PHYLOGENETIC ANALYSES OF SPECIES-SPECIFIC MACERGENS IN SOUTH AFRICAN EXPORTABLE VEGETABLES

BY

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DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

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DEDICATION

This thesis is dedicated to six indispensable people of my life; my lovely husband Oluwole Samuel Aremu, three jewels Favour, Mercy and Grace, my dearest daddy Amos Alade Amoo and late mummy Dorcas Mosunmola Amoo.

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GENERAL ABSTRACT

Macergens are pectinolytic bacteria that macerate plant parenchymatous tissues by releasing pectolytic enzymes resulting in total destuction of the plant. Vegetable is an essential source of nutrients commonly included in most of South African meals. However, it suffers serious threat from macergens in the field, transit and storage. This results in low productivity and great economic losses of the vegetable availability. The traditional taxonomic markers employed in identification and classification of these macergens are based on phenotypic, chemotaxonomic and genotypic characteristics which are not clear and reliable, and which do not have worldwide applicable criteria. These methods are tedious and time consuming. Hence, the use of modern molecular methods is required. Rapid detection of these macergens becomes imperative for higher productivity of these vegetables and for certification before importation and exportation of the vegetables in and out of the country. In this study, 16S rDNA nucleotides sequences of pectinolytic bacteria were retrieved from the GenBank and used in designing primers for easy identification of macergens. The nucleotide sequences were aligned using ClusterW via BioEdit and primers were designed using Primer3Plus Platform. The size and primer location for each species and Polymerase Chain reaction (PCR) product size were defined. The nucleotide sequences from this study were deposited into the GenBank and were assigned accession numbers (KJ784522-KJ784534; KP114439-KP114448; KM924134-KM924145; KP792433-KP792441; KU143750-KU143763; KP792442-KP792449; KP899920-KP899932; KU143764-KU143773). In addition, these nucleotide sequences were used in the construction of phylogenetic trees. The 10 primers designed were synthesized and used in quick detection of the macergens in 26 exportable vegetable samples from South Africa in a PCR reaction. All the ten primers designed as molecular markers to effectively detect macergens satisfied the conditions for good primers, as well as *in silico* and empirical specificity tests for pectinolytic gene. This is because they

were optimal for heterogeneity of macergens. Upon molecular characterization of the 16S rRNA sequences of these macergens, there exists variation in the number of base pairs as well as the percentage of G+C and also A+T content. Among the species of the macergens, 541 identical pairs of the nucleotide paired frequencies were found. The particular transitional and transversional pairs ratio obtained was 4.25. The most effective model for the present data set, determined by the evaluation of the maximum likelihood of twenty-four distinct nucleotide substitution patterns resulted in the T92+G having the lowest Bayesian understanding Criterion (BIC) as well as Akaike information criterion (AIC) scores, and distinct signatures were obtained. From the phylogenetic point of view, when distance and likelihood methods were utilized with the use of different algorithms, the trees inferred well-supported phylograms of macergens with high resolution of the inner branches. The different species of macergens like *Enterobacter* sp., *Lelliottia* sp, *Klebsiella* sp., *Citrobacter* sp., *Rautella* sp., *Yesina* sp., were found having similarity index greater than 98% with the primary macergens (*Pectobacterium* sp.). They all revealed that macergens are heterogeneous as they cut across different species. Fifteen probable novel species of the macergens namely: *Cedecea* sp. (KM924136), *Citrobacter* sp. (KM924138), *Pectobacterium* sp. (KM924140), *Rahnella* sp. (KM924143), *Lelliottia* sp. (KM924144), *Tatumella* sp. (KM924145), *Cronobacter malonaticus* (KP792435), *Enterobacter* sp. (KP792439), *Citrobacter* sp. (KP114441), *Pantoea* sp. (KP114444), *Pseudomonas* sp (KP114445), *Lelliottia* sp. (KP114447), *Tatumella* sp. (KP114448), *Enterobacter* sp. (KJ784522), *Raoutella* sp. (KJ784524), *Erwinia* sp. (KJ784532) and *Citrobacter* sp. (KJ784529) with distinct signatures were obtained in this study. In spite of the heterogeneity of these macergens, phylogenetic analyses revealed their similarities and evolutionary trends. The use of PCR assay with primers specific for 16S rDNA gene of macergens can be employed with minimal quantities of the vegetable tissues for prompt and rapid identification of the macergens in the various

vegetables for certification prior to selling to consumers. Hence, these macergens specific primers could be of use to the quarantine section of the Agricultural Department of the country for enhancement of macergens detection before exportation and importation of these vegetables.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background and Rationale

Bacteria that cause plant cell separation leading to tissue collapse, i.e., maceration of plant tissue, are called macergens (Beattie, 2007, Bhai et al., 2012). Tissue maceration, the most characteristic symptom of soft rot diseases in vegetable, begins as a small water soaked lesion that expands and intensifies until the tissue turns soft and watery. Externally, the surface may remain intact, although brown and depressed, or become covered in an oozing bacterial slime layer (Reddy, 2015). Foul odours are common due to the release of volatile compounds during tissue degradation. Most bacterial growth occurs after plant cell lysis in these diseases. Soft-rotting bacteria are notable for the speed at which they promote soft rot. Stored produce may liquefy in only a few hours. These pathogens typically invade through wound sites or natural openings such as lenticels and remain in the intercellular spaces and vascular tissues until the environmental conditions become suitable for disease development. At this time, they co-ordinately produce large amounts of exoenzymes, including cellulolytic enzymes, pectate lyases and pectin methylesterases. These dissolve the plant cell walls and pectin holding the plant cells together and cause tissue collapse and plant cell lysis (Liao, 2005).

Soft rot is a form of decay characterized by a watery transparency in infected leafy plant parts and watery disintegration of nonleafy plant materials (Liao, 2005). Soft rot of fleshy vegetables and ornamental plants could be caused by the bacterium *Erwinia* spp. The bacterium is a Gram negative rod, approximately 0.7 x 1.5 μm and has peritrichous flagella. It

is non-spore forming and facultative anaerobe. It grows well in nutrient agar and nutrient broth but not above 36°C. The soft rot bacteria can grow and are active over a range of temperatures from 3-35°C but are killed by extended exposure at about 50°C. Soft-rot *Erwinia* tend to initiate infection and decay at wound sites and once initiated, can speedily progress to total damage of the plant (Saranraj et al., 2012). Soft-rot *Erwinia* express four pectin-degrading extracellular enzymes: pectin lyase, polygalacturonase, pectin methyl esterase and pectate lyase. Of these enzymes, pectate lyase is mainly accountable for general decay of the plant tissues. *E. carotovora* has built-in redundancy for this seemingly acute pathogenicity feature, showing four distinct extracellular pectate lyase isozymes (Yap et al., 2005, Saranraj et al., 2012). Soft rot occurs globally any place where fleshy storage tissues of vegetables and ornamentals could be found (Mir et al., 2010, Chudasama and Thaker, 2014, Moawad and EL-Rahman, 2014). Potatoes, carrots, and onions are among the most affected vegetables along with tomato and cucumber (De Boer, 2003, Lucas and Campbell, 2012).

The primary species that cause soft rots are *E. chrysanthemi* and *E. carotovora* subsp. *carotovora*, which exhibit a broad host range and *E. carotovora* subsp. *atroseptica*, which infects primarily potatoes. These organisms have been renamed *Pectobacterium chrysanthemi*, *P. carotovorum* subsp. *carotovorum*, and *P. atrosepticum*, respectively (Villavicencio et al., 2011). However, these names have not yet been widely adopted. A range of opportunistic pathogens can also cause soft rot under some conditions, including *Bacillus* spp., *Clostridium* spp., *Pseudomonas marginalis* and *Pantoea agglomerans* (Beattie, 2007). The bacterium *E. carotovora* subsp. *carotovora* is a microorganism vastly damage plant tissues causing soft rot across a broad host range of vegetables and some fruits (Saranraj et al., 2012). *E. carotovora* subsp. *carotovora* is one of the various species of *Erwinia*

reported to infect and destroy plant tissues both pre- and postharvest resulting in the greatest damage of harvested vegetables (Amy, 2007, Saranraj et al., 2012).

Soft-rot *Erwinia* thrive well at temperatures of 20°C and above; this calls for a continuous cold chain from farm to final consumer to prevent macergens invasion (Saranraj et al., 2012). At a temperature of about 4°C and even below, fluorescent *Pseudomonads* (i.e. *P. fluorescens* and *P. viridiflava*), can decay plant tissue extensively. This is one explanation for the high prevalence of these bacteria on decayed vegetables at wholesale and retail markets. The soft-rotting fluorescent *Pseudomonads*, when considered together with soft-rot *Erwinia*, present a strong concern to fresh product business and fresh vegetables in particular, from the farm to retail and wholesale outlets (Omogbai and Ojeaburu, 2011).

The detection of these vegetable macergens is currently based on symptoms, host range, biochemical, serological and physiological properties, which are laborious and time consuming. However, there are currently no commercial agents available specifically for control and detection of soft rot (Dong et al., 2004). Hence, this study will relate design of primers centred on the 16S rDNA of the macergens and optimization of the polymerase chain reaction (PCR) conditions for rapid, specific and sensitive detection of the soft rot bacteria.

1.2 Research Problem

Despite advances in vegetable production and disease management, growers of vegetables face many challenges, out of which the major one is the damage caused by macergens. Macergens damage the tissues of vegetables thereby reducing the quality, yields, shelf-life and consumer satisfaction of these plants (Howard, 2013, Akhtar, 2015). They affect vegetable tissues in the field, in transit and in storage or during marketing, resulting in great economic losses (Lee et al., 2012). In today's market worldwide, there are extremely

high expectations for growers to provide ample supplies of high-quality, disease-free produce that have extended shelf-life (Garbutt, 2000, Cheverton, 2015). The traditional methods to identify these macergens are extremely slow, more complex and obsolete (Hawks, 2005). Identification but not addressing damage caused. Also, resistance genes active against macergens have been found in multiple host species, but their sequences and mechanisms remain unknown (Lebecka and Zimnoch, 2005), Hence, means of quick identification of these bacteria is essential.

1.3 The Significance of the Study

This work will enhance prompt and accurate diagnosis of macergens affecting vegetables in South Africa. As a result of this, vegetable crops with commercially acceptable levels of yield and quality will be practicable for the farmers. Also, this study will help in the understanding of the taxonomy of macergens to identify them accurately, understand their biology and ultimately to know the best method of controlling them. This ongoing research will help also in discovery of additional species with modern molecular methods to more precisely define and classify macergens, resulting in occasional but significant changes in previous taxonomic schemes of these macergens.

1.4 Purpose and Objectives of the Study

1.4.1 Purpose

The broad objective of this study is to design primers for detection of macergens in vegetables based on the 16S rDNA sequencing, which would facilitate rapid and easy identification of these bacteria.

1.4.2 Objectives of the Study

This study will be directed at:

- Construction of species- specific primers for the macergens.
- Molecular identification of macergens in South African Vegetables.
- Phylogenetic analysis of the macergens.

LIST OF PUBLICATIONS

Chapter 2: Classification and taxonomy of vegetable macergens. *Published in Frontier in Microbiology*, doi: 10.3389/fmicb.2015.01361

Authors: Bukola Rhoda Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

Chapter 3: Methods for the detection and quantification of vegetable macergens. *Accepted for publication as a book chapter. In: Agriculture, Ecology and Environment, Pawan, K.B., Babalola O.O. and C. Avnish (Eds.). Discovery Publishing House Pvt. Ltd., New Delhi-110002.*

Authors: Bukola Rhoda Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

Chapter 4: Construction of Specific Primers for Rapid Detection of South African Exportable Vegetable Macergens. *Published in International Journal of Environmental Research and Public Health. 2015; 12(10):12356-12370.*

Authors: Bukola Rhoda Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, did all the wet laboratory bench work, performed all the analyses, interpreted the results and wrote the first draft of the manuscript.

Chapter 5: Molecular characterization and phylogenetic construction of macergens using 16S Ribosomal RNA Gene. *This chapter has been submitted in this format for publication in Biological Opens.*

Authors: Bukola Rhoda Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, did all the wet laboratory bench work, performed all the analyses, interpreted the results and wrote the first draft of the manuscript.

Chapter 6: Comparative study of constructed macergens specific oligonucleotides for the selection of pectinolytic gene in different vegetables. *This chapter has been submitted in this format for publication in Open Biology Journal.*

Authors: Bukola Rhoda Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, did all the wet laboratory bench work, performed all the analyses, interpreted the results and wrote the first draft of the manuscript.

CHAPTER 2

CLASSIFICATION AND TAXONOMY OF VEGETABLE MACERGENS

Abstract

Macergens are bacteria capable of releasing pectic enzymes (pectolytic bacteria). These enzymatic actions result in the separation of plant tissues leading to total plant destruction. This can be attributed to soft rot diseases in vegetables. These macergens primarily belong to the genus *Erwinia* and to a range of opportunistic pathogens namely: the *Xanthomonas* spp, *Pseudomonas* spp., *Clostridium* spp., *Cytophaga* spp. and *Bacillus* spp. They consist of taxa that displayed considerable heterogeneity and intermingled with members of other genera belonging to the *Enterobacteriaceae.* They have been classified based on phenotypic, chemotaxonomic and genotypic characteristics which are obviously not necessary in the taxonomy of all bacterial genera for defining bacterial species and describing new ones. These taxonomic markers have been used traditionally as a simple technique for identification of bacterial isolates. The most important fields of taxonomy are based on clear, reliable and worldwide applicable criteria. Hence, this review clarifies the taxonomy of the macergens to the species level and reveals that their taxonomy is incomplete. For discovery of additional species, further research with the use of modern molecular methods like phylogenomics needs to be done. This can precisely identify and classify macergens, resulting in occasional, but significant, changes in previous taxonomic schemes of these macergens.

Keywords: Classification, macergens, pectolytic, proteolytic, species, taxonomy

2.1 Introduction

Macergens are soft rot causing bacteria, responsible for plant tissue maceration resulting in total tissue collapse (Bhai et al., 2012, Beattie, 2006). Soft rot diseases of vegetables are the most characteristic symptom of tissue maceration in a plant. These begin as small water soaked lesions that expand and intensify until the tissue turns soft and watery (Reddy, 2015). Apparently, the outer surface of the diseased plant might stay unbroken, while tanning and depressed, or enclosed in an exuding bacterial mucus layer (Heyman et al., 2013). Foul smells are common, owing to the discharge of explosive complexes through tissue degradation. Best bacterial growth follows plant cell lysis in these diseases (Rich, 2013). Soft-rotting bacteria are distinguished for the speed at which they stimulate soft rot. Warehoused crops may turn to liquid in only a few hours (Reddy, 2015). These pathogens usually enter through wound spots or natural openings such as lenticels and persist in the intercellular spaces and vascular tissues till the environmental conditions become fit for disease development. Parenchymatous tissues are macerated by massive quantities of pectic exoenzyme exudates produced during this period. These enzymes comprise of cellulolytic enzymes, pectate lyases and pectin methylesterases, which are responsible for the total tissue destruction (Parthiban et al., 2012).

Soft rot can be found worldwide, wherever ample storage tissues of vegetables and ornamentals are found (Golkhandan et al., 2013, Elbanna et al., 2014). Potatoes, carrots, and onions are among the most affected vegetables, along with tomatoes and cucumbers (Figure 2.1.) (Mir et al., 2010).

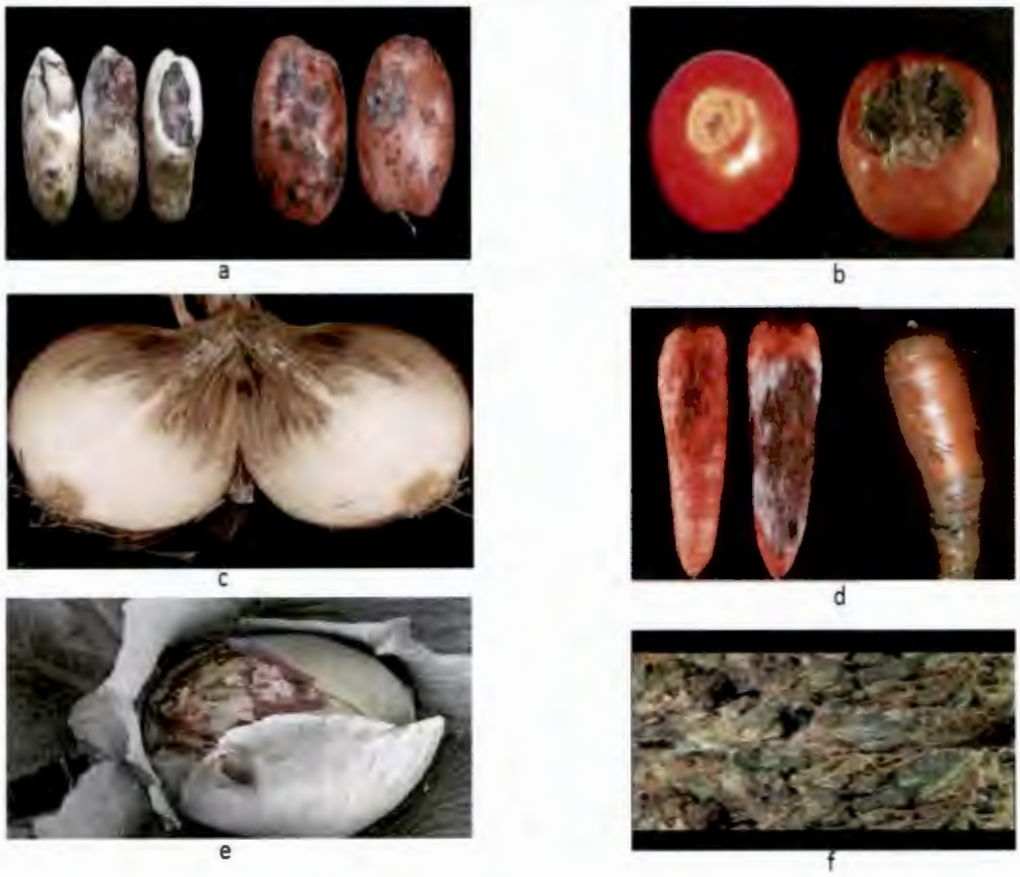

Figure 2.1: Unmarketable vegetable as a result of macergens infestation. (a). Potato with soft rot diseases; (b). Tomatoes affected by soft rot diseases; (c). Onions with soft rot disease; (d). Carrots with soft rot disease; (e). Cabbage with soft rot disease; (f). Spinach with soft disease.

Soft rot of fleshy vegetables and ornamental plants can be caused by more than six genera of pectolytic bacteria comprising; *Erwinia, Pseudomonas, Clostridium, Bacillus, Cytophaga* and *Xanthomonas* (Elbanna et al., 2014). The estimated rate of infection of macergens on harvested crops ranges from 15 to 30%. *Erwinia* are the major macergens causing tissue degradation in vegetables (Choi and Kim, 2013, Waleron et al., 2014). Although *Erwinia* are the primary macergens, it is not a single taxon. It is reclassified into genera such as *Pectobacterium* and *Dickeya* (Brady et al., 2012, Czajkowski et al., 2013, Nabhan et al., 2012). Macergens comprise of multiple groups ranging from the very complex *Pseudomonas*, a gamma-*Proteobacteria* to as diverse as *Bacillus* and *Clostridium* which are firmicutes. *Bacillus* spp., *Clostridium* spp., *Pseudomonas marginalis* and *Pantoea agglomerans* only cause soft rot when conditions are favourable to do so, thus they are secondary invaders called opportunistic pathogens (da Silva, 2013). Among all these pectolytic bacteria, soft rot *Erwinias* are the most important primary macergens that can macerate both the growing and harvested crop (Baz et al., 2012). All other bacteria are referred to as secondary because they can only destroy the parenchymatous tissues of plants under extreme environmental conditions, or secondary invaders after *Erwinias* or other pathogens have infected the plant.

These macergens infect and destroy plant tissues both pre- and postharvest and this species causes the greatest damage to harvested vegetables (Lee et al., 2012). There is need to ensure a continuous cold chain from immediately after postharvest to retail, for successful management of these ubiquitous spoilage bacteria that only thrive well at temperatures of 20°C and above (Tournas, 2005). The fluorescent *Pseudomonads* (*P. fluorescens* and *P. viridiflava*) can macerate plant parenchymatous tissues at a temperature below 4°C. This cause higher occurrence of these bacteria on decayed vegetables both at wholesale and retail markets. These soft-rotting fluorescent *Pseudomonads* and *Erwinia* therefore become the

major threat to commercial fresh product operations and fresh vegetables precisely, from the farm to retail and wholesale outlets (Saranraj et al., 2012). There are currently no commercial agents available specifically for controlling soft rot (Yaganza et al., 2014).

Despite advances in vegetable production and disease management, growers of vegetables face many challenges, a major one being the damage caused by macergens (Wu et al., 2012). Macergens damage the tissues of vegetables thereby reducing the quality, yield, shelf-life and consumer satisfaction of these plants (Akhtar, 2015). They usually cause great economic losses due to their ability to infect and macerate vegetable tissues at any point in time, be it in the field, transit, storage or marketing period (Lee et al., 2012). In the nature of today's worldwide market, there are extremely high expectations for growers to provide ample supplies of high-quality, disease-free produce that have extended shelf-life (Cheverton, 2015, Kewa, 2012). The traditional methods to identify these macergens are extremely slow, complex and obsolete (Hawks, 2005). Also, resistance genes active against macergens have been found in multiple host species, but their sequences and mechanisms remain unknown (Nykyri et al., 2012). Hence, means of quick identification of these bacteria are essential. The understanding of the taxonomy of these macergens will go a long way in shedding light to understanding their biology and ultimately to understand the best method of controlling them.

At present, there is very little knowledge available on the biology, ecology and epidemiology of macergens affecting vegetables in lowland and highland tropics. In order to increase crop production an assessment of the biology, ecology and epidemiology of these bacteria needs to be successfully implemented. Thus, this review focuses on the classification and taxonomy of the macergens to the species level. This is very important for exploration in biotechnology.

2.2 Types of Microorganisms on Vegetables

The majority of Gram negative rods identified from raw vegetables were fluorescent *Pseudomonads* spp., *Klebsiella* spp., *Serratia* spp., *Flavobacterium* spp., *Xanthomonads* spp., *Chronobacterium* spp. and *Alcaligenes* (Elbanna et al., 2014). In vegetables like broccoli, cabbage, mungbean sprouts and carrot, Gram positive rods were predominantly isolated (Andrews and Harris, 2000). *Coryneform* bacteria and catalase negative c*occi* were also predominatly isolated from broccoli, raw peas and raw sweet corn. In India, the mesophilic microflora of potatoes mainly comprised Gram positive bacteria, *Bacillus* spp., *Micrococcus* spp., fluorescent *Pseudomonads*, *Cytophaga* spp., *Flavobacterium* spp., *Xanthomonas* spp. and *Erwinia* spp. *Leuconostic meseteroides* was the most common and abundant species found in vegetables among lactic acid bacteria (Andrews and Harris, 2000)

2.3 Taxonomy of Macergens

2.3.1 Genus *Erwinia*

Erwinia belongs to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacteriales* and family *Enterobacteriacae*. For the past several decades, *Enterobacteria* that macerate and decay plant tissues, often referred to as the pectolytic Erwinias, were placed in genus *Erwinia*, and named after the eminent plant pathologist, Erwin F. Smith. They are non-spore forming, facultative Gram negative rod-shaped anaerobes of approximately 0.7 x 1.5μm in size with peritrichous flagella. This genus contains a diverse set of groups of organisms represented in Table 2.1. Since its establishment many new genera have been generated from *Erwinia*.

Table 2.1: List of Interesting *Erwinia* species

Erwinia species	Sources	References
E. amylovora	Apple, Pear	Ashmawy et al. (2015)
E. ananas	Honeydew Melon	Wells et al. (1987)
E. cacticida	Sunflower	Valenzuela-Soto et al. (2015)
E. carotovora	Carrots, Potatoes, Cucumbers, Tomatoes Lettuce	Nazerian et al. (2013), Akbar et al. (2015)
E. chrysanthemi	Potatoes	van der Wolf et al. (2014)
E. papaya	Papaya	Gardan et al. (2004)
E. cypripedii	Papaya	Leu et al. (1980)
E. herbicola	Tomatoes	Ibrahim and AL- Saleh (2010)
E. mallotivora	Papaya	Amin et al. (2011)
E. nigrifluens	Walnut, Hazelnut	Frutos (2010)
E. persicinus	Bananas, Cucumbers Tomatoes	O'Hara et al. (1998)
E. psidii	Guava, Eucalyptus	Pomini et al. (2005), Coutinho et al. (2011)
E. quercina	Oaks	Shang et al. (2015)
E. rhapontici	Rhubarb, Garlic, Tomato, Onions, Cucumber	Dowson (1941), Huang et al. (2003)
E. rubrifaciens	Walnut, Hazelnut	Frutos (2010)
E. stewartii	Sweet Corn	Roper (2011)
E. tracheiphila	Pumpkin, Watermelon	Sanogo et al. (2011)
E. uredovora	Rice	Yan et al. (2010)
E tasmiensis	Pear	Thapa et al. (2012)
E. bilingiae	Pear	Kube et al. (2005)
E. wasabiae	Potatoes	Moleleki et al. (2013)
E. brasiliense	Potatoes	van der Merwe et al. (2010)
E. betavasculorum	Sugarbeet	Nedaienia and Fassihiani (2011)
E. oleae	Olive	Moretti et al. (2011)
E. pyrifoliae	Pear	Shrestha et al. (2003)
E. atrosepticum	Potatoes	Kwasiborski et al. (2013)
E. uzenensis	Pear	Matsuura et al. (2012)
E. odoriferum	Chicory, Potato	Waleron et al. (2014)
E. piriflorinigrans	Pear	López et al. (2011)
E. toletana	Olive	Rojas et al. (2004)

2.3.2 Nomenclature of *Erwinia*

Traditionally two species (*Erwinia carotovora* and *Erwinia chrysanthemi*) are circumscribed as the important plant pathogenic strains, but have been reclassified into a new genus, *Pectobacterium*, with multiple species being proposed (Gardan et al., 2003). *Pectobacterium* spp. (Waldee, 1945) (formerly *Erwinia carotovora*) and *Dickeya* spp. (formerly *Erwinia chrysanthemi*) species are related to soft rot *Enterobacteria* pathogens with extensive host ranges (Onkendi and Moleleki, 2014). These species formerly were known as the soft rot *Erwinia* spp., but several studies have shown that the soft rot *Enterobacteria* and *E. amylovora*, the type strain of the *Erwinia* genus, are too divergent to be included in one clade (Barbé et al., 2014). Therefore, the soft rot *Erwinia* spp. were later divided into two new genera as *Pectobacterium* and *Dickeya* (Nabhan et al., 2013). *Pectobacterium* and *Dickeya* spp. are possessed by a wide range of hosts, because they have been isolated from many plant species, and in part because single strains are pathogens of numerous plant species under experimental conditions (Potrykus et al., 2014, Ngadze et al., 2012). Within the genus *Pectobacterium*, there are five major clades designated I, II, III, IV, and V, which differ from previous studies. These comprise five subspecies or species-level clades of *Pectobacterium* namely; *Pectobacterium carotovorum* subsp. *carotovorum* (syn. *Erwinia carotovorum* subsp. *carotovorum*) (Lugtenberg, 2014), *Pectobacterium atrosepticum* (syn. *Erwinia carotovorum* subsp. *atrosepticum*) *Pectobacterium wasabiae* (syn. *Erwinia carotovorum* subsp. *wasabiae*), *Pectobacterium betavasculorum* (syn. *Erwinia carotovorum* subsp. *betavasculorum*) and *Pectobacterium carotovorum* subsp. *brasiliense* (Nabhan et al., 2012, Hauben et al., 2005).

The reconstructed phylogenies agree that *P. atrosepticum*, *P. betavasculorum*, and *P. wasabiae* do form individual clades and place the *brasiliensis* strains in an individual clade.

Previous suggestions to separate the pectolytic *Enterobacteria* into the genus *Pectobacterium* have not found favour among phytobacteriologists. Initially the suggestion was made by Waldee (1945), who recommended the segregation on the basis of the unique pectolytic activity of the bacteria. Consequently, Hauben et al. (1998) revived the suggestion to support the proposal by adding evidence from the 16S ribosomal DNA sequence analysis of various plant-associated members of the *Enterobacteriacae*. Although the phenotypic characterization and analysis of a single DNA fragment might have been considered insufficient for the subdivision at the generic level, the DNA-DNA hybridization study conducted by Gardan et al. (2003) provides further stimulation to change in favour of the new nomenclature. Samson et al. (2005), have proposed several new species from new genus, *Dickeya* for *E. chrysanthemi*, consisting of six genomic species namely: *Dickeya dianthicola, D. dadantii, D. zeae, D. chrysanthemi, D. dieffenbachiae, D. paradisiaca.* A recently initiated multi-locus sequencing project, as well as DNA hybridization data from the 1970s, supports the transfer of *E. carotovora* and *E. chrysanthemi* to two separate genera as well as the elevation of some soft rot *Erwinia* subgroups to the species level (Brady et al., 2012).

All the phylogenetic analyses completed to date have suffered from the small number of strains available for some *Enterobacteria* species, which makes it difficult to determine the relatedness of these taxa. Unfortunately, the naming and re-naming of species has caused considerable confusion in the literature, resulting in manuscripts being published with names that were used for only a few years. Since *Erwinia* has remained the preferred name used in the literature the comprehensive phylogenetic study of the entire group of soft rot *Enterobacteria* remains uncompleted (Charkowski, 2006, Elbanna et al., 2014). *Pectobacterium carotovorum*, in the family *Enterobacteriaceae*, is a highly diverse species consisting of at least two valid names, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* and a suggested third taxon, *P. carotovorum* subsp.

brasiliense (De Boer et al., 2012). Despite the lack of valid publication on *carotovorum*, the *P. carotovorum* subsp. *brasiliense* name has been used in more than ten publications since first published in 2004 as *Erwinia carotovora* subsp. *brasiliense* (Ma et al., 2007). Assigning strains to this taxon was based mainly on the genetic information of the 16S-23S intergenic spacer region of the rRNA operon, partial sequence of 16S rRNA gene and multilocus sequence analysis (MLSA) of housekeeping genes and MALDI-TOF characterization (Wensing et al., 2012). Table 2.2 depicts the molecular methods employed in the characterization of *Pectobacterium* and *Dickeya* species. *Pectobacterium carotovorum* subsp. *brasiliense* was first described as causing blackleg disease on potatoes (*Solanum tuberosum* L.) in Brazil and has since been described as also causing soft rot in *Capsicum annum* L., *Ornithogalum* spp., and *Daucus carota* subsp. *sativus*. Strains of this taxon were isolated in the USA, Canada, South Africa, Peru, Germany, Japan, Israel and Syria (Moleleki et al., 2013, Ngadze et al., 2012).

Table 2.2: Molecular methods of identifying macergens

Macergens	Molecular Methods	Isolation Sources	References
Pectobacterium carotovora	AFLP, MLSA, MLST, PFGE, MALDI-TOF MS, qPCR	Potatoes	Nabhan et al. (2012), Ngadze et al. (2012), Šalplachta et al. (2015), Humphris et al. (2015)
Pectobacterium atrosepticum	AFLP, RFLP, RAPD, qPCR, MALDI-TOF MS	Potatoes	Ngadze et al. (2012), Duarte et al. (2004), Pritchard et al. (2013), Šalplachta et al. (2015)
Pectobacterium wasabiae	AFLP, MLST, RAPD, qPCR	horse radish, potatoes, crucifer	Avrova et al. (2002), De Boer et al. (2012), Kim et al. (2012)
Pectobacterium odoriferum	AFLP, MLSA, MLST	potatoes, celery	Avrova et al. (2002), Waleron et al. (2014)
Pectobacterium betavasculorum	AFLP, MLST, 16S rRNA, qPCR	Potatoes	(Avrova et al. (2002), De Boer et al. (2012)), van der Merwe et al. (2010), Humphris et al. (2015)
Pectobacterium brasiliense	MLST, 16S-23S rDNA, qPCR, MALDI-TOF MS	Potatoes	De Boer et al. (2012) Czajkowski et al. (2015), Werra et al. (2015)
Dickeya chrysanthemi	16S—23S rDNA, RFLP of recA, AFLP, rep-PCR, 16S rDNA, MLST, DNA–DNA hybridization, qPCR, MALDI-TOF MS	potatoes	Laurila et al. (2008), Waleron et al. (2002), Avrova et al. (2002), Sławiak et al. (2009), (Ma et al., 2007), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya dianthicola	rep-PCR, 16S rDNA, PFGE, MALDI-TOF MS, DNA–DNA hybridization, qPCR,	potatoes	Sławiak et al. (2009), Degefu et al. (2013), Šalplachta et al. (2015), Samson et al. (2005), Pritchard et al. (2013)

Dickeya dadantii	rep-PCR, 16S rDNA, PFGE, DNA–DNA hybridization, qPCR, MALDI-TOF MS	Potatoes,	Sławiak et al. (2009), Degefu et al. (2013), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya zeae	rep-PCR, 16S rDNA, RPLP, PFGE, DNA–DNA hybridization, qPCR, MALDI-TOF MS	Potatoes, maize	Sławiak et al. (2009), Samson et al. (2005), Degefu et al. (2013), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya dieffenbachiae	rep-PCR, 16S rDNA, AFLP, PFGE, DNA–DNA hybridization, MALDI-TOF MS	potatoes	Sławiak et al. (2009), Samson et al. (2005), Degefu et al. (2013), Šalplachta et al. (2015)
Dickeya paradisiaca	rep-PCR, 16S rDNA, AFLP, PFGE, qPCR, MALDI-TOF MS	Potatoes, banana, maize	Sławiak et al. (2009), Degefu et al. (2013), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya solani	rep-PCR, PFGE, RFLP, qPCR, MALDI-TOF	potatoes, tomato, maize,	van der Wolf et al. (2014), Degefu et al. (2013), Waleron et al. (2013a), Pritchard et al. (2013), Šalplachta et al. (2015)

PFGE: Pulse-field gel electrophoresis; 16S-23S intergenic transcribed region of the rRNA operon; MLSA: multilocus sequence analysis of housekeeping genes; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AFLP: amplified fragment length polymorphism; MLST: multilocus sequence tagging; RFLP: restriction fragment length polymorphism; RAPD: random amplification of polymorphic DNA; rep-PCR: repetitive sequence-based PCR.

2.3.3 Genus Pseudomonas

Genus *Pseudomonas* was first described in 1894 as one of the most diverse and ubiquitous bacterial genera whose species have been isolated worldwide from soil, decayed plant materials and rhizospheric region, quite a numerous plant species (Migula, 1894). They comprise a heterogeneous group of species which were grouped into five groups based on RNA homology (Saranraj et al., 2012). The RNA-homology group I belong to the fluorescent group because of their ability of producing pyoverdines. Pectolytic *Pseudomonas* belongs to this rRNA group I organism of gamma *Proteobacteria*. They are non-sporulating, Gram-negative, strict aerobic, rod-shape with polar flagella (Özen and Ussery, 2012). The strains of these bacteria called *P. marginalis* or *P. fluorescens* can be attributed to soft rot diseases in vegetables. The very complex groups of fluorescent, oxidase positive soft rot *Pseudomonas* are opportunistic macergens. Table 2.3 represents the molecular methods for the description of *Pseudomonas* species belonging to macergens.

Table 2.3: Molecular methods for the description of *Pseudomonas* species belonging to macergens

Macergens	Molecular Methods	Isolation Sources	References
Pseudomonas. fluorescens	RFLP ITS1, 16S rRNA gene, WC-MALDI-TOF MS	Wheat	Franzetti and Scarpellini (2007), Mulet et al. (2012)
Pseudomonas marginalis	16S rRNA	Onion	Achbani et al. (2014)
Pseudomonas putida	16S rRNA, MLSA	Potato	Delfan et al. (2012), Mulet et al. (2010)
Pseudomonas chlororaphis	16S rRNA, MLSA, WC-MALDI-TOF MS	Sugar beet, Spring Wheat	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas aureofaciens	16S rRNA, MLSA, WC-MALDI-TOF MS	Corn	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas syringae	16S–23S rDNA, 16S rRNA, MLSA	Kiwifruit, Tomato Cucumber,	Rees-George et al. (2010), Mulet et al. (2010)
Pseudomonas stutzeri	16S rRNA, MLSA	Ginseng	Mulet et al. (2010)
Pseudomonas aeruginosa	RFLP ITS1, 16S rRNA gene, MLST	Tomato, Celery Lettuce	Franzetti and Scarpellini (2007),
Pseudomonas pertucinogena	16S rRNA, MLSA	Wheat	Mulet et al. (2010)
Pseudomonas aurantiaca	16S rRNA, MLSA, WC-MALDI-TOF MS	Cotton	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas corrugata	rep-PCR fingerprinting, MLSA	Tomato	Trantas et al. (2015)
Pseudomonas cichorii	16S rRNA, MLSA	Tomato	Mulet et al. (2010)

16S-23S intergenic transcribed region of the rRNA operon; MLSA: multilocus sequence analysis of housekeeping genes; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AFLP: amplified fragment length polymorphism; MLST: multilocus sequence tagging; RFLP: restriction fragment length polymorphism; rep-PCR: repetitive sequence-based PCR

2.3.4 Nomenclature of *Pseudomonas*

The nomenclature of bacteria in the genus *Pseudomonas* has changed considerably during the last decennia. *P. marginalis* or *P. fluorescens* are pectinolytic bacteria that cause soft rot on a wide range of hosts. The taxonomic and phytopathogenic status of *P. marginalis* is not well known. However, these are biochemically and phenotypically indistinguishable from saprophytic strains of *P. fluorescens* biovars II, *P. putida*, and *P. chlororaphis* (now includes *P. aureofaciens*). Based on their ability to degrade pectin and macerate the plant parenchymateous tissues they are referred to as *P. marginalis*. Recently, based on 16S rRNA analysis Anzai et al. (2000) came up with 57 strains of *Pseudomonas sensu stricto* with seven subclusters: *P. syringae* group, *P. chlororaphis* group, *P. fluorescens* group, *P. putida* group, *P. stutzeri* group, *P. aeruginosa* group and *P. pertucinogena* group (Novik et al., 2015). Also, in the same genus *Pseudomonas*, some species have been found to be misclassified, for instance *P. aureofaciens* and *P. aurantiaca*, which were reclassified into *P. chlororaphis* (Peix et al., 2007).

Since the discovery of genus *Pseudomonas*, it has undergone several taxonomic changes not only as far as the number of species included, but also as far as the criteria used for their definition and delineation are concerned. In Bergey's Manual of Systematic Bacteriology's current edition, an extensive list of methods used in *Pseudomonas* taxonomy was integrated (Palleroni, 2005). These methods, which consist of cell morphology and structure, cell wall composition, pigment types, nutritional and metabolic characteristics, susceptibility to different compounds, antibiotic production, pathogenicity of other organisms, antigenic structure and genetic and ecological studies, revealed the efforts for characterizing *Pseudomonas* species. The phenotypic taxonomic markers comprise a set of tests, namely: cell shape, flagella type, consumption of carbon sources such as organic acids, polyalcohols and amino acids, ability to

grow in different culture conditions, antibiotic resistance, production of antibiotic substances and exocellular enzymes (Palleroni, 2005).

In *Pseudomonas* taxonomy, the effectiveness of chemotaxonomic studies has been confirmed, such as quinone systems, fatty acid, protein, polar lipid or polyamine profiles, which are usually useful in the taxonomy of most bacterial groups. Generally, *Pseudomonas* species were reclassified by chemotaxonomic markers into other genera such as *P. mephitica* into *Janthinobacterium lividum* (Kämpfer et al., 2008). Janse et al. (1992) used whole fatty acid analysis in the study of a broad collection of opportunistic phytopathogenic *Pseudomonas* to clarify the taxonomic position of some *P. marginalis* strains included in the *P. fluorescens* group. Also, Janse et al. (1992) reported that other bacteria (*P. putida*, *P. aureofaciens*, and *P. tolaasii*) within the fluorescent oxidase positive pseudomonads group also exhibit pectinolytic ability. Hence, they are referred to as *P. fluorescens* supercluster. The study of polyamine composition in *Proteobacteria* revealed putrescine as the main polyamine present in the *P. fluorescens* complex, thus helping in the delineation of species from this group. Recently, the polar lipid patterns of representative species of genus *Pseudomonas* were analysed, and which showed the presence of phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine as major polar lipids (Cámara et al., 2007).

Siderotyping, an interesting taxonomic tool, was used in characterizing fluorescent and then nonfluorescent *Pseudomonas* based on the isoelectrophoretic focusing. Characterization of the major siderophores and pyoverdines and determination of strains pyoverdine mediated iron uptake specificity led to characterization of several *Pseudomonas* strains at species level, through species-specific pyoverdines (Novik et al., 2015). Mass spectrometry for the determination of

molecular mass of pyoverdines has helped recently to improve siderotyping resolution power and accuracy (Meyer et al., 2008).

Currently fluorescent spectroscopy fingerprinting, the most modern techniques for biomolecules analysis, are being applied to *Pseudomonas* taxonomy, by emission spectra of three intrinsic fluorophores (NADH, tryptophan, and the complex of aromatic amino acids and nucleic acid), which have been able to differentiate *Pseudomonas* at genus level from *Burkholderia*, *Xanthomonas* or *Stenotrophomonas* with very high sensitivity, and moreover at species level *P. chlororaphis*, *P. lundensis*, *P. fragi*, *P. taetrolens* and *P. stutzeri* grouped separately from *P. putida*, *P. pseudoalcaligenes* and *P. fluorescens*, which correlate with the phylogenetic clusters earlier obtained by Anzai et al. (2000); Peix et al. (2007) and Tourkya et al. (2009).

Hence, other gene sequences like housekeeping genes have been used in the last decade as phylogenetic molecular markers in taxonomic studies such as the *rec*A, *atp*D, *car*A, *gyr*B, and *rpo*B, whose effectiveness has been demonstrated in genus *Pseudomonas* for species differentiation (Hilario et al., 2004). For instance, the effectiveness of *rpo*B has been reported in discriminating closely related *Pseudomonas*, with a phylogenetic resolution of the *rpo*B tree roughly three times higher than that of the 16S rRNA gene tree (Tayeb et al., 2005). These genes also enhanced differentiation of subspecies within *P. chlororaphis* (Hilario et al., 2004, Peix et al., 2007). Nevertheless, the analysis of housekeeping genes has not frequently been used so far in *Pseudomonas* species description; only *gyr*B, *rpo*B and *rpoD* have been integrated in the current description of *P. xiamenensis* (Lai and Shao, 2008).

16S-23S rRNA intergenic spacer is another phylogenetic marker used increasingly in taxonomic studies for discrimination of very closely related bacteria, at species and intraspecific levels, even at the strain level, because of its high variability both in size and sequence

(Sakamoto et al., 2001). This region can be amplified by using universal primers, and specific protocols (Locatelli et al., 2002). The efficacy of this phylogenetic marker has been reported in the differentiation of *Pseudomonas* species (Guasp et al., 2000). The selection of the minimal principles necessary for species delineation and description is selected for each bacterial genus by a committee created by experts in the given genus. The methods used in the taxonomy of the genus *Pseudomonas* and its related genera have been standardized by the subcommittee on the taxonomy. However, the minimal standards for genus *Pseudomonas* species description are yet to be cleared after the 2002 meeting of this subcommittee (De Vos and Yabuuchi, 2002). Hence, the new species description of this genus must be based on the general minimal standards for bacterial species characterization (Stackebrandt et al., 2002). These general minimal standards needed for the classification of new species and/or subspecies must comprise 16S rRNA sequencing, DNA-DNA hybridization, fatty acid analysis and phenotypic classification.

2.3.5 Genus *Xanthomonas*

The genus *Xanthomonas* belong to the family *Xanthomonadaceae*. This family is composed of ten genera that dwell in an extreme environment. The genus *Xanthomonas* belongs to the gamma proteolytic subdivision (Mbega et al., 2014). They are Gram-negative, aerobic, rod-shaped, motile, non-spore forming with a single polar flagellum, comprise of 27 species infecting more than 400 dicots and monocots plant species (Rodriguez et al., 2012).

2.3.6 Nomenclature of *Xanthomonas*

Traditionally, genus *Xanthomonas* is referred to as a taxon of pathogenic plant bacteria (Bradbury, 1984, Dye et al., 1974). *Xanthomonas* usually produce some extracellular polysaccharide namely: xanthan and xanthomonadin, a membrane-bound, brominated, aryl-

polyene, yellow pigment (Adriko et al., 2014). This yellow pigment is responsible for their pathogenicity and virulence (Subramoni et al., 2006). However, the yellow-pigmented *X.* spp. (*X. campestris*) are the only ones associated with tissue maceration of the post-harvest vegetables and fruits (Liao and Wells, 1987). They are opportunistic macergens because they invade through natural openings or after infection of the plant by *Erwinia* spp. Genetically, it can be differentiated into over 141 pathovars (pv.) based on specificity range (Swings and Civerolo, 1993). But *Xanthomonas* classification of *X. campestris* pathovar was based on the host pathogenicity system (Table 2.4).

Table 2.4: Macergens host pathogenicity

Macergens	Disease Symptoms	Host Range	References
Erwinia carotovora	Soft rot	Wide	Nabhan et al. (2012), Nabhan et al. (2013)
Erwinia carotovora ssp. *atrosepticum*	Soft rot	Potato	Baz et al. (2012), Ngadze et al. (2012)
Erwinia carotovora ssp. *brasiliensis*	Soft rot	Potato	Moleleki et al. (2013), Zhao et al. (2013)
Erwinia carotovora ssp. *carotovora*	Soft rot	Sugar beet	Waleron et al. (2013b)
Erwinia carotovora ssp. *odorifera*	Soft rot	Chicory	Lan et al. (2013)
Erwinia carotovora E. chrysanthemi	Soft rot	Wide	Brady et al. (2012)
Erwinia cypripedii	Brown rot	Cypripedium	Horst (2013)
Erwinia rhapontici	Crown rot	Rhubarb	Brady et al. (2012)
Erwinia carcinegiena	Soft rot	Giant cactus	Ma et al. (2007)
Pseudomonas marginalis	Soft rot	Lettuce, cabbage	Gašić et al. (2014)
Pseudomonas fluorescens	Soft rot	Pepper, potato	Bhai et al. (2012), Czajkowski et al. (2012)
Pseudomonas viridiflava	Soft rot	Carrot, Pepper,	Almeida et al. (2013), Mitrev et al. (2014)
Pseudomonas putida	Soft rot	Lettuce, Ginger	Krejzar et al. (2008), Moreira et al. (2013)
Xanthomonas campestris	Black rot	Crucifers	Kifuji et al. (2013), Vicente and Holub (2013)
Xanthomonas campestris	Soft rot	Tomato, pepper	Singh et al. (2012)
Xanthomonas. campestris aberrans	Soft rot	Brassica	Gupta et al. (2013)
Xanthomonas axonopodis vesicatoria	Soft rot	Tomato	Sharma and Agrawal (2014)
Xanthomonas axonopodis phaseoli	Black rot	Bean	Porch et al. (2012), Dutta et al. (2013)
Xanthomonas axonopodis dieffenbachia	Soft rot	Tomato, Pea	Ismail et al. (2012), Czajkowski et al. (2014)
Xanthomonas. axonopodis citri	Soft rot	Potato	Terta et al. (2012)

Initially, this genus underwent diverse taxonomic and phylogenetic studies based on their phenotype and host specificity, until Vauterin et al. (1995) revised the reclassification of *Xanthomonas* by DNA-DNA hybridization into 20 species based on their genomic relatedness. Phenotypic fingerprinting techniques such as 50S-polyacrylamide gel electrophoresis (50S-PAGE) of cellular proteins and gas chromatographic analysis of fatty acid methyl esters (FAME) reasonably supported these genomic groups to an extent. Hence, both techniques are useful tools in specific and interspecific differentiation of *Xanthomonas* levels (Rademaker et al., 2000).

Other analyses like Multi-Locus Sequence Analysis (MLSA) and Amplified Fragment Length Polymorphism (AFLP) were also used in characterisation of this genus, revealing the complexity and diversity of the genus previously descibed by DNA-DNA hybidization (Ferreira-Tonin et al., 2012, Hamza et al., 2012). Not very long, the phylogeny of species representing the principal lineages of the genus *Xanthomonas* were reported based on their genome (Rodriguez et al., 2012). The 16S ribosomal DNA sequences and MLSA classified *Xanthomonas* species into two major groups (Vicente and Holub, 2013). Group I comprising: *X. albilineans, X. hyacinthi, X. theicola, X. sacchari and X. translucens,* and Group II made up of *X. arboricola, X. axonopodis, X. bromi, X. campestris, X. cassavae, X. codiaei, X. cucurbitae, X. fragariae, X. hortorum, X. melonis, X. oryzae, X. pisi, X. populi, X. vasicola* and *X. vesicatoria* (Rodriguez et al., 2012). Thus, taxonomy of this genus is still subjected to debate since the last decade (Rodriguez et al., 2012, Lamichhane, 2014, Vandroemme et al., 2013).

2.4 Conclusion

The taxonomy of all these macergens is far from being complete because of the controversial issues arising from their classification, which was based on host pathogenicity

(Table 2.4). This may be affected by the sudden change in the ecosystem. This classification is not based on a scientific research perspective for defined taxa, and the consequences brought about by these macergens may become difficult to understand. It is majorly based on symptoms that are similar in all the macergens, and this is unreliable according to Sławiak et al. (2013). Although some scientific methods like MLSA were used for the classification, they have the limitation of single locus analysis. Thus, a proper classification is imperative, in order to reflect an understanding of their existing natural diversity and relationships among them. This will help plant breeders, farmers, and legislators to ensure quick and effective disease diagnosis and management, in order to avoid unnecessary destruction of economically valuable crops.

As a concluding comment, applauding further developments in molecular methods of analyzing macergens for a better classification of these macergens is of great paramount. However, any future progress in taxonomy as a scientific discipline will depend only on the availability of new experimental data that will broaden and refine the view on bacterial diversity.

CHAPTER 3

METHODS FOR THE DETECTION AND QUANTIFICATION OF VEGETABLE MACERGENS

Abstract

The major constraint facing vegetable production is the problem of controlling macergens (pectolytic bacteria) that macerate the plant tissues both on the field, in transit and in storage. They cause high economic losses, hence rapid identification of these bacteria needs to be done in order to prevent them from causing total damage to the plant. Formerly, culturing gram staining, growth characteristics, antibiogram, biochemical methods, fully or partly automated identification methods were basically the identification and isolation techniques used in the conventional methods which are mainly biochemical and phenotypic features that are slow and time consuming. This method is based on the morphological characterisation which include pigmentation, gram staining his review highlights some of the molecular methods that are more recently applied in rapid and quick identification of these marcergens.

Keywords: Conventional, detection, macergens, methods, molecular

3.1 Introduction

Growers of vegetables are continuously faced with the challenge of damages caused by macergens (Pérombelon, 2002). Macergens damage the tissues of vegetable thereby reducing the quality, yields and shelf-life and consumer satisfaction of these plants (Koike et al., 2006). They cause great economic losses of vegetables in the field, transit, storage and during marketing of these vegetables. The worldwide market nowadays expects farmers to supply crops of high quality, disease-free, with longer shelf life. In order to meet this great

demand, different ways of combating the macergens need to be employed. Unfortunately, all efforts to control these bacteria prove to be ineffective, hence the only means of meeting the world market demand is to prevent macergens from attacking these vegetables. This could be done by checking the vegetables coming in and out of the country for any traces of these macergens at the quarantine sector of ministry of Agriculture.

The traditional methods of identifying these macergens are extremely slow, complex and obsolete. The sequences and mechanisms of resistance genes that are active against macergens, found in multiple host species are not known (Lebecka et al., 2005). It is necessary to know which macergen is responsible for total tissues destruction in a particular host plant in order to ensure effective control measures of these macergens. However, the available methods commonly used cannot meet this present demand for rapid and accurate identification. Hence, means of quick identification of these bacteria is essential. Rapid and accurate identification of vegetable macergens is essential for modern agriculture, as it permits informed decision making with respect to potentially costly but necessary control measures that include quarantine and the destruction of infected plant material in the field (Gottwald et al., 2001).

Bacterial taxonomies were based on the various classification methods and as diagnostic methods evolved, they have been used in reclassification of different subspecies, species and even genera. The modern methods for identifying macergens, on the other hand, eliminate the necessity of confirmation with biochemical tests and always give instant and real time enumeration and quantification of these bacteria, thus they are robust, accurate and efficient. These methods basically use molecular biology approach involving PCR protocols, DNA microarray assay and immunoassay for macergens identification processes. These will

help in quantitative detection of macergens, for rapid monitoring of the vegetables at the quarantine sector of Department of Agriculture.

The detection of these vegetable macergens are currently based on symptoms, host range, biochemical, serological and physiological properties, which are laborious and time consuming. This chapter therefore highlights the various methods of prompt and accurate diagnosis of these vegetable macergens for effective production systems. As a result of this, vegetable crops with acceptable quality (freshness and taste) will be practicable for the farmers for better agronomic improvement of the plant. Also, this will help in the understanding of the taxonomy of macergens, to identify them accurately, understand their biology and ultimately to control them.

3.2 Vegetables

Vegetables are one of the most important food crops along with fruits and grains (Koike et al., 2006). They are extremely important in the human diet. They are highly perishable in nature; hence have a short shelf life. The internal tissues are very rich in nutrients, comprising 88% water, 8.6% carbohydrate, 1.9% protein, 0.3% fat, 0.84% minerals, while fat and water soluble vitamins are less than one percent, and their pH tends towards neutral an ideal condition for the growth and survival of many microorganisms. Thus, vegetables are a good substrate for a wide range of microorganisms including yeast, moulds and bacteria. Structurally they are composed of polysaccharides; namely cellulose, hemi cellulose, and pectin. As a result of this, starch is the principal storage polymer in vegetables. Microorganisms utilize host plant cells by degrading the polymer responsible for water release and other intercellular constituents for plant nourishment and growth with extracellular lytic enzymes (Barth et al., 2010, Miedes and Lorences, 2004). During fruit development, microorganisms can invade plant tissues, either via the calyx, stem or different

specialized water and gas exchange structures of leafy matter (Barth et al., 2010). Vegetables have a natural barrier in terms of their outer protective layer, coated with natural waxy cuticle that contain principally the cutin compound (Barth et al., 2010), hence successful colonization depends upon the ability of spoilage microbes to overcome these multiple natural protective barriers (Lequeu et al., 2003). Some spoilage microbes still have the ability to initiate and establish on healthy and undamaged plant tissue (Tournas, 2005).

Out of these microorganisms, bacteria have accounted for the major loss in harvested vegetables because they have more consequence in the spoilage of vegetables due to their rapid growth that out-compete fungi for the substrate readily available in vegetables and the intrinsic properties which enhance their growth. It is therefore a big problem for vegetable farmers of the world to detect these bacteria before damage is done to the plant (Bartz, 2006, Koike et al., 2006) in order to meet the constant market expectation for a high value of produce free from diseases.

3.3 Disease causing microorganisms in some vegetables

Both the Gram negative and positive bacteria can be found in the vegetable surface and tissues. The Gram negative rods commonly isolated from raw vegetables are fluorescent Pseudomonads spp., *Klebsiella* spp., *Serratia* spp., *Flavobacterium* spp., *Xanthomonads* spp., *Chromobacterium* spp. and *Alcaligenes* while the Gram positive rods are predominantly isolated from broccoli, cabbage, mungbean sprouts and carrots. *Coryneform* bacteria and catalase negative *cocci* can be isolated from broccoli, raw peas and raw sweet corn. In India, the mesophilic microflora of potatoes mainly comprise Gram positive bacteria, *Bacillus* spp., *Micrococcus* spp. as fluorescent *Pseudomonads*, *Cytophaga* spp., *F.* spp., *X.* spp. and *E.* spp. The most frequent and abundant species of lactic acid bacteria found on vegetables is *Leuconostic meseteroides* (Andrews and Harris, 2000).

3.4 Vegetable macergens

These are bacteria splitting the vegetable plant cell wall resulting in total tissue damage (Beattie, 2006). They are pectolytic bacteria that can destroy the cell wall barrier of the vegetable tissues. There are two major types of macergens; namely primary and opportunistic macergens.

3.4.1 Primary macergens

Erwinia species are the primary macergens because they possess the ability to invade both healthy and unhealthy or wounded plants. They have extensive host range as well as wide distribution (Pitman et al., 2010). These primary macergens are *E. chrysanthemi* and *E. carotovora* subsp. *carotovora,* which display a wide host range, and *E. carotovora* subsp. *atroseptica*, which infects basically potatoes (Toth et al., 2011, van der Wolf et al., 2014). These macergens can also be called *Pectobacterium chrysanthemi*, *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* respectively, although these are not generally accepted by taxonomists (Beattie, 2006).

3.4.2 Opportunistic macergens

These macergens can only invade plant tissues when the condition is favourable for their growth and the plant is unhealthy, that is having an opening which can serve as their route of entering to the plant tissues. A range of opportunistic macergens include: *Bacillus* spp., *Xanthomonas* spp., *Pseudomonas* spp. and *Cytophaga* spp.

3.5 Mechanism of infection

Soft-bacteria (macergens) are versatile in destroying plant tissues at a very fast rate to the extent that they liquefy the host tissues within a very short period of time (Beattie, 2007, Beattie, 2006). The mechanism and symptoms of infection of macergens are represented in

Figures 3.1 and 3.2 respectively. In addition, the cycle of expressing the symptoms of the diseased conditions are depicted in Figure 3.3.

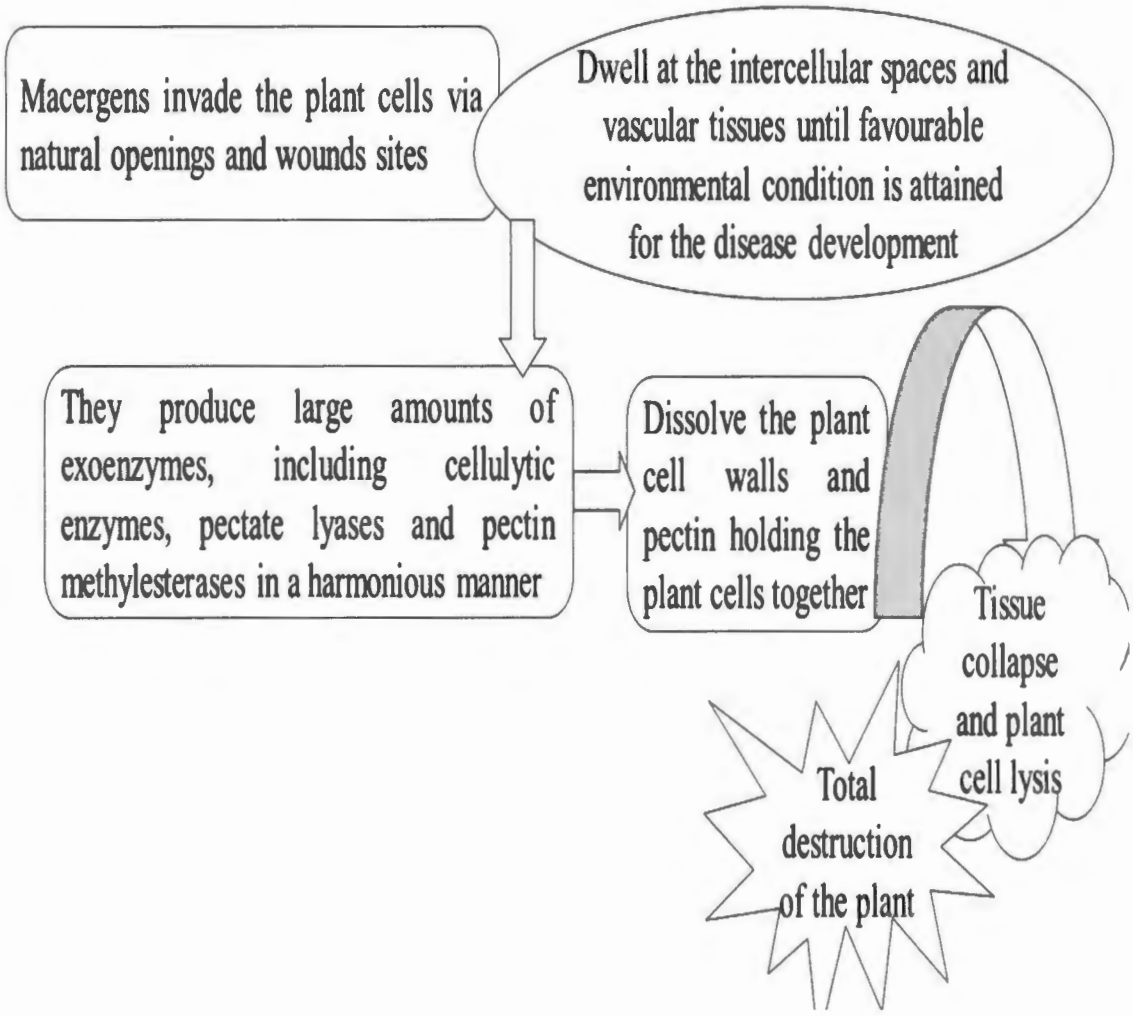

Figure 3.1: Mechanism of the soft rot disease

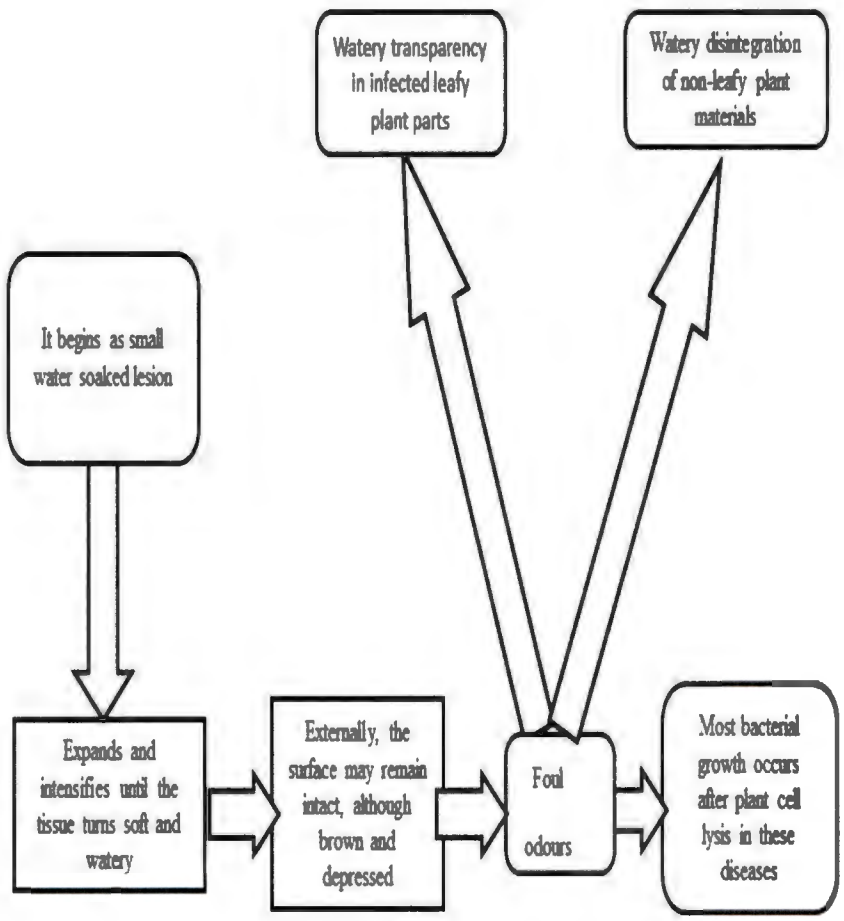

Figure 3.2: Symptoms of disease caused by macergens

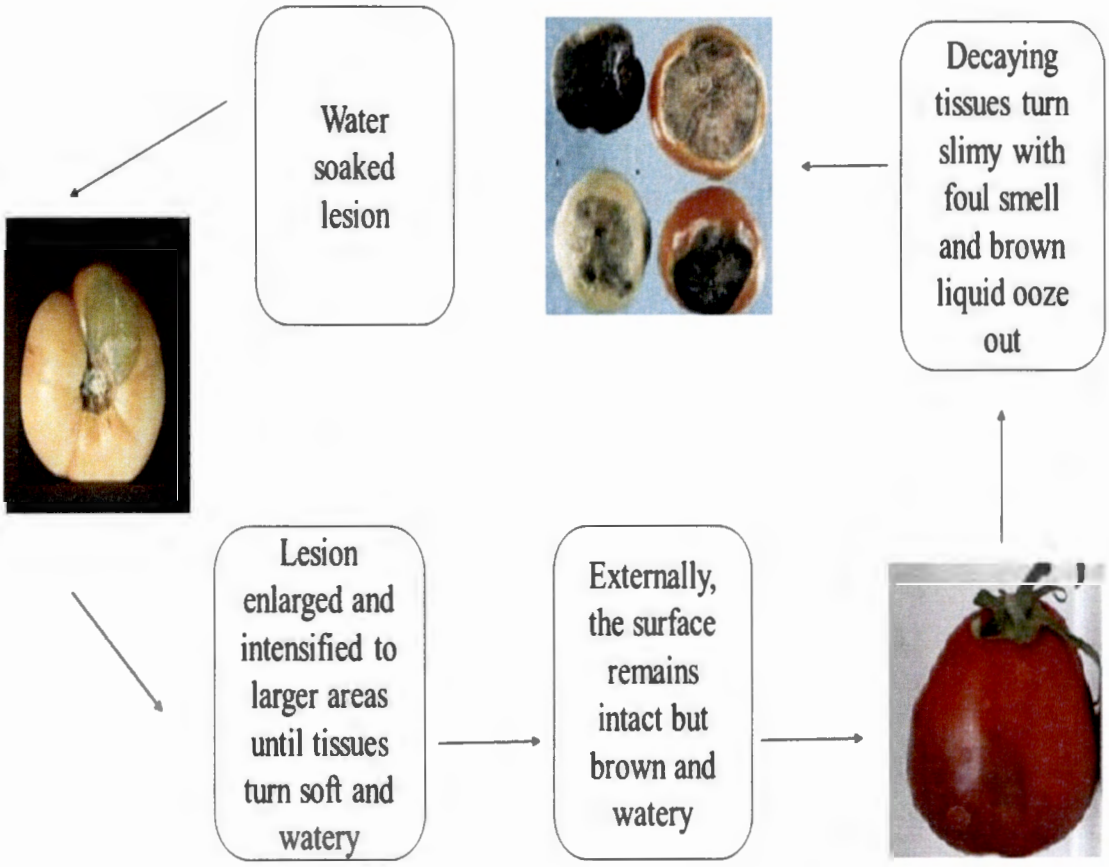

Figure 3.3: A cycle showing symptoms of macergens in tomato

3.6 Identification Methods

Selective detection and quantification of pectolytic bacteria from different habitats requires a broad knowledge of their epidemiological and ecological potentiality in different habitats (Hélias et al., 2012). There are two major methods of detecting macergens; namely traditional or conventional method and molecular or modern method (Table 3.1).

Table 3.1: Methods employed in macergens detection

Macergens	Plants	Methods	References
Erwinia sp.	carrot, potato, cucumber, onion, tomato, lettuce, sunflower, artichoke, chicory, leeks, spinach, broccoli, pepper	CVP medium, Chemical methods, RFLP, DNA hybridization, Serology, DNA sequencing, Enzyme profiling	(Cuppels and Kelman, 1974), (Gardan et al., 2003), (Darrasse et al., 1994, Fessehaie et al., 2003), (Fessehaie et al., 2003) (Yap et al., 2005)
Xanthomonas sp.	cabbage, cauliflowers, brussels, pepper	Chemical method, Fatty Acid Methyl Ester (FAME) Analysis, DNA fingerprinting methods, Polymerase chain reaction methods, DNA barcoding methods, Serological methods immunofluorescence or ELISA	(Weller et al., 2000), (Fessehaie et al., 2003)
		CVP medium, Conductimetry method, DNA sequencing, ELISA, PCR	(Fraaije et al., 1993)

3.6.1 Conventional Approach in Identification of Macergens

The conventional approach depends solely on the biochemical and phenotypical attributes of the macergens. This involves the use of physiological, biochemical and serological methods. Presently, phenotypical features, are used for the environmental tracking and identification of these primary macergens (*Erwinias*) through growth and pit formation in pectate selective media like crystal violet pectate medium at different temperatures. *E. carotovora* subsp. *atroseptica* thrives well at 27°C, *E. carotovora* at 27°C and 33.5°C, and *E. chrysanthemi* at 27°C, 33.5°C and 37°C (Toth et al., 2001, Hyman et al., 2001, Moleleki et al., 2013). Isolates may grow outside their expected range of temperature, making the differential temperature inaccurate, thus this identification method often remains unfit (Pérombelon, 2002). Their selectivity is in view of the consideration of crystal violet, which hinders the development of Gram-positive microorganisms and in the vicinity of pectin as the significant carbon source. Pectolytic bacteria produce pits in the medium, on account of their capacity to metabolize pectin (Hélias et al., 2012).

The biochemical method used for standard identification and taxonomy of macergens can only be applied to purified cultures, and allows the differentiation of all subspecies, but can take up to 14 days for the identification to be done, hence it is a time consuming method. Also, this method is very sensitive in the sense that if it is carried out by non- specialist laboratories it often gives indefinite identification (Verdonck et al., 1987).

There are a lot of other conventional approaches used for identification of soft rot *Erwinias* but they all have limitations in one area or another as with biochemistry and growth on CVP. These methods can be tedious and a lot of time is wasted in sample processing. The uses of serology for macergens are limited due to the high serological heterogeneity and cross-reactivity found among subspecies (De Boer and Sasser, 1986, Singh et al., 2000).

Serological techniques are basically inefficiency of the specificity of antibodies used and not the bacteria isolation, thus they are usually regarded as being inaccurate. Serological test is also based on the specificity of monoclonal and polyclonal antibodies which can be affected by the high number of serogroups among subspecies (Fraaije et al., 1997). Monoclonal antibodies have lower affinity for the antigens but are more specific than polyclonal antisera (Gorris et al., 1994). This technique is incapable of detecting very low bacterial populations, but can only identify epidemiologically significant bacterial populations. *Erwinia* species with the exception of *E. carotovora* have been differentiated and identified using fatty acid profiling method (Persson and Sletten, 1995).

However, these conventional methods are basically on biochemical and phenotypic attributes of these bacteria, but rapid and accurate detection of these macergens cannot be ascertained. This technique is not always conclusive with many drawbacks, and requires an expert to carry it out. As a result of this, isolates can be misinterpreted when effective results are not obtained. Hence, there is a need for alternative methods to fill these gaps that can be handled by any staff, whether skilled or unskilled.

3.6.2 Molecular Approach in Identification of Macergens

Many problems encountered by conventional methods of detecting microorganisms have been resolved through the emergence of molecular methods that are able to detect smaller quantities of these bacteria. As a result of this breakthrough, serology, enzymology, and metabolic analyses that were normally used for plant pathogenic bacteria identification are gradually being surpassed by these molecular techniques (Babalola, 2004). The standardized molecular techniques that have been used for bacteria identification are based on nucleic acid techniques which can be the use of probes or polymerase chain reaction (PCR). Clarification and acceleration of the in vitro amplification process of nucleic acid materialize

through PCR and other recently developed amplification methods (Bisen et al., 2012). These amplified products, referred to as amplicons can be characterized by different techniques, namely nucleic acid probe hybridization, restriction endonuclease followed by fragment analysis or direct sequence analysis. These fast methods of nucleic acid amplification and characterization have considerably expanded the distinctive tools of microbiologists (Tang et al., 1997).

3.6.2.1 Probing Method

Probes are the single stranded DNA or RNA molecules, usually labelled with a radioactive isotope, an enzyme or a fluorescent dye that binds to the complementary DNA sequences on the macergens of target which can be detected. They have been used for the detection of *Erwinia* spp., Pseudomonas spp. and *Xanthomonas* spp. The subtractive hybridization of the *Erwinia* genome was used to produce specific hybridization probes for detecting *Erwinia* (Charkowski, 2006). However, the invention of PCR-based methods that are simpler and cheaper meant that these probes for *Erwinia* strains detection and identification were not widely used. Macroarray techniques for identification and detection of soft rot bacteria (*Erwinia*) affecting potato were recently described (Charkowski, 2006, Fessehaie et al., 2003). This array is made up of 40 different 16 to 24-mer oligonucleotides which are homologue of the 3' end of the 16S rRNA gene and the 16S-23S spacer region. Macroarrays like these are moderately less expensive to deliver (Charkowski, 2006).

3.6.2.2 DNA Hybridization

DNA-DNA hybridization is a precise technique which unfortunately cannot be used for routine identification and characterization of a large quantity of macergens, particularly when substantial quantities of strains are concerned, due to its time-consuming factors (Toth et al., 2001, Vandamme et al., 1996). PCR-restriction fragment length polymorphism (PCR-

RFLP) analysis of a pectate lyase gene has been utilized, yet has been unsuccessful as a part of distinguishing all *E. carotovora* subspecies *carotovora* (Toth et al., 2001, Hélias et al., 2012). Amplified fragment length polymorphism and repetitive sequences can be utilized for fingerprinting of the macergens, but also involves computer analysis for the detection which may not be accessible to numerous laboratories (Toth et al., 2001, Louws et al., 1999).

3.6.2.3 Automated DNA Sequencing Technology

This is also fast and accurate for DNA product amplification. It determines the amplicon sequence, then analyses the DNA-sequence based on phylogenetic analysis to identify the macergens specifically. Currently, this can be achieved in two ways, namely: electrophoretic separation and solid-phase sequencing, which are based on the principle of polyacrylamide slab gels or glass capillaries and matrix determination respectively. In future, DNA sequence may be used for macergen strain typing because of their ready availability, and their data are very useful in phylogenetic placement. The sequence of *Xanthomonas axonopodis, X. campestris* and *X. oryzae* genomes have significantly helped in identifying virulence determinants of plant pathogenic bacteria (Subramoni et al., 2006, Qian et al., 2005). The genome sequences of one strain of each of *X. oryzae* and *X. axonopodis* and two strains of *X. campestris* have been completed and are accessible in the public domain (Lee et al., 2005, Subramoni et al., 2006). The genomes of several other *Xanthomonads* including *X. campestris* are also being sequenced (Büttner et al., 2003). These genome sequences have been analysed to identify genes that are peculiar to particular species or strains of *Xanthomonads* (Subramoni et al., 2006).

The phylogenetic relationships among *E.* spp. were studied using 16S rRNA gene to cross check the taxonomy of the *E. carotovora* subspecies *carotovora* (Toth et al., 2001), and do permit identification of species and subspecies although presently sequencing is not

applicable for routine identification. In addition, this method reaches its limits of sensitivity beneath the species level. The rRNA genes are isolated by the multicopy 16S-23S intergenic transcribed spacer (Sakamoto et al., 2001), which possesses greater sequence and length variation and is also suitable for differentiating below the species level and can be used in a simple PCR-RFLP-based test (Toth et al., 2001, Guasp et al., 2000, Sakamoto et al., 2001).

3.6.2.4 Polymerase Chain Reaction (PCR)

High specificity is being maintained dramatically as the nucleic acid amplification increases in sensitivity. The best developed and most commonly used nucleic acid amplification technique is PCR. The principle of operation of PCR depends on the capability of the polymerase DNA to copy a strand of DNA, by extension of complementary strands starting with a pair of closely spaced chemically synthesized oligonucleotide primers (Tang et al., 1997). A PCR assay consists of three major steps: DNA denaturation (separation of target DNA double strand), primer annealing at lower temperature (primer cling to their complementary target sequences) and extension reaction step (DNA polymerase extends the sequence between the primers) (Tang et al., 1997). The quantities of PCR product are doubled theoretically at the end of each cycle made up of three steps and this is done in a thermal cycler (Tang et al., 1997).

PCR-based method is used for rapid synthesis of millions of copies of specific DNA. The conserved ribosomal genes are now commonly used in microbial taxonomy and detection. There are two main categories of PCR-based assays for macergens; the first category detects a diseased plant sample invaded by macergens and the second category is employed in the characterization of the isolates (Charkowski, 2006). Numerous PCR-based detection assays were operated based on the *hrp* gene sequences and utilized for macergens like *P. syringae* pv *papulans* and *P. avellanae* (Loreti and Gallelli, 2002, Höfte and De Vos,

2006). These assays had started before the report of the newest *E. carotovora* subspecies *brasiliensis.*

No successful identification and diagnosis of *E. carotovora* by these primer sets is known (Charkowski, 2006). Most of these assays aforementioned were being utilized before the invention of the qPCR and arrays, and it is likely that these innovations will overrule various assays pronounced for macergens detection with time (Charkowski, 2006). However, the conventional PCR-based assays described, are able to type more strains than the real time PCR and array assays but these primer sets in future will be more useful macergens detection (Höfte and De Vos, 2006). As a result of the failure of conventional PCR to allow quantitative detection of the target macergens, real-time PCR with species-specific primers can give a precise quantification method through the measurement of the amount of PCR product in each cycle as fluorescence (intensity of SYBR Green I) (Sakamoto et al., 2001, Matsuki et al., 2004).

3.7 Conclusion

Current identification methods for macergens are both imprecise and time-consuming. The detection of these vegetable macergens are currently based on symptoms, host range, biochemical, serological and physiological properties, which are laborious and time consuming. However, there are currently no commercial agents available specifically for controlling macergens (Dong et al., 2004). Hence, there is a need for cheaper and rapid molecular techniques that can be handled by both skilled and unskilled personnel. This calls for further studies on how to produce primers based on the 16S rDNA genes of these macergens and optimization of the polymerase chain reaction (PCR) conditions for rapid, specific and sensitive detection of these macergens.

CHAPTER 4

CONSTRUCTION OF SPECIFIC PRIMERS FOR RAPID DETECTION OF SOUTH AFRICAN EXPORTABLE VEGETABLE MACERGENS

Abstract

Macergens are bacteria causing great damage to the parenchymatous tissues of vegetables on field, in transit and storage. To effectively investigate the diversity and distribution of these macergens, four specific primers for the rapid detection of these macergens were designed by retrieving 16S rDNA sequences of pectolytic bacteria from GenBank through National Center for Biotechnology Information (NCBI). These were aligned using ClusterW via BioEdit and primers were designed using Primer3Plus platform. The size and primer location for each species and PCR product size have also been defined. In order to evaluate their specificity, DNA template of known macergens (*Pectobacterium chrysanthermi*) and fresh healthy vegetable were used. These primers yielded expected size of approximately 1100 bp product only when tested with known macergens and no amplicons with fresh healthy vegetable. However rapid detection of macergens in the rotten vegetable samples was carried out using these primers. The nucleotides sequences of macergens identified were deposited into the GenBank and were assigned accession numbers. Hence, with these specific primers, macergens can be identified with minimal quantities of the vegetable tissues using molecular techniques, for future use at the quarantine section of the Agricultural Department of the country for quick and rapid detection of macergens before exportation.

Keywords: ClusterW, detection, macergens, pectolytic, primer, rapid, vegetable

4.1 Introduction

Several bacteria species that can macerate parenchymatous tissues of a wide range of plants, termed macergens, can occur in growing plants and on the harvested crop either in storage, transit or field. They cause great losses in the production and economy of the affected plant depending on the severity of the attack (Sobiczewski, 2008, Beattie, 2006). The different tissue maceration enzymes produced by these macergens result in rapid tissue degradation in plants (Saxena, 2015, Sharma et al., 2013, Liao, 2009). The macergens include pectolytic strains of bacteria belonging mainly to six genera namely *Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga*, and *Bacillus* (Pérombelon, 2002, Mikiciński et al., 2010). The activities of these macergens are tightly interwoven with agricultural efficiency and plant production, leading to great economic losses (Sobiczewski, 2008, Tarkowski and Vereecke, 2014).

For a complete diet, fruit and vegetables (leafy and fleshy vegetables) are essential. These items of our diet are being threatened by macergens both on the farm, transit and in storage, reducing their quality, yields, shelf-life and consumer satisfaction. If mistakenly eaten, it can result in food poisoning and allergens (Howard, 2013, Francis et al., 2012, Daniels, 2011, Montero-Calderón and Cerdas-Araya, 2011). In order to guide against this, early detection of these macergens needs to be considered. The conventional methods that have been in use are laborious and time consuming. No diagnostic primers are yet available to discriminate macergens (Dong et al., 2004).

Polymerase Chain Reaction (PCR) assays is the most sensitive of all the existing rapid methods, to detect microbial pathogens in many specimens (Yeni et al., 2014, Naravaneni and Jamil, 2005, Riffon et al., 2001). These include several critical steps such as Deoxy Ribonucleic Acid (DNA) extraction, PCR amplification and the detection of amplicons

through electrophoresis study. Hence, the need for rapid and accurate detection of these becomes imperative. Rapid detection of macergens in vegetables is becoming more critical and the development of rapid and sensitive methods is of great interest for human safety. Moreover, molecular techniques can be used to confirm the identity and the nature of the macergens, thus the major aim of this chapter is to design specific primers for rapid, accurate detection and identification of macergens.

4.2 Materials and Methods

4.2.1 Primer Design

All database searching was done through the website of the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/. Sequences retrieved were as follows: *Erwinia chrysanthemi* strain ICMP 9290 (EF530561), *E. chrysanthemi* strain Y4 (JQ867399), *E. chrysanthemi* strain 09-1 (HM222417), *E. chrysanthemi* strain H12 (GU252371), *Dickeya dadantii* strain SUPP2200 (AB713534), *D. dadantii* strain SUPP877 (AB713563), *D. dadantii* strain CFBP 1269 (NR_041921), *D. dadantii* strain SUPP2162 (AB713572), *D. dadantii* strain MAFF106634 (AB713545), *D. dadantii* stain MAFF301767 (AB713543), *D. dieffenbachiae* strain LMG 25992T (JF419463), *D. dadantii* subsp. *dieffenbachiae* (JX575747), *Dickeya.* sp. 0827-3 (HQ287574), *Dickeya.* sp. strain SUPP2451 (AB713550), *Pectobacterium chrysanthemi* strain 582 (AF373175).

These pectolytic macergens 16S rDNA nucleotide sequences from NCBI were saved FASTA files. FASTA files were copied into BioEdit files in the program BioEdit Sequence Alignments Editor, Version 7.0.9.0 (Hall, 2004). Multiple Sequence alignments (MSA) were performed using the ClustalW 2.0 algorithm (Larkin et al., 2007). Stringency was varied to achieve an alignment with a small number of gaps and mismatches. Altering the stringency was also done to yield as many regions with a high degree of sequence similarity as possible.

MSA's were consolidated based on obvious discrepancies (i.e. the presence of a pectolytic bacterium) and a lack of sequence similarity to the consensus. The lack of sequence similarity was measured subjectively and on a percent similarity basis when needed. Consolidated trials were then aligned with each other and sequences with low similarity were discarded.

They were then opened in BioEdit, to determine the highly conserved regions where primers can be designed for macergens. The primers were designed using the Primer3Plus interface (http://frodo.wi.mit.edu/) and the best primers were selected using criteria for good primer design (Innis and Gelfand, 2012, Rychlik, 1993). Proceeding to empirical testing, the selected primer sequences were checked for potential hairpins structure, self-dimer, cross-dimer, cross-homology, and tested for binding affinities to the priming sites (delta G values) using Gene infinity Platform. Their specificity were determined through *in silico* PCR in Gene Infinity platform. NCBI Blast was also used to see if the primers were able to give the target macergens. Finally, the best primers were synthesized by Integrated DNA Technology at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa.

4.2.2 Primer Development

Primers were tested with a gradient PCR machine from 47–59°C to test for varying annealing temperatures. Concentrations of $MgCl_2$ were varied from 1.0 mM to 4.0 mM and 10 ng of DNA was used per reaction tube. Reaction volumes of 50 µl consisted of 5 µl 10x Buffer, 10 mM dNTPs, 20 µg/ml BSA, 5U/µl Taq polymerase, 10 µM forward and reverse primers and enough nanopure water to fill reaction volume to 50 µl. The PCR began with a 94°C hot start for 10 min. The PCR cycles consisted of a 94°C melting temperature for 30 sec/cycle, a 47-59°C annealing temperature for 30 sec/cycle, and a 72°C polymerase elongation step for 1 min/cycle. The PCR ended with a 72°C elongation for 10 min and a holding period at 4°C for infinite time. Samples were loaded into a 1.6% agarose gel stained

with EtBr (Ethidium Bromide), 1 kb DNA ladders were loaded in 5 µl volumes, while 7 µl of sample was loaded with 2 µl of loading dye. The gel was allowed to run for 2 h at 60 V. Test results were visualized with a ChemiDoc™ MP System (Bio-Rad Laboratories US).

Primers were empirically checked for specificity, by using them to amplify a known macergens DNA template of *Pectobacterium chrysanthemi* (31 ng/µl) serving as positive control and fresh healthy vegetable DNA template as negative control. This was done in order to know if they really amplified the target region of pectolytic gene, and also to eliminate any possible contamination in the PCR assay.

4.2.3 Detection of Macergens from Vegetable Samples

The designed primers were used for detection of macergens after the specificity test.

4.2.3.1 Extraction of Metagenomic DNA from Vegetables

DNA was extracted from the twenty-six rotten South African vegetables (comprising white cabbage, straight baby marrow, beetroot, white button mushroom, straight small marrow, round baby marrow, red cabbage, iceberg lettuce, cauliflower, parsley, celery, potatoes, broccolis, spinach, spring onions, bell pepper, three different types of potatoes and carrot, two different types of tomatoes and onions) using ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) according to the manufacturer instructions.

4.2.3.2 PCR Amplification

The average amount of the DNA used as template for PCR was 1ng per reaction using the previously described conditions in this study. These were repeated at least twice, until the result was clear. PCR amplicons were analyzed by electrophoresis on 1% (w/v) agarose gel as above to confirm the expected size of the amplicons and visualized using ChemiDoc

Image Analyser while the remaining PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Germany).

4.2.3.3 DNA Sequencing

The Sequencing of the purified PCR products were done at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa with PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, USA) by following the manufacturer's instructions.

4.2.3.4 Sequence Analysis

ChromasLite version 2.33 software was used for the analysis of Chromatograms, (sense and antisense) resulting from sequencing reaction for good quality sequence assurance (Technelysium, 2004). The resulting chromatograms were edited using BioEdit Sequence Alignment Editor (Hall, 2004). After this the resulting consensus 16S rDNA sequences obtained were Blast in the NCBI (www.ncbi.nlm.nih.gov) database with the Basic Alignment Search Tool (BLASTn) for homology in order to identify the probable organism in question (Altschul et al., 1997). These sequences were deposited in the GenBank.

4.2.3.5 Phylogenetic Analysis

The phylogenetic analyses based on the 16S rDNA gene for pectolytic bacteria were further used to characterize the macergens in order to establish relationships among them. The partial 16S rDNA sequences obtained for the macergens were utilized in the search of reference nucleotide sequence available in NCBI GenBank database using BlastN algorithm (Altschul et al., 1997). Mafft version 7.0 was employed in the multiple alignment of nucleotide sequences (Katoh and Toh, 2010) while trees were drawn based on three major

techniques using MEGA 6 (Tamura et al., 2013). These techniques include: Distance based Neighbour-Joining (NJ) with cluster-based algorithm and Minimum Evolution with optimality-based algorithm used in calculating pairwise distance between sequences and group sequences that are most similar. Character based method (Maximum Likelihood) for comparing set of data against set of models of evolution to select the best model for the variation pattern of the sequences and lastly, parsimony (Maximum Parsimony) was utilized in getting the common descent based on the character of the entities, (Jill Harrison and Langdale, 2006).

4.3 Results and Discussions

The 16S rDNA gene of the pectolytic bacteria were chosen as the target genes for primer development because they are highly conserved regions of the bacteria and most reliable. They are present in all target organisms as single copy per genome and are improbable to undergo horizontal gene transfer. The significance of the alignment containing several pectolytic different bacterial species was that the developed primers would have a better chance of amplifying macergens community DNA as a whole. This means that our primers may encompass a broader range of species to be recognized by PCR analysis. These species could be bacteria that we had not considered during our development process. The primers were developed around bacterial species that can macerate plant tissues so that they could be used to amplify community DNA extracted from plants. Four primers sets were successfully developed, from the 16S sequences of the pectolytic bacteria downloaded for better performance. The designed primers tested in the Gene Infinity Platform for binding affinities to the priming sites (delta G values), showed that they did not have potential hairpins structure, self-dimer, cross-dimer and cross-homology. All the forward primer sets sequences are good due to their legitimate G/C clamp at the 3' end, their moderate melting

temperature, and their location past the 5' end of the coding sequence. These sequences are moderate in length, which facilitate specific binding to the target gene. The *in silico* PCR performed in the Gene Infinity platform revealed an excellent specificity of designed primers. Further primer specificity, in NCBI's Primer-BLAST also resulted in the target macergens. These are in line with the primer properties proposed by Dieffenbach et al. (1993), Innis and Gelfand (2012) and Wu et al. (1991) which resulted in excellent primer performances. Thus, generated macergens-specific PCR primers from 16S rDNA sequences of pectolytic bacteria with their properties and the locations are depicted in Table 4.1.

Table 4.1: The species-specific primers properties for polymerase chain reaction assay

Primer	Primer Set	Oligonucleotide Sequence	GC %	Tm	Length	Location	Position
M101F	Set 1	CGGACGGGTGAGTAATGTCT	55	56.5	20	16S	101-121
M1208R	Set 1	AAGGGCCATGATGACTTGAC	50	55.1	20	16S	1208-1180
M182F	Set 2	CGATCCCTAGCTGGTCTGAG	60	60.0	20	16S	182-202
M1190R	Set 2	TTATGAGGTCCGCTTGCTCT	50	60.0	20	16S	1190-1170
M180F	Set 3	GACGATCCCTAGCTGGTCTG	60	56.9	20	16S	180-200
M1190R	Set 3	TTATGAGGTCCGCTTGCTCT	50	56.0	20	16S	1190-1170
M57F	Set 4	GAGGAAGAAACCGGCGATAG	55	55.3	20	16S	57-77
M296R	Set 4	GGCGTATCCACCGATGTAAT	50	54.6	20	16S	296-279

In Figure 4.1, the sensitivities of PCR assay of the primers revealed that 1000-1200 bp product were obtained only when macergens specific primers were used to amplify the DNA of positive control in which the vegetable were exposed to *P. chrysanthemi*, and negative control that were not exposed to any macergens or microorganisms (DNA template of fresh healthy vegetable).

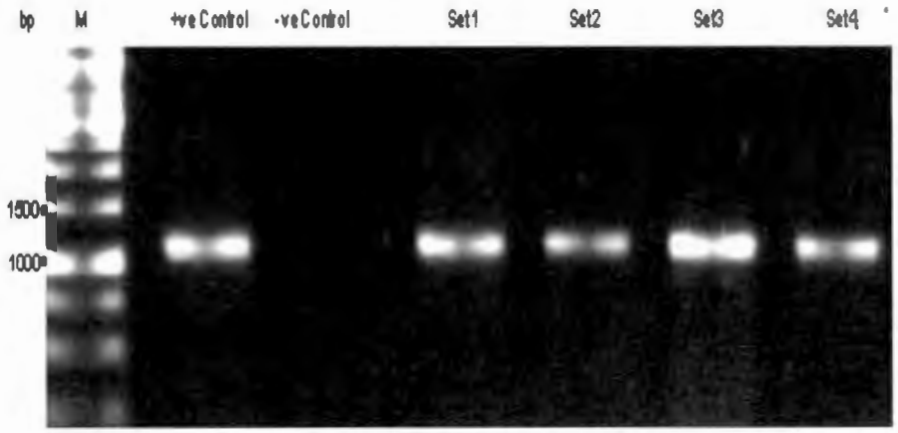

Figure 4.1: Agarose gel electrophoresis of PCR products of *Pectobacterium chrysanthermi* using the macergens specific primers (M101F+M1208R, M182F+M1190R, M180F+M1190R, M57F+ M296R) designed in this study which give the expected size of approximately 1100 base pairs. M: 1kb Molecular weight marker; Lane 1: Positive Control; Lane 2: Negative Control; Lane 3: Primer Set 1 (M101F+M1208R); Lane 4: Primer Set 2 (M182F+M1190R); Lane 5: Primer Set 3 (M180F+M1190R); Lane 6: Primer Set 4 (M57F+ M296R).

The result obtained from these group specific primers, on vegetable DNA samples, revealed their ability to amplify 16S rDNA product of the correct size exclusively from DNA of these vegetables. These are depicted in Figures 4.2, 4.3 and 4.4. Hence, there is clarity in the specificity of designed primers because they did not bind to the DNA template that is devoid of the target gene in question. As a result of this, they were able to detect macergens from the vegetable samples.

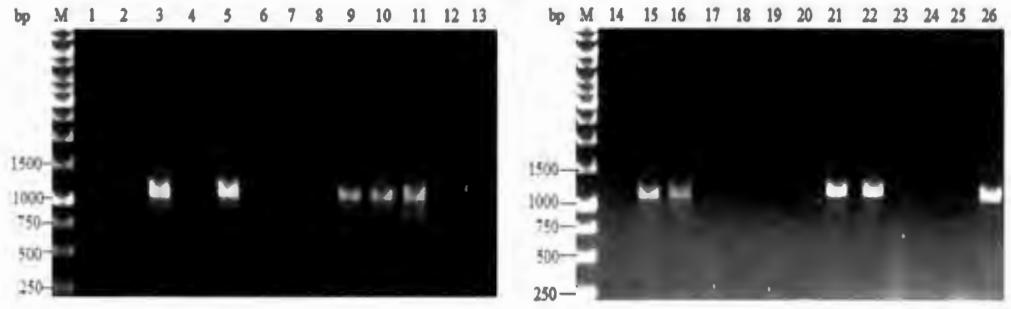

Figure 4.2: Ethidium bromide-stained gels of PCR amplification products obtained from different rotten vegetable samples using set1 and set 4 (M101F+M1208R and M57F+ M296R). Lanes 1, 2, 4, 6, 7, 8, 12, 13, 14, 17, 18, 19, 20, 23 and 24: No amplification; Lanes 3, 5, 9, 10, 11, 15, 16, 21, 22 and 26: Amplicon size ranges from 1000-1100bp. These macergens detected are represented in Table 4.2.

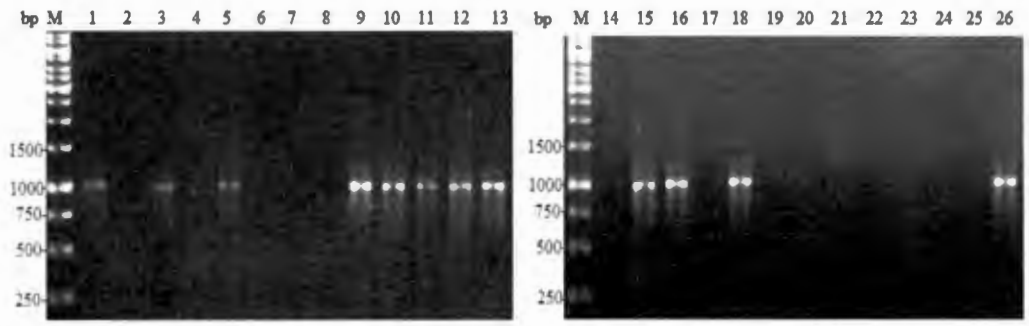

Figure 4.3: Ethidium bromide-stained gels of PCR amplification products obtained from different rotten vegetable samples using M182F+M1190R. Lanes 2, 4, 6, 7, 8, 14, 17, 19, 20, 21, 22, 23, 24 and 25: No amplification; Lanes 1, 3, 5, 9, 10, 11, 12, 13, 15, 16, 18 and 26 : Amplicon size of 1000bp. These macergens detected are represented in Table 4.3.

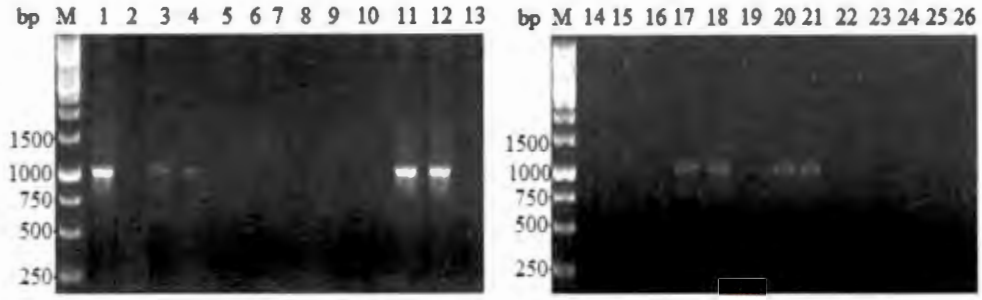

Figure 4.4: Ethidium bromide-stained gels of PCR amplification products obtained from different rotten vegetable samples using M180F+M1190R. Lanes 2, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 19, 22, 23, 24, 25 and 26: No amplification; Lanes 1, 3, 4, 11, 12, 17, 18, 20 and 21: Amplicon size ranges from 1000-1100bp. These macergens detected are represented in Table 4.4.

With the use of these designed primers, fourteen macergens were detected in sixteen vegetables out of twenty-six samples examined. *Enterobacter* sp., *Lelliottia* sp. and *Klebsiella* sp. were detected by all the primer sets. The most abundant out of all the macergens detected is *Citrobacter* sp. detected by primer set 1, 2, and 4 (Fig. 4.5). The sequences of the macergens detected were deposited in the GenBank. Also, the macergens detected by the primer pairs from the samples with their accession numbers are shown in Tables 4.2, 4.3 and 4.4 respectively.

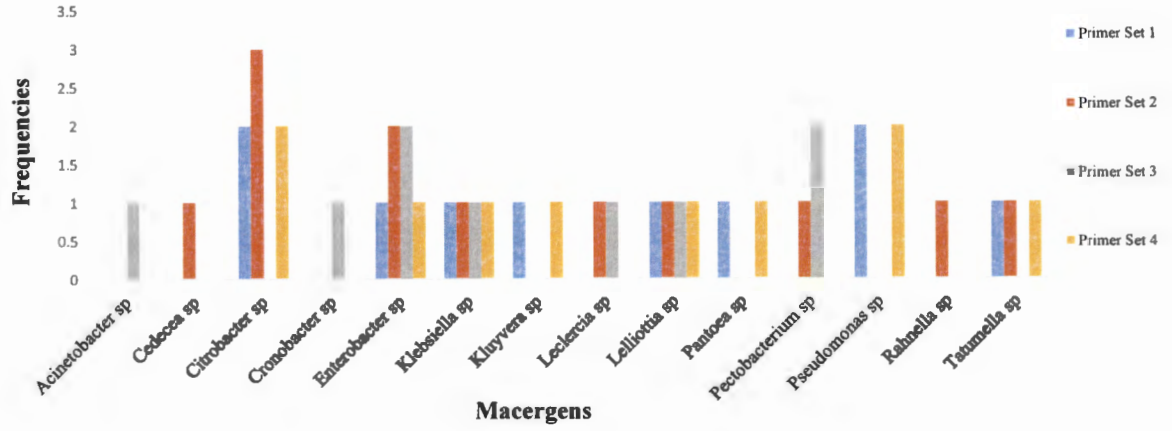

Figure 4.5: Selective frequencies of the primer with respect to the macergens in the vegetables.

Table 4.2: Macergens detected by set 1 and set 4 primers from the rotten vegetables

Lanes	Vegetable Source	Macergens	Clone Codes	Accession Number
3	Straight Baby Marrow	Uncultured *Kluyvera* sp.	M111	KP114439[a]
5	White Button Mushroom	Uncultured *Enterobacter* sp.	M112	KP114440[a]
9	Straight Small Marrow	Uncultured *Citrobacter* sp.	M113	KP114441[a]
10	Round Baby Marrow	Uncultured *Pseudomonas* sp.	M114	KP114442[a]
11	Red Cabbage	Uncultured *Klebsiella* sp.	M115	KP114443[a]
15	Parsley	Uncultured *Pantoea* sp.	M116	KP114444[a]
16	Potatoes	Uncultured *Pseudomonas* sp.	M117	KP114445[a]
21	Spinach	Uncultured *Citrobacter* sp.	M118	KP114446[a]
22	Spring Onions	Uncultured *Lelliottia* sp.	M119	KP114447[a]
26	Bell Paper	Uncultured *Tatumella* sp.	M120	KP114448[a]

[a] accession number assigned to macergens detected in this study

Table 4.3: Macergens detected by set 2 primers from the rotten vegetables

Lanes	Vegetable Source	Macergens	Clone Codes	Accession Number
1	White Cabbage	Uncultured *Enterobacter* sp.	M20	KM924134[a]
3	Straight Baby Marrow	Uncultured *Enterobacter* sp.	M21	KM924135[a]
5	White Button Mushroom	Uncultured *Cedecea* sp.	M22	KM924136[a]
9	Straight Small Marrow	Uncultured *Citrobacter* sp.	M23	KM924137[a]
10	Round Baby Marrow	Uncultured *Citrobacter* sp.	M24	KM924138[a]
11	Red Cabbage	Uncultured *Klebsiella sp.*	M25	KM924139[a]
12	Iceberg Lettuce	Uncultured *Pectobacterium* sp.	M26	KM924140[a]
13	Cauliflower	Uncultured *Citrobacter* sp.	M27	KM924141[a]
15	Parsley	Uncultured *Leclercia* sp.	M28	KM924142[a]
16	Potatoes	Uncultured *Rahnella* sp.	M29	KM924143[a]
18	Potatoes	Uncultured *Lelliottia* sp.	M30	KM924144[a]
26	Bell Pepper	Uncultured *Tatumella* sp.	M31	KM924145[a]

[a] accession number assigned to macergens detected in this study

Table 4.4: Macergens detected by set 3 primers from the rotten vegetables

Lanes	Vegetable Source	Macergens	Strains Codes	Accession Number
1	White Cabbage	Uncultured *Pectobacterium carotovorum*	M32	KP792433[a]
3	Straight Baby Marrow	Uncultured *Acinetobacter calcoaceticus*	M33	KP792434[a]
4	Beetroot	Uncultured *Cronobacter malonaticus*	M34	KP792435[a]
11	Red Cabbage	Uncultured *Klebsiella pneumoniae*	M35	KP792436[a]
12	Iceberg Lettuce	Uncultured *Pectobacterium* sp.	M36	KP792437[a]
17	Celery	Uncultured *Lelliottia amnigena*	M37	KP792438[a]
18	Potatoes	Uncultured *Enterobacter* sp.	M38	KP792439[a]
20	Potatoes	Uncultured *Leclercia adecarboxylata*	M39	KP792440[a]
21	Spinach	Uncultured *Enterobacter* sp	M40	KP792441[a]

[a] accession number assigned to macergens detected in this study

Identification of bacteria has often been difficult using traditional methods, but is easier by 16S rDNA sequencing (Clarridge, 2004). Although this has insufficient discriminating power in some genera, phylogenetic analysis allows us to exclude other species and genera. This can be used to eliminate the hypothetical cause of diseases in the quarantine section. The 16S rDNA constitutes a real step forward towards accurate identification with 85.8% of species level identification, as compare to the traditional methods that are slow and unreliable (Drancourt et al., 2000).

Furthermore, Figures 4.6, 4.7, 4.8 and 4.9 depict the analysis of phylogenetic relationships of thirty-one sequences of macergens detected alongside with twenty-seven 16S rDNA sequences of most closely related taxa retrieved from GenBank.

These relationships were based on three methods of phylogenetic tree, namely: distance, likelihood methods and parsimony. This was done in order to establish the proven resolution and statistical significance of the various treeing algorithm according to Konstantinidis and Stackebrandt (2013) and Tindall et al. (2010). The distance based method inferred the evolutionary relationship using NJ (Neigbour Joining) clustered-based algorithm and ME (Minimum Evolution) optimality-based algorithm. The concatenated NJ and ME showed the optimal of 46.60977 branch length with 207 position in the final dataset. Based on the cluster algorithm, NJ tree revealed the percentage of evolutionary relationship with the macergens based on the degree of differences between the sequences. The concatenated NJ and ME showed that M32 and M112 have very high homology of 100% with *Enterobacter ludwigii* and *Enterobacter* sp. respectively. Equally, M35, M40 and M118 also shared 100% homology in NJ and 99% in ME with *Rahnella genomosp.* In NJ, M20, M21, M31, M33, M37, M39 and M115 are closely related to *Kluyvera intermedia*, *Citrobacter murliniae*, *Cronobacter malonaticus*, *Leclercia adecarboxylata*, *Tatumella terrea* and *Enterobacter* sp.

respectively with 99% bootstrap value. But M21 and M33 are much more related in ME to *Citrobacter murliniae* and *Leclercia adecarboxylata* by 100% similarity value. Based on the two distance trees, M114 and 23 have 99% homology with *Rautella* sp., *Yesina murmii* and *Pectobacterium* sp. which is a well-known primary macergen (Nabhan et al., 2012). Equally, M28 and M36 also possessed 99% similarities with *Klebsiella michiganensis* and uncultured *Pectobacterium* sp. also a primary macergen (Brady et al., 2012). Also, M25 exhibited 99% evolutionary relationship in NJ and ME with *Citrobacter youngae* and *Enterobacter youngae*. In NJ, M27 expressed 94% homology while 93% was shown in ME with *Citobacter freundii*. NJ tree showed that M111 has 91% homology with *Rahnella genomosp* whereas 90% similarities was revealed by ME. These high bootstrap values expressed by the afore-mentioned macergens is beyond 70% borderline of degree of relatedness proposed by Wayne et al. (1987).

In addition to this, similarities expressed by these marcergens with the reference taxa belonging to different species, is due to their high similarity value which results in DNA reassociation values that fall below the 70% threshold values (Stackebrandt et al., 2002). This showed high genetic relatedness that is increasingly reliable because they cannot be wiped out overnight according to Konstantinidis and Stackebrandt (2013). In both NJ and ME, M22, M24, M26, M29, M30, M38, M113, M117, M119 and M120 form distinct clades with boostrap value less than 50% but are closer to *Enterobacter* sp. Also M34 and M116 have very low bootstrap value that is less than 50% but have closest relative to be *Citrobacter murliniae* and *Cronobacter malonaticus* respectively. These macergens did not cluster with any strains as a result of peculiarity of their nucleotide signature pattern (Togashi et al., 2001). This indicated that M22, M24, M26, M29, M30, M34, M38, M113, M116, M117,

M119 and M120 are novel macergens based on their distinctness (Konstantinidis and Stackebrandt, 2013).

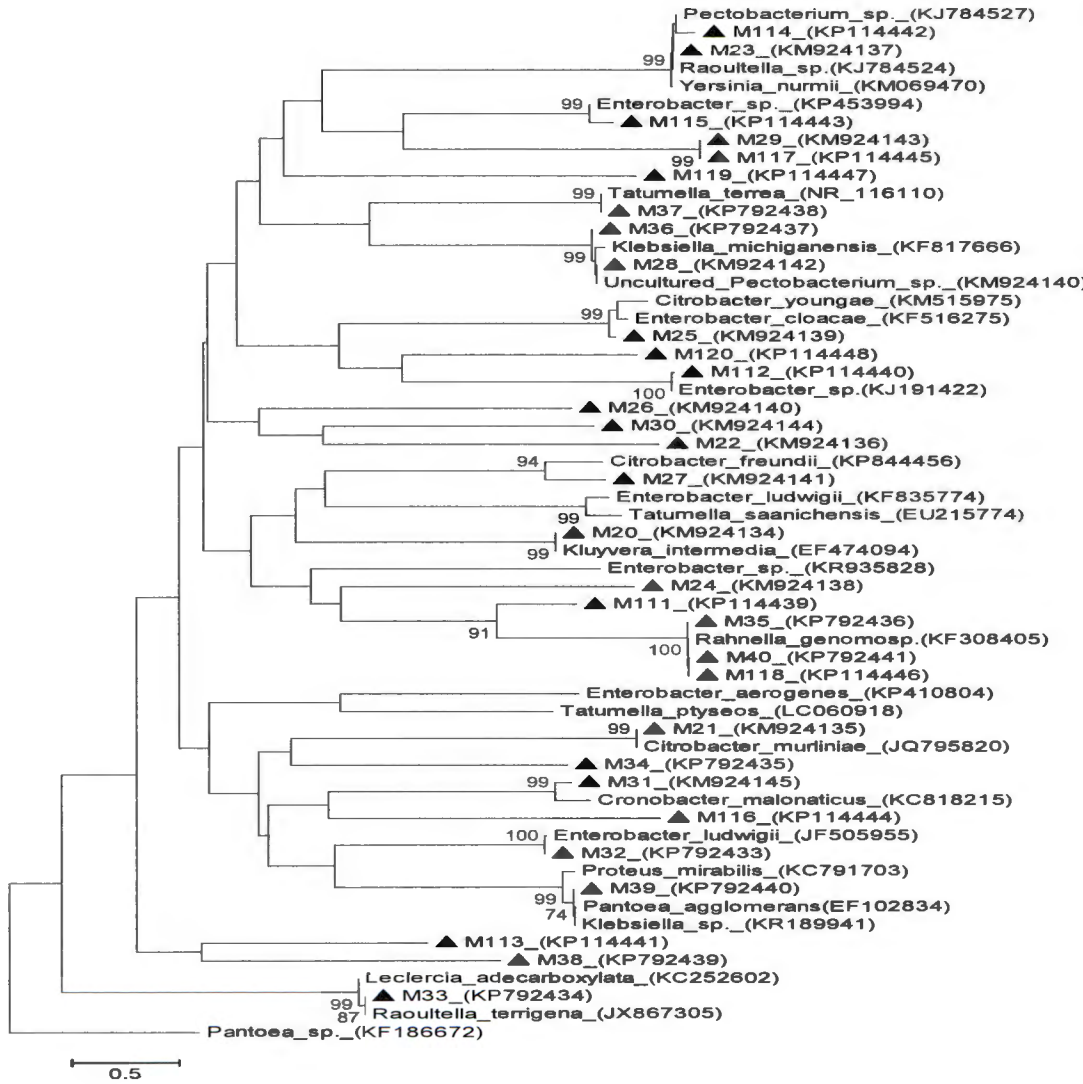

Figure 4.6: Neighbour Joining method of phylogenetic tree based on partial 16S rDNA gene sequence, showing the phylogenetic relationships between macergens and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 base substitution per site. *Pantoea* species was set as the out-group. Sequences obtained in this study are denoted with a triangle(▲).

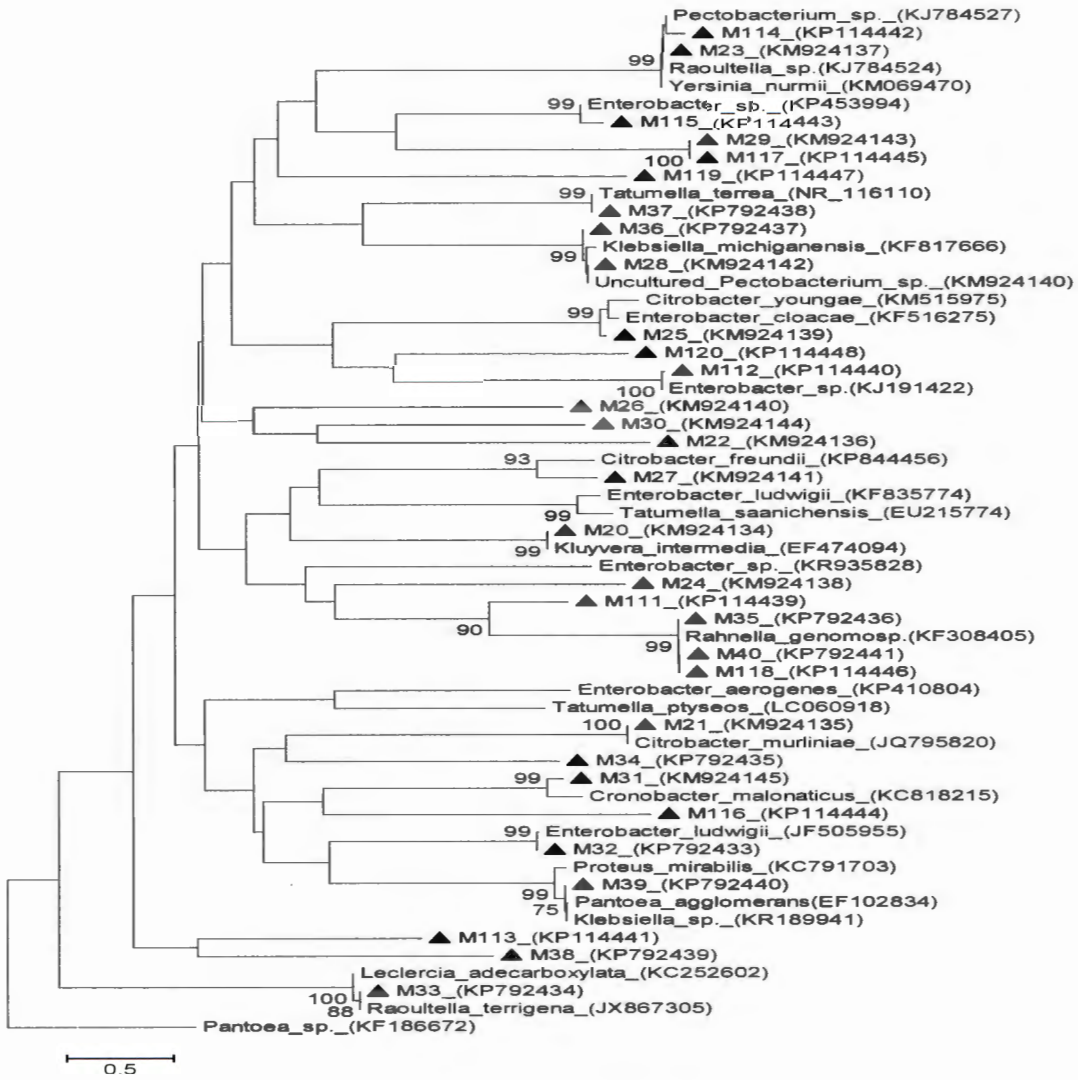

Figure 4.7: Minimum Evolution method of phylogenetic tree based on partial 16S rDNA gene sequence, showing the phylogenetic relationships between macergens and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 base substitution per site. *Pantoea* species was set as the out-group. Sequences obtained in this study are denoted with a triangle(▲).

The maximum likelihood method was based on Kimura-2-parameter model (Kimura, 1980). This showed the relatedness of macergens based on the discrete character shared with the reference taxa. The tree with the highest log likelihood of -9764.8523 was shown with 205 final data position. This tree showed that M35, M40, M118 are closely related to *Rahnella genomosp* with 99% bootstrap value. This result is exactly the same as ME distance tree based on optimality algorithm. Also, M111 has moderate similarities of 74% bootstrap value with *Rahnella genomosp.* M20 and M112 also have high homology of 96% with *Kluyvera intermedia* and *Enterobacter* sp. There is high relatedness of 98% bootstrap value between M21 and *Citrobacter murliniae. Enterobacter ludwigii* is closely related to M32 with 94% bootstrap value. *Pantoea agglomerans* and *Klebsiella* sp. are moderately similar to M39 with 89% while *Proteus mirabilis* have 88% homology with M39. *Klebsiella michiganensis* and uncultured *Pectobacterium* sp. have 93% homology with M28 and M36. Also, M33 expressed 92% similarities with *Raoultella terrigena* and *Lerclercia adecarboxylata.* Moderate relatedness of 81% was seen in M27 with *Citrobacter freundi*, 85% in M115 with *Enterobacter* sp. and 87% in M25 with *Enterobacter cloacae* and *Citrobacter youngae.* High level of similarities of 95% was also expressed in M23 and M114 with *Yersinia murmii*, *Raoultella* sp. and *Pectobacterium* sp., and 97% in M37 with *Tatumella terrea.* All these results are still in accordance with the distance based method with the exception of M31 that clustered with 99% homology in NJ and 100% homology in ME. This clustered with *Cronobacter malonaticus* in ML with the bootstrap value that is less than 50%. Hence, the relationship between *Cronobacter malonaticus* and M31 has been wiped out (Konstantinidis and Stackebrandt, 2013). It is not reliable because their DNA reassociation is above the threshold level based on results depicted by ML tree (Stackebrandt et al., 2002). ML tree also shows that macergens did not align with any of the reference taxa based on their uniqueness. These include: M22, M24, M26, M29, M30, M31, M34, M38, M113, M116,

M117, M119 and M120. These were classified as novel macergens with unique nucleotide signature pattern (Kalia et al., 2011, Naushad et al., 2014).

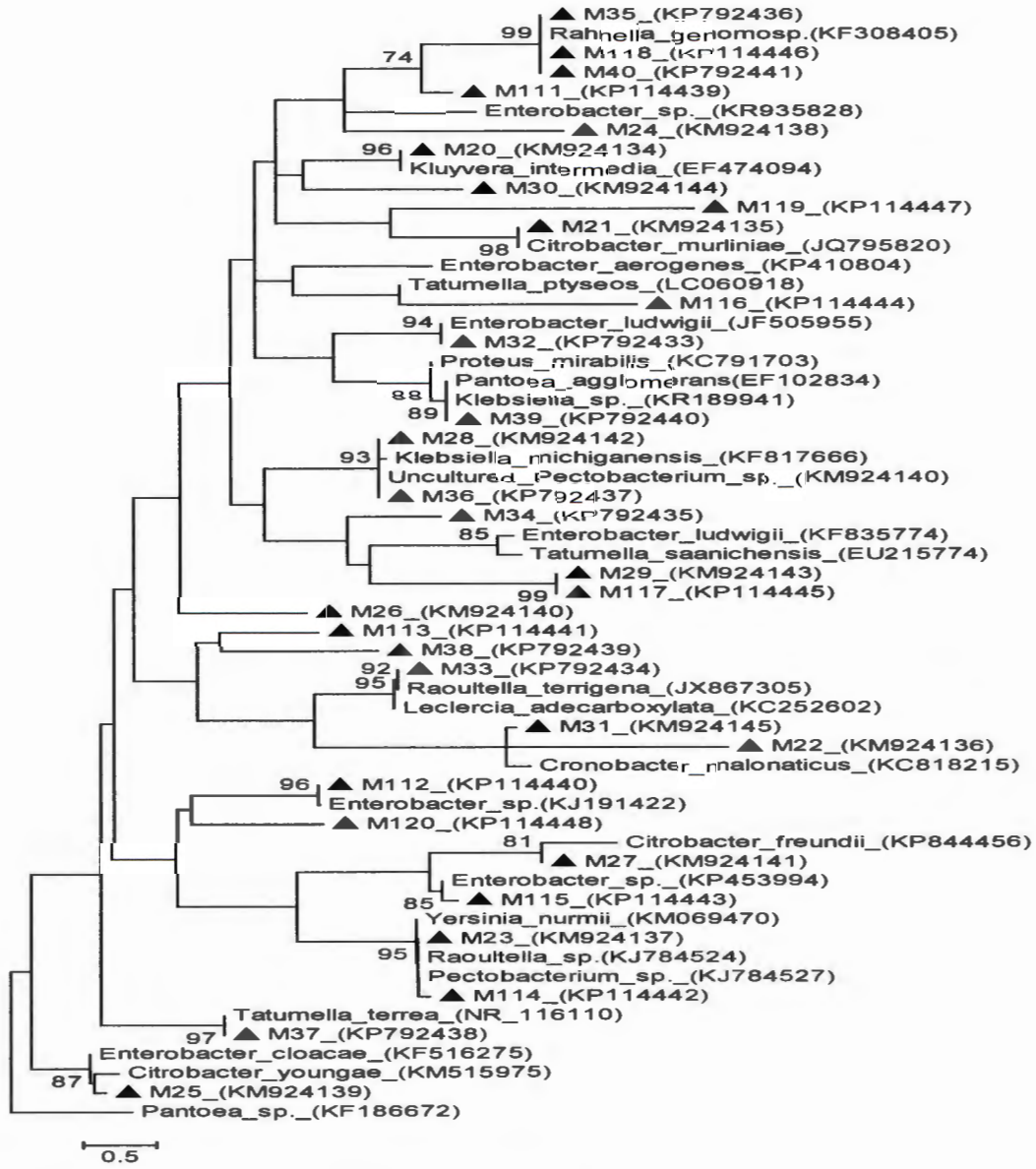

Figure 4.8: Maximum Likelihood phylogenetic tree based on partial 16S rDNA gene sequence, showing the phylogenetic relationships between macergens and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 nucleotide substitution per site. *Pantoea* species was set as the out-group. Sequences obtained in this study are denoted with a triangle(▲).

Figure 4.9 shows the Maximum Parsimony tree with 3336 parsimonious tree length. For all the sites, the index of consistency, retention and composite are 0.184353, 0.596351 and 0.109939 respectively. Based on the Subtree-Prunning-Regrafting algorithm (Nei and Kumar S., 2000) shown 205 position in the final dataset. This was used in the phylogenetic tree reconstruction, which resulted in seven clades. This resulted tree is much more similar to the distance and maximum likelihood trees described above.

In Clade I, M23 clustered with *Pectobacterium* sp. with moderate similarity value of 87%. On the other hand, M114 have very high similarity value of 100% with *Pectobacterium* sp. High similarities of 92% were also observed in M27 with *Citrobacter freundii* and 100% in M115 and *Enterobacter* sp. Similarly, M32 and M112 have 100% similarity value with *Enterobacter ludwigii* and *Enterobacter* sp. Also, M25 possessed 100% homology with *Citrobacter youngae* as well as 84% moderate homology with *Enterobacter cloacae*. *Tatumella terrea* have high 100% similarities with M37. Low similarities with bootstrap value that is less than 50% were obtained in M120, M26, M113 and M38. Clade III comprised, M28 and M36 with high homology of 100% with *Klebsiella michiganensis* and 95% with uncultured *Pectobacterium* sp. Bootstrap value less than 50% was observed in M34. In Clade IV, *Kluyvera intermedia* has 100% homology with M20 and less than 50% bootstrap value with M30. Clade V, *Citobacter murliniae* has high 100% similarity value with M21 and very low similarity value, less than 50% with M24. Clade VI, *Rahnella genomosp* showed high similarity value of 100% with M40. However, *Rahnella genomosp* shared moderate similarities of 74% with M111. In like manner, M35 and M118 expressed low similarities with *Rahnella genomosp* by bootstrap value less than 50%. Clade VII consists of M39 having high homology of 100% with *Proteus mirabilis* and 98% with *Klebsiella* sp. In the same manner, M31 also has 100% high homology with *Cronobacter*

malonaticus, while M22 expressed less than 50% low similarity value with *Cronobacter malonaticus*. Also in this clade M33 has 100% homology with *Leclercia adecarboxylata* and 92% with *Raoultella terrigena*, while M119 shared very low homology less than 50% with them.

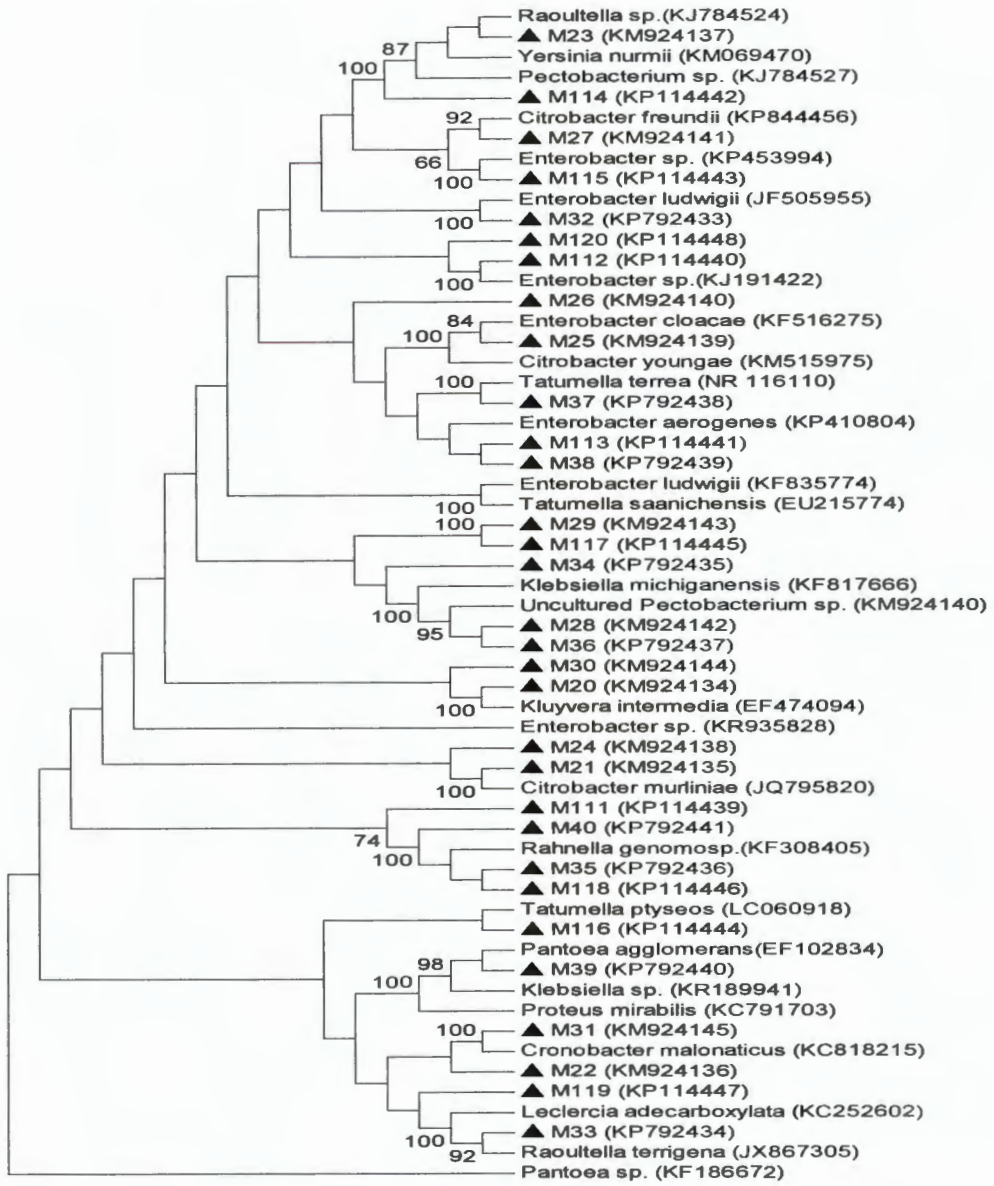

Figure 4.9: Maximum Parsimony phylogenetic tree based on partial 16S rDNA gene sequence, showing the phylogenetic relationships between macergens and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 nucleotide substitution per site. *Pantoea* species was set as the out-group. Sequences obtained in this study are denoted with a triangle(▲).

From the phylogenetic point of view, with the use of different algorithms, the trees inferred well-supported phylograms of macergens with high resolution of the inner branches. They all revealed that macergens are heterogeneous as they cut across different species. This is in line with Stackebrandt and Goebel (1994). Thus, it is not surprising that novel strains that do not cluster with the current known members of the previous macergens have emerged. The four oligonucleotide primer (M101F+M1208R, M182F+M1190R, M180F+M1190R, M57F+ M296R) designed in this study enhanced specificity for DNA from macergens, which provides a simple method for identifying macergens.

4.4 Conclusion

The four primers designed were able to produce amplicons of expected sizes upon PCR analysis; they were optimal for heterogeneity of macergens. In spite of the heterogeneity of these macergens, phylogenetic analyses revealed their similarities and evolutionary trends. The high degree of similarity between the sequences chosen, through many rounds of search and refinement, implies that the primers are specific for pectolytic gene. Since these primers were designed around bacterial species, it can be concluded that, they must be specific to the certain amount of pectinolytic bacteria in any plant samples. This method offers advantages over classical methods of detection, in the sense that the entire assay is fast, reliable, cost effective and no taxonomist is required before the identification is complete. This can be employed in analyzing and monitoring plant materials for macergens invasion in a quarantine section of the agricultural sector of a country before importation and exportation of these plants.

CHAPTER 5

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC CONSTRUCTION OF MACERGENS USING 16S RIBOSOMAL RNA GENE

Abstract

The 16S rDNA sequence was employed in the actual molecular characterization of macergens accountable for vegetable tissue collapse. Amplification involving 16S gene was performed through polymerase chain reaction. The primers employed for this amplification are forward primer: M101F: 5'-CGGACGGGTGAGTAATGTCT-3' and reverse primer: M1284R: 5'-TTATGAGGTCCGCTTGCTCT-3' developed in this particular study. The sequencing of the 16S rDNA amplicons was done at Inqaba Biotech, Pretoria using the ABI Prism Analyzer. There is certain variation within the16S rDNA sequence of these macergens based on the number of base pairs as well as the percentage of G+C and also A+T content. They had higher G+C content than A+T, an indication of their potential to undergo horizontal gene transfer. Among the species of the macergens, 541 identical pairs of the nucleotide paired frequencies were found. The particular transitional and transversional pairs ratio obtained was 4.25. The sequence similarity search amongst various other macergens was done using BlastN along with nucleotide sequences which were aligned correctly using the ClustalW within MEGA6 software package. The most effective model for the present data set, determined by the evaluation of the maximum likelihood of twenty-four distinct nucleotide substitution patterns, resulted in the T92+G having the lowest BIC as well as AIC scores. The phylogenetic tree constructed indicates the four clades in which macergens are clustered at 53%-86%. Four macergens *Enterobacter* sp. (WcVb1), *Raoutella* sp. (BeBb1), *Erwinia* sp. (PoLp1) and *Citrobacter* sp. (IlEd1) with distinct signatures were obtained. In the

constructed phylogenetic tree, it was discovered that macergens clustered with different species and were thus heterogeneous.

Key words: Macergens, nucleotide, pectolytic, transition, transversion

5.1 Introduction

The vast majority of the crucial vegetable crops of the entire world is increasingly being threatened by macergens due to the pectolytic impact elicited on the parenchymatous tissue of these vegetables through the increase of the numerous exoenzymes produced by these macergens. Most of these pectolytic effects are generally from the Type II secretion system of macergens. This specifically promotes greater influence on the rate of the invasion in the tissue of the vegetables, rendering it water soaked as well as slimy rotten. On account of this, tissue maceration macergens colonize and still have an absolutely broader range of host plants immediately after infection, consequently control of these macergens becomes challenging (van der Merwe et al., 2010, Vanjildorj et al., 2009). At the moment, chemical compounds are usually not readily available for resolving this challenge posed by the macergens on vegetables (Aysan et al., 2003, Czajkowski et al., 2011, Liplap et al., 2014, Akbar et al., 2015). This has triggered a proper exploration of the molecular level of macergens with regards to codon usage. This implies the use of various molecular attributes which include: DNA content, chromosomal as well as mitochondrial DNA organization. The mitochondrial genomes regarding bacteria tend to be generally small, circular DNA, yet can seemingly provide information into the deeper divergences as revealed by a research in which 16S rDNA sequences were employed to examine the actual affinities of the significant lineages. These mitochondrial genomes can easily fix deep-level phylogenetic issues (Rokas et al., 2003). Hence, the 16S large subunit of the ribosomal RNA is usually extremely useful for probing phylogenetic relationships involving distantly related taxa since a lot of regions

remain conserved or perhaps semi-conserved over large periods of time (Habeeb et al., 2011, Patwardhan et al., 2014).

In the long run, the ribosomal RNAs could be taken as molecular clocks. Normally, the rate of molecular evolution is somewhat constant after a while. Considering that the 16S rRNA molecule is usually typical of almost all life kinds, it becomes apparent to check the clock for any single molecule over numerous phyla (Sanjayan and Rama, 2013). The 16S rRNA gene, which often encodes the mitochondrial large ribosomal subunit (mt LSU) in bacteria, has been extensively utilized in the exploration of phylogenetic relationships in bacteria at most of the phylogenetic levels; familial level and as well as the genus level, and lower (Bakke and Johansen, 2002, Lindahl et al., 2013). The broad range of utility regarding 16S at different taxonomic levels shows that the differential rates of molecular evolution within 16S, drastically have an impact on the phylogenetic utility, on account of diverse functional restrictions (Krakowetz and Chilton, 2015, Sanjayan and Rama, 2013).

In this chapter, we present the molecular characterization of macergens, based on sequences of 16S ribosomal RNA through analysing their gene content, base composition, and nucleotide pair frequencies. Data exploration pertaining to related information on some related species was carried out, along with the reconstruction of a phylogenetic tree immediately after visualizing the best DNA model.

5.2 Materials and Methods

5.2.1 Genomic DNA Isolation

The genomic DNA extraction was done from the vegetable samples using ZR Soil Microbe DNA Miniprep™ according to the manufacturer's instructions.

5.2.2 Amplification and Sequencing of 16S rDNA

The 16S rDNA was amplified using the forward primer M101F: 5'-CGGACGGGTGAGTAATGTCT-3' and reverse primer M1284R: 5'-TTATGAGGTCCGCTTGCTCT-3' designed in this study. PCR amplification was carried out with the DNA template of bacteria from the vegetable samples in 50µl using PCR master mix with the following PCR conditions in Bio-Rad C100 Thermo Cycler; initial denaturation at 96°C for 3 min, denaturation at 96°C for 45 sec, annealing at 56°C for 30 sec, extension for 1 min at 72°C while further extension was done also at 72°C for 5 min. this was held at 4°C until used hence the total cycles is 35 cycles. The amplified product was checked for amplicons using gel electrophoresis studies and the best picture of the amplicons were taken using Chemdoc. These amplicons were sequenced using the ABI Prism Analyzer at Inqaba Biotech Pretoria. These 16S rDNA sequences were analysed with Chromas lite software, saved in Bioedit and their FASTA sequences were deposited in the GenBank database.

5.2.3 16S rDNA Sequence Analysis and Phylogenetic Construction

The 16S rDNA sequence determined was used as the query sequence in BLASTn similarity search in order to obtain the reference sequences of the 16S rDNA sequences of the macergens determined. The BLASTn was optimized for highly similar sequences (megablast), out of which the top sequences of significant alignment were chosen. The reference and the determined sequences were aligned using Mafft Multiple Alignment Software. These alignments were exported to MEGA Software (Tamura et al., 2011) after computation where the data menu was opened and the active data were explored for analysis in various sites such as conserved sites, parsimonious tool. The statistics of this nucleotide composition were analysed and exported automatically to Microsoft Excel 2007. Furthermore, the pairwise distances for the estimation of evolutionary divergence between

sequences were computed with the Best DNA model for aligning sequences. Neighbour-joining and Maximum Likelihood were employed to construct the phylogenetic trees. The stability of the relationship was assessed by performing bootstrap analyses of the neighbor-joining data based on 1000 replications. Under the rates and patterns option of MEGA software, the General Time Reversible (GRT) models with 5 discrete Gamma categories were opted out.

5.3 Result and Discussion

The nucleotide signature of the 16S rDNA sequences macergens were deposited at the GenBank database and assigned accession numbers. The accession numbers obtained for these sequences are depicted in Table 5.1.

Table 5.1: 16S rDNA gene of macergens in Genbank

Sources	Species	Species Codes	Accession Numbers
White Cabbage	*Enterobacter* sp.	WcVb1	KJ784522[a]
Potatoes	*Rahnella* sp.	PoGw1	KJ784523[a]
Beetroot	*Raoultella* sp.	BeBb1	KJ784524[a]
Tomatoes	*Klebsiella* sp.	ToGf1	KJ784525[a]
Tomatoes	*Enterobacter* sp.	ToRr1	KJ784526[a]
Onions	*Pectobacterium* sp.	OnFw1	KJ784527[a]
Round Baby marrow	*Citrobacter* sp.	BmEd1	KJ784528[a]
Iceberg lettuce	*Citrobacter* sp.	IlEd1	KJ784529[a]
Cauliflower	*Erwinia* sp.	CuPf1	KJ784530[a]
Parsely	*Ewingella* sp.	PaWf1	KJ784531[a]
Potatoes	*Erwinia* sp.	PoLp1	KJ784532
Celery	*Pantoea* sp.	CeDc1	KJ784533
Potatoes	*Lelliottia* sp.	PoMs1	KJ784534

[a] accession number assigned to macergens detected in this study

The base statistics for the gene are represented in Table 5.2. In the vegetable samples examined ten genera belonging to *Bacteriaceae* were detected. These exhibited variation in their nucleotide length. The longest nucleotide length was 1062 obtained in *Pantoea* sp. while the shortest was 938 seen in *Ewingella* sp. This is demonstrated in Figure 5.1. Also, the amount of G nucleotide present in each of the macergens is much greater in comparison with other bases. In addition, G+C percentage is greater in all the macergens as compared to A+T percentage which is in line with Pavlovic-Lazetic et al. (2014). Thus, ratio of GC: AT ranges from 1.15:1.26:1 in *Enterobacter* sp. The differences in their GC content is between 1% and 5%, an indication of homogeneity among the macergens which is in line with Goodfellow and O'Donnell (1993) and Zhou et al. (2014). Research has pointed out that bacteria possessed greater percentage of G+C when compared with A+T (Zhou et al., 2014). Perhaps this rendered their DNA molecule to be much more stable. This GC content usually ranges from 25% to 75% in bacteria (Pavlovic-Lazetic et al., 2014).

Table 5.2: Base statistics of 16S rDNA of macergens

Species	Length	A	G	C	T	Gap	Other	G+C%	A+T%	GC:AT
Enterobacter sp.	955	212	286	187	199	0	71	49.5	43.04	1.15:1
Rahnella sp.	982	247	316	214	205	0	0	53.97	46.03	1.17:1
Raoultella sp.	974	229	296	201	205	0	43	51.03	44.56	1.21:1
Klebsiella sp.	993	245	320	222	202	0	4	54.58	45.02	1.21:1
Enterobacter sp.	951	231	307	217	186	0	10	55.1	43.85	1.26:1
Pectobacterium sp.	961	216	290	191	193	0	71	50.05	42.56	1.17:1
Citrobacter sp.	961	234	307	225	193	0	2	55.36	44.43	1.25:1
Citrobacter sp.	927	226	300	212	187	0	2	55.23	44.55	1.24:1
Erwinia carotovora.	940	232	299	193	199	0	17	52.34	45.85	1.15:1
Ewingella sp.	938	210	284	185	188	0	71	50	42.43	1.17:1
Erwinia sp.	1004	245	329	226	203	0	1	55.28	44.62	1.24:1
Pantoea sp.	1062	264	338	243	215	0	2	54.71	45.1	1.21:1
Lelliottia sp.	1060	263	339	240	217	0	1	54.62	45.28	1.21:1

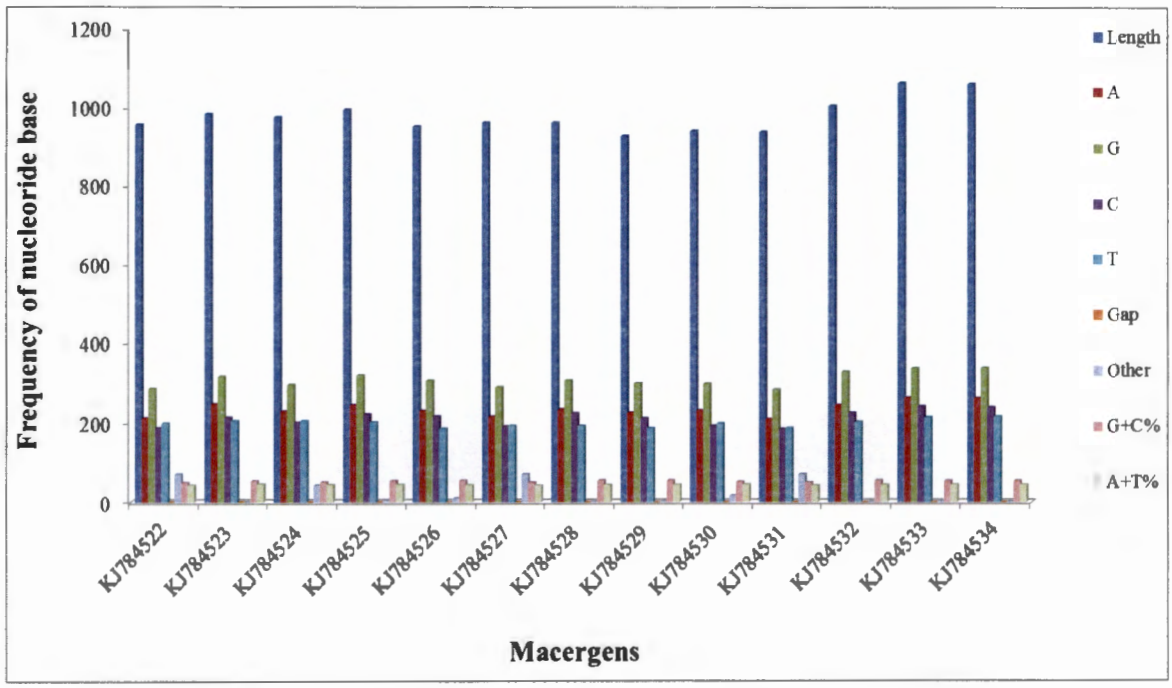

Figure 5.1: Bar chart showing macergens based on frequency of their nucleotide base composition

5.3.1 Sequence Similarity

The top sequence producing significant alignments was chosen from the similarity search conducted in Blastn for all the resulting 16S rDNA sequences of the macergens (Table 5.3). The Blast similarity search resulted in 30 hits of the references sequences. According to Sanjayan and Rama (2013) the expected value (e- value) symbolized the number of Blast hits that is expected to be obtained by chance with the observed score or higher. The 'e' values obtained for the entire similarity search are very small, revealing that the relationship among them is of high biological significance rather than just by chance. Likewise, good alignments transpire between the reference sequences and the observed macergens 16S gene sequences which depict the homology in them. The identity percentage ranges from 86% to 100% and this is considered as a good similarity index among the macergens and reference sequences. Table 5.4 showed the nucleotide frequencies percentage, which revealed that all the sequences possessed maximum percentage of G nucleotides (35%) than any other nucleotides present. The codon generally favoured G+C with a strong bias. However, 1st codon position showed that G ranges from 37% to 39% with an average of 38%. Also, in the 2nd and 3rd position, the G+C bias with an average of 31% to 33% was revealed. Moreover, this enhances the ability of macergens to under horizontal gene transfer.

Table 5.3: Top selected sequences with significant alignment with 16S gene sequences of macergens detected

Accession Numbers	Species	% Identity	Alignment Length	Mismatch	Gap Open	e value	Bit score
KJ784522	*Enterobacter sp.*	100	955	1	0	0	1530
KM253079	*Pantoea sp*	86	1404	1	1	0	1404
KM589031	*Enterobacter aerogenes*	86	1303	1	1	0	1184
KJ784523	*Rahnella sp.*	100	982	1	0	0	1772
JX867757	*Rahnella aquatilis*	100	1449	1	0	0	1772
JX162051	*Enterobacteriaceae bacterium*	100	1396	1	0	0	1772
DQ086780	*Serratia grimesii*	100	1353	1	0	0	1772
KJ784524	*Raoultella sp.*	100	974	1	0	0	1640
AB854618	*Uncultured Klebsiella sp.*	93	1455	1	0	0	1541
KJ599629	*Raoultella ornithinolytica*	93	1455	1	0	0	1541
HQ242728	*Raoultella terrigena*	93	1446	1	0	0	1541
KJ784525	*Klebsiella sp.*	100	993	1	0	0	1781
KM881701	*Klebsiella oxytoca*	99	1775	1	0	0	1775
KM096434	*Klebsiella pneumoniae*	99	1772	1	0	0	1772
KJ784526	*Enterobacter sp.*	100	951	1	0	0	1689
KF453771	*Enterobacter aerogenes*	98	1505	1	0	0	1665
KP717404	*Enterobacter xiangfangensis*	98	1374	1	0	0	1656
KJ784527	*Pectobacterium sp.*	100	961	1	0	0	1541
KJ685502	*Kluyvera cryocrescens*	86	1233	1	0	0	1213
DQ158205	*Averyella dalhousiensis*	86	1375	1	0	0	1211
KJ784528	*Citrobacter sp.*	100	961	1	0	0	1728
AB741678	*Citrobacter freundii*	99	1324	1	0	0	1714
KM515967	*Citrobacter braakii*	99	1446	1	0	0	1710
KM515975	*Citrobacter youngae*	99	1368	1	0	0	1701

KJ784529	*Citrobacter sp.*	100	927	1	0	0	1667
JX174253	*Leclercia sp.*	99	1546	1	0	0	1658
HQ242734	*Serratia ureilytica*	99	1411	1	0	0	1653
KP226572	*Erwinia sp.*	99	1451	1	0	0	1644
KJ784530	*Erwinia sp.*	100	940	1	0	0	1649
KJ939700	*Rahnella aquatilis*	97	1383	1	0	0	1590
KC951918	*Serratia grimesii*	97	1501	1	0	0	1590
KM891553	*Ewingella americana*	97	1246	1	0	0	1577
KJ784531	*Ewingella sp.*	100	938	1	0	0	1499
JN900058	*Erwinia persicina*	88	1428	1	0	0	1312
KF500096	*Erwinia rhapontici*	88	1438	1	0	0	1306
NR_104724	*Erwinia aphidicola*	88	1461	1	0	0	1303
KJ784532	*Erwinia sp.*	100	1004	1	0	0	1808
KJ210675	*Pantoea agglomerans*	99	1370	1	0	0	1772
KF913823	*Pantoea eucalypti*	99	1350	1	0	0	1772
HQ407241	*Pantoea vagans*	99	1210	1	0	0	1772
KJ784533	*Pantoea sp.*	100	1062	1	0	0	1911
AB089115	*Uncultured gamma proteobacterium*	99	1323	1	0	0	1911
KC951921	*Lelliottia amnigena*	99	1498	1	0	0	1902
KJ784534	*Lelliottia sp.*	100	1060	1	0	0	1909
KC139434	Enterobacter amnigenus	99	1420	1	0	0	1887

73 query sequences were generated in total

Table 5.4: Percentage of the nucleotide frequencies

Domain	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
WcVb1	19.5	22.2	23.4	34.9	513.0	17	20.2	23.8	38.7	168.0	21	27.8	18.3	33.1	169.0	20	18.8	27.8	33.0	176.0
OnFw1	18.9	22.4	23.9	34.7	518.0	17	20.1	24.1	38.5	174.0	19	28.8	19.4	32.4	170.0	20	18.4	28.2	33.3	174.0
KM253079	18.9	23.5	24.2	33.4	557.0	16	21.0	24.7	38.2	186.0	20	31.2	18.8	30.1	186.0	21	18.4	29.2	31.9	185.0
KM589031	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
PoGw1	19.6	22.4	24.4	33.6	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.4	30.6	186.0	22	17.3	28.1	33.0	185.0
JX867757	19.6	22.4	24.4	33.6	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.4	30.6	186.0	22	17.3	28.1	33.0	185.0
JX162051	19.6	22.4	24.4	33.6	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.4	30.6	186.0	22	17.3	28.1	33.0	185.0
DQ086780	19.6	22.4	24.4	33.6	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.4	30.6	186.0	22	17.3	28.1	33.0	185.0
BeBb1	19.4	22.2	24.6	33.8	536.0	17	20.0	25.0	38.3	180.0	21	29.4	19.2	30.5	177.0	21	17.3	29.6	32.4	179.0
AB854618	18.9	23.3	24.4	33.4	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.8	30.1	186.0	20	18.4	29.2	32.4	185.0
KJ599629	18.9	23.3	24.4	33.4	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.8	30.1	186.0	20	18.4	29.2	32.4	185.0
HQ242728	18.9	23.3	24.4	33.4	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.8	30.1	186.0	20	18.4	29.2	32.4	185.0
ToGf1	18.7	23.4	24.0	33.9	555.0	17	20.4	24.7	38.2	186.0	19	31.4	18.4	30.8	185.0	20	18.5	28.8	32.6	184.0
KM881701	18.7	23.5	23.9	33.9	557.0	17	20.4	24.7	38.2	186.0	19	31.7	18.3	30.6	186.0	20	18.4	28.6	33.0	185.0
KM096434	18.7	23.5	23.9	33.9	557.0	17	20.4	24.7	38.2	186.0	19	31.7	18.3	30.6	186.0	20	18.4	28.6	33.0	185.0
ToRr1	18.2	23.6	24.0	34.2	550.0	16	20.7	25.0	38.0	184.0	19	31.0	18.5	31.5	184.0	19	19.2	28.6	33.0	182.0
KF453771	18.1	24.1	23.7	34.1	557.0	16	21.0	24.7	38.2	186.0	19	31.2	18.3	31.2	186.0	19	20.0	28.1	33.0	185.0
KP717404	18.3	23.9	23.9	33.9	557.0	16	21.0	24.7	38.2	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
KJ685502	18.7	23.5	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
DQ158205	18.7	23.5	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
BmEd1	18.3	23.9	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
AB741678	18.3	23.9	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
KM515967	18.3	23.9	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
KM515975	18.3	23.9	24.1	33.8	557.0	16	21.0	25.3	37.6	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
IlEd1	18.7	23.4	24.0	33.9	555.0	16	21.1	24.3	38.4	185.0	20	30.8	18.4	30.8	185.0	20	18.4	29.2	32.4	185.0
JX174253	18.5	23.7	24.1	33.8	557.0	16	21.0	24.7	38.2	186.0	19	31.7	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
HQ242734	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
KP226572	18.3	23.9	23.9	33.9	557.0	16	21.0	24.7	38.2	186.0	19	31.7	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
CuPf1	19.7	21.9	24.1	34.3	543.0	17	18.9	25.6	38.3	180.0	20	30.0	18.9	31.1	180.0	22	16.9	27.9	33.3	183.0
KJ939700	19.6	22.4	24.6	33.4	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.9	30.1	186.0	22	17.3	28.1	33.0	185.0
KC951918	19.6	22.4	24.4	33.6	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.4	30.6	186.0	22	17.3	28.1	33.0	185.0
KM891553	19.6	22.4	24.6	33.4	557.0	18	19.4	25.8	37.1	186.0	19	30.6	19.9	30.1	186.0	22	17.3	28.1	33.0	185.0
PaWf1	19.3	21.7	23.7	35.3	507.0	16	20.0	24.7	39.4	170.0	21	28.6	17.3	32.7	168.0	21	16.6	29.0	33.7	169.0
JN900058	18.7	23.5	23.9	33.9	557.0	16	21.0	24.7	38.2	186.0	20	30.6	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
KF500096	18.5	23.7	23.9	33.9	557.0	16	21.0	24.7	38.2	186.0	19	31.2	18.3	31.2	186.0	20	18.9	28.6	32.4	185.0
KJ210675	19.0	23.3	24.1	33.6	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.3	30.6	186.0	21	18.4	28.6	32.4	185.0
PoLp1	19.2	23.0	24.1	33.6	556.0	17	20.4	25.3	37.6	186.0	20	31.2	18.3	30.6	186.0	21	17.4	28.8	32.6	184.0
KF913823	19.0	23.3	24.1	33.6	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.3	30.6	186.0	21	18.4	28.6	32.4	185.0
HQ407241	19.0	23.3	24.1	33.6	557.0	17	20.4	25.3	37.6	186.0	20	31.2	18.3	30.6	186.0	21	18.4	28.6	32.4	185.0
CeDc1	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
AB089115	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
NR 104724	18.9	23.2	24.3	33.6	556.0	17	20.4	25.3	37.6	186.0	20	30.8	18.4	30.8	185.0	20	18.4	29.2	32.4	185.0
KC951921	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
PoMs1	18.9	23.3	24.2	33.6	557.0	16	21.0	25.3	37.6	186.0	20	30.6	18.3	30.6	186.0	20	18.4	29.2	32.4	185.0
Avg.	18.9	23.2	24.1	33.8	552.9	17	20.4	25.1	37.8	184.6	20	30.8	18.6	30.9	184.4	20	18.3	28.7	32.6	183.9

5.3.2 Relative Synonymous Codon Usage (RSCU)

The codon usage always revealed information regarding molecular evolution of individual genes. Mostly amino acids are being coded by more than one synonymous codon as a result of frequent genetic code degeneration. The RSCU values represent the frequency of observing a particular codon with regard to when observed in the absence of any codon usage bias. According to McInerney (1998) RSCU values of less than 1 indicate that the codon is seldom used, while it is the other way round when it is greater than 1. Table 5.5 depicts the details of the RSVU for the 16S gene sequences of the macergens. In this study, the average codon expressed by all the macergens is 180. The AGG codon coding for Arginine is the most frequently used, followed by GUA and GUG coding for Valine.

Table 5.5: Relative Synonymous codon usage for macergens 16S gene

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	1.3	1.15	UCU(S)	1.9	1.08	UAU(Y)	0	0	UGU(C)	3.3	1.16
UUC(F)	0.9	0.85	UCC(S)	2.1	1.18	UAC(Y)	0.9	2	UGC(C)	2.4	0.84
UUA(L)	3	1.56	UCA(S)	1	0.56	UAA(*)	1.3	0.71	UGA(*)	3	1.64
UUG(L)	1.1	0.59	UCG(S)	1.7	0.97	UAG(*)	1.2	0.65	UGG(W)	5	1
CUU(L)	1.4	0.73	CCU(P)	4.9	1.62	CAU(H)	0	0	CGU(R)	3.5	1.23
CUC(L)	1.3	0.66	CCC(P)	2.3	0.76	CAC(H)	3	2	CGC(R)	0.9	0.33
CUA(L)	1.8	0.94	CCA(P)	2.9	0.96	CAA(Q)	4.7	1.22	CGA(R)	0.9	0.32
CUG(L)	2.9	1.52	CCG(P)	2	0.66	CAG(Q)	3	0.78	CGG(R)	1.9	0.68
AUU(I)	1	0.59	ACU(T)	5.7	1.46	AAU(N)	1	0.66	AGU(S)	I.9	1.08

AUC(I)	3	1.8	ACC(T)	3.9	1	AAC(N)	2	1.34	AGC(S)	2	1.13
AUA(I)	1	0.61	ACA(T)	2.8	0.71	AAA(K)	3	1	AGA(R)	4.2	1.48
AUG(M)	2.8	1	ACG(T)	3.3	0.83	AAG(K)	3	1	AGG(R)	5.6	1.97
GUU(V)	0	0	GCU(A)	3.8	0.72	GAU(D)	1	2	GGU(G)	6	1.12
GUC(V)	2	0.55	GCC(A)	3.1	0.6	GAC(D)	0	0	GGC(G)	3.3	0.61
GUA(V)	6.5	1.76	GCA(A)	6.2	1.19	GAA(E)	4.9	0.98	GGA(G)	5.1	0.96
GUG(V)	6.2	1.69	GCG(A)	7.8	1.5	GAG(E)	5.1	1.02	GGG(G)	7.1	1.31

5.3.3 Nucleotide Substitution among the Sequences

Determination of the nucleotide substitution per site (d) present amongst DNA sequences is crucial in the study regarding molecular evolution, taking into consideration the rates of transitional and transversional nucleotide substitution (transition-transversion bias) and the deviation of the G+C content from 0.5 (G+C-content bias), which are the two crucial parameters in the evaluation of nucleotide substitution per site (Tamura, 1992, Sanjayan and Rama, 2013). With this understanding the nucleotide substitution among the sequences of the macergens was revealed. The nucleotide pair frequencies (Table 5.6) deduced for 44 species revealed 541 identical pairs involving an overall total of 549 pairs, of which there were clearly 102 TT pairs, 185 GG, 124 CC as well as 131 AA. The rate of transition of Transitional Pairs versus Transversional pairs was 4.25.

Whenever DNA sequences result from a common ancestral sequence, the actual descendant sequences progressively diverge through nucleotide substitution (Sethuraman and Sanjayan, 2013). An effective way of measuring the degree of sequence divergence is the number of nucleotide sites of which this sequence is very different. This is estimated as the p-distance with regard to nucleotide sequences. It is employed to know the frequencies of various nucleotide pairs among the sequences. Since there are four nucleotides, there are 16 different types of nucleotide pairs. There are four pairs of identical nucleotides (AA, TT, CC, GG symbolized as O), four transition-type pairs (AG, GA, TC, CT expressed as P) along with the remaining eight transversion-type pairs represented as Q. The p distance with regard to nucleotide sequence was calculated to be 7 (i.e. 6+1), using p=P+Q. If nucleotide substitution takes place by chance, Q is likely to be almost two times greater than P whenever p is small, which is not the case in this present investigation.

In general, transition usually occurs more frequently than transversions. For that reason P could possibly be more than Q. Once the degree of divergence is small, the ratio (R) of transitions to transversions is usually estimated from the experimental values of P as well as Q. R is often 0.2-2 in lots of nuclear genes, however, mitochondrial DNA can be as high as 15 (Vigilant et al., 1991). In the present study the value of R was 4.25. The analysis of the p value deduced for the 1st, 2nd and 3rd codons was 0.02. This revealed that no synonymous substitution occurs in the first three codons.

Table 5.6: Nucleotide pair frequencies in macergens sequences: undirectional (10 pairs)

	Ii	si	sv	R	TT	TC	TA	TG	CC	CA	CG	AA	AG	GG	Total
Avg	541.00	6.00	1.00	4.25	102.00	4.00	0.00	0.00	124.00	1.00	0.00	131.00	2.00	185.00	548.93
1st	181.00	2.00	0.00	46.22	30.00	1.00	0.00	0.00	37.00	0.00	0.00	45.00	1.00	69.00	183.27
2nd	180.00	2.00	1.00	2.98	35.00	2.00	0.00	0.00	55.00	0.00	0.00	33.00	1.00	56.00	182.84
3rd	180.00	2.00	1.00	2.95	36.00	1.00	0.00	0.00	32.00	0.00	0.00	52.00	0.00	59.00	182.82

ii = Identical Pairs; si = Transitional Pairs; sv = Transversional Pairs; 1st, 2nd, 3rd Codon Position TC, AG – Transition; TA, TG, CA, CG – Transversion; TT, CC AA, GG – Identical Pairs

5.3.4 Molecular Evolutionary Genetic Analysis

The nucleotide sequences of the 16s rDNA were aligned utilising the ClustalW option present as a part of the MEGA6 programming. Every phylogenetic strategy makes assumptions concerning the system of DNA substitution (Felsenstein, 1988). An assumption usual to phylogenetic ways is a branching tree to portray the phylogeny of species (Huelsenbeck and Crandall, 1997). All methods of phylogenetic conclusion rely on their basic models. To have reliance in inferences, it is crucial to have confidence in the models (Goldman, 1993). On account of this, all strategies in light of unequivocal models of evolution ought to investigate which is the model that fits the information best. Models with the least BIC scores (Bayesian information criterion) are considered to portray the best substitution pattern quality. Table 5.7 supplies the full Maximum Likelihood of 24 diverse nucleotide substitution models.

The T92+G had the least BIC scores and for that reason was regarded as the finest model for the present data set. The T92+G (Tamura-3- parameter) model an extension of Kimura 2-parameter, corrects for a couple of hits, considering variations in transitional and transversional rates and G+C-content bias (Tamura, 1992). It assumes an equality of substitution rates amongst the sites. Yet another means of deciding on the most fitting model for a data set is to utilize the Akaike information criterion (AIC) (Akaike, 1974), which can be considered as the measure of data lost when a specific model is utilized to inexact fact. The AIC executes best-fit model determination by computing the probability of the proposed models, and imposing a penalty in view of the quantity of model parameters. Parameter-rich models cause a bigger penalty than extra easy models which cannot become excessively elaborate model. The best fitting model is the one with the smallest AIC value. The T92+G

had the smallest AIC value (Table 5.7). As a result of this T92+G is the best model for this study.

Table 5.7: Maximum Likelihood fits of 24 different nucleotide substitution models

Model	BIC	AICc	lnL	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)
T92+G	2395.4	1697.9	-760.56	n/a	0.05	7.66	0.214	0.214	0.286	0.286
K2+G	2396.2	1706.7	-765.96	n/a	0.05	7.63	0.250	0.250	0.250	0.250
T92	2397.1	1707.6	-766.41	n/a	n/a	7.53	0.214	0.214	0.286	0.286
K2	2398.0	1716.3	-771.79	n/a	n/a	7.53	0.250	0.250	0.250	0.250
HKY+G	2399.4	1686.1	-752.66	n/a	0.05	7.82	0.232	0.195	0.214	0.359
HKY	2400.9	1695.5	-758.36	n/a	n/a	7.53	0.232	0.195	0.214	0.359
T92+G+I	2404.5	1699.1	-760.15	0.12	0.05	7.68	0.214	0.214	0.286	0.286
K2+G+I	2405.9	1708.4	-765.84	0.03	0.05	7.64	0.250	0.250	0.250	0.250
T92+I	2406.6	1709.1	-766.16	0.06	n/a	7.53	0.214	0.214	0.286	0.286
TN93+G	2406.6	1685.4	-751.28	n/a	0.05	7.68	0.232	0.195	0.214	0.359
K2+I	2406.7	1717.1	-771.17	0.15	n/a	7.53	0.250	0.250	0.250	0.250

TN93	2408.3	1695.0	-757.10	n/a	n/a	7.53	0.232	0.195	0.214	0.359
JC+G	2408.6	1726.9	-777.11	n/a	0.05	0.50	0.250	0.250	0.250	0.250
HKY+G+I	2408.8	1687.6	-752.38	0.00	0.05	7.81	0.232	0.195	0.214	0.359
HKY+I	2410.0	1696.7	-757.96	0.10	n/a	7.54	0.232	0.195	0.214	0.359
JC	2410.3	1736.6	-782.92	n/a	n/a	0.50	0.250	0.250	0.250	0.250
TN93+I	2410.7	1689.5	-753.33	0.63	n/a	7.59	0.232	0.195	0.214	0.359
TN93+G+I	2416.6	1687.4	-751.28	0.00	0.05	7.68	0.232	0.195	0.214	0.359
JC+G+I	2418.2	1728.6	-776.93	0.05	0.05	0.50	0.250	0.250	0.250	0.250
JC+I	2419.3	1737.7	-782.46	0.11	n/a	0.50	0.250	0.250	0.250	0.250
GTR+G	2435.6	1690.6	-750.88	n/a	0.05	4.94	0.232	0.195	0.214	0.359
GTR	2437.3	1700.2	-756.69	n/a	n/a	4.92	0.232	0.195	0.214	0.359
GTR+G+I	2441.2	1688.3	-748.72	0.57	0.05	5.04	0.232	0.195	0.214	0.359
GTR+I	2443.5	1698.5	-754.82	0.39	n/a	4.92	0.232	0.195	0.214	0.359

GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

5.3.5 Maximum Likelihood Estimate of Gamma Parameter for Site Rates

It is an established fact that various amino acid residues of any protein could possibly have distinct functional restrictions in such a way that the substitution rate differs among the sites. The gamma distribution has been widely employed in modelling the rate of variation amongst the sites. Based on the gamma distribution, the substitution rate typically differs from site to site within a sequence. The state of this dissemination is either controlled by gamma parameter, otherwise called the shape parameter, or by assuming that a definite fraction of sites are evolutionarily invariability (+I). In the present evaluation, the estimated value of the shape parameter of the discrete Gamma Distribution is 0.05.

Perpetually, the assumption on the certain portion of the sites of evolutionary invariable was not employed in this study. Substitution pattern and rates were assessed under the Kimura 2-parameter model (Kimura, 1980). Relative values of instantaneous r should be regarded when assessing them. For ease, sum of r values is made equivalent to 100. The result for nucleotide frequencies in this study revealed that A = 23.22%, T/U = 19.52%, C = 21.38% as well as G = 35.88%. The ML values were given by a robotically tree topology computation. This gave the value of -756.258 for the Maximum Log likelihood. The analysis involved 44 nucleotide sequences with codon positions involving 1st+2nd+3rd+Noncoding. Almost all positions comprising gaps as well as missing data were wiped out. There were an aggregate of 469 positions in the final dataset. Evolutionary analyses were performed in MEGA6 (Tamura et al., 2013).

5.3.6 Distance matrix

The base substitutions numbers per site within the sequences are depicted in Table 5.8. The analysis involving 44 sequences by utilizing the Maximum Composite Likelihood model, with the exclusion of gaps and missing data from almost all the positions and

inclusion of 1st+2nd+3rd+Noncoding (codon position) resulted in 557 positions in the final dataset. Employing MEGA6 of Tamura et al. (2013) for the evolutionary analyses, revealed that some of the species have no evolutionary divergence by having the value of 0.00. The average divergence exhibited by these sequences is 0.014. This low value is an indication of insignificant evolution divergence among the 44 species studied, based on Tamura et al. (2004).

Table 5.8: Estimation of the evolutionary divergence between sequences

KJ78452																																												
KJ78452	0.01																																											
KM2530	0.02	0.03																																										
KM5890	0.01	0.02	0.01																																									
KJ78452	0.02	0.02	0.03	0.02																																								
JX86775	0.02	0.02	0.03	0.02	0.00																																							
JX16205	0.02	0.02	0.03	0.02	0.00	0.00																																						
DQ0867	0.02	0.02	0.03	0.02	0.00	0.00	0.00																																					
KJ78452	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02																																				
AB85461	0.01	0.02	0.01	0.01	0.03	0.03	0.03	0.03	0.01																																			
KJ59962	0.01	0.02	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.00																																		
HQ2427	0.01	0.02	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.00	0.00																																	
KJ78452	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01																																
KM8817	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.00																															
KM0964	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.00	0.00																														
KJ78452	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01																													
KF4537	0.02	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.00																												
KP7174	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01																											
KJ68550	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01																										
DQ1582	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00																									
KJ78452	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00																								
AB74167	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00																							
KM5159	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00																						
KM5159	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00																					
KJ78452	0.01	0.02	0.01	0.00	0.03	0.03	0.03	0.03	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01																				
JX17425	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00																			
HQ2427	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01																		
KP2265	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01																	
KJ78453	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02																
KJ93970	0.02	0.02	0.03	0.03	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00															
KC9519	0.02	0.02	0.03	0.02	0.00	0.00	0.00	0.00	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.03	0.00	0.00														
KM8915	0.02	0.02	0.03	0.03	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00	0.00	0.00													
KJ78453	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.02	0.03	0.02	0.03												
JN90005	0.01	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.02	0.03	0.03	0.03	0.00											
KF50005	0.02	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.02	0.03	0.03	0.03	0.00	0.00										
NR_104	0.02	0.03	0.01	0.01	0.03	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.02	0.03	0.03	0.03	0.00	0.00	0.00									
KJ78453	0.01	0.03	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01								
KJ21067	0.02	0.03	0.01	0.02	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.00							
KF91382	0.02	0.03	0.01	0.02	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.00	0.00						
HQ4072	0.02	0.03	0.01	0.02	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.01	0.02	0.02	0.02	0.00	0.00	0.00					
KJ78453	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.03	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02				
AB08911	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.03	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.00			
KC9519	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.03	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.00	0.00		
KJ78453	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.00	0.00	0.00	
KC1394	0.01	0.02	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.03	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.00	0.00	0.00	0.00

5.3.7 Evolutionary Relationships of Taxa Involving 16S rRNA Sequence

Maximum likelihood method utilized the sequence information specifically to reconstruct a tree, in this manner using data in particular nucleotide variations as opposed to summarizing changes with a genetic distance. Due to these distinctions, ML offers noteworthy statistical properties in comparison with genetic distance-based approaches, however it is rather more computationally intensive. In each of these phylogenetic estimators, the evolutionary history was once deduced in light of the Tamura 3-parameter model. In these techniques the tree branching sample was indistinguishable. In Fig 5.2, the tree with the highest log likelihood (-766.1249) is demonstrated. The percentage of trees in which the related taxa clustered together is demonstrated by the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances assessed using the maximum Composite Likelihood (MCL) method, and then making a choice on the topology with superior log likelihood value.

The PoGw1 and CuPf1 clustered with *Rahnella aqualitis*, *Enterobacteria bacterium*, *Serratia grimesii*, with low boostrap value of 62%. *Rahnella aquatilis* and *Ewingella americana* have 86% similarity with OnFw1. *Citrobacter freundii, Citrobacter braakii* and *Citrobacter youngae* expressed similarity value that is less than 50% with BmEd1. Likewise, PoMs1 and CeDc1 exhibit low similarity value of less than 50% with *Enterobacter aerogenes*, *Kluyvera cryocrescens*, *Averyella dalhousiensis*, *Serratia ureilytica*, *Uncultured gamma proteobacterium*, *Lelliottia amnigena*, and *Erwinia aphidicola*. *Enterobacter aerogenes*, *Pantoea agglomerans*, *Pantoea eucalypti*, and *Pantoea vagans* shared moderate similarity value of 86% with ToRr1. In addition, PaWf1 expressed low similarity value of 53% with *Erwinia persicina* and *Erwinia rhapontici*. Whereas, ToGf1 showed very low value that is less than 50% with *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Enterobacter*

xiangfangensis, *Leclercia* and *Erwinia* sp. Among these macergens only four of them did not cluster with any of the reference strains. These are: IlEd1 and PoLp1 closer to *Erwinia* sp. also WcVb1 and BeBb1 closer to *Raoultella terrigena.* The outcome of the phylogenetic relationships amongst these taxa indicates a steady topology with the most often acknowledged relationship. The existence of a moderate similarity value suggest divergence of the macergens (Konstantinidis and Stackebrandt, 2013). The similarity value less than 50% is an indication of probable novel species of the macergens according to Naushad et al. (2014).

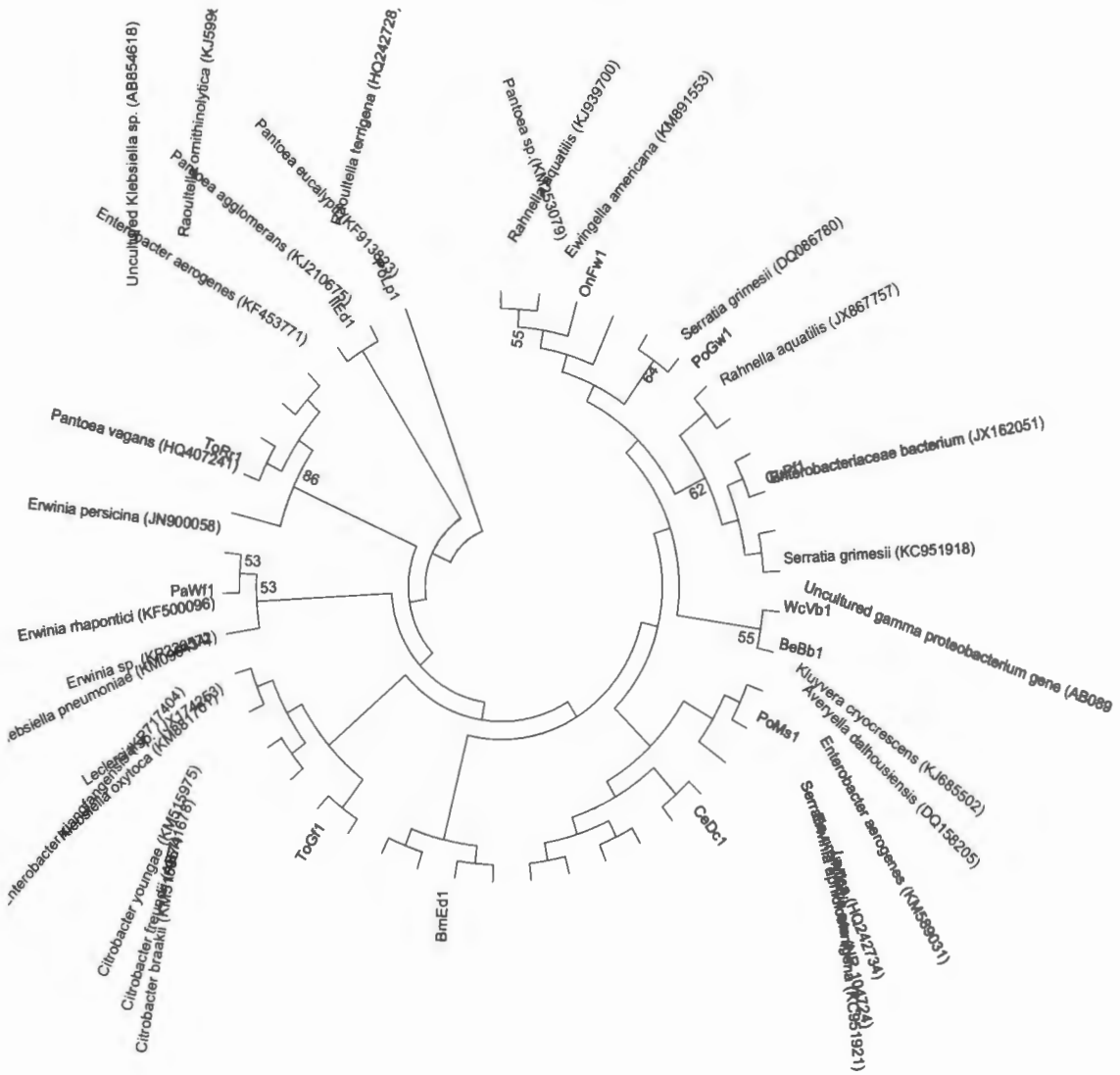

Figure 5.2: Maximum Likelihood phylogenetic tree based on partial 16S rDNA gene sequence, showing the phylogenetic relationships between macergens and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.002 nucleotide substitution per site. *Pantoea* species was set as the out-group.

5.4 Conclusion

Macergens could be recognized based on their 16S rRNA sequence configuration. Their 16S rRNA sequence varies in the cumulative number of base pairs in addition to the G+C and A+T content. Conversely, a sequence similarity of 86% to 100% was recorded. The identical pairs exhibited among the macergens from nucleotide paired frequencies was 541. The proportion of the transitional to transversional pairs was 4.25. Analysis of the maximum likelihood of 24 diverse nucleotide substitution configuration to reconstruct the phylogenetic tree, demonstrated that T92+G had the minimum BIC and AIC scores, subsequently this can be considered as the best model for the present data set. The phylogenetic tree constructed indicates the four clades in which macergens are clustered at 53%-86%. Four novel macergens *Enterobacter* sp. (WcVb1), *Raoutella* sp. (BeBb1), *Erwinia* sp. (PoLp1) and *Citrobacter* sp. (IlEd1) with distinct signatures were obtained.

CHAPTER 6

COMPARATIVE STUDY OF CONSTRUCTED MACERGENS SPECIFIC OLIGONUCLEOTIDES FOR THE SELECTION OF PECTINOLYTIC GENE IN DIFFERENT VEGETABLES

Abstract

The detection of macergens from vegetables is becoming imperative because of the threat they pose to human health and safety. Ten primers were designed as molecular markers to effectively detect macergens in vegetables. These primers were designed by the computational method (Primer3Plus platform). These were employed in the differential PCR assay and the resulting electrophoresed, sequenced and analyzed. All the primers designed satisfied the conditions for good primers as well as *in silico* and empirical specificity test for pectinolytic gene. These primers were compared for their efficiency in detecting macergens in vegetable samples. The performances of these primers range in ascending order from second primer set→fourth primer set→fifth primer set→seventh primer set→tenth primer set→ third primer set→sixth primer set→ eighth primer set→ second primer set→nineth primer set. Hence, primer set 9 (M580F and M877R) was chosen as the best of all the primers designed for rapid detection of macergens with expected amplicons of 375 bp and highest specificity.

Keywords: Macergens, pectinolytic, primer, rapid, specificity

6.1 Introduction

Macergens are pectinolytic bacteria responsible for absolute tissue degradation in plants (Bhai et al., 2012). They invade vegetable tissues on the field, in transit and storage causing great economic losses of the crops by the farmers. This results in low yield and loss of potential consumer values which consequently leads to economic losses (Wang et al., 2015). The most popular means of combating these macergens is the traditional techniques involving culturing, biotyping and serotyping which are erroneous and slow. The available means of detecting these macergens such as physical, chemical and biological methods are not efficient in that they fail to detect them in time before much harm is done to the plant (Aremu and Babalola, 2015b, Cheverton, 2015, Czajkowski et al., 2012). As a matter of fact, their early and prompt detection is of great paramount to ensure enough food, and if vegetables are to reach market in their healthy states, to promote human health (Aremu and Babalola, 2015a). This calls for the introduction of Polymerase Chain Reaction (PCR) that have been widely used for identification of pathogens in biology (Chuang et al., 2013).

For a PCR reaction, primers need to be constructed. Whenever primers are being designed a lots of constraints encompassing their functionality and specificity should be put into consideration. In this study, computational methods were employed in designing primers suitable for quick detection of macergens. In addition, these primers designed were compared to identify the best of all the primer sets, in terms of specificity and amplification efficiency, that can be of future use in detection of macergens from any type of vegetables and phylogenetic analysis.

6.2 Materials and Methods

6.2.1 Collection of Samples

Twenty-six vegetable samples were obtained from various cities in South Africa (Table 6.1). They were kept in a dark cupboard for 2 weeks in order to become rotten for macergens invasion, after which they were surface sterilized, and the inner parts were used for molecular analysis.

Table 6.1: Sources of vegetable samples

Serial number	Sample designation	Sample code	Selected vegetable	Place of collection
1	WcVb	M1	White Cabbage	Van Staden Boerdery, Bultfontein, Hartbeesfontein, North West
2	PoGw	M2	Potatoes	Greenway, Tarlton, Gauteng
3	SmEb	M3	Straight Baby Marrow	Eldorado, Aliwal North, Eastern Cape
4	BeBb	M4	Beetroot	Barlow Boerdery, Krokodidrift West, Brits, North West
5	WmSs	M5	White Button Mushroom	Silver Stream, Helderstroom, Western Cape
6	ToGf	M6	Tomatoes	Greenway, Tarlton, Gauteng
7	ToRr	M7	Tomatoes	ZZ2, Musina, Limpopo
8	OnFw	M8	Onions	Wildeklawer, Barkley West, Northern Cape
9	SmEbi	M9	Straight Small Marrow	Starke Ayres, Rosebank, Cape Town, Western Cape
10	BmEb	M10	Round Baby Marrow	Eldorado, Allanridge, Free State
11	RcVb	M11	Red Cabbage	Van Staden Boerdery, Bultfontein, Hartbeesfontein, North West
12	IlEd	M12	Iceberg Lettuce	Eldorado, Vorstershoop, North-West
13	CuPf	M13	Cauliflower	Leeupoort, Tala Valley, Kwazulu Natal

14	CtGf	M14	Carrot	Greenway, Tarlton, Gauteng
15	PaFw	M15	Parsely	Bloublommetjieskloof, Wellington, Western Cape
16	PoLp	M16	Potatoes	LangPlaas, Siyanda, Northern Cape
17	CeDc	M17	Celery	Dew Valley, Muldershift, Gauteng
18	PoMs	M18	Potatoes	Potato Pride, Eastern Free State
19	BrBb	M19	Broccolis	Meadowbrook, Drakensberg, KwaZulu Natal
20	PoBc	M20	Potatoes	Laucob Boerdery, Christiana, Western Cape
21	SpVs	M21	Spinach	Greenway, Tarlton, Gauteng
22	SoSd	M22	Spring Onions	ZZ2, Polokwane, Limpopo
23	OnBb	M23	Onions	Barlow Boerdery, Krokodidrift West, Brits North West,
24	CtBv	M24	Carrot	Barlow vorentoe, Hermon, Western Cape
25	CtLb	M25	Carrot	Langplaas Boerdery, Brits, North West
26	BpLb	M26	Bell Pepper	Laucob Boerdery, Christiana, Western Cape

WcVb: white cabbage from Boerdery, PoGw: potato from Greenway, SmEb: straight baby marrow from Eldorado, BeBb: beetroot from Barlow, WmSs: white button mushroom from Silver Stream, ToGf: tomatoes from Greenway Farm, ToRr: tomatoes from ZZ2, OnFw: onion from Wildeklawer Farm, SmEbi: small marrow from Starke Ayres, BmEb: baby marrow from Eldorado, RcVb: red cabbage from Van staden Boerdery, IIEd: iceberg lettuce from Eldorado, CuPf: cauliflower from Leeuport Farm, CtGf: carrot from Greenway Farm, PaFw: parselyfrom Bloublommetjieskloof Wellington, PoLp: potatoes from LangPlaas, CeDc: celery from Dew Valley, PoMs: potatoes from Potato Pride, BrBb: broccolis from Meadowbrook, PoBc: potato from Laucob Boerdery, SpVs: spinach from Greenway, SoSd: spring onions from ZZ2, OnBb: onion from Barlow Boerdery, CtBv: carrot from Barlow vorentoe, CtLb: carrot from Langplaas Boerdery, BpLb: bell pepper from Laucob Boerdery.

6.2.2 Metagenomic DNA Extraction

Direct DNA extraction of the total bacteria in the vegetable samples was conducted using ZR Fungal/Bacterial DNA MicroPrep™ Kit extraction kit (Zymo Research Corp., Irvine, CA 92614 US) following the manufacturer's protocol.

6.2.3 Primer Design

The 16S rDNA sequences of the genera belonging to macergens were retrieved from the NCBI database (www.ncbi.nlm.nih.gov/) and aligned with MAFFT multiple sequence alignment (http://mafft.cbrc.jp/alignment/server/). This was opened in MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0.) to locate the conserved region. The pairs of primer (both forward and reverse) were then generated using Primer3Plus platform (www.bioinformatics.nl/primer3plus/).

6.2.4 Assessment of the Designed primers

6.2.4.1 In-silico application

The designed primers were tested for properties of good primers and specificity for pectinolytic bacteria using the IDT platform and Gene Infinity respectively. Consequently, those primers with good primer criteria were synthesized at Integrated DNA Technology at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa.

6.2.4.2 Empirical application

The synthesized primers were used for amplification of a known macergens DNA template (*Pectobacterium chrysanthemi* (31 ng/µl)), a positive control and negative control (fresh healthy vegetable DNA template). The experimental application of these primers in the laboratory was conducted to ascertain ability to detect macergens in any of these samples

without amplifying any other non-pectinolytic bacteria. The resulting gel pictures were visualized with a ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules CA 94547, US).

6.2.5 Amplification of the PCR

The designed primers were used to amplify the 16S rDNA region of the pectinolytic bacteria of the metagenomic DNA from vegetable samples. The PCR gradient machine was used to optimize the best condition necessary for each primer pairs. The PCR reactions were performed using C 1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules CA 94547, US). The PCR were conducted in 50 µl reaction mixture with 10 µM for each primer, 10 mM dNTPs, 5 µl 10x thermophilic buffer, 1.0 mM to 4.0 mM $MgCl_2$ (25 mmol), 0.2 µl *Taq* DNA polymerase (5U/µl), 10 ng of DNA and enough nanopure water to fill reaction volume to 50 µl was used per reaction tube. The PCR cycles comprised of a 94°C melting temperature for 30 sec/cycle, a 47-59°C annealing temperature for 30 sec/cycle, and a 72°C polymerase elongation step for 1 min/cycle. The PCR completed with a 72°C elongation for 10 min and a holding period at 4°C for infinite time.

6.2.6 Gel Electrophoresis

The purified PCR products were analyzed using gel electrophoretic studies. The PCR products (7 µl of each sample) were electrophoresed on 1% (w/v) agarose gel (Sigma, Deisenhofen, Germany) stained with ethidium bromide (Bio-Rad Laboratories, Hercules CA 94547, US). A molecular weight marker (5 µl volumes) with a loading dye (Thermo Fisher Scientific Inc., Waltham, MA USA) of 2 µl was incorporated for amplicon sizes evaluation. Finally, electrophoresis gels were viewed on ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules CA 94547, US).

6.2.7 Sequencing

The amplicons were excised and purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., Irvine, CA 92614 US) to yield high-quality. The purified DNA were checked for nucleotide determination employing ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. The resulting chromatographs were also analyzed using bioinformatics tools and the nucleotide sequence of the 16S rDNA of the clones specific for macergens were determined with Basic Alignment Search Tool (BLAST) accessible at the ncbi-nlm-nih.gov website (Altschul et al., 1997). The FASTA sequences obtained from this study were later deposited in the GenBank database (www.ncbi.nlm.nih.gov/genbank/) to assign accession numbers.

6.3 Results

6.3.1 Primer Design

A total of ten primers (both forward and reverse) were designed and demonstrated in Table 6.2. These primers showed the criteria for good primers in the sense that their GC% ranges from 47.6 to 60, sequence length 20-23 bp and annealing temperature (T_m) ranges from 54.5 to 56.9 at the IDT platform using OligoAnalyser 3.1. Also they exhibited good absorbance at 260 nanometers OD_{260} values of nmole/OD260 from 4.38 to 5.62, µg/OD260 from 29.24 to 34.23 4.38-5.62 as well as molecular weight from 6090 to 7165.7 (Table 6.2). In addition to these, no primary secondary structure in terms of self-priming, potential hairpin formation, 3' complementarity and potential self-annealing sites was seen in all these primers.

Table 6.2: Sequences and properties of the designed oligonucleotide for PCR amplification

Primer	Primer Set	Oligonucleotide Sequence	GC %	Tm °C	Lt	L	M_w	nmole /OD260	µg/ OD260	Position
M101F	Set 1	CGG ACG GGT GAG TAA TGT CT	55	56.5	20	16S	6213.1	5.07	31.51	101-121
M1284R	Set 1	TTA TGA GGT CCG CTT GCT CT	50	56	20	16S	6090	5.62	34.23	1284-1304
M101F	Set 2	CGG ACG GGT GAG TAA TGT CT	55	56.5	20	16S	6213.1	5.07	31.51	101-121
M1208R	Set 2	AAG GGC CAT GAT GAC TTG AC	50	55.1	20	16S	6166.1	5.04	31.05	1208-1180
M182F	Set 3	CGA TCC CTA GCT GGT CTG AG	60	56.6	20	16S	6109	5.41	33.06	182-202
M1190R	Set 3	TTA TGA GGT CCG CTT GCT CT	50	56	20	16S	6090	5.62	34.23	1190-1170
M180F	Set 4	GAC GAT CCC TAG CTG GTC TG	60	56.9	20	16S	6109	5.4	33	180-200
M1190R	Set 4	TTA TGA GGT CCG CTT GCT CT	50	56	20	16S	6090	5.62	34.23	1190-1170
M57F	Set 5	GAG GAA GAA ACC GGC GAT AG	55	55.3	20	16S	6249.1	4.68	29.24	57-77
M296R	Set 5	GGC GTA TCC ACC GAT GTA AT	50	54.6	20	16S	6117	5.13	31.37	296-279

M451F	Set 6	TCG GAA TTA CTG GGC GTA AAG	47.6	54.8	21	16S	6510.3	4.75	30.94	451-472
M1176R	Set 6	CTG AGA TAG GGT TTC TGG GAT TG	47.8	54.9	23	16S	7165.7	4.46	31.93	1176-1199
M836F	Set 7	GCG GTG GAG TAT GTG GTT TA	50	54.7	20	16S	6259.1	5.01	31.34	836-856
M1032R	Set 7	CTC CTT AGA GTT CCC ACC ATT AC	47.8	54.5	23	16S	6894.5	4.8	33.07	1032-1055
M475F	Set 8	GCG TAG GTG GTG GTT TAA GT	50	54.9	20	16S	6259.1	5.04	31.55	475-495
M707R	Set 8	TCC AGT TCG CAT CGT TTA GG	50	54.9	20	16S	6099	5.38	32.79	707-727
M580F	Set 9	TGT AGC AGT GAA ATG CGT AGA G	45.5	54.8	22	16S	6863.5	4.38	30.06	580-602
M877R	Set 9	GTG GAT GTC AAG ACC AGG TAA G	50	55.1	22	16S	6848.5	4.38	30.01	877-899
M77F	Set 10	GAT GTG CCC AGA TGG GAT TAG	52.4	55.3	21	16S	6526.3	4.75	30.97	77-98
M322R	Set 10	GGT GCT TCT TCT GCG AGT AA	50	54.9	20	16S	6139	5.36	32.9	322-342

The *in silico* test revealed that the primer specificity test to the pectinolytic gene in macergens at platform of Gene Infinity all possessed optimality for macergens because their inability to amplify any other bacteria apart from the pectinolytic bacteria. Equally, the empirical test also depicted the ability of the primers to amplify only the positive DNA template of known macergens, whereas the negative healthy vegetable DNA template was not amplified. This is expressed in Figure 6.1.

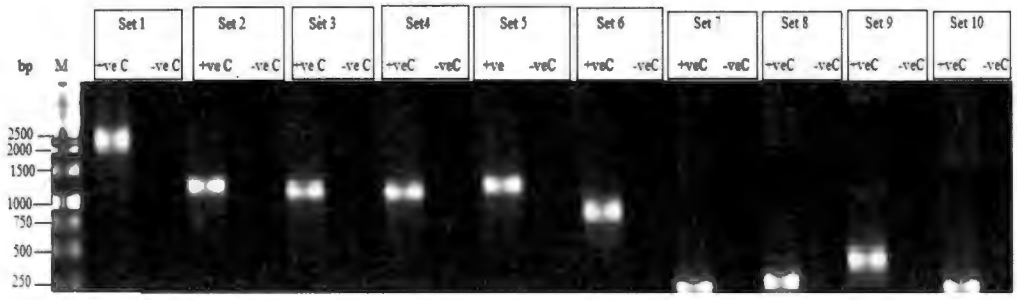

Figure. 6.1: Agarose gel electrophoresis of PCR products of *Pectobacterium chrysanthermi* (positive control) and healthy vegetable (negative control) using the macergens specific primers designed in this study which gave the expected sizes of each of the primers. M: 1kb molecular weight marker; +veC: positive control; -veC: negative control

6.3.2 Amplification of the PCR

The primers specifically amplified the pectinolytic region of the DNA template of the twenty-six samples and gave the exact amplicons for each primer set. This result is represented in Table 6.3. The electrophoresis visualized gel on ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules CA 94547, US) revealed the resulting expected amplicon sizes for each primer set (Figure 6.2). Furthermore, the amplification efficiency of macergens DNA template varied with the different primers, expressed in Figure 6.3. In the Figure 6.3, it is shown that primer set 9 has the highest amplification efficiency followed by the primer set 2 and primer set 6.

Table 6.3: Results of PCR amplification from each template DNA with each primer set

Source of template DNA[a]	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}
WcVb	+	–	+	+	–	+	+	+	+	–
PoGw	+	–	–	–	–	–	+	+	+	–
SmEb	+	+	+	+	+	+	–	+	+	–
BeBb	–	–	–	+	–	+	+	+	+	+
WmSs	+	+	+	–	+	+	–	+	+	+
ToGf	–	–	–	–	–	+	–	–	+	–
ToRr	+	–	–	–	–	–	–	–	+	–
OnFw	+	–	–	–	–	–	–	–	+	+
SmEbi	+	+	+	–	+	+	+	+	+	–
BmEb	–	+	+	–	+	–	–	+	+	–
RcVb	+	+	+	+	+	+	+	+	+	+
IlEd	+	–	+	+	–	+	–	+	+	+
CuPf	+	–	+	–	–	+	–	+	+	+
CtGf	+	–	–	–	–	–	–	–	–	–
PaFw	+	+	+	–	+	+	+	+	+	+
PoLp	+	+	+	–	+	–	+	–	+	+

CeDc	+	_	_	+	_	+	+	+	_	_
PoMs	_	_	+	+	_	_	_	+	+	_
BrBb	+	_	_	+	_	+	+	+	+	+
PoBc	+	_	_	+	_	+	+	_	+	+
SpVs	+	+	_	+	+	+	+	+	+	+
SoSd	+	+	_	_	+	+	_	+	_	_
OnBb	+	_	_	_	_	_	_	_	_	_
CtBv	_	_	_	_	_	+	_	_	+	_
CtLb	+	_	_	_	_	+	_	_	+	_
BpLb	_	+	+	_	+	_	_	_	+	_

[a] Sample designation are as in Table 1; P1: primer set1; P2: primer set2; P3: primer set3; P4: primer set4; P5: primer set5; P6: primer set6; P7: primer set7; P8: primer set8; P9: primer set9; P10: primer set10

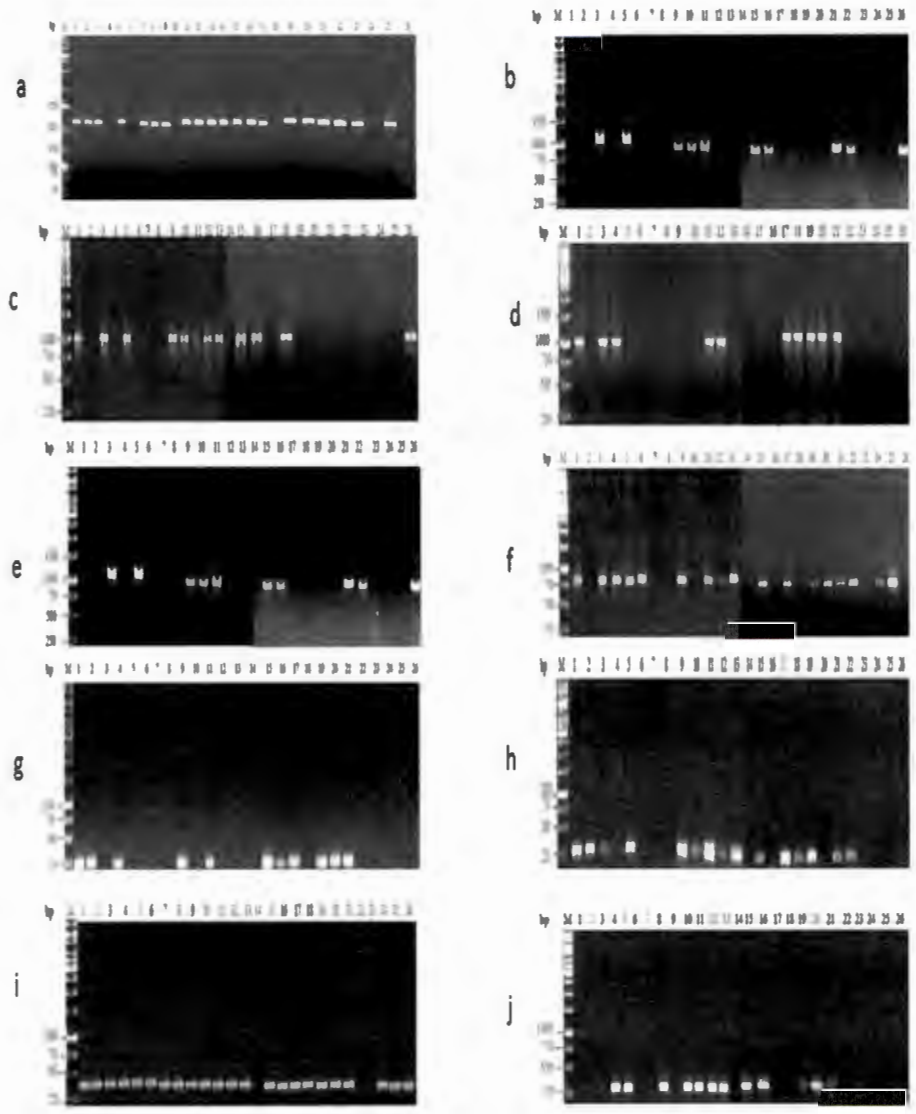

Figure 6.2: Comparison of the primers designed based on the amplification efficiency of macergens detected. a: primer set 1; b: primer set 2; c: primer set 3; d: primer set 4; e: primer set 5; f: primer set 6: g: primer set 7; h: primer set 8; i: primer set 9; j: primer set 10.

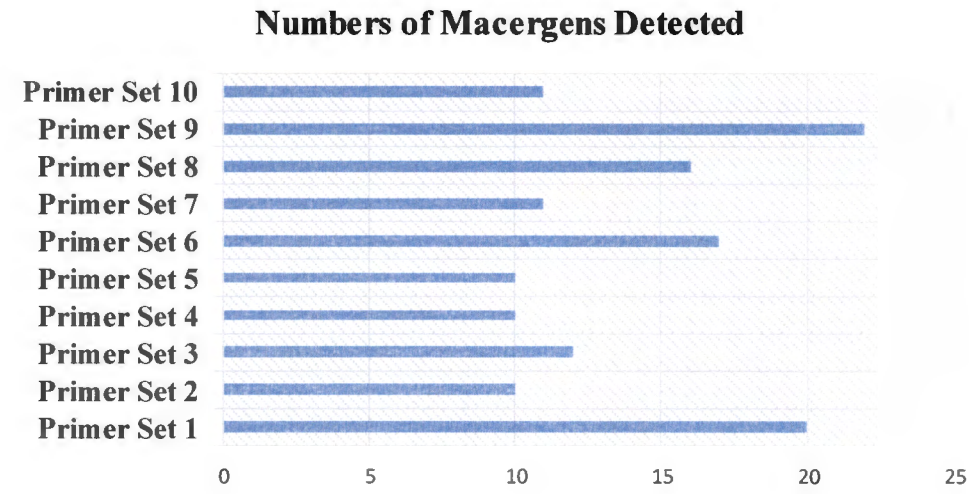

Figure 6.3: Amplification efficiency of the primers

6.3.3 Sequencing

The sequences of the 16S rDNA determined were assigned accession numbers. Some of the sequences were exactly the same and thus accession numbers were only assigned to their representatives. Thus the following in parenthesis are the accession numbers of the sequences obtained in this study. Primer set 1 (KJ784522-KJ784534); primer set 2 and 5 (KP114439-KP114448); primer set 3 (KM924134-KM924145); primer set 4 (KP792433-KP792441); primer set 6 (KU143750-KU143763); primer set 7 and 10 (KP792442-KP792449), primer set 8 (KP899920-KP899932); primer set 9 (KU143764-KU143773).

6.4 Discussion

All the ten designed primers satisfied the criteria for good primers. This enhanced their specific amplification, which leads to high yield of PCR product. Their lengths fall within the standard range (18-30 bp) for good primers of Innis and Gelfand (2012), which resulted in successful PCR reactions of the primers. Their lengths are long enough to enhance adequate specificity according to Ye et al. (2012). Also, they are neither too short nor too long to easily bind to the template at the annealing temperature (Wu et al., 2004). The GC contents of all the primers also propel good T_m values for the oligonucleotides. These attributes ensure the ability of one of the double DNA to dissociate into single stranded DNA enhancing duplex stability. The inability to form primary secondary structure increases the amplification efficiency by the availability of the primer to the amplification reaction, this is in line with (Vallone and Butler (2004)). The *in silico* and empirical applications of the primers revealed that the primers annealed to the pectinolytic target regions only, hence showing a good specificity for macergens. This is as a result of the above primer characteristics that gave a successful outcome of the PCR experiments (Chuang et al., 2013). Moreover, this result gave a more reliable species-specific primers that are highly specific for

pectinolytic bacteria without having secondary structure, mis-priming and primer dimer. Among all the designed primers the primer set 9 showed the best amplification efficiency in all the samples analysed for the macergens.

6.5 Conclusion

The ten primers designed promptly and efficiently optimized pectinolytic gene of macergens in vegetable samples. Conclusively, the primer properties such as GC content, primer length and length difference of a primer pair, PCR product size, stable secondary structures and primer T_m are essential for a successful PCR reaction. Hence, the amplification efficiency of the designed primers was high due to their primer properties. These primers can be employed in PCR assay for early detection of macergens from food samples.

CHAPTER 7

GENERAL CONCLUSION

The ten primers designed were able to amplify the 16S rDNA sequence of macergens for prompt and efficient macergens detection in vegetable samples. They all satisfied the conditions for good primers as well as the in silico and empirical specificity test for pectinolytic gene. However, best specificity was achieved with primer set 9, which produced amplicons of expected sizes upon PCR analysis and were optimal for heterogeneity of macergens. Since these primers were designed around bacterial species, we inferred that they must be specific for a certain amount of bacteria. Primer properties are essential for a successful PCR reaction. This method offers advantages over classical methods of detection, in the sense that the entire assay is fast, reliable, cost effective and no taxonomist is required before the identification is done.

Macergens could be recognized based on their 16S rRNA sequence configuration. There is variability in the cumulative number of base pairs of the 16S rRNA sequence in addition to the G+C and A+T content. The identical pairs exhibited among the macergens from nucleotide paired frequencies were 541. The proportion of the transitional to transversional pairs was 4.25. Analysis of the maximum likelihood of 24 diverse nucleotide substitution configurations to reconstruct the phylogenetic tree, demonstrated that T92+G had the minimum BIC and AIC scores, subsequently it can be considered as the best model for the present data set. The phylogenetic tree which was constructed indicates a similarity index that is greater than 98% in most of the species in these macergens. Fifteen probable novel macergens namely; *Cedecea* sp. (KM924136), *Citrobacter* sp. (KM924138), *Pectobacterium* sp. (KM924140), *Rahnella* sp. (KM924143), *Lelliottia* sp. (KM924144), *Tatumella* sp. (KM924145), *Cronobacter malonaticus* (KP792435), *Enterobacter* sp. (KP792439),

Citrobacter sp. (KP114441), *Pantoea* sp. (KP114444), *Pseudomonas* sp (KP114445), *Lelliottia* sp. (KP114447), *Tatumella* sp. (KP114448), *Enterobacter* sp. (KJ784522), *Raoutella* sp. (KJ784524), *Erwinia* sp. (KJ784532) and *Citrobacter* sp. (KJ784529) *Enterobacter* sp. (WcVb1), *Raoutella* sp. (BeBb1), *Erwinia* sp. (PoLp1) and *Citrobacter* sp. (IlEd1) with distinct signatures were obtained. This study revealed that macergens are heterogeneous because they cut across different species, but in spite of their heterogeneity, phylogenetic analyses revealed their similarities and evolutionary trends. In addition, structurally all the macergens detected possessed the ability to code for Arginine, an amino acid which is responsible for their irrevocable virulent behaviour but usually absent in non-virulent bacteria.

The understanding of existing natural diversity and relationships among macergens revealed by the detection method embarked in this study will help plant farmers and disease control agents to ensure quick and effective disease diagnosis and management, and to avoid unnecessary destruction of economically valuable crops. This can be employed in analyzing and monitoring plant materials for macergen invasion at the quarantine section of the Agricultural sector of the country before importation and exportation of these plants. It has also provided the knowledge about host resistance management strategies for plant breeders.

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