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Chapter 1

Introduction

1.1. BACKGROUND

Organic farming is a form of agriculture that relies on techniques such as crop rotation (growing a series of different types of crops in the same area in sequential seasons), green manure (crops have already been uprooted), compost and biological pest control (controlling pests by other living microorganisms). There are 37.2 million hectares of organic agricultural land reported in 2011 (FiBL & IFOAM, 2013). Organic food and drink sales reached almost 63 billion US dollars in 2011. Among the factors involving in the organic farming, compost has been used on farming for thousands of years ago.

In the composting, organic residues of plant and/or animal are biologically broken down by the microbial activities of bacteria, actinomycetes, and fungi to produce the compost product. Due to the oxidative action of microorganisms, composting temperature begins to increase with the progress of composting. Thermophilic temperature (up to 60^{o}C or 70^{o}C) maintained during composting process is expected to be effective to eliminate pathogens in the raw materials and ensure the safety of usage of the compost product. In a large scale composting, however, there are large temperature distributions such as core region with the highest temperature, center region with medium temperature and surface region with the lowest temperature. Therefore, pathogens containing in the raw composting materials are expected to be eliminated at the high temperature regions. However, some kinds of pathogens can survive at the medium temperature for a certain time. Hutchison et al. (2005) has shown the extended survival of *Salmonella*, *E. coli* O157:H7, and *Listeria* in static composting piles for more than 8 days at temperature above 55°C. Singh et al. (2011) showed that *E. coli* B and *Salmonella enterica* serovar Typhimurium Q survived for at least 9 days at 60^{o}C in a food waste composting. In addition, other factors in composting such moisture content, aeration, heap size, pH, populations of microflora, etc., may affect the self-heating at the beginning of composting. Consequently, some populations of pathogens may become acclimatized before lethal temperatures are reached, or even survive, for an extended period of time (Davis et al., 1992; Singh et al., 2011).

In this study, *E. coli* O157:H7 was chosen as the target pathogen. If the composting operation is not adequate, compost can be a source of *E. coli* O157:H7 contamination and human is easily infected by the *E. coli* O157:H7 through using agricultural products. *E. coli* O157:H7 causes approximately 73,000 illnesses in USA annually with the main epidemiological symptoms of severe diarrhea and hemolytic uremic syndrome. The largest *E. coli* O157:H7 outbreak was reported in January 1993 with more than 700 ill people and 4 children died (Rangel et al., 2002). Some foodborne outbreaks caused by *E. coli* O157:H7 in Japan was reported (Watanabe et al., 1999) in which the largest *E. coli* O157:H7 outbreak arose in primary schools in Okayama prefecture in May 1996 involving 468 patients, two of which accompanied hemolytic uremic syndrome and died (Japan Infectious Agents Surveillance Report, 1996). The *E. coli* O157:H7 outbreak has been also reported in many other countries (Isaacson et al., 1993; Chapman et al, 1987; Armstrong et al., 1996). Therefore, detection of *E. coli* O157:H7 in agricultural products and compost plays important role to contribute to prevention of foodborne outbreaks caused by *E. coli* O157:H7. The first detection method of *E. coli* O157:H7 is agar plate combined with serotyping kits. The method enables quantitative detection, however, it is expensive and time consuming. The second detection method is DNA-based method such as real-time PCR that is based on amplification of specific genes of *E. coli* O157:H7. The method also enables the quantitative detection, but, it is expensive. The third detection method is bacteriophage-based method that has been of great interest according to high host recognition specificity of bacteriophage (phage) and rapidity. However, the method is unquantifiable. In addition, pre-existing phage-based bioluminescent and fluorescent methods require specific and expensive apparatus to accomplish the detection. It makes the application of the phage-based methods conditional depending whether the apparatus are equipped. Therefore, a simple phage-based method that don't need specific and expensive apparatus to conduct the detection is necessary.

1.2. GOAL AND OBJECTIVES OF DISSERTATION

The goal of this study is to quantitatively evaluate the concentration of *E. coli* O157:H7 in various materials by developing a colorimetric phage-based method.

To achieve the overall objective, several specific objectives are taken into account in this study as follows:

1. To invent a new method for colorimetric detection of *E. coli* K12 using a recombinant T4 phage.
2. To develop a quantitative detection method used to detect *E. coli* O157:H7 in various samples.
3. To evaluate the fate of *E. coli* O157:H7 in composting by using the quantitative detection method.

The detailed discussion of each specific objective is consecutively presented in the following chapters 3, 4, and 5.

1.3. THE OUTLINE OF DISSERTATION

CHAPTER 1: INTRODUCTION

This chapter presents a statement of organic farming and the concern of possible contamination of *E. coli* O157:H7 in compost through the composting process. In addition, detection methods of *E. coli* O157:H7 are shown with their merits and demerits. Moreover, the significance and motivation to further seek a better detection method were also stated together with the goal and the specific objectives of the dissertation.

CHAPTER 2: LITERATURE REVIEW

This chapter reviews the literature on the organic farming and possible contamination of *E. coli* O157:H7 in compost product after composting. The literature review also include the introduction of relevant principles of *E. coli* and the foodborne outbreak caused by the *E. coli*. In addition to detection methods of *E. coli*, fundamental knowledge about phages and the previous phage-based detection methods of *E. coli* are reviewed.

CHAPTER 3: COLORIMETRIC DETECTION OF *ESCHERICHIA COLI* K12 BY USING A RECOMBINANT PHAGE

The aim of this chapter is to invent a new method for colorimetric detection of *E. coli* K12 using a recombinant T4 phage bearing the *cytochrome c peroxidase* gene (*ccp*) derived from *Saccharomyces cerevisiae*. The colorimetric detection of *E. coli* K12 using the recombinant T4 phage was examined. The oxidation activity towards the chromogenic substrate cytochrome c was demonstrated by the cytochrome c peroxidase (CCP) produced

from the T4ccp genome. The color change caused by the oxidation of the substrate was examined visually.

CHAPTER 4: QUANTITATIVE DETECTION OF *ESCHERICHIA COLI* O157:H7 USING A COMBINATION OF THE COLORIMETRIC PHAGE-BASED ASSAY AND MOST PROBABLE NUMBER TECHNIQUE

In this chapter, a method for the quantitative detection of *E. coli* O157:H7 using a combination of the colorimetric phage-based assay and most probable number technique was developed. Firstly, a recombinant phage PP01ccp carrying the *ccp* gene was constructed to detect the pathogenic *E. coli* O157:H7. Principle of the construction of PP01ccp was similar to that of T4ccp shown in the chapter 3. The PP01ccp was used to detect *E. coli* O157:H7 and color change derived from the experiment could be easily recognized by eyes. Secondly, a combination of the detection method based on PP01ccp and the MPN technique (as abbreviated as MPN-phage assay) was developed and successfully applied to quantify *E. coli* O157:H7 concentration in samples of apple juice, milk and cattle manure.

CHAPTER 5: QUANTITATIVE EVALUATION OF *ESCHERICHIA COLI* O157:H7 DURING CATTLE MANURE COMPOSTING

Two types of composting runs, with and without turning, were conducted. In both cases, the raw material was composted in three separate reactors set at 30, 50, and 70°C. In this chapter, the fate of *E. coli* O157:H7 during cattle manure composting was investigated using the MPN-phage assay. In addition, the meaning of the turning on the fate of *E. coli* O157:H7 during the composting was also understood.

CHAPTER 6: GENERAL CONCLUSIONS

With the results obtained from chapters 3, 4, and 5, the general conclusions associated with the safety of organic farming based on the new phage-based system were presented together with the recommendations for further applications of the method in this chapter.

Chapter 2

Literature review

This chapter presents the principles and benefits of organic farming and possible risk of contamination of pathogens in compost that is one of main factors of organic farming. Moreover, comprehensive review on related aspects of *E. coli* and phages was shown. Lastly, detection methods of *E. coli* were reviewed to understand merits and demerits of each method. Among the detection methods, phage-based detection methods were reviewed more detailed to demonstrate the merit of the phage-based method invented in this study.

2.1. ORGANIC FARMING

Organic farming is a form of agriculture that relies on techniques such as crop rotation (growing a series of different types of crops in the same area in sequential seasons), green manure (crops have already been uprooted), compost and biological pest control (controlling pests by other living microorganisms).

There are 37.2 million hectares of organic agricultural land reported in 2011 (FiBL & IFOAM, 2013). The regions with the largest areas of organic agricultural land are Oceania (12.2 million hectares, 33 percent of the world's organic agricultural land). Asia has 3.7 million hectares, 10 percent. The countries with the most organic agricultural land are Australia (12 million hectares), Argentina (3.8 million hectares), and the United States (1.9 million hectares). The following figure shows growth of the organic agricultural land by continent from 2005 to 2011. In spite of the slowdown in the global economy, international sales of organic product continue to rise, e.g., organic food and drink sales reached almost 63 billion US dollars in 2011. The market has expanded by 170 percent since 2002. Demand for organic products is mainly in North America and Europe; these two regions comprise more than 90 percent of sales.

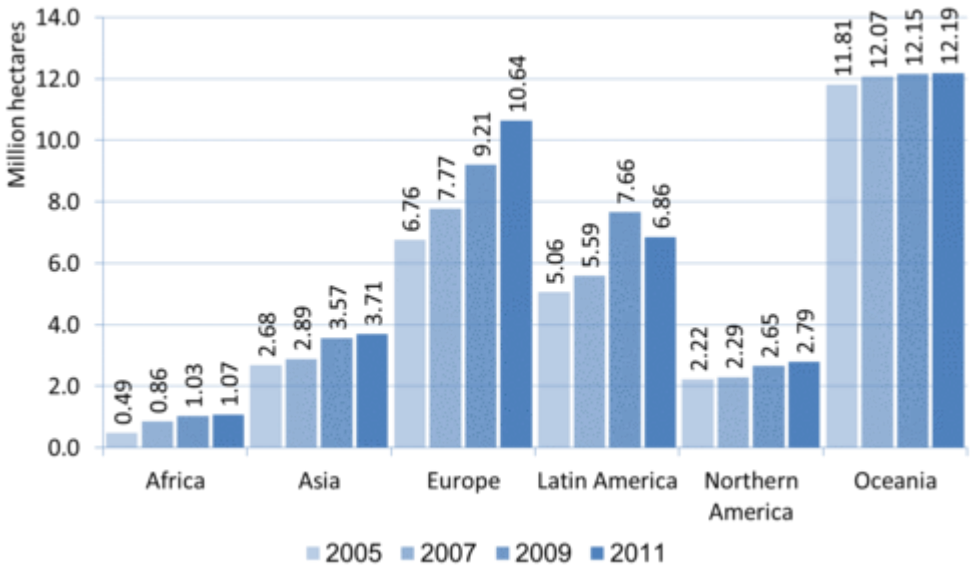

Figure 2.1. Growth of the organic agricultural land by continent (FiBL & IFOAM, 2013)

2.2. PREVALENCE OF PATHOGENS IN COMPOST SAMPLES

Compost has been used in the organic farming from a long time ago. Compost is a bio-fertilizer that is widely used in agriculture to increase soil quality in terms of nutrients, water holding capacity and to increase yield of crop. Compost is produced from composting process in which animal manures are normally used as composting materials. Among pathogenic bacteria in the animal manures, *E. coli* O157:H7 is one of the most concerned bacteria according to two main reasons. First reason is that infection of *E. coli* O157:H7 causes severe bloody diarrhea and hemolytic-uremic syndrome in human (Rangel et al., 2002). Secondly, in the animal manures, prevalence of *E. coli* O157:H7 is high compared to other pathogenic bacteria since animal manures are considered as the original source of the *E. coli* O157:H7. Zhao et al. (1995) surveyed prevalence of *E. coli* O157:H7 in dairy herds and reported that about 3.4% of fecal samples is positive with *E. coli* O157:H7 test and the *E. coli* concentration ranged from 10^3 to 10^5 CFU g^{-1}. Concentration of *E. coli* O157:H7 in fecal samples was also revealed in other researches with various ranges from 10^1 to 10^6 CFU g^{-1} (Ogden et al., 2002; Omisakin et al., 2003). Therefore, possible *E. coli* O157:H7 in animal manures is usually treated by the composting.

In the composting, organic residues of plant and/or animal are biologically broken down by the microbial activities of bacteria, actinomycetes, and fungi to produce compost product.

As composting progresses, sugars, starches, proteins, and fats containing in the raw composting materials are consumed by microbes for the production of cell mass, which generates the exhaust gas and water as by-product. Due to the oxidative action of microorganisms, composting temperature begins to increase with the progress of composting. Thermophilic temperature (up to 60ºC or 70ºC) maintained during composting process is expected to be effective to eliminate pathogens in the raw materials and ensure the safety of usage of the compost product. In a large scale composting, however, there are large temperature distribution such as core region with highest temperature and surface region with lowest temperature. Therefore, if the composting operation is not adequate, compost is a possible source of *E. coli* O157:H7 contamination, *E. coli* O157:H7 may survive at the low temperature region. If *E. coli* O157:H7 still remains in the compost product, it is easy to transmit into agricultural products and people are possibly infected by the *E. coli* O157:H7 by consuming the agricultural products.

Therefore, it is important to ascertain compost product free from pathogens. In the United States, Environmental Protection Agency (EPA) regulations for composting include either a minimum temperature of 55°C for 3 days in aerated static piles or in-vessel systems or for 15 days with 5 turnings in windrow systems (Singh et al., 2011). Although temperature is an essential factor during composting used to eliminate pathogens, extended survival of pathogens in composting has been reported. Hutchison et al. (2005) has showed the extended survival of *Salmonella, E. coli* O157:H7, and *Listeria* in static composting piles for more than 8 days at temperature above 55°C. Singh et al. (2011) showed that *E. coli* B and *Salmonella enterica* serovar Typhimurium Q survived for at least 9 days at 60 in a food waste composting. These studies indicated that the criteria set by the EPA shown above may not always be sufficient to ensure complete inactivation of pathogens within the entire composting pile. Pathogen inactivation during composting depends on many factors, besides the elevated temperature mentioned above, such as moisture content, carbon/nitrogen ratio (C/N), particle size, aeration, heap size, pH, and types and populations of indigenous microflora. Variations of these factors may affect the self-heating at the beginning of composting and the transition time from the mesophilic to the thermophilic phase of composting. Consequently, some populations of pathogens may become acclimatized before lethal temperatures are reached, or even survive, for an *extended* period of time (Davis et al., 1992; Singh et al., 2011).

2.3. TARGET BACTERIA IN THIS STUDY

2.3.1. General principles of *E. coli*

E. coli were discovered by German pediatrician and bacteriologist Theodor Escherich in 1885. *E. coli* is a Gram-negative, rod-shaped bacterium and it is normally found in the lower intestine of warm-blooded organisms including humans. *E. coli* can be grown easily and inexpensively in a laboratory setting. Therefore, *E. coli* is the most widely studied prokaryotic model organism and it is an important species in the fields of biotechnology and microbiology where it has served as the host organism for the majority of work with recombinant DNA. In laboratory, *E. coli* K12 together with *E. coli* B is normally used in biological researches, in which the complete genome of *E. coli* K12 was sequenced (Blattner et al., 1997). *E. coli* K12 is a non-pathogenic *E. coli* that was isolated from a stool sample of a patient convalescent from diphtheria (Bachmann, 1972). An extensive list of *E. coli* K 12 strain derivatives and their individual construction, genotypes and phenotypes information were described (http://ecoliwiki.net/colipedia/index.php/Category:Strains).

E. coli are originally from feces. However, by many routes, *E. coli* can transmit into environment, water, food samples and finally infect to human (Chekabab et al., 2012). Most *E. coli* strains are harmless, but some kinds are toxic that can cause serious diseases for humans (Natoro et al., 1998). The most popular disease caused by *E. coli* is diarrhea. The World Health Organization reported that about 5 million children die each year because of acute diarrhea (Harald Brussow, 2005). Toxic *E. coli* can be classified into 5 main groups such as Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC) diarrhea (Nataro & Kaper, 1998). Among five types of toxic *E. coli* above, EHEC strains are currently noted as a global health problem due to their outbreaks on over the world (Riley et al., 1983; Nataro & Kaper, 1998). The most common EHEC strain in food-borne outbreaks is *E. coli* O157:H7. ETEC is the leading cause of diarrhea for more than 25% of all diarrheal patients every year (Bui et al., 2008). EIEC does not produce toxins, but they severely damage the intestinal wall via mechanical cell destruction that resulted in some outbreaks in remote areas (Nataro & Kaper, 1998). EAEC was defined in 1987 and recognized as a serious pathogen which can cause persistent and acute diarrhea in children in developing countries. EAEC is transmitted by the fecal–oral route and by consumption of contaminated water and food. Some outbreaks have been reported with main reason of EAEC (Bui et al., 2008). The type EPEC is considered as one of reasons to also cause diarrhea. However, there are some EPEC serotypes as considered

as “new EPEC”. The association of these serotypes to diarrhea in children is not fully understood.

E. coli are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens. The somatic antigens are lipopolysaccharide and located at the surface of the cell wall. A total of 170 different somatic antigens, each defining a serogroup, are recognized currently. The flagellar antigens are protein and the capsular antigens are polysaccharide. A specific combination of O and H antigens defines the “serotype” of an isolate. Table 2.1 shows serotypes of the diarrheagenic *E. coli* strains.

Table 2.1. Serotypes characteristic of the diarrheagenic *E. coli* categories (Nataro & Kaper, 1998)

Category	Serogroup	Associated H antigen (s)
EHEC	O26	H11, H32, NM
	O55	H7
	O111ab	H8, NM
	O113	H21
	O117	H14
	O157	H7
ETEC	O6	H16
	O8	H9
	O11	H27
	O15	H11
	O20	NM
	O25	H42, NM
	O27	H7
	O78	H11, H12
	O128	H7
	O148	H28
	O159	H20
	O173	NM
EPEC	O55	H6, NM
	O86	H34, NM
	O111	H2, H12, NM
	O119	H6, NM
	O125ac	H21
	O126	H27, NM
	O127	H6, NM
	O128	H2, H12
EAEC	O3	H2
	O15	H18
	O44	H18
	O77	H18
	O111	H21
EIEC	O28ac	NM
	O29	NM
	O112ac	NM
	O124	H30, NM
	O152	NM
	O159	H2, NM
	O167	H4, H5, NM

2.3.2. *Escherichia coli* O157: H7

The recognition of EHEC as a distinct class of pathogenic *E. coli* resulted from the first observation reported by Riley et al. (1983), who investigated two outbreaks of a distinctive gastrointestinal illness characterized by severely bloody diarrhea. This was associated with the eating of undercooked hamburgers at a fast-food restaurant chain. Stool samples from these patients revealed a previously rarely isolated *E. coli* serotype, O157:H7. Another key observation by Karmali et al. (1983) showed the association of sporadic cases of hemolytic uremic syndrome (HUS) with fecal cytotoxin and cytotoxinproducing *E. coli* in stools. Thus, the two key clinical microbiological observations led to the recognition of a novel and increasingly important pathogenic *E. coli* O157:H7.

E. coli O157:H7 causes approximately 73,000 illnesses in USA annually with the main epidemiological symptoms of severe diarrhea and hemolytic uremic syndrome. The largest *E. coli* O157:H7 outbreak was reported in January 1993 with more than 700 ill people and 4 children died (Rangel et al., 2002). *E. coli* O157:H7 has become a national notifiable infection in USA in 1994 (Rangel et al., 2002). Some foodborne outbreaks caused by *E. coli* O157:H7 in Japan was reported (Watanabe et al., 1999) in which the largest *E. coli* O157:H7 outbreak arose in primary schools in Okayama prefecture in May 1996 involving 468 patients, two of which accompanied hemolytic uremic syndrome and died (Japan Infectious Agents Surveillance Report, 1996). The *E. coli* O157:H7 outbreak has been also reported in many other countries (Isaacson et al., 1993; Chapman et al, 1987; Armstrong et al., 1996). It was reported that foodborne has occupied up to 52% total transmission routes (the others is 21% unknown, 14% person-to-person, 9% waterborne water, 3% animal contact and 0.3% laboratory-related transmission route). In the transmission route of foodborne, *E. coli* O157:H7 could be detected in various types of meats, vegetables, juices, dairy products (Rangel et al., 2002). It is especially dangerous since *E. coli* O157:H7 can survive and growth at severe circumstances. The outbreaks have been reported in acidic foods such as mayonnaise and apple cider (Armstrong et al., 1996). The pH of these products is less than 4.6 that is considered to be low risk for transmission of pathogenic bacteria. However, *E. coli* O157:H7 can survive at pH as low as 2.0 (Miller & Kaspar, 1994; Conner & Kotrola 1995). In vegetables, it is shown that *E. coli* O157:H7 can grow on lettuce at temperature as low as 12°C (Abdul-Raouf et al., 1993). In water samples, *E. coli* O157:H7 can survive up to 10 months (Chekabad et al., 2013). Therefore, detection of *E. coli* O157:H7 in environmental, food and water samples play an important role contributing to prevent *E. coli* O157:H7 outbreaks.

2.4. BACTERIOPHAGES INFECTING *E. COLI*

2.4.1. General principles of bacteriophages

Bacteriophages (or phages) are viruses that infect only bacteria. In other words, they do not infect and make harm to human, animals and plants. Phages were discovered independently by British microbiologist Felix Twort in 1915 and by French-Canadian microbiologist Felix d'Hérelle in 1917 (Richard M., 1999). Phages use fibers to attach to specific receptors such as protein and lipopolysaccharide (LPS) molecules on the surface of the host bacterium. And then the phages inject their nucleic acids into the host chromosome. Phages can have either a "lytic" or a "lysogenic" lifecycle (Figure 2.2).

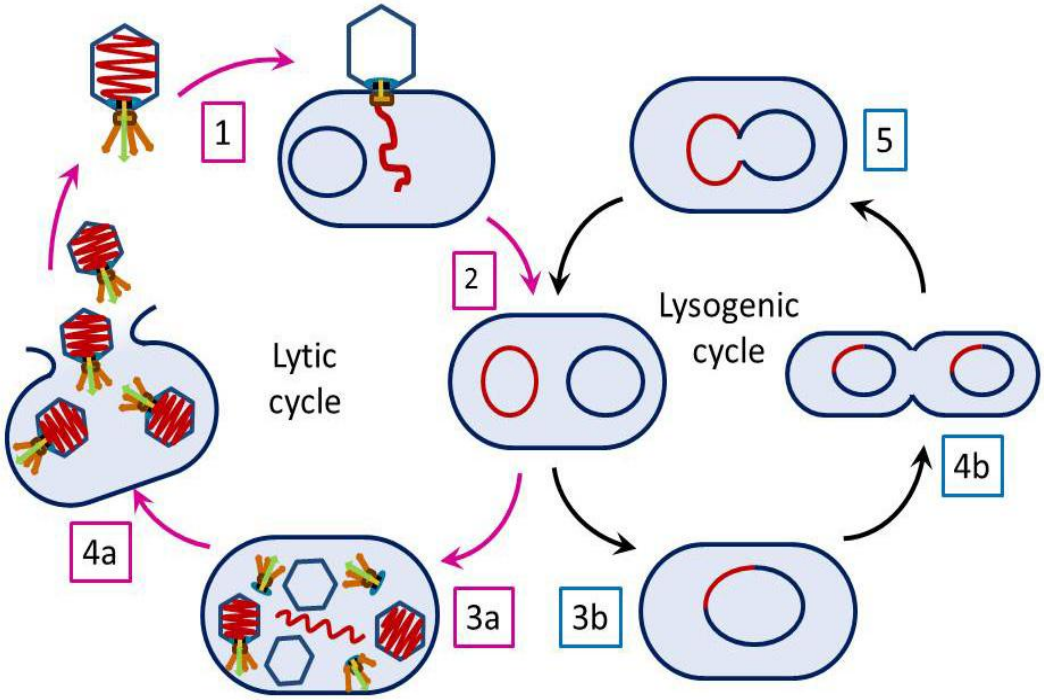

Figure 2.2. Two cycles of bacteriophage reproduction (Kurtböke, 2012)

1 - Phage attaches the host cell and injects genome; 2 – Phage genome enters lytic or lysogenic cycle; 3a – New phage genome and proteins are synthesized and virions are assembled; 4a – Cell lyses releasing virions; 3b and 4b – steps of lysogenic cycle: integration of the phage genome within the bacterial chromosome (becomes prophage) with normal bacterial reproduction; 5 – Under certain conditions the prophage excises from the bacterial chromosome and initiates the lytic cycle.

In the “lysogenic” lifecycle, phages are replicated together the host cell division and do not cause the lysis. On the other hand, in the “lytic lifecycle”, lytic phages use the host genetic machinery to amplify many new phages. The number of phages produced during infection of a single cell is called the burst size. When the burse size is sufficient (varying from 50 to 200 new phages), new phages will break and lyse the host cell. These released phages will continue infect to other host cells. It sounds that lytic phages are more applicable in treating bacteria than lysogenic phages. For instance, if the burst size is 200 new phages in the 1^{st} cycle, ideally, these 200 new phages can infect and produce 40 000 new phages in the 2^{nd} cycle. Similarly, there are 8 million new phages at the end of the 3^{rd} cycle and so on. Thus, the similar amount of host bacteria is expected to be killed by the amount of phages.

Classification of viruses is based on several factors such as phage morphology, host bacteria and genome type. The phage genome can be either DNA or RNA. The most of phages contain double strand DNA (dsDNA), while there are small phage groups with single strand RNA or DNA or dsRNA (Kutter & Sulakvelidze, 2004). There are a few morphological groups of phages such as filamentous phages, icosahedral phages without tails, phages with tails (Kurtböke, 2012) (Figure 2.3). There are more than 5500 phages that have been examined in the electron microscope (Ackermann, 2007).

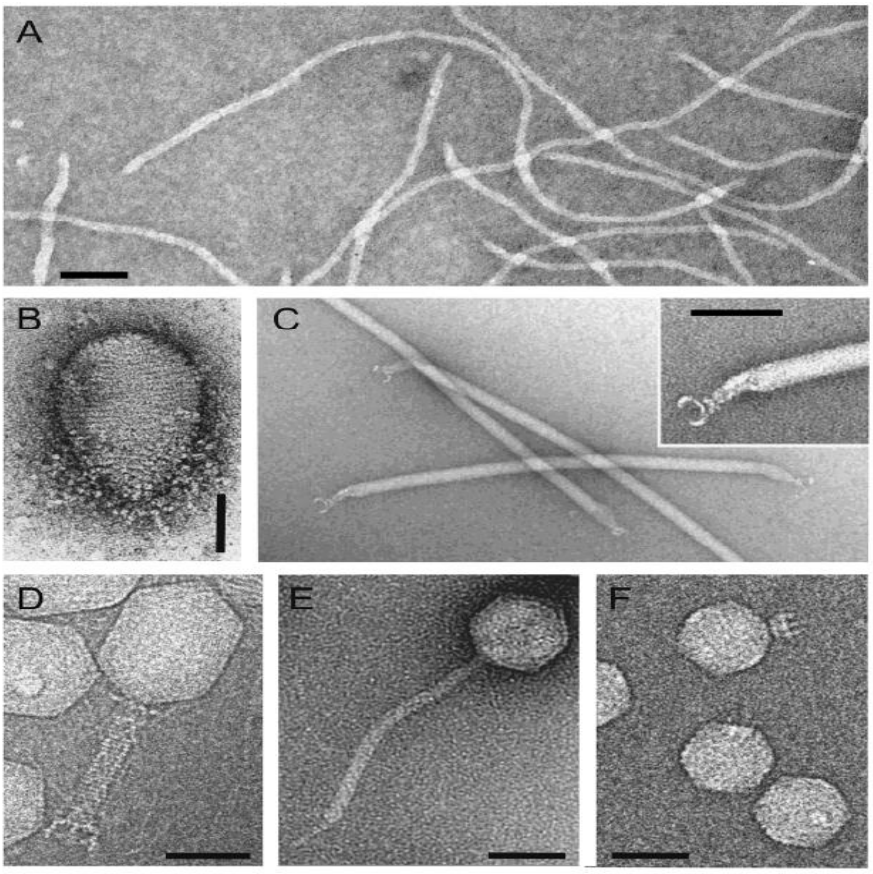

Figure 2.3. Images of bacteriophages (Kurtböke, 2012)

A – Filamentous phage B5 (*Inoviridae*)*;* B – dropletshaped phage (*Guttaviridae*);

C – Filamentous phage (*Lipothrixviridae*) with tail structures in native conformation;

D – Bacteriophage T4 (*Myoviridae)*; E – Bacteriophage SPP1 (*Siphoviridae*);

F – Bacteriophage P22 (*Podoviridae*). Bars are 50 nm

2.4.2. T4 bacteriophage

There are a variety of phages infecting *E. coli* that are defined as coli phages. Classification of coli phage based on the morphology is introduced into four main types involving Myoviridae, Podoviridae, Siphoviridae and filamentous phage. In this dissertation, phages belonging to myoviridae were studied. Therefore, this type of phage was introduced in details. There are 3 families of Myoviridae phages for *E. coli* such as T2, T4 and T6 that can be called as T-even phages. Their structures are similar each other that compose of polyhedral head, contractile tails and fibers to adhere surfaces of their *E. coli* host.

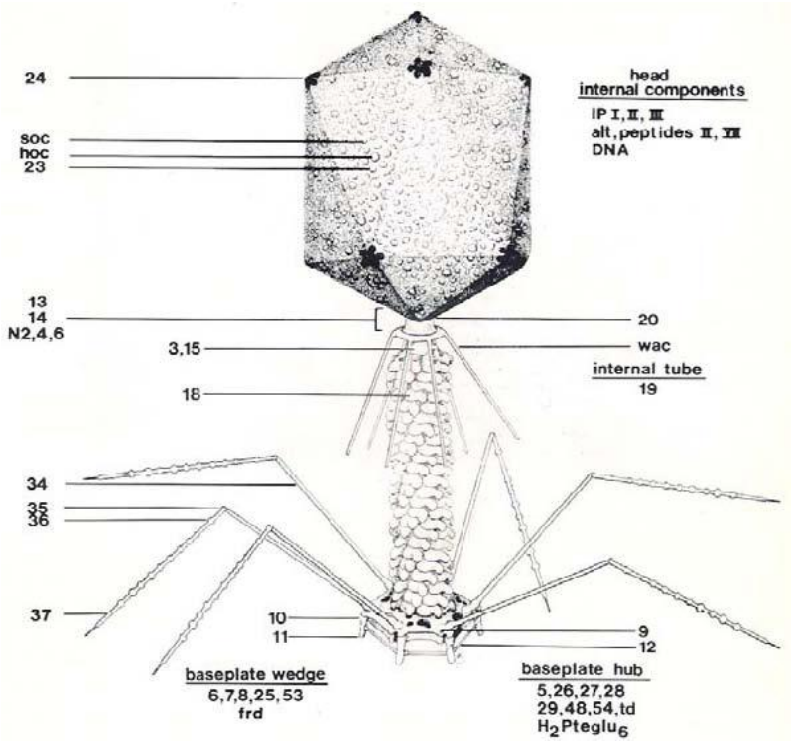

Figure 2.4. Structure of T4 phage (Li et al., 2007)

Like all viruses, T-even phages need an *E. coli* host cell to replicate their genetic material. In order to inject dsDNA into *E. coli* cells, the phage attaches to the surface of *E. coli* (at receptors) using the proteins on its tail (sensors). Among the three kinds of *E. coli* T-even phages, phage T4 has been well studied (Miller et al., 2003) (Figure 2.4).

The length of phage T4 DNA is 169-170 kbp and the DNA is located in an icosahedral head. Phage T4 is relatively large compared to other phages. It is approximately 200 nm long (while lengths of other phages are from 25 to 200 nm) and 90 nm wide. T4 motor packages DNA at a rate of up to 2000 bp/sec, the fastest reported to date of any packaging motor (Kurtböke, 2012). T4 phage carry out its lytic lifecycle (not lysogenic lifecycle). Attachment between phage T4 and *E. coli* occurs via interaction of the tip of the long tail fibers to outer membrane porin C protein or lipopolysaccharides.

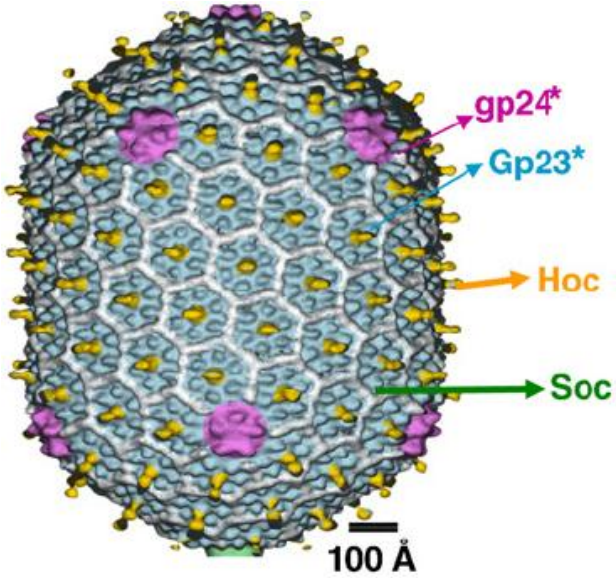

Figure 2.5. Structure of capsid of T4 phage (Li et al., 2007)

The capsid of the T4 phage is built with three essential proteins: gp23*, gp24* and gp20. The capsid also contains two non-essential outer capsid proteins, SOC and HOC, which decorate the capsid surface (Figure 2.5). Removal of the SOC and HOC proteins do not significantly affect growth rate and host specificity of the phage. This characteristic is a base of phage display techniques (Jiang et al,. 1997; Malys et al,. 2002; Tanji et al., 2004; Shivachandra et al., 2007; Li et al., 2007).

2.4.3. PP01 phage

Isolation of phage specific for *E. coli* O157:H7 is interested in phage researches. Relative abundant phages specific for *E. coli* O157:H7 were isolated from animal or human feces samples. A virulent phage named PP01 was isolated from 60 bovine, 52 swine and 5 chicken fecal samples by Morita et al. (2002). The PP01 phage has a high lytic activity. In addition, PP01 has a high host recognition specificity since it does not infect *E. coli* strains of other O-serogroups and K-12 strains. PP01 uses outer membrane protein OmpC as receptor to infect *E. coli* O157:H7. The research also revealed that PP01 is a member of the T-even phages. PP01 has been applied to create recombinant phages used in detection of target *E. coli* O157:H7 (Oda et al., 2004; Brigati et al., 2007; Ripp et al., 2008).

2.5. *E. COLI* O157:H7 DETECTION METHODS

2.5.1. Culture techniques

Agar medium that was usually used to detect *E. coli* O157:H7 is Sorbitol-MacConkey (SMAC) agar (Farmer & David, 1985; March & Ratnam, 1986). The agar medium contains sorbitol instead of lactose. *E. coli* O157:H7 is unable to ferment sorbitol and the color of *E. coli* O157:H7 colonies is translucent while other strains of *E. coli* are able to ferment sorbitol to produce acid and their color of colonies is pink or red (Figure 2.6). Based on the color difference, *E. coli* O157:H7 can be differentiated from other strains of *E. coli*. The method requires 24 to 72 h to confirm the presence of *E. coli* O157:H7 (Park et al., 1996). In order to increase of efficiency of eliminating background microbes, addition of tellurite and cefixime into SMAC agar to produce CT-SMAC agar has been recommended (Hussein & Bollinger, 2008) in which tellurite is result in oxidative activity eliminating gram-negative except *E. coli* and *Proteus* while cefixime inhibit most of gram-positive and gram-negative bacteria but do not affect *E. coli*.

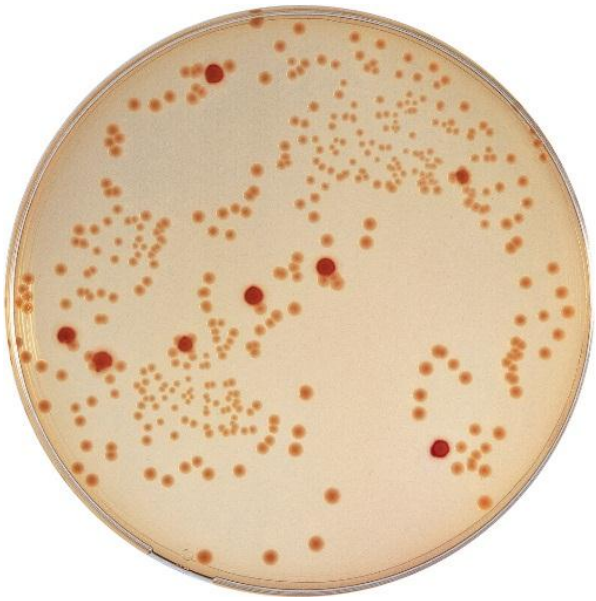

Figure 2.6. MacConkey Sorbitol agar (Biokar Diagnostics Co Ltd.)
(*E. coli* O157:H7: colorless or straw colonies and smaller;
E. coli non-O157: H7: red colonies and larger)

However, Hussein et al. (2008) demonstrated that the sorbitol-negative characteristic is not unique for *E. coli* O157:H7 strains by showing several Shiga toxin *E. coli* isolates of members of the O1: H2, O125: H2, O125: H19 and O158: H16 serotypes are also sorbitol-negative. It implied the inaccuracy of the SMAC agar-based methods although the methods are simple and inexpensive.

2.5.2. Immunoassay

The immunological assays used to detect *E. coli* O157:H7 are based on detection of O and H antigens. Many commercially available ELISA kits to detect *E. coli* O157 antigen directly in samples offer testing times of less than 1 hour (Dylla et al., 1995; Park et al., 1996). Park et al. (1996) showed that the ELISA kits had a sensitivity of 91.2% and a specificity of 99.5% that were higher than the SMAC agar method. Although the ELISA methods associated with O and H antigens are fast and sensitive, the cost of materials used is often high. The average cost per ELISA is approximately $2 to $3 (Park et al., 1996).

In real samples, small numbers of *E. coli* O157 present. Therefore, immunomagnetic separation (IMS) with commercially available magnetic beads coated with antibody against *E. coli* O157 has been utilized to concentrate the *E. coli* concentration. The specificity of the antibody together with the magnetic properties of the beads enables separation of the target

bacterium from the non-targeted bacteria. IMS has been used to detect *E. coli* O157 in food and water samples (Yu & Bruno, 1996; Goodridge et al., 1999; Brigati et al., 2008). With a 4-h pre-cultivation, IMS can support to detect as low as 10^2 CFU g^{-1} in stool samples (Karch et al., 1996). The IMS exhibited excellent performance with respect to the rapidity and selectivity of separation; however, the requirement of expensive immunomagnetic beads renders the method costly. Furthermore, additional assays for determining the binding of the bacteria to the beads are essential for completing the detection.

2.5.3. Polymerase Chain Reaction

In detection of *E. coli* O157 using PCR, primer probes have been usually designed based on *stx* genes (Nataro et al., 1998; Jinneman et al., 2003). In the PCR, at least two probes are usually used for *stx1* and *stx2* (Jinneman et al., 2003) and additional probes specific for *stx2c* and *stx2e* are required to detect strains containing these genes (Karch & Meyer, 1989; Paton et al., 2001; Tyler et al., 1991; Kawano et al., 2012). PCR methods have been used to detect *stx*-containing *E. coli* in food samples. Witham et al. (1996) showed that PCR method could detect approximately 10^5 CFU per reaction and as few as 0.5 CFU of Shiga-like toxin producing *E. coli* per gram could be detected in ground beef with only 12 h of enrichment in a broth. Besides PCR based on the *stx* genes, the method was applied to detect the *E. coli* using primers designed based on the gene *fliC* encoding the H7 antigen (Gannon et al., 1997). Cebula et al. (1995) developed a multiplex PCR assay that simultaneously identifies isolates of O157:H7 by using a multiple primers according to *stx* and *uidA* genes. Two of which were conserved with the *stx* genes and the third primer set was related to the *uidA* gene. Although O157:H7 isolates do not exhibit glucuronidase activity, they carry the *uidA* gene. Therefore, *E. coli* O157:H7 could be differentiated from other *E. coli* strains.

Real-time PCR approaches are now widely applied in microbial ecology to quantify the abundance of functional gene markers within the environment. Real-time PCR combines the traditional PCR with fluorescent detection technologies to record the accumulation of amplicons in 'real time' during each cycle of the PCR amplification. By detection of amplicons during the early exponential phase of the PCR, this enables the quantification of gene numbers when these are proportional to the starting template concentration (Smith & Osborn, 2009). Real-time PCR has been applied to quantify the *E. coli* O157:H7 in environmental samples. Ibekwe et al. (2002) applied real-time PCR to quantify *E. coli* O157:H7 in soil, manure, cow and calf feces, and dairy wastewater in an artificial wetland. Primers and probes were designed to amplify and quantify the *stx1*, *stx2* and *eae* genes of *E. coli* O157:H7. In this study,

quantification of *E. coli* O157:H7 in soil, manure, feces, and wastewater was possible when cell numbers were greater than 3.5 x 10^4 CFU g^{-1}. Elizaquível et al. (2012) employed real-time PCR to detect and quantify foodborne *E. coli* O157:H7 in fresh-cut vegetables.

2.5.4. Phage-based detection methods

2.5.4.1. Detection of *Escherichia coli* using non-engineered phages

1) Phage typing

The method is used to identify an unknown bacterium by using various types of phages with known host range. The phages will infect the bacterium sample individually and if the bacterium is within the host range of a certain phage, the plaque will be created on agar plate by plaque assay. Name of the unknown bacterium will be determined based on which phage forming the plaque. Phages used in phage typing test is called typing phages.

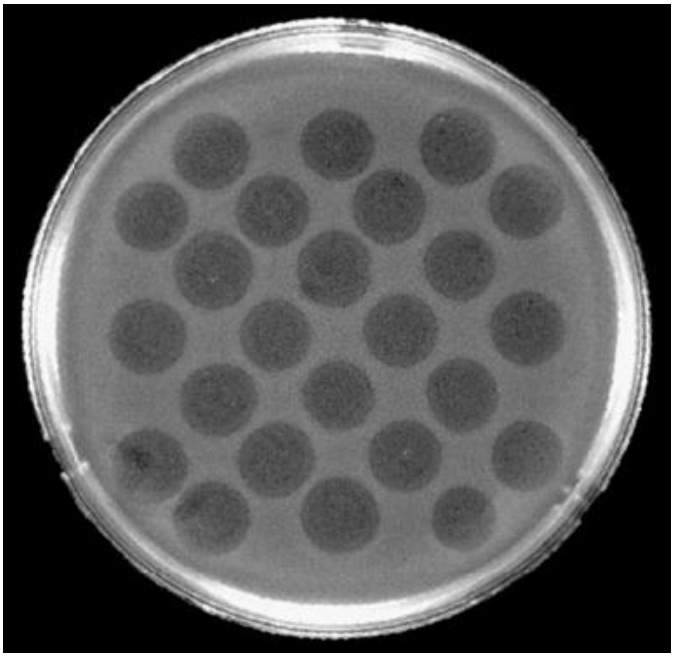

Figure 2.7. Phage typing (Smartt et al., 2012)

The typing phages should own high lytic activity, high specificity, and high lytic reaction stability. Phage typing is an important method for epidemiological diagnostics and surveillance. It has been applied to a various types of bacteria including *Escherichia* (Parisi et al., 1969; Ahmed et al., 1987; Khakhria R et al., 1990), *Staphylococcus* (Wentworth, 1963), *Yersinia* (Barker & Farmer, 1982), etc. Although the method requires no specialized equipment,

it requires large library of known host range phages to identify an unknown bacterium. Therefore, small laboratories may have difficulties in applying phage typing.

2) Phage-mediated cell lysis

The method is based on monitoring intracellular components that are released after phage infection into host bacteria occur. The release of these components is monitored and the presence or absence of the host bacteria will be indicated. Among intracellular components, intracellular adenosine triphosphate (ATP) is commonly used as a marker in the phage-mediated cell lysis method. ATP exists in all living bacteria at a relatively constant concentration. By conducting a bioluminescent assay using firefly luciferase as enzyme and the ATP released as one of substrates, the number of viable phage-specific host cells can be estimated. Because of the high host specificity of phages against host bacteria, other non-host cells containing in samples do not significantly interfere the assay. Another enzyme such as adenylate kinase is another marker in the phage-mediated cell lysis method. Adenylate kinase also exists in all living cells and it catalyzes the conversion of adenosine diphosphate (ADP) to ATP. With the present of adenylate kinase, the added ADP substrate will be conversed to ATP that can be measured quantitatively by the firefly luciferase assay as shown above. Kannan et al. (2010) used the phage-mediated cell lysis to detect *Escherichia coli* O157:H7 in ground beef. The assay used an O157-specific lytic bacteriophage CSLO157 to infect the *E. coli* O157:H7 in sample. After cell lysis, the released adenylate kinase was measured in terms of relative light units using luciferin-luciferase assay. Besides ATP and adenylate kinase, β-D-galactosidase also can be used as a marker in detection of *E. coli* using the phage-mediated cell lysis method. Neufeld et al. (2003) utilized a λ vir phage that specifically infects *E. coli* K12 and β-D-galactosidase was released after cell lysis. Appearance of *E. coli* was evaluated by measuring the released β-D-galactosidase activity in an enzyme assay using *p*-aminophenyl-*β*-D-galactopyranoside (β-PAPG) as a substrate. The product *p*-aminophenol (PAP) was oxidized at a carbon anode at 220V in an electrochemical system.

3) Phage amplification

Principle of the phage amplification detection is based on increase number of phages as indicator of a successful infection. At the end of phage infection process, phage will lyses host cells and release hundreds of new phage particles. Therefore, the presence or absence of a target bacterium in a sample can be examined by adding the phages specific for host bacterial cells and monitoring whether the increase of the phage appear (Hirsh & Martin, 1983). The

increase of the phage concentration is detected by by high-performance liquid chromatography (HPLC). However, the original phage amplification method is disadvantageous by the requirement of a large number of target bacteria present in the sample as well as the cost and complexity of HPLC analysis (Smartt et al., 2012). To overcome this disadvantage, an improved phage amplification method was reported as follows.

Firstly, phages specific for the target bacterium is added to sample. If the target bacterium exists in the sample, it will be recognized and infected by the phage (Figure 2.8, a & b). Before the host cells are lysed and progeny phages are released, a phagicidal reagent is added to inactivate any free phages present outside the host cells (Figure 2.8, c). The phagicidal reagent will not affect the phage particles inside the host cells. The virucide is then neutralized and a number of the host cells is added into the mixture and plaque assay is carried out. After incubation, the phages inside the host cells will replicate and release during cell lysis (Figure 2.8, d). The progeny phages then infect the added host cells and result in the formation of plaques. The formation of plaques indicate the presence of target cells in the original samples. The disadvantage of this improved phage amplification method is its dependence on the efficiency of the phagicidal reagent to inactivate all free phages. Any free phages that would not be inactivated will lead to false-positive results since the free phages can infect the added host cells. Favrin et al. (2003) utilized the IMS that specific binds to target host cells to separate them from the free phages without needing of the phagicidal reagent.

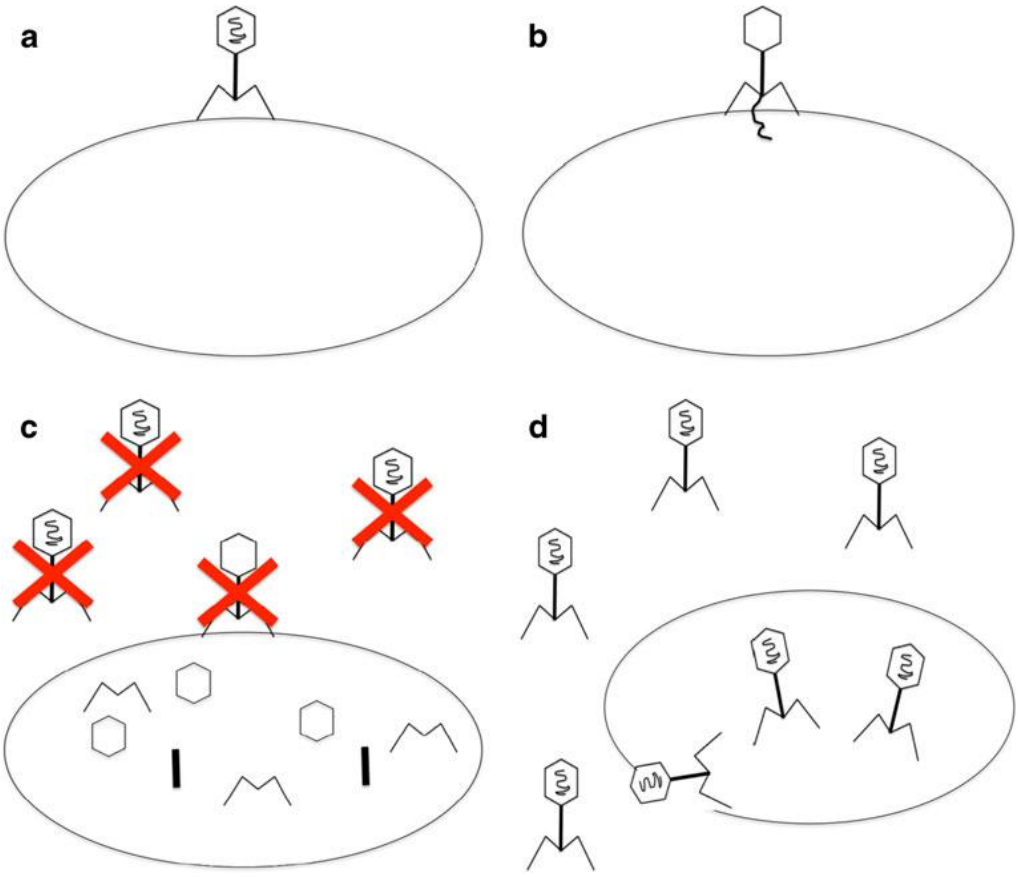

Figure 2.8. Phage amplification method (Smartt et al., 2012)
a – Adsorption of the specific phage; b – Injection of the phage DNA; c – Inactivation of free phages outside the host cells; d – Release of new phages after cell lysis

2.5.4.2. Detection of *Escherichia coli* using engineered phages

1) Fluorescent labeling of phage nucleic acid

Detection of *E. coli* can be conducted by using phages whose nucleic acid genomes are fluorescently labelled. Preparation of labelled phages is a multiple-step and quite complicated process (Goodridge et al., 1999a) using a commercially available dye designed for nucleic fluorescent. When genome of a phage was fluorescently labelled, the phage becomes "glowing" and can be detected by using epifluorescence microscopy or flow cytometry.

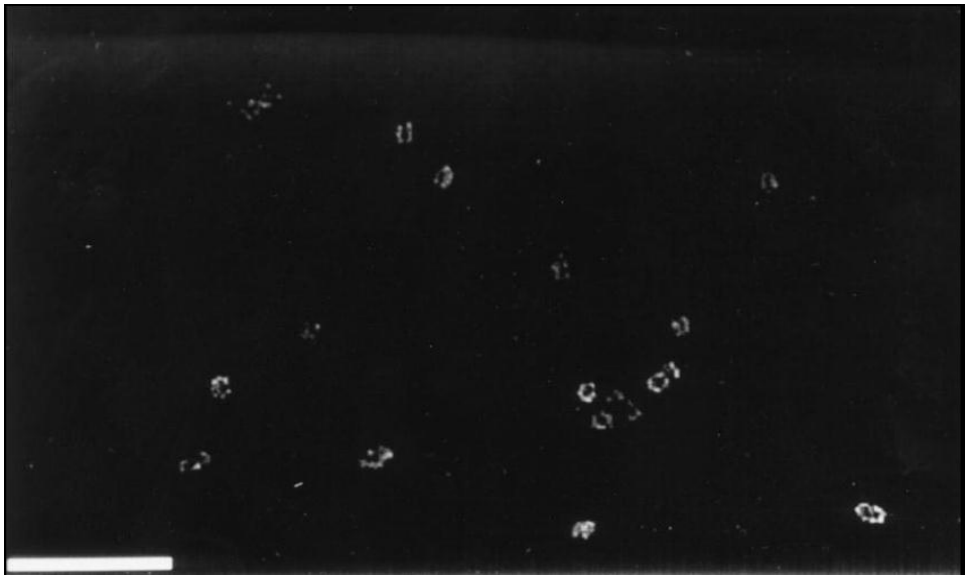

Figure 2.9. *E. coli* O157:H7 cells labelled with the fluorescent bacteriophage LG1. Bar = 10 μm. Magnification, x 1000 (Goodridge et al., 1999a)

Briefly, in the detection procedure, sample that may contain *E. coli* is mixed with fluorescently labelled phage. If *E. coli* is involved in the sample, the fluorescently labelled phage attaching on *E. coli* cells could be observed by epifluorescence microscopy or flow cytometry. In practice, immunomagnetic beads are commonly used to firstly collect *E. coli* cells in samples. Then the fluorescently labelled phage will bind onto *E. coli* on the beads and will be easily detected. However, false-positive labeling may occur since attachment of the labelled phage may occur to cells that don't belong to the normal host range of the labelled phage. In addition, the dyes designed for nucleic fluorescence are normally expensive (Tanji et al., 2004). Moreover, apparatus required for the assay such as epifluorescence microscopy or flow cytometry are specific and expensive.

2) Bioluminescent detection

Generally, bioluminescent- based reporter systems utilizes the bacterial (lux) and firefly (luc) luciferase reporter genes. The lux genes is a group involving five different genes such as luxC, luxD, luxA, luxB and luxE (luxCDABE). The *lux* genes are derived from *Vibrio* and *Photobacterium* genera of bacteria. The chemical equation of the bacterial luciferase catalyzed reaction is shown in the Figure 2.10 Substrates of the bacterial luciferase are reduced flavin mononucleotide ($FMNH_2$), molecular oxygen (O_2) and long chain fatty aldehyde (eight carbons or longer). Oxidation and reduction of substrates releases energy as blue/green light emission at wavelength approximately 490 nm. In the reaction, flavin mononucleotide and molecular oxygen exist in both eukaryotic and prokaryotic cells.

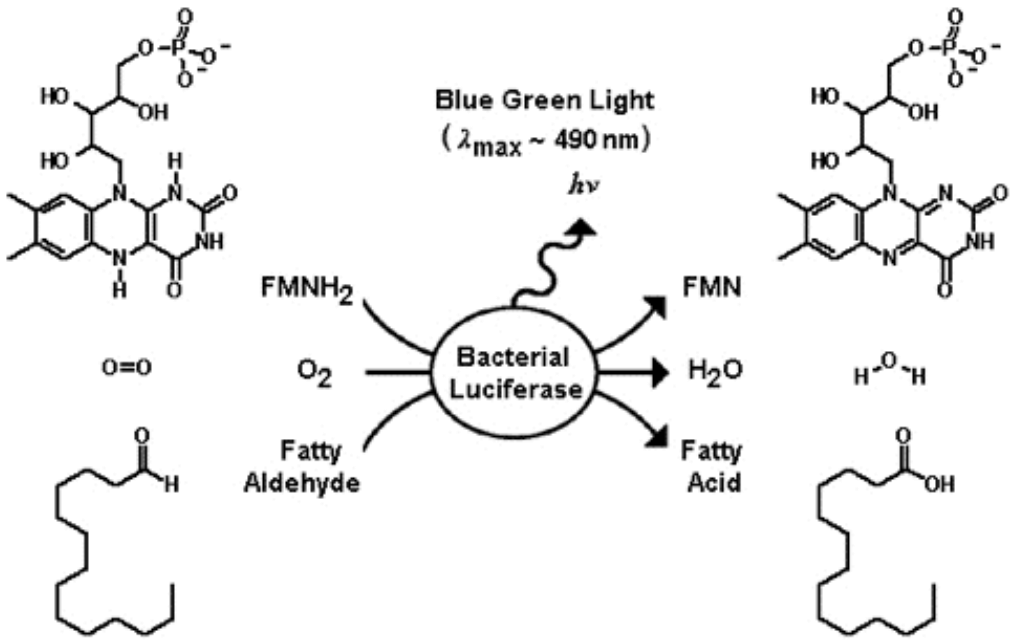

Figure 2.10. The chemical equation of the bacterial luciferase catalyzed reaction (http://www.photobiology.info/Lin.html)

In bioluminescent phage-based detection methods, normally, there are two approaches towards the *lux* reporter genes system. The first approach is to use the full luxCDABE operon that is inserted into a bacteriophage genome. The *luxAB* encode the proteins responsible for generating bioluminescence, while the reductase (*luxC*), transferase (*luxD*), and synthetase (*luxE*) genes encode proteins responsible for production of an aldehyde substrate that involved in the bioluminescent reaction as shown above (Figure 2.10). Generation of the light using this mechanism is fully autonomous without addition of external substrates. However, the size of the luxCDABE operon (approximately 6000 bp) is considered as too large to be inserted to a phage genome without any problem for head packaging of the phage (Smartt et al., 2012). To overcome this problem, a phage genome need to be engineered prior to the insertion of the luxCDABE operon (Smartt et al., 2012). The second approach is to insert only a partial of the full luxCDABE operon into a phage genome. Normally, the *luxAB* genes (approximately 2000 bp) are incorporated into a phage genome to synthesize the LuxAB protein. Then the bioluminescent reaction was performed by adding the external aldehyde substrate (Waddell & Poppe, 2000). Substrate addition is not always ideal because of single time point measurements.

To eliminate the disadvantages of both approaches as described above, a bioluminescent phage- based detection based on quorum sensing phenomenon was introduced (Brigati et al., 2007; Ripp et al., 2008). In this system, a reporter cell is used to carry the complete luxCDABE operon. Genome of a recombinant phage is incorporated by a foreign gene. By infection of the recombinant phage to the target *E. coli*, the protein derived from expression of the foreign gene will diffuse to the reporter cells and active the expression of luxCDABE operon. An example of this system was shown in the Figure 2.11.

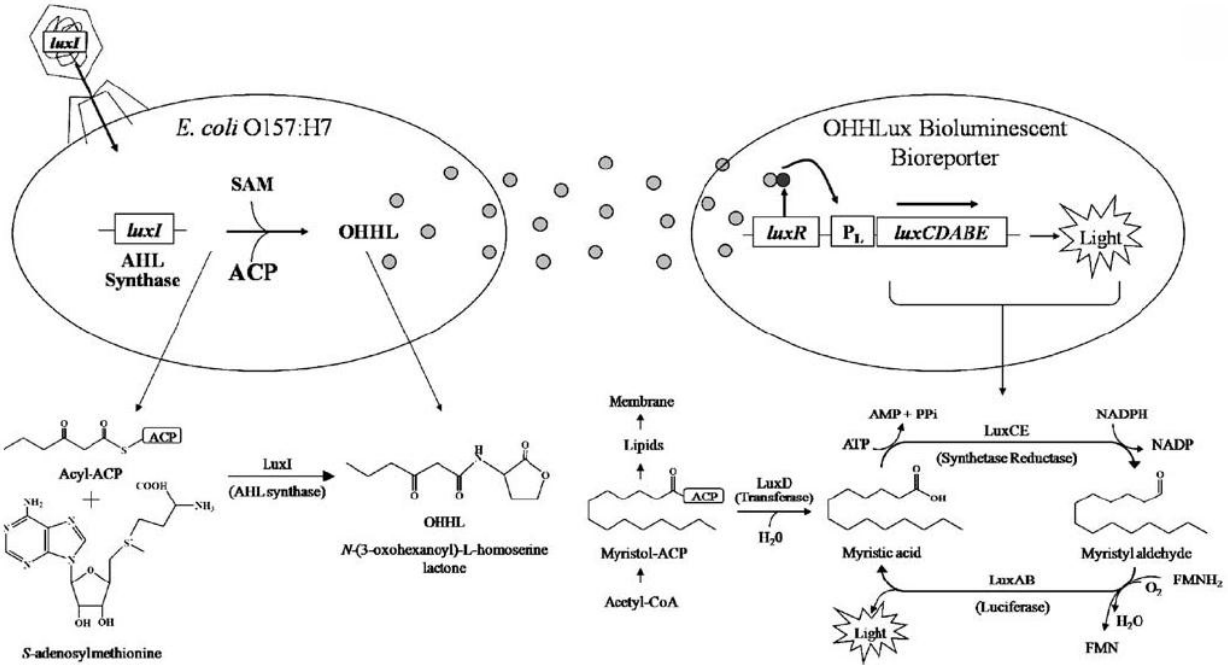

Figure 2.11. Anatomy of the binary phage reporter assay for the detection of *E. coli* O157:H7 (Ripp et al., 2008)

In this research, genome of the recombinant phage PP01-luxI was engineered to carry the *luxI* gene. Under the control of a strong phage promoter, upon infection of the PP01-luxI to specific *E. coli* O157:H7 host, the luxI is expressed and resulting in the synthesis of OHHL autoinducer from acyl-ACP and S-adenosyl methionine (SAM). OHHL diffuses extracellularly and interacts with the OHHLux bioluminescent bioreporter cells, specifically binding with the LuxR regulatory protein to trigger transcription of the luxCDABE operon and produce the light as described above.

Using this system was firstly described in the research of Brigati et al., (2007) and was successfully applied for detection of *E. coli* O157:H7 concentration of 10^1 CFU ml^{-1} in pure culture for 8.5 h (6-h pre-incubation and 2.5-h assay) (Ripp et al., 2008). But it was not possible to be applied for detection of *E. coli* O157:H7 in undiluted apple juice, no light was produced even at the highest concentration of bacteria and even the samples were brought to a neutral pH. When the sample was diluted 6 times by LB medium, for a concentration of 10^4 CFU ml^{-1}, the detection was accomplished in 7.5 h (6-h pre-incubation and 1.5-h assay). However, no significant bioluminescence was observed when *E. coli* concentrations were below 10^4 CFU ml^{-1}. In order to detect the *E. coli* concentrations below 10^4 CFU ml^{-1} in apple juice, some modifications were utilized in preparation of apple juice sample (Ripp et al., 2008) and it allowed the method can detect *E. coli* O157:H7 concentration of 10^1 CFU ml^{-1} for about 19 h (6-h pre-incubation and 12-h assay). The method could also be applied for detection of *E. coli* O157:H7 in tap water. But it was not successful in the detection of *E. coli* O157:H7 inoculums below 10^6 CFU ml^{-1} in ground beef since ground beef inherently contains OHHL autoinducer (Ripp et al., 2008).

Bioluminescent-based methods are known as more rapid compared to colorimetric-based methods. However, the main demerit of the bioluminescent-based methods is requirement of specific and expensive apparatus to detect the luminescent signal. These apparatus are not always equipped in laboratories especially in less developed areas.

3) Fluorescent detection

Green fluorescent protein (GFP) isolated from the jellyfish Aequorea Victoria is a well-known protein that was applied in fluorescent- based detection methods. The chromophore involving Threonine 65, Tyrosine 66, and Glycine 67 of the GFP can automatically cause fluorescence that is valuable in the fluorescent- based detection methods. Size of the *gfp* gene is relatively small (approximately 700 bp) and it is easily to be integrated into a phage genome without effect on the phage head packing. Tanji et al. (2004) introduce a fluorescence-based

detection method involved introducing the *gfp* gene into the lysozyme-inactivated T4 phage genome. The *gfp* gene was incorporated into *soc* gene location in the T4 phage genome. Production of the green fluorescent protein and consequently its accumulation in infected *E. coli* cells enabled selective observation under an epifluorescence microscope (Figure 2.12).

Oda et al. (2004) also incorporated *gfp* gene into either C- or N-terminal of *soc* gene of PP01 phage to produce either PP01-GFP/SOC or PP01-SOC/GFP, respectively. The recombinant phage was used to detect *E. coli* O157:H7. Fusion of GFP to SOC did not change the host range of the PP01. The binding affinity of the recombinant phages to the host cell increased. However, the stability of the recombinant phages in alkaline solution decreased. In order to detect *E. coli* in sewage water, Namura et al. (2008) engineered two type of phages such as IP008 and IP052. The phage genomes were firstly modified by deleting gene e responsible for lysozyme and inserting the *gfp* gene into the gene e location. It resulted in two recombinant phages such as IP008e-/GFP and IP052e-/GFP. However, the fluorescent intensity of *E. coli* cells infected with IP008e-/GFP and IP052e-/GFP was not enough for visualization of the cell. In order to increase the fluorescent intensity, insertion of GFP to SOC of IP008e-/GFP and IP052e-/GFP was carried out to produce IP008e-/2xGFP and IP052e-/2xGFP, respectively. Because of the broad host range to *E. coli* of the phages, *E. coli* in the natural environment could be detected using this system.

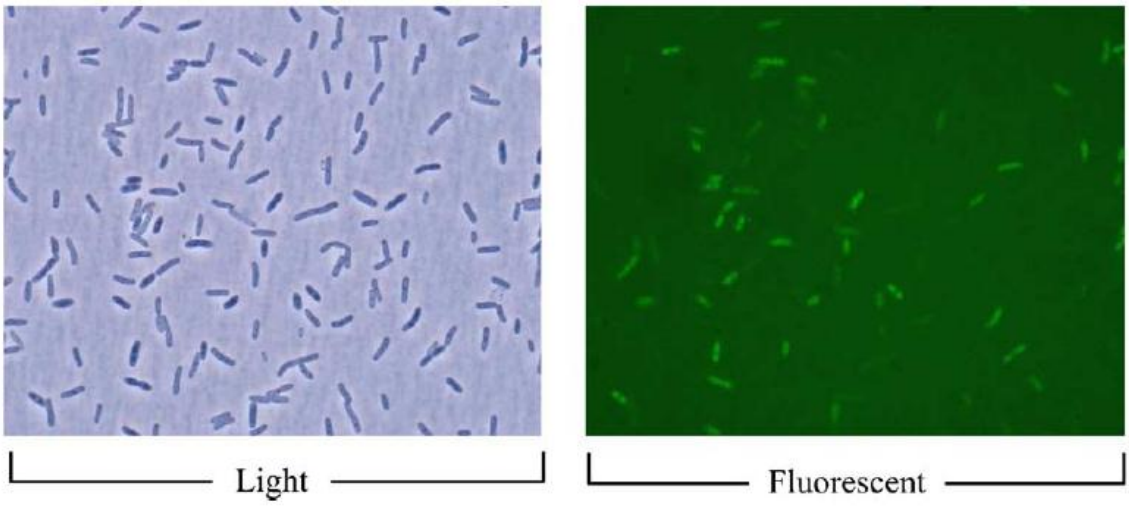

Figure 2.12. Optical and fluorescent microscope images of the mixture of *E. coli* K-12 and *P. aeruginosa* PAO1 mixed with T4e−/GFP phage (Tanji et al., 2004)

Besides the autofluorescence characteristic as described above, GFP also has an excellent stability and low toxicity. However, it requires exposure to an excitation light source to initiate its 509 nm fluorescent properties. Therefore, in the detection of *E. coli* using the fluorescent phage- based method, epifluorescence microscopy is required to detect the fluorescent signals. Because epifluorescence microscopy is a specific and expensive apparatus, it make the detection conditional.

Table 2.3 showed a summary of phage-based methods that were investigated in previous studies for detection of *E. coli* in terms of sample matrix, detection scheme, detection limit and response time.

Table 2.3. Phage-based methods for the detection of *E. coli*

Targeted pathogen	Sample matrix	Detection scheme	Detection limit	Response time	Reference
E. coli O157:H7	Ground beef	Phage-mediated cell lysis	<10 cells 25 g^{-1}	10 h	Kannan et al. (2010)
E. coli O157:H7	Ground beef	Phage amplification	2 CFU g^{-1}	23 h	Favrin et al. (2003); Smartt et al. (2012)
E. coli O157:H7	Raw milk	Labeling of phage DNA (YOYO-1 fluorescent dye, detected by epifluorescent microscope)	10 CFU mL^{-1}	12 h	Goodridge et al. (1999)
E. coli O157:H7	Ground beef	Labeling of phage DNA (YOYO-1 fluorescent dye, detected by epifluorescent microscope)	10 CFU g^{-1}	7 h	Goodridge et al. (1999)
E. coli	Sewage	Reporter gene (*gfp*)	NR	6 h	Namura et al. (2008)
E. coli O157:H7	Apple juice	Reporter genes (*luxI* & *lux R*)	1 CFU mL^{-1}	22 h	Ripp et al. (2008)
E. coli O157:H7	Tap water	Reporter genes ((*luxI* & *lux R*)	1 CFU mL^{-1}	12.5 h	Ripp et al. (2008)
E. coli O157:H7	Spinach	Reporter genes ((*luxI* & *lux R*)	1 CFU mL^{-1}	6 h	Ripp et al. (2008)
E. coli	Iceberg lettuce	Reporter genes ((*luxI* & *lux R*)	130 CFU ml^{-1}	22.4 h	Ripp et al. (2006)
E. coli O157:H7	Beef sirloin tip	Reporter gene (*lacZ*, colorimetric substrate)	10^3 CFU 100 cm^{-2}	12 h	Willford & Goodridge (2008)
E. coli O157:H7	Beef sirloin tip	Reporter gene (*lacZ*, luminescent substrate)	10^2 CFU 100 cm^{-2}	10 h	Willford & Goodridge (2008)

NR: Not reported

Chapter 3

Colorimetric detection of *Escherichia coli* K12 by using a recombinant phage

SUMMARY

A new rapid and simple method was developed for the detection of *Escherichia coli* K12 by constructing a recombinant T4 phage carrying the *cytochrome c peroxidase* gene derived from *Saccharomyces cerevisiae* (T4ccp) using which, the colorimetric detection of *E. coli* K12 was examined. The oxidation activity towards the chromogenic substrate cytochrome c was demonstrated by the cytochrome c peroxidase (CCP) produced from the T4ccp genome. The color change caused by the oxidation of the substrate could be visually perceived. The possibility of interference in the detection by the coexistence of other bacteria was assessed using *Pseudomonas aeruginosa* as a non-target bacterium and it was confirmed that the coexistence of *P. aeruginosa* caused no interference in the detection of *E. coli* K12.

Chapter 4

Quantitative detection of *E. coli* O157:H7 using a combination of the colorimetric phage-based assay and most probable number technique

SUMMARY

Colorimetric phage-based detection method was invented in the previous chapter. The method enabled the detection of *E. coli* based on the color change that is expected to be inexpensive compared to the previous phage-based detection methods. However, the method is still similar to the previous phage-based methods in terms of impossible quantification. In this chapter, quantitative detection of *E. coli* O157:H7 using a combination of the colorimetric phage-based assay and most probable number technique was developed. Firstly, a recombinant phage PP01ccp carrying the *ccp* gene was constructed to detect the pathogenic *E. coli* O157:H7. Principle of the construction of PP01ccp was similar to that of T4ccp shown in the previous chapter in which ccp gene was recombined into region between *g56* and *soc* genes in genome of the PP01 phage. The PP01ccp was used to *E. coli* O157:H7 in broth and color change derived from the experiment could be easily recognized by eyes. It confirmed that CCP expressed from the PP01ccp genome contributed substantially to the oxidation of the cytochrome c. Secondly, a combination of the detection method based on PP01ccp and the MPN technique (abbreviated as MPN-phage assay) was developed to quantify *E. coli* O157:H7 concentration in various samples of apple juice, milk and cattle manure. For apple juice and milk samples, the MPN-phage assay was tested with an *E. coli* O157:H7 concentration of 10^2 cells mL^{-1}. In the cattle manure, there are many background bacteria co-existing with *E. coli* O157:H7. Therefore, selection of proper antibiotics that suppressed growth of background bacteria but mostly did not affect growth of *E. coli* O157:H7 was investigated prior to the quantification. Then, antibiotics of cefixime and vancomycin were selected to be used in quantification of *E. coli* O157:H7 at two concentration of 10^2 and 10^5 cells g^{-1} in the cattle manure by the MPN-phage. The MPN-phage assay was successful in quantification of *E. coli* O157:H7 in the all types of samples.

Chapter 5

Quantitative evaluation of *E. coli* O157:H7 during cattle manure composting

SUMMARY

The fate of *E. coli* O157:H7 during a cattle manure composting was investigated using the MPN-phage assay. In large composting pile, temperature varies extensively as lowest at the surface of the pile, moderate near the bottom, and highest at the center. In this chapter, two types of composting runs, with and without turning, were conducted with the temperature of composting was set at 30, 50, and 70°C to simulate the temperature condition in the large pile. For the runs with turning, the material from the reactors was removed, mixed each other and redistributed daily, whereas for those without turning, the compost was agitated inside the reactors. The composting was sampled and examined the fate of *E. coli* O157:H7 by using the MPN-phage assay. Organic matter degradation during the composting was analyzed and compared between the composting with and without turning. The MPN-phage assay revealed that the *E. coli* O157:H7 was rapidly eliminated at composting at 70^{o}C and 50^{o}C for both with and without turning. For the composting at 30^{o}C, the difference of concentration of *E. coli* O157:H7 was large between the composting with and without turning. It indicated that the fate of *E. coli* O157:H7 was accelerated by effect of turning.