CHAPTER 3

EXPERIMENTS

3.1 Materials and Equipments

3.1.1 Bacterial strains

- *Escherichia coli*
- *Bacillus cereus*
- *Pseudomonas fluorescens* TISTR 358
- *Enterobacter aerogenes* TISTR 1468
- *Lactobacillus plantarum* TISTR 1265
- *Micrococcus luteus* TISTR 884
- *Serratia marcescen* TISTR 1354

The food spoilage bacteria were obtained from either Department of Biotechnology, Faculty of Agro-Industry, Chiangmai University or Thailand Institute of Scientific and Technological Research.

3.1.2 Chitosan

A commercial chitosan was purchased from Fluka Co Ltd. The characteristic of chitosan was shown in Appendix A.

3.1.3 Minced pork

Fresh pork was purchased from local market on the day of preparation, cut and minced with a blender.

3.1.4 Media and Chemical reagents

3.1.4.1 Microbiological media and diluent

(1) Nutrient agar and Nutrient broth (NA and NB)
(2) Eosin Methylene Blue agar (EMB)
(3) DeMan Rogasa and Sharp agar (MRSA)
(4) *Salmonella-Shigella* agar (SS agar)
(5) Plate count agar (PCA)
(6) *Bacillus cereus* medium (BCM)

The composition of microbiological media for the cultivation and enumeration, microbiological diluents for maintenance of the food spoilage bacteria were show in Appendix B.

3.1.4.2 Chemical reagents

Names of chemical reagents	Production company
HCl	MERCK
NaOH	MERCK
CH_3COOH	MERCK
K_2HPO_4	MERCK
$MgSO_4.7H_2O$	UNILAB
$MnSO_2.4H_2O$	Scharlau
Sodium acetate	Scharlau
diammonium citrate	MERCK
Peptone	HIMEDIA

Names of chemical reagents	Production company
Tryptone	HIMEDIA
Beef extract	HIMEDIA
Yeast extract	HIMEDIA
Peptone water	HIMEDIA
Glucose	FLUKA
Lactose	MERCK
Tween 80	LABCHEM
Crystal Violet	Riedal-deHaen
Methylene Blue	BAKER ANALYZED
Eosin Y	CARCO ERBA
Neutral Red	FLUKA
Bromocrecol purple	FLUKA
SS-agar powder	HIMEDIA
Ethanol 95%	

3.1.5 Equipments

Name of equipments	Production company
Analytical balance (4 digits)	OERTING
Analytical balance (2 digits)	OERTING
Autoclave Model ACV-3167	IWAKI
Autopipette	GIBSON
Controlled temperature shaker Model ES-W	KUHNER
Hot air oven	MEMMERT
Light microscope Model CH20	OLYMPUS
Magnetic stirrer	VELP SCIENTIFIC
pH meter Model C830	CONSORT
Purifier vertical clean beach	LABCONCO
Refrigerated centrifuge Model Super T21	SORVALL
Spectrophotometer Model V-530	GENESYS
Stomacher	MASTICATOR

Name of equipments	Production company
Vortex mixer (2 Genie)	BOHEMIA
Water bath Model 1255 PC	SHEL-LAB

3.2 Method

3.2.1 Bacterial strains and cultivation

The food spoilage bacteria from the TISTR culture collection or Department of Biotechnology were streaked onto different agar plates made up of nutrient agar, Eosin Methylene Blue (EMB) agar or DeMan Rogasa Sharpe (MRS) agar and incubated at 37°C or room temperature for 24 hr. A single colony on the plate was then used to inoculate onto nutrient or MRS slants. After that, the slants were incubated at 37°C or room temperature for 24 hr. Stock cultures were maintained at 4° C for future study. They were periodically subcultured by transferring to fresh agar medium every 4 weeks.

3.2.2 Preparation of chitosan solution

Two molecular weight chitosans were prepared for stock solutions with a concentration of 1.0% (w/v) by dissolving chitosan in 1 M glacial acetic acid. The mixture was stirred by a magnetic stirrer for 2 hours at room temperature for a complete dissolution. The stock solutions were, then, stored at cold room (4°C).

3.2.3 Antibacterial effect of chitosan on the growth of some food spoilage bacteria *in vitro* and determination of the minimum inhibitory concentration (MIC) of chitosan.

Stock cultures were initially used for fresh slant culture preparation. A loopful of this slant culture was used as an innoculum in 20 ml of nutrient or MRS broth (for *L. plantarum*). After inoculating, each culture was incubated at 37°C or room

temperature for 24 hr with shaking at 180 rpm and used as a starter for the next experiment step.

The effect of chitosan on the growth of some food spoilage bacteria in liquid media was assessed by a plate count assay. The chitosan stock solution (1.0% w/v) was added into nutrient or MRS broth to a final concentration of 0, 0.01, 0.02, 0.04, 0.06 and 0.08% (w/v) for a final volume of 10 ml. After that, pH values of the solutions were adjusted to 6.0. All of the mixture solutions were sterilized by an autoclave at 121°C for 15 minutes. Then 0.1 ml of bacterial cultures were inoculated into each chitosan concentration and incubated by shaking at 37°C or room temperature for 24 hr. Samples of bacterial cultures (1.0 ml) from each flask were diluted in test tubes of 0.85% (w/v) NaCl and viable cells were enumerated by a spread plate technique in duplication. Microorganisms on the plates were counted and number of colony forming units were calculated. The MIC was defined as the lowest concentration of chitosan required to completely inhibit bacterial growth after incubation at 37°C or room temperature for 24 hr.

3.2.4 Effect of pH on antibacterial activity of chitosan

Effect of pH on antibacterial activity of chitosan against *B. cereus* and *M. luteus* was monitored. Chitosan solution at 0.1% (w/v) in 1 M acetic acid was added to nutrient broth to give a final chitosan concentration of 0.01% (w/v) with a total volume of 50 ml. The pH values were adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 with 1 N HCl and 1 N NaOH before autoclaving. After that, 0.1 ml of overnight inocula was inoculated into each flask. Then these cultures were incubated with shaking at 180 rpm at 37°C for 24 hours. The microbial growth was measured as the turbidity of the solution by a spectrophotometer at 600 nm with 4 hr intervals, comparing the developed biomass against that of a blank in which no chitosan was added. Measurement of number of bacteria in each treatment was obtained from a standard graph of turbidity versus cell count.

3.2.5 Effect of temperature on antibacterial activity of chitosan

Chitosan solution with a concentration of 0.1% (w/v) from the stock solution was added to nutrient broth to give a final concentration of 0.01% (w/v). The pH of the broth was adjusted to 6.0 with 1 N HCl or 1 N NaOH before autoclaving. Then 0.1 ml of each tested bacterium was inoculated in the broth and incubated with shaking at 180 rpm for 24 hr at two different temperatures of 25 and 37°C. The growth of bacteria was measured in terms of optical density (OD) in a spectrophotometer at 600 nm. Samples were taken at 4 hr intervals.

3.2.6 Investigation of antimicrobial activity of chitosan on a mixed culture *in vitro*

Bacterial culture of *B. cereus* and *M. luteus* were used in this study. The cultures of two bacteria were incubated at 37°C for 24 hr, and then inoculated into nutrient broth containing a chitosan concentration of 0.01% (w/v) and nutrient broth without chitosan for control. The mixture cultures were incubated at 37°C and samples were taken after 24 hr incubation for enumeration of the viable cells by a spread plate technique which was done in duplicate. After incubation, the plates were counted and numbers of colony forming units were calculated.

3.2.7 The effect of bacteria cells on antibacterial activity of chitosan

A study on the number of colonies of starter culture that could be inhibited by chitosan was investigated. The experiments were carried out using 24 hr incubated bacteria growth in 200 ml nutrient broth. The cell were harvested by centrifugation at 4°C, 6000 rpm for 10 min. After a rinsing step, the microbial pellets were re-suspended in 10 ml nutrient broth for preparing a dilution series. The dilution series was 1, 1/4, 1/16 and 1/64 concentration. Simultaneously the actual starter cell count in each dilution before applying to the chitosan solution was determined by the plate count method. Each bacterial dilution was made a serial dilution and counted the growth colonies after a 24 hr incubation. Then samples from each dilution were

inoculated into nutrient broth with a chitosan concentration of 0.01% (w/v) and incubated at 37°C with shaking at 180 rpm. Samples were taken and measured for the growth of bacteria at 4 hr intervals for up to 24 hr by monitoring the turbidity in terms of optical density (OD) in a spectrophotometer at 660 nm. This method was a simplified version used by Darmadji and Izumimoto (1994).

3.2.8 Effect of chitosan for antimicrobial activity in minced pork

Fresh pork was purchased from a local market on the day of preparation. Meat was cut and minced with a blender. The minced meat was mixed with either 0%, 0.5% and 1% (w/v) of chitosan in two replicates. A 25 g meat sample of different chitosan concentrations was then packed and wrapped. They were placed in a refrigerator operating at 4°C for 0, 2, 4, 6, 8 and 10 days. These samples were used for microbial analyses. The 25 g sample was added to 225 ml of peptone water and homogenized with a stomacher for 30 sec. Duplicated samples (1 ml each) of the slurry were serially diluted in 9 ml of peptone water and each was spread-plated or pour-plated in duplicate as follows: PCA for total aerobic bacteria, MRSA for lactic acid bacteria, EMB for *E. coli* and SS agar for *Salmonella-Shigella* spp.