

Master's thesis for the degree of Life Science & Technology

Improvement of a Laboratory Seed-Train and Characterization of Quail Suspension Cell Line CCX.E10 for Cultivated Meat Production

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Abstract

Livestock production forms a threat to the environment and human health, while meat consumption increases due to global population and income growth. Commercial scale cultivated meat production provides a credible alternative due to its resemblance to the taste, texture and looks of conventional meat. In order to commercialize cultivated meat, major technological improvements are needed to scale-up its production from laboratory to commercial scale. This requires an animal cell line that can provide a food-grade product. Quail suspension cell line CCX.E10 is not genetically modified, is spontaneously immortalized and cultivated in animal component free medium. To research the growth kinetics of the cell line for commercial scale production investigation at a laboratory scale is required. Through this research a seed-train protocol to scale-up CCX.E10 cell bank to a scale-down model, namely a DASbox mini bioreactor system, was improved. Additionally, the growth kinetics of CCX.E10 were characterized during batch cultivation, while utilizing this system. A Design-of-Experiments approach was developed to understand what variables have the largest impact on growth kinetics of CCX.E10 to improve operational conditions. Input variables of the approach include the aeration rate, agitation rate and dissolved oxygen concentration of the bioreactor. This research found an exponential specific growth rate of 0.016 h^{-1} and viable cell density of 2.1×10^6 cells/mL for batch cultivation of cell line CCX.E10 in a DASbox bioreactor. A cell-specific lactate production rate 30% higher than the cell-specific glucose consumption rate was observed during the lag phase of cell cultivation. The growth kinetics derived during cell proliferation suggest that cell line CCX.E10 exhibits the Warburg effect. The findings indicate that the operational conditions for cultivated meat production based on CCX.E10 require further improvement to design a commercial scale bioprocess.

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1. Introduction

Since the beginning of this century, the continued negative impact of the livestock production on our environment has triggered heated debates [1]. The established concerns, namely poor animal welfare, are now supported by significant sustainability and human health issues. Already in 2015, the animal protein production accounted for approximately 12 percent of the total amount of carbon emissions globally. Conventional meat production claims the largest share of these emissions with a value of 67 percent [2]. Simultaneously, increasing population and income ensure a continuously growing total food demand inducing pressure on the agricultural sector. The upper-middle income countries alone are expected to account for 45 percent of total food consumption growth in 2034 compared to 2025 [3]. At the same time, the necessary intensification of livestock production and its low genetic diversity provide opportunities for pathogens to develop and transmit to humans. Of all emerging pathogens 75 percent occur as zoonotic diseases, while human pathogens are still being discovered every year [4]. These significant sustainability and human health issues originating from the agricultural food sector require alternatives and disruptive solutions to rapidly reduce the demand of animal-derived products.

Particularly, the protein in conventional meat produced by the livestock is regarded as an essential nutrient in a healthy diet [5]. Therefore, to minimize the mentioned issues of the livestock production it is crucial to find protein-rich alternatives for conventional meat [6]. Available alternatives are plant-based proteins, fermentation-derived proteins and cultivated meat. The first two increased their market share for over a decade. The latter makes commercial advances since 2020, when the food technology company Eat Just was granted approval for commercialization of cultivated chicken in Singapore [7].

The environmental and health benefits of plant-based protein products compared to animal-derived products are evident. Regardless, consumers retain their preferences for proteins that are inseparable from the taste, structure and looks of conventional animal meat [8, 9]. As a consequence, the potential of plant-based or fermentation-derived proteins might reach an end before it replaces significant parts of the conventional products. Plant-based protein consumption by itself is expected to displace only one-sixth of livestock by 2050 [10]. In contrast to plant-based alternatives, cultivated meat (CM) is manufactured to closely resemble conventional meat and the more complex structured meat products. Therefore, its production and commercialization hold significant potential to replace a substantial part of the agricultural sector [11, 12].

Although research into the various key elements of CM is ongoing, in order to commercialize CM, significant technological innovation is still required. These include cell line development, serum-free culture media development and scaffold design [13]. Several companies, such as Eat Just, Aleph Farms and Upside Foods are already or close to commercial scale production of CM, with more companies following [14, 15]. To ensure the continuance and long-term availability of CM production technology, research aimed at commercial scale bioprocessing is necessary [16, 17].

Bioprocess scale-up methods currently described in literature comprise multiple steps to expand cell culture volumes to certain larger scales, depending on the aim of the process. Such an operation is called a seed-train. It enables controlled volume increase where cultivation conditions are constantly monitored and adjusted to the bioprocess requirements. During the seed-train, cell growth conditions that are adapted to the optimal growth conditions of the employed cell line are essential to maintain an exponential cell growth regime and yield a high number of cells for product (Figure 1) [18-20].

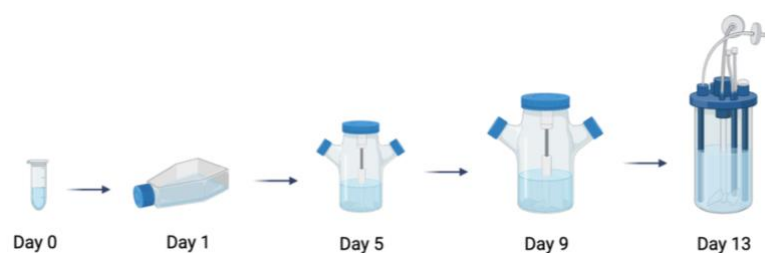


Figure 1. Example of a seed-train to scale-up the cultivation of CCX.E10 from laboratory scale (1 mL) to a bioreactor at pilot scale (5 L) with T- and spinner flasks.

In general, the final stage of the seed-train is performed in a bioreactor with a volume (>1000L) to produce enough cells for commercial scale production. Examining growth conditions for a particular cell line at this volume size is expensive and demanding. Therefore, it is advised to develop and optimize cell culture processes at a laboratory-scale (<5L) before proceeding to large-scale bioreactors to increase time and cost-efficiency [21, 22].

Currently, bioprocesses that employ animal cells mostly originate in a pharmaceutical manufacturing context. These processes and their associated scale-up methods have been examined for decades. Due to the low number of available seed-train literature for CM production, the already conducted pharmaceutical research serves as a substantial reference for optimization of a CM seed-train [23]. For instance, viral vaccine production, requires high standards for the animal cells to endure stressful conditions. The cells are used to an environment inside a living organism where there are numerous mechanisms in place to ensure optimal conditions. Therefore, vaccine production requires careful bioprocess handlings as well to ensure cell growth. Moreover, biosafety of the vaccine manufacturing is essential for commercial production [24, 25]. These requirements are recommended for CM production as well [26]. In contrast, significant differences lie in the objective of the production processes. While intensified CM production generally aims for high cell growth, the pharmaceutical industry usually employs animal cells to produce high viral titers [27].

The required bioprocess conditions differ highly for each animal cell line. Therefore, to select a cell line that exhibits potential for commercial scale CM production several qualities ask for consideration. Cells that grow in suspension instead of attached to a surface (adherent) show an improved feasibility. This is primarily because of the required area for adherent cells to grow. Converting a cultivation volume of a 1000 L bioreactor for suspension cells to the corresponding required surface area for cultivation of adherent cells results in a value of 1,500 km² [28]. Consequentially, in regard of required filling volume, scale-up efficiency and required laboratory handlings, commercial scale CM production requires suspension cells [22].

Furthermore, to commercialize CM for consumption a focus on food regulations and consumer acceptance is unquestionable. Preferably, the selected cell line is not genetically modified and has spontaneously reached an immortal state [23]. Lastly, prevention of animal harm must be considered. This requires the availability of animal-component-free media to cultivate the cell line of interest [29].

The quail suspension cell line CCX.E10 developed by Nuvonis (Austria) corresponds to each of these qualities[20]. Unfortunately, in literature only a few publications are available that describe this cell line. These all focus on vaccine manufacturing optimization [30]. A seed-train for and characterization of quail suspension cell line CCX.E10 for CM production is non-existent in literature. Bioprocesses utilizing this particular cell line do exist for vaccine manufacturing, but these publications aim for high virus titers instead of high cell growth [31, 32]. A seed-train workflow at a laboratory-scale is essential for efficient characterization of animal cell lines to advance CM bioprocess scale-up research.

To reach high viable cell densities (VCD) an understanding of the optimal cultivation conditions of the cell line is crucial. Distinguished factors limiting the exponential growth of cells include the depletion of nutrients, accumulation of waste metabolites and sub-optimal environmental conditions (pH, temperature, dissolved oxygen concentration). Examination of cell-specific rates and operational conditions provides insights to engineer strategies for commercial scale cultivation. In literature several process parameters for cultivation of cell line CCX.E10 are found. A paper that incorporated a Chinese Hamster Ovary (CHO) cell line showed the use of cultivation and scale-up methods that are similar to the methods available for the current study [33]. Together, these provide a guideline for seed-train and bioreactor cultivation improvement (Table 1).

The research goal of this report is to document an improved seed-train protocol based on existing process parameters to scale-up the cultivation of quail suspension cell line CCX.E10 from a cryopreserved cell bank (1 mL) to a multi-parallel stirred-tank bioreactor system (four vessels of 250 mL, DASbox®, Eppendorf, Germany). This leads to the following research question of this thesis.

1. What conditions ensure maximum cell growth rates and cell viability values above 90% of quail suspension cell line CCX.E10 during a seed-train from cell bank to DASbox bioreactor?

The DASbox bioreactor system presents a multi-parallel scale-down model for characterization of kinetic parameters and optimal operational conditions of cell line CCX.E10 in a bioreactor batch cultivation. This enables the investigation of the second and third research question.

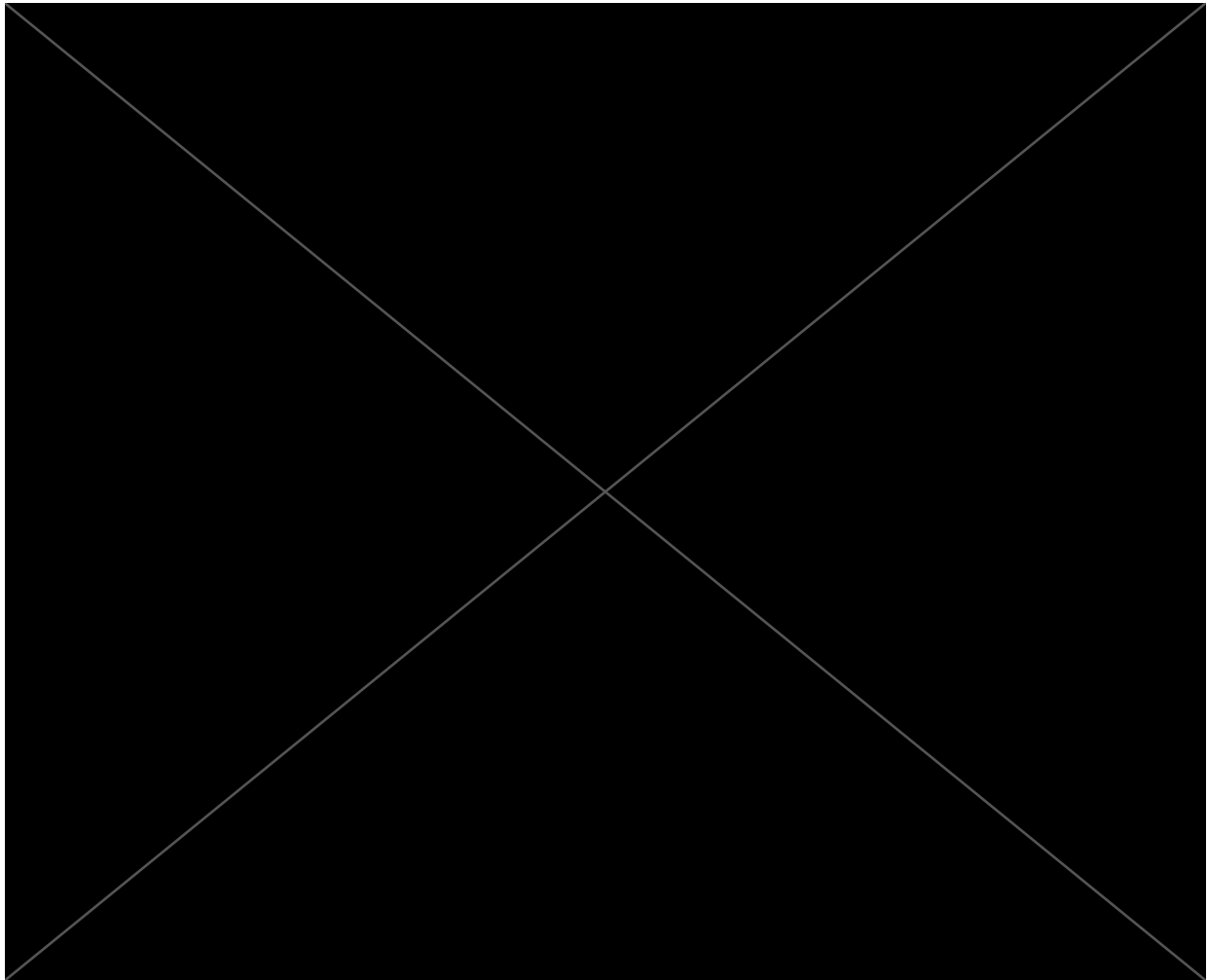
2. What operational conditions improve the cell-specific rates and yield-coefficients of quail suspension cell line CCX.E10 during exponential cell growth in a DASbox bioreactor?
3. What are the limiting factors for exponential cell growth of quail suspension cell line CCX.E10 during batch cultivation in a DASbox bioreactor?

This research aims to answer the research questions in two parts. The first part describes the improvement of a seed-train workflow to reach a high VCD of CCX.E10 to initiate the DASbox bioreactor system. The second part describes the characterization of cell-specific rates and yield-coefficients of CCX.E10 during batch cultivation in DASbox bioreactors. To initiate the DASbox system during the second part a certain minimum number of viable cells is required from the seed-train in the first part. Therefore, the two parts are interdependent to compose a complete seed-train for the characterization of quail suspension cell line CCX.E10 in a bioreactor scale-down model from a cryopreserved cell bank vial.

Table 1. Overview of animal cell cultivation parameter values found in literature for Quail Suspension Cell line CCX.E10 (QSC) or CHO cell line CHO-K1 in shake flask and stirred-tank reactor. Conditions employed by the manufacturer of cell line CCX.E10 are denoted by ‘*’. The cell-specific glucose consumption rate is denoted as ‘qGlc’. The cell-specific lactate and ammonium consumption rates are denoted as ‘qLac’ and ‘qNH4’, respectively.

Variable	Value	Cell line	Notes	Reference
Independent (input)				
Temperature [°C]	37	QSC		*
	37	QSC		[27, 30, 31]
	30 – 37	QSC		[20]
	37	CHO-K1		[33]
<u>Shake flask</u>		QSC	T- and spinner flask	*
		QSC	Baffled shake flask	[27, 30, 31]
		QSC	Spinner flask	[20]
		CHO-K1	Non-baffled	[33]
Inoculation density [$\times 10^6$ cells/mL]	0.05, 0.1 or 0.2	QSC	Depends on passaging	*
	0.8	QSC		[27, 30]
	0.2	CHO-K1		[33]
Vessel volume [mL]	500	QSC	Spinner flask	*
	125	QSC	Baffled flask	[27, 30]
	125	CHO-K1	Non-baffled flask	[33]
Filling volume (wv) [mL]	300	QSC		*
	50	QSC		[27]
	15	CHO-K1		[33]
Passaging (d)	2, 3 or 4	QSC		*
	3 – 4	QSC		[27, 30]
	3 – 4	CHO-K1		[33]
Shaking speed [rpm]	60	QSC	Spinner flask, acceleration to 125 rpm when VCD equals 0.5×10^6 cells/mL	*
CO ₂ incubator [%]	185	QSC	50 mm shaking diameter	[27, 30, 31]
	5	QSC		*
	5	QSC		[27, 30]
	5	CHO-K1		[33]
<u>Bioreactor</u>				
		QSC	DASgip (batch)	[30]
		QSC	DASgip (perfusion)	[27]
		CHO-K1	DASbox (batch)	[33]
Inoculation density [$\times 10^6$ cells/mL]	0.8	QSC		[27, 30]
	0.25	CHO-K1	DASbox	[33]
Vessel volume [L]	1	QSC		[30]
	3	QSC	DASgip	[27]
Filling volume (wv) [mL]	350	QSC		[30]
	1.3	QSC	DASgip	[27]
pH	7 – 7.4	QSC		[27]
	7.2	QSC		[30]
	7.2	CHO-K1		[33]
Agitation rate [rpm]	130	QSC		[30]
	180	QSC	DASgip	[27]
Aeration rate [sL/h]	3 – 9	QSC		[27, 30]
Dissolved oxygen conc. [%]	>50	QSC		[27, 30]
Dependent (output)				
<u>Shake flask</u>				
VCD [$\times 10^6$ cells/mL]	4.4	QSC		[30]
	3.2	QSC		[27]
Cell-specific growth rate [h^{-1}]	0.026 ± 0.003	QSC		[27, 30]
qGlc [$\times 10^{-11}$ mmol/(cell*h)]	-7.9 ± 0.6	QSC		[30]
qLac [$\times 10^{-11}$ mmol/(cell*h)]	8.6 ± 0.9	QSC		[30]
qNH4 [$\times 10^{-11}$ mmol/(cell*h)]	0.4 ± 0.04	QSC		[30]
<u>Bioreactor</u>				
VCD [$\times 10^6$ cells/mL]	4.2	QSC	batch	[30]
	20.3	QSC	perfusion	[27]
Cell-specific growth rate [h^{-1}]	0.022	QSC		[30]
qGlc [$\times 10^{-11}$ mmol/(cell*h)]	-15.8	QSC		[30]
qLac [$\times 10^{-11}$ mmol/(cell*h)]	16.4	QSC		[30]
qNH4 [$\times 10^{-11}$ mmol/(cell*h)]	0.6	QSC		[30]

2. Materials and methods



2.2 Shake flask cultivation

For all shake flask cultivations either the non-baffled Corning® (US) 125 mL Polycarbonate Erlenmeyer Flask or the baffled Corning® 125 mL Polycarbonate Erlenmeyer Flask were used. In case of the latter this is specifically mentioned. During shake flask cultivation the cells were incubated in a CO₂ incubator (Heracell™ 150i, Thermo Scientific, US) at 37°C and 5% CO₂ while shaking at determined setpoints conditions at an orbital shaking platform with shaking diameter of 19 mm (CO₂ Resistant Shaker, Thermo Scientific). All shake flask operations were performed inside a laminar flow cabinet (LAF) (MSC-Advantage Biological Safety Cabinet, Thermo Scientific) to prevent contamination of the culture.

In several experiments the growth of ‘adapted’ cells was analyzed. These cells were continuously cultivated in shake flasks prior to the experiments. The duration of the adaptation was not properly documented. Therefore, estimations are made of the adaptation duration. This is specified in the designated results sections. Several experiments involve the analysis of thawed cells. In this case, the growth analysis is initiated from inoculation of the shake flask with the thawed cells. Unless mentioned otherwise, all shake flask experiments are performed with triplicate biological replicates. The analysis methods and data processing described in a subsequent section are carried out for each shake flask cultivation experiment for samples withdrawn every 24 hours.

2.2.1 Procedure

2.2.1.1 Thawing

Cryopreserved cells are thawed in a water bath at 37 °C and added to a small amount incubated medium prior to inoculating a shake flask with the residual amount of the desired volume of SCGM. A more detailed protocol for cell thawing is documented (from Additional Materials, see Protocols, Shake Flask Cultivation – Cell bank thawing).

2.2.1.2 Passaging

After a 3 – 4 day cultivation cells are passaged by first centrifuging the culture for 10 min at 300 g in a centrifuge (Mega Star 600R, VWR, US). The supernatant with old medium is removed and fresh medium is added. Prior to passaging the cells, a cell count is performed. Medium is added to the cell pellet to transfer the desired number of cells to a flask filled with the residual volume of fresh medium (from Additional Materials, see Protocols, Shake Flask Cultivation – Passaging). The cell count method is described in a later section.

2.2.1.3 Sampling

Immediately starting after inoculation, culture samples are withdrawn every 24 hours of 500 μ L from the shake flask. The samples are analyzed for cell density, viability, cell diameter, morphology and osmolality. Afterwards, the culture samples are centrifuged in a benchtop centrifuge (Biofuge Pico, Heraeus, Germany) for 10 min at 5000 g to cryopreserve the supernatant for metabolite analysis. Samples of the complete cultivation are collected before performance of the analysis. The analysis methods are elaborated in a subsequent section. The data retrieved from the analyses is processed according to methods described in another subsequent section. A more detailed protocol for sampling is documented (from Additional Materials, see Protocols, Shake Flask Cultivation – Sampling).

2.2.2 Filling volume and shaking speed

The optimal shake flask cultivation setpoints for filling volume and shaking speed are examined. For determination of these conditions the description of a liquid distribution for optimal mass-transfer that was found in literature is adopted [36, 37]. The studies describe a configuration where a minimum amount of liquid covers the center point of a non-baffled shake flask, due to an orbital shaking movement [38]. Accordingly, it is assumed an excessive or insufficient shaking speed leads to sub-optimal gas-liquid mass transfer (Figure 2).

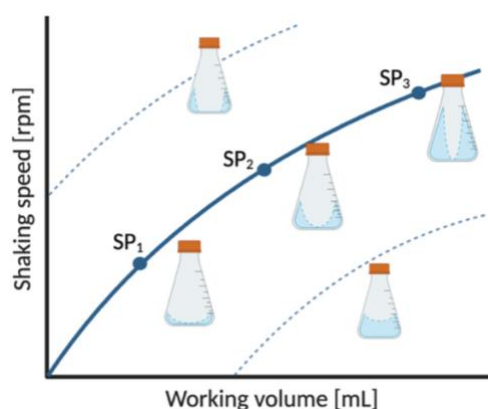


Figure 2. Varied filling volume and shaking speed setpoints in non-baffled shake flasks.

A thumb rule for shake flask cultivation is a filling volume between 10 and 30% of the total volume. Therefore, several shake flask filling volumes within this range are examined (Table 4).

Table 4. Filling volume setpoints determined based on filling volume percentages of a total volume of 125 mL.

Parameter	Setpoint				
Filling volume [%]	10	15	20	25	30
Filling volume [mL]	12.5	18.75	25	31.25	37.5

Non-baffled shake flasks that contained the specified filling volumes of SCGM are shaken on the shaking platform between 100 – 300 rpm, while increasing the shaking speed by steps of five rpm until the described optimal liquid distribution is approached. The movement of the liquid is captured by taking slow-motion recordings. The recordings are compared to determine the shaking speed for the optimal liquid distribution. The shaking speed and corresponding filling volume are documented. The conditions determined for non-baffled shake flasks are adopted for baffled shake flask cultivations as well.

2.2.3 Baffled versus non-baffled shake flasks

Cell growth in baffled and non-baffled shake flasks is compared. Prior to the comparison, the cells are cultivated in a non-baffled shake flask to adapt to the shake flask environment. The cells are passaged either to a baffled or non-baffled shake flask and subsequently, passaged in three-fold replicated flasks for either flask type with an inoculation density of 0.1×10^6 cells/mL. The cell growth is monitored from inoculation of the triplicates (Figure 3).

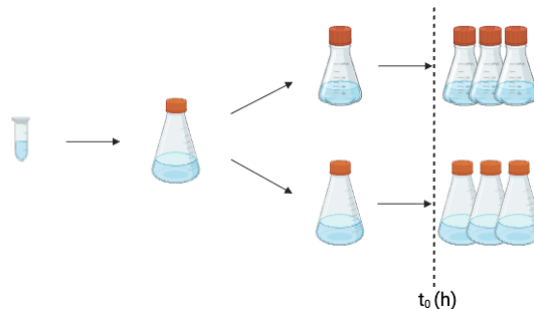


Figure 3. Experimental set-up to compare cell growth in baffled and non-baffled shake flasks in triplicates.

2.3 DASbox bioreactor system

Batch cultivations are performed in a DASbox multi-parallel stirred-tank reactor system. Due to availability, three out of four bioreactors are employed with a total volume of 250 mL each. Three campaigns are carried out with the DASbox. Each bioreactor experiment was performed in 3-fold technical replicates. A filling volume of 100 mL is maintained. The DASbox bioreactor cultivation is performed while utilizing the DASware software provided by Eppendorf. Setpoints for agitation, aeration, DO, temperature and pH are established in the software and controlled by a Proportional-Integral (PI) controller system. The agitation rate is controlled between 100 – 500 rpm. For agitation, a one pitched blade impeller with an outer diameter of 30 mm and maximum shaking inside diameter of 63.5 mm is used (counterclockwise). The aeration is used for control of the DO concentration between 20 – 100% and for control of pH by enrichment with CO₂. The aeration rate is controlled between 1 – 5 sL/h. The pH of 7.2 is further controlled by overhead addition of 7.8% NaHCO₃. The temperature setpoint was 37 °C (Appendix, Table 10). The setpoint values for aeration, agitation and DO concentration are established depending on the experiment. This is discussed in a subsequent section.

2.3.1 Procedure

2.3.1.1 Inoculation

A cell count is performed prior to centrifuging the total amount of culture in the flasks at the end of the seed-train. Based on the seed-train protocol, multiple flasks are used to grow cells for the DASbox cultivation. Therefore, the process of centrifuging the cells for 10 mins at 5000 g, removing supernatant and addition on top with subsequent culture is repeated for all flasks to combine the cells in a single tube. After the suspension in fresh medium, the DASbox bioreactors are inoculated with the cells in equal quantities with a sterile syringe through a male Luer Lock tubing port. Afterwards, the tubing port is flushed with air from inside the LAF using a sterile syringe (from Additional Materials, see Protocols, DASbox Operation – Inoculation).

2.3.1.2 Sampling

Samples of 1 mL in triplicates are extracted with a sterile syringe from the bioreactor utilizing the Luer Lock tubing port. The extracted volume is supplemented with incubated SCGM to maintain the filling volume of 100 mL. After sampling, the tubing port is flushed with sterile air. The samples are used for offline pH measurements with a pH probe (691 pH Meter, Metrohm, Switzerland) to validate the in-line probe pH values of the bioreactor monitored by DASware. Furthermore, cell density, viability, cell diameter, morphology and osmolality are analyzed. The residual of the sample is centrifuged for 10 min at 5000 g. The supernatant is cryopreserved. After collection of all samples of the experiment, a metabolite analysis is performed. For the DASbox sampling procedure a protocol is documented (from Additional Materials, see Protocols, DASbox Operation – Sampling). The analysis methods are detailed in a subsequent section.

2.3.2 Design-of-Experiments approach

A Design-of-Experiments (DoE) approach is developed to efficiently assess the impact of three variables in different combinations on cell growth in the DASbox bioreactor system. This way the total number of One-Factor-at-a-Time experiments is reduced. A fractional-factorial design was adapted for an initial screening of three selected variables which leads to a cubic research model (Figure 4).

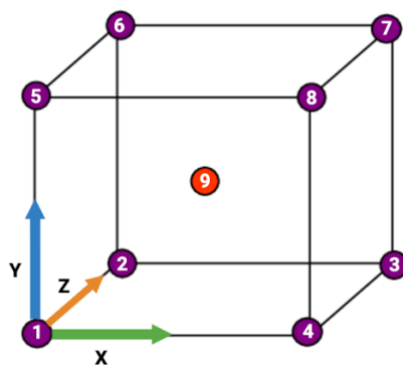


Figure 4. Fractional-factorial DoE approach comprising nine different setpoint conditions for three different variables with a center point that represents the mid-values for the selected maximum and minimum bound values of the variables.

This design tests the significance of the impact of the input variables on the output. The input parameters prioritized for investigation are the agitation rate, aeration rate and dissolved oxygen percentage. The output investigated is the viable cell density at the end of the exponential growth regime (Table 5). The minimum and maximum values for the variables are defined so they comprise a center point that approximates the operational conditions for the variables detailed previously (Table 1).

Table 5. Design-of-Experiments inputs for batch cultivations of cell line CCX.E10 in the DASbox mini bioreactor System. Experiment 9 is based on parameter values found in literature (Table 1).

Exp. No	1	2	3	4	5	6	7	8	9
Aeration rate (X) [sL/h]	1	1	5	5	1	1	5	5	3
Agitation rate (Y) [rpm]	100	100	100	100	500	500	500	500	300
Dissolved oxygen (Z) [%]	20	100	100	20	20	100	100	20	60

The JMP® 19 software from JMP Statistical Discovery is used to process setpoint combinations with the availability to run statistical tests. Based on these tests the software allows for understanding of the relationship between input and output variables. Due to time constraints, only five out of nine combinations of input parameter values (1 – 5) are examined within this research. Out of three DASbox campaigns the first included experiments 1, 6 and 9. The second included experiment 3, 7 with a repetition of 9. The final campaign compared only one variable, the aeration rate, while maintaining the agitation rate at 300 rpm and dissolved oxygen concentration at 60%. Therefore, no statistical analysis of the outputs is performed.

2.3.3 DASbox with shear agents

The effect of shear protective agents on cell growth during stationary phase of cell cultivation is examined. Agents Pluronic F-68 (PF68) (10%, Sigma-Aldrich) and Antifoam C Emulsion (ACE) (Sigma-Aldrich) are selected. PF68 is known for protecting cells against shear due to a physical mechanism that lowers interfacial tension [39, 40]. ACE is an agent that reduces bubble formation, which leads to less bubble shear affecting cell growth during bioreactor cultivation. Around 118 hours after inoculation of bioreactors during the first DASbox campaign, either 5 mL PF68 or 0.15 mL ACE (10x diluted) is added. A third bioreactor represented the negative control, without the addition of shear agents. Operational settings are adjusted to an aeration rate of 3 sL/h, agitation rate of 300 rpm and dissolved oxygen concentration of 60%. Culture samples are retrieved and analyzed 48 hours later.

2.3.4 Multi-well plate with shear agents

A 24-deep well plate is used for comparison of the impact of shear agent cytotoxicity. Concentrations of ACE (100x diluted) between 5 – 50 ppm are examined. Further, a concentration of 0.1% wv PF68 is examined, which equals 10,000 ppm [41]. The shear agents are added inside the LAF to prevent contamination. The concentrations of the agents are determined based on threshold values found in literature and manufacturer suggestions (Table 6) [42-44].

Table 6. Composition of cell culture and shear agents ACE and PF68 in a 24-well plate in triplicates.

Additive	Volume agent [ppm]	Volume agent [μ L]	Volume culture [mL]
None (negative control)	0	0	1
ACE	5	0.5	“
“	10	1	“
“	20	2	“
“	30	3	“
“	50	5	“
Pluronic F-68	10,000	10	0.9

The 24-deep well plate is incubated at conditions equal to the shake flasks, while using a clamp system (Clamp System CR1801h, EnzyScreen, The Netherlands) to prevent culture spillage. The shaking speed was calculated according to the following equation described in literature [45]:

$$n = \sqrt{\frac{2h}{d} \times \frac{5.6 \times 10^7}{(d_0 + \frac{d}{y})}} \quad (1)$$

Here, the shaking speed n is determined by the filling height (h), the internal well diameter (d), the shaking diameter (d_0) and the diameter factor (y). For the selected conditions a filling height of 3.91 mm, internal well diameter of 16 mm, shaking diameter of 19 mm and estimated diameter factor of 4 are maintained. Based on the calculation a shaking speed of 200 rpm is maintained. The cell growth and viability are measured at 24 and 48 hours after inoculation to observe cell behavior during the lag phase.

2.3.5 Ethanol quantification

Samples are extracted from the first DASbox campaign for quantification of ethanol in the culture. A High-performance liquid chromatography (HPLC) procedure is performed with the 1260 Infinity HPLC (Agilent Technologies, US). Water, Milli-Q and fresh SCGM samples are included as a reference during the procedure.

2.4 Analytical methods

2.4.1 Morphology

Cell morphologies are analyzed using a microscope (EVOS M3000) to look for contamination. During cultivation microscope images of the samples were compared to previously captured cultures that visualized healthy, unhealthy and contaminated cultures of CCX.E10.

2.4.2 Cell count

Directly preceding the analysis methods, the samples were thoroughly mixed with a vortex machine (MS2 Minishaker, IKA®, Germany) or by mixing the total liquid volume with a pipet. The cell count (cell/mL), cell viability (%) and cell diameter (μm) values are determined with the NucleoCounter (Chemometec, Denmark) to calculate the viable cell density. During performance of the seed-train workflow the Countess (Thermo Fisher Scientific) is used for cell counts and viability, as it is a less expensive method. The product of the cell counts and cell viability represents the VCD of the analyzed culture sample.

During the second DASbox campaign it was discovered that the NucleoCounter measures background noise. The NucleoCounter counts cell with AO/DAPI. This is a dual-fluorescence method, where AO penetrates alive and dead cells to stain the nuclei green. DAPI is only capable of entering dead cells and stains them blue. It is hypothesized that AO/DAPI stains certain component in SCGM. The NucleoCounter generated mostly dead cell counts of SCGM samples. To correct the measurements for the background noise blank measurements are added to the analysis during the final DASbox campaign. For the cultivations triplicate blank samples are measured that are withdrawn prior to inoculation of the bioreactor. The resulting cell viabilities and viable cell densities are processed by calculating the number of viable and of dead cells. These numbers are subtracted from the viable and dead cell numbers found for the cell growth experiment samples. This led to an increase in viability of approximately 3%.

2.4.3 Osmolality

The osmolality is measured with the Osmomat 030 (Gonotec, Germany) to monitor the stability of the metabolic environment and observe possible osmotic shifts due to nutrient depletion or toxicity from waste accumulation.

2.4.4 Metabolites

The extracellular metabolite concentrations of cultivation samples without cells are determined employing the BioProfile® Flex2 (Nova Biomedical, US). Samples of approximately 250 µL were analyzed for glutamine, glutamate, glucose, lactate, ammonia concentrations using the Chemistry Module (Nova Biomedical). To process the measurement error of the Flex2 in the standard deviation calculations, triplicate blank samples are withdrawn from the bioreactors prior to inoculation. This is not performed for the shake flask experiment. The measurement error was 5% maximally for all measurements.

The analysis methods are described in more detail in the composed protocols (see Protocols, Cell Culture Analysis Methods from Additional Materials).

2.5 Data processing

Most of the data was processed using Microsoft Excel. The VCD served as a proxy for the biomass concentration required for the following equations. The natural logarithm of VCD is plotted against time to determine the exponential growth rate.

$$\ln\left(\frac{VCD_t}{VCD_0}\right) = \mu \cdot (t - t_0) \quad (2)$$

The variable VCD_0 represents the VCD at the moment of inoculation. The equation yields a linear trend for the exponential growth phase. A trend through at least three datapoints and r^2 -value > 0.99 is considered to indicate an exponential growth regime. The corresponding gradient of the trend represents the exponential growth rate μ (h^{-1}). The doubling time of the exponential growth regime is calculated based on this growth rate.

$$T_d = \frac{\ln(2)}{\mu} \quad (3)$$

Additionally, the growth rate for each interval is determined. The maximum growth rate is determined from the calculated interval growth rates.

$$\mu = \frac{\ln(VCD_{n+1}) - \ln(VCD_n)}{t_{n+1} - t_n} \quad (4)$$

With the VCD and measured metabolite concentrations the apparent yields of the cultivation are determined based on equations found in literature [27].

$$Y_{X/S} = \frac{x(t_{n+1}) - x(t_n)}{c_S(t_n) - c_S(t_{n+1})} \quad (5)$$

$$Y_{X/P} = \frac{x(t_{n+1}) - x(t_n)}{c_P(t_{n+1}) - c_P(t_n)} \quad (6)$$

$$Y_{P/S} = \frac{c_P(t_{n+1}) - c_P(t_n)}{c_S(t_n) - c_S(t_{n+1})} \quad (7)$$

Where x is the viable cell density, c_S the substrate concentration and n the sampling time point. These yield coefficients are plotted against time to analyze the metabolic performance during cell growth.

Additionally, the cell-specific rates are calculated based on the number of cells per substrate or product yield coefficients:

$$q_S = \frac{\mu}{Y_{X/S}} \quad (8)$$

The rates are plotted against time to visualize and interpret the resulting regimes and gradients.

During this research the mean, standard deviation and standard deviation percentages are determined for all the retrieved data. A standard deviation above 34.1% indicates there is substantial fluctuation. These results are considered unreliable. Such results were not considered for this report.

The Flex2 and DASware data outputs are processed using Python scripts. A Python code editor (Visual Studio Code, US) is used. For both, Python scripts and Jupyter Notebooks were developed to clean, process and visualize the data (See Additional Materials, Python Scripts and Notebooks). The script used for Flex2 data processing determines the average metabolite concentrations and standard deviation for each datapoint. It detects and processes duplicates or triplicates. An option is provided to add measurement error to the standard deviations. The code defines the error from an analyzed reference sample to the standard deviation values. The script for the DASware data selects the desired variables and fills all the data gaps with the previously measured value if available. If not available, it takes the initially measured value for the preceding data gaps. Plots are created from the processed data of each selected variable against time.

2.6 Scale-up metrics

To correlate the shaking speed of the flasks to the agitation rate in the bioreactor the scale-up metric volumetric power input-ratio (P/V-ratio) is employed. Equations to determine the P/V-ratio are adapted from literature [38, 46, 47]. For the utilization of these equations, it is assumed the culture is shaken in-phase.

$$\frac{P}{V_L} = \bar{\varepsilon} \cdot \rho \quad (9)$$

The volumetric-power input $\frac{P}{V_L}$ in (W/m³) is related to the mean energy dissipation rate $\bar{\varepsilon}$ (W/kg) and density of the medium ρ (kg/m³).

$$\bar{\varepsilon} = Ne' \cdot n^3 \cdot d^4 \cdot V_L^{-2/3} \quad (10)$$

The average energy dissipation rate $\bar{\varepsilon}$ (W/kg) is calculated by the modified power number Ne' , the shaking speed n (s⁻¹), maximum inside shaking flask diameter d of the 125 mL Erlenmeyer shake flask (m) and the culture volume V_L (m³).

$$Ne' = 70 \cdot Re^{-1} + 25 \cdot Re^{-0.6} + 1.5 \cdot Re^{-0.2} \quad (11)$$

The modified power number Ne' is based on the dimensionless Reynolds number Re .

$$Re = \frac{\rho \cdot n \cdot d^2}{\mu} \quad (12)$$

The fluid density of SCGM is determined with a density meter (DMA™ 5000 M, Anton Paar, Austria). The dynamic fluid viscosity μ (Pa.s) is determined using a 1.5 g steel ball in a microviscometer (Lovis 2000 ME, Anton Paar).

Accordingly, the P/V-ratio derived for the shake flask conditions is used to determine the agitation rate N (rps) from the equation for P/V-ratio described in literature [48].

$$\frac{P}{V_L} = (N_p \cdot \rho \cdot N^3 \cdot d^5)/V_L \quad (13)$$

The agitation rate N (rps) is calculated from P/V-ratio, the dimensionless impeller power number (Newton number) N_p , the liquid density ρ (kg/m³), the impeller outer diameter d (m) and filling volume V_L (m³).

3. Results

3.1 Seed-train protocol

3.1.1 Filling volume and shaking speed

To find the optimal filling volume and shaking speed conditions in shake flasks, several filling volumes were examined at varying shaking speeds. Optimal liquid distributions were captured by camera for five filling volume setpoints (Appendix, Table 11). Based on the observations the optimal filling volume and shaking speed combinations were determined (Table 7).

Table 7. Optimal setpoints for filling volume and shaking speed in non-baffled shake flasks based on analyzed recordings).

Parameter	Setpoint				
Filling volume [mL]	12.5	18.75	25	31.25	37.5
Shaking speed [rpm]	140	175	205	270	300

A study that reported similar shake flask cultivation conditions used a filling volume of 12.5 mL (10% wv) with a shaking speed of 150 rpm [46]. Due to minimum VCD requirements for the inoculation of the DASbox bioreactor, a filling volume larger than 10% wv was preferred. Based on these considerations it was decided to proceed with a filling volume of 25 mL and shaking speed of 205 rpm as operational conditions for shake flask cultivation.

3.1.2 Baffled versus non-baffled shake flasks

To determine the growth rate, cell viability and VCD of CCX.E10 a cell growth curve was analyzed. The cells adapted to the shake flask environment for approximately 10 days before analysis was initiated. The cells were cultivated for 11 days in non-baffled (Figure 5) and baffled shake flasks (Figure 6) to compare growth rates and cell viabilities.

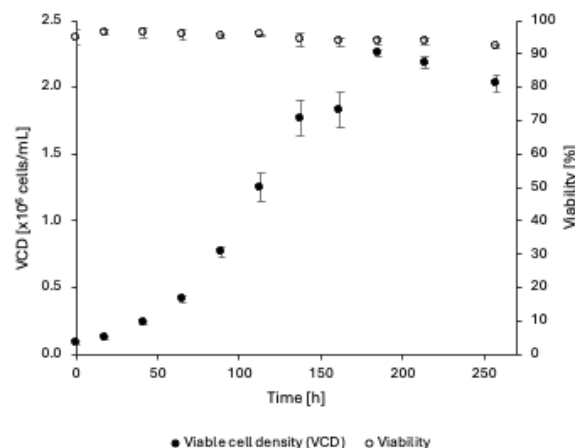


Figure 5. Growth curve for a 255 hour cultivation in non-baffled flasks with an inoculation cell density of 0.1×10^6 cells/mL (original data is available in Appendix, Table 12).

A lag phase of 18 hours was observed. The exponential growth regime was determined to be between 18 – 113 hours (Appendix, Figure 22). The exponential growth rate was 0.025 h^{-1} with a doubling time of 27.3 h. The maximum growth rate was 0.028 h^{-1} . The maximum VCD is 2.26×10^6 cells/mL. The cell viability ranged between 92.1 – 96.4%. After 113 hours a growth rate decrease was observed. Between 162 – 186 hours the growth rate increased again, before a stationary phase and death phase is observed. The osmolality ranged between 0.260 – 0.275 osmol/kg H_2O . In subsequent experiments the osmolality is only mentioned when it differs from the observed range.

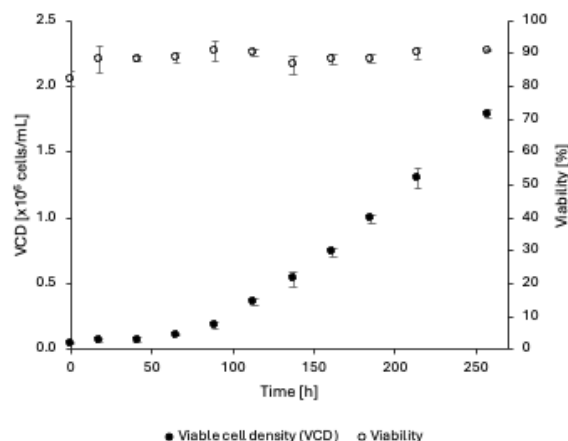


Figure 6. Growth curve for a 255 hour cultivation in baffled shake flasks with an inoculation cell density of 0.1×10^6 cells/mL (original data is available in Appendix, Table 14).

A lag phase of 65 hours was observed. The exponential cell growth phase lasted from 65 to 137 hours (Appendix, Figure 24). The growth rate was 0.023 h^{-1} with a doubling time of 29.7 h. The maximum growth rate was 0.027 h^{-1} . The maximum VCD was 1.79×10^6 cells/mL. The cell viability ranged between 82 – 90.8%.

Due to the shorter lag phase, higher growth rate and higher maximum VCD in non-baffled shake flasks compared to baffled flasks, it was decided to proceed the research with non-baffled shake flasks. Moving onward these are specified as ‘shake flasks’.

3.1.3 Inoculation density effect

The cell bank vials of CCX.E10 contain cells with a cell density of 7.5×10^6 cells/mL. This leads to an inoculation cell density of 0.3×10^6 cells/mL in shake flask with a filling volume of 25 mL. Cell growth was analyzed after inoculation of shake flasks with this cell density over a period of 3 days (Figure 7). The adaptation duration of the cells was approximately 20 days.

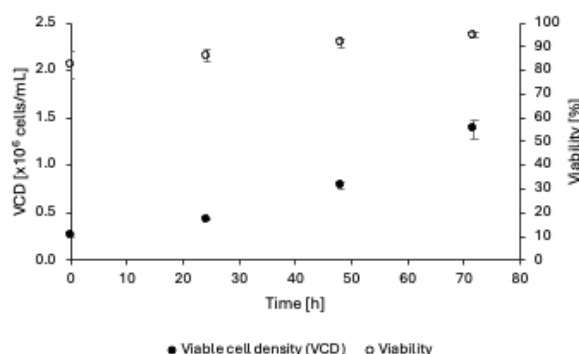


Figure 7. Growth curve analysis for 70 hours with an inoculation cell density of 0.3×10^6 cells/mL (original data is available in Appendix, Table 16).

A lag phase of 24 hours was observed. The exponential growth rate was determined at 0.025 h^{-1} during the exponential phase between 24 – 72 hours after inoculation (Appendix, Figure 22). The doubling time was 28.3 h. The maximum measured VCD was 1.38×10^6 cells/mL. The viability lied between 82.1 – 94.8 %. The found exponential growth rate was equal to the growth rate found previously when the cultivation was inoculated with a cell density of 0.1×10^6 cells/mL (Figure 5).

It was preferred to inoculate a shake flask with the total cell bank volume for subsequent experiments with thawed cells. Therefore, the decision was made to continue the improvements of the seed-train workflow with integrating the inoculation of shake flasks with a viable cell density of 0.3×10^6 cells/mL.

3.1.4 Cell growth thawed cells

The examined cells in previous experiments were adapted to shake flask conditions prior to cell growth analysis. To investigate whether CCX.E10 cells from the cell bank can reach an exponential growth regime and cell viabilities above 90%, the cell growth of thawed cells was analyzed (Figure 8).

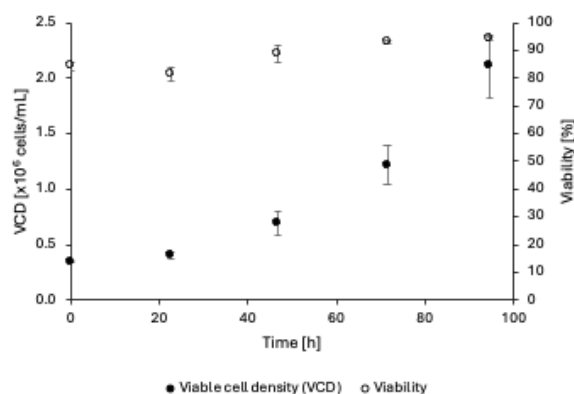


Figure 8. Growth curve for thawed cells for 95 hours with an inoculation cell density of 0.3×10^6 cells/mL (original data is available in Appendix, Table 18).

The growth rate of CCX.E10 was 0.023 h^{-1} and the doubling time 30.3 h during the exponential regime between 23 – 95 hours (Appendix, Figure 24). The maximum VCD was 2.11×10^6 cells/mL. The cell viability lied between 81.5 and 94.1%. Since the exponential phase and cell viability of 90% were reached according to the requirements, follow-up experiments were continued initiating with thawed cells.

3.1.5 Effect of shaking speed on thawed cells

To improve the cell growth and duration of the adaptation phase of CCX.E10 cells after vial thawing, a shaking speed of 60 rpm was compared to static cultivation, which was suggested by the cell line manufacturer. The viability was determined for both shaking conditions one day after vial thawing with an inoculum of 0.1×10^6 cells/mL. The cell viability after 1 day was 89% at 60 rpm compared to 81% for the static condition. Accordingly, it was decided to integrate a shaking speed of 60 rpm during the cultivation of thawed cells the first 24 hours of the seed-train instead of the static condition.

3.1.6 Seed-train

The seed-train that is composed after interpretation of the final laboratory results consists of three stages from cell bank to shake flask to DASbox bioreactor. The initial triplicate shake flasks are inoculated with thawed cells with an inoculation cell density of 0.1×10^6 cells/mL. The first 24 hours the flasks are shaken at 60 rpm. Afterwards the shaking speeds is maintained at 205 rpm. Cells are passaged every 4 days with an inoculation density of 0.1×10^6 cells/mL. The DASbox bioreactors are inoculated with a VCD of 0.6×10^6 cells/mL (Figure 9, Table 8).

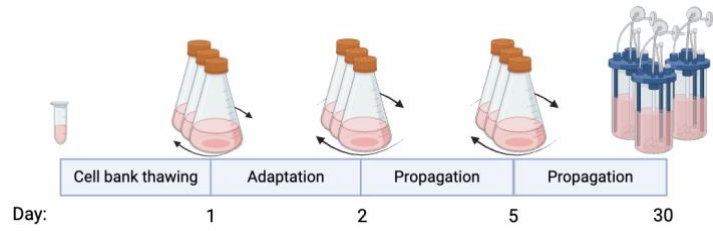


Figure 9. Seed-train for gradual volume increase from cell bank (1 mL) to DASbox bioreactor (300 mL).

Table 8. Seed-train workflow conditions.

Stage	Vessel	Total Volume [mL]	Filling volume [mL]	Inoculation cell density [$\times 10^6$ cells/mL]	Shaking speed [rpm]	Adaptation [d]
1	Cell bank	1.5	1	7.5	-	-
2	Shake flask	125 (x3)	25 (x3)	0.1 / 0.3	60 / 205	30
3	Bioreactor	250 (x3)	100 (x3)	0.6	-	-

3.1.7 Metabolite analysis

The metabolite concentrations measured during shake flask cultivation were plotted for an initial analysis of growth limiting factors, such as nutrient (glucose, glutamine) depletion and waste (lactate, ammonium) accumulation (Figure 10).

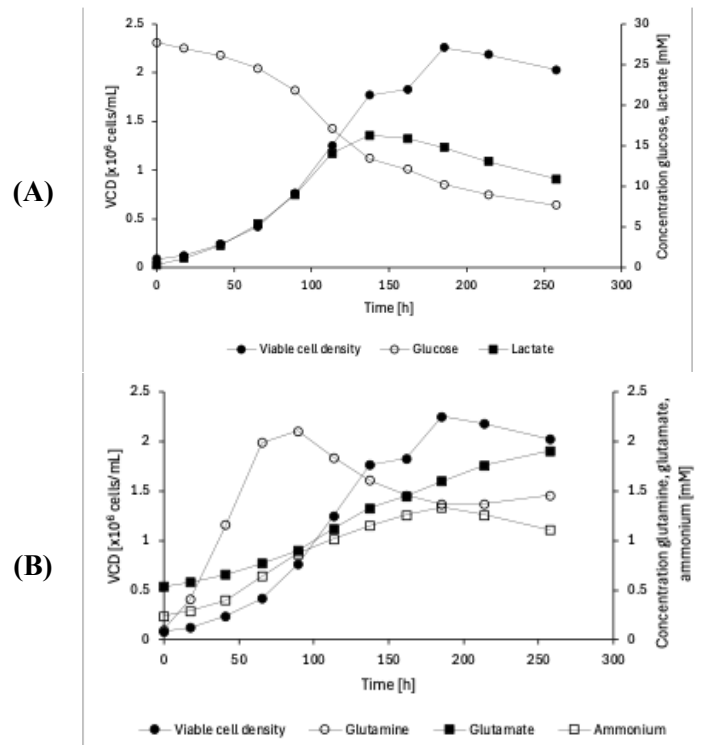


Figure 10. VCD and metabolite concentrations over time for cultivation in shake flasks for 11 days. (A) Concentrations of glucose and lactate until 30 mM. (B) Concentrations of glutamine, glutamate and ammonia until 2.5 mM. The exponential growth phase lasted between 18 – 113 hours.

During the exponential growth phase between 18 and 113 hours the concentration of the substrate glucose was reduced from 27 to 17.1 mM. No depletion of glucose was observed. The concentration of produced lactate reached a maximum at 137 hours of 16.28 mM. Afterwards, the concentration decreased. The concentration of produced ammonium increased until a value of 1.34 mM at 185 hours and decreased afterwards.

3.1.7.1 Cell-specific rates

To delve further in the metabolic regimes and shifts during the cultivation, the cell-specific consumption and production rates of glucose, lactate and ammonium were plotted against time (Figure 11).

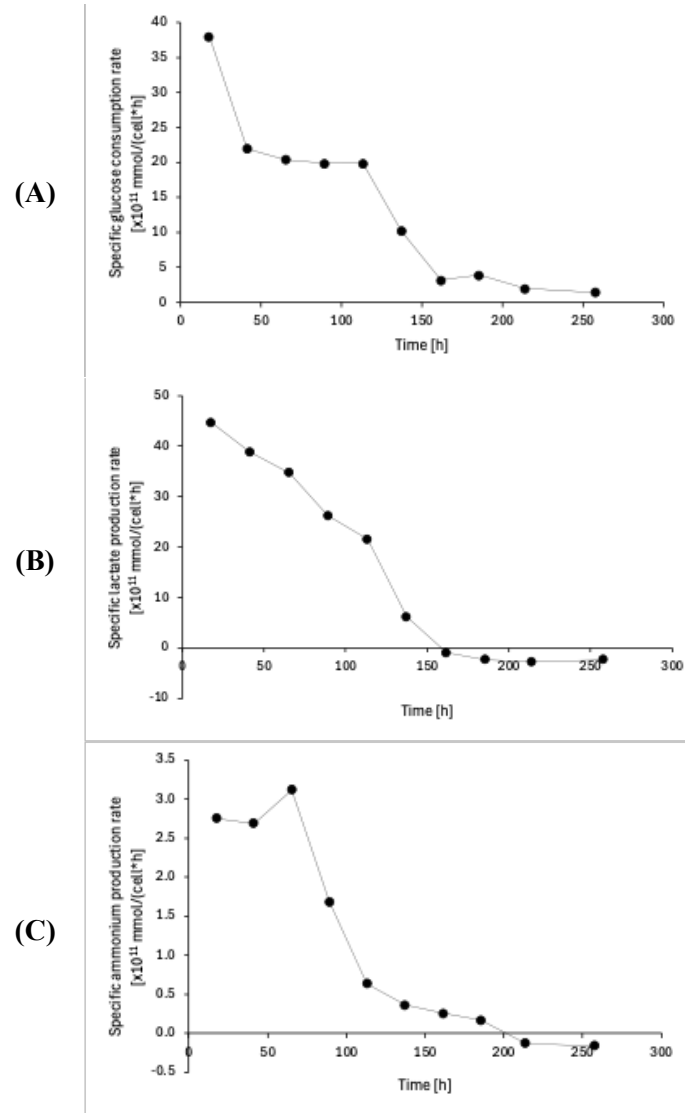


Figure 11. Cell-specific consumption and production rates of (A) glucose, (B) lactate and (C) ammonium during shake flask cultivation of CCX.E10 in SCGM. Exponential growth lasted between 18 – 113 hours.

A decrease of the cell-specific consumption and production rates of glucose and lactate was observed from the maximum values determined in the lag phase. The initial glucose rate was equal to 38×10^{11} mmol/(cell*h) after the initial 18 hours when it shifted downwards to 20×10^{11} mmol/(cell*h). From there, a consistent rate was observed between 41 – 113 hours, which likely represents the exponential cell growth phase. It shifted between 113 – 162 hours to 10×10^{11} mmol/(cell*h) after which it remained similar.

The initial lactate rate was 45×10^{11} mmol/(cell*h). It gradually decreased to 22×10^{11} mmol/(cell*h), until a downward shift between 113 and 137 hours to 6.2×10^{11} mmol/(cell*h). From 162 hours the rate decreased to below zero.

The initial ammonium consumption rate appeared consistent at 2.7×10^{11} mmol/(cell*h) from initiation to 41 hours. It increased to a maximum of 3.1×10^{11} mmol/(cell*h) between 41 – 65 hours. A downward shift was observed between 65 – 113 hours to 0.6×10^{11} mmol/(cell*h). Afterwards, the ammonium rate gradually decreased and between 186 – 220 it reduced to a rate below zero.

3.1.7.2 Apparent lactate over glucose yield

To further investigate the metabolic efficiency of the cell the yield-coefficient of lactate production over glucose consumption was plotted against time (Figure 12).

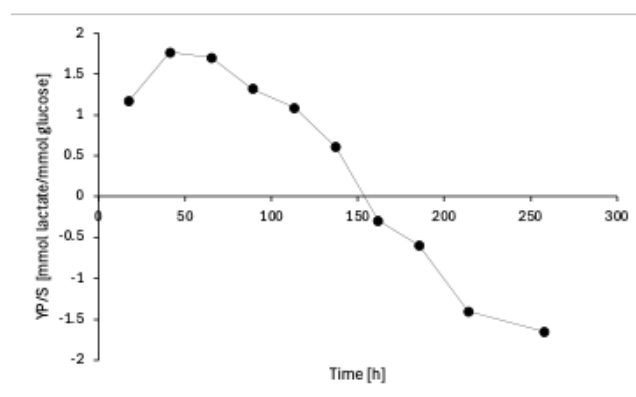


Figure 12. Yield-coefficient for lactate production over glucose consumption in mmol lactate per mmol glucose. Exponential growth lasted between 18 – 113 hours.

An initial value of 1.2 was observed for the yield-coefficient after the initial 18 hours. It increased to a maximum of 1.75 between 18 – 41 hours. Between 65 – 113 hours it gradually decreased towards a value of 1.1. The coefficient decreased below 1 between 113 – 137 hours. It decreased below 0 between 137 – 162 hours.

3.2 DASbox cell line characterization

3.2.1 Effect inoculation density of 0.05×10^6 cells/mL

To determine the specific rates of CCX.E10 during bioreactor cultivation the first DASbox campaign was initiated with an inoculation VCD of 0.05×10^6 cells/mL. The batch cultivations in the reactors were performed with three different agitation and aeration setpoints according to experiment number 1, 6 and 9 from the DoE approach (Table 5). For all cultivations this led to an apparent death phase before an exponential growth regime was discovered. The osmolality values of the bioreactor cultivations ranged between 0.280 and 0.300 osmol/kg H₂O. In subsequent experiments solely osmolality values outside of this range are mentioned. The cell viability values prior to inoculation were around 80%, thus below the required 90%. This suggests the cell viability was too low before the transfer and the cells were not able to recover at the new cultivation conditions in the bioreactor. This led to the hypotheses the cells endured too much stress due to the air bubbles, insufficient pH control or ethanol build up from sterile practices. Accordingly, follow-up experiments were performed.

3.2.1.1 Comparison effect ACE and PF68

To find out whether cells were able to recover and cell viabilities increase, shear agents were added to the bioreactors. The shear agents ACE and PF68 were added to separate bioreactors for comparison from the moment a stationary phase was observed 118 hours after inoculation. The cell viabilities before addition ranged between 35 – 57%. While it was observed the amount of foam immediately decreased after the addition of the protective agents, the cell viabilities maintained a decrease. This may indicate the cells had endured too much stress and were not able to recover anymore.

3.2.1.2 Ethanol presence in bioreactor

To determine the concentration of ethanol in the cell cultures during the batch cultivation and samples were analyzed with HPLC (Table 9).

Table 9. Ethanol quantities in cell culture samples withdrawn 170 hours after initiation batch cultivation in the bioreactor.

Sample	Ethanol conc. [mM]
SCGM	0
Unit 1	2.42
Unit 2	0
Unit 3	0.54

The maximum ethanol concentration was found in Unit 1 with a value of 2.42 mM. This is equal to 0.01% v/v. In literature, ethanol concentrations above 0.1% v/v showed inhibitory effects [49, 50]. This suggests other factors besides ethanol presence had a more significant impact on the observed cell growth regimes. Nevertheless, the sterile practices were adjusted to reduce the amount of ethanol entering the bioreactors during batch cultivation in the bioreactors.

Together, the results led to the hypothesis that the inoculation density of 0.05×10^6 cells/mL had been too low for the cells to endure the different conditions in bioreactors.

3.2.2 Effect inoculation density of 0.15×10^6 cells/mL

To investigate whether an increased inoculation cell density positively influences the cell growth of CCX.E10 the bioreactors were inoculated with 0.15×10^6 cells/mL. The bioreactors were operated at agitation, aeration and dissolved oxygen setpoints for experiment numbers 3, 7 and 9 from the DoE approach (Table 5). Unfortunately, for these operational conditions the exponential cell growth phase was not observed in the bioreactor. Since the cells were not able to recover after being transferred to the new environment, this led to the hypotheses that the mechanical shear negatively impacted the cell growth, that the formation of foaming led to shear stress and that the seeding cell density of 0.15×10^6 cells/mL was too low for the cells to create a steady growth environment. These hypotheses were further investigated.

3.2.2.1 Volumetric power input-ratio correlation

Batch cultivation in shake flasks repeatably resulted in observed exponential cell growth regimes, while it was not yet observed in bioreactors. To understand the differences of shear stress induced on the cells the shear from mechanical forces was correlated for both cultivation configurations. The scale-up metric of P/V-ratio was used to determine the agitation rate in the bioreactor inducing equivalent mechanical shear in the shake flasks (Section A.3.4). For the calculation the fluid density and viscosity of SCGM were determined at 1001 kg/m^3 and 0.732 mPa.s . These values were validated with values found in literature for other media that are suitable for animal cell lines suspension cultures [51].

Within the range of the assumed values of the impeller power number the calculation led to an agitation rate between 456 and 575 rpm. Within this range the mechanical shear stress was expected to correspond to the shear stress from the shake flask conditions. The calculated lower bound agitation rate of 456 rpm was higher than the earlier examined agitation rates of 100 and 300 rpm. The calculation suggested that the impact from mechanical shear stress on cell growth is not the root cause for the absence of exponential cell growth. With this calculation the shear stress from foaming and bubble breaks is not explained. To improve understanding of shear stress from bubble formation comparison of concentrations of foam reducing agents is required.

3.2.2.2 Effect ACE and PF68 on cell growth in multi well plate

The addition of the shear protective agents ACE and PF68 might protect cells from shear stress that originates from foam formation and bubble breaks during bioreactor cultivation. Although, the agent ACE is expected to exhibit cytotoxic effects that inhibit cell growth. To find the maximum concentration of ACE at which cell growth is not inhibited, the impact of several concentrations ACE on cell growth during cell cultivation in a multi well plate cultivation is compared. PF68 is not expected to inhibit cell growth and is added for validation (Figure 13).

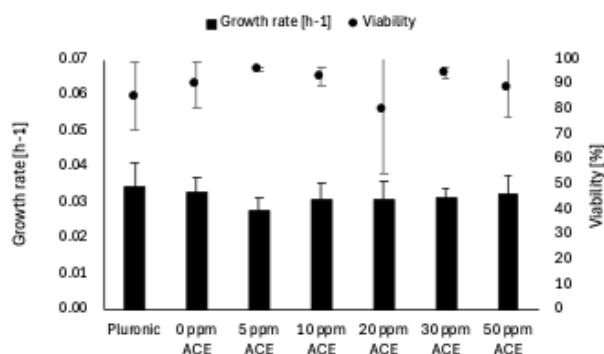


Figure 13. Growth rates and cell viabilities between between 45 and 110 hours after addition of PF68 and different concentrations ACE.

An increase in the concentration of ACE did not result in observed cell growth inhibition when comparing concentrations of 0 ppm and 50 ppm. The growth rate for 0 ppm is determined at 0.033 h⁻¹ with a cell viability of 90%. At 50 ppm the growth rate was 0.032 h⁻¹ with a viability of 89%. All growth rates ranged between 0.028 – 0.033 h⁻¹. This may indicate that the cells can grow in the presence of the cytotoxicity caused by the additive. The addition of PF68 showed a slightly higher cell growth of 0.034 h⁻¹. For the subsequent experiment, it was decided to add 10 ppm ACE each time foaming occurs in the bioreactor to remain below an accumulated concentration of 50 ppm and reduce foam formation over the duration of the bioreactor cultivation.

3.2.3 Effect inoculation density of 0.6x10⁶ cells/mL

Due to time constraints this was the final DASbox campaign of the research. Since the previous campaigns did not result in any observed exponential cell growth during cultivation, it was decided to abandon the DoE approach and perform the current campaign according to a One-Factor-At-A-Time experiment. The variable investigated was the aeration rate. Values of 1, 3 and 5 sL/h were examined. The cultivation was initiated with an inoculation cell density of 0.6x10⁶ cells/mL. The inoculum consisted of cells that were continuously cultivated for 27 days in shake flasks and passaged 6 times. The cells doubled more than ten times after thawing of the cell bank. It was expected that the cells were more adapted to cultivation conditions than the cells analyzed in the previous campaigns.

Cell growth was observed in one of the three bioreactors with a gas flow rate of 5 sL/h. This may indicate the operational conditions in the other bioreactors induced more shear stress, which is unexpected because the gas flow rates of 1 and 3 sL/h cause less bubble formation. Prior to inoculation, these two bioreactors were accidentally installed in the opposite position in the DASbox system. Measured outputs of one bioreactor led to control of the inputs of the other and vice versa. This resulted in extreme temperature values between 20 – 45 °C and pH values between 5.5 – 8.5. These extremes might have degraded growth factors or other components in the medium, which might be an explanation for the absent exponential growth phase for cultivation in these bioreactors. Accordingly, only the cultivation with a gas flow rate of 5 sL/h was further analyzed for cell growth and viability (Figure 14).

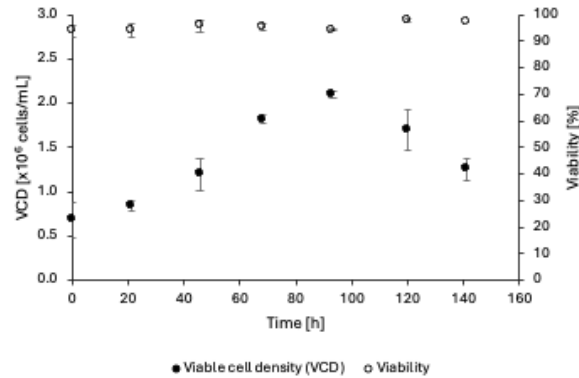


Figure 14. Growth curve for batch cultivation in a bioreactor for 170 hours with an inoculation cell density of 0.6×10^6 cells/mL. The operational conditions included an aeration rate of 5 sL/h, agitation rate of 300 rpm and dissolved oxygen concentration of 60%.

The exponential growth regime was observed between 21 – 93 hours. During the exponential growth phase, the cell growth rate was 0.016 h^{-1} and doubling time 42.3 h. The cell viability remained above 90%. The osmolality ranged between 0.269 and 0.292 osmol/kg H_2O . The maximum VCD was 2.10×10^6 cells/mL.

3.2.4 Metabolite analysis

Similarly as for shake flasks, the metabolite concentrations present during bioreactor cultivation were plotted for an initial analysis of growth limiting factors, such as nutrient (glucose, glutamine) depletion and waste (lactate, ammonium) accumulation (Figure 15).

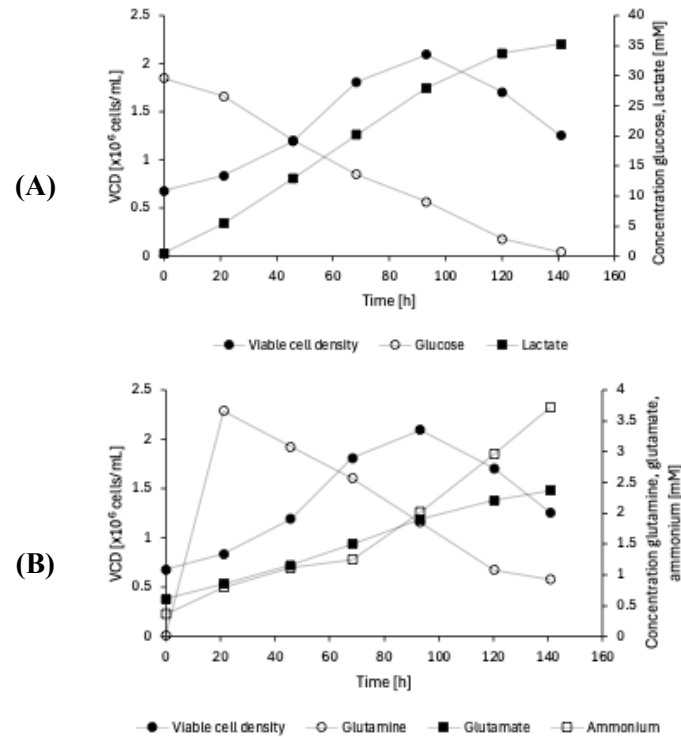


Figure 15. VCD and metabolite concentrations for a 7 day cultivation in a bioreactor. (A) Concentrations of glucose and lactate ranged until 40 mM. (B) Concentrations of glutamine, glutamate and ammonium ranged until 4 mM. The exponential growth phase lasted between 21 – 68 hours.

During the exponential growth phase, the substrate concentration of glucose decreased from 30 to 20 mM. Product concentrations observed at the end of the growth phase were 20 mM for lactate and 1.3 mM for ammonium.

3.2.4.1 Cell-specific rates

To analyze the metabolic regimes and shifts during bioreactor cultivation, the cell-specific consumption and production rates of glucose, lactate and ammonium were plotted against time (Figure 16).

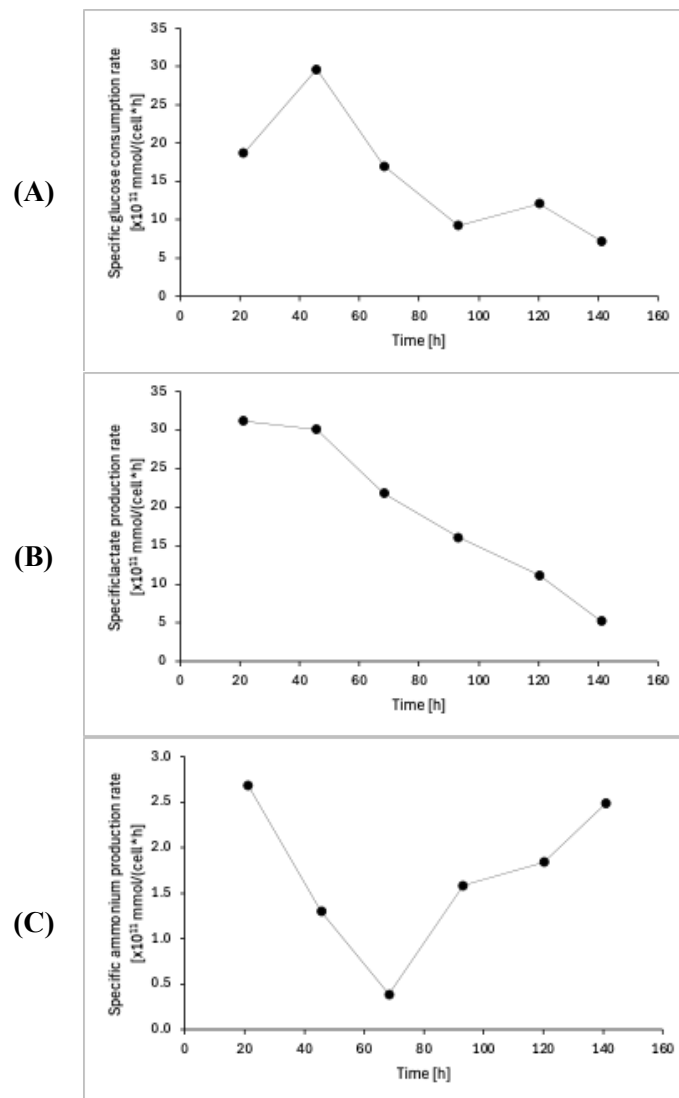


Figure 16. Cell-specific substrate consumption and waste production rates for (A) glucose, (B) lactate and (C) ammonium during bioreactor cultivation. The exponential growth phase lasted between 21 – 68 hours.

An increase of the cell-specific glucose consumption rate from 19×10^{11} to 30×10^{11} cell/(mol $\cdot$ h) was observed between 20 – 46 hours. Afterwards, the rate shifted downward between 46 – 93 hours to 9×10^{11} cell/(mol $\cdot$ h). From there, it increased to 12×10^{11} cell/(mol $\cdot$ h) until 120 hours. After, a maintained decrease was observed to 7.2×10^{11} cell/(mol $\cdot$ h).

The cell-specific lactate consumption rate was observed to be consistent around 31×10^{11} cell/(mol $\cdot$ h) until 46 hours, which may correspond to the exponential cell growth phase. Then it shifted down to 22×10^{11} cell/(mol $\cdot$ h) between 46 – 68 hours. Afterwards, the rate maintained a gradual decrease to 5×10^{11} cell/(mol $\cdot$ h) until 141 hours.

Initially, for the cell-specific ammonium rate a maximum value of 2.7×10^{11} cell/(mol $\cdot$ h) was observed. Afterwards, it decreased between 21 – 68 hours to 0.4×10^{11} cell/(mol $\cdot$ h). Subsequently, a shift upward is observed to 1.6×10^{11} cell/(mol $\cdot$ h) until 93 hours. The rate maintained an increase to a value of 2.5×10^{11} cell/(mol $\cdot$ h) until the end at 141 hours.

3.2.4.2 Apparent lactate over glucose yield

To further investigate the metabolic efficiency of the cell the yield-coefficient of lactate production over glucose consumption was plotted (Figure 17).

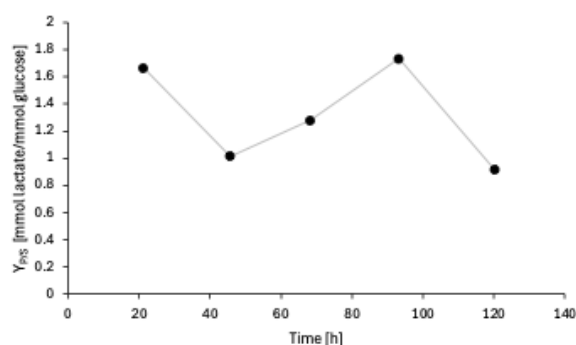


Figure 17. Yield-coefficient of the amount of product lactate over the amount of substrate glucose in mmol lactate per mmol glucose. The exponential growth phase lasted between 21 – 68 hours.

An initial lactate over glucose yield-coefficient of 1.67 was observed. It decreased to 1 between 21 – 46 hours. Between 46 – 93 hours it increased to a maximum value of 1.73, after which it decreased to a yield-coefficient value of 0.9.

The previous and last DASbox campaign allowed for refinement of the seed-train workflow (Table 8) and DASbox Standard-Operating-Procedure (from Additional Materials, see Protocols, DASbox procedure). The setpoint conditions and PI control methods employed while utilizing the DASware were condensed in an overview (see Appendix, Table 10).

4. Discussion

The research reflected in this paper addressed several research gaps by investigating the required conditions for a gradual volume increase during cultivation of cryopreserved cells of cell line CCX.E10 in shake flasks to inoculate a DASbox bioreactor system. Accordingly, the research was intended to show the potential of this cell line for commercial scale cultivated meat production. While previous studies investigated cultivated meat production based on various other animal cell lines, the quail suspension cell line CCX.E10 for the same purpose has not been investigated yet [52, 53]. Cultivated meat production requires bioprocess conditions that are of a quality suitable for human consumption. The cell line preferably is not genetically modified, does not require animal components in the growth medium and is spontaneously immortalized [20]. Although literature on large-scale bioprocesses that utilize cell line CCX.E10 is available, these studies focus on maximizing viral titer production for vaccines instead of prioritizing cell growth [27]. Moreover, a seed-train protocol that utilizes a DASbox bioreactor system for metabolic characterization of a cell bank was not available in our laboratories yet.

4.1 Key findings and comparison with existing literature

4.1.1 Metabolic behavior and waste accumulation

4.1.1.1 Lactate and ammonium accumulation

At the end of the exponential growth phase in shake flasks a concentration of 14 mM lactate and 1 mM ammonium was measured (Figure 10). In the bioreactor, concentrations of 20 mM lactate and 1.2 mM ammonium were observed (Figure 15). Afterwards, in both cultivations a stationary phase is observed. It is likely, that the accumulation of these waste metabolites inhibited cell growth. In literature a concentration of 20 mM lactate and 2 – 3 mM ammonium are adopted as a threshold for animal cell cultivation to limit cell growth [54]. The study elaborates that an inefficient overflow metabolism leads to lactate and ammonia production, where high concentrations of glucose lead to rapid conversion into lactate. Except for the measured lactate concentration in the bioreactor, the observed lactate and ammonium concentrations are below the thresholds. The paper highlights that the threshold differs per cell line and operating conditions. This may indicate the cell growth is affected by another factor beside waste accumulation.

4.1.1.2 Warburg effect

A lactate over glucose yield-coefficient above 1 was observed during the exponential cell growth of cultivation in both shake flask and bioreactor (Figure 12 and Figure 17). This suggests the cells produced more lactate than they consumed glucose during cell growth. In literature a similar observation is described as the Warburg effect [55, 56]. This effect is defined by the conversion of glucose to lactate to generate energy through aerobic glycolysis instead of oxidative phosphorylation, while sufficient oxygen levels are present. Through aerobic glycolysis 4 Adenosine Triphosphate (ATP) are generated per glucose. Through oxidative phosphorylation up to 36 ATP are generated without the production of toxic metabolites. This appears as an inefficient cell metabolism during cell proliferation. The Warburg effect might lead to cell growth inhibition from lactate accumulation. In an already mentioned paper that described the cultivation of CCX.E10, nutrient depletion seemed to influence cell growth instead of lactate accumulation [30]. This suggests the cells observed in the study did not exhibit the Warburg effect or the influence of the effect was less noticeable. The observations of the study likely differed due to distinct employed operating conditions, including bioreactor size, filling volume, agitation rate and aeration rate.

4.1.1.3 Glucose residue

At the end of the exponential phase, glucose concentrations of 17 and 14 mM were observed during cultivation in shake flask and bioreactor, respectively. At the beginning of cultivation glucose concentrations of 28 mM and 30 mM were observed (Figure 10 and Figure 15). Literature suggests that the available glucose concentration during cultivation influences the observation of the Warburg effect. It was demonstrated that high glucose concentrations ($>5\text{mM}$) lead to the production of lactate and low concentrations may lead to lactate consumption [57, 58]. In the current study, the specific lactate production rates observed in shake flask and bioreactor cultivation show a negative gradient from the start (Figure 11B and Figure 16B). This suggests the lactate consumption rate is influenced by another variable than solely the available glucose concentration.

4.1.2 Growth kinetics in shake flasks

In three varied shake flask experiments exponential growth rates between $0.023 - 0.025 \text{ h}^{-1}$ were observed for inoculation densities of 0.1×10^6 and 0.3×10^6 cells/mL with a filling volume of 25 mL and shaking speed of 205 rpm (Figure 5, Figure 7 and Figure 8). This suggests that the inoculation densities and operational settings allow for cell growth in shake flasks. The growth rates correspond to the growth rate range of $0.026 \pm 0.003 \text{ h}^{-1}$ found in literature [27, 30].

A maximum VCD of 2.3×10^6 cells/mL was observed at the end of the exponential growth phase for cultivation in shake flasks (Figure 5). For cultivation in baffled shake flasks a maximum VCD of 1.79×10^6 cells/mL was found (Figure 6). Prior to the shake flask analyses, the cells were cultivated for less than two weeks to adapt to shake flask conditions. In literature a VCD of 4.4×10^6 cells/mL is described [30]. This suggests that the employed operational conditions were sub-optimal for cell growth. Several reported operating conditions differed. In the study baffled shake flasks were inoculated with a cell density of 0.8×10^6 cells/mL. Additionally, the cells were adapted to the shake flask environment by continuously cultivating the cells for 30 days and passaging the cells 10 times before initiating the analysis. In the current research the VCD observed in baffled shake flasks was lower than the VCD in non-baffled shake flasks. This may indicate that not the shake flask type but the implementation of a cell adaptation of 30 days or the higher inoculation density of 0.8×10^6 cells/mL had caused the higher VCD described in literature.

4.1.3 Bioreactor performance

An inoculation of 0.6×10^6 cells/mL for cultivation in the DASbox bioreactor showed an exponential growth phase (Figure 14). This suggests that cultivation of cell line CCX.E10 requires higher inoculation cell densities than the 0.3×10^6 cells/mL in shake flasks. Literature reported an inoculation cell density of 0.8×10^6 cells/mL for stirred-tank reactors [27, 30]. This density is higher, which may indicate a further increase of the inoculation density might be beneficial for cell growth. Although, this implies the seed-train protocol requires integration of a prolonged cell proliferation or an additional cell passage.

Viabilities above 90% are observed at the beginning of the bioreactor cultivation (Figure 14). During the seed-train prior inoculation of the bioreactor a viability of 96.7% was measured (Appendix, Table 25). This may indicate that cells require a minimum viability of 90% during the seed-train before transferred to the bioreactor. In literature a viability above 90% is found during shake flask and bioreactor cultivation [30]. This corresponds to the found results and might validate the seed-train procedure to maintain a cell viability above 90%.

A maximum VCD of 2.1×10^6 cells/mL was observed (Figure 14). This suggests cell growth was inhibited. Literature reported a value of 4.2×10^6 cells/mL for batch cultivation in a stirred-tank reactor [30]. In this study the exponential growth phase transitioned to a stationary phase due to nutrient depletion of glucose and glutamine. In the current research nutrient depletion was not observed. It might differ due to the inoculation with cells adapted for over 30 days or the inoculation cell density of 0.8×10^6 cells/mL. This may indicate that the causes for cell growth inhibition in the current study did not apply to the cell growth found in literature.

The exponential growth rate during bioreactor cultivation was 0.016 h^{-1} (Figure 14). This may imply the operating conditions were not optimal. In literature a growth rate of 0.022 h^{-1} is reported [30]. The study employed a 1 L stirred tank reactor with a 350 mL filling volume at 130 rpm and aeration rate between 3 – 9 sL/h. This differs from the 250 mL reactor, 100 mL filling volume, 300 rpm and 5 sL/h employed in the current research (Appendix, Table 10). It suggests the setpoints for agitation, aeration and dissolved oxygen percentage were sub-optimal for exponential cell growth.

For all shake flask and bioreactor cultivations a lag phase of 20 hours was observed (Figure 5, Figure 7, Figure 8 and Figure 14). This may indicate a consistent adaptation of the cell line after passaging and transferring to a new environment. The exponential growth phase of cultivation in a shake flask lasted for 95 hours (Figure 5) and in the bioreactor for 72 hours (Figure 14). This suggests the cells behave differently in an alternate cultivation environment. In literature an exponential growth phase of 96 hours is found for cell cultivation in shake flasks and of 72 hours in a 1 L stirred-tank reactor at 130 rpm [30]. This corresponds to the found values of the current research. It is likely, that dissimilarities in operational conditions across different cultivation configurations lead to variance in the duration of exponential cell growth.

4.2 Limitations

4.2.1 Cell line adaptation

Previous comparison with research that described the cultivation of CCX.E10 in shake flasks and bioreactors, showed a distinct cell adaptation method was carried out [30]. The study described a 30 day cultivation, including 10 passages prior to shake flask analysis. The current study cultivated cells for 30 days with 6 passages prior to bioreactor cultivation (Appendix, Section A.3.6). The influence of an increased number of passages during shake flask adaptation was not investigated in the current research. Understanding of the possible positive impact of adaptation on cell growth and VCD requires additional experiments.

4.2.2 Seed-train and bioreactor conditions

The seed-train workflow prior to bioreactor cultivation that led to observed exponential cell growth of CCX.E10, was performed only once. The consistently observed exponential growth phases in shake flasks may indicate robustness of the seed-train protocol (Figure 5, Figure 7 and Figure 8). However, repetition of the identical seed-train is required to prove its reliability.

Out of the nine bioreactor experiments executed, only one experiment resulted in an observed exponential growth phase. Therefore, comparison of varied operational conditions across experiments is not possible. Investigation of the impact of different values of agitation rate, aeration rate and dissolved oxygen concentration on maximum VCD in bioreactor cultivation is limited.

4.2.3 Glutamine consumption

As mentioned previously, SCGM contains the component GlutaMAX. This is the brand name for the dipeptide L-alanyl-L-glutamine. It gradually releases glutamine in the presence of cells by the secretion of peptidases [35]. It is likely that during cultivation the glutamine concentration increases due to the gradual release, while it decreases due to nutrient consumption by the cells. Predicting the glutamine release is complicated due to the variables involved, varied glutamine consumption, VCD and operational conditions. This suggests specific glutamine consumption rates cannot be observed from glutamine concentration measurements alone, which limits understanding of the specific glutamine consumption rate. Moreover, it was found in literature that the lactate production seemed to be highly responding to the amount of glutamine present. It suggested that the lactate production is influenced by the glutaminolytic pathway as well, apart from aerobic glycolysis [59]. Together, it may indicate a method is required to quantify the concentration of glutamine to further understand the cell metabolism in the presence of GlutaMAX.

4.2.4 Mechanical and bubble shear

Another limitation is related to the induced shear stress during bioreactor cultivation. Based on the correlation of the P/V-ratio of shake flask cultivation to bioreactor cultivation an agitation rate of 450 rpm was found. This may correspond to the mechanical forces induced on cells from a shaking speed

of 205 rpm in shake flasks (Appendix, Section A.3.4). During bioreactor cultivation an agitation rate of 300 rpm was employed (Figure 14). This may indicate the agitation rate can be increased to induce the same amount of mechanical shear stress on the cells compared to shake flasks. Although, the bioreactor cultivation environment differs and shear stress is influenced by bubble shear from air sparging as well. An increased agitation rate increases the quantity of air bubbles and associated bubble breaks in the culture, which induces shear stress apart from mechanical forces [60]. This suggests that the aforementioned correlation of the P/V-ratio indicates only a share of the total amount of shear stress induced on the cells in a bioreactor. It may only be used as an approximation of the shear, while considering shear from air bubbles. Therefore, the interpretation of the influence of shear stress on cell growth remains limited.

4.2.5 Cytotoxicity of antifoaming agent

The addition of concentrations ACE between 5 – 50 ppm to cell culture showed no differences in specific growth rate compared to a negative control in a multi well plate (Figure 13). This may indicate that the cytotoxicity of the additive did not influence cell growth. A study that investigated the effects of ACE during bioreactor cultivation of animal cells suggested that a concentration higher than 10 ppm inhibits cell growth [61]. This might be different due to the operational conditions in a multi well plate or bioreactor. A multi well plate is not agitated or sparged with air. This may indicate cells experience increased shear stress in the bioreactor and a decreased resistance to cytotoxicity. Further research of the impact of antifoaming during bioreactor cultivation is required to understand the influence of the cytotoxicity on cell growth.

4.3 Implications for further research

4.3.1 Adaptation and passaging

In this research the cell adaptation in flasks involved less passages than cell adaptation of CCX.E10 described in literature [27, 30]. Although, another paper suggested that continuously cultivating and passaging cells increases the risk of genetic mutations [62]. It is advised to prevent changes in genotype continuous cell line cultivation by cultivating for 3 months maximum. Together, it might suggest that the current adaptation method of 27 days and 6 passages could benefit from additional passages and a longer duration. Research of an improved cell line adaptation procedure is recommended.

4.3.2 Operational conditions and control

Performance of the residual experiments of the DoE approach provided by this research is recommended. It may improve understanding of the interaction of the varied operating conditions and cell-specific rates and VCD. Literature suggests increased agitation and aeration rates cause metabolic alterations due to induced shear stress [63, 64]. This may indicate the operational conditions directly influence cellular metabolism. Therefore, through the DoE approach certain parameter setpoints might be found that lead to improved specific rates and VCD in bioreactor cultivation.

Lastly, PID control during bioreactor cultivation has shown to influence cell growth and specific lactate production rates when not properly tuned [65]. It is recommended for each set of operation conditions to tune the PI control prior to analysis. Therefore, a tuning protocol may be desired.

4.3.3 Glutamine quantification

A method is required to determine the accurate glutamine concentration during cell cultivation. A fluorescence detection method with precolumn derivatization of samples with o-phthalaldehyde in an HPLC system showed high sensitivity for detection of GlutaMAX [66-68]. As described in literature, this method might be suitable to acquire metabolite concentrations in cultivation samples. This method is recommended for further characterization of metabolic behavior of CCX.E10 during bioreactor cultivation.

4.3.4 Stoichiometric feeding

In literature stoichiometric feeding is described for the controlled feeding of nutrients such as glucose and glutamine [69, 70]. It suggested that this method leads to prolonged culture span and improved specific productivity based on the cellular stoichiometric composition and bioprocess requirements. This feeding strategy is recommended to improve the possibly observed metabolite overflow due to glucose concentrations. Additionally, the paper described that the model might be further enhanced by measuring the oxygen in the gas out-flow. This provides more insight on the required oxygen to control the aeration [71]. This is recommended to aim for aeration rate minimization to decrease bubble shear.

4.3.5 Shear agents

The performed multi well plate experiment on the impact of cytotoxicity of ACE on cell growth requires follow-up experiments in bioreactors. The antifoaming effect of ACE might reduce bubble breaks during bioreactor cultivation. Additionally, the physical mechanisms of PF68 to protect cells from bubble shear remain uncovered. It is recommended to further investigate the impact of the addition of these shear agents to reduce shear stress on cells during bioreactor cultivation and therefore improve cell growth.

4.4 Conclusion

The research reflected in this paper intended to answer three key research questions:

1. What conditions ensure maximum cell growth rates and cell viability values above 90% of quail suspension cell line CCX.E10 during a seed-train from cell bank to DASbox bioreactor?

This research provided a seed-train workflow to initiate the DASbox bioreactor system for parallel investigation and characterization of cell line CCX.E10 (Table 8, Figure 9). It consists of the cultivation of thawed cells in non-baffled shake flasks for 27 days with 6 passages. The operational conditions in the shake flask comprise a filling volume of 25 mL and shaking speed of 205 rpm. The initial cultivation with thawed cells is inoculated in triplicated flasks with an inoculation VCD of 0.1×10^6 cells/mL. The cells are passaged every 4 days with an inoculation VCD of 0.3×10^6 cells/mL. After 6 passages and observing a viability above 90% directly after passaging in flasks, the DASbox is inoculated with a VCD of 0.6×10^6 cells/mL.

2. What operational conditions improve the cell-specific rates and yield-coefficients of quail suspension cell line CCX.E10 during exponential cell growth in a DASbox bioreactor?

Characterization of the cell-specific rates and yield-coefficients of CCX.E10 during exponential cell growth in the DASbox bioreactor is performed. Due to time constraints, the cell growth analysis is performed for solely one set of operational conditions. These include an aeration rate of 5 sL/h, agitation rate of 300 rpm and dissolved oxygen concentration of 60%. To find conditions that result in improved cell-specific rates and yield-coefficients follow-up experiments are required.

3. What are the limiting factors for exponential cell growth of quail suspension cell line CCX.E10 during batch cultivation in a DASbox bioreactor?

The findings of this research resulted in the observation of two cell growth limiting factors. Firstly, the concentrations of the produced metabolites lactate and ammonium, between 14 – 20 mM and 1 – 1.2 mM, respectively, might have caused detrimental waste accumulation that likely resulted in cell growth inhibition. Secondly, the employed operational conditions in the bioreactor may have resulted in an amount of shear stress induced on the cells that led to growth inhibition. Likely, a combination of both observations negatively influenced the exponential cell growth.

By way of this research a seed-train workflow for a gradual volume increase from cell bank to shake flask to DASbox bioreactor is contributed. The study found cell-specific rates and yield-coefficients for glucose consumption, lactate production and ammonium production during shake flask and bioreactor cultivation of cell line CCX.E10. It provides a DASbox Standard-Operating-Procedure with DASbox PI-control parameter settings for dissolved oxygen concentration, aeration and pH. Lastly, it contributes Python scripts for data processing and visualization of Flex2 metabolite concentrations and DASware control parameter outputs.

The optimal operational conditions for aeration, agitation and dissolved oxygen concentration of cultivation of cell line CCX.E10 in a DASbox bioreactor remain unknown. Additionally, a procedure is required to quantify the amount of available GlutaMAX during cultivation to determine the cell-specific glutamine consumption rate and yield-coefficients for ammonium production over glutamine consumption. The improvement of shear stress induced during bioreactor cultivation might benefit from a detection method to determine the contribution from bubble shear. Lastly, the impact of the shear agents PF68 and ACE on cell-specific rates during bioreactor cultivation remains unknown.

The findings of this research matter for technological development of cultivated meat production for a few reasons. First of all, the provided seed-train workflow allows for a well planned characterization of CCX.E10 in the DASbox bioreactor system. This includes, data processing methods that can be used for efficient visualization of experimental output from the Flex2 and DASware. Secondly, the growth kinetics derived for bioreactor cultivation of CCX.E10 indicated what follow-up experiments are recommended to improve understanding of the optimal cell growth conditions. Lastly, the research suggested that the cell line might exhibit the Warburg effect. This matters because on that note a focus on specific bioprocess variables such as shear stress and nutrient provision is required to improve cell growth conditions for CCX.E10. Unfortunately, an approach to determine the cell-specific glutamine consumption rate was not found within the timeframe of this research. This matters for possible subsequent research goals that involve medium modifications or the development of feeding strategies. To conclude, the findings of this research matter for commercial scale cultivated meat production, because it showed that cell growth of CCX.E10 is possible in a scale-down bioreactor model and it provided a first understanding of the kinetic behavior of CCX.E10 in such an environment.

4.5 Declaration on the use of AI tools

Over the full duration of this research two AI tools were consulted. The Google search AI tool was used for superficial scientific insights and use of English. The sharing of sensitive data was prevented by using the tool for general questions only. GitHub Copilot was used with large language model GPT-5 mini for coding Flex2 and DASbox data analysis scripts. This tool ensures data security regarding confidential inputs. The shared sensitive data included data from Flex2 measurements and DASware output.

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Appendix

A.1 DASware settings

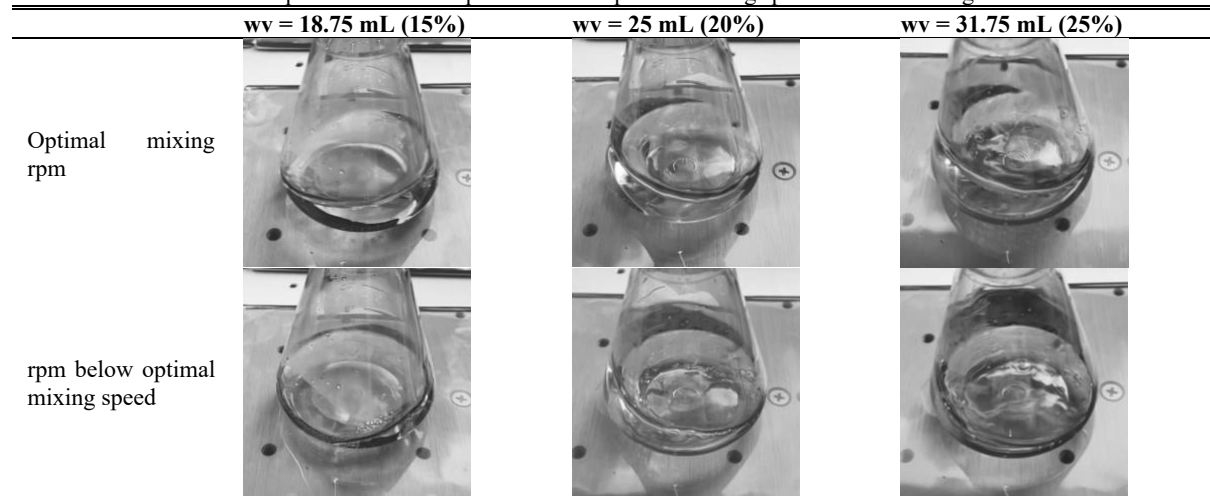
Table 10. Parameter inputs and control settings for DASbox bioreactor cultivation. X1 presents the start value of the controller output, X2 the end value of the controller output, Y1 the start value of the actuator and Y2 the end value of the actuator.

Parameter				Setpoint					
Filling volume				100 mL					
Temperature setpoint				Temperature setpoint	37 °C				
				P	15				
				Ti [s]	1,000				
pH setpoint				pH setpoint	7.2				
				Preset [%]	-5				
				P	50				
				Ti [s]	3,600				
				Deadband	0.05				
				AutoResetYi	true				
				X.out min [%]	-50				
				X.out max [%]	100				
Two-sided pH control				Pump B (base) [mL/h]	40				
				Base	7.8% NaHCO ₃				
				Dosage type	overhead				
				pH control profile, flow rate base	X1 [%]	0			
					Y1 [mL/h]	0			
					X2 [%]	100			
					Y2 [mL/h]	10			
				Acid	Acid	CO ₂			
				DO setpoint				DO setpoint	60
								Preset [%]	0
P	0.5								
Ti [s]	600								
X.out min [%]	-100								
X.out max [%]	100								
Agitation								Agitation [rpm]	300
				Direction	counterclockwise				
Gas flow				Gas flow [sL/h]	5				
				DO control profile, O ₂ concentration in gas mix	X1 [%]	-100			
					Y1 [%]	10			
					X2 [%]	0			
					Y2 [%]	15			
					X3 [%]	100			
					Y3 [%]	25.2			
				pH control profile, CO ₂ concentration in gas mix	X1 [%]	-100			
					Y1 [%]	100			
					X2 [%]	0			
					Y2 [%]	0			

A.2 Shake flask cultivation data

A.2.1 Shaking speed and filling volume

Table 11. Liquid distribution captured for the optimal shaking speed of varied filling volumes.



A.2.2 Baffled versus non-baffled shake flasks

Table 12. Cell growth analysis during non-baffled shake flask cultivation

	Timespan [h]	Osmolality [osmol/kg H ₂ O]	Viability [%]	Cell diameter [μm]	VCD [10 ⁶ cells/mL]
average	0.0	0.268	94.9	18.5	8.54E-02
st.dev		0.004	2.41		9.02E-03
st.dev [%]		1.5	2.54		
average	17.7	0.268	96.4	19.5	1.22E-01
st.dev		0.001	0.81		1.08E-02
st.dev [%]		0.5	0.84		
average	41.3	0.269	96.2	20.0	2.38E-01
st.dev		0.001	1.33		1.03E-02
st.dev [%]		0.5	1.38		
average	65.4	0.274	95.6	19.9	4.17E-01
st.dev		0.001	1.54		2.66E-02
st.dev [%]		0.3	1.61		
average	89.3	0.270	95.3	19.7	7.63E-01
st.dev		0.001	0.56		3.94E-02
st.dev [%]		0.4	0.58		
average	113.3	0.271	95.5	19.8	1.25E+00
st.dev		0.006	0.21		1.03E-01
st.dev [%]		2.4	0.22		
average	137.3	0.262	94.1	20.2	1.77E+00
st.dev		0.001	1.99		1.28E-01
st.dev [%]		0.4	2.11		
average	161.8	0.261	93.6	18.5	1.83E+00
st.dev		0.002	1.26		1.32E-01
st.dev [%]		0.7	1.34		
average	185.5	0.267	93.7	17.3	2.26E+00
st.dev		0.011	0.81		2.30E-02

st.dev [%]		4.1	0.86		
average	213.9	0.258	93.7	16.2	2.18E+00
st.dev		0.001	1.06		4.24E-02
st.dev [%]		0.5	1.13		
average	257.8	0.261	92.1	15.8	2.03E+00
st.dev		0.001	0.38		5.90E-02
st.dev [%]		0.5	0.41		

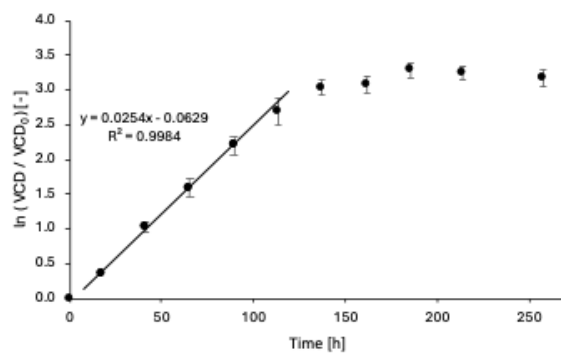


Figure 18. Exponential growth determination for cultivation in non-baffled shake flasks.

Table 13. Metabolite concentrations for cell culture samples of CCX.E10 in SCGM in non-baffled flasks. Values from cell culture samples in triplicates were measured with the Flex2 and averaged for visualization.

Timespan [h]	Glutamine [mmol/L]	Glutamate [mmol/L]	Glucose [mmol/L]	Lactate [mmol/L]	Ammonia [mmol/L]
0.0	0.10	0.54	27.68	0.30	0.24
17.7	0.41	0.58	26.99	1.11	0.29
41.3	1.16	0.66	26.09	2.70	0.40
65.4	1.99	0.77	24.52	5.37	0.64
89.3	2.11	0.90	21.80	8.96	0.87
113.3	1.84	1.12	17.10	14.06	1.02
137.3	1.61	1.33	13.45	16.28	1.15
161.8	1.46	1.45	12.06	15.87	1.26
185.5	1.37	1.60	10.19	14.76	1.34
213.9	1.37	1.76	8.96	13.03	1.26
257.8	1.46	1.91	7.66	10.88	1.11

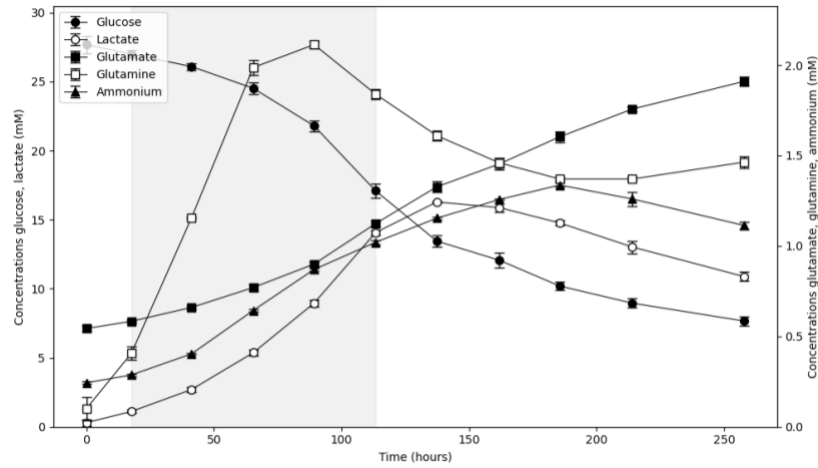


Figure 19. Metabolite concentrations during non-baffled shake flask cultivation. Exponential phase was between 18 and 113 hours (grey). Generated with Flex2_data_processing.py and Flex2_analysis (see Additional Materials, Python Scripts and Notebooks).

Table 14. Cell growth data for baffled shake flask cultivation.

	Timespan [h]	Osmolality [osmol/kg H ₂ O]	Viability [%]	Cell diameter [μm]	VCD [10 ⁶ cells/mL]
average	0.0	0.270	82.0	17.1	4.25E-02
st.dev		0.002	2.32		5.03E-03
st.dev [%]		0.7	2.83		
average	17.7	0.269	88.1	17.0	6.18E-02
st.dev		0.001	3.98		1.13E-02
st.dev [%]		0.4	4.52		
average	41.3	0.270	88.3	18.9	7.16E-02
st.dev		0.000	0.76		1.40E-02
st.dev [%]		0.2	0.86		
average	65.4	0.274	88.5	18.3	1.10E-01
st.dev		0.002	1.56		3.13E-03
st.dev [%]		0.8	1.76		
average	89.3	0.273	90.6	18.7	1.77E-01
st.dev		0.001	3.24		2.58E-02
st.dev [%]		0.5	3.57		
average	113.3	0.273	89.9	18.3	3.58E-01
st.dev		0.000	1.12		2.55E-02
st.dev [%]		0.2	1.24		
average	137.3	0.275	86.5	18.6	5.32E-01
st.dev		0.000	2.76		5.98E-02
st.dev [%]		0.2	3.19		
average	161.8	0.272	88.0	18.6	7.33E-01
st.dev		0.002	1.46		2.98E-02
st.dev [%]		0.6	1.66		
average	185.5	0.275	88.3	17.7	9.89E-01
st.dev		0.006	1.14		3.14E-02
st.dev [%]		2.4	1.29		
average	213.9	0.271	89.9	16.7	1.30E+00

st.dev		0.000	1.65		7.77E-02
st.dev [%]		0.2	1.84		
average	257.8	0.266	90.8	14.8	1.79E+00
st.dev		0.000	0.40		3.67E-02
st.dev [%]		0.00	0.45		

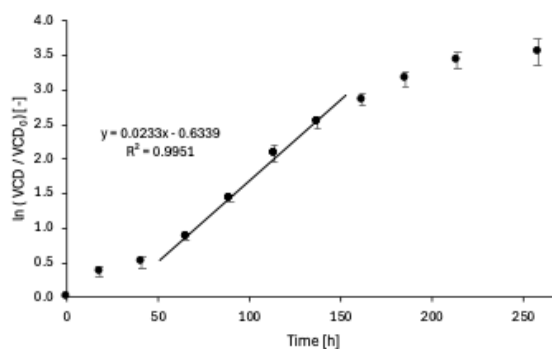


Figure 20. Exponential growth determination for cultivation in baffled shake flasks. The exponential phase was between 65 and 137 hours.

Table 15. Metabolite concentrations data for cell culture samples of CCX.E10 in SCGM in baffled flasks. Values from cell culture samples in triplicates were measured with the Flex2 and averaged for visualization.

Timespan [h]	Glutamine [mmol/L]	Glutamate [mmol/L]	Glucose [mmol/L]	Lactate [mmol/L]	Ammonia [mmol/L]
0.0	0.00	0.54	27.81	0.00	0.26
17.7	0.12	0.57	27.49	0.00	0.30
41.3	1.02	0.60	27.68	0.00	0.37
65.4	1.61	0.65	27.38	0.00	0.50
89.3	2.19	0.71	26.64	0.44	0.72
113.3	2.14	0.81	25.38	2.92	0.99
137.3	1.97	0.93	23.44	5.99	1.13
161.8	1.82	1.03	21.65	8.29	1.18
185.5	1.72	1.17	20.02	10.47	1.22
213.9	1.55	1.35	17.47	12.54	1.24
257.8	1.32	1.60	12.66	16.47	1.33

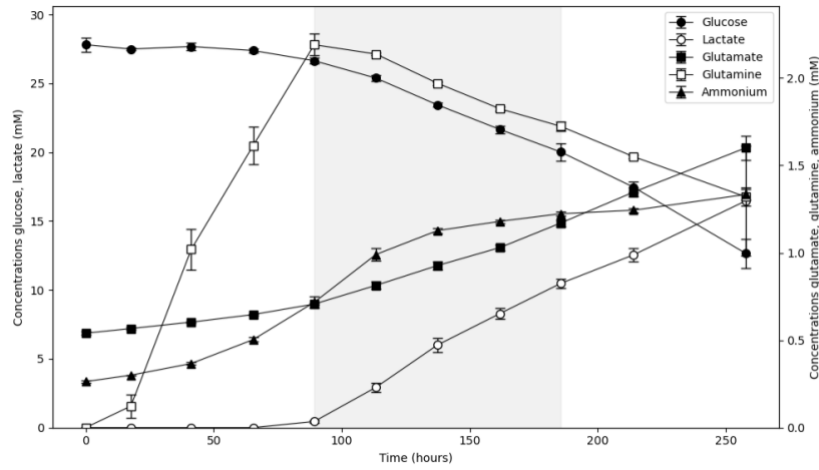


Figure 21. Metabolite concentrations in baffled flasks. The exponential phase was between 65 and 137 hours. Generated with Flex2_data_processing.py and Flex2_analysis (see Additional Materials, Python Scripts and Notebooks).

A.2.3 Inoculation density effect

Table 16. Cell count analysis after inoculation shake flask with 0.3×10^6 cells/mL.

	Timespan [h]	Osmolality [osmol/kg H ₂ O]	Viability [%]	Cell diameter [μm]	VCD [10 ⁶ cells/mL]
average	0.0	0.264	82.1	20.1	2.65E-01
st.dev		0.003	5.62		1.56E-02
st.dev [%]		1.3	6.85		
average	24.3	0.269	86.0	19.0	4.29E-01
st.dev		0.001	2.52		1.31E-02
st.dev [%]		0.2	2.93		
average	48.1	0.272	91.4	19.9	7.83E-01
st.dev		0.002	1.91		3.68E-02
st.dev [%]		0.7	2.09		
average	71.8	0.267	94.8	18.8	1.38E+00
st.dev		0.002	1.01		1.04E-01
st.dev [%]		0.8	1.07		

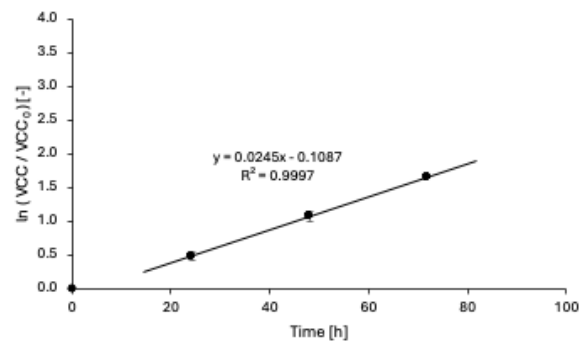


Figure 22. Exponential growth determination for cultivation in shake flasks inoculated with a density of 0.3×10^6 cells/mL. Exponential cell growth between 24 hours and 72 hours.

Table 17. Metabolite concentrations in non-baffled flasks inoculated with a density of 0.3×10^6 cells/mL.

Timespan [h]	Glutamine [mmol/L]	Glutamate [mmol/L]	Glucose [mmol/L]	Lactate [mmol/L]	Ammonia [mmol/L]
0.0	0.00	0.53	27.6	0.0	0.28
24.3	1.94	0.66	26.3	0.3	0.53
48.1	1.97	0.82	23.5	5.1	0.86
71.8	1.73	1.02	19.4	11.2	1.00

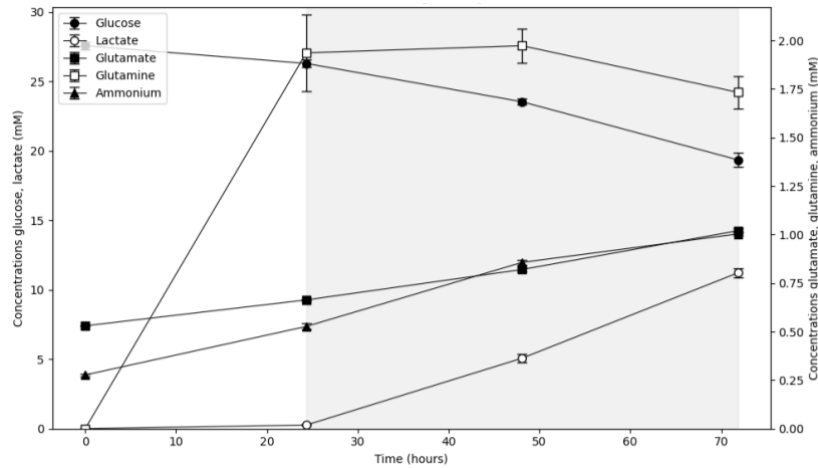


Figure 23. Metabolite concentrations over time in non-baffled after inoculation with 0.3×10^6 cells/mL. The exponential phase ranged between 24 and 72 hours (grey). Generated with Flex2_data_processing.py and Flex2_analysis (see Additional Materials, Python Scripts and Notebooks).

A.2.4 Effect vial thawing

Table 18. Cell counts shake flask cultivation with inoculation of 0.3×10^6 cells/mL thawed cells.

	Timespan [h]	Osmolality [osmol/kg H ₂ O]	Viability [%]	Cell diameter [μm]	VCD [10 ⁶ cells/mL]
average	0.0	0.265	84.4	19.0	0.344
st.dev		0.001	1.5		0.010
st.dev [%]		0.2	1.8		
average	22.7	0.270	81.5	19.4	0.402
st.dev		0.002	2.6		0.031
st.dev [%]		0.6	3.1		
average	46.8	0.271	88.9	19.8	0.696
st.dev		0.002	3.1		0.105
st.dev [%]		0.8	3.4		
average	71.5	0.266	93.1	19.1	1.214
st.dev		0.003	0.8		0.174
st.dev [%]		1.1	0.8		
average	94.6	0.261	94.1	18.4	2.110
st.dev		0.003	0.6		0.282
st.dev [%]		1.2	0.6		

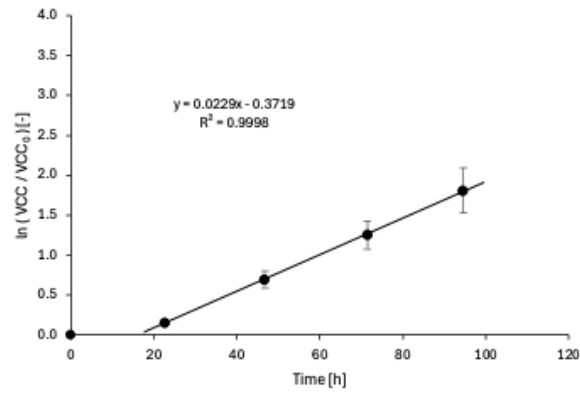


Figure 24. Exponential growth determination for cultivation in shake flasks inoculated with a density of 0.3×10^6 cells/mL thawed cells. Exponential cell growth between 23 hours and 95 hours.

Table 19. Metabolite concentrations in flasks after inoculation with 0.3×10^6 cells/mL of thawed cells.

Timespan [h]	Glutamine [mmol/L]	Glutamate [mmol/L]	Glucose [g/L]	Glucose [mmol/L]	Lactate [g/L]	Lactate [mmol/L]	Ammonia [mmol/L]
0.0	0.00	0.54	5.12	28.4	0.00	0.0	0.27
22.7	2.65	0.67	4.93	27.4	0.07	0.7	0.54
46.8	2.55	0.84	4.51	25.1	0.27	3.0	0.90
71.5	2.19	1.05	3.79	21.0	0.77	8.6	1.06
94.6	1.77	1.28	2.86	15.9	1.25	13.9	1.12

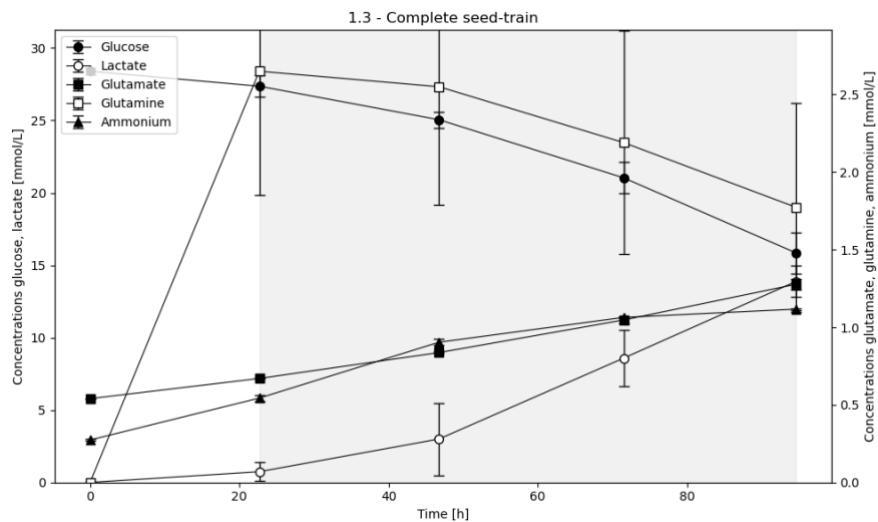


Figure 25. Metabolite concentrations over time in shake flasks with an inoculation cell density of 0.3×10^6 of thawed cells. Exponential growth was between 23 and 95 hours (grey). Generated with Flex2_data_processing.py and Flex2_analysis (see Additional Materials, Python Scripts and Notebooks).

A.3 DASbox cultivation data

A.3.1 Effect inoculation density of 0.05×10^6 cells/mL

Table 20. Seed-train prior to DASbox run.

Timespan [h]	Time difference [h]	Viability [%]	VCD [10^6 cells/mL]	Growth rate [h ⁻¹]	Doubling time [h]	Population doublings
0	0	89.5	0.145	-	-	-
17.8	17.8	88.0	0.149	0.0015	461.2	0.04
42.1	24.3	75.6	0.301	0.0298	23.2	1.05
					Total:	1.09

A.3.2 Ethanol presence in bioreactor

Table 21. Detected ethanol, glucose and lactate concentrations from HPLC analysis of cell culture samples taken after 170 hours. The glucose and lactate concentrations are added as a control and correspond to the analyzed metabolite concentrations.

Sample	Ethanol conc. [mmol/L]	Glucose conc. [mmol/L]	Lactate conc. [mmol/L]
SCGM	0	27.7	0
Unit 1	2.42	26.4	8.27
Unit 2	0	25.2	6.45
Unit 3	0.54	27.0	7.48

A.3.3 Effect inoculation density of 0.15×10^6 cells/mL

Table 22. Seed-train prior to DASbox inoculation. Note that a doubling time of 20.4h is reached.

Timespan [h]	Time difference [h]	Viability [%]	VCD ($\times 10^6$ cells/mL)	Growth rate [h ⁻¹]	Doubling time [h]	Population doublings
0.0	0.0	73.5	0.143	-	-	-
23.3	23.3	65.0	0.151	0.0024	294.1	0.079
46.3	22.9	67.3	0.194	0.0110	63.0	0.364
71.1	24.8	82.0	0.402	0.0294	23.6	1.052
90.9	19.8	91.5	0.787	0.0339	20.4	0.970
					Total:	2.46

A.3.4 Volumetric power input-ratio correlation

The P/V-ratio for shake flasks was determined based on the equations provided in Material and Methods section 2.5 and several known and retrieved parameter values. The Reynolds number approximates 20,000. Therefore, a turbulent flow was assumed. The impeller power number is assumed to range between 1 – 2.

Table 23. Parameter values required for the correlation of P/V-ratio of shake flask and bioreactor cultivation and calculation of the agitation rate in the bioreactor based on the ration.

Parameter	Value	Notes
Density, ρ [kg/m ³]	1000.6	SCGM, measured with viscometer
Shaking speed, n [s ⁻¹]	3.42	Equivalent to 205 rpm
Shaking flask diameter, d [m]	0.0635	
Culture volume, V_L [m ³]	1×10^{-4}	
Dynamic fluid viscosity, μ [Pa.s]	7.32×10^{-4}	SCGM, measured with viscometer
Newton number, N_p [-]	1 – 2	Assumed range for 45° angle Pitched blade impeller, sensitivity analysis performed for agitation rate calculation.
Impeller outer diameter, d [m]	0.03	

A.3.5 Effect ACE and PF68 on cell growth in multi well plate

Table 24. Cell growth analysis in presence of PF68 and ACE between 45 and 110 hours after addition.

Agent		Time difference [h]	Viability [%]	VCD [10 ⁶ cells/mL]	Growth rate [h ⁻¹]
Pluronic-68	average	67	85.3	0.199	0.034
	stdev		13.8	0.069	0.007
0 ppm ACE	average	67	89.8	0.259	0.033
	stdev		9.4	0.033	0.004
5 ppm ACE	average	67	95.8	0.214	0.028
	stdev		0.6	0.017	0.004
10 ppm ACE	average	67	93.0	0.196	0.031
	stdev		3.6	0.022	0.004
20 ppm ACE	average	67	80.0	0.143	0.031
	stdev		25.5	0.046	0.005
30 ppm ACE	average	67	94.3	0.175	0.031
	stdev		2.4	0.025	0.003
50 ppm ACE	average	67	88.8	0.182	0.032
	stdev		12.0	0.043	0.005

A.3.6 Effect inoculation density of 0.6x10⁶ cells/mL

Table 25. Seed-train parameter values with corrected viability and VCD values based on NucleoCounter measurements for baseline samples prior to inoculation presumed to contain SCGM only. The passages are shown in bold in the notes. Medium exchanges are treated as passages due to nutrient provision and removal of waste metabolites. NM = Not measured.

Replicas (n)	Time span [h]	Viability [%]	VCD [x10 ⁶ cells/mL]	Time difference [h]	Growth rate [h ⁻¹]	Doubling time [h]	Population doublings	Notes
3	0	89.5	0.145	0.0	-	-	-	thawed
3	18	88.0	0.149	17.8	0.0015	461.2	0.04	start 205rpm
3	42	75.6	0.316	24.3	0.0298	23.2	1.05	prior dasbox 1st run
3	210	NM	NM	-	-	-	-	Pooled leftovers in fresh medium
3	310	90.1	0.420	267.4	0.0049	141.5	1.89	Cell count
4	313	95.5	0.427	3.0	0.1146	6.0	0.50	half medium

								exchange
4	360	99.0	1.356	47.5	0.0244	28.4	1.67	cell count
1	450	83.0	0.525	90.0	-0.026	-26.68	-	8 mL leftovers in 25 mL fresh medium
1	503	96.0	1.301	52.7	0.0172	40.2	1.31	prior passage
2	504	90.0	0.925	0.8	0.4225	1.6	0.51	after passage
2	528	90.0	0.712	24.0	-0.0109	-63.6	-	cell count
2	552	95.5	1.354	24.8	0.0259	26.8	0.93	prior passage
5	553	85.9	0.403	0.7	-0.4428	-1.6	-	after passage
5	622	98.5	2.428	68.7	0.0261	26.5	2.59	prior passage
5	646	96.7	1.248	24.1	-0.0081	-85.2	-	after passage
Total:							10.5	

Table 26. Cell growth analysis of CCX.E10 in DASbox bioreactor after a cultivation at an agitation rate of 300 rpm, aeration rate of 5 sL/h and dissolved oxygen saturation of 60%.

	Timespan [h]	ACE 100x [mL]	Osmolality [osmol/kg H ₂ O]	Viability [%]	Cell diameter [μm]	VCD [10 ⁶ cells/mL]	Growth rate [h ⁻¹]	Doubling time [h]
average	0.0	30	0.279	89.6	19.7	0.68	-	-
stdev			0.012	2.7		0.20	-	-
stdev [%]			4.4	3.0				
average	21.2	0	0.278	90.4	19.1	0.84	0.010	97.3
stdev			0.004	2.1		0.06	0.006	
stdev [%]			1.3	2.3				
average	45.7	20	0.269	93.0	19.5	1.19	0.023	31.3
stdev			0.016	1.1		0.19	0.005	
stdev [%]			5.8	1.2				
average	68.3	15	0.271	93.5	17.9	1.81	0.044	15.9
stdev			0.006	0.3		0.05	0.002	
stdev [%]			2.1	0.3				
average	93.2	20	0.283	92.8	15.6	2.10	0.064	10.8
stdev			0.015	0.8		0.04	0.002	
stdev [%]			5.4	0.9				
average	120.2	20	0.284	93.5	14.1	1.70	0.084	8.3
stdev			0.003	1.0		0.23	0.006	
stdev [%]			1.0	1.1				
average	141.0	0	0.292	92.3	13.2	1.26	0.119	5.8
stdev			0.006	0.7		0.12	0.009	
stdev [%]			2.2	0.8				

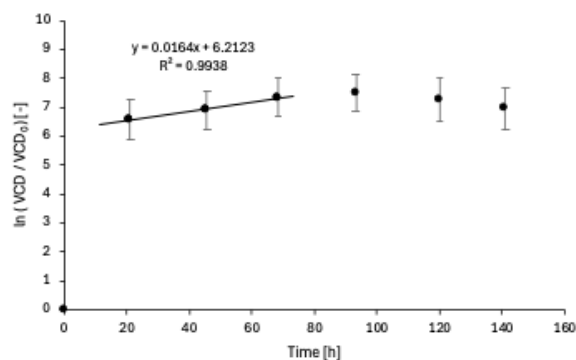


Figure 26. Exponential growth determination bioreactor cultivation. Exponential phase is between 21 – 68 hours.

Table 27. Metabolite concentrations in DASbox bioreactor

Timespan [h]	Glutamine [mmol/L]	Glutamate [mmol/L]	Glucose [mmol/L]	Lactate [mmol/L]	Ammonia [mmol/L]
0	0.02	0.61	29.6	0.5	0.37
21.2	3.66	0.86	26.6	5.5	0.80
45.7	3.08	1.16	19.3	12.9	1.12
68.3	2.57	1.50	13.6	20.2	1.25
93.2	1.85	1.90	9.1	28.0	2.02
120.2	1.08	2.21	2.9	33.7	2.96
141.0	0.93	2.37	0.7	35.3	3.72

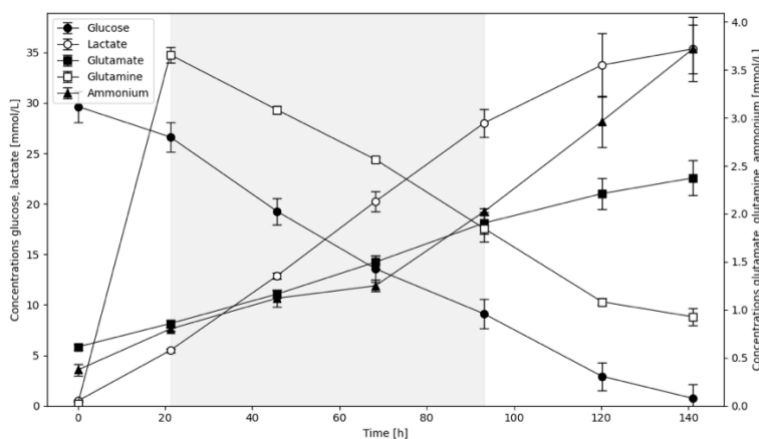


Figure 27. Metabolite concentrations over time during DASbox bioreactor cultivation with an agitation rate of 300 rpm, gas flow rate of 5 sL/h and dissolved oxygen saturation of 60%. Exponential phase between 20 and 45 hours (grey). Generated with Flex2_data_processing.py and Flex2_analysis (see Additional Materials, Python Scripts and Notebooks).

A.3.6.1 DASware PI control output

The PI control plots were generated with the Python functions in ‘DASdata_processing.py’ and notebook ‘DASdata_analysis.py’ (See Additional Materials, Python Scripts and Notebooks).

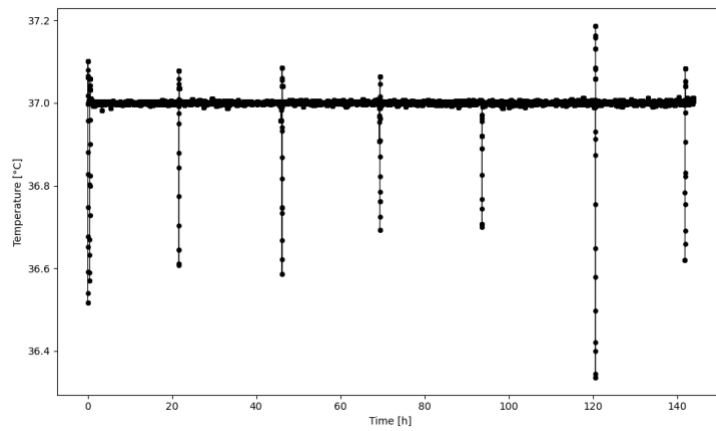


Figure 28. Temperature over time during DASbox bioprocess.

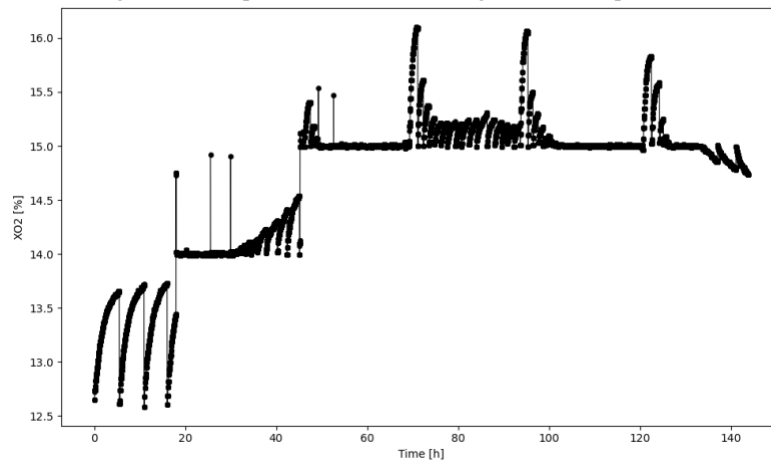


Figure 29. Percentage of oxygen in gas flow over time during DASbox bioprocess.

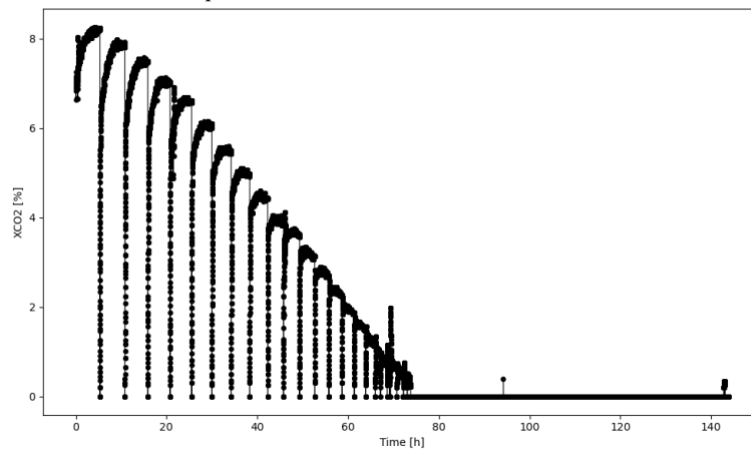


Figure 30. Percentage of carbon dioxide in gas flow over time during DASbox bioprocess.

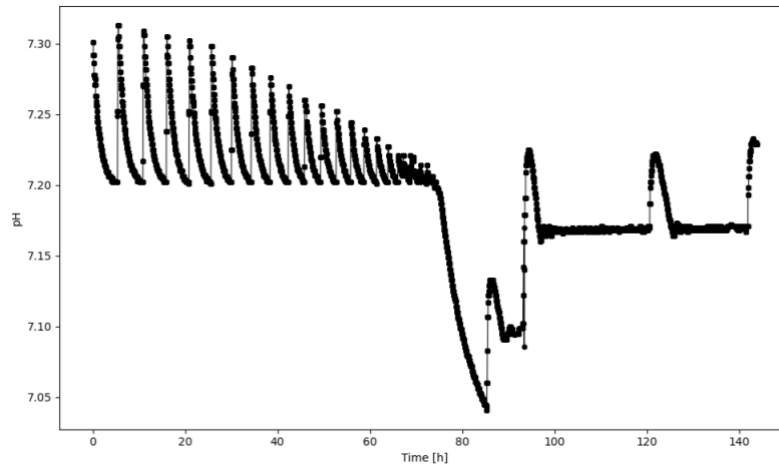


Figure 31. pH over time during DASbox bioprocess.

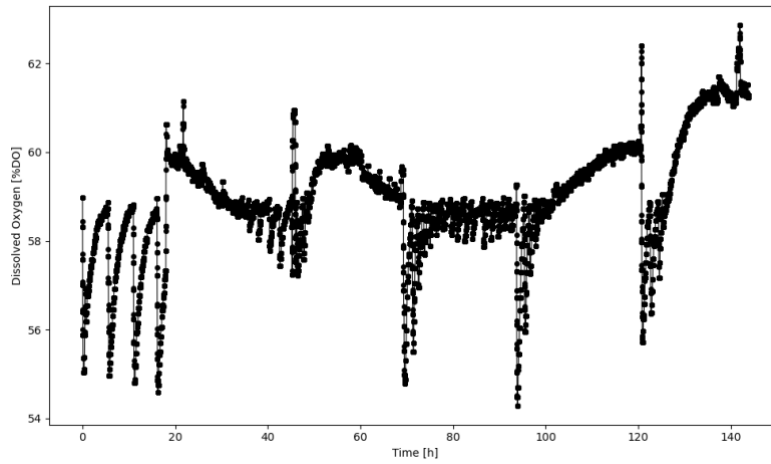


Figure 32. Dissolved oxygen percentage over time during DASbox bioprocess batch cultivation.