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Executive Summary of the Thesis

Scale-down perfusion bioreactor optimization for the production of therapeutic oligonucleotides.

Laurea Magistrale in Chemical Engineering - Ingegneria Chimica

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1. Introduction

Therapeutics RNA oligonucleotides are assuming increasing interest in the present day pharmaceutical landscape thanks to the possibility of extending the range of druggable targets from conventional proteins to RNAs and to the genome itself. The most promising method for the production of these molecules is represented by the *in-vivo* synthesis, that presents several advantage respect to chemical or *in-vitro* synthesis.

Rhodovulum Sulfidophilum purple marine bacterium proved to be a suitable host for the recombinant production of artificial RNA molecules thanks to the capacity of extracellular nucleic acid secretion and to the absence of observable RNases emissions in the culture environment [1, 3].

Nowadays, nucleic acids production by means of *R. Sulfidophilum* fermentation in perfusion bioreactors combines the characteristics of *in-vivo* synthesis with the advantages related to a continuous productive process.

In this work a scale-down optimization approach has been adopted for screening multiple operating conditions. Two micro-scale VCDmax experiments (plus one previously conducted by our research group) have been performed for testing the culture's behavior at various perfusion rates when exposed to different aeration conditions. The reliability of this method was cross-validated by comparing the results of spin-tube experiments with the data collected from benchtop-scale perfusion bioreactor.

Further measurements on nucleic acid concentration in the culture environment have been performed to relate the cells productivity with the operating conditions.

In future work, further benchtop-scale experiments are required for the assessment of performance parameters for high-densities cultivations and to provide samples for the quantitative and qualitative analyses of oligonucleotides productivity.

2. Materials and methods

2.1. Bacterial Strain

The bacterial strain used for this work is the purple phototrophic marine bacterium *R. Sulpfidophilum*, strain DSM 1374 provided by the research group of professor F. Sousa at *Universidade da Beira Interior*.

2.2. Media Formulation

Both the expansion media and the optimized growth media formulations derive from the semi-defined media proposed by Pereira et al. [3]. Figure 1 reports the optimized media used for all the experiments of this work [2].

Component	Formula	Concentration [g/L]
Triptone	-	0
Polypeptone	-	0
Yeast Extract	-	13
Sodium Chloride	NaCl	30
Potassium Phosphate Dibasic	K2HPO4	4
Potassium Dihydrogen Phosphate	KH2PO4	1
MilliQ	H2O	-
Glucose	C6H12O6	30
Magnesium Sulfate heptahydrate	MgSO4 · 7H2O	1
Calcium Chloride dihydrate	CaCl2 · 2H2O	0.05
Ammonium sulfate	(NH4)2SO4	1
Trace Element Solution(TES)	-	1[mL/L]

Figure 1: Optimized growth media formulation

2.3. Pre-experimental procedures

The cells of *R. Sulpfidophilum* were firstly used for the preparation of master- and working cell banks (MCB and WCB). The preparatory work required by each experiment consists in a cellular expansion aimed to produce a desired volume of bacterial culture with an adequate cell density to meet the experiment's planned initial conditions.

2.4. Scale-down model: VCDmax experiment

The micro-scale experiment exploited 50mL spin-tube bioreactors (TubeSpin; TPP, Trasadingen, Switzerland) for simulating the continuous perfusion operation through discontinuous daily activities according to the procedure proposed by Wolf et Al. [4]. The experiment was conducted by placing the spin-tube reactors inside the incubator (Adolf Kühner AG, Birsfelden, Switzerland) operating at the following parameters: temperature 30°C, relative humidity 90% and atmospheric CO2 concentration, and three separate runs have been performed at different shaking speed, 200-260-320rpm (orbital shaker, Thermo Fisher Scientific, Waltham, USA).
The Perfusion simulation consists in a sequence of 5 steps: 1) a 0.5ml sample is taken from the spin-tube, 2) the centrifugation (18500rcf, 12 minutes) separates the cells from the supernatant, 3) a precise liquid volume is harvested according to the PR, 4) the correct amount of fresh media is injected, 5) the cells are resuspended and placed in the incubator until the next working day.

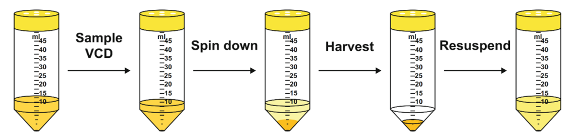


Figure 2: Procedure of scale-down VCDmax experiment [4].

The 0.5mL sample is used for VCD assessment by means of optical density analysis at 600nm (OD600) using a Cedex Bio analyzer (Roche Diagnostics AG, Basel, Switzerland), and of cell dry weight measurements (CDW). This was evaluated by centrifuging (>12000rpm, 6 minutes) and drying (t >48h) a known sample volume using 1.5mL Eppendorf Tubes (Eppendorf Safe-Lock microcentrifuge tubes, Sigma-Aldrich, USA) Centrifuge 5415R (Eppendorf, Hamburg), and a vacuum concentrator (Concentrator Plus, Eppendorf, Hamburg). The CDW value was measured by subtracting the tare from the final weight.
The supernatant from the perfusion procedure was frozen and stored in 15mL falcons (Corning 15 mL centrifuge tubes, Corning, USA) at -80°C for further analysis of glucose concentration and oligonucleotides content.
This procedure has been used for conducting three runs: 1) 200rpm shaking speed, perfusion rates tested: 0.05-0.1-0.2-0.3-0.4-0.5-0.7-1 RV/Day. 2) 260rpm, perfusion rates: 0.1-0.3-0.4-0.5-0.7-1 RV/Day. 3) 320rpm shaking speed, perfusion rates tested: 0.05-0.1-0.2-0.3-0.4-0.5-0.7-1 RV/Day.

2.5. Perfusion Benchtop Bioreactor

The equipment used for perfusion benchtop scale experiments is a DASGIP Parallel Bioreactor

System (Eppendorf AG, Hamburg, Germany) with a working volume of 2L. Online measurements of temperature, pH, dissolved oxygen (DO) and capacitance have been used to monitor and control the working conditions. The working volume was kept constant by monitoring the reactor weight in order to maintain the steady-state operation.
Operating conditions: 30°C, 20% dissolved oxygen (DO), 500rpm stirring speed, pH 7. Foam formation was controlled by discreet manual ainjection of 10% Antifoam B Emulsion (Sigma-Aldrich, USA). The perfusion operation was ensured using a Alternate Tangential Flow (ATF) retention device: the ATF perfusion controller (Repligen Corporation, Waltham, USA) was coupled with a 0.5 µm Spectrum MiniKros polyethersulfone hollow fiber filter.

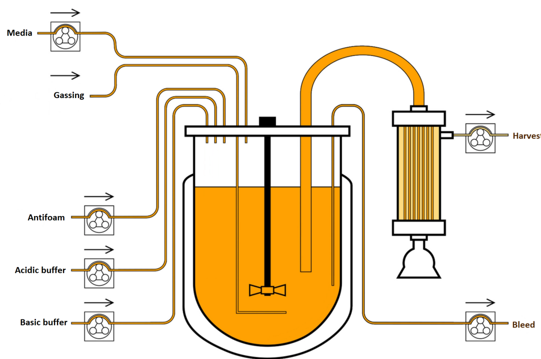


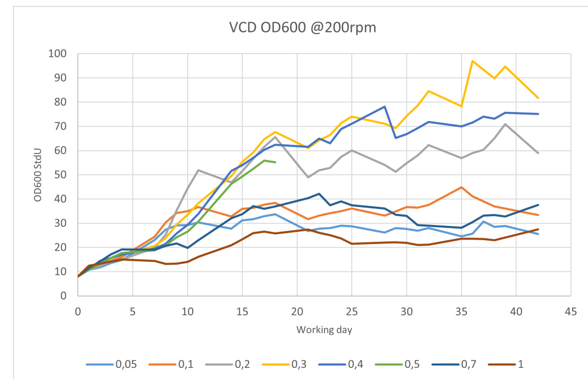
Figure 3: Perfusion benchtop-scale bioreactor layout.

2.6. Analytics of harvest samples

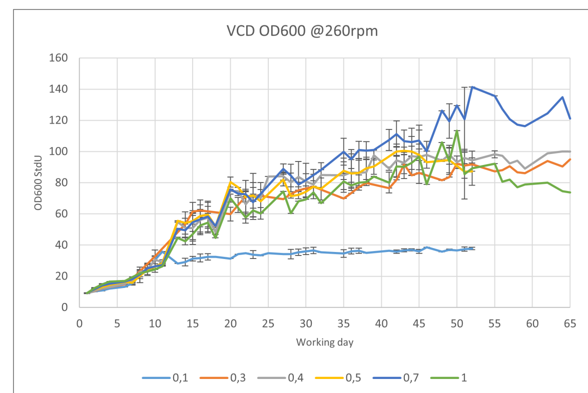
The concentration of glucose was determined using an Agilent 1200 HPLC RID system (Agilent Technologies Inc., Santa Clara, USA) equipped with an Organic Acid Analysis column (Aminex HPX-87H Ion Exclusion Column, BIO RAD, USA). The HPLC was also used for the measurement of the nucleic acid content after extraction in ethanol: the analyses have been repeated with an ion-pair reverse-phase Agilent AdvanceBio Oligonucleotide 2.7µm, 2.1 x 100mm column, and with a ZORBAX Eclipse Plus C18 3.5µm, 4.6 x 150mm paired with a LC/UV detection system (Agilent Technologies Inc., Santa Clara, USA).

3. Results and discussion

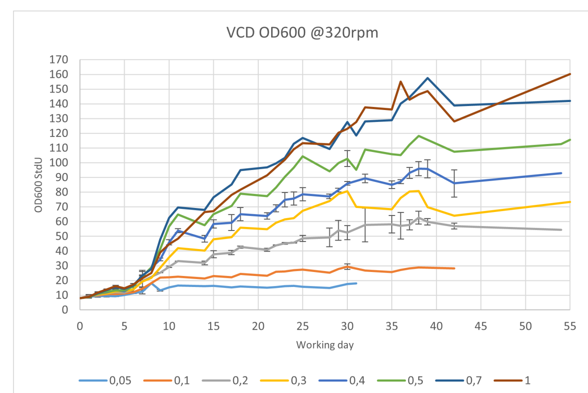
By comparing the results of all the three experiments it emerges that the shaking speed exert a strong influence on the behavior of the culture subjected to perfusion rates >= 0.4 RV/day: an increase in shaking speed determines an increase in the steady-state OD600, and thus a higher VCD.



(a)



(b)



(c)

Figure 4: Comparison of cellular growth and steady-state VCD for VCDmax experiments @ 200rpm (a), 260rpm (b), and 320rpm (c)

From the direct comparison of the relationship

between PR and VCDmax (figure 5 appears that all the experiments align on the same CSPRmin limiting condition, identifying the lowest PR to sustain a specific cell concentration: in these conditions: the nutrients supplied by the media represent the limiting substrate.
However 200 and 260rpm experiments proved that a higher PR does not necessarily translates in a higher VCD and demonstrate the existence of a second limiting conditions. It can be described as an upper limitation (CSPRmax) that is determined by the inhibition effect exerted by an excessive nutrients concentration in an environment with reduced oxygen concentration.

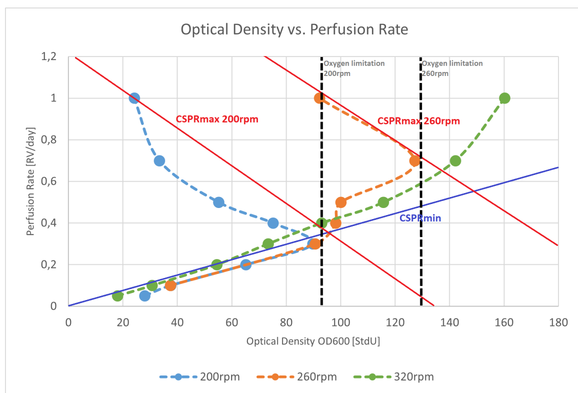
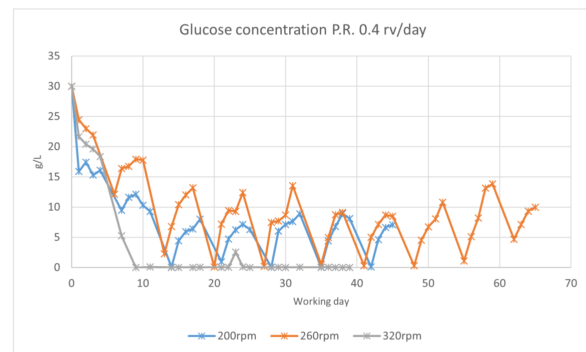
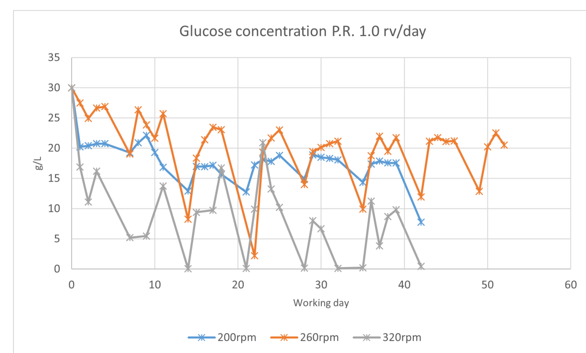


Figure 5: Maximum VCD vs. PR for VCDmax @200-260-320rpm.

The shaking speed governs the oxygen transfer to the cell culture thus the simplest hypothesis to explain the deviation from the CSPRmin boundary relies on the oxygen becoming the limiting substrate. According to this hypothesis all the experiments with PR >= 0.3 RV/day & 200rpm, and with PR >= 0.7 & 260rpm should draw a vertical limit on the OD vs. PR chart of figure 5.
The VCDmax is indeed determined by the balance among cell growth rate, cell death rate, and simulated bleed rate. Assuming that the growth rate µ follows the dynamics described by the Monod model equation, µ(S_ox) should be equal for all the experiments at the same shaking speed, resulting in the two vertical limits depicted with the black-dashed lines in the previous chart.
The experimental data does not sustain this hypothesis thus the glucose concentration in experiments with PR 0.4 and 1 RV/day has been analyzed for all the three shaking speeds. Data are collected in figure ref, where the indicators are placed on the days during which the perfusion simulation has been performed. When no nutrients are supplied for longer periods of time the concentration often drops to near-zero values.
From these analyses, it is clear that the experiments conducted at higher rpm better exploit the glucose due their higher VCD, resulting in a lower average concentration. In addition to this, considering the data collected for PR 1 RV/day on days where the culture had not received nutrients for more than 48h another important conclusion can be extrapolated: the drop in glucose concentration is more relevant for an increase in the shaking speed and this points out the lower metabolic activity of the 200rpm experiment



(a)



(b)

Figure 6: Comparison of glucose concentration for VCDmax experiments at 200, 260, and 320rpm for perfusion rates 0.4RV/day (a) and 1.0 RV/day (b)

The comparison between benchtop-scale perfusion bioreactor and VCDmax experiments for a perfusion rate of 0.4 RV/day highlights a significant difference in the transient phase from inoculum to steady-state: the cellular growth inside the bioreactor is much higher than the

one experienced at the micro-scale.
Regardless of this initial difference, it is possible to observe that the maximum steady-state VCD reached by the bioreactor is comparable to the one measured for VCDmax experiments at 260 and 320rpm, the only experiment that shows a lower cell density is the one conducted at 200rpm. This divergence may be explained by considering the relationships between nutrient concentration, perfusion rate, and the concentration of dissolved oxygen in the culture environment. Inside the bioreactor, the oxygen is kept to set-point by the online Dasgip control system and the culture never undergoes oxygen depletion. Thus, these results are consistent with the hypothesis of oxygen limitation and CSPRmax effect. The perfusion bioreactor indeed has no oxygen limitation hence it is expected that the cell culture obeys the CSPRmin limitation settling around a steady-state VCD that matches the higher-rpm VCDmax experiments. The experimental results prove this statement. As a consequence, it is possible to affirm that, even if the transient phase of the benchtop-scale bioreactor is not well-reproduced by the tube-spin experiment, the scale-down approach offers a reliable platform for experimenting different working conditions at perfusion rate 0.4 RV/day.

In the end, analytics method for the quantification of nucleic acids concentration inside the VCDmax harvest were performed. The trend resulting from spectrophotometry UV absorbance analysis offer the possibility of making hypothesis about the relation between RNA productivity, VCDmax, and PR. Figure 7 suggest that the productivity is affected more by the PR with respect to the VCD. However further analyses have to be performed to assess the nutrient-dependent production performance.

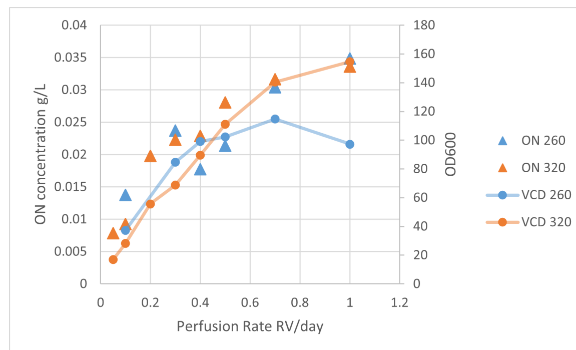


Figure 7: VCD and oligonucleotides concentration of VCDmax experiments at 260 and 320rpm and for PR ranging from 0.05 to 1 RV/day.

Following the process optimization approach, the supernatant removed from VCDmax experiments has been analyzed with different methods to assess the quality and the amount of RNA dissolved in the bacterial culture environment. In this way, it is possible to relate the perfusion rate and the shaking speed to the process productivity and determine whether the different growing conditions affect the quality of the products. The supernatant has been processed according to the RNA extraction procedure and the samples have been analyzed with the spectrophotometer and with the HPLC.

4. Conclusions

In conclusion, the scale-down model proved to be a powerful experimental procedure for the optimization of the continuous perfusion process with *R. Suplfidophilum*. The data collected from the first micro-scale VCDmax experiments provided substantial information about the behaviour of this bacterial strain subjected to different nutrients feeding rates. The VCDmax 200rpm experiment exhibits important limiting condition proving the existence of a lower limit related to insuffient nutrients supply, the CSPRmin line, and introducing the concept of substrate inhibition for higher perfusion rates, the CSPRmax. Further VCDmax experiments at 260 and 320rpm made it possible to identify the explanation of the inhibited growth in the relation between perfusion rates and culture oxygenation. The higher dissolved oxygen concentration enables the culture to fully exploit the nutrients supplied by the higher perfusion rates resulting in a larger maximum viable cell

density. This assessment is proved by the analysis conducted on the spent media: for a constant perfusion rate (>= 0.4 RV/day) the experiments performed at higher shaking speed showed a higher glucose consumption reaching null concentrations when nutrients were not supplied for longer period of time.
A satisfactory degree of reliability of the scale-down model was also proven by comparing the spin-tubes results with benchtop-scale perfusion bioreactor runs: the two experiments showed approximating steady-state VCD performances. Further benchtop scale perfusion experiments have to be performed in order to assess the maximum VCD achievable with different perfusion rates and to prove the viability of the continuous production.
The analysis of the oligonucleotides concentration in the harvest provided interesting results for posing hypothesis on the correlation among RNA productivity, steady-state VCD, and perfusion rate, rising the idea that the nucleic acid production might be more related to the amount of accessible nutrients than the cell density itself. However additional researches have to be performed, focusing on analysing a wider range of samples and testing the presence of the same trend at the benchtop scale.
In this sense, the future work may focus on performing perfusion experiments at benchtop-scale for assessing the stability of the culture at higher PR, and for investigating the oligonucleotides productivity.

References

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