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ISOLATION, MOLECULAR CHARACTERIZATION, AND ANTIMICROBIAL ACTIVITY OF THE MEMBERS OF THE FAMILY *BACILLACEAE* OBTAINED FROM SOIL SAMPLES

Ali Sevim[*], *Alperen Kutalmış*, *Cafer Yabaneri*

Address (es): Ali Sevim,
Department of Plant Protection, Faculty of Agriculture, Kırşehir Ahi Evran University, Kırşehir, Turkey, 40100.

*Corresponding author: ali.sevim@ahievran.edu.tr

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ABSTRACT

The soil is a very rich environment in terms of microorganism diversity and the microorganisms in the soil are the source for many secondary metabolites. Bacteria in the family Bacillaceae are commonly found in the soil and can maintain their vitality for many years because of their spore production. At the same time, the species in this family can produce secondary metabolites which have different functions. In this study, *Bacillus* or *Bacillus*-like bacteria were isolated from soil samples in wheat fields and they were identified based on their morphological and molecular features using 16S rRNA gene sequencing. In addition, the antimicrobial activities of the isolates were investigated against various important human pathogens. A total of twenty bacteria were isolated and all of them were identified as *Bacillus* sp., except for ES-18 which is *Lysinibacillus xylanilyticus*. Six isolates showed antibacterial activity at different levels and the most effective isolate, ES-18, showed antimicrobial activity against *Bacillus cereus, B. megaterium, B. subtilis*, *Stapyhlococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes*. MIC values of the isolate ES-18 were also determined against the related bacteria and the highest dilution factor was determined to be 2^{-1}. It was also determined that all effective dilutions were bacteriostatic. This is the first study showing antimicrobial properties of *L. xylanilyticus* and the results obtained from this study might be important for the discovery of new antimicrobial compounds.

Keywords: *Lysinibacillus*, Gene sequencing, 16S rRNA, Phylogeny, Antimicrobial activity

INTRODUCTION

The genus of *Bacillus* compromise more than 350 species, and the members of this genus are typically Gram-positive, endospore-forming, and aerobic or facultative anaerobic bacteria and they have rod-shaped cell morphology (**Turnbull, 1996; Caulier *et al.*, 2019**). Although this genus includes mostly Gram-positive and rod-shaped bacteria, some species are Gram-variable (**Maughan and Van der Auwera, 2011**). Because of many species in this genus which have different physiological characteristics, their endospore-forming ability, and their capacity to produce plenty of antimicrobial compounds, they can colonize and survive in most natural environments such as soil, marine environment with different characteristics, plants, and animals (**Turnbull, 1996; Caulier *et al.*, 2019; Achi and Halami, 2016**). The *Bacillus* genus have been undergone remarkable taxonomic changes and reclassification since advances in molecular biology, particularly on the genetic level, have brought out a very high genetic and phylogenetic heterogenous groups (**Cihan *et al.*, 2012; Fritse, 2004**). Among the molecular techniques and advances, especially the 16S rRNA sequence analysis has been widely used for the modern bacterial taxonomy and has a great influence on bacterial and *Bacillus* systematics (**Fritse, 2004; Woese, 1987; Ki *et al.*, 2009**). For instance, based on 16S rRNA sequence similarity, some bacteria classified in *Bacillus* genus exhibited distinguishable difference and this led to the reclassification of many *Bacillus* species into new genera such as *Lysinibacillus Brevibacillus*, *Paenibacillus* and *Geobacillus* (**Bhandari *et al.*, 2013**). Further detailed morphological, genetic, and molecular characterization studies of selected *Bacillus* species have been revealed the creation of more new genera such as *Amphibacillus*, *Gracilibacillus*, *Geobacillus* and *Marinibacillus* (**Xu and Cote, 2003**). Lately, the partial sequence of 16S rDNA gene and rRNA gene restriction digestion patterns have been widely used for the rapid identification of *Bacillus* species, and for also some the related genera (**Goto *et al.*, 2000; Joung and Cote, 2002**). Apart from 16S rRNA gene region, *rpoB* and *recA* gene sequences have shown to be successful for species identification of the members of the genus *Bacillus* (**Ahaotu *et al.*, 2013; Mohkam *et al.*, 2016**). Also, Rep-PCR was found to be discriminatory for further differentiation *Bacillus* isolates, even at determination of intra-specific genetic variability (**Ahaotu *et al.*, 2013**).

Since several *Bacillus* species or the related bacteria have a great diverse of metabolic diversity, can produce different enzymes, antibiotics, and metabolites and exhibit large variety of physiological properties, some species in this genus have many uses in medical, pharmaceutical, agricultural, and other industrial applications (**Celandroni *et al.*, 2019**). Several *Bacillus* species can produce plenty of secondary metabolites which have several different basic chemical structures and show a broad spectrum of antimicrobial or antibiotic activity against some important pathogens (**Sumi *et al.*, 2015**). Also, many studies showed that some members of the genus *Bacillus* produce several antimicrobial compounds such as lipopeptides, bacteriocins or other kind of peptides (**Sumi *et al.*, 2015; Stein, 2005; Zimina *et al.*, 2020**). Some antibiotics produced by *Bacillus* members are known to be widely used in veterinary and medicine. For example, bacitracin and polymyxin are two well-known and widely used antibiotics which mainly produced by *Bacillus* species (**Turnbull, 1996**). In addition, subtilin and subtilosin are well-known bacteriocins, produced by *B. subtilis*, which are active against many Gram-positive bacteria (**Jansen *et al.*, 1944; Zheng *et al.*, 1999**). Different laboratory techniques or methods could be used to appraise or screen the *in vitro* antibacterial or antifungal activity of an extract, or a pure compound obtained from different sources. The most common and well-known methods for evaluating antimicrobial activity are the disk-diffusion and agar dilution methods (**Balouiri *et al.*, 2016**). Apart from these methods, agar well diffusion method is broadly used to measure the *in vitro* antimicrobial activity of plant, microbial extracts or the other extracts obtained from different organisms (**Balouiri *et al.*, 2016; Magaldi et al., 2004**).

Today, infectious diseases are an important cause of deaths worldwide (**Guerrant, 1998**). However, the microorganisms that cause infection develop resistance to the drugs (especially antibiotics) used against them, and the World faces a significant increase in infections caused by antibiotic-resistant infection agents (**Ventola, 2015**). For example, in Europe, deaths caused by multidrug-resistance bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa were estimated at approximately 25.000 for each year (***Prestinaci *et. al.*, 2015**). Given the current prevalence of antibiotic-resistant pathogens worldwide, the discovery and development of new classes of powerful antibiotics with new inhibitory mechanisms is a necessity. Bacteria in the *Bacillus* genus can produce many antimicrobial peptides, and only a small fraction of the potentially produced antimicrobial molecules by the genus have been estimated to be identified. Therefore, it seems to be reasonable to select these bacteria as a good starting point in the search for new inhibitory substances. For this purpose, it is aimed to molecularly identify different Bacillaceae members isolated from soil samples and to investigate their antimicrobial activity against various human pathogenic bacteria and fungi. The results obtained are thought to be important in the discovery and identification of new antimicrobial agents.

MATERIAL AND METHODS

Isolation of bacteria

The bacterial isolates were isolated from soil samples collected from wheat fields in Kırşehir, Turkey. A total of ten soil samples were collected. 1 gr of soil for each sample was weighted and suspended in 3 ml of sterile PBS (Phosphate Saline Buffer). After suspending of soil samples, serial dilutions from 10^{-1} to 10^{-6} was prepared using sterile PBS for each sample. 100 µl suspension from each sample

was plated on *Bacillus* ChromoSelect Agar (Sigma-Aldrich, Missouiri, USA) and incubated at 30°C for two days in dark. At the end of the incubation period, *Bacillus* or *Bacillus*-like colonies were selected and transferred to nutrient agar (NA) (Sigma-Aldrich, Missouiri, USA) by streaking and incubated at 30°C for two days. Finally, single colonies were selected and stored at -20°C in 20% glycerol for further experiments.

Phenotyping

Each selected bacterial isolate was streaked on NA and incubated at 30°C for two days in dark. All morphological characters were determined by studying a single colony. Bacterial colony and cell morphologies were determined by using a stereo microscope or a binocular microscope (1000 × magnification), respectively. Gram staining procedure was followed according to the method of **Claus (1992)**. Endospore staining was performed based on the method described by **Prescott *et al.* (1996)**. The motility of cells was determined in semi-solid agar (**Soutourina *et al.*, 2001**). Negative staining was used to determine whether bacterial isolates had capsules or not.

Gene sequencing

For all genomic analyses, genomic DNAs for each bacterium were extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Invitrogen, Waltham, MA, USA) according to the manufacturer's recommendations. All genomic DNAs spectrophotometrically quantified, checked using agarose gel electrophoresis and stocked at -20°C for further analysis.
16S rRNA genes of the related bacterial isolates were amplified by the Polymerase Chain Reaction (PCR) from genomic DNAs using the primer pairs of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') as forward and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as reverse (**Lane, 1991**). PCR reactions for 50 µl volume contained 5 µL 10× *Taq* DNA polymerase reaction buffer, 1.5 µL 10 mmol/L from each dNTP, 1.5 µL 10 pmol each of the opposing oligonucleotide primers, 1,25 µL 5 U/ µL of *Taq* DNA polymerase (Fermentas, Waltham, MA, USA), 3 µL $MgCl_2$ and 1 µl genomic DNA as template. The final volume was completed to 50 µl by dH_2O. PCR amplification was performed with a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA). PCR conditions were set up as after initial denaturation for 5 min at 95°C, 35 cycles of denaturation (50 s at 94 °C), annealing (60 s at 55 °C), and extension (1,5 min s at 72 °C). A 7-min final extension at 72°C was provided at the end of the cycling steps, and then samples were maintained at 4°C. Water was used as template in negative control (**Demirci *et al.*, 2013**). The primer pairs of 518F (5′-CCAGC AGCCGCGGTAATACG-3′) and 800R (5′-TACCAGG GTATCTAATCC-3′) were used for sequence analysis (Macrogen) (**Sevim *et al.*, 2018**). 5 µl from each PCR product were loaded in 1-2 % agarose gel containing ethidium bromide and visualized under UV light after running at 90 V for 35 min with 100 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). PCR products whose accuracy were proven were selected and sent to Macrogen (The Netherlands) for sequence analysis.

Antimicrobial activity assay

Within the scope of the study, the antimicrobial effects of the isolated *Bacillus* or *Bacillus*-like isolates against various human pathogenic bacteria and fungi were also investigated by agar-well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS, 1993). Following bacterial and fungal pathogen were used: *Escherichia coli* ATCC 25922, *Yersinia pseudotuberculosis* ATCC911, *Bacillus cereus* 709 ROMA, *B. megaterium* DSMZ32, *B. subtilis* ATCC 6633, *B. spinosa* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 6057, *Klebsiella pneumoniae* ATCC 13883, *Listeria monocytogenes* NCTSC 11994, *Candida albicans* ATCC 60193, *C. tropicalis* ATCC 13803 and *C. glabrata* ATCC 66032. All bacteria and fungi to be tested were streaked on NA (for bacteria) and potato dextrose agar (PDA) (for fungi) to obtain single colonies and incubated at 37°C for 18 h for bacteria and 48 h for fungi. After that, all bacteria from single colony were first cultured in Nutrient broth (NB) (Sigma-Aldrich, Missouiri, USA) in a rotary shaker at 30°C for 18 h. After that, each strain was adjusted to approximately a concentration of 10^8 cells/ml based on 0.5 McFarland standard (**Gonelimali *et al.*, 2018**). Potato dextrose broth (PDB) (Sigma-Aldrich, Missouiri, USA) was used for fungal strains, and they were incubated at 37°C for two days. After that, each inoculum for bacteria and fungi to be tested was spread on Muller Hinton Agar (MHA) and Potato Dextrose Agar (PDA), respectively, with a sterile swab moistened with the bacterial and fungal suspensions. After spreading, all were air dried for two hours. After that, wells were drilled on the surface of the agar with a diameter of 6 mm with a sterile cork borer. *Bacillus* or *Bacillus*-like strains to be used were firstly inoculated in 3 ml of mueller hinton broth and incubated at 30 °C overnight. After incubation, they were centrifuged at 6,000 g for 15 min and supernatants were separately filtered with Millex-GV filters (0.22 µm, Merck, Darmstadt, Germany) to obtain cell free supernatant (CFS) (**Zimina *et al.*, 2016**). And then, 100 µl from each CFS were inoculated into the wells. All petri dishes were incubated at 37°C for 20 h and the antimicrobial activity was evaluated by measuring the inhibition zone (including the wells diameter) against the test organism. Wells containing the same volume of nutrient broth employed as negative controls while standard antibiotic solutions of kanamycin (50 µg/ml) and tetracycline (50 µg/ml) were used as the positive controls (**Gonelimali *et al.*, 2018; Zimina *et al.*, 2016; Dahiya and Purkayastha, 2012**). Tests were repeated three times and the standard deviation were provided.

Minimal inhibition concentration assay

The strain ES-18 was selected for MIC (Minimal Inhibitory Concentration) assay since it has the most effective antimicrobial result. MIC was determined using the microplate dilution method according to the study of Andrews (**Andrews, 2002**). To obtain the cell free supernatant for strain ES-18, the bacterial inoculation from single colony was done into 3 ml of nutrient broth and incubated at 30°C overnight. After that, 1 ml of this culture corresponding to 10^3 cfu/ml was inoculated into 99 ml nutrient broth and incubated at 30°C overnight. The culture was centrifuged at 8.000 g for 20 min and the supernatant was filtered with Millex-GV filters (0.22 µm, Merck, Darmstadt, Germany) to obtain CFS. After that, the serial dilution was made on the CFS with NB by two-fold dilution (2^{-1} to 2^{-8}). Then, 5 µl cell suspension (~10^7 cfu/ml) of fresh pathogenic bacterial cultures (*B. cereus* 709 ROMA, *B. megaterium* DSMZ32, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. faecalis* ATCC 6057and *L. monocytogenes* NCTSC 11994) were inoculated into each well. Tetracycline and nutrient broth were used as positive and negative control, respectively. The 96-well plates were incubated at 37˚C for 18 h. Finally, the growth in each well was evaluated by eye. The minimum dilution without the growth was indicated as the MIC value (**Baharudin *et al.*, 2021**).

Minimum bactericidal concentration assay

After 18 hours of inoculation in MIC experiments, approximately 5 µl were taken from the wells with no bacterial growth and spread on nutrient agar. The petri dishes were then incubated in the dark for 18 hours at 37°C. The concentration in the growing petri dishes was bacteriostatic, while the concentration in petri dishes without growth was evaluated as bactericidal (**Baharudin *et al.*, 2021**).

Data analysis

All sequences from the bacterial isolates were processed and edited by Bioedit software (**Hall, 1999**). They were first analyzed for the presence of chimeras using the UCHIME2 program from the National Center for Biotechnology Information (NCBI) database (**Edgar, 2016**). Sequences which were suspected to be chimeric were not included in phylogenetic analyses. All sequences were separately subjected to the BLAST search in NCBI database to determine percent similarity of the isolates with the most related sequences (**Altschul *et al.*, 1990**). After that, final sequences obtained from this study and downloaded from GenBank were aligned using Clustal W packed in Bioedit (**Hall, 1999; Thomson *et al.*, 1994**) for each gene region. Alignment positions that were high in insertions and/or deletions were removed. Finally, MEGA 11.0.10 software was used to construct the neighbor-joining tree with p-distance analysis (**Saitou and Nei, 1987; Tamura et al., 2021**). The tree was subsequently bootstrapped with 1.000 replicates to determine the strength of the internal branches (**Felsenstein, 1985**).
The antimicrobial activity measured as the inhibition zone were presented as mean ± SD of three replicates. One-way analysis of variance (ANOVA) and LSD multiple comparison test were performed by SPSS 16.0 software to determine significant differences among the bacterial isolates. P value lower than < 0.05 was considered as significant difference.

Accession numbers

All 16S rRNA gene sequences for each bacterium used in this study were deposited in GenBank and the GenBank *accession numbers* for 16S rRNA of the bacterial *isolates* are MW699448 to MW699467 (Table 2).

RESULTS

A total of twenty *Bacillus* or *Bacillus*-like isolates were obtained from soil samples using *Bacillus* ChromoSelect Agar. They were first identified based on their morphological characters. The colony of all isolates were cream color and rough. They were all Gram positive and endopsore-forming bacil. None of them had capsule and some of them (ES-10, 11, 12, 12, 14, 17, 20 and 22) were motile (Table 1).

Table 1 Morphological characteristics of *Bacillus* or *Bacillus*-like isolates

Isolates	Colony color	Colony shape	Shape of Bacteria	Gram Strain	Spore Strain	Place of Spore	Shape of Spore	Capsule	Motility	Turbidity *	Source
ES-1	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-2	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-3	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-4	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-6	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-7	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-8	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-9	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-10	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-11	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-12	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-13	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-14	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-15	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-16	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-17	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-18	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-19	Cream	Rough	Bacil	+	+	Central	Oval	-	-	Turbid	Soil
ES-20	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil
ES-22	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Soil

* When grown in nutrient broth

Morphological characterization of the isolates was also confirmed by 16S rRNA, *rpoB* and *recA* gene sequences. Based on 16S rRNA Blast search and phylogeny, all isolates were identified at genus level as *Bacillus* sp., except for the strain ES-18 which is *Lysinibacillus* sp. The Blast search results and the phylogram generated by 16S rRNA sequences were given on Table 2 and in Figure1, respectively. Since *Lysinibacillus* sp. ES-18 has the highest antibacterial activity, this isolate was compared with other *Lysinibacillus* species in NCBI GenBank using 16S rRNA sequencing and phylogenetic analysis. Accordingly, ES-18 was identified as *Lysinibacillus xylanilyticus* (Fig. 2).

Table 2 Percentage query coverage and identity of the bacterial isolates with their the most related bacterial species based on Blast search in GenBank using 16S rRNA gene sequences.

Isolate	**Isolate GenBank ID number**	**Species**	**GenBank ID number**	**Query coverage (%)**	**Identity (%)**
ES-1	MW699448	*Bacillus cereus* EB62	MH130346	100	99.79
		Bacillus sp. CKL3	MT197315	100	99.79
		Bacillus sp. CKL8	MT197314	100	99.79
		Bacillus thuringiensis FDB-10A	MH553093	100	99.79
ES-2	MW699449	*Bacillus* sp. CSB16	KX289457	100	99.93
		Bacillus kochii FJAT-46246	MK859993	100	99.86
		Bacillus sp. JSM 1685054	MG893121	100	99.86
		Bacillus sp. JSM 1685035	MG893120	100	99.86
ES-3	MW699450	*Bacillus* sp. HBUM206332	MT541005	100	99.86
		Bacillus cereus f24	KP411923	100	99.86
		Bacillus sp. BK51-2004	KJ186086	99	99.93
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.86
ES-4	MW699451	*Bacillus thuringiensis* K2	MK696253	100	99.93
		Bacillus thuringiensis ZLynn800-5	KY316426	100	99.93
		Bacillus sp. ZLynn500-27	KY316417	100	99.93
		Bacillus cereus ZLynn500-19	KY316412	100	99.93
ES-6	MW699452	*Bacillus* sp. PC-21	MG988287	100	99.93
		Bacillus toyonensis GT35	KY312777	100	99.93
		Bacillus cereus EB62	MH130346	100	99.86
		Bacillus sp. CKL3	MT197315	100	99.86
ES-7	MW699453	*Bacillus* sp. OP7	MK757670	100	99.93
		Bacillus wiedmannii Q8	MK719881	100	99.93
		Bacillus wiedmannii Q7	MK719880	100	99.93
		Bacterium R-54	KF475711	100	99.93
ES-8	MW699454	*Bacillus* sp. HBUM206332	MT541005	100	100
		Bacterium MIS_YJ_J02	MW037762	100	100

		Bacterium CAP_KM_H60	MW037721	100	100
		Bacterium R-15	KF475707	100	100
ES-9	MW699455	*Bacillus cereus* EB62	MH130346	100	100
		Bacillus sp. CKL3	MT197315	100	100
		Bacillus sp. CKL8	MT197314	100	100
		Bacillus thuringiensis FDB-10A	MH553093	100	100
ES-10	MW699456	*Bacillus* toyonensis FJAT-30000	MG905872	100	100
		Bacillus wiedmannii ER6	MT124531	100	99.93
		Bacillus wiedmannii EH20	MN750775	100	99.93
		Bacillus sp. 206312	MN539139	100	99.93
ES-11	MW699457	*Bacillus* sp. BK51-2004	KJ186086	100	100
		Bacillus proteolyticus D65_CV6R	MK883171	100	99.93
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.93
		Bacillus toyonensis NGB-SF113	MK318228	100	99.93
ES-12	MW699458	*Bacillus proteolyticus* D65_CV6R	MK883171	100	99.86
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.86
		Bacillus sp. BK51-2004	KJ186086	99	99.79
		Bacillus sp. HBUM206332	MT541005	100	99.72
ES-13	MW699459	*Bacillus proteolyticus* D103_CV6R	MK883200	100	100
		Bacillus proteolyticus D65_CV6R	MK883171	100	100
		Bacillus sp. BK51-2004	KJ186086	99	99.93
		Bacillus sp. HBUM206332	MT541005	100	99.86
ES-14	MW699460	*Bacillus* sp. HBUM206332	MT541005	100	99.86
		Bacillus cereus f24	KP411923	100	99.86
		Bacillus sp. BK51-2004	KJ186086	99	99.93
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.86
ES-15	MW699461	*Bacillus thuringiensis* L26	KU179338	100	99.72
		Bacillus sp. N5/665	LN680102	99	99.79
		Bacillus sp. SYW15	FJ601645	99	99.79
		Bacillus sp. 816OP10	MK757668	100	99.65
ES-16	MW699462	*Bacillus* sp. HBUM206332	MT541005	100	99.86
		Bacillus cereus f24	KP411923	100	99.86
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.86
		Bacillus proteolyticus D65_CV6R	MK88317	100	99.86
ES-17	MW699463	*Bacillus cereus* M2	JF836882	100	100
		Bacillus sp. CKL3	MT197315	100	100
		Bacillus sp. CKL8	MT197314	100	100
		Bacillus toyonensis FJAT-29971	MG905843	100	100
ES-18	MW699464	*Lysinibacillus* sp. mkj-14	KU159199	100	99.65
		Lysinibacillus xylanilyticus fwzy21	KF208475	100	99.65
		Lysinibacillus sp. Ce.BL.R.9	MT126521	100	99.65
		Lysinibacillus sp. Firmi-71	MH683160	100	99.51
ES-19	MW699465	*Bacillus cereus* EB62	MH130346	100	100
		Bacillus sp. CKL3	MT197315	100	100
		Bacillus sp. CKL8	MT197314	100	100
		Bacillus thuringiensis FDB-10A	MH553093	100	100
ES-20	MW699466	*Bacillus* sp. BK51-2004	KJ186086	100	100
		Bacillus sp. HBUM206332	MT541005	99	100
		Bacillus proteolyticus D103_CV6R	MK883200	100	99.93
		Bacillus toyonensis NGB-SF113	MK318228	100	99.93
ES-22	MW699467	*Bacillus* sp. RNT2	MT173794	100	99.93
		Bacillus sp. 206314	MN538871	100	99.93
		Bacillus sp. J16OP6	MN519545	100	99.93
		Bacillus cereus IAM7	MH815089	100	99.93

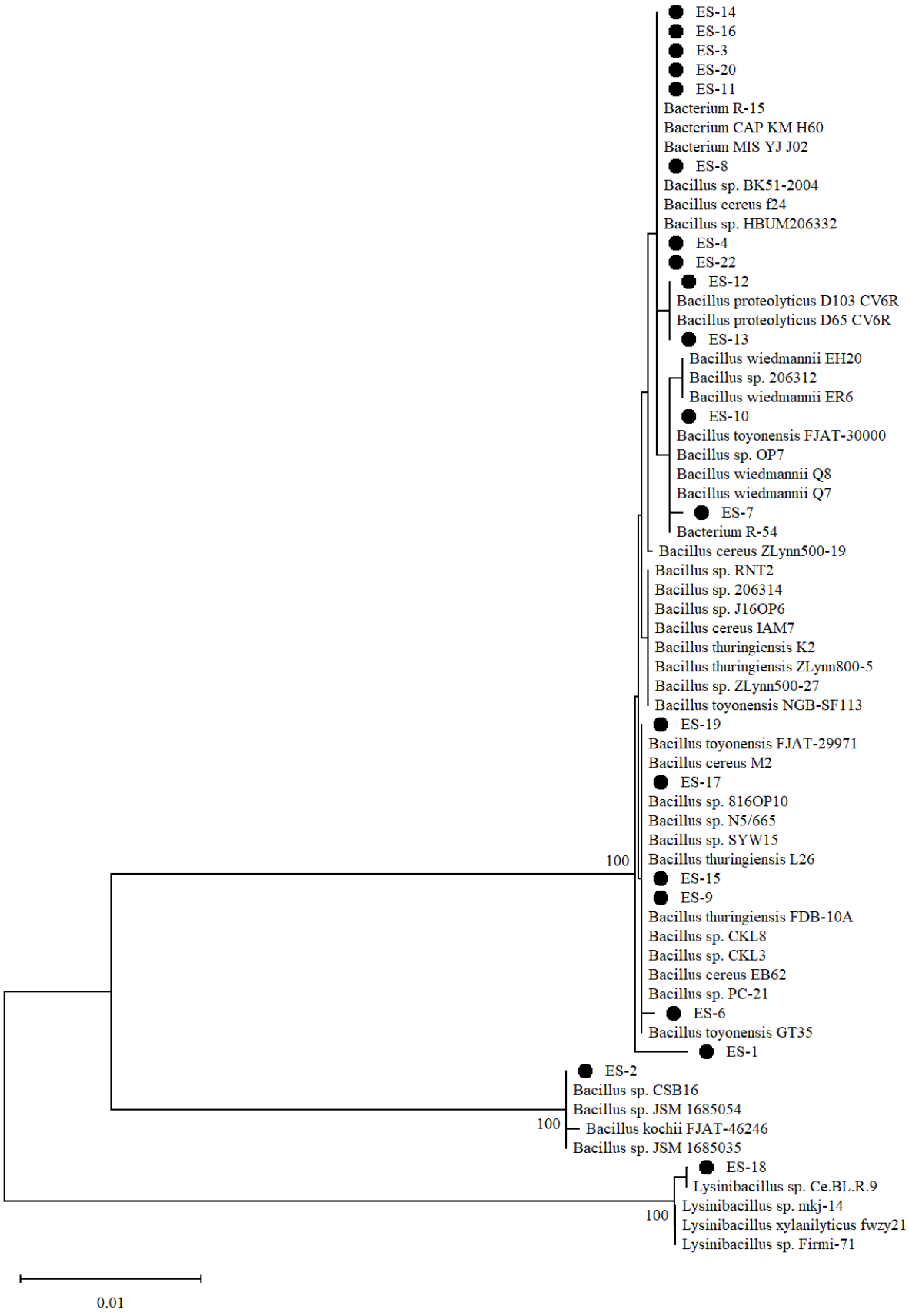

Figure 1 A phylogram showing phylogenetic relations of the bacterial isolates and their most related bacterial strains or species based on Blast search in GenBank.

The phylogram was constructed using clustering approach (the neighbor-joining analysis with p-distance correction) in MEGA 11 (**Tamura *et al.*, 2021**). Numbers indicate bootstrap values was inferred after 1.000 pseudoreplicates and bootstrap values with > 70% were indicated on each node. The bacterial isolates obtained from this study were indicated as black circle at the beginning of each isolate. The tree included a scale bar on the bottom of the phylogram indicating the degree of dissimilarity.

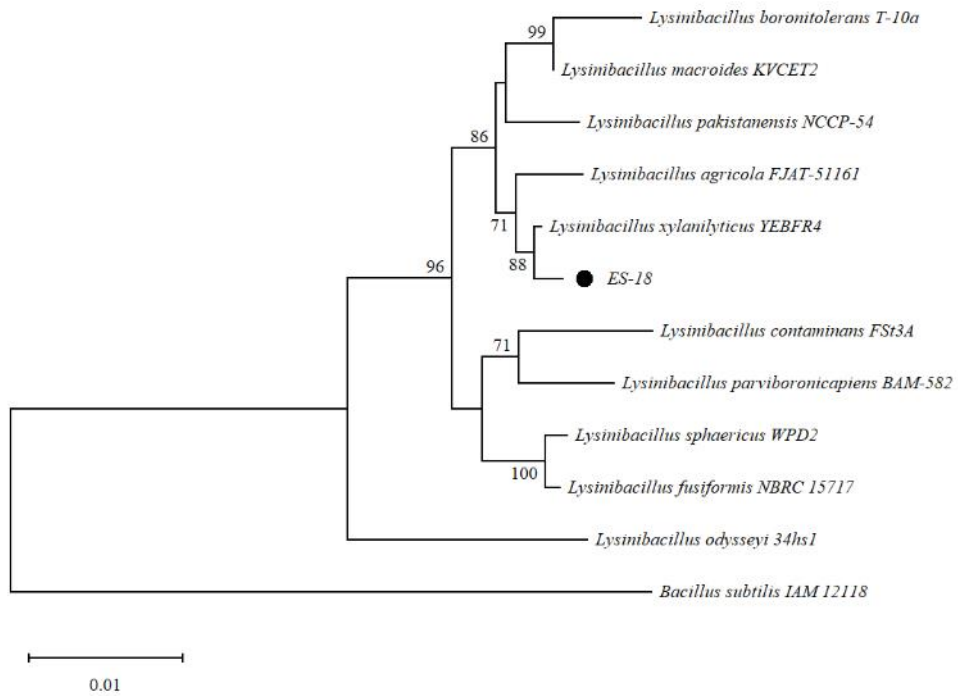

Figure 2 A phylogram showing phylogenetic position of the isolate ES-18 and other *Lysinibacillus* species in NCBI GenBank. The tree was constructed using clustering approach (the neighbor-joining analysis with p-distance correction) in MEGA 11 (**Tamura *et al.*, 2021)**. Numbers indicate bootstrap values was inferred after 1.000 pseudoreplicates and bootstrap values with > 70% were indicated on each node. The isolate ES-18 was indicated with black circle. The tree included a scale bar on the bottom of the phylogram indicating the degree of dissimilarity.

Some of the bacterial isolates used in this study were observed to have antibacterial activity against both Gram-positive and Gram-negative bacteria such as *B. cereus* 709 ROMA, *B. megaterium* DSMZ32, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. faecalis* ATCC 6057 and *L. monocytogenes* NCTSC 11994. None of them have any antifungal effects against *Candida* species (Table 3).

Table 3 Antimicrobial activity of *Bacillus* or *Bacillus*-like isolates used in this study

	Inhibition zone (mm)												
Isolates	**Ec**	**Yp**	**Bc**	**Bm**	**Bs**	**Sa**	**Ef**	**Kp**	**Lm**	**Bss**	**Ca**	**Cg**	**Ct**
ES-1	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-2	-	-	-	15 ± 1	15 ± 0	-	15 ± 0	-	15 ± 0	-	-	-	-
ES-3	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-4	-	-	15 ± 1	-	-	-	-	-	-	-	-	-	-
ES-6	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-7	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-8	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-9	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-10	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-11	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-12	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-13	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-14	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-15	-	-	15 ± 0	15 ± 0	15 ± 1	15 ± 0	15 ± 0	-	15 ± 1	-	-	-	-
ES-16	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-17	-	-	15 ± 1.7	15 ± 2.6	15 ± 0	16 ± 0	15 ± 1	-	14 ± 1	-	-	-	-
ES-18	-	-	15 ± 0	13 ± 0	13 ± 1	18 ± 1	15 ± 1	-	15 ± 0	-	-	-	-
ES-19	-	-	10 ± 1	10 ± 2	10 ± 1	15 ± 1	12 ± 0	-	-	-	-	-	-
ES-20	-	-	-	-	-	-	-	-	-	-	-	-	-
ES-22	-	-	-	-	-	-	-	-	-	-	-	-	-
MHB*	-	-	-	-	-	-	-	-	-	-	-	-	-
Kanamycin	-	-	-	-	-	-	-	-	-	-	-	40 ± 2	-
Tetracycline	40 ± 1	20 ± 0	40 ± 2	35 ± 1	35 ± 0	35 ± 0	45 ± 3	45 ± 1	30 ± 0	25 ± 1	-	-	-

* MHB (Mueller Hinton Broth) was used as negative control. Ec, *Escherichia coli*; Yp, *Yersinia tuberculosis*; Bc, *Bacillus cereus*; Bm, *Bacillus megaterium*; Bs, *B. subtilis*; Sa, *Staphylococcus aureus*; Ef, *Enterococcus faecalis*; Kp, *Klebsiella pneumoniae*; Lm, *Listeria monocytogenes*; Bss, *B. spinosa*; Ca, *Candida albicans*; Ct, *C. tropicalis* and Cg, *C. glabrata.*

The difference between *Bacillus* or *Bacillus*-like isolates, which had antibacterial activity against different types of the tested bacteria, was separately and statistically evaluated. There was a significant difference among isolates with respect to the activity against *B. cereus* 709 ROMA (df=4, F=15, p<0.05). The highest antibacterial activity was obtained from ES-4, ES-15, ES-17, and ES-18 (df=4, F=15, p<0.05). There was also significant different between the isolates in terms of the antibacterial activity against *B. megaterium* DSMZ32 (df=4, F=6, p<0.05). The highest activity was obtained from ES-2, ES-15, and ES-17 (df=4, F=6, p<0.05). As for *B. subtilis* ATCC 6633, the statistical difference was also determined and ES-2, ES-15, and ES-17 showed the highest activity (df=4, F=24, p<0.05). For *S. aureus* ATCC 25923, the significant difference was seen, and the isolate ES-18 showed the important activity which is the highest activity observed in this study (df=3, F=6, p<0.05). The isolates ES-2, ES-15, ES-17, and ES-18 showed statistically important activity against *E. faecalis* ATCC 6057 (df=4, F=5.4, p<0.05). Four isolates (ES-2, ES-15, ES-17, and ES-18) showed the activity against *L. monocytogenes* NCTSC 11994 but there is no significant difference among them (df=3, F=0.0, p<0.05).

MIC values of the isolate ES-18 were indicated as CFS dilution factor and determined as 2^{-1}, 2^{-1}, 2 (no dilution), 2 (no dilution), 2^{-1} and 2 (no dilution) against *B. cereus* 709 ROMA, *B. megaterium* DSMZ32, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. faecalis* ATCC 6057 and *L. monocytogenes* NCTSC 11994, respectively. It was also determined that CFS of the isolate ES-18 had bacteriostatic effect on the tested bacteria.

DISCUSSION

The recent emergence of antibiotic resistance in pathogenic bacteria, which are important for human and animal health, makes difficult to treat infections seen in the clinic and made the research and discovery of new antimicrobial substances as an important and still desirable topic. Members of the Bacillaceae family are one of the most commonly found bacteria groups on earth due to their resistance endospore (**Mandic-Mulec *et al.*, 2015**). At the same time, members of this family are an important source for producing a wide range of structurally diverse antimicrobial substances with rapid kill activity against various pathogens (**Zhao *et al.*, 201**8). Antibiotics, which constitute an important part of antimicrobial substances, are mainly produced by microorganisms living in the soil (**Stoica *et al.*, 2019**). For this purpose, the antimicrobial effect of twenty isolates in the Bacillaceae family isolated from the soil was investigated against various pathogenic bacteria.

Although no antifungal activity was obtained in this study, the highest antibacterial activity against some pathogens was obtained from *L. xylanilyticus* ES-18. The genus *Lysinibacillus* was first separated from *Bacillus* genus in 2007 and reclassified as a novel genus. Members of this genus consist of motile and rod-shaped cells that produce ellipsoidal or spherical-shaped endospores (**Ahmed *et al.*, 2007**). Until now, the bacteria involved in this genus have been reported to be important for showing insecticidal activity against various insects (*L. sphaericus*), heavy metal bioremediation (such as *L. sphaericus, L. fusiformis, L. xylanilyticus,* and *L. macrolides*) and plant growth stimulator and biological control of plant diseases in agriculture (such as *L. sphaericus, L. fusiformis, L. chungkukjangi*, and *L. xylanilyticus*) (**Ahsan and Shimizu, 2021**). *L. xylanilyticus* within the genus was first identified in 2010 with the ability to degrade xylan (**Lee *et al.*, 2009**). Antimicrobial studies related to this bacterium are relatively limited. **Suvega (2014)** investigated the antimicrobial properties of some marine *L. xylanilyticus* isolates and they found that some isolates showed both antibacterial and antifungal activity against plant pathogens. In a study, **Bibi et al. (2020)** determined that *L. xylanilyticus* EA370 isolated from a marine sponge *Suberea mollis* showed low level antifungal activity. In addition, a few studies indicate that some strains belonging to the genus *Lysinibacillus*, which are phylogenetically closest to *L. sphaericus* and *L. xylanilyticus*, showed antimicrobial activity against foodborne bacterial and fungal pathogens (**Ahmad *et al.*, 2014**).

CONCLUSION

We isolated and characterized twenty *Bacillus* and *Bacillus*-like bacteria from soil samples and tested them against important bacterial and fungal pathogens. All isolates were characterized by 16S rRNA gene sequencing. No isolate showed antifungal activity. However, some of isolates showed antibacterial activity and the isolate ES-18 had the highest antibacterial activity with moderate-level. Further studies should be carried out to purify and characterize the compound or protein produced by the ES-18 isolate, which causes antibacterial activity.

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