EVALUATION AND IMPROVEMENT OF ISOLATION PROTOCOLS OF PATHOGENIC *Vibrio parahaemolyticus* IN FROZEN PRAWNS

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EVALUATION AND IMPROVEMENT OF ISOLATION PROTOCOLS OF PATHOGENIC *Vibrio parahaemolyticus* IN FROZEN PRAWNS

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EVALUATION AND IMPROVEMENT OF ISOLATION PROTOCOLS OF PATHOGENIC *Vibrio parahaemolyticus* IN FROZEN PRAWNS

ABSTRACT

The evaluation of different protocols for isolation and enumeration of *Vibrio parahaemolyticus* from frozen prawn samples were developed in this study. The selectivity of CHROMagar™Vibrio (CV) and Thiosulphate-citrate-bile salts-sucrose agar (TCBS) media for *Vibrio parahaemolyticus* were examined using different enrichment methods and incubation temperatures. Direct plating on CV at 37°C resulted in significantly higher counts of *V. parahaemolyticus* compared to direct plating on TCBS agar at lower temperatures ($p<0.05$). There was a significant improvement in selectivity on TCBS agar using an enrichment process. Virulent isolates of *V. parahaemolyticus* (*tdh*$^-$/*trh*$^+$) were only recovered from one prawn sample out of 20 frozen prawn packs (5%) when grown on CV agar. REP-PCR molecular typing demonstrated that CV isolated more genetically diverse *V. parahaemolyticus* than TCBS agar. The carbon consumption of *V. parahaemolyticus,* including pathogenic and non-pathogenic (*tdh*$^-$/*trh*$^-$) isolates was studied using a phenotype microarray system (BIOLOG). Carbon utilization in *tdh*$^+$/*trh*$^-$isolates were significantly different from other isolates. This assists in the development and improvement of selective media for effective isolation of foodborne pathogens. In conclusion, the findings suggest that CV is a better selective medium and is less inhibitive for the isolation and detection of *V. parahaemolyticus* particularly virulent isolates from frozen prawn samples. This current work also recommended that using a direct-plating method on CV at 37°C could be an efficient alternative to MPN-PCR for enumeration of *V. parahaemolyticus* from frozen prawn samples.

Keywords: *Vibrio parahaemolyticus*, Foodborne pathogens, Food poisoning, Phenotype Microarray, Virulence genes

PENILAIAN DAN PENAMBAHBAIKAN PROTOKOL ISOLASI *Vibrio parahaemolyticus* BERPATHOGENIK DALAM UDANG BEKU

ABSTRAK

Penilaian protokol yang berbeza untuk pemencilan dan penghitungan *Vibrio parahaemolyticus* dari sampel udang beku telah dikaji. Selektiviti media CHROMagar™ Vibrio (CV) and Thiosulphate-citrate-bile salts-sucrose agar (TCBS) telah diperiksa menggunakan kaedah memperkayaan dan suhu inkubasi yang berlainan. Kaedah plat langsung pada CV pada suhu 37°C mengakibatkan peningkatan dalam pemencilan *V. parahaemolyticus* berbanding dengan plat langsung pada agar TCBS pada suhu yang lebih rendah ($p < 0.05$). Terdapat peningkatan yang ketara dalam selektiviti TCBS agar selepas menggunakan proses pengayaan. Strain virulen *V. parahaemolyticus* (*tdh*$^-$/*trh*$^+$) dalam kajian ini hanya ditemui daripada satu sampel udang daripada 20 pek udang beku (5%) daripada agar CV. Kajian molecular REP-PCR menunjukkan bahawa CV menghasilkan lebih banyak jenis *V. parahaemolyticus* yang berbeza dari segi genetik berbanding TCBS agar. Penggunaan karbon untuk isolat *V. parahaemolyticus* patogenik dan bukan patogenik *(tdh*$^-$*/trh*$^-$*)* telah dikaji menggunakan sistem fenotip '*microarray*' BIOLOG. Corak penggunaan karbon oleh isolat *tdh*$^+$/*trh*$^-$ jauh berbeza dengan strain lain. Ini membantu dalam perumusan dan penambahbaikan media selektif untuk pengasingan yang lebih berkesan terhadap patogen makanan.

Kesimpulanya, penemuan dari kajian ini mencadangkan bahawa CV adalah media yang lebih memilih dan tidak menghalang pemencilan dan pengesanan *V. parahaemolyticus* terutamanya pada pencilan yang virulen daripada sampel udang beku. Kajian ini juga mencadangkan bahawa penggunaan kaedah plat langsung ke atas CV pada 37°C boleh menjadi alternatif yang lebih cekap daripada kaedah MPN-PCR untuk penghitungan *V. parahaemolyticus* dari sampel udang beku.

Kata kunci: *Vibrio parahaemolyticus*, Patogen bawaan makanan, Keracunan makanan, Mikroarray fenotip, Gen virulen

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LIST OF SYMBOLS AND ABBREVIATIONS

&	and
°C	Degree Celsius
%	percentage
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-*2H*-tetrazolium chloride
DAPI	4´,6-diamidino-2-phenylindole
CTC	5-cyano-2,3-ditolyl-tetrazolium chloride
APW	Alkaline Peptone Water
bp	base pair
C. jejuni	*Campylobacter jejuni*
CV	CHROMagar™Vibrio
cfu/g	colony forming unit per gram
dNTPs	Deoxynucleotide Triphosphates
DNA	Deoxyribonucleic Acid
ddH_2O	double-distilled water
ERIC	Enterobacterial Repetitive Intergenic Consensus
et al.	et alia
FITC	Fluorescein Isothiocyanate
g	gram
h	hour

Log	Logarithm
LB	Luria-Bertani
$MgCl_2$	magnesium chloride
µl	microliter
µM	micromolar
ml	millilitre
Mm	millimolar
min	minutes
NADH	Nicotinamide-Adenine-Dinucleotide
NA	Nutrient Agar
OD	Optical Density
PM	Phenotype Microarray
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
REP	Repetitive Extragenic Palindromic sequence
RFLP	Restriction Fragment Length Polymorphism
sec	second
NaCl	sodium chloride
spp.	species

TRH	TDH-related haemolysin
TDH	Thermostable Direct Haemolysin
TCBS	Thiosulphate-Citrate-Bile Salts-sucrose agar
TBE	Tris-borate-EDTA
TE	Tris-EDTA
UV	ultraviolet
USA	United States of America
VBNC	viable but not culturable
V. cholerae	*Vibrio cholerae*
V. mimicus	*Vibrio mimicus*
V. vulnificus	*Vibrio vulnificus*
V	voltage

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CHAPTER 1: INTRODUCTION

1.1 Background of the study

Vibrio, a genus of Gram-negative bacteria, is abundant in seafood and water. Among the *Vibrio* species, *Vibrio parahaemolyticus* has been frequently reported to cause food poisoning in humans via the consumption of contaminated seafood (Atthasampunna, 1974; Su & Liu, 2007). *V. parahaemolyticus* is a Gram-negative, halophilic bacterial pathogen which is widely transmitted through seafood and seafood products. Consumption of raw, contaminated or poorly processed seafood has resulted in a high incidence of food poisoning associated with virulent *V. parahaemolyticus* in humans (Alam et al., 2003; Newton et al., 2012; Yu et al., 2013). In many countries, including the United States, Japan, and other Asian countries, *V. parahaemolyticus* was found to be the leading cause of human gastroenteritis associated with seafood consumption (Kaysner & DePaola, 2001; Newton et al., 2012); with *V. parahaemolyticus* as the causative pathogen in around one out of every four food poisoning cases in Japan (Alam et al., 2002). The symptoms of the infection include abdominal cramps, diarrhoea, nausea and fever (Baker-Austin et al., 2010). Although uncommon, *V. parahaemolyticus* has been reported to cause wound infections, ear infections or septicaemia in individuals with poor immune systems which can potentially be life-threatening (Su & Liu, 2007; Zhang & Orth, 2013). Research has shown that a considerable number of virulent species of *Vibrio* exist in environments such as marine waters, rivers, marine organisms and seafood (Ham & Orth, 2012; Velazquez-Roman et al., 2012).

As *V. parahaemolyticus* is widely disseminated in the marine environment, the major route of transmission is via the consumption of raw or undercooked seafood, particularly shellfish (Finkelstein, 1996). Thus, the detection of the bacterium is

important and many researchers have focused on evaluating and comparing the performance of different protocols for the isolation and detection of *V. parahaemolyticus* in seafood and environmental samples (Kim et al., 1999; Hara-Kudo et al., 2001; Wang et al., 2011) In recent years, conventional methods have been combined with molecular approaches to improve the detection of *V. parahaemolyticus* in seafood and marine waters (Blanco-Abad et al., 2009).

Resuscitation of injured or stressed *V. parahaemolyticus* from chilled or frozen seafood is often problematic. It is generally known that alkaline conditions with the presence of bile salts enhances the growth of *V. parahaemolyticus*, therefore Alkaline Peptone Water (APW) is generally used as the first step to enrich and resuscitate injured bacteria in seafood. The most widely used selective medium to detect and isolate *V. parahaemolyticus* is Thiosulphate Citrate Bile Sucrose (TCBS) agar, even though TCBS is more effective in the isolation of *Vibrio cholerae*. Standard culturing methods along with direct-PCR after enrichment with APW was effective; yet there are challenges that remain for the recovery and efficient isolation of injured or viable but not culturable (VBNC) pathogenic *V. parahaemolyticus* strains from frozen seafood. Hara-Kudo et al. (2001) developed a chromogenic agar medium for effective detection of *V. parahaemolyticus* from seafood. CHROMagar™Vibrio (CV) contains beta-galactosidase substrates and was found to be more accurate and specific than TCBS (Hara-Kudo et al., 2001; Di Pinto et al., 2012). Therefore, this study employed the use of different protocols including both CV and TCBS together with enrichment for an effective method for recovering pathogenic (*tdh*$^-$/*trh*$^+$ and *tdh*$^+$/*trh*$^-$) and non-pathogenic (*tdh*$^-$/*trh*$^-$) *V. parahaemolyticus* strains from frozen prawns.

This work also aims to explore the possibility to further improve the performance of selective media for detecting and isolating *tdh*$^-$/*trh*$^+$ and *tdh*$^+$/*trh*$^-$ strains of *V. parahaemolyticus* by comparing the growth of different strains in different NaCl

concentrations and the carbon utilization between pathogenic and non-pathogenic strains of *V. parahaemolyticus*. In spite of the numerous publications regarding the isolation of *V. parahaemolyticus* from various food sources, there are limited studies on the improvement of the selectivity of bacteriological media in detecting, isolating and differentiating *tdh*$^-$/*trh*$^+$ and *tdh*$^+$/*trh*$^-$ strains from *tdh*$^-$/*trh*$^-$ strains.

1.2 Objectives of the study

The objectives of this study are:

i) To assess the effects of enrichment step, incubation temperatures and different isolation media to isolate *V. parahaemolyticus*, particularly pathogenic strains, from frozen prawn samples.

ii) To determine the genetic diversity of *V. parahaemolyticus* isolated under different culture conditions.

iii) To compare carbon utilization profiles of pathogenic (*tdh*$^-$/*trh*$^+$ and *tdh*$^+$/*trh*$^-$) and non-pathogenic (*tdh*$^-$/*trh*$^-$) strains of *V. parahaemolyticus*.

CHAPTER 2: LITERATURE REVIEW

2.1 Characteristics of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a member of the genus *Vibrio* within the Vibrionaceae family, is a Gram-negative, salt requiring and non-spore forming bacterium (Drake et al., 2007; Nair et al., 2007). The bacterium has a curved rod-shape with 1.4-2.4 μm length and 0.5 - 0.8 μm width when viewed under microscopes. It is oxidase positive and a facultative anaerobe. There are two types of flagella in *V. parahaemolyticus* that help the bacteria survive in its environment. The polar flagellum helps the motility of the bacteria in liquid environments and the lateral flagellum supports the swarming movement of the bacteria in semi-solid environments (Shinoda et al., 1974, 1976; Shinoda & Okamoto, 1977; Butt et al., 2004; Yeung & Boor, 2004).

V. parahaemolyticus can be found in coastal and brackish water with temperatures of 15°C and above, and can be isolated from seafood, seawater and zooplankton (Kaneko & Colwell, 1973; Venkateswaran et al., 1989). This bacterium is able to lyse blood cells with β-type haemolysis activity on Wagatsuma agar. This activity is known as the Kanagawa phenomenon (KP) which is a virulence indicator of *V. parahaemolyticus*. The abundance of the KP^+ strains of *V. parahaemolyticus* in the environment is only 1-2% (Sakazaki et al., 1968; Miyamoto et al., 1969). Pathogenic strains of this bacteria specifically carry the Thermostable Direct Haemolysin (TDH) genes and/or TDH Related Haemolysin (TRH) genes that code the KP^+ strains (Honda et al., 1987). High pH values with a range of NaCl concentrations are generally suitable environmental conditions for the growth of *V. parahaemolyticus*. The bacteria has a wide temperature range for growth with growth occurring as low as 10°C, with a maximum of 44 - 45°C and an optimum of 37°C (Oliver et al., 1997).

2.2 Natural occurrence and habitat

V. parahaemolyticus can colonize many different hosts such as humans, fishes and seafood, such as shrimp, clams, octopus, sardines, codfish, crab, scallops and oysters (Barker, 1974; Su & Liu, 2007). *V. parahaemolyticus* can be found occurring in shellfish from coastal brackish waters as part of the shellfish natural microbiota (Yeung & Boor, 2004; Nair et al., 2007). The population of the bacteria rises during the warm seasons of the year, however the bacterial community tends to travel from zooplankton hosts in summer to sediments in winter (Kaneko & Colwell, 1975; Parveen et al., 2008). The prevalence of *V. parahaemolyticus* from seafood is high (Abd-Elghany & Sallam, 2013; Coly et al., 2013; Suffredini et al., 2014; Yano et al., 2014; Tra et al., 2015). Many countries in south Asia have reported cases of foodborne illnesses caused by *V. parahaemolyticus* due to raw seafood consumption, as well as other countries such as the United States, Japan, Korea and Europe (Rippey, 1994; Pan et al., 1996; Pan et al., 1997; Matsumoto et al., 2000; Yeung & Boor, 2004; Nair et al., 2007). *Vibrio* was first introduced by Pacini in 1854 and officially approved as "*Vibrio cholera* Pacini 1854" in 1965 (Frerichs & Shaheen, 2001). The first reported food poisoning incidence caused by *V. parahaemolyticus* bacteria was from Japan in 1950 (Fujino et al., 1953). After the first outbreak, this food poisoning associated with O3:K6 serotype (Okuda et al., 1997) expanded to other countries in the world (Serichantalergs et al., 2007; Velazquez-Roman et al., 2014). The early distribution of the *V. parahaemolyticus* outbreak in different continents is shown in Figure 2.1 (Okuda et al., 1997; Daniels et al., 2000; Ansaruzzaman et al., 2005; González Escalona et al., 2005; Nair et al., 2007).

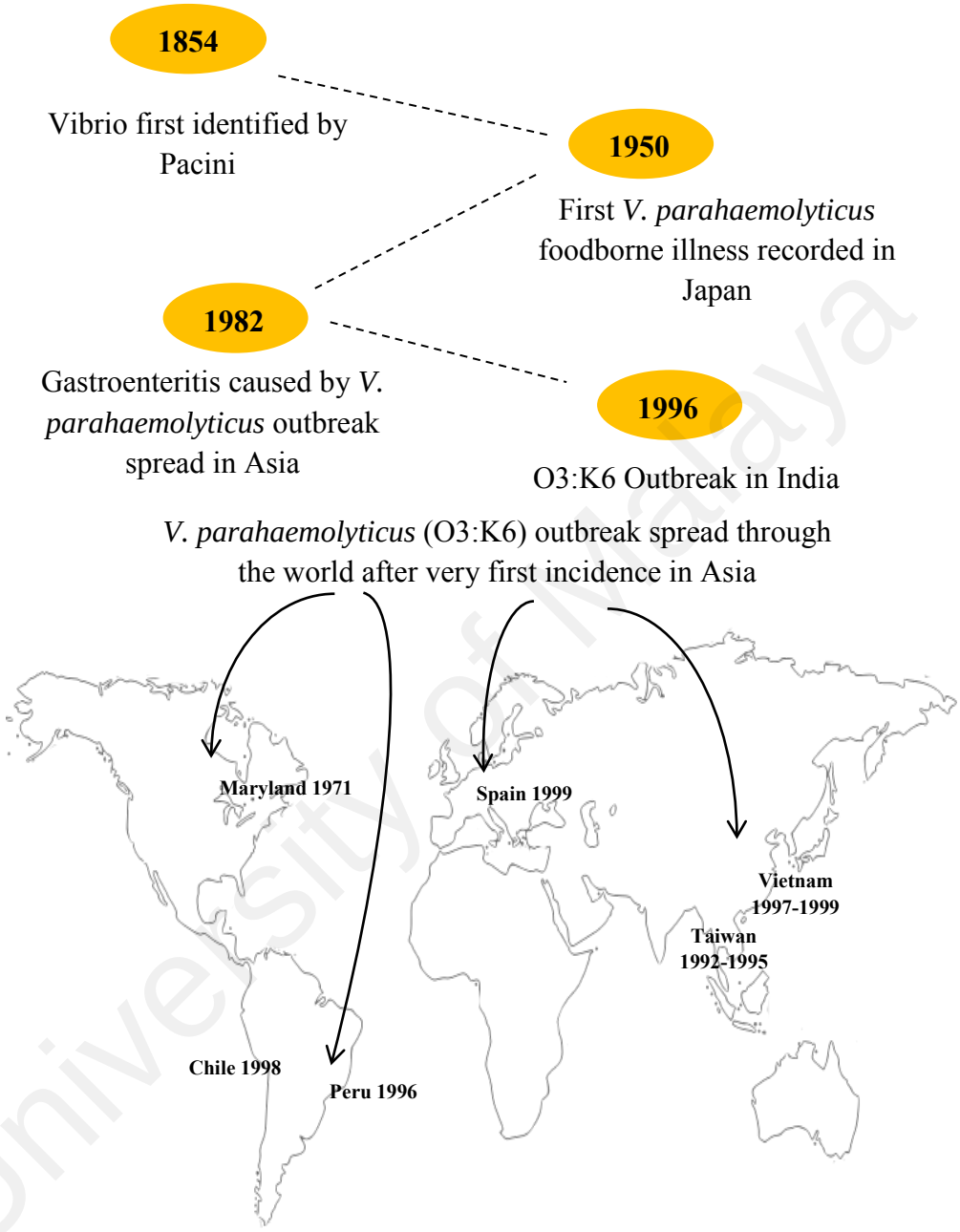


Figure 2.1: *V. parahaemolyticus* outbreaks begin to distribute around the world between 1996 to 2000 (Fujino et al., 1953; Okuda et al., 1997; Daniels et al., 2000; Frerichs & Shaheen, 2001; Ansaruzzaman et al., 2005; González-Escalona et al., 2005; Nair et al., 2007).

2.3 Diseases caused by *Vibrio parahaemolyticus*

V. parahaemolyticus can cause diseases in humans as well as in shellfish and other aquaculture organisms (Lavilla-Pitogo et al., 1998; Chen et al., 2000). It causes red disease (with up to 80% mortality) in tiger prawns (Chatterjee & Haldar, 2012). Acute hepatopancreatic necrosis disease (AHPND) in shrimp, known as early mortality syndrome, is spread throughout Southeast Asia to Mexico (Gomez-Gil et al., 2014; Nunan et al., 2014). This disease has caused significant losses in the shrimp farming industry (Food and Agriculture Organization of the United Nations, 2013) and *V. parahaemolyticus* has been identified as the main causative agent of AHPND (Tran et al., 2013). It is reported that plasmid-encoded binary toxins $PirA^{VP}$/ $PirB^{VP}$ is responsible for the primary cause of AHPND. *V. parahaemolyticus* was found to colonize the host tissue of shrimp after crossing the epithelial cells (Lightner, 1996). Yet, the virulence mechanism of *V. parahaemolyticus* in infected shrimp is not clear (Li et al., 2017). Some of the common symptoms of disease in fish caused by pathogenic *Vibrio* species include intestinal necrosis, anaemia, ascetic fluid, petechial haemorrhage in the muscle wall, liquid in the air bladder, etc. (Colwell & Grimes, 1984). Infection in humans is usually transmitted by eating raw or undercooked shellfish. *V. parahaemolyticus* causes Vibriosis with symptoms such as vomiting, diarrhoea, abdominal pain, nausea, fever, chills 2 to 48 h after consuming contaminated or undercooked food. The organism grows in the intestinal tract and produces one or more toxins that contribute to the symptoms. Gastroenteritis is a self-limited disease, whereas, wound infection and primary septicaemia are highly lethal conditions (40 to 60%) that occur most often in people with liver disease or low immunity conditions (Ray & Bhunia, 1996).

2.4 Pathogenicity and virulence

The virulence of *V. parahaemolyticus* is characterized by different factors (Table 2.1) such as endotoxin, enterotoxin, cytotoxin, protease, lipase, phospholipase, adhesive factor, thermostable direct haemolysins (TDH), and TDH related haemolysins (TRH) (Nakasone & Iwanaga, 1990; Nishibuchi et al., 1992; Tada et al., 1992; Kim & McCarter, 2000; Funahashi et al., 2002; Makino et al., 2003; Zhang & Austin, 2005). High amounts of rRNA and tRNA genes in aquatic *V. parahaemolyticus* will increase the growth rate and enhance the gastroenteritis consequences of the consumption of raw or contaminated seafood (Reen et al., 2006; Oliver et al., 2013). The expression of virulence factors is also dependent on external conditions as *V. parahaemolyticus* can grow rapidly in culture media and host cells when provided with adequate nutrient substrates. Vibriosis also tends to increase during warmer seasons when water temperatures and salinity rises, and the organic structure of the environment becomes suitable for virulence induction in *V. parahaemolyticus* strains (Caipang & Aguana, 2011).

TDH is known as the main virulence factor and the most marked virulence feature of *V. parahaemolyticus*, however the occurrence of the *tdh* gene in isolates retrieved from environmental samples is very scarce and is estimated to occur in less than 1% of samples (Deepanjali et al., 2005; Martinez-Urtaza et al., 2005; West et al., 2013). TDH creates pores in the host intestine and erythrocytes cells and facilitates the ion movement through these pores and ultimately causes cellular death. Intracellular activity of TDH to induce and change the secretion or concentration of chloride and calcium ions in rabbit models has been proven (Takeda, 1982; Nishibuchi et al., 1989; Raimondi et al., 1995; Takahashi et al., 2000; Hardy et al., 2004). However, the

accurate mechanism of the TDH virulence function in host cells is not clear (Baffone et al., 2005; Matsuda et al., 2010).

There are other supplementary virulence factors in *V. parahaemolyticus* such as chemotaxis, flagella, pilus, iron acquisition systems (pvuA, psuA), two type III secretion systems (T3SS1 and T3SS2) and a type VI secretion system (T6SS). *V. parahaemolyticus* has two plasmids (p03k6 and Psa19) and two circular chromosomes (I, II) in its genome with chromosome 1 encoding the T3SS1 in all *V. parahaemolyticus* strains while T3SS2 is encoded in chromosome 2 and only appears in *tdh*$^+$ strains (Makino et al., 2003; Ono et al., 2006). T3SS1 can be found in almost all clinical and environmental *Vibrio parahaemolyticus* strains (Paranjpye et al., 2012). The cytotoxic activity of T3SS1 has been proven by several studies (Zhang & Orth., 2013; Ritchie et al., 2012; Burdette et al., 2008). T3SS1 also effectively inhibits the ability of the host cells to phagocytose *Vibrio parahaemolyticus* (Jegga et al., 2011). T3SS2 is found in clinical isolates of *Vibrio parahaemolyticus* (Meador et al., 2007). The T3SS2 secretion system has shown cytotoxic activity in intestinal cells (Ritchie et al., 2010). Thermostable hemolysin (TLH) is one of hemolysins encoded by *tlh* gene in *Vibrio parahaemolyticus* (Wang et al., 2013). TLH is produced by all environmental and clinical strains of *Vibrio parahaemolyticus* (Bej et al., 1999). The sequencing of the whole genome of *V. parahaemolyticus* allows a deeper insight into the pathogenesis mechanisms of this bacteria as much of it remains unknown (Thompson & Swings, 2006).

Table 2.1: List of virulence factors of *V. parahaemolyticus* (Wang et al., 2015).

Effectors	Gene	Biological activity	Effects on host cells
Toxins			
TDH	*tdh*	Forms pores on cells	Cytotoxicity and enterotoxicity
TRH	*trh*	Forms pores on cells	Cytotoxicity and enterotoxicity
TLH	*tlh*	Hemolysin activity	Cytotoxicity
T3SS1 effectors			
Vop Q	vp1680	Forms pores and binds V-ATPase	Autophagy, cell lysis, MAPK activation, IL-8 secretion
Vop S	vp1686	Inhibition of Rho by AMPylation	Cells rounding, phagocytes invasion
VPA0450	vpa0450	Phosphatidylinositol phosphatase	Membrane blebbing, destabilization
Vop R	vp1683	Binds PIP2 in membrane	Promoting refolding of T3SS effectors
T3SS2 effectors			
Vop A/P	vpa1346	Inhibition of MAPK by acetylation of MKK	Blocking of phosphorylation and ATP binging
Vop T	vpa1327	Ras ADP-ribosylation	Cytotoxicity and yeast growth inhibition
Vop L	vpa1370	Actin nucleation	Stress fibers formation and cell shape changing
Vop C	vpa1321	Activation of Rac and CDC42 by deamidation	Invasion of non-phagocytic cells
Vop V	vpa1357	Actin binding and bundling	Enterotoxicity and blunting of villi
Vop Z	vpa1336	Inhibition of TAK1 and downstream MAPK and NF-kB	Enterotoxicity and colonization
VPA1380	vpa1380	Cysteine catalysis dependent on IP6	Toxicity in yeast

2.5 Isolation and identification of *Vibrio parahaemolyticus*

Standard protocols for the isolation and enumeration of vibrios have been previously established by the Food and Drug Administration (FDA) and the International Organization for Standardization (ISO) (Kaysner & DePaola, 2004; Yeung & Boor, 2004). The standard culturing method was introduced based on the ability of the bacteria to develop in alkaline environments and high amounts of bile salts. The optimum NaCl concentration for growth of *V. parahaemolyticus* in media is between 2% to 3% and the optimal growth temperature is 37°C (Elliot et al., 1995). Stress conditions such as cold and heat shock or an imbalance of nutrient availability will affect the growth of bacteria. Therefore, there are several inducers that influence the resuscitation of bacteria from injured or VBNC states caused by environmental shock such as amino acid mixtures, gas mixtures, rich media, growth temperatures and supernatants of growing cultures (Wong et al., 2004).

Enrichment techniques, using different enrichment media and/or broth in either one- or two-steps, are frequently used by many researchers despite these steps limiting the fast and reliable enumeration of the bacterial community from environmental samples (Simidu & Tsukamoto, 1980; Dupray & Cormier, 1983; Blanco-Abad et al., 2009). Bacterial growth can be monitored using both solid and liquid media. Cell counts can be done on solid media using spread plate or pour plate techniques (Singleton, 2004). Different methods employed such as traditional and modern isolation and detection of *V. parahaemolyticus* are shown in Table 2.2.

Table 2.2: Conventional and advanced methods that have been developed for detection of *V. parahaemolyticus.*

Conventional standard methods	Selective Media (TCBS)
	Biochemical Test
	MPN
	Chromogenic Media
	Biochemical Kits (API)
	PCR (monoplex-multiplex, qPCR)
Automated Advance Methods	GENIII BIOLOG
	Omnilog system (Biolog)
	VITEK
	Real time PCR

2.5.1 Enrichment techniques

Different selective media that utilizes a one-step or two-step enrichment techniques were used for the resuscitation and isolation of *V. parahaemolyticus* (Paydar et al., 2013). One such enrichment technique involves using Alkaline Peptone Water (APW) which provides a pH range of 8.5 - 9 (Kaysner & DePaola, 2004). In the two-step enrichment process, non-selective media such as Salt Trypticase Soy Broth (TSB) supplemented with 2% salt is recommended along with Salt Polymyxin Broth (SPB) as a selective medium. Polymyxin B sulphate inhibits the growth of Gram-positive bacteria present in the sample (Hara-Kudo et al., 2001). The incubation time for processed food samples is recommended to be between 6 – 8 h and 18 – 21 h (Donovan & Van Netten, 1995). Although enrichment for the recovery of vibrios is widely used by means of different steps using various media, the effectiveness of enrichment for the recovery of injured vibrio cells in stressed condition has not yet been ascertained.

2.5.2 Selective differential plating

A present, two selective media, TCBS and CHROMagar™Vibrio, have been developed for the isolation of vibrios. TCBS is frequently used for the isolation of *Vibrio* spp. This isolation medium provides alkaline conditions (pH=8.6) as the main selective property and the growth of different *Vibrio* spp. on TCBS is relatively reliant on the fermentation of sucrose. Sucrose positive strains produce yellow colonies on the green agar. Most vibrios form a green colony on TCBS agar and *V. cholerae* noticeably utilizes the sucrose and produces yellow colonies. TCBS has been specifically used for isolation of vibrios, however the media does not allow for a precise differentiation among the various species which can form green colonies such as *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* as they are visually indistinguishable. In some cases, *V. parahaemolyticus* also showed sucrose positive activity that would complicate the effective isolation of *V. cholerae*.

To improve the insufficient validity of the former selective media Hara-Kudo et al. (2001) developed a chromogenic medium for a more effective and rapid isolation of *Vibrio* spp. This medium contains beta-galactosidase substrates that effectively responds to discriminate vibrio strains. *Vibrio* colonies can produce three clearly different colorations on CV. Specifically, *V. parahaemolyticus* forms mauve to dark blue colonies on this agar medium (Hara-Kudo et al., 2001; Di Pinto et al., 2011).

2.5.3 Enumeration of *Vibrio parahaemolyticus*

Detection and enumeration of stressed, injured or VBNC cells has always been problematic and requires alternative protocols to be designed based on the environmental stresses and the structure of host cells during pre-harvest and post-harvest treatment of stressed samples. Initially, field microscopy techniques were used

to obtain direct counts of the viable cells and discriminates these cells from dead cells through changes in the cell morphology (Kogure et al., 1979). This was followed by fluorescence microscopy, which uses dyes such as DAPI, FITC, INT and CTC to stain cells. These dyes are also used to detect VBNC or injured cells, for example, DAPI stains live cells green whereas injured or dead cells are dyed orange (Holmberg et al., 1989; Villarino et al., 2000). A newly developed method called Live/Dead Baclight assay can detect and count total and actively viable cells in the sample using a fluorescent dye reaction (Stiefel et al., 2015). Aside from microscopy, molecular based techniques have also been used for detection and differentiation of dead and active cells inside samples. Real time quantitative PCR conjugated with ethidium monoazide (EMA) could detect live cells and differentiate them from dead cells (Yamazaki et al., 2008). Fluorescence in situ hybridization (FISH) was used with suitable rRNA probe sequences to detect the total bacteria and VBNC cells in a sample (Villarino et al., 2000). The MALDI-ToF/MS technique was also able to detect non-culturable and resuscitated cells (Kuehl et al., 2011).

The MPN-PCR technique made the enumeration of viable *V. parahaemolyticus* cells feasible. The method is culture-independent and monitors cell growth through replicate tubes in ten-fold dilutions (Blodgett, 2010). MPN-PCR provides enhanced estimation of samples containing low concentrations of microorganisms (<100/g) and food samples with visible particles in their texture that interfere with the plate count process. Further, the usage of serial dilutions is helpful to simplify enumeration of samples in a wide-range of concentrations. The MPN method estimates the results in a most probable number using ten-fold dilution and three replicates for each dilution to increase the accuracy of the method. In fact, the concept of the method is based upon positive/negative growth rates in the incubated nutrient broth tubes with the bacteria being randomly distributed in the sample (Cochran, 1950). The observed turbidity

(Positive/Negative) inside the tubes is read using an MPN Table to normalize the most probable number of microorganisms (positive) per unit volume of the original sample with 95% confidence interval. 10 cells per gram is generally considered as a normal distribution of *V. parahaemolyticus* cells in the sample, however this amount can exceed 1000 cells per gram in the same sample (Oliver et al., 1997). The MPN Table is established by the FDA-BAM (US Food and Drug Administration's Bacterial Analytical Manual (BAM)) (Blodgett, 2010).

2.5.4 Recovery of injured or stressed *Vibrio parahaemolyticus* from chilled and frozen seafood

Different methodologies have been tested for the detection of *V. parahaemolyticus* from seafood and environmental samples (Blanco-Abad et al., 2009). Conventional and molecular techniques have been developed for effective and improved detection of *V. parahaemolyticus* from environmental matrix. Detection protocols for injured cells under environmental stress or VBNC state needs more investigations. Bacterial cells will undergo injury after exposure to low temperatures of around 2°C. The sensitivity of cold-stressed cells is higher than non-stressed cells when they are exposed to selective enrichment broths. This lowers their capability to repair damages caused by cold-stress. However, when provided with a suitable environment, the cells are able to repair injuries that would otherwise prove lethal (Beuchat, 1978). Yet, it is not clear if the use of selective media containing different stressors will improve the recovery of injured cells from frozen seafood samples and to what extent enrichment helps these cells to revive cells or increase the sensitivity of detection. Therefore, it is crucial to study the differentiation between stressed and VBNC cells more precisely and then apply reliable

detection methods to quantify and resuscitate culturable, injured and VBNC cells (Di Pinto et al., 2011, 2015).

2.5.5 Molecular methods to identify and characterize *Vibrio parahaemolyticus*

There is extensive genetic diversity among *Vibrio* spp. that helps to discriminate between different species of *Vibrio* through specific nucleotide sequences. Different primers and gene regulators have been used in monoplex and duplex PCR assays for detection of *V. parahaemolyticus* and its pathogenic strains. Detection of *tl*, *tdh* and *trh* genes can be performed in monoplex (Wang & Levin, 2004) and multiplex PCR (Tada et al., 1992; Bej et al., 1999). The differentiation of pathogenic and non-pathogenic strains of *V. parahaemolyticus* was previously done in a multiplex PCR using *groEL*, *tdh* and *trh* genes (Hossain et al., 2013). Detection using *gyr B* (Venkateswaran et al., 1998) and *toxR* (Rosec et al., 2009; Vimala et al., 2010; Suffredini et al., 2014) was performed to assay total and virulent strains of *V. parahaemolyticus*. Other PCR methods includes a duplex PCR-hybridization-LightCycler® technique that was reported to be a fast and sensitive method for detection of *V. parahaemolyticus* (Lo et al., 2008). Real time PCR has also been applied to detect total *V. parahaemolyticus* and pathogenic strains in seafood samples (Blackstone et al., 2003; Raghunath et al., 2009).

Detection and characterization of vibrios and pathogenic strains using DNA based techniques is not always precise and perfectly specific, since there are some limitations such as cross reaction with other *Vibrio* spp. in multiplex PCR approaches or inhibitors inside samples that reduce the sensitivity of the detection. Moreover, the bacterial community in the sample cannot be quantitatively detected through PCR methods unless it is combined with MPN techniques. A recent development in DNA based techniques was introduced with a noticeable sensitivity and specificity for detection of *V. parahaemolyticus* and virulence genes (Nemoto et al., 2011; Zeng et al., 2014). Loop

mediated isothermal amplification (LAMP) (Notomi et al., 2000) can perform amplifications with lesser amount of DNA sample compared to PCR by targeting different segments of the goal genes in the sample using a single temperature cycle (Yamazaki et al., 2008; Nemoto et al., 2009; Yamazaki et al., 2010).

2.6 Molecular typing of *Vibrio parahaemolyticus*

V. parahaemolyticus strains can travel through seawater and shellfish to different regions around the world to cause outbreaks in food poisoning. Molecular typing has proved to be useful in this regard and allows researchers and public health authorities to understand and track the ecological distributions of *V. parahaemolyticus* and its pathogenic strains. Several molecular techniques have been developed for the typing of *V. parahaemolyticus*. Most of these technologies are time consuming and labour intensive. Techniques such as PFGE, RFLP, REP-PCR, ERIC-PCR and RAPD-PCR have been applied for DNA typing and genetic relatedness of *Vibrio* genes. All these techniques are based on the PCR assay to amplify specific and conserved DNA sequences of different length within hours.

Genetic variability of *V. parahaemolyticus* strains were previously examined through three different typing methods such as RAPD, REP-PCR and ERIC-PCR. A study by Maluping et al. (2005) found REP-PCR to be less reproducible compared to ERIC-PCR and RAPD techniques. In a study by Wong et al. (1999), molecular typing of *V. parahaemolyticus* was done using PFGE and RAPD methods. Both methods showed satisfying discriminative results, however the RAPD technique was less labour-intensive and faster than PFGE. In another study by Chen et al. (2012), four different molecular typing methods including ribotyping, ERIC-PCR, PFGE and sequence analysis of the *gyrB* gene was applied to characterize unrelated *V. parahaemolyticus*

from clinical samples. It was found that ERIC-PCR combined with the *gyrB* gene yielded improved discriminatory and rapid typing results. REP-PCR and ERIC-PCR methods using designed primers were compared in molecular typing of *V. parahaemolyticus* (Wong & Lin, 2001). All methods were reliable for subspecies typing and clustering analysis of *V. parahaemolyticus*. However, REP-PCR was more beneficial in terms of reproducibility of the fingerprints. REP-PCR produced fingerprints using primers with a length of 33 bp to amplify repetitive and highly conserved sequences (Versalovic et al., 1991).

2.7 Phenotype Microarray

A technology named Phenotype Microarray (PM) provides a series of data of metabolic abilities and phenotypic growth rate of microorganisms using different nutrient sources. This method, by means of various 96 well plates (PM1-20) containing different substrates, enables the analysis of phenotypic patterns of microorganisms through cell respiratory pathways (Bochner et al., 2001; Zhou et al., 2003; Bochner, 2009). Phenotype Microarrays were first established for the phenotypic assessment of *Escherichia coli* (Bochner et al., 2001; Zhou et al., 2003).

Phenotype Microarrays have since been applied to compare and identify phenotypic behaviour among same bacterial species in various environmental conditions (Rodrigues et al., 2011). For instance, the phenotype assessment (carbon sources and sensitivity to osmolytes and pH) of *Campylobacter jejuni* ATCC 33560 has been developed using PM which clarified the behaviour of *C. jejuni* under environmental changes (Tang et al., 2010). Likewise, the genomic diversity and relatedness of different *Streptococcus* species were evaluated through microarray-based comparative genomics (Hakenbeck et al., 2001).

The Phenotype Microarray application supports nearly 2000 culture condition varieties in which growth and metabolic activity of microorganism can be phenotypically measured. These sets of different culture conditions include substitutions of C-sources, N-source, P-and S-source, nutrient substrates and different culture environment with changing pH, ion and osmotic condition (Table 2.3). The basis of the method relies on a universal culture medium containing all the nutrient substrates essential for growth (e.g. C, N, P, S, K, Na, Mg, Ca, Fe, amino acids, pyrimidines, and vitamins). Each plate contains equal amounts of different substrates which can be used to assess the phenotypic characteristics of microorganisms. PM1 and PM2 microplates contain alternative C-sources (Figure 2.2).

Table 2.3: PM01 to PM20 sets of plate function.

Plate types	Mode of action category
PM01-PM02	Carbon source
PM03	Nitrogen source
PM04	Sulphur, Phosphorus sources
PM05	Nutritional supplement
PM06-PM08	Nitrogen utilization
PM09	Osmotic sensitivity, Toxicity
PM010	pH
PM011-PM020	Inhibitors, Chemical sensitivity

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

Figure 2.2: PM1 plate used in current study contains carbon source nutrients (http://www.biolog.com).

Strains that can catabolize the carbon substrates will uptake the carbon which will enter the respiratory pathway resulting in NADH production (Figure 2.3). Electrons will transfer through the electron transport chain from NADH to the tetrazolium dye. As cell respiration progresses, a purple colouration will appear in the well following the tetrazolium dye reduction (Bochner & Savageau, 1977).

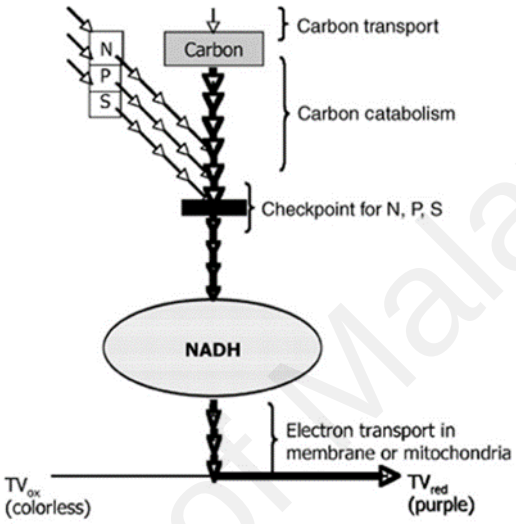


Figure 2.3: Respiratory pathway in carbon consumer strains (Bochner & Savageau, 1977).

The colour formation level in each well will be recorded by the Omnilog system (Biolog Inc., USA) during the incubation time using a camera inside an incubator with slots for up to 50 microplates (Bochner, 2003). The camera is attached to a computer containing a software that translates this into kinetic graphs. The kinetic graphs will be recorded as coloured lines to compare growth curves of the different strains. The Phenotype Microarray application can be used to enhance the assessment and analysis of the metabolic activity and phenotype characteristics of foodborne and environmental pathogens. Since isolation and identification of bacteria from food and environment is

challenging, the use of specific microplates for bacterial growth rate analysis in various nutrient sources provides an insight into their kinetic variation and phenotype profile.

Rapid analysis of several phenotypes among similar strains or other strains from different species delivers large quantities of raw data to the researcher. These microplates are commercially available by Biolog, Inc. (Hayward, USA) and enables researchers to track the growth kinetic trends of the organisms in various environment changes such as pH or different substrates which is similar to their natural habitat and therefor provides information on specific nutrient sources that are essential for the growth of certain bacterial species.

This technology supports the analysis of growth curves even in small quantities of bacterial samples. It can also be utilized to evaluate growth curves from only one colony or a community of aerobic or anaerobic bacteria in the presence of diverse nutrients (Koutny & Zaoralkova, 2005; Borglin et al., 2009). Moreover, the application of PM plates for the study of mutated strains provides detailed data about phenotypic changes in mutants compared to wild-type strains (Johnson et al., 2008; Atanasova & Druzhinina, 2010).

CHAPTER 3: MATERIALS AND METHODS

This study consisted of four parts. In part I, 14 different detection protocols of *V. parahaemolyticus* in frozen prawn samples were evaluated and compared (Table 3.1). In part II, isolated *V. parahaemolyticus* from one single frozen prawn sample using the 14 tested protocols in part I were then subjected for REP-PCR genotyping to study the genetic relatedness of *V. parahaemolyticus* isolates. In part III, the growth of *tdh*$^-$/*trh*$^-$ strains and *tdh*$^-$/*trh*$^+$ strains of *V. parahaemolyticus* in different NaCl concentration was conducted. Finally, in part IV, the carbon utilization profile of *tdh*$^-$/*trh*$^-$ strains, *tdh*$^+$/*trh*$^-$ strains and *tdh*$^-$/*trh*$^+$ strains *V. parahaemolyticus* were compared. The flow of this research study is outlined in Figure 3.1.

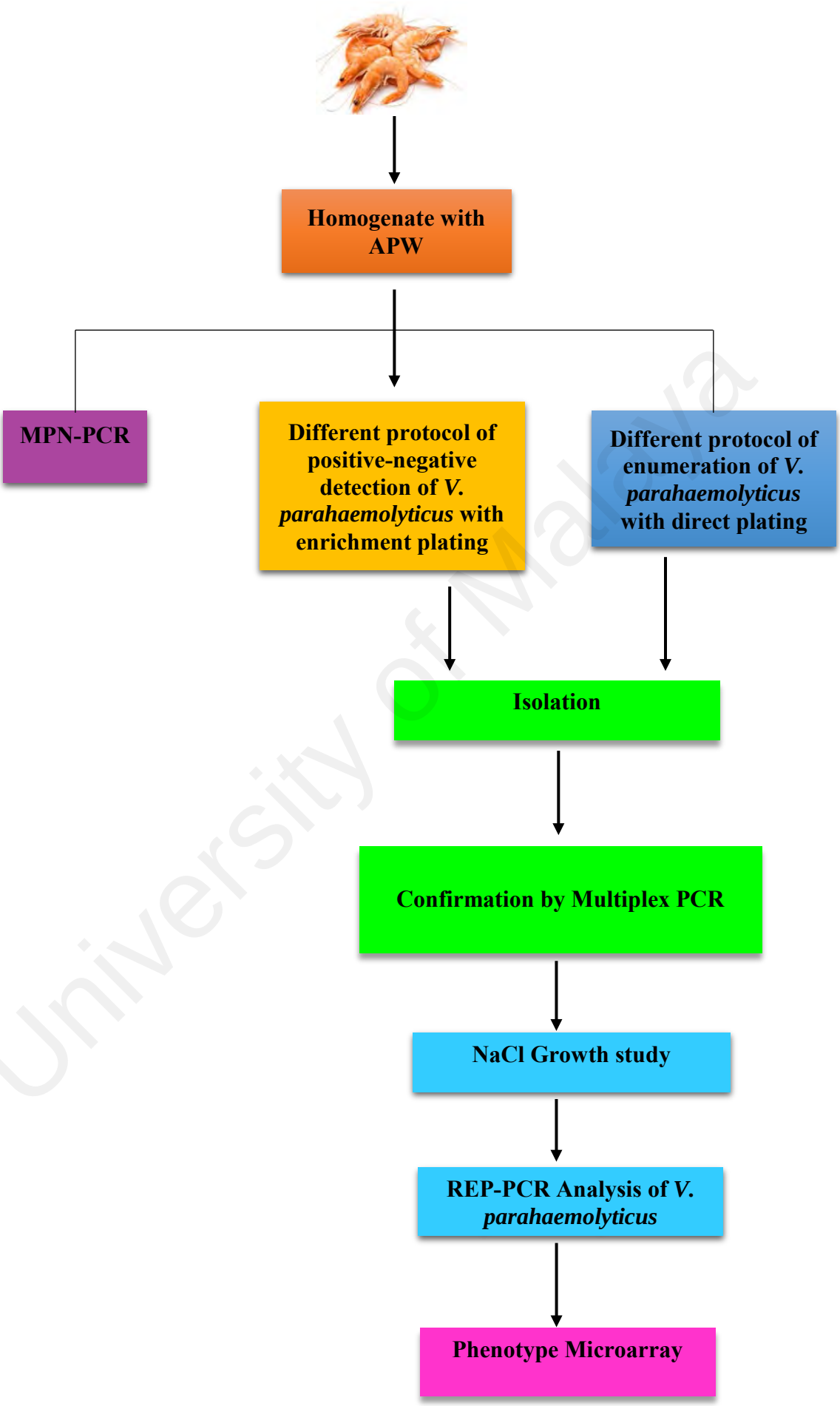


Figure 3.1: General flow of research experiment.

3.1 Frozen prawn sample preparation

Twenty packs (300 g each pack) of retailed frozen white Indian prawn (*Fenneropenaeus indicus*) were purchased from several markets in Kuala Lumpur from June to July 2013. The frozen prawns were transported on ice to the laboratory immediately. The sample was thawed at room temperature for 2 h before analysis was performed. After the frozen prawn sample was completely thawed, 25 g of prawn sample was added to 225 ml of sterile Alkaline Peptone Water (APW) (Merck KGaA, Germany) in a sterile stomacher bag. The sample was then homogenized using a stomacher for 3 min. The homogenate was then ready for enumeration and detection of *V. parahaemolyticus* using different protocols in this study.

3.2 Enumeration of *Vibrio parahaemolyticus* using a direct-plating approach

For enumeration of *V. parahaemolyticus* in frozen prawn samples by direct-plating, a total of six protocols (D30CV, D35CV, D37CV, D30T, D35T, D37T) were tested in this study (Figure 3.2, a). Three loops per plate (amounting to around 30 µl) of the homogenate was streaked directly onto CV (CHROMagar, Paris, France) and TCBS agar (Merck, KGaA, Germany) plates for selective isolation of *V. parahaemolyticus* strains. In total, for each sample, plates were prepared in duplicates with a total of six plates of CV and six plates of TCBS being inoculated. All plates were then incubated at 30°C (D30CV and D30T), 35°C (D35CV and D35T) or 37°C (D37CV and D37T) for 18 h. All the presumptive *V. parahaemolyticus* colonies grown on CV (mauve coloured colonies) and TCBS agar (green coloured colonies) were counted for the total number of *V. parahaemolyticus* (CFU/g) in frozen prawn sample. Five to ten presumptive *V.*

parahaemolyticus isolates were then randomly selected from each plate for further confirmation and characterization.

3.3 Detection of *Vibrio parahaemolyticus* in frozen prawn using enrichment-plating

To determine the presence or absence of *V. parahaemolyticus* in frozen prawn samples, eight protocols using an enrichment-plating approach with different incubation temperatures, duration of enrichment and selective media (ES30CV, ES37CV, ES30T, ES37T, EL30CV, EL37CV, EL30T, EL37T) were conducted in this study (Figure 3.2, b). In brief, the prepared homogenate (25 g of prawn in 225 ml APW) in a stomacher bag was subjected to enrichment with APW by incubating at 37°C for 6 h or 18 h. After the enrichment, 30 µl of the enriched sample was then streaked onto four plates of CV and four plates of TCBS. All plates were incubated at 30°C (ES30CV, ES30T, EL30CV, EL30T) or 37°C (ES37CV, ES37T, EL37CV, EL37T) for 18 h. The plates were then observed for the presence of presumptive colonies of *V. parahaemolyticus* (green coloured colony on TCBS and mauve coloured colony on CV). Five to ten presumptive *V. parahaemolyticus* isolates were randomly selected from each plate for further confirmation and characterization. Samples with positive detection of *V. parahaemolyticus* was scored positive for *V. parahaemolyticus*.

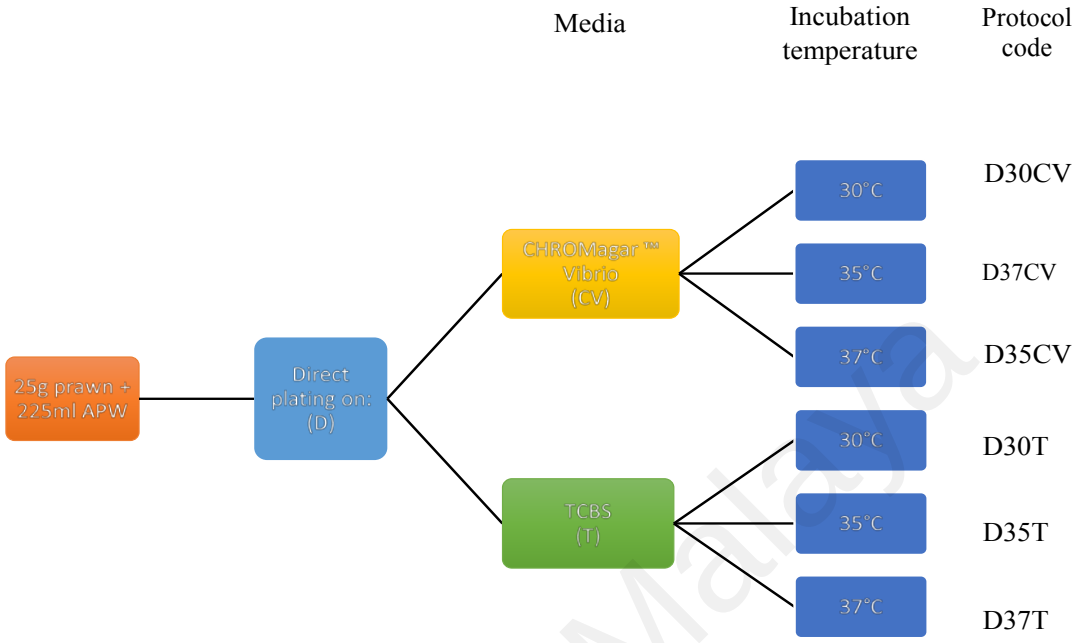
Gram stain was used to confirm the detection of *Vibrio parahaemolyticus* strains by first preparing a heat fixed smear of the bacteria on a glass slide. Crystal violet was added and allowed to stand for one min. The slide was washed with water, then Gram's iodine was gently added and allowed to stand for one min. The slide was rinsed again followed by a rapid decolorization using acetone for 2-3 sec. The counter stain, safranin, was then added and allowed to stand for 30 sec. After the final rinse with water, the slide was viewed under a light microscope for morphological observation of the cells.

Presumptive *Vibrio parahaemolyticus* strains appeared as curved or comma-shaped cells and are Gram negative (observed as a pink colour). All presumptive strains of *Vibrio parahaemolyticus* were also subjected to an oxidase test using the oxidase reagent. Positive strains of *V. parahaemolyticus* turned purple after exposure.

Table 3.1: Culture conditions for enumeration and detection of *V. parahaemolyticus* from frozen prawn using 14 different protocols.

Enrichment	Duration of enrichment	Plating media	Incubation temperature	Protocol code
Direct plating (No enrichment)		CHROMagar™Vibrio	30°C	D30CV
		CHROMagar™Vibrio	35°C	D35CV
		CHROMagar™Vibrio	37°C	D37CV
		TCBS	30°C	D30T
		TCBS	35°C	D35T
		TCBS	37°C	D37T
Enrichment in APW	6 h	CHROMagar™Vibrio	30°C	ES30CV
	6 h	CHROMagar™Vibrio	37°C	ES37CV
	6 h	TCBS	30°C	ES30T
	6 h	TCBS	37°C	ES37T
	18 h	CHROMagar™Vibrio	30°C	EL30CV
	18 h	CHROMagar™Vibrio	37°C	EL37CV
	18 h	TCBS	30°C	EL30T
	18 h	TCBS	37°C	EL37T

(a) Enumeration of *V. parahaemolyticus* from frozen prawn with direct plating



(b) Detection of *V. parahaemolyticus* in frozen prawn using enrichment plating

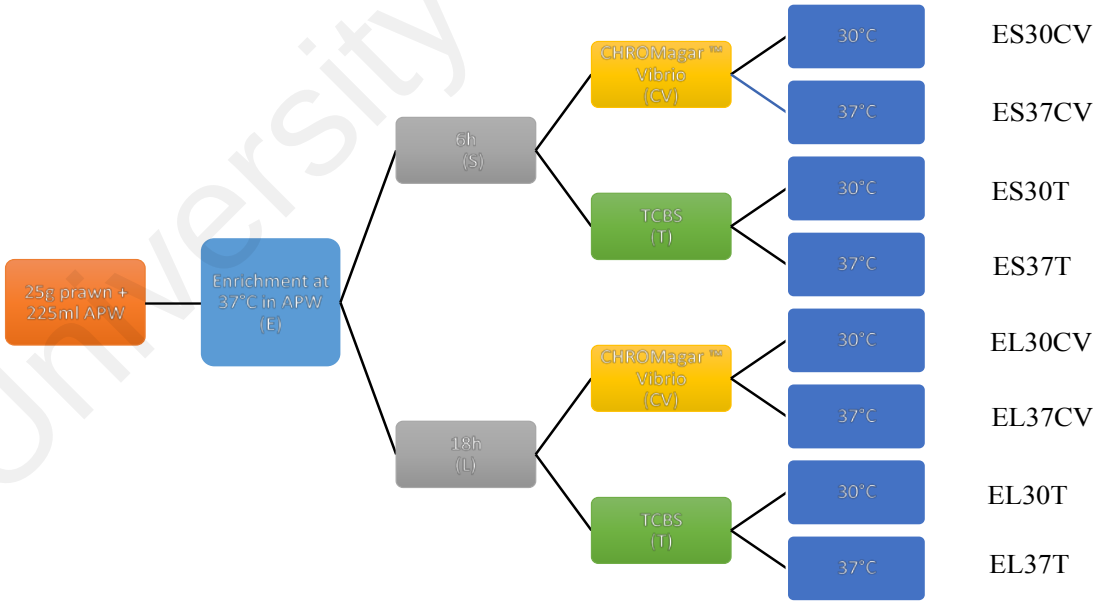


Figure 3.2: The flowchart shows the different culture conditions for enumeration of *V. parahaemolyticus* from frozen prawns from direct plating (a) and after enrichment plating (b).

3.4 Enumeration of *Vibrio parahaemolyticus* based on Most Probable Number-Polymerase Chain Reaction (MPN-PCR)

MPN-PCR was applied to enumerate *V. parahaemolyticus* in frozen prawns using *tl*, *tdh* and *trh* target genes in a multiplex PCR reaction. Three-tube MPN method was used to estimate the number of *V. parahaemolyticus* in prawn samples (Ray et al., 1978). The process of preparing serial dilutions for PCR detection is illustrated in Figure 3.3.

Twenty-five grams of prawn samples were added into 225 ml of APW and homogenized using stomacher. Three-tube dilution was used in this method (MPN-BAM) (Ray et al., 1978). Three tubes filled with 9 ml APW was prepared for each dilution in three replicates. One ml of the sample homogenate was added to nine ml APW in the first tube and yielded 10^{-1} dilution. Three-tubes serial dilution (10^{-1}, 10^{-2}, and 10^{-3}) was prepared as shown in Figure 3.3. All tubes were incubated at 37°C for 18 h. After incubation, all tubes were observed for turbidity and tubes that showed turbidity were then centrifuged for 1 min to spin down the bacterial cells. The resulting pellet was then washed twice using 500 µl of TE buffer. The washed pellet was then re-suspended in 100 µl purified sterile distilled water (dH_2O) and heated for 5 min at 99°C and immediately chilled on ice for 10 min. The treated tubes were then centrifuged for 1 min. The clear supernatant containing crude DNA was transferred into sterile tubes and used as DNA template for PCR. Multiplex PCR was then performed to detect *tl*, *tdh* and *trh* genes of *V. parahaemolyticus* strains as described as in section 3.5.1. The number of tubes detected positive by PCR at each dilution level were recorded and the MPN number of *V. parahaemolyticus* (MPN/g) for each sample was obtained by keying in the MPN code into MPN Calculator (Figure 3.4) that can be accessed freely from http://www.i2workout.com/mcuriale/mpn/ (Oblinger & Koburger, 1975).

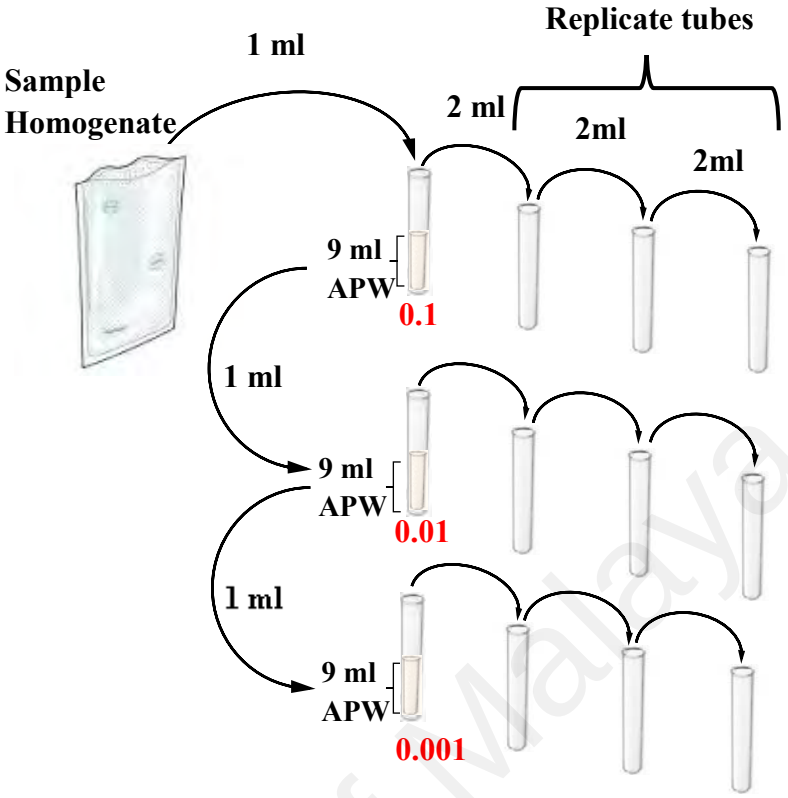


Figure 3.3: MPN three-tube preparation.

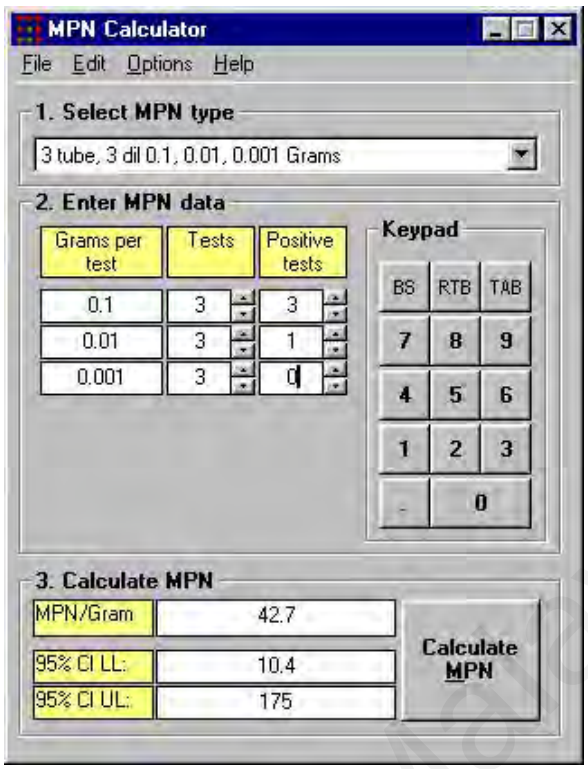


Figure 3.4: MPN calculator (http://www.i2workout.com/mcuriale/mpn/).

3.5 Confirmation of *Vibrio parahaemolyticus* isolates

After the presumptive *V. parahaemolyticus* isolates were confirmed with Gram staining, microscopy observation and oxidase test, the positive isolates were then subjected to PCR to re-confirm the identity as *V. parahaemolyticus* and to determine the virulence genes.

3.5.1 Molecular identification and confirmation of presumptive *Vibrio parahaemolyticus* using polymerase chain reaction (PCR)

Presumptive *V. parahaemolyticus* isolates were sub-cultured on Nutrient Agar (Merck, KGaA, Germany) with additional 1.5% NaCl. The boil-cell method was used to isolate the crude bacterial DNA. A purified single colony was suspended in 100 µl of

sterile distilled water in 1.5 ml Eppendorf tube and vortexed to resuspend the bacterial cells. The bacterial suspension was then heated at 99°C for 5 min and then placed on ice for 10 min. The boiled cell lysate was then centrifuged at 13,400 rpm for 3 min and the upper supernatant was transferred to a new sterile tube and used as DNA template for PCR detection of 16SrDNA, *tl*, *tdh* and *trh* gene.

Duplex PCR targeting 16SrDNA and *tl* genes was carried out in a multiplex PCR according to protocols by Bej et al. (1999) for confirmation of *V. parahaemolyticus* species. The 16SrDNA gene is present in all *Vibrio* species and *tl* gene is specific for *V. parahaemolyticus* (Table 3.2). The multiplex PCR was run in a total volume of 25 µl containing 5 µl of the DNA template (Table 3.3). Detection of *tdh* and *trh* genes using multiplex PCR assay was carried out according to protocols by Panicker et al., 2004, Tada et al., 1992 and Bej et al., 1999. An aliquot of 1X of PCR mixture contained ~10 ng of DNA template, 5 µM of tdh and trh primers, 1.0 nM of $MgCl_2$, 5 mM of each dNTPs and 1.0 U of Taq DNA polymerase (Promega, US) (Table 3.4). PCR for virulence gene detection was carried out using the following conditions: pre-denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and primer extension at 72°C for 30 sec, final extension at 72°C for 5 min (Table 3.5).The amplicon length of *tdh* and *trh* genes were 269 bp and 500 bp, respectively. All PCR products were run on 1.5% agarose gel electrophoresis using 0.5X TBE buffer and then stained with GelRed® nucleic acid stain (Biotium, Hayward, US). After staining for about 30 min PCR products were visualized under a UV light using a T-LUM25W Gel-Doc (UVP Inc., US). Some of the PCR amplicons were selected for sequencing to reconfirm their sequences. The sequencing service was outsourced to First BASE Laboratories, Malaysia. The sequences was aligned and compared using the Basic Local Alignment Search Tool (BLAST) with sequences

deposited in the National Center for Biotechnology Information (NCBI) database available at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Table 3.2: List of primers used in Multiplex PCR for detection of *V. parahaemolyticus* strains.

Target gene	Primers	Sequence	Amplicon size(bp)	References
16SrDNA	567F	GGCGTAAAGCGCATGCAGGT	120	
	680R	GAAATTCTACCCCCCTCTACAG		
tl	TL-F	AAAGCGGATTATGCAGAAGCACTG	450	Taniguchi et al., 1985, 1986
	TL-R	GCTACTTTCTAGCATTTTCTCTGC		
tdh	TDH-F	GTAAAGGTCTCTGACTTTTGGAC	269	Nishibuchi and Kaper, 1985
	TDH-R	TGGAATAGAACCTTCATCTTCACC		
trh	TRH-F	TTGGCTTCGATATTTTCAGTATCT	500	Honda and Lida, 1993; Honda et al., 1991
	TRH-R	CATAACAAACATATGCCCATTTCCG		

Table 3.3: Multiplex PCR reactions for amplification of 16SrDNA and *tl* genes.

Reagents	Stock Concentration	Reaction Concentration	Volume (µl)
DNA Template			5
Buffer	5X	1X	5
$MgCl_2$	25mM	1mM	1
dNTP mix	10mM	5mM	0.5
Primer1 (16SrDNA)	10µM	3.1µM	0.4
Primer2 (tl)	10µM	2.5µM	1
Taq DNA polymerase	5U/µl	1U	0.2
ddH_2O			Make up to 25
Total volume			25

Table 3.4: Multiplex PCR reaction mixture for amplification of *tdh* and *trh* virulence genes.

Reagents	Stock Concentration	Reaction Concentration	Volume (µl)
DNA Template			5
Buffer	5X	1X	5
$MgCl_2$	25mM	1mM	1
dNTP mix	10mM	5mM	0.5
Primer1 (tdh)	10µM	5µM	0.5
Primer2 (trh)	10µM	5µM	0.5
Taq DNA polymerase	5U/µl	1U	0.2
ddH_2 O			Make up to 25
Total volume			25

Table 3.5: Multiplex PCR conditions used for 16SrDNA/*tl* and *tdh*/*trh* based amplification.

Condition	Temperature (^{o}C)	Duration	Cycle
Pre-denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	55	30 s	
Extension	72	30 s	
Final extension	72	5 min	1

3.6 Repetitive Extragenic Palindromic-Polymerase Chain Reaction (REP-PCR) Finger printing

The REP-PCR typing method was performed to characterize the genetic diversity and similarity of the 39 confirmed *V. parahaemolyticus* isolates from a single pack of frozen prawn sample using different protocols in this study as this single prawn sample contained the *tdh*$^-$/*trh*$^+$ isolates. Thus, the *tdh*$^-$/*trh*$^+$ *V. parahaemolyticus* isolates were also included in the typing. This is carried out using primers that target the repetitive sequences in the genome (Navia et al., 1999; Lim et al., 2009). The REP-PCR was performed using 5X buffer (Promega, USA), 2.5 Mm $MgCl_2$, 10 mM of each dNTPs, 1.0 U of Taq DNA Polymerase (Promega, USA), 10 µM of REP primer and ~10 ng of template DNA in a final volume of 25 µl. The PCR conditions consisted of pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and primer extension at 72°C for 8 min and a final extension at 72°C for 8 min. The reagents are listed in Table 3.6 and conditions shown in Table 3.7. An agarose gel consisting of 1.7% agarose was prepared with 0.5X TBE buffer and used to differentiate REP-PCR bands. Electrophoresis was done using 100 V for about 5 to 6 h and the gel was then stained with GelRed® nucleic acid stain (Biotium, Hayward, US). After 30 min, the gel was visualized under UV light. The gel image captured on a T-LUM25W Gel-Doc (UVP Inc., US) was used for data analysis using BioNumeric version 6 (Applied Maths, Belgium). After normalizing the gel, a dendrogram was produced using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). 1 kb and 100 kb DNA ladder (Promega, US) was used as a DNA size marker.

Table 3.6: REP-PCR master mixture used for amplification.

Reagents	Stock Concentration	Reaction Concentration	Volume (µl)
DNA Template			5
Buffer	5X	1X	5
$MgCl_2$	25mM	2.5mM	2.5
dNTP mix	10mM	200µM	0.5
REP-Primer	10µM	0.5µM	1.25
Taq DNA polymerase	5U/µl	1U	0.2
ddH_2O			Make up to 25
Total volume			25

Table 3.7: REP-PCR amplification conditions.

Condition	Temperature (^oC)	Duration	Cycle
Pre-denaturation	94	4 min	1
Denaturation	94	1 min	35
Annealing	42	1 min	
Extension	72	8 min	
Final extension	72	8 min	1

3.7 Growth of *tdh*$^-$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ strains of *Vibrio parahaemolyticus* in different NaCl concentration

Six selected strains of *V. parahaemolyticus* (VP16, VP63, VP12, VP13-14, VP31, VP32) were evaluated for their growth characteristics in 1%, 2%, 3%, 5% and 10% of NaCl. In brief, bacterial inoculum with concentrations of 10^3 CFU/g were prepared by inoculating 5 ml of LB broth (Difco™, US) containing different NaCl concentration (1%, 2%, 3%, 5% and 10%) with fresh bacterial cultures grown on nutrient agar containing 1.5% NaCl. Then, 100 µl of bacterial inoculum with different NaCl concentration were transferred to flat-bottomed 96 well plates. Negative control without bacterial inoculum was included in the test. The growth rate was read at OD_{600nm} for every 15 min for 24 h using microplate reader (VersaMax™, California, US) at 37°C. The data collected was plotted against time using the SPSS software (Version 19). The experiments were conducted in duplicates.

3.8 Phenotype Microarray

In this study, carbon utilization of *tdh*$^-$/*trh*$^-$ strains (ATCC17802, VPD37CV, VPEL37CV), *tdh*$^+$/*trh*$^-$ strains (ATCC43996™, *tdh*$^+$/*trh*$^-$3TBW10), and *tdh*$^-$/*trh*$^+$ strains (*tdh*$^-$/*trh*$^+$D30CV, *tdh*$^-$/*trh*$^+$EL37CV) of *V. parahaemolyticus* were studied using Biolog PM system (SA, US). The details of the *V. parahaemolyticus* strains used in this study were described in Table 3.8.

Table 3.8: *V. parahaemolyticus* strains used in phenotype microarray study for their carbon utilization profiles.

Strain code	Virulence genes	Origin/ source of strain
ATCC43996	*tdh*$^+$/*trh*$^-$	ATCC
3TBW10	*tdh*$^+$/*trh*$^-$	Isolated from brackish water Matang, Perak, Malaysia in 2012.
VP31	*tdh*$^-$/*trh*$^+$	Isolated in study using protocol D30CV
VP13-14	*tdh*$^-$/*trh*$^+$	Isolated in study using protocol EL37CV
ATCC17802	*tdh*$^-$/*trh*$^-$	ATCC
VP16	*tdh*$^-$/*trh*$^-$	Isolated in study using protocol D37CV
VP63	*tdh*$^-$/*trh*$^-$	Isolated in study using protocol EL37CV

V. parahaemolyticus strains included in this study were streaked on nutrient agar containing 1.5% NaCl and incubated at 37°C overnight. A pure single colony of each strain was carefully picked using a moistened cotton swab to avoid contact with the surface of the media and then re-suspended into 10 ml of IF-0a fluid (Biolog Inc., USA) to reach 85% of bacterial cell density at OD 670 nm measured using a Biolog

turbidimeter. The cell suspension was then transferred into a sterile reservoir and 1% redox dye-D (Biolog Inc., USA), 1200 µl of 10% NaCl solution and 680 µl ddH_2o were added.

Each well in the 96-well microtiter plate of Biolog PM1 was inoculated with 100 µl of cell suspension using a multichannel pipette and inserted into the Omnilog PM machine (Biolog Phenotype Microarray, US) for incubation at 37°C for 24 h. The system read, recorded and plotted the colour density of each well. Cell growth was monitored every 15 min through the colour change that resulted from the tetrazolium reduction. The recording was carried out for 24 h. A1 zero option (negative control) was selected in Omnilog PM software during data analysis to remove the background noise. The procedure to set up the Omnilog software and load batches is described in a flowchart below (Figure 3.5).

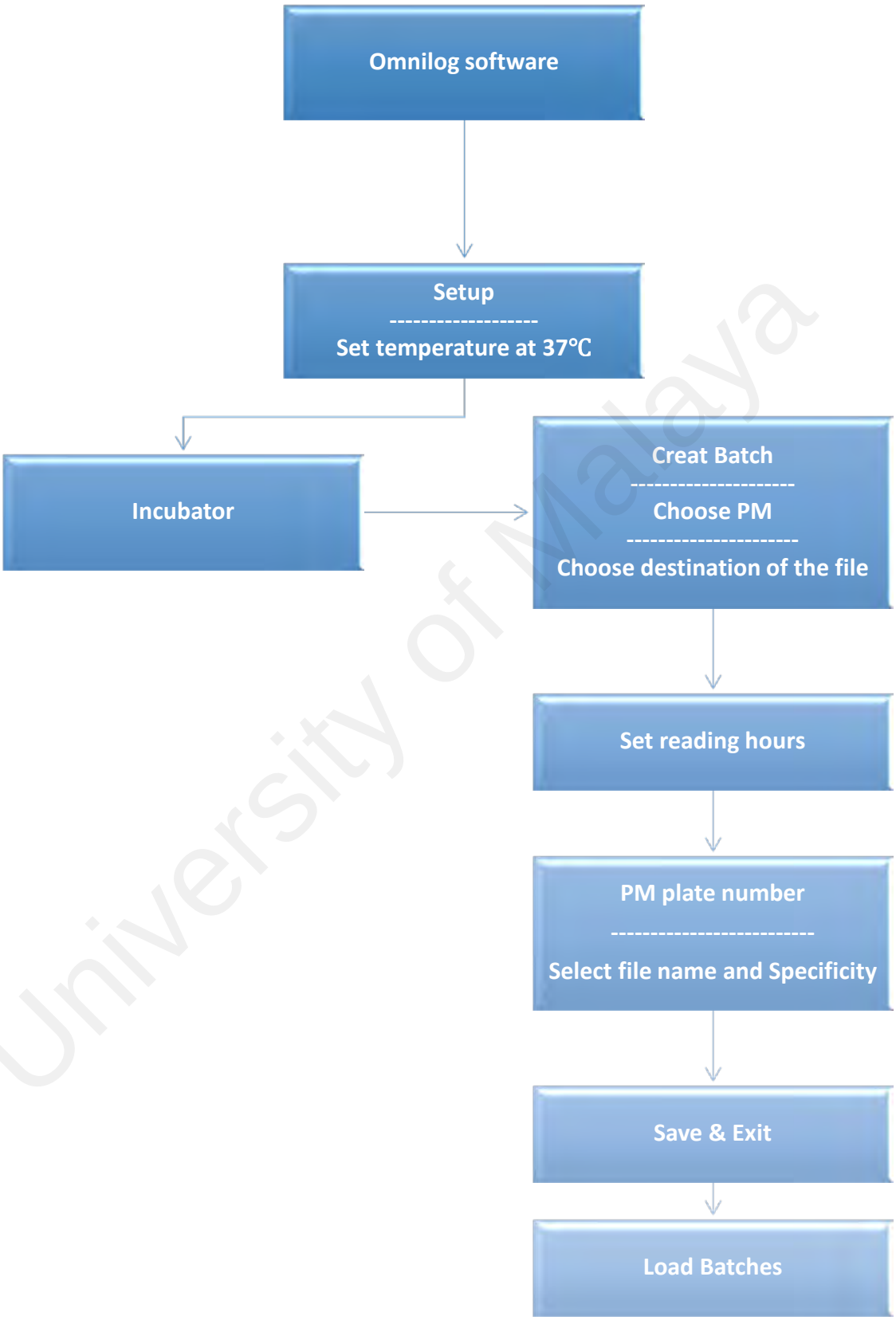


Figure 3.5: Different steps to set up Omnilog software and incubator.

3.9. Statistical analysis

Statistical analysis for enumerating and detecting *V. parahaemolyticus* and virulence genes in this study were generated in Microsoft Excel and SPSS version 10 (IBM, US). Significance testing was done using chi-square contingency test. REP-PCR analysis was performed using UPGMA (Unweighted Pair-Group Method using Arithmetic average).

CHAPTER 4: RESULTS

4.1. Effect of enrichment and incubation temperature on the performance of TCBS and CV in *Vibrio parahaemolyticus* isolation from frozen prawns

In general, isolation protocols using CV agar resulted in better positive predictive percentage for *V. parahaemolyticus* (ranged from 97.1 - 100%) compared to protocols using TCBS as isolating media (ranged from 88.5 - 100%) (Table 4.1). In other words, CV medium was more specific in detecting *V. parahaemolyticus* as compared to TCBS. The isolation of *V. parahaemolyticus* strains was least efficient using direct plating on CV at 35°C (97.1%), as lesser numbers of *V. parahaemolyticus* were isolated. Similarly, the protocol that used 6 h enrichment on TCBS at 30°C yielded fewer confirmed positive *V. parahaemolyticus* strains (88.5%). The performance of both media was affected by the incubation temperature and the enrichment step prior to isolation. The effect of the enrichment step was more pronounced on TCBS agar, in which direct plating of homogenate frozen prawn sample onto TCBS without enrichment yielded more false-positive isolates (n = 6) than with enrichment (n = 3) prior to plating (Table 4.1). The standard isolation protocols of overnight or 6 h enrichment in APW prior to streaking onto TCBS or CV with incubation temperature of 37^{o}C yielded good positive predictive percentage (100%); while decreasing the incubation temperature to 30^{o}C reduced the selectivity of the medium.

4.2. Detection and isolation of virulent *Vibrio parahaemolyticus* from frozen prawns via different isolation protocols

Out of the 20 frozen prawn samples tested, only one sample (5%) was detected positive for *tdh*$^{-}$/*trh*$^{+}$ *V. parahaemolyticus*; while *tdh*$^{+}$/*trh*$^{-}$ *V. parahaemolyticus* was not

detected in all the sample tested. Four isolates of *tdh*$^{-}$/*trh*$^{+}$ *V. parahaemolyticus* were recovered from the frozen prawn samples using CV as isolating media (D30CV, 3 isolates; EL37CV, 1 isolate) (Table 4.1). Out of eight mauve colonies randomly picked from CV following protocol D30CV, three colonies were confirmed as *tdh*$^{-}$/*trh*$^{+}$ *V. parahaemolyticus*; while one out of ten mauve colonies selected from EL37CV was *tdh*$^{-}$/*trh*$^{+}$ *V. parahaemolyticus*. The *tdh*$^{-}$/*trh*$^{+}$ gene was not detected in any of the 29 green coloured colonies recovered from TCBS agar plates from the positive prawn sample.

Table 4.1: Recovery of *V. parahaemolyticus* and virulent *strains* (*tdh*$^+$/*tdh*$^-$ and *tdh*$^-$/*trh*$^+$) from retailed frozen prawns using different isolation protocols.

Method	Total number of presumptive colony tested	Number of isolate confirmed as *V. parahaemolyticus*[a]	Number of isolates positive for *trh* gene [b]	Positive predictive percentage for *V. parahaemolyticus* (%) [c]	Percentage of *trh*$^+$ *V. parahaemolyticus* (%) [d]
D30CAV	87	87	3	100	3.5
D35CAV	34	33	0	97.1	0
D37CAV	69	69	0	100	0
ES30CAV	27	27	0	100	0
ES37CAV	14	14	0	100	0
EL30CAV	103	102	0	99	0
EL37CAV	94	94	1	100	1.1
Subtotal (CHROMagar™Vibrio)	**428**	**426**	**4**	**99.5**	**0.9**
D30TCBS	66	65	0	98.5	0
D35TCBS	31	29	0	93.5	0
D37TCBS	73	70	0	95.9	0
ES30TCBS	26	23	0	88.5	0
ES37TCBS	21	21	0	100	0
EL30TCBS	75	75	0	100	0
EL37TCBS	73	73	0	100	0
Subtotal (TCBS)	**365**	**356**	**0**	**97.5**	**0**
Total	**793**	**782**	**4**	**98.6**	**0.5**

[a] Presumptive isolate of *V. parahaemolyticus* was confirmed with species-specific PCR.
[b] All of the isolates was negative for *tdh* gene.
[c] Positive predictive percentage = number of isolate confirmed as *V. parahaemolyticus*/ total number of presumptive colony tested × 100%
[d] Percentage of *trh*$^+$ *V. parahaemolyticus* = number of isolates positive for *trh* gene/ number of isolate confirmed as *V. parahaemolyticus* × 100%

4.3. Comparison of *Vibrio parahaemolyticus* counts in frozen prawn using direct-plating and MPN-PCR protocols

The concentration of *V. parahaemolyticus* in frozen prawns as enumerated by direct-plating onto CV and TCBS showed an increasing trend with incubation temperature from 30°C to 37°C (Figure 4.1). The enumeration results yielded by CV (ranged from 3.56 to 3.95 log CFU/g) and TCBS (ranged from 3.53 to 3.83 log CFU/g) was not significantly different ($p>0.05$); however, the incubation temperature yielded significant differences in the *V. parahaemolyticus* counts in frozen prawn, particularly between incubation temperatures at 30°C and 37°C ($p<0.05$) (Figure 4.1). The count of *V. parahaemolyticus* in frozen prawn yielded by incubating at 37°C was 0.30 and 0.39 log CFU/g higher than samples incubated at 30°C with TCBS and CV, respectively (Figure 4.1). Almost half of the frozen prawn samples tested using MPN-PCR approach had >3 log MPN/g of *V. parahaemolyticus*, suggesting comparable results with the direct-plating approach (Figure 4.1). Nonetheless, the findings suggest that incubation temperature is a significant parameter for enumeration of *V. parahaemolyticus* in frozen prawns if a direct-plating method is to be used. On the other hand, enumeration of *V. parahaemolyticus* using the MPN-PCR approach was time-consuming and tedious compared to enumeration by direct-plating.

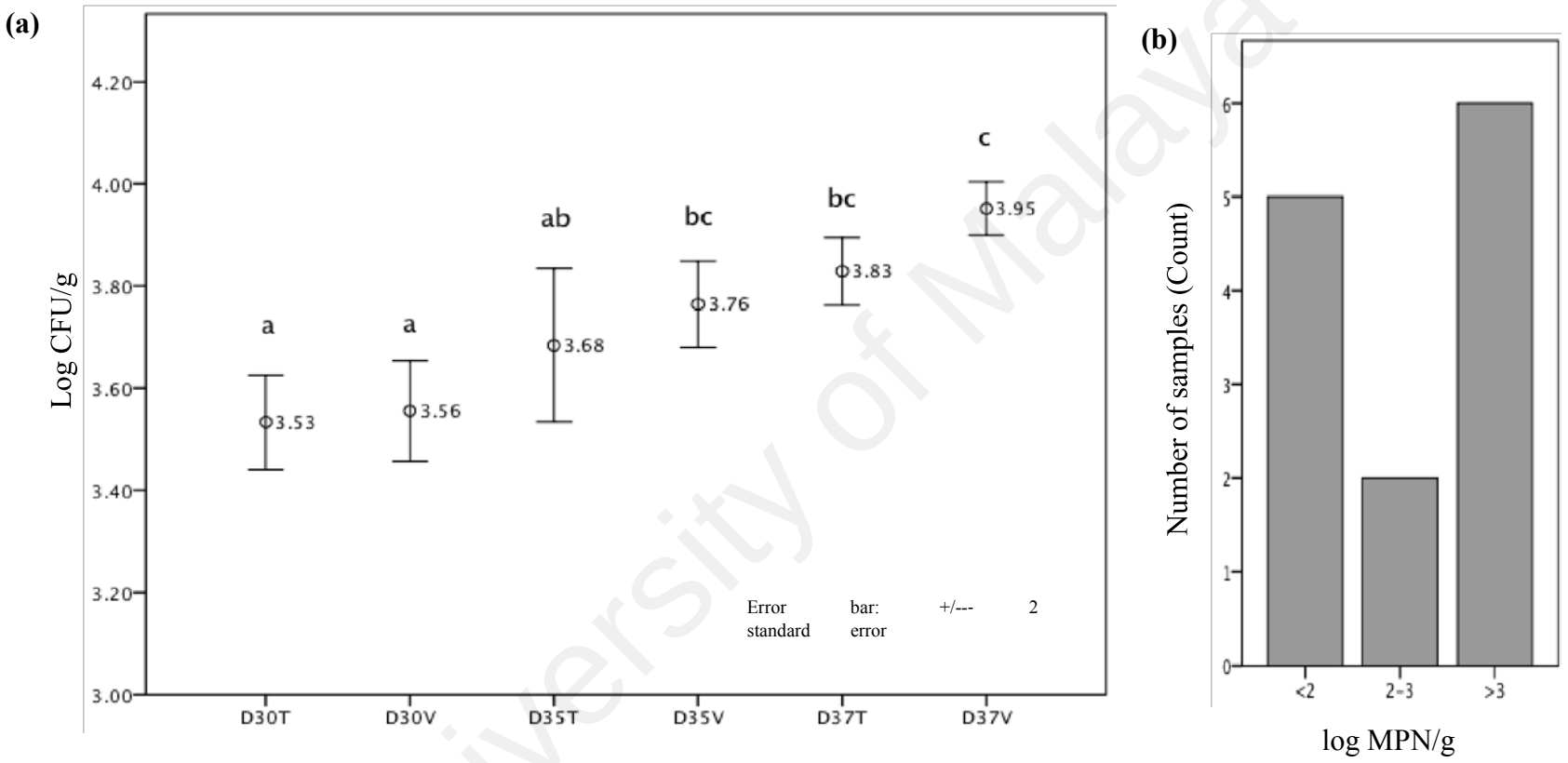


Figure 4.1: Enumeration of *V. parahaemolyticus* in individually packed frozen prawns obtained from local markets (n=20 for D30T, D30CV, D37T and D37CV; n=10 for D35CV and D35T; n=13 for MPN) by (a) direct plating on CV and TCBS and (b) MPN-PCR approach. Categories with different alphabet showed statistically significant differences with $p<0.05$ based on Tukey post-hoc analysis. D30CV, D35CV and D37CV are direct plating on CV with incubation at 30°C, 35°C and 37°C, respectively; D30T, D35T and D37T are direct plating on TCBS with incubation at 30°C, 35°C and 37°C, respectively.

4.4. Repetitive Extragenic Palindromic-Polymerase Chain Reaction (REP-PCR) fingerprinting of *Vibrio parahaemolyticus* isolates originated from a single frozen prawn sample using different isolation protocols

All 39 *V. parahaemolyticus* isolates from different protocols were grouped in 17 clusters with 90% similarity (Figure 4.2). Of these 17 REP clusters, 8 were single isolate clusters; and the rest consists of 2 to 8 isolates per cluster (Figure 4.2, a). It was found that REP cluster VI, XII and XIII consisted of isolates recovered from CV; REP cluster IX and XVII consisted of isolates recovered from TCBS. *V. parahaemolyticus* isolates in REP cluster I were yielded from direct-plating protocols; whereas isolates in REP cluster XIII and X were obtained from protocols that included an enrichment step (Figure 4.2, a). At 90% similarity, *V. parahaemolyticus* isolated from CV (n=19) and TCBS (n=20) were grouped into 12 (Figure 4.2, b) and 10 (Figure 4.2, c) clusters, respectively.

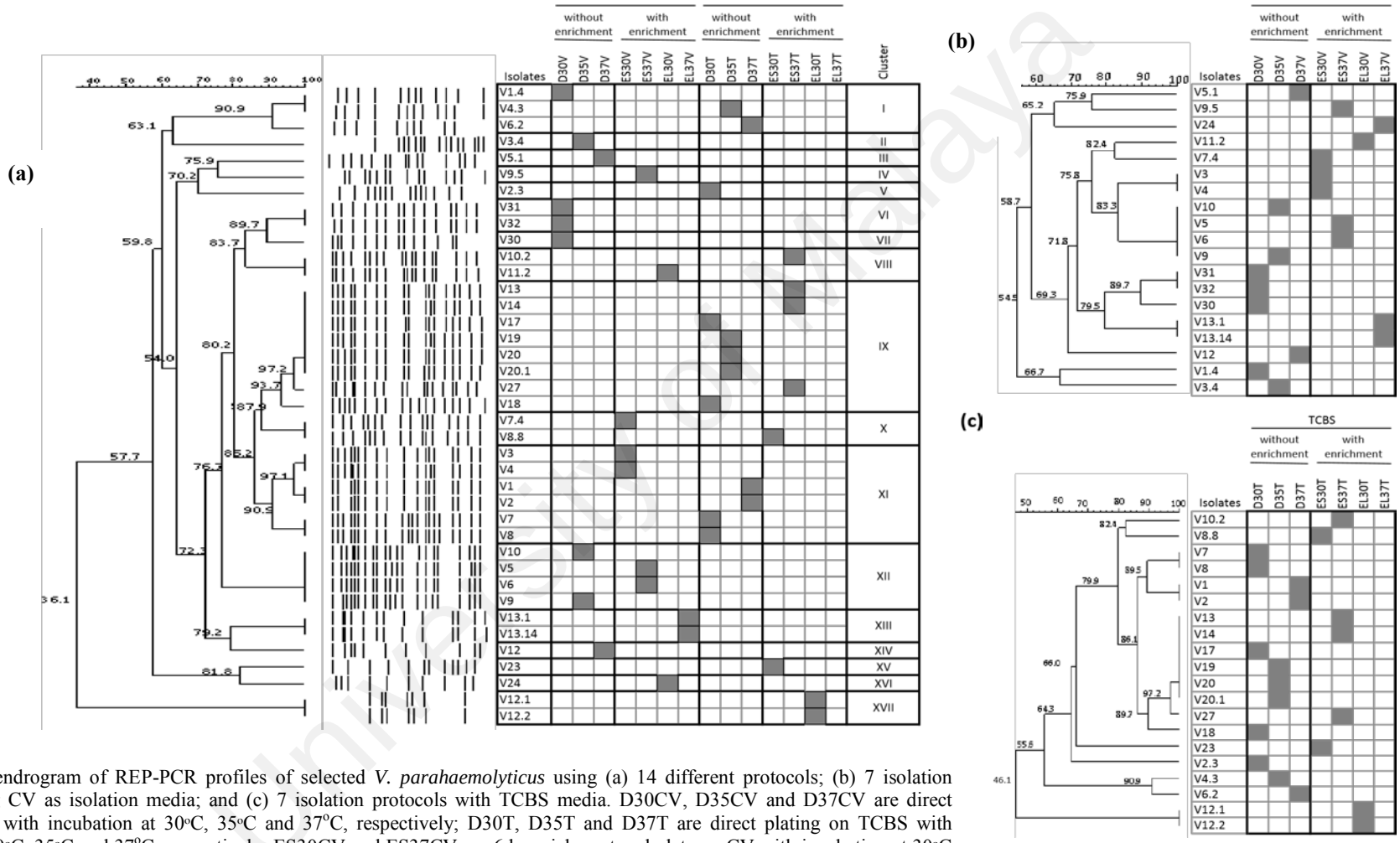


Figure 4.2: Dendrogram of REP-PCR profiles of selected *V. parahaemolyticus* using (a) 14 different protocols; (b) 7 isolation protocols using CV as isolation media; and (c) 7 isolation protocols with TCBS media. D30CV, D35CV and D37CV are direct plating on CV with incubation at 30°C, 35°C and 37°C, respectively; D30T, D35T and D37T are direct plating on TCBS with incubation at 30°C, 35°C and 37°C, respectively; ES30CV and ES37CV are 6 h enrichment and plate on CV with incubation at 30°C and 37°C, respectively; ES30T and ES37T are 6 h enrichment and plating on TCBS with incubation at 30°C and 37°C, respectively; EL30CV and EL37CV are 24h enrichment and plating on CV with incubation at 30°C and 37°C, respectively; EL30T and EL37T are 24h enrichment and plating on TCBS with incubation at 30°C and 37°C, respectively.

4.5. Growth of *tdh*$^-$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ *Vibrio parahaemolyticus* strains in nutrient broth with different NaCl concentration

The results showed that the range of NaCl concentration for most of the *V. parahaemolyticus* strains tested in this study was between 1 to 3% (Figure 4.3). Most of the strains had a highest growth rate that varied between 2% and 3% of NaCl in the first 5 h of incubation, except for VP63 that demonstrated a relatively lower growth rate in 1%, 2%, 3% and 5% NaCl concentration tested. None of the strains tested grew in nutrient broth with a concentration of 10% of NaCl concentration. It is generally observed that the growth rate was highest in 2% and 3%, followed by 1%, then 5% and no growth in 10% NaCl concentration. Also, it was observed in this study that the growth pattern of the three and *tdh*$^-$/*trh*$^+$ strains were quite similar to each other as compared to the highly variable growth pattern shown among the three *tdh*$^-$/*trh*$^-$ strains (Figure 4.3).

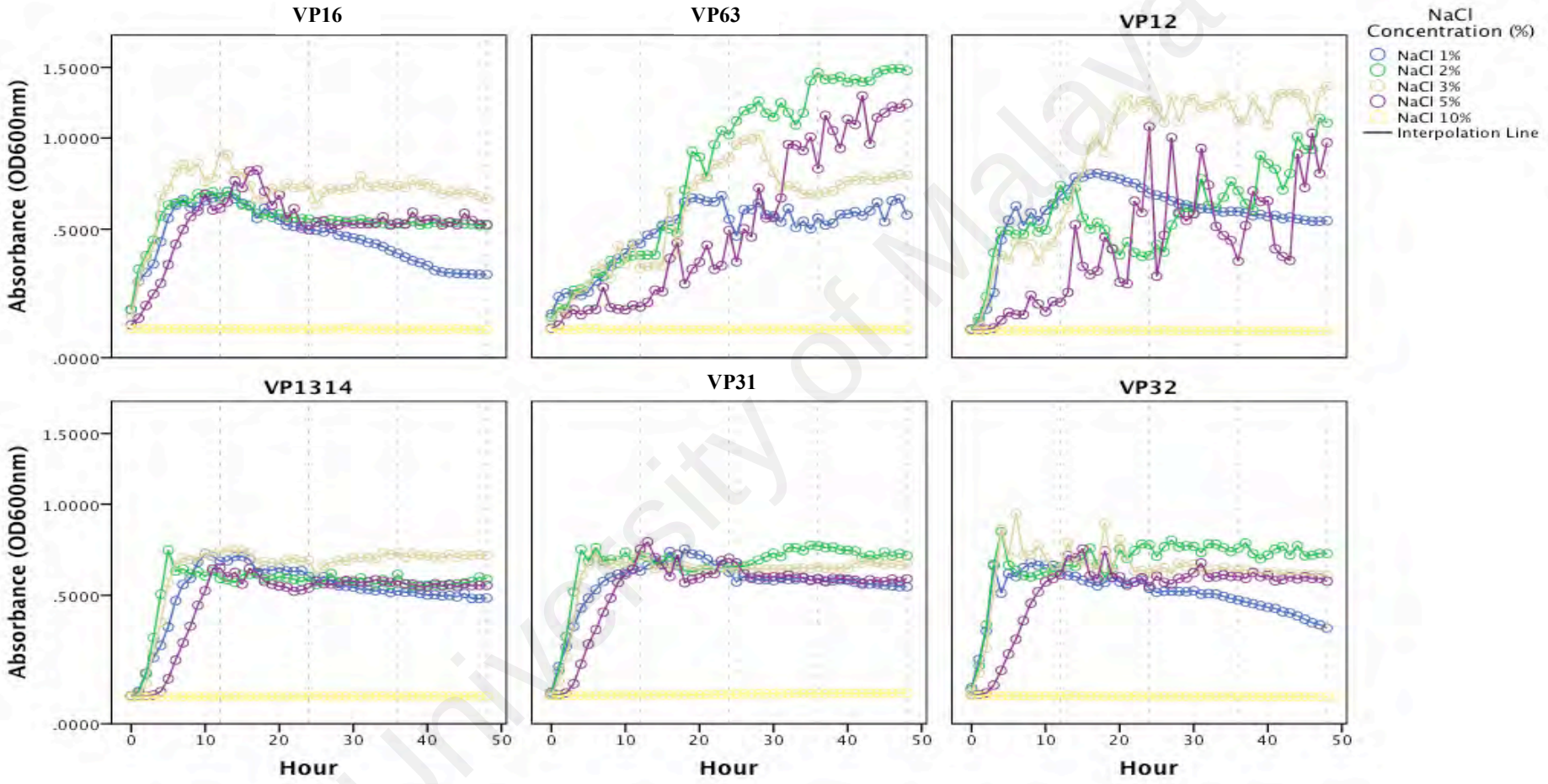


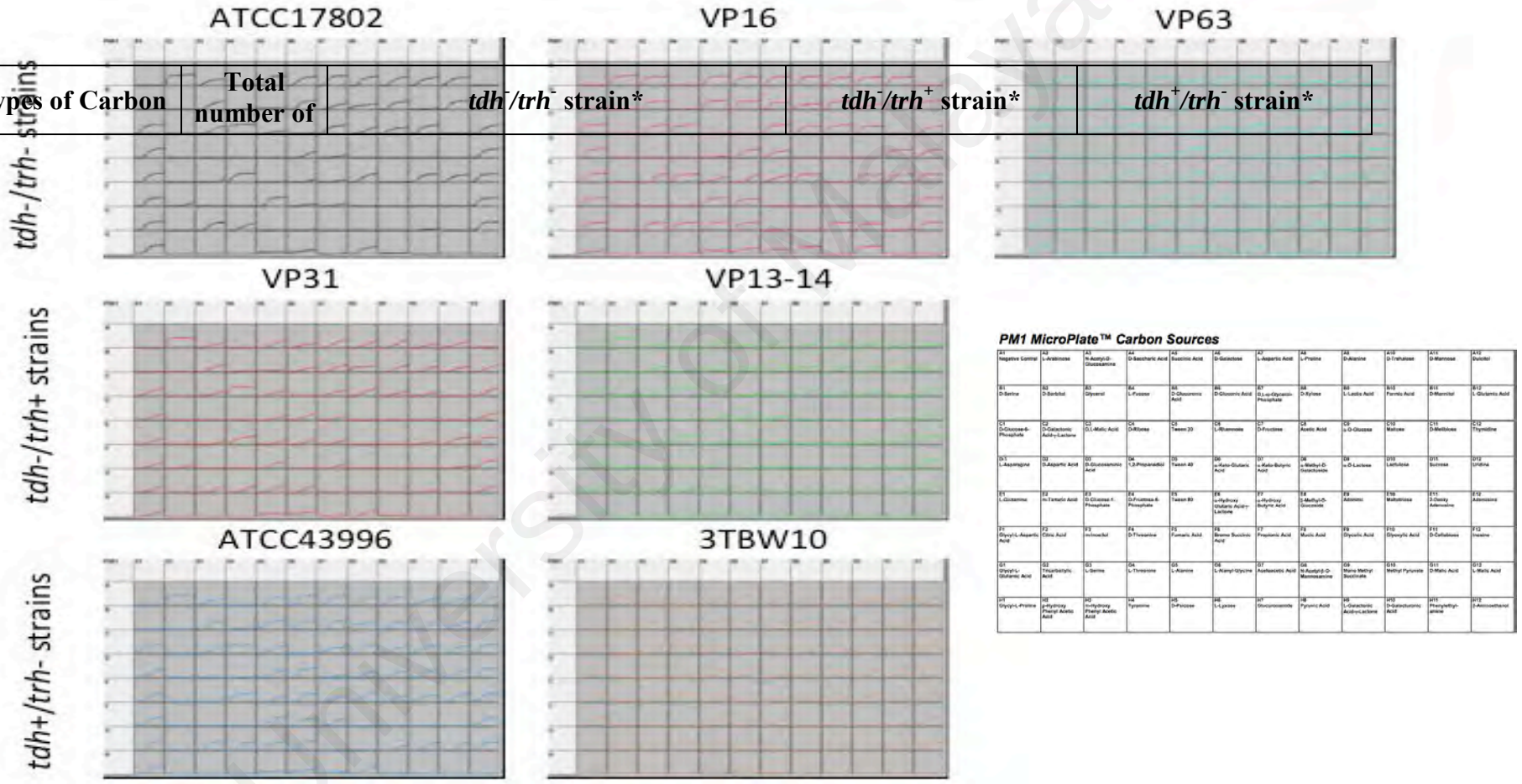
Figure 4.3: Growth pattern of *tdh*$^{-}$/*trh*$^{-}$ strains (VP16, VP63 and VP12) and *tdh*$^{-}$/*trh*$^{+}$ strains (VP1314, VP31 and VP32) of *V. parahaemolyticus* isolated from frozen prawn samples in NaCl 1%, 2%, 3%, 5% and 10% nutrient broth incubated at 37^{o}C.

4.6. Phenotype Microarray analysis

The phenotype microarray results showed that the carbon utilization capacity of *V. parahaemolyticus* strains included in this study varied from strain to strain, but with a certain degree of similarity among *tdh*$^-$/*trh*$^-$, *tdh*$^+$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ *V. parahaemolyticus* strains (Figure 4.4).

Out of the 95 carbon substrates evaluated, *tdh*$^+$/*trh*$^-$ *V. parahaemolyticus* (strain 3TBW10 and ATCC43996) used 70 (74%) and 68 (72%) carbons, respectively, and recorded the highest capacity compared to the other strains (Table 4.2). ATC17802 (*tdh*$^-$/*trh*$^-$ strain) demonstrated the lowest capacity in carbon utilization, with only a 47% (45 out of 95 carbons) utilization rate. Both strains of *tdh*$^-$/*trh*$^+$ (VP31 and VP1314) showed ability to grow in 56 out of 95 (59%) substrates as the sole carbon source at 37^{o}C under aerobic conditions (Table 4.2).

It is noteworthy to point out that dulcitol (well A12), D-melibiose (well C11), a-methyl-D-galactoside (well D08), a-D-lactose (well D09), lactulose (well D10), adonitol (well E09) and D-malic acid (well G11) could only be used by both strains of *tdh*$^+$/*trh*$^-$ *V. parahaemolyticus* (3TBW10 and ATCC43996); while b-methyl-D-glucoside (well E08) could only be used by both strains of *tdh*$^-$/*trh*$^+$ *V. parahaemolyticus* (VP31 and VP1314) (Table 4.3).



Types of Carbon	Total number of	*tdh*⁻/*trh*⁻ strain*	*tdh*⁻/*trh*⁺ strain*	*tdh*⁺/*trh*⁻ strain*

Figure 4.4: Growth curves of *tdh*⁻/*trh*⁻ strains (ATCC17802, VP16 and VP63), *tdh*⁻/*trh*⁺ strains (VP31 and VP13-14) and *tdh*⁺/*trh*⁻ strains (ATCC43996 and 3TBW10) of *V. parahaemolyticus* in different types of sole carbon sources measured with Biolog Omnilog Phenotype Microarray using Biolog PM1 plate.

Table 4.2: The number and percentage of different types of carbon substrates that could be catabolized by *tdh*$^-$/*trh*$^-$ (ATCC19802, VP16 and VP63), *tdh*$^-$/*trh*$^+$ (VP31 and VP13-14) and *tdh*$^+$/*trh*$^-$ (ATCC43996 and 3TBW10) strain of *V. parahaemolyticus* at 37^{o}C under aerobic condition.

		ATCC19802	**VP16**	**VP63**	**VP31**	**VP1314**	**3TBW10**	**ATCC43996**
Alcohol	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Amide	3	0 (0)	1 (33)	1 (33)	0 (0)	0 (0)	1 (33)	1 (33)
Amino acid	16	11 (69)	14 (88)	13 (81)	13 (81)	13 (81)	13 (81)	13 (81)
Carbohydrate	38	23 (61)	26 (68)	25 (66)	25 (66)	25 (66)	33 (87)	32 (84)
Carboxylic acid	32	10 (31)	17 (53)	18 (56)	15 (47)	15 (47)	19 (59)	18 (56)
Ester	1	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Fatty acid	3	1 (33)	1 (33)	2 (67)	3 (100)	3 (100)	3 (100)	3 (100)
Grand Total	**95**	**45 (47)**	**60 (63)**	**60 (63)**	**56 (59)**	**56 (59)**	**70 (74)**	**68 (72)**

* number of carbon substrate that was able to be catabolized (percentage of carbon substrates used over the number carbon substrates in the type group, %).

Table 4.3: Ability of *tdh*$^-$/*trh*$^-$ (ATCC19802, VP16 and VP63), *tdh*$^-$/*trh*$^+$ (VP31 and VP13-14) and *tdh*$^+$/*trh*$^-$ (ATCC43996 and 3TBW10) strains of *V. parahaemolyticus* to grow in 95 different types of substrates as sole carbon source at 37^{o}C aerobically. (code "0" and red for no growth observed while code "1" and green for growth observed).

Type of carbon	Carbon substrate	Well	CAS#	*tdh*$^-$/*trh*$^-$			*tdh*$^-$/*trh*$^+$		*tdh*$^+$/*trh*$^-$	
				ATCC19802	VP16	VP63	VP31	VP1314	3TBW10	ATCC43996
Alcohol	1,2-Propanediol	D04	CAS57-55-6	0	0	0	0	0	0	0
	2-Aminoethanol	H12	CAS141-43-5	0	0	0	0	0	0	0
Amide	Glucuronamide	H07	CAS3789-97-7	0	1	1	0	0	1	1
	Tyramine	H04	CAS60-19-5	0	0	0	0	0	0	0
	Phenylethylamine	H11	CAS156-28-5	0	0	0	0	0	0	0
Amino acid	L-Aspartic acid	A07	CAS3792-50-5	1	1	1	1	1	1	1
	L-Proline	A08	CAS147-85-3	1	1	1	1	1	1	1
	D-Alanine	A09	CAS338-69-2	1	1	1	1	1	1	1
	D-Serine	B01	CAS312-84-5	0	1	0	0	0	0	0
	L-Glutamic acid	B12	CAS6106-04-3	1	1	1	1	1	1	1
	L-Asparagine	D01	CAS70-47-3	1	1	1	1	1	1	1
	D-Aspartic acid	D02	CAS1783-96-6	0	0	0	0	0	0	0
	L-Glutamine	E01	CAS56-85-9	1	1	1	1	1	1	1
	Gly-Asp	F01		1	1	1	1	1	1	1
	D-Threonine	F04	CAS632-20-2	0	0	0	0	0	0	0
	Gly-Glu	G01	CAS7412-78-4	1	1	1	1	1	1	1
	L-Serine	G03	CAS56-45-1	1	1	1	1	1	1	1
	L-Threonine	G04	CAS72-19-5	1	1	1	1	1	1	1
	L-Alanine	G05	CAS56-41-7	0	1	1	1	1	1	1
	Ala-Gly	G06	CAS687-69-4	0	1	1	1	1	1	1
	Gly-Pro	H01	CAS704-15-4	1	1	1	1	1	1	1

Table 4.3, continued

Type of carbon	Carbon substrate	Well	CAS#	*tdh*⁻/*trh*⁻			*tdh*⁻/*trh*⁺		*tdh*⁺/*trh*⁻	
				ATCC19802	VP16	VP63	VP31	VP1314	3TBW10	ATCC43996
Carbohydrate	L-Arabinose	A02	CAS87-72-9	1	1	1	1	1	1	1
	N-Acetyl-D-Glucosamine	A03	CAS7512-17-6	1	1	1	1	1	1	1
	D-Galactose	A06	CAS59-23-4	1	1	1	1	1	1	1
	D-Trehalose	A10	CAS99-20-7	1	1	1	1	1	1	1
	D-Mannose	A11	CAS3458-28-4	1	1	1	1	1	1	1
	Dulcitol	A12	CAS608-66-2	0	0	0	0	0	1	1
	D-Sorbitol	B02	CAS50-70-4	0	0	0	0	0	0	0
	Glycerol	B03	CAS56-81-5	1	1	1	1	1	1	1
	L-Fucose	B04	CAS2438-80-4	0	0	0	0	0	0	0
	D,L-a-Glycerol Phosphate	B07	CAS3325-00-6	1	1	0	1	1	1	1
	D-Xylose	B08	CAS58-86-6	1	1	1	1	1	1	1
	D-Mannitol	B11	CAS69-65-8	1	1	1	1	1	1	1
	D-Glucose-6-Phosphate	C01	CAS3671-99-6	1	1	1	1	1	1	1
	D-Ribose	C04	CAS50-69-1	1	1	1	1	1	1	1
	L-Rhamnose	C06	CAS3615-41-6	0	0	0	0	0	0	0
	D-Fructose	C07	CAS57-48-7	1	1	1	1	1	1	1
	a-D-Glucose	C09	CAS50-99-7	1	1	1	1	1	1	1
	Maltose	C10	CAS69-79-4	1	1	1	1	1	1	1
	D-Melibiose	C11	CAS585-99-9	0	0	0	0	0	1	1
	Thymidine	C12	CAS50-89-5	1	1	1	1	1	1	1
	a-Methyl-D-Galactoside	D08	CAS3396-99-4	0	0	0	0	0	1	1

	a-D-Lactose	D09	CAS63-42-3	0	0	0	0	0	1	1
	Lactulose	D10	CAS4618-18-2	0	0	0	0	0	1	1
	Sucrose	D11	CAS57-50-1	0	1	0	0	0	1	1

Table 4.3, continued

Type of carbon	Carbon substrate	Well	CAS#	*tdh*$^-$/*trh*$^-$			*tdh*$^-$/*trh*$^+$		*tdh*$^+$/*trh*$^-$	
				ATCC19802	VP16	VP63	VP31	VP1314	3TBW10	ATCC43996
Carbohydrate	Uridine	D12	CAS58-96-8	1	1	1	1	1	1	1
	D-Glucose-1-Phosphate	E03	CAS56401-20-8	0	1	1	1	1	1	1
	D-Fructose-6-Phosphate	E04	CAS26177-86-6	1	1	1	1	1	1	1
	b-Methyl-D-Glucoside	E08	CAS709-50-2	1	1	1	0	0	1	1
	Adonitol	E09	CAS488-81-3	0	0	0	0	0	1	1
	Maltotriose	E10	CAS1109-28-0	1	1	1	1	1	1	1
	2`-Deoxyadenosine	E11	CAS16373-93-6	1	1	1	1	1	1	1
	Adenosine	E12	CAS58-61-7	1	1	1	1	1	1	1
	m-Inositol	F03	CAS87-89-8	0	0	0	0	0	0	0
	D-Cellobiose	F11	CAS528-50-7	0	0	1	1	1	1	0
	Inosine	F12	CAS58-63-9	1	1	1	1	1	1	1
	N-Acetyl-D-Mannosamine	G08	CAS7772-94-3	0	0	0	0	0	0	0
	D-Psicose	H05	CAS551-68-8	0	1	1	1	1	1	1
	L-Lyxose	H06	CAS1949-78-6	1	1	1	1	1	1	1
Carboxylic acid	D-Saccharic acid	A04	CAS576-42-1	0	0	0	0	0	0	0
	Succinic acid	A05	CAS6106-21-4	1	1	1	1	1	1	1
	D-Glucuronic acid	B05	CAS14984-34-0	0	1	1	0	0	1	1
	D-Gluconic acid	B06	CAS527-07-1	1	1	1	1	1	1	1
	L-Lactic acid	B09	CAS312-85-6	1	1	1	1	1	1	1
	Formic acid	B10	CAS141-53-7	0	1	1	1	1	1	1

	D-Galactonic acid-g-Lactone	C02	CAS2782-07-2	0	0	0	0	0	0	0
	D,L-Malic acid	C03	CAS6915-15-7	1	1	1	1	1	1	1
	Acetic acid	C08	CAS127-09-3	1	1	1	1	1	1	1
	D-Glucosaminic acid	D03	CAS3646-68-2	0	0	0	0	0	0	0
	a-Ketoglutaric acid	D06	CAS22202-68-2	1	1	1	1	1	1	1
	a-Ketobutyric acid	D07	CAS2013-26-5	0	1	1	1	1	1	1

Table 4.3, continued

Type of carbon	Carbon substrate	Well	CAS#	*tdh*$^-$/*trh*$^-$			*tdh*$^-$/*trh*$^+$		*tdh*$^+$/*trh*$^-$	
				ATCC19802	VP16	VP63	VP31	VP1314	3TBW10	ATCC43996
Carboxylic acid	m-Tartaric acid	E02	CAS147-73-9	0	0	0	0	0	0	0
	a-Hydroxyglutaric acid-g-Lactone	E06	CAS21461-84-7	0	1	1	1	1	1	1
	a-Hydroxybutyric acid	E07	CAS19054-57-0	0	1	1	1	1	1	1
	Citric acid	F02	CAS6132-04-3	1	0	1	1	1	1	0
	Fumaric acid	F05	CAS17013-01-3	1	1	1	1	1	1	1
	Bromosuccinic acid	F06	CAS923-06-8	0	1	1	0	0	1	1
	Propionic acid	F07	CAS137-40-6	0	0	1	1	1	1	1
	Mucic acid	F08	CAS526-99-8	0	0	0	0	0	0	0
	Glycolic acid	F09	CAS79-14-1	0	0	0	0	0	0	0
	Glyoxylic acid	F10	CAS563-96-2	0	0	0	0	0	0	0
	Tricarballylic acid	G02	CAS99-14-9	0	0	0	0	0	0	0
	Acetoacetic acid	G07	CAS3483-11-2	0	0	0	0	0	0	0
	Mono-Methylsuccinate	G09	CAS3878-55-5	0	1	1	0	0	1	1
	D-Malic acid	G11	CAS636-61-3	0	0	0	0	0	1	1
	L-Malic acid	G12	CAS138-09-0	1	1	1	1	1	1	1
	p-Hydroxyphenyl Acetic acid	H02	CAS156-38-7	0	0	0	0	0	0	0
	m-Hydroxyphenyl Acetic acid	H03	CAS621-37-4	0	0	0	0	0	0	0
	Pyruvic acid	H08	CAS113-24-6	1	1	1	1	1	1	1
	L-Galactonic acid-g-Lactone	H09	CAS1668-08-2	0	0	0	0	0	0	0
	D-Galacturonic acid	H10	CAS91510-62-2	0	1	0	0	0	0	0

Ester	Methylpyruvate	G10	CAS600-22-6	0	1	1	0	0	1	1
Fatty acid	Tween 20	C05	CAS9005-64-5	1	0	1	1	1	1	1
	Tween 40	D05	CAS9005-66-7	0	0	1	1	1	1	1
	Tween 80	E05	CAS9005-65-6	0	1	0	1	1	1	1

CHAPTER 5: DISCUSSION

5.1. Comparison of performance of the fourteen protocols in enumeration and detection of pathogenic *Vibrio parahaemolyticus* in frozen prawn samples

This recent work tried to evaluate the effect of enrichment and incubation temperatures on the performance of TCBS and CV for detection of pathogenic and non-pathogenic *V. parahaemolyticus* in frozen prawn samples, with the aim of developing an improved and more effective protocol for isolation of bacteria from frozen seafood.

There are numerous studies that compared the selectivity of TCBS and CV for isolation and detection of *V. parahaemolyticus* (Su et al., 2005; Blanco-Abad et al., 2009; Di Pinto et al., 2011; Canizalez-Roman et al., 2011; Yamazaki et al., 2011). CV had more discriminative results in most of studies compared to TCBS for detection of *V. parahaemolyticus* in clinical, environmental and food samples (Hara-Kudo et al., 2001; Su et al., 2005; Garcia et al., 2009; Di Pinto et al., 2011). Similarly, in our study, CV was more efficient and accurate than TCBS (Table 4.1) in terms of discrimination between *Vibrio* spp. and detection of pathogenic strains, however the resuscitation of injured and stressed *V. parahaemolyticus* from frozen seafood remained uncertain. In most studies, isolation and detection of *V. parahaemolyticus* using selective agar was carried out through a two-step enrichment prior to streaking onto selective media for example, samples are enriched with APW for 6 h or overnight for the recovery of *V. parahaemolyticus* isolates.

Based on the results obtained, the enrichment process with APW prior to culture on TCBS media improved the selectivity of this media and therefore decreased the false positive number of *V. parahaemolyticus* isolates on TCBS. In contrast, a two-step enrichment with APW did not significantly enhance CV function. Therefore,

performance of TCBS and CV were affected by the incubation temperature and enrichment process prior to isolation but the impact was stronger on TCBS agar, in which direct plating of homogenate frozen prawn sample onto TCBS without enrichment yielded more false-positive isolates than with enrichment prior to plating on TCBS (Table 4.1).

Differentiation on TCBS is not completely efficient. Some of the marine bacteria such as *Photobacterium* spp., *Chryseomonas* spp. and *Shewanella* spp. appear with the same characteristics and phenotypes with *V. parahaemolyticus* on TCBS (Blanco-Abad et al., 2009) Therefore, it is essential to provide an alkaline environment with APW to enrich the homogenate samples and increase the selectivity of TCBS media by inhibiting the growth of these competitive microflora and increase the number of alkalophilic bacteria such as *Vibrio* spp. in the sample.

In contrast, the use of enrichment steps prior to culture on CV did not increase the performance of this media. Since, the formulation of CV is highly specific and selective for *V. parahaemolyticus*, enrichment with APW prior to inoculation on this media made no difference in inhibiting competitive bacteria nor resulted in an increase of *V. parahaemolyticus* isolates.

From a total of 20 frozen prawn samples, virulent isolates carrying tdh^-/trh^+ *V. parahaemolyticus* were only detected from one sample using two protocols (D30CV and EL37CV) (Table 4.1). Three trh^+ genes were recovered from protocol D30CV and one trh^+ gene was identified from method EL37CV. The results of the present study indicate that CV performed as a better culture environment for resuscitation of pathogenic strains of *V. parahaemolyticus* in cold stress condition compared to TCBS. In fact, TCBS agar in this study presented an inhibitive effect on the recovery of pathogenic isolates. It is also reported by Raghunath and co-workers that some virulent strains of *V. parahaemolyticus* may not grow on TCBS (Raghunath et al., 2008).

This study has found that isolation of pathogenic *V. parahaemolyticus* (tdh^-/trh^+) in 30°C on CV was more efficient compared to isolation at higher incubation temperature such as 35°C and 37°C (Table 4.1). This is despite 30°C being considered to be a suboptimal temperature for *V. parahaemolyticus* isolates. This observation concurred with the studies by Lucht et al., (1998) and Abbis (1983). Lucht et al. (1983) showed that the suboptimal temperature could recover *E. coli* and *Salmonella* strains from irradiated food samples while Abbis (1983) showed that injured foodborne microorganisms could be resuscitated and had a higher survival rate when grown at suboptimal growth temperature. Lucht et al., (1998) suggested that the lower temperature reduces the pressure on the cell to divide and replicate. This allows resources to be directed towards repairing cells that are damaged during stress conditions.

Also, enrichment steps could increase the number of colonies of other *Vibrio* species as well as the already larger population of non-pathogenic *V. parahaemolyticus* than pathogenic *V. parahaemolyticus* and thus mask the isolation of pathogenic strains. Nonetheless, the low prevalence and low number of pathogenic *V. parahaemolyticus* in frozen prawn samples tested in this study has restricted the conclusion that direct-plating on CV with 30°C incubation temperature offered better chances to detect pathogenic *V. parahaemolyticus*. Yet, culturability of pathogenic isolates under stress and injury remained complicated and unclear. Hence, there is an extensive need for more discovery and findings regarding their metabolic activity and physiological characteristics.

Accordingly, Jiang and Chai (1996) reported that in low temperature starvation, morphologic changes from rods to spheres occurred in Kanagawa positive isolates of *V. parahaemolyticus*. These strains lost their culturability faster than Kanagawa negative

isolates. As a result, virulent strains of *V. parahaemolyticus* could display different physiological and metabolic characteristics compared to nonvirulent strains.

It is common to assume that foodborne pathogens sustain different degrees of sublethal injury when exposed to heat, refrigeration and freezing. However, although ice crystal formation during freezing causes physical damage to bacterial cells, freezing is considered the least "effective" inactivation approach compared to refrigeration, starvation and heating (Wesche et al., 2009). Many constituents of food, such as certain sugars, peptide and proteins in prawns, could act as cryoprotectants to *V. parahaemolyticus* (Mackey, 1984). Therefore, the necessity for an enrichment procedure prior to isolation to repair, resuscitate and increase the number of stressed or injured cells in frozen prawn needs to be reconsidered. Our findings clearly demonstrated that direct-plating on CV and TCBS yielded almost comparable count of *V. parahaemolyticus* with the MPN-PCR method (Figure 4.1). According to the results in Figure 4.1, 37°C proved to be the most suitable growth temperature as it yielded the highest *V. parahaemolyticus* counts from direct-plating of frozen prawn samples on CV. At time of writing, there is no available data or similar work that allows for comparison.

Although both CV and TCBS support the growth of *V. parahaemolyticus* and have both been used intensively for isolation of *V. parahaemolyticus* in foods, the chemicals and nutrients used in both media to suppress background microflora and select *Vibrio* spp. are different (Kobayashi et al., 1963; Hara-Kudo et al., 2001; Di Pinto et al., 2011). TCBS agar contains sodium thiosulfate and sodium citrate to inhibit Enterobacteriaceae; bile salts inhibit most Gram-positive bacteria in foods; while its alkaline condition and sucrose enhance the growth of *Vibrio* spp. On the other hand, although CV agar has an alkaline pH, it uses chromogenic compounds as selective indicators and does not contain sucrose to enrich *Vibrio* spp. These variations could support the growth of a different subset of *V. parahaemolyticus* present in food samples. To investigate this

postulation, this study typed all the *V. parahaemolyticus* isolates recovered from a single frozen prawn sample using various protocols with REP-PCR to compare genetic diversity. From this, it was found that *V. parahaemolyticus* isolates obtained from CV demonstrated more diverse genetic profiles than those isolated from TCBS (Figure 4.2, b and c), which means that TCBS could be inhibitive to certain strains of *V. parahaemolyticus*. The clustering analysis of REP-PCR profiles revealed that grouping of *V. parahaemolyticus* isolates was affected by the type of isolation media used in this study. The findings suggested that different protocols and isolating media used might be only selective for a subset of *V. parahaemolyticus* population present in the samples. Genetic profiling of strains obtained using different protocols could introduce bias in analysis and needed careful interpretation.

5.2. Growth of *tdh*$^-$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ strains of *V. parahaemolyticus* in different NaCl concentration

The six *V. parahaemolyticus* strains included in this study showed that the optimum NaCl concentration for growth were about 2-3% (Figure 4.3). It is generally known that *V. parahaemolyticus* is a moderate halophile and requires a minimum of 0.5% of NaCl for growth (Palasuntheram, 1981). In this study, none of the strains were able to grow at 10% NaCl, which is substantiated by previous studies that have reported *V. parahaemolyticus* growth in salt conditions between 1 to 9% NaCl (Whitaker et al., 2010). Although there were no obvious differences observed between both groups of *tdh*$^-$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ strains in growth response toward NaCl concentration, it is noteworthy to point out that the growth response among the *tdh*$^-$/*trh*$^+$ strains were more stable and similar as compared to the growth response among the three *tdh*$^-$/*trh*$^-$ strains, in which VP63 and VP12 had the lowest growth rates at 5% NaCl (Figure 4.3). While

this different growth response toward different NaCl concentrations could be solely a strain specific response, the differences could indicate that *tdh*$^-$/*trh*$^+$ strains are more tolerable to changes in NaCl concentration and therefore survive better in the environment and human host compared to the non-pathogenic strains. *V. parahaemolyticus*, as a marine bacterium, needs to be able to adapt to changes in salinity for survival in brackish water (0.5% to less than 3.5%), off-shore water (>3.5% salinity) and within oysters. As such, an optimum NaCl concentration of around 2 - 3% would be expected for strains isolated from these environments. Other than that, pathogenic *V. parahaemolyticus* is also able to survive in human hosts that has low pH and salinity. Therefore, tolerance to a wide range of NaCl concentration might offer better adaptability for survival particularly to pathogenic strains. Nonetheless, more study is needed to clarify this speculation.

5.3. Variable carbon utilization among *tdh*$^-$/*trh*$^-$, *tdh*$^+$/*trh*$^-$ and *tdh*$^-$/*trh*$^+$ strains of *Vibrio parahaemolyticus*

In this study, the carbon utilization capacity was variable among the strains tested. The *tdh*$^+$/*trh*$^-$ strains were found to be most efficient in catabolizing carbohydrates, ranged from 84% to 87% of carbohydrates tested, which was relatively higher compared to the other strains (ranged from 61% to 68%). Both *tdh*$^+$/*trh*$^-$ strains demonstrated the highest capacity in up taking and using different carbon substrates as sole carbon sources for growth, suggesting that this genotype (*tdh*$^+$/*trh*$^-$) could have added advantage for survival in various niches in nature, and marine and human hosts. On the other hand, both genotypes (*tdh*$^-$/*trh*$^+$ and *tdh*$^-$/*trh*$^-$) had indifferent carbon utilization capacity that ranged from 47% to 63% (Table 4.2). Based on the PM data analysis, the *V. parahaemolyticus* strains with the *tdh*$^-$/*trh*$^+$ genotype utilized a lesser number of carbon

substrates as compared to strains with the *tdh*$^{+}$/*trh*$^{-}$ genotype. This implies that its requirement for growth was lesser. This could explain why strains with the *tdh*$^{-}$/*trh*$^{+}$ genotype could grow better than the *tdh*$^{+}$/*trh*$^{-}$ strains. In our isolation of virulent *V. parahaemolyticus* from frozen prawn samples, the higher prevalence of *tdh*$^{-}$/*trh*$^{+}$ could be due to *tdh*$^{-}$/*trh*$^{+}$ *V. parahaemolyticus* having a higher occurrence in seawater and prawns from this region (Table 4.1). It is also noteworthy to point out that only pathogenic *V. parahaemolyticus* (*tdh*$^{+}$/*trh*$^{-}$ and *tdh*$^{-}$/*trh*$^{+}$ strain) were able to grow on all the fatty acids tested (Tween 20, 40 and 80) again suggesting added advantage to survive in gastrointestinal tract that has high concentration of fatty acids.

Knowledge on variable capacity to use different carbon sources for growth among *tdh*$^{-}$/*trh*$^{-}$, *tdh*$^{+}$/*trh*$^{-}$ and *tdh*$^{-}$/*trh*$^{+}$ strains of *V. parahaemolyticus* could be explored further to assess the effect of substituting different carbon substrates in bacteriological media to enhance detection and isolation of pathogenic *V. parahaemolyticus*, as well as in the selectivity for specific pathogenic strains and rapid differentiation of pathogenic strain from non-pathogenic strains of *V. parahaemolyticus*.

CHAPTER 6: CONCLUSION

In conclusion, findings from this study supported previous studies that CHROMagar™Vibrio (CV) performed better than Thiosulphate Citrate Bile Sucrose (TCBS) in foodborne *V. parahaemolyticus* detection in terms of its ability to distinguish *V. parahaemolyticus* from other bacteria. It was also less inhibitive to *V. parahaemolyticus* and had a higher recovery rate for pathogenic strains. This study suggested that enumeration of *V. parahaemolyticus* in frozen prawns could be conducted via direct-plating on CV, which significantly reduces the testing time and cost. Nonetheless, more work is required to further investigate and improve the recovery of pathogenic *V. parahaemolyticus* in seafood.

The result of REP-PCR genotyping of *V. parahaemolyticus* isolates obtained from one single frozen prawn sample using different protocols suggested that the genotyping profiles of isolates using different isolation protocols might not be accurate for comparison as different protocols might have different selectivity towards strains with certain characteristics in term of phenotypic and genotypic characteristics. This study also suggested that TCBS could be inhibitive to certain strains of *V. parahaemolyticus.*

Although this study did not find the growth response towards different NaCl concentration to be significantly different between tdh^-/trh^- and tdh^-/trh^+ strains of *V. parahaemolyticus*, the slight variation in the growth pattern could indicate that the tdh^-/trh^+ strains are more tolerable to changes in salinity and therefore survive better in broader niches. This speculation was again supported by the results obtained from phenotype microarray assay used in this study, in which the pathogenic strains (tdh^-/trh^+ and tdh^+/trh^-) were able to use more carbon substrates than the non-pathogenic strains. The findings from the phenotype microarray also provides a starting point for future

works to improve the performance of bacteriological media for the detection and isolation of pathogenic *V. parahaemolyticus*.

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