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Polysaccharide purification from *Haemophilus influenzae* type b through tangential microfiltration

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ABSTRACT

Haemophilus influenzae type b (Hib) is a human pathogen that causes meningitis in infants worldwide. Capsular polysaccharide linked to a protein has been used as an efficient vaccine, and this approach has reduced the incidence of Hib disease since its inclusion in national immunisation campaigns. The traditional polysaccharide downstream process is based on several ethanol precipitations, treatment with detergents and centrifugation. The aim of this study was to introduce tangential microfiltration (TMF) in the place of centrifugation to simplify handling and to scale up the process. The purity of the polysaccharide was RP_{NA} = 1747.2 and RP_{Prt} = 196.1 for nucleic acid and protein, respectively, meeting the quality requirements for this polysaccharide. Moreover, the polysaccharide was recognised by at specific antibody, and the ribose and phosphate contents were within the expected limits. Thus, we established a process for the purification of capsular polysaccharide produced by *H. influenzae* type b that is effective, robust and feasible to be scaling up.

1. Introduction

Encapsulated *Haemophilus influenzae* type b (Hib) causes invasive diseases, such as septicemia, meningitis and lower respiratory tract infections, in children less than two years old, causing death or permanent disabilities, including mental retardation and deafness (Moxon and Vaughn, 1981; Danovaro-Hollyday, Garcia, Quadros, Tambini, & Andrus, 2008). Type b capsular exopolysaccharide composed of polyribosylribitol-phosphate (PRP), traditionally regarded as the critical virulence determinant because it confers resistance to phagocytosis and complement mediated host defences (Moxon and Kroll, 1990; Sukupolvi-Petty, Grass, & Geme, 2006).

Alternatively, purified PRP is used as an antigen in the vaccine formulation against Hib diseases. However, the polysaccharide induces T-cell independent protection; therefore, it has low efficiency in children. To elicit immunological memory, particularly in infants younger than 2 years old, PRP was chemically linked to a protein, stimulating a T-cell dependent anti-polysaccharide response (Ada & Isaacs, 2003; WHO, 2000). After introduction of the conjugated vaccine to outline infant immunisation schedules, the number of *H. influenzae* type b isolates in children younger than 2 years old has decreased from 92 in 2000 to 30 in 2005 (Danovaro-Hollyday et al., 2008).

The process used to isolate and purify PRP from Hib is complex, involving multistep repeated ethanol precipitations (inflammable), anionic detergent (pollutant), and phenol (corrosive and toxic) to obtain a final product with the purity required by regulatory authorities (Gottschlich, Liu & Artenstein, 1969; Frasch, 1990; Lee, 1994; Kuo, 1980; Rienstra, Scattergood, Edgar, & Sitrin, 1991; WHO, 2000). Furthermore, the extensive use of centrifugation and ultracentrifugation makes this process expensive, due to the capital and energy employed. A simpler method for pathogenic bacterial polysaccharide purification was adopted as an alternative, reducing the ethanol precipitation steps to two (30% and 80%, v/v) and replacing the phenol treatment and ultracentrifugation steps with enzymatic treatments with endonuclease and proteases associated with detergents and tangential ultrafiltration in the first and last steps (Tanizaki et al., 1996; Gonçalves et al., 2003, 2007; Takagi et al., 2008).

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The inclusion of the Hib vaccine in the national immunisation schedule has increased the demand for this polysaccharide, and high production costs highlight the need to develop an efficient and less expensive method of purification. Membrane technologies, which are high throughput and display ever-improving selectivity, have the potential to fulfil the increased demands placed on downstream processing. Recently, Albani, Silva, Takagi, and Cabrera-Crespo (2012) customized the purification process described above to purify the capsular polysaccharide produced by *H. influenzae* type b introducing two types of detergents treatment during the tangential flow filtration and diafiltration steps (Albani et al., 2012).

In this study, we established an alternative purification process of the capsular polysaccharide produced by *H. influenzae* b, replacing the centrifugation step with tangential microfiltration, creating a process that is simple to handle, as well as technically and economically suitable for scaling up.

2. Material and methods

2.1. Microorganism

H. influenzae type b strain GB 3291 was purchased from the Department of Bacteriology of the Institute Adolfo Lutz (São Paulo, Brazil).

2.2. Up stream processing – cultivation of bacterium

Fed-batch cultivations were performed in a bioreactor Bioflo 5000 (New Brunswick Scientific Co., USA) with 80 litres of nominal volume and 60 litres of culture. The first steps were performed in batch cultures containing 15 L of MMP medium, as described by Takagi, Cabrera-Crespo, Zangirolami, Raw, & Tanizaki (2006), and the cultures were inoculated to achieve an $OD_{540\,nm}$ of 0.1 (Takagi et al., 2006). The cultures were grown under the following conditions: pH 7.5 (controlled using 5M NaOH), temperature 37 °C, air supply of 1.0 vvm, agitation at 200–800 rpm and pO_2 controlled at 30% of air saturation. The cultures were fed with a concentrated solution of glucose (20%; a carbon source) enriched with nitrogen represented by yeast extract (20%) when all the glucose contained in the batch medium was exhausted (i.e, 9.5 hour of cultivation with a flow rate of 1.2 L/h for 12 hour resulting in the final volume approximately 60 litres.

2.3. Downstream processing – polysaccharide purification

Bacterial cells were removed from the culture broth using a continuous tubular centrifuge with automatic piston discharge (APD75 Celeros Inc., MI, USA) at 20,000 × *g*, 4 °C with a feed flow rate of ~200 mL/min. The clarified fraction was named Supernatant (Spr).

2.3.1. 1st concentration/diafiltration 100 kDa

Supernatant (Spr) was then concentrated to 1/10 of the initial volume using a tangential ultrafiltration membrane with a 100 kDa NMWCO (Nominal Molecular Weight Cut-Off), 2.5 m^2 filtration area with an inlet pressure of 7–10 psi and a maximum transmembrane pressure of 7.5 psi (Pellicon 2 maxi cassette, Millipore, MA, USA). Sequentially, the concentrate was submitted to 3 washing steps with six volumes of each following buffers: (1) 25 mM Tris–HCl pH 7.5 containing 150 mM NaCl; (2) 25 mM Tris–HCl pH 7.5 containing 0.3% (w/v) sodium deoxycholate (DOC) and 2 mM ethylenediamine tetraacetic acid (EDTA); and (3) 25 mM Tris–HCl pH 7.5. The final concentrate of 100 kDa (1CTUF100 – first concentration on tangential ultrafiltration at 100 kDa) was divided into 12 fractions of ~0.5 L and frozen at −20 °C. To establish the purification process, each fraction of 0.5 L was considered to be one fermentation run consisting of five litres of culture.

2.3.2. 1st ethanol precipitation 30%

Ethanol precipitation at 30% was performed according to the method described by Albani et al. (2012), however the insoluble fraction was separated using tangential flow microfiltration through a hollow fibre of 0.2 μm pore size with an inlet pressure of 3 psi (CFP-2-E-5A-ID 1.0 mm, 0.12 m^2 filtration area, 30 cm length GE Healthcare, MA, USA). During the microfiltration test, the feed stream was pumped from the tank using a peristaltic pump (easy-load masterflex I/P model 77601-10). The retentate was recycled back to the feed tank, concentrated to approximately 0.25 L and washed with six volumes of fresh 30% EtOH solution to recover a majority of the soluble PRP into the microfiltrate fraction (1TMF), and the insoluble material of 30% ethanol precipitation was discarded.

Microfiltrate fraction – 1TMF (first tangential microfiltration) containing the soluble PRP was collected and concentrated by tangential ultrafiltration with a membrane of 50 kDa NMWCO; (0.18 m^2 filtration area) and defined as 2CTUF50 (second concentration on tangential ultrafiltration at 50 kDa).

2.3.3. 2nd ethanol precipitation 80%

The pH of 2CTUF50 was adjusted to 5.8 using acetic acid, and it was submitted to ethanol precipitation at 80% according to Albani et al. (2012). Ethanol solution (second tangential microfiltration – 2TMF, a waste fraction) was discarded by tangential flow microfiltration through a hollow fibre of 0.2 μm pore size and the precipitated PRP was solubilised in purified water and recirculated using the same hollow fibre system with the microfiltrate line closed to equilibrate the membrane and initiate microfiltration. In this step, the concentrated fraction containing insoluble materials (impurities) was discarded, and the microfiltrate fraction contained the PRP (3TMF – third tangential microfiltration). This fraction was further concentrated as described above and named 3CTUF50 (third concentration on tangential ultrafiltration at 50 kDa).

2.4. Enzymatic treatment

The pH of the 3CTUF50 fraction was adjusted to 7.5 using Tris–HCl buffer containing 2 mM $MgCl_2$ and 20 mM NaCl, and it was submitted to enzymatic treatment in the presence of endonuclease and proteases. Then, it was isolated according to the method described by Albani et al. (2012) generating purified PRP. Pure polysaccharide was distributed (~20 mL) in pre-weighted 50 mL centrifuge tubes , frozen at −80 °C and submitted to lyophilisation for further characterisation and analysis of quality control. Fig. 1 shows the flux diagram of the purification process described above.

2.5. Analytical methods

2.5.1. Optical density

The presence or absence of bacteria in the supernatant after cell separation was monitored by measurement at 600 nm ($OD_{600\,nm}$).

2.5.2. Polysaccharide determination

The PRP was measured by the modified Bial method using ribose as the standard (Ashwell, 1957); the concentration of PRP was calculated by multiplying the ribose concentration by 2.55 (Crisel, Baker, & Dorman, 1975).

2.5.3. Protein determination

The protein (Prt) concentration was measured by Lowry's method.

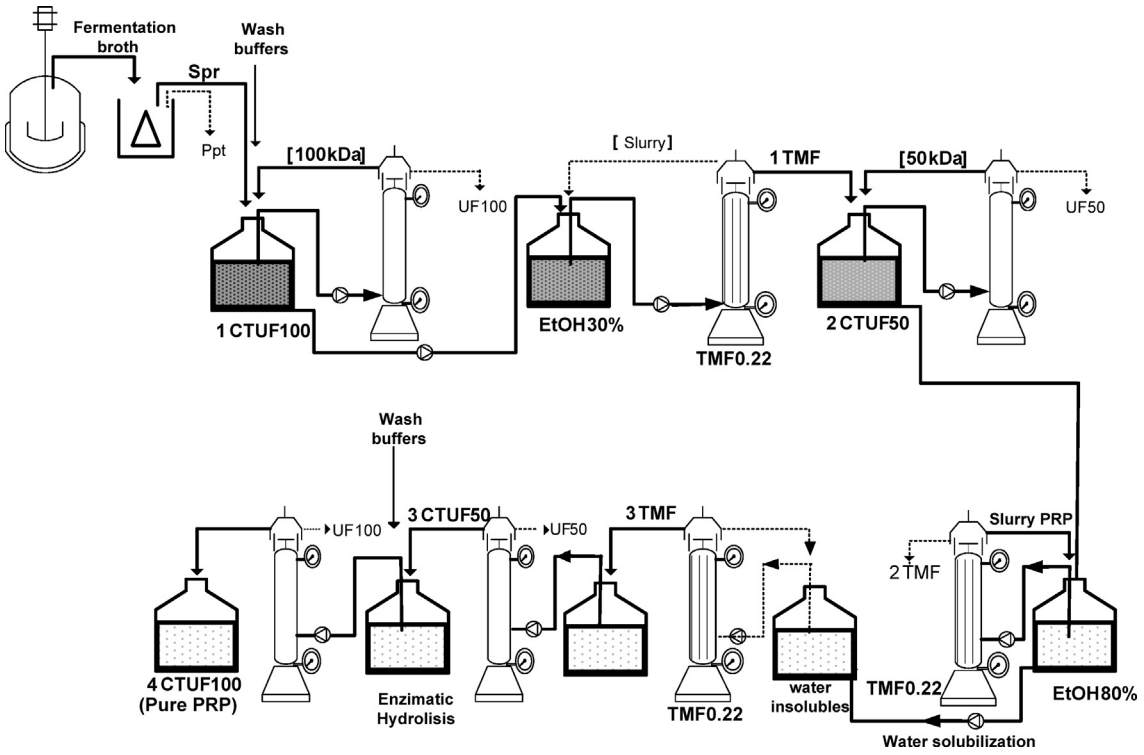

Fig. 1. Schematic diagram of the PRP purification process.

2.5.4. Nucleic acid determination

The nucleic acid (NA) concentration was determined by measuring the absorbance 260 nm, where a value of $OD_{260\,nm} = 1.0$ corresponded to 50 μg NA/mL (WHO, 1981).

2.5.5. Phosphorus determination

Phosphorus was measured by the ascorbic acid method (Chen, Toribara, & Warner, 1956)

2.5.6. Rocket imunoelectrophoresis

Rocket imunoelectrophoresis was used as an identity test to recognise the specific antibody (Laurell, 1956). Rachel Schneerson – Eunice Kennedy Shiriver National Institute for Child Health and Human Development – USA, kindly provided antibody against PRP.

2.5.7. Molecular mass determination

The molecular mass of PRP was determined by size exclusion chromatography (SEC) connected to a RID detector (RID-10 Shimadzu) using two column TSK gel (TOSOH Bioscience) connected in series (40 °C and flow rate of 0.6 ml/min). The mobile phase was a solution consisting of the following: NaCl, 150 mM; Na_2HPO_4, 10 mM; e NaN_3, 0.02%; and pH 7.5 (adjusted with HCl 6 N). The elution time of the PRP sample was converted in molecular mass (kDa) using a standard curve built with the following Dextran standards: 1.5; 6; 10; 40; 70; 229 and 2000 kDa.

The number average molecular mass (M_n) and the mass average molecular mass (M_w) were calculated from the heights of the chromatographic signal at each elution time, as specified in the equations below, where h_i is the height of the signal and M_i is the respective molecular mass calculated from the elution time (Meunier, 1997). The ratio M_w/M_n defines the molar-mass dispersity, $Đ_M$, which represents how broad the polymer distribution is (Stepto, 2009).

$$M_n = \frac{\sum h_i}{\sum h_i/M_i}$$

$$M_w = \frac{\sum h_i M_i}{\sum h_i}$$

2.6. Identify and purity test

A known amount of lyophilised PRP was weight obtain a solution of 1 mg/ml PRP, which was used for all analysis dedicated to purity and identity testing.

2.7. Purification table

For all purification steps we calculated the recovery of PRP, relative purity (RP) and purification factor (PF). The PRP concentration is presented as milligram of polysaccharide per litre of culture broth, recovery refers to the percentage of PRP obtained in each purification steps with respect to the initial fraction (Spr) or previous one (represented by brackets), and it represents the amount of PRP in the step*100/amount PRP in the Spr or previous step. RP refers to the relationship between the amount of polysaccharide and contaminants (mg PRP/mg contaminant with NA or Prt as contaminants). PF expresses how many times the relative purity improved in relation to the first step or previous one (RP_{step}/RP_{Spr}).

3. Results and discussion

Vaccines play an important role in protecting humans against life, threatening diseases, but the material used for vaccination must be in accordance with well-defined quality standards. Tangential filtration technology is widely used in the biotechnology industry, especially in pharmaceutical downstream processes. Economically, it is an attractive method for separating biomolecules, allows for processes to be performed in closed and sterile conditions, is easy to handle, environmental friendly and scaling up

Table 1
Evaluation of the PRP purification process.

	Purification steps	PRP (mg/L)	PRP recovery (%)	RP_{Prt}	PF_{Prt}	RP_{NA}	PF_{NA}
Initial	Supernatant	1924.0	100 (100)	0.7	1.0	0.3	1.0
	1CTUF100	844.9	44 (44	1.7	2.6 (2.6)	7.2	21.9 (21.9)
Intermediary	1TMF	662.8 ± 58.8	34 ± 4.4 (78)	5.1 ± 1.1	7.8 (3.0)	17.4 ± 3.4	53.2 (2.4)
	2CTUF50	543.7 ± 60.0	28 ± 1.8 (83)	10.2 ± 1.9	15.4 (2.0)	43.3 ± 12.4	132.3 (2.5)
	3TMF	560.6 ± 35.3	29 ± 3.1 (103)	17.1 ± 5.6	25.9 (1.7)	104.3 ± 27.4	318.9 (2.4)
	3CTUF50	502.3 ± 84.7	26 ± 3.1 (90)	25.9 ± 6.9	39.1 (1.5)	429.6 ± 110.0	1312.8 (4.1)
Final	4CTUF100 (Pure PRP)	275.7 ± 83.1	14 ± 4.3 (54)	196.1 ± 61.8	296.8 (7.6)	1747.2 ± 383.8	5339.6 (4.1)

Value represents the average from 5 experiments ± standard deviation; RP-relative purity (protein, nucleic acids); RP_{Prt} = mg PRP/mg Prt, RP_{NA} = mg PRP/mg NA; PF-purification factor (protein, nucleic acids); $PF = (PR_{step}/PR_{Spr})$; $PF = (PR_{step}/PR_{Spr})$. Values in brackets refer to the previous purification step.

is feasible (Meacle, Aunins, Thornton, & Ann Lee, 1999; Reis and Zydney, 2007; Li et al., 2011).

3.1. Downstream process of polysaccharide produced by H. influenzae b

In this work, the downstream protocol of capsular polysaccharide of *H. influenzae* type b was carried out based on tangential flow filtration technology. The cell separation is made by continuous flow centrifugation because the use of tangential flow microfiltration for cell separation of *H. influenzae* type b retained more than 50% of PRP in the retentate fraction (data not shown). This behaviour was not observed for *S. pneumoniae* polysaccharides from serotype 23 and 6B, where cell separation by tangential micro-filtration efficiently recovered the majority of polysaccharide in the microfiltrate fraction (Gonçalves et al., 2003, 2007). However, cross flow microfiltration for cell separation deserves special attention due to the fouling, cell deposition and polarisation effects that affect the efficiency of the membranes. Moreover, the cultivation conditions and polymers characteristics have been the subject of investigations to improve cell separation and filtration performance (Haarstrick, Rau, & Wagner, 1991; Harscoat, Jaffrin, Bouzerar, & Courtois, 1999; Zhou, Ni, Huang, & Zhang, 2006).

It was not possible to use cross flow microfiltration, because 60 litres of culture broth fermentation was submitted to cell separation through continuous flow centrifugation. A majority of cells were observed ($OD_{600\,nm} = 2.8$) in the supernatant compared to that from conventional centrifugation ($OD_{600\,nm} = 0.14$). In our process, these cells were removed by pre filtration of 25 and 3 μm in tandem before proceeding to the downstream process.

Table 1 shows the evaluation of the polysaccharide downstream process divided into three main groups: initial, intermediary and final stage of purification.

3.1.1. Initial stage – first concentration/diafiltration 100 kDa

The initial sample used analyse the relative purities, purification factor and polysaccharide recovery during the downstream process was the supernatant (Spr). In this step, the polysaccharide concentration was 1924 mg PRP/L of culture with relative purities of $RP_{NA} = 0.3$ and $RP_{Prt} = 1.7$, for nucleic acid and protein, respectively. A sample from the first concentration (1CTUF100) displayed purification factors of 2.6 and 7.6 for protein and nucleic acid, respectively, however, it also showed a low global recovery in PRP (44%).

The supernatant fraction of PRP from *H. influenzae* and other Gram-negative bacteria contains a phospholipid at the reducing terminus (lipo-PRP). The presence of a phospholipid enables the aggregation of lipo-PRP with hydrophobic molecules from the outer membrane of the cell, nucleic acid and proteins, resulting in an apparent high molecular mass for the PRP (Kuo, Doelling, Graveline, & McCoy, 1985; Arakere, Lee, & Frasch, 1994). In the classical polysaccharide purification process, lipo-PRP was removed by the selective ethanol fractionation, however the loss was approximately 70% (Lee, 1994).

During the diafiltration step, the aggregated molecules can dissociate in the presence of detergents such as sodium deoxycholate (DOC), decreasing its apparent molecular size and crossing through the 100 kDa membrane.

3.1.2. Intermediary stages – selective ethanol fractionation

The intermediary stages of purification comprise the ethanol fractionation at 30% and 80%. The centrifugation step after 30% ethanol precipitation was replaced with hollow fibre tangential microfiltration, resulting in $RP_{NA} = 17.4 \pm 3.4$ and $RP_{Prt} = 5.1 \pm 1.1$, which was similar to that found by Albani et al. (2012) with value of 23.8 and 6.6, respectively, however we observed a slight difference in recovery, 34.4% when using microfiltration compared to 63.2% when using centrifugation. In our previous PRP purification protocol using the centrifugation method, the supernatant of the 30% ethanol fraction at displayed a cloudy, red-brownish colour that gradually disappeared during the purification process. In contrast the fraction after the first tangential microfiltration, 1TMF, as slightly cloudy and displayed a pale yellow colour.

The slurry concentrate of 30% ethanol was diafiltered with 6 volumes of fresh solution of 30% ethanol to recover as much PRP as possible into the 1TMF, resulting in a large volume. An ultrafiltration step of 50 kDa NMWCO (2CTUF50) was performed resulting in $RP_{Prt} = 10.2 \pm 1.9$ and $RP_{NA} = 43.3 \pm 12.4$, with a PRP recovery of 28%. This step was introduced to reduce the volume of 1TMF, but surprisingly, the purity increased approximately 2 fold with PF_{Prt} 2.0 and PF_{NA} 2.5 (Table 1).

Following purification, the 1MFT fraction was submitted to 80% ethanol fractionation in the cold room for 12 hours to precipitate the polysaccharide. Ethanol was removed through a hollow fibre (0.22 μm cut-off) and the precipitated polysaccharide was solubilised in purified water. The insoluble impurities were retained in the concentrated fraction of the microfiltration membrane, and ~100% of the polysaccharide was recovered in the microfiltrated (3TMF) fraction after exhaustive washing with water resulting in the relative purities of $P_{Prt} = 17.1$ and $RP_{NA} = 104.3$. The degree of PRP purification was similar to that obtained in the previous study for proteins, however the nucleic acids purity achieved the value established by the regulatory authorities (WHO, 2000).

3.1.3. Final stage – polishing of polysaccharide using enzymes treatment

A concentration step at 50 kDa was introduced for (3CTUF50), resulting in a recovery of 90% in relation to the previous step and an $RP_{Prt} = 25.9 \pm 6.9$ and $RP_{NA} = 429.6 \pm 110.0$, with PF_{Prt} 1.5 and RP_{AN} 4.1. This fraction was further treated with nuclease and proteases, hydrolysates were removed by tangential ultrafiltration (4CTUF100), yielding purified PRP with relative purities of: $RP_{Prt} = 196 \pm 62$ and $RP_{NA} = 1747 \pm 384$, in accordance with WHO

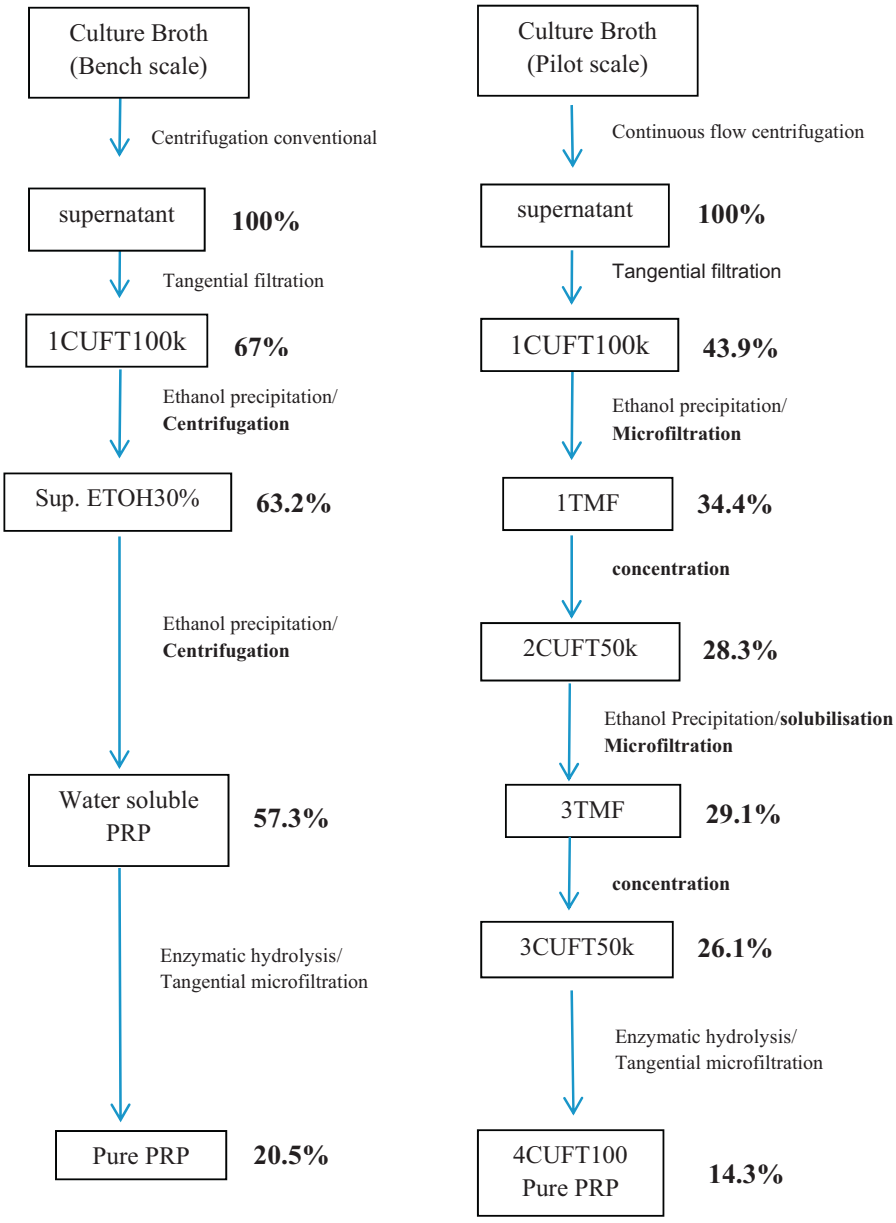

Fig. 2. Comparison between the centrifugation and microfiltration processes for polysaccharide purification.

requirements of RP ≥ 100 for both parameters. The polysaccharide recovery step yielded 54%, with a global recovery of 14%.

3.2. Comparison between the centrifugation and microfiltration purification process

Replacing the centrifugation steps used to remove the insoluble material with tangential microfiltration using hollow fibre has produced consistent results that were comparable to centrifugation considering that the upstream process was at the Pilot scale, and the cells were separated through a continuous flow centrifuge, meaning the supernatant was more contaminated in the bacterial cell. Fig. 2 shows the comparative flow diagram of both processes and the polysaccharide recovery for each steps.

In both processes, the first and last ultrafiltration steps (1CTUF100 and 4CTUF100) presented nearly 50% PRP loss, compared to the previous step (Fig. 2). Albani et al. associated this low PRP recovery with the polydispersed characteristic of the polymer; its size distribution varies according to the production conditions and fermentation time. According to the polysaccharide structure, which is similar to the RNA molecule, the polysaccharide is more stable at pH 7.0 or less (Egan, Schneerson, Werner, & Zon, 1982; Li and Breaker, 1999). Additionally, stability tests performed in parallel with polysaccharide purification indicated that the polysaccharide is more stable as the pH value is reduced (paper in preparation). Considering that polysaccharide cultivation was performed at 37 °C and pH 7.5, this may contribute to the loss of PRP during the first concentration (1CTUF100) because the molecular mass can be hydrolyse during the cultivation process; moreover the washing buffer used during the first concentration was prepared at pH 7.5. The second step that reflected considerable loss of PRP was after the enzymatic treatment, in which the best condition for the hydrolysis occurred at pH values above 7.5, and a portion of preparations were incubated at 37° overnight.

Despite low of PRP recovery in both processes, the purity of the final product was better using microfiltration, highlighted by the relative purity values and the transparent appearance of the pure PRP.

Table 2
Physical–chemical characterisation of purified polysaccharide.

Requirements	WHO	Purified PRP
Ribose (%)	>32	36.9 ± 1.4
Phosphate (%)	6.8–9.0	8.0 ± 0.3
Protein (wt Prt/wt PRP*100)	≤1%	0.36% ± 0.17
RP_{Prt} (wt PRP/wt Prt)	≥100	196 ± 62
Nucleic acid (wt NA/wt PRP*100)	≤1%	0.09% ± 0.04
RP_{AN}(wt PRP/wt NA)	≥100	1747 ± 384

Despite its traditional problems, such as fouling and polarisation effects, flow filtration technology is more flexible than centrifugation due to its ability to vary the design of the membrane modules to adapt to the specific product being separated or purified, and the whole system of membranes is more compact, which makes it easier to scale up.

The next challenge is to improve polysaccharide recovery by considering the stability of the polysaccharide at different pH and temperature, as well as culture conditions for polysaccharide production and purification. Evaluation of purification process based on different culture conditions should be performed to confirm the consistency and robustness of the process.

3.3. Purity and identity test

The final purified polysaccharide fraction was lyophilised and a solution of 1 mg/ml was prepared to confirm its purity and identity before proceeding to the conjugation process. The content based on ribose and phosphate was consistent and within the limits (Table 2). An immunological method, rocket immunoelectrophoresis, was used for the identity test Burro serum against PRP was recognised by the pure polysaccharide molecule, as illustrated in the Fig. 3 (WHO, 2000). The PRP presented the following molecular mass: $M_n = 369.6 \pm 29.7$ and $M_w = 520.4 \pm 48.5$ kDa; the dispersity ($Đ_M = 1.41 \pm 0.03$) was closer to the distribution of uniform polymer ($Đ_M = 1$) than broad distribution ($Đ_M \sim 2$), according to Stepto (2009). Thus, the quality of the polysaccharide obtained is in adequate for chemical conjugation to the protein.

Fig. 3. Identity of the purified polysaccharide. Rocket imunoelectrophoresis of pure PRP with 5, 10, 15 and 20 μg of PRP per spot.

4. Conclusions

We utilised a hollow fibre cartridge membrane for the purification PRP polysaccharide, making it possible to replace the centrifugation with TMF. Pure polysaccharide was achieved, as determined by nucleic acid and protein with $RP_{NA} = 1747.2$ and $RP_{Prt} = 196.1$, respectively. The lyophilised polysaccharide material was white in colour, and the content of ribose and phosphate were within in the range established by the WHO. Moreover, the polysccharide irecognised the specific antibodies in an identity test. Thus, the established process for purifying PRP produced by *H.influenzae* type b is effective, consistent and feasible for scaling up.

Acknowledgments

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