CHAPTER III

METHODOLOGY

3.1 Research overview

The research was divided into three phases including the optimal oil treatment condition of chitosan, the effectiveness of oil biodegradation by bacteria isolated from soils, and oil-in-water emulsion treatment by chitosan immobilized bacteria. The last phase was to compare the three types of oil-in-water emulsion treatment technique. Research flow chart was illustrated in Figure 3.1.

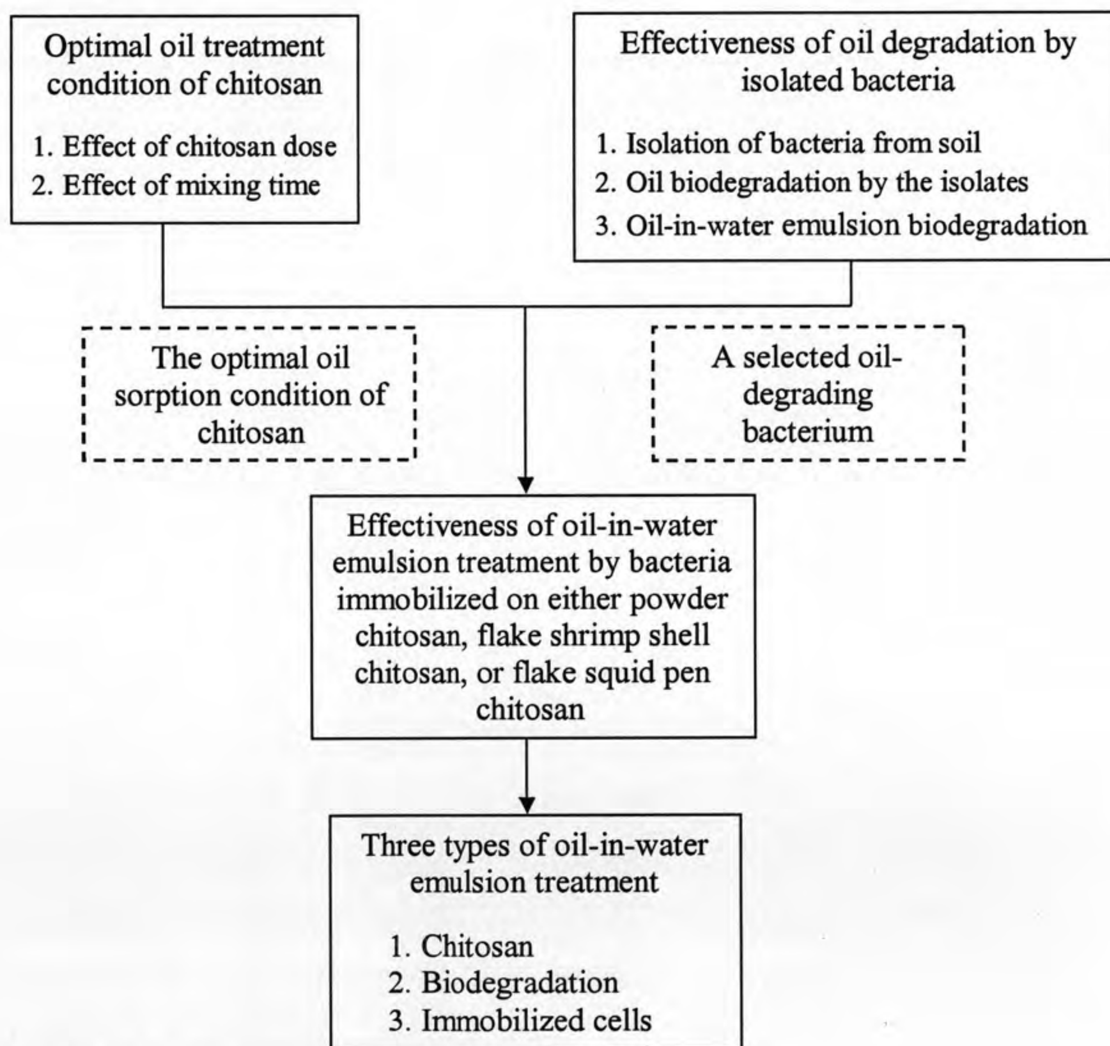


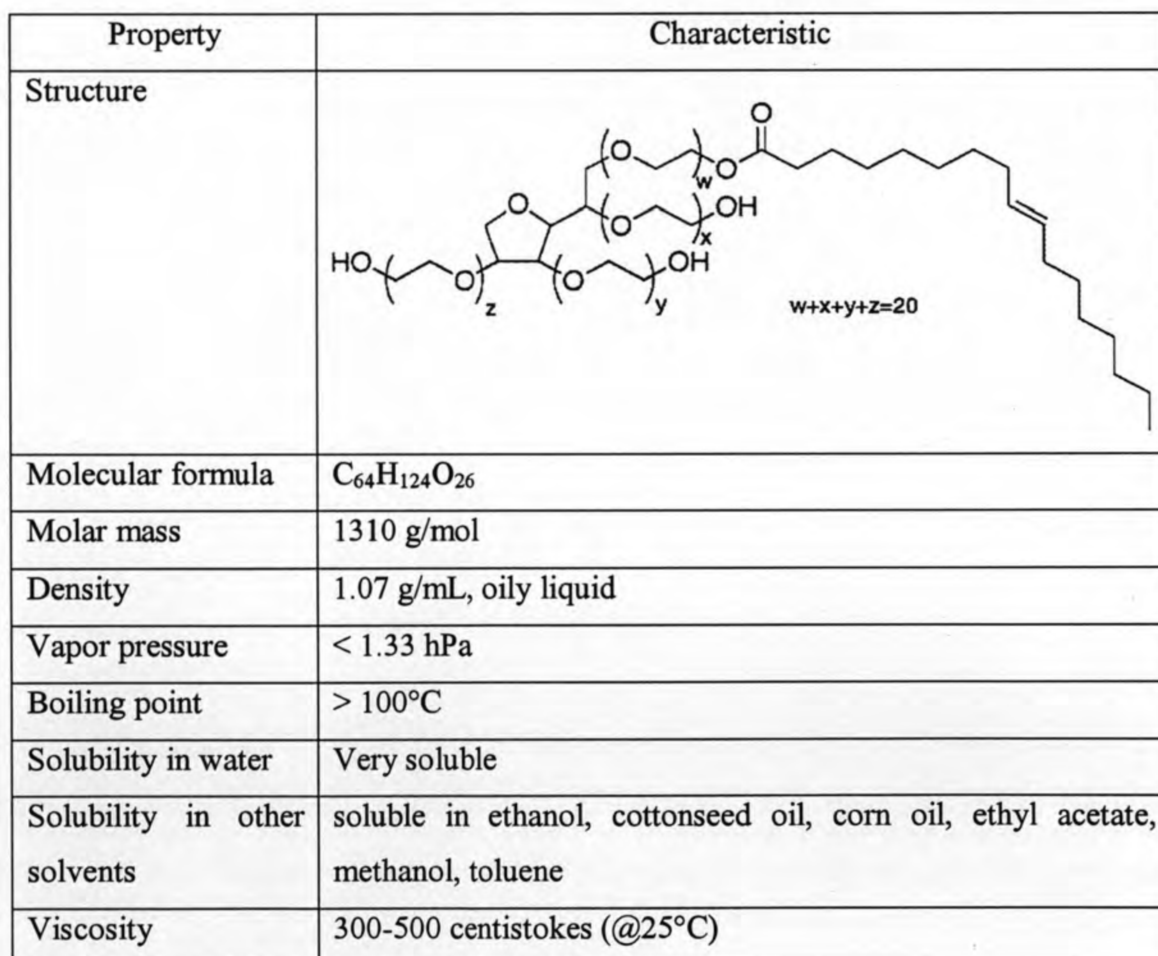
Figure 3.1 Flow chart of the research.

3.2 Materials

3.2.1 Chemicals

1. Tween80 was purchased from Merck Cooperation Ltd.
2. Chitosan powder grade was purchased from Aldrich Cooperation Ltd.
3. Flake shrimp shell chitosan and flake squid pen chitosan were obtained from Center for Chitin-Chitosan Biomaterials (CCB), Chulalongkorn University, and National Metal and Materials Technology Center (MTEC), Thailand, respectively.
4. Lubricant oil, PTT V-120 manufactured by Petroleum Authority of Thailand (PTT).

Table 3.1 Physical and chemical properties of tween80

Property	Characteristic
Structure	
Molecular formula	$C_{64}H_{124}O_{26}$
Molar mass	1310 g/mol
Density	1.07 g/mL, oily liquid
Vapor pressure	< 1.33 hPa
Boiling point	> 100°C
Solubility in water	Very soluble
Solubility in other solvents	soluble in ethanol, cottonseed oil, corn oil, ethyl acetate, methanol, toluene
Viscosity	300-500 centistokes (@25°C)

Source: Merck (2005), http://www.merck.co.th

Table 3.2 Specifications of sorbent materials

Sorbent	Shape	Granulometry (mesh)	Cost (baht/kg)	Degree of Deacetylation (%)
Chitosan from Merck Chemical	Powder grade	>60	56,000	75
Chitosan from shrimp shell	Coarse flake	<6	1000	>85
Chitosan from squid pen	Coarse flake	<6	1400	>85

3.2.2 Soil samples

Soil samples used in the research were collected from Chanthaburi province. The rich soil samples were collected from five areas that have different activities and have a lot of biodiversity. Thus, these soil samples have the possibility to provide the oil-degrading bacteria for this research. Sources of five soil samples were summarized in table 3.3.

Table 3.3 Sources of soil samples

Soil sample	Source of soil
1	Soil from Srabab Mountain
2	Soil from canal
3	Soil which has a mushroom grow on it
4	Soil under a mangosteen tree
5	Soil from a rubber plantation

3.2.3 Phosphate buffer solution

The buffered inorganic salts solution at pH 7.0 contained per 1 L deionized water: $MgSO_4.H_2O$ 0.2 g, KH_2PO_4 4.4g, $Na_2HPO_4.12H_20$ 18.1 g, NH_4CI 1.0 g. (Setti *et al.*, 1998).

3.2.4 Culture media

1. Carbon Free Mineral Medium (CFMM) was used to culture bacteria. The composition of CFMM was 0.2 g $MgSO_4$, 1 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, and 5 mL trace element in 1000 mL of distilled water. The composition of trace element is 24 g EDTA, 4 g NaOH, 2 g $MgSO_4.7H_2O$, 0.8 g $ZnSO_4.7\ H_2O$, 0.8 g $MnSO_4.4\ H_2O$, 0.2 g $CuSO_4.5\ H_2O$, 4 g $Fe_2(SO_4)_3.5H_2O$, 20 g Na_2SO_4, 0.2 g $Na_2MoO_4.2H_2O$, and 1 mL H_2SO_4 in 1 L distilled water. All the media and solution was autoclaved at 1 atm for 15 min.

2. LB (Luria-Bertani) was used as rich medium for determined the amount of total bacteria.

3.2.5 Equipments

1. Iatroscan^{TM} MK-616s, Mitsubushi Kagaku Iatron, INC., Tokyo, Japan
2. Rotary vacuum evaporator, EYELA, Japan
3. Oven, Contherm Scientific, New Zealand
4. Ultrasonicator FS4000, Decan Ultrasonics, England
5. Vortex mixer Gene 2, Scientific Industries, USA
6. Autoclave, Kakusan, Japan
7. Spectrophotometer UV-160A, Shimadzu, Japan
8. Hot air oven D06063, Memmert, Germany
9. Incubator 30 °C BE800, Memmert, Germany
10. Freezer -20 °C MDF-U332, Sanyo Electric, Japan
11. Freezer -70 °C ULT1786, Forma Scientific, USA
12. Blender SK-380, SKG Electric, Thailand

3.3 Procedure

3.3.1 Synthetic oil-in-water emulsion preparation

SKG Blender with 350 W, 13,000 rpm motor and maximum capacity of 1.5 L was used for preparing stock oil-in-water emulsion. One mL of Oil (PTT V-120) was injected into the blender and mixed with 800 mL distilled water and 100 mL 0.1% emulsifier (tween 80) for 1 min. The admixture was later stabilized by stirring for 10

min and diluted to 1 L with distilled water (Panpanit, 2001; Kloet *et al.*, 2001). This concentration of stock oil-in-water emulsion was equivalent to 994 mg/L with respect to the specific gravity of lubricant oil. This mixture was diluted to 200 mg/L, which represents an oil concentration in actual car wash wastewater (Panpanit, 2001). The oil-in-water emulsion remained stable during the experimental run.

3.3.2 Oil and water sorbent performance of chitosan

The test was made according to the procedure of ASTM F726-06 (2006) and Setti *et al.*, (1998). 300 mg dry chitosan was added to 25 mL phosphate buffer pH 7.0 in a 125 mL flask. After 1 h of shaking at 150 rpm, 50 μL lubricating oil was added and shaking immediately for another hour. The cycle was repeated until oil film seen to be formed on the free water surface at the end of a shake period. The purpose of this test was to determine the optimum oil sorbent capacity of the matrixes in the competing presence of water. Oil adsorbancy was defined as the ratio of oil adsorbed (Os) to dry adsorbent weight (So),

$$\text{Oil adsorbancy} = Os/So$$

The water sorbent capacity of the matrixes was carried out by weighing the damp sorbents (Sw) after the first shake period in the absence of crude oil. Water adsorbancy was calculated as a ratio of water adsorbed to dry sorbent weight (So) as follows,

$$\text{Water adsorbancy} = (Sw - So)/So$$

where Sw was the saturated adsorbent weight of water.

3.3.3 Oil sorption by chitosan

3.3.3.1 Effect of chitosan dosage

The synthetic oil-in-water emulsion was prepared as in 3.3.1 and diluted to 200 mg/mL. 50 mL of 200 mg/L oil-in-water emulsion was transferred into 250 mL

flasks and the different concentrations of chitosan between 0.1 to 10 g/l were added to each flask. The sample was mixed for 60 min at 200 rpm on orbital shaker and allowed to settle with sedimentation time of 60 min. Then, the residual oil in water was extracted and analyzed for the quantities of oil. The amount of emulsion in the sample after treatment was analyzed by measuring its turbidity (Bratskaya *et al.*, 2005).

3.3.3.2 Effect of mixing time

The effect of mixing time on the oil adsorption of chitosan was analyzed using the optimized chitosan dosage from the previous study, mixing rate of 200 rpm, and sedimentation time of 60 min. The mixing time was varied between 5 to 80 min. Then, the residual oil in water was extracted and analyzed for the quantities of oil. Oil-in-water emulsion after treatment was analyzed for the turbidity.

3.3.4 Oil degradation by isolated bacteria

3.3.4.1 Oil-degrading bacteria isolation

10 g of each soil samples was mixed with 100 mL CFMM media containing 5 mg/mL lubricating oil and shaken at 200 rpm, room temperature for 5 days. Cultures were enriched by transferring into new CFMM media when a change in lubricating oil was observed. The process was repeated several times. Bacterial isolates were purified by spreading on CFMM agar that has lubricating oil spread on surface (Mohamed, 2006). Bacterial isolates were re-streaked on LB agar in order to confirm the purity of isolates. If isolate gave only one characteristic colony, the isolate was confirmed to be purified.

3.3.4.2 Inoculums preparation

The bacterial isolates were cultured in CFMM agar that had lubricating oil spreading on agar surface for 5 days. Then, colonies were brought to 300 mL CFMM broth containing 5 mg/mL lubricating oil for 5 days. Cells were centrifuged at 8,000 rpm, 4°C for 10 minute and washed using 0.85% sodium chloride solution. To remove the remaining oil, the cells was resuspended in sodium chloride solution and incubated for

overnight at room temperature, 200 rpm. The cell suspensions were washed and adjusted the optical density (OD) to 1.0 at 600 nm.

3.3.4.3 Biodegradation test

30 mL cell suspensions from 3.3.4.2 were added to 125 mL flask containing lubricating oil with the final concentration of 25 mg/mL. The flask with only saline solution and oil was used as control and all flasks were done in triplicates. All flasks were shaken at 200 rpm, room temperature for 20 days. Then, the sample was analyzed for the amount of residual oil. The strain that provide highest activity was selected to determine the oil-in-water degradability over time.

Oil-degrading ability of the selected strain was examined with 200 mg/L oil-in-water emulsion. Each sample was collected and analyzed for the amount of residual oil in water at 0, 1, 4, 12, and 24 h. Number of bacteria during the experiment was also determined by total plate count to determine the relationship between oil removal and bacteria survival.

3.3.5 Oil-in-water emulsion treatment by chitosan-immobilized bacteria

3.3.5.1 Production of immobilized cells

To immobilize the selected oil-degrading bacteria on chitosan, the bacteria was cultured together with chitosan in CFMM with lubricating oil as the sole source of carbon and energy. Oil-degrading bacteria cells suspension was prepared as in 3.3.4.2. Then, 1.25 g sterilized chitosan was incubated with 500 mL cell suspensions in 1,000 mL flask with 0.25% (v/v) of oil (Gentili, 2006). The suspension was shaken at 150 rpm and room temperature for 5 days. Subsequently, the immobilized cultures was washed with mineral salt (MS) medium and filtrated through sterilized filter paper Whatman No.4 for removing unattached cells. A filtrated culture material was air dried in sterile hood for 4 hours.

3.3.5.2 Oil-in-water emulsion biodegradation by immobilized cells

Three forms of chitosan used for the immobilization of Ch2 bacteria were powder chitosan, flake shrimp shell chitosan, and flake squid pen chitosan. The

chitosan-immobilized cells were later tested with oil-in-water emulsion in order to know which chitosan form was the suitable media for immobilization and provided the highest oil-in-water emulsion removal efficiency. Each dried immobilized cells were transferred to 250 mL sterile flask containing 50 mL of 200 mg/L oil-in-water emulsion. The initial concentration of immobilized cells was 1.0 g/L. To determine the effectiveness of chitosan-immobilized cells for a long term application, 200 mg/L lubricating oil was further added daily to the oil-in-water emulsion until finish the study. After finish the treatment, samples of chitosan immobilized with bacteria were taken from the flasks to analyze for the number of attached bacteria. The amount of residual oil in water phase was also analyzed.

At the end of experiment, three forms of oil-in-water emulsion treatment were examined (Figure 3.2): (I) sorption by chitosan, (II) degradation by the isolated bacteria, and (III) sorption and degradation by chitosan-immobilized cells. Then, the residual oil in water was determined to compare the removal efficiency of each treatment.

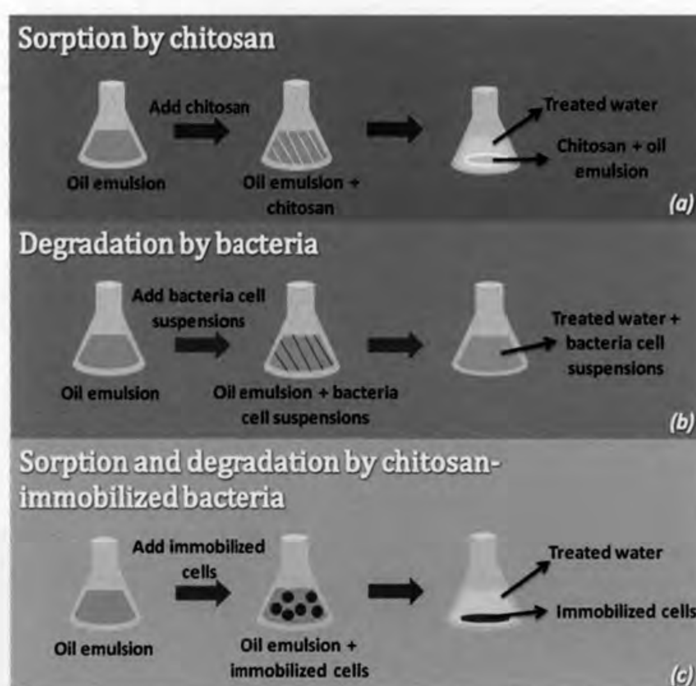


Figure 3.2 Brief procedure of oil-in-water emulsion removal by three treatment types: (a) sorption by chitosan, (b) degradation by the isolated bacteria, and (c) sorption and degradation by chitosan-immobilized cells.

3.4 Analytical methods

3.4.1 Quantitative analysis of oil-in-water emulsion

Amount of oil residual in water was extracted by chloroform and quantified by Thin Layer Chromatography with flame ionized detector (TLC-FID). To ensure complete extraction of oil to chloroform phase, NaCl was added to the sample after treatment at the final concentration of 0.25 M (Rajesh *et al.*, 2005). The mixture was then mixed with chloroform at the ratio equal to 1:5 and intensively shaken for 30 min. The extraction was repeated twice. Stearyl alcohol was used as internal standard by adding 2 mL of 6.25 mg/mL stearyl alcohol to the sample. The chloroform phase was transferred into an evaporation bottle. Then, the solvent was evaporated by an evaporator until the volume was around 1 mL. The extract was applied to Chromarods and developed in solvents of increasing polarity to separate saturated and aromatic hydrocarbon from resin/asphaltenes (Maruyama *et al.*, 2003). The chromarod development conditions were *n*-hexane for 10 cm (25 min), dichloromethane (DCM) for 6.5 cm (2 min) and 4 cm (5 min), and DCM/methanol (95/5, v/v) for 1 cm (1 min). Finally, the amount of oil on the Chromarods was quantified using the FID of the Iatroscan with scan speed equal to 30s/scan (normal scan). Flow rate of hydrogen for the FID were 160 mL/min. Retention time of stearyl alcohol, saturates, aromatics, resin, and asphaltenes were approximately 0.35, 0.13, 0.24, 0.42, and 0.47 min, respectively.

3.4.2 Quantitative analysis of oil adsorbed in chitosan

Amount of oil adsorbed in chitosan was quantified by Thin Layer Chromatography with flame ionized detector (TLC-FID). Chitosan after treatment was mixed with chloroform at chloroform/chitosan phase ratio equal to 1:1 and intensively vortex to ensure complete extraction of oil to chloroform phase. Stearyl alcohol 12.5 mg was added as internal standard. The chloroform phase was separated and evaporated by an evaporator until the volume was around 1 mL. The extract was applied to Chromarods and then developed as same as 3.4.1.

3.4.3 Turbidity of oil-in-water emulsion

Turbidity was measured via the optical density (OD) of oil-in-water emulsion at 500 nm using Lambda 800 UV-VIS Spectrometer (Perkin-Elmer). OD_{500} of untreated emulsion exposed to the same condition but without chitosan addition was measured as control (Bratskaya *et al.*, 2005). Blank was the distilled water.

3.4.4 Determination of the amount of lubricating oil-degrading bacteria and total bacteria

Plate count technique was used for determining the amount of oil-degrading bacteria and total bacteria. The 100 μl of ten-fold serial dilution of cell suspensions was placed on CFMM agar containing lubricating oil to determine amount of oil-degrader and on LB agar to determine the amount of total bacteria. The number of bacteria per mL (CFUs) of culture was calculated.

3.4.5 Determination of the number of attached viable bacteria

To assess the number of attached viable bacteria, the samples of chitosan immobilized bacteria was taken from the flasks and analyzed according to Gentili *et al.* (2006). Briefly, the sample was washed with CFMM medium and filtrated through sterilized filter paper. A filtrated culture material was air dried in laminar flow cabinet for 4 hours. Then, 0.01 g dried immobilized cells of each sample were enumerated by resuspending in CFMM 9.99 mL and leave to rehydrate for 2 min. The suspensions was sonicated for another 2 min and shaken vigorously on a vortex mixer for 3 min. The process was repeated twice. The suspension was centrifuged and the pellet was resuspended. Aliquots containing 10 μL was plated on LB agar plate and incubated at room temperature for 1 week. Then, bacteria colonies were counted and the viable count was referred to the dry weight of chitosan (Gentili *et al.*, 2006).

3.4.6 Standard curve of lubricating oil

The standard curve of lubricant oil was prepared by adding lubricant oil into 5 mL volumetric flask. Chloroform was used as the solvent to dissolve lubricating oil. Stearyl alcohol was used as internal standard by adding 1 mL of 25mg/mL of stearyl alcohol to the standard lubricant oil solution. Concentrations of standard lubricant were 1, 5, 15, 25, and 30 mg/mL. The standard lubricant oil solution was analyzed by TLC-FID technique as same as 3.4.1. The standard curve was plotted between ratio of area (lubricant oil/stearyl alcohol) and ratio of concentration (lubricant oil/stearyl alcohol).

3.4.7 Scanning electron microscope (SEM) analysis

SEM photograph of powder, flake shrimp shell, and flake squid pen chitosan before and after immobilized were analyzed at Scientific and Technological Research Equipment Centre (STREC), Chulalongkorn University, Thailand. Firstly, samples were soaked in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h. Secondly, samples were washed by phosphate buffer and distilled water for 10 min 2 times. Thirdly, samples were dehydrated by ethanol 30%, 50%, 70%, 90%, and 100% for 10 min 3 times, respectively. Fourthly, samples were dried by critical point dryer. Finally, all of dried chitosan samples were coated with gold (Au) before SEM analysis.