CHARACTERISATION OF ESBL-PRODUCING ENTEROBACTERIACEAE OF FOUR LOCAL VEGETABLES

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CHARACTERISATION OF ESBL-PRODUCING ENTEROBACTERIACEAE OF FOUR LOCAL VEGETABLES

By

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I would like to dedicate my thesis to my beloved Mother.

Thanks, Mom.

ABSTRACT

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Toh Wai Keat

The outbreak associated with ESBL-producer is well-documented worldwide and the number of cases is on the rise. The purpose of this study was to PCR amplify and characterise ESBL homologous genes, *bla*-CTX-M and *bla*-PER-2 genes from the ESBL-producing Enterobacteriaceae. Bacterial isolates with different morphological characteristics were isolated from tomato (*Lycopersion esculentum*), cucumber (*Cucumis sativus*), celery (*Apium graveolens*) and broccoli (*Brassica oleracea*) using MacConkey agar supplemented with 50 μg/ml ampicillin. Following isolation of bacteria, several preliminary biochemical tests were carried out. Twenty five bacteria isolates were shown to be Gram-negative, catalase positive, oxidase negative and facultative anaerobes, fulfilling the preliminary criteria for being an Enterobacteriaceae. Based on the antibiotic susceptible test, nine bacterial isolates were observed to be resistant towards multiple antibiotics. A potential ESBL-producer (TM P2) was identified with resistant towards antibiotics classified as penicillin, monobactam, 3rd generation cephalosporins and showed a positive phenotypic test result via combined disc

methodology. Biochemical characterisation using API 20E demonstrated the presumptive identification of TM P2 and CE B2 as being *Enterobacter cloacae* and *Klebsiella pneumonia ssp pneumonia*, respectively. PCR amplification using *bla*-CTX-M degenerate primers was performed and a DNA fragment corresponding to the expected size of about 590 bp was obtained from TM P2. BlastX alignment search showed a gene fragment which had a high similarity to metabolite-proton symporter of *Enterobacter cloacae* instead of desired *bla*-CTX-M gene fragment. This observation might be due to the use of unspecific degenerate primers and inappropriate PCR condition. On the other hand, no *bla*-PER-2 homologous genes were successfully amplified using *bla*-PER-2 specific primers. For future studies, the only ESBL-producer (TM P2) obtained in this study will be further characterised using other ESBLs gene specific primers.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UTAR or other institutions.

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APPROVAL SHEET

This project report entitled "**CHARACTERISATION OF ESBL-PRODUCING ENTEROBACTERIACEAE OF FOUR LOCAL VEGETABLES**" was prepared by TOH WAI KEAT and submitted as partial fulfilment of the requirement for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

I hereby certified that **TOH WAI KEAT** (ID No: **08ADB04004**) has completed this final year project entitled "**CHARACTERISATION OF ESBL-PRODUCING ENTEROBACTERIACEAE OF FOUR LOCAL VEGETABLES**" supervised by Asst. Prof. Dr. Choo Quok Cheong (Supervisor) _____ (Co-supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisors to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	Arginine Dihydrolase
AMY	Amygdalin
ARA	Arabinose
BHIB	Brain Heart Infusion Broth
BPW	Buffered Peptone Water
$CaCl_2$	Calcium Chloride
CIT	Citrate
ESBLs	Extended-spectrum beta-lactamases
GEL	Gelatinase
GLU	Glucose
H_2S	Hydrogen Sulfide
IND	Indole
INO	Inositol
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
LDC	Lysine Decarboxylase
MAC	MacConkey Agar
MAN	Mannitol
$MgCl_2$	Magnesium Chloride
MH	Muller Hinton
MOB	Mobility

N_2	Nitrogen
NO_2	Nitrous Oxide
ODC	Ornithine Decarboxylase
O-F	Oxidation-Fermentation
ONPG	O-NitroPhenyl-β-D-Galactopyranosidase
PCR	Polymerase Chain Reaction
psi	Pound-force per Square Inch
RHA	Rhamnose
SAC	Saccharose
SOR	Sorbitol
TDA	Tryptophane Deaminase
URE	Urease
VP	Voges Proskauer
VRBG	Violet Red Bile with Glucose
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Foodborne diseases caused by microbes are a widespread and growing public health problem worldwide. According to a report by World Health Organization (WHO) in 2004, a large number of people are affected by discomforts and diseases due to the consumption of contaminated food every year. Based on the new estimates from the Centers for Disease Control and Prevention (CDC), about 48 million people get sick, 128,000 are hospitalised and 3,000 die each year from foodborne disease caused by major pathogens and unspecified agents in America (Scallan, Griffin, Angulo, Tauxe & Hoekstra, 2011a; Scallan et al., 2011b).

Raw vegetables have been shown to contribute to vegetable-borne outbreaks for at least a century (Beuchat, 1996). Several factors are believed to be the potential causes that contributed to the microbial contaminations of vegetables, including contaminated soil amendments, fertilizers, water used in horticulture, processes of harvesting, transporting, further processing and handling the vegetables (Food and Agriculture Organization of the United Nation/World Health Organization, 2008).

Bacteria that cause infections are extremely resilient and they have developed ways to resist antibiotics due to the increased usages and often misuses of existing antibiotics, both in human and agriculture (Todar, 2008a). A high level of

antibiotic resistance is often related to the member of the Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae (Johnson, Kuskowski, Smith, O'bryan & Tatini, 2005), the largest group of medically important Gram-negative, catalase positive, oxidase negative and facultative anaerobic bacteria (Health Protection Agency, 2010).

ESBL-producing Enterobacteriaceae have been related to most of the recently documented foodborne outbreaks which are associated with microbial contamination. For instance, in 2006, DeNoon reported cases of *Escherichia coli* serotype 0157:H7 food poisoning with associated to the consumption of fresh-cut spinach involving about 200 victims, inclusive of three dead persons. In 2008, a *Salmonella* outbreak in certain raw, red tomatoes had occurred and infected at least 167 people, including 23 people being hospitalised in 17 states of the United States (Hitti, 2008).

ESBL-producing Enterobacteriaceae might become a major threat to human health as the infections caused by these bacteria have increasingly been reported worldwide in the last decade (Rodriguez-Villalobos et al., 2010). Professor Christian Ruef pointed out that as ESBL-producing Enterobacteriaceae has the ability to inactivate almost entire of the beta lactam antibiotics, infections can only be treated via injections or infusions due to their increased toxicity. In other word, reserved antibiotics which are available outside of hospitals may no longer be able to be used to treat the infection (Laarmann, 2010). Without a proper

antibacterial therapy to cure these infections, it may result in a high rate of patient mortality worldwide (Pitout, 2010).

In this study, four different types of vegetable that are often consumed raw and having a high prevalence of foodborne diseases linked to the consumption were selected. The objectives of this study include:

- To determine the antibiotic sensitivity of Enterobacteriaceae cultivated from the four selected raw vegetables.
- To screen and confirm the presence of potential ESBL-producing bacteria among the cultivated Enterobacteriaceae.
- To characterise the potential ESBL-producing Enterobacteriaceae by using several preliminary biochemical tests and API 20E kit.
- To amplify *bla*-CTX-M and *bla*-PER-2 homologous genes from Enterobacteriaceae.
- To determine the presence of *bla*-CTX-M and *bla*-PER-2 homologous genes using DNA sequencing methodology.

CHAPTER 2

LITERATURE REVIEW

2.1 Vegetables Consumption and Its Health Issues

The World Health Organization (WHO) recommends the consumption of at least five 100 g portions of fruits and vegetables daily to stay healthy. According to WHO (2002), a diet of low fruit and vegetable intake is contribute to about 31% of ischaemic heart disease and 11% of stroke worldwide.

Having diets rich in fruits and vegetables is vital for health as they are packed with various types of phytochemical such as flavonoids, alkaloids and terpenes, just to name a few (Tyagi, Singh, Sharma & Aggarwal, 2010). Consuming vegetables that contain high level of phytochemicals with antioxidant property may provide a better protection against metabolic dysfunction, gastro-duodenal pathogenesis, premature aging, inflammation, rheumatoid arthritis, atherosclerosis, cancer and neuro-degeneration (Prakash & Gupta, 2009). Regular consumptions on vegetables may also reduce the risk of getting eye-related diseases such as cataract and age-related macular degeneration due to the presence of lutein and zeatin (Rajasekaran, Sivagnanam & Xavier, 2008). Lastly, the indigestible fiber which is widely available both in fruits and vegetables can trigger the regular bowel movement and thus constipation can be relieved (Lembo & Camilleri, 2003).

As phytochemicals may be destroyed by processing techniques or heating, it is advised to consume vegetables raw in order to retain the bioavailability of phytochemicals (Tyagi et al., 2010). Nowadays, raw vegetables have been habitually consumed due to expectation of improved food functionalities (Isshiki, 2007)

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2.2 Emergence of Foodborne Illnesses

A foodborne disease outbreak can be defined as an incident which two or more persons experience a similar illness, usually gastrointestinal, after ingestion of contaminated food and epidemiological analysis implicates the food as the source of the illness (Boyd, 1995). It may occur as apparently sporadic cases, as small clusters of cases, or as point source outbreaks which may be small or large and may last from hours to months.

Between the years of 1990 and 2005 in the United State, contaminated vegetables had contributed towards 260 outbreaks out of a total of 5,316 outbreaks of illnesses related to specific food (DeWaal & Bhuiya, 2007). Thus, although the chance of contracting a foodborne disease via consumption of raw vegetables is low, there are still risks available (Sapers, Gorny & Yousef, 2006) due to the surfaces of raw vegetables often carry a large number of bacteria as bacteria are able to colonise the nooks, the crannies or even the pores of the vegetables (Food Safety Net, 2010).

To date, the world's largest reported vegetable-borne outbreak occurred in Japan in 1996 where over 11,000 people were affected and involved the death of three children. Furthermore, more than 6000 cases were culture-confirmed as *Escherichia coli* serotype O157:H7 infection which was associated with radish sprouts (Ministry of Health and Welfare of Japan, 1997).

2.2.1 Infections and Pathogenicity of Gram-negative Bacteria

The pathogenicity found in many species of Gram-negative Enterobacteriaceae is often related to the lipopolysaccharide (LPS) layer of the Gram-negative bacterial cell envelope. Studies indicated that the presence of endotoxin properties residing largely in the lipid portion of LPS, called lipid A. They are responsible for the toxicity whereas the carbohydrate portion of LPS, the core polysaccharide and the O-specific polysaccharide, are responsible for the immunogenicity (Madigan, Martinko, Dunlap & Clark, 2009).

The physiological activities of LPS are mediated mainly by lipid A. Endotoxins are released from the bacterial cell envelope and greatly contribute to the symptoms and pathologies of the diseases encountered. They include fever, diarrhea, inflammation or even death which may occur in the presence of large doses of endotoxin (Todar, 2008b; Todar, 2008c; Madigan et al., 2009). For instance, *Escherichia coli* secrete endotoxins, called enterotoxins, which activate adenylate cyclase within the cells of the small intestine, and cause diarrhea (Levinson & Jawertz, 1992).

As for the polysaccharide side chains of LPS, their involvements in the expressions of virulence, possibly due to the O-specific polysaccharide, could allow organisms to adhere specifically to certain tissues, and allow resistance to antibodies and phagocytes. Also, the hydrophilic O-specific polysaccharide could act as water-solubilising carriers for endotoxins (Todar, 2008b).

2.2.2 Enterobacteriaceae

The members of the family Enterobacteriaceae are commonly known as enterics as they inhabit the intestinal tracts of human and other animals (Tortora, Funke & Case, 2010). Enterics can be divided into three groups depending on the major anatomic location of disease, such as pathogens found both within and outside the enteric tract like *Escherichia coli* and *Salmonella;* pathogens mainly found within the enteric tract like *Shigella*; and pathogens found outside the enteric tract like *Klebsiella*, *Enterobacter*, *Serratia* and *Proteus* (Levinson & Jawertz, 1992).

Enterics have fimbriae which enable them adhere to surfaces or mucous membranes. The presence of specialized sex pili aids in the exchange of genetic information, for example the antibiotic resistance factor (R factors), between the same or different families of the enteric. Moreover, they also produce protein called bacteriocins which aid in maintaining the ecological balance of various enteric in intestines (Boyd, 1995; Tortora et al., 2010).

Moreover, enterics are all facultative anaerobes with no cytochrome oxidase and possess catalase enzymes except for *Shigella dysenteriae* type 1 (Health Protection Agency, 2010). A battery of biochemical tests has been specially designed for the identification of Enterobacteriaceae such as citrate utilization (CIT) test, Voges-Proskauer (VP) test and gelatin (GEL) test, will differentiate all of the pathogenic Enterobacteriacaea. Nowadays, commercial identification kits like API 20E kit are used to identify Enterobacteriaceae instead of using conventional methods to avoid time consuming and labor intensive (Boyd, 1995).

Research showed that a wide range of Enterobacteriaceae such as *Salmonella*, *Shigella*, and *Escherichia coli* are able to contribute to the occurrence of foodborne diseases as their toxins can be transmitted via food (Scientific Committee on Food, 2002). They reside mainly in the intestinal tracts of animals, and are more likely to contaminate raw fruits and vegetables through contact with feces, sewage, untreated irrigation water or surface water (Tambekar & Mundhada, 2006).

2.3 Antimicrobial Agents

Antimicrobial agents are chemical compounds used in medical intervention in an infection, to actively inhibit or kill a pathogen (Forbes, Sahm & Weissfeld, 2002). Generally, antimicrobial agents can be divided into two major categories. Chemotherapeutic agents, which are chemically synthesized is the first group and

the second major group will be the antibiotics, a substances produced by certain groups of microorganisms to treat certain bacterial infections (Todar, 2009).

Antimicrobial agents can be classified as either bactericidal or bacteriostatic drug. A bactericidal drug kills bacteria whereas a bacteriostatic drug inhibits the growth of bacteria but does not kill it. Thus, the use of bactericidal drug, sometimes, is more essential compared to bacteriostatic drug in clinical situations for curing purposes (Levinson & Jawertz, 1992) as it allows rapid elimination of bacteria and a decreased possibility of resistance development or infection recurrence (Finberg et al., 2004).

2.3.1 Antibiotics as Antimicrobial Agents

Antimicrobial chemotherapy began with Fleming's discovery of penicillin in 1929, and the discovery of sulfonamides as a chemotherapeutic agent by Domagk in 1935. Up to date, several hundreds of antibiotics have been isolated from microorganisms over the years. Only a few of them with selective toxicity such as beta lactam antibiotic, streptomycin, just to name a new are clinically useful as they have minimal or no toxicity to human (Todar, 2009).

2.3.1.1 Spectrum of Antimicrobial Activity of Antibiotics

Bacterial spectrum can be defined as the range of bacteria that can be affected by a particular antibiotic. Some antibiotics, for example tetracycline, can be used to treat a wide range of bacteria and thus known as broad-spectrum antibiotic (Black,

1996). Others which can only inhibit selected group of bacteria, for example penicillin G, are called narrow-spectrum antibiotics (Tortora et al., 2010).

2.3.1.2 Mechanisms of Action of Antibiotics

A total of five mechanisms are used to explain the actions of antibiotics. The five mechanisms mentioned include the inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, metabolite synthesis and lastly the alternation of cell membrane (Madigan et al., 2009).

All beta lactam antibiotics such as penicillins, cephalosporins, carbapenems and monobactams are inhibitors of cell wall synthesis. In general, beta lactam drugs will interfere with the final linking of the peptidoglycan rows by inhibiting transpeptidases, results in degradation of peptidoglycan and osmotic rupture of bacterial cells (Levinson & Jawertz, 1992).

Protein inhibitor antiobiotics such as tetracyclines and aminoglycosides act on ribosomal 30S subunit by blocking tRNA binding to ribosomes, thus interrupting the function of initiation complex and causing misreading of mRNA, respectively. Meanwhile, chloramphenicols, erythromycin and clindamycin act on ribosomal 50S subunit by blocking peptidyltransferase step of protein synthesis, translocation and the formation of peptide bond respectively (Levinson & Jawertz, 1992; Katzung, 1998). Due to the differences between bacterial and human

ribosomal proteins, RNAs and associated enzymes, protein inhibitor antibiotics do not interfere with the synthesis of proteins in human (Levinson & Jawertz, 1992). Next, both sulfonamides and trimethoprims serve as inhibitors of metabolite precursor synthesis (Madigan et al., 2009) as they are competitive inhibitors of *para*-aminobenzoic (PABA) and dihydrofolate reductase, respectively. Drugs mentioned will disrupt the synthesis of folate, which is an essential coenzyme in its reduced form for nucleic acid and protein synthesis (Ryan, 1994).

The inhibition of nucleic acid includes inhibition of DNA or RNA synthesis. Quinolones are bactericidal drugs that block bacterial DNA synthesis and replication by inhibiting DNA gyrase which is the enzyme responsible for supercoiling, nicking and sealing bacterial DNA (Ryan, 1994). On the other hand, the selective mode of action of rifampins, a type of drugs to cure tuberculosis caused by *Mycobacterium tuberculosis*, is based on blocking mRNA synthesis by bacterial RNA polymerase (Levinson & Jawertz, 1992).

Lastly, polymyxin B and colistemethate, also known as polymyxin E, are known for causing disorganization of the cell membrane (Baron, 1996). The positively-charged free amino acid groups contained in polymyxins serve as cationic detergents and thus able to disrupt the phospholipid structure of the cell membrane (Levinson & Jawertz, 1992).

2.4 Beta Lactam Drugs

The activities of beta lactam drugs are either bactericidal or bacteriostatic, greatly depending on the types of drug and the bacterial species or strains (Satta et al., 1995). Studies indicate that the bactericidal effect of beta lactam drugs is mainly related to the unregulated activities of autolysins (Handwerger & Tomasz, 1985) while the bacteriostatic effect is depending on the inactivation of the enzymes which are involved in the late stage of peptidoglycan synthesis (Spratt, 1975; Waxman & Strominger, 1983).

There are four major groups in beta lactam antibiotics, include penicillins, cephalosporins, carbapenems and monobactams. Each group of the drugs contains a beta lactam ring which is linked to different additional ring except for monobactams. For instance, an additional thiazolindine ring for penicillins, cephem nucleus for cephalosporin, a double ring structure for carbapenems while none for monobactams (Samaha-Kfoury & Araj, 2003). This property helps in differentiating which groups the beta lactam antibiotic belongs to.

2.4.1 Penicillins

Penicillin is one of the earliest discovered and widely used antibiotic agents, derived from the *Penicillium* mold by Fleming in 1929 and being produced from cultures of *Penicillium notatum* in 1940 by Chain, Florey and their associates (Katzung, 1998). Penicillin is used mainly to treat bacterial infections in the past and thus, most bacteria are resistant to penicillin.

As one of the member of beta lactam drugs, penicillin has a basic structure of a beta lactam ring linked to a thiazolidine ring (Ryan, 1994). In general, it is a bactericidal drug which kills bacteria by inhibiting the synthesis of cell wall (Levinson & Jawertz, 1992).

2.4.2 Cephalosporins

Cephalosporins were isolated from *Cephalosporium* molds. They are similar to penicillin chemically, in mode of action, and toxicity, but exist differently in structures (Levinson & Jawertz, 1992).

Cephalosporins can be classified into four major generations, which are the 1st generation, 2nd generation, 3rd generation and 4th generation, merely depending on their spectrums of antimicrobial activity. Generally, 1st generation cephalosporins have better activity against Gram-positive bacteria whereas the later generation of cephalosporins shows improved activity against Gram-negative aerobic bacteria (Katzung, 1998).

2.4.3 Beta-lactamase Inhibitors

A combination of a beta-lactamase inhibitor with a substrate beta lactam drug provides one strategy to overcome beta-lactamase-mediated resistance (Livermore, 1993). Beta-lactamase inhibitors include clavulanic acid, sulbactam and tazobactam, have little or no antimicrobial activity but they are potent inhibitors

of many (but not all) beta-lactamases and thus they can protect the beta lactams drugs from inactivation by the enzymes mentioned (Katzung, 1998).

Both clavulanic acid and sulbactam are highly effective against staphylococcal penicillinase and TEM type beta-lactamases but it showed lesser efficiency in inhibiting cephalosporinase (Ryan, 1994). Meanwhile, tazobactam has moderate activity against some class I chromosomally-mediated beta-lactamases, especially that of *Morganella morganii*, but not that of *Enterobacter cloacae* (Livermore, 1993). As for inhibiting CTX-M type beta-lactamses, these enzymes are inhibited better by the tazobactam compared to others like sulbactam and clavulanaic acid (Bradford et al., 1998; Tzouvelekis, Tzelepi, Tassios & Legakis, 2000).

2.5 Development of Antibiotic Resistance

Use of antibiotics is critical in treating bacterial infections. Years of misuse and overuse of these antibiotics have fueled a major increase in prevalence of multidrug resistance pathogens (Katzung, 1998). Any kind of antimicrobial used, be it for human, animal or plant health purposes, can lead to emergence of resistance and further promote the dissemination of resistant bacteria and resistance genes (Newell et al., 2010).

Plasmids harboring multiple antimicrobial-resistance determinants (R plasmids) can be readily transferred horizontally between bacteria from animals, human and food (Kruse & Sorum, 1994). When the R plasmids were discovered in Japan in

the 1950s, many of them already contained resistance genes for aminoglycosides, tetracycline, chloramphenicol, and sulfonamides. Thus, multidrug resistance can be transferred to a susceptible bacterium in a single conjugation event. Moreover, R plasmids are stably maintained and usually transferred between bacterial cells at a very high efficiency (Nikaido, 2009).

To date, the most significance in development of antibiotic resistance involves the evolution of beta-lactamases which results in limited antibiotics option can be used in therapeutic treatment (Paterson & Bonomo, 2005).

2.5.1 Classification of Beta-lactamases

Beta-lactamases are any of various enzymes that are produced by Gram-negative bacteria and hydrolyse beta lactam rings, thereby able to inactivate penicillin and cephalosporin antibiotics. They are most commonly being classified according to two general schemes, the Ambler molecular classification scheme (Ambler, 1980) and the Bush-Jacoby-Medeiros functional classification system (Bush, Jacoby & Medeiros, 1995). The Ambler scheme divides beta-lactamases into four major classes, namely class A to D according to the protein homology but not according to phenotypic characteristics (Ambler, 1980). Later on, Bush-Jacoby-Medeiros classification has been developed in 1995 to integrate the functional molecular characteristics of beta-lactamases.

Class A enzymes consist of a large family, including staphylococcal penicillinases, TEM, SHV, CTX-M and ESBLs. They are found in Gram-negative as well as Gram-positive bacteria while the other three classes are mainly found in Enterbacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp. (Alba et al., 2002). TEM and SHV of class A enzymes are the forerunners of most of the ESBLs which arise from the enzymes due to point mutation (Queener, Webber & Queener, 1986).

Compared to the other three classes of beta-lactamases which consist of serine enzymes, class B enzymes are consisting of zinc-metallo enzymes (Bunyak, 2006). This class of enzymes preferentially inhibits cephalosporins (Queener et al.,1986) yet they are inhibited by ethylenediamine tetraacetic acid (EDTA), 2, 6-pyridinedicarboxylic acid, succinic acid and mercaptocarboxylate (Alba et al., 2002).

Class C enzymes encompass the chromosomal AmpC cephalosporinases (Livermore, 1998). AmpC beta-lactamases which are found naturally on chromosomes of *Enterobacter* spp. and *Citrobacter* spp. can be readily spread to *Escherichia coli* and *Klebsiella* spp., causing these isolates to be resistant to the oxyimino-cephalosporins, cefoxitin, cefotetan and beta-lactamase inhibitor but not to carbapenems (Kader & Nasimuzzaman, 2001).

Lastly, class D enzymes, also known as the OXA class enzymes, are scattered in Enterobacteriaceae as plasmid-borne types (Livermore, 1998). All class D enzymes significantly hydrolyze amino and carboxypenicillins but inactivate cloxacillin and oxacillin poorly (Poirel, Naas & Nordmann, 2010).

For Bush-Jacoby-Medeiros classification, it puts 178 beta-lactamases into four groups based on the inhibitor and substrate profiles (Bush et al., 1995; Chaudhary & Aggarwal, 2004). Thus, this classification scheme is of much immediate relevance to the physician or microbiologist in a diagnostic laboratory (Paterson & Bonomo, 2005).

2.5.2 The Extended-spectrum Beta-lactamases (ESBLs)

Extended-spectrum beta-lactamases (ESBLs) are a rapidly evolving group of beta-lactamase. This extends the spectrum of beta-lactam antibiotics susceptible to hydrolysis by these enzymes. The ESBLs are usually plasmid encoded and these plasmids responsible for ESBLs production frequently carry genes encoding resistance to other drug classes. Hence, only limited antibiotic can be used in the treatment of infections caused by ESBL-producing organisms (Paterson & Bonomo, 2005).

ESBLs were first observed in 1983 in isolates of *Klebsiella ozaenae* in Germany (Paterson & Bonomo, 2005). Thereafter, a majority of cases reported in the first decade after the discovery of ESBL were mainly from France (Philippon, Labia, & Jacoby, 1989).

A commonly used working definition is that the ESBL is beta-lactamase belonging to group 2be in the Bush-Medeiros-Jacoby system and to class A in the Ambler system. They are capable of conferring bacterial resistance to the penicillins, 1st generation, 2nd generation, and 3rd generation cephalosporins, and monobactam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by beta-lactamase inhibitors (Paterson & Bonomo, 2005).

Today, over 150 different ESBLs have been described. Most of them are derivatives of TEM or SHV enzymes due to the points mutation (Gly238→Ser, Glu240→Lys, Arg164→Ser, Arg164→His, Asp179→Asn, and Glu[Asp]104→Lys) within plasmid or transposon genes encoding TEM and SHV type beta-lactamases (Perez, Endimiani, Hujer & Bonomo, 2007; Ryan, 1994).

2.5.2.1 CTX-M Type ESBLs

An increasing number of non-TEM or SHV ESBL lineages have recently been described. For instance, CTX-M beta-lactamases is a new family in class A ESBLs, characterised at the beginning of the 1990s in the first reports of MEN-1 (CTX-M-1) enzyme (Barthelemy, Peduzzi, Bernard, Tancrede & Labia, 1992).

Based on the changes in amino acid sequences identities, CTX-M beta-lactamases are divided into five clusters, CTX-M 1 branch, CTX-M 2 branch, CTX-M 8 branch, CTX-M 9 branch and lastly, CTX-M 25 branch (Naas, Oxacelay &

Nordmann, 2007). CTX-M enzymes probably originated by horizontal gene transfer and subsequent mutation, from the chromosomal AmpC beta-lactamases of *Klyuvera ascorbata*, which is 99% homology with CTX-M-2 (Humeniuk et al., 2002; Poirel et al., 2002).

CTX-M beta lactamases evolved in a different way compared to TEM and SHV beta-lactamases. The choromosomal beta-lactamase found in different species of *Kluyvera* spp. was transferred into the plasmids, which are then transferred into *Klebsiella* spp. and *Escherichia coli* through conjugation (Poirel, Kampfer & Nordman, 2002). Also, CTX-M beta-lactamases are different from TEM and SHV beta-lactamases as there are only 40% identities (Tzouvelekis et al., 2000).

CTX-M beta-lactamases preferentially hydrolyze cefotaxime than ceftazidime but some of them such as CTX-M-15 and CTX-M-19 hydrolyze ceftazidime efficiently and thus may complicate their phenotypic recognition (Pitout, et al., 2005). Furthermore, several studies found out that these enzymes are inhibited better by the beta-lactamases inhibitor, tazobactam compared to others like sulbactam and clavulanic acid (Bradford et al., 1998; Tzouvelekis et al., 2000).

2.5.2.2 PER Type ESBLs

PER enzymes are class A beta-lactamases and have a similar kinetic behavior to other ESBLs of the same class (Celenza et al., 2006). They share only about 25 to

27% homology with known TEM and SHV type ESBLs (Pateson & Bonomo, 2005).

The first successful isolation of PER-1 enzyme was from *Pseudomonas aeruginosa* strain (Nordmann et al., 1993) and followed by *Salmonella enterica serovar Typhimurium* and *Acinetobacter* spp. isolates in Tukey (Vahaboglu et al., 1995; Vahaboglu et al., 1997). Followed by the successful isolation in Turkey, it was also detected in the following countries such as France (Poirel et al., 1999), Italy (Pagani et al., 2004), Belgium (Claeys, Verschraegen, Baere & Vaneechoutte, 2000) and Korea (Yong et al., 2003).

The PER-2 enzyme, which shares 86% homology to PER-1, was first identified in Argentina in a *Salmonella serovar Typhimurium* strain (Bauernfeind et al., 1996) and followed by detection in *Escherichia coli, Klebsiella pneumonia*, *Proteus mirabilis* (Bauernfeind et al., 1996), and *Vibrio cholera* later on (Petroni et al., 2002). Up to 2007, the PER-2 enzyme was reported exclusively in South America countries only (Power et al., 2007).

2.6 ESBLs Detection Methods

ESBLs detection methods can be divided in two major ways, by clinical microbiology techniques and molecular detection methods. ESBLs can be difficult to detect because they have different levels of activities against various

cephalosporins and that is the reason why the choice of antibiotics to test is vital (Centers for Disease Control and Prevention, 2010).

Although several methods have been proposed for the detection of ESBLs in clinical isolates, it is important to note that none of the methods that rely on phenotypic expression of the beta-lactamase will detect every ESBL producing isolate (Bradford, 2001).

According to National Committee for Clinical Laboratory Standards (1999), all penicillins, cephalosporins, and aztreonam should be reported as resistant if an ESBL is detected. Moreover, each *Klebsiella pneumoniae*, *Klebsiella oxytoca*, or *Escherichia coli* isolate should be considered a potential ESBL-producer if their inhibition zones based on antibiotic susceptible test results are as follows: cefpodoxime $\leq$ 22 mm, ceftazidime $\leq$ 22 mm, aztreonam $\leq$ 27 mm, cefotamixe $\leq$ 27 mm and lastly, ceftriazone $\leq$ 25 mm.

2.6.1 Clinical Microbiology Techniques

Methods which are utilized similarly to standard susceptibility tests based on the Kirby-Bauer disc diffusion test methodology are the most convenient clinical detections for ESBLs. Primary screening is done by detecting the synergy effects between a beta-latamase inhibitor, indicator, cephalosporin (Jitsurong & Yodsawat, 2006).

Combined disc method used in this study is a recently developed method from double disc method with ability to detect presence of ESBL. A difference of more than 5 mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate acid disc is taken to be the phenotypic confirmation of ESBLs production (Clinical and Laboratory Standard Institute, 2007).

Compared to other conventional methods such as double disc approximation test described by Jarlier and co-researchers (1988), and E test (Cambridge Diagnostics Services Ltd, Cambridge, UK), the combined disc method might be the most ideal way in primary screening for ESBLs as it is cost effective and having a high specificity. Several researches pointed out the disadvantages of double disc method such as it requires an accurate separation in between the discs and thus variation may occur (Jabeen, Zafar & Hasan, 2003). Furthermore, the optimal distance needed is varying on different bacterial strains (Livermore & Brown, 2001). As for E test, Florijin, Nijssen, Schmitz, Verhoef and Fluit (2002) found out that it often yields unsatisfied result.

Combined disc method has been found to perform well for the detection of ESBLs in *Escherichia coli* and *Klebsiellae* spp. (Carter, Oakton, Warner and Livermore, 2000). However, for a bacterium which has inducible AmpC beta lactamases, it might give false positive results as the enzymes mentioned are able to mediate resistance to cephalosporin and beta lactamase inhibitor (Jacoby, 2009).

2.6.2 Molecular Detection Methods

As the clinical microbiology methods are a presumptive identification of ESBLs, molecular detection methods are preferably to be carried out to confirm the presence of ESBL genes. DNA probes method that were specific for TEM (Arlet & Philippon, 1991; Gallego, Umaran, Garaizar, Colom & Cisterna, 1990) and SHV enzymes (Arlet & Philippon, 1991) were used in the early detection of beta-lactamases.

Detection using DNA probes is rather labor intensive and it was replaced by polymerase chain reaction (PCR) with oligonucleotide primers that are specific for a beta-lactamase gene. These primers are usually chosen to anneal to regions where various point mutations are not known to occur (Bradford, 2001).

The standard method to detect all variant of ESBL is by nucleotide sequencing the suspected genes (Bradford, 2001). However, this can give different results depending on the method used. A study established by Bradford in 1999 showed that the standard dideoxy-chain termination method resulted in errors in the interpretation of the SHV nucleotide sequence and the derived amino acid sequence that had been reported previously whereas the automated thermal cycling method was better and gave consistent results in the correct sequences for that particular gene.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Vegetable Samples

This study was conducted using tomato, cucumber, celery and broccoli. All of the vegetable samples were bought from Tesco Kampar, Perak, one hour before sampling.

3.1.2 Materials and Equipments

Chemical reagents and equipments required in this study were provided by the Department of Biological Science, Perak campus of University Tunku Abdul Rahman. All of the chemical reagents were readily obtainable from the Microbiology and Molecular Biology Laboratories while the equipments were assessable in the Final Year Project Laboratory. Details of the materials and equipments used are listed in Table 3.1 and Table 3.2, respectively.

Table 3.1 List of Materials Used and Their Suppliers in this Study

Materials	Suppliers
Brain Heart Infusion Broth (BHIB), Commercial Antibiotic Discs (cefotaxime/clavanulate & ceftazidime/clavanulate), Muller Hinton (MH) Broth, Oxidation – Fermentation	BD
Ampicillin Powder	Bio Basic Inc
API 20E kit	BioMerieux
Absolute Ethanol	Copens Scientific
100 bp DNA Ladder, DNA loading dye, Isopropyl-beta-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-beta-D-galactophyranoside (X-Gal)	Fermentas
dNTP, DNA-spin™ Plasmid DNA Purification Kit, Magnesium Chloride ($MgCl_2$), $MgCl_2$ Free PCR buffer, *Taq* Polymerase,	iNtRON
Buffered Peptone Water, Gram's Crystal Violet Solution, Luria-Bertani (LB) Agar, Luria-Bertani (LB) Broth, Safranine Solution	MERCK
Commercial Antibiotic Discs (Except for Cefotaxime/Clavanulate & Ceftazidime/Clavanulate), MacConkey (MAC) Agar, Muller Hinton (MH) Agar	Oxoid
pGEM-T® Easy Vector System	Promega
Violet Red Bile Glucose (VRBG) Agar, Oxidation-Fermentation (O-F) Basal Medium	Pronadisa
QIAquick™ Gel Purificarion Kit	QIAGEN
D-glucose	Rdeh
SP6 Promoter Primer, T7 Promoter Primer	Research Biolabs
Gram's Iodine	R & M Chemicals
Mineral Oil	Sigma
1 kb DNA Ladder, 100 bp DNA Ladder, Agarose Powder, *bla*-CTX-M Forward and Reverse Primers, *bla*-PER-2 Forward and Reverse Primers	Vivantis

Table 3.2 List of Equipments Used and Their Suppliers in this Study

Equipments	Suppliers
Electronic Balance	Adventurer™ Pro
PCR Machine (Thermal cycle)	Biometra
Spectrophotometer	BIO-RAD SmartSpec™
Incubator Shaker	Hettich Zentrifugen
Autoclave Machine	HIRAYAMA
Laminar Flow Hood	Isocide™
Light Microscope	Leica
Incubator, Water Bath	Memmert
Centrifuge Machine, Microcentrifuge, Nanodrop 1000, Electrophoresis Power Supply	Thermo Electron Corporation
UV Transilluminator	UVP

3.2 General Methodology

3.2.1 Preparation of Media and Reagents

Powdered-form growth media were dissolved in sterile deionised water, following instructions as per provided respectively. All of the media, agar and reagent were sterilised before using. Media and agar that required additional components were mixed well before using. Molten agar was poured onto petri dishes after autoclaving and allowed to solidify before being stored at room temperature. All the reagents were kept at room temperature except in cases where storage at 4°C or lower is required such as antibiotic discs and ampicillin were stored in freezer at -20°C. Methods for media and buffer preparation are stated in Table 3.3.

Table 3.3 Preparation of Media Used in this Study

Media	Media Type	Method of preparation
Ampicillin containing media (50µg/ml)	-	An amount of 500 µl of ampicillin stock (50 mg/ml) was added into 1L of media which is cooled down to around 50°C. The media was then well mixed.
Ampicillin stock solution (50mg/ml)	Antibiotic	An amount of 0.05 g of ampicillin powder was dissolved in 1ml of sterile deionised water. The solution was then filter-sterilized into eppendorf tube, wrapped with parafilm and stored at -20°C.
Brain Heart Infusion Broth (BHIB)	Broth	37.0 g of BHIB powder was dissolved and topped up to 1L using sterile deionised water.
Buffered Peptone Water (BPW) broth	Broth	25.5 g of BPW powder was dissolved and topped up to 1L using sterile deionised water.
Luria-Bertani (LB) agar	Molten agar	37.0 g of LB agar powder was dissolved and topped up to 1L using sterile deionised water.
Luria-Bertani (LB) broth	Broth	25.0 g of LB broth powder was dissolved and topped up to 1L using sterile deionised water.
Muller Hinton (MH) agar	Molten agar	38.0 g of MH agar powder was dissolved and topped up to 1L using sterile deionised water.
Muller Hinton (MH) broth	Broth	22.0 g of MH broth powder was dissolved and topped up to 1L using sterile deionised water.
MacConkey (MAC) agar	Molten agar	52.0 g of MAC powder was dissolved and topped up to 1L using sterile deionised water.
O-F (Oxidation-Fermentation) Basal Medium	Semi-solid agar	9.8 g of O-F basal medium powder was dissolved and topped up to 1L using sterile deionised water.
Violet Red Bile Glucose (VRBG) agar	Molten agar	41.5 g of VRBG powder was dissolved and topped up to 1L using sterile deionized water.

3.2.2 Sterilisation

Sterilisation of all media and reagents were accomplished either through autoclaving or filter sterilisation. Liquid media were autoclaved at 121°C at 15 psi for 15 minutes whereas heat-liable media such as ampicillin and glucose were filter-sterilised using a 0.20 μm syringe filter. As for glasswares, pipette tips, microcentrifuge tubes and other heat-stable apparatuses, they were autoclaved at 121°C at 15 psi for 20 minutes. Autoclaved apparatuses were placed into 70°C oven for drying purposes.

3.3 General Classification of Bacteria Strains

3.3.1 Sampling of Vegetable Samples

All vegetable selected for this study were picked with a clean plastic bag in order to avoid contamination from sampler. Prior to the vegetable sampling, working area and equipments were sterilised using 70% (v/v) ethanol solution. Sterilisation was carried out before and after handling each different type of vegetables. The vegetable samples were washed using distilled water to remove soil residues and spoiled parts were trimmed off. Only parts that will be eaten raw were chopped into small pieces. About 5 g of the chopped vegetable samples were transferred into Brain-Heart Infusion Broth (BHIB) and Buffered Peptone Water (BPW) broth supplemented with 50 μg/mL ampicillin. They were then incubated at 37°C overnight in incubator shaker with 200 rpm agitation. Overnight incubated cultures were then subjected to a ten-fold serial dilution using sterile deionised water. A volume of 100 μl of each dilution was poured and spread evenly on

MacConkey (MAC) agar plates which were supplemented with 50 μg/mL of ampicillin and incubated overnight at 37°C. After incubation, each colony with different morphology were picked and streaked on Violet Red Bile Glucose (VRBG) agar plates which were also supplemented with 50 μg/mL of ampicillin. The plates were then incubated overnight at 37°C. Colonies were sub-cultured to Luria-Bertani (LB) agar plates with 50 μg/mL of ampicillin.

3.3.2 Preliminary Characterisation of Bacteria Isolates

Gram staining and several biochemical tests such as catalase test, oxidase-fermentation (O-F) test and oxidase test were carried out with all of the isolated bacteria. Later on, selected potential ESBL-producing bacterial strains will be further identified using API 20E (BioMerieux). All the tests were performed following the instructions stated in the manufacturer's manual.

3.3.2.1 Differential Staining – Gram's Staining

A single colony of pure isolated bacterium was retrieved using an inoculation loop and placed on a clean glass slide to form a thin smear over the glass slide. The bacterial smears were heat-fixed by passing the glass slide through the flame of the bunsen burner. The heat-fixing process has to be fast as overheating may cause distortion of the shapes of the bacterial cells. The surface of the slides containing bacteria smears were then flooded with crystal violet dye for 30 seconds. The slides were then washed under running water to remove excess crystal violet dye. Gram's iodine was dripped onto the smears for 30 seconds.

After washing off Gram's iodine dye with tap water, 95% (v/v) alcohol was evenly distributed on the slides and washed off after 10 seconds for decolourisation purposes. Bacterial smears were then counterstained by safranin for 30 seconds and then rinsed off with tap water. The slides were tilted to drain off the excess water and allowed to air-dry before being examined microscopically using oil immersion lens microscope. Colour and morphology of the bacterial cells were recorded.

3.3.2.2 Catalase Test

The bacteria isolates were first streaked onto LB agar supplemented with 50 μg/mL ampicilin and allowed to grow overnight. A few drops of 3 % hydrogen peroxide (H_2O_2) were added to the colonies on the plate. Presence of bubbles indicates positive reaction.

3.3.2.3 O-F (Oxidation-Fermentation) Test

A young colony of bacterium was picked and inoculated into 2 tubes of O-F basal medium supplemented with 1 % glucose. Sterile mineral oil was added into one of the inoculated tube to create an anaerobic condition while the other tube remained as aerobic condition. The inoculated tubes were incubated at 37°C for 48 hours. A colour change in medium, from green to yellow in both tubes after incubation indicates that the bacterium is a fermentative bacterium.

3.3.2.4 Oxidase Test

A drop of oxidase reagent was dripped on a filter paper. A single colony of young bacterium was picked and placed on the top of the oxidase reagent on filter paper. An appearance of violet in colour on the filter paper indicates positive reaction whereas negative reaction is signified by no colour change on the filter paper

3.4 Antibiotic Susceptible Test

The bacteria isolates were incubated in Mueller Hinton (MH) broth overnight at 37°C. The turbidity of inoculums was adjusted to a 0.5 McFarland standard. They were subsequently incubated for a longer period of time or diluted with sterile deionised water if the turbidity was too light or too heavy, respectively. A sterile cotton swab was dipped into the inoculums and excessive fluid was removed by rotating the cotton swab using firm pressure against the wall of tube. The cotton swab was then swabbed 3 times over the entire MH agar surface by rotating the plate approximately 60° each time to ensure an even distribution and confluent growth. Fifteen different commercial antibiotic discs were used. They include aztreonam (30 μg), cefotaxime (30 μg), cefotaxime/clavanulate (30 μg/10 μg), ceftazidime (10 μg), ceftazidime/clavanulate (30 μg/10 μg), cefpodoxime (10 μg), ceftriazone (30 μg), amoxicillin/clavulanate (20 μg/10 μg), oxacillin (1 μg), ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), trimethoroprim/sulfamethozaxole (25 μg) and gentamicin (10 μg). The discs were placed on MH agar with not less than 2 cm apart. The agar plates were

then incubated at 37°C overnight. Diameter of clear zones around each antibiotic disc were measured and recorded.

3.5 Biochemically Characterisation of Bacterial Isolates

Two bacterial isolates which showed a positive phenotypic result (TM P2 & CE B2) were further analysed by using API 20E kit (BioMerieux). An incubation box was prepared and 5 ml of deionised water was distributed into the honeycombed wells of the tray to create a humid atmosphere. An API 20E strip was then placed in the incubation box. A single young colony was removed by pipette and diluted in 5 ml deionised water. The suspension was emulsified to achieve a homogeneous state and distributed into the individual tubes of the strip accordingly. The strip was tilted slightly forward to avoid bubbles formation. The incubation box was then closed and incubated at 37°C for 18 to 24 hours. The results of all of the tests were recorded and interpreted by referring to apiweb™.

3.5.1 O-NitroPhenyl-β-D-Galactopyranosidase (ONPG) Test

The bacterial suspension was distributed into the ONPG tube and incubated for 18 to 24 hours. A yellow colour formed after incubation indicates a positive reaction whereas colourless indicates a negative reaction.

3.5.2 Arginine Dihyrolase (ADH) Test, Lysine Decarboxylase (LDC) Test, Ornithine Decarboxylase (ODC) Test, Hydrogen Sulfide (H_2S) Test, and Urease (URE) Test

The bacterial suspension was distributed into the tubes accordingly. Mineral oil was added into all tubes to create an anaerobic condition and incubated for 18 to 24 hours. For ADH test, LDC test and ODC test, a red or orange colour formed after incubation indicates a positive reaction while a yellow in colour indicates a negative result. For H_2S test, a black deposit and thin line formed after incubation indicates a positive reaction, a colourless or greyish in colour was considered as a negative reaction.

3.5.3 Citrate Utilization (CIT) Test, Voges - Proskauer (VP) Test, Gelatin (GEL) Test

The bacterial suspension was distributed into both tubes and cupules accordingly and incubated for 18 to 24 hours. For CIT test, a blue-green or blue colour formed after incubation indicates a positive reaction whereas a pale green or yellow colour signifies a negative reaction. For VP test, both VP 1 and VP 2 reagents were added into the tubes, one drop each. A pink or red colour formed 10 minutes after adding the reagents mentioned indicates a positive reaction while a colourless or pale pink colour formed indicates a negative reaction. For GEL test, a diffusion of black formed after incubation indicates a positive reaction whereas no diffusion formed signified a negative reaction.

3.5.4 Tryptophan Deaminease (TDA) Test

The bacterial suspension was distributed in the tube and incubated for 18 to 24 hours. A drop of TDA reagent was added. A reddish-brown colour formed immediately after adding the reagent signifies a positive reaction while a yellow colour indicates a negative reaction.

3.5.5 Indole (IND) Test

The bacterial suspension was distributed in the tube and incubated for 18 to 24 hours. A drop of James reagent was added. A pink colour developed in the whole cupule immediately after adding the reagent indicates a positive reaction whereas a colourless, pale green or yellow colour signifies a negative reaction.

3.5.6 Glucoes (GLU) Test, Mannitol (MAN) Test, Inositol (INO) Test, Sorbitol (SOR) Test, Rhamnose (RHA) Test, Saccharose (SAC) Test, Melibiose (MEL) Test, Amygdalin (AMY) Test, Arabinose (ARA) Test

The bacterial suspension was distributed in the tubes accordingly and incubated for 18 to 24 hours. A yellow colour formed after incubation indicates a positive reactive whereas a blue or blue-green colour signifies a negative reaction.

3.5.7 Nitrate Reduction

A drop for each of the NIT 1 and NIT 2 reagents were added into the GLU tube and incubated for 2 to 5 minutes. A red colour formed after incubation indicates a positive result whereas a yellow colour signifies a negative result. About 2 to

5 mg of zinc reagent was added into GLU tube. The tube which remains yellow in colour after incubation for 5 minutes indicates a positive reaction whereas an orange or red colour formed after incubation signifies a negative reaction.

3.6 Total DNA Extraction

In this study, the fast boil method (Holmes & Quigley, 1981) was used to extract the total DNA. Bacterial cells were cultured in 5 ml of LB broth overnight at 200 rpm agitation. An amount of 1.5 ml of the bacterial culture was centrifuged at 14,000 rpm for 15 minutes at room temperature. The supernatant was discarded whereas pellet was then re-suspended in 300 µl of sterile deionised water and centrifuge at 14,000 rpm for 10 minutes at room temperature. The supernatant was the removed. The remaining suspension was then boiled at 100°C for 10 minutes and cooled on ice immediately for 3 to 5 minutes. The samples were then centrifuged at 14,000 rpm for 2 minutes. The supernatant was then being transferred to a new sterile microcentrifuge tube and stored at -20°C until future use. The concentration and purify of DNA obtained were measured using Nanodrop 1000 (Thermo Scientific).

3.7 Polymerase Chain Reaction (PCR)

3.7.1 Oligonucleotides for PCR Amplification

A set of degenerate primers (CTXM_F and CTXM_R), were used in PCR amplification of the beta-lactamase CTX-M gene while a set of specific primers

(PER2_F and PER2_R), were used in PCR amplification of the beta-lactamase PER-2 gene. Details and sequences of the primers were stated in Table 3.4.

Table 3.4 Primers Sequences used in *bla*-CTX-M and *bla*-PER-2 Homologous Genes Amplification

Primer	Primer Sequences (5' to 3')	Length (base)
CTXM_F	5' – ATG TGC AGY ACC AGT AAR GT – 3'	20
CTXM_R	5' – TGG GTR AAR TAR GTS ACC AGA – 3'	21
PER2_F	5' – GTA GTA TCA GCC CAA TCC CC – 3'	20
PER2_R	5' – CCA ATA AAG GCC GTC CAT CA – 3'	20

Legend : F: Forward; R: Reverse
Note : Y: C/T; R: A/G; S: G/C

3.7.2 Preparation of PCR mixtures for ESBLs (*bla*-CTX-M and *bla*-PER-2) Homologous Genes Amplification

The PCR reactions were carried out by using a non-gradient PCR thermal cycler (Biometra). All PCR reactions were conducted in 25µl aliquots containing 1X $MgCl_2$ free PCR buffer (iNtRON), 2 mM $MgCl_2$ (iNtRON), 0.04 U/ml *Taq* DNA polymerase (iNtRON), 0.2 mM dNTP mix (iNtRON), 400 nM of each forward and reverse primers, respectively, 0.46 ng/µl of DNA template and ddH_2O.

3.7.3 Optimisation of the PCR Amplification

PCR reaction was carried out for the amplification of ESBLs gene using a non-gradient PCR thermal cycler (Biometra). The annealing temperature was optimised for generating the desirable amplified products.

The PCR amplification for *bla*-CTX-M homologous gene was performed using the condition stated below:

94°C	:	5 minutes	Initial denaturation	1 cycle
94°C	:	45 seconds		
50°C /53°C/55°C	:	45 seconds	Annealing temperature	30 cycles
72°C	:	45 seconds		
72°C	:	10 minutes	Final extension step	1 cycle

The PCR amplification for *bla*-PER-2 homologous gene was performed using the condition stated below:

94°C	:	10 minutes	Initial denaturation	1 cycle
94°C	:	30 seconds		
55°C	:	30 seconds	Annealing temperature	25 cycles
72°C	:	30 seconds		
72°C	:	5 minutes	Final extension step	1 cycle

3.7.4 Gel electrophoresis of PCR Products

An aliquot of 3 µl of the PCR product was mixed with 1µl of loading dye and analyzed using 1.5% (w/v) agarose gel electrophoresis stained using ethidium bromide and visualized under the UV transilluminator. A 100 bp DNA ladder (Fermentas) was used as molecular weight marker when the expected sizes of the PCR products are below a 1 kb whereas 1 kb DNA ladder (Vivantis) was used when the expected sizes of PCR products are above 1 kb. The electric current

used was 80 V and switched off when the loading dye reached ¾ of the length of the agarose gel.

3.8 Cloning of PCR Product

3.8.1 Agarose Gel Purification

Amplified PCR products were purified using QIAquick™ Gel Purificarion Kit (QIAGEN) following the manufacturer's manual. DNA was electrophoresed in a 1.5% (w/v) agarose gel and viewed under UV transilluminator. The DNA fragment was excised from the gel using a clean and sharp scalpel. The gel slide was weighted in a 1.5 ml microcentrifuge tube, 3 volumes of Buffer QG were later added onto 1 volume of gel. The mixture was then incubated at 50°C for 10 minutes or until the gel was completely dissolved. The sample was then applied to a QIAquick™ spin column which placed on a 2 ml collection tube, centrifuged for 2 minutes at 13,000 rpm and the flow-through was discarded. Subsequently, 0.75 ml of Buffer TE was added to the spin column, centrifuge for 2 minutes at 13,000 rpm and the flow-through was discarded. The spin column was then spin again for another 2 minutes at 13,000 rpm to ensure all of the ethanol from Buffer TE was completely removed. The spin column was then placed on a sterile 1.5 ml microcentrifuge tube and 30 µl of Buffer EB was added to the center of the QIAquick™ membrane to elute the DNA. It was left standing for 5 minutes before being centrifuged for 2 minutes at 13,000 rpm. The concentration of purified DNA was measured using Nanodrop 1000 and stored the purified DNA at -20°C.

3.8.2 Ligation of Purified DNA to pGEM-T® Easy Vector

An amount of 3 µl of PCR products, 1 µl of pGEM-T® Easy Vector (Promega, 50 ng), 1 µl of T4 ligase (Promega, 3 U/µl) and 5 µl of 2X Rapid Ligation Buffer (Promega) were mixed together and incubated at 4°C overnight.

3.9 Transformation and Plasmid Extraction

3.9.1 Preparation of JM109 Competent Cells

A single colony of *E.coli* strain JM109 was inoculated into 5 ml of LB broth and incubated at 37°C overnight with agitation at 200 rpm. An amount of 300 µl of the overnight inoculum was transferred into 15 ml of fresh LB medium. The inoculums were further agitated (200 rpm) at 37°C until it reached an OD_{600} of between 0.5-0.6. The cells were pelleted by centrifugation at 5,000 rpm for 5 minutes at 4°C. Cells were subsequently resuspended in 2 ml of 0.1 M of cold $CaCl_2$ and incubated on ice for 2 hours.

3.9.2 Transformation of Competent Cells

An amount of 3 µl of ligation mixture was mixed gently with 200 µl of competent cells in a sterile pre-cold 1.5 ml microcentrifuge tube. The mixture was placed on ice for 1 hour before being subjected to heat-shocking for exactly 90 seconds at 42°C. The tube was immediately placed on ice for another 5 minutes. An amount of 900 ml of LB broth was added into the tube and then incubated at 37°C for 45 minutes in incubator shaker with 80 rpm agitation. The cells were pelleted by centrifugation at 8,000 rpm for 10 minutes. An amount of 900 ml of supernatant

was removed and pellet was resuspended in remaining supernatant. Lastly, the mixture was plated onto LB agar plate which was supplemented with 50 µl/ml ampicillin. For blue-white selection of the transformed bacteria, selection was performed on the 50 µl/ml ampicillin-supplemented LB agar plate that were spread with 20 µl of IPTG (100 mM) and 20 µl of X-Gal (50 mg/ml). For positive and negative control plates, competent cells were transformed with and without pUC19, respectively. The plates were then incubated overnight at 37°C. White colonies formed were then cultured onto a new plate which was spread with 20 µl of IPTG (Fermentas, 100 mM) and 20 µl of X-Gal (Fermentas, 50 mg/ml) for confirmation and further screening purposes.

3.9.3 Colony PCR

A colony was gently touched with a sterile toothpick and placed into a standard PCR mixtures consisting of 1 X $MgCl_2$ free PCR buffer (iNtRON), 2 mM $MgCl_2$ (iNtRON), 0.04 U/ml *Taq* DNA polymerase (iNtRON), 0.4 µM of each SP6 and T7 promoter primers (Research Biolabs), 200 µM dNTP mix (iNtRON) and ddH_2O to a final volume of 25 µL. PCR reaction was carried out using non-gradient PCR thermal cycler (Biometra). After PCR amplification, the PCR products were assessed in a 1.5% (w/v) agarose gel. Details and sequences of the primers are stated in Table 3.5. PCR mixtures consisting of 1 X $MgCl_2$ free PCR buffer (iNtRON), 2 mM $MgCl_2$ (iNtRON), 0.04 U/ml *Taq* DNA polymerase (iNtRON), 0.4µM of each SP6 and T7 promoter primers (Research Biolabs),

200µM dNTP mix (iNtRON) and ddH_2O to a final volume of 25 µL. Details and sequences of the primers were stated in Table 3.5.

Table 3.5 Primers Sequences Used in Colony PCR

Primer	Primer Sequences (5' to 3')	Length (base)
T7	5' – TAA TAC GAC TCA CAT TAG GG – 3'	20
SP6	5' – ATT TAG GTG ACA CTA TAG – 3'	18

The PCR amplification for colony PCR was performed using the condition stated below:

96°C:	3 minutes	Initial denaturation	1 cycle
96°C:	30 seconds		
50°C:	15 seconds	Annealing temperature	30 cycles
60°C:	150 seconds		
60°C:	10 minutes	Final extension step	1 cycle

3.9.4 Plasmid Extraction

Plasmid extraction was performed with DNA-spin™ Plasmid DNA Purification Kit (iNtRON) following the methodology given by the manufacturer. A single freshly streaked transformed bacterial colony was picked and inoculated in a 5 ml LB broth supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C with agitation 180 rpm. Bacterial cells were pelleted using 3 ml of overnight incubated broth by centrifugation at 13,000 rpm for 30 seconds at room temperature. Supernatant was discarded. Pellet was resuspended completely in

250 µl of Resuspension Buffer by pipetting until no clumps of the cell pellet remain. An amount of 250 µl of Lysis Buffer was added to the resuspended cells. The tube was mixed gently by inverting it for several times. The cells were incubated at room temperature for up to 5 minutes. An amount of 350 µl of chilled Neutralization Buffer was added and mixed gently by inverting the tube several times. The tube was then centrifuged at 13,000 rpm for 10 minutes at 4 °C. DNA-spin™ column was inserted into a 2 ml collection tube. A maximum of 750 µl of supernatant formed after centrifugation was transferred promptly into the column and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the column was placed into the same collection tube. An amount of 500 µl of Washing Buffer A was added into the column and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the column was placed into the same collection tube. An amount of 700 µl of Washing Buffer B was added into the column and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the column was placed into the same collection tube. An additional centrifuge at 13,000 rpm for 60 seconds was performed to remove the residual ethanol completely. The collection tube was discarded and the column was placed on a sterile 1.5 ml microcentrifuge tube. An amount of 50 µl of Elution Buffer was added to the upper reservoir of the column, left for 1 minute before centrifuging the tube assembly at 13,000 rpm for 60 seconds. Eluted DNA was stored at -20°C.

3.10 DNA Sequencing and Analysis

3.10.1 Sequencing of the Extracted Recombinant Plasmid

Extracted recombinant DNA plasmid was outsourced to 1st BASE Laboratories Sdn. Bhd. for cycle sequencing using specific primers, T7 and SP6.

3.10.2 BlastX Alignment Analysis

The resulting DNA sequences obtained were aligned with BlastX software which is available on the NCBI, National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

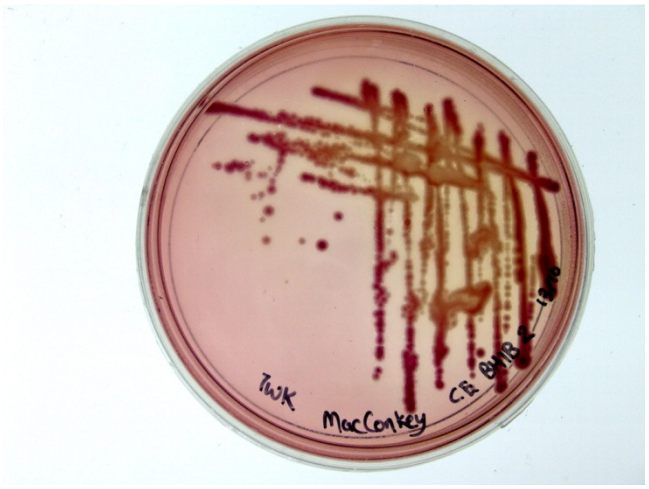
CHAPTER 4

RESULTS

4.1 Cultivation and Isolation of Gram-negative Bacteria

In this study, selection of Gram-negative bacteria was performed on MacConkey (MAC) agars supplemented with 50 μg/ml of ampicillin. A total of 25 morphologically different bacterial isolates were isolated. They include 8 bacterial isolates from tomato, 8 bacterial isolates from cucumber, 5 bacterial isolates from celery and 4 bacterial isolates from broccoli. The individually isolated bacterial isolates were then streaked on Violet Red Bile Glucose (VRBG) agars supplemented with 50 μg/ml of ampicillin to obtain pure strain isolates. Figure 4.1 (a) and Figure 4.1 (b) illustrates the bacterial single isolates on MAC and VRBG agars supplemented with 50 μg/ml of ampicillin, respectively.

(a)



(b)

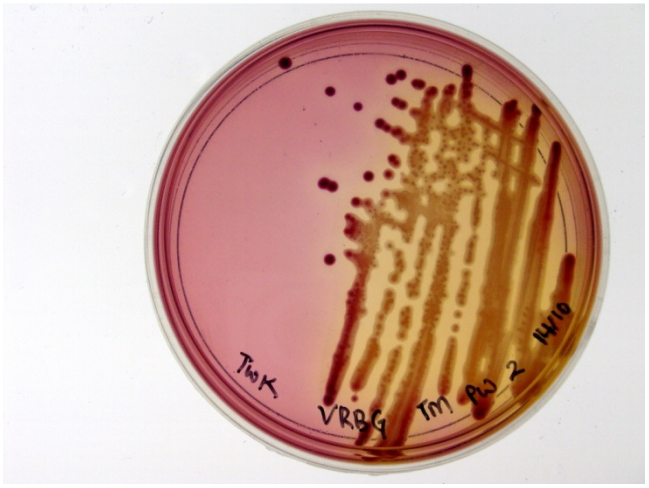


Figure 4.1 Bacterial Single Isolates on both MAC and VRBG Agar Supplemented with 50 µg/ml of Ampicillin

Image shows (a) bacterial single isolates on MAC agar supplemented with 50 µg/ml of ampicillin and (b) bacterial single isolate on VRBG agar supplemented with 50 µg/ml of ampicillin.

4.2 Preliminary Characterisation of Bacterial Isolates

All bacterial isolates selected were characterised based on their morphological and biochemical characteristics. Validation of the isolates for Gram-negative bacteria based on their Gram's reaction was performed. Further identification was carried out using several preliminary biochemical tests as well as using API 20E kit (BioMerieux). Table 4.1 and 4.2 summarise the morphological characteristics of each bacterial isolate on both MAC and VRBG agars supplemented with 50 μg/ml of ampicillin.

4.2.1 Differential Staining – Gram's Staining

Bacterial cells that retained the purple colour of crystal violet dye after Gram's staining will be classified as Gram-positive bacteria whereas bacterial cells which take up the reddish pink colour of counter stain safranin dye will be classified as Gram-negative bacteria. All bacterial isolates were stained pink in colour after performing Gram's staining. Therefore, it can be concluded that all of them were Gram-negative bacteria. Out of 25 bacterial isolates, 20 bacterial isolates were rod-shaped while other 5 were coccobacillus. Figure 4.2 illustrated the images of bacterial cells viewed under oil immersion objective of the microscope (magnification of 1000x) after Gram's staining whereas the morphology and colour of the bacterial cells observed was tabulated in Table 4.3.

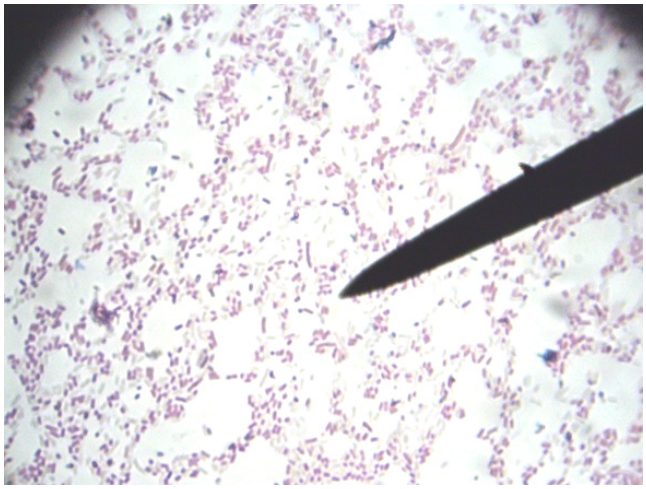
Table 4.1 Appearance and Morphology of Bacterial Isolates Isolated from Tomato and Cucumber on MacConkey (MAC) Agar and Violet Red Bile Glucose (VRBG) Agar

Vegetable Source	Assigned Code	Appearance and Morphology of Colony						
		Colour		Shape	Elevation	Edge	Opacity	Texture
		MAC	VRBG					
Tomato	TM B1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	TM B2	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	TM B3	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	TM B4	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	TM B5	Ivory	Violet	Circular	Convex	Entire	Opaque	Smooth
	TM P1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	TM P2	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	TM P3	Ivory	Violet	Circular	Convex	Entire	Opaque	Smooth
Cucumber	CC B1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	CC B2	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	CC B3	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	CC B4	Ivory	Violet	Circular	Convex	Entire	Opaque	Smooth
	CC P1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	CC P2	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	CC P3	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	CC P4	Ivory	Violet	Circular	Convex	Entire	Translucent	Glistening

Table 4.2 Appearance and Morphology of Bacterial Isolates Isolated from Celery and Broccoli on MacConkey (MAC) Agar and Violet Red Bile Glucose (VRBG) Agar

Vegetable Source	Assigned Code	Appearance and Morphology of Colony						
		Colour		Shape	Elevation	Edge	Opacity	Texture
		MAC	VRBG					
Celery	CE B1	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	CE B2	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	CE B3	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	CE P1	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
	CE P2	Pink	Violet	Circular	Convex	Entire	Opaque	Smooth
Broccoli	BR B1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	BR B2	Ivory	Violet	Circular	Convex	Entire	Opaque	Smooth
	BR P1	Pink	Violet	Circular	Convex	Entire	Translucent	Glistening
	BR P2	Ivory	Violet	Circular	Convex	Entire	Opaque	Smooth

(a)



(b)

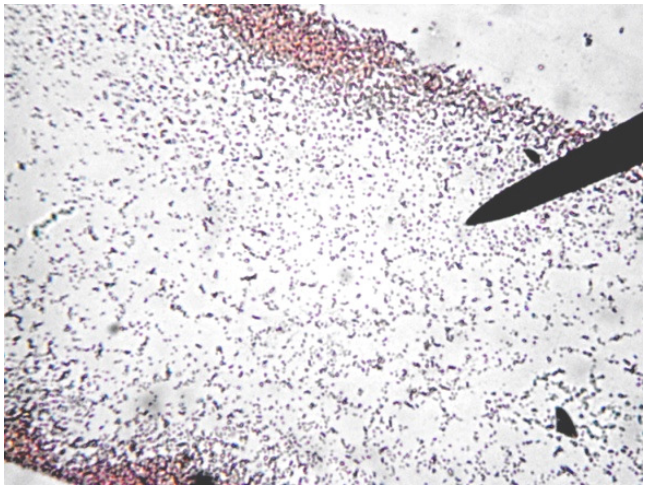


Figure 4.2 Bacterial Cells Viewed Under Oil Immersion Objective of the Microscope (Magnification of 1000X) after Gram's Staining

Images of (a) rod-shaped bacteria and (b) coccobacillus bacteria stained in pink.

Table 4.3 Microscopic Observation (Magnification of 1000X) of Bacterial Isolates after Gram's Staining

Bacterial Isolate (Code)	Morphology	Colour	Gram's Reaction
TM B1	Rod	Pink	Gram-negative
TM B2	Rod	Pink	Gram-negative
TM B3	Rod	Pink	Gram-negative
TM B4	Coccobacillus	Pink	Gram-negative
TM B5	Rod	Pink	Gram-negative
TM P1	Rod	Pink	Gram-negative
TM P2	Rod	Pink	Gram-negative
TM P3	Rod	Pink	Gram-negative
CC B1	Rod	Pink	Gram-negative
CC B2	Rod	Pink	Gram-negative
CC B3	Rod	Pink	Gram-negative
CC B4	Coccobacillus	Pink	Gram-negative
CC P1	Rod	Pink	Gram-negative
CC P2	Rod	Pink	Gram-negative
CC P3	Rod	Pink	Gram-negative
CC P4	Rod	Pink	Gram-negative
CE B1	Rod	Pink	Gram- negative
CE B2	Rod	Pink	Gram-negative
CE B3	Rod	Pink	Gram-negative
CE P1	Coccobacillus	Pink	Gram-negative
CE P2	Rod	Pink	Gram-negative
BR B1	Coccobacillus	Pink	Gram-negative
BR B2	Rod	Pink	Gram-negative
BR P1	Coccobacillus	Pink	Gram-negative
BR P2	Rod	Pink	Gram-negative

4.2.2 Catalase Test

For the catalast test, bubble formation (Figure 4.3) indicates the presence of catalase enzyme which is used to detoxify a cell of hydrogen peroxide (H_2O_2). All the 25 bacterial isolates formed bubbles after few drops of 3 % H_2O_2 were added and thus showing positive result in this biochemical test.

4.2.3 O-F (Oxidation-Fermentation) Test

All the 25 bacterial isolates turned both open tube (aerobic condition) and closed tube (anaerobic condition) of the inoculated O-F basal medium supplemented with 1 % glucose into yellow in colour (Figure 4.4) after incubation for 24 to 48 hours. Thus, they were fermentative bacteria.

4.2.4 Oxidase Test

All the 25 bacterial isolates were classified as oxidase negative as no colour change occurring after adding single colonies of the bacterial isolates separately onto the oxidase reagent on filter papers.

Figure 4.3 Catalase Test

Image showed the formation of bubbles on bacterial isolate after adding few drops of 3 % hydrogen peroxide (H_2O_2).

Figure 4.4 O-F (Oxidation-Fermentation) Test

Image showed O-F test with negative control tube (left), open tube (middle) and closed tube (right). Both open tube (aerobic condition) and closed tube (anaerobic condition) turned into yellow in colour in the O-F test.

4.3 Antibiotic Susceptible Test

Interpretation of the Zone Diameter Interpretative Standards of Clinical Laboratory Standard Institute (CLSI) was used as reference to determine the resistance of the bacteria as “susceptible”, “intermediate” or “resistance” in this study towards 15 types of commercial antibiotic disc. Table 4.4, 4.5, 4.6 and 4.7 showed the collective measurements of the inhibition zone and interpretation of bacteria isolates isolated from tomato, cucumber, celery and broccoli towards the antibiotic agents, respectively whilst a summary of antibiotic susceptible test on all bacterial isolates obtained was tabulated in Table 4.8.

All the bacteria isolates were demonstrated resistant towards ampicillin and oxacillin. Among the 25 bacteria isolates, some of them showed additional resistance towards other antibiotics. Based on the result obtained, a total of nine isolates showed resistance towards more than one class of first-line antibiotics.

In this study, cefotaxime/clavulanate and ceftazidime/clavulanate were used for the detection of ESBL-producer based on phenotypic disc testing by comparing the inhibition zone of the cephalosporin tested in combination with clavulanic acid versus its inhibition zone when tested alone. If an inhibition zone of the cephalosporin tested in combination with clavulanic acid was larger or equal to 5 mm of its inhibition zone when tested alone was observed, the particular isolate was classified as phenotypic positive isolate. Out of 25 bacterial isolates, only two isolates, TM P2 and CE B2 demonstrated a positive phenotypic result.

Table 4.4 Antibiotic Susceptible Test of Bacterial Isolates Isolated from Tomato

Bacterial isolate (code)	TM B1	TM B2	TM B3	TM B4	TM B5	TM P1	TM P2	TM P3
Type of antibiotics	**Clear zone diameter (mm)**							
Aztreonam	26 (S)	29 (S)	30 (S)	28 (S)	30 (S)	34 (S)	18 (I)	25 (S)
Cefotaxime	26 (S)	29 (S)	28 (S)	28 (S)	25 (S)	32 (S)	14 (R)	26 (S)
Cefotaxime/Clavulanate	26	29	29	29	25	32	20	26
Ceftazidime	22 (S)	24 (S)	24 (S)	25 (S)	23 (S)	28 (S)	11 (R)	25 (S)
Ceftazidime/Clavulanate	24	25	26	26	24	29	16	25
Cefpodoxime	24 (S)	25 (S)	26 (S)	21 (S)	23 (S)	29 (S)	6 (R)	22 (S)
Ceftriazone	24 (S)	25 (S)	28 (S)	25 (S)	22 (S)	31 (S)	14 (I)	20 (S)
Amoxycillin/Clavulanate	19 (S)	20 (S)	21 (S)	18 (S)	20 (S)	26 (S)	11 (R)	20 (S)
Oxacillin	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)
Ampicillin	11 (R)	13 (R)	7 (R)	9 (R)	9 (R)	9 (R)	6 (R)	10 (R)
Chloramphenicol	20 (S)	22 (S)	28 (S)	22 (S)	25 (S)	28 (S)	21 (S)	24 (S)
Ciprofloxacin	24 (S)	26 (S)	30 (S)	25 (S)	30 (S)	29 (S)	28 (S)	25 (S)
Tetracycline	22 (S)	22 (S)	24 (S)	21 (S)	24 (S)	25 (S)	22 (S)	23 (S)
Trimethroprim/Sulfamethozaxole	20 (S)	22 (S)	20 (S)	23 (S)	19 (S)	26 (S)	24 (S)	20 (S)
Gentamicin	15 (S)	18 (S)	19 (S)	15 (S)	17 (S)	19 (S)	16 (S)	17 (S)

Note: R: Resistant, I: Intermediate, S: Susceptible

Table 4.5 Antibiotic Susceptible Test of Bacterial Isolates Isolated from Cucumber

Bacterial isolate (code)	CC B1	CC B2	CC B3	CC B4	CC P1	CC P2	CC P3	CC P4
Type of antibiotics	**Clear zone diameter (mm)**							
Aztreonam	30 (S)	29 (S)	28 (S)	25 (S)	29 (S)	29 (S)	25 (S)	28 (S)
Cefotaxime	29 (S)	29 (S)	25 (S)	26 (S)	26 (S)	25 (S)	25 (S)	20 (S)
Cefotaxime/Clavulanate	29	30	25	26	26	25	25	22
Ceftazidime	25 (S)	25 (S)	25 (S)	20 (S)	24 (S)	25 (S)	20 (S)	22 (S)
Ceftazidime/Clavulanate	25	25	25	21	25	25	20	22
Cefpodoxime	26 (S)	23 (S)	22 (S)	25 (S)	14 (R)	14 (R)	22 (S)	15 (R)
Ceftriazone	27 (S)	23 (S)	22 (S)	25 (S)	25 (S)	25 (S)	23 (S)	22 (S)
Amoxycillin/Clavulanate	20 (S)	11 (R)	10 (R)	20 (S)	10 (R)	11 (R)	19 (S)	10 (R)
Oxacillin	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)
Ampicillin	6 (R)	8 (R)	10 (R)	9 (R)	10 (R)	9 (R)	6 (R)	6 (R)
Chloramphenicol	24 (S)	24 (S)	23 (S)	24 (S)	20 (S)	19 (S)	28 (S)	24 (S)
Ciprofloxacin	29 (S)	28 (S)	28 (S)	30 (S)	34 (S)	20 (I)	28 (S)	28 (S)
Tetracycline	21 (S)	22 (S)	22 (S)	24 (S)	11 (R)	17 (I)	23 (S)	15 (I)
Trimethroprim/Sulfamethozaxole	23 (S)	23 (S)	24 (S)	18 (S)	28 (S)	16 (S)	18 (S)	26 (S)
Gentamicin	18 (S)	17 (S)	17 (S)	18 (S)	20 (S)	19 (S)	15 (S)	19 (S)

Note: R: Resistant, I: Intermediate, S: Susceptible

Table 4.6 Antibiotic Susceptible Test of Bacterial Isolates Isolated from Celery

Bacterial isolate (code)	CE B1	CE B2	CE B3	CE P1	CE P2
Type of antibiotics	**Clear zone diameter (mm)**				
Aztreonam	24 (S)	20 (S)	25 (S)	22 (S)	26 (S)
Cefotaxime	24 (S)	20 (S)	28 (S)	24 (S)	24 (S)
Cefotaxime/Clavulanate	24	23	28	24	25
Ceftazidime	20 (S)	18 (S)	23 (S)	18 (S)	22 (S)
Ceftazidime/Clavulanate	24	23	25	22	24
Cefpodoxime	24 (S)	21 (S)	25 (S)	24 (S)	24 (S)
Ceftriazone	24 (S)	20 (S)	26 (S)	25 (S)	25 (S)
Amoxycillin/Clavulanate	16 (I)	18 (S)	23 (S)	17 (I)	18 (S)
Oxacillin	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)
Ampicillin	6 (R)	10 (R)	9 (R)	6 (R)	6 (R)
Chloramphenicol	6 (R)	26 (S)	25 (S)	6 (R)	6 (R)
Ciprofloxacin	20 (S)	24 (S)	24 (S)	22 (S)	22 (S)
Tetracycline	6 (R)	22 (S)	23 (S)	6 (R)	7 (R)
Trimethroprim/Sulfamethozaxole	6 (R)	24 (S)	26 (S)	6 (R)	6 (R)
Gentamicin	15 (S)	14 (I)	16 (S)	15 (S)	16 (S)

Note: R: Resistant, I: Intermediate, S: Susceptible

Table 4.7 Antibiotic Susceptible Test of Bacterial Isolates Isolated from Broccoli

Bacterial isolate (code)	BR B1	BR B2	BR P1	BR P2
Type of antibiotics	**Clear zone diameter (mm)**			
Aztreonam	28 (S)	34 (S)	27 (S)	33 (S)
Cefotaxime	27 (S)	26 (S)	27 (S)	28 (S)
Cefotaxime/Clavulanate	28	26	28	29
Ceftazidime	23 (S)	21 (S)	23 (S)	24 (S)
Ceftazidime/Clavulanate	25	25	24	28
Cefpodoxime	24 (S)	21 (S)	26 (S)	21 (S)
Ceftriazone	28 (S)	25 (S)	27 (S)	24 (S)
Amoxycillin/Clavulanate	21 (S)	12 (R)	21 (S)	24 (S)
Oxacillin	6 (R)	6 (R)	6 (R)	6 (R)
Ampicillin	6 (R)	6 (R)	7 (R)	8 (R)
Chloramphenicol	25 (S)	28 (S)	25 (S)	28 (S)
Ciprofloxacin	24 (S)	29 (S)	22 (S)	28 (S)
Tetracycline	19 (S)	25 (S)	22 (S)	25 (S)
Trimethroprim/Sulfamethozaxole	21 (S)	21 (S)	23 (S)	22 (S)
Gentamicin	14 (I)	18 (S)	15 (S)	14 (I)

Note: R: Resistant, I: Intermediate, S: Susceptible

Table 4.8 Summary of Antibiotic Susceptibility Test of Gram-negative Isolates from Four Local Vegetables Samples

Type of antibiotics	No (%) of resistant isolates from				
	All (n=25)	Tomato (n=8)	Cucumber (n=8)	Celery (n=5)	Broccoli (n=4)
Aztreonam	1[a] (4.0)	1[a] (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Cefotaxime	1 (4.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Cefotaxime/Clavulanate	1 (4.0)	1[c] (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Ceftazidime	1 (4.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Ceftazidime/Clavulanate	2 (8.0)	1[c] (12.5)	0 (0.0)	1[c] (12.5)	0 (0.0)
Cefpodoxime	4 (16.0)	1 (12.5)	3 (37.5)	0 (0.0)	0 (0.0)
Ceftriazone	1[a] (4.0)	1[a] (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Amoxycillin/Clavulanate	9[b] (36.0)	1 (12.5)	5 (62.5)	2[a] (40.0)	1 (25.0)
Oxacillin	25 (100.0)	8 (100.0)	8 (100.0)	5 (100.0)	4 (100.0)
Ampicillin	25 (100.0)	8 (100.0)	8 (100.0)	5 (100.0)	4 (100.0)
Chloramphenicol	3 (12.0)	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)
Ciprofloxacin	1[a] (4.0)	0 (0.0)	1[a] (12.5)	0 (0.00)	0 (0.0)
Tetracycline	6[b] (24.0)	0 (0.0)	3[b] (37.5)	3 (60.0)	0 (0.0)
Trimethroprim/Sulfamethozaxole	3 (12.0)	0 (0.0)	0 (0.0)	3 (60.0)	2 (50.0)
Gentamicin	2[a] (8.0)	0 (0.0)	0 (0.0)	0 (0.0)	2[a] (50.0)

[a]These isolates demonstrated intermediate sensitivity to the antimicrobial agent.
[b]This number includes isolated demonstrating resistance and intermediate sensitivity.
[c]These isolates demonstrated a ≥ 5mm zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone, indicating it as a ESBL-producer based on phenotypic disc test.

4.4 Biochemically Characterisation of Bacterial Isolates

4.4.1 API 20E Kit

The result of each biochemical tests obtained via API 20E kit for both TM P2 and CE B2 was summarised in Table 4.9. It was observed that 22 tests out of 26 tests showed the same results for both TM P2 and CE B2. The 22 tests are ONPG, CIT, H_2S, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA, OX, NO_2, N_2, McC, OF-O and OF-F tests.

For ONPG, CIT, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA, NO_2, N_2, McC, OF-O and OF-F tests, both TM P2 and CE B2 showed positive reaction results whereas for H_2S, TDA, IND, GEL and OX tests, both bacterial isolates showed negative reaction results.

The other 4 tests showed different results (ADH, LDC, ODC and IND tests). For ADH and ODC tests, TM P2 showed positive reaction results whereas CE B2 showed negative reaction results. For LDC and IND tests, CE B2 showed positive reaction results while TM P2 showed negative reaction results. Figure 4.5 showed the API multi-test system strips for both TM P2 and CE B2.

(a)

(b)

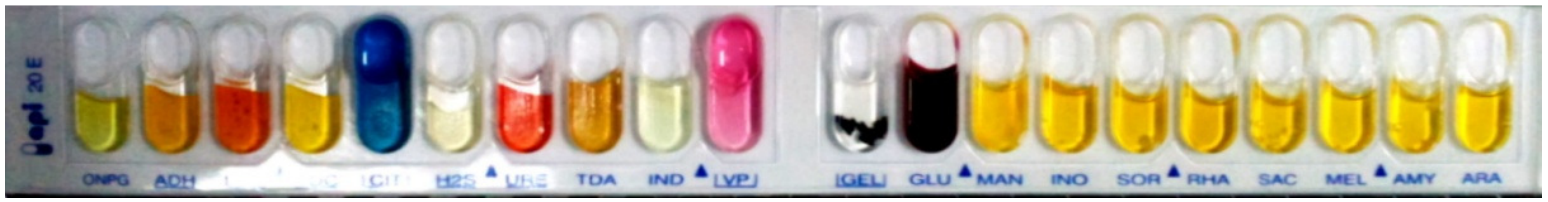


Figure 4.5 API Multi-test System Strips

Images showed API multi-test system strip (a) on top containing inoculums of TM P2 while strip (b) at below containing inoculums of CE B2.

Table 4.9 Summary of Biochemical Tests

Biochemical tests		Bacterial code	
		TM P2	CE B2
β - galactosidase	ONPG	+	+
Arginine dihydrolase	ADH	+	-
Lysine decarboxylase	LDC	-	+
Ornithine decarboxylase	ODC	+	-
Citrate utilization	CIT	+	+
H_2S production	H_2S	-	-
Urease production	URE	-	+
Tryptophan deaminase	TDA	-	-
Indole production	IND	-	-
Voges-Proskauer	VP	+	+
Gelatin hydrolysis	GEL	-	-
Glucose	GLU	+	+
Mannitol	MAN	+	+
Inositol	INO	+	+
Sorbitol	SOR	+	+
Rhamnose	RHA	+	+
Sucrose	SAC	+	+
Melibiose	MEL	+	+
Amygdalin	AMY	+	+
Arabinose	ARA	+	+
Oxidase production	OX	-	-
NO_2 production	NO_2	+	+
N_2 production	N_2	+	+
MacConkey agar	McC	+	+
Oxidation	OF-O	+	+
Fermentation	OF-F	+	+

Legend: +: Positive, -: Negative

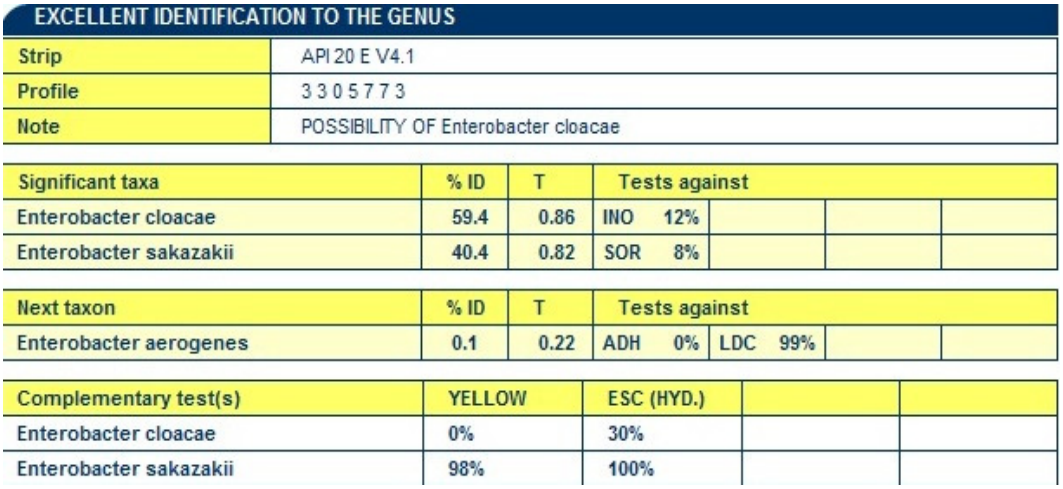
4.4.2 Identification of Bacterial Isolates

Based on the identification result obtained from apiweb^{TM}, TM P2 was identified as bacteria from the genus of *Enterobacter*. Two probable bacteria strains for TM P2 where may be either *Enterobacter clocae*, which has identity of 59.4% and T value of 0.86, or *Enterobacter sakazakii*, with an identity of 40.4% and T value of 0.82. On the other hand, for CE B2, it showed significant taxa of *Klebsiella pneumonia ssp pneumonia*, which has the identity of 97.6% and T value of 1.0. However, it also has the possibility to be *Raoultella planticola*. Thus, further identifications are needed to validate the identity of the bacterial isolates. Figure 4.6 showed the print screen images of the results for both TM P2 and CE B2 obtained from apiweb^{TM}.

4.4.3 Further Identifications of Bacterial Isolates TM P2 and CE B2

Both TM P2 and CE B2 were further tested with the complementary tests suggested by apiweb^{TM}, yellow test and methyl red test, respectively to validate their identity. In yellow test, TM P2 was streaked onto LB agar and incubated overnight at 37°C. The colour of colonies observed was ivory (Figure 4.7). Hence, it indicates that it has a bigger possibility to be *Enterobacter clocae* rather than *Enterobacter sakazakii*. As for CE B2, methyl red test was performed. A negative reaction result was obtained (Figure 4.8), indicated that it has a bigger possibility to be *Klebsiella pneumonia ssp pneumonia* rather than *Raoultella planticola*.

(a)



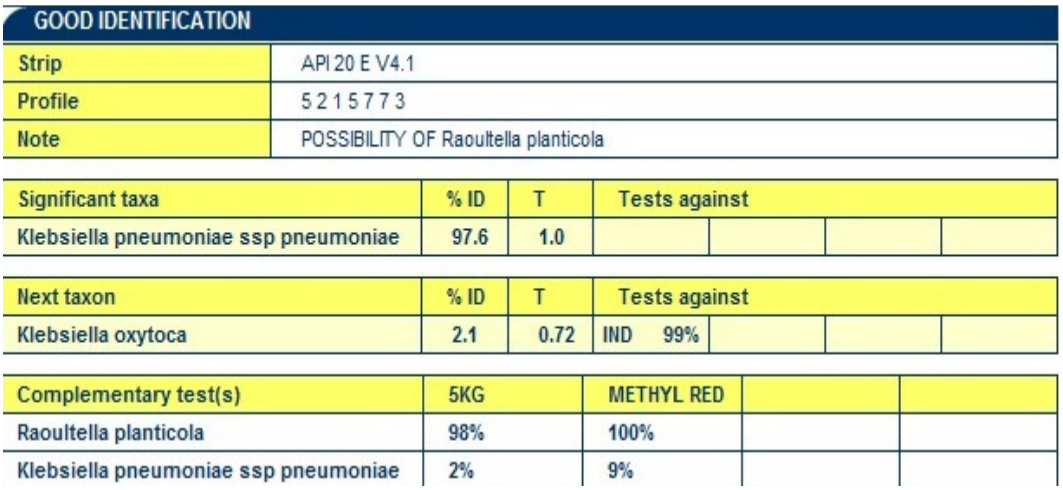
EXCELLENT IDENTIFICATION TO THE GENUS	
Strip	API 20 E V4.1
Profile	3 3 0 5 7 7 3
Note	POSSIBILITY OF Enterobacter cloacae

Significant taxa	% ID	T	Tests against			
Enterobacter cloacae	59.4	0.86	INO 12%			
Enterobacter sakazakii	40.4	0.82	SOR 8%			

Next taxon	% ID	T	Tests against			
Enterobacter aerogenes	0.1	0.22	ADH 0%	LDC 99%		

Complementary test(s)	YELLOW	ESC (HYD.)		
Enterobacter cloacae	0%	30%		
Enterobacter sakazakii	98%	100%		

(b)



GOOD IDENTIFICATION	
Strip	API 20 E V4.1
Profile	5 2 1 5 7 7 3
Note	POSSIBILITY OF Raoultella planticola

Significant taxa	% ID	T	Tests against			
Klebsiella pneumoniae ssp pneumoniae	97.6	1.0				

Next taxon	% ID	T	Tests against			
Klebsiella oxytoca	2.1	0.72	IND 99%			

Complementary test(s)	5KG	METHYL RED		
Raoultella planticola	98%	100%		
Klebsiella pneumoniae ssp pneumoniae	2%	9%		

Figure 4.6 Result of Identification of Bacterial Isolates Obtained from apiweb™

Images show the print screen result from apiweb™ of bacterial isolate (a) TM P2 and (b) CE B2.

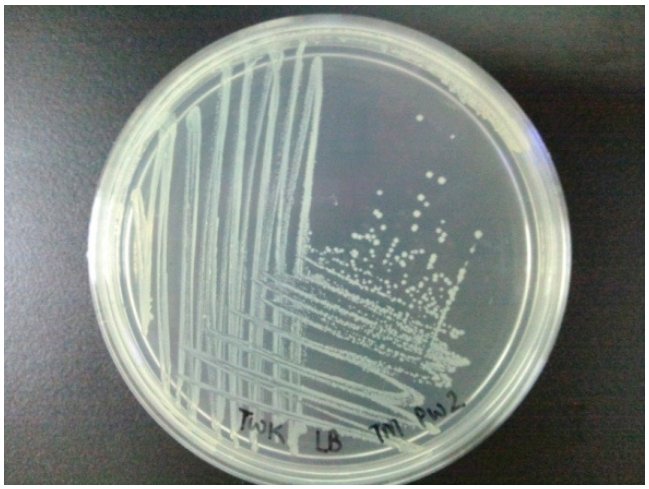


Figure 4.7 Observation of TM P2 on LB Agar Plate

Image showed colonies of TM P2 on LB agar plate which were ivory in colour.

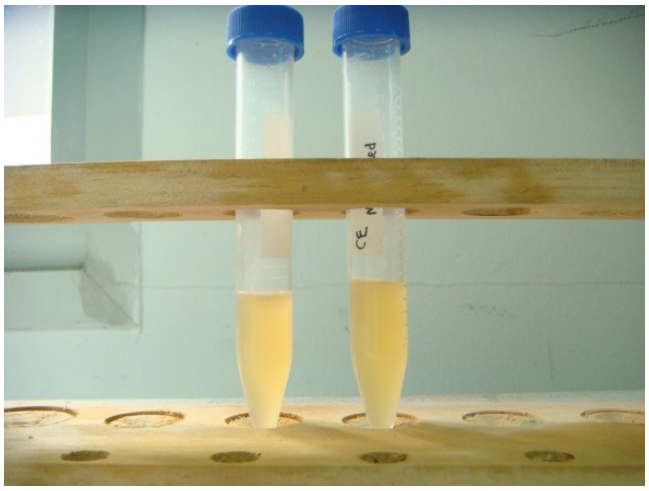


Figure 4.8 Methyl Red Test of CE B2

Image showed methyl red test of CE B2 with negative control tube on left and CE B2 methyl red test tube on the right.

4.5 Analyses of PCR Product and Agarose Gel Electrophoresis

4.5.1 Purity of Extracted DNA

DNA extracted from all the bacterial isolates was considered as relatively pure as their A_{260}/A_{280} ratio was within the range of 1.8 to 2.0 except for TM P1, CC P1, CE P1 and BR B2. The A_{260}/A_{280} ratio of TM P1, CC P1 and BR B2 were slightly higher than 1.8, indicates that they might be contaminated with RNA. For CE P1 with the A_{260}/A_{280} ratio of 1.69, it indicates that the DNA might be contaminated with residual protein (Sambrook & Russell, 2001). The A_{260}/A_{280} ratio and concentration of bacterial isolates were tabulated in Table 4.10.

4.5.2 *bla*-CTX-M PCR Products and Gel Purified PCR Products

Figure 4.9 showed the agarose gel electrophoresis image of 12 PCR products from TM B1, TM B2, TM P1, TM P2, CC B1, CC B2, CC P1, CC P2, CE B1, CE B2, BR B1 and BR B2, amplified using *bla*-CTX-M degenerate primers with annealing temperature of 50°C. Other 13 PCR products were not being showed in Figure 4.9 as the results were negative.

Lane 1 is the negative control and as expected, no product was observed. On the other hand, only PCR product from TM P2 corresponded near to the expected approximately 590 bp was successfully amplified. However, smear and primer-dimer appearing indicates that the PCR condition might not be optimised yet. Thus, the DNA of TM P2 was re-amplified again with the annealing temperatures of 53°C and 55°C. Figure 4.10 (a) showed agarose gel

electrophoresis image of PCR products from TM P2 using annealing temperature of 50°C, 53°C, and 55°C. The optimum temperature for *bla*-CTX-M degenerate primer was determined to be at 53°C as the least smear and primer-dimer were observed at this temperature.

Subsequently, the resulting PCR product from TM P2 amplified by *bla*-CTX-M degenerate primers using annealing temperature of 53°C was purified as describe in Section 3.7.1. The concentration and purity of gel-purified product was measured by Nanodrop 1000, showed reading of 8.5 ng/µl and 1.85, respectively. Image of agarose gel electrophoresis for the gel-purify PCR product was shown in Figure 4.10 (b).

4.5.3 *bla*-PER-2 PCR Products

None of the PCR product mentioned corresponded to the expected size of 740 bp was successfully amplified. Thus, it was presumed that all the 25 isolates obtained in this study did not possess any *bla*-PER-2 homologous genes.

Table 4.10 A_{260}/A_{280} Ratio and Concentration of Bacterial Isolates DNA Obtained using Nanodrop 1000

Bacterial Isolates (code)	A_{260}/A_{280}	Concentration (ng/µl)
TM B1	1.82	323.47
TM B2	1.89	677.34
TM B3	1.80	410.41
TM B4	1.99	786.34
TM B5	1.84	542.31
TM P1	2.02	375.86
TM P2	1.86	475.60
TM P3	1.84	496.94
CC B1	1.92	508.91
CC B2	1.94	702.99
CC B3	1.98	912.32
CC B4	1.88	519.85
CC P1	2.03	894.96
CC P2	1.90	778.95
CC P3	1.92	696.12
CC P4	1.81	503.40
CE B1	1.82	429.41
CE B2	1.85	719.79
CE B3	1.92	329.60
CE P1	1.69	393.32
CE P2	1.81	516.87
BR B1	1.89	370.19
BR B2	2.09	772.30
BR P1	1.92	728.54
BR P2	1.94	429.31

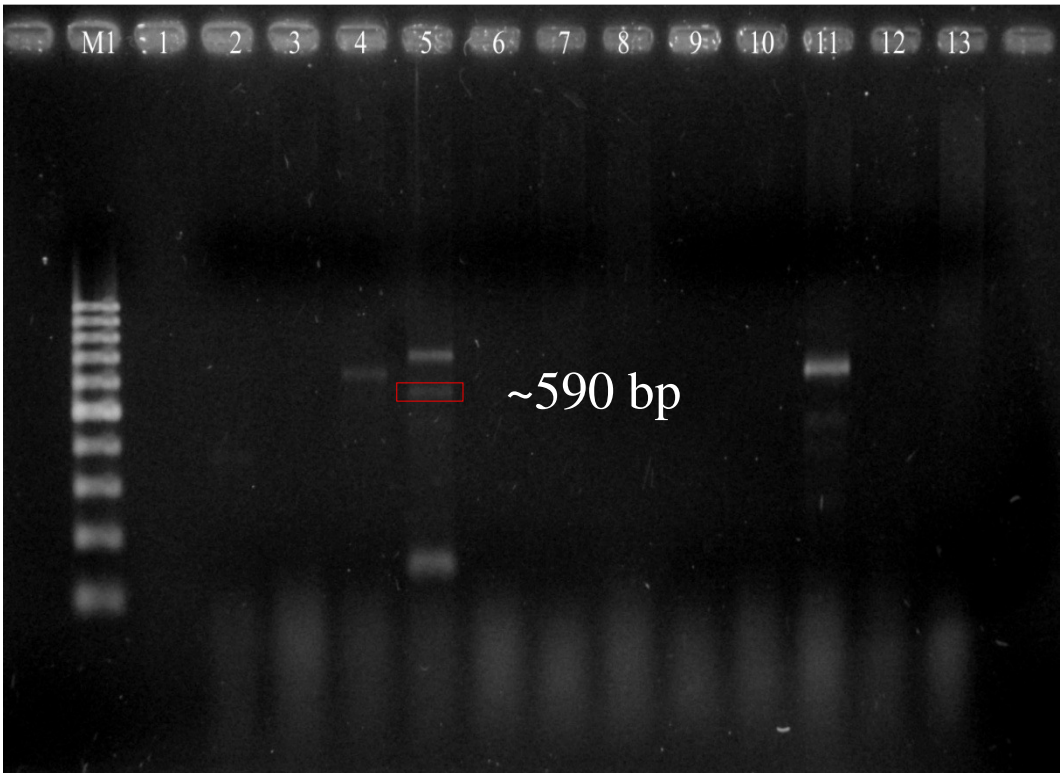


Figure 4.9 Representative Gel Electrophoresis Image of PCR Products Amplified using *bla*-CTX-M Degenerate Primers with Annealing Temperature of 50°C

Lane M1 is the 100 bp DNA ladder (Fermentas). Lane 1 represents the negative control. Lane 2 to 13 represents the PCR products of bacterial isolates TM B1, TM B2, TM P1, TM P2, CC B1, CC B2, CC P1, CC P2, CE B1, CE B2, BR B1 and BR B2 amplified using *bla*-CTX-M degenerate primers, respectively. Only PCR product from TM P2 in lane 5 showed a band with the size about 590 bp.

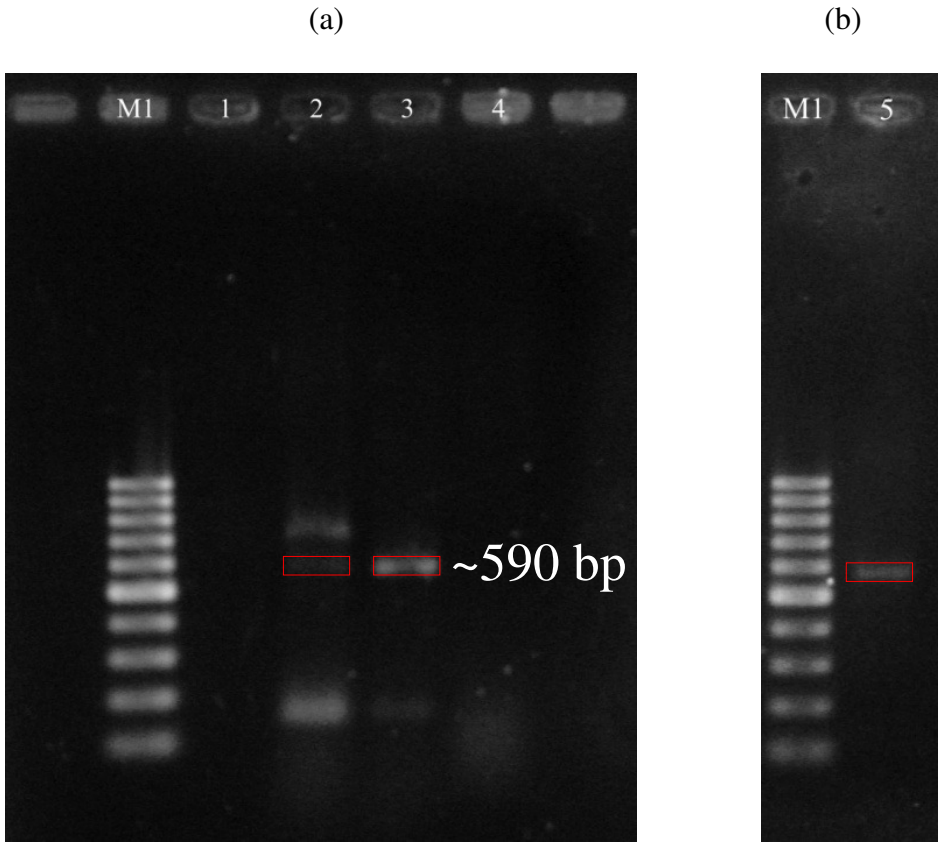


Figure 4.10 Gel Electrophoresis Images for the Optimisation of PCR Reaction for *bla*-CTX-M Degenerate Primers and Gel-purified PCR Products

Image showed (a) Optimisation of PCR Reaction for *bla*-CTX-M Degenerate Primers and (b) Gel-purified PCR Products. Lane M1 is the 100 bp DNA ladder (Fermentas) while Lane 1 represents the negative control. Lane 2 to 4 represents the PCR products of TM P2 amplified with different annealing temperatures of 50°C, 53°C and 55°C, respectively. Lane 3 showed the most efficient PCR amplification with least smear and primer-dimer formation. Lastly, lane 5 represents the gel-purified PCR products obtained in lane 3, showed a distinct band with the size of about 590 bp.

4.6 Transformation

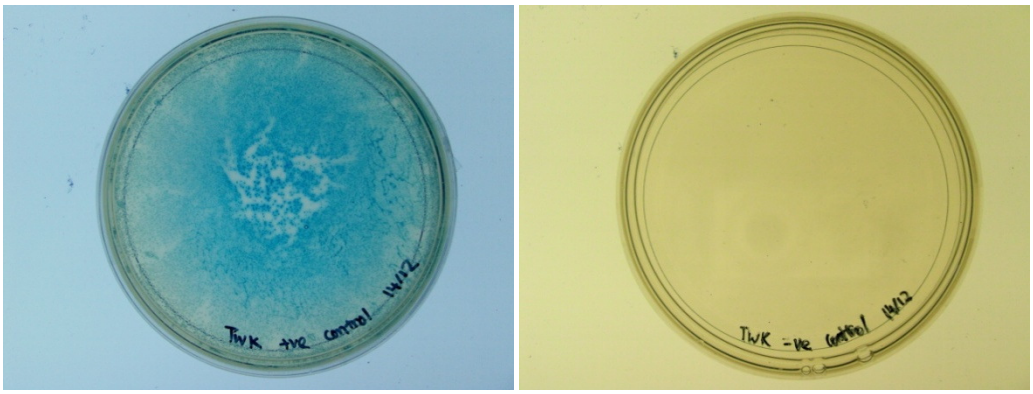
4.6.1 Screening of the Presence of Insert in the Vector

The transformation process was performed as mentioned in Section 3.9.2. As shown in Figure 4.11, white and blue colonies formation representing the transformed and untransformed bacterial cells, respectively. On the other hand, the massive number of blue colonies presented on positive control plate indicated the high efficiency of competent cells whereas no colony formation on negative control plate showed proper aseptic techniques were being applied during the transformation process.

4.6.2 Colony PCR

A potential white colony of transformed competent cells which suspected to harbour *bla*-CTX-M gene fragment was assessed. Colony PCR were performed as mentioned in Section 3.9.3. Figure 4.12 showed the results for colony PCR. No product was amplified in the absence of DNA as shown in Lane 1, indicating that the PCR process is contaminant-free and the products amplified were probably the desired PCR products. The amplification of untransformed bacterial cells, blue colony was showed in lane 2 with the expected size of around 180 bp whereas the amplification of the white colony which suspected carrying *bla*-CTX-M gene fragment was shown in lane 3 the expected product size of approximately 780 bp. Recombinant plasmids were extracted from the white colony and DNA sequencing was performed.

(a) (b)



(c)

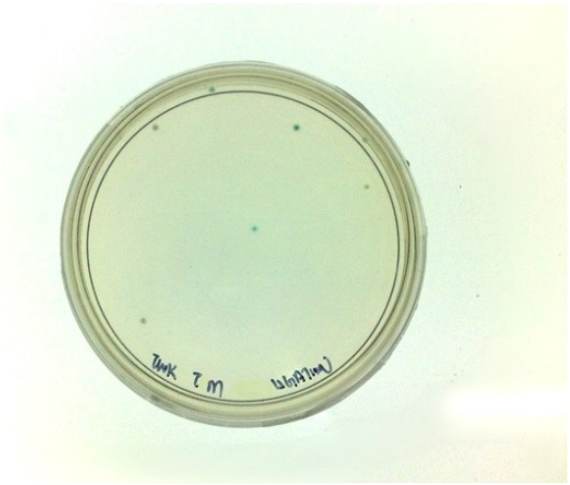


Figure 4.11 Positive, Negative Control Plates and Transformation Plate

Plates A and B indicate the positive and negative controls of the transformation process respectively. A massive number of blue colonies were formed on positive control plate while no colony was presented on negative control plate. On the other hand, obvious blue and white colonies were observed on the transformation plate, plate C.

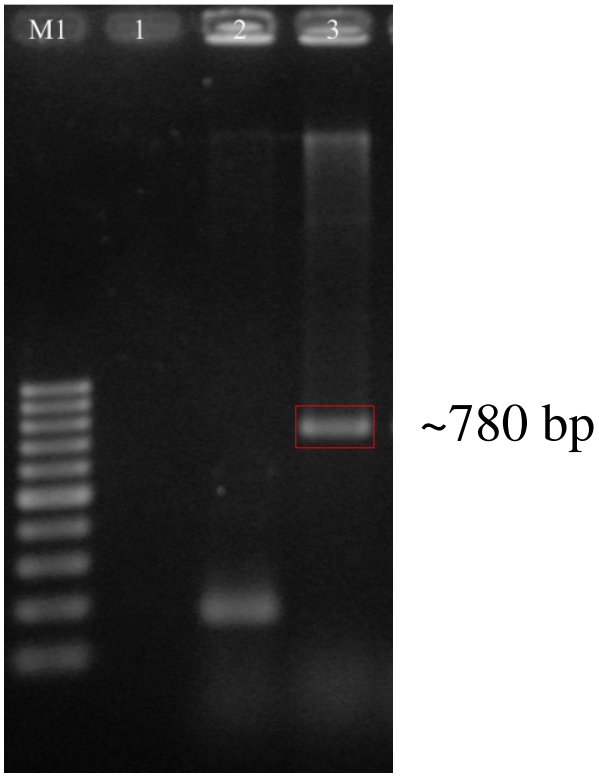


Figure 4.12 Gel Electrophoresis Image of Colony PCR Amplification

The amplified colony PCR prodcucts were expected to have a product size of approximately 780 bp. Lane M1 is the 100 bp DNA ladder (Fermentas) while Lane 1 represents the negative control. Lane 2 represents the amplification of blue colony, shows a clear band approximately to the size of 180 bp whereas Lane 3 represents the amplification of the white colony, and shows a clear band of the size of around 780 bp.

4.7 DNA Sequencing

Sequencing result obtained was reported as reliable sequences by 1st Base laboratories. The nucleotide sequence of each sample was analysed using BlastX program which search the protein database. Table 4.11 tabulated the obtained BlastX results for the amplified samples using *bla*-CTX-M degenerate primers.

Table 4.11 Top Five BlastX Alignments from TM P2 Forward Sequence Amplified using *bla*-CTX-M Degenerate Primers

BlastX identity	Accession	Identities (%)	Score (bits)	Query coverage (%)	Expect value
• **Proline/glycine betaine transporter [*Citrobacter* spp.]**	ZP 04558676.1	91	257	72	6e-67
• **Metabolite-proton symporter [*Enterobacter clocae*]**	CBK844563.1	99	254	68	3e-66
• **Proline/glycine betaine transporter [*Escherichia coli* O157:H7]**	ZP 0386445.1	99	254	68	3e-66
• **Proline/glycine betaine transporter [*Escherichia coli* 1_1_43]**	ZP 04871198.1	93	254	71	4e-66
• **Proline/glycine betaine transporter [*Escherichia coli* CFT073]**	NP 756966.1	93	254	71	4e-66

CHAPTER 5

DISCUSSION

5.1 Cultivation and Isolation of Gram-negative Bacteria

In this study, Gram-negative bacteria were cultivated and isolated from four types of raw vegetables. Brain-Heart Infusion Broth (BHIB), Buffered Peptone Water (BPW) broth were used for cultivation purpose while MacConkey (MAC) agar and Violet Red Bile Glucose (VRBG) agar were used for selection and isolation purposes.

BHIB, a type of general non-selective medium was used to cultivate bacteria from raw vegetables as it supplies the nutritional requirements for growth of microorganisms and able to maintain the osmotic balance of the medium. On the other hand, BPW was used in this study as it is rich in nutrients and produces high recovery rates for injured bacteria and intense growth. Furthermore, the phosphate buffer system prevents bacterial damage due to changes in the pH of the medium as vegetable specimens in general have a low buffering capacity (Zimbro, Power, Miller, Wilson & Johnson, 2009).

Both Gram-positive and Gram-negative bacteria were able to grow equally in BHIB and BPW. Thus, the cultivated Gram-negative bacteria were isolated and validated using MAC agar and VRBG agar, respectively. A positive validation

was indicated by their ability to grow on VRBG agar. The growth of Gram-positive bacterium was inhibited by bile salts and crystal violet dye in both MAC and VRBG agar. (Flournoy, Wongpradit & Silberg, 1990).

MAC and VRBG are also a differential medium. MAC has the ability to differentiate lactose and non-lactose fermenting bacteria based on the colour of the colonies. For instance, lactose fermenting bacteria, their colonies will appear in pink on the MAC agar plate as shown in Figure 4.1 (a). However, it cannot differentiate non-lactose and non-glucose fermenting bacteria (Pompei, Berlutti, Thaller"b, Ingiannia & Sattac, 1996). In order to solve this problem, VRBG was used. As such, non-lactose fermenting but glucose fermenting Gram-negative bacterium was differentiated using VRBG agar (Zimbro et al, 2009). The formation of purple-red colonies as shown in Figure 4.2 (b) indicated the occurrence of glucose fermentation.

5.2 Differential Staining – Gram's Stain

The Gram's staining method was named after Christian Gram, a Danish bacteriologist. It is the most widely used method nowadays in differentiating bacteria into two major groups, i.e., Gram-positive and Gram-negative, on the basis of their Gram's reactions (Harley & Prescott, 2002).

Gram-negative bacteria are stained in pink after Gram's staining as the lipopolysaccharide layer of Gram's negative bacteria was disrupted by the alcohol

wash, resulting the CV-I complexes. This is formed by the combination crystal violet dye and iodine it is washed out from the cells. Thus, they remain colourless until it is counterstained by safranin, after which appear in pink. On the other hand, Gram-positive bacteria have a thicker peptidoglycan cell wall than Gram's negative bacteria, resulting in the CV-I complexes cannot be washed out from the cells. As a result, Gram-positive bacteria retain the dye and appear in purple. (Tortora et al., 2010)

All the 25 isolates were validated as Gram-negative bacteria after Gram's staining. In this study, only young culture cells were being used in Gram's staining in this study as the Gram's reaction is most consistent when it is used on young bacterial cells.

5.3 Catalase Test

Catalase test is to detect the presence of catalase enzyme by the decomposition of hydrogen peroxide to release oxygen and water in most of the cytochrome-containing aerobic and facultative anaerobic bacteria (Doelle, 1969) except *Streptococcus* and *Enterococcus* spp.. The presence of catalase enzyme is relatively important to both aerobic and facultative anaerobic bacteria as hydrogen peroxide is formed as an oxidative end product of the aerobic breakdown of sugars. A high amount of hydrogen peroxide will result in cell death as it is highly toxic (MacFaddin, 2000). All bacterial isolates were catalase positive, which was in tally with the theoretically identification characteristic of Enterobacteriaceae.

5.4 O-F (Oxidation-Fermentation) Test

The O-F test, also known as the "oxferm" test, is used to determine either oxidative or fermentation routes is used for a bacterium in metabolizing glucose and other carbohydrates. Oxidative organisms can only metabolize carbohydrates under oxidative reaction while facultative anaerobes can metabolites carbohydrates in both oxidative and fermentative reactions (Brown, 2001). As the end product of metabolizing a carbohydrate is an acid, it can be detected by the pH indicator which present in the O-F medium. All bacterial isolates obtained turned the colour of the medium from green into yellow throughout both open and closed tubes (Figure 4.4), indicated all of them were actually fermenting organisms.

5.5 Oxidase Test

The oxidase test can be used to determine if an organism possesses the cytochrome oxidase enzyme. The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride, a redox dye in its reduced form, acts as an artificial electron acceptor and forms indophenol blue in the presence of oxidase enzyme. All 25 bacterial isolates were oxidase negative, indicating that they lacked of cytochrome C oxidase.

5.6 Antibiotic Susceptible Test

All the 15 types of commercial antibiotic disks mentioned in Section 3.3.3 were used in this study to test the presence of multiple resistance genes. In addition,

aztreonam, cefotaxime, ceftazidime, cefpodoxime, ceftriazone, ampicillin, oxacillin, ceftazidime/clavulanate, cefotaxime/clavulanate and amoxicillin/clavulanate were also used for the investigation of the presence of potential ESBL-producer.

As stated by Finch, Greenwood, Norrby and Whiteley (2003), a bacterial isolate can be labeled as multidrug-resistant if it was reported to be resistant towards at least two of the first-line agents. In this study, nine isolates were labeled as multi-drug-resistant, namely TM P2, CC P1, CC P2, CC P4, CE B1, CE P1, CE P2, BR B1 and BR P2 as they have the ability to resist more than one type of the first-line antibiotics.

The presence of ESBL genes can be detected via the phenotypic test, which is comparing the inhibition zone diameter for 3rd generation cephalosporins tested in combination with clavulanic acid versus its zone when it tested alone based on the antibiotic susceptible test. Based on the result obtained, a total of 2 bacterial isolates, TM P2 and CE B2 showed a positive result for the phenotypic test.

According to Rawat and Nair (2010), an ESBL-producer demonstrates resistance towards penicillins, 1st generation, 2nd generation, 3rd generation cephalosporins and monobactam but not the cephamycins or carbapenems. Thus, only TM P2 alone fulfilled all the criteria mentioned as it is resists towards oxacillin, ampicillin and amoxicillin/clavulanate which belong to the class penicillin;

aztreonam which belongs to the class of monobactam; cefotaxime, ceftazidime, cefpodoxime and ceftriazone which belong to the class of 3rd generation of cephalosporins. As for CE B2, it was just suspected as a normal beta lactamase-producer as it did not shows resistance towards the mentioned antibiotics.

In short, after analysing the detection results among penicillin, monobactam, 3rd generation cephalosporins and the phenotypic test of all the 25 bacterial isolates, it can be concluded that only TM P2 has a big possibility of being a potential ESBL-producer whereas bacteria CE B2 might not be a ESBL-producer as it did not fulfills the requirements for being an ESBL-producer although it showed a positive result for the phenotypic test.

5.7 Identification of Bacterial Isolates

To identify an organism using API 20E kit, the profiles obtained was compared to the profiles of the taxa in the database. The accuracy of API identification is greatly dependent on the percentage of ID (identity) and the T index obtained from the tested bacterial profile. The percentage of ID is the relative proximity to the different taxa of the database and it allows us to determine whether the profile obtained is close to a taxon. On the other hand, T index represents the proximity to the most typical profile in each of the taxa. In this study, bacterial isolates TM P2 and CE B2 were further characterised using API 20E kit.

Based on the identification result obtained from apiweb^{TM}, TM P2 was able to identify up the genus level. The result indicated that TM P2 might be *Enterobacter clocae*, which has identity of 59.4% and T value of 0.86, or *Enterobacter sakazakii*, which has identity of 40.4% and T value of 0.82 as shown in Figure 4.6 (a). A possible reason explaining why both identity percentages are relatively low as there is only 19% differences in both species due to *Enterobacter sakazakii* has a biochemical profile that very similar to that of *Enterobacter clocae* (Nazarowec-White, Farber, Reiji, Cordier & Schotrst, 2003). According to Farmer and co researchers (1980), *Enterobacter sakazakii* was named as "yellow-pigmented *Enterobacter clocae*" in the past, showing that colonies colour of *Enterobacter sakazakii* are yellow in colour whereas *Enterobacter clocae* is non-pigmented in general (Sakazaki, 1974). As a result, the colonies colour of TM P2 are needed to be observed to further identify the bacterial identity and the colour being observed was ivory in colour (Figure 4.7). Thus, it gives a better indication that TM P2 might to be *Enterobacter clocae* rather than *Enterobacter sakazakii*.

On the other hand, CE B2 showed significant taxa of *Klebsiella pneumonia ssp pneumonia*, which has the identity of 97.6% and T value of 1.0. There is also a possibility that it could be *Raoultella planticola* as shown in Figure 4.6 (b). As demonstrated by Alves, Silva Dias, Dias de Castro, Riley and Moreira (2006), the appropriate identification of *Klebsiella* spp. is not easily achieved in most clinical microbiology laboratories due to several species

especially *Klebsiella pneumonia* and *Raoultella planticola* sharing a similar biochemical profile. Several findings (Monnet, Freney, Brun, Boeufgras, & Fleurette, 1991; Westbrook, O'Hara, Roman & Miller, 2000) showed that some isolates that were being classified as *Klebsiella pneumonia* could in fact be *Raoultella planticola*. Thus, a complementary methyl red test, suggested by apiweb^{TM}, was carried out. It showed negative reaction result (Figure 4.8), which indicates that it has a bigger possibility of being *Klebsiella pneumonia ssp pneumonia* as compared to *Raoultella planticola*.

5.8 Total DNA Extraction

In this study, fast boil method (Holmes & Quigley, 1981) was used to extract total DNA from the bacterial isolates as this method is fast, inexpensive, repeatable, does not need any specific reagents and yield reasonable results (Sadeghi, Najafabadi, Abedi & Dehkordi, 2008). The concentration of total DNAs extracted are relatively high as they ranged from 323.47 ng/µl to 912.32 ng/µl. The purity of the total DNA mentioned was relatively pure. An absorbance ratio of 260 nm to 280 nm around 1.8 indicates of a pure DNA, whereas ratio which was less than 1.8 and more than 2.0 indicates of protein contamination and RNA contamination, respectively (Sambrook & Russell, 2001). Twenty one of 25 bacterial total DNAs absorbance ratios of 260 nm to 280 nm were ranged between 1.8 to 2.0 while the other four were just slightly deviated from the pure DNA range.

5.9 PCR Amplification

All the reagents and PCR conditions contributed crucial roles during the PCR amplification. *Taq* DNA polymerase, assay buffer, deoxynucleoside triphoshates, stabilizing agents, and primers aid in the amplification of the DNA template *in vitro* to get a detectable quantities (Singh & Kumar, 2001). Moreover, the parameters of PCR also play a vital role in the amplification of desired products. During PCR amplification, template-independent primer interactions can take place that give rise to some of the non-specific products like primer dimers (Rychlik, 1995).

Initially, the annealing temperature used for PCR amplification of *bla*-CTX-M genes was 50°C. However, smearing as well as primer dimers were observed after agarose gel electrophoresis (Figure 4.9). This phenomenon might be due to the PCR condition was not specific enough. According to Rychlik, Spencer, and Rhoads (1990), empirical experiments have to be performed to determine the optimal temperature of PCR amplification. Thus, optimisation of *bla*-CTX-M primer was carried out with the annealing temperature ranging from 50°C to 55°C, as mentioned in Section 3.6.3. PCR amplification using annealing temperature of 53°C was shown to demonstrate optimal annealing temperature as it gave the least primer dimmers smearing (Figure 4.10).

On the other hand, the PCR amplification conditions for *bla*-PER-2 genes used were as suggested by the study by Petroni et al. (2002). Similar set of PCR

primers were also utilised as well. Based on the result obtained, none of the 25 isolates showed any detectable gene amplification on the agarose gel electrophoresis image. It was presumed that all of the 25 bacterial isolates were *bla*-PER-2 negative. As the sample size of this study was relatively small, it was not be able to conclude that there is no other *bla*-PER-2 producer. Several literatures demonstrated that *bla*-PER-2 producers were only restricted to South America (Celenza et al., 2006; Power et al., 2007). Further investigations such as optimising the other PCR parameters or the use of another set of *bla*-PER-2 gene specific primers could be performed to validate the presence or absence *bla*-PER-2 gene in all bacteria isolates. In order to warrant that there are no other *bla*-PER-2 producers, a larger sampling size is required.

5.10 Transformation

Transformation is a process which involves the acquisition of naked DNA from the extracellular environment, and the ability to undergo transformation is term genetic competence (Chen & Dubeau, 2004). Transformation process was performed to facilitate the DNA uptake of chemically treated *Escherichia coli* JM 109 competent cells, using $CaCl_2$ transformation method after the gel purification process (Sambrook & Russell, 2001).

As introduced by Mandel and Higa (1970), Ca^{2+} chemical transformation is a suitable and economical method to induce the DNA uptake of *Escherichia coli*. Since high concentration of Ca^{2+} will influence on the phospholipids composition

on cells, pre-incubation of bacteria cells in chilled $CaCl_2$ will render the cell membranes to be more permeable, resulting from the breaking down of the outer membrane. As a result, it facilitates the uptake of heterogeneous DNA during the heat shock treatment. Also, the culture must be collected in early logarithmic phase and cell density should be low in order to increase the efficiency of transformation.

Transformed cells were plated on LB agar containing ampicillin together with X-gal and IPTG, to enable the screening and selection purposes. Ampicillin was chosen due to the presence of ampicillin resistant gene present within the pGEM®-T Easy vector that used in this study. By doing so, undesirable growth of other microbes can be eliminated. Positive control plate (Figure 4.11) indicates the high efficiency of competent cells and chemicals, with inserted pUC19 vector. Both blue and white colonies were presented on the transformation plate, illustrates high transformation efficiency. The formation of blue colonies was due to the absence of recombinant DNA in the bacteria cells whereas the formation of white colonies indicates the recombinant DNA was inserted successfully into the transformed bacteria.

5.11 BlastX Alignment Search

In this study, the extracted recombinant DNA plasmid of TM P2 was sequenced. Both forward and reverse DNA strands were sequenced using T7 and SP6 primers,

respectively. The DNA sequences were then aligned using BlastX analysis software as provided by NCBI.

Instead of getting the desired *bla*-CTX-M genes, BlastX alignments search showed highest similarity with proline/glycine betaine transporter of *Citrobacter* spp. (91%), followed by metabolite-proton symporter of *Enterobacter cloacae subsp. cloacae* (99%) (Refer to Table 4.11). None of the BlastX alignment search showed similarities to *bla*-CTX-M genes sequences. Several factors might attribute to this incident. First of all, according to Pagani et al. (2003) findings, the primers used for amplification of *bla*-CTX-M genes was a set of degenerate primers which were designed based on the conserved regions of *bla*-CTX-M genes. This set of degenerate primers was able to amplify various types of *bla*-CTX-M genes, including *bla*-CTX-M-1 to *bla*-CTX-M-30, *bla*-TOHO-1 to *bla*-TOHO-3, *bla*-FEC-1, *bla*-UOE-1, and *bla*-UOE-2 (Pagani et al., 2003). Also, literature stated that degenerate primers have a higher probability of causing undesired and possible permutation which is likely to be recognized other as gene product, hence non-specific or undesired product might be amplified out (Dieffenbach & Dveksler, 2003).

Further analysis of the DNA sequenced revealed that instead of amplifying the desired gene product, the reverse primer (CTXM_R primer) acted as both forward and reverse primers in the PCR amplification. The sequence of CTXM_R (5'– TGG GTR AAR TAR GTS ACC AGA –3'), consist of degenerate

oligonucleotides where R could be adenine or guanine whist S could be be either guanine or cytosine. Thus, it serves as forward primer with the sequence of 5' – TGG GTG AAA TAG GTG ACC AGA – 3'and as reverse primer with the sequence of 5' – TGG GTA AAG TAA GTG ACC AGA – 3'. After analysing the sequences, it showed that there were only three single nucleotide differences (as highlighted in red) in both forward and reverse primers served by CTXM_R in this amplification. Hence, it can be concluded that the *bla*-CTX-M degenerate primers used in this study was not specific enough to detect *bla*-CTX-M genes in bacterial isolate TM P2.

By calculating using the nearest-neighbor thermodynamic theory, the melting temperature of CTXM_F and CTXM_R were approximately 47.7°C to 51.8°C and 48.5°C to 54.4°C, respectively. As a rule of thumb, the annealing temperature of the primers used should be 5°C lower than its respective melting temperature (Dieffenbach & Dveksler, 2003). However, the annealing temperature of 53°C was used in this study and this might caused CTXM_F failing to anneal to its specific site on the desired genes as the annealing temperature used was higher than the melting temperature of CTXM_F.

Lastly, based on the BlastX alignment result, the sequence aligned with high similarity to the metabolite-proton symporter of *Enterobacter cloacae*. The resultant alignment demonstrated a high similarity of 99% identity and a low E-value of 3e-66. The bacterial isolate was identified as *Enterobacter cloacae* via

apiweb™ as shown in Figure 4.6 (a). Analysis of the BlastX alignment result was in accordance with the result obtained from apiweb™ where the metabolite-proton symporter was also from *Enterobacter cloacae*.

5.12 Future Studies

ESBL homologous genes are constantly evolving due to various selection pressures and the ability of the microorganism to adapt. Thus, the origin and identity of the antibiotic resistance bacteria should be determined. Characterising the bacterial pool found on vegetables will lead us to a better understanding of the relationship between vegetables and foodborne diseases. For future studies, more variety and a larger sampling number of vegetables from different sources should be considered for a more detailed investigation.

To detect the ESBL homologous genes, several aspects in this study can be modified and improved. First of all, more antibiotic discs can be included in antibiotic susceptible test. For instance, tazobactam should be tested on bacteria isolates suspected to harbor *bla*-CTX-M genes as it yields better result compared to other beta lactamases inhibitors (Bradford et al., 1998; Tzouvelekis et al., 2000). Moreover, a more specificity set of primers can be designed for the detection of the genes of interest. A suitable approach can include nested PCR where it would enhance the specificity of amplifying the *bla*–CTX-M homologous genes.

Lastly, TM P2 will be tested for other ESBL genes as it is shown to be a potential ESBL-producer. The 16S rRNA gene will be sequenced out in future studies to further validate its identity too.

CHAPTER 6

CONCLUSIONS

Four different types of vegetable (tomato, cucumber, celery and broccoli) were subjected to characterise for the presence of multiple antibiotics resistance and ESBL genes. A total of 25 bacterial isolates with resistance to ampicillin were isolated based on their different morphological characteristics. All isolates were found out to be Gram-negative, catalase positive, oxidase negative and facultative anaerobes, fulfilling the preliminary criteria of Enterobacteriaceae.

Based on the antibiotic susceptible test, all isolates were resistant towards antibiotics classified as penicillin. Nine isolates were demonstrated to be multidrug-resistant bacteria. Although both TM P2 and CE B2 showed positive phenotypic result, only TM P2 is classified as a potential ESBL-producer. CE B2 did not show any resistance towards aztreonam and amoxicillin/clavulanate and hence did not fulfil the criteria of being ESBL-producer. TM P2 and CE B2 were biochemically characterised as *Enterobacter cloacae* and *Klebsiella pneumonia ssp pneumonia*, respectively.

No *bla*-CTX-M and *bla*-PER-2 genes were amplified successfully from all the bacterial isolates using *bla*-CTX-M degenerate primers and *bla*-PER-2 gene specific primers in this study. Although a clear band with the expected size of

approximately 590 bp was successfully amplified using *bla*-CTX-M degenerate primers from TM P2, BlastX alignment showed alignment to metabolite-proton symporter. The result could be due to the non-specific amplification of target gene and the use of non-optimised PCR condition.

TM P2 will be further characterised using various ESBL genes specific primers in future to verify whether it is a true ESBL-producer. Moreover, 16S rRNA gene sequencing will also be performed for further characterisation.

REFERENCES

Alba, J., Ishii, Y., Galleni, M., Frere, J. M., Ito, M., & Yamaguchi, K. (2002). Cefcapene Inactives Chromosome-encoded Class C β-lactamases. *Journal of Infection and Chemotherapy*, *8*(3), 207-210.

Alves, M. S., Silva D. R., Dias de Castro, A. C., Riley, L. W., & Moreira, B. M. (2006). Identification of Clinical Isolates of Indole-positive and Indole-negative *Klebsiella* spp. *Journal of Clinical Microbiology*, *44*(4), 3640-3646.

Ambler, R. P. (1980). The Structure of β-lactamases. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *289*, 321-331.

Arlet, G., & Philippon, A. (1991). Construction by Polymerase Chain Reaction and Intragenic DNA Probes for Three Main Types of Transferable β-lactamases (TEM, SHV, CARB). *FEMS Microbiology Letters*, *82*(1), 19-25.

Barthelemy, M., Peduzzi, J., Bernad, H., Tancrede, C., & Labia, R. (1992). Close Amino Acid Sequence Relationship between the New Plasmid-mediated Extended-spectrum β-lactamases MEN-1 and Chromosomally Encoded Enzymes of *Klebsiella oxytica*. *Biochimica et Biophysica Acta*, *1122*, 15-22.

Baron, S. (1996). *Bacterial Resistance*. Galveston: The University of Texas Medical Branch.

Bauernfeind. A., Stemplinger, I., Jungwirth, R., Mangold, P., Amann, S., Akalin, E., et al. (1996). Characterisation of β-lactamases. *Antimicrobial Agents and Chemotherapy*, *40*(3), 616.620.

Beuchat, L. R. (1996). Pathogenic Microorganisms Associated with Fresh Produce. *Journal of Food Protection*, *59*(2), 204-216.

Black, J. G. (1996). *Microbiology*. *Principles and Applications* (3rd ed.). New Jersey: Prentice Hall.

Boyd, R. F. (1995). *Basic Medical Microbiology* (5th ed.). Boston: Little, Brown and Company.

Bradford, P. A. (1999). Automated Thermal Cycling is Superior to Traditional Methods for Nucleotide Sequencing of *bla*-SHV Genes. *Antimicrobial Agents and Chemotherapy*, *43*(12), 2960-2963.

Bradford, P. A. (2001). Extended-spectrum β-lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Treat. *Clinical Microbiology Reveiws*, *14*(4), 933-951.

Bradford, P. A., Yang, Y., Sahm, D., Grope, I., Gardovska, D., & Storch, G. (1998). CTX-M-5, A Novel Cefotaxoime-hydrolysing β-lactamases from an Outbreak of *Salmonella typhimurium* in Latvia. *Antimicrobial Agents and Chemotherapy*, *42*(8), 1980-1994.

Brown, A. E. (2001). *Benson: Microbiological Applications, Laboratory Manual in General Microbiology* (8th ed.). Boston: McGraw-Hill Publishers.

Bunyak, J. D. (2006). Understanding the Longevity of β-lactam Antibiotics and of Antibiotic/ β-lactamases Inhibitor combinations. *Biochemical Pharmacology*, *71*(7), 930-940.

Bush, K., Jocoby, G. A., & Medeiros, A. A. (1995). A Functional Classification Scheme for Beta-lactamases and Its Correlation with Molecular Structure. *Antimicrobial Agents and Chemotherapy*, *39*(6), 1211-1233.

Carter, M. W., Oakton, K. J., Warner, M., & Livermore, D. M. (2000). Detection of Extended-spectrum β-lactamases in *Klebsiella* with the Oxoid Combination Disk Method. *Journal of Clinical Microbiology*, *38*(11), 4228-4232.

Celenza, G., Pellegrini, C., Caccamo, M., Segatore, B., Amicosante, G., & Perilli, M. (2006). Spread of *bla*CTX-M-type and *bla*PER-2 β-lactamases Genes in Clinical isolates from Bolivian Hospital. *Journal of Antimicrobial Chemotherapy*, *57*, 975-978.

Centers for Disease Control and Prevention. (2010). *Laboratory Detection of Extended-spectrum β-lactamases (ESBLs)*. Retrieved December 1, 2010, from http://www.cdc.gov/HAI/settings/lab/lab_esbl.html

Chaudhary, U., & Aggarwal, R. (2004). Extended-spectrum β-lactamases (ESBL) – An Emerging Threat to Clinical Therapeutics. *Indian Journal of Medical Microbiology*, *22*(2), 75-80.

Chen, I., & Dudnau, D. (2004). DNA Uptake during Bacterial Transformation. *Nature Reviews. Microbiology*, *2*, 241-249.

Claeys, G., Verschraegen, G., Baere, T., & Vaneechoutte, M. (2000). PER-1 β-lactamases-producing *Pseudomonas aeruginosa* In an Intensive care unit. *Journal of Antimicrobial Chemotherapy*, *45*, 924-925.

Clinical and Laboratory Standards Institute. (2007). *Performance Standards for Antimicrobial Suseptibility Testings*. *17th Information Supplement. CLSI/NCCLS Document M100-S16*. Wayne, PA: Clinical and Laboratory Standards Institute.

DeNoon, D. J. (2006). *E. coli* Fears Prompt Lettuce Recall. *WebMD Health News*. Retrieved December 15, 2010, from http://www.webmd.com/food-recipes/news/20061009/e-coli-fears-prompt-lettuce-recall

DeWaal, C. S., & Bhuiya, F. (2007). *Outbreak Alert! 2007*. Retrieved December 20, 2010, from http://www.cspinet.org/foodsafety/outbreak_alert.pdf

Dieffenbach, C. W., & Dveksler, G. S. (Eds). (2003). *PCR Primer: A Laboratory Manual* (2nd ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Doelle, H. W. (Ed). (1969). *Bacterial Metabolism*. London: Academic Press.

Farmer, J. J. III, Asbury, M. A., Hickman, F. W., Berner, D. J., & the Enterobacteriaceae Study Group. (1980). *Enterobacter sakazakii*: A New Species of "Enterobacteriaceae" Isolated from Clinical Specimens. *International Journal of Systematic Bacteriology*, *30*(3), 569-584.

Finberg, R. W., Moellering, R. C., Tally, F. P., Craig, W. A., George, A. P., Dellinger, E. P., et al. (2004). The Importance of Bacterial Drugs: Future Directions in Infectious Disease. *Clinical Infectious Disease*. *34*, 1314-1320.

Finch, R. G., Greenwood, D., Norrby, S. R., & Whiteley, R. J. (2003). *Antibiotic and Chemotherapy: Anti-infective Agents and their use in Therapy.* United States: Walter Burns Saunders.

Florijin, A., Nijssen, S., Schmitz, F-J., Verhoef, J., & Fluit, A. C. (2002). Comparison of E Test and Double Disk Diffusion Test for Detection of Extended-spectrum β-lactamases. *European Journal of Clinical Microbiology & Infectious Diseases*, *21*(3), 241-243.

Fluornoy, D. J., Wongpradit, S., & Silberg, S. L. (1990). Facilitating Identification of Lactose-fermenting Enterobacteriaceae on MacConkey Agar. *Proceedings of the Oklahoma Academy of Science*, *70*, 5-8.

Food and Agriculture Organization of the United Nations/World Health Organization. (2008). *Microbiological Hazards in Fresh Leafy Vegetables and Herbs: Meeting Report*. Retrieved December 15, 2010, from http://www.fao.org/ag/agn/agns/jemra/Jemra_Report%20on%20fresh%20leafy%20vegetables%20and%20herbs.pdf

Food Safety Net. (2010). *Does Washing Vegetable Get Rid of Bacteria?* Retrieved December 20, 2010, from http://foodsafety.suencs.com/archives/11461

Forbes, B. A., Sahm, D. F., & Weissfeld, A.S. (2002). *Bailet and Scott;s Diagnostic Microbiology* (11th ed.). Missouri: Mosby.

Gallego, L., Umaran, A., Garaizar, J., Colom, K., & Cisterna, R. (1990). Digoxigenin-labeled DNA Probe to Detect TEM Type β-lactamases. *Journal of Microbiological Methods*, *11*(3-4), 261-267.

Handwerger, S., & Tomasz, A. (1985). Antibiotic Tolerance among Clinical Isolates of Bacteria. *Reviews of Infectious Diseases*, *7*(3), 368-386.

Harley, J. P., & Prescott, L. M. (2002). *Laboratory Exercises in Microbiology* (5th ed.). London: McGraw-Hill Publishers.

Health Protection Agency (2010). Identification of Enterobacteriaceae. National Standard Method BSOP ID 16 Issue 3. Retrieved November 1, 2010, from http://www.hpa-standardmethods.org.uk/documents/bsopid/pdf/bsopid16.pdf

Hitti, M. (2008). *Salmonella* Tomato Warning Expanded. *WebMD Health News*. Retrieved December 15, 2010, from http://www.webmd.com/foodrecipes/foodpoisoning/news/20080610/salmonella-tomato-warning-expanded

Holmes, D. S., & Quigley, M. (1981). A Rapid Boiling Method for the Preparation of Bacterial Plasmids. *Analutical Biochemistry*, *114*, 193-197.

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labiam, R., & Philipponm, A. (2002). Beta-lactamases of *Kluyvera ascorbata*, Probable Progenitors of Some Plasmid-encoded CTX-M types. *Antimicrobial Agents Chemotherapy*, *46*, 3045-3049.

Isshiki, K. (2007). Eating Raw Food as a Culture and Issue with Its Safety. *Foods & Food Ingredients Journal of Japan*, *212*(8).

Jabeen, K., Zafar, A., & Hasan, R. (2003). Comparison of Double Disc and Combined Disc Method for the Detection if Extended-spectrum β-lactamases in Enterobacteriaceae. *Journal of the Pakistan Medical Association*, *53*(11), 534-536.

Jacoby, G. A. (2009). AmpC Beta-lactamases. *Clinical Microbiology Review*, *22*(1), 161-182.

Jarlier, V., Nicholas, M. H., Fournier, G., & Philippon, A. (1988). Extended Broad-spectrum Beta-lactamases Conferring Transferable Resistance to Newer Beta-lactam Agents in Enterobacteriaceae: Hospital Prevalence and Susceptibility Patterns. *Reviews of Infectious Diseases*, *10*, 867-878.

Jitsurong, S., & Yodsawat, J. (2006). Prevalence of Extended-Spectrum Beta-Lactamases (ESBLs) Produced in Blood Isolates of Gram-negative in a Teaching Hospital in Southern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, *37*(1), 31-35.

Johnson, J. R., Kuskowski, M. A., Smith, K., O'bryan, T. T., & Tatini, S. (2005). Antimicrobial-resistant and Extra Intestinal Pathogenic *Escherichia coli* in Retail Foods. *Journal of Infectious Diseases*, *191*, 1040-1049.

Kader, A. A., & Nasimuzzaman, M. (2001). Antimicrobial Resistance Patterns of Gram-negative Bacteria Isolated from Urine Cultures in Almana General Hospital. *Annals of Saudi Medicine*, *21*(1-2), 110-112.

Katzung, B. G. (Ed.). (1998). *Basic & Clinical: Pharmacology* (7th ed.). Stamford: Appleton & Lange.

Kruse, H., & Sorum, H. (1994). Transfer of Multiple Drug Resistance Plasmids between Bacteria of Diverse Origins in Natural Microenvironments. *Applied And Environmental microbiology, 60*(11), 4015-4021.

Laarmann, K. (2010). ESBL: A greater danger than MRSA? *European Hospital*. Retrieved December 15, 2010, from http://www.european-hospital.com/en/article/7342-ESBL_A_greater_danger_than_MRSA.html

Lembo, A., & Camilleri, M. (2003). Chronic Constipation. *The New England Journal of Medicine, 349*, 1360-1368.

Levinson, W. E., & Jawetz, E. (1992). *Medical Microbiology & Immunology: Examination & Board Review* (2nd ed.). Norwalk: Appleton & Lange.

Livermore, D. M. (1993). Determinants of the Activity of β-lactamase Inhibitor Combination. *Journal of Antimicrobial Chemotherapy*, *31*, 9-21.

Livermore, D. M. (1998). β-lactamases-mediated Resistance and Opportunities for Its Control. *Journal of Antimicrobial Chemotherapy*, *41*(4), 25-41.

Livermore, D. M., & Brown, D. F. J. (2001). Detection of Beta-lactamase-mediated Resistance. *Journal of Antimicrobial Chemotherapy*, *48*(Suppl1), 59-64.

MacFaddin, J. F. (Ed). (2000). *Biochemical tests for Identification of Medical Bacteria* (3rd ed.). Philadelphia: Lippincott Williams and Wilkins.

Madigan, M. T., Martinko, J. M., Dunlap, P. V., & Clark, D. P. (2009). *Brock Biology of Microorganisms* (12th ed.). New Jersey: Pearson Higher Education.

Mandel, M., & Higa, A. (1970). Calcium-dependent Bacteriophage DNA Infection. *Journal of Molecular Biology*, *53*, 159-162.

Ministry of Health and Welfare of Japan, National Institute of Infectious Diseases and Infectious Disease Control Division (1997). Verocytotoxin Producing *Escherichia coli* (Enterohaemorrhagic *E. coli*) Infection, Japan, 1996-June 1997. *Infectious Agents Surveillance Report*, *18*, 153-154.

Monnet, D., Freney, J., Brun, Y., Boeufgras, J. M., & Fleurette, J. (1991). Difficulties in Identifying *Klebsiella* strains of Clinical Origin. *Zentbl. Bakteriol*, *274*, 456-464.

Naas, T., Oxacelay, C., & Nordmann, P. (2007). Identification of CTX-M-Type Extended-spectrum β-lactamase Genes Using Real-Time PCR and Pyrosequencing. *Antimicrobial Agents and Chemotherapy*, *53*(1), 223-230.

Nikaido, H. (2009). Multidrug Resistance in Bacteria. *Annual Review of Biochemistry*, *78*, 119-146.

National Committee for Clinical Laboratory Standards. (1999). *Performance standards for antimicrobial susceptibility testing*. *NCCLS Approved Standard M100-S9*. Wayne PA: National Committee for Clinical Laboratory Standards.

Nazarowec-White, M., Farber, J. M., Reiji, M. W., Cordier, J. L., & Schotrst, M. V. (2003). *Enterobacter sakazakii*. In *International Handbook of Foodborne Pathogens*. CRC Press.

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., et al. (2010). Foodborne Disease – The challenges of 20 Years Ago Still Persist while New Ones Continue to Emerge. *International Journal of Food Microbiology*, *139*, 3-15.

Nordmann, P., Ronco, E., Nass, T., Duport, C., Michel-Briand, Y., & Labia, R. (1993). Characterization of a Novel Extended-spectrum Beta-lactamase from *Psuedomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, *37*(5), 962-969.

Pagani, L., Dell'Amico, E., Migliavacca, R., D'Andrea, M. M., Giacobone, E., Amicosante, G., et al. (2003). Multiple CTX-M-type Extended-spectrum β-lactamases in Nosocomial Isolates of Enterobacteriaceae from a Hospital in Northen Italy. *Journal of Clinical Microbiology*, *41*(9), 4264-4269.

Pagani, L., Mantengoli, E., Migliavacca, R., Nucleo, E., Pollini, S., Spalla, M., et al. (2004). Multifocal Detection of Multidrug-resistant *Pseudomonas aeruginosa* Producing the PER-1 Extended-spectrum β-lactamase in Northern Italy. *Journal of Clinical Microbiology*, *42*, 2523-2529.

Paterson, D. I., & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: A Clinical Update. *Clinical Microbiology Reviews*, *18*(4), 657-686.

Perez, F., Endimiani, A, Hujer, K. M., & Bonomo, R. A. (2007). The Continuing Challenge of ESBLs. *Current Opinion in Pharmacology*, *7*(5), 459-469.

Petroni, A., Corso, A., Melano, R., Cacace, M. L., Bru, A. M., Rossi, A., et al. (2002). Plasmidic Extended-spectrum Beta-lactamases in *Vibrio cholera* O1 El Tor Isolates in Argentina. *Antimicrobial Agents and Chemotherapy*, *46*, 1462-1468.

Philippon, A., Labia, R., & Jacoby, G. (1989). Extended-spectrum Beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *33*(8), 1131-1136.

Pitout, J. D., Nordmann, P., Kevin, B., Laupland, K. B., & Poirel, L. (2005). Emergence of Enterobacteriaceae Producing Extended-spectrum β-lactamases (ESBLs) in the Community. *Journal of Antimicrobial Chemotherapy*, *56*(1), 52–59.

Pitout. J. D. (2010). Infection with Extended-spectrum Beta-lactamases Producing Enterobacteriaceae: Changing Epidemiology and Drug Treatment Choices. *Drugs*, *70*(3), 313-333.

Poirel, L., Kampfer, P., & Nordmann, P. (2002). Chromosome-encoded Ambler Class A Beta-lactamase of *Kluyvera georgiana*, a Probable Progenitor of a Subgroup of CTX-M Extended-spectrum Beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *46*, 4038-4040.

Poirel, L., Karim, A., Mercat, A., Thomas, I. L., Vahaboglu, H., Richard, C., et al. (1999). Extended-spectrum β-lactamase-producing Strain *of Acinetobacter baumannii* Isolated from a Patient in France. *Journal of Antimicrobial Chemotherapy*, *43*(1), 157-158.

Poirel, L., Naas, T., & Nordmann, P. (2010). Diversity, Epidemiology, and Genetics of Class D β-Lactamases. *Antimicrobial Agents and Chemotherapy*, *54*(1), 24-38.

Pompei, R., Bertutti, F., Thaller"b, M., C., Ingianni, A., & Statta, G. (1996). A Modified MacConkey Medium which Allows the Recognition of Enterobacteriaceae from Other Gram-negative Bacteria on Primary Culture Plates. *Journal of Microbiological Methods*, *25*(2), 271-278.

Power, P., Di Conza, J., Rodríguez, M., Gutkind, G., Ayala, A. A., Casellas, J. M., et al. (2007). Biochemical Characterization of PER-2 and Genetic Environment of bla_{PER-2}. *Antimicrobial Agents and Chemotherapy*, *51*(7), 2359-2356.

Prakash, D., & Gupta, K., R. (2009) The Antioxidant Phytochemicals of Nutraceutical Importance. *The Open Nutraceuticals Journal*, *2*, 20-35.

Queener, S. F., Webber, A. & Queener, S. W. (Eds). (1986). *Beta-lactam Antibiotics for Clinical Use*. New York: Marcel Dekker.

Rajasekaran, A., Sivagnanam, G., & Xavier, R. (2008). Nutraceutical as Therapeutic Agents: A Review. *Research Journal of Pharmacy and Technology*, *1*(4), 328-340.

Rawat, D., Nair, D. (2010). Extended-spectrum β-lactamases in Gram-negative Bacteria. *Journal of Global Infection*, *2*(3), 263-274.

Rodriguez-Villalobos, H., Bogaerts, P., Berhin, C., Bauraing, C., Deplanol, A., Montesinos, I., et al. (2010). Trends in Production of Extended-spectrum β-lactamases among Enterobacteriaceae of Clinical Interest: Results of a Nationwide Survey in Belgian Hospitals. *Journal of Antimicrobial Chemotherapy*, *66*(1), 37-47.

Ryan, K. J. (Ed.). (1994). *Sherris Medical Microbiology: An Introduction to Infectious Diseases* (3rd ed.). Stamford: Appleton & Lange.

Rychlik, W., Spencer, W. J., & Rhoads, R. E. (1990). Optimization of the Annealing Temperature for DNA Amplification in Vitro. *Nucleic Acids Research, 18*(21), 6409-6412.

Rychlik, W. (1995). Selection of Primers for Polymerase Chain Reaction. *Molecular Biotechnology*, *3*(2), 129-134.

Sadeghi, H. M. M., Najafabadi, A. J., Abedi, D., & Dehkordi, A. J. (2008). Identification of an Isolate of *Pseudomonas aeroginosa* Deposited in PTCC as a PHA Producer Strain: Comparison of Three Different Bacterial Genomic DNA Extraction Methods. *Journal of Biological Sciences*, *8*(4), 826-830.

Sakazaki, R. (1974). *Enterobacter cloacae*. In *Bergey's Manual of Determinative Bacteriology* (8th ed.). Baltimore: The Williams & Wilkins Co.

Samaha-Kfoury, J. N., & Araj, G. F. (2003). Recent Developments in β-lactamses and Extended-spectrum β-lactamses. *British Medical Journal, 327*(7425), 1209-1213.

Sambrook, J., & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sapers, G. M., Gorny, J. R., & Yousef, A. E. (2006). *Microbiology of Fruits and Vegetables*. Boca Raton: CRC Press.

Satta, G., Cornaglia, G., Mazzariol, A., Golini, G., Valisena, S., & Fontana, V. (1995). Target for Bateriostatic and Bactericidal Activities of β-Lactam Antibiotics Against *Escherichia coli* Resides in Different Penicillin-Binding Proteins. *Antimicrobial Agents and Chemotherapy*, *39*(4), 812-818.

Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V., & Hoekstra, R. M. (2011a). Foodborne Illness Acquired in the United States—Unspecified Agents. *Emerging Infection Diseases. 17*(1). Retrieved January 25, 2011, from http://www.cdc.gov/eid/content/17/1/16.htm

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M-A., Roy, S. L., et al. (2011b). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infection Diseases. 17*(1). Retrieved January 25, 2011, from http://www.cdc.gov/eid/content/17/1/7.htm

Scientific Committee on Food. (2002). Risk Profile on the Microbiological Contamination of Fruits and Vegetables Eaten Raw. *SCF/CF/FMH/SURF/Final.* European Commission.

Signh, V. K., & Kumar, A. (2001). PCR Primer Design. *Molecular Biology Today*, *2*(2), 27-32.

Spratt, B. G. (1975). Distinct Penicillin Binding Proteins Involved in the Division, Elongation and Shape of *Escherichia coli* K12. *Proceedings of the National Academy of Sciences USA*, *72*(8), 2999–3003.

Tambekar, D. H., & Mundhada, R. H. (2006). Bacteriological Quality of Salad Vegetables Sold in Amravati City (India). *Journal of Biological Sciences*, *6*(1), 28-30.

Todar, K. (2008a). Bacteria Resistance to Antibiotics. In *Todar's Online Textbook of Bacteriology* (p.1). Retrieved December 15, 2010, from http://www.textbookofbacteriology.net/resantimicrobial.html

Todar, K. (2008b). Bacterial Endotoxin. In *Todar's Online Textbook of Bacteriology* (p.1). Retrieved December 20, 2010, from http://textbookofbacteriology.net/endotoxin.html

Todar, K. (2008c). Bacterial Structure in Relationship to Pathogenicity. In *Todar's Online Textbook of Bacteriology* (p.1). Retrieved December 20, 2010, from http://textbookofbacteriology.net/BSRP.html

Todar, K. (2009). Antimicrobial Agents in the Treatment of Infectious Disease In *Todar's Online Textbook of Bacteriology* (p.1). Retrieved December 20, 2010, from http://textbookofbacteriology.net/antimicrobial.html

Tortora, G. J., Funke, B. R., & Case, C. L. (2010). *Microbiology: An Introduction* (10th ed.). San Francisco: Pearson Benjamin Cummings.

Tyagi, S., Singh, G., Sharma, A., & Aggarwal, G. (2010) Phytochemicals as Candidate Therapeutics: An overview. *International Journal of Pharmaceutical Sciences Review and Research*, *3*(1), 53-55.

Tzouvelekis, L. S., Tzelepi, E., Tassios, P. T., & Legakis, N. J. (2000). CTX-M-type Beta-lactamases: An Emerging Group of Extended-spectrum Enzymes. *International Journal of Antimicrobial Agents*, *14*(2), 137-143.

Vahaboglu, H., Hall, L. M. C., Mulazimoglu, L., Dodanli, S., Yildirim, I., & Livermore, D. M. (1995). Resistance to Extended-spectrum Cephalosporins, Caused by PER-1 ß-lactamase, in *Salmonella typhyimurium* from Istanbul. *Turkey Journal of Medical Microbiology*, *43*, 294-295.

Vahaboglu, H., Oztürk, R., Aygün, G., Coşkunkan, F., Yaman, A., Kaygusuz, A., et al. (1997). Widespeard Detection of PER-1-type Extended-spectrum ß-lactamases among Nosocomial *Acinetobacter* and *Pseudomonas aerurginosa* Isolates in Turkey: A Nationwide Multicentre Study. *Antimicrobial Agents and Chemotherapy*, *41*, 2265-2269.

Waxman, D. J., & Strominger, J. L. (1983). Penicillin Binding Proteins and the Mechanism of Action of Beta-lactam Antibiotics. *Annual Review of Biochemistry*, *52*, 825–869.

Westbrook, G. L., O'Hara, C. M., Roman, S. B., & Miller, J. M. (2000). Incidence and Identification of *Klebsiella planticola* in Clinical Isolates with Emphasis on Newborns. *Journal of Clinical Microbiology*, *38*, 1495-1497.

World Health Organizatio. (2002). *The World Health Report, Reducing risks, promoting Health*. Geneva: World Health Organization.

World Health Organization. (2004). WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe. *Eurosurveillance*, *8*(1). Retrieved December 15, 2010, from http://www.eurosurveillance.org/viewarticle.aspx?articleid=2356

Yong, D., Shin, J. H., Kim, S., Lim, Y., Yum, J. H., Lee, K., et al. (2003). High Prevalence of PER-1 Extended-spectrum β-lactamase-producing *Acinetobacter* spp. in Korea. *Antimicrobial Agents and Chemotherapy*, *47*, 1749-1751.

Zimbro, M. J., Power, D. A., Miller, S. M., Wilson, G. E., & Johnson, J. A. (Eds) (2009). *Difco™ & BBL™ Manual. Manual of Microbiological Culture Media* (2nd ed.). Spark, Maryland: Becton, Dickinson and Company.