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The Structural Characterization of Extracellular Polysaccharide from *Enterococcus faecium* M20

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HIGHLIGHTS

- The extracellular polysaccharide (EPS) from *Enterococcus faecium* M20 was characterized.
- The degradation of polysaccharide was in three steps and it was thermally stable.
- SAXS data showed that the bacterial EPS possessed the lamellar structure.
- The investigation of structure of its EPS may be extremely important to find a specific way for treatment of its infection.

Abstract: *Enterococcus faecium* is one of the well-known human pathogens producing biofilm. Todays, it is an important nosocomial infection agent and is spreading rapidly worldwide. As the first step of the infection, this bacterium adheres to tissue or devices with EPS fibrils, and then the biofilm grows. Therefore, investigation of structure of its EPS is extremely important to find a specific way for treatment of its infection. In this study, we isolated and investigated the physicochemical properties extracellular polysaccharide (EPS) from *E. faecium* M20 obtained from Denizli Public Hospital. The exopolysaccharide of M20 strain was purified by methanol/ethanol extraction method and then analyzed by SAXS, TGA, HPLC and SEM. Carbohydrate and protein were main compounds in the isolated EPS. Moreover, the uronic acid content was found to be high. The HPLC analysis indicated that the EPS consisted of glucose+maltitol, fructose and sorbitol+ksilitol. According to SAXS data, the EPS of M20 possessed a lamellar structure and prolate core shell with a major core radius of 1644.1 Å, a major shell thickness of 121.9 Å, and a bilayer thickness of 12.6 Å. The natural polysaccharide structure was more stable within aqueous (0.5%, w/v) and serum albumin solutions at room temperature. These results may be used to develop new strategies in future for eradicating biofilms of this pathogen bacterium.

Keywords: *enterococcus faecium*; exopolysaccharide; SAXS; HPLC; SEM.

INTRODUCTION

Biofilm is a complex structure created by the microorganism to protect itself from chemical, physical and biological factors. Large number of microorganisms can form this structure and so they are resistant to environmental factors by the help of biofilm. Moreover, biofilm formation is a strong response at the cellular level for microbial survival against environmental factors for pathogens and plays a critical role in the initiation and progression of infection. One of the strategies for preventing microbial infection is to inhibit biofilm formation. But, this is so difficult because the mechanism of biofilm formation varies from organism to organism. Therefore, a detailed understanding of formation and structure of biofilm has a critical importance for fighting bacterial infection. Extracellular polysaccharides (EPS) are the main matrix of biofilm. A wide variety of microorganisms including bacteria, cyanobacteria, marine microalgae, lactic acid bacteria and yeast can produce EPS [1-5]. EPS is extremely important in the attachment of bacteria to surfaces and thus development of biofilms. In addition to role in bacterial attachment, they are involved in colony formation of microorganisms, flocculation, cell-cell recognition, formation of barrier to protect from environment conditions and water retention for dehydration [6,7]. Especially, the electrostatic interactions contribute to EPS adsorption between bacteria and minerals in soil and aquatic environments, and that interactions influence on bacteria adhesion, mineral transport, biofilm formation, mineral dissolution, biomineralization and heavy metals remediation [8-10]. For example, according to Do and coauthors (2020), EPS produced by *Rahnella* sp. LRP3 has possessed significant effect in the remediation of Cu contaminated soil [11]. In addition, EPS also has unique biological properties such as larvicidal, antibiofilm, antibacterial, antiviral and immunoregulatory effects [12-14]. All of these properties of EPSs are affected by their physicochemical properties and three-dimensional structure. Therefore, recent studies have been mainly focused on investigation of the three-dimensional structure and chemical ingredients of EPS with different techniques. For example, techniques such as SAXS, XRD, SEM, NMR etc. have been used for the detailed investigation of polysaccharide structures and morphologies [15-19]. In this regard, we aimed to characterize the three-dimensional configuration of EPS purified from clinical-isolate *Enterococcus faecium* M20 with SAXS in this study. *E. faecium* is an opportunistic pathogen and its infections are predominantly seen in immunocompromised patients. Therefore, these infections became increasingly prominent in intensive care, burns, oncology and organ transplant units of hospitals [20]. As the first step of the infection, bacteria adhere to tissue or devices with EPS fibrils, and then the biofilm grows [21]. That's why, investigation of structure of its EPS is extremely important to find a specific way for treatment of its infection. According to the current literature, there is no information about the EPS structure of this pathogen bacterium including SAXS. It is well known that; the polysaccharides of pathogens usually are not detected by the host immune system. Moreover, they have important role in attachment of host cells. In addition to these, the diversity of the polysaccharide and protein complexes in pathogens gives them the ability of mimicking the host surface. Therefore, detailed analysis EPS by different techniques may be important to prevent attachment of pathogens to host cells. By this way, the infection of pathogens may be prevented by designing new therapeutic substances that destroy EPS of pathogens. Todays, new strategies to treat biofilm related infections such as use of enzymes that degrade the biofilm matrix components in combination with antibiotics were suggested [22]. Similarly, the small molecule inhibitors, antimicrobial peptides and quaternary ammonium amphiphiles were investigated for the eradication and inhibition of biofilms as some alternative biofilm-target agents [23-25]. Undoubtedly, the biofilm inhibition success of these strategies is closely related to the composition and structure of exopolysaccharides. In the light of this information, by the help of detailed analysis of EPS in biofilms, new biomaterials that are resistant to biofilm formation may be designed and may be used for production of medical devices. Therefore, these results may be used to develop therapeutic substances and biomaterials for eradicating biofilms of this pathogen bacterium.

MATERIAL AND METHODS

Bacterial strain and screening of biofilm

The strain M20 isolated from human blood sample in Denizli Public Hospital was used in this study. For molecular identification of the strain, 16S rRNA sequence analysis was done in Gazi University, Life Sciences Research and Application Center (Ankara, Turkey). The bacterial strain was stocked from the Bacteriology Laboratory in Department of Biology, Pamukkale University. To verify biofilm formation of isolate, Congo red

agar method was performed according to the protocol of Freeman and coauthors 1989 [26]. The Congo red stain was added 0.8 g/l in Brain heart infusion Agar (BHA) and black-colored colonies were evaluated as biofilm positive.

Microtiter plate test

Quantitative biofilm formation was determined by Microtiter plate test as described by Jain and Agarwal that was modified in our laboratory conditions [27]. The cell culture at 0.5 McFarland turbidity standards was dispensed into each well of 96-well plates in the presence of 200 µL TSB (Merck) and diluted TSB at 1:100 and 1:200 ratios. Plates were incubated at 37 ˚C for different incubation time periods. Then, the wells were washed 3 times with sterile Phosphate buffer saline (PBS) to remove non-adherent cells and were air-dried. After that, wells were stained with 1% crystal violet (100 µL) for 15 minutes and washed again with PBS to remove unbound stain. Finally, 33% glacial acetic acid was added to each well and then the OD of each well was read on a microplate reader (Optic ivymen system 2100-C) set to absorbance of 600 nm. The biofilm increments were calculated as a percentage using the following formula:

$$\% = 100 \times (OD_{final} - OD_{initial}) / OD_{initial} \quad (1)$$

Extracellular polysaccharide (EPS) purification and characterization

EPS was obtained from bacterial pellet according to the method of Hung and coauthors 2005 [28]. M20 strain was incubated in 1/200 diluted TSB medium. The cell culture was centrifuged at 3500 rpm for 30 min. The proteinase solution (final conc. 0.5 mg/L) was added in supernatant and kept at 37˚C and 70 rpm for 12 h to remove proteins. To form a precipitate, the cold alcohol mixture (95% ethanol and 5% methanol) was added and kept for 12 h in the refrigerator. Then, the precipitate was collected with 0.22 µm pore diameter membrane (Millipore membrane filter, HAWP04700) using Sartorius membrane filtration device. After the precipitate has been dissolved with water, NaCl (final concentration of 30g/L) was added and treated with a cold alcohol mixture for 12 h in the refrigerator. This step was repeated three times. The final precipitate was dissolved with 10-20 mL of dH_2O and dialyzed against water. This procedure described above for the supernatant was also applied to the pellet as well. The pure EPS was freeze-dried (LYOQUEST, -85, Telstar Technologies, Spain) for the further analyses. The EPS from the supernatant and pellet was then combined and stored as a powder. The total of carbohydrate amount in freeze-dried EPS was detected by the method of phenol sulfuric acid. Glucose was used as a standard [29]. The protein amount in EPS was determined by modified Lowry method [30]. Uronic acid content was measured by using Hung and Santschi (2001) that was optimized in our laboratory conditions. For this purpose, 1.0 mg of EPS was dissolved in 0.4 mL of distilled water and 40 µL of 2 M sulfamic acid pH 1.6 (Reagent A), 2.4 mL of 75 mM sodium tetraborate in 96% sulfuric acid (Reagent B) and 30 µL of 0.15% of m-hydroxydiphenyle prepared in 0.5% of NaOH solution (Reagent C) were added to EPS contained solution. The developed color was measured at 532 nm. The slope of standard curve generated by using different concentration of glucuronic acid was used for the calculation of the amount of uronic acid in our EPS sample. Details of the assay were given in Hung and Santschi (2001) [31]. The monosaccharide content of EPS was determined by HPLC (The Central Laboratory, Molecular Biology and Biotechnology R&D Centre, Middle East Technical University, Ankara, TURKEY). Varian Prostar HPLC System, Varian Carbohydrates Ca column (300x6.5mmx3/8) and Refractive Index (RI) detector were used for this analysis. The separations were carried out at 90˚C using a flow rate of 0.5 mL/min was injected. Dr. Ehrenstorfer Certified Reference Standards were used as a standard.

Small angle X-ray scattering (SAXS)

SAXS analysis was carried out as described in our previous study [18]. 0.005 g EPS of M20 strain was placed in 1000 µL aqueous and 50 µL serum albumin solutions. Samples were measured for 10 minutes at room temperature using the system's sample holder.

Scanning electron microscopy (SEM)

For SEM analysis, the lyophilized EPS was overlaid on aluminum stamps and coated with gold-palladium approximately 30 min. Then, the samples were analyzed in QUANTA 250-FEG SEM Instrument at the Izmir Institute of Technology (Izmir, Turkey).

RESULTS

Determination of biofilm formation

The M20 strain was 99% identical to *Enterococcus faecium* (GenBank numbers: KJ726575.1, KM186186.1, KJ958412.1 and KJ702575.1). Its biofilm formation was verified by using Congo red agar. Microtiter plate test was used for quantifying biofilm formation. The rates of biofilm formation by M20 depended on the time and the nutrient levels in the medium. Biofilm formation in diluted medium (1:100 and 1:200 diluted) was greater than undiluted. The maximum biofilm formation was observed at 96 hours in 1:200 diluted TSB (92.30%) (Table 1).

Table 1. Quantitative biofilm formation of *Enterococcus faecium*.

Medium	Biofilm (%)	Time (h)
TSB	20.95 ± 0.55	48 ± 0.0
TSB (1/100)	55.92 ± 0.49	144 ± 2.0
TSB (1/200)	92.30 ± 5.0	96 ± 2.0

Biochemical composition

Biochemical composition and sugar content of isolated EPS were also determined throughout in this study. It was found that carbohydrate and protein were the major organic compounds with a concentration of 227.8 mg/g and 153.7 mg/g, respectively. Moreover, uronic acid was found to be 40.5 mg/g in M20 EPS. HPLC analysis shown that EPS was consisted of glucose+maltitol, fructose, sorbitol+ksilitol (Tables 2 and 3, Figure 1).

Table 2. The composition of EPS produced by *Enterococcus faecium* M20

	Parameter (mg/g EPS)		
	Total carbohydrates	**Uronic Acid**	**Protein**
Freeze-dried EPS	227.8 ± 4.4	40.5 ± 0.05	153.7 ± 1.0

Table 3 The sugar composition of EPS produced by *Enterococcus faecium* M20 (µg/mL)

Glucose+Maltitol	Fructose	Sorbitol+Ksilitol
5	6.7	6.7

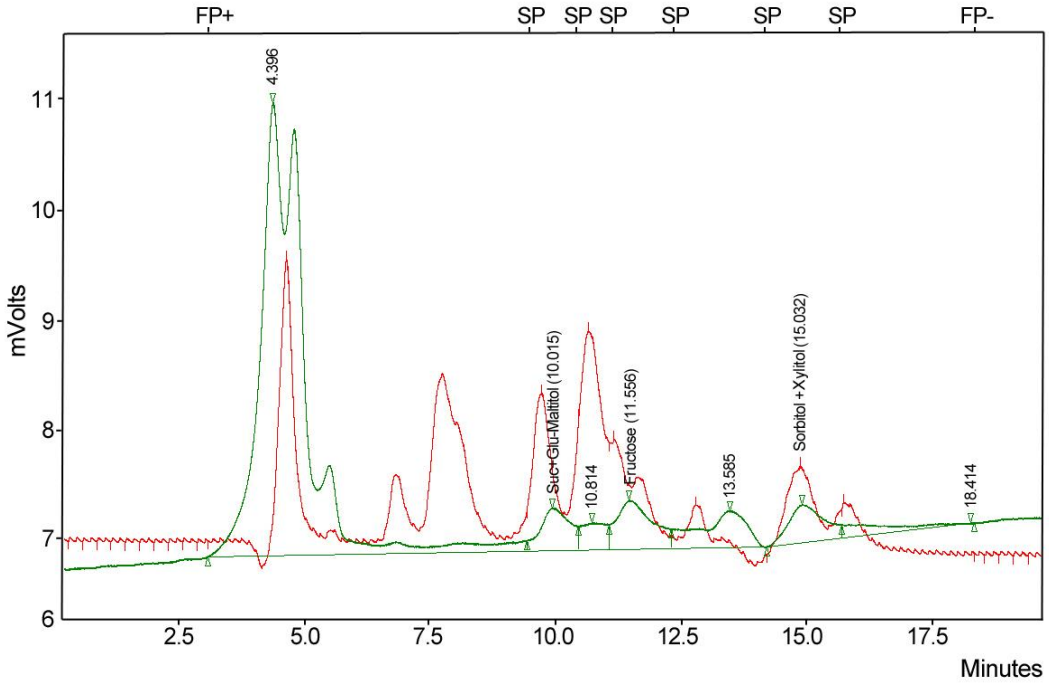

Figure 1. Chromatogram analysis of EPS produced by *Enterococcus faecium* M20 (red line: 0.005 mg/L sugar standard mixture, green line: bacterial EPS).

Small angle X-ray scattering (SAXS)

In the present study, we also performed structural analysis of the bacterial EPS with SAXS analysis by using a HECUS-SAXS system3 (Hecus X-ray systems, Brucker Austria GmbH, Graz, Austria). The HECUS-SAXS system3 consists of CuKα (λ = 1.54 Å) radiation, position sensitive detectors (~54 µm resolution, 1024 channels) and line collimation operated at 50 kV and 50 mA. In aqueous and serum albumin solutions, formations of EPS exhibit structural changes depending on different physical conditions. Therefore, 0.005 grams pure particulate EPS of M20 strain was placed in 1000 µl aqueous (pellet) and 50 µl serum albumin (pellet A) solutions. In the SAXS system, our samples were carried out at room temperature for a time interval of 600 seconds. Figure 2 presents the SAXS profiles of freeze-dried EPS of M20 as compared to aqueous (a), serum albumin (b) and each other (c), respectively. SAXS profiles can be fitted a suitable shape with model-independent approximations [32,33]. We also fitted data understandably well using the lamellar with the sum of prolate core shell and core shell spherical model. Moreover, it was found that the EPS structure was stable in aqueous (0.5% w/v) and serum albumin solutions at room temperature. Figure 3 represents the SAXS data for EPS in aqueous and serum albumin solutions, at room temperature and the same pH value. Under tested conditions, the SAXS data indicated formation of EPS for both cases. The big hump recorded in q = 0.1–0.4 Å-1 was nanoscale structural information (Figure 2). Assuming spherical EPS, the radius of hydrodynamic RH obtained for EPS decreased from 29.1 ± 0.5 to 26.7 ± 0.5 Å with the addition of serum albumin as a solvent. Under these conditions, the samples of EPS possessed the structure of lamellar and prolate core-shell with the major core-radius of 1644.1 ± 5.0 Å, the shell-thickness of 121.9 ± 2.4 Å, and the bilayer-thickness of 12.6 ± 0.7 Å with the adding of aqueous serum albumin. In presence of serum, the samples of EPS possessed the lamellar and prolate core-shell with the major core-radius of 1623.6 ± 5.2 Å, shell-thickness of 101.1 ± 2.1 Å, and the bilayer-thickness of 6.8 ± 0.6 Å (Table 4 and 5). The prolate structural model was schematically given in Figure 4.

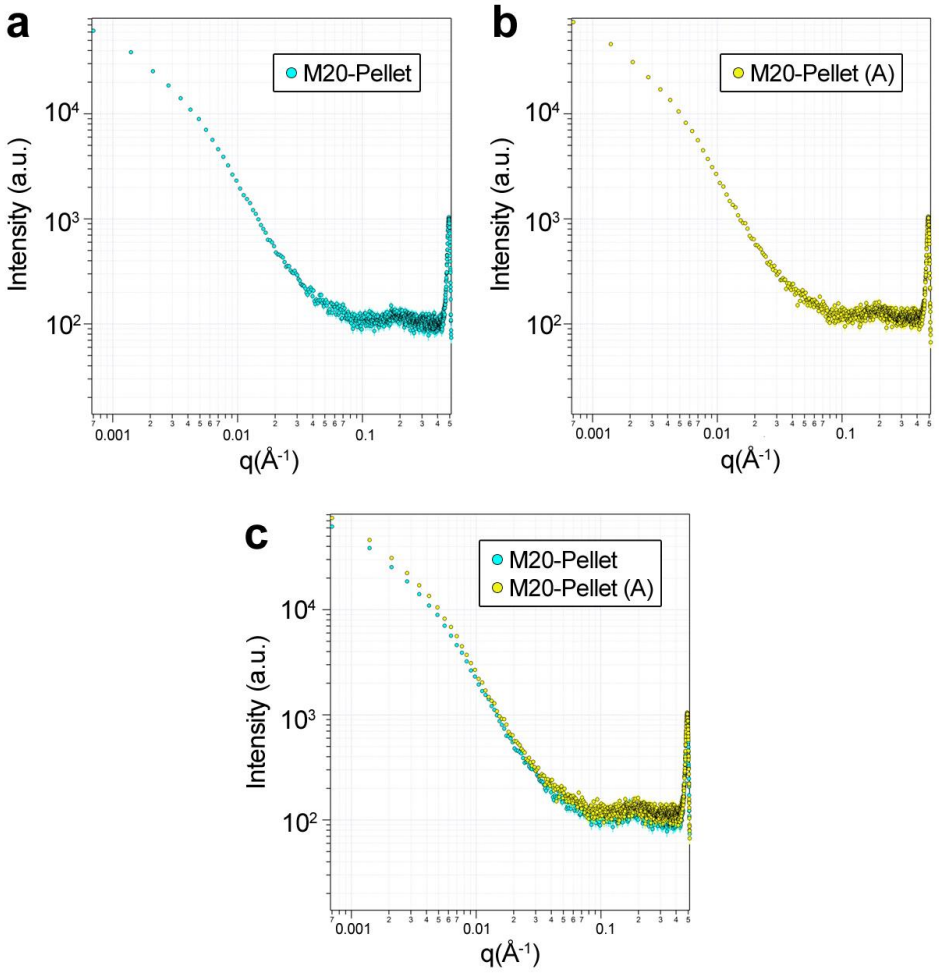

Figure 2. SAXS graphics pattern of bacterial EPS in aqueous (a), serum albumin (b) and each other (c) of solvents.

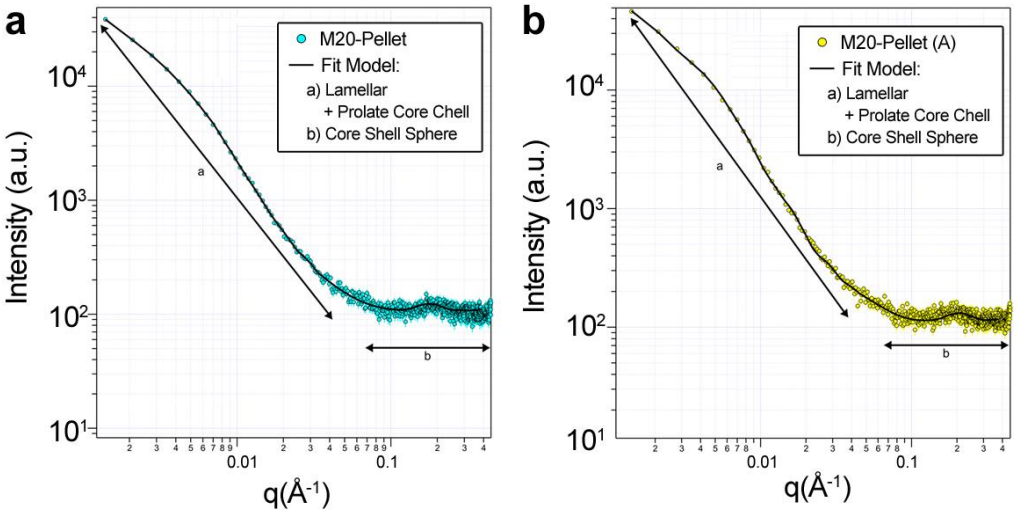

Figure 3. The SAXS data are fitted (solid curves) using lamellar + prolate core shell model for zone a, and core shell sphere model for zone b.

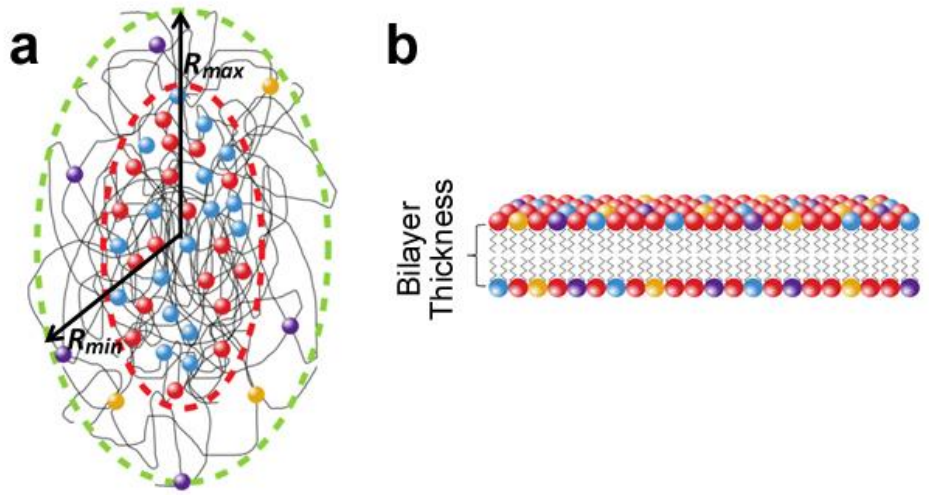

Figure 4. (a) In the schematic representation of the model, Rmax and Rmin are the major and minor radius, (b) A proposed prolate structural model for the EPS with the bilayer thickness of the membrane presentation, respectively.

Table 4. The structural information of bacterial EPS of M20 in aqueous (EPS) and serum albumin added solutions (EPS A) was obtained by analyzing the SAXS data in Zone a.

Zone a	EPS	EPS (A)
Bilayer thickness (Å)	12.6	6.8
Major core radius (Å)	1644.1	1623.6
Minor core radius (Å)	312.8	290.2
Major shell thickness (Å)	121.9	101.1
Minor shell thickness (Å)	235.9	221.1
SLD core ($Å^{-2}$)	2.0×10^{-6}	1.8×10^{-6}
SLD shell ($Å^{-2}$)	1.4×10^{-6}	1.6×10^{-6}
SLD solvent ($Å^{-2}$)	4.4×10^{-6}	4.1×10^{-6}

Table 5. The structural information of bacterial EPS of M20 in aqueous (EPS) and serum albumin added solutions (EPS A) was obtained by analyzing the SAXS data in Zone b.

Zone b	EPS	EPS (A)
Core radius (Å)	17.1	15.8
Shell thickness (Å)	12.0	10.9
Core SLD ($Å^{-2}$)	2.5×10^{-5}	2.5×10^{-5}
Shell SLD ($Å^{-2}$)	1.3×10^{-5}	1.3×10^{-5}
Solvent SLD ($Å^{-2}$)	9.5×10^{-6}	9.4×10^{-6}

Scanning electron microscope analysis (SEM)

Bacterial cells morphologies and their interactions with EPS were determined by SEM analysis. SEM analysis results showed that M20 cells were present both alone and in dense clusters within the polymeric carbohydrate matrix (Figure 5a and 5b). The cells connected to each other with EPS fibers (Figure 5c and 5d). Moreover, it has been found that the EPS matrix is mostly densified lamellae (CL) and rarely extruded layers (RL).

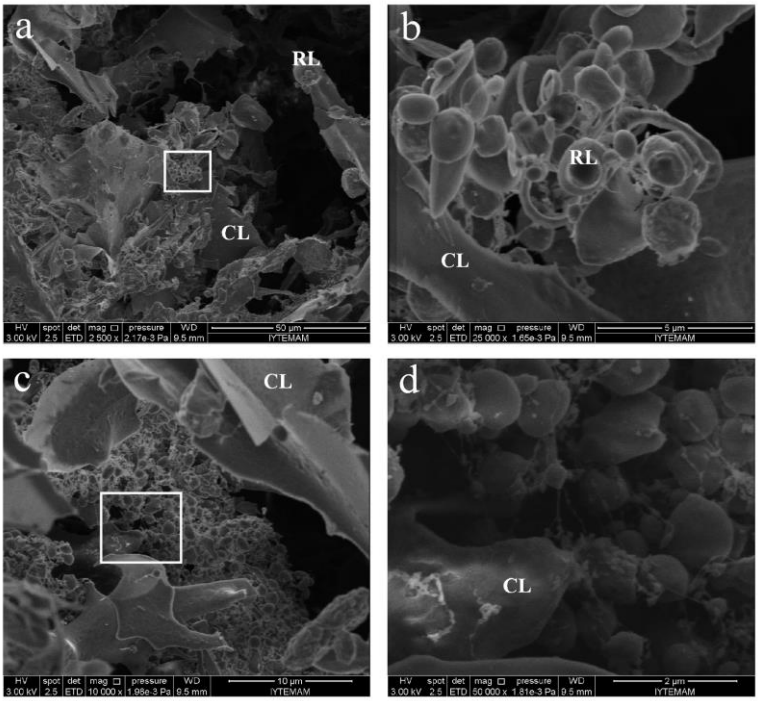

Figure 5. SEM photomicrographs. (a–b) M20 cells within the matrix; (c–d) M20 cells connecting with EPS fibers. CL and RL indicate condensed lamellas and rolled layers, respectively.

DISCUSSION

Enterococci are major human pathogens due to cause nosocomial infections. They are being detected frequently in community-acquired infections and they show partial or complete resistance to many antibiotics. Moreover, these bacteria create antibiotic resistance with remarkable new mechanisms and rapidly transfer this resistance to new generations. Especially, *E. faecium* was emerged as a main causative agents of infection in humans in the 1970s, and, in the past two decades, they spread rapidly worldwide as it developed resistance against important antibiotics such as glycopeptides [34,35]. *Enterococcus faecium*-biofilm positive pathogen naturally produce biofilm exopolysaccharide matrix. The first step of the infection, bacteria adhere to tissue or non-living surfaces with EPS fibrils, and then the biofilm grows [21,36]. It is known that the bacterial EPS is responsible for the formation of the biofilm matrix. Moreover, there are promising studies about EPS in the context of novel antimicrobial and delivery system approaches and strong penetration of antimicrobial agents in biofilms. Today, matrix degradation is achieved by increasing the sensitivity of the remaining biofilm to antimicrobial therapy, especially thanks to EPS targeting strategies [37,38]. In addition to these, the effect of wound healing and antitumor by the EPS produced by *Bacillus licheniformis* PASS26 was reported [39]. Therefore, analysis of EPS structure is extremely important to prevent infection. In this regard, we isolated and analyzed of EPS from *E. faecium* M20. Content of protein, carbohydrate and uronic acid of EPS was determined. According to biochemical analysis, 1 g freeze-dried EPS contained 227.8 mg carbohydrate and 153.7 mg protein. It is well established that the carbohydrate and protein are the main components of the bacterial EPS [18,28,40-42]. Moreover, different bacterial strains may have different protein content in their EPS. For example, the EPS produced by *P. fluorescens* Biovar II (ATCC no. 55421) has less protein content as compared to our EPS material [28]. In addition to carbohydrate and protein, high amount of uronic acid was found in M20's EPS. Its amount varies species to species. For example, the amount of uronic acid in EPS purified from *Pseudomonas fluorescens* Biovar II was found to be high [28]. On the other hand, our previous reports have shown that the amount of uronic acid in EPS extracted from *Bacillus* species is very low [18,42]. Contrary to our previous studies, the amount of uronic acid in EPS of *E. faecium* M20 was found to be quite high (40.5 mg). This result was very close to findings of Hung and coauthors [28]. There is limited information about the content of uronic acid of EPS obtained from pathogen bacteria in the literature. Uronic acids can give unique properties to EPS. For example, bacterial cells are resistant to acid hydrolysis due to presence of high uronic acid. Furthermore, cells can have attached to numerous surfaces including metals by the help of carboxylic groups in uronic acids [43-47]. According to Tsuneda and coauthors (2001), acidic polysaccharides such as uronic acid played a major role in bioaggregation ability of *Nitrosomonas europaea* and bacterial aggregation capacity was influenced in presence of uronic acid [48].

Also, the bacterial EPS rich in uronic acid was resistant to mineralization by microorganisms and had a long residence time in global oceans [49]. Therefore, high content of uronic acid may be one of the factors for progressive infection of these pathogen bacterial strains. But further studies were needed to test this possibility.

HPLC analysis showed that our EPS contains glucose+maltitol, fructose and sorbitol+ksilitol. Some researchers reported that the monosaccharide composition of EPS isolated from different strains were different. For example, the EPS of *Enterococcus faecium* K1 was indicated to consist of mannose, glucose and galactose [50]. FTIR analysis showed that EPS from *E. faecium* strains was homopolymer of glucose [51]. Based on the thin layer chromatography, EPS of *E. faecium* MC13 was found to be a heteropolysaccharide, composed of galactose and glucose sugar units [52]. Moreover, the capsular polysaccharides from *E. faecalis* and *E. faecium* were contained glycerol teichoic acid-like molecules with a carbohydrate backbone structure of 6-a-D-glucose-1-2 glycerol-3-PO4 with substitution on carbon 2 of the glucose with an a-2-1-D-glucose residue [53]. While *Bacillus thermoantarcticus* strain synthesized a sulfated heteropolysaccharide composed exclusively of mannose and glucose [54], the polymer produced by *Bacillus* sp MC6B-22 contained amino sugars and uronic acids [55]. In another study, Gupta and coauthors(2019) reported that the carbohydrate composition and monomeric sugars of EPS from *Cupriavidus* sp. ISTL7 were glucose, xylose, sorbitol and fructose by GC–MS analysis [56]. All of these results revealed that the different EPS from different strains has quite variable sugar composition.

There are many studies about determination of the EPS, there is limited data to characterize the surface of EPS. In the present study, SAXS analysis was performed to find the conformation of EPS of *E. faecium* M20-clinic isolate. Fitting to the analyzed SAXS results showed that the aqueous solution results were consistent with that of serum albumin. Given the aggregation behavior of the M20, a more compact EPS with distinctive core-shell structure and bilayer-thicknesses is formed. In our previous paper, we showed structure of *Bacillus pseudomycoides* U10 EPS with SAXS and reported that the layer thickness of U10 EPS changed under different growth conditions [18].

The bacterial cells were embedded in EPS matrix as a single cell or dense clusters. In addition, it has been determined by SAXS analysis that the EPS matrix is concentrated in a layer around the cells. Also, SEM photomicrographs strongly suggested that M20 strain was able to form EPS fibers and the cells were tightly attached with these fibers to each other. All of these data were consistent to each other. Bhat and Bajaj [50] denoted that the surface morphology of EPS from *E. faecium* K1 was flake like uneven and compact structure without pores.

CONCLUSION

In conclusion, the physicochemical properties of EPS from *Enterococcus faecium* M20 were analyzed by using SAXS for the first time. Results of this study indicated that EPS matter comprised two main substances, mainly polysaccharide and protein in a poorly crystalline state. In addition to these, bacterial cells were embedded in EPS matrix as a single cell or in dense clusters. This structure also was confirmed by SAXS. Also, exopolysaccharide from M20 was also rich by uronic acid. Therefore, it is suggested antibacterial compounds that target acidic polysaccharides such as uronic acid or inhibition of their production may be beneficial to combat bacteria that produce uronic acid-rich biofilm matrix. But further studies will be needed to investigate interaction of bacterial EPS with different biomolecules including human matrix proteins.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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