CHAPTER II

MATERIALS AND METHODS

1. Microorganism

Streptococcus pyogenes (group A) strain C 203 S was obtained from the National Streptococcal Reference Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

The maintenance condition was modified from the method of Josephine and Bohnhoff (82) as follow :

The original culture was obtained in a frozen state. It was thawed and then cultivated by direct streaking on blood agar plates (see Appendix I-1). The plates were incubated overnight at 37°C. Some isolated colonies were selected and suspended in 5 ml Todd Hewitt broth (see Appendix I-3). After overnight incubation at 37°C, the culture was centrifuged at 3,000 rpm (Damon, IEC Division, DPR - 600 centrifuge) for 30 min at 4°C and the bacteria were resuspended in 2 ml maintenance medium (see Appendix I-2). This culture was mixed and dispensed into sterile ampoules with a nine-inch (23 cm) Pasteur pipette, using five to six drops per ampoule. The ampoules were plugged with cotton and sealed with parafilm. The remaining culture was streaked on blood agar for purity tests. The stock cultures were kept at -70°C (Forma Scientific, Bio Freezer, (U.S.A.) until used.

2. Culture Conditions

Blood agar plates were used for purity tests at every step

of cultivation.

2.1 Preparation of Inoculum

As shown in Figure 4.1, the stock culture (from section 1) were allowed to liquify. By using a nine-inch (23 cm) Pasteur pipette, three or four drops of the culture were inoculated in 5 ml Todd Hewitt broth. The tubes were incubated overnight at 37°C. The bacteria were then streaked on blood agar plates. After 24 hr incubation at 37°C, the bacteria were suspended in 2 ml 0.85 % normal saline solution (see Appendix II-3) (39, 46) in colorimeter tubes (12 x 75 mm) to an optical density (OD) of 0.25 at 550 nm (Junior II spectrophotometer, model 6/35, Coleman, Maywood, Illinois, U.S.A.). Uninoculated 0.85 % normal saline solution was used to adjust the spectrophotometer to the zero point (modified from the method of Slade and Knox) (42).

2.2 Preparation of Starter

As illustrated in Figure 4.2, one drop of inoculum (from section 2.1) from a No. 21 hypodermic needle was used to inoculate in each 10 ml Todd Hewitt broth (modified from the method of Slade and Knox) (42) in 20 x 150 mm test tubes. The cultures were incubated for the specified times (see Chapter III, section 1) at 37°C. These cultures were "Starters" for the preparation of SLO.

2.3 Preparation of Streptolysin O (modified from the method of Vejjajiva) (68).

At the end of the incubation of starters (from section 2.2), total growth were estimated by optical density at

550 nm, as shown in Figure 4.3. Uninoculated Todd Hewitt broth was used as a blank (42). The remaining starters were then inoculated in 250 ml Erlenmeyer flasks containing 100 ml Todd Hewitt broth. After being incubated for the specified times at 37°C, total growth was observed by spectrophotometer as described above. The remaining culture fluids were centrifuged at 4°C. Centrifugation should clear the cultures completely to facilitate filtration, and usually required a speed of 3,000 rpm for 30 min. The supernate was then sterilized by membrane filtration and pH measuring was done by an electric pH meter (Radiometer, PHM 83 Autocal pH meter, Copenhegen, Denmark). The filtrate was preserved by adding merthiolate in a dilution of 1 : 10,000 (or 1 ml of 1 % merthiolate per 100 ml filtrate) (see Appendix II-2). This crude SLO should be stored at 4 - 10°C for a month before titration.

3. Growth Cycle of Streptococcus pyogenes (Group A) C 203 S in Todd Hewitt Broth

After preparation of inoculum according to the method described in section 2.1, twenty drops of inoculum from a No. 21 hypodermic needle were inoculated in 500 ml Erlenmeyer flask containing 200 ml Todd Hewitt broth. The flask was incubated at 37°C and the culture was sampled every half an hour to determine the optical density, at the wave length of 550 nm, which represented bacterial growth (85). Uninoculated Todd Hewitt broth was used as a blank (42).

The method was done in duplicate. Then, the means of the optical density at 550 nm from the two runs were plotted against the

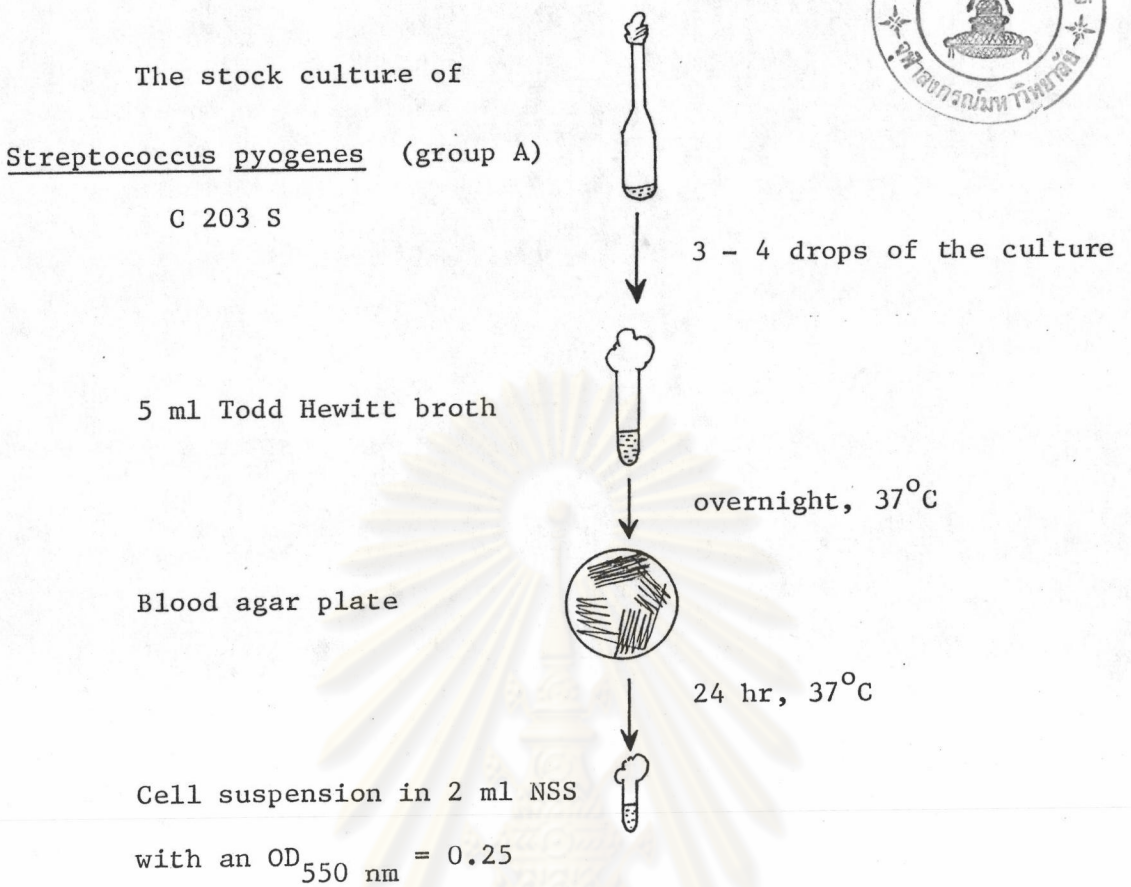

Figure 4.1 Preparation of Inoculum

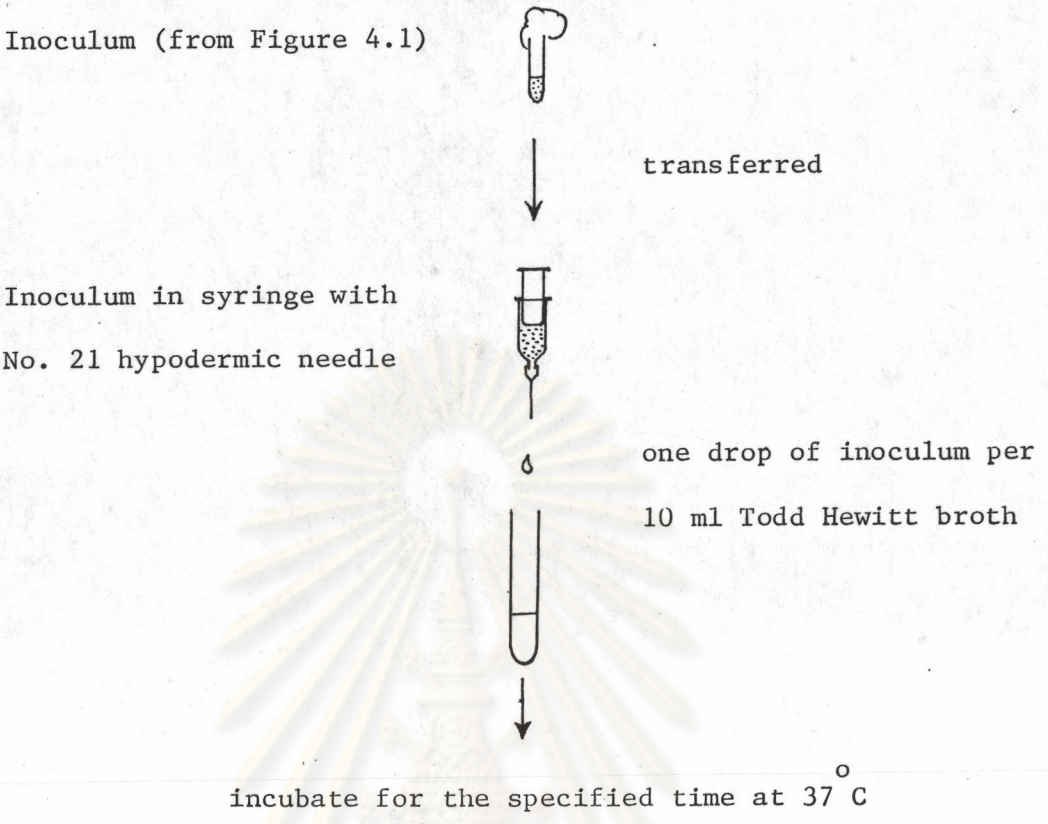

Figure 4.2 Preparation of Starter

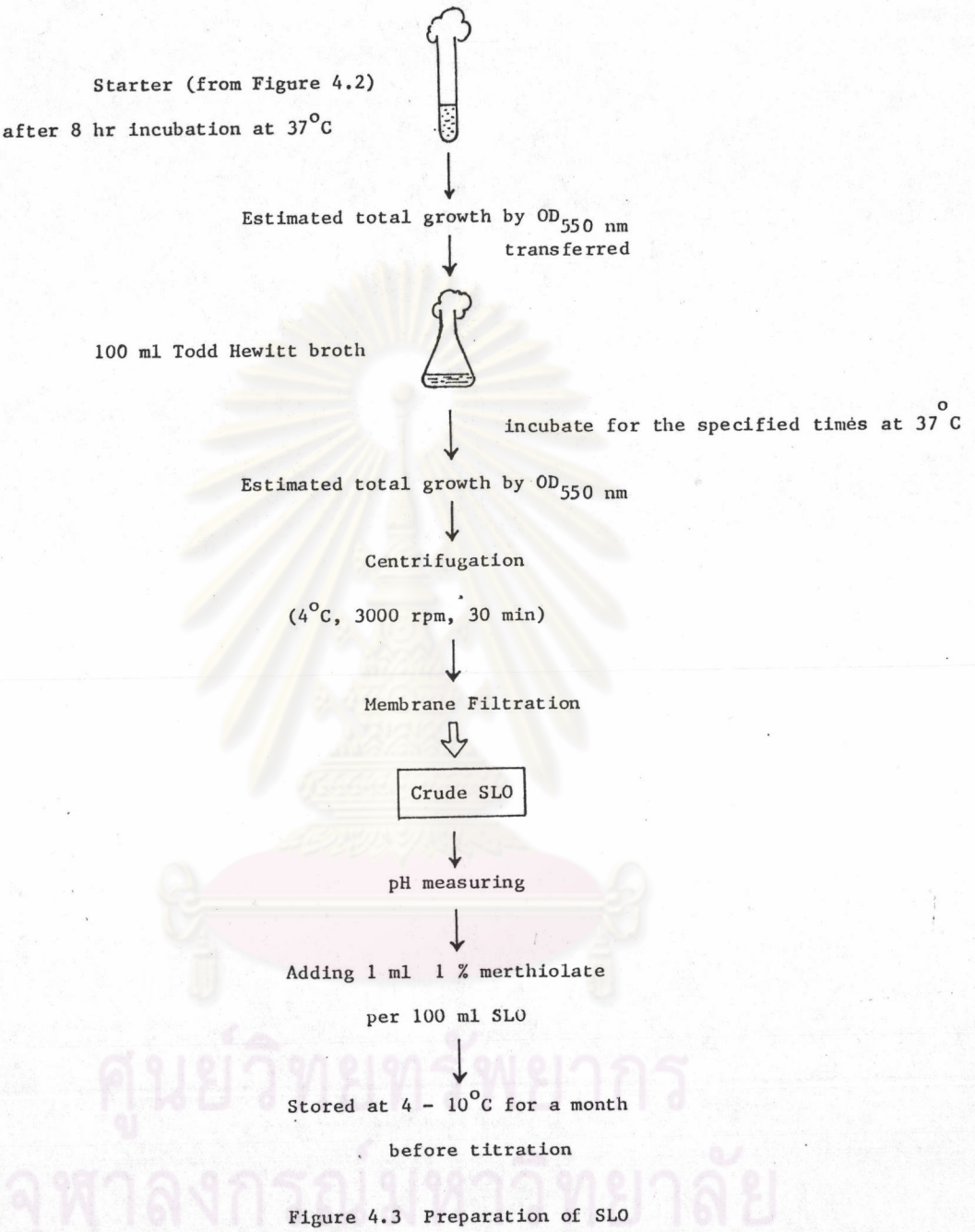

Figure 4.3 Preparation of SLO

Figure 4 The Overall Procedures for Streptolysin O Production

cultivation times, as shown in Figure 5.

4. Optimization for High Streptolysin O Production

Each of the methods in this section was done two times in one run. The results shown in Chapter III, section 2 were the means of the duplication.

4.1 The Effect of the Incubation Period

In order to determine the optimal incubation period for SLO production ; inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for the incubation period of the final step of the cultivation (section 2.3) which was varied as follows, 0, 2, 4, 6, 8, 10 and 6, 9, 12, 15, 18, 21, 24 hr. Then titration of each sample of crude SLO was done in duplicate according to the method described in section 5 of this chapter.

4.2 The Effect of the Age of Starter

In this experiment ; inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for

- the age of the starter (section 2.2) which was varied as follows, 5, 8, 10, 12 and 18 hr

- the incubation period of the final step of the cultivation (section 2.3) which was 4 hr according to the result in Chapter III, section 2.1

Titration of each sample of crude SLO was then done in duplicate according to the method described in section 5.

4.3 The Effect of the Size of Starter

For studying the effect of the size of starter for SLO production ; inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for

- the size of the starter (section 2.2) which was varied as follows, 5, 10 and 15 % of fresh medium in the final step of the cultivation (section 2.3)

- the incubation period of the final step of the cultivation which was 4 hr according to the result in Chapter III, section 2.1

Titration of each sample of crude SLO was done in duplicate according to the method described in section 5.

4.4 The Effect of Temperature

Inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for the following conditions in the last step of the cultivation (section 2.3)

- incubation temperature which was 25 and 37^{o}C so as to compare the potency of SLO obtained from these two conditions

- incubation period which was 4 hr according to the result in Chapter III, section 2.1

Then titration of each sample of crude SLO was done in duplicate according to the method described in section 5.

4.5 The Effect of the Initial pH

So as to determine the optimal initial pH for SLO production ; inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for the following conditions in the final step of the cultivation (section 2.3)

- the pH of 100 ml Todd Hewitt broth which was adjusted before sterilization by using 1 M NaOH and 1 M HCl, to yield the pH of 6.6, 7.2, 7.8 and 8.2 after sterilization

- incubation period which was 4 hr according to the result in Chapter III, section 2.1

Titration of each sample of crude SLO was then done in duplicate according to the method described in section 5

4.6 The Effect of Carbondioxide (CO_2) and Agitation

Inoculum, starter and SLO were prepared according to the method described in section 2.1, 2.2 and 2.3, respectively, except for the following conditions in the final step of the cultivation (section 2.3)

- for studying on the effect of CO_2 and agitation, the cultural conditions were varied in four different ways,

A. incubated in atmospheric CO_2 and

A-1. standstill

B-1. stirred with a magnetic stirrer at approximately 140 rpm

B. incubated in 5 % CO_2 and

B-1. standstill

B-2. stirred with a magnetic stirrer at approximately 140 rpm

- incubation period which was 4 hr according to the result in Chapter III, section 2.1

Titration of each sample of crude SLO was then done in duplicate according to the method described in section 5

5. Titration of Streptolysin O (Spectrophotometric method)

Reagents

1. Reduced SLO (see Appendix II-4)
2. Standard ASO Serum (see Appendix II-7)
3. SLO Buffer (see Appendix II-5)
4. Standardized suspension of erythrocytes (see Appendix II-8)

Method : Modified from the procedure of Vejjajiva (68) and Rotta & Facklam (48)

1. The standard ASO serum was diluted with SLO buffer so as to obtain 1 Todd unit per ml

2. Various amounts of SLO buffer were pipetted into tubes according to Table 6 (It might be more than ten tubes).

3. After centrifugation, 1 ml of the clear supernate from each tube was diluted with 2 ml of distilled water. The transmittance at the wave length of 520 nm was determined and converted into hemolysis according to the table (see Appendix IV, Table 13). The

end point of the titration was indicated by the test tube dilution whose homolysis value is nearest to 50 % (48).

In this study, that end point was indicated by the test tube dilution whose transmittance value is nearest to 46.5 % T, according to Appendix IV, Table 13.

Controls

1. Red Cell Control and Serum Control

The supernate after centrifugation should display no hemolysis.

2. SLO Control

This tube should be completely hemolyzed.

Table 6 Methodology for the Titration of SLO (68)

Reagent	Controls			Tube Number											
	Red Cell	Serum	SLO	1	2	3	4	5	6	7	8	9	10	→	→
SLO Buffer (ml)	0.75	0.25	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	→	→
Reduced SLO (ml)	-	-	0.75	1	1	1	1	1	1	1	1	1	1	1	1
Shake gently to mix and then transfer															
ml - transfer	-	-	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Standard ASO 1 Todd unit/ml (ml)	-	0.5	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Shake gently to mix Incubate at 37°C for 15 min															
Standard Suspension of Erythrocytes (ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Shake gently Incubate at 37°C for 45 min, shaking after first 15 min Centrifuge tubes for 5 min at 1,500 rpm															
International Units (or dilution 1:)				1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	→	→

The proportions of reduced SLO and SLO buffer in the dilution tubes were according to the series :-

Tube 1 1 plus 0.1

Tube 2 1 plus 0.2

Tube 3 1 plus 0.3

etc.

The steps in this series, increased by 0.1 of the original volume of SLO in the dilution tubes, gave sufficient accuracy in titration (69).

The stronger the lysin (SLO), the higher the dilution in unit volume (= 0.25 ml) that would lyse the standard volume of red cells in the presence of unit volume (= 0.5 ml) of the diluted standard ASO serum containing 1 unit per ml (69). The end point of the titration was the tube containing just sufficient lysin that still caused 50 % hemolysis (observable with spectrophotometer) after being exactly neutralized by the standard ASO at 37°C within 1 hr (total).

The potency of SLO expressed in International Units (48) was the reciprocal of the highest dilution of reduced SLO showing 50 % hemolysis, as described above. (For example, if the ninth tube (Table 6) was the end point of the titration, the SLO contained 1.9 International Units and it would be used in the dilution 1 : 1.9 ; i.e., one part of reduced SLO and 0.9 parts of SLO buffer, for the determination of ASO titers in the patient's serum by the spectrophotometric method.) (48)