CHAPTER 3

MATERIALS AND METHODS

3.1 Composting

Matured compost was prepared by composting a mixture of 75% grass clippings and 25% cow dung (Navarani, 2009; Siti Aishah; 2011). The materials were uniformly mixed to ensure even distribution of microbes for optimum composting. Heap method was used and composting was carried out under a shade. Water was added to the compost mixture to maintain the moisture level at 60 %. Aerobic condition was maintained by manual turning of composting mixture with daily mixing for the first 8 days and then mixing on alternate days. Temperature of the composting mixture was measured daily using electronic thermometer (Model Oregon Scientific SA880SSX). The moisture content was determined gravimetrically by oven drying compost at 104°C for 24 hours and expressed as the mass ratio of water to drying compost, following the ASTM (2004) procedure. The pH of the compost was measured using the pH meter model HANNA HI 8424. Further Chemical Analysis was conducted on the compost in duplicates according to the standard procedures as described in the results.

Plate 3.1: Matured Compost from grass clippings

3.2 Isolation of methanotrophs.

A modified Nitrate Mineral Salt (NMS) medium was used. The medium contained the following, per 1,000 ml of distilled water: $NaNO_3$, 850 mg; K_2SO_4, 170 mg; $MgSO_4 \cdot 7H_2O$, 37 mg; and $CaCl_2 \cdot 6H_2O$, 7 mg. After autoclaving, 10 ml of phosphate buffer solution, 0.5 ml of trace element solution, and 1 ml of iron solution were added. The trace element solution (pH 4.0) contained the following, per liter of distilled water: $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2 \cdot 4H_2O$, 3 mg; H_3BO_4, 30 mg; $Na_2MoO_4 \cdot 2H_2O$, 3 mg; $CaCl_2 \cdot 6H_2O$, 20 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; and $CuCl_2 \cdot 2H_2O$, 1 mg. The phosphate buffer contained 1.4 g of KH_2PO_4 and 3.6 g of Na_2HPO_4 in 100 ml of distilled water, pH 6.8. The iron solution contained 1.12 g of $FeSO_4 \cdot 7H_2O$ in 5 ml of 0.25 M H_2SO_4 in 100 ml of distilled water.

Methanotrophs were isolated in liquid culture, as well as, on agar plates. Enrichment cultures (3% [vol/vol] CH_4 atmosphere, NMS medium) from landfill soil samples where previously exposed to CH_4 atmosphere were made and transferred several times to the NMS media. Cells from these cultures were diluted in NMS medium (10-fold dilution) with a 3% (vol/vol) methane atmosphere to obtain pure cultures of methanotrophs. Cells from enrichment cultures medium were also spread on agar plates. The plates contained NMS medium and 1% (wt/wt) Noble agar.

After 2 weeks of incubation in 3% (vol/vol) CH_4, milky colonies from the agar plates were transferred into liquid medium. The absence of heterotrophic contaminants was tested by using complex agar (pH 7.4) containing the following, per liter of distilled water: meat extract, 0.5 g; Bacto Peptone, 0.5 g; yeast extract, 0.1 g; KH_2PO_4, 0.1 g; NaCl, 50 mg; and agar, 15 g. The uniform cell shape of pure cultures was examined by microscope (Martin *et al*., 1998). The three cultures isolated from above method are shown in Plate 3.2- Plate 3.4.

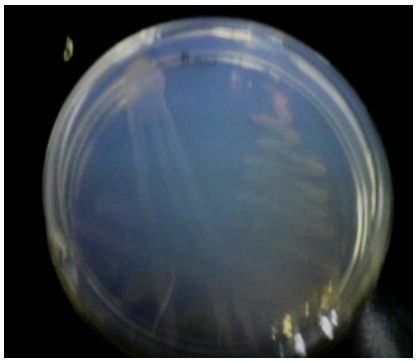

Plate 3.2 : *Methylomonas* sp

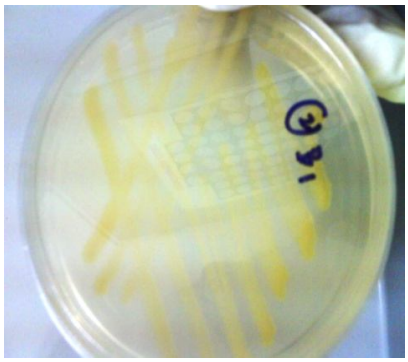

Plate 3.3 : *Methylococcus* sp 1

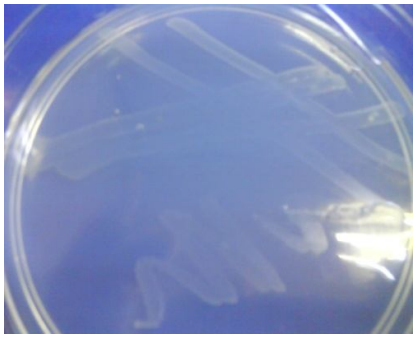

Plate 3.4 : *Methylococcus* sp 2

3.3 The bacteria culture preparation

3.3.1 Preparation of the individual methanotrophic culture

Three types of bacteria isolated from landfill soil showed in Plate 3.1, Plate 3.2 and Plate 3.3 were prepared separately as individual culture from the above method and single colony was directly inoculated into 10ml NMS media in Wheaton bottles and sealed with rubber septa and aluminium foil. 3% (v/v) of CH_4 was then introduced and incubated for 2 weeks until the culture turned turbid.

3.3.2 Preparation of the methanotrophic bacterial mixed culture.

Three types of bacteria isolated from the above method are prepared as mixed culture at a ratio of 1:1:1 and were directly inoculated into 10ml NMS media in Wheaton bottle sealed with rubber septa and aluminium foil and then 3% (v/v) CH_4 was introduced and incubated for 2 weeks until the culture turned turbid.

3.4 Batch experiment on CH_4 Oxidation activity with Matured compost

Batch experiments were carried out using Wheaton bottles (Plate 3.5). All experiments were performed in triplicates. 20g of compost were transferred into 125ml bottle and sealed with rubber septa and aluminium seal to ensure gas tight. Then 15 ml of air from the headspace of the Wheaton bottle was withdrawn using an airtight syringe and replaced with 10 ml of O_2 gas (99.98% purity) and 5 ml of CH_4 (99.9% purity). This amount provided a mixing ratio of approximately 4% of CH_4 (v/v) and 8% O_2 (v/v) in headspace. O_2 gas was added into the bottles to ensure that aerobic conditions prevailed during the experiment. The concentration of CH_4, O_2 and CO_2 in the headspace was measured daily using Gas chromatography (Model Shimadzu 8A) (Plate 3.6 and Plate 3.7).

Plate 3.5: Wheaton bottles experiment with compost

Plate 3.6: Gas chromatography

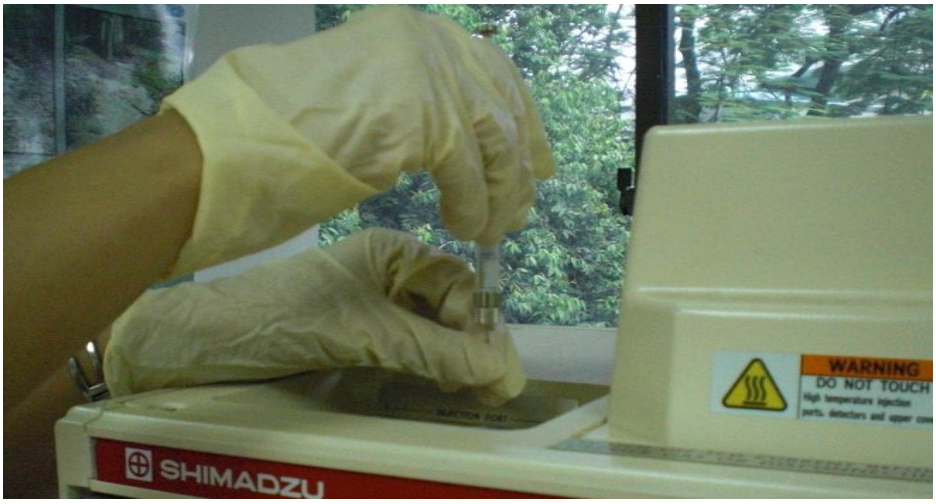

Plate 3.7: Injection of gas sample to GC

3.5 CH_4 depletion efficiency by pure cultures of methanotrophs

Three types of methanotrophic bacteria isolated and identified as *Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2 were tested for CH_4 consumption. Pure cultures of methanotrophs (single colony) was inoculated in 10ml of NMS broth in 125ml wheaton bottles and sealed with rubber septa and aluminium seal to ensure it is

gas tight. Then 15 ml of air from the headspace of the Wheaton bottle was withdrawn using an airtight syringe and replaced with 10 ml of O_2 gas (99.98% purity) and 5 ml of CH_4 (99.9% purity). The concentration of CH_4, O_2 and CO_2 in the headspace was measured daily using Gas chromatography (Model Shimadzu 8A).

3.6 Batch experiment on CH_4 oxidation activity with addition of methanotrophic bacteria individual culture to the compost at different concentration

Batch experiments were carried out as described in 3.4 using 3 types of methanotrophic bacteria culture added at different The moisture content was maintained at 60% v/v and incubated at 35°C.

3.6.1 *Methylomonas* sp

Batch experiments were carried out as described in Section 3.4 using *Methylomonas* sp culture at different concentrations ranging from 2 X 10^7 CFU/g to 14.33 X 10^7 CFU/g.

3.6.2 *Methylococcus* sp 1

Batch experiments were carried out as described in Section 3.4 using *Methylococcus* sp 1 culture at different concentrations ranging from 6.33X 10^7 CFU/g to 14X 10^7 CFU/g.

3.6.3 *Methylococcus* sp 2

Batch experiments were carried out as described in Section 3.6 using *Methylococcus* sp 2 culture at different concentrations ranging from 3 X 10^7 CFU/g to 15.66 X 10^7 CFU/g.

3.7 Batch experiment carried out at different varying parameters for individual cultures

Batch experiments were carried out as described in Section 3.4. The specific methanotrophic bacteria culture was added at fixed concentration individually (*Methylomonas* sp -7 X 10^7 CFU/g, *Methylococcus* sp 1-10.33 X 10^7 CFU/g or *Methylococcus* sp 2 - 7.33 X 10^7 CFU/g) and carried out at different varying parameters.

3.7.1 Influence of temperature on the CH_4 oxidation rate with the addition of *Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2 individually

Batch experiments were carried out as described in Section 3.4. The specific methanotrophic bacteria culture was added at fixed concentration individually as described 3.7. The moisture content was maintained at 60% v/v and incubated at different incubator ranging from 30-60°C (Mor *et al*., 2006). All experiments were compared with control.

3.7.2 Influence of moisture content on the CH_4 oxidation rate with the addition of *Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2 individually

Batch experiments were carried out as described in Section 3.4. All experiments were performed in triplicates. The moisture content of the compost was adjusted by adding demineralised water for increasing or decreased by evaporating the water content to the desired level ranging from 30% to 70% v/v (Mor *et al*., 2006). The specific

methanotrophic bacteria culture was added as described in 3.7. Samples are incubated at 35°C. All experiments were compared with control.

3.7.3 Influence of pH on the CH_4 oxidation rate with the addition of *Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2 individually.

Batch experiments were carried out as described in Section 3.4. All experiments were performed in triplicates. The pH of the compost was adjusted by adding HCI or NaOH to obtain pH range from 5-8 (Mor *et al*., 2006). The specific methanotrophic bacteria culture was added as described in 3.7. Samples are incubated at 35°C and moisture was maintained at 60% v/v.

3.8 Batch experiment: Methane oxidation rate with the addition of mixed culture of *Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2.

Batch experiments were carried out as described in Section 3.4. Mixed culture (*Methylomonas* sp, *Methylococcus* sp 1 and *Methylococcus* sp 2 at ratio of 1:1:1) were added at concentration range of 2.33X 10^7 CFU/g to 11.33X 10^7 CFU/g.

3.9 Batch experiment carried out at different parameters with mixed cultures

3.9.1 Influence of temperature on the CH_4 oxidation rate with the addition of mixed culture

Batch experiments were carried out as described in Section 3.4. Methanotrophic bacteria mixed culture was added at fixed concentration of 5.33X 10^7 CFU/g**.** The

moisture content was maintained at 60% v/v and incubated at different incubator ranged from 30-45°C. All experiment was compared with control.

3.9.2 Influence of moisture content on the CH_4 oxidation rate with the addition of mixed culture

Batch experiments were carried out as described in Section 3.4. Methanotrophic bacteria mixed culture was added at fixed concentration of 5.33X 10^7 CFU/g. The moisture content of the compost is adjust by adding demineralised water for increasing and decreased by evaporating the water content to the desired level ranged from 30% to 80% v/v. Samples are incubated at 35°C. All experiment was compared with control.

3.9.3 Influence of pH on the CH_4 oxidation rate with the addition of mixed culture.

Batch experiments were carried out as described Section in 3.4. Methanotrophic bacteria mixed culture was added at fixed concentration of 5.33X 10^7 CFU/g. The pH of the compost is adjusted by adding HCI or NaOH to obtained pH ranged from 5-8. Samples are incubated at 35°C. All experiment was compared with control.

3.10 Batch experiment with different concentration of CH_4

Batch experiments were carried out as described in Section 3.4 with the addition of CH_4 at different concentration range 4%-16% CH_4 in headspace. Methanotrophic bacteria individual culture or mixed culture was added at fixed concentration. Samples are incubated at 35°C. All experiment was compared with control.

3.11 Batch experiment: Study with compost and sterilized compost with addition of Individual cultures or mixed cultures at fixed amounts and fixed parameters.

Batch experiments were carried out as described in Section 3.4 with compost or sterilized compost. Methanotrophic bacteria individual culture or mixed culture was added at fixed concentration. Samples are incubated at 35°C. All experiment was compared with control.

3.12 Batch experiment with different bacteria type combination

Batch experiments were carried out as described in Section 3.4. Methanotrophic bacteria culture was added at different concentration and combination as described in Table 3.1.

Table 3.1: Bacterial Combination

Combination (Ratio 1:1)	Bacterial concentration range
A- *Methylomonas* sp and *Methylococcus* sp 1	2 X 10^7 CFU/g to 15.33 X 10^7 CFU/g.
B- *Methylomonas* sp and *Methylococcus* sp 2	2X 10^7 CFU/g to 15.33 X 10^7 CFU/g.
C- *Methylococcus* sp 1 and *Methylococcus* sp 2	3X 10^7 CFU/g to 18 X 10^7 CFU/g.

3.13 Bacterial counting

Triplicates samples from each samples were withdrawn at end of the experiment for all the experiment set up in this study. Serially diluted samples (0.1ml) were plated on NMS agar medium for 2 weeks at 35°C and the colonies were counted using Colony Counter.

3.14 Phase II: Column experiment

Column (Plate 3.8) is one meter high and fabricated with 10mm thick PVC with an internal diameter of 0.14m. The sampling ports were embedded in the columns at an interval of 0.1m for gas sampling at different heights. Column reactor experiment (Plate 3.9) is an advance stage in CH_4 mitigation studies and aimed to determine the suitable biocover height for field application.

These experiments represent in determining the suitable height of the compost with the addition of methanotrophic bacteria bacterial at fixed rate separately (*Methylomonas* sp-7 X 10^7 CFU/g, *Methylococcus* sp 1-10.33 X 10^7 CFU/g and *Methylococcus* sp 2 - 7.33 X 10^7 CFU/g, mixed culture - 5.33 X 10^7 CFU/g), the optimum concentration from Wheaton Bottle experiments and tested at different parameters such as temperature, moisture content for the optimum CH_4 oxidation. Compost at total of 7.5kg was added with the culture at the fixed rate based from the bottle experiments outputs and are placed into the columns and the top columns were sealed with 5mm thick plexiglass to ensure the air tight. At the bottom of the column ,

4% of CH_4(v/v) which accounts for 707ml and ,8% of O_2 which accounts for 1414ml are introduced using a flow meter through an inlet. The gas samples at each port were analyzed using gas chromatography (GC) for CH_4, O_2, and CO_2.

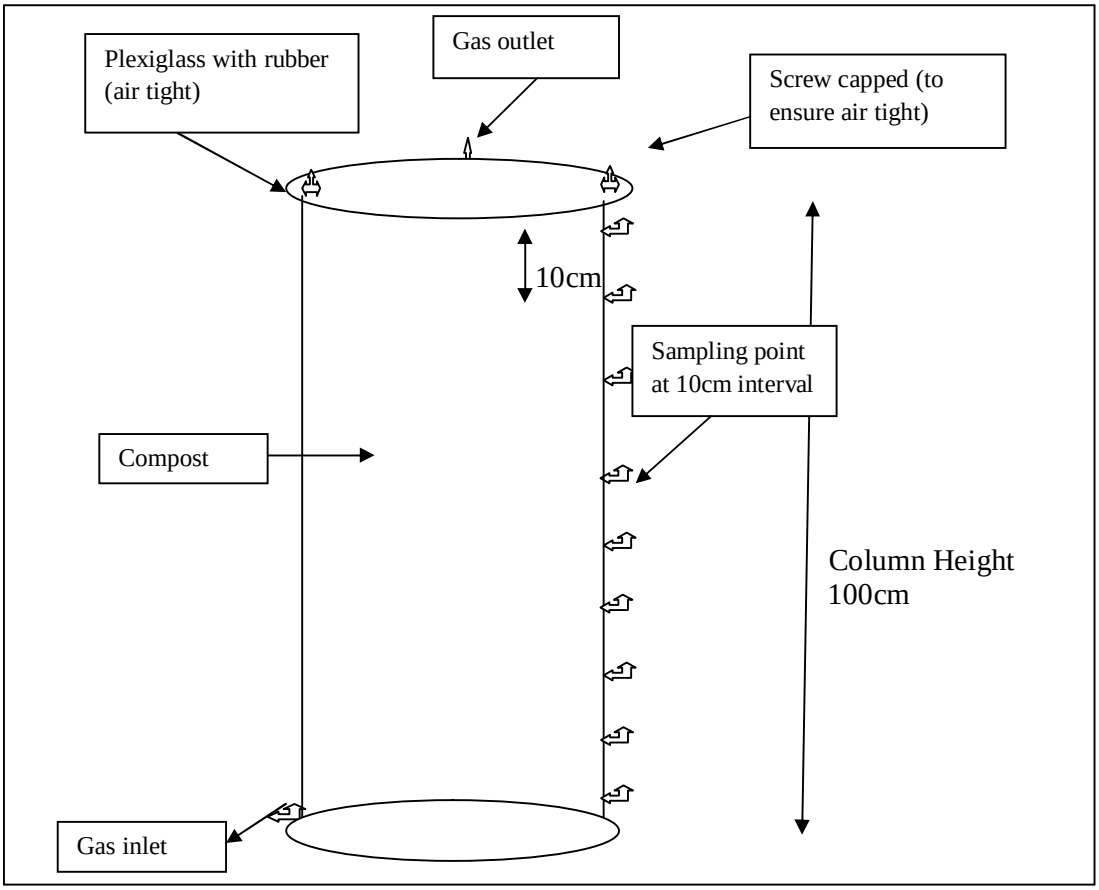

Plate 3.8: Schematic picture of a column

Plate 3.9: Column

3.14.1 Column experiment tested at different incubation temperature

Column experiments were carried out as described in Section 3.14 with addition of mixed culture (ratio 1:1:1) at fixed concentration 5.33X 10^7 CFU/g to compost and incubated at different temperature ranged from 30˚C to 40˚C.

3.14.2 Column experiment tested at different moisture content.

Column experiments were carried out as described in Section 3.14 with addition of mixed culture (ratio 1:1:1) at fixed concentration 5.33X 10^7 CFU/g to compost and adjusted the moisture content ranged of 50%-70% v/v.

3.15 Column experiment tested with and without daily O_2 input

Column experiments were carried out as described in Section 3.14 with addition of mixed culture (ratio 1:1:1) at fixed concentration 5.33X 10^7 CFU/g to compost (Temperature 35˚C and Moisture content 60% (v/v). Experiments were carried out and compared with and without daily 8% O_2 supply.

3.16 The calculation of the CH_4 oxidation rate

CH_4 Oxidation rate Calculation

The CH_4 Oxidation rate = $\frac{(CH_4)_0 - (CH_4)_n}{W \times N}$ ——————Equation 3.1

$(CH_4)_0$= Initial concentration of CH_4 (ml) injected

$(CH_4)_n$ = Concentration of CH_4 at a time n (ml)

W= the amount of compost (g)

N= time taken for complete methane oxidation (hours)

3.17 Kinetic modeling studies

Further analysis on methane oxidation capacity was conducted with the kinetics model as described by the Michaelis-Menten equation (Pawloska, 2006):

$$R_p = R_{max} \frac{1}{1 - (K_m / C)} \quad \text{(Equation 3.2)}$$

Where

R_p = potential methane oxidation rate (ml/d)

R_{max} = maximum methane oxidation rate (ml/d)

K_m = half-saturation reaction rate (ml/d)

C = initial CH_4 concentration (%) (fixed at 4% v/v)

Since C is a constant (4% v/v) for all batch incubation, C was eliminated from Equation 2 to modify the kinetics, where R_p is now described as follows:

$$R_p = R_{max} \frac{1}{1 - (K_m)} \quad \text{(Equation 3.3)}$$

3.18 Bacteria characterization and Identification

The bacteria isolated from landfill soil were characterized by microscopic techniques and biochemical test using test kit. The identities of the isolates were determined by comparing the characteristics with the Bergeys Manual (Bowman *et al.*, 1993).