MOLECULAR CHARACTERISATION OF *bla*-TEM HOMOLOGOUS GENES FROM *Enterobacter sp.* AND *Serretia sp.*

By

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ABSTRACT

MOLECULAR CHARACTERISATION OF *bla*-TEM HOMOLOGOUS GENES FROM *Enterobacter sp.* AND *Serretia sp.*

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The purpose of this study is to screen, characterize and clone the *bla*-TEM and *bla*-OXA gene fragments from Enterobactericeae bacteria isolated from common raw vegetables. A total of 22 morphologically different bacteria were isolated from green capsicum (*Capsicum annuum*), Beijing cabbage (*Brassica rapa*), mixed vegetable salad and China cauliflower (*Brassica oleracea*) samples using MacConkey agar supplemented with 50 µg/mL ampicillin. Gram staining and preliminary biochemical tests were carried out to validate the bacterial isolates as gram negative, oxidase negative, catalase positive and fermentation reaction. Subsequently, the remaining 19 bacterial isolates were subjected to the antibiotic susceptibility tests to demonstrate potential strains that carry multiple antibiotic resistant genes. API 20E Bacterial Identification Kit was done and the bacterial isolates S 3 and S 5 were presumed to be *Enterobacter cloacae* and *Serratia odorifera,* respectively. Total DNA were extracted and subjected to PCR amplification using *bla*-TEM and *bla*-OXA primers. Using the *bla*-TEM specific primers, three bacterial isolates (S 3, S 5 and S 7) were shown to contain the gene fragments with an expected size of 870 bp. On the other hand, the gene fragment

with an expected size of 430 bp was not amplified when *bla*-OXA specific primers were used. The amplified fragments were gel-purified and ligated into pGEM-T Easy Vector. Transformation into *E. coli* JM109 bacteria cells was performed and screening was based on blue-white selection. Subsequently, colony PCR was used on selected ligated white colonies. Amplification yielding the expected size of 1040 bp were subjected to plasmid extraction and subsequently outsourced for DNA sequencing. BlastX analyses for bacterial isolate S 3 illustrated high similarity to *bla*-TEM of *Enterobacter cloacae*, while for bacterial isolate S 5 illustrated high similarity to *bla*-TEM of *Serratia marcesceus.* Consequently, pairwise alignment between bacterial isolate S 3 and S 5 showed highly similar with 99 % identities.

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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 "**MOLECULAR CHARACTERISATION OF *bla*-TEM HOMOLOGOUS GENES FROM *Enterobacter sp.* AND *Serretia sp.***" was prepared by TAN WEI CHEAT and submitted as partial fulfilment of the requirement for the degree of Bachelor of Science (Hons) in Biotechnology at University Tunku Abdul Rahman.

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PERMISSION SHEET

I hereby certified that **TAN WEI CHEAT** (ID No: **08ADB05023**) has completed this final year project entitled "**MOLECULAR CHARACTERISATION OF *bla*-TEM HOMOLOGOUS GENES FROM *Enterobacter sp.* AND *Serretia sp.***" 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.

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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
DNA	Deoxyribonucleic Acid
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

In recent times, a changing trend in human diet is observed. Nowadays, people are more health conscious and paid attention to the food that they consume. Some of the food, vegetables in particular, is now being eaten raw. For instance, lettuce, radish, cabbage, cucumber and tomatoes are chosen to be consumed raw instead of cooked (Isshiki, 2007). This type of diet is becoming more popular as people believe that this is the best way to retain much of the nutrients in fresh greens (Schultz & Callow, 2010).

Nevertheless, the increasing popularity of raw eating habit has posed several health hazards. Cases of food borne diseases or some food-related health issues are being reported more frequently than before. In 1990s, the Center for Disease Control and Prevention stated that up to 12% of foodborne diseases were caused by consumption of raw vegetables (Buck, Walcott & Beuchat, 2003). Surprisingly, the pesticides or antibiotics which are normally used in agricultural field are now no longer effective or have lesser impacts towards a variety of bacteria (Lerner & Lerner, 2003). The adaptation of bacteria towards a wide range of antibiotics out alarming and caused illnesses to people consuming raw greens.

Among the various antibiotics resistance strains, microorganisms from the family Enterobacteriaceae is the one of the most common diseases causing microbes (Johnson, Kuskowski, Smith, O'Bryan & Tatini, 2005). This phenomenon is primarily due to bacteria that are found inhabiting the vegetables, causing the emergence of various food borne illnesses. Such resistances are normally developed following the mutation occurring in the genes of these bacteria (Levy, 1992). It is evident that a new group of enzyme, the extended-spectrum beta lactamase developed in certain type of Enterobacteria via mutation is found to be responsible for their resistance towards a wider range of beta-lactam antibiotics such as ampicillins and cephalosporins (Al-Jesser, 2006; Paterson & Bonomo, 2005). The emergence of resistant bacterial strains caused difficulties in treatment of infections (Lindberg, Alderberth & Wold, 2004).

Molecular diagnoses as well as biochemical tests are effective means to identify the types of bacteria causing various illnesses. The beta-lactame genes are utilized extensively to identify the DNA sequences responsible for the resistant gene in the bacterial isolates (Haber & Adhya, 1987). BLAST analysis and interpretation of the obtained DNA sequences for the identification of the type of bacteria causing the illness. Further confirmation step such as 16S RNA gene sequencing and phylogenetic trees construction can be performed.

The objectives of this study are:

- To look at the prevalence of antibiotic resistance genes in the bacterial isolates from raw vegetable samples
- To screen for extended spectrum beta lactamase (ESBL) genes in bacterial isolates
- To make presumptive identification of Enterobacteriaceae using Gram staining, biochemical tests, antibiotic susceptibility test and API 20E Bacterial Identification Kit
- To amplify and sequence *bla*-TEM and *bla*-OXA homologous genes

CHAPTER 2

LITERATURE REVIEW

2.1 Intake of Vegetables and Its Health Issues

The inclusion of vegetables as part of a diet is crucial in order to sustain better health. The food pyramid constructed by the U.S. Department of Agriculture (USDA) recommended a daily consumption of vegetables of about 3 to 5 servings (Brandy, Lindquist, Herd & Goran, 2000). It is evident that, by consuming correct proportion of vegetables in daily meals, certain diseases such as cardiovascular diseases, hypertension, macular degeneration and some types of cancer can be prevented (Brown, Rimm & Seddon, 1999; Hung, Joshipura & Jiang, 2004). Based on the data collected from the Nurses' Health Study and the Health Professionals' Follow-Up Study, individuals who consume the greatest amount of vegetables (20 percent) had less chance to develop coronary heart diseases than those with the lowest vegetables consumption (He, Nowson, Lucas & MacGregor, 2007). Furthermore, British researchers found that lung cancer risk are diminished more than 40 percent by elevating the intake of beta-carotene, particularly in carrot from 1.7 to 2.7 milligrams every day. Another study also indicates that women who take raw carrots were 5 to 8 times less probable to suffer from breast cancer compared to women who did not eat carrots (Patrick, 2000).

Raw vegetable diet is already a common practice nowadays. People believe that there is a higher nutritional value in uncooked greens compared to those which are cooked (Isshiki, 2007). Among all cooking methods, boiling results in the greatest loss of nutrients in vegetables as water-soluble nutrients can be transferred into water and lost when water is removed. It is estimated that 75% of the vitamin C and folate, 70% of the thiamine and potassium, 65% of the vitamin B6, 55% of the niacin and sodium, 50% of the vitamin B12, 45% of the riboflavin and copper, 40% of the iron and magnesium, and 35% of the vitamin A and phosphorus are lost from the process of boiling and draining vegetables (Schultz & Callow, 2010). Besides that, high-temperature cooking procedures may also cause denaturation to the enzymes necessary for digestion found in greens (Tang et al, 2008). Ultimately, these reasons constitute to the increasing habit of raw vegetable diet among people. However, problems arise pertaining to this habit when the cases of food-borne illness intensified in recent years.

2.2 Emergence of Food-borne Diseases

The outbreaks of human infections are mainly microbial infections associated with raw vegetables consumption and this had happened with increased frequency during the past few decades. According to a report released by the U.S. Centers for Disease Control (UCDC), about 48 million people became ill due to the intake of contaminated food, resulting in 128,000 cases of hospitalization and 3000 death cases annually in the United States (PhysOrg.com, 2010; World Health Organization, 2007). In Canada, an estimation done by public health experts

shows 11 to 13 million food-borne disease cases occurred annually in the nation (Hall et al, 2005).

Consumption of food and drinks which are contaminated with bacteria, viruses and parasites can result in food-borne illness. Raw vegetables have been recognized as a major source of pathogens because most of the pathogens are not completely exterminated during cooking process but still residing on their surface (Washington State Department of Health, 2009). Surveillance on vegetables has denoted that these food can be contaminated with various bacterial pathogens such as *E. coli O157:H7, Salmonella*, *Shigella*, *Campylobacter* and *Listeria monocytogenes* (Tauxe, 2002; Zacharia, Kamitani, Muhimbula & Ndabikunze, 2010).

Campylobacteriosis is an example of widespread food-borne infection. In the United States, *Campylobacter jejuni* became the major cause of acute diarrheal illness and resulted in more than 2 million cases of gastroenteritis every year (Doyle, 2010; World Health Organization, 2007). Acute health consequences of campylobacteriosis are severe abdominal pain, diarrhea, fever and nausea. In 1996, one of the biggest outbreaks of *E. coli* 0157 had resulted in 200 people became ill and 20 of them died in Wishaw, Scotland (Pennington, 2009). Besides that, the Illinois Department of Health reported a recent *Salmonella* sprouts outbreak in the country which had caused 57 residents to become ill due to contaminated sprouts on sandwiches served in a restaurant (Falkenstein, 2010).

Vomiting, nausea, headache, fever, abdominal pain and diarrhea are some of the symptoms of Salmonellosis (World Health Organization, 2007). In addition, other cases regarding food and water-borne diarrhoeal illnesses that cause 2.2 million deaths worldwide annually are also being highlighted in a World Health Organisation (WHO) report, indicating the severity of this issue (Harrington, 2010).

The crisis is further exacerbated when great changes happened in the mode of processing and distribution of food. Nowadays, foods are no longer manufactured in small plants but are processed in big factories in huge quantity. With this changing trend, the probability of food contamination during mass production and packaging increases, which will in turn cause the prevalence of food-borne disease outbreaks (Doyle, 2010). Following the increase in global food trade, subsequently, the risk for cross-border transmission of these infectious diseases are also elevated (Kaferstein, Motarjemi & Bettcher, 1997).

2.2.1 Enterobacteriaceae

Enterobacteriaceae is a family of Gram-negative bacteria which usually populate the intestines of human and animals (Falagas & Karageorgopoulos, 2009; Health Protection Agency, 2007). They are widely distributed in the ecosystem and can be found in soil, water, animals or plants. *Citrobacter sp., Enterobacter sp., Escherichia sp., Klebsiella sp. and Salmonella sp.* are some common genera of Enterobacteria family (Health Protection Agency, 2007). A variety of

Enterobacteriaceae species are able to bring about pneumonia and urinary tract infections. For instance, *Klebsiella pneumoniae* is usually involved in respiratory diseases. Besides that, they are also identified as the main reason of wound and other nosocomial infections. In the United States, these bacteria are estimated to claim 100,000 lives annually. Moreover, several pathogenic strains of Enterobactericeae also produce exotoxins which could be a major cause of diarrhea and body fluid loss (Falagas & Karageorgopoulos, 2009). Contaminated food and water may be a mode of transmission for these bacteria. Over the years, the incidence of antibiotic resistance of Enterobacteriacae is rising continually with an increase of the extended-spectrum beta-lactamases (ESBLs) that spread into the community, reaching a worrying level worldwide (Paterson, 2006).

2.3 Antimicrobial Agents

Antimicrobial agents are widely utilised in medicinal approaches to destroy or interfere with pathogen growth in an infection (Li, Mehrota, Ghimire & Adewoye, 2007; Todar, 2009a). They can generally be classified into two categories, namely antibiotics and chemotherapeutic agents (Todar, 2009b) Antibiotics are biologically originated agents that are derived from certain microorganisms whereas chemotherapeutic agents are substances that are synthesized chemically in the laboratory (Forbes, Sahm & Weissfeld, 2002; Mayer, 2010).

In a host body, bacterial infections are normally proliferated when bacteria grow and multiply indefinitely to cause a massive health hazard (Levy, 1998).

Antimicrobial agents are thus used to effectively disrupt some particular vital processes needed for normal growth and division of bacteria in order to inhibit the infections (Baron, 1996). The term bactericidal is given to the type of antimicrobial agents that totally destroy the bacteria. Meanwhile, bacteriostatic agents refer to substances that hinder the development of bacteria but usually do not eradicate them. Generally, bactericidal is more favourable to be used in an infection, especially chronic diseases that are lethal (Todar, 2009a). On the other hand, bacteriostatic agents normally require the host's defense mechanism to be incorporated in order to efficiently get rid of the bacteria (Levinson, 2004). Besides bacteria, other organisms that are affected by antimicrobials are parasites, fungi and some particular types of viruses.

2.3.1 Antibiotics Usage

Antibiotics were the first antimicrobial agents. Over the decades, antibiotics have been used broadly to treat various kinds of diseases, particularly bacterial infections. Antibiotics are low-molecular-weight biological derivatives of certain microorganisms which have positive clinical impacts on a vast variety of microbes causing diseases (Madigan & Martinko, 2006). In general, different types of antibiotics have distinct effects on various levels of illnesses, ranging from mild discomforts to critical, life-threatening diseases. The action of antibiotics depends on their spectrum of activity. Broad-spectrum antibiotics have their effects on a wider range of microbes compared to narrow-spectrum antibiotics that target only a certain type of microorganism. In specific infection,

narrow-spectrum antibiotics are chosen to be used instead of broad-spectrum agents in order to reduce the probability of developing resistance among the microorganisms (Todar, 2009). Today, there are over 100 known antibiotics. Some main groups of the antibiotics are penicillins, sulfonamides, tetracyclines, macrolides, fluoroquinolones and aminoglycosides (Pallasch, 2003).

2.3.2 Antibiotics: Mechanism of Activity

Antibiotics have their therapeutic actions via some specific mechanisms. The five main mechanisms involve the inhibiting the manufacture of cell wall, prohibition of protein synthesis, forbiddance of nucleic acid production, inhibition of cytoplasmic membrane and antimetabolites (Levinson, 2004).

Penicillin is an example of beta-lactam antibiotics that act as the inhibitor to the synthesizing of bacterial cell wall (Pallasch, 2003). By binding to and restraining the enzymes transpeptidase and carboxypeptidase which are necessary in the final step of peptidoglycan systhesis, these penicillins act as bactericidal, attacking the peptidoglycan layer of the bacterial cell wall. As a result, the bacteria will be unable to survive in hypotonic environment due to the damaged layer affecting its rigidity (Neu & Gootz, 1996). Other examples of the antibiotics that act in this way are vancomycin, cycloserine and cephalosporins (Miller, 2002). Apart from that, antibiotics such as the macrolides, tetracyclines, chloramphenicol and aminoglycosides disrupt the protein synthesis inside the bacterial cell (Alanis, 2005). For instance, the tetracyclins which is a type of bacteriostatic perform their

action by reversibly attach to the 30S ribosome and prevent the binding of aminoacyl-t-RNA to the acceptor site on the 70S ribosome (Mechant & Vithlani, 1986). Furthermore, by interfering with the production of nucleic acids, certain types of antibiotics like rifampin and quinolones have their cidal or static impacts towards certain types of bacteria (McClane, Mietzer, Dowling & Phillips, 1999). A good example of this is the antibiotic ripamfin, a bactericidal that is widely utilized in the treatment of *Mycobacterium avium* complex infections and tuberculosis. They act by attaching to the DNA-dependent RNA polymerase and prohibit the initiation of RNA synthesis (Paramasivan, 1994). Other than this, certain antibiotics also have their action in targeting the bacterial call membrane. The structure and function of bacteria membranes are disturbed in such a way that affecting the integrity of both the bacterial cytoplasmic and outer membrane, causing death to the cells. Clinically, the antibiotics polymyxins are effectively used against Gram-negative bacteria usually for urinary tract infections caused by *Pseudomonas* strains that are gentamicin, carbenicillin and tobramycin resistant (Hancock & Speert, 2000). Last but not least, another mechanism of antimicrobial action is through antimetabolites. For example, in the inhibition of folic acid production, trimethoprim, methotrexate, pyrimethamine are used to prevent the formation of tetrahydrofolic acid by binding to dihydrofolate reductase (Hazra & Tripathi, 2001; Rao & Venkatachalam, 1998).

2.3.3 Example of Antibiotics

2.3.3.1 Ampicilins

Over the years, ampicillin, a type of beta-lactam antibiotic is being utilized widely to deal with bacteria-causing illnesses (Stamm, McKevitt & Counts, 1987). It is a type of broad spectrum antibiotics that target a wider range of bacteria. Besides being efficient against Gram-positive microbes like *Staphylococci* and *Streptococci*, Ampicillin has its action towards Gram-negative microorganisms, for example, *H. influenzae*, *Coliforms* and *Proteus spp* (Wenzler, Schmidt-Eisenlohr & Daschner, 2003).

Ampicillin belongs to the penicillin group of beta-lactam antibiotics. By having an amino group inside its molecular structure, Ampicillin is distinguished from the penicillin. It is the amino group that allows the antimicrobial to pass through the outer membrane of Gram-negative bacteria (Wenzler, Schmidt-Eisenlohr & Daschner, 2003). This drug operates as a competitive inhibitor of transpeptidase which is a type of enzyme required by bacteria during the final stage of their cell walls synthesis. Eventually, the cell undergoes lysis and died (Neu & Gootz, 1996). In research field, Ampicillin is usually applied as a selective agent in molecular biology to verify the uptake of certain genes by bacteria (Stamm, McKevitt & Counts, 1987).

2.4 Evolution of Antibiotic Resistance

The use of antimicrobial agents especially antibiotics to fight against disease-causing pathogens are a common practice since twentieth century (Todar, 2008). As drug usage become prevalent, antibiotics have greater impact over a vast variety of microbes including bacteria. Nevertheless, due to the fact that bacteria are capable of evolving over time and thus develop resistance against these antibiotics, treatment of bacteria-causing illnesses becomes progressively tough (Tenover, 2006). In medicine field, the rise in bacterial resistance towards broad spectrum antibiotics poses numerous problems (Ghotaslou, Jodati & Manzary, 2010). Illnesses that are once treatable with antibiotics are now becoming increasingly complicated due to the evolved resistant strains among bacteria. These diseases include tuberculosis, septicaemia, wound infections, gonorrhoea and childhood ear infections (Todar, 2008).

The alarming rise in drug resistance among bacteria is primarily due to the extensive usage of antibiotics in various fields. In the United States, over 60% of antibiotics are utilized for agricultural purposes, accounting for about 18,000 tons of antibiotics transferred to the environment annually (Witte, 1998). Besides that, the bacterial resistant strains have also developed drastically following the misuse and overuse of clinical antibiotics. It is being discovered by some recent studies that the rise in resistance of ESBLs-producing Enterobactericeae is mainly due to the elevating prescriptions of third and forth generations of cephalosporin (Ghotaslou, Jodati & Manzary, 2009).

Selective pressure like antibiotics exposure can cause some bacteria to evolve over time and develop resistant traits against certain antibiotics. Upon exposure to antibiotics, some bacteria are able to survive and adapt to the altered environment which will in turn allow them to develop resistant gene among themselves. Mutation in the bacterial DNA is the major cause of resistant gene development and is significant for the continued evolution of acquired resistance genes (Bush, Banerjee & Gaynes, 1994). For example, over 100 variants of the TEM family of beta-lactamases is formed due to mutation. Apart from that, *Mycobacterium tuberculosis* and *Helicobacter pylori* have also developed resistance to certain antibiotics like fluoroquinolones and oxazolidinones from mutation (Woodford & Ellington, 2007).

Bacteria can transfer their resistant strains coded in their DNA from one to another through a process called horizontal gene transfer. Basically, 3 mode of gene transfer can happen which are transformation, transduction and conjugation (Clewell, 2008). Transformation occur when free DNA from one bacterium is set free and being incorporated into the other bacterium. Meanwhile, the mode of DNA transmission which involves the aid of viruses as vectors is termed transduction. Another mechanism of gene transfer is known as conjugation whereby the DNA is shifted from one another when the bacteria cells are in contact (Roberts, 1996). It is evident that horizontal gene transfer has happened to a large extent over time based on genome analyses on various bacterial species.

For instance, bacteria like *Enterococcus faecalis* are found to have more than 25% of foreign DNA in its genome (Clewell, 2008).

The multi-resistant feature of certain bacteria results in the existence of super bacteria. Super bacterium refers to a bacterium that contains various resistance genes in its cell. They are resistant against several types of antibiotics and are hardly destroyed (The Korea Times, 2010). Recently, the emergence of high-level vancomycin resistance in *Staphylococcus aureas* and the frequent appearance of multiple drug-resistant strains of *Mycobacterium tuberculosis* have resulted in rising concern among the public (Clewell, 2008) In addition, 50 cases regarding the infection by super bacteria had also been reported in the United Kingdom. It was found that these bacteria managed to synthesize an enzyme called NDM-1that can confer resistant to carbapenems, which is the strongest antibiotic group (Star City News, 2010).

2.4.1 Beta-Lactamase and The Extended-Spectrum Beta-Lactamase

Beta-lactamases are produced by almost all Gram-negative bacteria, including the family of Enterobacteriaceae which have the function of inactivating the activities of a specific type of antibiotic (Buynak, 2006). These enzymes play an important role in developing resistance towards beta-lactam antibiotics like penicilins, cephamycins, and carbapenems (Samaha-Kfoury & Araj, 2003). Beta lactamase enzyme hydrolyzes the beta-lactam ring of the antibiotics, causing alteration in the antibacterial molecular structure hence deactivating the antibiotics. As a result,

beta-lactamases allow the bacteria producing them able to be resistant against the dysfunctional antibiotics (Buynak, 2006).

Extended-spectrum beta-lactamases (ESBLs) are a new group of enzymes detected in the mid-1980s which confer resistance to an extended-spectrum of cephalosporins besides penicillins by hydrolyzing them with an oxymino side chain (Dbaibo, 2000). Mutations along the genes TEM-1, TEM-2, or SHV-1 have caused a change to the amino acid sequence on the enzymes' active site (Jain & Mondal, 2008). These ESBLs are normally plasmid associated and result in resistance towards a vast number of antibiotics (Joshi, Litake, Ghole, & Niphadkar, 2003).

2.4.2 Categorization of Beta-Lactamases

Beta-lactamases can be classified into four major groups, namely class A, B, C and D. Examples of class A beta lactamases are TEM, SHV and CTX-M beta lactamases (Lee, Bae & Lee, 2010).. In 1965, the TEM-1 enzyme was first discovered from an *E. coli* isolate and is the most common beta lactamase detected in Enterobacteriaceae for the time being (Livermore, 1995). Furthermore, the TEM-1 also accounts for more than 50% resistance among the AmpR *E. coli* clinical isolates. In addition, all the anti-gram-negative-bacterium penicillins except temocillin and narrow-spectrum cephalosporins, cefamandole, and cefoperazone are attacked by the TEM-1 (Fronze et al., 1995).

A good example of class B beta lactamase is Metallo-beta-lactamase (NDM-1). NDM-1 is usually synthesized by Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumonia,* and can transfer this gene from one bacterial strain to another by the process of horizontal gene transfer (Janny, 2010). The infections caused by bacteria producing this enzyme are hard to cure as these bacteria are commonly susceptible to tigecycline and polymyxins only. It is this enzyme that allows the bacteria to become resistant towards a wide variety of beta-lactam antibiotics (Janny, 2010).

AmpC-type beta-lactamases are under the group of class C beta lactamase. Extended-spectrum cephalosporin-resistant Gram-negative bacteria produce this kind of enzymes commonly. Typically, they are found in Gram-negative bacteria like *Citrobacter*, *Serratia* and *Enterobacter* species inside their chromosome (Pfaller, & Segreti, 2006). Besides that, plasmid-associated class C beta lactamases are also being detected most often in the isolates of *K. pneumoniae* and other naturally AmpC^{-} species including *K. oxytoca*, *Salmonella* and *P. mirabilis* (Philippon, Arlet, & Jacoby, 2002).

Class D includes OXA beta-lactamases which has its function by hydrolyzing oxacillin and related anti-staphylococcal penicillins (Livermore, 1998). This OXA beta-lactamases develops resistance to ampicillin and cephalothin and is recognized by their high hydrolytic action towards oxacillin and cloxacillin. Moreover, these enzymes are also barely prohibited by clavulanic acid. Generally,

these OXA-type ESBLs can be discovered in *P. aeruginosa* (Naas & Nordmann, 1999).

2.4.3 The *bla* Gene

In bacteria, the *bla* gene codes for beta lactamase protein and is responsible for resistant towards ampicillin (Seeberg & Wiedemann, 1984). Through the process of DNA replication, bacteria can pass along the resistance genes to other bacteria and this method is termed vertical gene transfer (Todar, 2009a).

Studies have demonstrated the mechanism of transmission of the chromosomal bla gene from *Enterobacter cloacae* to *Escherichia Coli* using RP4::Mini-Mu. The results show the dominance *E. cloacae* resistant bla gene over the susceptible organisms' genes (Seeberg & Wiedemann, 1984). Apart from that, a report in the J*ournal of Antimicrobial Chemotherapy also mentioned that there is a* combination of bla CTX-M-15, bla OXA-1and bla TEM-1 being discovered in strains of *Escherichia coli* from the UK, India and Canada (Boyd & Mulvey, 2006).

2.5 Identification of Unknown Bacteria by Phylogenetic Classification

Unknown bacteria can be identified using phylogenetic classification based on rRNA gene (rDNA) sequences (Subbotin, Madani, Krall, Sturhan & Moens, 2005). This is mainly due to the rRNA molecules that contain greatly conserved sequences disseminated with sections of more variable sequences. The intention

to identify bacteria-type pathogens becomes easier and more effective with the advancement in polymerase chain reaction (PCR) technique. Thus, probes of a particular bacterial phylogenetic group can probably be created in order to meet the aim of recognizing unknown bacteria using this process (Jalava et al., 2000).

There are several steps in recognizing an unknown bacterial species using its 16s rRNA sequence. These include retrieve similar sequences; align the sequences and the subsequent phylogenetic analysis (Christen, 2006). By using rRNA phylogeny, a particular sequence from the bacterial rDNA which is either amplified straightaway from clinical samples or is isolated from bacteria in pure cultures is able to be placed somewhere in the bacterial phylogeny during the recognizing process (Ludwig & Schleifera, 1994).

Gene sequencing carried out with 16S rRNA is extremely useful in classifying bacteria. However, its low phylogenetic power at the species level and poor discriminatory power for several genera make this technique less appealing (Janda & Abbott, 2007). This is so as a number of researchers discovered that some bacteria groups which comprise of the family *Enterobacteriaceae*, mycobacteria, the *Acinetobacter baumannii-A. calcoaceticus* complex, *Achromobacter*, *Stenotrophomonas*, and *Actinomyces have* difficulties in resolution at the genus or species level with 16S rRNA gene sequencing data. Thus, in order to resolve these taxonomic problems, DNA relatedness studies are usually required (Janda & Abbott, 2007). In Finland, a vast variety of bacterial PCR targeting rRNA genes

(rDNAs) was used to interpret 536 clinical samples acquired from 459 hospitalized patients during a 4-year study period (Jalava et al., 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Vegetable Samples

This study was conducted using four types of vegetable, namely green capsicum (*Capsicum annuum*), Beijing cabbage (*Brassica rapa*), mixed vegetable salad and China cauliflower (*Brassica oleracea*). The vegetable samples were obtained from Tesco Kampar, Perak, 1 to 2 hours prior to sampling.

3.1.2 Chemical Reagents and Equipments

The chemical reagents and equipments used were provided by the Department of Biological Science, Universiti Tunku Abdul Rahman. The chemical reagents and equipments were readily obtained from the Microbiology, Molecular Biology and Final Year Project Laboratories. The lists of materials and equipments used together with their brand and manufacturer were tabulated in Table 3.1 and 3.2.

Table 3.1: List of materials used and their brands and manufacturers

Materials	Brand & Manufacturer
1kb DNA ladder, 100bp DNA ladder, Agarose powder, blaCTX-M forward and reverse primers, blaPER2 forward and reverse primers	Vivantis
100bp DNA ladder, DNA loading dye, Isopropyl-beta-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-beta-D-galactophyranoside (X-Gal)	Fermentas
Absolute ethanol	Copens Scientific
Ampicillin powder	Bio Basic Inc
API 20 E kit	BioMerieux
Brain Heart Infusion Broth (BHIB), Commercial antibiotic discs (cefotaxime/clavanulate & ceftazidime/clavanulate), Muller Hinton (MH) Broth, Oxidation – Fermentation	BD
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
D-glucose	Rdeh
dNTP, DNA-spin™ Plasmid DNA Purification Kit, Magnesium Chloride ($MgCl_2$), $MgCl_2$ free PCR buffer, Taq polymerase	iNtRON
Gram's Iodine	R & M Chemicals
Mineral oil	Sigma
pGEM-T® Easy Vector system	Promega
QIAquick™ Gel Purificarion Kit	QIAGEN
SP6 promoter primer, T7 promoter primer	Research Biolabs
Violet Red Bile Glucose (VRBG) agar, Oxidation-Fermentation (O-F) basal medium	Pronadisa

Table 3.2: List of equipments used and their brand and manufacturer

Equipments	Brand & Manufacturer
Autoclave machine	HIRAYAMA
Centrifuge machine, Microcentrifuge, Nanodrop 1000, Electrophoresis power supply	Thermo Electron Corporation
Electronic balance	Adventurer™ Pro
Incubator, Water bath	Memmert
Incubator shaker	Hettich Zentrifugen
Laminar flow hood	Isocide™
Light microscope	Leica
PCR machine (Thermal cycle)	Biometra
Spectrophotometer	BIO-RAD SmartSpec™
UV transilluminator	UVP

3.2 General Methodology

3.2.1 Preparation of Media and Reagents

All media and buffers were prepared using distilled water and kept in Scott bottles. Molten agar was prepared and poured onto Petri dishes or other tubes after autoclaving and allowed to cool before storing for future use. Biochemical tests agar and reagents were prepared 2 weeks in advance. Media that needed to be pre-mixed with additional components should mix well while in preparation. The prepared media and agar were stored at room temperature unless storage at 4 °C is

stated. All media prepared as well as media containing antibiotics can be used up to a month.

3.2.2 Sterilization

The liquid media and apparatus used were sterilized through autoclaving or filtration. The heat stable liquid media and apparatus were autoclaved at 121°C at 15 psi for either 15 or 20 minutes. Autoclaved items were dried at 70°C in an oven prior to use. For heat sensitive medium such as ampicillin and glucose, they were filter-sterilized through a 0.20 µm membrane filter.

3.3 Cultivation and Isolation of Bacterial Isolates

3.3.1 Sampling of Vegetable Samples

The vegetable samples were picked from the supermarket using a clean plastic bag to avoid contamination from the sampler's hands. The knife, tray and gloves were sterilized with 70% (v/v) ethanol before and after handling each sample. The vegetable were washed with distilled water to remove dirt and soil residues and further chopped into smaller pieces. Each 5 g of chopped sample was put into two different medium, BHIB and BPW medium supplemented with ampicillin (50 µg/mL). They were incubated overnight in a shaker at 37°C with 200 rpm. The overnight cultures were then subjected to ten-fold serial dilution using sterile distilled water. A volume of 100 µL of each dilution was spread on MacConkey agar supplemented with ampicillin (50 µg/mL) and incubated overnight at 37°C. After incubation, each colony type with different morphological was picked and

streaked on VRBG agar supplemented with ampicilin (50 µg/mL) and incubated overnight at 37°C. The bacterial isolates were further sub-cultured to LB agar.

3.4 Characterization of Bacterial Isolates

All bacterial isolates were subjected to Gram staining and selected biochemical tests such as catalase test, oxidase test and oxidation-fermentation test. Bacterial isolates from the Enterobacteriaceae family comes are gram negative, oxidase negative, catalyse positive and glucose-fermenting strains. Bacterial isolates tested and fulfilled the criteria of Enterobacteriaceae family were further characterized using antibiotic susceptibility tests.

3.4.1 Gram Staining

A single colony of bacterial isolate was picked using an inoculation loop. A thin smear was made on a clean glass slide by mixing the inoculum with tiny drop of sterile distilled water. The bacterial smear was heat-fixed by passing it through the flame of the Bunsen burner several times. The slide with bacterial smear was flooded with crystal violet dye for 1 minute and washed under running tap water. The slide was then covered with Iodine for another 1 minute. After washing the iodine with tap water, 95% (v/v) ethanol was added onto the smear for 30 seconds and rinsed off with tap water. Lastly, the slide was counterstained with safranin dye for 1 minute and then rinsed. Excess water was removed and the slide was allowed to air-dry. The slide was then examined under the oil-immersion microscope.

3.4.2 Biochemical Tests

3.4.2.1 Catalase Test

A fresh and young culture was prepared by streaking bacterial isolates on LB agar supplemented with ampicillin (50 µg/mL) and incubated overnight at 37°C. A few drops of 3% hydrogen peroxide were added on the top of colonies on the plate. The appearance of bubbles indicated positive reaction while the non-bubbles appearance signified negative reaction.

3.4.2.2 Oxidase Test

A fresh and young culture was prepared by streaking bacterial isolates on LB agar supplemented with ampicillin (50 µg/mL) and incubated overnight at 37°C. An oxidase reagent was dropped on a filter paper. A single colony of bacterial isolate was picked using the inoculation loop and placed on top of the reagent on the filter paper. The appearance of strong violet colour indicated positive reaction while the opposed signified negative reaction.

3.4.2.3 Oxidation-Fermentation (O-F) Test

A single colony of bacterial isolate was picked and inoculated into two tubes of O-F basal medium. One tube was covered with mineral oil to create an anaerobic environment while the other tube was left uncovered to create aerobic environment. The tubes were then incubated at 37°C for 48 hours. The medium colour change indicated the ability of bacteria to utilize the carbohydrate provided.

3.4.3 Antibiotic Susceptibility Tests

The bacterial isolates were inoculated in Muller Hinton (MH) broth overnight at 37°C. A sterile cotton swab was soaked into the broth and then streaked over the MH agar surface entirely. The inoculum density should be appropriate, not to over- or under-inoculate the plate. The antibiotic discs were placed on the agar with a distance of approximately 2 to 3cm between each other. The bacterial isolates were tested against 15 types of antibiotics such as aztreonam, cefotaxime, cefotaxime/clavulanate, ceftazidime, ceftazidime/clavulanate, cefpodoxime, ceftraizone, amoxycilin/clavulanate, oxacilin, ampicillin, chloramphenicol, ciprofloxacin, tetracycline, trimethroprim/sulfamethozaxole and gentamicin. The agar plates were then incubated at 37°C for 24 hours. The zone clearing on the plates were then observed. Zone of inhibition implies the sensitivity of bacteria towards the antibiotics and these interpretations are based on Zone Diameter Interpretative Standard of Clinical Laboratory Standard Institute (CLSI).

3.4.4 API 20E Bacterial Identification Kit

A fresh and young culture was prepared by streaking bacterial isolates on LB agar supplemented with ampicillin (50 µg/mL) and incubated overnight at 37°C. A single colony of fresh culture was picked using micropipette and diluted in 5 mL sterile distilled water. The suspension was pipetted several times to achieve a homogenous state and used immediately after preparation. The API 20E strip consists of 20 microtubes containing dehydrated substrates. The bacterial suspension was distributed into the tubes accordingly. This was done by tilting the

strip slightly forward to avoid formation of bubbles. The incubation box was closed and incubated at 37°C for 18 to 24 hours. During incubation, metabolism produces colour changes that either spontaneous of revealed by adding reagents. The results obtained from the biochemical tests were translated into a seven-digit octal code and further interpreted by referring to the apiweb.

3.4.4.1 O-Nitrophenl-β-D-Galactopyranosidase (ONPG) Test

The bacterial suspension was distributed into the microtube and incubated for 18 to 24 hours. A yellow colour indicated a positive reaction while colorless indicated a negative reaction.

3.4.4.2 Arginine Dihydrolase (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 and overlaid with mineral oil to create an anaerobic environment and incubated for 18 to 24 hours. For ADH, LDC, ODC and URE tests, a red or orange colour indicated a positive reaction while a yellow colour indicated a negative reaction. For H_2S test, a black deposit or thin line indicated positive reaction while a colourless or greyish indicated negative reaction.

3.4.4.3 Citrate Utilization (CIT) Test, Voges Proskauer (VP) Test, and Gelatin (GEL) Test

The bacterial suspension was distributed into the tubes. For CIT test, the formation of blue-green or blue colour indicated positive reaction while a pale-green or yellow colour indicated negative reaction. For VP test, one drop each of VP 1 and VP 2 reagents were added to the tubes and incubated at least for 10 minutes. A pink or red colour indicated a positive reaction while a slightly pink colour appearing after 10 minutes indicated a negative reaction. For GEL test, a diffusion of black pigment indicated a positive reaction while no diffusion indicated a negative reaction.

3.4.4.4 Tryptophan Deaminase (TDA) Test

The bacterial suspension was distributed into the tube and incubated. A drop of TDA reagent was added to the tube. A reddish brown colour indicated a positive reaction while a yellow colour indicated a negative reaction.

3.4.4.5 Indole (IND) Test

The bacterial suspension was distributed into the tube and incubated. A drop of James reagent was added to the tube. A pink colour indicated a positive reaction while colourless or greyish indicated negative reaction.

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

The bacterial suspension was distributed into tubes and incubated. For GLU test, a yellow or greyish colour indicated a positive reaction while a blue or blue-green colour indicated a negative reaction. For MAN, INO, SOR, RHA, SAC, MEL, AMY, and ARA tests, a yellow colour indicated a positive reaction while a blue or blue-green colour indicated a negative reaction.

3.4.4.7 Nitrate Reduction

A drop of each NIT 1 and NIT 2 reagents were added to GLU tube and incubated for 2 to 5 minutes. A red colour indicated positive reaction (NO_2) while a yellow colour indicated negative reaction (N_2).

3.5 Total DNA Extraction

The fast boil method was used in extracting the total DNA (Holmes and Quigley, 1981). Bacterial cells were inoculated in 5 mL of LB broth with ampicillin (50 µg/mL) and incubated overnight at 37°C with 200 rpm. An amount of 1.5 mL of the overnight culture was centrifuged at 15,000 rpm for 15 minutes at room temperature. The supernatant was discarded. The pellet was then resuspended in 300 µL of sterile distilled water. It was then boiled in the water bath at boiling water for 10 minutes and cooled on ice immediately for 3 to 5 minutes. The sample was centrifuged at 15,000 rpm for 2 minutes. The suspension or

supernatant was then transferred to a new micocentrifuge tube and stored at -20°C for future use. The DNA concentration and the A_{260}/A_{280} purity were determined using Nanodrop 1000 Spectrophotometer.

3.6 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

3.6.1 Oligonucleotides for PCR Amplification

Table 3.3: The *bla*-TEM and *bla*-OXA primers were used in PCR amplification

Primer Names	Length (base)	Primer Sequences	Expected Fragment Size
bla-TEM-F	20	5'-ATGAGTATTCAACATTTCCG-3'	867 bp
bla-TEM-R	20	5'-CTGACAGTTACCAATGCTTA-3'	
bla-OXA-F	20	5'-TTTTCTGTTGTTTGGGTTTT-3'	427 bp
bla-OXA-R	20	5'-TTTCTTGGCTTTTATGCTTG-3'	

3.6.2 Preparation of PCR mixtures

The detection of *bla*-TEM and *bla*-OXA gene was performed using PCR amplification. All PCR reactions were conducted in 25µl aliquots containing 1X PCR Buffer, 2mM $MgCl_2$, 0.04U/ml Chromo *Taq* Polymerase, 0.2mM dNTP, 400 nM of each forward and reverse primer, 200ng/µl of DNA template and ddH_2O. A negative control was prepared with no DNA template added and included in every PCR batch to ensure no contamination. After the PCR mixtures well-mixed and prepared, the samples were placed in a pre-programmed thermal

cycler. The parameters used for both *bla*-TEM and *bla*-OXA gene amplification were shown in Table 3.4.

Table 3.4: PCR amplification parameters for *bla*-TEM and *bla*-OXA gene amplification

Stage	Temperature	Duration	Cycle
Initial Denaturation	94 °C	5 minutes	1
Denaturation	94 °C	45 seconds	
Annealing	50 °C	45 seconds	30
Extension	72 °C	45 seconds	
Final Extension	72 °C	10 minutes	1

3.6.3 Gel Electrophoresis of PCR Products

An aliquot of 2 µL of PCR product was mixed with 1 µL of loading dye. The mixture was then loaded and electrophoresed in 1.5% (w/v) agarose gel. The 100 bp DNA ladder (Vivantis) was used as the molecular weight marker in every agarose gel. The gel electrophoresis was carried out at 80 V for 40 minutes. When the gel electrophoresis was completed, it was stained with ethidium bromide and viewed under U.V. irradiation.

3.7 Agarose Gel Purification

A volume of 50 µL of PCR products was loaded and separated by gel electrophoresis on 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. The corresponding gene fragment was excised and purified using the *Gel Extraction Kit* (GeneJET). The purified DNA was electrophoresed to verify the appropriate fragment size purified. The concentration and A_{260}/A_{280} purity were measured using Nanodrop 1000 Spectrophotometer. The purified DNA was then stored at -20°C for future use.

3.8 Ligation and Transformation

3.8.1 Ligation with pGEM-T EasyVector

The purified DNA fragments were ligated with pGEM-T Easy Vector as described by the manufacturer (Promega). The ligation mixture was prepared by mixing 3 µL of the purified DNA fragments, 1 µL of pGEM-T Easy vector (50 ng), 1 U of T4 ligase (3 U/µL) and 5 µL of 2X Rapid Ligation Buffer. The mixture was then incubated overnight at 4°C. The ligated product was subsequently stored at – 20°C until use.

3.8.2 Preparation of Competent Cells

A single colony of *E. coli* JM109 strain was inoculated into 5 mL of LB broth and incubated overnight at 37°C with agitation of 200 rpm. An amount of 300 µL of overnight inoculum was transferred into 15 mL of fresh LB medium. The inoculums was further agitated at 200 rpm at 37°C until it reached an OD_{600} of

between 0.5 – 0.6. The cells were pelleted by centrifugation at 4000 *g* for 5 minutes at 4°C. Cells were subsequently resuspended in 2 mL of cold $CaCl_2$, and incubated on ice for at least 2 hours. The competent cells were kept on ice before proceeding to the transformation process.

3.8.3 Transformation

In a sterile 1.5 mL microcentrifuge tube placed on ice, 3 µL of ligation mixture (Section 3.8.1) was gently mixed with 200 µL of *E. coli* JM109 competent cells. In another tube labeled positive control, 1 µL of pUC19 vector (0.1 µg/mL) was mixed with 200 µL of *E. coli* JM109 competent cells. For negative control, no pUC19 vector was added to the competent cells. The mixtures were then incubated on ice for 1 hour which were subsequently subjected to heat-shock at 42°C for exactly 90 s. The tubes were immediately incubated on ice for an additional 5 minutes. An amount of 800 µL LB medium was added to each tube and the mixtures were incubated at 37°C for 45 minutes (with shaking at 75 – 80 rpm). The cells were pelleted at 6000 *g* for 10 minutes and resuspended in 100 µL LB medium. Lastly, the cells were plated onto agar plates containing ampicillin (50 µg/mL). All LB agar plates were spread with 20 µL of IPTG (100 mM) and 20 µL of X-gal (50 mg/mL). The plates were incubated for 16 – 18 hours at 37°C.

Colonies were observed on overnight plates. Selected colonies were picked and patched onto a fresh LB agar plate supplemented with ampicillin (50 µg/mL),

IPTG (100mM) and X-Gal (50mg/mL) for further confirmation of the colour development.

3.9 Colony PCR

The selected colonies were subjected to colony PCR. The forward primer and reverse primer used were T7 promoter and SP6 promoter primers. The sequences of primers were shown in Table 3.5. PCR mixtures consisting of 1X Go*Taq* Colorless Flexi buffer, 2mM $MgCl_2$, 0.04U/ml Chromo *Taq* Polymerase, 0.4µM of each T7 promoter primer (forward primer) and SP6 promoter primer (reverse primer), 200µM dNTP mix and ddH_2O to a final volume of 25µL.

A colony was picked using a sterile toothpick and placed into the PCR mixture. A negative control was prepared with no colony added to the PCR tube and included in every colony PCR batch to ensure no contamination. After the PCR concoctions were mixed and prepared, the samples were placed in a pre-programmed thermal cycler. The parameters used were shown in Table 3.6.

Table 3.5: Sequences of the T7 and SP6 promoter primers

Primer Names	Primer Sequences
T7 (Forward primer)	5'-TAATACGACTCACTATAGGG -3'
SP6 (Reverse primer)	5'-ATTTAGGTGACACTATAG -3'

Table 3.6: Colony PCR parameters

Stage	Temperature	Duration	Cycle
Initial Denaturation	96°C	3 minutes	1
Denaturation	96°C	30 seconds	
Annealing	50°C	15 seconds	30
Extension	60°C	2.5 minutes	
Final Extension	60°C	10 minutes	1

3.10 Plasmid Extraction

A single colony was picked from a freshly streaked bacterial plate and inoculated into 5 mL of LB broth with ampicillin (50 µg/mL). The culture was incubated overnight with shaking. The *DNA-Spin Plasmid DNA Purification Kit* (iNtRON) was used to purify the plasmid DNA from bacterial culture. The protocol of purification was based on the manufacturer's manual. The purified DNA was then performed with gel electrophoresis to confirm the presence of plasmid. The concentration and A_{260}/A_{280} purity were measured using Nanodrop 1000 Spectrophotometer. The purified plasmid was then stored at -20 °C for future use.

CHAPTER 4

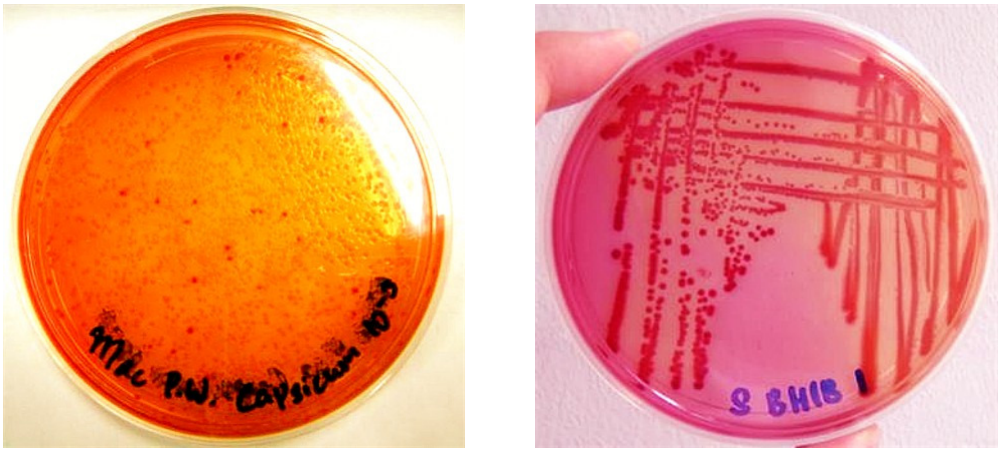
RESULTS

4.1 Cultivation and Isolation of Bacterial Isolates

Bacteria were cultivated from green capsicum, Beijing cabbage, mixed vegetable salad and China cauliflower samples using BHIB and BPW selective medium and subsequently plated on MacConkey agar supplemented with ampicillin (50 µg/mL). A total of 22 morphologically different bacteria were isolated from vegetable samples where green capsicum and Beijing cabbage samples contributed 5 bacterial isolates each, mixed vegetable salad sample contributed 8 bacterial isolates and China cauliflower sample contributed 4 bacterial isolates. The bacterial isolates were then streaked on VRBG agar supplemented with ampicillin (50 µg/mL) to obtain pure isolates. They were further streaked on LB agar for strain maintaining and morphological observations purpose. The diagram of plates was shown in Figure 4.1.

In order to characterize the bacterial isolates, their morphological characterizations were observed in the aspects of color, shape, elevation, edge, opacity and texture. The results were summarized in Table 4.1 and 4.2.

(a) (b)



(c)

Figure 4.1: Bacterial isolates on MacConkey, VRBG and LB agar plates.

(a) Bacterial isolates performed with ten-fold serial dilution and plated on MacConkey agar supplemented with ampicillin (50 µg/mL). (b) Bacterial isolates streaked from MAC agar onto VRBG agar supplemented with ampicillin (50 µg/mL). (c) Bacterial isolates streaked on LB agar supplemented with ampicillin (50 µg/mL).

Table 4.1: Appearance and morphology of bacterial isolates from green capsicum and Beijing cabbage samples observed in MAC, VRBG and LB agar.

Vegetable Sample	Bacterial Isolate	Appearance and Morphology of Colony							
		Color			Shape	Elevation	Edge	Opacity	Texture
		MAC	VRBG	LB					
Green capsium	CP 1	Ivory	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	CP 2	Ivory	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	CP 3	Pink	Pink	Ivory	Circular	Convex	Entire	Opaque	Glistening
	CP 4	Pink	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	CP 5	Ivory	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
Beijing cabbage	CB 1	Ivory	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	CB 2	Ivory	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	CB 3	Ivory	Pink	Ivory	Irregular	Convex	Curled	Translucent	Wrinkled
	CB 4	Ivory	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	CB 5	Ivory	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening

Table 4.2: Appearance and morphology of bacterial isolates from mixed vegetable salad and China cauliflower samples observed in MAC, VRBG and LB agar.

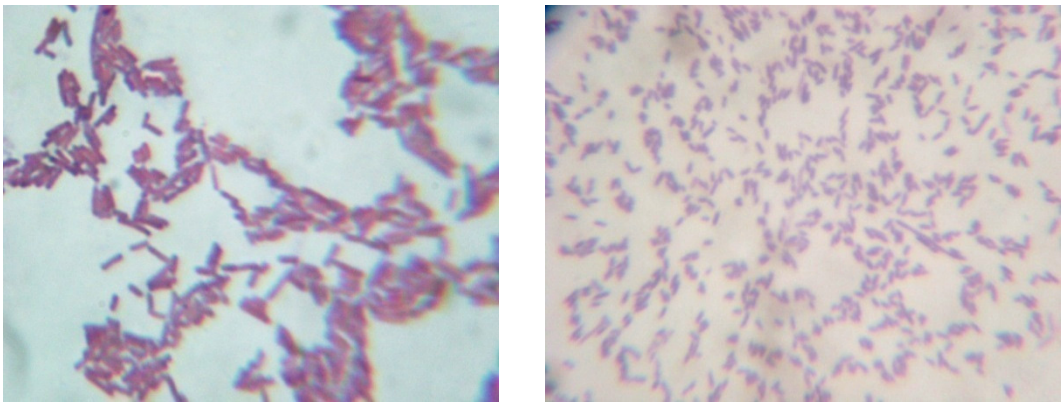
Vegetable Sample	Bacterial Isolate	Appearance and Morphology of Colony							
		Color			Shape	Elevation	Edge	Opacity	Texture
		MAC	VRBG	LB					
Mixed vegetable salad	S 1	Pink	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	S 2	Colorless	Pink	Ivory	Circular	Convex	Entire	Transparent	Glistening
	S 3	Pink	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	S 4	Ivory	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	S 5	Pink	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	S 6	Colorless	Pink	Ivory	Circular	Convex	Entire	Transparent	Glistening
	S 7	Pink	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	S 8	Ivory	Pink	Ivory	Circular	Convex	Entire	translucent	Smooth
China cauliflower	CF 1	Ivory	Pink	Ivory	Circular	Convex	Entire	Opaque	Smooth
	CF 2	Colorless	Pink	Ivory	Circular	Convex	Entire	Translucent	Glistening
	CF 3	Ivory	Pink	Ivory	Circular	Convex	Entire	Translucent	Smooth
	CF 4	Colorless	Pink	Ivory	Circular	Convex	Entire	Opaque	Glistening

4.2 Characterization of Bacterial Isolates

4.2.1 Gram Staining

Gram staining was carried out to validate the bacterial isolates as gram negative strain based on their cell wall compositions. Gram positive bacteria will be stained purple as they are able to retain the crystal violet dye, while gram negative bacteria will be stained pink as they being decolorized and stained by the safranin dye. All bacterial isolates were observed under 1000x oil immersion objective of the microscope and was illustrated in Figure 4.2. The results of microscopic examination were tabulated in Table 4.3.

(a) (b)



Magnification: 1000X Magnification: 1000X

Figure 4.2: Bacterial isolates viewed under oil immersion objective of the microscope.

Images show bacterial isolates CB 2 and CB 3 with (a) bacillus shape and (b) coccobacillus shape respectively. Both figures illustrated bacterial isolates stained pink in color and indicated gram negative reaction.

Table 4.3: Microscopic observations of bacterial isolates after Gram staining

Vegetable Sample	Bacterial Isolate	Color	Conformation	Reaction
Green Capsicum	CP 1	Pink	Coccobacillus	Gram Negative
	CP 2	Pink	Coccobacillus	Gram Negative
	CP 3	Pink	Coccobacillus	Gram Negative
	CP 4	Pink	Coccobacillus	Gram Negative
	CP 5	Pink	Coccobacillus	Gram Negative
Beijing Cabbage	CB 1	Pink	Coccobacillus	Gram Negative
	CB 2	Pink	Bacillus	Gram Negative
	CB 3	Pink	Coccobacillus	Gram Negative
	CB 4	Pink	Coccobacillus	Gram Negative
	CB 5	Pink	Bacillus	Gram Negative
Mixed Salad	S 1	Pink	Coccobacillus	Gram Negative
	S 2	Pink	Coccobacillus	Gram Negative
	S 3	Pink	Coccobacillus	Gram Negative
	S 4	Pink	Bacillus	Gram Negative
	S 5	Pink	Coccobacillus	Gram Negative
	S 6	Pink	Coccobacillus	Gram Negative
	S 7	Pink	Coccobacillus	Gram Negative
	S 8	Pink	Bacillus	Gram Negative
China Cauliflower	CF 1	Pink	Coccobacillus	Gram Negative
	CF 2	Pink	Bacillus	Gram Negative
	CF 3	Pink	Coccobacillus	Gram Negative
	CF 4	Pink	Bacillus	Gram Negative

4.2.2 Biochemical Tests

4.2.2.1 Catalase Test

This test was used to identify the presence of catalase enzymes in the bacterial isolates. Formation of bubbles indicated a positive reaction and the opposed signified a negative reaction. The bubbles formation was showed in Figure 4.3 and the results were tabulated in Table 4.4. All 22 bacterial isolates showed catalase positive and thus corresponded to the characteristic of Enterobacteriaceae family.

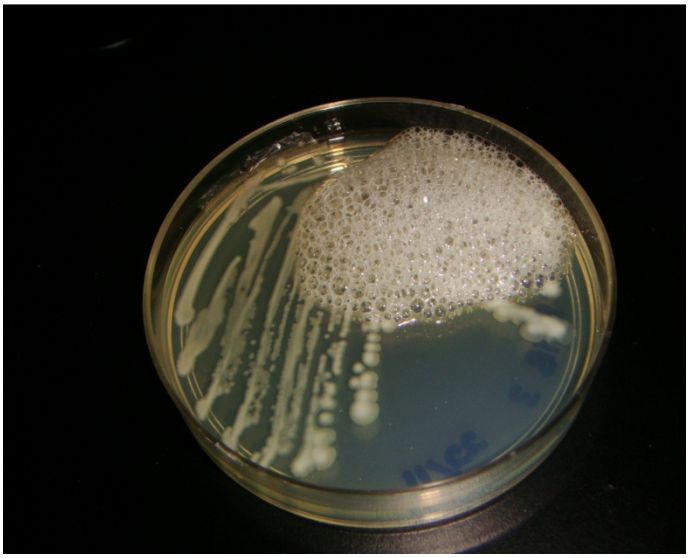


Figure 4.3: Catalase test.

Presence of bubbles formation after adding few drops of hydrogen peroxide indicated a catalase positive.

Table 4.4: Observation for catalase test of bacterial isolates.

Vegetable Sample	Bacterial Isolate	Observation	Reaction
Green Capsicum	CP 1	Formation of gas bubbles.	Catalase Positive
	CP 2	Formation of gas bubbles.	Catalase Positive
	CP 3	Formation of gas bubbles.	Catalase Positive
	CP 4	Formation of gas bubbles.	Catalase Positive
	CP 5	Formation of gas bubbles.	Catalase Positive
Beijing Cabbage	CB 1	Formation of gas bubbles.	Catalase Positive
	CB 2	Formation of gas bubbles.	Catalase Positive
	CB 3	Formation of gas bubbles.	Catalase Positive
	CB 4	Formation of gas bubbles.	Catalase Positive
	CB 5	Formation of gas bubbles.	Catalase Positive
Mixed Salad	S 1	Formation of gas bubbles.	Catalase Positive
	S 2	Formation of gas bubbles.	Catalase Positive
	S 3	Formation of gas bubbles.	Catalase Positive
	S 4	Formation of gas bubbles.	Catalase Positive
	S 5	Formation of gas bubbles.	Catalase Positive
	S 6	Formation of gas bubbles.	Catalase Positive
	S 7	Formation of gas bubbles.	Catalase Positive
	S 8	Formation of gas bubbles.	Catalase Positive
China Cauliflower	CF 1	Formation of gas bubbles.	Catalase Positive
	CF 2	Formation of gas bubbles.	Catalase Positive
	CF 3	Formation of gas bubbles.	Catalase Positive
	CF 4	Formation of gas bubbles.	Catalase Positive

4.2.2.2 Oxidase Test

This test was used to identify the presence of respiratory enzymes, cytochrome oxidase in the bacterial isolates. Formation of strong violet color indicated a positive reaction and the opposed signified a negative reaction. The results of oxidase test were showed in Figure 4.4 and tabulated in Table 4.5. All 22 bacterial isolates showed oxidase negative and thus corresponded to the characteristic of Enterobacteriaceae family.

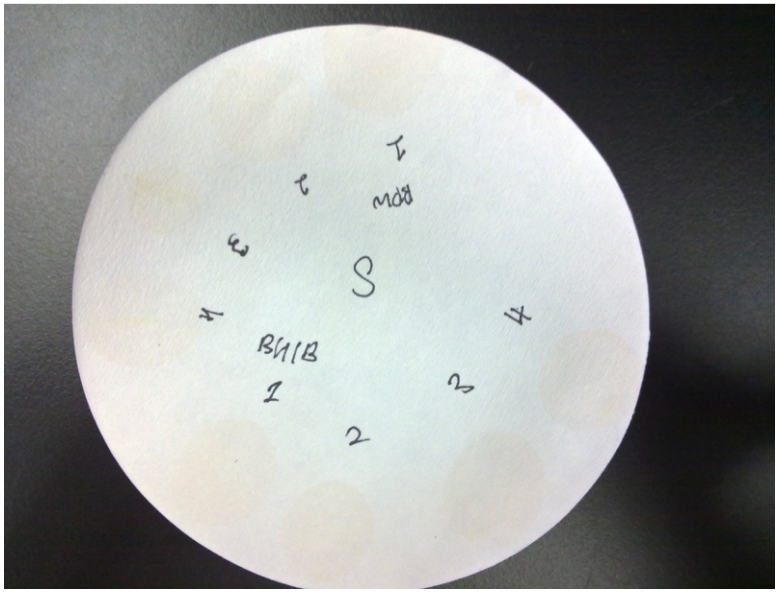


Figure 4.4: Oxidase test.

No formation of strong violet color after placing a single colony on top of oxidase reagent indicated oxidase negative.

Table 4.5: Observation of oxidase test for bacterial isolates.

Vegetable Sample	Bacterial Isolate	Observation	Reaction
Green Capsicum	CP 1	No strong violet color formed.	Oxidase Negative
	CP 2	No strong violet color formed.	Oxidase Negative
	CP 3	No strong violet color formed.	Oxidase Negative
	CP 4	No strong violet color formed.	Oxidase Negative
	CP 5	No strong violet color formed.	Oxidase Negative
Beijing Cabbage	CB 1	No strong violet color formed.	Oxidase Negative
	CB 2	No strong violet color formed.	Oxidase Negative
	CB 3	No strong violet color formed.	Oxidase Negative
	CB 4	No strong violet color formed.	Oxidase Negative
	CB 5	No strong violet color formed.	Oxidase Negative
Mixed Salad	S 1	No strong violet color formed.	Oxidase Negative
	S 2	No strong violet color formed.	Oxidase Negative
	S 3	No strong violet color formed.	Oxidase Negative
	S 4	No strong violet color formed.	Oxidase Negative
	S 5	No strong violet color formed.	Oxidase Negative
	S 6	No strong violet color formed.	Oxidase Negative
	S 7	No strong violet color formed.	Oxidase Negative
	S 8	No strong violet color formed.	Oxidase Negative
China Cauliflower	CF 1	No strong violet color formed.	Oxidase Negative
	CF 2	No strong violet color formed.	Oxidase Negative
	CF 3	No strong violet color formed.	Oxidase Negative
	CF 4	No strong violet color formed.	Oxidase Negative

4.2.2.3 Oxidation-Fermentation (O-F) Test

This test is used to detect the formation of acid by either oxidation or fermentation utilization of glucose. The test was conducted with O-F basal medium with glucose. The production of acid turned the agar into yellow while no production of acid caused the agar remained green. Bacteria with oxidative metabolism may or may not produce acid at aerobic condition, but will not produce acid at anaerobic condition. Bacteria with fermentation metabolism will produce acid for both aerobic and anaerobic conditions. The tubes of O-F test were shown in Figure 4.5 and the results were tabulated in Table 4.6. Only 19 out of 22 bacterial isolates showed fermentation metabolism which corresponded to the characteristic of Enterobacteriaceae family.

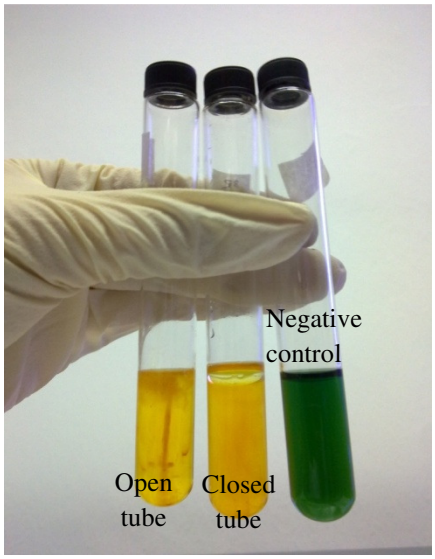


Figure 4.5: Oxidation-Fermentation test.

The open and closed tube with the production of yellow color indicated fermentation metabolism.

Table 4.6: Observation for oxdidation-fermentation test of bacterial isolates.

Vegetable Sample	Bacterial Isolate	Observation		Reaction
		Aerobic Condition	Anaerobic Condition	
Green Capsicum	CP 1	Yellow	Yellow	Fermentation
	CP 2	Yellow	Yellow	Fermentation
	CP 3	Yellow	Yellow	Fermentation
	CP 4	Yellow	Yellow	Fermentation
	CP 5	Yellow	Yellow	Fermentation
Beijing Cabbage	CB 1	Yellow	Yellow	Fermentation
	CB 2	Yellow	Yellow	Fermentation
	CB 3	Yellow	Yellow	Fermentation
	CB 4	Yellow	Yellow	Fermentation
	CB 5	Yellow	Yellow	Fermentation
Mixed Salad	S 1	Yellow	Yellow	Fermentation
	S 2	Yellow	Yellow	Fermentation
	S 3	Yellow	Yellow	Fermentation
	S 4	Yellow	Yellow	Fermentation
	S 5	Yellow	Yellow	Fermentation
	S 6	Yellow	Yellow	Fermentation
	S 7	Yellow	Yellow	Fermentation
	S 8	Yellow	Yellow	Fermentation
China Cauliflower	CF 1	Yellow	Green	Oxidation
	CF 2	Yellow	Green	Oxidation
	CF 3	Yellow	Yellow	Fermentation
	CF 4	Yellow	Green	Oxidation

4.2.3 Antibiotic Susceptibility Tests

The bacterial isolates were tested against 15 types of antibiotic agents and the interpretation of inhibition zone was referred to the Zone Diameter Interpretative Standards of Clinical Laboratory Standards Institute (CLSI) as susceptible, intermediate or resistance. The antibiotic susceptibility test plate was shown in Figure 4.6 and the results were tabulated in Table 4.7 to 4.11. All 19 bacterial isolates showed resistant towards oxacilin and ampicillin. Among these isolates, 11 of them showed a difference of ≥ 5mm between the zone of inhibition of a single disc and in combination with clavulanic acid were considered as potential ESBL producer.

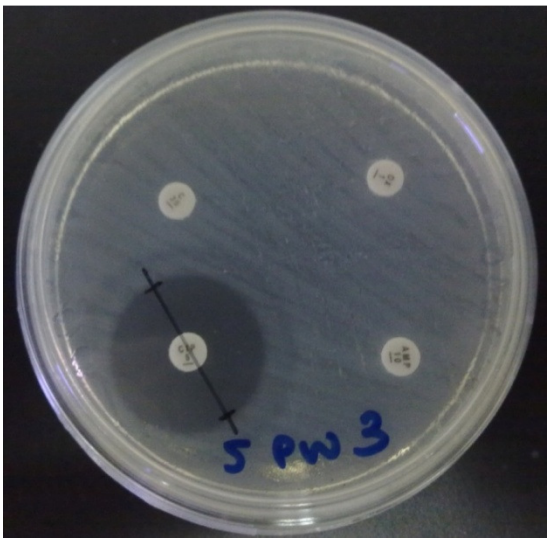


Figure 4.6: Antibiotic susceptibility test

MH agar plate with various commercial antibiotic discs placed on top of it. The zone of inhibition around the discs was observed after 24 hours incubation.

Table 4.7: Antibiotic susceptibility of bacterial isolates from green capsicum sample.

Antibiotic Disc		Bacterial Isolate				
		CP 1	**CP 2**	**CP 3**	**CP 4**	**CP 5**
Aztreonam	ATM	S (25 mm)	S (30 mm)	S (25 mm)	S (30 mm)	S (31 mm)
Cefotaxime	CTX	I (18 mm)	S (25 mm)	S (26 mm)	S (29 mm)	S (30 mm)
Cefotaxime / Clavulanate	CTX / CLA	S (20 mm)	S (30 mm)	S (27 mm)	S (30 mm)	S (31 mm)
Ceftazidime	CAZ	R (14 mm)	S (25 mm)	S (22 mm)	S (24 mm)	S (25 mm)
Ceftazidime / Clavulanate	CAZ / CLA	S (26 mm)	S (30 mm)	S (24 mm)	S (26 mm)	S (26 mm)
Ceftpodoxime	CPD	S (27 mm)	S (24 mm)	S (24 mm)	S (25 mm)	S (25 mm)
Ceftriazone	CRO	S (28 mm)	S (28 mm)	S (26 mm)	S (27 mm)	S (26 mm)
Amoxycilin / Clavulanate	AMC	S (25 mm)	S (22 mm)	S (23 mm)	S (22 mm)	S (26 mm)
Oxacilin	OX	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)
Ampicilin	AMP	R (10 mm)	R (13 mm)	R (11 mm)	R (6 mm)	R (6 mm)
Chloramphenicol	C	S (22 mm)	S (24 mm)	S (25 mm)	I (15 mm)	S (23 mm)
Ciprofloxacin	CIP	S (29 mm)	S (30 mm)	S (25 mm)	R (14 mm)	S (21 mm)
Tetracycline	TE	S (24 mm)	S (23 mm)	S (22 mm)	S (23 mm)	S (20 mm)
Trimethroprim / Sulfamethozaxole	SXT	S (30 mm)	S (28 mm)	S (25 mm)	S (25 mm)	S (25 mm)
Gentamicin	CN	S (16 mm)	S (18 mm)	I (14 mm)	S (16 mm)	S (16 mm)

Note: S: susceptible; I: intermediate; R: resistance

Table 4.8: Antibiotic susceptibility of bacterial isolates from Beijing cabbage sample.

Antibiotic Disc		Bacterial Isolate				
		CB 1	**CB 2**	**CB 3**	**CB 4**	**CB 5**
Aztreonam	ATM	R (13 mm)	S (30 mm)	S (24 mm)	R (13 mm)	S (23 mm)
Cefotaxime	CTX	I (15 mm)	S (29 mm)	I (21 mm)	I (15 mm)	S (16 mm)
Cefotaxime / Clavulanate	CTX / CLA	S (18 mm)	S (30 mm)	S (23 mm)	S (20 mm)	S (23 mm)
Ceftazidime	CAZ	R (12 mm)	S (23 mm)	I (17 mm)	R (13 mm)	S (20 mm)
Ceftazidime / Clavulanate	CAZ / CLA	S (19 mm)	S (26 mm)	S (17 mm)	S (17 mm)	S (26 mm)
Ceftpodoxime	CPD	R (10 mm)	I (20 mm)	R (9 mm)	R (10 mm)	R (6 mm)
Ceftriazone	CRO	I (14 mm)	S (29 mm)	S (22 mm)	R (13 mm)	R (6 mm)
Amoxycilin / Clavulanate	AMC	I (16 mm)	R (10 mm)	I (14 mm)	I (14 mm)	R (6 mm)
Oxacilin	OX	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)
Ampicilin	AMP	R (10 mm)	R (6 mm)	R (6 mm)	R (12 mm)	R (6 mm)
Chloramphenicol	C	I (14 mm)	S (24 mm)	S (23 mm)	R (9 mm)	S (23 mm)
Ciprofloxacin	CIP	S (23 mm)	S (30 mm)	S (32 mm)	S (23 mm)	S (24 mm)
Tetracycline	TE	S (20 mm)	S (24 mm)	S (26 mm)	S (17 mm)	S (20 mm)
Trimethroprim / Sulfamethozaxole	SXT	S (20 mm)	S (33 mm)	S (25 mm)	S (25 mm)	S (22 mm)
Gentamicin	CN	S (17 mm)	S (21 mm)	S (17 mm)	S (17 mm)	S (16 mm)

Note: S: susceptible; I: intermediate; R: resistance

Table 4.9: Antibiotic susceptibility of bacterial isolates from mixed vegetable salad sample.

Antibiotic Disc		Bacterial Isolate							
		S 1	**S 2**	**S 3**	**S 4**	**S 5**	**S 6**	**S 7**	**S 8**
Aztreonam	ATM	S (28 mm)	S (30 mm)	S (29 mm)	S (30 mm)	S (27 mm)	S (29 mm)	S (25 mm)	S (28 mm)
Cefotaxime	CTX	S (26 mm)	S (27 mm)	S (25 mm)	S (25 mm)	S (27 mm)	S (30 mm)	S (24 mm)	S (26 mm)
Cefotaxime / Clavulanate	CTX / CLA	S (26 mm)	S (31 mm)	S (27 mm)	S (26 mm)	S (28 mm)	S (29 mm)	S (24 mm)	S (27 mm)
Ceftazidime	CAZ	S (23 mm)	S (26 mm)	S (23 mm)	S (21 mm)	S (20 mm)	S (24 mm)	S (20 mm)	S (21 mm)
Ceftazidime / Clavulanate	CAZ / CLA	S (25 mm)	S (31 mm)	S (28 mm)	S (25 mm)	S (26 mm)	S (25 mm)	S (22 mm)	S (26 mm)
Ceftpodoxime	CPD	S (26 mm)	S (26 mm)	I (20 mm)	S (21 mm)	S (23 mm)	S (25 mm)	I (19 mm)	S (23 mm)
Ceftriazone	CRO	S (26 mm)	S (32 mm)	S (26 mm)	S (26 mm)	S (26 mm)	S (26 mm)	S (24 mm)	S (32 mm)
Amoxycilin / Clavulanate	AMC	S (24 mm)	S (26 mm)	R (10 mm)	I (14 mm)	S (20 mm)	S (22 mm)	R (10 mm)	S (22 mm)
Oxacilin	OX	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)	R (6 mm)
Ampicilin	AMP	R (13 mm)	R (10 mm)	R (6 mm)	R (6 mm)	R (9 mm)	R (10 mm)	R (6 mm)	R (6 mm)
Chloramphenicol	C	S (28 mm)	S (26 mm)	R (6 mm)	S (23 mm)	S (29 mm)	S (24 mm)	R (6 mm)	S (25 mm)
Ciprofloxacin	CIP	S (21 mm)	S (34 mm)	S (30 mm)	S (28 mm)	S (28 mm)	S (30 mm)	S (24 mm)	S (26 mm)
Tetracycline	TE	I (18 mm)	S (27 mm)	R (6 mm)	S (24 mm)	S (25 mm)	S (25 mm)	R (6 mm)	S (25 mm)
Trimethroprim / Sulfamethozaxole	SXT	S (25 mm)	S (26 mm)	R (6 mm)	S (16 mm)	S (27 mm)	S (29 mm)	R (6 mm)	S (22 mm)
Gentamicin	CN	S (17 mm)	S (18 mm)	S (17 mm)	S (15 mm)	S (17 mm)	S (17 mm)	S (17 mm)	S (17 mm)

Note: S: susceptible; I: intermediate; R: resistance

Table 4.10: Antibiotic susceptibility of bacterial isolate from China cauliflower sample.

Antibiotic Disc		Bacterial Isolate CF 3
Aztreonam	ATM	I (20 mm)
Cefotaxime	CTX	I (22 mm)
Cefotaxime / Clavulanate	CTX / CLA	S (23 mm)
Ceftazidime	CAZ	S (22 mm)
Ceftazidime / Clavulanate	CAZ / CLA	S (29 mm)
Ceftpodoxime	CPD	R (6 mm)
Ceftriazone	CRO	I (20 mm)
Amoxycilin / Clavulanate	AMC	R (6 mm)
Oxacilin	OX	R (6 mm)
Ampicilin	AMP	R (6 mm)
Chloramphenicol	C	R (12 mm)
Ciprofloxacin	CIP	S (25 mm)
Tetracycline	TE	I (15 mm)
Trimethroprim / Sulfamethozaxole	SXT	S (18 mm)
Gentamicin	CN	S (18 mm)

Note: S: susceptible; I: intermediate; R: resistance

Table 4.11: Classification of antibiotics and results of antibiotic susceptibility tests of bacterial isolates.

No	Antibiotic Disc		No (%) of Resistant Bacterial Isolates from			
			Green Capsicum (n=5)	Beijing Cabbage (n=5)	Mixed Salad (n=8)	China Cauliflower (n=1)
1	Aztreonam	ATM	0 (0)	2 (40)	0 (0)	1[a] (100)
2	Cefotaxime	CTX	1[a] (20)	3[a] (60)	0 (0)	1[a] (100)
3	Cefotaxime / Clavulanate	CTX / CLA	1[c] (20)	2[c] (40)	3[c] (37.5)	0 (0)
4	Ceftazidime	CAZ	1 (20)	3[b] (60)	0 (0)	0 (0)
5	Ceftazidime / Clavulanate	CAZ / CLA	2[c] (40)	2[c] (40)	5[c] (62.5)	0 (0)
6	Ceftpodoxime	CPD	0 (0)	5[b] (100)	2[a] (25)	1 (100)
7	Ceftriazone	CRO	0 (0)	3[b] (60)	0 (0)	1[a] (100)
8	Amoxycilin / Clavulanate	AMC	0 (0)	5[b] (100)	3[b] (37.5)	1 (100)
9	Oxacilin	OX	5 (100)	5 (100)	5 (100)	1 (100)
10	Ampicilin	AMP	5 (100)	5 (100)	5 (100)	1 (100)
11	Chloramphenicol	C	1[a] (20)	2[b] (40)	2 (25)	1 (100)
12	Ciprofloxacin	CIP	1 (20)	0 (0)	0 (0)	0 (0)
13	Tetracycline	TE	0 (0)	0 (0)	3[b] (37.5)	1[a] (100)
14	Trimethroprim / Sulfamethozaxole	SXT	0 (0)	0 (0)	2 (25)	0 (0)
15	Gentamicin	CN	1[a] (20)	0 (0)	0 (0)	0 (0)

Note:

[a] Bacterial isolates demonstrated intermediate sensitivity toward antibiotic disc.

[b] Bacterial isolates demonstrated resistance and intermediate sensitivity.

[c] Bacterial isolates demonstrated a ≥ 5mm zone diameter for either antibiotic disc tested in combination with clavulanic acid versus its zone when tested alone, indicating it as a ESBL producing.

4.2.4 API 20E Bacterial Identification Kit

Two bacterial isolates were selected for API 20E bacterial identification. They are bacterial isolates S 3 and S 5. Figure 4.7 showed API strip with multi biochemical tests to identify the bacterial isolates. The summary of biochemical tests' reactions were shown in Table 4.12, while the identification results using apiweb were shown in Table 4.13.

(a)

(b)

Figure 4.7: API Multi-test Strip

Bacterial identification of (a) S 3, (b) S 5 using API 20E kit.

Table 4.12: Summary of biochemical test reactions.

Tests		Bacterial Isolates	
		S3	S5
β-galactosidase	ONPG	+	+
Arginine Dihydrolase	ADH	+	-
Lysine Decarboxylase	LDC	-	+
Ornithine Decarboxylase	ODC	+	+
Citrate Utilization	CIT	+	-
H_2S Production	H_2S	-	-
Urease	URE	-	-
Tryptophan Deaminase	TDA	-	-
Indole Production	IND	-	+
Voges-Proskauer Reaction	VP	+	+
Gelatinase	GEL	-	+
Glucose	GLU	+	+
Mannitol	MAN	+	+
Inositol	INO	-	-
Sorbitol	SOR	+	+
Rhamnose	RHA	+	+
Sucrose	SAC	+	+
Melibiose	MEL	+	+
Amygdalin	AMY	+	-
Arabinose	ARA	+	+
NO_2 Production	NO_2	+	+
N_2 Production	N_2	-	-

Note: +: Positive reaction; -: Negative reaction

Table 4.13: Identification result using apiweb.

Bacterial Isolate	Significant taxa	Percentage Identity
S 3	*Enterobacter cloacae*	96.7 %
S 5	*Serratia odorifera*	99.1 %

4.3 Analyses of PCR Product and Agarose Gel Electrophoresis

4.3.1 Purity of Extracted Total DNA

Total DNA were extracted from the bacterial isolates that fulfilled the characteristics of Enterobacteriaceae family. Bacterial isolates CB 4, S 2 and S 6 had A_{260} / A_{280} values lower than 1.8 which are 1.69, 1.71 and 1.75 respectively, which indicate residual protein contamination. Bacterial isolate S 4 had A_{260} / A_{280} value higher than 2.0 which is 2.03 and this indicates slightly RNA contamination. The other bacterial isolates showed A_{260} / A_{280} values ranging from 1.8 – 2.0 which are indication of pure DNA. The absorbance readings and concentration of the extracted total DNA were tabulated in Table 4.14.

Table 4.14: Absorbance and total DNA concentrations of bacterial isolates.

Vegetable Samples	Bacterial Isolate	A_{260} / A_{280}	Concentration (ng / µL)
Green Capsicum	CP 1	1.80	518.76
	CP 2	1.80	490.21
	CP 3	1.91	460.88
	CP 4	1.86	516.08
	CP 5	1.85	561.05
Beijing Cabbage	CB 1	1.87	397.99
	CB 2	1.86	525.07
	CB 3	1.85	550.69
	CB 4	1.69	261.89
	CB 5	1.80	422.38
Mixed Salad	S 1	1.89	392.46
	S 2	1.71	558.73
	S 3	1.87	399.27
	S 4	2.03	711.91
	S 5	1.94	418.41
	S 6	1.75	492.68
	S 7	1.93	723.83
	S 8	1.97	457.13
China Cauliflower	CF 3	1.90	1282.93

4.3.2 DNA Analyses of Bacterial Isolates

Total DNA from the 19 bacterial isolates were PCR amplified using *bla*-TEM and *bla*-OXA primers. Three out of 19 bacterial isolates, S 3, S 5 and S 7 were successful in amplifying and gave the expected band size of 870 bp. This DNA size corresponded to the expected gene fragment size of *bla*-TEM. None of 19 bacterial isolates gave the approximate expected band size of 430 bp when amplified using *bla*-OXA primers. The results may indicate the absence of *bla*-OXA gene fragments in the bacterial strains. The gel electrophoresis image of PCR products was shown in Figure 4.8.

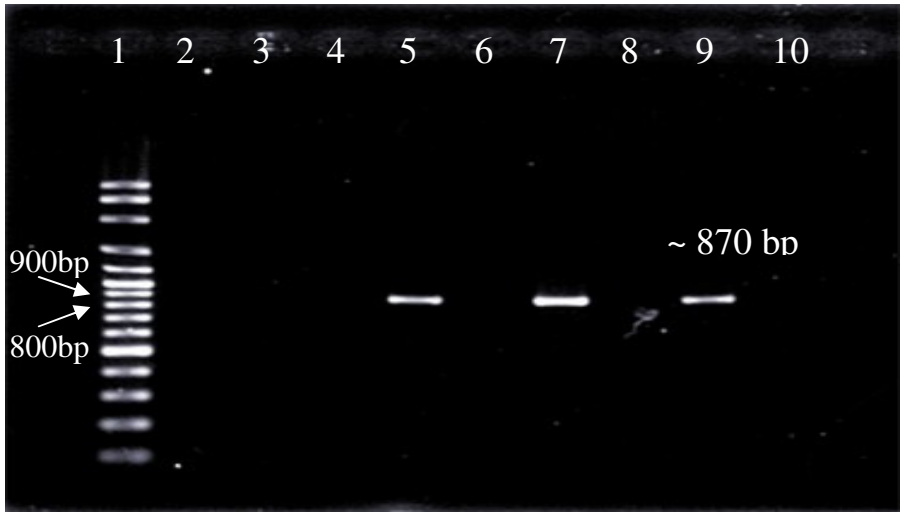


Figure 4.8: PCR products amplified using *bla*-TEM primers

Lane 1 is the 100 bp DNA ladder (Vivantis). Lane 2 is the negative control. Lanes 3 to 10 are the PCR products for bacterial isolates S 1, S 2, S 3, S 4, S 5, S 6, S 7 and S 8, respectively amplified using *bla*-TEM primers. The bacterial isolates S 3 (lane 5), S 5 (lane 7) and S 7 (lane 9) showed band size approximately 870 bp.

4.4 Gel-Purified PCR Products

The PCR products of bacterial isolates S 3, S 5 and S 7 were gel-purified using the *GeneJET Gel Extraction Kit (GeneJET).* The electrophoresis image of gel-purified PCR products was shown in Figure 4.9. The absorbance readings and concentration of the gel-purified DNA were illustrated in Table 4.15.

Table 4.15: Absorbance readings and concentration of the gel-purified PCR products.

Bacterial Isolate	A_{260} / A_{280}	Concentration (ng / µL)
S 3	1.81	12.79
S 5	2.03	10.89
S 7	1.97	9.35

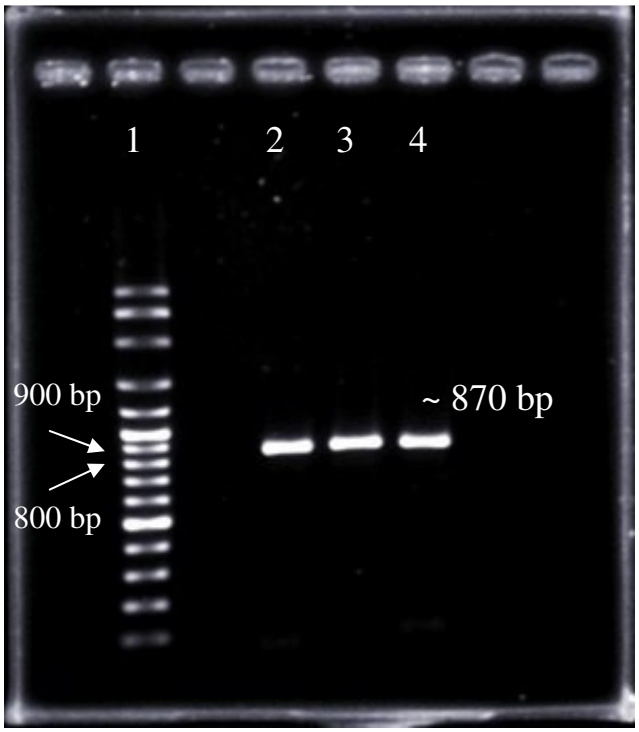


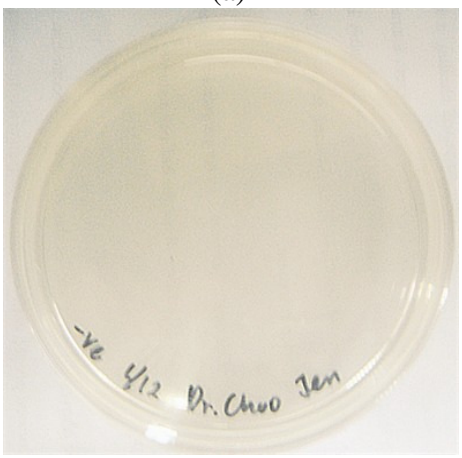
Figure 4.9: Gel-Purified PCR Products

Lane 1 is the 100 bp DNA ladder (Vivantis). Lane 2 to 4 are the gel-purified PCR products for bacterial isolates S 3, S 5 and S 7, respectively. The DNA size is approximately 870 bp.

4.5 Tansformation of *E. coli* JM109

The gel-purified PCR products from bacterial isolates S 3 and S 5 were ligated into pGEM-T Easy Vector (Promega) and were subsequently transformed. The transformation plates were shown in Figure 4.10. No growth observed on the negative plate indicated that no contamination occurred (Figure 4.10 (a)). The positive control plate showed a lawn of blue colonies indicated high transformation efficiency (Figure 4.10 (b)). For ligation mixture plate, white colonies were interspersed in between blue colonies (Figure 4.10 (c)).

(a)



(b)

(c)

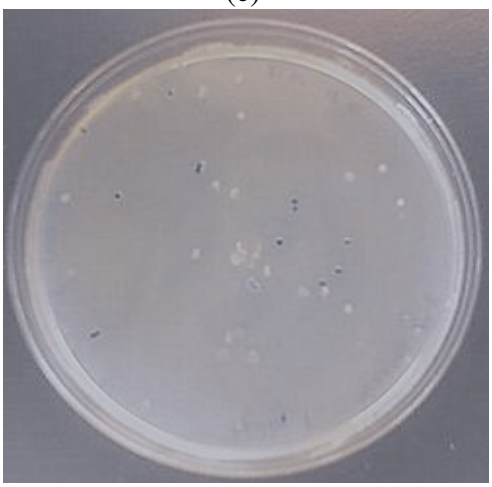


Figure 4.10: Transformation Plates

(a) Negative control plate; (b) Positive control plate; and (c) Ligation mixture plate with the gel-purified PCR product from bacterial isolate

4.6 Colony PCR

Selected colonies from ligation mixture plate were carried out using colony PCR methodology. The primers used are T7 and SP6 primers. Results from blue colony gave the approximate size of 180 bp, indicating no insert, while the white colony gave the approximate size of 1040 bp, indicating the presence of insert with the desired gene fragment. The electrophoresis image of colony PCR products was shown in Figure 4.11.

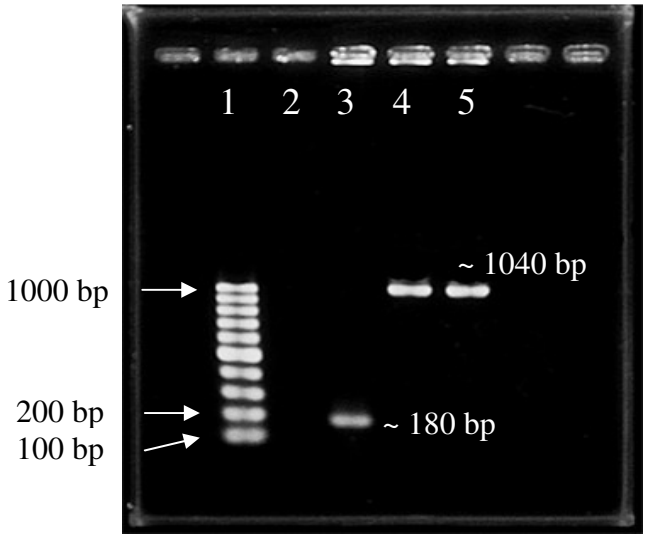


Figure 4.11: Gel Electrophoresis of Colony PCR Products

Lane 1 is the 100 bp DNA ladder (Fermentas). Lane 2 is the negative control. Lane 3 is the colony PCR products of blue colony. Lane 4 and 5 are the colony PCR products of white colonies from bacterial isolates S 3 and S 5, respectively.

4.7 Plasmid Extraction

White bacterial colonies that were confirmed to contain the DNA fragment of interest were subsequently subjected to plasmid extraction. The recombinant plasmids corresponding to gene fragments from bacterial isolate S 3 and S 5 were reassigned as pTWC_S3 and pTWC_S5, respectively. They were subjected to agarose gel electrophoresis and a band approximately 3100 bp was observed in Figure 4.12. The absorbance readings and concentration of the plasmid extraction were illustrated in Table 4.16.

Table 4.16: Absorbance readings and concentration of the plasmids extracted.

Plasmid	A_{260} / A_{280}	Concentration (ng / µL)
pTWC_S3	1.93	241
pTWC_S5	2.00	215

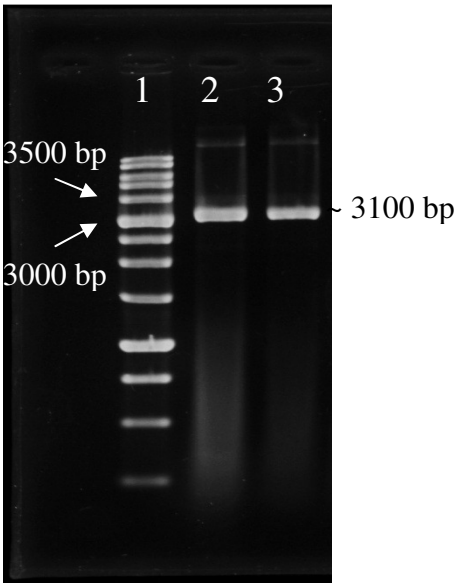


Figure 4.12: Gel Electrophoresis of Plasmid Extraction

Lane 1 is the 1kb DNA ladder (Fermentas). Lane 2 and 3 are the recombinant plasmids pTWC_S3 (lane 2) and pTWC_S5 (lane 3), respectively.

4.8 DNA Sequencing

The plasmids pTWC_S3 and pTWC_S5 were sequenced using T7 and SP6 promoter primers and the DNA sequences were shown.

(a) Bacterial isolate S 3 using T7 promoter primer

ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCG
GCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAA
AGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTAC
ATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCG
CCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGC
TATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCA
ACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT
ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGT
AAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACT
GCTGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGC
TAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT
GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACG
AGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCG
CAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAAC
AATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACT
TCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACT
GGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGA
CGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGC
TGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAG

(b) Bacterial isolate S 5 using SP6 promoter primer

CTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCG
ATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTG
TAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTG
CTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTT
ATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAG
TGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG
CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTCTGCGC
AATGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTC
GTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGC
GAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCC
TTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCAGCAGTGTT
ATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCA
TGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC
AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG

CCCGGCGTCAACACGGGATAATACCGCACCACATAGCAGAACT
TTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACT
CTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCA
CTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGC
GTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAA
AGGGAATAAGGGCGACACGGAAATGTTGAATACTCAT

4.9 Bioinformatics Analysis

4.9.1 BlastX Analysis

The DNA sequences were analyzed using BlastX alignment. Both sequences corresponded to beta lactamase *bla*-TEM gene with the identity between 98% - 99%. The results of BlastX were shown in Table 4.17.

Table 4.17: BlastX alignment results

Bacterial Isolate	Blast Identity	Accession	Percentage Identity	Score (bits)	E value
S 3	Beta-lactamase TEM-141 [*Enterobacter cloacae*]	AAX56615.1	99 %	540	1e-151
	Inhibitor-resistant beta-lactamase TEM-80 [*Enterobacter cloacae*]	AAM15527.1	98 %	538	4e-151
	Beta-lactamase TEM-157 [*Enterobacter cloacae*]	ABI81768.1	98 %	537	6e-151
S 5	Beta-lactamase [*Serratia marcescens*]	ADD96657.1	98 %	538	4e-151

4.9.2 Pairwise Alignment

Gene fragments from bacterial isolates S 3 and S 5 were aligned using pairwise alignment. The percentages of identity showed 99%. The dissimilar nucleotides were underlined and highlighted in red color.

```
>lcl|12803 P1
Length=868

 Score = 1592 bits (862),  Expect = 0.0
 Identities = 866/868 (99%), Gaps = 0/868 (0%)
 Strand=Plus/Plus

Query  1    ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCT  60

Query  61   GTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA  120

Query  121  CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCC  180

Query  181  GAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCC  240

Query  241  CGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTG  300

Query  301  GTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA  360

Query  361  TGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATC  420

Query  421  GGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT  480

Query  481  GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATG  540

Query  541  CCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCT  600
            |||||||||||||||||||| ||||||| ||||||||||||||||||||||||||||||
Sbjct  541  CCTGCAGCAATGGCAACAACATTGCGCAGACTATTAACTGGCGAACTACTTACTCTAGCT  600

Query  601  TCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  TCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGC  660

Query  661  TCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCT  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  TCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCT  720

Query  721  CGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTAC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  CGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTAC  780
```

```
Query  781  ACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781  ACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCC  840

Query  841  TCACTGATTAAGCATTGGTAACTGTCAG  868
            ||||||||||||||||||||||||||||
Sbjct  841  TCACTGATTAAGCATTGGTAACTGTCAG  868
```

CHAPTER 5

DISCUSSION

5.1 Cultivation and Isolation of Bacteria

Bacteria were cultivated and isolated from vegetable samples through the use of medium such as Brain Heart Infusion Broth (BHIB) medium, Buffered Peptone Water (BPW) medium, MacConkey (MAC) agar and Violet Red Bile Agar with Glucose (VRBG). These medium were supplemented with ampicillin (50 µg/mL) to allow survival of bacteria isolates with antibiotic resistance.

BHIB media is a nutrient media used to cultivate and isolate both fastidious and non-fastidious bacteria from vegetables. BHIB medium contain infusion of beef heart and calf brain, peptone and dextrose mixture that support the growth of microorganisms (Hardy Diagnostics, 2011a).

BPW media is a non-selective, preliminary enrichment nutrient media used to isolate pathogenic Enterobacteriaceae, particularly *Salmonella sp.* Compositions of BPW medium consists of peptones and phosphate buffer mixture and thus come with the functions of repairing damage cells and facilitating the recovery of *Salmonella sp* (Hardy Diagnostics, 2011b).

Both Gram positive and negative bacteria can equally grow in BHIB and BPW medium, thus MAC serve as selective media to isolate out Gram negative bacteria. MAC contains bile salt, crystal violet dye, neutral red dye, lactose and peptone mixture. Presence of crystal violet and bile salts can inhibit the growth of Gram positive bacteria, while presence of lactose allow the differentiation of bacteria that are able to ferment lactose based on colony color. Pink colony appeared indicates lactose fermenting bacteria and has the potential of being Enterobacteriaceae (Flournoy, Wongpradit and Silberg, 1990).

Colony types were streaked to single colonies on VRBG media. VRBG is modified from MAC media where composition of lactose is replaced with glucose. VRBG has the function similar to MAC, yet the replacement with glucose serve as source of carbohydrate and allow a better recovery rate for bacteria (Hardy Diagnostics, 2011c).

A total of 22 morphologically different bacterial isolates obtained from green capsicum, Beijing cabbage, mixed salad and China cauliflower samples. All bacterial isolates were recognized as Gram negative bacteria with ampicillin antibiotic resistance and showed potential Enterobacteriaceae bacteria.

5.2 Characterization of Bacterial Isolates

5.2.1 Differential Staining – Gram Staining

Gram staining was developed and named after Hans Christian Gram, a Danish bacteriologist in 1800's and serve as the basis for the identification of bacteria which divides them into two major groups, namely Gram Positive and gram negative bacteria (Harley and Prescott, 2002).

The staining involves a series of dyes that stain the bacteria either pink or purple to indicate their Gram's reaction. Crystal violet dye serves as the primary stains that passes through the cell wall and cell membrane and stain both Gram positive and negative bacteria cells purple. Iodine acts as a mordant that increases the affinity or attraction between cells and crystal violet dye. The 95% ethanol used for decolourizing purpose where it washes away the unretained dye from Gram negative bacteria cells. At this stage, Gram positive bacteria stained purple while Gram negative bacteria appeared in colorless form. Thus, the safranin serves as the counterstain to stain the decolorized cells in a different colour from that of the primary stain.

Gram positive bacteria with thick peptidoglycan layer on its outermost cell wall structure enable it to retain crystal violet dye and stained purple, while Gram negative bacteria with thinner peptidoglycan layer undergo decolorizing and stained pink by safranin.

All 22 bacterial isolates were examined under microscope showed either bacillus or coccobacillus shape with a shade of pink. They were confirmed as Gram negative bacteria and showed potential Enterobacteriaceae bacteria.

5.2.2 Biochemical Tests

5.2.2.1 Catalase Test

Under aerobic condition, some bacteria breakdown the sugar and produce hydrogen peroxide as an oxidative end product. Hydrogen peroxide is a powerful oxidizing agent, thus its accumulation is highly toxic to bacteria and can lead to the death of cells. Hence, bacteria able to produce the catalase enzyme for surviving purpose by decomposing hydrogen peroxide to water and oxygen. Most aerobes and facultative anaerobes have the ability of producing catalase enzyme, while anaerobes lacks of such ability (Taylor and Achanzar, 1972).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

All the bacterial isolates were observed as catalase positive through the formation of bubbles. This indicates that they able to produce catalase enzyme and showed potential Enterobacteriaceae bacteria.

5.2.2.2 Oxidase Test

The cytochrome oxidase is the respiratory enzyme that is present in aerobic bacteria and able to catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (oxygen). The oxidase reagent, namely N, N, N, N-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron

acceptor for the enzyme oxidase. It oxidized to form purple compound called Wurster's blue (Health Protection Agency, 2010).

Characteristically, Enterobacteriaceae bacteria are oxidase negative and lack of cytochrome oxidase. From the results obtained, all 22 bacterial isolates did not show formation of purple compound. This indicates oxidase negative and thus corresponded to characteristic of Enterobacteriaceae bacteria.

5.2.2.3 Oxidation-Fermentation Test

Bacteria can metabolize the carbohydrate source by oxidation (aerobic) or / and fermentation (anaerobic). Bacteria that can undergo both oxidation and fermentation metabolisms are called facultative anaerobes. OF media was supplemented with glucose as the carbohydrate source for both aerobic and anaerobic condition. If the bacteria are able to metabolize the glucose in the condition present, acid produced will turn the media into yellow color. In vice versa, media that remain green indicates that metabolism did not occur. The bacteria with oxidative metabolism will display yellow media in aerobic condition but green media in anaerobic condition. The bacteria with fermentative metabolism will display yellow agar in both conditions (Hugh and Leifson, 1953).

Typically, Enterobacteriaceae bacteria are facultative anaerobic. From the results obtained, 19 out of 22 bacterial isolates showed fermentation metabolisms and corresponded to Enterobacteriaceae's characteristic. Another three bacterial

isolates, namely CF 1, CF 2 and CF 4 showed oxidation metabolisms which is no longer potential Enterobacteriaceae bacteria.

5.2.3 Antibiotic Susceptibility Test

Bacteria can form resistance to antibiotics in the form of intrinsic resistance o acquired resistance. Intrinsic resistance means that the bacteria were resistant to an antibiotic even before its introduction while acquired resistance means that the bacteria were originally susceptible to an antibiotic and later became resistant. The development of acquire antibiotic resistance can occur by either mutation or genetic material exchanging among similar or related species. The ESBLs gene developed by Enterobacteriaceae family is the best example to demonstrate the concept of acquires resistance.

Antibiotic susceptibility tests can serve as a preliminary test to detect the potential ESBL-producing strains. When bacterial isolates tested against antibiotic disc, the potential ESBL-producer will resistance towards ceftazidime, cefotaxime, and aztreonam. Bacterial isolates that demonstrated a $\geq$ 5mm zone diameter for both cefotaxime and ceftazidime tested in combination with clavulanic acid versus its zone when tested alone, indicating it is a potential ESBL producer. Besides, resistance toward ampicillin also gave indication that there is potentially present of *bla* gene coding for beta-lactamases (Rasheed et al., 1997).

The zone of inhibition implies the sensitivity of bacteria towards the antibiotic. The interpretation is based on the Zone Diameter Interpretative Standards of Clinical Laboratory Standard Institute (CLSI) as either susceptible, intermediate or resistance.

From the results obtained, all 19 bacterial isolates showed resistant towards oxacilin and ampicillin. Among these isolates, 11 of 19 them showed a difference of ≥ 5mm between the zone of inhibition of a single disc and in combination with clavulanic acid were considered as potential ESBL producer.

Some of the results obtained may not be accurate due to inconsistence parameters. The degree of diffusion of the antibiotic in the plate, the different thickness of poured agar, the inoculums volume, and the time of incubation can contribute to the difference in results interpretation (Tilton, Leiberman and Gerlach, 1973).

In order to obtain more reliable and accurate results, the parameters in performing the tests should be consistent. The agar medium should be prepared with a uniform depth of approximately 4 mm. The inoculums density should be standardized by referring to 0.5 McFarland standard. The antibiotic disc must be pressed down to ensure complete contact with the agar surface to allow optimum diffusion (Tilton, Leiberman and Gerlach, 1973).

5.2.4 API 20E Bacterial Identification Kit

The bacterial isolates S 3 and S 5 that successful in amplifying the desire gene fragment were subsequently identified using API 20E Bacterial Identification Kit.

The purpose of o-nitrophenyl-ß-D-galactopyranoside (ONPG) test is used to detect the presence or absence of the enzyme ß-galactosidase in the bacteria. The presence of two enzymes, permease and ß-galactosidase, are required in the lactose fermentation. The enzyme permease permits the lactose enter the bacterial cells, while the enzyme ß-galactosidase hydrolyses lactose to form galactose and glucose. The ONPG reagent is structurally similar to lactose. The used of ONPG does not require the activity of permease. Thus, the present of ß-galactosidase will cleave the colourless ONPG into galactose together with the yellow compound, namely o-nitrophenol (Isenberg, 1992). From Table 4.13, both bacterial isolates showed ONPG positive indicated the present of ß-galactosidase.

The arginine dihydrolase (ADH) test is used to determine the bacteria that can metabolize arginine to produce alkaline amide and ammonia under anaerobic conditions. The activity of arginine dihydrolase involve arginine iminohydrolase, citrulline ureidase and ornithine decarboxylase (the production only limited to certain species of bacteria cells). From Table 4.13, bacterial isolate S 3 showed ADH positive, while bacterial isolate S 5 showed oppose reaction.

The lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) tests are used to determine the ability of bacteria to decarboxylate lysine or ornithine to

produce alkaline amines under anaerobic conditions. The lysine is converted to cadaverine, while the ornithine is converted to putrescine. Bacterial isolate S 3 showed LDC negative and ODC positive, while bacterial isolate S 5 showed positive results for both tests.

The citrate (CIT) test is used to identify the bacteria that are able to utilize citrate as the sole carbon source for energy in the presence of oxygen. The citrate metabolism involves two enzymes, namely citrate permease and citrase. The enzyme citrate permease permits the transport of citrate into the bacterial cells, while the enzyme citrate breaks down citrate into acetic acid and oxalocetic acid. The oxaloacetate will further metabolized to yield pyruvate and carbon dioxide. Ammonium phosphate salts are included in the media to serve as a nitrogen source. Bacteria will then utilize nitrogen and release ammonia. The alkaline ammonia raises the pH and changes the indicator to blue color (MacFaddin, 2000). From the results obtained, bacterial isolate S 3 showed CIT positive, while bacterial isolate S 5 showed CIT negative.

The hydrogen sulfide (H_2S) test is used to detect the production of H_2S by bacterial cells. In the present of amino acid containing sulfur such as cysteine and methionine, some bacteria can liberate H_2S. The media used is in the form of sodium thiosulfate with ferric ammonium citrate. H_2S will react with sodium thiosulfate to yield ferric ammonium citrate. This will eventually form the black

deposit that blackening the medium. Both bacterial isolates showed H_2S negative indicated no involvement in production of H_2S.

The urease (URE) test is used to determine the ability of bacteria cells to cleave urea into ammonia, carbon dioxide and water. The reaction will turn the medium alkaline and eventually raise the pH. The urease can be detected due to colour changes of pH indicator in medium. Both bacterial isolates showed URE negative indicated no involvement in production of urease.

The tryptophan deaminase (TDA) test is used to determine the ability of bacteria to deaminate aromatic amino acid such as tryptophan to yield aromatic derivatives of pyruvic acid. The end-product is detected by adding the ferric chloride. The production of reddish brown color indicated positive reaction. Both bacterial isolates showed TDA negative indicated no production of tryptophan deaminase.

The indole (IND) test is used to detect the present of indole in bacterial cells. Tryptophan is hydrolyzed by tryptophanase to yield indole, pyruvic acid and ammonia (MacFaddin, 2000). Indole can be detected by adding an aldehyde reagent that condenses with the pyrrole ring in indole to produce a quinoidal compound that is red in color. Bacterial isolate S 3 showed IND negative while, bacterial isolate S 5 showed IND positive indicated present of indole.

The Voges Proskauer (VP) test is used to detect the production of acetyl methyl carbinol from glucose. Acetyl methyl carbinol is an intermediate compound found in bacteria as the end-product of glucose fermentation. It can be detected by addition of potassium hydroxide to produce diacetyl. Subsequently, it reacts with guanidine residues present in peptone to produce pink colour (Leboffe and Pierce, 2006). Both bacterial isolates showed VP postitive indicated there was production of acetyl methyl carbinol.

The gelatin (GEL) test is used to test the ability of bacteria to break down gelatin. Enzyme gelatinase catalyze the breaking down of gelatin and thus allow the diffusion of particles throughout the cupule. Bacterial isolate S 3 showed GEL negative, while bacterial isolate S 5 showed GEL positive.

The glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA) tests are used to determine the ability of bacteria to produce acid by undergoing fermentation metabolism. As mentioned, Enterobactericeae bacteria are facultative anaerobes. They have the capability of fermenting most of the glucose source. From the results obtained, bacterial isolate S 3 showed positive to all tests accept INO, while bacterial isolate showed positive to all tests accept INO and AMY.

The identification results using apiweb were shown in Table 4.14. Bacterial isolate S 3 was identified as *Enterobacter cloacae* with the percentage identity of 96.7%, while bacterial isolate S 5 was identified as *Serratia odorifera* with the percentage of 99.1%. The minimum acceptable identification of API 20E Kit must show the percentage of identity greater or at least equal to 80%. Thus, we can conclude that the identification results obtained are reliable.

The API 20 E system contains a few limitations. The system can only used for the identification of Enterobacteriaceae and non-fastidious Gram negative bacteria, it cannot be used to identify other organisms or even exclude their presence. Besides, glucose reaction for some bacteria may revert from positive to negative and this may affect the percentage of identity. Lastly, only pure culture of the bacteria can give the reliable identification result.

5.3 Analyses of PCR Product and Agarose Gel Electrophoresis

5.3.1 Total DNA Extraction

Total DNA of the bacterial isolates was extracted using fast boil method (Holmes and Quigley, 1981). The cells were lyzed partially to allow the escape of plasmids, while most chromosomal DNA trapped in the cell debris. The trapped chromosomal DNA was removed by denaturing at high temperature. Re-annealing took place on ice incubation where supercooling allow the plasmids to re-associate, whereas chromosomal DNA remain denatured. Lastly,

centrifugation was used to discard the chromosomal DNA, together with cell debris.

The concentrations and A_{260} / A_{280} values of extracted total DNA were determined and shown in Table 4.15. Bacterial isolates CB 4 and S 2 had A_{260} / A_{280} values lower than 1.8 which are 1.69 and 1.71 indicate slightly protein contamination. Bacterial isolate S 4 had A_{260} / A_{280} value higher than 2.0 which is 2.03 indicates slightly RNA contamination. The other bacterial isolates showed A_{260} / A_{280} values ranging from 1.8 – 2.0 indicate pure DNA. Besides, the DNA concentrations ranging from 250 to 1450 ng/µL indicated very high concentrations.

5.3.2 DNA Analyses of Bacterial Isolates

The Polymerase Chain Reaction (PCR) is the molecular method developed by Kary Mullis in the 1980s used to amplify and made a huge number of copies a specific region of a DNA strand. With the presence of primer sets targeted on specific DNA region, DNA polymerase (Taq polymerase) can amplify the desired sequence by adding the nucleotides to the 3'-OH group of preexisting primers.

Conditions of PCR play a critical role in the amplification of desired sequence. Optimization of PCR conditions such as annealing temperature and time were carried out at 50ºC for 45 s to allow annealing of primers to DNA template. The

extension temperature depends on the DNA polymerase used was adjusted to 72ºC which is the optimum temperature for the activity of *Taq* polymerase.

The total DNA was carried out with PCR amplification using *bla*-TEM and *bla*- OXA primers where the results were shown in Figure 4.8 and 4.9 respectively. In the amplification using *bla*-TEM primer, 3 out of 19 bacterial isolates, namely S 3, S 5 and S 7 gave a band of approximate 870 bp in gel electrophoresis image which were corresponded to the expected size of 867 bp. Successful amplification of *bla*-TEM genes with no smearing observed indicated PCR condition selected was optimum and suitable for *bla*-TEM primer.

When *bla*-OXA primers were used for PCR amplification, no band was observed for all bacterial isolates. The failure of *bla*-OXA genes amplification may due to unoptimized PCR condition such as unspecific annealing temperature. After various PCR optimization condition were tried and yet still no band observed, we then can make a presumptive conclusion that the 19 bacterial isolates do not contain any desired *bla*-OXA genes.

5.3.3 Gel-Purified PCR Products

Bacterial isolates S 3, S 5 and S 7 that gave the expected size of *bla*-TEM genes during PCR amplification were subsequently gel purified. In Figure 4.10, the bands with approximate size of 870 bp indicated the presence of purified PCR products. Sharp and distinct bands with no smearing indicated homogenous DNA

fragments. The absorbance readings and concentration of the gel-purified DNA were illustrated in Table 4.16. Bacterial isolates S 3 and S 7 showed pure purified DNA with A_{260} / A_{280} values ranging from 1.8 – 2.0, while bacterial isolates S 5 showed slightly RNA contamination with A_{260} / A_{280} values higher than 2.0. The concentrations of purified DNA for these three bacterial isolates were considered very low which ranged from 9 to 13 ng/µL. The purity and concentration of purified DNA are the crucial factors that may affect the degree of DNA sequences ligated into the vector.

5.4 Ligation and Transformation

5.4.1 Ligation with pGEM-T Easy Vector

The term DNA ligation is the concept of joining linear DNA fragments together with covalent bonds, while the term recombinant DNA is the joining or recombining of DNA fragments to new DNA molecules (Vento & Gillum, 2002). The pGEM-T Easy Vector was used to ligate desired PCR fragments due to several reasons. The pGEM-T Easy Vector was designed with 3'T overhangs. This advantage will prevent the re-circularization of vector and yet provide a compatible overhang for the ligation of PCR fragments. The used of *Taq* polymerase in PCR amplification will definitely generate a single 3'A overhang at the PCR fragments and this will complement to the single 3'T overhang at the vector. Besides, the vector designed with the selectable marker genes such as *lacZ* gene provides the recognition and selection advantage.

5.4.2 Tansformation of *E. coli* JM109

Transformation is a process where a foreign DNA was introduced into a living cell. For example, *E. coli* has the ability to take up a small amount of DNA under normal condition. Thus, to introduce foreign DNA into bacteria effectively and efficiently, it has to undergo certain modification by some form of physical or chemical treatment in order to enhance their ability to uptake DNA. Bacteria cells that have undergo are known as competent cells.

Transformed cells were plated in LB agar medium containing ampicilin antibiotics, together with X-Gal and IPTG to enable screening and selection of the desired colonies. Three plates were prepared for the blue-white screenings which were negative control plate, positive control plate and ligation plate.

In the positive control plate, the aim is to test the efficiency of the competent cells. The pUC19 vectors contain the ampicillin resistant gene and therefore transformed *E. coli* with the vector hence will cause the appearance of blue colonies in the ampicillin plate. In the negative control plate, the purpose is to test on the effectiveness and efficiency of the antibiotics and also aseptic technique. Therefore, the result of the plate have no growth occurred as the cell cannot survive in ampicillin medium and no sign of contamination.

In the pGEM-T Easy Vector- ligation plate, *E. coli* is transformed with pGEM-T Easy Vector. The blue colony indicates the non-recombinant as it carried the self-

ligated plasmid whereas the white colony indicates the successful recombinant as it carried the recombinant plasmid. Colonies that carry the self-ligated vector will appear as blue as it growed in the medium because it is able to produce β-galactosidase and cleave the substrate X-gal. Whereas the successful ligation (transformed *E. coli*) of the foreign DNA insert will disrupt the Lac Z frame and hence it cannot code for the β-galactosidase.

In Figure 4.11, no growth was observed on the negative plate indicated that no contamination occurred. The positive control plate showed a lawn of blue colonies indicated high transformation efficiency. For ligation mixture plate, several white colonies interspersed with blue colonies were observed.

5.5 Colony PCR

Colony PCR was carried out to further confirm the presence of recombinant DNA in the transformed cells and the gel electrophoresis image was shown in Figure 4.12. PCR amplification using T7 and SP6 primers gave additional 176 bp due to amplified of restriction sites in pGEM-T Easy Vector. The selected blue colony gave the approximate band size of 180 bp indicated E.coli cells transformed with pGEM-T without insert. The selected white colonies from bacterial isolates S 3 and S 7 ligation plates gave the approximate band size of 1040 bp indicate there was insertion with approximately 870 in size. Thus, we can conclude that PCR fragments from both bacterial isolates showed successful transformation.

5.6 Plasmid Extraction

White colonies that were confirmed to contain the DNA fragment of interest were subjected to plasmid extraction. In Figure 4.13, the bands with approximate size of 3100 bp indicated the present of extracted plasmid. The expected fragment size was about 3900 bp. This discrepancy could be explained that the plasmids extracted are in the supercoiled forms. Few bands were observed indicated different conformations of plasmid occurred.

The absorbance readings and concentration of the extracted plasmid were illustrated in Table 4.17. Plasmid pTWC_S3 and pTWC_S5 showed pure plasmid DNA with A_{260} / A_{280} values ranging from 1.8 – 2.0. The concentrations of the extracted plasmids pTWC_S3 and pTWC_S5 were considered high which were 241 and 215 ng/µL, respectively.

5.7 Bioinformatics Analysis

5.7.1 BlastX

Extracted plasmid pTWC_S3 and pTWC_S5 were sequenced using T7 and SP6 primers. The DNA sequences with 868 bp in size were considered reliable as both forward and reverse sequences showed 100% complimentary to each other. Subsequently, both DNA sequences were carried out BlastX analysis through the NCBI webpage.

The BlastX results of bacterial isolates S 3 and S 5 were shown in Table 4.17. The DNA sequence from bacterial isolate S 3 showed highly similar to *Enterobacter cloacae* beta-lactamase TEM sequences with the percentages of either 98% or 99%. The score bits ranging from 537 to 540 were considered very high and indicated high definitive of sequence identities. These results corresponded to the result of API 20E Bacterial Identification Kit where indicated bacterial isolate S 3 is *Enterobacter cloacae.*

For bacterial isolate S 5, the DNA sequence showed highly similar to *Serratia marcescens* beta-lactamase TEM sequences with the percentage of 98%. The score bit of 538 indicated very high definitive of sequence identity. In comparison to the result of API 20E Bacterial Identification Kit, only genera of the bacterial isolate corresponded. Further test such as 16S RNA can be performed to confirm the identity of bacterial isolate S 5 for better result interpretation.

5.7.2 Pairwise Alignment

In pairwise alignment of nucleotides, the result showed high similarity for the DNA sequences with 99% identities, where 866 out of 868 bases were identical. No gap was observed in the alignment, thus both amplified *bla*-TEM genes are different *bla*-TEM gene fragments from two different bacterial isolates. Differences in two bases may due to single-nucleotide mutation.

5.8 Prevalence of Antibiotics Resistance in *Enterobacter cloacae*

Since 1983, ESBLs was reported progressively in the Europe after its first identification. Until today, a broad range of bacteria are found to produce ESBLs, and one of the common species is *Enterobacter cloacae*. Clinically, *E. cloacae* is well-known for causing an extensive variety of illnesses such as meningitis, urinary tract infections, gastrointestinal infections and sepsis. The highly occurrence of the diseases is primarily due to the establishment of ESBL strains in the bacteria species which confers resistant to a diverse range of antibiotics, including ampicillin, erythromycin and rifampicin. This increasing extent of antibiotics resistance has made *E. cloacae* to be listed as one of the few bacteria that has to be monitored in healthcare sectors (Haryani et al., 2008). Corresponding to the various data reported, it has been demonstrated in this paper, that the disease-causing *E. cloacae* and *S. odorifera* were able to be successfully identified and isolated from several vegetables samples. Hence, the results obtained can be utilised as a useful reference for further investigation in coping with the crisis of bacterial resistant as well as better controlling of foodborne epidemics in the future.

5.9 Future Studies

The ESBL genes associated with the antibiotic resistance are constantly evolving and tend to create significant therapeutic problems in the future. Future studies are important to investigate the evolution of ESBL genes as well as the identity of antibiotic resistant bacteria. The information on the transmission and circulation

of antibiotic resistant genes may provide understanding and guidance towards the treatments for multi-resistant infections.

By screening the ESBL genes, a larger sample size of vegetables would permit better investigation on the bacteria pool in understanding their relation to food-borne diseases. A wider range of antibiotics can also be used to screen the bacterial isolates.

The bacterial isolates with successful amplification of *bla* gene fragments can be used to perform 16S rRNA gene sequencing. This method allows further identification and conformation of bacteria identities. Furthermore, the construction of phylogenetic trees of Enterobacteriaceae can also be done in order to determine their phylogenetic relationships and natural affiliations.

CHAPTER 6

CONCLUSION

In charactering the ESBLs genes of Enterobactericeae bacteria, four types of raw vegetables were selected and analyzed. A total of 22 bacterial isolated were isolated and showed ampicillin resistant. These isolates were then subjected to preliminary biochemical tests. Nineteen bacterial isolates fulfilled the requirements of Enterobacteriaceae family which are Gram negative, catalase positive, oxidase negative and fermentation reaction. When antibiotic susceptibility tests were performed, all 19 bacterial isolates showed resistant towards oxacilin and ampicilin and 11 out of 19 bacterial isolates showed a difference of $\geq$ 5mm between the zone of inhibition of a single disc and in combination with clavulanic acid were considered as potential ESBL producer.

Only three isolates, S 3, S 5 and S 7 were found corresponding to *bla*-TEM gene, while none of isolates was corresponded to *bla*-OXA gene. The API 20E Kit illustrated that bacterial isolates S 3 and S 5 were *Enterobacter cloacae* and *Serratia odorifera,* respectively. The DNA sequencing results were carried out BlastX and pairwise alignment analysis. BlastX results showed DNA sequences of both bacterial isolates corresponded to bla-TEM beta-lactamase gene. Bacterial isolate S 3 illustrated high similarity to beta-lactamases TEM of Enterobacter *cloacae*, while bacterial isolate S 5 illustrated high similarity to beta-lactamases

TEM of *Serratia marcesceus.* Consequently, pairwise alignment between bacterial isolate S 3 and S 5 showed highly similar with 99 % identities.

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