CHAPTER III

MATERIALS AND METHODS

3.1 Sources of microorganisms

Twenty seven strains of identified bacteria, *Bacillus subtilis,* were provided by N. Maneechote and 16 isolates of denitrifying bacteria were from K. Smartivutikoon. In addition, bacteria isolated from other sources in this study were from decomposed vegetables, soil around food waste dumping area, soil around hot spring areas from Sankampang, Chiang Mai, Thailand and the compost obtained from The Royal Project, Chitralada Palace.

3.2 Culture and selective media

3.2.1 Media for cellulase activity

The solid medium for screening cellulase producing bacteria (CMC agar, Hankin and Anagnostakis, 1977) was composed of (per Liter): $(NH_4)_2SO_4$, 1 g; CMC (carboxymethylcellulose), 5 g; Yeast extract, 1 g and agar, 10 g. The cellulase enzyme production medium (Krishna, 1999) was composed of (per Liter): Na_2HPO_4. $7H_2O$, 1.18 g; KH_2PO_4, 0.9 g; NaNO3, 1 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; Yeast extract, 0.5 g; casein hydrolyzate, 0.5 g and 10 g of cellulose powder, pH 7.0.

3.2.2 Media for protease activity

The medium used for screening protease producing bacteria was composed of (per Liter): peptone, 5 g; yeast extract, 3 g; skim milk, 12.5 g and agar, 12 g. Colonies producing clear zone were collected. The medium for protease production was composed of (per Liter): peptone 10 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.4 g; $MnSO_4.H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g and yeast extract, 1 g; at pH 7.0 (Bayoudh, 2000).

3.2.3 Media for lipase activity

Solid medium for screening lipase-producing bacteria (Kouker and Jaeger, 1987) was composed of (per Liter): nutrient broth, 8 g; NaCl, 4 g; rhodamine B 0.01 g; olive oil, 25 g and agar, 10 g; pH 7.0. The liquid medium was composed of ($g.L^{-1}$): soybean meal, 20 g; peptone, 10 g; soluble starch, 10 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 1 g and $CaCO_3$, 5 g.

3.3 Chemicals and instruments

3.3.1 Chemicals

di-Ammonium sulfate ($(NH_4)_2SO_4$) (Merck, Germany)

3% Hydrogen peroxide solution (Vitayasom, Thailand)

Agar powder (Agar-agar, Chili)

Avicel, PH 101 (Fluka, Ireland)

Calcium carbonate ($CaCO_3$) (APS, Australia)

Carboxymethylcellulose (CMC) (Merck, Germany)

Casein hydrolyzate (Merck, Germany)

Congo-red (APS, Australia)

di-Sodium hydrogen phosphate anhydrous, Na_2HPO_4. (Carlo, Italy)

Ferrous sulfate hecta hydrate ($FeSO_4.7H_2O$) (Merck, Germany)

Peptone (Merck, Germany)

Polyvinyl alcohol (Carlo, Italy)

di-Potassium hydrogen phosphate (K_2HPO_4) (Carlo, Italy)

Potassium chloride (KCl) (Merck, Germany)

Magnesium sulfate hecta hydrate ($MgSO_4.7.H_2O$) (Merck, Germany)

Manganese sulfate mono hydrate ($MnSO_4.H_2O$) (Merck, Germany)

Nutrient agar (NA) (Schalau, Spain)

Nutrient broth (NB) (Difco, USA)

Olive oil pure solution (CO-PH, Thailand)

Rhodamine B (APS, Australia)

Sodium chloride (NaCl) (Carlo, Italy)

Sodium hydroxide (NaOH) (Carlo, Italy)

Soluble starch (Himedia, India)

Sulfanilic acid (Schalau, Spain)

Tetramethyl-para-phenylenediamine dihydrochloride (TMPD) (Merck, Germany)

Yeast extract (Schalau, Spain)

3.3.2 Instruments

1. Hot air oven (Binder, Germany)
2. Balancer, model BP 2100S (Sartorius, Sweden)
3. Shaker, 1010 (Heidolph Instrument, Germany)
4. pH Meter, model 250 (Denver Instrument. Ltd., Co., Germany)
5. Autoclave (Diamond, Taiwan)
6. Laminar flow, Clean Model (Lab service ltd., part, Thailand)
7. Spectrophotometer, model DR/2010 (Hach, USA)
8. Incubator (EHRET, Germany)
9. Microscope (Olympus, Japan)
10. Centrifuge (Hettech, Germany)
11. Spectrophotometer, model DR/2010 (Hach, USA)

3.4 Screening of bacteria

Bacteria from all 4 types of samples, namely solid waste, soil from food dumping area, hot spring areas and compost, were similarly screened with the same method. Ten grams of each sample was separately placed in 100 ml of sterile water with constant stirring to obtain the homogenous solution. Then the samples were subjected to serial dilution to 10^{-6} fold concentration. The diluted solutions were spread on agar plate suitable for each enzyme activity assay. The spread plates were incubated at room temperature (28°C) for 24 hours. In addition, the bacteria provided by N. Maneechote and K. Smartivutikoon were screened for three types of enzymes activities. The colonies were later selected for each activity assay as follows.

3.4.1 Cellulase-producing colonies

The diluted solutions were spread on CMC agar and incubated for 24 - 48 hours. (Hankin and Anagnostakis, 1977 cited in Moungkaew, 2001). Approximately 5 ml of congo red were poured on the medium and left stand for 1 hour. Then the clear zones were observed by measuring the ratio of clear zones and colonies and the colonies were picked. Pure cultures were grown on nutrient agar for further activity testing.

3.4.2 Protease-producing colonies

The diluted solutions were spread on skim milk agar following the method of Bayoudh *et al* (2000). The clear zones producing colonies were selected for further assay.

3.4.3 Lipase-producing colonies

The diluted solutions were spread on Rhodamine B agar following the method of Kouker and Jaeger (1987). After 24 hours of incubation, the plates were placed under the UV lamp for the detection of the fluorescence colonies. The orange fluorescence colonies were then picked for further assay.

3.5 Assay of enzyme activity

3.5.1 Cellulase activity

The method follows that of Chen *et al* (2004). The selected colonies were subcultured in the nutrient broth for 24 hours at 28°C. Cultivations were conducted in 50 ml of cellulase enzyme production medium in 250 ml conical flasks. Then, they were continuously shaken at room temperature (28°C) for 72 hours. The cultured media were later centrifuged at 8,000 rpm for 20 minutes and the supernatants were collected for the assay of cellulase activity. The reducing sugar concentrations in the solution were quantitatively measured by the DNS methods as described by Ghose (1987). Firstly, 0.5 ml crude enzyme was added to 0.5 ml of 2% CMC solution used as substrate. The sample was incubated in waterbath at 50°C for 60 minutes. The reaction was stopped with 3 ml DNS (3, 5-dinitrosalicylic acid) and then boiled up for 5 minutes. The sample was spectrophotometrically determined for the reducing sugar content at 540 nm compared to the glucose standard. The reaction without crude enzyme was used as blank. One unit of cellulase activity equals one micromole per minute of glucose converted. The activity of enzyme was calculated as

$$\text{Cellulase activities} = \frac{0.0926}{\text{Enzyme concentration to release 1.0 mg glucose}} \text{ units/ml}$$

3.5.2 Protease activity

The method follows that of Bayoudh, *et al* (2000). The selected colonies were separately subcultured in 50 ml nutrient broth and grown for 24 hours at 28°C. The cultivations were conducted in 50 ml protease production medium for 24 hours. at 28°C. Then the cultures were centrifuged at 8,000 rpm for 20 minutes and the supernatants were collected for the protease assay using the azocasein method (Samel *et al,* 1990). Twenty µl of crude enzymes were incubated at 30°C for 30 minutes in a mixture containing 230 µl of 2% (w/v) azocasein, and 150 µl of 0.2 M tris buffer pH 7.0. To terminate the reaction, 1.2 ml of 10% trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 minutes at room temperature. Then 1.4 ml of 1M NaOH was added and the solution was mixed thoroughly prior to measuring the absorbance at 440 nm. One unit enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 1.0 in 60 minute. The reaction without crude enzyme was used as blank.

3.5.3 Lipase activity

The method follows that of Sztajer and Maliszewska (1988). The selected colonies were separately subcultured in 50 ml nutrient broth and incubated for 24 hours at 28°C. The cultivations were conducted in 50 ml lipase production medium for 72 hours at 28°C, then the cultures were centrifuged at 8,000 rpm for 20 minutes and the supernatants were collected for the lipase assay. The 10 ml reaction mixture consisted of 5 ml olive oil- polyvinyl alcohol (PVA) emulsion (10% (v/v) olive oil in 2% PVA), 4 ml of 0.05 M Tris-HCl buffer (pH 7.0), and 1 ml of culture supernatant. The mixture was incubated at 30°C for 30 minutes with continuous shaking. Enzyme reaction was terminated by addition of 20 ml of acetone-ethanol mixture (1:1 v/v). Released acids were titrated with 0.05 M NaOH in the presence of thymolphthalein. All experiments were carried out in triplicates. One unit of lipase activity was defined as the amount that liberated 1 μmol of free fatty acid per minute under the experimental conditions. The reaction without crude enzyme was used as blank.

3.5.4 Protein concentration

Protein concentration was determined by the method of Lowry et al (1951) using bovine serum albumin (BSA) as the standard.

3.6 Identification of selected colonies

3.6.1 Characterization of bacterial colonies and cells

The selected colonies were grown on nutrient agar at room temperature (28 °C) for 24 hours. The characters of colonies were observed and the morphology of bacterial cell were observed under light microscope at 1000 times magnification.

3.6.2 Chemical test

3.6.2.1 Gram stain

The selected colonies were smeared on glass slide and stained with crystal violet solution (85% dye) then left at room temperature for 1 minute. Then samples were later stained with iodine solution and destained with ethanol alcohol 95 %, and stained again with safranin O solution and observed under the light microscope for gram detection.

3.6.2.2 Oxidase test

One-half ml of 1% TMPD was dropped on the filter paper and the selected colonies were streaked to observe the purple color of the colonies along the streaked line.

3.6.2.3 Catalase test

The selected colonies were separately smeared on a glass slide and 2 drops of 3% H_2O_2 were dropped on the slide. The gas production was detected.

3.6.3 Biochemical test

The selected colonies were subcultured on nutrient agar and in nutrient broth for biochemical tests following the methods of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) which consisted of the followings:

3.6.3.1 Simmon's citrate test
3.6.3.2 Urease test
3.6.3.3 Nitrate / nitrogen gas production
3.6.3.4 Esculin hydrolysis
3.6.3.5 Voges Prosauer test
3.6.3.6 Gelatinase test
3.6.3.7 Glucose assimilation
3.6.3.8 Mannitol utilization test
3.6.3.9 D-Xylose utilization
3.6.3.10 Rhamnose utilization
3.6.3.11 L-Arabinose utilization
3.6.3.12 Salicin utilization
3.6.3.13 Arginine decarboxylase
3.6.3.14 Adonitol utilization
3.6.3.15 Sucrose utilization
3.6.3.16 Egg yolk
3.6.3.17 Ornithine Decarboxylase
3.6.3.18 TSI/H_2S
3.6.3.19 Acetate
3.6.3.20 Fructose utilization

3.7 Antagonistic test of selected bacteria

Selected of bacteria were streaked across each other on nutrient agar plate. Bacterial antagonism was detected from the colonies of bacteria that inhibited growth of the others.

3.8 Solid waste composting with selected bacteria

3.8.1 Substrate preparation

Vegetables, pork and used oil (palm oil for cooking) were mixed in the ratio of 1:1:1 w/w, representing cellulose, protein and lipid sources, namely synthetic waste. The kitchen wastes were used as home food waste. Substrates were cut into small pieces. Autoclaved substrates were used for control.

3.8.2 Bacterial preparation

Twelve isolates of bacteria were selected for biofertilizer production containing 4 isolates of cellulase producing bacteria, 4 isolates of protease producing bacteria and 4 isolates of lipase producing bacteria. The selected bacteria were grown in 100 ml of nutrient broth in 250 ml conical flasks, shaken 150 rpm for 24 hours and the growth of the bacteria was observed by measuring the absorbance at 540 nm.

3.8.3 Biofertilizer production

Twelve isolates of selected colonies of bacteria were mixed together and inoculated to each pot containing 3 kg of substrates (1:1:1 w/w of vegetables, pork and used oil). The experiment consisted of 6 pots (treatments);

Pot 1: home food waste;

Pot 2: home food waste inoculated with mixed culture of bacteria;

Pot 3: Synthetic waste composed of cellulose: protein: lipid in the ratio of 1:1:1 w/w;

Pot 4: Synthetic waste inoculated with mixed cultured of bacteria;

Pot 5: Autoclaved synthetic waste;

Pot 6: Autoclaved synthetic waste inoculated with mixed cultured of bacteria.

All treatments were conducted in the closed pot system and were located at the green house.

3.8.4 Termination of composting

The composts were left stand for 30 days. During that time, they were stirred every 2 days for 1 week and every 5 days after 1 week. The temperature and pH in the biofertilizer pots were measured every 3 and 5 days, respectively. After 30 days of composting, moisture content, C:N ratio and nutritional values (nitrogen, phosphorus and potassium) were measured (Thammakhate, 1997 cited in Moungkaew, 2001).

3.9 Test of the biofertilizer efficiency

3.9.1 Soil and plant preparation

Soils were autoclaved at 121°C and 15 pound/square inch for 20 minutes. Testing plant was Chinese spinach (Slender amaranth; *Amaranthus viridis* L). One-week seedlings were transplanted to the 6-inch diameter pots with 500 g soil.

3.9.2 Testing of the biofertilizer

The experiment designed was randomized block design (RBD) with 8 replicates. The biofertilizers were diluted in water at the ratio of 1:500 (recommended by Land Development Department). One hundred and fifty ml of diluted liquid biofertilizer were provided to the plants every morning after 1 week of transplanting. The experiments consisted of 7 treatments;

A: Diluted liquid biofertilizer pot 1

B: Diluted liquid biofertilizer pot 2

C: Diluted liquid biofertilizer pot 3

D: Diluted liquid biofertilizer pot 4

E: Diluted liquid biofertilizer pot 5

F: Diluted liquid biofertilizer pot 6

G: Control (water)

Plant heights were measured every 3 days. After 30 days of planting, all plants were harvested and fresh weight was measured. The data analysis was analyzed by ANOVA using program SPSS version 10.0.