A new chemically defined medium for cultivation of *Streptococcus pneumoniae* Serotype 1

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A chemically defined culture medium for the production of S. pneumoniae was developed. The performance of the synthetic medium for cultivation of S. pneumoniae serotype 1 was evaluated by comparing biomass production in this medium with biomass produced by growing the bacteria in trypticase soy broth (TSB) medium. Bacterial growth was monitored by measuring the optical density (OD), change in the pH of the medium and glucose consumption. Production of capsular polysaccharide was confirmed by using a specific latex reagent in culture supernatant. The performance of the synthetic medium analyzed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) on intact cells showed an improvement of the resolution of samples/matrix mixture and a very homogeneous spectrum which confirmed the feasibility of growth of S.pneumoniae in the chemically defined medium (CDM).

Keywords: *Streptococcus pneumoniae*, capsular polysaccharide, chemical defined culture medium.

Abbreviations: CDM: Chemically defined medium; CPS: Capsular polysaccharide; GMP: Good manufacturing practices; MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight spectrometry; PCV: Pneumococcal conjugate vaccine; TSB: Tryptic soy broth.

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Introduction

S.pneumoniae is a major bacterial pathogen worldwide. It is an important cause of serious invasive infections including meningitis, bacteremia, and pneumonia and less serious, but more common infections including acute otitis media and sinusitis [1, 2, 3]. Control of the pathogen is mainly achieved by the use of existing vaccines. In the past few decades several vaccines have been developed and are available in the market, which are formulated with a limited number of polysaccharides corresponding to the prevalent serotypes. A vaccine consisting of a mixture of 23 capsular polysaccharides (CPS) obtained from different pathogenic strains of *S. pneumoniae* was earlier developed [4]. This vaccine is effective against the disease, except in infants, in the elderly or in immunocompromised people [5]. It provides serotype-specific protection, and covers 23 of the more than 90 existing serotypes.

The fact that pneumococcal vaccines are less effective in these populations has driven research towards achieving an increase of its immunogenicity, such as conjugated vaccines like PCV7 and PCV13 [6,7]. These vaccines are

developed by attaching 7 or 13 polysaccharides antigens to carrier proteins to enhance the immune response of the capsular antigens.

The capsular polysaccharide is the main virulence factor and the main component of existing vaccines, thus vaccine production largely relies on cultivation of the pathogen in appropriate conditions. This represents a central problem because the pathogen has very high metabolic requirements which lead to the use of complex media for its culture.

The biotechnological production of medicinal products requires the use of Good Manufacturing Practices (GMPs), which comprise the part of Quality Assurance that ensures products strictly manufactured and controlled consistently to the established quality requirements for use (safety and efficacy). Particularly, vaccine production must be carried out with a quality level that consistently renders products that meet the pre-established standards at all stages of the process, ensuring the efficacy and harmlessness of the finished product.

Despite their importance in public health, little is known about the culture media used for the production of these bacterial antigens, the literature is scarce and not updated, or at least it is not openly available for the research community.

A partially defined culture medium for *Streptococcus penumoniae* was described in 1942 and since then, several authors [8, 9] had improved its formulation, but all maintained the casein or the soy hidrolyzate as nitrogen and the carbon sources [10, 11, 12]. The most chemically defined culture medium used is the one described by Van De Rijn [13] i.e. for cultivations of *Streptococcus mutans* [14] but there are to our knowledge no complete chemical defined medium described for the cultivation of *S. pneumoniae*.

The presence of undefined components is unsuitable for commercial vaccine production since they do not meet some of the GMP regulatory requirements, as well as the downstream antigen purification processes become more complex.

The best known media to grow *S. pneumoniae* are the Brain-Heart Infusion (BHI), the Todd-Hewitt and Tryptic Soy Broth (TSB) media, containing of supplements such as yeast extract, or other sources of carbon and nitrogen, even when those sources may be of vegetal origin. According to the GMP standards during the fermentation to produce vaccine antigens or biological products, the medium must contain only those essential components that allow the maximum cell density and the reproducibility of the process.

Here, we present results of a novel culture medium, free of undefined components for cultivation of *S. pneumoniae*, and evaluated it with a strain of *S. pneumoniae* serotype 1, one of the serotype with the highest incidence in pneumococcal disease in several region of the world and mainly associated with invasive disease [3]. Furthermore we report the composition of this synthetic medium, the size of the initial inoculum and the conditions of culture for the production of *S. pneumoniae* serotype 1, able to optimize the biomass production in conditions that enable an easy extraction and purification of capsular polysaccharide, using a process that conforms to GMP standards.

Materials and Methods

Streptococcus pneumoniae serotype 1 strain 2741 and serotype 14 strain 5287 used in this study were obtained from The National Centre of *Streptococcus* (Alberta, Canada). TIGR 4 serotype 4 (strain BAA 334) was obtained from ATCC. Working stocks were prepared from cultures in Todd Hewitt Yeast Broth according to the protocol from Bacterial Respiratory

Pathogens UAB (Birmingham, AL, USA) and maintained at –80°C.

Tryptic Soy Broth (TSB) medium was prepared according the specifications of the manufacturer (Oxoid, Basingstoke, and Hampshire, England). The chemically defined medium (CDM) components were obtained from the following sources: inorganics salts, galacturonic acid and glucose were from Fluka, and amino acids and vitamins from Sigma-Aldrich.

MALDI matrix 3, 5-Dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) and peptide calibration standard mix were purchased from Bruker Daltonics (Billerica, MS, USA).

Defined Medium composition and preparation

The components of the synthetic medium are listed below from 1 to 5. The compounds were added in groups and each group was dissolved completely before addition of the next.

1. Basal Medium contains amino acids as follows (mg/L): L-tryptophane,35; glycine, 65; L-cystine, 166; L- tyrosine, 144; L-lysine, 230; L-valine, 173; L-leucine, 230; L-isoleucine, 170; L-threonine, 120; L-methionine, 73; L-aspartic acid, 184; L-proline, 43; L-histidine hydrochloride, 55; L-arginine hydrochloride, 125; L-phenylalanine, 125; L-serine, 235.

   This basal medium also contain salts (g/L) such as monobasic potassium phosphate 5.5, monobasic sodium phosphate 3.2 and dibasic sodium phosphate 7.3.

2. The solution of vitamins was prepared dissolving the components (mg/L), in distilled water as follows: biotine, 0.15; nicotinic acid, 100; pyridoxal, 100; calcium pantothenate, 500; thiamine, 100; riboflavine, 100; adenine sulfate, 1000; uracil, 1000. The mixture was aliquoted and maintained at -20°C.

3. The solution of salts was prepared dissolving the components (g/L) in distilled water as follows: magnesium sulphate 7 H_2O, 250; ferrous sulphate 7 H_2O, 2.5; zinc sulphate 7 H_2O, 0.4; manganese sulphate, 0.2 and chlorhydric acid 10 ml.

4. One liter of vitamins, salts and growth factors solution was prepared as follows: 200 ml of the vitamins solution (2)*, 40 ml of the salts solution (3)* and the growth factors: L-glutamine, asparagines, and choline chlorydrate in the following amounts, 12.5 g, 2 g, and 0.2 g respectively.

   * The numbers (2) and (3) are the solutions previously prepared.

5. A sodium bicarbonate ($NaHCO_3$) and thioglycolic acid solution was prepared as follows: 1 g of $NaHCO_3$ and 1ml of thioglicolic acid (80%) were mixture in 25 ml of distilled water. This unstable mixture was prepared and immediately added to the medium prior to the sterilization process.

A volume of one liter of this complete medium was prepared as follows: 900 ml basic medium, 50 ml of vitamins, salts, and growth factors solution, 12.5 g/L of glucose and 25 ml bicarbonate/thioglicolic acid solution were mixture in distilled water. The pH was adjusted to 7.2, the solution was sterilized by filtration through a 0.22 µm-pores-size membrane and placed at 37°C in 5% CO_2 atmosphere.

Culture of *S.pneumoniae*

S. Pneumoniae was cultured 12 hours in triptic soy broth (TSB) in a 5% CO_2 atmosphere. The bacterial mass was collected, washed twice with synthetic culture medium and dispensed into flasks with either TSB or CDM. Inoculums of 100 and 300 µl of a bacterial suspension containing 7 x 10^9 cells/ml (based in McFarland's scale) were used to start the cultures in one liter flasks. Samples were taken every 3 hours for 9 hours and at the end of the cultivation time at 24 hours.

Analytical methods

Biomass growth was followed by measuring optical density at 600 nm in an Ultraspec 1000 spectrophotometer (Amersham Biosciences). The pH in the samples was controlled and maintained near to 7.0, by addition of 0.1 N NaOH.

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 titer was determined by a specific latex reagent [15] in the culture supernatants samples and the protein content was determined respectively [16].

The quality and reproducibility of growing in the different media were analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) obtained from whole bacteria, on a Microflex LR MALDI-TOF (Bruker Daltonics, Billerica, MS, USA) with a 337 nm nitrogen laser operated in positive ion reflectron mode with delayed extraction and optimized in the m/z range of 0 to 20 kDa. Calibrations were performed with a peptide calibration standard mix (Bruker Daltonics). The laser was fired 100 times at each of ten locations for each sample well on a 96 well plate for a cumulative 1000 shots per sample well taken at 30 % intensity.

At the time of analysis, the cells grown in both media were washed twice in saline, centrifuged 5 minutes at 5,000 rpm and resuspended in saline again to a concentration of approximately 1.0 OD_{600}/ml [17]. At this time 1 µl of each of the bacterial suspension was mixed pipetting up and down with 1 µl of matrix solution (sinapinic acid 10 mg/ml in sterile H_2O with 1% TFA) at ratios of 1:1. The sample and matrix mix was spotted onto a 96 well stainless steel plate and allowed to air dry for 15 minutes at room temperature.

Results and Discussion

Initial experiments were carried out using two strains of *S. pneumoniae* (2741 of serotype 1 and 5287 of serotype 14), that have high incidence in pneumococcal disease. Both virulent strains grew adequately in the synthetic medium (data not shown for strain 5287 of serotype 14). The medium contains all the components of various other media described for the cultivation of *S. pneumoniae* or others microorganisms [10, 11, 12, 14], but it is devoid of any component of animal or vegetal origin. Van De Rijn and collaborators used a defined medium similar to the one we present here. We worked to achieve a better and more reproducible results in the cultivation of *S. pneumoniae* than the ones we found using complex medium. To know the components and their concentrations leads to a controlled environment during the cultivation of the microorganisms. The new synthetic medium presented here is the result of repeated studies where we systematically work to achieve higher growth yields for *S. pneumoniae* using small inoculums and a minimal adaptation time giving as a result higher densities than those obtained using the complex medium but avoiding the use of not defined components. The growth properties for *S. pneumoniae* serotype 1 were compared using the synthesized chemical defined medium (CDM) and the trypticase–soy medium (TSB). Cultures were developed for 24 hours and bacterial growth in both media was estimated by optical density. As it can be seen in Figure 1 biomass yield was higher at the end of logarithmic phase in the CDM compared with TSB, reaching a value of 1.3 ± 0.07 in contrast with 0.8 ± 0.03 of the TSB. The results were obtained in three independent experiments and despite of the initial bacterial inoculum used. Overall, the results demonstrate that the synthetic medium allows increasing the total biomass and reduces the lag phase demonstrating a good potential for optimizing the production of these antigens and reducing the fermentation time. The results obtained were the same when the bacteria were grown

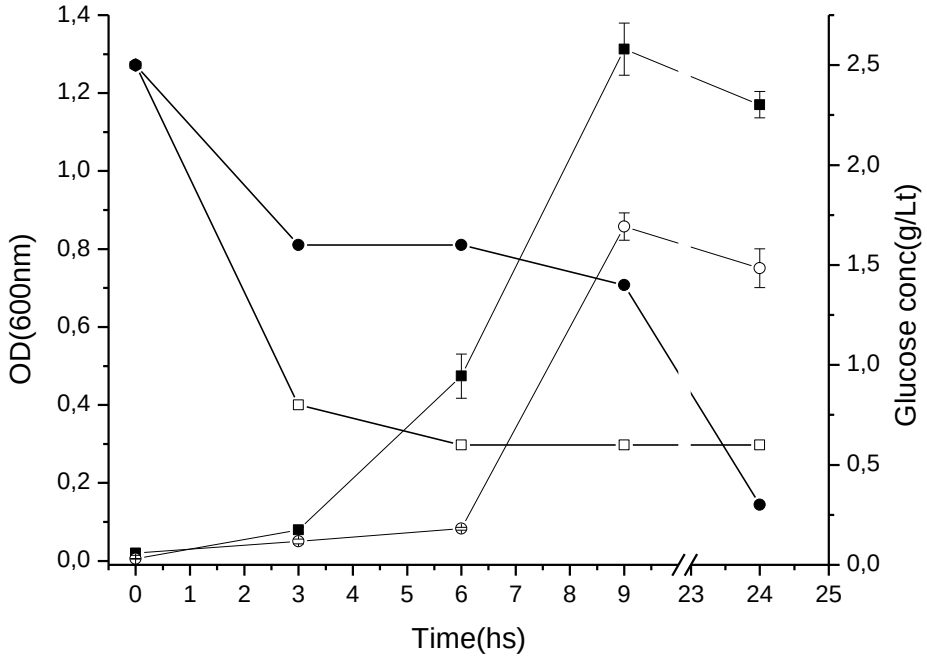

Figure 1. Growth profile of S. pneumoniae in TSB (○) and in CDM (■). Glucose consumption in TSB (●) and in CDM (□). Standard desviation (±SD) was calculated over three independent experiments.

in a biofermentor instead of flasks (data not shown).

At the same time, Figure 1 also shows the time profile of glucose consumption in the two media. By 14 hours more than 95% of the glucose had been depleted from both media. However it could be observed that in the CDM the glucose consumption was depleted before the growth reaches its maximum. The observation of the rapidly nutrient uptake suggests eventually less interference for the bacterial growth in CDM. It is therefore apparent that the specific culture environment must be taken in consideration before comparing bacterial growth. The pH changes during culture of *S. pneumoniae* was measured and showed a similar decline at the same times of cultivation in both media, indicating that the bacterial metabolism do not experiment changes when grown in CDM as compared with TSB medium. Furthermore, the pH was maintained at 7.0 for most of culture time, as it has been reported to be the optimum pH for the production of type 1 polysaccharide [11] and a key parameter to obtain high biomass yields.

The production of capsule polysaccharide in both cultures was determined by a specific latex reagent [15]. Figure 2 shows a direct relationship between latex titer and bacterial growth. However when the bacteria is grown in TSB Figure 2 a, the latex titer reached its maximal value at 6 hours of culture and then declines, whereas it is maintained consistently high in CDM culture (Figure 2 b). This behavior could be explained by the presence of components in the TSB medium that interfere with latex particles, a behavior that was previously reported for strain 14 of *S. pneumoniae* [12].

The protein content was higher for TSB (0.35 ± 0.02 mg /ml) than for CDM media (0.2 ± 0.01 mg/ml), suggesting a major purity of the

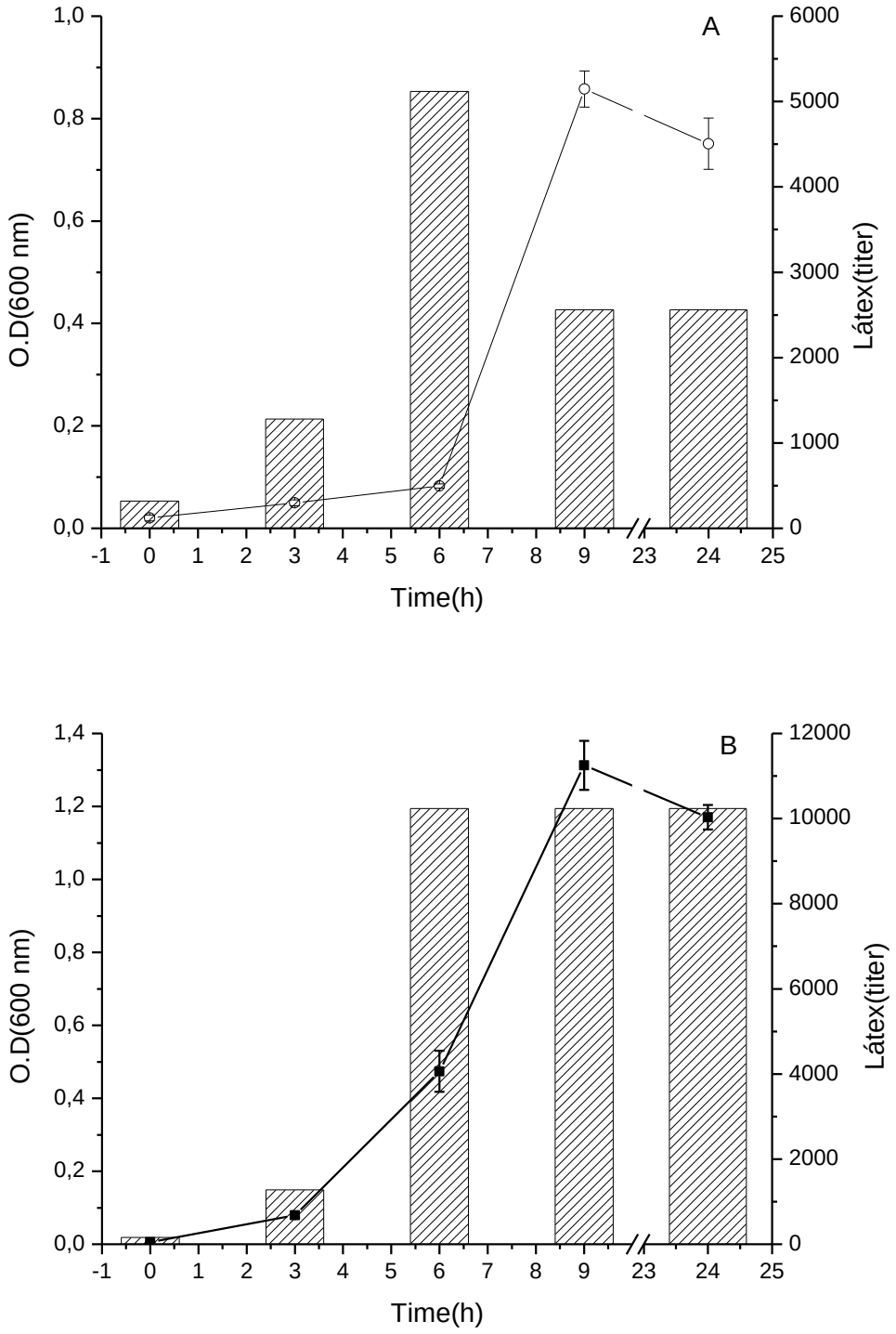

Figure 2. Latex titer in TSB (A) and CDM (B). Standard desviation (±SD) was calculated over three independent experiments.

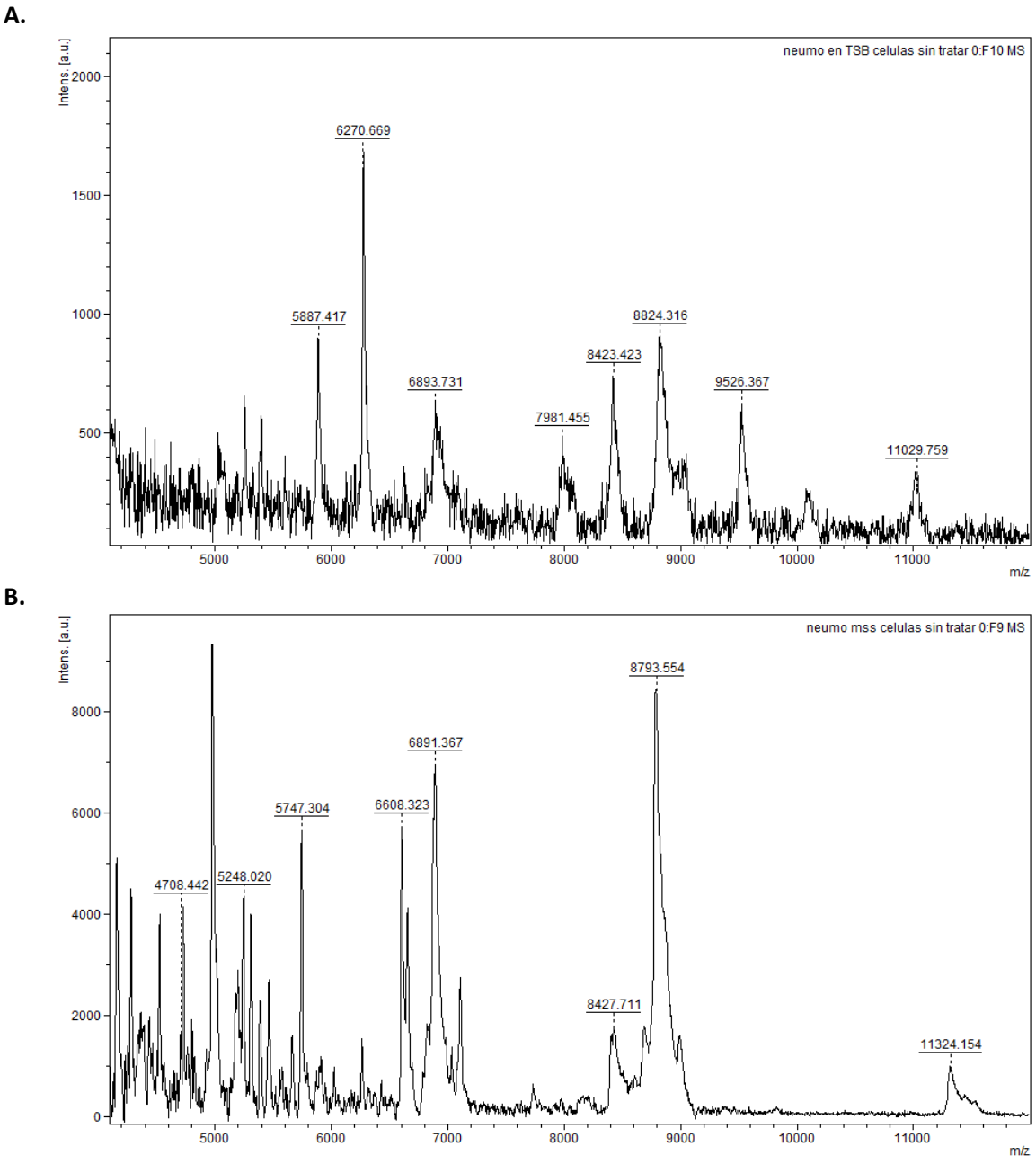

Figure 3. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry on S.pneumoniae intact cells cultured in a) TSB and b) CDM.

product obtained in CDM given that impurities on the medium can interfere with the results obtained.

In addition mass spectrometry on intact cell microorganisms was performed and the spectra obtained for both media shows in Figure 3a and 3b. It can be seen that the intensity of the peaks found in cells grown in CDM is greater; this may be due to less interference of the medium components which allow a better resolution of the peaks and greater intensities. This technique is used to identify different species by obtaining a fingerprint spectrum i.e.

Staphylococci [18] and Helicobacter [19]. Early work highlighted the significant effect that variation in culture media, incubation conditions, and length of incubation had on the spectra produced [17]. Therefore, in order to observe if there were any changes on the fingerprint spectra on the intact cell of the *S. pneumoniae* cultivated in these two different media, we used MALDI-TOF technique. In the course of these studies and under conditions used, a greater uniformity of samples obtained from the cells grown in the CDM was observed; they showed a more homogeneous sample/ matrix mixture and provided spectra of higher intensity and greater reproducibility. It confirms that the use of CDM improves the resolution and accuracy of samples.

Conclusions

The results observed demonstrate that the chemically defined medium is capable of supporting the growth of *S. pneumoniae*. This is a fastidious microorganism that requires rich media for its development. The possibility of using a medium not containing components of animal or vegetal origin is a change in usual cultivation techniques. A chemically defined medium is inherently more reproducible than a complex one. Furthermore a chemically defined medium enables discrete analysis of the effect of each component and strict control of medium formulation through identity and purity testing of raw materials. Even if the scaling process may represent high raw material costs, this is compensated by the better quality of the product free of debris that hinders the process of purification of the antigen. The product obtained should have a higher quality that makes it suitable for use in human vaccines.

The fact that other strains of *S. pneumoniae* were cultivated and successfully growth in the CDM, demonstrate that this medium could be suitable to cultivate others strains of this microorganism.

The ability to recover increased quantities of antigens in the defined medium described in this study will further aid investigators in their study of mechanisms of action of the components and in their attempts to develop a more efficacious human vaccine.

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