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(54) Benævnelse: **FREMGANGSMÅDE TIL KONTROL AF MOLEKYLVÆGT AF STREPTOCOCCUS PNEUMONIAE-POLYSACCHARID VED ANVENDELSE AF CARBON**

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DESCRIPTION

FIELD OF THE INVENTION

[0001] The invention relates to methods for increasing the molecular weight of isolated *Streptococcus pneumoniae* capsular polysaccharides having a phosphodiester linkage between saccharide repeat units.

BACKGROUND

[0002] In the preparation of multivalent conjugate pneumococcal vaccines directed to the prevention of invasive diseases caused by the organism *Streptococcus pneumoniae* (also known as pneumococcus), selected *Streptococcus pneumoniae* serotypes are grown to supply polysaccharides needed to produce the vaccine. The cells are grown in fermentors with lysis induced at the end of the fermentation by addition of sodium deoxycholate or an alternate lysing agent. The lysate broth is then harvested for downstream purification and the recovery of the capsular polysaccharide which surrounds the bacterial cells. After conjugation with a carrier protein, the polysaccharide is included in the final vaccine product and confers immunity in the vaccine's target population to the selected *Streptococcus pneumoniae* serotypes.

[0003] Polysaccharide size is a quality attribute assayed for in each preparation batch and must be appropriately controlled. Traditional processing has involved using NaOH (sodium hydroxide) as a base titrant during fermentation. The use of NaOH has the advantage of being able to lower the pH of the deoxycholate lysate without foaming to remove protein and improve filtration. This material is subjected to centrifugation followed by filtration to remove most of the solids down to a 0.45 μm nominal size. However, such traditional processing methods result in lower molecular weight polysaccharide (< 450 kDa) for serotypes having a phosphodiester linkage between saccharide repeat units (*e.g.,* 6A, 6B, 19A, and 19F).

[0004] Accordingly, improved methods for the recovery of high molecular weight capsular polysaccharide from cellular *Streptococcus pneumoniae* lysates, in particular lysates containing *Streptococcus pneumoniae* serotype 6A, 6B, 19A, or 19F polysaccharides, are needed.

[0005] WO0168903 discloses methods of modulating capsular polysaccharide production in *Streptococcus pneumoniae.* In particular a method of producing capsular polysaccharide from *Streptococcus pneumoniae* by maintaining a gas having a subatmospheric concentration of oxygen in contact with the growth medium.

[0006] US2007184071 discloses an immunogenic composition having 13 distinct polysaccharide-protein conjugates and optionally, an aluminum-based adjuvant. Each conjugate contains a capsular polysaccharide prepared from a different serotype of

Streptococcus pneumoniae (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) conjugated to a carrier protein. Methods for making an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 19A polysaccharide are also provided in which the serotype 19A polysaccharide is co-lyophilized with a carrier protein and conjugation is carried out in dimethyl sulfoxide (DMSO) via a reductive amination mechanism.

[0007] EP0497524 discloses a type-specific capsular polysaccharide preparations from *Streptococcus pneumoniae,* having on average less than about 1000 oligosaccharide repeat units per molecule, polydispersities between 1.0 and 1.4, intrinsic viscosities between 0.6 and 3.0 dL/g, and less than 3% contamination of type-specific polysaccharide by group-specific C-polysaccharide, produced by a process.

[0008] US2006228381 discloses a process for reducing the protein content and preserving the capsular polysaccharide content in a complex cellular *Streptococcus pneumoniae* lysate broth prior to purification is described. Utilizing pH reduction after cellular lysis has resulted in a purified polysaccharide that consistently meets the protein specification, and higher recovery yields of polysaccharide during the purification process.

[0009] WO2008118752 disclosed a shortened process for producing a solution containing substantially purified capsular polysaccharides from a cellular *Streptococcus pneumoniae* lysate broth. Ultrafiltering and diafiltering a clarified *S. pneumoniae* lysate followed by pH adjustment to less than 4.5, preferably about 3.5, precipitated at least 98% of the protein in the solution without seriously affecting polysaccharide yield.

BRIEF SUMMARY OF THE INVENTION

[0010] Improved methods for the recovery of high molecular weight capsular polysaccharides from cellular *Streptococcus pneumoniae* lysates containing serotypes having a phosphodiester linkage between saccharide repeat units are provided. In one method, CO_2 is supplied to a fermentation culture of a *Streptococcus pneumoniae* serotype containing a phosphodiester linkage between saccharide repeat units. Accordingly, in one embodiment of the invention, the method includes the steps of: 1) preparing a fermentation culture of *Streptococcus pneumoniae* bacterial cells that produce capsular polysaccharides comprising a phosphodiester linkage between repeat units; 2) supplying CO_2 to the fermentation culture, wherein supplying CO2 to said fermentation culture comprises a first addition of NaHCO3 to the fermentation culture at 10-50mM and a second addition of Na2C03; 3) lysing the bacterial cells in the fermentation culture; and 4) isolating *Streptococcus pneumoniae* capsular polysaccharides from the fermentation culture, wherein the molecular weight of said isolated *Streptococcus pneumoniae* capsular polysaccharide is at least 480 kDa, and wherein said *Streptococcus pneumoniae* capsular polysaccharides are serotype 19A.

[0011] Supplying CO_2 to the fermentation culture includes a first addition of $NaHCO_3$ and a

second addition of Na_2CO_3. In an embodiment, the molecular weight of the isolated *Streptococcus pneumoniae* capsular polysaccharide is at least 700 kDa. Further disclosed herein is a solution containing high molecular weight isolated *Streptococcus pneumoniae* capsular polysaccharides in which the polysaccharides comprise phosphodiester linkages between repeat units is provided, where the solution is produced by the method described above.

[0012] A method is provided for producing a solution containing high molecular weight isolated *Streptococcus pneumoniae* serotype 19A capsular polysaccharides. The method includes the steps of: 1) preparing a fermentation culture of *Streptococcus pneumoniae* bacterial cells that produce serotype 19A capsular polysaccharides; 2) supplying CO_2 to the fermentation culture, wherein supplying CO2 to said fermentation culture comprises a first addition of NaHCO3 to the fermentation culture at 10-50mM and a second addition of Na2CO3; 3) lysing the bacterial cells in the fermentation culture; and 4) isolating *Streptococcus pneumoniae* serotype 19A capsular polysaccharides from the fermentation culture; whereby a solution containing high molecular weight isolated *Streptococcus pneumoniae* serotype 19A capsular polysaccharides is produced, wherein the molecular weight of said isolated *Streptococcus pneumoniae* capsular polysaccharide is at least 480 kDa. Furthre disclosed is a solution containing high molecular weight isolated *Streptococcus pneumoniae* serotype 19A capsular polysaccharides is provided, where the solution is produced by the method described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Figure 1 shows the optical density (OD), base and glucose levels during the fermentation phase with Na_2CO_3 as titration base from laboratory studies at 3 L scale. Base in grams is divided by 10 for plotting purposes.

Figure 2 shows the optical density (OD), base and glucose levels during the fermentation phase with NaOH as titration base from laboratory studies at 3 L scale. Base in grams is divided by 10 for plotting purposes.

Figure 3 shows total protein and polysaccharide results at different pH adjustments for alternate base feeds of Na_2CO_3 or NaOH.

DETAILED DESCRIPTION OF THE INVENTION

[0014] *Streptococcus pneumoniae* are Gram-positive, lancet shaped cocci that are usually seen in pairs (diplococci), but also in short chains or as single cells. They grow readily on blood

agar plates with glistening colonies and display alpha hemolysis unless grown anaerobically where they show beta hemolysis. The cells of most pneumococcal serotypes have a capsule which is a polysaccharide coating surrounding each cell. This capsule is a determinant of virulence in humans, as it interferes with phagocytosis by preventing antibodies from attaching to the bacterial cells. Currently there are more than 90 known pneumococcal capsular serotypes identified, with the 23 most common serotypes accounting for approximately 90% of invasive disease worldwide.

[0015] As a vaccine, the pneumococcal polysaccharide coat can confer a reasonable degree of immunity to *Streptococcus pneumoniae* in individuals with developed or unimpaired immune systems, but a conjugated protein with polysaccharide allows for an immune response in infants and elderly who are also most at risk for pneumococcal infections. The pneumococcal cells are grown in fermentors with lysis induced at the end of the fermentation. The lysate broth is then harvested for downstream purification and the recovery of the capsular polysaccharides.

[0016] Polysaccharide size is a quality attribute assayed for in each preparation batch and must be appropriately controlled. The molecular weight for serotypes having a phosphodiester linkage between saccharide repeat units (*e.g.*, 6A, 6B, 19A, and 19F) is affected by parameters of the fermentation process. The methods of the present invention allow for the recovery of high molecular weight capsular polysaccharides from cellular *Streptococcus pneumoniae* lysates containing serotype 19A.

[0017] In the development of the present methods, the concentration of HySoy and choice of base titrant were modified in an attempt to modify final polysaccharide yields and molecular weights. Four fermentation schemes were tested. The first used a baseline NaOH process with 28 g/L HySoy. The second used 20% sodium carbonate as the base titrant and 20 g/L HySoy. The third combined advantages of the first two approaches by introducing carbonate through the batching of sodium bicarbonate and using a mixed NaOH/carbonate base titrant. The fourth approach used carbonate as the base titrant with a 10 mM bicarbonate addition to bolster growth.

[0018] Using NaOH as the base titrant during fermentation had the advantage of being able to lower the deoxycholate lysate to pH 5.0 without foaming to remove protein and improve filtration, but resulted in lower molecular weight polysaccharide (< 450 kDa). Na_2CO_3 provided higher molecular weight but had foaming issues if the pH of the deoxycholate lysate was lowered. At a higher pH hold step of 6.6, the fermentations using Na_2CO_3 formed a gel-like material, with subsequent filtration problems. Minimizing the amount of Na_2CO_3 by using a blend of NaOH and Na_2CO_3 as a pH titrant provided the molecular weight size benefits of Na_2CO_3 while eliminating foaming and filtration problems due to the sudden release of large amounts of CO_2. The use of 20% Na_2CO_3 (w/v) as the base titrant with a 10 mM $NaHCO_3$ addition to bolster growth (fourth approach) produced consistent, high molecular weight polysaccharides at high yield.

[0019] The present invention thus provides improved methods for the recovery of high molecular weight capsular polysaccharides from cellular *Streptococcus pneumoniae* lysates containing serotype 19A. CO_2 is supplied to a fermentation culture of a *Streptococcus pneumoniae* serotype 19A. Accordingly, in one embodiment of the invention, a method for producing a solution containing high molecular weight isolated *Streptococcus pneumoniae* capsular polysaccharides that comprise phosphodiester linkages between repeat units is provided, which includes the steps of: 1) preparing a fermentation culture of *Streptococcus pneumoniae* bacterial cells that produce capsular polysaccharides comprising a phosphodiester linkage between repeat units; 2) supplying CO_2 to the fermentation culture, wherein supplying CO2 to said fermentation culture comprises a first addition of NaHCO3 to the fermentation culture at 10-50mM and a second addition of Na2C03; 3) lysing the bacterial cells in the fermentation culture; and 4) isolating *Streptococcus pneumoniae* capsular polysaccharides from the fermentation culture;
whereby a solution containing high molecular weight isolated *Streptococcus pneumoniae* capsular polysaccharides with phosphodiester linkages between repeat units is produced, wherein the molecular weight of said isolated *Streptococcus pneumoniae* capsular polysaccharide is at least 480 kDa, and wherein said *Streptococcus pneumoniae* capsular polysaccharides are serotype 19A. Further disclosed herein is a solution containing high molecular weight isolated *Streptococcus pneumoniae* capsular polysaccharides with phosphodiester linkages between repeat units, where the solution is produced by the method described above.

[0020] The methods of the invention produce high molecular weight *Streptococcus pneumoniae* capsular polysaccharides from serotype 19A. As used herein, "high molecular weight" refers to molecular weights that are at least about 480 kDa, about 490 kDa, about 500 kDa, about 510 kDa, about 520 kDa, about 525 kDa, about 550 kDa, about 575 kDa, about 600 kDa, about 625 kDa, about 650 kDa, about 675 kDa, about 700 kDa, about 725 kDa, about 750 kDa, about 775 kDa, about 800 kDa, about 825 kDa, about 850 kDa, about 875 kDa, about 900 kDa, about 925 kDa, about 950 kDa, about 975 kDa, or about 1000 kDa.

[0021] Supplying CO_2 to the fermentation culture includes adding $NaHCO_3$ to the fermentation culture. $NaHCO_3$ additions of 10-50 mM is used, such as 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, or 50 mM. Supplying CO_2 to the fermentation culture also includes adding Na_2CO_3 to the fermentation culture. Na_2CO_3 additions of 0.1 M-2.0 M can be used, such as 0.1 M, 0.2 M, 0.4 M, 0.6 M, 0.7 M, 0.9 M, 1.0 M, 1.5 M, 1.8 M, or 2.0 M. A weight/volume (w/v) equivalent can also be used, such as 5% (w/v) Na_2CO_3, 10% (w/v) Na_2CO_3 or 20% (w/v) Na_2CO_3. Supplying CO_2 to the fermentation culture includes a first addition of $NaHCO_3$ and a second addition of Na_2CO_3 to the fermentation culture.

[0022] Within the methods of the present invention, the bacterial cells may be lysed using any lytic agent. A "lytic agent" is any agent that aids in cell wall breakdown and release of autolysin which causes cellular lysis including, for example, detergents. As used herein, the term

"detergent" refers to any anionic or cationic detergent capable of inducing lysis of bacterial cells. Representative examples of such detergents for use within the methods of the present invention include deoxycholate sodium (DOC), N-lauryl sarcosine (NLS), chenodeoxycholic acid sodium, and saponins.

[0023] In one embodiment of the present invention, the lytic agent used for lysing bacterial cells is DOC. DOC is the sodium salt of the bile acid deoxycholic acid, which is commonly derived from biological sources such as cows or oxen. DOC activates the LytA protein, which is an autolysin that is involved in cell wall growth and division in *Streptococcus pneumoniae.* The LytA protein has choline binding domains in its C-terminal portion, and mutations of the *lytA* gene are known to produce LytA mutants that are resistant to lysis with DOC.

[0024] Although there is no evidence that the use of DOC during *Streptococcus pneumoniae* polysaccharide purification poses a health risk, the use of such biologically derived reagents could raise potential regulatory concerns. Accordingly, in one embodiment of the present invention, the lytic agent used for lysing bacterial cells is a non-animal derived lytic agent. Non-animal derived lytic agents for use within the methods of the present invention include agents from non-animal sources with modes of action similar to that of DOC (*i.e.,* that affect LytA function and result in lysis of *Streptococcus pneumoniae* cells). Such non-animal derived lytic agents include, but are not limited to, analogs of DOC, surfactants, detergents, and structural analogs of choline, and may be determined using procedures as described in the Experimental section herein below. In one embodiment, the non-animal derived lytic agent is selected from the group consisting of decanesulfonic acid, tert-octylphenoxy poly(oxyethylene)ethanols (*e.g.* Igepal® CA-630, CAS #: 9002-93-1, available from Sigma Aldrich, St. Louis, MO), octylphenol ethylene oxide condensates (*e.g.* Triton® X-100, available from Sigma Aldrich, St. Louis, MO), N-lauryl sarcosine sodium (NLS), lauryl iminodipropionate, sodium dodecyl sulfate, chenodeoxycholate, hyodeoxycholate, glycodeoxycholate, taurodeoxycholate, taurochenodeoxycholate, and cholate. In another embodiment, the non-animal derived lytic agent is NLS.

[0025] Within the methods of the present invention, *Streptococcus pneumoniae* capsular polysaccharides are isolated using standard techniques known to those skilled in the art. For example, following fermentation of bacterial cells that produce *Streptococcus pneumoniae* capsular polysaccharides, the bacterial cells are lysed to produce a cell lysate. The capsular polysaccharides may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, precipitation, ultra-filtration, and column chromatography (See, for example, U.S. Patent App. Pub. Nos. 20060228380, 20060228381, 20070184071, 20070184072, 20070231340, and 20080102498).

[0026] The process changes described above allow for the recovery of high molecular weight capsular polysaccharides from cellular *Streptococcus pneumoniae* lysates containing serotype 19A. This is a robust improvement of the fermentation/recovery process that can greatly enhance the production of pneumococcal polysaccharides.

[0027] The following examples are offered by way of illustration.

EXAMPLES

[0028] Selected *Streptococcus pneumoniae* serotypes were grown to supply polysaccharides needed to produce vaccines for active immunization against invasive disease caused by *Streptococcus pneumoniae* due to capsular serotypes included in the vaccine. The cells were grown in fermentors with lysis induced at the end of the fermentation. The lysate broth was then harvested for downstream purification and the recovery of the capsular polysaccharides. Because polysaccharide size is a quality attribute assayed for in each preparation batch, polysaccharide size must be appropriately controlled. The molecular weight for serotypes having a phosphodiester linkage between saccharide repeat units (*e.g.,* 6A, 6B, 19A, and 19F) was found to be affected by parameters of the fermentation process. The following example describes studies relating to the supply of CO_2 during fermentation of *Streptococcus pneumoniae* serotypes having a phosphodiester linkage between repeat units to improve polysaccharide molecular weight.

Example 1: Carbon Dioxide Supply Effect on Polysaccharide Molecular Weight

Fermentation

[0029] Laboratory runs were performed in 3 L Braun Biostat B fermentors (B. Braun Biotech, Allentown, PA). They were filled with 1.8 L of HYS medium (20 g/L HySoy, 2.5 g/L NaCl, 0.5 g/L KH_2PO_4, 0.013 g/L $CaCl_2 \cdot 2H_2O$, 0.15 g/L L-Cysteine HCl). The fermentors were then autoclaved for 60 minutes at 121°C. After cooling, either 40 or 60 mL/L of a 50% Glucose + 1% Magnesium Sulfate (w/v) (DMS) solution was added to each unit. If required, sodium bicarbonate was added prior to inoculation.

[0030] Two 2 L seed bottles containing 1 L of HYS media were inoculated with Type 19A or Type 6A frozen seed stocks and incubated at 36°C without shaking for approximately 6-8 hours. Inoculation of the fermentors was performed with a volume of 100 mL (~5.2% v/v) aliquoted from a bottle with an OD_{600} between 0.3-0.9 and pH between 4.75-5.60. The fermentation temperature and pH were controlled at the desired setpoints. The standard conditions of 36°C, 1 L/min air overlay, pH controlled to 7 and agitation of 75 rpm were used. Two impellers were placed at the low and middle positions on the agitator shaft. A bottle containing the appropriate base titrant (3 N NaOH, 3 N NaOH blended with various concentrations of $NaHCO_3$, 3 N NaOH blended with various concentrations of Na_2CO_3 and $NaHCO_3$, and 20% Na_2CO_3) was hooked up for automatic pH control. The fermentors were sampled at various time points for external pH, OD_{600}, glucose, polysaccharide, and protein.

The runs were terminated when the glucose concentration was near depletion, or no increase in OD over time was noted.

Optical Density (OD_{600}) Measurement

[0031] The cellular density of the fermentation broth was determined by reading the absorbance of the samples at 600 nm using a Shimadzu (Columbia, MD) UV-1601 (2 nm bandwidth) or Spectronics (Westbury, NY) Genesys 5 spectrophotometer (5 nm bandwidth). The unit was blanked with the HYS medium diluted with de-ionized (DI) water to match the dilution required of the sample. The sample was diluted to keep the absorbance below a reading of 0.4, which is well within the linear range of the spectrophotometer.

Glucose Concentration

[0032] Glucose levels were determined by centrifuging out the cells and using the supernatant straight or 3x diluted with DI water. The samples were analyzed on a Nova Biomedical (Waltham, MA) BioProfile 400.

Polysaccharide Analysis

[0033] Samples were taken at the final fermentation reading and treated with 12% sodium deoxycholate (DOC) to a concentration of 0.13% (w/v) and gently agitated. They were held between 8-24 hours at 5°C then pH adjusted to 5.0 with 50% acetic acid to precipitate out most of the DOC and protein. After another hold interval of 12-24 hours at 5°C, the samples were centrifuged (14000 rpm, Sorvall (Thermo Fisher Scientific, Waltham, MA) SS34 rotor, 10 min at 15°C). The pH of the supernatant was adjusted to 6.0. The supernatant was then filtered through 0.45 µm Pall (East Hills, NY) HT Tuffryn Membrane syringe filters (low protein binding). The filtered product was analyzed by high-performance size exclusion chromatography (HPLC-SEC) using standard methodology well known in the art (see, *e.g.*, Aquilar, M. "HPLC of Peptides and Proteins: Methods and Protocols" Totowa, NJ: Humana Press (2004)).

Protein Analysis

[0034] Protein levels were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods well known in the art (see, *e.g.,* Walker, J.M. "The Protein Protocols Handbook" Totowa, NJ: Humana Press (2002)). The filtered cell lysate (supernatant) as prepared above was aliquoted into microfuge tubes at 65 µL/tube. Additions of reducing agent (10 µL dithiothreitol (DTT)) and NuPAGE® (Invitrogen, Carlsbad, CA) 4x lithium dodecyl sulfate (LDS) sample buffer (25 µL) were made to each sample. The samples

were vortexed and heated for 10 minutes prior to 10 µL/lane loading on NuPAGE® 4-12% Bis-Tris 12 well gels. The gels were run in NuPAGE® MES-SDS buffer at 150 V limiting for approximately 60 minutes and subsequently stained using the Zoion staining protocol (Zoion Biotech, Worcester, MA). Sample analysis was performed using an UVP Imager (UVP Inc., Upland, CA) with LabWorks™ (UVP Inc.) V.3 software to obtain approximate concentrations of specific protein bands of interest. Bovine Serum Albumin (BSA) Fraction V was used to develop a protein standard curve to calculate the approximate protein values of the samples (in lysed cell broth).

Molecular Weight Analysis

[0035] Fermentation samples of 1-2 liters were treated with 12% sodium DOC to a concentration of 0.13% (w/v) with agitation at 200 rpm. Samples were held between 8-24 hours at either 5°C or 20°C. The samples were then pH adjusted to 5.0 or 6.6 with 50% acetic acid to precipitate out most of the DOC and protein. After another hold interval of 12-24 hours at 5°C, the samples were centrifuged (11000 rpm, Sorvall (Thermo Fisher Scientific, Waltham, MA) SLA-3000 rotor, 15 min at 10°C). The supernatant samples were then pH adjusted to 6.0 with 3 N NaOH, and filtered using 0.45 µm Millipore (Billerica, MA) MP60 filters. The samples were then subjected to a modified purification process consisting of 100K molecular weight cut-off (MWCO) diafiltration (5x concentration followed by 7.5x diafiltration with DI water), 0.1% HB precipitation, and carbon filtration. The purified material was then subjected to Multi Angle Laser Light Scattering (MALLS) analysis.

Fermentation Process Study

[0036] Based on previous studies, the fermentation process was optimized by switching from Na_2CO_3 to NaOH as the base titrant. Use of NaOH allowed the recovery pH to be lowered to 5.0 resulting in significant protein precipitation. Na_2CO_3 will release CO_2 at low pH (< 6.6) creating foam formation. The impact of base titrant on Type 19A polysaccharide and protein levels was examined. Two 3 L fermentors were set up with one fermentor serving as the original process control, using 20% Na_2CO_3 solution (w/v) as the base feed. The other fermentor used 3 N NaOH as the base feed.

[0037] During the recovery phase, cells were lysed in the fermentor with DOC (final concentration 0.13% (w/v)) with the fermentor held at 36°C for 30 minutes. Following this step, the lysate was held overnight with agitation at ambient temperature (22°C). After the lysate hold, the lysate was pH titrated through a range from unadjusted to 4.5 with samples pulled at various pH setpoints. These samples were held overnight at ambient temperature prior to being processed and analyzed for polysaccharide and protein concentrations. The OD, base and glucose levels during the fermentation phase are shown in Figure 1 and Figure 2. The major difference was a higher final OD for the carbonate run.

[0038] The effect of post DOC lysate pH adjustment on total protein levels was also examined, and is shown in Figure 3. The lower pH levels reduced the protein load in cell free broth for both the NaOH run and the Na_2CO_3 run. The lower pH (< 6.6) had no negative impact on the polysaccharide yield. The fermentation analysis results served as an indication that the NaOH base feed was an acceptable alternative to the process using the Na_2CO_3 base feed during fermentation, but produced lower yields than what was obtained with the Na_2CO_3 feed.

Effect of Base Titrant on 19A and 6A Molecular Weight

[0039] A set of fermentations at the 3 L scale were performed to determine if the base titrant, HySoy concentration and pH hold step affected serotype 19A molecular weight. The molecular weight determination was performed using MALLS assay following the modified purification process. Results are shown in Table 1. For serotype 6A, only the base titrant was evaluated. Results are shown in Table 2.

Table 1. Impact of base titrant on 19A molecular weight (L29331-94)

Run No.	pH/Temp	HySoy	pH Hold	Base	MALLS (kDa)
D	7.0/36°C	28 g/L	5.0	3 N NaOH	340
E	7.0/36°C	20 g/L	5.0	3 N NaOH	350
F	7.0/36°C	20 g/L	5.0	20% Na_2CO_3	713
H	7.0/36°C	20 g/L	6.6	20% Na_2CO_3	713

Table 2. Impact of base titrant on 6A molecular weight

Run No.	Base	MALLS (kDa)
Lab 1	3 N NaOH	662
Lab 2	20% Na_2CO_3	1189
Pilot 1	3 N NaOH	500
Pilot 2	20% Na_2CO_3	950

Effect of Bicarbonate and Mixed Base pH Titrant

[0040] In the first study (Runs L29331-122 and -139), varying levels of initial sodium bicarbonate and base blends of sodium hydroxide and sodium carbonate were used in conjunction with a pH 5.0 hold step after the DOC hold step. The initial bicarbonate additions ranged from 10-50 mM and the sodium carbonate added to 3N sodium hydroxide for the base titrant ranged from 0.2-1.8 M. One run contained 50 mM initial bicarbonate and used NaOH as a base titrant. The carbonate levels at the end of these fermentations ranged from 14-111 mM. Serotype 19A molecular weights ranged from 520 to 713 kDa. Run parameters and results are

shown in Table 3.

Table 3. Na_2CO_3 vs. mixed base as pH titrant

Run No.		$NaHCO_3$ (mM)	Base		MALLS (kDa)	PS Yield (mg/mL)
			Na_2CO_3	NaOH		
Part I L29331-122 20 g/L HySoy	E	0	20%	0	759	0.836
	F	10	0.2 M	3 N	520	0.308
	G	10	0.4 M	3 N	648	0.538
Part II L29331-139 28 g/L HySoy	H	10	0.9 M	3 N	563	0.334
	C	20	0.9 M	3 N	662	1.027
	D	20	1.8 M	3 N	611	0.903
	G	50	0.9 M	3 N	713	0.924
	H	50	0 M	3 N	713	1.051

[0041] A second study (L29331-159 and -185) used initial bicarbonate additions of 15-30 mM and base blends using 0.4-1.0 M Na_2CO_3. The carbonate levels at the end of fermentation ranged from 24-62 mM. Serotype 19A molecular weights ranged from 502 to 763 kDa. Run parameters and results are shown in Table 4.

Table 4. $NaHCO_3$ with mixed base as pH titrant

Run No.	HySoy/ DMS	$NaHCO_3$ (mM)	Na_2CO_3/NaOH	MALLS (kDa)	PS Yield (mg/mL)
G2	28 g/L/ 60 mL/L	15	1.0 M/ 3 N	657	0.853
H2	28 g/L/ 60 mL/L	15	0.4 M/ 3 N	605	0.755
C	20 g/L/ 60 mL/L	20	0.4 M/ 3 N	571	0.386
E	20 g/L/ 60 mL/L	20	1.0 M/ 3 N	763	0.439
F	20 g/L/ 60 mL/L	25	0.7 M/ 3 N	462	0.382
G	20 g/L/ 60 mL/L	30	0.4 M/ 3 N	502	0.355
H	20 g/L/ 60 mL/L	30	1.0 M/ 3 N	594	0.415

Comparison of Mixed and Pure Carbonate Titration Base Fermentation Processes

[0042] An experiment was performed to compare the base blend process (0.7 M Na_2CO_3/3 N

NaOH) to the carbonate titrant process (20% Na_2CO_3 solution, w/v). Results (Table 5) confirmed that the molecular weight from the carbonate titrant process was higher and more consistent (778, 781 kDa) than the molecular weight from the base blend process (561-671 kDa). Polysaccharide yield was also higher with the Na_2CO_3 process.

Table 5. Run L29399-1 Na_2CO_3 vs. mixed base

Run No.	$NaHCO_3$ (mM)	Base		MW (kDa)	PS Yield (mg/mL)
		Na_2CO_3	NaOH		
C	25	0.7 M	3 N	565	1.106
D	25	0.7 M	3 N	561	0.908
E	25	0.7 M	3 N	612	0.894
G	25	0.7 M	3 N	671	0.873
F	0	20%	0	778	1.282
H	0	20%	0	781	1.249

Pilot Scale Runs

[0043] Several serotype 19A pilot scale (100 L) runs with various base titrants were performed. The molecular weight determination was performed using MALLS assay following a complete purification process and is reported from the final purified batch. Results are shown in Table 6.

Table 6. Impact of base titrant on 19A molecular weight at pilot scale

Fermentation Batch	Titration Base	Purification Batch	FBC MALLS (kDa)
RRP19A-0008	3 N NaOH	L26563-10	390
RRP19A-0009	3 N NaOH	L26563-11	380
IPPPN19A-005	3 N NaOH/0.6 M Na_2CO_3	L26260-37	492
IPPPN19A-006	3 N NaOH/0.6 M Na_2CO_3	L26260-38	480
IPPPN19A-007	3 N NaOH/0.6 M Na_2CO_3	L26260-39	490
IPPPN19A-014	20% Na_2CO_3	L26260-49	580
IPPPN19A-016	20% Na_2CO_3	L26260-50	559
IPPPN19A-017	20% Na_2CO_3	L26260-51	599

Effect of Base Titrant and Overlay on 19A Molecular Weight

[0044] A set of fermentations at the 3 L scale were performed to determine if the base titrant and atmospheric overlay affected the molecular weight. The molecular weight determination was performed using MALLS assay following the modified purification process. Results are shown in Table 7.

Table 7. Impact of base titrant and overlay on 19A molecular weight

Run No.	Base	Overlay	MALLS (kDa)
Control	3 N NaOH	Air	350
C	0.7 M Na_2CO_3	Air	855
D	1.5 M Na_2CO_3/1.5 N NaOH	Air	710
E	3 N NaOH	100% CO_2	634
F	3 N NaOH	50% CO_2	646
G	3 N NaOH	20% CO_2	567
H	3 N NaOH	10% CO_2	547

[0045] The article "a" and "an" are used herein to refer to one or more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

[0046] All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains.

[0047] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims.

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til fremstilling af en opløsning, som indeholder isolerede *Streptococcus pneumoniae*-kapselpolysaccharider med høj molekylvægt, hvor polysaccharriderne omfatter phosphodiesterbindinger mellem gentagelsesenheder, hvilken fremgangsmåde omfatter:

a) at fremstille en fermenteringskultur af *Streptococcus pneumoniae*-bakterieceller, som producerer kapselpolysaccharider, der omfatter en phosphodiesterbinding mellem gentagelsesenheder;

b) at levere CO_2 til fermenteringskulturen; hvor levering af CO_2 til fermenteringskulturen omfatter en første tilsætning af $NaHCO_3$ til fermenteringskulturen ved 10-50 mM og en anden tilsætning af Na_2CO_3;

c) at lysere bakteriecellerne i fermenteringskulturen; og

d) at isolere *Streptococcus pneumoniae*-kapselpolysaccharider fra fermenteringskulturen;

hvor molekylvægten af det isolerede *Streptococcus pneumoniae*-kapselpolysaccharid er mindst 480 kDa, og hvor *Streptococcus pneumoniae*-kapselpolysaccharriderne er serotype 19A.

2. Fremgangsmåde ifølge krav 1, hvor lysering af *Streptococcus pneumoniae* i fermenteringskulturen omfatter tilsætning af natriumdeoxycholat til fermenteringskulturen.

DRAWINGS

Figure 1

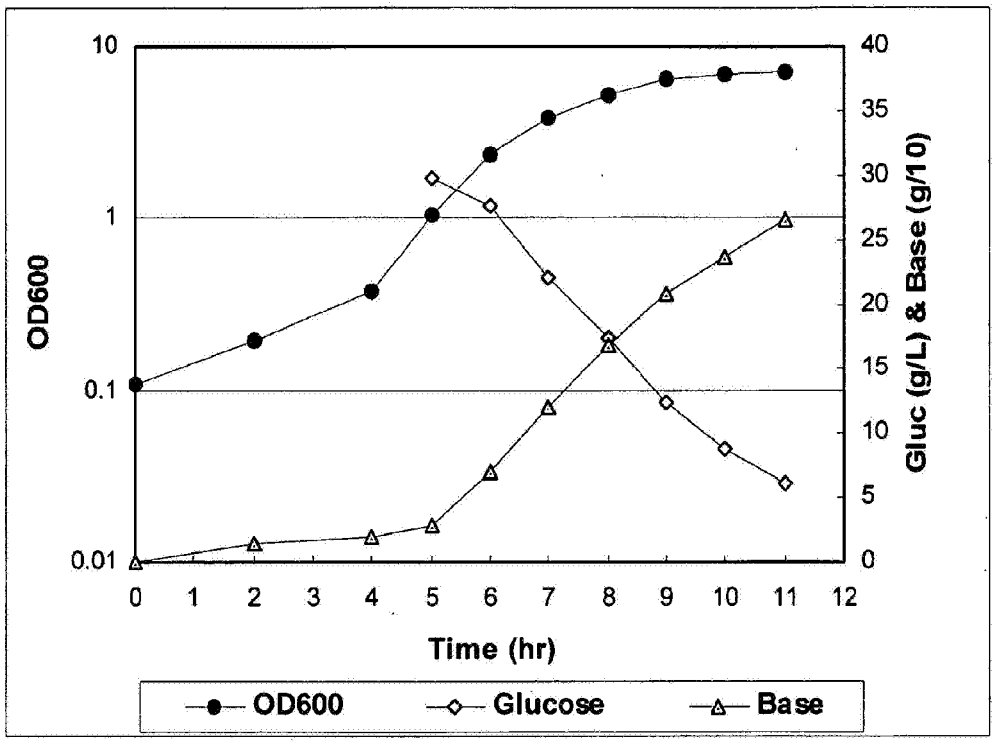


Figure 2

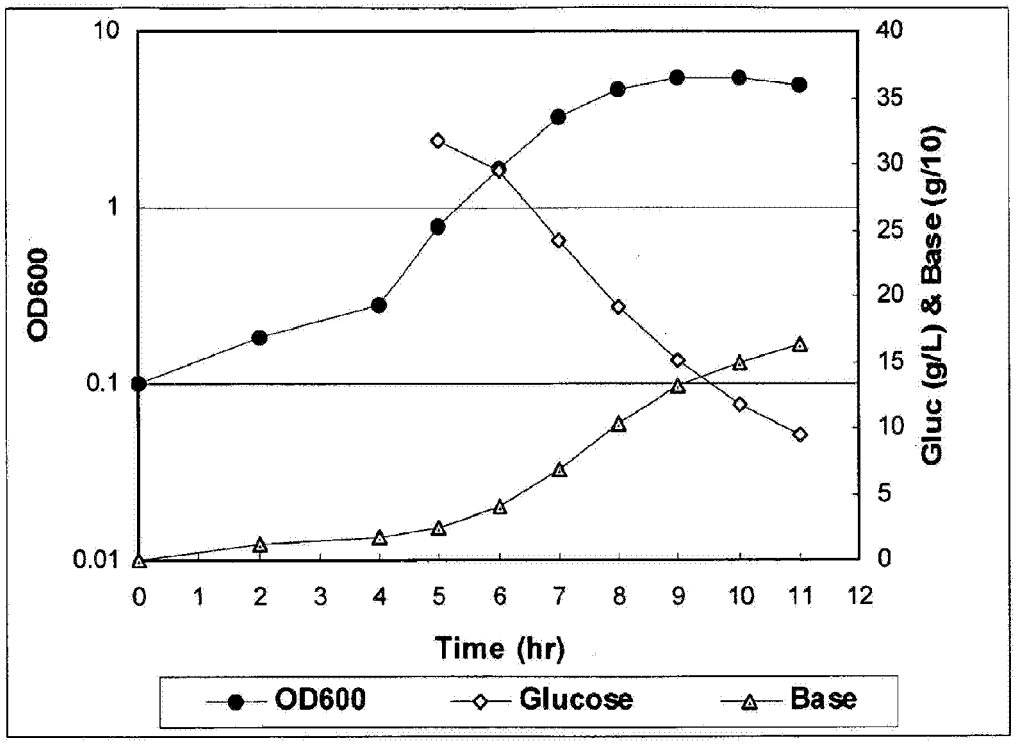
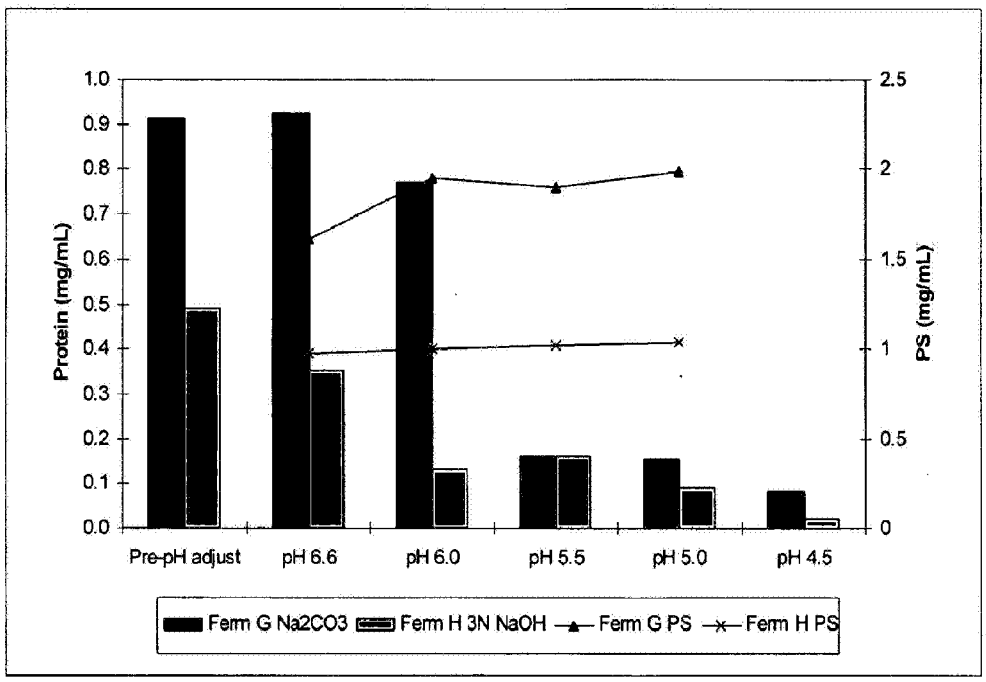

10
1
0.1
0.01
OD600
40
35
30
25
20
15
10
5
0
Gluc (g/L) & Base (g/10)
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hr)
OD600
Glucose
Base

Figure 3


Protein (mg/mL)
PS (mg/mL)
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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
2.5
2
1.5
1
0.5
0
Pre-pH adjust
pH 6.6
pH 6.0
pH 5.5
pH 5.0
pH 4.5
Ferm G Na2CO3
Ferm H 3N NaOH
Ferm G PS
Ferm H PS