CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Chemicals

Materials and chemicals used in this research are listed as follows.

1. Nisin from *Streptococcus lactis* was purchased from A.C.S., Xenon Limited Partnership, Thailand.
2. Gelatin powder was purchased from Ajax Finechem, Australia.
3. MRS agar was purchased from White Group Public Co. Ltd., Thailand.
4. MRS broth was purchased from White Group Public Co. Ltd., Thailand.
5. Tryptic Soy Agar (TSA) was purchased from S.R. LAB. Co. Ltd., Thailand.
6. Tryptic Soy Broth (TSB) was purchased from S.R. LAB. Co. Ltd., Thailand.
7. Bacto Agar was purchased from White Group Public Co. Ltd., Thailand.
8. Sodium chloride (NaCl) (A.R. grade) was purchased from White Group Public Co. Ltd., Thailand.
9. Glacial hydrochloric acid (HCl) (A.R. grade) was purchased from S. R. LAB. Co. Ltd., Thailand.
10. Glacial acetic acid (Ac) (A.R. grade) was purchased from Lab Scan, Thailand.
11. Glutaraldehyde (Glu) (A.R. grade) was purchased from A.C.S, Xenon Limited Partnership, Thailand.
12. Dibasic sodium phosphate (H_2NaPO_4) (A.R. grade) was purchased from S. R. LAB. Co. Ltd., Thailand.

13. Glycerol (A.R. grade) was purchased from White Group Public Co. Ltd., Thailand.

3.2 Tested Microorganisms Preparation

1. *Lactobacillus plantarum* TISTR 850 was inoculated in MRS broth and incubated in anaerobic condition at 37 °C until bacteria grew up to mid-log phase (10^7-10^8 CFU/mL).

2. *Staphylococcus aureus* ATCC 25923 was inoculated in TSB and incubated at 37 °C until bacteria grew up to mid-log phase (10^7-10^8 CFU/mL).

3. *Listeria monocytogenes* DMST 17303 was inoculated in TSB and incubated at 37 °C until bacteria grew up to mid-log phase (10^7-10^8 CFU/mL).

4. *Salmonella* Typhimurium ATCC 13311 was inoculated in TSB and incubated at 37 °C until bacteria grew up to mid-log phase (10^7-10^8 CFU/mL).

The bacterial growth was measured by spectrophotometer at 600 nm every hour. The growth curves were plotted between absorbance at 600 nm (Y-axis) and time in hour (X-axis) (see appendix A; Figures A1 – A8). The average amount of bacteria was determined by spread plate method (see appendix A; Tables A1 - A4). Bacteria at mid-log phase were diluted to 10^6 CFU/mL for used in subsequent experiments.

3.3 Instruments

1. Incubator (Model 800, Memmert GmbH and Co. KG., Western Germany)

2. Incubator shaker (Model SK-737, Amerex Instruments, Inc., USA)

3. Orbital shaker (Innova Model, New Brunswick C0., Inc., USA)

4. Autoclave (Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan)

5. Hot air oven (Model UC 30, Memmert GmbH and Co. KG., Western Germany)

6. Spectrophotometer (Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, USA)

7. Laminar flow 'clean' (Model V6, Lab Service Ltd., Part)

8. Cold room (Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K)

9. 4-Digit precision weighting balance (Model AG 204, Mettler Toledo, Switzerland)

10. Microwave (Model 000502174, Thai Cityelectric Co. Ltd., Thailand)

11. Hot plate stirrer (Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)

12. Vortex mixer (Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, USA)

13. Water bath (Model WB14, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)

14. High voltage power supply (Model D-ES30PN/M692, Gamma High Voltage Research, Florida, USA)

15. Viscometer (Brookfield DV-III, Brookfield Engineering Laboratories, MA, USA)

16. Conductivity meter (Orion 160, Orion Research Inc., Beverly, MA, U.S.A)

17. Universal testing machine (Lloyd Model LRX, Fareham, Hans, UK)

18. Scanning electron microscope (JEOL model JSM-6400 LV, Peabody, MA, USA)

19. Ion sputtering (JEOL JFC-1100E, Peabody, MA, USA)

20. Image analyzer software (Image J, JEOL, USA)

3.4 Experimental Procedures

3.4.1 Determination of Nisin Properties

3.4.1.1 Minimum Inhibitory Concentration (MIC) of Nisin

Nisin was dissolved in 0.02N HCl at different concentrations (0.01, 0.05, 0.1, and 0.2 g/mL) and transferred into 11 test tubes, which contained 4.5 mL MRS broth at the volume spectied in Table 3.1 to reach final concentration of 0 – 5 mg/mL. Each tube was inoculated with *Lactobacillus plantarum* TISTR 850 (10^6 CFU/mL). This experiment was performed in 2 replications. The details of the experimental procedure are shown in Figure 3.1.

Table 3.1 Determination of final nisin concentration.

Tube	1	2	3	4	5	6	7	8	9	10	11
Final nisin concentration (mg/mL)	0	0.05	0.1	0.15	0.2	0.25	0.3	0.5	1	2	5
MRS broth (mL)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
0.01 g/mL nisin in 0.02 N HCl (μL)	0	25	50	75	100	125	150	0	0	0	0
0.05 g/mL nisin in 0.02 N HCl (μL)	0	0	0	0	0	0	0	50	100	0	0
0.1 g/mL nisin in 0.02 N HCl (μL)	0	0	0	0	0	0	0	0	0	100	0
0.2 g/mL nisin in 0.02 N HCl (μL)	0	0	0	0	0	0	0	0	0	0	125

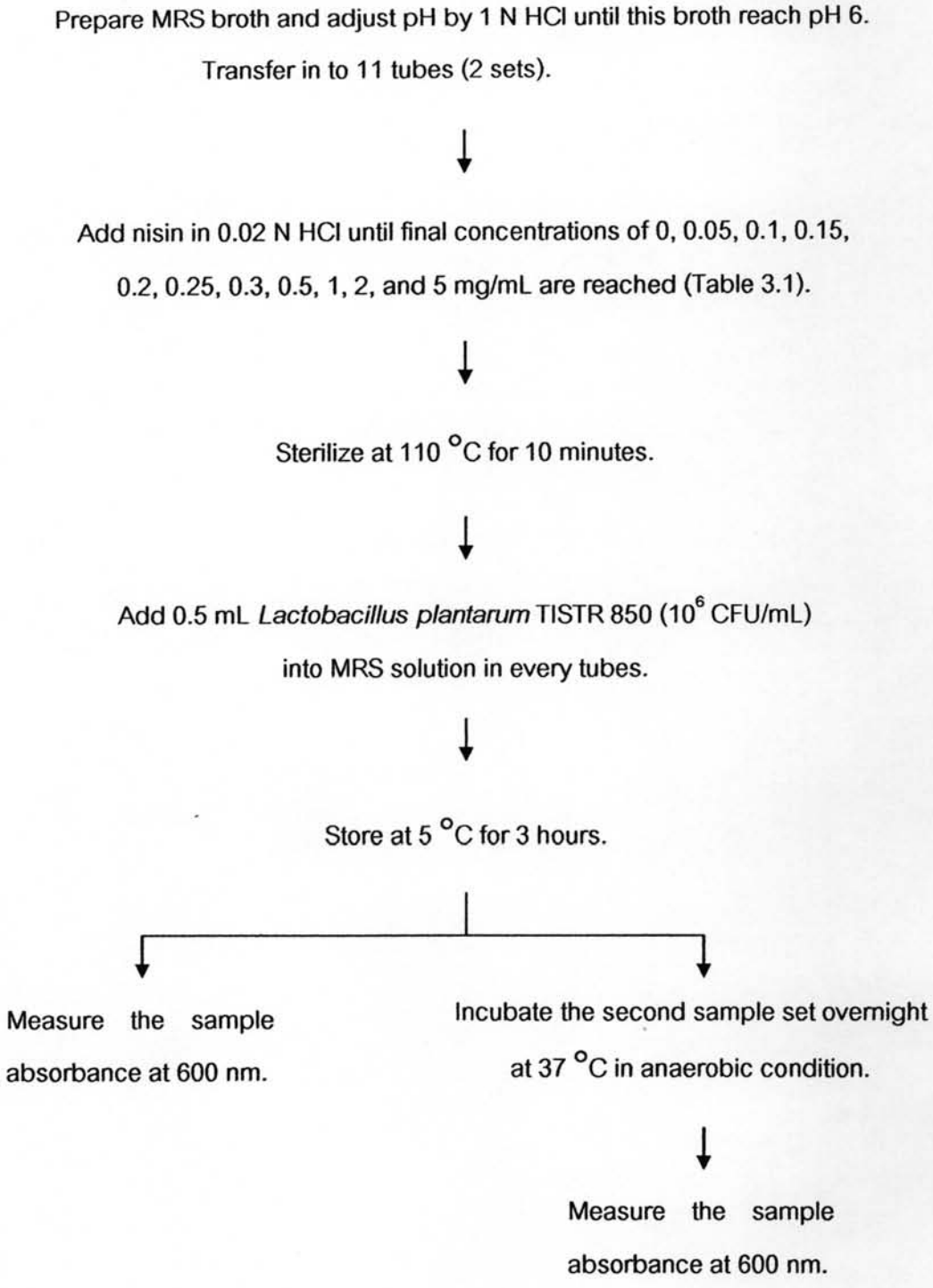

Figure 3.1 Determination of minimum inhibitory concentration of nisin.

3.4.1.2 Determination of Nisin Stability

Two test tubes containing 5 mL of 0.28 mg/mL nisin solution in each tube were prepared by dissolving 1.4 mg nisin in 5 mL of 0.02N HCl. The first test tube was heated at 120 ^{o}C for 30 minutes and the second test tube was let stand at room temperature (without heating) before they were tested for inhibition effect against *Lactobacillus plantarum* TISTR 850 using agar diffusion technique. The experiment was conducted in 4 replications.

3.4.1.3 Determination of Nisin by Agar Diffusion Technique

The agar diffusion bioassay is the most widely used method for the quantification of nisin, due to its high sensitivity, simplicity, and cost-effectiveness. This method is based on the measurement of the inhibition zone produced by nisin-sensitive microorganisms. The size of the zone is affected by many factors, such as nisin-sensitivity of the strain, amount of added agar and surfactant, and pre-diffusion step. The procedure shown in Figure 3.2 was modified from that of Dawson et al. (2003).

Inoculate 100 µL *Lactobacillus plantarum* TISTR 850
(10^6 CFU/mL) into a plate.

↓

Add 20 mL sterilized MRS soft agar
(MRS broth + 1% agar) into the plate.

↓

Pour plate and cool to room temperature
to allow agar solidification.

↓

Make 4 test wells using a sterile 5 mm diameter cork corer

↓

Dispense standard nisin or test solutions into individual wells
(50 µL per well).

↓

Store at 5 °C for 3 hours.

↓

Incubate plates overnight at 37 °C in anaerobic condition
until growth of the test organism was observed.

↓

Measure the diameter of inhibition zone (cm)
using a vernier caliper.

Figure 3.2 Procedure for determination of nisin by agar diffusion technique.

3.4.2 Determination of Effects of Nisin Concentration and Diameter of Gelatin Nanofiber on Nisin Release

3.4.2.1 Production of Gelatin Nanofibers and Antimicrobial Gelatin Nanofibers

- Preparation of gelatin-nisin solution

Gelatin-nisin solutions (22% w/v gelatin concentration and 0% – 3% w/w initial nisin concentration) were prepared by dissolving 2.2 g of gelatin in 8.37 mL of 70:30 v/v acetic acid: distilled water before adding 0 g to 0.066 g of nisin. Gelatin-nisin solutions (3% w/w nisin concentration and 20% - 24% w/v gelatin concentrations) were prepared by dissolving 2.0 g, 2.2 g, and 2.4 g of gelatin in 8.52 mL, 8.37 mL, and 8.22 mL of 70:30 v/v acetic acid: distilled water (See Appendix-B), respectively. 0.066 g of nisin was added in each solution. The solutions were stirred at room temperature (~ 25 ^{o}C) in order to obtain homogeneous gelatin-nisin solution.

- Characterization of gelatin-nisin solution

The pH of the gelatin-nisin solution was determined by a pH meter at 25 ^{o}C. The viscosity of the solution was measured by a Brookfield viscometer (model DV-III, Brookfield Engineering Laboratories, MA, USA) with spindle no. 21 at a rotational speed of 78 rpm at 25 °C. The conductivity of solution was determined by a conductivity meter (model Orion 160, Orion Research Inc., Beverly, MA, USA) at 25 ^{o}C. All measurements were carried out in 3 replications.

- Electrostatic spinning process

15 mL of Gelatin-nisin solution was loaded into a glass syringe, which was held on a stand as shown in Figure 2.4. The syringe tip was connected to the positive lead from a high voltage power supply (Model D-ES30PN/M692, Gamma high

voltage research, Florida, USA) via an alligator clip. Grounded counter electrode was connected to aluminum foil or metal drum that was used as a collector. The high voltage supply was 15 kV and the distance between the metal syringe needle and collector was 20 cm. The solution was electrospun upon applying an electric current. To collect enough samples for nanofiber characterization, the electrostatic spinning process was carried out for 5 minutes. However when a metal drum was used as the collector, it took 3 days to produce 37-149 μm thick nanofiber mat.

- Characterization of Gelatin Nanofibers and Antimicrobial Gelatin Nanofibers

The gelatin nanofiber and antimicrobial gelatin nanofiber mat was coated with gold by an Ion sputtering (JEOL JFC-1100E, Peabody, MA, USA) at 10 mA for 4 minutes. Their morphology was characterized by a scanning electron microscope (JEOL model JSM-6400 LV, Peabody, MA, USA). Average nanofiber diameter was analyzed by using an image analyzer software (Image J, JEOL, USA).

Figure 3.3 displays the process for electrostatic spinning of gelatin nanofiber and antimicrobial gelatin nanofiber.

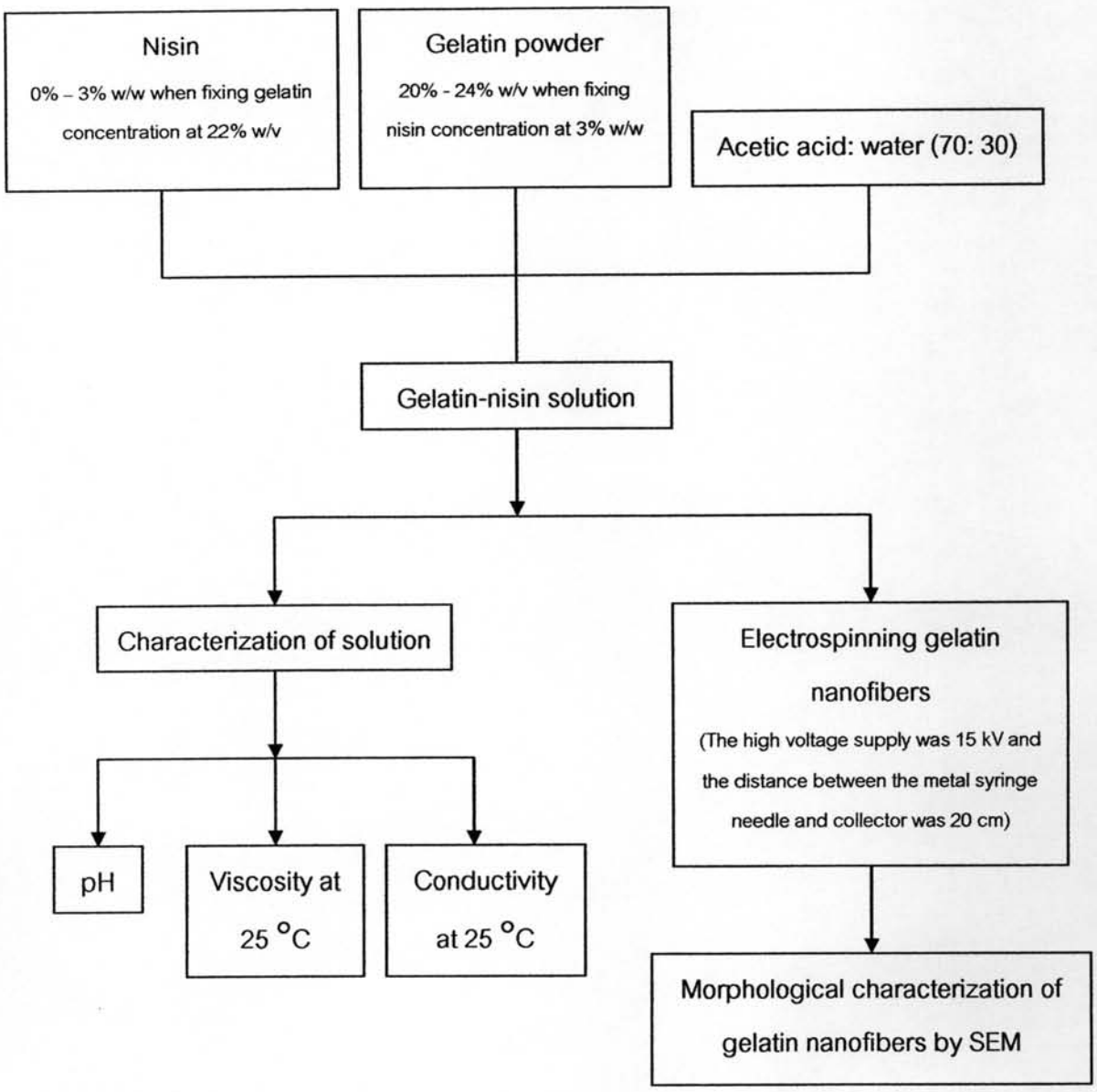

Figure 3.3 Electrospinning process for the production of gelatin nanofibers and antimicrobial gelatin nanofibers using various nisin concentrations and various gelatin concentrations.

3.4.2.2 Crosslinking of Gelatin Nanofibers and Antimicrobial Gelatin Nanofibers Mats

Gelatin nanofibers and antimicrobial gelatin nanofibers mats were crosslinked by saturated glutaraldehyde vapour at 37 °C for 5 minutes following the method that was modified from that of Matthews et al. (2002) and Zhang et al. (2006). The crosslinking process was carried out by placing the air-dried gelatin-nisin nanofibrous membrane in a chamber containing 250 mL of aqueous glutaraldehyde solution. The membranes were placed on a shelf in the chamber and crosslinked by saturated glutaraldehyde vapour at 37 °C for 5 minutes.

3.4.2.3 Determination of Mechanical Properties of Crosslinked Gelatin Nanofibers and Crosslinked Antimicrobial Gelatin Nanofibers Mats

Tensile strength, Young's modulus, and elongation of crosslinked gelatin nanofiber and crosslinked antimicrobial gelatin nanofiber mats (width x length = 10 mm x 80 mm) were determined by a universal testing machine (Lloyd Model LRX, Fareham, Hans, UK) using a 50 N load cell. A cross-head speed of 10 mm/min was used for all of the specimens tested. The gauge length was 30 mm. The thickness of the samples for crosslinked nanofibers mats were in the 47.8 - 132.8 μm range. All measurements were carried out in 5 replications.

3.4.2.4 Construction of Nisin Standard Curve

Standard nisin solutions (0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/mL) were prepared in sterile 0.02 N HCl. The solutions then tested for inhibitory effect to *Lactobacillus plantarum* TISTR 850 (10^6 CFU/mL) by agar diffusion technique as described previously in section 3.4.1.3. The experiment was done in 2 replications. The nisin standard curve plot between the width of inhibition zone (cm)

(Y-axis) and log of nisin concentration in $x10^{-2}$ mg/mL (X-axis) was made for each replication (see appendix C). Regression analysis was used to determine the standard equation, which was used to calculate the concentration of nisin in antimicrobial gelatin nanofibers. The R^2 for standard curve in 0.02 N HCl should be 0.99.

3.4.2.5 Determination of Concentration of Nisin in Antimicrobial Gelatin Nanofibers Mats

Initial concentration of nisin in antimicrobial gelatin nanofibers mats which were produced by the methods in section 3.4.2.1 was measured by extraction the nanofiber mat with 0.02 N HCl. Nisin concentration in the extract was determined using by agar diffusion technique as described in section 3.4.1.3 (Dawson et al, 2003). The experiment was conducted in 4 replications. The details for each procedure are shown in Figure 3.4.

Grind nanofiber mat samples using a blender
and weight into a 0.25±0.005 g portion

↓

Extract the ground sample using 45 mL of 0.02 N HCl
at 75 °C for 10 minutes.

↓

Cool and filter the sample through Whatman no. 1 paper filter.

↓

Adjust the volume to 50 mL using 0.02 N HCl
in a volumetric flask.

↓

Determine the concentration of nisin in the acid filtrate by
using the agar diffusion technique described previously (section 3.4.1.3).

Figure 3.4 Procedure for determination of nisin concentration in antimicrobial gelatin nanofibers mats.

3.4.2.6 Statistical Analysis

All data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Statistical analysis was performed using the Statistical Package for Social Science (SPSS for windows version 10.0, SPSS Inc., Chicago, IL, USA).

3.4.3 Determination of Effects of Temperature and Water Activity (a_w) on Nisin Release

3.4.3.1 Determination of Effect of Temperature on Nisin Release

The apparatus and procedure for the diffusion tests was modified from that of Dawson et al. (2003). A 6x6 cm^2 crosslinked antimicrobial gelatin nanofiber mat was conditioned at 96% - 98% RH in a desiccator containing saturated solution of dibasic sodium phosphate at ambient temperature (25 °C) for 16 - 18 hours. The crosslinked antimicrobial gelatin nanofiber mat was then immersed in 50 mL of sterile distilled water in a flask. The flask was placed in an incubator at 5, 25, 35, and 45 °C. Samples of 500 µL were taken with a micropipette after 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 hours at 5 °C, after 5, 10, 15, 20, 25, 30, 40, 50 minutes, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, and 48 hours at 25 and 35 °C, and after 5, 10, 15, 20, 25, 30, 40, 50 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 24 hours at 45 °C. The concentration of nisin in the water was determined by using the agar diffusion technique as described in section 3.4.1.3. The concentration of nisin which released into water was expressed as the width of inhibition zone (cm). The experiment was done in 4 replications.

3.4.3.2 Determination of Effect of Water Activity (a_w) on Nisin Release

Water-glycerol mixtures with various a_w (0.992, 0.975, and 0.955) were prepared by dissolving 0%, 8%, and 16% glycerol in sterile distilled water, respectively. A 6x6 cm^2 crosslinked antimicrobial gelatin nanofiber mat was conditioned at 96% - 98% RH in a desiccator containing a saturated solution of dibasic sodium phosphate at ambient temperature (25 °C) for 16 - 18 hours. The crosslinked antimicrobial gelatin nanofiber mat was then immersed in 50 mL of water-glycerol mixtures in a flask. The flask was placed in an incubator at 25 °C. Samples of 500 µL were taken with a micropipette after 5, 10, 15, 20, 25, 30, 40, 50 minutes, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, and 48 hours at 25 °C. The concentration of nisin in the water was determined by using the agar diffusion technique as described in section 3.4.1.3. The

concentration of nisin which released into water was expressed as the width of inhibition zone (cm). The experiment was done in 4 replications.

3.4.4 Determination of Effects of Crosslinked Antimicrobial Gelatin Nanofiber Mat on the Inhibition of Bacterial Growth

A crosslinked antimicrobial gelatin nanofiber mat prepared in section 3.4.2.2 was tested for its ability to inhibit the growth of pathogens which were *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* DMST 17303, and *Salmonella* Typhimurium ATCC 13311 compared with the crosslinked gelatin nanofiber mat that did not contain nisin. The experiment was performed in 3 replications. The details of the experimental procedure, which was modified from that of Hoffman et al. (2001), are shown in Figure 3.5.

Add 15 mL of the inoculums (10^6 CFU/mL) to each of the flasks containing the crosslinked antimicrobial gelatin nanofiber mat (0.08 g) or crosslinked gelatin nanofiber mat (0.08 g).

↓

Put the flasks on an orbital shaker (50 rpm) and orbitally shake at room temperature (~25 °C).

↓

Collect 100 µL samples after 0, 2, 4, 8, 12, 24, and 48 hours, dilute, and spread in triplicate on TSA agar plate.

↓

Incubate the plates at 37 °C until the growth of the test organism was observed.

↓

Count the number of colonies on each plate and report as CFU/mL.

Figure 3.5 Procedure for the determination of the effects of crosslinked antimicrobial gelatin nanofiber mat on the inhibition bacterial growth.

3.4.5 Determination of Antimicrobial Activity of Crosslinked Antimicrobial Gelatin Nanofibers Mats after 5 Months Storage

The crosslinked antimicrobial gelatin nanofibers mats produced in section 3.4.2.2 were stored in a desiccator at room temperature (~ 25 °C) for 5 months. The activity of this mat was determined following section 3.4.2.5. The experiment was performed in 8 replications.