Development of microfluidic reactors for the continuous production of extracellular metabolites

Eduardo João Silva Brás
ejsbras@gmail.com

Instituto Superior Técnico, Lisboa, Portugal

October 2015

Abstract

The large costs of the biotechnological industry has lead many groups to look at the possibility of miniaturizing certain aspects of the optimization process, namely using microfluidic devices for cell screening purposes. This work takes another step forward by focusing on the development and optimization of a microbioreactor for the continuous fermentation of *Saccharomyces cerevisiae* in order to produce the extracellular protein invertase. 4 designs were developed and 1 of them was optimized in order to limit cell division and maximize production. CFD simulations were taken into account when designing the chips. An increase in production of 11 times is achieved by changing several aspects of the culture medium used, such as pH, concentration and shear stress of the liquid. The microfluidic device is than tested in terms of longevity, being able to work for at least 32h of continuous perfusion. The micro reactor was then compared to macroscale fermentations in terms of production and physical parameters, where the need for future improvements on the device are demonstrated. The integration of a downstream processing step is attempted by including a chromatography channel into the same device as the microbioreactor which would allow for the continuous production and then extraction of the product of interest.
Keywords: Fermentation, Invertase, Microbioreactor, Microfluidics, Plant-on-a-chip.

1. Introduction

1.1. Microfluidics and the Biotechnology Industry

Fermentation and downstream processing technologies are vital part of several biotechnological industries, so the reduction of expenses in these areas are essential for a profitable industry. [1]

With this in mind several research groups have focused their attention on minaturizing optimization procedures due to the large expenses imposed by industrial scale systems. This minaturization goes from the use of shake flasks and 96-well plates to emulate fermentation processes as well as extraction and purification steps to the use of microfluidic devices capable in operating in the μL and even nL scale. [2] [3] [4]

Several microfluidic devices have been developed in order to perform cell screening assays but there aren't many which focus on being a platform for production. The only relevant work for this purpose comes from Garcias' work. [5] [6]

The proposed work is to fabricate and optimize a microfluidic platform capable of growing cells and extraction of a extracellular protein in a continuous fashion.

1.2. PDMS

The substrate of choice to produce the microreactor is poly(dimethylsiloxane) (PDMS). PDMS benefits from several features that are useful in a biological application, such as optical transparency, biocompatibility, gas permeability and the fact that it's main disadvantage, hydrophobicity, can be easily overcome by using different types of surface modifications. [7] [8]

1.3. *Saccharomyces cerevisiae* and Invertase

The biological model chosen was *Saccharomyces cerevisiae*, otherwise known as baker's yeast due it being a very well known biological species and for its production of the extracellular protein invertase. [9]

β-fructofuranosidase, EC 3.2.1.26 more commonly known as invertase is an enzyme that catalyzes the break down of sucrose into it's monomers, glucose and fructose.

Invertase is an important enzyme in the food industry and can be obtained from several organisms, for example bees synthesize invertase in order to produce honey. The main source of industrial invertase is baker's yeast due to it's non-pathogenic and non-toxicogenic behavior. [10]

It's importance comes from the fact that inverted sugar is sweeter than regular sucrose solutions, hence lowering production costs for the industry. Enzymatic conversion using invertase is more appealing than acid hydrolysis due to it's higher yield and because of the colorless solution that is produced. Inverted sugar solutions obtained form acid hydrolysis also tends to crystallize quicker than that of which is obtained by using invertase, which can become troublesome in the food industry in order to ensure that food remains soft for a longer time. [10] [11]

1.4. Reynolds Number

The Reynolds number (Re) is an adimensional number which indicates the state of turbulence of a fluid. Typically Re below 10^5 are considered laminar regime, while values above that are considered turbulent regime. A high Re usually means that there is good mixing of the fluid due to its own turbulence which enhances heat and mass transfer. The Re can be calculated with Equation 1

$$Re = \frac{L \times \rho \times v}{\mu} \tag{1}$$

Where L is the characteristic dimension of the system, ρ is the fluid density, v is the fluid speed and μ is the dynamic viscosity.

1.5. Shear Stress

Shear stress (τ) is the parallel force exerted on a boundary, in other words, for liquids, τ is the force the liquid exerts on a surface or object by flowing parallel to it and can be generally calculated using Equation 2

$$\tau = \mu \frac{\partial v}{\partial x} \tag{2}$$

With this it's possible to calculate for instance, the forces applied onto the surface of cells in a fermentation and try to understand if this has an impact on the culture.

1.6. Peclet Number

The Peclet number (Pe_L) is the reason between advective transport rate and diffusive transport rate and can be calculated in terms of mass transport or in terms of heat transfer. For mass transport Pe_L can be calulated using Equation 3

$$Pe_L = Re \times Sc = Re \times \frac{\mu}{D \times \rho} \tag{3}$$

Where Sc is the Schmidt number which is the ratio between momentum diffusivity and mass diffusivity, D is the diffusion coefficient. In the case of heat transfer the Prandtl number is used instead of Sc.

1.7. Phase number and Froude number

To assure agitation parameters are adequate for a shake flask the Phase number (Ph) and the Froude number (Fr_a) can be calculated, as seen in the following equations.

$$Ph = \frac{d_{Mix.\ Table}}{d} \times [1 + 3 \times log\,(Re_f)] \tag{4}$$

$$Re_f = \left(\frac{Re\pi}{2} \times \left(\sqrt{1 - \frac{4}{\pi} \left(\frac{V_L^{1/3}}{d} \right)^2} \right)^2 \right) \tag{5}$$

$$Fr_a = \frac{(2\pi \times n_{Mix.\ Table})^2 \times d_{Mix.\ Table}}{2g} \tag{6}$$

When Ph>1.26 and Fr_a>0.4, the shaking conditions are adequate for the experiment at hand.

2. Materials and Methods

2.1. Microfluidic Construction

Reactors were designed using AutoCAD software and optimized in terms of dimensions and structure placement using the laminar flow module of COMSOL in order to obtain a uniform flow of liquid inside the device.

This design was used to create a aluminum hard mask through soft lithography. A 1.3 μm thick layer of positive photoresist (PR) (PFR7790G) was spin coated onto the glass covered with aluminum. This is than exposed according to the AutoCAD design in the Direct Write Laser machine (Laserarray DWL 2.0). The sample is than baked and developed (TMA 238 WA) and is ready for chemical etching of the aluminum (Gravure Aluminum Etchant; Technic, Microchemicals).

The aluminum mask is than used to create the SU-8 mold which is a negative PR, SU-8 2015 and 2050 (Microchem) were used depending on the height desired. The PR is spin coated on a clean silicon wafer and than pre-baked to eliminate the solvent, leaving a uniform layer of PR. Next the PR is exposed to UV light through the hard mask in order to generate a photo acid, the time for this step was optimized through trial and error for the desired heights. After exposition, the sample is baked again in order to help the reaction of the photo acid in order to make the exposed areas more durable to withstand the development step. [12] After the post-exposure bake the substrate is ready for development, which consists of dipping the substrate into propylene glycol mono-methyl ether acetate (PGMEA, Sigma-Aldrich) for 1 or 15 min, depending on the desired height, than washing with IPA to

remove any excess PGMEA. What this does is remove all the none exposed areas of SU-8 giving the desired topography to create the microfluidic structures. After development the mold is hard baked for around 1h at 150 C to harden the mold.

PDMS is than prepared at a ratio of 1:10 with its curing agent (Sylgard 184 silicon elastomer kit, Dow-Corning) and poured on top of the mold and baked for 1-1.5 h.

After punching the necessary holes needed for the inlets and outlets the structures are sealed by applying a weak plasma for 1 min using a Harrick Plasma PDC-002-CE machine and joining both halves.

2.2. Cell Preparation

Cells were cultured over 3 days in a petri dish with solid culture medium (LB Agar, NZYTECH) and than transfered to a liquid medium composed of sucrose (20 g/L; Fischer-Chemical), yeast extract (20 g/L; Himedia) and peptone (7.5 g/L, Bacto Peptone) where it grew for 12-14 h at 30C in a sterile falcon shaking at 250 rpm. [13]

2.3. Microfluidic Perfusion Assays

The setup included a microscope equipped with a AmScope MD600 camera which was connected to my personal laptop using AmScope software to visualize. A thin transparent hotplate (Okolab) was used to maintain temperature at 30 C, while a syringe pump (New Era Pump Systems Inc.) fitted with custom made adapters for using more than one syringe (1 mL, CODAN) was used to push the liquids inside the microfluidic structure.

The syringes were topped with 20 ga luer stubs (INSTECH) and connected to BTPE 90 tubing (INSTECH) and connected to the microfluidic device using a metal adapter (SC20/15, INSTECH).

All perfusion assays started with a cell seeding step, where a cell suspension of O.D.=0.1 was injected inside the chamber at a flow rate of 1.5 μL/min while growth medium was injected at a flow rate of 0.5 μL/min to prevent the occurrence of back flow of the cells into the medium inlet. Once the microfluidic device was successfully inoculated (10-20% of occupation) the cell flow was gradually reduced until stopped with the inlet then being sealed off, using a metal plug, followed by washing the structure by flowing growth medium at 4.5 μL/min for 5 min to remove any residual cells that would get attached to the PDMS in non-trapping regions and then the growth medium flow was adjusted for the needs of the experiment. Invertase production and cell variation was monitored by recovering samples and pictures, respectively, every 90 min.

For the dilution experiments the growth medium mas diluted with Milli-Q water until the desired concentration was achieved. In the experiments using bovine serum albumin (BSA) a solution of 1% of BSA (Sigma Aldrich, A2153) in phosphate buffer saline (PBS) and injected into the channel at 0.5 μL/min for 5 min before inserting the cells.

For the pH experiments a citric acid-phosphate buffer was prepared covering several pH's. The composition of this buffer can be found in Table 1.

Table 1: Different Buffer compositions used.

pH	Acetic Acid (%)	Sodium Phosphate (%)
5.23	50.7	49.3
6.20	37.9	62.1
7.12	17.7	82.3
8.00	95.8	4.2

2.4. Longevity assay

After finishing the optimization of the fermentation conditions for the microbioreactor perfusion assays were performed until the structure stopped working, this could be achieved due to the reactor clogging due to cell buildup, cell death or a cease in production. This experiment was performed with the same setup as the previous perfusion experiments, with the only difference being the use of a 2.5 mL syringe.

2.5. Macro Scale Fermentation Assays

For the batch fermentation a 250 mL shake flask was filled with 100 mL of non-diluted growth medium with the pH adjusted to 4.5 using the citric acid buffer and than seeded with 10 mL of a concentrated cell suspension. The flask was kept in an orbital shaker at 30 C and 250 RPM, with 150 μL samples retrieved periodically, inside a sterile laminar flow chamber. The optical density of these samples were measured and the invertase activity was measured. This experiment was performed for 53 hours.

The continuous fermentation was carried out in an improvised setup which consisted in a 1L Schott flask connected to a filtered air compressor. This large flask was filled with the same culture medium used in the batch fermentation and was connected to a smaller vessel of 25 mL through tubing and a peristaltic pump (Watson Marlow). The smaller vessel is where the fermentation was taking place and was connected to an outlet tube which used the same pump and also to a thermal bath (LAUDA, Eco-silver) in order to maintain a temperature of 30 C. The samples retrieved were treated in the same fashion as the batch fermentation.

2.6. Invertase Activity Measurements

Initially the DNS method adapted from Millers work [14] was used, but due to the high sample volumes required, the DNS method didnt allow for

a precise analysis of invertase activity. So a second protocol was developed that used the glucose measuring capabilities of a personal glucose meter (PGM). The device used was a Glucocard ™ MX by A.MENARIN diagnostics, it's main features are summarized in Table 2

Table 2: Glucocard ™ MX specifications.

Glucocard ™ MX	
Sample Volume (μL)	0.3
Measurement Interval (mg/dL)	10-600
Test Duration (s)	5

The protocol developed consisted in diluting 1 μL of sample in 4 μL of a 50 mg/L sucrose solution. This mixture was then maintained at 52 C for 15 min so the sucrose could be cleaved. After this, samples were immediately measured using the PGM or dipped in boiling water for around 3 min depending on the amount of samples for measurement. This last step is to exclude any false values due to the samples waiting before measurements, by raising the temperature to 100 C, the enzymes were denatured and the reaction stopped, allowing a more precise result.

By obtaining the amount of sugars produced the activity was calculated using Equation 7

$$Act = \frac{C_{measured} \times F_{Correction} \times F_{Dilution}}{180.36 \times t_{reaction} \times 100} \quad (7)$$

2.7. Cell Counting

To monitor cell variation, the image analysis software named Image J was used. The main idea behind this process was to calculate the area occupied by the cells and divide it by the average cell area to give an estimate of the number of cells present at any given moment. To do this in a systematic and rapid manner a macro was developed.

2.8. Microchromatography

Dietylaminoetyl-sepharose (DEAE-sepharose GE Health Care) beads were packed into a channel, this structure has 2 heights, a chamber area were the beads are inserted into with 100 μm and also a thinner channel with 20 μm to allow liquid to flow without dragging the beads out of the channel.

The beads were mixed with a solution of PEG 8000 33% in phosphate buffer saline (PBS) at a ratio of 20 μL to 100 μL, then 50 μL were inserted into a pipette tip which was then inserted into the larger whole of the microchannel. The beads were then pulled into the channel using a syringe pump with a pulling function at a flow rate of 15 μL/min. To remove the viscous PEG, 100 μL of PBS was used in the same fashion as the beads. After washing the beads the pipette tip was removed and spiked invertase solutions were pushed into the channel through the larger inlet, with the resulting flow through solution being recovered for activity measurements. The final step was to elute the captured invertase using a 500 mM NaCl buffer solution, pH=7.5, fractions of this elution buffer were recovered and analyzed.

3. Results

3.1. Reactor Design

Four reactor designs were developed during this work, but only one was chosen to further optimize which can be found in Figure 1.

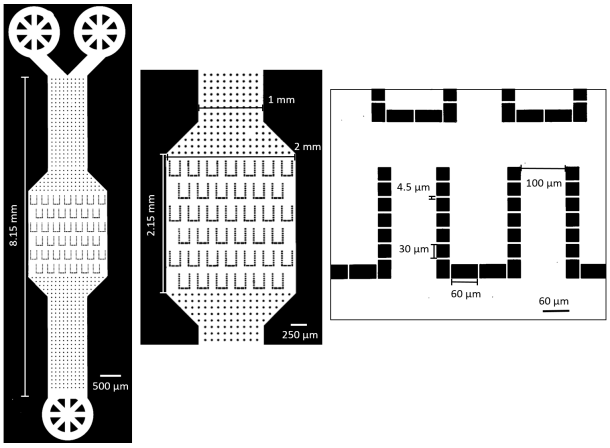

Figure 1: Detailed view of the optimal reactor geometry with the respective dimensions.

With these dimensions and a height of $9\mu m$, the volume of the reactor was calculated to be V= 45.2 nL. This design proved to have both the highest and most reproducible production conditions.

3.2. Flow rate Optimization

The first parameter considered was the flow rate of the device to see how the shear stress τ and medium renovation impacted cell growth and invertase production. The results obtained for these experiments can be seen in Figure 2.

By taking into consideration the flow rate (FR) the total production (TP) for the 9h of experimentation can be calculated using Equation 8, Act_i is the activity at a given time point (t_i).

$$TP = FR \times \sum \left[(t_i - t_{i-1}) \times \frac{Act_i + Act_{i-1}}{2} \right] \quad (8)$$

With this it's concluded that the optimal flow rate is FR=0.8μL/min, achieving a total production of 182.5x10^{-3} U and a specific growth rate of 0.191 h^{-1}.

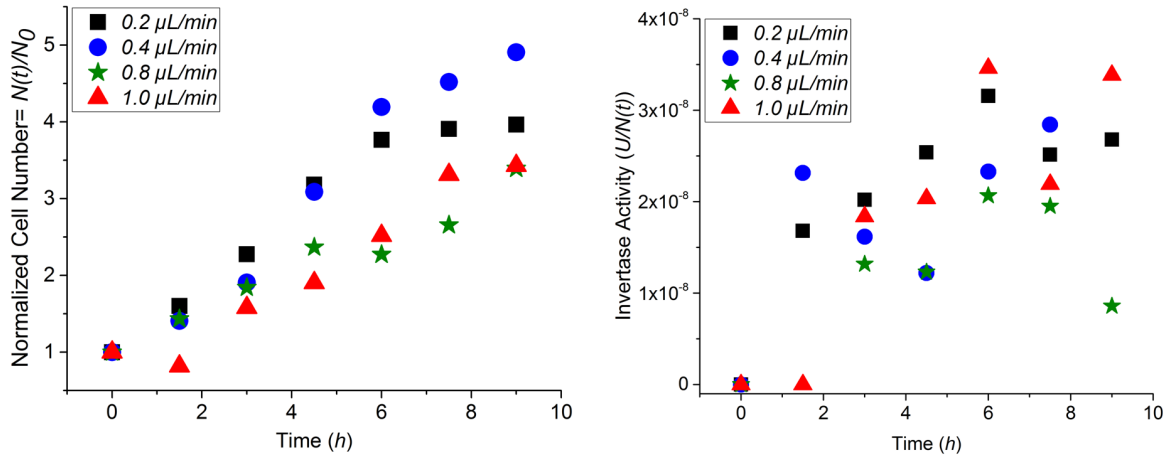

Figure 2: Results obtained from the flow rate studies. On the left the normalized cell growth is presented while on the right invertase activity per cell is shown.

With this result the Reynolds number (Re) and τ are calculated, using Equation 1 and Equation 2. The residence time of the reactor is also calculated obtaining the value of TR= 3.43s.

The results obtain indicate that laminar flow is present (Re<10^5) and as expected the shear stress is extremely low when compared to macroscale vessels. [15]

3.3. Medium Dilution

Next set of experiments consisted in diluting the medium used to test if the lack of nutrients would increase competition between cells and overall production. Results can be seen in Figure 3.

By analyzing the results it's concluded that the optimal condition is diluting the medium 500x, in which an average production of 2.45x10^{-7} U and a specific growth rate of 0.106 h^{-1} were obtained.

3.4. Impact of BSA

To understand if the fact that the cells were in contact with a non-biological substrate had any impact on the growth and invertase production, the surface was coated with BSA and the results can be found in Figure 4.

The average productivity without BSA coating the surface was higher (2.87x10^{-7} U) and growth was lower (k=0.226 h^{-1}) than when using BSA. The growth is explained by the fact that being BSA a biomolecule it's expected that cells would grow better when in contact with it when compared to growing on top of a polymer. To explain the production levels, it's thought that there may be some unspecific binding of invertase to BSA preventing it's elution and generating a pseudo immobilized enzyme reactor which would reduce the need for more invertase production.

3.5. pH Studies

Up until this point the pH of the culture medium was ignored and medium was being prepared with Milli-Q water. A range of pH's are then tested, with results presented in Figure 5.

The best pH to maintain cell number and increase production is pH= 6.20 for which a average production of 2.67x10^{-7} U and a specific growth rate of k= 0.06 h^{-1}. It's important to say that the changes in pH didn't affect production in a significant manner excluding for the value of pH= 8.0, however the presence of buffer had severe impacts on the growth rate of the cells. This is thought to be caused by the presence of citric acid which as been proven to inhibit growth in yeast. [16]

3.6. Longevity Assay

After optimization an experiment was performed in which the perfusion would only stop when production ceased or due to external issues. This experiment ran for 32h, being forced to stop due to the presence of a contamination. The results for cell growth and production can be seen in Figure 6. The results suggest that the reactor is in a steady-state of operation by displaying a stable cell number and production profile throughout the majority of the experiment. The initial variations are due to the existence of a lot of cells in non trapping areas which are than washed out of the reactor.

3.7. Macroscale Comparison

To further validate the system developed, macroscale assays were performed, one batch and one continuous fermentation. The results for cell growth and invertase production are presented in Figure 7.

The growth and production profiles obtained are

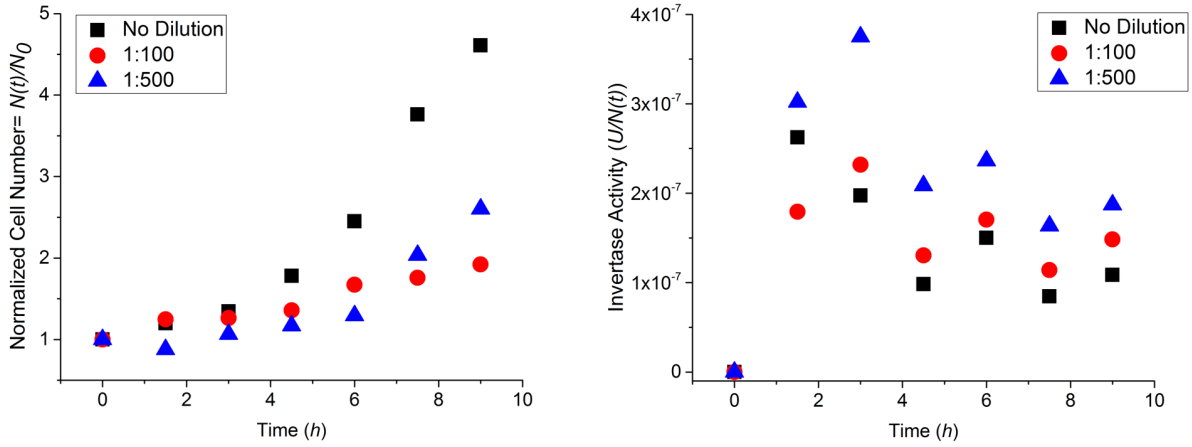

Figure 3: Results obtained from the dilution studies. On the left the normalized cell growth is presented while on the right invertase activity per cell is shown.

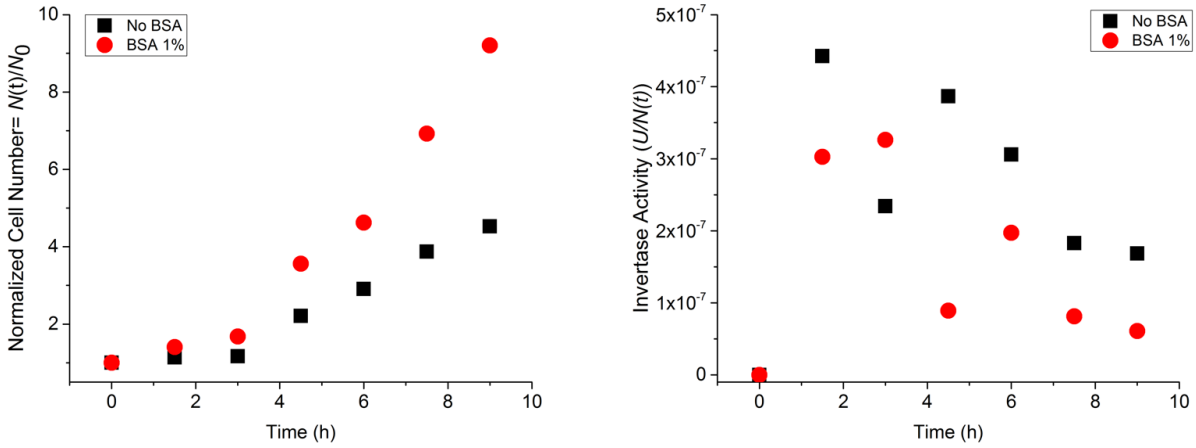

Figure 4: Results obtained from the BSA studies. On the left the normalized cell growth is presented while on the right invertase activity per cell is shown.

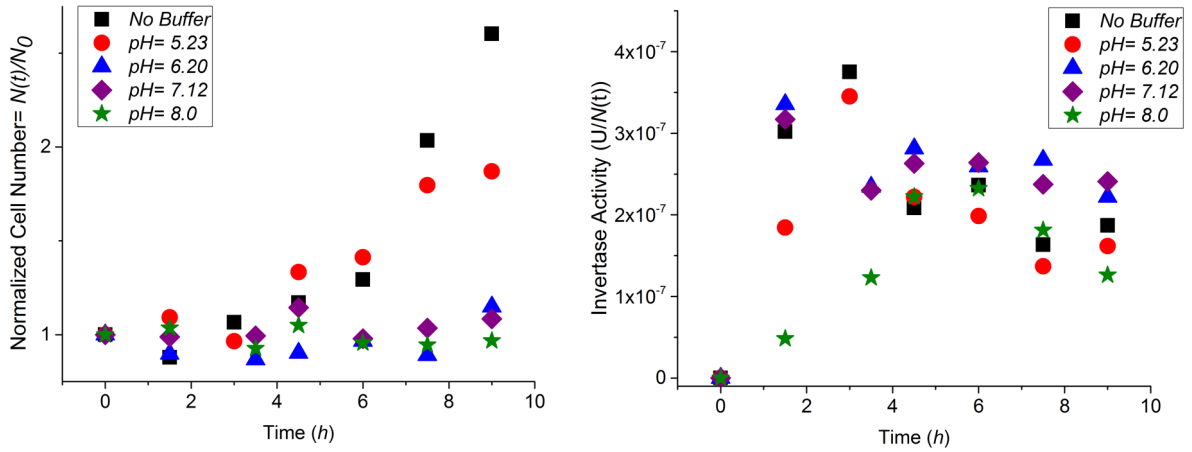

Figure 5: Results obtained from the pH studies. On the left the normalized cell growth is presented while on the right invertase activity per cell is shown.

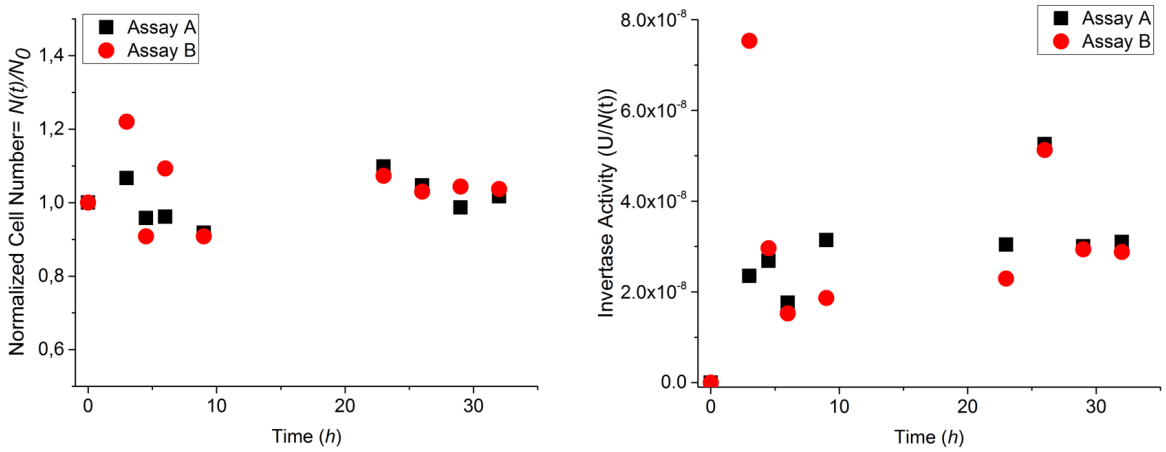

Figure 6: Cell growth in the microreactors during the 32h assay. Cell fluctuation in the beginning can be explained by cells in non-trapping areas being washed away.

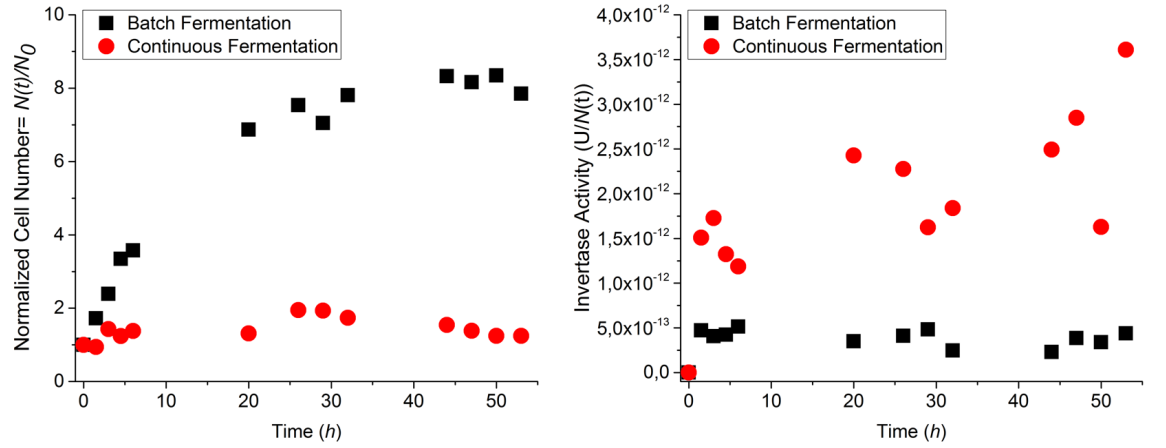

Figure 7: Cell growth results obtained with the macroscale fermentations, at t=3h the flow rate of the continuous vessel was increased and at t=20h the rotation of the agitator was also increased

Table 3: Comparison between the macro and microscale modes of operation, depending on how the analysis is conducted different conclusions are obtained.

	Batch	Continuous	Microfluidic (Average)
Total Invertase Produced, 32h (U)	1215.83	2150.84	0.233
Medium Used (mL)	110	64.83	1.54
Production (U/mL)	11.05	33.18	0.15
Production (U/cell)	$4.81\text{x}10^{-13}$	$1.55\text{x}10^{-12}$	$2.86\text{x}10^{-8}$
Sucrose Used (mg)	2200	1296.54	0.0614
Production (U/mg sucrose)	0.533	1.66	3.79
N of Microreactors to equal hourly production	5226	9244	-
Residence Time	-	19.15h	3.43s
Re	$2.91\text{x}10^{4}$	282	2.11
Shear Stress (N/m^2)	<1.072	$<2.31\text{x}10^{-2}$	$<6.59\text{x}10^{-4}$
Pe_L	$4.47\text{x}10^{7}$	$4.33\text{x}10^{5}$	$3.62\text{x}10^{3}$
Phase N	3.97	-	-
Fr_a	$2.01\text{x}10^{6}$	-	-

what is expected for these modes of operation. In the case of the continuous fermentation the residence time of 19.15h is what is thought to be responsible for the production cycles registered.

Both scales were compared in terms of production in order to assess which would be the best mode to operate in, these results are summarized in Table 3

In terms of amount of medium necessary the macroscale is much more productive than the microscale, however in terms of nutrients used and cell productivity, the microreactor is a better bet.

To better understand the systems the Re and τ were calculated for the macroscale and also the Pe_L, Ph and the Fr_a were calculated. The results obtained are also summarized in Table 3.

From this is concluded that only the continuous fermentation was in a state of turbulence and was not limited by mass and heat transport as in the case of the continuous run. The microfluidic structure has a very short residence time, when compared with the continuous fermentation, when this is and the low productivity per volume of culture medium it's easy to conclude that the product is extremely diluted in this vessel. All 3 reactors are in a situation where advection is more important than diffusion in terms of mass transport due to the high values of Pe_L.

3.8. Microchromatography

In an attempt to include a DSP step in the same chip as the production a microchromatography column was attempted and despite the protocol developed being proved to be efficient at capturing and eluting the protein of interest the final inclusion of the process in the same chip failed due to structural problems which required more time to solve.

4. Conclusions

A total of 4 reactor designs were created, all of them possessing the capability to produce invertase in a continuous fashion, however one design stood out and was optimized in order to leave the cells in a state of limited cell division while maximizing production. In terms of cell growth, the specific growth rate was reduced 3.7 times from 0.221 h^{-1} to 0.06 h^{-1} while production increased 4.68 times from $182.5\text{x}10^{-3}U$ to $854.8\text{x}10^{-3}U$, during a 9 hour production run.

The system was maintained working for 32h, in which a steady-state of operation was rapidly achieved. The microfluidic device proved to be very robust in terms of production maintaining a very stable output of the product of interest.

While the traditional means of production proved to be economically more viable, the micro reactor developed proved to be able to better use the cells for production having production per cell levels of several orders of magnitude higher than in the macroscale. Nonetheless it can not be forgotten that those vessels weren't optimized during this work so the comparison is merely an indication of how these 2 scales fair against each other. The most ideal way to compare the structures would be to perform a perfusion fermentation, but the equipment required wasn't available at the time. It's important to say that despite being created with the intention to serve as a production platform it can also be used to screen and optimize conditions for macroscale assays.

The final part of this work was to include a step of purification into the production chip in order to be one step closer to a future "plant-on-a-chip". Sadly this objective wasn't achieved, however it was proved to be possible through experiments in a con-

trolled environment.

In order to take this device to the next level, the main focus of the work would be to first optimize the chromatography step in order to maximize the capture of the invertase produced and than focus on the possibility of scaling-out the device, in other words create a platform where several of these structures would be able to function in parallel or even in series.

If medium consumption is as low as it is thought to be several approaches to reduce production cost from that perspective can be used, such as diluting even further the medium used, partially recirculating the medium into the device or have several/longer reactors in series where the one reactor would be fed by the previous one until exhausting the nutrients completely or until a high concentration of inhibitory metabolites would be present in the medium.

Once the operational conditions had been thoroughly optimized the addition of instrumentation would be crucial, such as sensors to control the number and state of the cells in the traps, oxygen and pH sensors to fully monitor the conditions at play inside the vessel and an automated way to elute the captured product of interest (assuming chromatography would remain as the main DSP step) without stopping the production part. A way to integrate several pumping lines for the medium would also be crucial in order to turn this into a more economical device.

As a final remark, it's important to state that similar systems to this can be used for different types of cell lines and for different types of products of interest, as long as all the necessary changes are made.

Acknowledgements
The author would like to thank everyone at INESC-MN and IBB, with a special thanks to João Tiago Fernandes and Ruben Soares. The author also wishes to thank the advisors of the project, Professors João Pedro Conde and Pedro Fernandes.

References

[1] Mads Gruenberg, Clemens Posten, and Markus Rueckel. Optimization of fermentation processes, October 30 2001. US Patent App. 10/016,616.

[2] Jonathan I Betts and Frank Baganz. Miniature bioreactors: current practices and future opportunities. *Microbial Cell Factories*, 5(1):21, 2006.

[3] Marco PC Marques, Filipe Carvalho, Salomé Magalhães, Joaquim MS Cabral, and Pedro Fernandes. Screening for suitable solvents as substrate carriers for the microbial side-chain cleavage of sitosterol using microtitre plates. *Process Biochemistry*, 44(5):556–561, 2009.

[4] Sunil Chhatre and Nigel J Titchener-Hooker. Review: Microscale methods for high-throughput chromatography development in the pharmaceutical industry. *Journal of chemical technology and biotechnology*, 84(7):927–940, 2009.

[5] Lucía D Garza-García, Leydi M Carrillo-Cocom, Diana Araiz-Hernández, Pedro Soto-Vázquez, Julián López-Meza, Eduardo J Tapia-Mejía, Sergio Camacho-León, Erika García-López, Ciro A Rodríguez-González, and Mario M Alvarez. A biopharmaceutical plant on a chip: continuous micro-devices for the production of monoclonal antibodies. *Lab on a Chip*, 13(7):1243–1246, 2013.

[6] Lucía D Garza-García, Erika García-López, Sergio Camacho-León, María del Refugio Rocha-Pizaña, Felipe López-Pacheco, Julián López-Meza, Diana Araiz-Hernández, Eduardo J Tapia-Mejía, Grissel Trujillo-de Santiago, Ciro A Rodríguez-González, et al. Continuous flow micro-bioreactors for the production of biopharmaceuticals: the effect of geometry, surface texture, and flow rate. *Lab on a Chip*, 14(7):1320–1329, 2014.

[7] Jinwen Zhou, Amanda Vera Ellis, and Nicolas Hans Voelcker. Recent developments in pdms surface modification for microfluidic devices. *Electrophoresis*, 31(1):2–16, 2010.

[8] Jinwen Zhou, Dmitriy A Khodakov, Amanda V Ellis, and Nicolas H Voelcker. Surface modification for pdms-based microfluidic devices. *Electrophoresis*, 33(1):89–104, 2012.

[9] Horst Feldmann. *Yeast: molecular and cell biology*. John Wiley & Sons, 2011.

[10] Uroš Andjelković, Srdjan Pićurić, and Zoran Vujčić. Purification and characterisation of saccharomyces cerevisiae external invertase isoforms. *Food chemistry*, 120(3):799–804, 2010.

[11] Emine Akardere, Büşra Özer, Evran Bıçak Çelem, and Seçil Önal. Three-phase partitioning of invertase from baker's yeast. *Separation and Purification Technology*, 72(3):335–339, 2010.

[12] Vânia C Pinto, Paulo J Sousa, Vanessa F Cardoso, and Graça Minas. Optimized su-8 processing for low-cost microstructures fabrication

without cleanroom facilities. *Micromachines*, 5(3):738–755, 2014.

[13] Gonalo Forjaz. Towards the continuous production of bacterial metabolites in microfluidic environment. Master's thesis, Instituto Superior Tcnico, 2015.

[14] Gail Lorenz Miller. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 31(3):426–428, 1959.

[15] YS Cao and GJ Alaerts. Influence of reactor type and shear stress on aerobic biofilm morphology, population and kinetics. *Water Research*, 29(1):107–118, 1995.

[16] Marina Karelina Nielsen and Nils Arneborg. The effect of citric acid and ph on growth and metabolism of anaerobic saccharomyces cerevisiae and zygosaccharomyces bailii cultures. *Food microbiology*, 24(1):101–105, 2007.