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RESEARCH

Functional characterization and biotechnological applications of exopolysaccharides produced by newly isolated *Enterococcus hirae* MLG3-25–1

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Abstract
This study investigated the potential applications of *Enterococcus hirae* MLG3-25–1 exopolysaccharides (EPS), with a focus on their isolation, identification, production, and functional characteristics. After the bacterial strain was cultured in De Man–Rogosa–Sharpe (MRS) medium containing 1% glucose at 37 °C, the EPS was refined, and the highest yield of 0.85 mg/mL was achieved at the 24-h incubation period. *Enterococcus hirae* MLG3-25–1 was found to be able to produce EPS. The study explored the microstructure of the EPS, which resembles polysaccharide sheets with smooth surfaces, through scanning electron microscope (SEM) analysis. Through Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) analysis, the chemical composition, aligning with glycosidic bond characteristics, has been deciphered. Furthermore, the antimicrobial and antibiofilm activities against pathogenic bacteria, particularly *Bacillus* sp., demonstrated potential applications in combating antibiotic resistance. The EPS exhibited notable antioxidant activity (89.36% DPPH scavenging), along with high water-holding capacity (575%), emulsifying activity, and flocculation activity, suggesting its potential as a stabilizing agent in the food industry. Overall, this study provides a comprehensive characterization of *Enterococcus hirae* MLG3-25–1 EPS, emphasizing its diverse applications in antimicrobial, antioxidant, and food-related industries. These findings lay the groundwork for further exploration and utilization of this EPS in various sectors.

Keywords *Enterococcus hirae* MLG3-25-1 · Exopolysaccharides · SEM · FT-IR · NMR

Introduction

Exopolysaccharides (EPS) stand out as intricate, long-chain polymers crafted by a variety of microorganisms, capturing attention for their unparalleled physical and chemical attributes (Ruffing and Chen 2006; Angelin and Kavitha 2020). This uniqueness positions them as appealing candidates for a vast array of applications in both industrial and biomedical realms. Beyond their already recognized functional properties—such as viscosity, emulsifying, gelling, and stabilizing capabilities—EPS showcase an additional layer of versatility (Kaur and Dey 2023; Pérez-Ramos et al. 2016). The structural diversity inherent in EPS allows for precise use of their properties to meet the demands of specific applications. These microbial-derived polymers combining monosaccharides and their molecular architecture can undergo manipulation through methods like genetic engineering or fermentation conditions optimization (Andrew and Jayaraman 2020). This diversity grants EPS extraordinary flexibility, facilitating customization for targeted functions across diverse sectors. In the food industry, for instance, EPS finds purpose as a thickening agent, elevating the texture and mouthfeel of products. The pharmaceutical realm benefits from their application in controlled drug delivery systems, leveraging their capacity to encapsulate and shield active compounds (Tabernero and Cardea 2020). Moreover, in the cosmetic industry, EPS plays a pivotal role in formulating stable emulsions and gels, enriching the overall performance of skin care products (Waoo et al. 2023). As ongoing research in this field progresses, the horizon of potential

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applications for exopolysaccharides is poised to expand, promising innovative solutions across a spectrum of scientific and industrial domains.

The study of EPS production by microorganisms has been thorough, with a specific emphasis on bacteria, fungi, and algae (Osemwegie et al. 2020). Lactic acid bacteria (LAB) distinguish themselves as a significant group within the microorganisms involved in EPS production. This category includes well-known strains like *Lactobacillus*, *Streptococcus*, and *Pseudomonas*, each demonstrating the capability to generate EPS (Heredia-Ponce et al. 2020), (Koo et al. 2010, Lee et al. 2022). Among these bacterial strains, *Enterococcus hirae* has emerged as a fascinating subject of study due to its unique EPS-producing abilities (Jia et al. 2022). As a gram-positive bacterium commonly inhabiting the gastrointestinal tract of both humans and animals, *Enterococcus hirae* has been extensively researched for more than just its EPS production (Jayamanohar et al. 2018b).

Enterococcus hirae is renowned for its capacity to produce bacteriocins, which are antimicrobial peptides (P. Sharma et al. 2020a, b). These peptides showcase potential applications in diverse fields, ranging from food preservation to healthcare. The antimicrobial properties of bacteriocins make them valuable for inhibiting the growth of harmful microorganisms, contributing to the extension of the shelf life of food products. Additionally, the healthcare industry explores the use of bacteriocins for their antimicrobial activity against pathogenic bacteria, opening avenues for novel therapeutic interventions (Unban et al. 2022).

The synergy between the beneficial attributes of lactic acid bacteria, exemplified by *Enterococcus hirae*, and the versatile applications of EPS adds a layer of complexity and richness to the potential benefits derived from these microorganisms. As research in this field progresses, the collaborative efforts of LAB and EPS are likely to yield even more innovative solutions, bridging the gap between industrial applications and human well-being.

Enterococcus hirae has also been found to produce EPS with diverse functional and biological properties. The EPS produced by *Enterococcus hirae* has been shown to have high viscosity, emulsifying, and stabilizing abilities, making them attractive for use in the food industry (Jia et al. 2022). Additionally, they have been found to have potential antioxidant, immunomodulatory, and prebiotic effects, which could have applications in the healthcare and biotechnology industries (Kavitake et al. 2024).

Despite the potential industrial and biomedical applications of EPS produced by *Enterococcus hirae*, there is still limited information on their structural, functional, and biological properties. Therefore, a comprehensive characterization of the EPS produced by *Enterococcus hirae* is essential for understanding their potential applications and optimizing their production (Jayamanohar et al. 2018b).

In this research paper, we aimed to provide a comprehensive characterization of the EPS produced by *Enterococcus hirae* MLG3-25–1, isolated from green tea, a specific strain of *Enterococcus hirae*. Here, we investigated the biological properties of these EPS, including their potential antioxidant, antimicrobial, and immunomodulatory effects. Furthermore, the evaluation of their functional properties, including their emulsifying and stabilizing abilities, was done to consider their potential use in the food industry. Overall, this research aimed to provide a thorough understanding of the EPS produced by *Enterococcus hirae* MLG3-25–1 and their potential applications in various industries and healthcare. The results of this study could provide important insights into the optimization of EPS production and the development of novel applications for these complex biomolecules.

Materials and methods

Sample collection

Green tea sample was collected from a super shop in Shahebbazar Rajshahi, Bangladesh, and brought to the Microbiology Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi.

Bacterial isolation

The bacterial isolation process was carried out as per protocols provided by HiMedia laboratories. Five grams of green tea sample was then fermented overnight in water. Following the fermentation process, after vortexing the green tea, MRS media was used to enumerate *Lactobacillus* species. Thereafter, the sample was spread-plated onto MRS agar media and cultured for 48 h at 37 °C. A single colony was then selected and cleaned with a quadrant streak method. After that, the bacterial strains that had been purified were kept in storage at − 80 °C with 50% glycerol added for further experiments (Angmo et al. 2016).

16S rRNA identification

Following the protocol of Naeem et al. (2018), the genomic DNA of the pure bacterial isolate was extracted. The single colony of the bacteria was suspended in 20 µL of TE buffer (Tris EDTA) and processed in a thermocycler at 95 °C for 10 min. At 6000 rpm (2–3 min), the sample was centrifuged, and the resulting supernatant was used as a DNA template. The 16S rRNA present in the extracted template DNA was then amplified. Polymerase chain reaction (PCR) was carried out by using universal reverse and forward primers, namely, 1510R (50-GGCTACCTT GTTACGA-30) and 9F (50-GAGTTTGATCCTGGCTCA

G-30). Conditions for PCR were set as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 1 min and annealing at 50 °C for 1 min, followed by extension at 72 °C for 1.5 min and the final extension at 72 °C for 5 min. After the completion of PCR, the amplified PCR products were sent for 16S rRNA sequencing through a commercial sequencing service of Invent Dhaka, Bangladesh. Using BLAST and the Gene Bank Internet service, it was possible to identify the bacterial strains down to the species level. The 16S rRNA sequence data was uploaded to the GenBank database (https://submit.ncbi.nlm.nih.gov). Then, MEGA-X software was utilized for phylogenetic and molecular evolutionary study.

Extraction and purification of EPS

One liter of MRS broth was prepared and supplemented with 10% glucose for achieving EPS. After sterilizing it at 121 °C and 15 psi pressure for 15 min, 100-µL preculture was added to the media and inoculated in an orbital shaker at 37 °C for 24 h. Following centrifugation of the culture (8000 × g for 20 min at 4 °C), the supernatant was recovered and treated with a concentration of 14% trichloroacetic acid (TCA) to denature the protein composition. The TCA treatment completely removes all proteins and contaminants (Sørensen et al. 2022). The culture was then homogenized in an orbital shaker at 37 °C for 30–40 min before spinning at 8000 × g for 20 min at 4 °C. Then, the supernatant was collected which was deemed as the crude EPS, and cool pure ethanol (twofold of the supernatant volume) was added before incubating at 4 °C for 48 h. Then, the mixture was again centrifuged at 8000 × g rpm for 20 min at 4 °C, and the pellet was collected and then dissolved in deionized water and dialyzed for 24 to 48 h (12–14 kDa). After that, the precipitate was washed with ethanol and dried as desired purified exopolysaccharide (Bajpai et al. 2016). Then, the purified exopolysaccharide was stored at 4 °C for further analysis.

Characterization of EPS

Quantification of EPS

The quantification study of purified EPS from *Enterococcus hirae* MLG3-25–1 was done for different incubation periods of the bacterial culture at 37 °C in an orbital shaker (1, 3, 5, and 7 days). The production procedure of EPS was then completed, and dry weight was determined by using the electric balance (Al-Abbasi 2018).

Surface visualization of the EPS through scanning *electron* microscope (SEM)

SEM was used to examine the microstructure and surface appearance of the EPS at acceleration voltages of 15.0 kV and magnifications of 130, 400, and 1000. Before SEM viewing, the lyophilized EPS sample was attached to the SEM samples with conductive tape and covered with a coating of 10-nm Au (Yang et al. 2018).

Fourier transform infrared (FT-IR) spectroscopy of purified EPS

The structural analysis of purified EPS was investigated using Fourier transform infrared (FT-IR) spectroscopy to identify the spread of functional groups. The EPS of *Enterococcus hirae* MLG3-25–1 dried at 80 °C for 6 h. Then, MGL3 EPS was obtained using the KBr method for the FT-IR spectrum. The polysaccharide samples were mixed into KBr pellets at the sample: KBr ratio 1:400 and heated at 120 °C for 12 h. The Fourier transform infrared spectra were collected on a Bruker Vector 22 device in the region of 4000–400 cm^{-1}, at a resolution of 4 cm^{-1}, and analyzed using Bruker OPUS software (Wang et al. 2010).

NMR analysis

^{1}H NMR spectra analysis of EPS was performed using Bruker AVANCE AV-500 spectrometer (Bruker Group, Fällanden, Switzerland) operated at 400. The purified EPS sample was dissolved in D_2O at 25 °C with concentrations of 5 mg/mL (for ^{1}H NMR). Chemical shifts were expressed in parts per million (ppm) (Choudhuri et al. 2020).

Antibacterial test

Using the agar well diffusion method with some modifications (A. Sharma et al. 2021), the antibacterial potential of the EPS of *Enterococcus hirae* MLG3-25–1 was assessed against three pathogenic bacteria: *Bacillus* sp., *Escherichia coli*, and *Staphylococcus aureus*. Using gentamycin as the baseline medication, the EPS was tested for its generation of antibacterial activity against these microorganisms. The indicator bacteria were planted onto Mueller–Hinton agar plates. Following that, 100 µL of the EPS solution was added to the wells (5 mm) that were created in the agar plates. After that, plates were incubated for 48 h at 37 °C. A distinct zone of inhibition was then measured in millimeters.

Antibiofilm assay

Inhibition of biofilm formation assay

In order to enable the formation of biofilm at the bottom of the wells, 100 µL of the chosen bacterial strains (*Bacillus* sp., *Escherichia coli*, *Staphylococcus aureus*) was allowed to grow on wells of a 96-well microtiter plate (Tarsons, India) filled with 100 µL of Luria–Bertani (LB) liquid medium. The plate was then incubated at 37 °C for 24 h without shaking. Following the incubation period, the plate underwent two rinses with double distilled water, air drying, and oven drying for 60 min at 37 °C. Following washing, the biofilm was dyed for 15 min with 200 µL of crystal violet (0.1%). After that, 150 µL of 100% ethanol was used to fix the biofilm for 5 min. In the end, a measurement at 595 nm was used to quantify 100 µL of the dilution (Ga 2011). The co-incubation approach, which followed the previously outlined protocol, was used to determine the impact of the bacteria's MLG3-25–1 EPS on the production of biofilms. The bacterial inoculum was introduced to 100 µL of EPS solution. The formula used to compute the biofilm disruption percentage was as follows.

$$Disruption\ percentage = (OD\ control - OD\ sample) \times 100/control$$

Visualization of disruption of biofilm formation through scanning *electron* microscope (SEM)

Bacteria were cultivated in an incubator for a 24-h period while they were let to grow on glass slides (5 × 5 mm) and co-incubated with the EPS, as per the previously presented methodology. Biofilms produced without the presence of EPS were used as a control. Following the incubation period, three 0.1% PBS washes were performed on each test sample. After that, 2.5% glutaraldehyde was used for fixation, which was done for 30 min at room temperature and then overnight incubation. Before they were dehydrated, the slides were cleaned three more times using PBS. Several ethanol volumes were used to dehydrate the sample, starting at 30%, 50%, 70%, 80%, and 100%, and each volume was incubated for 10 min. Next, the material is incubated for 1 h at 100% ethanol. Next, the adhesive tape is applied to the SEM stub, and the bacterial sample is added to the tape to visualize the disruption of the biofilm (Ammar 2017).

Antioxidant test

To test for antioxidant activity, 2.5 mg of the lyophilized crude EPS of *Enterococcus hirae* MLG3-25–1 was then reconstituted in 1 mL of sterile distilled water, filtered using a Millipore filter, and then used in the DPPH radical scavenging experiment. After combining 2 mL of the composition with 2 mL of freshly prepared DPPH solution, the mixture was left at room temperature and dark for 30 min. At 517 nm, the absorption was detected. As a control, ascorbic acid was employed. The following equation was used to compute the scavenging activity (Ansari et al. 2022). The following equation was used to compute the scavenging activity.

Using a GENSYS 10S UV–VIS Thermo scientific spectrophotometer, the OD value was determined. The percentages of antioxidants were determined using the equation.

$$Scavenging\ rate\ (\%) = 1 - (A_{sample} - A_{blank})A_{control} \times 100\%$$

Water-holding capacity

The water-holding capacity (WHC) of purified EPS was investigated. Initially, an EPS solution was prepared for this test by immersing a 0.04-g sample in 2 mL of deionized water and vortexing the mixture for 2 min. The mixture was centrifuged for 25 min at 10,000 rpm after that. Water that was unbound and not confined by EPS was disposed of. Pre-weighted filter paper was used to hold the entire EPS material in order to guarantee complete water drainage. In this way, the amount of EPS that precipitated was calculated (K. Sharma et al. 2020a, b). The percentage of WHC was calculated using the following formula:

$$WHC\ (\%) = total\ sample\ weight\ after\ water\ absorption \times 100 / weight\ of\ sample\ (dry\ weight\ basis) \times 100$$

Emulsification activity test

The following procedure was applied for two distinct EPS solution concentrations and two distinct types of oil in order to calculate the emulsifying activity (EA). In a glass tube, 2 mL each of olive and soybean oil (1% and 1.5% w/v) and 2 mL of EPS solution were combined. Following a 2-min vortex, the mixture was kept at 25 °C for the next 1, 24, and 48 h. Next, the following formula was used to determine EA_1, EA_{24}, and EA_{48}.

$$EA\% = E_1/E_0 \times 100$$

where E_0 represented the mixture's full height and E_1 the emulsifying layer's height (Ye et al. 2018).

Flocculation activity

In order to determine the flocculation activity of the EPS, 8 mL of activated charcoal (6 mg/mL) and 0.5 mL of calcium chloride solution (7.0 mM) were combined with EPS

samples (0.2–1.0 mg/mL). Following a 2-min vortex, the reaction mixture was allowed to sit at room temperature (25 °C) for a duration of 10 min. Following the incubation time, 1 mL of the mixture from the upper portion was taken, and the absorbance at 550 nm was determined (Ali et al. 2022).

$$Flocculating\ activity(\%) = (blank - sample/blank) \times 100$$

Results

Bacterial isolation and molecular identification

The LAB was first cultured using Selective De Man, Rogosa, and Sharpe (MRS), which was also utilized as a master plate. The pure isolate of the LAB strain was chosen based on the plate's colony characteristics. Following that, the molecular identification of the isolated pure culture was done using the 16S rRNA gene sequence analysis (Sanjay et al. 2020).

The isolated bacterium's 16S rRNA gene sequence exhibited 96.91% similarity with *Enterococcus hirae* MLG3-25–1 against 16S rRNA gene sequences of other organisms that had previously been uploaded to the NCBI, and the isolate was identified as *Enterococcus hirae* MLG3-25–1 (Fig. 1).

Extraction and quantification of EPS

At first, *Enterococcus hirae* MLG3-25–1 yielded more EPS than other strains. Consequently, the MLG3-25–1 strain was chosen for additional study. The strain MLG3-25–1 dry EPS yield under early incubation conditions was determined to be 0.85 mg/mL (Table 1). To increase EPS production, the impact of different fermentation times—such as 1, 3, 5, and 7 days—on EPS generation was studied.

Table 1 Effect of incubation periods on EPS production

Time	Weight (mg/mL)
1 day	0.85 ± 0.1
3 days	0.79 ± 0.08
5 days	0.62 ± 0.06
7 days	0.43 ± 0.02

Structural and functional characteristics of EPS

Scanning *electron* microscopic (SEM) analysis

SEM was used to examine the microstructure and surface appearance of the EPS that was produced from *Enterococcus hirae* MLG3-25–1. The EPS revealed that the irregular structure mimics sheets of polysaccharide overlaid with some dispersed pieces and appeared as smooth surfaces with pores under 5000 magnifications, showing sheet and compact structure (Fig. 2).

FT-IR analysis

The FT-IR analysis results are displayed in Fig. 3. The peaks display several compounds (Table 3). The peak as attributed at 812.15 cm^{-1} and at 1065.89 cm^{-1} reflected the presence of α- and β-configurations of mono-sugars (Kozarski et al. 2011). The band at 1240.40 cm^{-1} was considered to be an O–H bond (Wang et al. 2012). The peak at 1414.21 cm^{-1} appeared as a C–H stretching vibration (Mathivanan et al. 2021). The peak at 1645.60 cm^{-1} could be attributed to the vibration of the C = O groups (López-Ortega et al. 2020). The stretching vibration of C–H was assigned as a peak signal at 2929.29 cm^{-1} (Kavita et al. 2011). The broad

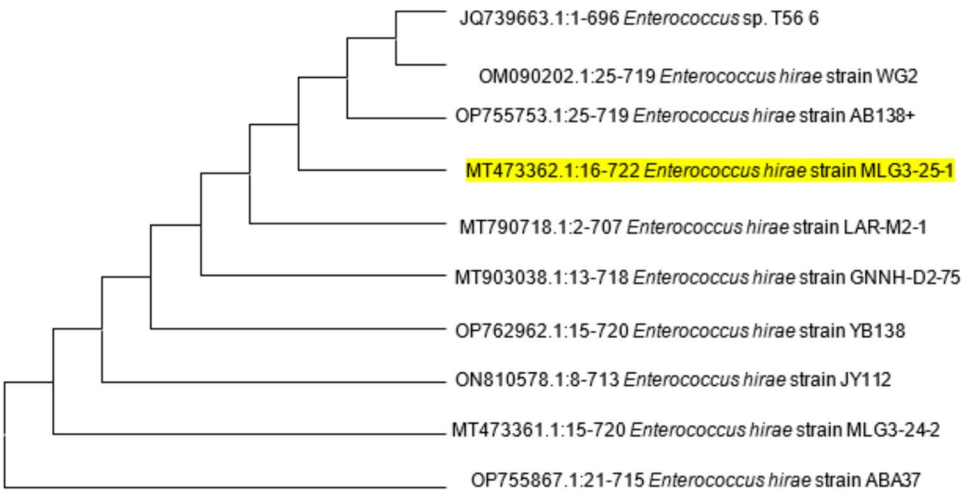

Fig. 1 Phylogenetic tree of *Enterococcus hirae strain* MLG3-25–1

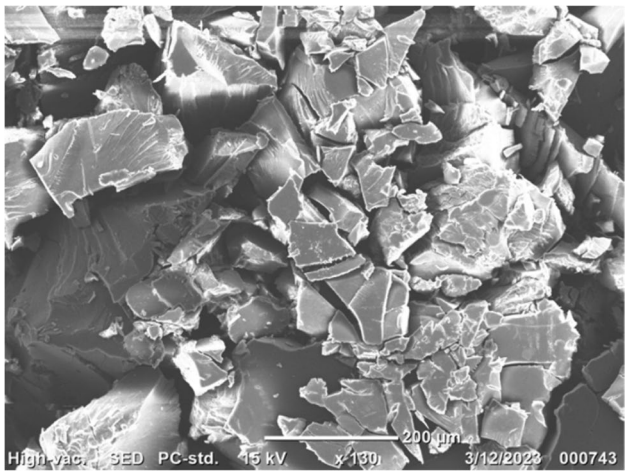

Fig. 2 SEM image showing the exterior morphology of the EPS produced from *Enterococcus hirae* MLG3-25–1

spectrum at 3429.47 cm^{-1} was deliberated to a wide number of stretching O–H groups in the EPS-MLG3-25–1 structure (Sardari et al. 2017).

NMR analysis

The ^{1}H NMR spectrum of the EPS was analyzed, revealing distinct signals indicative of its structural composition. The 500 MHz ^{1}H NMR spectrum of EPS showed 14 signals at the anomeric region. The signals were assigned as 1 to 14 in ascending order of chemical shift 3.263, 3.584, 3.683, 3.822, 3.948, 3.990, 4.037, 4.140, 4.698, 4.968, 5.017, 5.031, and 5.215 ppm (Fig. 4). The ^{1}H NMR analysis of the EPS identified β-anomeric sugar residues for signals 1–9 and α-anomeric sugars for signals 10–12. The presence of fucose was confirmed by a δ 1.21 shift for $–CH_3$. The ^{1}H NMR analysis of EPS offers valuable information regarding its chemical structure.

Antimicrobial activity test

The *Enterococcus hirae* MLG3-25–1 EPS exhibited varying degrees of antibacterial efficacy against three pathogenic bacteria. The most vulnerable species to *Enterococcus hirae* MLG3-25–1 EPS was discovered to be *Bacillus* sp., with a zone of inhibition of 11.33 mm in diameter, significantly greater ($P < 0.05$) than other species. *S. aureus* and *Escherichia coli* were next, measuring 10.66 mm and 8.33 mm, respectively (Fig. 5).

Antibiofilm test

Biofilm formation assay

One of the primary reasons for antibiotic resistance is the development of biofilms. The microbes were all capable of forming biofilms. Table 2 shows the biofilm-forming ability of the three selected bacteria.

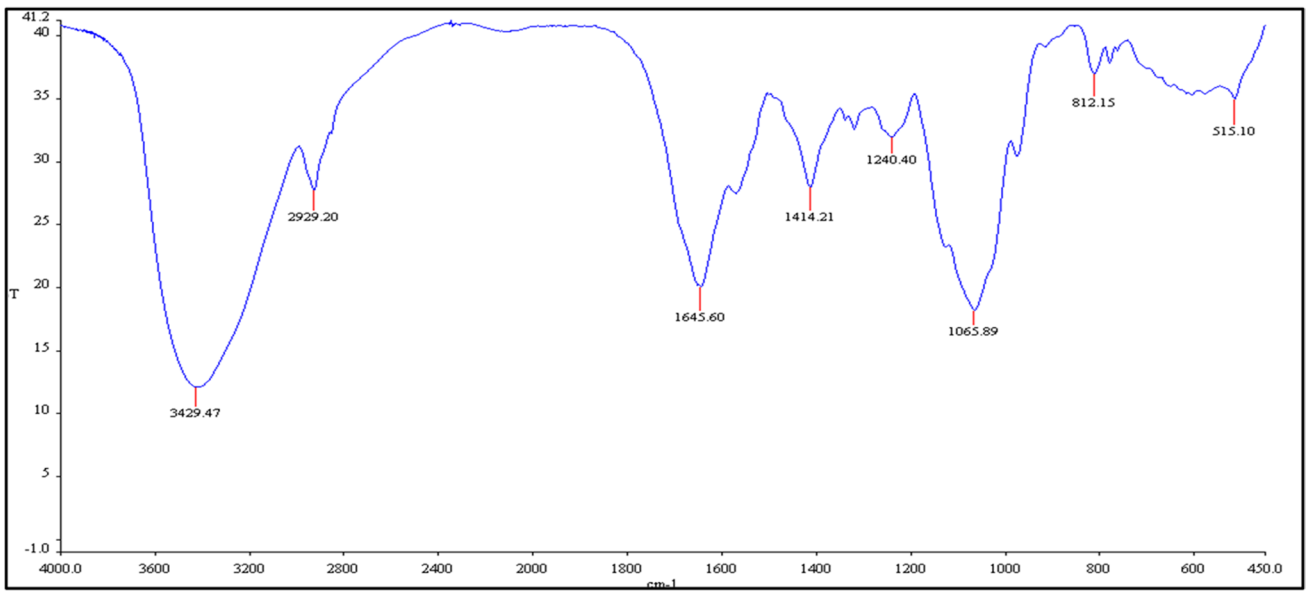

Fig. 3 The pictorial diagram of FT-IR analysis of the exopolysaccharide produced from *Enterococcus hirae* MLG3-25–1

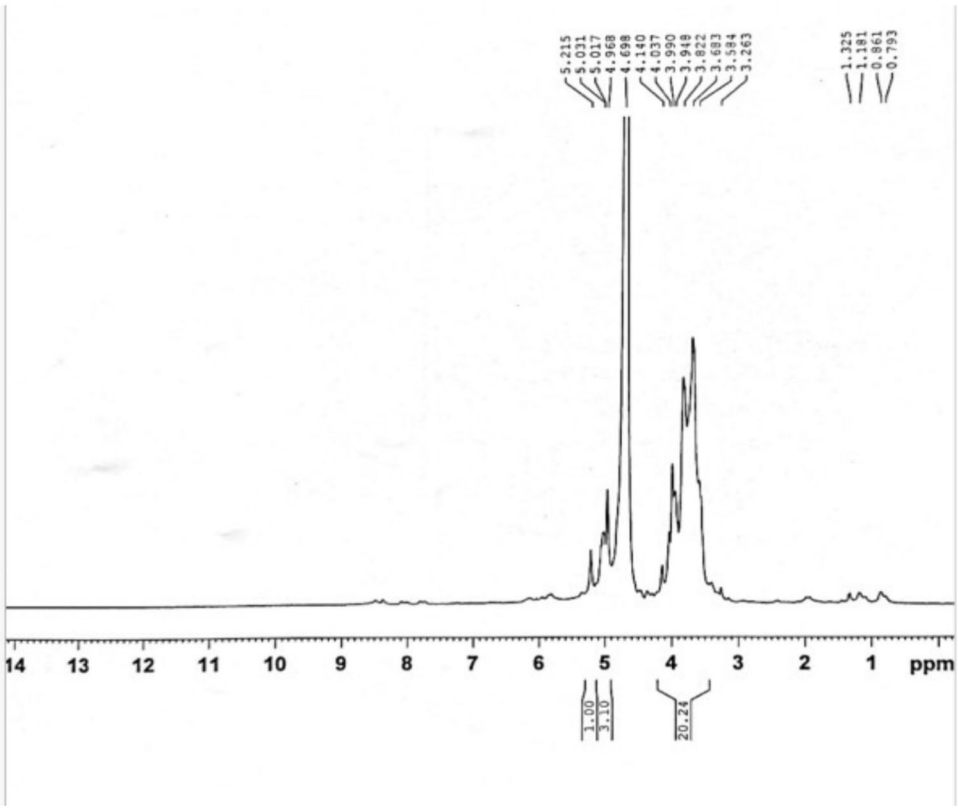

Fig. 4 The pictorial diagram of NMR analysis of the *Enterococcus hirae* MLG3-25–1 EPS

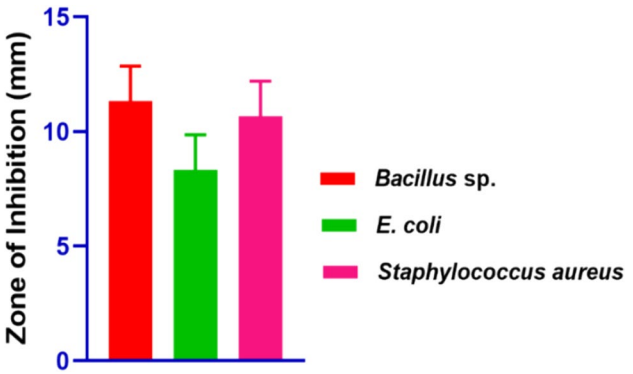

Fig. 5 Antimicrobial activity test of the bacterial strain against three selected bacteria

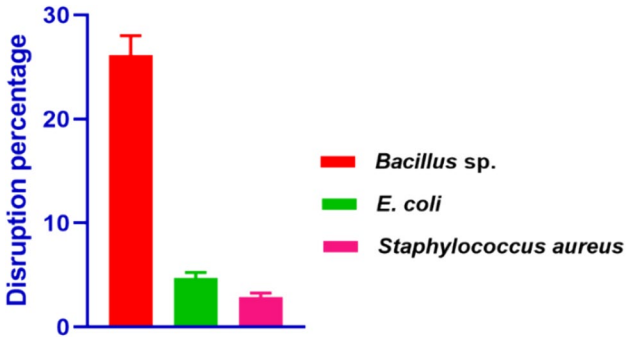

Fig. 6 Biofilm Inhibition by the EPS of *Enterococcus hirae* MLG3-25–1

Table 2 The biofilm formation efficacy of the selected bacterial strains

Name of the bacteria	Range	Result
Bacillus sp.	OD > 4 × OD cutoff	Strong
Escherichia coli	OD > 4 × OD cutoff	Strong
Staphylococcus aureus	OD > 4 × OD cutoff	Strong

Biofilm inhibition by the EPS of *Enterococcus* hirae MLG3-25–1

The findings showed that strains treated with EPS greatly decreased the production of biofilms, whereas the least antibiofilm activity was shown against *Staphylococcus aureus*, at about 2%. There was a notable 27% inhibition of *Bacillus* sp. adhesion to the microtiter plates. Figure 6 displays the EPS's capacity to suppress biofilm formation against pathogenic bacteria.

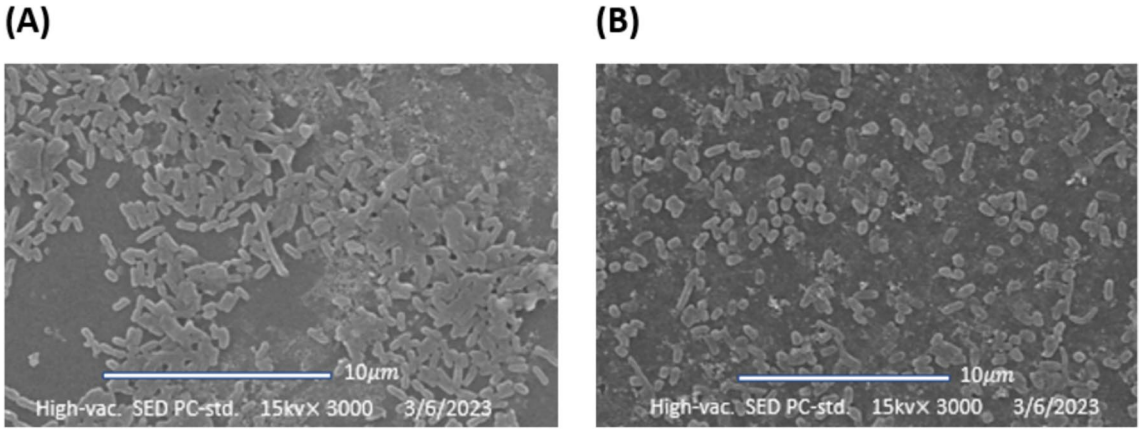

Fig. 7 Scanning electron micrographs. **A** Biofilm formed by *Bacillus* sp. **B** Biofilm disruption by co-incubation with *Bacillus* sp. and the EPS of *Enterococcus hirae* MLG3-25–1

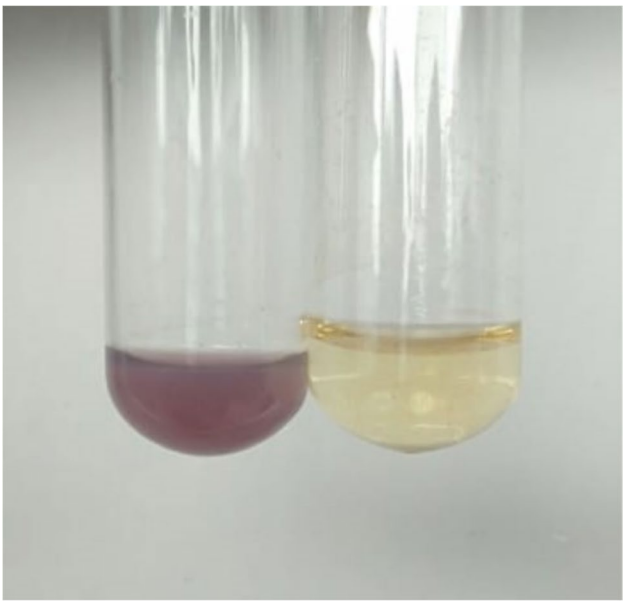

Fig. 8 Antioxidant activity test

Table 3 WHC test result for the isolate

Properties	Percentage of activity
WHC	575

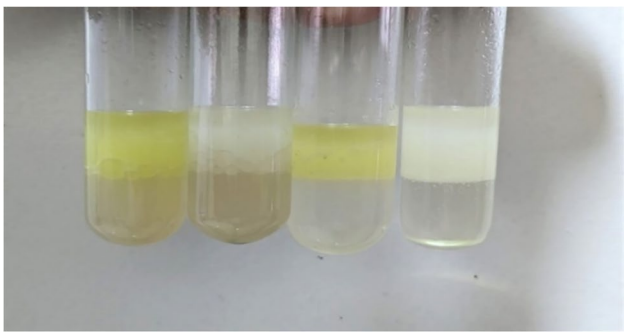

Fig. 9 Results of the emulsification index test

Visualization of disruption of biofilm formation through scanning *electron* microscope

Biofilm formed by *Bacillus* sp. showed a clustered structure (Fig. 7A). On the contrary, the biofilm structure was broken down by co-incubating *Bacillus* sp. with the EPS of *Enterococcus hirae* MLG3-25–1 (Fig. 7B).

Antioxidant activity test

For the EPS of *Enterococcus hirae* MLG3-25–1, an antioxidant test was performed. According to the results, the DPPH scavenging activity of the EPS was 89.36% (Fig. 8).

Water-holding capacity (WHC)

The *Enterococcus hirae* MLG3-25–1 EPS showed a WHC of 575% WHC. Results are given in Table 3.

Emulsifying activity

An efficient emulsifier should be able to retain at least 50% of an emulsion's primal capacity for 24 h after creation. The two tested concentrations (1.5% w/v and 1% w/v) of olive oil showed better results than soybean oil. The percentage

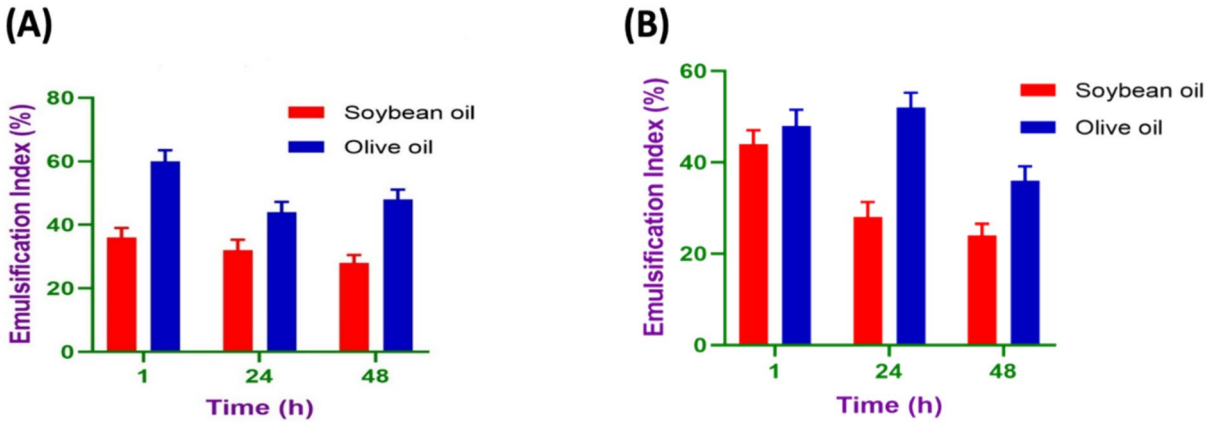

Fig. 10 The graphical presentation of the emulsification index test result (1.5% w/v) (**A**) and (1% w/v) (**B**)

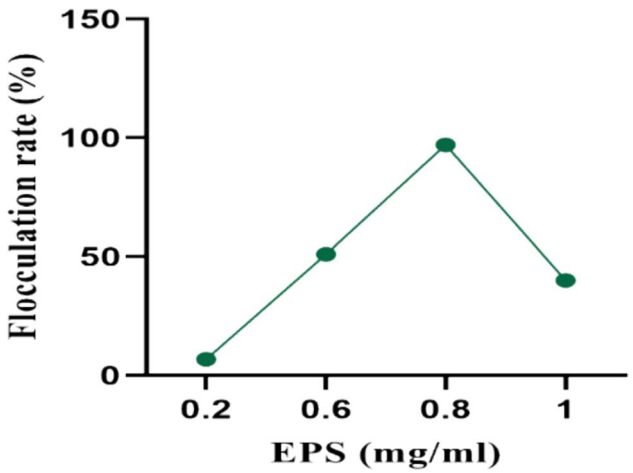

Fig. 11 Flocculation capacity of the *Enterococcus hirae* MLG3-25–1 EPS in four different concentrations

activity of the emulsification index test is presented in Figs. 9 and 10.

Flocculation activity test

The percentage of flocculation activity showed 6.8%, 51%, 97%, and 40% for 0.2, 0.6, 0.8, and 1.0 mg/mL of EPS samples respectively. This data revealed a first increase to fall pattern in the region of 0.2–1.0 mg/mL. The peak flocculation rate of the EPS occurred at 0.8 mg/mL (Fig. 11).

Discussion

There is a growing interest among researchers to explore novel bacterial exopolysaccharides (EPSs). This is because several microbial EPSs, including xanthan, sphingans, and cellulose, have gained commercial significance. Some of the bacterial EPSs mentioned above are used in various medicinal applications, such as antibacterial and tissue engineering composites or frameworks, due to their biocompatibility and apparent non-toxicity (Dwivedi 2018). Lactic acid bacteria, particularly *Enterococcus* sp., produce EPS, biomolecules with unique properties. These EPS exhibit various functional and biological characteristics, including antioxidant, antibacterial, anticancer, and prebiotic potentials (Ozturkoglu-Budak et al. 2023). The structural and thermal properties of *Enterococcus* EPS are studied using different techniques. The diverse applications of *Enterococcus* EPS in food, pharmaceuticals, biomedical, and environmental fields make them valuable for commercial use (Kavitake et al. 2023).

The presented findings unveil a comprehensive exploration of the characteristics and potential applications of *Enterococcus hirae* MLG3-25–1. The choice of LAB isolation was driven by their diverse applications, particularly in the food industry. Molecular identification through 16S rRNA sequencing established the strain's identity as *Enterococcus hirae* MLG3-25–1, demonstrating a 96.91% sequence identity with known entries in the NCBI GenBank database. The construction of a phylogenetic tree further solidified the bacterial isolate's taxonomic position.

EPS production has been observed to be affected by the incubation period (Chug et al. 2021). The EPS production capabilities of *Enterococcus hirae* MLG3-25–1 were a crucial point of this investigation. The observed variation in EPS output over different time intervals highlighted the dynamic nature of EPS production under different incubation conditions. The highest yield was recorded on the first day of incubation (24 h), suggesting an early optimal harvesting time followed by a death phase that causes a drop in the EPS production (Oleksy-Sobczak et al. 2020).

SEM is recognized as a potent instrument for investigating the morphological characteristics of polysaccharides and can be employed to unveil their physical properties (Lei et al. 2019). The EPS derived from *Enterococcus hirae* MLG3-25–1 exhibited a surface characterized by smooth cubes. Moreover, SEM analysis revealed that the EPS comprised a uniform matrix. Our result is in agreement with the EPS structure of *Lactobacillus paraplantarum* KM1 (K. Sharma et al. 2020a, b). Additionally, our EPS was observed to have a compact porous structure, which is consistent with the findings of *Enterococcus hirae* KX577639 (Jayamanohar et al. 2018a). Numerous studies have demonstrated that the compact and porous nature of EPS can enhance the physical and pseudoplastic properties of food applications (Ahmed et al. 2013; Saravanan and Shetty 2016; Wang et al. 2010).

A useful tool for characterizing the functional classes of biological macromolecules is FT-IR. FT-IR analysis delved into the chemical composition of the EPS, revealing characteristic peaks corresponding to functional groups such as O–H stretching, C = O stretching, and glycosidic bonds. Our result matches with the recent findings of LAB strains (Jia et al. 2022) (Ermiş et al. 2020). These insights provide valuable information for understanding the structural properties of the EPS. The chemical structure of EPS can be better understood by examining its ^{1}H NMR analysis. The ^{1}H NMR analysis of EPS offers valuable information regarding its chemical structure, facilitating further studies into its properties and potential applications in various fields. β-Anomeric sugar residues were detected in signals 1–9 and α-anomeric sugars in signals 10–12 by ^{1}H NMR analysis of the EPS. A δ 1.21 shift for $–CH_3$ indicated the presence of fucose. These findings are in accordance with standard literature (Agrawal 1992; Sørensen et al. 2022). The strong signal detected at 4.9 was identified as α-(1 → 6) glycosidic branching links, which are identical to the moieties found in *Enterococcus hirae* KX577639 EPS (Jayamanohar et al. 2018a). Additionally, similar results were found in *Lactobacillus plantarum* DM5 (Das and Goyal 2014) and *Leuconostoc lactis* (Saravanan and Shetty 2016). In terms of food, the antimicrobial activity of strains of *Enterococcus* spp. is crucial. *Enterococci* bacteria produce a number of distinct antimicrobial agents, including hydrogen peroxide (O'Hanlon et al. 2011), lactic acid (Sun et al. 2015), bacteriocins (Avnİ Kırmacı et al. 2016), and bacteriocin-like compounds (Valyshev 2014). The antimicrobial and antibiofilm activities of the EPS were assessed against pathogenic bacteria. Notably, *Bacillus* sp. exhibited high susceptibility to EPS, indicating its potential as an antimicrobial agent. Furthermore, the EPS demonstrated biofilm inhibition against selected bacterial strains especially against *Bacillus* sp., presenting an avenue for combating biofilm-associated antibiotic resistance. As no antimicrobial and antibiofilm effect of our strain is not available, it was not possible to compare. However, several strains of *Enterococcus* demonstrated antimicrobial and antibiofilm efficacy against *Bacillus* sp. along with *E. coli* and *S. aureus* (Hajikhani et al. 2021; Kanmani et al. 2013). The antioxidant activity of *Enterococcus hirae* MLG3-25–1 EPS showcased a significant DPPH scavenging percentage of 89.36%. However, no result is available for this strain but the bacteria from a similar genus *Enterococcus faecium* WEFA23 (Jia et al. 2019) and *Enterococcus faecalis* NOC219 (Özdemir 2023) showed similar results to us justifying our study. Furthermore, this antioxidant potential positions the EPS as a promising candidate for applications in functional foods and nutraceuticals.

Microbial polymers are gaining prominence as stabilizing agents in the food industry because they exhibit key functional qualities, specifically a high solubility index and the ability to hold onto water effectively (Jayamanohar et al. 2018b). The functional properties of the EPS were further evaluated through water-holding capacity (WHC) and emulsifying activity tests. The remarkable WHC of 575% highlights its capability as a water-retaining agent, while the efficient emulsifying activity indicates its potential use in emulsion-based applications in industries. *Enterococcus hirae* KX577639 from feces showed 202.04% holding capacity (Jayamanohar et al. 2018b), and in another study, *Enterococcus* sp. showed 882.5% water-holding efficacy (Jiang et al. 2021). Overall, in this study, the novel exopolysaccharides (EPS) produced by *Enterococcus hirae* MLG3-25–1 have significant functional properties and biological activities. This makes EPS a valuable candidate for various industrial applications, especially in the food and nutraceutical sectors.

Conclusion

In conclusion, *Enterococcus hirae* MLG3-25–1 was isolated and evaluated to produce exopolysaccharides, and various physiological and biological parameters of the isolated EPS were investigated. Results revealed that *Enterococcus hirae* MLG3-25–1 exhibited good emulsifying properties and water-holding capacity. Moreover, the antimicrobial and antibiofilm capacities of the EPS sample showed potential activity against *Bacillus* sp. These characteristics make *Enterococcus hirae* MLG3-25–1 a potential compound for food industries. However, some in vivo studies are needed to examine its potential use in human health.

Author contribution MMM: Designed the research, performed all experiments and drafted original manuscript; FSS: Performed experiment and drafted manuscript; SI: Performed experiment and analysed data; SZ: Provided resources, reviewed the script; MSU: Provided

resources, edited the script; MAS: Designed the research, reviewed and edited the script and supervised the whole work.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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