EXTRACELLULAR POLYSACCHARIDE PRODUCTION IN *BACILLUS LICHENIFORMIS* SVD1 AND ITS IMMUNOMODULATORY EFFECT

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Bacillus licheniformis SVD1 exhibited highest production of three different polysaccharides when sucrose was used as the carbon source for polysaccharide production and yeast extract was used as the nitrogen source. Polysaccharides were characterized using size exclusion chromatography (SEC), thin layer chromatography (TLC), gas chromatography with mass spectrometry (GCMS), and Fourier Transform Infrared (FTIR) analysis. Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) were used to examine the topography of the cells and polysaccharides. The cell-associated polysaccharides were composed of galactose, while two different polysaccharides were present in the extracellular medium, one of 2,000 kDa (EPS1), consisting of fructose monomers and identified as a levan with (2→6)-linkages and (1→2)-branching linkages. The other extracellular polysaccharide (EPS2) consisted of mannose and galactose and had a range of sizes as identified through SEC. All three polysaccharides displayed an immune modulatory effect as measured using Interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα).

Keywords: Bacillus licheniformis; Glycocalyx; Immunomodulatory; Levan; Multi-enzyme complex; Polysaccharide; Xylanases

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INTRODUCTION

Microorganisms produce extracellular polysaccharides that are either associated with the surface of the cell, capsular polysaccharides (CEPS), or found within the supernatant as exopolysaccharides (EPS), in which case it is also called slime (De Vuyst *et al.* 2001; Liu *et al.* 2010). These polysaccharides have several important applications in nature and industry. They form part of biofilms, where they function as an ion exchanger to trap and concentrate nutrients and to protect bacteria within the biofilm from antibacterial agents (Costerton 1999). Due to their rheological properties, bacterial polysaccharides are valued in the dairy industry as viscosifying, stabilizing, gelling, and emulsifying agents (Liu *et al.* 2010). Bacterial polysaccharides are also used as bioflocculants, bioabsorbents, heavy metal removal agents, and for drug delivery (Liu *et al.* 2010). They have also been demonstrated to have antitumor, antiviral, immunostimulatory, and anti-inflammatory properties (Liu *et al.* 2010).

Bacterial polysaccharides have very complex structures compared to plant polysaccharides. They do not have a uniform chemical composition and may differ substan-

tially from organism to organism (Erlandsen *et al.* 2004). The composition and structure is determined by the nutrients and carbon source within the growth medium, specifically divalent cation concentration and carbon-nitrogen ratio (Costerton 1999).

B. licheniformis SVD1 has been studied in our laboratory and found to produce a multi-enzyme complex (MEC) of 2,000 kDa with mainly hemicellulolytic activity (Van Dyk *et al.* 2009b, 2010). This micro-organism produced extensive extracellular polysaccharides, visible as distinct mucoid colonies, some of which appeared to be closely associated with the MEC. It was hypothesized that the polysaccharides may be involved in formation of the MEC in *B. licheniformis* SVD1. This study was, therefore, undertaken to investigate extracellular polysaccharide formation in *B. licheniformis* SVD1, which will be followed by a future study into the relationship between this polysaccharide and the proteins in the MEC.

EXPERIMENTAL

Organism and Culture Conditions

B. licheniformis SVD1 was routinely maintained on nutrient broth and stored as glycerol stocks at -20 ºC. The following optimized medium was used for production of extracellular polysaccharides: 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4$ (anhydrous), 40 g/L sucrose, and 10 g/L yeast extract.

Determination of Carbon and Nitrogen Requirements for Extracellular Polysaccharide Production

Two types of methods were utilized to determine the carbon and nitrogen requirements for production of extracellular polysaccharides. An initial screening method was based on the presence of mucoid colonies on agar plates as an indication of extracellular polysaccharide production (Liu *et al.* 2010). Agar plates were prepared in triplicate with different carbon sources namely glucose, arabinose, sucrose, xylose, cellobiose, galactose, and mannose, each at a concentration of 10 g/L. Serial dilutions were made of a log phase culture of *B. licheniformis* SVD1, plated and incubated at 37 ºC for 24 h. Plates were then assessed for growth and production of mucoid colonies. An assessment of the optimal nitrogen source was performed in a similar manner using yeast extract, peptone, tryptone, ammonium chloride, ammonium sulphate, and sodium nitrate at a concentration of 10 g/L.

Based on the results from the initial screening method, further flask studies were conducted to determine the carbon and nitrogen requirements for production of extracellular polysaccharides. Carbon sources that displayed good slime production were glucose, sucrose, cellobiose, and mannose, and these were selected for further investigation. Each of these carbon sources was used at different concentrations of 5, 10, 20, and 40 g/L in 50 mL of media. These were cultured at 37ºC with shaking for 48 h. Cultures were centrifuged at 12,000 g for 10 min, and the pellets and supernatants separated. The pellets were washed with 0.9% NaCl and then resuspended in 3 mL of 0.9% NaCl. The supernatants were precipitated with 3 volumes of absolute ethanol and left overnight at 4 ºC. After centrifugation at 12,000 g for 10 min, the pellets were resuspended in 5 mL distilled water and dialyzed against distilled water. Using the phenol-sulfuric acid method, total sugars associated with the cell pellets and in the supernatant were measured and compared.

In the same manner, 50 mL cultures using different nitrogen sources were used to determine the sugars associated with the cells and in the supernatant of cultures containing 10 g/L yeast extract, 10 g/L peptone, or 5 g/L yeast extract plus 5 g/L peptone. Only these nitrogen sources were used, as no mucoid colonies were detected using tryptone or inorganic sources of nitrogen.

Extracellular Polysaccharide Production over Time

Based on the screening methods as described above, a further experiment was conducted to determine the growth and production of extracellular polysaccharides over time. A sucrose culture (40 g/L) with yeast extract (10 g/L) in a 400 mL volume was prepared, inoculated, and incubated for a period of 96 h with shaking at 150 rpm. Samples of 30 mL were removed at various time periods, the pellet and supernatant were separated, and the polysaccharides in the supernatant were precipitated in the same manner as described above. Sugars associated with the cells and present in the supernatant were measured using the phenol-sulphuric acid method. Growth was determined by measuring cell optical density at 600 nm.

Phenol-Sulphuric Acid Assay

Total sugars were determined as glucose equivalents according to the modified method of Dubois *et al.* (1956) and Masuko *et al.* (2005). Samples containing sugars (100 μL) were placed in Eppendorf tubes. Concentrated sulphuric acid (300 μL) was added to the samples, followed by 60 μL of a 5% (w/v) phenol solution. Eppendorf tubes were vortexed, then heated at 90 ºC for 10 min and cooled down before removing 250 μL and taking readings on a microplate reader at a wavelength of 490 nm.

To determine the sugars in the fractions after size exclusion chromatography, a modified method was used based on Masuko *et al.* (2005). Samples of fractions (50 μL each) were placed in microtiter plate wells, to which 150 μL of concentrated sulphuric acid was added. This was followed by 30 μL of a 5% (w/v) phenol solution, after which readings were taken at 490 nm without any heating. While this method by Masuko *et al.* (2005) produced high standard deviations and was not suitable for accurate determination of sugar concentration, it allowed qualitative determination of sugars in the fractions from the size exclusion chromatography.

Electron Microscopy

Samples were prepared for visualization using transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM). Samples from a *B. licheniformis* SVD1 culture were removed at different time periods and prepared according to the method of Erlandsen *et al.* (2004). For TEM, samples were centrifuged and cells washed in 0.9% (w/v) NaCl. For FESEM, samples from cell cultures were vacuum-filtered using nylon membranes. The membrane was then cut into small pieces (5x5 mm) and prepared for microscopy. Both sets of samples were suspended in 2% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.15 M potassium phosphate buffer containing the following cationic dyes: 0.15% (w/v) alcian blue, 0.15% (w/v) ruthenium red, 0.15% (w/v) safranin O, 0.15% (w/v) alcian blue plus 0.15% (w/v) ruthenium red, 0.0075% (w/v) L-lysine hydrochloride, and 0.15% (w/v) alcian blue plus 0.0075% (w/v) L-lysine hydrochloride. After primary fixation in aldehyde with cationic dyes for 20 h, samples were washed in 0.15 M potassium phosphate buffer and post-fixed in 1% (w/v) OsO_4 in 0.15 M potassium phosphate buffer containing 1.5% (w/v)

potassium ferrocyanide. Samples were then dehydrated in ascending concentrations of ethanol.

For FESEM, membrane samples were prepared by critical point drying with CO_2, mounted on adhesive carbon film and coated with ~1 nm of carbon. Samples were examined using a Nova NanoSEM 230 field emission SEM with a landing energy of ≤2 keV.

For TEM, samples were embedded in Araldite-TAAB 812 resin and dried for 36 h at 60 ºC and then cut with a microtome into sections of approximately 100 nm thickness. Sections were mounted on copper grids (300 mesh) and viewed on a JEOL 1210 transmission electron microscope.

Purification of Extracellular Polysaccharides

A culture of *B. licheniformis* SVD1 was incubated for 48 h at 37 ºC with shaking. The culture was then centrifuged at 12,000 g for 15 min, and the supernatant was precipitated with a 3x volume of absolute ethanol. The supernatant with ethanol was kept at 4 ºC overnight before it was centrifuged at 12,000 g for 15 min. The pellet was resuspended in distilled water, and 3% (w/v) pepsin was added. This solution was incubated at 37 ºC for 24 h before the solution was heated at 100 ºC for 10 min. After boiling, the solution was centrifuged at 12,000 g for 15 min to remove the denatured protein. The supernatant was dialyzed against several changes of distilled water before it was lyophilized. This fraction was designated as the EPS.

Polysaccharides associated with the cells (capsular polysaccharides or CEPS) were purified by suspending the pelleted cells in 0.1 M NaOH and stirring for 3 h. The cells in NaOH were then centrifuged at 12,000 g for 15 min. The supernatant containing the CEPS was precipitated with 3x volume of absolute ethanol. After incubation at 4 ºC, the supernatant was centrifuged at 12,000 g for 15 min. The pellet was dialyzed against several changes of distilled water and was then lyophilized. This fraction was designated as the CEPS.

Protein Determination

Protein determination was performed using the method of Bradford (1976). Readings were taken at 595 nm, and the protein concentration was calculated according to a standard curve using bovine serum albumin (BSA) as a standard.

Size Exclusion Chromatography (SEC)

Samples of the EPS, after removal of protein, were suspended in small volumes of 50 mM NaCl, loaded on a Sepharose 4B column (50 cm x 2.5 cm), and eluted using 50 mM NaCl with 0.03% NaN_3. Elutions were collected as 3 mL sized fractions. Fractions were analyzed for sugars using the phenol-sulfuric acid assay. Two main peaks were collected as exopolysaccharide 1 (EPS1) and 2 (EPS2).

Trifluoroacetic Acid (TFA) Hydrolysis

Samples were hydrolyzed using 2 M TFA for 1 h (EPS1) and 6 h (EPS2 and CEPS) at 100 ºC on a digital dry bath. TFA was removed using a rotary vacuum evaporator and samples suspended in distilled water.

Thin Layer Chromatography (TLC)

Hydrolyzed samples were applied to Silica Gel 60 F254 HPTLC plates. Plates were developed with acetonitrile:water (9:1, v/v). To detect carbohydrates, plates were stained with a *p*-anisidine/phthalic acid (1.23%:1.66%) (w/v) stain in 95% ethanol, air-dried, and then heated at 110 ºC until spots appeared (approximately 5 to 10 min).

Fourier Transfer Infrared (FTIR) analysis

The mid-infrared absorption frequencies (4000 to 700 cm^{-1}) of purified, freeze-dried samples of EPS1, EPS2, CEPS, as well as levan (from *Zymomonas mobilis*, Sigma), were recorded using a Perkin-Elmer Spectrum 100 FT-IR spectrometer equipped with a universal attenuated total reflectance (ATR) accessory.

Gas Chromatography with Mass Spectrometry (GCMS) Analysis

For GCMS analysis, TFA-hydrolyzed samples were derivatized with methoxy amine and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and the sugars analyzed as trimethylsilyl (TMS) derivatives. Analysis was carried out with an Agilent 6890 N Gas Chromatograph (equipped with a HP5 column: 30 m, 0.25 mm ID, 0.25 μm film thickness), and an Agilent 5975 Mass Spectrometer. Derivatives were separated with a helium flow of 1 ml/min with a 1 μL injection, 1:10 split, and a temperature program starting at 70 ºC, ramped at 1 ºC per min until 76 ºC, then ramped to 310 ºC and held for 8 min.

GCMS Linkage Analysis

For GCMS linkage analysis, 1 mg quantities of the selected samples were methylated (CH_3I) (35 drops), hydrolyzed with TFA (500 µL, 2 M), reduced ($NaBD_4$) (10 mg in 500 µL DMSO), and acetylated (Ac_2O) (500 µL). The partially methylated alditol acetates were extracted into dichloromethane and analyzed on an Agilent 6890N GC (equipped with a HP5 column: 30 m, 0.25 mm ID, 0.25 μm film thickness) and Agilent 5975 Mass Spectrometer. The instrument settings were as follows: injector temperature: 280 °C; injection volume: 1 μL; split ratio: 1:10; constant flow: 1 mL/min; carrier gas: helium; MS transfer: 280 ºC EI+; electron energy: 70 eV; scanning mass range: 50 to 550 m/z; Solvent delay: 6 min. The temperature program started at 50 ºC (held for 2 min), ramped at 40 ºC per min until 130 ºC (held for 2 min), then ramped at 4 ºC per min to 250 ºC and held for 10 min.

Immune Response due to Polysaccharides

Blood samples from three, healthy donors were collected and diluted (1:10) using RPMI 1640 medium with added penicillin/streptomycin. EPS1, EPS2, and CEPS were dissolved in DMSO and 100 µL added at different concentrations (12.5, 25, 50, 100, and 200 μg/mL) to the blood/RPMI medium (final DMSO concentration 0.1%). Controls were included with medium only, medium with 0.1% DMSO (C), and lipopolysaccharide (LPS from *E. coli* O128:B12, Sigma) at a concentration of 5 μg/mL as a positive control (PC). The blood/RPMI medium containing the polysaccharides and controls were incubated at 37 ºC for 24 h. Diluted blood samples were used to determine the white blood cell count for each donor using a haemocytometer. Cells were stained using 0.1% (w/v) Brilliant Green in 2% (v/v) acetic acid. Blood cell counts were used to normalize results. After a 24 h exposure period, the 24 well plates were centrifuged at 900 g for 5 min at room temperature. Approximately 900 μL of the supernatant was removed from

each well and placed in Eppendorf tubes. These samples were stored at -80 ºC and used for detection of interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα) using ELISA kits (EBioscience). The protocol as provided by the manufacturer was followed. To determine whether any of the polysaccharides at the concentrations tested was cytotoxic, the MTT assay was used (Mosmann 1983). Results were statistically analyzed using Microsoft Excel ANOVA.

RESULTS AND DISCUSSION

Determination of Carbon and Nitrogen requirements for Extracellular Polysaccharide Production

An initial screening of carbon and nitrogen sources was carried out based on an assessment of the growth and mucoid colonies produced on agar plates. The best carbon and nitrogen sources for extracellular polysaccharide production were selected for further investigation. Of the carbon sources, sucrose, glucose, cellobiose, and mannose exhibited the highest percentage of mucoid colonies as well as good growth. Therefore, further flask experiments were conducted to differentiate between these carbon sources. Of the nitrogen sources, the inorganic sources resulted in poor growth and had no mucoid colonies. Tryptone as a nitrogen source also resulted in an absence of mucoid colonies. Therefore, further experiments were carried out with yeast extract and peptone in different combinations.

In the flask studies, the highest production of CEPS and EPS was found in sucrose cultures as demonstrated in Table 1. A 2% concentration of sucrose resulted in the highest amount of CEPS at 17.1 mg, while a 4% sucrose culture gave the highest EPS production of 54.96 mg.

Table 1. The Effect of Carbon Source and Concentration on Total Production of CEPS and EPS in *B. licheniformis* SVD1 (values are means n = 3 ± SD)

Carbon Source	% Carbon	Total Sugar in CEPS (mg)	Total Sugar in EPS (mg)
mannose	0.5%	0.47 ± 0.01	4.59 ± 0.07
	1.0%	0.63 ± 0.25	3.88 ± 0.02
	4.0%	8.18 ± 0.15	6.87 ± 0.09
glucose	0.5%	1.87 ± 0.05	4.33 ± 0.06
	1.0%	1.33 ± 0.01	4.29 ± 0.001
	2.0%	1.97 ± 0.001	5.37 ± 0.10
	4.0%	3.90 ± 0.07	5.42 ± 0.06
cellobiose	0.5%	1.61 ± 0.001	3.86 ± 0.02
	1.0%	1.80 ± 0.004	4.21 ± 0.06
	2.0%	2.48 ± 0.04	5.12 ± 0.03
	4.0%	5.27 ± 0.07	4.23 ± 0.004
sucrose	0.5%	3.19 ± 0.02	9.65 ± 0.07
	1.0%	7.91 ± 0.03	16.03 ± 0.03
	2.0%	17.10 ± 0.11	51.96 ± 0.02
	4.0%	11.04 ± 0.01	54.96 ± 0.004

It has been clearly indicated in the literature that the carbon source as well as the carbon concentration has an impact on polysaccharide production and yield (Cerning *et*

al. 1994) and that different sugars produce different polysaccharides. This was also apparent in the current study, where sucrose resulted in the highest polysaccharide yield, similar to another study using *B. licheniformis* (Liu *et al.* 2010). The sucrose concentration affected the type of polysaccharide produced, with CEPS and EPS yields being highest at different sucrose concentrations.

Further investigation of the best nitrogen source for extracellular polysaccharide production was carried out. The best cell growth was achieved with a culture containing 0.5% yeast extract and 0.5% peptone. However, with this nitrogen source, very low levels of CEPS and EPS were measured. Using 0.5% or 1% yeast extract in the medium resulted in similar levels of EPS being produced. However, in the culture medium using 1% yeast extract, higher growth as well as higher levels of CEPS production was achieved (data not shown). These results indicate that the source of nitrogen was an important factor. No polysaccharides were produced on inorganic nitrogen sources. Of the complex nitrogen sources, peptone and tryptone also appeared to suppress polysaccharide production, while yeast extract initiated polysaccharide production. Many studies have found complex nitrogen sources to be the best for polysaccharide production (Liu *et al.* 2010).

The carbon:nitrogen (C:N) ratio has been said to have an important impact on polysaccharide production (Costerton 1999) and has been investigated by a number of researchers. Many researchers have indicated that EPS was only produced under conditions where high levels of the carbon source were available under conditions of nitrogen limitation (DuGuid and Wilkinson 1953; Sutherland 2001). However, some studies have found that the C:N ratio did not influence EPS or CPS production (Bonet *et al.* 1993). Others found that the C:N ratio only had an impact on the molecular weight of the EPS, with higher complex nitrogen levels producing higher levels of the low molecular weight EPS (DeGeest & DeVuyst 1999; Marshall *et al.* 1995). In the current study, it was found that a change in C:N ratio did not affect the yield of EPS, although it had an impact on the CEPS yield. It is therefore clear that the issue of the C:N ratio is far more complex and requires further investigation.

Polysaccharide Production over Time

To determine polysaccharide production over time, samples were taken from a 400 mL culture containing 4% sucrose and 1% yeast extract. In Fig. 1 it can be observed that CEPS reached a maximum level during the logarithmic phase of growth, while a reduction in the sugars associated with the cells was observed during the stationary phase after 40 h.

EPS production increased during the logarithmic phase of growth but remained fairly constant once stationary phase of growth was reached. In reports in the literature, Bonet *et al.* (1993) found that both CEPS and EPS were produced at the end of logarithmic phase, while Larpin *et al.* (2002) found that highest EPS production took place in the middle of the logarithmic phase.

The result in the current study, where the EPS formation followed the same trend as the growth curve, was also found by DeGeest and DeVuyst (1999). The different trends found with respect to the production of CEPS and EPS could be linked to a nutrient limitation.

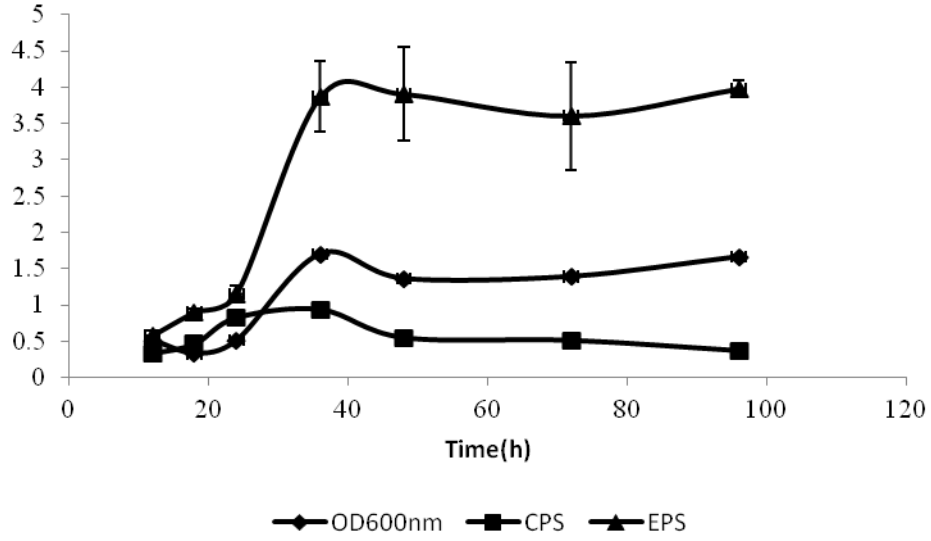


Fig. 1. Graph displaying the growth of a 4% sucrose culture of *B. licheniformis* SVD1 over time as measured using optical density at 600 nm, by the presence of sugars associated with the cells (CEPS), and present in the supernatant (EPS). Data points represent means ± SD, n = 3.

Electron Microscopy

TEM and FESEM were used to visualize the polysaccharides associated with the cells, as well as those present in the intercellular matrix. Various cationic dyes were used to stabilize the polysaccharides based on the method by Erlandsen *et al.* (2004). In the presence of the cationic dyes, the surface topography of the cells and the features of the extracellular polysaccharides were enhanced.

Based on the FESEM (Fig. 2 A, C, E) and TEM (Fig. 2 B, D, F) images, certain features of the extracellular polysaccharides can be observed. A fibrous network between cells appeared to link cells together (Fig. 2, denoted (a)). It can furthermore be observed that extensive cell surface detail was visible, which in some cases appeared to form part of the fibrous network (Fig. 2, denoted (b)). A further feature is the nodule-like cell protrusions which in some cases appear to be detached from the cells and trapped in the fibrous network of polysaccharide between the cells (Fig. 2, denoted (c)).

The fibrous network as observed with FESEM appeared very similar to FESEM micrographs of *Pseudomonas putida* G7 (Kachlany *et al.* 2001), which the authors concluded to be EPS that collapsed due to the dehydration in preparation of the samples. However, based on SEM photomicrographs of various flagellated bacteria and biofilms (Erlandsen *et al.* 2003; Morikawa *et al.* 2006), it is also probable that the fibrous network observed in this study could be multiple flagella.

The protuberances on the cells resemble cellulosomes such as found in *Clostridium thermocellum* (Bayer and Lamed 1986); such protuberances have been previously reported for *B. licheniformis* SVD1 in a study on its multi-enzyme complex formation (Van Dyk *et al.* 2009a). This would require further investigation as it is not possible at this stage to distinguish the levan from the other polysaccharides formed by this organism.

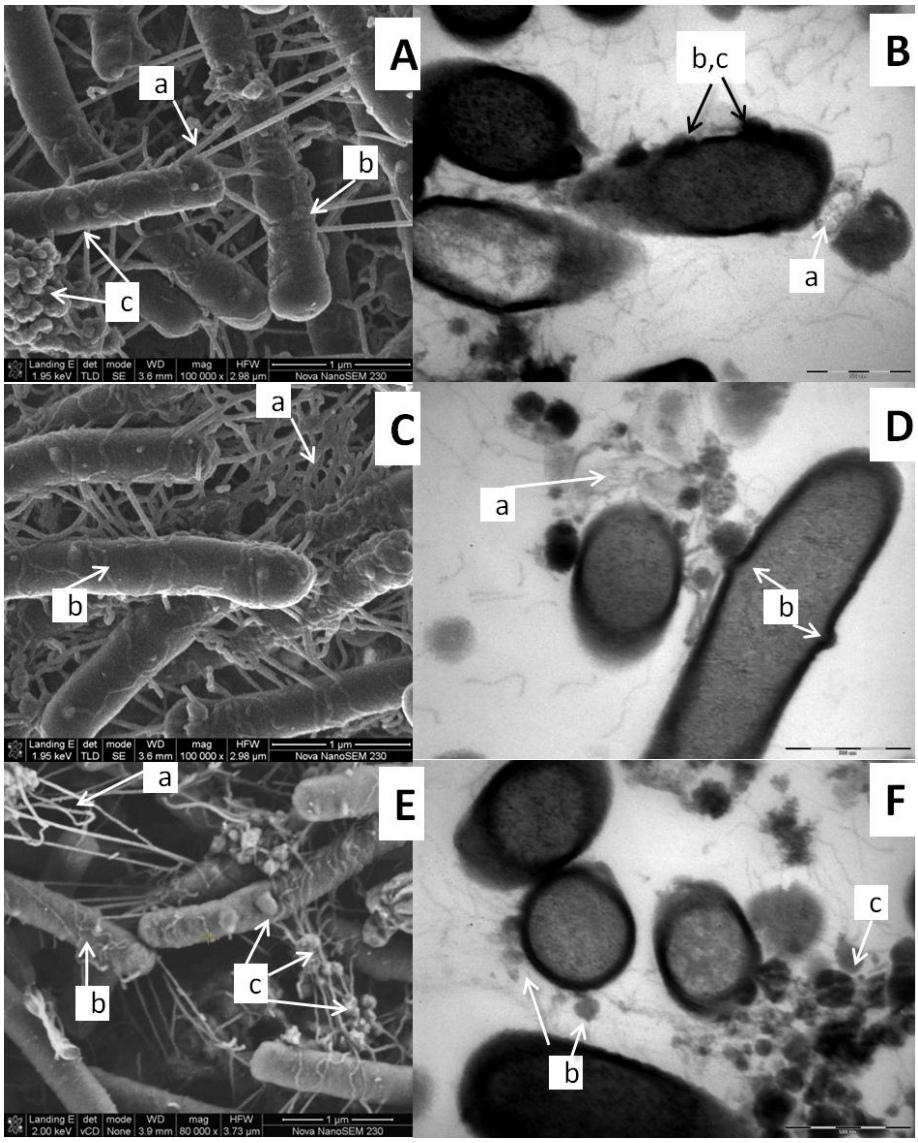


Fig. 2. Characteristics and features of EPS and CEPS visualized using FESEM (A, C, and E) and TEM (B, D, and F). (a) - fibrous network of EPS around and between cells clearly showing the attachment of these to the cells, (b) – cell surface detail, (c) – round globular features of EPS formed on cell surface and present in large quantities between cells.

Purification of EPS and CEPS

The initial purification step fractionated total polysaccharides into EPS and CEPS, after which the EPS was further separated into EPS1 and EPS2 using SEC with Sepharose 4B resin. Total EPS purified from a 4% sucrose culture yielded 1.9 g/L of polysaccharides based on measurement of sugars using the phenol-sulphuric acid method. The total EPS also contained 38.8 mg/L of protein, which was removed through pepsin digestion. After the pepsin digestion and removal of protein, EPS was separated into two fractions, EPS1 and EPS2, based on the presence of sugars in the fractions (Fig. 3A). EPS1 eluted at a similar position to dextran blue (2,000 kDa) and was therefore identified as a polysaccharide of approximately 2,000 kDa. EPS2 eluted over a broad range of fractions of various sizes from 70 to 700 kDa. Several studies have also isolated different size polysaccharides (DeGeest and DeVuyst 1999; Marshall *et al.* 1995). DeGeest and

DeVuyst (1999) found a high molecular weight polysaccharide of $1.8x10^6$, as well as a low molecular weight polysaccharide of $4.1x10^5$ kDa.

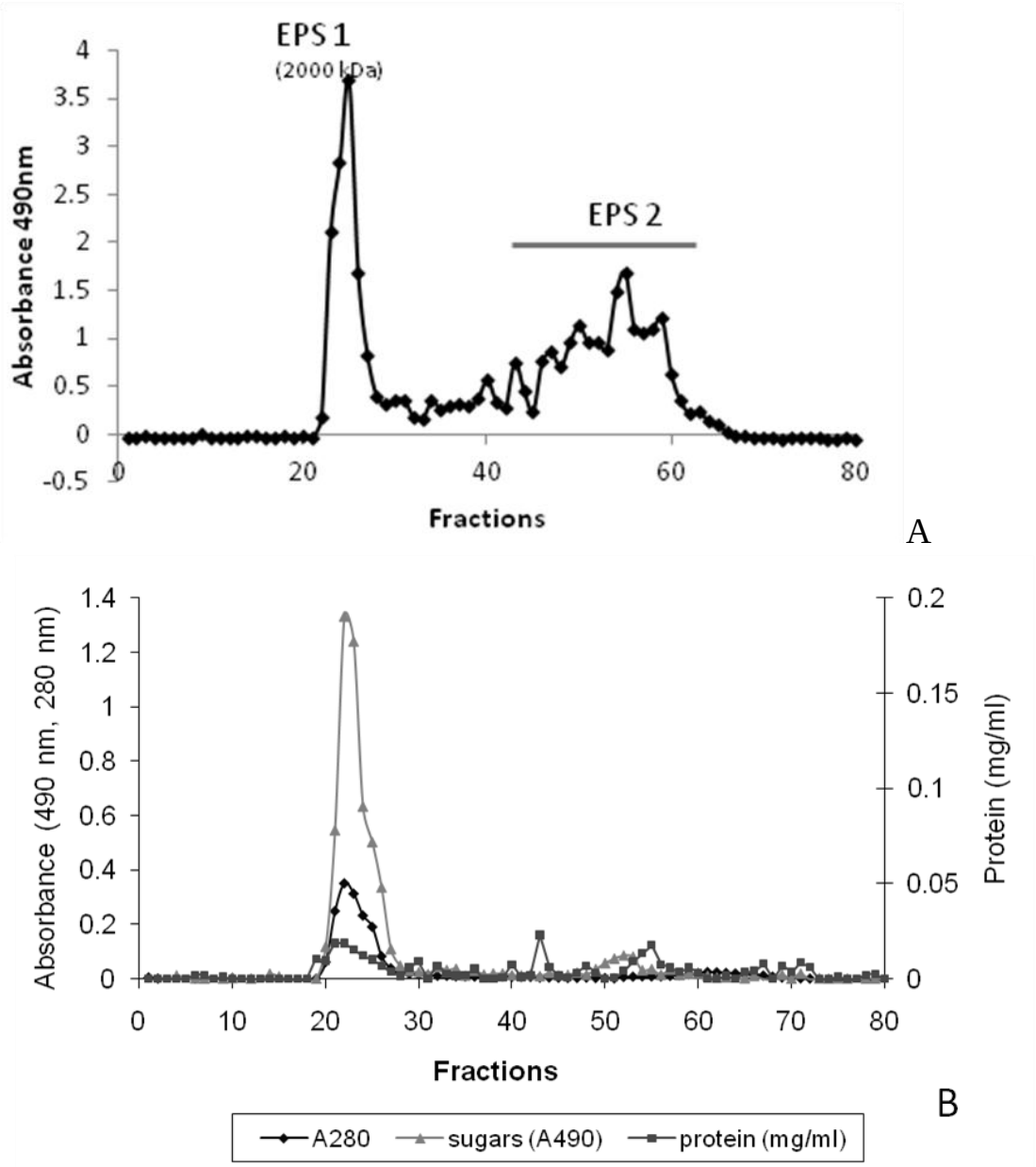


Fig. 3. Sepharose 4B chromatograms from purification of polysaccharides. Fig. 3A is the chromatogram displaying purification of EPS1 and EPS2 after removal of protein through pepsin digestion. Fig. 3B is a chromatogram of EPS1 before removal of protein, indicating that the polysaccharides and protein displayed an identical elution. The Sepharose 4B column was 50 x 2.5 cm, and fractions of 3 mL were collected (flow rate 40 mL/h). Blue dextran (2,000 kDa) (Fraction 20), thyroglobulin from bovine thyroid (670 kDa) (Fraction 47) and laccase from *Trichoderma reesei* (70 kDa) (Fraction 64) were used as standards.

EPS1 eluted in the identical position to the MEC previously isolated from *B. licheniformis* SVD1 in previous studies (Van Dyk *et al.* 2009b). Prior to the pepsin digestion the EPS1 and protein eluted on Sepharose 4B exhibited a protein peak in the

same position (Fig. 3B). EPS1 may therefore be associated with the proteins at 2,000 kDa, and this will be further investigated.

Analysis of Composition of Polysaccharides

Various analytical techniques were used to characterize the polysaccharides isolated from *B. licheniformis* SVD1 with the focus on EPS1. TLC was carried out after TFA hydrolysis of EPS1, EPS2, and CEPS to determine the sugar composition of these fractions. The results indicated that these three polysaccharide fractions had very different compositions, with EPS1 and CEPS composed of a single, but different sugar, putatively fructose and galactose, respectively, while EPS2 had a composition with two sugars, putatively mannose and galactose. Putative identification using TLC was followed up with GCMS analysis to confirm the sugar composition.

GCMS results of hydrolyzed, derivatized polysaccharides indicated that EPS1 consisted of D-fructose, while EPS2 was composed of D-mannose, and D-galactose, and CEPS of D-galactose.

Linkage analysis was only conducted on EPS1, as this polysaccharide was the main focus of this study. Methylation, acid hydrolysis, reduction, and conversion into alditol acetates were performed, followed by analysis of products using GC-MS. The fragmentation patterns of the GC-MS peaks were identified as 2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methyl hexitol, and 1,2,5,6-tetra-*O*-acetyl-(2-deuterio)-3,4-tri-*O*-methyl hexitol.

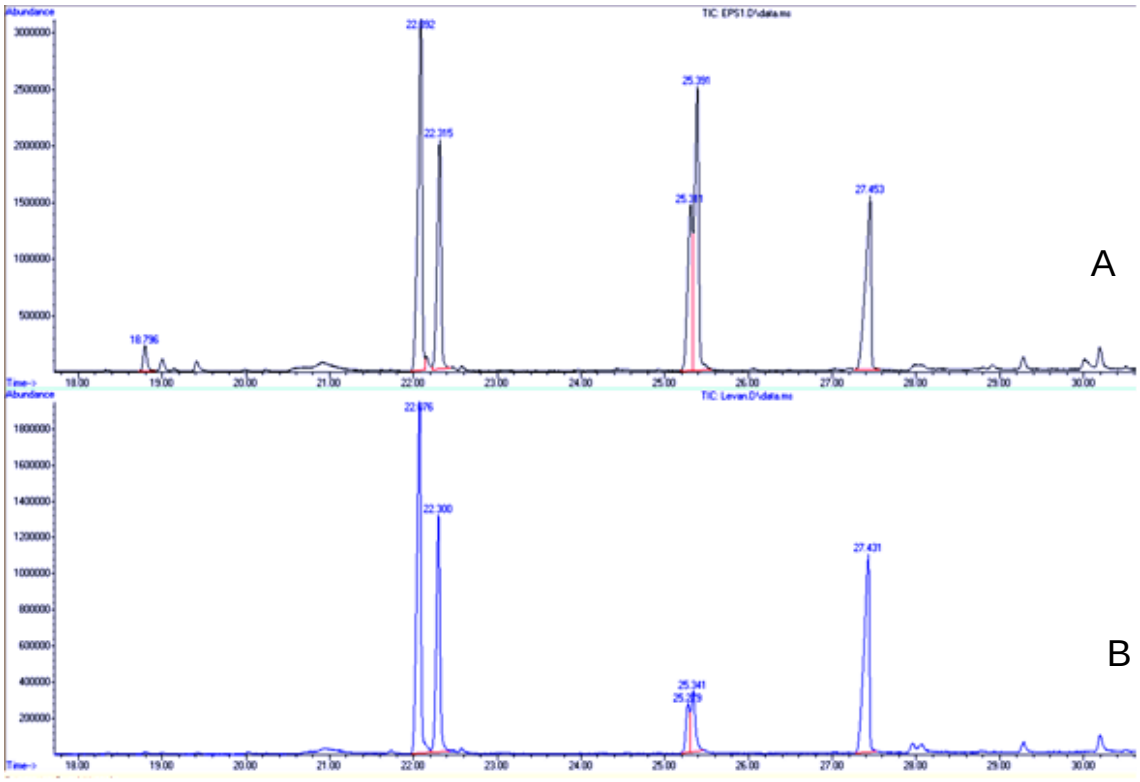
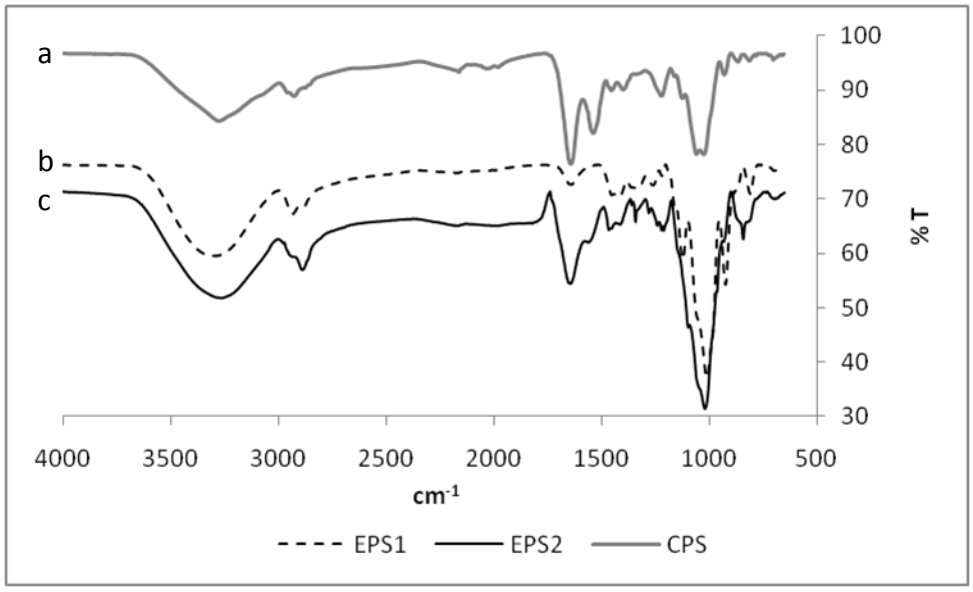


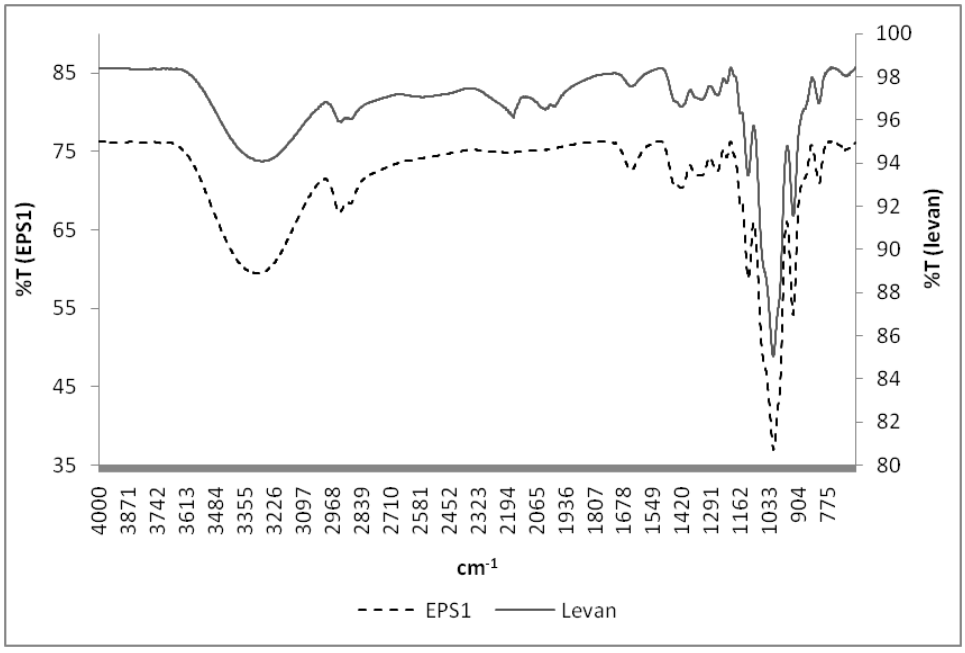
Fig. 4. GCMS linkage chromatograms of EPS1 (Fig. 4A) and commercial levan from *Zymomonas mobilis* (Fig. 4B)

Based on the reference spectra in Carpita and Shea (1989), and the fact that the MS fragmentation patterns corresponded to that of a commercial levan from *Zymomonas mobilis*, it was confirmed that EPS1 was a fructose polymer with the backbone having a (2→6)-linkage and branching formed by a (1→2)-linkage. The peaks corresponding to the (1→2)-linkage, indicated a larger extent of branching in EPS1 (Fig. 4A) compared to the commercial levan (Fig. 4B).

Analysis was further carried out using ATR-FTIR, and the results (Fig. 5A) confirmed that EPS1, EPS2, and CEPS were all polysaccharides, as the infrared spectra displayed distinctive characteristics of polysaccharides.



A



B

Fig. 5. ATR-FTIR spectra of (A): CEPS(a), EPS1(b) and EPS2(c) and (B): and a comparison of ATR-FTIR spectra of EPS1 and levan from *Zymomonas mobilis* (B)

All three compounds displayed a broad band between 3000 and 3500 cm^{-1}, which is distinctive of the hydroxyl stretching vibration, while the band between 2800 and 2900 cm^{-1}, present in all three compounds, is ascribed to the C-H stretching and bending vibrations (Kucukasik *et al.* 2010). The bands present at 1600 cm^{-1} are generally indicative of amide bonds (Bramhachari *et al.* 2007). The region between 1000 and 1200 cm^{-1} is considered the fingerprint region for carbohydrates and is different for each carbohydrate (Grube *et al.* 2002). It is dominated by ring vibrations overlapping with stretching vibrations of (C–OH) side groups and glycosidic band vibrations (C–O–C) (Grube *et al.* 2002).

As the structure of EPS1 was suspected to be levan, the ATR-FTIR spectrum of EPS1 was compared with a commercial levan, and the results are displayed in Fig. 5B. From this comparison, it appeared that the structure of EPS1 was very consistent with that of levan, with only the small bands at 1600 cm^{-1} differing between the two compounds, probably due to higher levels of protein present in levan. Thus, we were able to conclude that EPS1 was definitely a levan similar to the levan produced by *Zymomonas mobilis*.

Polysaccharide production is very common in all bacteria and has also been reported in various strains of *B. licheniformis*, although the composition of the polysaccharides displayed substantial variation. Li *et al.* (2009) reported a polysaccharide containing mannose, galactose, xylitol, rhamnose and galacturonic acid, which was produced using starch and yeast extract. Maugeri *et al.* (2002) produced an EPS from *B. licheniformis* consisting of mannose using glucose as a carbon source. Singh *et al.* (2011) used a strain of *B. licheniformis* to produce two polysaccharides of different sizes composed of glucose, galactose, mannose and arabinose.

Levan is produced by many microorganisms (Ghaly *et al.* 2007; Poli *et al.* 2009) and is a homopolymer consisting of fructose monomers with β-(2→6)-linkages, with branching often occurring as β-(2→1)-linkages. In bacteria, levan is synthesised and polymerised outside the bacterial cell, and has been implicated in the formation of biofilms (Koczan *et al.* 2009). Bacterial levan is produced by the transfructosylation reaction of levansucrase (EC 2.4.1.10) (Ammar *et al.* 2002), although the same enzyme is also able to catalyse hydrolysis reactions.

Levan has many applications in various industries, including the medical/ pharmaceutical industry, where it is used as a blood plasma extender, hypocholesterolemic agent, tablet binder and antitumor agent. It is also used in the food industry as a sweetener, emulsifier, stabilizer and thickener and encapsulating agent. Other applications of levan include its use as an adhesive, cosmetic, plugging agent, and surface finishing agent (Ghaly *et al.* 2007).

The levan structure and size is not uniform and varies depending on the microorganism and the culture conditions. Sucrose is generally the basis for levan production, while yeast extract has been used as a nitrogen source (De Oliveira *et al.* 2007), similar to that used in this study. Although some authors have used very high concentrations of sucrose, Hettwer *et al.* (1995) indicated that at concentrations higher than 10% sucrose, the hydrolysis reaction of the levansucrase was preferred to the levan formation reaction. The molecular weight and branching affects the functioning of the levan (Calazans *et al.* 1997; Kucukasik *et al.* 2010). As levan is an important and very useful polysaccharide, it may be useful to investigate ways in which levan production in *B. licheniformis* SVD1 may be increased to become the predominant polysaccharide.

Immunostimulatory Response

The polysaccharides isolated in this study were investigated for their effect on the immune response in human blood samples, as this has been demonstrated to be an important application of bacterial polysaccharides. EPS1 and EPS2 stimulated IL6 expression even at low levels of 12.5 μg/mL, while CEPS stimulated IL6 levels in a dose-dependent manner (Fig. 6A). EPS1 suppressed TNFα levels in a dose-dependent manner, while CEPS increased TNFα levels (Fig. 6B). EPS2 induced TNFα production at all concentrations tested, and even at concentrations as low as 12.5 μg/mL, induction of TNFα levels was similar to the LPS control.

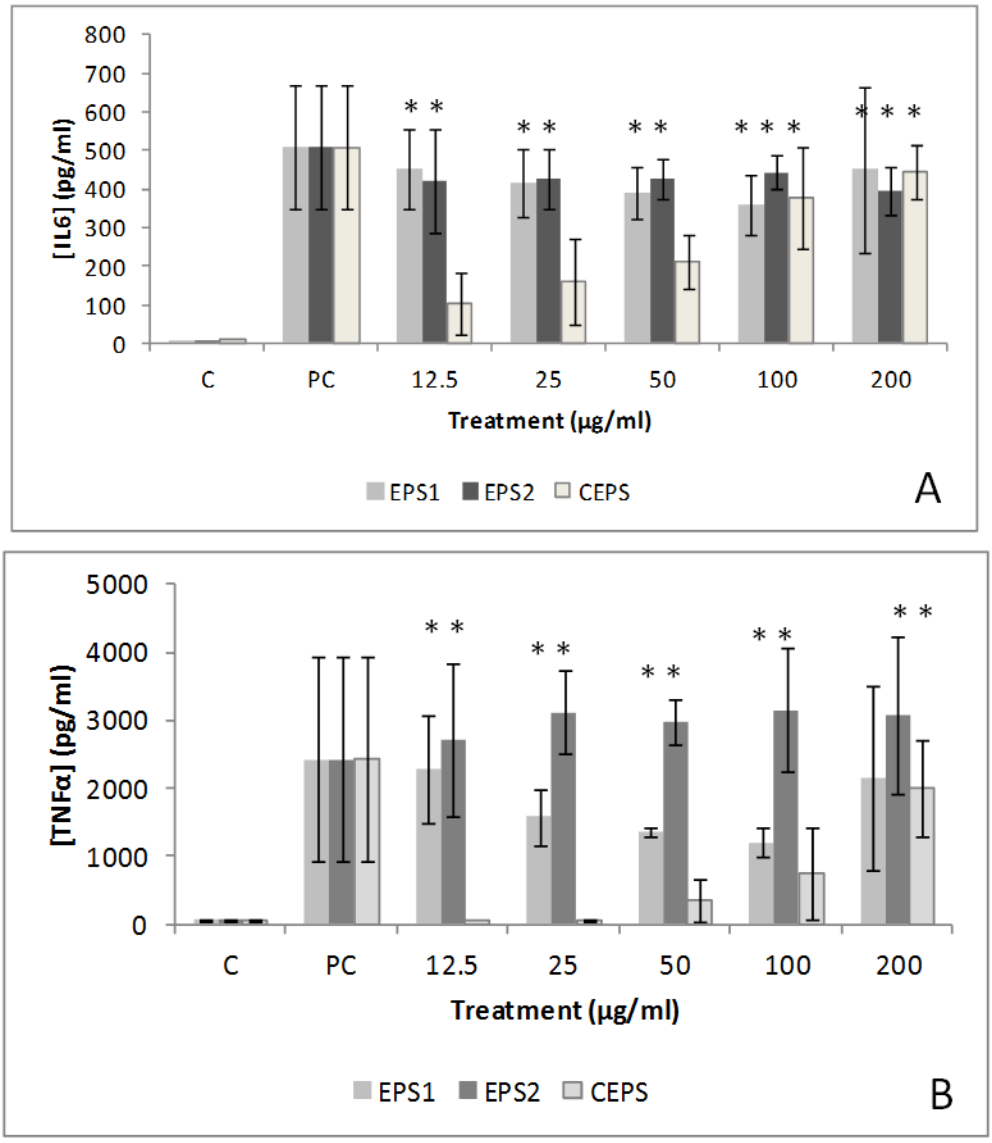


Fig. 6. Induction of IL6 (A) and TNFα (B) by EPS1, EPS2 and CEPS. C = medium control, PC = positive control (LPS) concentration (n = 3). * P ≤ 0.01 relative to C

Based on the results from the MTT assay, none of the polysaccharides displayed cytotoxicity at any of the concentrations tested (12.5 to 200 μg/mL).

Surface or capsular polysaccharides and/or lipopolysaccharides in bacteria elicit an immune response that confers a protection against diseases caused by such bacteria (Weintraub 2003). These polysaccharides have therefore been used in vaccinations to build up resistance to infection, although these have some limitations (Weintraub 2003). However, bacterial polysaccharides that are not associated with the cell surface have also been found to have immunomodulatory, anti-cancer, and antiviral effects (Arena *et al.* 2009; Liu *et al.* 2010). Thus they may have application as a natural immune modulator. Arena *et al.* (2009), for example, investigated the ability of bacterial polysaccharides to enhance immunity of cells against herpes simplex virus (HSV), a virus that is able to block the production of cytokines "in order to evade the immune response of the host". They utilised an extracellular polysaccharide of *B. licheniformis* to enhance cytokine production in cells and enhance the immune response against HSV infection (Arena *et al.* 2009). The specific antiviral effect was linked to the pattern of cytokines induced by the polysaccharide. In the current study, the three different polysaccharides, EPS1, EPS2, and CEPS displayed a different response in enhancement of cytokine production, with EPS1 suppressing TNFα levels while EPS2 and CEPS increased TNFα levels. Further *in vivo* and *in vitro* studies can be conducted to determine the full immunomodulatory potential of these polysaccharides and the specific pattern of cytokine production.

In the current study, EPS1 was identified as a levan polysaccharide. Levan has been identified in several studies as an important immune modulator and was found to display antitumor activity (Yoon *et al.* 2004) and prebiotic properties (Dal Bello *et al.* 2001). The extent of branching in levan has also been demonstrated to have an impact on its anti-tumor activities. Therefore it is important to study new sources of bacterial levans, as they may have different anti-tumor properties. This will be further investigated in future work.

CONCLUSIONS

1. *Bacillus licheniformis* SVD1 produced three different extracellular polysaccharides.
2. The optimal carbon and nitrogen sources for polysaccharide production were sucrose and yeast extract.
3. A cell-associated polysaccharide (CEPS) consisted of mainly galactose monomers, while the two extracellular polysaccharides consisted of a large polysaccharide of 2,000 kDa (EPS1), confirmed to be a levan with (2→6)-linkages in the main chain and (1→2)-linkage branching. A second extracellular polysaccharide (EPS2) was determined to be composed of mannose and galactose.
4. All three polysaccharides (EPS1, EPS2, and CEPS) displayed an immune modulatory effect as measured using interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα).

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