Reduced Formation and Elimination of *Salmonella typhimurium* Biofilm Using Crude Garlic Extract
การลดและกำจัดไบโอฟิล์มของ *Salmonella typhimurium* โดยใช้สารสกัดจากกระเทียม

Presented in the 3rd International Conference for Value Added Agricultural Products (3rd FerVAAP Conference)

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Abstract

Salmonellae enterica serovar Typhimurium is a common food-borne pathogen that causes the deadly salmonellosis in human. *Salmonella* produces biofilm as its protection against antimicrobial agents and sanitizing agents on the varieties of surfaces. Crude garlic extract represented the ability to prevent or to reduce a formation of *Salmonella* biofilm on surfaces of glass and plastics; polystyrene (PS) or polyvinyl chloride (PVC) based on the crystal violet assay and visual observation. The ability of the crude garlic extract to eliminate the mature *Salmonella* biofilm formed on glass surface was slightly lower than that of 200 ppm sodium hypochlorite, a common industrial disinfectant, with the P-values of 0.0005 and 0.0001 for the treatment with the extract and sodium hypochlorite, respectively. Both solutions performed similarly on the polystyrene surface with the P-value of 0.06.

Keywords: *Salmonella typhimurium*, Biofilm, Crude garlic extract

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Introduction

Salmonella typhimurium is gram negative bacterial pathogen that causes Salmonellosis disease in human and animal. Salmonellosis causes an abdominal pain, vomiting and inflammatory diarrhea in the patient which probably leads to the death in immunocompromised people. Human acquires *S. typhimurium* through the consumption of contaminated animal products. Meat industry, dairy industry and poultry products are accounted as the principal reservoirs of salmonellae in the worldwide at which mediate the disease transmission to the consumers (D'Aoust, 1989). The survival of *S. typhimurium* in the food industry environment is prolonged because of its ability to develop the biofilms on the surfaces of industrial equipments which are major sources of food contamination and disease transmission (Giaouris and Nychas, 2006; Hood and Zottola, 1997). Microbial colonization on biotic or abiotic surfaces and embedded under extracellular polymeric substance (EPS) as called as biofilm has been found in a wide range of environmental conditions (O'Toole et al., 2000). Biofilms can develop on various kinds of surface materials including glass, stainless steel, plastic and rubber that are the main materials in the food processing where nutrients, ions and other organic materials are presence to promote the growth (Chae and Schraft, 2000). Therefore, biofilms commonly contaminate industrial pipelines, drains, standing water tank, food contact surfaces and floors where the inappropriate method of sanitizing has been applied in the industrial clean up. The presence of EPS of the mature biofilms also provide a protective environment for the bacteria therefore, the biofilms are generally more tolerant to disinfectants or sanitizers than the planktonic cells (Gilbert et al., 2001).

S. typhimurium has an ability to form on glass, plastic, rubber and stainless steel materials in the industries (Carballo, 2000; Ronner and Wong, 1993). The biofilm of *S. typhimurium* was found to be more resistant to sanitizers than the planktonic cells. The routine cleaning with the chemical sanitizer in the food industry represents a slightly effect on the elimination of *S. typhimurium* mature biofilm (Mettler and Carpentier, 1998). The chemical sanitizers also cause various health effects and material corrosions. The alternative natural compounds have been studied extensively on their effect on the biofilm development and mature biofilm elimination (Baveja et al., 2004). Garlic (*Allium sativum*) has been tremendously grown in Thailand. Over the last decade, the antimicrobial activity of garlic has been investigated on its effect against food spoilage bacteria and food-borne pathogens. Garlic has been shown to be a potential compound that is applicable to use in the preservation of processed foods due to the less health effect and low price. Garlic contains a high amount of allicin that is believed to play an important role in the antimicrobial activity. Garlic has been shown to have effects on the biofilm developments of many microorganisms via the interrupting of quorum sensing systems that are required for the mature biofilm developments (Bjarnsholt et al., 2005; Rasmussen et al., 2005).

The purpose of this study was to analyze on the effect of crude garlic extract on the development of *S. typhimurium* biofilm on glass and plastic materials. This study also focused on the potential of using the crude garlic extract in the elimination of *S. typhimurium* mature biofilm in comparison to sodium hypochlorite. The microscopic observation and crystal violet staining the attached cells were applied in this study to observe the biofilm formation.

Materials and Methods

Bacterial strain and cultivation medium

S. typhimurium (strain TISTR 292) was obtained from Thailand Institute of Scientific and Technological Research (TISTR). The culture was maintained in -20°C frozen storage. Prior to the use, the culture was grown on trypticase soy agar (TSA) and transferred to trypticase soy broth (TSB) at 37°C for 24 hours and subcultured twice. The final culture with the optical density (OD_{600}) of approximately 1.0 was used for the biofilm studies. The medium used in biofilm studies was 20-time diluted TSB (dTSB) (Stepanovic et al., 2004)

Crude garlic extract

The fresh garlic bulbs were peeled and chopped into small pieces. The chopped garlic 150 g was submerged in 150 ml of toluene and stirred on the shaker (180 rpm) at room temperature for 24 hours. Then, the mixture was filtered through Whatman no.1 filter paper. The total of 150 ml of sterile water was added into the previously extracted solute. The mixture was stirred for another 24 hours at room temperature on the shaker. After which, the aqueous phase and organic phase were allowed to form. The aqueous phase was separated from the organic phase using a separatory funnel and sterile filtered with Millex GS filter unit 0.22 μm (MF-Millipore MCE Membrane). The aqueous phase was used in this study as a crude garlic extract (Rasmussen, 2005).

Preparation of the test surface materials

The tests were carried out using the flat surfaces of polyvinyl chloride (PVC) and glass slides and the surfaces of glass and polystyrene (PS) tubes. Glass tube was prepared by rinsing with distilled water then air-dried and autoclaved at 121°C for 15 minutes. Polystyrene (PS) tube was prepared by soaking with 95% ethanol for 30 minutes. The tubes were air- dried in the laminar flow for 30 minutes before they were used in the test.

All the flat surfaces used in this study had the dimension of 22 mm x 22 mm. PVC slide was prepared by submerging in 95% ethanol for 30 minutes, while the glass slide was prepared by rinsing with distilled water, air-dried and then autoclaved at 121°C for 15 minutes. All slides were air-dried in the laminar flow for 30 minutes before using in the test.

The effect of crude extract on the growth of the planktonic cells

The flasks containing TSB supplemented with 20, 60 and 100 mg/ml of the garlic extract were inoculated with *S. typhimurium.* They were incubated at room temperature on a shaker at an agitation rate of 180 rpm. The maximum specific growth rate (μm) of the planktonic cultures under various conditions were measured based on the optical density level (OD_{600}nm). The appropriate concentration of crude garlic extract that applied in this study was selected from the minimal effect on the specific growth rate of planktonic cells though the P- value determination.

Quantitative analysis on the biofilm formation

The quantitative analysis on the effect of crude garlic extract on the biofilm formation or cell adhesion was performed using crystal violet staining of the attached cells. The quantitative assays on the effect of crude garlic extract on *S. typhimurium* biofilm formation were performed in glass and PS tubes. The total of 1% (v/v) of the bacterial culture ($OD600_{nm} \approx 1.0$) was inoculated into 2 ml of dTSB,

dTSB plus sterile distilled water (as a control, sterile distilled water was added in the equal volume of the crude garlic extract and dTSB plus the appropriate concentration of crude garlic extract (treatment). Tubes were prepared for 3 days test with the media replacements every 24 hours. Biofilms were allowed to develop under the static condition at 37°C. Biofilm developments of all conditions were quantified on day 1, day 2 and day 3 by crystal violet staining (1% w/v) and spectroscopic determination. At each time point, the supernatant of the tubes were aspirated and rinsed the tubes 3 times with distilled water and fixed by drying on the bench top until they were fully dried out. The total of 2 ml of 1% crystal violet was added into each tube to stain for 25 minutes. The excess stain was washed off for 3 times with distilled water. The crystal violet that stained the attached cells was destained with 2 ml of 95% ethanol by leaving at the room temperature for 30 minutes. The optical density ($OD595_{nm}$) of 1 ml destained solution was examined using a spectrophotometer (Djordjevic, 2002). The absorbance value is positively correlated to the amount of the bacterial adhesion or biofilm. All tests were performed in triplicate and the absorbance readings were averaged.

Visual observation on the biofilm development

The biofilms were allowed to develop on PVC and glass slides for 3 days by submerging the slides on the plates containing 20 ml of dTSB, dTSB with sterile distilled water added (as a control, sterile distilled water was added in the equal volume as added crude garlic extract in the test one) and dTSB with the appropriate concentration of crude garlic extract. All three different conditions were inoculated with 1% (v/v) *S. typhimurium* culture (at $OD600_{nm}$ about 1.0). The biofilms were allowed to develop at 37°C under the static condition. Fresh media were replaced every 24 hours. Daily sampling was carried out where each surface was carefully removed with sterile forceps and slightly air-dried in the laminar flow. The biofilm development in each condition was examined under the brightfield microscope with the magnification of 1000x (individual cell attachment stage) and 400x (biofilm stage). All experimental assays were performed in triplicate.

Effects of Crude garlic extract and Sodium hypochlorite on mature biofilms

S. typhimurium biofilms were allowed to develop on the surfaces of glass and PS tubes by inoculation 1% (v/v) of the bacterial culture ($OD600_{nm}$ about 1.0) to the tubes containing 2 ml of dTSB. On day 3 post- inoculation, the planktonic cells were aspirated out and biofilms or attached cells were remained on the surface of the tubes. The mature biofilms on both surface materials were submerged with dTSB, dTSB with sterile distilled water added (as a control), dTSB with the appropriate concentration of crude garlic extract and dTSB with 200 ppm sodium hypochlorite. The mature biofilms were submerged with the active compounds for 3, 5 and 24 hours. The biofilm amounts under all treatments were examined using crystal violet staining method as previously described. All tests were performed in triplicate and the absorbance readings were averaged.

Statistical analysis

The mean values and standard deviations of all replication were calculated using Excel software. The differences were identified based on the P-values from unpaired t-test.

Result and Discussion

The purpose of this study was to study on the effect of crude garlic extract to reduce and eliminate *S. typhimurium* biofilm, therefore, the non-growth inhibition concentration of the crude garlic extract on the planktonic *Salmonella* cells was selected for the study in order to allow the growth of planktonic cells to develop biofilms. The maximum specific growth rates of the planktonic cell under the three concentrations of garlic extracted (20, 60 and 100 mg/ml) were analyzed based on the optical density ($OD600_{nm}$). The P- values were determined in comparison to the control (no addition of crude garlic extract). In the medium containing 60 mg/ml of crude garlic extract represented a non significant effect on the growth while 100 mg/ml represented the P-value that close to the value of significant effect (P-value close to 0.05) (Table 1). The concentration of 20 mg/ml showed the less effect on the planktonic cell growth, moreover the low concentration of garlic extract also represented no effect on the biofilm from crystal violet analysis (data not shown). Thus, the concentration of 60 mg/ml of crude garlic extract was selected as an appropriate concentration to use in this study.

Table 1. Maximum specific growth rate (μ_m) of the planktonic cell growths under the presence of crude garlic extracts. The P-Value was calculated in comparison to the μ_m of the control (without the addition of crude garlic extract).

Concentration of garlic extract (mg/ ml)	μ_m, $hours^{-1}$	P-Value
Control	1.058	-
20	0.847	0.3
60	0.697	0.208
100	0.473	0.075

The quantitative analysis of biofilm formation has been analyzed in many microorganisms by using crystal violet stain the total attached biomass on the surface (O'Toole et al., 1999). Therefore, the staining was selected to measure the reduction of biofilm under the garlic treatment. Figure 1 demonstrated the biofilm formations as referred as bacterial attachment of *S. typhimurium*, which were obviously developed on the air- liquid interface in glass and polystyrene tubes. The biofilms were obviously seen after staining with crystal violet. As *S. typhimurium* is a facultative aerobic bacterium, the air- liquid interface could be a preference area for the bacterial attachment that benefits the cells in terms of oxygen supply and nutrient accessibility from the top as compared to the bottom of the tube (Figure 1).

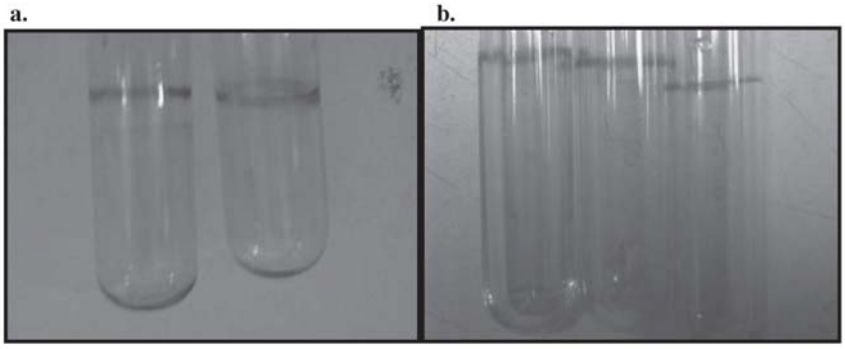


Figure 1. Representative biofilm formation of *S. typhimurium* in glass tube (a) and polystyrene tube (b) after staining with 1% crystal violet solution.

The results on the effect of garlic extract on biofilm formation (Figures 2) showed that the crude garlic extract had an effect on the biofilm formation of *S. typhimurium* from the early stage of biofilm development and dramatically interrupted the mature biofilm formation in the later stage on both glass and PS as indicated from the significant reduction in OD_{595nm} value on day 3 in both materials. Comparing between Figure 2A and 2B, *Salmonella* attached better with the higher strength to the hydrophobic material (PVC) than the hydrophilic material (glass) with or without treatment with garlic extract. This was due to the high hydrophobic property of its mature biofilm (Carballo, 2000). Although the presence of garlic extract showed the marked decreased in the number of attached cells (biofilm) from day 1, the significant effect of the crude garlic extract was found on day 3. This could probably explain the garlic extract represented a greater effect on the later stage of biofilm development than the beginning. Garlic extract has shown to be a quorum sensing inhibitor (QSI) in *Pseudomonas aeruginosa* biofilm formation (Rasmussen et al., 2005). Since, quorum sensing is the cell to cell communication that is required for the differentiation of the individual cell to the mature biofilm (Davies et al., 1998). Therefore, the presence of garlic extract was speculated to interrupt the mature biofilm formation in *S. typhomurium* that the biofilm amount also dramatically diminished according to the significant reduction in the OD_{595nm} value of the treated culture when compared to the untreated ones on both materials.

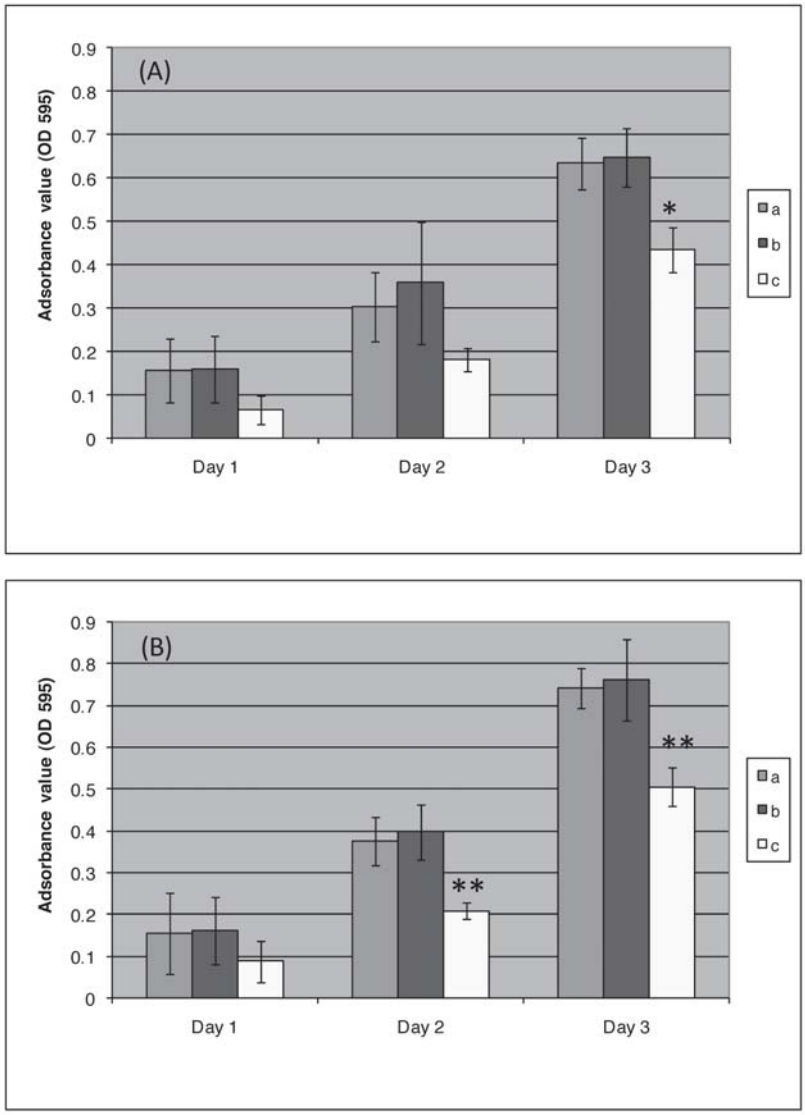


Figure 2. Absorbance values representing the amount of the bacterial attachment on glass surface (A) and polystyrene surface (B) from day 1 to day 3 when the cultures were grown in 3 different conditions; a) dTSB, b) dTSB with the supplement of sterile distilled water and c) dTSB with the crude garlic extract supplement (60 mg/ml). Each assay was completed in triplicate. The P value of garlic treatment was analyzed by unpaired t- test. Significant differences were indicated by the asterisk when one asterick means P is < 0.05 and two astericks mean P is < 0.01.

The further visual observations of biofilm formation on glass and PVC (plastic) slides showed no visual detection of *S. typhimurium* biofilm after 2 days under the treatment with the garlic extract while the biofilm started forming on day 2 under the untreated conditions (Figure 3 and 4). The results under the direct observations correlated to the previous indirect observations where the significant reduction

on the bacterial attachment was found on day 3. In the untreated condition, the tertiary structure of mature biofilm was completely observed on day 3 where the attached cells were embedded under the matrix or extracellular polymeric substrance (EPS) while the mature biofilm was not found under the treated condition. The results from the direct observation also supported our speculation that the garlic extract interrupted the mature biofilm formation of *S. typhimurium*. Garlic extract can be considered as an effective compound to prevent the mature biofilm formations on glass and plastic surfaces.

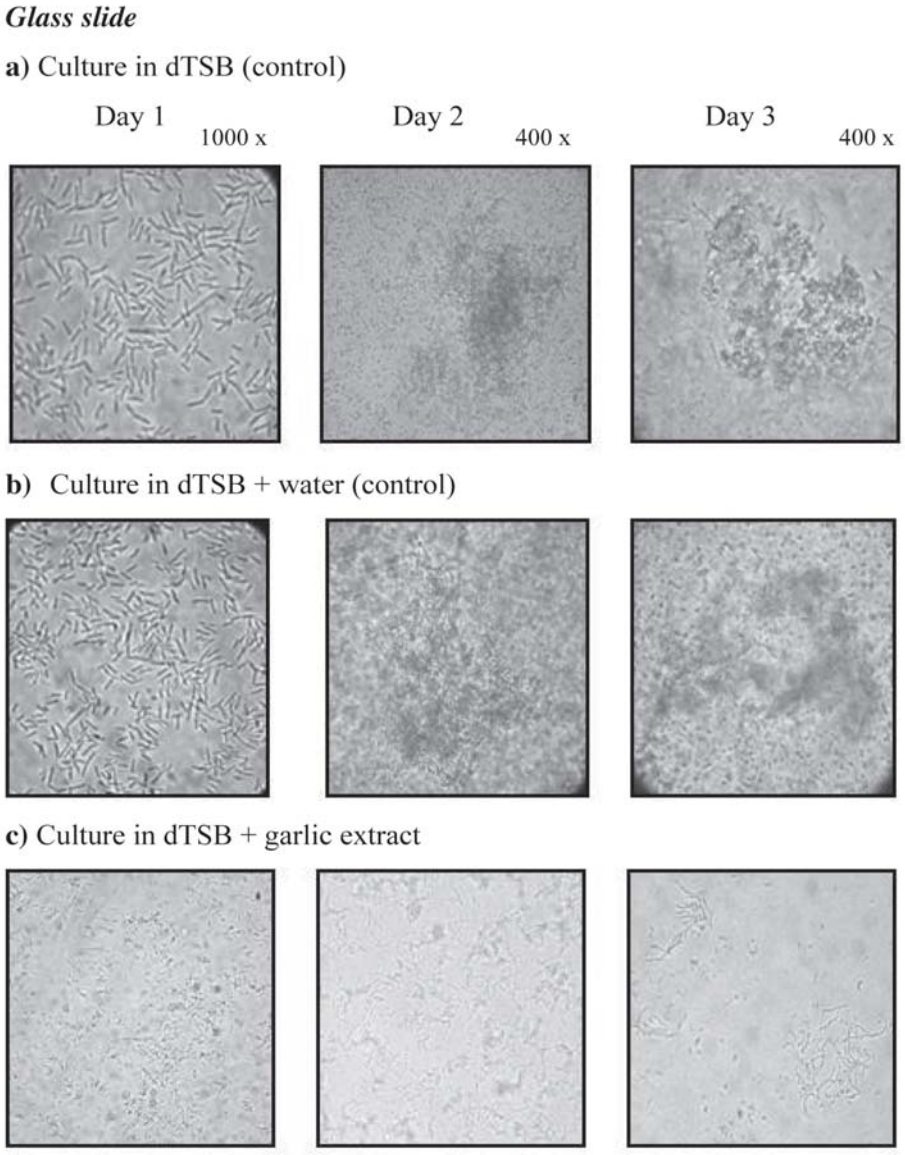
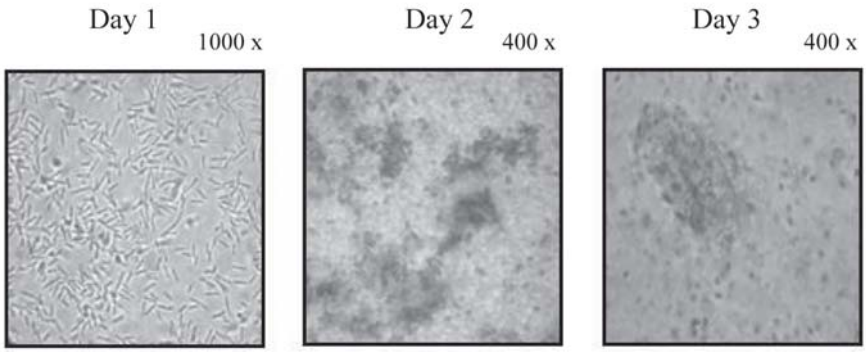


Figure 3. The direct observation of biofilm development on the glass slides under the bright-field microscope and the surface attached cells were captured at the magnification of 1000x and 400x. The mature biofilm represented as the dark region against the light background.

Polyvinyl chloride (PVC) slide

a) Culture in dTSB (control)



b) Culture in dTSB + water (control)

c) Culture in dTSB + garlic extract

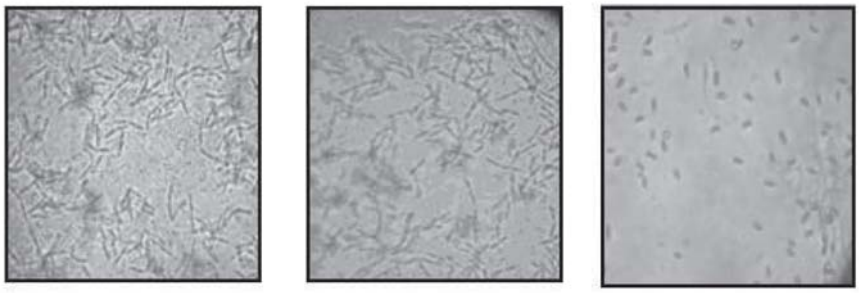


Figure 4. The direct observation of biofilm development on the polyvinyl chloride slides under the bright-field microscope and the surface attached cells were captured at the magnification of 1000x and 400x. The mature biofilm represented as the dark region against the light background.

The ability of the garlic extract to eliminate the mature biofilm (3 days old) on glass and polystyrene surfaces was analyzed and compared with a common industrial disinfectant, sodium hypochlorite (NaClO) (Figure 5 and 6). The ability of crude garlic extract to eliminate the mature biofilm on glass surface was slightly lower than those treated with sodium hypochlorite with the P- value of 0.0005 for the treatment with garlic extract and 0.0001 for sodium hypochlorite treatment under 24 hours treatment. Both solutions performed similar effect on polystyrene surface with the P-value of 0.06 which were non- significant effects under 24 hours treatment. The ability of the solutions on the reduction of mature biofilm on the glass material was much higher than on the polystyrene material as *Salmonella* strains were found to attach in higher numbers to the more hydrophobic material (PVC). This was supported by Carballo, (2000) who reported that 73% of the mature biofilms were hydrophobic. Therefore, hydrophobic interaction between *S. typhimurium* mature biofilm and hydrophobic material as polystyrene should be even stronger than the hydrophobic interaction of the mature biofilm with hydrophilic surface as glass material which burdened the sanitizing process. In addition, both solutions are hydrophilic that are more accessible to the hydrophilic compound as in glass material, so, the solutions represented the greater effect on the mature biofilm over the glass than the polystyrene. Therefore, the effectiveness of sanitizers in the reduction of bacterial adherence was also dependent upon the hydrophobicity and hydrophilicity of bacteria, materials and reagents.

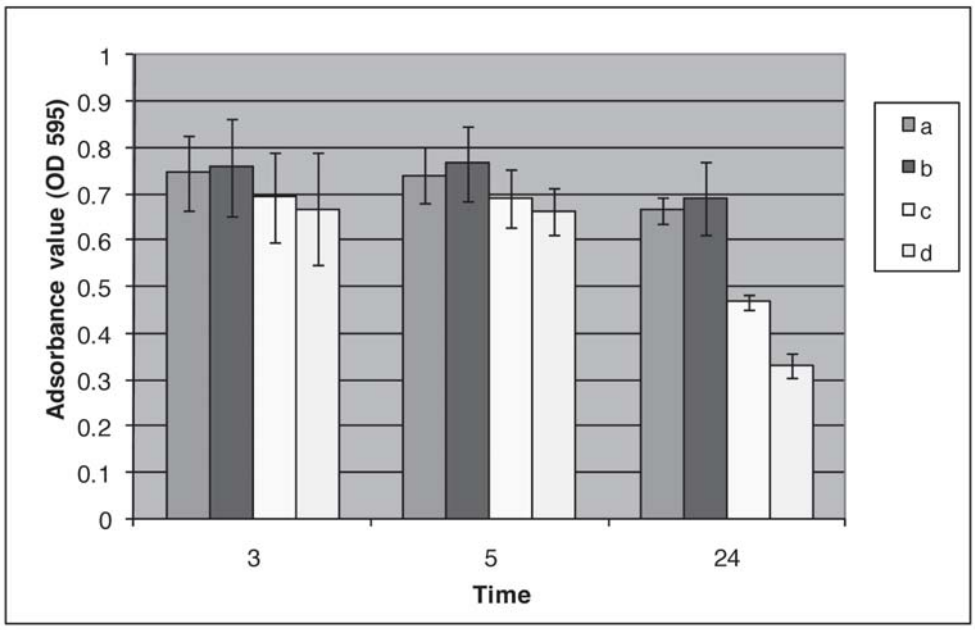


Figure 5. Comparison in the biofilm reduction on glass surface when using the garlic extract (c) and sodium hypochlorite (NaOCl) (d) after the treatments of 3 days old mature biofilms for 3, 5 and 24 hours. The controls were dTSB (a) and dTSB + water (b). The P value of garlic treatment and NaOCl were analyzed by unpaired t- test to compare with the untreated condition (a), the P-values of 0.0005 and 0.0001 respectively.

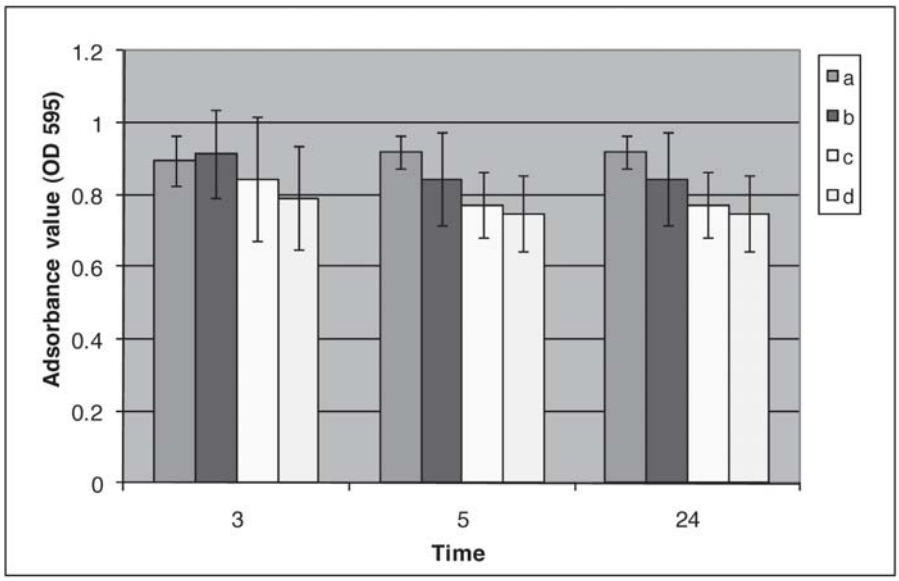


Figure 6. Comparison in the biofilm reduction on polystyrene surface when using the garlic extract (c) and sodium hypochlorite (NaOCl) (d) after the treatments of 3 days old mature biofilms for 3, 5 and 24 hours. The controls were dTSB (a) and dTSB + water (b). The P value of garlic treatment and NaOCl were analyzed by unpaired t- test to compare with the untreated condition (a), the P-values of both compounds were equal as 0.06.

Conclusion

In conclusion, we have shown that the garlic extract prevented the formation of *S. typhimurium* mature biofilm on glass and plastic (polystyrene or polyvinyl chloride) surfaces. The ability of garlic extract in the elimination of mature biofilm on the glass surface was slightly lower than that of sodium hypochlorite and the ability of both solutions were similar on the plastic surface. However, the removal of mature biofilm from the plastic surface was harder than the glass surface especially when garlic extract and sodium hypochlorite were applied after the mature biofilms had been developed. Therefore, garlic extract should be considered as a natural sanitizer that can be used in the food industry in order to prevent the mature biofilm formations of *S. typhimurium*. However, the strong odor of garlic is probably a big concern on its application in the food industry. The isolation of the pure active compound from the garlic with the removal of strong odor is prospected to be more useful for the industrial application in the future. This study has shown the potential of using the crude garlic extract to eliminate the mature biofilm of the pathogenic *Salmonella*. The use of this natural extract is beneficial in terms of health concern and less effect on the material corrosion.

Acknowledgement

The thesis committee from Biotechnology faculty, Assumption University and Assistant Professor Dr. Mallika Boonmee are gratefully acknowledged for their useful feedbacks on this study.

Reference

Baveja, J. K., Willcox, M. D., Hume, E. B., Kumar, N., Odell, R. & POOLE-WARREN, L. A. 2004. Furanones as potential anti-bacterial coatings on biomaterials. **Biomaterials** 25: 5003-12.

Bjarnshlt, T., Jensen, P. O., Rasmussen, T. B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H. P., Rygaard, J., Moser, C., Eberl, L., Hoiby, N. & Givskov, M. 2005. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. **Microbiology** 151: 3873-80.

Carballo, E. S. A. J. 2000. Attachment of *Salmonella spp.* and *Listeria monocytogenes* to stainless steel, rubber and polytetrafuor- ethylene: the influence of free energy and the e¡ect of commercial sanitizers. **Food Microbiology** 17: 439- 447.

Chae, M. S. & Schraft, H. 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. **Int J Food Microbiol** 62: 103-11.

D'aoust, J.-Y. 1989. *Salmonella*, New York, Marcel Dekker, Inc.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. **Science** 280: 295-8.

Djordjevic, D., M. Wiedmann & L. A. Mclandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. **Appl Environ Microbiol** 68: 2950-295.

Giaouris, E. D. & Nychas, G. J. 2006. The adherence of *Salmonella enteritidis* PT4 to stainless steel: the importance of the air-liquid interface and nutrient availability. **Food Microbiol** 23: 747-52.

Gilbert, P., Das, J. R., Jones, M. V. & Allison, D. G. 2001. Assessment of resistance towards biocides following the attachment of micro-organisms to, and growth on, surfaces. **J Appl Microbiol** 91: 248-54.

Hood, S. K. & Zottola, E. A. 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int **J Food Microbiol** 37: 145-53.

Mettler, E. & Carpentier, B. 1998. Variations over time of microbial load and physicochemical properties of floor materials after cleaning in food industry premises. **J Food Prot** 61: 57-65.

O'toole, G., Kaplan, H. B. & Kolter, R. 2000. Biofilm formation as microbial development. **Annu Rev Microbiol** 54: 49-79.

O'toole, G. A., Pratt, L. A., Watnick, P. I., Newman, D. K., Weaver, V. B. & Kolter, R. 1999. Genetic approaches to study of biofilms. **Methods Enzymol** 310: 91-109.

Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Kote, M., Nielsen, J., Eberl, L. & Givskov, M. 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. **J Bacteriol** 187: 1799-814.

Ronner, A. B. & Wong, A. C. L. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N-rubber. **J Food Prot** 56: 750-758

Stepanovic, S., Cirkovic, I., Ranin, L. & Svabic-Vlahovic, M. 2004. Biofilm formation by *Salmonella spp.* and *Listeria monocytogenes* on plastic surface. **Lett Appl Microbiol** 38: 428-32.