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EXOPOLYSACCHARIDE SYNTHESIS BY *Bacillus thuringiensis* HA1 USING CARBON SOURCES FROM THE SUGARCANE AGROINDUSTRY

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ABSTRACT

Exopolysaccharides are biopolymers produced by bacteria and have characteristics that make them suitable for applications in the pharmaceutical, environmental, and food industries. However, exopolysaccharide production faces challenges like high production costs. Therefore, strategies such as culture conditions improvement, strain selection, and the use of low-cost carbon sources have emerged as alternatives to improve exopolysaccharide production. In this work, the capability of *Bacillus thuringiensis* HA1 to produce exopolysaccharides using low-cost carbon sources (commercial sucrose, molasses, and panela) was explored. The production conditions were evaluated as follows: fermentation time (0–86 h), initial pH (5–9), temperature (31–43 °C), carbon sources (commercial sucrose, molasses, and panela), and concentration of carbon sources (50–350 g L^{-1}). The settled conditions to assess the carbon sources were 60 h, 37 °C, and pH 7.5. Exopolysaccharide production was higher using commercial sucrose (23.54 mg mL^{-1}), followed by molasses (8.62 mg mL^{-1}) and panela (6.37 mg mL^{-1}). The sucrose sample showed similarity to a glucan-type exopolysaccharide, since the presence of peaks at 1000–1200 is characteristic of C–O–C glycosidic linkages, while the molasses sample showed similarity to the standard levan. These results were achieved without pretreating the carbon sources, thus allowing the process to be economically feasible. To date, *Bacillus thuringiensis* has not been reported as a producer of two types of exopolysaccharides using different carbon sources.

Keywords: levan, biopolymers, molasses, commercial sucrose, submerged culture.

INTRODUCTION

Exopolysaccharides (EPSs) are high-molecular-weight carbohydrate biopolymers produced by microorganisms (Nadzir *et al*., 2021). EPSs function as protective mechanisms against environmental factors and are key for adaptation, survival, and

other functionalities (Osemwegie *et al*., 2020). These polysaccharides are known for their biocompatibility, nontoxicity, and unique functionalities, making them valuable in healthcare and biomedical applications such as drug delivery systems, healing and tissue engineering, collagen stimulants and anti-aging agents, antioxidants and skin care agents, cholesterol or triglyceride reducers, antibiotics to promote cytotoxic activity for colon and breast cancer, immune-stimulatory agents, and stabilizers in formulations.

Probiotic-derived EPSs exhibit health-promoting properties by improving the human digestive system, thus contributing to health and well-being (Aziz *et al*., 2022; Pourjafar *et al*., 2022; Wu *et al*., 2022; Ahuja *et al*., 2023; Waoo *et al*., 2023). In the food industry, EPSs are applied as texture enhancers, viscosity agents, and moisture retainers in products such as low-fat dairy, gluten-free bakery items, and fermented meats (Abarquero *et al*., 2021; Pourjafar *et al*., 2023). In the environmental field, EPSs have been used in remediation strategies to address heavy metal contamination and organic pollutant bioremediation (Balíková *et al*., 2022).

EPS production can be achieved by bacterial fermentation (Saadat *et al*., 2019). Microorganisms from the genera *Leuconostoc*, *Zymomonas*, *Halomonas*, and *Bacillus* have been confirmed as EPS producers (Braga *et al*., 2022; Erkorkmaz *et al*., 2022; Vega-Vidaurri *et al*., 2022). Several *Bacillus* species, such as *B. subtilis*, *B. licheniformis*, and *B. megaterium*, have been identified as EPS producers, most notably of levan, a fructan biopolymer with significant prebiotic and antioxidant properties (Díaz-Cornejo *et al*., 2022). However, EPS production faces challenges such as high production costs, low yields, and purity constraints (Erkorkmaz *et al*., 2022; Zhang *et al*., 2023). Therefore, approaches including strain selection, genetic engineering, culture condition optimization (initial pH, temperature, and fermentation time), and the use of cheap carbon sources have emerged as alternatives to improve EPS production (Nguyen *et al*., 2020; Zhang *et al*., 2023).

Many agro-industrial co-products such as fruit pomace and husk, lignocellulosic biomass, and molasses have been used for EPS production (Wang *et al*., 2025). However, the pretreatment of carbon sources like fruit waste or lignocellulosic biomass is necessary to ensure sugar availability, which increases EPS production costs (Pérez-Contreras *et al*., 2025). Although pretreatment may improve yields, Wang *et al*. (2025) found that out of 65 studies, 37 did not use pretreatments, and submerged fermentation was employed in 63 studies. Therefore, unpretreated sugar agro-industrial products and by-products such as sucrose, molasses, and panela can serve as alternatives for EPS production, as they are abundant and constitute important raw materials (Ni *et al*., 2022; Venkatesh *et al*., 2023).

Sucrose is a natural sweetener widely used for human consumption and has been studied as an enzyme production inducer, leading to the production of bioproducts like sucrose isomers, oligosaccharides, and monosaccharides (Ni *et al*., 2022). Molasses is a dark-brown, viscous liquid rich in proteins, inorganic salts, trace elements, and high sugar concentrations (Liang *et al*., 2022). Panela, known in Latin America as a

traditional unrefined sweetener or non-centrifugal sugar, contains phenolic acids, flavonoids, minerals, and bioactive compounds (Zidan and Azlan, 2022). Therefore, the objective of this work was to investigate the capability of *Bacillus thuringiensis* HA1 to synthesize EPSs using cheap carbon sources (commercial sucrose, molasses, and panela) produced by the sugarcane agroindustry.

MATERIALS AND METHODS

Exopolysaccharides (EPSs) were produced using *Bacillus thuringiensis* HA1, obtained from the Laboratory of Applied Microbial Biotechnology at the Postgraduate College Campus Córdoba. The strain was initially cultured on nutrient agar (23 g L^{-1}) in slanted tubes and incubated at 37 °C for 24 h. Subsequently, the reactivated culture was transferred to 250 mL Erlenmeyer flasks containing nutrient broth (8 g L^{-1}) and incubated at 37 °C for an additional 24 h under continuous agitation at 150 rpm. The resulting biomass was used as the inoculum (Gayosso-Sánchez *et al*., 2024).

Strain conservation was performed by cultivating *B. thuringiensis* HA1 as described above, followed by biomass recovery through centrifugation at 7500 × g and two washes with sterile water. The biomass was then resuspended in sterile water (5 mL), and 1 mL aliquots were transferred to cryovials containing 30 % glycerol and sterile crystal spheres. The cryovials were stored at 4 °C until use (Vega-Vidaurri *et al*., 2022). Prior to EPS production, the conserved *B. thuringiensis* HA1 strain was reactivated.

Exopolysaccharide production

EPS production was carried out using commercial sucrose under submerged culture conditions in 120 mL glass vessels of identical shape. The kinetics of EPS production were evaluated in a formulated medium based on commonly reported components (g L^{-1}): commercial sucrose (100), yeast extract (5), sodium chloride (5), dipotassium phosphate (1), and magnesium sulphide (0.2). The culture conditions were established at an initial pH of 7.5 using 0.1 M sodium phosphate buffer, an inoculum concentration of 1 × 10^6 CFU mL^{-1}, a temperature of 37 °C, and agitation at 150 rpm. Samples were collected at regular intervals of 0, 12, 24, 36, 48, 60, 72, and 84 h (Long *et al*., 2024).

One factor at a time approach for exopolysaccharide production

The influence of several factors on EPS production by *B. thuringiensis* HA1 was evaluated through a series of experiments. Temperature effects were assessed at 33, 35, 37, 39, 41, and 43 °C under an initial pH of 7. Subsequently, the effect of initial pH was evaluated at 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9, using the optimal temperature determined in the previous assays (Long *et al*., 2024). The effect of carbon sources on EPS production was evaluated using commercial sucrose, molasses, and panela. Carbon source concentrations were analyzed at 50, 100, 150, 200, 250, 300, and 350 g L^{-1}, maintaining the temperature and pH conditions determined previously (Gudiña *et al*., 2022). All experiments were conducted in triplicate.

Exopolysaccharide recovery

The submerged culture was centrifuged at 7500 × g for 15 min at 4 °C, and EPSs were recovered from the supernatant by precipitation with chilled ethanol at a ratio of 1:3 (v/v). The mixture was stored overnight at -4 °C to achieve maximum precipitation. The EPSs were resuspended in distilled water and washed three times with cold acetone to remove monosaccharide residues, followed by a second precipitation with chilled ethanol. EPSs were then recovered by centrifugation at 7500 × g for 15 min and lyophilized at -50 °C for 8 h under 0.18 bar using a Labconco FreeZone 4.5 system. EPSs were quantified by dry weight (Zhang *et al*., 2025).

Sugar profiles of carbon sources by chromatographic analysis

Sugar quantification was performed using a high-resolution liquid chromatograph (HPLC) Thermo Finnigan Surveyor equipped with a Surveyor LC system, autosampler, and RI Surveyor Plus detector. Separation was achieved using a Phenomenex Rezex RNM-Carbohydrate Na^{+2} column (300 × 7.8 mm) with Milli-Q double-distilled water as the mobile phase. The column temperature was maintained at 80 °C, the flow rate at 0.4 mL min^{-1}, and the detector temperature at 37 °C. Prior to injection, samples were diluted and filtered through PHENEX PTFE Acrodisc filters (25 mm, 0.2 μm pore). Sugar concentrations were determined using calibration curves of sucrose, glucose, and fructose (Sigma-Aldrich) in the range of 200–1000 ppm. Results are reported in g L^{-1} (Gayosso-Sánchez *et al*., 2024).

Total phenolic content determination on carbon sources

The total phenolic content (TPC) of the carbon sources was spectrophotometrically determined using the Folin-Ciocalteu method, with gallic acid (Sigma-Aldrich, St. Louis, MO, USA) as the standard. Results were expressed as milligrams of gallic acid equivalents per gram (mg GAE g^{-1}). The reaction mixture was prepared by combining 250 μL of 1 N Folin-Ciocalteu reagent with 250 μL of the carbon source sample. After 8 min, 1250 μL of 7.5 % Na_2CO_3 and 480 μL of distilled water were added, and the mixture was incubated in the dark for 30 min. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific Biomate 3S UV-Vis, WI, USA) (Jiménez-Morales *et al*., 2024).

Exopolysaccharide characterization

The EPSs produced by *B. thuringiensis* HA1 under submerged culture conditions were characterized by Fourier-transform infrared spectroscopy (FT-IR) using attenuated total reflectance (ATR) with a zinc selenide crystal and a resolution of 4 cm^{-1}. Spectra were recorded in the mid-infrared region from 400 to 4000 cm^{-1}. Levan from *Erwinia herbicola* (Sigma) and inulin from *Dahlia* tubers (Sigma) were used as reference standards. The obtained spectra were processed using Origin 6.1 software (OriginLab Corporation, USA) (Zhang *et al*., 2025).

Statistical analysis

To evaluate the effect of each independent variable (temperature, initial pH, carbon source type, and concentration), differences among treatment means were analyzed for significance using analysis of variance (ANOVA) followed by Tukey's test in Minitab 17.

RESULTS AND DISCUSSION

Exopolysaccharide production

EPS production over time was initially evaluated using commercial sucrose (100 g L^{-1}). EPS synthesis began after 12 h (0.79 mg mL^{-1}) and reached a maximum at 60 h (1.77 mg mL^{-1}) (Figure 1). Biomass production also started after 12 h (0.09 mg mL^{-1}), reaching its highest value at 24 h (0.16 mg mL^{-1}), and *B. thuringiensis* HA1 growth remained constant until 72 h (0.164 mg mL^{-1}).

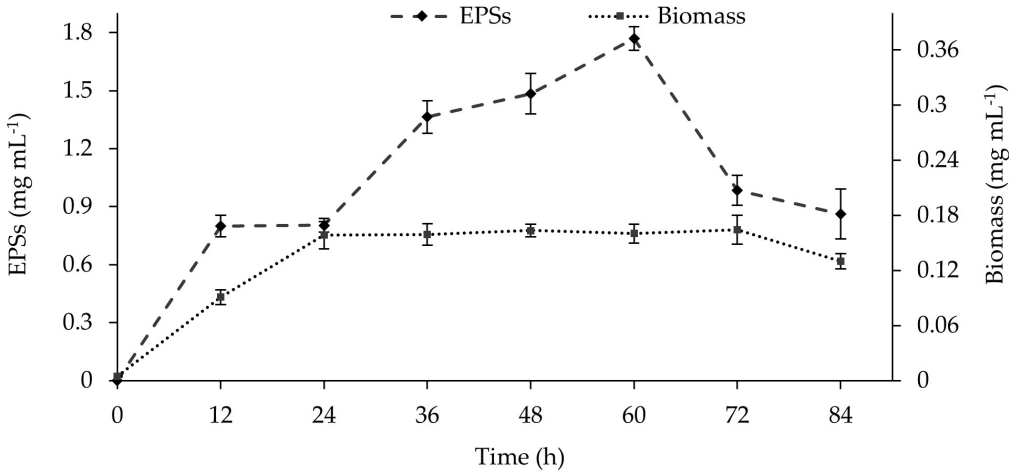

Figure 1. Kinetics of exopolysaccharide (EPS) production by *Bacillus thuringiensis* HA1 in submerged culture using commercial sucrose as carbon source (bars indicate standard error).

The EPS production obtained in this study was higher than that reported by Zhang *et al*. (2021), who observed a maximum of 0.609 g L^{-1} using *Lactobacillus paracasei* as the inoculum. Similarly, Mıdık *et al*. (2020) reported a maximum EPS production of 0.515 g L^{-1} after 120 h using *Lactobacillus plantarum* MF460, while Kumar *et al*. (2020) achieved a maximum EPS production of 1.3 mg mL^{-1} after 72 h with *Pediococcus acidilactici* NCDC 252. Song *et al*. (2021) reported EPS production of 1.62 g L^{-1} after 72 h of fermentation using *P. acidilactici* M76 and black raspberry extract as the carbon source. However, the values obtained in this study were lower than those reported by Gudiña *et al*. (2022), who achieved 6.1 g L^{-1} of EPSs after 144 h using *Rhizobium viscosum* CECT908. These differences in EPS production may be attributed to the selected strain and its

intrinsic capacity for EPS synthesis. Notably, *B. thuringiensis* HA1 achieved maximum EPS production in a shorter fermentation time compared to most reported studies.

Influence of temperature on exopolysaccharide production

The highest EPS production was observed at 37 °C (3.62 mg mL^{-1}), followed by 39 °C (3.19 mg mL^{-1}). At 33, 35, 41, and 43 °C, EPS production values were 1.14, 1.15, 1.2, and 1.12 mg mL^{-1}, respectively, while the lowest production was recorded at 31 °C (0.8 mg mL^{-1}). Biomass production reached its maximum at 33 °C (0.53 mg mL^{-1}) and was lowest at 41 and 43 °C (0.15 mg mL^{-1}) (Figure 2).

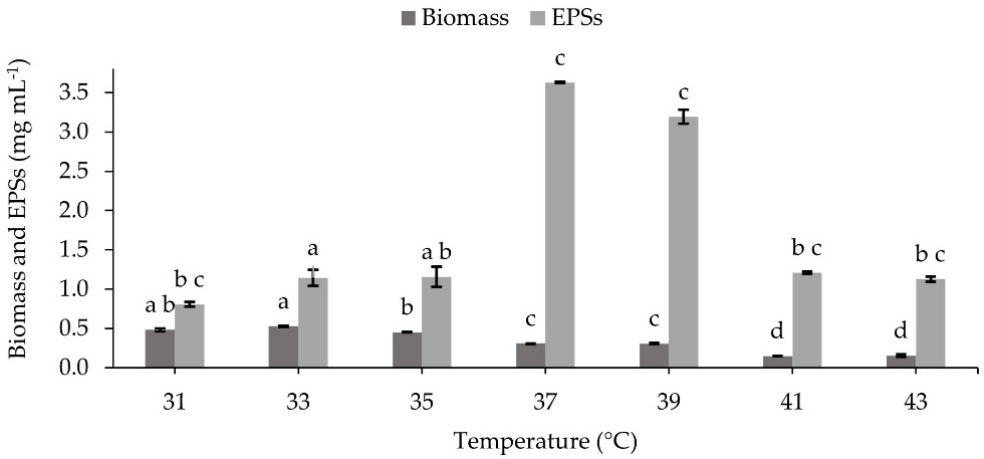

Figure 2. Effect of temperature on exopolysaccharide (EPS) production using *Bacillus thuringiensis* HA1 (bars indicate standard error).

These results were higher than those reported by Zhao and Liang (2023), who achieved a maximum of 186.77 mg L^{-1} at 37 °C using *Lactiplantibacillus plantarum* M5, but lower than those reported by Upadhyaya *et al*. (2024), who reported 9.99 g L^{-1} at 30 °C using *Bacillus tequilensis*. These results confirm that temperature is a key factor influencing EPS production and that optimal temperatures vary among bacterial strains (Upadhyaya *et al*., 2024).

Influence of pH on exopolysaccharide production

The maximum EPS production (3.14 mg mL^{-1}) was achieved at pH 7.5, whereas the lowest (0.11 mg mL^{-1}) occurred at pH 5. In contrast, maximum biomass production (1.09 mg mL^{-1}) was observed at pH 7, while the lowest (0.04 mg mL^{-1}) was also recorded at pH 5 (Figure 3). These demonstrate how pH has a direct influence on the metabolic processes involved in EPS production.

These results were higher than that reported by Cheng *et al*. (2019), who achieved a maximum of 1.5 g L^{-1} at pH 7.2 using *L. plantarum* LPC-1 as the inoculum. However,

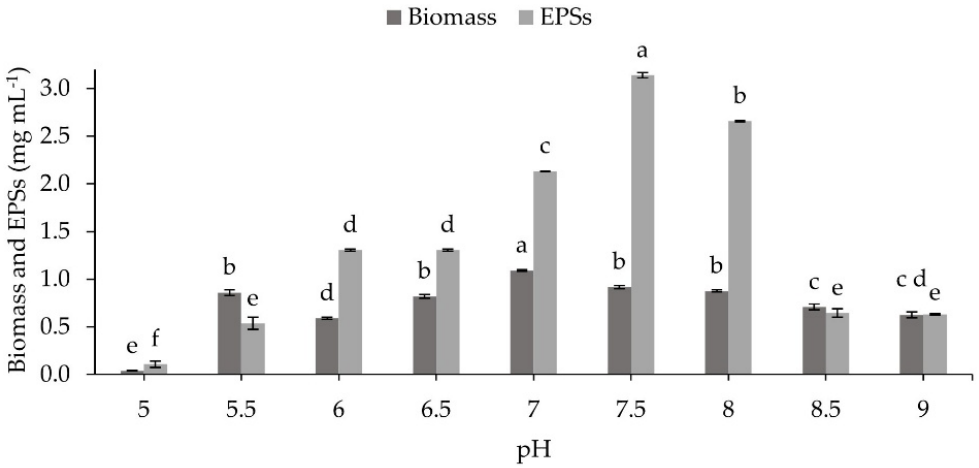

Figure 3. Effect of initial pH on exopolysaccharide (EPS) production by *Bacillus thuringiensis* HA1 (bars indicate standard error).

the values were lower than those reported by Asgher *et al*. (2021), who reached a maximum EPS production of 9.006 g L^{-1} at pH 7 using *Bacillus licheniformis* under optimized conditions. These comparisons indicate a wide variation in optimal pH for EPS production, reflecting the strain-specific capacity to adapt to different environmental conditions (Mıdık *et al*., 2020).

These conditions have an influence not only on biomass growth but also on metabolite production, directing fermentation toward two possible outcomes: programmed cell death, a genetically controlled process of self-destruction triggered by unfavorable conditions for growth, or enhanced EPS synthesis through enzyme expression, enabling biofilm formation that protects microorganisms from the fermentation environment (Ju *et al*., 2022; Naseem *et al*., 2024).

Influence of carbon sources and their concentration on exopolysaccharide production

The influence of carbon source type (commercial sucrose, molasses, and panela) and concentration on EPS and biomass production was evaluated. The maximum EPS production (23.54 mg mL^{-1}) was achieved using sucrose at 50 g L^{-1} (Figure 4). When molasses was used as the carbon source, the highest EPS production (8.62 mg mL^{-1}) occurred at 100 g L^{-1}, whereas panela yielded a maximum EPS production of 6.37 mg mL^{-1} at 50 g L^{-1}. Regarding biomass production, the highest value was observed with molasses at 250 g L^{-1} (3.87 mg mL^{-1}), followed by panela at 300 g L^{-1} (4.18 mg mL^{-1}). In contrast, sucrose resulted in a lower maximum biomass production of 1.06 mg mL^{-1} at 300 g L^{-1}.

EPS production using commercial sucrose was higher than that reported by Santos and Cruz, who obtained 19.8 g L^{-1} using *Leuconostoc mesenteroides* with sucrose as the

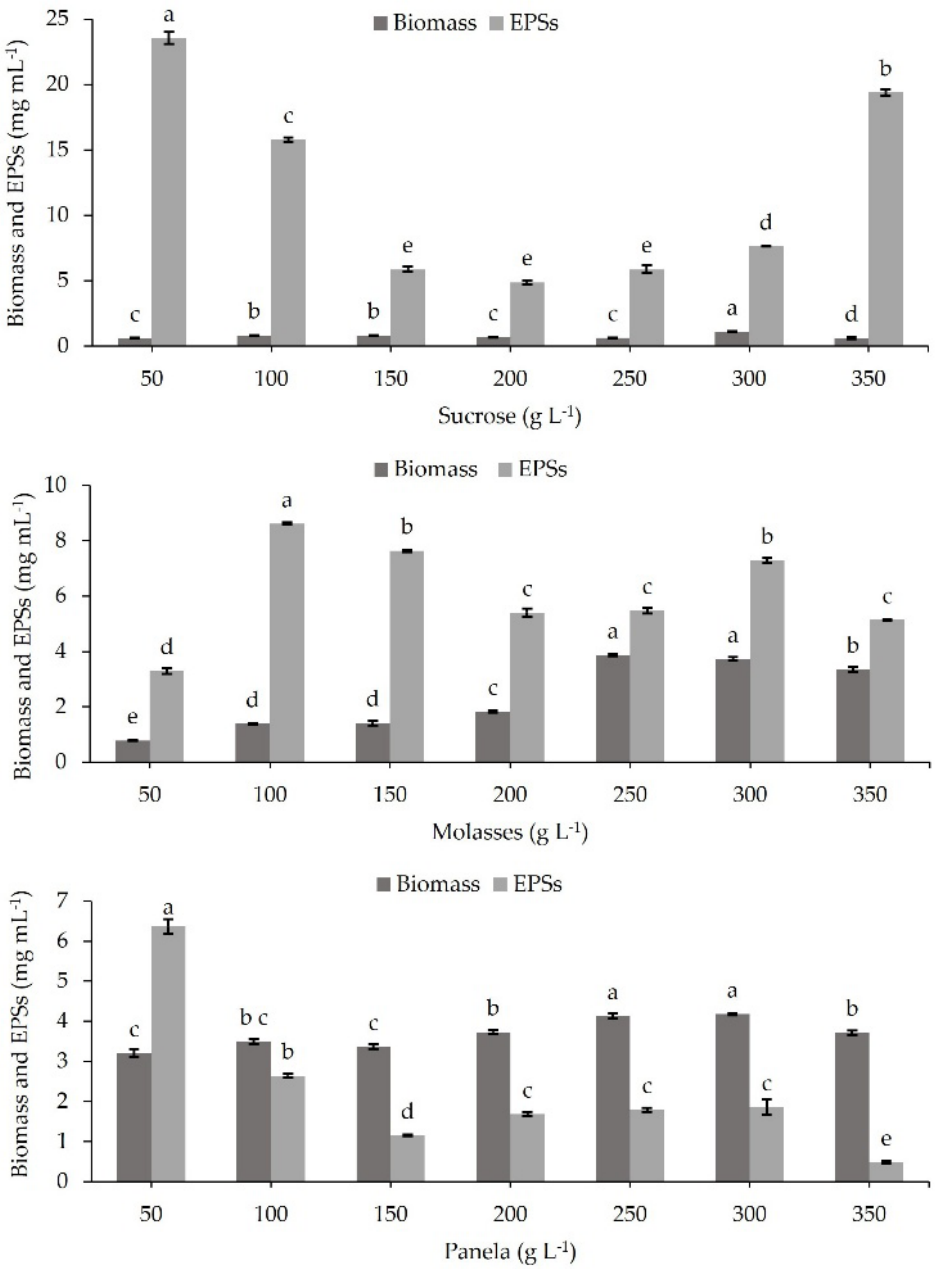

Figure 4. Effect of the carbon sources and their concentration on exopolysaccharide (EPS) production by *Bacillus thuringiensis* HA1 (bars indicate standard error).

carbon source. Similarly, Erkorkmaz *et al*. reported a maximum EPS production of 24.5 g L^{-1} using industrial sugar beet sucrose, which is comparable to the production observed in the present study without carbon source pretreatment.

Production values using molasses were higher than that reported by Asgher *et al*. (2021), who achieved 2.855 g L^{-1} using sugarcane molasses and *B. licheniformis* mutants. Under optimized conditions, the same authors reported 9.006 g L^{-1}, which is close to the maximum EPS production obtained in this study using unpretreated molasses. Gudiña *et al*. (2022) reported a maximum EPS production of 6.1 g L^{-1} using sugarcane molasses and *R. viscosum* CECT908. In contrast, Mehta *et al*. (2023) reported 18.5 g L^{-1} using pretreated sugarcane molasses and *Bacillus megaterium* KM3, while Liang *et al*. (2022) reported the highest EPS production (48.45 g L^{-1}) using sugarcane molasses and *Leuconostoc citreum* B-2. These results indicate that EPS yield is not exclusively dependent on the carbon source, as strain selection is also a determining factor in EPS production.

Overall, previous studies demonstrate that sugarcane molasses is a suitable alternative carbon source for reducing EPS production costs. For instance, Gudiña *et al*. (2022) reported that their process was approximately 30 times less expensive than a conventional production process using glucose as the carbon source.

Total phenolic contents and sugar profile determination of carbon sources

Sugar profile and total phenolic content were analyzed to evaluate the response of *B. thuringiensis* HA1 to the different carbon sources. Commercial sucrose contained 94 % disaccharide and showed no detectable phenolic compounds. In contrast, molasses consisted of 18 % sucrose, 1 % glucose, and 6 % fructose, with a phenolic content of 19.5 mg GAE g^{-1}. Panela exhibited a soluble sugar composition of 45 % sucrose, 22 % glucose, and 33 % fructose, and a phenolic content of 2.4 mg GAE g^{-1}.

The sugar profile indicated a direct correlation between sucrose concentration and EPS production, as maximum EPS synthesis was achieved using commercial sucrose. This can be attributed to the role of sucrose as a metabolic inducer, promoting the expression of enzymes involved in the polymerization of homopolysaccharides for the synthesis of α-glucans or β-fructans (Jurášková *et al*., 2022; Kaur and Dey, 2022; Yu *et al*., 2022). In contrast, the presence of glucose and fructose in molasses and panela may have negatively affected EPS production. This phenomenon may be due to monosaccharides being primarily used by bacteria as a carbon source for biomass production, rather than EPS synthesis (Bruni and Terrel, 2022). Consistently, the highest biomass production was observed when molasses and panela were used as carbon sources, suggesting a negative correlation between microbial growth and EPS production due to the redirection of metabolic flux toward primary metabolism and glycolysis (Qiu *et al*., 2023).

It was also observed that in the carbon sources where phenolic compounds were found, the production of EPSs was lower. Although polyphenols can stimulate probiotic bacterial growth (Chen *et al*., 2023), the combined presence of readily assimilable monosaccharides and phenolic compounds may explain the reduced EPS production observed when molasses and panela were used as carbon sources.

Exopolysaccharide characterization by Fourier-transform spectroscopy

Spectra obtained from EPSs produced using different carbon sources were compared with those of inulin from *Dahlia* tubers and levan from *E. herbicola* (Figure 5). The spectrum showing the highest similarity corresponded to levan from *E. herbicola*. Characteristic levan bands include the O–H stretching vibration at 3400 cm^{-1}, the C–H stretching vibration at 2900 cm^{-1}, indicating the presence of aliphatic chains, the C=O stretching vibration at 1650 cm^{-1}, the C–O–C and C–O–H stretching vibrations associated with glycosidic linkages in the 1050–1150 cm^{-1} region, and the fructofuranosyl ring vibrations at 900–950 cm^{-1}.

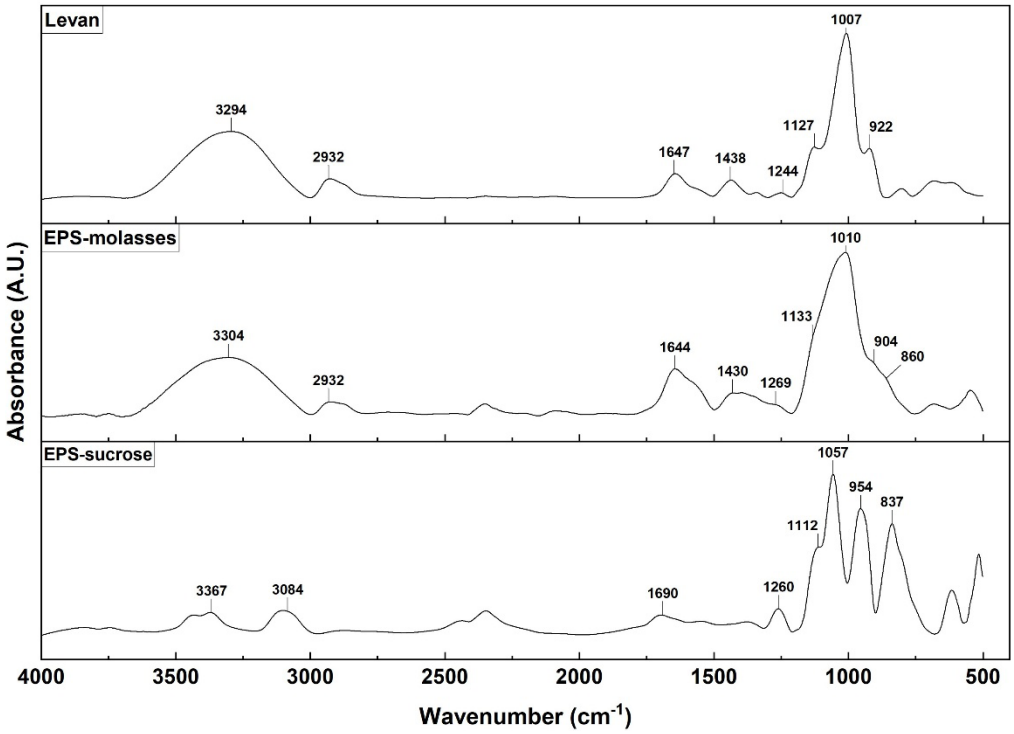

Figure 5. Fourier transform infrared spectra of the exopolysaccharides (EPS) produced by *Bacillus thuringiensis* HA1 compared to levan standard reference.

The EPS produced using molasses as the carbon source exhibited these characteristic bands, with the O–H band at 3304 cm^{-1}, the C–H band at 2932 cm^{-1}, the C=O band at 1644 cm^{-1}, the C–O–C and C–O–H stretching vibrations at 1133 and 1010 cm^{-1}, and the fructofuranosyl ring vibration at 904 cm^{-1}. These distinctive bands are consistent with those reported by Hertadi *et al*. (2020), Thakham *et al*. (2020), and Bruni and Terrell (2022), in which levan produced by *Escherichia coli* BL21 (DE3), *Bacillus siamensis*, and *Gluconobacter japonicus*, respectively, was characterized using FT-IR spectroscopy.

The EPS produced using sucrose showed an O–H band at 3367 cm^{-1}, a slightly shifted C–H band at 3084 cm^{-1}, a C=O band at 1690 cm^{-1}, C–O–C and C–O–H stretching vibrations at 1112 and 1057 cm^{-1}, a fructofuranosyl ring vibration at 954 cm^{-1}, and an additional band at 837 cm^{-1}. Although these bands correspond to an EPS, comparison with dextran and inulin standards did not reveal spectral similarity.

CONCLUSIONS

Bacillus thuringiensis HA1 exhibited remarkable metabolic flexibility, a trait of significant value for industrial biotechnology applications. This study demonstrated that the biosynthetic output of the strain can be directed by the composition of the carbon source, with the ability to produce levan when sugarcane molasses was used. In contrast, the use of commercial sucrose shifted the metabolism toward the production of a distinct glucan-like exopolysaccharide. These findings highlight both the influence of carbon source selection and the capacity of *B. thuringiensis* HA1 to valorize agro-industrial coproducts into diverse value-added products.

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