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ชื่อโครงการ Biodegradation of leaf compost by thermophilic cellulolytic bacteria isolated from compost

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

การย่อยสลายทางชีวภาพของปุ๋ยหมักใบไม้โดยใช้แบคทีเรียย่อยสลายเซลลูโลสที่ชอบอุณหภูมิสูง ซึ่งคัดแยกจากกองปุ๋ยหมัก

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Biodegradation of leaf compost by thermophilic cellulolytic bacteria isolated from compost

Miss Chavisa Sathirawisankit

Project Submitted in Partial Fulfillment of Requirements for
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By	Miss Chavisa Sathirawisankit
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Project Advisor	Supawin Watcharamul, Ph.D.

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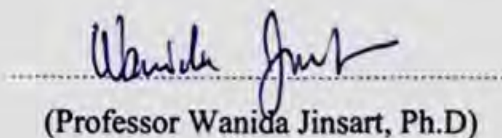
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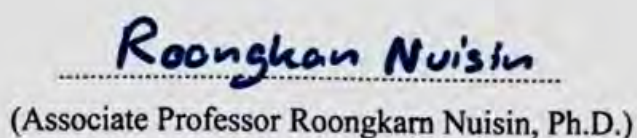
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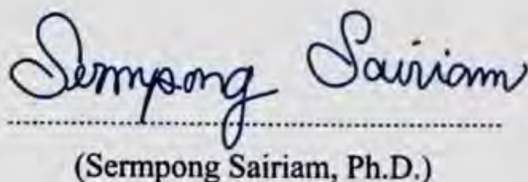
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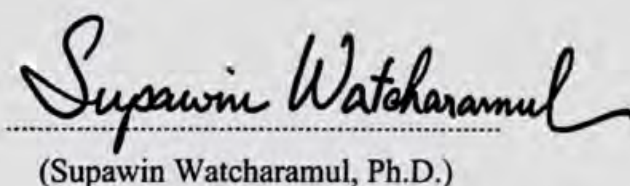
(Supawin Watcharamul, Ph.D.) Project Advisor

ชวิศา สถิรวิสาลกิจ : การย่อยสลายทางชีวภาพของปุ๋ยหมักใบไม้โดยใช้แบคทีเรียย่อยสลาย
เซลลูโลสที่ชอบอุณหภูมิสูงซึ่งคัดแยกจากกองปุ๋ยหมัก
อาจารย์ที่ปรึกษา : อ.ดร.ศุภวิน วัชรมูล

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อคัดแยกและคัดเลือกแบคทีเรียมีความสามารถในการย่อยสลายเซลลูโลสได้ที่อุณหภูมิสูง และเพื่อประเมินประสิทธิภาพของแบคทีเรียสายพันธุ์ที่ผ่านการคัดแยกและคัดเลือก ในการย่อยสลายปุ๋ยหมักใบไม้ แบคทีเรียถูกคัดแยกจากปุ๋ยหมักใบไม้ 4 ชนิด ประกอบด้วยปุ๋ยหมักอายุ 7 วัน 1 เดือน 2 เดือน และ 3 เดือน โดยเป็นปุ๋ยหมักจากใบไม้ที่เก็บรวบรวมจากภายในจุฬาลงกรณ์มหาวิทยาลัย จากการคัดแยกเชื้อแบคทีเรียโดยใช้อาหารเลี้ยงเชื้อแข็งที่มีคาร์บอกซีเมทิลเซลลูโลสเป็นแหล่งอาหารสำหรับจุลินทรีย์ ได้แบคทีเรียมาทั้งสิ้น 79 สายพันธุ์ หลังจากทำการคัดเลือกแบคทีเรียที่มีความสามารถในการย่อยสลายเซลลูโลสโดยการย้อมสีอาหารเลี้ยงเชื้อแข็งด้วยสารละลายไอโอดีน พบว่ามีแบคทีเรีย 44 สายพันธุ์ที่สามารถย่อยสลายเซลลูโลสที่อุณหภูมิสูงได้ โดยอุณหภูมิซึ่งใช้ในการคัดและคัดเลือก ตลอดจนการทดสอบการย่อยสลายกำหนดไว้ที่ 60 องศาเซลเซียส แบคทีเรียที่สามารถย่อยสลายเซลลูโลสได้สามารถสังเกตได้จากการปรากฏวงใสรอบโคโลนีของแบคทีเรีย ค่าความสามารถในการย่อยสลายซึ่งคำนวณจากขนาดเส้นผ่านศูนย์กลางของวงดังกล่าวของแบคทีเรียทั้ง 44 สายพันธุ์ถูกนำมาวิเคราะห์ทางสถิติเพื่อจัดลำดับความสามารถในการย่อยสลายของแบคทีเรีย พบว่ามีแบคทีเรียทั้งสิ้น 11 สายพันธุ์ที่มีค่าความสามารถในการย่อยสลายสูงกว่าควอร์ไทล์ที่ 3 และแบคทีเรียสายพันธุ์ D08 มีค่าความสามารถในการย่อยสลายสูงที่สุด โดยมีค่าเท่ากับ 4.92 แบคทีเรียทั้ง 11 ชนิดถูกเลือกเพื่อทำการศึกษากิจกรรมของเอนไซม์เซลลูเลส เพื่อประเมินว่าแบคทีเรียสายพันธุ์ใดมีความสามารถในการย่อยสลายสูงที่สุด และจะถูกนำมาใช้ในการประเมินประสิทธิภาพในการย่อยสลายปุ๋ยหมักใบไม้ แต่เนื่องจากสถานการณ์การแพร่ระบาดของเชื้อไวรัสโควิด-19 ในประเทศไทยทำให้ไม่สามารถดำเนินการศึกษาต่อจนบรรลุวัตถุประสงค์ที่ตั้งไว้ได้ครบ

คำสำคัญ: แบคทีเรียย่อยสลายเซลลูโลสที่ชอบอุณหภูมิสูง, ปุ๋ยหมักใบไม้, การย่อยสลายทางชีวภาพ

Chavisa Sathirawisankit : Biodegradation of leaf compost by thermophilic cellulolytic bacteria isolated from compost
Project Advisor : Supawin Watcharamul, Ph.D.

This study aims to isolate and screen bacteria with degrading cellulose at high temperature capacity and to investigate the effectiveness of isolated bacteria in fresh leaf compost degradation. Bacteria were isolated from 4 types of leaf compost consisting of compost Day 7, Month 1, Month 2, and Month 3 which the main raw material of composting was leaf debris collected from Chulalongkorn University. Bacterial strains were isolated using carboxymethyl cellulose (CMC) media, and 79 bacterial strains were obtained. After that, cellulolytic bacteria were screened using Gram's iodine technique. There were 44 strains of bacteria can decompose cellulose at high temperatures (60ºC). Cellulolytic bacteria were screened by observing the presence of clear zone around each bacterial colony. Hydrolysis capacity (HC) values, which is calculated from the diameter of the clear zone of all 44 strains were analyzed statistically to rank HC values from all bacteria strains. It was found that 11 bacterial strains had greater HC value than the third quartile and D08 showed the highest HC value at 4.92. All 11 bacterial strains were selected for the cellulase assay to assess which bacterial strain had the highest degradability and used for investigating the efficiency in fresh leaf compost decomposition. Due to the epidemic of Covid-19 in Thailand unfortunately, this study was unable to achieve all the proposed objectives.

Keywords: thermophilic cellulolytic bacteria, leaf compost, biodegradation

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Chavisa Sathirawisankit

CHAPTER 1

INTRODUCTION

At Chulalongkorn University, there is a large quantity of leaf debris as lignocellulosic waste each day. Poor management and disposal may lead to various problems such as visual pollution, bad odors, and pathogen increase due to the leaf spoilage. Improper burning is also causing air pollution (particulate matter and smoke) and residual ash. Composting is the method to manage large amounts of leaf debris at Chulalongkorn University. From the interview, leaf debris is collected from various areas in the university, brought into the grinder to a small size to reduce the time to decompose, and mixed with soil, manure and, bio-fermented extract. The composting process usually takes around 3 months before the compost can be used as a fertilizer for any gardening purposes in the university.

Composting is a biological degradation of organic substrates carried out by microbial population under humidity, solid-state, self-heating, and aerobic conditions (Baharuddin *et al.*, 2010; Liu *et al.*, 2011) to produce carbon dioxide, water, minerals, and stable organic material called compost (Insam and de Bertoldi, 2007). During composting, there are 4 phases occur: (1) mesophilic (25-40°C) which easily degradable compounds like sugar and protein are degraded by microorganisms leading to a rapid increase in temperature; (2) thermophilic phase (35-65°C) which decomposition continues rapidly; (3) cooling phase (secondary mesophilic phase); and (4) maturation phase (Abdel-Rahman *et al*., 2016; Insam and de Bertoldi, 2007; Liu *et al.*, 2011). Leaf debris can be degraded and mineralized into the form of beneficial nutrients and compounds by the microorganisms in the compost pile.

Cellulose, an important component of leaf debris (Akhtar, Goyal, and Goyal, 2015), is the chain of beta-D-glucose with a polymerization degree of 40,000 and link each glucose molecules by the β-1,4-glycosidic bond (Insam and de Bertoldi, 2007). Cellulose can be degraded by using the group of enzymes consist of (1) endo-1,4-β-

glucanase, randomly hydrolyzes the β-1,4 glycosidic bond creating a long chain with free ends; (2) exo-1,4-β-glucanase, separates long chains into cellobiose; and (3) β-glucosidase, cleavages cellobiose into glucose monomers (Insam and de Bertoldi, 2007; Salah, Ibrahim, and El-Diwany, 2007). These enzymes are produced from microorganisms living in compost piles which has an ability to utilize cellulose as an energy and carbon source (Baharuddin *et al.*, 2010).

Cellulose is most degraded during the thermophilic phase, during the degradation of the organic substrate, heat is released from the mineralization process causing the rise of temperature inside the compost pile. Biodegradation at high temperatures occurs mainly by bacterial activities because other types of microbes, especially fungi, are unable to withstand temperatures that may reach 70ºC (fungi usually inactive at temperatures above 55ºC). The thermophilic phase has many advantages, such as improving the hydrolysis reaction of cellulosic substrates, higher mass-transfer rate leading to higher substrate solubility, increase the decomposition rate which the temperature increases by 10°C will cause a double degradation rate (Salah, Ibrahim, and El-Diwany, 2007), and the reduction of the contamination risk with other microbes, especially pathogens (Rastogi *et al.*, 2010). Thermophilic bacteria, the domain group during the thermophilic phase, is capable of performing and producing thermo-tolerance enzymes which cause an increase in degradation rate, less the amount of enzyme needs, and longer half-life (Baharuddin *et al.*, 2010).

The conventional spontaneous composting process can take several months (Liu *et al.*, 2011), various methods have been developed to reduce timing and enhance composting processes such as mechanical-biological composting, co-composting using additive and microbial inoculums (Gabhane *et al.*, 2012). In addition to time requirement, the large amount of leaf debris and limited composting area makes large quantities of leaf debris have not been processed yet. The inoculation of effective cellulose-degrading bacteria isolated from the same compost pile is of interest because it makes the process efficiently in the term of composting time and compost quality (Abdel-Rahman *et al.*, 2015) and unnecessary require electricity to operate composting reactor, additive, or external bacterial strain that might be effected to local bacterial community. *Geobacillus* spp. is a dominant bacterial species that can be isolated from compost and

has high efficiency to decompose cellulose at high temperatures (Acharya *et al*., 2011; Baharuddin *et al*., 2010; Rastogi *et al*., 2010; Sarkar *et al*., 2010). Fang *et al.* (2001) and various studies have reported the effect of inoculation with thermophilic cellulolytic bacteria to enhance biological activity and biodegradation efficiency, and lead to a rapid decrease in C/N ratio which an indicator of compost maturity.

For the reason mentioned, this study aims to use leaf compost as the source of effective thermophilic cellulolytic bacteria and determine the efficiency of isolated bacteria in fresh leaf compost degradation.

1.1 Objectives

1) To isolate and screen thermophilic cellulolytic bacteria from leaf compost.
2) To investigate the efficiency of using isolated bacteria in fresh leaf compost degradation.

1.2 Scope of study

Leaf compost samples were collected from leaf compost piles at Chulalongkorn University and used as the source of bacteria and raw material for degradation study. The criteria used for microorganism selection are hydrolysis capacity (HC) value and cellulase activity of each bacterial isolate. In leaf compost biodegradation study, parameters that used to detect the degradation progress are total organic carbon (TOC), total Kjeldahl nitrogen (TKN), and C/N ratio. This study duration was between August 2019 – May 2020 (10 months).

1.3 Expected outcome

Effective thermophilic cellulolytic bacteria could be obtained from leaf compost and has an efficiency to degrade fresh leaf compost.

CHAPTER 2

LITERATURE REVIEW

2.1 Composting

Composting is a decomposition process of organic material by various microbial populations under moist, aerobic, and controlled conditions. During the process, microorganisms consume oxygen and break down organic materials (e.g. carbohydrates, proteins, cellulose, and lignin) into carbon dioxide, water, inorganic compounds, and stable organic material called compost. Composting is a self-heating process, a heat generated by decomposition reactions and microbial activities is also released from a compost pile. Composting can use to handle organic waste such as industrial wastes, municipal wastes, yard wastes, and even though food wastes. Biodegradation process in composting leads to a decrease in the volume of waste and produces a useful product. Appropriate design and management of composting can control odors, bioaerosols, or other problems that may be an environmental impact. In addition, separating organic waste from other wastes for composting can also reduce the amount of waste to landfill. (Epstein, 1997).

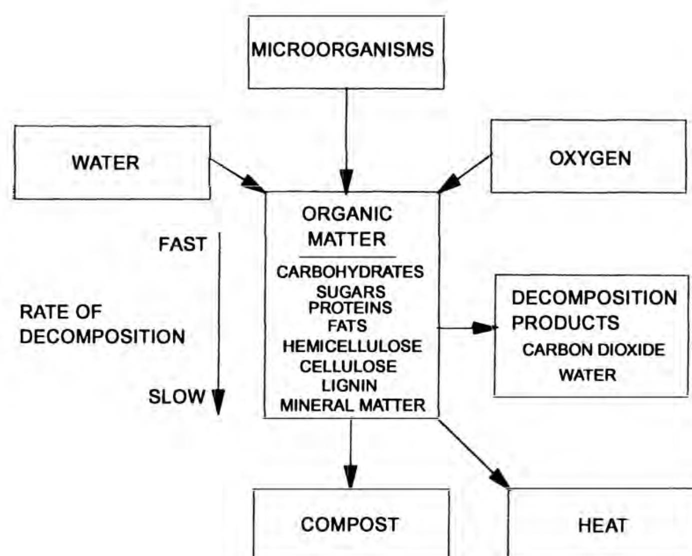

Figure 2.1 The composting process (Epstein, 1997)

2.1.1 Factors affecting composting process

There are many factors affect the composting process and some factors are also affect by the process progress.

2.1.1.1 Oxygen and aeration

Oxygen is essential for microbial activities and a transformation of an organic compound. Minimum oxygen content is 5% of pore space in compost (Pace, 1995). In condition with limited oxygen, organic materials degradation tends to be anaerobic that causing odors (from acid, methane, and other volatile compounds production). Aeration may be necessary in some cases in order to maintain a proper oxygen concentration in the compost pile. However, the aeration must not be too much because it affects to a reduction of the temperature heat and moisture loss out of the compost pile (Guo *et al*., 2012) and may result in degradation activities.

2.1.1.2 Temperature

Temperature is a major factor in the composting process as it determines a group of microorganisms that degrade organic materials in a compost pile. A change of temperature in the composting process is a function of degradation activities. There are two ranges of temperatures that occur the process; mesophilic and thermophilic (over 45ºC). Temperature is rising in the initial of the process, leads to microbial population changing from mesophilic microbes to thermophilic microbes and affects to organic materials decomposition rate. Previous studies have reported various optimum temperature for effective degradation, the maximum decomposition rate is in temperatures range of 50-60ºC. In addition to decomposition rate, high temperature can destroy pathogens, weed seeds, and fly larvae in composting material resulting in sanitation of compost (Avnimelech *et al*., 2004).

Temperature is related to aeration rate and moisture content. Excessive aeration and moisture lead to a reduction in temperature that affects microbial activity, composting time, and compost maturity.

2.1.1.3 Moisture

Water is produced during biodegradation of organic matter and microbial activities resulting in the moisture of compost and loss through evaporation. Moisture content plays an important role to dissolve nutrient and mineral elements that necessary to metabolic activities of microorganisms (Guo *et al*., 2012). The optimum moisture content for composting is in a range between 40-65% (Pace, 1995). Lower moisture has effects on solubility of nutrient, viability, and movement of microorganisms. On the other hand, moisture more than 60%, small pores between compost particles is be replaced by water which affects the air movement within the compost pile. The limit of air movement resulting in a lack of oxygen concentration leads to an anaerobic condition. During composting, generated heat and water evaporation make compost material dry out. Proper management is necessary by watering the compost to maintain the moisture content in the optimum range (Richard *et al*., 2002). Apart from oxygen and airflow, moisture directly affects compost temperature. Wiley and Pierce (1955) have reported the highest temperature was achieved at 55-69% moisture content while 72-77% moisture resulting in the lowest temperature (Epstein, 1997).

An important factor affects moisture content is aeration. Excessive aeration causes moisture loss from compost. From the information data mentioned above, there is relationship between aeration, temperature, and moisture content. Therefore, proper management is required to balance the optimum range of 3 factors to achieve the most effective composting.

2.1.1.4 C/N ratio

Carbon and nitrogen are important nutrients for microorganisms. Microbes utilize carbon as a source of energy for cellular growth and microbial activities, while nitrogen is a major element for protein production. Carbon and nitrogen are provided to microbial communities from organic material degradation. Carbon is the main component of all organic substrate, but the amount of nitrogen varies according to a type of raw material (for example, food wastes has higher nitrogen content than yard wastes). The ideal C/N ratios of raw material for composting are between 25:1 to 30:1. C/N ratios below 20:1, nitrogen is rich, carbon is utilized rapidly resulting in insufficient amounts to stabilize nitrogen. In this case, nitrogen is transformed into ammonia which causing an unpleasant odor. For C/N ratios above 40:1, the amount of nitrogen is not enough for the microbial growth and activities of microbes, especially enzymes production which plays an important role in organic material decomposition (Epstein, 1997; Pace, 1995).

2.1.1.5 Raw material

Type of raw materials and their characteristics are related to particle size, moisture content, and C/N ratio. Rate of raw material decomposition depends on the particle size, smaller particles degrade easily than large ones because there are higher surface areas for attach by microbes and degrade by enzymes produced from microbes. However, the material size that is too small may reduce the porosity of the compost leads to a lack of air movement within the compost pile. Pace (1995) has mentioned, the optimal particle diameter for composting is approximately 1/8-2 inches. That is why in some cases, raw materials have to be put into a grinder to minimize the diameter or mix raw material with high porous materials such as chopped coconut shells or saw dust.

2.1.2 Phases of composting process

The progress of an enclosed composting process can be divided into 4 phases, using a different temperature in each phase as a basis (Insam and de Bertoldi, 2007).

1) Mesophilic phase: The first phase called the mesophilic phase which the temperature around 25-40ºC. In this phase, easily degradable organic materials such as sugars; small molecular carbohydrates; and proteins are degraded by primary decomposer including fungi; actinobacteria; and bacteria. The main microbial populations that function during this phase are mesophilic microorganisms. In addition to biodegradation by microbes, in this phase, organic material undergoes physical degradation by mesofauna such as worms; mites; and millipedes, resulting in the materials becoming smaller; more surface area; and more easily to degrade. The decomposition and microbial growth results in a rapid increase in temperature within the compost pile and entering the thermophilic phase

2) Thermophilic phase: In the second phase of composting, the temperature in the thermophilic phase approximately 40-65ºC. When the temperature rises, mesophilic microbes are inactive and some of them die off. Group of microbes which play a role in degradation in this phase are the microbes that can survive and function at a high temperature referred to thermophilic microorganisms. The decomposition during the period occurs rapidly causing the temperature rising to achieve 60ºC. At this temperature, actinobacteria and thermotolerance and thermophilic bacteria is the main decomposer because fungi are able to function at the temperature of 35-50ºC. A special advantage of the thermophilic phase is hygienization. At high temperature, pathogens, weed seeds, and insect larvae are destroyed.

3) Cooling phase (secondary mesophilic phase): In the third phase of composting, the temperature drops to below 40ºC. At lower temperatures, mesophilic bacteria with surviving spores recolonize and play a role in degradation. In this phase, the most common group of microbes are bacteria and fungi that degrade starch and cellulose.

4) Maturation phase: The last phase of composting, organic materials are almost completely decomposed except recalcitrant and stable compounds such as lignin and humus. Normally, compost in this phase has a large proportion of fungi and low bacterial population. Mature compost which ready to an application is rich in required plant nutrients in available form, active carbon to degradation is low, and plant and human pathogens are absent. Ideal C/N ratio of mature compost is around 10. Observed physical characteristics of effective compost are dark brown or black, felling small granular; spongy; or fine fibrous, and smell of mold or like good soil (Mathur, 1998).

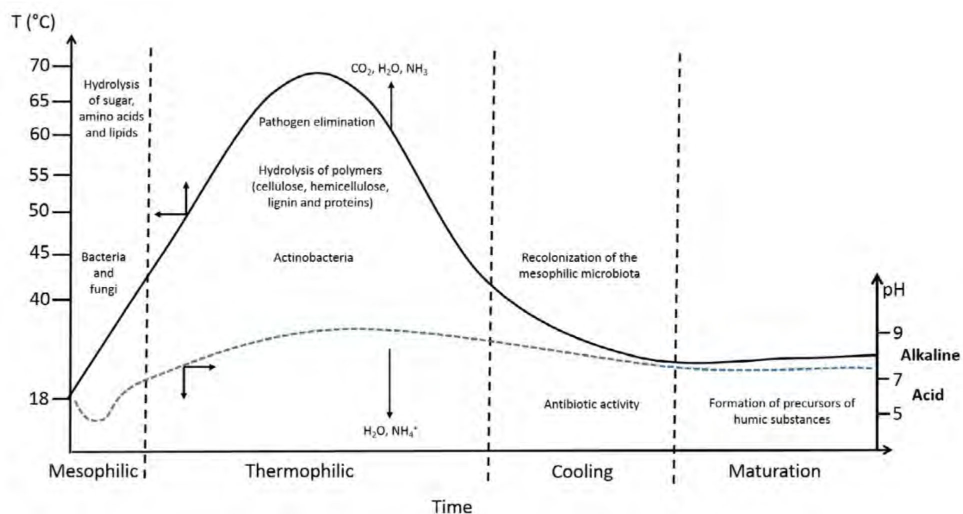

Figure 2.2 The change of temperature during composting process

(Sánchez, Ospina, and Montoya, 2017)

2.2 Microorganisms in compost

2.2.1 Actinobacteria

Actinobacteria or actinomycetes are filamentous Gram-positive bacteria which are discernible by naked eye in think mats and play a crucial role to degrade lignin and complex cellulose. Actinobacteria can grow at neutral and alkaline pH in mesophilic and thermophilic conditions but can grow and function well in a temperature range of 50-60ºC, so actinobacteria can be assumed that they are thermophilic or thermotolerance microorganisms. During the thermophilic phase, actinobacteria work with bacteria to decompose lignin and cellulose (available carbon) into unavailable carbon. Actinobacteria

that found in compost comprise *Saccharomonospora viridis*, *Streptomyces thermovulgaris*, *S. lincolnesis*, *S. variegatus*, *S. lusitanus*, *Actinobifida chromogena*, *Thermoactinomyces bulgaris*, *Micropolyspora faeni*, *Pseudonocardia thermophila*, *Thermomonaspora curvata*, *Th. viridis*, *Th. sacchari*, *Micromonaspora carbonacea*, and other (Insam and de Bertoldi, 2007; Sánchez *et al*., 2017). Kausar *et al*. (2011) studied on rice straw degradation by actinobacteria by isolating actinobacteria from rice straw compost and found that *M. carbonacea* was the best in degradation. Moreover, the inoculation of this actinobacteria species to a large scale of the composting process can reduce the process time.

2.2.2 Bacteria

Bacteria play an important role in organic matter decomposition in compost production. Various communities of bacteria are found at different phases of the process, which vary according to the temperature inside the compost pile and difference in functions. At the beginning of the process, mesophilic bacteria such as *Lactobacillus* spp.; and *Acetobacter* spp.is responsible for producing organic acids (Golueke, Card, and McGauhey, 1953). Bacteria are the main group of microbes in the thermophilic phase due to the diversity of the species and large quantities, responsible for decomposition of cellulose which is an important component of raw materials for composting. Various previous studies reported about thermophilic cellulose-degrading bacteria found in compost including *Bacillus stearothermophilus*, *S. brevis*, *B. sphaericus*, *B. subtilis*, *B. licheniformis*, *Bacillus* spp., *Geobacillus* spp., and *Clostridium thermocellum* (Insam and de Bertoldi, 2007). Another important function of bacteria in compost production is the transformation of nitrogen compounds into available forms for plants. During the first mesophilic phase, protein and other nitrogenous compounds are converted into amino acids. Afterward, amino acids are transformed into ammonia through the ammonification process by ammonifying bacteria resulting in pH inside compost pile increases at the beginning of the process. Composting that is an aerobic process, nitrogen-fixing bacteria reduce nitrogen in the atmosphere and convert into ammonia. Pepe, Ventorino, and Blaiotta (2013) have reported N-fixing bacteria isolated from compost to comprise of *Stenotrophotomonas*, *Xanthomonas*, *Pseudomonas*, *Klebsiella*, *Alcaligenes*, *Achromobacter*, and *Caulobacter*. Ammonia is converted into nitrites and

nitrates via the nitrification process by ammonia-oxidizing bacteria (AOB). *Nitrosomonas europaea* has been reported as domain AOB species in the thermophilic phase of composting. Ultimately, nitrite-oxidizing bacteria (NOB) convert nitrites into nitrates which are stable and available products for plants. Moreover, some of phosphate-solubilizing bacteria (PSB) and potassium-solubilizing bacteria (KSB) are found in composting to transform organic P and K into available forms for plant (Sánchez *et al*., 2017).

2.2.3 Fungi

Fungi are most often found in the mesophilic phase (at the beginning and late of the composting). Early in the process, fungi play an important role in the degradation of lignin which is the main component of lignocellulosic materials (raw materials for composting). Fungi also function together with bacteria in cellulose degradation. There are 3 phyla of fungi found in compost, Zygomycetes; Ascomycetes; and Basidiomycetes. From previous studies, it was found that fungi found in the mesophilic phase such as *Mortierella turficola*; *Mucor miehei*; *Chaetomium elatum*; *Armillaria mellea*; *Clitopilus insitus*; *Pleurotus ostreaus*; *Lentinus lepideus*; and *Fomes* sp. However, some species can survive and function in thermophilic conditions (temperature not over 55ºC) such as *Rhizomucor* spp.; *Chaetomium thermophilum*; *Thermoascus aurantiacus*; *Thielavia thermophilia*; *Dactylomyces crustaceus*; and *Aporothielavia leptoderma* (Insam and de Bertoldi, 2007). Fungi play another role to transform organic phosphorus into inorganic forms through phytase (enzyme in hydrolysis of phytate) production. In composting raw materials (plant debris) that contain phytate, fungi responsible to decompose and release orthophosphate (Sánchez *et al*., 2017).

2.3 Cellulose

Cellulose, the most abundant lignocellulosic polymer in plant biomass with approximately 35-50% of plant dry weight, often found in combination with hemicellulose and lignin in the plant cell walls. Aside from plants, cellulose is also produced by some algae, fungi, and bacteria (Akhtar, Goyal, and Goyal, 2015; Huber *et al*., 2012; Lynd *et al*., 2002; Sun *et al*., 2016). Cellulose is a linear syndiotactic homopolymer of D-glucose. Two glucose molecules are combined by β- (1,4)-glycosidic

bonds and generating disaccharide called cellobiose, therefore, cellulose is a polymer with a repeating unit of cellobiose as shown in **Figure 2.3**. The degree of polymerization (DP) is an indicator of the polymer chain length, which widely ranging from 100-14,000 depending on a source of the cellulose. Cellulose structure consists of many hydroxyl groups which can form intra- and intermolecular hydrogen bond within and between cellulose molecules. The hydrogen bonding network resulting in a crystalline structure and makes it insoluble in water and recalcitrant (Suhas *et al*., 2016; Marshall, 1985; Klemm *et al*., 2005). However, some positions of cellulose structure are arranged in a disordered chain, this region called amorphous which can be hydrolyzed easier than the crystalline region.

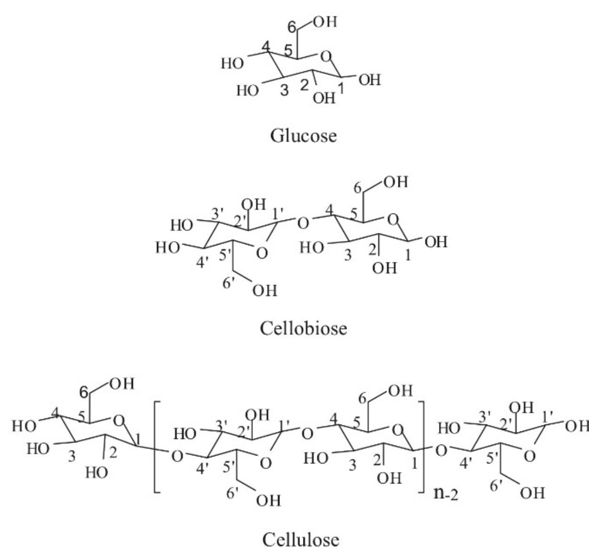

Figure 2.3 Structure of glucose, cellobiose, and cellulose (Suhas *et al*., 2016)

2.4 Cellulose degradation by microorganisms

Cellulose can be degraded by various cellulolytic microorganisms, which are utilized cellulose as cell components and an energy source for cell growth, most belong to fungi and bacteria. Moreover, some anaerobic protozoa and slime mold have an ability to degrade cellulose too. Cellulolytic microorganisms are often found in terms of a consortium with other species in synergistic relationships to complete cellulose degradation to carbon dioxide, water, and other inorganic substance (Béguin and Aubert, 1993). Cellulose degradation occurs by multiple enzymes called cellulase

secreted by cellulolytic microbes as shown in **Figure 2.4**. There are 3 major types of enzymes act on hydrolyzed β-1,4-glycosidic bond of cellulose: (i) endo-β-1,4-glucanases, (ii) exo-β-1,4-glucanases or cellobiohydrolases, and (iii) β-glucosidases (Pérez *et al*., 2002). At first, endoglucanases randomly cut cellulose polysaccharide chain at amorphous region into various lengths of oligosaccharide and releasing new chain ends. The second process, glucose or cellobiose are separated from reducing or nonreducing ends of oligosaccharide by exoglucanase. In addition, crystalline cellulose can also be degraded by exoglucanase. Then, β-glucosidases break down cellobiose into glucose monomer (Lynd *et al*., 2002), and in the end, glucose is converted into carbon dioxide and water. Furthermore, an anaerobic biota such as manure; compost; sewage sludge; soil; and sediment, cellulose can be degraded by anaerobic cellulolytic microbes and produce carbon dioxide; water; and methane as the final product. Beside crystalline structure that affects to cellulose is recalcitrant polymer, there are numerous factors effect on cellulose hydrolysis such as degree of polymerization; surface properties of cellulosic substance; pH; temperature; and nutrients (El-Fadel, Findikakis, and Leckie, 1996).

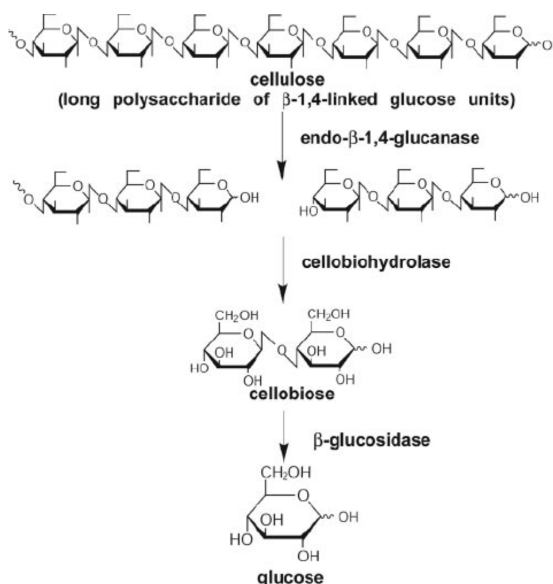

Figure 2.4 Cellulose degradation mechanism involving enzymes (Xie *et al*., 2007)

2.4.1 Cellulose degradation by thermophilic microorganisms

Temperature is one of the factors affects cellulose degradation. El-Fadel *et al.* (1996) have mentioned, a hydrolysis rate constant of cellulosic materials is believed to increase with increasing temperature. However, some cellulolytic bacteria may lose their activities when temperature increase (Jung, Pyong, and Hee, 1999). Thermophilic cellulolytic microorganisms are a great deal of interest dues to they are resistant to high temperatures and did not lose their activities. Thermophilic microbes have an ability to grow at temperature above 50ºC and secrete highly active and thermostable enzymes. Thermostable enzymes have several potential advantages in cellulosic material hydrolysis: (i) improved hydrolysis of the cellulosic substrate leads to shorter hydrolysis time, (ii) higher activity, resulting in decreasing the amount of enzyme needed (iii) higher stability, (iv) risk of contamination decrease, (v) decreased cost and energy for cooling treatment in some industrial process (Bhalla *et al*., 2013; Rastogi *et al.,* 2010; Viikari *et al.,* 2007). Previous studies have reported species of fungi and bacteria produced thermostable enzymes. For example, thermophilic cellulolytic bacteria mostly belonging to *Bacillus*; *Geobacillus*; *Caldibacillus*; *Aciothermus*; *Calsacellum*; and *Clostridium* (Bhalla *et al*., 2013).

2.5 Measurement of cellulase activity

3,5-dinitrosalicylic (DNS) reagent is a method for determination of cellulase enzyme activity by measuring the reducing sugar concentration in a solution. The principle of this method is a cellulose is decomposed by the cellulase generating reducing sugar, 3,5-dinitrosalicylic acid can be reduced to 3-amino-5-nitrosalicylic (ANS) acid by reducing sugar in alkali and heated condition as illustrated in **Figure 2.5**. The solution color changes from a yellow color of the DNS to an orange color of the ANS. The color intensity depends on the concentration of reducing sugar in the solution, which can be measured using spectrophotometer 575 nm. In addition to 3,5-dinitrosalicylic acid, the DNS reagent consists of Rochelle salt (Potassium sodium tartrate), to prevent the reagent from dissolving oxygen; phenol, to maximize the quantity of color; and bisulfite, to stabilize the color in present of phenol (Miller, 1959).

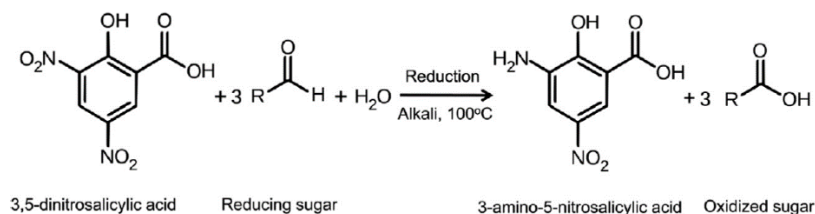

Figure 2.5 The reaction of 3,5-dinitrosalicylic acid with reducing sugar (Thongprajukaew *et al., 2014)*

Moreover, a hydrolysis capacity (HC) value is a basic method that can be used to measure cellulase activity. When culturing cellulolytic microbes on a solid agar which consists of a cellulosic substrate, the microbes can be degraded the substrate as an energy source and create the microbial colony. After flooding the agar plate with colorizing reagent, that can react with the cellulosic substrates, such as Gram's iodine or Congo red, the clearing zone will appear around the colony. The clearing zone can represent the area that the cellulosic substrate is degraded by cellulase secreted from microbes. HC value is calculated as the sum of colony and clearing zone diameter divided by colony diameter (Awasthi *et al*., 2018). Microorganisms with high HC values tend to be high cellulase activity.

2.6 Microbial study

2.6.1 Culture medium

Culture media suitable for the type of microbes are very necessary for cultivation, enrichment of microbial population, and other microbial studies. Culture medium contains required elements and nutrients for microbial growth consist of as follows (Kokare, 2008):

1) Water: Mostly important for the microbial living because it is the main proportion of total cell weight, dissolve the nutrients and other elements, and transport substances into the cells

2) Carbon: Microbes require carbon as an energy source for cell growth and their activity. Microbes can be classified according to the type of energy source requirement such as cellulolytic microbes which can degrade carbon in the form of cellulose for use as an energy source.

3) Nitrogen: Many microbial activities, such as cell division, enzyme production, or protein synthesis, require nitrogen. In culture media, nitrogen is added in the form of protein, amino acid, or inorganic salt. Ingredients such as peptone, yeast extract, and meat extract are the source of nitrogen for some medium.

4) Other elements: Sulphur, phosphorus, sodium, potassium, magnesium, iron, and calcium (in the form of inorganic salt) are added into the media in small quantities. Some of these elements are components of biomolecules in the microbial cells and some of which are involved in activities within the cell.

2.6.2 Streak plate technique

Streak plate technique is used to isolate bacterial colonies from mixed populations. Bacterial colonies are streaked on agar surface by using a wire loop. Firstly, the loop is sterilized by burning with fire until the loop is red and allow it cool. The colony is picked on the loop and close parallel streak on the plate as area 1. For area 2 and 3, the loop is sterilized and let it cool again. After streaking for 4-5 times as shown in **Figure 2.6** and incubate the plate, a single colony of bacterial strain appears. The colony may be picked and re-streak again to isolate pure culture.

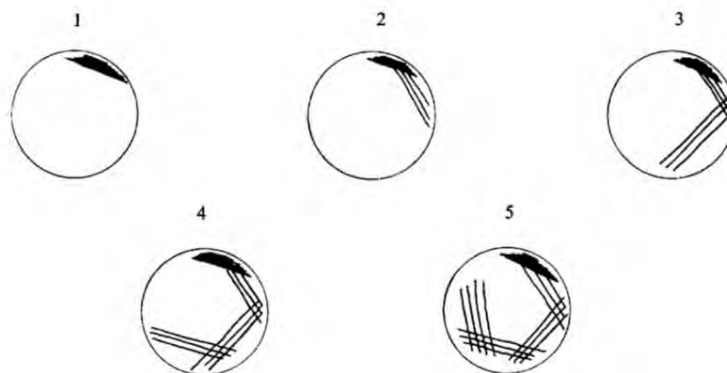

Figure 2.6 Streak plate technique (Harrigan and McCance, 1966)

2.6.3 Morphology study

2.6.3.1 Colony morphology

The bacterial colonies can be observed by culturing bacteria on solid media. Shape (shape of the colony seen from the top view), elevation (lifting characteristic observed by the side view), and margin (edge of the colony) are main elements reported in colonial morphology studies as shown as **Figure 2.7**. However, there are other elements that are considered consisting of size (colony diameter); chromogenesis (color of colony); opacity (transparent, translucent, or opaque); surface texture (smooth, rough, dull, or glistening); consistency; emulsifiability; and odor (Harrigan and McCance, 1966).

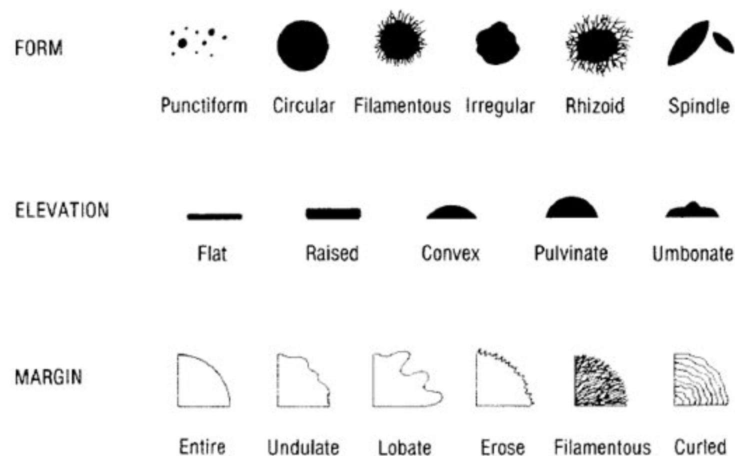

Figure 2.7 Type of bacterial colony (Pelczar, 1957)

2.6.3.2 Cell morphology

Each bacterial species has its own specific cell characteristics which can be observed by staining bacterial cell and observe through a high magnification microscope.

Shape of bacteria

Bacterial cell shape can be divided as follows (illustrated in **Figure 2.8**)

1) Cocci: Small bacterial cells with round, oval or spherical shape (from kokkos meaning berry in Greek).

2) Bacilli: Stick-like or rod-shaped cells with rounded or swollen ends (From baculus meaning rod). In some case in which the width and length of cells are equal, this type of bacteria is called coccobacilli.

3) Vibrios: Curve rod or comma-shaped cells

4) Spirilla: Longer rigid rod cells with a curved shape to a corkscrew-like or coil spiral.

5) Spirochetes: Flexuous, more coiled, and motile spiral cells.

6) Actinomycetes: Branching filamentous bacteria.

7) Microplasma: Round or oval shaped cell with interlacing filamentous.

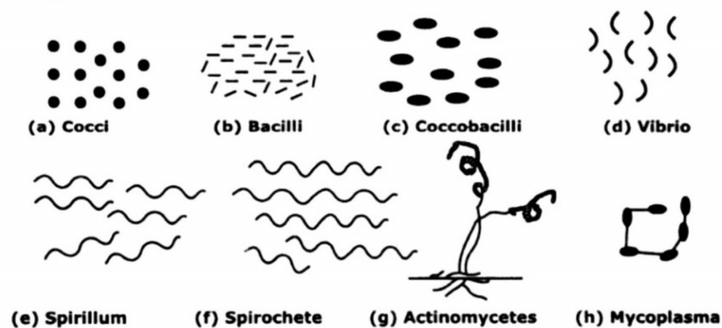

Figure 2.8 Bacterial cell shape (Kokare, 2008).

Cell arrangement

According to cell division of bacterial cells, there are a variety of cell arrangements. Cocci and bacilli are the bacteria that arranged in many different forms, as shown in **Figure 2.9**.

1) Arrangements of cocci

a. Diplococci: Cells divide in one plane and join as a pair.

b. Streptococci: Cells divide in one plant and arrange in a long chain.

c. Tetracocci: Cells divide in two planes and arrange in a group of four cells.

d. Staphylococci: Cells divide in three planes and arrange in irregular cluster.

e. Sarcinae: Cells divide in three planes and arrange in a group of eight in cubic form.

2) Arrangements of bacilli

a. Diplobacilli: Cells arrange as a pair.

b. Streptobacilli: Cells arrange in a long chain.

c. Palisades: The bent at the point of division resulting in cells resemble as a picket fence or Chinese letter structure.

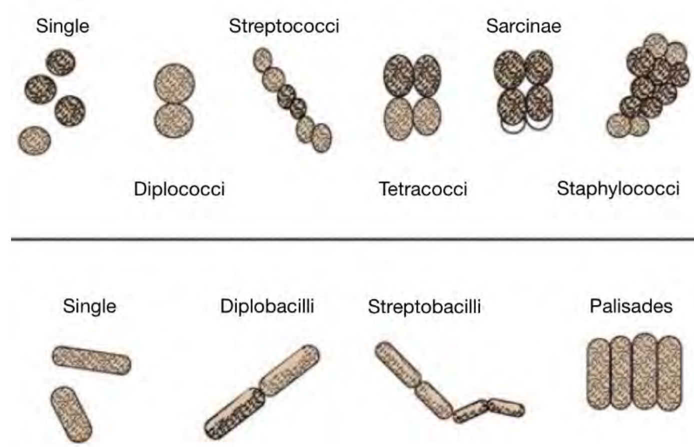

Figure 2.9 Arrangements of cocci and bacilli (adapted from www.brainkart.com)

2.6.3.3 Gram's staining method

Gram's staining is a basic method that can classify bacteria into 2 types, Gram-positive bacteria and Gram-negative bacteria, by using the difference of color attached to the bacterial outer membrane. The cell wall of Gram-positive bacteria contains a layer of peptidoglycan that can be attached by crystal violet dye and observed as purple color on the cell wall by using a microscope. On the other hand, the cell wall of Gram-negative bacteria is lipopolysaccharide layer, the color of crystal violet in this layer can be washed off by decolorizing solution (alcohol or acetone). When dyeing with Safranin, the outer membrane of Gram-negative bacteria is observed to be red (Beveridge, 2000). Gram's staining procedure is described in section **3.7**.

2.7 Related research

Traditional composting process takes several months, microbial inoculum is one method that can increase the decomposition rate and reduce the time takes in the process. Jusoh, Manaf, and Latiff (2013) studied the effect of effective microorganisms (EM) on rice straw composting. They hypothesized that EM can increase the decomposition rate, mineralization process, and enhance microbial activities. The study has shown that the decomposition rate and mineralization of compost with EM (C_1) are higher than without EM (C_2) due to a decrease in total organic carbon (TOC) and C/N ratio. TOC in C_1 was sharply decreased between day 15-30 while the decreasing rate is lower in C_2. At the end of the study, the TOC in C_1 and C_2 was decreased by 49.1% and 36.3%, respectively. C/N ratio in the range of 10-20 indicates compost maturity. C1 compost was faster mature than C2, C/N ratio at day 45 of the process of C1 and C2 compost are around 15.0 and 25.0, respectively. In addition, nitrogen; potassium; and phosphorus content of compost applied with EM is significantly higher than that compost without EM ($p < 0.05$) which can be assumed that EM produce higher quality compost.

From the advantages of biodegradation of lignocellulosic material at high temperatures mentioned in **2.4.1**. Thermophilic cellulolytic microorganisms and their thermotolerance enzymes are widely applied in composting. Thermophilic bacteria are more interesting than other thermophilic microbes because they can survive and active at high temperatures and a variety of species.

Acharya *et al*. (2012) studied the species of thermophilic cellulolytic bacteria isolated from a compost pile. All compost samples were collected at a thermophilic state (temperature more than 50ºC), serial diluted, spread on CMC agar plate, and incubated at 55ºC. One percent of Congo red was used to determine the isolated that can hydrolyze cellulose in agar. Bacterial isolates were identified by the method described in Bergey's Manual of Systematic Bacteriology. They reported that *Amphibacillus* spp., *Bacillus licheniformis*, *Bacillus* spp., *Bacillus subtilis*, *Cellulomonas cellulans*, *Clostridium* spp., *Geobacillus* spp., and *Penibacillus* spp. are

bacterial strains obtained from the compost which Bacillus subtilis have the highest clearing zone of hydrolysis.

There are many studies about using thermophilic cellulolytic bacteria for enhance composting process in terms of timing and compost quality. The study of Sarkar *et al*. (2010), the use of isolated thermophilic cellulolytic bacteria from compost (a mixture of green leafy vegetable, rice straw, and cow dung) for biodegradation of vegetable waste compost. The results have shown that *Geobacillus* is domain bacterial species in the thermophilic phase of composting. The tests were divided into 2 sets, *control* (without inoculum bacterial population) and *treatment* (inoculum added). After the inoculation, total bacterial population of all *treatment* is significantly higher than *control* sets. In this study, the temperature in the composting process peaked in day 2 and still in the thermophilic phase until day 10, in which the elevated temperature is related to enzymatic activities. Dehydrogenase activities increased during the thermophilic phase until achieved the maximum activities at day 10 in both sets with 61% enzyme activities higher in *treatment*. Total organic carbon (TOC), micro-Kjeldahl nitrogen, and C/N ratio was measures to estimate the degradation process and found that C/N ratio of *treatment* is 20% less than *control*. They concluded that the inoculum of thermophilic bacteria led biodegradation of the composting process more efficiently, especially in the thermophilic phase.

Abdel-Rahman *et al*. (2016) studied about inoculum *B. licheniformis* and *B. sonorensis* in rice straw composting which gave the results in the same direction as Sarkar *et al*. (2010) study and also indicated that *treatment* sets became mature compost (C/N ratio less than or equal to 20) before *control* sets.

CHAPTER 3

MATERIAL AND METHOD

3.1 Source of microorganisms

Thermophilic cellulolytic bacteria were isolated from leaf compost at Chulalongkorn University (13°44′32.2″N 100°31′33.2″E) on 20th January 2020.

3.2 Raw material and source

Compost sample

Four types of compost sample (Day 7, Month 1, Month 2, and Month 3) were collected from compost piles at Chulalongkorn University and used for isolation and screening of thermophilic cellulolytic bacteria. Fresh leaf compost was collected from the same compost pile at day 0 of composting process and used as substrates for investigate degradation efficiency of the isolated bacteria. Leaf debris, the main component of leaf compost, mostly were Chamchuri (*Samanea saman*) leaf.

3.3 Culture Media[1]

3.3.1 Carboxymethyl cellulose (CMC) broth

CMC broth was a minimum nutrient liquid medium that contained only inorganic salts and CMC as carbon and energy sources. CMC broth was use for cultivation and enrichment of bacteria, to screening bacteria that have an ability to utilize and degrade cellulose.

3.3.2 Carboxymethyl cellulose (CMC) agar

CMC agar was a specific growth solid media for bacteria which can utilize and degrade cellulose as carbon and energy sources. CMC agar was used for isolation bacterial colony and Gram’s iodine staining test.

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[1] Formulas and preparations are shown in Appendix A

3.3.3 Luria-Bertani (LB) agar slant

Luria-Bertani (LB) agar was a solid medium for cultivation and maintenance of bacteria for genetic molecular study. It is widely used due to rich of nutrients and easy to prepare. LB agar slant was solid slope media used for preservation of bacterial culture.

3.4 Chemicals and reagents, and special instruments

3.4.1 Chemical and reagents[2]

The chemicals and reagents used in this study were analytical grade. Composition and preparation of each reagent were described in **Appendix B**.

3.4.2 Special instrument

The special instruments used in this study were include

- Autoclave: HVA-85, Hirayama, Japan
- High-speed centrifuge: SorvallTM LegendTM XT/XF Centrifuge, The United States of America
- Hot air oven: D 06062, Model 700, Memmert, Germany
- Incubator: Model 600, Mammert, Germany
- Incubator shaker: 10X 400 Environmental Shaker, The United Kingdom
- Laminar flow
- Light microscope: Olympus CHL, Japan
- Precision digital scale balance: 40SM-200A, Precisa, Switzerland
- Spectrophotometer: 1200, Labomed, inc., The United States of America
- Vernier caliper
- Vortex mixer

3.5 Compost sampling and cultivation procedures

Four types of compost with different age (Day 7, Month 1, 2, and 3 of composting process) were collected from the compost pile at Chulalongkorn University at 15 cm dept using sterile plastic bags to laboratory and stored at 4°C to reduce bacterial activity before using in bacterial isolation. In addition, temperature was

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[2] Formulas and preparations are shown in Appendix B

measured *in situ* to determine environmental condition and some of the compost samples were taken to analyze moisture content and pH.

3.5.1 Isolation and screening of thermophilic cellulolytic bacteria

Carboxymethyl cellulose (CMC) broth media was used to bacterial cultivation and enrichment (Sarkar *et al*., 2010). Ten grams of each compost samples were inoculated in 100.0 mL culture media and incubated at 60°C on 200 rpm shaking condition for 3 days. After 3 days, 45.0 mL new CMC broth were inoculated with 5 mL bacterial culture and enriched under the same condition. After 3 cycles of enrichment, culture broths were serial diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} concentration and 0.1 mL of each dilution were spread on CMC agar consist of same compositions as CMC broth with 2% agar and incubated at 60°C for 3 days.

All of the colonies obtained from spread plate technique were observed the colony morphology (shape, elevation, margin, and color) and picked up using a wire loop to streaked on new CMC agar and incubated at 60°C for 3 days to isolate pure culture. Some of each bacterial colony were preserved on Luria-Bertani (LB) ager slant at 4°C for further study. After 3 days, each isolate was spotted on new CMC plates and flooded with Gram's iodine solution for 3-5 min (Kasana *et al*., 2008). Cellulolytic bacteria can be able to utilize CMC as carbon and energy source and develop a hydrolytic zone of clearance around a bacterial colony. Hydrolysis capacity (HC value) was calculated as described in **3.6.1**. Bacterial isolates with high HC values were selected to determine cellulase activity.

3.6 Procedures of chemical analysis

3.6.1 Hydrolysis capacity measurement

From Gram's iodine test, hydrolysis zone of clearance was visible. Diameter in mm of the zone and bacterial colony was measure using Vernier caliper. Hydrolysis capacity (HC value) was calculated as the sum of colony and substrate degradation zone diameter divided by total colony diameter (Awasthi *et al*., 2018) as shown in **Equation 3.1**. All HC values obtained from all bacterial colony were analyzed statistically using SPSS software (Version 22.0). The criteria used for selection of bacteria was HC value at

third quartile (Q_3) (Di Benedetto *et al*., 2019). Bacteria isolates with greater HC value than Q_3 were selected for further studies.

$$\text{Hydrolysis capacity} = \frac{\text{colony and clear zone diameter}}{\text{colony diameter}} \tag{3.1}$$

3.6.2 Determination of cellulase activity

A loopful of each selected bacterial isolate was inoculated in 10.0 mL CMC broth and incubated at 60°C, 200 rpm shaking condition for 3 days. After that, the broths were centrifuged at 14,000 rpm; 4°C for 20 min to remove bacterial cells, clear solutions containing cellulase were obtained (Kim *et al*., 2004). 3,5-Dinitrosalicylic acid (DNS) reagent was used to measured enzymatic activity of cellulase (Miller, 1959). The analysis procedures were performed in serial step as follows:

1. Three mL DNS reagent were added to 3 mL the supernatant solutions and blank solution (CMC broth without bacterial culture), heated the mixtures in boiling water for 5 min.
2. After boiled, the mixtures were added with 1 mL Rochelle salt (potassium sodium tartrate) solution to stabilize the color and cooled to room temperature under running tap water.
3. The mixtures absorbance was measured at 575 nm using spectrophotometer and determined reducing sugar production of each bacterial by comparing with glucose standard curve (preparation was described in **Appendix B**).

After comparing absorbance of the mixtures with glucose standard curve, concentration of reducing sugar (mg/mL) in the solutions were obtained. Bacterial isolate with the highest reducing sugar production was selected for bacterial morphology study and degradation ability study.

3.7 Bacterial morphology study

Selected bacteria were characterized their shape, arrangement, and Gram type by Gram's staining method. The process would be performed in steps as follows:

1. A drop of sterile distilled water on slide was and smeared with a loopful of bacteria and fixed by heat.
2. Crystal violet was flooded to cover the smeared area for 1 min and washed gently with distilled water.
3. Flooding the smeared with Gram's iodine for 1 min and rinsed gently with distilled water.
4. Decolorizing agent (mixture of 95% alcohol and acetone) was flooded for 15-20 sec and rinsed with distilled water.
5. The slide was flooded with Safranin dye after decolorization for 30 sec and washed with distilled water.
6. The remaining water on the slide was gently wiped off with tissue paper until the slide was dry.
7. The stain attachment on cell wall, shape, arrangement of bacterial cells were observed under a light microscope in 100X magnification (using with an immersion oil for microscope).

3.8 Degradation efficiency test

Fresh leaf compost (leaf debris mixed with other composting materials) at Day 0 of the process was obtained from the same compost pile which is the source of bacterial culture using sterile plastic bag and stored at 4ºC until experiments. The sample was subjected to analyzed initial pH, total organic carbon (TOC), and total Kjeldahl nitrogen (TKN). The degradation experiments were performed in 250.0 mL Erlenmeyer flask and carried out in triplicate. The testing procedures were as following order (Awasthi *et al*., 2018):

1. The selected bacterial culture was enriched in CMC broth at 60°C, 200 rpm for 3 day.
2. Three flasks containing 100.0 g compost were inoculated with 10.0 mL of bacterial culture as *treatment* while one flask without microbial inoculum was

set as a *control*. All flasks were incubated at 60°C for 15 days in aerobic condition.

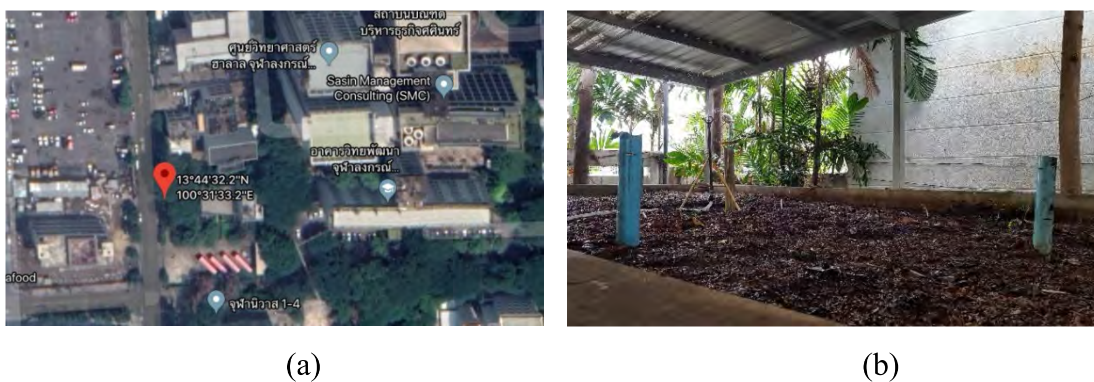
3. During the degradation, an aliquot of compost was subjected at Day 3, 6, 9, 12, and 15 to analyze pH, TOC, and TKN to detect degradation progress.
4. The values obtained from the analysis were plotted as the graph to determine the degradation by the selected bacteria.

CHAPTER 4

RESULTS

4.1 Compost sampling

Leaf compost sample was collected at 15 cm dept from compost pile which is managed by the Office of Physical Resources Management, Chulalongkorn University (13º44′32.2″N 100º31′33.2″E) on Monday 20th January 2020 as shown in **Figure 4.1**. At the time of sampling, the weather was quite cool and humid with ambient temperatures and humidity around 28ºC and 76%, respectively. There are 4 types of compost samples consisting of Day 7, Month 1, Month 2, and Month 3 compost. Physical and chemical properties (including compost temperature, pH, and %moisture) of each compost were measured. The results showed that the temperatures of Day 7, Month 1, Month 2, and Month 3 compost at a depth of 15 cm are 55.3,48.3, 37.3, and 30.3ºC, respectively. Month 2 compost has the highest moisture at 66.4%, followed by Month 1, Month 3, and Day 7 compost which has the moisture content of 63.4; 61.7; and 61.5%, respectively. From the measurement of pH using a pH-indicator strip, pH values of all types compost were similar in a range of 5-6. The results shown in **Table 4.1**. The physical characteristic of each type of compost samples was clearly different. Day 7 compost can be observed the texture of leaves and petioles. The color of this compost was brown to dark brown, which is the color of dried leaf debris. In Month 2 compost can be observed that the size of leaf debris was explicitly smaller than Day 7 compost. However, the color of both compost samples was resembled. The texture of compost samples was obviously different from other in Month 3 compost, ready-to-use compost has a fine texture like the soil, not be seen the nature of leaves, and the color was dark brown or black as illustrated in **Figure 4.2**.



(a) (b)

Figure 4.1 Sampling site (a) Coordinates of compost piles (b) Compost pile

Table 4.1 Physical characteristics of compost samples

Age of compost	Compost temperature (ºC)	pH	Moisture (%)
7 days	55.3	5	61.5
1 month	48.3	5	63.4
2 months	37.3	5	66.4
3 months	30.3	6	61.7

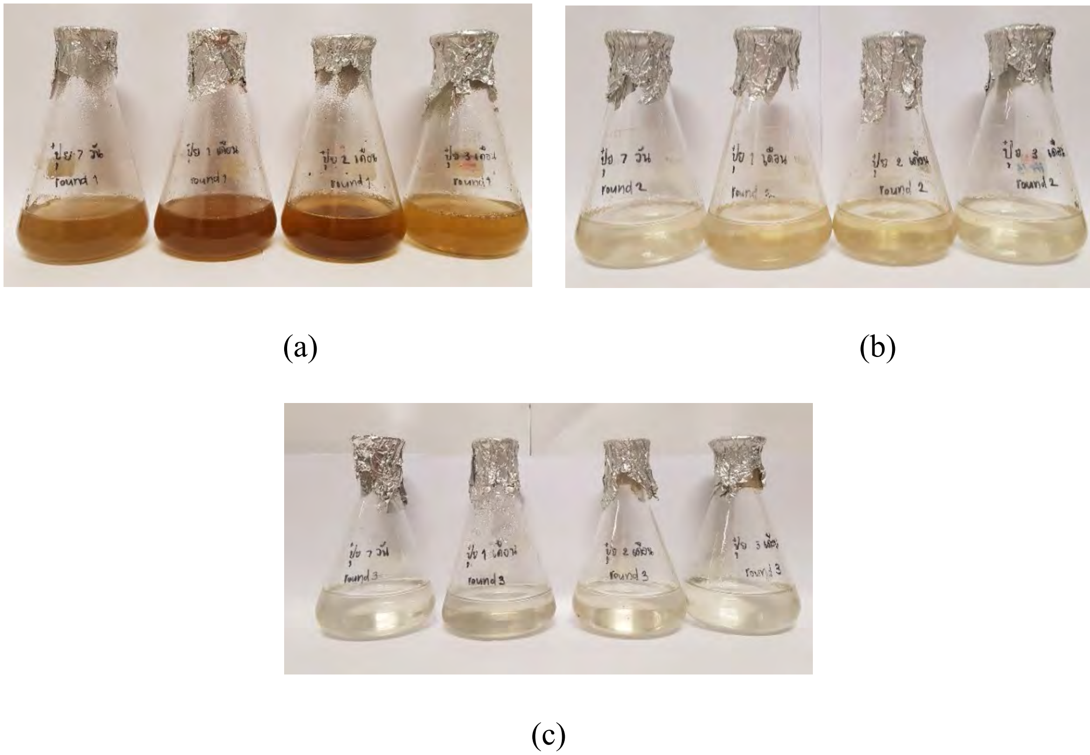
(a) (b)

(c) (d)

Figure 4.2 Compost samples (a) Day 7 compost (b) Month 1 compost |(c) Month 2 compost (d) Month 3 compost

4.2 Isolation and screening of thermophilic cellulolytic bacteria

Four types of compost samples were cultivated and 3 cycles enriched 1% CMC broth, the process was taken 12 days as shown in **Figure 4.3**. Along the process of enrichment, bacteria which use only cellulose as carbon and energy source can grow and multiply. The third enrichment was serial diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} concentration. Each dilutes solution was spread on CMC agar and incubated at 60ºC. After incubation for 3 days, the colony of cellulose degrading bacteria was appeared on agar as shown in **Figure 4.4**. Bacterial colony morphology of all colonies was observed and presented in **Table C-1** in **Appendix C**.



(a) (b)

(c)

Figure 4.3 Bacterial enrichment in 1% CMC broth (a) First cycle (b) Second cycle (c) Third cycle

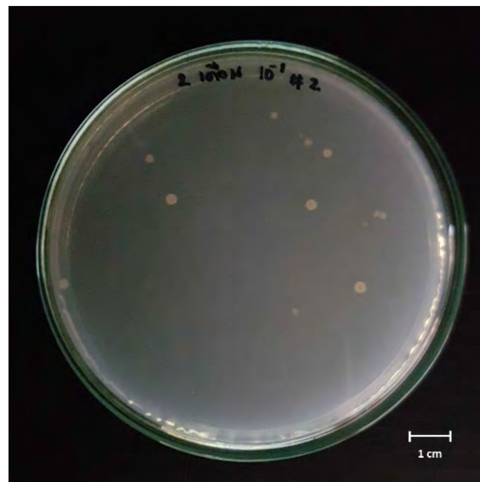


Figure 4.4 Bacterial colony from Month 2 compost at concentration 10^{-1} on CMC agar

Seventy-nine colonies were obtained on CMC agar and named as A01-A16, B01-B06, C01-C36, and D01-D21 for colonies from Day 7, Month 1, Month 2, and Month 3 compost, respectively. To isolate single colony, all the colonies were selected to streak on new CMC agar and incubated at the same condition. There were 50 colonies were obtained on the plates as shown in **Figure 4.5**.

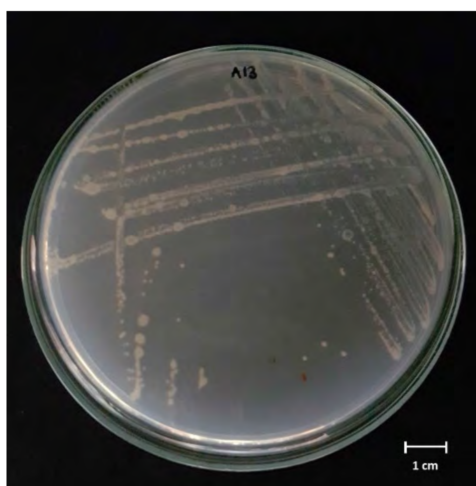


Figure 4.5 Single colony of cellulose degrading bacteria obtained from Day7 compost by streak plate method

After that, each colony was spotted on new CMC agar to test cellulose degradation capacity by flooding the plates with Gram's iodine. The clear zone around the colony was appeared as shown in **Figure 4.6** and measured clear zone diameter for calculating hydrolysis capacity (HC) value. There are 44 colonies that had hydrolysis capacity on agar plate and shown the clear zone around the colony as shown in **Table D-1** in **Appendix D**. The result showed that D08 strain obtained the highest HC value at 4.92. After statistic analyzed, the result was shown as box plot graph as illustrated in **Figure 4.7**, HC value at third quartile (Q_3) was 2.55, and there were 11 bacterial colonies with HC value greater than Q_3as presented in **Table 4.2**. These 11 bacteria were chosen to analyze cellulase activities. Colony morphologies of all colonies obtained by spreading bacterial culture solutions on CMC agar were observed, then, the colony morphologies of top 11 selected bacteria were presented in **Table 4.3**.

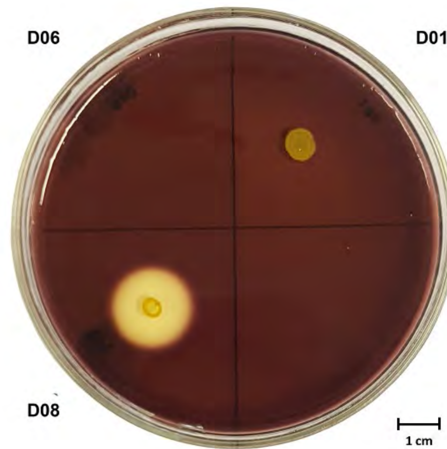


Figure 4.6 The clear zone around cellulose degrading bacteria colony after flooding with Gram's iodine

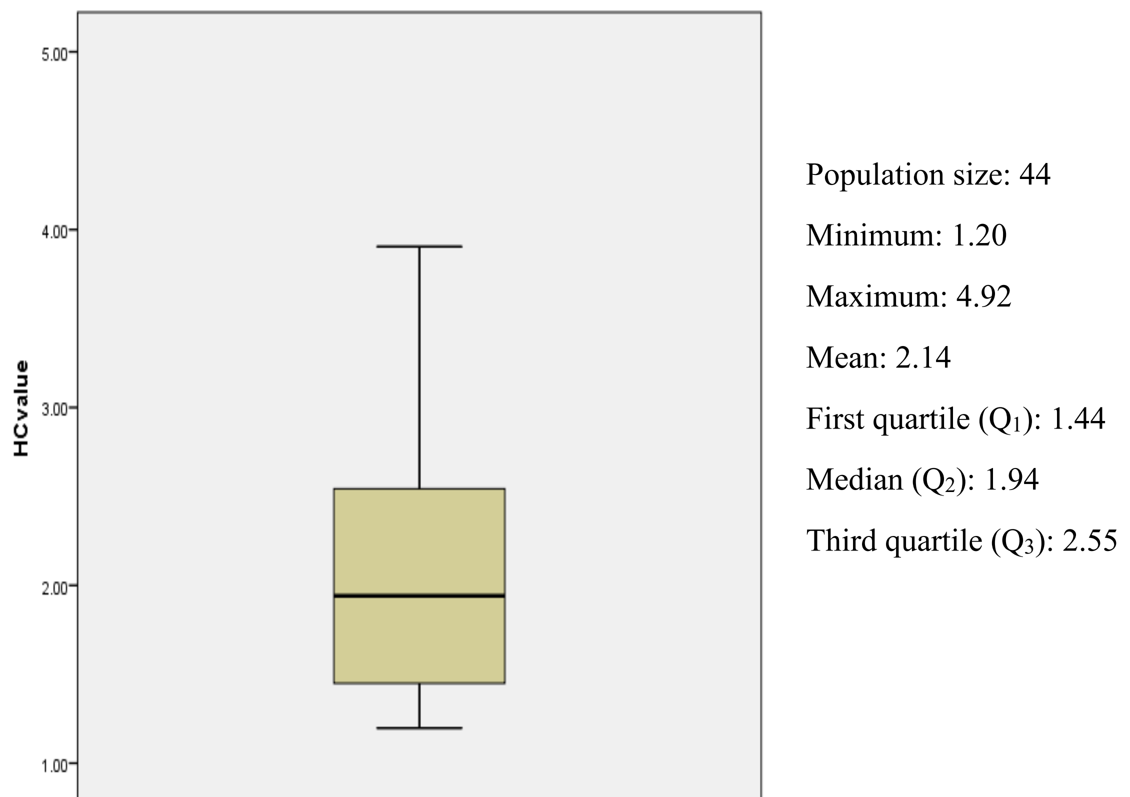


Figure 4.7 Box plot graph from statistical analysis of hydrolysis capacity (HC) value

Table 4.2 Hydrolysis capacity (HC) value of selected thermophilic cellulolytic bacteria

Bacterial colony	Hydrolysis capacity (HC) value
A03	2.55
C25	2.81
C02	2.88
B04	3.06
C33	3.12
A15	3.26
C18.1	3.29
B06	3.63
B03	3.69
C35	3.90
D08	4.92

Table 4.3 Bacterial colony morphology of selected bacteria thermophilic cellulolytic bacteria.

Bacterial colony	**Colony morphology**			**Color**
	Shape	Elevation	Margin	
A03	Circular	Convex	Entire	White
C25	Circular	Flat	Entire	Brown
C02	Circular	Flat	Undulate	White
B04	Circular	Flat	Entire	Yellow
C33	Circular	Flat	Entire	Brown
A15	Circular	Flat	Entire	Yellow
C18.1	Circular	Flat	Entire	White
B06	Circular	Convex	Entire	White
B03	Circular	Convex	Entire	White
C35	Circular	Flat	Entire	Brown
D08	Circular	Flat	Entire	Brown

Due to the intensifying domestic situation of COVID-19 caused the university temporarily closed, it was inconvenient to conduct the experiments about cellulase activity, bacterial morphologies, and degradation study.

CHAPTER 5

DISCUSSION & CONCLUSION

5.1 Compost sampling

Leaf composting process at Chulalongkorn University is resembled to passively aerated static pile composting. The compost piles are quite large, with a volume of each pile about 400 m^3 (2 m high, 5 m wide, and 10 long). The main raw material for composting is leaf debris collected on the campus, grinded leaf mixed with soil, manure, chopped coconut shell, and bio-fermented extract. Composting raw materials are gradually added up in everyday until full capacity of the piles. In order to aeration, the compost is occasionally turned up. Nevertheless, due to the large size of the compost pile, some parts could not be turned up and exposed to the air, then the use of plastic pipes with many small drilled holes in the compost pile to ventilated the air and released the heat. The heat generated by the decomposition causes a drying of compost from the evaporation of water. The compost piles were therefore watered every 2-3 days by spraying water droplets on the piles top to increase the humidity.

The temperature of each compose was *in situ* measured at a compost pile in 15 cm depth. The result showed that Day 7 compost achieved the highest temperature at 55.3ºC, while the other compost had lower temperatures and the lowest in Month 3 which the temperature was 30.3ºC. The reason that Day 7 compost has the highest temperature compared to other compost when measured at the same depth is the volume of compost in this pile is considerably lower than other piles, the depth at the temperature was measured is closer to inner zone of compost than the others. Base on temperature distribution, main three temperature regions can be classified in passively aerated static compost pile, (i) inner zone or the middle center, (ii) transition region, and (ii) outer zone. In the middle center, the temperature can be reached the maximize at 60-70ºC (Insam, and de Bertoldi, 2007; Fernandes *et al*., 1994; Poulsen, 2010).

Month 1; Month 2; and Month 3 compost pile, the temperature was measured at the outer zone which temperature is lower. The temperature of Month 2 is below a thermophilic range because on the day that measured, Month 2 compost was watered. Month 3 compost had the lowest temperature at 30.3ºC due to entering the maturation phase, which the temperatures are decreased.

The moisture content of Day 7 was 61.5%, which is an appropriate value for the composting process (40-60%). While Month 1 and Month 2 compost samples had greater moisture content than suitable value resulting from watering. Month 1 compost was watered a previous day before samples were collected as a result of 63.4% moisture. The highest moisture content was found in Month 2 compost at the value of 66.4%, according to watering while collecting the samples. Watering to the compost helps to keep the humidity not too low until microbial activities are inhibited (below 35%). However, it must be cautious, the humidity not too high until a lack of oxygen within the compost pile. At the end of the composting, mature compost should have the moisture content approximately 40% (Stentiford, 1996). While the moisture content of Month 3 compost (ready-to-use compost) was 61.7% but is expected to be unlikely affected the use of compost.

At an initial of the composting, small molecule organic substances are decomposed by primary decomposer leads to organic acids production, resulting in the pH of the compost is mildly acidic (pH around 5). Over time, pH increases due to ammonia generated from the degradation of nitrogen-containing substances. Throughout the process, pH varies in the neutral range (Liu *et al*., 2011; Sánchez *et al*., 2017). All compost samples have the pH trend that is consistent with the principle mentioned, Day 7 compost (beginning of the process) with the pH of 5, while the pH of Month 1; Month 2; and Month 3 were 6.

The physical characteristics of the compost have changed with the increasing duration of composting, size of raw materials was smaller when the time pasted. The texture of Day 7 compost was rough, can be observed in the nature of leaves and petioles. As the decomposition proceeded, the texture of the Month 1 and Month 2 became finder, the size of leaf debris was smaller. Three months passed, organic

materials were almost completely decomposed. The texture of the Month 3 compost was fine resembled to loam, color and smell likely to good quality soil. The color of the compost changed from the brown color of the leaf debris to the dark brown or black color of humus, which is a stable organic substance obtained from the composting process. From Figure 4.2, it could be observed that the texture of the compost sample was obviously changed in Month 1 compost. It was possible that biodegradation in compost pile occurs the most when the time passed for a month.

5.2 Isolation and screening of thermophilic cellulolytic bacteria

Mineral salt broth with carboxymethyl cellulose (CMC) is widely used for cultivation thermophilic cellulolytic bacteria from compost (Abdel-Rahman *et al*., 2015; 2016; Acharya *et al*., 2012; Baharuddin *et al*., 2010; Ng *et al*., 2009; Sarkar *et al*., 2010). This broth contains various inorganic salts which are a source of trace elements for bacteria, with CMC being the only source of carbon and energy. Bacteria that cannot use cellulose as an energy source cannot grow and multiply in this media, cellulolytic bacteria are obtained. CMC broth with bacterial culture was spread on CMC agar containing 2% agar. Due to culturing bacteria at high temperature, solid must consist of 2% agar, which makes the media more solidity, to reduce the evaporation of water from the culture media. There were 79 bacterial colonies obtained after an incubation of CMC agar at 60ºC for 3 days. All bacterial colonies were streaked on new CMC agar, and 50 isolates were obtained. To screen thermophilic cellulolytic bacteria, an easy method is flooded CMC plates with a solution that has an ability to form a colored complex with cellulose such as Congo red and Gram's iodine solution. The zone of clearance would appear in an area that cellulose is hydrolyzed by cellulase produced from bacteria. Cellulose degradability of each bacteria is reported by hydrolysis capacity (HC) value. Comparing to other reagents, Gram's iodine notably engenders the sharpness and distinct clear zone around the colonies (Kasana *et al*., 2008). From the study, there were 44 bacterial isolates that enhanced the clear zone around their colony. HC values obtained from every isolate were analyzed statistically and found that there were 11 isolates obtained HC value greater than third quartile (Q_3). D08 isolate was achieved outstanding HC value from others, with the value equal to 4.92 while C35; the second; was 3.90.

The texture of the compost and the probability of the highest biodegradation when the time passed for a month had been related to HC values. Four bacterial isolates with hydrolysis capacity could be isolated from Month 1 compost sample. Among them, there were 3 isolates had HC value greater than Q_3 accounting for 75.0%. While bacteria strains isolated from Day 7, Month 1, and Month 3 compost samples were 15.4%, 31.2%, and 9.1% percent that obtained HC values greater than Q_3, respectively. Therefore, it could be extrapolated that high cellulase hydrolysis capacity of bacterial leads to higher biodegradation of cellulose in the compost pile.

5.3 Conclusions

This study isolated thermophilic cellulolytic bacteria from 4 types of leaf compost at Chulalongkorn University and screened effective bacteria using hydrolysis capacity (HC) value as criteria. There were 44 bacterial isolates isolated from leaf compost with the ability in cellulose degradation from 50 pure culture of bacterial strains, accounting for 88.0%. Bacterial strain D08 was obtained the highest HC value and has the highest possibility to be an effective thermophilic cellulolytic bacteria isolated from leaf compost for further study. Due to the epidemic of COVID-19, it was not possible to complete the study as intended objectives.

5.4 Suggestion

Culture media was used in this study consisted of only inorganic salts and CMC. To enhance bacterial growth, yeast extract may be added into the medium (Abdel-Rahman *et al.*, 2015; Baharuddin *et al.*, 2010) because of yeast extract contains nitrogenous compounds that is essential for bacterial growth and activities. This study can be afterward developed by investigating the appropriate amount of bacterial inoculation to compost. Moreover, it is also possible to study the comparison of compost degradation efficiency between the addition of *ex situ* and *in situ* isolated microbes.

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ABBREVIATION

ºC	=	Degree Celcius
cm	=	Centimeter
CMC	=	Carboxymethyl cellulose
DNS	=	3,5-Dinitrosalicylic acid
g	=	Gram
HC value	=	Hydrolysis capacity value
LB agar	=	Luria-Bertani Agar
rpm	=	Round per minute
m	=	Meter
m^3	=	Cubic meter
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
nm	=	Nanometer
Q_3	=	The third quartile
sec	=	Second
TOC	=	Total organic carbon
TKN	=	Total Kjeldahl nitrogen
w/v	=	Weigh/volume

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APPENDICES

APPENDIX A - Media

Formula

1. **Carboxymethyl cellulose (CMC) broth**

Formula per liter

K_2HPO_4	0.5	g
KH_2PO_4	0.5	g
$(NH_4)_2SO_4$	1.0	g
$MgSO_4 \cdot 7H_2O$	0.1	g
$CaCl_2$	0.1	g
NaCl	6.0	g
CMC*	10.0	g
Distilled water	1.0	l

Final pH 7.0±0.2

Preparation of this medium would be discussed later

*Only use as a substrate for cellulolytic bacteria

2. **Carboxymethyl cellulose (CMC) agar**

Formula per liter

K_2HPO_4	0.5	g
KH_2PO_4	0.5	g
$(NH_4)_2SO_4$	1.0	g
$MgSO_4 \cdot 7H_2O$	0.1	g
$CaCl_2$	0.1	g
NaCl	6.0	g
CMC*	10.0	g
Agar**	20.0	g
Distilled water	1.0	

Final pH 7.0±0.2

Preparation of this medium would be discussed later

*Only use as a substrate for cellulolytic bacteria

**Only use 2% agar for thermophilic bacteria

3. **Luria-Bertani (LB) agar slant**

Formula per liter

Tryptone	10.0	g
Yeast extract	5.0	g
NaCl	10.0	g
CMC*	10.0	g
Agar**	20.0	g
Distilled water	1.0	l

Final pH 7.0±0.2

Preparation of this medium would be discussed later

*Only use as a substrate for cellulolytic bacteria

**Only use 2% agar for thermophilic bacteria

Preparation

For all media, all components were added in a glass beaker, dissolved with distilled water and heated on a hotplate stirrer until all the components completely dissolved. The solution was transferred to an Erlenmeyer flask and covered with cotton plug and aluminum foil before sterilized in an autoclave at 121ºC for 20 minutes.

After sterilized, medium number 2 was poured into Petri dishes about 15-20 mL per plate and left the solutions to become solid. For medium number 3, the solutions were poured into test tubes for 7-10 mL per tube. The tubes were placed in an incline and allowed the solutions to solid. All the processes mentioned were performed in a laminar flow cabinet.

APPENDIX B - Reagents

Formula

1. **DNS reagent**

   Formula per liter

3,5-Dinitrosalicylic acid	10.0	g
Phenol	2.0	g
Sodium sulfite	0.5	g
Sodium hydroxide	10.0	g

2. **40% Rochelle salt**

   Formula per liter

Potassium sodium tartrate	400.0	g

3. **Crystal violet**

   Solution A: 2.0 g Crystal violet dissolved in 20.0 mL 95% ethanol

   Solution B: 0.8 g ammonium oxalate dissolves in 80.0 mL distilled water

4. **Safranin O**

   Step 1: 2.5 g Safranin O dissolves in 100.0 mL 95% ethanol

   Step 2: 10.0 mL solution in Step 1 added to 90.0 mL distilled water

5. **Gram's iodine solution**

   Formula per 300 mL

Iodine	1.0	g
Potassium iodide (KI)	2.0	g

6. **Decolorizer**

   Formula per liter

Ethanol	500.0	mL
Acetone	500.0	mL

Preparation

All components were completely mixed and dissolved in distilled water. After that, the reagents were stores in the amber glass bottle to reduce the reaction with light.

Preparation of glucose stock solutions for glucose standard curve

Glucose stock solutions were prepared from 1% w/v glucose solution in CMC broth. The solution was serial diluted until 0.1 mg/mL concentration. All of the stock solutions were added with DNS reagent as the steps according to Miller (1995). The absorbance of each concentration was plotted as the glucose standard curve.

APPENDIX C - Isolation and screening of thermophilic cellulolytic bacteria

APPENDIX C in page 52-56

Please contact my project advisor for further information.

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APPENDIX D – Hydrolysis capacity value

APPENDIX D in page 58-63

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BIOGRAPHY

Miss Chavisa Sathirawisankit was born in Bangkok on the 8th of May, 1998. She achieved the degree of high school from Kasetsart University Laboratory School Center for Educational Research and Development and entered Chulalongkorn University as a freshman of Department of Environmental Science, Faculty of Science in 2016. When she was sophomore, she received the opportunity and experience for training at Waste and Hazardous Substances Management Bureau, Pollution Control Department. Please feel free to contact her at maychavi@hotmail.com.