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ORIGINAL PAPER

Production of a high molecular weight levan by *Bacillus paralicheniformis*, an industrially and agriculturally important isolate from the buffalo grass rhizosphere

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Abstract A new exopolysaccharide (EPS) producing Gram-positive bacterium was isolated from the rhizosphere of *Bouteloua dactyloides* (buffalo grass) and its EPS product was structurally characterized. The isolate, designated as LB1-1A, was identified as *Bacillus paralicheniformis* based on 16S rRNA gene sequence and phylogenetic tree analysis. The EPS produced by LB1-1A was identified as a levan, having β(2→6) linked backbone with β(2→1) linkages at the branch points (4.66%). The isolate LB1-1A yielded large amount (~42 g/l) of levan having high weight average molecular weight (*Mw*) of 5.517×10^7 Da. The relatively low degree of branching and high molecular weight of this levan makes *B. paralicheniformis* LB1-1A a promising candidate for industrial applications.

Keywords *Bacillus paralicheniformis* · Exopolysaccharide · Fructan · Fructooligosaccharide · Levan

Introduction

Bacillus paralicheniformis is emerging as an industrially important bacterium due to its potential for being used as a probiotic bacterium (Makled et al. 2019; Xiao et al. 2019; Zhao et al. 2020), as a biopreservative due to the production of antimicrobial compounds (Ahire et al. 2020) and for plant

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growth-promoting properties and control of pathogens in plants (Valenzuela-Ruiz et al. 2019; Wang et al. 2017). Though several reports are available on antimicrobials and plant growth properties of *B. paralicheniformis*, to date no data are available on fructan production by this industrially and agriculturally important bacterium.

Microbial fructans are fructose-based exopolysaccharides (EPS) further classified as inulin and levan based on the glycosidic linkages in the main chain. In inulin, the fructose units are linked through β(2→1) glycosidic bonds. In levan, the fructose units are predominantly connected by β(2→6) glycosidic bonds and some are linked by β(2→1) glycosidic bonds at branch points. The amount of β(2→1) linkages may vary among different levan structures (Venugopal 2011). Fructans are produced by specific organisms belonging to all three domains of life including bacteria, fungi, plants, and archaea (Kırtel et al. 2019). Microbial levan molecules generally have higher molecular weights than plants, and applications of levan are highly dependent on its molecular weight (Öner et al. 2016; Ortiz-Soto et al. 2019). Additionally, the time required for EPS production and the high cost of downstream processing for plant and algae predisposes the levan industry towards microbial production systems.

Levan finds a wide range of applications in many industrial fields such as in detergents, brewing, downstream processing, cosmetics, encapsulating agents, flavor enhancers, stabilizing agents, and wastewater treatments (Öner et al. 2016). In addition, levan is associated with a plethora of health benefits such as maintenance of gut health and prevention of chronic lifestyle diseases. It also has immunological, antioxidant, anti-carcinogenic and mineral absorption properties and is involved in the lipid regulation, which significantly contribute to its prebiotic potentials (Adamberg et al. 2015; Al-Sheraji et al. 2013; Liu et al. 2010; Peshev and Van den Ende 2014; Srikanth et al. 2015). Moreover, fructooligosaccharides (FOSs; low molecular weight fructan oligomers having 2–10 fructosyl residues) can be fermented by most of the known bifidobacterial strains that use them as carbon sources (Rossi et al. 2005).These FOSs are widely regarded as highly butyrogenic fibers (Tuncil et al. 2017).

Levans produced by rhizosphere bacteria can alter the physical and chemical properties of the root-adhering soil. For instance, they assist in the significant aggregation and stabilization of the rhizosphere (Amellal et al. 1999) and help in creating a microaerobic environment for nitrogen fixation by providing a barrier for oxygen diffusion (Velázquez-Hernández et al. 2011). It has been demonstrated that levan biofilm matrices act as extracellular nutrient reservoirs for *Bacillus subtilis* under starvation conditions, protect the bacteria from desiccation, and shield the community from predatory organisms (Dogsa et al. 2013). Exploration of levan production in rhizosphere bacteria would generate more knowledge in this direction.

The emerging plethora of applications in different sectors makes levan an important industrial commodity. However, the cost of producing levan through microbial culture would be very high with complex downstream process, if its yield is low. Therefore, screening of high yielding strains would be important for its exploitation at industrial scale. Here, we report the production of large amount of a high molecular weight levan by a *B. paralicheniformis* strain LB1-1A isolated from *Bouteloua dactyloides* (buffalo grass) rhizosphere. The outcome of this study will not only enhance the understanding about the mechanism of biofilm formation and root colonization of this bacterium but also render a new source of levan production for commercial exploitation.

Materials and methods

Isolation of levan producing bacteria

A soil sample was collected from the rhizosphere of *Bouteloua dactyloides* (commonly known as buffalo grass) grown in a garden of NIGBE Faisalabad, Pakistan. Rhizosphere soil was aseptically transferred into the saline water (0.85 g NaCl in 100 ml dH_2O). Serial dilutions and streak plate methods were used repeatedly for obtaining single colonies of pure cultures of EPS-producing bacteria on LB (NaCl, 10 g/l; yeast extract, 5 g/l; tryptone 10 g/l) and LB-agar media supplemented with sucrose (20 g/l) as a carbon source. These LB-agar-sucrose plates were incubated at 37 °C and observed for the appearance of mucoid colonies. The colonies were aseptically transferred to test tubes containing LB broth supplemented with sucrose (20 g/l). These test tubes were incubated

under aerobic conditions at 37 °C for 24 h to obtain the growth of bacteria for preservation of the cultures and genomic DNA extraction for molecular identification. PCR amplification with primers PHr (5′-TGC GGCTGGATCACCTCCTT-3′) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) followed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) was applied to distinguish different strains of the same species using the method described by Idris et al (2004) and Xu et al (2015) (data not shown). Consequently, two EPS producing bacterial strains were screened out. Based on its capability to produce large amount of levan, one bacterial isolate, designated as LB1-1A, was selected for the present study. Salt tolerance of the isolate LB1-1A was also checked by growing it on LB medium containing different sodium chloride concentrations (0–10%).

Identification and phylogenetic analysis of isolate LB1-1A

The isolate was observed under the phase contrast microscope for cell morphology.

The isolate LB1-1A was phylogenetically placed using its full length 16S rRNA gene sequence. For this purpose, its genomic DNA was extracted using Thermo Scientific GeneJET Genomic DNA Purification Kit (#K0721) following the supplier's instructions. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using DreamTaq Green PCR Master Mix (#K9021) and universal primers FD1 [5′-AGAGTTTGATCCTGGCTCAG-3′] and rP1 [5′ -ACGG(ACT)TACCTTGTTACGACTT-3′] (Akhtar et al. 2008). The PCR product (~1500 bp) was purified using Thermo Scientific™ GeneJet PCR Purification Kit (#K0701), cloned in pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Thermo Scientific™) and sequenced commercially by Macrogen, Inc. (Seoul, Korea). The obtained sequence was analyzed using nucleotide BLAST available in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic relationship of LB1-1A with other related bacteria was inferred by phylogenetic tree constructed on the basis of full length 16S rRNA gene sequences using Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker 2019), as described previously (Meier-Kolthoff et al 2014).

Production, isolation and purification of EPS

LB broth supplemented with 20% (w/v) sucrose was prepared and sterilized by autoclaving as already described above. The autoclaved broth was inoculated with 5% (v/v) of the inoculum and incubated at 37 °C for 48 h in a rotatory shaker at 100 rpm. At the end of the fermentation time, the culture was centrifuged at 8000 × *g* for 15 min and the supernatant was collected. Trichloroacetic acid (14% w/v) was added to the supernatant followed by incubation at 23 °C for 40 min (50 rpm) and then centrifugation at 4 °C for 10 min to deproteinate the solution (Abid et al. 2019). EPS was then precipitated by adding 2 volumes of ice cold absolute ethanol and collected by centrifugation at 6000 × *g* for 10 min. To remove any residual sucrose, the EPS pellet was washed twice by re-dissolving in distilled water, precipitation by ethanol, and centrifugation as mentioned above. The final product was dissolved in distilled water and freeze-dried to obtain purified EPS.

Thin-layer chromatography (TLC) of the EPS product

EPS in the fermented broth was identified by spot test on a silica gel 60 plate (F_{254}; Merck) as described previously by Anwar et al (2010). Briefly, 2 µL of supernatant was analyzed on TLC plate using butanol/ethanol/water (5:5:3) as a mobile phase and run in a TLC tank for 6 h. The mobile phase on the TLC plate was evaporated under warm air for 10 min. Carbohydrate spots on the chromatogram were visualized by spraying a mixture of urea developing solution (100 ml water-saturated butanol, 3.0 g urea, 5.9 ml phosphoric acid, 5 ml ethanol) and developing in a heating incubator at 120 °C for 15 min. A mixture of sucrose, 1-kestose, and nystose was used as standard.

Sugar composition analysis

For sugar analysis, the purified EPS product (10 mg/ml) was hydrolysed with 2 M trifluoroacetic acid (TFA) at 50 °C for 30 min (Xu et al. 2016). The hydrolysis reaction was stopped by the addition of barium carbonate ($BaCO_3$) powder in excess amount (Zhang et al. 2014) when the pH of the reaction mixture was adjusted to 7. The hydrolysed product was filtered through a 0.22 µm membrane and analyzed by high-performance liquid chromatography (HPLC)

fitted with Bio-Rad Aminex HPX-87P ion exclusion column (Bio-Rad, Hercules, CA) and coupled with a refractive index detector (Model 2414, Waters Corporation, Milford, MA). The HPLC samples were eluted with B-pure purified water at 85 °C with a flow rate of 0.6 ml/min according to Chen et al (2013).

^{13}C-NMR spectrometry of the fructan product

To determine the linkage type of isolated EPS, a lyophilized sample was dissolved in dimethyl sulfoxide (DMSO) and the spectrum was recorded on a Bruker Avance AV-III 300 MHz spectrometer at 25 °C. The experiment was carried out at an operating frequency of 75.47 MHz and chemical shifts were expressed in parts per million (ppm) with respect to the DMSO peak (39.51 ppm).

High-pressure anion-exchange chromatography (HPAEC)

Isolate LB1-1A was cultured in LB medium supplemented with 200 g/l sucrose and incubated at 37 °C for 48 h, as described above. The supernatant was collected and separation of FOSs was carried out by HPAEC with Integrated Pulsed Amperometric Detection (HPAEC-IPAD) as previously described (Vergauwen et al. 2000). Identification of carbohydrates was performed using D-glucose, D-fructose, sucrose, 1-kestose, 6-kestose, neo-kestose, maltose, maltotriose and nystose as standards.

Linkage analysis

The EPS sample was derivatized to its partially methylated alditol acetates (PMAA) following the method described by Nasir et al (2020). Briefly, the lyophilized sample was methylated with iodomethane and hydrolysed using trifluoracetic acid. Reduction of the hydrolysed residues was performed with sodium borodeuteride ($NaBD_4$) in ammonia water and partially methylated alditol residues were acetylated using Ac_2O. Myo-inositol was used as an internal standard. Finally, the derivatized EPS sample was dissolved in acetone and analyzed by gas chromatography-mass spectrometry. The PMAAs were identified by comparing the results of MS fragments with the published literature (Gojgic-Cvijovic et al. 2019; Nasir et al. 2020).

Molecular weight determination

The molecular weight of EPS was determined by gel permeation chromatography coupled with a multi-angle laser light scattering detector (MALLS–GPC) according to the method previously described (Erkorkmaz et al. 2018) with slight modifications. Briefly, the chromatography system was equipped with an ultra-hydrogel linear (0.78 × 30 cm, Waters) column and analysis was performed at 22 °C. The mobile phase was composed of 0.1 M of $NaNO_3$ in a 2% (v/v) acetic acid aqueous solution and it was supplied to the system with a flow rate of 0.8 ml/min. The sample concentration was adjusted to 0.5 mg/ml and the sample was filtered through a 0.45 µm filter prior to injection into the system. Phosphate-buffered saline was used as a solvent and an injection volume of 100 µl was used.

Results

Isolation and identification of bacteria

LB1-1A, isolated from the rhizosphere of buffalo grass, was selected for this study due to its ability to produce a large amount of EPS as indicated by its slimy and mucoid colonies (Online Resource: Fig. S1). The amount of levan production was also optimized before selecting the specific isolate for further study. For levan biosynthesis, optimum temperature, initial pH, sucrose concentration and aeration rate were determined to be 25 °C, 6.0, 300 g/L and 150 rpm respectively. The striking feature of the isolate LB1-1A is that it gives a very high yield of levan (~42 g/l) under optimized conditions. Additionally, the isolate was able to grow effectively up to 5% salt concentrations, though maximum growth was observed at 0.5% NaCl concentration, indicating it has halotolerant characteristics.

The cells of LB1-1A were found to be Gram-positive, endospore forming motile rods, sometimes forming short chains. For taxonomic analysis, the full length 16S rRNA gene sequence of LB1-1A was compared with other sequences using BLASTn. Further, a phylogenetic tree was constructed based on 16S rRNA gene sequences of closely related type strains, as shown in Fig. 1. In the BLASTn search, the isolate LB1-1A showed

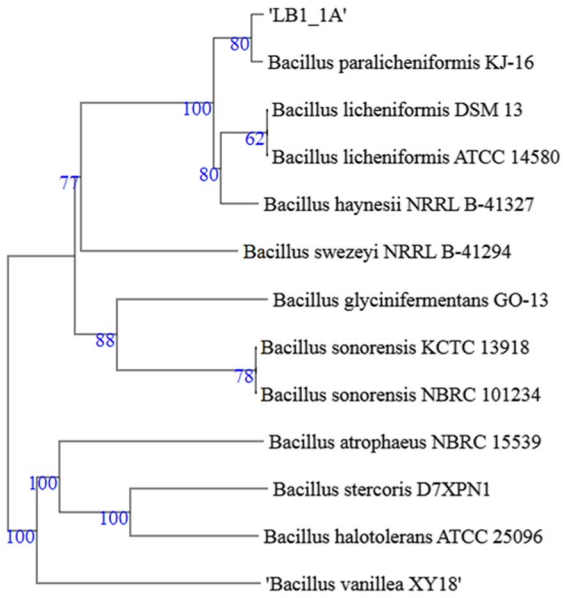

Fig. 1 Phylogenetic tree of *B. paralicheniformis* LB1-1A generated by Type (Strain) Genome Server (TYGS). The tree was inferred with FastME 2.1.6.1 (Lefort et al 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from 16S rRNA gene sequences. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications

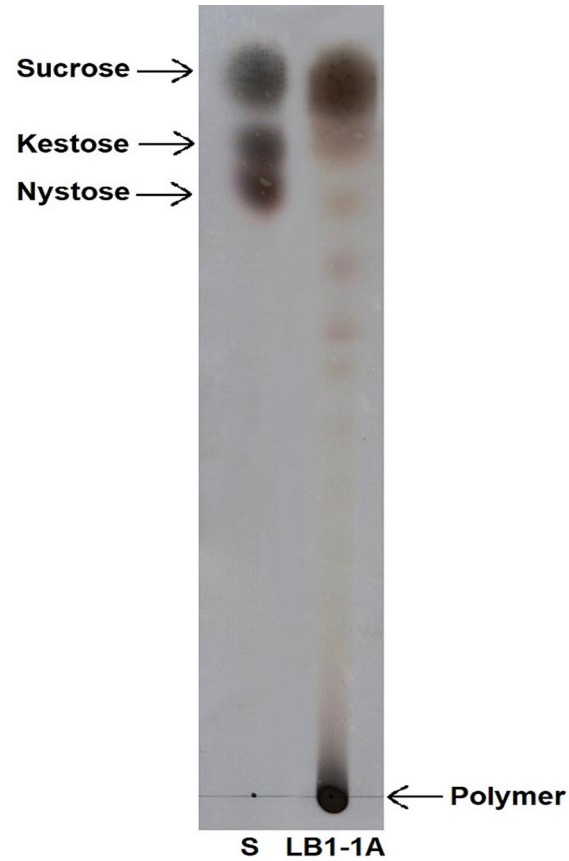

Fig. 2 Thin layer chromatographic analysis of the products synthesized by the growing cells of *B. paralicheniformis* LB1-1A. S: standard oligosaccharides; LB1-1A: *B. paralicheniformis* culture broth sample taken after 48 h of fermentation

99.87–100% identity to various *Bacillus paralicheniformis* strains. In the phylogenetic tree also, the LB1-1A clustered closely with *B. paralicheniformis* (type strain KJ-16) in a distinct clade confirming the isolate to be a *B. paralicheniformis* strain (Fig. 1). The resultant full length 16S rRNA gene sequence of LB1-1A was submitted to GenBank under accession No. ON115208. Further, the whole genome of the isolate has also been sequenced (data to be published separately) and this Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JALJCM000000000. In comparison with the type strain *B. paralicheniformis* KJ-16, the calculated genomic digital DNA Hybridization (dDDH) (https://www.dsmz.de/services/online-tools/genome-to-genome-distance-calculator-ggdc) and Average Nucleotide Identity (ANI) values (Kostas Lab server, http://enve-omics.ce.gatech.edu/ani/) of 89.5 and 98.78%, respectively, also confirm LB1-1A to be a *L. paralicheniformis* strain.

EPS product analysis

Supernatant from the culture broth of *B. paralicheniformis* LB1-1A was subjected to TLC analysis specific for detecting fructan products and it was observed that the isolate produced fructans and a wide range of FOSs in addition to 1-kestose and nystose (Fig. 2). The fructan and FOSs were further characterized by ^{13}C NMR and HPAEC as described below.

The sugar compositional analysis in HPLC chromatogram (Fig. 3a) indicates two peaks at different retention times, a weak peak observed at 12.7 min and a strong peak at 17.075 min. Comparing their retention times with standards showed that the product contained both glucose and fructose, but was overwhelmingly composed of fructose by mass; these

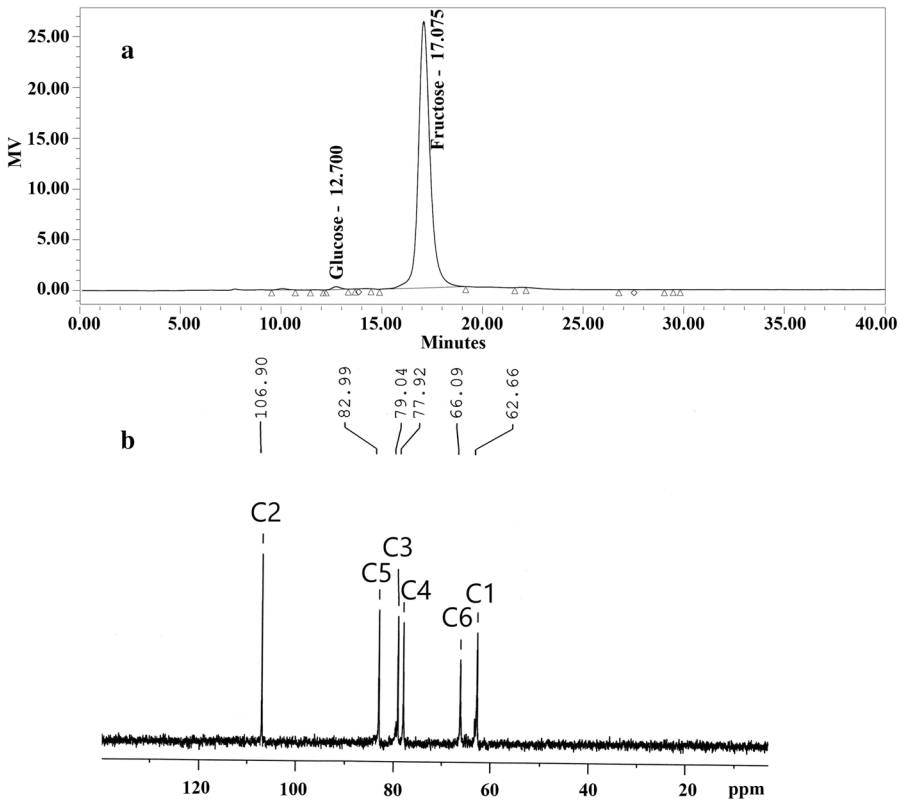

Fig. 3 **a** High performance liquid chromatogram of the hydrolysate of exopolysaccharide produced by *B. paralicheniformis* LB1-1A. The large peak at 17.075 min retention time shows that the major component of the polymer was fructose. **b** The.^{13}C NMR spectrum of purified exopolysaccharide produced by *B. paralicheniformis* LB1-1A recorded in dimethyl sulfoxide. Chemical shift values of the six carbon atoms of fructosyl units of the polysaccharide are given in parts per million with respect to DMSO peak (39.51 ppm)

data further confirmed that the EPS product was of the fructan type.

The ^{13}C-NMR spectrum of the fructan (Fig. 3b) shows chemical shifts in the range of 62.65–106.89 ppm that describes the polysaccharide nature of carbons in carbohydrate rings. The spectrum showed six broad resonance signals at 106.90 ppm (C2), 82.99 ppm (C5), 79.04 ppm (C3), 77.92 ppm (C4), 66.09 ppm (C6), and 62.66 ppm (C1). The relative positions of the signals confirmed the identification of EPS as levan.

To analyze the composition of the FOSs produced by *B. paralicheniformis*, we analyzed the sample with HPAEC-IPAD (Fig. 4). With the commonly used standards, 1-kestose, nystose and some unidentified oligosaccharides that eluted corresponding to maltose

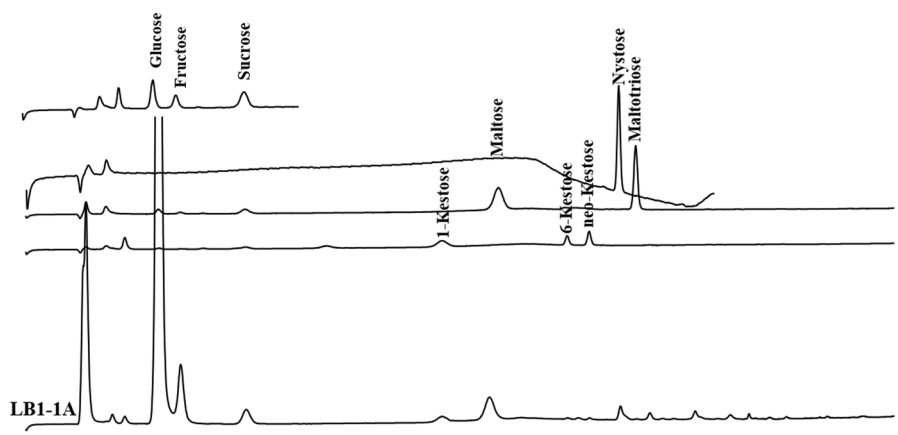

Fig. 4 High performance anion exchange (HPAE) chromatographic analysis of fructooligosaccharides synthesized by growing cells of *B. paralicheniformis* LB1-1A. The analysis was carried out on an HPAE chromatograph coupled with integrated pulsed amperometric detector

and maltotriose peaks were detected. Some other, higher DP FOSs, present in small amounts, could also not be identified. However, the main products obtained under these conditions were small carbohydrates, being di-, tri- and tetrasaccharides.

Linkage analysis

Methylation analysis of the EPS was carried out to determine the linkage types between fructosyl residues at branching points, in addition to linkages in the backbone of the EPS. Figure 5a shows the total ionization chromatogram (TIC) of the derivatives obtained. Signal peaks that appeared on the TIC were further identified by its fragmentation spectra (Fig. 5b), and the results are summarized in Table 1. EPS derivatives consisted of three types of D-fructofuranosyl residues, where signal peak with the lowest retention time at 11.210 min was identified as terminal linked Fru*f* residues: 2,5-di-O-acetyl-(2-deuterio)-1,3,4,6-tetra-O-methyl mannitol/glucitol. The signal peak with the highest abundance (14.657 min) confirmed the presence of (2→6)-linked Fru*f* residues which yielded 2,5,6-tri-O-acetyl-(2-deuterio)-1,3,4-tri-O-methyl mannitol/glucitol sugar derivatives, while 1,2,5,6-tetra-O-acetyl-(2-deuterio)-3,4-di-O-methyl mannitol/glucitol represents (2→1)-linked Fru*f* residues at the branching points (19.075 min). The peak

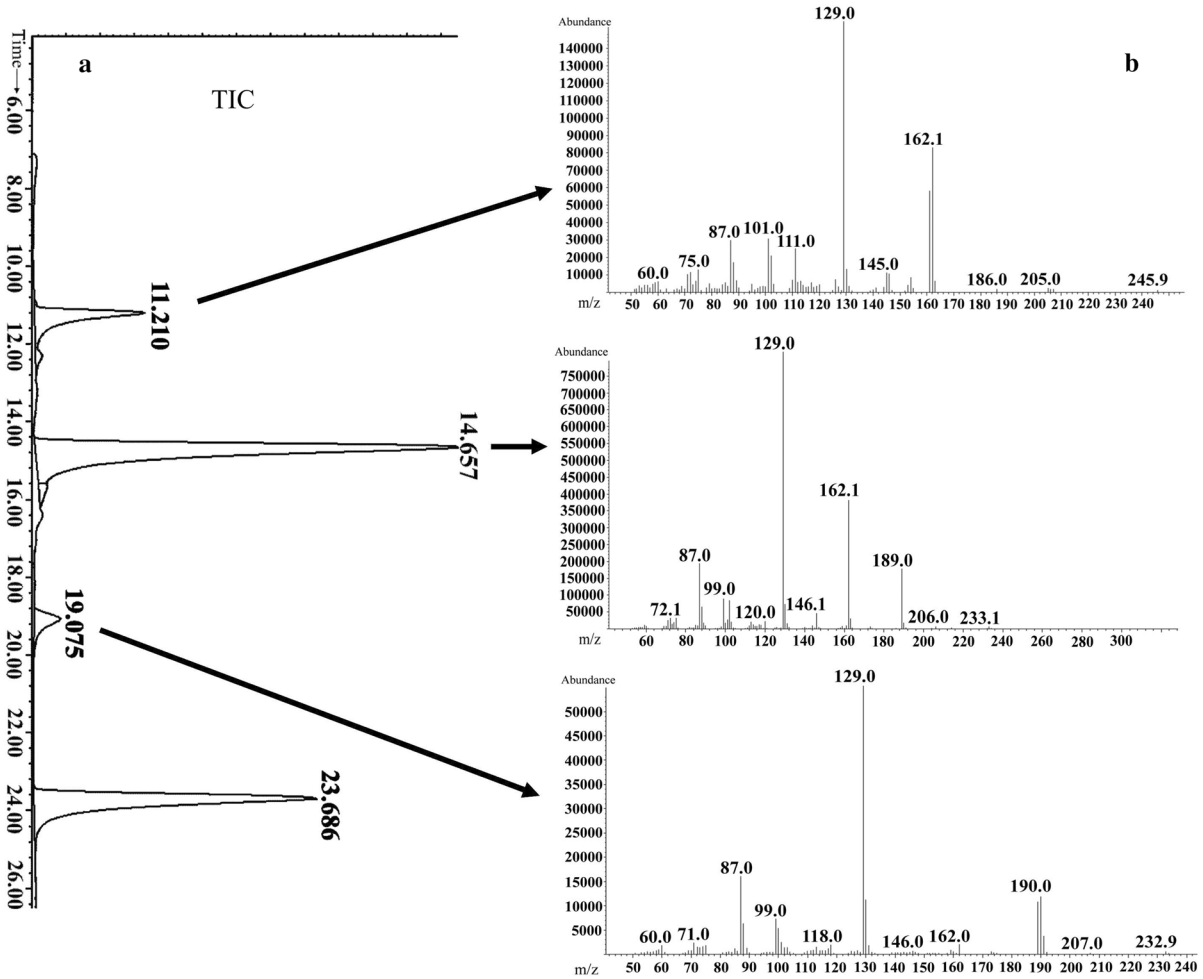

Fig. 5 Linkage analysis by methylation of the exopolysaccharide produced by *B. paralicheniformis* LB1-lA. The partially methylated alditol acetates (PMAAs) were analyzed by a gas chromatograph coupled with mass spectrometer. **a** Total ionization chromatogram; **b** mass spectrometric fragments

Table 1 Glycosidic linkages of *B. paralicheniformis* LB1-1A levan determined by methylation analysis and GC–MS profiles

Signal peaks	Diagnostic fragments	Sugar derivatives	Deduced residues	Mol%
11.210 min	87, 101/102, 129, 161/162 & 205/206	2,5-di-O-acetyl-(2-deuterio)-1,3,4,6-tetra-O-methyl mannitol/glucitol	Terminal-Fru*f*	18.2 ± 0.2
14.657 min	87, 129, 162, 189, 206 & 233	2,5,6-tri-O-acetyl-(2-deuterio)-1,3,4-tri-O-methyl mannitol/glucitol	(2 → 6)-Fru*f*	77.14 ± 0.5
19.075 min	87, 129, 189/190, & 233/234	1,2,5,6-tetra-O-acetyl-(2-deuterio)-3,4-di-O-methyl mannitol/glucitol	(2 → 1)-Fru*f*	4.66 ± 0.1

with the highest retention time at 23.686 min denotes the internal myo-inositol standard. Consequently, the EPS from *B. paralicheniformis* LB1-1A was deduced to be a levan-type molecule consisting of 77.15% (2 → 6)-linked Fru*f* residue in the backbone and 4.66% (2 → 1)-linked Fru*f* branched residues.

Molecular weight determination

B. paralicheniformis LB1-1A was found to synthesize a macromolecular polysaccharide with weight-average molecular weight (M_w) of 5.517×10^7 Da, number-average molecular weight of (M_n) 3.331×10^7 Da and polydispersity index ($PD = M_w/M_n$) of 1.66 (Online Resource Fig. S2).

Discussion

Though several microbes capable of producing levan have been obtained from various sources, the discovery and characterization of EPS synthesizing microorganisms from unexplored niches will add to the versatility of EPSs and their diverse range of applications. Specifically, this work aimed to identify and characterize the bacterial EPS produced by a rhizospheric isolate. The isolate grew well and produced EPS under aerobic conditions in LB media supplemented with sucrose. On solid medium, it formed colonies having a slimy texture, which is an indication of EPS (levan) production as reported previously (Han et al. 2016; Moussa et al. 2017; Nasir et al. 2020). When grown in liquid medium, the isolate was observed to synthesize tremendously viscous broth. The isolate was identified as *B. paralicheniformis*. Previously, we have used the EPS product from this isolate as an additive in poultry feed for health improvement of the birds (Ashfaq et al. 2020). *B. paralicheniformis* is also known to positively influence the plant growth and vitality, and is commonly recognized as PGPR (Valenzuela-Ruiz et al. 2019; Wang et al. 2017). Though EPS synthesis has been reported in several *Bacillus* species, *B. paralicheniformis* has never been explored for the characterization of its EPS which adds further to the novelty of this work. This study is the first comprehensive work on the production and characterization of EPS from this bacterium.

Structural characterization of EPS

The EPS produced by *B. paralicheniformis* LB1-1A was identified by ^{13}C NMR spectroscopy. The carbon chemical shifts were attributed to β-configured fructofuranose units in comparison with carbon chemical shifts of the standard methyl-glycoside (Bock and Pedersen 1983). In ^{13}C NMR spectra, fructans (inulin and levan) have characteristic distribution of the chemical shift signals due to the stereoelectronic effects of different glycosidic linkages between fructosyl units. This distribution of chemical shifts has been used to classify the fructans as inulin and levan (Anwar et al. 2010, 2008; Kırtel et al. 2019). Crucial among these are the chemical shift signals of Carbon 6 (C6) and Carbon 1 (C1), which are directly linked to the glycosidic bonds in levans and inulins, respectively. Due to the characteristic downfield shifts of the carbons, the difference in chemical shifts between C6 and C1 is small for inulins (~ 1 ppm), while this difference ranges from 3.0 to 3.6 in levan-type fructans. Conversely, the chemical shift differences between Carbon 3 (C3) and Carbon 4 (C4) are small in levans (~ 1 ppm) but they are farther apart in inulin-type fructans. Based on these characteristic differences, the obtained results correspond to the peak positions for β(2 → 6) linked fructose residue that describes the polysaccharide nature of the carbons in the carbohydrate rings. The detection of β(2 → 6) glycosidic bonds validates the production of levan-type EPS by

the isolate. These findings are similar to the ^{13}C NMR spectra reported for levan type fructans of *B. licheniformis* (Xavier and Ramana 2017; Kırtel et al., 2021), *Brachybacterium sp.* (Djurić et al. 2017) and *Bacillus aryabhattai* (Nasir et al. 2020).

FOSs production by the isolate

Besides levan production, *B. paralicheniformis* LB1-1A was found to produce FOSs. The main products obtained under these experimental conditions were small carbohydrates, being di-, tri- and tetrasaccharides, as depicted by HPAEC-IPAD analysis. These FOS products are of narrow range as compared to FOSs from *Bacillus aryabhattai* GYC2-3 isolated from *Taraxacum* spp. plant in our previous study (Nasir et al. 2020). Varying diversity and range of FOSs from other strains of the *Bacillus* genus have been reported (Li et al. 2015). This indicates the species-specific formation of a wide variety of FOSs with different functions that can be elucidated involving *in vivo* and *in vitro* experiments. HPAEC results also indicated that, after 48 h of incubation, most of the sucrose was utilized by the isolate. A huge peak of glucose reflected the EPS glycosyl residue stoichiometry and was consistent with the production of EPS directly from sucrose.

LB1-1A produces a moderately branched levan

Methylation analysis of the LB1-1A levan further elaborated its fine structure, including linkages in the main chain as well as the branching pattern. Analysis of PMAAs by GC–MS suggests that fructose unit is the chief constituent of the main chain of the EPS produced by the *B. paralicheniformis* LB1-1A isolate, while branched chains or substituted groups are present at the (2→1) positions of the fructose units. GC–MS analysis also supports the assignment of ^{13}C NMR spectrum. The reduction of C-2 of fructose with $NaBD_4$, allowed the discrimination between (2→6)-linked Fru*f* and (2→1)-linked Fru*f* of levan and inulin, respectively (Hellerqvist et al. 1990). Apart from that, sometimes the presence of 1,5-anhydro-2,3,4,6-tetra-O-methyl-D-glucitol can also be detected in levans, which indicates the presence of terminal α–D-Glc (terminal glucose) (Lopez et al. 2003). According to Van den Ende (2013), fructans may or may not contain terminal glucose units. Nevertheless, in our case, (Fig. 5), a small glucose peak could be observed after terminal-Fru*f* peaks in the chromatogram at 12 min retention time. The extent of branching measured for levan of *B. paralicheniformis* LB1-1A is in accordance with that reported for levan described by Dong et al (2015). Some bacterial levans are reported to have high amount of branching such as: 14.3% for *B. licheniformis* 8-37-0-1 (Liu et al. 2010), 10.5% for *B. subtilis* (Benigar et al. 2014) and ~ 12% for *P. polymyxa* B-18475 (Han and Clarke 1990). However, some bacterial levans are listed as linear with a low (< 2%) degree of branching, e.g. levan from acetic acid bacteria (Jakob et al. 2013) and lactic acid bacteria (Ahmad et al. 2022).

High molecular weight levan produced by LB1-1A

Many bacterial species are known to produce levans with different molecular weights and molecular size distributions. Compared to previously reported levans from *Bacillus* genus, M_w of *B. paralicheniformis* LB1-1A levan i.e. 5.517×10^7 Da is comparable to the M_w 5.317×10^7 Da of *B. aryabhattai* levan (Nasir et al. 2020) while relatively larger than that reported for *B. subtilis sp.* and *B. subtilis* natto, which produced levan having M_w of 1.66×10^4 Da with PD = 2.17 and M_w between 9 and 2000 kDa (Ghoneim et al. 2016; Wu et al. 2013), respectively. Higher PD values indicate broad molecular weight distributions, whereas narrow molecular weight distribution (1.66) was detected for levan of *B. paralicheniformis* LB1-1A indicating a relatively homogenous molecule. The applications of fructans are highly influenced by their molecular weights and physicochemical properties (Xu et al. 2018; Ortiz-Soto et al. 2019). For instance, Yoo and coworkers observed both in *in vitro* and *in vivo* studies that levans with high molecular weights (710,000 Da, 570,000 Da and 380,000 Da) were superior at inhibiting tumor cell lines as compared to levan with low molecular weight (40,000 Da), which showed poor antitumor activity (Yoo et al. 2004). Similarly, high molecular weight levan has been commonly used as an encapsulating agent, stabilizer, emulsifier and thickener due to its specific rheological and physicochemical properties (Goncalves et al. 2015). High MW levan produced by *Z. mobilis* levansucrase exhibited a strong in vitro antibacterial

effect (Byun et al. 2014), while anti-tumor activities of levan were observed depending on its molecular size (Calazans et al. 2000). Therefore, the high MW levan with relatively low degree of branching, produced by *B. paralichenisformis* LB1-1A could prove to be a promising candidate for industrial applications.

Conclusions

In this study, with the motivation to find a new fructan-producing bacterium, *B. paraicheniformis* was isolated from the rhizosphere of buffalo grass. This study demonstrates that the moderately halotolerant *B. paralicheniformis* LB1-1A is a new source for EPS and oligosaccharide production and a gateway for new perspectives and applications. Based on the monosaccharide composition, ^{13}C NMR, and linkage analysis, the structure of EPS was confirmed as levan which is composed of fructose with the β-(2,6) linkages. Another striking feature of this isolate is that it gives a high molecular weight (5.517×10^7 Da) levan having relatively low degree of branching i.e. 4.66%, which adds further to its novelty. The isolate's association with plants and demonstrated role in plant growth promotion, its probiotic properties, and now its levan production capability makes *B. paralicheniformis* attention worthy of further investigations for diverse applications.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevent financial or non-financial interests to disclose.

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