ASSESMENT OF GENES THAT PLAY ROLE IN PHENOLIC COMPOUND DEGRADATION IN OLIVE ORCHARD MICROBIOME

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

In Molecular Biology and Genetics

by

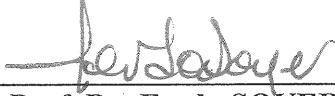
Ertuğrul ACAR

October, 2019

İZMİR

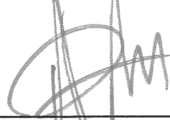
We approve the thesis of **Ertuğrul ACAR**

Examining Committee Members:



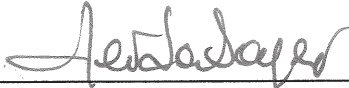
Assoc. Prof. Dr. Ferda SOYER DÖNMEZ
Department of Molecular Biology and Genetics,
İzmir Institute of Technology

Assoc. Prof. Dr. Özden YALÇIN ÖZUYSAL
Department of Molecular Biology and Genetics,
İzmir Institute of Technology

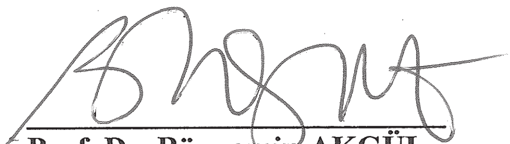


Asst. Prof. Dr. Ayşe Banu DEMIR
Faculty of Medicine
İzmir University of Economics

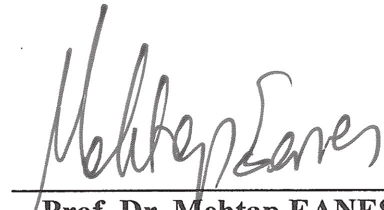
10 October 2019



Assoc. Prof. Dr. Ferda SOYER
Supervisor, Department of Molecular
Biology and Genetics
İzmir Institute of Technology



Prof. Dr. Bünyamin AKGÜL
Head of Department of Molecular
Biology and Genetics



Prof. Dr. Mehtap EANES
Dean of the Graduate School of
Engineering and Sciences

ACKNOWLEDGMENTS

I would like to thank my supervisor Assoc Prof. Dr. Ferda SOYER DÖNMEZ for her guidance, understanding, and support throughout this study.

I would also like to express my gratitude to Assoc. Prof. Özden YALÇIN ÖZUYSAL and Asst. Prof. Ayşe Banu DEMİR for being the committee members for my thesis.

My special thanks go to my dear friends Özgün Öykü ÖZDEMİR and Deniz KEMAN who worked with me in the Molecular Bacteriology Laboratory for their sincere help offers, friendships, and moral support.

I would like to express my grateful thanks to Elif GEYİK from Genometri Biotechnology Research and Development Company, İzmir, Turkey for valuable support and help during my thesis study.

I should also state my thanks to Gökhan HARDAL and Mehmet Salih YILDIZ for their priceless friendships through years and for their supports and motivations.

Finally, I would like to thank my family members Fatma ACAR, Elif Fulya ERBOSNALI, Funda ŞAHİN for their unconditional love, belief, support, and enormous encouragement.

ABSTRACT

ASSESMENT OF GENES THAT PLAY ROLE IN PHENOLIC COMPOUND DEGRADATION IN OLIVE ORCHARD MICROBIOME

The olive tree (*Olea Europea L.*) is one of the most important fruit trees in Mediterranean countries. Its products, olive oil and table olives, are important components of the Mediterranean diet and widely consumed all around the World. Olives and virgin oil provide a rich source for phenolic compounds. The plant phenolics are secondary metabolites, and possesses several common biological and chemical properties. In this study, microorganisms were collected from soil, olive leaves, fruits, and Olive fruit fly larva and Olive mill wastewater (OMWW). They were characterized by 16S rRNA analysis. The microorganisms that were tolerant to phenolic compounds were selected in order to seek which genes were associated with the phenolic compound degradation. The genes related to the degradation of the selected organisms were identified by Sanger Sequencing and the level of phenol-degrading gene expression were aimed to be compared by using qPCR and Droplet Digital PCR (ddPCR).

Microorganisms which degrade phenolic compounds can be harnessed for the purpose of bioremediation. However, the number of defined phenolic compound degrading microorganisms is still low in the literature. For this reason, many different microorganisms were used at the same time for bioremediation. Investigation of olive orchard microorganisms and phenolic-degrading genes might benefit bioremediation in the future.

In this study, 8 different bacterial strains were identified and characterized from olive orchards. After that, their phenol hydroxylase and catechol 1,2 dioxygenase genes tried to be sequenced with primers designed by using of reference strains in NCBI database.

ÖZET

ZEYTİNLİK MİKROBİYOMUNDA FENOLİK MADDE İNDİRGENMESİNDE ROL ALAN GENLERİN DEĞERLENDİRİLMESİ

Zeytin ağacı (Olea Europea L.) Akdeniz ülkelerindeki en önemli meyve ağaçlarından biridir. Bu ağaçların ürünleri, zeytin yağı ve sofralık zeytin Akdeniz beslenme biçimin en önemli bileşenleri oluşturur ve dünya çapında tüketilir. Zeytin ve zeytin yağı fenolik bileşikler adına zengin bir kaynak sunar. Bitkisel fenolik bileşikler ikincil metabolitler olarak birtakım ortak biyolojik ve kimyasal özellikle barındırırlar. Topraktan, zeytin yapraklarından, zeytinlerden, Akdeniz meyve sineğinin larvasından ve zeytin fabrikası atık suyundan toplanan mikroorganizmalar, 16S rRNA analizi ile karakterize edilmiştir. Fenolik bileşiklerin yıkımıyla ilgili genlerin izolasyonu için fenolik bileşiklere toleransı olanlar seçilmiştir. Seçilen organizmaların yıkımla ilgili genleri sekanslanarak saptanmıştır ve gen ekspresyonu seviyesi qPCR ve Droplet Digital PCR (ddPCR) ile karşılaştırılması amaçlanmıştır.

Fenolik bileşikleri yıkabilen mikroorganizmalar çevresel iyileştirme sürecine kullanılabilir. Ancak, farklı çeşitlilikte fenolik bileşikleri yıkan mikroorganizmaların sayısı hala az oldiğu için birden fazla mikroorganizma çevresel iyileştirme çalışmalarında aynı anda kullanılmaktadır. Zeytinliklerdeki mikroorganizmaların araştırılması çevresel iyileştirme için potansiyel vaat etmektedir.

Bu çalışmada, zeytin bahçelerinden izole edilen 8 farklı bakteri suşu tanımlanmış ve karakterize edilmiştir. Fenol hidroksilaz ve katekol 1,2 dioksijenaz genleri NCBI veri tabanında referans suşlar kullanılarak tasarlanan primerler kullanılarak sekanslanmaya çalışıldı.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES x

ABBREVIATIONS xii

CHAPTER 1. LITERATURE REVIEW 1
1.1. Phenolic Compounds 1
1.1.1. Phenolic compounds other than phenolic acids 1
1.1.2. Phenolic acids 4
1.1.3. The Beneficial Properties of Phenolic Compounds 5
1.2. Olive Orchard and Its Phenolic Properties 7
1.2.1. Phenolic Compounds in Olive Fruits 8
1.2.2. Phenolic Compounds in Olive Oil 9
1.2.3. Phenolic Compounds in Olive Mill Wastewater 9
1.3. Microorganisms that Degrade Phenolic Compounds 10
1.4. The Genes Responsible for Phenolic Compound Degradation 12
1.4.1. Phenol Hydroxylase 13
1.4.2. Catechol Dioxygenases 15
1.5. Olive Mill Wastewater (OMW) 16
1.6. Introduction 17

CHAPTER 2. MATERIALS AND METHODS 19
2.1. Sample Collection and Preparation 19
2.2. Preparation of MMSM 19
2.3. Isolation and Selection of the Samples 20
2.4. Morphological Characteristic of Selected Bacterial Strains 21
2.5. Phenolic Compound Tolerance Test 22
2.6. DNA Extraction of Isolated Strains by Using CTAB Method 22
2.7. PCR Amplification of Partial 16S rRNA Gene 23
2.8. Purification of amplified 16S rRNA gene of Isolated Strains 24

2.9. 16S rRNA Sequencing and Analysis ... 24
2.10. Primer design for the amplification of genes relating to phenolic compound degradation ... 25
2.11. RNA Isolation and cDNA Synthesis of Selected Strains ... 25

CHAPTER 3. RESULTS AND DISCUSSION ... 27
3.1. Isolation And Identification Of The Microorganisms From Olive Orchard ... 27
3.2. The Assessment Of The Genes Responsible For Phenolic Compound Degradation ... 37

CHAPTER 4. CONCLUSION ... 44

REFERENCES ... 46

APPENDIX-A Phylogenetic Tree Of 16S rRNA Partial Sequence Of Isolated Strains ... 56

LIST OF FIGURES

Figure **Page**

Figure 1. The general chemical structures of phenolic acid group…..........…………4

Figure 2. The most common phenolic compounds in the olive orchard……..………..8

Figure 3. Ortho- and meta-cleavage of the catechol…………...……………………..16

Figure 4. Schematic representation of the phenolic compound tolerance test………...22

Figure 5. Agarose gel analysis of PCR amplified partial 16S rRNA gene region of the isolated strains……………………......……………...…………...30

Figure 6. Phylogenetic tree of 16S rRNA partial sequence of isolated strains............32

Figure 7. Design of the degenerate primer pairs of Cat12-F and Cat12-R…...............38

Figure 8. PCR amplification of phenol hydroxylase gene from the genomic DNA of selected strains…………......…………..………...…………………39

Figure 9. PCR amplification of phenol hydroxylase gene from the cDNA of selected strains…...………….…………………………………….……..…..40

Figure 10. PCR amplification of catechol 1,2-dioxygenase gene from the cDNA of selected strains……………………......……………………………41

Figure 11. Electropharogram of the catechol 1,2-dioxygenase gene sequences of the isolates…..………………..……………………………………......42

Figure 12. Multiple sequences alignment of the catechol 1,2-dioxygenase genes of selected strains…………….……….…………………………..…42

Figure 13. Phylogenetic tree of 16S rRNA partial sequence of Larvae1 strain…...…56

Figure 14. Phylogenetic tree of 16S rRNA partial sequence of Larvae7 strain.......…56

Figure 15. Phylogenetic tree of 16S rRNA partial sequence of Larvae8 strain………57

Figure 16. Phylogenetic tree of 16S rRNA partial sequence of Larvae9 strain………57

Figure 17. Phylogenetic tree of 16S rRNA partial sequence of Larvae11 strain…...…57

Figure **Page**

Figure 18. Phylogenetic tree of 16S rRNA partial sequence of Larvae14 strain……58

Figure 19. Phylogenetic tree of 16S rRNA partial sequence of OMWW1 strain…......58

Figure 20. Phylogenetic tree of 16S rRNA partial sequence of OMWW7 strain…....58

Figure 21. Phylogenetic tree of 16S rRNA partial sequence of OMWW8 strain…....59

Figure 22. Phylogenetic tree of 16S rRNA partial sequence of Soil1 strain…...……59

Figure 23. Phylogenetic tree of 16S rRNA partial sequence of Soil3 strain...………59

Figure 24. Phylogenetic tree of 16S rRNA partial sequence of Soil4 strain...………60

Figure 25. Phylogenetic tree of 16S rRNA partial sequence of Soil5 strain……...…60

Figure 26. Phylogenetic tree of 16S rRNA partial sequence of Soil7 strain...………60

Figure 27. Phylogenetic tree of 16S rRNA partial sequence of Soil8 strain……...…61

Figure 28. Phylogenetic tree of 16S rRNA partial sequence of Soil12 strain...……...61

Figure 29. Phylogenetic tree of 16S rRNA partial sequence of Soil13 strain…….....61

Figure 30. Phylogenetic tree of 16S rRNA partial sequence of Soil15 strain.......….62

Figure 31. Phylogenetic tree of 16S rRNA partial sequence of Soil16 strain……….62

Figure 32. Phylogenetic tree of 16S rRNA partial sequence of Soil19 strain……......62

Figure 33. Phylogenetic tree of 16S rRNA partial sequence of Soil24 strain...……...63

Figure 34. Phylogenetic tree of 16S rRNA partial sequence of Soil28 strain........….63

Figure 35. Phylogenetic tree of 16S rRNA partial sequence of Soil29 strain………..63

Figure 36. Phylogenetic tree of 16S rRNA partial sequence of Soil30 strain………64

LIST OF TABLES

Table **Page**

Table 1. The classification of the phenolic compounds according to their carbon number……..……………………………………………………...…..2

Table 2. The list of the phenolic compound degrading microorganisms that are isolated from contaminated area……………...………………..……...14

Table 3. The composition of Modified Mineral Salt Medium……………...………..20

Table 4. The list of the primer pairs used in this study……………………………….26

Table 5. The morphological and biochemical characterization of phenolic compounds degrading isolates……………………………………….……29

Table 6. The list of species which indicated highest homology with our isolated strain…………….…………………………………………………..31

LIST OF ABBREVIATIONS

3-HPAA	3-Hydroxyphenylacetic Acid
BLAST	Basic Local Alignment Search Tool
CA	Caffeic Acids
cDNA	Complementary DNA
CT	Condense Tannins
CTAB	Cetyl Trimethylammonium Bromide
ddPCR	Droplet Digital PCR
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ETC	Electron Transport Chain
FA	Ferulic Acids
g/L	Gram\Liter
GA	Gallic Acid
HCAs	Hydroxycinnamic Acids
HT	Hydrosoluble Tannins
LAB	Lactic Acid Bacteria
MAR	Multi-Antibiotic-Resistant
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MMSM	Modified Mineral Salt Medium
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
OD	Optical Density
OMWW	Olive Mill Wastewater
OWW	Olive Washing Water
PA	p-Coumaric Acids

PAH	Polycyclic Aromatic Carbon
PCR	Polymerase Chain Reaction
PCR-TGGE	Polymerase Chain Reaction-Temperature Gradient Gel Electrophoresis
p-HBA	p-Hydroxybenzoic Acid
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SA	Salicylic Acid
SDS	Sodium Dodecyl Sulfate
TE	Tris-EDTA
TMAC	Tetramethyl Ammonium Chloride
UNIPROT	Universal Protein Resource
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV filter	Ultraviolet Filter
VA	Vanillic Acid

CHAPTER 1

LITERATURE REVIEW

1.1. Phenolic Compounds

Phenolic compounds are defined chemically as compounds that have one or more aromatic ring structures with the attachments of hydroxyl groups. These compounds are also naturally occurring substances and various types of phenolic compounds are produced by the plant as secondary metabolites. Secondary metabolites can be found in all components of the plants like leaves, flowers, and fruits [1]. Phenolic compounds are the most abundant secondary metabolites in the plant and there are many different using terms in the literature that refer to these compounds such as phenols, phenolics, phenylpropanoids, plant phenolics, polyphenols, and biophenols. The terms 'Plant phenolics and polyphenols' are usually used to refer to the natural secondary metabolites[2].

Phenolic compounds are classified in different ways because they cover a very large and diverse group of organic compounds[1–3]. The first classification method has been developed and used for the phenolic compound by Swain and Bate-Smith in 1962 [4]. They categorized the phenolic compounds by looking at their prevalence and classify in common and less common. Later, Harborne and Simmonds (1964) classified the phenolic compounds according to the number of constitutive carbon atoms in the molecules (Table 1). This classification is the simplest and the most suitable one for the plant phenolics due to a large number of compounds (There are thousands isolated and identified chemical compounds)[5]. Moreover, phenolic compounds were classified into three groups by Ribereau-Gayon in 1972. These groups are widely distributed phenols (1), less widely distributed phenols (2) and phenolic compounds as the polymers (3) [6].

1.1.1.Phenolic compounds other than phenolic acids

Phenols are organic compounds that have a benzene structure consisting of a hydroxyl functional group. Simple phenolic compounds are the phenols that can have one

or more substituents on different carbon atoms. *Ortho*, *Meta* and *Para* addition of different radicals on benzene rings can form a variety number of simple phenolics. *Ortho*,

Table 1. The classification of the phenolic compounds according to their carbon number [4].

Structure	Class
C_6	Simple phenolics
C_6-C_1	Phenolic acids and related compounds
C_6-C_2	Acetophenones and phenylacetic acids
C_6-C_3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C_6-C_3	Coumarins, isocoumarins and chromones
C_{15}	Chalcones, aurones, dihydrochalcones
C_{15}	Flavans
C_{15}	Flavones
C_{15}	Flavanones
C_{15}	Flavanonols
C_{15}	Anthocyanidins
C_{15}	Anthocyanins
C_{30}	Biflavonyls
C_6-C_1-C_6, C_6-C_2-C_6	Benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	Quinones
C_{18}	Betacyanins
Lignans, neolignans	Dimers or oligomers
Lignin	polymers
Tannins	Oligomers or polymers
Phlobaphenes	Polymers

meta, and *para* refers to a substituent addition on a benzene ring at the positions 1,2-, 1,3- and 1,4-, respectively. Here, the hydroxyl group on the benzene ring is the directing group in structure. For instance, Catechol (1,2-dihydroxybenzene) is an ortho-directed simple phenolics and phloroglucinol (1,3,5-trihydroxy benzene) is a meta directed simple phenolics [7].

Flavonoids are another important subgroup of polyphenolic secondary metabolites found in higher vascular plants, particularly in the flowers, leaves, and barks. They are especially abundant in the fruits, grains, and nuts. They play important roles in the biological activities of plants like the colors of fruits and flowers, the aroma of fruits, the defense mechanisms, protection from biotic and abiotic stress and as a unique UV filter. Flavonoids have basically C6-C3-C6 structural skeleton that consists of two aromatic C6 rings and a heterocyclic ring with one oxygen atom and classified into six subgroups [5,6,8,9].

Coumarins are a group of secondary metabolites widely distributed in nature. They can be found mostly in fruits and flowers, particularly seeds, roots, leaves and stems in higher plants [10]. They play an important role in the defense mechanisms of the plants against the herbivore and microorganisms. Coumarin and its derivatives are usually found free forms in the plants as polar structures. Their simplicity and versatility make then interesting compounds for a wide range of application. There are many types of research that indicate the coumarin compounds are one of the best candidates as drugs due to strong pharmacological activity, low toxicity, lower side effects, higher bioavailability and antioxidant, antimicrobial activity [10,11].

Tannins are one of the most important secondary metabolites are defined as high molecule weight polyphenols varied in the range between 500 Da and 3000 Da. They are found in the leaves, the bark, the wood of plants and they can bind to the proteins to form a tannin-protein complex. These complexes associated with the plant defense mechanisms can be both soluble and insoluble [12]. Tannins are separated into two main groups as Hydrosoluble Tannins (HT) and Condense Tannins (CT). Hydrosoluble tannins contain a carbohydrate group, generally D-glucose. They are found in the plants with lower concentrations than Condense Tannins. Condense tannins are the most common types of tannins in nature. Tannins are distinguishable features when they are compared other polyphenols. Having antioxidant activity, being a larger molecule and being able to bind to proteins and pigments are several of these different properties [12,13].

Lignins are the second most abundant biopolymers after cellulose on the earth. They are one of the larger polyphenols which play an important role in supporting and conducting tissue of the plants. Also, lignins provide hydrophobicity which promotes water transport through the vascular tissue. Their chemical complexity and lack of regulatory provide rigidity to the cell wall. Thus, they act as a physical barrier against other organisms such as insects and fungi. Lignans are another type of polyphenol

molecule that contains two phenylpropanoid units and forms an 18-carbon skeleton. Also, different functional groups can be added to the lignans' structures by the plants. Lignans are responsible for providing rigidity, strength and water impermeability for cell membranes [2,3].

1.1.2. Phenolic acids

Phenolic acids are one of the largest subclasses of secondary metabolites widely distributed throughout the plant kingdom. Phenolic acids also called phenol carboxylic acids in chemistry are phenols that have a phenolic ring and one or more organic carboxylic acid as a functional group. They are usually present conjugated with structural components of the plant like lignin, larger organic molecules like polyphenols (flavonoids), or smaller organic molecules like sugar or organic acids [14]. The phenolic acids are classified into three groups depending on the carbon atoms of the lateral chain that attached to the phenolic ring are C6-C3, C6-C2, and C6-C1 compounds. However, phenolic acids are assumed that separated into two groups, because C6-C3 (derived from the hydroxycinnamic acid) and C6-C1 (compounds with a hydroxybenzoic structure) exist in all plant and plant-derived foods such as fruits, vegetables, and grains (Figure 1) [15,16]. Approximately one-third of the phenolic compounds in the human's diet is provided by phenolic acids. Phenolic acids are universally distributed in plants. A great number of studies relating to biological, chemical, agricultural and medical aspects of these compounds [6].

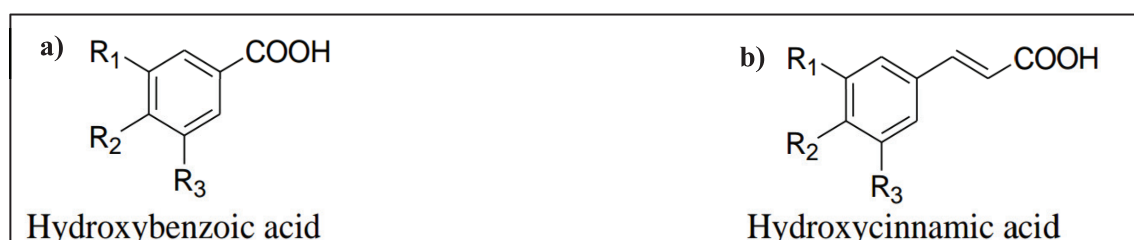


Figure 1. The general chemical structures of phenolic acid groups. a) Hydroxybenzoic acids and b) Hydroxycinnamic acids.

The hydroxybenzoic acids and derivatives have a general structure of C6-C1 that are characterized by the presence of carboxylic group on phenolic rings. There are four commonly occur acids: p-hydroxybenzoic acid (p-HBA), salicylic acid (SA), gallic acid (GA) and vanillic acid (VA). Variations in the structures hydroxybenzoic acids can be formed by hydroxylations and methylations of the aromatic ring in different positions. These phenolic acids usually present in soluble form and conjugate with sugars or organic acids. Also, they can be attached to cell wall fractions such as lignin [17,18].

The hydroxycinnamic acids (HCAs) are an important class of phenolic compounds are synthesized by the phenylpropanoid pathway. These compounds have C6-C3 general structure and p-coumaric acids (PA), caffeic acids (CA), ferulic acids (FA), sinapic acids(SIA), and their esterified/ etherified conjugates such as chlorogenic acids (figure-x). Chemical structures of these compounds are indicated in Figure. They are rarely present in free form and mostly associated with the other molecules to form glycosylate derivatives and esterified hydroxy acids like shikimic acids and tartaric acids. They are present in all parts of fruits and vegetables although the highest concentrations are found in the outer part of ripe fruits [19–21].

1.1.3. The Beneficial Properties of Phenolic Compounds

Phenolic compounds are involved in many important functions for plants. Phenolic compounds have hydroxyl and carbonyl groups differs from molecule to molecule can bind particularly heavy metals like iron and copper. They can chelate metallic ions and suppress the involvement of these ions in Fenton reactions. On the other hand, free radical scavenging ability of the phenolic compounds provides antioxidant activity to the plants. Previous studies indicate that there is a correlation between the number and the position of hydroxyl groups on the rings and the degree of the antioxidant activity [22]. Plants are a rich source of nutrients for different organisms such as bacteria, fungi, and insects. These organisms are a great threat to the plants. Hence, various defensive mechanisms against harmful effects of these organisms like chemical, structural and protein-based precautions are developed by plants. As chemical precautions, the plants produce a large variety of phenolic compounds (Phenolic acids and polyphenols, etc.) During the threatening, the phenolic compounds are rapidly

accumulated at the infection site to stop the harmful organisms before they cause great damage [2]. Phenolic compounds in different parts of the plant (leaves and fruits) provide unique taste, flavor, and health-promoting properties [23]. For this reason, increasing the phenolic composition in these plants can improve their quality. They are also important components for determination of the fruit and flower colors in plants. They play a crucial role in the regulation of plants growth and reproduction as chemical messengers. They are generated as a reaction to environmental factors such as light, chilling, and pollution[14].

Besides the fact that phenolic compounds have important functions for plants, they exhibit various biological properties that affect human health beneficially. Previous studies reported that phenolic compounds are linked to antioxidant, anti-inflammatory, anti-allergic, anti-carcinogenic, and antimicrobial activities. Phenolic compounds as natural products can be considered better antioxidants than synthetic alternatives. Thus, the past decade has seen the rapid development of the identification of novel antioxidants from a natural resource. Moreover, previous research has indicated that phenolic contents have a positive impact on antioxidant activity. Several studies have revealed that free radicals cause many diseases such as cancer, diabetes, neurodegenerative, aging-related and cardiovascular diseases[3,10,24–26].

Multi-antibiotic-resistant (MAR) pathogens related infections are the major threat for worldwide health concern. Majority of the antibiotics used conventionally have become inactive because of the drug resistance caused molecular evolution. Hence, it has revealed that an urgent necessity to develop new classes of antimicrobial agents to control resistant bacteria. The antimicrobial properties of the plant have been used in traditional medicine to fight fever and overcome infections. The chemical structure of phenolic compounds, especially, the position of substitution and the type of radical group on the benzene ring and the length of the saturated chain [27,28].

Taguri et *al.* (2006) analyzed the relationship between the antimicrobial activity of 26 different phenolic compounds and their structures by using 26 species. Although many kinds of research have been carried out on the antimicrobial activity of phenolic compounds, the results were not able to compare with each other due to the application of different methods and using various bacterial strains. For this reason, they used both a wide variety of polyphenols and many bacteria to demonstrate the antibacterial profile by using the same minimum inhibitory concentration (MIC) method. The results revealed that there was a relatively simple structure– antimicrobial activity relationship and

pyrogallol derivative compounds were more potent antimicrobial agents than others like catechol [29].

Most studies in the field of natural antimicrobial agents have focused on phenolic acids and their impact on several crucial pathogens such as *P. aeruginosa*, *S. aureus, Listeria monocytogenes, and Enterococcus faecalis*. In 2013, Borges et *al.* published an article in which they described the antibacterial activity and mechanism of action of ferulic and gallic acids on selected pathogens (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Listeria monocytogenes).* MIC and Minimum bactericidal concentration (MBC) were examined for four strains [30].

In contrast, very few studies have investigated the antimicrobial activity of phenolic compounds found in the olive orchard on bacterial strains. To determine the effects on several microorganisms related with human intestinal and respiratory tract infections: (1) *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* (gram positive), (2) *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (gram negative) and (3) *Candida albicans* and *Cryptococcus neoformans* (fungi) [31]. Pereira et al (2007) extracted phenolic compounds from olive leaves. They identified and quantified seven different phenolic compounds present in olive leaves and determined antimicrobial activity of these extracts on selected strains. The results indicated that olive leaves extracts have antimicrobial and antifungal activity even at low concentration [31].

1.2. Olive Orchard and Its Phenolic Properties

Olive trees (*Olea europaea L.*) are one of the oldest and the most valuable fruit trees throughout the history of humanity. The new studies indicate that olive trees were among the oldest cultivated trees in the world and they were domesticated firstly in the Asia Minor that today includes Israel, Palestine, Jordan, Lebanon and Syria and then spread the rest of the Mediterranean lands between 8000 and 6000 years ago. Nowadays, The Mediterranean region provides almost 98% of olive tree plantation. In mythology, Athens and Romans believed that the olive was a gift for the Greeks [32,33].

Research on plant phenolic compounds has a long tradition due to the various function of these compounds both the plants (antioxidant activity, antimicrobial activity, fruit, and flower characteristic like color, aroma etc.) and humans (beneficial effect on

human health anti-inflammatory, antioxidative, anticarcinogenic, antimicrobial and protection against chronic diseases) [34–36]. In the past several decades, a large number of existing studies in the broader literature have examined the relationship between the consumption as different products as olive oil and table olives and their functions on a variety of diseases. Because of the Mediterranean diet is based on olive oil, it is one of the reasons why some cancers and chronicle heart diseases are lower in countries where they are applied than in other regions [37].

Olives contain a wide range of secondary metabolites. Three phenolic compounds in olive oil are found in more high concentration as compared to other phenolic compounds. These phenolic compounds are glycoside oleuropein, hydroxytyrosol, and tyrosol (Figure 2) [38] which are structurally very similar compounds [37]. In addition to these compounds, many different types of phenolic compounds have been identified. Also, the phenolic compositions in different parts of an olive tree like fruits, leaves and seeds and olive oil and olive mill wastewater may show different characteristics [32,35,39].

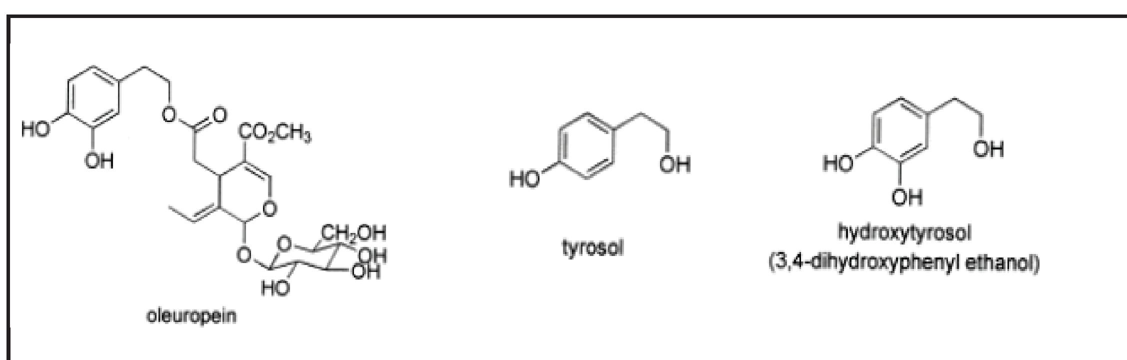


Figure 2. The most common phenolic compounds in the olive orchard[38].

1.2.1. Phenolic Compounds in Olive Fruits

The list of phenolic compounds in olives is constantly updated with the increase of the analytical methods. Secoirioids derive from oleosides are the most common compounds in olive fruits. Oleuropein, the ester of elonolic acids with hydroxytyrosol, is the most abundant secoirioids which gives the bitterness to the olive fruits. Demethyloleuropein and ligstroside are the other main secoirioids [31,33]. Moreover, several various oleuropein derivatives such as oleuropein aglycon, hydroxytyrosol, and tyrosol glucosides are found in olive fruits. In addition to the secoirioids, they contain phenolic

acids as benzoic, cinnamic and phenylacetic acid derivatives. Vanillic acid, sinapic acid, syringic acid, coumaric acid, and caffeic acids are some of these phenolic acids [39]. The level of them in olives vary according to the type and the growth area of olive fruits. Luteolin-7-glycoside, apigenin, and luteolin are the main phenolic compounds of olives as flavonoids. There is a relationship between the concentration level of these flavonoids and fruit maturation. Tyrosol and hydroxytyrosol are the second most common phenolic compounds in olive fruits. They are also present as free alcohols in different parts of olives like the peel, the pulp, and the seeds.

1.2.2. Phenolic Compounds in Olive Oil

The phenolic compound content of olive oil is different from olive fruit due to the process of olive oil production. Tyrosol, hydroxytyrosol, and their derivatives are the major phenolic compounds in olive oils. Several phenolic compounds in olive oil include vanillic acid, syringic acid, p-coumaric acid, o-coumaric acid, gallic acid, caffeic acid, 4-acetoxy-ethyl-1,2-dihydroxybenzene, 1-acetoxy-pinoresinol, apigenin, oleuropein and elenolic acids. The content of the flavonoids of olive oil may vary due to the type of extraction system and the process condition. Phenolic compounds in olive oils are important for the stability and the taste. Although high phenolic content provides a long shelf life for the oil, this may cause negative effects the aroma of the oil like the bitter taste [37,40].

1.2.3. Phenolic Compounds in Olive Mill Wastewater

The phenolic composition of the OMWW may also be variable because of the effects of many external factors such as the type of olives, the geographical region that they grow, the extraction methods, and the climate of the area. During the olive oil process, the release of the enzymes from the fruit's cells changes the profile of phenolic compounds in OMWW [41]. Hydroxylated and methoxylated phenolic acids are mostly found in the phenolic profile of OMWW. Also, the phenolic compounds with higher molecular weight increase due to the oxidation of these monomeric compounds. For

instance, while the initial color of OMWW is reddish, the color then turns the black because of the polymerized tannins under sunlight in evaporation pools [42,43]. Other phenolic compounds found in olive and olive oil are found in wastewater such as oleuropein, tyrosol, caffeic acid, gallic acid, and etc.

1.3.Microorganisms that Degrade Phenolic Compounds

Phenolic compounds are found widespread in nature. They can be found in environmental pollutants as well as many industrial wastes of oil, plastics, steel, pharmaceutical, petroleum refineries, and phenolic resin industries. Numerous microorganisms utilize phenol and phenol derivatives as a sole carbon in their energy metabolism. Phenolic compounds have been determined as highly toxic to most of the organisms both prokaryotic and eukaryotic. Therefore, many methods have been developed to remove phenol from industrial wastewater. These methods include activated carbon extraction, chemical oxidation, enzymatic treatment and degradation by the microorganism. Among all these methods, biological degradation was superior due to complete degradation of phenol [44].

The mesophilic aerobic microorganisms, mainly the genus *Pseudomonas,* have the ability to degrade phenol. Thermophilic bacteria, *Bacilli*, has been reported to harness phenol as an energy source and can degrade phenol at 60-65ºC. A cold-tolerant bacterium, *Pseudomonas putida,* has been reported to degrade phenol at 10ºC and play role in the degradation of phenols in low-temperature industrial wastewater in which mesophilic phenol-degrading bacteria have a limited activity [45].

The aerobic degradation pathways of the phenol include hydroxylation of phenol to catechol which serves as ring-cleaving enzymes hence, includes *ortho*- and *meta*-cleavage pathways. Degradation of phenol by *Pseudomonas putida* EKII and produced phenol derivatives were assessed and the specificity of phenol hydroxylase enzyme has been reported by the degradation and transformation of cresols and chlorophenol, *ortho*- and *meta*-substituted phenols [45].

In a mixture of pollutants, microorganisms may degrade the compounds in different rates. Degradation of compounds may be performed simultaneously as well as the presence other compounds may prevent utilization of another compound. To become

more precise, the degradation of a second compound may be prevented by transcriptional regulation until the first compound was fully exhausted. *Arthrobacter chloropenolicus* has been reported to show preference in degrading phenolic compounds when tested with a growth media containing 4-bromophenol, 4-chlorophenol, and 4-nitrophenol [46].

Phenol can be spread to the soil such as plant root exudates, leaf leachates or products of plant tissue decomposition process. Although microorganisms that present in soil degrade phenolic compounds, phenols have been reported to accumulate in soils and prevent the growth of neighboring plants. *P. putida* 4CD1, *P. nitroreducens* 4CD2, *P. putida* 4CD3, and *R. glutinis* 4CD4 have been isolated from bamboo *(Bambusa chungii*), pine (*Pinus massoniana*) and rice *(Oriza sativa*) soils and have been reported to decompose *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde [46]. Zhang et *al.* reported the isolation and characterization of microorganisms from the soils of the natural forest of bamboo and pine contains a high concentration of phenolic compounds. Four microbes were isolated and their abilities to grow in a medium containing p-coumaric acid as carbon source were examined. The identification of these strains was done by biochemical analysis and sequencing of their 16S and 18s rRNA. Biodegradation experiments with 1 g/L of p-coumaric acid were also performed. They showed that all strains were capable to degrade 1g/L of p-coumaric acid within 48 hours. Moreover, the results confirm that these strains effectively degraded different phenolic acids like ferulic acid and 3-hydroxyphenylacetic acid (3-HPAA).

Apart from the phenol degrading ability of microorganisms, the presence of phenolic acids may exhibit an inhibitory effect on microorganism's growth. Therefore, some of the phenolic acids have been exploited as antimicrobial agents in the food industry. The olive tree is an important agricultural tree in Mediterranean countries due to its products of olive and olive oil. Olive consumption has been reported as healthy due to by being a rich source of phenolic acids and olive's antioxidant capacity. A 100 g of the olive may confer up to 80 mg of phenolic acids, and the variety of phenolic compound differ according to the cultivar, the location, and the ripeness degree. Thus, there are several studies in literature related to the identification of microorganisms which can degrade phenolic compounds from the olive orchard, especially the olive mill wastewater[47].

Nine phenolic compounds (oleuropein, hydroxytyrosol, tyrosol, vanillic, p-hydroxybenzoic, sinapic, syringic, protocatechuic and cinnamic acids) which are found in olive oil have been tested on inhibition of *L. plantarum's* growth. Sinapic and Syringic

have been reported to display the highest inhibition on *L. plantarum* by having a concentration range in between 12.5 to 50 mM and only oleuropein and protocatechus have been reported to be degraded by *L. plantarum* strains [48].

Maza-Marquez et *al.* carried out the characterization of the bacterial community of olive washing water (OWW) by both cultivation-dependent and -independent methods. PCR-TGGE method was used to reveal variations of the bacterial community. On the other hand, several strains were isolated from OWW and cultivated within an increasing amount of polyphenols containing a medium. The strains with higher phenol degrading capacity were selected and identified through 16S rRNA gene sequencing as *Raultella terrigena* and *Pantoea agglomerans*. These strains indicated that they were able to degrade up to 93% of OWW treatment [48].

In recent years, with the increase of environmental pollution, the number of published studies about the phenolic compound degrading microorganisms is increasing very rapidly and the new resistant species are discovered. Other researches about phenolic compound degrading microorganisms are listed below at Table 2 [48].

1.4. The Genes Responsible for Phenolic Compound Degradation

Phenol and its derivatives are one of the major pollutants due to their presence in a variety of industrial effluents such as crude oil industry, ceramic and steel plants, petrochemicals, perfumes, and pharmaceuticals. Environmental pollution gradually increases which causes continuous worsening in the quality of life. These factors enforce scientists to search for an urgent and effective solution to protect the human health and the nature. Bioremediation is one of the most preferred methods to remove phenols induced environmental pollutant. Bioremediation is a process involving the use of living microorganisms to remove contaminants from soil, water, and other environment types. The process is separated into three main parts: (1) Transforming and alteration, (2) Degradation into simpler compounds, and (3) Conversion into simpler molecules (H_2O, CO_2, H_2, etc.) [49].

In recent years, the number of research about bioremediation of phenolic compounds is dramatically increased. Several kinds of microorganisms have the enzyme systems that provide to break down the diverse aliphatic and aromatic toxic compounds.

Knowing their abilities about degrading phenol and phenolic derivatives is necessary to screen species with high-degradation activity by intensive efforts. Specific microbial enzymes provide microorganisms a capacity that helps transformation xenobiotics into compounds that are able to contribute to the normal growth cycle of the organism. Therefore, many researchers pay attention to the analysis of enzyme reactions about degradation and detoxification of phenol pollutants. Microorganisms can use phenol as a carbon and energy source due to its accessibility in lots of different habitats. It is possible to find both aerobic and anaerobic microorganism that can complete the consumption of phenol process [49,50].

The number and location of the different substitution groups obscure the mechanism of aromatic compound degradation. Phenol-degrading aerobic bacteria can produce non-toxic intermediate compounds from phenol and these compounds can join the Tricarboxylic acid cycle through ortho- or meta- pathways of degradation. Mono-hydroxylation at the ortho-position of the aromatic ring takes the first step of both of those pathways. Monooxygenase (phenol hydroxylase) is an enzyme that catalyzes these reactions and it has a crucial role in the aerobic degradation of monoaromatic compounds. Aromatic monooxygenases can be categorized as monocomponent and multicomponent enzyme forms. Modern methods of protein molecule analysis have found the significant diversity and specific functional characteristics of monooxygenases that have an ability to convert different aromatic compounds. Catechol and its derivatives are the first intermediate products of phenolic compound degradation. Catechol dioxygenase enzymes are responsible for the catalyze of cleavage of the aromatic ring in microorganisms. The product which is formed as a result of catec hol dioxygenase activity converted to harmless product by cis, cis-muconate cyclase enzyme[49].

1.4.1.Phenol Hydroxylase

The attachment of a hydroxyl group at the ortho-position of the aromatic ring is catalyzed by phenol hydroxylase, so phenol is hydroxylated to catechol. An enzyme in the category of NADP-dependent flavin monooxygenase catalyzes this reaction, which creates the initial step in the degradation of aromatic compounds in microorganisms.

Table 2. The list of the phenolic compound degrading microorganisms that are isolated from contaminated area [51].

Bacteria	Type of Sample	Reference
Acinetobacter Pseudomonas Comamonas	Wastewater and activated the sludge of petroleum chemical plant	Agarry et *al.*
Pseudomonas flurescences	NA	Sharma et *al.*
Acinetobacter	Paper mill effluent and sediment core of pulp	Shourian et *al.*
Acinetobacter calcoaceticus	Wastewater of the coal industry	Sandhu et *al.*
Alcaligenes Acinetobacter Rhodococcus	Trees of *Fraxinus pennsylvanica* near of a swine production facility	Liu et *al.*
***Sphingomonas* sp**	Activated sludge and phenol contaminated soil	Zhang et *al.*
Pseudomonas putida Rhodotorula glutinis	The soil of natural forest of pine and bamboo	Li et *al.*
Enterobacter Acinetobacter Serratia Stenotrophomonas Arthrobaster Bacillus	Wastewater of cork manufacturing	Castillo et *al.*
Proteus mirabilis Citrobacter freundi	Oil contaminated soil	Mohite et *al.*
Bacillus cereus	Wastewater of coking industry Na	Lu et *al.* Liu et *al.*
Thermoanaerobacterium thermosaccharolyticum Thermoanaerobacterium aciditolerans Desulfotomaculum* sp. *Bacillus coagulans Clostridium uzonii	Palm oil mill effluent	Mamimin et *al.*
Ochrobacterium	Soil of wetland	Chen, C et *al.*
Sphaerobacter Acinetobacter baumannii Comamonas testosterone Novospingobium naptalenivorans	Activated sludge in coking plant	Chen, XH et *al.*
Alcaligenes faecalis	Steel plant	Subhasis et *al.*

Phenol hydroxylase makes the hydroxylation of hydroxyl-, amino-, halogen-, or methyl-substituted phenols faster[52,53].

Catechol could be also hydroxylated by phenol hydroxylase and pyrogallol was produced. If there is a high concentration of phenol and it is the only substrate for the enzyme, pyrogallol can be observed. It could have resulted from the inhibition of phenol hydroxylase and the specificity changes of the enzyme in the presence of excess phenol [49].

The first publication about the cloning, sequencing, and expression of *T. cutaneum* phenol hydroxylase gene in *E. coli* was in 1992. Researchers realized some differences (e.g. higher substrate specificity in the partially purified enzyme from *C. tropicalis ATCC 46491* than the enzyme belonging to *T. cutaneum*) in the enzyme structure of phenol hydroxylase that was isolated from different yeast species. The focus point is the enzyme inducibility to finding the relationship between the aromatic substrate type and phenol hydroxylase activity. The effect of reaction conditions such as pH and temperature and the substrate specificity for the activity of the enzyme that was isolated from *C. maltosa* was examined by testing phenol, catechol, resorcinol, p-cresol, and benzoic acid as substrates. As a result, phenol (1 g/L) was found as the best inductor while the lowest enzyme activity was in the use of p-cresol as a substrate [54,49].

1.4.2. Catechol Dioxygenases

Catechol dioxygenases are a group of iron-containing enzymes that play a key role in the biodegradation of aromatic compounds especially phenol and its derivatives by an aromatic ring cleavage. For this reason, these enzymes are common in soil microorganisms. Catechol dioxygenases are classified into two groups as ortho- and meta-pathway enzymes according to the orientation of cleavage of the aromatic ring. Catechol 1,2-dioxygenases (the ortho-pathway) cleavage results in the production of cis, cis-muconic acids while catechol 2,3-dioxygenase (the meta-pathway) produces muconic semi aldehyde (Figure 3)[55,49].

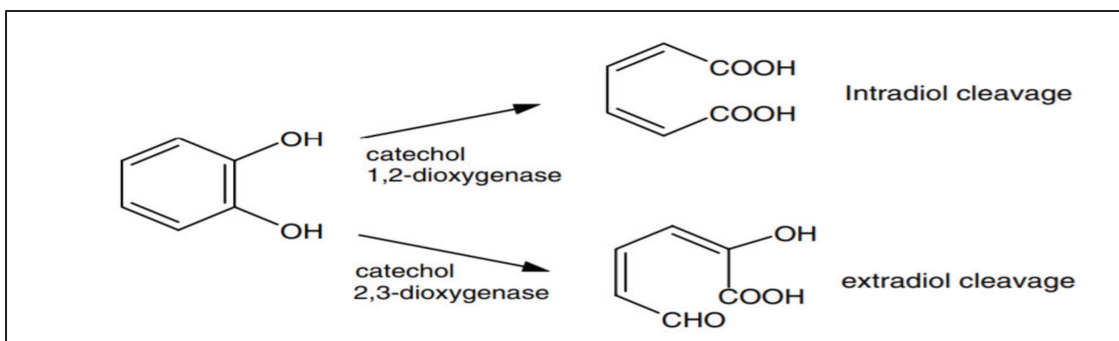


Figure 3. Ortho- and Meta-cleavage of the catechol by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase enzymes. (Intradiol-Ortho, Extradiol-Meta) [55].

Catechol 1,2 dioxygenases use non-haem Fe (III) for cleavage of the aromatic ring at ortho position whereas catechol 2,3 dioxygenase use non-haem Fe (II) or different metal ions with two-valent for cleavage in connection with a hydroxyl group. Previous research has indicated that meta-pathway enzymes are generally thought to have more enzyme activity than the ortho-pathway enzymes. Several studies have revealed that catechol 2,3 dioxygenases enzymes (extradiol dioxygenases) have broad substrate specificity and more structural variants and take part in more metabolic pathways [55].

1.5.Olive Mill Wastewater (OMW)

Olive mill wastewater (OMW) is produced in the process of olive oil extraction and it has serious polluting properties mainly due to high organic and high phenolic contents. When chemically characterized, tyrosol is detected as the most abundant phenolic compound, followed by catechol. OMW has been demonstrated to have lethal and sub-lethal effects on some model organisms such as *Vibrio fischeri, Danio rerio and Triticum aestivum in concentrations of* $EC_{50} = 0.24\%$ of OMW sample final concentration, $EC_{50} = 7.05\%$, $EC_{50} = 11.58\%$, respectively. Tested organisms had decreased root and sprouts elongation, as well as increased mortality, higher abnormality rate, decreased hatching and pigmentation formation rate [47,56,57].

OMW causes a reduction in soil fertility and damage nearby ecosystems. In a study where olive-mill wastewater (OMW) distribution in durum wheat were reported, OMW caused necrosis of the leaves and resulted in a slow emission of secondary stems

of wheat. [2] In another study where the impact of OMW to marine environment was investigated, it was found that there were pre-pathological alterations in tissues of mussels [47].

In order to reduce pollution concerns of disposing OMW to soil or marine environments, there were some high cost treatments were proposed. However, the effective treatment of OMW requires many physical, chemical and biological techniques such as filtration, adsorption, and treatment to clear solids, color, odor and polyphenols. The most efficient technique to reduce OMW pollutants was demonstrated as 'membrane technology' due to being cost-effective [57,58].

1.6. Introduction

Olive (*Olea europapaea* L.) is a species of Mediterranean climate which is a fruit of the family of Oleaceae. Archaeological finds indicate that olive has been known since 6000 B.C. Today, Olive is cultivated in different regions of the world especially in the Mediterranean basin. Olive is an important constituent of the Mediterranean diet with the consumption of a large amount of table olive and olive oil. There is a significant number of phenolic compounds in various part of the olive such as leaves, seeds and especially fruits. Also, Olive wastewater formed in the process of olive oil production possesses high amounts of phenolic compounds which is an environmental problem today.

The investigation of the microorganisms, resistant to phenolic compounds, is an important area about phenolic compound degradation. During the past 30 years, with the growing industry in parallel with the increasing population, the wastes that cause environmental pollution, increased rapidly. Industrial wastes consist of various chemical and physical substances. Phenol and its derivatives are one of the most important and harmful chemical wastes in nature. The pollution of phenolic compounds affects both the health of organisms negatively and disrupts the balance of nature considerably. For this reason, numerous studies have attempted to minimize the harmful effects of these compounds with bioremediation. Bioremediation is a process to eliminate or minimize the number of hazardous wastes from the contaminated area by using mostly microorganisms and sometimes using microbial and plant enzymes. Although bioremediation is the most effective and environmentally friendly method among the

methods used, it has several disadvantages. Only a few of the microorganisms used to affect the large mass of organic compounds and requiring a long time to show their full effect are two of the important disadvantages of bioremediation. In recent years, there is a large volume of published research to overcome these disadvantages. For instance, finding microorganisms resistant to phenolic substances from soil and water of various types of industrial waste, investigation of the enzymes responsible for degradation and development of effective recombinant enzymes are some of the investigation areas for bioremediation.

We know that olive orchard is a rich region in the phenolic compound matter. As far as we know, no previous research has investigated isolation and identification of bacteria found in olive orchard microbiome. Only a few studies in the literature demonstrated the microbial profile for only olive mill wastewater. Identification of these organisms was done with 16S rRNA sequencing method.

In this study, we focused on the microbial profile of the soil and the gut of the olive fruit fly larvae in an olive orchard with the microbial profile of the OMWW. Our hypothesis was that if the microorganisms can live in the olive orchard, then they should have resistance against phenolic compounds and should have the required genes for phenolic compound degradation. In this study, the main aim is to investigate the bacterial strains have phenolic compound degradation ability and the genes used in the degradation metabolism in olive orchard microbiome.

CHAPTER 2

MATERIALS AND METHODS

2.1. Sample Collection and Preparation

Soil and Larvae samples were collected on campus at Izmir Institute of Technology in İZMİR and olive mill wastewater (OMWW) produced during olive oil processing have collected the pool of wastewater from Eğlen village in Karaburun. Sterile water was added to the collected soil near the roots of the olive tree all under water and incubated for overnight. For Larvae samples, the olives, we expected to have larvae of Olive fruit fly were collected. Then, olives were opened with lancet under sterile condition and larvae were transferred in sterile Eppendorf contain PBS (Phosphate Buffer Saline) solution. After that, the larvae with PBS solution were homogenized by using glass homogenizer to be ready for inoculation. OMWW (1 g) was mixed with 50 ml of broth media and incubated for 48 hours at room temperature.

2.2. Preparation of MMSM

Modified Mineral Salt Medium (MMSM) was prepared according to the below list in Table 3. Chemicals in media were separated into the groups to prevent the precipitation during autoclave for broth medium.

The pH of the medium was adjusted to 8.0 and the medium was autoclaved at 121°C (Nüve) for 15 minutes. After autoclave, the solutions were collected into the new bottle when they are cooled. When its temperature was approximately 50-60°C, 0.3044 g/L of 3-HPAA (3-hydroxyphenylacetic acid) (Sigma Aldrich, PN: H49901) were added to do reach 2 mM final concentration.

Table 3. The composition of Modified Mineral Salt Medium.

Groups	Chemicals	Amount(g/L)
Solution-I	KH_2PO_4 (Sigma Aldrich, PN: P5655)	1,5
	K_2HPO_4 (Sigma Aldrich, PN: 1551128)	0,5
	NaCl (Sigma Aldrich, PN: 31434)	0,2
	Yeast Extract (Fluka, PN: 70161)	2,0
Solution-II	NH_4Cl (Sigma Aldrich, PN: 254134)	0,5
	$MgSO_4.7H_2O$ (Sigma Aldrich, PN:63138)	0,5
Solution-III	$MnSO_4.H_2O$ (Sigma Aldrich, PN: M8179)	0,5
	$Fe_2(SO_4)_3$ (Sigma Aldrich, PN: 307718)	0,01
	$(NH_4)_2S_2O_8$ (Sigma Aldrich, PN: 248614)	0,01
	$NaMoO_4.2H_2O$ (Sigma Aldrich, PN: 331058)	0,001
	$CuSO_4.H_2O$ (Sigma Aldrich, PN: 209198)	0,01
Solutıon-IV	$CaCl_2$ (Sigma Aldrich, PN: 449709)	0,5
Solution-V	NH_4NO_3 (Sigma Aldrich, PN:221244)	0,2
***For Solid Medium**	Agar(Fluka, PN: 05039)	20

*Only used for MMSM agar.

*For MMSM agar, all chemicals were added in the same bottle and then autoclaved.

2.3. Isolation and Selection of the Samples

Serial dilution was done from 10^0 to 10^{-3} for prepared soil, homogenized larvae or OMWW samples. Then, 100 µl of these samples from each dilution were plated on MMSM plates containing 2 mM 3-HPAA by spread plate technique and all the plates were incubated at 30°C (Nüve, PN: N025) for 48 hours. After incubation, phenotypically different colonies from the plates were selected and subcultured in fresh MMSM agar containing 2 mM 3-HPAA. In this way, the microorganisms which have no resistance against phenolic compounds were eliminated.

2.4. Morphological Characteristic of Selected Bacterial Strains

The morphological characteristic of the selected bacterial strains was examined according to firstly their colony morphology such as shape, size, and color, and then their cell morphology (shape and arrangement) and gram characteristic.

Gram staining was performed as described by Reade (1985) wherein a loopful of culture or a colony, resuspended in a loopful of water, was spread on a glass microscope slide. The bacteria were dried onto the slide by holding it over the Bunsen burner. Crystal violet was then applied to the slide and left for 60 seconds. The slide was rinsed with water and Lugol solution was added and left for 30 seconds. It was then rinsed again with water and decolorized with decolorization solution for 15 seconds. It was then washed again and counter-stained with Fuchsin for 60 seconds, washed with water, and dried approximately ten minutes for observing under a microscope. If the bacteria stained purple color, it was taken as an indication of the presence of a Gram-positive bacterium while if they stained pink color, it was taken as an indication of the presence of a Gram-negative bacterium.

Oxidase test to check the presence of the electron transport chain (ETC) by using artificial electron acceptor (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) (Sigma Aldrich, PN: 87890) instead of oxygen. This acceptor changes the color to purple if it takes the electron from the last element cytochrome oxidase in the ETC. With a sterile loop, a small amount of colony of isolated culture from an agar plate was placed on sterile filter paper. Then, a drop of oxidase test reagent onto the paper. The positive reactions turn the bacteria color to purple immediately or in 30 seconds.

Catalase enzyme is produced by the microorganisms that live in aerobic condition mediates the breakdown of hydrogen peroxide (H_2O_2) (Sigma Aldrich, PN: H1009) into H_2O and O_2. Hydrogen peroxide has a bactericidal effect for microorganisms and catalase enzyme neutralize its toxic effect by breaking down the compound. To test the microorganisms that possess the catalase enzyme, a small inoculum of isolated culture was mixed with a drop of 3% of hydrogen peroxide onto a slide and it was observed that bubbles are formed or not.

2.5. Phenolic Compound Tolerance Test

Phenol tolerance test was applied to select the more tolerated strains. Four different MMSM broth mediums were prepared which contained 2 mM, 3mM, 4mM and 5 mM of 3-HPAA. Firstly, single colony inoculation of the isolated bacterial strains was performed in 4 ml of 2 mM MMSM broth medium. Then, all the cultures were incubated for 48 hours at 30°C. After incubation, optical density (OD) measurement was done at 600 nm by using Thermal multiscan Spectra Reader (Finland) and the data was recorded. After that, 200 µl of these cultures were transferred to 3.8 ml of 3 mM 3-HPAA containing MMSM (5% inoculation). Again, the tubes were incubated at 30°C for 48 hours. This procedure was continued until inoculation of the cultures into 5 mM 3-HPAA containing MMSM as indicated the schema in Figure 3. This process was applied to all isolated colonies.

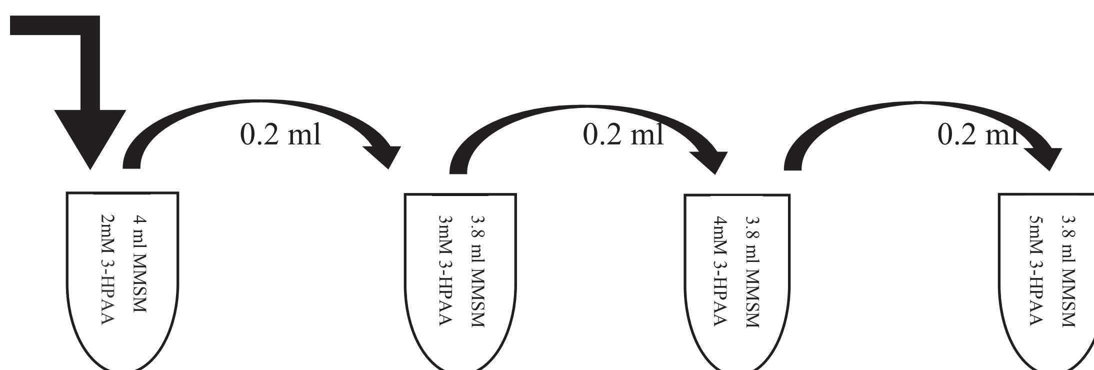


Figure 4. Schematic representation of the phenolic compound tolerance test.

2.6. DNA Extraction of Isolated Strains by Using CTAB Method

Isolated bacterial strains were inoculated into a 5 ml broth medium and incubated at 30°C for 48 hours. 1.5 ml of each liquid culture were transferred into an Eppendorf tube that centrifuged for 2 minutes at 14000 g. The remaining liquid cultures were centrifuged until the whole liquid in tubes were finished. Then, supernatants were discarded. The pellets were resuspended with 500 µl of TE (Tris-EDTA) buffer (Multicell, PN:809-215-CL) by pipetting. To lyse the cells, 30 µl of 10% SDS (Sodium dodecyl sulfate) (Sigma Aldrich, PN: 436143) and 4 µl of 20 mg/ml proteinase K (Sigma

Aldrich, PN: AM2546) were added and mixed well. The Eppendorf tubes were incubated for an hour at 37°C. Then 100 µl of 5 M NaCl (Sigma Aldrich, PN: 31434) addition was followed by addition of 80 µl of CTAB/NaCl solution. After mixing thoroughly, the tubes were incubated at 65°C for 10 minutes. An equal volume (approximately 0.7 ml) of Phenol/Chloroform/Isoamyl Alcohol (25:24:1) was added, mixed thoroughly, and centrifuged for 5 minutes at maximum speed. Aqueous, viscous supernatants were removed to the fresh Eppendorf tubes for each sample, and an equal volume of Chloroform/Isoamyl Alcohol (24:1) was added. The tubes were centrifuged for 5 minutes at maximum speed. The supernatants were transferred to the fresh tubes and 0.6 volume of Isopropanol (Sigma Aldrich, PN: 90764) were added to precipitate the nucleic acids and centrifuged at maximum speed for 15 minutes at 4°C after two hours incubation at -20°C. The washing was performed with ice-cold 70% ethanol (Sigma Aldrich, PN:32221) (preferably at -20°C) and centrifuged for 5 minutes at room temperature. The supernatants were removed carefully, and the pellets were allowed to air dry for overnight. Finally, the pellets were dissolved with 100 µl TE buffer. DNA concentrations were measured with UV absorption at 260 nm by using Nanodrop. The isolated genomic DNA samples were stored at -20°C until used for further tests.

2.7. PCR Amplification of Partial 16S rRNA Genes

After DNA extraction of isolated bacterial strains with CTAB method, 700-800 bp of 16S rRNA sequences were amplified by using universal E8F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and universal E1115R reverse primer (5'-AGGGTTGCGCTCGTTG-3') [59]. The final volume of PCR reactions for each isolated bacterial strain was adjusted to 50 µl. The following components with required concentration were mentioned below for PCR reactions: 2.5U Taq Polymerase (Fermentas, PN: EP0402), 2 mM $MgCl_2$, 10 µM dNTPs (Fermentas, PN: R0192), 1 µM of forward, 1 µM of reverse primer, 1x Taq Polymerase buffer and finally approximately 1 µg of total genomic DNA were added. The reaction volume was completed with Nuclease-free water to 50 µl. The amplification reactions of 16S rRNA genes were performed with the following conditions. 1 cycle of predenaturation at 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 2 minutes

which continue with a final extension step at 72°C for 15 minutes. For all PCR reactions, 5 µl of amplified 16S rRNA gene of isolated bacterial strains were confirmed on agarose gel electrophoresis (2% w/v).

2.8. Purification of amplified 16S rRNA gene of Isolated Strains

After PCR reactions, PCR products were purified with ExoSap-IT (Thermo, PN: 78201.1.ML) which includes Exonuclease I and Shrimp Alkaline Phosphatase enzymes. They are used for enzymatic cleanup by hydrolyzing excess primers and nucleotides. 2 µl of ExoSap-IT were mixed with 5 µl of PCR product of each strain. ExoSap reaction was performed at 37°C for 15 minutes (enzymes activation) and then at 80°C for 15 minutes (Inactivation) Now, The PCR product was ready for downstream reactions.

Sequencing reactions were performed by using Bigdye™ Terminator v3.1 cycle sequencing kit (Applied Biosystem, PN: 4337455). The reactions were performed according to the kit manual for all isolated strains.

Sephadex spin column for cleanup of PCR products was the last part of the purification before sequencing. Sephadex G-50 powder (GML, PN: S6022) was added to 42 ml dH_2O and swirled to mix fully. Then, the mix was allowed for polymerization at 4°C for an hour. Spin columns were placed in 1.5 ml collection tubes and 700 µl of polymerized Sephadex were added. The tubes were centrifuged at 4700 rpm for two minutes on benchtop centrifuge. The flow-through in collection tubes were discarded and spin columns were placed again. Bigdye reaction products were applied to the Sephadex column and centrifuged at 4700 rpm for 2 minutes. Now, all the samples were ready for sequencing.

2.9. 16S rRNA Sequencing and Analysis

Each purified PCR was loaded on a 96-well reaction plate (Applied Biosystem). Sequencing was performed with capillary electrophoresis method by using 3130XL genetic analyzer (Applied Biosystem) under 3130 POP7-BDTV3-KB electrophoresis condition and the results were analyzed by using BLAST which is most frequently used

and most reliable bioinformatics algorithm to search sequence similarity for the identification of unknown sequences. rRNA sequence of isolated strains was loaded BLAST one by one and homology search was performed by using the NCBI database.

2.10. Primer design for the amplification of genes relating to phenolic compound degradation

PCR primers were designed to amplify the sequences of phenol hydroxylase and catechol 1,2-dioxygenase genes. A number of related genes were aligned and the areas of relative homology selected for the primer design internally to the individual genes. It was sometimes necessary to design degenerate primers (more than one nucleotide in a particular position). These are as the following: R=A+G, S=C+G Y=C+T, M=A+C, W=A+T, V=G+A+C, B=C+G+T and N=A+G+C+T. All primers were commercially synthesized by Sentegen (Ankara). The sequences of the related genes from the selected strains that are close relation with the isolated strains were collected by using the UNIPROT and NCBI database. Then, Multiple sequences alignment of nucleotide sequences of the selected strains were performed by using the T-coffee program to determine the conserved regions on the sequences. The forward and the reverse primers of the related genes were designed in accordance with the Tm values and GC ratios. The primer pairs were listed in Table 4.

2.11. RNA Isolation and cDNA Synthesis of Selected Strains

The selected strains are inoculated at the same condition and then total RNA was isolated using the High Pure RNA Isolation Kit (Roche, PN:11 828 665 001). RNA quantity was determined by using the QuantiFluor® RNA system (ProMEGA, PN: E3310). cDNA synthesis was performed with SensiFAST cDNA synthesis kit (Bioline, PN: BIO-65054). The same amount (10 ng) of RNA were added into cDNA reaction for each strain to quantify and compare the gene expression levels of selected strains.

Table 4. The list of the primer pairs used in this study.

Primer Pairs	Sequence of the Primers	Product Length
	Phenol Hydroxylase	
PhenolH-F **PhenolH-R**	5'-GCCAGTACATCAATATCGAGTTGCC-3' 5'-TTCTGTTCCAGAAGATCCAGGATCATC-3'	~289 bp
pheU-F **pheU-R**	5′-CCAGGSBGARAARGAGARGAARCT-3′ 5′-CGGWARCCGCGCCAGAACCA-3	~620 bp
PHE-F **PHE-R**	5'-GCWCTACCYNCACWTGABGY-3' 5'-CRCGSAGYTTGGCVTMRAG-3'	
Bacphe-F **Bacphe-R**	5'- CCCCGACTACAAGGCAAGTT-3' 5'- GATCGGCAGACCGTAGTGAG-3'	~300 bp
Curto-F **Curto-R**	5'-GGAGTTGACCTCTTCGAGGC-3' 5'- GTCGTGTTGTATGAGCAGCG -3	~292 bp
	Catechol 1, 2-dioxygenase	
Catec-F **Catec-R**	5'-TCTGGCACGCCAATACCC-3' 5'-AGGTTGATYTGGGTGGTCA-3'	~260 bp
C12-F **C12-R**	5'-GCCAACGTCGACGTCTGGCA-3' 5'-CGCCTTCAAAGTTGATCTGCGTGGT-3'	~280 bp
Cat12-F **Cat12-R**	5'-GACGACGGCRAGAKCM-3' 5'- GSCAYGATGCTGCGCAMG-3'	~212 bp

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation and Identification of the Microorganisms from Olive Orchard

The soil samples and olive fruit fly larvae collected from the olive orchard in IYTE and OMWW took from Eğlen village in Karaburun were examined for the presence of microorganisms that were resistant to phenolic compounds. All samples were enriched in Modified Mineral Salt medium (MSMM) containing 2 mM of 3-HPAA as a phenolic compound for the first selection of the resistant strains. Thirty bacterial strains from soil samples, sixteen bacterial strains from olive fruit fly larvae and eight bacterial strains from OMWW were isolated. They were individually inoculated for enrichment with MMSM containing of 3-HPAA and subjected to a tolerance test that was performed according to the method 3.5. for the selection of highly resistant strains. The strains which had tolerance to 5 mM of 3-HPAA were selected for further investigations of the study (Table 4).

After the selection of 3-HPAA tolerant strains, several biochemical and morphological tests were applied to all isolates. As biochemical tests, catalase and oxidase tests were carried out and then gram staining were performed. The results were represented in Table 5 for all strains in this study.

According to the results, all strains had a rod shape except for the isolate named OMWW1 as expected , because, rod-shaped bacteria are the most abundant type of bacteria followed by cocci and spiral, respectively in soil [60]. Soil is a diverse environment and different types of living organisms such as plant, animal, fungi, and bacteria exist. It is known that the cell shape is governed due to some cellular events such as nutrient access, cell division and segregation, attachment to surfaces, active motility, and escape from predators. There is a correlation between motility a bacterial shape morphologically [61]. The main theoretical premise behind this correlation is that the requirement of physically and energetically on cell shape. For example, a 0.2 μm change in cell diameter changes extraordinarily the energy required to be a factor of 10^5 for chemotaxis. Thus,

energy use compels the cell to be rod shape with a certain ratio of width and length for highly motile bacteria [62]. Soil is also suitable for rod-shaped bacteria due to environmental conditions. Cocci- and spiral-shape bacteria are found near the surface and viscous environmental condition generally. As we can see in Table 5, all rod-shaped bacteria are also motile. On the other hand, OMWW1 strain was the only cocci-shaped and non-motile bacterium among all isolated strains.

Gram stain determination indicated that the majority of the isolated strains were gram-negative (~76 %), while a few (~ 24%) of the isolated strains were gram-positive. A gram-positive strain for 15 of soil isolates, 3 gram-positive strains for 6 larvae isolates and 2 gram-positive strains for 3 olive mill wastewater isolated are identified. Thus, we can say that environmental changes affect the dynamics of the bacterial community. Catalase test results demonstrated that all isolated strains were catalase positive. Catalase positive strains were mostly aerobic microorganisms because catalase enzyme is involved in the breakdown of hydrogen peroxide and provides oxygen release. For this reason, aerobic bacteria tend to be catalase positive. Anaerobic organisms do not need oxygen and oxygen might be toxic for them. Moreover, aerobes are found in dry soil dominantly due to the requirement of oxygen while anaerobes are tent to be found mostly in wet condition and poorly drained soils [63]. Thus, it was expected that the strains which were isolated from the soil would be aerobic. For remaining isolated strains, the conditions were quite aqua rich which was suitable for anaerobes. However, these strains were also catalase positive. Therefore, these strains could be facultative anaerobes. The oxidase test was performed to identify the strains which produce cytochrome c oxidase enzyme or not. Cytochrome c oxidase enzyme is present in electron transport chain (ETC) and ETC uses oxygen as a terminal acceptor in respiration. According to the results of this study, seven isolates were oxidase positive. Oxidase positive strains possess ETC system which requires oxygen as an acceptor. Briefly, oxidase positive strains were absolutely aerobic. On the other hand, we can't say that oxidase negative strains were strictly anaerobes. Anaerobes, facultative anaerobes and also aerobes may be oxidase negative. The oxidase test indicates whether the bacteria only has the cytochrome c oxidase enzyme or not [64]. These bacteria can use different oxidases for respiration in ETC.

Table 5. The morphological and biochemical characterization of phenolic compounds degrading isolates

SAMPLES	Cell Morphology			Biochemical Characteristics	
	Shape	Gram Staining	Motility	Catalase test	Oxidase test
Soil1	Rod	Negative	Motile	+	-
Soil3	Rod	Negative	Motile	+	-
Soil4	Rod	Negative	Motile	+	-
Soil5	Rod	Negative	Motile	+	+
Soil6	Rod	Negative	Motile	+	-
Soil7	Rod	Negative	Motile	+	+
Soil8	Rod	Negative	Motile	+	-
Soil12	Rod	Negative	Motile	+	-
Soil13	Rod	Negative	Motile	+	-
Soil15	Rod	Negative	Motile	+	-
Soil16	Rod	Positive	Motile	+	+
Soil19	Rod	Negative	Motile	+	-
Soil24	Rod	Negative	Motile	+	-
Soil28	Rod	Negative	Motile	+	-
Soil29	Rod	Negative	Motile	+	-
Soil30	Rod	Negative	Motile	+	-
Larvae1	Rod	Negative	Motile	+	-
Larvae7	Rod	Positive	Motile	+	+
Larvae8	Rod	Positive	Motile	+	+
Larvae9	Rod	Negative	Motile	+	-
Larvae11	Rod	Negative	Motile	+	-
Larvae14	Rod	Positive	Motile	+	+
OMWW1	Cocci	Positive	Non-motile	+	-
OMWW7	Rod	Negative	Motile	+	-
OMWW8	Rod	Positive	Motile	+	+

After morphological and biochemical characterization, 16S rRNA gene sequencing was performed for genus and species identification of the isolated strains. The degree of the relationship was also demonstrated by comparisons of the sequences. organisms. To identify at the level of genus and species level, application of molecular genetic techniques is required to solve the problems with taxonomic issues. Partial sequence analysis of 16S rRNA is the most important and easiest way to determine what species that the isolated strain belongs to.

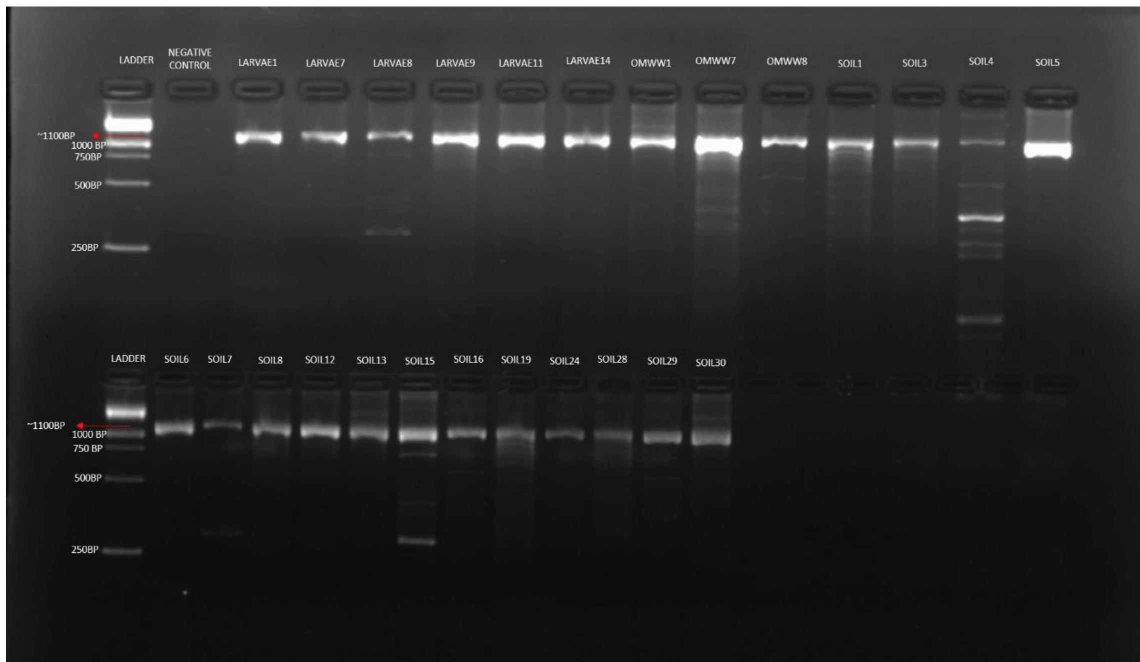


Figure 5. Agarose gel analysis of PCR amplified partial 16S rRNA gene region of the isolated strains by using Amplitaq Gold polymerase (Applied Biosystems). Line 1 & Line 16: 1 kb DNA size marker (GeneDirex) (250, 500, 750, 1000, 1500 bp….). Line 2-15 & Line 17-28: ~1100 bp PCR products (16S rRNA gene).

16S rRNA gene encodes the component of the small subunit of the ribosome and ribosome cannot be translated mRNA without 16S rRNA. All bacteria have 16S rRNA and it is the most conserved sequence of all bacteria. It comprised of both conserved and variable regions, this makes it easy to target a wide variety of bacteria with universal primers. It is also the most studied sequence; so many sequence inputs are available in the NCBI database Species-level identification was defined as a 16S rDNA sequence similarity of ≥99% between unknown sequence and reference strain sequence in GenBank [65]. Identification at the genus level requires a sequence similarity of ≥ 97% with the

reference strain, while similarity score of <97 was thought insufficient for genus determination. A similarity value between ≥ 95% and < 97% was considered sufficient for family level identification [66].

In this study, to identify the isolated strains according to the 16S rRNA sequence analysis, genomic DNA of the strains were used as a template to amplify 16S rRNA sequences by the pair of E8F forward primer and E1115R reverse primer which were able to amplify nearly 1100bp [59]. As shown in Figure 4, expected length of fragments were indicated on the 2% of agarose gel. After that, PCR products of each isolated strain were purified and sequenced. Obtained sequences were submitted to NCBI database and BLAST search were performed to retrieve sequences of related strains for each isolated strains. Based on 16S rRNA gene sequence analysis, it was found that all the isolated strains were represented in eight different species. Phylogenetic trees of isolated strains were constructed one by one with maximum likelihood method in MEGA X software, indicating the relationship of the isolated strains with the already published strains (Appendix). Additionally, a large phylogenetic tree was constructed to show the relationship between all isolated strains and eight different species with several similar strains (Figure 5).

The results obtained from the BLAST analysis of sequences are presented in Table 6. It is apparent from in the table that similar bacterial strains can live in different environments. For instance, Soil16, Larvae7, 8 and 14 were the members of the same *Bacillus sp.* strain and Soil3, Larvea1, 9 and 11 had similar (>99%) 16s rRNA sequences with *Pantoea sp.* strain.

Table 6. The list of species which indicated highest homology with our isolated strain according to the BLAST results of 16S rRNA gene sequences.

Isolated Strains	Reference Strain
Soil1, Soil4, Soil6,Soil8, Soil12, Soil13, Soil19, Soil24, Soil28, Soil29, Soil30	*Enterobacter sp*. strain
Soil3, Larvae1, Larvae9, Larvae11	*Pantoea sp.* strain
Soil5, Soil7	*Pseudomonas sp.* strain
Soil15	*Pseudoescherichia Vulneris sp.* strain
Soil16, Larvae7, Larvae8, Larvae14	*Bacillus sp*. Strain
OMWW1	*Enterococus sp.* strain
OMWW7	*Basillus Ginsenghumi sp.* strain
OMWW8	*Curtobacterium sp.* strain

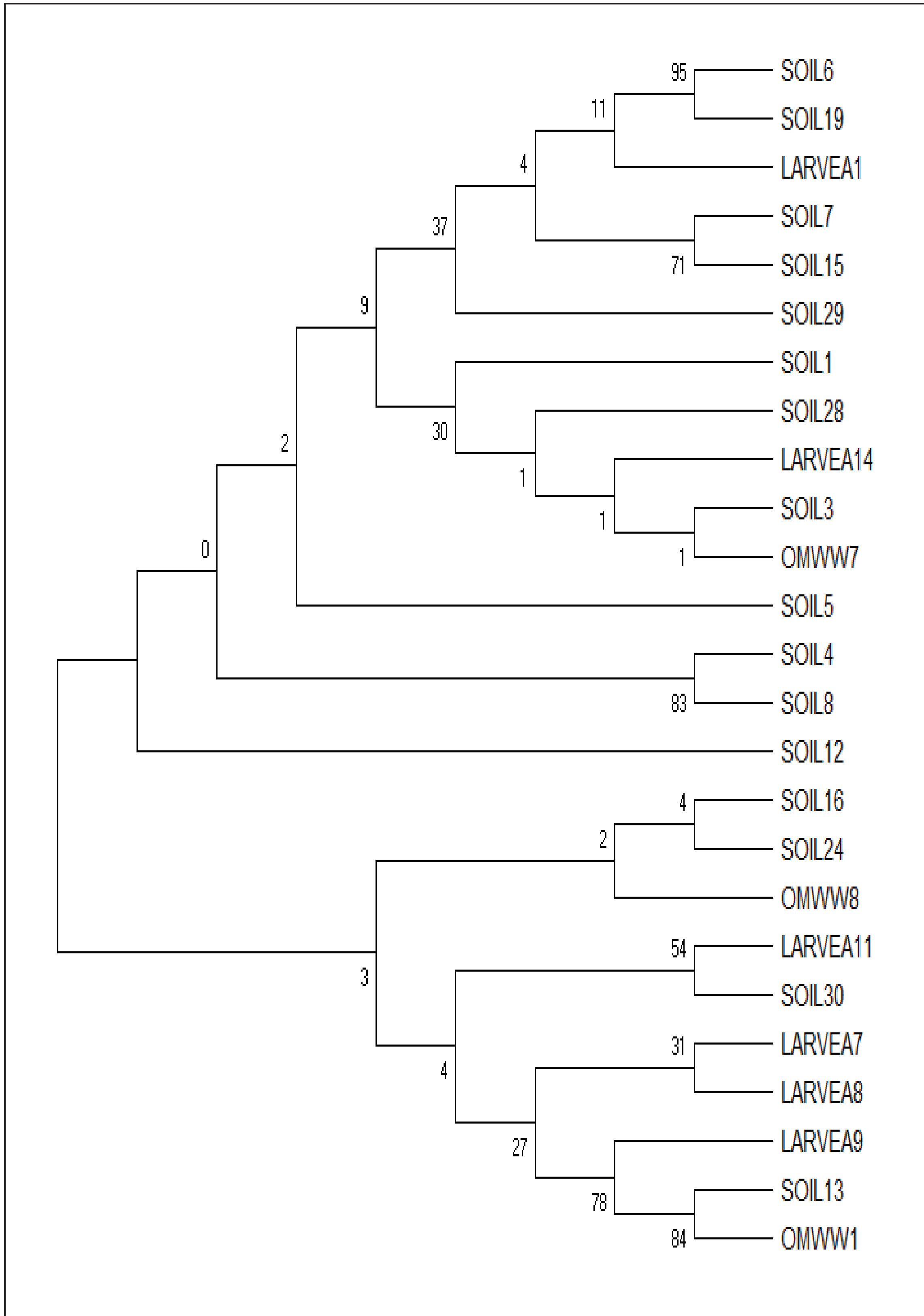


Figure 6. Phylogenetic tree of 16S rRNA partial sequence of isolated strains. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA X software.

According to the BLAST results, most of the isolated strains belong to the *Enterobacter* species (the sequences had similarity higher than %99.5 with *Enterobacter cloacae* (MG274288.1)*, Enterobacter asburiae* (AP019533.1) and *Klebsiella Oxytaca* (KX396021.1)) in soil sample. Although many of them were the same and closer identity to *Klebsiella oxytaca* species*,* our isolated strains were not categorized in this species level group. Because, we found that all these strains were motile according to the motility test. *Klebsiella oxytoca* is a non-motile, gram-negative rod-shaped bacterium while Enterobacter (*Enterobacter cloacae, Enterobacter hormaechei, and Enterobacter asburiae*) are motile, gram-negative and rod-shaped bacteria [59]. Therefore, morphology test was usefully complemented the molecular analysis results and we included these isolated species into the *Enterobacter* group. *Enterobacter* are common in the nature; they are widely distributed in soil, water, and sewage [67]. They are also found in plants and humans. Thus, it was expected that most of the isolated bacteria belong to these species. Because, *Enterobacter sp*. strains can help promote plant growth in several ways; (1) helping the host to overcome toxic effects of environmental pollution and (2) affecting nodule formation by legumes, fixing nitrogen and produce plant hormones [68]. Also, the *Enterobacter* species can be pathogenic for plants [69]. These factors may explain why most of the bacteria isolated from soil were *Enterobacter*.

Moreover, previous studies have reported the investigation of members of the genus *Enterobacter* which play a role in the degradation of phenolic compounds and other aromatic compounds [44,45,70]. Ammar et *al*. (2005) aimed to isolate bacteria which can grow on OMWW and which were especially able to degrade monomeric aromatic compounds, having a toxic effect on microflora in the sludge plant. For this reason, bacteria from the *Enterobacteriaceae* group were isolated. They tried to determine the capacities of these bacteria in degrading some monomeric aromatic compounds [58]. In another study, Thomas et *al*. (2001) tried to isolate stable and metabolically versatile denitrifying bacteria which can degrade phenolic compounds. They investigated the degradation of phenol and other phenolic compounds by a defined mixed culture of *Alcaligenes faecalis* and *Enterobacter* species. Mixed bacterial culture was able to degrade up to 600 ml/L of phenolic compound. Also, their study demonstrated the potential of the defined culture to degrade other phenolic compounds like o-cresol, 1, 2-dihydroxybenzene (catechol), o-hydroxybenzoic acid, 2, 5-dihydroxybenzoic acid and 2, 3-dihydroxybenzoic acid [44].

Phylogenetic analysis on the basis of 16S rRNA gene sequence confirms that the isolated strains Soil5 and Soil7 belong to the Pseudomonas species with 100% similarity. The similarity was the same for different *Pseudomonas sp.* strain (*Pseudomonas koreensis strain CF30* (MK697064.1), *Pseudomonas vancouverensis strain MS88* (MK696982.1), *Pseudomonas lactis strain FW305-C-5* (MK402978.1) and more). *Pseudomonas* is one of the most studied species of bacteria and it is well known as a species that populate a wide range of niches due to the metabolic and physiological diversity. This diversity provides *Pseudomonas* the adaptation to different environments and resistance against adverse conditions generated by abiotic and biotic factors like high and low temperature, moisture, oxygen and nutrients availability, antibiotics, toxic organic compounds, etc. [71]. As mentioned in the literature review, members of the genus Pseudomonas have reported for degradation of a variety of phenolic compounds [72–75]. A lot of studies performed previously demonstrated that the members of *Pseudomonas* genus are known to be the best degrader of phenol and phenolic compounds (Collins et al., 2005, Hamitouche et al., 2012). *Pseudomonas aeruginosa* (El-Sayed et al., 2003), *Pseudomonas fluorescence* (Agarry et al., 2008). *Pseudomonas putida* (Ravikumar et al., 2011) and many other *Pseudomonas* strains were identified and characterized by phenol degradation and tolerance. *Pseudomonas* belongs to the most abundant genus of bacteria in the bioremediation process [44]. There is a large amount of published studies describing the isolation of various Pseudomonas strains in contaminated soil and wastewater [44,74]. It is well known that Pseudomonas is an organism with an ability to metabolize even the most complex polymers and it has many induced enzymes responsible for a high degree of convergences. It is because the non-specificity of the induced enzymes provides simultaneous utilization of different organic substrates without redundant genetic coding for enzyme induction [50]. Therefore, we expected to isolate *Pseudomonas* species in the soil of olive orchard since an olive tree with leaves, fruits, and stems have many different organic compounds from simple to complex.

According to the 16S rRNA gene sequencing, four isolates (Soil3, Larvae1, Larvae9, and Larvae11) were 99% similar to *Pantoea sp*. strains. The phylogenetic tree indicates a strong association with the genus *Acinetobacter* as shown in Appendix Figure-a-b-c-d. *Pantoea* is a member of the *Enterobacteriaceae* family and ubiquitous in nature, especially soil. There are more than 20 *Pantoea* species, with some of them like *Pantoea agglomerans* and *Pantoea ananatis* are opportunistic pathogens associated with human infections. On the other hand, *Pantoea* strains mostly have many benefits to plants by

fighting against plant pathogens, helping the growth of plants and also their use in bioremediation [76]. The soil isolates of *Pantoea* have been demonstrated to have capabilities that include the degradation of toxic organic compounds like petroleum hydrocarbons into less harmful compounds [77], metal reduction [78] and solubilization of insoluble inorganics. It was expected that *Pantoea* species was present among the bacteria that were isolated in this study. It is because the members of these strains are known to degrade complex organic compounds as mentioned in the literature review [42].

Based on our results, Soil16, Larvae7, Larvae, and Larvae14 shared 100% sequence similarity of 16S rRNA gene (99.8% similarity for Larvae14) with both *Bacillus pumilus strain K11 (*MK696262.1) and *Bacillus safensis strain K1 (*MK696252.1). *Bacillus pumilus* and Bacillus *safensis* can not be distinguished from each other with other *Bacillus* strains simply by using 16S rRNA gene sequences. For instance, *B. pumilus, B. safensis, B. stratosphericus, B.altitudinis* and *B. Aerophilus* possess nearly the same 16S rRNA gene sequence (over 99.5% similarity). Therefore, it was not possible to determine which species belong to these four strains. Dokic et *al. (*2011) described the isolation of gram positive bacteria from soil capable of degradation odf aromatic compounds (phenol, toluene, biphenyl, naphthalene and etc.). Four *Bacillus sp.* were isolated as *Bacillus MEGAterium* (CP001982), *Bacillus pumilus* (JF769748), *Bacillus cereus* (CP001746) and *Bacillus simplex* (GU086427). Their enzymes which responsible for the degradation of phenolic compounds were analyzed for both sequence and activity. These strains demonstrated very high homology with our isolated *Bacillus* strains. They found that these strains have phenol hydroxylase and catechol 1,2-dioxygenase enzymes which show ortho-pathway degradation [79]. Also, a number of studies were examined phenolic compound degrading *Bacillus* strains [80]. All considered together, these results suggest that there is an association between *Bacillus* strains and phenolic compound degradation. Therefore, it seems that the isolation of *Bacillus* strains in olive orchard was expected for this study.

Soil15 isolate have >99% similar to *Pseudescherichia vulneris* strain. This strain belongs to the *Enterobacteriaceae* family and shows all the characteristic attributes of this family. It is found in variety of environments like soil, water, on plants, human wound and respiratory tract. There has been little information about this strains. Only several research about the infection appear on human wounds were carried out. In addition, no research has been found related to the degradation of phenolic compounds or other aromatic compounds by using *Pseudescherichia vulneris* strain [81].

The isolate OMWW1 exhibited more than 99% homology with *Enterococcus faecalis* strain and Enterococcus *faecium*. Enterococci are ubiquitous microorganisms that is a facultative anaerobe, non-motile, Gram-positive bacterium and could be found everywhere; in water, plant, soil, foods, and gastrointestinal tract of humans and animals[82,83]. Our finding was unexpected firstly because we generally know that *Enterococcus* are important members of gut communities in many animals and also opportunistic pathogens that cause millions of infections annually. On the other hand, the results of several researches indicate the infection of different plants by this strain. For instance, Ajay et al. reported that three *E. faecalis* strains (FA-2-2, V583, and OG1RF) that are capable of infecting the leaves and roots of the model plant species *Arabidopsis thaliana*, causing plant mortality in 7 days after inoculation. Moreover, the genus *Enterococcus* belongs to lactic acid bacteria (LAB) and previously used as starters in food fermentation due to their biotechnological traits [84]. In 2018, Dimitrios *et al*. described the fermentation of table olives by enterococcus strains. They found that enterococci from Cypriot table olives should be considered as a new source of potential starter cultures for fermented products, having possibly promising technological and the probiotic attributes[84]. Numerous studies have also attempted to explain the bioremediation activity of *Enterococcus* and their resistance against different phenolic compounds in the literature. *Enterococcus* strains were isolated from many different pollutant areas like crude oil, sludge and wastewater [85–87]. Thus, these strains are able to survive various harmful environment. Overall, these results supported that the isolation of these strain from OMWW is expected due to the suitability of the environment for *Enterococcus* strain.

Another strain OMWW7 isolated from OMWW were identified as *Bacillus ginsenghumi* with higher than 99% homology. *B. ginsenghumi* is a Gram-positive, aerobic or facultative anaerobic, non-motile, endospore-forming bacterial strain which was initially isolated from a soil sample of a ginseng field in Pocheon Province (Korea) and named by Ten et al. in 2006. They demonstrated that the highest 16S rRNA gene sequence similarities were found with *Bacillus shackletonii* LMG 18435T (97.6%), *Bacillus acidicola* DSM 14745T (96.9%), *Bacillus sporothermodurans* DSM 10599T (96.5%), and *Bacillus oleronius* DSM 9356T (96.5%). They also found that the strain utilized a lot of different carbon source for growth [88]. Ginseng is known to have various biological properties and pharmacological properties, such as, anticancer, antiemetic, antioxidant, and anti-proliferative properties, as well as other health benefits. These biological and

pharmacological properties are strongly related to the phytochemicals present in ginseng, including phenolic compounds. Like olive, ginseng is one of the plants which contains the most phenolic compounds in its structure [89,90]. Hence, the environmental conditions can be considered close both olive and ginseng orchard. For this reason, our isolated strain can be considered as closely similar species.

The last one from isolated strain was OMWW7 strain from OMWW. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain OMWW7 was related most closely to species of the genus *Curtobacterium* like *Curtobacterium flaccumfaciens*, in the family *Microbacteriaceae*. *Curtobacterium* is Gram-positive and short rod-shaped soil organisms that that cause disease on a variety of plants [91]. *Curtobacterium* sp. were listed among the genera of hydrocarbon degrading bacteria grow in the presence of polycyclic aromatic carbons (PAHs) [70,92]. Rajaei et al. isolated indigenous oil-degrading bacterial strains from wild oat rhizosphere (oil contaminated soil). Based on their 16S rRNA gene sequences results, one of the isolated strain was *Curtobacterium spp.* They found that the presence of catabolic genes like alkane monooxygenase (alkM), alkane hydroxylase (alkB) and catechol 2, 3-oxygenase (xylE), responsible for biodegradation of the alkanes and aromatic petrochemical compounds were detected in contaminated area. They claimed that the present study revealed the adaptability of microbes to the rhizospheric area and subsequently their great potential to be exploited for cleaning up hydrocarbon contaminated sites [92]. Similarly, Andrew (2014) isolated the organisms with oil degrading activity from petroleum exploration areas in Uganda. After phenotypic identification of the bacterial isolates, one of the isolates were identified as *Curtobacterium* sp. with 95% identity. Also, the morphological and biochemical characters were consistent [93].

3.2. The Assessment of the Genes Responsible for Phenolic Compound Degradation

The scope of this investigation was to isolate the microorganisms from olive orchard with the persistent ability to degrade phenol as well as other phenolic compounds and assess their genes related to the phenol degradation. These functional genes can be used as a marker to assess the catabolic potential of bacteria in bioremediation. In the first part, studies demonstrated the potential of isolated strains to degrade phenolic compounds

by doing tolerance test. As mentioned in the literature review, similar degradation capabilities have been also demonstrated for the microorganisms close to strains we isolate.

Within the range of our reference search, there has been several researches of primers for the detection of phenol hydroxylase and catechol 1,2-dioxygenase genes until now. The majority of these research was related to mostly *Pseudomonas sp., Acinetobacter sp. and Marinobacter sp.* We isolated 25 bacteria within 8 different genera and only one of them belongs to the pseudomonas genus. Although the primer pairs in the literature was not consistent for our isolated strain, we selected a primer pairs for both phenol hydroxylase and catechol 1, 2-dioxygenase from literature (PheU-F-R and C12-F-R). If our isolates were phylogenetically related to these genus, we could have constituted excellent material for assessing micro diversity of phenol hydroxylase and catechol 1,2-dioxygenase genes of phenolic compounds degrading microorganisms isolated from an olive orchard. Moreover, several of primer pairs were designed to amplify both phenol hydroxylase and catechol 1, 2-dioxygenase region based on the selection of annotated sequence database from close species. An example of primer design was shown in Figure 6.

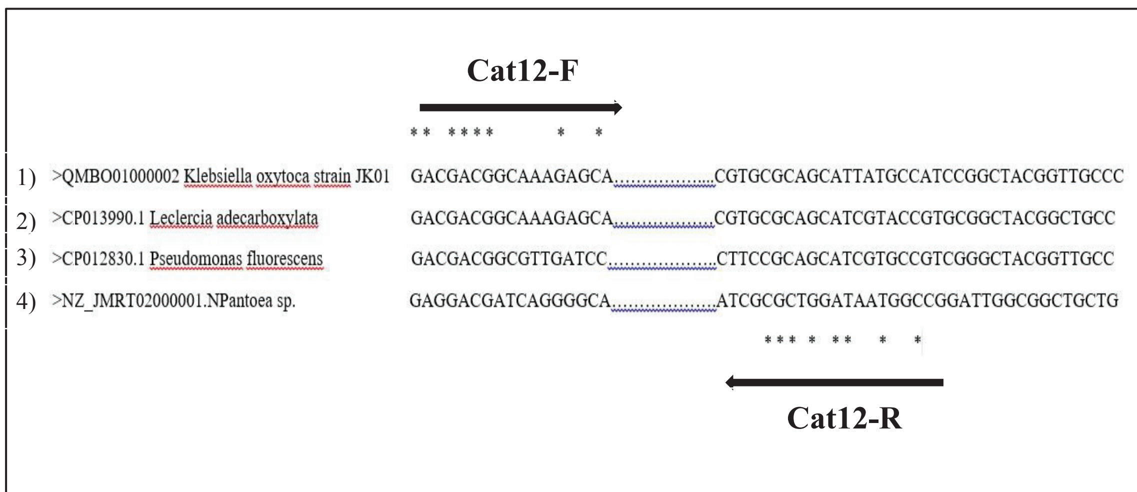


Figure 7. Design of the degenerate primer pairs of Cat12-F and Cat12-R by multiple aligment of 4 different strain catechol 1,2-dioxygenases**.** **1.** *Klebsiella oxytoca* strain JK01, **2.** *Leclercia adecarboxylata* strain USDA-ARS-USMARC, and **3.** *Pseudomonas fluorescens* strain FW300-N2E3 **4.** *Pantoea* sp. 3.5.1 NZ_JMRT02000001.N

To amplify the conserved region of phenol hydroxylase gene, the gradient PCR were firstly performed for all primer pairs with positive control (*P. aeruginosa*) by using

genomic DNA. Due to unsuccessful results, PCR reaction conditions were changed by using different additive like dimethyl sulfoxide (DMSO), tetramethyl ammonium chloride (TMAC) and betaine for optimization. However, most of the primer pairs did not work properly and produce the desired bands with PCR reactions followed by an agarose gel analysis. For Phenol hydroxylase gene, five different primer pairs were used to find the gene if located in the isolates. As mentioned in the literature review, this gene was the first enzyme found in the degradation of phenolic compounds[53,94–99]. In addition, the isolated strains have resistance against phenolic compounds due to the growth area (olive orchard) and tolerance test with the phenolic compound. Thus, the isolates should have this gene in their chromosomes. However, it was not possible to amplify the gene region with designed primer pairs by using total genomic DNA (Figure 7).

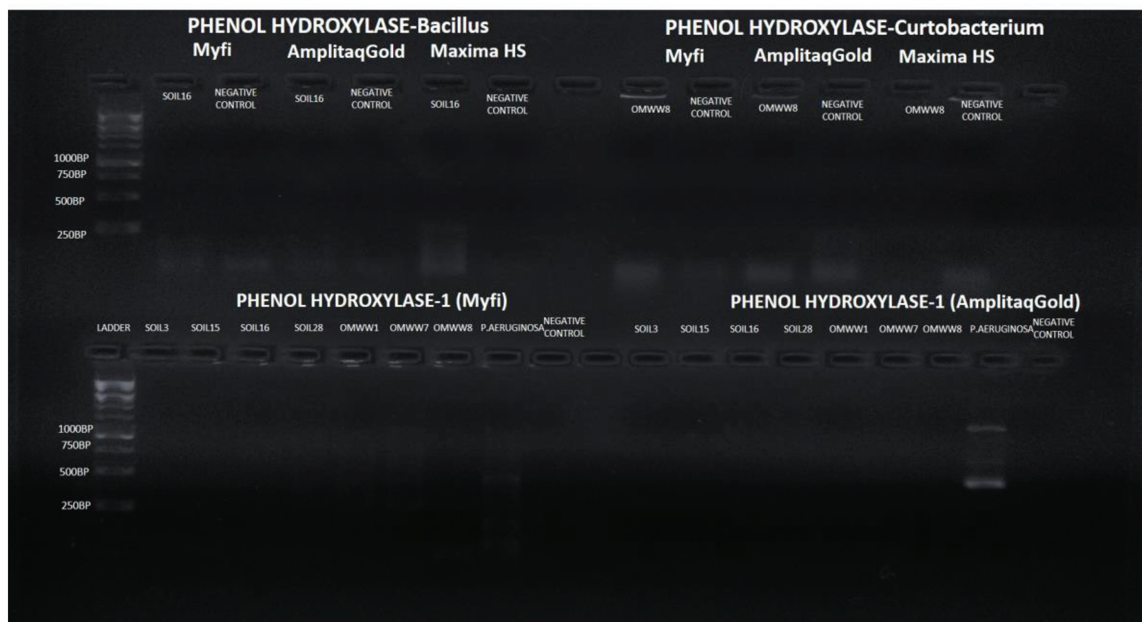


Figure 8. PCR amplification of phenol hydroxylase gene from the genomic DNA of selected strains with three different primer pairs by using Amplitaq Gold polymerase (Applied Biosystems), MyFi (Bioline), and Maxima HS (Fermentas). Line 1 & Line 16: 1 kb DNA size marker (GeneDirex) (250, 500, 750, 1000, 1500 bp….). (Phenol hydroxylase 1-PheU-F and PheU-R, Phenol hydroxylase-Bacillus- Bacphe-F and Bacphe-R, Phenol hydroxylase-Curtobacterium- Curto-F and Curto-R primer pairs)

Due to unsuccessful trials with chromosomal DNA as the template, it was decided to use cDNA as the template in these PCR reactions. Then the RNA isolation were performed and cDNA were produced. Only PheU primer pairs produced bands after a PCR reaction with cDNA as the template however single bands were not obtained for all strains (Figure 8). Only, Soil16 produced the single band for ~750 bp and positive control produced multiple band with the desired band for ~600 bp. These fragments were subjected to sequencing; however, it was resulted no analyzable data due to the excess bands and dimers.

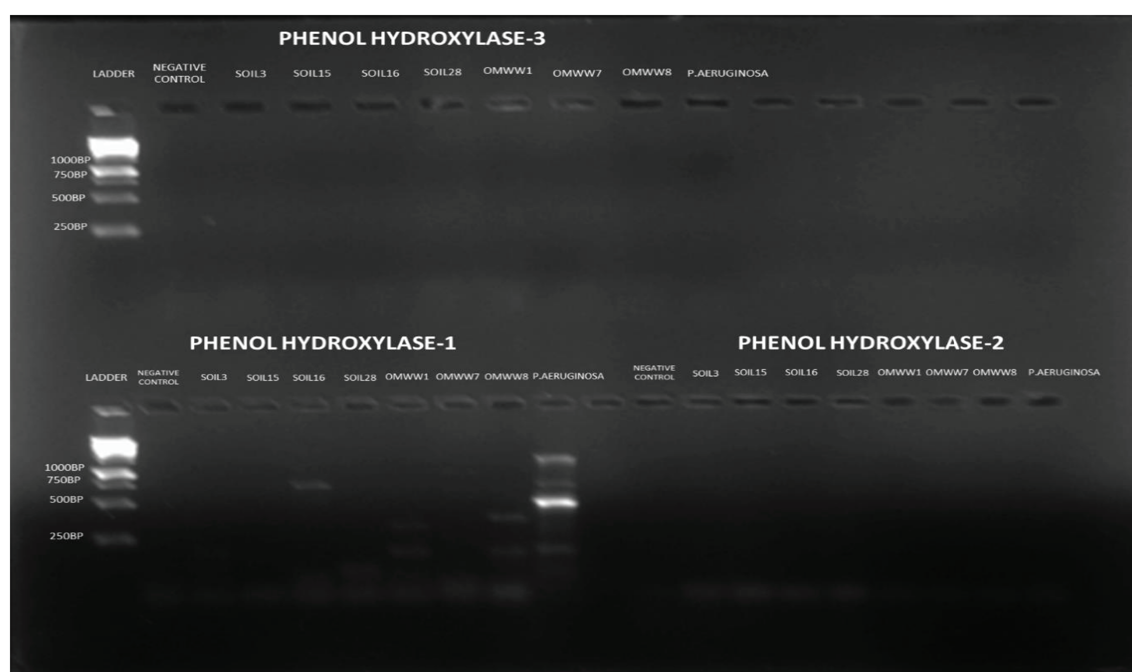


Figure 9. PCR amplification of phenol hydroxylase gene from the cDNA of selected strains with three different primer pairs by using Amplitaq Gold polymerase (Applied Biosystems). Line 1 & Line 16: 1 kb DNA size marker (GeneDirex) (250, 500, 750, 1000, 1500 bp….). (Phenol hydroxylase 1-PheU-F and PheU-R, Phenol hydroxylase-2=PhenolH-F and PhenolH-R s, and Phenol hydroxylase-3=Phe-F and Phe-R primer pairs).

Three different primer pairs were used for amplification of catechol 1,2-dioxygenase gene in the isolated strains. Firstly, PCR reactions were applied by using genomic DNA as it is done for phenol hydroxylase gene. However, no bands were obtained for all strains except for the positive control. On the other hand, PCR trial with the cDNA as the template resulted a band production with the desired size for two of the designed primers however, it was not possible to get rid of the formation of the primer dimers and multiple bands. The results of PCR product of the gene for all three primer

pairs with the cDNA as the template is presented in Figure 9. The bands were obtained for CAT12 and Catec12 primer pairs. Then, the sequencing analysis were performed for five strain which labeled with red rectangular in Figure 9.

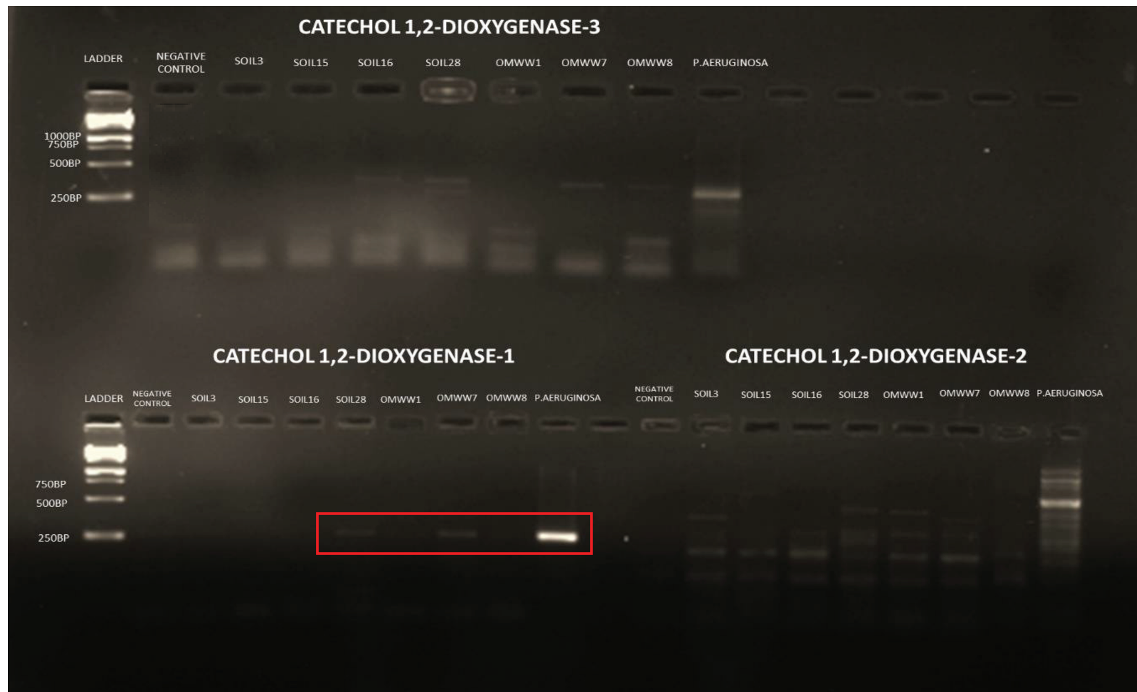


Figure 10. PCR amplification of catechol 1,2-dioxygenase gene from the cDNA of selected strains with three different primer pairs by using Amplitaq Gold polymerase (Applied Biosystems). Line 1 & Line 16: 1 kb DNA size marker (GeneDirex) (250, 500, 750, 1000, 1500 bp….). (Catechol 1,2-dioxygenase-1=CAT12-F and CAT12-R, Catechol 1,2-dioxygenase-2=C12-F and C12-R, Catechol 1,2-dioxygenase-3= Catec12 -F and Catec12-R primer pairs).

After sequencing of the PCR product of catechol 1,2-dioxygenase genes of selected strains, the results were analyzed by using BLAST program. A search for homology in gene banks (GenBank/NCBI for genes) indicated that the partial nucleotide of catechol 1,2-dioxygenase had high similarity with each other for all these five strains in Figure 10. According to the BLAST results, Soil28, OMWW7, OMWW8, and positive control displayed higher homology with catechol 1,2-dioxygenase of the *Pseudomonas* genus (OMWW7-95%OMWW8-95%, Soil28-%98 and positive control (*P. aeruginosa*)-99%). For OMWW1 strain, higher homology was obtained for catechol 1,2-dioxygenase gene of *Klebsiella aerogenes* strains with 92% homology. In addition, multiple sequences alignments were performed to find the relationship of sequences obtained from the isolated strains. As shown in Figure 11, the homology was very high between sequences.

Several of the sequences was short do to the low quality and yield of the PCR product as seen in the Figure 9.

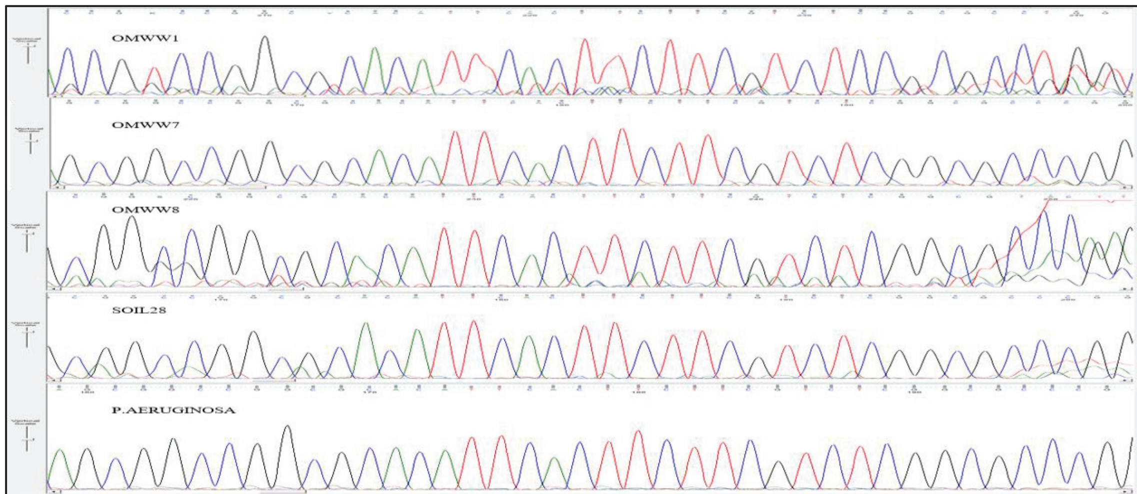


Figure 11. Electropharogram of the catechol 1,2-dioxygenase gene sequences of the isolates Soil28, OMWW1, OMWW7, OMWW8 and P.aeruginosa (positive control).

Figure 12. Multiple sequences alignment of the catechol 1,2-dioxygenase genes of selected strains by using T-coffee program.

We know that these strains were different from each other according to the partial 16S rRNA gene sequences. Also, the PCR product were not obtained with the same primer pairs except for the positive control when genomic DNA were used. On the other hand, the bands were obtained for several strains when cDNA were used. A possible

explanation for these results may be horizontal gene transfer between species. A horizontal gene transfer could be accomplished by conjugation, transformation, and transduction both in vitro and under natural conditions. In this context, bacterial plasmids encoding resistance genes, degradative genes, and tolerance to stress conditions are of much significance. Soil ecosystems harbor an extremely broad diversity of microbiota reflecting plant type, soil type, soil management regime, and other factors. The physical environment of soil is quite heterogeneous in terms of gaseous, liquid or solid phases. Also, abiotic factors (temperature, oxygen, pH and etc.) and biotic factors (antagonistic, commensal, mutualistic relationships) in soil may affect positively the frequencies of horizontal gene transfer. Thus, it seems possible that these results are due to the finding of high homology gene sequences for different strains.

CHAPTER 4

CONCLUSION

The present study was designed to isolate and identify phenolic compound degrading bacteria from olive orchard and also for the assessment the related genes for phenolic compound degradation. We selected resistant strains among all bacterial strain based on tolerance tests up to 5 mM of 3-HPAA. In order to identify the selected strains, 16S rRNA gene analysis were performed. As a result, they were identified as into 8 different strains due to some of the isolated strains come from the same species. The investigation of 16S rRNA has shown that most of the selected strains were identified and used in many previous studies related to phenolic compound degradation experiments such as *Pseudomonas sp., Bacillus sp., and Pantoea sp.* On the other hand, this is the first time that *Curtobacterium sp*. has been explored for degradation of phenolic compounds. It was also shown that a strain was identified with 99.63% identity as *Bacillus ginsenghumi sp.* which isolated and identified from ginseng orchard. The phenolic compound degradation capacity of this strain was also indicated firstly in this study. Taken together, these results suggest that these two strains were

In the assessment of the related genes part of the study, to be able to find the nucleotide sequence of phenol hydroxylase and catechol 1, 2-dioxygenase genes, we carefully designed pairs of degenerate primer based on the highly conserved regions in the selected similar species with our identified strains. For the genomic DNA, the gene regions were not amplified with all primer pairs except for positive control. The degenerate primers might be one of this reason for unsuccessful result due to the inadequate consensus region of sequence of these genes in database. On the other hand, sequencing of hydroxylase and catechol 1, 2-dioxygenase were performed for cDNA of selected strains and positive control, the amplification of catechol 1,2-dioxygenase were successful for some of the selected strains and positive control. Their sequence identities were more than 90% for the same species (*Pseudomonas aeruginosa sp.*) except for OMWW1 (92%-*Klebsiella aerogenes sp.*). Our work suggests that there is no correlation between the phylogenetic groupings of phenol-degrading bacteria and their phenol

degrading genes genotypes possibly due to extensive horizontal gene transfer of this functional gene. Genes that encode the degradation of organic compounds are often located on plasmids and, consequently, they can be spread by horizontal gene transfer into ecologically competitive and indigenous bacterial populations.

Future trials should assess the measurement of phenolic compound degradation capacity o phenolic compound pollutant. Therefore, the new and more resistant strain might explore to use in bioremediation, especially OMWW pollutant. In addition, more work will need to be done to determine degradation related genes of selected strains by using gene expression and protein experiments. More information on these genes would help us to establish a greater degree of accuracy on this matter. Thus, recombinant production of these enzymes might be done with higher degradation capacity than currently used phenolic degrading microorganisms.

REFERENCES

(1) Baruah, J. B. *Chemistry of Phenolic Compounds: State of the Art*; 2011.

(2) Kabera, J. N.; Semana, E.; Mussa, A. R.; He, X. Pvv0Plant Secondary Metabolites: Biosynthesis, Classification, Function and Pharmacological Properties. *J. Pharm. Pharmacol.* **2014**, *2*, 377–392.

(3) Ho, C.-T. Phenolic Compounds in Food. **2009**, No. 4, 2–7. https://doi.org/10.1021/bk-1992-0506.ch001.

(4) Vermerris, W.; Nicholson, R. *Phenolic Compound Biochemistry*; 2006. https://doi.org/10.1007/978-1-4020-5164-7.

(5) Tsimogiannis, D.; Oreopoulou, V. Classification of Phenolic Compounds in Plants. *Polyphenols in Plants* **2018**, 263–284. https://doi.org/10.1016/b978-0-12-813768-0.00026-8.

(6) Pereira, D. M.; Valentão, P.; Pereira, J. A.; Andrade, P. B. Phenolics: From Chemistry to Biology. *Molecules* **2009**, *14* (6), 2202–2211. https://doi.org/10.3390/molecules14062202.

(7) Gutiérrez-Grijalva, Erick & Ambriz-Pérez, Dulce & Leyva-López, Nayely & Castillo, Ramon & Heredia, José. (2016). Review: Dietary phenolic compounds, health benefits and bioaccessibility. Archivos Latinoamericanos de Nutrición. 66. 87-100.

(8) Ramawat, K. G.; Mérillon, J. M. Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes. *Nat. Prod. Phytochem. Bot. Metab. Alkaloids, Phenolics Terpenes* **2013**, No. January, 1–4242. https://doi.org/10.1007/978-3-642-22144-6.

(9) Panche, A. N.; Diwan, A. D.; Chandra, S. R. Flavonoids: An Overview. *J. Nutr. Sci.* **2016**, *5*. https://doi.org/10.1017/jns.2016.41.

(10) Access, O. We Are IntechOpen , the World ' s Leading Publisher of Open Access Books Built by Scientists , for Scientists TOP 1 %. *Intech* **2016**, *i* (tourism), 13. https://doi.org/http://dx.doi.org/10.5772/57353.

(11) Wang, D. S. Y. Secondary Metabolites from Plants. **2012**, 1–52.

(12) Crozier, A.; Jaganath, I. B.; Clifford, M. N. Phenols, Polyphenols and Tannins: An Overview. *Plant Second. Metab. Occur. Struct. Role Hum. Diet* **2007**, No. i, 1–24.

https://doi.org/10.1002/9780470988558.ch1.

(13) Factors, S. P.; Amirjani, M. R. Int . J . Forest , Soil and Erosion , 2012 2 (1): 50-58 ISSN 2251-6387 © February 2012 , GHB ' s Journals , IJFSE , Shabestar , Iran Full Length Research Paper. *Int. J.* **2012**, *2* (February), 50–58.

(14) M. Akram. Flavonoids and Phenolic Acids: Role and Biochemical Activity in Plants and Human. *J. Med. Plants Res.* **2012**, *5* (32), 6697–6703. https://doi.org/10.5897/jmpr11.363.

(15) Ramawat, K. G.; Mérillon, J. M. Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes. *Nat. Prod. Phytochem. Bot. Metab. Alkaloids, Phenolics Terpenes* **2013**, No. August 2015, 1–4242. https://doi.org/10.1007/978-3-642-22144-6.

(16) M. Akram. Flavonoids and Phenolic Acids: Role and Biochemical Activity in Plants and Human. *J. Med. Plants Res.* **2012**, *5* (32). https://doi.org/10.5897/jmpr11.363.

(17) Pietta, P.; Minoggio, M.; Bramati, L. Plant Polyphenols: Structure, Occurrence and Bioactivity. *Stud. Nat. Prod. Chem.* **2003**, *28*, 257–312. https://doi.org/10.1016/S1572-5995(03)80143-6.

(18) Robbins, R. J. Phenolic Acids in Foods: An Overview of Analytical Methodology. *J. Agric. Food Chem.* **2003**, *51* (10), 2866–2887. https://doi.org/10.1021/jf026182t.

(19) Alam, M. A.; Subhan, N.; Hossain, H.; Hossain, M.; Reza, H. M.; Rahman, M. M.; Ullah, M. O. Hydroxycinnamic Acid Derivatives: A Potential Class of Natural Compounds for the Management of Lipid Metabolism and Obesity. *Nutr. Metab.* **2016**, *13* (1). https://doi.org/10.1186/s12986-016-0080-3.

(20) Vinholes, J.; Silva, B. M.; Silva, L. R. Hydroxycinnamic Acids (HCAS): Structure, Biological Properties and Health Effects. *Adv. Med. Biol.* **2015**, *88* (July), 1–345.

(21) Alam, M. A.; Subhan, N.; Hossain, H.; Hossain, M.; Reza, H. M.; Rahman, M. M.; Ullah, M. O. Hydroxycinnamic Acid Derivatives: A Potential Class of Natural Compounds for the Management of Lipid Metabolism and Obesity. *Nutr. Metab.* **2016**, *13* (1), 1–13. https://doi.org/10.1186/s12986-016-0080-3.

(22) Rozi, Z. A. Medern Web Design. **2016**, *2* (4), 16.

(23) Tomás-Barberán, F. A.; Espín, J. C. Phenolic Compounds and Related Enzymes as Determinants of Quality in Fruits and Vegetables. *J. Sci. Food Agric.* **2001**, *81* (9), 853–876. https://doi.org/10.1002/jsfa.885.

(24) Kumar, S.; Pandey, A. K. HÓA HỌC VÀ HÓA SINH FLAVONOID.Pdf. **2013**, *2013*. https://doi.org/10.1016/S1572-5995(05)80065-1.

(25) Huang, M. T.; Ho, C. T.; Lee, C. Y. *Phenolic Compounds in Food and Their Effects on Health II: Antioxidants and Cancer Prevention.*; 1992.

(26) Ho, C.-T.; Lee, C.; Huang, M.-T. *Phenolic Compounds in Food and Their Effects on Health: Volume I: Analysis, Occurrence, and Chemistry*; 1992.

(27) Hsieh, P. C.; Mau, J. L.; Huang, S. H. Antimicrobial Effect of Various Combinations of Plant Extracts. *Food Microbiol.* **2001**, *18* (1), 35–43. https://doi.org/10.1006/fmic.2000.0376.

(28) Quave, C. L.; Health, H. Antibiotics from Nature: Traditional Medicine as a Source of New Solutions for Combating Antimicrobial Resistance. **2016**, 1–20.

(29) Taguri, T.; Tanaka, T.; Kouno, I. Antimicrobial Activity of 10 Different Plant Polyphenols against Bacteria Causing Food-Borne Disease. *Biol. Pharm. Bull.* **2004**, *27* (12), 1965–1969. https://doi.org/10.1248/bpb.27.1965.

(30) Borges, A.; Ferreira, C.; Saavedra, M. J.; Simões, M. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids Against Pathogenic Bacteria. *Microb. Drug Resist.* **2013**, *19* (4), 256–265. https://doi.org/10.1089/mdr.2012.0244.

(31) Pereira, A. P.; Ferreira, I. C. F. R.; Marcelino, F.; Valentão, P.; Paula, B.; Seabra, R.; Estevinho, L.; Bento, A.; Pereira, J. A. Phenolic Compounds and Antimicrobial Activity of Olive (. *Molecules* **2007**, 1153–1162.

(32) Boskou, D.; Blekas, G.; Tsimidou, M. Phenolic Compounds in Olive Oil and Olives , D . Boskou , G Blekas and M . Tsimidou. *Curr. Top. Nutraceutical Res.* **2005**, *3* (2), 125–136.

(33) Ryan, D.; Robards, K. Phenolic Compounds in Olives. *Analyst* **1998**, *123* (5), 31–44. https://doi.org/10.1039/a708920a.

(34) Franco, M. N.; Galeano-Díaz, T.; López, Ó.; Fernández-Bolaños, J. G.; Sánchez, J.; De Miguel, C.; Gil, M. V.; Martín-Vertedor, D. Phenolic Compounds and Antioxidant Capacity of Virgin Olive Oil. *Food Chem.* **2014**, *163* (November 2015), 289–298. https://doi.org/10.1016/j.foodchem.2014.04.091.

(35) Ryan, D.; Robards, K. Critical Review. Phenolic Compounds in Olives. *Analyst* **2005**, *123* (5), 31R. https://doi.org/10.1039/a708920a.

(36) Markín, D.; Duek, L.; Berdícevsky, I. In Vitro Antimicrobial Activity of Olive Leaves. *Mycoses* **2003**, *46* (3–4), 132–136. https://doi.org/10.1046/j.1439-0507.2003.00859.x.

(37) Tuck, K. L.; Hayball, P. J. Major Phenolic Compounds in Olive Oil: Metabolism and Health Effects. *J. Nutr. Biochem.* **2002**, *13* (11), 636–644. https://doi.org/10.1016/S0955-2863(02)00229-2.

(38) ŞAHAN, Y.; ÇELİK, G.; DOĞANGÜN, E. ORGANİK VeKonvansiyonel OlarakÜretilen SizmaZeytinyağlarinin BaziFenolİçeriklerinin Karşilaştirilmasi. *Uludağ Univ. J. Fac. Eng.* **2017**, *22* (3), 179–186. https://doi.org/10.17482/uumfd.372458.

(39) Alagna, F.; Mariotti, R.; Panara, F.; Caporali, S.; Urbani, S.; Veneziani, G.; Esposto, S.; Taticchi, A.; Rosati, A.; Rao, R.; et al. Olive Phenolic Compounds: Metabolic and Transcriptional Profiling during Fruit Development. *BMC Plant Biol.* **2012**, *12* (1), 1. https://doi.org/10.1186/1471-2229-12-162.

(40) Schneider, S. Quality Analysis of Extra Virgin Olive Oils – Part 6 Nutritive Benefi Ts – Phenolic Compounds in Virgin Olive Oil.

(41) Ntougias, S.; Bourtzis, K.; Tsiamis, G. The Microbiology of Olive Mill Wastes. *Biomed Res. Int.* **2013**, *2013*, 1–16. https://doi.org/10.1155/2013/784591.

(42) Maza-Márquez, P.; Martínez-Toledo, M. V.; González-López, J.; Rodelas, B.; Juárez-Jiménez, B.; Fenice, M. Biodegradation of Olive Washing Wastewater Pollutants by Highly Efficient Phenol-Degrading Strains Selected from Adapted Bacterial Community. *Int. Biodeterior. Biodegrad.* **2013**, *82*, 192–198. https://doi.org/10.1016/j.ibiod.2013.03.025.

(43) Bhuyan, D. J.; Basu, A. Or d Fr an on Bu y r P Potential Health Benefits and Toxicity l U Phenolic Compounds Se Y. **2017**, No. May 2018.

(44) Thomas, S.; Sarfaraz, S.; Mishra, L. C.; Iyengar, L. Degradation of Phenol and Phenolic Compounds by a Defined Denitrifying Bacterial Culture. *World J. Microbiol. Biotechnol.* **2002**, *18* (1), 57–63. https://doi.org/10.1023/A:1013947722911.

(45) Khorasani, A. C.; Mashreghi, M.; Yaghmaei, S. Study on Biodegradation of Mazut by Newly Isolated Strain Enterobacter Cloacae BBRC10061: Improving and Kinetic Investigation. *Iran. J. Environ. Heal. Sci. Eng.* **2013**, *10* (2), 1. https://doi.org/10.1186/1735-2746-10-2.

(46) Unell, M.; Nordin, K.; Jernberg, C.; Stenström, J.; Jansson, J. K. Degradation of Mixtures of Phenolic Compounds by Arthrobacter Chlorophenolicus A6. *Biodegradation* **2008**, *19* (4), 495–505. https://doi.org/10.1007/s10532-007-9154-2.

(47) Mann, J. Microbial Bioremediation of Olive Mill Wastewater. **2011**, No. August.

(48) Landete, J. M.; Rodriguez, H.; Curiel, J. A.; de las Rivas, B.; de Felipe, F. L.; Muñoz, R. *Degradation of Phenolic Compounds Found in Olive Products by Lactobacillus Plantarum Strains*; Elsevier Inc., 2010. https://doi.org/10.1016/B978-0-12-374420-3.00043-7.

(49) Krastanov, A.; Alexieva, Z.; Yemendzhiev, H. Microbial Degradation of Phenol and Phenolic Derivatives. *Eng. Life Sci.* **2013**, *13* (1), 76–87. https://doi.org/10.1002/elsc.201100227.

(50) Ahmad, S. A.; Syed, M. A.; Arif, N. M.; Yunus, M.; Shukor, A.; Shamaan, A. Isolation , Identification and Characterization of Elevated Phenol Degrading. **2011**, *5* (8), 1035–1045.

(51) Nawawi, N. M.; Ahmad, S. A.; Shukor, M. Y.; Syed, M. A.; Ibrahim, L. Isolation and Characterization of Phenol-Degrading Microorganism : Recent Advances. *J. Environ. Bioremediation Toxicol.* **2014**, *2* (1), 11–22.

(52) Mohanty, S. S. Microbial Degradation of Phenol: A Comparative Study. **2012**, No. January, 137.

(53) Silva, C. C.; Hayden, H.; Sawbridge, T.; Mele, P.; De Paula, S. O.; Silva, L. C. F.; Vidigal, P. M. P.; Vicentini, R.; Sousa, M. P.; Torres, A. P. R.; et al. Identification of Genes and Pathways Related to Phenol Degradation in Metagenomic Libraries from Petroleum Refinery Wastewater. *PLoS One* **2013**, *8* (4), 1–11. https://doi.org/10.1371/journal.pone.0061811.

(54) Long, Y.; Yang, S.; Xie, Z.; Cheng, L. Identification and Characterization of Phenol Hydroxylase from Phenol-Degrading Candida Tropicalis Strain JH8 . *Can. J. Microbiol.* **2014**, *60* (9), 585–591. https://doi.org/10.1139/cjm-2014-0417.

(55) Wang, C. H.; Lu, J. W.; Wei, H. H.; Takeda, M. Functional Model for Intradiol-Cleaving Catechol 1,2-Dioxygenase: Synthesis, Structure, Spectra, and Catalytic Activity of Iron(III) Complexes with Substituted Salicylaldimine Ligands. *Inorganica Chim. Acta* **2007**, *360* (9), 2944–2952. https://doi.org/10.1016/j.ica.2007.02.034.

(56) Hanafi, F.; Mountadar, M.; Etahiri, S.; Fekhaoui, M.; Assobhei, O. Biodegradation of Toxic Compounds in Olive Mill Wastewater by a Newly Isolated Potent Strain: <I>Aspergillus Niger</I> van Tieghem. *J. Water Resour. Prot.* **2013**, *05* (08), 768–774. https://doi.org/10.4236/jwarp.2013.58078.

(57) Rahmanian, N.; Jafari, S. M.; Galanakis, C. M. Recovery and Removal of Phenolic

Compounds from Olive Mill Wastewater. *JAOCS, J. Am. Oil Chem. Soc.* **2014**, *91* (1), 1–18. https://doi.org/10.1007/s11746-013-2350-9.

(58) Ammar, E.; Nasri, M.; Medhioub, K. Isolation of Enterobacteria Able to Degrade Simple Aromatic Compounds from the Wastewater from Olive Oil Extraction. *World J. Microbiol. Biotechnol.* **2005**, *21* (3), 253–259. https://doi.org/10.1007/s11274-004-3625-y.

(59) Turner, S.; Pryer, K. M.; Miao, V. P. W.; Palmer, J. D. Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit RRNA Sequence Analysis. *J. Eukaryot. Microbiol.* **1999**, *46* (4), 327–338. https://doi.org/10.1111/j.1550-7408.1999.tb04612.x.

(60) Wafula, E. N.; Kinyua, J.; Kariuki, D.; Muigai, A.; Mwirichia, R.; Kibet, T. Morphological Characterization of Soil Bacteria in Ngere Tea Catchment Area of Murang'a County, Kenya. *Int. J. Life Sci. Res.* **2015**, *3* (1), 2348–313.

(61) Young, K. D. The Selective Value of Bacterial Shape. *Microbiol. Mol. Biol. Rev.* **2006**, *70* (3), 660–703. https://doi.org/10.1128/mmbr.00001-06.

(62) Young, K. D. Bacterial Morphology: Why Have Different Shapes? *Curr. Opin. Microbiol.* **2007**, *10* (6), 596–600. https://doi.org/10.1016/j.mib.2007.09.009.

(63) Liu, G.; Jin, M.; Cai, C.; Ma, C.; Chen, Z.; Gao, L. Soil Microbial Community Structure and Physicochemical Properties in Amomum Tsaoko-Based Agroforestry Systems in the Gaoligong Mountains, Southwest China. *Sustain.* **2019**, *11* (2), 1–14. https://doi.org/10.3390/su11020546.

(64) Steubing, P. M. Chapter 6 Isolation of an Unknown Bacterium from Soil. *Education* **1993**, 81–113.

(65) Janda, J. M.; Abbott, S. L. 16S RRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *J. Clin. Microbiol.* **2007**, *45* (9), 2761–2764. https://doi.org/10.1128/JCM.01228-07.

(66) Benga, L.; Benten, W. P. M.; Engelhardt, E.; Köhrer, K.; Gougoula, C.; Sager, M. 16s Ribosomal DNA Sequence-Based Identification of Bacteria in Laboratory Rodents: A Practical Approach in Laboratory Animal Bacteriology Diagnostics. *Lab. Anim.* **2014**, *48* (4), 305–312. https://doi.org/10.1177/0023677214538240.

(67) O'Hara, C. M.; Steigerwalt, A. G.; Hill, B. C.; Farmer, J. J.; Fanning, G. R.; Brenner, D. J. Enterobacter Hormaechei, a New Species of the Family Enterobacteriaceae Formerly Known as Enteric Group 75. *J. Clin. Microbiol.* **1989**, *27* (9), 2046–2049.

(68) Khalifa, A. Y. Z.; Alsyeeh, A. M.; Almalki, M. A.; Saleh, F. A. Characterization of the Plant Growth Promoting Bacterium, Enterobacter Cloacae MSR1, Isolated from Roots of Non-Nodulating Medicago Sativa. *Saudi J. Biol. Sci.* **2016**, *23* (1), 79–86. https://doi.org/10.1016/j.sjbs.2015.06.008.

(69) Wei, Y.; Yang, Y.; Zhou, L.; Liu, Z.; Wang, X.; Yang, R.; Su, Q.; Zhou, Y.; Zhao, J.; Yang, J. Genome Sequence of Enterobacter Cancerogenus YZ1. *Genome Announc.* **2013**, *1* (1), 3108. https://doi.org/10.1128/genomea.00023-13.

(70) Zhao, X.; Liu, M.; Liu, Y.; Li, Y.; Zhang, S.; Du, K. Aerobic Degradation of 4-Monobrominated Diphenyl Ether: Identifying and Characterizing Three Strains Isolated from Populus Tomentosa Phyllosphere. *Polish J. Environ. Stud.* **2017**, *26* (3), 1385–1392. https://doi.org/10.15244/pjoes/68154.

(71) Zhang, Z. Y.; Pan, L. P.; Li, H. H. Isolation, Identification and Characterization of Soil Microbes Which Degrade Phenolic Allelochemicals. *J. Appl. Microbiol.* **2010**, *108* (5), 1839–1849. https://doi.org/10.1111/j.1365-2672.2009.04589.x.

(72) Reardon, K. F.; Mosteller, D. C.; Bull Rogers, J. D. Biodegradation Kinetics of Benzene, Toluene, and Phenol as Single and Mixed Substrates for Pseudomonas Putida F1. *Biotechnol. Bioeng.* **2000**, *69* (4), 385–400. https://doi.org/10.1002/1097-0290(20000820)69:4<385::AID-BIT5>3.0.CO;2-Q.

(73) Margesin, R.; Bergauer, P.; Gander, S. Degradation of Phenol and Toxicity of Phenolic Compounds: A Comparison of Cold-Tolerant Arthrobacter Sp. and Mesophilic Pseudomonas Putida. *Extremophiles* **2004**, *8* (3), 201–207. https://doi.org/10.1007/s00792-004-0378-3.

(74) Hinteregger, C.; Leitner, R.; Loidl, M.; Ferschl, A.; Streichsbier, F. Degradation of Phenol and Phenolic Compounds by Pseudomonas Putida EKII. *Appl. Microbiol. Biotechnol.* **1992**, *37* (2), 252–259. https://doi.org/10.1007/BF00178180.

(75) Shourian, M.; Noghabi, K. A.; Zahiri, H. S.; Bagheri, T.; Karballaei, G.; Mollaei, M.; Rad, I.; Ahadi, S.; Raheb, J.; Abbasi, H. Efficient Phenol Degradation by a Newly Characterized Pseudomonas Sp. SA01 Isolated from Pharmaceutical Wastewaters. *Desalination* **2009**, *246* (1–3), 577–594. https://doi.org/10.1016/j.desal.2008.07.015.

(76) Coutinho, T. A.; Venter, S. N. Pantoea Ananatis: An Unconventional Plant Pathogen. *Mol. Plant Pathol.* **2009**, *10* (3), 325–335. https://doi.org/10.1111/j.1364-3703.2009.00542.x.

(77) Dutkiewicz, J.; Mackiewicz, B.; Lemieszek, M. K.; Golec, M.; Milanowski, J. Pantoea Agglomerans: A Mysterious Bacterium of Evil and Good. Part IV. Beneficial Effects. *Ann. Agric. Environ. Med.* **2016**, *23* (2), 206–222. https://doi.org/10.5604/12321966.1203879.

(78) Hasan, S. A.; Jabeen, S. Degradation Kinetics and Pathway of Phenol by Pseudomonas and Bacillus Species. *Biotechnol. Biotechnol. Equip.* **2015**, *29* (1), 45–53. https://doi.org/10.1080/13102818.2014.991638.

(79) Engineering, G. Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11010. **2011**, *63* (4), 1057–1067. https://doi.org/10.2298/ABS1104057.

(80) Banerjee, A.; Ghoshal, A. K. Phenol Degradation by Bacillus Cereus: Pathway and Kinetic Modeling. *Bioresour. Technol.* **2010**, *101* (14), 5501–5507. https://doi.org/10.1016/j.biortech.2010.02.018.

(81) Brenner, D. J.; McWhorter, A. C.; Knutson, K. L.; Steigerwalt, A. G. Escherichia Vulneris: A New Species of Enterobacteriacea Associated with Human Wounds. *J. Clin. Microbiol.* **1982**, *15* (6), 1133–1140.

(82) Byappanahalli, M. N.; Nevers, M. B.; Korajkic, A.; Staley, Z. R.; Harwood, V. J. Enterococci in the Environment. *Microbiol. Mol. Biol. Rev.* **2012**, *76* (4), 685–706. https://doi.org/10.1128/mmbr.00023-12.

(83) Erginkaya, Z.; Yurdakul, N. E.; Karakaş, A. The Properties of Enterococcus Faecium and Enterococcus Faecalis as a Starter and Probiotic Cultures. *Gıda* **2007**, *32* (3), 137–142.

(84) Jha, A. K.; Bais, H. P.; Vivanco, J. M. Enterococcus Faecalis Mammalian Virulence-Related Factors Exhibit Potent Pathogenicity in the Arabidopsis Thaliana Plant Model. *Infect. Immun.* **2005**, *73* (1), 464–475. https://doi.org/10.1128/IAI.73.1.464-475.2005.

(85) Chen, Y. S.; Yanagida, F.; Shinohara, I. Isolation and Identification of Lactic Acid Bacteria from Soil Using an Enrichment Procedure. *Lett. Appl. Microbiol.* **2005**, *40* (3), 195–200. https://doi.org/10.1111/j.1472-765X.2005.01653.x.

(86) Anagnostopoulos, D. A.; Bozoudi, D.; Tsaltas, D. Enterococci Isolated from Cypriot Green Table Olives as a New Source of Technological and Probiotic Properties. *Fermentation* **2018**, *4* (2). https://doi.org/10.3390/fermentation4020048.

(87) El-Bestawy, E. A.; Gohar, Y. M.; Wefky, S. M. Antimicrobial Phenolic

Compounds from Enterococcus Faecium S29 (EU 158188): Characterization and Production Optimization. *Int. J. Environ. Sci. Technol.* **2017**, *14* (3), 497–508. https://doi.org/10.1007/s13762-016-1161-6.

(88) Ten, L. N.; Im, W. T.; Baek, S. H.; Lee, J. S.; Oh, H. M.; Lee, S. T. Bacillus Ginsengihumi Sp. Nov., a Novel Species Isolated from Soil of a Ginseng Field in Pocheon Province, South Korea. *J. Microbiol. Biotechnol.* **2006**, *16* (10), 1554–1560.

(89) Chung, I. M.; Lim, J. J.; Ahn, M. S.; Jeong, H. N.; An, T. J.; Kim, S. H. Comparative Phenolic Compound Profiles and Antioxidative Activity of the Fruit, Leaves, and Roots of Korean Ginseng (Panax Ginseng Meyer) According to Cultivation Years. *J. Ginseng Res.* **2016**, *40* (1), 68–75. https://doi.org/10.1016/j.jgr.2015.05.006.

(90) Parikh, M.; Raj, P.; Yu, L.; Stebbing, J.-A.; Prashar, S.; Petkau, J.; Tappia, P.; Pierce, G.; Siow, Y.; Brown, D.; et al. Ginseng Berry Extract Rich in Phenolic Compounds Attenuates Oxidative Stress but Not Cardiac Remodeling Post Myocardial Infarction. *Int. J. Mol. Sci.* **2019**, *20* (4), 983. https://doi.org/10.3390/ijms20040983.

(91) Yamada, K.; Komagata, K. Taxonomic Studies on Coryneform Bacteria: V Classification of Coryneform Bacteria. *J. Gen. Appl. Microbiol.* **1972**, *18* (6), 417–431. https://doi.org/10.2323/jgam.18.417.

(92) Seyedi, S. M.; Rajaei, S.; Raiesi, F.; Shiran, B.; Raheb, J. Characterization and Potentials of Indigenous Oil-Degrading Bacteria Inhabiting the Rhizosphere of Wild Oat (Avena Fatua L.) in South West of Iran. *Iran. J. Biotechnol.* **2013**, *11* (1), 32–40. https://doi.org/10.5812/ijb.9334.

(93) Bay, O. Al of Micro Obiology R B Can Nker Pa Athoge Ens Cou Uld Co Mplica Ate the e Man Ageme Ent of Phytop Ra Blac Ck Pod of Coc Coa. **2014**, *8* (33), 3094–3100. https://doi.org/10.5897/A.

(94) Tian, M.; Du, D.; Zhou, W.; Zeng, X.; Cheng, G. Phenol Degradation and Genotypic Analysis of Dioxygenase Genes in Bacteria Isolated from Sediments. *Brazilian J. Microbiol.* **2017**, *48* (2), 305–313. https://doi.org/10.1016/j.bjm.2016.12.002.

(95) Nešvera, J.; Rucká, L.; Pátek, M. Catabolism of Phenol and Its Derivatives in Bacteria: Genes, Their Regulation, and Use in the Biodegradation of Toxic Pollutants. *Adv. Appl. Microbiol.* **2015**, *93*, 107–160.

https://doi.org/10.1016/bs.aambs.2015.06.002.

(96) Haffter, P.; Granato, M.; Brand, M.; Mullins, M. C.; Hammerschmidt, M.; Kane, D. A.; Odenthal, J.; Van Eeden, F. J. M.; Jiang, Y. J.; Heisenberg, C. P.; et al. The Identification of Genes with Unique and Essential Functions in the Development of the Zebrafish, Danio Rerio. *Development* **1996**, *123*, 1–36.

(97) Davis, B. L. IDENTIFICATION AND CHARACTERISATION OF BACTERIAL GENES ASSOCIATED WITH RESISTANCE TO AND / OR DEGRADATION OF ENVIRONMENTAL POLLUTANTS By. **2011**.

(98) Nacke, H.; Will, C.; Herzog, S.; Nowka, B.; Engelhaupt, M.; Daniel, R. Identification of Novel Lipolytic Genes and Gene Families by Screening of Metagenomic Libraries Derived from Soil Samples of the German Biodiversity Exploratories. *FEMS Microbiol. Ecol.* **2011**, *78* (1), 188–201. https://doi.org/10.1111/j.1574-6941.2011.01088.x.

(99) Litova, K. Sequence Analyses of Genes Coding Key Enzymes Responsible for Degradation of Phenolic Compounds by Aspergillus Fumigatus Strain AL3. **2015**, 195–203.

APPENDIX-A

PHYLOGENETIC TREE OF 16S RRNA PARTIAL SEQUENCE OF ISOLATED STRAINS

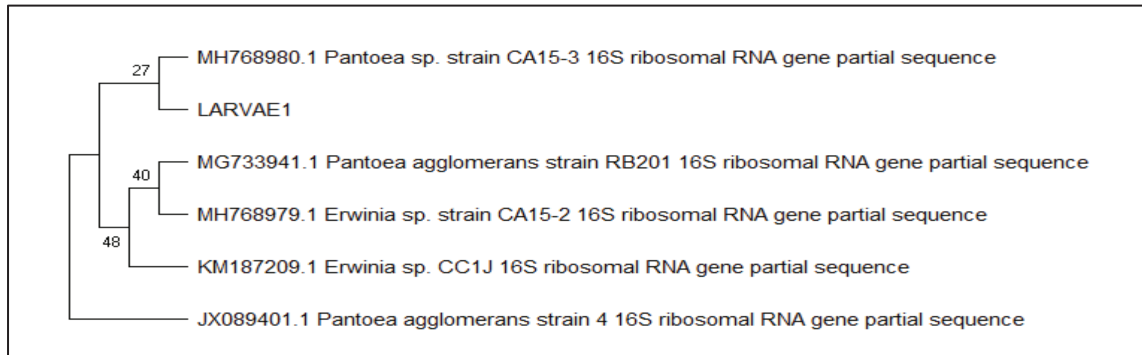


Figure-13: Phylogenetic tree of 16S rRNA partial sequence of Larvae1 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

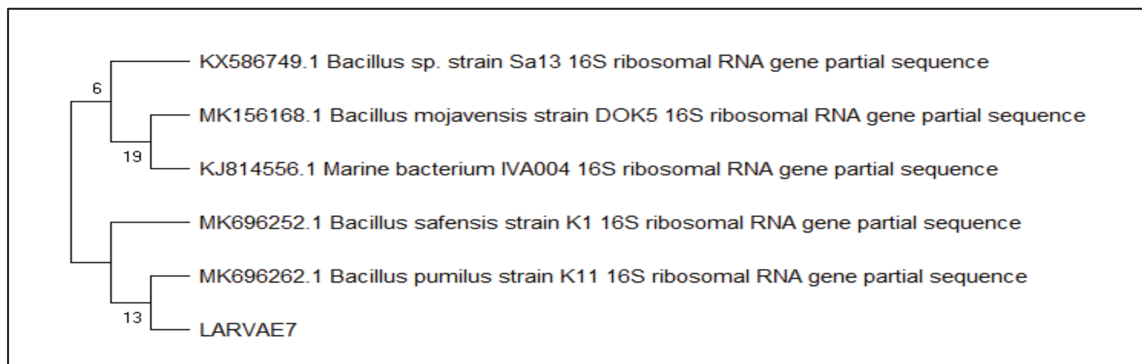


Figure-14: Phylogenetic tree of 16S rRNA partial sequence of Larvae7 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

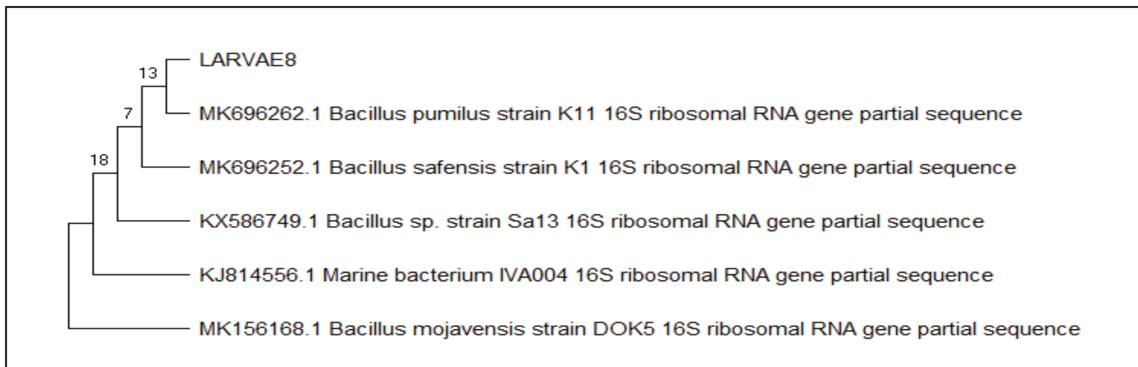


Figure-15: Phylogenetic tree of 16S rRNA partial sequence of Larvae8 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

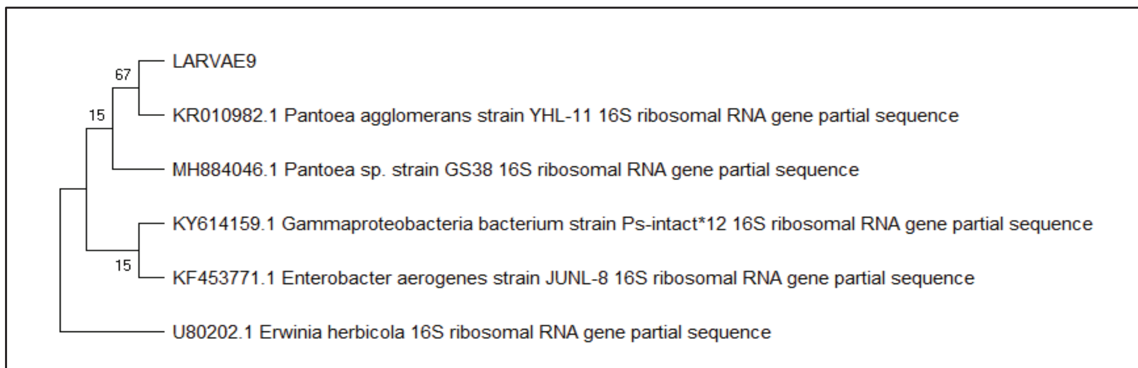


Figure-16: Phylogenetic tree of 16S rRNA partial sequence of Larvae9 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

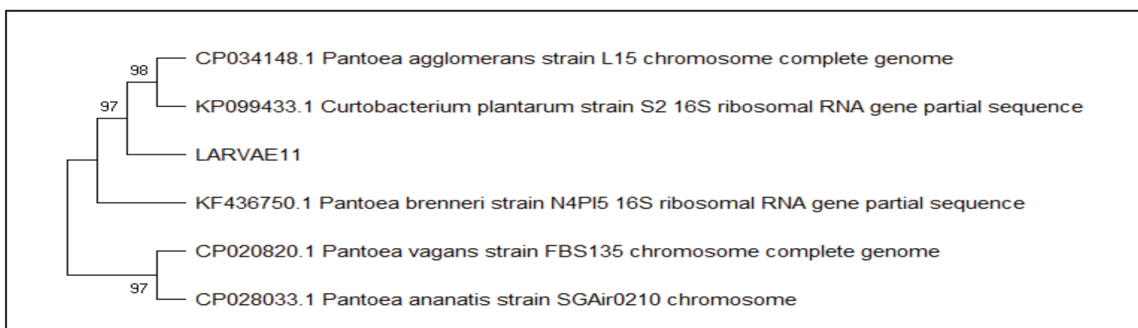


Figure-17: Phylogenetic tree of 16S rRNA partial sequence of Larvae11 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

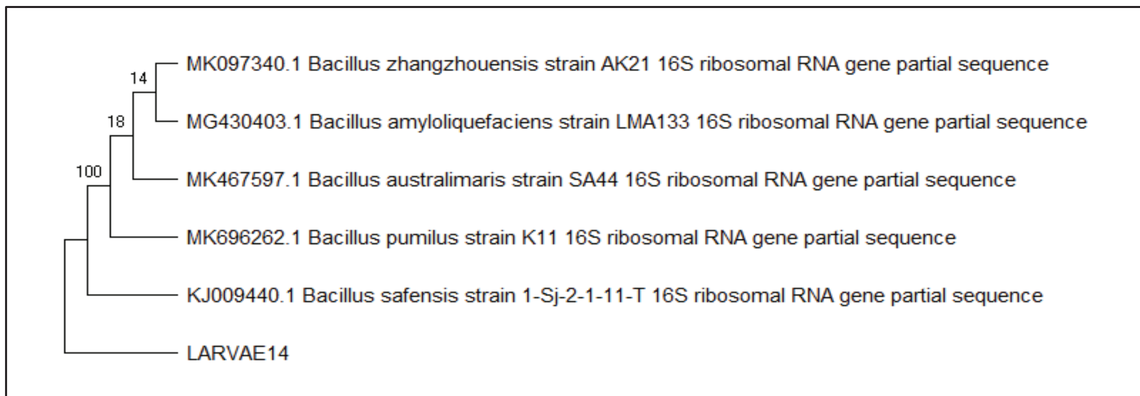


Figure-18: Phylogenetic tree of 16S rRNA partial sequence of Larvae14 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

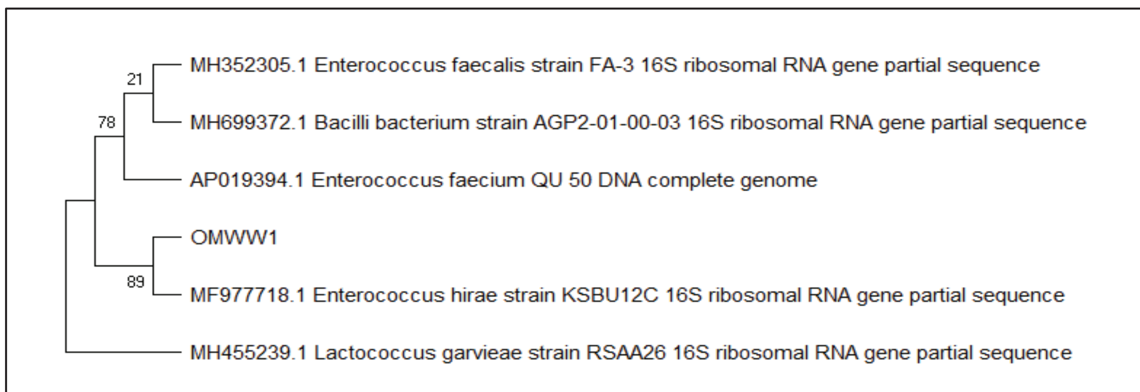


Figure-19: Phylogenetic tree of 16S rRNA partial sequence of OMWW1 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

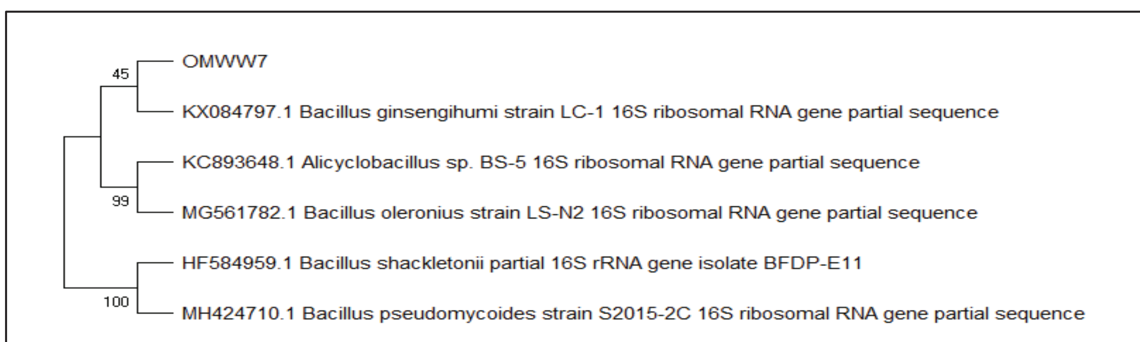


Figure-20: Phylogenetic tree of 16S rRNA partial sequence of OMWW7 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

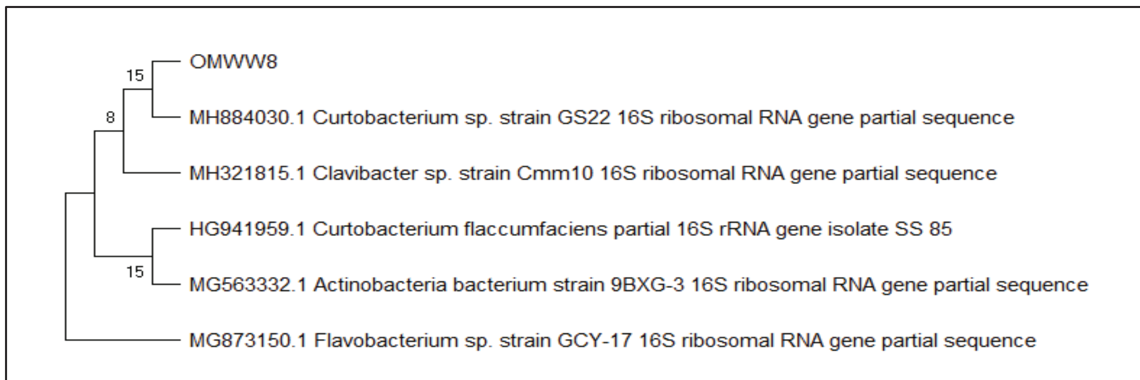


Figure-21: Phylogenetic tree of 16S rRNA partial sequence of OMWW8 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

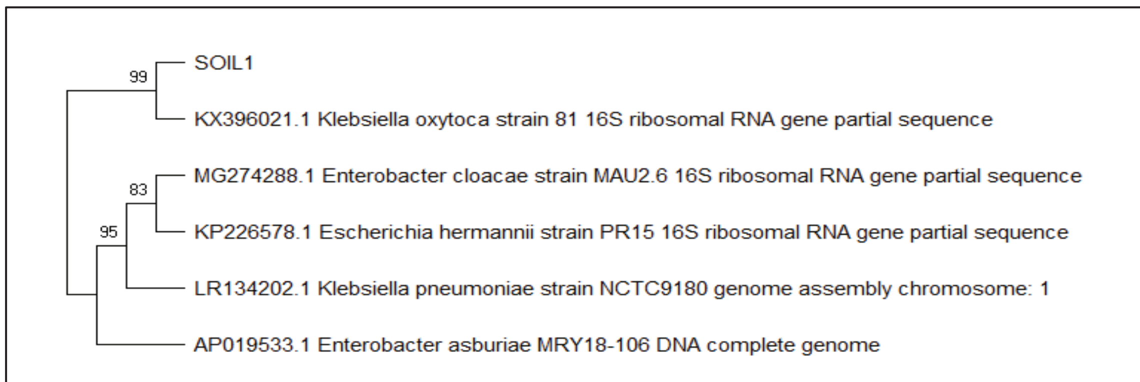


Figure-22: Phylogenetic tree of 16S rRNA partial sequence of Soil1 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

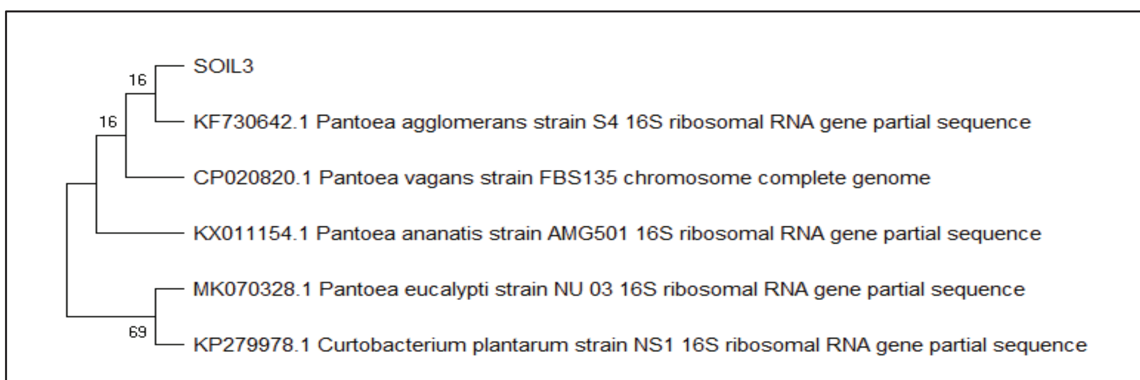


Figure-23: Phylogenetic tree of 16S rRNA partial sequence of Soil3 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

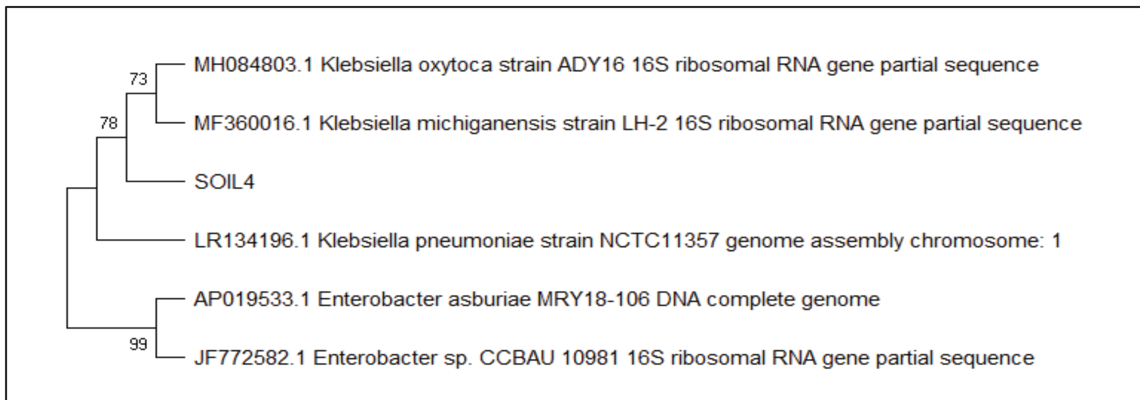


Figure-24: Phylogenetic tree of 16S rRNA partial sequence of Soil4 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

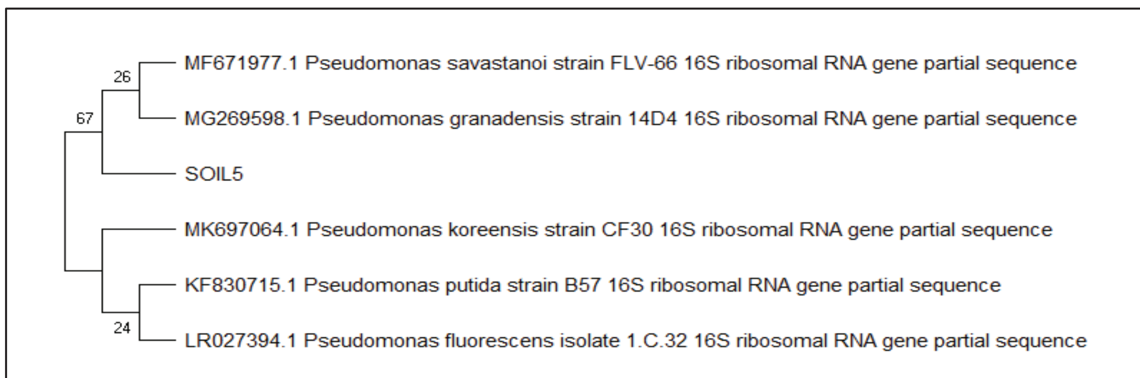


Figure-25: Phylogenetic tree of 16S rRNA partial sequence of Soil5 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

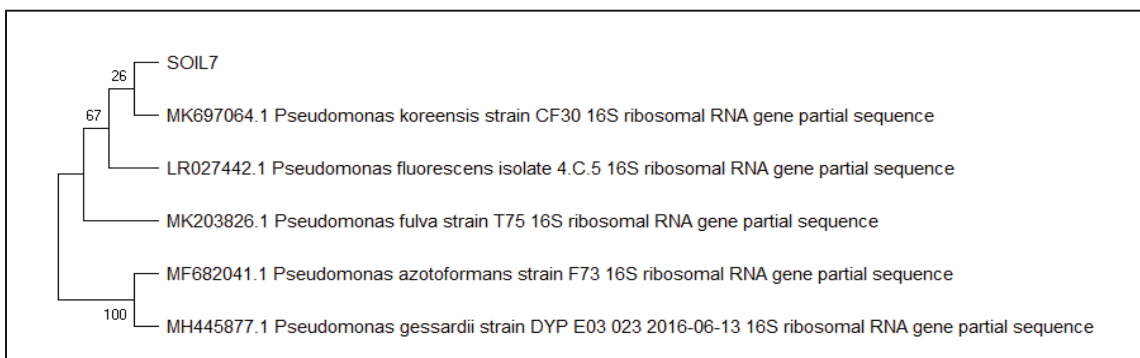


Figure-26: Phylogenetic tree of 16S rRNA partial sequence of Soil7 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

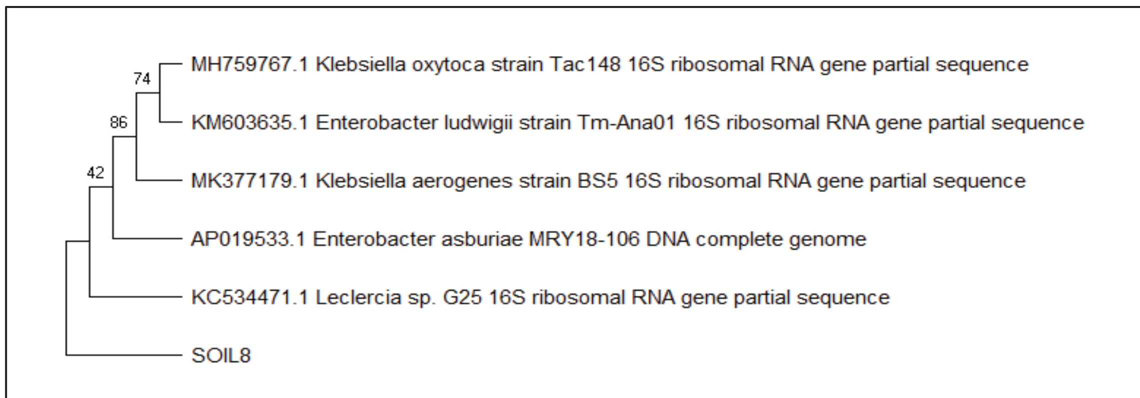


Figure-27: Phylogenetic tree of 16S rRNA partial sequence of Soil8 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

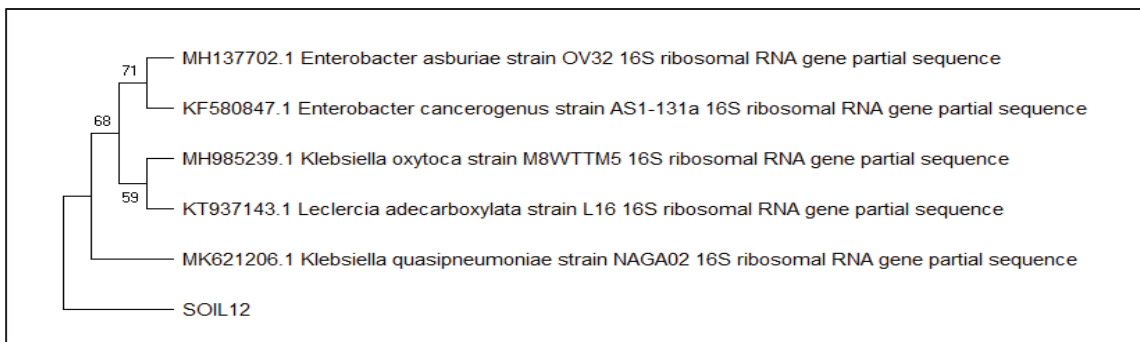


Figure-28: Phylogenetic tree of 16S rRNA partial sequence of Soil12 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

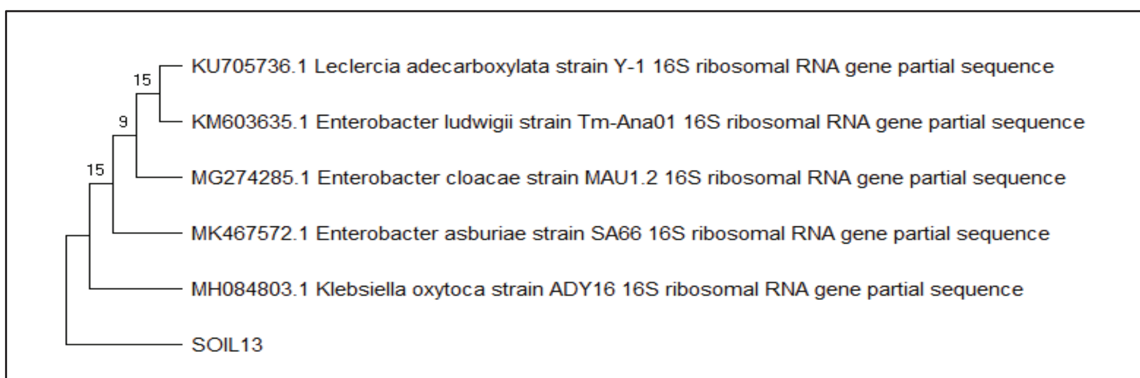


Figure-29: Phylogenetic tree of 16S rRNA partial sequence of Soil13 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

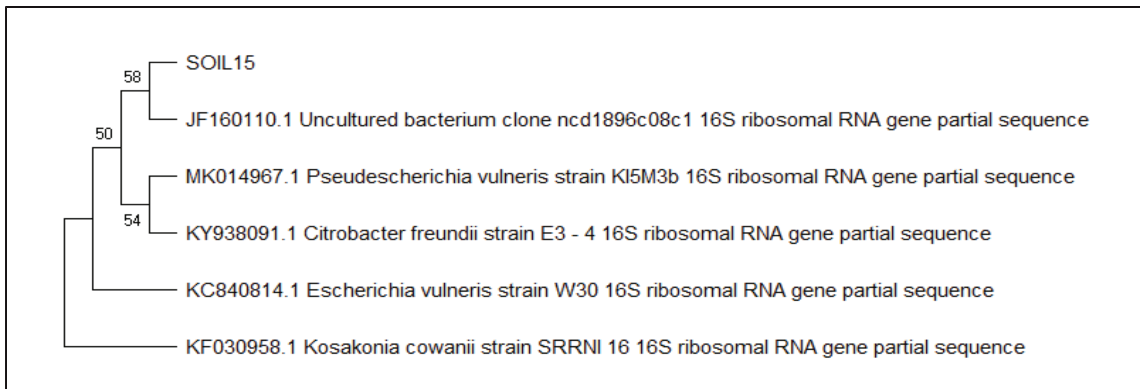


Figure-30: Phylogenetic tree of 16S rRNA partial sequence of Soil15 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

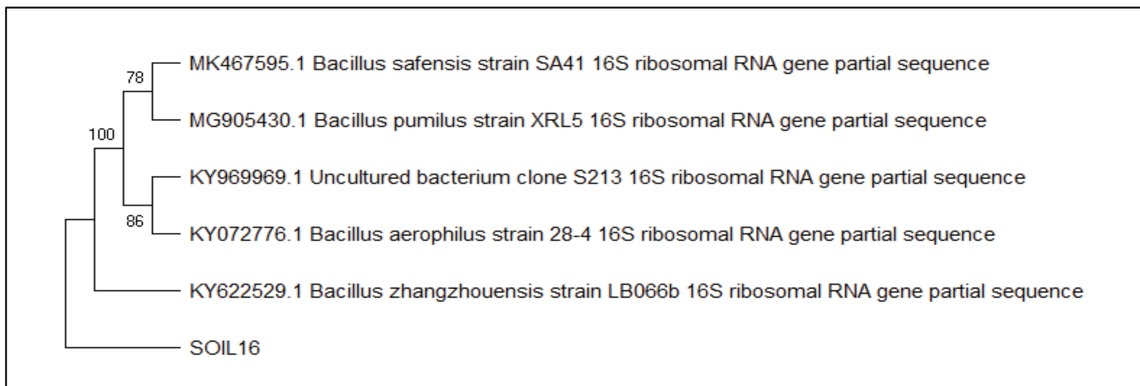


Figure-31: Phylogenetic tree of 16S rRNA partial sequence of Soil16 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

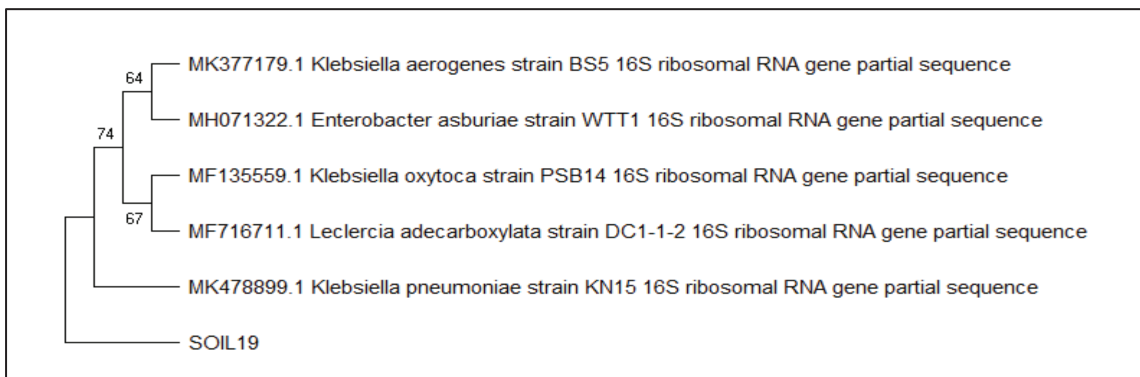


Figure-32: Phylogenetic tree of 16S rRNA partial sequence of Soil19 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

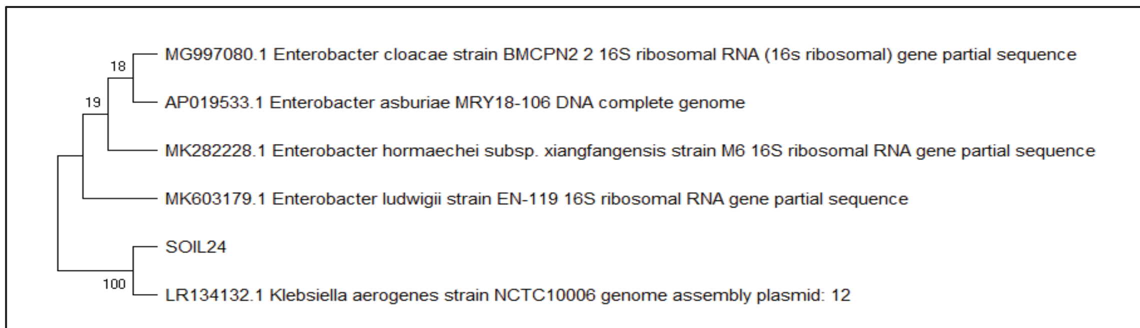


Figure-33: Phylogenetic tree of 16S rRNA partial sequence of Soil24 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

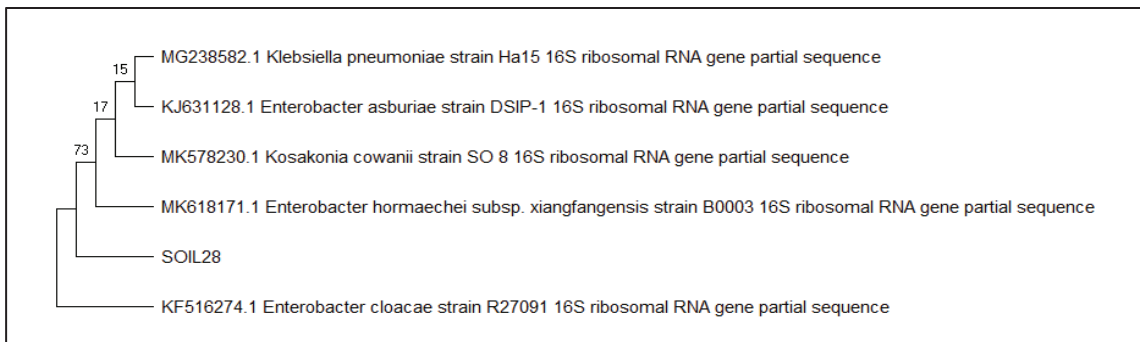


Figure-34: Phylogenetic tree of 16S rRNA partial sequence of Soil28 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

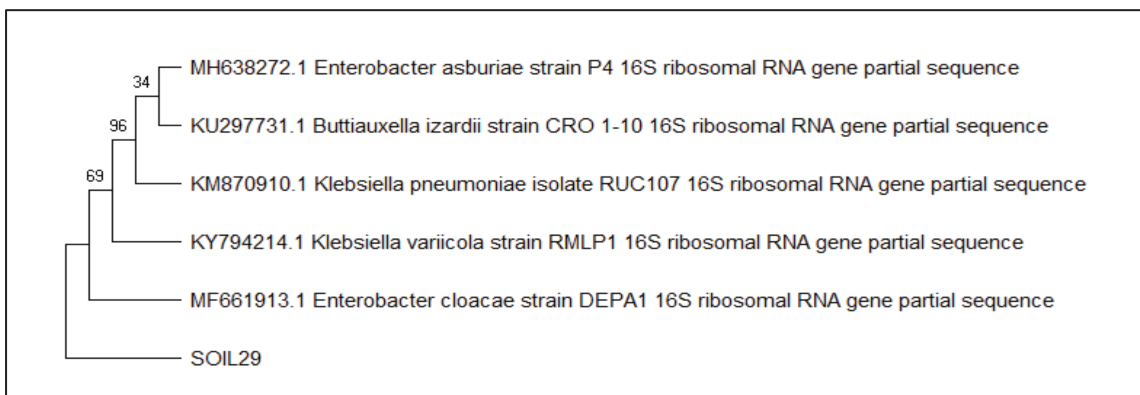


Figure-35: Phylogenetic tree of 16S rRNA partial sequence of Soil29 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.

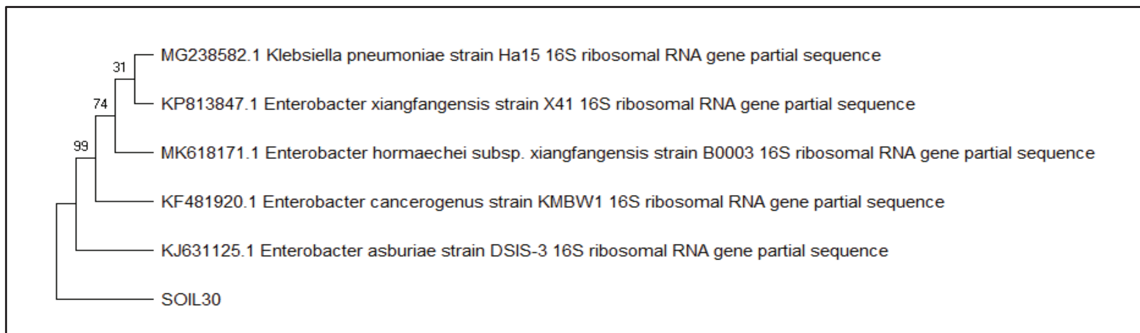


Figure-36: Phylogenetic tree of 16S rRNA partial sequence of Soil30 strain. The tree was constructed using sequences of the comparable region of 16S rRNA sequences available in the NCBI database by using MEGA-X software. Maximum likelihood method was used to show tree topology.