CHAPTER IV

RESULTS AND DISCUSSIONS

1. Optimization of AHL detection and extraction

1.1 Optimization of AHL detection by colorimetry method

The colorimetry method for AHL detection was proposed by Yang et al in year 2006. This technique used multiscanner spectrophotometer which micro volume sample (20 – 50 μl) was required. In order to use simple UV absorbance spectrophotometer for AHL analysis, this colorimetry method could be modified by increasing sample volume to approximately 1 ml.

In this study, the colorimetry method using simple spectrophotometer was evaluated for analysis of AHL in broth media system. Volume of sample included Reagent I and Reagent II were increased for twenty folds (800, 1000 and 1000 μl, respectively) until the total volume was sufficient for the spectrophotometer detection. The total volume of 2.8 ml was therefore used for the absorption reaction of AHL-ferric chloride complex in this experiment.

In preliminary experiment, occurring of yellow to dark brown color would present after approximately 10 minutes after adding Reagent II into AHL standard solution. The color-complex were absorbed a maximum wavelength of 520 nm (λ_{max} = 520) scanned by spectrophotometer. Therefore, the condition for wavelength absorption measurement through the study were λ_{max}520 (OD_{520}), and 2.8 ml of total sample volume.

The evaluation of AHL analysis by colorimetry method was conducted by varied AHL standard solutions (0 to 1600 nmol/800 μl) in D.I. water. The concentration levels were plotted against their absorbance (O.D.) as shown in Figure D1 in Appendix D.

According to Figure D1 in Appendix D, %RSD of OD_{520} measured from AHL standard solution in concentrations range 0 to 30 nmol/800 µl were higher than 15 (Table D1, Appendix D). Since the measurements of OD_{520} from these AHL concentrations exhibited higher error than acceptable limit proposed by USFDA guideline (2001). Therefore these data were excluded from the calibration curved. In the other hand, AHL concentrations in range 1400 – 1600 nmol were also excluded even their %RSD were acceptable. This was due to their OD_{520} influenced the coefficient of determination (R^2) of the whole linear line lower than 0.9850 (Figure D1 Appendix D).

Therefore, AHL concentration in range of 30 to 1300 nmol were used for building the calibration curve. The equation representing the relationship of the calibration line was Y = 0.0002 X + 0.0575 and the coefficient of determination (R^2) was 0.9935 (Figure 4.1).

Since OD_{520} represented intensity of color-complex from a reactive of AHL and ferric chloride, it would determine an AHL concentration in determination sample system.

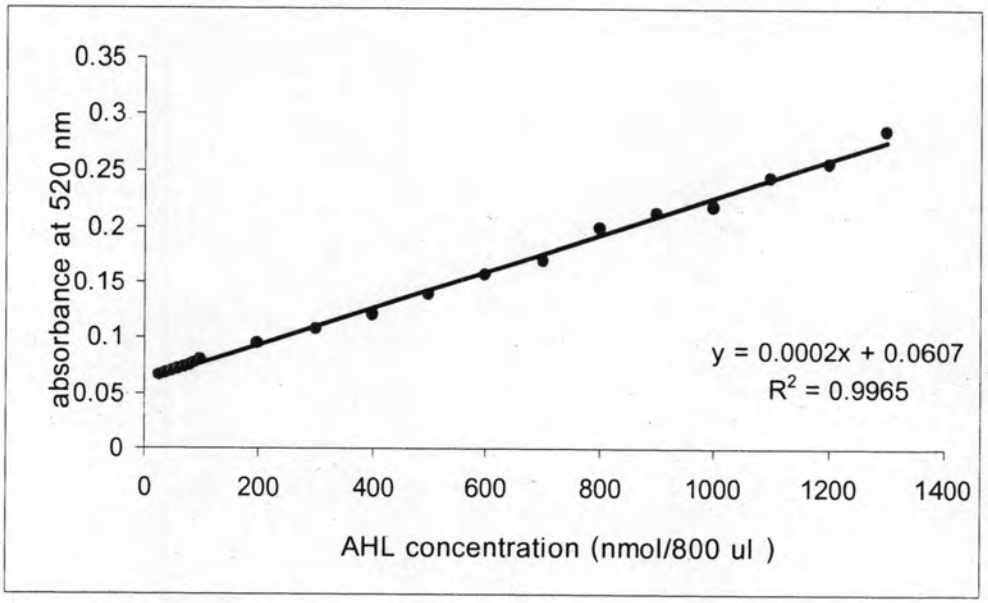


Figure 4.1 The calibration curve of AHL concentrations (30 – 1300 nmol/800 µl) in deionized water and their absorbance values (λ_{max} = 520 nm)

1.2 Optimization of AHL extraction methods

1.2.1 Extraction of AHL standard from NB

Chemical signal in environment is generally present in low concentration (Schupp et al, 2005). Therefore, the signal molecule in sample was normally extracted, purified and concentrated before AHL determination. Liquid-liquid extraction method (LLE) have been widely used to extract and purify AHL from sample matrix. Organic solvent reported for the AHL extraction were ethyl acetate, methanol, dichloromethane or chloroform. (van Brussel et al, 1985; Pearson et al, 1994; Schripsema et al, 1996; Shaw et al, 1997; Jacobi et al, 2003; Frommberger et al, 2004; Schupp et al, 2005).

In this study, preliminary experiment was evaluated for the optimization of AHL extraction by LLE method using different organic solvent as stated above. It was found that methanol could not be used for AHL extraction in aqueous sample because there was no separation between liquid sample and methanol phase. Ethyl acetate and dichloromethane were not applicable because their evaporation rates were low. This resulting in AHL lost during the evaporation process.

Chloroform shows to be a potential organic solvent. It could be used to extract AHL from water-containing sample because of its nonpolar property. This property makes chloroform to completely separate from aqueous phase, such as broth media used in this study. In addition, chloroform also has low boiling point, it therefore volatiles rapidly. This allows the solvent evaporation step to be performed rapidly.

According to the preliminary experiment, chloroform was selected to use as an organic solvent for AHL extraction. Therefore in this study, an optimum volume of chloroform for the extraction was evaluated. This was done by spiking known concentration of AHL standard into NB then determined %AHL recovery after the extraction, as shown in Table 4.1. %recovery was calculated from Equation 4.1 shown below.

$$\% \text{ recovery} = \frac{\text{absorbance of the extracted AHL}}{\text{absorbance of AHL standard}} \times 100 \qquad (4.1)$$

From Table 4.1, OD_{520} and %recovery of the extraction method using 1 ml of chloroform were the significantly lowest. Two ml of chloroform exhibited 96.60 of %recovery which was not statistically different from %recovery of 3 ml chloroform. Based on %recovery, 2 ml of chloroform could be an optimum volume for AHL extraction from NB. Therefore this volume was selected to use for AHL extraction through this study.

Table 4.1 %recovery obtained from the AHL extraction using various volumes of chloroform

volume of chloroform (ml)	OD_{520} [A]	% recovery
1 ml	0.0742 ± 0.0018	68.92
2 ml	0.1046 ± 0.0010	97.23
3 ml	0.1039 ± 0.0016	96.60

[A] mean ± SD of OD_{520} from 2 replication

1.2.2 Extraction of bacterial AHL from bacterial cultures

The optimum condition for the extraction method was also evaluated for bacterial AHL extraction from broth culture.

Mid-log culture of two strains of *V.parahaemolyticus* and one strain of *P.aerunginosa* were used for AHL extraction from cell-free supernatant by chloroform. These represented AHL producing reference strains. *E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811 supernatant cultures were also tested for AHL extraction. These bacteria represented AHL non-producing reference strains.

In order to investigate AHL producing property, bacteria strains were mostly cultured in media broth to middle and late logarithmic phase of cell growth (Shaw et al, 1997; Gram et al, 1999; Blossler and Gray, 2000). AHL was detectable when cell numbers reached mean value of approximately 10^6 CFU/ml (Bruhn et al, 2004; Gram et al, 2005). According to these previous reports, therefore in this study, mid-log cultures of

reference strains containing approximately 10^7 - 10^8 CFU/ml were used to evaluate the extraction.

Table 4.2 OD_{520} of bacterial AHL extracted from broth cultures

cultures	OD_{520} [A]	TPC (logCFU/ml)
V.parahaemolyticus DMST 22092	0.0736 ± 0.0009	9.99 ± 0.07
V.parahaemolyticus DMST 22093	0.0724 ± 0.0015	10.12 ± 0.06
P.aerunginosa ATCC 27853	0.0713 ± 0.0009	9.80 ± 0.05
E.coli ATCC 25922	0	10.26 ± 0.02
Salmonella sp. ATCC 13811	0	8.47 ± 0.01

[A] mean of OD_{520} ± SD of 2 replication

Results as shown in Table 4.2, OD_{520} observed in extracts of AHL-producing cultures solution containing colorimetry Reagent I and Reagent II indicated that there were color complex of ferric chloride reaction occurred. In contrast, OD_{520} of 0 observed in extracts of non AHL producing culture solution containing colorimetry Reagent I and Reagent II indicated that non of color complex was occurred. These results indicated that any substances available in cell culture would not interfere the AHL determination. Therefore, the procedure included AHL extraction and detection by colorimetry could be used for bacterial AHL analysis.

1.3 Sample preparation for direct determination of AHL by colorimetry method

As stated in 1.2, AHL extraction using chloroform was performed in order to purify and concentrate AHL signals for the analysis efficientcy improvement. However, the extraction step made the procedure more complicate and possibly caused the loss of AHL during the process.

Therefore, to develop a faster and less laborious method for the AHL analysis, AHL determination by directly measuring the absorbance of AHL-ferric molecule in NB without performing the extraction step, so called "direct AHL determination", was evaluated.

1.3.1 Evaluation of direct AHL determination in nutrient broth

This study was to evaluate the efficientcy of the method for direct AHL determination by omitting the extraction step. AHL standard was spiked into NB. Reagent I and Reagent II were then directly added allowing ferric-AHL complex reaction to be occurred before subjected to absorbance measurement. As proposed in the guidance for industry, bioanalytical method validation, U.S. department of health and human service (May 2001), the calibration curve should be prepared in the same biological matrix as the sample in intended study. To meet this guidence, another calibration curve of AHL standard in NB, concentration in range 0 to 1300 nmol/800 μl, was constructed. AHL-spiked NB were directly subjecting to absorbance measurement after ferric chloride reaction. The AHL concentrations were plotted against their absorbance as shown in Figure D2 (Appendix D).

According to Figure D2 in Appendix D, results corresponded to the results as reported in Figure 4.1, relationship of OD_{520} and AHL concentration in range of 0 to 20 and 1400 to 1600 nmol were excluded as previous described. Again, only AHL concentration in range of 30 to 1300 nmol were used to construct the calibration curve of direct AHL determination. The equation for the relationship of $Y = 0.0002 X + 0.0594$ and the coefficient of determination (R^2) of 0.9981 (Figure 4.7) shown in Figure 4.2.

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

The direct bacterial AHL determination was evaluated its efficiency by testing on cell-free supernatants of both AHL producing and non-producing bacterial strains as used in 1.2.2. OD_{520} of ferric-AHL complex in cell-free supernatant and TPC of cultures were determined and shown in Table 4.3.

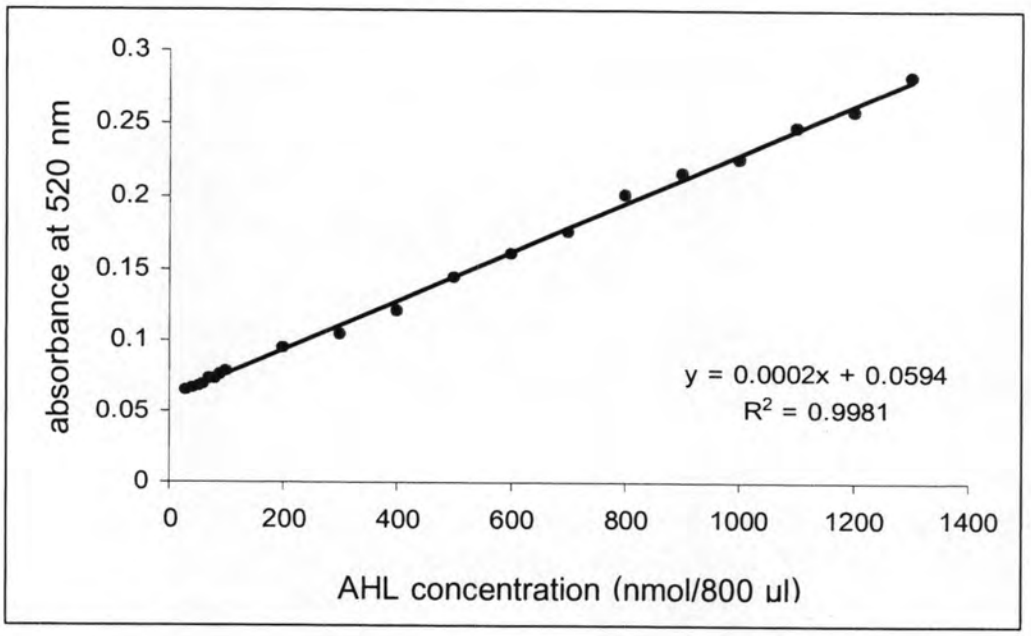


Figure 4.2 The calibration curve of AHL concentrations in range 30 – 1600 nmol/800 µl and their absorbance values (λ_{max} = 520 nm) directly measure in NB

Table 4.3 TPC and OD_{520} of bacterial AHL in cell-free supernatants measured by direct AHL determination

cultures	TPC [A] (logCFU/ml.)	OD_{520}
V.parahaemolyticus DMST 22092	9.84 ± 0.08	0.0803 ± 0.0011
V.parahaemolyticus DMST 22093	9.83 ± 0.08	0.0750 ± 0.0004
P.aerunginosa ATCC 27853	9.07 ± 0.06	0.0730 ± 0.0004
E.coli ATCC 25922	10.02 ± 0.04	0
Salmonella sp. ATCC 13811	8.81 ± 0.07	0

[A] mean ± SD of 2 replication

According to the Table 4.3, there was no color complex in the cell-free supernatant of non AHL producing cultures while there were color complex observed in that of AHL producing cultures. These results suggested that substances in NB could not form color complex with ferric chloride. Therefore, the direct determination of AHL in cell-free supernatant culture could be used in further study.

The AHL determinations by ferric chloride complexation from cell-free supernatant of bacterial culture with or without further extraction were optimized in this study. However, these methods needed to be validated in their accuracy, precision, sensitivity and selectivity before applying in AHL analysis of unknown sample.

2. Validation of AHL extraction and colorimetry method

Quantification analysis of AHL was proposed by several previous studies. Most of the methods such as bioassay and TLC relied on specificity of AHL receptor of genetically engineering indicator strains. However, non of indicator strains containing receptor for all type of AHLs was constructed. Direct method for quantification of total AHL presented in sample had still not been available. Recently, Yang et al (2006) proposed new AHL quantification methodology based on colorimetry method. This technique based on chemical reaction between lactone group and ferric ion resulting in coloring complex. As all type of AHL contained lactone group, this colorimetry method therefore detected all AHL signals. However, this report evaluated colorimetry analysis subjecting to determine commercial AHL standards, and there were no report about quantification of AHL produced in bacterial culture based on this colorimetry analysis until now. Therefore, this study aimed to validate the detection of bacterial AHL by two colorimetry analysiss developed as Procedure A and Procedure B, as described in material and method in chapter 2. Validation parameters included accuracy, precision, sensitivity and selectivity. The validations were conducted by following USFDA guideline (2001).

2.1 Accuracy

The accuracy is an exactness of the result in compare with true value obtained by performing the same method. The exactness value usually exhibits in term of %recovery.

In this study, two colorimetry analysis procedures (Procedure A and Procedure B, section 2 of chapter 3) were used to quantify AHL extracted from broth media and AHL

existing in broth media. The accuracy values were statistically analysed as %recovery (Equation 3.1).

2.1.1 Procedure A for AHL determination

AHL-spiked NB in concentration range of 0 to 800 nmol/ml were used to evaluate an accuracy of Procedure A for AHL analysis. AHL was extracted from NB and purified before subjecting to measure absorbance of AHL color complex. OD_{520} of each AHL concentration was compared to calibration curve (Figure 4.1). The same tested experiment was repeated in another day. The results were shown in Table 4.4.

%recovery indicated a difference of measured AHL concentration extracted from D.I. water and broth media as listed in Table 4.4. OD_{520} of the AHL concentration in range 0 to 800 nmol/ml were approximately 0.279 to 12.1200. This study was also repeated by conducting the similar experiment in day 2. The results also revealed a similarly range of %recovery. According to USFDA guideline (2001), an acceptable accuracy limit shall be within ±15%. Therefore, the accuracy of this procedure was an acceptable for determination of AHL extracted from NB in concentration ranges 0 to 800 nmol/ml.

Table 4.4 : %recovery of AHL in NB determination by Procedure A

AHL concentrations (nmol/ml)	nominal absorbance[A]	OD_{520}			
		day 1		day 2	
		mean [B]	% recovery	mean [B]	% recovery
0	0	0	0	0	0
100	0.0800	0.0777[a] ± 0.0014	97.08	0.0779[a] ± 0.0020	97.400
200	0.0962	0.0934[b] ± 0.0007	97.18	0.0934[b] ± 0.0019	97.1607
300	0.1076	0.1041[c] ± 0.0013	96.71	0.1018[c] ± 0.0018	94.6097
400	0.1204	0.1165[d] ± 0.0010	96.82	0.1172[d] ± 0.0019	97.3494
500	0.1395	0.1393[e] ± 0.0009	99.84	0.1391[e] ± 0.0030	99.7419
600	0.1579	0.1548[f] ± 0.0009	98.07	0.1559[f] ± 0.0008	98.7393
700	0.1704	0.1666[g] ± 0.0010	97.78	0.1686[g] ± 0.0009	99.0493
800	0.1995	0.1942[h] ± 0.0005	97.38	0.1954[h] ± 0.0019	97.9544

[a,b,c,...] values with significantly difference in each row are indicated by different letters ($P < 0.05$ by Duncan's new multiply range test)

[A] absorbance value calculated from calibration curve (Figure 4.1)

[B] mean ± SD of 5 replication

2.1.2 Procedure B for AHL determination

This procedure was developed for faster AHL determination by performing a direct absorbance measuring of AHL-ferric complex in broth media without extraction and purification steps. AHL-spiked NB concentration in range from 0 to 800 nmol/ 800 μl were used to evaluate an accuracy of exploided Procedure B. OD_{520} of each AHL concentration was compared to calibration curve (Figure 4.2). The accuracy value was reported in term of %recovery as shown in Table 4.5.

Table 4.5 : %recovery of AHL in NB determination by Procedure B

AHL concentrations (nmol/ml)	nominal absorbance[A]	OD_{520}			
		day 1		day 2	
		mean [B]	% recovery	mean [B]	% recovery
0	0	0	0	0	0
100	0.0792	$0.0790^{a} \pm 0.0011$	99.70	$0.0785^{a} \pm 0.0022$	99.0909
200	0.0957	$0.0959^{b} \pm 0.0014$	100.23	$0.0962^{b} \pm 0.0014$	100.5016
300	0.1046	$0.1048^{c} \pm 0.0014$	100.22	$0.1053^{c} \pm 0.0016$	100.6982
400	0.1208	$0.1206^{d} \pm 0.0014$	99.82	$0.1211^{d} \pm 0.0021$	100.2318
500	0.1439	$0.1434^{e} \pm 0.0017$	99.69	$0.1429^{e} \pm 0.0023$	99.3396
600	0.1600	$0.1590^{f} \pm 0.0020$	99.43	$0.1596^{f} \pm 0.0020$	99.7687
700	0.1751	$0.1748^{g} \pm 0.0018$	99.82	$0.1730^{g} \pm 0.0024$	98.7778
800	0.2006	$0.2001^{h} \pm 0.0024$	99.79	$0.2006^{h} \pm 0.0021$	100.0249

[a.b.c.] values with significantly difference in each column are indicated by different letters ($P < 0.05$ by Duncan's new multiply range test)

[A] OD_{520} values calculated from calibration curve (Figure 4.1)

[B] mean ± SD of 5 replication

According to Table 4.5, %recovery of every spiked AHL concentration were in range -6 to 3%, which were not over ±15%. The relative difference of the test in day two still remained under ±15%. Since an accuracy of this analysis procedure met the USFDA guideline, therefore it was acceptable for using to determine AHL in media broth concentrations in range 0 to 800 nmol/800 µl.

2.2 Precision

As stated in literature review, the precision of the method is a consistency of the results obtained by performing the same method. The consistency value generally indicates in term percent of standard deviation (%RSD). In this study, AHL-spiked NB and cell-free supernatant from culture were used to validate the precision of Procedure A and Procedure B.

2.2.1 Procedure A for AHL determination

AHL standard spiked in NB concentrations ranged from 0 to 800 nmol/ml were extracted and determined AHL. Mean of OD_{520} of five replication of each concentration was calculated. The precision value was reported in term of %RSD as shown in Table 4.6.

Bacterial AHL in cell-free supernatant collected from different bacterial cultures were determined through Procedure A. The results were shown in Table 4.7.

According to Table 4.6 and 4.7, %RSD of OD_{520} obtained from every determinations were under ±15%, which met the USFDA guideline (2001). In addition, when interday precision was further investigated in AHL-spiked NB, the similar results were found. Therefore, Procedure A could be used as a precision AHL determination method.

Table 4.6 OD_{520} and %RSD of standard AHL in NB determination by Procedure A

AHL concentrations (nmol/ml.)	OD_{520}			
	day 1		day 2	
	mean [A]	%RSD	mean [A]	%RSD
0	0	0	0	0
100	0.0777[a] ± 0.0014	1.8584	0.0779[a] ± 0.0020	2.5770
200	0.0934[b] ± 0.0007	0.7850	0.0934[b] ± 0.0019	2.0174
300	0.1041[c] ± 0.0013	1.2411	0.1018[c] ± 0.0018	1.7993
400	0.1165[d] ± 0.0010	0.8218	0.1172[d] ± 0.0019	1.6134
500	0.1393[e] ± 0.0009	0.6430	0.1391[e] ± 0.0030	2.1354
600	0.1548[f] ± 0.0009	0.5814	0.1559[f] ± 0.0008	0.5322
700	0.1666[g] ± 0.0010	0.5902	0.1686[g] ± 0.0009	0.5121
800	0.1942[h] ± 0.0005	0.2758	0.1954[h] ± 0.0019	0.9785

[a,b,c,...] values with significantly difference in each row are indicated by different letters ($P < 0.05$ by Duncan's new multiply range test)

[A] mean ± SD of 5 replication

Table 4.7 %RSD and OD_{520} obtained from the determination of bacterial AHL in cell-free supernatants by Procedure A

cultures	OD_{520}	
	mean [A]	%RSD
V.parahaemolyticus DMST 22092	0.0805 ± 0.0013	1.56
V.parahaemolyticus DMST 22093	0.0780 ± 0.0031	3.93
P.aerunginosa ATCC 27853	0.0710 ± 0.0012	1.72

[A] mean ± SD of 5 replication

2.2.2 Procedure B for AHL determination

AHL standard in NB and bacterial AHL in cell-free supernatant samples were directly measured for absorbance of AHL-ferric complex following Procedure A. Precision values of the method in term of %RSD were shown in Table 4.8 and 4.9.

Table 4.8 OD_{520} and %RSD of standard AHL in NB determination by Procedure B

AHL concentrations (nmol/800 µl)	OD_{520}			
	day 1		day 2	
	mean [A]	%RSD	mean [A]	%RSD
0	0	0	0	0
100	0.0790[a] ± 0.0011	1.4373	0.0785[a] ± 0.0022	2.8140
200	0.0959[b] ± 0.0014	1.4378	0.0962[b] ± 0.0014	1.4839
300	0.1048[c] ± 0.0014	1.2686	0.1053[c] ± 0.0016	1.4721
400	0.1206[d] ± 0.0014	1.1453	0.1211[d] ± 0.0021	1.7380
500	0.1434[e] ± 0.0017	1.1967	0.1429[e] ± 0.0023	1.6133
600	0.1590[f] ± 0.0020	1.2462	0.1596[f] ± 0.0020	1.2481
700	0.1748[g] ± 0.0018	1.0020	0.1730[g] ± 0.0024	1.3734
800	0.2001[h] ± 0.0024	1.2155	0.2006[h] ± 0.0021	1.0516

[a,b,c,...] values with significantly difference in each row are indicated by different letters ($P < 0.05$ by Duncan's new multiply range test)

[A] mean ± SD of 5 replication

Table 4.9 OD_{520} and %RSD of bacterial AHL in cell-free supernatant determination by Procedure B

culture	OD_{520}	
	mean [A]	%RSD
V.parahaemolyticus DMST 22092	0.0840 ± 0.0035	4.190337
V.parahaemolyticus DMST 22093	0.0822 ± 0.0022	2.676399
P.aerunginosa ATCC 27853	0.0722 ± 0.0019	2.614777

[A] mean ± SD of 5 replication

From Table 4.8 and 4.9, %RSD of absorbance of every AHL in NB concentration range of 0 to 800 nmol/800 µl and supernatant cultures were under ±15%, and %RSD of interday precision analysis was also similar. Therefore, the Procedure B could be a precise analysis for both AHL in NB and bacterial AHL in culture supernatant.

2.3 Sensitivity

The sensitivity is the lowest limit of detection with an acceptable accuracy and precision of the method. In this section, both AHL-spiked NB and cell-free supernatant was used for validating the sensitivity of Procedure A and Procedure B.

2.3.1 Procedure A for AHL determination

Sensitivity of Procedure A in determination of AHL standard in NB was validated by measuring absorbance values of color complex in the AHL-spiked NB solution at the concentration range of 10 to 100 nmol/ml. Since the sensitivity of the method is the lowest detection limit as stated above, therefore to evaluate the sensitivity of the method, %recovery and %RSD of each determination were calculated and shown in Table 4.10.

Sensitivity of Procedure A in determination of bacterial AHL in cell-free supernatants were also validated by measuring absorbance of the color complex in gravimetrical diluted cultures containing cell populations (TPC) ranged from 3 to 7 logCFU/ml. The lowest detection limit with an acceptable. %RSD of OD_{520} were evaluated in term of total viable cell (TPC) as shown in Table 4.10.

Table 4.10 %RSD and %recovery obtained from standard AHL in NB determination by Procedure A

AHL (nmol)	nominal absorbance[B]	OD_{520}		
		mean [A]	% RSD	%recovery
10	0.0037	0.0059 ± 0.0058	97.9445	160.541
20	0.0419	0.0288 ± 0.0200	71.5511	68.7829
30	0.0663	0.0655 ± 0.0016	2.4736	98.7934
40	0.0671	0.0664 ± 0.0013	2.0041	99.0164
50	0.0689	0.0677 ± 0.0015	2.2107	98.2584
60	0.0702	0.0690 ± 0.0011	1.5876	98.2906
70	0.0732	0.0717 ± 0.0019	2.6842	97.924
80	0.0754	0.0737 ± 0.0018	2.4612	97.7719
90	0.0777	0.0760 ± 0.0014	1.7829	97.8636
100	0.0807	0.0800 ± 0.0020	2.4997	99.1822

[A] mean ± SD of 5 replication

[B] OD_{520} calculated from calibration curve (Figure 4.1)

Table 4.11 %RSD obtained from bacterial AHL in cell-free supernatant determination by Procedure A

dilutions	*V.parahaemolyticus* DMST 22092			*V.parahaemolyticus* DMST 22093		
	TPC [A]	OD_{520}		TPC [C]	OD_{520}	
		mean [B]	%RSD		mean [D]	%RSD
0	7.15 ± 0.08	0.0754 ± 0.0011	1.4138	7.34 ± 0.04	0.0659 ± 0.0018	2.7171
9:10	7.12 ± 0.05	0.0737 ± 0.0006	0.8009	7.29 ± 0.05	0.0659 ± 0.0008	1.2753
8:10	7.03 ± 0.06	0.0706 ± 0.0010	1.4497	7.21 ± 0.06	0.0650 ± 0.0009	1.4205
7:10	6.90 ± 0.09	0.0673 ± 0.0010	1.5127	7.00 ± 0.06	0.0623 ± 0.0007	1.1031
6:10	6.64 ± 0.06	0.0630 ± 0.0010	1.5445	6.86 ± 0.06	0.0528 ± 0.0016	3.0650
5:10	6.35 ± 0.03	0.0556 ± 0.0020	3.5215	6.54 ± 0.06	0.0243 ± 0.0136	56.145
4:10	5.96 ± 0.07	0.0196 ± 0.017	88.9820	6.12 ± 0.06	0.0608 ± 0.0044	72.7398
3:10	5.45 ± 0.07	0.0037 ± 0.0052	141.4472	5.54 ± 0.09	0.0055 ± 0.0081	147.4298
2:10	4.70 ± 0.06	0.0099 ± 0.0133	133.9873	4.87 ± 0.05	0.0068 ± 0.0101	148.9484
1:10	3.74 ± 0.06	0.0051 ± 0.0088	174.0567	3.83 ± 0.08	0.0016 ± 0.0033	200.5996

[A,B,C,D] mean ± SD of 5 replication

According to Table 4.10, apart from the determination in AHL concentration ranged from 30 to 100 nmol/ml, %RSD and %relative absorbance obtained from all determinations were under ± 15.

From Table 4.11, %RSD of OD_{520} determined in *V.parahaemolyticus* DMST 22092 supernatant were under ± 15 if TPC in the culture ranged from 6.65 to 7.15 logCFU/ml. When evaluated by using *V.parahaemolyticus* DMST 22093 as a test, the similar results were found.

Based on the sensitivity of Procedure A evaluated in this study, the procedure could be used to analyze AHL in NB at concentration low to 30 nmol/ml. the procedure could detect AHL production from *V.parahaemolyticus* culture containing population approximately 6 logCFU/ml.

2.3.2 Procedure B for AHL determination

AHL-spiked NB prepared as described in 2.3.1 were used to evaluate the sensitivity of p Procedure B. Results were shown in Table 4.12.

The similar testes of bacterial cultures were also used to evaluate the sensitivity of Procedure A to bacterial AHL determination. The results were shown in Table 4.13.

According to Table 4.12 and 4.13, the results were similar to the result of Procedure A (Table 4.10 and 4.11). The %RSD and %recovery obtained from determination of AHL in NB at the concentration ranged from 30 to 300 nmol/800 µl were in acceptable range. Therefore, the sensitivity of Procedure B for determination of AHL in NB was 30 nmol (equal to 30 µM).

The procedure could determine AHL produced from *V.parahaemolyticus* in the culture containing population approximately 6 logCFU/ml, which was similar to sensitivity of Procedure A.

Table 4.12 %RSD and %recovery obtained from standard AHL in NB determination by Procedure B

AHL (nmol/800 µl)	nominal absorbance[B]	OD_{520}		
		mean [A]	%RSD	%recovery
10	0.0037	0.0041 ± 0.0051	125.5022	109.7297
20	0.0419	0.0257 ± 0.0190	73.1411	61.2888
30	0.0663	0.0655 ± 0.0016	2.3828	98.7330
40	0.0671	0.0663 ± 0.017	2.6600	98.8376
50	0.0689	0.0655 ± 0.0016	3.2111	97.0972
60	0.0702	0.0669 ± 0.0021	2.9929	98.5470
70	0.0732	0.0721 ± 0.0018	2.5253	98.5246
80	0.0754	0.0734 ± 0.0021	2.8562	97.3475
90	0.0777	0.0761 ± 0.0014	1.8883	97.9408
100	0.0807	0.0797 ± 0.0022	2.6953	98.7856

[A] mean ± SD of 5 replication

[B] OD_{520} calculated from calibration curve (Figure 4.1)

Table 4.13 : %RSD obtained from bacterial AHL in cell-free supernatant determination by Procedure B

dilutions	*V.parahaemolyticus* DMST 22092			*V.parahaemolyticus* DMST 22093		
	TPC [A]	OD_{520}		TPC [C]	OD_{520}	
		mean [B]	%RSD		mean [D]	%RSD
0	7.15 ± 0.16	0.0769 ± 0.0009	1.1448	7.92 ± 0.04	0.0721 ± 0.0009	1.2920
9:10	7.12 ± 0.08	0.0758 ± 0.0008	1.10278	7.85 ± 0.08	0.0702 ± 0.0008	1.2045
8:10	7.03 ± 0.11	0.0736 ± 0.0009	1.2581	7.73 ± 0.07	0.0683 ± 0.0009	1.3514
7:10	6.9 ± 0.04	0.0716 ± 0.0010	1.4446	7.54 ± 0.09	0.0658 ± 0.0009	1.3118
6:10	6.66 ± 0.09	0.0686 ± 0.0007	1.0280	7.33 ± 0.07	0.0420 ± 0.0021	4.9142
5:10	6.35 ± 0.07	0.0656 ± 0.0013	1.9963	7.05 ± 0.03	0.0201 ± 0.0043	21.3845
4:10	5.96 ± 0.06	0.0138 ± 0.0156	89.2796	6.66 ± 0.09	0.0066 ± 0.0044	65.677
3:10	5.45 ± 0.08	0.0065 ± 0.0061	93.9413	6.12 ± 0.03	0.0025 ± 0.0037	147.7024
2:10	4.71 ± 0.06	0.0053 ± 0.0073	137.6641	5.45 ± 0.13	0.0004 ± 0.0007	194.0472
1:10	3.73 ± 0.12	0.0021 ± 0.0031	149.5268	4.44 ± 0.06	0.0006 ± 0.0009	135.5868

[A, B, C, D] mean ± SD of 5 replication

2.4 Selectivity

As stated in literature review, selectivity is the ability of the method to select and quantify the analytes in the sample. Therefore, selectivity validation of Procedure A and Procedure B was tested whether any substance in NB or broth culture would interfere the AHL reaction or compete to bind with ferric chloride leading to false negative or false positive result.

2.4.1 Procedure A for AHL determination

Positive and negative control samples were used to test the specificity of the method. Positive control such as AHL-spiked NB, cell-free supernatant from AHL producing strain cultures and negative control such as AHL-free NB and the supernatant from AHL negative producing strain cultures samples, were conducted an extraction and purification before AHL determination. OD_{520} of five replication were calculated to find %RSD and shown in Table 4.14.

Table 4.14 OD_{520} values and %RSD obtained from AHL in negative and positive control samples determination by Procedure A

	samples	OD_{520}	
		mean [A]	%RSD
positive control samples	AHL-spiked NB (300 nmol/ml)	0.1020 ± 0.0017	0.82
	V.parahaemolyticus DMST 22092	0.0801 ± 0.0012	1.75
	V.parahaemolyticus DMST 22093	0.0795 ± 0.0007	1.01
	P.aerunginosa ATCC 27853	0.0802 ± 0.0011	1.57
negative control samples	AHL-free NB	0	0
	E.coli ATCC 29522	0	0
	Salmonella sp. ATCC 13811	0	0

[A] mean ± SD of 5 replication

As results shown in Table 4.14, AHL color complex represented OD_{520} of all positive control samples were determined with %RSD under ± 15%, whereas the color

complex of negative control samples were not observed (OD_{520}= 0). The results indicated that non of any substance presented in NB or bacterial culture could interfere this AHL determination system although typical component of NB are mainly composed by simple sugars and proteins. Since simple sugar in form of glucose and amino acid polypeptide in protein do not contain lactone group in their molecular structure, therefore they will not react with ferric ion to form coloring complex. Therefore false positive detection resulting from the interference of simple sugar and protein should not be occurred. Moreover, in this study, NB already reacted with ferric chloride was normally used as a blank for the absorbance determination. Therefore the coloring complex which might generate in NB was already eliminated. In bacterial broth culture, there are some bacterial metabolites such as amino acid and polysaccharide possibly releases to broth media and still remains in the broth after centrifugation. However, absorbance values determined in the broths were not observed. This result indicated that these metabolites might not contain lactone or lactone-liked, which could form complex with ferric ion, in their molecules.

These results could confirm that the selectivity of procedure A to AHL in NB determination was acceptable.

2.4.2 Procedure B for AHL determination

To validate the selectivity of the method for direct determination of AHL in NB (Procedure B), negative and positive samples as described in 2.4.1 were used to test and the results are shown in Table 4.15.

The results as shown in Table 4.15 corresponded to the result of Procedure A (Table 4.14) in which similar absorbance values were determined from positive control samples, whereas absorbance values, equal to 0, still determined from negative control samples.

These indicated that any substances and a bacterial metabolites possibly presented in NB as described above did not interfere the absorbance determination system. Therefore these interferences were not necessarily eliminated.

In conclusion, the selectivity of AHL determination method based on Procedure B was also acceptable.

From this study, based on statistical data of each validation parameters, both Procedure A and Procedure B were potential to use as the AHL determination methods for further study. However, when considered the simplicity of the method, Procedure B was more advantageous since it was more rapid and less laborious. Therefore, Procedure B was selected to use for the further works.

Table 4.15 OD_{520} and %RSD obtained from AHL determination in negative and positive control samples by Procedure B

	samples	OD_{520}	
		mean [A]	%RSD
positive control samples	AHL-spiked NB (300 nmol/ml)	0.1053 ± 0.0015	1.4281
	V.parahaemolyticus DMST 22092	0.0835 ± 0.0011	1.3106
	V.parahaemolyticus DMST 22093	0.0812 ± 0.0011	1.2961
	P.aerunginosa ATCC 27853	0.0842 ± 0.0009	1.0437
negative control samples	AHL-free NB	0	0
	E.coli ATCC 29522	0	0
	Salmonella sp. ATCC 13811	0	0

[A] mean ± SD of 5 replication

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

Several previous researches mentioned that AHL production could be induced or suppressed by intrinsic factor such as strains and growth phase, and extrinsic factor such as environmental conditions. For instance, the research of Blossler and Gray (2000) who mentioned that concentration and profile of AHL generated from *V.fisheri* MJ1 were influenced by growth stages. The proportion of 3-oxo-C6-HSL, C6-HSL and C8-HSL generated by this strain during late exponential was different from stationary phase. In the other hand, some environmental factors, for example, salt containing in cultural media and temperature did not associate with AHL production as the report of Buchholtz et al (2005). This report demonstrated that *V.anguillarum* growing, in culture

media containing different salt concentrations and under different temperatures, generated similar AHL profiles analyzed by HPLC-MS.

The study in this section therefore investigated factors possibly influencing AHL production of *V.parahaemolyticus*. There were growth phases, strains and cultivation conditions considered to be investigated whether they affected its AHL production in *V.parahaemolyticus*.

3.1 Growth stages

To study the influence of growth stage on AHL production of *V.parahaemolyticus*, the correlation of cell populations and AHL concentrations during growth stage were investigated. Two strains of *V.parahaemolyticus* (*V.parahaemolyticus* DMST 22092 and *V.parahaemolyticus* DMST 22093) were cultured for 29 hours as described in materials and methods section 3.1. TPC and AHL concentrations in the cultures were observed every 1 hour. The results are shown in Figure 4.3 and 4.4.

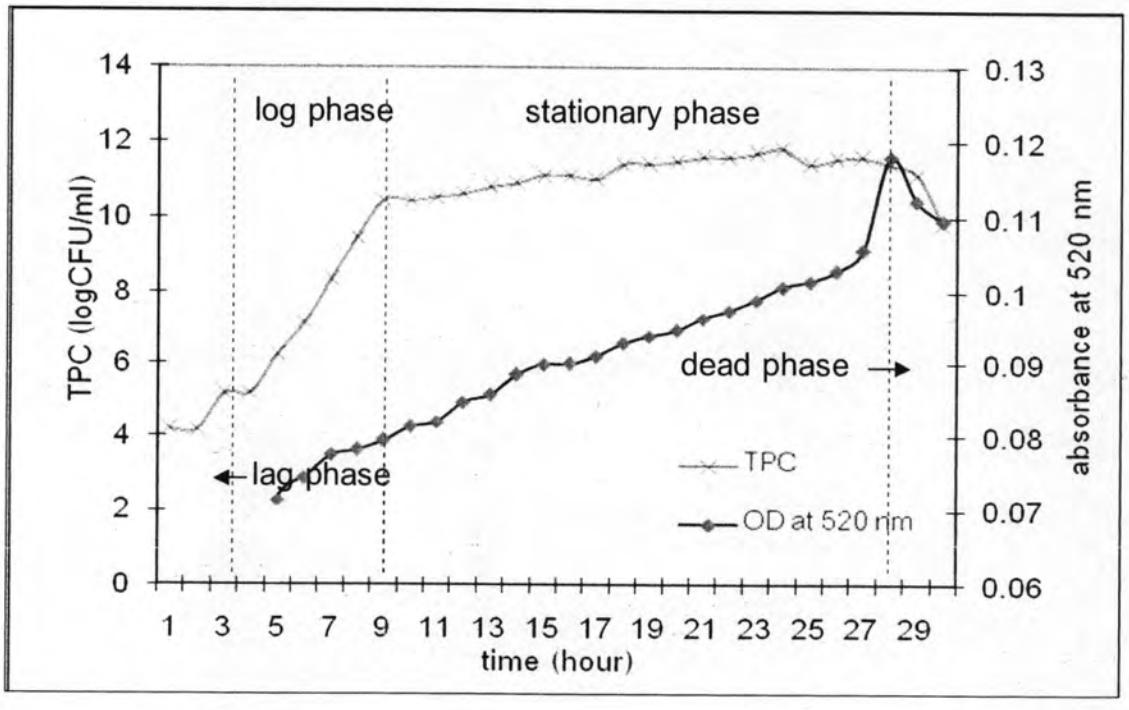


Figure 4.3 AHL production of *V.parahaemolyticus* DMST 22092 during growth phase

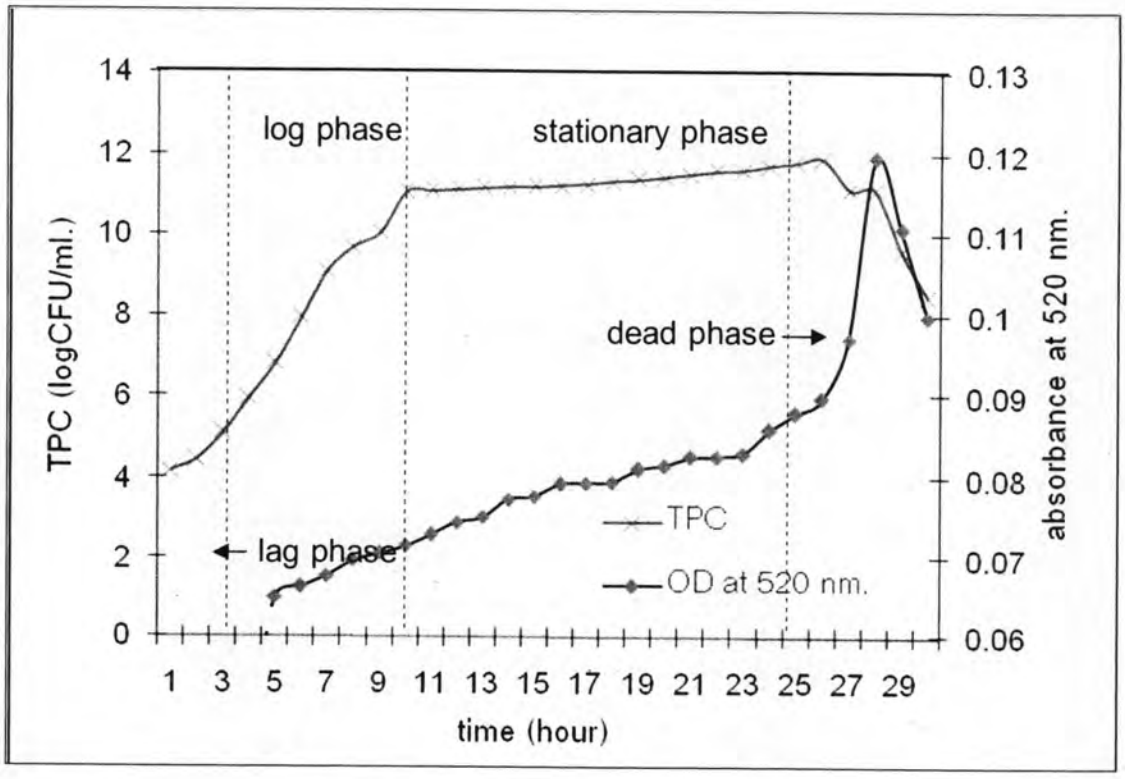


Figure 4.4 AHL production of *V.parahaemolyticus* DMST 22093 during growth phase

According to Figure 4.3, during lag phase (the 0, 1st hours), with initial cell population of 4.16 logCFU/ml, AHL color complex was likely observed (OD_{520} = 0.0004) in the culture but the coefficient of variation (%RSD) of the OD_{520} was unacceptable (over ± 15%, Table D3 in Appendix D). This result could arise from two possibilities. Firstly, sensitivity of the method might not be high enough to detect AHL which in fact it did present in the culture. Secondly, this detection might be due to a false positive result. For instance, interferences that could react with ferric chloride formed similar AHL color complex giving a false positive result. Therefore to solve this possibility, any substance in cell free supernatants of the lag phase culture of non AHL-producing strains (*E.coli* ATCC 29522 (the 0 hour) and *Salmonella* sp. ATCC 13811 (the 0 hour)) were tested and used AHL-free NB as blank. It was found that OD_{520} of both samples were equal to zero (Table D5 and D6, Appendix D). This study also investigated cell-free supernatant from the lag culture of another AHL producing strain, *P.aerunginosa* ATCC 27853 (the 0, 1st hours) (Figure D3 in Appendix D). The OD_{520} measured in cell-free supernatant of lag phase of this bacteria were higher than OD of blank and %RSD was also over ± 15 (Table D7 in Appendix D). Therefore, it could be concluded that wide range of %RSD

came from the sensitivity of the method. Hence, it is confirmed that AHL could be produced during lag phase of *V.parahaemolyticus* culture. However, to further prove, other AHL detection which had higher sensitivity than colorimetry method such as nano-HPLC-MS should be applied for this investigation.

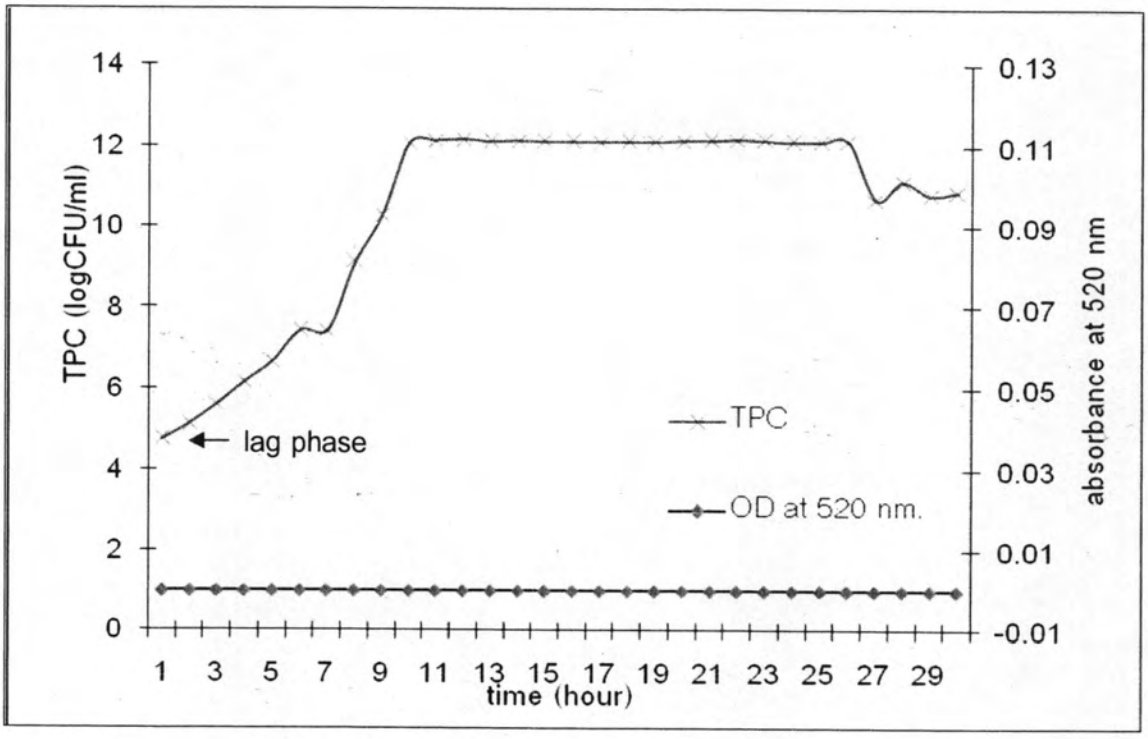


Figure 4.5 AHL production of *E.coli* ATCC 25992 during growth phase

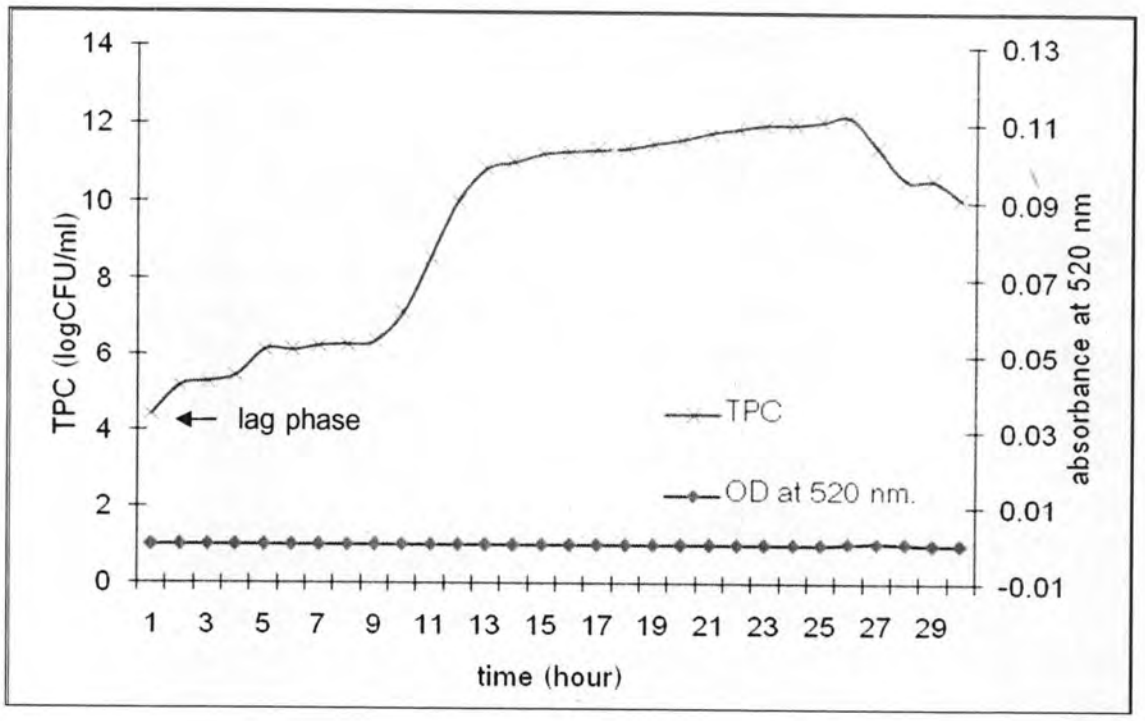


Figure 4.6 AHL production of *Salmonella* sp. ATCC 13811 during growth phase

During log phase (the 2nd to 8th hours), when cell population increased to 6.21 logCFU/ml (Figure 4.3), AHL color complex was detected with OD_{520} 0.0712 and %RSD was an acceptable (%RSD = ± 2.69). The population increasing rate represented in term of slope of linear regression line (m = 0.900) was faster than an increasing rate of AHL product. AHL production increasing rate during stationary phase (m = 0.001) was non significant different from the rate in log phase while population increasing rate dropped (m = 0.07). This result indicated that AHL production rate could not relate to growth rate of *V.parahaemolyticus* DMST 22092. This result was inconsistent with previous researches which reported that the concentration of AHL signal increased at the same rate as the increase in cell number in *S.liquefaciens* and *En.agglomerans* (Gram et al, 1999), and *S.proteomaculans* B5a and *E.carotovora* ATCC 39048 (Ravn et al, 2001). The researches stated that in these bacteria, when AHL concentration in the system reached threshold level, signal molecule would induce an expression of other phenotypic expression to allow the adaptation of cell to new environment but did not induce the signal production, therefore the AHL production rate was relied on growth rate of bacteria. This AHL production was so called "constitutive". However, many

studies proposed that, AHL generation of some bacterial species, AHL-signal molecules influence to induce the signal production. This autoinducible circuit resulted in a significant increasing of tremendous amount of AHL per unit of cell and was named as "up-regulation production" (Fuqua et al, 1994; Latif et al, 1995; Kuo et al, 1996;Greenberg, 1997). Therefore, the results from these two stages of growth indicated that the AHL production character of *V.parahaemolyticus* corresponded to the up-regulation production.

Interestingly, during late stationary phase of *V.parahaemolyticus* DMST 22092 (the 26^{th} - 27^{th} hours, Figure 4.3), AHL was extraordinary increased, leading the slope of linear regression line to 0.012. The possibility associated this phenomenon could be explained as following, in the late stationary phase, conditions of the culture were changed such as depletion of nutrients and accumulation of toxic substance released from dead cell. This caused stress to bacterial cells and lead to over-release of signal molecule. The environmental stress induction of quorum sensing signal molecule was documented by some reports. They indicated that the response of bacteria to oxidative stress were partially controlled through quorum sensing system (Hassett et al., 1999; McDougald et al, 2001; McDougald et al, 2002; Lumjiaktase et al, 2006). This possibly induced the cell to over-release AHL.

The decreasing of AHL concentration was observed at first hour of dead phase (the 27^{th} hours, Figure 4.3). In this stage, the pH of the culture was changed to alkaline side (pH = 7.4 to 7.7). Since lactone group in AHL structure is not stable under alkaline condition and partially degrade when pH reaches 7.5 and completely decomposed when pH over 8.5 (Ravn et al, 2001; Yate et al, 2002; Yang et al, 2006). Therefore the reduction of AHL through dead phase possibly came from the degradation of lactone ring due to alkaline condition of the culture.

In addition, as presented in Figure 4.4, the relationship of AHL production and population increasing rate found in *V.parahaemolyticus* DMST 22093 culture through all growth phases was also similar to *V.parahaemolyticus* DMST 22092 (Figure 4.3). AHL was observed in lag phase (the 0 hour) but %RSD of OD_{520} measured was over ± 15 (Table D4, Appendix D). During log phase, AHL concentration was acceptably detected (0.0651, %RSD = ± 1.31) at cell population of 6.82 logCFU/ml and gradually increased

through log and stationary phase of growth. Interestingly, the extraordinary increasing of AHL concentration also presented in early dead phase (the 27^{th} to 28^{th} hours, m = 1.04). At the second hour of dead phase (the 29^{th} hours), AHL was then suddenly decreased. The results obtained from these two strains could be represented as a model of AHL production in *V.parahaemolyticus*.

According to the model of AHL production found in this study, growth phase of bacteria could not directly influence to AHL production. Whereas cultivation conditions such as oxidative stress generated at the late stationary phase shown as an obvious a significant factor associated with AHL production. Moreover, this was the first report which demonstrated that model of AHL signaling system in *V.parahaemolyticus* was more similar to up-regulation model than constitutive induction. However, to confirm this conclusion, more number of *V.parahaemolyticus* strains should be further investigated.

3.2 strain

Various *V.parahaemolyticus* strains, were study using three non-pathogenic strains isolated from seafood and three pathogenic strains isolated from patient stools, were used for investigation of influences of strain on AHL production. Bacteria were cultured in NB for 48 hours. One ml of cultures were collected at the 12^{th}, 24^{th} and 48^{th} hours to determine TPC and AHL concentration. Due to the variation of the TPC, therefore each culture TPC was normalized to a number of 100. Subsequently, AHL concentration of each culture's normalized TPC was compared and presented in term of %measured absorbance. The results were shown in Table 4.16. %measured absorbance was calculated by Equation 4.2 showing below

$$\%\text{measured absorbance} = \frac{OD_{520}\text{ value}}{\text{TPC (logCFU/ml)}} \times 100 \qquad (4.2)$$

According to the data in Table 4.16, %measured absorbance of all strains cultivated for the 12^{th} hours were in ranged from 0.6812^{b} to 0.7149^{a}. %measured absorbance of all strains were not significantly different except of *V.parahaemolyticus* DMST 22092 culture. However, At the 24^{th} hours, %measured absorbance of all strains

were also not significantly different. The % measured absorbance range were 0.8553[a] to 0.8739[a]. These results indicated that interspecies of *V.parahaemolyticus* exhibited the similar property AHL production. These finding agreed with a previous research. Buchholtz et al (2005) reported that AHL production from different *V.anguillarum* serotypes were similar, which indicated that AHL production of these bacteria could be strain-independent.

Table 4.16 %measured absorbance of bacterial AHL in the 12th, 24th and 48th hours of *V.parahaemolyticus* cultures

V.parahaemolyticus strains	% measured absorbance		
	the 12th hours[A]	the 24th hours[A]	the 48th hours[A]
non-pathogenic			
DMST 22092	0.7502[a] ± 0.0347	0.8739[a] ± 0.0118	3.0283[a] ± 0.1628
DMST 22093	0.6812[b] ± 0.0182	0.8640[a] ± 0.0121	1.9445[b] ± 0.0617
ATCC 17802	0.7112[b] ± 0.0231	0.8599[a] ± 0.0104	2.6879[c] ± 0.0333
pathogenic			
DMST 23797	0.6951[b] ± 0.0760	0.8667[a] ± 0.0108	4.1989[d] ± 0.2124
DMST 23798	0.6902[b] ± 0.0137	0.8561[a] ± 0.0089	2.0472[b] ± 0.0539
DMST 23799	0.7149[b] ± 0.0197	0.8553[a] ± 0.0172	2.4647[e] ± 0.0865

[a, b, c,...] value with significantly difference in each column are indicated by different letters. ($P < 0.05$ by Duncan's new multiply range test)

[A]mean ± SD of 3 replication

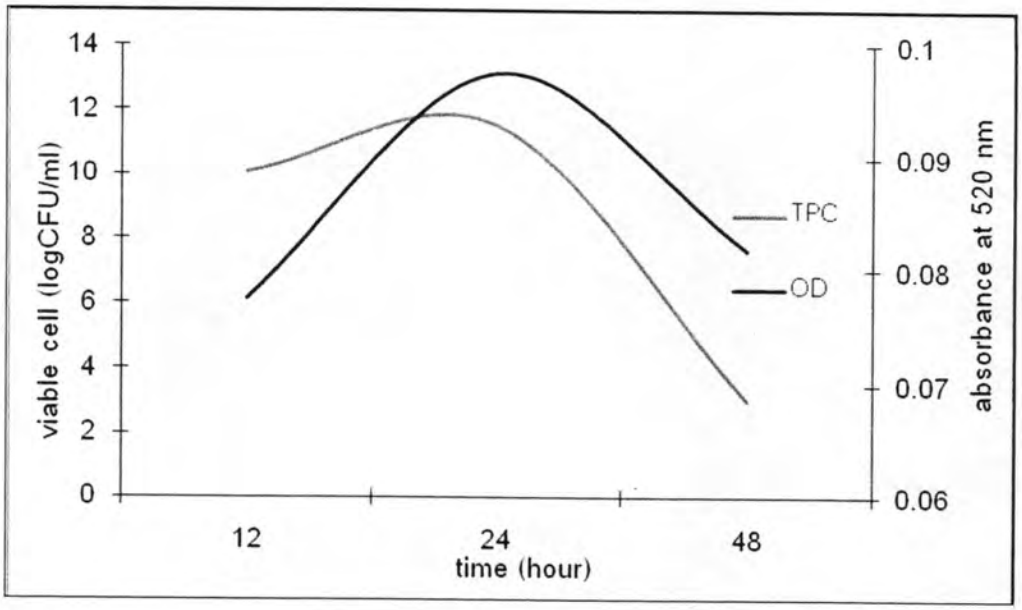


Figure 4.7 TPC and OD_{520} of *V.parahaemolyticus* ATCC 17802 culture in NB for 48 hours

However, the culture of the 48th hours, %measured absorbance of all strains were significant different. In addition, the %measured absrbance widely ranged from 1.9445 ^{b}- 4.1989 ^{d}, which was affected from variation of TPC (viable populations) remaining in these dead phase cultures (1.23 to 3.03 logCFU/ml).

As described in section 3.1, the cell in this dead phase condition would not generate AHL, and AHL remaining in the cultures could be partially degraded due to alkaline condition causing by substances generated from dead cells (Figure 4.7). Therefore, the variation of %measured absorbance (calculated from TPC and OD_{520}) of all strain cultures could not be due to influences of strains. Whereas the cultural conditions changed during cultivation period and the number of population presenting in the culture could associated to the AHL production.

In conclusion, the results of this section displayed that AHL production of *V.parahaemolyticus* could be strain-independence.

3.3 Cultivation conditions

The cultivation conditions such as media composition, salt concentration and temperature were varied. Reference strains (*V.parahaemolyticus* ATCC 17802 and *V.parahaemolyticus* DMST 23798) were cultured in each cultivation condition for 12

hours. TPC and amount of AHL were then evaluated. Data in term of %measured absorbance were shown in Table 4.17 below.

According to Table 4.17, % measured absorbance of *V.parahaemolyticus* ATCC 17802 cultured in different types of media were not statistically different. % measured absorbance investigated in temperature 15°C and room temperature (28 – 30 °C) were not also significantly different. %measured absorbance of bacteria cultured in NB containing 0.5 and 3% sodium chloride, which were %NaCl as found in typical media culture and marine environment, respectively, was not statistically different. % measured absorbance of 8% NaCl culture was statistically different from both 0.5 and 3% NaCl. Interestingly, *V.parahaemolyticus* ATCC 17802 cultured in NB containing 8% NaCl exhibited high OD_{520} than others whereas TPC in this salt concentration was the lowest (data not shown). Generally, culturing in media containing 8% NaCl is the main approach for isolation of *V.parahaemolyticus*, since it is only food associated pathogen which can survive and grow in media containing 8% salt (Bacteriological analytical manual online (BAM), USFDA, May 2004; Hatthayananon, Department of Medical Science). This condition could cause stress to *V.parahaemolyticus*, retarding growing rate of the cell resulting in the lower TPC relative to others cultivation conditions as shown in the table. In the other hand, this condition could induce the cell to generate larger amount of AHL (Sewald et al, 2007). Consequently, this higher %measured absorbance than those culture containing 0.5% NaCl for 27.79%, was therefore observed in the culture containing 8% NaCl.

The results from this experiment demonstrated that intrinsic factor such as bacterial strain and extrinsic factors, such as media compositions and temperatures, could not influence on AHL production of *V.parahaemolyticus*. High salt concentration (8% NaCl) was found to be an important factor inducing *V.parahaemolyticus* to significantly produce AHL. However, this explanation was based on quantitative analysis. Therefore, in further study, more definitive analysis, HPLC, was used to investigate the influences of factors as evaluated above. The influences of these factors on AHL composition profile were qualitative analysis by HPLC.

Table 4.17 % measured absorbance of *V.parahaemolyticus* strains in different cultivation conditions

environmental conditions		% measured absorbance	
		V.parahaemolyticus ATCC 17802	*V.parahaemolyticus* DMST 23798
media nutrition	NB	0.8599 ^{ab} ± 0.0124	0.8461 ^{a} ± 0.0888
	LB	0.8447 ^{a} ± 0.0112	0.8503 ^{a} ± 0.0133
salt concentrations (%w/v)	0.5	0.8882 ^{b} ± 0.0124	0.8710 ^{a} ± 0.0039
	3	0.8828 ^{b} ± 0.0142	0.8805 ^{a} ± 0.0749
	8	1.1351 ^{c} ± 0.0367	1.1027 ^{b} ± 0.0194
temperatures (^{o}C)	15	0.8256 ^{a} ± 0.0213	0.8326 ^{a} ± 0.0129
	RT^{A}	0.8599 ^{ab} ± 0.0124	0.8461 ^{a} ± 0.0089

$^{a, b, c,...}$ value with significantly difference in each column are indicated by different letters. ($P < 0.05$ by Duncan's new multiply range test)

^{A} room temperature

4. Characterization of AHLs produced from *V.parahaemolyticus* by HPLC

In previous studies, the colorimetry method could analyze only the total amount of AHLs generated by *V.parahaemolyticus*. It could not identify the type of AHLs because this method was based on the determination of complex between ferric chloride and lactone group containing in all types of AHLs. Therefore, the colorimetry method is not a qualitative analysis for AHLs.

Type of AHLs could be determined by many qualitative methods. HPLC has been used in several studies for separating the signaling compounds produced from bacteria (Winson et al, 1995; Middleton et al, 2002; Yate et al, 2002; Morin et al, 2003; Frommberger et al, 2004; Buchholtz et al, 2005; Rasch et al, 2005; Li et al, 2006). However, the sample preparation in AHLs characterization by HPLC required further improvement since the separated AHLs peaks were interfered by other substances from media culture. Moreover, the chromatography conditions have not been well developed.

The objectives of this study were to optimize the protocol for characterization of AHLs produced from *V.parahaemolyticus* by HPLC and to use that protocol to investigate the influences of intrinsic and extrinsic factors on the type of AHL production in *V.parahaemolyticus*.

4.1 Optimization of HPLC condition

In this study, gradient profiles used for a mobile phase; acetonitrile, were tested in order to produce a high resolution of AHL peaks. The concentration of acetonitrile was increased from 5% to 30% within three different time intervals: 15, 18, and 20 minutes. 3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL) as an AHL standard was dissolved in deionized water (0.6 mg/ml). Ten microliters of the 3-oxo-C6-HSL solution was then injected into HPLC system. The chromatograms of 3-oxo-C6-HSL eluted from three different gradient profiles were shown in Figure 4.8.

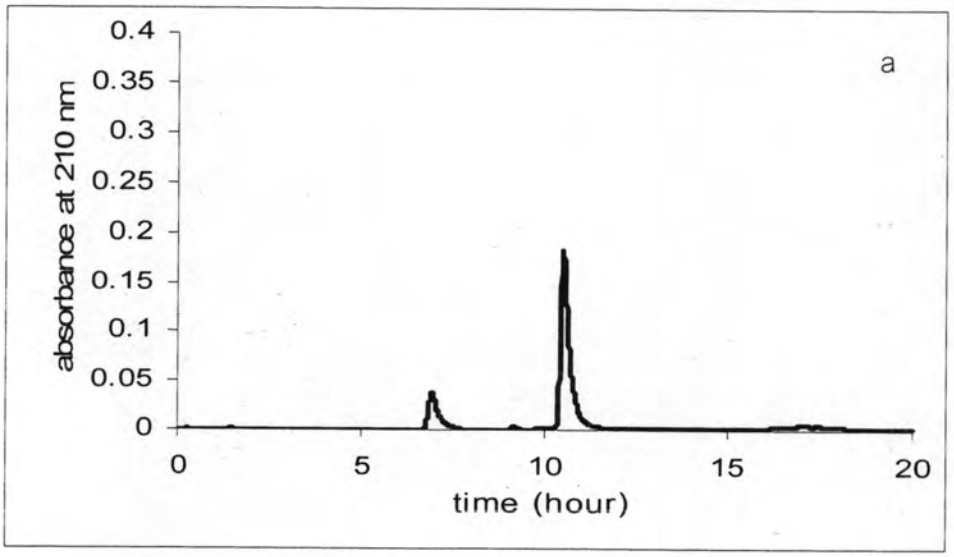


Figure 4.8 Chromatograms of 3-oxo-C6-HSL eluted with acetonitrile which its concentration increasing from 5% to 35% within 20 (a), 18 (b) and 15 (c) minutes

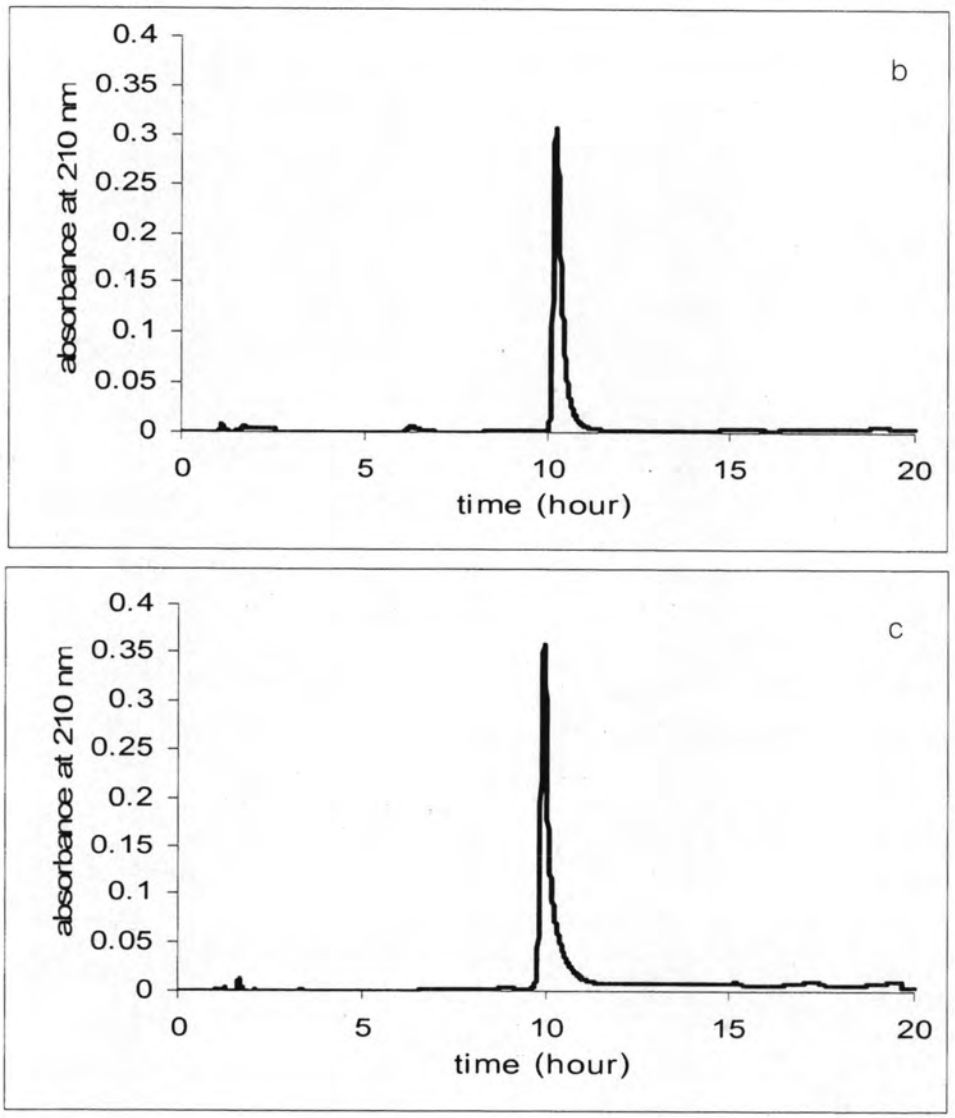


Figure 4.8 Chromatograms of 3-oxo-C6-HSL eluted with acetonitrile which its concentration increasing from 5% to 35% within 20 (a), 18 (b) and 15 (c) minutes (continue)

The chromatogram of 3-oxo-C6-HSL eluted with acetonitrile which its concentration increasing from 5% to 35% within 20 minutes (Figure 4.8a) showed similar profile as the reference chromatogram from the manufacture (Sigma-Aldrich, USA). There were two peaks at 6.9 and 10.52 minute. The peak at 6.9 minute was lower than the peak at 10.52 minutesand could be just the impurity. This impurity peak was lower when the acetonitrile concentration increasing at a faster rate (Figure 4.8b and 4.8c). The peak at 10.52 minute was the peak of 3-oxo-C6-HSL which eluted faster and shifted from 10.52 minute to10.22 and 9.95 minute when the acetonitrile concentration

increasing at a faster rate within 20 minute to18 and 15 minute, respectively (Figure 4.8b and 4.8c). This early shifting of the 3-oxo-C6-HSL peak might be due to the change in polarity of the mobile phase. When acetonitrile concentration was higher, the mobile phase would be more apolar. As a result, the molecule with non-polar substitution group such as 3-oxo-C6-HSL would eluted out from the column packed with non-polar substance faster.

Comparing all three gradient profiles, the gradient profile which the concentration of acetonitrile increasing from 5% to 35% within 15 minutes obtained the highest peak of 3-oxo-C6-HSL (Figure 4.8c). Therefore, this gradient profile would be used for the HPLC analysis of AHLs in further study. The detail of the optimized gradient profile is shown in Table 4.18.

Table 4.18 The optimized gradient profile for the HPLC analysis of AHLs in further study

Mobile phase (acetonitrile : water) (v/v)	time (minute)
5 : 95	0
35 : 65	15
100 : 0	20
100 : 0	25
5 : 95	30

4.2 Optimization of AHLs extraction from bacterial colonies

The extraction assays of AHLs from bacteria were evaluated in order to obtain a good HPLC chromatogram. The first assay was performed by extracting AHLs from bacteria cultured in broth media (NB). Broth media containing both bacteria cells and their excreted AHLs was mixed with chloroform in a separatory funnel. The chloroform fraction supposedly containing AHLs was then separated through the funnel bottom. The chloroform was evaporated and the concentrated AHLs mixture was then filtrated and directly injected into the HPLC system. The obtained chromatograms showed a complex peak pattern; as a result, the peaks of AHLs could not clearly identified (data

not shown). This complex pattern was also shown even in the chromatogram of pure NB. This could be implied that the interfering peaks were the peaks of substances in NB. These substances might have a similar polarity as AHLs and dissolved with chloroform.

The second AHLs extraction assay was developed in order to eliminate the interfering peaks from NB. Instead of culturing in NB, bacteria were cultured in agar plate and the bacteria colonies were directly suspended in water. The mixture was then filtrated and injected into the HPLC system. Comparing to the previous extraction assay, the chromatogram from this method should have less interfering peak because the bacteria colony would have much less impurities and higher AHLs content than the NB. Chloroform was not used to extract AHLs in this method because chloroform could break up the bacteria cell membrane and the cell metabolites could release out and contaminate with AHLs. AHL molecule consists of both hydrophilic and hydrophobic groups. Therefore, it can dissolve in both chloroform and water. According to preliminary test (data not shown), the ratio between the amount of colonies and water to obtain an optimum AHL concentration for HPLC analysis was 2 full-loops of colonies suspending in 100 µl of water.

In this study, AHL-producing bacteria (*V.parahaemolyticus* DMST 22092, *V.parahaemolyticus* DMST 22093 and *P.aerunginosa* ATCC 27853) and non AHL-producing bacterias (*E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811) were cultured on nutrient agar for 24 hours. Two full-loops of colonies were suspended with 100 µl D.I. water as described in material and method section (Table 3.4). Cell-free supernatants were prepared and injected into HPLC system. The chromatograms of the water-extract from all bacteria colonies cultured on NA were illustrated in Figure 4.9 to 4.13.

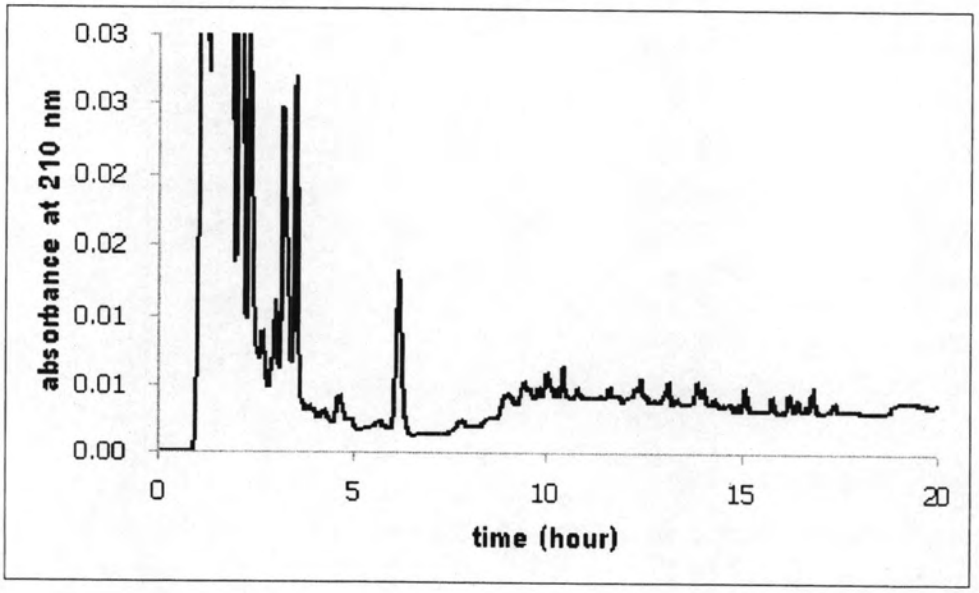


Figure 4.9 Chromatogram of water-extract from *V.parahaemolyticus* DMST 22092 colonies cultured on NA for 24 hours

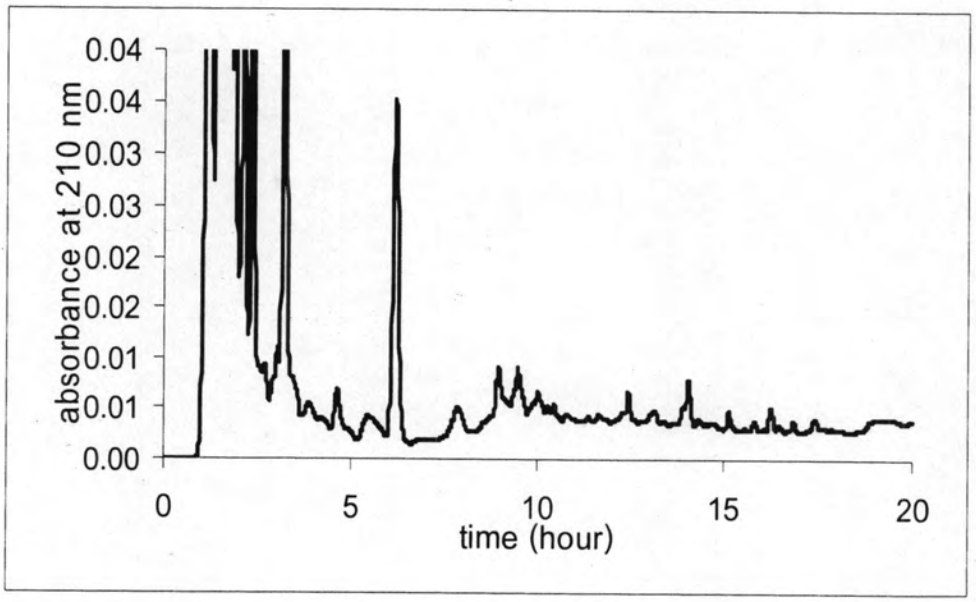


Figure 4.10 Chromatogram of water-extract from *V.parahaemolyticus* DMST 22093 colonies cultured on NA for 24 hours

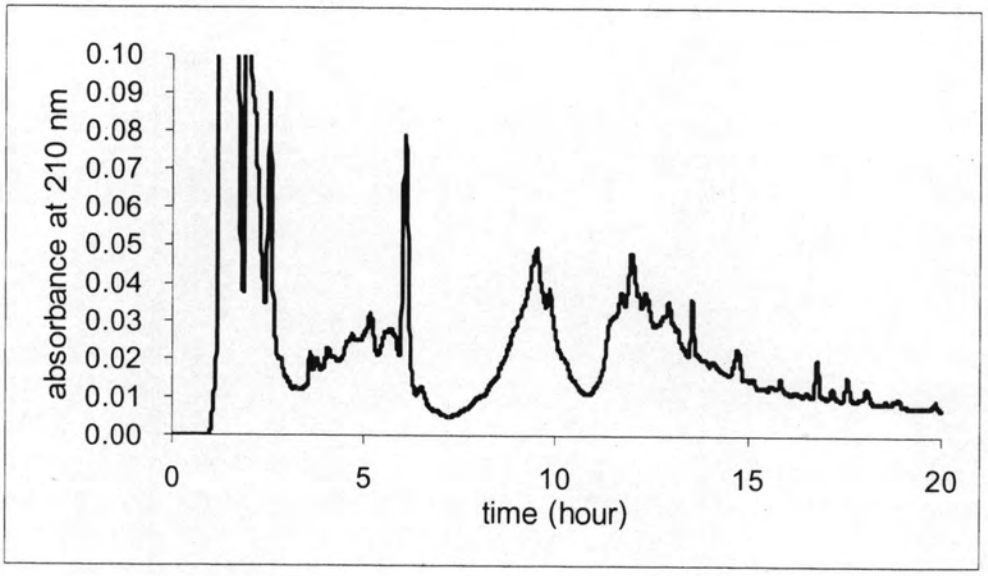


Figure 4.11 Chromatogram of water-extract from *P.aerunginosa* ATCC 27853 colonies cultured on NA for 24 hours

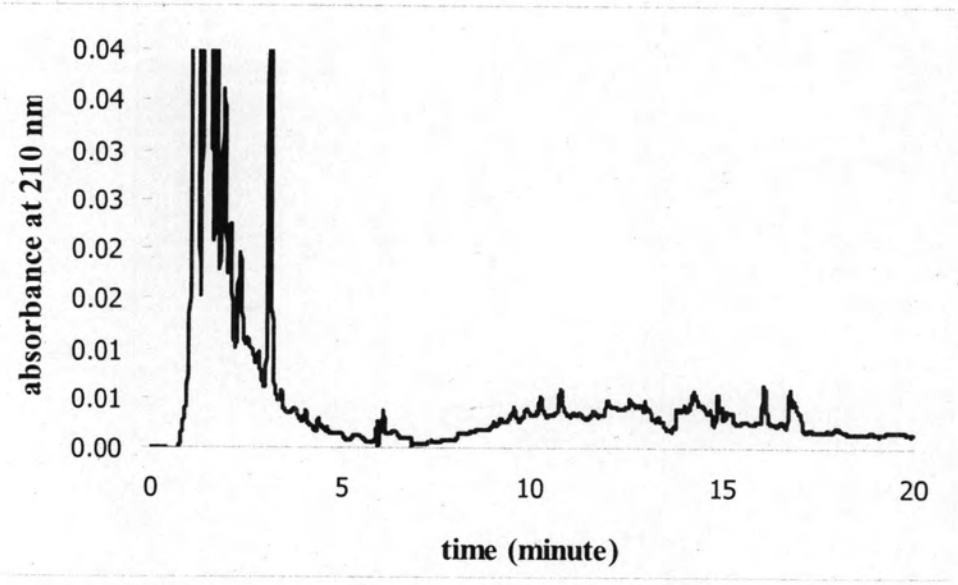


Figure 4.12 Chromatogram of water-extract from *E.coli* ATCC 25922 colonies cultured on NA for 24 hours

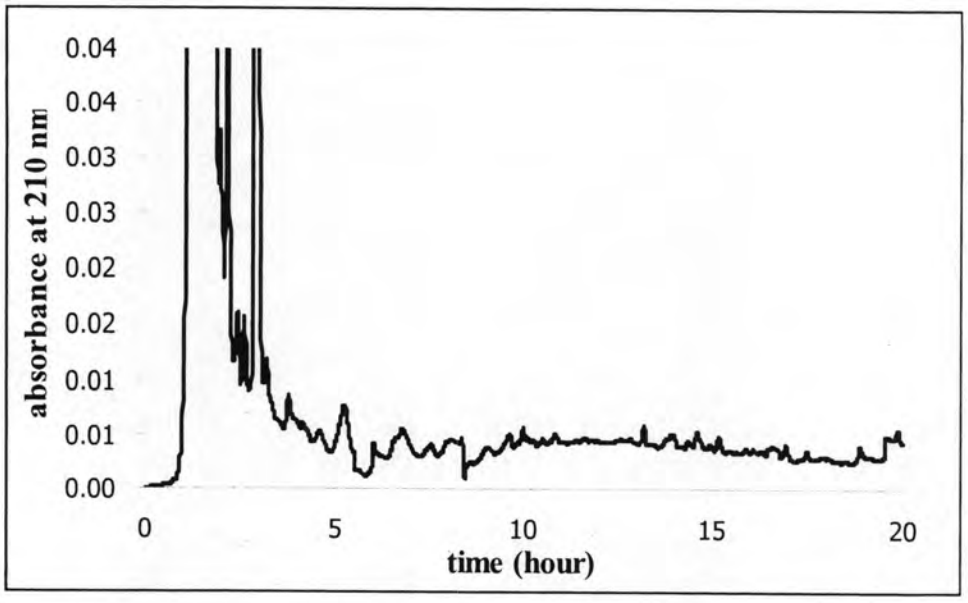


Figure 4.13 Chromatogram of water-extract from *Salmonella* sp. ATCC 13811 colonies cultured on NA for 24 hours

The chromatograms of water-extract from both *V.parahaemolyticus* DMST 22092 and *V.parahaemolyticus* DMST 22093 colonies were have similar profile with one major peak at 6.2 minute (Figure 4.9 and 4.10). Previous studies reported that *V.parahaemolyticus* produced only one AHL type used as AI-1 signal molecule which was 3-hydroxybutanoyl-homoserine lactone (3-hydroxyl-C4-HSL) (Bassler, Greenberg, and Stevens, 1997 and Henke and Bassler, 2004). Compared to 3-oxo-C6-HSL used as AHL standard in previous section, 3-hydroxyl-C4-HSL had a shorter acyl side chain resulting in a more polar molecule. Therefore, 3-hydroxyl-C4-HSL should elute out earlier than 3-oxo-C6-HSL which had a retention time at 9.95 minute (Figure 4.8c). Based on these information, the major peaks of chromatograms in Figure 4.9 and 4.10 could be 3-hydroxyl-C4-HSL peaks which had a retention time at 6.2 minute. All peaks eluted before 4 minutes possibly could be ghost peaks due to a change in pressure during injection because these peaks also existed in the standard 3-oxo-C6-HSL chromatograms (Figure 4.8). The minor peaks after 10 minutes might be due to the interferences from colonies.

The chromatogram of water-extract from *P.aerunginosa* ATCC 27853 colonies probably had three major peaks at 6.42, 9.95, and 12.15 minute (Figure 4.11). Previous

works proposed that *P.aerunginosa* produced three different types of signaling molecules; namely, C4-HSL, 3-oxo-C6-HSL, and 3-oxo-C12-HSL (Gambello, Kaye and Iglewski, 1991; Passador et al, 1993; Pearson et al, 1994; Latif et al, 1995; Winson et al, 1995; latif et al, 1996; Lewenza et al, 1999) According to the chromatograms of 3-oxo-C6-HSL (Fig. 4.13c) and the chromatograms of water-extract from both *V.parahaemolyticus* DMST 22092 and *V.parahaemolyticus* DMST 22093 colonies (Figure 4.9 and 4.10), the peaks at 6.42 and 9.95 minute should be the peak of C4-HSL, 3-oxo-C6-HSL, respectively. The peak at 12.15 minute probably was the peak of 3-oxo-C12-HSL due to its longer acyl side chain compared to that of 3-oxo-C6-HSL. As a resulting, it had less polar than 3-oxo-C6-HSL and should elute out at a later retention time. However, the peak identification need to be verified by injecting more AHL standards or employing other more precise analysis technique such as HPLC equipped with mass spectroscopy.

Middleton et al (2002) studied the AHLs production of *P.aerunginosa* and reported that 3-oxo-C12-HSL and C4-HSL were the major cognate signal molecules while 3-oxo-C6-HSL was produced in smaller quantities. Their results were inconsistence with this study in that C4-HSL seemed to be the major signaling molecule while 3-oxo-C6-HSL and 3-oxo-C12-HSL were the minor signals. However, the transportation of 3-oxo-C12-HSL across membrane was difficult as this molecule had a long hydrophobic acyl side chain (Fuqua and Greenberg, 2002). Pearson, Van delden and Iglewski (1999) was also reported that inside the *P.aerunginosa* cell contained 3-oxo-C12-HSL threefold higher than outside the cell. The chromatograms of water-extract from *E.coli* ATCC 25922 and *Salmonella* sp. ATCC 13811 (Fig. 4.12 and 4.13, respectively) were shown complex noise peak patterns without any major AHL peak found in the previous chromatograms of water-extract from AHL-producing bacteria . These results could support the precision of these AHL extraction and HPLC analysis methods. The presence of noise peaks could come from the impurities in colonies extract.

The chromatograms of both AHL producing and non-producing bacteria in this study suggested that this AHL extraction method could be used in the HPLC analysis of the type of AHL produced from *V.parahaemolyticus*. This extraction technique reduced

interfering peaks of exogeneous substances such as nutrients in broth media. However, the purity of AHL extract need to be improved in order to eliminate the noise peaks.

4.3 Factors associated with the type of AHL produced by *V.parahaemolyticus*

In the AHL production study, the effects of both strains and cultivation conditions on the amount of AHL produced by *V.parahaemolyticus* were investigated. The study showed that both factors did not significantly affect the amount of produced AHL. The objective of this study was to investigate how the type of AHL produced by *V.parahaemolyticus* was influenced by those both factors.

4.3.1 Effects of growth phase and *V.parahaemolyticus* strains on the type of AHL

Pathogenic (DMST 23799) and non-pathogenic (DMST 22092) of *V.parahaemolyticus* were characterized their produced AHLs by HPLC analysis. Strains were cultured on NA for 24, 48, and 72 hours. The colonies water-extract prepared by the method as described in the previous section was then subjected to HPLC analysis. The chromatogram results were shown in Figure 4.14 and 4.15.

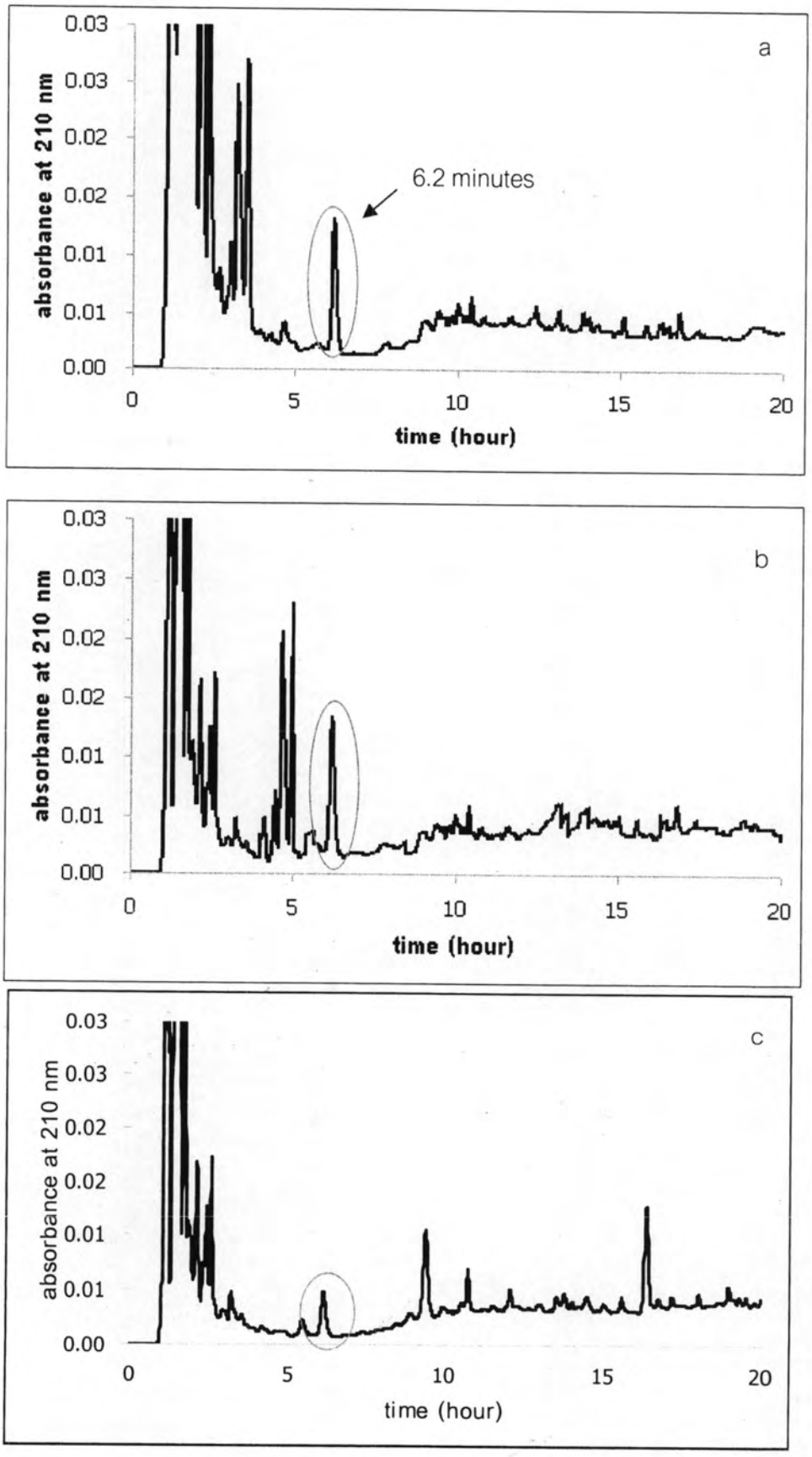


Figure 4.14 Chromatograms of *V.parahaemolyticus* DMST 22092 colonies cultured on NA for 24 (a), 48 (b) and 72 hours (c)

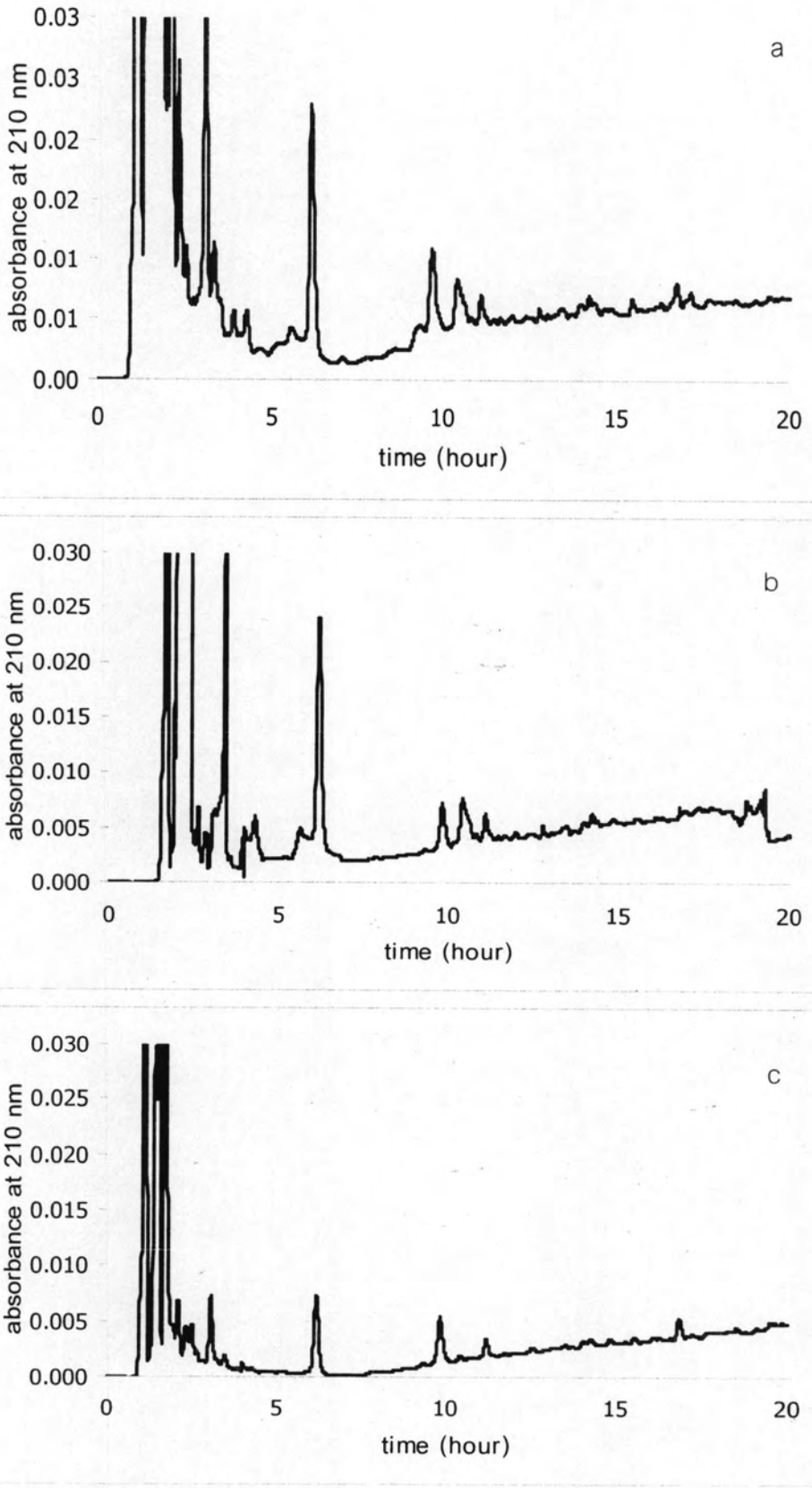


Figure 4.15 Chromatograms of *V.parahaemolyticus* DMST 23799 colonies cultured on NA for 24 (a), 48 (b) and 72 hours (c)

In this study, different incubation times were tested in order to indirectly investigate the influence of the growth phase on the profile of produced AHLs. The chromatograms of water-extract from *V.parahaemolyticus* DMST 22092 incubated for 24 hours (Figure 4.14a) showed the same profile as that shown in the previous section (Figure 4.9). Therefore, the major peak at 6.2 minute should be the peak of 3-hydroxy-C4-HSL. When the incubation time increased to 48 hours, the peak height of 3-hydroxy-C4-HSL was slightly increased (Figure 4.14b). However, the amount of 3-hydroxy-C4-HSL peak was significantly decreased after 72-hours incubating. These changes in the amount of produced AHL at different incubation time were in accordance well with the result of the previous study about the amount of produced AHLs at different growth phase (section 3.1). The amount of AHL was highest at late stationary phase of bacteria cultured in broth media or cultured for 48 hours on nutrient agar. Similarly, AHL decreased at the dead phase of bacteria cultured in broth media or cultured for 72 hours on nutrient agar. The increasing of AHL content at the middle of growth should be the result of accumulated AHL produced during cultivating. The reduction of AHL content at the dead phase could be explained by two possible rationales. Firstly, after cell death, cell membrane would break and the metabolites in cell would spread into surrounding media. These metabolites contained enzyme which could degraded AHL molecule resulting in smaller AHL fraction. Secondly, at the dead phase, the nutrient source for cell growth was insufficient; therefore, bacterial could consume AHL as carbon and nitrogen source. However; based on the chromatogram of water-extract from *V.parahaemolyticus* DMST 22092 incubated for 72 hours (Figure 4.14c), the first rationale seemed to be more likely to occur than the second rationale. In this chromatogram; beside the 3-hydroxy-C4-HSL peak, there were other major peaks near 9.5, 11, and 16.5 minutes. These later peaks might be the AHL hydrophobic residues from enzymatic degradation.

The water-extract from *V.parahaemolyticus* DMST 23799 had a similar chromatogram profile as that of *V.parahaemolyticus* DMST 22092 (Figure 4.14 and 4.15). Moreover, the incubation time also influenced on the AHL content of *V.parahaemolyticus* DMST 23799 in the same fashion as on that of *V.parahaemolyticus* DMST 22092. According to Figure 4.15, *V.parahaemolyticus* DMST 23799 would produce the highest

amount of 3-hydroxy-C4-HSL when it was cultured on NA for 48 hours and lowest when it was cultured for 72 hours. This trend could be explained by the same rationale previously discussed. Besides these two strains of *V.parahaemolyticus*, other strains were also studied and showed their water-extract chromatogram profiles in the same manner as those two strains (Figure E1 to E4, Appendix E).

4.3.1 Effects cultivation conditions on the type of AHL

Influences of cultivation conditions in terms of nutrient source, salt concentration, and incubation temperature on the type of produced AHL were investigated in this section. *V.parahaemolyticus* DMST 23798 and *V.parahaemolyticus* ATCC 17802 were cultured on agar media for 24 hours, otherwise indicated. The water-extract from colonies was prepared then subjected to HPLC analysis.

4.3.2.1 Effect of nutrient source

V.parahaemolyticus DMST 23798 was cultured on four different agar media: NA, PCA, LB agar and TCBS. The chromatograms of the water-extract from colonies were depicted in Fig. 4.16.

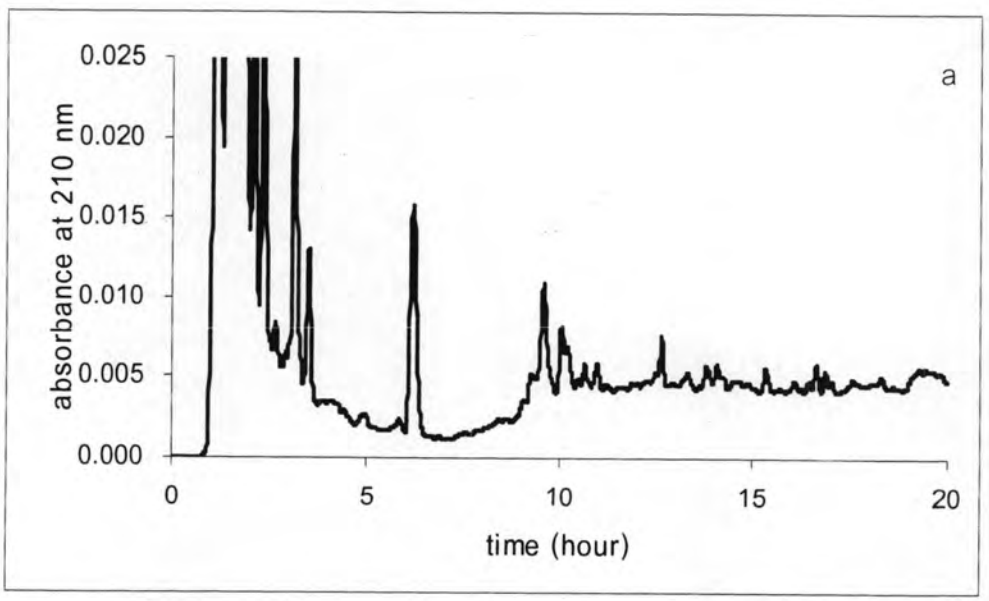
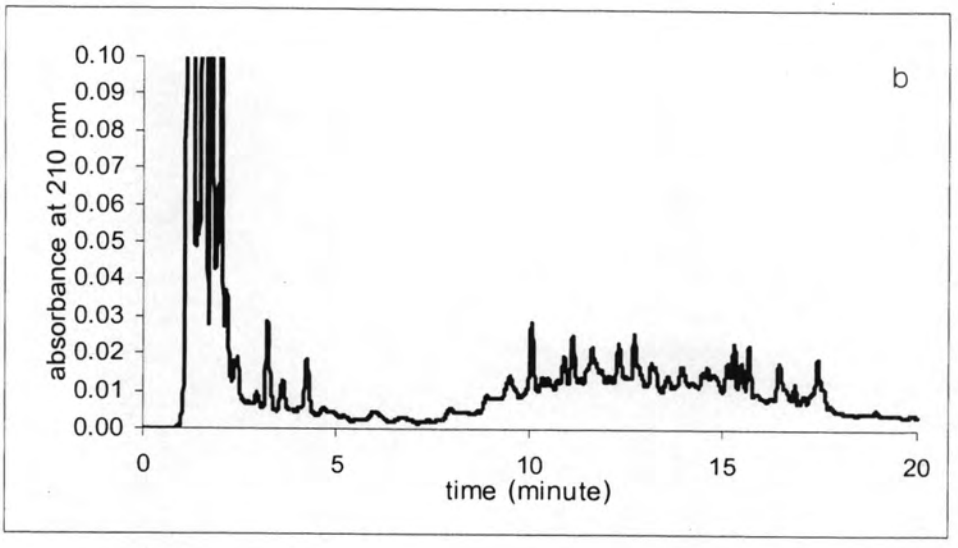
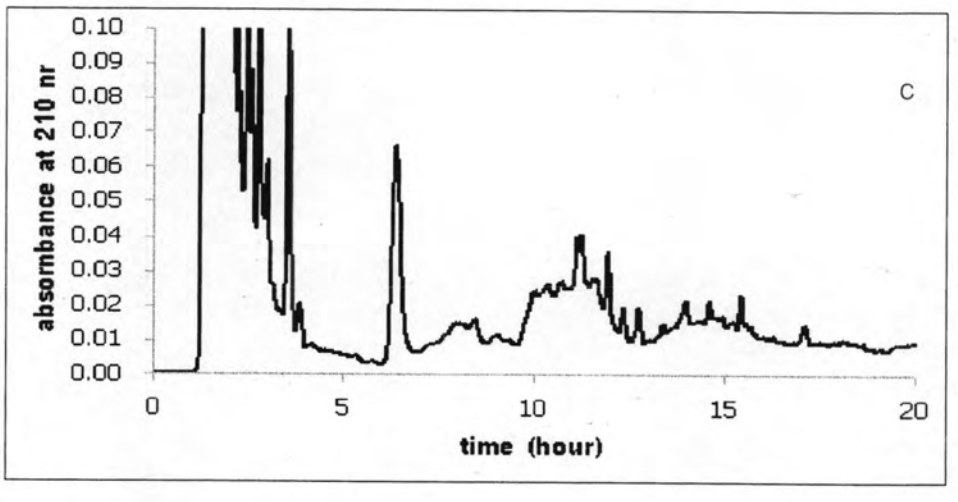


Figure 4.16 Chromatograms of *V.parahaemolyticus* DMST 23798 colonies cultured at room temperature for 24 hours on different agar media: NA (a), PCA (b), TCBS (c), and LB agar (d)





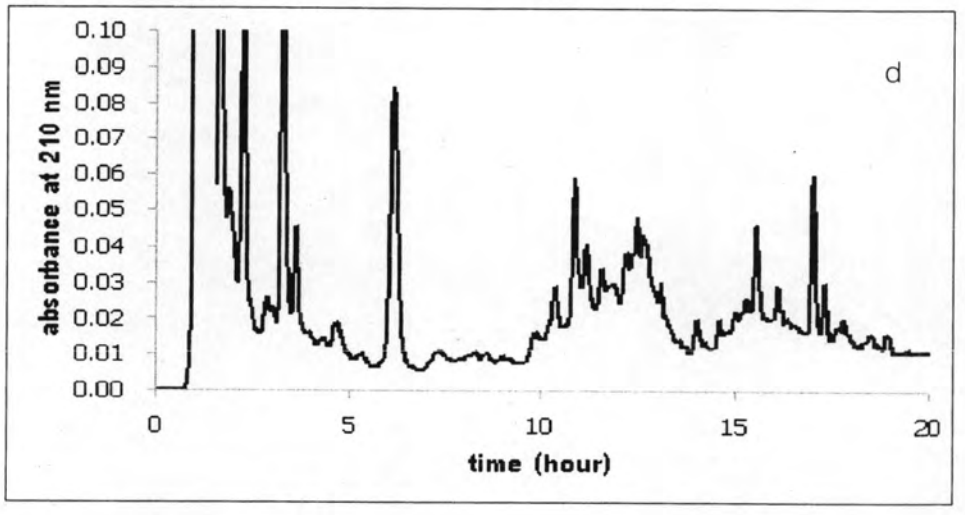


Figure 4.16 Chromatograms of *V.parahaemolyticus* DMST 23798 colonies cultured at room temperature for 24 hours on different agar media: NA (a), PCA (b), TCBS (c), and LB agar (d) (continue)

The chromatograms of water-extract from *V.parahaemolyticus* DMST 23798 colonies cultured on different media had the same major peak at 6.2 minute (Figure 4.21). According to the previous discussion, this peak could be the peak of 3-hydroxyl-C4-HSL. However, the height of 3-hydroxyl-C4-HSL peak was different among all chromatograms. The chromatograms of water-extract *V.parahaemolyticus* DMST 23798 colonies cultured on NA and PCA had a much lower 3-hydroxyl-C4-HSL peak than those of water-extract *V.parahaemolyticus* DMST 23798 colonies cultured on TCBS and LB (Figure 4.21). *V.parahaemolyticus* DMST 23798 produced the highest amount of 3-hydroxyl-C4-HSL when it was cultured on LB agar (Figure 4.21). These results were relevant with the colony size of *V.parahaemolyticus* DMST 23798 cultured on different agar type. The one cultured on PCA had a relatively smaller in its colony size than the one cultured in other agars (data not shown). This study suggested that *V.parahaemolyticus* DMST 23798 could produce 3-hydroxyl-C4-HSL in most of nutrient source but with different amount depending on its growth rate. Similar effects of the nutrient source on the chromatogram profile of the water-extract from *V.parahaemolyticus* ATCC 17803 colonies cultured on different type of agar media were also found (Figure E5 in Appendix E).

4.3.2.2 Effect of salt concentration

V.parahaemolyticus DMST 23798 was cultured for 24 hours on three NAs different in salt concentrations: 0.5%, 3%, and 8% NaCl. The chromatograms of the water-extract from the colonies were shown in Figure 4.17.

The major peak at 6.2 minute or the peak of 3-hydroxyl-C4-HSL appeared in all chromatograms of water-extract from *V.parahaemolyticus* DMST 23798 cultured on NA in different salt concentrations (Figure 4.17). This result suggested that *V.parahaemolyticus* DMST 23798 could produce 3-hydroxyl-C4-HSL in all different salt concentrations. However, the salt concentration seemed to influence on the AHL content. The hight of 3-hydroxy-C4-HSL peak when the salt concentration in agar was higher (Figure 4.17). This positively dependent relationship between the salt concentration and the AHL production could be due to the salt inducing AHL production of *V.parahaemolyticus* as described previously in 3.3. These effects of salt concentration

on the AHL production also found in *V.parahaemolyticus* ATCC 17802 (Figure E6 in Appendix E).

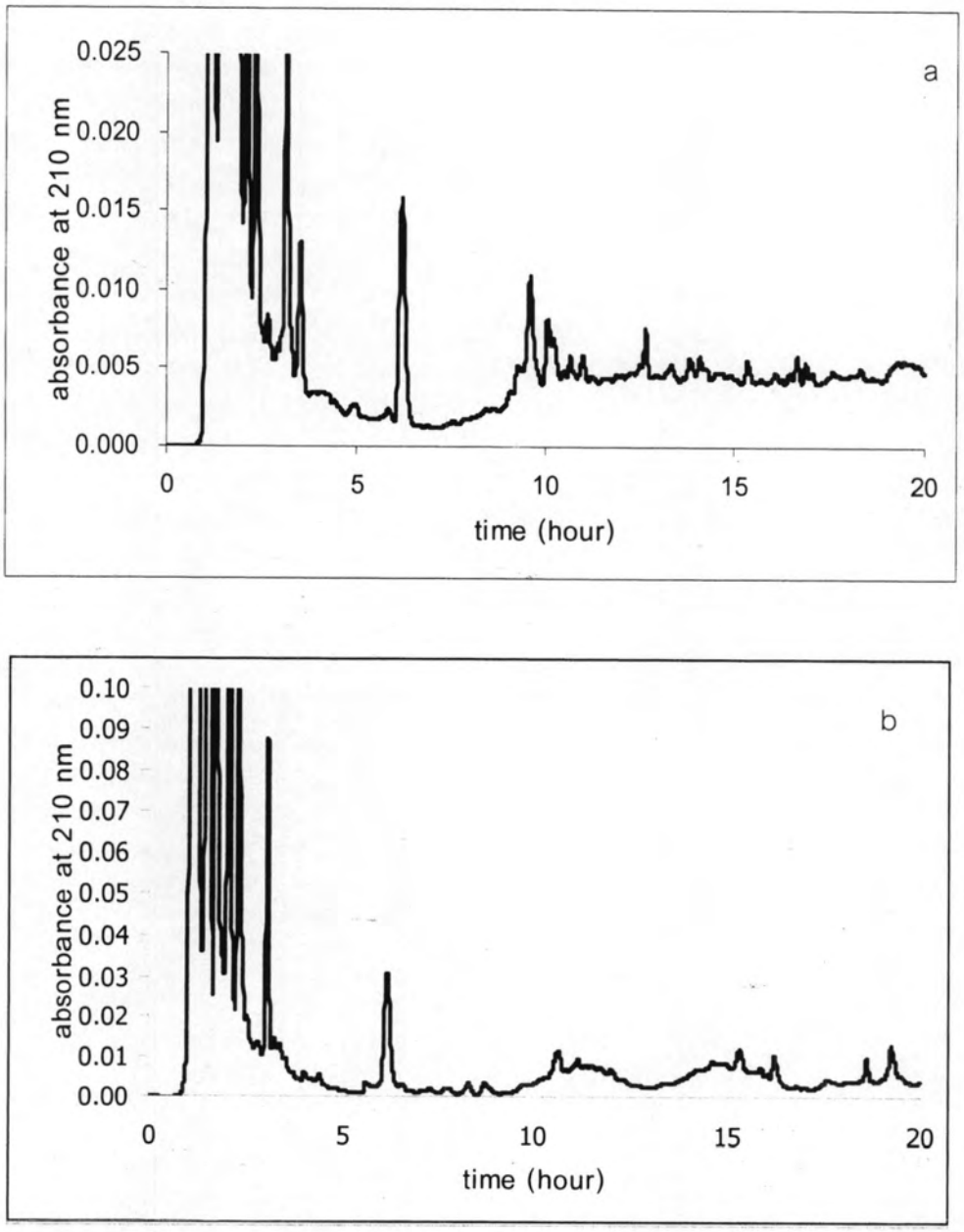


Figure 4.17 Chromatograms of the water-extract from *V.parahaemolyticus* DMST 23798 colonies cultured for 24 hours on three NA different in salt concentrations: 0.5% (a), 3% (b), and 8% NaCl (c)

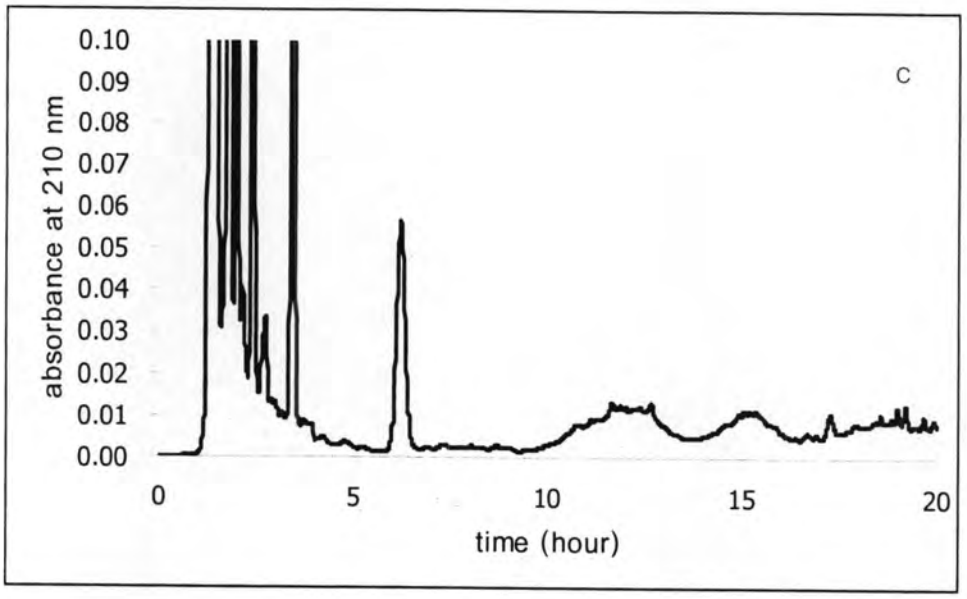


Figure 4.17 Chromatograms of the water-extract from *V.parahaemolyticus* DMST 23798 colonies cultured for 24 hours on three NA different in salt concentrations: 0.5% (a), 3% (b), and 8% NaCl (c) (continue)

4.3.2.3 Effect of incubation temperature

V.parahaemolyticus DMST 23798 was cultured on NA for 48 hours at 15 °C and room temperature. The chromatograms of the water-extract from the colonies were shown in Figure 4.18.

At 24 hours, no growth was observed when *V.parahaemolyticus* DMST 23798 was incubated at 15 °C (data not shown). Therefore, the incubation time was extended to 48 hour. The chromatograms of water-extract from *V.parahaemolyticus* DMST 23798 colonies incubated at both temperatures had the same major peak at 6.2 minute (Figure 4.18). This peak could be the peak of 3-hydroxyl-C4-HSL as previously described. Therefore, *V.parahaemolyticus* DMST 23798 still produced the same type of AHL even at the uncommon temperature. However, the height of 3-hydroxyl-C4-HSL peak was lower when *V.parahaemolyticus* DMST 23798 was incubated at 15 °C. This could be due to the slower growth rate of *V.parahaemolyticus* DMST 23798 incubated at a low temperature. These effects of incubation temperature on the AHL production also found in *V.parahaemolyticus* ATCC 17802 (Figure E7 in Appendix E).

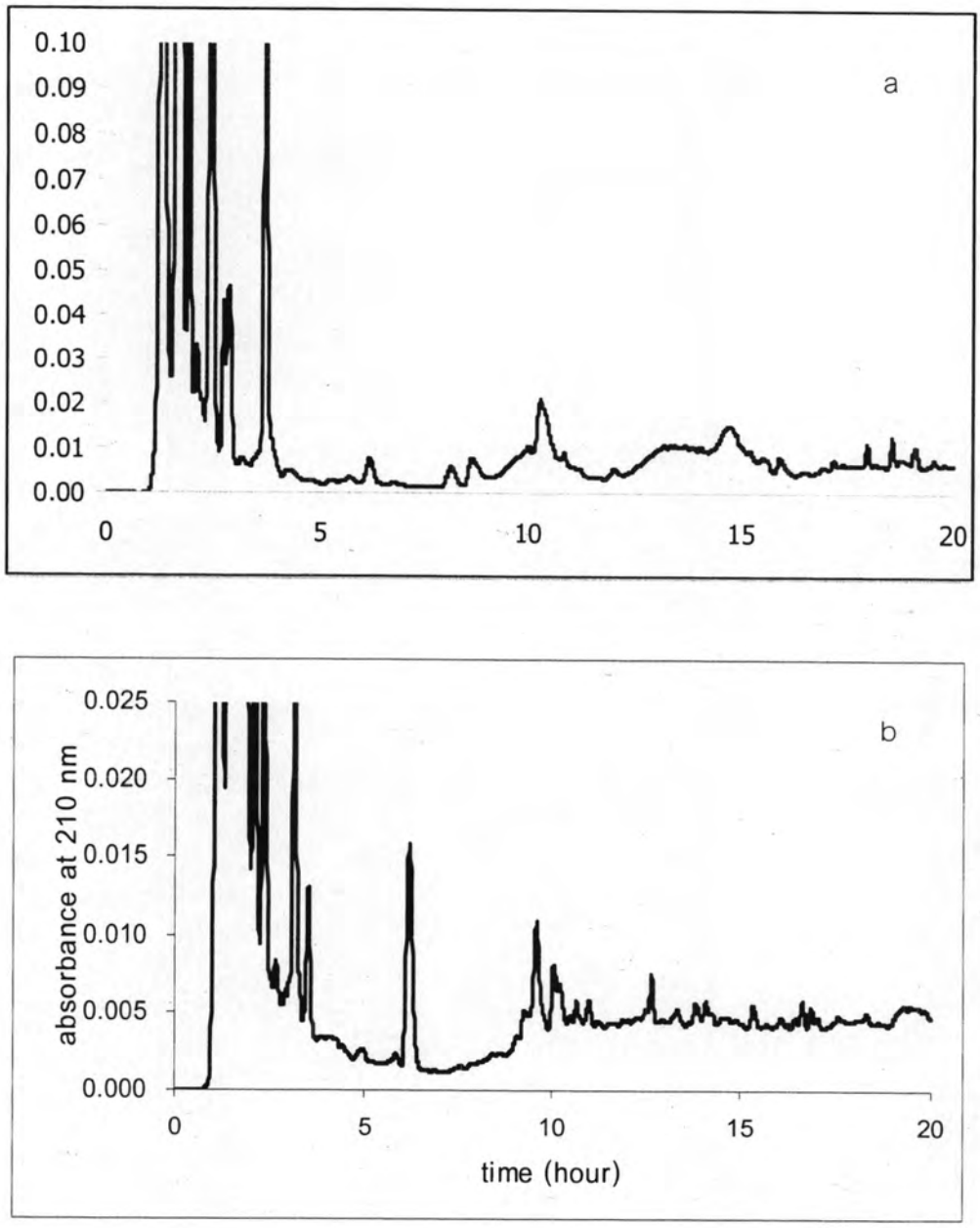


Figure 4.18 Chromatograms of water-extract from *V.parahaemolyticus* DMST 23798 colonies cultured on NA for 24 hours at 15 °C (a) and room temperature (b)

In this study, HPLC analysis for characterization of AHL produced from bacteria was developed and evaluated. Extraction of AHL directly from bacteria colonies provided a good resolution of AHL chromatogram without interference from impurity. This developed HPLC technique was convenient, reproducible, and precise and was used for further study. Both pathogenic and non-pathogenic strains of *V.parahaemolyticus* produced the same AHL type which was 3-hydroxy-C4-HSL.

V.parahaemolyticus produced only 3-hydroxy-C4-HSL as its AHL in all growth phases but with the highest amount of 3-hydroxy-C4-HSL during the stationary phase. Nutrient source, salt concentration, and incubation temperature did not affect the type of AHL produced from *V.parahaemolyticus* but did affect the amount of produce 3-hydroxy-C4-HSL. This study suggested that the produced AHL could be used as a finger print for identifying unknown bacteria culture due to its independence on strains and cultivation conditions.

5. Evaluation and application of AHL analysis procedure for *V.parahaemolyticus* determination

The objective of this experiment was to develop *V.parahaemolyticus* isolation and identification methods through AHL production property. Based on the nature of *V.parahaemolyticus* which could grow in medium containing 8% NaCl, and as previous described in sections 3.3 and 4.3.2, 8% NaCl could suppress bacterial growth but it was likely to induce *V.parahaemolyticus* AHL production. In addition, when %measured absorbance of 8%NaCl peptone water was compare to 8%NaCl NB cultivated under the same condition, it was found that %measured absorbance of peptone water was higher than NB approximately 42% (data not shown). This demonstrated that 8% NaCl peptone water could better used to induce the AHL production of *V.parahaemolyticus* than NB. Therefore, 8%NaCl could be a proper enrichment selective media for cultivation of *V.parahaemolyticus* in order to activate the AHL production along with the isolation of the *V.parahaemolyticus* from the other bacteria.

In addition, the acceptable microbiological quality criteria of viable *V.parahaemolyticus* cell in seafood was lower than 200 logCFU/g (Department of Medical Science, Ministry of Public Health, Thailand). Hence, this initial population level was used to evaluate *V.parahaemolyticus* determination through this study.

Therefore in this study, single and mixed strains of pathogenic *Vibrio* sp. generally found in seafood (*V.parahaemolyticus* DMST 23798, *V.cholerae* DMST 2873, *V.vulnificus* ATCC 27562) were selected to evaluate the selective media. Bacteria culture containing initial population of 2 logCFU/ml were cultured in peptone water (1%)

containing 8% NaCl for 26 hours. TPC and AHL were determined every 2 hours. Results were shown in Figure 4.19 to 4.20.

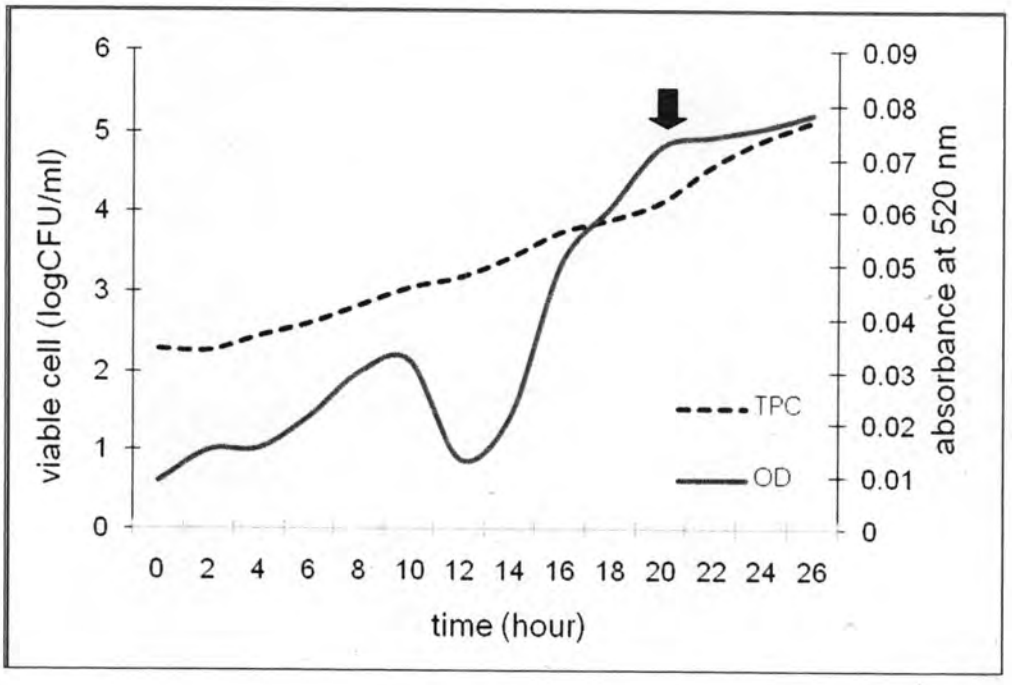


Figure 4.19 TPC and OD_{520} of *V.parahaemolyticus* DMST 23798 culture in 1% peptone water containing 8%NaCl

From Figure 4.19, TPC and OD_{520} were determined in the culture of *V.parahaemolyticus* DMST 23798 collected for every 2 hours. The first two hours TPC did not change. These indicated that there was no growth during this period, since bacteria adapted their cell to new environment. AHL in the culture of this hour was detected (OD_{520}= 0.0092) but %RSD of OD_{520} was over ± 15 (Table F1, Appendix F). This could be due to the sensitivity limit of the method as described in section 3.1. At the 4^{th} hours of cultivation time, the increasing in TPC was observed and TPC were increased through the 26^{th} hours of cultivation time (slope, m = 0.11). AHL was still detected with unacceptable %RSD through 18^{th} hours. The AHL was correctly measured at the 20^{th} hour (SD < ±15%, point arrow) and gradually increased through the 26^{th} hours (m = 0.001).

Therefore, based on this methodology, *V.parahaemolyticus* in the single culture containing initial population 2 logCFU/ml could be determined within 20 hours.

To confirm the growing properties of the other bactria that could interfere the *V.parahaemolyticus* isolation and its AHL production in the system. *V.vulnificus* and *V.cholerae* were also subjected to culture in 8%NaCl peptone water under similar cultivation conditions. The results are shown in Figure 4.20 and 4.21, respectively.

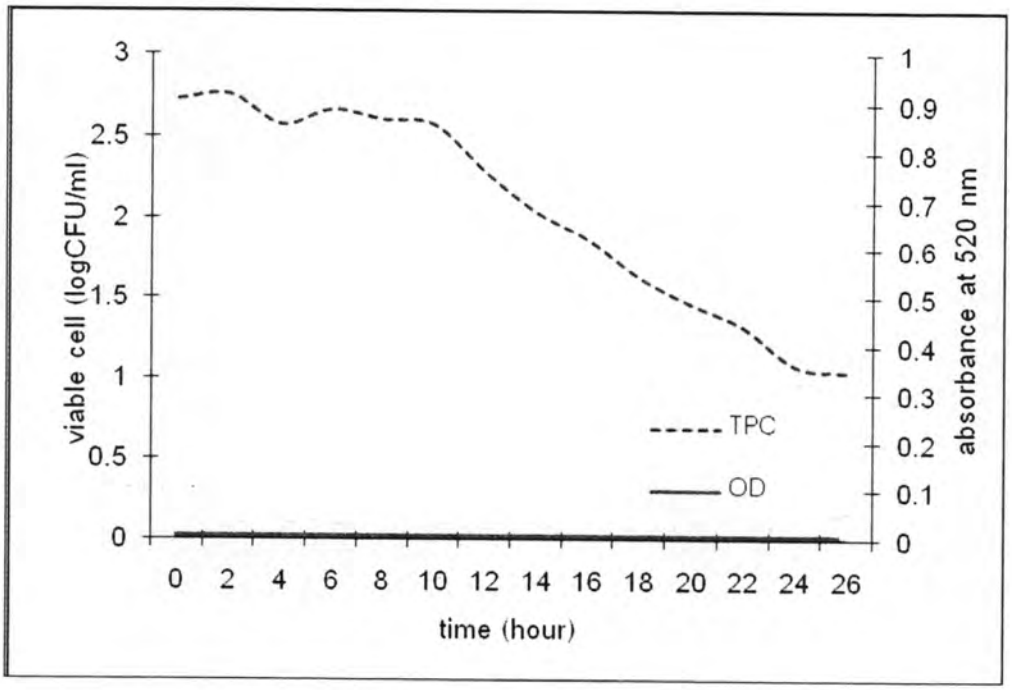


Figure 4.20 TPC and OD_{520} of *V.vulnificus* ATCC 27562 culture in 1% peptone water containing 8%NaCl

According to Figure 4.25, after cultivating *V.vulnificus* 2.84 logCFU/ml, the population did not significantly reduce during the 0 to 9th hours. The population began to reduce at the 10th hours and relatively decreased through the end of cultivation time.

AHL in the culture were not observed through the cultivation times (OD_{520} = 0). This confirmed that substances generated from *V.vulnificus* at any growth stages in 8%NaCl peptone water could not interfere AHL detection system of this *V.parahaemolyticus* determination methodology.

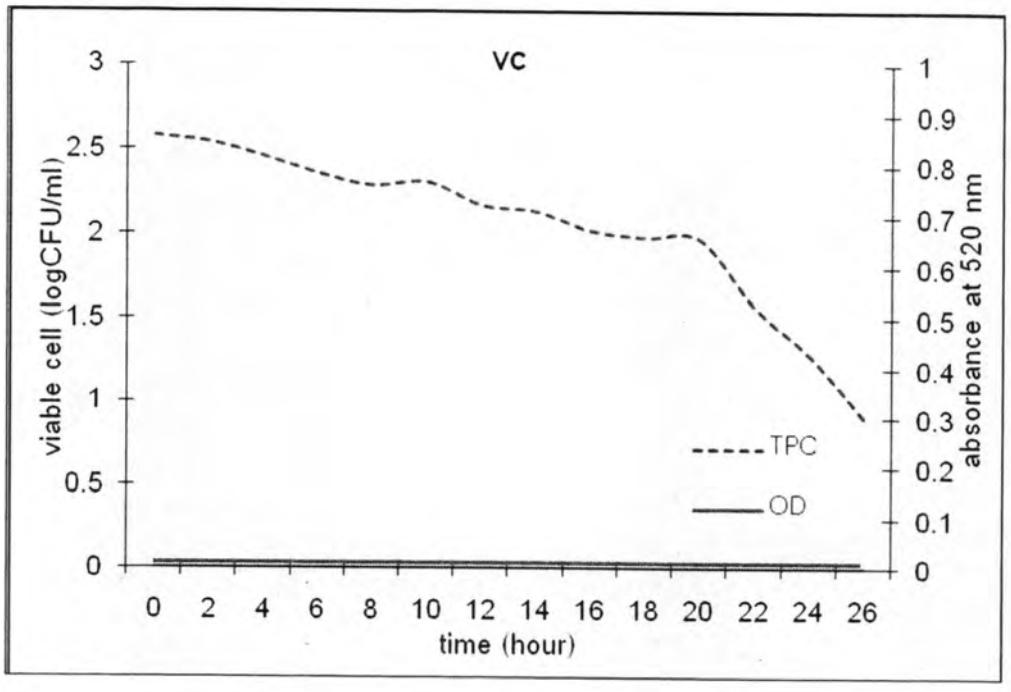


Figure 4.21 TPC and OD_{520} of *V.cholerae* DMST 2873 culture in 1% peptone water containing 8%NaCl

For the cultivation of *V.cholerae* (Figure 4.21), the reduction of population during cultivation period was also similar to *V.vulnificus*. However, it was found that *V.cholerae* population slightly decreased during the 0 to 20th hours. This reduction rate remained constant in longer period than *V.vulnificus*. Again, based on absorbance determination results, it could be concluded that any substance generated from this bacteria could not interfere AHL detection system for *V.parahaemolyticus* determination methodology developed in this study.

To evaluate the influences of the other bacteria on the growth and AHL production of *V.parahaemolyticus*, the mixture of *V.parahaemolyticus* and another 2 species, *V.vulnificus* or *V.cholerae*, 2 logCFU/ml were cultured in 8%NaCl peptone water under the similar cultivation system as previous described. The results are shown in Figure 4.22 and 4.23.

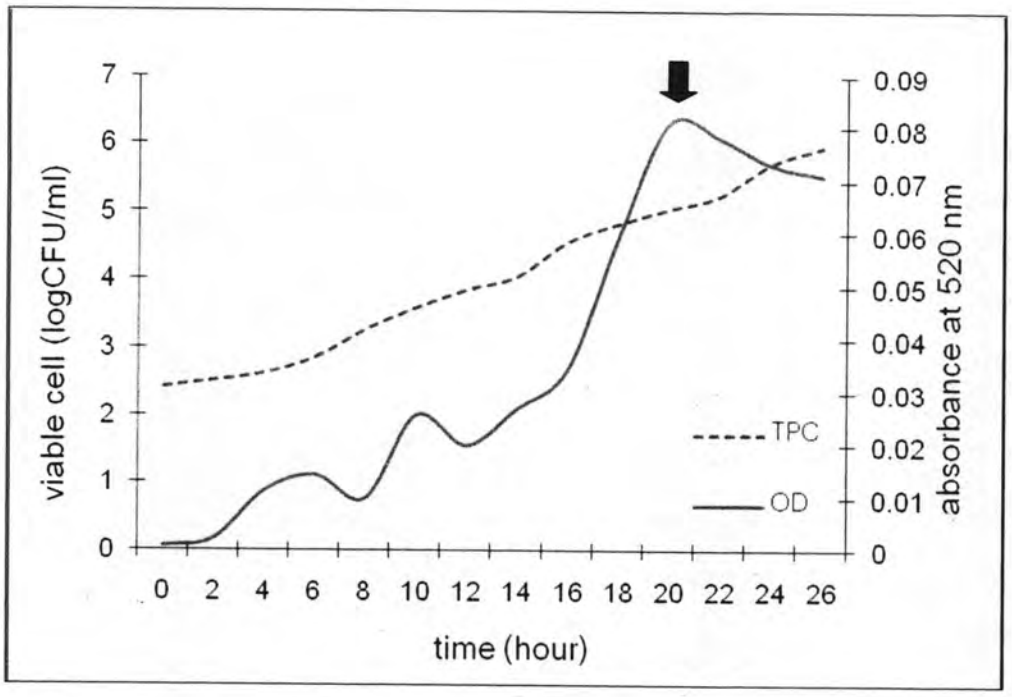


Figure 4.22 TPC and OD_{520} of *V.parahaemolyticus* DMST 23798 and *V.vulnificus* ATCC 27562 mixed culture in 1% peptone water containing 8%NaCl

In Figure 4.22, AHL was also detected at the beginning of the co-culture of *V.parahaemolyticus* DMST 23798 and *V.vulnificus* ATCC 27562 but the %RSD of determination was over ± 15 (Table F4 in Appendix F). From 0 hour, population increased through the cultivation times. TPC increasing rate was low at the first 18 hours (m = 0.06) and started to be faster from 18th to 26th hours (m = 0.3). AHL could be increasingly detected at the 2nd hours but %RSD was over ± 15. During the 2nd to 18th hours, %RSD of the AHL determinations were still over ± 15. AHL was acceptable determination (%RSD < ± 15) at the 20th hours. Interestingly, the reduction in AHL concentration during the 22nd to 26th hours of the cultivation time was observed. This AHL production properties of *V.parahaemolyticus* DMST 23798 in co-culture with *V.vulnificus* ATCC was different from a single culture as shown in Figure 4.26.

However, it could be observed that amount of AHL in single culture of *V.parahaemolyticus* and co-cultured with the other species could be acceptable detected at the same cultivation period which was the 20th hours. This results demonstrated that 2 logCFU/ml of *V.parahaemolyticus* in single and mixed cultures could be determined by this method within 20 hours.

When AHL concentration at the 20th hours of single culture was compared to the mixed culture, it was found that amount of AHL in the mixed culture (OD_{520} = 0.0827) was larger than the single culture (OD_{520} = 0.0725). This result demonstrated that the presenting of another species in the culture could influence on the AHL production of *V.parahaemolyticus*.

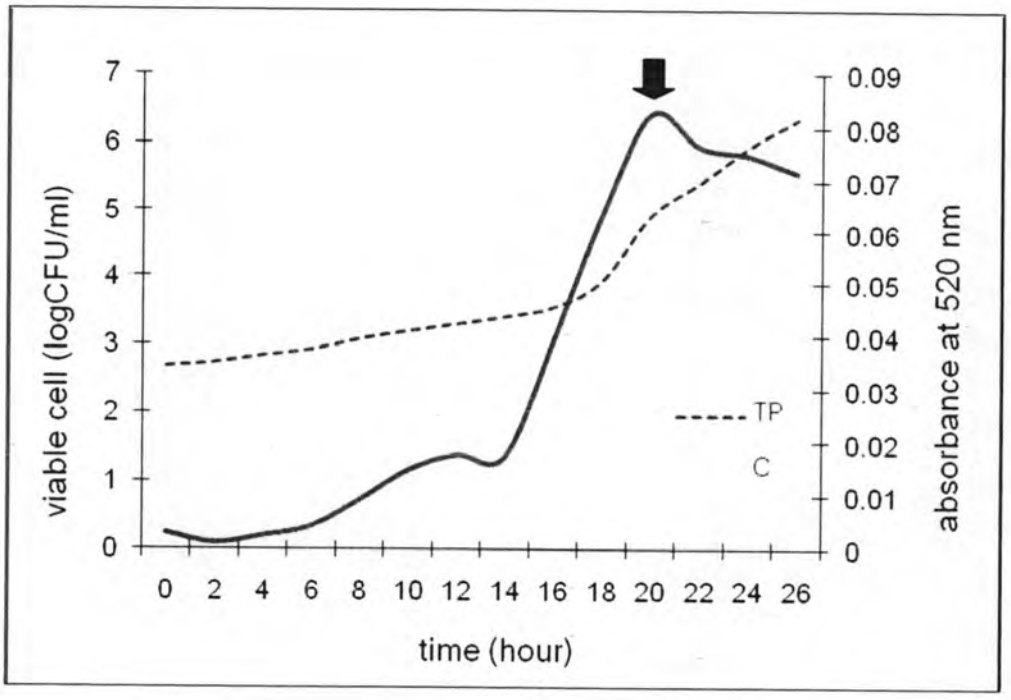


Figure 4.23 TPC and OD_{520} of *V.parahaemolyticus* DMST 23798 and *V.cholerae* DMST 2873 mixed culture in 1% peptone water containing 8%NaCl

Similar AHL production property was also observed in co-culture of *V.parahaemolyticus* DMST 23798 and *V.cholerae* DMST 2873 (Figure 4.23). From 0 hour, TPC constantly increased through the cultivation time (m = 0.29). AHL were detected during the 2nd and 18th hours with wide range of %RSD (over ± 15, Table F5 in Appendix F). The acceptable AHL detection (%RSD < ± 15) also began at the 20th hours. At this cultivation time, larger amount of AHL (OD_{520}= 0.0811) from single culture was also determined.

In conclusion, this finding confirmed that the other species presented in the cultural system could influence AHL production of *V.parahaemolyticus*.

V.parahaemolyticus in co-cultured with *V.vulnificus* or *V.cholerae* could generate larger AHL amount approximately 13% than the single culture of *V.parahaemolyticus*. Two logCFU/ml of the *V.parahaemolyticus* in single and mixed culture could be determined within 20 hours by the methods developed in this study.

From the results shown in Figure 4.19, 4.22 and 4.23 demonstrated the amount of AHL generated in the mixed cultures were larger than in single culture. To illustrate these finding, curves of OD_{520} determined in all cultures were compared as shown in figure 4.24

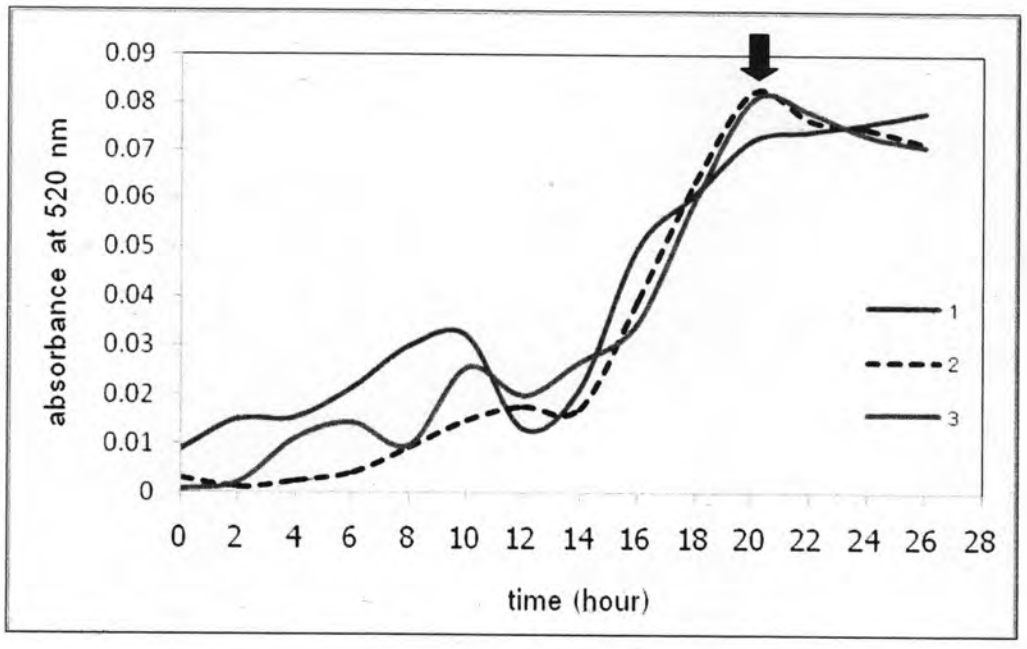


Figure 4.24 OD_{520} observed from *V.parahaemolyticus* in single culture of *V.parahaemolyticus* DMST 23798 (1) and mixed cultures of *V.parahaemolyticus* DMST 22092 and *V.vulnificus* ATCC 27562 (2), and *V.parahaemolyticus* DMST 22092 and *V.cholerae* DMST 2873 (3)

According to Figure 4.24, detected AHL during the 0 to 18th hours were shown in dash line. At first 12 hours, the AHL trend line of single and mixed cultures did not show any relationship to each other. The increasing of AHL began at the 14th hours of cultivation time. The maximum AHL concentrations of all cultures exhibited at the 20th hours, where %RSD of the determinations were also acceptable (%RSD < ± 15, Table

F1, F4 and F5 in Appendix F). After the 20th hours, AHL in both mixed culture reduced through the end of cultivation period.

In conclusion, the cultivation media, 1%peptone containing 8%NaCl could be used for growing *V.parahaemolyticus* and inducing its AHL production. The other *Vibrio* species presented in the culture system (such as *V.vulnificus* and *V.cholerae*) could influence on the AHL production.

From the finding in this section, the existing of *V.vulnificus* and *V.cholerae* in the culture system of *V.parahaemolyticus* was likely to induce the AHL production of the *V.parahaemolyticus*. The investigation in mechanisms of AHL production influenced from the other bacteria, and relationship of initial populations and AHL detectable time should be further studied. The data obtained can be used to further improvement of this AHL determination methodology.

Based on its properties in inducing the growth and AHL production of *V.parahaemolyticus*, this medium system could be a potential enrichment selective media for *V.parahaemolyticus* isolation before subjecting to identify by AHL determination method as developed in this study.

Therefore, following flow diagram (Figure 4.25) is proposed as a new strategy for isolation and identification of *V.parahaemolyticus*. This method could be an alternative for a simple identification of *V.parahaemolyticus*.

From the results in section 4, since cultural conditions and strains could not affect the type of AHL produced from *V.parahaemolyticus*. Therefore, another alternative method which could be rapid qualitative identification for *V.parahaemolyticus* based on HPLC analysis is also proposed (Figure 4.26).

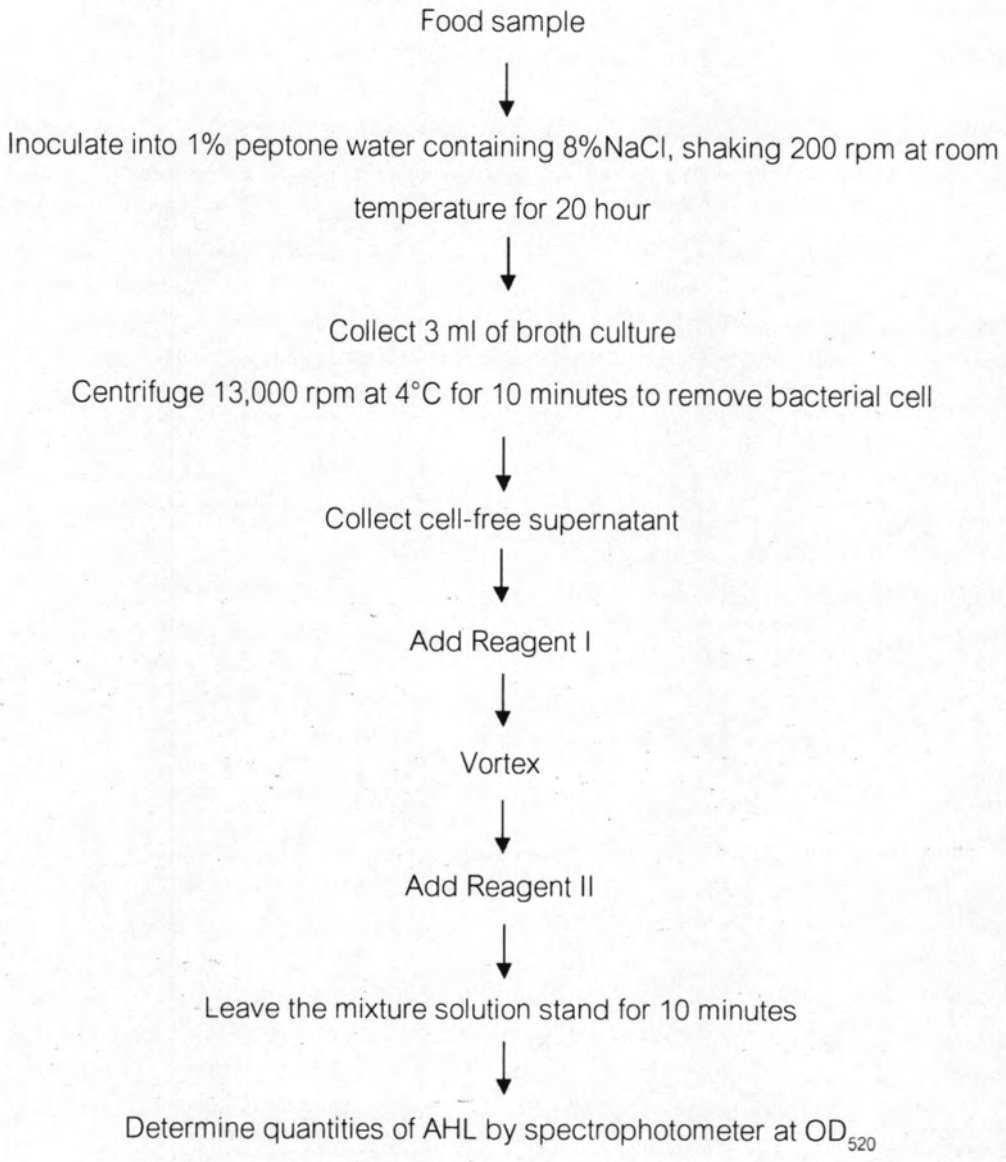


Figure 4.25 Direct colorimetry procedure proposed for *V.parahaemolyticus* determination based on its AHL producing properties

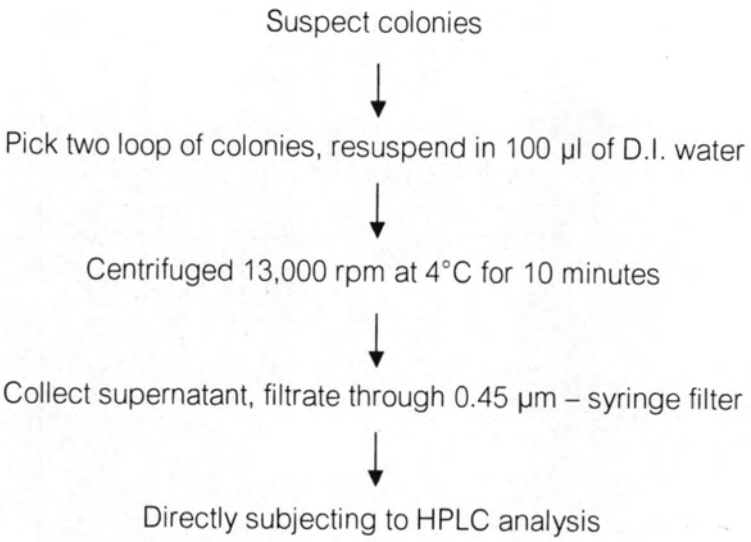


Figure 4.26 HPLC analysis procedure proposed for *V.parahaemolyticus* determination based on its AHL producing properties