DOCTORAL THESIS

# Composting of food waste with Chinese medicinal herbal residues as a bulking agent to produce a high-end organic fertilizer with antipathogenic effect

Zhou, Ying

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# COMPOSTING OF FOOD WASTE WITH CHINESE MEDICINAL HERBAL RESIDUES AS A BULKING AGENT TO PRODUCE A HIGH-END ORGANIC FERTILIZER WITH ANTIPATHOGENIC EFFECT

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# Composting of Food Waste with Chinese Medicinal Herbal Residues as A Bulking Agent to Produce A High-end Organic Fertilizer with Antipathogenic Effect

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**for the degree of**

**Doctor of Philosophy**

**Principal Supervisor: Prof. Jonathan WONG W. C.**

**Hong Kong Baptist University**

**April 2015**

# DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of Ph.D. at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: _____

Date: April 2015

## Abstract

Composting is a sustainable method to deal with huge amount of daily organic waste due to its robustness and easy operation. However, food waste (FW) as the main material in composting has disadvantages such as the heterogenous properties, high foreign matters contamination, high moisture content, low C/N ratio, poor structure, low porosity and high acidity during the initial phase of composting. These shortcomings not only influence degradation efficiency but also cease the composting process. Therefore, a bulking agent is required to increase the porosity and adjust the moisture content as well as C/N ratio of the composting mixture (Wong et al., 2010). For previous research, sawdust (SD) and tree barks were commonly used as the bulking agent in composting system but the demand for sawdust and tree barks significantly increased the cost of the composting process, and this has stimulated the demand of alternative substitutes. Therefore, the ideal situation is to find the bulking agent which is not only suitable for composting but is also a waste. Traditional Chinese medicine is widely used nowadays and huge amount of residues are accumulated and treated in landfilling (Wang and Li, 2013). According to previous research, only 5% of the active ingredients can be extracted from the medicinal plants which means there are still a large fraction of active ingredients remain in the herbal residues (Wu et al., 2013). In addition to the bulking property of Chinese medicinal herbal residues (CMHRs), it is assumed mature CMHRs compost have the ability to hinder regular metabolic pathway of phytopathogens after land application (Bernal-Vicente et al., 2008).

The first experiment of this study investigated the formula between food waste, sawdust and CMHRs in order to achieve efficient composting. The experimental results demonstrated positively the use of CHMRs is a suitable candidate to co-compost with food waste. In terms of biodegradation decomposition efficiency and compost maturity, the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) showed the best performance among all treatments with 67% organic matter degradation and 157% seed germination index. Only well-matured composting product can suppress plant diseases in soil since it has some microorganisms which can inhibit phytopathogens. The treatment 5:5:1 (FW: SD: CHMRs, dry wt. basis) also reached maturity but with a longer composting period; however, it was the treatment which could accommodate the highest quantity of food waste. The log copy number of the bacterial population was 7-8 initially, which decreased and stabilized along the composting. Results revealed that the CHMRs can be used as a bulking agent with

food waste, and a dry weight ratio of 1:1:1 (FW: SD: CHMRs) would be optimum to achieve higher organic decomposition and faster maturity. However, the initial lower microbial population in the treatment, though without any adverse effect on the overall microbial decomposition, will warrant further work to indicate the total population is not a practical means to illuminate the effective microbial decomposition. Besides, the advantage in using CHMRs will need further experiment to indicate its potential pathogen suppression capability.

Humification during co-composting of food waste, sawdust and CMHRs was investigated to reveal its correlation with compost maturity. The huge decrease in the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) of aliphatic organics in humic acids (HA) demonstrated the degradation of the readily available organics, while an increase in aromatic functional groups indicated the maturity of compost. Disappearance of hemicellulose and weak intensity of lignin in the CMHRs treatments indicated that the lignin provided the nucleus for HA formation; and the CMHRs accelerated the compost maturity. Humic acid to fulvic acid (HA/FA) ratio of 1:1:1 treatment was the highest at the end of composting and showed a clear correlation with compost maturity as also evidenced through the presence of higher aromatic functional groups in the HA fraction. Pyr-TMAH-GC-MS results indicated that dominant groups were aliphatic and alicyclic esters and ethers at the early composting stages in all treatments. Long chain fatty acids were broken down into smaller molecular compounds earlier in treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis), resulting from the faster decomposition rate. The complicated ring-structure components appeared dominantly at the later phase of composting. The peak intensities in treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) indicated that the composts became mature earlier than the other two treatments. In brief, the treatment with dry weight ratio 1:1:1 had greatest humification degree with more cyclic structures and stable final products at the end of composting.

Water and acetone extract of composts with food waste and CMHRs were tested with their antipathogenic effect on two kinds of commonly found phytopathogens, Alternaria *solani* (A. *solani*) and Fusarium *oxysporum* (F. *oxysporum*). Seventeen bacterial species and 22 fungal species were isolated and identified as prevalently existed microbes during composting process. The results of $MIC_{50}$ indicated that the treatment with dry weight ratio 1:1:1 (FW: SD: CHMRs, dry wt. basis) required least concentration of composts extraction to kill half quantity of the phytopathogens, 16% for A. *solani* and 22% for F. *oxysporum* extracted by acetone. The phytopathogen suppression capacity of composts was partially due to antagonistic abilities from some of the isolated microorganisms as well as the inhibition of active compounds. As shown in the comparison, the interfere/compete between antagonistic microorganisms

and target pathogens were more powerful than individually influenced by chemical compounds. However, the influencing factors should not be considered independently since antagonistic interactions between microbes in composts and phytopathogens are highly dependent on the abiotic properties of the composts and the alternative environment. In a word, the antipathogenic effects from composts were synergism of both antagonism and chemical factors.

Suppressive capacity on phytopathogens is one of the major function of mature composts and the antipathogenic effect was stimulated when CMHRs was used as the bulking agent in composting process. The abiotic inhibitory rates of treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) indicated that more powerful bioactive components were remained at the end of composting than in the treatment 5:5:1 and control which had no CMHRs but plastic beads as the bulking agent. Hence sensitive and comprehensive analytical technique of ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-QTOF-MS) was utilized to acquire a better understanding of the complicated structures of final composting products. Seven dominant among 22 active compounds with antibacterial/antifungal properties were obtained in the treatments with CMHRs while 17 kinds of compounds with higher contents were shared in all treatments, which should be derived from food waste. The bioactive components from CMHRs composting were mainly from the groups of alkaloids, flavonoids and coumarins.

Mature composts were used as biofertilizer to protect plants (*Brassica chinensis* and *Lycopersicon esculentum*) from phytopathogenic infection. This study showed the crop yields were increased with the addition of mature CMHRs composts to acid soil, and 5% CMHRs compost was the optimum application rate, while at the higher application rate of 10% (dry weight basis, w/w) plant growth was inhibited which might be due to the higher salt contents and the phytotoxicity of alkaloids, flavonoids and coumarins in the CMHRs. According to the biomass results, *Brassica chinensis* was more sensitive to the inhibitory effect of phytopathogen inoculation, while nutrient supply was to a less extent due to the short growth period as compared to *Lycopersicon esculentum*. The present study showed clearly that mature compost provided *Lycopersicon esculentum* and *Brassica chinensis* sufficient nutrients such as nitrogen and phosphorus. Additionally, the advantage of using mature CMHRs compost as a soil conditioner was also observed for blocking phytopathogenic infection from plant roots. The mechanism was mainly derived from the bioactive components in mature CMHRs compost which inhibited phytopathogenic activities in soil. Many identified compounds were alkaloids, flavonoids and coumarins which have powerful antifungal and antibacterial abilities and most of them maintained during growth period though their amounts reduced greatly due to their photolytic and

pyrolytic properties. Therefore, mature CMHRs compost can be the substitute to reduce the usage of fungicides and its associated environmental hazards. The present study demonstrates clearly the beneficial effects of using CMHRs as a bulking agent to co-compost with food waste with the additional phytopathogens suppression property.

Therefore, it is concluded that Chinese medicinal herbal residues can be a good choice of bulking agent in food waste composting system. Organic matter degradation and humification process were accelerated by CMHRs addition and mature CMHRs compost had antipathogenic effect and protect plants from infection.

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# CHAPTER ONE

# AIM AND OBJECTIVES

## 1.1 Introduction

Landfill disposal of municipal solid waste was 9600 tons per day in Hong Kong during 2012; of which 39.8% were food wastes (HKEPD, 2014). Currently landfilling is the only means for food waste arising in Hong Kong. However, landfill disposal not just used up a lot of valuable land space but in the same time landfill becomes the major source of leachate, odours, and greenhouse gases.

Composting is a suitable method to treat organic waste due to its robustness and easy operation. Despite the intensive composting research in the past decades, food waste composting is still in the developing level due to problems such as the heterogenous properties of food waste, high foreign matters contamination, high moisture content, low C/N ratio, poor structure, low porosity and high acidity during the initial phase of composting. Onset of acidity during this initial phase is due to the rapid decomposition of readily biodegradable substances such as carbohydrates and fats which hamper the composting process. Our recent study revealed that the

successful operation of food waste composting depends on the mixing of substantial amounts of bulking agents eg. sawdust and tree barks, to increase the porosity and adjust the moisture content as well as C/N ratio of the composting mixture (Wong et al., 2010). However, the demand for sawdust and tree barks significantly increased the cost of the composting process, and this has stimulated the demand of alternative substitutes. Therefore, the ideal situation is to find the bulking agent which is not only suitable for composting but is also a waste.

According to the statistics from relative marketing research, there are more than 1636 kinds of Chinese medicines well known and used in China and more than 50000 institutions and clinics providing traditional Chinese medicine (TCM) services (Wang and Li, 2013). Consumption amount of botanic Chinese medicine reaches 70 million tonnes annually basis on such huge number of industries, resulting in millions of tonnes of vegetal medicinal residues (including water). Most of these residues turn into wastes and as reported only 5% of the active ingredients can be extracted from the medicinal plants which means there are still a large fraction of active ingredients remain in the herbal residues (Wu et al., 2013). According to previous research, the prevalent antifungal bioactive compounds in TCM have been reported as phenolic compounds including alkaloids, flavonoids, coumarins, saponins, monoterpene glycosides, diterpenoids, triterpenoids and steroids; some of them are quite aquous

stable while others have photolytic and pyrolytic properties (Dragojevic et al., 2011; Yang et al.; 2009). The mechanism is mainly attributed to chemical functional groups in TCM hinder regular metabolic pathway of phytopathogens (Bernal-Vicente et al., 2008).

## 1.2 Research objectives and outline of the present study

This project aims at demonstrating the feasibility of using Chinese medicinal herbal residues (CMHRs) collected from local Chinese medicine clinics as bulking agents for food waste composting to reduce the operation cost of composting as well as to develop composting product with added benefit of anti-phytopathogens property derived from CMHRs. The research has been divided into five phases. A flow chart showing the experimental approaches adopted in this study is illustrated in Fig. 1-1.

**PHASE I Develop an ideal formulation and conditions for co-composting of food waste and CMHRs**

Objectives: to develop the optimum ratio between food waste, sawdust and CMHRs, achieving high efficiency of composting by using CMHRs as the bulking agent.

**PHASE II Evaluate the composts maturity and monitor the dynamics of functional groups during humification process**

Objectives: to correlate the functional groups of the composting mass at various phases of composting and composts maturity when CMHRs was supplemented as a bulking agent with the understanding that the rate of humification can reflect the rate of compost maturity.

**PHASE III Investigate the potential impact of antibacterial/antifungal substances in mature composts with CMHRs in both abiotic and biotic aspects**

Objectives: to study the dynamics of bacteria and fungi existed in composts and the antipathogenic effect (both antagonistic and abiotic factors) of final mature composts on the two specific phytopathogens, *A. solani* and *F. oxysporum* (in vitro).

**PHASE IV Monitor the dynamics of bioactive components during co-composting of food waste and CMHRs**

Objectives: to identify the changes in profiles of bioactive compounds during the co-composting of CHMRs and food waste with different CMHRs mixing ratios and elucidate their antifungal properties.

**PHASE V Investigate the quality of final composting products derived from food waste and CMHRs co-composting; as well as its effects on the growth of plants and inhibition of soil phytopathogenic microorganisms**

Objectives: to evaluate the effectiveness of the antipathogenic properties of the mature CMHRs composts on planting of *Lycopersicon esculentum* and *Brassica chinensis* while soil was inoculated with *A. solani* and *F. oxysporum* in certain concentration.

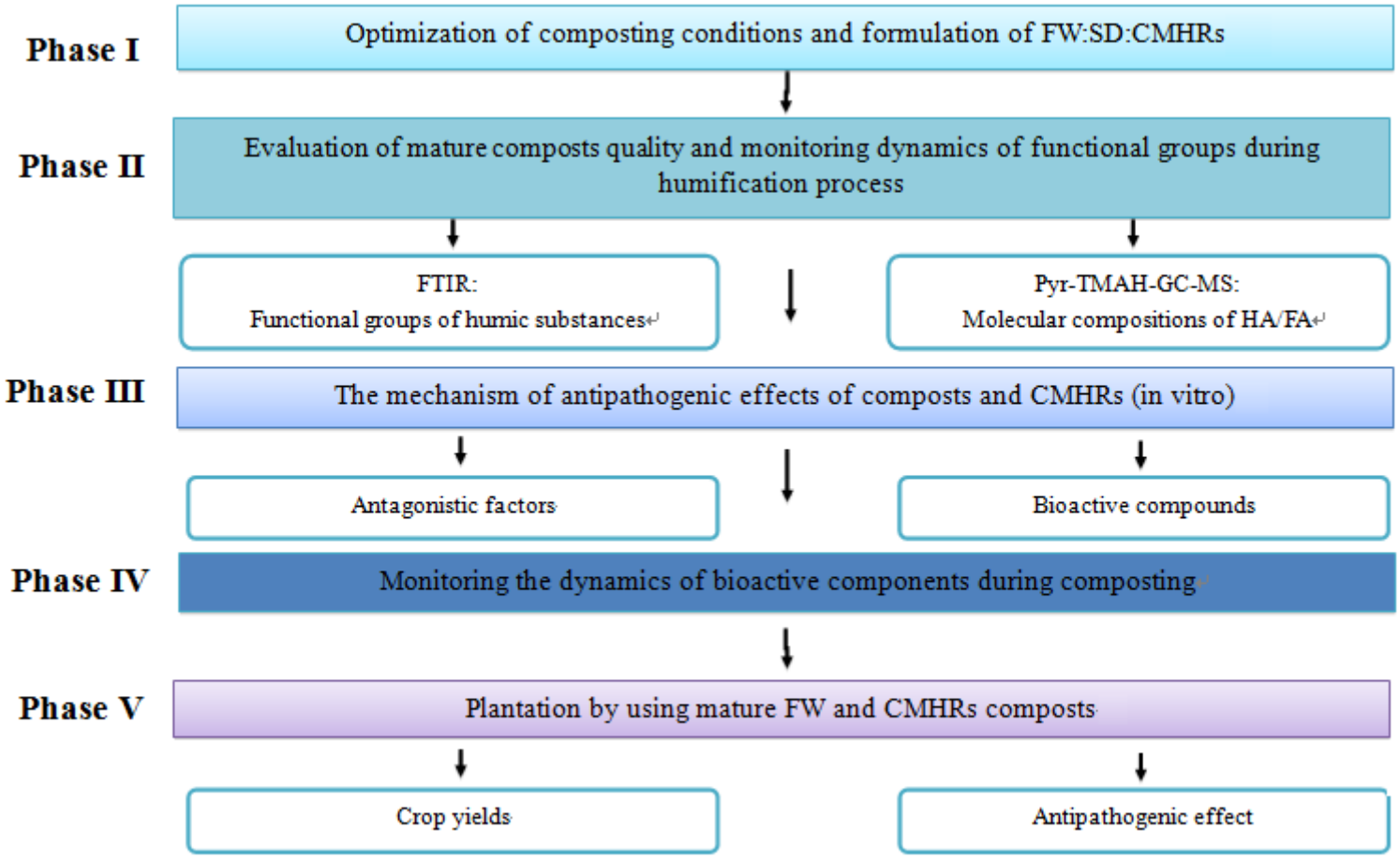


Figure 1-1. The flow chart of the experimental approaches in this study.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 General Introduction

### 2.1.1 Definition

There is no unique definition of composting. As Haug (1993) defined, “composting is the biological decomposition and stabilization of organic substrates, under conditions that allow development of thermophilic temperatures as a result of biologically produced heat, to produce a final product that is stable, free of pathogens and plant seeds, and can be beneficically applied to land”.

### 2.1.2 Treatments of Municipal Solid Waste

Landfill

Landfill is the most widely used treatment which is a place for the deposit of the waste onto or into land. Although this method is not outdated, it has many disadvantages. The site choice is very limited which must have minimum distance of 2000 meters from urban area for eventual urban development. The site should locate separately from either water currents in general or low permeable soil. However, it should not be too far from the collection area theoretically in order to save the transportation cost. Besides, visual impact should be minimized and leachate is a serious problem due to its extreme value of pH, COD and ammonium nitrogen. Once

the capacity of landfill is exhausted, it must be closed (Woon and Lo, 2014).

Incineration

Incineration is the convert controlled combustion of municipal solid waste into inert materials. The initial waste volume can be reduced by 90% while the weight is estimated around 70% (Lam et al., 2010). The negative sides of incineration include air pollution and it is costly to build an incinerator. However, traditional methods of treatment, including landfill and incineration, will cause environmental pollution.

Composting

Composting is the aerobic biological decomposition of organic substances under controlled conditions. This process aims at the stabilization of the organic substances, converting it into a mature and stable product which can be used as an alternative source of biofertilizer on land application, avoiding the accumulation of toxicity of manufactured chemical fertilizers (Wong et al., 1999; Wong et al., 2009). The final products are rich in organic substances which can promote plant growth. The time commitment may be the biggest disadvantage of this method. For compost to decay uniformly and properly, the composting period can last as long as more than two months. The value of the final product must be approved to firm the leading position in the market. But composting is the only sustainable way to deal with the huge amount of waste people daily produce. Although composting may only have effcient degradation on the putrecsible food waste rather than flammable organic waste and inorganic waste, it is the most suitable treatment in China. More food waste is generated daily in China than in western countries, therefore putrecsible organic waste is the most huge problem. Composting manages the problem with less cost and simpler techiques, comparing to landfilling and incineration.

### 2.1.3 Bulking agents in composting

Composting process is always aerobic and microorganisms ultilize oxygen to meet their request for activities. Bulking agents are organic or inorganic material with fitting sizes, with both function of making the matrix structure suitable for air passage and buffering initial conditions for microbial activities in efficient composting (Wong et al., 2011). Bulking agents are added to the composting system to assess the stable effect of the process, implemented by various ratios of bulking agents and food waste. Food waste can be composted much more efficiently with initial C/N ratio between 25-40, pH of 6-8 and the moisture content around 55%; however food waste is acid and has lower C/N ratio and higher moisture content; bulking agents are a prerequisite in composting for absorbing leachate, providing proper porosity and buffering initial pH and C/N ratio of composting matrix (Chang and Chen, 2010). Proper selection and optimum quantity of bulking agents are required to minimize the purchasing and transportation cost.

Studies on different bulking materials by Adhikari et al.(2008) and Haug et al. (1993) showed various physical and chemical properties of sawdust, hey, cardboard paper and paper water by comparing their absorption capacities, particle densities and C/N ratios. Different bulking agents contain various contents of readily available carbon source which may influence the biodegradation kinetics and composting performance due to microbial immobilization of nitrogen and the metabolic heat generation to reach the thermophilic temperature. Additionally, adjustment of moisture content also depends on the properties of bulking agents.

## 2.2 Basic concepts of composting

### 2.2.1 Composting process

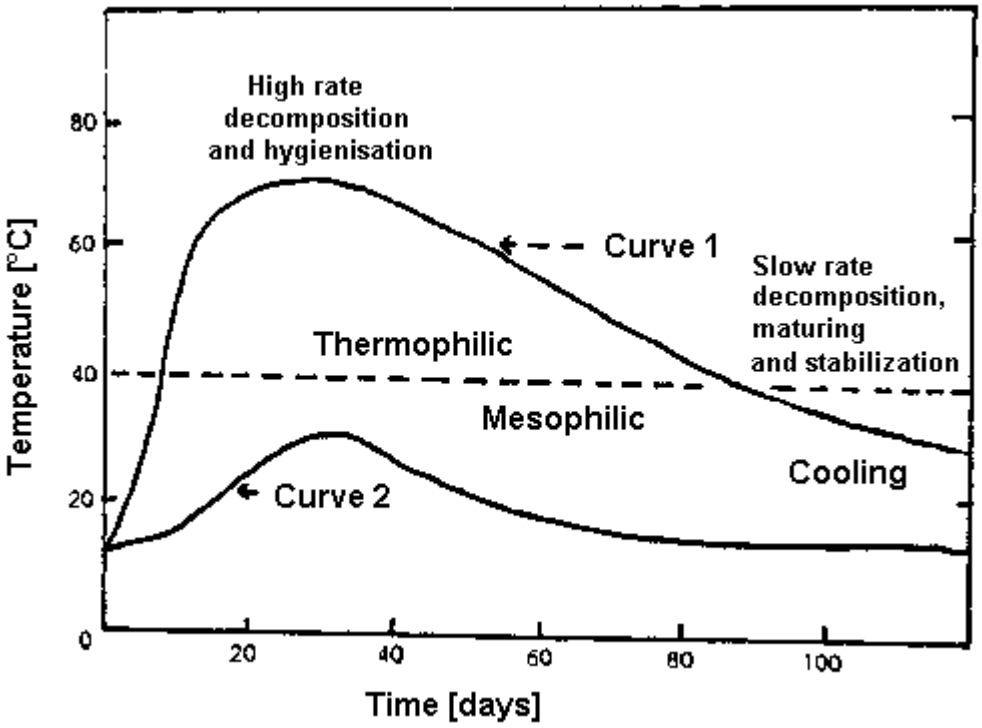


Figure 2-1. Composting Process.

(http://www.earthineer.com/blog/19122/bokashi-composting-part-1-basics)

As shown in Fig. 2-1, microorganisms such as facultative and strict aerobic bacteria, fungi and actinomycetes assimilate complicated organic substances and transfer them into inorganic nutrients during decomposition. Mature composts contain stabilized carbon sources and the thermophilic temperature eliminates potential phytopathogens before utilizing the final composting products as biofertilizer to land application (Ozores-Hampton et al., 1998; Huang et al., 2000).

Mesophilic phase

The temperature of the mesophilic phase is between 15°C to 40°C and predominant microbial groups usually include mesophilic bacteria such as Pseudomonas spp., Bacillus spp., and Achromobacter spp. (Zibilzke et al., 2005).

Enough abundance of readily available compounds and oxygen provide microbes proper environment to be active and reproduce in large quantities. Heat is produced from organic degradation under microbial activity which arises compost temperature.

Thermophilic phase

The thermophilic phase starts when temperatures elevate to 40°C and thermophiles take over the degradation process (Gray et al., 1971). A constant temperature maintaining higher than 40°C is compulsory. Common thermophiles include aerobic bacteria eg. Bacillus spp., Steptomyces spp. and Thermoactinomyces spp. so that enough oxygen needs to be introduced by mixing frequently (Zibilzke et al., 2005). The center temperature of the composting matrix remains at 60°C to 70°C for 3 to 15 days before thermophilic phase is finished (Gray et al., 1971; Maynard et al., 2000). During this phase, more complicated organic substances such as lignin, cellulose and hemicellulose are broken down into small molecular compounds. The thermophilic temperatures eliminate harmful microorganisms, especially phytopathogens which are infections for crops (Zibilzke et al., 2005).

Curing phase

When the organic substrates are exhausted, the curing phase occurs as the central temperature of composting matrix decreases below 60°C and then approaches ambient temperatures. The mesophilic organisms reappear when the temperature drops back to 40°C. The maturing phase takes up majority time period and requires a couple of months. During this phase, the mesophilic organisms dominate in the composting mass while condensation and polymerization of organic substances take place (Gray et al., 1971). By the end of curing phase, the mesophilic microbes break down deleterious metabolic intermediates such as acetic acid and phenolic compounds. The final product is mature compost which is a stable and complex of humic acid (Zibizke

et al., 2005).

### 2.2.2 Chemical and physical factors influence the composting process

Various factors influence the efficiency of composting process. Temperature and decomposition rate of composting is highly influenced by microorganism activities, which is affected by moisture, oxygen, pH, carbon, nitrogen and phosphorus content in composting matrix (Epstein et al., 1997).

Temperature

Heat generates by decomposition of organic substances under microbial activities, arising the temperature of compost system. A direct positive relation between temperature and rate of oxygen consumption has been detected which results in faster the decomposition rate. Composting matrix temperatures between 50°C to 60°C suggest efficient decomposition while temperatures higher than 60°C inhibit the most active microbial activity. Therefore, the optimum temperature range of rapid composting is between 50°C and 60°C. Large volumes of organic substances allow interior temperatures to rise to 55°C to 60°C within a few days of after composting starts; and maintain the thermophilic level for about two weeks following gradually decreasing and finally dropping to ambient temperature. This circumstance of temperature change along with time shows the decomposition and stabilization phases in composting proceeds. As shown in Fig. 2-2, the decomposition rate among the stabilized, finished compost products is slow with little amount of heat generation (Hog Producers Sustainable Farming Group, 1996).

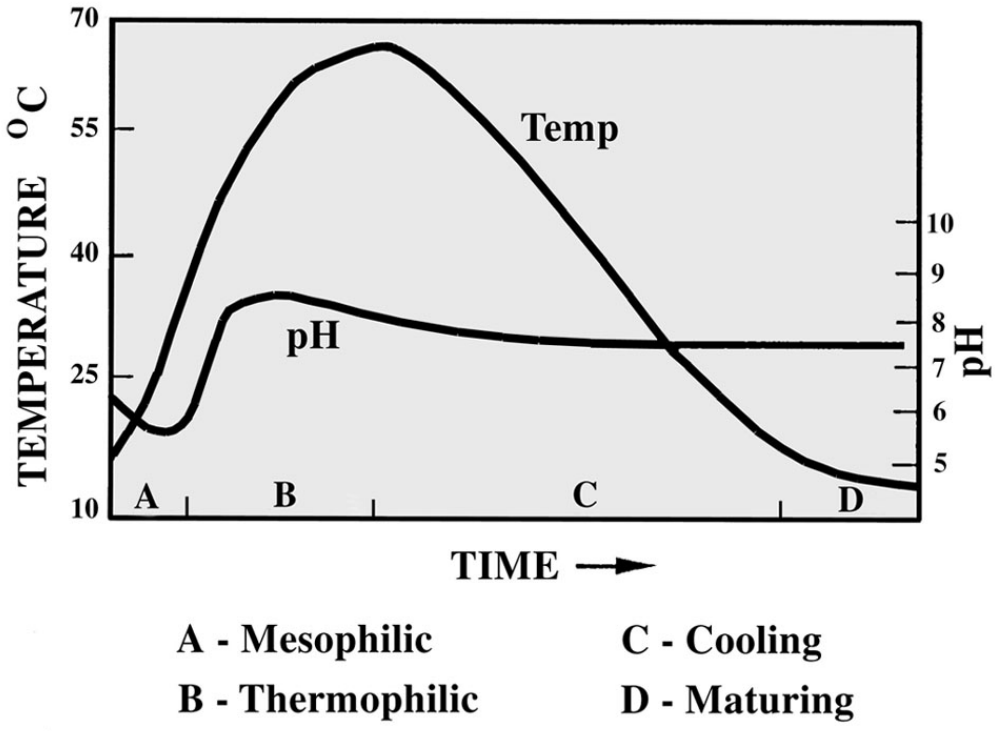


Figure 2-2. Composting temperature and pH variation with time (Haug, 1993).

Aeration

Aeration provides adequate amount of fresh air to the center of the compost matrix since highly effective aerobic decomposition of organic substances only occurs in the presence of sufficient oxygen. Regular mixing referred to as turning, enhances aeration in compost mass. Initial mixing of feedstocks usually introduces enough air for composting to start and oxygen requirements are maximized during the initial weeks of most vigorous activity. Porosity and moisture content are the most critical factors influence the air movement through the compost matrix (Wong et al., 2012).

Moisture

Environmental humidity takes an essential role in microbial metabolism and

influences the oxygen supplement indirectly since microorganisms can only utilize organic molecules which are soluble in water. The moisture content from 40% to 60% provides adequate water without aeration limitation. If moisture content is less than 40%, bacteria activities slow down and entirely cease if below 15%; when the moisture content exceeds 60%, nutrients are leached while air volume is reduced, as well as odors are produced (due to anaerobic conditions) so that decomposition rate is slowed down (Wong et al., 2009). High humidity can be controlled by turning and mixing frequently which allows air to fill in the system and loosen the materials for better draining and air drying. Addition of bulking agent such as sawdust or finished compost can also be the solution to excess moisture problem. Additional water can be added if the moisture content is below optimum amount. A more effective method includes turning the mass and rewet materials in the composting process since most bulking agents have properties of absorbing water only on their surface (Illmer and Schinner, 1997).

pH

Composting may proceed effectively within a range of acidity-basicity however initial pH below 6.0 may inhibit decomposition. Quantity of heat production is limited when pH<6.0 for the reason that bacteria activities is not vigorous until it climbs up to a more desirable level (Wong et al., 2009). The optimum pH for microbial activities involved in composting is from 6.5 to 7.5. Reduction of pH in initial composting is due to the release of organic acids and followed increment is contributed to the production of ammonia from nitrogenous compounds. At the end of the composting, the final products always reach a stable pH around 7-8, no matter what the pH values measured in the initial materials.

Nutrients

Carbon (C) and nitrogen (N) compounds are the most important nutrient sources which influence limitation of the composting in either excessive or insufficient condition, even worse if the carbon-to-nitrogen (C/N) ratio is incorrect. Microorganisms process chemical reaction in composting by digesting oxycarbide as an energy source and ingest nitrogen for protein synthesis. The optimum ratio between these two elements should be approximate 30:1 (dry weight basis, w/w) for microbial activity. Many researches indicate C/N ratios between 25:1 to 40:1 can achieve an efficient composting (Wang et al., 2013). Sawdust is a good source of carbon and other inexpensive sources of carbon include municipal waste and shredded newsprint or cardboard. The nitrogen decomposition is achieved slowly if the C/N ratio is too high; however, when the C/N ratio is too low, much nitrogen will lose in the form of ammonia gas to the atmosphere, which results in odor problems and lack of nutrient source for microbes. Besides, phosphorus, sulfur, calcium, magnesium and other trace elements also take important roles in cell mechanism.

## 2.3 Microbiology of composting

### 2.3.1 Microbial activities during decomposition

Bacteria are the smallest living organisms but take up the majority position of microbial decomposition in composting. Billions of microorganisms are found in each gram of composts and bacteria take up 80 to 90% of them. All decomposition and heat generation associated reactions occur in composting are due to bacteria activities. Bacteria have single-celled structure, rod-shaped bacilli, sphere-shaped cocci or spiral-shaped spirilla. They are air-introduced and normally it is not necessary to add

bacteria manually to the composting matrix. As mentioned in 2.2.1, various types of bacteria participate in the composting, thriving at different phases contributed to different temperatures and on different substrates.

Actinomycetes are filamentous bacteria which are lack of nuclei as other bacteria but grow multicellular filaments like fungi. During the composting, actinomycetes are able to break down tough woody materials through their enzymes so that they play an important role which cannot be substituted in complex organic substances degradation, such as cellulose, lignin, chitin and proteins.

Fungi include molds and yeasts, and most fungi are considered as saprophytes since they absorb nutrient sources from dead organic plant materials. During composting, fungi are requisite because they break down tough debris and provide optimum condition for bacteria to continue the decomposition process once most of the cellulose has been exhausted. Antagonistic relations between fungi and bacteria are indispensable for composting. Fungi species are numerous during both mesophilic and thermophilic phases of composting process.

### 2.3.2 Phytopathogens

The hazards of plant pathogens have been concerned nowadays. The major sources of infection are relevant to animal and human activities, municipal and biosolid waste treatments as well as plant effluents. Phytopathogens are able to survive in the soil and transmit from ground water to surface water, because of their small sizes and properties. The moisture and temperature situations provide them good living conditions, resulting in a huge problem of crop yield reduction. It is indicated that phytopathogens even arise the outbreak chance of potential health risk

of animal and human being (Gerba et al., 2004). With the development of modern technologies, the recycle of municipal solid waste has been undergone in an increasing trend. The amount of biosolids for landfill has been declined since more and more waste is recycled into agricultural land use.

Table 2-1. Pathogens in municipal solid waste [a].

| Categories | Pathogen of concern | Diseases or symptoms for organism |
|---|---|---|
| Viruses | Enteroviruses | acute gastroenteritis |
| | Noroviruses | acute diarrhea |
| | Hepatitis a virus | infectious hepatitis |
| | Poliovirus | poliomyelitis |
| | Rotavirus | acute gastroenteritis with severe diarrhea |
| | Adenoviruses | respiratory tract infections, gastroenteritis |
| Bacteria | Escherichia coli | gastroenteritis |
| | Faecal coliforms | gastroenteritis |
| | Faecal streptococci | gastroenteritis |
| | Salmonella | salmonellsis |
| | Shigella | bacillary dysentery |
| | Yersinia | acute gastroenteritis |
| Protozoa | Cryptosporidium | gastroenteritis, cryptosporidiosis |
| | Toxoplasma gondii | toxoplasmosis |
| | Entamoeba histolytica | acute enteritis |

[a] Adapted from Gerba et al (2011); Gerba et al (2004).

Waste is full of pathogenic microbes such as enteric viruses and true thermophilic enteric bacteria (Table 2-1) which are expected to survive longer in municipal solid waste after landfills (Gerba et al., 2011). The pathogenic microorganisms in municipal solid waste is non-infectious eventually after composting, depending largely upon the elevated temperatures and moisture while it is possible for them to survive from a few days to several months in landfill (Mehta et al., 2014).

### 2.3.3 Antipathogenic effect of mature organic waste composts

Only well-matured composts have the capacity of suppressing plant diseases after utilized as biofertilizers in soil due to their antipathogenic effect from both biotic and abiotic aspects. Heat generated during the active phase of composting is considered as the main cause of pathogen eradication while environmental acidity is another reason of microbial selection (Blaya et al., 2013). The biotic influences of phytopathogen control are mainly contributed to microbial antagonism including the production of antibiotics and parasitism as well as competition for nutrients but not chemical compounds (Luo et al., 2010; Suárez-Estrella et al., 2007). For instance, several recent studies have proved the antagonistic capacity of *Aspergillus niger* on different plant pathogens (Danon et al., 2010; Rai and Upadhyay, 2002; Kandhari et al., 2000). The presumable reason why isolated microbes surpass plant pathogens is that they grow faster than any other microorganisms indigenous to the compost (Phae et al, 1990). *Trichoderma* is also widely induced in compost to control different plant diseases infected by phytopathogens such as *Fusarium* spp., *Phytophthora* spp., *Sclerotinia* spp., *Rhizoctonia* spp. and *Pythium* spp. Trichoderma spp. produces organic acids which decrease soil pH, promoting their own growth and negatively influencing phytopathogenic activity, especailly on *Fusarium* wilt severity (Bernal-Vicente et al., 2008). The mechanism was attributed to pH adjustment function of *Trichoderma* spp. which reduced the availability of nutrients such as iron and induced siderophore production and competition for this mineral, resulting in nutrient competition among microbes, especifically against *F. oxysporum* (Segarra et al., 2010). Addition to fungi, some species of copiotrophic bacteria also have biocontrol capacities with mycelial growth blocking functions, such as *Pseudomonas* and *Bacillus* (Pane et al., 2013).

## 2.4 Carbon and Nitrogen transformation during composting

Various kinds of carbon and nitrogen exist and transform during composting, eg. the water-soluble, acid-hydrolysable and non-hydrolysable. The amounts of the water-soluble and acid-hydrolysable carbon and nitrogen decrease during composting while non-hydrolysable carbon remained relatively constant and non-hydrolysable nitrogen increase dramatically. The ammonium ($NH_4^+$-N), known as water-soluble forms of nitrogen, decreases at the initial stage of composting; while an increment of nitrate ($NO_3^-$-N) and nitrite ($NO_2^-$-N) towards the end of composting.

### 2.4.1 Carbon transformation

During composting, the reduction matrix weight is mainly due to the mineralization of the organic substances. During these phases, the organic carbon fractions have enormously transformation, either transferring to dissolve form or utilized by microbes for their activities. Along with the total carbon content reduction, the humic substances are formed and accumulated, promoting the mature degree of composts (Wong et al., 2006). It is reported in Huang et al. (2006) that C and H decreased while N and O increased of humic acids. Well humified composts protect land crops from toxic compounds found in immature composts which are harmful for plant growth.

According to Garcia et al's (1991) study, carbon fractions transform drastically during initial composting phases and become much slower after thermophilic phase. This result illustrated that microorganisms are more active during the initial

composting period where carbon mineralization takes place principally. During compost maturation, small quantity of carbon fractions which are biodegraded easily and maintained in the compost matrix, were lost and the organic substances was stabilized. The increasing content of humic substances during the composting is owing to either the formation of humic acid-like substances or the separation of these substances from other more complex carbon compounds.

### 2.4.2 Nitrogen balance

Nitrogen is more complicated because some of the nitrogen is lost in the forms of $NH_3$, $N_2$ and $NO_2$, which are either major constituent of air or not recorded. Additionally, organic and inorganic forms of nitrogen such as ammonium-N, nitrite-N and nitrate-N are defined overall as total nitrogen content in the solid and liquid phase. Since $NH_4$-N has phytotoxic limitation on seed germination and root elongation, low amount of extractable ammonium provides necessary growing environment to the crops. Most of the nitrogen exists in the form of organic nitrogen, normally as part of the protein and simple peptides in the composting matrix. Microbes can only use dissolved form of nitrogen which is undergone ammonification process, for their activities. For instance, dissolved ammonium can be utilized by microorganisms as the main nitrogen source in the composting matrix and transformed into inorganic nitrogen in alternative situations. Nitrate is formed when temperature decrease to $40^oC$ with the activation of nitrifying bacteria; microorganisms utilize nitrate as the oxygen source with absent of oxygen, leading to denitrification and ceasing nitrification. Ammonium and nitrate are the most important forms of nitrogen which plant roots absorb directly in crop planting (Monedero et al., 2000). Alternatively,

when undergone the condition of high temperature with the pH up to 7.5 (thermophilic phase of composting), the ammonia volatilizes as nitrogen loss.

## 2.5 Evaluation of compost maturity

During composting, readily degraded organic substances are transferred by microorganisms as a nutritive source into inorganic compounds. The transformed, slowly-degradable compounds, intermediate breakdown products and the cell walls of dead microorganisms are considered as humic substances which can be hugely found in final composts after decomposition. The increasing demand of mature composts as the soil conditioner is introduced by the requirements of utilization of humic acid to soil ecology, fertility and structure as well as its beneficial effects on plant growth (Wong et al., 2009). Additionally, the natures of humic acid accumulated during composting have a staple effect on the compost quality. The increment is observed in polycondenzed structures and more stable organic substances in mature composts. The phenomenon can be good instruction of the humification degree processes in organic waste composting (Wei et al., 2007).

Compost is for alternative use, depending on which mature level it has reached. Plants grow slowly and crops are turn to be damaged if immature composts are applied to the agricultural land. The reason is due to insufficient biodegradation of organic substances result in oxygen competing or causing phytotoxicity to plants because of high levels of organic acids, high C/N ratio, extreme pH and salts content (Wu et al., 2000). The characters of immature compost determine its application on covering ground undergoing bioremediation or landfill sites.

### 2.5.1 Physical characters

The color of composts become darker since humic substances are formed during composting. Generally, temperature of composts maintain at ambient level during turning once becoming thoroughly mature and not returnable to anaerobiosis during storage. The moisture content of mature compost is relatively low with dramatically reduction in both weight and volume. Unpleasent and acid smell of wastes disappear with much more uniform partical size since organic acids accumulate in early phases of composting is broken down by acidophile. The acid environment with low pH is also positive for fungal growth and the breaking down lignin and cellulose. However, acid accumulation can lower the pH<4.5 if the composting system becomes anaerobic, inhibiting microbial activities severely. In such cases, sufficient amount of aeration is compulsory to repair acid environment to acceptable ranges.

### 2.5.2 Microbiological dynamics

<u>Heat production and respiration</u>

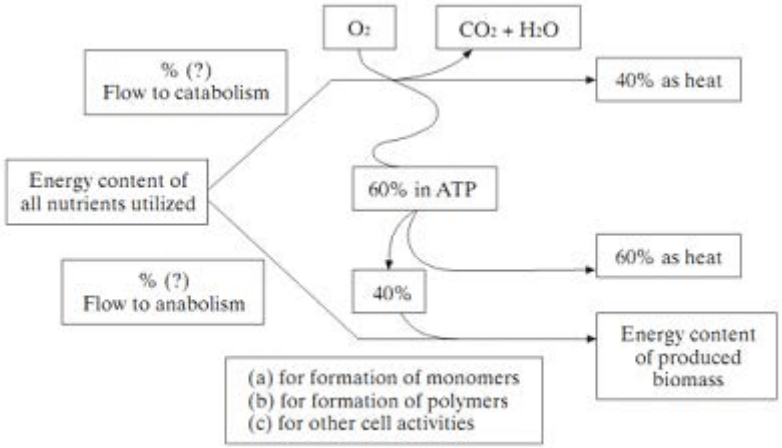


Figure 2-3. Energy flow in aerobic metabolism of microorganisms (Kutzner, 2008).

Any metabolism from microbes to human beings inevitably produces heat as a consequence result of the second law of thermodynamics. Fig. 2-3 represents that only some portion of the consumed energy can be transformed into useful work like biosynthesis, while the other energy is liberated as heat to increase the entropy of the surroundings.

Oxygen and carbon are utilized by microorganisms for their activities, producing carbon dioxide and energy. This process can be inclined to anaerobic without sufficient amount of oxygen and produces undesirable odors, such as the rotten-egg smell due to sulfureted hydrogen gas. Surprisingly, aerobic microbes can survive with the oxygen concentrations as low as 5% though oxygen takes up 21% in atmosphere. Oxygen concentration > 10% is considered to be optimum for supporting aerobic composting. Some compost systems provide adequate oxygen supplement continually through natural diffusion and convection while other systems require active aeration, providing by blowers or through turning or mixing the compost materials.

Microbial dynamics

As described in Section 2.2.1, changes in microbial constitution during composting process have been estimated greatly. The amount of mesophilic and thermophilic bacteria are varied numerously in different phases during composting as well as nitrogen-fixing bacteria and lignin degradation microorganisms. However, microbial parameters are not reliable for the evaluation of composting maturity because populations of microorganisms are influenced by environmental condition easily, such as temperature, moisture, acidity and nutrients.

### 2.5.3 Chemical characters

C/N ratio

Carbon-Nitrogen (C/N) ratio is the most common and critical parameter to evaluate the compost maturity since it provides a direct estimation of the biologically degradation. The initial C/N ratio around 30 is optimum for composting process since microorganisms contain autologous C/N ratio around 30. Microorganisms implement degradation in the most effective way when environmental C/N ratio is similar to their own.

The C/N ratio is considered as the symbol of assess of the biodegradability of organic substances and compost maturity. C/N ratio decrease during composting either for the reason that carbonaceous and nitrogenous materials are transformed into more stable complex organic forms which chemically and biologically resemble humic substances by microbial activities; or utilized by microbes for their metabolism and activities, resulting in the microbial biomass synthesis (Guo et al., 2012). Additionally, carbon dioxide and water are produced and volatilize into the atmosphere (Wong et al., 2009).

Humic substances

Humic substances comprise the most important fraction of organic substances because of their unique properties, such the capacity to interact with metal ions, the ability to buffer pH and act as a potential source of nutrients for crops. Humic substance in composts mainly consists of humic acid (HA), fulvic acid (FA) and humin. According to Hsu's research, total humic acid accumulated during the composting, from 28% to 44% of the organic substances after 33 days and stabilized at this value until the end while the fulvic acid content gradually decreased along humification process (Hsu et al., 1999). Hsu also suggested that the most effective factor influenced the humification process was the type of raw material for

composting. The ascendant trend of HA/FA represents the degree of humification and maturity of composts. Initially, fresh compost matrix is consisted of low amount of humic acid and relative more fulvic acid. As microbial reactions start in composting, the percentage of humic acid and the fulvic acid exchange gradually due to a continuous release of the easily biodegradable substances broken down during the thermophilic phase.

### 2.5.4 Plant research

Seed germination

Seed germination and plant growth bioassay are the most common techniques used to evaluate phytotoxicity of the final composts. Phytotoxicity has been introduced as the association of immaturity of final composts and plant health can be improved by reducing the amount of organic acids. Phytotoxicity is described as an intoxication of living plants by substances present in the growth medium and accumulated in plant tissue. There are large variations among bioassays and plant species. Fuentes et al (2004) observed that seed germination has been regarded as a less sensitive method than root length when used as a bioassay for the phytotoxicity evaluation. According to Araujo and Monteiro (2005), the bioassay by only examining seed germination is relatively low sensitive to many toxic substances. The reason is that many chemicals are absorbed by seeds but assimilating its nutritional requirements internally from seed stored materials while it is effectively isolated from the environment. The root lengths are more reliable of proving the optimum applied concentration of composts since the roots are responsible for absorption and accumulation of metals (Mitelut et al., 2011).

Plant growth with addition of mature compost as biofertilizer

Agricultural application of the final mature composts raises both nutrient content and nutrient availability in the soil. Comparing to only three kinds of main nutrients (nitrogen, phosphorus and potassium) in most of frequently used chemical fertilizers; composts also provide micronutrients, which are also expressed as trace elements, such as iron, zinc and manganese. Moreover, mature compost increases nutrient availability because of its buffering capacity as soil conditioner, providing an optimal pH which makes nutrients available to plants and controlling toxins and phytopathogenic attack in the soil.

Mature compost has a function of conditioning all types of the soil properties. Taking the clay soils as an example, composts support tightly packed soil particles a chance to be separated and increase air passage for plant roots. On the contrary to clay soils, composts fill in empty spaces between particles in sandy soil which increases water retention and improves nutrient availability. Preferable fertility is contributed to the conditioned soil structure since young plant roots can penetrate into the earth deeply and absorb all the nutrients to satisfy their requirements.

Land application of mature compost to soil greatly controls the incidence of plant disease and insect pests. The benefit is due to compost properties of increasing the quantities of beneficial microorganisms (fungi and bacteria) in soil. It is shown that antagonistic effects of these positive creatures in mature composts reduce phytopathogenic infections of root rot, ashy stem blight and chili wilt (Verma et al., 2007). Composts can be an effective form of plant pest biocontrol since they maintain billions of beneficial microorganisms which are competitive to negative phytopathogens.

## 2.6 Chinese medicinal herbal residues

### 2.6.1 Background of Chinese medicinal herbal residues

There are over three hundred kinds of Chinese medicinal herbs are commonly used today. Fifty fundamental herbals widely used are listed in Table 2-2 (wikipedia, 2012).

Table 2-2. Fifty fundamental Chinese medicinal herbals commonly used.

| Binomial nomenclature | Chinese name | English Common Name (when available) |
|---|---|---|
| Agastache rugosa | huò xiāng (藿香) | Korean Mint |
| Alangium chinense | bā jiǎo fēng (八角枫) | Chinese Alangium Root |
| Anemone chinensis | bái tóu weng (白头翁) | Chinese anemone |
| Anisodus tanguticus | shān làng dàng (山莨菪) | - |
| Ardisia japonica | zǐ jīn niú (紫金牛) | Marlberry |
| Aster tataricus | zǐ wǎn (紫菀) | Tatar aster, Tartar aster |
| Astragalus propinquus | huáng qí (黄芪) or běi qí (北芪) | Chinese astragalus |
| Camellia sinensis | chá shù (茶树) or chá yè (茶叶) | Tea Plant |
| Cannabis sativa | dà má (大麻) | Cannabis |
| Carthamus tinctorius | hóng huā (红花) | Safflower |
| Cinnamomum cassia | ròu gùi (肉桂) | Cassia, Chinese Cinnamon |
| Cissampelos pareira | xí shēng téng (锡生藤) or (亞乎奴) | Velvet leaf |
| Coptis chinensis | duǎn è huáng lián (短萼黄连) | Chinese Goldthread |

| | | |
|---|---|---|
| Corydalis ambigua | yán hú suǒ (延胡索) | Fumewort |
| Croton tiglium | bā dòu (巴豆) | Purging Croton |
| Daphne genkwa | yuán huā (芫花) | Lilac Dahpne |
| Datura metel | yáng jīn huā (洋金花) | Devil's Trumpet |
| Datura stramonium | zǐ huā màn tuó luó (紫花曼陀萝) | Jimson Weed |
| Dendrobium nobile | shí hú (石斛) or shí hú lán (石斛兰) | Noble Dendrobium |
| Dichroa febrifuga | cháng shān (常山) | BlueEvergreen Hydrangea, Chinese Quinine |
| Ephedra sinica | cǎo má huáng (草麻黄) | Chinese ephedra |
| Eucommia ulmoides | dù zhòng (杜仲) | Hardy rubber tree |
| Euphorbia pekinensis | dà jǐ (大戟) | Peking spurge |
| Flueggea suffruticosa(formerly Securinega suffruticosa) | yī yè qiū (一叶秋) | - |
| Forsythia suspensa | liánqiáo[136] (连翘) | Weeping Forsythia |
| Gentiana loureiroi | dì dīng (地丁) | - |
| Gleditsia sinensis | zào jiá (皂荚) | Chinese Honeylocust |
| Glycyrrhiza uralensis | gān cǎo (甘草) | Licorice |
| Hydnocarpus anthelminticus | dà fēng zǐ (大风子) | Chaulmoogra tree |
| Ilex purpurea | dōngqīng (冬青) | Purple Holly |
| Leonurus japonicus | yì mǔ cǎo (益母草) | Chinese motherwort |
| Ligusticum wallichii | chuān xiōng (川芎) | Szechuan lovage |

| | | |
|---|---|---|
| Lobelia chinensis | bàn biān lián (半边莲) | Creeping Lobelia |
| Phellodendron amurense | huáng bǎi(黄柏) | Amur cork tree |
| Platycladus orientalis(formerly Thuja orientalis) | cèbǎi (侧柏) | Chinese Arborvitae |
| Pseudolarix amabilis | jīn qián sōng (金钱松) | Golden Larch |
| Psilopeganum sinense | shān má huáng (山麻黄) | Naked rue |
| Pueraria lobata | gé gēn (葛根) | Kudzu |
| Rauwolfia serpentina | shégēnmù (蛇根木), cóng shégēnmù (從蛇根木) | Sarpagandha, Indian Snakeroot |
| Rehmannia glutinosa | dìhuáng (地黄) | Chinese Foxglove |
| Rheum officinale | yào yòng dà huáng (药用大黄) | Chinese or Eastern rhubarb |
| Rhododendron tsinghaiense | Qīng hǎi dù juān (青海杜鹃) | - |
| Saussurea costus | yún mù xiāng (云木香) | Costus |
| Schisandra chinensis | wǔ wèi zi (五味子) | Chinese Magnolia Vine |
| Scutellaria baicalensis | huáng qín (黄芩) | Baikal Skullcap |
| Stemona tuberosa | bǎi bù (百部) | |
| Stephania tetrandra | fáng jǐ (防己) | Stephania Root |
| Styphnolobium japonicum | huái (槐), huái shù (槐树) | Pagoda Tree |
| Trichosanthes kirilowii | guā lóu (栝楼) | Chinese Cucumber |
| Wikstroemia indica | liǎo gē wáng (了哥王) | Indian stringbush |

Chinese medicinal herbs can be classified either by temperature characteristics,

namely hot, warm, cold, neutral, and aromatic or the taste property, namely sour, bitter, sweet, spicy and salty. It was reported that many kinds of diseases can be cured by Chinese medicinal herbs, from common flu to cancers even in ancient times. Typically, one batch of Chinese medicines includes one or two main ingredients that target the illness with other ingredients to adjust the formula according to the patient's disease pattern personally. Ingredients also play the role of counteracting the toxicity or side-effects of the main ingredients; additionally, some herbs require other substances as catalysts. Overall, the balance and interaction of all the ingredients are considered more important than the effect of a single ingredient.

The polyphamaceutic character of Chinese medicines results in thousands of bioactive components found in medicinal waste and 40% of them can be left in residues after normal decoction. Waste management of CMHRs focus mainly on landfilling, with only few records indicated the potential property of CMHRs as biofertilizer. It is reported the growth promotion effect of CMHRs on edible fungi was positive if appropriate quantity added (Cai and Gao, 2009). Treatments with overdose of CMHRs represent poor growth with less absorption of nutrients, even suffocation, degeneration and death in latter phases. It resulted from the larger viscosity of CMHRs which leads to poor air permeability in soil. Good water holding capacity of CMHRs is another factor resulted in mycelium death since hindering aeration lead to lack of oxygen supplement (Yu et al, 2006). Ding et al. (2010) used CMHRs as the compound fertilizer to cultivate tomato and carrot and achieve better yields. Comparing to the control group, tomato yield was increased from 22.4% to 43.2% with vitamin C and total sugar contents dramatically inclined (10.5% to 43.6% and 3.0% to 20.2%, respectively). Better crop yield was found in carrot as 31.2% to 57.0%; with vitamin C content increased 9.0% to 31.6% and total sugar 8.6% and 23.8%.

Herbal extracts are widely used in the Chinese medicine clinics in Hong Kong in decades. Presently, herbal residues after decoction in the clinics are mainly disposed through landfilling as wastes. These herbal residues mainly contain fibrous plant materials which are bulky in nature and consist of high carbon contents. The disposal of CMHRs causes a significant source of greenhouse gases upon landfilling. Therefore, diverting these residues from incineration and subject to alternative waste treatments would reduce the air pollution with acidic gases, dioxins and furans, recycling carbon source (Akter et al, 2002).

### 2.6.2 Common chemical compounds in Chinese medicinal herbal residues

Chinese medicines consist of plant components, animal components and some mineral herbs. Among three compositions, plant herbals account for more than 87%. As reported, alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins are the most important chemical constitutions in Chinese medicines (Wang and Fang, 2009; Ye et al., 2009; Yang et al., 2009; Zou et al., 2008). From Ma et al's (2004) and Ding et al's (2010) result in Table 2-3, the dregs of a decoction are rich in organic carbon and organic substances. Flavonoids are the main secondary chemical constitution in CMHRs (Ma et al., 2004).

Table 2-3. General content of primary chemical compositions in Chinese medicinal herbal residues (%) [a].

| Total organic carbon | Total organic substances | Total phosphorus | Protein | Starch | Reducing sugar | Sucrose | Fiber |
|---|---|---|---|---|---|---|---|
| 47.5 | 81.89 | 0.0162 | 6.766 | 1.3 | 2.627 | N/A | 25.72 |

[a] Adapted from Ma et al. (2004).

The active ingredients extracted from CMHRs are rich in a variety of organic nutrients and retained a certain amount of trace elements, including amino acids, crude protein, crude fiber and silicon, manganese, aluminium, zinc, barium, chromium, magnesium, iron. Some kinds of inorganic elements and a small amount of vitamins also provide great development value. The hypothesis of addition of CMHRs in composting includes enhancing plant growth and controlling the survival rate of spore germination of phytopathogenic microorganisms.

### 2.6.3 Comparison of characteristics between Chinese medicinal herbal residues and green waste

Table 2-4. Main physico-chemical characteristics of the raw materials.

| Parameters | CMHRs | Green waste |
|---|---|---|
| Moisture content, % | 60 | 64 |
| pH | 5.5 | 6.0 |
| Total Kjeldahl nitrogen (g/kg) | 1.55 | 1.50 |
| Total organic carbon (g/kg) | 48.0 | 52.8 |
| C/N ratio | 29.6 | 35.2 |
| Ash (g/kg) | 93.1 | 83.3 |

[a] Adapted from Jouraiphy et al. (2005); Yu et al. (2006).

Table 2-4 lists the main physico-chemical characteristics of CMHRs and green waste, both of which can be the alternative bulking agent in composting. There is no significant difference between CMHRs and green waste. The moisture content, pH

and C/N ratio of both raw materials are within the range of the optimum requirement of composting. However, green wastes contain several varieties of heavy metals which may result in toxicity if transformed from crops to human beings or animals, such as zinc, lead, cadmium, chromium and copper (Whittle & Dyson, 2002; Yuan et al, 2009).

### 2.6.4 Inhibitory effect of CMHRs on soil pathogens in agricultural application

Fungi live primarily in the intercellular spaces of the host's tissue for the sake of obtaining support, protection and food from the phloem rich in nutrient. In the meantime, fungi produce one or more antibiotics which can protect the hosts from pathogenic infections. Regardless of their functions in the plant, the antibiotics have provided commercial applications in both control of some phytopathogens in agricultural industry or new drug discovery. So far, several bioactive components from entophytic Phoma have been reported. These include phomallenic acids A, B, and C; eupenoxide; phomodione as antibiotics; alkaloids phomacins A, B, and C; prenylated polyketides epoxyphomalin A and B as anti-tumor agents; and pleofungins A, B, C, and D as inositol phosphorylcer amide synthase inhibitors (Wang et al. 2012).

It is reported that CMHRs could be used as the soil improvement agent in soil and had inhibitory effect on both powdery mildew and damping off on cucumber (Segarra et al., 2010). Table 2-5 showed that 14 days after germination, the incidence of damping-off has been reduced by 2.50%, 2.78% and 2.69% separately according to addition of biological additives, CMHRs and sawdust in the soil. Recovery rate of the powdery mildew is 7.5%, 9.7% and 14.3% respectively, with disease index of 19.7%.

Table 2-5. Inhibitory effects of biological additives, Chinese medicinal herbal residues and sawdust.

| Base materials, % | Morbidity | Recovery rate |
|---|---|---|
| Biological additives | 2.50 | 7.5 |
| CMHRs | 2.78 | 9.7 |
| Sawdust | 2.69 | 14.3 |

As shown in Cai et al's results (2009), it is illuminated among all treatments, CMHRs was used as main material in the culture medium reached the optimum productivity, with the biotransformation efficiency of 35.7% which increased by 19.9% to the control group (cotton seed hulls as main material in the culture medium). The mixing culture medium with 50% CMHRs and 50% cotton seed hulls followed the highest output, with the biotransformation efficiency of 21.6% which increased by 53.8% to the control group. The productivity of the straw mushroom increased with the ratio of CMHRs increasing in the culture medium which implied nutritional and physical characters of the CMHRs are more proper to the growth requirements for straw mushroom.

### 2.6.5 Global research situation

However, to address the problem of agricultural fertilizers usage and bring sustainable development challenges, there is an ascending demand of ecological agriculture and organic agriculture in developed countries. Nowadays there are more than 100 countries are focusing on the research of organic agriculture globally, which is the most active area in food industry with the increasing rate of approximately 20% per year.

About 60% to 70% of organic products are imported by developed countries

such as EU countries, the United States and Japan as a major consumer. It brings a wonderful opportunity of export of organic agricultural products for developing countries like China. It also has brought chances for the promotion of organic fertilizer.

According to Wang et al's research, CMHRs were used to compost with oil cake (2009). Generally, treatments with high percentages of oil cake took longer time for temperature arising and had shorter thermophilic phase. Therefore, their maturing phase was lagged and all these appearances were due to toxic substances in oil cake which inhibited microorganism activities. A rapid aerobic composting process can be achieved by adding CMHRs alone and duration of high temperature lasted more than 15 days. Total amounts of nitrogen, phosphorus and pH of the final composts tend to increase with the increment of CMHRs content (Wang et al., 2008).

Nowadays, research field of composting with CMHRs is lack of published paper. Many reports indicated the most popular use of CMHRs is the cultivation of edible mushrooms and medicine mushrooms. According to the existing literature, edible and medicinal fungi which can be cultivated by CMHRs include cap fungi, straw mushroom, needle mushroom, pleurotus eryngii, dried mushroom, coprinus, agrocybe aegerita, glossy ganoderma, Schizophyllum, hericium erinaceus, black fungus and pleurotus nebrodensis (Wu et al., 2011).

### 2.6.6 Comparison of composting products by using Chinese medicinal herbal residues and municipal solid waste in global countries

Table 2-6. Parameters of composting by using Chinese medicinal herbal residues and municipal solid waste in global countries.

| Parameter | Unit | Compost by CMHRs only | Compost by Municipal Solid Waste | | |
|---|---|---|---|---|---|
| | | | China[a] | US[b] | Germany[c] |
| Organic substances | g/kg | 532 | 300 | 434 | 360 |
| N | g/kg | 37.2 | 5 | 12.6 | 9.3 |
| $P_2O_5$ | g/kg | 14.9 | 5 | 10 | 12.8 |
| $K_2O$ | g/kg | 10.2 | 9.7 | 6.2 | 12.4 |
| pH | - | 7.0 | 6-8.5 | 6.7-7.6 | - |
| Na (dm) | g/kg | 0.3 | - | 4.8 | 1.5 |
| Ca (dm) | g/kg | 12.8 | - | 47 | 49 |
| Mg (dm) | g/kg | 2.4 | - | 4.9 | 6.8 |
| Fe (dm) | mg/kg | 2480 | - | - | - |
| Mn (dm) | mg/kg | 177 | - | - | - |
| Cu (dm) | mg/kg | 19 | 250-500 | 212 | 80-480 |
| Zn (dm) | mg/kg | 63 | 500-1000 | 583 | 565-1255 |
| Ba (dm) | mg/kg | 5.8 | - | - | - |
| B (dm) | mg/kg | 27 | - | - | - |

a. Wang et al, 2008; b. Petruzzelli et al, 1989; c. Vogtmann et al, 1989.

As shown in Table 2-6, the organic substances achieve 532 g/kg in the final composting product by using CMHRs only, higher than municipal solid waste composting in China, US, and Germany. The final composts with CMHRs addition also contained abundant nutritive elements which could be ideal soil conditioner and was essential for crop growth.

## 2.7 Conclusions

Among all kinds of waste treatments, composting is the most sustainable one which can be defined as the aerobic biological decomposition of organic substances under controlled conditions. This process aims at the stabilization of the organic substances, converting it into a product that can be used in agriculture. After three

phases of composting, the final products are rich in humic substances, which are essential for crop growth. The quality of the final composts is relative to several factors; C/N ratio is the most impactive one, followed by the temperature, moisture content and pH.

Bulking agent is one of the most important parts in composting for the reason of influencing the reaction efficiency. Bulking agents are organic or inorganic material with fitting sizes, with the function of making the composting matrix structure suitable for air passage and water holding. Sawdust is one of the most commonly used bulking agents for its proper properties such as water absorption capacity and high carbon content. However, the expense of sawdust is creasing as the time of global economic development. CMHRs, residues after decoction of Chinese medicine, also can be utilized as bulking agent in composting. Some bioactive components left in CMHRs have the inhibitory effects on phytopathogens in soil and provide good soil conditions for crop growth.

Evaluation of compost maturity can be identified by various physical and chemical characters. C/N ratio is commonly used, expressing the maturity level of composting degradation. Amount of oxygen utilized and carbon dioxide output by microorganisms are other indicators. There are many regulations to evaluate whether the composting product has achieved the standard, by testing the contents of carbon, nitrogen, phosphorus and trace elements.

Mature compost is rich in humic substances which is essential for plant growth. Final products of composting can be used as ideal fertilizer and avoid harvest-limited problem of agricultural products. Inferior strengths of normal fertilizers include low availability of nitrogen and environmental pollution due to huge loss. Fertilizers are able to adjust C/N ratio in the soil for the sake of providing optimum survival

condition to beneficial microorganisms. The soil microbial activities are improved by the superior competition of beneficial microorganisms.

Herbal extracts are widely used in the Chinese medicine clinics in Hong Kong. Chinese medicines consist of plant components, animal components and some mineral herbals. Among three compositions, plant herbals account for more than 87%. Chinese medicines contain various active chemical compounds such as alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins. Some of the active elements have the inhibitory function of phytopathogenic microorganisms in soil and improve the quality of crop growth.

# CHAPTER THREE

# DEVELOPING OPTIMUM MIX FORMULA FOR FOOD WASTE AND CHINESE MEDICAL HERBAL RESIDUES CO-COMPOSTING

## 3.1 Introduction

Municipal solid waste disposed of in landfills was 8,996 tons per day in Hong Kong during 2011, of which 42.3% were food wastes (HKEPD, 2011). Due to the putrescible property of food wastes, it is a major source of leachate, odours and greenhouse gases emission of landfill. Diversion for landfill disposal, food waste should be separated and treated by biological means to conserve the remaining resources in the waste. Composting provided an appropriated method to treat the organic waste due to its robustness and environmentally friendliness. Compost product is rich in humic substances and contains nutrients, which are essential for plant growth. Despite the intensive composting research in the past decades, food waste composting is still facing a number of questions not yet solved, due to the poor structure, high moisture content, low carbon-to-nitrogen (C/N) ratio, low porosity as well as the high acidity during the initial stages of the composting (Wong et al., 2009). It is essential to have good bulking agents such as sawdust and tree barks to improve the structure and porosity, and adjust the C/N ratio of the composting mass to achieve good composting efficiency (Adhikari et al., 2008; Mayabi, 2010). However, the use of sawdust and tree barks significantly increases the cost for the composting process, and therefore it is the objective of the present study to find cheap alternative bulking agents.

The quantity of Chinese medicine consumption by such huge number of industries reaches more than 65 million tons annually, resulting in millions of tons of Chinese medicine herbal residues (CHMRs) on wet weight basis (Zou et al, 2008). The decoction of the plant materials forms the essential part of most Chinese medicines which are extracted once or twice at 100 $^{o}$C leaving the residual leaves or shoots as waste. Nowadays the most common treatment method for the CHMRs is landfilling, which can result in both environmental pollution and the waste of available resources (Yu et al, 2006). These CHMRs contain mainly fibrous plant materials which are bulky in nature and consist of high carbon contents. The disposal of CHMRs represents a significant source of greenhouse gases upon landfilling. Therefore, diverting these residues from landfilling and subject to alternative waste treatments would reduce the greenhouse gas emission from the landfills (Liang et al., 2009).

Research on composting of CHMRs is lacking, although some other usage have been published (Zou et al., 2008). Many reports indicated that the most popular use of CHMRs is the cultivation of edible mushrooms and medicinal mushrooms (Wu et al., 2011; Yu et al, 2006). Nowadays, researchers are committing to search for new bulking agents, for instance, sawdust, clay residues etc. in order to not only optimize the composting condition but also reduce the cost (Jolanun and Towprayoon, 2010). However, the use of CHMRs as a bulking agent with other organic wastes, especially with food waste has never been attempted. Besides, the CHMRs also have the probability of containing anti-pathogenic effects, adding value to the compost produced from this waste. It is reported that phytopathogens are able to survive in the soil and transmit from ground water to surface water, because of their small sizes and characters. The moisture and temperature situations provide them good living

conditions, resulting in a huge problem of limiting the harvest efficiency. It is indicated that the outbreak of phytopathogens will even a potential health risk to animal and human being (Gerba et al., 2004). During the thermophilic phase of composting, plant pathogens are eliminated and the organic substances which are toxic to plants are transformed into nontoxic organic substances. Only well-matured composting product can suppress plant diseases in soil since it has some microorganisms which can inhibit phytopathogens (Wong and Selvam, 2009). It is well known that CHMRs contained numbers of antibacterial and antifungal compounds. The mature composts containing CHMRs could enhance the growth inhibition ratio of plant pathogens and control the survival rate of their spore germination. Additionally, CHMRs have the effect of suppressing the potential re-growth of bacterial and fungi (Zhu et al., 2006; Zhu et al., 2007). Recently, a couple of studies attempted to research on composting of CHMRs alone and the effect of such compost on the crop growth (Liang et al., 2009). However, the feasibility of composting of food waste with CHMRs has not been investigated yet. Composting products with CHMRs could be an ideal modifying agent or even botanical pesticide in soil.

Therefore, the aim of this study was to demonstrate the feasibility and efficiency of CHMRs, collected from Chinese medicine clinics, as a bulking agent for food waste composting. Since the moisture content of the CHMRs is higher than the optimum moisture required for the composting, the food waste was initially mixed with sawdust (SD) and composted with CHMRs. The organic degradation and maturity of compost was monitored to assess the efficiency of composting; while other composting parameters were monitored to follow the performance of the overall composting process.

## 3.2 Material and methods

### 3.2.1 Substrates and composting

The CHMRs were collected from the clinic of the School of Chinese Medicine in Hong Kong Baptist University for a period of one month to obtain representative samples of characterization of basic properties. The daily collection lasted for one month at various seasons in order to overcome the heterogeneity and carbon/nitrogen content variation of the residues. Food waste was artificially prepared using rice, bread, cabbage and boiled pork in the ratio of 13:10:10:5 on fresh weight basis and the raw materials were cut into 0.5 $cm^3$ to keep the bulk density around 0.5 kg/L (Wong et al., 2009). The sawdust was purchased from Wong Yee Kee CO Ltd, Hong Kong. Selected properties of the raw materials are presented in Table 3-1.

Table 3-1. Selected physicochemical properties of the synthetic food waste, sawdust and the composting mix used in the study.

| Parameters | Food waste | Sawdust | Chinese medicinal herbal residues | |
|---|---|---|---|---|
| | | | Summer | Winter |
| Moisture content (%) | 59.0 (0.02) | 7.24 (0.03) | 63.0 (0.13) | 65.1 (0.16) |
| Total organic carbon (%) | 45.5 (1.70) | 52.9 (0.91) | 48.0 (0.41) | 47.0 (0.48) |
| Total Kjeldahl nitrogen (%) | 3.28 (0.04) | 0.59 (0.04) | 1.62 (1.32) | 1.55 (0.08) |
| C/N | 13.9 (0.35) | 89.8 (4.56) | 29.6 (2.21) | 30.3 (2.67) |

The food waste, sawdust and CMHRs were mixed in the ratio of 5:5:1, 2:2:1 and

1:1:1 (dry weight basis, w/w), while food waste and sawdust mixed at 1:1 (dry weight basis, w/w) served as the control to reveal the influence of CMHRs on the humification process, i.e additions of CHMRs were 1 kg, 2 kg and 3.5 kg (Table 3-2). For the control treatment, 1 kg plastic beads were added as bulking agent instead of CHMRs. Lime, at 2.25% (w/w, dry weight basis), was added to all the treatments based on our previous research to all treatments except for the additional treatment with the dry weight ratio of 2:2:1 (w/w, dry weight basis) to reveal the necessary of lime amendment. The C/N ratio of all the treatments was between 25 and 26; while the initial moisture contents were between 55% and 60%. About 7.5 kg of composting mixture was prepared for each treatment and composted for 56 days in the 20-L bench-scale composting reactors, which are made of stainless steel (Fig. 3-1). The composting system is connected with the composting control system, and the temperature and $CO_2$ emission were continuously monitored using temperature probes fixed inside the reactor and an in-line WMA-2 $CO_2$ gas analyzer (PP systems, Herts, UK) and were recorded by the AIDCS System. The schematic diagram of the composter was described in a previous publication (Wong et al., 2009). About 200 g of sample for each treatment was collected on day 0, 3, 7, 14, 21, 28, 42 and 56; and the composting masses were thoroughly mixed on these sampling days and additionally on days 10, 35 and 49. The entire experiment was repeated three times with CMHRs collected in various seasons in order to obtain the triplicates in each tank.

Figure 3-1. The composting system consisted of 20-L tanks connected with digital panel.

Table 3-2. Details of the treatments used in the study.

| Treatments | Dry weight ratio (Food waste:sawdust:CMHRs) | Lime (%) | Chinese medicinal herbal residues (kg) | C/N ratio |
|---|---|---|---|---|
| T1 | 1 : 1 : 0 | 2.25 | 0.0 | 25 |
| T2 | 5 : 5 : 1 | 2.25 | 1.0 | 25 |
| T3 | 2 : 2 : 1 | 2.25 | 2.0 | 26 |
| T4 | 2 : 2 : 1 | 0.00 | 2.0 | 26 |
| T5 | 1 : 1 : 1 | 2.25 | 3.5 | 26 |

### 3.2.2 Chemical Analyses

Parameters analyzed include pH, electrical conductivity (EC), total organic substances, total organic carbon (TOC), total Kjeldahl nitrogen (TKN), extractable ammonium ($NH_4^+$-N), nitrite, nitrate, total phosphorus and seed germination index. Moisture content was determined after drying samples at 105 $^oC$ for 24 h and total organic substances was determined after igniting the oven-dried sample at 550 $^oC$ for 16 h. The TOC, TKN, $NH_4^+$-N, nitrite and nitrate was analyzed following the standard methods for testing compost materials (TMECC, 2003). The ammonia emitted from the reactor was absorbed by boric acid and titrated against 0.1 N HCl solution. Cress (*Lepidium sativum L.*) seed germination index was determined as described in HKORC (2005).

### 3.2.3 Determination of bacterial population

The treatments with the dry weight ratio of 5:5:1, 1:1:1 and control were chosen for further determination of bacterial population, based on the reason that significant

differences could be observed based on the mixing ratio. Genomic DNA from 0.3 g fresh sample was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden) following the manufacturer's recommendation and used as the template for polymerase chain reaction (PCR) amplification. DNA products were checked in 2% agarose gel (Ultrapure, MB grade, USA) in TAE buffer at 100 V, along with the DNA ladder of 100 bp.

The quantitive PCR mixture (20 μL), containing 10 μL SYBR Premix Ex Taq II (Takara, Dalian), 7 μL nuclease free water, 1 μL of each forward/reward primers (338F, ACTCCTACGGGAGGCAG and 805R, GACTACCAGGGTATCTAATCC; 10 μmol/L each) of (Tech Dragon Ltd.) and 1 μL product template (about 20 ng), was used in q-PCR analysis of bacterial population.

The q-PCR analyses were performed using Agilent Technologies Stratagene Mx3000P (USA) with the following temperature profile: initial denaturation 95 $^{o}$C for 5 min, 34 cycles of 95 $^{o}$C for 30 s, 55 $^{o}$C for 30 s, 72 $^{o}$C for 1 min, and final dissociation 95 $^{o}$C for 1 min, 55 $^{o}$C for 1 min, 95 $^{o}$C for 1 min. DNA products were checked in 2% agarose gel (Ultrapure, MB grade, USA) in TAE buffer at 100 V, along with the DNA ladder of 1000 bp to confirm the amplicons.

### 3.2.4 Data analyses

Analyses were performed in duplicate samples and the mean values and standard deviation on dry weight basis, unless otherwise indicated are presented. The data were processed using SigmaPlot 11.0.

## 3.3 Results and discussion

### 3.3.1 Changes in total organic substances and $CO_2$ evolution during composting

As shown in Table 3-1, properties of CMHRs collected in different reasons had no significant variation which indicated CMHRs can be repeatable used in composting experiment. The treatment with dry weight ratio 1:1:1 exhibited long thermophilic phase (>55 $^{o}$C) for three weeks while the control and treatment with 5:5:1 (w/w) completed within two weeks (Fig. 3-2a). The temperature fluctuated when no lime was added to the treatment due to acid inhibition. During composting, the weight reduction occurs mainly due to the degradation of the organic substances. The organic decomposition rates were 56.7%, 48.2%, 61.4% and 67.2% in the control, 5:5:1, 2:2:1 and 1:1:1 treatments, respectively, and the increase in CHMRs addition indeed increased the organic decomposition. Among all the treatments, higher reduction of organic substances (67.2%) was observed in the treatment 1:1:1 with 2.25% lime. Without CHMRs addition, the total organic substance degraded was about 56.7% at the end of composting. The addition of 2.25% lime (w/w) decreased the initial organic substances content by 2-4% than the treatment without lime (Fig. 3-2b). Results indicate that the CHMRs can be a suitable bulking agent for composting and its dry weight ratio of FW: SD: CMHRs should be more than 2:2:1 as revealed by the percent organic degradation achieved.

Carbon dioxide evolution gave a good indication of the microbial activity of the composting process. As presented in Fig. 3-2 (c,d), the daily and cumulative $CO_2$ evolution in all the treatments increased dramatically for the first two weeks due to the rapid degradation of easily degradable organic compounds and then decreased afterwards similar to other study (Kwon and Lee, 2004; Wong et al., 2009). Among treatments containing CHMRs with lime addition, the more the CHMRs added, the

higher the cumulative $CO_2$ evolution indicating the suitability of CHMRs in promoting the microbial activities through its bulking capacity. Carbon fractions transformed drastically during thermophilic composting phases and became much slower during the curing stage (Garcia et al., 1991), indicating that microorganisms are more active during the initial period of time where carbon mineralization takes place principally (Hsu et al., 1999). During compost maturation, only a small quantity of the carbon fractions was lost due to their stabilized nature of the organics (Garcia et al., 1991). During the third week, $CO_2$ evolutions of the treatment with mixing ratio of 2:2:1 without lime addition and that with 1:1:1 were much higher than other treatments due to more efficient degradation, resulting in higher organic carbon decomposition (Wong et al., 2009).

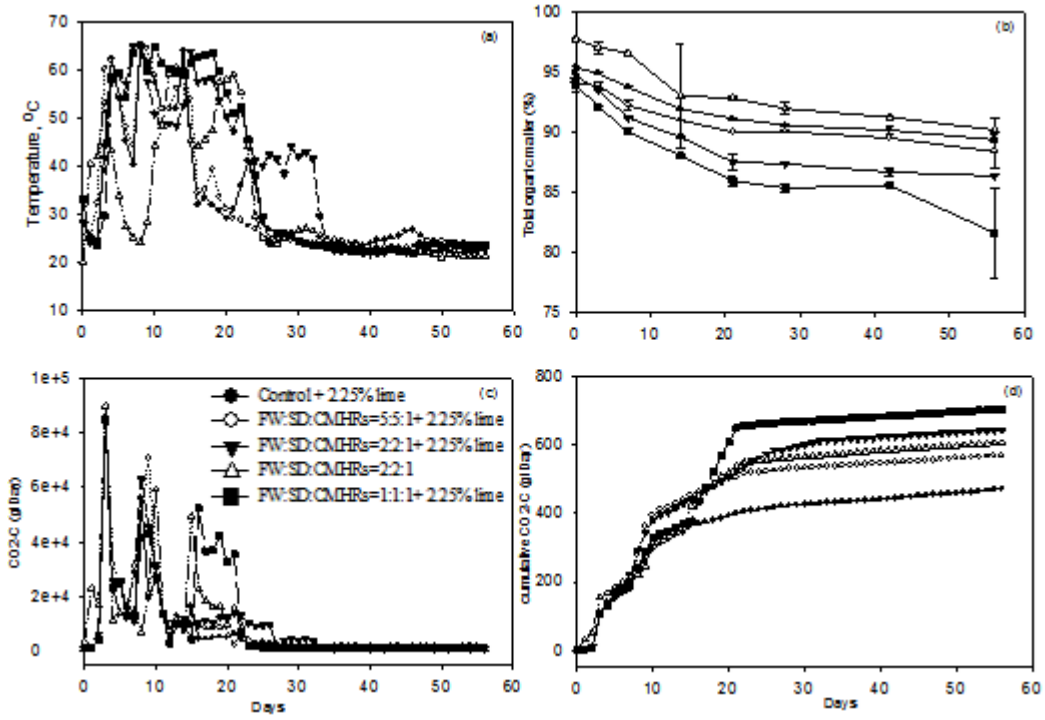


Figure 3-2. The temperature (a), total organic substances (b) and daily and cumulative carbon dioxide evolution (c,d) in various treatments with different material ratios during co-composting of food waste and CHMRs.

### 3.3.2 Changes in pH and EC

Addition of lime increased the initial pH of the composting mass to around 12 and then decreased in the first week due to organic acids generation as a result of the decomposition of organic substances in food waste under high temperature condition (Cheung et al., 2010). The ascendant temperature is the result of rapid breakdown of the readily available organic substances and nitrogenous compounds by microorganisms. Treatments with lime addition achieved longer and more stable thermophilic phase for around two weeks while the temperature of the treatment without lime fluctuated as a result of acid generation. Moreover, heat generated during the active phase of the composting process is the main cause of pathogen destruction (Fig. 3-3a). Wu et al (2012) reported that the herbal residues had a positive influence on the composting system, indicating the long thermophilic phase.

Besides, ammonium ion assimilated into microbial biomass is also one of the reasons why composting matrix became acid (Monedero et al., 2001). Treatment with dry weight ratio 2:2:1 without lime addition required longer time before reaching neutral pH due to volatile fatty acid based inhibition. After 21 days, pH values of all treatments were around 8. During composting process, decomposition rate increased when pH value rose to 7 to 9. Lime at 2.25% is enough for buffering against organic acid formation and was able to maintain the pH within the optimum range for microbial growth as reported earlier (Wong et al., 2009).

Electrical conductivity (EC) reflects the salinity of the composting matrix. High EC poses limitation on the potential application of the compost (Ren et al., 2010). The initial EC of treatment with dry weight ratio 2:2:1 without lime was much lower than the other treatments because no lime was added to the composting mass (Fig. 3-3b).

EC increased with increasing composting time which is likely due to the release of soluble degradation products, such as ammonium, volatile fatty acid and etc. Treatment without lime addition showed the highest EC value around Day 21 because low pH improved the solubility of cations. The decreasing trend of EC in all the treatments after 21 days could be explained as precipitation of cationic salts into carbonate form due to $CO_2$ equilibration and absorption of cations onto the solid matrix (Wong et al., 1996).

Electrical conductivity indicates the composting end product quality whether they are acceptable for land application. An EC value of 4 mS $cm^{-1}$ should have no inhibitory effect on plant growth (Wong et al, 2009). The treatments with dry weight ratio 2:2:1 and 1:1:1 showed EC values of 3.92 and 4.05, respectively; however, ECs of the treatment 5:5:1 and control were slightly higher than the salinity tolerant limit for sensitive crops. This indicated direct concentration of salt quantities of these composts may inhibit plant growth.

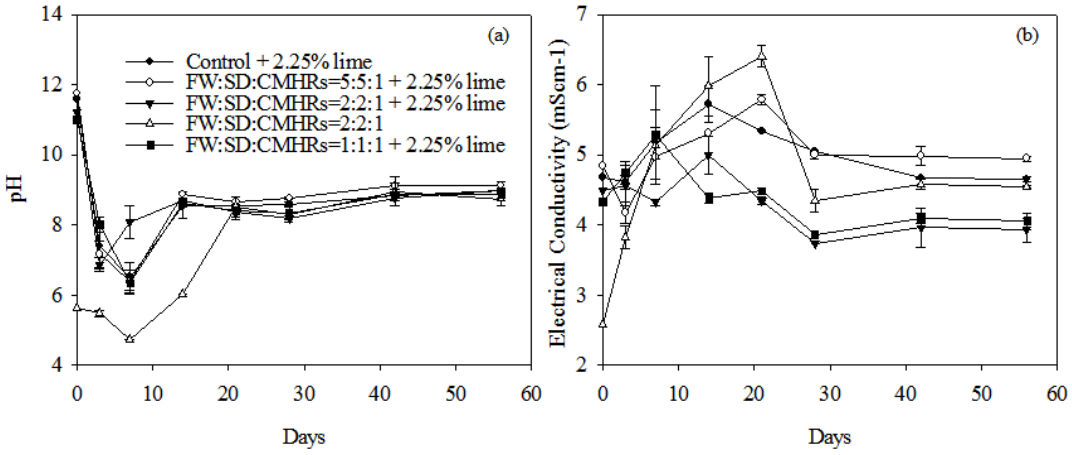


Figure 3-3. Changes of pH (a) and EC (b) values in various treatments with different material ratios during co-composting of food waste and CHMRs.

### 3.3.3 Nitrogen dynamics during composting process

Total nitrogen content increased about 0.13 to 0.44% (dry weight basis) during the composting process (Fig. 3-4a). The increase during the first week was mainly due to concentration effect coming from the net loss of dry mass through reduction in organic substances. However, from day 7 to 21, the TKN content decreased due to the large amount of ammonia emission. Following that, total nitrogen concentration increased again due to the reduction in $NH_3$ loss and the changes tended to be less (Ren et al., 2010). As the organic degradation of treatment 1:1:1 with lime addition was the highest, a higher concentration of TKN was observed as compared to other treatments.

The initial low pH of the treatment without lime addition hindered the ammonification process. As to other treatments, liming of organic nitrogen promoted the growth of microorganism and facilitated the ammonification. This explains the sharp increase in $NH_4^+$-N concentration (Fig. 3-4c) and ammonia emission (Fig. 3-4b) during thermophilic phase in all treatments except the treatment without CaO addition due to the change from organic nitrogen to extractable ammonium (Wong et al., 2009). The volatilization of ammonia gas is more favourable at high temperature and pH. In the treatment 2:2:1 without lime, ammonia emission was only observed just before day 18 because of poor ammonification. As shown in Fig. 3-4b, cumulative $NH_3$ evolution of the treatment without lime addition and the treatment highest CHMRs amendment were lower than other treatments within the initial two weeks. This was respectively due to the low initial pH value and the high CHMRs amendment inhibited the microbial activities. On the other hand, addition of CHMRs might have a nitrogen preservation effect because mineralized nitrogen and organic substances

combined and precipitated as humus during humification process (Cayuela et al., 2009). The sharp decline of extractable ammonium after 21 days is due to volatilization loss and immobilization of microorganisms (Polpprasert, 1989). Ammonium can turn into nitrate when temperature decreases to 40 $^{o}$C with the activation of nitrifying bacteria. Concentrations of inorganic forms of N, ammonium and nitrate are the most important because plant roots can absorb this form of nitrogen directly (Monedero et al., 2001).

Quantities of nitrite and nitrate were extremely low until the latter stage of composting (Fig. 3-4d and Fig. 3-4e). The initial low levels were due to inhibition of excess amount of ammonia the growth of nitrifying bacteria (He et al., 2007). The nitrite and nitrate contents increased throughout the composting process due to the transformation from ammonium-N under the reaction of nitrifying bacteria. On Day 56, the nitrite and nitrate contents of treatment with dry weight ratio 5:5:1 were slightly higher than other treatments, which did not show any significant difference. Additionally, it was reported by McClung (1985) that nitrification could be inhibited by high salinity which agreed to the result in the treatment (dry weight ratio 2:2:1 without lime) with low pH and high EC.

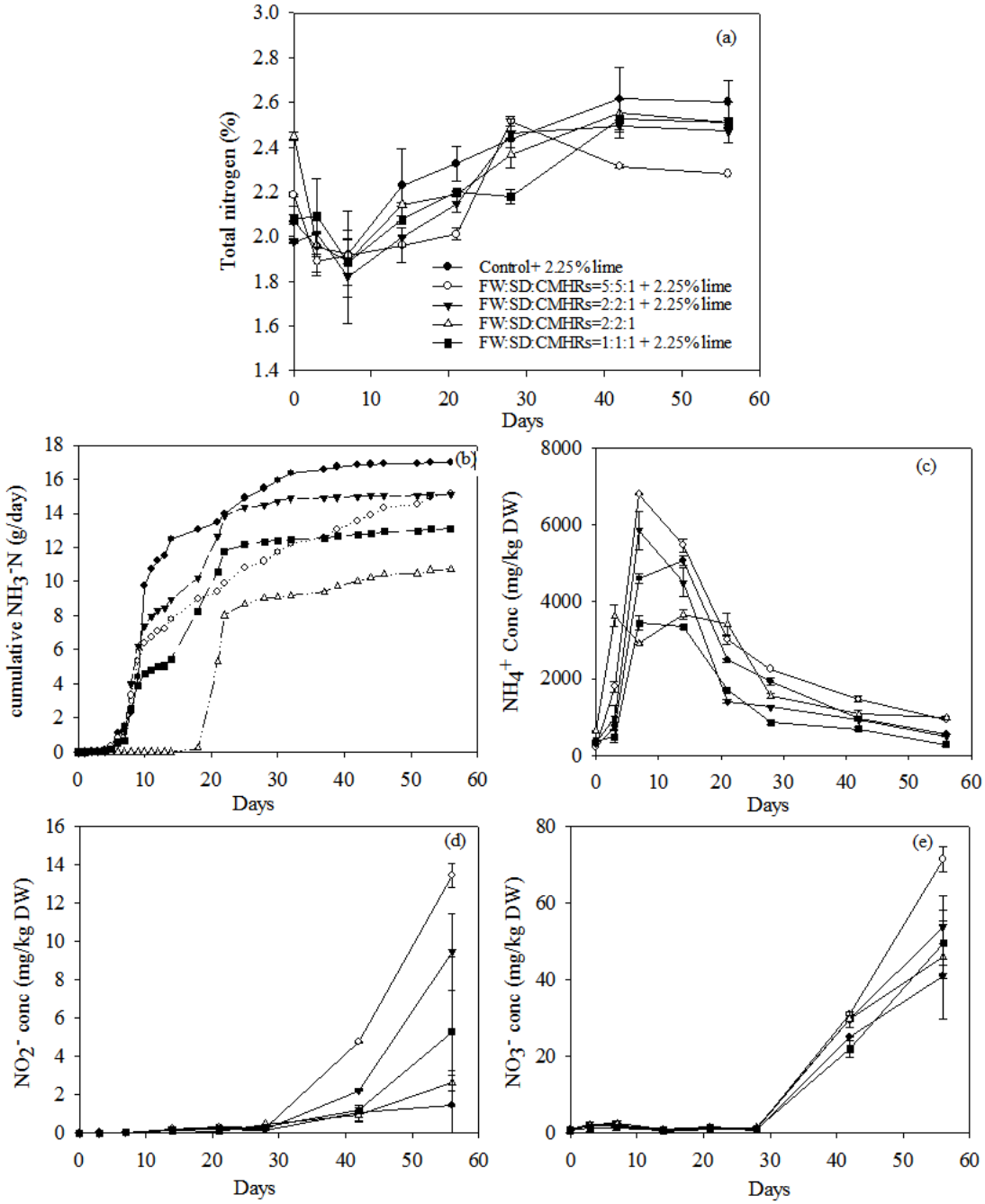


Figure 3-4. Changes of total nitrogen (a), cumulative ammonia emission (b), extractable ammonium (c), nitrite content (d) and nitrate content (e) in various treatments with different material ratios during co-composting of food waste and CHMRs.

### 3.3.4 Total phosphorus and total potassium

Table 3-3. Comparison of the nutrient contents of the food waste composts obtained from the present study with Chinese medicinal herbal residues (CHMRs) as a bulking agent and those from municipal solid wastes.

| Parameter | Unit | Control[d] | 5:5:1[d] | 2:2:1[d] | 2:2:1 No lime[d] | 1:1:1[d] | Municipal solid waste composting | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | China[a] | USA[b] | Germany[c] |
| N | g/kg | 26.0 | 22.8 | 24.7 | 25.1 | 25.1 | 5.0 | 12.6 | 9.3 |
| $P_2O_5$ | g/kg | 6.2 | 8.7 | 15.6 | 13.6 | 20.2 | 5.0 | 10.0 | 12.8 |
| $K_2O$ | g/kg | 14.6 | 14.7 | 15.7 | 15.7 | 18.3 | 9.7 | 6.2 | 12.4 |

a: Wang et al., 2004; b: Mays and Giordano., 1989; c: Vogtmann and Fricke., 1989.

d: Treatment details is presented in Table 3-2.

Besides nitrogen, phosphorus and potassium are also important fertility indicators for mature compost evaluation (Chang et al, 2007). The increase in total phosphorus and potassium was contributed by the dry weight loss of composting mass due to organic substances biodegradation. Therefore, the higher the decomposition rate, the larger the increase in total phosphorus and potassium. Treatments with the addition of CHMRs had higher contents of total phosphorus and potassium than the control treatment because herbal residues contained phosphorus and potassium even after decoction process (Wang et al, 2008). Treatment with dry weight ratio 1:1:1 (8.7 g/kg of $P_2O_5$ and of 14.7 g/kg $K_2O$) and 5:5:1 (20.2 g/kg of $P_2O_5$ and of 18.3 g/kg $K_2O$) provided significantly more nutrients for plant growth than the control (6.2 g/kg of $P_2O_5$ and of 14.6 g/kg $K_2O$) ($p<0.05$). Comparing to the composts from municipal solid waste, the co-composting of food waste with CHMRs resulted in higher nutrients (N, P and K) in the final composting products (Table 3-3).

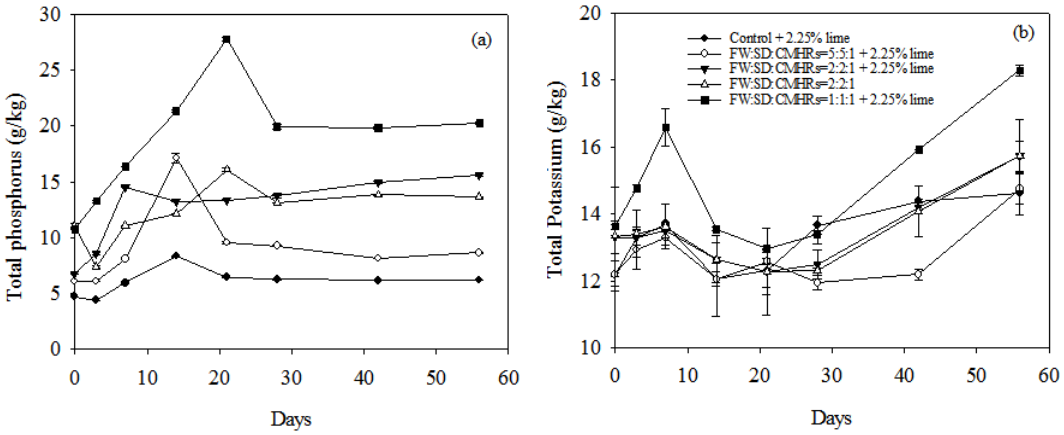


Figure 3-5. Changes of total phosphorus (a) and total potassium (b) in various treatments with different material ratios during co-composting of food waste and CHMRs.

### 3.3.5. Compost maturity

#### 3.3.5.1. Carbon/nitrogen ratio

C/N ratio of the solid phase composting mass was traditionally used for evaluating the compost maturity (Fang et al., 1999). The initial C/N ratio was around 25 on day 0 that declined to about 16 to 20 (Fig. 3-6a). The rapid decrease in C/N ratio in the first week could be due to the rapid reduction in carbon while an increase in total nitrogen. All treatments have more or less the same C/N ratio at the end of the composting process except treatment with a dry weight ratio of 1:1:1, which was significantly lower (C/N 16, $p<0.05$) indicating better degradation. During composting, carbonaceous and nitrogenous materials are transformed into more stable complex organic forms, which chemically and biologically resemble humic substances. The amount of humus accumulates varies in different processes. Some of the chemical ingredients of organic residues undergo rapid degradation as a result of microbial activities, resulting in the microbial biomass synthesis and the resistant humus-like complex formation. Therefore, the carbon and nitrogen are turned into highly complex molecules (Francou et al., 2005). A final C/N ratio of below 20 is normally considered as satisfactory for final composting product maturity (Wong et al., 2009).

#### 3.3.5.2 Cress seed germination test

Cress seed germination index (GI) is another method for assessing compost maturity in a biological way. Phytotoxic components in composting product may

hinder plant growth (Mitelut et al., 2011). The phytotoxic components were produced predominantly during thermophilic stage and the phytotoxicity is minimal when the germination index exceeds 80% (Bernal et al., 1998). Ammonium ions and low molecular weight organic acids constitute the phytotoxic substances. All treatments with lime addition reached 80% GI value after 50 days (Fig. 3-6b). The treatment with dry weight ratio 1:1:1 resulted in 157% GI at the end of composting while the treatment with dry weight ratio 5:5:1 resulted in 126%. However, the compost from the treatment without lime addition did not reach maturity in term of GI after 56 days of composting, indicating that a longer composting period is needed to reach maturity. This might be due to the acidic condition, which inhibited the microbial activity. The low ammonium level of this treatment elucidated the inefficient decomposition rate.

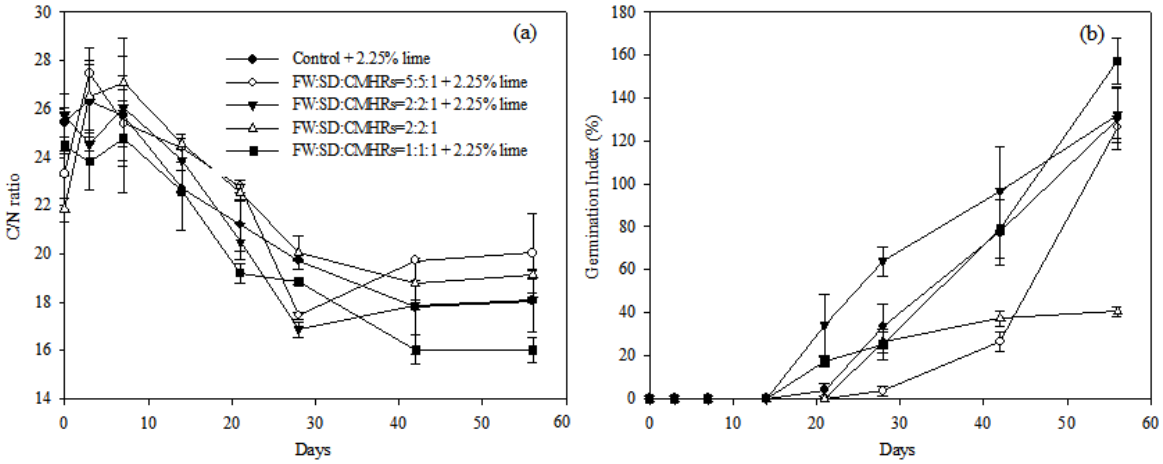


Figure 3-6. Changes of C/N ratio (a) and germination index (b) values in various treatments with different material ratios during co-composting of food waste and CHMRs.

### 3.3.6 Change in bacterial population during composting process

The log copy number of the bacterial population was 7-8 initially and increased during the first week since the readily available carbon source and prevalence mesophilic temperature provided the microbes appropriate living conditions for their rapid reproductions (Selvam et al., 2012). The initial quantity of total bacteria in the treatment 1:1:1 was 7.1 which was lower than the other treatments (7.9 for treatment 5:5:1 and 8.0 for control) since more CMHRs prohibited microbial growth due to more bioactive components with antipathogenic effects and high lignin content in CMHRs was difficult for microbes processing mineralization as nutrient substrate. On the Day 7, the sudden environmental condition change resulted from the organic substance decomposition progress and temperature risen limited various kinds of bacteria while some selectively promoted (Zhang et al., 2011). During the thermophilic phase, the ammonifying bacteria broke organic nitrogen into ammonium actively since the C/N ratio between 20 to 25 provided a proper condition for their activities.

The bacterial quantities gradually declined after thermophilic phase for the reason of the high temperature (> 55 $^{o}$C) sanitized the mesophilic microbes. At the mature phase, the treatment 1:1:1 contained the lowest bacterial population (copy number of 5.2), which was significantly lower than that of the treatment 5:5:1 (7.1) and control (7.5) since less food waste proportion provided less readily nutrients for their activities ($p<0.05$). During this phase, the correlation was also found between bacteria population, ammonium and nitrate contents since dominant bacterial flora utilized the remnant carbon and nitrogen components such as cellulose and lignin (Bernal et al., 2009; Kowalchuk et al., 1999). For the bacteria which cannot

metabolize them, the malnutrition limited their growth and reproduction (Zhang et al., 2002). The nitrifying bacteria converted ammonium, which is toxic to plants by bio-oxidate reactions, to nitrite and further nitrate (final form of nitrogen which is uptaken by crops). As the nutrient source was exhausted and composts became mature, the microbial community tended to be stable. Nitrogen-fixing bacteria played also an important role in the curing phase and promoted the quality of composts after land application (Meunchang et al., 2005).

### 3.4 Conclusions

The experimental results demonstrated positively the use of CHMRs is a suitable candidate to co-compost with food waste. In terms of decomposition efficiency and compost maturity, the treatment with dry weight ratio 1:1:1 (with 2.25% lime) showed the best performance among all treatments with 67% organic substances degradation and 157% seed germination index. Oppositely, the treatment with dry weight ratio 2:2:1 without lime addition can not reach mature level due to acidity. Only well-matured compost can suppress plant diseases in soil since it has some microorganisms which can inhibit phytopathogens. The treatment 5:5:1 also reached maturity but with a longer composting period; however, it was the treatment which could accommodate the highest quantity of food waste. The log copy number of the bacterial population was 7-8 initially, which decreased and stabilized along the composting. Results reveal that the CHMRs can be used as a bulking agent with food waste, and a ratio of 1:1:1 with 2.25% lime would be optimum to achieve higher organic decomposition and faster maturity. However, the initial lower microbial population in the treatment, though without any adverse effect on the overall

microbial decomposition, will warrant further work to indicate the total population is not a practical means to illuminate the effective microbial decomposition. Besides, the advantage in using CHMRs will need further experiment to indicate its potential pathogen suppression capability. Additionally, qualitative and quantitative dynamics of functional groups will be monitored during humification process in order to demonstrate the feasibility and efficiency of CMHRs in composting since research on composting with CMHRs is lacking of publications.

# CHAPTER FOUR

# EVALUATION OF HUMIC SUBSTANCES DURING CO-COMPOSTING OF FOOD WASTE, SAWDUST AND CHINESE MEDICINAL HERBAL RESIDUES

## 4.1 Introduction

Municipal solid waste disposed in landfills was 8,996 tons per day in Hong Kong during 2011; of which 39.8% were food wastes (HKEPD, 2012). Composting is a sustainable way to process the huge quantity of food waste, which has negative effects on environment upon landfilling, such as production of landfill leachate and emission of odors from greenhouse gases. Bulking agents are important components of composting mix, especially for feedstock with high nitrogen contents and high bulk density such as food wastes. Sawdust was the widely used bulking agent in the composting systems; while use of another waste material, Chinese medicinal herbal residues (CMHRs) as a supplement bulking agent along with sawdust was demonstrated in our previous study (Chapter 3). However, CMHRs compost is an innovative research that has limited research findings, therefore humification process needs to be monitored in order to fill the gap of knowledge on its effect on compost maturation process.

The humic substances are a cluster of polymers of organic materials with varying molecular weights resulted from the actions of microorganisms and enzymes during humification process. They have variable charges and contain a variety of functional groups such as carboxylic, phenolic and hydroxylic compounds. It

normally contains one or more aromatic nucleus connecting more than one reactive functional groups of the outer most structure and plays an important role due to its ability to affect the water holding capacity, pH and nutrient dynamics in the soil (Stevenson, 1994). Thus investigating the nature of humic substances in compost is important in terms of predicting its efficiency during application. Despite previous published data, there is no uniform agreement of the structures and dynamics of the components of humic substances in the final products, mainly as a consequence of the differences in the composting substrate and operating conditions. There are two ways of humic substances generation (Lopez et al., 2002): (1) lignin derivatives oxidized from side chains of lignin constitute the core structure of humic substances under microbial activities, and (2) the polymerization of the monomers through microbial metabolism. Therefore, the composting mix containing higher contents of lignin may accelerate the formation of stable humic substances. Generally, the bulking agents such as the widely used sawdust can provide the lignin; however, the degradation rate of sawdust was normally slow. In contrast, CMHRs after decoction may be easily accessible for microbial attack and the lignin contents might be easily available for the formation of humic substances; however, no report on this issue is available from the literature and deserves to be investigated.

Humic substances include humic acid (HA), fulvic acid (FA) and other components such as humin. However, HA and FA represent the majority of the humic substances and have been the foci of studies focusing on the transformation of humic substances. Humic acid is a series of polymer polycondensates with different molecular weights, and has the capacity of polydispersity, which is one of the general characteristics of macromolecular substances. Soil with HA is usually well buffered because of their dissociation of acidic functional groups (Campitelli et al., 2003). In

contrast, FA includes compounds with smaller molecular weight, high activity and high oxidation level. Fulvic acid contains lower mass fraction of carbon, hydrogen and nitrogen but higher oxygen when compared with HA. In general, the immature composts contain high content of FA and relatively low content of HA while the HA dominate the mature composts (Tuomela et al., 2000; Garcia et al., 1992). After composting, the HA content is significantly improved resulting from the stability of organic matter, indicating that the composts are mature enough for land application.

Carbon/Nitrogen (C/N) ratio is widely used as the indicator for compost maturity; however, it may not be a stand-alone parameter to indicate the maturity and should be considered along with other maturity and stability parameters. Nevertheless, composts rich in HA or having high HA/FA ratio could be considered to assess the compost maturity as well as to assess the impact of compost on subsequent soil application because HA and FA elicit different responses in the soil regulating carbon and nitrogen dynamics.

Non-destructive method such as Fourier transform infrared (FTIR) spectroscopy has been used to monitor the functional groups and transformation between HA and FA during composting (Spaccini and Piccolo, 2009). The functional groups revealed using FTIR can be used to predict the groups of compounds, which in turn can be used to assess the correlation between humification and compost maturity. Analysis of humic substances was reported previously with other bulking agent such as sawdust (Tuomela et al., 2000); however influence of CMHRs on the humification has never been investigated. Molecular compositions of humic substances can be further characterized using off-line pyrolysis with the tetramethylammonium hydroxide (TMAH) followed by gas chromatography-mass spectrometry (Pyr-TMAH-GC-MS) for quantitative analysis. The GC profile gives the resemblant evidences of the

molecular characteristics of the precursor of functional groups in the composting mass (Fuentes et al., 2010). The application of thermochemolysis with TMAH of HAs allowed monitoring the methylated counterparts of highly polar compounds such as fatty acids, alcoholic, phenolic and aromatic compounds by solvolysis and methylation of ester and ether bonds (Spaccini and Piccolo, 2007). CMHRs represent a major source of solid waste which mainly consists of woody and leafy materials suitable to be used as a bulking agent for composting with food waste. Since the usage of CMHRs as a bulking agent in composting system is a new approach, there is no report available on the humification, especially on the changes in molecular composition of HA during the co-composting of food wastes with CMHRs. Therefore, the aim of this paper was to apply the Pyr-TMAH-GC-MS to evaluate the molecular changes occurring in HA extracted from compost at various phases of biological maturity since humic substances are a good indicator of compost maturity.

Therefore, the aim of this paper was to correlate the functional groups of the composting mass at various stages of composting and maturity of the composts when CMHRs was supplemented as a bulking agent with the understanding that the rate of humification can reflect the rate of compost maturity.

## 4.2 Material and methods

### 4.2.1 Experimental design and composting conditions

The CMHRs were collected from the school of Chinese Medicine, Hong Kong Baptist University. The daily collection lasted for about one month to minimize species variation of CMHRs samples. A synthetic food waste prepared by mixing 1.3

kg boiled rice, 1 kg bread, 1 kg cabbage and 0.5 kg boiled pork, as described previously (Wong et al., 2009), was used in this experiment. Selected physicochemical properties of the substrates used were presented in Table 4-1. The food waste, sawdust and CMHRs were mixed in the ratio of 5:5:1 and 1:1:1 (dry weight basis, w/w), while food waste and sawdust mixed at 1:1 (dry weight basis, w/w) served as the control to reveal the influence of CMHRs on the humification. The initial moisture contents of the composting mixtures mentioned above were adjusted to 55-60% while obtaining the C/N ratio of about 25. Besides, based on our previous experience with food waste composting (Wong et al., 2009; Wang et al., 2013), 2.25% lime (dry weight basis, w/w) was added to all the treatments to alleviate the onset of low pH during the initial stages of composting. About 7.5 kg of composting mixture was prepared for each treatment and composted for 56 days in 20-L composting reactors, which were connected to a computer control system with WMA-2 gas analyzer for on-line measurement of carbon dioxide (PP systems, Herts, UK) and temperature; and the data were logged continuously. Further details of the composting reactors can be obtained from our previous report (Wong et al., 2009). Aeration rate was set at 0.5 L/min/kg composting mass (dry weight) through an aerator pump for the first two weeks; then the rate of aeration was reduced to 0.25 L/min/kg based on the $O_2$ requirement as observed in our previous studies. Periodically the composting mass in each reactor was mixed and about 200 g of samples from each treatment collected on day 0, 7, 28 and 56 were used to extract the humic substances.

Table 4-1. Selected physicochemical properties of the synthetic food waste, sawdust and the composting mix used in the study.

| Parameters | Food waste | Sawdust | Chinese medicinal herbal residues |
|---|---|---|---|
| Moisture content (%) | 59.0 (0.02) | 7.24 (0.03) | 63.0 (0.13) |
| Total organic carbon (%) | 45.5 (1.70) | 52.9 (0.91) | 48.0 (0.41) |
| Total Kjeldahl nitrogen (%) | 3.28 (0.04) | 0.59 (0.04) | 1.62 (1.32) |
| C/N | 13.9 (0.35) | 89.8 (4.56) | 29.6 (2.21) |

Values represent mean and standard deviation (n = 3).

### 4.2.2 Extraction of humic substances

The humic substances from the compost mass were extracted following the procedure of Huang et al. (2006). Air-dried sample was extracted with a proportional mix of 0.1 M $Na_4P_2O_7 \cdot 10H_2O$ and 0.1 N NaOH, with a solid: extractant ratio of 1:10 (w/v, dry weight basis) after shaking at room temperature for 24 h. The supernatant containing soluble humic substances were collected after centrifugation at 25,931 x *g* for 15 min, the procedure was repeated for two more times and the extracts were pooled together. The pH of the extractant was adjusted to 7.0 with 0.5 M HCl and the total organic carbon (TOC) of the humic substances was analyzed using Walkey-Black method (Nelson and Sommers, 1982). The humic acid (HA) and fulvic acid (FA) from the extracted humic substances were extracted as follows: the pH of the humic substance solution was adjusted to 1.0 with 3 M HCl, allowed to stand for layer separation overnight at room temperature and centrifuged at 25,931 x *g* for 15 min. The pellet (precipitate) contained the HA while the supernatant contained the FA.

The HA was washed with 0.05 M HCl several times and the pH was adjusted to 7.0. The TOC contents of HA and FA were estimated using Walkey-Black method.

### 4.2.3 Fourier transform infrared (FTIR) spectroscopy

The HA and FA solutions were freeze-dried and about 200 mg potassium bromide was added to 1 mg sample and ground until mixture became homogeneous, which was pressed to a lubricous slice under the pressure of 10 $t/cm^{-2}$ (Carver Hydraulic Press Equipment, USA). The samples were scanned within the detection range between 4000 and 350 $cm^{-1}$ by the Nicolet 550 Magna-IR spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with OMNIC software (Wei et al., 2007).

### 4.2.4 Off-line pyrolysis TMAH-GC-MS

Air-dried sample (10 g) was extracted with a solid: extractant ratio of 1:10 (w/v, dry weight basis) with a mix of $Na_4P_2O_7 \cdot 10H_2O$ and NaOH (1:1) as extractants at room temperature. The mixture was shaking for 24 h. The pH of extract was adjusted to 1.0 using 6 M HCl. The supernatant (FA) and precipitate (HA) were separated using centrifugation at 26,000 *g* and collected separately before freeze-dried at -80 $^{o}C$ (TMECC, 2003).

Tetra-methyl ammonium hydroxide (TMAH 25% in methanol) solution (1 ml) was added to 300 mg of freeze dried HA sample, dried under a gentle stream of nitrogen and ignited at 400 $^{o}C$ for 30 min (Barnstead Thermolyne 21100 furnace). Pneumatolytic samples were collected by a series of three triangular flasks containing 50 ml of chloroform. The collected solutions were then concentrated to 1 ml using a

rotary evaporator. Chemical structures of functional groups of HA were resoluted and identified using gas chromatography-mass spectrum. A DB-5 capillary column (60 m×0.25 mm; film thickness, 0.25 mm) was used with a PE Turbomass-Gold quadrupole mass spectrometer. The temperature program was 60 $^{o}$C for 1 min, then raised at the rate of 7 $^{o}$C min$^{-1}$ to 320 $^{o}$C and maintained for 10 min. The injector temperature was set at 280 $^{o}$C and the split-injection mode had a 30 ml min$^{-1}$ of split flow. Mass spectra were obtained in EI mode (70 eV) scanning in the range of 45–550 m/z, with helium as a carrier gas at 1.90 ml min$^{-1}$. Identification of functional groups in humus was based on comparison of mass spectra with the NIST-library database (Spaccini and Piccolo, 2009).

### 4.2.5 Chemical analyses

A 1:10 aqueous extract of compost was used to analyze the pH, dissolved organic carbon (DOC) and soluble organic nitrogen (SON) as described previously (Huang et al., 2006). The total Kjeldahl nitrogen (TKN) and total organic carbon (TOC) were determined following the methods of TMECC (2003) and Walkey-Black method as described by Nelson and Sommers (1982), respectively.

### 4.2.6 Statistical analysis

Analyses were performed in duplicate samples and the mean values and standard deviations on dry weight basis were presented. The data were processed using SigmaPlot 11.0 and IBM SPSS statistics 19 while the significance of the differences were tested using Duncan multiple rage test at $p<0.05$.

## 4.3 Results and discussion

### 4.3.1 Co-composting of the food waste, sawdust and CMHRs

All the three treatments showed a good composting performance since adequate buffering materials (lime), bulking agents (sawdust or sawdust with CMHRs), aeration and moisture were provided. During food waste composting, initial intensive acidification was the major bottleneck severely affecting the organic degradation and subsequently the microbial activities. The pH of the composting mass clearly indicated that good composting performance was achieved (Chapter 3). During the first week, the pH decreased due to the formation of volatile fatty acids (VFAs) as a result of microbial activities; the composting process could be inhibited by low pH (<6.0), resulting in the accumulation of VFAs that further enhanced the inhibition of the organic matter degradation (Nakasaki et al., 1993; Wong et al., 2009). However eventually the pH increased and reached to ~8.0 after 21 days and was stable around this level during the rest of the composting.

The TOC content decreased along the composting period. As shown in Fig. 4-1a, during the initial composting stage (0 to 14 days), organic carbon was rapidly decomposed mainly as a result of utilization of easily biodegradable organic matter by the microbes. Once easily degradable organics were utilized, cellulose, hemicellulose and lignin dominated in the organic matter and became the nutrient source for microbial activities thus the rate of degradation became slow (Yamada and Kawase, 2006). The reduction in TOC after 56 days of composting was 35.1% for 1:1:1 treatments which was significantly higher ($p<0.01$) than the 18.9% for 5:5:1, and

20.1% for control. The slight increments of total nitrogen contents in all treatments (0.4% for treatment 1:1:1, 0.1% for treatment 5:5:1 and 0.5% for the control) were due to concentration effect. The reduction of total carbon and increment of total nitrogen resulted in the decreasing trend of C/N ratio during composting process, indicating the composts were tend to be more stable and mature.

During the early stages of the composting, the rapid degradation of readily available compounds in composts generated smaller, soluble molecules which constituted the dissolved fraction (Zmora-Nahum et al., 2005) and the concentrations were higher. The initial DOC concentration varied due to the different ratios of raw materials in the composting mix and decreased dramatically at the end of composting. The 1:1:1 treatment achieved the greatest reduction of DOC during composting as shown in Fig. 4-1b, followed by the control treatment without CMHRs while the 5:5:1 treatment had the least decomposition. Relationship between the DOC content and compost maturity was investigated by many researchers; and the reduction of DOC represented the compost maturity and stability (Garcia, et al, 1991; Goyal, et al., 2005; Huang et al., 2004; Hue and Liu, 1995; Laor and Avnimelech, 2002). The final DOC contents of the composting mass from all the treatments were around 10 g/kg, a value suggested for matured composts by Hue and Liu (1995).

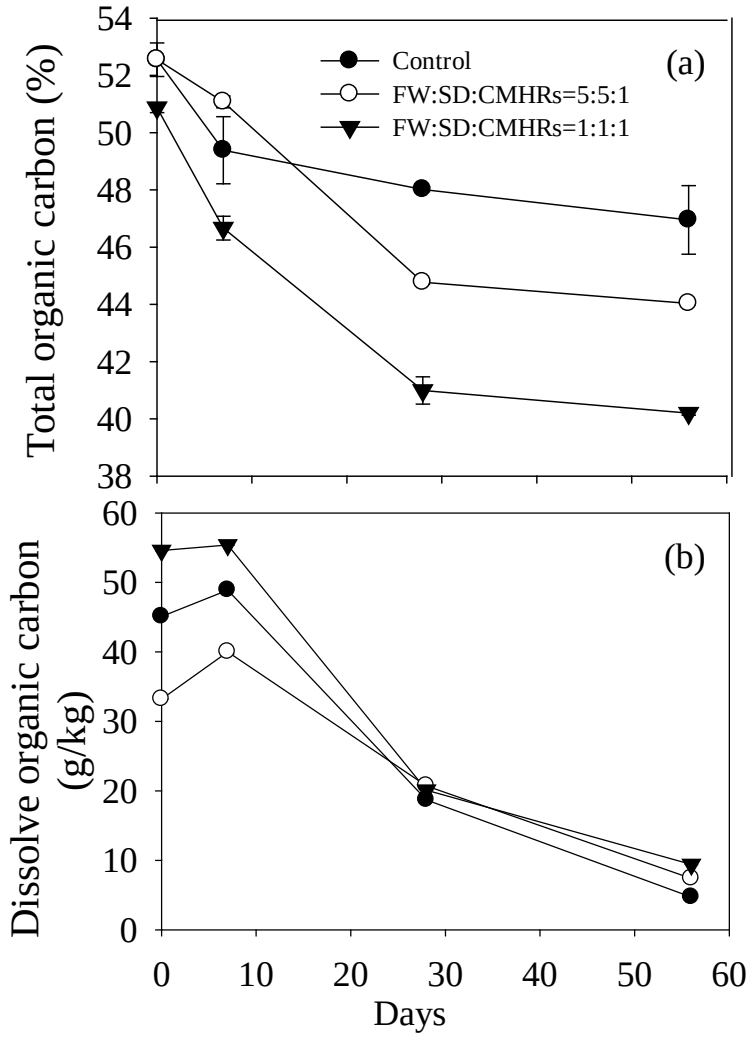


Figure 4-1. Changes in TOC (a) and DOC (b) contents during the food waste, sawdust and CMHRs co-composting.

The C/N ratio is one of the most widely used compost maturity parameters. $C/N_{solid}$ ratio less than or equal to 20 is considered as a satisfactory level for compost maturity (Hirai et al., 1983). The soluble C/N ratio ($C/N_{soluble}$) showed a similar decreasing trend as that of $C/N_{solid}$ in all treatments during composting. After 56 days, the lowest C/N ratios in both solid and soluble fractions were observed in the 1:1:1 treatment, 16 and 2.4 (Fig. 4-2a and 4-2b), respectively. C/N values were higher of control and 5:5:1 treatment, $C/N_{Solid}$ of 18.1 and 20.0; and $C/N_{soluble}$ of 3.1 and 4.3, respectively. $C/N_{soluble}$ can be used as an indicator for compost stability since composting involves biochemical reactions occurring mainly in the aqueous phase. These results were consistent with the previous report (Huang et al., 2006), in which the $C/N_{soluble}$ ratio decreased dramatically from a wide range of 42.94 -52.97 to a narrow range of 2.39 - 4.32 at the end of pig manure composting. The $C/N_{soluble}$ of 5-6 is normally considered as the satisfactory level to indicate the maturity (Chanyasak and Kubota, 1981); thus the $C/N_{soluble}$ of all treatments in this study met the requirement of compost maturity.

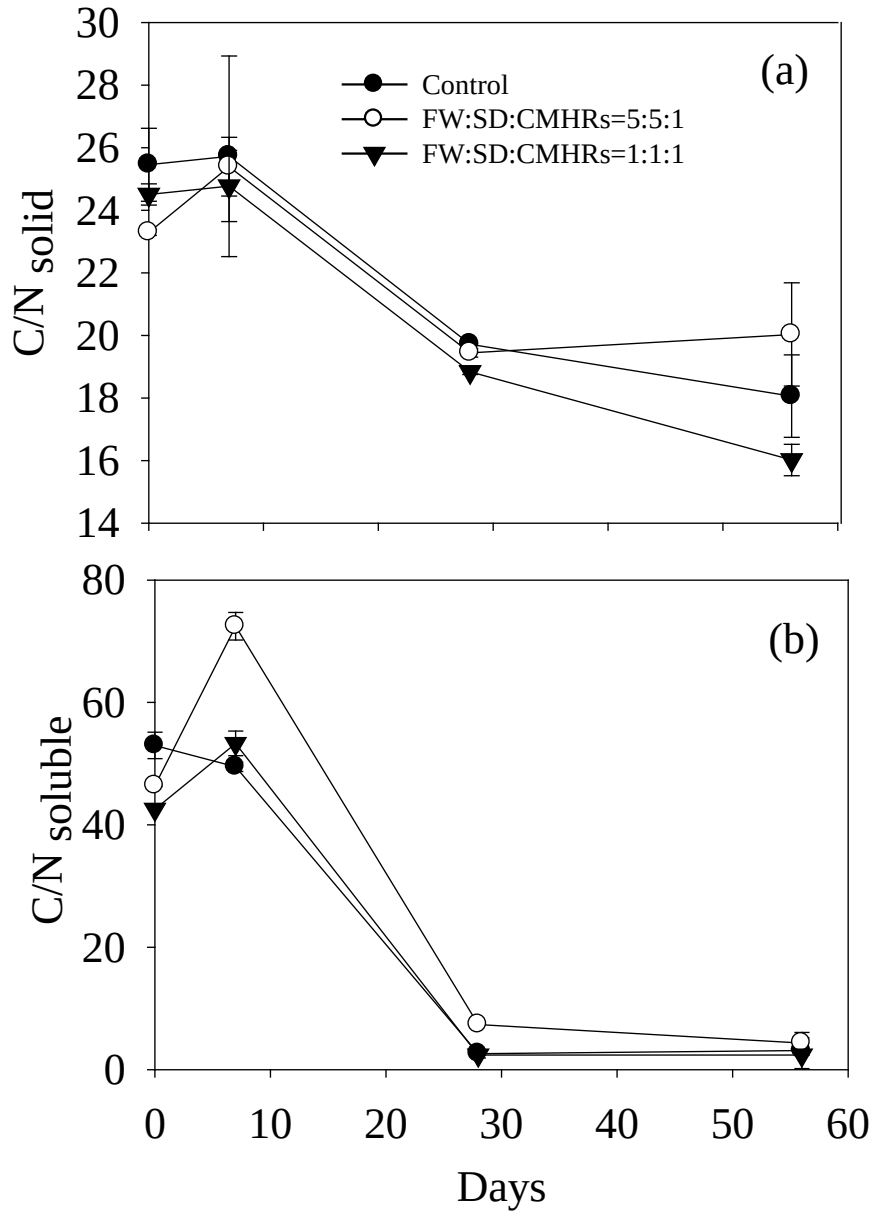


Figure 4-2. Changes in $C/N_{solid}$ ratio (a) and $C/N_{soluble}$ ratio (b) during the food waste, sawdust and CMHRs co-composting.

### 4.3.2 Analysis of humic substances

Humic substances are mainly derived from lignin, polysaccharides and nitrogenous components, and can be divided into two main components: HA and FA (Watteau and Villemin, 2011). As shown in Fig. 4-3a, the contents of humic substances declined from 5.06% to 1.48%, 9.42% to 2.91% and 8.10% to 3.97% in control, 5:5:1 and 1:1:1 treatments, respectively. Contents of humic substances decreased possibly due to the dramatic decrease of FA in all treatments (Fig. 4-3c). On the contrary, HA contents increased from 1.22% to 1.54% and 0.96% to 2.67% in 5:5:1 and 1:1:1 treatments, respectively (Fig. 4-3b); whereas in the control decreased from 0.68% to 0.40% with a higher HA content after two weeks of composting. Efficient composting results in rapid break down of organic materials, producing HA during composting process. The slightly higher HA content of control treatment in the initial two weeks may be attributed to higher content of readily available organic matter from food waste which could be easily decomposed at that time, resulting in the HA formation speed faster than degradation. Additionally, CMHRs contained more fibre-structure components such as lignin, which are known to provide more stable phenolic compounds required as starting material for humification processes (Lopez et al., 2002). Alternatively, the HAs were generated from other forms of humic substances, such as FA. The similar fluctuations were also found in Smidt et al.'s research in which kitchen waste was used, due to initial instability of HA formation and transformation under the influence of microbial reaction and also affected by the thermophilic temperature (2008). The quantity of the HA contents observed in this study were similar to the levels reported by Huang et al. (2006) and Xiong et al. (2010).

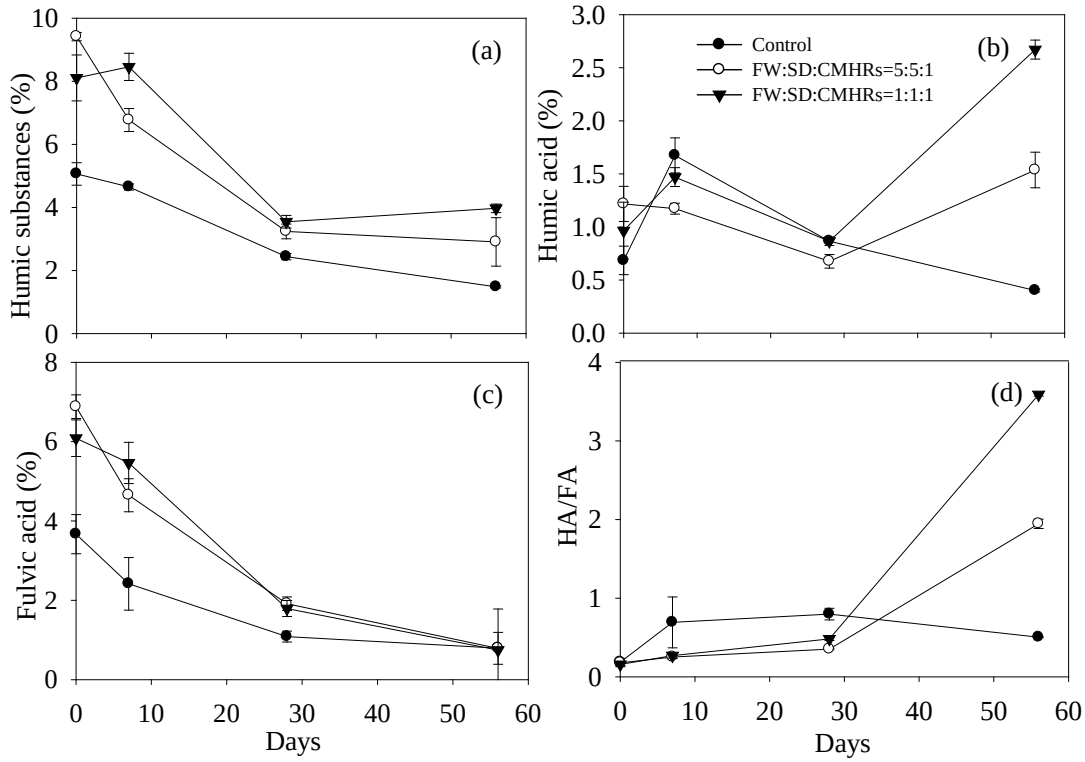


Figure 4-3. The extractable humic substances (a), humic acid (b), fulvic acid (c) and HA/FA ratio (d) during the food waste, sawdust and CMHRs composting.

The FA contents decreased from 3.67% to 0.80%, 6.88% to 0.79% and 6.09% to 0.74% in control, 5:5:1 and 1:1:1 treatments, respectively (Fig. 4-3c). These results were similar to those described by Huang et al. (2006). Due to the presence of more acid functional groups and lower molecular weight, the water solubility of the FA is higher than HA; thus FA content was relatively higher at the initial composting phase as reported before (Fukushima et al., 2009) due to relative high mobility and availability of compounds; and the immature condition of composts. A value less than 1% of FA in final compost implied that easily available carbon in the composts was reduced and the stability of the composts increased. During composting, the microbes utilized FA for their metabolism and involved in the organic matter transformation towards HA. It is reported that to some extent the FA are precursors for the formation of HA (Doane et al., 2003). The degradation of the readily available organic substances, including the FA, provided energy to the microorganism. The bio-oxidation of these compounds resulted in the production of substances with more stable structures in mature composts.

The humic acids-to-fulvic acid (HA/FA) ratio is widely used to describe the relative speed of HA and FA transformation as well as the maturity of the final composts (Dev and Antil, 2011). As shown in Fig. 4-3d, the HA/FA ratio increased in all treatments with hugest change in the 1:1:1 treatment (from 0.1 to 3.6), followed by the 5:5:1 treatment (from 0.2 to 2.0) and the control treatment (from 0.2 to 0.5). Increasing differences among treatments were significant ($p<0.05$). During the humification process, the lignin in the treatment 1:1:1 provided rich substrates for aromatization and oxidation. As a result, the cores of humic substances were constructed and oxygen-containing HA functional groups increased. The complicated ring structures in HA had positive correlation with compost maturity and humification

degree (Fukushima et al., 2009). Additionally, the composting condition turned to be alkalescence and substrates had higher molecular weights as the composts were aged; this situation catalyzed the degradation of FA due to its property of containing acidic functional groups and lower molecular weight. The FA also condensed to HA during mineralization, resulting in sharp increase in HA.

### 4.3.3 Fourier transform infrared (FTIR) spectroscopy analysis

FTIR is one of the most important and efficient techniques for monitoring the changes in the functional groups during humification of composting. The absorption peaks, that express the chemical bonds stretch and bending vibration after energy level transition, explain the changes of chemical structures.

Table 4-2. Assignment values of absorption bands of humic acid in FTIR spectra.

| Wavelength / $cm^{-1}$ | Assignment |
|---|---|
| 3300-3500, 3000 | O-H stretch of hydrogen bond in carboxylic groups, alcohols and phenols |
| 2920-2840 | Aliphatic methylene groups in lipids and fat |
| 1650 and 1290 | C-N stretch of Amide I band in protein |
| 1520 | Amide II |
| 1460-1400 | Alkyl compounds of hemicellulose and lignin |
| 1230 | Amide III |
| 1100-1000 | C-O stretch of carbohydrate and polysaccharide |
| 880 | C-C stretch of aromatic compounds |

The FTIR spectra of the HA extracted from the composting masses are presented in Fig. 4-4. The major absorbance bands in HA spectra observed are shown in Table 4-2. The reduction in C-H bond intensity appeared at 1400 $cm^{-1}$ representing lignin decomposition while aromatic skeletal bond at 1100 $cm^{-1}$ of hemicellulose disappeared in the treatments with CMHRs at latter composting phases. Spaccini and Piccolo (2009) also observed such disappearance of hemicellulose during composting. Day 28 was a mark that –OH groups (3500 and 3300 $cm^{-1}$) and aliphatic methylene groups (2920-2840 $cm^{-1}$) gradually disappeared, corresponding to the degradation of the lipid, protein and polysaccharides from food waste (Huang et al., 2006; Ravindran et al.. 2011); and the change was more obvious in the control treatment since food waste represented a higher ratio. Amide bands (I, II and III) and peaks in carbohydrate and polysaccharides region also clearly reduced as the consequence of food waste degradation. The control treatment with high food waste ratio had the least change in carboxylic group which was consistent with the decrease of HA content since the presence of carboxylic groups enhanced the HA the ability to form complexes with ions. The reduction of –OH, $-CH_2$, $-CH_3$ indicated an efficient degradation of organic matter. The treatment 1:1:1 had sharper peak at 880 $cm^{-1}$ as the aromatic skeletal, indicating the compost became most uniform and mature. The 1:1:1 treatment contained more lignin and hemicellulose since CMHRs are mainly of plant materials with high fiber content; therefore, stronger absorption band at 1100-1000 $cm^{-1}$ was observed. In brief, the contents of aliphatic and carboxylic groups from large molecular compounds decomposed during composting while quantities of aromatic groups appeared and the changes were more obvious in 1:1:1 treatment indicating the composting product was more mature and stable and CMHRs accelerated the humification.

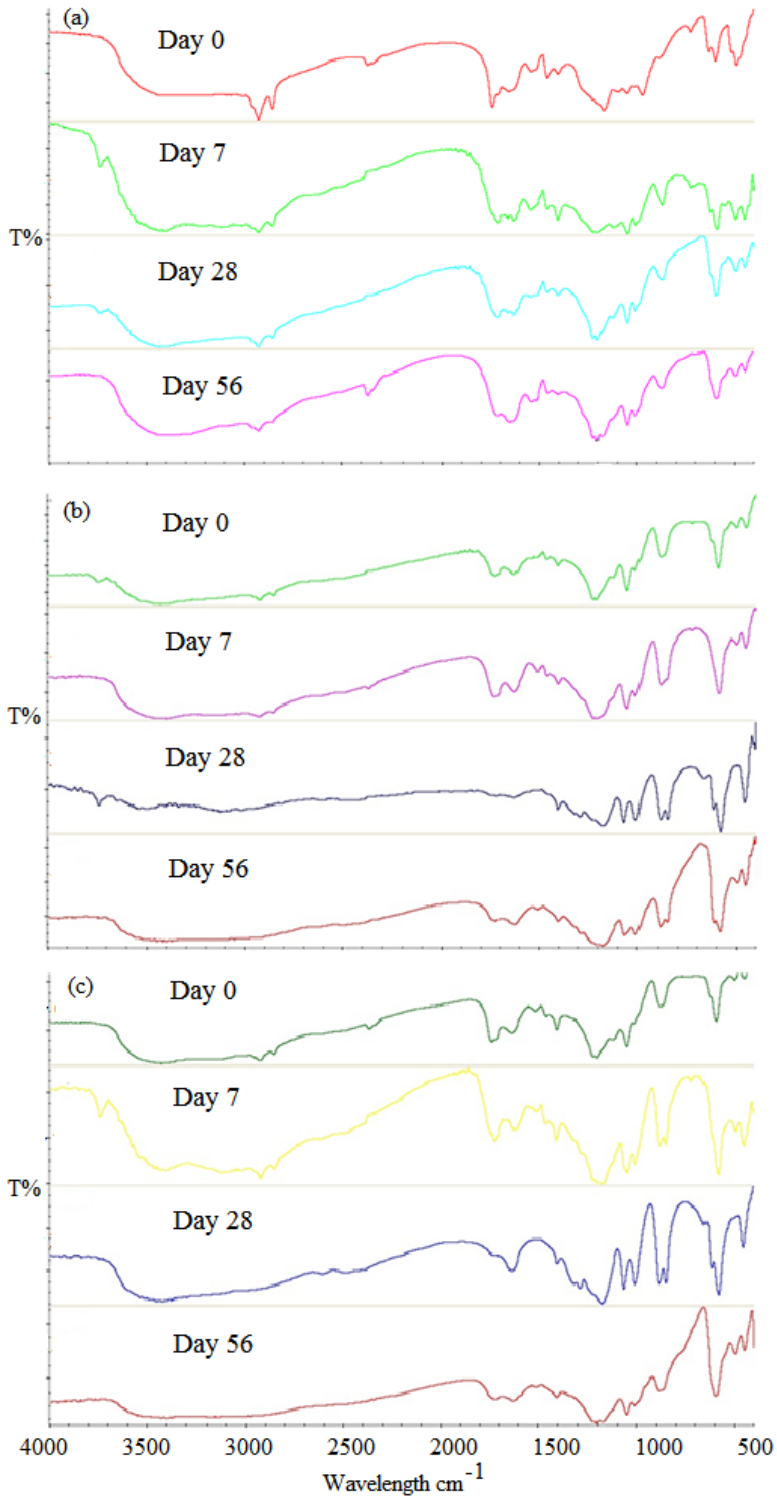


Figure 4-4. FTIR images of humic acids of (a) Control without CMHRs, (b) FW:SD:CMHRs 5:5:1 (dry weight basis) and (c) FW:SD:CMHRs 1:1:1 (dry weight basis) during different stages of composting.

Table 4-3. Assignment values of absorption bands of fulvic acids in FTIR spectra.

| Wavelength / $cm^{-1}$ | Assignment |
|---|---|
| 3400 | O-H and N-H stretch of hydrogen bond in carboxylic groups, alcohols and phenols |
| 2490 | S-H group in alkyl mercaptans |
| 1630 | C=C vibrations of aromatic compounds |
| 1290 | C-O stretch in epoxides |
| 1170 | C-O stretch in alcohols |
| 1070 | C-O stretch of carbohydrate and polysaccharide |
| 880-850 | C-H in aromatic compounds |

The FA spectrum was similar with HA and as shown in Table 4-3, during the initial composting stages, the FTIR spectra of FA of both treatments with herb residues confirmed the strong absorption at 3400 $cm^{-1}$ (–OH and –NH stretch) and 880 $cm^{-1}$ to 850 $cm^{-1}$ (–CH in aromatic compounds) characteristic of aromatic compounds (Baddi et al, 2004). On Day 28, a progressive disappearance of aliphatic and carboxylic groups (1177 $cm^{-1}$ for C-O stretch and 1070 $cm^{-1}$ for polysaccharides) was evident and became more homogeneous. After 56 days, both aromatic and aliphatic groups were reduced, except the control treatment, indicating that the rate of FA degradation can indicate the stabilization of the composting mass. This is consistent with the observation that the treatment 1:1:1 showed the rapid maturity followed by 5:5:1 and control. The changes in the FA during composting were relatively unstable for all treatments and a similar fluctuation was also observed by Huang et al. (2006). The FTIR spectra results indicated that the 5:5:1 treatment had sharper and stronger absorption peaks and the result concluded the maturity was

slightly slow and required longer composting time. Besides, the immature composts showed sharper and stronger peaks at wave length of 1177 $cm^{-1}$ and 1070 $cm^{-1}$, both of which are relevant to food waste degradation; thus their concentrations can be used to assess the compost maturity.

Rich lignin content in the treatment with CMHRs provided sufficient substrate for polymerization, transforming organic substances to HA. As the humification proceeded, the aliphatic and carboxylic groups in HA were replaced by aromatic compounds. The carbon degradation of control treatment was the lowest (20.1%); and less efficient conversion between HA and FA was also observed. More aromatic ring structures observed in the treatment 1:1:1 indicated that the final composts were more stable and mature. High molecular weight compounds increased as the level of humification increased eventually producing more polycondenzed HA during composting. The analyzed functional groups revealed that the stabilization process involves steady incorporation and transformation of organic substances between HA and FA.

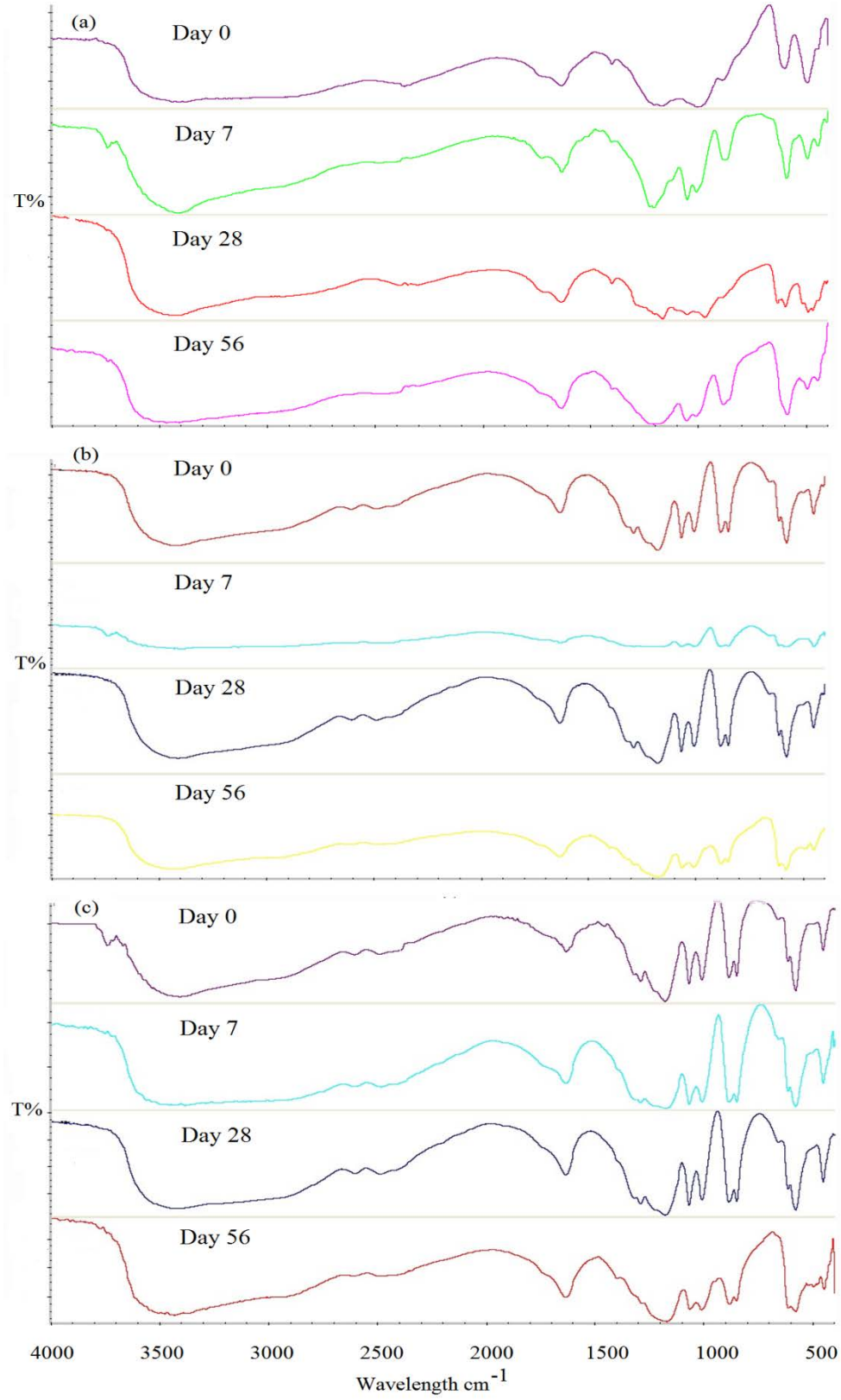


Figure 4-5. FTIR images of fulvic acids of (a) Control without CMHRs, (b) FW:SD:CMHRs 5:5:1 (dry weight basis) and (c) FW:SD:CMHRs 1:1:1 (dry weight basis) during different stages of composting.

### 4.3.4 Off-line Pyr-TMAH-GC-MS analysis

The coupling technique of gas chromatography and mass spectrometry is an efficient method for organic compounds identification. Off-line Pyr-TMAH-GC-MS is well applied for further detecting the changes of compounds during composting (Amir et al., 2006).

The typical compounds in HA chromatographic outcomes derived from Pyr-TMAH-GC-MS are listed in Table 4-4. More than 100 compounds were identified by thermochemolysis of HA, most of which were mono-, di- and tri-hydroxy acids, fatty acids, dicarboxylic acids, phenolic acid esters and ethers, which can be considered as the conjecture of original HA structure. Large range of fatty acids released from food waste and CMHRs, resembled with previous report, which also used composts from domestic organic wastes and green wastes as target materials (Spaccini and Piccolo, 2009).

For the control treatment with no CMHRs addition, the initially dominant fatty acid methyl ester was isomers of octadecanoic acid, methyl (C19). The 11-Eicosenoic acid, methyl ester (C21) existed after Day 7 in the composting process. The C15 and C17 iso- and anteiso- (i, a) compounds typically rooted from bacterial action while even-numbered long compounds (C20 to C26) originated from plants (Perry et al., 1979). The aliphatic compounds occurred right at the beginning of the composting process till the end. The fatty acid compounds have the ability to provide the substrates for aromatization, resulting in promoting the humification process. From the peak intensities of fatty acids and fatty acid methyl esters, it is clear that the quantities of fatty acids decreased during composting while the relative amount of the fatty acid methyl ester showed an increasing trend before Day 28 (Table 4-4). The

amounts of the methyl esters of the control treatment were relatively stable after Day 28; while the short-chain fatty acids disappeared from the early stage of composting process since they were more easily degraded by microbes. The ring structures of the control treatment included 9,10-Anthracenedione, 1-amino-2-me and benzoic acid on Day 56. The quantity of lignin components mostly released by thermochemolysis after Day 7 in the control treatment closely resembles that obtained for lignocellulose fractions of plant tissues and plant debris due to the higher content of sawdust which contains high amount of lignin (Vane et al., 2001; Spaccini and piccolo, 2007).

Table 4-4. The main thermochemolysis products released from humic acid of compost.

| RT (min) | Compounds | Control Treatment | | | | Treatment 5:5:1 | | | | Treatment 1:1:1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | D0 | D7 | D28 | D56 | D0 | D7 | D28 | D56 | D0 | D7 | D28 | D56 |
| 17.18 | 1-Undecene, 8-methyl- | | | | | | * | * | * | | * | | * |
| 17.24 | Cyclooctane, methyl- | * | | | | | | | | | | * | |
| 21.08 | 3-Tridecene, (Z)- | | | | | | | | | | | * | |
| 21.37 | Cyclododecane | | | | | | | | | | * | | |
| 23.90 | Cyclopentane, 1-pentyl-2-propyl- | | | | | | | * | | | | | |
| 25.07 | Hexadecane nitrile | | | | | | * | | | * | * | | |
| 25.09 | Pentadecane nitrile | | | | | * | | | | * | * | | |
| 25.11 | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | | | | | | | | * | | | * | * |
| 25.45 | Hexadecanoic acid, methyl ester | * | * | * | * | * | * | * | * | * | * | * | |
| 26.28 | Tridecanoic acid | | | | | | * | * | | * | | * | * |
| 26.31 | Tetradecanoic acid | | | | | | | | | | * | | |
| 26.66 | 3,5-di-tert-Butyl-4-hydroxyphenylpropionic acid | | | | | | | | | | | * | |
| 26.80 | 9-Acetylphenanthrene | | | | | * | | * | * | | * | | |
| 26.86 | Ethanone, 1-(9-anthracenyl)- | | | | | | | | | | | * | * |
| 26.95 | Propanoic acid, 3-mercapto-, dodecyl ester | | | | | * | | * | * | * | * | | * |
| 29.16 | 9-Tricosene, (Z)- | | | | | | | | | * | | | |
| 29.20 | Dodecane, 2,6,11-trimethyl- | | | | | * | | | | | | | |
| 29.42 | Octadecanoic acid | * | * | * | * | | | | | * | * | * | * |
| 29.66 | 9,11-Octadecadienoic acid, methyl ester, (E,E)- | | * | * | * | * | | | | * | * | | |
| 29.90 | 1-Octadecene | * | | | | | | | | * | | | |
| 31.24 | Eicosenoic acid, methyl ester | | * | * | * | * | | | | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 31.52 | 2(3H)-Furanone, dihydro-5-tetradecyl- | | | | | * | * | | | * | | | * |
| 31.59 | 1,3-Xylyl-15-crown-4, 2,3-.pinanedioxyboryl- | | | | | | | * | | | | | * |
| 31.75 | 1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-di methyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta.,10a. alpha.)]- | | | | | | | | | | | | * |
| 32.44 | Hexanedioic acid, bis(2-ethylhexyl) ester | | | | | * | * | * | | * | | | * |
| 33.23 | Oleic Acid | * | | | | | | | | | | | |
| 33.43 | N,N-Dimethyldecanamide | | | | * | * | * | | * | * | | | |
| 34.34 | 1,2-Benzenedicarboxylic acid, ditridecyl ester | | | | | | | * | | | | | * |
| 36.28 | 9,10-Anthracenedione, 1-amino-2-me | | | * | * | | | | | | | | |
| 38.90 | Benzoic acid | | | | * | | | | | | | | |
| 39.43 | Benzenamine, 4-octyl-N-(4-octylphenyl)- | | | | | | | * | | | | * | * |
| 39.65 | 1,2-Benzenedicarboxylic acid, diisodecyl ester | | | | | * | | | | | | | * |
| 40.37 | 5-Methyl-Z-5-docosene | | | | | | * | | | | | | |
| 40.94 | Tetracosanoic acid, methyl ester | | * | | | | | | | | | | |
| 41.79 | Cyclopentane, (4-octyldodecyl)- | | | | | | | * | | | | | |
| 48.38 | Propanoic acid, 3,3'-thiobis-, didodecyl ester | | | | * | * | * | * | * | * | * | * | * |

* Compounds can be observed.

As for the treatment with dry weight ratio 5:5:1 on Day 0, more long-chain fatty acids and their esters such as iso- and hexadecanoic acid, methyl ester (C16), iso- and 9,11-Octadecadienoic acid, methyl ester (C18) and eicosenoic acid, methyl ester (C20) were observed. On the other hand, tridecanoic acid (C13) and hexanedioic acid, bis (2-ethylhexyl) ester (C22) existed in both treatments with CMHRs after Day 7 while 9-Octadecenoic acid (Z)-, methyl ester (C20) were found along the composting process. More long-chain fatty acids were observed in the treatment 1:1:1 and 5:5:1 because CMHRs contain the long-chain fatty acids themselves. Short chain fatty acid esters were also found in both treatments with CMHRs along the composting process, eg. propanoic acid, 3,3'-thiobis-, dodecyl ester and propanoic acid, 3-mercapto-, dodecyl ester, which might be due to efficient decomposition of organic substances into smaller molecular compounds by microorganisms during the thermophilic phase (Yamada and Kawase, 2006). The metabolic pathway of hydrocarbons acted distinctly. For instance, 1-Undecene, 8-methyl- appeared after Day 7 for both treatment with CMHRs addition while Pentadecane nitrile could only be observed at the initial composting period. Furthermore, the alkyl group of Dodecane, 2,6,11-trimethyl- existed in the treatment with dry weight ratio 5:5:1 specially. Some cyclanes, such as cyclopentane, (4-octyldodecyl)- and cyclopentane, 1-pentyl-2-propyl- were much more stable and were observed in the treatment with less CMHRs until the end of composting process. These aliphatic compounds were derived from lipids of food waste. Along the composting process, long-chain fatty acids were either broken down into smaller molecular compounds by microbial activity or reduced in concentrations. The peak intensities of hydrocarbon and alkyl groups also decreased due to the decomposition of organic substances. However, some compounds with complex ring structures such as

2(3H)-Furanone,dihydro-5-tetradecyl-,7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien e-2,8-dione, 1,3-Xylyl-15-crown-4, 2,3-.pinanedioxyboryl-, 9-Acetylphenanthrene, 1,2-Benzenedicarboxylic acid, ditridecyl and ditridecyl ester and Benzenamine, 4-octyl-N-(4-octylphenyl) were observed in both mature composts with CMHRs, except the fact that some of them appeared earlier in the treatment 1:1:1 (Day 28). The saturated, unsaturated and aromatic hydrocarbons existed in 56-day old composts could be linked to the stability and maturity of these composts.

The treatment with dry weight ratio 1:1:1 had similar composition of aliphatic compounds but contained more long-chain fatty acids (tetradecanoic acid, C14) (Fig. 4-6). The reason was that CMHRs contain thousands of compounds with very complex structures. Cyclododecane and cyclooctane were the additional alkyl compounds in treatment 1:1:1 as well as 3-Tridecene, 9-Tricosene, (Z)-, 1-Octadecene and 5-Methyl-Z-5-docosene as the alkenes. The nitrogen-containing compounds such as N,N-Dimethyldodecanamide were also observed during the initial composting phase. Nitrogen-containing compounds, typically amino compounds, were considered to be released from protein/amino acid by pyrolysis (Chiavari and Galletti, 1992, Hendricker and Voorhees, 1998). The amino compounds consisted of relatively simple structures during the initial phase of composting; however they were replaced by the cyclic N-containing compounds after Day 7. Pentadecane and hexadecane nitrile, N,N-Dimethyldodecanamide appeared in both of the treatments with dry weight ratio of 5:5:1 and 1:1:1. The results suggested that the amino acids and proteins were easily degraded in the earlier period of the composting process, which was consistent with the FTIR analyses of these samples. Ring structures such as ethanone, 1-(9-anthracenyl)-, 9-Acetylphenanthrene, 1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester,

[1R-(1.alpha.,4a.beta.,10a. alpha.)]-, 2(3H)-Furanone, dihydro-5-tetradecyl- were found in treatment with dry weight ratio 1:1:1 on Day 56. More molecular ring structures were found at the later stage of composting process in treatment 1:1:1, indicating compost from this treatment was more mature than the other two treatments.

In brief, readily available organic compounds like short chain fatty acids and carboxylic groups were degraded at the initial composting phase. Long chain fatty acids and lignin were broken down into smaller molecular mass along the composting process and provided the essential core frames for polymerization of humus with complicated ring structures. At the end of composting by comparing mature composts, the treatment with dry weight ratio 1:1:1 had earlier breakdown time of aliphatic compounds and more aromatic complex. It was derived from the long maintenance of thermophilic phase and most efficient decomposition rate as well as rich source of lignin from fibre texture of CMHRs.

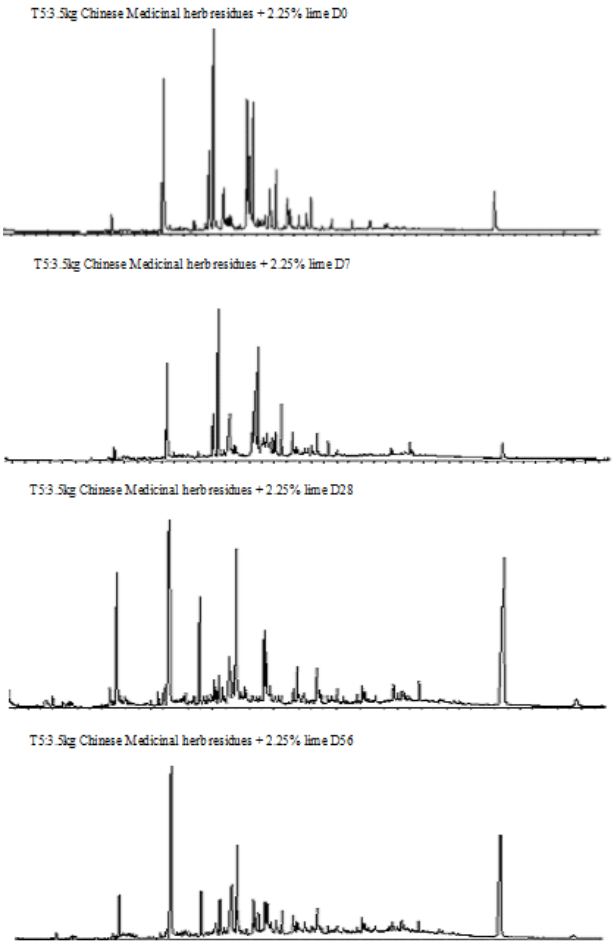


Figure 4-6. A representative Pyr-TMAH-GC-MS chromatogram of HA obtained from the treatment with dry weight ratio 1:1:1 during different phases of composting.

## 4.4 Conclusions

HA/FA ratio of treatment with dry weight ratio 1:1:1 was the highest at the end of composting and showed a clear correlation with compost maturity as also evidenced through the presence of higher aromatic functional groups in the HA fraction. FTIR results clearly demonstrated that the reduction of aliphatic and carboxylic groups and the increment of aromatic groups can be considered to indicate the stability and maturity of the compost and an efficient humification. Higher lignin content of the CMHRs enhanced and accelerated the humification due to the supplementation of lignin and its derivatives that formed the nucleus of HA. Pyr-TMAH-GC-MS results further indicated that dominant groups were aliphatic and alicyclic esters as well as ethers at the early composting phase in all treatments. Long chain fatty acids were broken down earlier into smaller molecular compounds in treatment 1:1:1, resulting from the faster decomposition rate. The complicated ring-structure components appeared dominantly at the later phase of composting. The peak intensities in treatment 1:1:1 indicated that the composts became mature earlier than the other two treatments. In brief, the treatment with dry weight ratio 1:1:1 had greatest humification degree with more cyclic structures and stable final products at the end of composting. Additionally, antipathogenic capacity of composts will be monitored during composting in order to confirm extra benefit of using CMHRs as a bulking agent.

# CHAPTER FIVE

# SUPPRESSIVENESS OF MATURE COMPOSTS WITH FOOD WASTE AND CHINESE MEDICINAL HERBAL RESIDUES AGAINST TWO PHYTOPATHOGENS

## 5.1 Introduction

Soil-borne pathogens are the main threat to the agricultural crop loss. Among all phytopathogens, *Alternaria solani* (*A. solani*) and *Fusarium oxysporum* (*F. oxysporum*) are two common pathomycetes which cause many plant diseases to cultivated crops, such as early blight and vascular wilt on potato/tomato (Pastor et al., 2012; Roncero et al., 2003). A host plant may be infected by multiple phytopathogens, resulting in unhealthy symptoms such as damping-off etc. The application of fungicides has serious negative effects of potential risks on both environment and human health (Brimner and Boland, 2003). Nowadays, more and more researchers focus on the biological control of phytopathogens in the long term of environmental friendly. Abiotic characters of soil, such as pH and nutrient contents can influence the general suppression; however, the microbial activities such as antagonism and mutualism are the most important factors, which keep the microflora balance in soil (Bernal-Vicente et al., 2008). During the past decade, compost is known as a fertilizer, which is rich in nutrients and, consequently, also has the ability to reduce the incidence of plant diseases by suppressing soil-borne diseases as the soil conditioner (Pugliese et al., 2008).

Composts as organic fertilizers are widely used for decades, promoting the

long-term productivity of agroecosystem. The soil amendments with composts addition provide sufficient nutrients to plants as well as modifying the soil microbial community (Termorshuizen et al., 2006). Their physical, chemical and biological characters on phytopathogenic control obtained positive achievements (EL-Masry et al., 2002; Serra-Wittling et al., 1996). The microbial community composition plays an important role in degradation, ammonification and mineralization of food waste composting. The bacterial and fungal dynamics, and their enzymatic activities were largely influenced by physico-chemical properties of composts, such as temperature, initial pH, carbon/nitrogen ratio and dissolve organic carbon (DOC) (Wong et al., 2011). However, there is a lack the co-composting by using CMHRs and food waste is lack of published papers on using CMHRs and food waste as co-composting materials, neither in China nor globally. As a matter of fact, CMHRs, which collected after normally decoction, are rich in compounds with antibacterial and antifungal effects as reported by previous researches (Chu et al., 2006; Feng et al., 2001; Wang et al., 2001; Yang et al., 2009; Zhu et al., 2006; Zhu et al., 2007). Mature composts have been proved the capacity of controlling various soil-borne diseases and based on our previous research, the mature composts containing CMHRs as a bulking agent showed greater degree of humification, encouraging further study of their role as bio-pesticide functionality (Chapter 4).

The purpose of this research was aim to investigate the antipathogenic effect of final composts on the two specific phytopathogens, *A. solani* and *F. oxysporum*. The mechanism of suppressiveness was determined by testing the antipathogenic ability (both antagonistic and abiotic properties) of the composts, including the fungal growth inhibition test by using sterile-treated products, dynamics of bacteria and fungi in composts and bacterial/fungal (isolated from composts) antagonism against the two

phytopathogens.

## 5.2 Material and method

### 5.2.1 Experimental design and composting conditions

The compost samples were collected from previous experiments and the composting materials and conditions were briefly listed in Chapter 3 (Section 3.2). The treatments involved in this experiment included dry weight ratios of 5:5:1 and 1:1:1 (FW: SD: CMHRs), while food waste and sawdust mixed at 1:1 (dry weight basis, w/w) served as the control to reveal the influence of CMHRs on antipathogenic effect. The treatments with the dry weight ratio of 5:5:1, 1:1:1 and control were chosen for further determination of bacterial population, based on the reason that significant differences could be observed based on the mixing ratio. Samples from each treatment collected on Day 0, 7, 28 and 56 were used to monitor the dynamics of active compounds periodically.

### 5.2.2 Reagents and phytopathogens

Acetone was purchased from Tedia (USA) and water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The phytopathogens, *A. solani* and *F. oxysporum* were purchased from the Centraalbureau voor Schimmelcultures, Netherland.

### 5.2.3 Dynamics of bacterial and fungal communities during composting process

5.2.3.1 DNA extraction

Genomic DNA from 0.3 g fresh sample was extracted and DNA extraction was proceeded by using QIAamp DNA stool mini kit (Qiagen, Hilden) following detail procedure of Section 3.2.3 in Chapter 3.

5.2.3.2 Plasmid construction

The PCR products of the V3 region of the bacterial 16S rDNA and ITS1 region of the fungal 18S rDNA were purified by Wizard_SV Gel and PCR Clean-Up System (Cat.# A9281, Promega), and then cloned in competent *Escherichia coli* DH5a using the pGEM-T easy vector system according to the manufacturer's instructions (Promega, Madison, WI, USA). Positive transformants were selected by blue-white screening on plates after ligation and transformation process. DNA concentration was measured by using Nanodrop spectrophotometer which saved as standards for subsequent quantitive PCR reactions.

5.2.3.3 Real-time PCR

The plasmid DNA of 16S rDNA and 18S rDNA were serially diluted for making standard curve. The quantitive PCR mixture (20 μL) of fungal DNA, containing 10 μL SYBR Premix Ex Taq II (Takara, Dalian), 7 μL nuclease free water, 1 μL of each forward/reward primers (NS1, GTAGTCATATGCTTGTCTC; Fung, ATTCCCCGTTACCCGTTG; 10 μmol/L each) of (Tech Dragon Ltd.) and 1 μL product template (about 20 ng), was used in q-PCR analysis of fungal population. The q-PCR analyses were performed using Agilent Technologies Stratagene Mx3000P (USA) with the following temperature profile: initial denaturation 95 $^{o}$C for 5 min, 40 cycles of 95 $^{o}$C for 30 s, 55 $^{o}$C (bacteria)/47 $^{o}$C (fungi) for 30 s, 72 $^{o}$C for 1 min, and final dissociation 95 $^{o}$C for 1 min, 55 $^{o}$C (bacteria)/47 $^{o}$C (fungi) for 1 min, 95 $^{o}$C for 1 min. DNA products were checked in 2% agarose gel (Ultrapure, MB grade, USA) in

TAE buffer at 100 V, along with the DNA ladder of 1000 bp to confirm the amplicons.

### 5.2.4 Isolation, molecular identification and quantification of prevalently visual bacteria and fungi in various composting processes

The composts (1 g fresh samples) were suspended on sterilized Ringer solution (1/10 w/v) and serial dilutions method was used to quantify the colony forming units (CFU) of universal bacteria and fungi. After shaking for 30 min, 100 μL suspension was placed on tryptose soya agar with cycloheximide (100 mg/L) for bacteria population counting and potato dextrose agar (rose bengal 50 mg/L, streptomycin 100 mg/L) for fungi. The TSA plates were incubated at 37 $^{o}$C for 24 h while incubation at 28 $^{o}$C for 72 h for PDA plates before plate counting (Bernal-Vicente et al., 2008).

5.2.4.1 DNA extraction

Bacterial and fungal colony which repetitively existed more than three times within one plate with various colors, shapes, types of growth and hyphal pigment detected according to their macroscopic and microscopic features were isolated on TSA and PDA for cultivation respectively. Isolated pure bacteria and fungi DNA were extracted and purified using QIAamp DNA stool mini kit (Qiagen, Hilden) following the manufacturer's recommendation and used as the template for polymerase chain reaction (PCR) amplification.

5.2.4.2 PCR amplification

The PCR mixture (25 μL), containing 12.5 μL SYBR Premix Ex Taq II (Takara, Dalian), 9.5 μL nuclease free water, 1 μL of each forward/reward primers (bacteria: 338F, ACTCCTACGGGAGGCAG and 805R, GACTACCAGGGTATCTAATCC; fungi: NS1, GTAGTCATATGCTTGTCTC; Fung,

ATTCCCCGTTACCCGTTG; 10 μmol/L each) of (Tech Dragon Ltd.) and 1 μL product template (about 20 ng), was used in PCR analysis of bacterial population (Muyzer et al., 1993; May et al., 2001).

The PCR amplification of isolated bacteria were performed using Agilent Technologies Stratagene Mx3000P (USA) with the following temperature profile: initial denaturation 95 $^{o}$C for 1 min, 34 cycles of 94 $^{o}$C for 45 s, 55 $^{o}$C for 45 s, 72 $^{o}$C for 1 min, and final dissociation 72 $^{o}$C for 10 min, 4 $^{o}$C for 30 min.

The PCR protocol for 18S ribosomal gene (isolated fungi) began with an initial denaturation at 94 $^{o}$C for 5 min, followed by 94 $^{o}$C with a 30 s extension time, 47 $^{o}$C for 45 s, and 72 $^{o}$C for 1 min, 35 cycles; with a final extension at 72 $^{o}$C for 10 min; 4 $^{o}$C with a 30 min.

5.2.4.3 Cloning and sequencing

For cloning library analysis, purified isolated bacteria and fungi PCR amplicons were performed by directly cloned in competent *Escherichia coli* using the pGEM-T easy vector system (Promega, Madison, WI, USA). Positive transformants were selected by blue-white screening on plates after ligation and transformation process. Plasmid DNA was amplified and sequenced using T7 and Sp6 primers before sending for identification (Techdragon, Hong Kong). Similarities of isolated bacteria and fungi were performed in GenBank using the BLAST program and matched the maximum identity as the first identification criteria.

### 5.2.5 Antipathogenic test

CMHRs and composts (30 g fresh samples) were extracted by using distilled water with solid: extractant ratio of 1:10 (w/v, dry weight basis) to prepare original

extractants with the concentration of 1 g/ml. Extractants were diluted to various concentrations and mixed with 20 ml PDA to obtain culture matrix of 1 ppm, 10 ppm, 100 ppm and 500 ppm (0.1%, 1%, 10% and 50%). The acetone extractions were dried by inert gases and redissolved to the same concentrations before mixed with 20 ml PDA. *A. solani* and *F. oxysporum* cakes (diameter of 1 cm) were inoculated in the middle of the plates and reverse cultivation for 9 days at 28 $^{o}$C. Distilled water of same amount was used for the blank test. The diameters of pathogenic colony sizes were measured by using straight cross method daily (Bernal-Vicente et al., 2008). The inhibition rate was calculated by formula below and the $MIC_{50}$ results were obtained from plotting by growth inhibition diameters. Each test was carried out in triplicates to allow statistical comparisons between treatments. Abiotic tests followed the same way but the samples were filtered through 0.2 μm membrane filters in order to eliminate the microorganisms.

$$\text{Inhibition rate (\%)} = \frac{\text{Colony diameter of control} - \text{Colony diameter of treatment}}{\text{Colony diameter of control}} \times 100$$

### 5.2.6 Bacterial and fungal antagonism against *A. solani* and *F. oxysporum*

The isolated bacteria was placed in the middle of a PDA plate and after incubation at 25 $^{o}$C for 24 h, a PDA disc (diameter of 1 cm) of phytopathogen was placed at 2 cm length from the bacterial solution line, incubated at 25 $^{o}$C for 7 days. As to fungal, phytopathogens were placed at 4 cm distance in a PDA plate, incubated at 28 $^{o}$C for 7 days. The control test of each pathogen was carried out by placing pathogens in Petri dishes without any isolated microbes. Biocontrol result was positive if pathogens colony sizes were smaller than 50% of the control. Antibiosis

was considered when expand of pathogenic mycelium was inhibited by the isolated bacteria or fungi. Mycoparasitism antagonism represented the interaction between pathogens and the tested microbes (Bernal-Vicente et al., 2008).

### 5.2.7 Statistical analysis

Analyses were performed in triplicate samples and the mean values and standard deviation on dry weight basis are presented. The data were processed using SigmaPlot 11.0 and IBM SPSS statistics 19 while the significance of the differences were tested using Duncan multiple rage test at $p<0.05$.

## 5.3 Results and discussion

### 5.3.1 Microbial dynamic changes during composting process

The variations of microbial community depended largely on the changes of physico-chemical factors during composting process. At the initial phase of composting, microbial population in all treatments were similar due to the same environmental source. The log copy number of both universal bacteria and fungi increased during the first week since the readily available carbon source and prevalence mesophilic temperature provided the microbes appropriate living conditions for their rapid reproductions (Selvam et al., 2012). The initial total bacterial population in the treatment 1:1:1 was 7.1 (Fig. 5-1) which was lower than 7.9 for treatment 5:5:1 and 8.0 for the control. This can be explained by the higher amount of CMHRs in the treatment 1:1:1 which prohibited microbial growth due to

higher amount of bioactive components with antipathogenic effects and higher lignin content in the CMHRs making it difficult for microbes to mineralize it as nutrient substrate for microbial growth. During thermophilic phase, the correlation was also found between bacteria population, ammonium and nitrate contents, but not with fungal species (Bernal et al., 2009). The nitrifying bacteria converted ammonium to nitrite and further nitrate (final form of nitrogen which is uptaken by crops). As the nutrient source was exhausted and composts became mature, the microbial community tended to be stable. Nitrogen-fixing bacteria played also an important role in the cooling phase and promoted the quality of composts after land application. At the maturation phase (Fig. 5-1a), the treatment 1:1:1 contained lowest bacterial population (copy number of 5.2) followed by the treatment 5:5:1 (7.1) and the control (7.5) since less food waste proportion provided less readily nutrients for their activities. However as shown in Fig. 5-1b, the treatment 1:1:1 limited the log copy number of fungi to 5.4 on Day 56, comparing to the treatment 5:5:1 was 6.1 and control of 6.2. During this process, the fast-growing bacteria might have the capacity of competing and hindering the fungal growth. Fungi proliferated at cooling phase when the temperature was decreasing and pH was alkalescent (Vargas-Garcia et al, 2010). At maturation phase, the fungal population was reduced slightly due to major environmental factors, such as microbial antagonisms, antibiosis, moisture content and stable acidity. Similar results have been obtained in Hassen et al's research (2001). The fungal population was the lowest for the treatment 1:1:1 since CMHRs in the final compost still controlled the fungal population effectively. In general, degradative capability of microbes could be achieved through their enzymatic actions such as the hydrolysis of complex macromolecules that constitute the organic wastes. As a result, readily available water-soluble compounds that support microbial growth are released

from composts, promoting the continuity of the process (Vargas-Garcia et al., 2010).

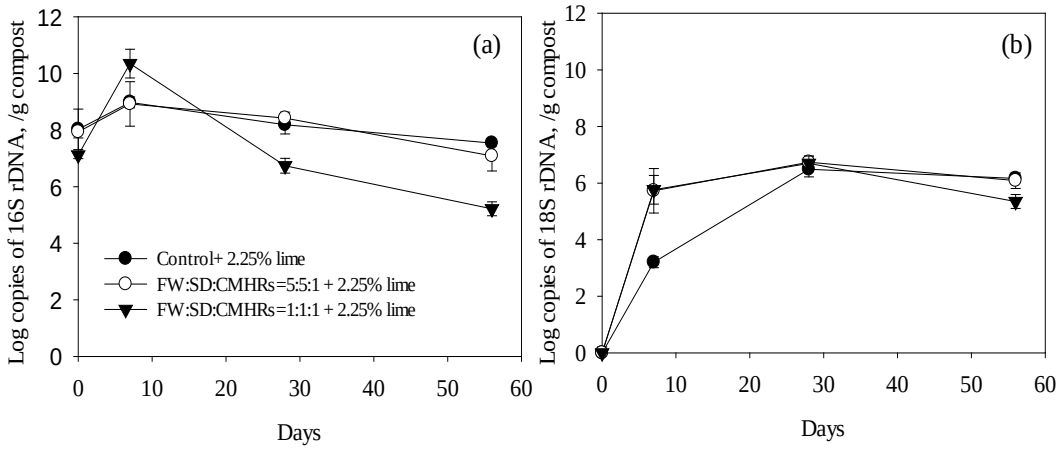


Figure 5-1. Bacterial 16S rDNA (a) and fungal 18S rDNA (b) copy number of 1 g composting mass during the composting period.

### 5.3.2 Sequences identification

Seventeen bacterial species and 22 fungal species were isolated and identified as prevalently existed microbes during composting process (Table 5-1-5-3).

Table 5-1. Isolated bacteria (1-17) and fungi (1-22) at various composting phases.

| | Bacteria | Similarity | | Fungi | Similarity |
|---|---|---|---|---|---|
| 1 | *Alcaligenes* sp. | 100% | 1 | *Absidia blakesleeana* | 100% |
| 2 | *Bacillus licheniformis* | 99% | 2 | *Acremonium flavum* | 100% |
| 3 | *Bacillus massiliensis* | 99% | 3 | *Aspergillus fumigatus* | 100% |
| 4 | *Bacillus thermocloacae* | 99% | 4 | *Aspergillus niger* | 100% |
| 5 | *Bacterium* sp. | 99% | 5 | *Basipetospora chlamydospora* | 100% |
| 6 | *Brevundimonas naejangsanensis* | 98% | 6 | *Candida tropicalis* | 100% |
| 7 | *Clostridium cellulolyticum* | 100% | 7 | *Doratomyces stemonitis* | 100% |
| 8 | *Clostridium thermocellum* | 99% | 8 | *Emericella nidulans* | 100% |
| 9 | *Flavobacterium mizutaii* | 100% | 9 | *Eurotium repens* | 99% |
| 10 | *Flavobacterium odoratum* | 100% | 10 | *Fusarium oxysporum* | 99% |
| 11 | *Hydrogenobacter* spp. | 100% | 11 | *Microascus longirostris* | 99% |
| 12 | *Lysinibacillus* sp. | 100% | 12 | *Microascus trigonosporus* | 100% |
| 13 | *Paenibacillus residui* | 100% | 13 | *Mucor pusillus* | 100% |
| 14 | *Pennisetum glaucum* | 100% | 14 | *Myceliophthora thermophila* | 100% |
| 15 | *Pseudomonas* sp. | 100% | 15 | *Penicillum decumbens* | 100% |
| 16 | *Solibacillus* sp. | 100% | 16 | *Penicillum janthinellum* | 100% |
| 17 | *Streptomyces flavoviridis* | 100% | 17 | *Pithoascus langeronii* | 100% |
| | Bacteria | 100% | 18 | *Sporotrichum thermophile* | 100% |
| | | | 19 | *Talaromyces thermophilus* | 100% |
| | | | 20 | *Taloromyces emersonii* | 100% |
| | | | 21 | *Thermoascus auranticus* | 100% |
| | | | 22 | *Thermomyces lanuginosus* | 100% |

Prevalently existed microbial species in composts were much closer with most related sequences at the initial composting phase than the later phase. Little overlap between initial/thermophilic and cooling/mature phases due to thermotolerant

capability of some of the microorganisms. Nevertheless few pathogens can survive along composting process, representing efficiency of composting to remove pathogens (Bonito et al., 2010). Temperature plays the key role resolved the capacities of microbial competition and survival rates (Ishii et al., 2000).

Table 5-2. Isolated prevalent bacteria in various composting phases.

| Bacterial species | | D0 | | D7 | | D28 | | D56 | |
|---|---|---|---|---|---|---|---|---|---|
| | | $10^5$ CFU/g, dry weight basis | STD | $10^5$ CFU/g, dry weight basis | STD | $10^5$ CFU/g, dry weight basis | STD | $10^5$ CFU/g, dry weight basis | STD |
| Treatment Control R1 | *Alcaligenes* sp. | - | | - | | 3600 | 4 | 450 | 2 |
| | *Bacillus massiliensis* | 2500 | 3 | 22000 | 2 | 5000 | 5 | 870 | 5 |
| | *Bacillus thermocloacae* | 1300 | 3 | 7600 | 17 | 0 | 0 | 0 | 0 |
| | *Bacterium* sp. | - | | - | | 0 | 0 | 1100 | 4 |
| | *Brevundimonas naejangsanensis* | - | | - | | 460 | 13 | 100 | 2 |
| | *Clostridium thermocellum* | 3400 | 13 | 9600 | 16 | - | | - | |
| | *Flavobacterium odoratum* | 1900 | 6 | 23000 | 2 | - | | - | |
| | *Flavobacterium mizutaii* | 1600 | 5 | 15000 | 3 | - | | - | |
| | *Paenibacillus residui* | - | | - | | 2000 | 3 | 140 | 2 |
| | *Pennisetum glaucum* | - | | - | | 1900 | 6 | 350 | 2 |
| | *Pseudomonas* sp. | 0 | 0 | 18000 | 2 | 2600 | 5 | 490 | 3 |
| Treatment 5:5:1 R2 | *Bacillus licheniformis* | 2500 | 4 | 24000 | | 5000 | 4 | 230 | 9 |
| | *Bacillus massiliensis* | 1500 | 2 | 16000 | 4 | 8700 | 7 | 330 | 7 |
| | *Bacillus thermocloacae* | - | | 10000 | 3 | - | | - | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Bacterium* sp. | - | | - | | - | | 270 | 5 |
| | *Clostridium thermocellum* | 2700 | 3 | 9300 | 9 | - | | - | |
| | *Streptomyces flavoviridis* | 2000 | 8 | 24000 | 3 | - | | - | |
| | *Hydrogenobacter spp.* | - | | - | | 4500 | 3 | - | |
| | *Lysinibacillus* sp. | - | | - | | - | | 160 | 3 |
| | *Pennisetum glaucum* | - | | - | | 2400 | 2 | 37 | 2 |
| | *Streptomyces flavoviridis* | - | | - | | 5800 | 7 | 200 | 8 |
| Treatment 1:1:1 R5 | *Bacillus licheniformis* | 260 | 7 | 63000 | 13 | 150 | 3 | 5 | 1 |
| | *Bacillus massiliensis* | 210 | 2 | 63000 | 6 | 200 | 4 | 6 | 0 |
| | *Bacillus thermocloacae* | - | | 46000 | 5 | - | | 3 | 0 |
| | *Clostridium cellulolyticum* | 130 | 5 | - | | - | | - | |
| | *Clostridium thermocellum* | 490 | 19 | 17000 | 5 | - | | - | |
| | *Lysinibacillus* sp. | - | | - | | 60 | 2 | 2 | 0 |
| | *Pennisetum glaucum* | - | | - | | 17 | 2 | 1 | 2 |
| | *Solibacillus* sp. | - | | - | | 6 | 2 | 0 | 0 |
| | *Streptomyces flavoviridis* | 220 | 9 | 53000 | 3 | - | | 3 | 1 |

Table 5-3. Isolated prevalent fungi in various composting phases.

| Fungi species | | D7 | | D28 | | D56 | |
|---|---|---|---|---|---|---|---|
| | | $10^5$ CFU/g, dry weight basis | STD | $10^5$ CFU/g, dry weight basis | STD | $10^5$ CFU/g, dry weight basis | STD |
| Treatment Control R1 | *Aspergillus fumigatus* | 0 | 1 | - | | - | |
| | *Basipetospora chlamydospora* | - | | 100 | 1 | 100 | 2 |
| | *Candida tropicalis* | - | | 16 | 7 | 15 | 3 |
| | *Emericella nidulans* | 0 | 0 | - | | - | |
| | *Eurotium repens* | - | | 13 | 4 | 9 | 3 |
| | *Fusarium oxysporum* | - | | 1 | 1 | 4 | 1 |
| | *Mucor pusillus* | 0 | 0 | - | | - | |
| | *Myceliophthora thermophila* | 0 | 1 | - | | - | |
| | *Penicillum decumbens* | - | | 3 | 0 | - | |
| | *Penicillum janthinellum* | - | | 3 | 1 | - | |
| | *Pithoascus langeronii* | - | | 7 | 4 | 16 | 0 |
| | *Sporotrichum thermophile* | 0 | 2 | - | | - | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Thermomyces lanuginosus* | 0 | 1 | - | | - | |
| Treatment 5:5:1 R2 | *Absidia blakesleeana* | - | | - | | 10 | 2 |
| | *Aspergillus fumigatus* | 14 | 2 | 60 | 5 | 60 | 4 |
| | *Basipetospora chlamydospora* | - | | 37 | | 27 | 6 |
| | *Microascus longirostris* | - | | - | | 6 | 2 |
| | *Microascus trigonosporus* | - | | - | | 4 | 2 |
| | *Myceliophthora thermophila* | 1 | 0 | - | | - | |
| | *Pithoascus langeronii* | - | | - | | 16 | 2 |
| | *Sporotrichum thermophile* | 18 | 3 | - | | - | |
| | *Taloromyces emersonii* | 6 | 1 | 12 | 1 | - | |
| | *Talaromyces thermophilus* | 13 | 2 | - | | - | |
| | *Thermomyces lanuginosus* | 2 | 1 | 14 | 2 | - | |
| Treatment 1:1:1 R5 | *Acremonium flavum* | - | | - | | 2 | 0 |
| | *Aspergillus niger* | 14 | 1 | 13 | 2 | 13 | 4 |
| | *Doratomyces stemonitis* | - | | 5 | 0 | 4 | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Emericella nidulans* | 1 | 0 | - | | - | | |
| *Fusarium oxysporum* | - | | 0 | 1 | 1 | 0 | |
| *Myceliophthora thermophila* | 3 | 1 | - | | - | | |
| *Pithoascus langeronii* | - | | - | | 3 | 0 | |
| *Sporotrichum thermophile* | 20 | 4 | - | | - | | |
| *Taloromyces emersonii* | 3 | 2 | 3 | 0 | - | | |
| *Talaromyces thermophilus* | 16 | 4 | - | | - | | |
| *Thermoascus auranticus* | - | | 1 | 0 | - | | |
| *Thermomyces lanuginosus* | 2 | 0 | - | | - | | |

As shown in Fig. 5-2 and 5-4, changes of microbial diversities of each treatment were quantified during composting. Fungal population on Day 0 was below the detection limit, which is likely due to the dominance in bacterial population and the potential antifungal effect of CMHRs. There were mainly four major bacterial species existed on Day 0: *Bacillus*, *Clostridium*, *Flavobacterium* and *Streptomyces*. *Bacillus* was present in all treatments which agreed to previous research that it has been widely found to be distributed in various composting raw materials such as garden, domestic and food wastes (Dees and Ghiorse, 2001). As listed in Table 5-2, *Bacillus*, *Clostridium*, and *Flavobacterium* averagely shared bacterial population in the control treatment, while *Bacillus* was dominant in treatment 5:5:1 ($4.0*10^7$ CFU/g, 46%) and *Clostridium* in treatment 1:1:1 ($6.2*10^7$ CFU/g, 47%).

On Day 7, the increment of temperature > 55 $^oC$ eliminated most mesophiles and caused a shift in the microbial diversity from mesophilic to thermophilic population (Bonito et al., 2010). Thermophilic bacteria species such as *Bacillus* spp. increased to $3.0*10^8$ CFU/g, $5.1*10^8$ CFU/g and $17.2*10^8$ CFU/g on dry weight basis (31%, 60% and 71%) in treatment control, 5:5:1 and 1:1:1, which was 8, 13 and 363 folds to Day 0 respectively. Treatments with CMHRs shared the same bacterial species in different populations; both *Clostridium* and *Streptomyces* were observed in smaller population in treatment 1:1:1 since it contained less food waste and more CMHRs, inhibiting bacterial growth. During the thermophilic phase, the ammonifying bacteria (*Bacillus*, *Clostridium*, *Pseudomonas* and *Streptomyces*) became the dominant population for the ammonification process, converting cycling of nitrogen (Chen et al., 2014; Meunchang et al., 2005; Sepers, 1981). Fungi which commonly parasitized in food waste composts such as *Aspergillus*, *Sporotrichum* and *Talaromyces* were observed in all treatments, which was agree to Neher et al's research that these fungi spread their

population with sufficient nutrient source and proper environmental condition (2013). Treatment 1:1:1 contained the highest populations ($3.5*10^5$ CFU/g, 59%) of Deuteromycetes (*Aspergillus* and of *Sporotrichum*), which had the function of cellulose hydrolysis, accelerating the degradation of CMHRs and promoting the maturity of compost. The existence of *Talaromyces* ($1.3 *10^5$ CFU/g of 5:5:1 and $1.6*10^5$ CFU/g of 1:1:1) should promote lignin degradation positively since its isolation is lignocellulose related, resulting in weight/volume reductions and humic substances production in treatments with CMHRs addition (Keshwani et al., 2009). Bioactivity of *Talaromyces* accelerated the humification process of composting and the largest changes of humic acids content was observed in the treatment 1:1:1 (0.96% to 2.67%), followed by treatment 5:5:1 (1.22% to 1.54%) and control (0.68% to 0.40%) (Chapter 4).

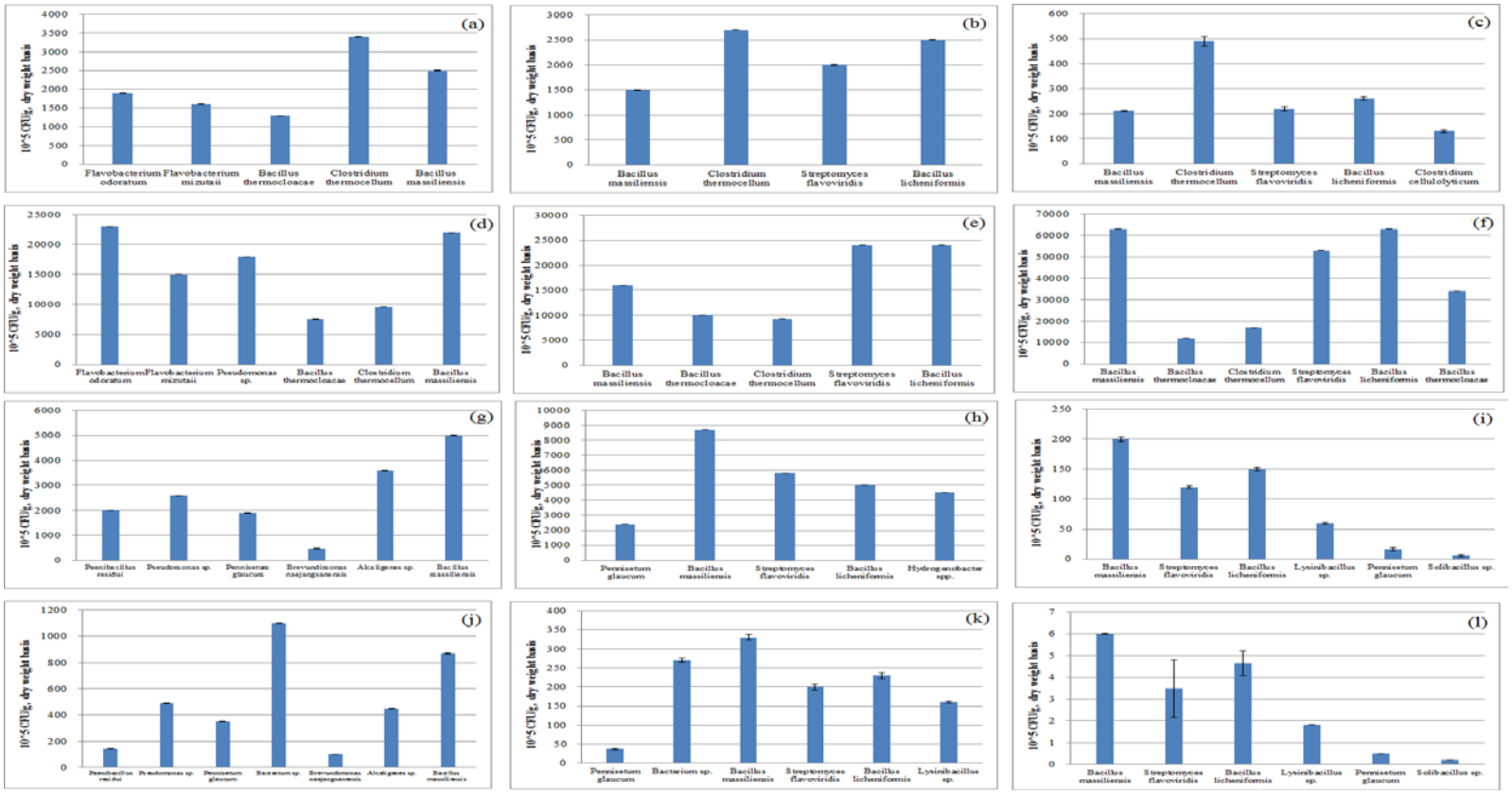


Figure5-2. Prevalently visual bacteria during composting (a: Day 0 R1; b: Day 0 R2; c: Day 0 R5; d: Day 7 R1; e: Day 7 R2; f: Day 7 R5; g: Day 28 R1; h: Day 28 R2; i: Day 28 R5; j: Day 56 R1; k: Day 56 R2; l: Day 56 R5; R1: Control; R2: FW:SD:CMHRs=5:5:1, dry wt. basis; R5: FW:SD:CMHRs=1:1:1, dry wt. basis).

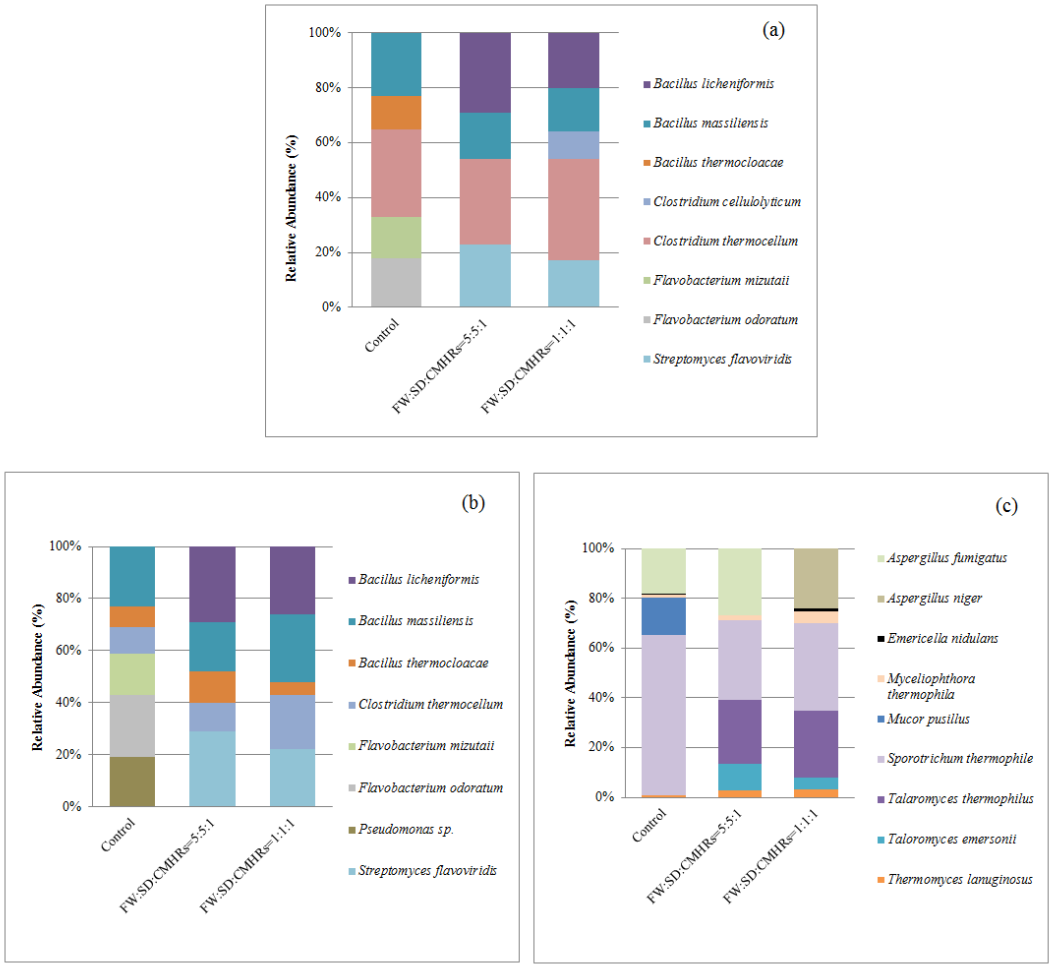


Figure 5-3. Composition of isolated bacteria and fungi in initial and thermophilic phase of composting (a: bacterial diversities on Day 0; b: bacterial diversities on Day 7; c: fungal diversities on Day 7).

The thermophilic bacterial population gradually declined with a decrease in temperature due to the replacement by mesophilic bacteria which became dominant at the cooling phase (Fig. 5-4a). In this phase, *Bacillus* spp. was still dominant in all treatments (32%, 52% and 64% for the treatment control, 5:5:1 and 1:1:1) due to their thermotolerant (above 60 $^{o}$C) properties (Zhang et al., 2011). Additionally, the appearances of *Bacterium*, *Alcaligenes* and *Streptomyces* were also widely selected by compost environment during cooling and mature phases, which agreed to the results of Zhang et al's (2002). In addition to the thermotolerant property, advanced functionalities of these bacteria include cellulase, chitinase, protease activities, producing indole acetic acid and nitrogen-fixing ability to result in more stable and mature composts. The hydrolytic enzymatic production and secondary metabolites secretion of these bacteria also promoted their antagonistic characters against soil-borne phytopathogens (Lin et al., 2013). Nevertheless, the universal fungal community expanded after thermophilic phase due to gradually released nutrient source from composts and lack of bacterial antagonism after high temperature. As shown in Fig. 5-3b, *Basipetospora* ($2.2*10^6$ CFU/g, 70%) tended to be maximum in the control treatment while *Aspergillus* spp. with lignin degradation property was dominant in treatment 5:5:1 ($2.7*10^6$ CFU/g, 49%) and 1:1:1 ($2.9*10^6$ CFU/g, 58%) respectively (Huang et al., 2010). *Mucor pusillus* in the control treatment disappeared after thermophilic phase and did not recur even under mesophilic temperature due to lack of polysaccharolytic property (Chang and Hudson, 1967). *Penicillium* ($1.2*10^5$ CFU/g, 4%) was observed only in the control treatment since it is commonly found as contaminants in foods (Pitt et al., 2000). From cooling phase, microbial species tended to be stable; both bacterial and fungal species maintained the same till the end of composting.

As illustrated in Table 5-2, thermophilic *bacilli* were prevalently observed in mature composts, which played an important role in composting microbial ecology. Fungal activities are closely associated with lignocellulosic degradation with relative low moisture content preference (Mehta et al., 2014). Several studies reported *Aspergillus* ($1.3*10^5$ CFU/g of *Aspergillus fumigatus* in treatment 5:5:1, 49% and $6.0*10^5$ CFU/g of *Aspergillus niger* in treatment 1:1:1, 56%) as dominant species in mature phase of composting which are in accord with the results in this study as listed in Table 5-3 (Grewal et al., 1988; Ryckeboer et al., 2003). This result suggested that the mature phase of compost system could be considered as simulated oligotrophic environment of soil. Both bacterial and fungal species in the treatments with CMHRs addition shared more similarity at the later phase of composting (Fig. 5-2). The reason may originate from the substrate of lignin left in CMHRs and the dominant microbial groups might be highly selected by the available substrates.

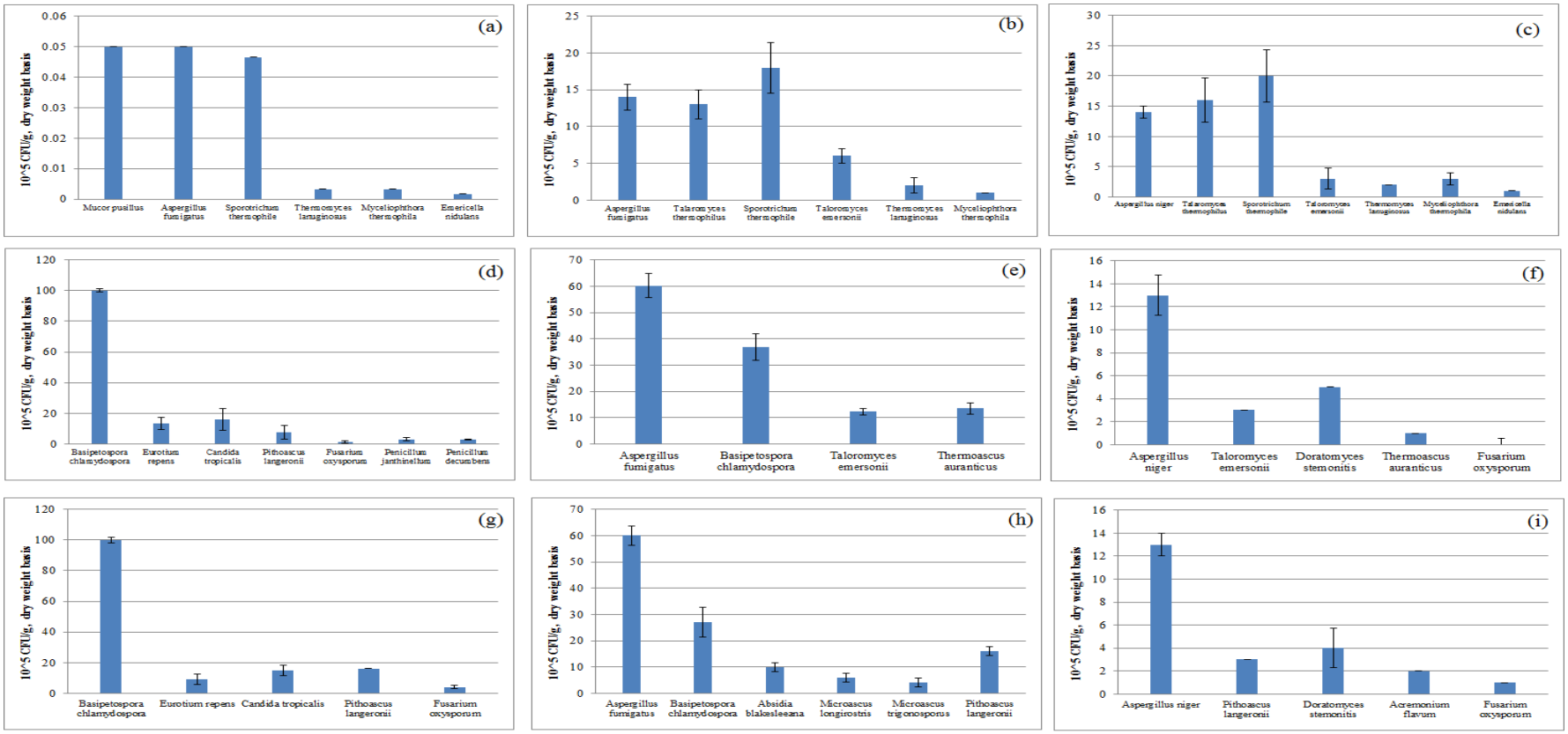


Figure 5-4. Prevalently visual fungi during composting (a: Day 7 R1; b: Day 7 R2; c: Day 7 R5; d: Day 28 R1; e: Day 28 R2; f: Day 28 R5; g: Day 56 R1; h: Day 56 R2; i: Day 56 R5; R1: Control; R2: FW:SD:CMHRs=5:5:1, dry wt. basis; R5: FW:SD:CMHRs=1:1:1, dry wt. basis).

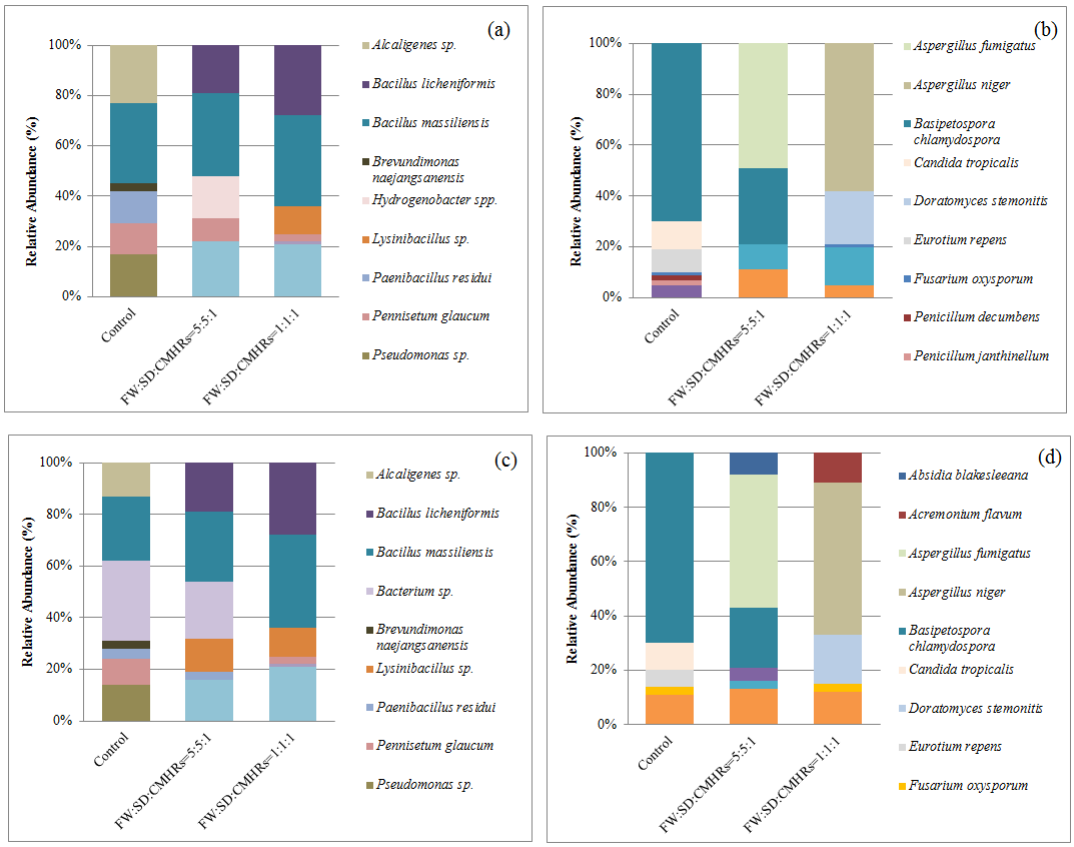


Figure 5-5. Composition of isolated bacteria and fungi in cooling and mature phase of composting (a: bacterial diversities on Day 28; b: fungal diversities on Day 28; c: bacterial diversities on Day 56; d: fungal diversities on Day 56).

### 5.3.3 Antipathogenic efficiency of CHMR extracts

In vitro experiments were processed to explore the antipathogenic effect of mature composts (Fig. 5-6). The fungal species of *A. solani* and *F. oxysporum* were selected as the soil borne phytopathogens since they cause vegetables with plant diseases such as early blight and stem lesions, commonly cultivated in Hong Kong (Champeil et al., 2004).

Table 5-4. Suppressiveness (%) of CMHRs and mature composts against *A. solani* and *F. oxysporum.*

| | | *A. solani* | | | | *F. oxysporum* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Water extraction | | Acetone extraction | | Water extraction | | Acetone extraction | |
| | | Relative Inhibition ratio, % | $MIC_{50}$ mg/mL | Relative Inhibition ratio, % | $MIC_{50}$ mg/mL | Relative Inhibition ratio, % | $MIC_{50}$ | Relative Inhibition ratio, % | $MIC_{50}$ mg/mL |
| CMHRs | | 30.5 (1.8) | 182 (7.6) | 75.6 (0.1) | 13 (0.9) | 35.7 (0.5) | 332 (7.7) | 54.3 (1.5) | 61 (2.0) |
| Day 56 (Total) | Control | 25.0 (0.9) | 108 (9.0) | 34.8 (0.6) | 120 (3.0) | 27.3 (0.7) | 190 (5.4) | 35.4 (1.4) | 176 (3.7) |
| | 5:5:1 | 28.3 (0.4) | 40 (1.0) | 36.4 (0.5) | 34 (1.6) | 34.8 (0.5) | 66 (3.0) | 35.4 (0.8) | 66 (2.9) |
| | 1:1:1 | 58.0 (0.5) | 28 (0.2) | 75.8 (0.1) | 16 (1.0) | 47.7 (1.2) | 30 (1.0) | 59.0 (0.9) | 22 (0.5) |
| Day 56 (Abiotic) | Control | 20.0 (0.5) | 178 (9.6) | 30.6 (1.1) | 139 (9.2) | 23.5 (0.2) | 198 (2.3) | 30.6 (0.2) | 192 (2.4) |
| | 5:5:1 | 22.3 (0.7) | 69 (2.0) | 38.5 (0.5) | 40 (2.2) | 25.5 (0.2) | 72 (1.9) | 32.6 (0.4) | 89 (1.0) |
| | 1:1:1 | 49.3 (0.5) | 42 (3.4) | 71.7 (0.5) | 21 (0.4) | 39.7 (0.5) | 49 (0.7) | 40.7 (0.9) | 45 (0.3) |

* Total: effects from both chemical compounds and antagonism from isolated microorganism in composts; Abiotic: effects from only chemical compounds in composts; MIC: Minimum Inhibitory Concentration.

As shown in Table 5-4, the acetone had better efficiency of extraction than distilled water since the organic solvent extracts more organic compounds from matrix due to the principle that the similar substance is more likely to be dissolved by each other. Mature composts had the capacity of inhibiting the pathomycetes growth (Suárez-Estrella et al., 2013). The acetone extraction of the treatment 1:1:1 showed the best performance of suppressing fungal growth (total), with the inhibition ratio of 75.8% and 59.0% for *A. solani* and *F. oxysporum* respectively. This result was higher than the other two treatments, even close to the antipathogenic effect of the pure CMHRs extraction (75.6% and 54.3%). The biotic results of the treatment 1:1:1 showed much lower antifungal capacity than CMHRs since concentrations of bioactive compounds were much lower in mature CMHRs composts. However, antagonistic properties of mature CMHRs composts contributed strong power to antifungal effect, comparing to CMHRs (Bernal-Vicente et al., 2008). Therefore, the suppressive efficiency of mature composts was replaced by the increment of the check-and-balance mechanism of microorganisms in composts. The results of $MIC_{50}$ indicated that the treatment 1:1:1 required least concentration of composts extraction to kill half quantity of the phytopathogens, 16% for *A. solani* and 22% for *F. oxysporum* extracted by acetone. The pathogen control was due to both the anti-fungal compounds of composts and competition of microbial diversities (Serra-Wittling et al., 1996).

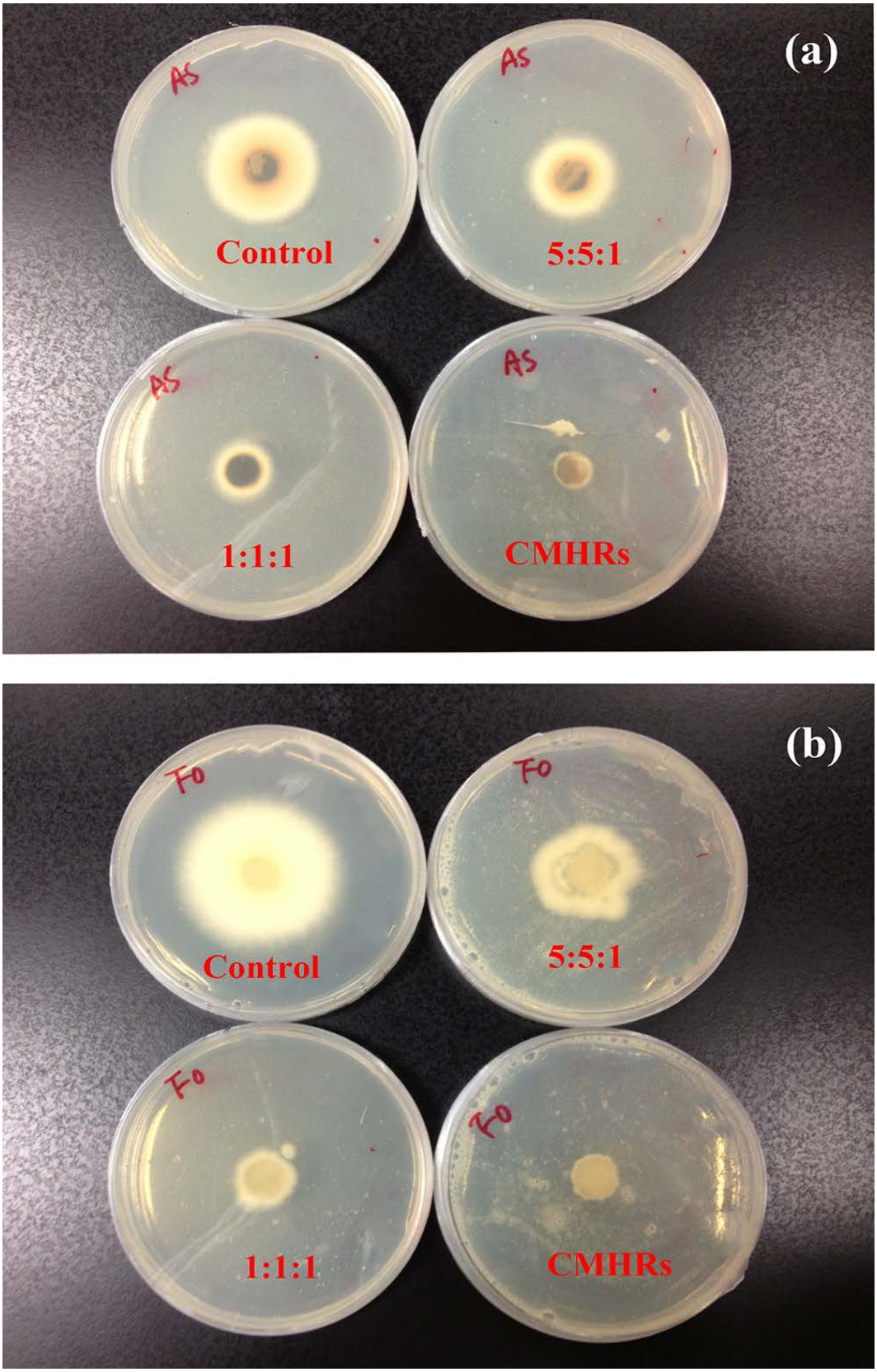


Figure 5-6. Antipathogenic effects of mature composts (Day 56) in various treatments (control, 5:5:1 and 1:1:1), comparing with pure CMHRs extraction.

### 5.3.4 Antagonistic mechanism

Isolated bacteria or fungi were inoculated in the center of plates and *A. solani* or *F. oxysporum* were placed in the same line with 2 cm distance in between, in order to find out the mutualistic or competitive relations between them (Fig. 5-7). For the control treatment without CMHRs only 17% of the isolated bacteria had inhibition on *A. solani* inhibition while no significantly influenced on *F. oxysporum* was noted. About 17% and 20% of isolated bacteria and fungi respectively from the treatment with 5:5:1 mixing ratio had impact on *A. solani*. The treatment with 1:1:1 mixing ratio performed better biocontrol results of both phytopathogens, about 30% for *A. solani* and 20% for *F. oxysporum* (Table 5-5). The antagonism results indicated the appearance of antibiotic-like compounds production by some microorganisms (Cuesta et al., 2012). Bacillus, *Streptomyces* and *Pennisetum* spp. had anti-fungal property due to their chitinolytic character and the ability of antifungal proteins production (Cui et al., 2012; Khamna et al., 2009). The former two genera maintained quantitive advantages in treatment 1:1:1 (64% and 21% respectively). Except bacterial properties mentioned above, fungi also had biocontrol mechanism of mycoparasitism on phytopathogenic control (De Boer et al., 1998). As shown in Table 5-4, isolated fungi contributed more to reduce pathogens due to their ability to distribute their mycelia, occupying greater interaction surface. For instance, several recent studies have proved the antagonistic capacity of *Aspergillus niger* on different phytopathogens which took up 56% identified fungi in the treatment 1:1:1 (Danon et al., 2010; Kandhari et al., 2000; Rai and Upadhyay, 2002).

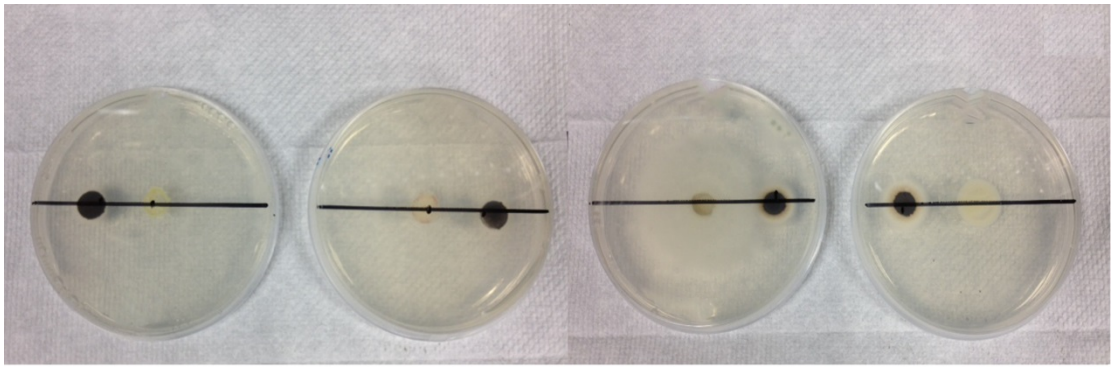

Figure 5-7. Antagonistic activities between isolated microorganisms (center) and selected phytopathogens (left or right).

Table 5-5. Bacteria and fungi quantities, number of isolated colonies, percentage of those with specific activities against *A. solani* and *F. oxysporum.*

| | Control | 5:5:1 | 1:1:1 |
|---|---|---|---|
| *Bacterial mechanisms of each positive antagonist A. solani (% respect total isolated)* | | | |
| Biocontrol | 17 b | 17 b | 29 a |
| Antibiosis | 0 | 0 | 0 |
| Mycoparasitism | 14 c | 17 b | 33 a |
| *Bacterial mechanisms of each positive antagonist F. oxysporum (% respect total isolated)* | | | |
| Biocontrol | 0 b | 0 b | 20 a |
| Antibiosis | 17 c | 33 b | 71 a |
| Mycoparasitism | 14 b | 33 a | 33 a |
| *Fungal mechanisms of each positive antagonist A. solani (% respect total isolated)* | | | |
| Biocontrol | 0 c | 20 b | 33 a |
| Antibiosis | 50 b | 40 c | 67 a |
| Mycoparasitism | 50 c | 80 b | 100 a |
| *Fungal mechanisms of each positive antagonist F. oxysporum (% respect total isolated)* | | | |
| Biocontrol | 0 | 0 | 0 |
| Antibiosis | 25 c | 40 b | 50 a |
| Mycoparasitism | 50 c | 60 b | 75 a |

* Data followed by the same letter indicate no significant difference among the data at $p<0.05$.

Additionally, it is reported that bacteria used as effective biocontrol elimination of plant diseases usually belong to the genera *Pseudomonas*, *Bacillus* and *Streptomyces* and the latter two of which could be positively observed in the treatment 1:1:1 (65% and 21% respectively) (Edwards et al., 1994). Antagonistic situation between isolated microorganisms and *A. solani*/*F. oxysporum* were shown in Fig. 5-8 and 5-9, in which microbial species names can be tracked in Table 5-1 according to numbers marked on petri dishes. Faster grew fungal species such as *Aspergillus* spp. ($6.0*10^6$ CFU/g, 49% in treatment 5:5:1 and $1.3*10^6$ CFU/g, 56% in treatment 1:1:1) could inhibit the growth of lignocellulolytic microbes under the circumstance of absence of CMHRs, resulting in less biocontrol effect in the control treatment. From an overall perspective, significant differences ($p<0.05$) were observed in the treatment 1:1:1, representing the highest percentages of antibiosis mycoparasitism effect on both pathogens, followed by treatment 5:5:1 while the control treatment had least impact. The suppression of pathogens was probably due to the extracellular lytic enzyme production (Bernal-Vicente et al., 2008).

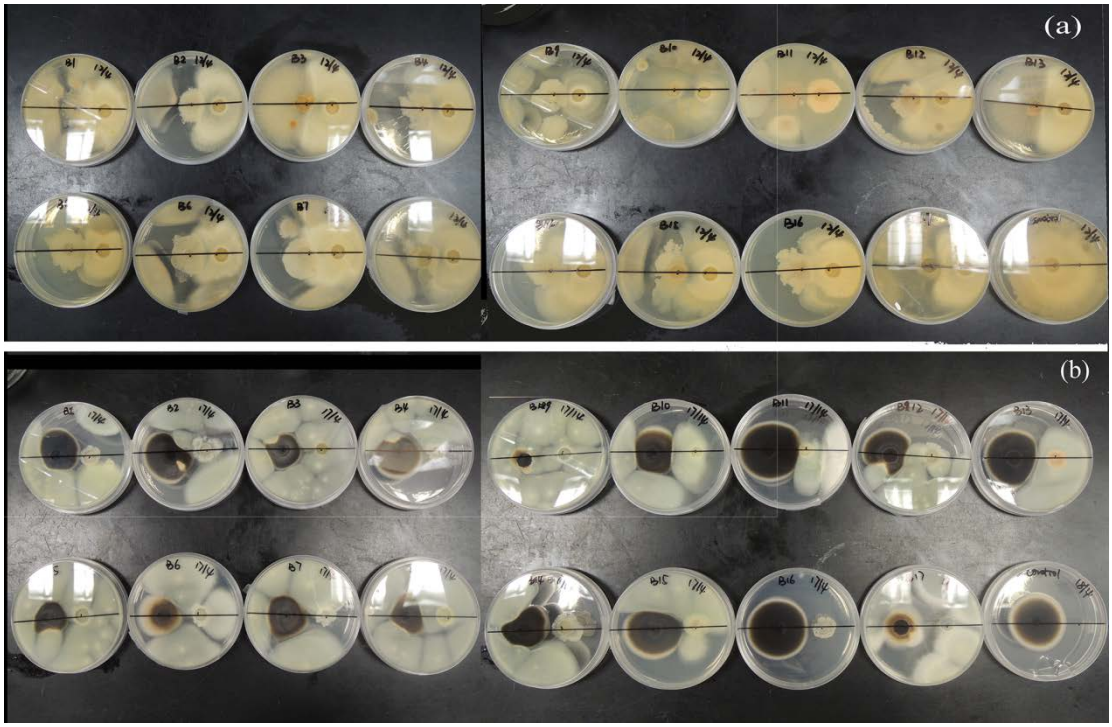


Figure 5-8. Antagonistic results of isolated bacteria (1-17) at various composting phases on *A. solani* (right fungi) (a) and *F. oxysporum* (left fungi) (b).

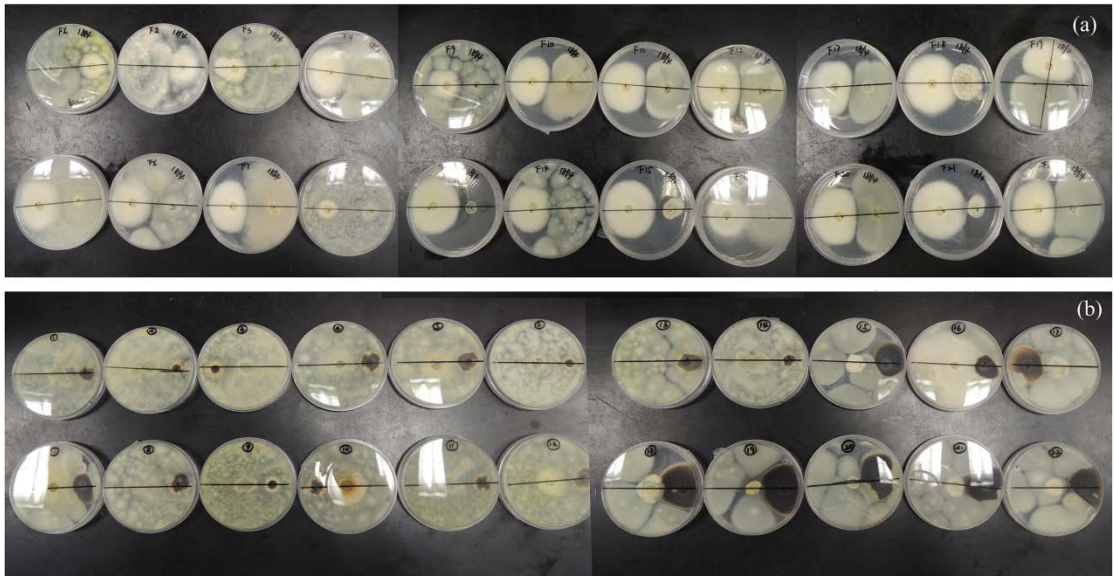


Figure 5-9. Antagonistic results of isolated fungi (1-22) at various composting phases on *A. solani* (left fungi) (a) and *F. oxysporum* (right fungi) (b).

### 5.3.5 Abiotic pathogenic inhibition

After sterilization, the antipathogenic efficiencies of mature composts increased along with the percentage of CMHRs addition and highest suppressiveness was observed in the treatment 1:1:1 (Table 5-4). The treatment 1:1:1 efficiently reduced growth diameters of both phytopathogens in acetone extraction and the inhibition ratios were 71.7% and 40.7% for *A. solani* and *F. oxysporum* respectively. The inhibition rate of both phytopathogens of control treatment was 30.6% while the treatment 5:5:1 achieved higher value, 38.5% for *A. solani* and 32.6% *F. oxysporum* respectively. It is illustrated that some chemical compounds in treatments with CMHRs addition had positive capacities on *A. solani* and *F. oxysporum* as the abiotic factor. Complicated composition in CMHRs as medicinal plants hindered the pathogenic growth, according to the reports of their antimicrobial activities (Silva and Fernandes 2010). The mechanism included disintegration of cytoplasmic membrane and active transport and coagulation of the cell content (Burt, 2004). The $MIC_{50}$ results showed no huge differences between abiotic and antagonistic effect of acetone extracts against the target pathogens, which indicated both factors were powerful to limit the phytopathogen growth (Serra-Wittling et al., 1996).

## 5.4 Conclusions

During composting, both microbial populations and diversities changed dramatically along with variable environmental factor. The universal bacteria and fungi in compost were highly influenced by temperatures with mesophilic and thermophilic levels. More CMHRs contained more microbial suppressive compounds

which inhibited microbial growth, resulting in the treatment 1:1:1 contained least copy number of bacteria (5.2) and fungi (5.4) in mature compost compared to the treatment 5:5:1 (7.1 for bacteria and 6.1 for fungi) and control treatment (7.5 for bacteria and 5.2 for fungi). However, isolated microbes from treatment 1:1:1 had powerful properties of fermenting (*Clostridium* and *Streptomyces*) and degradation on lignin derivates (*Talaromyces*) from CMHRs, promoting composting degradation and humification process.

It is confirmed that mature composts had the suppressive ability against phytopathogens such as *A. solani* and *F. oxysporum*. The treatment 1:1:1 showed most powerful inhibition on phytopathogens due to high concentration of bioactive compounds from CMHRs (abiotic factor) as well as various beneficial bacteria and fungi (*Bacillus, Streptomyces, Pennisetum* spp. and *Aspergillus*) with anti-fungal properties (biotic factor) due to their chitinolytic character and the ability of antifungal proteins production. Faster grew fungal species such as *Aspergillus* spp. shared high population in treatment 1:1:1, which inhibited the growth of lignocellulolytic microbes under the circumstance of absence of CMHRs, resulting in less biocontrol effect in the control treatment. From an overall perspective, significant differences ($p<0.05$) were observed in the treatment 1:1:1, representing the highest percentages of antibiosis mycoparasitism effect on both pathogens, followed by treatment 5:5:1 while the control treatment had least impact. The suppression of pathogens was probably due to the extracellular lytic enzyme production (Bernal-Vicente et al., 2008). However, microorganisms are sensitively influenced by environmental condition such as temperature and moisture, so that bioactive components in mature composts are more dependable as abiotic factor which impacts phytopathogenic control. Therefore, further analysis of bioactive components in

mature composts will be monitored in order to follow the mechanism of phytopathogenic suppression.

# CHAPTER SIX

# IDENTIFICATION AND CHANGES OF BIOACTIVE COMPOUNDS DURING CO-COMPOSTING OF FOOD WASTE AND CHINESE MEDICINAL HERBAL RESIDUES USING UPLC-QTOF-MS

## 6.1 Introduction

Composting is one of the most eco-friendly methods for treating the organic fraction of municipal solid waste. Degradation of the organic substances during composting reduces both the volume of waste and the toxic compounds that are harmful for plant growth after land application (Sundberg et al., 2013). Based on the previous experiments (Chapter 3), CMHRs can be used as a bulking agent in the composting system that improved the compost maturity and humification process. About 40% of the active compounds can be left in CMHRs after the normal decoction, and some of them still have antibiotic effect (Yang et al., 2009). Due to a rich source of bioactive phytochemicals, CHMRs can be converted or used as a source to value-added products. For example, CHMRs could be used to prepare composting with anti-pathogenic properties against plant pathogens. The co-composting of food waste and CMHRs would yield valuable compost with added properties such as antipathogenic effect since CMHRs contain enzyme inhibitors, herbicides, bioinsecticides, etc (Shen et al., 2010). However, hitherto co-composting of CMHRs with food waste has not yet been investigated. The composition of bioactive compounds of CHMRs might change over the course of composting, and thus their property might also change. Therefore, to understand the fate of bioactive compounds

and organic residues from CMHRs, comprehensive analytical studies are urgently required.

The identity and structural information on the bioactive compounds present in CHMRs are essential to elucidate their functional aspects. As the Traditional Chinese medicine comprises of a complicated mixture of different phytochemicals (plant secondary metabolites), the structural elucidation of CHMRs is much more complicated. However, recent developments in advanced analytical tools such as ultra-performance liquid chromatography (UPLC) coupled with mass spectrometry (MS) have become more and more popular for isolation and characterization of known and even unknown compounds in CMHRs or food. Chromatographic fingerprinting as well as quantitative analysis of target components exist in very low concentrations can be performed using UPLC coupled to time-of-flight mass spectrometry (TOF-MS) via selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) (Lacorte and Fernandez-Alba, 2005; Wang and Feng, 2009; Wu et al., 2013; Yang et al., 2011; Zheng et al., 2010). The tandem mass spectrometry is commonly used to obtain the fragments information of isolated chemical structures with high selectivity and sensitivity (Ahn et al., 2008; Dai et al., 2009; Han et al., 2007; Li et al., 2010; Liang et al., 2013; Liu et al., 2011; Shang et al., 2012; Shibano et al., 2007; Zheng et al., 2008).

The aim of this chapter was to elucidate the changes in profiles of bioactive compounds during the co-composting of CHMRs and food waste with different CMHRs mixing ratios. Solvent extracts of the compost samples were monitored by UPLC–Q-TOF/MS coupled with the Agilent MassHunter Work station software for qualitative analysis. The dominant bioactive components in different composting

phases were also identified by matching the mass spectra with the TCM LC-MS library.

## 6.2 Material and method

### 6.2.1 Composts and treatments

The CMHRs were collected from the School of Chinese Medicine, Hong Kong Baptist University. The daily collection lasted for about one month to minimize species variation of CMHRs samples. A synthetic food waste was artificially prepared using boiled rice, bread, cabbage and boiled pork (13:10:10:5, w/w fresh weight basis), as reported previously (Wong et al., 2009). The food waste, sawdust and CMHRs were mixed in the ratio of 5:5:1 and 1:1:1 (dry weight basis, w/w), while food waste and sawdust mixed at 1:1 (dry weight basis, w/w) served as the control to reveal the influence of CMHRs on the humification. The initial composting conditions and properties of starting materials were described in detail in Chapter 3. Compost samples (200 g) collected on Day 0, 7, 28 and 56 were used to monitor the dynamics of active compounds.

### 6.2.2 Antipathogenic test

The phytopathogens, *Alternaria solani* and *Fusarium oxysporum* were purchased from the Centraalbureau voor Schimmelcultures, Netherland. CMHRs and composts (30 g fresh samples) were extracted by using distilled water with solid: extractant ratio of 1:10 (w/v, dry weight basis) to prepare original extractants with the

concentration of 1 g/ml. Extractants were diluted to various concentrations and mixed with 20 ml PDA to obtain culture matrix of 1 ppm, 10 ppm, 100 ppm and 500 ppm (0.1%, 1%, 10% and 50%). The acetone extractions were dried by inert gases and redissolved to the same concentrations before mixed with 20 ml PDA. A. *solani* and *F. oxysporum* cakes (diameter of 1 cm) were inoculated in the middle of the plates and reverse cultivation for 9 days at 28 $^{o}$C. Distilled water of same amount was used for the blank test. The diameters of pathogenic colony sizes were measured by using straight cross method daily (Bernal-Vicente et al., 2008). The inhibition rate was calculated by formula below and the $MIC_{50}$ results were obtained from plotting by growth inhibition diameters. Each test was carried out in triplicates to allow statistical comparisons between treatments. Abiotic tests followed the same way but the samples were filtered through 0.2 μm membrane filters in order to eliminate the microorganisms.

$$\text{Inhibition rate (\%)} = \frac{\text{Colony diameter of control} - \text{Colony diameter of treatment}}{\text{Colony diameter of control}} \times 100$$

### 6.2.3 Chemicals and reagents

HPLC grade acetonitrile was purchased from E-Merck (Darmstadt, Germany). HPLC grade formic acid (purity 96%), acetone (purity 99.5%) and 80% ethanol were purchased from Tedia (USA). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Standard compounds were accurately weighed (36.68 mg and 20 mg for berberine and osthole, respectively) and dissolved in a mixture acetone and 80% ethanol at 10:1 (v/v).

### 6.2.4 Sample preparation for UPLC-QTOF-MS test

Compost samples were oven dried at 55 °C to constant weight and 1 g sample was accurately weighed and transferred to clean glass bottles/Erlenmeyer flasks. Then extraction was performed using different solvents such as acetone and 80% ethanol with solid: solvent ratio of 1:50 (w/v). Samples were sonicated for 1 h in an ultrasonicator (Branson, USA) and then centrifuged at 25,931 x *g* for 15 min; the supernatant was filtered through 0.45 μm membrane filters before HPLC injection.

### 6.2.5 UPLC-TOF-MS analysis

UPLC–Q-TOF/MS analysis was performed on an UPLC coupled to an Agilent 6540 ultra-high definition accurate mass quadrupole time-of-flight spectrometer (UPLC–Q-TOF/MS, Agilent Technologies, USA). Separation was obtained by gradient elution on a C18 analytical column (100 mm × 2.1 mm, 1.7 μm, ACQUITY UPLC® BEH, Waters, USA) preceded by a C18 pre-column (5 mm × 2.1 mm, 1.7 μm, Van Guard™ BEH, Waters, USA) at room temperature (20 $^{o}$C). The mobile phase was (A) water and (B) acetonitrile both containing 0.1% (v/v) formic acid and delivered at 0.35 mL/min according to the following linear gradient: 0-5 min, 5-15% B; 5-20 min, 15-100% B; isocratic 100% B for 5 min then back to 5% B within 5 min. The injection volume was 5 μL. Mass spectra were acquired by full scan from m/z 100-1000. Both positive and negative ion modes were analyzed with the following operating conditions: dry gas ($N_2$) temperature 300 $^{o}$C, dry gas flow rate 7 L/min; nebulizer pressure 45 psi; Vcap 4000; fragment or voltage 140 V.

### 6.2.6 Statistical analysis

Analyses were performed using triplicate samples and the mean values and standard deviations on dry weight basis were presented. Peak assignment was performed using database of TCM Library, which was provided by the School of Chinese Medicine, Hong Kong Baptist University. Data analysis was performed using Agilent MassHunter Work station software Qualitative Analysis (version B.04.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc., 2011). The data correlation analysis were processed using SigmaPlot 11.0 and IBM SPSS statistics v19 while the significance of the differences were tested using Duncan multiple rage test at $p<0.05$.

## 6.3 Results and discussion

### 6.3.1 Optimization of the sample preparation method and UPLC-TOF-MS conditions

Ultrasonication is often considered as widely used method to extract compounds of interest in TCM analysis (Han et al., 2007; Liu et al., 2011; Yang et al., 2011; Zheng et al., 2010). The advantages of ultrasonication include maintaining integrality of target compounds from ionization, hydrolysis and oxidation during extraction as well as high efficiency of the extraction and consumption of solvent.

During extraction process, acetone or 80% ethanol solutions are often used as solvents for high extraction efficiency. The extraction method was evaluated by monitoring the extraction efficiency based on two compounds commonly found in

TCM, berberine (alkaloid) and osthole (coumarin). The results given in Fig. 6-1 indicated that acetone exhibited more efficient extraction than 80% ethanol in the treatment with the highest CMHRs application rate, allowing efficient extraction of all target analytes with high yields. The mechanism follows the similar solubility principle so that the non-polar property of acetone matches the properties of the natural compounds in Chinese medicine (Killedar and More, 2012; Ncube et al., 2008; Yang et al., 2011).

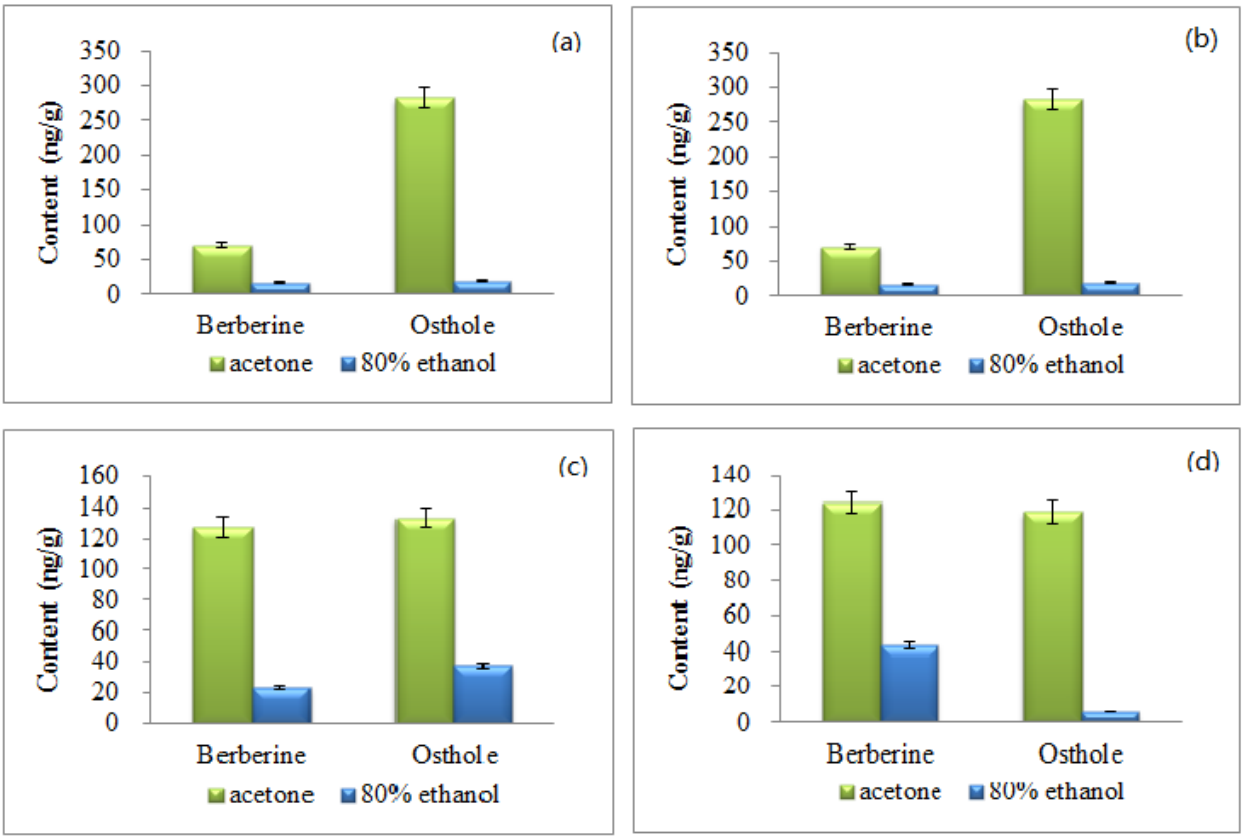


Figure 6-1. The optimization of extraction solvents for berberine and osthole in the treatment 1:1:1 (FW: SD: CMHRs): (a) D0, (b) D7, (c) D28 and (d) D56 (Data of the contents are expressed as mean ±S.D. (n=3).).

Optimized chromatographic conditions were achieved after several trials with various gradient elutions and both ionization modes with better resolution of adjacent peaks within a short time. The gradients of mobile phases and positive mode ionization were determined referred to several references and preliminary results of total ion chromatography (TIC) (Lin and Harnly, 2009; Shang et al., 2012; Wang and Feng, 2009). It was shown that the addition of 0.1% of formic acid promoted the ionization efficiency under $ESI^+$ mode and achieved the higher sensitivity, less interferences and best resolution especially to complex compositions of herbal products (Yang et al., 2009; Zheng et al., 2010).

### 6.3.2 Pathogenic suppression capability of composts

According to previous results (Chapter 5), the mature compost in the treatment with dry weight ratio of 1:1:1 (Day 56) showed the strongest prohibitive effect on *A. solani* and *F. oxysporum*. At this ratio, the growth inhibitions were 75.8% and 59.0% for *A. solani* and *F. oxysporum* respectively, comparing to 36.4% and 35.4% for treatment 5:5:1 and 34.8% and 35.4% for the control. This inhibition was even close to the anti-pathogenic effect of the pure CMHRs extracts (75.6% for *A. solani* and 54.3% for *F. oxysporum*). The reasons might be due to the anti-pathogenic compounds and extracellular lytic enzymes of CMHRs (Bernal-Vicente et al., 2008). It is also indicated that with the exclusion of antagonistic interference, the fungal inhibitory abilities of bioactive components increased along with an increase in the percentage of CMHRs addition. The treatment with 1:1:1 ratio efficiently reduced fungal growth by 71.7% and 40.7% for *A. solani* and *F. oxysporum* respectively. The inhibition rates of treatment 5:5:1 were 38.5% and 32.6% on *A. solani* and *F. oxysporum* respectively

while the control treatment obtained only 30.6% for both pathogens. This result is consistent with the observation from the research of Liu et al. (2012). The higher amount of CMHRs in the composts represented more active components which showed a wider spectrum of activities against phytopathogens since multiple varieties of herbs enhanced the inhibition effects on pathogens. According to the report of Yang et al. (2009), the prevalent antifungal bioactive constituents in TCM were phenolic compounds including alkaloids, flavonoids, coumarins, saponins, monoterpene glycosides, diterpenoids, triterpenoids and steroids (2009).

### 6.3.3 Dynamics of bioactive components during composting

Bioactive compounds are fully scanned and identified by using Chinese medicine Library from School of Chinese Medicine in Hong Kong Baptist University. Unlike the GC technique, compounds are not directly identified by matching the patterns of fragments, in which mainly determined by the percentages of the fragment intensity. UPLC-MS technique identify unknown compounds by collecting information of fragments and select characteristic peaks with automatic calculation of dimer, trimer, $Na^+$, $K^+$, $OH^-$ etc in positive and negative pattern respectively (Liu et al., 2009). From data processing, the retention time, formula, parent, fragment ion masses, taxonomy and antibacterial/antifungal capacities of 39 bioactive compounds in total were identified by cross comparison with Traditional Chinese Medicine Library. As shown in Table 6-1 and 6-2, seventeen components were identified in all treatments (from food waste source) while another 22 bioactive compounds were only observed in treatments with CMHRs (from CMHRs source) during composting.

Table 6-1. Bioactive compounds in control as determined using UPLC–Q-TOF/MS.

| Peak | Rt (min) | Identification | Formula |
|---|---|---|---|
| 1 | 7.757 | Angeliticin A | $C_{20}H_{22}O_7$ |
| 2 | 7.757 | Chasmanthin | $C_{20}H_{22}O_7$ |
| 3 | 8.811 | Fawcettiine | $C_{18}H_{29}NO_3$ |
| 4 | 9.203 | 2,3,4-Trimethyl-5-phenyloxazolidine | $C_{12}H_{17}NO$ |
| 5 | 10.038 | Sanleng acid | $C_{18}H_{34}O_5$ |
| 6 | 11.428 | Gossypetin hexamethyl ether | $C_{21}H_{22}O_8$ |
| 7 | 12.121 | Isosinensetin | $C_{20}H_{20}O_7$ |
| 8 | 13.227 | Iso-mucromatol | $C_{18}H_{18}O_5$ |
| 9 | 13.227 | 4,7-Dihydroxy-5-methoxyl-6-methyl-8-formyl-flavan | $C_{18}H_{18}O_5$ |
| 10 | 13.349 | 3-O-cis-p-Coumaroyl alphitolic acid | $C_{40}H_{56}O_6$ |
| 11 | 13.377 | Albopetasin | $C_{20}H_{28}O_3$ |
| 12 | 14.263 | alpha-Eleostearic acid | $C_{18}H_{30}O_2$ |
| 13 | 14.263 | Chrysanthenone | $C_{10}H_{14}O$ |
| 14 | 14.263 | Tetrahydrobungeanool | $C_{18}H_{33}NO_2$ |
| 15 | 14.606 | Isopetasin | $C_{20}H_{28}O_3$ |
| 16 | 15.007 | Abietic acid | $C_{20}H_{30}O_2$ |
| 17 | 15.402 | Cyclotetradecan-1-one | $C_{14}H_{26}O$ |

Table 6-2. Bioactive components found only in the treatments with CMHRs addition as determined using UPLC–Q-TOF/MS.

| Peak | $t_R$ (min) | Identification | Formula |
|---|---|---|---|
| 18 | 7.642 | Mesuein | $C_{28}$ $H_{34}$ $O_{15}$ |
| 19 | 7.642 | Hesperidin | $C_{28}$ $H_{34}$ $O_{15}$ |
| 20 | 8.484 | 5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarin*# | $C_{16}$ $H_{18}$ $O_5$ |
| 21 | 8.484 | 7-Methoxy-8-(1'-methoxy-2'-hydroxy-3?methyl-3'-butenyl) coumarin*# | $C_{16}$ $H_{18}$ $O_5$ |
| 22 | 11.416 | Nobiletin | $C_{21}$ $H_{22}O_8$ |
| 23 | 11.416 | Cimicifugc acid* | $C_{20}$ $H_{20}$ $O_7$ |
| 24 | 12.103 | (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroses elin*# | $C_{21}$ $H_{22}$ $O_8$ |
| 25 | 12.103 | 5,7,2',4',6'-Pentamethoxyflavone | $C_{20}$ $H_{20}$ $O_7$ |
| 26 | 12.111 | Chimaphylin* | $C_{12}$ $H_{10}$ $O_2$ |
| 27 | 13.393 | Glutimic acid# | $C_{20}$ $H_{34}$ $O_4$ |
| 28 | 13.661 | Dihydrokaranone*# | $C_{15}$ $H_{22}$ O |
| 29 | 13.528 | Oxyberberine* | $C_{20}$ $H_{17}$ N $O_5$ |
| 30 | 13.528 | Oxoglaucin | $C_{20}$ $H_{17}$ N $O_5$ |
| 31 | 14.099 | (2R,3R)-2,3-Dihydro-2-(4-hydroxyphenyl)-5-methoxy-3-m ethyl-7-propenylbenzofuran | $C_{19}$ $H_{20}$ $O_3$ |
| 32 | 14.099 | Parakmerin A | $C_{19}$ $H_{20}$ $O_3$ |
| 33 | 14.228 | Cumic alcohol | $C_{10}$ $H_{14}$ O |
| 34 | 14.229 | Linolenic acid*# | $C_{18}$ $H_{30}$ $O_2$ |
| 35 | 15.427 | Ichthyotherol | $C_{12}$ $H_{14}$ $O_2$ |
| 36 | 15.427 | Lachnophyllol acetate | $C_{12}$ $H_{14}$ $O_2$ |
| 37 | 15.488 | Isotanshinone II# | $C_{19}$ $H_{18}$ $O_3$ |
| 38 | 15.500 | Tanshinone IIA*# | $C_{19}$ $H_{18}$ $O_3$ |
| 39 | 17.202 | Muscone* | $C_{16}$ $H_{30}$ O |

* Compounds increased in treatment 5:5:1 during composting; # Compounds with araised contents in treatment 1:1:1 (FW:SD:CMHRs, dry wt. basis).

Microorganisms in the composting mass are highly influenced by complicated environmental factors such as temperature, moisture and pH. In contrast, the bioactive components with antipathogenic properties present in composts are the key factor determining the survival of phytopathogens since most of the compounds are not readily decomposed and could inhibit soil borne phytopathogens with their functions such as producing phytoalexin and inducing apoptosis of pathogens (Zhang et al., 2011). The dynamics of these compounds can be deduced from the comparison of the obtained simultaneous extractable compounds chromatograms (ECC) of the various composts in different composting phases as shown in Fig. 6-2 - 6-4.

Seventeen kinds of bioactive components were identified to be dominant in all treatments during composting (Table 6-1) and eleven of them had the antibacterial/antifungal abilities (Ammar et al., 2013; Bandara et al., 1990; Hegedus and Marx, 2013; Kocsis et al., 2009; Xu et al., 2009; Zabka and Pavela, 2013; Zhang et al., 2014). These compounds should be derived from food waste, for instance, plant materials from cabbage, and the quantities were fluctuated by the intermediate products of organic degradation during composting (Kosseva, 2013). The dynamics of these compounds were similar, decreasing dramatically in the first week and increased slightly before declining again. The reason was that most of these compounds are pyrolytic and easily degradable under high temperature and microbial activities during thermophilic phase of composting. The slight increments on Day 7 could be due to the accumulation from the decomposition of readily available components. The bioactive components declined again gradually afterwards due to the nutritive utilization by huge amount of microbes (Wang et al., 2009). Among all bioactive compounds in control treatment, only contents of Fawcettiine, 2,3,4-Trimethyl-5-phenyloxazolidine, Gossypetin hexamethyl ether and Isosinensetin increased at the end of composting.

With CMHRs addition, only the former two compounds (Fawcettiine and 2,3,4-Trimethyl-5-phenyloxazolidine) were observed with increasing amounts. Most bioactive components with antibacterial/antifungal property identified were the major constituents of ketons (Angeliticin A, Chrysanthenone, Cyclotetradecan-1-one), esters (Chasmanthin), ethers (Gossypetin hexamethyl ether), terpenoids (Isopetasin), alkaloids (Tetrahydrobungeanool), flavonoids (4,7-Dihydroxy-5-methoxyl-6-methyl-8-formyl-flavan) and some acids (Sanleng acid, alpha-Eleostearic acid and Abietic acid). The contents of Fawcettiine, 2,3,4-Trimethyl-5-phenyloxazolidine, Gossypetin hexamethyl ether and Isosinensetin accumulates more than initial amounts in control treatment at the end of composting, while the former two compounds were found to be araised in treatment 5:5:1 and only 2,3,4-Trimethyl-5-phenyloxazolidine increased in 1:1:1 (FW:SD:CMHRs, dry wt. basis). However, Fawcettiine and 2,3,4-Trimethyl-5-phenyloxazolidine had no antipathogenic properties which were belonged to alkaloids and oxazolidine respectively.

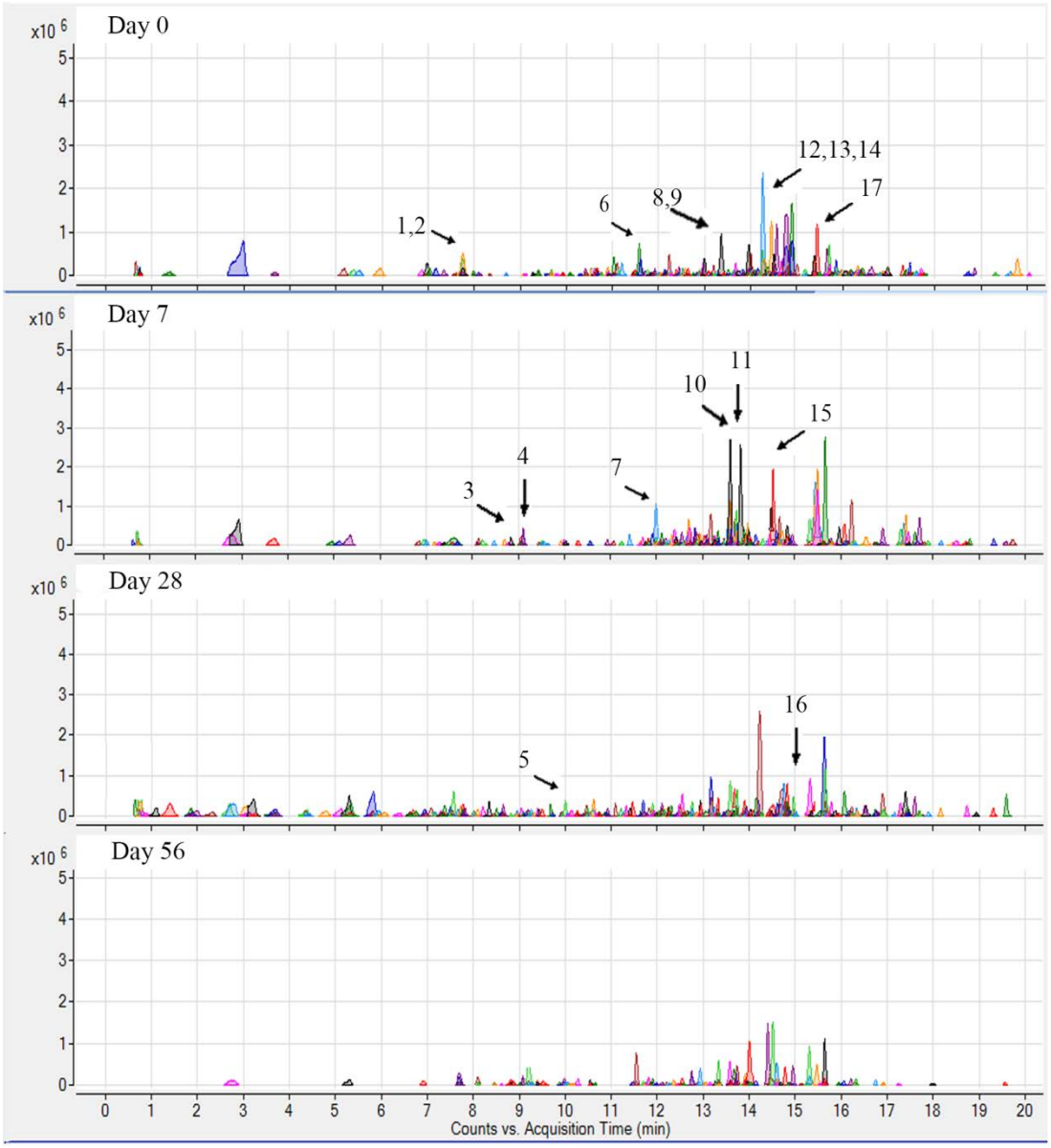


Figure 6-2. Extractable compounds chromatograms (ECC) of the control treatment with seventeen bioactive components identified in all treatments during composting process as listed in Table 6-1 (1-17) ESI (+) TOF-MS with peaks in random colors.

Additional evidence regarding more interesting bioactive components that have antibiotic or antiviral effects were found in the CMHRs treatments with addition (Table 6-2); though not all the detailed structural fragmentation or metabolic pathway of herbal bioactivities were perfectly known since the compositions of TCM are so complicated. Many specific compounds have been characterized from the crude extracts for structural determination of TCM by researchers previously; however, the dynamics of the bioactive components during composting process is still lacking in the relative research field. Rapid screening and structural characterization by UPLC-TOF-MS technique showed that up to 22 different bioactive components were obtained in the treatments 1:1:1 and 5:5:1 only with varying quantities. Most of the compounds were unstable under high temperature, resulting in dramatic reduction in contents within the first week while the formation or transformation of complicated components and the microbial utilization of those compounds contributed to the subsequent slight increment and gradual decline of these bioactive components (Meng et al., 2014; Wang et al., 2010). Amounts of 10 compounds (marked with * in Table 6-2) in treatment 5:5:1 increased during degradation while only 8 components (marked with # in Table 6-2) arised in treatment 1:1:1. It was shown that more bioactive compounds with increasing content observed in treatments with CMHRs addition, which indicated chemical components in CMHRs were more complicated during biodegradation. With the decomposition of compounds with complicated structures into simpler molecules, quantities of specific compounds may have a possibility to be enhanced. Most bioactive components present in the roots and rhizomes of plant materials exhibit pharmacological actions (Yang et al., 2009). In the treatment 5:5:1, seven kinds of bioactive components with higher quantities were observed that include (3'R, 4’R)-3'-epoxyangeloyloxy-4'-acetoxy-3',

4’-dihydroseselin, linolenic acid, nobiletin, cimicifugic acid, 5,7,2',4',6'-pentamethoxyflavone, tanshinone IIA and 7-methoxy-8-(1'-methoxy-2'-hydroxy-3-methyl-3'-butenyl) coumarins (Fig. 6-5a). The contents of these compounds were higher in the treatment 1:1:1; and (3'R, 4’R)-3'-epoxyangeloyloxy-4'-acetoxy-3', 4’-dihydroseselin played the leading role among all the bioactive components. The aqueous stability of coumarin dimers could be the reason providing higher availability of this compound (Dragojevic et al., 2011).

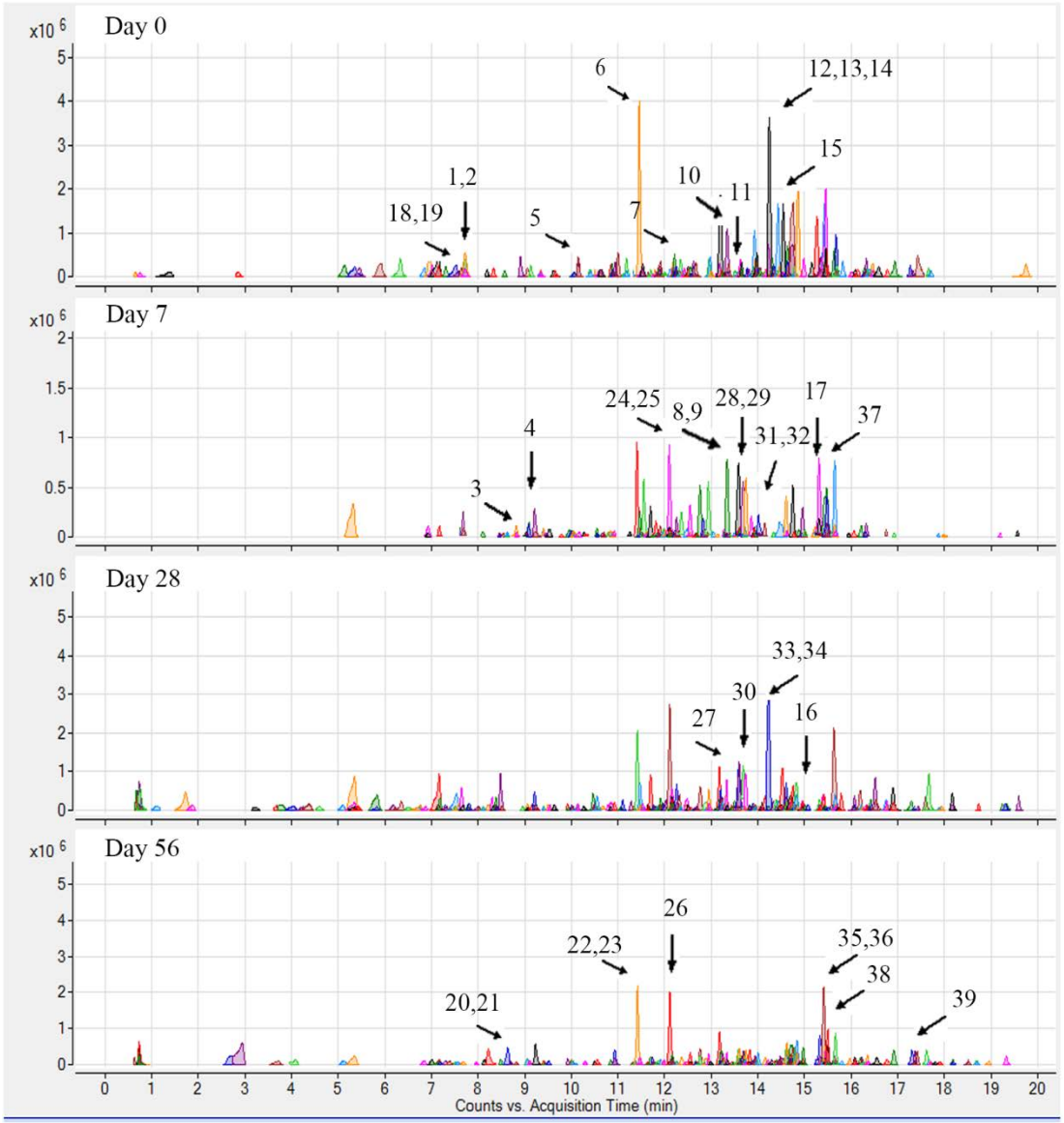


Figure 6-3. Extractable compounds chromatograms (ECC) of the treatment 5:5:1 with 22 bioactive components identified in treatments with CMHRs addition during composting process as listed in Table 6-2 (1-22) ESI (+) TOF-MS with peaks in random colors.

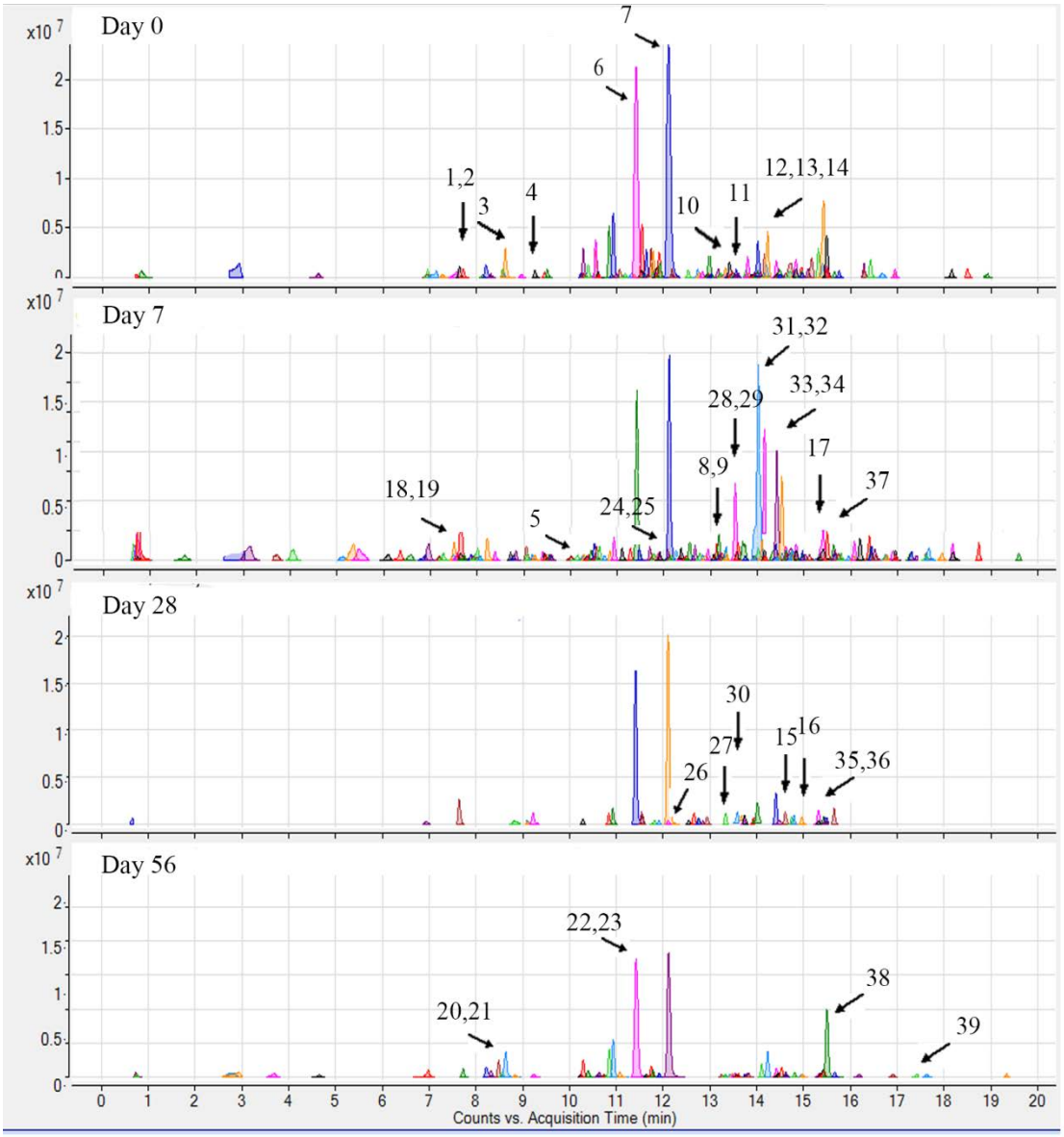


Figure 6-4. Extractable compounds chromatograms (ECC) of the treatment 1:1:1 with 22 bioactive components identified in treatments with CMHRs addition during composting process as listed in Table 6-2 (1-22) ESI (+) TOF-MS with peaks in random colors.

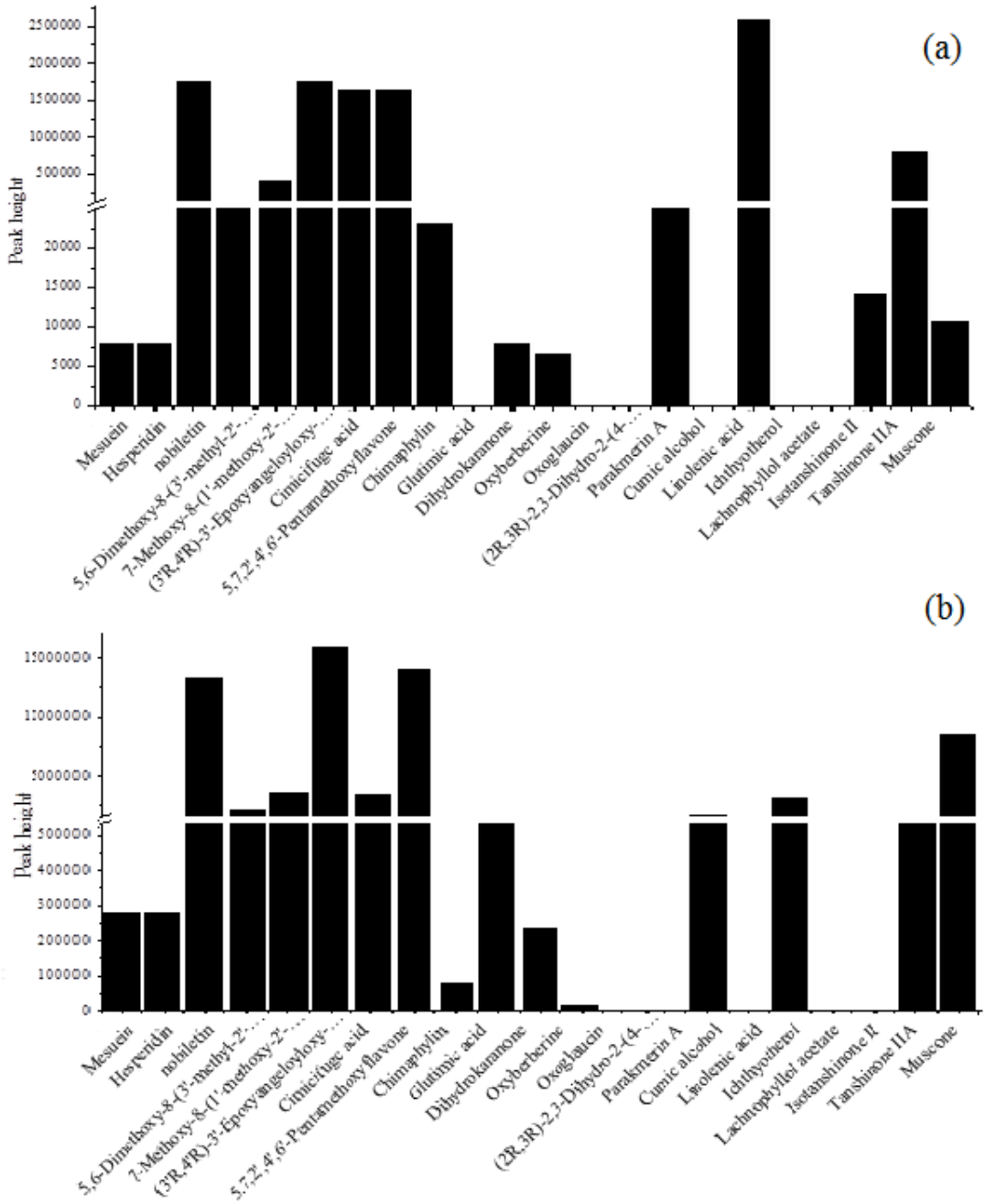


Figure 6-5. Bioactive components available in the compost treatment 5:5:1 (a) and 1:1:1 (b) after 56 days of composting.

### 6.3.4 Correlation between identified bioactive components and phytopathogenic effect during composting

As shown in Table 6-3, there are 21 identified bioactive components positively and significantly correlated with inhibition of *A. solani* / *F. oxysporum*. All of them have antipathogenic abilities except 2,3,4-Trimethyl-5-phenyloxazolidine. These results imply that treatments with CMHRs (5:5:1 and 1:1:1, dry weight ratio) showed potentially significant phytopathogenic control and functional compounds were mainly belonged to alkaloids, flavonoids, coumarins, ketones and acids.

Table 6-3. Pearson’s correlations between the phytopathogens and bioactive components identified from compost samples.

| | Family | Antifungal effect | *A. solani* | | *F. oxysporum* | |
|---|---|---|---|---|---|---|
| | | | * (p<0.05) | ** (p<0.01) | * (p<0.05) | ** (p<0.01) |
| Tetrahydrobungeanool | Alkaloids | Y | | √ | √ | |
| Oxyberberine | Alkaloids | Y | - | | | √ |
| Mesuein | Flavonoids | Y | √ | | - | |
| Hesperidin | Flavonoids | Y | √ | | - | |
| Nobiletin | Flavonoids | Y | √ | | - | |
| 5,7,2',4',6'-Pentamethoxyflavone | Flavonoids | Y | | √ | - | |
| Isotanshinone II | Flavonoids | Y | | √ | | √ |
| Tanshinone IIA | Flavonoids | Y | | √ | | √ |
| (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroseselin | Coumarins | Y | - | | - | |
| 5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarin | Coumarins | Y | | √ | √ | |
| 7-Methoxy-8-(1'-methoxy-2'-hydroxy-3-methyl-3'-butenyl)coumarin | Coumarins | Y | | √ | - | |
| 2,3,4-Trimethyl-5-phenyloxazolidine | Alkanes | N | - | | | √ |
| Chasmanthin | Ester | Y | √ | | √ | |
| Gossypetin hexamethyl ether | Ether | Y | - | | | √ |
| Linolenic acid | Acid | Y | √ | | √ | |
| Cimicifugc acid | Phenolic acid | Y | | √ | - | |
| Glutimic acid | Protein | Y | √ | | | √ |
| Parakmerin A | Lignan | Y | | √ | | √ |
| Angeliticin A | Ketones | Y | - | | | √ |
| Chrysanthenone | Ketones | Y | - | | √ | |
| Dihydrokaranone | Ketones | Y | | √ | | √ |

“-” means none of available.

### 6.3.5 Properties of dominant chemical profiling in composting process

As shown in Table 6-3, bioactive compounds positively influenced the antipathogenic effect was mainly from alkaloids, flavonoids, coumarins and ketones. These compounds are known to have fungal inhibition capabilities (Gao and Li, 2009; Han et al., 2007; Zheng et al., 2008; Zheng et al., 2010).

#### 6.3.5.1 Alkaloids

Tetrahydrobungeanool were identified in all treatments while oxyberberine was observed only in CMHRs compost. After composting for eight weeks, 4% of tetrahydrobungeanool remained in control and 2% in CMHRs composts. In case of oxyberberine about 2% of oxyberberine was left in both treatments with CMHRs addition. Alkaloids can be widely found in vegetables and TCM which are inhibitory to fungal and bacterial pathogens and induce apoptosis by the mechanisms resulting from their ability to interact with phytopathogenic proteins and DNA (Hu et al., 2014).

#### 6.3.5.2 Flavonoids

Flavonoids are the mostly common compounds with significant antimicrobial activities, especially against *Aspergillus*. Over 15,000 flavonoids have been separated and identified from plants (Cushnie and Lamb, 2005; Han et al., 2007; Xiao et al., 2014). Six kinds of flavonoids with high antimicrobial potential abilities were identified in treatments with CMHRs, including mesuein, hesperidin, nobiletin,

5,7,2',4',6'-pentamethoxyflavone, isotanshinone II, tanshinone IIA.

Mesuein is a flavanone glycoside from *Mesua ferrea* with antifungal properties (Alam et al., 1987). Mesuein is relatively unstable since only 4% and 27% left in treatment 5:5:1 and 1:1:1 respectively after composting due to its photolytic properties. The peel of citrus fruits is rich in flavanones and many polymethoxylated flavones (PMFs) with pharmacological potential. Ortuño et al. (2006) and Ho and Kuo (2014) reported that hesperidin (4% and 27% left in treatment 5:5:1 and 1:1:1, respectively) and nobiletin (37% and 83% left in treatment 5:5:1 and 1:1:1, respectively) was the most effective compound with fungal inhibition property which can be found in all varieties of *Citus sinensis*. Nobiletin and 5,7,2',4',6'-pentamethoxyflavone (15% and 79% left in treatment 5:5:1 and 1:1:1, respectively) are considered as phytoalexins in phenolic group of some *Citrus* species (Ortuño et al., 2006). The antifungal effect of these compounds can be explained by permeability change of cytomembrane and cell wall fragility induced by PMFs inhibiting chitin synthase. Tanshinone IIA belongs to the group of lipophilic diterpenoid tanshinones. It was reported to possess pronounced antibacterial and antifungal properties which hold potential applications in pharmaceutical and medicinal industry (Zhao et al., 2011). Tanshinones are present in the root of danshen (*Salvia miltiorrhiza*) and yinxing (*Ginkgo biloba*) with pharmacological actions of anti-inflammation and antioxidation (Yang et al., 2009). Additionally, they are sensitive to light and can be photosynthesized (Cushnie and Lamb, 2005), resulting in 15 to 56 folds of increase in treatments of 5:5:1 and 1:1:1 respectively.

#### 6.3.5.3 Coumarins

Coumarins are secondary metabolites of plants and widely distributed in the in families of Umbelliferae, Rutaceae, Leguminosae and Compositae. They are well known due to their broad-spectrum antibacterial and antifungal properties (Murray et al., 1991; Stein et al., 2006). The amount of 7-methoxy-8-(1'-methoxy-2'-hydroxy-3-methyl-3'-butenyl) coumarin and 5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarin increased during composting since coumarins were metabolized by microorganisms. The interaction between coumarins and microbes has been proved and the metabolic transformation could be proceeded under the microbial activities. For example, *Mucor spinosus* is able to transform coumarone via hydroxylation, dehydrogenation, demethylation and glycosylation reactions. Better water solubility and bioactivities of derivatives of coumarins indeed provide significant potential to the bacterial/fungal control (Li and Chan, 2013). In addition to the light-dependent mechanism of fungal control, coumarins are inhibitors of the ATP hydrolysis and DNA supercoiling reactions catalyzed by DNA gyrase. Therefore, ATP hydrolysis of phytopathogen is blocked at the Gyrase B subunit (Sardari et al., 2000).

As shown in Fig. 6-5b, the pyranocoumarin, (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroseselin was observed in the highest quantity (120% of initial content) at the end of composting in treatment 1:1:1. This compound could be isolated from the dry root of *Peucedanum praeruptorum* (Apiaceae) (Wu et al., 2003), which was one of the widely used plant material in the TCM for the cure of cough and upper respiratory infections for decades in China (Song et al., 2013). It was also reported by Chen et al. (2002) that the extracts of *Peucedanum praeruptorum* had antifungal capacity and (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroseselin was one of the major

constituents with pharmacological properties (Song et al., 2013). It was reported *A. niger* and *A. fumigatus* were found to be most resistant (MIC 0.625 mg/ml) to pyranocoumarin. This result is in agreement with the results presented in Section 5.3.2.1 (Table 5-1) that *A. niger* and *A. fumigatus* were observed in the treatments with CMHRs addition. The antifungal mechanism of the pyranocoumarin may be due to the α-methylene-γ-lactone moiety present in the side chain and conduce implication in bioactivity in the sesquiterpene lactones (Kumar et al., 2012).

#### 6.3.5.4 Ketones

Angeliticin A was completely degraded during composting in all treatments while 2%, 3% and 9% of chrysanthenone remained in control, 5:5:1 and 1:1:1 treatments, respectively. Dihydrokaranone was observed only in CMHRs treatments and contents were reduced by 90% and 70% in treatment 5:5:1 and 1:1:1, respectively. Ketones influence the development of pseudohyphae and induce significant changes in the protein composition of some fungi, resulting in inhibiting their growth (Kocsis et al., 2009).

#### 6.3.5.5 Acids

Fatty acids extracted from plant species are considered as one of the main ingredients in pharmaceutical products. Linolenic acid and cimicifugic acid were widely known as the fatty acid from medicinal plants with antibacterial and antifungal actions (Akbar, et al., 2011; Borrelli et al., 2003). Linolenic acid is commonly found in *Schotia brachypetala* while cimicifugic acid is one of the phenolic components of

the rhizomes/roots of *Cimicifuga* species (He et al., 2006; McGaw, et al., 2002). These compounds alter the membrane fluidity which leads to fungal cell death due to leakage of intracellular components. Meanwhile, fungal protein synthesis is also blocked preventing their reproduction (Oliveira et al., 2014).

## 6.4 Conclusions

This study showed the changes of chemical profiles of the bioactive components during 56 days of co-composting of food waste and CMHR. Compared to the 5:5:1 food waste: sawdust: CHMRs (dry weight basis), the treatment with 1:1:1 mixing ratio exhibited the highest inhibiting effect on the growth of phytopathogens (*A. solani* and *F. oxysporum*), which is likely due to the residual bioactive components in mature compost with effective antibacterial/antifungal functions. Among the 22 kinds of bioactive components with antipathogenic properties found only in the treatments with CMHRs addition, only seven were the dominant species. Of which (3'R, 4’R)-3'-epoxyangeloyloxy-4'-acetoxy-3', 4’-dihydroseselin and linolenic acid were observed in the highest quantities in the mature compost of treatments with 1:1:1 and 5:5:1 mixing ratio, respectively. Although there were 17 kinds of active compounds identified from all the treatments, only 11 of them possessed the suppressive capacities against fungi. Thus, these compounds are believed to be the major active compounds against the two fungal phytopathogens studied in this investigation. This affirms the results obtained from the compost extract antipathogenic properties in Chapter 3. Additional crop growth experiment will be performed to give direct evidence on the antipathogenic capacity of mature composts following their use in land application.

# CHAPTER SEVEN

# ANTI-PATHOGENIC PROPERTIES OF FOOD WASTE-CHINESE MEDICINAL HERBAL RESIDUES CO-COMPOST– GREENHOUSE EXPERIMENT

## 7.1 Introduction

Mature composts derived from sewage sludge, poultry manure and vermicompost have been commonly used as soil conditioners and/or biofertilizers, providing nutrients and better physical properties to soil. The advantages of using compost as biofertilizer include increasing soil pH, preventing soil erosion, promoting beneficial soil microbial activities, reducing the demands for chemical fertilizers and pesticides as well as diverting organic wastes out of landfills (Wei and Liu, 2005). Decomposed organic substances in mature compost slowly release nutrients which can be absorbed by plants, resulting in improvement of agroecosystem productivity (McLauchlan, 2006). Besides, organic substances in mature compost promote ecological functions such as water and nutrient holding capacity, resistance to compaction and erosion, infiltration and aeration (Whalen et al., 2003). Additionally, one of the most important beneficial advantageous of composts in land application is the suppression of pathogenic microorganisms, enhancing the plant health; and the antipathogenic effect was further improved if Chinese medicinal herbal residue (CMHR) was one of the raw materials in composting (Section 5.3.2.3). As mentioned in Section 5.3.2.1, the mature CMHRs compost is rich in fungal biological control agents (BCAs), which inhibits a wide range of plant pathogens (Verma et al., 2007).

The suppressive mechanism could be derived from both interaction between the phytopathogens and saprophytic microflora exist in the mature compost and the presence of bioactive components with antipathogenic capacities in the mature composts. Both the composition and population of saprophytic microflora are highly dependent on the degree of organic waste degradation (Cotxarrera, et al., 2002). The antagonistic effects involved are antibiosis, mycoparasitism and competition for nutrients. It is indicated that both abiotic and biotic factors play irreplaceable role in the compost-amended suppression to wilt diseases.

Among all soilborne diseases, the early blight and Fusarium wilt caused by *Alternaria solani* and *Fusarium oxysporum* respectively (Fig. 7-1 and 7-2) are the most serious ones in the world (Koné et al., 2010; Park and Oyaizu, 2002). These pathogens spread rapidly on their preferent hosts of tomato and cabbage respectively, causing serious economic loss due to crop damage globally each year. Tomato early blight is a common disease derived caused by *Alternaria solani*, which infects the plant leaves (Fig. 7-1a), stems, petiole, twig and fruits (Fig. 7-1b) under favorable condition, resulting in defoliation, drying off of twigs and premature fruit drop as well as stem lesion. Spores (Fig. 7-1c) of *Alternaria solani* can survive long time in soil and mycelia (Fig. 7-1d) grow under proper environmental condition to infect fruits (Khan et al., 2012; Patel et al., 2011). This disease leads to up to 80% yield losses and 30% increases in fungicide requirement (Grigolli et al., 2011). The Fusarium wilt is a prevalent plant disease which spreads from infected transplants and seeds; sometimes water and planting equipment are also the sources of infection. The plant leaves turn yellow (Fig. 7-2a) and finally damp off with rotten fruits (Fig. 7-2b) since the pathogen infects the roots of plants, blocking water and nutrients from soil. *Fusarium oxysporum* can infect plants by mycelia (Fig. 7-2d) or the penetration of germinating

spores (Fig. 7-2c) into the plant's root tips or wounds (Roncero et al., 2003). An effective fungicide for Fusarium wilt is still unavailable which promotes the requirement of biocontrol treatment (Borrero et al., 2006).

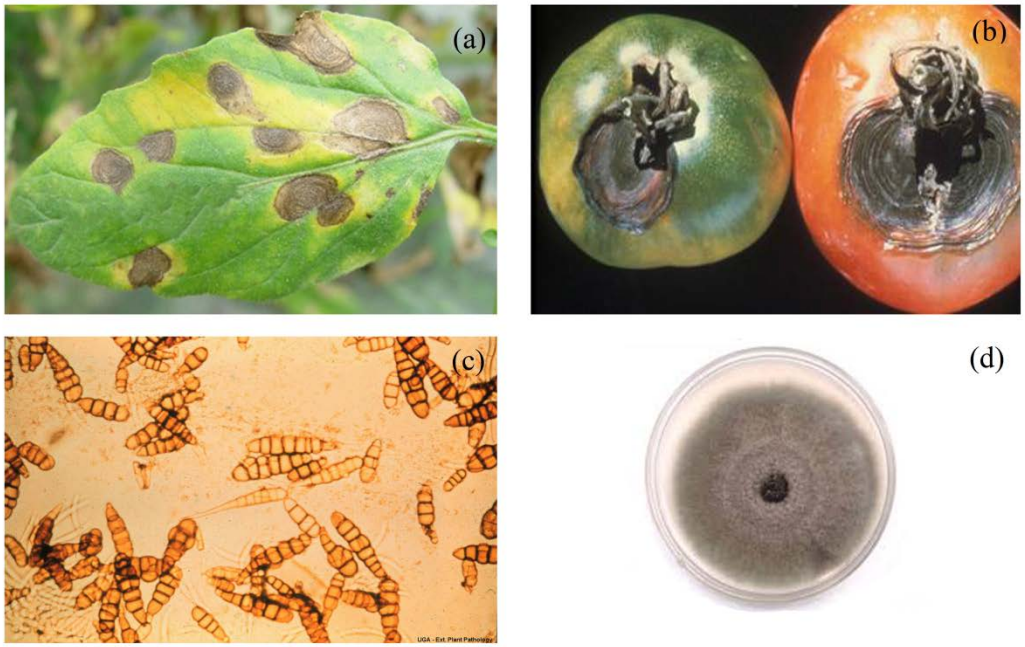


Figure 7-1. Leaves and fruits after infected by *Alternaria solani*. (a) infected leaves, (b) infected cabbage, (c) spores and (d) fungal morpha. (Source: http://www.doctorfungus.org/thefungi/Fusarium_oxysporum.php)

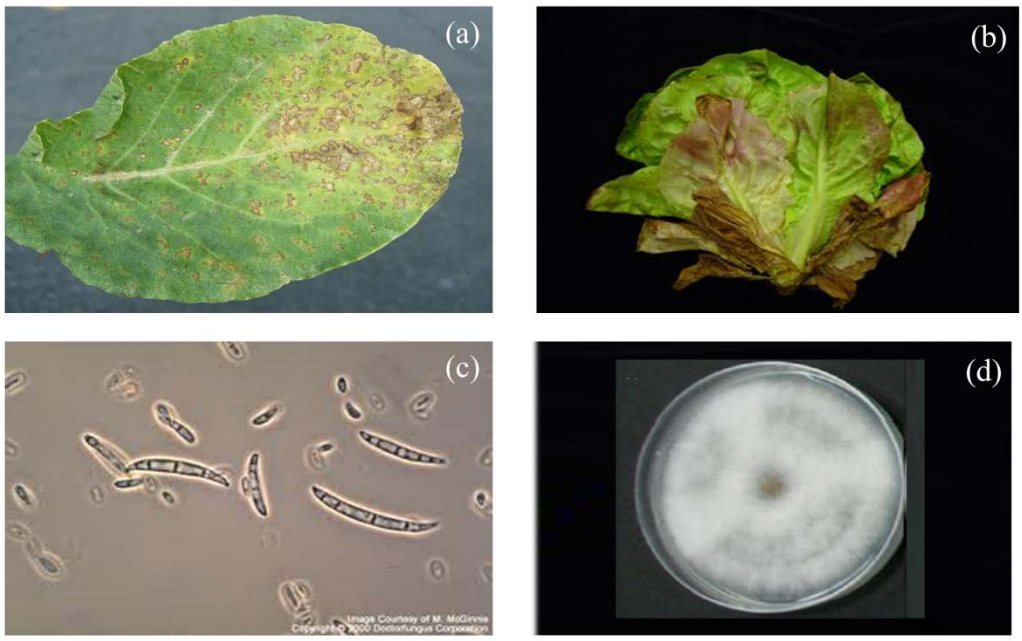


Figure 7-2. Leaves and fruits after infected by *Fusarium oxysporum*. (a) infected leaves, (b) infected tomato, (c) spores and (d) fungal morpha. (Source: http://plantpath.caes.uga.edu/extension/plants/vegetables/tomatoearlyblight.html)

Several studies reported the antipathogenic capacities of mature composts were derived from organic molecules such as humic, phenolic or bioactive compounds (Hoitink et al., 1997, Siddiqui et al., 2008; Spatafora and Tringali, 2012), as well as enzymes produced by bacteria and fungi existed in composts, which could eliminate phytopathogens in soil as fungal bioagent. The mechanism of plant diseases suppression was mainly attributed to the stimulation of antagonists (especially from antibiotic, mycoparasitism, competitive capacities of *Trichoderma* and *Pseudomonas* spp. in composts) in the plant rhizosphere and unidentified chemical factors and humic acid content in the composts, the induction of systemic resistance in plants, etc (Joshi et al., 2009). Although many kinds of composts such as vermicompost，spent mushroom compost and compost from other organic wastes have been used to test the suppressive effects on soil-borne and foliar diseases on various crops infected by fungi such as *Rhizoctonia solani* and *Phaeoisariopsis griseola*, there is a lack of information about the antipathogenic effect of CMHRs for land application. Hence, the aim of this study was to evaluate the effectiveness of the antipathogenic property of the CMHRs composts in soil inoculated with *A. solani* and *F. oxysporum.*

## 7.2 Material and methods

### 7.2.1 Plant materials

Cherry tomato (*Lycopersicon esculentum*) and Chinese cabbage (*Brassica chinensis*) were selected for this experiment due to their adaptability to tropical, warm and wet conditions of Hong Kong. The seeds were bought from local seed supplier was surface sterilized by soaking in 1% NaOCl for 5 min then washed by sterile water

for 5 times before used.

### 7.2.2 Soil and mature composts

The soil was collected from the top 15 cm layer from a non-cultivated area of a local vegetable field (Produce Green Foundation) in the New Territories, Hong Kong. The soil was air-dried in the greenhouse, ground and sieved through a 2 mm sieve before used in the plant growth experiment.

Table 7-1. Physicochemical properties of soil, food waste (FW) compost and Chinese medicinal herbal residues (CMHRs) and food waste co-compost.

| Parameters | Soil | FW compost | CMHRs co-compost |
|---|---|---|---|
| pH | 4.33 (0.08) | 8.08 (0.40) | 7.95 (0.47) |
| EC (dS/cm) | 0.24 (3.54) | 5.45 (0.43) | 5.34 (0.21) |
| TOC (%) | 0.92 (0.29) | 37.79 (4.19) | 38.98 (6.12) |
| TKN (mg/kg) | 852 (100) | 13560 (855) | 17400 (2032) |
| TP (mg/kg) | 702 (200) | 7198 (668) | 8796 (671) |
| TK (mg/kg) | 9.54 (0.30) | 14.6 (0.45) | 18.3 (0.16) |

Values in parentheses are standard deviation (n=3).

Mature compost derived from food waste and Chinese medicinal herbal residues co-composting process (CMHRs compost) was obtained from our previous experiment. The selected chemical properties of the composts are presented in Table 7-1, while the physicochemical parameters are presented in Chapter 3. For comparison purpose, mature food waste compost from the same experiment was also used in this experiment.

### 7.2.3 Pathogenic culture collections

Two types of phytopathogenic fungi were chosen as sources of infection: *Alternaria solani* (CBS 110.41) (Centraalbureau voor Schimmelcultures, Netherland) causes early blight on tomato (Madden et al., 1978; Vloutoglou et al., 2000) while *Fusarium oxysporum* (CBS 186.53) is one of the major pathogens causing wilt disease on cabbage (Heitefuss et al., 1960; Alam et al., 2011).

### 7.2.4 Inoculum

Spores of *F. oxysporum* and *A. solani* were obtained after their growth on PDA (potato dextrose agar, Oxoid, 39 g $L^{-1}$, rose Bengal 50 mg $L^{-1}$, streptomycin 100 mg $L^{-1}$) plates for 9 days, washed with sterile distilled water and filtered through 3-layer sterile gauze. The spore concentration was determined by counting using hemocytometer and the density of the conidia was adjusted to $1x10^6$ spores/ml for *F. oxysporum* and $1x10^5$ spores/ml for *A. solani*.

### 7.2.5 Greenhouse experimental design

Mature CMHRs compost was applied at a rate of 2, 5 and 10% (dry weight basis, w/w) with the soil in pots of 10.2 cm in diameter for cabbage and 15.2 cm in diameter for tomato. CMHRs compost amendment at 5% was equivalent to an application rate of 150 kg N /ha that is commonly used for crop growth (Wong et al., 1996). Mature food waste compost at 5% application was also prepared while soil was used as the control treatment. All treatments received inoculation by mixing the spore suspensions with the 1/4 of the top soil/compost mixtures with a density of $10^6$

spores/g of *F. oxysporum* and $10^5$ spores/g of *A. solani* into soil or soil-compost mix. *Fusarium oxysporum* was applied to cabbage plants while *A. solani* was applied to tomato plants. Soil alone as the control treatment was prepared to show the normal growth without pathogens infection and one treatment of soil with chemical fertilizer (Schultz, USA) with a total nitrogen input of 1525 mg/kg (dry weight basis) was also included. All soil control treatment received a fertilizer treatment of 150 kg N/ha in the form of slow release fertilizer Osmocote. Three replicates were maintained for each treatment and the replicates were randomly arranged on the greenhouse table.

Two to three seeds were sown in each pot and after emergence, one best-growing seedling was allowed to grow while others were removed after 3-5 days. For each of the treatments, the pathogenic infection symptoms were monitored daily. Soil was maintained at the field moisture by weighing daily. Cherry tomato (*Lycopersicon esculentum*) and Chinese cabbage (*Brassica chinensis*) were planted for 7 and 9 weeks respectively before harvesting.

### 7.2.6 Chemical analyses

The biomass was calculated by determining the dry weights (dry at 55$^{o}$C until constant weight) of the plants after harvesting (Kozlowski and Pallardy, 2002).

The nutrients such as different forms of nitrogen and phosphorus and pH, EC with water extraction (1:2 w/v) in soil were tested in each treatment before planting and after harvesting. The soil samples were dried and ignited in the oven at 550 $^{o}$C for 16 h for total organic matter tests; total Kjeldahl nitrogen (TKN), ammonium and total phosphate were determined by TMECC method (2003). The chemical analyses were carried out using filtered extracts of the soils. $NH_4$-N was analyzed using the

indophenol blue method; $NO_2$-N and $NO_3$-N by copperized cadmium reduction method (Keeney and Nelson, 1982); and $PO_4$-P using the molybdenum blue method (Olsen and Sommers, 1982). After harvest, changes of nitrogen and phosphorus, biomass production as well as copy numbers of phytopathogens were analyzed.

### 7.2.7 Determination of *Alternaria solani* and *Fusarium oxysporum* populations

Genomic DNA from 1.0 g fresh soil sample of all treatments, after harvesting the plants, was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden) following the manufacturer's recommendation and used as the template for quantitative polymerase chain reaction (q-PCR) amplification. The PCR mixture (20 μL), containing 10 μL SYBR Premix Ex Taq II (Takara, Dalian), 7 μL nuclease free water, 1 μL of 10 μmol/L each of forward and reverse primers (Fn-1, 5'-TACCACTTGTTGCCTCGGC-3' and Fn-2, 5'-TTGAGGAACGCGAATTAAC-3' for *F.* oxysporum; AS1: 5'-GCTCCCACTCCTTCCGCGC-3' and AS2: 5'-GGAGGTGGAGTTACCGACAA-3' for A. *solani*) (Tech Dragon Ltd., Hong Kong) and 1 μL DNA template (about 20 ng), was used in q-PCR analysis of fungal population (Kumar et al., 2013; Zhang et al., 2005).

The q-PCR analyses were performed using Agilent Technologies Stratagene Mx3000P (USA) with the following temperature profile: initial denaturation 95 $^{o}$C for 10 min, 40 cycles of 94 $^{o}$C for 15 s, 47 $^{o}$C for 10 s, 72 $^{o}$C for 20 s, and final dissociation 95 $^{o}$C for 1 min, 47 $^{o}$C for 1 min, 95 $^{o}$C for 1 min. The standard plasmid DNA was constructed as described in the Section 5.2.3.2. In addition, the PCR products were separated on 1.0% agarose gel stained with 0.5 ml SYBR Safe DNA gel stain at 12 V for 30 min. A non-template control was included in every run. DNA

products were checked in 2% agarose gel (Ultrapure, MB grade, USA) in TAE buffer at 100 V, along with the DNA ladder of 1000 bp to confirm the amplicons.

### 7.2.8 Sample preparation for UHPLC/Q-TOF/MS

UHPLC-MS analysis was performed on an UHPLC coupled to an Agilent 6540 ultra-high definition accurate mass quadrupole time-of-flight spectrometer (UHPLC–Q-TOF/MS, Agilent Technologies, USA). Soil samples were dried at 55 °C to constant weights and extracted using acetone with the ratio as solid: solvent of 1:50 (w/v). After sonicating for 1 h in an ultrasonicator (Branson, USA), centrifuging at 25,931 x *g* for 15 min and filtrating through 0.45 μm membrane filters, the extracts were subjected to full scan analysis with gradient elution using water and acetonitrile both containing 0.1% (v/v) formic acid as mobile phase as follows: 0-5 min, 5-15% B; 5-20 min, 15-100% B; isocratic 100% B for 5 min then back to 5% B within 5 min. The injection volume was 5 μL and flow rate was 0.35 mL/min. Peak assignment was performed by using database of TCM Library, available at the School of Chinese Medicine, Hong Kong Baptist University. Data analysis was performed using Agilent MassHunter Work station software Qualitative Analysis (version B.04.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc., 2011). Detailed information of equipment and chromatographic conditions are presented in Sections 6.2.3-6.2.5.

### 7.2.9 Statistical analysis

Analyses were performed on triplicate samples and the mean values and standard deviations on dry weight basis are presented. The data were processed using

SigmaPlot 11.0 and IBM SPSS statistics 19 while the significance of differences were tested using Duncan multiple rage test at $p<0.05$.

## 7.3 Results and discussion

### 7.3.1 Soil pH and electrical conductivity

Addition of CHMR compost demonstrated a positive pH buffering effect on the acidic soil and the soil pH increased significantly from ~4 to ~7 with an increase in CHMR compost amendment up to a maximum of 10% (Fig. 7-3a and 7-3c). A neutral pH (pH 7-9) is optimum for nitrifying bacteria and nitrogen-fixing bacteria (Rifat et al., 2010). Moreover, maintaining high pH is an essential factor of devitalizing phytopathogens in soil. Food waste compost also achieved the same level of pH buffering capacity (pH of 7.0) as that of CHMR compost at 5% application rate.

Both FW and CHMR composts had higher EC due to large quantities of soluble salts in food waste (Section 4.3.2), and their application significantly increased the soil EC, with an increase in food waste content in the initial composting mix. However, the ECs of all compost application rates were all below the limit for sensitive crops of 2 mS $cm^{-1}$ (Richards and McCarty, 1954). After the plant growth, EC decreased significantly as compared with the initial EC indicating the removal of soluble salts by the plants.

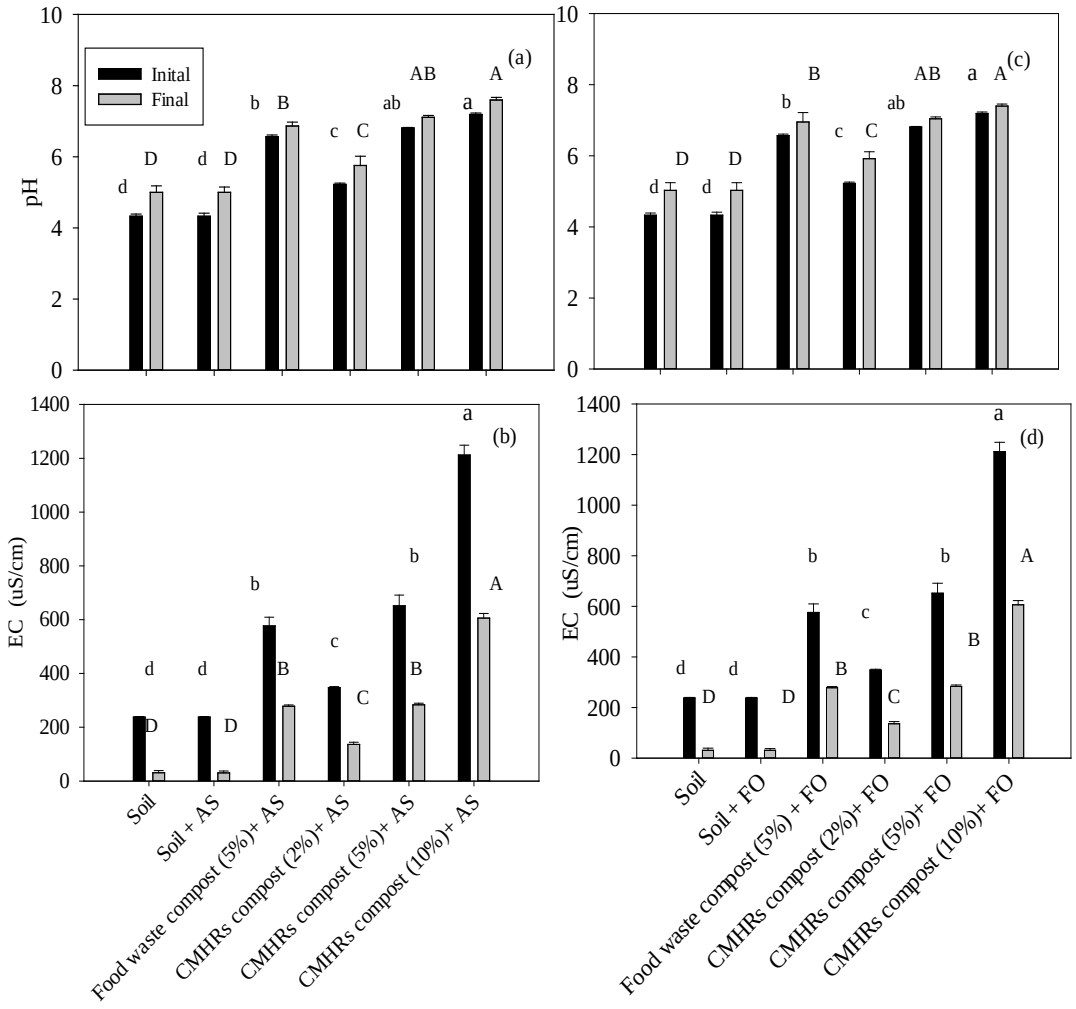


Figure 7-3. Changes of pH and electrical conductivity of soil, amended with food waste compost and CMHR compost at different application rates, before and after the cultivation of *Lycopersicon esculentum* (a) and (b), and *Brassica chinensis* (c) and (d). AS- *Alternaria solani*; FO- *Fusarium oxysporum*. Similar letter above bars of the same parameter of each plant species indicate that treatments did not demonstrate significant difference by Duncan multiple range test at $P < 0.05$ (lowercase letter: differences between treatments before the plant growth; uppercase letter: differences between treatments after harvesting the plant).

### 7.3.2 Dynamics of nitrogen and phosphorus in soil

Nitrogen is essential for crop growth as it is required for the synthesis of enzymes, proteins, chlorophyll and DNA/RNA. The migration and mobility of various forms of nitrogen are different, for instance, nitrate migrate by mass flow while ammonium by diffusion (Song and Li, 2006). Active beneficial bacteria in the soil play an important role in transformation, mobilization and solubilization of nitrogen (Rifat et al., 2010).

As shown in Fig. 7-4, changes in the total N contents of the control and the soil treatment with pathogens inoculation did not differ significantly, probably due to poor plant growth. On the contrary, treatments with compost amendment had significantly higher total N contents due to the high nitrogen contents (1.3% of FW compost and 1.7% of CMHRs compost) of the compost. $NH_4^+$ and $NO_3^-$ subsequently influence the level of nitrogen in soil amended for plant growth, both total and inorganic nitrogen. During nitrification, $NH_4^+$ is oxidized into $NO_2^-$ and subsequently to $NO_3^-$ which is the final and major form of available inorganic N for plant uptake while more than 90% soil N is in organic form (Tan, 2005). Among all treatments with pathogen inoculation, dramatic reductions of $NH_4^+$ and $NO_3^-$ were observed in treatments with 5% and 10% CMHRs compost amendment for both Chinese cabbage and cherry tomato, due to higher N mineralization and utilization by plants. The reason can be explained as anti-pathogenic properties of CMHRs compost protected the plants roots from pathogenic infection therefore healthy roots absorbed more nutrients from soil. This allowed a better root growth and subsequently enhanced nutrient uptake (Song and Li, 2006). $NH_4^+$ and $NO_3^-$ were considered as the most important N sources that can be taken up plants. As shown in Fig. 7-4, the absorption preferences of both *L.*

*esculentum* and *B. chinensis* were combinative use of the two N sources with superior to $NO_3^-$. The treatments with soil had lower pH which negatively influenced inorganic N absorption so that least amount of $NO_3^-$ was uptaken while media pH of treatments with FW and CMHRs composts were positively improved $NO_3^-$ absorption. However, excess high content of $NH_4^+$-N in 10% CMHRs treatment limited plant growth due to higher food waste salinity. The reason was that ammonium penetrates plant membrane and enters cells directly through diffusion, resulting in an increment of cytoplasmic pH and inhibiting glutamate synthase activity as the primary cause (Li et al., 2013).

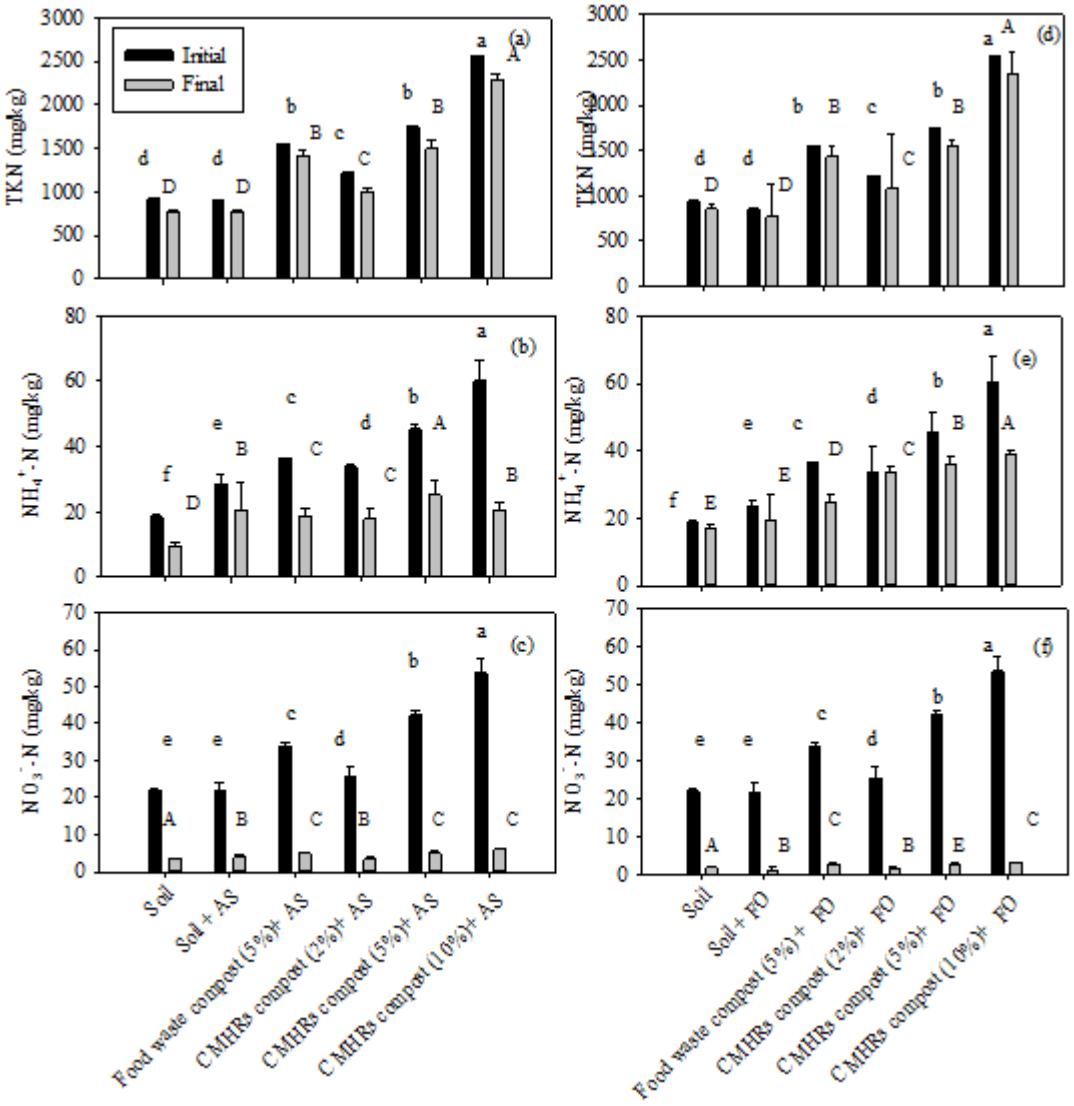


Figure 7-4. Changes of TKN (a), extractable ammonium (b) and nitrate (c) in the soil before and after the growth of *Lycopersicon esculentum* (a, b and c, respectively) and *Brassica chinensis* (d, e and f, respectively). AS- *Alternaria solani*; FO- *Fusarium oxysporum*. Similar letter above bars of the same parameter of each plant species indicate that treatments did not demonstrate significant difference by Duncan multiple range test at $P < 0.05$ (lowercase letter: differences between treatments in initial phase; capital letter: differences between treatments in final phase).

Phosphorus is the other critical macronutrients for crop growth, which is less mobile than nitrogen, and the transformation among the various forms of phosphorus is dominated by physical rather than biological processes. However, initial phosphorus concentration was significantly increased with the increment in compost amendment (1.2 to 2.1 folds), providing crops sufficient nutrient source for their growth (Fig 7-5). Food waste compost with application rates of 2% and 5%, 10% CMHRs compost had higher contents of extractable P due to their neutral pH values. The treatments with CMHRs compost were observed with much more reduction of total phosphorus (tomato: 35% for 2% CMHRs compost, 25% for 5% CMHRs compost and 47% for 10% CMHRs compost; cabbage: 3% for 2% CMHRs compost, 19% for 5% CMHRs compost and 29% for 10% CMHRs compost); while increasing the contribution of extractable phosphorus in the treatments of 2% CMHRs compost (19% for tomato and 4% for cabbage), 5% CMHRs compost (20% for tomato and 16% for cabbage) and 10% CMHRs compost (21% for tomato and 16% for cabbage), especially in cherry tomato. The reductions of total phosphorus were mainly due to plant removal of available phosphorus degradated by microbial activities, such as *Aspergillus* sp. and *Penicillium* sp. and highest population of *Aspergillus* sp. was observed in treatment 1:1:1 Section 5.3.2.1. The loss tendency of total phosphorus was similar to the findings of Liu et al. (2014). It indicates that abundant organic P must be transformed to inorganic P before it is available for plants uptake and crop removal is the largest contributor of phosphorus depletion since it is not volatile (Wong et al., 1999). These beneficial microorganisms in CMHRs treatments also mediate the solubilization of insoluble inorganic phosphorus by secreting enzymes such as phosphatase, phosphonoacetate hydrolase and phytase in the presence of sufficient carbon and nitrogen sources which CMHRs compost provided in the soil

(Pradhan and Sukla, 2005). According to Wang et al. (1999), soluble phosphorus was chelated with $Ca^{2+}$ to form $Ca^{3-}$-P and $Ca^{4-}$-P in acid condition which was less soluble; while solubility of available phosphorus also decreased under high pH condition, conjugating with Fe and Al. As a result, huge amount of soluble P was available in the 5% FW compost, 5% and 10% CMHRs compost treatments, providing sufficient soluble phosphorus for crop growth.

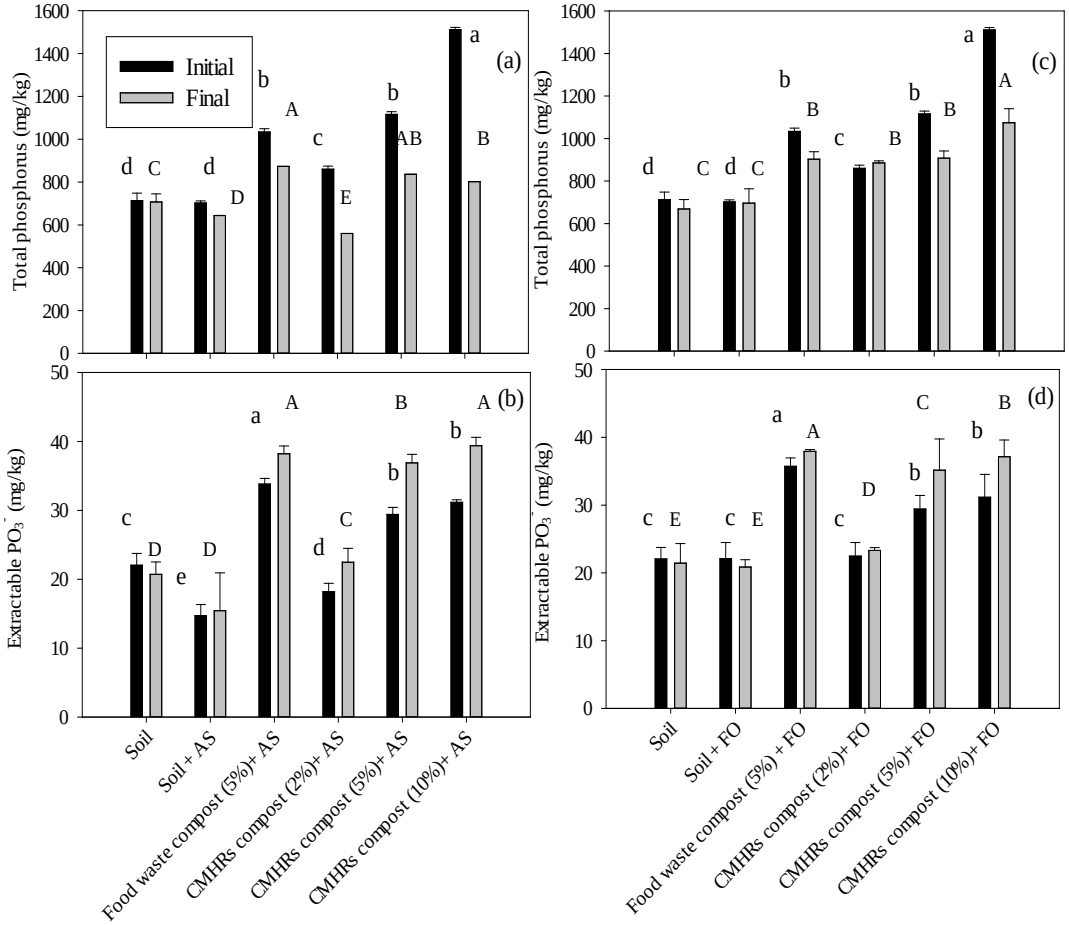


Figure 7-5. Changes of total phosphorus (a) and extractable phosphorus (b) of Lycopersicon esculentum; total phosphorus (c) and extractable phosphorus (d) of Brassica chinensis during cultivation respectively (AS: *Alternaria solani*; FO: *Fusarium oxysporum* ). Similar letter above bars of the same parameter of each plant species indicate that treatments did not demonstrate significant difference by Duncan multiple range test at $P < 0.05$ (lowercase letter: differences among treatments in initial phase; capital letter: differences among treatments in final phase).

### 7.3.3 Plant biomass production and nutrients uptake

The control soil without *A. solani* inoculation had higher dry weight yield of cherry tomato (2.36 g $pot^{-1}$) than the soil inoculated with *A. solani* (1.52 g $pot^{-1}$) (Fig. 7-6a)- confirming the crop damage by phytopathogen. Similarly, dry weight yield of Chinese cabbage in the soil without inoculation of *F. oxysporum* was 0.47 g $pot^{-1}$, which was significantly higher than the 0.39 g $pot^{-1}$ observed in soil treatment with fungal inoculation (Fig. 7-6b). The treatment without pathogen inoculation produced higher biomass since phytopathogens inhibited nutrient absorption from environment by plant roots. However, for *B. chinensis*, the growth in the control soil was even better than the one with 5% FW compost, indicating the short growth period and the pathogenic effect were higher than the nutrient effect. After inoculation, even addition of 5% FW compost showed significant preponderance on biomass production to soil with pathogen. The reason should be due to sufficient nutritional sources such as nitrogen and phosphorus and microbes with antagonistic properties as well as the inhibition effect of bioactive compounds. Plants grew 2.5 folds larger than soil treatment without pathogens when fertilizer was added. For *L. esculentum*, the long growing period did counteract against the negative effect of pathogens on plant growth as evidenced by the high yield with 5% FW compost amended soil as compared to the control; while biomass production of fertilizer treatment was 1.5 folds higher than only soil.

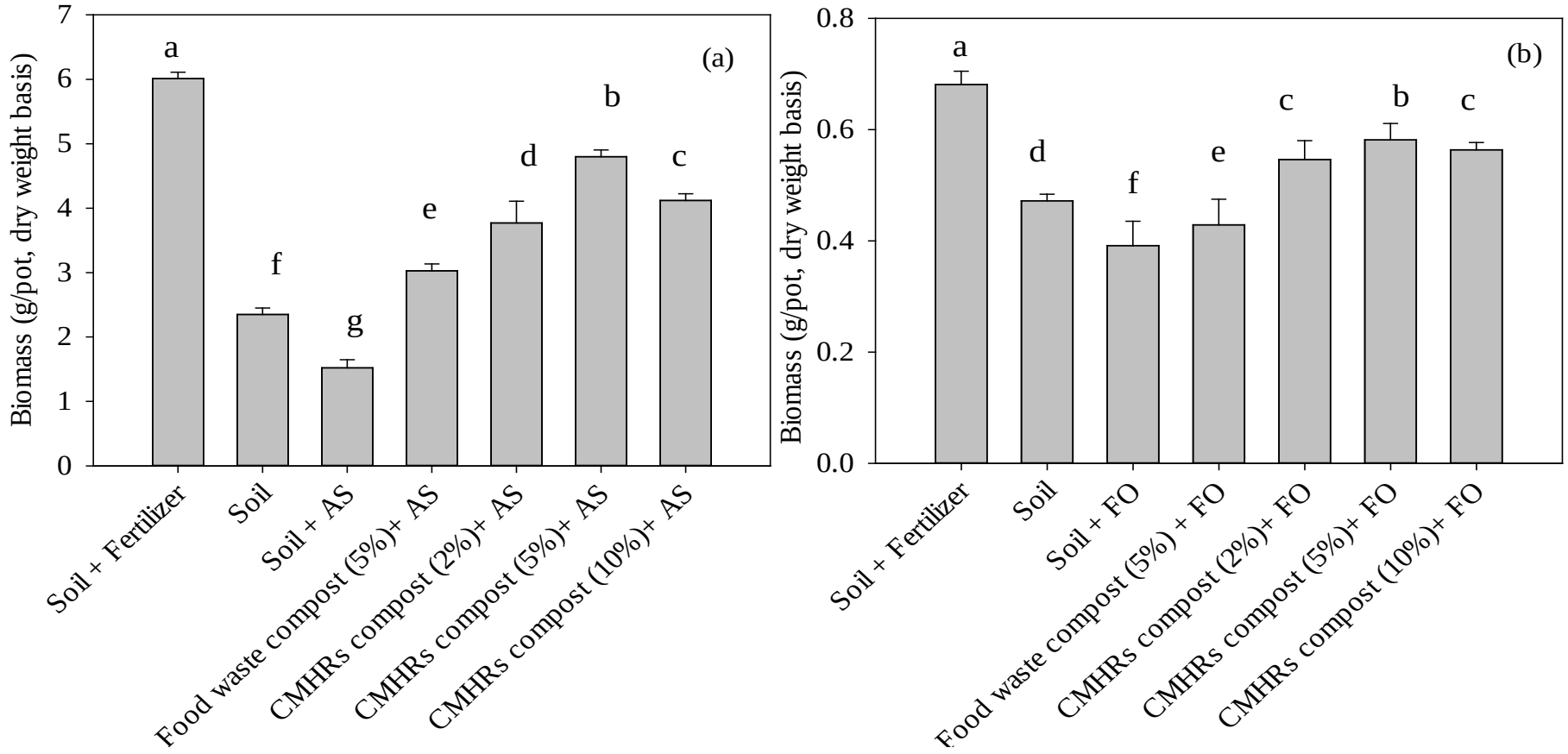


Figure 7-6. The dry weight yields of *Lycopersicon esculentum* and *Brassica chinensis* in soil with various application rates of mature compost (AS: *Alternaria solani*; FO: *Fusarium oxysporum* ).


Figure 7-7. The photo of *Lycopersicon esculentum* and *Brassica chinensis* in soil with various application rates of mature compost (F: fertilizer; AS: *Alternaria solani*; FO: *Fusarium oxysporum*).

In contrast, with the inoculation of plant pathogens, the biomass of treatments with 5% CMHRs compost showed the highest biomass production for both crops (4.80 g $pot^{-1}$ for cherry tomato and 0.95 g $pot^{-1}$ for Chinese cabbage) which agreed with Wong and Wong (1990) that > 10% of mature compost as soil amendment decreased crop yields due to the negative impact from high food waste salinity. *Brassica chinensis* with 5% and 10% CMHRs compost addition (with F. *oxysporum* inoculation) in soil even grew better than the treatment of soil without F. *oxysporum* inoculation. This result indicated that *B. chinensis* was more sensitive to nutrient source than pathogenic infection because of the short growth period, comparing to *L. esculentum*. Moreover, potentially available nutrients in mature CMHRs compost gradually release the nutrients into bulk soil and were absorbed by healthy roots. For example, the nutrient uptake of 5% CMHR treatment with pathogen inoculation was 1.6 fold and 2.4 fold higher the control for *B. chinensis* and *L. esculentum*, respectively.

As shown in Fig. 7-8, the soil treatment of *L. esculentum* without pathogenic inoculation took up more nitrogen and phosphorus than infected one since phytopathogens damaged the roots of crops which are the most important part of absorbing nutrients from environment while no significant different was observed in *B. chinensis* which result was agreed to biomass production, indicating *B. chinensis* was more sensitive to nutrient source than pathogenic infection because of the short growth period.

For *L. esculentum*, soil treatments with and without *A. solani* almost absorbed same amount of nitrogen (11 mg and 8 mg); however, in treatments with CMHRs compost addition of 2%, 5% and 10%, N increments reached 2.8, 5.3, 4.8 folds (29 mg, 40 mg and 34 mg) than control respectively and FW compost also enhanced

nitrogen uptake by 2.9 folds (18 mg) than that of the soil treatments. Phosphorus uptake by the plants grown in control soil was 1.3 folds higher than pathogen inoculated soil treatment. In CMHRs compost treatments, the P uptake increased to 3.4 to 4.7 folds (from 2 mg to 4 mg) than soil with *A. solani* inoculation. For *Brassica chinensis*, 10% CMHRs compost took up more nutrients (5.3 and 14.4 folds than control, 21 mg of N and 4 mg of P), followed by 5% (3.8 and 14.1 folds than control, 15 mg of N and 4 mg of P), 2% CMHRs compost (2.2 and 9.5 folds than control, 9 mg of N and 2 mg of P) and 5% FW compost (1.9 and 6.0 folds than control, 8 mg of N and 2 mg of P). Besides, when *F. oxysporum* was absent in the soil treatment, nitrogen and phosphorus uptakes were much higher (1.5 and 12.0 folds than soil with *F. oxysporum*).

Nutrition uptake is highly depend on root health and it has been proved that mature FW compost had more powerful antifungal effect than soil (Mehta et al., 2014), furthermore mature CMHRs compost addition had better inhibition capacity on phytopathogenic growth and protected plants root from infection.

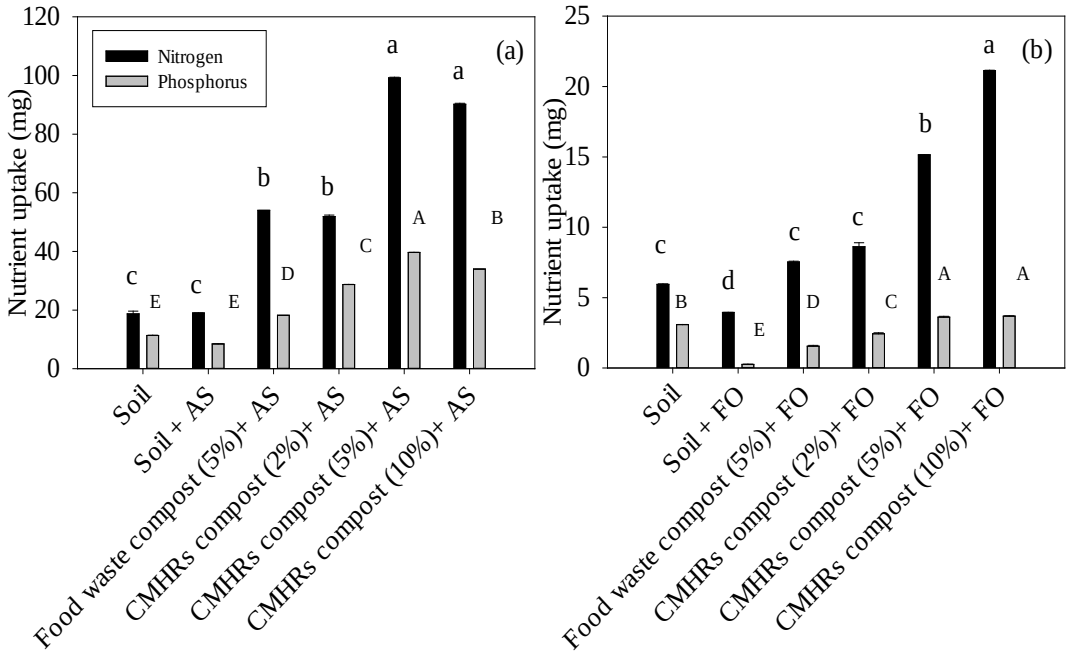


Figure 7-8. The nitrogen and phosphorus uptake of *Lycopersicon esculentum* and *Brassica chinensis* in soil with various application rates of mature compost (AS: *Alternaria solani*; FO: *Fusarium oxysporum* ). Similar letter above bars of the same parameter of each plant species indicate that treatments did not demonstrate significant difference by Duncan multiple range test at $P < 0.05$ (lowercase letter: differences among treatments in initial phase; capital letter: differences among treatments in final phase).

### 7.3.4 Populations of *Alternaria solani* and *Fusarium oxysporum*

Among all treatments, 5% and 10% of mature CMHRs compost significantly reduced the populations of *A. solani* and *F. oxysporum* comparing to control treatment due to higher pH which was not propitious for fungal growth as well as high contents of bioactive compounds. The microbes in CMHRs composts were more antagonistic to *A. solani* and *F. oxysporum* that could possibly provide better protection of crop root surface from infection by phytopathogens through both competitive effect and production of antimicrobial agents (Chapter 5). Additionally, the bioactive components originated from the CHMR, such as linolenic acid, cimicifugic acid, nobiletin, 5,7,2',4',6'-pentamethoxyflavone, tanshinone IIA, coumarins and (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroseselin that exist in the mature compost also showed antibacterial and antifungal capacities (Section 6.3.3). Higher application rates of CMHRs compost into the soil should have exerted potential antipathogenic effect that enhanced the crop health and biomass production.

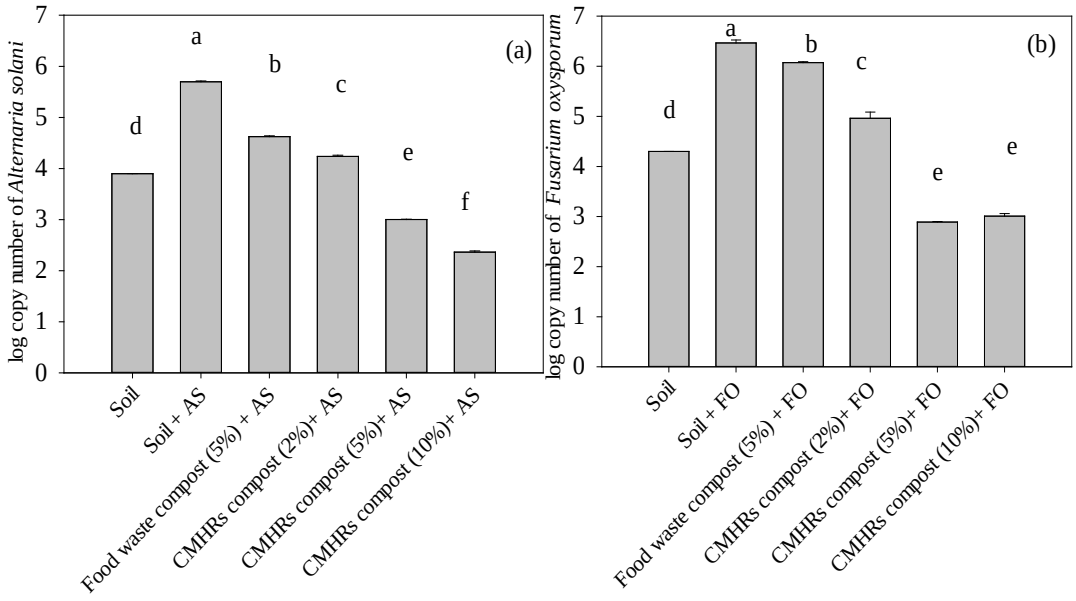


Figure 7-9. Quantification of *Alternaria solani* (a) and *Fusarium oxysporum* (b) with various application rates of compost.

### 7.3.5 Bioactive components existed in mature composts added soil during plant growth

As presented in Section 6.3.2, CHMR composts showed inhibitory effect on *Alternaria solani* and *Fusarium oxysporum* growth was mainly due to the antifungal activity of bioactive compounds from CMHRs. However, it was not clear whether these CHMR derived bioactive compounds applied to the soil through CHMR composts could persist in the soil for about 7 to 9 weeks during plant growth. As shown in Fig. 7-10 – 7-12, it was indicated that no active compounds was observed in soil and bioactive compounds in compost treatments broken down gradually during plant growth. In previous research (Chapter 6, Table 6-1 and 6-2), 17 different bioactive compounds were identified to be dominant in both food waste and CMHRs composts while 22 bioactive compounds were only observed in CMHRs compost. These compounds were quantified before and after plant growth since most of these compounds had antipathogenic capacity and fungal control depends on the residual concentration of these active compounds after land application.

As shown in Table 7-2, the residual bioactive compounds after plant harvest were identified and quantified, and correlated with *A. solani* and *F. oxysporum* population in order to find out decisive compounds for fungal control. More than 50% of the compounds maintained in different treatments for *Brassica chinensis* planting except Angeliticin A, Chasmanthin, Fawcettiine, 4,7-Dihydroxy-5-methoxyl-6-methyl-8-formyl-flavan and 3-O-cis-p-Coumaroyl alphitolic acid while most of compounds disappeared in *Lycopersicon esculentum* after three more weeks cultivation and only Iso-mucromatol and Tetrahydrobungeanool was observed at about 43% and 49% respectively, of the

initial concentration before planting. Meanwhile as listed in Table 6-2, bioactive compounds derived from CMHRs degraded relatively less. For *Brassica chinensis*, bioactive compounds were maintained from 1% to 81% with all Chimaphylin disappeared; Mesuein, Dihydrokaranone and Isotanshinone II was observed to be about 57%, 70% and 101% respectively, of the initial levels in 5% CMHRs compost treatment after *B. chinensis* growth. One percent to 41% of compounds were degraded with *L. esculentum* growth with no Chimaphylin or 5,7,2',4',6'-Pentamethoxyflavone existed at the end of planting; while 41% of Dihydrokaranone left in 5% CMHRs compost treatment after *Lycopersicon esculentum* growth. Some of the compounds (Table 6-1) should be mainly derived from food waste such as vegetables and meat which contain phenolic acid, flavonoids and tannins (Liu et al., 2008). However, these compounds were easily degraded either under microbial activities in soil matrix or due to their protein property or instability in non-neutral environment (Kosseva, 2013). Bioactive compounds in CMHRs compost degraded due to oxidative and polymerization reactions under their photolytic and pyrolytic properties (Hu et al., 1999).

Table 7-2. Pearson's correlations of disease suppression between the phytopathogenic populations and bioactive components identified from compost samples.

| | Family | Antifungal effect | AS PDA | | AS (plant growth) | | FO PDA | | FO (plant growth) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | * (p<0.05) | ** (p<0.01) | * (p<0.05) | ** (p<0.01) | * (p<0.05) | ** (p<0.01) | * (p<0.05) | ** (p<0.01) |
| Tetrahydrobungeanool | Alkaloids | Y | | √ | | √ | √ | | √ | |
| Oxyberberine | Alkaloids | Y | - | | | | | √ | √ | |
| Mesuein | Flavonoids | Y | √ | | - | | - | | | |
| Hesperidin | Flavonoids | Y | √ | | - | | - | | | |
| Nobiletin | Flavonoids | Y | √ | | √ | | - | | | |
| 5,7,2',4',6'-Pentamethoxyflavone | Flavonoids | Y | | √ | | √ | - | | | |
| Isotanshinone II | Flavonoids | Y | - | | | | √ | | √ | |
| Tanshinone IIA | Flavonoids | Y | - | | | | √ | | √ | |
| 5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarin | Coumarins | Y | | √ | | √ | √ | | - | |
| 7-Methoxy-8-(1'-methoxy-2'-hydroxy-3-methyl-3'-butenyl)coumarin | Coumarins | Y | | √ | | √ | - | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroselin | Coumarins | Y | - | | | | - | | √ | |
| 2,3,4-Trimethyl-5-phenyloxazolidine | Alkanes | N | - | | | | | √ | √ | |
| Chasmanthin | Ester | Y | √ | | √ | | √ | | √ | |
| Gossypetin hexamethyl ether | Ether | Y | - | | | | | √ | √ | |
| Linolenic acid | Acid | Y | √ | | √ | | √ | | - | |
| Cimicifugc acid | Phenolic acid | Y | | √ | | √ | - | | | |
| Glutimic acid | Protein | Y | √ | | √ | | | √ | | √ |
| Parakmerin A | Lignan | Y | | √ | | √ | | √ | | √ |

“-” means none of available.

As shown in Table 7-2, 13 bioactive components were positively correlated with *A. solani* populations control ($p<0.05$) on *Lycopersicon esculentum* growth. Comparing to previous in vitro results (Section 6.3.3), two compounds were excluded (Mesuein and Hesperidin) in the correlation with *A. solani* inhibition. This result may be due to their more easily degradable properties. For *Brassica chinensis*, 11 bioactive components were positively correlated with *F. oxysporum* growth ($p<0.05$). Angeliticin A, Chrysanthenone, 5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarins and Linolenic acid had no correlation with *F. oxysporum* comparing to previous antipathogenic tests on PDA plates. In general, 87% and 79% of correlated bioactive components were left respectively after the growth of *L. esculentum* and *B. chinensis* for 7 to 9 weeks.

As shown in Fig. 7-10 - 7-12, compounds decomposed up to 88% during the growth period of *B. chinensis* (except Angeliticin A, 4,7-Dihydroxy-5-methoxyl-6-methyl-8-formyl-flavan and 3-O-cis-p-Coumaroyl alphitolic acid, which were not observed in mature composts); only 49% of compounds were left as the highest amount during the growth period of *L. esculentum* and more compounds disappeared including Chrysanthenone, Isopetasin, Abietic acid and Cyclotetradecan-1-one. This is likely due to the longer growth period of *L. esculentum* experiment. It is also well known that herbal components is sensitive that temperature, humidity, light and air can make bioactive compounds in TCM change, transform or even deteriorate (Hu and Xu, 2014).

Comparing the ECC graph of all treatments, it is easily observed that several peaks within the time range between 14 and 15 min. These compounds were unidentified by TCM data base which means these non-targeted compounds were derived from soil. Most of these compounds were hardly decomposed during plant

growth which could be ignored in respect of antipathogenic effect.

In general, the bioactive components with antipathogenic capacities inhibited *A. solani* and *F. oxysporum* growth effectively since 85% of them had capacities to inhibit phytopathogens. Larger amounts of these bioactive components were quantified in the treatment with 5% CMHRs compost addition, resulting in the log population of *A. solani* was well controlled to eliminate 50% while 54% for *F. oxysporum* at end of plant growth (Fig. 7-9). Most of these compounds are belonged to alkaloids (Oxyberberine and Oxoglaucin), flavonoids (Mesuein, Hesperidin, Nobiletin, 5,7,2',4',6'-Pentamethoxyflavone, Isotanshinone II and Tanshinone IIA), coumarins (5,6-Dimethoxy-8-(3'-methyl-2'-oxobutyl) coumarin, 7-Methoxy-8-(1'-methoxy-2'-hydroxy-3’methyl-3'-butenyl) coumarin and 3'R,4'R)-3'-Epoxyangeloyloxy-4'-acetoxy-3',4'-dihydroseselin), ketones (Angeliticin A, Chrysanthenone and Dihydrokaranone) and acids (Linolenic acid and Cimicifugc acid) which have powerful antifungal and antibacterial abilities. As a result (Fig. 7-7), among all treatments with phytopathogens inoculation, 5% CMHRs had yielded the highest biomass (4.8 for *L. esculentum* and 0.6 for *B. chinensis*) since bioactive components positively protected plant roots from fungal infection while high contents of alkaloids, flavonoids and coumarins in the treatment of 10% CMHRs might lead to phytotoxicity to plant growth (Nebo et al., 2014).

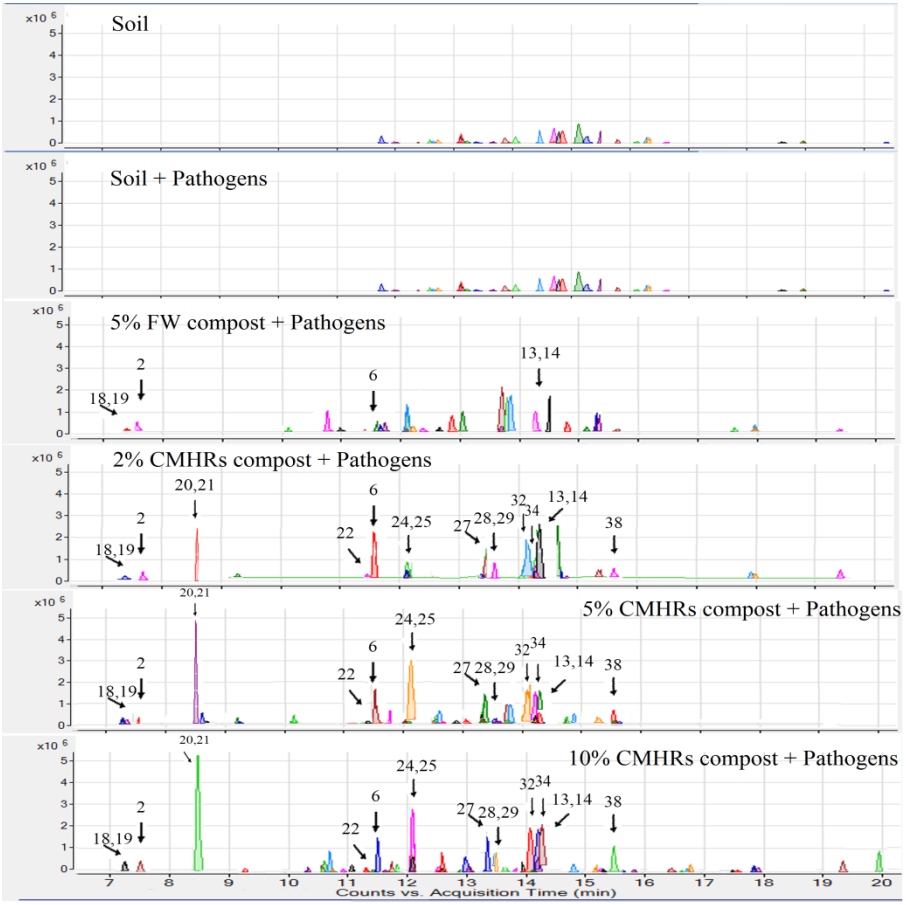


Figure 7-10. Extractable compounds chromatograms (ECC) of the soil and soil with different compost applications before planting *Brassica chinensis*. ESI (+) TOF-MS with peaks in random colors.

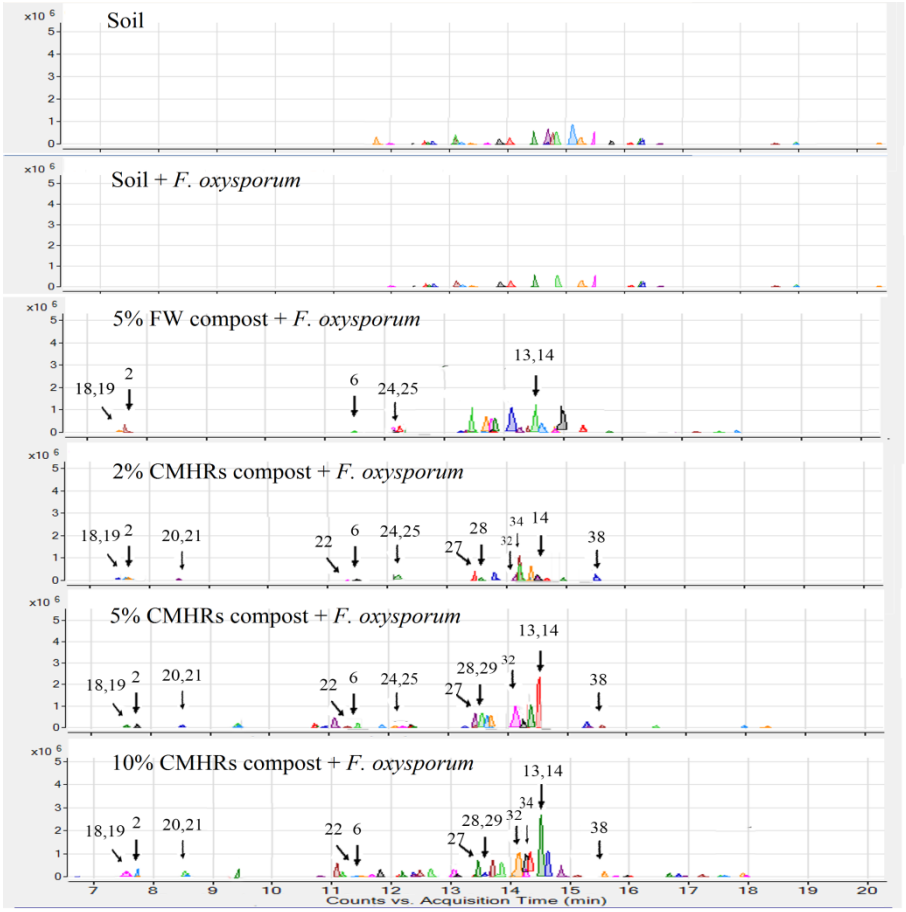


Figure 7-11. Extractable compounds chromatograms (ECC) of treatments at final phase of *Brassica chinensis* planting for identification: ESI (+) TOF-MS with peaks in random colors.

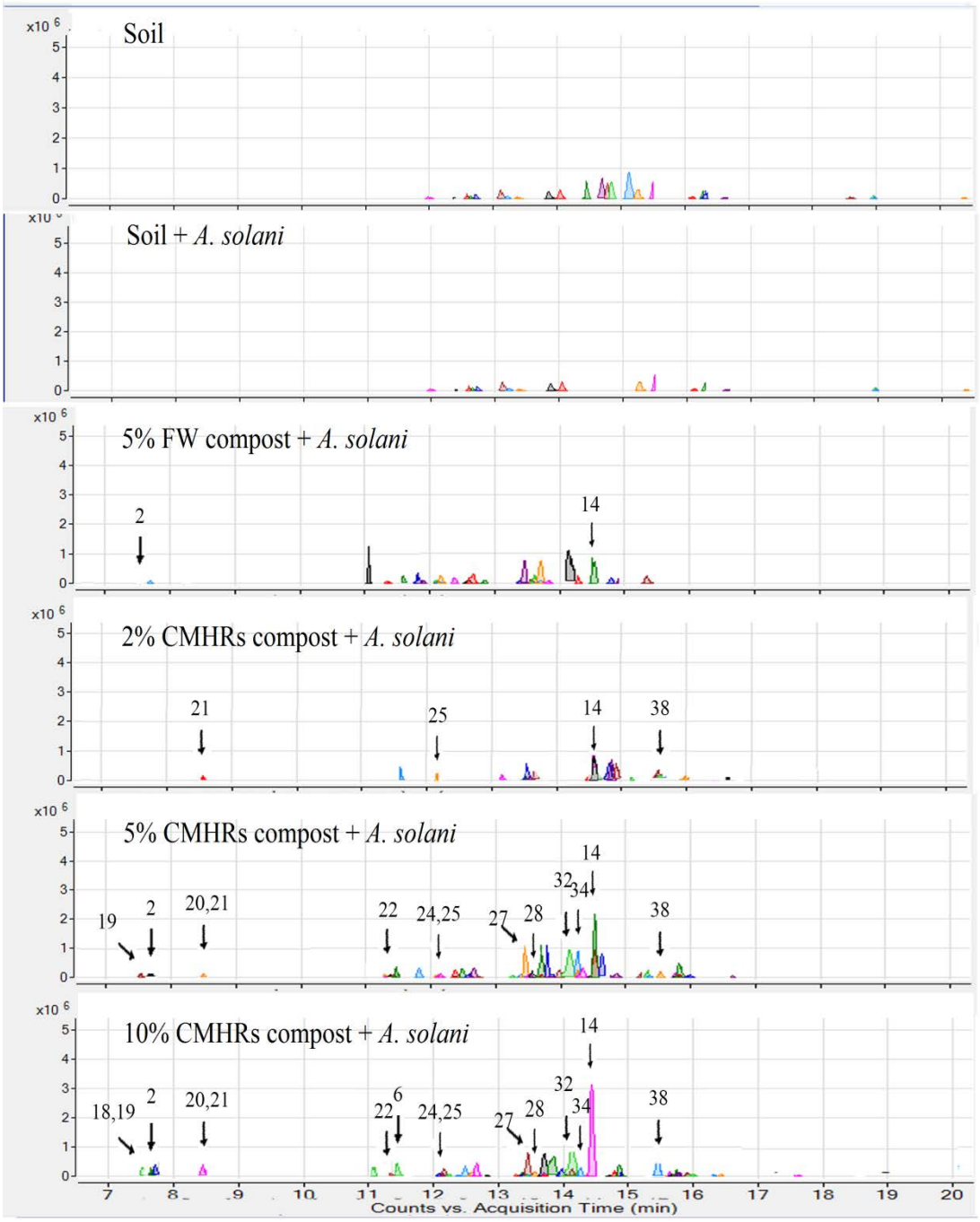


Figure 7-12. Extractable compounds chromatograms (ECC) of treatments at final phase of *Lycopersicon esculentum* planting for identification: ESI (+) TOF-MS with peaks in random colors.

## 7.4 Conclusions

This study showed that the crop yields were increased with the addition of CMHR composts to acidic soil, and 5% compost application was the optimum, while at the higher application rate of 10% (dry weight basis, w/w), plant growth was inhibited which might be due to the higher salt contents and the phytotoxicity of alkaloids, flavonoids and coumarins in the CMHRs. *Brassica chinensis* was more sensitive to the inhibitory effect of phytopathogen inoculation, while nutrient supply was to a less extent due to the short growth period as compared to *Lycopersicon esculentum*. This study clearly showed that mature compost provided *L. esculentum* and *B. chinensis* sufficient nutrients such as nitrogen and phosphorus. Additionally, the advantage of using CMHR compost as a soil conditioner was the protection against the fungal pathogen. The main contributor of this protection was due to the bioactive compounds of the CMHRs compost. Identified bioactive compounds belonged to alkaloids, flavonoids and coumarins, which have powerful antifungal and antibacterial abilities and most of them persist during plant growth though their quantities reduced greatly as highest amount of 81%. Therefore, CMHR compost can be a potential alternative to reduce the usage of fungicides and its associated environmental hazards. The present study clearly demonstrates the beneficial effects of using CMHR compost to enhance the plant biomass yield and control the pathogenic incidence.

# CHAPTER 8

# GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

## 8.1 Introduction

Composting is one of the most sustainable methods to treat food waste which has grown tremendously all over the world, including big cities like Hong Kong (HKEPD, 2014). This treatment prevents the leachate, odours, and greenhouse gases production from disposal organic wastes through landfilling (Haug, 1993). The major disadvantages of using food waste as a raw material for composting include high moisture content, low C/N ratio, poor structure, low porosity and high acidity, which will slow down or even cease the composting process (Wong et al., 2010). To improve the poor structure of food waste composting, good bulking agents with proper function will be needed. Chinese medicine herbal residues (CHMRs) as a waste represents a good bulking agent for food waste composting it was estimated about 65 million tons produced annually in China on wet weight basis (Zou et al, 2008). High fibrous and woody nature of CHMRs makes it bulky and it also consists of high carbon contents, which could be source of air pollution with acidic gases, dioxins and

furans after incineration (Akter et al, 2002). The initial composting conditions were improved by addition of CMHRs with high carbon content and the bulky property; CMHRs could provide a proper co-composting material to food waste which can not be composted alone due to its properties of low pH and C/N as well as high moisture content. Besides, it contains bioactive compounds which is the most important additional function of hindering phytopathogenic growth (Yang et al.; 2009).

The overall aims of my Ph.D. research were to study the feasibility of CMHRs as a bulking agent in the composting process and to evaluate any antipathogenic capability in the CMHRs compost. This study consisted of five phases:

Phase I focused on developing the optimum ratio between food waste, sawdust and CMHRs, achieving high efficiency of composting by using CMHRs as the bulking agent;

Phase II investigated the correlation of the change of functional groups of humic substances during the humification process of the composting mass and composts maturity when CMHRs was supplemented as a bulking agent; and

Phase III of the research verified the dynamics of bacteria and fungi existed during the compost during composting process and the antipathogenic effect (both antagonistic and abiotic factors) of final mature composts on two specific phytopathogens, A. *solani* and F. *oxysporum* (in vitro);

Phase IV was to identify the changes in profiles of bioactive compounds during the co-composting of CHMRs and food waste with different CMHRs mixing ratios with the purpose to elucidate their antifungal properties; and

Phase V was to evaluate the effectiveness of the antipathogenic properties of the mature CMHRs composts on planting of *Lycopersicon esculentum* and *Brassica chinensis* while soil was inoculated with *A. solani* and *F. oxysporum* in certain concentrations. Based on results obtained in these studies, this chapter presents a general discussion, conclusions and recommendations for the future studies.

## 8.2 Formulation and conditions for co-composting of food waste and CMHRs

The availability of bulking agent is always a factor limiting composting as a viable technology for organic waste management, which depends on the regional waste product availability. Chinese medicinal herbal residues (CHMRs) not only represented a good bulking agent to provide air passage for microorganisms but might also possess antipathogenic properties. Therefore, the first experiment (Chapter 3) was designed to develop optimum raw material ratios of composting between food waste, sawdust and CMHRs. CHMRs were composted with food waste (FW) and sawdust (SD) mixture at a mixing ratio of 5:5:1, 2:2:1 or 1:1:1 (FW: SD: CHMRs, dry

wt. basis). Lime, at 2.25%, was used to buffer the initial low pH due to natural anaerobic decomposition during storage. After composted in computer controlled batch composter for 56 days, the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) exhibited higher organic matter degradation rate of 67%, which was 17.5% higher than that of the control treatment while germination index was 157%, indicating the more effective composting. This indicates the better maturation for the treatment with 1:1:1 ratio. The reason can be explained that the bulky structure of CMHRs provided more air passage in composting matrix and sufficient oxygen made microorganisms more active to break down organic substances. During the thermophilic phase, total bacterial population in the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) was highest and as a result, carbonaceous and nitrogenous materials are transformed into more stable complicated organic forms effectively, which chemically and biologically resemble humic substances to achieve maturity level of composting under rapid degradation by microbial activities.

### 8.3 Evaluation of the composts maturity and monitoring the dynamics of functional groups during humification process

Humification during co-composting of food waste, sawdust and CMHRs was

investigated to reveal its correlation with compost maturity (Chapter 4). Results obtained from previous studies suggested that treatments with the dry weight ratio of 5:5:1, 1:1:1 (FW: SD: CHMRs, dry wt. basis) and control were chosen for further research, based on the reason that significant differences of compost maturity could be observed. Humic acid/fulvic acid (HA/FA) ratio of the control and treatment at 5:5:1, and 1:1:1 (FW: SD: CHMRs, dry wt. basis) mixing ratio increased to 0.5, 2.0 and 3.6, respectively at the end of composting; which represented rapid break down of organic substances by active microorganisms resulted from the bulky structure of CMHRs to induce sufficient oxygen for microbial activities, producing HA during composting process. Besides, CMHRs contained more fibre-structure components such as lignin, which are known to be the core structure of humic substances. Therefore, microorganisms in the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) processed gradually released nutrient source to build up complex humic substances efficiently.

Non-destructive method such as Fourier transform infrared (FTIR) spectroscopy has been used to monitor the functional groups and transformation between HA and FA during composting (Spaccini and Piccolo, 2009). The decrease in aliphatic organics in HA during composting demonstrated the degradation of the readily available organics corresponding to the degradation of the lipid, protein and

polysaccharides from food waste (Huang et al., 2006; Ravindran et al, 2011); and the change was more obvious in the control treatment since food waste represented a higher ratio. The obvious increment in aromatic functional groups in the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) indicated the maturity of compost. The reason was derived from the disappearance of hemicellulose and weak intensity of lignin in the CMHRs treatments, indicating the positive correlation between lignin and the nucleus for HA formation. The mechanism was that high content of lignin in the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) provided rich substrates for aromatization and oxidation. As a result, the cores of humic substances were constructed and oxygen-containing HA functional groups increased. The complicated ring structures in HA had positive correlation with compost maturity and humification degree (Fukushima et al., 2009). Molecular compositions of humic substances can be further characterized using off-line pyrolysis with the tetramethylammonium hydroxide (TMAH) followed by gas chromatography-mass spectrometry (Pyr-TMAH-GC-MS) for quantitative analysis. Pyr-TMAH-GC-MS results further indicated that initially dominant aliphatic and alicyclic esters groups were gradually replaced by complicated ring-structure components appeared dominantly at the later phase of composting. The peak intensities of hydrocarbon and alkyl groups from food waste decreased dramatically especially in control treatment due to the decomposition

of organic substances. The treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) accelerated the decomposition due to more active microorganism activities, breaking organic substances into smaller molecular compounds, which provided source for aromatization. Cyclic N-containing compounds were observed after Day 7 and more complicated structures were found in the treatment with CMHRs, especially in 1:1:1 treatment. The mechanism was that long chain fatty acids (C15 to C26) mainly from FW and lignin in CMHRs were broken down into smaller molecular mass along the composting process and provided the essential core frames for polymerization of humus with complicated ring structures. More aromatic ring structures were observed at a later stage of the composting process, which could be a good indicator of composts maturity. In brief, the treatment with dry weight ratio 1:1:1 (FW: SD: CHMRs, dry wt. basis) had greatest humification degree with more cyclic structures and stable final products at the end of composting.

### 8.4 Investigation of the potential impact of antibacterial/antifungal substances in mature composts with CMHRs in both abiotic and biotic aspects

Both humification process and antipathogenic effect of composts were inseparable related to microbial activities during composting (Smidt et al., 2008).

Microbial populations in all treatments were similar since they shared the same environmental source at the initial composting phase, while the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) contained least bacteria and fungi in mature phase. The reason was due to higher amount of bioactive components with antipathogenic effects and higher lignin content in the CMHRs making it difficult for microbes to mineralize it as nutrient substrate for microbial growth. The results of $MIC_{50}$ indicated that the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) required least concentration of composts extraction to kill half quantity of the phytopathogens, 16% for A. *solani* and 22% for F. *oxysporum* extracted by acetone. The phytopathogen suppression capacity of composts was partially due to antagonistic abilities from some of the isolated microorganisms as well as the functions of bioactive compounds.

To investigate the antipathogenic effect in composts, 17 kinds of prevalently visible bacteria and 22 kinds of fungi were isolated from composts in various phases and both antagonistic and abiotic influences were monitored (Chapter 5). It was revealed that isolated microbes from treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) had powerful properties of nitrogen fixing (*Bacillus*, *Bacterium*, *Alcaligenes* and *Streptomyces*), fermenting (*Clostridium* and *Streptomyces*) and degradation on lignin derivates (*Talaromyces*), all of which promoted composting maturity and humification process. Their chitinolytic character and the ability of antifungal proteins production

promoted their antagonistic characters against soil-borne phytopathogens (Cui et al., 2012; Khamna et al., 2009). It is also confirmed that the treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) showed most powerful antipathogenic effect on A. *solani* and F. *oxysporum* due to high population of *Aspergillus* which are widely known to interfere/compete with the growth and/or survival of plant pathogens as biological control due to its ability to distribute mycelia, occupying greater interaction surface as well as its lignin degradation property. Therefore, CMHRs compost amendments effectively modified microbial community composition and enhanced the competition and/or antagonism among microorganisms, leading to a reduction in phytopathogenic activity.

As shown in the comparison (Table 5.4), the interfere/compete between antagonistic microorganisms and target pathogens were more powerful than individually influenced by chemical compounds. However, the influencing factors should not be considered independently since antagonistic interactions between microbes in composts and phytopathogens are highly dependent on the abiotic properties of the composts and the alternative environment. Therefore, further analysis of bioactive components in mature composts will be monitored in order to follow the mechanism of phytopathogenic suppression.

## 8.5 Monitoring the dynamics of bioactive components during co-composting of food waste and CMHRs

Suppressive capacity on phytopathogens is one of the major function of mature composts and the antipathogenic effect was stimulated when CMHRs was used as the bulking agent in composting process based on previous experiments (Chapter 5). The abiotic inhibitory rates of treatment 1:1:1 (FW: SD: CHMRs, dry wt. basis) indicated that more powerful bioactive components were remained at the end of composting than in the treatment 5:5:1 (FW: SD: CHMRs, dry wt. basis) and control which had no CMHRs but plastic beads as the bulking agent. Hence sensitive and comprehensive analytical technique of ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-QTOF-MS) was utilized to acquire a better understanding of the complicated structures of final composting products (Chapter 6). Sample preparation method was optimized by using ultrasonicated extraction and acetone as the solvent. Optimized chromatographic conditions were achieved by using positive mode ionization with mobile phases as water and acetonitrile, both of which containing 0.1% (v/v) formic acid. Samples (5 μL) were delivered at 0.35 mL/min according to the following linear gradient: 0-5 min, 5-15% B; 5-20 min, 15-100% B; isocratic 100% B for 5 min then back to 5% B within 5 min. Mass spectra were

acquired by full scan from m/z 100-1000.

Twenty-two kinds of bioactive compounds with antibacterial/antifungal properties were only obtained in the treatments with CMHRs addition while 17 kinds of compounds with higher contents were shared in all treatments during composting, which should be derived from food waste and believed to be the abiotic factor of antipathogenic effect in food waste compost. Most of the bioactive compounds contents were dramatically reduced during composting due to their photolytic and pyrolytic properties (Meng et al., 2014; Wang et al., 2010). Among the 22 kinds of bioactive compounds with antipathogenic properties found only in the treatments with CMHRs addition, of which (3'R, 4’R)-3'-epoxyangeloyloxy-4'-acetoxy-3', 4’-dihydroseselin and linolenic acid were observed in the highest quantities in the mature compost of treatments with 1:1:1 and 5:5:1 (FW: SD: CHMRs, dry wt. basis), respectively. The aqueous stability of coumarin dimers could be the reason providing higher availability of this compound (Dragojevic et al., 2011).

There were 21 identified bioactive components positively and significantly correlated with inhibition of *A. solani* / *F. oxysporum*, which were mainly from the groups of alkaloids, flavonoids and coumarins. All of them have antipathogenic abilities except 2,3,4-Trimethyl-5-phenyloxazolidine. Alkaloids can be widely found in vegetables and TCM which are inhibitory to fungal and bacterial pathogens and

induce apoptosis by the mechanisms resulting from their ability to interact with phytopathogenic proteins and DNA (Hu et al., 2014). The antifungal effect of flavonoids can be explained by permeability change of cytomembrane and cell wall fragility induced by PMFs inhibiting chitin synthase (Zhao et al., 2011). Better water solubility and bioactivities of derivatives of coumarins indeed provide significant potential to the bacterial/fungal control (Li and Chan, 2013). In addition to the light-dependent mechanism of fungal control, coumarins are inhibitors of the ATP hydrolysis and DNA supercoiling reactions catalyzed by DNA gyrase. Therefore, ATP hydrolysis of phytopathogen is blocked at the Gyrase B subunit (Sardari et al., 2000).

### 8.6 Investigate the quality of final composting products derived from food waste and CMHRs co-composting; as well as its effects on the growth of plants and inhibition of soil phytopathogenic microorganisms

Results from previous experiment indicated that powerful antipathogenic effect of mature CMHRs compost were derived from bioactive compounds in CMHRs (Chapter 6); therefore in the last phase, the mature CMHRs composts (FW: SD: CHMRs of 1:1:1, dry wt. basis) was applied to soil inoculated with *A. solani* and *F. oxysporum* with various application rates in order to investigate the pathogenic

inhibition efficiency (Chapter 7). This study showed the initial pH and total nitrogen content as well as crop yields were increased with the addition of mature CMHRs composts to acid soil, and 5% CMHRs compost was the optimum application rate, while at the higher application rate of 10% (dry weight basis, w/w) plant growth was inhibited which might be due to the higher salt contents. According to the biomass results, *Brassica chinensis* was more sensitive to the inhibitory effect of phytopathogen inoculation, while nutrient supply was to a less extent due to the short growth period as compared to *Lycopersicon esculentum*. During nitrification under microbial activities, $NH_4^+$ was degraded into $NO_2^-$ and subsequently $NO_3^-$ which is the final and main form of inorganic N for crop uptake; as well as the less mobile phosphorus transformation between total and extractable forms. Significant reductions $NH_4^+$ and $NO_2^-$ as well as extractable P were observed in treatments 5% CMHRs compost which was due to stronger nutrient absorption abilities of healthier roots as well as sufficient nitrogen and phosphorus sources. It was clearly shown the advantage of using mature CMHRs compost as a soil conditioner to block phytopathogenic infection from plant roots. The copy number of A. *solani* was limited to 2.4 CFU/g of 5% CMHRs compost and 3.0 CFU/g of 10% CMHRs compost, comparing to 5.7 CFU/g of control for *Lycopersicon esculentum*; while 2.9 CFU/g of 5% CMHRs compost and 3.0 CFU/g of 10% CMHRs compost, comparing to 6.5

CFU/g of control on F. *oxysporum* for *Brassica chinensis*). The mechanism was mainly derived from the bioactive components in mature CMHRs compost which effectively inhibited phytopathogenic activities in soil. Thirteen and 11 out of 21 kinds of bioactive compounds were positively correlated to A. *solani* and F. *oxysporum* maintained in soil after planting. Most of these identified compounds were belonged to groups of alkaloids, flavonoids and coumarins which have powerful antifungal and antibacterial abilities and most of them maintained during growth period though their amounts reduced greatly due to their photolytic and pyrolytic properties. Therefore, mature CMHRs compost can be the substitute to reduce the usage of fungicides and its associated environmental hazards. The present study demonstrates clearly the beneficial effects of using CMHRs as a bulking agent to co-compost with food waste with the additional phytopathogens suppression property.

## 8.7 Recommendations for future research

Based on the results obtained, CMHRs can be used as the bulking agent in composting system and dry weight ratio of 1:1:1 (FW: SD: CHMRs) positively enhanced the degradation of organic substances in composting. 5% of mature CMHRs compost improved growth of *Lycopersicon esculentum* and *Brassica chinensis* as well

as eliminated phytopathogens (A. *solani* and F. *oxysporum*) after land application due to both impact factors of antagonism and bioactive compounds. Further studies are needed to investigate the co-composting of food waste and CMHRs in pilot scale by using composter, for instance dealing with waste 100 kg/day. Besides, potential toxic effect of CMHRs compost in land application can be another possible research area since it is said that medication always has a side effect. Further research can make sure the plants are safely edible by using the optimum application rate of CMHRs compost as biofertilizer. Moreover, a wider range of broad spectrum soil borne phytopathogens should be carefully considered. Additionally, bioactive compounds are the main factors to inhibit phytopathogenic growth; however most of them disappeared during planting due to their photolytic and pyrolytic properties (Hu et al., 1999). Further work can focus on how to preserve these components for longer time in soil.

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