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Production and Characterization of Extracellular Polysaccharide from *Anoxybacillus flavithermus* AAG-61 with Resistance to Sulfamethoxazole and Ofloxacin

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Abstract

Extracellular polysaccharide producers from Kursunlu (Manisa) thermal spring were identified and a total of nine isolates were selected. One isolate was selected based on its strong mucoid structure and identified as *Anoxybacillus flavithermus* (AAG-61) by MALDI-TOF MS. *A. flavithermus* AAG-61 had multiple antibiotic resistance to both Sulfamethoxazole, and Ofloxacin. The polysaccharide produced by *A. flavithermus* AAG-61 was purified and characterized. It was an anionic heteropolysaccharide containing rhamnose, mannose, arabinose, and glucose. The polysaccharide was water-soluble and had a concentration of 3.1 g/L carbohydrate and 0.11 g/L protein. The EPS consisted of 32% carbon, 7% nitrogen, 5% hydrogen, and traces of sulfur (0.6%). The presence of protein and sulfur in the polysaccharide was confirmed by FT-IR. Zeta potential, mobility, and conductivity values were -8.3 (± 0.5) mV, -0.65 (± 0.04) µmcm/Vs, and 1.57 (± 0.08) mS/cm. The crystallinity pattern was also determined by XRD analysis. No effect on the viability of the human fibroblast cell line was observed.

Keywords: *Anoxybacillus flavithermus*, antibiotic resistance, extracellular polysaccharide

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Introduction

The class *Anoxybacillus* was named by Pikuta et al. [1]. They expressed that the phenotypic, biochemical and molecular attributes of bacteria got from manure were different from those of the genus *Bacillus*. Based on this, the bacterium *Anoxybacillus pushchinoensis* K1^T was separated from the genus *Bacillus* and included in the new genus *Anoxybacillus*.

The model strain of the class *Anoxybacillus* was *A. pushchinoensis* K1^T. The suffix 'anoxi' signifies 'anaerobic' and was used because *A. pushchinoensis* was believed to be an anaerobic bacterium when it was first described. Nonetheless, Pikuta et al. [2] later resolved that it was a facultative anaerobe, not an obligate anaerobe, and the obligate anaerobic characterization of the *Anoxybacillus* was changed to aero-tolerant or facultative anaerobe.

All species belonging to the class *Anoxybacillus* have been thermophilic. Thermophilic bacteria are microorganisms that can adjust to extreme temperature conditions where other bacteria cannot develop. These organisms have been vital in biotechnology, especially on the grounds that they have biochemical substances that are resistant to high temperatures. The temperature resistance of enzymes and other proteins of thermophilic microorganisms was higher than that of mesophilic ones. Crucial amino acid substitutions at only a few points in the enzyme structure empowered the enzyme to acquire a temperature-resistant three-dimensional structure The fact that thermophiles have lipids rich in saturated fatty acids ensures that their cytoplasmic membranes remain stable and functional at high temperatures [3].

A large portion of the bacteria having a place with this genus have been isolated from thermal springs [4], mud, manure and geothermal soils [5-7]. *A. contaminans* strain was isolated from contaminated gelatin [8]. Individuals from this genus are Gram-positive, endospore-forming bacteria. They are moderately thermophilic (37-60°C), motile or immobile, and exhibited alkaliphilic or alkatolerant properties. The distinguished types of this genus are *A. flavithermus, A. kestanbolensis, A. ayderensis, A. pushchinoensis, A. voinovskiensis, A. gonensis, A. contaminans, A. kamchatkencillus kamchatkenolyticus, A. geothermalis* [9, 10].

Microorganisms can produce abundant extracellular polysaccharides (EPS). Microbial EPS is a complex biopolymer comprising essentially of polysaccharides as well as protein, nucleic acid, lipid and humic substance. The backbone of microbial EPS is carbohydrate (polysaccharides) and they can be named as homo-polysaccharide or hetero-polysaccharide as per their structure. The carbohydrate content of exopolysaccharide can be affected by a few variables during production and extraction, but the main factors

are the microorganism, carbon source, nutrients (nitrogen and phosphorus), and extraction method [11].

The polysaccharide production of *Anoxybacillus* sp. was first introduced by Zhao et al. [12] with biosorption capacity. Another extracellular polysaccharide from Sulfamethoxazole-resistant *A. pushchinoensis* G11 had both antibiofilm and antitumor activities [13], and this was the first report on the polysaccharide production from *A. pushchinoensis* and its biological activity. *A. gonensis* YK25 had the ability to produce sulfated-polysaccharide with anticancer activity [14]. The aim of the study was to isolate the thermophilic bacterium with the strongest mucoid structure, to identify of the isolate and to carry out the production, purification and characterization of the polysaccharide. The novelty of this study stems from the first report that *A. flavithermus* is a polysaccharide producer and has multiple antibiotic resistance.

Materials and methods

Screening of bacteria in water samples

Water and sludge samples were gathered from Kursunlu (Manisa) thermal spring in Turkey. Samples were collected by sterile glass bottles and taken by aseptically. The collected samples (5 mL) were incubated in nutrient broth (NB) to enrich the microbial flora. This mass was diluted with physiological water (by sequential dilution between 10^{-1}-10^{-8}) and each dilution was spread on nutrient agar (NA) medium and incubations were performed. All the incubations through the study was performed at 60 °C for 48 hours. The initial elimination of isolates was based on shape and color differences of colony on these agar plates.

Determination of polysaccharide production potential

The next step was to produce polysaccharides extracellularly in Tryptic soy broth (TSB), For this purpose, isolates selected according to colony differences were transferred to Tryptic soy agar (TSA) for preparation of inoculum. An inoculum ratio of 1 % was used to initiate polysaccharide production in 100 mL of TSB under optimal conditions of the strain when the OD_{600} value reached at 0.1. The isolate (strain) with the longest mucoid structure on TSA and the highest yield in TSB was selected as polysaccharide producer. The strain was frozen (-86°C) in NB supplemented with 15 % (v/v) glycerol, for further studies [13].

Identification of bacterial isolate

Identification of the strain was carried out by Matrix assisted laser desorption ionization (MALDI-TOF MS) technique (Centre for Implementation and Research of Plant Health Clinic, Mustafa Kemal University, Hatay) with expert guidance. The optimum growth conditions of the strain in NB were determined at different temperature (10-70 °C and pH 7- 1 % NaCl), pH (3-11 and 60 °C-1 % NaCl), and NaCl (1-10 % and 60 °C - pH 7) values. Antibiotic susceptibilities of the strain against Amoxicillin, Cefoperazone, Chloramphenicol, Kanamycin, Netilmicin, Ofoxacin, and Sulfamethoxazole were determined by disc diffusion technique [15].

Partial purification of polysaccharide

Cell debris was eliminated by centrifugation at 4500 rpm for 10 min. Trichloroacetic acid (4 % (w/w)) was added to the crude extract and stirred at 4 °C for 4 hours to remove contaminants. Chilled ethanol was added to precipitate the polysaccharide. The mixture was kept overnight (4 °C) and lyophilized after precipitation of the polysaccharide [16].

Solubility

The solubility of lyophilized polysaccharide (10 mg) was determined in 2 mL of different solvents (water, hexane, ethanol, methanol, chloroform, and dimethyl sulfoxide)., pellet formation was observed after vortex mixing for about 2 min.

Concentrations and spectrum scanning

The carbohydrate concentration and protein content of polysaccharide was determined in microplate by phenol sulfuric acid and Biuret method, respectively. Briefly, 50 µL polysaccharide sample was mixed with 25 µL phenol and 125 µL concentrated sulfuric acid and incubated at 100 °C for 5 min. [17]. After cooling, absorbance values at 490 nm were evaluated according to standard values (R^2 = 0.94). For protein determination, 40 µL polysaccharide sample was mixed with 210 µL Biuret solution and incubated at 37°C for 30 min. After this time, the absorbance values at 540 nm were used to calculate the protein concentration (R^2 = 0.99). Glucose and bovine serum albumin (BSA) were standards. A spectrum scan was also performed between 200–1000 nm.

Elemental analysis

The elemental composition of the polysaccharide was analyzed by using an elemental analyzer (TruSpec® Micro- CHNS analyzer) without acid hydrolysis. Nitrogen, carbon, hydrogen, and oxygen were analyzed in order to estimate the elemental composition of the polysaccharide.

Functional group analysis

The polysaccharide sample (2 mg) was mixed with 100 mg of potassium bromide (KBr) and then crushed and added to a 1 mm pellet to perform a Fourier-transform infrared spectrum scanning (PerkinElmer Spectrum Version 10.5.2) in the frequency range of 400–4000 cm^{-1}.

Monosaccharide composition

The monosaccharide composition of the polysaccharide was analyzed by gas chromatography (GC) [18]. For hydrolysis, 1 mg of polysaccharide was treated with 500 µL of 2 M trifluoroacetic acid at 120 °C for 120 min. Reduction and acetylation were conducted by addition of $NaBH_4$ (20 mg) in 1 mL of 2 M NH_4OH to the residue and the solution was heated for 60 min at 60 °C, then neutralized with acetic acid, evaporated to dryness with a stream of N_2 at 40 °C, and evaporated five times by adding 500 µL methanol. The residue was acetylated with 200–250 µL of pyridine and 200–250 µL of acetic anhydride at 100–120 °C for 20 min, partitioned with water and CH_2Cl_2, and the resulting alditol acetates were analyzed by GC with flame ionization detection (FID) and coupled to MS. Glucose, fructose, galactose, rhamnose, arabinose, xylose and mannose were standard sugars and they were also exposed to the same procedure.

X-ray diffraction determination

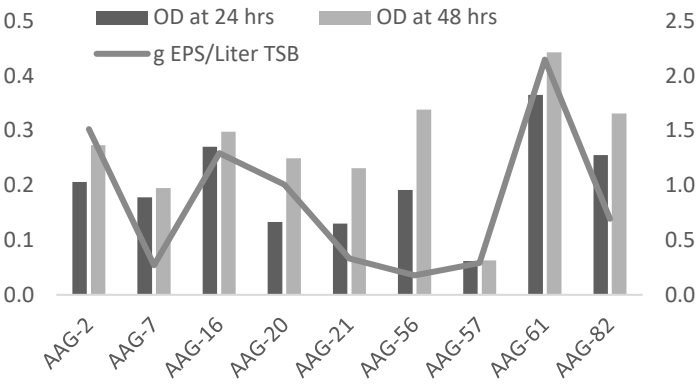
The polysaccharide (~100mg) was analyzed on Bruker D8 DISCOVER XRD device at an increase rate of 2°/min between 30-100° depending on the angle of incidence [13].

Zeta potential

The determination of zeta potential of the polysaccharide was performed with Malvern/Zetasizer Nano ZSP with a system temperature of 25°C, a count rate at 176 kcps and a measurement position of 2 mm. The study was repeated three times.

Cytotoxicity

The cytotoxicity assay of the polysaccharide was performed at the Pharmaceutical Application and Research Center, Bezmialem University, Istanbul. Briefly, cell viability was tested by MTT assay on CCD-1079Sk fibroblast cell line ($1x10^4$ cells/well). Cultivation parameters were 37 °C, 5 % CO_2 and 24 hours. DMEM-F12 (containing 10 % FBS and 1 % penicillin/streptomycin) was used as control. Results were obtained in triplicate and analyzed by accuracy analysis with GraphPad Prism 5.



Results and discussion

Figure 1. Optical densities of isolates from water samples during polysaccharide production

Serial dilutions of water samples were performed and a total of nine isolates were found to be able to produce extracellular polysaccharides (**Figure 1**). The results showed that, strain AAG-61 produced the highest amount of polysaccharide and had the longest mucoid structure on agar plate.

This strain was identified by MALDI-TOF MS technique based on ionization of biomolecules and showed similarity to *Anoxybacillus flavithermus* with a log score of 1.19. Therefore, it was referred to as *A. flavithermus* AAG-61 in the following sections.

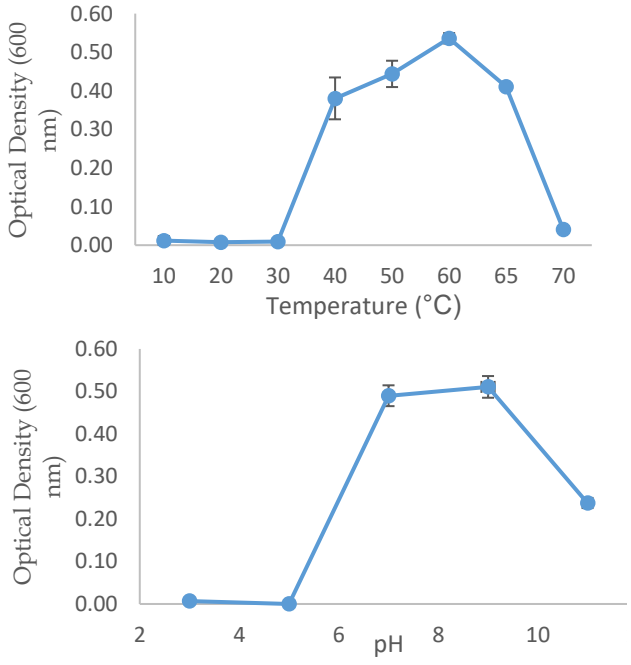


Figure 2. Determination of temperature and pH parameters for optimum growth of *Anoxybacillus flavithermus* AAG-61

The optimum growth conditions of *A. flavithermus* AAG-61 were also determined and the results were given in **Figure 2**. *A. flavithermus* AAG-61 was able to survive between 30-70°C and 5-11 pH. However, the optimum

Table 1. Inhibition of *A. flavithermus* AAG-61 by different antibiotics

Antibiotic		Resistance	Inhibition diameter	Control (*E. coli* O157:H7 35150)
Amoxicillin	(30 µg/disk)	-	3.9 mm (± 0.3)	15 mm (± 1.6)
Cefoperazone	(75 µg/disk)	-	5.2 mm (± 0.3)	28 mm (± 1.2)
Chloramphenicol	(30 µg/disk)	-	3.4 mm (± 0.3)	16 mm (± 1.2)
Kanamycin	(30 µg/disk)	-	2.0 mm (± 0.1)	88 mm (±1.1)
Netilmicin	(30 µg/disk)	-	2.8 mm (± 0.3)	90 mm (±1.3)
Ofloxacin	(5 µg/disk)	+	-	88 mm (±1.4)
Sulfamethoxazole	(50 µg/disk)	+	-	89 mm (±1.2)

* Experiments were carried out on 90 mm diameter petri dishes.

temperature and pH were 60°C and pH 7-9, while the salt requirement was determined at 2% NaCl concentration. *Anoxybacillus* sp. had a wide range of pH between 3-11 and temperature between 50-95°C [19]. Tramice et al. [20] cultivated *A. amylolyticus* at 60°C and a pH of 5.6. The growth temperature of *A. flavithermus* obtained from geothermal areas in the Indian Himalaya is 60°C [21]. Panosyan et al. [22] found that the optimum growth parameters of *A. karvacharensis* were 60-65°C, 8-9 pH and 1-1.5% NaCl, but concentrations higher than 2.5% were toxic.

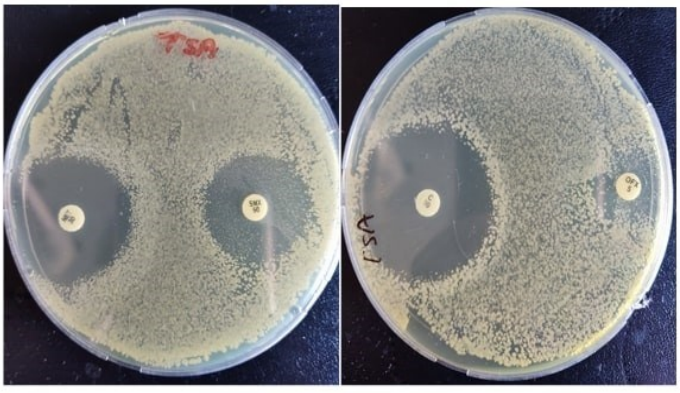


Figure 3. Antibiotic resistance of *A. flavithermus* AAG-61 to Sulfamethoxazole (a) and Ofloxacin (b)

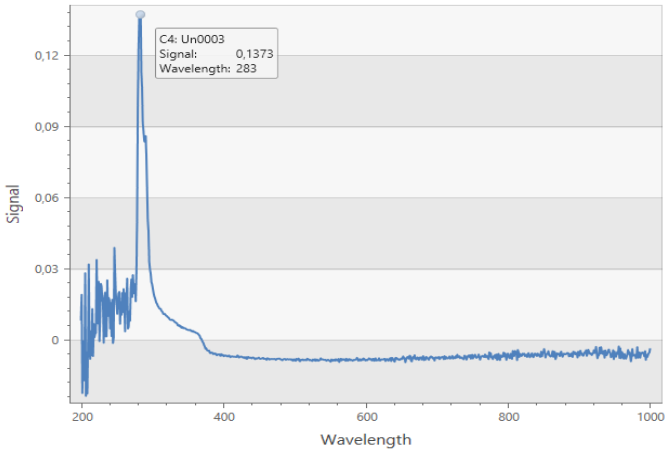


Figure 4. Spectrum scanning of EPS from A. flavithermus AAG-61

Antibiotic susceptibility of *A. flavithermus* AAG-61 was determined and remarkable results were obtained. Among the antibiotics tested, *A. flavithermus* AAG-61 was sensitive to Amoxicillin, Cefoperazone, Chloramphenicol, Kanamycin, and Netilmicin but resistant to Sulfamethoxazole, and Ofloxacin (**Table 1** and **Figure 3**). Many studies on *Anoxybacillus* sp. resulted in sensitivity to different antibiotics [5, 23-25]. However, *A. flavithermus* AAG-61 had multiple antibiotic resistance and no studies on the antibiotic susceptibility of *A. flavithermus* has been conducted so far [13].

Extracellular polysaccharide (EPS) production was induced in TSB medium EPS was precipitated and purified from *A. flavithermus* AAG-61 after cultivation. Solubility was tested in different solvents but was found to be soluble only in water. Total carbohydrate and protein concentrations were determined in a 96-well microplate. The original method of Dubois et al. [26] was evaluated according to the Masuko [17]. The aim was to achieve high precision and accuracy while minimizing the generation of toxic waste. The total carbohydrate concentration was 3.1 g/L and the protein concentration was 0.11 g/L. EPS had a strong spectrum at 283 nm (no peak at 260 nm) indicating the presence of protein substituents in the polysaccharide chain (**Figure 4**).

Elemental analysis confirmed the presence of protein in the chain. It was composed of 32% carbon, 7% nitrogen, 5% hydrogen, and traces of sulfur (0.6%). The thermophilic bacterium *Bacillus licheniformis* BITSL006 produced polysaccharide containing 49% carbon 5% nitrogen 0.8% sulfur, and others [27].

The nitrogen content also reinforced the results obtained from both protein analysis and infrared spectra. According to FT-IR analysis (**Table 2** and **Figure 5**), EPS had nine distinct peaks showing similar functional groups as in an ordinary microbial polysaccharide. In particular, the band at 3277.35 cm^{-1} corresponded to sugar residues while the stretches at 1624 and 1524 cm^{-1} were indicative of amide groups [28]. A different stretching was observed at 1231 cm^{-1} due to sulfate residues in the polysaccharide structure [29].

Table 2. Frequency and transmittance values of EPS from *A. flavithermus* AAG-61

Functional groups	Frequency (cm^{-1})	Transmittance (%)
Intracellular hydrogen bond or hydroxyl group	3277.35	87.81
C-H stretching in sugar ring	2925.83	87.38
C=O stretches of amide (I) bond	1624.27	75.88
C-N stretches of amide (II) bond	1524.7	85.17
C-H stretching deformation and C-O-H bending vibrations	1453.71	92.31
C-H stretching deformation and C-O-H bending vibrations	1331.67	92.17
S=O stretching of weak bands	1231.24	87.94
C–C and C–O vibrations in pyranose form of sugars and C–O–C vibration in glycoside band	1044.88	82.25
typical for α and β configurations in pyranose form	829.28	85.81
related to the carbohydrate skeletal vibrations	676.92	84.15

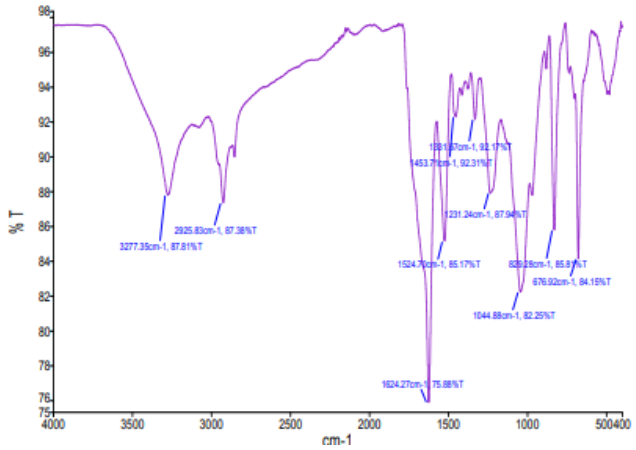


Figure 5. FT-IR spectrum of EPS from A. flavithermus AAG-61

Monosaccharide composition of EPS from *A. flavithermus* AAG-61 was revealed by gas chromatography to determine the components of polysaccharide chain. It was heteropolysaccharide and consisted of rhamnose, mannose, arabinose, and glucose in a ratio of 2.1-0.3-1-0.2, respectively. *Geobacillus toebii* ArzA-8 produced polysaccharide containing mannose, galactose, glucose, and arabinose (1/0.5/0.2/0.05) [30].

The zeta potential, mobility and conductivity of EPS were -8.3 (± 0.5) mV, -0.65 (± 0.04) µmcm/Vs and 1.57 (± 0.08) mS/cm, respectively. The negative region of the zeta potential value was indicative of anionic charge, therefore the EPS from *A. flavithermus* AAG-61 was an anionic hetero-polysaccharide.

XRD analysis was carried out to determine the phase identification and EPS was found to have a uniform structure with large extended peaks (strong crystallinity) and no amorphous regions (**Figure 6**). *Geobacillus* sp. produced polysaccharide with crystalline structure [31].

Finally, a normal human fibroblast cell line (CCD-1079Sk) was used in this study to evaluate the toxicity of EPS derived from *A. flavithermus* AAG-61 in this study (**Figure 7**) and there was no significant effect on the cell line.

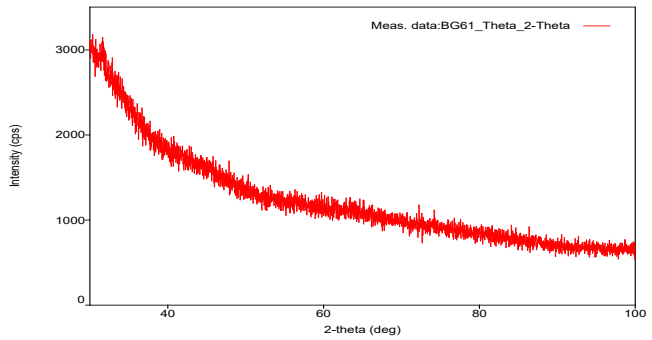


Figure 6. XRD pattern of EPS from *A. flavithermus* AAG-61

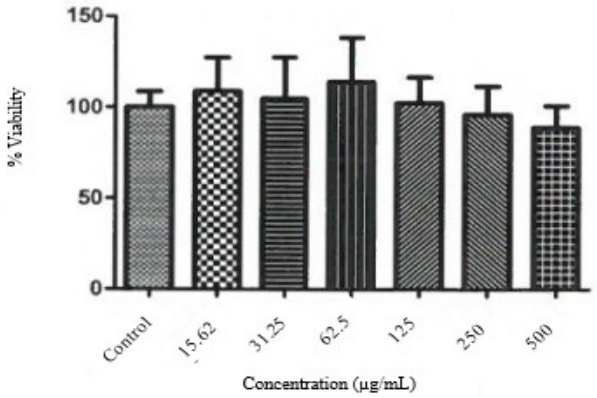


Figure 7. The cytotoxicity assay of EPS from *A. flavithermus* AAG-61 on human fibroblast cell line (CCD-1079Sk)

Conclusion

Antibiotic resistance is a vital and important issue because, whether natural or adaptive, it threatens public health and accumulates in nature. Antibiotic resistance and resistance genes are current issues for thermophilic microorganisms and trying to understand the nature of the antibiotic resistance mechanism in thermophilic bacteria might be guiding to understand their adaptation to extreme conditions or their polysaccharide production as a defense mechanism. in this study, *A. flavithermus* AAG-61 isolated from thermal springs was found to have multiple antibiotic resistance. For further studies, it would be better to investigate in depth that the relation between polysaccharide production and antibiotic resistance mechanism.

Thermophilic regions are home to diverse and valuable products due to their extreme growing conditions. Microbial polysaccharides have different application areas due to their chemical, physicochemical and biological properties. Polysaccharide production of *A. flavithermus* AAG-61 reinforces this fact by its uniform structure, anionic nature and non-toxicity on human fibroblast cell line. These features of polysaccharide from *A. flavithermus* AAG-61 and its usage can be investigated in the concept of different industrial areas such as food, material science, pharmacology, and medical. This study concludes that extreme conditions and the potential of microorganisms need to be studied in detail to improve knowledge about their products and survival secrets.

Competing Interests
No competing interests were disclosed

Ethical approval
None.

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Ethical Approval and Consent
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