CHAPTER II
LITERATURE REVIEW

1. Quorum sensing (QS)

Quorum sensing is a type of cell to cell signaling involving the production of pheromone-like compound called autoinducers (AIs). These AIs act as "bacterial language" for communication in their own groups or between groups. Many AI molecules diffuse through cell membrane to outer environment, or vise versa. Therefore, accumulation of AHL in the environmental system as well as in bacterial cell occurs. When the accumulation reaches threshold level, induction of gene expression is occurred. This QS phenomenon has been described as cell density-dependent system, allowing bacteria to sense their own population as well as other bacteria in environment (Bassler, 2002). The first evidence about QS signaling system was proposed by McVittie et al in year 1962. The study demonstrated that fruiting body formation of Myxococcus xanthus was regulated by some bacterial signaling. About three years later, the first direct experimental evidence of the production of signal molecule by microorganisms was reported by Tomasz (1965). The study revealed that *Pneumococcus* produced a signal molecule and released into growth medium, resulting in expression of pneumococcal competence in some stage of growth. Moreover, variable of the organism was due to the accumulation of some extracellular substances.

Consequently, many following researches have been reported that QS signal is used to regulate some specific phenotype expression, such as virulence, growth, sporulation, toxin production, enzyme production, biofilm formation and this regulation occurs only when the bacteria reaches high cell density (Nealson et al, 1970; Eberhard et al, 1981; Bodman and Farrand, 1995; Eberl et al, 1996; Wood et al, 1997; Davies et al, 1998; Ravn et al, 2001; Lynch et al, 2002; Podbielski and Kreikemeyer, 2005; Martinez et al, 2005). These studies also revealed that many microorganisms were used different signal molecule for the communication. However, the regulation steps of all QS molecules were similar as described below.

1.1 QS regulation

Process of QS regulation of bacterial cell included 3 steps of QS production, accumulation and reception as depicted in Figure 2.1 (Podbielski and Kreikemeyer, 2004).

1.1.1 QS production, the signal molecules are produced from an expression of specific gene(s) and then transported through cell membrane. Once molecules are outside, most of them diffuse into environment but some molecules are bounded to surface of bacterial producing cell.

1.1.2 QS accumulation, the accumulation could be due to the production of a molecule at a constant rate. Moreover, decreasing of available space for bacteria and signal molecules in close environment, or an impermeable structure in close vicinity of bacteria are both also lead to the increasing of QS signal concentration.

1.1.3 QS reception, bacteria sense the signals from the outside. Signals move from the environment through bacterial cell membrane by two mechanisms. The small molecule will be uptaken by diffusion. The large molecule will be uptaken through cell by active transport. In some case, the sensing occurs at cell surface by sensor protein. Bacterial sensing of signal molecule takes place when the signal is produced but the regulation of specific gene will occur only when signal concentration reach threshold level.

1.2 Groups of QS signaling molecules

The signaling molecule groups were systemically reviewed by Podbielski and Kreikemeyer (2003). QS signaling molecule have been divided into 3 groups (AI-1, AI-2 and AI-3) based on their molecular structure as following

1.2.1 Acyl homoserine lactone (AHL); AI-1, or AHL, is the first QS signaling that has been reported. This QS signal is the most widely studied. The

signaling group is used among Gram negative bacteria to communicate in their own groups. However, for some bacteria, *Pseudomonas aerunginosa*, AHL are used for specific interspecies communication.

1.2.2 Furanosyl borate or nonboronated diester (AI-2 molecules); AI-2 is assumed to be hightly conserved communication in bacteria. Many of Gram negative and positive bacteria, such as *V. harveyi*, *V. cholorae* and *Escherichia coli* produce this type of QS signal. AI-2 likely to uses for interspecies communication. Signal transportation into bacterial cell occurs through either ribose transporter or membrane-located sensor system. Other types of QS signal in this group is heptyl-hydroxy-quinolones (PQSs), which size and lipophilic property are similar to AHL. PQSs are produced by Gram negative bacteria (*P.aerunginosa*) and freely diffuse through bacterial membrane.

1.2.3 Oligo- and cyclic dipeptide; these 2 type of QS signals are chemically diverse from other groups above. Cyclic dipeptide signal is produced by some Gram negative bacteria. These molecules diffuse freely through bacterial cell membrane. Oligopeptides are produced in both of Gram negative and Gram positive bacteria. In Gram positive bacteria, oligopeptide sensing system (step 3, Figure 2.1) occurs at cell membrane surface so called externally sensed. However, in some cases, the signals are active transport by permease enzyme and the sensing occurs inside bacterial cell. These oligopeptides are the most important QS signal in Gram positive bacteria.

Sample structures, groups of QS producing bacteria, signal uptake and sensing location were shown in Table 2.1

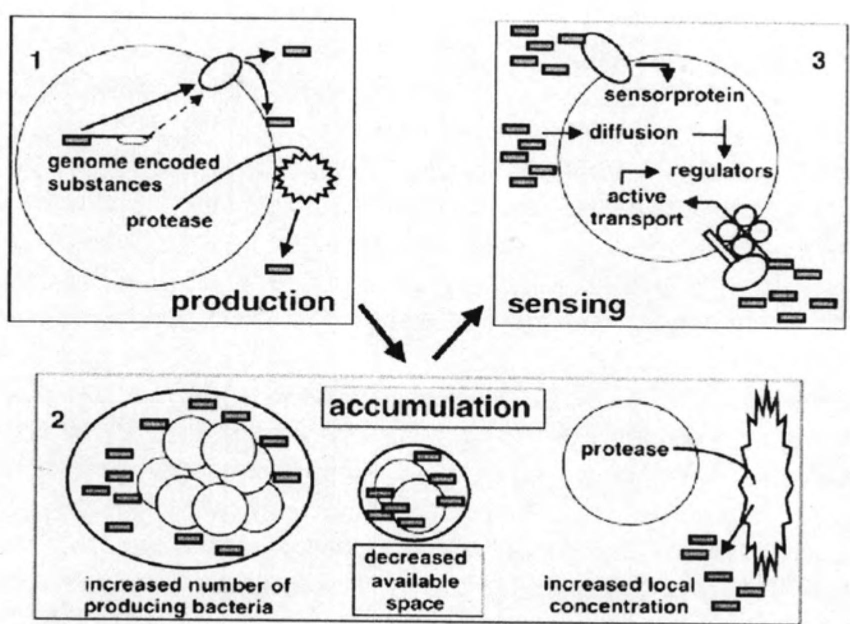

Figure 2.1 Three steps in quorum sensing regulation

Source Podbielski and Kreikemeyer, 2004

Table 2.1 Quorum sensing signal molecules.

groups	structures	producing bacteria	uptaking pathways	locations of sensing
AHL		Gram-negative	diffusion	Intracellular
AI-2		Gram positive / negative bacteria	active transport	extra / intracellular
cyclic dipeptides		Gram negative	diffusion	intracellular
oligopeptides		Gram positive / negative bacteria	active transport	extra / intracellular

Source Podbielski and Kreikemeyer, 2004

2. QS in Gram negative bacteria

Even the first evidence of bacterial communication had been found earlier. But well intensive study of this phenomenon was commenced by the work of Nealson, Platt and Hastings in year 1970. The Gram negative light producing marine bacterial *V.fischeri* was studied in that, why its luminescence system only occurs in the middle of log phase but does not occur in other phases of growth. The luminescence system is controlled by luciferase enzyme. From this study, they found that the enzyme production was controlled by genetic mechanism. Luciferase gene was repressed in freshly inoculated culture. But during period of growth, activation and synthesis of luciferase were rapidly occurred. This phenomenon was referred as "autoinduction". After that, the autoinduction was further studied by Nealson and Hasting (1979). They reported that self-produced bacteria autoinducer was control the luminescence and concluded that bacteria used chemical signals to communicate between its group. In the next three years, an autoinducer molecule was isolated from cell-free supernatant of *V.fischeri* MJ-1. HPLC chromatogram of this autoinducer indicated the structure of N-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanone (N-(β-ketocaproyl) homoserine lactone); 3-oxo-C6-HSL), one of AHL derivatives (Eberhard et al, 1981).

The luminescence system in *V.fischeri* was further investigated. Engebrecht et al (1983) and Silverman (1984) explained that luciferese operon in *V.fischeri*, *luxICDABE*, was regulated by two regulatory proteins called LuxI and LuxR. This marine bacterial - regulatory circuit became one of the fundamental models for the following quorum sensing studies.

From the first reported about QS-dependent of light-emission phenotype in *V.fischeri*, QS regulation and its physiological impact in Gram negative bacteria have been intensively studied in great detail. QS circuit identified in most Gram negative bacteria were resemble to *V.fischeri* circuit, which used AHL as signaling molecule and had two regulatory proteins, LuxI and LuxR. The members of LuxI and LuxR proteins family from Gram negative bacteria, for example, were TraI/TraR in *Agrobacterium tumefaciens*, YenI/YenR in *Yersinia enterocolitica* (Fuqua and Eberhard,1999), LasI/LasR and RhlI/RhlR in *P.aerunginosa* (Miller and Bassler, 2001). In some AHL

regulatory, non-LuxI/LuxR type were found, such as LuxM/LuxN in *V.harveyi* (Bassler et al, 1993). Some bacteria such as Escherichia coli used LuxR homologs, SdiA to regulate cell division (Gambello and Iglewski, 1991). As comparisons of amino acid sequence from LuxI-type protein of different bacteria, level of conservation ranged from 28 to 34%. LuxI and its homologues direct the synthesis of cognate AHLs associated with a given quorum-sensing circuit (Swift et al, 1996).

Figure 2.2 General structure of N-3-acylated homoserine lactone
Source Gram et al, 2002

AHLs are lipophilic, therefore, short acyl chain AHL can freely diffuse through bacterial membrane. For AHL molecules containing long acyl chain, active transport is used for transportation of signals through cell membrane. Common variations of the N-acyl side chain structure include chain length and the nature of the substituent (usually oxo-) at the C3 position of acyl side chain (Fuqua, Winas and Greenberg, 1994; Meighen, 1994; Salmond et al, 1995; Sitnikof, Schineller and Baldwin, 1995; Winson, 1995) (Figure 2.2). These variations determine the biological properties of the AHL within a given population. In the environmental system, bacteria live in mixed cultures, where they can expect to encounter AHLs from a number of rival or symbiotic species. For example, several bacteria produce the same AHL signal molecule, although, in each it is clearly used to regulate the expression of different biological properties (Table 2.2). Other bacteria have been shown to produce multiple AHLs, each having different effects on phenotype. Therefore, possibilities have been existed for bacteria to regulate genes according to a number of population - based parameters, by varying the physical and biological properties of the signal molecule (Swift et al, 1996).

Table 2.2 : Example of AHL phenotypic regulation in Gram negative bacteria

organisms	signals	regulated phenotype(s)	source
Aeromonas hydrophila	C4-HSL	serine protease and metalloprotease production	Swift et al, 1997
Ae.salmonicida	C4-HSL	exoprotease production	Swift et al, 1999
A.tumefaciens	3-oxo-C8-HSL, C4-HSL	Ti-plasmid conjugation transfer	Piper et al, 1993
Berkholdaria cepacia	C8-HSL	protease and siderophore production	Lewenza et al, 1999
Chromobacterium violaceum	C6-HSL	violacein pigment, hydrogen cyanide, antibiotics, exoprotease and chitinolytic enzyme	Chernin et al, 1998
Erwinia carotovora	3-oxo-C6-HSL	Carbapenem antibiotic sysnthesis (through CarI/R)	Jones et al, 1993,
	3-oxo-C8-HSL	exoenzyme synthesis (through ExpI/R)	Bainton et al, 1992
E.chrysanthemi	3-oxo-C6-HSL, C6-HSL	regulator of pectinase synthesis	Nasser et al, 1998
Pseudomonas aerofaciens	3-oxo-C6-HSL, 3-oxo-C12-HSL	Phenazine antibiotic biosynthesis	Wood et al, 1997

Table 2.2 : Example of AHL phenotypic regulation in Gram negative bacteria (continue)

organisms	signals	regulated phenotype(s)	Source
P.aerunginosa	3-oxo-C12-HSL, C4-HSL	Virulence and biofilm development	Pearson et al, 1994
Rhizobium etli	multiple, unconfirmed	restriction of nodule number	Pearson et al, 1995
R.leguminosarum	C6-HSL, C8-HSL	nodulation	Rosemeyer et al, 1998
Serratia liquefaciens	C4-HSL	swarmer cell differentiation, exoprotease	Cubo et al, 1992
Vibrio anguillarum	3-oxo-C10-HSL	unknown	Eberl et al, 1996
V.fischeri	3-oxo-C6-HSL, C8-HSL	luminescence and colonization	Milton et al, 2001
V.harveyi	3-OH-C4-HSL	biolumiunescence, exopolysaccharide production, siderophore and metalloprotease	Lupp and Ruby, 2004 Bassler et al,1993
Yersinia enterocolitica	C6-HSL	unknown	Throup et al, 1995
Y.pseudotuberculosis	C8-HSL	unknown	Atkinson et al, 1999

2.1 AHL producing bacteria in food

According to Table 2.2, AHL producing organisms has been found in various groups of bacteria such as marine bacteria (*V.fischeri, V.harveyi*), plant pathogenic bacteria (*A.tumefaciens, E.carotovora*), food spoilage bacteria (*En.agglomerans*) and food pathogenic bacteria (*Y.enterocolitica*). Even QS in bacteria has been widely studied for over 40 years. But for food-related bacteria, attention of their QS property just began only for the last decade.

In year 1999, Gram et al proposed that many Gram negative bacteria isolated from food produced AHLs. About 75% of the phychrotrophic strains of *Enterobacteraiecae* isolated from cold-smoked salmon and vacuum chilled meat produced detectable level of AHLs when cell density reached 106 CFU/ml. Major signaling molecules of these *Enterobacteraiecae* was 3-oxo-C6-HSL. Revn et al (2001) reported that 147 strains out of 148 of *Enterobacteriaceae* isolated from chilled vacuum-packed food produced AHLs. Most strains produced AHLs more than 1 type. This report also confirmed that 3-oxo-C6-HSL was the predominant signals used in *Enterobacteriaceae* QS regulation.

In the study of Flodgaard et al (2003), two strains of *Enterobacteriaceae* were isolated from cold-smoked salmon, *S.proteomaculans* B5a and *En.agglomerans* B6a produced 3-oxo-C6-HSL. In *En.agglomerans* B6a, another signal molecules (3-oxo-C8-HSL) was detected. Interestingly, this bacteria did not produced 3-oxo-C6-HSL as the major signal molecules. This bacteria always produced 3-oxo-C6-HSL and 3-oxo-C8-HSL in a 1:9 ratio when cultured in different conditions (carbon source, temperature, pH, salt concentration and co-existing lactic acid bacteria microflora). In food sample, this bacteria still produced the similar ratio of QS. These shown the consistency of AHL production in this bacterial strain. AHL production in food and AHL regulation of some specific bacterial phenotype during food storage was also relative stable.

More evidence about the AHL-mediated gene regulation in bacteria associated with food spoilage or food toxicity had been found. Kievit and Iglewski (2000) proposed that exoenzyme production in *E.carotovora* was controlled by AHL signal. This phytopathogenic bacteria causes soft rod in commercial vegetable. It used QS

regulation to ensure that exoenzyme production would occur only when cell density is sufficient for successful plant tissue destruction. In the same year, the report of Whan et al (2000) suggested that milk spoilage by post-contamination of *Pseudomonas* species after pasteurization might be involved in the QS regulation of exoenzyme activity.

2.2 Factors associated with AHL production in Gram negative bacteria

After the existing of AHL producing bacteria and their AHL production in food have been reported recently, a number of investigations about the intrinsic and extrinsic factors associated with AHL production. Several studies have demonstrated that altering growth conditions or growth phase can influence the amounts of AHL produced by an organism (Atkinson et al., 1999, Geisenberger et al., 2000; Lithgow et al., 2001, Blosser-Middleton and Gray, 2001, Byers et al., 2002).

In consideration of growth phase, Yate et al (2002) reported that C6-HSL and 3-oxo-C6-HSL produced from *Y.pseudotuberculosis* were detected at mid- to late-logarithmic-phase cultures for higher quantities than stationary phase. Similarly, *P.aeruginosa* stationary-phase cultures grown under the same conditions also contained much lower levels of C4-HSL and 3-oxo-C12-HSL. They suggested that this decreasing could be due to the pH-dependent hydrolysis of AHL molecule. The release of ammonia from aerobic metabolism during the growth of bacterial culture resulted in an increasing of media pH. As lactone ring in AHL molecule is unstable under alkaline condition (Byers et al, 2002). Therefore, in the alkaline pH achieved in stationary-phase LB medium, the open-ring form accumulates. However, this AHL reduction during growth phase did not observed in some bacteria. The AHL production of *S.proteamaculans* strain B5a was not significantly different at the different cell densities (Flodgaard et al, 2003).

Temperature seemed to be one of extrinsic factors influenced AHL production. Schaefer et al. (1996) studied the effect of the temperature on the enzyme activity of LuxI, the autoinducer synthase enzyme that acts in the production of AHL. Optimal activity of the enzyme at 20–30 °C was described, whereas only a 10% of the maximal

activity at 37 °C was reported. Three years later, more evidences about temperature influence with bacteria AHL production was proposed by Atkinson et al (1999). They reported that AHL profile of *Y.pseudotoberculosis* changed with temperature. Moreover, the involvement of temperature with AHL stability has been reported. Gram et al. (1999) proposed that low temperature and the pH typically found in food products (pH < 7) would help stabilize the compounds allowing them to accumulate. Yates et al. (2002) also showed that the stability of AHLs decreased as the temperature was increased from 22 to 37 °C.

For some bacteria, such as *R.leguminosarum*, the media composition can affect the types of AHL produced (Geisenberger et al. 2000; Lithgow et al., 2001). Correspond to these reports, C4-HSL production in *Ae.hydrophila* was affected by environmental factors such as temperature and composition of medium (glucose concentration) (Martinez et al, 2005).

These reports revealed that QS system in some bacteria were affected by both of intrinsic (such as growth phase) and extrinsic factors (such as temperature and media composition). However, the influence of these and other factors with AHL production or profiles of many other bacteria have not been investigated. Thus, further study in other bacteria should be investigated.

3. *Vibrio parahaemolyticus*

The genus *Vibrio* is found freely in aquatic environment or associates with marine animal as microflora in fish, as symbiont in fish and squid, in particular, as pathogen causing disease in fish coral crustaceans as well as human. At least 12 strains of *Vibrio* cause disease to human by the ingestion or wound infection. Non-cholera *Vibrio* pathogens are considered as importance pathogens. They are transmitted by handling or consuming contaminated seafood or by exposure of open wound to the aquatic environment. One of the most significant non-cholera human pathogen that widely distribute in the marine environment is *V.parahaemolyticus* (Su and Lui, 2007).

V.parahaemolyticus is Gram negative, rod shape bacteria. This bacteria living in sea and brackish water. Therefore, it can be generally isolated from seafood. *V.parahaemolyticus* is oxidase positive, facultatively aerobic, and non spore-forming bacteria. Like other members of the genus *Vibrio*, this species is motile by a single, polar flagellum. This bacteria is food pathogen causing gastroenteritis. Infection occurs via ingestion raw or undercooked seafood (such as oyster, which is the predominant cause) or wound infection. The strains isolated from patient mostly produce thermostable direct hemolysin toxin. Serotype of *V.parahaemolticus* can be devided from O-antigen (cell wall antigen, 13 type) and K-antigen (capsule antigen, 71 type). The serotype that reported as pathogenic strains are O3:K6, O1:K25, O1:KUT, O4:K8, and O4:K68 (Bhuiyan et al, 2002).

The first outbreak of *V.parahaemolyticus* was reported in Japan by Fujino in year 1950 (Fujino et al, 1953). This outbreak was caused by raw fish consuming. From that point, the outbreaks were found worldwide. Because of seawater temperatures in summer and early fall season are appropriate for the growth of bacteria, the outbreak becomes intense in these periods. Reports from Japan, China, Taiwan, India and most of Asian and America countries indicated that more than 50% of food borne disease, particulary in fishery product, was caused by *V.parahaemolyticus*. This bacteria was also reported to cause 34% of wound infection and 5% of blood infection.

During year 2000 - 2002, The Epidemiology Bureau of Thailand reported that *V.parahaemolyticus* was the pathogen causing gastroenteritis for the first rank. In year 2000, The National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health Thailand demonstrated that 31.7% which was the largest portion isolated of bacterial strains isolated from diarrhea patient stool were *V.parahaemolyticus*. And during 2001 – 2003 the number were 15.8%, 17.7% and 14.8%, respectively. NIH database (2001-June 2004) shown that the serotype O3:K6 was detected from food (fresh raw saba) for 1.3%, and it was the largest number of isolated from gastroenteritis patient stool. Outbreak incidents of pathogenic *V.parahaemolyticus* strains from NIH database are shown in Table 2.3. In addition, the first 3 strains mostly isolated from patients were O3:K6 (27.6%), O4:K8 (10.8%), O1:KUT (10.3%). The foodborne

V.parahaemolyticus strains intensively found in seafood were O1:KUT (10.3%) and O3:KUT (9.0%) (Hatthayananon, NIH, Available from http://www.dmsc.moph.go.th). This data indicate that the outbreak of gastroenteritis caused by pathogenic *V.parahaemolyticus* strains expanded, the serotype of pathogenic *V.parahaemolyticus* changed and the thermostable direct hemolysin strains was isolated from seafood.

Therefore, for disease prevention, the detection of *V.parahaemolyticus* in seafood is important to fully control the risk posed by the implicated food. To date, the detection of *V.parahaemolyticus* in seafood products is mostly performed by conventional cultural methods, following by biochemical identification of the isolates. The description of this and other new methods were detailed as following

Table 2.3 Situation of *V.parahaemolyticus* outbreak in Thailand between years 2000 – 2003

year	location	*V.parahaemolyticus* serotype
2000	Nonthaburi province	O3:K6, O3:KUT and O4:K8
April 2003	Mueng Thong Thani	O3:K6
August 2003	Rio-ed province	O1:K25
September 2003	Trang province	O4:K9, O1:KUL and O3:K6

Source Hatthayananon, NIH, Available from http://www.dmsc.moph.go.th

3.1 Detection of *V.parahaemolyticus*

The methods for detecting *V.parahaemolyticus* were excellently reviewed by Su and Lui (2007). In their study, the detection methods ranged from standard conventional to newly developed method were described.

3.1.1 Most probable number (MPN)

US Food and Drug Administration (FDA's Bacteriological Analytical Manual chapter 9, May 2004) describes MPN method as commonly method used for the detection of *V.parahaemolyticus* in food. This analytical procedure for enumeration of *V.parahaemolyticus* are presented in Figure 2.3. Isolation of *V.parahaemolyticus* from other bacteria is done by using selective thiosulfate-citrate-bile salts-sucrose agar (TCBS). Round (2-3 mm in diameter), green or blue-green colonies on TCBS are suspect colonies. However, this selective agar can not isolate *V.vulnificus*, another important foodborne bacteria. Therefore, further analysis with biochemical tests such as 3-galactosidase activity must be taken for conformation. The result may not be obtained within 4-5 days.

The other widely used method in European countries is the International Organization for Standardization (ISO) cultural method (ISO 8914:1990). In this ISO method, the samples must be incubated in 2 enrichment media for 78 hours, or in case of deep-frozen products, for 18 hours. The enriched samples are then plated on TCBS and triphenyltetrazolium chloride soya tryptone agar (TSAT). The smooth, green on TCBS or the smooth, flat, dark red on TSAT with approximately 2-3 mm diameter in both case are the suspected colonies. The result must be further confirmed by biochemical tests.

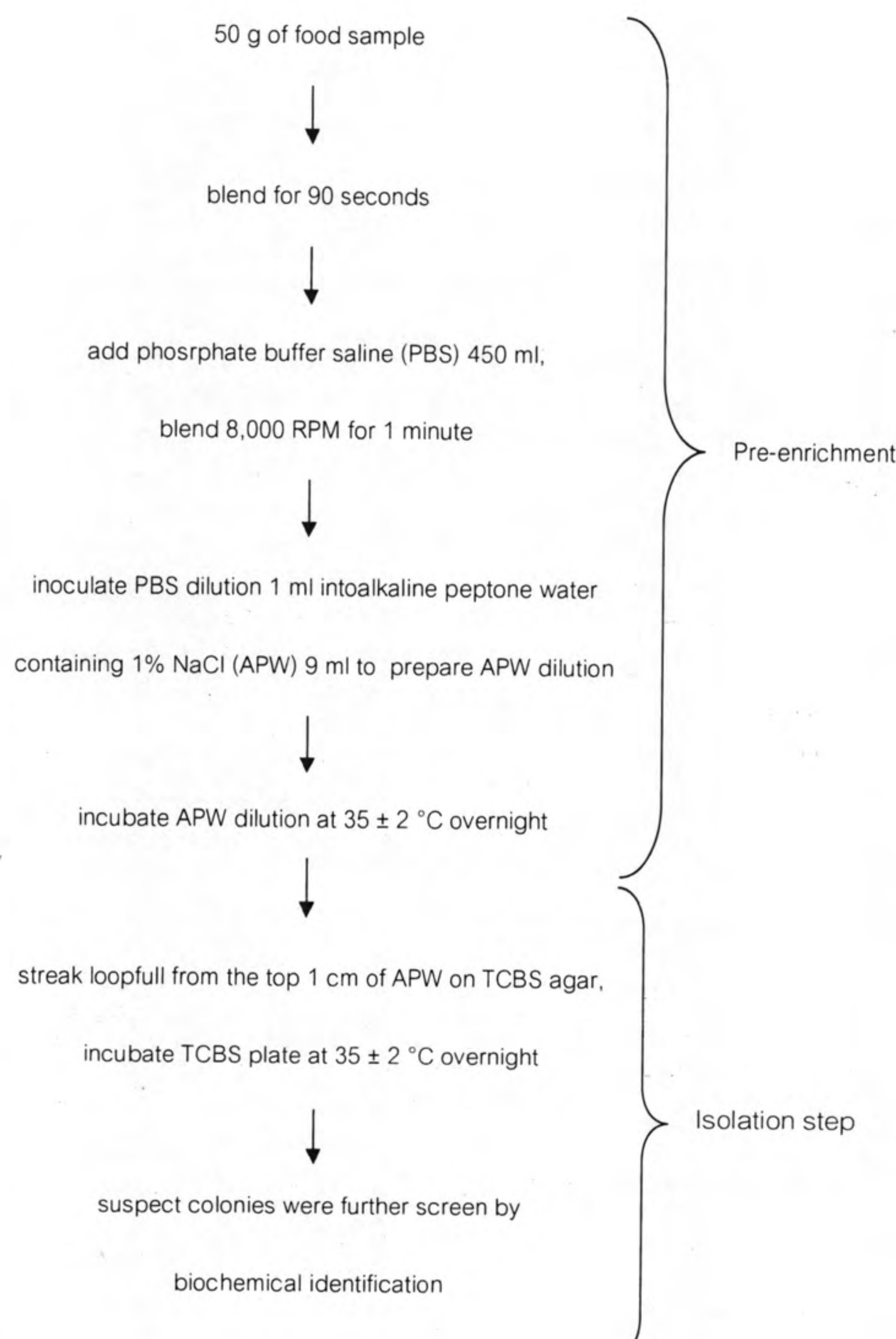

Figure 2.3 Standard MNP method for *V.parahaemolyticus* determination in food sample
Source Modified from FDA's Bacteriological Analytical Manual chapter 9, May 2004

3.1.2 Polymerase chain reaction (PCR)

In 1992, Tada et al developed PCR procedure for detection of specific genes (*tdh* and *trh*) finding in *V.parahaemolyticus* virulent strains. However, detection of

V.parahaemolyticus that excluding *tdh* and *trh* gene could not be done. Additional, sensitivity of the method is reduced in normal fecal samples.

A multiplex PCR procedure reported by Bej et al in 1999 could be used for detecting total and virulent *V.parahaemolyticus* in shellfish. The sensitivity of detection was at least 1-10 cells per gram of alkaline peptone water enriched homogenized sample for 8 hours.

Recently, Kaufman et al (2004) has developed a quantitative real-time PCR assays for detecting total loading of *V.parahaemolyticus* in oyster tissue and mantle fluid. This rapid method could quantify *V.parahaemolyticus* within 1 hour of sampling time.

3.1.3 DNA hybridization

In addition, apart from PCR assays, the molecular methodology DNA–DNA hybridization methods were also developed for specific detection of *V.parahaemolyticus*. Recently, two non-radioactive probes (alkaline phosphatase (AP)-labeled and digoxigenin (DIG)-labeled probes) were developed for specific and sensitive detection of *tlh* gene of *V.parahaemolyticus* (McCarthy et al., 1999). Based on the AP- and DIG-labeled probe methods, two direct plating procedures using AP-and DIG-labeled probes to detect the tlh gene were subsequently developed for detecting total *V.parahaemolyticus* (Gooch et al, 2001). These detection procedures could be completed in 1–2 days. However, it may require skilled workers to conduct the tests because both procedures involve colony lift, hybridization, and colorimetric detection, with an additional preparation of probe and membrane if the digoxigenin-labeled probe method is used.

More recently, Banerjee et al. (2002) developed a rapid DNA probe method for detecting *V.parahaemolyticus* grown on hydrophobic grid membrane filters (HGMF). Although the method was reported capable of detecting *V.parahaemolyticus* in 1 day after an enrichment process. It is a complicate detecting system involving DNA isolation, synthesis of DIG-labeled probes, preparation of *V.parahaemolyticus* primers, and colony hybridization

3.1.4 Chromogenic medium

Recently, a chromogenic medium (Bio-Chrome Vibrio medium) was developed to allow differentiation of *V.parahaemolyticus* from other *Vibrio* species based on formation of unique purple colonies on the medium. Growth of *V.parahaemolyticus* on Bio-Chrome Vibrio medium (BCVM) can easily be distinguished from blue green colonies formed by growth of *V.vulnificus, V.cholerae* and *V.mimicus*. BCVM has more specific and accurate than TCBS (Duan and Su, 2005)

Until now, the detection method of *V.parahaemolyticus* in food samples still has same drawbacks. Several methods require high skill technicians, or special instrument. Some are labor-intensive and time-consuming. For the used in food industry, the other new method has to be develop for more reliable, easy and quick detecting of *V.parahaemolyticus*

4. QS in *Vibrio* sp.

Many of the *Vibrio* members are likely to utilize QS in regulation of some specific response and these systems are function of their populations. One of the most intensive studied QS producing bacteria is *V.harveyi*, the marine bacteria that share a symbiosis with the Hawaiian bobtail squid *Euprymma scolopes*. This Gram negative bacterial QS circuits contains two regulatory proteins, LuxI and LuxR, similar to QS system in *V.fischeri* as describe earlier in title 2 of this section (Milton, 2006). The QS in *V.harveyi* are detailed as follow.

4.1 V.harveyi

V.harveyi is a luminescent bacterium that associated with disease in cultured shrimp worldwide (Lui and Lee, 1999). This bacterial is also associated with eye disease in several fishes which are haemorrhagic spots near the fin and mouth of farmed sole, and necrosis in packhorse rock lobster. Extracellular products and siderophore from V.harveyi culture isolated from infected fish or shellfish are cytotoxic when injected into

healthy animal. Expression of several specific phenotypes of this bacteria are quorum sensing regulation.

Quorum sensing system in *V.harveyi* composes of three parallel cell signaling systems to regulate bioluminescence, metalloprotease, siderophore, and exopolysaccharide production (Bassler et al., 1993; Henke and Bassler, 2004; Mok et al., 2003; Lilly and Bassler, 2000). Also, negatively type III secretion (TTS) system involving in virulence of bacteria is controlled by QS. Model of *V.harveyi* quorum sensing system is showing in Figure 2.4. LuxM/N system used N-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL) as AI-1 signal molecule. AI-2 signal, 3A-methyl-5,6-dihydro-furo(2,3D)(1,3,2) dioxaborole-2,2,6,6A-tetraol, was utilized by LuxS/PQ system. The last system was CqsA/S that utilizes the CAI-1 molecule. The chemical structure of CAI-1 is still unknown. Each signal molecule was different from others and worked synergistically in gene regulation.

At low cell-population density and absence of signal molecule, LuxN, LuxQ, and CqsS transferred phosphate to shared phoshotransferase (LuxU). LuxU was then transmitted the phosphate to response regulator domain, LuxO, and the phosphor-LuxO repressed expression of luxR. At high-cell density, LuxN, LuxQ, and CqsS produce signal molecules and bind to their sensors. 3-hydroxy-C4-HSL and CAI-1 molecule bind directly to LuxN and CqsS, respectively. The AI-2 molecule firstly interacted with periplasmic protein LuxP before bind to LuxQ. All of this leads to the inactivation of LuxO. Therefore, luxR was translated. LuxR bind at luxCDABE promoter and induced bioluminescence and others as described above. LuxR also control the expression of at least three gene clusters encoding TTS system involving in virulence of bacteria. In quorum sensing repressed TTS system at high cell density (Milton, 2006).

4.2 The QS in *V.cholerae*

V.cholerae is a human pathogen and an example for waterborne disease (Colwell, 2004). The hallmark of cholera is a profuse "rice water" diarrhea that is a result of cholera toxin (CT) production. CT, an ADPribosylating toxin, is the most critical

virulence factor made by *V.cholerae* (Kaper et al., 1994 and Dickinson and Lencer, 2003).

According to report of Miller et al (2002), the QS regulation in *V.cholerae* was similar to *V.harveyi*. The system consisted of AI-2 LuxS/PQ system, CqsA/S and third system which was not yet characterized. Non of AI-1 -liked was found in *V.cholerae* indicating that this bacteria did not produce AHL molecules. LuxS/PQ and CqsA/S system functioned similarly to *V.harveyi* (Milton et al, 2006). The downstream regulatory cascade LuxU. LuxO and LuxR were relative to those finding in *V.harveyi* but there was a little difference, as LuxR replaced by HapR in *V.cholerae* (Camara et al, 2002). System 3 was believed to bind directly on LuxO and activated a single regulatory cascade. Thus, all three systems converged at LuxO. Figure 2.5 represents QS system in this pathogenic bacteria.

4.3 The QS in V.vulnificus

V.vulnificus has three main biotypes. Biotype 1 is associated with human disease, biotype 2 prevails in eel infections, and biotype 3 is found in human vibriosis due to fish handling. Human disease caused by *V.vulnificus* is correlated with contaminated seafood or seawater and is characterized by primary septicaemias, wound infections, and gastrointestinal illnesses (Milton, 2006).

V.vulnificus had been demonstrated to possess the *V.harveyi* – like AI-2 system (McDougald et al, 2000, 2001). No homologues of AHL systhaes were found in this bacteria and no AHL signaling molecules had been detected (Kim et al., 2003). The downstream regulatory cascade LuxU, LuxO and the LuxR transcription regulator, SmcR, was also found. Moreover, the regulation cascade in *V.vulnificus* was predicted to be a similar cascade as found in *V.harveyi* (Lenz et al., 2004). The QS system in *V.vulnificus* is shown in Figure 2.7.

4.4 The QS in *V.parahaemolyticus*

First evidence about quorum sensing system in *V.parahaemolyticus* was reported by Greenberg et al in 1979. The study proposed that culture fluid of

V.parahaemolyticus induced the lux expression *V.harveyi*. The work of Greenberg was supported by Bassler, Greenberg and Stevens in year 1997 which studied about cross-species induction of luminescence in *V.harveyi*. The results revealed that *V.parahaemolyticus* produced some distinct substances that could induce both of signaling system 1 and system 2 in *V.harveyi*. The *V.harveyi* AI-1 induced molecule from *V.parahaemolyticus* could be extracted by organic solvent. Moreover, several other species of bacteria produced AI-2-like activity but there was only *V.parahaemolyticus* which produced an *V.harveyi* AI-1-like activity. A possible explanation was, system 1 was the higher-specificity, higher-sensitivity system used to monitor the environment in *V.harveyi* group, and system 2 was lower-specificity, lower-sensitivity system used for monitor the environment for the other species of bacteria.

In 2004, Henke and Bassler set the experiment to study about QS regulated genes and TTS gene cluster regulation by quorum sensing system in *V.harveyi*. The study about LuxR homolog reported by McCarter in 1998, and completed genome sequence of *V.parahaemolyticus* proposed by Makino (2003) were compared with TTS gene sequence in this study. The comparison revealed that the TTS system in *V.parahaemolyticus* was very similar to *V.harveyi*, and *V.parahaemolyticus* possessed all the genes encoding *V.harveyi* - like QS system. However, *V.parahaemolyticus* genome lacked luciferase operon was found in *V.harveyi*. Further study shown that the *V.harveyi* - like quorum sensing system presented in *V.parahaemolyticus* and this system controlled TTS.

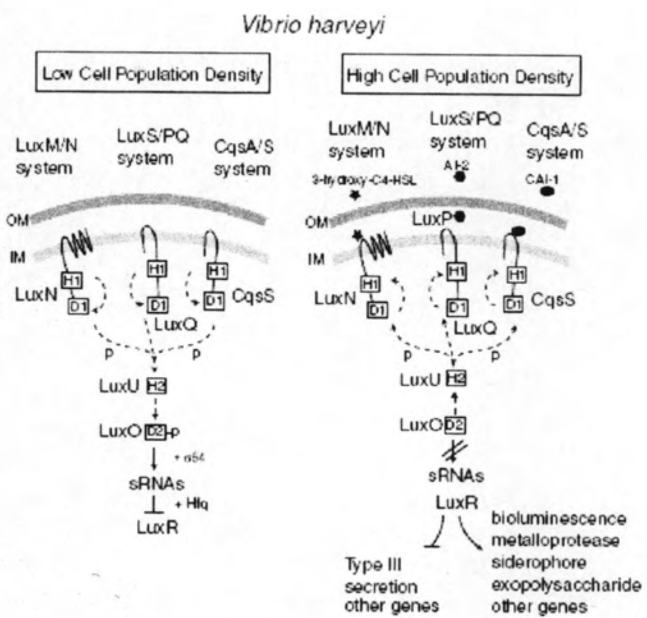

Figure 2.4 Model of quorum sensing system in *V.harveyi* at low and high cell density

Source Milton, 2006.

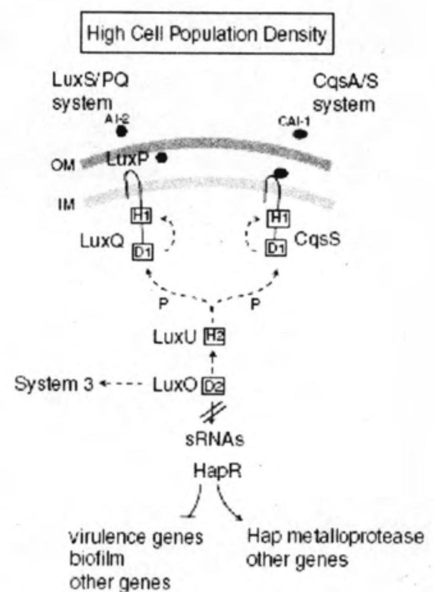

Figure 2.5 Model of quorum sensing system in *V.cholerae* at high cell density

Source Milton, 2006.

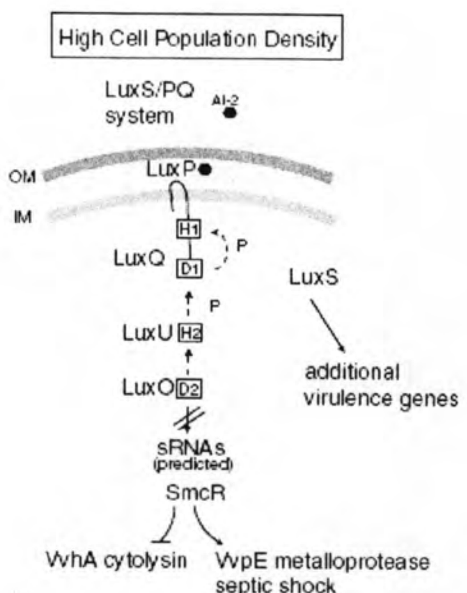

Figure 2.6 Model of quorum sensing system in V.vulnificus at high cell density

Source Milton, 2006.

5. The detection of AHL

The determination of AHLs is currently of great interest, and the development of reliable and sensitive analytical methods for their structural characterization can provide supporting information to the elucidation of their biological significance and activities (Cataldi, 2007). Here, reviews about AHL detection methods are described.

5.1 Bioassay

Bioassay, or for the full word, biological assay, is the scientific experimental method which usually used to measure effect of substance on living organisms. Bioassay can be used for quantitative or qualitative analysis. For the AHL bioassay, genetically constructed indicator strains (the genetically bacterial strains used as indicator of AHL signals) are used. These indicator strains do not express AHL synthase, but contained AHL transcriptional activator (LuxR homolog) and an AHL-activated promoter fused to a reporter gene(s) such as lacZ or luxCDABE (Shaw et al., 1997; Singh and Greenstein, 2005). In the present of AHL in the system, an expression

of given characteristic in indicator strains were induced. Table 2.4 shown review of the indicator strains using in AHL bioassay.

Disadvantages of the bioassay are 1) bioassay appropriates for using in research work only, as this method has to use the skilled operator, and indicator strains are genetically unstability. 2) For the use in real sample, using of more than 2 or 3 indicator strains is necessary. 3) The overall detection time is more than 2 days.

5.2 Thin layer chromatography (TLC)

The specificity of AHL signal molecule is conferred by the length and the substitution group at carbon 3 of the acyl side chain as described previously (Figure 2.2). This property is useful for the detection and identification of AHL molecule by TLC. The AHL molecules are separated by TLC and detected by overlaying the developed plate with a culture of bacterial indicator strains.

The detection and characterization of AHL signal molecules by this technique was firstly reported by Shaw et al (1997). C18 reversed-phase TLC plate couple with A.tumefaciens strain NT1 (pDCI41E33) which harbouring lacZ were developed. Testing in AHL standards, this method could separate 10 standard compound in single sample. The sensitivity was identical by pmol level for most standards, but it could not detect 3-oxo-C4, C4 and C12-HSL, at least in pmol level.

This method has its own limitation, which include the limit of the indicator strains to detect various of AHL signals, the amount of AHL molecules must be present at level detectable by reporter, tailing spots of 3-oxo- and/or 3-hydroxyl- molecule would mask presenting of the 3-unsubstituted at the same acyl-chain length.

Table 2.4 The indicator strains and their response to AHL signal molecules

year	researchers	indicator strains	response
1998	Winson et al	*E.coli* MT102	bioluminescence
2000	Blosser and Gray	*C.violaceum* CV0blu	purple color
2001	Andersen et al	*E.coli* MT102	GFP
2002	Middleton et al	*E.coli* JM109	bioluminescence
2003	Jacobi et al	*E.coli* (pSB403)	bioluminescence
2004	Jafra and Wolf	*E.coli* JB534-MT102	GFP
2005	Chambers et al	*A.tumefaciens* A136	blue color
2005	Singh and Greenstein	*A.tumefaciens* NTL4	blue color

5.3 Liquid chromatography- mass spectrometry (LC-MS)

High performance liquid chromatography (HPLC) is an effective method for differentiate each of AHL molecule from the unknown sample and for the preparation of AHL molecule for structural analysis. Bainton et al (1992) and Chhabra et al (1993) used C8 reversed-phase column and gradient or isocratic mobile phase elution (e.g. acetonitrile-water) to separate the AHL molecules. The separated molecules were subjected to MS and nuclear magnetic resonance spectroscopy for structural identification.

From that time, using of LC-MS method to analyzed AHL signal molecules was widely recognized, and the condition was modified. Frommberger et al (2004) developed device that could be used to both of pre-concentration and separation of sample. Using labolatory-made, miniaturized (75µL i.d.) reversed-phase nano-liquid

chromatography column couple with microelectrosprey-ionization ion trap mass spectrometry, it made this method extreamly robust and cost-effective.

Bruhn et al (2004) were used C18 reversed-phase (particle size, 3μm; 50 by 2 mm.) HPLC column couple with positive-electrospray high-resolution mass spectroscophy was used to detect 3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) in pure culture of *Hafnia alvei*.

5.4 Colorimetry detection

Yang et al (2006) had developed new AHL detection by colorimetric method. This method was modified from the work of Goddu, LeBlanc and Wright (1955), which developed method for determination of ester, anhydrides and other hydrolysable derivatives of carboxalic acid. The modified colorimerty method was quick, easy, and used only the simple spectrometer to detect the AHL molecules in sample matrix.

This colorimetry method based on reaction of lactone group with ferric chloride resulting in UV absorptionable yellow to dark brown complex. Lactone is a cyclic ester product containing both of alcohol and carboxylic groups in same molecule. Many research groups demonstrated that lactone group was presented in all types of AHL structure (Miller and Bassler, 2001; Fuqua and Greenberg, 2002; Jacobi et al, 2003; Buchholtz et al, 2005; Pillai and Jesudkasen, 2006).

In alkaline condition, treated with sodium hydroxide, lactone in AHL structure is hydrolyzed to the straight chain ester (Figure 2.8). Once lactone ring of AHL molecule opens, the carboxyl side chain of corresponding homoserine can react with hydroxylamine resulting in hydroxamic acid formation (Figure 2.9a) (Goddu et al, 1955; Yang et al, 2006). The hydroxamic acid product forms a colored chelate complex in the presence of ferric chloride in system and this complex can be determined by spectrophotometer. (Figure 2.9b).

Figure 2.7 AHL hydrolysis under alkaline condition. N-octanoly-D/L-homoserine lactone is hydrolyzed under alkaline condition. The hydrolysis product is N-octanoly-D/L-homoserine.

Source Englmann et al (2007)

$$RCOOR' + NH_2OH \longrightarrow RCONHOH + R'OH \quad 4.2a$$

$$3\,RCONHOH + FeCl_3 \longrightarrow (RCONHO)_3Fe + 3\,HCl \quad 4.2b$$

Figure 2.8 Colorimetry reaction of hydrolyzed AHL. 4.9a ; reaction of straight chain ester with hydroxylamine resulting in hydroxamic acid formation. 4.9b; the colorimetry reaction of hydroxamic acid with ferric chloride . The colored chelate complex product can be determined by spectrophotometer.

Source Glagovich, Organic Qualitative Analysis Outline, available online at http://www. chemistry.ccsu.edu/glagovich/teaching/316/qualanal/tests/ hydroxamic.html.

According to Figure 2.10, complex of ferric and most aliphatic hydroxamic acid had a maximum absorbance wavelength at 530 nm (λ max = 530 nm.). But for the complex of ferric with ester containing conjugated double bond or more than one carboxyl group might has λ max at slightly different wavelengths (Goddu et al, 1955). The complex of alkaline treated AHL with ferric are yellow to dark brown solution, and has λ max at 520 nm. The color of solution is depended on lactone concentrations.

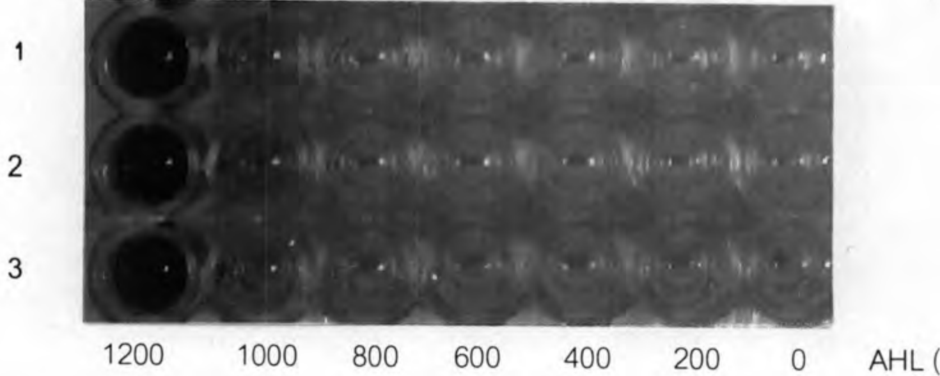

Figure 2.9 The yellow to dark brown color of ferric-AHL complex in AHL concentration ranging of 0 to 1200 nmol. Row1; 2-hexanoyl-3-hydroxymethyl butanolide, Row 2; 2-(6-methyl-l-hydroxyheptyl)-3-hydroxymethyl buthanolide, Row 3; N-3-0xooctanoyle-homoserine lactone (3-oxo-C10-HSL).

Source Yang et al, 2006

One of the importance parts in every analytical method is sample preparation. The preparation step increases efficiency of the detection method, as it consists of isolation, concentration and purification of signal molecules from other substances. Therefore, before AHL detection is performed, sample should be prepare as described following.

5.5 Sample preparation

In order to detects AHL presenting in sample, sample preparation before subjecting to determination method should be done. For the AHL detection, AHL extraction was done in order to purify and concentrated signal molecules.

The extraction could be done by using lot of organic solvents. For solid sample, 100% methanol followed by either acidified ethyl acetate, acidified ethyl acetate, acetonitrile-water or dichloromethane-ethyl acetate extraction might be used (Anderson et al., 2001; Jacobi et al., 2003; Bruhn et al., 2004; Buchholtz et al., 2005 and Schupp et al., 2005, respectively). Acetonitrile-chloroform (5:1) and chloroform might be used to extract AHL from liquid sample (Shaw et al, 1997; Frommberger et al, 2004, respectively). Bioassays, TLC and colorimetry might not need sample preparation

before analysis, because their selectivity, or its own separation system (in TLC technique), are effective enough.

6. Analytical method validation

As non of standard procedure for method validation was proposed, guidance for industry, bioanalytical method validation recommended by USFDA was used as a guideline.

In bioanalytical experiment, published analysis methods are often modified to fit the requirement of each study or the laboratory instrument. This modification may leads to changing the analytical method performance. Therefore, the validation of modified method is necessary to ensure that this method is suitable and reliable for the intend analytical application.

In typical method development and establishment for bioanalytical method, determination of selectivity, accuracy, precision, recovery and calibration curve should be done. The analyst have to judge that how much additional validation from previously is needed, which might be consider from the changing level of method or the specific type of analytical method used (USFDA, 2004)

6.1 The level of validation

6.1.1 Full validation

Full validation is used when the new bioanalytical method developed and implemented for the first time, or when using new kind of sample.

6.1.2 Partial validation

When the already validated bioanalytical methods are modified, partial validation should be done. Range of partial validation is from only intra-assay accuracy and precision determination to a nearly full validation.

6.1.3 Cross-validation

Comparison of validation parameters of two or more bioanalytical methods are used in same study or across different study is cross-validation. Cross-validation might be used when the original method is the reference for the revised method.

6.2 The parameter for bioanalytical method validation

6.2.1 Selectivity

Selectivity is the ability of analytical method to select and quantify the analyte in the sample. The blank sample in appropriate matrix from at least six source should be collected to the selectivity determination, and selectivity should be ensure at the lower limit of quantification (LLOQ).

6.2.2 Accuracy

The accuracy refers to the closeness of the mean test result and the true value. Accuracy is determined by measure at least five sample, which know its concentration, per one level of concentration, and a minimum of three concentration in expected range are recommended. The difference of nominal and tested value (%Recovery) of the result, which should be within 15% from true value, serves as the accuracy. Only the mean value at LLOQ that SD should not more than 20%.

6.2.3 Precision

The precision describe the closeness of each batch of analyte measurement when the multiple aliquots of sample are measured repeatedly. The minimum of five determination, and at least three concentration in expected range should be determined the precision. The relative standard deviation (%RSD), which tells the precision, should not exceed 15% except at LLOQ that CV should not reach 20%. Precision can further be divided into within-run repeatability, which is the precision during a single analytical time, and between-run repeatability, which is the precision with time and may involve different analysts, equipment, reagent, and laboratories.

6.2.4 Recovery

The recovery is the detector response obtain from an amount of analyte add to and extracted from the analytical matrix, compare with the response from the true concentration of pure standard. Recovery need not be 100%, but the recovery of an analyte and of internal standard should be consistent, precise, and reproducible.

6.2.5 Standard curve

Standard curve is the relationship between the instrument response and the concentration of an analyte. The calibration curve should be prepared from the same procedure as the sample by spiking know amount of analyte to the sample matrix to get the concentration in expected range in a particular study. A blank sample (matrix sample without internal standard), zero sample (matrix sample with internal standard), and other non-zero standard including LLOQ should be determined.

In this experiment, the evaluation of colorimetry method for bacterial AHL in broth culture determination was performed. However, since the first report of colorimetry determination proposed by Yang et al in year 2002, modification and evaluation of this method for bacterial AHL determination was still not be done. Therefore, validation of the method before application usage must be tested.

In order to evaluate the V.parahaemolyticus detection by its quorum sensing property, there were still some gaps of knowledge which has to be investigated as follow.

i) Which AHL detection method is used? Is it efficient and suitable enough for a food microbiological determination application?

ii) How about V.parahaemolyticus AHL production? Are intrinsic and extrinsic factors effect the signal production?

iii) Do the AHL signals produced by V.parahaemolyticus represent an existing of this bacteria in food sample?

To answer these questions, a set of experiments was designed. Material and methodology used in this study were informed in the next chapter, Materials and Methods.