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Isolation and Molecular Identification of *Bacillus boroniphilus*sp.nov., Isolated from Dishwasher

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Abstract

Although the dishwashers and specialized cleaning agents are designed to assure conditions for cleaning the inlet, the dishwasher itself may still be a reservoir of unwanted microbiota. In this study, we isolated and molecular identified two isolates of*Bacillus boroniphilus*sp.nov., from domestic dishwashers. A total of 51 bacterial isolates were isolated from samples collected from six domestic old household dishwasher in Mosul/Iraq. The swab collecting, followed by bacterial cultivation and identification revealed that two isolates were belonged to a novel Bacillus species *Bacillusboroniphilus*sp.nov. and were designated as KAAI1 and KAAI2 isolated from drain water part and basket part respectively. The results of phylogenetic analyses showed that *B.boroniphilus* KAAI1and KAAI2 were most related to Bacillus sp. (GQ284386), *Bacillus subterraneus* (KT719820) based on the 16S RNA genes tree. The two isolates demonstrated differences for Boron tolerance. For the ability to grow on free B different media, colonies growth were observed for KAAI1 isolate where's no growth for KAAI2 isolate. The 16S rRNA gene of KAAI1and KAAI2 novel isolates were deposited in the GenBank database (NCBI) under the accession numbers (MW193122) and (MW193123) respectively. This study concluded that the two isolates belong to a novel species *B. boroniphilus*KAAI1and KAAI2is a boron-tolerant bacterium that also requires this element for its growth. Dishwashers environments with high temperatures and contained the element boron in varying quantities in a detergents, this features makes it an excellent candidate for being a model organism to investigate the boron-bacterium relationship using molecular and biochemical approaches.

Key words: *Bacillus boroniphilus,* Dishwasher environment, Bacterial Boron, Boric acid-bacterium.

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1. Introduction

In developed countries, electric dishwashers have become an essential piece of household appliances. They are admired for their cost-effective features, such as water conservation, as well as the ease with which they can replace the daily routine of cutlery, hand-washing dishes, and other kitchen accessories. Nonionic detergents, polycarboxylates, and phosphonates, as well as a fragrances and mixture of hydrolysing enzymes, are popular ingredients in dishwasher cleaning agents. Despite the fact that advanced cleaning agents and dishwashers are built to ensure that the inlet is cleaned, the dishwasher itself may be a source of microbiota.

(Zupančič, Turk, Črnigoj, Avguštin, &Gunde-Cimerman, 2019). The dishwasher, like other food processing environments and industrial facilities, has a microbial surface that is vulnerable to biofilm production. (Banaszczyk et al., 2017).

Microbial community growth is generally caused by organic particles of food remnants and moist environments, particularly when the dirty inlet is washed the next day or stored for an extended period of time rather than being cleaned immediately. Unwanted signs such as a bad odor, colored sludge, or limescale, which cause technical problems with in dishwasher, all may be the product of algae, bacteria, and fungi forming biofilms. Listeria sp., Salmonella sp., Staphylococcus sp., and *Escherichia coli* are the most common foodborne pathogens found in biofilms (Banaszczyk et al., 2017). In the swab material collected from domestic dishwashers, yeast-like opportunistic fungi or Candida have been discovered. On the process boundaries, the various microorganisms present in the biofilm microbiological reservoir are adsorbed (Döğen et al., 2013). Extracellular production of metabolic by-products from the microorganism communities also helps to keep the biofilm stable. As a result, it can involve polysaccharides, proteins, phospholipids, and nucleic acids, which make contaminants much more difficult to remove, even with the use of special disinfectants designed for use within dishwashers (Mah& O'Toole, 2001),

Boron, in the presence of perborate tetrahydrate or sodium perborates, is applied to disinfectants because it has peroxygen bonds that can deliver oxidizing bleaching agents to solutions. Sodium perborate, on the other hand, must be hydrolyzed in hot water unless an enhancer is available. Increased boron rates in waste water have been recorded as a result of disinfectants containing sodium perborate. Since it creates hydrogen peroxide at lower

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temperatures, sodium percarbonate has been used as a replacement mainly in Europe. The amount of boron consumed has changed as a result of this substitution. (Parks & Edwards, 2005; Rathore).

Boron is a necessary micronutrient for plant, as it helps them preserve the stability of their cell walls. It is also required for the wellness of some animals as an ultra-trace function. (Rowe &Eckhert, 1999). It was initially reported that Boron is essential for some cyanobacteria (Mateo, Bonilla, Fernandez-Valiente, & Sanchez-Maeso, 1986). Furthermore, it is believed to really be toxic to living tissue when present in concentrations over a certain level (Çöl, Özkeserli, Kumar, Özdağ, &Alakoç, 2014; Nable, Bañuelos, & Paull, 1997).

An extremely boron-tolerant gram-positive, rod-shaped bacterium, motile was recently isolated from boron-containing soil in the Hisarcik region of Kutahya, Turkey. The bacterium was identified as belonging to the Bacillus genus and given the name *B. boroniphilus* depending on phenotypic, phylogenetic analyses, and chemotaxonomic. (Ahmed, Yokota, & Fujiwara, 2007; Birbir, Cicek, Caglayan, & Aslan, 2013). A number of boron-tolerant soil bacteria, including B. subtilis, were studied in a more recent study. The boron uptake of boroniphilus, the most resistant species of all, was measured after pulse exposures to low and high levels of boron in the growing medium. The much more tolerant the species, the less protoplasmic boron content it had, according to statistical analysis of the data. As a result, a high boron efflux or / and exclusion process in the tested bacteria was proposed as a boron tolerance mechanism. (Ahmed & Fujiwara, 2010).

Dishwashers environments with high temperatures and contained the element boron in varying quantities in a detergents, this feature makes it an excellent candidate for being a model organism to investigate the boron-bacterium relationship. Concerning all above and for the first time, this study aimed to isolate and molecular identify a boron tolerant bacterium *B. boroniphilus*from the domestic dishwasher's environments.

2. MATERIALS AND METHODS

2. 1. Microorganisms cultivation and microscoping

The Tryptic Soy Broth (TSB) medium preparation was from Scharlau Microbiology (Barcelona, Spain), other reagents like boric acid, sodium chloride, bacteriological agar and

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dyes for bacterial-cell staining were from Sigma-Aldrich (St Louis, MO, USA).Microorganisms cultivation and plate streaking were performed using standard microbiology methods. All incubations were made in a New Brunswick Scientific (Boulevard, CT, USA) or Binder (Tuttlingen, Germany) microbiology incubators.

2.2 Sample collection and isolation of bacteria:

Sample collected from six dishwasher of different types which used for periods of time ranging from several months to years in Mosul city/ Iraq, where the conventional cleaning products were used. The treatment of dishwashers were performed according to (Banaszczyk et al., 2017) with modifications. Briefly, dishwasher samples included six category: Upper basket, Lower basket, Cutlery rack, Main filter, Fine filter and Drain water Figure 1. Samples were collected using sterile swab sticks (BIONOVO, Poland) from the previous six sites of each dishwasher, and then cultured on MacConkey agar, nutrient agar, mannitol salt agar, methylene blue agar and SSA, the plates were then incubated at 37 ºC, 24-48 h. Isolates were purified to obtain pure colonies. The microbiological properties were determined using the Gram-staining method, slide glass preparations were observed under an Olympus CX21FS1 light microscope with total 1200x magnification. Slides of isolates were prepared to find out the total number of gram positive and gram negative bacteria and to observe the phenotypic shape of the cells. Two isolates KAAI1 from drain water part and KAAI2 from basket part which fitted to the description of genus Bacillus in Bergey's Manual of Systemic Bacteriology by analysis of colony appearance, cell morphology, where chosen for further molecular identification using 16S rRNA.

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Figure 1: dishwasher isolated part included six category: Upper basket, Lower basket, Cutlery rack, Main filter, Fine filter and Drain water

2.3 DNA extraction, 16SrRNA Gene Amplification, Sequencing and Phylogenetic Trees Construction

The isolates KAAI1 from drain water part and KAAI2 from basket part were grown for 24 h at 37°C on a rotary shaker (250 rpm) in a 50 ml falcon tube containing 20 ml of Tryptic Soy Broth (TSB). The procedure of G- spin DNA extraction kit (intron biotechnology, Korea) was then followed for DNA extraction. Polymerase chain reaction (PCR) of 16S rRNA genes amplification from genomic DNA of both isolates were performed following the protocol of Maxime PCR PreMix kit (i-Taq).For each PCR reaction 5µl of Taq PCR PreMix , 1µl of 10 picomols/µl of each conserved primers 1250 F (5'- AGAGTTTGATCCTGGCTCAG- 3') and 1250R (5'- GGTTACCTTGTTACGACTT- 3') from IDT (Integrated DNA Technologies company, Canada) , 1.5µl of DNA template and 16.5 µl ddH2O for a total volume of 25µl.

The optimum condition of the PCR amplification cycle for detection gene listed in Table 1, Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the agarose gel (Figure 2).

Table 1: The optimum condition of the PCR amplification cycle

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min.	1 cycle
2-	Denaturation -2	95°C	45sec	35 cycle
3-	Annealing	58°C	45sec	
4-	Extension-1	72°C	45sec	
5-	Extension -2	72°C	7 min.	1 cycle

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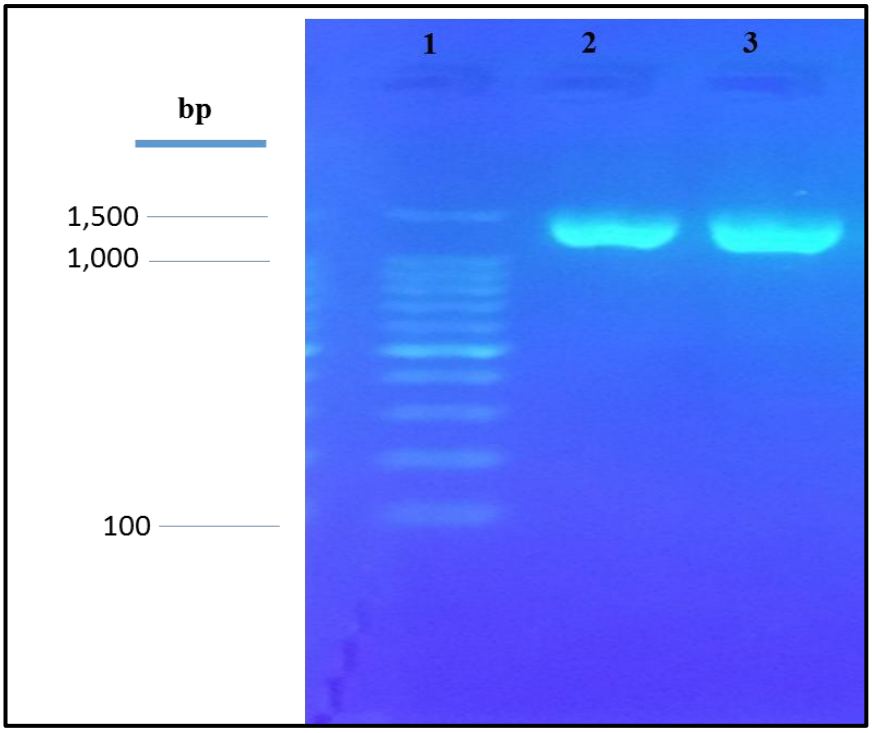

Figure 2: Agarose gel electrophoresis of 16S rRNA gene amplified from isolatesKAAI1 andKAAI2. Lane 1: 1,500 bp DNA ladder; lane 2: strain KAAI1, lane 3: strain KAAI2.

2.3.1 Purification of PCR Products

PCR products was purified using QIAquik PCR purification kit (QIAGEN) by following the manufacturer's instructions. Briefly, 5 volumes of Binding Buffer were added to 1 volume of the PCR product, mixed well and then placed in a QIAquick spin column, followed by centrifugation for 30-60 s to bind the DNA, flow-through was discarded and the column was placed back in to the collection tube. DNA was washed with 0.75 ml of wash buffer and was centrifuged again for 30-60s. The flow-through was discarded and the tube was centrifuged for additional 1 min. The column was then placed in a clean 1.5 ml centrifuge tube. For DNA elution, 50 µL of Elution Buffer was added to the center of the QIAquik membrane and the tube was centrifuged for 1 min. Purified PCR product was sent for sequencing (Macro gene/Korea Sequencing Service).

2.3.2 Phylogenetic Analyses Based on 16S rRNA Gene Sequences

The obtained DNA sequences were compared to sequences available online in the gen bank database (http://www.ncbi.nlm.nih.gov). A homology search was performed using bioinformatics tools available online, i.e. BLAST (www.ncbi.nlm.nih.gov/BLAST). Sequences shared the highest similarity with both *Mesobacillusboroniphilus*KAAI1

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and*Mesobacillusboroniphilus*KAAI2 were retrieved and downloaded in FASTA format. A maximum-likelihood (Felsenstein, 1981) phylogenetic tree for each 16S gene of *Mesobacillusboroniphilus*KAAI1 and*Mesobacillusboroniphilus*KAAI2 were constructed using the program MEGA version 7.0, from the aligned sequences using Multiple Sequence Comparison by Log Expectation (MUSCLE) software. The 1000 bootstrap replicates of the original sequence data were run to assess the confidence value of individual branches. The complete 16S rRNA gene sequences for two selected isolates were deposited in the GenBank database.

2.4 Boron tolerance assay

The method of (Kato, Miwa, Takano, Wada, & Fujiwara, 2009) with modification was followed to demonstrate Boron (B) tolerance of the novel isolates *Bacillus boroniphilus* (KAAI1 and KAAI2)in comparison with *Bacillus cereus* (control) obtained from microbiology laboratory/ Biology department/ University of Mosul, the isolates were cultured on TSA medium supplemented with 0, 50, 100, 200 µmol / L boric acid, followed the incubated plates at 37 ºC, 24-48 h.

2.5 The ability of novel isolates *Bacillus boroniphilus* to grow on free B different media

For the ability of novel isolates *Bacillus boroniphilus* (KAAI1 and KAAI2)to grow without boron on different media, MacConkey, EMB and Blood agar were assayed.

3. Results

Streaking on the cultured media plates showed that dishwasher's parts are populated with diverse bacterial communities dominated by gram-negative bacteria. All the six sites of each dishwasher, were residentially colonized by bacteria, with a diverse profile for microbiological species (data not shown). In total, 51 bacterial isolates were obtained and showed higher cultivable bacterial diversity. On average, 19 isolates were gram positive, while 32 isolates were gram negative (Table 2).

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Part of dish washer	Number of isolates	Gr+	Gr-
Main filter (M)	6	4	2
Fine filter (F)	23	9	14
Drain water (D)	18	5	13
Baskets (B)	4	1	3
Total number	51	19	32

Table 2.Dishwasher microflora:Microscope imaging of Gram-stained bacterial population, collected from the model dishwasher, Main filter, Fine filter, Drain water and Baskets.

3.1 Nucleotide Sequence Accession Numbers

The two rare isolates designated as *Mesobacillusboroniphilus*KAAI1 and*Mesobacillusboroniphilus*KAAI2and their ribosomal 16S gene sequences were deposited in the NCBI Genbank database under the accession numbers MW193122 and MW193123 respectively.

3.2 16S rRNA gene sequences and phylogenetic analysis for the isolates

Amplifications of 16S rRNA genes of the chosen isolates exposed a PCR product sized at 1500 bp when analysed through 1% agarose gel-electrophoretic procedure as shown in (Figure 2). BLAST analyses on partial 16S r RNA sequences of each isolates were performed with the GenBank BLAST (http//www.ncbi.nlm.nih.gov) search tool. *Mesobacillusboroniphilus*KAAI1 and*Mesobacillusboroniphilus*KAAI2 exhibited 95-to-98% similarity with the sequences in the NCBI GenBank database. (Figure 3) depicts the Neighbour-Joining tree (NJ) of isolates as assembled in MEGA ver. 7. The scale symbolises 0.01 substitutions for each nucleotide position. *P. mirabilis* (KX898582) was employed as the out-group.

A neighbor-joining tree based on the highest similar 16S rRNA gene sequences showed that the *Mesobacillusboroniphilus*KAAI1 and*Mesobacillusboroniphilus*KAAI2 is the most closely related strain to Bacillus sp. (GQ284386), *Bacillus subterraneus* (KT719820), *Bacillus thioparans* (MG705670) and Bacillus boroniphilus (MG705737).

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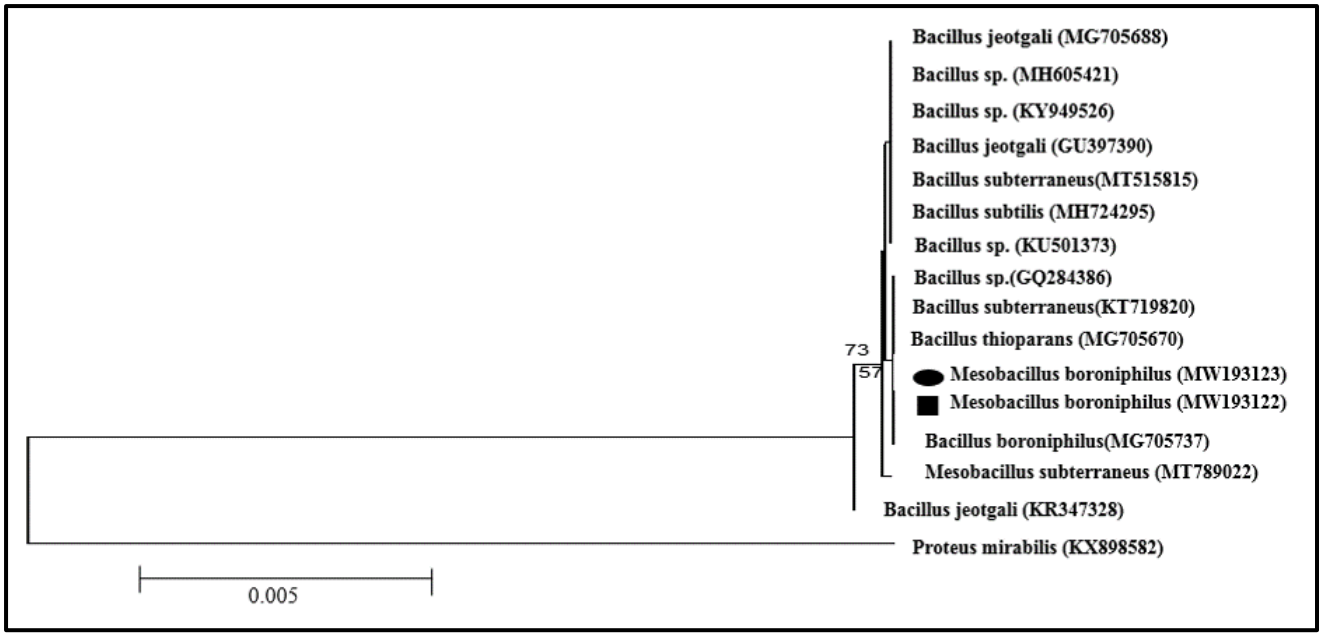

Figure 3: Evolutionary relationships of taxa.The evolutionary history was inferred using the Neighbor-Joining method. Next to branches are the ratio of duplicate trees where the related taxa clustered together with bootstrap test (1000 replicates). Formula of Kimura 2-parameter was used to measure the evolutionary distances, which are measured in numbers of base substitutions per location. A total of 16 nucleotide sequences were examined. Gaps and incomplete data were removed from all roles. Evolutionary analyses were conducted in MEGA ver. 7.

3.3 Boron tolerance assay

The isolated novel isolates *Bacillus boroniphilus* (KAAI1 and KAAI2)from naturally B containing dishwasher of the drain water and basket area demonstrated boron requirement for the growth, and differences for B tolerance. KAAI1 isolate showed single colonies on free TSB media and TSB media supplemented by 50 and 100 µmol / L boric acid after growth for 24 h, while KAAI2 isolate only grew slightly on plates of 50 µmol / L boric acid after 24 h, with compared with *Bacillus cereus* where used as control, the cultures not showed any growth by boric acid concentrations. (Table 3) (Figure 5).

Table 3: Growth of *Bacillus boroniphilus* isolates (KAAI1, KAAI2) and *Bacillus cereus* as control at different levels of boric acid supply (µmol / L) in TSA medium.

Isolates	**TSA media + boric acid**			
	0 µmol / L	**50 µmol / L**	**100 µmol / L**	**200 µmol / L**
KAAI1	growth	growth	Growth	No growth
KAAI2	No growth	growth	No growth	No growth
Bacillus cereus (control)	growth	No growth	No growth	No growth

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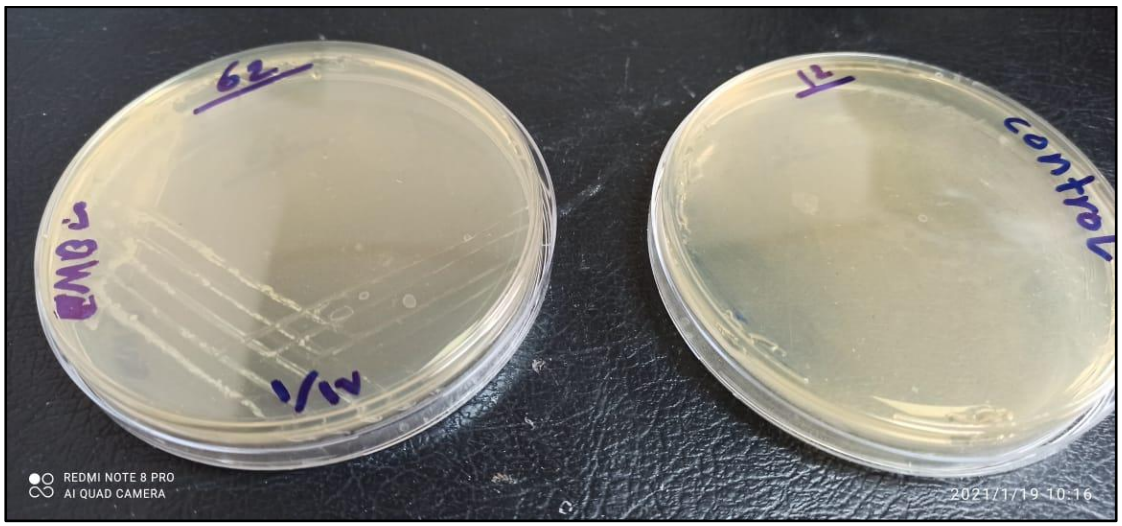

Figure 5: Left:*Bacillusboroniphillus* cultured on TSA medium supplemented with 100 µmol / L boric acid, growth slightly after 24 h . Right: *Bacillus cereus* (control)on TSA medium supplemented with boron,no growth.

3.4 The ability of novel isolates of *Bacillus boroniphilus* to grow on free B different media

To investigate whether the novel isolates *Bacillus boroniphilus* (KAAI1 and KAAI2)to grow on free B different media, different media, MacConkey, EMB and Blood agar were used, the colonies growth were observed for KAAI1 isolate where's no growth for KAAI2 isolate supporting that this novel isolate need B for growth (Figure 6).

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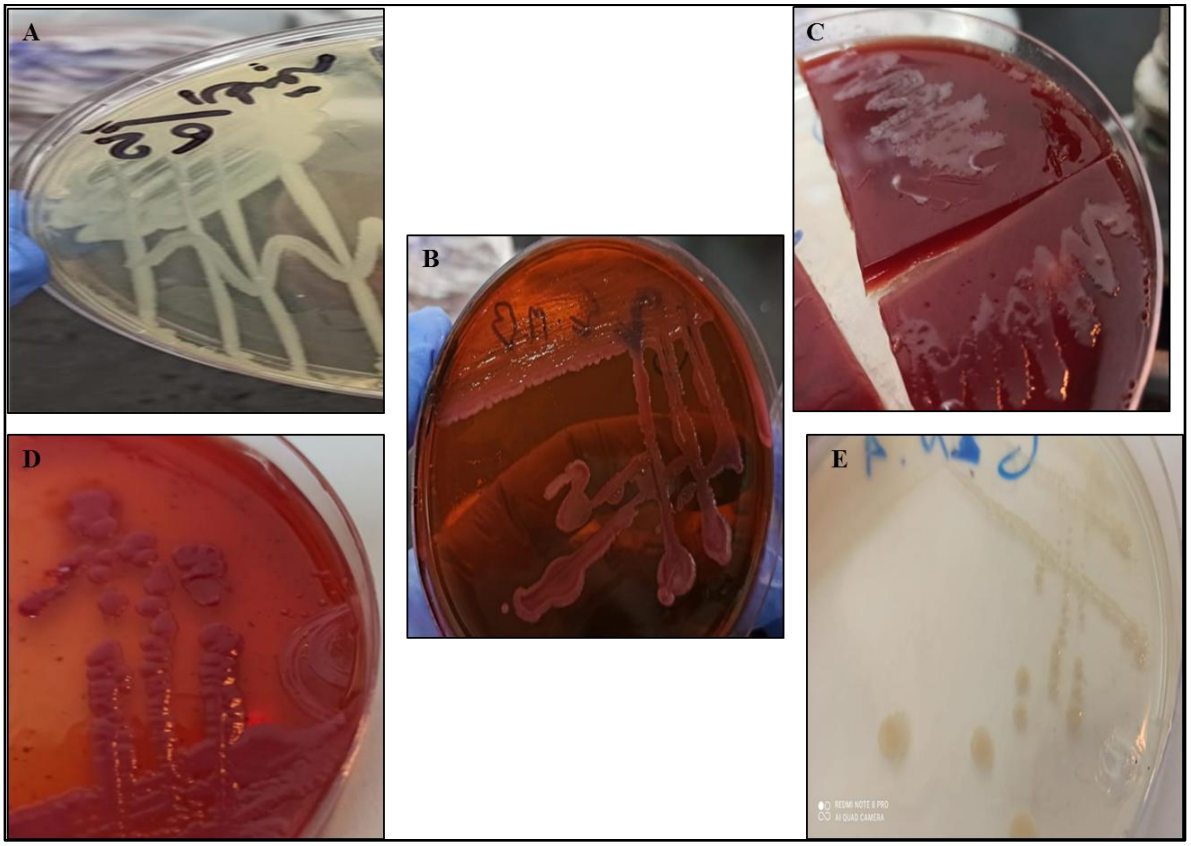

Figure 6: The ability of novel isolate *Bacillus boroniphilus* KAAI1 to grow on free B different media (A): TSA media (B) EMB media (C) Blood media (D) Mackonkey media (E) Nutrient media.

Discussion

To detect the internal microflora of an old dishwasher that was used for over ten years with commonly available dishwasher cleansing items. Upper basket, Lower basket, Cutlery rack, Main filter, Fine filter, and Drain water were the origins of swab collection in the old dishwasher. The sampling locations for microbes were chosen with care, since they are the most important sections for collecting soil and food that are still exposure to water or are in continuous contact with water (drain hose). Microbiological streaking on plates is the most difficult to monitor because these points of the dishwasher ensure the conditions needed for microorganism proliferation, which resulted in massive bacterial growth at 37 °C, as shown in Table 2.

The largest number of isolates was obtained in the Fine filter of the washing machine, as it is the most contaminated part, as the isolates of Gram negative bacteria was greater than the Gram-positive bacteria, this is axiomatically, considering that the negative bacteria for the

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Gram pigment are more tolerant of harsh conditions such as high temperatures and cleaning powders.

Among the 51 bacterial isolates that were obtained from the used dishwasher, 2 isolates were*Bacillus boroniphilus*(KAAI1 and KAAI2). Phylogenetic tree showing inter-relationship of the two strains (KAAI1 and KAAI2) of *Bacillus boroniphilus* sp. nov.

A neighbor-joining tree based on the highest similar 16S rRNA gene sequences showed that the (KAAI1 and KAAI2) isolates is the most closely related strain to *Bacillus boroniphilus*(MG705737), *Bacillus thioparans*(MG705670) with identity of 99 %, followed by 98 % with *Bacillus* sp.(GQ284386) and *Bacillus subterraneus*(KT719820).

Bacillus boroniphilus(MG705737) was isolated from naturally B containing soil of Hisarcik area in the Kutahya Province, Turkey (Ahmed et al., 2007). While *Bacillus thioparans*(MG705670) isolated by (Rodríguez-Tirado, Green-Ruiz, & Gómez-Gil, 2012) from the bacterial consortium used in a continuous culture system for wastewater treatment operating in a double-walled culture vessel. *Bacillus* sp. (GQ284386) and *Bacillus subterraneus*(KT719820) were isolated from a systematic study of the prokaryotic diversity Ghats samples (water, sediment and mangrove swamp sediment (Unpublished data)

The isolates which dominate these habitats shared that they were environments with high temperatures and contained the element boron in varying quantities, and this is what also characterized the environment of the current research samples.

In this study, the novel isolates *Bacillus boroniphilus*(KAAI1 and KAAI2) were investigated to assess their ability to grow on media containing different concentrations of boric acid 0, 50, 100, 200 µmol / L, the two isolates showed different ability to grow with B, KAAI1 isolate was able to grow on free and 50 and 100 µmol / L boric acid, while KAAI2 isolate grown only in 50µmol / L boric acid, that is most probably due to B carried over from the B contained inoculum in addition to B contained in the nutrient agar, while the limited initial growth in the cultures without adding B may be due to B contained in the medium being quickly depleted until growth completely stopped.

The main boron-tolerance mechanism in plants has been identified as boron efflux. (Hayes & Reid, 2004). Slight boron stress was used as an external stimuli during the early exponential growth phase of yeast development, and the findings were promising, similar results were observed. (Kaya et al., 2009). *B. boroniphilus*, on the other hand, has a slightly

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higher degree of boron tolerance than those organisms. Thus, Ahmed and Fujiwara (2010) assessed the boron concentration of a variety of boron-tolerant soil bacteria exposed to boron to explore bacterial boron tolerance mechanisms, with *B. boroniphilus* strain T-17 s being the most tolerant. In that research, Bacillus subtilis strain ISW 1214 has been used as a control, and results of the tests revealed that the lower a bacterial species' protoplasmic boron concentrations, the higher its boron resistance level.

Boric acid's toxicity to living cells at high amounts is a well-known phenomena. (Nable et al., 1997). The only example of Boron's physiological role at the molecular level that has been documented is in plants, where it generates esters with a cisdiol moiety in rhamnogalacturonan-II, which is necessary for stability and integrity. (Reguera et al., 2010); Even so, since rhamnogalacturonan -II has yet to be detected in bacteria, the molecular basis for Boron's essentiality in these species is unknown at this time. It's unclear what causes these species' high B tolerance. B tolerance in plants is known as the efflux of excessive B from its cell, which keeps the cell's B concentration low (Reid, 2014). However, because these novel species can tolerate several times further B toxicity than plants, it's possible that they employ a different strategy to cope with the harsh conditions. It's not unexpected that only some novel species need B as an important component while others may not, as B requirements differ between species, as shown by an evolutionary study of Boron acquisition of an essential role in plant metabolic (Lovatt, 1985). The findings of B tolerance and the need for the novel strains provide a genetic resource for defining the genes necessary for the B tolerance process in bacteria due to its small genome size. These genes may be useful for cloning in a variety of many other organisms, including crop species that grow in high-B soil. The research of genotype in *B. boroniphilus* sp. nov.could be important in delving into the biochemical functions of *B. boroniphilus* sp. nov.

Boron is essential for species from at least three different phyla within the kingdom Eubacteria Proteobacteria, Actinobacteria, and Cyanobacteria. An antibiotic developed by Streptomyces, bacteria belonging to the phylum Actinobacteria, was the first boron biomolecule discovered.This, as well as other boron-containing bacterial products from many other Actinobacteria organisms, is linked to the organism's immunological protection. Boron is available for many dinitrogen-fixing bacteria with heterocysts in the phylum Cyanobacteria. Boron is thought to bind with the hydroxyl groups in the glycolipid inner layer of heterocysts to stabilize it. (Bonilla, Garcia-González, & Mateo, 1990), and it was an important element during the initial evolution of life, as evidenced by the heterocystous

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Cyanobacteria, which were the dominant organisms during the Middle Pre-Cambrian Period. Boromycin and related antibiotics were the first natural biomolecules discovered to contain boron. Boromycin was discovered in an African soil specimen from a species of Streptomyces antibioticus. (Hunt, 2003). Its connection with the cytoplasmic membrane causes the permeability barrier for potassium ions to be broken. (Pache&Zähner, 1969). Aplasmomycin is a third boron-containing antibiotic released by a marine isolate of the *Streptomyces griseus* bacterium (Pache&Zähner, 1969), when given orally to mice infected with *Plasmodium berghei*, it has inhibitory effect against Gram positive bacteria *in vitro* and plasmodium *in vivo*.

(Chen et al., 2002) referred that a boron containing bio molecule developed by a bacterium that is not an antibiotic but rather a cell to cell communication signal. Bacteria communicate with each other by exchanging extracellular signaling molecules known as autoinducers (AI). Quorum sensing is a mechanism that enables bacterial communities to organize gene expression for community cooperative processes including antibiotic development and virulence factor expression. AI-2 is a bacterial protein that contains one boron atom per molecule and is formed by a variety of bacteria. It is produced from S-ribosylhomocysteine, which is a ribose moiety. *Vibrio harveyi*, a gliding bioluminescent marine bacterium, expresses and binds autoinducers-2 (AI-2). The protein LuxP, which consists of two similar domains bound by a three-stranded hinge, is the principal receptor and sensor for AI-2 in *V. harveyi*. The AI-2 ligand forms a furanosyl borate diester complex with in deep slit between the two domains.

An active boron transport may also be necessary for bacteria to obtain boron in boron-deficient environments or to escape the cytotoxic effect of excessive boron. The boron transporter in bacteria is still a mystery, and no obvious AtBor1 homologs have been found in bacterial genome sequence databases. (Miwa & Fujiwara, 2009).

However, to our knowledge, there are no reports in the literature on isolate Boron tolerance bacteriam*Bacillus boroniphilus* from dishwasher environment. Further detailed investigations including genetic studies are necessary to clarify the molecular mechanism of boron resistance in *B. boroniphilus*.

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