CHAPTER III

MATERIALS AND METHODS

I Experimental materials and equipments

1. Materials

a) Luna C18(2) 100A. 150 x 4.6 mm HPLC column, Phenomenex, USA

b) Security guard semi-prep gard cartridge system 10mm ID for HPLC colomn, Phenomenex, USA

c) HLC-disk 25 GF/PP 0.45 µm, Kanto Chemical Co., Inc., Japan

d) Cotton swobs, Thai Gauze co., LTD., Thailand

e) 3 ml syringe, BD, Singapore

2. Equipments

a) Cooled CO_2 incubator, Gallenkamp, Germany

b) Biomedical freezer, Sanyo MDF 236, Japan

c) pH meter, Cyberscan 1000, USA

d) Microscopy, Olympus CH 30, Japan

e) Colony counter, Gallenkamp, Germany

f) Vortex mixer, Lab-line Instrument Inc., USA

g) Laminar flow hood, BVT 123 Issco, USA

h) Centrifuge, Micro22R Hettich, Germany

i) Microwave, KOR-63-D7 Daewoo, Korea

j) Incubator shaker, SW23 Julabo, USA

k) Spectrophotometer, V530 Jasco, USA

l) Balance, AB 204 Mettler Todledo, Switzerland

m) Balance , RP 310S Satorious, Germany

n) HPLC, 600 Waters, USA

o) HPLC software, Empower software Waters, Ireland

p) Computer, IBM, USA

q) 250 µl microsyringe, MS R250, Ito corp., Japan

r) Gyrotory shaker, Model G2 New Brunswick Scientific, USA

s) Incubator shaker, Lab-line Instrument Inc., USA

t) Autoclave, SS320 Tomy, USA

u) Hot-air oven, Binder, Germany

v) 10 µl autopipette, lenpipette Labsystems, Finland

w) 50 µl autopipette, Finnpipette Step Adjustable Labsystem, Finland

x) 1000 µl autopipette, LMS, Japan

y)

3. Chemicals

a) Sodium chloride, analytical grade, Ajax Finechem, USA

b) Sodium hydroxide, analytical grade, Merk, Germany

c) Ferric chloride, analytical grade, Asia Pacific Specially Chemical Limited, Singapore

d) Hydroxylamine hydrochloride, analytical grade, Fluka, USA

e) *N*-(β-ketocaproyl)-homoserine lactone (3-oxo-C6-AHL), HPLC grade, Sigma-Aldrich, Germany

f) Trifluoroacetic acid, HPLC grade, Sigma-Aldrich, USA

g) Acetonitrile, HPLC grade, Merck, Germany

h) Chloroform, analytical grade, Ajax Finechem, USA

i) Methanol, HPLC grade, Merck, Germany

j) Ethanol, analytical grade, Ajax Finechem, USA

k) 37% Hydrochloric acid, analytical grade, Reidel-de Haen, Germany

l) Glycerol, analytical grade, Fluka, USA

4. Bacterial culture media

a) Plate count agar (PCA), Difco, France

b) Thiosulfate Citrate Bile Sucrose Agar (TCBS), Merck, Germany

c) Nutrient agar (NA), Himedia, USA

d) Peptone from casein, Merck, Germany

e) Tryptone, Merck, Germany

f) Yeast extract, Difco, France

5. Bacterial culture

a) *Vibrio parahaemolyticus* DMST 22092 (isolated from seafood) (Department of Medical Science, Thailand)

b) *Vibrio parahaemolyticus* DMST 22093 (isolated from seafood) (Department of Medical Science, Thailand)

c) *Vibrio parahaemolyticus* ATCC 17802 (Department of Medical Science, Thailand)

d) *Vibrio parahaemolyticus* DMST 23797 (isolated from patient stool) (Department of Medical Science, Thailand)

e) *Vibrio parahaemolyticus* DMST 23798 (isolated from patient stool) (Department of Medical Science, Thailand)

f) *Vibrio parahaemolyticus* DMST 23799 (isolated from patient stool) (Department of Medical Science, Thailand)

g) *Vibrio vulnificus* ATCC 27562 (Department of Medical Science, Thailand)

h) *V.chlorelae* DMST 2873 (Department of Medical Science, Thailand)

i) *Pseudomonas aeruginosa* ATCC 27853 (Department of Medical Science, Thailand)

j) *Escherichia coli* ATCC 25922 (culture collection unit, Chualongkorn Hospital)

k) *Salmonella* sp. ATCC 13811 (culture collection unit, Chualongkorn Hospital)

II Experimental procedures

1. Optimization of AHL detection and extraction

1.1 Optimization of AHL detection by colorimetry method

AHL standard stock solution (0.005 M, A1 in Appendix A) was added into deionized water (D.I. water) 800 µl in different concentration (10 - 1600 nmol) (A2, Appendix A). Non-AHL D.I. water was also used as 0 nmol AHL solution. Preparations of Reagent I and Reagent II used for colorimetry determination were shown below (B3, Appendix B).

Reagent I was the mixture of 2M hydroxylamine hydrochloride and 3.5 M. sodium hydroxide (1:1).

Reagent II was the mixture of 10% ferric chloride in 4 M hydrochloric acid : 95% ethanol (1:1).

The detection of AHL signal molecules was done according to the report of Yang et al (2006) (Figure C1, Appendix C) with a modification as shown in figure 3.1.

The calibration curve of AHL concentration in D.I. water and absorbance at 520 nm (OD_{520}) were plotted. The value of OD_{520} and AHL concentration that had a linear relationship would be used for calibration curve. The experiment was done in 2 replication.

1.2 Optimization of AHL extraction method

1.2.1 Extraction of AHL standard from NB

Various volume of chloroform (1, 2 and 3 ml) were added to 1 ml of sample, then mixed thoroughly for 30 seconds. These mixing steps were operated twice. After vortex mixing, the samples were left until the separation was observed. The chloroform layer was transferred to another tube before subjecting to evaporation.

AHL pellet was kept at -35 °C before determination of AHL by colorimetry method as shown in title 1.1. This experiment was conducted in 2 replication.

1.2.2 Extraction of bacterial AHL from bacterial cultures

The AHL producing reference cultures of *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093 and *P.aerunginosa* ATCC 27853 were used for the optimization of AHL extraction method. The non AHL producing strains of *E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811 were also optimized.

Bacterial strains were cultured in NB 10 ml, orbital shaking 200 rpm in room temperature for 6 hours. This culture would be mid-log of growth phase, consisting approximate 10^7 - 10^8 CFU/ml.

Cell-free supernatants from mid-log culture were collected by transferring culture 1 ml to centrifuge 13,000 rpm at 4 °C for 10 minutes. The cell-free supernatants were then subjected to determine AHL concentrations by colorimetry method as described in 1.1. The experiment was tested for 2 replication.

1.3 Sample preparation for direct determination of AHL by colorimetry method

1.3.1 Evaluation of direct AHL determination in nutrient broth

AHL standard solution in NB concentration ranged from 0 to 1600 nmol/800 µl were prepared. Non-AHL NB was used as blank. All of AHL-spiked NB were directly subjecting to determine AHL concentrations by colorimetry method as described in 1.1. The experiment was tested for 2 replication.

1.3.2 Evaluation of direct bacterial AHL determination in cell-free supernatant

Cell-free supernatants from mid-log culture of AHL producing strains *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093 and *P.aerunginosa* ATCC 27853 were prepared as described in the section of 1.2.2. *E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811 were also evaluated as non AHL producing strains.

The concentrations of AHL in cell-free supernatant were determined by colorimetry follow the procedure described in figure 3.1. The experiment was set into 2 replication.

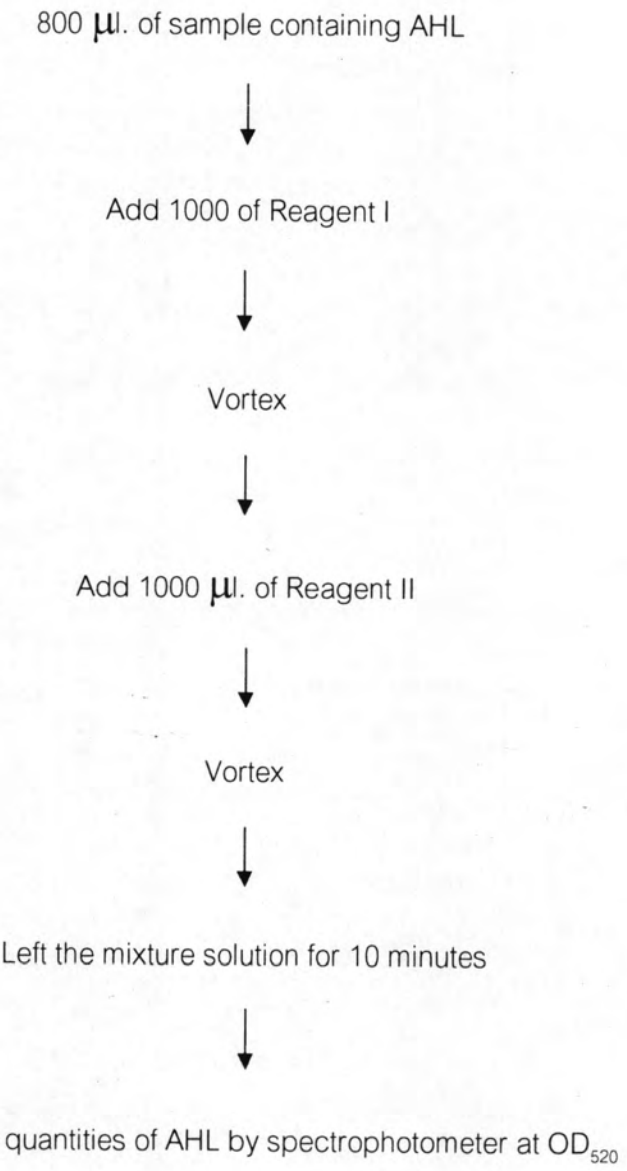

Figure 3.1 Procedure for colorimetry determination of AHL quantification

2. Validation of AHL extraction and colorimetry method

To check validity of both extraction and colorimetry, accuracy, precision, selectivity and sensitivity of the methods were investigated. These were done following the bioanalytical method validation, guidance for industry of United State department of health and human services, food and drug administration (USFDA, MAY 2001).

AHL determination setting up into two procedures were evaluated (Figure 3.2). The first procedure included a step of AHLs extraction from culture broth, purification

and determination by colorimetry. The second procedure was a directly determination of AHL in broth culture.

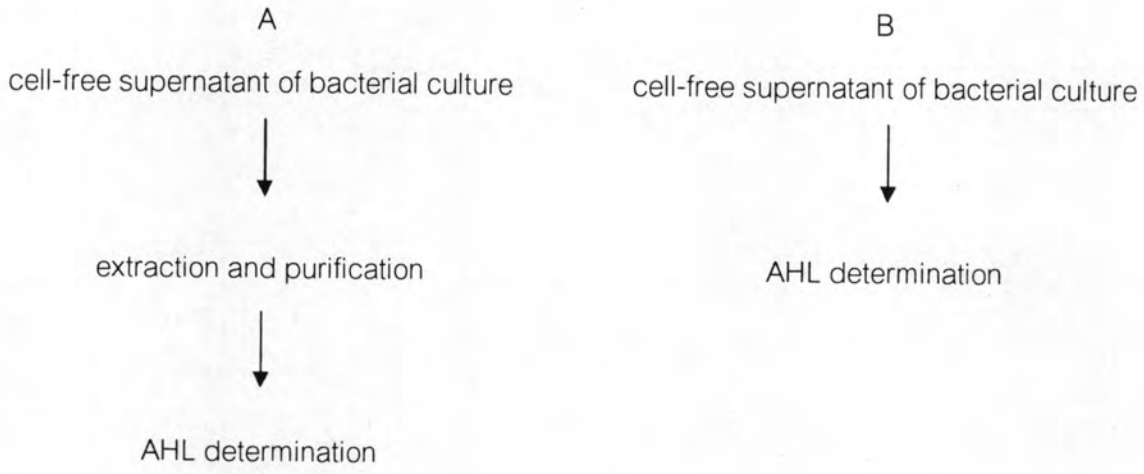

Figure 3.2 AHL determination procedures: Procedure A, AHL in sample was extracted and purified before determined AHL; Procedure B, sample was directly determined for AHL.

2.1 Evaluation for accuracy

The closeness of the mean test results to the true value were evaluated for accuracy validation.

2.1.1 Procedure A for AHL determination

AHL stock solution (0.1 M) was added into NB in different concentrations (100, 200, 300, 400, 500, 600, 700 and 800 nmol/ml, respectively) NB without AHL was used as a negative control sample in this experiment.

The AHL in NB was extracted following the optimized method as demonstrated in 1.2.1. The pellets of AHL obtained from extraction was resuspensed in D.I. water (800 µl) before subjecting to determination for AHL concentration by the optimized colorimetry method from 1.1.

AHL concentrations in NB tested in this experiment were nine levels and each concentration was conducted for 5 replication. Therefore, the sample size of this

experiment was 45 (n = 9x5). The accuracy was expressed as %recovery of measured and nominal values (Equation 3.1). An estimate of the accuracy (%) for each concentration should be lower than 15%.

$$\%\text{recovery} = \frac{\text{measured value x 100}}{\text{nominal value}} \quad (3.1)$$

2.1.2 Procedure B for AHL determination

AHL stock solution (0.1 M) was added into NB 800 µl in different concentrations (100, 200, 300, 400, 500, 600, 700 and 800 nmol, respectively) NB without AHL was used as a negative control sample in this experiment. AHL solution in each concentration was then directly subjecting to AHL determination.

AHL concentrations were nine levels and each concentration was conducted for 5 replication. Therefore, the sample size of this experiment was 45 (n = 9x5). The accuracy at each concentration was expressed as %recovery as described above (2.1.1).

2.2 Precision

The precision evaluated by determining the repeatability of the methods which assessed precision during a single analytical run and between-run. Both of AHL standard and AHL from bacterial cultures were evaluated.

2.2.1 Procedure A for AHL determination

AHL standard solution in NB in different concentrations as described in 2.1.1 were prepared, including NB without AHL, used as a negative control sample in this experiment.

Cell-free supernatants from mid-log cultures of *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093 and *P.aerunginosa* ATCC 27853 were prepared as described in the section of 1.2.2. AHL produced from each strain was then extracted from cell-free supernatant as described in 1.2.1. The concentrations of both

AHL standard and bacterial AHL obtained from the extractions were measured by colorimetry.

AHL standard concentrations in NB were nine levels and each concentration was conducted for 5 replication (n = 45). The precision of extraction method of AHL produced from bacteria was evaluated in three strains of bacteria, and each culture was operated for 5 replication (n = 15).

The precision of both extraction methods were reported in term of % relative standard deviation (%RSD).

2.2.2 Procedure B for AHL determination

NB containing AHL standard (0 – 800 nmol) and cell-free supernatants were prepared, and directly subjecting to determine AHL concentrations by colorimetry.

The precision of both methods were statistically analyzed according to 2.2.1.

2.3 Sensitivity

The lowest concentrations of AHL standard detected by the methods were evaluated. The sensitivity of the method was reported in term lower limit of quantification.

2.3.1 Procedure A for AHL determination

AHL standard in NB in different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nmol/ml, respectively) were prepared and processed for AHL extraction and colorimetry determination.

Mid-log culture of *V.parahaemolyticus* DMST 22092 and *V.parahaemolyticus* DMST 22093 were gravimetrically diluted in NB to give the final cell population in range of 10^3 to 10^7 CFU/ml. Cell population in each dilution was confirmed by performing total plate count (TPC).

Cell-free supernatant of each dilution was collected and processed AHL extraction and colorimetry determination.

AHL standard concentrations in NB were ten levels and each concentration was conducted for 5 replication (n = 50). The sensitivity of extraction method of AHL

produced from bacteria was evaluated in two strains of bacteria, and each culture was operated for 5 replication (n = 10).

2.3.2 Procedure B for AHL determination

The similar samples as described in 2.4.1 were prepared and directly processed AHL colorimetry determination. Similar statistical analysis methodology also performed.

2.4 Selectivity

Specificity of the method was evaluated to test the selectivity by analyzing of negative control samples comparing to positive control samples.

2.4.1 Procedure A for AHL determination

Positive control sample of AHL-spiked NB (300 nmol/ml) and cell-free supernatant of AHL producing strains (*V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093 and *P.aerunginosa* ATCC 27853) were prepared and subjecting to extraction. Also, negative control of non AHL-spiked NB and AHL non-producing strains (*E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811) were investigated. The tests were operated for 5 replication (n = 35).

2.4.2 Procedure A for AHL determination

Similar samples of both AHL standard and cell-free supernatants in 2.3.1 were prepared and directly subjecting to determine AHL concentrations.

3. Investigation of factors associated with AHL production of *V.parahaemolyticus*

3.1 Growth stages

Mid-log culture of *V.parahaemolyticus* DMST 22092 and *V.parahaemolyticus* DMST 22093 were prepared and used as starter culture. Then, the culture was inoculated into NB 10 ml and shook 200 rpm at room temperature until optical density

reached to 0.1 (OD_{600}). The cell suspension 1 ml was inoculated into 100 ml NB medium and shaking 200 rpm at room temperature for 29 hours. Every 1 hour, the cultures were collected for TPC determination.

Cell-free supernatant in each hour were collected and directly subjecting to determine AHL concentration by colorimetry method. The experiment was conducted for 3 replication.

3.2 Strains

Mid-log culture of *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093, *V.parahaemolyticus* ATCC 17802, *V.parahaemolyticus* DMST 23797, *V.parahaemolyticus* DMST 23798 and *V.parahaemolyticus* DMST 23799 were cultured in NB 50 ml and shook 200 rpm in room temperature. The cultures were collected for every 12, 24 and 48 hours. TPC determinations were performed.

Cell-free supernatant in each time was collected and directly processed for AHL colorimetry determination. The test was conducted in 3 replication.

3.3 Cultivation condition

The cultivation conditions investigated in this experiment were media, salt concentrations and temperatures. Mid-log culture of *V.parahaemolyticus* ATCC 17802 and *V.parahaemolyticus* DMST 23798 (10 ml) were cultured in varied cultivation condition as described in Table 3.1. In each condition, culture was shook 200 rpm for 24 hours.

TPC was investigated similarly as performed in 3.2. AHL colorimetry was also determined similarly. The process was performed in 3 replication.

Table 3.1 The investigated cultivation condition of *V.parahaemolyticus*

cultivation conditions	varied condition as:
3.3.1 media	NB and LB (A1, Appendix A)
3.3.2 sodium chloride concentrations	NB supplemented with 0.5, 3 and 8% sodium chloride
3.3.3 temperatures	15 °C and room temperature

4. Determination of AHL profile of *V.parahaemolyticus* by HPLC

4.1 Optimization of HPLC condition

AHL standard solution was prepared by dissolved AHL crystal (0.6 mg) in 1 ml. of D.I. water to use for HPLC condition optimization.

The AHL standard solution was analyzed by high performance liquid chromatography (HPLC) on a Waters 600E multisolvent delivery system equipped with , Luna C_{18} II column, 100A 150 x 4.6 mm (Phenomenex). The HPLC detector was Waters 2487 Dual Absorbance. Separation was performed with water (MilliQ) containing TFA (50 µl/l) and acetonitrile.

HPLC condition was conducted according to an instruction of the AHL standard manufacturer (Sigma-Aldrich Co., C2 and Table C1 in Appendix C). An optimum time of gradient varied in Table 3.2 was evaluated. Flow rate was adjusted to 1.5 ml/minute.

The volume of injection was 10 µl. After running, the column were equilibrated for 10 minutes. The separated analytes were detected at 210 nm. The experiment was tested in 3 replication.

Table 3.2 The gradient profile used to optimize HPLC condition for the study of AHL pattern

gradient profile (acetonitrile:water)	minute		
	profile 1	profile 2	profile 3
5 : 95	0	0	0
35 : 65	20	18	15
100 : 0	25	23	20
100 : 0	30	28	25
5 : 95	32	30	30

4.2 Optimization of AHL extraction from colonies

The AHL positive strains of *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093, *P.aerunginosa* ATCC 27853 and AHL negative strains (*E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811) were streaked onto NA media plate and inoculated at room temperature for 24 hours. After that, the isolated colonies were picked by sterile cotton swob and spreaded onto another NA plate, incubating 24 hours at room temperature.

The 24 hours colonies were picked using sterile loop and suspended in D.I. water as the ratio shown in Table 3.3.

The cell-suspension was centrifuged 13,000 rpm at 4 °C for 10 minutes. Supernatant containing AHLs was then filtered through 0.45 micron syringe filter to remove cell debris and impuritants, stored at -35 °C for HPLC analysis. The study was conducted in 3 replication.

Table 3.3 The ratio of colonies to the volume of D.I. water used to optimization the AHL extraction condition from bacterial colonies

AHL extracted colonies	volume of water
1 foll-loop	100 µl
	500 µl
2 full-loop	100 µl
	500 µl

4.3 Factors associated with AHL pattern of *V.parahaemolyticus* in agar media

4.3.1 Strains

Colonies of *V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093, *V.parahaemolyticus* ATCC 17802, *V.parahaemolyticus* DMST 23797, *V.parahaemolyticus* DMST 23798 and *V.parahaemolyticus* DMST 23799 were prepared as described in 5.1, excepted the incubation time was 24, 48 and 72 hours.

The colonies in each incubation time was collected to AHL extraction and AHL profile of each strain was analyzed by HPLC by optimized procedure of 5.1 and 5.2, respectively. The analysis was repeated three times.

4.3.2 Cultivation condition

Each of *V.parahaemolyticus* DMST 23798 and *V.parahaemolyticus* ATCC 17802 strain was spreaded onto various agar plates conditioning as shown in Table 3.4.

AHL was extracted from colony and supernatant was directly subjecting to AHL profile determination by HPLC. The experiment was done for three replication.

Table 3.4 The cultivation condition used to determine the AHL pattern by HPLC

cultivation conditions	varied conditions as:	control conditions
media	NA, PCA, TCBS and LB agar[A]	incubation at room temperature
sodium chloride concentrations	NA supplemented with 0.5, 3 and 8% sodium chloride	incubation at room temperature
temperatures	15 °C and room temperature	cultured on NA agar plate

[A]A2 in Appendix A

5. Evaluation and application of colorimetry method for detection of AHL from *V.parahaemolyticus* in pre-enrichment selective media

Nowadays, detection of *V.parahaemolyticus* in food was still relied on standard MPN method, which time-consuming and the interpreting of the result was easily incorrect. Therefore, in this part, there was an attempt to develop the isolation and detection of *V.parahaemolyticus* in food based on their unique AHL production property.

V.parahaemolyticus is halophilic bacterial which required 1-3% of sodium chloride in culture media for optimum growth. 8% sodium chloride inhibited the commonly found food-pathogen *Vibrio* species, except *V.parahaemolyticus*. From this unique salt-torolence property, peptone water containing 8% sodium chloride was used as selective culture of *V.parahaemolyticus*. This detection was performed after pre-enrichment the food sample in alkaline peptone water.

The investigation was performed in 2 systems. There were single culture of *Vibrio* strains and mixed culture of *V.parahaemolyticus* with other *Vibrio* strains as shown in Table 3.3. The reason for choosing these bacteria was that they commonly found in seafood, also they were well-documented as human pathogens.

Mid-log culture of *V.parahaemolyticus* DMST 23798, *V.chlorelae* DMST 2873 and *V.valnificus* ATCC 27562 were serially diluted to 10^{-3} dilution. Diluted cell suspension of *V.parahaemolyticus* DMST 23798 was mixed with either of *V.chlorelae* DMST 2873 or *V.valnificus* ATCC 27562 as described in Table 3.3. Then 1 ml of both single and mixed

culture were inoculated into 100 ml of 1% peptone water containing 8% sodium chloride (A3, Appendix A), shaking 200 rpm at room temperature.

Cell-free supernatants were collected every two hour for 26 hours and then subjected to AHL determination by colorimetry method. The experiment was tested in 3 replication.

Table 3.5 Bacterial strains and mixing ratio used to evaluated the colorimetry method for AHL detection

bacterial strains	mixing ratio
V.parahaemolyticus DMST 23798	100% of single culture
V.chlorelae DMST 2873	100% of single culture
V.valnificus ATCC 27562	100% of single culture
V.parahaemolyticus DMST 23798 + *V.chlorelae* DMST 2873	1:1 (500 µl:500µl)
V.parahaemolyticus DMST 23798 + *V.valnificus* ATCC 27562	1:1 (500 µl:500µl)