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Bacterial Biodiversity of Industrial Soils from Aydın and Trabzon Province

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Abstract: The aim of this study was to determine bacterial biodiversity of industrial soils from Aydın and Trabzon Province using morphological, cultural, biochemical and molecular methods. Factory wastes, detergents, pesticides and gasoline are accepted as pollutants to the environment. In this study a total of 65 samples which can tolerate xylene, acetone, chloroform and methanol were acquired. According to the Bergey's Manual of Systematic Bacteriology morphological, cultural and biochemical tests, 48 of these samples were found to be *Bacillus sp*. and 17 of them were found to be *Pseudomonas sp*. For molecular identification 16S rDNA-PCR method was used. Seven different genera and a total of sixteen species were found as a result of the study.

Key Words: Bacteria, 16S rRNA, biodiversity soil, Aydın, Trabzon, Turkey

1. Introduction

Biodiversity is the foundation of ecosystem services to which human well-being is intimately linked [1]. It is one of the basic components of nature and it ensures the survival of earth by all means. Biodiversity depends on the climatic conditions and areal components of the region. The presence of the pollutants diminishes the bacterial biodiversity as well as overall biodiversity.

Soil biodiversity influences a huge range of ecosystem processes that contribute to the sustainability of life on earth [2]. Soil biodiversity maintains critical and key processes such as carbon storage, nutrient cycling, plant species diversity, soil fertility, soil erosion, nutrient uptake by plants, formation of soil organic matter, nitrogen fixation, biodegradation of organic materials, reducing hazardous waste, production of organic acids that weather rocks, and control of plant and insect populations through natural biocontrol [3, 4, 5]. Biodiversity and soil are strongly linked, because soil is the medium for a large variety of organisms, and interacts closely with the wider biosphere. Conversely, biological activity is a primary factor in the physical and chemical formation of soils [6]. The main role of soil microorganisms is to recycle organic matter which stemmed from in and above-ground organisms, even after their

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passing away. Both natural and agricultural vegetation boundaries correspond closely to soil boundaries, even at continental and global scales [7].

In our day soil pollution can be traced back to xenobiotic activities. The rapid development of the industry caused rapid disposal of the wastes and this caused serious problems for the environment. Factory wastes, detergents, pesticides and gasoline are the main pollutants of the environment. Various technologies have been developed for remediation of contaminated soil/ sediments [8]. One of these clean up options is the usage of biological elements such as bacteria. This process is called bioremediation [9]. Some microorganisms can digest certain organic chemicals. Bacteria that live in these kinds of soils can also be used to clean these pollutants from soil. The aim of this study is to determine bacterial biodiversity of industrial soils from Aydın and Trabzon Province of Turkey. Bacterial species which found in this study can be used as biological cleaners in polluted soils.

2. Material and Methods

2.1. Sample Collection

Samples were collected aseptically from industrial sites, sewer sites, city dump and factory dump sites. Locations are Değirmendere Industrial Site/TRABZON, Değirmendere Sewer/TRABZON, Değirmendere Site/TRABZON, Menderes River Site/AYDIN, Nazilli City Dump/AYDIN.Samples were taken in March, 2014. Samples were collected in the 50 ml sterile falcon tubes and kept in a portable refrigerator and brought to the laboratory. Samples were added in the enrichment media that were prepared in advance.

2.2. Bacterial Isolation

Enrichment of the bacteria was made by adding 1% of chloroform, methanol, xylene and acetone to the soil samples and leaving them to incubation at 30 ºC for five days. This process repeated three times. A series of dilutions were made from enriched samples up to 10^{-6}. Bacterial growth was realized on Plate Count Agar (PCA) and Pseudomonas Selective Agar at 30 ºC for 48 h. Samples which gives between 30-300 colonies were chosen. Each different colony was isolated and stocked in skim milk.

2.3. Classical Identification

Morphological, cultural and biochemical identifications were made according to the Bergey's Manual of Systematic Bacteriology [10]. For identification Gram staining, lactose, sucrose, mannitol, citrate, nitrate reduction, starch hydrolase and gelatin hydrolase tests were made.

2.4. Molecular Identification

DNA isolation of the samples was made according to De Boer and Ward (1995) [11]. After isolations DNA concentration and purity was measured with nanodrop spectrometer (Thermo Scientific). Their purity values were between the values of 1.73 and 2.20. For PCR 16S universal rDNA primers were used (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-CGG TTA CCT TGT TAC GAC TT-3') [12]. 16S rRNA PCR reactions were carried out at initial denaturation 95 ºC 5 min, denaturation 94 ºC 40 sec, annealing 50 ºC 40 sec, extension 72 ºC 40 sec with 35 cycles and final extension at 72ºC 10dk. Reagents 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. Evolutionary analysis and tree construction were made with MEGA 7.0 software The evolutionary history was inferred using the Maximum Parsimony method [13-15].

3. Results and Discussion

3.1. Classical Identification

In the present study, a total of 65 samples that can tolerate xylene, acetone, chloroform and methanol were obtained. Consistent with morphological, cultural and biochemical tests, 48 of these samples were found to be *Bacillus sp*. and the rest were found to be *Pseudomonas sp*. *Pseudomonas sp*. is a Gr(-) rod shaped bacteria (Fig.1) with glycerol, lactose, sucrose and mannitol fermentation abilities, produces H_2S, hydrolyses gelatin and is citrate positive (Table 1, Fig.2) while *Bacillus sp*. were Gr (+) rod shaped (Fig.1), endospore forming bacteria with catalase, glucose, sucrose, mannitol, gelatin hydrolisation and citrate positive (Table 2, Fig.2).

Table 1. Classical identification of *Pseudomonas* species from Aydın and Trabzon Province industrial soils.

Source	Gram Staining	Cell Shape	G	L	S	M	NR	SH	H_2S	C	GH	Bacteria
T-3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-3Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
A-2Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Xylene	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-2 Methanol	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Chloroform	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-3Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.
T-1 Acetone	_	Rod	_	_	_	_	+	_	+	+	+	*Pseudomonas* sp.

G: Glycerol, L: Lactose, S: Sucrose, M: Mannitol C: Citrate, NR: Nitrate Reduction, SH: Starch Hydrolyse, GH: GelatinHydrolyse
T1:Değirmendere Industrial Site/TRABZON, T2:Değirmendere Sewer/TRABZON, T3:Değirmendere Site/TRABZONA1:Menderes River Site/AYDIN, A2:Nazilli City Dump/AYDIN,

Table 2. Classical identification of *Bacillus* species collected from Aydın and Trabzon Province.

Source	Gram Staining	Cell Shape	G	L	S	M	NR	SH	H_2S	C	GH	Ct	Bacteria
A-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-2 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-2 Chloroform	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Acetone	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-1 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
A-1 Methanol	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
T-3 Xylene	+	Rod	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.

G: Glycerol, L: Lactose, S: Sucrose, M: Mannitol, C: Citrate, NR: Nitrate Reduction, SH: Starch Hydrolyse, GH: GelatineHydrolyse, Ct: Cathalase

T1:Değirmendere Industrial Site/TRABZON, T2:Değirmendere Sewer/TRABZON, T3:Değirmendere Site/TRABZONA1:Menderes River Site/AYDIN, A2:Nazilli City Dump/AYDIN,

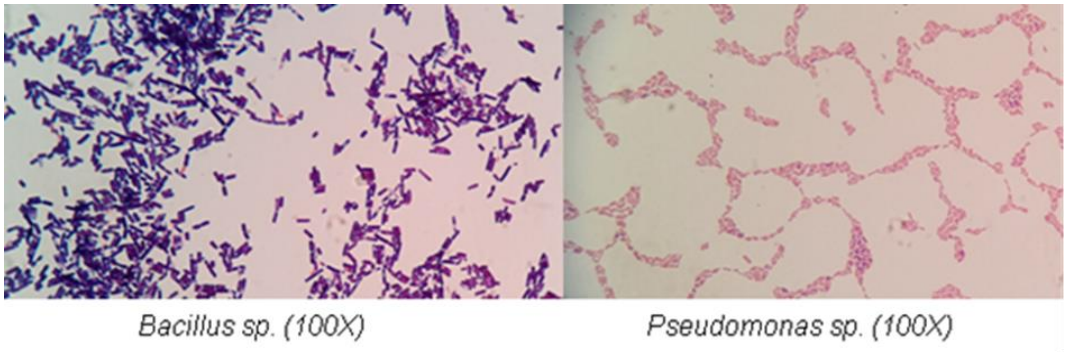

Fig. 1. Gram staining of *Bacillus* and *Pseudomonas* species

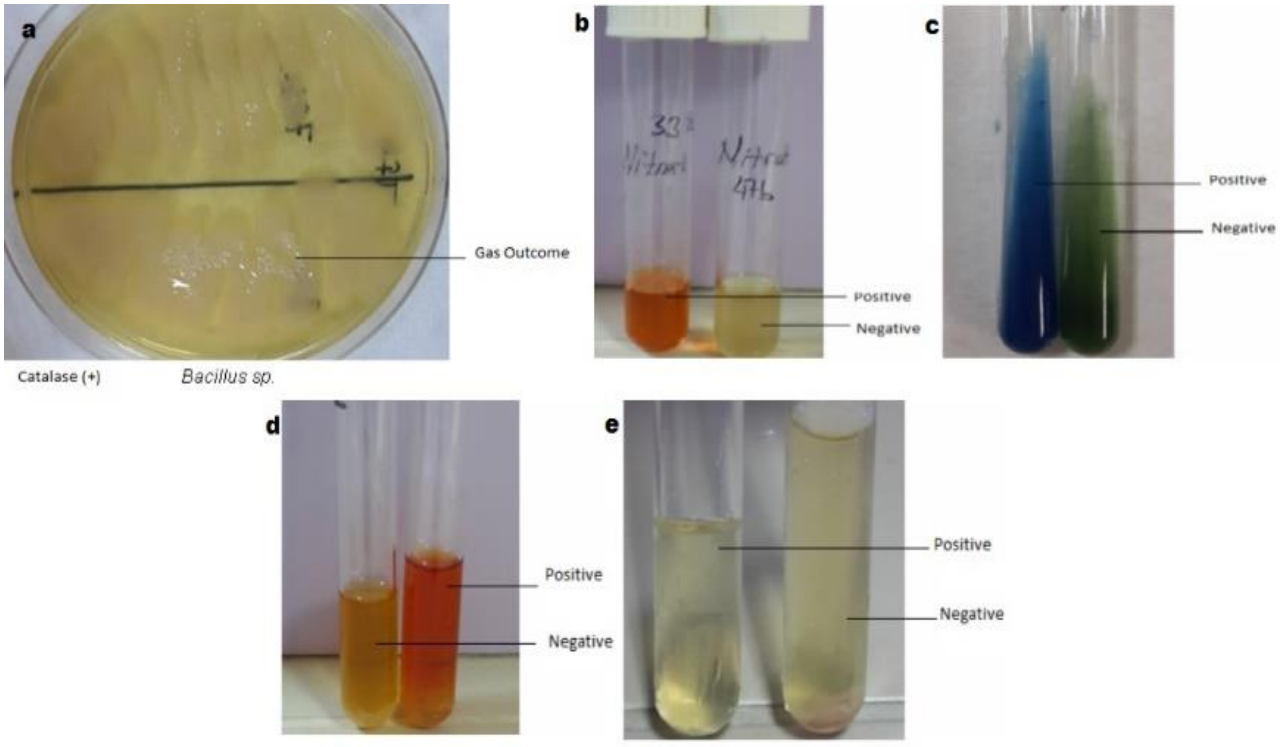

Fig. 2. Biochemical test results of the samples. (a: Catalase test, b: Nitrate reduction test, c: Citrate test, d: Voges-Proskauer test, e: Gelatin hydrolase test)

3.2. Molecular Identification

16S rDNA primers were used for molecular identification (Fig.3). After amplification and agarose gel electrophoresis PCR results of these samples were send to the sequencing (GATC BioTech, Germany).Molecular identification was made by comparing sequence results with GenBank using BLASTn software. Our analysis of the industrial soils of Trabzon and Aydın Province were showed that there were not only *Bacillus* and *Pseudomonas* species but seven different genera and sixteen species were present (Table 3).

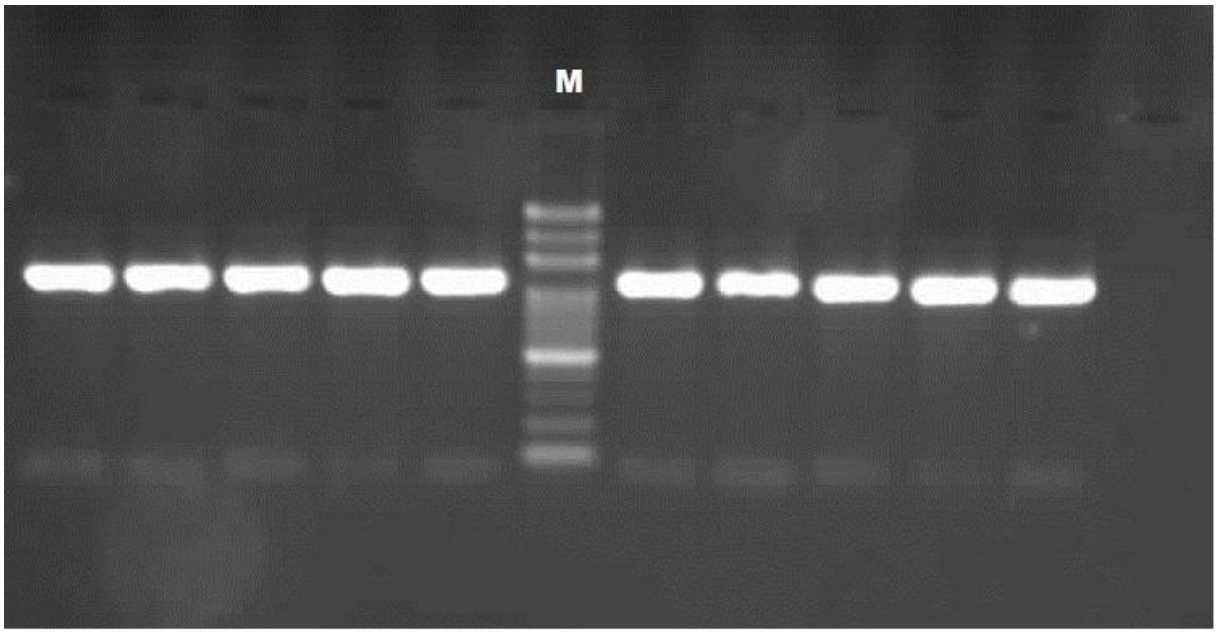

Fig. 3. 16SrDNA-PCR products. (M: 100 bp DNA ladder)

Table 3. Molecular identification of the species from Aydın and Trabzon Province industrial soils.

Name of The Species	Province	Number of Strains	Accession No
Bacillus stratosphericus	Trabzon	1	KC866366.1
Pseudomonas sp.	Trabzon	1	KT005263.1
Bacillus cereus	Trabzon	6	KF836529.1 JF736841.1 KF494191.1 KP729612.1 JX629271.1 JQ824137.1
Bacillus megaterium	Trabzon	1	KT222849.1
Arthrobacter oxydans	Trabzon	2	LN774368.1 KC934768.1
Bacillus anthracis	Trabzon	2	KP813675.1 KP813664.1
Bacillus thuringiensis	Trabzon	2	KT965080.1 KT986127.1
Corynebacterium tuberculostearicum	Trabzon	1	KT805279.1
Pseudomonas brassicacearum	Trabzon	1	KP851953.1
Bacillus pumilus	Trabzon	1	KP851957.1
Staphylococcus epidermidis	Trabzon	1	KX349995.1
Pseudomonas putida	Trabzon	1	KX349990.1
Pseudomonas plecoglossicida	Trabzon	1	KX082839.1
Bacillus megaterium	Aydın	4	KJ526882.1
Bacillus cereus	Aydın	3	KT897915.1 GQ855296.1 FJ763650.1
Bacillus subtilis	Aydın	1	KP184704.1
Bacillus subterraneus	Aydın	1	KT719810.1
Aerococcus viridans	Aydın	1	GQ161096.1
Microbacterium aerolatum	Aydın	1	LN774527.1
Staphylococcus hominis	Aydın	1	HM163532.1
Arthrobacter oxydans	Aydın	1	EU086783.1

MEGA 7 software was used for evolutionary analysis. Maximum parsimony method was used to infer evolutionary history. Maximum parsimony tree was shown in Figure 4. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).All positions with less than 95% site coverage were eliminated.

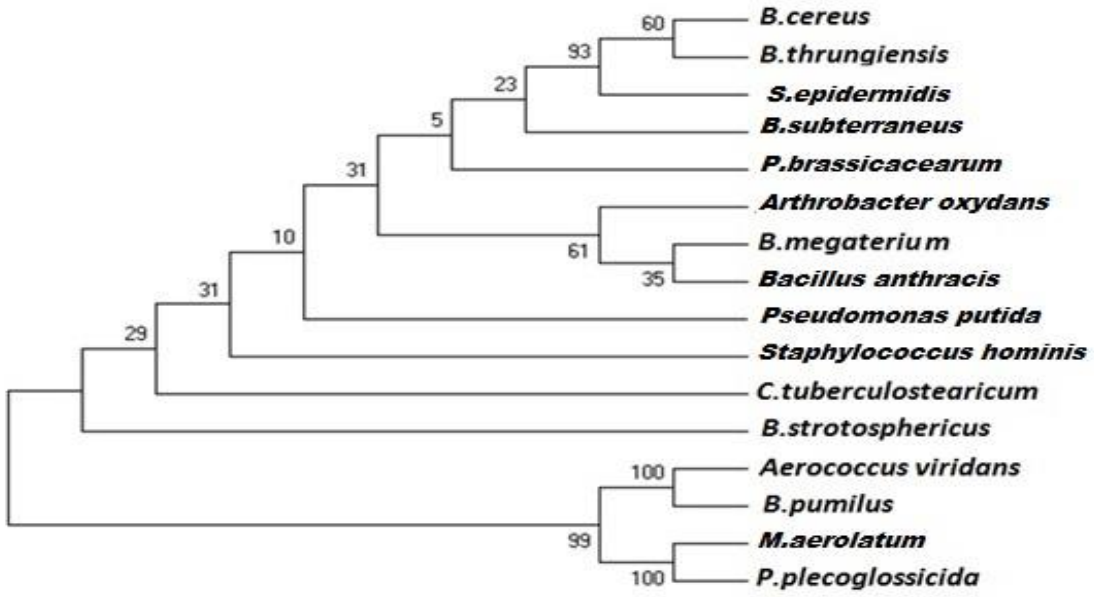

Fig. 4. Maximum Parsimony analysis of taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

It is a fact that the microbial diversity of the soil is immense but pollutants have an inhibitory effect on this diversity. In contrast to normal soil samples, Bornemann and Triplett (1997) found 98 bacterial and 2 archaea species in Eastern Amazonia, while Jesus et al. (2009) found 654 bacterial species in non-industrial soils [16, 17]. This can clearly be seen in our study. Bacterial diversity was reduced to seven genera and sixteen species because of the pollutants.

According to morphological, cultural and biochemical test only two genera can be identified but molecular identification showed that there is seven genera in fact. This dictates the importance of molecular methods in identification of organisms once more. Classical identification methods allowed us to identify on the genus level while molecular methods let us to identify on the species level. Once more this shows us the insufficiency of the classical identification methods and the necessity of molecular methods. The failure of the detection of *Arthrobacter oxydans* with classical methods is normal since this bacterium can show different properties, e.g. gram staining, cell shape, between young and old colonies [18].

Soil hosts a large variety of *Bacillus* species. Even in industrial soils *Bacillus* species are abundant as nine of the sixteen species belongs to the genus *Bacillus*. This can be inferred as the members of the genus *Bacillus* have high tolerance to the environmental conditions. This is due to the spore formation ability of the genus.

4. Conclusion

In this study we aimed to determine the bacterial biodiversity of industrial soils from the two Province of Turkey. We used morphological, biochemical and molecular methods for that purpose. Morphological and biochemical methods gave us two genera, *Bacillus* sp, and *Pseudomonas* sp. while molecular method led us to seven genera and sixteen species. Most abundant genus in the soils are *Bacillus* species as they can form spores.

It is not a surprise that molecular methods can give more accurate results than morphological and biochemical methods as they use DNA sequence knowledge. Since these bacterial species can live in polluted soils they can also be used as biological cleaners. More studies are required for that purpose.

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