroxidase (LiP) (EC 1.11.1.14)

The assay was done by monitoring the oxidation of dye Azure B in the presence of H_2O_2 (Archibald, 1992; Arora and Gill, 2001). The reaction mixture contained (final concentration) sodium tartarate buffer (50 mM, pH 3.0), Azure B (32 μM), H_2O_2 (100 μM) and 0.5 ml of enzyme extract. The reaction is initiated by adding 0.5 ml of H_2O_2. One unit of enzyme activity is equivalent to an absorbance decrease of 0.1 unitsmin^{-1} ml^{-1}.

Mn peroxidase (MnP) (EC 1.11.1.13)

MnP assay is based on the oxidation of phenol red (Orth et al., 1993). Five ml of reaction mixture contained 1.0 ml sodium succinate buffer (50 mM, pH 4.5), 1.0 ml sodium lactate (50 mM, pH 5.0), 0.4 ml manganese sulphate (0.1 mM), 0.7 ml phenol red (0.1 mM), 0.4 mlH_2O_2 (50 μM), gelatin 1 $mgml^{-1}$ and 0.5 ml of enzyme extract. The reaction was initiated by adding H_2O_2 and conducted at 30°C. One ml of reaction mixture was taken and

40 µl of 5 N NaOH was added to it. Absorbance was taken at 610 nm. After every minute the same steps were repeated with 1 ml of the reaction mixture up to 4 min. One unit of enzyme activity is equivalent to an absorbance increase of 0.1 unitsmin^{-1} ml^{-1}.

Laccase (Lac) (EC 1.10.3.2)

Laccase production was assessed by measurement of enzymic oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\in$ = 3.6×10^4 cm^{-1} M^{-1}) (Bourbannais and Paice, 1988). The reaction mixture contained 300 µL of extracellular fluid, 300 µL of 1 mM ABTS and 0.1 M Na Acetate buffer (pH 4.5). Oxidation of ABTS was measured by determining the increase in absorbance of the mixture on a Shimadzu UV-160 spectrophotometer at 420 nm. One Unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol ABTS in 1 min. To determine the error associated with this technique, 3 samples were run 10 times each. A variance of less than 10% was found and in light of the high degree of confidence associated with this assay, all subsequent samples were done in duplicate.

3.2.5 Batch experiment

Batch experiments were conducted to find out the optimum pH, optimum dose and optimum contact time for each co-substrate. Optimum conditions are defined as conditions where maximum color removal was achieved.

a) Biomass growth of immobilized and mobilized fungi

To find the biomass growth, 10 pieces of polyurethane foam covered with fungal mycelium after four days of initial growth in PDB (as above) were placed in 100 mL of leachate. The growth of fungi was determined everyday by weighing the flask and comparing it with the initial weight for 15 days. This provided the biomass growth in both attached and suspended form. Co-substrates (glucose, corn starch and cassava) at different concentrations were also added to see the effect on growth of fungi. For mobilized fungi, the wet weight of the fungal mass was determined by weighing the total weight of flasks with the fungal ball and the leachate sample and comparing them with the weight of flasks before the fungal mobilization until the weight is constant. However, the batch experiment with mobilized fungi was conducted with leachate 3 only. In addition, co-substrates (glucose, corn starch and cassava) at 3 g/L and one without co-substrate were also varied to see the effect on growth of fungi. Experiments were carried out for 15 days. All processes were done under sterile conditions at ambient air temperature.

b) Effect of pH on color removal

Ten pieces of PUF with immobilized fungi were put in each flask containing 100 ml of leachate. The pH in each flask was varied by using 1N NaOH and 1N HCl solution to pH 3, 4, 5 and one flask was used as a control. All flasks were shaken at 150 rpm for 24 hours. The flasks were plugged with cotton and covered with aluminum foil. Experiments were carried out for 1 to 5 days and color removal was observed. The changes in pH were observed and recorded daily. All processes were done under sterile conditions and at ambient temperature. Effects of pH on color removal experiment were conducted with leachate 1, 2, and 3 only.

c) Effect of co-substrates concentration and contact time

Leachate (100 ml) was adjusted to the optimum pH of 4 and 10 pieces of PUF were then added. Concentration of co-substrate (glucose, cassava, and corn starch) was varied at 1, 2, 3 g/l. For glucose, higher co-substrate concentrations at 4 and 5 g/l were also tested. In one flask, no co-substrate was added (control). All flasks were shaken at 150 rpm for 15 days to find the effect of contact time. The leachate was filtered and color removal was monitored.

d) Reuse of immobilized *T.versicolor* BCC 8725

The reuse of immobilized fungi experiment was conducted with leachate 3 only. This was carried out to investigate the efficiency of reusing immobilized fungi, *T.versicolor* BCC 8725, with 3 g/l of each co-substrate. New leachate was introduced into the batch reactor after 7 days (cycle). Color removal was observed throughout the experiment.

3.2.6 Continuous experiment

Batch experiments were used for preliminary investigations and finding the operational parameters, but in practice, technical systems normally used continuous experiment to obtain a factual design model of the system.

Continuous experiments were conducted to evaluate a flow-through system at optimum condition found from batch experiment as well as its potential for actual operation with *T. versicolor* BCC 8725 with glucose 3 g/L as a co-substrate.

a) Continuous operation

An assembly of the reactor was constructed with acrylic sheet with the inner dimension of 5 cm width, 28.7 cm length and 35 cm height. Three baffles were put as partitions in each reactor to have an up-down flow as shown in Figure 3.2 in order to increase the contact time. Each baffle was 2.9 mm thick and baffles were put closely to the bottom to allow only the liquid (not the PUF immobilized fungi) to pass through. The water level was maintained 5 cm below the top so the liquid volume is 3L. Small hole was made on the top for PUF sampling to measure the biomass growth of fungi. Air diffusers were put at the bottom of each compartment to provide sufficient air. The reactor was packed with immobilized fungi attached on PUF (200 pieces) in each compartment (total 800 PUF).

To study the effect of organic loading on decolorization efficiency, experiments were conducted with 5-times diluted leachate and concentrated leachate. The influent (raw leachate) with an additional carbon source (glucose 3g/L for concentrated leachate and 600 mg/L for 5 times diluted leachate) and adjusted pH (pH=4) was fed continuously into the reactor from the top which flowed down and up in a zigzag direction until the effluent was out from the bioreactor to provide sufficient contact time for reaction. The incoming flow rate of leachate was adjusted to 0.4 mL/min so that the retention time is 5 days. Treated leachate was continuously recycled (at the rate of 0.4 mL/min) and the remaining was an effluent (0.4 mL/min) while retaining the fungus. According to Figure 1, the influent flow is 0.4 ml/min and effluent flow is equal to influent flow. Although the recycle leachate is 0.4 ml/min, the influent flow to reactor after one cycle is 0.8 ml/min. In this condition, the influent flow would increase and the contact time will decrease, but the influent loading is

reduced as the leachate gets diluted due to recycling of treated leachate. Throughout the experiment, treated leachate was analyzed at the outlet of the bioreactor for color, BOD and COD reduction. The experiment was performed at ambient temperature (30-33ºC) and continued until removal efficiency became almost stable. Each cycle was run for 5 days. New leachate was introduced in the next cycles using the same immobilized fungi to study the efficiency of reuse of fungi in consecutive cycles. The continuous bioreactor with leachate recirculation is shown in Figure 3.2.

Reactor design

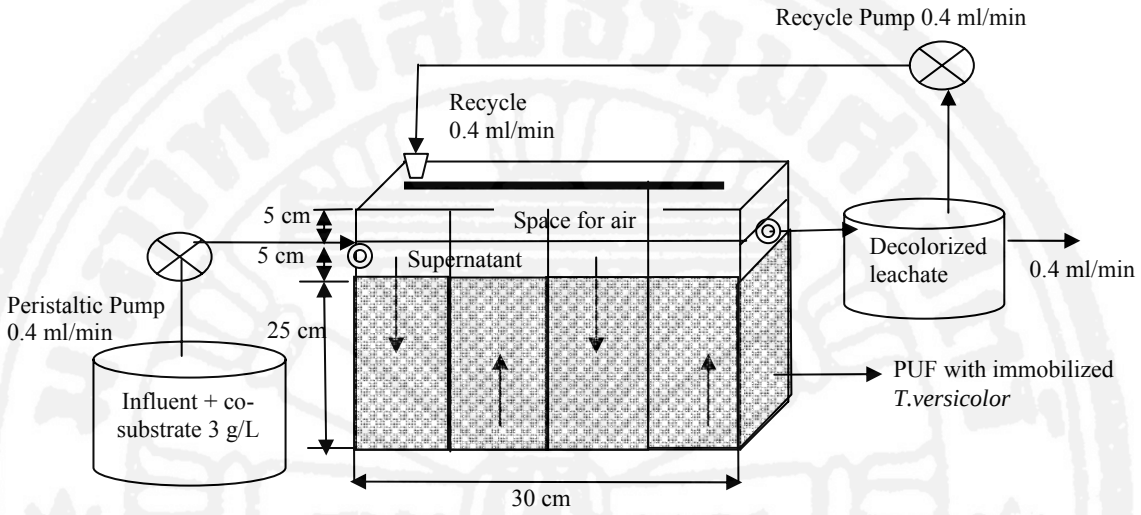

Figure 3.1 Vertical flow baffled bioreactor

b) Biomass growth of immobilized *T.versicolor* BCC 8725 (continuous experiment)

After fungal immobilization, the dry weight (dried at 80°C for 48 h) of one piece out of 800 pieces of immobilized fungi on PUF was measured and used as a representative. Dry weight of one piece of PUF with immobilized fungi was also measured after each cycle of 5 days and was compared with initial weight to find the biomass growth. Suspended fungal cells that were not attached were not counted. All processes were done under sterile condition at ambient air temperature (30-33°C).

3.2.7 Determination of leachate characteristics at optimum condition

Removal of color, BOD, COD, and heavy metals were investigated after fungal treatment and the results were compared with the initial value.

The overview of the preparation process and the overview of the experiment procedures are shown in Figure 3.3 and 3.4.

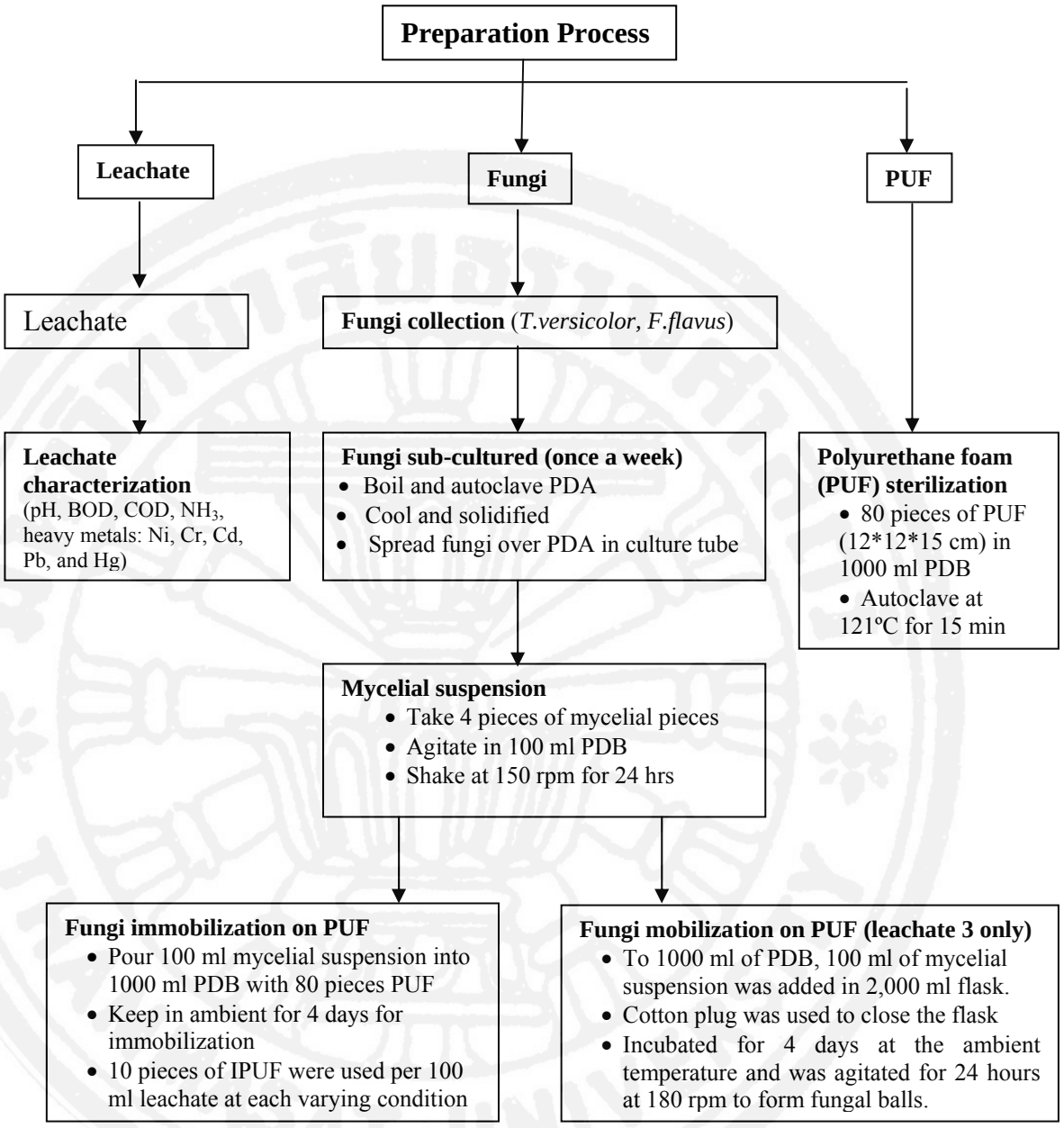

Figure 3.2 The overview of the preparation process

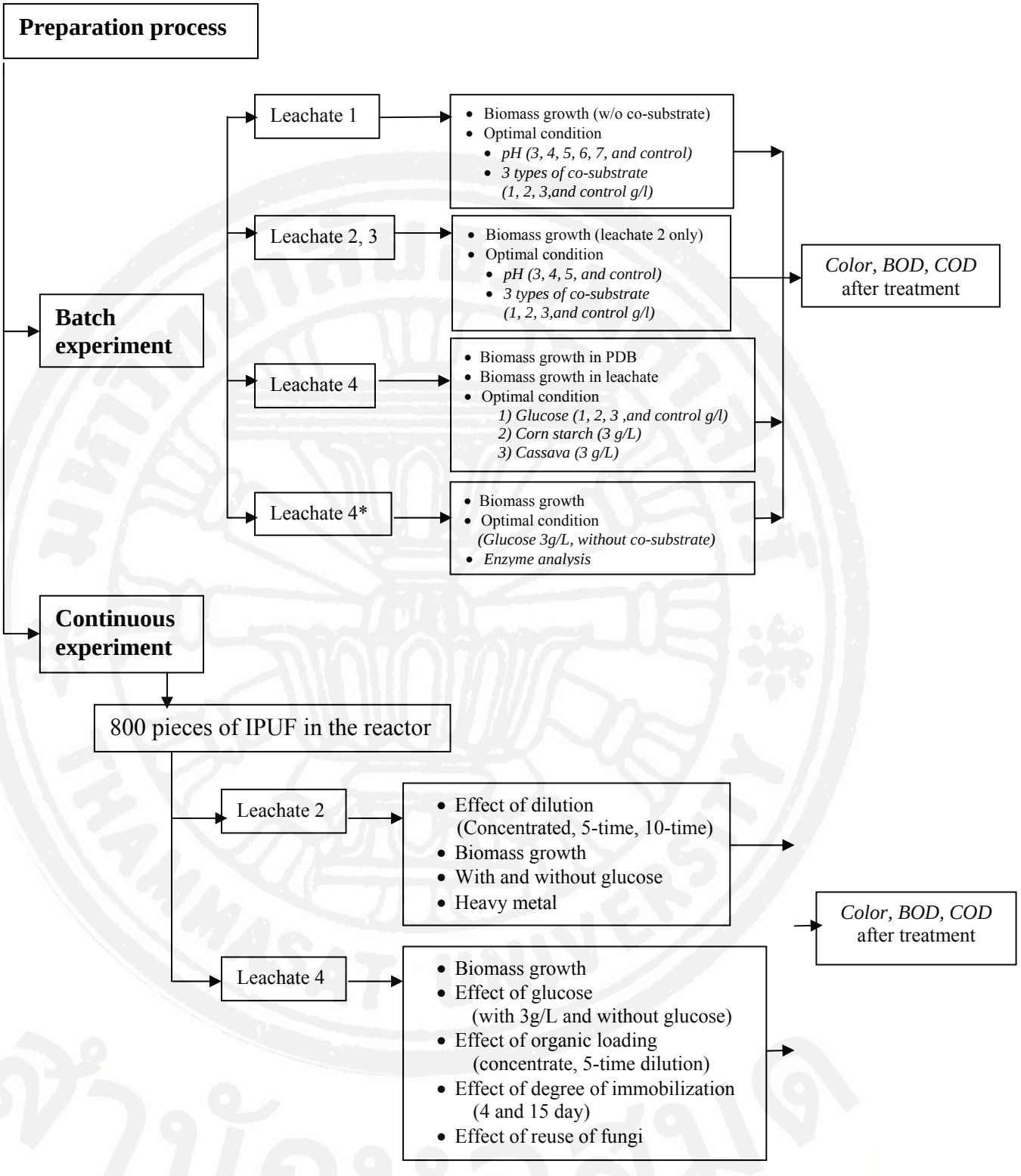

Figure 3.3 The overview of the experiment procedures