TÉCNICO LISBOA

Thesis to obtain the Master of Science Degree in

Biological Engineering

June 2015

Towards the continuous production of bacterial metabolites in microfluidic environment

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ARTICLE INFO

Keywords:

Microreactor
Invertase
Saccharomyces cerevisiae
Optimization

ABSTRACT

In the present work, a continuous microreactor was designed to study the effect of miniaturization on bioreactors for the production of invertase from S. cerevisiae MM01 in a microfluidic environment. In order to simplify the analysis and considering typical production strategies at laboratory scale, 250 ml baffled flasks and a 2.5 l batch bioreactor were used as bench-scale models for the perfusion microreactor. In order to improve invertase production, the effect of different compounds in the culture media such as carbon source, nitrogen source (organic and inorganic), metal ion, polyether, amino acid and buffer on invertase production and cell growth were screened at bench-scale before scaling-down the process. Maximum invertase activity for baffled flask reactor was registered after 32 hours of process time (1.40 U/ml), immediately before stationary phase, in the non-buffered optimized medium (20 g/l sucrose, 20 g/l yeast extract and 7.5 g/l bacto peptone) at 30 ºC, 200 rpm and with an initial pH of 6.0. After microfabrication the optimized medium was tested for dilution factors of 1, 10, 100 and 1000. Maximum invertase activity was registered after perfusion of the most diluted medium for 54 hours (7.94 U/ml) at pH 6.0 and 30 ºC.

1. Introduction

Invertase (EC. 3.2.1.26) is an enzyme with the systematic name β-D-fructofuranosidase that promotes the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranoside [1]. There are several microbial sources of invertase, but yeast invertases are more common in the food industry. Its preferred substrate is sucrose but invertase is also able to catalyse the hydrolysis of other carbon sources such as raffinose, stachyose and inulin [2]. Through catalysis of the hydrolysis of sucrose to D-glucose and D-fructose (Eq. 1), a mixture called inverted sugar syrup is obtained, which is sweeter and does not crystallize as easily as sucrose [3].

$$\text{Sucrose} + H_2O \xrightarrow[\text{Invertase}]{} \text{D-Glucose} + \text{D-Fructose} \quad (1)$$

The *S. cerevisiae* yeast cell is a rich source of both intracellular and extracellular invertase. Extracellular invertase exists in the form of a glycosylated periplasmic protein with nine or ten N-glycosidically linked oligosaccharides which correspond to the de-repressed form of the enzyme and is regulated by catabolic repression. Intracellular invertase exists in the form of a cytosolic non-glycosylated protein and corresponds to the repressed form of invertase. High concentrations of glucose in the culture medium repress production of the enzyme, whereas the use of sucrose or raffinose as carbon source allows de-repression of invertase synthesis [2].

For aerobic reactors, *S. cerevisiae* production of free invertase usually occurs in the presence of sucrose as a carbon source for an optimum temperature and pH level of 30 ºC and 6.0 respectively [4–6]. Contrary to most enzymes, invertase exhibits high activity over a broad range of acid pH (3.5 - 6.0), with an optimum temperature and pH level at 50 ºC and 4.5 [7–9].

The prospect of manipulating small amounts of fluids moving in narrow channels (microfluidics) has attracted the attention of researchers from different fields. Microfabricated bioreactor technology is anticipated to offer numerous advantages. Properties of microfluidic systems offer an improved heat and mass transfer that allow an increased control of reaction conditions that result in higher yields than those obtained with industrial or bench-scale reactors [10]. By scaling-down macroscopic systems and taking advantage of the possibility of massive parallel processing, some microfluidic systems enable high-throughput biological experiments. Specific effects of laminar

flow at the micron scale enable spatial control of liquid composition at subcellular resolution, fast media and temperature change, and single cell handling and analysis. Microfluidic technology allows studies of cell behaviour from single to multicellular organism level with precise and localized application of experimental conditions, otherwise unreachable using macroscopic tools [11]. The current most popular technology for the fabrication of microfluidic devices for cell culture applications is based on the soft-lithography of Polydimethylsiloxane (PDMS). The ability to fabricate microfluidic devices in a few hours, together with a few intrinsic properties such as biocompatibility, low-cost, transparency, low autofluorescence, and the possibility to mould with a high resolution of a few nanometres, remains very attractive for research teams [12–14].

Soluble enzyme microreactors take advantage of the physical properties of non-aqueous solvents together with the manipulation of microfluidic channel configurations. One example are enzymatic reactors with liquid-liquid parallel flow that can offer the possibility of separating phases at the end of the channel. This could be achieved by chemical treatment of one of the exits to become more hydrophilic or hydrophobic, or by changes in the flow rate ratio to obtain a stable interface at the outlet. Another example are enzymatic reactors with non-parallel liquid-liquid flow that can offer additional improvement of mass transfer between phases. This could be achieved by internal vortex flow caused by shearing motion, or enhanced surface area to volume ratio by contacting immiscible fluids in the form of segmented or droplet flow [15].

In microfluidic biocatalytic processes, enzymes can be immobilized for different purposes, but in some cases, soluble enzyme production from cells is preferred since enzyme extraction and purification can be avoided, and cells can have a longer lifetime than enzymes. Cell immobilization is critical for applications that focus on the genetic, physiological and biochemical aspect of an individual cell. Other processes such as biotransformations, also rely on immobilization for product recovery, cell stabilization, protection against shear forces, among other favourable physiological alterations [16]. Cell immobilization techniques used in microfluidic systems include the use of dam structures to hydrodinamicaly trap cells, which is particularly suitable for the development of biosensors [17].

In this work, a continuous microreactor was designed to produce free extracellular invertase from immobilization of *S. cerevisiae* yeast cells. As proof of concept, the cell culture medium was optimized in microtiter plates, followed by scale-up to baffled flask and aerated batch bioreactor. Once cell culture medium was optimized and invertase activity profiles were determined, the bioreactor was miniaturized to a PDMS perfusion microreactor.

Nomenclature

Abs	Spectrophotometer absorbance
ANS	3-amino,5-nitrosalicylic acid
BP	Bacto Peptone
CRS	Concentration of reducing sugars
DF	Dilution factor
DNS	3,5-dinitrosalicylic acid
DO	Dissolved oxygen
DWL	Direct Write Laser
EC	Enzyme Commission number
ITO	Indium tin oxide
k_La	Volumetric oxygen transfer coefficient
$MW_{glucose/fructose}$	Glucose or Fructose molecular weight
OD	Optical density
OTR_{max}	Maximum oxygen transfer rate
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PR	Photoresist
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
Q	Air flow rate
rpm	Revolutions per minute
U	Enzyme unit defined in this study as the amount of invertase that catalyses the production of 1 µmol of glucose (or fructose) per minute
V_L	Filling volume
$V_{reaction}$	Reaction volume
V_{sample}	Sample volume
vvm	Volume of gas per culture volume and minute
W	Wetting tension between liquid and microtiter plate material
YE	Yeast Extract

2. Materials and methods

2.1. Materials

Agar for yeast cultures was acquired from José M. Vaz Pereira S.A.; D-Glucose anhydrous, sucrose, bacto peptone, and sodium citrate tribasic dihydrate (purity ≥ 99 %) were purchased from Fisher Scientific. Yeast nitrogen base was obtained from Difco. Yeast extract powder was acquired from HiMedia Laboratories. Urea (99.5% purity), calcium chloride (granular 5-15 mm) and citric acid (purity ≥ 99 %) were purchased from Merck. Ammonium chloride was obtained from CHEM-LAB; PEG 3350 was acquired from SYNCOM; and L-Histidine (purity ≥ 99 %) was purchased from Sigma-Aldrich.

2.2. Cell density determination

Bench-scale experiment samples were immediately measured after collection for optical density measurement at 600 nm with the aid of a UV-VIS spectrophotometer (Hitachi) for biomass concentration quantification. In microfluidic experiments, cell count was estimated by calculating the cell cluster area and individual cell area with the aid of an optical microscope (MD600, AmScope), its image capture software (AmScope MD600, Scopetek) and an image processing program (ImageJ).

In this work, cell dry weight was not determined in the available time.

2.3. Sucrose hydrolysis

Samples collected for enzyme activity were immediately stored at -20 ºC for a maximum period of 24 h (bench-scale experiments) and 72 h (microfluidic experiments). Protocol for sucrose hydrolysis and determination by DNS colorimetric assay was based on the method developed by Miller, but adapted to 2.2 ml 96-well polypropylene microtiter plates (System Duetz, EnzyScreen) [18]. 250 µl sample (from bench-scale experiments) with no dilution or 220 µl sample with a dilution factor of 11 (20 µl sample and 200 µl sterilized water) from microfluidic experiments were added to 100 g/l sucrose with acetate buffer (pH 4.5) in a 1:1 ratio, in order to reach a final concentration of 50 g/l sucrose. Catalysis of the hydrolysis reaction was triggered at the invertase optimum temperature (50 ºC) in a thermostatic bath for 1 h, in order to convert sucrose to the reducing sugars. The activity unit in this study was defined as the amount of invertase capable of catalysing the production of 1 µmol of glucose or fructose per minute.

2.4. DNS colorimetric assay

Quantification of glucose and fructose was tested by oxidizing the free carbonyl group in the reducing sugars, which in turn reduce 3,5-dinitrosalicylic acid (DNS) to 3-amino,5-nitrosalicylic acid (ANS) under alkaline conditions at 90ºC. The product is a reddish-brown colour which absorbs light at 540 nm.

For bench-scale samples, 100 µl of hydrolysed reducing sugars were taken from the previous reaction with a dilution factor of 5 (20 µl sample and 80 µl sterilized water) and immediately added to DNS in 1:1 ratio to stop the hydrolysis reaction, due to the high alkalinity of DNS. For microreactor samples, a dilution factor of 1 (100 µl of hydrolysed reducing sugars and 100 µl of DNS) was used instead. The mixture was then boiled at 100ºC for 5 min to trigger the redox reaction and for the ANS to fully develop a reddish-brown colour. Afterwards, 500 µl sterilized water was added to the solution, from which 200 µl were taken to a 250 µl PVC 96-well microtiter plate (Corning) for absorbance measure at 540 nm in a microplate reader (SpectraMax Plus 384, Molecular Devices). A 50 g/l sucrose blank in sterilized water (without enzyme) was used as a reference sample in order to apply the same protocol for both bench-scale and microfluidic samples due to the low sample volume of the latter.

2.5. Invertase activity calculation

After the enzymatic assay, absorbance measured in the microplate reader was converted to concentration of produced reducing sugars with the aid of a calibration regression line previously calculated in the lab:

$$CRS = (0.5267 \times Abs - 0.0362) \times DF \quad (2)$$

where CRS [g/l] is the concentration of reducing sugars produced per hour, Abs is the spectrophotometer absorbance at 540 nm, and DF is the dilution factor used for the sample. CRS was then converted to invertase activity [U/ml]:

$$\text{Invertase activity} = \frac{CRS \times V_{reaction} \times 10^6}{MW_{glucose/fructose} \times V_{sample} \times 1000 \times 60} \quad (3)$$

where $V_{reaction}$ [ml] is the reaction volume containing the sample volume V_{sample} [ml] together with the 100 g/l sucrose volume added, and $MW_{glucose/fructose}$ [g/mol] is the molecular weight of glucose or fructose (=180.16 g/l). For bench-scale experiments, relative invertase activity [$U/g_{wet\ cell}$] was calculated based on the values obtained for invertase activity and biomass concentration [$g_{wet\ cell}/l$]:

$$\text{Relative activity}_{bench\text{-}scale} = \frac{\text{Invertase activity}}{\text{Biomass concentration}} \times 1000 \quad (4)$$

For microreactors, an alternative method using cell count was formulated for relative invertase activity [$U/g_{wet\ cell}$]. Calculation is based on the values obtained for invertase activity [U/ml], estimated number of cells inside the microchannel, sample volume (V_{sample}) and average *S. cerevisiae* wet cell weight from the literature (= 7.9 x 10^{-11} g/cell) [19]:

$$\text{Relative activity}_{microreactor} = \frac{\text{Activity} \times V_{sample}}{\text{Nº cells} \times \text{Average wet cell weight}} \quad (5)$$

Specific invertase activity ($U/mg_{protein}$) was not calculated in this work due to several problems with the Bradford protein assay.

2.6. Microtiter plate screening

Yeast cell culture medium was optimized in a squared 11 ml 24-well polypropylene microtiter plate with a sandwich cover (System Duetz, EnzyScreen), in order to maximize invertase activity and minimize biomass concentration. Control medium was composed of 20 g/l sucrose, 3 g/l yeast extract and 5 g/l bacto peptone initially adjusted to pH 6.0 without a buffer solution. Separate addition of the following components and/or concentrations were tested: 20 g/l glucose (replaced sucrose); 10 g/l yeast extract; urea (2 and 5 g/l); 0.5 g/l ammonium chloride; 0.2 g/l calcium chloride; 2 g/l PEG 3350; L-histidine (0.2 and 2 g/l); and 0.1 M citrate buffer (pH 6.0). The cell culture was incubated in a 25 mm orbital shaker (Agitorb, Aralab) with a working volume of 2 ml at 30 ºC and 200 rpm for 24 hours.

A second optimization was also performed to determine the optimum yeast extract/bacto peptone concentration ratio in order to maximize invertase activity and minimize biomass concentration. In order to achieve it, 15 different mixtures with concentrations of 2.5; 5; 10; 15; and 20 g/l yeast extract were added to concentrations of 2.5; 5 and 7.5 g/l bacto peptone, together with 20 g/l sucrose, and incubated in the same conditions.

Microtiter plate samples were collected at the end and measured for invertase activity and biomass concentration.

2.7. Baffled flask fermentation

After optimization, fermentations in 250 ml flasks with 4 baffles (Duran) were carried out in three different media: the optimized Medium A (20 g/l sucrose, 20 g/l yeast extract and 7.5 g/l bacto peptone); and the previously screened Medium B (20 g/l sucrose, 10 g/l yeast extract and 5 g/l bacto peptone) and Medium C (20 g/l sucrose, 2.5 g/l yeast extract and 2.5 g/l bacto peptone). The pH level was initially adjusted to 6.0 without a buffer solution before incubation in a 25 mm orbital shaker with a working volume of 50 ml at 30 ºC and 200 rpm for 86 h.

Baffled flask samples were collected in order to monitor biomass concentration, invertase activity and pH level.

2.8. Batch bioreactor fermentation

A 2.5 l aerated and mechanically agitated batch bioreactor (Minifors, Infors HT) with a working volume of 1.2 l containing the optimized Medium A initially adjusted to pH 6.0, was inoculated by a reactor seed with an inoculum volume of 10% (v/v) in relation to the final medium fermentation volume. The reactor seed was incubated for 12 h in a 25 mm orbital shaker at 30 ºC and 200 rpm before transfer.

The pH value of the bioreactor was set to be automatically controlled to keep the values between 4.0 and 6.0 to avoid cell growth inhibition. The pH control was done through 2 M H2SO4 and 2 M NaOH addition. Automatic control of temperature and oxygen levels was performed at 30ºC with a minimum dissolved oxygen tension of 30% of air saturation, achieved by aeration at an initial air flow rate of 1.6 vvm and an initial agitation rate of 300 rpm. Due to a malfunction of the DO sensor, agitation rate and air flow rate were manually adjusted every time the cells showed heavy signs of oxygen limitation. As a result, samples for biomass concentration and invertase activity were taken at irregular intervals.

2.9. Microreactor design and fabrication

A single layer microchannel (8 mm x 200 µm x 9.5 µm) with squared PDMS traps with 5 µm gaps for cell retention was designed with the help of AutoCAD software (Autodesk) and SolidWorks software (Dassault Systèmes) and its features were exposed with the aid of a Direct Write Laser (Lasarray) onto a glass hard mask with a 0.2 µm Al and a 1.5 µm positive PR (photoresist) layer. The SU-8 mould was fabricated by spin coater deposition of 9.5 µm of negative PR SU-8 2015 (Microchem) and UV exposure of the hard mask. PDMS with curing agent in a 10:1 (w/w) ratio was poured on the mould and cured for 70 ºC for 1 h 30 min. The device was closed with a 500 µm PDMS film created by spin coater deposition, and sealed by corona discharge treatment.

2.10. Microreactor fermentation

Once fabricated, the device was connected to sterilized 1 ml syringes with Teflon® polytetrafluoroethylene (PTFE) tubing and mounted on syringe pumps (New Era Pump Systems, Inc.). Temperature control was maintained via a heated glass plate coated with ITO (H401-M-FRAME-GLASS, Okolab) at 30 ºC. Cell growth was monitored with an optical microscope (MD600, AmScope) together with its imaging software (AmScope MD600, Scopetek).

Microreactor fermentation was achieved through three stages: a priming stage, a cell loading stage, and a medium perfusion stage. First, the microchannel was primed with filtered 1% BSA (10 mg/ml) at a flow rate of 1 µl/min, in order to purge other residual liquids or gases, suppress enzyme adhesion to PDMS surfaces, remove possible contaminants and debris, and eliminate any dead volumes that might affect subsequent stages. Second, the cell loading stage contained yeast cells previously cultivated for 12 h that were loaded into the microchannel at a flow rate of 0.5 µl/min for brief moments. Finally, the medium perfusion phase used the optimized Medium A with 0.1M phosphate buffer (pH 6.0), flowed at 0.5 µl/min with a dilution rate of 1, 10, 100 and 1000. Since some cells were retained in the inlet or were not detained by the PDMS traps, sampling and imaging started only once all cells were inside the trap area, and only once a minimal amount of cells were spotted at the inlet.

Samples were collected for invertase activity measure, and cell growth was monitored by imaging software.

3. Results and discussion

3.1. Microtiter plate screening

In order to avoid microchannel clogging and improve medium diffusion to the cells after miniaturization, due to rapid cell proliferation, the growth medium was optimized for high invertase activity and low biomass concentration (Table 1).

The level of extracellular invertase activity is regulated by glucose repression. Invertase synthesis is repressed with the presence of glucose and de-repressed with the presence of sucrose in the medium [2,20]. For this reason, sucrose utilization as a carbon source had a higher invertase activity value than glucose utilization as a carbon source.

For organic nitrogen source, a concentration increase from 3 g/l to 10 g/l of yeast extract doubled biomass concentration and relative invertase activity. It is likely that the amount of organic nitrogen in the control medium was not enough for suitable cell growth. Sucrose metabolism shows a specific physiological response to the presence of nitrogen source, which would also explain the rise in relative invertase activity [21].

Urea addition in optimum concentration can induce urease in sufficient amount to promote invertase production, but in high concentrations it has a denaturing effect towards proteins. Relative invertase activity showed little change for a concentration of 2 g/l

and was reduced to half for a concentration of 5 g/l, probably due to enzyme denaturation [22].

Table 1 – Biomass concentration and relative invertase activity of the first optimization step of the growth medium. The effect of the addition of carbon sources, organic and inorganic nitrogen sources, metal ion, amino acid, polyether, and buffer solution in the growth medium was tested. Control had a composition of 20 g/l sucrose, 3 g/l yeast extract and 5 g/l bacto peptone.

Supplement Concentration (g/l)		Biomass Concentration (g/l)	Relative Invertase Activity (U/g wet cell)
Control		7.85	48.15
Glucose	20	6.82	18.86
Yeast extract	10	15.43	97.71
Urea	2	8.20	47.13
	5	8.42	27.36
NH_4Cl	0.5	8.07	49.80
$CaCl_2$	0.2	5.47	35.87
L-Histidine	0.2	7.65	49.68
	2	6.89	43.09
PEG 3350	2	7.40	59.06
Citrate Buffer	0.1 M (pH 6.0)	8.03	34.73

Ammonium chloride is often used in the food industry (E510) as an additive and as an inorganic source of nitrogen for *S. cerevisiae*. It is known to have a positive effect on invertase activity in low concentrations [23,24]. Addition of 0.5 g/l ammonium chloride however, hardly increased both biomass concentration and relative invertase activity.

Although yeast is capable of synthesizing amino acids from nitrogen sources, addition of specific amino acids is known to promote invertase synthesis. The most important amino acids are glutamic acid, aspartic acid, and asparagine for yeast cell growth, and methionine, alanine and cysteine for invertase activity [24,25]. However, unknown at the time and due to the regular use of L-histidine in yeast cell culture protocols, it was chosen instead. For low concentrations of L-histidine (0.2 g/l), there was hardly any change, while for high concentrations (2 g/l), invertase activity was aggravated. L-histidine has shown to be involved in *S. cerevisiae* cytotoxicity by directly interacting with copper ions and reduce its availability after incorporation into the cells. In this case, for media with low copper content, it is possible that cell growth and invertase activity were hindered in the presence of high concentrations of the amino acid [26].

Calcium chloride in low concentrations has been reported to greatly improve enzyme activity, most likely due to the significant role of inorganic cations in the regulation of invertase in the cell wall [4,24,27]. However, in this study the effect proved to be significantly inhibitory for invertase activity. High concentrations of calcium are reported to strengthen the cell wall by inducing wall shrinkage and making it less extensible, which in turn reduces substrate uptake by the cell. This would affect cell growth and repress invertase synthesis by accumulating hydrolysed glucose outside the cell wall [27]. Another explanation is that the addition of calcium ions promoted flocculation among yeast cells that were not properly dispersed by an agitation of 200 rpm in a 2 ml working volume inside the microtiter plate well. This could also be verified by the significant drop in biomass concentration. Flocculated cells would share the same limited microenvironment, which would limit the availability and access of sucrose molecules to each individual cell. This limited amount of sucrose in the microenvironment would be swiftly hydrolysed to glucose, which would in turn repress invertase synthesis [2,28].

Polyethylene glycol (PEG) is a hydrophilic non-ionic polymer used for many biochemical and industrial applications. By binding weakly to proteins, PEG with a high-molecular weight (6000 and above) or low-molecular weight in high concentrations (e.g. 400 g/mol at 200 g/l) are capable of forming stable complexes with enzymes, even in the presence of ethanol [29,30]. PEG 3350 at 2 g/l showed a slight decrease in biomass concentration and a great increase in relative invertase activity, probably due to the increased enzyme stability. Another explanation could be that PEG precipitated the enzyme and removed it from the cell microenvironment. This would enable sucrose hydrolysis and glucose production away from the cells, which in turn would reduce invertase synthesis repression [2,31].

The importance of a buffer solution in the medium was tested for a 0.1M citrate buffer (pH 6.0). The optimum pH of *S. cerevisiae* for free invertase production is 6.0 and citrate buffer is widely used in several invertase studies with *S. cerevisiae* fermentations due to its wide acid buffer capacity [3,4,24,32]. Still, in the presence of a buffer solution, relative invertase activity dropped significantly. It is possible that for the *S. cerevisiae* MM01 strain, optimum pH for invertase production is lower than what was recorded in the literature. Another possibility is that invertase was more stable for more acidic pH levels in non-buffered medium. Optimal pH for enzyme activity is 4.5, which could have had some influence in invertase stability.

After the first stage of growth medium optimization, results showed that yeast extract had the largest influence in invertase production. Since the combined use of bacto peptone and yeast extract on fermentation medium is widespread and to avoid increasing the complexity of the optimization with the addition of PEG, ratio between yeast extract and bacto peptone was studied instead.

For high concentrations of nitrogen sources, biomass concentration severely decreased, while invertase production slightly increased, which in turn significantly improved individual cell efficiency at producing invertase (Table 2). Although an excess of organic nitrogen is known to inhibit cell growth, results showed a positive contribution in invertase synthesis [33]. It is possible that a low concentration of cells promoted invertase synthesis by reducing glucose production in the microenvironment. Since the increase in activity was not substantial, sucrose concentration

could have been maintained for longer periods of time and preserved de-repression of enzyme production [2,20]. In a reactor with limited amount of substrate, the effect of a rise in invertase production from an increased number of cells would be swiftly outweighed by the repression of the pathway from glucose accumulation. Be that as it may, the effect is contradictory when compared to the results obtained for 10 g/l yeast extract in the previous optimization. In the first screening, biomass concentration increased with the addition of nitrogen content, which hints towards the influence of other factors. Moreover, the biomass concentration values are far lower than those registered in the second screening. Thus, although cells were collected after the same period of incubation in the two sets of experiments, the growth stage may have not been exactly the same, due to unnoticed inconsistencies regarding the physiological state of the inoculum, which may have eventually disturbed the time frame of growth. Following the trend, 20 g/l sucrose, 20 g/l yeast extract and 7.5 g/l bacto peptone was chosen as the optimized medium (Medium A).

Table 2 - Biomass concentration, invertase and relative invertase activity of the second optimization step of the growth medium. Effect of yeast extract and bacto peptone ratio and concentration was tested. Values are shaded from lowest (orange) to highest (green).

Biomass Concentration (g/l)		Bacto Peptone (g/l)		
		2.5	5	7.5
Yeast Extract (g/l)	2.5	28.23	24.95	24.75
	5	25.78	25.07	24.57
	10	22.52	19.42	17.25
	15	16.32	16.33	16.58
	20	14.80	15.27	15.23

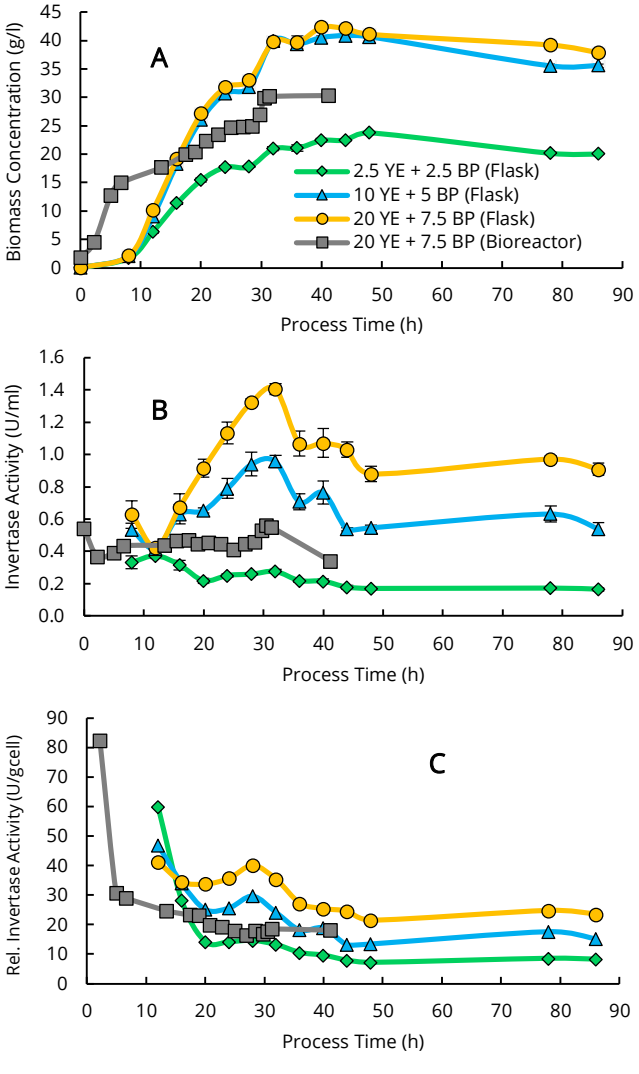
Invertase Activity (U/ml)		Bacto Peptone (g/l)		
		2.5	5	7.5
Yeast Extract (g/l)	2.5	0.63	0.64	0.69
	5	0.74	0.76	0.77
	10	0.84	0.81	0.75
	15	0.77	0.76	0.83
	20	0.80	0.88	0.88

Relative Invertase Activity (U/g wet cell)		Bacto Peptone (g/l)		
		2.5	5	7.5
Yeast Extract (g/l)	2.5	22.26	25.79	27.79
	5	28.54	30.26	31.55
	10	37.25	41.76	43.56
	15	47.32	47.10	50.01
	20	53.78	57.99	57.88

3.2. Baffled flask fermentation

Once the fermentation medium was optimized, the yeast cells were cultivated in baffled flask to determine and compare cell growth and invertase activity profiles with the microreactor. In order to gain further information about the implications of increasing the amount of organic nitrogen source, the profiles were studied with the optimized medium and two other media with less amount of yeast extract and bacto peptone.

In baffled flasks, a higher nitrogen content led to greater cell concentrations, which in turn led to a rise in invertase activity, as opposed to microtiter plates. One likely explanation could be that the geometry and agitation rate of the baffled flask promoted a better dispersion of sucrose and glucose in the medium, thus improving sucrose diffusion to the cells, and glucose removal from the cell microenvironment. However, due to the difference in vessel geometry, parameters such as agitation rate, pH value, oxygen transfer, and inoculum concentration could have had different effects in each cell culture. Therefore, the only viable conclusion available from comparing both vessels, is that high nitrogen media results in greater invertase activity values.



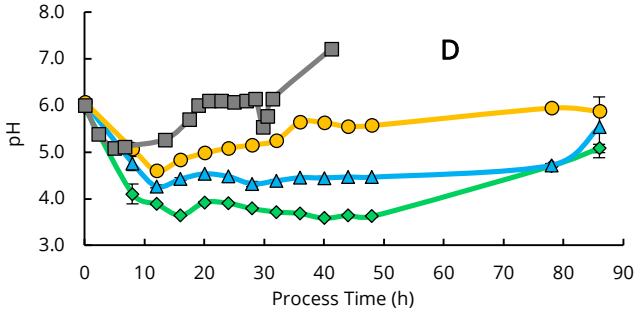


Figure 1 – Biomass concentration (A), invertase activity (B), relative invertase activity (C), and pH (D) profiles of baffled flask and bioreactor fermentations for media with different nitrogen content.

Baffled flasks are commonly used to enhance maximum oxygen transfer rate (OTR_{max}) during cultivation. For low shaking frequencies they have a higher k_La value than unbaffled shake flasks. Considering that 150 ml baffled flasks (70 ml filling volume) in a 30 mm orbital shaker at 200 rpm result in a k_La increase of 1.62 when compared to unbaffled flasks in the same condition, then for a k_La of 104 h^{-1} for a 250 ml unbaffled shake flask in this study, although in different conditions, should lead to a rough estimation of k_La 168 h^{-1} for the baffled flask [34,35]. In order to ensure the same oxygen transfer conditions as the baffled flask, shaking frequency in microtiter plate should have had been at least 450 rpm However for high shaking frequencies, out-of-phase cell culture liquid can cause splashes, which could contaminate the membrane in the cover and lower gas permeability. Without a method to measure oxygen transfer coefficients directly, such as the hydrogen sulphide approach, it is uncertain just how high the k_La coefficient is for baffled flasks.

During the lag phase of the baffled flask culture, the relative invertase activity was initially high until it decreased to more stable values throughout the experiment (Figure 1C). This was attributed to the large amount of invertase produced by few cells in the presence of high quantities of sucrose and low quantities of glucose. Invertase activity reached a maximum immediately before stationary phase at 32 h (Figure 1B). Depletion of carbon source lowered invertase production to a fixed amount, which was maintained throughout the experiment. Since there was no apparent enzyme denaturation, it is likely that yeast cells released proteases to degrade invertase in order to generate assimilable nitrogen compounds [36]. Degradation stopped at 48 h, after which a fixed amount of invertase was maintained.

The pH of the medium was initially adjusted to 6. After inoculation, production of carbonic and organic acids from cellular respiration are responsible for acidifying the medium at the start. Once the exponential phase starts, the production of ammonia from yeast extract and bacto peptone by a large amount of cells was enhanced and increased the pH levels (Figure 1D). Its effect was stronger for high concentrations of nitrogen sources and could possibly have outweighed the effect from cellular respiration in the case of Medium A [37]. For Medium C, pH levels under 4.0 could have inhibited cell growth. Nevertheless, the short supply of nitrogen components was more likely to have had a larger impact in reducing cell duplication rate. Invertase production and invertase stability were promoted for optimum pH values of 6.0 and 4.5 respectively. Since no buffer solution was added, the possibility that pH levels could have swayed the invertase activity profile still remained.

3.3. Batch bioreactor fermentation

Since no oxygen limitation is expected for microfluidic experiments due to the gas permeability of PDMS, a controlled bioreactor fermentation was performed in order to produce a more consistent biomass concentration and invertase activity profile. However, due to a DO sensor malfunction, agitation rate and air flow rate were manipulated manually based on sample measurement and pH values.

With an initial agitation rate of 300 rpm and an air flow of 1.6 vvm, cell doubling time during the initial exponential phase was 1.80 h until 5 h of process time, when cell growth suffered inhibition (Figure 1A). With a doubling time of 25.88 h, cell duplication was halted and pH levels increased, which revealed oxygen limitation, most likely due to the production of ethanol by alcoholic fermentation. Together with ammonia production, ethanol contributed to the culture alkalinisation. In order to increase oxygen supply, at 16 h of process time, agitation rate and air flow rate were adjusted to 400 rpm and 2.0 vvm. However, hardly any change was detected. The pH level continued to increase until the upper limit (pH 6.0), set by the pH controller.

After 29 h, agitation rate was increased to 600 rpm in a last effort. The cell culture entered a new but short exponential phase with a faster duplication rate (7.78 h doubling time). After the adjustment, pH level immediately dropped to pH 5.54, but rapidly increased to pH 6.0 after 1 h (Figure 1D). This suggests that cell culture returned to aerobic conditions without oxygen limitation, and for a moment, the sudden production of organic and carbonic acids led to a drop in pH during new exponential phase. However the effect was likely swiftly offset by the production of ammonia and ethanol. Stationary phase was reached after 31 h. However, since the pH controller failed at that time, there was no addition of acid, which allowed the medium to reach pH levels above 7.0. It is uncertain if the stationary phase was caused by substrate limitation, pH inhibition, shear stress or even oxygen limitation.

For an agitation rate and air flow rate of 300 rpm 1.6 vvm, the bioreactor had an estimated k_La value of 55 h^{-1}. Subsequent escalations to 400 rpm and 2.0 vvm, and 600 rpm and 2.0 vvm led to estimated k_La values of 95 h^{-1} and 186 h^{-1}. Since the latter entered the stationary phase with a biomass concentration of 30 g/l and baffled flask showed no apparent signs of oxygen limitation with a maximum biomass concentration of 40 g/l, it is possible that the true k_La value of the baffled flask could be slightly higher than 186 h^{-1}. However, due to the accumulation of toxic components,

high pH level, or shear stress, other factors that contributed to cell growth inhibition in the bioreactor, it is uncertain. Since the agitation rate is already maximum for safety reasons, the k_La value could only be easily improved with the same equipment and volume, by increasing the air flow rate to a maximum of 3 vvm (k_La 210 h^{-1}), which is allowed for 1 litre aerobic bioreactors [38].

Bioreactor invertase activity (Figure 1B) did not follow the same profile as Medium A and Medium B from the baffled flask culture. In fact, a brief increase in invertase production was only registered around 2 h and 29 h of process time with proper supply of oxygen. It is evident that invertase synthesis is repressed under oxygen limitation. When the cell culture is faced with a low oxygen supply, yeast cells tend to use the alcoholic fermentation pathway, instead of the citric acid cycle. Since the cell uses several amino acids such as aspartate and glutamate (or by-products from these amino acids) for the production of invertase, that are produced in the citric acid cycle, it is possible that under oxygen limitation, amino acid synthesis is severely inhibited and the cells must rely instead on the amino acids supplied by the nitrogen source in the medium for invertase synthesis [39].

3.4. Microreactor fermentation

To determine miniaturization efficiency when comparing a microreactor with bench-scale batch reactors, invertase activity was measured for several growth medium dilutions. In this work, due to the irregular profile in the 1.2 l batch bioreactor, the baffled flask was chosen instead as the bench-scale batch reactor model.

In order to compare a batch reactor with a continuous reactor, some assumptions had to be made. Extracellular invertase produced in batch mode would be suspended in the medium, while invertase produced in perfusion mode would be washed away and collected from the microreactor outlet. Therefore, to compare both reactors, samples taken from the microreactor had to be added together to simulate the total amount of enzyme one would find suspended in a batch reactor. This method was necessary, since it was not possible to compare a continuous microreactor with a bench-scale batch reactor otherwise. One alternative would be protein purification for quantification, but such a task would be time consuming.

Microreactor experiments were performed until the PDMS sealing was broken and cell culture medium leaked. This was attributed to pressure build-up inside the microchannel from clogging of cells or PDMS debris. Long duration of the run containing perfused medium diluted 1000 times was due to low cell density. Medium without dilution was performed separately on another fabricated device at a different time, which might explain some differences on cell count and invertase profile.

Cell growth in the microreactor seemed to follow an exponential growth profile despite possible entry and exit of cells during the run. Some cells were able to escape the traps and be washed way, which would explain the reduction in the medium diluted 10 times at 24 h of process time (Figure 2A). Moreover, cell duplication did not just occur inside the traps near the inlet as it was expected. Some cells became trapped in debris or adhered to the microchannel wall which over time duplicated unrestrained. The cell clumps would then dislodge and adhere further downstream, only to again duplicate with less spatial limitation in the PDMS traps, thus promoting further exponential growth despite spatial limitation. It is likely that throughout the process there was no oxygen limitation, and that an average height of 1 cm of PDMS in the device was enough to allow gas transfer. It was also estimated that a filled microchannel contained around 70.000 yeast cells.

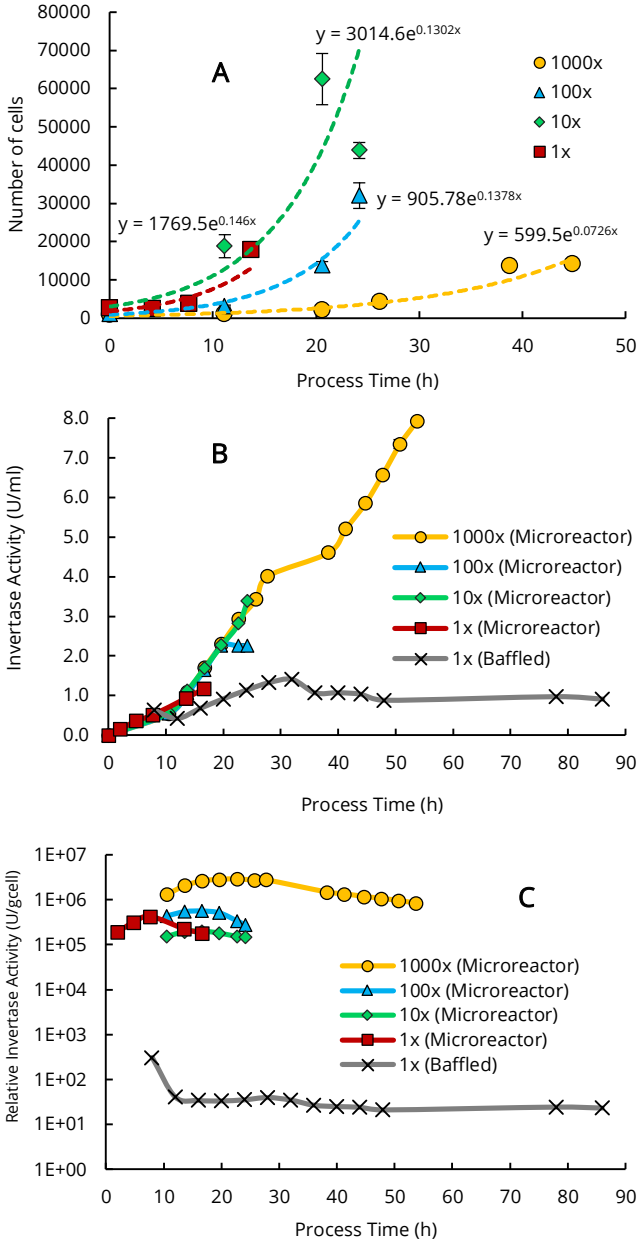


Figure 2 – Estimated number of cells inside the microchannel for several dilution factors (A). Invertase activity (B) and relative invertase activity (C) profiles of microreactor fermentation for several dilution factors and baffled flask fermentation with the optimized Medium A.

Invertase activity profile in the microreactor (Figure 2B) followed the same trend for all dilutions despite the different amount of cells present in each experiment. In a perfusion reactor with constant supply of nutrients and a constant removal of waste products it was expected that enzyme production would follow the cell growth profile. However, only the cells in direct contact with the medium were more likely to produce invertase. This could explain the apparent constant production of invertase throughout the experiment despite the dilution. The only condition for viable production of invertase is enough nutrient renewal in the cell microenvironment, which is confirmed up to a dilution factor of 1000. With a continuous flow, invertase synthesis is always de-repressed, since sucrose is abundant and glucose is constantly being removed from the cell microenvironment.

After 32 h of perfusion, the microchannel with medium diluted 1000 times had already produced 3 times more invertase (4.30 U/ml) than the maximum invertase activity registered for baffled flask at the same time (1.40 U/ml). After 54 h of perfusion, the microchannel produced almost 6 times more (7.94 U/ml) than the maximum invertase activity for the baffled flask. A continuous supply of growth medium meant that yeast cells received an excess of nutrients in a constant environment without the consequences of accumulating toxic compounds. Moreover, the continuous presence of sucrose in the perfusion medium stimulated the production of invertase without glucose repression. With a more efficient medium diffusion to the cells, it is possible that invertase synthesis could be further multiplied.

Since enzyme production was constant for all dilutions in the microreactor and attributed to the first row of cells in direct contact with the medium, relative invertase activity increased for dilutions with a lower number of cells. Therefore, cells that were no longer being supplied with medium, did not produce invertase and were simply dead space that contributed to channel clogging, given the limited amount of room inside the microreactor. The reproducibility issues in the microreactor experiment with a dilution factor of 1 could be attributed to different velocity profiles from small irregularities in the microfabrication process that might be relevant enough to alter the results.

4. Conclusion

A continuous microreactor for the production of invertase was developed and characterized. Yeast cells were trapped and separated from the perfused product for high dilution factors with low cell content. During miniaturization, conditions for culture medium composition and temperature were maintained. Differences in pH levels were overlooked due to pH profiling with a non-buffered cell culture. Changes in mass and gas transfer were disregarded due to the difference in vessel geometry and guaranteed only for experiment viability.

Optimized cell culture medium (20 g/l sucrose, 20 g/l yeast extract and 7.5 g/l bacto peptone) increased invertase activity by 38 %, reduced biomass concentration by 39 % and increased relative invertase activity by 124 % in microtiter plates when compared to the control medium initially found on lab protocols. Low cell content with high invertase production, was considered to be very beneficial under microfluidic conditions, in order to avoid microchannel clogging and promote longer runs without hindering production. Maximum invertase production in baffled flasks with the optimized medium was registered around 32 h of process time (1.40 U/ml), immediately before reaching stationary phase. After 54 hours of perfusion, invertase activity was almost 6 times higher in the continuous microreactor (7.94 U/ml) with a dilution factor of 1000, when compared to the maximum invertase activity in the baffled flask.

Invertase production on microreactors was superior to batch reactors, which shows incredible potential for process parallelization due to small culture volumes. However, for more consistent results in the future, comparison with a fed-batch reactor or a continuous packed bed reactor with immobilized invertase is needed. Moreover, the use of a buffer solution, together with the determination of cell dry weight and specific invertase activity would also contribute for more reliable results.

5. References

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