Effect of Garlic Extract on Salmonella typhimurium Biofilm

By

Ms. Ramida Jongsupangkarat

A Special project submitted to the Faculty of Biotechnology,
Assumption University in part fulfillment of the repuirements for
the degree of Bachelor of Science in Biotechnology
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Abstract

Salmonella typhimurium is a foodborne pathogen which contaminates food by cross contamination and unsanitary process. This bacteria causes salmonellosis that affect to health and finally causes death. Normally, most bacteria can be treated by bleaching agent or antibiotic, but when bacteria join into a community known as biofilm, bacteria become harder to be eliminated. In addition, sanitizer and antibiotic are not efficient enough due to the price of antibiotic and health concern of sanitizer. The alternative substance from the nature as garlic that has antimicrobial property was studied in this research. This research was conducted by using rapid method to verify the bacterial attachment on to surfaces which are glass and polystyrene, and to determine the suitable concentration of garlic that can inhibit the biofilm development. The comparison of the elimination ability with sanitizer (sodium hypochlorite) was compared with the garlic extract. The 2% *S. typhimurium* was inoculated in glass tube and polystyrene tube contained 1/20 TSB. The bacterial attachment was measured based on staining the attached cells with 1% crystal violet. The staining of crystal violet indicates the amount of biofilm formed on the surface that normally occurred at the air-liquid interface. Consequently, biofilm can form more onto polystyrene by hydrophobic interaction, and 60 mg/ml garlic extract concentration was minimum inhibition concentration for an inhibition of *S. typhimurium* biofilm. Therefore, the elimination effect of garlic extract was compared with NaOCl at the concentration that is commonly applied in the industrial clean up. The study showed that garlic extract represented less elimination effect to the mature biofilm than NaOCl. The study was based on the absorbance value which showed to be lower in NaOCl treatment than the garlic extract treatment that illustrated the lower in the bacterial attachment when it was compared to the treatment by garlic extract. Although garlic is not the best option as NaOCl for elimination of mature biofilm, but garlic is safe in term of health concerned.

Acknowledgement

I would like to show my deep gratitude towards many people that has continuously support me throughout the years of studies and experiments.

Firstly, Dr.Tatsaporn Todhanakesem, from the faculty of Biotechnology, Assumption University for her guidance advises, and patience which lead me through this project.

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Lastly, I greatly appreciate my parents for the inspiration and everything. I would not be able to come this far without them.

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Contents

List of Tables

List of Figures

Introduction

Biofim formation is a microbial colonization that has been studied for many years (Scott and Edmund,1997). It is a microbial cells population growing on a surface and enclosed in extracellular matrix (Stepanovic et al,2004). It causes the corrosion on the contact surface. There are many factors presence in biofilm attachment such as temperature, pH, nutrient limited or surface conditioning. Quorum sensing which is known as acylhomoserine lactones (acyl-HSLs) becomes important in biofilm formation as it involves the communication of bacterial cells. Different types of bacteria have different signals which are very specific, as a consequence, bacteria has unique behavior. After the attachment, extracellular polymeric substance or exopolysaccharide (EPS) is secreted out. EPS composes of carbohydrate, protein and lipid that act as protective substance from harsh condition and also antibiotic or bleaching agent, causing biofilm hardly eliminated.

Biofilm can be produced by various types of microorganisms included in both gram positive and gram negative bacterium. *Salmonella spp.* are gram negative facultative motile bacterium. They are one of the most important foodborne pathogens, so they cause a lot of infections in food processing environment. *Salmonella typhimurium* is one of the most common *Salmonella* serovars causing Salmonellosis infection. There are many outbreaks from *Salmonella spp.*, based on the research, it is estimated that 1.4 million cases of Salmonellosis occurs in each year in U.S., and 95% of those cases are foodborne-related, and 31% are food-related death (Marler,2007). One suspicious reason is that *Salmonella spp.* form biofilm within the food processing equipments, causes post-contamination on food. When organic substances move along a very narrow zone between liquid phase and surface of the pipe with low velocity, causing these organic materials settle and deposit to the surface. Bacteria which are free floating in the pipe be able to attach to these organic deposits by using flagella, and secrete out the protective shield known as exopolysacharide (Vanessa, 2008). This contamination in food becomes more concerned because people are more realized in food sanitation. Food claimed contaminated is a big issue that impact to the acceptance of consumers and product image. Many organizations have been established to inspect and approve the food safety, but outbreak cases are still high

and cause serious illness as mentioned. Thus, many industries try to prevent the contamination in food by eliminating the biofilm formation.

Antibiotic has ability to inhibit biofilm formation but its price is very expensive, so other alternative substances are created to eliminate biofilm formation. Besides antibiotics, some chemicals can also inhibit biofiom formation but it might remain in the food equipments that affect to the consumer health. For example, the uses of sodium hypochlorite for sanitation in the industry can cause skin and eyes irritation and corrosion to the equipments. Thus, this experiment try to use the natural compound to inhibit biofilm formation, focusing on *Salmonella* biofilm in order to reduce the use of chemical and antibiotics. Garlic is chosen as it is very well-known for anti-fungal and anti-microbial activity (Thomas et al., 2005). Garlic has allicin which has been reported that it has ability to block quorum-sensing of bacteria, resulting the reduction of bacteria formation. However, there is no research focus on the inhibition of garlic over *Salmonella typhimurium* biofilm formation.

If this experiment was conducted successfully, It would provide benefits on many food industry fields in term of natural antimicrobial compound coated on the equipment. It takes advantages on the safety, or even cost expenses.

Objectives

1. To study on the formation ability of *S. typhimurium* under glass and polystyrene surfaces
2. To study antimicrobial effect of garlic extract against *S. typhimurium* and identify the optimum concentration of garlic extract on the biofilm inhibition
3. To compare the effect of garlic extract with the Sodium hypochlorite in the elimination of *S. typhimurium* mature biofilm

Literature Review

Salmonella spp.

Salmonella or common name salmonellae is a genus of rod-shaped Gram-negative enterobacteria which usually mobile by means of peritrichous flagella. It is a facultative intracellular anaerobes. Many *Salmonella* strains are capable of growth on a chemically defined medium, can use citrate as a carbon source, and are aerogenic (gas producing). Typical strains ferment neither lactose nor sucrose, and with few exceptions produce abundant H_2S. The optimum temperature is between 35 to 37°C. The slow growth can be observed at 5°C and maximum growth temperature ranged 45 to 47°C. The recent evidence indicates the prolonged exposure of mesophilic strains to thermal stress condition results in the mutant capable of growth at 54°C. The optimum pH is between 6.5 to 7.5 but growth can occur between pH 4 and 9.0. Water activity that can observe the growth ranged between a_w 0.999 and 0.945, and no growth support under a_w 0.93 (Anonymous, 2008). Salmonellae are inhibited in the presence of 3 to 4 % NaCl, bacterial salt tolerance increases with increasing temperature in the range of 10 to 30 °C.

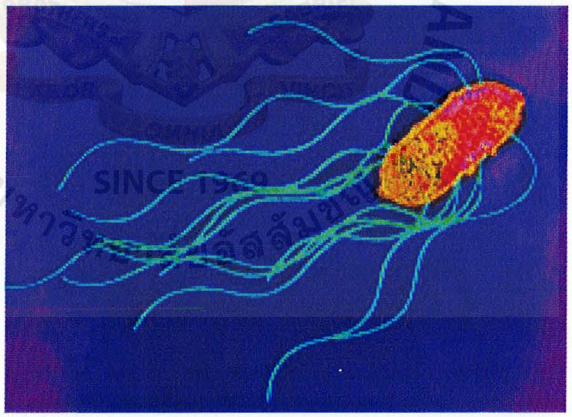


Figure 1 : *Salmonella* image (Kenneth, 2005)

The cell envelop of *Salmonella* and other gram-negative bacteria is unique (Efstathios and George,2006). It is a complex structure composed of 3 morphological layers which are cytoplasmic membrane, membranous structure and outer membrane or L-layer at the outer surface of cell (Osborn et al., 1972). The outer membrane

fraction of *Salmonella typhimurium* contained approximatly 60% of the protein of the total membrane, 50 % of the phospholipid, and 90% of lipopolysaccharide (Osborn et al., 1972).

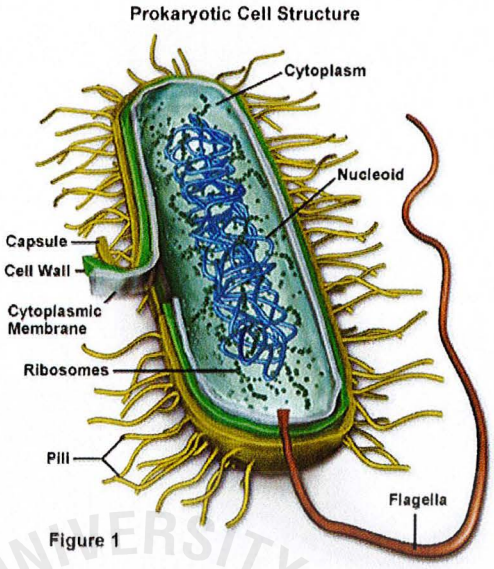


Figure 2 : Cell structure of *Salmonella* (Anonymous,2003)

Salmonella are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection or intoxication (Kenneth, 2005). *Salmonella* can be found in contaminated food during slaughter, cross contamination and unsanitary food handling practice. The reservoirs of *Salmonella* are egg and poultry. Moreover, contaminated water, milk, milk product, beef, fruit, vegetable and dairy product are also common source. *Salmonella* colonizes in the intestine and translocates across the intestinal epithelium via 3 routes which are invasion of enterocytes, invasion of epithelial cell known as M cells and through dentritic cells that intercalate epithelial cells. These invasions promote inflammatory to host that benefit the intestinal pathogen and competitive advantage over native flora (Michael, and Dirk, 2008). *Salmonella typhimurium* is one in the three main serovars of *Salmonella enterica* that is the most common cause of food poisoning. It can cause typhoid in mice, but for human, it causes diarrhea, abdominal cramps, vomiting and nausea. *Salmonella* infections can cause death if they are not treated with antibiotic because *Salmonella* have mechanism to prevent our immune system (Stanley and Rob, 1999). Like other foodborne illness, the symptom of *Salmonella* infection are nausea,

vomiting, abdominal cramps, diarrhea or bloody diarrhea, fever, and headache (Joel, 2008). The sign or symptoms of *Salmonella* infection appear 12 to 36 hours after ingestion of the contaminated food product (Viviane, Denise, and Jacyr, 2004).

Salmonella Outbreak

Salmonellosis is one of the most common causes of foodborne infections worldwide. In United State reported that 1.4 million people are affected by salmonellosis yearly, causing around 16,800 hospitalizations (1.2%) and 600 deaths (Ribeiro *et al.*, 2006). Human acquired *Salmonella enteritica* infection by eating contaminated animal products which usually associated with eggs and poultry (Viviane, Denise, and Jacyr, 2004). Eggs can be contaminated in chicken cloaca or by transovarian infection. Clean egg is the major risk of infection because it can transmit *Salmonella* if eaten raw or undercooked. Moreover, eaten poultry when meat is cooked and cooled down and eaten as cold, or after reheating can associated with *Salmonella enterica.* Among the *Salmonella enterica* serovars commonly implicated in most of the major outbreaks are serovars Enteritidis, Typhimurium, Heidelberg, Newport and Infantis (Toshio, William, and Hideo, 2002). There are many reports focus on the *Salmonella* outbreak. In the year 2001, Denmark reported an outbreak of *Salmonella Schwarzenground* infections in Denmark and the United States that was traced to contaminated chickens in Thailand. In 2002, a total of 16,580 cases caused by foodborne agents were diagnosed in the United States. From these, *Salmonella* was the most common infectious agent (6,028 cases). Moreover, the consumption of contaminated duck eggs has been reported as the cause of salmonellosis outbreaks in Italy, Thailand and United States (Ribeiro *et al.*, 2006). National Health Laboratory in Luxembourg had studied the emergence of *Salmonella enterica* monophasic serovar which was responsible to human cases in New York, Spain, Brazil, Thailand and Taiwan. It reported that 66% were *S. enteritidis* and 20% were *S. typhimurium* (Mossong, 2006). Many institutes in Thailand also realized on the *Salmonella* outbreak, so many researches were conducted. In 1999, Food and Agriculture Organization (FAO) and World Health Organization (WHO) focused on the risk assessment for *Salmonella* in fermented pork sausage or Nham. They found that *Salmonella* manage themselves to survive during the fermentation and cause the

adverse heath effects to consumers. The infection occurred after ingestion of food with *Salmonella* of about 10^4 to 10^6 cells (Tuitemwong *et. al*, 2004) Between 1990 – 1997, the phage type of *S. enteritidis* was verified to clarify the potential for human infection by this bacteria in chicken meat, human and poultry. PT4 was consistently the most common PT among those found in human and poultry isolates from Thailand (Boonmar *et al.,* 1998). *S. typhimurium* was also studied in Thailand. After isolation by PCR assay, the seven isolates positive duplex PCR was *S. typhimurium* DT104 which is considered zoonotic and life-threatening, and commonly associated with multiple-drug-resistant (MDR) characteristics (Amavisit, Boonyawiwat, and Bangtrakulnont, 2005).

Biofilm formation

Microbial biofilm began study last 2 decades ago. In natural aquatic systems, bacteria are found to attach to the surfaces freely (George, Heidi, and Roberto, 2000). A biofilm is a population of one or more organisms attached to each other and a surface as a bacterium-initiated matrix. This matrix provides a stability to biofilm itself and to be able to resist on anti-microbial agents (Prouty et al.,2001). This bacterial communities in nature play a key role in the production and degradation of organic matter (Mary and George,2000).

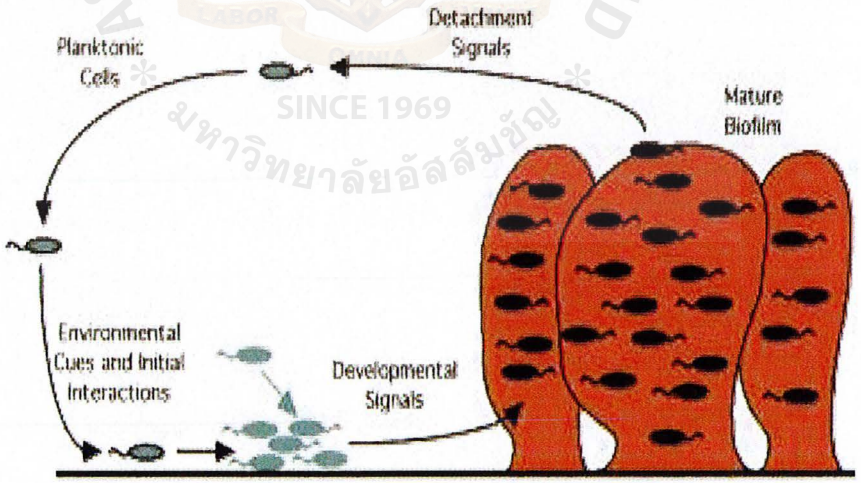


Figure 3 : Four stages of biofilm formation: Initial attachment, Microcolony formation, Mature biofilm and Detachment (O'Toole, Kaplan, and Kolter, 2000)

Attachment is the first stage of biofilm formation that occurs when microorganisms (planktonic form) come close enough to the surface held weakly by electrostatic forces (Scott and Edmund,1997). There are two stages of bacterial cell attachment which are reversible and irreversible attachment (Brenda, 2007). Reversible attachment occurs where individual cells secret long, string-like polymers which weakly anchor the cell to the surface. This causes the colonization of the surface as the attached cells multiply and spread over the surface creating a conditioning film of minimal coverage allowing cells to detach from the surface. Irreversible attachment occurs when the cells adhere and begin excreting extracellular polymeric substances, or exopolysaccharides (EPS). EPS is a long polymer chain made up of carbohydrates, proteins and lipids in long threadlike molecules that acts as the structural grid of the matrix which creates permanent cellular attachment to the colonizing surface, overcoming even charge repulsion between the cells and substratum. Once rigidly cemented to the surface, the EPS matrix is established, and the microcolony expands through cellular division and subsequent EPS production (Brenda, 2007). The ability of cell to perform the initial attachment depends on the environmental factors such as temperature, pH, nutrient, genetic factor, motility function etc. Simple chemical could affect for the behavior of bacteria during their attachment in term of cell-surface interactions (George, Heidi, and Roberto,2000). The important for initial attachment of bacteria is flagella which help in swimming for propulsion, referred as "flagella-mediated motility", but gram-negative motile bacteria as *Salmonella* has only 1% of genome devoted to flagella function. Another form of bacterial motility is a twitching motility that due to the extension and reaction of pili, another appendage. This motility is important in both formation of microcolonies and spreading of biofilm because it occurs when cells are attached to the surface and bacteria themselves across the surface (O'Toole, Kaplan, and Kolter, 2000).

After the initial attachment, cells start growing as a monolayer to form microcolonies. There are two associated properties for bacteria adaptation to life in biofilm. Those are the increased synthesis of EPS which composes of polysaccharides, proteins, nucleic acids, lipid/phospholipids or humic substances, and the structure itself provides a protective environment from harsh condition. EPS contains inonic groups which confer net negative or positive charges on the polymer at near neutral pH. Their negative charge due to the presence of carboxyl groups of non-carbohydrate (Wuertz, Bishop, and Wilderer, 2003). Normally, the extracellular proteins contribute

to the hydrophobic properties as their high proportion of the hydrophobic amino acids, and the combination of acidic and hydrophobic amino acids lead to the electrostatic and hydrophobic interaction (Wuertz, Bishop, and Wilderer, 2003). Bacteria can develop other properties such as increasing the resistance to UV light, antibiotic, extreme pH, irradiation. The biofilm development also facilitates the genetic exchange between microorganisms that live under the biofilm structure. Moreover, it also altered biodegradative capabilities, and secondary metabolite production (George, Heidi and Roberto,2000). Biofilm maturation has been demonstrated that Quorum-sensing molecules known as acylhomoserine lactones (acyl-HSLs) are required to regulate the biofilm structure (Mary and George,2000). QS system is a cell-cell communication which helps bacteria to keep track of their number (Thomas et al., 2005). Bacteria release signalling molecules to the environment to measure the concentration of the molecule in a population as whereby the accumulation of signalling molecules enable a single cell to sense the number of bacteria (cell density). There are many different types of bacteria living together, so various classes of signalling molecules released. Thus, they cannot necessarily talk to all other bacteria. This QS enables bacteria to co-ordinate their behaviour. When the surrounding condition changed, bacteria need to respond quickly in order to survive. It is very important for pathogenic bacteria during infection of a host (e.g. humans, other animals or plants) to co-ordinate their virulence in order to escape the immune response of the host in order to be able to establish a successful infection. (Bassler and Losick (2006); Williams et al. (2007); Diggle et al. (2007)).

Large population of biofilm leads to the detachment stage which may come from the starvation as nutrient limited, and accumulation of toxic. Detachment is the process of removal of biomass of biofilm in order to balance the biofilm growth. There is a reported that enzyme alginate lyase may play an important role in *P. aeruginosa*. The overexpression of alginate lyase can speed up the rate of detachment and cell sloughing from biofilm.

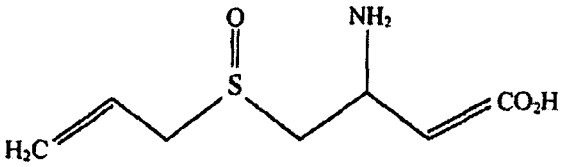
Salmonella biofilm formation

Salmonella biofilm formation has been studied by many methods such as transmission electron microscopy, epifluorecent microscopy or Microbial adhesion to hydrocarbon method to determine stages or proper conditions.

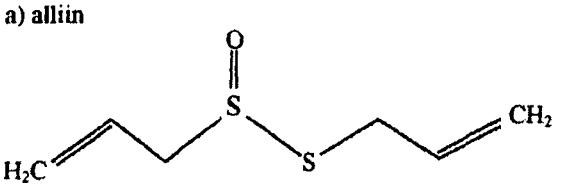
The machanisms for *Salmonella* biofilm formation starts with the initial attachment to the surface, microcolony formation, development of a three-dimensional community structure and maturation and detachment (Prakash *et al.*, 2005). Free floating cells with the presence of flagella and pili approach to the surface so close, so their motility slow down and they form a transient association with the surface. The surface should be rough and more hydrophobic. Bacteria become stable for microcolony formation after adhering. They mutiply while releasing the intercommunicated chemical signal among bacterial cells known as quorum sensing. Once the signal intensity exceeds a certain threshold level, genetic mechanism underlying EPS production are activated. Thus, bacteria multiply and embed under EPS matrix which give rising to the microcolony formation with the transcription of some specific genes. These are required for EPS synthesis. The extracellular matrix has the formation of water-filled channels as the primitive circulatory system, delivering nutrient and removing waste. To become a productive member in biofilm community, bacteria repress the synthesis of flagella and become destabilized and get encased within the EPS. This reinforce the biofilm structure as the gene response to the EPS synthesis are upregulated 3 -5 fold in the recently attached cells vs planktonic counterparts whereas genes required for flagellin and pilin are down-regulated (Prakash *et al.*, 2005). Occasionally, some bacteria are shed from biofilm or some stop producing EPS and are released to the surrounding environment. Detachment occurs as a result of nutrient level or quorum sensing or shearing of biofilm aggregates because of flow effect. As a result of increasing of thickness of EPS and anaerobic condition develops, the film detaches and sloughs off from the surface. Polysacchaaridase which is specific for EPS may be produced during different phases of biofilm growth and lead to the detachment (Prakash *et al.*, 2005).

Properties of Garlic

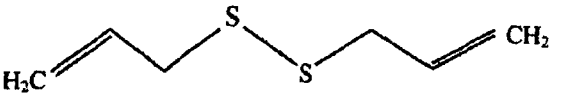
Garlic (*Allium sativum*) is a species in the family Alliaceae. It is one of the most popular spices in the world. It was believed to originate from Central Asia, although no wild form is known (Gernot, 2008). Garlic has been consumed as a spice and medicine for thousand of years. (Muhsin and Amina,2007). Over the last decade, the antimicrobial activity of garlic and garlic derived organosulfur compounds was widely investigated against food spoilage bacteria and food-borne pathogens. Besides, garlic showed the effective antioxidant activity in vivo and vitro. Garlic-rich organosulfur compounds and their precursors (allicin, diallyl sulfide and diallyl trisulfide) are believed to play a key role in these biological effects (Sallam, Ishioroshi and Samejima,2004). It has been found in many therapeutic functions including antimicrobial, anticardiovascular, immunostimulatory and hypoglycaemic activities. (Muhsin and Amina, 2007). Allicin (diallyl disulphide oxide) is a volatile molecule with characteristic of garlic odor. The volatile property of allicin minimizes any loss during studies of antimicrobial effect (O'Gara, Hill and Maslin,2000). There is the presence of transformation of alliin (S-2-propenyl-L-cysteine sulfoxide) to allicin which is extremely rapid process by the oxygenation of alliin which is a stable precursor in the presence of phosphopyridoxal enzyme allinase within a garlic clove. Garlic is odor free until it designed as a defense mechanism against invading pathogens. The active allicin molecule also has very short half-life for keeping the defence machamism rapid and localised, the rest alliin preserved for future attack (Muhsin and Amina,2007). Wills reported that allicin is an inhibitor of sulfhydryl metabolic enzymes and suggested that its antimicrobial properties are due to specific interference with –SH group (Edward, and Vincent,1985). It has been reported that the development of resistance to betalactam antibiotics is 1000 fold easier than the development of resistance to allicin, making garlic more competitive for therapeutic use (Muhsin and Amina,2007). In addition, garlic has been presented the advantages over the use of bleaching agent or antibiotic: (i) cheaper price as garlic can be grown in Thailand and easy to find, (ii) no health effect or corrosion on the equipment as garlic is a natural compound that has antimicrobial property, and (iii) domestic used.



a) alliin



b) allicin (diallyl thiosulphinate)



c) diallyl disulphide

Figure 4 : The chemical structure of compound found in garlic clove (O'Gara, Hill, and Maslin, 2000)

Garlic extract plays important role in the Quorum sensing system. There is a reported in mouse that when garlic extract is present, QS is blocked, and the adequate immune respond is mounted. Polymorphonuclear leukocytes (PMNs) become more active, bacterium are more efficiently eliminated (Thomas et al., 2005).

Fresh garlic extract had been reported against *Candida albicans* which is a yeast germ that can cause human infection that resist to many available medications. Garlic can affect to the germ in different way depend on the stage of growth. Low concentration required before it attaches to the surface, but after germ attaches to the surface, the higher concentration is needed for inhibition (Jennifer, 2004.). Moreover, *Candida albicans* was studied in three phases which were planktonic, adherent, and sessile phases on silicone elastomer disk. The activity of garlic decreases as *C. albicans* biofilm develops (Jennifer, James and Robin, 2005).

Rapid method

Rapid method is a detection of pathogens and other microbial contaminants in food. It makes the detection and identification faster, more convenient, more sensitive, and more specific than conventional assays. Almost all rapid methods are designed to detect a single target, which makes them ideal for use in quality control program to quickly screen large number of food samples for the presence of a particular pathogen or toxin. This method can be done in a few minutes to a few hours (Anonymous, 2001).

Crystal violet (CV) staining, a colorimetric method, has been widely used to measure biofilm formation because of its amenability to large screening procedures. It is particularly screening assay for surface adhesion-deficient mutants by measuring the absolute amount of biofilm formed by CV staining. To search for antibiofilm compounds in growth-inhibitory, garlic, the CV assay must be modified to measure the amount of biofilm formed relative to overall growth. It is generally assumed that CV binds proportionally to biomass, although there are multiple physical, chemical, and biological factors that could influence the binding of CV to biofilms. The factors involve the structural factors that effect to the dye diffusion, the difference of morphological and physiological in each cells that influence the binding of dye and the chemical interaction of garlic extract and crystal violet (Niu and Gilbert, 2004.)

The mechanism of CV staining is crystal violet dissociates and gives positive charge. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV ion which has positive charge interacts with negatively charged components of bacterial cells and stains the cells purple (Davies *et al.*, 1983). Moreover, CV can absorb more light, so it provides high sensitivity for detection. Thus, this method is a quantifying biofilm density that commonly used in quantifying biofilms on many researches, including *Salmonella* biofilm investigation.

Sanitizer (NaOCl)

Sodium hypochlorite (NaOCl) is a compound that used on for surface purification, bleaching, odor removal and water disinfection. Liquid bleaching agents was developed based on sodium hypochlorite by the Frenchman Berthollet in the year around 1785. It was first produced by Javel company, and the product known as "liqueur de javel" which was used to bleach cotton (Anonymous.,2008). It is a clear, slightly yellowish solution with a characteristic odor. It is an unstable with the evaporation of chloride at a rate of 0.75 grams per day from the solution. The sanitizer for domestic use contains 5% sodium hypochlorite which has pH around 11. It also contains a concentration 10-15% sodium hypochlorite with a pH around 13, but at this concentration, it causes burn and corrosive. Normally, chlorine concentration of 50 to 200 parts per million (ppm) is recommended to disinfect food contact surfaces in the industry. The contact time for the dose that does not exceed 200 ppm, no rinsing of the surface is required. If using a solution stronger than 200 ppm, rinse the surface with clean water after a few minutes of application, and do not let the chlorine solution stay in contact with equipment for more than 30 minutes or it could corrode. The bactericidal activity increases with longer exposure time. The temperature also affects to the antimicrobial activity as the warmer temperature, the higher of antimicrobial ability, but chlorine compounds may release chlorine gas which is toxic. The potential of corrosion also increases as temperatures go up (Anonymous. 2008). Thus, the killing efficacy was observed as 100 fold increased at 20 – 45 ^{o}C (Sirtes et al., 2007). It is because NaOCl is a strong oxidator which can react with flammable compounds and reductors as shown in the equation : $NaOCl + H_2O \rightarrow HOCl + NaOH$

Hypochlorous acid is formed that can be divided into hydrochloric acid (HCl) and oxygen (O). The oxygen atom is a very strong oxidator. Sodium hypochlorite has an effect against bacteria, viruses and fungi as it can disinfect the same way as chlorine does. It can block any resistance of microorganism. It can prevent the biofilm.

Health effects of sodium hypochloride

Sodium hypochlorite can cause various health effect. The skin or eyes contact with this compound causes redness and pain. After prolonged exposure, the skin can become sensitive. People who expose to sodium hypochlorite by inhalation of aerosols can have sore throat and coughing. If people swallow sodium hypochlorite, it affects stomach ache, a burning sensation, diarrhea ,coughing, a sore throat and vomiting. Sodium hypochlorite is poisonous for water organisms. It is mutagenic and very toxic when it comes in contact with ammonium salts (Anonymous,2008). Therefore, the natural compound is an alternative sanitizing agent that could be extensively used in the future.

Besides NaOCl which is a chloride base, other compounds are used as sanitizers such as (i) iodine compound which is the combination of iodine and solubilizing agent that is effective against all bacteria especially in acidic condition without any toxic or irritating to skin, (ii) quaternary ammonium compounds (QAC) are nontoxic and effective against some bacteria but slow acting against some common spoilage bacteria, (iii) acid-anionic surfactants are the combination of acid usually phosphoric acid with surface active agents, and effective against bacteria only below pH 2.5 but it is nontoxic, stable, and noncorrosive to stainless steel, and (iv) peracetic acid solution is a mixture of peracetic acid, acetic acid and hydrogen peroxide that effective against all microorganisms, including bacterial spores, and can be corrosive to the skin (Anonymous, 1999:2008).

Surface attachment

Salmonella has flagella as the main component on surface adherence. The adhesion to a solid surface depends on the attractive force and repulsion force at the same time. These physiochemical forces of attraction and repulsion included the electrostatic interaction, van der Waals forces, dipole interaction, chemical bonding and hydrophobic interaction (McEldowney and Fletcher, 1986). Besides the forces, other factors involved cell hydrophobicity, cell surface charge, and EPS production also affect bacterial attachment. The bacterial attachment has two events which are the bacterium encounters the surface and come close enough for attachment, and the bacterial outer surface adhered to the surface (Fletcher and Loeb, 1979). Most bacteria have a net negative charge, as most solids do, thus, the electrostatic repulsion tends to prevent close approach between surfaces. Therefore, adhesion should be promoted by increased electrolyte concentration or reduction in charge difference between two opposing surface. *Salmonella* strains showed higher hydrophobicity when compared with other bacterium as *Listeria monocytogenes* strains, so it can attach on hydrophobic surface as bacteria mostly formed highest on more hydrophobicity material (Sinde and Carballo, 2000).

This study focuses on *S. typhimurium* biofilm formation on glass and polystyrene surface.

Polystyrene

Polystyrene is a strong plastic created from erethylene and benzene. It is a colorless or yellowish oily liquid. It was discovered by a German apothecary called Eduard Simon in the year 1839. He isolated a substance from natural resin which later realized that it comprised of long chains of styrene molecules, was a plastic polymer. commonly used to make foam board or beadboard insulation, concrete block insulation, and a type of loose-fill insulation, which consists of small beads of polystyrene.

There was a research on the role of electrostatic and hydrophobic interaction of four bacterial species (*Pseudomonas fluorescens, Enterobacter cloacae, Chromobacterium* sp., and *Flexibacter* sp.) onto polystyrene substrata (McEldowney

and Fletcher, 1986). The data indicates that the electrolytes could influence adhesion through their effect on hydrophobic interactions, since the increased electrolyte concentrations tend to increase the strength of interaction. Moreover, hydrophobic interaction showed in bacterial adhesion to the inert substrata as polystyrene is largely hydrophobic (McEldowney and Fletcher, 1986). Thus, both electrostatic and hydrophobic interactions can play role on adhesion, but bacteria itself has an alternative mechanisms for adhesion depends on condition and the surface.

Glassware

A glass is an inorganic non metallic material. It does not have a crystalline structure. Glassware that we used in the lab is borosilicate glass which composed of 70% - 80% Silica (SiO_2) and 7% - 13% Boric oxide (B_2O_3) with small amounts of the alkali Sodium Oxide (soda) (Na_2O) and Aluminum Oxide (AI_2O_3), and it removed out of alkali ions by exposure to water vapor at high temperature. This kind of glass has exceptional resistance to thermal shock because it has a low coefficient of expansion ($3.3 \times 10^{-6} K^{-1}$) with a high softening point. The maximum recommended working temperature (short time) is 500°C. Borosilicate glass is widely used in the field of photomistry as it has optical property with the ability to transmit light through the visible range of the spectrum and in the near ultra-violet range. It enables to resist strongly on water, acids, salt solutions, halogens and organic solvents, and moderately resist to alkaline solutions (Anonymous, 2008).

The surface characteristic as glass was studied on the attachment of Marine Pseudomonad. Glass was observed to have a low number of attached bacteria, and also have negative surface charge in seawater. This charge is consistent with their chemical structure, involving oxyanions at the surface (Fletcher and Loeb, 1979). Most bacteria appear to bear negative surface charge, thus, the adhesion to the positive platinum or neutral germanium was higher attachment than glass. Therefore, surface charge is suggested to be a major factor. Once, the hydrophobic surface which is the favorable attachment was converted to hydrophilic, negatively charged surface, the result showed the decreased rate of bacterial attachment to a surface (Fletcher and Loeb, 1979). Fletcher and Loeb noted that large number of bacteria attach to hydrophobic surface with little or no surface charge, moderate number attached to hydrophobic metals with positive charge or neutral charge, and very few

attached to the hydrophilic negatively charged surface (Mahdavi, Jalali and Kasra, 2008).

Salmonella spp. strains can adhere in higher numbers to more hydrophobic material such as rubber and plastic (Stepanovic et al,2004). The hydrophobicity of *S. enteritidis* was 73% which leads to the formation of biofilm on polystyrene than glass as there was a report on the cells attachment on glass and polystyrene equals to 1.68 x 10^7 and 2.07 x 10^7 cfu cm^{-2}, respectively (Mahdavi, Jalali and Kasra, 2008). However, *Salmonella spp.* still can grow on the hydrophilic material such as glass and stainless steel, but they need optimum oxygen and nutrient condition.

Nutrient availability is one of the factor that can enhance *S. enteritidis* to form biofilm with greater density of cells. Cells can grow well and produce more biofilm on the nutrient-limited medium as there is the maximum expression of agfD promoter under aerobic condition (Stepanovic et al, 2004), thus, oxygen and poor medium with low nutrient supply would be the other two factors that affect on the biofilm formation. It was found that biofilm development was sensitive to oxygen availability with 2-3 log greater count on partially exposure than fully submerged (Efstathios and George, 2006). In nutrient limited medium, bacteria may enhance the adherence. The increased of adherence can alter the cell surface characteristic which lead to cells come closely to the surface where nutrient scavenging take place (Scott and Edmund, 1997). There was a research indicates the quantity of *Salmonella* biofilm formed on 1/20 TSB equal to 72.9% whereas on TSB, BHI or MB medium with the same strain has only 27.1% (Stepanovic *et al.*, 2004)

Materials and Methods

1. Materials and Chemicals

1.1 Culture

- *Salmonella typhimurium* (TISTR 292) was purchased from Thailand Institute of Scientific and Technological Research (TISIR) isolated from Chicken

1.2 Antimicrobial agent

- Garlic
- Sodium hypochlorite

1.3 Medias

- Trypticase Soy Agar (TSAYE, Merck, Germany)
- Trypticase Soy Broth (TSBYE, Merck, Germany)

1.4 Chemicals

- Toluene (Fisher Scientific, UK)
- 1 % Crytal violet
- 70% ethanol alcohol (v/v)
- 95% ethanol alcohol (v/v)
- Sterile distilled water

1.5 Equipment

- Analytical balance (Ohause, Analytical plus 210S)
- Autoclave (Hirayama, Model HA 300 MII, Japan)
- Blender (Kassel blender KBL 550, China)
- Cuvette (Rungthip, Thailand)
- Filter paper NO. 4 110 mm Ø (Filter paper, Whatman, England)
- Incubator (Jouan, EB 280)
- Laminar flow (Dwyer Mark II, Clean Model H2, USA)
- Micropipette 0.1-2.5 µl, 10-100 µl and 100-1000 µl (Biohit Proline, Germany)
- Polystyrene tube (Evergreen Scientific, USA)
- Shaker (KikaLabortechnik, KS 501 D, Germany)
- Spectophotometer (Spectronic, GENESYS 5, Milton Roy, USA)
- Vacuum pump (Vacuubrand, Germany)
- Various glassware (Pyrex)

2.Experimental Procedures

2.1 Media Preparation

The mediums used for biofilm development were Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB) and 1/20 Trypticase Soy Broth. TSA and TSB were used for preparing the master culture and working culture. 1/20 Tryptone Soy Broth was used for biofilm development. All media need to be sterile by autoclave under 121°C for 15 minutes.

2.2 Garlic extraction

Stem and dead leaves were prior removed from the garlic bulbs. The fresh garlic bulb was blended into small pieces, and weighted to get 150 g of fresh garlic chop. Then, 300 ml of toluene was added and left in a chemical hood for overnight. The suspension was filtered through the Whatman no.1 filter paper to remove out the garlic, and 150 ml of sterile water was added. The mixture was stirred by the shaker at 190 rpm for 24 hr at room temperature. After two phases were formed (the organic phase was separate out of water phase), the water phase was further sterile filtered by vacuum machine and used as raw extract. The garlic extract bottle was covered by the aluminum foil to prevent the oxidation from light that caused the detrioration.

This experiment, garlic concentration used was varied into 20 mg/ml, 40 mg/ml and 60 mg/ml, 80 mg/ml and 100 mg/ml for growth curve determination (Appendix A). At 20 mg/ml, 40 mg/ml and 60 mg/ml were used for biofilm formation (Appendix A).

2.3 Sodium hypochlorite preparation

The concentration of sodium hypochlorite that used in this experiment was 200 ppm, so NaOCl was diluted with sterile distilled water (Appendix A).

2.4 Culture preparation

S. thyphimurium was streaked by cross streak method on trypticase soy agar, and incubated at 37°C for 24 hours. The single colony was transferred to 5 milliliter trypticase soy broth and incubated at 37°C for 24 hours, created the bacterial suspension in the tube. This suspension was further used as a working culture. Two percent of working culture at OD ~ 1.0828 was inoculated in 20 millitiler 1/20 trypicase soy broth for growth curve determination, and also inoculated in 2 milliliter 1/20 trypicase soy broth for biofilm formation.

2.5 Growth curve measurement

The flasks contained culture at different concentration of garlic which were 20 mg/ml, 40 mg/ml and 60 mg/ml, 80 mg/ml and 100 mg/ml were placed onto the shaker which was operated all the time during the experiment. The sample was measured every hour to measure the growth by spectrophotometer at wavelength 595 nm until it reached stationary phase.

2.6 Biofilm formation

2.6.1 To test garlic concentration

The sterile glass tube and disposal polystyrene tube were used as surface for biofilm attachment. Three different treatments were performed. The first treatment was 2% culture with 2 ml of 1/20 dilution of trypticase soy broth. The second treatment was 2% culture with 2 ml of 1/20 dilution of trypticase soy broth, and water added in the same volume as garlic as a control. The third treatment was 2% culture with 2 ml of 1/20 dilution of trypticase soy broth, and each concentration of garlic extract which are 0.04 milliliter of 20 mg/ml, 0.08 milliliter of 40 mg/ml and 0.12 milliliter of 60 mg/ml. The media was replaced everyday to provide a sufficient nutrient. At each day, the culture was taken to measure by rapid method everyday until day 4.

2.6.2 To test the comparison between garlic and NaOCl concentration

The 2 ml of 1/20 dilution of trypticase soy broth in sterile glass tube and disposal polystyrene tube containing 2% culture were used for biofilm development. The culture was grown until day 3 by replacing media everyday. Then, 0.12 milliliter 60 mg/ml garlic extract and NaOCl were added, and after CV staining, measure the absorbance at 3 hr, 5 hr and 24 hr in order to measure the among of cell attachment on the surface after two different treatments were applied.

2.7 Rapid method (Crystal violet staining)

The liquid culture in the tube was removed to remain only the attached cell which refers to biofilm. The tubes were washed softly with distilled water and dried for 20 minutes. The tube was stained by 1% crystal violet and left for 25 minutes. Then crystal violet was removed, the tubes were rinsed with distilled water for 4 times or until no crystal violet remained in a tube except the stained one which was similar like a ring on the surface around the tube at the media level. The tube was dried for 15 minutes. Crystal violet was destained by 95% ethanol that left for 30 minutes. The absorbance value was measured at wavelength 595 nm by taking 1 ml solution to measure the optical density.

Result

1. The effect of various concentration of garlic extract over the growth of *S. typhimurium* planktonic cells

Growth curve was constructed to determine the suitable garlic concentration to use in further experiment. Five different concentrations of garlic extract which are 20 mg/ml, 40 mg/ml and 60 mg/ml, 80 mg/ml and 100 mg/ml were used. The growth of cells was measured the absorbance value at wavelength 595 nm. Specific growth rate at each concentration was calculated to compare the affect of each garlic extract concentration on the growth of planktonic cells.

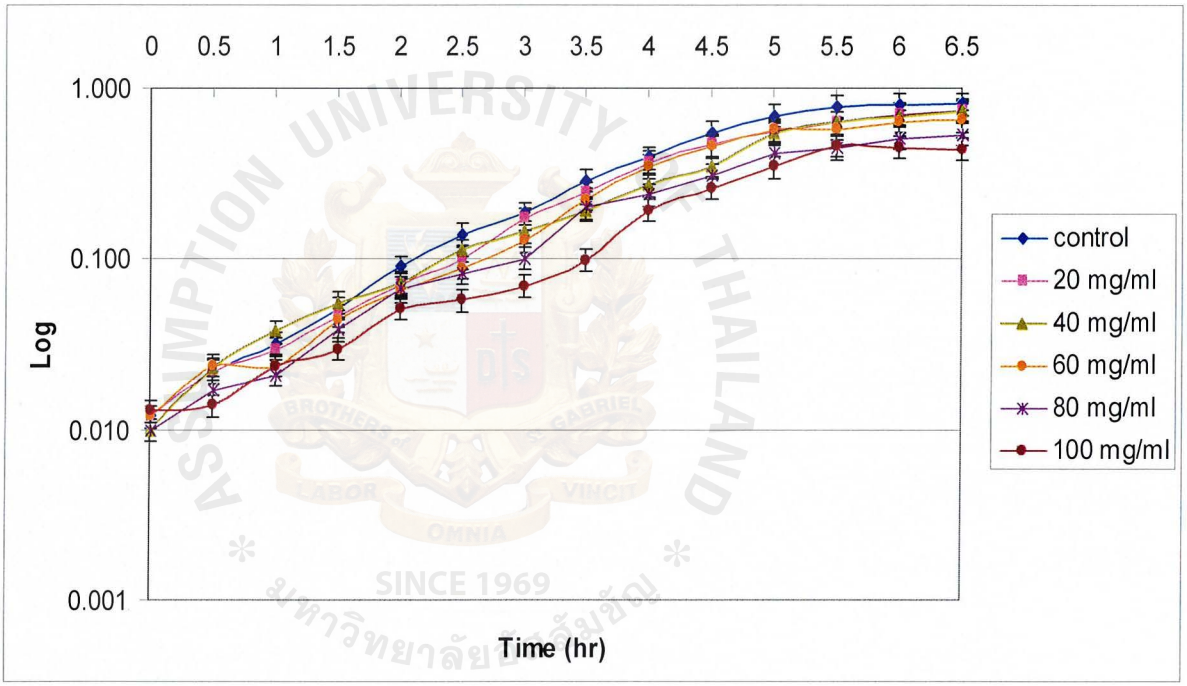


Figure 5 : *S.typhimurium* growth curve at different garlic extract concentrations: control without garlic extract, 20 mg/ml, 40 mg/ml and 60 mg/ml, 80 mg/ml and 100 mg/ml.

Table 1 : Specific growth rate value (μ) at six conditions of garlic extract and P-value

Concentration of garlic extract	**Specific growth rate (μ) (hr^{-1})**	**P-value**
control	1.058	
20 mg/ml	0.847	0.3
40 mg/ml	0.826	0.266
60 mg/ml	0.697	0.208
80 mg/ml	0.54	0.129
100 mg/ml	0.473	0.075

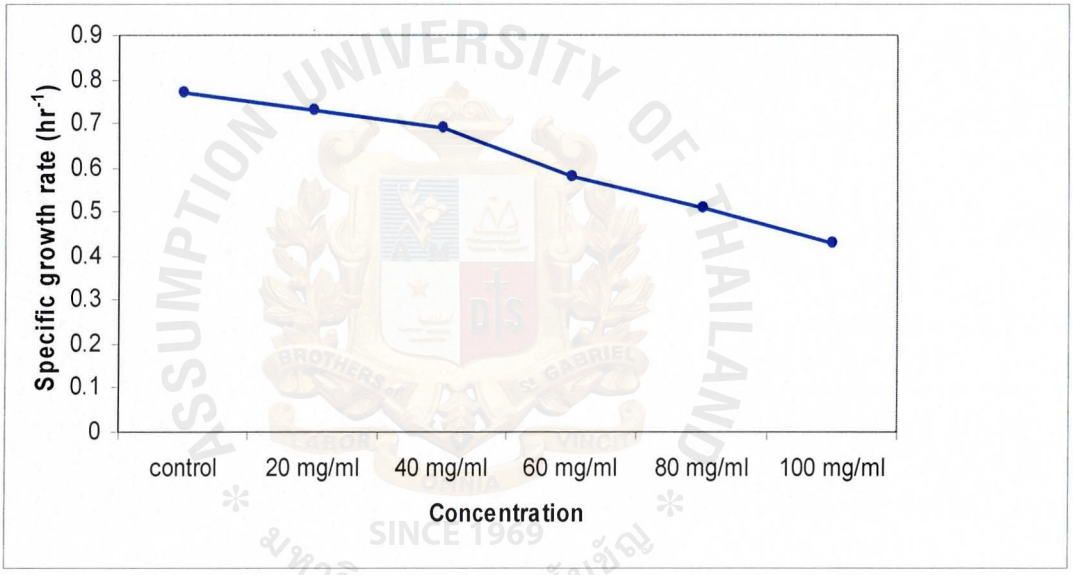


Figure 6 : The relationship of specific growth rate against each concentration of garlic extract

Five garlic extract concentrations were studied. Logarithm graph was plotted to show the trend of growth and determine specific growth rate of under six conditions which are control, 20 mg/ml, 40 mg/ml and 60 mg/ml, 80 mg/ml and 100 mg/ml. The negative control was set up without the addition of garlic in the culture condition, and different amounts of garlic extract added according to the concentrations as tests. When compared with wild type, at garlic extract concentration

of at 20 mg/ml, 40 mg/ml and 60 mg/ml had a very small effect to the planktonic growths from the μ values. From the μ values, 80 mg/ml and 100 mg/ml garlic extract concentration showed the dramatic decrease in the growths, and the end of the graph drops a little bit, indicates the death of cells. Specific growth rates were inversely proportional to the concentration of the garlic extracts. Specific growth rates were gradually dropped until 40 mg/ml concentration with specific growth rate values are 1.058, 0.847 and 0.826, respectively. At the concentration of 20 mg/ml and 40 mg/ml represented the less effect on *S. typhimuriun* planktonic cells. The specific growth rate of 60 mg/ml is 0.697 which was the medium level among 5 garlic extract conditions. At this concentration, the growth of planktonic cells was not absolutely reduced as cells still alive and allowed planktonic growth, thus, 60 mg/ml garlic extract concentration seemed to be a suitable concentration for further study. At 80 mg/ml and 100 mg/ml, specific growth were dramatically reduced to 0.54 and 0.473 which showed a strong effect on the planktonic cell growth. The concentration that further used in the study of garlic effect on *S. typhimurium* biofilm must not represent a great impact to the planktonic cells. Therefore, the concentration 20 mg/ml 40 mg/ml and 60 mg/ml were chosen for the further study.

2. The study of various garlic extract concentration on S. typhimurium biofilm formation by rapid method

Crystal violet staining is one of the rapid method that has been used to study the biofilm formation. It was used to detect biofilm formation under three different concentrations of garlic extracts which are 20 mg/ml, 40 mg/ml and 60 mg/ml on glass and polystyrene surface. *S. typhimurium* was grown in 1/20 TSB under the 3 different gatlic concentrations. The biofilm formations were observed every single day from day 0 to day 4. The CV stains the attached cell which is referred to the amount of biofilm formation.

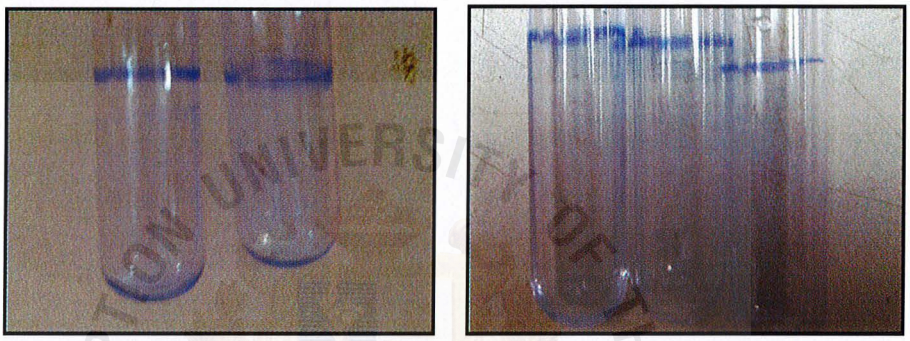


Figure 7 : CV staining on biofilm in both glass tube (a) and polystyrene tube (b)

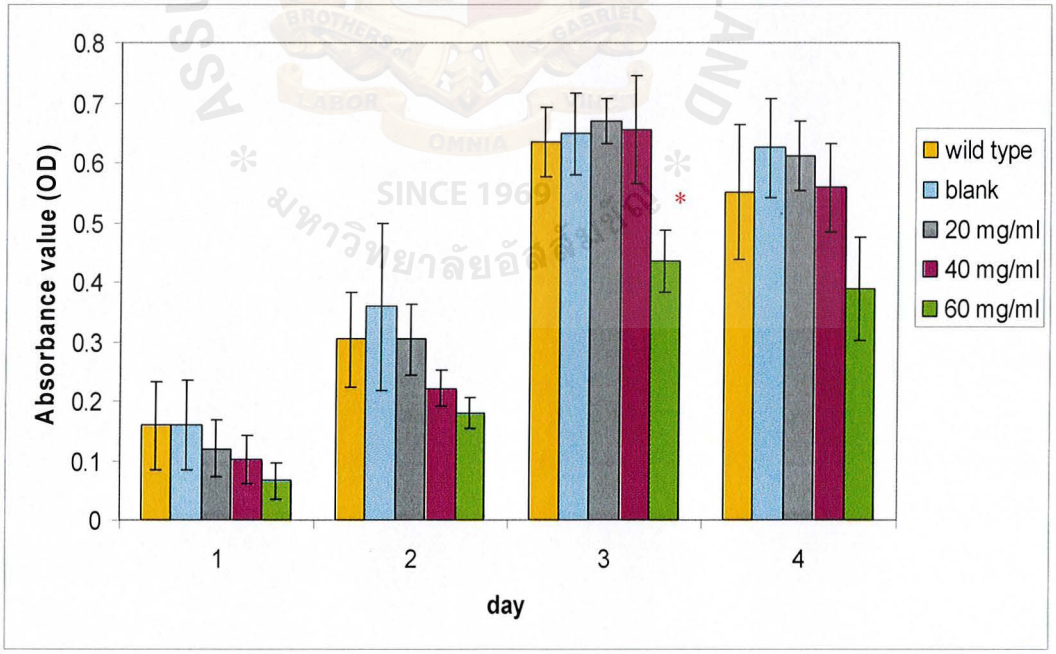


Figure 8 : *S. typhimurium* biofilm formation on glass surface at three concentrations of garlic extract (20 mg/ml, 40 mg/ml and 60 mg/ml) for 4 days.

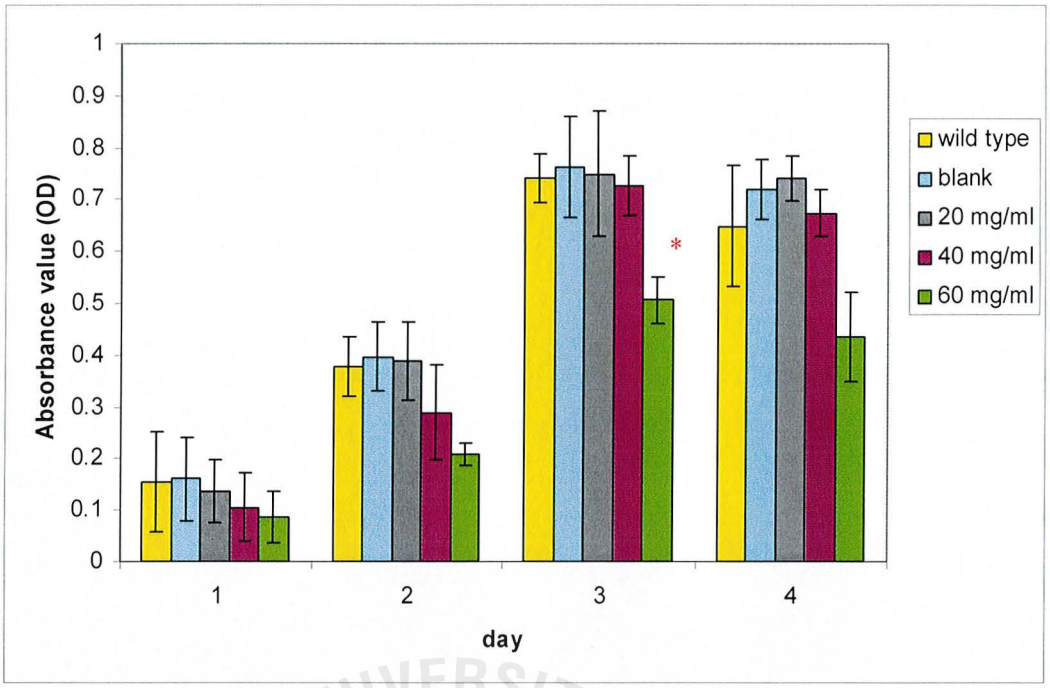


Figure 9 : *S. typhimurium* biofilm formation on polystyrene surface at three concentrations of garlic extract (20 mg/ml, 40 mg/ml and 60 mg/ml) for 4 days.

Table 2 : The optical density of biofilm formed on glass and polystyrene

Day / Surface	Glass	Polystyrene
Day 1	0.158	0.154
Day 2	0.303	0.377
Day 3	0.635	0.741
Day 4	0.551	0.648

The effect of garlic extract concentration on *S. typhimurium* biofilm formation was observed by having wild type (1/20 TSB + 2% *S. typhimurium*) and blank (1/20 TSB + 2% *S. typhimurium* + sterile water) as control. Three different garlic extract concentrations that had been reported on no effect on the planktonic cells were chosen. The optimum concentration for an inhibition of *S. typhimurium* biofilm formation was determined based on the dramatic reduction of the biofilm which related to the

dramatic reduction of the OD value. In both of glass and polystyrene surfaces showed the same patern of inhibitions. Day 1, in all conditions of the extract showed the reduction on biofilm formations but none of them creates significant difference. Day 2, the inhibition was detected more clearly on 40 mg/ml and 60 mg/ml garlic concentration but 20 mg/ml did not have any inhibition. Day 3, the inhibition did have on 60 mg/ml garlic concentration and there was a significant difference between wild type and garlic extract at the concentration 60 mg/ml. From day 1 to day 3, biofilm formation kept increasing in each day in every condition, and be able to form more on polystyrene surface than glass surface or about OD~0.1 difference as shown in table 2. Day 4, biofilm formation reduced in every condition. This might be caused by the cell detachment. However, the inhibition could be detected obviously on 60 mg/ml garlic concentration. Thus, the minimum concentration of garlic extract that can be used in minimizing the biofilm formation was 60 mg/ml.

3. The comparison between 60 mg/ml garlic extract concentration and 200 ppm sodium hypochlorite on the *S. typhimurium* biofilm formation by rapid method.

As the concentration of garlic 60 mg/ml showed a significant effect on *S. typhimurium* biofilm formation, therefore its biofilm elmination ability was compared with bleaching solution which normally uses in the industry at concentration 200 ppm. This is to verify whether it has elimination ability against *S. typhimurium* biofilm formation as NaOCl. This concentration of garlic extract has been considered to be a minimum concentration for inhibition of biofilm as it showed a significant reduction in *S. typhimurium* biofilm formation in 4 days experiment. *S. typhimurium* was inoculated in 1/20 TSB and biofilm developed for 3 days prior addition of garlic extract and NaOCl to measure the elimination ability on *S. typhimurium* biofilm formation. The effect of garlic on the mature biofilm was observed at 3, 5 and 24 hours after the exposure to garlic extract.

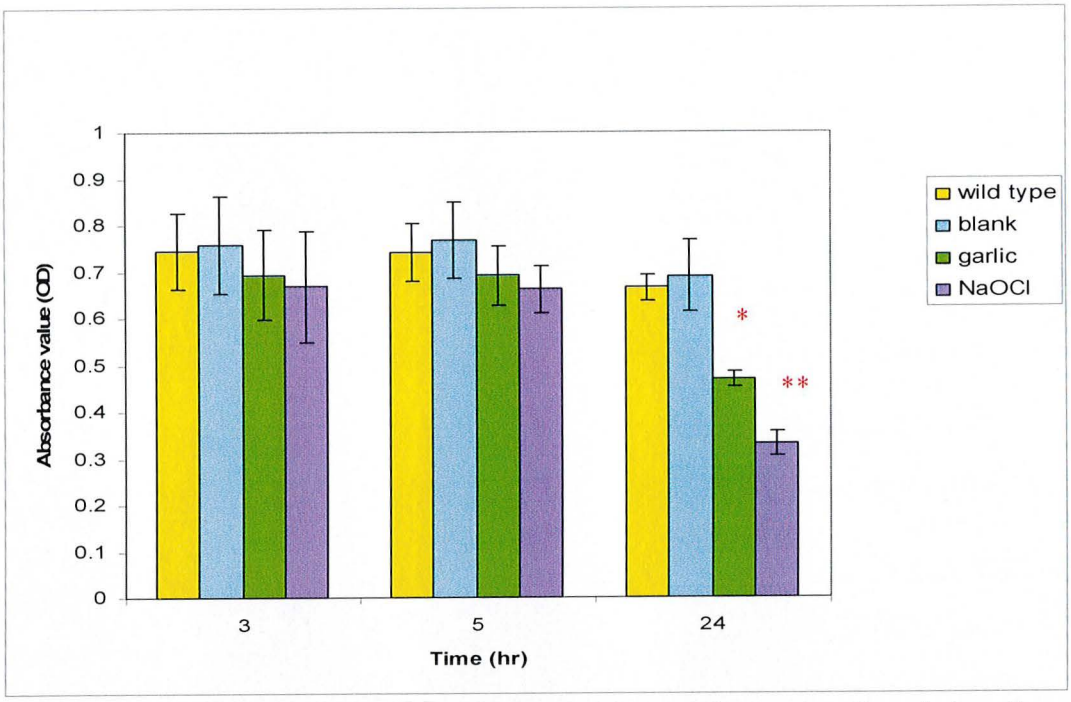


Figure 10 : *S. typhimurium* biofilm formation on glass surface under 60 mg/ml garlic extract concentration and 200 ppm sanitizer addition.

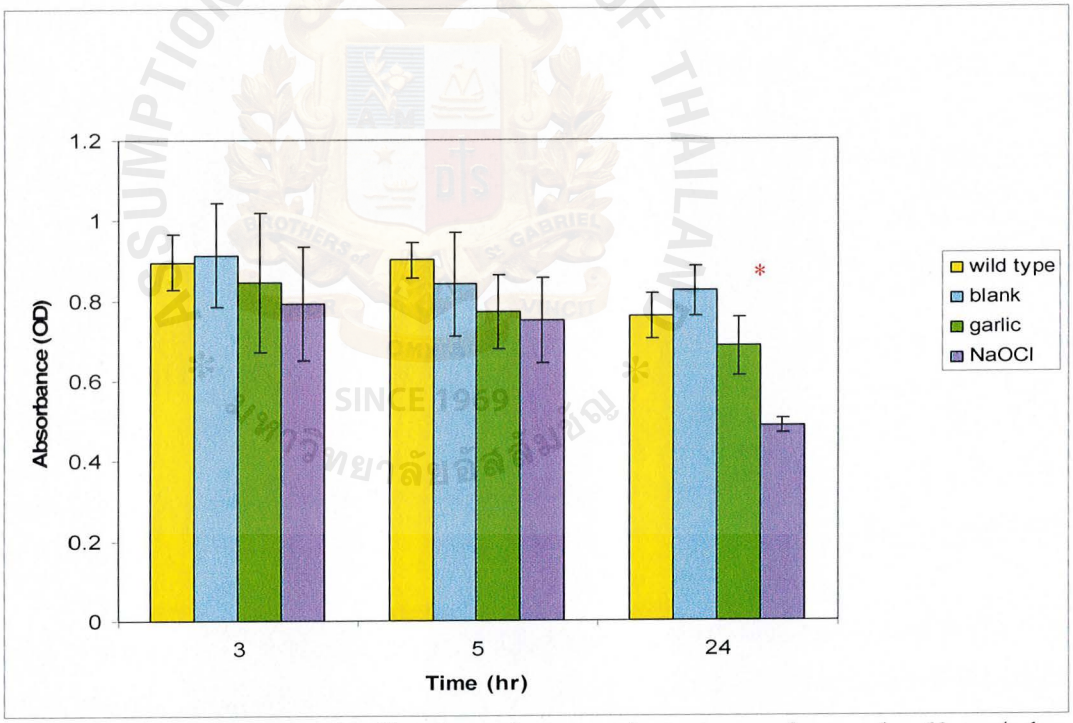


Figure 11 : *S. typhimurium* biofilm formation on polystyrene surface under 60 mg/ml garlic extract concentration and 200 ppm sanitizer addition.

From figure, the inhibition occurred on both glass surface and polystyrene surface in every period of inspecting time. On 24 hr submersion on mature biofilm with garlic and NaOCl represented the highest detachment of biofilm when compared to 3 hr and 5 hr, so time exposure affects to the capability of elimination of both garlic and NaOCl. The longer the exposure, the higher the detachment. However, the elimination potential of garlic was not as much as NaOCl. Based on statistic analysis, garlic had significant difference whereas NaOCl had highly significant difference on glass, and garlic had no significant difference whereas NaOCl did on polystyrene.

Discussion

Biofilm is layers of bacterial growth that attach to the surface and develop an exopolysaccharide as a protective shell to protect them from detergents and sanitizers (Vanessa,2008). Biofilm becomes one of the problems in the industry because of the dead zone in food equipment or pipe line in the production process. At the dead zone, biofilm can form easily, and hardly be eliminated by any sanitizing agents. Development can occur along the pipe line. Biofilm composes of water channel that allows water, nutrient to get in to feed bacteria underneath the biofilm structure. Other bacteria can be developed in a biofilm as a community which probably includes a pathogen. Water bacteria as *Salmonella spp.* is most common biofilm former in food industries. The biofilm can cause a food contamination once it detacks from the surface which also leads to the disease transmission to the consumers. *Salmonella spp.* is food-borne pathogen which causes gastroenteritis or enteric typhoid fever (Michael and Dirk,2008) to human. Therefore, many industries try to solve biofilm problem by using the sanitizers which is the best way of elimination but the use of sanitizers can cause the corrosion to the surface and have bad effect to human health. Another substance is antibiotic, but due to the high price and easier to development of bacterial resistance, antibiotic is not suitable to the industrial clean up. This research was studied on garlic extract as an antimicrobial to inhibit biofilm formation on glass surface and polystyrene surface in order to reduce the usage of sanitizers. Moreover, garlic has 1000 fold harder in development of resistance compared with antibiotic. Various concentrations of garlic extract were applied in order to study on its effect on biofilm development of *S. typhimurium*. The rapid method (crystal violet staining) was used to obtain the best inhibiting concentration, and further compare the elimination capability with industrial sanitizing agent (sodium hypochlorite) on *S. typhimurium* biofilm formation.

The research was begun with the effect of garlic on *S. typhimurium* planktonic cells. The growth of cells under five concentrations (20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml) of garlic extract were compared with the control to identify the minimum inhibition concentration that must not affect to planktonic growth but can inhibit the biofilm formation. Then, at this concentration could be applied in the elimination of mature biofilm. The specific growth rate (μ) is the increase of cell mass

per unit cell mass per unit time (Anonymous, 2008). At garlic concentration 20 mg/ml and 40 mg/ml, the μ values were 0.847 and 0.826 which were not much different from the wild type that had μ value equals to 1.058. The concentrations might be too low to effect the cells obviously, therefore, they might not show any effect to the biofilm because biofilm was protected by the exopolysaccharide that act as protective layers for detergent and other sanitizers.

The μ value became slightly different from wild type that was treated with 60 mg/ml garlic extract concentration. At this concentration, the μ value reduced to 0.697, but, this concentration was not dramatically inhibit the growth of all planktonic cells which lead to cells' death because just small number of planktonic cells were inhibited by the garlic. Therefore, there should be planktonic cells left for entering the biofilm development process. This concentration was in the middle among five garlic extract concentrations which showed the less reduction of planktonic cells, however, the reduction of planktonic cells by 60 mg/ml garlic extract concentration was not as much as 80 mg/ml and 100 mg/ml garlic extract concentration.

Garlic extract had a great impact on the planktonic cell growths at concentration 80 mg/ml and 100 mg/ml. The μ values were 0.54 and 0.473, respectively. Their specific growth rate values represented significant decrease from the wild type without any treatment. Growth trend of these two concentrations were significantly differences from other four conditions (control, 20 mg/ml, 40 mg/ml and 60 mg/ml). Growth drop a bit at the end of graph indicate death phase of planktonic cells. It could be demonstrated that the increased of garlic extract concentration affected directly to the *S. typhimurium* planktonic cells growth. These two concentrations were not suitable for the purpose of this research as they affect cells since the planktonic stage.

However, the first three concentrations (20 mg/ml, 40 mg/ml and 60 mg/ml) were used to investigate the effect of garlic on *S. typhimurium* biofilm formation because *S. typhimurium* planktonic cells were not much affected which future used to study on the effect of garlic over the biofilm formation. This study was done under the sufficient nutrient supplied and optimum condition.

S. typhimurium biofilm formation was studied by using a rapid method. Crystal violet was used for staining the bacterial attachment on the surface which refers as a biofilm to determine the biofilm formation under each condition. There were five conditions involved which were wild type without adding garlic extract, blank without adding garlic extract (only sterile distilled water which acted as a solvent was added) and other three concentrations of garlic extract at various concentration (20 mg/ml, 40 mg/ml 60 mg/ml). TSB was diluted in the ratio 1:20, and used as a medium for biofilm formation since this formula was reported as a optimum formula for *S. typhimurium* biofilm development (Stepanovic *et al.*, 2004). It has less amount of nutrient which probably induces the biofilm development because the lack of nutrient help cells associated closely to the surface (Scott and Edmund, 1997). The formation of *S. typhimurium* biofilm was observed along 4 days under glass and polystyrene surfaces. Biofilm form on glass surface and polystyrene surface were in similar fashion that developed from initial attachments to be the mature biofilm and finally the detachments were found in both materials. The van der Waals forces, polar or Lewis acid base and electrostatic interaction between the surface of object and the cell membrane of *S. typhimurium* facilitate the bacterial attachment. The interactions originate from all cells body and from more specific, localized adhesion sites such as protein and cell surface (Oh *et al.*,2007.). Bacterial surface charge is a result of charged functional groups on lipopolysaccharides that plays role in bacterial intersection with solid surface which generally carries a negative charge under physiological conditions. Hydrophobic of flagella is another reason because polystyrene represents hydrophobic surface, and commonly hydrophobic interact to each other. Moreover, the hydrophobicity of *Salmonella* was as high as 73%, so it can attach to the hydrophobic material (Mahdavi, Jalali and Kasra, 2008). Therefore, this study also indicated that the biofilm formation on polystyrene was higher than glass.

The study also found that there was a violet color ring from crystal violet at the air-liquid interphase as shown in figure can demonstrate that enhanced activation of traits associated with attachment and surface colonization of bacteria because bacteria can access to gaseous (oxygen) and liquid (nutrient) phase (Efstathios and George, 2006). The stronger of staining indicated the greater the biofilm formation. In

every condition, biofilm kept forming until day 3, then on day 4, biofilm dissociated, causing the reduction of the absorbance value that shown in the result.

On day 1, the bacterial attachment was in the low level which probably represented the initial stage of biofilm development where the planktonic cell attach on the surface. The flagella might play an important role in this step. *S. typhimurium* planktonic cells colonized onto the conditioning films by using flagella. Flagella made up of protein flagellin that provides cells with the ability of motility and adherence. The effect of garlic extract was detected clearly in every concentration because less *S. typhimurium* planktonic cells attached to the surface. However, the inhibitions were not significant different from the untreated condition.

On day 2, the density of *S. typhimurium* biofilm became higher as the staining of crystal violet at the ring on the intersection between liquid and air was darker also showed the high OD value after the destaining with ethanol. This might be explained from the microcolony formation after the initial attachment. After initial attachment of biofilm formation, *S. typhimurium* cells tried to extent their colonization. QS plays an important role in cell tracking. *S. typhimurium* releases some signal molecules (acyl HSLs) to communicate with others to join the biofilm community. At this stage, EPS was produced higher as a larger community which distributed to the stability of the biofilm structure. The polysaccharide production resulted from the excess of carbohydrate, especially simple sugar in the growth medium that enhanced cells to form the colonies, not just attachment (Osborn et al.,1972). The secretion of EPS led to the trapping of nutrient substance feeding to cells, and also helped to trap and attach cells because the charge and the neutral polyshaacharide that can react to the bacterial cell membrane. Moreover, the thicker of EPS layer caused the harder of inhibition of biofilm formation. There was no inhibition at 20 mg/ml garlic concentration because less amount of garlic caused lower property of inhibition when the biomass in biofilm community increased. Other two concentrations (40 mg/ml and 60 mg/ml) showed the inhibition over the biofilm formation because garlic extract probably block the Quorum sensing molecule, so cells hardly follow up with each other, causing the reduction of biofilm formation. The study of garlic extract was done in *S. typhimurium* biofilm study. It was found to inhibit the QS communication.

On day 3, biofilm became mature biofilm as the highest absorbance values were found in all five conditions. The amount of *S. typhimurium* biofilm formation on this day was as twice as the amount on day 2, indicating the highest microorganism joined in their community. QS and EPS also presented in this stage (George, Heidi and Roberto, 2000). The inhibition of garlic extract showed only on 60 mg/ml concentration which was significantly different from the wild type on both glass surface and polystyrene surface. It showed that this garlic concentration has high efficiency to inhibit *S. typhimurium* mature biofilm.

On day 4, biofilm dissociated was found which relevant to the decrease in the absorbance values in every condition. However, at 60 mg/ml garlic concentration still demonstrated the greatest inhibition on the bacterial attachment. The dissociation is the last part in biofilm formation. Normally, EPS act as the barrier for biofilm protection, but the channel on biofilm surface help as the entrance to transport nutrient, O_2 and other trance element to cells inside. Once, the mature biofilm has an old thick layers of EPS which block the transport of nutrient to the microorganism inside causing the starvation stage. In addition, the coverage of EPS also traps the toxic waste inside affecting the biofilm hardly survive. Thus, Cells try to balance the biofilm growth by removing some biomass known as detachment which relates to the result of this study in day 4 (Duangkamol, 2008).

Therefore, 60 mg/ml garlic extract concentration is the best concentration for inhibition of biofilm formation with the less effect on the planktonic cells. Biofilm reduction was observed clearest at this concentration when compared with other two concentrations. The staining at the ring around the surface of air-liquid interphase was lightest. This might be explained by the inhibition of quorum sensing. The amount of inhibitory compound in garlic extract at this concentration was high enough to inhibit the quorum sensing between cells. Cells lose tracking, causing less ability of bacterial free cells to become a sessile form or biofilm form. With this concentration of garlic, this study was further conducted to compare the elimination capability with sanitizer (sodium hypochloride) which is normally used in the industrial clean up.

The elimination abilities of garlic extract and sanitizer were tested on the three days old bacteria. The elimination effects was observed along 3 periods of time after submerged for 3 hours, 5 hours and 24 hours in garlic extract and sanitizer (NaOCl).

The sanitizer of both garlic extract and NaOCl were best detected on 24h hours and NaOCl showed a greater inhibition than garlic extract. Generally, when NaOCl dissolved in water, it dissociates into sodium (Na^+) and hypochlorite (OCl^-) ions. A small fraction of the hypochlorite ions reacts with water to form hydroxide ions(OH^-) and hypochlorous acid (HOCl). Hypochlorous acid and hypochlorite ions are both strong oxidizers that can react and disrupt a wide variety of important biological compounds, such as DNA, fatty acids, proteins, and cholesterol. However, hypochlorous acid molecule is an active component as hypochlorite ions cannot easily cross the cell membrane, due to their negative charge. Some scientists believe that HOCl is able to disrupt cell functions in term of interferes with the metabolism of Adenine, one of the "bases" of DNA and RNA, and also a component of ATP. Moreover, chemical agents as detergent or sanitizers may attack and destroy the matrix (Vanessa, 2008). It shows that NaOCl have strongly effect directly to the bacterial cells to form on biofilm, so the inhibition of NaOCl occurred greater than garlic that blocks the QS which controls the biofilm formation by directing the expression of exopolysaccharide biosynthesis genes (Efstathios and George, 2006). Although the use of sanitizer as sodium hypochlorite gives more effective on elimination of *S. typhimurium* biofilm formation but it also provides the bad effect to people health and the equipment maintenance. Using NaOCl causes the corrosion to the equipments, and skin/eyes irritation, moreover, the remaining of the chemical affect to the stomach ache, diarrhea or vomiting. Therefore, garlic extract probably be considered as an alternative sanitizing agent that can be used in the industry in the future. It is also an abundant compound that is easily found in over country.

Conclusion

Biofilm is a microbial colonization onto the surface that included both the adhesion of bacteria and also the matrix of extracellular material produced by bacteria. The formation of biofilm protects the bacteria from severe environmental conditions. It causes a serious problem in industrial, environmental and medical issues. This biofilm causes food post-contamination contributing to lower shelf-life product and health problem due to pathogen biofilm forming as *S. thiphimurium.* Garlic was chosen to perform the inhibition on *S. thiphimurium* biofilm formation, and detected by crystal violet staining. As a consequence of increased and decreased in absorbance values, S. typhimurium biofilm could be divided into 3 stages: initial attachment, mature biofilm development and biofilm detachment based on the rapid method. Moreover, biofilm usually formed at the air-liquid interphase because of nutrient and oxygen available. *S. thiphimurium* biofilm formed more on polystyrene surface than glass surface because of hydrophobic interaction on the cell membrane which plays a significant role on the hydrophobic surface attachment as in polystyrene.

The minimum inhibition concentration of garlic extract was 60 mg/ml. At this concentration the mature biofilm was inhibited. The reduction of biofilm indicates that this concentration of garlic extract might interrupt the quorum sensing that plays important role to keep cells to join their community. However, the elimination effect of garlic extract on the mature biofilm was not as high as sanitizer (sodium hypochlorite) in the concentration of 200 ppm that commonly uses in the industry. After biofilm became a mature biofilm on day 3, both sanitizers were used but NaOCl showed the decreased of biofilm formation more clearly. NaOCl can dissociate to hypochlorous acid molecule which is the main active that disrupt the cell function at the metabolism of Adenine.

Nevertheless, garlic provides more benefit in term of health effect because garlic is a product from the nature, so it is not harmful or produces any toxic accumulation in human health whereas sanitizer is a chemical compound that causes irritation to health and corrosion to equipment. In the future, the active compound of garlic extract might be isolated to use as a sanitizer which probably be more competible with the NaOCl.

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Appendix A

- Preparation 1/20 TSB

  Water 1000 ml with TSB 30 g

  Water 100 ml with TSB $\frac{30x100}{1000} = 3$ g

  Preparing 1000 ml of solution : $\frac{1}{20}$ x 1000 = 50 ml of solution

  Then, 50 ml TSB taken to make 1000 ml of 1/20 TSB

- Preparing 2% culture of *S. typhimurium* in 20 ml 1/20 TSB

  $\frac{2}{100}$ x 20 = 0.4 ml of *S.typhimurium*

- Preparing 2% culture of *S. typhimurium* in 2 ml 1/20 TSB

  $\frac{2}{100}$ x 2 = 0.04 ml of *S.typhimurium*

- Find amount of garlic extract as 60 mg/ml in 20 ml of media

  $C_1V_1 = C_2V_2$

  1 g V_1 = 0.06 mg x 20 ml of media

  V_1 = 1.2 ml of garlic extract solution (V/V)

- Find amount of garlic extract as 60 mg/ml in 2 ml of media

  $C_1V_1 = C_2V_2$

  1 g V_1 = 0.06 mg x 2 ml of media

V_1 = 0.12 ml of garlic extract solution (V/V)

- Find amount of bleaching agent as 200 ppm from stock solution 13%

200 ppm = 200 mg/1000 ml = 2g/10000ml

$C_1V_1 = C_2V_2$

$0.13\ g/ml\ V_1 = 2 \times 10^{-4} g/ml \times 500\ ml$ of sterile water added

V_1 = 769 µl

Appendix B

Table 3 : The absorbance values of each condition for *S. typhimurium* planktonic growth

Time (hr)	control	20 mg/ml	40 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml
0	0.012	0.012	0.01	0.012	0.01	0.013
0.5	0.022	0.022	0.023	0.024	0.017	0.014
1	0.032	0.03	0.038	0.024	0.021	0.024
1.5	0.052	0.047	0.056	0.044	0.039	0.03
2	0.091	0.072	0.073	0.066	0.068	0.052
2.5	0.141	0.101	0.113	0.09	0.083	0.058
3	0.187	0.173	0.145	0.129	0.102	0.07
3.5	0.289	0.245	0.195	0.225	0.201	0.099
4	0.394	0.371	0.272	0.351	0.24	0.193
4.5	0.551	0.47	0.348	0.459	0.313	0.262
5	0.694	0.558	0.548	0.573	0.42	0.347
5.5	0.779	0.633	0.632	0.583	0.45	0.466
6	0.802	0.706	0.696	0.64	0.511	0.452
6.5	0.812	0.749	0.739	0.647	0.537	0.441

Table 4 : The absorbance values of each condition within 4 days for biofilm formation on glass surface

	day 1			AVG	SD	day2			AVG	SD
wild type	0.155	0.233	0.085	0.158	0.074	0.337	0.361	0.212	0.303	0.080
blank	0.135	0.245	0.1	0.160	0.076	0.471	0.406	0.2	0.359	0.141
20 mg/ml	0.112	0.172	0.077	0.120	0.048	0.298	0.364	0.246	0.303	0.059
40 mg/ml	0.09	0.146	0.066	0.101	0.041	0.254	0.217	0.192	0.221	0.031
60 mg/ml	0.054	0.1	0.043	0.066	0.030	0.168	0.211	0.165	0.181	0.026

	day3			AVG	SD	day4			AVG	SD
wild type	0.567	0.675	0.664	0.635	0.059	0.436	0.663	0.553	0.551	0.114
blank	0.571	0.682	0.691	0.648	0.067	0.535	0.695	0.645	0.625	0.082
20 mg/ml	0.639	0.712	0.658	0.670	0.038	0.546	0.658	0.631	0.612	0.058
40 mg/ml	0.589	0.621	0.756	0.655	0.089	0.497	0.641	0.538	0.559	0.074
60 mg/ml	0.392	0.421	0.492	0.435	0.051	0.307	0.378	0.478	0.388	0.086

Table 5 : The absorbance values of each conditions within 4 days for biofilm formation on polystyrene surface

	day 1			AVG	SD	day2			AVG	SD
wild type	0.111	0.266	0.086	0.154	0.098	0.431	0.316	0.383	0.377	0.058
blank	0.109	0.254	0.12	0.161	0.081	0.457	0.408	0.327	0.397	0.066
20 mg/ml	0.1	0.207	0.1	0.136	0.062	0.469	0.376	0.321	0.389	0.075
40 mg/ml	0.079	0.18	0.058	0.106	0.065	0.363	0.317	0.186	0.289	0.092
60 mg/ml	0.076	0.142	0.044	0.087	0.050	0.233	0.198	0.196	0.209	0.021

	day3			AVG	SD	day4			AVG	SD
wild type	0.781	0.689	0.753	0.741	0.047	0.569	0.593	0.782	0.648	0.117
blank	0.772	0.659	0.851	0.761	0.097	0.696	0.678	0.785	0.720	0.057
20 mg/ml	0.806	0.611	0.831	0.749	0.120	0.702	0.785	0.736	0.741	0.042
40 mg/ml	0.751	0.661	0.769	0.727	0.058	0.623	0.708	0.691	0.674	0.045
60 mg/ml	0.473	0.487	0.557	0.506	0.045	0.509	0.339	0.457	0.435	0.087

Table 6 : The absorbance values for the comparison between garlic extract and NaOCl along 3 periods of time which were 3, 5 and 24 hours on glass surface

a) 3 hours

	3			AVG	SD
wild type	0.696	0.839	0.701	0.745	0.081
blank	0.647	0.856	0.771	0.758	0.105
garlic	0.6	0.689	0.791	0.693	0.096
NaOCl	0.529	0.73	0.746	0.668	0.121

b) 5 hours

	5			AVG	SD
wild type	0.727	0.687	0.809	0.741	0.062
blank	0.679	0.783	0.839	0.767	0.081
garlic	0.62	0.742	0.711	0.691	0.063
NaOCl	0.608	0.668	0.708	0.661	0.050

c) 24 hours

	24			AVG	SD
wild type	0.633	0.686	0.676	0.665	0.028
blank	0.734	0.736	0.602	0.691	0.077
garlic	0.484	0.452	0.468	0.468	0.016
NaOCl	0.36	0.326	0.307	0.331	0.027

Table 7 : The absorbance values for the comparison between garlic extract and NaOCl along 3 periods of time which were 3, 5 and 24 hours on polystyrene surface

a) 3 hours

	3			Average	SD
wild type	0.825	0.964	0.898	0.896	0.070
blank	0.952	1.02	0.772	0.915	0.128
garlic	0.649	0.984	0.9	0.844	0.174
NaOCl	0.645	0.931	0.797	0.791	0.143

b) 5 hours

	5			Average	SD
wild type	0.867	0.951	0.942	0.920	0.046
blank	0.754	0.784	0.992	0.843	0.130
garlic	0.698	0.742	0.874	0.771	0.092
NaOCl	0.638	0.756	0.852	0.749	0.107

c) 24 hours

	24			Average	SD
wild type	0.867	0.951	0.942	0.920	0.046
blank	0.754	0.784	0.992	0.843	0.130
garlic	0.698	0.742	0.874	0.771	0.092
NaOCl	0.638	0.756	0.852	0.749	0.107