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Streptococcus agalactiae serotype Ia capsular polysaccharide production from a Brazilian strain

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ABSTRACT

The pathogen *S. agalactiae*, categorized as Lancefield group B streptococci (GBS), is a Gram-positive bacterium, encapsulated, anaerobic facultative bacillus, which occurs in pairs or short chains. It was responsible for more than 319,000 cases of infections in neonates. Early and late streptococci, in the course of an illness or medical condition, are infections that have high mortality and morbidity rates, with an incidence of 2.0 to 5.0 per 1000 live births in North America, Europe, and Australia. The infection of newborns usually happens through transmission from infected mothers during and after childbirth, with GBS being among the critical bacterial infections in the Brazilian epidemiological picture. *S. agalactiae* strains isolated from different regions of Brazil, indicate a great diversity among the isolated serotypes but with a higher incidence of serotype Ia. Publications report that, in pregnant women in Brazil, the prevalence of serotype Ia has been constant between 2002 and 2018, followed by serotypes II and IV. Serotypes Ib, III, and V also appear, but with some fluctuations in different regions of the country. The pathogen which causes invasive disease are usually surrounded by a polysaccharide capsule and is its major virulence factor, being that the key antigen in protective response triggered by polysaccharides and, more recently, protein-polysaccharide conjugate vaccines. The development of antibiotic resistance by these microorganisms to different antibiotics has been the guideline in leading therapeutic options against these infections including the use of prophylactic vaccines in pregnant women, immunosuppressed or older people. Such prophylactic alternatives can be constructed from capsular polysaccharides.

Based on the experience in the production and purification of Neisseria meningitidis polysaccharides for vaccines, Bio-Manguinhos developed a process for the production, purification and characterization of *S. agalactiae* Ia polysaccharides. In the present study, the capsular polysaccharide from the strain isolated in clinical material of a public hospital, in Rio de Janeiro – Brazil, was obtained from simple batch cultures, with purification process achieving about 20 mg g^{-1} in yield for cell dry weight (CDW) and ca. of 100 % of purity. Hence, to prove that the CPS of GBS Ia can be consistently produced, purified, and characterized by NMR, HRGC-MS, and SEC-MALS – from such strain – to be used in vaccine productions against *S. agalactiae*.

1. Introduction

The pathogen *S. agalactiae* is a Gram-positive bacterium, encapsulated, anaerobic facultative bacillus, that occurs in pairs or short chains. Globally, it was responsible for more than 319,000 cases of infections in neonates, with more than 90,000 deaths associated with that just in 2015 (Carreras-Abad et al., 2020; Seale et al., 2017). Also known as Lancefield group B streptococci (GBS), it appears as a significant threat of invasive infection in neonates, characterizing a problem common to several countries (Johri et al., 2006, 2013; Seale et al., 2017). Early-onset and late-onset *streptococci* infections have high mortality and morbidity rates, with an incidence of 2.0 to 5.0 per 1000 live births in

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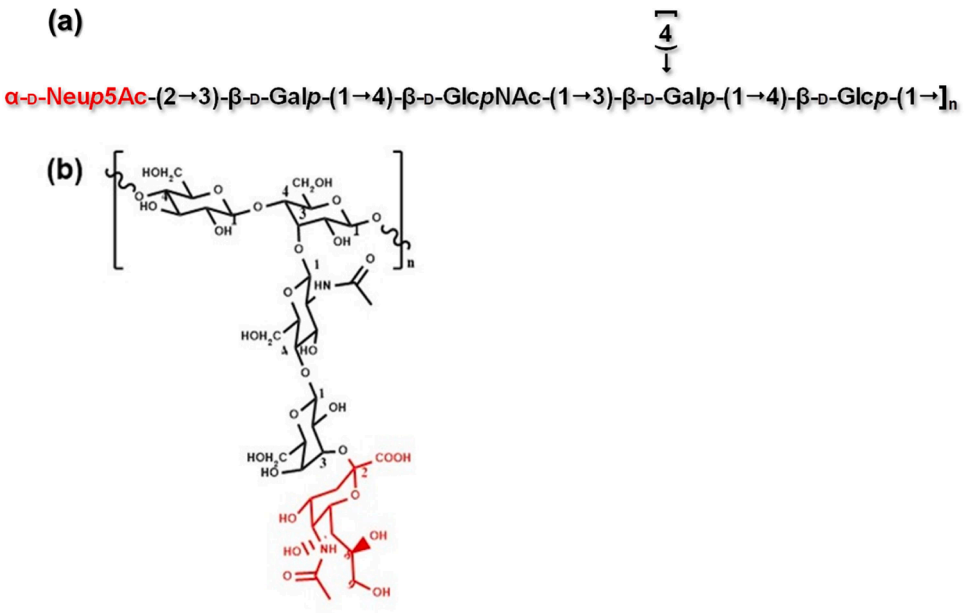

Fig. 1. *S. agalactiae* monomer structure and connectivity: (a) – Formal text notation for CPS of S. agalactiae hetero-monomer;(b) – Structural representation for CPS of S. agalactiae monomer, with Neu5Ac highlighted in red.

North America, Europe, and Australia. The infection of newborns usually happens through transmission from infected mothers during and after childbirth (Melin & Efstratiou, 2013). *S. agalactiae* is among the critical bacterial infections in the Brazilian epidemiological picture. A study of genotypic and phenotypic diversity conducted with 392 strains of *S. agalactiae*, isolated between 1980 and 2006, indicates that the serotypes prevalent in the country have been Ia (25.9 %), V (17.9 %), III (14.2 %), II (13.6 %), with around 30 % nontippable (Johri et al., 2013). More studies by Dutra et al. (2014), in which strains isolated from different regions of Brazil were genotyped, indicate a great diversity among the isolated serotypes but with a higher incidence of serotype Ia. Publications report that, in pregnant women in Brazil, the prevalence of serotype Ia has been constant between 2002 and 2018, followed by serotypes II and V. Serotypes Ib, III, and IV also appear, but with some fluctuations in different regions of the country (Botelho et al., 2018; do Nascimento et al., 2019). In Rio de Janeiro State - Brazil, results for 3647 women screened over eight years (Botelho et al., 2018) showed the detection of GBS anogenital colonization in 26.2 %. The serotype Ia was identified as the most prevalent from the contaminated group, found in about 37.3 % of the cases.

The ten antigenically distinct serotypes (Ia, Ib, II-IX) are identified by the chemical structure of its polysaccharide capsule. This taxonomical classification defines as Ia the complex capsular polysaccharide from group B *Streptococcus agalactiae* (CPS of GBS Ia), a high molecular weight polymer consisting of a repeating unit of pentasaccharide being formed by two units of $_{D}$-galactose ($_{D}$-Gal*p*), one unit of $_{D}$-glucose ($_{D}$-Glc*p*), one unit of *N*-acetyl-$_{D}$glucosamine ($_{D}$-Glc*p*NAc) and *N*-acetylneuraminic acid – sialic acid – ($_{D}$-Neu*p*5Ac). The lateral branching of the polymeric chain may or may not be sialylated, which results in different yields of sialic acid for different strains or species, and Neu*p*5Ac is well known as an antiphagocytic factor for many bacterial species, including the GBS group, also contributing to adhesion, colonization etc. (Charland et al., 1996; Hussain et al., 2013) (Fig. 1). The polysaccharide sialylation level plays an important role in the CPS since it is associated with microorganism virulence, as shown by Kazatchkine et al. (1979). Their papers show that the relative abundance of sialic moieties in CPS can or cannot activate the human alternative complement pathway that contributes to opsonization, phagocytosis, and lysis of the invader pathogen, being quite important in the immunologic scenario.

The capsule is the main virulence factor of this microorganism what helps in the progression of the disease by hindering phagocytosis, except in the presence of antibodies with opsonizing activity. The pathogen virulence is complex and multifactorial, which is a necessary causative issue of opportunistic infections in pregnant women, newborns, and the elderly people clientele (Maisey et al., 2008). The use of penicillin has been the most frequent option in treating *S. agalactiae* infections due to the sensitivity of these bacteria to β-lactams. However, antibiotic resistance has been increasingly observed in the same way as for *Streptococcus pneumoniae* (Plainvert et al., 2023). Substantial resistance to macrolides, tetracyclines, lincosamides, and fluoroquinolones has been described, indicating the Antibiotic Resistance (AR) phenomenon occurring for 3rd generation drugs. The development of AR by these microorganisms to different antibiotics has been the guideline in leading therapeutic options against these infections and includes the use of prophylactic vaccines in pregnant women, immunosuppressed, and older people, which can be constructed from capsular polysaccharides (Baker et al., 1988; Dutra et al., 2014; Johri et al., 2013).

Protection against these organisms is highly dependent on circulating serum antibodies due to the rapid development of the disease after infection, which can result in death within hours (Sadarangani, 2018). While vaccination stimulates the development of B cell memory, it takes several days after exposure to the pathogen for a detectable immune response to occur.

Most B-cell responses are T-cell dependent. Nevertheless, studies have demonstrated that capsular polysaccharides generate T-cell independent immune responses (Siegrist, 2013; Siegrist & Labert, 2016; Williams, 2011). As a result, formulations of polysaccharide-based immunogens may lack the development of specific antigens and immunological memory. Notably, despite the effectiveness of such formulations in adults, they prove ineffective in protecting children under 2 years of age, likely due to the immaturity of their immune systems (Astronomo & Burton, 2010; Jones, 2005).

Currently, only a few vaccines are still on the market based solely on polysaccharides. They are designed for immunization against pneumococcal diseases, infections by meningococci, *Salmonella* Typhi, and *H. influenzae* (Hamborsky et al., 2015).

Associated with issues such as the lack of promotion of memory cells and the inefficiency of these vaccines for infants under 2 years, it is important to highlight that capsular polysaccharides (CPS) also contribute to the characterization of various serotypes within microbial

species. The virulence or pathogenicity of a serogroup may be linked to its serotype, and cross-immunogenicity is not necessarily found between serogroups (Jones, 2005). Consequently, a vaccine formulation based on a CPS related to a serotype of a microbial species may not necessarily provide protection against the entire spectrum of serogroups related to that microorganism. The common approach is formulating a vaccine for each serotype or combining multiple antigens in a single formulation, establishing the concept of a combined or multivalent vaccine (Astronomo & Burton, 2010; Mohanty & Sai Leela, 2014).

Building upon the previously emphasized limitations of polysaccharide vaccines, the strategy of conjugating a polysaccharide or oligosaccharide with a protein signifies a strategic evolution. This conversion of a T-independent antigen into a T-dependent one holds the promise of augmenting the induced immune response. As a result, this approach not only facilitates the generation of more specific antibodies but also fosters the establishment of Bcell-mediated memory, even in young children, which can provide rapid responses upon administration of future vaccine doses (Avci, 2013; Lesinski & Westerink, 2001). There are still no licensed vaccines against GBS although there are some in advanced stages of clinical studies, and it is important to highlight that the protective mechanisms against this pathogen are not yet as well established as for microorganisms like *N. meningitidis, S. pneumoniae* and *H. influenzae* (Absalon et al., 2021; Kasper et al., 1999; Sadarangani, 2018). Based on the expertise gained in the production and purification of *N. meningitidis* polysaccharides for vaccines, Bio-Manguinhos/Fiocruz has developed a preliminary process for the production, purification, and characterization of *S. agalactiae* Ia polysaccharides. In this current investigation, the capsular polysaccharide from strain RS72, isolated from clinical material obtained from a public hospital in Rio de Janeiro, Brazil, was acquired through simple batch cultures.

The objective of this study is to demonstrate that the capsular polysaccharide (CPS) of Group B Streptococcus (GBS) Ia can be successfully produced, purified, and characterized using NMR, HRGC-MS, and SEC-MALS techniques. The CPS derived from strain RS-72 is intended for use in subsequent vaccine production against *S. agalactiae*.

Although the CPS of GBS Ia structure is well-known (Carlo et al., 1985; Jennings et al., 1983; Yamamoto et al., 1999), its analysis is firstly required to ensure its hetero-monomer composition and to qualify and quantify Neu*5*Ac yield in the Brazilian strain, since the high yield of Neu*5*Ac is directly related to the virulence factor (Lewis et al., 2006).

In the present study, the capsular polysaccharide the strain isolated from clinical material of a public hospital in Rio de Janeiro – Brazil, was obtained from simple batch cultures. Hence, to prove that the CPS of GBS Ia can be consistently cultivated, harvested, purified, and characterized by NMR, HRGC-MS, and SEC-MALS – from such strain – to be used in vaccine productions against *S. agalactiae*.

2. Material and methods

2.1. General reagents

The CPS of GBS Ia acetylation reactions were made using, as references, monosaccharide standards including d-glucose (D-Glc), d-galactose (D-Gal), d-*N*-acetylneuraminic acid (D-Neu*5*Ac) and d-*N*-acetylglucosamine (D-Glc*N*Ac) purchased from Merck Brazil reagents. The substances *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), ethanol, and cetyltrimethylammonium bromide (CTAB) were purchased from Merck Brazil reagents as well and were analytical grade.

2.2. Tangential flow filtration apparatus

All tangential filtration processes were accomplished using a Centrasette™ Lab Tangential Flow Filtration System, Cytiva (formerly Pall Lab) apparatus, and Merck Millipore Pellicon® 2 Biomax® Microfiltration modified polyethersulfone cassettes.

2.3. Strain

Clinical bacterial strain isolate of *Streptococcus agalactiae* serotype Ia (strain RS-72) was kindly supplied by Dr. Lucia Martins Teixeira (Instituto de Microbiologia Professor Paulo Góes, Universidade Federal do Rio de Janeiro, Brazil). The use of the strain was registered in the National System for Management of Genetic Heritage and Traditional Knowledge as *Streptococcus agalactiae* serotype Ia (SISGEN; ABEE 191), linked to the Brazilian Ministry of the Environment, together with the Genetic Heritage Management Council.

2.4. Culture media

Fermentation was performed in a medium described by Carlo et al. (1985) with some modifications, which was composed of 30 g L^{-1} tryptic soy broth of non-animal origin, 22.5 g L^{-1} glucose, and 10 g L^{-1} yeast exact, and whose pH was adjusted to 7.2 with 5 mol L^{-1} NaOH solution. The medium for the inoculum and bioreactor was sterilized by filtration through a 0.22 μm pore size filter.

2.5. Inoculum preparation and fermentation

For inoculum preparation, cells were streaked on 5 % (v/v) sheep-blood Columbia agar plates and incubated at 37°C in a 5 % CO_2 atmosphere (Thermo Scientific incubator, Series II with jacket). After 20–24 h, the strain was subcultured for confluent growth (approximately 10–20 cfu) in the center of sheep-blood Columbia agar plates and incubated for 20–24 h at 37 °C with 5 % of CO_2. The cultures were transferred from plates to four 500 mL shaker flasks containing 250 mL of modified medium and incubated at 37 °C on a rotary shaker at 60 min^{-1} (New Brunswick, Innova 44) until the optical density (OD) reached a value of 1.0 – 1.5 at 675 ηm (VWR, UV-3100 PC). This culture was then transferred to four 5 L shaker flasks containing 2250 mL of the same medium and cultivated under the conditions previously described. The contents from four shaker flasks, corresponding to an initial optical density around 0.10 - 0.15 at 675 ηm, were employed to inoculate the 150 L (total capacity) bioreactor (Sartorius Stedim, Biostat D) containing 90 L Merck medium. Reactor cultivations were conducted, without air addition, at 37 °C, 50 min^{-1} for 24 h, and the pH was controlled at 7.2 by adding 5 mol L^{-1} NaOH (von Hunolstein et al., 1993). Microscopic examinations were conducted using the Gram technique (Gregersen, 1978) to verify possible contaminations.

2.6. Inoculated media analytical methods

At regular intervals of time during cultivation, 15 mL samples were harvested and divided into two aliquots: (i) 2.7 mL to follow bacterial cell growth; (ii) 10.0 mL was centrifuged at 13,776*xg*, 30 min, 4 °C (Beckman Coulter, Avanti J-30I); the cell-free supernatants were filtered through 0.22 μm membrane (frozen at -20 °C) and used to measure glucose concentrations. Bacterial growth during cultivation was followed by optical density at 675 ηm, using the culture medium as blank. Samples were taken in two hours and diluted in the culture medium if the $OD_{675\eta m}$ was higher than 0.5. The Glc concentrations were measured using the enzymatic-colorimetric assay oxidase enzyme kit (Interteck Katal, Brazil).

2.7. Inactivation of fermentation and primary recovery polysaccharide (base treatment)

A solution of 10 mol L^{-1} of sodium hydroxide was added to the cell culture to a final concentration of 0.8 mol L^{-1}, incubated at 37 °C for 36 h with mixing (200 min^{-1}) (Berti et al., 2016). Inactivated cell harvest was carried out using the disc-stack centrifuge (AlfaLaval, LAPX4045GP/TGP) at 9000 min^{-1}, 25 °C, and a 100 L h^{-1} flow rate. The cell suspension was discarded, and the supernatant (S1) followed the

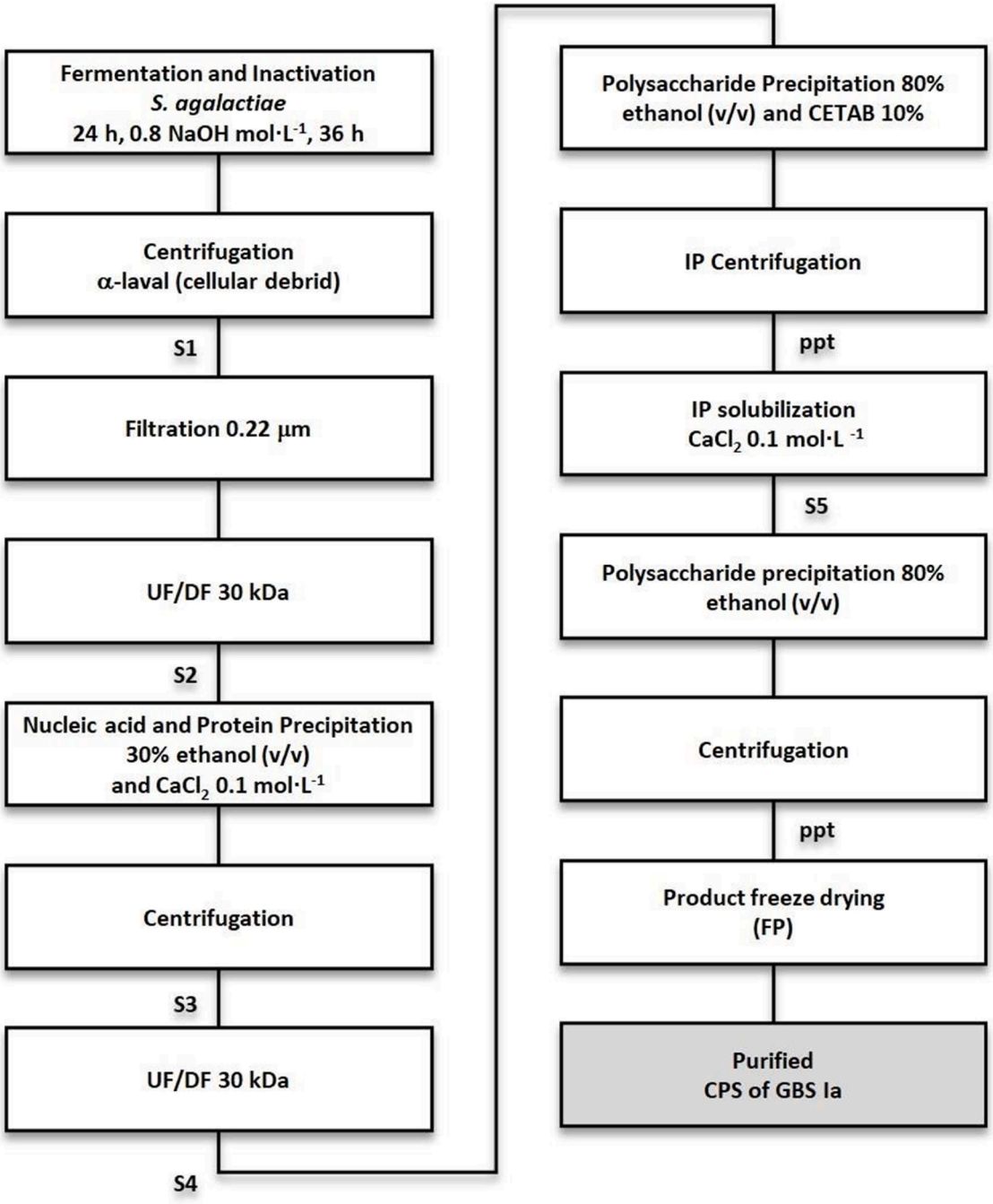

Fig. 2. Purification flowchart for CPS of GBS Ia.

downstream process. To verify inactivation, samples of bacterial suspension from the bioreactor were spread in sheep-blood Columbia agar plates and incubated for 20–24 h at 37 °C with 5 % CO_2.

2.8. Downstream processes

The supernatant (S1) was then submitted to a microfiltration stage using a 0.22 μm membrane cassette, followed by concentration via tangential filtration through a 30 kDa membrane cassette, with the final volume being reduced to the equivalent of 10 % of the initial volume (S2). The concentrated phase was collected and diafiltrated against pure water (PW) using a 30 kDa membrane cassette until achieving pH = 7.4, around 25 diavolumes. The diafiltrated and neutralized solution (S2) was treated to protein and nucleic acid precipitation by adding *quantum satis* of ethanol 100 % and calcium chloride ($CaCl_2$) to reach 30 % and 0.1 mol L^{-1} concentrations, respectively. The resulting biphasic mixture was centrifuged at 9000 rpm and 4 °C for 30 min, with supernatant retention (S3) and disposal of the precipitate. S3 was once more diafiltrated using a 30 kDa membrane cassette against PW until it passed a chlorine test, with a volume reduction to achieve 10 % relative to S1 (S4) as the final volume. The chlorine test is performed by mixing 1 mL of 0.1 mol L^{-1} $AgNO_3$ solution with 9 mL of S4 at the beginning of the diafiltration process, with one diavolume (identifying the chlorine presence by white precipitation formed), and at the end, with no residual precipitate observed. Cetyltrimethylammonium bromide (CTAB) was then added to S4 until precipitation of the CTAB-CPS of GBS Ia complex and centrifuged at 9000 rpm and 4 °C for 30 min, with the recovery of precipitate as intermediate product (IP). IP was dispersed in $CaCl_2$ 0.1

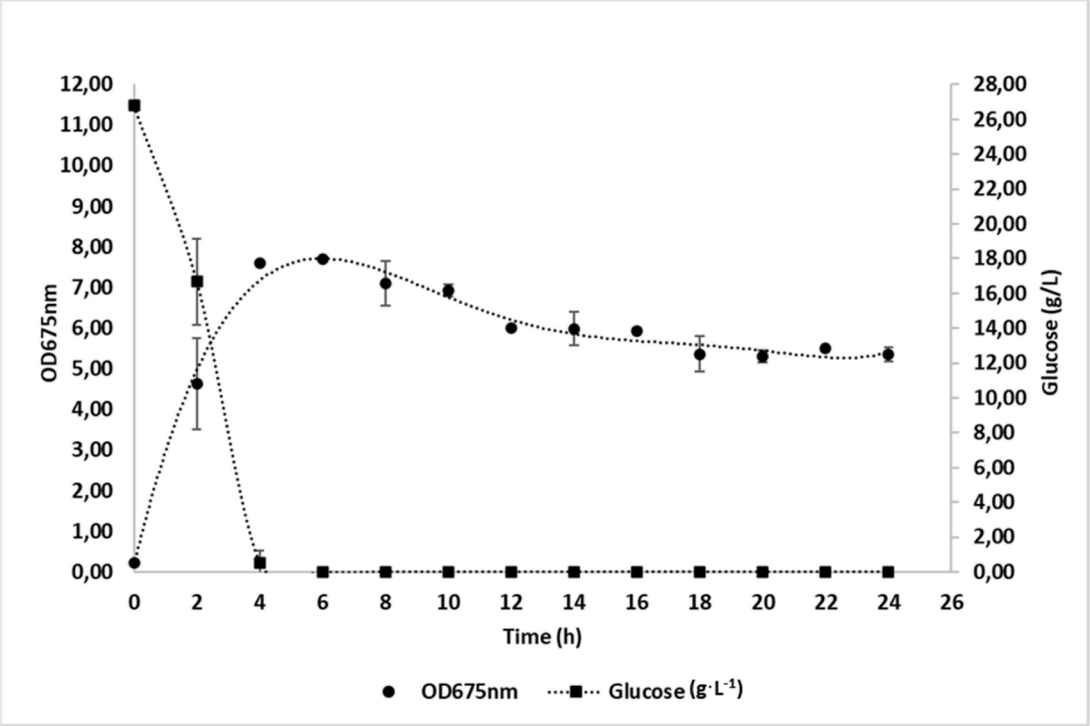

Fig. 3. Growth and glucose profiles from batch cultivation of *S. agalactiae* Ia strain RS-72 in 150 L bioreactor Sartorius Stedim, Biostat D. Average and standard deviation of two independent experiments.

mol L^{-1} until its complete solubilization (S5) – ca. 20–40 mg mL^{-1} – after which ethanol 100 % was incorporated into S5 to reach 80 % (v/v) in ethanol content. The new precipitate was separated by centrifugation (at 9,000 rpm and 4 °C for 30 min) and freeze-dried (FP). FP is analyzed for yields in saccharides, proteins, nucleic acids, and sialic acid (Fig. 2).

2.9. Weight average molecular weight determination

The weight average molecular weight of CPS of GBS Ia was determined by a multi-angle light scattering detector (MALS) on a DAWN HELEOS instrument and an Optilab® rEX refractive index (RI) detector (Wyatt Technology Corp.). These were connected to an HPLC SHIMADZU LC40 apparatus presenting a TSK G5000PWxl column, having PBS 0.1 mol L^{-1} as mobile phase, pH = 7.0, flow rate of 0.500 mL min^{-1}, at 658 ηm of laser wavelength, being executed twice. In the experiment, the apparatus was connected in the following order: SEC-MALS-RI. The SEC separated materials passed through the MALS detector, which used a 685 ηm laser, followed by the RI detector.

2.10. CPS of GBS Ia hetero-monomer composition

An aliquot of 250 μL of CPS of GBS Ia in an aqueous solution (2 μg $μL^{-1}$) and 200 μL of sorbitol (1 μg $μL^{-1}$), used as internal standard, were added in a reaction vial, and then freeze-dried. The sample was hydrolyzed by commercial 0.5 mol L^{-1} HCl·MeOH (Supelco®), sealed, and heated for 24 h at 80 °C. The alcoholic phase was extracted with three aliquots of 500 μL of heptane (Merck™). The acid was then neutralized by 20 mg of Ag_2CO_3 (Merck™) and re-*N*-acetylated with 25 μL of distilled acetic anhydride by storing the tube in the darkness at 25 °C for 6 h. The sample was then centrifuged (5000 rpm, 4 °C, 5 min), and the supernatant was transferred to a clean reaction vial and evaporated under a nitrogen stream. The per-O-trimethylsilyl derivatives were prepared by adding 50 μL of pyridine and 50 μL of the reagent *N,O*-Bis-(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA: TMCS; 99:1 Sigma-Aldrich™) and let stand at room temperature for one hour before analysis.

The commercial standard solution was prepared by adding in the reaction vial an aliquot of 200 μL of a standard solution (1 μg $μL^{-1}$) (Gal, Glc*N*Ac, Glc, and Neu*5*Ac) containing 200 μL of sorbitol (1 μg $μL^{-1}$) as internal standard. It was derivatized and analyzed in the same analytical conditions.

2.11. Spectrophotometric analysis

The CPS of GBS Ia purity was determined based on Dübois spectrophotometric method for reducing sugars (DuBois et al., 1956). To an aliquot of 2 mL of CPS of GBS Ia 2.5 mg mL^{-1} solution is added 0.1 mL of phenol 80 % (w/w), and 5 mL of H_2SO_4 conc. at 110 °C for 15 min, with resultant solution absorbance been read at 490 ηm, using a calibration curve with a $_d$-glucose + *d*-Galactose solution (1:2, respectively). The purity by Neu*5*Ac yield was determined based on Svennerholm method (Svennerholm, 1957), with adding to an aliquot of 100 μL from an 1 mg mL^{-1} of CPS of GBS Ia solution 2 mL of an resorcinol reagent (2.2 mg mL^{-1} in HCl conc. plus 0.25 mL of $CuSO_4$ 0.1 M) at 110 °C for 15 min, extracted with 3 mL of 1-pentanol, and the organic phase absorbance read at 450 ηm, using Neu*5*Ac in the calibration curve, as well.

Protein content was determined based on Lowry method (Waterborg, 2009), with adding to an aliquot of 100 μL from an 1 mg mL^{-1} of GBS Ia solution 3 mL of an alkaline cooper reagent (100 parts of a solution containing 2 % of Na_2CO_3, 0.4 % of NaOH, 0.16 % sodium tartrate, and 1 % of SDS plus 1 part of a solution containing 4 % of $CuSO_4 \cdot H_2O$) at 25 °C for 15 min, add 0.3 mL of Folin-Ciocalteu reagent (diluted 1:1 with H_2O) at 25 °C for 45 min. The following developed color absorbance was read at 660 ηm. The BSA was used in calibration curve.

Nucleic acids content was determined by spectrophotometric analysis for direct reading absorbance at 260 ηm from a sample in 0.2 mol L^{-1} NaOH, with 1.000 AU being equivalent to 37 μg mL^{-1} of nucleic acid.

2.12. General NMR spectroscopy procedure

For NMR experiments, a mass of 15 mg of CPS of GBS Ia was

dissolved in D_2O (D_2O 99 % + 0.05 % DSS-*d6*, Cambridge™) and freeze-dried three times. The lyophilized material was dissolved one last time in D_2O, and the analysis was carried out on a Bruker Avance 400 MHz (^1H) spectrometer with a 3 mm gradient probe at 25 °C; the ^1H NMR spectrum was acquired with 128 scans; Heteronuclear Single Quantum Coherence (HSQC) were processed, and the spectra assignment was performed using the Bruker Biospin™ software, version 3.

2.13. General chromatographic procedure

The gas-chromatography analysis used an Agilent 6890 N gas chromatograph with a flame ionization detector (FID). A volume of 1 μL of the sample was injected by an autosampler in splitless injection mode, using an HP (DB- 5) column, 30 m x 320 μm x 0.50 μm. The injector was set at 220 °C, and the oven temperature gradient was 120 °C to 240 °C at 2 °C min^{-1}, and it was held at 240 °C for 10 min. The carrier gas was helium at a 1.6 mL min^{-1} flow rate.

2.14. General gas chromatography-mass spectrometry (HRGC-MS) procedure

The HRGC-MS analysis was performed in a gas chromatograph-mass spectrometer system (Agilent™, 6890 N/5973) with an online computer library (Wiley 275; Agilent™). The sample was injected in an HP-INNOWAX (DB-5MS) column, 30 m x 220 μm; 0,25 μm, split mode (1:20) using helium as carrier gas (1.5 mL min^{-1}). The injector was set at 220 °C with the oven temperature range from 120 °C to 240 °C at a rate of 2 °C min^{-1}. The mass spectrometry acquisition parameters included full scanning mode, in a range of *m/z* 70,700, in electron ionization mode (70 eV).

3. Results and discussion

3.1. The cell growth profile

Fig. 3 illustrates the temporal pattern of both cell growth and residual glucose. No lag phase was observed, signifying that the cells promptly progressed into the exponential growth phase, indicative of their adaptation to the culture conditions. The capsular polysaccharide is produced during bacterial growth. Generally, a small amount is released into the culture medium and another amount is cell-bound. The procedure followed Ross et al. (1999) conditions and had as objective to figure out both polysaccharide contents: in the media supernatant; and in the media biomass. In this context, the harvest was executed at the 24th hour as a strategy to release a greater amount of polysaccharide into the supernatant. According to von Hunolstein et al. (1993), no discernible impact on the degradation of both the polysaccharide released into the supernatant and that bound to the cell was observed in connection with the depletion of glucose in the media over an extended period of 24 h.

The value of the specific growth rate (0.49 h^{-1}) was three times lower than obtained in this work (1.48 h^{-1}), suggesting that it would be possible to obtain a greater amount of polysaccharide in this last study. The residual glucose concentration dropped to zero at the 4th cultivation hour (beginning of stationary phase) indicating a limitation of this substrate.

3.2. Spectrophotometric results

The polysaccharide was analyzed to evaluate the downstream process, ensure the elimination of nucleic acid and residual protein, and calculate the purity. The nucleic acid was analyzed by direct spectrophotometric analysis as direct measurement, and the protein content was determined by Lowry assays (Waterborg, 2009) by establishing calibration curves with BSA as an analytical standard. The same way, values for *N*-acetylneuraminic acid (sialic acid) were determined following the Svennerholm method (Svennerholm, 1957), and total reducing sugars determined by Dübois method (DuBois et al., 1956). The values used as specifications were based on theoretic stoichiometric calculations and similar references from the WHO (World Health Organization, 2013). The CPS of GBS Ia obtained from all three batches of RS-72 strain cultivated, harvested, and purified presented consistent results for Dübois, Svennerholm, nucleic acids, and protein content assays by Lowry. All the analyses were performed in triplicate, and the bias was calculated. The results listed in Table 1 show satisfactory outcomes for what is expected for Neu5Ac and reducing sugars as purity indicators, according to its percentual presence in the CPS of GBS Ia monomer. The consistency of the purification process can also be confirmed by the data in Table 1.

Table 1
Analytic results for batches A, B, and C of CPS of GBS Ia from RS-72 strain.

	Reference (%)	Batch A (%)	Batch B (%)	Batch C (%)
Total Red. Sugars (%)	50.5	53.5 ± 1.2	49.7 ± 1.3	53.0 ± 1.0
Nucleic acid (%)	≤ 2	1.8 ± 0.07	1.2 ± 0.04	1.7 ± 0.04
Protein%	≤ 3	1.4 ± 0.15	1.5 ± 0.04	1.1 ± 0.05
Yield (%)	-	104.8 ± 0.8	98.7 ± 0.9	104.9 ± 0.2
Neu5Ac (%)	-	29.9 ± 0.3	28.5 ± 0.5	26.7 ± 0.3
MW (kDa)	-	126.7 ± 11.0	121.4 ± 5.2	114.5 ± 8.1
Đ	-	6 %	2 %	0.3 %

The downstream approach was able to significantly reduce the level of impurities and build up polysaccharide yields, with the precipitation methodology associated with salt content control being capable of improving such levels. In addition, the content of Neu5Ac was shown to be near the theoretically expected (around 31 %), with reducing sugar content reaching the expected values.

Given the absence of a dedicated reference material (World Health Organization, 2018, 2023), the pursuit of an enhanced and more standardized purification process stands as a scientific and pragmatic imperative. Consequently, the parameters employed for characterizing the CPS of GBS Ia, specifically from the RS-72 strain, were aligned with those established to produce well-known conjugate vaccines (World Health Organization, 2013).This is particularly relevant in the context of optimizing the growth, harvest, and purification stages of the CPS derived from a Brazilian GBS strain. Achieving a higher purity grade in this process not only addresses current challenges but also enhances the prospects of producing meticulously tailored vaccines with improved efficacy and safety profiles.

The present work was capable of to define the Brazilian strain produce around 20 $mg_{cps}\ g_{cdw}^{-1}$ and its purity ca. 100 % as reducing sugars, what seems promising in terms of polysaccharide extraction. Constantino and coworkers (2013) highlights that there is an idiosyncratic variability of polysaccharide yields as function of its strain, with values ranging from 10 to 30 $mg_{cps}\ g_{cdw}^{-1}$ or more. With that piece of information, further research will be done to enhance those results.

Conventional literature on the purification of Group B Streptococcus (GBS) polysaccharides typically reports yields consistent with data from widely studied polysaccharides (Anderson et al., 2016; Yuan et al., 2014). Nevertheless, considering that publications on this subject commonly attain elevated purity levels post-polysaccharide activation, the current findings presented in this study represent a notable advancement in terms of achieving heightened purity of polysaccharides prior to the activation process.

3.3. Molecular weight determination by MALS results for CPS of GBS Ia

To characterize the purified polysaccharide weight-average molar

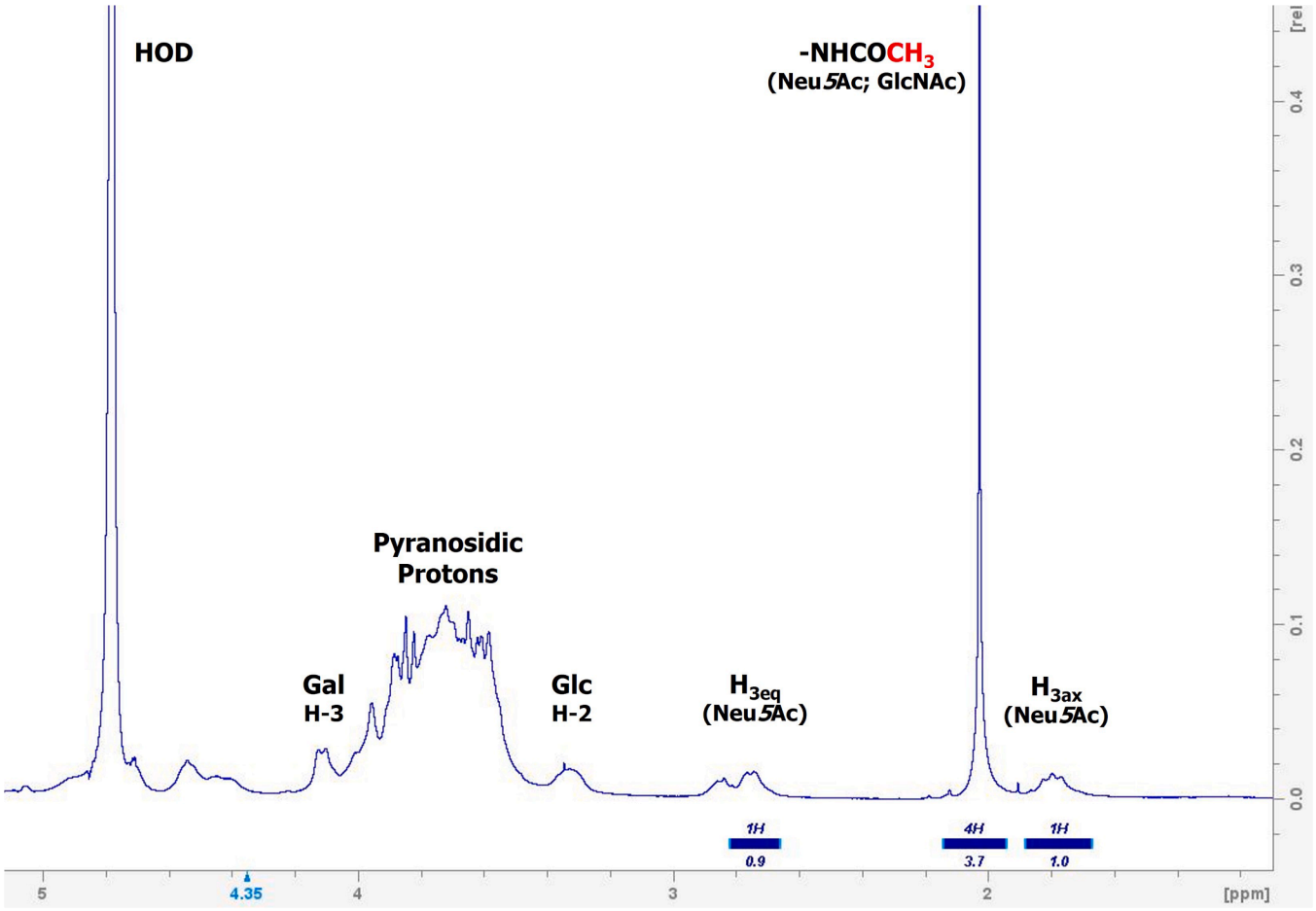

Fig. 4. ^{1}H NMR of CPS of GBS Ia in Deuterium oxide D_2O 99 % + 0.05 % DSS - d6, acquired in Bruker Advance 400 MHz equipment, at 298 K.

mass (M_w), its polydispersity, and aggregate formation, the molecular weight - determined by Multi-Angle

Light Scattering (MALS) - showed that M_w for the samples A, B, and C was in the range of 121–145 kDa. No evidence of aggregate formation was observed. From the same analysis, it is possible to perceive the sample homogeneity by a dispersity (Đ) ranging between 1.06–1.18 (0.3–6.0 % of dispersity). According to IUPAC Recommendations (2009), the Dispersity is a dimensionless ratio defined by Đ and is a derived quantity from M_w M_N^{-1} (since M_N = relative molar mass). Such quantity expresses the grade of dispersion of a polymer, with values of $Đ \approx 1.0$ indicating a more uniform polymer weight distribution (Stepto, 2009). The employed multi-angle light scattering (MALS) technique, coupled with size-exclusion chromatography (SEC) and refractive index (RI) detector, provide absolute molecular weight. The specific refractive index increment (dn/dc) was determined experimentally as 0.140 mg mL^{-1}. The analysis was performed in triplicate.

3.4. *Per-trimethylsilylation of CPS of GBS Ia and hetero-monomer composition*

In the hetero-monomer compositional analysis, the glycosidic linkages were cleaved by methanolysis, and the monosaccharides were then derivatized, generating their per-O(trimethylsilyl)-methylated derivatives that were analyzed by HRGC-FID and HRGC-MS adapted from DeJongh (1969). All the results were certificated by data obtained from commercial standards $_d$-Galactose (Gal), *N*-Acetyl-$_d$-Glucosamine (Glc*N*Ac), $_d$-glucose (Glc), and *N*-Acetyl-$_d$-neuraminic acid (Neu5Ac), that were analyzed in the same conditions.

HRGC-FID analysis was able to isolate the sample and commercial standard per-*O*(trimethylsilyl)-methylated derivatives under chromatographic conditions. The neutral monosaccharides, with an anomeric center, showed four chromatographic peaks relative to pyranose and furanose rings in their alpha and beta anomeric configurations. The analytes were previously differentiated by their retention time, and the more intense peak was chosen for the fragmentation profile identification of the analytes. $_d$-glucose (27.2 min), $_d$-galactose (26.3 min) derivatives present a very similar fragmentation profile since they only differ in the absolute configuration of C-4

3.5. *NMR spectroscopy to CPS of GBS Ia*

The CPS of GBS Ia sample was analyzed by one- and two-dimensional NMR, aiming to support the data obtained by chemical composition analysis in a cross-sectional characterization strategy. The spectra obtained by NMR for CPS purified from GBS Ia are consistent with expectations. The nature of the saccharides that form the polysaccharide hetero-monomer follows what was anticipated according to data already published in current literature (Pinto & Berti, 2014). Although no Neu5Ac could be observed in HRGCFID, it was possible to identify the methylene hydrogens chemical shift relative to its axial and equatorial protons at C-3 position with δ_{3ax} at 1.79 ppm and δ_{3eq} at 2.75 ppm, showing the alpha configuration, at ^{1}H NMR (Fig. 4). The methyl group from Neu5Ac and Glc*N*Ac overlapped at 2.0 ppm, with the core signals ranging from 3.25 to 4.25 ppm to pyranosidic protons. Therefore, the characterization of sialic acid in the polysaccharide structure was performed by adopting an orthogonal analytical approach, commonly described in the literature for several vaccine polysaccharides (Jennings et al., 1983; Oliver et al., 2013; Pinto & Berti, 2014).

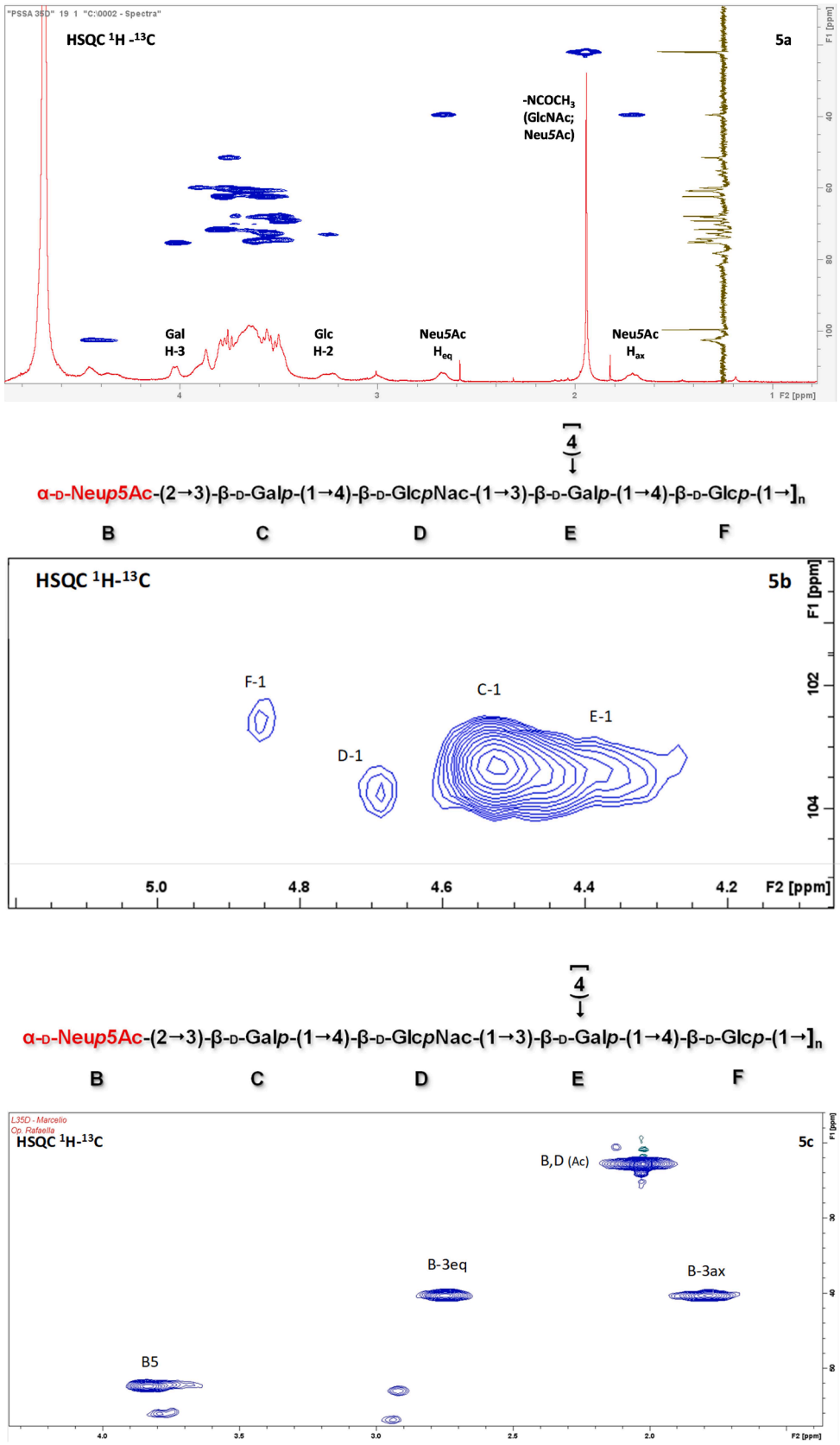

Fig. 5. 5a: ^{1}H x ^{13}C HSQC of CPS of GBS Ia, acquired in Bruker Advance 500 MHz equipment, D_2O at 298 K. 5b-5c: ^{1}H x ^{13}C HSQC expansions – (5b) anomeric region, and (5c) signal characteristic of Neu5Ac.

The anomeric ^{1}H region of the spectrum (4.4–5.6 ppm) is overlapped by the residual HOD resonance, but the anomeric protons can be observed in the HSQC spectrum (Fig. 5a), highlighted in the spectrum expansion in Fig. 5c.The main indications are described in Fig. 5(b-c), as expansions of 5a spectrum. In the Heteronuclear Single Quantum Coherence spectrum (^{1}H x ^{13}C HSQC), the cross-peak correlation was identified, with the $3H_{ax}$, $3H_{eq}/C_3$ chemical shift at (1.80, 2.75/40.2 ppm), H5 at (3.84/52.4 ppm) – characteristic of Neu*5*Ac residue – and the protons of acetyl moieties of Glc*N*Ac and Neu*5*Ac that are overlapped at (2.03/22.4 ppm).

The sialic acid moiety assignment, in ^{1}H x ^{13}C HSQC spectrum, was supported by Keresztesi (2022) and Pinto and Berti (2014) experiments. The main indications are described in Fig. 5(b-c), as expansions of 5a spectrum. In the ^{1}H x ^{13}C HSQC spectrum, the crosspeak correlation was identified, with the E-3_{ax}, E-3_{eq} chemical shift at (1.80, 2.75/40.2 ppm), E-5 at (3.84/52.4 ppm) – characteristic of Neu5Ac residue – and the protons of acetyl moieties of GlcNAc and Neu5Ac that are overlapped at (2.03/22.4 ppm).

Additionally, the assignment of anomeric cross-peaks correlation in 1H - 13C HSQC was performed based on Keresztesi (2022) showing C-1 at (4.52/103.3); d-1 at (4.70/103.8); E-1 at (4.38/103.3) and F-1 at (4.86/102.6). The cross-peaks correlation data is compatible with β-configuration. It was not possible to determine the spin sequence of each carbohydrate unit in the structure. Therefore, the assignment was supported by literature data (Fig. 5c) (Berti & Ravenscroft, 2015; Keresztesi, 2022; Pinto & Berti, 2014).

4. Conclusions

The *S. agalactiae* Brazilian RS-72 strain was cultivated and harvested, and its capsular polysaccharide was purified using successive precipitation methods and salt control content with success and in good yields for purity. Three batches were well characterized through spectrophotometric, chromatographic, and NMR techniques. The presence of sialylation was observed as analytical data, and consistency in the CPS molecular weight was shown through the SEC-MALS technique. Therefore, the presented results showed a successful process to obtain a CPS of GBS Ia to be used in bioconjugation reactions and subunit vaccine synthesis.

CRediT authorship contribution statement

Ellen Jessouroun: Project administration, Conceptualization, Methodology, Writing – original draft. **Marcélio de Moura Oliveira:** Investigation, Writing – original draft. **Bárbara Araújo Nogueira:** Investigation. **Ozéias de Lima Leitão:** Resources, Investigation. **Elizabete Pereira de Figueredo:** Resources, Investigation. **Maria de Lourdes M Leal:** Investigation, Writing – review & editing. **Marilza Batista Corrêa:** Supervision, Methodology, Writing – review & editing. **Bárbara V. Silva:** Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No dataset was used for the research described in the article.

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