bioengineering

MDPI

Article

Simple Fed-Batch Strategy for Production of Capsular Polysaccharide by *Haemophilus influenzae* b at Pilot Scale

Mateus Ribeiro da Silva [1,2], Silvia Maria Ferreira Albani [1], Joaquin Cabrera-Crespo [1,†], José Geraldo da Cruz Pradella [3] and Mickie Takagi [1,*]

[1] Process Development Laboratory, Development and Innovation Center, Butantan Institute, São Paulo 05503-900, SP, Brazil; mat_biomed@yahoo.com.br (M.R.d.S.); silalbani@hotmail.com (S.M.F.A.)
[2] Bioprocess Laboratory, University of Campinas, UNICAMP, Campinas 13083-970, SP, Brazil
[3] Bioprocess Laboratory—IP&D, University of the Paraiba Valley, UNIVAP, São José dos Campos 12000-000, SP, Brazil; jpradella51@gmail.com
* Correspondence: mickie.takagi@butantan.gov.br; Tel.: +55-11-2627-9720
† In memoriam.

Abstract

Haemophilus influenzae b (Hib) is a pathogenic bacterium that causes meningitis worldwide, mainly in children less than two years old. The capsular polysaccharide b (PRP) is an essential antigen for vaccine formulation. This study aimed to develop a high-yield, technically accessible production strategy for PRP production to facilitate vaccine manufacturing in non-profit laboratories. Various fed-batch cultivation strategies were evaluated to address metabolic limitations and identify a robust, simplified process suitable for seamless scale-up to pilot scale. Glucose limitation strategies did not reduce inhibitory acetic acid accumulation due to deficiencies in Hib's respiratory chain, whereas oxygen availability was identified as critical parameter. Increasing the specific air flow from 0.5 to 1.0 vvm in constant fed-batch (Cfb) resulted in a 33% yield increase, reaching 1706.40 mg PRP.L^{-1}. However, the highest PRP concentration was achieved using exponential fed-batch with cell recycling (EfbCR), resulting in 1879.28 mg PRP.L^{-1}. Although EfbCR offered high productivity, the Cfb strategy emerged to be the most technically feasible and robust solution and was successfully scaled up to an 80 L bioreactor, achieving 1885 mg PRP.L^{-1}. These results advance understanding of PRP production by Hib and provides valuable insight into an efficient and simplified strategy for producing this key/vital vaccine antigen. The findings support the potential for cost-effective local production in public health initiatives.

Keywords: fed-batch cultivation; scale-up; capsular polysaccharide; *Haemophilus influenzae*; vaccine; pilot-scale

Check for updates

Academic Editor: Giovanni Vozzi

Received: 30 November 2025
Revised: 12 January 2026
Accepted: 23 January 2026
Published: 20 February 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Haemophilus influenzae (Hi) is responsible for life-threatening infections that can lead to serious illness, such as pneumonia, epiglottitis, and, in the most severe cases, meningitis, particularly in children less than two years old [1]. Encapsulated strain of *H. influenzae* are classified into six serotypes (a–f) based on their capsular polysaccharide composition, however serotype b is the primary cause systemic infection in humans [2]. The capsular polysaccharide serotype b is the outermost layer of Hib, and it is composed of units of polyribosylribitol phosphate (PRP) repeating units [3]. This polysaccharide protects the bacterium from phagocytosis, and in some cases, they allow the microorganism to invade the bloodstream.

Pure PRP is not protective for children and elderly people, and it fails to induce immunological memory. An efficient vaccine requires the covalent conjugation of PRP to carrier protein [3]. However, the production cost remains high due to the complexities of large-scale bacterial cultivation for PRP, alongside the multiple stages of purification, toxoid production and purification, chemical conjugation, and the subsequent purification of the PRP-toxoid conjugate.

The Hib-conjugate vaccine was introduced in the official Brazilian c Brazilian immunization schedule in 1999, and more than a 90% reduction in the number of meningitis cases was observed since then. Five years later, incidence had decreased by 98% in children under five years of age. In Brazil, the surveillance laboratory reported the impacts of the vaccine against Hib being incorporated into the immunization program. In 1990, Hib was responsible for 98% of the meningitis cases isolated from cerebrospinal fluid or blood; however, this prevalence dropped to 59% between 2000 and 2008 [4–8]. Around the world, Hib persists as a disease which requires monitoring and a sustained vaccination program [9–13]. Indeed, whereas there is no vaccination, the Hib persists, on the other hand, countries that incorporate the vaccine against Hib in their immunization program, they have nearly eliminated the considerable childhood morbidity and mortality associated with Hib infection [14–17].

The first reports concerning the industrial production of Hib polysaccharide by date back to 1984, most of which were patents [18]. Carty et. al., 1985 [19] introduced a complex medium based on soybean peptone (MP) and yeast extract. They evaluated superficial aeration at constant agitation of 750 rpm, submerged batch cultivation at 200 rpm producing 258 mg PRP.L^{-1} and 91 mg PRP.L^{-1} respectively with both condition controlled at pH 7.0 and temperature of 36 °C. Merrit et al. [20] performed fed-batch cultivation of *H. influenzae* type b working at bench and pilot scale utilizing agitation 400–900 rpm, pH 7.3 and a temperature of 36.5 °C. The specific airflow rate was maintained between 0.6–0.8 volume of air per volume of medium per minute (vvm). The feed medium consisted of glucose (10% *w*/*v*) and yeast extract (20% *w*/*v*) with the feeding rate adjusted to maintain a maximum specific growth rate near 0.50 h^{-1}. This strategy yielded polysaccharide concentration of 1190 mg PRP.L^{-1} and 1300 mg PRP.L^{-1}, respectively for bench and pilot scale. Takagi et al. [21] reported that the capsular polysaccharide was growth-associated across three cultivation modes: batch, fed-batch and repeated batch achieving with 132 mg PRP.L^{-1}. To further improve yields, Takagi et al. [22] modified the MP medium by increasing the concentrations of growth factors Hemin and NAD to 30 and 15 mg.L^{-1}, respectively and named as MMP. Under these conditions, batch cultivation with intermittent glucose addition and superficial aeration produced lower polysaccharide levels than in the submerged condition. While, polysaccharide production was similar at 10 or 30% of air saturation, pH control proved to be indispensable for improving polysaccharide production, achieving 980 mg PRP.L^{-1}. Pillaca-Pullo [23] demonstrated the robustness of scaling Hib production from 1.5 L to 75 L using k_La (52 h^{-1}) as a scale-up criterion—achieving a high final concentration of 1400 mg PRP.L^{-1} in fed-batch mode. While Solidade et al. [24] explored continuous cultivation to enhance productivity and simplify manufacturing. Although this strategy drastically increased the production rate to 165 mg PRP.L^{-1}.h^{-1} at a dilution rate of 0.23 $^{-1}$, it resulted in a lower final PRP titer (874 mg PRP.L^{-1}) compared to fed-batch yields. Given that fed-batch offers superior final concentration while continuous culture provides better time-efficiency, there is a clear impetus to explore alternative cultivation strategies.

This study evaluates the production of the *H. influenzae* type b capsular polysaccharide using various cultivation strategies in to determine the most technically feasible cultivation process to scale up.

2. Materials and Methods

2.1. Strain

Haemophilus influenzae type b strain GB3291 was obtained from the Microorganism Collection Center at Adolph Lutz Institute, São Paulo—Brazil. The master and working lots were prepared according to Takagi et al. [21,22] and stored at −80 °C.

2.2. Culture Medium

The cultivation medium MMP was prepared according to Takagi et al. [21,22], containing (per liter): soy peptone (10.0 g) and yeast extract UF, (5.0 g)—BD Difco, glucose (5.0 g) and NaCl (5.0 g)—Merck (Rahway, NJ, USA), K_2HPO_4 (2.5 g), Na_2HPO_4 (13.1 g), $NaH_2PO_4.H_2O$ (3.3 g), NAD (0.015 g), Hemin (0.030 g)—Sigma Aldrich (St. Louis, MO, USA). Feed medium was composed by MMP medium where glucose and yeast extract were increased to 200 $g.L^{-1}$. The MMP medium was sterilized by filtration in a Millipore system with a 0.22 µm membrane previously autoclaved at 120 °C for 15 min and aseptically transferred to the fermentation vessel or Erlenmeyer flasks.

2.3. Inoculum

Pre-inoculum was prepared by transferring 100 µL of bacterium suspension (1.5×10^{-9} $CFU.mL^{-1}$) kept at −80 °C into 500 mL Erlenmeyer flask containing 100 mL of sterile MMP medium. After incubation for 8 h at 37 °C under a 5-6% CO_2 atmosphere, the resulting cell suspension was transferred to a 1 L Erlenmeyer flask with 200 mL of culture medium. After incubation at 37 °C and 200 rpm (Shaker series 25—Eppendorf-New Brunswick Scientific Co., Edison, NJ, USA) for 14 h, a known volume of the inoculum was transferred to the bioreactor, to achieve an initial optical density ($OD_{540\,nm}$) of 0.1.

2.4. Experiments

Experiments were carried out in bioreactor (Bioflo 2000 and Bioflo 5000—Eppendorf-New Brunswick Scientific Co., Enfield, CT, USA) with nominal capacities of 5 L, 10 L and 80 L. The working volumes were 3.5 L (or 4.5 L), 6.5 L and 45 L of MMP culture medium, respectively. The temperature was maintained at 37 °C and the pH was controlled at 7.5 through the addition of 5 M NaOH solution. Dissolved oxygen (pO_2) was kept at 30% air saturation by adjusting agitation speed and the aeration rate (0.2 to 1.0 vvm), depending on the experiment. Online cultivation monitoring and data acquisition were performed using LabView (Bioflo 2000) or Biocommand (Bioflo 5000) software. Samples were taken every hour to measure dry cell weight (DCW) as well as concentrations of organic acids, glucose and polysaccharide.

Experiments were carried out as follows:

(a) Intermittent fed-batch cultivation (Ifb): performed with an initial volume of 4.5 L to 5 L bioreactor. Glucose 50% solution was added intermittently after complete glucose consumption to restore the concentration to 5 $g.L^{-1}$. The specific air flow was maintained at 0.2 vvm and 0.5 vvm for Ifb0.2 and Ifb0.5, respectively;

(b) Constant rate fed-batch cultivation with an initial volume of 3.5 L to 5 L bioreactor. The feeding flow rate was set to 77 $mL.h^{-1}$ and specific air flow rate at 0.5 vvm (Cfb0.5);

(c) Constant rate fed-batch cultivation with initial volume of 6.5 L to 10 L bioreactor. The feeding flow rate of 131 $mL.h^{-1}$ and specific air flow rate at 1.0 vvm (Cfb1.0);

(d) Exponential fed-batch cultivation (Efb): conducted with initial volume of 6.5 L to 10 L bioreactor. The exponential feeding flow rate ranged from 50 $mL.h^{-1}$ up to 172 $mL.h^{-1}$ and air flow 1.0 vvm (Efb1.0);

(e) Exponential fed-batch cultivation with cell recycling (EfbCR): performed with initial volume of 6.5 L to 10 L bioreactor. The feeding flow rate was adjusted to maintain

a specific growth rate (μ) at 0.2 h^{-1}. Bacteria were recycled by cross flow filtration using a hollow fiber membrane (0.1 μm, model #CFP-2-E-5 A, 1200 cm^2 Cytiva) with a specific air flow rate of 1.0 vvm (EfbCR1.0);

(f) Pilot scale constant fed-batch cultivation (PSCfb): conducted with an initial volume of 45 L to 80 L bioreactor. The feeding flow rate of 1.087 $L.h^{-1}$ and the specific air flow rate was 0.5 vvm (PSCfb0.5).

The specific cell growth rates applied during the feeding phases of the experiments (d) Efb1.0 and (e) EfbCR1.0 were determined based on growth rate profiles obtained from the assays and the parameters generated by AnaBio software [25]. In the AnaBio analysis, product formation (acetic acid—HA) associated with cell growth as well as the Levenspiel's model incorporating product inhibition were used to describe the growth kinetic of Hib (Equations (1)–(4)). Cultivation parameters:

$$\frac{dC_X}{dt} = \left(\mu - \frac{F}{V}\right)\cdot C_X, \quad (1)$$

$$\frac{dC_S}{dt} = \left(\frac{F}{V}\right)\cdot(C_{SF} - C_S) - \left(\frac{1}{Y_{\frac{X}{S}}}\right) + \left(\frac{\alpha}{Y_{\frac{PRP}{S}}}\right)\cdot\mu\cdot C_X, \quad (2)$$

$$\frac{dC_P}{dt} = (\alpha\cdot\mu)\cdot C_X - \left(\frac{F}{V}\right)\cdot C_P, \quad (3)$$

$$\mu = \mu_{max}\cdot\left(\frac{C_S}{K_S + C_S}\right)\cdot\left(1 - \frac{C_P}{C_P^*}\right)^n, \quad (4)$$

where, μ is specific cell growth rate (h^{-1}); μ_{max} is the maximum specific cell growth rate (h^{-1}); α is the pseudo-stoichiometric coefficient for growth-associated acetic acid formation; K_S is the saturation constant ($g.L^{-1}$); CX is the cell concentration ($g.L^{-1}$); CS is the substrate concentration ($g.L^{-1}$); CP is the product concentration ($g.L^{-1}$); CP* is the critical value of product concentration for inhibition by product ($g.L^{-1}$); V is the initial volume (L); CSF is the substrate concentration of the feed medium ($g.L^{-1}$); F is the feed rate ($L.h^{-1}$); n is the parameter for Levenspiel's model; $Y_{X/S}$ or $Y_{PRP/S}$ are the yield of biomass or PRP per gram of consumed glucose ($g.g^{-1}$), $Y_{PRP/X}$ is the specific PRP, grams of PRP per gram of biomass ($g.g^{-1}$).

3. Analytical Methodology

3.1. Biomass Concentration

Ten-milliliter samples were withdrawn from the bioreactor and distributed into pre-weighed centrifuge tubes in duplicate. The tubes were centrifuged at 12,000× *g* at 4 °C for 10 min. The resulting pellet was then resuspended in 10 mL of 0.15 M NaCl and centrifuged again under the same conditions. The supernatant from the first was stored at –20 °C for further analysis, while the second supernatant was discarded. The washed cell pellets were dried at 60 °C until a constant weight was reached, allowing calculation of the dry biomass concentration in $g{\cdot}L^{-1}$.

3.2. PRP Concentration

Ten-milliliter samples were withdrawn from the bioreactor and centrifuged at 12,000× *g*, 4 °C for 10 min. The supernatant was then dialyzed against distilled water for 24 h using a membrane with cut off of 12–14 kDa; with the water being changed every 2 h to remove low molecular weight molecules. Polysaccharide concentration was measured using the modified Bial method, with ribose as the standard [26]. The Bial's method was modified to include a hexose removal step based on Drury's study [27] PRP

concentration was determined by multiplying the ribose concentration by 2.55, according to the PRP structural formula reported by Crisel [28].

3.3. Glucose and Acetic Acid Concentrations

The concentrations of glucose and acetic acid were determined by High Performance Liquid Chromatography (HPLC) coupled with UV and RID detectors. Cell-free culture samples of the culture were diluted five or ten-fold with 50 mM sulfuric acid solution, filtered through a 0.22 µm membrane and injected into an Aminex HPX-87 H column (Bio Rad Europe GmbH, Basel, Switzerland). A 5 mM sulfuric acid solution was used as the mobile phase, with flow rate of 0.6 mL.min^{-1} at 60 °C. Glucose and of acetic acid concentrations were calculated using software Class VP software, version 6.2.

3.4. Determination of Kinetic Parameters

Maximum specific cell growth rate, conversion factor and productivity were determined by Equations (5)–(10):

Maximum Specific cell growth rate (μ_{max})

$$\mathrm{Ln}\frac{Cx}{C_{Xo}} = \mu_{max}t \tag{5}$$

where C_x is the biomass concentration (g.L^{-1}) at time t and C_{xo} is the biomass concentration in the initial time (t = 0) in the bioreactor.

3.5. Conversion Factors Yield

$$Y_{PRP/Glu} = \frac{(C_{PRP} - C_{PRP-0})}{(C_{Glu-0} - C_{Glu})} \tag{6}$$

$$Y_{DCW/Glu} = \frac{(C_{DCW} - C_{DCW-0})}{(C_{Glu-0} - C_{Glu})}, \tag{7}$$

$$Y_{PRP/DCW} = \frac{(C_{PRP} - C_{PRP-0})}{(C_{DCW} - C_{DCW-0})} \tag{8}$$

$$Y_{HA/Glu} = \frac{(C_{HA} - C_{HA-0})}{(C_{Glu-0} - C_{Glu})} \tag{9}$$

where C_{PRP} is the PRP concentration (mg.L^{-1}) at time *t*, C_{PRP_0} is the initial PRP concentration (t = 0); C_{DCW} is the biomass concentration (g.L^{-1}) at time t and $C_{DCW\text{-}0}$ is the biomass concentration in the initial (t = 0); C_{HA} is the acetic acid concentration (g.L^{-1}) at time *t* and $C_{HA\text{-}0}$ is the initial acetic acid concentration (t = 0); C_{Glu} is the glucose concentration (g.L^{-1}) at time *t* and $C_{Glu\text{-}0}$ is the initial glucose concentration (t = 0).

3.6. Productivity

$$P_{PRP} = \frac{(C_{PRP} - C_{PRP-0})}{t_f}, \tag{10}$$

where C_{PRP} is the PRP concentration (mg.L^{-1}), C_{PRP_0} is the initial PRP concentration (t = 0); and t_f is the final cultivation time.

3.7. Glucose Feed Rate (FR)

$$FR = F \cdot CSF/V, \tag{11}$$

where C_{SF}—concentration of glucose in the feed solution (mg.L^{-1}); F—volumetric feeding flow rate (L.h^{-1}) and V—is the incremented volume (L) in the bioreactor.

4. Results

In this work, different cultivation strategies were evaluated to assess PRP production, and kinetics parameters based on cell growth, acetate formation (HA) and PRP production were analyzed. The most technically feasible cultivation strategy was then scaled up to a pilot bioreactor to compare and evaluate the process robustness.

Figure 1 shows the kinetics profiles of biomass, glucose consumption, PRP and acetate formation in experiments conducted using intermittent fed-batch 0.2 vvm (Ifb0.2) and 0.5 vvm (Ifb0.5), and constant fed-batch 0.5 vvm (Cfb0.5 vvm) and 1.0 vvm (Cfb1.0 vvm). At the end of the (Ifb0.2), experiment, biomass concentration reached 4.70 g DCW.L^{-1}, while acetic acid and capsular polysaccharide concentrations were 9.44 g HA.L^{-1} and 636.25 mg PRP.L^{-1}, respectively. In the (Ifb0.5) condition, glucose consumption was approximately 33% higher than at 0.2 vvm resulting in an increase biomass and PRP production, reaching 5.17 g DCW·L^{-1} and 786.37 mg PRP.L^{-1}, respectively.

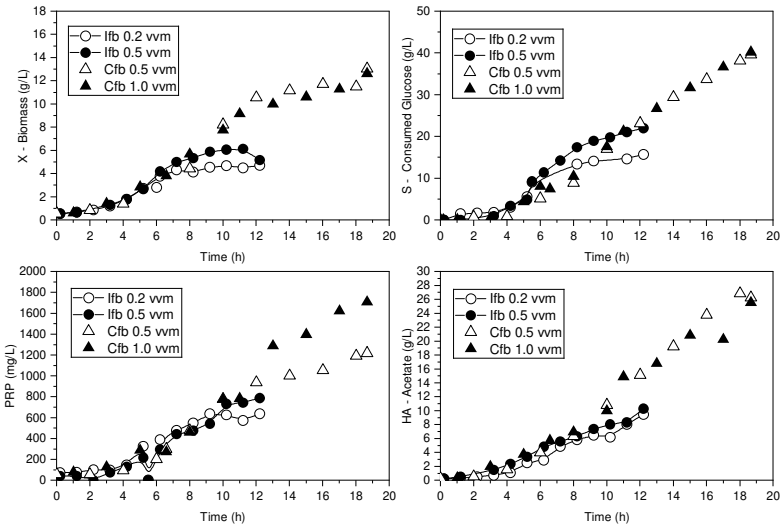


Figure 1. Profile of biomass, PRP, acetic acid production and glucose consumption for Intermittent fed-batch cultivation with specific air flow rate at 0.2 and 0.5 vvm and Constant fed- batch cultivation with specific air flow rate at 0.5 and 1.0 vvm.

Constant fed-batch cultivation (Cfb) was performed in order to improve cell density. As shown in Figure 1, Cfb0.5, produced 1217.53 mg PRP.L^{-1}, an increase of 54.82% compared to the intermittent feeding strategy. The glucose consumption was approximately twice as high (39.65 g.L^{-1}) as that observed in as intermittent fed-batch cultivation (22.00 g.L^{-1}) and resulted in a two-fold increase in biomass and acid metabolite of 12.05 g DCW.L^{-1} and 27.23 g HA.L^{-1}, respectively.

Constant fed-batch with an aeration airflow rate of 1.0 vvm (Cfb1.0) was performed to evaluate the effect of increased aeration on cell growth and polysaccharide production. This experiment was carried out in 10 L bioreactor, which has a volume 1.8 times larger than the previous 5 L biorreactor. To assess the production process at different scales, the feeding flow rate for the 10 L bioreactor was set to 131 mL·h^{-1}, proportional to the 77 mL.h^{-1} (glucose feed rate, Equation 11). Under these conditions, bacterial growth was exponential for nearly 13 h before transitioning to linear growth until the end of the cultivation. Glucose consumption (40.2 g.L^{-1}) was very similar to that of the Cfb0.5. However, polysaccharide production was enhanced, reaching 1706.40 mg PRP·L^{-1} compared to 1217.53 mg PRP·L^{-1}

in Cfb0.5. Acetic acid formation was slightly lower at 25.52 g HA·L^{-1} as illustrated in Figure 1.

The concentration of acetic acid was approximately 10 g HA.L^{-1} in the intermittent fed-batch experiment and increased to around 26.23 g HA.L^{-1} in constant fed-batch cultivation. To reduce acetic acid accumulation and prevent potential inhibition of cell growth, an exponential fed-batch cultivation (Efb) was carried out with a controlled, limited glucose supply.

Figure 2 presents the profile of biomass, residual glucose, PRP and acetic acid production of the Efb1.0 experiment in which the feeding rate was adjusted to maintain the specific growth rate (μ_{set}) constant at 0.07 h^{-1}. For the Efb**1.0** cultivation, the setpoint for the specific μ_{set} was established at the minimum value observed during previous Cfb1.0 experiments. This parameter was calculated and monitored using the AnaBio 1.0 software [25]. In this configuration, glucose was limited up to 16.5 h, after which residual glucose began to accumulate in the medium.

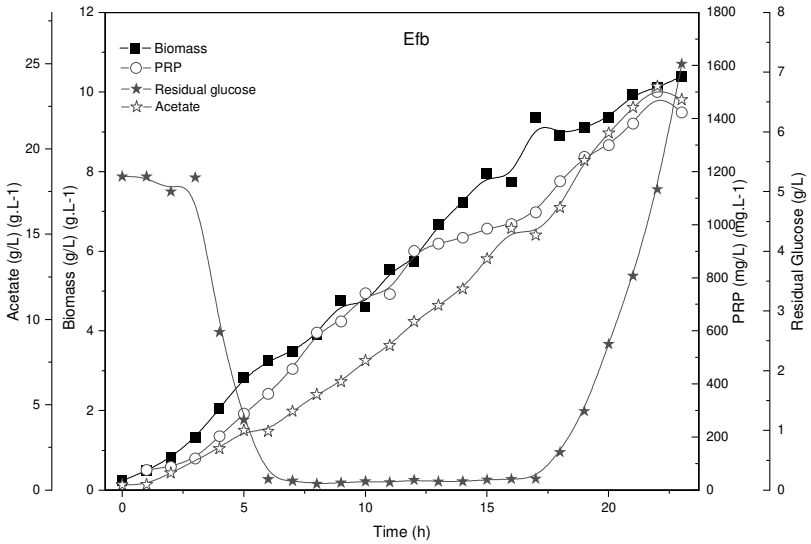


Figure 2. Exponential fed-batch cultivation (μset = 0.07 h−1). Profile of biomass, PRP and acetic acid production and residual glucose concentration.

By the end of the cultivation, biomass concentration reached 10.4 g DCW.L^{-1}, while PRP production reached 1423.2 mg PRP.L^{-1}, corresponding to a PRP productivity of 61.88 mg PRP.L^{-1}.h^{-1}. Acetate was continuously produced during the Efb1.0, even during glucose-limited phase, reaching a final concentration of 23.7 g HA.L^{-1}.

Since acetic acid production appears to be inherent to Hib metabolism, occurring even under aerobic conditions and limited glucose supply, other strategies for its removal from the culture broth were explored. For this purpose, an exponential fed batch cultivation with -cell recycling via hollow fiber membrane was performed, the process parameters are shown in Figure 3. The feed flow rate was adjusted to maintain the specific growth rate (μ_{set}) at 0.2 h^{-1} while the specific air flow rate kept at 1.0 vvm. The selected value of μ_{set} 0.2 h^{-1} represented an intermediate value between those used in exponential fed-batch cultivation (Efb1.0 μ_{set} = 0.07 h^{-1}) and the maximum value simulated by AnaBio software for the constant fed-batch (Cfb1.0 μ = 0.39 h^{-1}). When the acetic acid concentration reached approximately 15 g HA.L^{-1}, the same level at which glucose began to accumulate in the previous experiment (Figure 2). A one-time removal of 1.5 L of cell-free broth was performed, followed by an exponential perfusion process using the same flow rate as the feed flow.

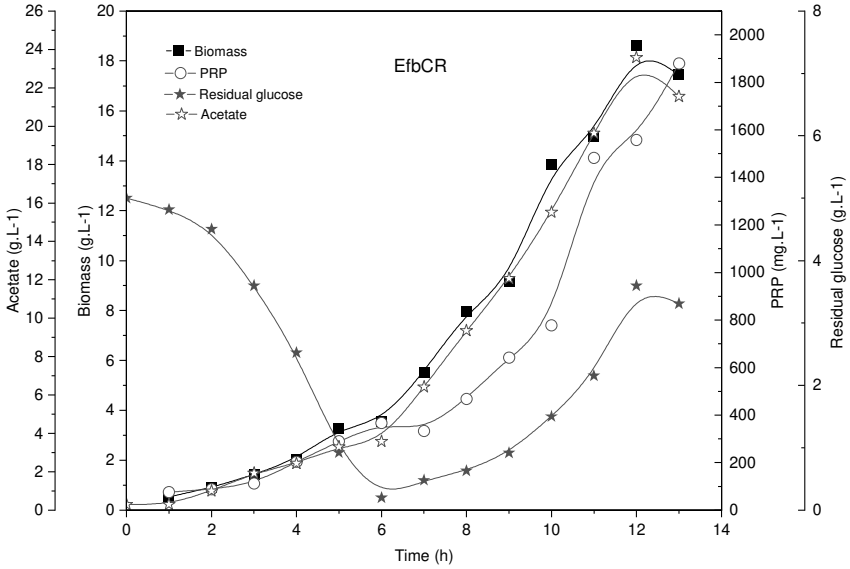


Figure 3. Exponential fed-batch cultivation with cell recycling (μ_{set} = 0.2 h^{-1}). Profile of biomass, PRP and acetic acid production and residual glucose.

Acetic acid during the EfbCR1.0 experiment was estimated indirectly based on NaOH consumption, using a calibration curve previously established from the Efb1.0 assay. At the end of the cultivation, biomass reached 18 g DCW.L^{-1}, while acetic acid and PRP concentrations were 3 g HA.L^{-1} and 1879.28 mg PRP.L^{-1} (Figure 3), respectively. The microfiltration system removed approximately 39% of culture volume as cell-free permeate, corresponding to the removal of 18.5% of the total acetic acid produced. Compared to the Cfb1.0 experiment, cell growth and PRP production in EfbCR1.0 increased by 38% and 12%, respectively.

Table 1 summarizes the scalability parameters applied in the scales of 5, 10 and 80 L scales for constant fed-batch cultures.

Table 1. Parameters for scaling up, PRP volumetric production and productivity.

Parameters	Unit	Bench Scale		Pilot Scale
		5 L	10 L	80 L
H/D		2.1	2.1	2
Diam. Impeller	cm	6.0	8.4	16.35
Air specific flow rate	vvm	0.5	1	0.5
Time of cultivation	h	18	18	20
Time of feeding	h	12	13.2	13.5
V_i–V_f	L	3.5–4.4	6.5–8.0	45–60
Feed rate	mL h^{-1}	77	131	1087
PRP	mg L^{-1}	1217	1706	1885
Productivity	mg $L^{-1}.h^{-1}$	65.22	91.41	83.77

H—vessel heigh; D—vessel diameter; V_i—initial volume; V_f—final volume.

5. Discussion

Scaling the industrial production of the PRP antigen is essential to address the global demand for Hib vaccines, particularly within developing countries [23]. Therefore, understanding the mechanism of its production and improve the cultivation parameters are critical steps toward achieving efficient, large-scale manufacturing [29–31].

Takagi et al. [22] found that maintaining the dissolved oxygen (pO_2%) at either 10% or 30% did not affect polysaccharide production when glucose was fed intermittently. In Ifb0.2 (Figure 1), the observed PRP production was comparable to that reported by

Takagi et al. [22] However, in the Ifb0.5 condition, increasing the specific air flow to 0.5 vvm led to a 10% increase in cell growth and a 34% increase in volumetric PRP production.

According to Takagi et al. [22] the synthesis of the *H. influenzae* type b capsular polysaccharide is closely associated to cell growth. Therefore, achieving a higher product yield requires attaining a high cell density [32]. The cultivation mode most commonly used to reach high cell densities is fed batch [33]. To this end, constant fed-batch cultivation was performed to improve cell density. Constant fed-batch cultivation was carried out with a specific airflow rate of 0.5 vvm and 1.0 vvm. Polysaccharide production was higher in latter condition, reaching 1217.53 mg PRP.L^{-1} and 1706.40 mg PRP.L^{-1} at the end of the cultivation for Cfb0.5 and Cfb1.0 respectively (Figure 1).

The concentration of acetic acid was approximately 10 g HA.L^{-1} in the intermittent fed-batch experiment (Figure 1) and increased to around 27 g HA.L^{-1} in the constant fed-batch cultivation. To reduce acetic acid accumulation and prevent potential inhibition of cell growth, an exponential fed batch cultivation was carried out with a controlled, limited glucose supply (Figure 2). In this approach, the substrate was fed in a way that prevented its accumulations in the culture medium; thereby minimizing for formation of unwanted metabolic [32].

Acetate was continuously produced during the EfbC1.0 cultivation, even during glucose-limited phase. When the acetic acid concentration reached approximately 15 g HA.L^{-1}, growth inhibition was evident from the decline in the growth rate profile, (Figure 3). This behavior reflects the fact that *Haemophilus influenzae* b possesses a unique metabolite profile The tricarboxylic acid (TCA) cycle in Hib is incomplete, lacking three genes encoding citrate synthase, aconitase and isocitrate dehydrogenase. Consequently, carbohydrate catabolism in Hib primarily follows the glycolytic pathways. The catabolism of carbohydrate in Hib occurs mainly by glycolytic pathway, resulting in pyruvate formation. Due to the absence of the initial enzymes of TCA cycle enzymes, acetate is formed by the action of phosphate acetyltransferase and acetate kinase, even at aerobic conditions [34,35]. Metabolic flux analysis of Hib has shown that acetate production persists even at very low specific growth rates. This indicates that acetate formation is tightly linked to the organism's central metabolism and may not be eliminated unless genetic modifications are made to disrupt the acetate production pathway [36].

The exponential fed-batch cultivation with limited glucose supply (Efb1.0) resulted in $Y_{HA/S}$ of 0.6 gHA.gglucose^{-1} and the $Y_{PRP/S}$ of 38 mg PRP.g glucose^{-1}. The maximum biomass concentration was 10 g DCW.L^{-1}, with PRP production reaching 1432.2 mg PRP.L^{-1} and a productivity of 61.88 mg PRP.L^{-1}.h^{-1}. In contrast, the exponential fed-batch with cell recycling and perfusion (EfbCR) removed approximately 19% of the total acetic acid produced, and led to improved performance, achieving 18 g DCW.L^{-1} of biomass, 1879.28 mg PRP.L^{-1}, and a productivity of 144.56 mg PRP.L^{-1}.h^{-1}. The $Y_{HA/S}$ and $Y_{PRP/S}$ for EfbCR were 0.5 g HA·g^{-1} glucose and 38 mg PRP·g^{-1} glucose, respectively. However, maintaining precise feed and perfusion flow rates is difficult challenging, even at bench scale. Additionally, cell recycling poses significant operational difficulties, including the need to maintain sterility in the cross-flow filtration system and the risk of membrane fouling over time.

Consequently, constant fed-batch cultivation emerges as the most pragmatic choice. It circumvents the technical complexities associated with perfusion hardware while maintaining high product concentrations that continuous cultures [24] fail to reach, all within a simplified operational framework suitable for industrial scale-up (Figure 1).

Table 2 summarizes the main kinetic parameters obtained from the different cultivation strategies in this study. The maximum specific growth rate (μ_{max}) was calculated in the

exponential batch phase of each cultivation. Results indicate that increasing the specific airflow rate from 0.2 to 1.0 vvm had only a modest effect on μ_{max}.

Table 2. Conversion factors, productivity and maximum specific growth rate of the different fed batches cultivation strategies.

Scale	Experiments	Vol L	PRP mg.L^{-1}	$Y_{PRP/DCW}$ mg.g^{-1}	$Y_{PRP/S}$ mg.g^{-1}	$Y_{DCW/S}$ g.g^{-1}	$Y_{HA/S}$ g.g^{-1}	P_{PRP} mg.L^{-1}.h^{-1}	μ_{max} h^{-1}
Bench	$Ifb_{0.2}$	5	636.25	63	23	0.27	0.5	52.15	0.33
	$Ifb_{0.5}$		786.37	86	33	0.25	0.44	64.46	0.34
	$Cfb_{0.5}$		1217.53	94	33	0.33	0.7	65.22	0.28
	$Cfb_{1.0}$	10	1706.40	191	44	0.29	0.6	91.41	0.30
	$Efb_{1.0}$		1423.20	105	38	0.26	0.6	61.88	0.44
	$EfbCR_{1.0}$		1879.28	101	38	0.25	0.5	144.56	0.44
Pilot	$PSCfb_{0.5}$	80	1885	180	140	0.60	0.8	83.77	0.40

Cultivation at 0.2 vvm with intermittent glucose addition resulted in $Y_{PRP/S}$ (23 mg PRP.g glucose^{-1}) slightly lower than the obtained at 0.5 vvm of 33 mg PRP.g glucose^{-1}. Additionally, cultivation at 0.5 vvm (Ifb0.5) resulted in higher cell density (6 g DCW.L^{-1}), greater polysaccharide production (786.37 mg PRP.L^{-1}) than 0.2 vvm, and improved polysaccharide productivity (64.46 mg PRP.L^{-1}.h^{-1})

Constant fed-batch cultivation at 1.0 vvm with a feeding rate of 131 mL.h^{-1} achieved higher PRP production (1706.40 mg PRP.L^{-1}) and productivity (91.41 mg PRP·L^{-1}.h^{-1}) higher than the corresponding experiment at 0.5 vvm and 77 mL.h^{-1}, which reached 1217.53 mg PRP.L^{-1} and 65.22 mg PRP.L^{-1}.h^{-1}, respectively. The $Y_{PRP/S}$ value for Cfb1.0 was 44 mg PRP.g^{-1} glucose, compared to 33 mg PRP.g^{-1} glucose, in the Cfb0.5 experiment. At 0.5 VVM, the $Y_{HA/S}$ was 0.7 g HA.g^{-1} glucose, compared to 0.6 g HA.g^{-1} glucose, with acetate production remaining nearly the same between both conditions, between 25 and 26 g.L^{-1}.

As show Table 2, EfbCR1.0 emonstratedd good PRP production, and productivity, however its preparation and execution were complex. The hollow-fiber module required chemical sterilization after each use and during tangential microfiltration, membrane fouling occurred reducing permeate flow.

Based on the results (Table 2), the fed-batch with constant feeding (Cfb) was the second performance, with good specific PRP production by unit of biomass. Moreover, it is technically simple to implement at large scale. For this reason, Cfd was scaled up in Pilot scale.

The scale-up criterion used from bench scale (5 L or 10 L) to the pilot scale (80 L) was based on maintaining a constant impeller tip speed (V_{tip}) and ensuring dissolved oxygen levels remained at 30% saturation (Table 1). At pilot scale, the air flow rate was maintained at 0.5 vvm due to gas flow limitations inherent to pilot and production scales, and to mitigate challenges such as excessive foam formation. To enhance oxygen transfer, the bioreactor was operated under slight overpressure (0.5 bar). The pilot-scale constant rate fed-batch cultivation, operated with a feed flow rate of 1087 mL.h^{-1} and an air flow rate of 0.5 vvm, achieved a maximum polysaccharide production of 1885 mg PRP.L^{-1}. The kinetic profile for cell growth at pilot scale was similar to that observed in bench-scale experiments. However, PRP production followed a linear trend, likely due to oxygen limitation during cultivation (Figure 4). Productivity reached 83.77 mg PRP.L^{-1}.h^{-1} comparable to the value obtained at bench scale under constant feeding conditions. The yields were $Y_{PRP/S}$ 140 mg PRP.g glucose^{-1} and $Y_{HA/S}$ 0.8 g HA.g glucose^{-1}, as shown in Table 2.

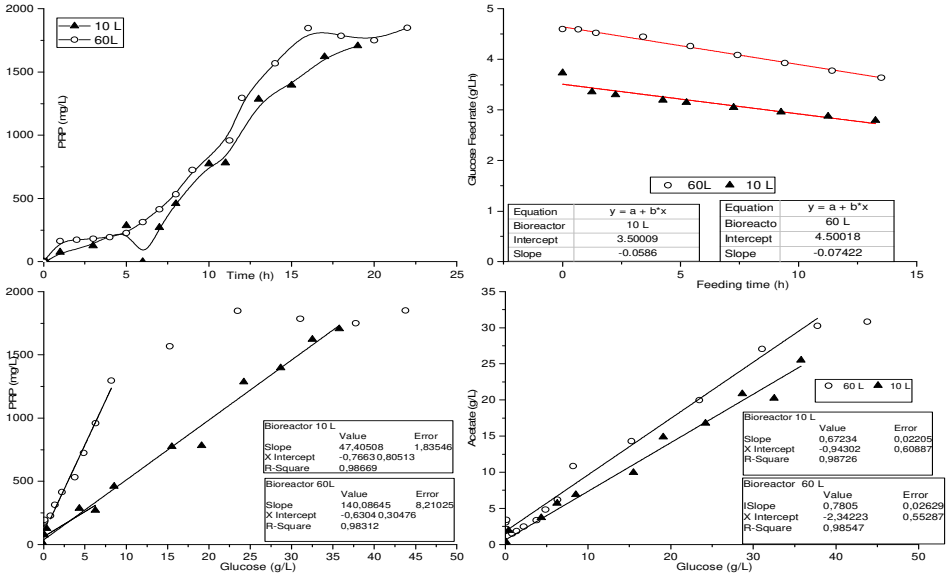


Figure 4. Comparison of kinetics profiles between constant fed-batch performed in bench and pilot scale.

6. Conclusions

In this study, different cultivation strategies were evaluated for the production of capsular polysaccharide (PRP) by *H. influenzae* type b. The most technically feasible approach was successfully scaled up to assess process robustness at pilot scale. The strategies investigated were based on extensive experience in PRP production using *H. influenzae* b cultures. Among the various factors affecting bacterial growth and capsule formation, two main strategies were tested to minimize acetic acid accumulation: increasing aeration to enhance aerobic metabolism and limiting glucose availability. While these approaches have shown promising results in recombinant *E. coli* cultures [31,32], they proved ineffective for Hib; glucose-limited cultivations failed to reduce acetic acid accumulation. Deficiencies in the respiratory chain or citric acid cycle likely contribute to the organism's inability to respond to environmental modifications intended to improve yield and efficiency. Among the strategies evaluated, constant fed-batch cultivation with air supply at 0.5 vvm and 1.0 vvm yielded 1217.53 mg PRP.L^{-1} and 1706.4 mg PRP.L^{-1}, respectively, and demonstrated operational simplicity. Ultimately, a simple fed-batch strategy with constant feed flow was selected as the most suitable method for producing capsular polysaccharide from the fastidious Hib. This approach was successfully scaled up to a pilot bioreactor with a nominal capacity of 80 L, achieving a maximum PRP concentration of 1885 mg.L^{-1} which is comparable to bench-scale results. These findings confirm the scalability and robustness of the selected process.

Author Contributions: Conceptualization: M.T. and M.R.d.S.; Methodology: M.T., S.M.F.A. and M.R.d.S.; Validation: M.T. and M.R.d.S.; formal analysis: M.T., J.C.-C. and M.R.d.S.; investigation: M.T., J.G.d.C.P., J.C.-C. and M.R.d.S.; writing—original draft preparation: M.T.; writing—review and editing: M.T., J.G.d.C.P., M.R.d.S. and J.C.-C.; visualization: M.T. supervision and project administration: M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by State of São Paulo Research Foundation (FAPESP) São Paulo, Brazil, grant number 2007/50082-2, Butantan Foundation and Foundation for Administration Development (FUNDAP).

Data Availability Statement: The data submitted for this manuscript are deposited in the library of the Federal University of São Carlos and can be accessed via the link: https://repositorio.ufscar.br/items/ddf60919-a581-4249-82f3-d2ad4ce32b9c (accessed on 20 November 2025). It is still possible

to contact the authors via email: mat_biomed@yahoo.com.br (personal); maribe@taltech.ee (professional); mickie.takagi@butantan.gov.br (professional) or even by the ORCID ID –0000-0001-8203-6960 Mateus Ribeiro da Silva; 0000-0002-0978-4070 —Mickie Takagi; 0000-0002-2902-0983 Jose Geraldo da Cruz Pradella. If you need further information on Haemophilus influenzae type b, you can find it in the Butantan Institute repository via the following link. Permanent URI for this community https://repositorio.butantan.gov.br/handle/butantan/1909 (accessed on 20 November 2025).

Acknowledgments: The authors gratefully acknowledge the financial support of the State of São Paulo Research Foundation (FAPESP) São Paulo, Brazil, Butantan Foundation and Foundation for Administration Development (FUNDAP). The authors also like to thank Lourivaldo Inácio de Souza, Inês do Amaral Maurelli and Ana Maria Rodrigues Soares for their technical assistance. This work is dedicated to the memory of Joaquín Cabrera Crespo, whose expertise were fundamental to the success of the Hib project.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Cfb	Constant rate fed-batch cultivation
$Cfb_{0.5}$	Constant rate fed-batch cultivation at 0.5 VVM
$Cfb_{1.0}$	Constant rate fed-batch cultivation at 1.0 VVM
DCW	Dry cell weight
Efb	Exponential fed-batch cultivation
$Efb_{1.0}$	Exponential fed-batch cultivation at 1.0 VVM
$EfbCR_{1.0}$	Exponential fed-batch cultivation with bacteria recycling at 1.0 VVM
FR	Glucose feed rate
HA	Acetic acid
Hib	*Haemophilus influenzae* type b
HPLC	High Performance Liquid Chromatography
Ifb	Intermittent fed-batch cultivation
$Ifb_{0.2}$	Intermittent fed-batch cultivation at 0.2 VVM
$Ifb_{0.5}$	Intermittent fed-batch cultivation at 0.5 VVM
MP	Complex medium based on soybean peptone
TCA	Tricarboxylic acid
$OD_{540\,nm}$	Optical density at 540 nm
pO_2	Dissolved oxygen
PRP	Polyribosylribitol phosphate
$PSCfb_{0.5}$	Pilot Scale Constant rate fed-batch cultivation at 0.5 VVM
VVM	Volume of air per volume of medium per minute
μ	Specific growth rate
μ_{max}	Maximum specific cell growth rate
μ_{set}	Defined specific cell growth rate
$V_{_tip}$	Constant impeller tip speed

References

1. Peltola, H. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21 st century: Global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. *Clin. Microbiol. Rev.* **2000**, *13*, 302–317. [CrossRef]
2. Moxon, E.R.; Kroll, J.S. Type b capsular polysaccharide as a virulence factor of *Haemophilus influenzae*. *Vaccine* **1988**, *6*, 112–115. [CrossRef]
3. Pichichero, M.E. Protein carriers of conjugate vaccines. Characteristics, development, and clinical trials. *Hum. Vaccines Immunother.* **2013**, *9*, 2505–2523. [CrossRef]
4. Miranzi, S.S.C.; Moraes, S.A.; Freitas, I.C.M. Impact of the *Haemophilus influenzae* type b vaccination program on HIB meningitis in Brazil. *Cad. Saúde Pública* **2007**, *23*, 1689–1695. [CrossRef]

5. Ribeiro, G.S.; Lima, J.B.T.; Reis, J.N.; Gouveia, E.L.; Cordeiro, S.M.; Lobo, T.S.; Pinheiro, R.M.; Ribeiro, C.T.; Neves, A.B.; Salgado, K.; et al. *Haemophilus influenzae* meningitis 5 years after introduction of the *Haemophilus influenzae* type b conjugate vaccine in Brazil. *Vaccine* **2007**, *25*, 4420–4428. [CrossRef] [PubMed]
6. Agrawal, A.; Murphy, T.F. *Haemophilus influenzae* infections in the *H. influenzae* type b conjugate vaccine era. *J. Clin. Microbiol.* **2011**, *49*, 3728–3732. [CrossRef] [PubMed]
7. Zanella, R.C.; Bokermann, S.; Andrade, A.L.S.S.; Flannery, B.; Brandileone, M.C.C. Changes in serotype distribution of *Haemophilus influenzae* meningitis isolates identified through laboratory-based surveillance following routine childhood vaccination against *H. influenzae* type b in Brazil. *Vaccine* **2011**, *29*, 8937–8942. [CrossRef] [PubMed]
8. Cleland, G.; Leung, C.; Cheong, J.W.S.; Francis, J.; Heney, C.; Nourse, C. Pediatric invasive *Haemophilus influenzae* in Queensland, Australia, 2002–2011: Young Indigenous children remain at highest risk. *J. Paediatr. Child Health* **2018**, *54*, 36–41. [CrossRef]
9. Yang, Y.; XiangPan, X.; Cheng, W.; Yang, Y.; Scherpbier, R.W.; Zhu, X.; Chen, Y.; Zhou, Y.; Jiang, Q. *Haemophilus influenzae type b* carriage and burden of its related diseases in Chinese children: Systematic review and meta-analysis. *Vaccine* **2017**, *35*, 6275–6282. [CrossRef]
10. Yang, Y.; Wang, Y.; Yang, D.; Dong, S.; Yang, Y.; Zhu, X.; Chen, Y.; Zhou, Y.; Jiang, Q. Factors associated with uptake of *Haemophilus influenzae* type b vaccination in Shanghai, China. *BMC Pediatr.* **2019**, *19*. [CrossRef]
11. xii Wang, S.; Tafalla, M.; Hanssens, L.; Dolhain, J. A review of *Haemophilus influenzae* disease in Europe from 2000–2014: Challenges, successes and the contribution of hexavalent combination vaccines. *Expert Rev. Vaccines* **2017**, *16*, 1095–1105. [CrossRef] [PubMed]
12. Steens, A.; Stanoeva, K.R.; Knol, M.J.; Mariman, R.; de Melker, H.E.; van Sorge, N.M. Increase in invasive disease caused by *Haemophilus influenzae* b, the Netherlands, 2020 to 2021. *Eurosurveillance* **2021**, *26*, 2100830. [CrossRef] [PubMed]
13. Slotved, H.-C.; Johannesen, T.B.; Stegger, M.; Dalby, T.; Fuursted, K. National Danish surveillance of invasive clinical *Haemophilus influenzae* isolates and their resistance profile. *Front. Microbiol.* **2023**, *14*, 1307261. [CrossRef] [PubMed]
14. Slack, M.; Esposito, S.; Haas, H.; Mihalyi, A.; Nissen, M.; Mukherjee, P.; Harrington, L. *Haemophilus influenzae* type b disease in the era of conjugate vaccines: Critical factors for successful eradication. *Expert Rev. Vaccines* **2020**, *19*, 903–917. [CrossRef]
15. Gilsdorf, J.R. Hib Vaccines: Their impact on *Haemophilus influenzae* type b disease. *J. Infect. Dis.* **2021**, *224*, 321–330. [CrossRef]
16. Tønnessen, R.; García, I.; Debech, N.; Lindstrøm, J.C.; Wester, A.L.; Skaare, D. Molecular epidemiology and antibiotic resistance profiles of invasive *Haemophilus influenzae* from Norway 2017 n2021. *Front. Microbiol.* **2022**, *13*, 973257. [CrossRef]
17. Hani, E.; Abdullahi, F.; Bertran, M.; Eletu, S.; D'Aeth, J.; Litt, D.J.; Fry, N.K.; Ladhani, S.N. Trends in invasive *Haemophilus influenzae* serotype b (Hib) disease in England: 2012/13 to 2022/23. *J. Infect.* **2024**, *89*, 106247. [CrossRef]
18. Hilleman, M.R.; Tai, J.Y.; Tolman, R.L.; Vella, P.P. Coupled *H. influenzae* type b vaccine. US Patent 4459286 A, 1984.
19. Carty, C.E.; Mancinelli, R.; Hagopian, A.; Kovach, F.X.; Rodriguez, E.; Burke, P.; Dunn, N.R.; McAleer, W.L.; Maigetter, R.Z.; Kniskern, P. Fermentation studies with *Haemophilus influenzae*. *Dev. Ind. Microbiol.* **1985**, *26*, 763–767.
20. Merritt, J.; Allard, G.; Toole, L.O.; Swartz, R.; Licari, P. Development and scale-up of a fed-bach process for the production of capsular polysaccharide from *Haemophilus Influenzae*. *J. Biotechnol.* **2000**, *81*, 189–197. [CrossRef]
21. Takagi, M.; Cabrera-Crespo, J.; Baruque-Ramos, J.; Zangirolami, T.; Raw, I.E.; Tanizaki, M. Characterization of polysaccharide production of *Haemophilus influenzae* type b and its relationship to bacterial cell growth. *Biochem. Biotechnol.* **2003**, *110*, 91–100. [CrossRef]
22. Takagi, M.; Cabrera-Crespo, J.; Zangirolami, T.C.; Raw, I.; Tanizaki, M. Improved cultivated conditions for polysaccharide production by *H. influenzae* type b. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 182–188. [CrossRef]
23. Pillaca-Pullo, O.; Vieira, L.D.; Takagi, M. Scale-up of capsular polysaccharide production process by *Haemophilus influenzae* type b using kLa criterion. *Bioengineering* **2022**, *9*, 415. [CrossRef] [PubMed]
24. Solidade, L.S.; Vieira, L.D.; Takagi, M. Improving *Haemophilus influenzae* Type b Polysaccharide Productivity Through Continuous Culture for Pentavalent Vaccine Manufacturing. *Fermentation* **2025**, *11*, 622. [CrossRef]
25. Oliveira, C.M.; Jesus, C.D.F.; Ceneviva, L.V.S.; Silva, F.H.; Cruz, A.J.G.; Costa, C.B.B.; Badino, A.C. Anabioplus: A new package for parameter estimation and simulation of bioprocesses. *Braz. J. Chem. Eng.* **2017**, *34*, 1065–1082. [CrossRef]
26. Ashwell, G. Colorimetric analysis of sugar. *Methods Enzymol.* **1957**, *3*, 87–90. [CrossRef]
27. Drury, H.F. Identification and estimation of pentoses in the presence of glucose. *Arch. Biochem.* **1948**, *19*, 455–466. [PubMed]
28. Crisel, R.M.O.; Baker, R.S.; Dorrman, D.E. Capsular polymer of *Haemophilus influenzae* type b. I. structural characterization of the polymer of strain Eagan. *J. Biochem. Chem.* **1975**, *250*, 4926–4930. [CrossRef]
29. Cintra, F.O.; Takagi, M. Kinetic models of exopolysaccharide production by *Haemophilus influenzae* type b. *Vaccines Vaccin. J.* **2020**, *3*, 114.
30. Cintra, F.O.; Takagi, M. Instability and depolymerization of the exopolysaccharide produced by *Haemophilus influenzae* type b during fermentation. *Vaccines Vaccin. J.* **2022**, *3*, 117.
31. Arsang, A.; Tabata Baie, A.; Vaziri, F.; Nejati, M.; Zolfaghari, M.R.; Fateh, A.; Rahimi Jamnani, F.; Bahrmand, A.R.; Siadat, S.D. Optimization of large scale production of *Haemophilus influenzae* type b polyribosyl-ribitol phosphate. *Minerva Biotechnol.* **2017**, *29*, 17–23. [CrossRef]

32. Lee, S.Y. High cell density culture of *Escherichia coli*. *Trends Biotechnol.* **1996**, *14*, 98–105. [CrossRef]
33. Korz, D.J.; Rinas, U.; Hellmuth, K.; Sanders, E.A.; Deckwer, W.D. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J. Biotechnol.* **1995**, *39*, 59–65. [CrossRef]
34. Fleischmann, R.D.; Adams, M.D.; White, O.; Clayton, R.A.; Kirkness, E.F.; Kerlavage, A.R.; Bult, C.J.; Tomb, J.F.; Dougherty, B.A.; Merrick, J.M.; et al. Whole-genome random sequencing and assembly of *Haemophilus influenza* Rd. *Science* **1995**, *269*, 496–512. [CrossRef]
35. Raghunathan, A.; Price, N.D.; Galperin, M.Y.; Makarova, K.S.; Purvine, S.; Picone, A.F.; Cherny, T.; Xie, T.; Reilly, T.J.; Munson, R., Jr.; et al. In silico metabolic model and protein expression of *Haemophilus influenzae* strain Rd KW20 in rich medium. *OMICS* **2004**, *8*, 25–41. [CrossRef]
36. Silva, M.R.; Portela, C.A.F.; Albani, S.M.F.; Paiva, P.R.; Tanizaki, M.M.; Zangirolami, T.C. Experimental design and metabolic flux analysis tools to optimize industrially relevant *Haemophilus influenzae* type b growth medium. *Biotechnol. Prog.* **2017**, *33*, 1508–1519. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.