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

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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 Bacillaceae-like bacteria were isolated from soil samples in wheat fields and they were identified based on their morphological and molecular features using 16S RNA 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 xylanitica. 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, Staphylococcus 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 2³. It was also determined that all effective dilutions were bacteriostatic. This is the first study showing antimicrobial properties of L. xylanitica 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 or coccoid forms (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 (Maughan and Van der Auwera, 2011). 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-resistant 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, Missouri, 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, Missouri, 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 were spectrophotometrically quantified, checked using agarose gel electrophoresis and stored 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’-TACGGYTACCTTGTTGAATCCT-3’) as reverse (Lane, 1991). PCR reactions for 50 µl reaction mixture contained 10 µL Taq DNA polymerase reaction buffer, 1.5 µL 10 mM each of the opposing oligonucleotide primers, 1.25 µL 5 U/µL of Taq DNA polymerase (Fermentas, Waltham, MA, USA), 3 µL MgCl2 and 1 µL genomic DNA as template. The final volume was completed to 50 µl by ddH2O. 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 (Demirei et al., 2013). The primer pairs of 518F (5’-CCAGCAGGCGGCTGATATTG-3’) and 806R (5’-TACGGYTACCTTGTTGAATCCT-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 staining with the CFS (Sigma, Aldrich, USA). Each strain was adjusted to 0.5 McFarland standard. 5 µl of kanamycin (50 µg/ml) and tetracycline (50 µg/ml) were used as the positive controls (Gonellinalli et al., 2018; Zimina et al., 2016; Dahiya and Purkayastha, 2012). Tests were repeated three times and the standard deviation was provided.

**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 ATCC91, Bacillus cereus 709 ROMA, B. megaterium DSMZ322, B. subtilis ATCC 6633, B. spinosa ATCC 6633, Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 6057, Klebsiella pneumoniae ATCC 13883, Listeria monocytogenes NCSTC 11994, Candida albicans ATCC 60193, C. tropicalis ATCC 13803 and C. glabrata ATCC 60302. 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, Missouri, USA) in a rotary shaker at 30°C for 18 h. After that, each strain was adjusted to approximately a concentration of 10⁶ cells/ml based on 0.5 McFarland standard (Gonellinalli et al., 2018). Potato dextrose broth (PDB) (Sigma-Aldrich, Missouri, USA) was used for fungal strains, and they were incubated at 37°C for 2 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, all bacterial and fungal strains 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.

**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 10⁶ 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 2⁻³). Then, 5 µl cell suspension (~10⁶ cfu/ml) of fresh pathogenic bactericules (B. cereus 709 ROMA, B. megaterium DSMZ322, B. subtilis ATCC 6633, S. aureus ATCC 25923, E. faecalis ATCC 6057 and L. monocytogenes NCSTC 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 minimal 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 rDNA 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 characteristics. The colony of all isolates were cream color and rough. They were all Gram positive and endospore-forming bacil. None of them had capsule and some of them (ES-10, 11, 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 ID number | Species | GenBank number | ID | Query coverage (%) | Identity (%) |
|---------|-------------------|---------|----------------|----|---------------------|--------------|
| ES-1    | MW699448          | Bacillus cereus EB62 | MH130346 | 100 | 99.79              |
|         |                   | Bacillus sp. CLK3  | MT197315 | 100 | 99.79              |
|         |                   | Bacillus sp. CLK8  | MT197314 | 100 | 99.79              |
|         |                   | Bacillus thuringiensis FDB-10A | MH553093 | 100 | 99.79              |
| ES-2    | MW699449          | Bacillus sp. CSB16 | KX289457 | 100 | 99.93              |
|         |                   | Bacillus kochi 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 cereus f24 | KP411923 | 100 | 99.96              |
|         |                   | Bacillus sp. BK51-2004 | KJ136068 | 99  | 99.93              |
|         |                   | Bacillus proteolyticus D103_CV6R | MK883200 | 100 | 99.86              |
| ES-4    | MW699451          | Bacillus thuringiensis K2 | MK696283 | 100 | 99.93              |
|         |                   | Bacillus thuringiensis ZLYm800-5 | KY316426 | 100 | 99.93              |
|         |                   | Bacillus sp. ZLYm500-27 | KY316417 | 100 | 99.93              |
|         |                   | Bacillus cereus ZLYm500-19 | KY316412 | 100 | 99.93              |
| ES-6    | MW699452          | Bacillus sp. PC-21 | MG988287 | 100 | 99.93              |
|         |                   | Bacillus toyomensis GT35 | KY317777 | 100 | 99.93              |
|         |                   | Bacillus cereus EB62 | MH130346 | 100 | 99.86              |
|         |                   | Bacillus sp. CLK3  | 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              |
|         |                   | Bacillus R-54 | KF475711 | 100 | 99.93              |
| ES-8    | MW699454          | Bacillus sp. HBUM206332 | MT541005 | 100 | 100                |
|         |                   | Bacillus MIS YJ J02 | MW037762 | 100 | 100                |
| Strain Name | Accession Number | Similarity | Percentage |
|-------------|------------------|------------|------------|
| Bacillus CAP_KM_H60 | MW037721 | 100 | 100 |
| Bacillus R-15 | KF475707 | 100 | 100 |
| Bacillus cereus EB62 | MH130346 | 100 | 100 |
| Bacillus sp. CLK3 | MT197315 | 100 | 100 |
| Bacillus sp. CLK8 | MT197314 | 100 | 100 |
| Bacillus thuringiensis FDB-10A | MH553093 | 100 | 100 |
| Bacillus toyonensis FIAT-30000 | MG905872 | 100 | 100 |
| Bacillus wiedmannii ER6 | MT124531 | 99.93 | 99.93 |
| Bacillus wiedmannii EH20 | MN750775 | 99.93 | 99.93 |
| Bacillus sp. 206312 | MN530139 | 99.93 | 99.93 |
| Bacillus sp. BK51-2004 | KJ186086 | 100 | 100 |
| Bacillus proteolyticus D65_CV6R | MK883171 | 99.93 | 99.93 |
| Bacillus proteolyticus D103_CV6R | MK883200 | 99.93 | 99.93 |
| Bacillus toyonensis NGB-SF113 | MK318228 | 99.93 | 99.93 |
| Bacillus proteolyticus D65_CV6R | MK883171 | 98.86 | 98.86 |
| Bacillus proteolyticus D103_CV6R | MK883200 | 98.86 | 98.86 |
| Bacillus sp. HBUM206332 | KJ186086 | 99 | 99.72 |
| Bacillus proteolyticus D103_CV6R | MK883200 | 98.86 | 98.86 |
| Bacillus toyonensis L26 | KU179338 | 97.2 | 97.2 |
| Bacillus sp. N5/665 | LN680102 | 99 | 99.79 |
| Bacillus sp. SYW15 | FJ601645 | 99 | 99.79 |
| Bacillus sp. 816OP10 | MK757668 | 99.65 | 99.65 |
| Bacillus sp. HBUM206332 | MTS41005 | 98.86 | 98.86 |
| Bacillus cereus f24 | KP411923 | 99.86 | 99.86 |
| Bacillus sp. BK51-2004 | KJ186086 | 99 | 99.93 |
| Bacillus proteolyticus D103_CV6R | MK883200 | 98.86 | 98.86 |
| Bacillus proteolyticus D65_CV6R | MK883171 | 99.93 | 99.93 |
| Bacillus cereus M2 | JF836882 | 100 | 100 |
| Bacillus sp. CLK3 | MT197315 | 100 | 100 |
| Bacillus sp. CLK8 | MT197314 | 100 | 100 |
| Bacillus toyonensis FIAT-29971 | MG905843 | 100 | 100 |
| Lysinibacillus sp. mkj-14 | KU159199 | 99.65 | 99.65 |
| Lysinibacillus xylanilyticus fwy21 | KF208475 | 99.65 | 99.65 |
| Lysinibacillus sp. Cc.BL.R.9 | MT126521 | 99.65 | 99.65 |
| Lysinibacillus sp. Firms-10 | MH883160 | 99.65 | 99.65 |
| Bacillus cereus EB62 | MH130346 | 100 | 100 |
| Bacillus sp. CLK3 | MT197315 | 100 | 100 |
| Bacillus sp. CLK8 | MT197314 | 100 | 100 |
| Bacillus thuringiensis FDB-10A | MH553093 | 100 | 100 |
| Bacillus sp. BK51-2004 | KJ186086 | 100 | 100 |
| Bacillus sp. HBUM206332 | MTS41005 | 99 | 100 |
| Bacillus proteolyticus D103_CV6R | MK883200 | 99.93 | 99.93 |
| Bacillus toyonensis NGB-SF113 | MK318228 | 99.93 | 99.93 |
| Bacillus sp. RNT2 | MT173794 | 100 | 99.93 |
| Bacillus sp. 206312 | MN538871 | 100 | 99.93 |
| Bacillus sp. J16OP6 | MNS519545 | 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 |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Isolates | Ec | Yp | Be | Bm | Bs | Sa | Ef | Kp | Lm | Bss | Ca | Cg | Ct |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| ES-1 | - | - | - | - | 15 ± 1 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | - | - | - | - |
| ES-2 | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - | - |
| ES-3 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-4 | - | - | - | 15 ± 1 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | - | - | - | - |
| ES-5 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-6 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-7 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-8 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-9 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-10 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-11 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-12 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-13 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-14 | - | - | - | - | 15 ± 1 | 15 ± 0 | - | - | - | - | - | - | - |
| ES-15 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | - | - | - |
| ES-16 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | - | - |
| ES-17 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | - |
| ES-18 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 |
| ES-19 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 |
| ES-20 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 |
| ES-21 | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 |
| MHB* | - | - | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 | 15 ± 0 |
| Kanamycin | 40 ± 1 | 20 ± 0 | 40 ± 2 | 35 ± 1 | 35 ± 0 | 35 ± 0 | 35 ± 0 | 45 ± 3 | 45 ± 1 | 30 ± 0 | 25 ± 1 | 40 ± 2 |

* MHB (Mueller Hinton Broth) was used as negative control. Ec, Escherichia coli; Yp, Yersinia tuberculosis; Be, Bacillus cereus; Bm, Bacillus megaterium; Be, 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 Bacillller-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 DSMZ22 (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). For B. subtilis ATCC 6057, the statistical difference was also determined and ES-2, ES-15, and ES-17 showed the high 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 NCTC 11994 but there is no significant difference among the isolates (F=0.0, p>0.05).

MIC values of the isolate ES-18 were indicated as CFS dilution factor and determined as 2, 2.1, 2 (no dilution), 2 (no dilution), 2 and 2 (no dilution) against B. cereus 709 ROMA, B. megaterium DSMZ22, 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 new common sources of groups on earth due to their resistance to harsh environments (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., 2018). 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 in showing insecticidal activity against various insects (L. sphaciaeus), heavy metal bioremediation (such as L. sphaciaeus, L. fusiformis, L. xylanilyticus, and L. macrolides) and plant growth stimulator and biological control of plant diseases in agriculture (such as L. sphaciaeus, L. fusiformis, L. chungkakjangi, 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. sphaciaeus and L. xylanilyticus, showed antimicrobial activity against foodborne and fungal pathogens (Ahmed 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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