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# Biodiversity of Bacteria Isolated from Different Soils

**Fatma YAMAN[1], Betül AKTAŞ[1], Mustapha TOURAY[1], Esin POYRAZOĞLU ÇOBAN[1,*], H. Halil BIYIK[1]**

Adnan Menderes University, Department of Biology, Aydın, TURKEY

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**Abstract**: The aim of this study was to determine the biodiversity of PHB producing bacteria isolated from soils where fruit and vegetable are cultivated (onion, grape, olive, mulberry and plum) in Aydın providence. Morphological, cultural, biochemical, and molecular methods were used for bacteria identification. These isolated bacteria were identified by 16S rRNA sequencing and using BLAST. The following bacteria *Bacillus thuringiensis* (6), B*acillus cereus* (8), *Bacillus anthrachis* (1), *Bacillus circulans* (1), *Bacillus weihenstephanensis* (1), *Pseudomonas putida* (1), *Azotobacter chroococcum* (1), *Brevibacterium frigoritolerans* (1), *Burkholderia sp.* (1), *Staphylococcus epidermidis* (1), *Streptomyces exfoliatus* (1), *Variovorax paradoxus* (1) were found. The Maximum Likelihood method was used to produce a molecular phylogenetic analysis and a phylogenetic tree was constructed. These bacteria can produce polyhydroxybutyrate (PHB) which is an organic polymer with commercial potential as a biodegradable thermoplastic. PHB can be used instead of petrol derivated non-degradable plastics. For this reason, PHB producing microorganisms are substantial in industry.

**Keywords**: PHB; bacteria; 16S rRNA; biodiversity; soil

## 1. Introduction

Plastics products are indispensable from automobiles to medicine in our lives. We use plastics and synthetic polymers produced from petrochemicals. As synthetic plastics are persistent in the environment, plastic materials are a substantial source of environmental pollution and damage natural habitats. Several 100,000 tons of plastic are disposing of marine environments every year and accumulate in certain oceanic regions. In this case, approximately 1.000.000 sea creatures are dies every year [1]. With the advancement of biotechnological research, the production of environmentally friendly plastic has been on the rise. These plastics are produced by microorganisms. Polyhydroxyalkanoates (PHA) are bacterial plastics. PHA's are synthesized by many prokaryotic and eukaryotic microorganisms in the appropriate growth conditions. Polyhydroxybutyrates (PHB) is the most significant member of polyhydroxyalkanoates. PHB was first described by Lemoigne in *Bacillus megaterium* [2]. PHB is an energy and carbon reserve material in microorganisms. PHB is accumulated as intracellular granules by bacteria such as *Bacillus spp*., *Azotobacter spp*., *Pseudomonas spp*. PHB is synthesized (when nutritional elements such as N, P, S, O, or Mg are deficient in the presence excess carbon source [3]. PHB granules are insoluble in water, relatively resistant to

*Corresponding Author: +90 256 218 2000; Fax: +90 256 213 5379
E-mail address: epoyrazoglu@adu.edu.tr

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hydrolytic degradation, biocompatible and nontoxic. It has good oxygen permeability and ultra-violet resistance. It also has a Melting point of 175ºC and glass transition temperature 2ºC [4]. PHB has many obvious applications in the manufacture of packaging containers, bottles, wrapping films, bags and the like. Some important medical uses are serving a surgical pins, stables, wound dressing, bone replacements and plates, and biodegradable carriers for the long-term release of medicines [5].

Isolation and identification of PHB producing bacteria from environments such as soil, sea water, aerobic and anaerobic sewage is important since these bacteria (*Bacillus sp*, *Alcaligenes sp*., *Pseudomonas sp*., *Streptomyces sp*., *Azotobacter sp*., *Burkholderia sp*. *Brevibacterium sp*.) are used in industry [6]. İdentification of PHB producing bacteria using classical taxonomic methods is unreliable and time consuming. As a result, most researchers use molecular methods as 16s rRNA-PCR, ERIC -PCR, REP-PCR for bacterial identification. Sujatha et al. [7] identified *Pseudomona ssp*. LDC-5 using 16S rDNA gene sequence and researched PHB production. López-Cortés et al. [8] isolated PHB-producing bacteria in a polluted marine microbial mat and identified using 16S rDNA gene sequence. As result, they found *Bacillus*, *Staphylococcus*, *Paracoccus*, *Micrococcus*, *Rhodococcus* and *Methylobacterium*. Mauti et al. [9] carried out molecular identification of soil bacteria by 16S rDNA sequence and identified as *Burkholderia cenocepacia*.

In this study, the biodiversity of PHB producing bacteria isolated from soils where fruit and vegetable are cultivated (onion, grape, olive, mulberry and plum) in Aydın providence was investigated.

## 2. Experiment

### 2.1. Collection of samples soils

Various soil samples were collected from nine different gardens and lawn soil of Aydin providence in Turkey. The garden soil samples used in this study were collected from 0-15 cm layer.

### 2.2. Isolation of microorganisms

One gram of each sample was suspended in 99 mL of sterile distilled water and shaken. The samples were heated at 80ºC for 5 min in water bath for *Bacillus sp.* isolation from soil. Mannitol Agar Medium (10g mannitol, 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.2 g $FeCl_3.6\ H_2O$, 0.005 g), Pseudomonas Selective Agar (Difco) and Nutrient Agar (Sigma) were used for isolation of *Azotobacter sp*., *Pseudomonas sp*. and *Bacillus sp*. respectively. Later, the liquid media were serially diluted in sterile 0.85 % physiological saline (NaCl) solution and the dilutions from $10^{-1}$ to $10^{-6}$ were plated on Agar Medium. Plates were incubated at 28-37 ºC for 24-48 h. After incubation different colony were isolated separately and stored in skim milk [10].

### 2.3. Identification of microorganisms

Morphological, cultural and biochemical identifications were made according to the Bergey's Manual of Systematic Bacteriology [11]. For molecular identification, DNA extraction was done using Green and Sambrook protocol [12]. DNA concentrations and purity was measured using a nanodrop spectrometer (Thermo Scientific). 16S rRNA PCR reactions were carried out at initial denaturation 95ºC 5min, denaturation 94ºC 40sec, annealing 50ºC 40 sec, extension 72ºC 40 sec with 35 cycles and final extension at 72ºC 10dk. Reagents used and their concentrations were 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM $MgCl_2$ and 1U Taq polymerase with the final volume of 25 µl. PCR products were sent to the sequencing (GATC BioTech, Germany) after electrophoresis at 1.4% agarose jel at 90 V 40 min.

### 2.4. Phylogenetic analysis of isolates

Phylogenetic tree was constructed using the Maximum Likelihood method [13] .Using BLASTn software our sample sequences were referenced against sequences found in GENBank. Sequences were aligned with ClustalW program which is inside MEGA 7.0 software [14].

## 3. Results and Discussion

17 bacterial species were identified and the results were *Bacillus sp*., (1) *Pseudomonas sp*. (1), *Azotobacter sp*. (1), *Brevibacterium sp*. (1), *Burkholderia sp*., *Staphylococcus epidermidis*, (1) *Streptomyces exfoliates*, (1) *Variovorax paradoxus* (1) (Table 1).

*Pseudomonas sp*., *Azotobacter sp*., *Burkholderia sp*. and *Variovorax paradoxus* are Gr (-), rod shaped bacteria (Fig. 1a, b, c, d). *Bacillus sp*., *Brevibacterium sp*., *Streptomyces exfoliates* are Gr (+), rod shaped bacteria and *Staphylococcus epidermidis* is Gr (+), coc shaped bacteria (Fig. 2 a, b, c, d).

**Table 1.** Characterization and number of bacteria isolated from different soils.

| Bacteria | Number of isolates | Characterizations |
|---|---|---|
| *Bacillus sp.* | 17 | Gr(+), rod shaped bacteria |
| *Pseudomonas sp.* | 1 | Gr(-), rod shaped bacteria |
| *Azotobacter sp.* | 1 | Gr(-), rod shaped bacteria |
| *Brevibacterium sp.* | 1 | Gr(+), rod shaped bacteria |
| *Burkholderia sp.* | 1 | Gr(-), rod shaped bacteria |
| *Staphylococcus epidermidis* | 1 | Gr(+), coc shaped bacteria |
| *Streptomyces exfoliatus* | 1 | Gr(+), rod shaped bacteria |
| *Variovorax paradoxus* | 1 | Gr(-), rod shaped bacteria |

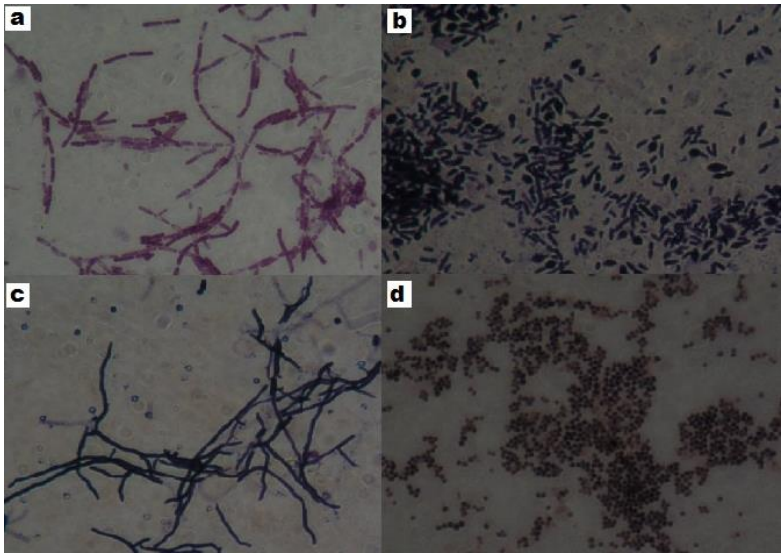


**Figure 1.** Image of bacteria under microscopic view on magnification x100.
***a***. *Bacillus sp*. ***b.*** *Brevibacterium sp*. ***c***. *Streptomyces exfoliates* ***d***. *Staphylococcus epidermidis*

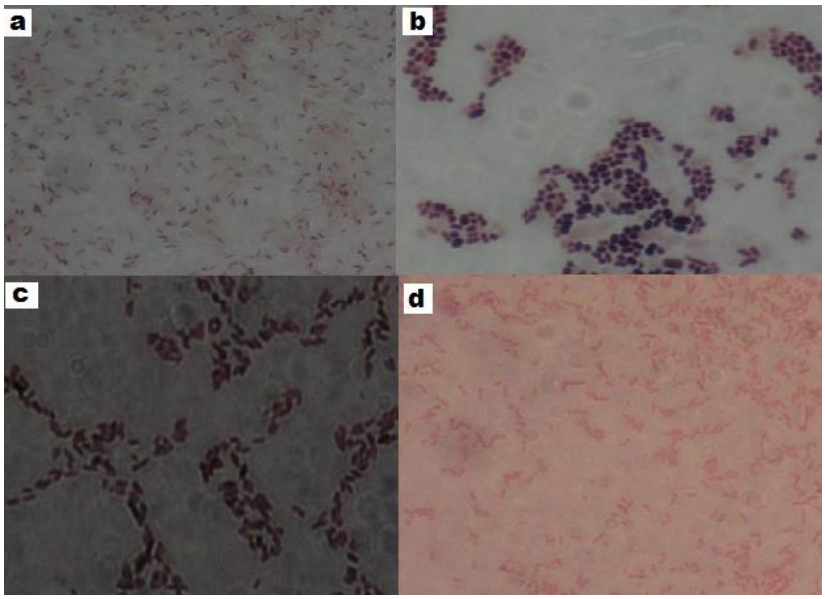


**Figure 2.** Microscopic view of bacteria magnification x100.
***a.*** *Pseudomonas sp*. ***b.*** *Azotobacter sp*. ***c.*** *Burkholderia sp*. ***d.*** *Variovorax paradoxus*

PCR results of these samples were sent to GATC Biotech, Germany for sequencing. Amplification of the 16S rRNA gene showed that 61 isolates were PHB produced bacteria. Molecular identification was made by comparing sequence results with Gene bank using BLASTn software. The analysis of the soil samples of Aydın providences showed that there were twelve species with accession number (Table 2).

**Table 2.** Molecular identification of the species isolated from soils where fruit and vegetable are cultivated (onion, grape, olive, mulberry and plum) in Aydın providence.

| Name of The Species | Name of Samples | Number of Strains | Accession No |
|---|---|---|---|
| *Bacillus thuringiensis* | Plum, Onion,Grape,Mulberry | 6 | KU179338.1<br>FJ981909.1<br>JN590251.1<br>KT714039.1<br>KJ784474.1<br>JQ685228.1 |
| *Bacillus cereus* | Olive, Onion,Grape, Mulberry | 8 | KX941838.1<br>KX301062.1<br>EF185296.1<br>GQ365209.1<br>KF831393.1<br>KT922033.1<br>FJ763651.1<br>KU179339.1 |
| *Bacillus anthrachis* | Olive | 1 | CP008846.1 |
| *Bacillus circulans* | Olive | 1 | KT983982.1 |
| *Bacillus weihenstephanensis* | Mulberry | 1 | KT363050.1 |
| *Pseudomonas putida* | Plum | 1 | KU977141.1 |
| *Azotobacter chroococcum* | Plum | 1 | KX108861.1 |
| *Brevibacterium frigoritolerans* | Olive | 1 | HQ202870.1 |
| *Burkholderia sp.* | Onion | 1 | JQ917954.1 |
| *Staphylococcus epidermidis* | Onion | 1 | KX019832.1 |
| *Streptomyces exfoliatus* | Plum | 1 | LN774329.1 |
| *Variovorax paradoxus* | Onion | 1 | AB622222.1 |

A neighbour-joining phylogenetic tree was constructed by MEGA 7.0 software from a partial 16S rDNA sequence of the bacterial isolates obtained in this study with selected sequences downloaded from GenBank, shown in Fig. 3. The ClustalW program in MEGA 7.0 was used to align the sequences. These isolates found were *B. thuringiensis* (6), *B. cereus* (8), *B. anthrachis* (1), *B. circulans* (1), *B. weihenstephanensis* (1), *P. putida* (1), *A. chroococcum* (1), *Brevibacterium frigoritolerans* (1), *Burkholderia sp.* (1), *Staphylococcus epidermidis* (1), *Streptomyces exfoliatus* (1), *Variovorax paradoxus* (1) (Fig. 3).

Many researchers have isolated and used molecular methods such as 16s rRNA-PCR, ERIC-PCR, REP-PCR in identifying PHB producing bacteria from different soil samples.

Nubia et al. [15] isolated PHB produced bacteria from soil and identified the isolates by partially sequencing the 16SrRNA gene. Singh and Palmar [16] carried out the biodiversity of PHB produced bacteria and found two novel species as *Rahnella aquatilis* and *Stenotrophomonas maltophilia*. Aarthi and Ramana [17] isolated PHB producing bacteria from garden soil and characterized based on their 16S rRNA gene sequences. They identified species as *Bacillus mycoides* DFC1, *Bacillus cereus* DC1, *Bacillus cereus* DC2, *Bacillus cereus* DC3 and *Bacillus cereus* DC4.PHB-producing bacterial strains were isolated from Antarctic soils and characterizated as *Pseudomonas spp*. and *Janthinobacterium* spp. by 16S rRNA gene sequence analysis by Goh and Tan [18]. Reji et al. [19] executed molecular identification using 16S r- RNA sequencing and the organism was identified as *Bacillus cereus*. Dul'tseva et al. [20] isolated bacteria of the genus *Variovorax* from the *Thioploca* mats of Lake Baikal and identified as *V. paradoxus*, *V. soli*, *V. ginsengisoli*, and *V. boronicumulans*, *V. dokdonensis* using 16S rRNA gene nucleotide sequences. Tonouchi et al. [21] isolated *Brevibacterium yomogidense sp.* from a soil sample conditioner made from poultry manure.Panigrahi and Badveli [22] researched the isolation and screening of soil bacteria PHB production and they observed that red soil was able to produce maximum yield of PHB. Ciesielski et al.[23] carried out molecular identification of polyhydroxyalkanoates-producing bacteria isolated from enriched microbial community and identified *Bacillus sp*.,*Microbacterium sp*., *Citrobacter sp*., *Aeromonas sp*., *Caulobacter sp*., *Sphingomonas sp*.Mazinani et al. [24] isolated Azotobacter sp. from soil samples and identified as *A. chroococcum*, *A. beijerinckii* and *A.vinelandii* using16S r- RNA sequencing. Chandani et al. [25] isolated *Bacillus sp*. from soil samples in Municipal Waste Areas and the isolate was characterized as *Bacillus tequilensis* NCS-3 based on 16S rRNA gene sequence. It was examined that Bacillus tequilensis NCS-3 produces PHB in different carbon and nitrogen sources, pH and temperatures. Prakash et al. [26] isolated a bacterium from soil of coastal region of India and identified as *Burkholderia pseudomallei* using 16S rRNA gene amplification. Agrawal et al. [27] reported that twenty four isolates of *Pseudomomas putida* isolated from soil samples and observed phenotypic characterization of these isolates. Osman et al. [28] isolated *Microbacterium* from soil sample and carried out molecular characterization as the 16s rRNA gene Biradar et al. [29] researched PHB producing *Bacillus* species isolated from agricultural soil and characterized using 16S r-RNA sequencing. Hall et al. [30] isolated *Burkholderia* species from soils in the Southern United States and diversed as *B. cenocepacia*, *B. cepacia*, *B. contaminans*, *B. diffusa*, *B. metallica*, *B. seminalis*, *B. vietnamiensis*. Kıran et al. [31] studied producing PHB from bacteria isolated from contaminated soils. Isolates were identified as *Bacillus anthracis* (IBB) and *Bacillus subtilis* with 16S rRNA gene amplification and phylogenetic relationship. Hoseinabadi et al. [32] carried out using16S rRNA sequencing identification of poly β-Hydroxybutyrate over-producing bacteria and identified as *Bacillus coagulans*. Hassan et al. [33] isolated *Bacillus sp*. from Egypt and examined production of PHB by *Bacillus* sp. N-2.

The aim of this study was to isolate and identify, using 16S rRNA sequencing methods, the PHB producing bacteria from garden soil samples. According to these; it has been showed that morphological methods are not always adequate and confidential for identification of species. Thus, both morphological and molecular methods for identification of bacteria were used. It can be seen that, in recent years, molecular identification gained more importance.

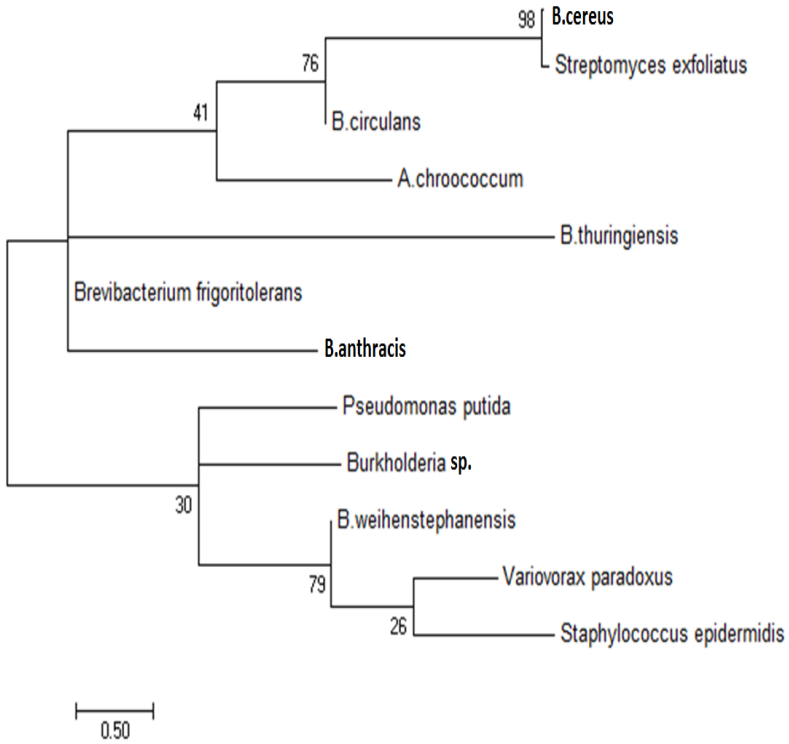


**Figure 3.** Molecular Phylogenetic analysis by Maximum Likelihood method.

Using the Tamura based model of the maximum likelihood method the evolutionary history was found and the as shown in the figure is the highest log likelihood. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths representıng the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon posıtıons were the usual trıplets wıth some non codıng nucleotıdes. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 166 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

## 4. Conclusion

In this study isolation of PHB producing bacteria from soils where fruit and vegetable are cultivated (onion, grape, olive, mulberry and plum) in Aydın providence were carried out. In addition, we obtained twelve various bacteria as *Bacillus thuringiensis*, B*acillus cereus, Bacillus anthrachis*, *Bacillus circulans*, *Bacillus weihenstephanensis*, *Pseudomonas putida*, *Azotobacter chroococcum*, *Brevibacterium frigoritolerans*, *Burkholderia sp.*, *Staphylococcus epidermidis*, *Streptomyces exfoliatus*, *Variovorax paradoxus* based on nucleotide homology and phylogenetic analysis. These bacteria can be used as PHB producers in industry.

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