Process intensification for production of *Streptococcus pneumoniae* whole cell vaccine

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Abstract

The available pneumococcal conjugate vaccines provide protection against only those serotypes that are included in the vaccine, which leads to a selective pressure and serotype replacement in the population. An alternative low-cost, safe and serotype-independent vaccine was developed based on a non-encapsulated pneumococcus strain. This study evaluates process intensification to improve biomass production and shows for the first time the use of perfusion-batch with cell recycling for a bacterial vaccine production. Batch, fed-batch and perfusion-batch were performed at 10 L scale using a complex animal component-free culture medium. Cells were harvested at the highest optical density, concentrated and washed using microfiltration or centrifugation to compare cell separation methods. Higher biomass was achieved using perfusion-batch, which removes lactate while retaining cells. The biomass produced in perfusion-batch would represent at least 4-fold greater number of doses per cultivation than in the previously described batch process. Each strategy yielded similar vaccines in terms of quality as evaluated by Western blot and animal immunization assays, indicating that, so far, perfusion-batch is the best strategy for the intensification of pneumococcal whole cell vaccine production, since it can be integrated to the cell separation process keeping the same vaccine quality.

1. Introduction

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium that causes human diseases as otitis, sinusitis, pneumonia, meningitis and sepsis. Current pneumococcal vaccines confer protection by generating antibodies to capsular polysaccharides of prevalent serotypes. In order to be effective in children, these polysaccharides have to be covalently linked to carrier proteins, producing conjugate vaccines (Pollard et al., 2009). Despite the availability of these vaccines, the burden of pneumococcal disease remains high due to two main reasons: i) the high price of conjugate vaccines limiting accessibility to the majority of the world population; ii) serotype replacement observed in all countries with universal pneumococcal vaccination programs (Weinberger et al., 2011), as a consequence of limited number of polysaccharides included in vaccine formulations in face of more than 95 known serotypes (Geno et al., 2015).

A promising alternative vaccine is the pneumococcal whole cell vaccine (PWCV), which is composed of a non-encapsulated strain that was genetically modified to delete the autolysin gene and substitute the wild-type hemolytic toxin pneumolysin with a detoxified derivative (Malley et al., 2001 and Lu et al., 2010a). Due to the absence of any polysaccharide capsule, in theory this vaccine could provide broad coverage against all serotypes. Moreover, PWCV induces protection against pneumococcal nasopharyngeal colonization and invasive disease in mice (Lu et al., 2010b). For its production, a culture medium free of animal compounds has been developed (Liberman et al., 2008), as well as the production and inactivation processes following current good manufacturing practices (cGMP) requirements. The cost of PWCV production was estimated to be

low, since the process established is relatively simple compared to other pneumococcal vaccines (Gonçalves et al., 2014).

Despite the success in producing the PWCV in cGMP conditions, the high number of doses necessary to immunize the population imposes further developments. In this context, the intensification of the production process of PWCV is worthy to pursue. According to Babi et al. (2016), process intensification can be defined as a process that achieves high efficiency of process equipment, reduction of cost and high yields. Many studies evaluate process intensification for different valuable products, as proteins (Berenjian et al., 2014) and viral vaccines (Tapia et al., 2016); here we evaluate process intensification of PWCV.

Process intensification for increasing pneumococcal cell density is particularly challenging due to the lactic acid produced during fermentation, which inhibits cell growth (Xu et al., 2006). The pneumococci, as other lactic acid bacteria (LAB), are strictly fermentative and nutritionally fastidious bacteria, hence carbohydrates normally are their energy source and the main end-product is lactate, which is also responsible for growth inhibition (Carvalho et al., 2013). Depending on the redox balance, pneumococcus can also metabolize carbohydrates to mixed-acid fermentation, producing acetate and formate (Yesilkaya et al., 2009). In addition to the end-product inhibition, the batch process suffers also from rather low productivity due to the long auxiliary time (Lu et al., 2016).

In the industry, continuous cultures with membrane cell-recycle systems have proven to be efficient for commercial production of lactic acid (Wee and Ryu, 2009, Min-tian et al., 2005, Kwon et al., 2001, Tejayadi and Cheryan, 1995) and biomass of *Lactobacillus casei*(Aguirre-Ezkauriatza et al., 2010). In contrast to batch fermentation, this operation mode allows the removal of the lactic acid produced, eliminating its inhibitory effects and increasing cell growth, while also improving productivity of lactic acid and biomass by decreasing the auxiliary time. Moreover, the use of bioreactor connected to the tangential flow microfiltration membrane meets two principles of process intensification: integration of operations and integration of functions (Lutze, 2010). For these reasons, we developed a process that uses the same principle of the continuous culture with cell-recycling, i.e., uses a microfiltration membrane to remove the inhibitors and to return the cells to the bioreactor. Since our goal was to increase cell biomass, we did not perform the continuous process during the steady-state, but integrated the up and downstream processing by employing the same microfiltration system for cell-recycling and cell separation when the highest biomass was reached. Thus, this integrated process was called here perfusion-batch, as it differs from the conventional continuous culture with cell-recycling.

Despite the advances of lactic acid production in continuous culture with cell recycling and the advantages of this system to increase cell density, to the best of our knowledge neither the strategy of continuous culture with cell-recycling nor the so-called perfusion-batch has not been applied to date for *S. pneumoniae* culture. The work presented here aimed at comparing the former processes developed for PWCV production in batch (Gonçalves et al., 2014) and fed-batch (Liberman et al., 2011) with the production in the perfusion-batch using hollow fiber membranes for cell-recycling and cell separation. We also compared microfiltration using hollow-fibers and centrifugation to perform cell separation procedures in order to address intensification on downstream process for batch and fed-batch as well. To evaluate whether the different fermentation and separation procedures would affect vaccine quality, we compared the efficacy of vaccines produced via these methods in mouse models.

2. Material and methods

2.1 Microorganism

The *S. pneumoniae* RM200 (Rx1 PdT Δ*lytA*) strain is derivative of the spontaneous non-encapsulated Rx1, which was genetically engineered for autolysin deficiency (Δ*lytA*) to improve cell density and had pneumolysin substituted by a non-toxic pneumolysoid derivative, PdT, with three amino acid substitutions (W433F, D385N, and C428G) to reduce any potential toxicity (Lu et al., 2010a).

2.2 Culture media, buffers and solutions

The composition of all culture media was based on the complex animal component-free culture medium

described by Liberman et al. (2008) and is shown on Table 1. BHI-blood agar plates, containing 5% (v/v) sheep blood, 15 g/L agar and 37 g/L of brain heart infusion (Difco, BD), were employed for bacterial enumeration, controlling culture purity and assessing hollow fiber membrane integrity. Automatic pH control of cultivation in the reactor was obtained by addition of 5 M NaOH. The cell washing and harvesting buffer was lactated Ringer's solution with glucose composed of 5 g/L NaCl, 0.3 g/L KCl, 0.2 g/L $CaCl_2.2H_2O$, 3 g/L sodium lactate and 2 g/L glucose. All reagents were of analytical grade.

2.3 Cultivation strategies

All processes were carried out with 10 L medium, except for fed-batch fermentation (initial volume of 8 L) in the bioreactor BioFlo410 (New Brunswick Scientific Company Inc., Edison, NJ, USA) with automatic pH control, temperature and stirring speed. The inoculum was prepared from a cryopreserved working cell bank, 750 µL inoculated in 500 mL of the same medium used in the batch phase in glass bottle and incubated statically at 36.5 ºC until the cell growth reached an optical density (OD) at 600 nm of approximately 2.0. This culture was then introduced into the bioreactor in order to obtain an initial OD around 0.1. The bioreactor cultivation was performed at 36 ºC (± 1 ºC) and the pH was automatically controlled at 7.0. The stirring speed was controlled at 150 rpm. Nitrogen was sparged throughout the fermentation at a flow rate of 0.1 vvm. Polypropylene glycol (Fluent Cane 114, Brenntag, Germany) 30% (v/v) was used as an antifoam agent. Fed-batch and perfusion-batch processes were operated in batch mode until 3-3.5 h and the feeding or perfusion operation started when the OD reached approximately 4.0. Previously, the feeding medium was sparged with N_2 in order to decrease the oxygen concentration. The flow-rate was 0.5 L/h for fed-batch process. The initial medium volume in fed-batch was 8.5 L, including the inoculum volume, and the mean final volume was 9.05 L after 3 h of feeding or 6 h of cultivation, when the highest OD was reached in this process. In the perfusion-batch , the working volume of the reactor was maintained at a constant value (10 L) by supplying the feeding medium with the same flow-rate as the permeate of the membrane, i.e., 7.3 L/h, which represents a $D = 0.63$ h^{-1}, until the highest OD was achieved, then the cells were harvested and washed as described below (item 2.5). A diagram for all three processes integrated with the cell separation system is shown in Figure 1.

2.4 Cell recycling

A polysulfone hollow fiber membrane with 0.1 µm of pore size and 0.92 m^2 of total filtration area (CFP-1-E-35A, GE Healthcare, Little Chalfont, Buckinghamshire, UK) was employed to remove metabolites as organic acids and return all cells to the bioreactor in the perfusion-batch process, without bleeding. The hollow fiber removed also medium nutrients, which were replaced by the feeding medium 1 from 3 h to 5 h and feeding medium 2 from 5 to 9 h (Table 1). Diaphragm pressure gauges were installed at the hollow fiber inlet and outlets and a peristaltic pump was used for continuous recirculation of fermentation broth through the hollow fiber system (Easy-load tubing pump, EMD Millipore, Merck KGaA, Darmstadt, Germany). The inlet pressure was kept below 10 psi during the operation. Additionally, two peristaltic pumps were used to control permeate flow-rate and feeding flow-rate (504U and 323U, respectively, Watson-Marlow Fluid Technology, Cornwall, UK) at the same flow rates in order to keep the reactor volume constant (Figure 1).

2.5 Cell separation and inactivation

Cell mass was harvested after OD 6.0 was reached, the same as for the GMP production (Gonçalves et al., 2014), or after reaching the highest OD in each operation mode. Two methodologies were applied in order to compare different cell separation methods: tangential microfiltration or centrifugation. The same system described for cell recycling in the perfusion-batch was used for tangential microfiltration (Figure 1). Cells were concentrated to OD 20-50, and then washed 6 times with the same volume of washing buffer. After washing, cell suspensions were adjusted to approximately OD 30. To evaluate the centrifugation method, samples of 33 mL were harvested by centrifugation at 1930 *g* for 20 min at 18 °C (RC5C, Sorvall, Du Pont Company, Newtown, CT), and then cell pellets were vortex-homogenized with 33 mL of washing buffer. This step was performed 6 times, and cell suspensions were adjusted to approximately OD 30.

After cell washing, cell inactivation was performed as previously standardized (Gonçalves et al., 2014) with

1:4000 (v/v) β-propiolactone (BPL, Sigma-Aldrich) for 30 h at 4° C mixing at 150 rpm (TE-140, Tecnal, Piracicaba, SP, Brazil). Then, the residual BPL was hydrolyzed by incubation at 37 ºC for 2 h mixing at 180 rpm (Series 25 Incubator Shaker, New Brunswick Scientific).

2.6 Analytical methods

Culture samples were taken during the process approximately every 30 min. Cell density was monitored by measuring the OD using a Spectrophotometer (U-1800, Hitachi High-Technologies, Tokyo, Japan). Dry cell weight was evaluated using previously weighed conical tubes, in which 30-45 mL culture samples were inactivated with 2-5% (v/v) formaldehyde (Synth, Diadema, SP, Brazil), depending on the cell density, for up to 18 h at room temperature, and then washed once with PBS by centrifuging at 3200 *g* for 20 min (5810R, Eppendorf). The cell pellets were dried at 60 °C for at least 2 days until constant weight. Purity was verified by Gram staining and plating samples on BHI-blood agar. Plates were incubated for 3 days at 36 °C into anaerobic jars to check colony morphology, purity and viability. For the analysis of residual sugar and metabolites, 2 mL samples were centrifuged at 17,530 *g* for 5 min (Mikro 120, Andreas Hettich GmbH & Co., Tuttlingen, Germany), and supernatants were stored at -20 ºC. Glucose, lactate and acetate were analyzed by high performance liquid chromatography (HPLC, SCL-10AVP, Shimadzu Corporation, Kyoto, Japan) using an Aminex HPX 87H column (300 x 7.8 mm, BioRad Laboratories Inc., Hercules, CA, USA), and 5 mM H_2SO_4 as mobile phase at 0.6 mL/min and 60 °C. The refraction

index detection (RID) was used for glucose analysis and UV detection at 210 nm for organic acids. Chromatograms were analyzed by Class VP software, version 6.14 SP2 (Shimadzu). Finally, the integrity of the hollow fiber membrane was evaluated by plating the filtrate on BHI-blood agar during the process.

2.7 Vaccine quality evaluation

After inactivation, final products were evaluated as described by Gonçalves et al., 2014. Briefly, aspect, pH, OD, bacterial and fungal sterility, endotoxin level were determined. Total and soluble protein contents were measured by Kjeldahl and Lowry, respectively. Also, bacterial identity was evaluated before inactivation.

The protein production profile was analyzed by Western blot to check if there was any difference between fermentation strategies. Briefly, 20 µL of vaccines (50 µg of each sample) were loaded onto precast 4 to 12% Bis-Tris gels (NuPAGE, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) and separated by electrophoresis. The proteins were transferred onto a nitrocellulose membrane (Biorad) and probed using different antibodies against specific pneumococcal proteins: SP0785, SP2070, SP2145, SP1572 (known as pneumococcal protective protein A - PppA), Pneumolysoid (PdT) and Pneumococcal surface protein A (PspA) or anti-Pneumococcal Whole Cell Vaccine (PWCV) polyclonal serum. Bands were visualized with the Super Signal West Pico Chemiluminescent Substrate Kit and exposed in CL-X Posure Film (both from Thermo Fisher Scientific, Waltham, MA, USA).

Immunogenicity and potency of the vaccines were also evaluated. Groups of female mice (C57BL/6J from Jackson Laboratories, Bar Harbor, Maine, USA) received one or two (at two-week interval) subcutaneous doses of 100 µg of vaccine preparations adsorbed onto 200 µg of aluminum hydroxide (Alum - $Al(OH)_3$; Brenntag North America, Reading, PA, USA). Mice were anesthetized with isoflorane and bled after 12 days of immunization in order to evaluate antibody and IL-17A production according to Campos et al. (2017). One week after bleeding, animals were anesthetized and received a lethal dose of 10^6 CFU of serotype 3 *S. pneumoniae* strain WU2 intranasally and illness monitored for 7-8 days. Any ill-appearing animal (defined prior to the initiation of any of the studies) was immediately and humanely euthanized. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Boston Children's Hospital.

2.8 Kinetics analysis

The mass balance and kinetic parameters were calculated according to the following equations:

$D = F_{\text{feed}}/V_{\text{sys}}$ (1)

$S_{\text{total}} = S_0 + S_{\text{feed}} - S_{\text{res}} - S_{\text{samp}} - S_{\text{perm}}$(2)

$$\text{acet}_{\text{total}} = \text{acet}_0 + acet_{\text{res}} + \text{acet}_{\text{samp}} + \text{acet}_{\text{perm}} \quad (3)$$

$$\text{lac}_{\text{total}} = \text{lac}_0 + lac_{\text{res}} + \text{lac}_{\text{samp}} + \text{lac}_{\text{perm}} \quad (4)$$

$$Y_{\text{acet}} = \text{acet}_{\text{total}}/S_{\text{total}} \quad (5)$$

$$P_x = \frac{X_{\max} - X_0}{t - t_0 \text{ x } V_{\text{consumed}}} \quad (6)$$

$$P_{\text{lac}} = \frac{\text{lac}_{\max} - \text{lac}_0}{t - t_0} \text{(for batch and fed-batch)} \quad (7)$$

$$P_{\text{lac}} = \text{lac}_{\max} \times D \text{ (for perfusion-batch)} \quad (8)$$

Where D is the dilution rate (h^{-1});F_{feed} is the feed flow rate (L/h);V_{sys} is the culture volume (L) of the system, including bioreactor, hollow fiber and tubing;S_{total} is total amount of glucose consumed (g);$acet_{total}$ and lac_{total} are the total amount of acetate (g) and lactate produced (g), respectively;$Y_{acet/S}$ is the acetate yield on glucose (g acetate produced/g glucose consumed); P_x is the biomass volumetric productivity (g dry cell weight/L.h) andP_{lac} is the lactate volumetric productivity (g lactate/L.h); X is the biomass (g dry cell weight); t is the time of cultivation (h); $\text{V}_{\text{consumed}}$ is the total medium used in the process. The index *0* indicates the initial condition, *res* is the residual amount inside the bioreactor,*samp* is the amount removed in sampling, *perm* is the amount present in hollow fiber permeate, *max* is the maximum value reached.

Linear regression fit was applied to calculate the angular coefficient, which corresponds to the yield coefficients on glucose: $Y_{X/S}$ is the biomass yield (g dry cell weight produced/ g glucose consumed), and $Y_{lac/S}$ is the lactate yield (g lactate produced/g glucose consumed). The linear regression was also performed to calculate the lactate yield on biomass ($Y_{lac/X}$, g lactate /g dry cell weight). The maximum specific growth rate (μ_{max} , h^{-1}) was calculated by the angular coefficient of linear regression fit of Ln(DO) vs. time in the first 3 h of cultivation (batch phase of all three processes).

The total protein of the whole cell vaccine obtained in each fermentation was calculated by multiplying the volume of concentrated bulk product by the total protein concentration measured by Kjeldahl. Then, to estimate the number of doses, the total protein amount was divided by 0.3 mg, which represents an estimate of the human dose of this vaccine (ClinicalTrials.gov, 2014).

2.9 Statistical analysis

Each culture was performed at least in triplicate. The mean of values and standard deviation is presented in the figures. All parameters were analyzed by one-way ANOVA and the means were compared by Tukey's Multiple Comparison Test. Statistical differences between IgG antibody titers and IL-17A production were evaluated by the Mann-Whitney *U*test. Animal survival after challenge was analyzed by Kaplan-Meier method and the log-Rank test to compare the curves. For all analyses, $P<0.05$ was considered to represent statistical significance.

3. Results and discussion

3.1 Batch fermentation

Batch cultivation was conducted at 10 L bioreactor as previously described for the production of cGMP lots at 60 L (Gonçalves et al., 2014). Cells were harvested at the same OD ˜ 6.0, to evaluate process reproducibility, downscale methods and have a sample product as a standard. Furthermore, batch fermentation was conducted to the highest OD to verify if harvesting at a higher cell density would interfere with vaccine quality. Figure 2 shows that the highest OD was 9.0-10.0; at this time-point (5 h) all glucose had been consumed. Dry cell weight was 4.15 ± 0.33 g/L at the highest OD, higher than the 1.26 g/L yield obtained in the culture of encapsulated strain 23F for polysaccharide production (Gonçalves et al., 2002) and the 1.13 g/L yield of strain 14 (Leal et al., 2011). The disruption of the*lytA* gene (which encodes for one of the autolysins of pneumococcus) in the strain used in this study has contributed to the ability to achieve higher biomass production. At the highest OD, lactate production was 19.0 ± 0.7 g/L and acetate production was 10.3 ± 1.1 g/L, values that were similar to other pneumococcal strains (Gonçalves et al., 2002; Gogola-Kolling et

al., 2014). At 3.5 h, when cultures reached OD 6.0, the concentration of lactate and acetate were about 10.0 g/L and 4.5 g/L, respectively, similar to cGMP conditions at 60 L (Gonçalves et al., 2014).

3.2 Fed-batch fermentation

Different concentrations of medium components have been previously tested, and the optimal condition was used in this study for the fed-batch process (Liberman et al., 2011). Figure 2 shows that the highest OD was between 14-15, thus 1.5 fold higher than in the batch process. However, dry cell weight was not statistically different, 4.43 ± 0.17 g/L, perhaps because the medium was more concentrated for Soytone in the batch process (20 g/L) than in fed-batch (5 g/L), which could have affected the physiology of the microorganism. Since the OD of the culture is a measurement of light scattering, which can vary according to size, density, opacity and complexity of cells, changes in the medium composition could alter the relation between OD and dry cell weight.

Compared to the batch process, which had no glucose at the end of cultivation, there was some glucose remaining (6.9 ± 1.5 g/L) at the beginning of the stationary phase (5 h of cultivation) in the fed-batch process. Since a higher OD was reached, we hypothesize that the bacteria stopped growing because of high concentrations of inhibitor products, of which lactate would be a reasonable possibility, as observed by Callewaert and De Vuyst (2000), during fed-batch cultivation of another Gram-positive bacteria, *Lactobacillus amylovorus* . Lactate production in the fed-batch process was 28.4 g/L, which was 1.2 to 1.4 fold higher than in the batch process. The same increase in lactate production was observed when Ding and Tan (2006) compared batch and fed-batch processes for cultivation of *Lactobacillus casei.* On the other hand, acetate production was 1.4 to 2.3 fold lower in fed-batch than in batch process.

3.3 Perfusion-batch with cell-recycling integrated to cell separation

To evaluate our hypothesis that high lactate production was responsible for the interruption of bacterial growth in the fed-batch process, perfusion-batch with cell recycling was performed in order to remove lactate, supply depleted nutrients and keep the cells inside the vessel. Moreover, the aim was to intensify the process and obtain the highest viable biomass; thus, this process was interrupted when the highest OD was reached (approximately after 9 h of cultivation), cells were then washed with the washing buffer and harvested to prepare the vaccine. For this reason, the perfusion was not operated as a continuous cultivation and the culture did not reach the steady-state, and the main advantage of this process over the others was the integration of up and downstream processing.

As presented in Figure 2, the batch phase was performed until 3 h, when the OD reached 4.0. Then, the feeding and removal of medium were initiated at the same flow-rate 7.3 L/h ($D = 0.63$ h^{-1}). At OD 12, after 5 h of cultivation, a concentrated medium for glucose, soytone and yeast extract, was supplied at the same flow-rate (Medium 2, Table 1), until 9 h of cultivation, when the highest OD was reached 29.8 ± 4.1. This OD was 3 times higher than in the batch process performed in this study and 2 times higher than fed-batch. Moreover, it was 5 times higher than the OD reached in the cGMP lots (Gonçalves et al., 2014). When perfusion-batch was compared to continuous processes with cell-recycling of other Gram-positive lactate-producing bacteria, this OD was 6 times lower than*Lactobacillus paracasei* , using a similar D (0.6 h^{-1}) (Xu et al., 2006). Dry cell weight (11.3 ± 1.4 g/L) was 2.5 fold higher than batch (4.15 ± 0.33 g/L) and fed-batch (4.43 ± 0.17 g/L). However, it was lower than other LAB cultivated in continuous process with cell-recycling, such as *Lactobaccillus delbrueckii* (118 g/L) and *Lactococcus cremoris* (88 g/L) (Chang et al., 1994), or *Streptococcus cremoris* (81.5 g/L) (Taniguchi et al., 1987). These differences can be explained by the fact that they are different bacteria, or because other media, dilution rates, and cell separation systems were applied. Moreover, *S. pneumoniae* RM200 might not have reached the maximum OD due to the accumulation of inhibitory metabolites in the vessel or in the absence of some nutrient.

Lactate production rose progressively during the cultivation until 21.9 ± 0.9 g/L at 8 h, when glucose concentration increased in the vessel. Acetate production increased until the end of the batch phase, then remained constant until 4 h, when the concentration started to raise again, reaching 5.52 ± 0.42 g/L at 7 h. After 7.5 h, acetate concentration decreased again, coincidently with the decline in lactate production.

Around 8 h of cultivation, the decreased consumption of glucose and production of lactate and acetate indicated that the microorganism stopped growing, and we proceeded with cell washing and inactivation at 9 h cultivation.

It is worth to note that perfusion process could be further investigated in order to develop a continuous culture with cell-recycling. To this aim, longer fermentation runs, genetic stability, different dilution and bleeding rates should be evaluated. However, the most challenging for continuous whole-cell vaccine production would be to perform the downstream process for harvesting and washing the biomass at the same time as operating the cell-recycling during the continuous culture. In this case, there should be two different microfiltration systems: one for cell-recycling and another for cell separation, because the fermented broth with inhibitory metabolites has to be removed and cells have to be washed with lactate Ringer's solution immediately after harvesting, before inactivation. This immediate downstream processing is very important to reach the vaccine quality for soluble protein content (Gonçalves et al. 2014). Moreover, a cost-effectiveness analysis should be done in order to verify the viability of a process with two microfiltration or other cell separation systems operating at the same time.

3.4 Comparison of production processes

When all processes were compared, we observed that the exponential growth phase occurred during the first 3 h in each case. Until this point, all processes were in the batch phase, and for this reason, they presented the same μ_{max} , 1.17 ± 0.06 h^{-1}, which was similar to other pneumococcus strains (Gogola-Kolling et al., 2014; Liberman et al., 2008).

As expected, glucose consumption was almost 4 times higher in the perfusion-batch, since the cultivation time is longer than in batch (Figure 3). Moreover, the production of lactate was almost 5 times higher and biomass was 3 times greater, measured by OD and dry cell weight. Furthermore, the production of lactate was almost 1:1 of glucose consumption. Dry cell weight was greater in batch process until 4 h, then, it became higher in the perfusion-batch due to growth arrest in the batch process. Table 2 compares our results from the batch, fed-batch and perfusion-batch.

All processes presented an equivalent average of biomass yield on glucose, with statistically similar $Y_{X/S}$. The mean $Y_{X/S}$ was 0.15 g dry cell weight / g glucose, similar to other LAB as *Lactobacillus delbrueckii* in continuous process with cell recycling (Ohleyer et al., 1985),*Lactobacillus casei* in batch, fed-batch and continuous process without recycling (Aguirre-Ezkauriatza et al., 2010), and similar to other pneumococcus (Liberman et al., 2008).

Total acetate production was higher in the perfusion-batch process, but $Y_{acet/S}$ was statistically higher for the batch process (0.41 g acetate/g glucose), which indicates the nutritional limitation of the batch culture, since acetate production occurs mainly in low glucose concentration (Carvalho et al., 2013).

$Y_{lac/S}$ and $Y_{lac/X}$ were significantly higher in the perfusion-batch process. $Y_{lac/S}$ obtained here in the perfusion-batch (0.97 g lactate/g glucose) was as high as reported for LAB used in lactate manufacture, such as *L. delbrueckii* subsp. *delbrueckii* ,*L. paracasei* and *L. lactis* subsp. *lactis* , which presented $Y_{lac/S}$ = 0.91 g/g (John et al., 2007), or *Lactobacilllus* sp. strain RKY2, which presented $Y_{lac/S}$ = 0.93-0.97 g/g in continuous process with cell recycling with D = 0.04-0.36 h^{-1} (Wee and Ryu, 2009). Although the conversion of glucose into lactate was high, $Y_{lac/X}$ was lower than other LAB as *Lactococcus lactis* (Parente et al., 1994), indicating the conditions employed here favored cell growth rather than lactate production, which is in accordance with our goal.

The perfusion-batch exhibited productivity 2 fold lower for biomass (P_X) due to the higher culture medium volume used in this process, and 3.5 fold higher for lactate (P_{lac}) in comparison to batch and fed-batch protocols. The P_X of perfusion-batch was also lower when compared to other pneumococcus strain (Gogola-Kolling et al., 2014), and other *Streptococcus* (Taniguchi et al., 1987). WhereasP_{lac} observed in all processes here was similar to other studies using LAB, such as in the batch process of*Enterococcus faecalis* (Wee et al., 2004) or continuous process of serotype 14 pneumococcus strain (Gogola-Kolling et al., 2014), it was lower

than other LAB cultivated using continuous process with cell-recycling, as *Lactobacillus paracasei* (Xu et al., 2006),*Lactobacillus helveticus* (John et al., 2007) or*Lactobacillus rhamnosus* (Kwon et al., 2001), which is in accordance with our goal of optimizing cell growth, but not the lactate production.

Here, we also estimated the total protein production and number of vaccine doses (Table 2). The perfusion-batch integrated to the cell separation had almost threefold more protein in the concentrated product, as observed for biomass production, which could generate 3 times more human vaccine doses per lot than the simple batch process harvested at OD 10. Despite the fact that batch processes consume less glucose and other reagents, and spend less time to produce one lot (considering cultivation and downstream process), as observed in Table 3, it would be necessary to perform 3 batch processes to obtain the same biomass as in 1 perfusion-batch (Figure 4). As consequence, the number of doses produced by lot in the perfusion-batch will be also about 3 times higher. Therefore, the batch protocol would be more expensive due to the cost with medium, reagents, cleaning process, employees, infrastructure, etc. Consequently, the perfusion-batch would minimize downtime, which is also costly. In addition, the perfusion-batch would produce 4 times more doses than the previously developed simple batch process, in which the cells were harvested at lower concentration, when OD reached 6.0 (Gonçalves et al., 2014).

3.5 Quality of vaccines

After cultivation, bacteria were harvested as previously described and samples were analyzed according to standardized parameters (Gonçalves et al., 2014). They all met the established Specification Criteria for Acceptance (data not shown), including the parameter of the percentage of soluble protein, which should be less than 15% (Lu et al., 2010a), indicating that bacterial death was mediated by BPL rather than by starvation. We also analyzed the protein profile of each lot produced using different fermentation strategies by Western blot (Figure 5). A panel of antibodies induced against potential vaccine candidates such as PspA, PdT and PppA, or IL-17-inducing proteins, such as SP0785, SP2070, SP2145 and SP1572, or even anti-PWCV sera were used. We observed that all lots presented similar amounts of specific proteins, with the expected size, independently of which process was used, and all were comparable to our standard, the original vaccine lot produced in 60 L in a batch process (Gonçalves et al., 2014).

In order to verify if different fermentation/downstream strategies would produce effective vaccines, different preparations were evaluated for the induction of IgG antibodies and IL-17A production in immunized animals. Mice immunized with PWCV from lots prepared by any of the fermentation strategies were equally immunogenic, producing high titers of IgG and IL-17A (Figure 6A and B). In fact, PWCV obtained from the perfusion-batch integrated to cell separation induced statistically higher antibodies and IL-17A titers than the standard vaccine, the cGMP lot produced at 60 L (Gonçalves et al., 2014) (Figure 6A and B, 2 and 6). This may be somehow due to a higher quality of the vaccine produced in the integrated process, as 100 µg of total protein was given as vaccine dose to all animals. One hypothesis is that the constant removal of inhibitory metabolites throughout the integrated process led to lower acetate and lactate concentration at the end of the culture, which would be beneficial not only for the cell growth, but also for the production of important antigens. On the other hand, metabolites accumulated in batch and fed-batch processes, probably affecting antigen synthesis besides inhibiting cell growth. Potency of these vaccines was evaluated by challenge in the fatal aspiration model. All lots independently of the fermentation strategy protected mice against challenge (Figure 6C), indicating that the process intensification did not impact the quality of vaccine.

The results of different downstream processes and storage temperatures evaluation were also presented in Figure 6. Again, all lots were equally immunogenic and induced high IgG and IL-17A titers (Figure 6D and E). The lot obtained from the fed-batch process was the only one that induced higher IL-17A titers than the positive control (cGMP lot produced at 60 L) (Figure 6E, 2 and 7). Vaccine produced in batch process with bacteria harvested by microfiltration induced statistically higher IL-17A titers than the lot using the same fermentation strategy and bacteria harvested by centrifugation (Figure 6E, 10 and 11). It is worth noting that, after heating for BPL degradation, the final product obtained using centrifugation for cell separation was not as homogenous as before heating, whereas the same product obtained by microfiltration

was homogenous before and after heating for BPL degradation. When centrifugation was applied, small particles or clumps were observed after heating for BPL degradation. These clumps were not present during the washing steps and after cell inactivation period, 30 h at 4 °C. We conclude that this phenomenon was only observed when bacteria were harvested by centrifugation, thus this methodology may compromise the quality of the final product. Nevertheless, all lots protected > 90% of immunized mice (Figure 6F).

4. Conclusion

In our studies, perfusion-batch with cell recycling integrated to the cell separation process was the best promising strategy for the production of PWCV, producing 3-fold higher biomass than batch and fed-batch processes and 4-fold greater number of doses than the previously described batch process, in which cells were harvested at OD 6.0. The perfusion-batch strategy supported the growth of *S. pneumoniae* RM200 by removing inhibitory metabolites from the culture and supplying nutrients. The integration of the perfusion with cell separation system could be a cost-effective alternative to produce high amounts of PWCV doses, using the same space and equipment as batch or fed-batch cultures, also diminishing the auxiliary time. Therefore, the process intensification achieved in this study has high potential for scale up and bulk production of PWCV, as well as of other whole-cell vaccines, which could be especially important to attend an epidemiological emergency, when a high number of vaccine doses are needed in a very short period of time. Moreover, the potential impact of process intensification was carefully evaluated with respect to the quality of the final product and no quality differences among the vaccines were observed. Therefore, the perfusion-batch cultivation with cell recycling integrated to the cell separation process should be explored for large-scale production of PWCV for human immunization.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 – Scheme for all three processes, batch, fed-batch and perfusion-batch with cell-recycling of *S. pneumoniae* RM200 in a 10 L bioreactor, including the microfiltration system used in downstream process for cell separation, washing and concentration. The feeding flask (number 3) was not used for batch. The microfiltration system (numbers 6 and 7) was applied only for downstream processing (cell separation, washing and concentration) in batch and fed-batch processes. For perfusion-batch, microfiltration system (numbers 6 and 7) was used during cultivation for cell-recycling and for downstream processing. Feeding flow-rates: 0.5 L/h, measured in flask 3 for fed-batch, and 7.3 L/h, measured in flasks 3 and 7, for perfusion-batch.

Figure 2 – Time profile for batch (n = 6 runs, top), fed-batch (n = 7 runs, middle) and perfusion-batch (n = 3 runs, bottom) fermentation of *S. pneumoniae* RM200 in a 10 L bioreactor: residual glucose (), residual

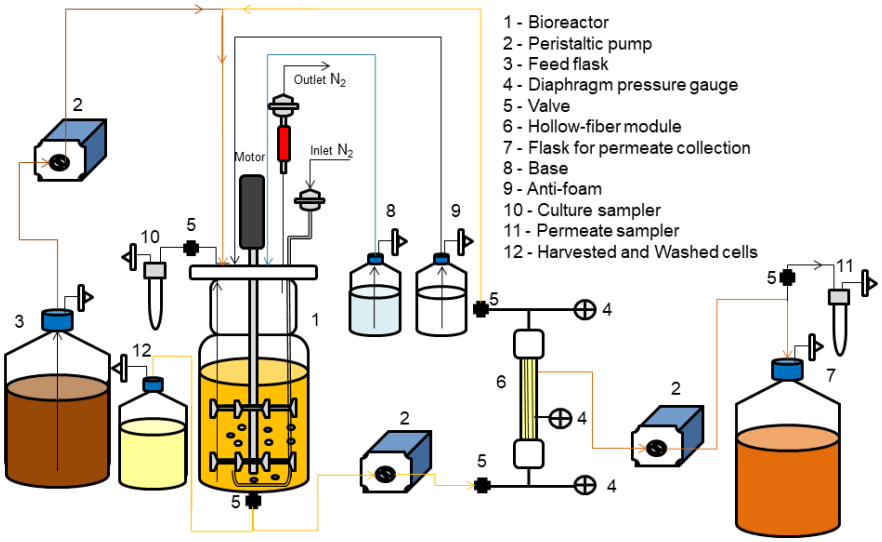
lactate () and residual acetate () measured by HPLC; biomass measured by optical density at 600 nm () and dry cell weight (). Each point represents mean of values and bars represent standard deviation. Dot line indicates the start of the fed-batch mode (middle) or the perfusion mode with feeding medium 1 (bottom). Dash line indicates the start of the perfusion with feeding medium 2 (bottom).

Figure 3 – Comparison of 3 fermentation protocols to total glucose consumption, lactate production, biomass production measured by dry cell weight and optical density at 600 nm: batch (), fed-batch () and perfusion-batch (). Each point represents mean of values and bars represent standard deviation.

Figure 4 – The total cell biomass (dry cell weight) produced per run (each point represents a run of batch (), fed-batch () and perfusion-batch ()) versus the time for running each lot, which was calculated according to Table 3. The horizontal dot line shows when batch and fed-batch produce the same amount of biomass obtained in perfusion-batch.

Figure 5 – Western blot analysis of different PWCV preparations probed with specific antibodies indicated in the left corner of the figure. Antibodies were produced against purified pneumococcal proteins or anti-PWCV polyclonal serum was used. Lane 1: Engineering lot 007/09 produced at 60 L bioreactor was used as positive control (Gonçalves et al., 2014); Lane 2: Batch fermentation performed at 10 L bioreactor as Engineering lot and cells harvested at similar OD (6.5); Lane 3: Batch fermentation with cells harvested at the highest OD (10); Lane 4: Fed-batch fermentation with cells harvested at OD 6; Lane 5: Fed-batch fermentation with cells harvested at the highest OD (14); Lane 6: Perfusion-batch fermentation with cells harvested at the highest OD (30). Numbers at right indicate the molecular weight in kDa.

Figure 6 – Immunogenicity and potency evaluation of PWCV. A-C) Comparison of different fermentation strategies by the administration of two subcutaneous doses of PWCV in two weeks interval. D-F) Comparison of different downstream processes and storage temperatures by the administration of one subcutaneous dose of PWCV. One PWCV dose is composed of 100 μg protein + 200 μg Alum. Mouse blood was collected twelve days after the last immunization. A and D) IgG antibody induction*in vivo* ; B and E) IL-17A production *in vitro* ; C and F) Survival after challenge. Numbers represent different PWCV lots used in immunization assay: 1- adjuvant Alum alone (negative control); 2- Engineering lot 007/09 produced at 60 L bioreactor (positive control , prepared as described by Gonçalves et al., 2014); 3- Batch fermentation () with cells harvested at the highest OD (10); 4- Batch fermentation () with cells harvested at OD 6.5; 5- Fed-batch fermentation () with cells harvested at the highest OD (14); 6- Perfusion-batch fermentation () with cells harvested at the highest OD (30); 7- Fed-batch fermentation () with cells harvested at the highest OD (14) using hollow fiber system and preserved at -80°C; 8- Same fed-batch lot () preserved at 4°C; 9- Fed-batch fermentation () with cells harvested at the highest OD (14) by centrifugation; 10- Batch fermentation () with cells harvested at OD 6.5 by hollow fiber system and preserved at -80°C; 11- Batch fermentation () with cells harvested at OD 6.5 by centrifugation and preserved at -80°C. Bars represent median. Significant difference is presented between adjuvant group and PWCV or between vaccines obtained from different fermentation strategies when indicated. Statistics was calculated by the Mann-Whitney U test and shown by asterisks: * $P< 0.05$, ** $P < 0.01$, and *** $P< 0.001$. Survival was analyzed by Kaplan-Meier method and the log-Rank test to compare the curves.



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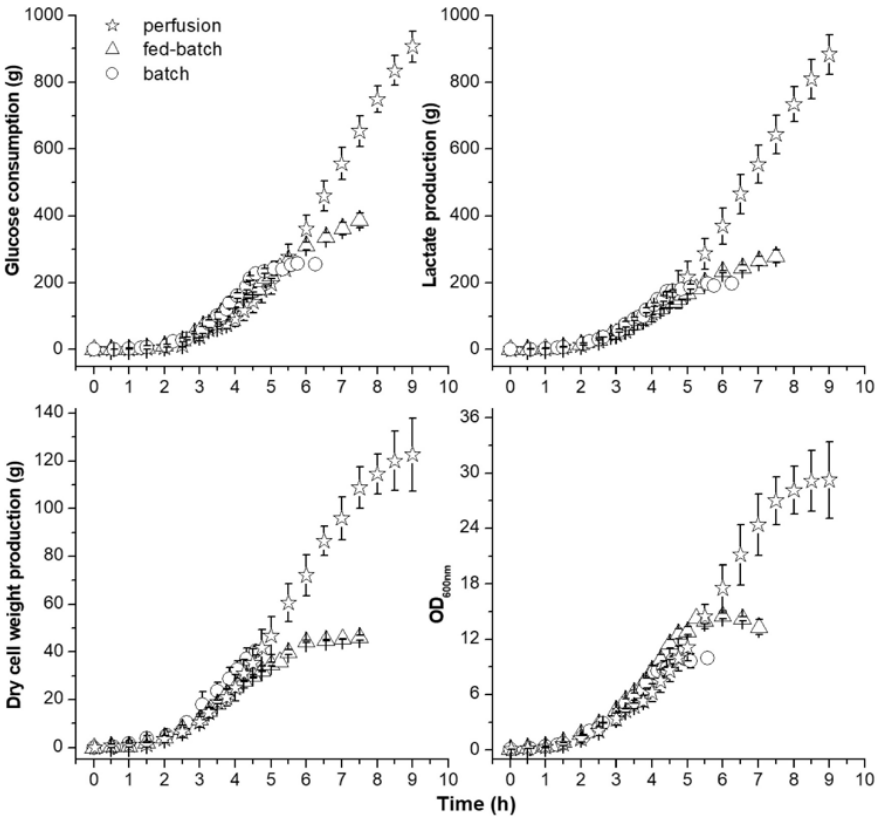

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