CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganism

Candida tropicalis CMU 10 was isolated from a soil sample and identified based on the biochemical characteristics by Faculty of Associated Medical Science, Chiang Mai University. The yeast culture was maintained on a mineral salt agar slope at 4 ºC prior to a regular sub-culturing on monthly basis.

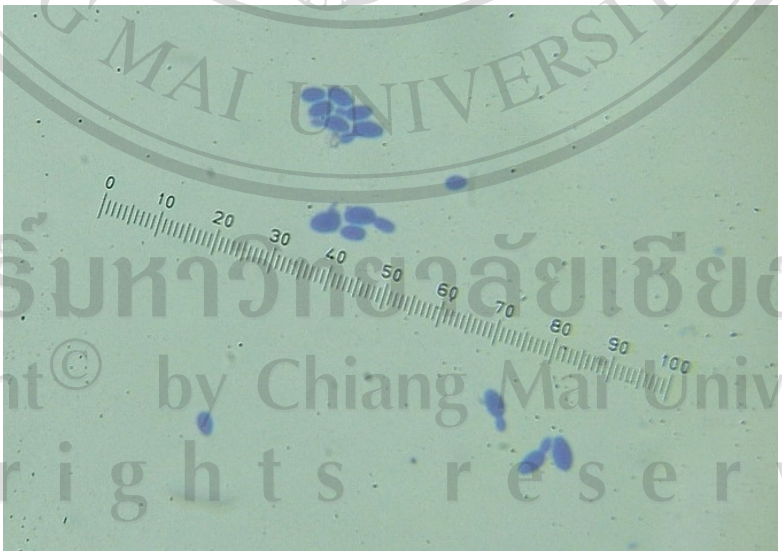

Figure 3.1 Morphology of *C. tropicalis* CMU 10 under light microscope.

3.2 Media

3.2.1 The medium for maintenance of phenol-degrading microorganism was mineral salt (MS) agar (Appendix A1).

3.2.2 The medium for cultivation of phenol-degrading microorganism was mineral salt (MS) broth (Appendix A2).

3.2.3 The medium for inoculum preparation was yeast malt (YM) broth (Appendix A3).

3.2.4 Phenol solution

A phenol stock solution was prepared by dissolving phenol pellets in deionized water (Appendix A4). The stock solution was added to the growth medium according to a specified concentration.

3.3 Inoculum preparation

C. tropicalis CMU 10 was grown in YM broth containing 200 mg/l phenol at 37 ºC in a shaking condition of 120 rpm for 24 h.

3.4 Phenol degradation experiments

3.4.1 Selection of optimal temperature for microbial growth

One hundred milliliters of the MS medium with 100 mg/l phenol in 250 ml Erlenmeyer flasks were inoculated with 1 ml inoculum and incubated at 20, 30, 37, 40, 42 and 45 ºC with shaking speed of 120 rpm. Samples were taken every 3 h. All experiments were done in triplicate and the average values were reported.

3.4.2 Effect of physical and nutritional

Unless otherwise stated, the 100 ml volumes of the MS medium with 1,000 mg/l phenol in 250 ml Erlenmeyer flasks were inoculated with 1 ml inoculum and subjected to the following factors.

3.4.2.1 Effect of phenol concentration : The medium contained 350, 500, 750, 1000 and 1250 mg/l phenol

3.4.2.2 Effect of pH level : The initial pH of the medium was adjusted to 5, 6, 7, 8 and 9.

3.4.2.3 Effect of glucose concentration : The medium contained 0, 0.16, 0.31, 2.5, 5 and 10 mM glucose.

3.4.2.4 Effect of organic acids : The medium contained 10 mM of citric, lactic, malic and succinic acids.

3.4.2.5 Effect of metal ions : The medium contained 0.04 mM of Co^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Zn^{2+} ions.

The culture was incubated at 37ºC with shaking speed of 120 rpm. Samples were taken every 24 h. All experiments were done in triplicate and the average values were reported.

3.5 Cell immobilization procedure

C. tropicalis CMU 10 was harvested after 12 h of growth in 1 litre of YM medium. The cells pellet obtained by centrifugation at 6000 rpm for 10 min at 4 ºC was used for immobilization in alginate and agar matrices.

The cell pellet was resuspended in a physiological saline (0.85% NaCl) and the corresponding suspension was adjusted to 4.0 unit of optical density measured at 600 nm (OD600). This was followed by mixing with 4% (w/v) sodium alginate in the ratio of 1 to 3. The mixture was added drop-wise to a 0.2 M $CaCl_2$ solution to form a spherical beads of alginate entrapped cells. The gel beads formed were left in the solution for 1 h prior to separation by filtration and washed with deionized water before experimental uses.

Agar entrapment of cells was carried out in sterile 4% (w/v) agar saline solution at 50°C. The cell suspension was mixed with 4% agar in the ratio of 1 to 3. The final mixture was poured into petri dishes and allowed to solidify. The gel was then cut in to pieces with an average volume of 0.025 cm^3.

3.6 Repeated batch degradation of phenol

The MS media were inoculated with free cells (0.5 g dry weight/300 ml) and immobilized cells (50 g of beads/300 ml). The effect of initial phenol concentrations on biodegradation rate in a repeated batch mode was investigated at 37 °C and pH 8.

The first repeated batch experiment was started using 300 ml cultivation medium containing 1,000 mg/l phenol. In the subsequent batch, when phenol was almost consumed, 70% of the liquid volume in that particular sequence was exchanged for the equal volume of fresh medium containing 250 mg/l higher phenol concentration than the former sequence.

3.7 Analytical methods

3.7.1 Determination of phenol concentration

Phenol was estimated by a direct photometric method (Greenberg *et al*., 1992), which was based on a rapid condensation with 4-aminoantipyrine, followed by oxidation with potassium ferricyanide under alkaline conditions to give a red colour product (Appendix B). The absorbance of sample was measured at 500 nm and phenol quantity was calculated from a standard calibration curve (Appendix C2).

3.7.2 Determination of cell density

The suspended biomass concentration was determined by a UV spectrophotometer (Shimadzu 1601, Japan). The absorbance of each sample was measured at 600 nm and the biomass concentration was calculated from a standard calibration curve (Appendix C1).

3.7.3 Determination of pH level

pHs of samples were measured using pH-meter (Cyberscan 500)

3.7.4 Calculation of average phenol degradation rate

The average rate of phenol degradation was calculated by dividing the total amount of phenol consumed with time required for total consumption of phenol (Kar *et al*., 1996).

$$\text{Average phenol degradation rate} = \frac{\text{Amount of phenol consumed}}{\text{Time required for complete consumption of phenol}}$$