



# Characterization and biological activities of a novel exopolysaccharide produced from *Geobacillus thermodenitrificans* HBB 111 strain

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## Abstract

In this study, EPS production conditions of *Geobacillus thermodenitrificans* HBB 111, a thermophilic microorganism, were optimized and the amount of produced EPS (EPS 111) was found to be 44.0 mg/L. EPS 111 was purified using ion exchange chromatography and gel filtration chromatography, and a single type of exopolysaccharide was obtained. The structure of the purified EPS 111 was evaluated by TLC, FTIR, NMR, and GC–MS, and it was observed that it contained hexose (glucose, fructose, galactose and mannose) and pentose sugars. From the SEM photographs, it was understood that EPS 111 had an amorphous, rough, and layered structure. It was found that purified EPS 111 had low cytotoxicity (2.3%) and exhibited high antioxidant activity and remarkable antidiabetic, prebiotic and fibrinolytic activities. It is very valuable that the purified EPS 111 in this study offers multiple biological activities compared to the thermophilic EPSs reported in the literature and has a high potential for use in biotechnological and biomedical fields.

**Keywords** Thermophilic bacteria · Exopolysaccharide · Biological activity · Chemical characterization

## Introduction

Microbial polysaccharides are secreted by the cell as capsular polysaccharides or exopolysaccharides. Exopolysaccharides are high molecular weight natural polymers that are produced by plant, algae, fungi, and bacteria (Dilna et al. 2015; Staudt et al. 2004). Exopolysaccharides (EPS) are large polymers of glycosides linked by glycosidic bonds. Their molecular weights are high and range from 100 to 2000 kDa. The monomeric ratio and additive degree provide

them with electrical charge (cationic, neutral, anionic), chain conformation (straight, branched) that gives them their hydrophilic or hydrophobic nature, and their unique physicochemical and functional characteristics (López-Ortega et al. 2021). EPSs are polysaccharides consisting of long-chain, branched, repeating sugars or sugar derivatives. They are classified into two groups depending on the composition of the repeating units and the biosynthesis pathway: homopolysaccharides (HoPS) and heteropolysaccharides (HePS) (Mende et al. 2016).

Although many bacteria can produce exopolysaccharides, bacteria isolated from extreme environments have become more promising because of their adaptation to harsh conditions. In exopolysaccharide production, thermophilic bacteria provide various advantages such as shorter fermentation time, better mass transfer and reduced viscosity of the fermentation broth. It has been reported in the literature that *Bacillus* and *Geobacillus* genera are good EPS producers (Panosyan et al. 2018; Radchenkova et al. 2013; Wang et al. 2021). Examples of EPS production by thermophilic bacteria in the literature are as follows, *Parageobacillus thermantarcticus ex Bacillus thermantarcticus*, (Nicolaus et al. 2004), *Geobacillus tepidamans* (Wang et al. 2017), *Geobacillus* sp. TS3-9, (Wang et al. 2017), *G. toebii* (Panosyan et al. 2018; Radchenkova et al. 2013), *G. thermodenitrificans* (Panosyan

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et al. 2018), *Aeribacillus pallidus*, (Radchenkova et al. 2013), *Anoxybacillus kestanbolensis*, (Radchenkova et al. 2013), *Brevibacillus thermoruber* (Yıldız et al. 2014), *A. puschinoensis* (Genç et al. 2021), *Geobacillus* sp. WSUCF1 (Wang et al. 2021).

Exopolysaccharides have strong biological activities. EPSs show remarkable antimicrobial activity against both gram-positive and gram-negative bacterial pathogens. Its antagonistic activity against bacterial pathogens has been reported in many studies (Ayyash et al. 2020; Rani et al. 2018; Saha et al. 2004; Wu et al. 2010). EPSs can also have an antioxidant activity, and they significantly delay or inhibit oxidation at low concentrations, in foods and body. These compounds protect the body against the different types of oxidative damage caused by reactive oxygen species (Saha et al. 2004). Besides, EPSs can have an antidiabetic role by inhibiting alpha amylase enzyme (Dilna et al. 2015). Recently, the prebiotic effect has become even more important. The number of probiotic bacteria is increased by prebiotics. These are non-digestible food ingredients that are beneficial to the host by triggering the growth of bacterial species already present in the colon. Thus, prebiotics improve host health (Tsuda & Miyamoto 2010). Moreover, EPSs with fibrinolytic activity are important (Al-Nahas et al. 2011) due to their potential in medical for paralyzed patients. Finally, some of EPSs are known to prevent biofilm formation. Microbial biofilm studies have started to attract attention in recent years. Bacteria in the biofilm exhibit high resistance to antibiotics, disinfectants, and host immune system clearance. Biofilm is very important in the medical, environmental and industrial field (Stepanović et al. 2007).

In this study, the EPS production conditions (time, temperature, pH, NaCl concentration, sugar type and sugar concentration) of a thermophilic bacterium HBB 111 (identified as *Geobacillus thermodenitrificans* HBB 111) were optimized, and the obtained exopolysaccharide was purified by ion exchange chromatography and gel filtration chromatography. Characterization of the purified exopolysaccharide was carried out by TLC, FTIR-ATR, NMR, GC-MS, SEM, and SEM-EDX techniques and hemolytic activity, antimicrobial activity, antioxidant activity, alpha-amylase enzyme inhibition activity, prebiotic activity, fibrinolytic activity and antibiofilm activity properties were examined.

## Materials and methods

### Bacterial isolate and identification

A thermophilic bacterium, HBB-111 was previously isolated by Dr. Gamze Başbülbul (Başbülbul et al. 2009, 2012) and used for EPS production. Bacterial isolate was stored at  $-20\text{ }^{\circ}\text{C}$  and grown on Tryptic Soy Agar (TSA)

medium at  $55\text{ }^{\circ}\text{C}$  for regrowth. Genomic DNA sample of HBB-111 was isolated using easyDNA genomic DNA isolation kit (R-Tech, Turkey) according to manufacturer instructions. 20F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1390R (5'-GACGGGCGGTGTGTACAA-3') primers were used to amplify 16S rDNA gene by PCR. Amplicon was sequenced by a company (MEDSANTEK) and after alignment with the sequences from NCBI database closest phylogenetic relative of isolate was determined (<http://www.ncbi.nlm.nih.gov>).

### Isolation and purification of EPS

For preincubation in TSB medium, thermophilic bacteria were incubated overnight at  $55\text{ }^{\circ}\text{C}$  in a 180 rpm shaking incubator for 24 h. The preincubated culture was inoculated in 5% volume of modified basal 2 medium (Wang et al. 2021). Thermophilic bacterium was incubated with 20 g/L sucrose modified basal medium 2 (B2) for 24 h at  $55\text{ }^{\circ}\text{C}$ . The effects of time, temperature, pH, NaCl concentration, sugar type and sugar concentration on the EPS production of the thermophilic isolate were examined and EPS production conditions were also optimized. Then, culture supernatant was taken and autoclaved, and then the volume (300 mL) was reduced to 10 mL by evaporation in an incubator at  $70\text{ }^{\circ}\text{C}$  for 3 days (Li et al. 2015; Patwal and Baranwal 2021; Radchenkova et al. 2013). Trichloroacetic acid (TCA) solution was added to the concentrated supernatant with a final concentration of 4% and incubated in a mixer at room temperature for 30 min. The culture was then centrifuged at  $2500\times g$  for 10 min. Pure alcohol, twice its volume, was added to the supernatant and kept at  $+4\text{ }^{\circ}\text{C}$  for 24 h for precipitation. It was then centrifuged at  $2500\times g$  for 10 min. The pellet was dissolved with distilled water and lyophilized and stored at  $-80\text{ }^{\circ}\text{C}$  for analysis (Bajpai et al. 2016; Wang et al. 2015). While the amount of sugar in the produced raw EPS samples was determined by the phenol-sulfuric acid method (Masuko et al. 2005), the amount of protein was determined by the Bradford method (Bradford 1976).

EPS purification was first performed by ion exchange chromatography column (1,2 cm  $\times$  50 cm) with Diethylaminoethyl (DEAE)-Cellulose by using 1.0 mL of EPS (40 mg/mL). 25 mL of solutions were passed through the column as NaCl gradient (0.1/0.25/0.5/0.75/1 M) at flow rate 1.0 mL/min. Secondly, both size separation and desalting were performed by gel filtration chromatography column (1,2 cm  $\times$  30 cm) with Sephadex G-100 at flow rate 0.5 mL/min. The column was loaded with 15 mL EPS obtained from ion exchange chromatography. Following, 25 mL of distilled water was passed through the column and samples were collected in 5 mL fractions each.

## Determination of the chemical structure of EPS

Thin layer chromatography was used to analyze the products formed as a result of the sulfuric acid hydrolysis of EPS (Dilna et al. 2015; Jouault et al. 2001). 5  $\mu$ L of different sugars (2 mg/mL of maltose, galactose, fructose, glucose, sucrose) and hydrolyzed EPS sample at 2 mg/mL concentration were applied to the layer and air-dried. A 5:3:2 mixture of *n*-butanol: ethanol: water was prepared for the mobile phase and added to the thin layer chromatography tank, the dried layer was placed in the tank and the samples were carried out. After the run-up, the sheet was air-dried. For the dyeing process, 0.2% orcinol prepared in sulfuric acid: methanol 1:9 mixture was used. The color change was observed by spraying the dye solution and keeping it in an oven at 100 °C for 10 min.

FTIR-ATR spectroscopy is widely used for the preliminary determination of functional groups in the structure of EPSs. FTIR-ATR spectroscopy of thermophilic EPS was performed using the FTIR-ATR device (Perkin Elmer Spectrum2). For this purpose, the FTIR-ATR spectrum was taken by placing 5 mg of exopolysaccharide on the sample loading part and the obtained spectra were evaluated (Seveiri et al. 2020). NMR analysis was performed for the structural examination of the produced EPS by using the NMR device (Agilent Technologies Varian 400). In order to determine the monosaccharide content of the purified exopolysaccharide by GC-MS analysis, 10 mg of EPS sample was hydrolyzed with 2 M 2 mL trifluoroacetic acid (TFA) for 2 h at 100 °C. After hydrolysis, TFA was evaporated. Then, methanol was added to the sample and the sample was evaporated again (Amiri et al. 2019). Chromatograms were taken by injecting 1  $\mu$ L of the hydrolysate into the GC-MS device (Shimadzu QP2010 ultra). The GC-MS operation conditions: carrier gas: helium; flow rate: 1.0 mL/min; temperature: 250 °C. SEM analysis of the surface morphology and structure of the purified EPS (10 mg) was examined with the SEM (Zeiss Sigma 300) device. For this purpose, the sample was coated with gold and given to the SEM-EDX device. Obtained SEM photographs and EDX analysis were evaluated.

## Determination of biological activities of EPS

Hemolytic activity was first evaluated qualitatively on blood agar as described by Filik and Kubilay (2020). Briefly, the medium was prepared by adding 5 mL of sheep blood sample to 100 mL of basal medium and poured into petri dishes. Then EPS solutions (0.025–0.50–1, 0–2.5 and 5.0 mg/mL) were filled into the wells by the agar well method and incubated at 37 °C for 24 h. The hemolytic activity test was also quantitatively performed according to the method applied by Abinaya et al. (2018). For this purpose, EPS solutions were added to each test tube and

mixed by gently inverting. PBS was used as blank and distilled water was used as control. After separating the supernatant, absorbance was measured at 540 nm, and hemolysis percentage (%) was calculated (Guezennec et al. 2012; Li et al. 2018).

The agar well diffusion method was used to examine the antimicrobial activity. Test microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) were incubated overnight at 37 °C on Nutrient agar (NA). *Candida albicans* was incubated overnight at 28 °C on Potato Dextrose Agar (PDA). The next day, suspensions at 0.5 McFarland turbidity were spread onto Müller Hinton Agar (MHA) petri dishes. Gentamicin (10  $\mu$ g) and flucanazole (25  $\mu$ g) discs were used as control for bacteria and yeast, respectively. Exopolysaccharide (1,5,15 mg/mL) was added to the wells as 100  $\mu$ L. Zones of inhibition around the wells were measured after 24 h of incubation (El Essawy et al. 2016).

Determination of DPPH radical scavenging activity was made according to Blois (1958) (Cao et al. 2020). BHT and ascorbic acid were used as standard antioxidants. The percent inhibition value was calculated from the formula  $\% \text{ inhibition} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$  ( $A_{\text{control}}$ : Absorbance of control,  $A_{\text{sample}}$ : Absorbance of sample/standard antioxidant), and the  $IC_{50}$  values were calculated. Determination of reducing power described by Lin et al. (2012) was performed.

Alpha-amylase enzyme (from *Aspergillus oryzae*) inhibition activity assay was performed as described method by Dilna et al. (2015). Results are expressed as percent inhibition of  $\alpha$ -amylase and calculated according to the formula,  $\% \text{ inhibition} = (A_{\text{control}} - A_{\text{test}}) / A_{\text{control}} \times 100$  (OD control; acarbose, OD test; EPS).

Prebiotic activity of EPS was determined by using *Lactobacillus acidophilus* 66, *L. bulgaricus* 118 and *L. plantarum* DSM 20174 bacteria and examining bacterial culture densities in liquid medium (El Essawy et al. 2016).

The fibrinolytic activity of EPS was investigated by modifying the USP 28-NF 23 pharmacopeia (2005) method. For this purpose, blood clot lysis was monitored using sheep blood and lysis levels were expressed at 5 diagnostic levels (+1, +2, +3, +4, +5) (Li et al. 2014; Saha et al. 2004).

The antibiofilm activity was performed as described method by Stepanović et al. (2007). Antibiofilm activity of EPS was tested on biofilm-forming bacteria *Escherichia coli* (3055), *Klebsiella pneumoniae* (5108), *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (RN4220). 96-well microplates were used to assess the biofilm formation of bacterial strains by spectrophotometrically at 595 nm (Li et al. 2014). The antibiofilm activity was expressed as percentage inhibition of biofilm formation.

## Statistical analysis

Data were statistically analyzed to determine statistical differences in the effects of the tested concentrations by one-way analysis of variance (ANOVA) using SPSS 23.17.0 software.  $p < 0.05$  value was used to identify statistically significant differences. Experimental results were expressed as the means of three parallel experiments  $\pm$  standard deviation (S.D.).

## Results and discussion

### BLAST result

As a result of BLAST analysis, the similarity rate of HBB 111 with other species in the database was determined. HBB 111 showed highest (94,35%) homology to *Geobacillus thermodenitrificans* strain ArzA-9/1 the isolate after alignment of 580 bp section of 16S rDNA gene. The sequence data was deposited in GenBank, under accession number OR896966.

### Isolation and purification of EPS

A limited number of thermophilic bacteria have been reported to be EPS producers in the literature. For example, Panosyan et al. (2018) determined that only two of the 16 thermophilic bacilli isolated from the Arzakan (Armenia) geothermal springs were EPS producers. One of these species was reported to be 99.7% similar to *G. thermodenitrificans* subsp. *calidus* F84bT (ArzA-6), and the other was reported to be 98.9% similar to *G. toebii* NBRC 107807 T (ArzA-8) (Panosyan et al. 2018). Similarly, in our study, the EPS producer bacterium was the *Geobacillus* species.

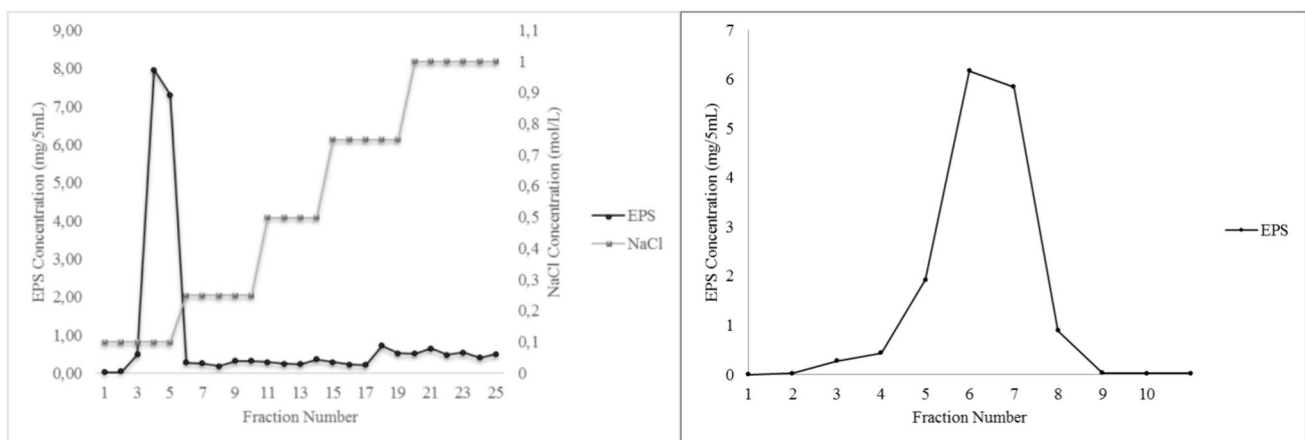
The effects of time, temperature, pH, NaCl concentration, sugar type and sugar concentration on the EPS production

conditions of the *Geobacillus thermodenitrificans* HBB111 isolate were examined, and the optimum EPS production conditions were selected as 24 h incubation, 55 °C, pH 8.0, NaCl-free medium and 2.4% lactose-containing medium (Fig. S1). The amount of EPS produced under these conditions was found to be 44.0 mg/L. The amount of protein in the isolated raw EPS sample was calculated as 1.9 mg/L. The obtained EPSs were lyophilized, dried and stored for use in chromatographic processes. Purification of EPS from the raw sample was carried out using ion exchange chromatography and then gel filtration chromatography, and the results are presented in Fig. 1. The obtained results showed that 0.1 M NaCl concentration was suitable for EPS purification and the presence of a single exopolysaccharide as a result of purification. The purification percentage of ion exchange chromatography was calculated as 39.4% and the purification percentage of gel filtration chromatography was calculated as 94.1%. The initial EPS concentration loaded on the columns was chosen as 40 mg/mL, and the amount of EPS obtained as a result of the chromatographic steps was found to be 14.84 mg/mL. As a result of the purification processes, the amount of protein in the exopolysaccharide sample was not determined. The purified EPS was named as EPS 111 and stored for characterization.

### Characterization of EPS

Thin layer chromatography of EPS 111 was investigated comparatively with standard sugars of maltose, galactose, fructose, glucose, and sucrose, and shown in Fig. S2. From the thin layer chromatogram, it is seen that the exopolysaccharide contains glucose, fructose, galactose sugars because of sulfuric acid hydrolysis.

FTIR-ATR analysis was performed for the characterization of purified EPS 111 and is shown in Fig. 2A. In the FTIR analysis of EPS, the band at  $3261\text{ cm}^{-1}$  is due to



**Fig. 1** Purification of EPS 111 by ion exchange chromatography (A) and gel filtration chromatography (B).

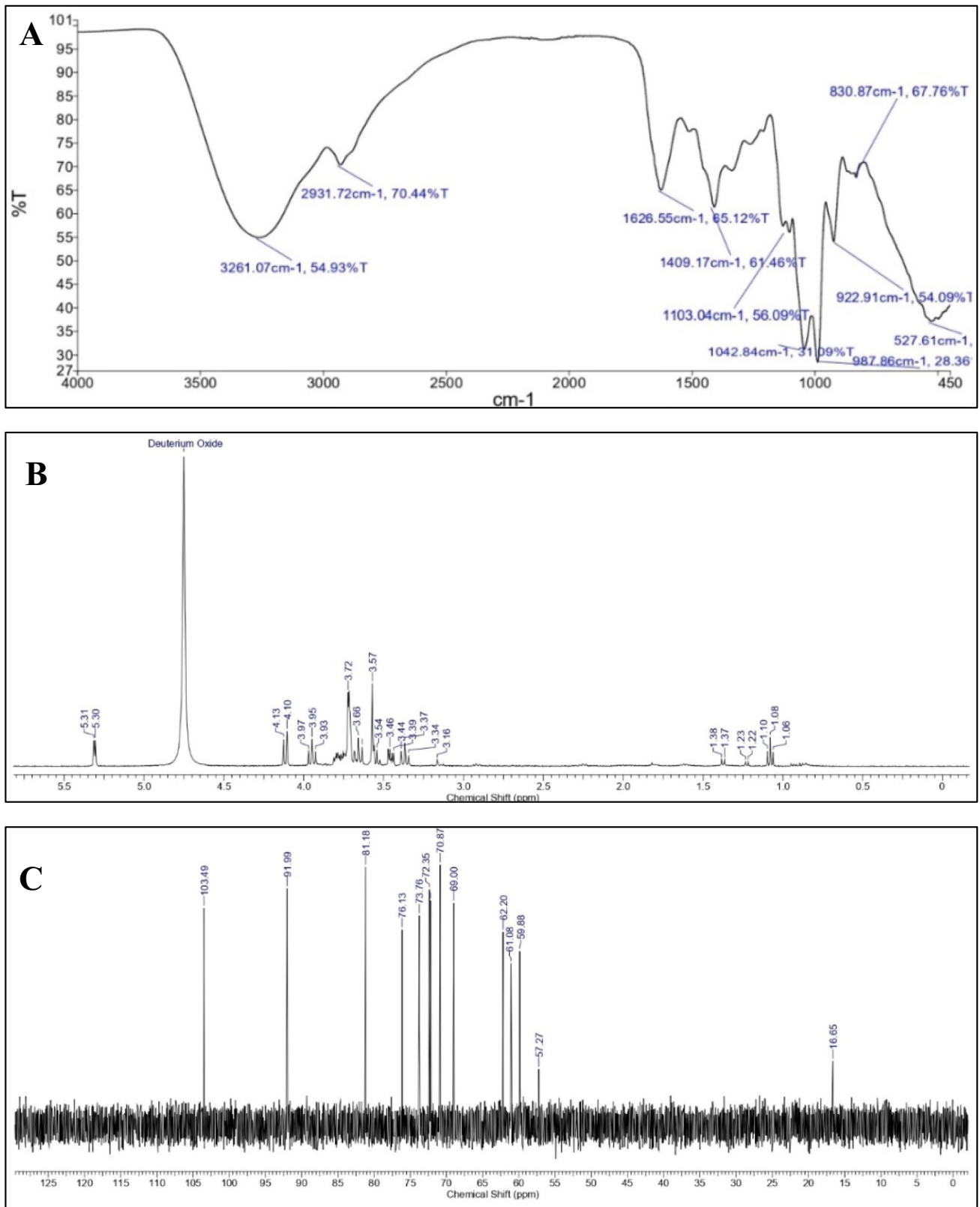


Fig. 2 A FTIR-ATR spectrum, B Proton NMR analysis, C <sup>13</sup>C NMR analysis of EPS 111.

stretching vibrations of the O–H groups of sugars and the band at  $2931\text{ cm}^{-1}$  is due to C–H stretching vibrations. The vibrations seen at  $1626$  and  $1409\text{ cm}^{-1}$  belong to the asymmetric and symmetric stretching vibrations of the carboxyl (–COOH) groups. Peaks at  $1103$  and  $1042\text{ cm}^{-1}$  are due to C–O–H stretching vibration and C–O–C stretching vibration of the pyranose ring, respectively (Hu et al. 2021). The peak at  $830\text{ cm}^{-1}$  is due to the  $\alpha$ -glycosidic bond. It is the fingerprint area between  $800$ – $1200\text{ cm}^{-1}$  and is useful in diagnosing different sugars (Amiri et al. 2019). In addition, Li et al. reported that the peak at  $922\text{ cm}^{-1}$  originates from the  $\beta$ -pyranose ring of a hexose and the  $\beta$ -pyranose ring of the peak mannose units at  $830\text{ cm}^{-1}$  (Li et al. 2014).

Proton NMR and  $^{13}\text{C}$  NMR analysis results of exopolysaccharides are shown in Fig. 2B and C, respectively. Two signals between  $4.5$  and  $5.5$  ppm as a result of proton NMR represent anomeric C. Both of these may belong to the  $\alpha$ -pyranose ring. On  $^{13}\text{C}$  NMR, the signal seen at  $103.49$  ppm indicates  $\beta$ -anomeric C, the signal seen at  $91.99$  ppm indicates  $\alpha$ -anomeric C, and the signal seen at  $81.18$  indicates furanosidic C shifts. In addition, the signals seen between  $60$  and  $80$  ppm are due to shifts of C2–C6 carbons (Hu et al. 2020).

GC–MS analysis of EPS 111 was performed, and the obtained chromatogram is presented in Fig. 3. When the GC–MS chromatogram is examined, hydroxymethyl furfural (RT 9432 min) and levulinic acid (pentanoic acid, 4 oxo, RT 4746 min) peaks are seen at different retention times. It has been reported in the literature that hexose sugars

(especially glucose) are hydrolyzed in TFA to form hydroxymethyl furfural and levulinic acid (Marzialetti et al. 2008). It is also known that pentose sugars form furfural by acid hydrolysis and may result from the breakdown of a furfural-5-methyl pentose sugar, which occurs in 3.66 min. The peak at 5,436 min in the chromatogram belongs to maltol. It is known that maltol is formed via dehydration by Maillard reaction as a result of heating reducing sugars with amino acids (Okur and Seydim 2011; Yıldız et al. 2010).

The surface structure and morphology of EPS 111 were examined by SEM device. For this purpose, photographs of the exopolysaccharide were taken at different magnifications (100X, 1.00KX, 5.00KX, 10.00KX) and are shown in Fig. 4A–D, respectively. The photographs obtained showed that the exopolysaccharide has an amorphous, rough, and layered dense polymeric surface. The obtained EDX result is presented in Fig. 4E, revealing that the exopolysaccharide is densely composed of C and O.

### Biological activities of purified EPS

Hemolytic activity test was performed to determine whether the EPS 111 were cytotoxic after purification. As a result of qualitative cytotoxicity method (Fig. 5A and B) no hemolytic activity was detected for EPS 111. Figure. 5C represents hemolytic potential of EPS 111 with 5 different concentrations ( $25$ – $500$ – $1000$ – $2500$ – $5000\text{ }\mu\text{g}/\text{mL}$ ), while Fig. 5B represents positive control/*S. pyogenes*. Quantitative cytotoxicity tests of EPS 111 were

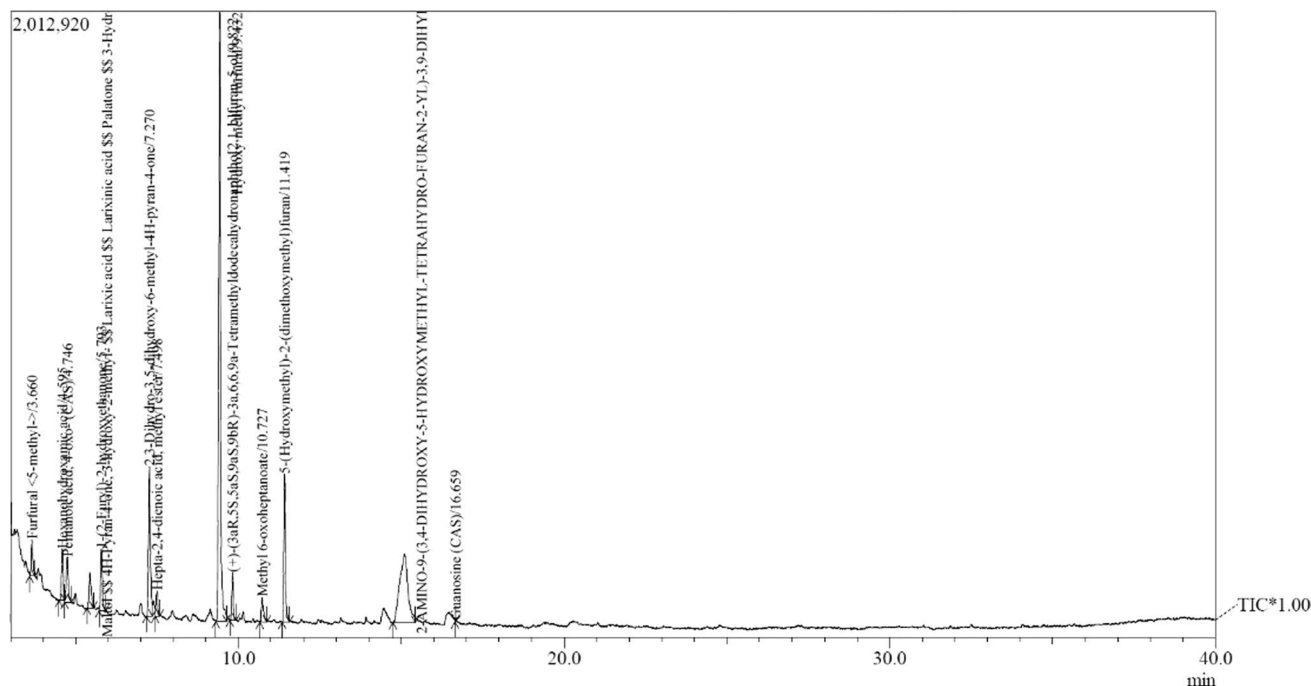
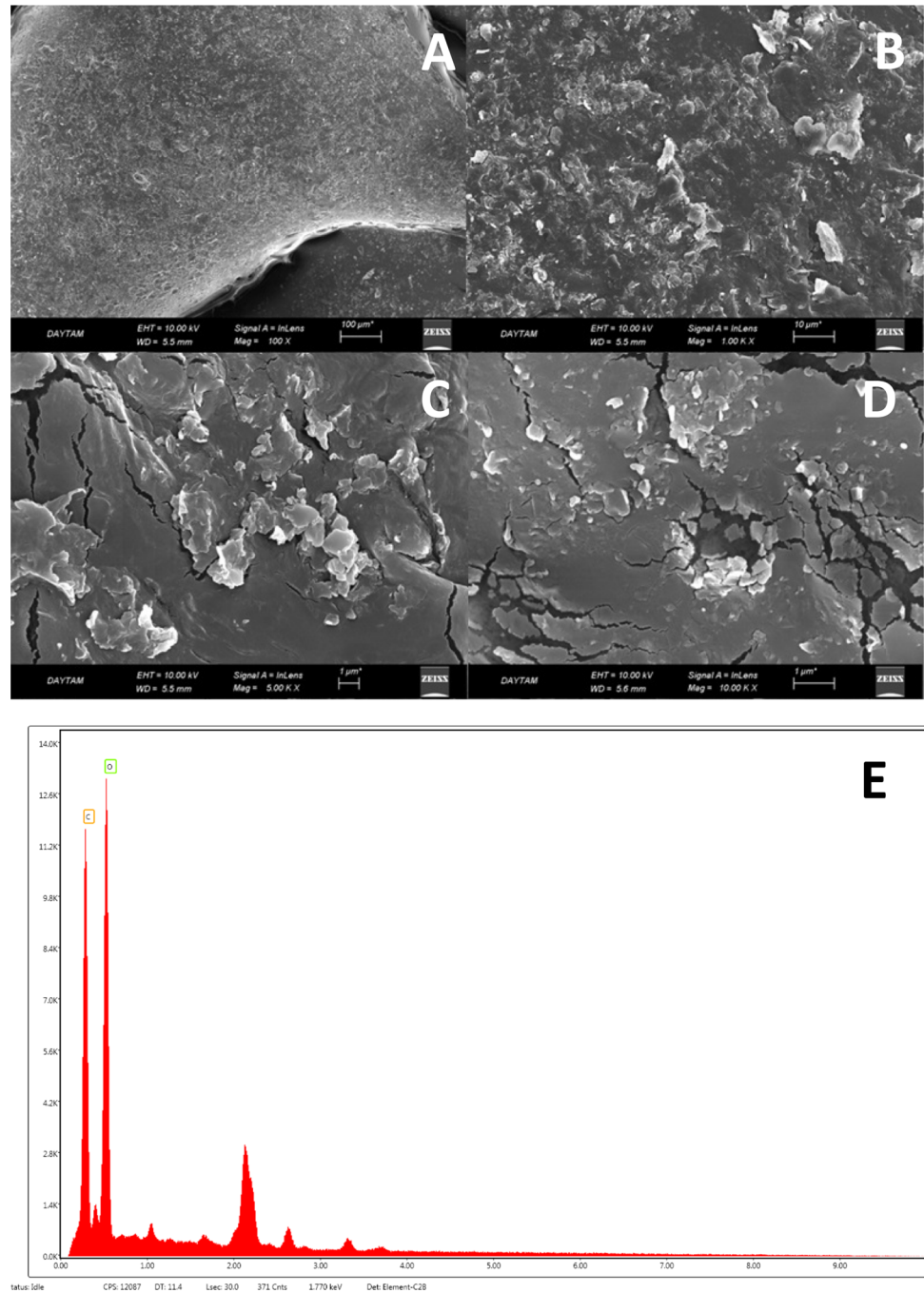


Fig. 3 GC–MS chromatogram of EPS 111.

**Fig. 4** Scanning electron microscope (SEM) images **A** 100X, **B** 1.00KX, **C** 5.00KX, **D** 10.00KX, and **E** Energy dispersive X-ray spectroscopy (EDX) analysis of EPS 111

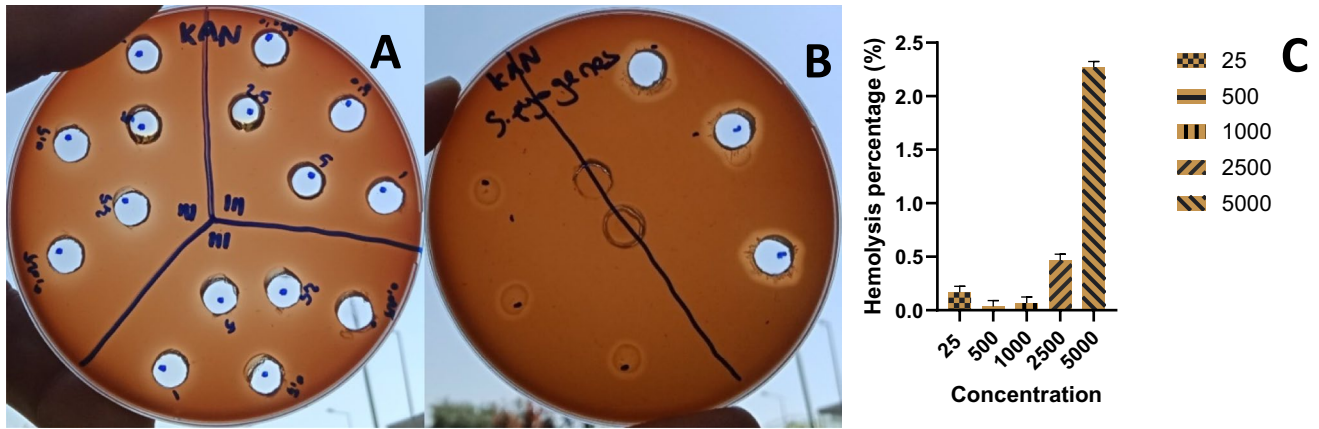


evaluated according to its hemolytic potential on red blood cells, and results are given in Fig. 5C. It has been reported that the allowable level of hemolysis for biomaterials is 5%. EPS 111 showed 2,3% cytotoxicity at 5000  $\mu\text{g}/\text{mL}$  concentration, so it was evaluated as not cytotoxic.

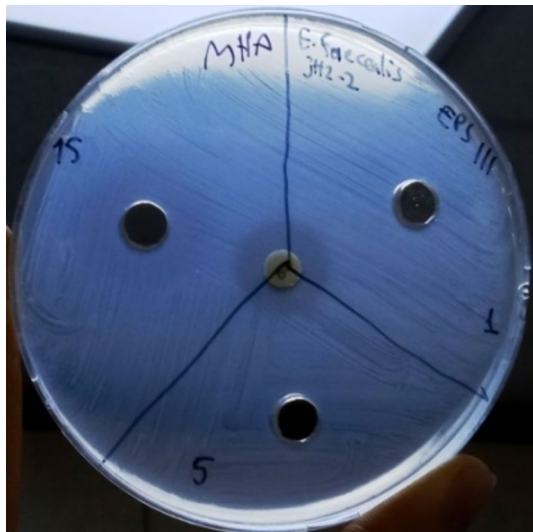
The antimicrobial activity of EPS 111 against *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, *P. vulgaris*, *C. albicans* pathogens was investigated, and it was determined that EPS 111 demonstrated antimicrobial activity only against *E. faecalis* (Fig. 6). EPS 111 showed antimicrobial

activity at the concentration of 15 mg/mL, with the zones of 21 mm.

To evaluate the antioxidant activity of EPS 111, DPPH radical scavenging activity and reducing power were carried out. The DPPH radical scavenging activity of the exopolysaccharide was demonstrated in Fig. 7A, and it was observed that the % inhibition rate increased with increasing EPS concentration and the  $\text{IC}_{50}$  value was calculated as 585  $\mu\text{g}/\text{mL}$ . The reducing power of exopolysaccharides was also studied, and experimental results are given in Fig. 7B. In the



**Fig. 5** Qualitative determination of cytotoxicity of EPSs by hemolytic potential test (A: EPS 111, B: *P.C./S. pyogenes*).C Hemolysis percentage of EPS 111. Values are means of three replicates with standard error bars shown.

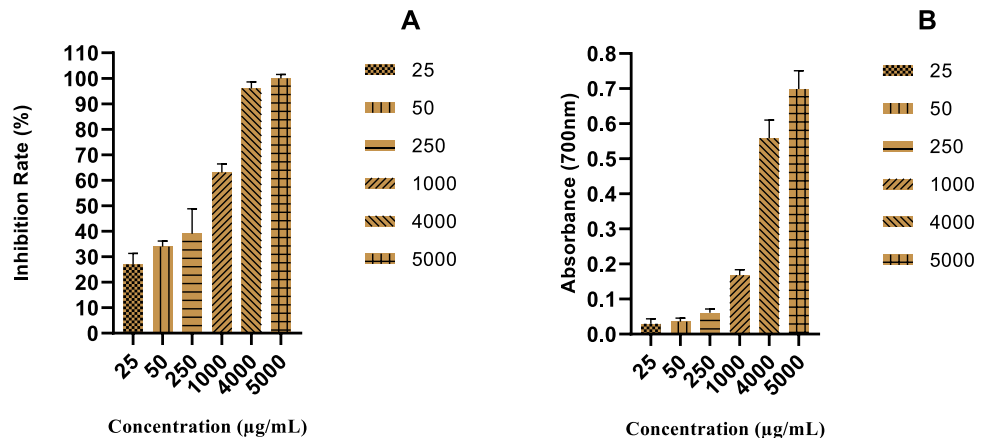


**Fig. 6** Agar well diffusion test of EPS 111 on *E. faecalis* JH2-2 bacteria.

reducing power analysis, the color change that occurs with the reduction of  $Fe^{3+}$  to  $Fe^{2+}$  is examined as an indicator of antioxidant activity. In the light of the results obtained, it was seen that the reducing power increased with the increase of EPS concentration.

The determination of  $\alpha$ -amylase inhibition activity was performed to evaluate the potential of EPS 111 for use in the antidiabetic field. The  $\alpha$ -amylase inhibition activity of exopolysaccharides was measured at the concentration of 800  $\mu$ g/mL. While acarbose, used as a standard, showed 37% activity at 1000  $\mu$ g/mL concentration, EPS 111 showed the inhibition activity 12% at 800  $\mu$ g/mL. The prebiotic activities of EPS 111 were investigated using lactic acid bacteria and enteric bacteria. EPS was used at the concentration of 2 mg/mL. Prebiotic indexes against *L. acidophilus* 66, *L. bulgaricus* 118 and *L. plantarum* DSM 20174 were found to be 4.33, 4.70 and 5.36, respectively. Prebiotic indexes being greater than 1 showed that EPS 111 has prebiotic activity. The fibrinolytic activity of

**Fig. 7** A DPPH radical scavenging activity, and B Reduction Power of EPS 1





EPS 111 in the study was investigated to determine their capacity to dissolve blood clots and thus their potential as drugs. Evaluation of fibrinolytic activity results was made by considering the amount of clot fragmentation between (+ 1 and + 5) levels. The results are given in Table 1. Clot fragmentation activity pictures of EPS 111 demonstrated in Fig S3andS4. When the results obtained were examined, it was seen that EPS 111 had moderate (+ 3) fibrinolytic activity.

Antibiofilm activity of EPS 111 was tested against different bacteria, and the results are given in Fig. 8. It is thought that the antibiofilm activity occurs by the EPS preventing the attachment of pathogenic bacteria and thus the formation of biofilm. EPS inhibits the initial attachment and association of bacteria. It does this by reducing cell–cell surface communication and weakening cell surface modifications (Kim and Kim 2009). In this study, it was determined that the

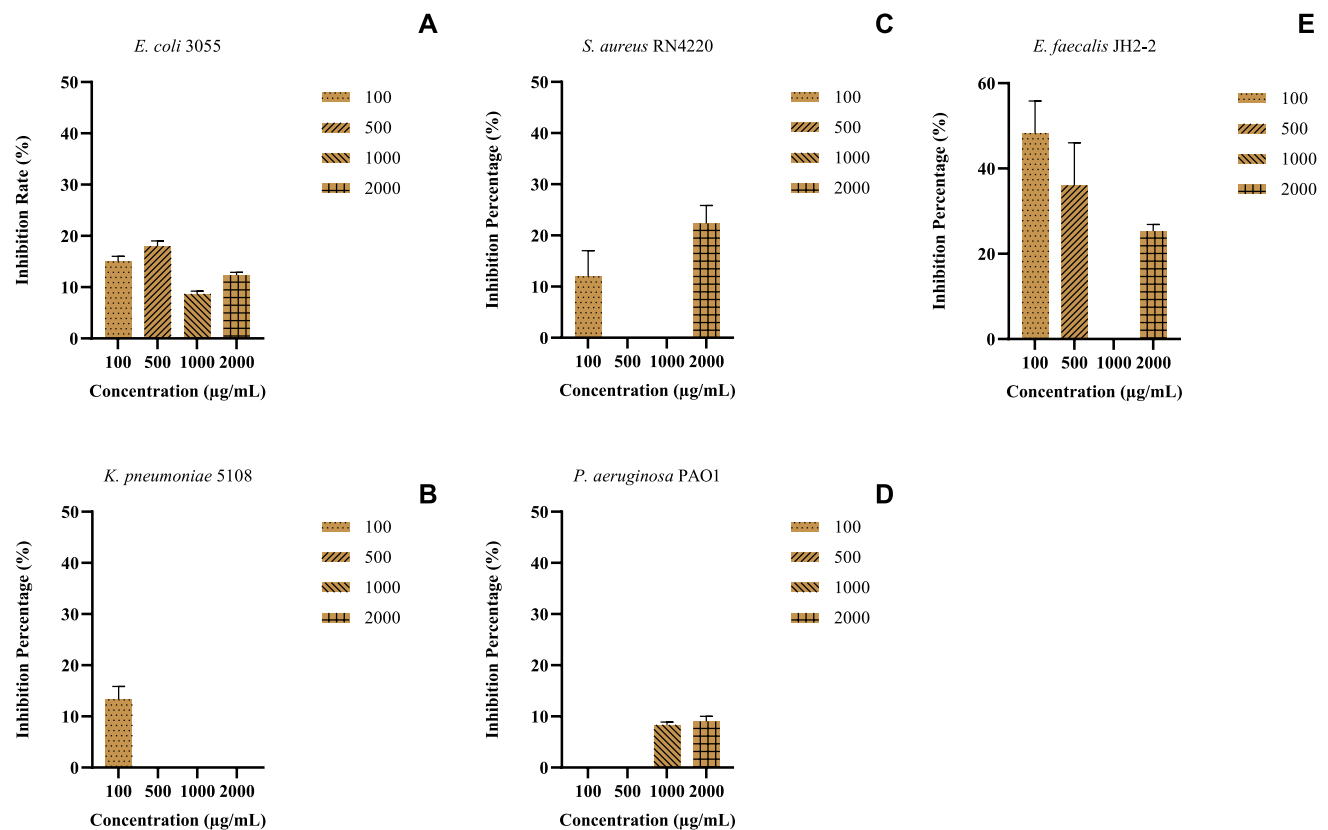
biofilm formation of *E. faecalis* was most inhibited. Inhibition rate of EPS 111 at 100 µg/mL concentration was 41%.

Thermophilic bacteria generally exhibit a high growth rate. This leads to a shortening of their doubling times and offer advantage for metabolite production (Panikov et al. 2003). Among the thermophilic endospore forming bacteria, the highest EPSs production was observed for the *Anoxybacillus sp.* R4-33 strain (1083 mg/L)isolated from a radon hot spring in China (Zhao et al. 2014), followed by *B. thermoruber* strain 423 (863 mg/L) isolated from Gradechnista hot spring in Bulgaria (Yildiz et al. 2014).

The structural analysis revealed that heteropolysaccharide composition of EPS111 was similar to those from other thermophilic bacteria. (Table 2). Moreover, Panosyan et al. 2018, stated that, thermophilic EPSs are heteropolysaccharides which composed of glucose, mannose and galactose generally as we detected for EPS111.

**Table 1** Fibrinolytic activity results of EPS 111 (2 mg/3 mL), P.C. (positive control: Actilyse 50 mg), N.C (negative control)

	EPS 111	P.C	N.C	EPS 111	P.C	N.C
				15 day		
Clot fragmentation	2	3	1	3	4	1



**Fig. 8** Antibiofilm activity of EPS 111 on **A** *E. coli* 3055. **B** *K. pneumoniae* 5108. **C** *S. aureus* RN4220. **D** *P. aeruginosa* PAO1, **E** *E. faecalis* JH2-2. Values are means of three replicates with standard error bars shown.

**Table 2** EPS production yields, chemical compositions and biological activities of EPSs from thermophilic bacilli

EPS producer	Biological Activity	Temperature (°C)	Time (h)	pH	Carbon source	Yield (mg /L)	References	EPS chemical composition
<i>Geobacillus thermodenitrificans</i> HBB 111	Non-cytotoxic, Antimicrobial, Antioxidant, Antibiofilm, Alpha-amylase inhibition, Prebiotic activity	55	26	8.0	Lactose	44	This study	Glucose/Fructose/Galactose/Mannose/Riboses
<i>Geobacillus</i> sp. TS3-9	Antioxidant, Antitumor activity	55	25	8.0	Lactose	87	(Wang et al. 2017)	Mannose/ Glucose/ Rhamnose
<i>G. tepidanmans</i> V264	Anti-cytotoxic (Brine shrimp test)	60	12	7.0	Maltose	111,4	(Kambourova et al. 2009)	Glucose/Galactose/ Fucose/Fructose
<i>G. thermodenitrificans</i> ArzA-6	NR	65	18	7.0	Fructose	76	(Panosyan et al. 2018)	Mannose/Galactose/ Arabinose/Fructose/Glucose
<i>G. toebii</i> ArzA-8	NR	65	18	7.0	Fructose	80	(Panosyan et al. 2018)	Mannose/Galactose/ Glucose/Arabinose
<i>Anoxybacillus pushchinoensis</i> G11	Antimicrobial, Antioxidant, Antitumor, Antibiofilm activity	60	48	8.0	Sucrose	18.4 (g dried EPS/L)	(Genc et al. 2021)	Arabinose/ Fructose/ Glucose/ Galactose
<i>Aeribacillus pallidus</i> 418	NR	55	13	7.0	Maltose	130	(Radchenkova,et al. 2013)	EPSI, Mannose/ Glucose/ Galactosamine/Glucoseamine/Galactose/ Ribose EPSII, Mannose/ Galactose/Glucosamine/Galactoseamine/Ribose/ Arabinose
<i>Geobacillus thermodenitrificans</i> B3-72	Antiviral activity	65	72	7.0	Glucose	70	(Arena et al. 2009)	Mannose/Glucose
<i>G. toebii</i>	NR	58	NR	NR	Sucrose	50	(Radchenkova et al. 2013)	NR
<i>P. thermantarcticus</i>	NR	65	24	6.0	Mannose	400	(Manca et al. 1996)	Mannose/Glucose
<i>Anoxybacillus</i> sp. R4-33	NR	55	48	8.0	Glucose	1083	(Zhao et al. 2014)	Mannose/Glucose
<i>Brevibacillus thermoruber</i> 423	NR	55	24	6.5	Maltose	863	(Yildiz et al. 2014)	Glucose/Galactose/ Galactosamine/ Mannose/Mannosamine
<i>Geobacillus</i> sp. WSUCF1	Non-cytotoxic Antioxidant activity	60	12	7.0	Glucose	525.7	(Wang et al. 2021)	EPSI, Mannose/ Glucose EPSII, Mannose

The biological activities of thermophilic EPSs have been studied less than those excreted by mesophilic ones. Despite of the relatively low yield of EPS111, as far as we know, this is the most detailed report regarding an EPS producing thermophile in terms of biological activities. As can be seen

from Table 2, the antioxidant, antimicrobial and antitumor activities of some thermophilic EPSs have been reported in the literature. However, in our study, a total of seven biological activities of EPS111 were examined and detected. Purified EPS 111 had low cytotoxicity (2.3%) and exhibited high

antioxidant capacity and remarkable antidiabetic, prebiotic and fibrinolytic activities.

## Conclusions

In recent years, studies on thermophilic bacteria producing EPS have been increasing and the biological activities of the produced exopolysaccharides have been demonstrated and characterized. Thermophilic EPSs are promising in these areas due to their biological and therapeutic potential, as well as their use as functional food ingredients. In this study, optimization of EPS production by *Geobacillus thermodenitrificans* HBB 111 was done and EPS 111 was purified using ion exchange and gel filtration chromatography techniques. Purified EPS was characterized, and its biological potential was examined by different methods such as hemolytic activity, antimicrobial activity, antioxidant activity, antidiabetic activity, prebiotic activity, fibrinolytic activity and antibiofilm activity. It was observed that the purified EPS in this study had multiple biological activities, unlike those in the literature. The use of natural source EPSs instead of synthetic or compounds with predicted toxicity is valuable in terms of biotechnology and biomedicine, and the EPS111 purified in this study has the potential to be used in this context.

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**Data availability** The data of this study is available upon request.

## Declarations

**Conflict of interest** The authors declare to have no conflict interests regarding the publication of the article.

## References

- Abinaya M, Vaseeharan B, Divya M, Sharmili A, Govindarajan M, Alharbi NS, Kadaikunnan S, Khaled JM, Benelli G (2018) Bacterial exopolysaccharide (EPS)-coated ZnO nanoparticles showed high antibiofilm activity and larvicidal toxicity against malaria and zika virus vectors. *J Trace Elem Med Biol* 45:93–103. <https://doi.org/10.1016/j.jtemb.2017.10.002>
- Al-Nahas M, Darwish M, Ali A, Amin M (2011) Characterization of an exopolysaccharide-producing marine bacterium, isolate *Pseudomonas* sp. *AM Afr J Microbiol Res* 5(22):3823–3831
- Amiri S, Mokarram RR, Khiabani MS, Bari MR, Khaledabad MA (2019) Exopolysaccharides production by *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. lactis BB12: optimization of fermentation variables and characterization of structure and bioactivities. *Int J Biol Macromol* 123:752–765
- Arena A, Gugliandolo C, Stassi G, Pavone B, Iannello D, Bisignano G, Maugeri TL (2009) An exopolysaccharide produced by *Geobacillus thermodenitrificans* strain B3-72: antiviral activity on immunocompetent cells. *Immunol Lett* 123(2):132–137
- Ayyash M, Abu-Jdayil B, Itsaranuwat P, Galiwango E, Tamiello-Rosa C, Abdullah H, Esposito G, Hunashal Y, Obaid RS, Hamed F (2020) Characterization, bioactivities, and rheological properties of exopolysaccharide produced by novel probiotic *Lactobacillus plantarum* C70 isolated from camel milk. *Int J Biol Macromol* 144:938–946
- Bajpai VK, Majumder R, Rather IA, Kim K (2016) Extraction, isolation and purification of exopolysaccharide from lactic acid bacteria using ethanol precipitation method. *Bangladesh J Pharmacol* 11(3):573–576
- Basbulbul, G., Biyik, H.Halil. (2009). Characterization and purification of bacteriocins produced by thermophilic bacteria isolated from various natural springs. [Doctorate thesis, Aydın Adnan Menderes University].
- Başbülbül Özdemir G, Biyik HH (2012) Isolation and characterization of a bacteriocin-like substance produced by *Geobacillus toebii* strain HBB-247. *Indian J Microbiol* 52:104–108
- Blois MS (1958) Antioxidant determinations by the use of a stable free radical. *Nature* 181(4617):1199–1200
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254
- Cao C, Li Y, Wang C, Zhang N, Zhu X, Wu R, Wu J (2020) Purification, characterization and antitumor activity of an exopolysaccharide produced by *Bacillus velezensis* SN-1. *Int J Biol Macromol* 156:354–361
- Dilna SV, Surya H, Aswathy RG, Varsha KK, Sakthikumar DN, Pandey A, Nampoothiri KM (2015) Characterization of an exopolysaccharide with potential healthbenefit properties from a probiotic *Lactobacillus plantarum* RJF4. *LWT Food Sci Technol* 64(2):1179–1186
- El Essawy AK, Abu Shady HM, Abu El Kher AM, Helal MM (2016) Antimicrobial, anticoagulation, fibrinolytic and prebiotic activities of exopolysaccharide produced by marine *Klebsiella* spp. *Egypt J Exp Biol (bot)* 12(2):267–274
- Filik N, Kubilay A (2020) *Aeromonas hydrophila* balık patojeninde biyosensör suşlar aracılığıyla çevreyi algılama sisteminin tespiti ve virülens faktörleri determination of quorum sensing system via biosensor strains and virulence factors in fish pathogen *aeromonas hydrophila*. *Ege J Fish Aquat Sci* 37(1):29–36
- Genc B, Taskin M, Adiguzel A (2021) Exopolysaccharide of *Anoxybacillus pushchinoensis* G11 has antitumor and antibiofilm activities. *Arch Microbiol* 203:2101–2118
- Guezennec J, Herry JM, Kouzayha A, Bachere E, Mittelman MW, Fontaine MNB (2012) Exopolysaccharides from unusual marine environments inhibit early stages of biofouling. *Int Biodeterior Biodegrad* 66(1):1–7
- Hu X, Li D, Qiao Y, Wang X, Zhang Q, Zhao W, Huang L (2020) Purification, characterization and anticancer activities of exopolysaccharide produced by *Rhodococcus erythropolis* HX-2. *Int J Biol Macromol* 145:646–654
- Hu S-M, Zhou J-M, Zhou Q-Q, Li P, Xie Y-Y, Zhou T, Gu Q (2021) Purification, characterization and biological activities of exopolysaccharides from *Lactobacillus rhamnosus* ZFM231 isolated from milk. *LWT* 147:111561
- Jouault SC, Chevolut L, Helley D, Ratiskol J, Bros A, Sinquin C, Roger O, Fischer AM (2001) Characterization chemical modifications and in vitro anticoagulant properties of an exopolysaccharide produced by *Alteromonas infernus*. *Biochim Et Biophys Acta (BBA)-gen Subj.* 1528(2–3):141–151

- Kambourova M, Mandeva R, Dimova D, Poli A, Nicolaus B, Tommonaro G (2009) Production and characterization of a microbial glucan, synthesized by *Geobacillus tepidamans* V264 isolated from Bulgarian hot spring. *Carbohydr Polym* 77(2):338–343
- Kim Y, Kim SH (2009) Released exopolysaccharide (r-EPS) produced from probiotic bacteria reduce biofilm formation of enterohemorrhagic *Escherichia coli* O157: H7. *Biochem Biophys Res Commun* 379(2):324–329
- Li W, Ji J, Rui X, Yu J, Tang W, Chen X, Jiang M, Dong M (2014) Production of exopolysaccharides by *Lactobacillus helveticus* MB2-1 and its functional characteristics in vitro. *Lwt—Food Sci Technol* 59(2):732–739
- Li Y, Li Q, Hao D, Jiang D, Luo Y, Liu Y, Zhao Z (2015) Production, purification, and antibiofilm activity of a novel exopolysaccharide from arthrobacter sp B4. *Prep Biochemi Biotechnol* 45(2):192–204
- Li C, Lin F, Sun W, Wu F-G, Yang H, Lv R, Zhu Y-X, Jia H-R, Wang C, Gao G (2018) Self-assembled rose bengal-exopolysaccharide nanoparticles for improved photodynamic inactivation of bacteria by enhancing singlet oxygen generation directly in the solution. *ACS Appl Mater Interfac* 10(19):16715–16722
- Lin R, Liu H, Wu S, Pang L, Jia M, Fan K, Jia S, Jia L (2012) Production and in vitro antioxidant activity of exopolysaccharide by a mutant, *Cordyceps militaris* SU5-08. *Int J Biol Macromol* 51(1–2):153–157
- López-Ortega MA, Chavarría-Hernández N, del Rocío López-Cuellar M, Rodríguez-Hernández AI (2021) A review of extracellular polysaccharides from extreme niches an emerging natural source for the biotechnology. from the adverse to diverse. *Int J Biol Macromol* 177:559–577
- Manca MC, Lama L, Improta R, Esposito E, Gambacorta A, Nicolaus B (1996) Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. *Appl Environ Microbiol* 62(9):3265–3269
- Marzalletti T, Valenzuela Olarte MB, Sievers C, Hoskins TJ, Agrawal PK, Jones CW (2008) Dilute acid hydrolysis of Loblolly pine: a comprehensive approach. *Ind Eng Chem Res* 47(19):7131–7140
- Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC (2005) Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Anal Biochem* 339(1):69–72
- Mende S, Rohm H, Jaros D (2016) Influence of exopolysaccharides on the structure, texture, stability and sensory properties of yoghurt and related products. *Int Dairy J* 52:57–71
- Nicolaus B, Moriello VS, Lama L, Poli A, Gambacorta A (2004) Polysaccharides from extremophilic microorganisms. *Orig Life Evol Biosph* 34:159–169
- Okur ÖD, Seydim ZG (2011) Geleneksel dolaz peynirinde bazı karakteristik özelliklerin belirlenmesi. *Ege Üniv Ziraat Fak Derg* 48(2):113–117
- Panikov N, Popova N, Dorofeev A, Nikolaev YA, Verkhovtseva N (2003) Growth of the thermophilic bacterium *Geobacillus uralicus* as a function of temperature and pH: an SCM-based kinetic analysis. *Microbiology* 72:277–284
- Panosyan H, Di Donato P, Poli A, Nicolaus B (2018) Production and characterization of exopolysaccharides by *Geobacillus thermodenitrificans* ArzA-6 and *Geobacillus toebii* ArzA-8 strains isolated from an Armenian geothermal spring. *Extremophiles* 22(5):725–737
- Patwal T, Baranwal M (2021) *Scenedesmus acutus* extracellular polysaccharides produced under increased concentration of sulphur and phosphorus exhibited enhanced proliferation of peripheral blood mononuclear cells. *3 Biotech* 11(4):1–9
- Radchenkova N, Vassilev S, Panchev I, Anzelmo G, Tomova I, Nicolaus B, Kancheva M, Petrov K, Kambourova M (2013) Production and properties of two novel exopolysaccharides synthesized by a thermophilic bacterium *Aeribacillus pallidus* 418. *Appl Biochem Biotechnol* 171(1):31–43
- Rani RP, Anandharaj M, Ravindran AD (2018) Characterization of a novel exopolysaccharide produced by *Lactobacillus gasseri* FR4 and demonstration of its in vitro biological properties. *Int J Biol Macromol* 109:772–783
- Saha K, Lajis N, Israf D, Hamzah A, Khozirah S, Khamis S, Syahida A (2004) Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *J Ethnopharmacol* 92(2–3):263–267
- Seveiri RM, Hamidi M, Delattre C, Sedighian H, Pierre G, Rahmani B, Darzi S, Brasselet C, Karimitabar F, Razaghpoor A (2020) Characterization and prospective applications of the exopolysaccharides produced by *rhodosporidium babjevae*. *Adv Pharma Bull* 10(2):254
- Staudt C, Horn H, Hempel D, Neu T (2004) Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms. *Biotechnol Bioeng* 88(5):585–592
- Stepanović S, Vuković D, Hola V, BONAVENTURA GD, Djukić S, Čirković I, Ruzicka F (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *Staphylococci*. *APMIS* 115(8):891–899
- Tsuda H, Miyamoto T (2010) Production of exopolysaccharide by *Lactobacillus plantarum* and the prebiotic activity of the exopolysaccharide. *Food Sci Technol Res* 16(1):87–92
- Wang J, Zhao X, Yang Y, Zhao A, Yang Z (2015) Characterization and bioactivities of an exopolysaccharide produced by *Lactobacillus plantarum* YW32. *Int J Biol Macromol* 74:119–126
- Wang L, Zhang H, Yang L, Liang X, Zhang F, Linhardt RJ (2017) Structural characterization and bioactivity of exopolysaccharide synthesized by *Geobacillus* sp. TS3-9 isolated from radioactive radon hot spring. *Adv Biotechnol Microbiol* 4:1–8
- Wang J, Salem DR, Sani RK (2021) Two new exopolysaccharides from a thermophilic bacterium *Geobacillus* sp. WSUCF1: characterization and bioactivities. *New Biotechnol* 61:29–39
- Wu M-H, Pan T-M, Wu Y-J, Chang S-J, Chang M-S, Hu C-Y (2010) Exopolysaccharide activities from probiotic bifidobacterium: immunomodulatory effects (on J774A 1 macrophages) and antimicrobial properties. *Int J Food Microbiol* 144(1):104–110
- Yasar Yildiz S, Anzelmo G, Ozer T, Radchenkova N, Genç S, Di Donato P, Kambourova M (2014) *Brevibacillus themoruber* a promising microbial cell factory for exopolysaccharide production. *J Appl Microbiol* 116(2):314–324
- Yıldız O, Şahin H, Meryem K, Aliyazıcıoğlu R, Tarhan Ö, Sevgi K (2010) Maillard reaksiyonları ve reaksiyon ürünlerinin gıdalardaki önemi. *Akademik Gıda* 8(6):44–51
- Zhao S, Cao F, Zhang H, Zhang L, Zhang F, Liang X (2014) Structural characterization and biosorption of exopolysaccharides from *Anoxybacillus* sp. R4–33 isolated from radioactive radon hot spring. *Appl Biochem Biotechnol* 172:2732–2746

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