CHAPTER III

MATERIALS AND METHODS

3.1 SOURCES OF MICROORGANISMS

3.1.1 Samples

Thirty-four samples were collected during January 1997 to April 1998. They were soil, sediment, sludge, canal water and black muddy water, etc, and were collected from industry plant, sewage treatment plant and other sites. Total isolate bacteria were obtained 346 strains. The details of all samples were shown in **Appendix A.**

3.1.2 Bacterial References Strains

The bacterial references strains were obtained from MIRCEN (Microbiological Resource Center), Thailand, namely, ***Pseudomonas aeruginosa*** (TISTR 0781), ***Escherichcr coli*** (TISTR 0780. ATCC 8739). They were used for confirmation of identification of selected bacterial strains.

3.2 CHEMICALS, REAGENTS AND INSTRUMENT

3.2.1 Chemicals and Reagents

All chemicals used in this study were analytical grades, e.g., $CdCl_2$ H_2O (Merck, Darmstadt, Germany), $CuSO_4$ $7H_2O$ (Merck), $ZnSO_4$ $7H_2O$ (Merck), $MnCl_2$ $4H_2O$, (M&B, Dagenham, England), $AgNO_3$, (Merck), $K_2Cr_2O_7$, (Merck), $NiSO_4$ $6H_2O$, (M&B), Hydrochloric acid (Merck), Sodium hydroxide, Acetone, (HPLC grade, Genzyme, Suffolk,

England), Glucose (Merck), Bovine Serum Albumin (BSA; Sigma, USA), Concentration Sulfuric acid, BDH (Poole, England), Sodium alginate (Carlo Erba, MI, USA), Calcium chloride (Merck), Cd Standard solution (Merck), Cu Standard solution (Merck), Mn Standard solution (Merck) dyes and reagent of Gram's stain, Folin-Ciocalteau (Lowry's reagent), Indian ink for capsule stain and Phenol reagent

3.2.2 Instrument

Incubator (Memmert GmbH, Model 700, Schwabach Germany), Oven (Memmert, GmbH, Model 700, Schwabach, Germany) Shaker Incubator (Lab-Line Instrument, Melrose Park, Illinois, USA), Autoclave (Labo Autoclave, Sanyo Electric, Japan, grateful thanks to Viriya Insurance, Co., Thailand, for donation of two sets to the Department of General Science, Faculty of Science, Chulalongkorn University), Microwave (Sanyo Electric, Japan, grateful thanks to Thanes, Co., Thailand, for donation to the Department of General Science), pH meter (Model pH scan 1, Singapore), Spectrophotometer (Spectronic 20 genesys, USA), Centrifuge (Hettich EBA 8S, Germany), High Speed Refrigerated Centrifuge (ALC Model 4293R, Italy), Atomic Absorption Spectrophotometer (Smith-Hiiefje 4000 series, USA). Refrigerator (Samsung, Korea, grateful thanks to Becthai, Co., Thailand for donation to the Department of General Science.

3.3 MEDIA*

3.3.1 Culture Media

Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, Michigan, USA) was the semi-solid medium that was used for isolated purify bacteria and maintained stock culture. Fluid growth medium was Tryptic Soy Broth (TSB) for study of growth rate and EPS production.

3.3.2 Selective Media

Triple Sugar Iron agar (TSI; Delost, 1997), MacConkey agar (MCA; Delost, 1997), Simmons citrate agar (SCA; Delost, 1997), Salmonella-Shigella agar (SS;Delost,1997), MacConkey-Inositol-Potassium Tellurite (MCIK; Tomas,Ciurana and Jofre, 1986), Pseudomonas Selective Isolation Agar (PSIA; Krueger and Sheikh, 1987), Motility test medium (Delost, 1997), MR-VP broth (Delost, 1997).

3.3.3 Medium for Metal Resistance Test

Minimal medium (Laddaga and Silver, 1985; Higham and Scawen, 1985; Silhavy and others, 1984) was used for heavy metal resistance test such as $CdCl_2.H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $AgNO_3$, $NiSO_4.H_2O$ and K_2CrO_4.

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* Formulas and preparation of all media are shown in **Appendix B.**

3.4 ANALYSES OF SOME HEAVY METALS

3.4.1 Cadmium

Standard solution have 1000 milligram/liter (mg/L) Cd concentration that were diluted to a concentration range approximately 0-2 mg/L for linear standard curve at absorbance 228.8 nanometer (nm) by flame atomic absorption. Also, all sample were diluted to the same concentration range (Rand, Greenberg and Taras, 1976).

3.4.2 Copper

Standard solution have 1000 milligram/liter (mg/L) Cd concentration that were diluted to a concentration range approximately 0-4 mg/L for linear standard curve at absorbance 324.7 nm by flame atomic absorption. Also, all samples were diluted to the same concentration range (Rand, Greenberg and Taras, 1976).

3.4.3 Manganese

Standard solution have 1000 milligram/liter (mg/L) Cd concentration that were diluted to a concentration range approximately 0-3 mg/L for linear standard curve at absorbance 279.5 nm by flame atomic absorption. Also, all samples were diluted to the same concentration range (Rand, Greenberg and Taras, 1976).

3.5 STAIN AND BIOCHEMICAL TEST

Gram's stain and indian ink were used for identification, shape, and EPS production of selected bacterial strains. After, some biochemical test were done such as MCA, TSI, SCA, SS, MCIK, PSIA and Motility test medium.

3.6 SAMPLING AND CULTIVATION PROCEDURES

3.6.1 Sampling Procedure

Sample of soils, waters, mud and sediments in the sterile bottle were collected from the polluted and non-polluted areas. Those samples were preserved at low temperature (4 °C) in the icebox for transport and then stored in the refrigerator until the screening and selection of Cd-resistance bacterial strains were performed.

3.6.2 Isolation of Cd- resistance Bacteria

Serial dilution of samples were spread both on TSA mixed with 100 µg/ml cadmium and TSA, duplicate. Count of colony (CFU/ml) were read after 24 hour incubate at 37 °C. Colony bacteria were purified by streak again on the same medium until single colony.

3.6.3 Cadmium Resistance Test

The agar dilution method with a multiple inoculation system was chosen for the determination of high concentration resistance to heavy metals (Nieto, Fernandez-Castillo, Marquez, Ventosa, Quesada and Ruiz-Berraquero, 1989; Koneman, Allen, Dowell and Sommers, 1984). A loopful amount of bacterial was inoculated into TSB 4 ml. After 24 hr incubation at 37 °C, 0.2 ml of culture was transferred into 2 ml TSB incubate 4 h. After, each culture containing 0.5-3.2x10^6 organisms from exponentially growing was transferred to multi-inoculation plate and sterile multi-inoculator (with 21 spots) was immersed and placed on surface of minimal medium (control) and minimal medium with different concentration cadmium, perform in at least triple. All minimal medium plate were incubated for 18-24 hr at 37 °C, the result were transferred by

make score compare with control (free cadmium); +4=good growth, +3=pass,+2=almost pass, +1=poor, 0=dead cell.

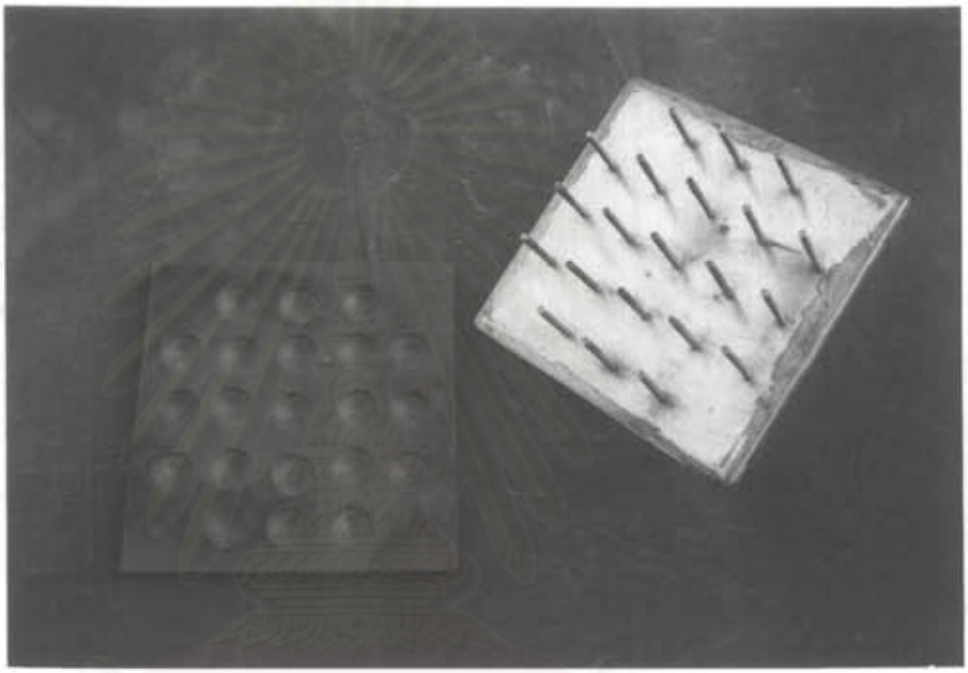

Figure 3.1 Multi-inoculator 21 spots.

3.6.4 Identification of Selected Bacteria.

3.6.4.1 Biochemical Test and Selective Medium.

First, 5 strains were cultured on selective medium, MacConkey agar and TSI. All strain were gram negative because they can growth on MacConkey agar and observed from microscopy. Following oxidase and catalase and other biochemical test were proceeded of sequence in **Figure 3.2**.

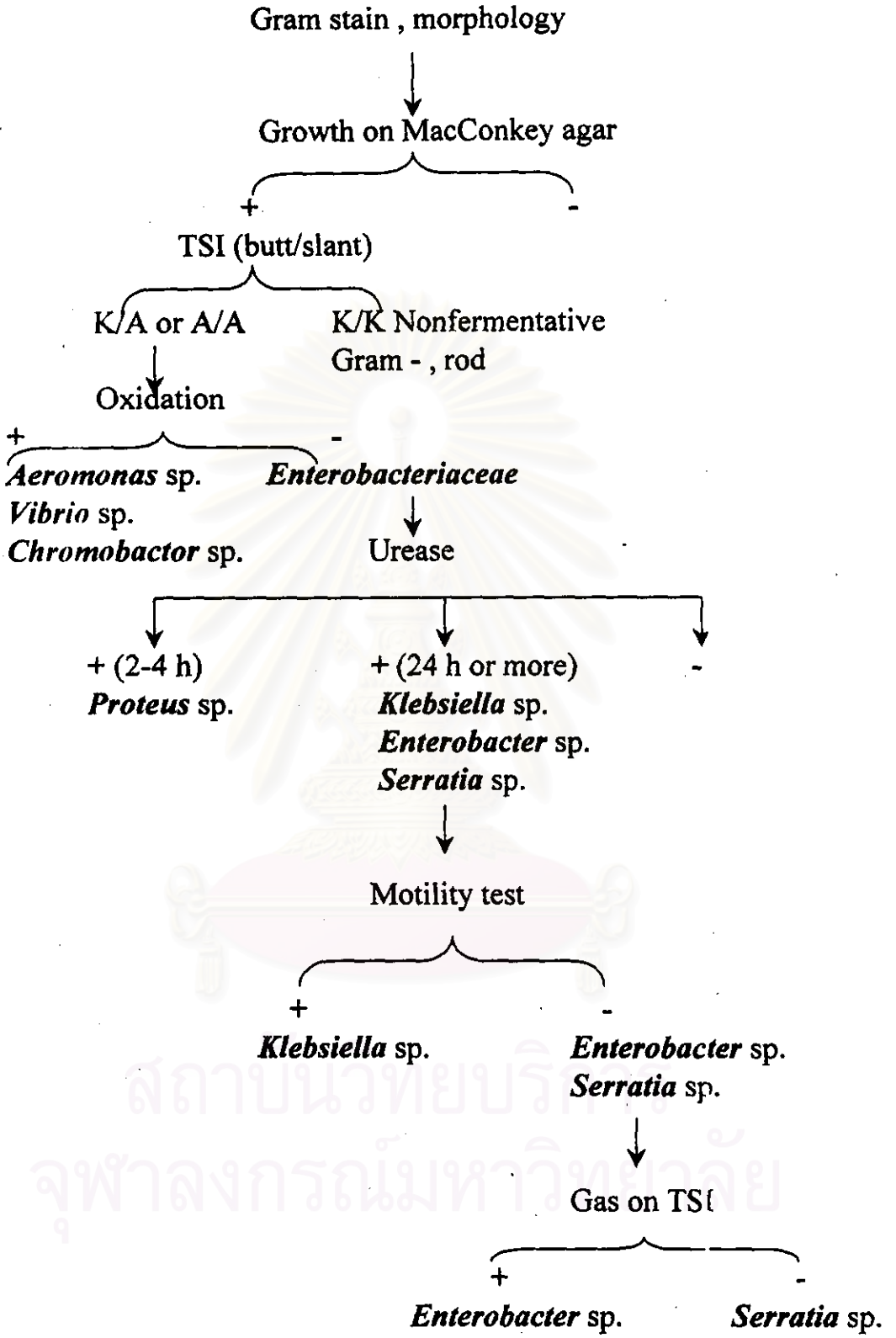

Figure 3.2 Sequence of bacteria identification by some biochemical test (Delost, 1997; Orskov and Orskov, 1982 ; Domingue, 1983).

3.6.4.2 EPS-Producing Bacteria

For capsule production, cadmium-resistance bacterial strains were stained by indian ink and viscosity of the culture broth were also observed (Margaritis and Pace, 1985; Norberg and Enfors, 1982).

3.7 EFFECTS OF SOME GROWTH FACTORS ON CADMIUM-RESISTANCE BACTERIAL ISOLATES.

3.7.1 Effect of pH

A loopful was inoculated into TSB 4 ml, at 37 °C for 18-24 hr. The 2 ml culture were transferred into 250 ml erlenmeyer flask containing 50 ml of TSB (adjusted pH 2-12) and incubate at 37°C, shake 120 rpm, sample 5 ml were collected to determine growth rate when hour at 12 (using spectrophotometer at A600 nm). Relation between time of growth versus absorbance was plotted for determination growth rate.

3.7.2 Effect of Temperature

The experiment was conducted in the same manner as in 3.7.1. except temperature of incubate culture was changed to 30,37, 40, 45, and 50 °C.

3.8 EXTRACTION OF EXOPOLYSACCHARIDE.

Random bacterial strain number 205 was cultured into TSB 4 ml, at 37°C for 18-24 hr. After, 2ml cultures were transferred to 500 ml erlenmeyer flask containing 200 ml TSB , shake 120 rpm, 24 hr. Then they were centrifuged at high speed for EPS extraction. The high speed was varied to 10000 g, 15000 g, 20000 g, 25000g and 30000g at 4 °C, 15 minutes. The supernatant obtained were added with four volumes of

ice-acetone to precipitate the EPS. The pellet obtained was dried in oven at 105 °C, 4 hr for define proportion with amount of EPS . The complete precipitation may take up to 18-24 hr at 4 °C. A precipitate is recovered by low-speed centrifugation. The characteristic of EPS was shown in **Figure 3.3** and dried in oven at 105 °C, 4 hr. (Brown and Lester, 1980;Boyle and Reade, 1983;Congregado and others, 1985; Lambert, 1988; Kim and others,1994; Williams and Wimpenny, 1977; Bonet, Simon-Pujol and Congregado, 1993).

Analysis of quantitative and qualitative of EPS by

(i) The amount of EPS were determined by weight.

(ii)The total carbohydrate contents were determined by the phenol-sulfuric acid method (Dubois and others, 1956). (detail has shown in **Appendix C**)

(iii) Protein contents were determined by method of Lowry and others (1951). For measures the degree of cellular disruption (see **Appendix D**).

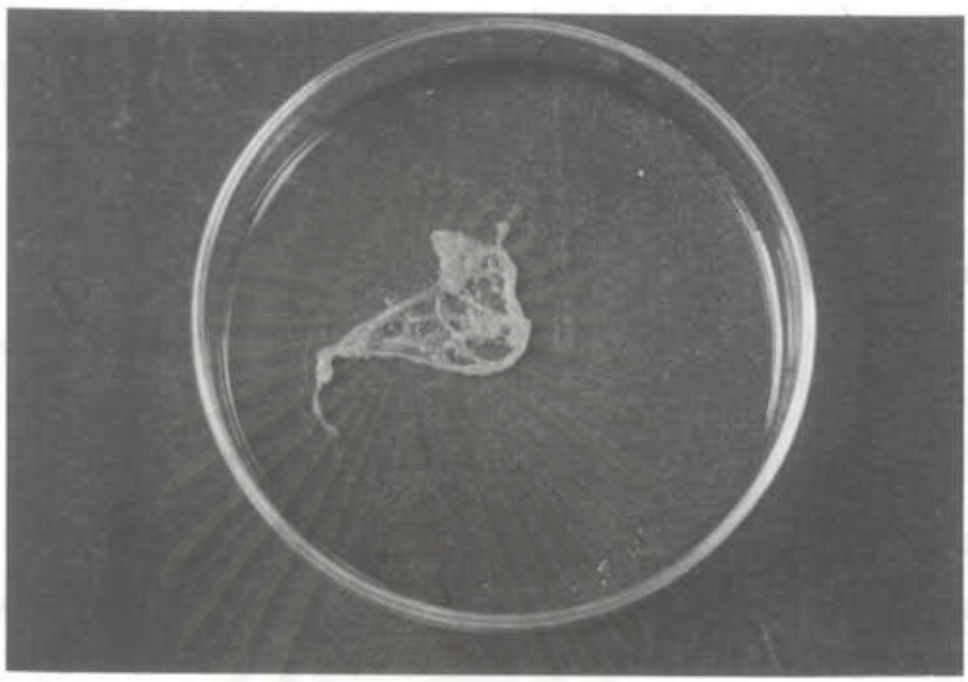

Figure 3.3 The characteristic of EPS.

3.9 OPTIMUM CONDITION FOR EPS PRODUCTION

For EPS production, one loopful of the culture of bacterial strains were incubated into a test tube containing 4 ml of TSB medium, at 37 °C for overnight. After, 2 ml of cultures were transferred to 500 ml erlenmeyer flask containing 200 ml of same medium. The factor were varied follow as:

3.9.1 Effect of initial pH

Initial pH of TSB were adjusted to 5,6,7,8 and 9 before incubate of culture. When culture age was 24 hr, the amount of EPS were detected, duplicates.

3.9.2 Effect of temperature

The experiment was conducted in the same manner, except temperature of incubate culture was changed to 30,37, 40,45 and 50 °C, duplicates

3.9.3 Effect of incubation period

10 ml of cultures were collected to check the amount of EPS at incubate time 24,48 and 72 hours, duplicates.

3.9.4 Effect of initial cadmium concentration

Vary initial cadmium concentration in TSB medium were made 0,10,20, and 100 ml/l and quantitative of EPS were detected at incubate 24 hr.

All of factor on EPS production, EPS were extracted by high speed centrifugation method at 20000 g.

3.10 ACCUMULATION OF CADMIUM AND OTHER METALS

3.10.1 Effect of Initial Cd Concentration to Cd Adsorption by Viable Cells.

All culture were growth for 24 hr in 100 ml of TSB at 37 °C on rotary shaker 120 rpm. The amount of equal viable cells were harvested by centrifugation at 6000 g for 15 min at 4 °C. The cells were added with vary Cd concentration to 10, 50 and 100 mg/l per mg dry weight cells. Uptake equilibrium was achieved within 5-30 min. The cells were removed by centrifugation at 6000 g for 15 min and determination of residual Cd level in supernatant by Atomic Absorption Spectrophotometer (AAS) analysis.

3.10.2 Cadmium

The viable cells, dead cells, and crude-extracted EPS, that were obtained from nearly the amount of cells were added with 10 mg/l $CdCl_2$ per 10 mg dry cells. Later 30 min, the cells were separated by centrifugation at 6000 g 4 °C 15 min and determination of residual Cd level in supernatant by AAS analysis.

3.10.3 Copper

The experiment was conducted in the same manner as in 3.10.2 , except 10 mg/l $CuSO_4$ .Solution was instead of 10 mg/l $CdCl_2$ solution.

3.10.4 Manganese

The experiment was conducted in the same manner as in 3.10.2 , except 10 mg/l $MnSO_4$ .Solution was instead of 10 mg/l $CdCl_2$ solution.

3.11 IMMOBILIZATION OF THE SELECTED BACTERIAL STRAINS.

Randomly, strain of bacteria number 205 and 273 were cultured overnight in 250 ml TSB medium at 37 °C, 120 rpm. The cultures were harvested by centrifugation at 6000 g for 15 min and the pellet was suspended in 20 ml of deionized H_2O (dH_2O). The resuspended cells were added to 125 ml of 2% high viscosity sodium alginate in dH_2O and mixed. The control was 20 ml dH_2O without cells. The cells were immobilized by forcing the cell-alginate mixture through needle into 125 ml of 1.47 % $CaCl_2$ solution. The beads formed

(**Figure 3.4**) were allowed to harden for 2 hr, stabilized with 250 ml of 1% polyethyleneimine (PEI), pH 5.0 in dH_2O, for 5 min and washed with 3 L dH_2O. After stabilization the immobilized cells were placed into 10,50, and 100 mg/l $CdCl_2$ solutions and at the first time, 2 ml of supernatant were separately detected the Cd remain between 0-30 min at room temperature. To prove decrease metal adsorption efficiency, the beads were treated with 0.48% nitrilotriacetic acid , pH 6.0, to recover adsorbed Cd. Following the beads washed, and used for recover adsorbed Cd again. This method was repeated 3 times.

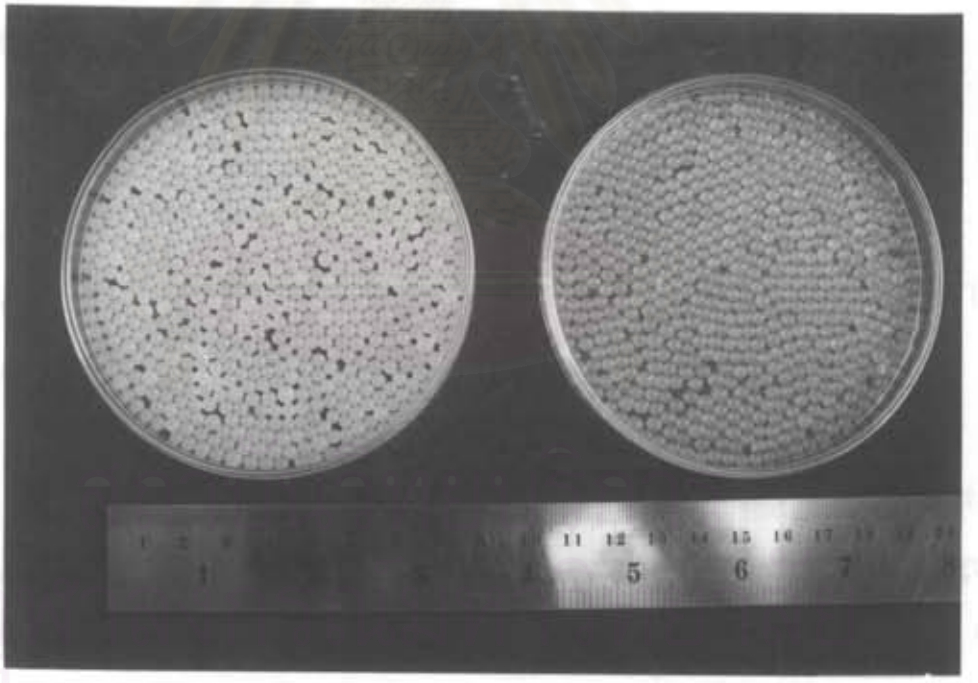

Figure 3.4 The characteristic of calcium alginate beads with cells(left), and without cells(right).