CHAPTER III

EXPERIMENTAL

This section involves the equipment and chemical reagents used in the research. The main experimental procedures will be explained in 4 parts: 1) preparation of solution for electrospinning, 2) chitosan nanofibers fabrication by electrospinning technique, 3) characterization of the obtained chitosan nanofibers, 4) characterization of nanofibers and cell attachment on chitosan nanofibers.

3.1 Chemical Reagents

1. Chitosan (Biolife, Thailand; Mw~100 kDa, 400 kDa and 760 kDa)
2. Poly(vinyl alcohol) (Ajax Finechem, Australia; Mw~ 80 kDa)
3. Glacial acetic acid (Analytical grade; BDH, England)
4. Sodium hydroxide (Analytical grade; Merck Ltd., Thailand)
5. Sodium chloride (Analytical grade; Merck Ltd., Thailand)
6. Potassium dihydrogen phosphate (Analytical grade; Merck Ltd., Thailand)
7. Potassium hydrogen phosphate (Analytical grade; Merck Ltd., Thailand)
8. LIVE/DEAD® *Bac*Light™ Bacterial viability kit (Model L7012, Molecular Probes, Invitrogen Corporation, USA.)

3.2 Preparation of Electrospinning Solution

3.2.1 Hydrolyzed chitosan

For a set of experiments to verify the effect of chitosan hydrolysis on the formation of nanofibers, chitosan with molecular weight of 760,000 g/mol was used. Hydrolysis was done by using 50% NaOH solution. The mixture of 1:25 (w/v) chitosan/NaOH solution was heated at 95°C for various period of time, in the range of 0-48 h. Each sample was strained and rinsed with distilled water before being dried at 60°C for 6 h. The electrospinning solution was then prepare by redissolving the

hydrolyzed chitosan in 90% wt. acetic acid solution under magnetic stirrer until the solution became clear and homogeneous.

3.2.2 Preparation of chitosan/PVA blend

For the fabrication of chitosan/PVA composite fibers, predetermined amount of chitosan was firstly dissolved in 90% wt. acetic acid solution under magnetic stirring overnight at room temperature. At the same time, an aqueous solution of 10% wt. PVA was prepared at 80°C. Then, chitosan solution and PVA solution were blended together at predetermined blending ratio, under constant stirring by a magnetic stirrer, until homogeneous solution of Chitosan/PVA was obtained. The blending chitosan to PVA solution is shown in Table 3.1.

Table 3.1 The blending chitosan to PVA solution

Chitosan 100kDa		Chitosan 400kDa		Chitosan 760kDa	
CS content (%w/v)	PVA content (%w/v)	CS content (%w/v)	PVA content (%w/v)	CS content (%w/v)	PVA content (%w/v)
0	0.10	0	0.10	0	0.10
0.004	0.08	0.003	0.08	0.002	0.08
0.008	0.06	0.006	0.06	0.004	0.06
0.012	0.04	0.009	0.04	0.006	0.04
0.016	0.02	0.012	0.02	0.008	0.02
0.020	0	0.015	0	0.010	0

3.3 Electrospinning of the Prepared Solution

A typical electrospinning set up was consisted of a capillary through which the solution to be electrospun was forced; a high voltage source with positive polarity, which injects charge in to the solution; and a grounded collector (see Figure 2.2). Electrospinning of the prepared solution was done according to the procedure described in literatures. Briefly, the solution was placed into a 10 ml syringe with a

stainless steel needle (diameter of 25mm). Then, the electrical field with a potential of 25 kV across the distance of 10 cm between the tip of the needle and the collector plate was supplied to the solution, which consequently produced ultrathin fibers travelling from the needle to the collector.

3.4 Characterizations

3.4.1 Characterization of electrospinning solution

Two main factors have been reported to affect electrospinability of the solution, i.e, viscosity and conductivity of the solution. The viscosity of the electrospinning solution was measured by a Brookfield Viscometer (model DV-II+ Programmable) at the condition of shear rate equal to 1.00 rpm and the spinning time of 1 minute. On the other hand, the electrical conductivity of the solution was measured by conductivity meter (model LC116, Mettler Toledo Instruments, China).

3.4.2 Characterization of electrospun fibers

The morphology of the electrospun fibers was observed under a scanning electron microscope (JEOL model JSM-6301F and HITACHI model S3400). The diameter of the electrospun nanofibers was measured with SemAfore image analyzing program. For each experiment, average fiber diameter and size distribution were determined from the data of about 100 measurements of the randomly selected fibers.

3.5 Cell Attachment

3.5.1 Mineral medium

The mineral medium was used for screening, isolation and cultivation of bacterial cells. The mineral medium was comprised of media and trace element.

I. Media

The media was prepared from:

Na_2HPO_4	1.4196 g
KH_2PO_4	1.3609 g
$MgSO_4.7H_2O$	0.0985 g
$CaCl.H_2O$	0.0059 g

All components were dissolved in 1 liter of distilled water and adjusted pH to 7 by 1N NaOH. The mineral medium was autoclaved at 121°C for 15 minutes.

II. Trace element

H_3BO_3	0.116 g
$FeSO_4.7H_2O$	0.278 g
$ZnSO_4.7H_2O$	0.115 g
$MnSO_4H_2O$	0.169 g
$CuSO_4H_2O$	0.038 g
$CoCl_2.6H_2O$	0.024 g
MoO_3	0.010 g

Trace element solution was separately prepared from the media as a stock solution. All components were dissolved in 100 ml of distilled water. The solution was autoclaved at 121°C for 15 minutes. The sterile trace element was supplemented in mineral medium at the condition of 0.15(v/v).

III MMSAY

Stock I	4 ml
Distilled water	96 ml
Trace element(0.1%)	0.1 ml
Yeast(0.1%)	0.1 g
AmS(1mM)	0.1 ml
Sucinate(4mM)	0.1 ml

MMSAY medium was dissolved in 100 ml of distilled water and its was autoclaved at 121°C for 15 minutes.

IV. Luria Bertani medium (LB)

Trypton	10 g
Yeast Extract	5 g
NaCl	10 g

LB medium was dissolved in 1 liter of distilled water and adjusted to pH 7.0. Then it was autoclaved at 121°C for 15 minutes.

3.5.2 Starter preparation and inoculation

The streak plate method is a rapid and simple technique of mechanically diluting a relatively large concentration of microorganisms to a small, scattered population of cells. It isused to obtain isolated colonies on a large part of the agar surface, so that desired species can then be brought into pure culture. Proper streaking of plates is an indispensable tool in microbiology. In most cases, a closed inoculating loop is used for streaking plates. Streak plates are made from a broth culture, an agar slant or from an agar plate. A loopful of inoculum is transferred from the source and put on the agar surface. A small spot is spreaded during the initial transfer. The first phase of the streaking pattern is begun. Several basic pattern are shown in Figure 3.1. The three-phase streaking pattern is recommended for beginners because it is most likely to give satisfactory results with suspensions having a wide range of microbial density. Single drop from the loop as it was rubbed along the agar surface can be developed into separate colonies in the inocubator for 20 hours. *Acinetobacter baylyi* strain GFJ2 was incubated at room temperature while *Brevibacillus agri* strain 13 was incubated at 45°C.

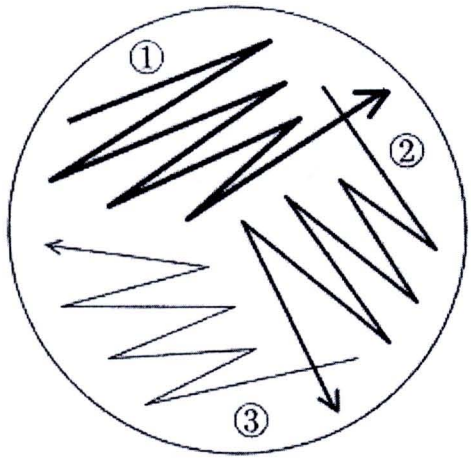

Figure 3.1 Streak plate pattern.

To inoculate a single colony onto broth medium, the sterilized inoculated loop was used to pick a single colony in the streak plate and place into the broth medium. The incubated medium was placed into an incubator shaker with 250 rpm operated at room temperature for 20 hours for *Acinetobacter baylyi* strain GFJ2 while *Brevibacillus agri* strain 13 was shaken at 45°C, 250 rpm for 20 hours. After that, bacteria grew in saturated manner in the broth medium. This is called a starter which was used as a bacterial stock.

3.5.3 Bacterial immobilization

Cell immobilization onto the electrospun chitosan nanofibers was tested by the following procedure. A blank aluminum foil was used as a control reference. All samples were sterilized under UV radiation prior to test with bacteria.

In this research, two strains of bacteria, i.e., *Brevibacillus agri* strain 13 and *Acinetobacter baylyi* strain GFJ2, were used to test the cell immobilization onto the electrospun chitosan nanofibers. In order to prepared the bacterial cells, 5 ml from broth medium were used for the cell proliferation in a 100 ml of medium (1% inoculum). The incubation was done for 20 h, under constant shaking at 250 rpm at 45°C for *Brevibacillus agri* strain 13 and at room temperature for *Acinetobacter*

baylyi strain GFJ2. After that, the medium was centrifuge at 5000 rpm for 15 minutes. The high density cells were placed into 20 ml of medium solution.

For the test of bacterial attachment, 100 µl of the bacterial cells solution was dropped onto 1.5x1.5 cm^2 piece of the testing sample in the agar plate. The agar plate was then taken into an incubator. The incubating temperature was 45°C for *Brevibacillus agri* strain 13 and room temperature for *Acinetobacter baylyi* strain GFJ2. After the predetermined incubation period, in the range of 0-48 h, the sample was taken out and rinsed with 0.5 ml (0.1 M, pH=7.04) phosphate buffer to remove unattached cells, twice. In order to determine the bacterial cells attached on chitosan nanofibers, the optical density of the initial bacterial cell solution and that of the washed solution was measured by spectrophotometer at 600 nm (OD_{600}). The Colony Forming Unit (CFU) was calculated from a standard calibration curve for *Acinetobacter baylyi* strain GFJ2 and *Brevibacillus agri* strain 13. Finally the sample was dried before subjected to the observation via SEM.

3.5.4 Bacterial viability

Conventional methods for examining cell viability and morphology utilize different fluorescent dyes to preferentially stain viable or dead cell. The LIVE/DEAD *Bac*Light Bacterial viability kit provides a novel two color fluorescence assay of bacterial viability by utilize mixtures SYTO9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain , propidium iodide to differentiate between cells with intact (viable) and compromised (dead or injured) membranes. The LIVE/DEAD *Bac*Light kit has been investigated extensively to easily, reliably and quantitatively distinguish live and dead bacteria analysis with a fluorescence microscopy [10]. It not only helps to monitoringcell viability and morphology but also proves reliable for both gram-positive and gram-negative bacteria.

Viability testing of cells attached onto the electrospun chitosan nanofibers tested by Live/Dead® *Baclight*™ *Bac*terial Viability Kit (model L7012). This kit is well suited for microscopic and quantitative analyses.

The *Bac*terial Viability kit consists of two nucleic acid stain, i.e., the green-fluorescence 3.34 mM SYTO9 dye solution in DMSO (Component A) and the red-fluorescence 20 mM Propidium iodide dye solution in DMSO (Component B). For preparation of the dye stock solution, two dye components were combined at the ratio between Component A and Component B of 1:1 in a microfuge tube. The dye mixture was added with 3μL for each mL of 1M NaCl, and mixed thoroughly.

For the test of bacterial viability, 50 μL of the dye solution was dropped onto 1.5x1.5 cm^2 piece of the testing sample in the LB plate. The sample was then incubated for 15 minutes. The incubating condition was room temperature in the dark for both *Brevibacillus agri* strain 13 and *Acinetobacter baylyi* strain GFJ2. After incubation period, the sample was taken out and observed in a fluorescence microscope (Olympus model BX-51). The fluorescence from both live (green fluorescence) and dead (red fluorescence) cells were detected using excitation wavelength of 470 nm. The emission for the green and red channel was detected at the wave length of 510-540 nm and 620-650 nm, respectively. For each experiment, the populations of both live bacteria and dead bacteria were determined from the data of about 3 points from the randomly selected area.