CHAPTER III

MATERIALS AND METHODS

3.1 Chemical

Aminopyrene (Aldrich, St. Louis, U.S.A.) was used to interact with nitrite in acid solution to produce a positive standard mutagen in the Ames test. D-Biotin, ammonium sulfamate ($NH_2SO_4NH_4$) were purchased from Sigma Chemical Company (St.Louis, Missouri, U.S.A.). Magnesiumsulfate heptahydrate ($MgSO_4.7H_2O$), sodiumchloride (NaCl), hydrochloric acid (HCl), potassium chloride (KCl), L-histidine monohydrochloride, citric acid monohydrate GR ($C_6H_8O_7$.H2O), di-sodium hydrogenphosphate (Na_2HPO4), and Bacto agar were supplied by E. Merck (Darmstadt, Germany). D(+)-Glucose monohydrate, crystal violet indicator and sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4.H_2O$) were bought from Fluka AG (Buch, Switzerland). Oxoid mutrient broth No.2 was supplied by Oxoid Ltd., (Basingstoke, Hants, England). Sodium di-hydrogen phosphate was furnished by May & Baker Ltd., (Degenham, England). Ampicillin sodium was furnished by Vesco Pharmaceutical Ltd., (Bangkok, Thailand). Sodium nitrite ($NaNO_2$) and di-potassium hydrogen phosphate anhydrous (K_2HPO_4) were purchased from BDH Chemicals Ltd., (Poole, England).

3.2 Experimental Design

The experimental design for overall investigation to elucidate the mutagenic modification effect of mushroom samples is shown in Figure 6. Beef boiled with various amounts of edible mushrooms were studied for their mutagenicity with and without nitrite treatment. Mushroom extracts and beef concentrate were tested for their mutagenicity with and without nitrite treatment. Then, antimutagenicity against nitrosated beef concentrate of each mushroom extract was also conducted.

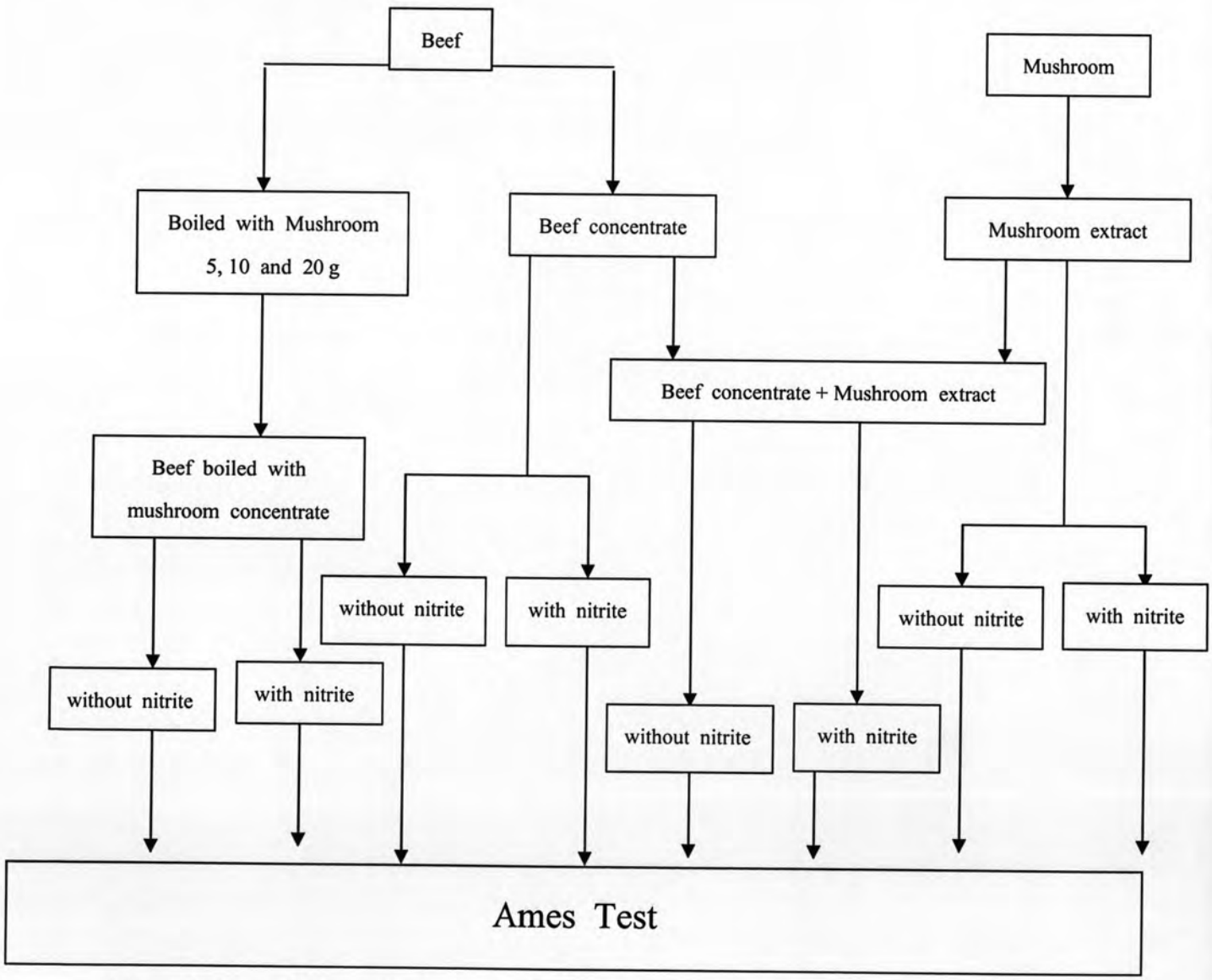

Figure 6 Overall investigations to elucidate the effect of mushroom samples

3.3 Sample Preparations

3.3.1 Beef Concentrate

Beef (loin) was purchased from a supermarket in Bangkok (Central chidlom), Thailand (Febuary, 2006). It was choped into small pieces. Beef (100 g) was boiled with 200 ml distilled water for 6 hours at 60°C with occasionally stirring. Then, aqueous solution was kept cold in refrigerator for 12 hours. The solidified fat was removed and the solution was filtered through the filter paper. The filtrate was evaporated by using vacumm rotary evaporator at 40°C. The residue was stored in a refrigerator. Beef concentrate was prepared by dissolving it in distilled water (1g/ 5 ml) and filtered through a sterile 0.22 μm millipore filter.

3.3.2 Mushroom Extracts

Button mushroom, dried shiitake mushroom, abalone mushroom and oyster mushroom were purchased from a supermarket in Bangkok (Central chidlom), Thailand (Febuary, 2006). Button, abalone and oyster mushrooms were washed with tap water and dried in hot air oven for 2-3 days at 40°C. Each dried mushroom (button, shiitake, oyster and abalone) was choped into small pieces in a home-used blender and boiled with distilled water (1:10 W/V) for 1 hour with occasionally stirring. The aqueous solution was filtered through filter paper. The filtrate was evaporated by using a vacumm rotary evaporator at 40°C. The residue was stored in a refrigerator. Each mushroom extract was prepared by dissolving it in distilled water (1g/ 5 ml). All samples were filtered through a sterile 0.22 μm millipore filter.

3.3.3 Concentrates from Beef Boiled with Selected Edible Mushrooms

Each dried mushroom was choped into small pieces in a home-used blender. Beef (100 g) and various amounts (5, 10 or 20 g) of each mushroom were boiled together with 200 ml distilled water for 6 hours at 60°C with occasionally stirring. The aqeous solution was kept cold in refrigerator for 12 hours. The solidified fat was removed and the solution was filtered through the

filter paper. The filtrate was evaporated by using a vacumm rotary evaporator at 40°C. The residue was stored in a refrigerator. Each concentrate of beef boiled with mushroom was prepared by dissolving it in distilled water (1 g/ 5 ml) and filtered through a sterile 0.22 μm millipore filter.

3.4 Mutagenicity Testing

3.4.1 The Bacterial Tester Strains

Salmonella typhimuium tester strains used in this study were histidine-dependent strains (His^-) TA98 and TA100 which were capable of detecting frameshift mutation and base-pair substitution respectively. Both strains were kindly provided by Assoc. Prof. Dr. Kaew Kangsadalumpai of the Institute of Nutrition, Mahidol University, Thailand. The tester strains were manipulated as suggested by Maron and Ames (1983). Overnight cultures of bacteria inoculated from frozen stock culture in Oxoid nutrient broth No.2 at 37°C were used for mutagenesis assay (Appendix). Cultures were kept in refrigerator until use.

3.4.2 Nutrient Agar

3.4.2.1 Preparation of a Minimal Agar Plate

Minimal agar containing 1.5% Bacto agar was autoclaved and then was mixed with 2% sterile glucose and Vogel-Bonner medium E stock salt solution (VB salt) (appendix). About 30 ml of molten agar was poured on to the sterile petri dish. It was left until solidified and was stored at 37°C in the incubator for 48 hours.

3.4.2.2 Preparation of Top Agar

Top agar containing 0.6% Bacto agar and 0.5% Sodium chloride was autoclaved and was stored at 45°C. Before use, 10%(v/v) of a sterile solution of 0.5 mM. histidine and biotin was added to the molten top agar and then was maintained at 45°C in the water bath.

3.4.3 Mutagenicity of Beef Concentrate

An aliquot (10, 20, 40 and 80 µl) of beef concentrate from experiment 3.3.1 was added to the tube containing appropriate amount of distilled water to obtain the final volume of 200 µl. Dilute HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable. The distilled water was added to obtain the final volume of 1000 µl. Then the tube with stopper was shaken at 37°C for 4 hours and stopped by placing the tube in an ice bath for 1 min. Distilled water was added to the reaction mixture to obtain the final volume of 1250 µl , and then the reaction tube was immersed in an ice bath for 10 min.

The preincubation method suggested by Yahagi *et al.* (1975) was used throughout this study. For the determination of direct mutagenicity, 100 µl of each culture of tester strain was added to a test tube containing 100 µl of different concentrations of each extract from edible mushroom and 500 µl of $NaPO_4KCl$ buffer (pH 7.4). The mixture was incubated at 37°C in a shaking water bath for 20 min. Then, 2 ml of molten top agar (45°C) was added, mixed well and poured onto a minimal glucose agar plate and incubated at 37°C in darkness for 48 hours. The number of histidine revertant colonies were counted. Non-toxic concentrations were those with no difference in the number of spontaneous revertants, size of the colonies, and intensity of the background lawn compared to the negative control. The procedure is shown in Figure 7.

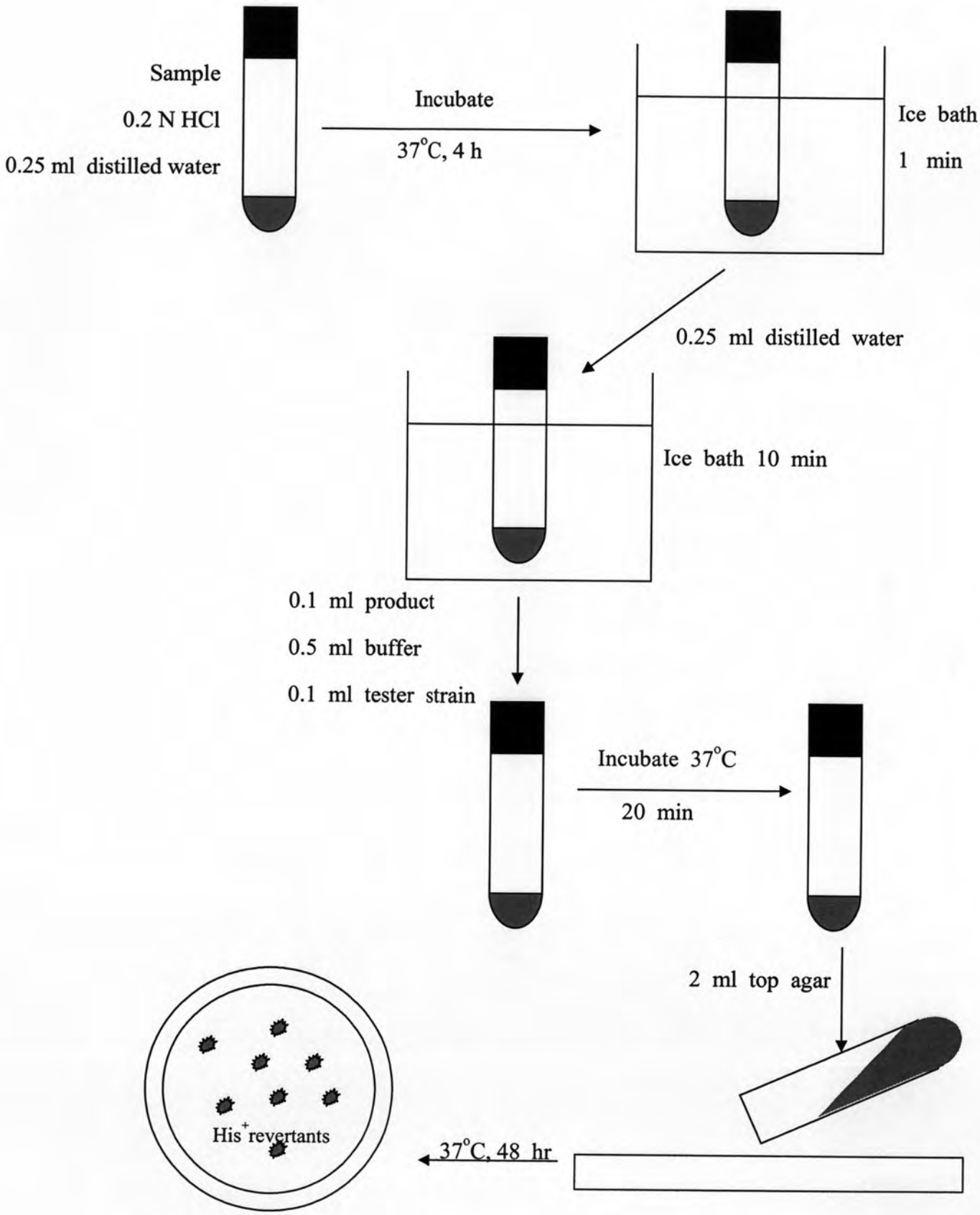

Figure 7 Steps to determine the mutagenicity of food samples using the Ames mutagnicity test (pre-incubation modification) in the absence of S-9 mix.

3.4.4 Mutagenicity of Nitrite Treated Beef Concentrate

An aliquot (10, 20, 40 and 80 µl) of beef concentrate from experiment 3.3.1 was added to the tube containing appropriate amount of distilled water to obtain the final volume of 200 µl. Dilute HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable. Adding 250 µl of 2M sodium nitrite started the reaction. The final volume was 1000 µl. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Then, 250 µl of 2M ammonium sulfamate was added to the reaction mixture to decompose the residual nitrite and then the reaction tube was immersed in an ice bath for 10 min. Then, the mixture was determined for its mutagenicity by Ames test as described in Figure 8.

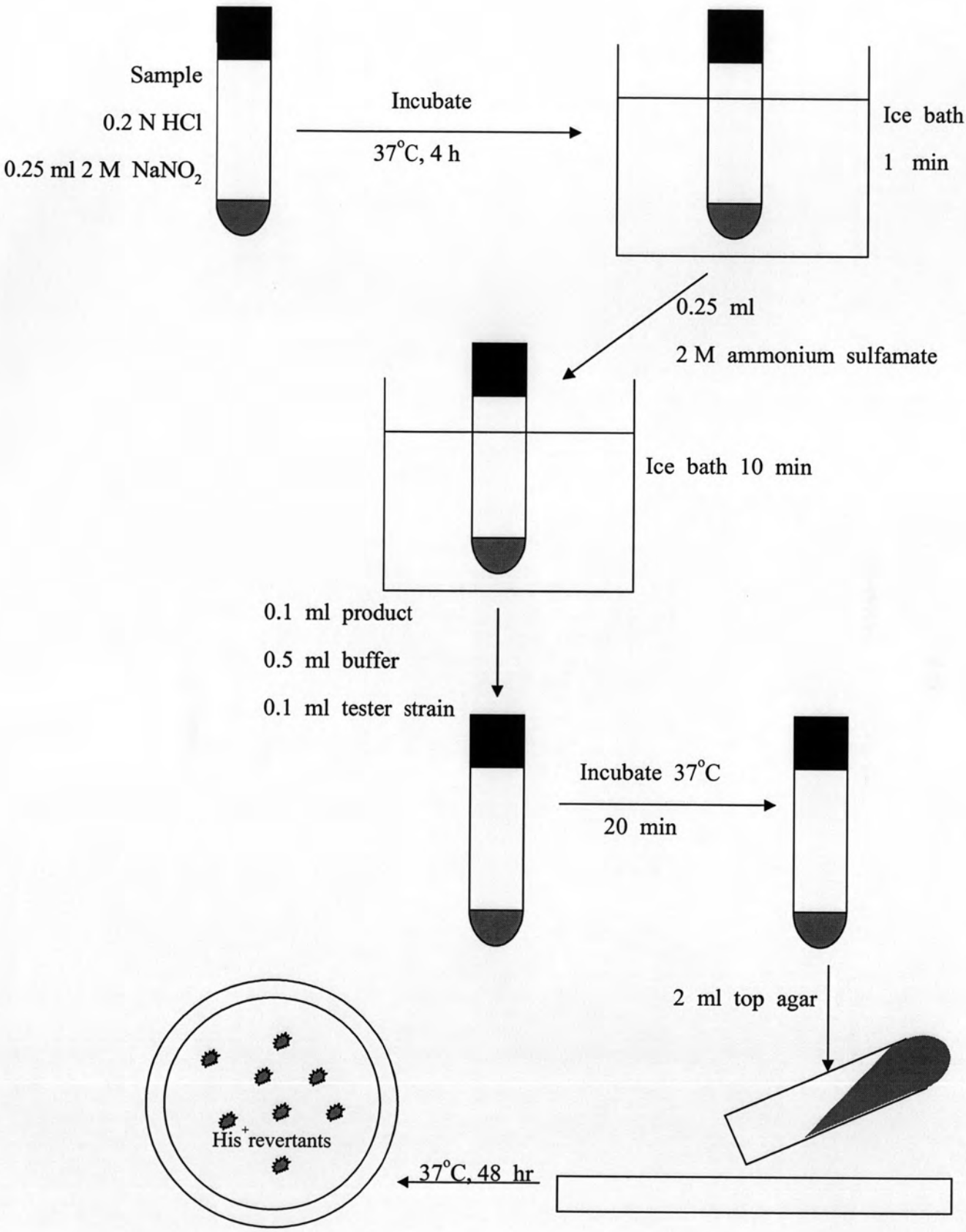

Figure 8 Steps to determine the mutagenicity of nitrite treated food samples using the Ames mutagnicity test (pre-incubation modification) in the absence of S-9 mix

3.4.5 Mutagenicity of Extracts from Selected Mushrooms Treated with and without Nitrite

The extracts from each selected mushroom in different amounts (10, 50, 100, 200 µl) from experiment 3.3.2 were introduced into the tube containing appropriate volume of distilled water to obtain the final volume of 200 µl. The experiment procedures to obtain the mutagenicity of non-nitrite and nitrite treated of these concentrates were carried out following the diagrams as shown in Figure 7 and 8 respectively.

3.4.6 Mutagenicity of Concentrates from Beef Boiled with Various Amounts of Each Selected Mushroom Treated with and without Nitrite

An aliquot (10, 20, 40 and 80 µl) of each concentrate from beef boiled with various amounts of edible mushroom (button, shiitake, oyster and abalone mushrooms) from experiment 3.3.3 was added to the tube containing appropriate amount of distilled water to obtain the final volume of 200 µl. The experiment procedures to obtain the mutagenicity of non-nitrite and nitrite treated of these concentrates were carried out following the diagrams as shown in Figure 7 and 8 respectively.

3.4.7 Mutagenicity Modification of Extracts from Various Edible Mushrooms on Mutagen Formation of Beef Concentrate

Beef concentrate and each mushroom extract were mixed together and treated with nitrite in acidic condition as follows: 80 µl of beef concentrate solution (1g/ 5 ml) and different amounts of each extract of mushroom (10, 50 and 100 µl) were introduced into the tube containing appropriate volume of distilled water to obtain the final volume of 200 µl. Then, the experiment was conducted followed the procedures of Figure 7 for without nitrite treatment and Figure 8 for with nitrite treatment.

3.5 Data Manipulation

1) The mutagenicity of each sample was presented as number of histidine revertants per plate. The data were reported as means with standard deviation of six plates from two different experiments.
2) To compare the degree of mutagenicity of samples among different experiments, the results are presented as mutagenicity index (MI) which was calculated from

$$\frac{\text{Average value of number of histidine revertants per plate of sample}}{\text{Average value of spontaneous revertants}}$$

Sample expressed its mutagenicity higher than 2 times of spontaneous revertants with dose-response relationship was evaluated mutagenic.

3) Percentage of modification (either increase or decrease on mutagenicity of direct mutagens) of edible mushrooms on mutagenicity induced by direct-acting mutagen was calculated as following.

$$\text{Percentage of modification} = \frac{\text{Expected MI} - \text{Actual MI}}{\text{Expected MI}} \times 100$$

Expected MI = Mutagenicity index calculated from the summation of mutagenicity index of nitrosated beef concentrate and nitrosated mushroom extracts.

Actual MI = Mutagenicity index of the nitrosated mixture of beef concentrate and mushroom extracts.