Black Sea Journal of Engineering and Science
doi: 10.34248/bsengineering.957791

Open Access Journal
e-ISSN: 2619 – 8991

Research Article
Volume 5 - Issue 2: 54-61 / April 2022

MOLECULAR CHARACTERIZATION OF GRAM NEGATIVE ISOLATED FROM WALNUT (*Juglans regia* L.) RHIZOSPHERE

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Abstract: In this study, 10 Gram negative bacteria isolated from the rhizosphere soil of walnut (*Juglans regia*) orchard in the Kırşehir (Central Anatolia of Turkey) were identified at species level by classical and molecular methods. It was determined that 99% of phylogenetic similarities with bacterial isolates were closely related to the isolated Gram negative bacterial isolates. Ten isolates were morpho-biochemically and molecularly characterized and on the basis of 16S rDNA sequencing were identified as 3 *Enterobacter cloacae*, 3 *Enterobacter ludwigii*, 2 *Escherichia coli*, 1 *Enterobacter hormaechei* and 1 *Plesiomonas shigelloides*. Gram negative bacteria showed enzymes activities. Except for P. shigelloides and E. coli, other Gram negative bacteria showed lipase, protease, cellulase, amylase, gelatinase and pectinase activities at 4 °C and 28 °C. As a result, the current research suggests that strains isolated from walnut rhizosphere soil can be used as bio-fertilizing and bio-control agents.

Keywords: Gram negative bacteria, 16S rRNA sequence, *Juglans regia* L., Rhizosphere

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Received: June 25, 2021
Accepted: February 03, 2022
Published: April 01, 2022

Cite as: Erdem B, Sevim E, Sevim A. 2022. Molecular characterization of gram negative isolated from walnut (*Juglans regia* L.) rhizosphere. BSJ Eng Sci, 5(2): 54-61.

1. Introduction

Walnut (*Juglans regia* L.) is a valuable tree nut from the *Juglandaceae* family, native to a wide geography including the Near East and Turkey. Walnuts are grown in all agricultural ecoregions in Anatolia due to their suitability to the various climates. Walnuts are generally produced in dry regions of Central Anatolia and some sections of Southeastern Anatolia in Turkey because irrigation is not frequent. Walnuts grown well in clayey, sandy soils with high organic matter soils. Recent research has found that when different plants are grown in the same soil, they can build rhizosphere microbiomes that are distinct to diverse microbial populations (Glick and Gamalero, 2021). Plants interact with a wide range of microbes, including bacteria found in the intestine. Members of the *Enterobacteriaceae* family are saprophytes that live as parasites on plants and animals.

Many studies have shown that *Enterobacteriaceae* are associated with plants and may have beneficial effects on plant development (Lodewyckx et al., 2002). *E. radicincitans*, *E. arachidis*, *E. oryzae* and *Enterobacter* sp. CBMB30, which is isolated from wheat phyllosphere, peanut rhizosphere and rice endosphere, are known support plant growth (Peng et al. 2009; Madhaiyan et al., 2010). The rhizosphere is a complex environment with beneficial and pathogenic bacteria that play an important role in the biogeochemical cycle of organic matter and mineral nutrients (Lagos et al., 2015).

In the agricultural management, there are many applications using *Enterobacteriaceae* members in the production of recombinant protein and non-protein products, control of infectious diseases, anticancer agents, biowaste recycling, biocontrol and bioremediation. As a result, genome-based phylogeny and genomics must be more exact in identifying Enterobacteriaceae members as well as defining genera and species within this family (Octavia and Lan, 2013).

Enterobacter can be found in terrestrial and aquatic environments (Halda-Alija et al., 2001) such as soil, water, sewage, vegetables and fruits, plants (Egamberdieva et al. 2008). The genus *Enterobacter* contains plant-related bacteria that promote plant growth. *E. ludwigii* belongs to the *E. cloacae* complex and has been reported to contain not only human pathogens but also plant-related strains (Yousaf et al., 2011). Gram negative bacteria including *E. radicincitans*, *E. oryzae*, and *E. ludwigii* are determined as plant associated growth-promoting bacteria (de Melo Pereira et al., 2012). *E. ludwigii* has been reported as a bacterium that promotes plant development and is plant related to biocontrol (Shoebitz et al., 2009). Proteobacteria were detected in the root endosphere (63%) and rhizosphere (54%) of

naturally grown walnut trees (Bai et al., 2020). Different genera of bacteria, including *Bacillus*, *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Azospirillum*, *Azotobacter* and *Serratia*, possess a range of properties, including P-solubilization, N2-fixation and the ability to produce cytokinins, antibiotics and hydrolytic enzymes that have been shown to increase yield in wheat, rice, maize, sugar beet and canola (Hayat et al., 2012). Zang, et al. (2015) identified 11 strains of phosphate solubilizing bacteria in walnut rhizosphere by 16S rDNA which belonged to 5 genera and they were namely *Pseudomonas*, *Staphylococcus*, *Planomicrobium*, *Microbacterium*, and *Acinetobacter*. Liu et al. (2014) have suggested that the inoculation of *B. cereus* L90 interferes with the suppression of stress conditions (salinity and temperature) the biological characteristics of walnut rhizosphere soil.

Soil bacteria have the ability to produce a variety of extracellular enzymes such as amylase, protease, lipase, pectinase, cellulase, and chitinase. Bacteria can be manipulated to produce commercially important enzymes in organic compound synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation (Logeswaran et al., 2014).

Bacterial amylase is more efficient than amylase from plants and animals because of its short growing time (Mishra and Behera 2008).

From the findings of the present study, the better source could be searched further to find potential bacterial isolates capable of producing the industrially significant and widely used amylase enzyme (Madhav et al., 2011).

Biotechnological applications of alkaline pectinases include textile processing, coffee, and tea fermentation, industrial wastewater pre-treatment containing pectin materials (Hoondal et al., 2002). Acid pectinases are used in the juicing industry to improve production, reduce viscosity, and clarify juice (Lara-Márquez et al., 2011). Despite the ecological importance and high economic value of the walnut tree, there are no studies on the bacteria associated with rhizosphere. Research on the rhizosphere bacteria of walnut not only explored our understanding of possible uses as plant growth promoting agents, but also their biocontrol potential in vitro.

In this study, the objectives are to find the molecular characterization of the bacteria in the rhizosphere soil of the walnut region around Kırşehir.

2. Material and Methods

2.1. Collection of Soil Samples

The samples were collected from the lands grown in walnuts in the districts of Kırşehir where located in 38°50'- 39°50' northern latitudes and 33°30'-34°50'east longitudes. The sampling was done in June 2017. Soil samples were collected from walnut orchards in Kırşehir were brought to the laboratory by ensuring cold chain. Taking 1 g of the soil samples, 10 ml of Nutrient Broth medium was taken and incubated at 30 ˚C for 24-48 hours, and then each breeding culture was transferred to Nutrient Agar and incubated at 30 ˚C for 24-48 hours. Pure colonies after incubation were stored as Gram negative bacteria.

2.2. Isolation and Identification of Bacteria from Walnut Rhizosphere Soil

The rhizosphere soil sample was taken 1 g and diluted serially (10^4-10^6). Diluted suspensions (0.1 mL) were spread over pre-cast nutrient agar medium and incubated at 30 °C for 24-48 hours. Then suspect colonies were selected. Some morphological and biochemical properties of the isolates were determined. In morphological characterization, colony color and form of isolated colonies were examined. Cell shape, size and Gram reaction were also noted. The various biochemical characterization indole production, methyl red test, Voges-Proskauer reaction, citrate utilization test, oxidase test, catalase production, acid production and H_2S production was carried out according to the Bergey's Manual of Systematic Bacteriology, volumes 1 and 2 (Krieg and Holt 1986; Sneath et al. 1986).The biochemical properties of the isolates were performed using API test kits (API 20E, Biomeriux).

2.3. Molecular Characterization of Gram Negative Isolates and Enhancement of the 16S rRNA Gene by PCR

The molecular characterization of bacterial isolates was carried out by 16S rRNA sequencing. Genomic DNA of the isolates was purified according to the procedure of Sambrook et al., (1989). 16S rRNA genes were amplified from genomic DNA obtained from each isolate with the help of UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTCA-3') forward and UNI16S-R (5'-ATGGTACCGTGTG ACGGGCGGTGTGTA) reverse primers. Conditions for PCR reactions were established according to Beffa et al. (1996). The replication process was carried out in 200 µl tubes, "BioRad thermocycler". 5 µl of the PCR products obtained were run on a 1.0% agarose gel and visualized after staining with ethidium bromide (0.5 µg/ml). PCR products were sent to Macrogen (S. Korea) Company for DNA sequencing analysis.

2.3. Data Analysis

All 16S rRNA sequences obtained were arranged with the BioEdit (Version 7.09) (Hall, 1999) program and their percentage similarities with other 16S rRNA sequences in GenBank were determined by BLAST in NCBI GenBank. The data obtained here were used to verify the morphological descriptions of the isolates.

Cluster analysis of 16S rRNA sequences was performed with the ClustalW program using the BioEdit program in the same way, and 1444 bp of the data obtained from this was used in neighbor joining (NJ) analysis with the help of MEGA (version 5) (Tamura et al., 2011) phylogenetic program. Aligment gaps were considered as missing data. The reliability of the generated dendrograms was tested with 1000 replicates by bootstrap analysis using the MEGA 5.0 program.

2.4. Enzymatic Activity of the Bacterial Isolates

Enzyme production was determined using a diffusion method involving colonies grown on solid media with a specific substrate. Each isolate was inoculated at 4 and 28oC and tests were done in duplicate. The open regions around the colonies were considered as an indicator of enzymatic activities and measured in mm as the difference between the halo and the diameter of the colony.

Extracellular enzyme activity of the bacterial isolates such as lipase, proteinase, cellulose, amylase, gelatinase and pectinase was also determined. Lipase activity was screened on rhodamine B (Sigma) agar plates including olive oil (Kouker and Jaeger 1987). Protease activity was determined on agar plates including skim milk (Yu et al., 2009). Cellulose activity was determined on nutrient agar plates which included carboxymethyl cellulose (0.5%). After 2 days of incubation, cellulose activity was determined with Congo red staining. Formation of clear zones around the colonies was assessed as an indication of enzyme activity (Teather, 1982). Starch hydrolysis test was used to detect amylase activity (Yu et al., 2009). To screen pectinolytic bacteria, bacteria that formed larger halos were selected after addition of 1% cetyltrimethylammonium bromide (CTAB) to MP medium (Schaad et al., 2001). The production of extracellular gelatinase was determined using culture media, with gelatin (Seeley et al., 1991).

3. Results

3.1. Imaging of Genomic DNA in Agarose Gel

Genomic DNA fragments isolated from the isolates were carried out in a 1% agarose gel at 100 V for 15-20 minutes. After execution, genomic DNAs were visualized under UV light (Figure 1).

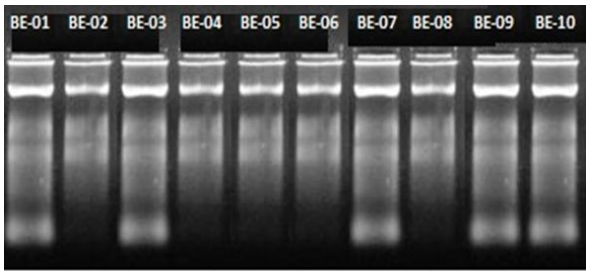

Figure 1. Genomic DNA image of gram negative isolates.

3.2. Imaging of 16S rRNA Gene Fragments in Agarose Gel

After the 16S rRNA PCR performed in the study, PCR products were carried out in 1% agarose gel. As a result of agarose gel electrophoresis, approximately 1600 bp PCR fragments were visualized (Figure 2).

3.3. Molecular Identification of Gram Negative Isolates

The 16S rRNA index analysis of the isolates was done by MACROGEN. These sequences were compared with the 16S rRNA genes of other bacteria existing in Gen Bank, and similarities were detected in the face (Table 1).

Table 1. 16S rRNA gene similarities of gram negative isolates (submitted to NCBI, USA)

Isolate	Species	Gen Bank ID Number	Query coverage (%)	Identity (%)
BE-01	*Enterobacter cancerogenus* 46	KF254599	100	99
	Enterobacter sp. AC3	KX608919	100	99
	Enterobacter cloacae SL11	KP058541	100	99
BE-02	*Enterobacter cloacae* EC1	KJ210326	99	99
	Enterobacter cloacae A2014-212	LC689714	99	99
	Enterobacter cloacae DX6	KM406493	99	99
BE-03	*Enterobacter cloacae* DX6	KM406493	99	99
	Enterobacter cloacae EC1	KJ210326	99	99
	Enterobacter sp. VTAN51	JN886722	98	99
BE-04	*Enterobacter hormaechei* HDYM-06	EF428236	100	93
	Enterobacter hormaechei STY31	HQ220153	100	93
	Enterobacter hormaechei ATY25	HQ219944	100	93
BE-05	*Enterobacter ludwigii* RCB319	KT260531	100	99
	Enterobacter ludwigii RCB308	KT260520	100	99
	Enterobacter ludwigii FGC63	KF358445	100	99
BE-06	*Enterobacter ludwigii* RCB319	KT260531	100	99
	Enterobacter ludwigii RCB308	KT260520	100	99
	Enterobacter ludwigii FGC63	KF358445	100	99
BE-07	*Enterobacter ludwigii* RCB319	KT260531	100	99
	Enterobacter ludwigii RCB308	KT260520	100	99
	Enterobacter ludwigii FGC63	KF358445	100	99
BE-08	*Plesiomonas shigelloides* XS-9	KP284552	100	99
	Plesiomonas shigelloides JT-0601	JN571746	100	99
	Plesiomonas shigelloides CIFRI	KX986915	100	99
BE-09	*Escherichia coli* RRL-36	JQ398845	100	99
	Escherichia coli JXZ-12	JF496560	99	99
	Escherichia coli ATY26	HQ219945	99	99
BE-10	*Escherichia coli* RRL-36	JQ398845	100	99
	Escherichia coli JXZ-12	JF496560	99	99
	Escherichia coli ATY26	HQ219945	99	99

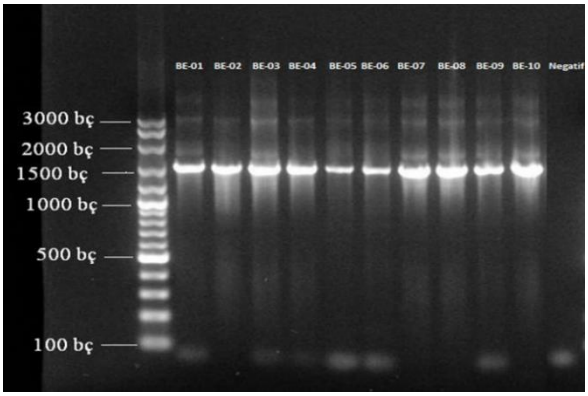

Figure 2. Image of 16S rRNA fragments of gram negative isolates.

Phylogenetic similarities with bacteria that 10 Gram negative bacterial isolated from walnut rhizosphere samples that were closely related were determined. According to the dendogram obtained, the highest bacterial isolate BE-01 of Enterobacter sp., the highest *Enterobacter cloacae* of the bacterial isolates BE-02 and BE-03, the most *Enterobacter hormaechei* of BE-04, and the most *Enterobacter ludwigii* of the bacterial isolates BE-05, BE-06 and BE-07, bacterial isolates BE-08 were the most similar to *Plesiomonas shigelloides*, bacterial isolates BE-09 and BE-10 were mostly similar to Escherichia coli (Figure 3).

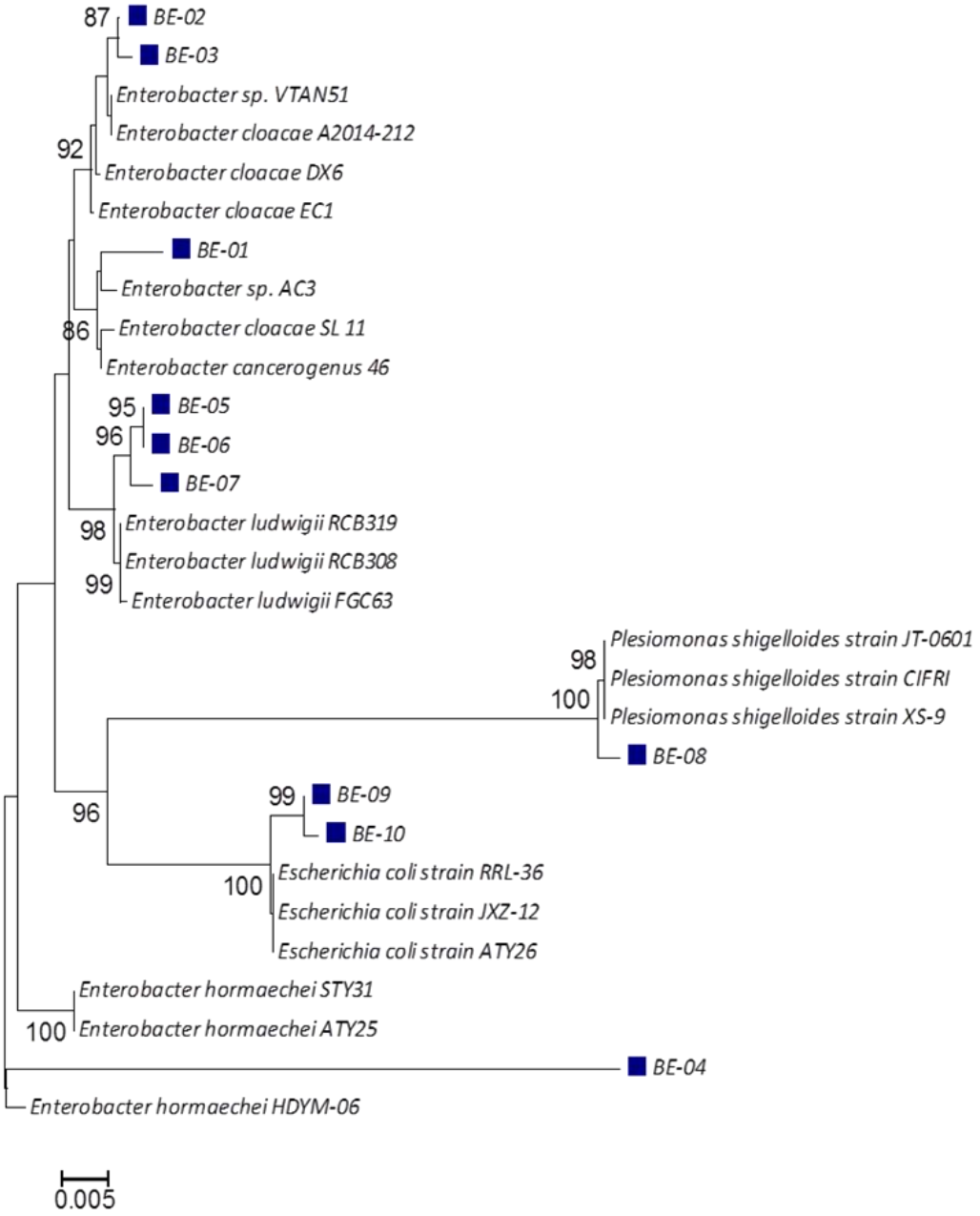

Figure 3. Pyhlogenetic analysis of the bacterial isolates from the rhizosphere soil of walnut and their closely related 10 bacterial species based on the partial sequence of the 16S rRNA gene. Dendogram was made using the neighbor-joining (NJ) method using the MEGA 5.0 phylogenetic program. The numbers next to the nodes show the bootstrap values. The scale below the figure shows the degree of similarity.

According to the molecular characterization studies, the determination results of Gram negative isolates at the species level are given in Table 2.

Table 2. Species determination results of Gram negative bacterial isolates

Isolates	Identified species
BE-01	*Enterobacter* sp.
BE-02, BE-03	*Enterobacter cloacae*
BE-04	*Enterobacter hormaechei*
BE-05, BE-06, BE-07	*Enterobacter ludwigii*
BE-08	*Plesiomonas shigelloides*
BE-09, BE-10	*Escherichia coli*

3.4. Screening for Extracellular Enzyme Activities

For screening of enzymatic activities, we worked with 10 bacteria isolates (Table 3). The size of halos and colonies were used to compare enzymatic activities and colony growth. *P. shigelloides* and *E. coli* had no detectable lipase, protease, cellulase, amylase, gelatinase and pectinase activities at 4 and 28 °C. *Enterobacter* sp., *E. cloacae*, *E. hormaechei* and *E. ludwigii* had lipase, protease, cellulase, amylase, gelatinase and pectinase activities 10-18 mm inhibition zone diameter at 4 and 28 °C. The isolates with the highest lipase activity are *E. hormaechei* and *E. ludwigii*. This isolates exhibited high lipase activity at 4 °C (15 mm) and at 28 °C (18 mm*). E. cloacae* had high pectinase activity at 28 °C (18 mm). *E. cloacae* and *E. hormaechei* had cellulase and gelatinase activities, but activity was found only at 28 °C (15 and 18 mm respectively). E. cloacae exhibited high amylase and lipase activity (12 mm) at 4 °C.

Table 3. Extracellular enzyme activities measured of diatemer and the colony halo in mm at 4 and 28 °C

		Lipase		Protease		Cellulase		Amylase		Gelatinase		Pectinase	
	Temperature (°C)	4	28	4	28	4	28	4	28	4	28	4	28
Isolates	Identified species												
BE-01	*Enterobacter* sp.	10	15	12	15	-	-	-	-	-	-	12	12
BE-02	*E. cloacae*	12	16	-	-	-	15	12	18	12	18	12	18
BE-03	*E. cloacae*	12	16	-	-	-	15	12	18	12	18	12	18
BE-04	*E. hormaechei*	15	18	12	15	-	15	12	16	-	18	12	12
BE-05	*E. ludwigii*	15	18	12	14	-	-	12	17	-	-	-	-
BE-06	*E. ludwigii*	15	18	12	14	-	-	12	17	-	-	-	-
BE-07	*E. ludwigii*	15	18	10	15	-	-	15	17	-	-	-	-
BE-08	*P. shigelloides*	-	-	-	-	-	-	-	-	-	-	-	-
BE-09	*E. coli*	-	-	-	-	-	-	-	-	-	-	-	-
BE-10	*E. coli*	-	-	-	-	-	-	-	-	-	-	-	-

-= absence of clear zone around the colony, += presence of clear zone around the colony.

4. Discussion

Walnuts can be used to discover the diversity of bacteria in their rhizosphere and to isolate the growth of plants under nutritional stress conditions since these trees have known to have rich rhizosphere for microrganism. The use of PGPR's can be successfully used as bio-fertilizers for sustainable plant production.

In this study, Gram negative bacteria isolated from walnut rhizosphere were compared to the sequence homology of the species using classical and molecular (16S rRNA gene region sequence) methods, and the species with the highest homology were found. Overall 10 bacteria BE-01, BE-02, BE-03, BE-04, BE-05, BE-06, BE-07, BE-08, BE-09 and BE-10 were isolated from the walnut rhizosphere soils of Kırşehir.

Endophytic bacteria were isolated from different tissues of walnut trees that 16S rRNA gene-based phylogenetic analysis revealed that strains showed 99%-100% similarity to *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Roseomonas* and *Streptomyces* genera (Ghorbani and Harighi, 2018).

4.1. Bacterial Flora

A total of 10 isolates that walnut rhizosphere soil was isolated from the soil and are given in Table 1. The five species found that 3 *E. cloacae*, 3 *E. ludwigii*, 2 *E. coli*, *1 E. hormahechei* and 1 *P. shigelloides* were isolated.

Common bacterial species that *E. cloacae*, *E. ludwigii* and *E. hormahechei* isolated. Other species, 1 *P. shigelloides* and 2 *E. coli* were also made in isolation. The biochemical properties of the isolates were performed using API test kits (API 20E, Biomeriux). These results highlight the potential of Enterobacter strain B-14 to be used in the Cleanup of contaminated pesticide waste in the environment (Singh et al., 2004). Our findings are supported by Bai et al. (2020) who found proteobacteria 54% dominant in walnut trees rhizosphere, and confirmed by Zang et al. (2015) who isolated high number of bacteria from walnut rhizosphere by 16S rDNA which belonged to genera and they were namely *Pseudomonas*, *Staphylococcus*, *Planomicrobium*, *Microbacterium*, and *Acinetobacter*.

4.2. Molecular Identification of Gram Negative Isolates

When comparing 16S rRNA index analyzes of isolates, 99% similarity was detected. The isolates were identified by amplifying their 16S rRNA gene sequences of different lengths. The partial sequences of nucleotides were compared with the available sequences from NCBI database and the sequences showing >99% similarity were retrieved by BLAST-N program (NCBI; www.ncbi.nlm.nih.gov/BLAST). The sequences submitted to NCBI and their accession numbers and number of base pairs amplified are indicated in Table 1. Phylogenetic analysis revealed that the bacterial isolates resembled with many reference sequences existing in the global bacterial gene pool and accordingly were identified on the basis of maximum sequence homology and phylogeny with the global reference sequences (Figure 1).

The bacterial isolates belonged to three genera namely *Enterobacter*, *Escherichia* and *Plesiomonas*. The two isolates were identified as *E. cloacae* (strains BE-02 and BE-03), *E. coli* (strains BE-09 and BE-10) while three isolates each were identified as *E. ludwigii* (strains BE-05, BE-06 and BE-07). One isolate each was identified as *Enterobacter* sp.(strain BE-01), *E. hormaechei* (strain BE-04) and *P. shigelloides* (strain BE-08).

Dar et al. (2018) have reported the presence of species level *B. licheniformis*, *B. tequilensis*, *B. cereus*, *B. subtilis*, *M. luteus*, *M. yunnanensis* and *Micrococcus* sp. as defined by 16S rDNA sequencing of rhizobacteria isolated from walnut (Juglans regia) rhizosphere in Western Himalayas. Researchers have reported that glyphosate degrading E. cloacae K7 may be useful for developing a biotechnology for the cleanup and restoration of glyphosate polluted soils (Kryuchkova et al., 2014). Previous results reported that *Pseudomonas* and *Enterobacter* strains potential PGPR inorganic fertilizers and could serve as bio fertilizer (Hayat et al., 2012).

The literature review revealed that no bacterial isolation studies were conducted on walnut rhizosphere soils based on molecular characterization, especially in the central Anatolia region, and the current study was first carried out in the central Anatolia region.

4.3. Extracellular Enzyme Activities

Enzymes can break down compounds and have various applications in biotechnology (food, beverage, personal and home care, agriculture, bioenergy, detergent, textiles, paper, pulp, biofuels, leather and pharmaceuticals) (Fasim et al., 2021). Therefore, screening of enzyme producing bacteria and characterization of the produced enzyme are important in biotechnology.

In this study, the ability of bacterial isolates to produce lipase, protease, cellulase, amylase, gelatinase and pectinase was screened using a special medium for each enzyme. *E. cloacae* BE-02 and *E. cloacae* BE-03 did not produce protease and geletinase. *E. ludwigii* BE-05, *E. ludwigii* BE-06 and *E. ludwigii* BE-07 showed no celluase, gelatinase and pectinase activity. Isolate BE-01 (*Enterobacter* sp.) exhibited lipase, proteinase and pectinase activity.

As a result, among the bacterial isolates, their production of lipase, protease, amylase and pectinase enzymes at 4 and 28 °C showed large clear halo around the bacterial colony in the special medium.

In addition, walnut rhisozphere soil can be a potentially economic source of various enzyme producing bacteria and when utilized properly this source can contribute to the growing needs of enzyme in various industries.

The percentage of amylolytic activity of bacteria in the garbage soils was higher than in the orchard soils. The reason for this may be the excess of starchy waste materials in the garbage soils (Saha et al., 2019). Pectinolytic enzymes that catalyze the breakdown of substrates containing pectin are widespread. Pectinases have potential applications in various industries, including food, animal feed, textile, paper, and fuel (Abdollahzadeh et al., 2020).

In this study, ten bacterial isolates were collected from Kırşehir city walnut orchard rhizosphere and four isolates showed the most pectinase activity. The biochemical and molecular test results showed that the four screened bacteria were *Enterobacter* sp. BE-01 and named *E. cloacae* BE-02, *E. cloacae* BE-03 and *E. hormaechei* BE-04.

Ten bacteria were isolated and characterized from walnut rhizosphere soil of walnut fields. Since bacteria are available in walnut fields, they could be beneficial for controlling insectised in the walnut fields through conservation of such biological control agents.

5. Conclusion

Various microorganisms in nature, which are used in various industrial applications such as enzyme production, fabric manufacturing, bioremediation and pharmaceutical production. Soil microorganisms play an important role in the release of nutrients that can be used by plants. This study revealed the diversity and isolation of microorganisms found in walnut rhizosphere soil. Further research is needed for production, optimization, purification and characterization of enzyme by these bacterial isolates and possible biotechnological application of the enzyme.

In conclusion, we isolated 10 bacterial strains from the walnut rhizosphere soil and purified, characterized and identified these strains by 16S rRNA gene sequencing. These bacterial strains were identified as belonging to the genera *Escherichia*, *Enterobacter* and *Pleisomonas*. Gram negative strains can be used to grow walnut trees as bio-fertilizing and bio-control agents after the PGPR potential has been determined. The existence of such microorganisms suggests that they can be utilized in future application such as controlling plant diseases, phytoremediation of contaminated soils or enhancing plant growth.

Author Contributions

BE: initiated the research idea, developed, organized,

analyzed and interpreted the data and wrote the manuscript. ES: supervised the research, suggested the research methods, and structured the paper. AS: designed the study and interpretation of the results and edited the manuscript. All authors reviewed and approved the manuscript

Conflict of Interest

The author declared that there is no conflict of interest.

Acknowledgements

The authors acknowledge the financial support by the Kırşehir Ahi Evran University Scientific Research Projects Coordination Unit (PYO-FEN.4001.15.005) to carry out this study.

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