CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Media

- NIH Thioglycollate Broth, purchased from Difco Laboratories, U.S.A.
- Nutrient Agar (NA), purchased from Difco Laboratories, U.S.A.
- Nutrient Broth (NB), purchased from Difco Laboratories, U.S.A.
- Tryptic Soy Broth (TSB), purchased from Difco Laboratories, U.S.A.

3.1.2 Chemicals

- 3, 5-Dinitrosalicylic acid, purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
- Albumin from bovine serum, minimum 98% electrophoresis, purchased from Sigma-Aldrich Co., Inc., Singapore.
- Ammonium oxalate, purchased from Ajax Chemicals, Australia.
- Calcium carbonate, ($CaCO_3$), purchased from Merck KGaA, Germany.
- Carboxymethyl cellulose, (CMC), purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
- Cellobiose powder, purchased from Sigma-Aldrich Co., Inc., Singapore.
- Citric acid monohydrate, ($C_6H_8O_7.H_2O$), purchased from UNIVAR, Australia.
- Congo red dye, purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.

- Copper sulphate pentahydrate, ($CuSO_4.5H_2O$), purchased from Ajax Finechem PTY Ltd., Australia.
- Crystal violet dye, purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
- Dipotassium hydrogenphosphate, (K_2HPO_4), purchased from Merck KGaA, Germany.
- Disodium Carbonate, (Na_2CO_3), purchased from Merck KGaA, Germany.
- Ethanol absolute, (C_2H_5OH), Analytical grade, purchased from Scharlau Chemie S.A., Spain.
- Ethylenediamine tetraacetic acid, (EDTA), purchased from Sigma-Aldrich Co., Inc., Singapore.
- Folin-Ciocalteu's phenol reagent, purchased from Merck KGaA, Germany.
- Glacial hydrochloric acid, (HCl), Analytical grade, purchased from Merck KGaA, Germany.
- Glucose, ($C_6H_{12}O_6$), purchased from Merck KGaA, Germany.
- Glycerol, Analytical grade, purchased from Merck KGaA, Germany.
- Hydrogen peroxide, (H_2O_2), purchased from Merck KGaA, Germany.
- Iodine crystal, purchased from Merck KGaA, Germany.
- Iron (II) sulfate heptahydrate, ($FeSO_4.7H_2O$), purchased from Merck KGaA, Germany.
- Lysozyme, purchased from Sigma-Aldrich Co., Inc., Singapore.
- Magnesium sulphate heptahydrate, ($MgSO_4.7H_2O$), purchased from Merck KGaA, Germany.
- Malachite green powder, purchased from Merck KGaA, Germany.
- Malt Extract, purchased from Lab Scan Analytical Sciences, Thailand.
- Peptone, purchased from Difco Laboratories, U.S.A.

- Potassium chloride, (KCl), purchased from Merck KGaA, Germany.
- Potassium iodide, (KI), purchased from Merck KGaA, Germany.
- Potassium sodium tartrate tetrahydrate, ($C_4H_4KNaO_6$. $4H_2O$), purchased from Merck KGaA, Germany.
- Potassium tartrate, ($C_4H_4K_2O_6$. $4H_2O$), purchased from Merck KGaA, Germany.
- Proteinase K, purchased from Fermentas International Inc., Canada.
- Safranin powder, purchased from Merck KGaA, Germany.
- Sodium chloride, (NaCl), Analytical grade, purchased from Merck KGaA, Germany.
- Sodium dodexyl sulfate, (SDS), purchased from Fluka, Sigma-Aldrich Co., Inc.,Singapore.
- Sodium hydroxide, (NaOH), purchased from Merck KGaA, Germany.
- Trizma base, minimum 99.9% titration, purchased from Sigma-Aldrich Co., Inc., Singapore.
- Whatman filter Paper No.1, purchased from Whatman, Germany.
- Yeast extract, purchased from Bio Springer, France.

3.1.3 Identification Kits

- API 20 E, purchased from Biomérieux, France.
- API 50 CHB, purchased from Biomérieux, France.
- Genome DNA Simax Kit, purchased from Beijing SBS Genetech Co., Ltd., China.

3.2 Instrument and Apparatus

- Autoclave: Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan.
- Cold room: Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K.
- 4-Digit precision weighting balance: Model AG 204, Mettler Toledo, Switzerland.
- DNA thermo cycler TP 600: TaKaRa Bio Inc., Otsu, Shiga, Japan.
- Electrophoresis chamber set: Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland.
- Freezer: Sharp, model FC27 (-20°C), Japan.
- Gel documentation system: Bio-Rad Laboratories Gel Doc TM XR, California, U.S.A.
- High speed refrigerated centrifuge: Beckman Coulter TM Avanti J-30I, Palo Alto, California, U.S.A.
- Hot air oven: Model UC 30, Memmert GmbH and Co. KG., Western Germany.
- Hot plate stirrer: Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand.
- Incubator: Model 800, Memmert GmbH and Co. KG., Western Germany.
- Incubator shaker: Model SK-737, Amerex Instruments, Inc., U.S.A.
- Kubota refrigerated microcentrifuge 6500: Kubota Corporation, Tokyo, Japan.
- Laminar flow 'clean': Model V6, Lab Service Ltd., Thailand.
- Microscope: Model CH 30RF200, Olympus Optical Co., Ltd., Japan.
- Orbital shaker: Innova Model, New Brunswick Co., Inc., U.S.A.
- Pipetteman: Gilson, France.
- pH meter: Mettler-Toledo International Inc., New York, U.S.A.

- Spectrophotometer: Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, U.S.A.
- Stomacher: Masticator, BEC-Thai, Thailand.
- Vortex mixer: Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, U.S.A.
- Water bath: Model WB14, Memmert GmbH and Co. KG., Western Germany.

3.3 Methodology

3.3.1 Termite Sample Collection

Lower termites and higher termites were obtained from Nakhon Pathom province. Random sampling of lower termites from an anthill and higher termites from a coconut wood were collected as a source of cellulose-degrading bacteria.

3.3.2 Nomenclature and Classification of Termites

Lower and higher termites were fixed in 95 vol.% ethanol in order to be identified by Dr. Duangkhae Sitthicharoenchai, Department of Biology, Faculty of Science, Chulalongkorn University.

3.3.3 Screening of Cellulose-Degrading Bacteria

3.3.3.1 Isolation of Cellulase-Producing Bacteria from Lower Termites

a) Isolation of cellulase-producing bacteria from dead termites

Schedorhinotermes sp., lower termite, were collected from anthill from Nakhon Pathom province. These termites died before the experiment because they lacked of water. Ten termites were surface-sterilized in 70 vol.% ethanol and then washed in normal saline solution. The termites were dropped in a stomacher bag, which contained 9 ml normal saline solution, and then homogenized by using a stomacher.

The termite-containing bag was enriched by incubating at the ambient condition for 4 hours, and then 0.5 ml of solution from the termite-containing bag was transferred to a test tube that contained 10 ml of carboxymethyl cellulose broth medium, CMC broth, and incubated at 37°C for 1 day. 0.5 ml of culture was transferred to fresh CMC broth and incubated at the same above conditions for six times.

The enriched cultures and their dilutions were spread on carboxymethyl cellulose agar medium, CMC agar, and incubated at 37°C for 1 day. After that, isolated colony was repetitively streaked on CMC agar for 1 day at 37°C.

The pure colonies of bacteria were spread on CMC agar at 37°C for 1 day. These pure colonies were flooded with 0.1 wt./vol.% congo red solution for 10 minutes and then washed by 0.1 M NaCl. After that, the clear-zone showing colonies were collected and further determined for hydrolysis capacity value or HC value.

b) Isolation of Cellulase-Producing Bacteria from Living Termites

The same type of lower termites was used in this experiment. Twenty living termites were prepared before enrichment by using procedures, which were used with dead termites. The termite-containing bag was enriched by incubating at the ambient condition for 4 hours, and then 0.5 ml of solution from the termite-containing bag was transferred to a test tube, which contained 10 ml of CMC broth, and incubated at 37°C for 1 day. 0.5 ml of the culture from culture was transferred to fresh CMC broth and incubated at the above conditions for six times.

The enriched cultures and their dilutions were spread on CMC agar and incubated at 37°C for 1 day. After that, isolated colony was repetitively streaked on CMC agar for 1 day at 37°C.

The pure colonies of bacteria were spread on CMC agar at 37°C for 1 day. These pure colonies were flooded with 0.1 wt./vol.% congo red solution for 10 minutes and then washed by 0.1 M NaCl. After that, the clear-zone showing colonies were collected and further determined for HC value.

3.3.3.2 Isolation of Cellulase-Producing Bacteria from Higher Termites

Microcerotermes sp., higher termite, were fed with coconut wood for 1 day before the experiment. The isolation was separated with 3 conditions—aerobic, 2 steps anaerobic, and aerobic with anaerobic pretreatment step—for determining the cellulase-producing bacteria.

For aerobic condition, one-hundred termites were weighed by a precised balance. After that, they were surface-sterilized in 70 vol.% ethanol for 15 minutes and then washed in normal saline solution. These termites were divided into 2 groups. The first fifty termites were ground and divided into 10 tubes, each of which contained 10 ml of 5 g l^{-1} Whatman filter paper No.1 containing 65 modified DSMZ broth medium 1 (Wenzel *et al.*, 2002). The remaining termites were also ground and divided in 10 tubes, each of which contained 10 ml of 65 modified DSMZ broth medium 1, which contained CMC 5 g l^{-1}. The inoculated media of each group were aerobically incubated under 2 conditions—37°C and room temperature—for 1 month.

For 2 steps anaerobic condition, twenty termites were weighed by a precised balance. After that, they were surface-sterilized in 70 vol.% ethanol for 15 minutes and then washed in normal saline solution. The termites were dropped and ground in a stomacher bag, which contained 9 ml normal saline solution. The termite-containing bag was enriched by incubating at the ambient condition for 1 hour, and then the 0.5 ml of solution from the termite-containing bag was transferred to a test tube that contained 7 ml of NIH Thioglycollate broth and incubated at 37°C for 1 day. 0.5 ml of culture was transferred to fresh NIH Thioglycollate broth and incubated at the same above conditions for six more times. After that, 0.5 ml of each dilution was transferred to 7 ml of fresh NIH Thioglycollate broth , which contained CMC 5 g l^{-1} and Whatman filter paper No.1 5 g l^{-1}. These inoculated media were incubated at 37°C for 1 month. The experiment was done in duplicate.

For aerobic with anaerobic pretreatment step condition, twenty termites were prepared and inoculated in NIH Thioglycollate broth as the 2 steps anaerobic condition. After six dilutions, 0.5 ml of each dilution was transferred to 7 ml of fresh 65 modified DSMZ broth medium 1, which contained Whatman filter

paper No.1 5 g l^{-1} and CMC 5 g l^{-1}. These inoculated media were incubated at 37°C for 1 month. The experiment was done in duplicate.

The enriched cultures of each condition were streaked on 65 modified DSMZ agar medium 3 (Wenzel *et al.*, 2002) and incubated at 37°C for 1 day. These samplings were done 5 times. The first 4 times was done every 5 days, and the last time was 10 days. The isolated colonies from each condition were repetitively streaked on 65 modified DSMZ agar medium 3 until all of isolates were pure colonies.

The pure colonics of bacteria of each condition were spotted on 65 modified DSMZ agar medium 3 and incubated at 37°C for 1 day. These pure colonies were flooded with 0.1 wt./vol.% congo red solution for 10 minutes and then washed by 0.1 M NaCl. Colonies surrounded by the clear zone were selected as cellulase-producing isolates (Teather and Wood, 1981).

3.3.3.3 Determination of Hydrolysis Capacity Value (HC value)

The selected pure colonies, which were isolated from both lower termite and higher termite, were single spotted on 65 modified DSMZ agar medium 3 and incubated at 37°C for 1 day. All colonies were measured for the diameter of colony, after that, they were flooded with 0.1 wt./vol.% congo red solution for 10 minutes and then washed by 0.1 M NaCl. Clear-zone appearance and diameter of each colonie were measured and calculated for HC value, which was calculated from the clear zone diameter divided by the colony diameter. The highest HC value of isolate from each condition was selected as the effective isolates, which was further determined for cellulase enzyme activity and tolerance in ionic liquid.

3.3.4. Cellulase Enzyme Activity Assay

3.3.4.1 Preparation of Crude Enzyme for Enzymatic Assay

Single colony of effective isolate was grown in 50 ml of Tryptic Soy Broth (TSB) medium, pH 7.2, and incubated at 37°C, 200 rpm for 24 hours. 0.5 ml of inoculum medium was transferred into 7 flasks of 250 ml Erlenmeyer flasks, which contained 50 ml of production medium (65 modified DSMZ broth medium 3) and incubated at the same condition. The supernatants of production mediums were collected at 4, 8, 10, 12, 16, 20, and 24 hours by using

centrifuge at 8000 rpm, 4°C for 10 min, and then these supernatants were used as the crude enzyme for determining cellulase enzyme activities—endoglucanase activity, exoglucanase activity, and β-glucosidase activity—and protein concentrations. The experiments were also performed at different tempartures—40, 45, and 50°C—for determining the effect of temperature on the cellulase enzyme activities and protein concentrations.

3.3.4.2 Quantitative of Cellulase Enzyme Assay

Endoglucanase activity was done by the method described by Mandels *et al.* (1971). Exoglucanase activity was done by the method described by Ghose (1987), with some modifications. β-glucosidase activity was done by the method described by Kubicek (1982). Figures 3.1–3.3 show the procedures of each cellulase enzyme activity assay.

The amount of released reducing sugar, which was referred to the cellulase enzyme activities, was measured by the DNSA method (Miller, 1959) by using glucose as the standard. 1 Units (U) of cellulase activity is defined as an amount of the enzyme yielding 1 μmol of glucose within 1 min under the assay condition. The enzymatic assays of each collected supernatant were performed in triplicate.

The amount of protein in each collected supernatant was determined in triplicates by using the method of Lowry *et al.* (1951). Figure 3.4 shows procedure for determination.

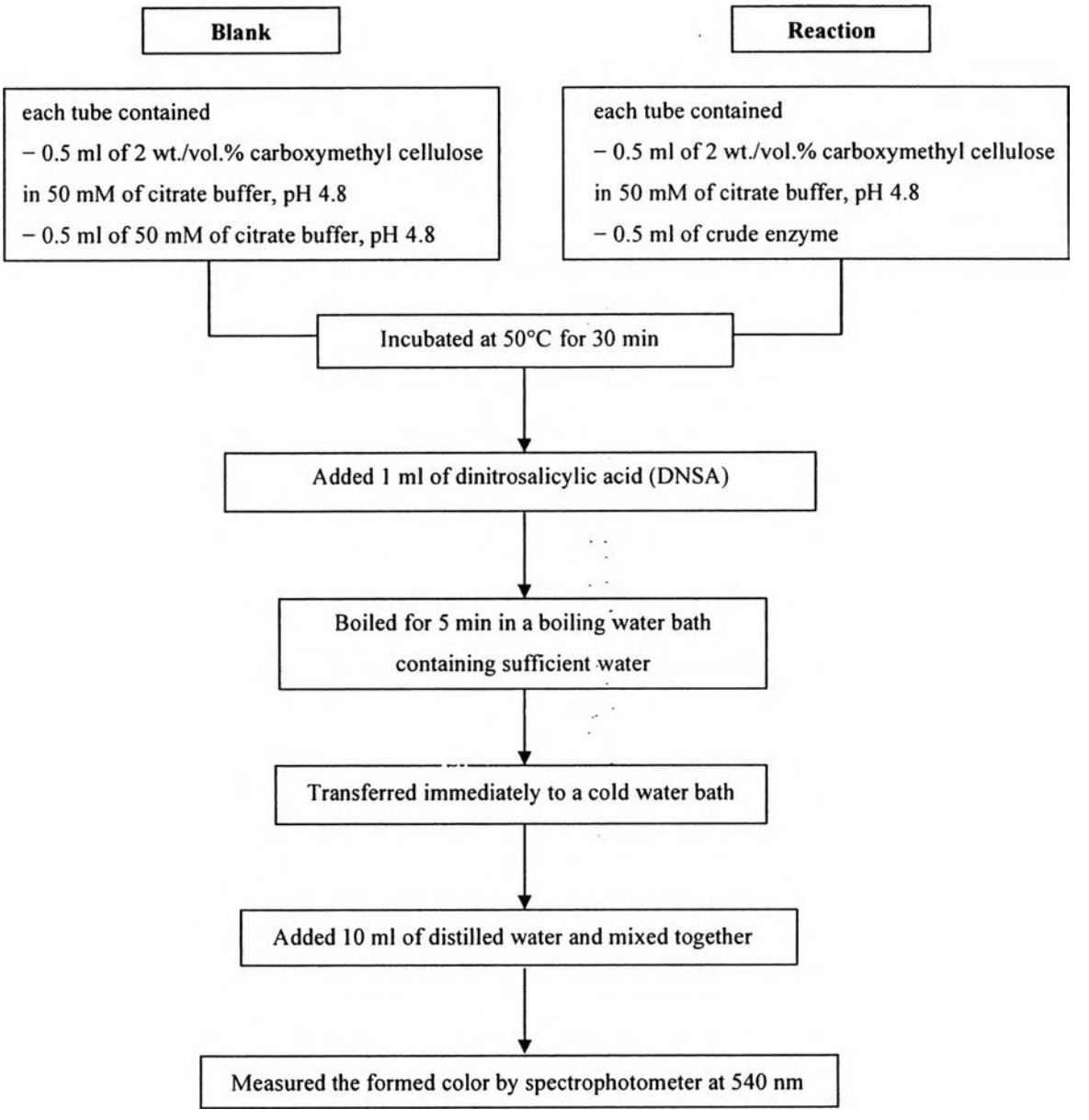

Figure 3.1 Procedure of endoglucanase activity assay.

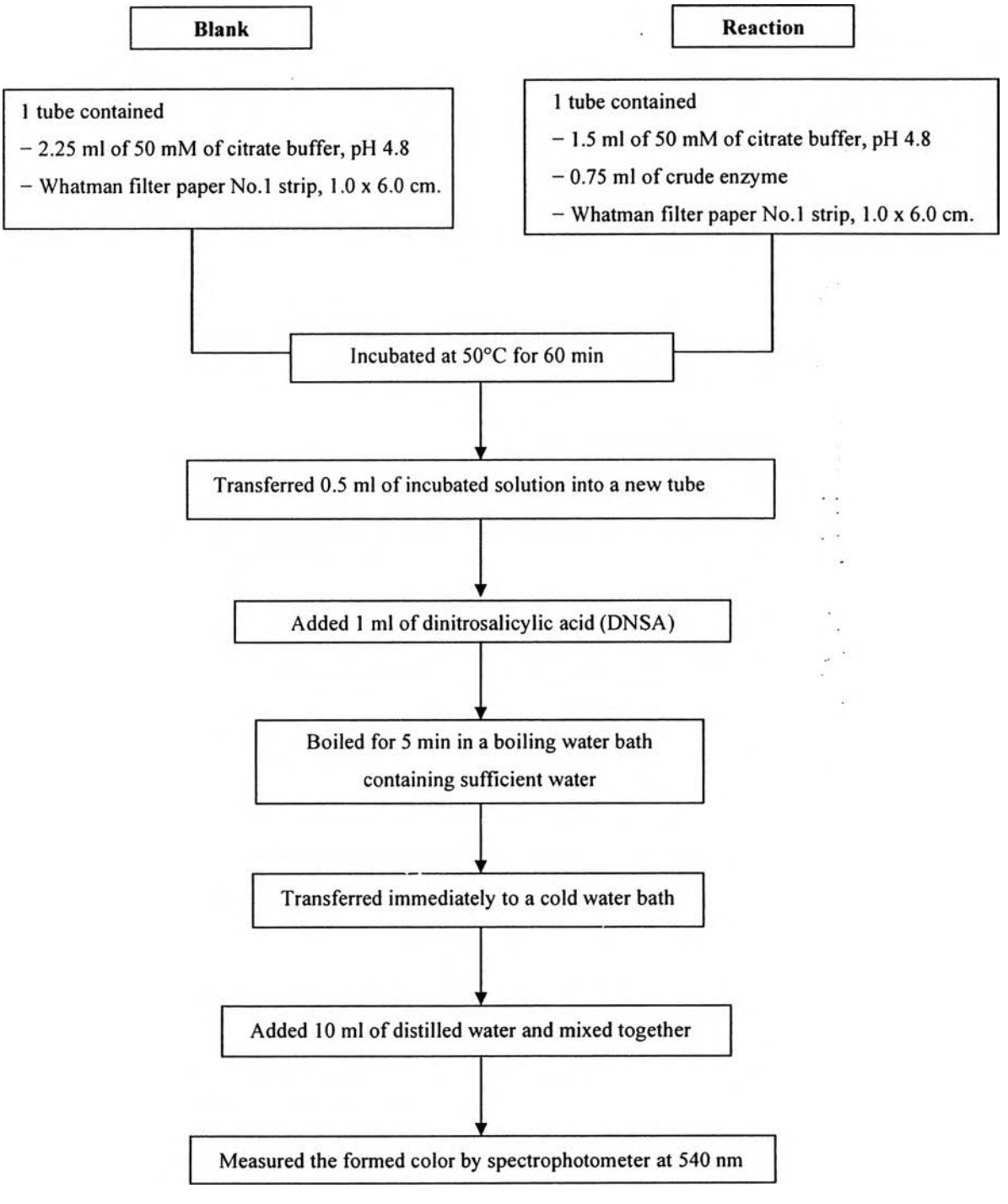

Figure 3.2 Procedure of exoglucanase activity assay.

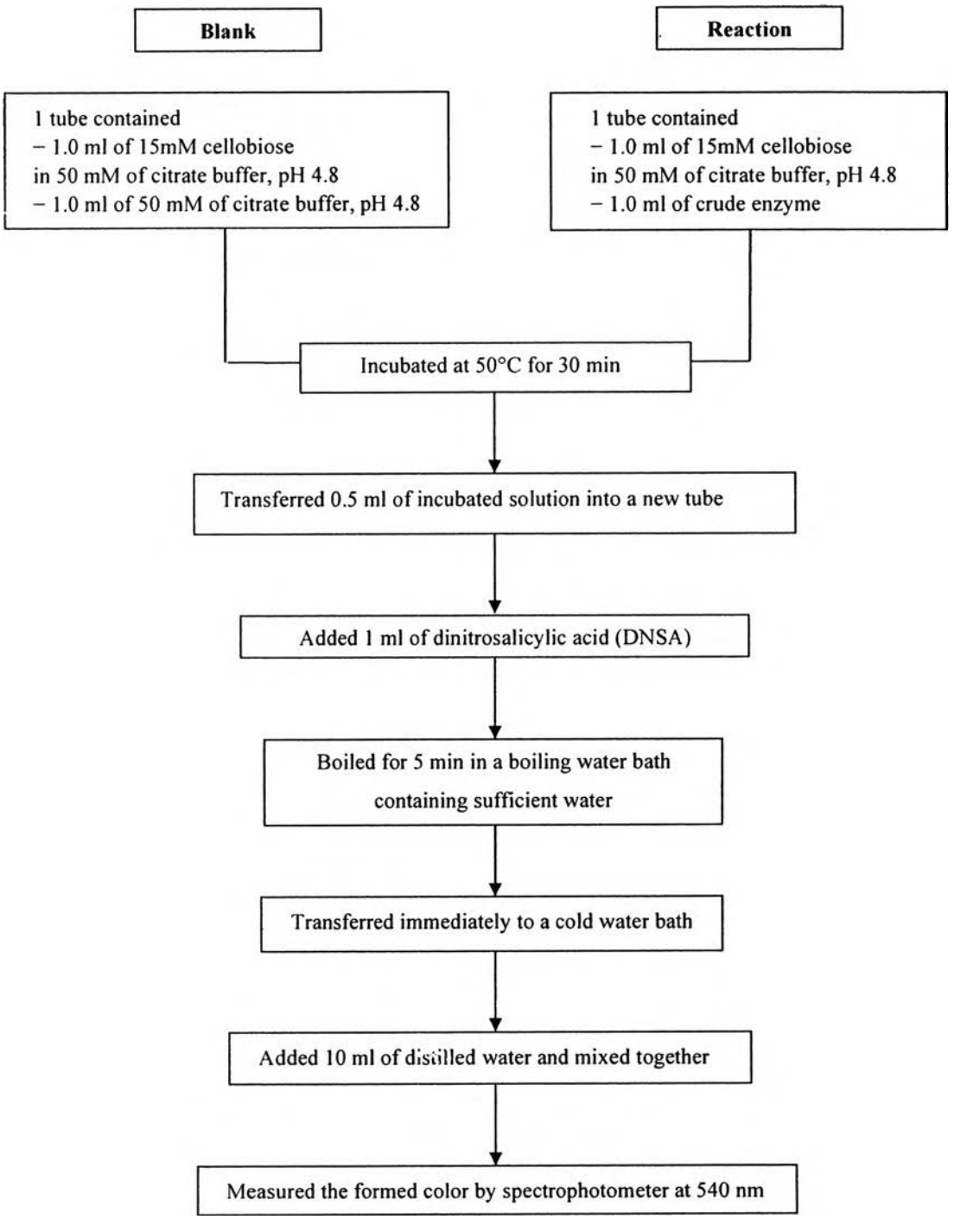

Figure 3.3 Procedure of β-glucosidase activity assay.

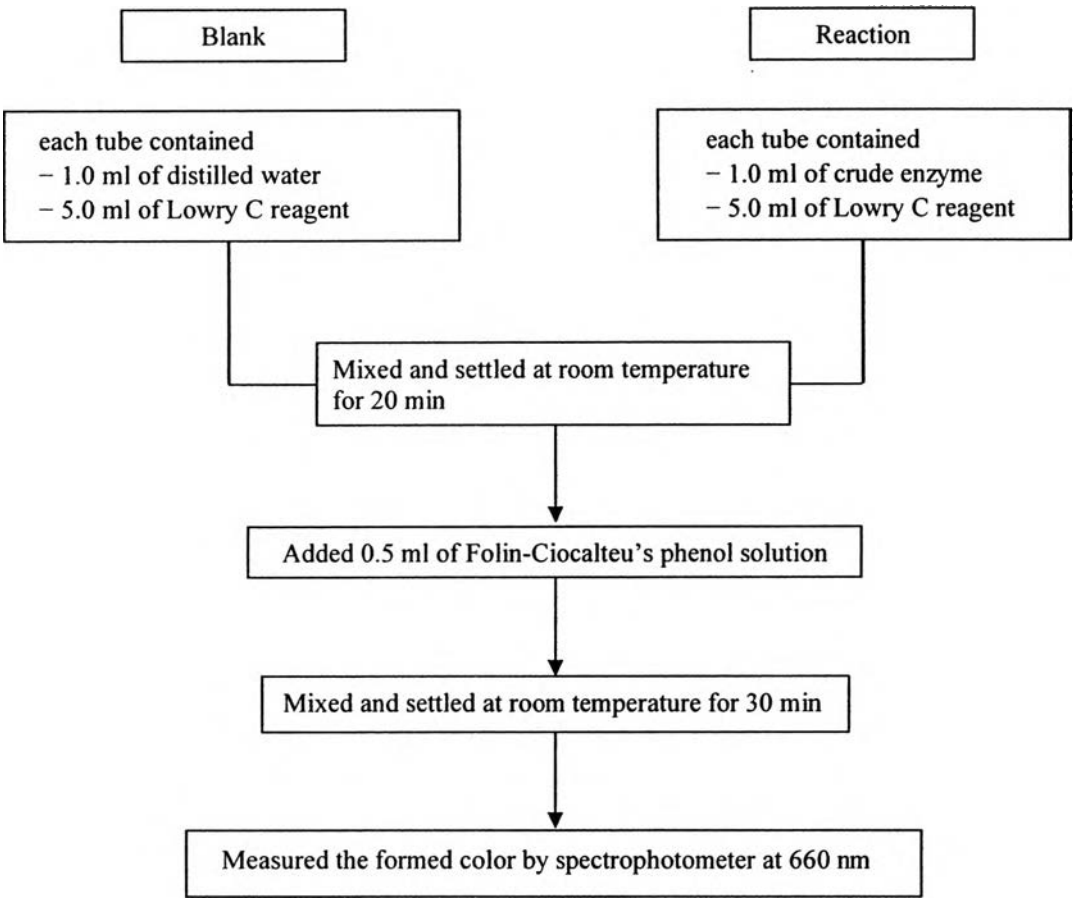

Figure 3.4 Procedure of protein determination of each supernatant.

3.3.5 Determination of the Specific Growth Rate (μ) and Tolerance of Cellulase-Producing Bacteria in the Presence of Ionic Liquid

The effective isolate from each condition was determined for their tolerance to the presence of ionic liquid, 1-butyl-3-methylimidazolium chloride or [BMIM]Cl, which was used to enhance the accessibility of cellulase enzyme into the cellulose chain.

The selected isolates were grown in a tube, which contained 3 ml of 65 modified DSMZ broth medium 3, at 37°C, 200 rpm for 24 hours. After that, 30 μl of the culture medium was transferred to 3 ml of fresh 65 modified DSMZ broth medium 3 in new sterile tubes containing various amounts of the ILs, [BMIM]Cl, at (0.1, 0.5, 1.0, 5.0, or 10.0 vol.%). One culture of each strain without [BMIM]Cl

served as a control. The tolerance determinations of each [BMIM]Cl concentrations were performed in triplicates by measuring the optical density at 550 nm within 24 hours. The specific growth rate (μ) of each isolate was calculated for investigating the effect of concentration of [BMIM]Cl on growth rate of bacteria.

3.3.6 Nomenclature and Classification of Bacteria from Determinative Bacteriology

3.3.6.1 Preliminary Identification by Microbiological Methods

The effective cellulase-producing bacterial colonies were grown on 65 modified DSMZ agar medium 3 at 37°C for 1 day. After that, they were examined for their colonial appearance, pigmentation, cell shape, Gram's staining, and spore forming under microscopic examination. Moreover, the oxidase test and catalase test were also performed.

3.3.6.2 Preliminary Identification by Biochemical Methods

The identifications of selected isolates were performed and checked using API 50 CHB and API 20 E test kit of Biomérieux, France. The identification methods were followed by the Biomérieux instructions.

3.3.6.3 DNA Base Composition

The effective cellulase-producing bacteria were grown on 65 modified DSMZ broth medium 3 at 37°C, 200 rpm for 24 hours before DNA extraction. Genomic DNA was extracted by using Genome DNA Simax Kit (Beijing SBS Genetech Co., Ltd., China). The method was used according to the manufacturer's instructions.

The polymerase chain reaction (PCR) analysis was followed the procedure, which was described by Horn *et al.* (1991) with some modifications. The PCR amplification was carried out in a 10 μl mixture in a DNA thermo cycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan). The conditions consisted of 35 cycles of initial denaturation at 94°C for 1 min, denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min (Hauben *et al.*, 1997). The forward and reverse primers were as follows (Bayane *et al.*, 2005):

Primer 1: 16F27: 5'-AGAGTTTGATCCTGGCTCAG-3'

Primer 2: 16R1522: 5'-AAGGAGGTGATCCAGCCGCA-3'

The PCR products were visualized using gel electrophoresis (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland) of 10 µl of the reaction mixture in a 2 wt./vol.% agarose gel in 0.5x of Trizma-EDTA buffer or TE buffer. DNA was made visible by 0.5 µg/ml of Ethidium bromide staining and ultraviolet transillumination. After that, the PCR products were purified and sequenced 16S rDNA by Macrogen Inc. (Seoul, South Korea).