CHAPTER 3

MATERIAL AND METHOD

3.1 Chemical reagents and equipments

3.1.1 Chemical reagents

Chemical reagents	Production companies
Acid dichromate	**Wako**
Agarose gel	**Merck**
Agar	**Helicopter**
p-Aminodimethyl laniline oxalate	**Phamacia**
Ammonium ferrous sulphate ($(NH_4)_2SO_4.FeSO_4.6H_2O$)	**Merck**
Ammonium molybdate	**Merck**
Ammonium paramolybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	**Merck**
Antimony potassium tartrate	**Merck**
Ascorbic acid	**Aldrich**
Azocasine	**Sigma**
Boric acid (H_3BO_3)	**Sigma**
Bromocresol green	**Phamacia**

Bromocresol purple	**Merck**
Bromothymol blue	**Merck**
Calcium carbonate ($CaCO_3$)	**Fisher**
Calcium nitrate anhydrous ($Ca(NO_3)_2$)	**Promega**
Casamino acid	**Merck**
Casein	**Merck**
Carboxy methyl celulose	**Promega**
Citric acid	**Phamacia**
Congored	**Merck**
Dextrose	**Fisher**
3,5-Dinitrosalicylic acid	**Merck**
Dipotassium hydrogenphosphate (K_2HPO_4)	**Merck**
Disodium sulphate	**Merck**
DNA marker	**Fermentas Life Sciences, USA**
Ethanol	**Fisher**
Ethidium bromide	**Merck**
Ethylenediaminetetraacetic acid (EDTA)	**Merck**
Ferrous sulphate ($FeSO_4.7H_2O$)	**Fisher**
Glacial acetic acid	**Promega**
Glucose	**Merck**
Hydrochloric acid (HCl)	**Promega**

Hydrogen peroxide	**Fisher**
Isoplant DNA extraction kit	**Promega**
Lanthanum chloride heptahydrate ($LaCl_3.7H_2O$)	**Merck**
Loading dye	**Fermentas Life Sciences, USA**
Lugol's iodine	**Promega**
Magnesium oxide	**Fisher**
Magnesium sulphate ($MgSO_4$)	**Merck**
Malt extract	**Merck**
Maltose	**Merck**
Mastermix	**Promega**
Methyl red	**Merck**
∝-Naphthol	**Sigma**
N-napthyl ethelene diamine hydrochloride	**Sigma**
Nitric acid (HNO_3)	**Fisher**
p-Nitrophenyl laurate	**Merck**
PCR-purification kit	**Takara, Japan**
Peptone	**Merck**
Perchloric acid ($HClO_4$)	**Sigma**
Phenol	**Sigma**
0-Phenanthroline	**Merck**
Phosphate	**Merck**

Potassium chloride	**Fisher**
Potassium dihydrogen orthophosphate	**Merck**
Potassium dihydrogen phosphate (KH_2PO_4)	**Merck**
Potassium nitrate (KNO_3)	**Merck**
Potassium phenolphthalein disulphate	**Sigma**
Primers 27F	**Operon**
Primers 520R	**Operon**
Primers ITS I	**Operon**
Primers ITS 4	**Operon**
RNase	**Nippon Gene**
Rose bengol	**Sigma**
Selenium(Se)	**Merck**
Skim milk	**Sigma**
Sodium chloride	**Sigma**
Sodium dichromate dehydrate ($Na_2Cr_2O_7$)	**Sigma**
Sodium nitrate ($NaNO_3$)	**Sigma**
Sodium potassium tartrate	**Merck**
Sodium sulphate (Na_2SO_4)	**Merck**
Soluble starch	**Sigma**
Sulfuric acid (H_2SO_4)	**Fisher**
Sulphanilamide	**Phamacia**
Toluene	**Phamacia**

Tributyrin	**Merck**
Trichloroacetic acid	**Promega**
Tris base	**Promega**
Trypticase	**Sigma**
Tryptone	**Sigma**
Tween-80	**Phamacia**
Tyrosine	**Sigma**
Yeast extract	**Merck**

3.1.2 Equipments

Equipments	Production companies
Analytical Balance Model AS64	**Ohaus Corporation**
Autoclave Model 8000DSE	**Thermo Scientific**
Autopipettes 200-1000 µL	**Wheaton Science Products**
Autopipettes 20-200 µL	**Wheaton Science Products**
Compound Microscope Model Advanced 1000 Series	**Fisher Science Education**
Deep freezer Model 8458	**Forma Scientific**
Hot air oven	**Binder**
Hot plate Model PC-620	**Corning**

Incubator Model 30M	**Thermo Scientific**
Incubator shaker Model I-24	**Brunswick**
Kjeldahl Digester Model 25	**Labconco**
Kjeldahl Distillation unit Model 6-Place	**Labconco Corporation**
Laminar air flow Model BHA 120&BHA 180	**BH 2000 Series Class II Biological Safety Cabinets**
Magnetic stirrer Model RCH-3L	**Eyela**
Microwave Model M764	**Philips**
PCR Sprint Thermal Cycler Model SPRINT	**Thermal hybrid**
Plate centrifuge Model 5810R	**Eppendorf**
pH & EC meter Model 210Aplus	**Thermo Scientific**
Refrigerated Centrifuge Model EBA 12 R	**Hettich**
Refigerature	**Toshiba**
Spectrophotometer Model 1200	**Thermo Scientific**
Sonicator Model 2510 DTH	**Thermo Scientific**
Stereo microscope Model S96054	**Labo America**
Transluminator	**Biorad**
Water Bath Shaker Model R76	**Thermo Scientific**

3.2 Experimental methods

3.2.1 Collection of samples

Twenty different sources of soils, 5 samples of soil culture, 5 samples of commercial compost and 2 samples of liquid bio-fertilizer were collected from the North of Thailand. The samples were transferred to laboratory and stored at 4°C in refrigerator till analysis.

3.2.2 Selection of microorganisms capable of cellulase-, amylase-, protease- and lipase activity

For selection of thermophillic cellulose-, starch-, protein- and lipid-degrading microorganisms, the 10 g of each samples was weighed and diluted in 90 mL of 0.85% NaCl by dilution-plate method (Johnson and Curl, 1972) on carboxy methyl cellulose agar medium (CMC agar) (carboxy methyl cellulose, 4 g; agar, 15 g; distilled water, 1 L [pH 6.8]), starch agar medium (peptone, 5 g; yeast extract, 1.5 g; soluble starch, 2.0 g; NaCl, 5.0 g; agar, 15 g; distilled water 1 L [pH 6.5]), skim milk agar medium(peptone, 5 g; yeast extract, 2.5 g; skim milk powder, 1 g; glucose 1 g; agar, 15 g; distilled water 1 L [pH 7.0]) and tributyrin agar medium (peptone, 5 g; yeast extract, 3 g; agar, 15 g; tributyrin 10 mL; distilled water 1 L [pH 7.5]), respectively and incubated at 50°C for 24-72 h. Then isolated microoganisms were screened for their activities on plates as follows:

Cellulolytic activity

Congo red overlay method (Ruijssenaars and Hartmans, 2000) was used to determine cellulolytic activity. The plates were flooded with 0.1% aqueous

congo red for 10 min and then washed with 1 M NaCl solution. A clear zone around the colony of oragnisms indicated CMC hydrolysis (Figure C1-A of appendix C).

Amylolytic activity (Hols *et al*, 1994)

The ability to degrade starch was used as the criterion for determination of ability to produce amylolytic enzymes. After incubation the plates were flooded with Lugol's iodine solution and a clear zone around the colony of organisms indicated amylolytic activity (Figure C1-B of appendix C).

Proteolytic activity (Nuria, 1967)

After incubation, degrading skim milk appeared as a clear zone on opaque agar medium. A clear zone around the colony of organisms indicated protein hydrolysis (Figure C1-C of appendix C).

Lipolytic activity (Sierra, 1957)

The formation of lipolytic enzymes appeared a clear zone of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. A clearing zone around the colonies indicated lipid hydrolysis (Figure C1-D of appendix C).

The colonies producing clear zones of more than 1 cm in diameter on media used were picked up and purified by streaking on nutrient agar (beef extract, 3 g; peptone, 5 g ;agar 15 g; distilled water, 1 L [pH 7.0]) plate. Pure cultures were collected on nutrient agar slant at 4°C for stock culture.

3.2.3 Enzymes assays

Preparation of crude enzyme

Selected microorganisms were incubated in 100 mL broth of CMC

medium, starch medium, skim milk medium and tributyrin medium in 250 mL erlenmeyer flasks and incubated at 50°C in incubator shaker at 250 rpm for 24 h for bacteria, 72 h for actinomycetes and fungi. After incubation, the broth cultures were centrifuged at 8,000 ×g at 4°C for 15 min, and the supernatant was collected to determine enzyme activity as follows:

3.2.3.1 Cellulase assay

Carboxy methyl cellulase (CMCase) activity was assayed by measuring the release of reducing sugars (Miller, 1959).

The assay mixture was prepared by thoroughly mixing 1 mL of enzyme control (crude enzyme, 500 µL; 0.05 M citrate buffer, 500 µL [pH 4.8]), 1 mL of substrate control (1% CMC, 500 µL; 0.05 M citrate buffer, 500 µL [pH 4.8]) and 1 mL of enzyme substrate (crude enzyme, 500 µL; 1% CMC in 0.05 M citrate buffer, 500 µL [pH 4.8]) together.

The assay mixture was incubated in water bath at 50°C for 60 min and stopped by dipping into ice bath. Then, 500 µL of distilled water, 2 mL of 3,5-dinitrosalicylic acid solution (DNS) (3,5- dinitrosalicylic acid, 5 g; NaOH, 5 g; Na_2SO_3, 0.25 g; phenol, 1 g; adjust to a final volume of 500 mL with distilled water) and 1 mL of 40% Na-K tartrate were added. Spectrophotometer was used to measure the absorbance of sample at 550 nm compared with the blank (distilled water, 500 µL; 0.05 M citrate buffer, 500 l µL[pH 7.0]).

The amount of glucose released from the carboxy methyl cellulose by the action of the CMCase was determined from a standard curve prepared by 0–10 mg

mL^{-1} glucose. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugar per 1 min under the assayed conditions. Cellulase activity was calculated by the following equation:

$$\text{Units mL}^{-1}\text{ enzyme} = \frac{(0.185)\ (\text{reducing sugar released, mg})}{(0.5)}$$

3.2.3.2 Amylase assay

Amylase activity was assayed by measuring the release of maltose in reaction mixture of crude enzyme and 2% soluble starch (Miller, 1959).

The reaction mixture was prepared by thoroughly mixing 1 mL of enzyme control (crude enzyme, 500 μL; 0.2 M phosphate buffer, 500 μL [pH 7.0]), 1 mL of substrate control (2% soluble starch, 500 μL; 0.2 M phosphate buffer, 500 μL [pH 7.0]) and 650 μL of enzyme substrate (crude enzyme, 150 μL; 2% soluble starch, 500 μL) together.

Reaction mixture was incubated in magnetic shaking water bath (Varimag type E, Germany) at 600 rpm and 50°C for 30 min. Three mL of DNS reagent was added into each tube, covered with glass bead and boiled for 15 min. Then, 1 mL of 40% Na-K tartrate was added into each tube. Spectrophotometer (Thermospectronic, Genesys 20, USA) was used to measured the absorbance of sample at 540 nm compared with the blank (0.2 M phosphate buffer [pH 7.0]).

Reducing sugar released by the action of enzyme on the starch substrate was determined from a standard curve prepared with 0 – 10 mg mL^{-1} glucose. One unit of hydrolyzing activity was defined as the amount of enzyme required to produce

1 μmol of glucose-equivalent reducing sugar in 1 min. Amylase activity was calculated by the following equation:

$$\text{Units mL}^{-1}\text{ enzyme} = \frac{(0.185)\ (\text{reducing sugar released, mg})}{(0.5)}$$

3.2.3.3 Protease assay

Protease activity using azocazein as substrate was determined as described by Brock *et al*. (1982).

The assay mixture was prepared by thoroughly mixing 390 μL of blank (2%(w/v) azocazein substrate, 240 μL; 0.5 M phosphate buffer, 150 μL [pH 7.0]) and 410 μL of enzyme substrate (crude enzyme, 20 μL; 2% (w/v) azocazein substrate, 240 μL; 0.5 M phosphate buffer, 150 μL [pH 7.0]) together.

The assay mixture was incubated in water bath at 50°C for 2 h and stopped the reaction by adding 1.2 mL of 10% trichloroacetic acid (TCA) and waited for 15 min. Then, reaction mixture was centrifuged at 10,000 rpm and 4°C for 5 min. One milliliter of supernatant was mixed with 1 mL of 1 M NaOH and measured the absorbance at 440 nm.

One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 440 nm equivalent to 1 μmol of tyrosine in one minute under the assay conditions. Protease activity was calculated by the following equation:

$$\text{Units mL}^{-1}\text{ enzyme} = \frac{(\text{mmol Tyrosine equivalents released})\ (A)}{(C)\ (B)\ (D)}$$

Where:

A = Total volume (in milliliters) of assay

B = Time of assay (in minutes) as per the unit definition

C = Volume (in milliliter) of enzyme used

D = Volume (in milliliters) used in colorimetric determination

3.2.3.4 Lipase assay

Hydrolytic activity was evaluated spectrophotometrically on emulsified *p*-nitrophenyl laurate (*p*-NPL) according to Markweg-Hanke *et al.*(1995).

One volume of a 16 mM solution of *p*-NPL in acetonitrile was suddenly mixed before use with nine volumes of a 50 mM phosphate buffer, pH 7.5 before use. The 1 mL of this mixture was pre-equilibrated at 25°C in the 1 mL cuvette of a spectrophotometer. The reaction was started by adding 10 µL of enzyme solution appropriately diluted with the 50 mM phosphate buffer, pH 7.5. The variation of the assay's absorbance at 410 nm against a blank solution without enzyme was monitored for 2 min.

The reaction rate was calculated from the slope of the absorbance versus time curve by using an apparent molar extinction coefficient of 12.75×10^6 cm^2 mol^{-1} for *p*-nitrophenol (*p*-NP), which was determined from the absorbance values of standard solutions of *p*-NP in the reaction mixture. One unit of activity is the amount of enzyme that produces 1 µmol of *p*-NP per minute under the assay conditions.

3.2.4 Identification of effective microorganisms

All isolates were identified based on morphological and biochemical characteristics and their DNA sequencing determinants.

3.2.4.1 Morphological and biochemical characteristics of bacterial isolates GB 12, LPC 2, LHE 3 and BS 1

A loopful of bacterial culture was transferred to a drop of tap water on slide. Spread the culture to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a 1 cm diameter. Air-dry the culture and heat-fix it over a gentle flame. These fixed cultures were stained by the Gram's method (Syndney *et al*., 1982). Add about 5 drops of crystal violet stain over the fixed culture. Let stand for 60 seconds. Pour off the stain and gently rinse the excess stain with a stream of water from a faucet. Add about 5 drops of the iodine solution on the smear, enough to cover the fixed culture. Let stand for 30 seconds. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface. Add a few drops of decolourizer so that the solution trickles down the slide. Rinse it off with water after 5 seconds. Counter stain with 5 drops of the safranin solution for 20 seconds. Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water and air-dried. Examine the finished slide under a microscope.

Bacteria were also identified using standard biochemical tests according to Bergey's manual of systematic bacteriology (Sneath *et al*., 1986) as follows:

Shake tube cultures

For observing colony formation in deep agar cultures, especially of anaerobic or microaerophilic organisms, dispense glucose agar in 20 mL amounts in tubes 20-25 mm diameter. Melt and cool to approximately palms, remove one loopful of test organisms to inoculate into deep agar, and gentle vortex the culture. Allow to set and incubate at 37°C for 24-48 h. Submerged colonies will develop and will be

distributed as follows: obligate aerobes grew only at the top of the medium; obligate anaerobes only near the bottom; microaerophiles grow near but not at top; facultative organisms grow uniformly throughout the medium.

Sulphatase test

Some organisms can split ester sulphates. Grow the organism in NA medium containing 0.001 mol L^{-1} potassium phenolphthalein disulphate at 37°C for 14 days. Add a few drops of ammonia solution. A pink colour indicates the presence of free phenolphthalein.

Nitratase test

Test organisms in nitrate broth medium were inoculated and incubated overnight. A dense suspension of the culture in 0.01 mol L^{-1} $NaNO_3$ in phosphate buffer, pH 7.0 was prepared and incubated at 37°C for 24 h. The organisms were cultured in a nutrient broth with a few drops of 1% $NaNO_3$ solution and incubated for 4 h. The nutrient broth was acidified with a few drops of 1 N HCL and added 0.5 mL each of a 0.2% solution of sulphanilamide and 0.1% N-(naphthyl) ethylene diamine hydrochloride. A pink colour denotes nitratase activity.

Catalase test

The test organisms were inoculated on the nutrient agar slant which a mixture of equal volumes of 1% tween-80 and 20-vol hydrogen peroxide were added to the culture. Clear bubbles, appearing within 30 sec, indicate catalase activity.

Oxidase test

The test organisms were inoculated on the nutrient agar slant. The culture was incubated on nutrient agar slopes at 37°C for 24-48 h. A few drops each of

freshly prepared 1% aqueous *p*-aminodimethylaniline oxalate and 1% ∝ - naphthol in ethanol were added and the mixture was allowed to run over the growth. A deep blue colour shows a positive reaction.

Oxidation fermentation test

Two tubes of Hugh and Leifson's medium (Medium I: peptone, 2 g; NaCl, 5 g; K_2HPO_4, 0.3 g; agar, 3 g; 15 mL of 0.2% bromothymol blue; glucose, 10 g; distilled water 1 l and Medium II: tryptone, 10 g; yeast extract, 1 g; glucose, 10 g; agar, 2 g; 20 mL of 0.2% bromocresol purple; distilled water 1 L) were heated in boiling water for 10 min to drive off oxygen, cooled and inoculated; incubated one aerobically and the other either anaerobically by sealing the surface of the medium with 2 cm of melted Vaseline at 37°C for 24 h.

Oxidative metabolism: acid in aerobic tube only.

Fermentative metabolism: acid in both tubes.

3.2.4.2 Morphological characteristics of fungal isolates LHE 12 and LHE 10

Fungi were identified by their morphological characteristics, according to Barnett (1988), Ellis (1993) and Sutton (1980) by using slide culture technique. Therefore, a block of sterile agar was cut out of a Petri dish and placed upon a sterile slide resting on a bent glass tube within a sterile Petri dish. A few spores of a fungus are inoculated at the edges of the sterile agar block and topped with a sterile cover-glass. A disc of moist filter-paper in the dish maintains humidity for the culture and incubated at 30°C for 72 h. The shape of mycelium and spore was observed by using microscope.

3.2.4.3 Morphological characteristics of actinomycete isolate LPA 15

Actinomycete was identified by morphological characteristics according to Holt (1994).: The growth and colony characters (color of vegetative and aerial mycelium and soluble pigments) of isolate LPA 15 grown on yeast-malt extract agar (yeast extract, 4.0 g; malt extract, 10.0 g; glucose, 4.0 g; agar, 15.0 g; distilled water 1 L) and incubated at 45°C for 72 h was studied. The shape of mycelium and spore chains was observed by using microscope.

3.2.4.4 Identification by DNA sequencing for bacteria and actinomycetes using 16S rDNA sequence determination

DNA Extraction

Cells were cultured in 5 mL nutrient broth for bacterial growth and nonsporulating broth(casamino acid, 20 g; yeast extract, 4 g; soluble starch, 20 g; distilled water 1 L) (Sanglier *et al*., 1992) for actinomycete growth at 37°C for 18 h. The pellet cells were washed and collected by centrifugation at 10,000 × *g* for 2 min. DNA was extracted from pellet cells using the Isoplant DNA extraction kit following the manufacturer's protocol without any modification. Add 300 µL of solution I and mixed by pulse-vortexing for 2 min**.** Then 150 µL of solution II was added and mixed totally using vortex, the mixture was incubated in 50°C water bath for 15 min. After incubated, add 150 µL of solution III and mixed by paulse-vortexing for 2 min. The suspension was placed on ice for 15 min. The mixture was then centrifuged at 10,000× *g* at 4°C for 15 min. Transfer 300 µL of upper layer to new microcentrifuge tube. DNA was precipitated with 1200 µL of absolute ethanol and placed on ice for 15 min.

After that, the mixture was centrifuged at 10,000× *g* at 4°C for 10 min. When centrifuged, absolute ethanol was discarded and the pellet DNA was dried in vacuum. The 50 µL of TE buffer (tris base, 0.12 g; EDTA, 0.0372 g; adjust to volume at 100 mL with distilled water [pH 7.5]) and 1 µL of RNase were added to suspend the dried DNA. The final step, the concentration of genomic DNA was measured by spectrophotometer at wavelengths of 260 nm. The reading at 260 nm allowed to calculate the concentration of nucleic acid in the following equation:

Concentration of DNA (ng μL^{-1}) = A_{260} × 50 × dilution factor

PCR amplification

The DNA was tested for PCR-amplifiable DNA with 16S rRNA-universal primers. The 16S rRNA gene of the bacterial and actinomycete isolates was amplified with universal primers. The primers 27F (forward primer, 5' AGAGT TTGATCCTGGCTCAG-3') and 520R (reverse primer, 5'-ACC GCGGCKGCTG GC-3') (Operon, Germany) were used. The amplification reaction was carried out in a 50 µL reaction volume, containing 25 µL of mastermix, 1 µL of genomic DNA, 2 µL of each primer and 20 µL of autoclaved distilled water. PCRs were carried out in a thermocycler (PCR Sprint Thermal Cycler) under the following conditions: the program was initiated by denaturation at 94°C for 5 min following by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, one cycle of 72°C for 5 min, and holding at 4°C until they were collected.

Gel electrophoresis

After amplification, the amplified product was run on a 1.5% agarose gel therefore, 1.5 g of agarose powder was weighted and put it into a glass beaker, add

100 mL of 1× TAE buffer(tris base, 4.84g; glacial acitic acid, 1.14 g; 0.5 M EDTA, 2 mL [pH 7.5]). The mixture was stirred on a hot plate until agarose was dissolved and solution was cleared. Allow solution to cool about 55°C before pouring. Then the cooled gel solution was poured into tray to a depth of about 5 mm. Allow gel to solidify about 20 minutes at room temperature. The tray was placed in electrophoresis chamber and covered with 1× TAE buffer. The samples were prepared for electrophoresis as follows as, add 1 μL of 6× gel loading dye for every 5 μL of DNA solution and mixed well. For DNA marker preparation, 1 μL of DNA marker was mixed well with 1 μL of loading dye and 4 μL of sterile distilled water. The 7 μL of DNA marker was loaded into the first well and the samples were loaded into the next well. Then, gel electrophoresis was performed at 100 volts until dye markers have migrated an appropriate distance. After electrophoresis, the gel was took off from the tray and it was stained with ethidium bromide solution for 10 min. DNA bands were visualized under UV light using transluminator.

Purification of PCR products

The DNA sequencing PCR products were purified to remove excess primer using TaKaRa SUPREC^{TM}-PCR (TaKaRa Biomedical). There were following steps of procedure: pipette 50 μL of PCR product and 400 μL of TE buffer onto the column and put it in a microcentrifuge tube and centrifuged at 4000× g for 15 min. After centrifugation, the supernatant was discarded and DNA in the column was washed with 20 μL of TE buffer. The column was inverted and put it into a new microcentrifuge tube and then centrifuged at 4000× g for 2 min to collect the purified PCR product. The purified PCR product was sent to the First Base Laboratories company at Malaysia for

sequencing of 16s rRNA. The sequences were compared to 16S rRNA gene sequences in the public database using the BlastN program. Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, National Library of Medicine) (http://www.ncbi.nlm.nih.gov/) by using the BLAST searching program.

3.2.4.5 Identification by DNA sequencing for fungi using ITS1-5.8S rDNA-ITS2 sequence determination

DNA extraction

Cells were cultured in 5 mL potato dextrose broth at 37°C for 48 h. The pellet cells were washed and collected by centrifugation at 10,000 × g for 2 min. Before DNA extraction, the pellet cells were freezed at −80 °C for 60 min and thawed at 65°C for 30 min. For further extraction, DNA extraction kit was used as above mentioned 16s rDNA extraction method.

PCR amplification

The eluted DNA was tested for PCR-amplifiable DNA with ITS1-5.8S rDNA-ITS2 rRNA universal primers. The ITS1-5.8S rDNA-ITS2 rRNA gene of the fungal isolates were amplified with universal primers. The primers ITS I (forward primer, 5'-TCCGTAGGTGAACCTGC-3') and ITS 4 (reverse primer, 5'-ACCGCGGCKG CTGGC-3') (Operon, Germany) were used. The amplification reaction was carried out in a 50 μL reaction volume containing 25 μL of mastermix, 1 μL of genomic DNA, 2 μL of each primer and 20 μL of autoclaved distilled water. To carry out PCRs in a thermocycler (PCR Sprint Thermal Cycler) under the conditions for PCR the steps were as follows: initial denaturation of DNA at 95°C for 3 min and then 35 cycles of

three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 52°C for 1 min, and extension at 72°C for 2 min. The samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles and holding at 4°C until they were collected.

Gel electrophoresis and purification of PCR products

The methods of gel electrophoresis and purification of PCR products can be followed by above described those for bacteria and actinomycetes.

3.2.5 Compost inoculum development for composting

Inoculum

Inocula from effective microorganisms were performed as follows: selected strains of *Bacillus subtilis* GB 12, *Bacillus subtilis* LPC 2, *Bacillus subtilis* BS 1, *Bacillus subtilis* LHE 3, *Streptomyces regensis* LPA 15, *Aspergillus flavus* LHE 12 and unknown fungus LHE 10 were used as effective bio-degrading microorganisms.

Inoculum preparation

Each of bacteria cultures was reactivated by using a sterile loop to take it from a slant culture tube. A loopful of bacterial slant culture was inoculated into 10 mL of Nutrient Broth (NB)(beef extract, 3 g; peptone, 10 g; NaCl, 5 g; distilled water 1 L [pH 7]) and incubated at 45°C for 24 h. To prepare a starter culture, 10 mL over night culture was inoculate into a 1000 mL Erlenmeyer flask with 500 mL nutrient broth. The flask was shaked on a reciprocal rotary shaker at 45°C for 48 h. The broth culture of each flask was harvested by centrifuge at 6000×g for 15 min. The supernatant was decanted and the cells were re-suspended in 10 mL of 0.1% steriled

peptone with pH 7.0. The cell concentration was determined by preparing a serial ten-fold dilution, spreading (0.1 mL) onto triplicate NA plates, which were incubated for 24 h at 45°C, and counting colony-forming units (CFU) developed on the agar plates. The amount of bacteria in harvesting culture was calculated as CFU g^{-1}.

Cultures of fungi were grown on Potato Dextrose Agar (PDA) (Potato, 200 g; glucose, 20 g; distilled water 1 L) for 5 days at 30° C and then the mycelia or spores were transferred into 250 mL Erlenmeyer flask containing 100 g of sterile sorghum and rice bran in 4:1 ratio as solid state fermentation and incubated at 30°C for 7 days. Spore counts were determined with a hemacytometer and were expressed as number of spores per gram.

For actinomycete, the culture grown on starch casein agar (SCA) (soluble starch, 10 g; casein, 0.3 g; KNO_3, 2.0 g; NaCl, 2.0 g; K_2HPO_4, 2.0 g; $MgSO_4$ $7H_2O$, 0.005 g; $CaCO_3$, 0.02 g; $FeSO_4$ $7H_2O$, 0.01 g; agar, 12.0 g; distilled water, 1 L) for 3 days at 45°C and then the colony was transfers into 20×30×10 cm^3 plastic box containing 100 g of sterile oat meal as solid state fermentation and incubated at 45°C for 7 days. The cell concentration was determined by preparing a serial ten-fold dilution, spreading (0.1 mL) onto triplicate SCA plates, which were incubated for 72 h at 45°C, and counting colony-forming units (CFU) developed on the plates. The amount of actinomycete was calculated as CFU g^{-1}.

All the microbial cultures (bacteria:fungi:actinomycete in ratio 1:1:1) were mixed with holder material compound of sterile compost powder and rice bran in the ratio 4 to 1. Inoculum, so called compost inoculum, was dried in hot air oven at 45°C for 24 h. The dried compost inoculum was cooled down at room temperature, and then it was packed tightly in aluminum foil bag (Figure C2 and C3 of Appendix C).

3.2.6 Assessment of microbial density of compost inoculum

The microbial density of compost inoculum were set up three different concentrations of each effective microorganisms i.e. 10^5 CFU g^{-1} for formula 1, 10^6 CFU g^{-1} for formula 2 and 10^7 CFU g^{-1} for formula 3 (Table 7). To compare the effectiveness of compost inocula on the degradation of plant material, using rice straw as the representative of plant material were decomposed by compost inocula formula 1, 2 and 3 compared with the commercial compost inoculum, Market brand with 10^6 CFU g^{-1}. One-hundred grams of sterile rice straw in 1,000 mL Erlenmeyer flask was inoculated with 0.001 g of each compost activators. During decomposition, the flask was incubated at 45°C for 56 days and maintained moisture content of 70% by spraying sterile water. Each treatment was carried out in duplicate. Various parameters analyses as shown in Table 7.

Table 7 Concentrations of effective microorganisms in compost inocula

Compost inocula	Amount of microorganisms (CFU g^{-1} rice straw)
Market brand	10^6
Formula 1	10^5
Formula 2	10^6
Formula 3	10^7

3.2.7 Composting process

This studies of composting were conducted both in laboratory and field experiment. The compost material consisted of plant wastes, cow's manure and urea fertilizer in the ratio 100:10:0.2 was tested for composting.

3.2.7.1 Laboratory experiment

The 100 g of composting materials (rice straw: cow's manure: urea fertilizer in ratio 100: 10: 0.2) and each of compost inocula i.e. Market brand from commercial compost inoculum and own compost inoculum, so called CMU (Formula 2), were put in 1000 mL Erlenmeyer flask. Composting material with out compost inoculum was used as control. There were 3 treatments with 3 replications. During composting process of 8 weeks the composting temperature was controlled at 45°C in an incubator and the moisture content of 70% was maintained by adding sterile water. Analytical parameters of composting as shown in Table 8 were measured.

3.2.7.2 Field experiment

In field study, the quantity of raw material (sawdust: cow's manure: urea fertilizer in ratio 100: 10: 0.2) for each piles was 1 ton which was mixed with compost inoculum (100 g) i.e. Market brand and CMU and the moisture content adjusted to approximately 70 % before piling. A triplicate series of windrows was set up with manual turning. Each pile was pyramid in shape, about 2 m in base-width and 1.5 m in height (Tiquia *et al*., 1996) (Figure C4 of Appendix C). Aeration and moisture content of 70% were provided by turning the piles every 7 days by hand (Figure C5 of Appendix C). During the composting process, ambient temperature and temperature at a depth of 60 cm in these piles were first monitored every 2 days in the first week, then afterwards every 10 days before turning till end composting period (for 8 weeks). Analytical parameters (Table 8) were performed.

Table 8 Physicochemical and biological parameters used for analyses of samples

Parameters	Sampling interval (days)		
	Rice straw decomposition	Laboratory experiment	Field experiment
Total count of mesophilic bacteria	-	-	10
Total count of thermophilic bacteria	7	7	10
Total count of mesophilic fungi	-	-	10
Total count of thermophilic fungi	7	7	10
Total count of mesophilic actinomycete	-	-	10
Total count of thermophilic actinomycete	7	7	10
Total count of cellulose-degrading mesophiles	-	-	10
Total count of cellulose-degrading thermophiles	7	7	10
Moisture content (%)	-	-	10
pH	7	7	10
Electrical conductivity(ms/cm)	*	-	-
Total organic carbon (%)	*	10	10
Total Nitrogen (%)	*	10	10
Total Phosphorus (%)	*	-	-
Total Potassium (%)	*	-	-
Total Calcium (%)	*	-	-
Total Magnesium (%)	*	-	-
Germination index (%)	*	-	-

* Initial and final day

3.2.8 Analytical methods

Various analytical methods used for the determination of physical, chemical and biological parameters are described below.

3.2.8.1 Microbiological analysis

During the composting process the growth of microflora at various stages of decomposition was also determined. Bacteria, fungi, actinomycetes, both

mesophile and thermophile, and cellulose-degrading microorganisms were determined using the spread-plate method. In the determination of amount of microorganisms 10 g of sample was diluted with 90 mL of sterile phosphate buffer, pH 7.0 and the dilution was well mixed. The mixture was left to stand at room temperature for 15 min and then this dilution was subjected to a serial ten-fold dilution using sterile phosphate buffer, pH 7.0. From each dilution, 0.1 ml aliquot was spread onto trypticase soy agar medium (BBL Microbiology Systems) for bacteria (trypticase peptone, 17 g; phytone peptone, 3 g; NaCl, 5 g; K_2HPO_4, 2.5 g; glucose, 2.5 g; agar, 20 g; distilled water, 1 liter [pH 7.3]), rose Bengal agar medium for fungi (peptone, 5 g; dextrose, 10 g; KH_2PO_4, 1 g; $MgSO_4$, 0.5 g; agar, 15 g; rose Bengal, 35 mg; distilled water, 1 L [pH 5.2]), starch casein agar medium for actinomycetes (soluble starch, 10 g; casein, 0.3 g; KNO_3, 2g; K_2HPO_4, 2 g; $MgSO_4$. $7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4$. $7H_2O$, 0.01 g; agar, 15 g; distilled water 1 L [pH 7.0]) and CMC agar medium for cellulose-degrading microorganisms by duplicate. The incubation temperatures were 30°C for mesophiles and 50°C for thermophiles. The incubation time was 24 h for mesophilic bacteria, 48 h for thermophilic bacteria, and 72 h for mesophilic and thermophilic fungi, actinomycetes and cellulose-degrading microorganisms, respectively. Culture in plates which showed between 30 - 300 colonies was chosen and colony counts were calculated as CFU g^{-1}.

3.2.8.2 Moisture content

Aluminium pans were pre-dried at 100°C for 15 h in a hot air oven and cooled in a desiccator. Pre-dried aluminium pans were weighed by analytical balance (Sartorius, AS64) then put 10 to15 g of wet compost sample in a prepared aluminium pans and weighed. Compost sample was placed in hot air oven (Binder) at 105°C

overnight. After drying, sample was removed and place in a desiccator to cool for 1 h, after that weighed the sample. The percentage of moisture content of compost sample was calculated by the following equation:

$$\% \text{ Moisture content} = \frac{(C-B)-(A-B)}{(C-B)} \times 100$$

Where:

A = weight of dried sample + dish, g

B = weight of dish, g

C = weight of wet sample + dish, g

3.2.8.3 pH

A 10 g of wet compost sample were transferred into a beaker and then added 25 mL distilled water and magnetic stirrer bar. After that, the beaker was placed on an magnetic stirrer (Eyela, RCH-3L) and stirred for 15 min. the pH of the stirred sample was measured by pH meter (Metrohm, 713).

3.2.8.4 Electrical conductivity

A 10 g of wet compost sample were transferred into a beaker and then added 25 mL distilled water and magnetic stirrer bar. After that, the beaker was placed on an magnetic stirrer and stirred for 15 min. The electrical conductivity (EC) of the stirred sample was measured by electrical conductivity meter (Thermo Scienctific, 210Aplus).

3.2.8.5 Total organic carbon

Compost sample containing 10-20 mg carbon was sufficient dried, grinded and accurately weighed in a 500 mL Erlenmeyer flask. Twenty five milliliters

of 66.7 mM acid-dichromate mixture ($Na_2Cr_2O_7$, 20.0 g in 50 mL of distilled water; (98% m m^{-1}) H_2SO_4, 400 mL; 70% $HCLO_4$ (1.70 g mL^{-1}), 140 mL; distilled water, 410 mL) was added into the flask and simmer for 2 h on a hot plate. While the sample was refluxing, the ferrous ammonium sulphate solution (98% m m^{-1} H_2SO_4, 5 mL in 1500 mL of distilled water; $(NH_4)_2SO_4.FeSO_4.6H_2O$, 314 g; distilled water, 500 mL) was standardized against the dichromate mixture. Two successive blank digestions were carried out by heating for 10 min using 25 mL of acid-dichromate. After cooled down, approximately 100 mL of distilled water were added, followed by 10 drops of ferroin indicator (o-phenanthroline, 3.71 g; $FeSO_4.7H_2O$; 1.74 g; distilled water, 250 mL) were added. The reaction mixture was titrated with the 0.4 M ferrous ammonium sulphate solution until the indicator changed from blue-green to reddish-grey. Organic carbon content was calculated by the following equation:

$$\textbf{Carbon content(mg C g}^{-1}\textbf{ air-dry compost)} = \frac{30(1 - x/y)}{\text{Soil mass(g)}}$$

Where:

x = residual acid-dichromate titre, mL

y = standardizing titre, mL

3.2.8.6 Total nitrogen

One to three grams of sample was placed in a dry digestion tube. Then, 5 mL of digestion mixture (Na_2SO_4, 50 g; Se, 0.5 g; conc. H_2SO_4, 500 mL) was added and swirled to wet the entire compost sample. The digestion tubes were placed in the digestion block. After completed digestion, 75 mL of 10 N NaOH was added into the sample. A 125 mL Erlenmeyer flask with 10 mL of H_3BO_3 plus indicator (10 g of H_3BO_3 was placed into 475 mL of distilled water and stired. 10 mL of mixed indicator

(bromocresol green, 0.099 g; methyl red, 0.066 g; ethanol, 100 mL) was added into H_3BO_3 solution. The solution was adjusted with 0.1 M NaOH until the solution turns reddish-purple (pH 4.8-5.0). Finally, made up to 1 L with deionized water and mixed it thoroughly.) was placed under the condenser so that the tip of the condenser was immerged in the H_3BO_3 plus indicator and distillated. Twenty five milliliters of distillate was collected and titrated with 0.025 N H_2SO_4 (0.01 M. The color changed at the end point was from green to pink. Percentage of total nitrogen was calculated by the following equation:

$$\textbf{Total Nitrogen (\%)} = \frac{\textbf{mL (sample-blank)} \times \textbf{M} \times \textbf{2.8}}{\textbf{Mass of sample (g)}}$$

1 mL of 0.025 N H_2SO_4 was equivalent to 0.28 mg of nitrogen.

Where:

M = concentration of H_2SO_4 (N).

3.2.8.7 Total phosphorus

Five milliliters of phosphorus working standard solution and 1 mL of 1.5 M H_2SO_4 (98% m/m H_2SO_4, 80 mL in 800 mL of distilled water) were added into a conical flask and swirled the solution to release CO_2. Then, 20 mL of 0.15 % m/v ammonium molybdate reagent (1.2 % m/v ammonium molybdate reagent, 1 vol.; distilled water, 8 vol.) and 5 mL of 1.5 % m/v ascorbic acid solution (freshly preparation before use) were added, swirled to mix and allowed to stand for 30 min for color development. The absorbance at a wavelength of 440 nm was measured by spectrophotometer. The color was stable for several hours. A graph relating absorbance to μg P was plotted. The absorbance values should be approximately 0 to

0.8 for the 0 and 35 μg standards respectively. The 0.4 g of compost sample was placed in Erlenmeyer flask, then, added 10 mL of acid mixture (conc. HNO_3: conc. H_2SO_4: conc. $HClO_4$ 5:1:2) and digested in digestion apparatus. The reaction mixture was made up to 50 mL with distilled water, mixed thoroughly, and centrifuged. Five milliliters of the compost extract were placed in a conical flask, followed by 5 mL of 1.5 M sulphuric acid. The other reagents i.e. 1.2 % m/v ammonium molybdate reagent (ammonium paramolybdate ($(NH_4)6Mo_7O_{24}.4H_2O$)), 24 g; antimony potassium tartrate, 0.6 g; distilled water, 1,200 mL; 98% m/m H_2SO_4, 296 mL and diluted to 2000 ml), 1 mg P ml^{-1} phosphorus stock standard solution (dry potassium dihydrogen orthophosphate, 0.879 g in distilled water; 36% m/m HCl, 1 mL and diluted to 200 ml; toluene, 1 drop), 20 μg ml^{-1} phosphorus intermediate standard solution (1 mg mL^{-1}phosphorus stock standard solution, 10 mL and diluted to 500 mL of distilled water; toluene, 1 drop), 0-7 μg P ml^{-1} phosphorus working standard solutions (20 μg mL^{-1} phosphorus intermediate standard solution, 0, 5, 10, 15, 20, 25, 30 and 35 mL diluted into 100 ml volumetric flasks) were added to the first sample. In addition, only the ammonium molybdate reagent was added in the duplicate. The absorbance was measured at 440 nm by spectrophotometer. The percentage of phosphorus was calculated by the following equation:

$$\textbf{Total phosphorus (\%)} = \frac{\textbf{mg P (from graph)} \times \textbf{1000}}{\textbf{Mass of sample (g)}}$$

3.2.8.8 Total Potassium

Five grams of the powdered compost sample was weighed into a 600 mL beaker, approximately added 400 mL of distilled water, covered with a watch glass, placed on a hot plate and boiled, then continued boiling gently for 30 min.

Allowed to cool, then transferred it with washings to a 1000 mL volumetric flask, made up to the mark and mixed. Filtered into a dry sample container, rejected the first 50 mL filtrate. A 10 mL of aliquot was pipetted into a 100 mL volumetric flask, made up to the mark and mixed. Analyze using a flame photometer (THERMO SCI, 3P), set up according to the manufacturer's instructions. A series of K standard solution containing 0, 50, 100, 200, 300 and 400 μg mL^{-1} (pipetted 0, 5, 10, 20, 30 and 40 mL from potassium stock standard solution 1000 μg mL^{-1}; and diluted to 100 mL with distilled water) were used to determine K in samples. The percentage of potassium was calculated by the following equation:

$$\textbf{Total potassium (\%)} = \frac{\textbf{mg K (from graph)} \times \textbf{1000}}{\textbf{Mass of sample (g)}}$$

3.2.8.9 Total calcium and magnesium

Five grams of compost sample was transferred into 100 mL glass beaker, then added 2 mL of ammonium ethanoate solution (Dilute approximately 230 mL glacial ethanoic(acetic) acid to 1000 mL. Dilute approximately 220 mL ammonia solution(ammonium hydroxide) approximately 35% mm^{-1} NH to 1000 mL in a fume cupboard. Mix together in a 5000 mL graduated beaker and adjust the pH to 7.0 using ethanoic acid added using a disposable polyethylene Pasteur pipette. Stir with a glass rod between additions, but allow solution to become still before reading the pH. Dilute to 4000 mL and transfer to a polythene storage bottle.), stirred and stood overnight. The contents were transferred to a filter funnel fitted with a 125 mm Whatman No. 44 filter paper and held in a 250 mL volumetric flask. The beaker with ammonium ethanoate reagent from a wash bottle was washed to remove all the sample, then added

successive 25 mL of reagent to leach the sample in funnel, allowing it to drain between additions. With the collected leachate volume approaching 250 mL, removed the funnel to a rack place in a 250 mL conical flask, and made up the volume to the 250 mL mark with reagent and retained for analysis of exchangeable bases. The sample in the funnel was washed free of excess reagent by five successive additions of 95% ethanol, allowed draining between washings. A wash bottle containing ethanol enabled the interior surface of the funnel, the outside of the stem, the exposed surface of the paper and the soil to be thoroughly washed. Any remaining ammonium ethanoate was elevated the final calcium and magnesium value. The funnel was now placed in a 100 mL volumetric flask and leached with successive 25 mL of potassium chloride solution (KCl, 100 g in water and made up to 1000 mL, added HCl, 2.5 mL to check the pH which was approximately 2.5), allowing draining between additions, until nearly 100 ml has been collected. Make up to the mark and retain for determination of calcium and magnesium. A 20 mL of extracted compost sample and standard solutions ([Calcium standard: $Ca(NO_3)_2$, 2.05 g; HCl, 1 mL; adjusted to volume at 500 mL with distilled water][Magnesium standard: magnesium oxide, 1.6581 g; adjusted to volume at 1 L with distilled water) were added into 50 mL beakers, and then pipetted 1 mL releasing agent solution ($LaCl_3.7H_2O$, 2.68 g made up to 100 mL with distilled water) into each beaker and mix. Then the absorbance was measured at 422 nm for calcium and at 285 nm for magnesium. The percentage of calcium and magnesium were calculated by the following equation:

$$\textbf{Total calcium (\%)} = \frac{\textbf{mg C (from graph)} \times \textbf{1000}}{\textbf{Mass of sample (g)}}$$

$$\text{Total magnesium (\%)} = \frac{\text{mg Mg (from graph)} \times 1000}{\text{Mass of sample (g)}}$$

3.2.8.10 Germination Index

Germination index, to assess phytotoxicity, was evaluated by means of a seed germination test using cress (*Lepidium sativum* L.). The germination tests were carried out for 24 h in the dark at 27°C. Seeds were placed in Petri dishes on sterile filter paper wetted with 1 mL of either filter-sterilized aqueous extract from compost or distilled water for the control assay. Fifteen plates, each with seven seeds, were prepared for both the control (germination only on sterile water) and the treatment (germination on the 30% dilution of compost aqueous extract). Compost extract was obtained from each compost and adjusted to a moisture content of 60% by maintainence of the samples at 250 atm for 15 min with a hydraulic press (Zucconi *et al*., 1985). Germination index (GI) was calculated by the following equation:

$$\text{Germination Index} = \frac{A \times B}{C \times D}$$

Where:

A= Mean of seeds germinated for assay with compost sample

B = Mean root length for assay with compost sample

C= Mean of seeds germinated for control assay

D= Mean root length for control assay