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Features of bacterial growth and polysaccharide production of *Streptococcus pneumoniae* serotype 14

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The effect of several cultivation conditions on the kinetics of bacterial growth and polysaccharide production of *Streptococcus pneumoniae* serotype 14 was studied. The presence in the supernatant of serotype-specific CPS (capsular polysaccharide) during growth was followed by size-exclusion HPLC and, in parallel, confirmed by using a specific latex reagent. The agitation level did not affect the production behaviour, whereas pH maintenance above 6 strongly enhanced both growth and CPS production throughout the cultivation period in flasks. Production of high-molecular-mass polysaccharide was found to be maximal between 5 and 6 h of cultivation, at the end of the exponential phase. By laser light scattering, 90 % of this purified CPS product showed a M_w (molecular mass) range from 350 to 1500 kDa, with an average M_w of 921 kDa. Extending the culture to 24 h gave rise to a clear shift of the M_w distribution of the polysaccharide to values lower than 100 kDa. These findings may have strong implications for the large-scale manufacture of the polysaccharide and the associated conjugate vaccine.

Introduction

Streptococcus pneumoniae is a capsulated bacterial pathogen responsible for mortality in the elderly and morbidity and mortality in the infant population all over the world. The capsular polysaccharides of pneumococci have been shown to be essential for their virulence [1]. Based on this, vaccines that include a number of the most prevalent capsular serotypes have been developed in the last decades. The classical 23-valent pneumococcal polysaccharide vaccine [2], still in use, is prepared as a mixture of purified CPSs (capsular polysaccharides) obtained from pathogenic strains of *S. pneumoniae*. This vaccine confers effective protection against the disease on youngsters and adults, but not so for infants, the main population at risk [3]. As a means of overcoming this limitation, various polysaccharide–protein conjugated vaccines against this pathogen have been under development [4,5] and currently one is licensed in the U.S.A. [6].

For both types of vaccine, highly purified pneumococcal capsular polysaccharides are needed. Determination of the time profiles of cell growth and polysaccharide production of the bacterium during cultivation is important, since it can dictate the optimal harvest time in terms of simplicity and economy of downstream operations. However, few reports can be found in the literature concerning such a kind of study for bacterial polysaccharides. Cultivation conditions of bacterial growth have been explored for the optimal polysaccharide production of serotype 1 [7] and, more recently, for the production of serotype 23F [8]. Also for serotype 19A [9], growth conditions were studied with regard to the structure and immunogenicity of the polysaccharide.

An epidemiological study for the Latin American region [10] has shown that serotypes 1, 5, 6A, 6B, 14, 19A, 19F and 23F are involved in 70–75 % of the cases of pneumoccocal infection. We have undertaken the study of bacterial growth and polysaccharide production for serotype 14, the most prevalent type in Latin America and worldwide, and also one with a high antibiotic resistance [11]. Several relevant features of bacterial growth and production of the CPS-14 polysaccharide were observed and are reported here in connection with the M_w (molecular mass) of the polysaccharide obtained and the yield of the overall process.

Materials and methods

Selection of bacterial strain and culture medium

The first bacterial cultivation experiments with *S. pneumoniae* serotype 14 were carried out with clinical isolates provided by Dr Maria Hortal de Peluffo, Pereira Rossell Hospital, Montevideo, Uruguay, which were grown in 125 ml flasks for 8 h, using Bacto™ Todd–Hewitt broth

Key words: capsule, capsular polysaccharide, fermentation, pneumoccocus, *Streptococcus pneumoniae*, vaccine.

Abbreviations used: CPS, capsular polysaccharide; M_w, molecular mass; SEC-HPLC size-exclusion HPLC; SEC-MALLS/RI, size-exclusion multi-angle laser light scattering/refractive index; SSM, semi-synthetic medium; TSB, tryptic soy broth.

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(Difco). The final supernatant of these cultivations was used to develop the initial latex reagent necessary to monitor the presence of polysaccharide in subsequent runs [12]. On entering the SIREVA (System for Regional Vaccine Production) programme for the Americas [10], the National Centre for Streptococcus, Ottawa, Canada, provided our laboratory with three different reference strains of CPS-14, namely numbers 1871, 2721 and 5782. Stock cultures of these were grown for 16 h at 36 °C in a 5% CO_2 incubator, and were kept frozen at –80 °C in the same medium containing 30% (v/v) glycerol.

The capacity of each of these strains for polysaccharide production was evaluated by latex titration of the polysaccharide in the supernatant of cultivation experiments, carried out with two culture media: TSB (tryptic soy broth) and the SSM (semi-synthetic medium) of Hoeprich [13]. The Todd–Hewitt medium was discarded, since it was considered not appropriate for systematic scale-up cultivation with production purposes.

The SSM components were obtained from the following sources: casein hydrolysate was from Difco, inorganic salts and glucose were from Fluka, and amino acids and vitamins were from Sigma–Aldrich. After adjusting the pH to 7.6, the medium was sterilized by filtration through a 0.22-μm-pore-size membrane and was kept at 0–4 °C until used.

Standard polysaccharide

A sample of pure CPS-14 polysaccharide was generously provided by Dr Chris Jones, National Institute of Biological Standards and Control, Potters Bar, U.K., and was used in the present study as the reference in the HPLC runs.

Analytical methods

Biomass growth was followed by measuring attenuance (*D*) at 620 nm in an Ultraspec 1000 spectrophotometer (Amersham Biosciences). In a given set of experiments, biomass dry mass was also determined in parallel as follows: 5 ml culture broth samples were centrifuged at 1789 ***g*** for 60 min at 10 °C. The bacteria-containing pellet was washed twice by resuspending in 0.9% NaCl and centrifuging as before and then it was dried at 60 °C until constant weight. A high correlation was found between both methods in the exponential phase and the corresponding data in the results section were expressed by attenuance reading. Residual glucose level was measured during the experiments directly on the original samples by the 'Accutrend' device (Boehringer–Mannheim), which was previously calibrated with a glucose oxidase kit (Wiener Laboratories). The CPS titre was determined by a specific latex reagent [12] on the supernatant of samples after extensive dialysis against distilled water. Protein concentrations were determined by the BIC (bicinchoninic acid) assay (Pierce) on the same samples.

Aliquots of the supernatant-dialysed samples were also analysed for polysaccharides by SEC-HPLC (size-exclusion HPLC) in a Zorbax GF-250 column, 4.6 mm diameter $\times$ 250 mm long (Agilent Technologies), at a flow rate of 2 ml/min and detection at 210 nm in a 1100 Series HPLC (Hewlett–Packard), equipped with an autosampler and a UV variable wavelength monitor (the molecular size exclusion of this column for globular proteins is, according to the manufacturer, of the order of 900 kDa. For linear polysaccharides the size exclusion of the same column is significantly lower. We have estimated it to be approx. 100 kDa). Chromatograms were processed with the Hewlett–Packard software. The M_w distribution of the pure polysaccharide was determined by SEC-HPLC coupled with a refractive index detector (RID-10A; Shimadzu) and a three-angle SEC-MALLS/RI (size-exclusion multi-angle laser light scattering/refractive index) detector (Wyatt Technologies), using a Superose 12 HR 10/30 column, irrigated with a pH 7.0 buffer (K_2HPO_4/KH_2PO_4; 20 mM), supplemented with NaCl (130 mM) at a flow rate of 0.4 ml/min. The M_w calculations were done using a refractive index increment, d*n*/dc, of 0.139 ml/g [14]. The identity of the final purified CPS-14 product was confirmed by the ^{1}H-NMR spectrum of a sample dissolved in ^2H_2O in a Bruker DPX-400 spectrometer, and comparison with authentic samples, as described previously [15,16].

Precultivation

At the time of the experiment, a frozen ampoule of *S. pneumoniae* strain number 5782 (see the Results section) was opened and grown on a blood agar plate for 24 h at 37 °C in a 5% CO_2 atmosphere. After checking its purity by using Gram stain, this culture was propagated in four to six blood/agar tubes, which were left overnight (16–18 h) under the same conditions. The agar surface of each tube was washed with 2 ml of sterile culture medium and the pooled washings were transferred to four 50 ml tubes containing 25 ml of SSM (see the Results section) each. The tubes were incubated for 3–5 h at 37 °C in a 5% CO_2 atmosphere until an attenuance reading of approx. 1–2 at 620 nm was obtained.

Growth kinetics in flasks

The effect of various experimental conditions on the growth curves was repeatedly tested on different days, with high reproducibility. Typically, four 250 ml flasks containing 150 ml of SSM were inoculated with approx. 10% (v/v) of the culture from the precultivation step. Then, 4 ml samples of the cultivation medium were taken along the run. The first sample was taken at zero time (P_0) from each flask and all flasks were allowed to grow at 37 °C under pre-established different conditions. The other samples were

Table 1 CPS-14 production of three strains of *S. pneumoniae* in two culture media, determined by latex titre

		Latex titre		
Medium	Strain number…	1871	5287	2721
TSB		1/32	1/128–1/256	1/64
SSM		Not detectable	1/256	1/2

taken every hour from 2 to 8 h (P2–P8) and, in several runs, an additional sample was taken at 24 h after inoculation (P24). After determining the attenuance, the samples were immediately heat-inactivated (60 °C, 5 min) and then centrifuged (5000 ***g***, 15 min). Subsequently, the glucose level and pH were determined in the supernatant. During the run, and according to the design of the experiment, glucose was incorporated by adding 2–3 ml of a concentrated, sterile glucose solution (20 %, w/v) to the flask. At the same time, in these runs the pH was adjusted by the addition of a 2 M NaOH solution to the flask, as required to keep the pH above 6. The effects of CO_2 atmosphere (0 or 5 %), agitation (0 or 50 rev./min) and pH level above 6 (yes or no) were investigated.

At the end of the run, the samples were dialysed for 36–48 h against distilled water, and the CPS titre was determined by a latex agglutination test [12]. Aliquots of the same samples were analysed by SEC-HPLC. Collected fractions from the first three SEC-HPLC peaks for samples taken at an intermediate point (P5) of two runs were also tested with the latex reagent. Pure CPS-14 was isolated from several end culture supernatants as reported elsewhere [12,15].

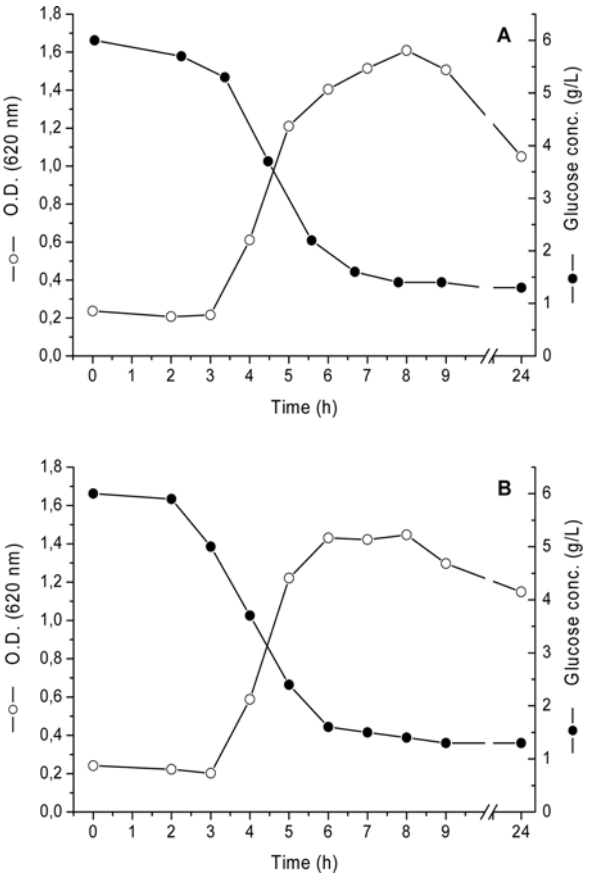

Figure 1 Effect of agitation on biomass growth and glucose consumption during cultivation of *S. pneumoniae* in flasks

(**A**) Static condition. (**B**) Agitation (50 rev./min). conc., concentration; O.D. (620 nm), D_{620}.

Results

Selection of bacterial strain and culture medium

Table 1 shows the results of the CPS-14 production of the three reference strains tested in the two culture media used, as determined by their latex titre in the cultivation supernatant. Clearly, strain number 5782 presented the highest CPS titres in both culture media used and was therefore selected for the subsequent experiments. On the other hand, the SSM medium was chosen instead of the classic TSB because, in addition to having a more defined composition, TSB tended to form polymeric compounds upon autoclaving and also interfered strongly with the latex determination of the polysaccharide.

Time-dependence of bacterial growth and CPS production

The time profiles of bacterial growth and glucose level of *S. pneumoniae* serotype 14 corresponding to static and low-speed agitation conditions (50 rev./min) are presented in Figures 1(A) and 1(B) respectively. Very little difference is apparent between both conditions. Figure 2 shows the effect of pH control on the corresponding curves for bacterial growth and glucose level for 50 rev./min stirring under 0 or 5 % CO_2. When pH was kept above 6, a substantially larger biomass production was achieved and higher glucose consumption was observed (Figure 2B) as compared with the experiments without pH control (Figure 2A). The glucose concentration increase observed at 5–6 h cultivation time in the pH-controlled experiments is caused by the external addition of glucose, as was needed to make up for its larger consumption under this condition so as to avoid total depletion.

Figure 3 shows the SEC-HPLC chromatogram of a sample corresponding to an intermediate point (P5) of a given kinetic run, superimposed on the corresponding results from the latex agglutination test, performed separately for each of the first three peaks. As is apparent from the Figure, the CPS titre corresponding to the first peak

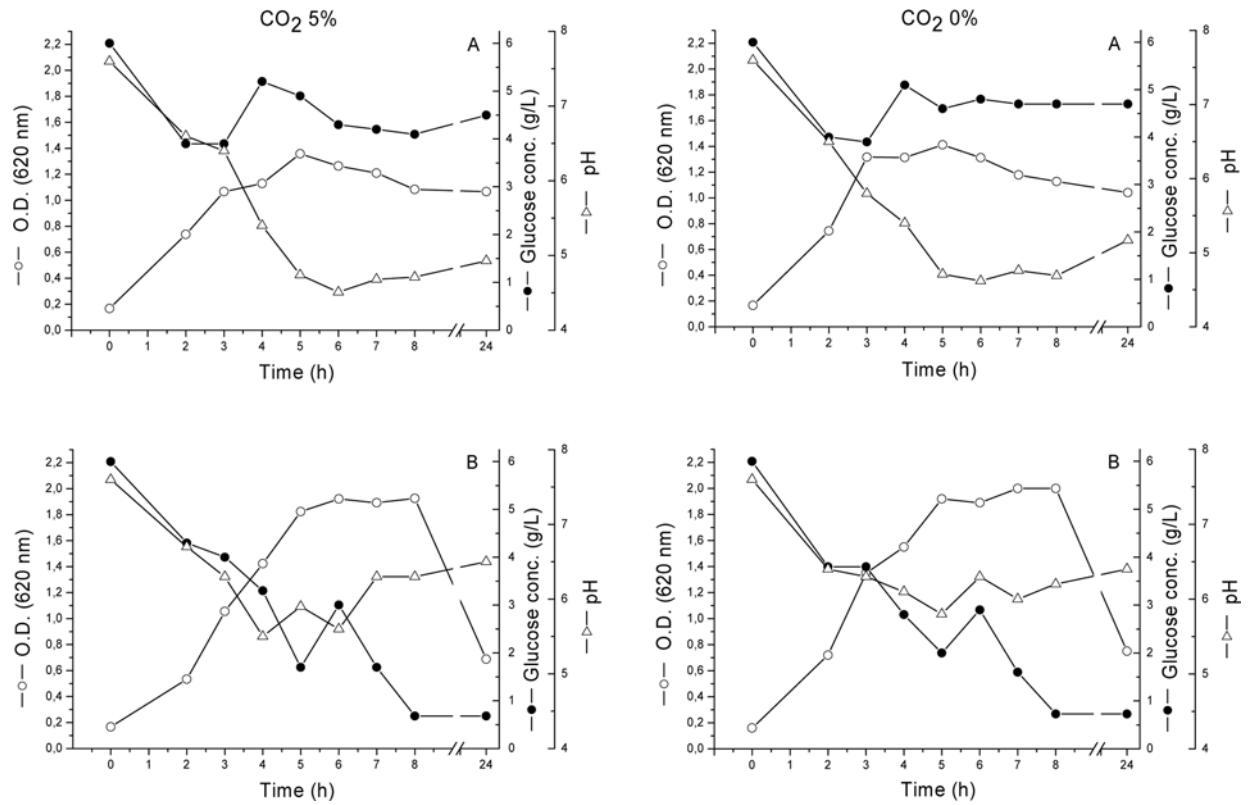

Figure 2 Effect of CO_2 atmosphere and pH control during cultivation of *S. pneumoniae* in flasks: (A) no pH control and (B) with pH control

conc., concentration; O.D. (620 nm), D_{620}.

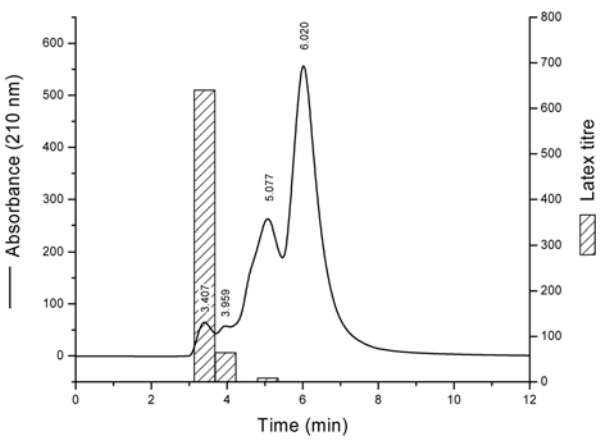

Figure 3 Latex titre of first HPLC chromatogram peaks of an intermediate sample during cultivation of *S. pneumoniae* in flasks

was more than 10 times higher than the corresponding value for the second one, showing that most of the polysaccharide elutes in the void volume, and was therefore selected in all samples as representative of the main CPS fraction.

Figure 4 shows the effect of pH control on CPS production followed by two methods: latex agglutination and percentage of total chromatogram area that corresponds to the major polysaccharide peak, eluted at the void volume in the SEC-HPLC analysis. In accordance with the results in Figure 2 for biomass growth, the line for the CPS latex titre in the pH-controlled experiment, Figure 4(B), indicates a CPS production level consistently higher than that without pH control (Figure 4A) with a slower increase in the percentage CPS peak area. In both Figures 4(A) and 4(B), on the other hand, an initial increase in all lines is observed up to 5–6 h cultivation time, but those for the percentage CPS peak area go down thereafter, whereas the latex titre stays constant. After 24 h of cultivation, a significant decrease in the fractional area of the CPS peak, in parallel with an increase in the latex titre, is evident.

Figure 5(A) shows the SEC-HPLC chromatograms at 210 nm for sequential samples (P2–P8 and P24) obtained during a culture period of 24 h with pH control and 5% CO_2. A steady shift to longer retention times is apparent for the main polysaccharide peak (see expansion on Figure 5B), which is more pronounced for cultivation times beyond 5 h (data points P5–P8 and P24).

Figure 6 shows the typical M_w distribution observed for purified CPS-14 polysaccharide obtained from 8 h cultivation

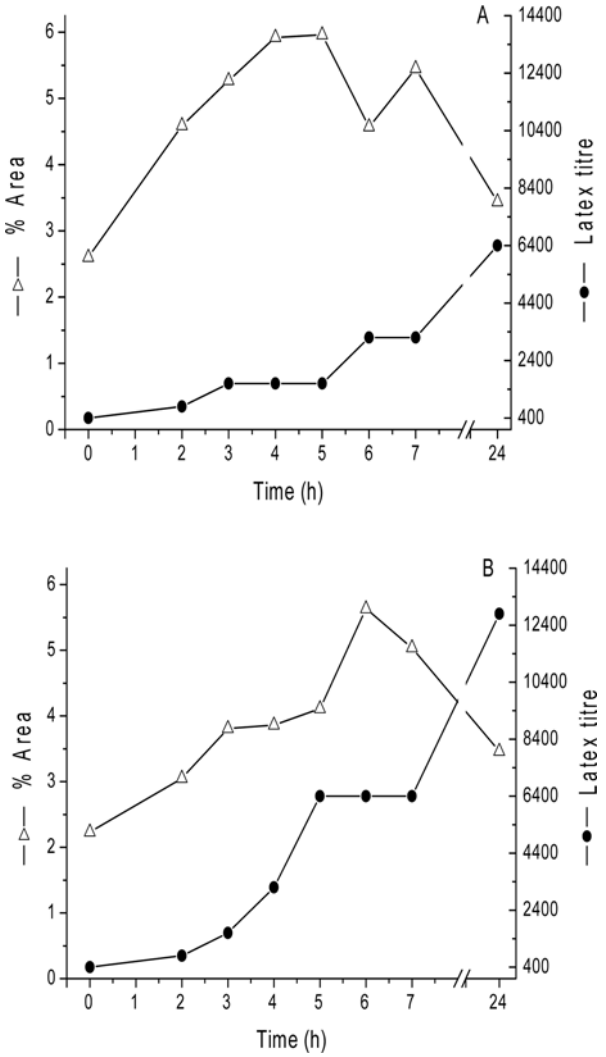

Figure 4 Effect of pH control on the production of CPS-14 followed by latex agglutination titre and SEC-HPLC during cultivation of *S. pneumoniae* in flasks: (A) no pH control and (B) with pH control

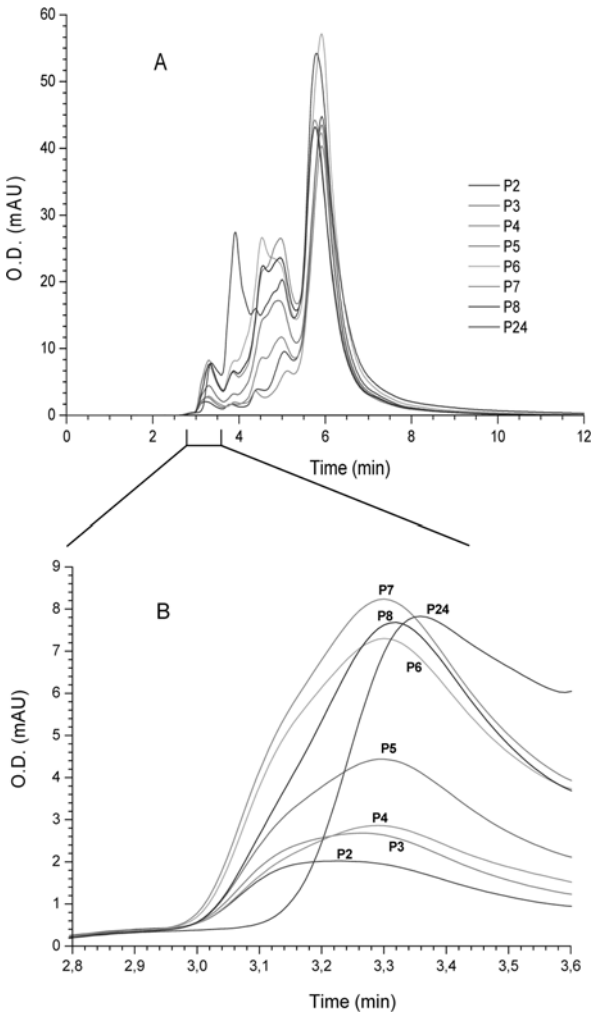

Figure 5 SEC-HPLC chromatograms of sequential samples taken during a typical cultivation run of *S. pneumoniae* in flasks with pH control: (A) whole profile and (B) CPS-14 peak (inset)

O.D. (mAU), absorbance (milli-absorbance units).

runs, measured by SEC-MALLS/RI. A normal M_w distribution is observed, with an average M_w of 921 kDa, and 90% of the samples having an M_w from 0.35×10^6 Da to 1.5×10^6 Da.

Discussion

Determination of the time profiles of bacterial growth and capsular polysaccharide production during cultivation is important, since it can indicate the optimal harvest time in terms of simplicity and economy of downstream operations. In turn, determining the effect of process parameters on these profiles provides the tools to achieve best results. For the case of *S. pneumoniae* serotype 14, Figure 1 indicates that the effect of agitation, that is, cultivation under static or stirred conditions, is not relevant in terms of biomass growth and glucose consumption. However, a minimum agitation level should be considered preferable, since it allows the homogeneous distribution of added nutrients and/or reagents to control pH level. Also the effect of keeping an artificial CO_2 atmosphere in the headspace of the flasks (Figure 2) does not induce appreciable differences in the behaviour of the culture in terms of glucose consumption and biomass growth, either with or without pH control. Working under an atmosphere with a low CO_2 partial pressure is probably more important in the precultivation stages, i.e. during the propagation of the culture from the seed. On the other hand, by comparing Figures 2(A) and 2(B), one can see that keeping the pH value above

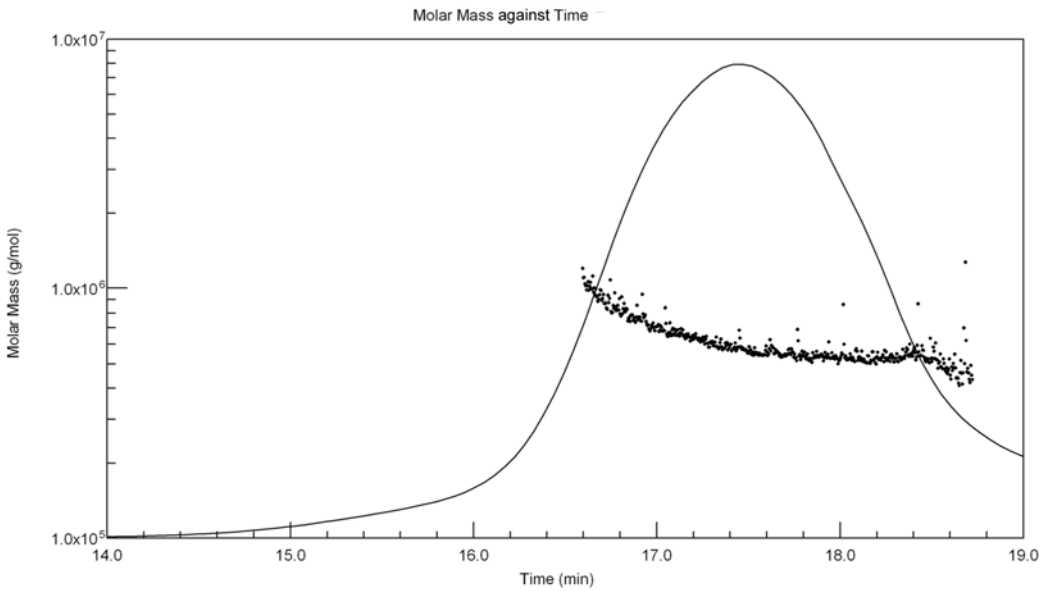

Figure 6 M_w distribution of purified CPS-14 determined by SEC-MALLS/RI

6 has a profound effect on the culture, enhancing both biomass growth and CPS production. Although this is a parameter routinely controlled in bioreactors, quite often experiments carried out at a small scale in the laboratory omit this aspect and therefore give rise to erroneous results during scale-up. Dahlgren et al. [17] have recommended pH control by buffering the cultivation medium to mimic the behaviour of a model system in a scale-down experiment.

The curves for CPS content in the supernatant compared with cultivation time, assessed by the specific latex reagent in Figure 4(B), indicate an optimal harvest time at 5–6 h, since a plateau is reached, in coincidence with a maximum level of the percentage CPS peak area in the SEC-HPLC analysis. Thereafter, an apparent discrepancy arises between the CPS level determined by the latex titre and the percentage CPS peak area by SEC-HPLC: while towards the end of the run, decreasing percentage peak areas are shown, the same sample exhibits first a constant and then an increased latex titre. As a possible explanation, we have reasoned that breakdown of the polysaccharide into smaller size fragments would reduce the content of CPS at the void volume peak, while at the same time it may not affect, or even could increase, the latex titre for the whole sample, since the same mass amount of polysaccharide would generate a higher molar concentration of active species. Late-phase degradation of the cell wall may also be contributing to these effects.

That this is a plausible mechanisms is supported by the HPLC chromatograms of sequential samples taken during an entire run, shown in Figure 5. There one can observe a steady shift to higher retention times, i.e. lower M_w values, of the corresponding polysaccharide peaks as the cultivation run progresses. Also, surprisingly, a significant change in chromatogram profile is evident for the supernatant obtained after 24 h of culture (P24), where a major peak appeared immediately after the void volume, indicating an average M_w of <100 kDa for polysaccharides. This fact, together with the clear decrease in the percentage peak area of the void volume peak and a 2-fold increase in latex titre, suggests CPS-14 polysaccharide degradation as cultivation time elapses. The dispersion in M_w of the pure CPS obtained is also reflected in the distribution measured by SEC-MALLS/RI for the CPS obtained from 8 h cultivation runs, shown in Figure 6. This can have important consequences for the large-scale manufacture of the CPS and the associated conjugate vaccine, because uniformity in M_w is desirable to obtain consistency between lots. Possible alternatives to achieve this would be the separation of the lower-M_w fractions and chemical hydrolysis of the larger ones in order to obtain a more uniform and sharp M_w distribution of the CPS product.

In conclusion, we have confirmed that pH control in laboratory experiments is essential to obtain cultivation results useful for scale-up, as this was particularly evident for the case of *S. pneumoniae* serotype 14 considered here. On the other hand, by the use of two monitoring techniques for the CPS in parallel, we were able to observe that harvest time not only affects the overall production

yield, but more intriguingly, it also determines the M_w distribution of the product. In this sense, the indicated value of 5–6 h as the optimal harvest time applies only to a polysaccharide product with a high M_w. In longer runs, since the average M_w strongly decreases with cultivation time, by fine-tuning the harvest time it would be possible to achieve a more uniform M_w distribution and, at the same time, a higher molar concentration of active units, giving rise to a potentially better yield of the conjugate vaccine. Uniformity in the M_w distribution of the polysaccharide, on the other hand, is desirable for the manufacture of the corresponding conjugate vaccine in order to achieve consistency of production. It is felt that valuable insights into the cultivation behaviour of other pneumoccocal serotypes can be achieved by an approach similar to that reported in the present study.

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