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Use of Mother-Daughter Multi-Bioreactor Systems for Studies of Steady State Microbial Growth Space

STEN ERM

TTÜ PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology

Supervisor: Professor Raivo Vilu
Tallinn University of Technology
Department of Chemistry and Biotechnology
Division of Chemistry

Co-supervisor: Senior Scientist Kaarel Adamberg
Tallinn University of Technology
Department of Chemistry and Biotechnology
Division of Food Technology

Opponents: Professor Peter Neubauer
Technische Universität Berlin
Institute of Biotechnology

Professor Juris Vanags
Riga Technical University
Faculty of Materials Science and Applied Chemistry

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DECLARATION *Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree elsewhere.*

Sten Erm

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Ema-tütre multi-reaktorite süsteemide kasutamine mikroorganismide püsiseisundis kasvuruumi uurimisel

STEN ERM

TTÜ KIRJASTUS

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ABSTRACT

Microbial physiology analysis is important in research and production optimisation and is realised by studying the effect of an array of environmental parameters by means of cultivations. When choosing the cultivation method typically non-steady state batch cultivation is preferred, as it is short in time. However, choosing non-steady state cultivation method can compromise the quality of the physiological state of microbial culture, blurring the effect of the environmental parameter studied. Steady-state cultivation methods (chemostat) provide more precise data but are characterised by long stabilisation times, rendering them unpopular. Quasi-steady state cultivation methods (A-stat) reduce the stabilisation period but do not exclude it.

Herein we describe a concept of steady-state growth space analysis using a bioreactor system, termed the mother-daughter (MD) system, where starting steady state cultivations with no stabilisation period is possible. This system operates by stabilising microbial culture in one reactor (mother-reactor) and distributing the stable culture to other reactors (daughter-reactors). Starting cultivation experiments in the daughter-reactors from the precisely defined physiological state allows instant quantitative physiology analysis. The system is not limited to a single transfer, and it is expected that the physiological state of microbes in the mother-reactor changes in quasi-steady state, by using changestat cultivations. Thus, when physiology analysis in the daughter-reactors is complete and the old culture discarded, they can be re-filled with culture in new physiological state (from the mother-reactor). By repeating transfers and combining different changestat cultivations, the effect of many environmental parameters can be analysed.

For the MD concept to work the physiological state of culture must remain unaltered during the culture transfer process. Statistical comparison of population characteristics of the microbial population before and after culture transfer showed the physiological state did remain unchanged. Further, an A-stat experiment started in the MD system provided statistically equal results to a reference A-stat experiment conducted in a separate bioreactor, showing that growth space scanning experiments are possible in the MD system. Similarly, a recombinant protein production in the MD system coincided well with the same production process undertaken in a separate bioreactor.

To estimate the potential advantages of using the MD system we compared a series of theoretical production experiments in separate bioreactors and the MD system. Experiments in the MD system were indeed faster if the number of experiments exceeded the number of reactors in the system. Further, the calculations show that the daughter-reactors should be ~10 times smaller than the mother-reactor to avoid introducing extra time due to the variable volume cultivation needed for biomass accumulation.

In current work, we used the concepts of growth space and physiological state of cell populations to develop efficient cultivation schemes in the MD system. While quantitative analysis of the physiological response to even a few environmental parameters remains a tedious task even in the MD system, the use of it theoretically reduces experiment time. Further, devising experiments in the MD system necessitates thinking about growth space, thus it can be viewed an educational tool as well

KOKKUVÕTE

Erinevate keskkonnatingimuste juures mikroobide füsioloogia uurimine on oluline nii baasteaduses kui ka tootmisprotsesside optimeerimisel. Kultivatsioonimeetodite valikul on tihti määravaks katse kestvus, seetõttu eelistatakse perioodilist kultiveerimist, mis aga hägustab vastavust uuritava keskkonnaparameetri ja tulemusena saadavate bioloogiliste parameetrite vahel. Püsiseisundis opereerivad kultivatsioonimeetodid (kemostaat) tagavad võimaluse füsioloogilist seisundit kvantitatiivselt hinnata, ent neid iseloomustab pikk stabilisatsiooni aeg, mis teeb nad mitteatraktiivseks. Kvaasi-püsiseisundis opereerivad kultivatsioonimeetodid küll vähendavad stabilisatsioonifaasi pikkust, kuid ei tee seda olematuks.

Käesolevas töös kirjeldati püsiseisundis oleva mikroobide kasvuruumi uurimiseks väljatöötatud bioreaktorite süsteemi (ema-tütarreaktor süsteemi), mis võimaldab katsete alustamist eelstabiliseeritud mikroobikultuuriga. Süsteemi tööpõhimõte seisneb mikroobikultuuri stabiliseerimises ühes reaktoris (emareaktor) ning järgnevas kultuuri ülekandes süsteemi teistesse reaktoritesse (tütarreaktorid), misläbi on viimastes võimalik alustada kohe peale ülekannet kvantitatiivset füsioloogia analüüsi. Muutes kontrollitult emareaktoris oleva kultuuri füsioloogilist seisundit ning teostades korduvalt kultuuri ülekandeid tütarreaktoritesse on võimalik kombineerida erinevaid kultiveerimismeetodeid, seega saab uurida paljude keskkonnaparameetrite mõju.

Ema- tütarreaktor süsteemi kontseptsiooni funktsioneerimiseks on tarvilik, et kultuuri ülekanne ei mõjutaks rakkude füsioloogilist seisundit. Selles veendumiseks analüüsiti matemaatilise statistika vahenditega rakupopulatsiooni parameetreid enne ja pärast kultuuri ülekannet. Samuti näitas statistiline analüüs, et A-staatse katse tulemused ema-tütarreaktor süsteemis ei erine tavasüsteemis saadutest. Lisaks näitas ema-tütarreaktor süsteemis teostatud rekombinantse valgu tootlikkuse analüüs head korrelatsiooni tavasüsteemis teostatud analoogse protsessiga.

Hindamaks ema-tütarreaktor süsteemi efekti katseaja vähendamisel, võrreldi mitmete teoreetiliste katsete pikkust antud süsteemis katsete pikkusega tavasüsteemis. Tulemustest selgub, et ema-tütarreaktor süsteemis teostatud katsed on lühemad, kui katsete arv ületab reaktorite arvu ning tütarreaktori maht on ~10 korda väiksem emareaktori mahust. Viimane on vajalik, et kultuuri ülekandel tarvilik ruumala suurendamise faas ei suurendaks katseaega.

Doktoritöös kasutati kasvuruumi ning füsioloogilise seisundi kontseptsioone, et töötada välja efektiivseid kultivatsiooniskeeme ema-tütarreaktor süsteemis. Kuigi juba mõnede keskkonnaparameetrite kvantitatiivse mõju kindlaks tegemine on vaevarikas töö ka ema-tütarreaktor süsteemis, kestab see teoreetiliselt siiski lühema aja, kui tavasüsteemis. Lisaks on ema-tütarreaktor süsteemi kasutamisel ka hariduslik moment, kuna seda rakendades ei ole katsete planeerimine mõeldav ilma kasvuruumi kontseptsiooni süüvimata.

LIST OF PUBLICATIONS

I Nahku, Ranno; Valgepea, Kaspar; Lahtvee, Petri-Jaan; Erm, Sten; Abner, Kristo; Adamberg, Kaarel; Vilu, Raivo. **Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures**. Journal of Biotechnology, 145(1):60-65 (2010)

II Erm, Sten; Adamberg, Kaarel; Vilu, Raivo. **Multiplying steady-state culture in multi-reactor system.** *Bioprocess and Biosystems Engineering, 37(11):61-70, (2014)*

III Erm, Sten; Abner, Kristo; Seiman, Andres; Adamberg, Kaarel; Vilu, Raivo. **Study of cells in the steady state growth space.** *Subramanian: Continuous Bio-manufacturing, Wiley, In press*

IV Erm, Sten; Adamberg, Kaarel; Vilu, Raivo. **Efficient growth space scanning and perturbation analysis of microbial processes using multi-bioreactor systems.** *Bioprocess and Biosystems Engineering (submitted)*

Summary of Author's Contribution

In publication I the author helped to perform the experimental work, analysed the data, and wrote the manuscript.

In publication II the author designed the experiments, performed the experimental work, analysed the data, and wrote the manuscript.

In publication III the author wrote the manuscript.

In publication IV the author designed the experiments, performed the experimental work, analysed the data, and wrote the manuscript.

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ABBREVIATIONS

ANN – Artificial Neural Network

CRISPR/Cas9 – Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9

DO – Dissolved Oxygen

FET – Field Effect Transistor

FU – Fluorescence Unit

GABA – Gamma-Aminobutyric acid

GFC – Gas Flow Controller

GFP – Green Fluorescent Protein

GS – Growth Space

IPTG – Isopropyl β-D-1-thiogalactopyranoside

MAGE – Multiplex Automated Genomic Engineering

MD – Mother–Daughter (system)

MTP – Micro Titer Plate

NSSGS – Non-Steady State Growth Space

OTR – Oxygen Transfer Rate

PAT – Process Analytical Technology

PFR – Plug Flow Reactor

pHU – pH units

QbD – Quality by Design

QSS – Quasi–Steady State

RNA – Ribonucleic Acid

SSGS – Steady State Growth Space

LIST OF SYMBOLS

μ – Specific growth rate [h^{-1}]

μ_{crit} – Critical specific growth rate [h^{-1}]

μ_{max} – Maximal specific growth rate [h^{-1}]

μ_{set} – Specific growth rate set-point [h^{-1}]

a – Acceleration rate in A-stat [h^{-2}]

C_{GFP} – Concentration of the GFP [$FU \cdot L^{-1}$]

D – Dilution rate [h^{-1}]

F – Pumping rate [$mL \cdot min^{-1}$]

K_S – Affinity coefficient [$mmol \cdot L^{-1}$]

O_{ace} – Acetic acid production per biomass [$mmol \cdot g^{-1}$]

O_{aceasp} – Acetylaspartic acid production per biomass [$mmol \cdot g^{-1}$]

O_{dho} – Dihydroorotic acid production per biomass [$mmol \cdot g^{-1}$]

O_{lact} – Lactic acid production per biomass [$mmol \cdot g^{-1}$]

P – Product concentration [$mmol \cdot L^{-1}$]

q_{ace} – Specific acetate production rate q_{ace} [$mmol \cdot g^{-1} \cdot h^{-1}$]

q_{CO2} – Specific carbondioxide production rate [$mmol \cdot g^{-1} \cdot h^{-1}$]

Q_{GFP} – Volumetric productivity of GFP [$FU \cdot L^{-1} \cdot h^{-1}$]

q_{main} – Glucose need for maintenance [$mmol \cdot sec^{-1} \cdot L^{-1}$]

S – Substrate concentration [$mmol \cdot L^{-1}$]

S_{res} – Residual substrate concentration [$mmol \cdot L^{-1}$]

t – time [h]

t90 – Time to reach 90 % of response (for a sensor)

V – Culture volume [L]

X – Biomass concentration [$g \cdot L^{-1}$]

Y'_{O2} – Biomass yield based on oxygen [$Cmol \cdot Cmol^{-1}$]

Y_{XS} – Biomass yield based on substrate [$g \cdot g^{-1}$]

τ – Residence time (time necessary to dilute the culture twofold at constant dilution rate) [h]

1 INTRODUCTION

The technological advances of genetic engineering (such as Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9 (CRISPR/Cas9), Multiplex Automated Genomic Engineering (MAGE)) have made possible easy introduction of basically any mutation in the target genome, increasing substantially the number of strains to be analysed in physiology studies and in bioprocess optimisation (Gallagher et al., 2014; Peters et al., 2016). The initial screening of mutants is typically done using high-throughput cultivations in technologically simple micro-titer plates (MTPs). However, as the MTPs are not suitable for in-depth physiology studies the more promising strains are further characterised in well-instrumented bioreactors. While the preparation of mutants and initial screenings are fast, the elaboration of physiology in bioreactors is not, thus creating a bottleneck (Alvin et al., 2013). The problem is that bioreactors used for quantitative physiology studies are typically less suitable for parallelization due to the need for more advanced process control, resulting in tens rather than thousands of simultaneously operating units. At the same time, the number of cultivation conditions that need to be analysed is substantial, as at least substrate, growth rate, temperature, pH effects should be studied. In detailed physiology studies culture in steady state should be used, the achievement of which is time-consuming, therefore further exacerbating the slowness issue (Daranlapujade et al., 2008). The steady state conditions are, however, very necessary if truly quantitative cause and effect relationships between biological and environmental conditions are to be achieved (Bull, 2010). Further, steady state cultivations need to be studied as continuous manufacturing is applied increasingly in the industry. The reason for the interest is that steady state cultivations can be more productive and easier to automate than batch or fed-batch cultivations. It is also simpler to apply in steady state the requirements of process analytical technology (PAT) and quality by design (QbD) (Hernandez, 2015; Konstantinov and Cooney, 2015; Warikoo et al., 2012). Steady state cultivations are also useful in development and validation of physiology models, due to the constant concentrations.

The gold standard of steady state cultivation is the chemostat, which however includes the unavoidable stabilisation phase making the method time-consuming. The stabilisation time can be reduced by using changestat cultivations, in which the changes in the environment are introduced at a low enough rate so that the physiology of the cells can adapt (Paalme et al., 1995). Further, by choosing the correct changestat method, the effect of practically any environmental parameter can be elaborated (Kasemets, 2003). For instance, an array of reactors running different changestat cultivations could be used to map the physiological response to all relevant environmental parameters. However, each of these cultivations would still need initial stabilisation phase, which is especially time-consuming in experiments where the physiology analysis entails disruption of the established steady state, thus destabilising the culture and leading to the need for a new stabilisation if another steady state is to be analysed. An example of such perturbation experiment would be the induction of recombinant protein production.

An alternative to using in physiology analysis a significant number of individual reactors in parallel is to use an interconnected multi-reactor network (Erm et al., 2014). In

this system, the culture is stabilised only once in one reactor, which is then used to periodically fill the working volumes of other, connected reactors with culture in the precisely defined state. This approach effectively allows starting cultivations from steady state physiology with no or minimum stabilisation time, which in turn allows achieving comprehensive growth space analysis by combining (in the different reactors of the system) different changestat and chemostat-based cultivation methods. The set-up is particularly useful in physiology disrupting experiments, as it allows decoupling biomass growth and perturbation reactors.

The aim of the thesis was to develop and evaluate the bioreactor system where cultivations in steady state start with pre-stabilised culture transferred from another reactor (the MD system). In the experimental part, the emphasis was on the comparison of physiology before and after culture transfer, as the physiology must remain unaltered for the concept to work. Changestat and production experiments undertaken in the MD system were compared statistically with equal experiments conducted in separate reactors. The time-efficiency (decrease in experiment time) of using the MD multi-reactor systems was quantified in theoretical comparisons of the experiment time upon different cultivation scenarios conducted in traditional chemostat bioreactors and the MD system.

2 LITERATURE REVIEW

2.1 The Concepts of Physiological State and Growth Space

Cell population manifests its physiology in its physiological state, which is characterised by fluxes of substrates and products, by all cellular processes and metabolism, and by the molecular components of biomass and their arrangement. The physiological state of cells in culture is based on the average physiological state of a population of asynchronously growing cells inside the bioreactor. Actually, each cell in a bioreactor can have its physiological state with the temporal (e.g. cell cycle) and spatial (e.g. cellular compartmentalisation) properties and models exist to describe these states (Abner et al., 2014; Donachie, 1968; Kretschmer and Schwille, 2016). Herein, if not indicated otherwise, the physiological state refers to that of the cell population in culture, not individual cells. The term "physiological state of cell population" was introduced in 1958 by Malek, who viewed it as the combined outcome of the environment, genetics of the organism and culture history, defining it in 1975 as: "physiological state of microbial populations is a term including the expressed properties and states as well as the latent potentialities which, depending on conditions in the medium, are reflected in a certain mode of reproduction and in an ability to respond to changes in these conditions by specific changes in the metabolism, thereby creating a new quality, either in the form of changed morphology or in the form of physiological and biochemical processes, often accompanied by formation of specific metabolites" (Malek, 1975). The environment stands for the availability and concentration of macro- and micronutrients, values of temperature and pH, etc. The effect of culture history is harder to quantify – it means that previous physiological states affect the current one; that biological properties (such as expression profile) can act as inputs modifying cellular behaviour. Essentially culture history can lead to the possibility of several physiological states at the same environmental parameter values, which complicates physiological state analysis. Generally, the culture history component should be minimal when the effect of environmental conditions on physiological and technological parameters is studied, as then the consequences of controllable environmental parameters on physiology can be uniquely determined. Culture history's impact is minimised by running well-controlled cultivations according to specific algorithms in dedicated bioreactors, discussed in more detail in the sections below.

The physiological state is a useful concept in studying cellular metabolism, evaluating organisms production potential, or in bioprocess control as it provides a measure to categorise cellular behaviour. Malek initially defined the term as a qualitative measure, but several authors have added quantitative nature to it. The physiological state could be determined unambiguously by measuring the concentrations of all components and fluxes of all substances in the culture with taking into account their change in time, which however is not practically possible. Therefore, typically, the physiological state determination is based on a limited number of parameters, and both biological and environmental ones have been used. For instance, Locher et al used on-line determined variables (pH, CO_2, O_2, redox potential, fluorescence, temperature) and their time derivatives to evaluate the physiological state in chemostat cultivation (Locher et al., 1990). They viewed the physiological state determination as pattern recognition, similar to that used

in face recognition algorithms. The use of online environmental variables has been contested by Konstantinov and Yoshida, who regarded the information provided insufficient to describe the behaviour of complex biological systems (Konstantinov and Yoshida, 1989). They proposed to base the physiological state determination on an array of physiology reflecting population characteristics (i. e. parameters such as specific productivities of metabolites, growth yields, specific growth rate), and in some cases, their time derivatives (Konstantinov and Yoshida, 1989). It is common to consider similar physiological states to represent the same subset; for instance, Konstantinov and Yoshida formulated a physiological state space comprised of sub-spaces, within which the population characteristics did not change significantly. It is noted that different subspaces (such as 'situation of optimal productivity') were defined based on expert knowledge. During continuous cultivation, the culture in particular physiological state was assigned automatically to the corresponding subspace, and the most suitable process control algorithm could be applied (Konstantinov and Yoshida, 1989). The off-line methods of molecular biology allow in-depth determination of physiological state. Stephanopoulos et al determined physiological state by applying different methods of linear algebra and statistics to expression patterns of ribonucleic acid (RNA) and proteins, in some cases supplemented with population characteristics (Stephanopoulos, 2002). For instance, they used the physiological state of cells to evaluate the polyhydroxyalkanoates production potential, to map different stages of cancer, to differentiate between responses of photosynthetic bacteria to cycles of different light availability (Stephanopoulos, 2002). The population characteristics have been linked to larger scale systems biology analysis (transcriptome, proteome, metabolome) in evaluating physiological state change during growth rate and pH change of bacteria in changestat cultivations (Lahtvee et al., 2009; Nahku et al., 2010; Valgepea et al., 2010). When comparing different physiological states often statistical analysis is used. For instance statistical methods with visual observations and Artificial Neural Network (ANN) models using offline and online data with online determination of product protein and chaperonin GroEL in interferon alpha production study were used to distinguish three physiological states of batch culture by Feng (Feng and Glassey, 2000).

Related to the physiological state is the concept of growth space of culture, which is defined as a combination of environmental parameter values and the corresponding physiological states where growth rate exceeds zero. It can be viewed as an n-dimensional space formed by the projections of environmental and biological variables (Adamberg et al., 2015). Analysis of growth space (if not stated otherwise, the growth space refers to that of the population, not of individual cells) is equal to the analysis of physiological states as each point in the growth space is represented by particular physiological state – the growth space can be viewed as a continuum of physiological states. Therefore the effect of culture history can shape the growth space as it can introduce new parameters (new axis) affecting it. It is important to note that theoretically growth space is of fixed shape, and by setting the same environmental parameter values and using the same organism with the same history, the same physiological state (point in growth space) must be reached. In reality, achieving the desired physiological state will be biased due to uncertainty related to cultivation instruments, and more notably by differences in starting physiology (from culture history). While the effect of culture history can theoretically be taken into account, it can significantly complicate the measuring of the physiological states and determining the growth space. However, there is a special

subset of growth space – the steady state growth space – in which the physiological state is set almost solely by the environmental parameters (the inherent "noise" in the regulation of biological systems will result in some unavoidable variation in physiological states (Pilpel, 2011)). The model steady state of culture is characterised by unchanging concentrations of different molecules inside and outside of the cells in culture, which is the consequence of near-unique interdependence between environmental conditions and steady state flux patterns of biochemical processes. An analogy is in thermodynamics where state variables set the thermodynamic state uniquely if the system is in thermodynamic equilibrium.

Steady state cultivations provide best means to fix the physiological state but are time-consuming, as after each change in environment stabilisation is needed to obtain the next steady state. However, if the change in environment is small enough, the internal machinery of the cells is able to 'keep up' with the changing environment, and the environmental parameters value will determine the physiological state. This is termed quasi-steady state (QSS), and cell population in it is equal to steady state cell population. As some parameter is changing in QSS a new environmental parameter – the rate of change of environmental parameter or acceleration rate – is introduced as an input, which can affect the physiological state. To maintain QSS, the change in the environment must be smaller than the rate of adaption of the micro-organisms studied, otherwise changes in metabolism and physiology can be induced (Adamberg et al., 2009; Kasemets, 2003). However, if the acceleration rate is correct, the QSS cultivation methods decrease cultivation times significantly while providing culture in distinct physiological states (Adamberg et al., 2015). If a correct acceleration rate is used in QSS, the steady state must follow upon halting the acceleration. Of course, QSS cannot be maintained forever, as an exit from the steady state must follow when the biological capacity of the cells is exhausted.

The growth space can be visualised using mathematical models, which are very useful to describe and understand cause and effect dependencies in bioprocesses. The Monod equation (Eq. 2) presented in Figure 1 A is an example of two-dimensional growth space where a biological parameter (specific growth rate) depends only on the residual substrate concentration (Monod, 1949). According to this model, altering residual substrate concentration results in movement in growth space (change of physiological state). By adding the effects of pH and substrate inhibition to the Monod equation a three-dimensional representation of growth space can be obtained, as depicted in Figure 1 B (modified from (Adamberg et al., 2015), with permission). Cultivation methods operating in steady or non-steady state can be used to "scan" this growth space. However, in non-steady state conditions, the position in growth space (the physiological state) is difficult to maintain (although there can exist a period during which the change in pH and substrate concentration does not significantly alter the physiological state of the culture). Furthermore, in non-steady state growth space (NSSGS) the metabolic changes constantly happening can introduce uncontrolled new environmental parameters affecting the physiological state, thus introducing new axis in the growth space and blurring the cause-effect relationships between physiology and the environment. Essentially obtaining fixed shape of growth space in non-steady state is difficult. On the contrary, in steady state conditions, the steady state growth space (SSGS) is of fixed shape – in the example of Figure 1 pH and residual substrate concentrations can be set, and according to the model, the physiological state is set. By including additional environmental parameters,

the SSGS is made increasingly complex. Also during steady-state bioprocess new factors affecting growth space can emerge as environmental variable values are altered (for instance acetate production in the overflow metabolism of *E. coli*) but after stabilisation the SSGS must assume new fixed shape according to the physiological state obtained.

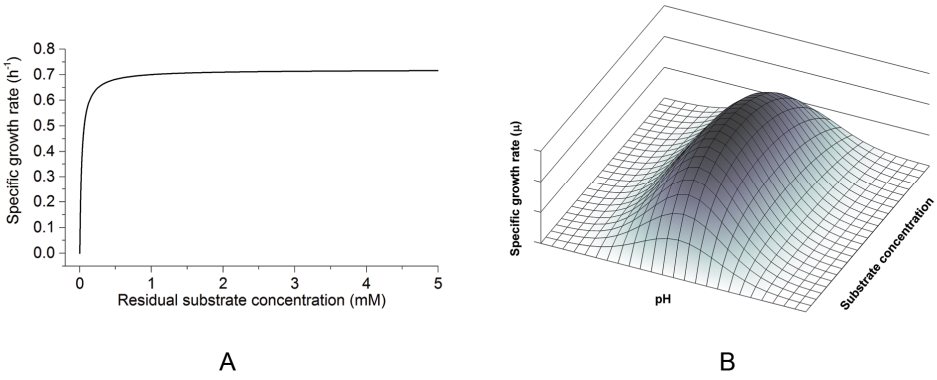

Figure 1. Models of growth space of cells. A – The Monod model is a representation of 2D growth space. B - 3D representation of theoretical growth space combining residual substrates and pH effects.

The growth space contains those parameter values that result in biomass increase, i.e. growth rate exceeds zero (Adamberg et al., 2015; Lahtvee et al., 2009). A broader definition of life space is used herein to include additionally those environmental parameter values that allow for metabolism but do not permit biomass growth. Examples of microorganisms' physiology in this 'non-growth space' are: i) strong induction of recombinant protein expression leading to cessation of growth (Marisch et al., 2013), ii) cultivating microorganisms at C-flow sufficient only for maintenance energy (Paalme et al., 1997), iii) 'viable but not culturable' cells (Oliver, 2010). The life and growth space has been further divided into subsets, best known is probably design space used in QbD initiative, which is a range of process parameters values validated for quality in biological manufacturing process (Rathore and Winkle, 2009).

2.2 Cultivation Methods to Analyse the Growth Space

Human beings have controlled (with varying success) the physiology of microorganisms since ancient times (Mcgovern et al., 2004). The currently available well-controlled and well-modelled cultivation methods are a product of series of research emanating from chemical engineering and microbiology. In the 20th century several key cultivation methods were described, such as chemostat by Monod and Szilard (separately), turbidostat by Bryson, fed-batch by Yoshida, changestat by Paalme and Vilu (Monod, 1949; Novick, Aaron, Szilard, 1950; Paalme et al., 1995; V. Bryson and Szybalsky, 1952; Yoshida et al., 1973). In bioprocess optimisation, all the parameters having an important effect on productivity should be determined, and their impact on physiological state analysed, effectively the relevant portion of life space needs to be looked into (Neubauer 2013).

Analysis of life space is complicated by the need to study the effect of many parameters affecting physiology, including temperature, pH, pO_2, nutrient availability (Madigan, 2015). In addition to analysing the parameter values that are optimal for the microbe, values at the edges of growth space should be studied in certain cases. For instance in the production phase of gamma-aminobutyric acid (GABA) low pH values need scanning, as it is known that glutamic acid conversion to GABA depends on pH (Li et al., 2010). As another example, it has been shown that during recombinant β-galactosidase production *E. coli* should grow at high growth rate to obtain more ribosomes for the protein synthesis (Sandén et al., 2003). At the same time, the regions of life space where production is hindered should be avoided, an example being glucose overflow metabolism at high growth rate in case of *E. coli*, a condition which can be detrimental for the production of recombinant proteins (Jensen and Carlsen, 1990). For obtaining detailed models of physiological states, large segments of the growth space should be scanned to get sufficient data. These examples show that there is a need to be able to scan the whole of growth space, which is possible only by combining different cultivation methods.

2.2.1 Material balance for Bioprocess

Cultivation methods (especially steady state methods) are very important in growth space analysis as they allow reducing the need to determine which physiological results derive from culture history. While controlling some environmental parameters (like pH and temperature) is easy, it is more complicated in the case of the growth rate, which has a major effect on the physiological state (Bull, 2010). Algorithms for growth rate control are derived from the cultivation's material balance (Eq. 1) providing the mathematical apparatus to describe the behaviour of biomass, substrates and products during bioprocess. Further, the material balance deserves somewhat detailed consideration as its components are used herein to obtain most of the population characteristics for evaluating physiological states. Although more elaborate models exist, commonly simple un-segregated and unstructured models are considered sufficient for deriving material balance (Enfors, 2011; Sonnleitner, 1991).

$$\frac{d(S \cdot V)}{dt} = F_{in} \cdot S_{in} - F_{out} \cdot S_{out} - \frac{\mu \cdot X \cdot V}{Y_{xs}} \qquad (1)$$

Material balance of substrate in stirred tank reactor (STR) with no biomass reflux, no maintenance energy and no biomass death. Where X [$g \cdot L^{-1}$] is biomass concentration, V [L] is culture volume, t [h] is cultivation time, F [$L \cdot h^{-1}$] is pumping rate, S [$mol \cdot L^{-1}$] is substrate concentration, μ [h^{-1}] is specific growth rate of biomass, calculated as $\frac{dX}{X * dt}$, Y_{XS} is the biomass yield [$g_{Biomass} \cdot g_{Substrate}^{-1}$], subscripts 'in' and 'out' denote the streams into and out of the bioreactor.

From the material balance, it is seen that growth rate must be controllable by tuning the in- and outflow of limiting substrate (defined as the substrate which depletion will result in growth arrest, other substrates are in surplus). If all other environmental parameters remain constant, the growth rate will depend on residual substrate S concentration, for example, according to the empirical Monod equation (eq. 2) (Monod, 1949). Although the coefficient K_S used in the model has been shown to vary with changing μ

(Ferenci, 2008) Monod equation and its modifications are very useful in describing growth rate behaviour.

$$\mu = \frac{\mu_{max} \cdot S_{res}}{K_S + S_{res}} \qquad (2)$$

Monod equation, where μ_{max} is the maximal growth rate [h^{-1}], K_S is the affinity coefficient [$mmol \cdot L^{-1}$], S_{res} is the residual concentration of growth limiting substrate [$mmol \cdot L^{-1}$] (the same as S_{out} in Eq. 1).

2.2.2 Periodical, Semi-Periodical and Continuous Cultivation Methods

Cultivation methods can be defined based on how the residual substrate concentration of Eq. 2 is obtained into periodic (batch), semi-periodic (fed-batch) or continuous (chemostat), which differ based on the inflow and outflow conditions and more importantly on the quality of process control. All cultivation methods can be further classified into sub-categories based on variations in control of feeding and other environmental parameters. Table 1 presents an overview of most often used cultivation methods applied in growth space analysis.

Table 1. Material balance, main algorithms and apparatus for control of growth rate and environmental parameters for different cultivation methods. We note that each method has several modifications available. For fed-batch and continuous cultivations, the equations are correct if $\frac{dS}{dt} = 0$, which is true when culture is in stabilised state.

Method / parameter	Material balance for substrate	Specific growth rate (μ)	Feeding control algorithm	Environmental parameter control	Most important auxiliary devices	Cultivation specifics
Batch	$\frac{dS}{dt} = -\frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = -\frac{dS \cdot Y_{xs}}{dt \cdot X \cdot V}$	N/A	Z=const, where Z is for instance pH, T, etc.	Sensors	+ - Fast, easy to parallelize, technologically simple, suitable for use in miniaturised systems - - control of physiological state difficult
Fed-batch	$\frac{d(S \cdot V)}{dt} = F_{in} \cdot S_{in} - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot S_{in} \cdot Y_{xs}}{X \cdot V}$	$F_{in} = \frac{\mu_{set} \cdot (X_0 \cdot V_0) \cdot e^{\mu_{set} t}}{S_{in} \cdot Y_{xs}}$	Z=const, where Z is for instance pH, T, etc.	Sensors, inflow, control algorithms	+ - Good control of physiological state, Possibility to achieve high biomass densities, suitable for use in miniaturised systems - - Physiological state cannot be maintained infinitely, technologically more difficult than batch
Chemostat	$\frac{d(S \cdot V)}{dt} = F_{in} \cdot (S_{in} - S_{out}) - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot (S_{in} - S_{out}) \cdot Y_{xs}}{X * V}$	$F_{in} = \mu_{set} \cdot V$	Z=const, where Z is for instance pH, T, etc.	Sensors, inflow, outflow, volume control, control algorithms	+ - Excellent control of physiological state, steady state cultivation - - Stabilisation of physiological state takes time, technologically complex, does not operate in substrate excess
A-stat	$\frac{d(S \cdot V)}{dt} = F_{in} * (S_{in} - S_{out}) - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot (S_{in} - S_{out}) \cdot Y_{xs}}{X \cdot V}$	$F_{in} = (\mu_0 + a \cdot t) \cdot V$	Z=const, where Z is for instance pH, T, etc.	Sensors, inflow, outflow, volume control, control algorithms	+ - Possibility to alter growth rate continuously, QSS cultivation - - Initial stabilisation needed, physiological state can depend on acceleration rate, technologically complex
D-stat	$\frac{d(S \cdot V)}{dt} = F_{in} \cdot (S_{in} - S_{out}) - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot (S_{in} - S_{out}) \cdot Y_{xs}}{X \cdot V}$	$F_{in} = \mu_{set} \cdot V$	$Z = Z_0 + a \cdot t$, where Z is for instance pH, T, etc.	Sensors, inflow, outflow, volume control, control algorithms	+ - Possibility to alter environmental parameter Z continuously at fixed dilution rate, QSS cultivation - - Initial stabilisation needed, physiological state can depend on acceleration rate, technologically complex
Auxostat	$\frac{d(S \cdot V)}{dt} = F_{in} \cdot (S_{in} - S_{out}) - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot (S_{in} - S_{out}) \cdot Y_{xs}}{X \cdot V}$	$F_{in} = \mu_{high} \cdot V$ or $F_{in} = \mu_{low} \cdot V$	Z=const, where Z is for instance pH, T, etc.	Sensors, inflow, outflow, volume control, control algorithms, biomass control	+ - Operates in substrate excess, excellent control of physiological state, steady state cultivation - - Stabilisation of physiological state takes time, technologically complex
Auxoaccelerostat	$\frac{d(S \cdot V)}{dt} = F_{in} \cdot (S_{in} - S_{out}) - \frac{\mu \cdot X \cdot V}{Y_{xs}}$	$\mu = \frac{F_{in} \cdot (S_{in} - S_{out}) \cdot Y_{xs}}{X \cdot V}$	$F_{in} = \mu_{high} \cdot V$ or $F_{in} = \mu_{low} \cdot$	$Z = Z_0 + a \cdot t$, where Z is for instance pH, T, etc.	Sensors, inflow, outflow, volume control, control algorithms, biomass control	+ - Possibility to alter environmental parameter Z continuously in substrate excess, QSS cultivation - - Initial stabilisation needed, physiological state can depend on acceleration rate, technologically complex

2.2.3 Batch Cultivation

Nearly all cultivations start as a batch culture, where the microorganisms inoculated into growth medium adapt to the conditions and start to grow exponentially. Typically, growth continues until some substrate is exhausted or product becomes toxic, this is followed by growth arrest phase. The control of the physiological state is difficult to achieve in batch cultivation. It is heavily influenced by the effect of culture history as concentrations of biomass, substrate, and products change (Monod, 1949). Reproducibility can be an issue in batch cultivation, for instance, CO_2 productivity landscape has been shown to depend on the size of inoculum (Sonnleitner, 1991).

In a theoretical model of batch cultivation a single limiting substrate controls the growth rate, with the effect of other substrates and products being negligible. The physiological state thus changes only due to growth rate, and this change can be visualized as moving in direction of decreasing substrate concentration in Figure 1 A. Thus, even in model batch cultivation the growth rate is constantly changing, meaning that physiological state control is lacking or difficult to achieve. There is a period of balanced growth during the exponential phase which can be denoted as steady state (in relation to biomass constituents) and has been used in physiology studies (Gerosa et al., 2015). However, the region where substrate concentration limits growth can be difficult to analyse in batch, as it is brief in time. Further, growth is influenced by the changing concentrations of metabolites and non-limiting substrates, therefore complicating the determination of the physiological state. Limited control of growth rate of microbes can and has been applied in batch cultivation by using different substrates or environmental parameter values, and several fundamental discoveries of physiology have been made this way (Schaechter et al., 1958). However, the extent of balanced growth is limited. It was suggested by Monod to last while biomass concentration remained below 0.25 $g \cdot L^{-1}$ (Monod, 1949). Other studies have shown balanced growth ending (growth rate starting to change) above OD=0.3 (approximately 0.1 $g \cdot L^{-1}$) in LB medium; physiology change above OD 0.5 has been noted as expression of alternative transcription factor σ^{24} increased (Lange and Hengge-Aronis, 1994; Sezonov et al., 2007).

Because of the difficulties in physiology control, the results obtained in a batch cultivation are mainly of qualitative or semi-quantitative nature (Bull, 2010; Le Marc et al., 2005). The ease of parallelisation makes batch cultivation common in initial clone screening experiments, although the use of cultivation methods more similar to the final production process has been suggested (Bareither and Pollard, 2011; Panula-Perälä et al., 2008). Still batch cultivation will remain the most often-used cultivation method, as the technological setup is simple.

2.2.4 Fed-Batch

Semi-periodical or fed-batch cultivation can be used to achieve high density biomass in a controlled manner; it is therefore much used in bioprocesses (Yang, 1996). Typically fed-batch is started a batch cultivation, once the C-source is exhausted controlled feeding is started. Exponential feeding control algorithm in Table 1 denotes that microbial growth is achieved by providing limited amounts of substrate to the culture, maintaining constant residual substrate concentration. The controlled growth rate is desirable to avoid metabolic peculiarities of the host (such as overflow metabolism), or not to introduce

growth inhibition by the concentrated substrate. The reduced growth rate is also used to keep clear of technological limitations of the bioreactor hardware such as mass transfer of oxygen or heat removal (Yang, 1996).

The constant residual substrate concentration results in constant growth rate (Figure 1 A) thus the culture is in steady state in relation to substrate concentration. Still, the physiological state during a fed-batch cultivation is typically not constant, as the increasingly concentrated biomass leads to changes in the environmental conditions in the reactor (such as ionic strength, osmolarity). For instance cell volume has been reported to decrease in the course of a fed-batch, and yellow fluorescent protein degradation has been shown to depend on biomass density (Paalme et al., 1990; Takahashi et al., 2014). Therefore, fed-batch cultivations with increasing biomass densities are not the best means of steady-state growth space analysis. However, it must be included if in production the final bioprocess will be fed-batch.

2.2.5 Chemostat

The gold standard for control of the physiological state – the chemostat – was invented in 1950 (Monod, 1950; Novick, Aaron, Szilard, 1950). It operates by keeping all parameters (both environmental and biological) constant at steady state, thereby nullifying the effect of culture history (Sonnleitner, 1991). Theoretically, the experiment time seizes to be an independent variable and culture can be kept in fixed physiological state indefinitely, although in practice mutations render this impossible (Ferenci, 2008). Still, a stabilised chemostat is arguably the best method to obtain well-determined steady state physiology, with which all other cultivation methods can be compared.

Chemostat starts as batch cultivation, in which after reaching some trigger condition medium inflow and outflow is started. From Table 1 it is seen that the growth rate of microorganisms in chemostat is established by providing feed and outflow at a constant rate; more specifically the feeding rate is equal to the multiplicative of growth rate setpoint and culture volume. The term $\frac{F_{in}}{V}$ is defined as dilution rate, D (h^{-1}) and in steady state, this will set the residual substrate concentration and thus specific growth rate μ.

Theoretically, every residual substrate concentration on the graph in Figure 1 A can be obtained, but only after stabilisation period which typically lasts for five residence times (τ) (Daranlapujade et al., 2008). We define one residence time as the time needed to dilute the culture in the reactor twofold. An example of growth space study using chemostat cultivation is presented in Figure 2 A.

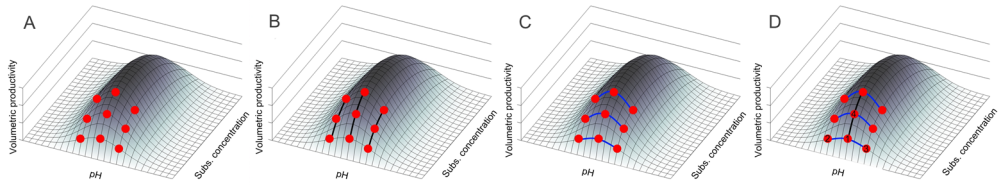

Figure 2. Theoretical volumetric productivity (can be recombinant protein production) as a function of pH and residual substrate concentration. A – D depict cultivation methods to analyse the growth space: A – chemostat, B – A-stat, C – D-stat, D – combination of A-stat with D-stat in the mother-daughter (MD) system.

Culture in fixed physiological state in chemostat is used extensively in physiology research. Ishii et al 2007 observed little change in physiological state on the level of transcriptome, proteome and metabolome in chemostat cultures of different single gene knockouts, but reported active regulation of enzyme levels to perturbations in dilution rate (Nobuyoshi Ishii, Kenji Nakahigashi, Tomoya Baba, 2007). The unchanging nature of chemostat data is also very suitable and has been widely used for modelling purposes, or for obtaining productivity data (Hellmuth, 1994; Holms, 1996).

Chemostat and continuous manufacturing is used more and more in the industry as it is more productive and easier to automate than batch or fed-batch cultivations. Further, it is convenient to apply the process analytical technology (PAT) and QbD requirements in continuous cultivation (Hernandez, 2015; Konstantinov and Cooney, 2015; Warikoo et al., 2012).

An inherent weakness of chemostat cultivation is the long stabilisation period and the discrete nature of the results, as physiological parameters are analysed in single points in the steady state growth space (Figure 2 A). After one dilution rate is studied, another is applied, which can lead to washout if the ramp in growth rate is too high – it was stated already in 1975 that the magnitude of feeding rate increase in chemostat is a source of uncertainty (Dunn and Mor, 1975). Further, the growth space which can be analysed by chemostat is restricted to the substrate limited regions – running chemostat near μ_{max} has proven to be difficult as any increase in glucose influx can result in overflow metabolism and consequently in culture washout (Paalme et al., 1995). Chemostat applies selective pressure to the culture, resulting in enrichment of mutants with faster substrate consumption rate (smaller K_s value, of increase in biomass yield) (Novick Aaron, 1950; Rabbers et al., 2015). For this reason, it has been argued that in reality, no steady state exists (Ferenci, 2008). However, chemostat has been used in numerous studies lasting hundreds of hours with no apparent mutation (Hoskisson and Hobbs, 2005).

A special case of chemostat cultivation is one without outflow. This can also be regarded fed-batch at constant growth rate and biomass density (the μ-stat). This method can be used to obtain QSS physiological state statistically representing chemostat conditions (Erm et al., 2014). Means to increase biomass amount in QSS are critical in the mother-daughter (MD) cultivations (discussed in detail in Materials and Methods section). This method can be used also to obtain QSS culture in small-scale minireactors, as there is no need for monitoring culture volume, provided that the inflow pump operates according to the pre-set exponential profile.

2.2.6 A-stat

The accelerostat (A-stat) is an example of a changestat cultivation developed to analyse the effect of specific growth rate on physiology in steady state conditions but faster and with better resolution than with chemostat (Paalme et al., 1995). A-stat starts as chemostat in which after stabilisation the feeding rate is not constant but changes according to the Eq. 3.

$$D = D_0 + a_D \cdot t \qquad (3)$$

Algorithm for A-stat operation. A-stat starts as chemostat where after stabilisation of culture the dilution rate is increased. D_0 – initial dilution rate [h^{-1}], $a_D = \frac{dD}{dt}$ – acceleration rate [h^{-2}], t – cumulative time during which the A-stat algorithm is applied [h]

Unlike chemostat which fixes the physiological state at one particular residual substrate concentration, the A-stat is expected to change the residual substrate concentration, resulting in the trajectory in growth space visualised in Figure 1 A and Figure 2 B. Due to the continuous movement in growth space the data obtained is also continuous with high information density. As growth rate is changing, the physiological state of microbes cultivated in the A-stat cultivation does not represent steady state but QSS. By applying proper acceleration rate A-stat has proven to provide continuous, high quality data; for instance in the elaboration of metabolic switch points during growth rate increase and productivity analysis (Hoekema et al., 2014; Valgepea et al., 2010; van der Sluis et al., 2001). By stopping the acceleration the A-stat cultivation must turn into chemostat cultivation, this can be used to monitor if the acceleration rate applied is sufficiently low (Adamberg et al., 2009).

2.2.7 D-stat

Another changestat operating in substrate limitation in QSS is the D-stat, whereby the effect of some environmental parameter other than growth rate can be analysed at fixed dilution rate (Kasemets, 2003; van der Sluis, 2002). D-stat is started as chemostat in which after stabilisation an environmental parameter (e.g. pH) denoted by Z is changed according to the algorithm presented in Eq. 4. Figure 2 C illustrates growth space scanning using D-stat cultivation. D-stat will remain in QSS growth space until the changing rate of environmental parameter exceeds a critical value beyond which the biological adaption rate cannot keep up. Then growth rate will become smaller than the dilution rate and washout will follow. Until that moment, the data from D-stat is continuous QSS data with high resolution.

$$Z = Z_0 + a_Z \cdot t \qquad (4)$$

Operational algorithm for D-stat cultivation. Z denotes an environmental variable (e.g. pH), Z_0 is the value of that variable at the onset of the cultivation algorithm, a_z is the rate of change of the environmental parameter, t is the time from the onset of the D-stat algorithm.

D-stat is a useful cultivation method to analyse the effect of an environmental parameter on physiology at fixed dilution rate – it has been used to study metabolic pathways, determine critical values of environmental parameters, studying co-consumption of substrates (Kasemets, 2003; Valgepea et al., 2010; van der Sluis, 2002).

2.2.8 Auxostat

The chemostat based cultivation methods will fail if growth space in the μ_{max} range is studied. For that section of growth space, auxostat based methods should be used. Steady state growth space analysis at μ_{max} is achieved by employing continuous cultivation methods whereby biological parameter correlated to biomass is held constant by diluting

excess culture with feed while providing the cells with ample substrate. As residual substrate concentration is high then according to Figure 1 A the growth rate must be high as-well. Historically the first auxostat maintained turbidity of the culture constant (termed turbidostat), but other parameters, such as pH and CO_2 have also been exploited (Bull, 2010; V. Bryson and Szybalsky, 1952). Auxostat cultivations have been used in metabolic flux analysis, to select for mutants, to analyse the effect of biomass concentration on heterologous protein degradation, and (Holms, 1996; Takahashi et al., 2014; V. Bryson and Szybalsky, 1952).

2.2.9 Auxo-accelerostat

Auxostat is conceptually similar to chemostat in that it allows analysing a single physiological state at a time, and analysis of another physiological state requires a new stabilisation. To change the physiological state continuously in QSS at μ_{max} an auxo-accelerostat cultivation method can be used. Auxo-accelerostat operates in substrate excess while keeping some biological parameter (correlated to biomass growth) constant by diluting the culture with feed. The physiological state is changed by altering some environmental parameter continuously according to Eq. 4. Auxo-accelerostat has been used to study at μ_{max} the stress response of *S. cerevisiae* toward linearly increasing NaCl or ethanol concentrations; and to study the metabolism of *S. cerevisiae* at linearly increasing temperature or tryptone concentration (Kasemets, 2003; Nisamedtinov et al., 2008).

2.3 Combining Different Cultivation Methods in Growth Space Analysis

The use of advanced changestat cultivations has been shown to reduce experiment time while providing continuous steady state equal data, but these cultivations are started as chemostat, therefore including substantial idle time during initial stabilisation (Adamberg et al., 2015). At the same time several different changestat cultivations are needed to analyse the different areas of the growth space – in substrate limitation A-stat needs to be used to analyse growth rate, D-stat to study the effects of pH, T, inhibitors, etc.; and the cultivation methods must change to auxo-accelerostat methods to analyse conditions at substrate excess (at μ_{max} conditions). Several combinations of environmental parameters must be analysed in the comprehensive screening of the growth space. Further, it can be possible that different acceleration rates have to be used. In total, this results in substantial stabilisation time, which could be spared if changestat cultivations would start from steady state in a multi-bioreactor system where culture from a mother-reactor is used to periodically fill daughter-reactors (the system is discussed in detail in Materials and Methods section). In the MD system different cultivations from the precisely defined physiological state in the daughter-reactors can be started, for instance, A-stat in the mother-reactor can be turned into two D-stats in the daughter-reactors (Figure 2 D). In addition to allowing starting different changestat or steady state cultivations with no stabilisation, the MD system can be used to combine growth space scanning experiments with physiology perturbing experiments, which are necessary for productivity studies and time consuming in traditional changestat and chemostat cultivations due to the need to start a new experiment after each perturbation.

2.4 Perturbation Analysis in Physiology Research

Typically, the introduction of non-steady state will complicate physiology analysis; however, there are several examples in physiology and productivity studies where steady state is deliberately perturbed and non-steady state introduced. This controlled exit from the steady state into the non-steady state is termed herein as perturbation experiment. Experiments where perturbations have been applied include mutant phenotype analysis, determining maximal growth rates or productivities, studying accumulation of polyhydroxyalkanoates, in balancing medium composition, heterogeneity analysis, and antibiotics efficacy analysis (Ishii et al., 2007; Lara et al., 2009; Levin and Udekwu, 2010; Spadiut et al., 2013).

Applying the perturbation will alter the molecular machinery and metabolite fluxes in the cell in more or less permanent manner, thus to obtain a new steady or QSS a new experiment or at least stabilisation is required, which makes repeated perturbation analysis time consuming. Starting perturbation experiments from the steady state without the need for stabilisation would substantially decrease the total experiment time. This is especially relevant if many physiological states need to analysed, such as in the analysis of recombinant protein production. Induction of recombinant protein production clearly disrupts the physiological state, as metabolic imbalance, stringent response, growth cessation, plasmid loss, etc. can be introduced (Carneiro et al., 2013; Marisch et al., 2013). The productivity of proteins is affected by numerous pre- and post-expressions conditions, which are typically experimentally analysed as productivity can vary depending on the host and the product. For instance, Curless et al reported increased production of interferon upon pre-induction growth rate increase while Shin reported little effect of pre-induction growth rate on human mini-proinsulin production (Curless et al., 1990; Shin et al., 1997). Post-expression medium effect has been reported by Tsai et al, who reported no production of insulin like growth factor-1 in mineral medium but were able to produce 30 $mg \cdot L^{-1}$ in temperature induced system in rich medium, as the high nitrogen content of the feeding protected the product from proteolysis (Yang, 1996). Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration increase in IPTG inducible expression systems has been shown to result in a linear increase in b-galactosidase activity when IPTG concentration was increased from 0.01 – 1 mM (Yang, 1996). However, IPTG can result in negative synergistic effect as IPTG can exacerbate the toxic effects of products (Dvorak et al., 2015).

The examples above illustrate the need to decouple growth space scanning from perturbation analysis. This has been realised in multi-bioreactor systems where biomass growth and perturbation experiments take place in separated bioreactors (Erm et al., 2014; Fricke et al., 2013, 2011; Shin et al., 1997). Once perturbation analysis in the perturbation unit is complete, the culture within can be discarded and replaced with culture from the growth unit in order to study the effect of another perturbation. We emphasize that in order to continue in the daughter-reactors experiments with culture in the physiological state established in the mother-reactor the transfer process itself must not introduce any disturbances. The transfer of culture from one reactor to several without altering the physiology of the organism was defined as multiplying the physiological state of the organism (Erm et al., 2014).

2.5 Multi-Bioreactor Systems and their Applications

Using reactors in series to gain high conversion rates is well established in chemical engineering (Coker, 2001). Similarly, the concept has been applied successfully in bio-engineering (Curless et al., 1991; Fricke et al., 2011; Shin et al., 1997). The idea is to prepare culture in one reactor of the system and to transfer an aliquot of that culture into another reactor for physiology or productivity analysis. The focus of prior works is mainly on starting cultivation experiments faster due to the increased amount of inocula, and to separate biomass growth from physiology disrupting experiments – strict control of the physiological state prior and after culture transfer has not been addressed.

A technologically simplest example of culture transfer is the repeated batch where overnight culture is inoculated into fresh media, resulting in severe changes in environmental conditions, which is no problem if the physiological state of the cells is not of particular interest. This technique was successfully used by Lenski et al in mutation analysis of *E. coli* (started in 1988, by 2016 had undergone 65 000 generations) resulting in increased fitness and size, and most strikingly the ability to consume citrate in aerobic conditions (Blount et al., 2008).

Culture transfer is not limited to shake flasks. For example, coupling STR to plug flow reactor (PFR) is often used to simulate the heterogeneities reported in industrial scale bioreactors. Lemoine et al used two PFRs connected to STR to study the effects of high feed concentration and oxygen limitation zones to the metabolism of *Corynebacterium glutamicum* in fed-batch cultivation, noting lactate accumulation in the PFR (Lemoine et al., 2015). Differently from previous Visser et al described a STR coupled to PFR in which the exit stream of PFR was not fed back to the STR (Visser et al., 2002). The device was called 'BioScope' and was designed to study short-term effects (during 90 seconds) of chemically induced perturbations.

There are several examples where culture from one STR is transferred into another STR. These systems differ based on whether the transfer of biomass is continuous or periodical. A continuous transfer of biomass from STR operated in the chemostat at 37 ^{o}C into the second chemostat operated at a higher temperature (47.5 ^{o}C) and 10 % higher dilution rate was used by Lüders et al to study the effect of constant heat shock (Lüders et al., 2009). A similar method was described by Curless et al who described recombinant interferon production in a two STR system where the first unit was used for biomass production, and the second was used for constantly inducing the recombinant product (Curless et al., 1990). In addition to continuous transfer also periodic biomass transfer between growth reactor and induction reactor has been realised previously (Fricke et al., 2011; Shin et al., 1997). Shin et al used periodic transfers from growth tank to induction tank and obtained 10 – 44 % better volumetric productivity of modified human insulin produced by *E. coli* compared to single stage operation (Shin et al., 1997). The cultivation in the growth tank was fed-batch cultivation with changing growth rate, needed to obtain the correct amount of culture at the time when the induction reactor was ready for transfer. The feeding was maintained constant in the induction tank after induction. Fricke et al engineered a conceptually similar but technologically superior system, where design of experiments was used for optimisation of production of malaria vaccine by *Pichia pastoris* (Fricke et al., 2013, 2011). They described inoculating six smaller scale (1 L) bioreactors with identical culture from one bigger (5 L) bioreactor, with varying

conditions in the 1 L reactors. The cultivation methods used were fed-batch in the growth tank, followed by batch cultivation in the production tanks.

The examples considered illustrate that prior works do not focus on multiplying the physiological state using culture transfer. For instance, in the work by Fricke et al the bacterial culture was diluted following the culture transfer. They also applied 20 ºC in the induction tank, while 30 ºC was used in the growth tank (Fricke et al., 2011). Shin et al used different growth rates in the growth and production tanks, Curless et al varied the growth temperature (Curless et al., 1991; Shin et al., 1997). The apparatus of culture transfer is not mentioned, nor is the culture transfer time; both are however critical to keep the steady state. The physiological states before and after culture transfer have not been compared statistically.

2.6 Hardware for Physiological State Control

Analysis of life space in well-defined physiological state is impossible without the proper apparatus for mass and heat transfer, sensors and actuators for precise measurements and control with software to apply cultivation algorithms at necessary speed. If any of these components malfunctions, new physiology affecting parameters can emerge, potentially resulting in uncontrolled exit from the steady state. For all hardware components, there are several options, most relevant examples relating to growth space analysis are discussed below. Emphasis is on technical solutions that find use in miniature bioreactors, as the MD system is most efficient when miniature bioreactors are used as daughter-reactors (discussed in Results and Discussion).

2.6.1 Bioreactor Configuration and Size (Mass Transfer)

To avoid gradients in the culture liquid proper mass transfer (characterised by k_La) inside the bioreactor is crucial. The gold standard with which most bioreactor set-ups are compared is the laboratory scale STR with Rushton turbine providing sufficient mass transfer (k_La ~400 h^{-1}) to rule out all possible physiological state disturbances due to culture heterogeneities, at least at low to moderate biomass densities (Bower et al., 2012; Krommenhoek, 2007; van Leeuwen et al., 2009). Further, the reactor operating at ~1 L range provides enough biomass for most on- or offline analysis. However, these reactors are difficult to parallelize, their set-up time is large, and they consume substrate in correlation with their volume (Ali et al., 2012; Alvin et al., 2013). For these reasons multiplexed miniature bioreactors (V<100 mL) leading to increased parallelization and decreased amounts of consumables are actively researched (Lattermann and Büchs, 2015). The mass transfer capabilities of the minireactors are typically the best in mini-STRs (k_La range 50 – 1440 h^{-1}), followed by MTPs (k_La range 100 – 400 h^{-1}) and shake flasks (k_La range 20 – 100 h^{-1}) (Kensy et al., 2009; Lamping et al., 2003; Puskeiler et al., 2005; Zhang et al., 2008). Further, there are options to use magnetically stirred Duran bottles as reactors (k_La ~200 h^{-1}) or custom made bioreactors fabricated from test-tubes, mixed either by gassing or by magnetic stirring (Li et al., 2014; Matteau et al., 2015; Takahashi et al., 2014). A problem of shaken reactors is the need to stop shaking and therefore introduce disturbance in the physiological state upon sampling. While designing apparatus for growth space study the size of the sample needed must also be kept in mind,

for instance minimally 3-5 mg of monoclonal antibodies have been suggested necessary for titer determination (Bareither and Pollard, 2011).

2.6.2 Environmental Parameters O_2, pH and T

Typical parameters determined on-line during bioprocess are O_2, pH and T; with relatively large polarographic and potentiometric sensors used for O_2 and pH and contact thermometers for temperature (Sonnleitner, 1991). The accuracy and response time of these sensors is considered sufficient for controlling steady-state, but their size limits their use in miniature systems (Sonnleitner, 1991). In the latter miniaturised versions of traditional sensors, optical or field effect transistor (FET) based sensors are applied (Ali et al., 2012; Kirk and Szita, 2013). The miniaturised versions of traditional sensors tend to be fragile. Optodes rely on fluorescence or absorption change, which depends on pH or pO_2 level, they are robust, easy to parallelize, with fast response time (msec), they need no calibration and no traditional reference sensor. However, they tend to have narrow reading frame (5-8 pHU), are destroyed by autoclaving, can display cross sensitivity to ions, can need some initial equilibration time (Biechele et al., 2015; Gernaey et al., 2012; Janzen et al., 2015; Krommenhoek, 2007; Lattermann and Büchs, 2015).

FET sensors are robust, small size, mechanically strong, with high sensitivity and fast response time (t90 below 1 sec) (Gernaey et al., 2012; Krommenhoek, 2007; Schäpper et al., 2009). However, FET sensors are relatively expensive which limits their use.

If, for example, a microtiter plate type bioreactor array is used in cultivation then control of pH is achieved either by dispensing titrant sequentially into the wells (Schmideder et al., 2015) or by dedicated dispensing systems (one dispenser per one well) (Ali et al., 2012). The periodic dispensing inherently introduces lag time between two consecutive pH regulation steps, which can be a problem for maintaining well controlled physiological state. Continuous dispensing can be realised by pumps (Ali et al., 2012) or by valves (Funke et al., 2010). However, periodical measurement and control is more economical as less hardware per reactor is required. Even more economically feasible are systems where the buffered medium is used to keep pH in the desired range (Klein et al., 2013; (Li et al., 2014). As an example, Klein studied the respiro-fermentative growth in buffered chemostat at different growth rates with good results, as in this case, the dissolved oxygen has much more impact on physiology comparative to the small pH change (Klein et al., 2013).

The measurement of dO_2 using optodes can be periodical or continuous, for instance the use periodical pO_2 determination by commercial robotic system the Biolector has been used to monitor the growth of *E. coli* in batch successfully and fed-batch cultivation in MTPs by (Funke et al., 2010) (Wilming et al., 2014). There are versions of oxygen measurement stations in which shaking/stirring is stopped upon measurement, which can result in anaerobiosis (Betts et al., 2014). However, if maximum process control is desired a dedicated sensor with the corresponding apparatus is the method of choice (Schmideder et al., 2015). Control of oxygen concentration in miniature systems is often absent, although examples with cascade control (stirring and aeration) exist (Bareither et al., 2013). We note that if the exact oxygen concentration is of no interest and oxygenation is sufficient, the physiological state control with no oxygen sensor is possible.

Temperature measurement in miniature systems typically employs Pt made thermistors (Krommenhoek, 2007). Temperature control is most often achieved by setting the temperature of the whole system by placing the units into an incubator or using water jacket, although individual reactor control has been described (Bareither et al., 2013) (Ali et al., 2012) (Wewetzer et al., 2015) (Schmideder et al., 2015).

2.6.3 Substrate Addition

Similarly to laboratory scale STRs, dispensing substrate to mini-and microreactors can be realised by peristaltic and syringe pumps, but also micro peristaltic pumps, micro membrane pumps, microvalves are employed (Bower et al., 2012; Gebhardt et al., 2011). The flow can be periodical or continuous, the first resulting in pulsed feeding (Puskeiler et al., 2005; Schmideder et al., 2015; Takahashi et al., 2014). Pulsed flow can disrupt the physiological state in substrate limited conditions but can be tolerated in cultivation methods operating substrate excess (auxostat, auxo-accelerostat). When less accurate, steadily decreasing specific growth rate control is acceptable, the enzymatic release of C-source monomers or diffusion of the substrate can be used (Wilming et al., 2014)

3 AIMS

The aims of the thesis were as follows:

1) develop a prototype system for multiplying steady state physiological state of *E. coli*;

2) evaluate the maintenance of the physiological state of cell population during the preparatory phases of culture transfer and compare the physiological state of the cell population in cultivations conducted in the MD system and traditionally;

3) analyse the efficiency of the MD system in growth space and perturbation analysis theoretically;

4) analyse the technological needs and possibilities for creating an automated MD system for growth space analysis.

4 MATERIALS AND METHODS

Publications I, II, and IV cover in detail the used Materials and Methods, below an overview is given.

4.1 Strains, Inoculation Procedure and Culture Media

In the A-stat cultivation of Publication I *E. coli* K-12 MG1655 strain (λ-, F-, rph-1, Fnr+; Statens Serum Institute (SSI), Reference number: C 438-01) was used. In the A-stat cultivation of Publication II *E. coli* K-12 MG1655 (λ-, F-, rph-1, Fnr+; Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), DSM No.18039) was used. In both A-stat cultivations, the inoculation procedure was as follows: 5 mL of glucose containing mineral medium was added to the lyophilized culture, incubated at 37 ºC for 24 h. A syringe was used for inoculation of pre-grown inocula into the bioreactor.

GFP production analysis (Publication IV) was carried out using kanamycin resistant Escherichia coli BL21(DE3) pET42-GFPuv (Clontech). Inocula was prepared by outplating lyophilized culture on kanamycin-containing LB Petri dishes followed by overnight incubation at 37 ºC. About three colonies were used to inoculate Erlenmeyer flask containing 15 mL of modified mineral medium (contained acid hydrolyzed casein as N-source). The culture was incubated for eight hours at 37 ºC at shaker operated at 240 rpm. A Syringe was used for inoculation of pre- grown inocula into the bioreactor, medium used was the same as in the Erlenmeyer flask. Production of the GFP was induced by adding IPTG (final concentration 0.5 mM).

The cultivation media in case of A-stat cultivation was mineral medium with added glucose (details in Publication I and Publication II). In the GFP production experiments in chemostat phase, the cultivation media was mineral medium with glucose, with additional casamino acids added. In the fed-batch phase the same, but 20 x concentrated media was used (details in Publication IV).

4.2 Analytical Methods

Biomass concentration was determined by multiplying the optical density of the culture with a predetermined conversion factor. Metabolites were determined by high-pressure liquid chromatography, fluorescence by fluorimeter (details in Publication I, II and IV).

4.3 Cultivation Methods

The A-stat cultivation was started as chemostat ($D=0.1\ h^{-1}$), in which after stabilisation of five residence time at constant conditions the A-stat algorithm (Eq. 3) was applied (acceleration rate $0.01\ h^{-1}$ was used). The GFP production was started as chemostat ($D=0.1\ h^{-1}$), in which following stabilisation for five residence times an exponentially fed fed-batch cultivation ($\mu_{set}=0.1\ h^{-1}$) was started. Simultaneously with the start of fed-batch, the heterologous protein production was induced by adding IPTG. The same procedure was repeated at dilution rate $D=0.4\ h^{-1}$.

4.4 Cultivation System

The cultivation system consisted of three modified 1.25 L glass bioreactors (Applikon). Actuators and sensors were managed using EZ controller (Applikon) which was in turn controlled by BioXpert XP or W7 software (Applikon). Bioreactors were equipped with in situ pH, T, pO_2, OD sensors; temperature was kept at 37 ^oC using heating blanket and pH at 7 using 2 M NaOH and 2 M H_2SO_4. The culture was aerated at a flow rate 150 $mL \cdot min^{-1}$ (A-stat) or 200 $mL \cdot min^{-1}$ (GFP production); gas mass flow controllers (GFC) set the flow. In the case of variable volume fermentation and the GFP production, the dO_2 level was maintained at ~40 % by sparging additionally pure oxygen into the culture medium according to a PI algorithm using another GFC. For in-line gas analysis (O_2 and CO_2) Bluesense gas sensors were used. Dilution rate was calculated by on-line weighting the inflow and the bioreactor using scales (Sartorius). Rushton turbine was used for mixing at 800 rpm. In order to keep the culture liquid during variable volume cultivation as homogeneous as possible three turbines were used on the stirrer shaft, separated from each other on the stirring axis by one impeller diameter. Data collection interval for the critical variables (weights, dO_2) was 1 second, for other variables it varied from 10 seconds to 5 minutes.

4.5 The MD System

The MD system was designed to transfer culture from one reactor to several without altering the physiology of the organism. We define this operation as multiplying the physiological state of the organism. The system consists of at least on mother- and at least one daughter-reactor. The set-up operates by maintaining culture in steady state physiology in the mother-reactor, and by transferring culture in this precisely defined physiological state into daughter-reactors where changestat or perturbation experiments could be run. Ideally, no stabilisation period in the daughter-reactors is needed. Figure 3 depicts a generic cultivation scheme in the MD-system.

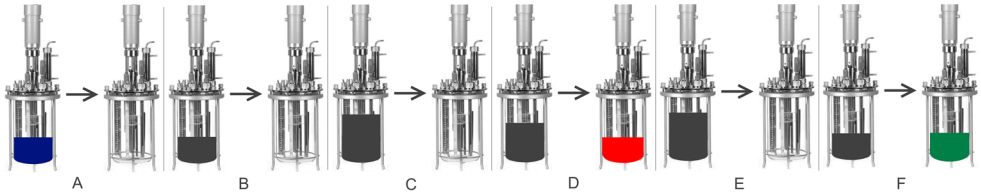

Figure 3. MD scheme in a generic set-up in two laboratory STRs. A – batch and stabilisation in the mother-reactor. B – changestat cultivation to bring the culture in the mother-reactor into the physiological state to be analysed. C – variable volume cultivation without altering the physiological state. Culture transfer into the daughter-reactor completes this phase. D – experiment one in the daughter-reactor. Red indicates that the physiological state in the daughter-reactor is different from that of the mother-reactor. Note that the physiological state in the mother-reactor remains unaltered, and volume increase phase has started. E – the daughter-reactor has been drained, cleaned and sterilised. The mother-reactor has reached the volume necessary to fill the working volume of the daughter-reactor. This phase ends with culture transfer into the daughter-reactor. F – experiment two in the daughter-reactor. Green indicates that the physiological state in the daughter-reactor is different from that of the mother-reactor.

4.6 Population Characteristics to Quantify the Physiological State

The equations to calculate the population characteristics of cell culture used in the thesis to evaluate the physiological state of the cells are presented below. Both the on- and offline data was splined with a cubic spline.

In continuous cultivations, the specific growth rate of the cells was calculated according to Eq. 5.

$$\mu = \frac{d(X \cdot V)}{X \cdot V \cdot dt} + D \qquad (5)$$

Specific growth rate μ [h^{-1}], where X is biomass concentration [$g \cdot L^{-1}$], V is culture volume [L], t is time [h], D is dilution rate [h^{-1}]

In continuous cultivations, the production per biomass of metabolites carbon dioxide (O_{CO2}), acetic acid (O_{ace}), acetylaspartic acid (O_{aceasp}), dihydroorotic acid (O_{dho}) and lactic acid (O_{lact}), all mmol·g^{-1}, were calculated according to the general Eq. 6:

$$O_P = \frac{D \cdot P + \frac{dP}{dt}}{D \cdot X + \frac{dX}{dt}} \qquad (6)$$

Production of product P per biomass O_P [mol·g^{-1}], where P is product concentration [mmol·L^{-1}]

In continuous cultivations the specific carbon dioxide production rate q_{CO2}, specific GFP production rate q_{GFP}, both mmol·g^{-1}·h^{-1} was calculated according to general Eq. 7.

$$q_P = O_P \cdot \mu \qquad (7)$$

Specific production rate of product P, q_P [mmol·g^{-1}·h^{-1}]

In fed-batch cultivation, the specific growth rate μ was calculated based on Eq. 8.

$$\mu = \frac{d(X \cdot V)}{X \cdot V \cdot dt} \quad (8)$$

Specific growth rate μ in the fed-batch cultivation.

In fed-batch cultivation, the volumetric productivity of GFP Q_{GFP} was calculated based on Eq. 9.

$$Q_P = \frac{d(C_{GFP} \cdot V)}{V \cdot dt} \quad (9)$$

Volumetric productivity of GFP Q_{GFP} [$FU \cdot L^{-1} \cdot h^{-1}$], where C_{GFP} is the concentration of the GFP [$FU \cdot L^{-1}$], FU stands for fluorescence units.

In fed-batch cultivation, the specific productivity of GFP q_{GFP} during the fed-batch cultivation was calculated based on Eq. 10

$$q_P = \frac{d(C_{GFP} \cdot V)}{X \cdot dt} \quad (10)$$

Specific productivity of GFP q_{GFP} [$FU \cdot g^{-1} \cdot h^{-1}$], where C_{GFP} is the concentration of the GFP [$FU \cdot L^{-1}$], determined as fluorescence.

4.7 Statistical analysis

The physiological state of cell population should not change due to culture volume increase and culture transfer. The physiological state was evaluated by comparing with statistical analysis the population characteristics of culture in the different phases of cultivation in the MD system. At D=0.1 h^{-1} ANOVA (single factor) was used to compare the culture of initial chemostat phase, volume increase phase, and chemostat phase after volume increase. At D=0.1 h^{-1} ANOVA (single factor) was also used to compare culture before and after culture transfer. At D=0.4 h^{-1} t-test was used to compare population characteristics during initial chemostat phase and volume increase phase. At dilution rate D=0.4 h^{-1} t-test was used to compare population characteristics before and after culture transfer. Similarly, in comparing A-stat cultivation carried out in the MD system with A-stat conducted traditionally, a t-test was used to compare individual data-points at equal dilution rates in both setups, and the number of comparisons where the data was statistically from the same population was compared to the total number of data points compared.

5 RESULTS AND DISCUSSION

Cultivation methods and tools providing steady state metabolism of cells (e.g. chemostat) form the backbone of quantitative cell physiology studies and optimisation of the continuous manufacturing processes. Due to the low throughput the concept of 'single reactor with culture in steady state' was re-thought in current thesis, replaced with a concept of a multi-reactor system where mother-reactors operating in changestat cultivations scan the steady state growth space and transfer culture (without disturbing its physiological state) into daughter-reactors for independent growth space scanning or perturbation experiments.

Quantitative physiology analysis in an MD multi-reactor system consists of the following critical elements: changestat cultivations for navigation in growth space, means to increase the volume of microbial culture to be transferred, culture transfer from the mother-reactor into daughter-reactors without altering its physiological state and following experiments in the latter. These elements were developed and experimentally tested in current thesis. Particular emphasis was placed on physiological state analysis before and after culture transfer, as the physiology must remain in QSS for the growth space analysis in multi-reactor system concept to work. Additionally, the time-length of selected theoretical experiments characterising the MD system were evaluated and compared with the time-length of reference cultivations providing the same data (chemostat in single reactors). The limitations of the technological set-up for growth space analysis in the MD multi-reactor system were described, and different bioreactor configurations for controlling the physiological state evaluated.

5.1 Growth Space Analysis in the MD System (Publications I, II, and III)

5.1.1 Growth Space Scanning and Perturbation Experiments in the MD System

Advanced changestat experiments are powerful cultivation methods in the scanning of the microbial SSGS. For instance, it is possible to devise an experiment whereby a trajectory in growth space is analysed by first changing growth rate in an A-stat cultivation, followed by a switch to changing pH in D-stat cultivation (Figure 4 A). It is emphasised that upon the switch no stabilisation of culture is necessary. Theoretically, a single fermentation running sequentially different changestat cultivations could scan the necessary portion of SSGS, including the regions at μ_{max} where the cultivation methods would need to be auxo-accelerostats. However, using a single reactor result in the need to move back and forth in growth space, rendering this approach time consuming; thus, several reactors operated in parallel should be used to obtain the results faster. Scanning the same segment of growth space with two reactors is presented in Figure 4 B, where, however, two stabilisations are required to run the two changestat experiments. A third option is to continue the D-stat cultivation from the physiological state obtained in the A-stat with no stabilisation – but in a different reactor. This option is possible in the MD system, illustrated in Figure 4 C.

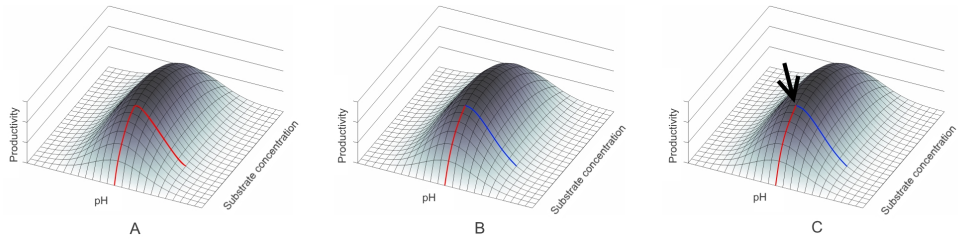

Figure 4. A – single reactor is assumed to scan the SSGS first in A-stat followed by D-stat (change in the method is seen in direction change of the red line). B – two reactors are used for SSGS analysis, one operating in A-stat (red) and the second in D-stat (blue). C – two reactors in the MD system are used for SSGS scanning, mother-reactor operating in A-stat (red) and daughter-reactor in D-stat (blue). The black arrow indicates culture transfer.

The advantage of using the MD system is that it enables moving in growth space by combining cultivation methods. For example, while a D-stat cultivation is conducted in the daughter-reactor, the culture in the mother-reactor can be maintained in chemostat, and once the D-stat cultivation is finished (and "D-stat culture" discarded) that reactor can be inoculated anew with QSS culture from the mother-reactor, to conduct further growth space analysis. This can mean running the D-stat anew, but at altered acceleration rate, for instance initially screening for the potential metabolic effect at higher acceleration rate to find the interesting growth space region, and then detailing that region at reduced acceleration rate. Comprehensive SSGS analysis can be achieved in the MD system as basically any QSS fermentation can take place in the mother unit, and similarly, the nature of processes ran in the daughter units is not limited, provided that they are started with QSS established in the mother unit. Theoretically, complex MD system set-ups with multiple mother- and daughter-reactors can analyse the whole of the growth space in well-controlled and efficient manner.

For the MD concept to work, it is necessary that the physiological state remains unaltered in the process of culture transfer. The physiological state must be equal in the mother- and daughter-reactor for just a brief period after culture transfer – after that the physiological state can be changed in a controlled manner.

5.1.2 Determining the Physiological State

The determination of physiological state based on all measurable parameters is currently impossible and impractical. Typically major determinants of physiology (such as oxygen uptake rate, carbon dioxide evolution rate, specific productivities, expression of proteins or ribonucleic acids (RNA)), measured on- or offline, are used to identify physiological state (Konstantinov and Yoshida, 1989; Stephanopoulos, 2002). In current thesis, the physiological state was determined based on population characteristics quantifying specific productivities (as denoted in the methods section). Instrumental analysis and cultivation control hardware error unavoidably introduces uncertainty in the determination of physiological state. Therefore statistical significance testing (e.g. $P>0.05$) was used to evaluate if the culture from different cultivations represented the same or different population. Theoretically, chemostat cultivation is superior in fixing the physiological state,

therefore, in current work, the different cultivation methods and phases were compared (where possible) with population characteristics from this standard method.

5.1.3 Example of Physiological State Analysis in A-stat Cultivation

In Publication I the suitability of a changestat cultivation (A-stat) in SSGS scanning was tested and the constantly changing physiological state evaluated by monitoring population characteristics. Further, the physiological state was analysed in depth by recording transcriptome changes at two growth rates. The results show that A-stat cultivation in conjunction with modern methods of molecular biology provides high-quality data that compares well with the data from chemostat cultivations made by other groups (discussed below). Publication I denotes specific results of the transcriptome changes recorded, herein only the major highlights characteristic of the A-stat cultivation method are presented.

It has been shown previously that A-stat is the method of choice to obtain growth rate related biotechnological parameters (Paalme et al., 1995). This is seen in Figure 5 where the onset of overflow metabolism at the critical specific growth rate (μ_{crit}, indicated by an increase of acetate concentration) and maximal specific growth rate (μ_{max}, indicated by a decrease of biomass concentration) at are clearly visible. The μ_{crit} was determined to be 0.34±0.02 h^{-1} and μ_{max} at 0.48±0.02 h^{-1} with good reproducibility (three A-stat cultivations compared, a=0.01 h^{-2}).

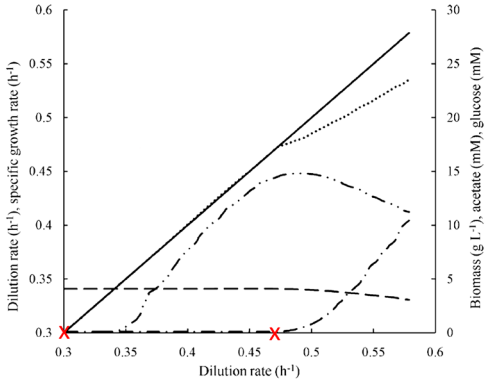

Figure 5. A-stat cultivation of *E. coli* K12 MG1655 with linearly increasing dilution rate, with acceleration rate a=0.01 h^{-2}. The solid line represents dilution rate (h^{-1}), dotted line – specific growth rate (h^{-1}), dashed line – biomass concentration ($g \cdot L^{-1}$), dashed dot line–glucose concentration (mM), dashed dot dot line – acetate concentration (mM). Red x marks the points of transcriptome measurement.

In Publication I, the transcriptome before and after μ_{crit} (D=0.3 h^{-1} and 0.47 h^{-1}) was compared. Higher growth rate led to catabolite repression, as alternative to glucose transporters were down-regulated 3-20 fold. The results show that *E. coli* will produce many alternative transporters at low glucose concentration, which is an important aspect to consider in bioprocessing and modelling. While the high-affinity ABC transporters induced at lower glucose concentrations are effective, they consume ATP in the progress.

The glucose effect has been reported in other works based on chemostat, showing that data obtained in A-stat is accurate (Ferenci, 2008).

At higher growth rate, the gluconeogenesis-related transcription decreased. These changes decrease the pool of phosphoenolpyruvic acid, which is a precursor for acetic acid production, possibly indicating an attempt to decrease acetate production. Still, as specific growth rate exceeded μ_{crit}, acetate started accumulating in the culture medium, the effect of this was noted as periplasmic acid stress chaperones (hdeAB) and glutamic acid decarboxylase (gadW) were upregulated respectively three- and twofold. Similar chaperone changes upon acid stress have been reported previously (Tramonti et al., 2008). Acetate consumption and transport related acetyl-CoA synthetase (acs) genes were strongly repressed at higher growth rate (>20 fold), which explains the acetate accumulation. The important role of acs in acetate accumulation was shown on the level of proteome and metabolome in further studies using changestat cultivations (Valgepea et al., 2010).

Downregulation of several Krebs cycle operons was recorded at higher growth rate (averagely 2-fold downregulated), such as sdhCDAB, sucABCD and fumAC. Similar results have been obtained by other groups (Vemuri et al., 2006).

To conclude this section, it can be stated that A-stat, and changestat cultivations in general, are powerful practical tools for SSGS scanning. The reproducibility of the experiments was very good, ~3 % for $\mu_{critical}$ and ~4.2 % for μ_{max} (three experiments compared). A-stat compares well with chemostat as the data from Publication I coincide well with the results reported by other research teams (Ferenci, 2008; Vemuri et al., 2006). Further, the comparison of A-stat and chemostat has been shown to be excellent (Lahtvee et al., 2011; Valgepea et al., 2010). While the nature of analysis renders transcriptome data not as continuous as off-gas or optical density monitoring, transcriptome (with other -omics) analysis can be very import in growth space analysis as it provides essential information for cellular models. A-stat cultivations (and changestat cultivations in general) enable to gather this data faster than traditional cultivations methods, with much better resolution (Adamberg et al., 2015).

5.1.4 Comparison of A-stat Cultivations Started from Traditional and MD Cultivations

We used ANOVA analysis to test if A-stat started from QSS established in the MD system provides statistically equal results to an independently conducted A-stat cultivation in a separate bioreactor (statistical methods are described in Materials and Methods and in Publication II). The cultivations were run at the same environmental parameter values, but in the MD system, the A-stats started in the daughter-reactors after culture transfer. The parameters compared were specific carbon dioxide production rate q_{CO2} ($mmol \cdot g^{-1} \cdot h^{-1}$), metabolite production per biomass of acetic acid (O_{ace}), acetylaspartic acid (O_{aceasp}), dihydroorotic acid (O_{dho}), lactic acid (O_{lact}), all $mmol \cdot g^{-1}$) presented in Figure 6. It was seen that for q_{CO2}, O_{ace}, O_{aceasp}, O_{dho} the data obtained was statistically the same at most growth rates compared (with $P>0.05$), however for O_{lact} it was not (with $P<0.05$). In the case of lactate, low concentrations (ranging from 0.05 to 0.11 mM) can explain the deviation; and based on the results we conclude that A-stat cultivation starting from the QSS is possible and provides same data to an A-stat experiment started traditionally. It

is further assumed that the culture at any growth rate in an A-stat cultivation can be transferred from the mother-reactor to the daughter-reactors at any dilution rate below μ_{max} without perturbing the physiological state, although the A-stat cultivations in the MD system were started at only at a single growth rate in chemostat at D=0.1 h^{-1}. We base this assumption on many previous reports showing the equality of chemostat and A-stat with acceleration rate 0.01 h^{-2} with *E. coli*.

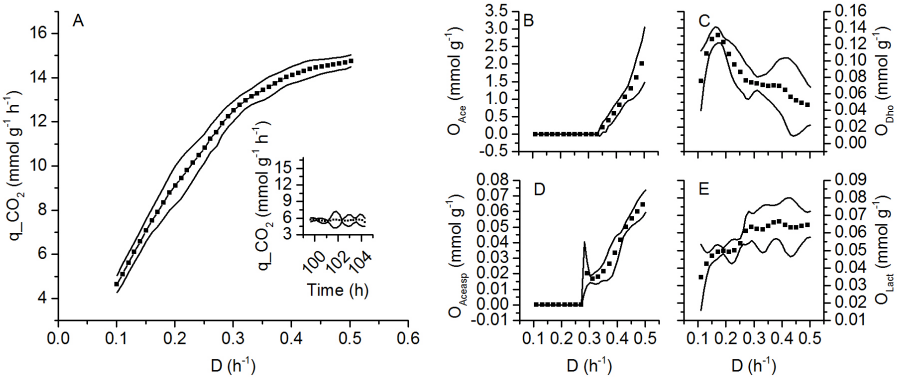

Figure 6. Change of physiological state in A-stat cultivations conducted in the MD system and traditionally in a single reactor. Left A depicts average specific carbon dioxide production rate q_{CO2} with experimentally determined standard deviation in four A-stat experiments (three from MD and one from control A-stat). The average relative standard deviation, presented by continuous line, was ~5 %; a similar value was obtained from three chemostat experiments at D=0.1 h^{-1} (inset on A). Right B – E present averaged flux values of metabolites per biomass from four A-stat experiments (three MD and one control A-stat)

5.1.5 Acceleration Rate in Changestat Cultivations

Although for *E. coli* K12 MG1655 the acceleration rate 0.01 h^{-2} has been shown to be small enough to maintain QSS, the acceleration rate used in changestat cultivations deserve some further discussion. For a changestat experiment to provide QSS data, the effect of change imposed on the cells must be slow enough so that the organism can adapt. Adaption in this context stands for adjusting the cellular machinery (transcriptome, proteome, metabolome) to deal with the changed environment. It has been shown that using too high acceleration rate will result in non-steady state conditions, for instance, manifested in early washout of culture (Adamberg et al., 2009). At the same time, the acceleration rate should be high enough to complete the experiment in reasonable time. A correlation linking acceleration rate with μ_{max} ($a=0.01\text{-}0.04\cdot\mu_{max}$) of the organism has been proposed and proven to work in many examples, this provides a good starting point for selecting the acceleration rate for uncharacterised organisms or mutants (Kasemets, 2003; Paalme et al., 1995; Valgepea et al., 2010). It is noted that the acceleration of 0.001 h^{-2} has been reported to be the fastest which can be applied while maintaining steady state growth during A-stats with yeasts (van der Sluis et al., 2001). On the other hand, less than 5 % difference between the QSS A-stat values and steady state chemostat values were reported with very high acceleration rate of $0.28\cdot\mu_{max}$ in case of microalga *Thalassiosira pseudonana* cultivation, while reducing experiment time 94 %

(Hoekema et al., 2014). A 'fool-proof' method of testing whether the acceleration rate applied is lower than the adaption rate of the organism is to switch during changestat cultivation into chemostat cultivation and to monitor physiology on-line. If all parameters stay constant, the physiological state was in QSS. This empirical testing is possible in the MD system, by running in the mother-reactor a changestat cultivation while at regular interval transferring culture into daughter-reactors for steady state monitoring in chemostat cultivations.

5.1.6 GFP Production in MD System

An inherent problem with physiology analysis in changestat cultivations is that they do not allow further growth space scanning once introduction of non-steady state disrupts the QSS. This perturbation of physiological state is typical in recombinant protein production, where after obtaining enough biomass a switch from biomass growth to protein production is made (Marisch et al., 2013). During production optimisation, several physiological states before the induction need to be analysed, and as a new experiment is necessary after each perturbation, the total experiment time will be substantial. Productivity analysis in the edges of the growth space (where growth is not optimal) exacerbates the long experiment time issue, as low acceleration rates are needed to remain in the SSGS.

In addition to the need to study different physiological states before introducing the perturbation, there is typically a need to study several various environmental conditions after the perturbation as well. If after perturbation growth is absent, studying the more general life space replaces the studying of growth space. Perturbation analysis in the MD system where experiments start with cells in the precisely determined physiological state would allow analysing several post-induction scenarios in reduced time, regardless where in the life space the organism under analysis is.

The option to combine growth space scanning experiments in the mother-reactor with perturbation experiments in the daughter-reactors was tested in the MD system, using IPTG induced GFP production as an example. We chose recombinant protein production as this can represent a typical perturbation experiment, resulting in the physiological state to exit from the QSSGS. We compared the specific productivity of GFP during the induced phase in two set-ups – in single reactors and the MD system. In the single reactors, the culture was stabilised in chemostat, after which the perturbation was introduced, cultivation switched to fed-batch mode and the effects on physiology monitored. In the MD system, the perturbation experiment in fed-batch cultivation was run in the daughter-reactor after culture transfer while the mother-reactor operated in chemostat. There was a one-hour chemostat phase in the daughter-reactors after transfer and before the induction, during which the constancy of on-line variables was monitored. In both the reference and MD systems the pre-given growth rate was equal in the chemostat and fed-batch phases, and two different growth rates (0.1 h^{-1} and 0.5 h^{-1}) were analysed.

Prior to the induction, the bacterial growth followed the pre-set profile. However, after induction at higher feeding rate, the specific growth rate decreased during the induced period (five hours) practically linearly from 0.5 h^{-1} to 0.035 h^{-1}, with acetate detected in the medium three hours after induction (Figure 7 A). The high feeding rate combined with decreasing growth rate resulted in increased glucose flux per biomass, leading to

the onset of overflow metabolism. The decline in growth rate is most likely due to the metabolic burden introduced by the recombinant protein production (Carneiro et al., 2013). Initially higher specific and volumetric productivity at higher feeding rate was observed (Figure 7 B and C), which can be explained by increased translational power at the moment of induction. It has been shown that cells at growth rate 0.5 h^{-1} contained ~500 % the ribosomes of cells at growth rate 0.1 h^{-1} (Sandén et al., 2003). By the last time-point post-induction, the cumulative impact of stress from recombinant protein and acetate resulted in a decrease in specific productivity of GFP (Figure 7 B). The effect above is also seen in the volumetric productivity of GFP at higher feeding rate, as it decreased 25 % in the last hour post-induction (Figure 7 C). We note that GFP production at higher growth and feeding rate resulted in ~25 – 40 % higher fluorescence per gramme of biomass rather constantly throughout the experiment despite the decreased volumetric productivity (data not shown).

The lower feeding rate never resulted in overflow metabolism, and the specific growth rate of bacteria followed the pre-set growth profile. As a result, the volumetric productivity of GFP at lower feeding rate increased near linearly throughout the induction phase.

The data obtained showed that the specific productivity of GFP (qGFP, $FU \cdot g^{-1} \cdot h^{-1}$) in traditional single reactors and the MD system at both growth rates was very well comparable. The correlation coefficients were 0.99 and 0.97 for feeding rates 0.5 h^{-1} and 0.1 h^{-1}, respectively (Publication IV), thus we conclude that the MD system was suitable for perturbation analysis started from QSS.

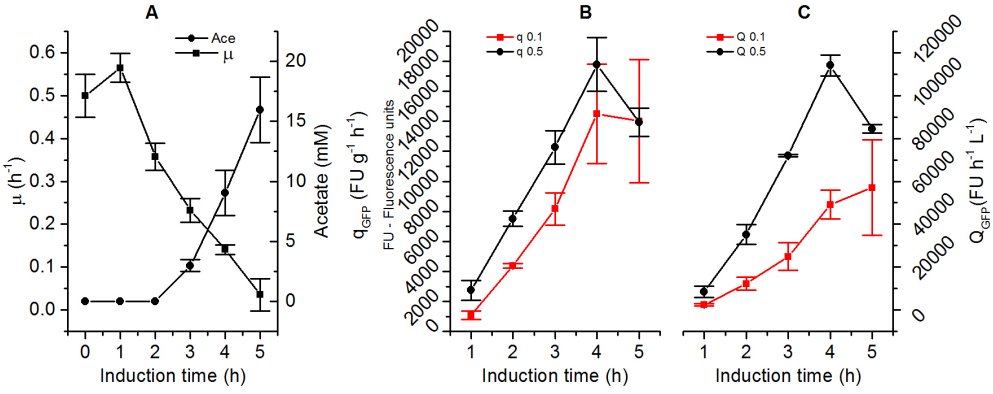

Figure 7. A – Specific growth rate and acetate concentration in higher (D=0.5 h^{-1}) feeding rate fed-batch experiment. B – specific productivity of GFP (determined as fluorescence) at high and low (D=0.1 h^{-1}) feeding rate experiments. C - Volumetric productivity of GFP. The error bars represent standard deviation for three experiments, two started in the MD system, and one started from traditional chemostat.

5.1.7 Analysis of MD Specific Phases

Starting any experiment in the daughter-reactors is preceded by preparatory phases in the mother-reactor – the volume increase to obtain sufficient biomass, and by culture transfer to fill the working volume of the daughter-reactors. Multiplying QSS physiolog-

ical state is only possible if these preparatory phases do not cause any disturbance. Therefore, the physiological state before and after culture volume increase, and before and after culture transfer were compared statistically.

5.1.7.1 Variable Volume Cultivation

As explained in the Materials and Methods section, chemostat phase preceded and succeeded the volume increase phase. In Publication II at growth rate 0.1 h^{-1} the population characteristic specific carbon dioxide production rate q_{CO2} was monitored during the three phases (chemostat at 300 mL, volume increase, chemostat at 900 mL) using ANOVA. Data shows that the q_{CO2} remained statistically constant throughout the phases analysed, with P value > 0.05 (Figure 8 A, Publication II). However, when the specific productivities of small metabolites lactic acid, acetyl aspartic acid, dihydroorotic acid, and carbamoyl aspartic acid were compared during the initial chemostat and variable volume D-stat phases, the physiological state remained unaltered in the case of lactic acid and acetyl aspartic acid and did not remain unaltered with dihydroorotic acid and carbamoyl aspartic acid. The cause can be the use of small concentrations in the calculations leading to high relative uncertainty in the HPLC analysis (or hardware related technical issues discussed in section 5.1.7.3). To avoid the error introduced by the small concentrations, the same three phases were statistically analysed at increased growth rate, at D=0.4 h^{-1}, with specific acetate production rate q_{ace} compared. T-test analysis confirmed that based on q_{ace} the three phases of cultivation indeed represent the same population, with P value > 0.05 (Figure 8 B, Publication II).

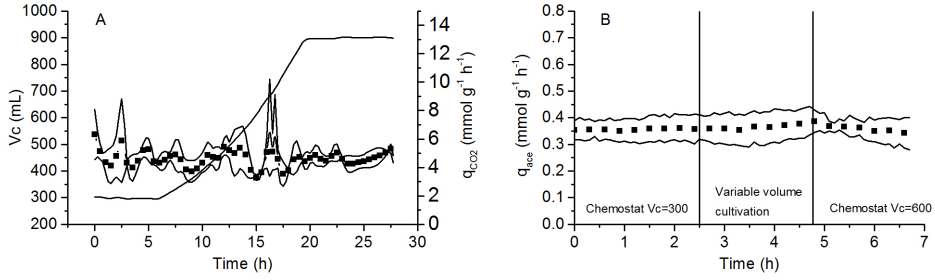

Figure 8. Left A depicts average specific carbon dioxide production rate q_{CO2} from three variable volume experiments at dilution rate 0.1 h^{-1}. Experimentally determined average relative standard deviation was 15 %. Three phases of the cultivation can be distinguished based on culture volume (initial chemostat at volume 300 mL, variable volume cultivation with increasing volume from 300 to 900 mL, chemostat at 900 mL). Right B depicts average specific acetate production rate q_{ace} of three experiments at dilution rate 0.4 h^{-1}.

5.1.7.2 Culture Transfer

The second unit operation necessary for multiplication of the physiological state is the culture transfer from the mother-reactor into the daughter-reactors. The possible effects introduced by culture transfer were investigated by comparing culture parameters before and after culture transfer with ANOVA, which showed that q_{CO2} before and after culture transfer was statistically similar with P>0.05 (Publication II). In the case of the unaltered

physiological state, the concentrations of metabolites before and after culture transfer should remain unaltered. Statistically, this was the case for orotic acid, dihydroorotic, and lactic acid but not with carbamoyl aspartic acid (Figure 9), measurement uncertainty at small concentrations (or hardware related technical issues discussed in section 5.1.7.4) can explain the dissimilarity in case carbamoyl aspartic acid.

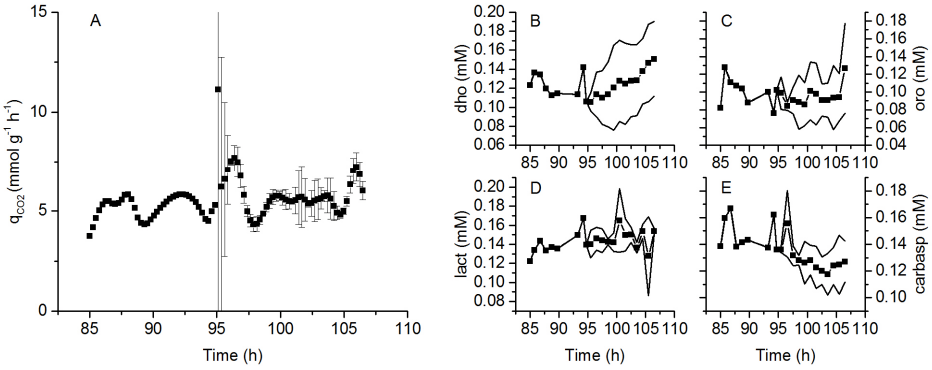

Figure 9. The physiological state before and after culture transfer. Culture transfer at hour 95 coincides with the appearance of error bars (represent standard deviation). The data after culture transfer shows the average of three experiments – one in the mother- and two in the daughter-reactors. Left A represents specific carbon dioxide production rate qCO_2 values. The large deviation after culture transfer in period ~95–96 h is an artefact due to splining error, omitted from statistical analysis. Right figures B–E show concentration profiles of minor metabolites before and after the culture transfer. The thin continuous line represents standard deviation.

5.1.7.3 Technical Details Affecting Variable Volume Cultivation

Possible technological source of variability in population characteristics during the D-stat with increasing volume at dilution rate 0.1 h^{-1} was analysed. The obvious effectors are the environmental parameters dilution rate, pH, and T, with respective relative standard deviations from setpoint values of 10, 0.12, and 0.13 %. The most fluctuating one is the dilution rate, which can cause some disturbance to the well-established chemostat physiology. Appling better quality pumps should reduce bias resulting from dilution rate fluctuations.

Mass transfer during volume increase phase can also affect the physiological state of the culture. In current work, there were three Rushton turbines on the stirrer's shaft, which submerged in succession as the level of liquid rose. In the beginning of variable volume cultivation only a single turbine was in the culture, and as the liquid level rose there had to exist a period when less energy was put into the fluid (when the second turbine was not yet submerged, but the culture volume had increased). Fluctuations in dissolved oxygen profile reflected these differences in mass transfer. However the mixing time remained at all cases below two seconds and dissolved oxygen concentration never decreased below 20 %, thereby culture homogeneity can be regarded sufficient (data not shown). Still, upon operating at higher growth rates and elevated biomass densities the mass-transfer capabilities of the reactor will have an effect on the physiological state of the culture.

Another source error during volume increase phase lies in the algorithm used to achieve the volume increase. It derives from the material balance (Eq. 1) that if there is no outflow and $\frac{dX}{dt} = 0$ during the volume increase phase, μ must equal D and QSS of typical changestat cultivation is established. To maintain constant μ the feeding must be exponential, typically achieved by using the equation proposed by Curless, presented in Eq. 11 (Curless et al., 1991).

$$F = V_0 \cdot \mu \cdot e^{\mu \cdot t} \qquad (11)$$

Algorithm for increasing culture volume at constant biomass concentration. Where V_0 - initial culture volume [mL], μ - specific growth rate [h^{-1}], t – time [h].

In current work, a more robust control algorithm denoted by Eq. 12 controlled the pumping rate during the volume increase phase, but also during the chemostat and A-stat phases. Upon switch from A-stat to volume increase phase the outflow pump simply automatically turned off and the exponential feeding based on Eq. 12 provided the culture volume necessary for filling the daughter-reactors. It is noted that a modification of Eq. 12 (culture volume calculated based on total inflow and outflow) has been used previously to obtain steady state insect cells for virus production (Drews, M.; Kasemets, K.; Nisamedtinov, I.; Paalme, 1998).

$$F_{in} = D_{set} \cdot V_{current} \qquad (12)$$

The equation used for maintaining constant dilution rate during volume increase phase. Where F_{in} stands for pump flow rate into the reactor, D_{set} denotes the pre-given dilution rate and $V_{current}$ marks the actual culture volume.

Eq. 12 is very practical as it will take into account any bias in culture volume, e.g. due to sampling. On the other-hand the nature of feeding according to it will result in a staircase like flow profile, inherently introducing an error. The reason is that pumping rate is calculated based on culture volume at the start of control interval and will remain constant until next control interval starts. However, the pumping rate should increase throughout the control interval according to an exponential function. A larger control interval must, therefore, result in larger bias. While effort should be input to decrease any source of uncertainty, it is noted that use of 1-second control interval resulted in ~0.008 % error by the time the culture volume necessary to fill the daughter-reactors (three times the initial volume) had been obtained and is, therefore, negligible.

5.1.7.4 Technical Details Affecting Culture transfer

For unbiased physiology during culture transfer, the culture inside the transfer tube must be free from any stress caused by uncontrolled environment changes. Ideally, the transfer should be instant, with minimal residence time. The transfer line should be empty, with no residue from previous transfers. The time before substrate depletion and thus the allowed time spent in the transfer tube will depend on the organism's physiology, biomass density, the growth rate, the nature and concentration of the substrate. For instance, with *E. coli* at biomass concentration 1 $g \cdot L^{-1}$ and growth rate 0.5 h^{-1} the residual oxygen of 8

$mg \cdot L^{-1}$ will be consumed in 37.8 seconds (Y'_{O2}=0.825 $Cmol_{bio} \cdot Cmol_{O2}^{-1}$, based on data from (Krahe, 2003)). This period exceeds the one liquid will spend in the tube during transfer (0.3 seconds for tube volume 5.88 mL and transfer rate of 20 $mL \cdot sec^{-1}$). However, oxygen will be depleted in 0.3 seconds if the biomass density at equal growth rate will be ~125 $g \cdot L^{-1}$. Another critical parameter is the residual glucose, which will be consumed by 1 $g \cdot L^{-1}$ *E. coli* culture growing at 0.5 h^{-1} in ~17 sec (Y_{XS}=0.5 $g_{bio} \cdot g_{subs}^{-1}$, q_{main}=0.222 $mmol \cdot sec^{-1} \cdot L^{-1}$, Ks = 0.028 $mmol \cdot L^{-1}$, μ_{max}=1 h^{-1}, data based on (Enfors, 2011)). To deplete the glucose in the 0.3 seconds needed for transfer, the biomass concentration would need to be ~60 $g \cdot L^{-1}$.

Once culture transfer from the mother-reactor to the daughter-reactor starts it will take time until the liquid level will reach the sensors in the latter, during which time pH and dO_2 control is inaccurate (was switched off). The minimal volume needed for measurements with the sensors was 150 mL, which was reached in 7.5 sec at biomass transfer rate of 20 $mL \cdot sec^{-1}$, during which time pH of dO_2 did not change markedly.

Another source of unavoidable bias in dilution rate during culture transfer will stem from the pumping rate error due to a fixed length of control interval. Ideally, the pumping rate should decrease continuously according to the decreasing culture volume. However, the software calculates pumping during one cycle based on the culture volume at the start of the control interval, meaning that the dilution rate is larger than it should be at the end of control interval. The error introduced by various control interval's lengths at the end of the particular interval is depicted in Table 2. It is visible that the dilution rate bias introduced by the finite reading interval becomes larger at lower volumes as the proportion of culture transferred in comparison with total culture volume will be larger then. This error will grow as the culture transfer speed is increased if the control interval stays constant.

Table 2. Dilution rate error due to constant pumping rate during control interval. The numeric value shown is that obtained at the end of control interval (at the start of control interval the error will be zero, as then new culture volume value is recorded). Starting volume was 900 mL, μ_{set} was 0.5 h^{-1}, culture transfer rate was 20 $mL \cdot sec^{-1}$ and the control interval varied between one, 5 and 10 seconds. Bold indicates the settings used in current study.

Time (sec)	Vc (mL)	Dilution rate error when control interval 10 sec, %	Dilution rate error when control interval 5sec, %	**Dilution rate error when control interval 1 sec, %**
0	900	0.00	0.00	**0.00**
1	880	2.27	2.27	**2.27**
2	860	4.65	4.65	**2.33**
3	840	7.14	7.14	**2.38**
4	820	9.76	9.76	**2.44**
5	800	12.50	12.50	**2.50**
6	780	15.38	0.00	**2.56**
..	..	..	..	**..**
10	700	28.57	11.43	**2.86**
11	680	0.00	14.71	**2.94**
12	660	3.03	0.00	**3.03**
..	..	..	..	**..**
21	480	41.67	12.50	**4.17**
22	460	0.00	17.39	**4.35**
23	440	4.55	22.73	**4.55**
24	420	9.52	0.00	**4.76**
25	400	15.00	5.00	**5.00**
..	..	..	..	**..**
29	320	43.75	31.25	**6.25**
30	300	53.33	0.00	**6.67**

The preferred options to reduce the error in dilution rate introduced by the culture transfer is to use smaller control interval for pump flow control and reducing the culture transfer time by transferring a smaller amount of culture, stating the need for minireactors as daughter-reactors. In case the previous options are not possible a longer culture transfer interval is a solution. However, depletion of substrates during culture transfer must be avoided. Another option would be to transfer the working volume in a short time period, by using apparatus operating like a syringe. In order to avoid large (although one-time) error in feeding rate, the feeding should be stopped during initiation of transfer and re-established after the transfer is complete.

5.2 Efficiency of the MD System in Changestat Cultivations (Publication IV)

An infinite number of different cultivation scenarios are possible in the MD system, which however will fall into three general categories. The simplest would be to continue in the daughter-reactor in the steady state physiological state established in the mother-reactor, which is a pre-requisite for any other cultivation started in the daughter-reactors. Next option is to introduce in the daughter-reactor in a controlled and reproducible manner non-steady state physiological state by perturbing the culture, defined as perturbation analysis. The perturbation can be a result of the addition of antibiotic in drug efficacy testing, or addition of IPTG in the analysis of recombinant protein productivity. A third option would be to keep the cells in the daughter-reactor in QSS physiology to move in the SSGS by applying changestat cultivation algorithms. Of course, combinations of the three are also possible.

Herein four hypothetical examples of cultivations in the MD system were analysed to evaluate the time-efficiency of the system; the setup of the cultivations is described in Table 3. The same examples were theoretically run in reference chemostat systems. Time-efficiency of the MD system was calculated by dividing cultivation time in the MD system with cultivation time in the reference system. The last paragraph of the section analyses the effect of relevant engineering parameters (dilution rate, the number of experiments, and the number of reactors) on the time-efficiency of the MD system.

Table 3. Overview of the theoretical experiments evaluated for time-efficiency.

Experiment description	Reactor set-up in the reference system	Description of cultivation process used in the reference system	Reactor set-up in the MD system	Description of cultivation process used in the MD system	Perturbation analysis length
30 different perturbations[a] applied in chemostat[b] at D=0.2 h^{-1}.	3 reactors operated in parallel in chemostat.	Stabilisation of culture in all reactors in chemostat, followed by perturbations in all reactors. A new experiment is started for next three perturbation analysis. The process is repeated to conduct all of the 30 perturbation experiments.	3 reactors, one mother-reactor (operated in chemostat) and two daughter-reactors (for perturbation analysis).	Stabilisation of culture in chemostat in the mother-reactor, followed by transfer of culture into daughter-reactors where perturbations are applied[c]. Following perturbation analysis, the culture in the daughter-reactors is discarded and daughter-reactors cleaned in situ. Procedure is repeated until 30 perturbations are analysed	One residence time (τ)[b] = 5 h
Single perturbation[d] at 10 different growth rates (0.1 h^{-1} to 1 h^{-1}, with step 0.1 h^{-1})	2 reactors operated in parallel in chemostat.	Stabilisation of culture in two reactors in chemostat, one at D=0.1 h^{-1}, the second at D=0.2 h^{-1}. Perturbations in both reactors follow. Culture is discarded, and new experiments at increased dilution rates (D=0.3 h^{-1} and 0.4 h^{-1}) started for next perturbation analysis. The procedure is repeated for all indicated dilution rates.	2 reactors, one mother-reactor (operated in A-stat[e] and one daughter-reactor (for perturbation).	The culture in the mother-reactor is stabilised in chemostat at D=0.1 h^{-1}, followed by smooth growth rate increase in A-stat cultivation. At D interval 0.1 h^{-1} culture is transferred to the daughter-reactor for perturbation analysis. Following perturbation analysis, the culture in the daughter-reactors is discarded and the daughter-reactors cleaned *in situ*. By repeating the process, the perturbation experiments at indicated growth rates are conducted.	One residence time
Single perturbation at 10 different growth rates (0.1 h^{-1} to 1 h^{-1}, step 0.1 h^{-1}) at 11 different pH values (0 % - 100 %, step 10 %).	5 reactors operated in parallel in chemostat.	Stabilisation of culture in five reactors in chemostat, followed by perturbation analysis in all reactors, after which culture is discarded, and new experiments at new growth rate / pH combinations started. By repeating the procedure at the remaining growth rate and pH values, the 110 perturbation analysis are done.	5 reactors – reactor one for A-stat, reactors two and three for D-stat, reactors four and five for perturbation analysis.	Stabilisation of culture in chemostat in the mother-reactor followed by smooth growth rate increase in A-stat cultivation. Transfer of culture to two daughter-reactors at D interval 0.1 h^{-1}. In the two daughter-reactors pH is changed from 0 % to 100 % in D-stat cultivation[f]. At 10 % pH interval culture is transferred from the D-stat reactors to the next two daughter-reactors for perturbation analysis. After scanning the pH effect, the culture in D-stat is discarded and replaced with culture from A-stat operating now at increased dilution rate. By repeating transfers at 110 combinations of growth rate and pH the 110 perturbation experiments are conducted.	Five hours
A-stat for detection of μ_{max}.	Two reactors operated in parallel in A-stat.	Growth space scanning in two A-stat experiments with acceleration rates 0.013 and 0.011 h^{-2}	Two reactors operated in A-stat with different acceleration rates (based on Table 1 in Appendix).	GS scanning in A-stat cultivation is started simultaneously in two reactors with different acceleration rates. Once the reactor with higher acceleration rate reaches apparent μ_{max} the culture within is discarded and replaced with culture from the second reactor (the one with lower acceleration rate and thus higher μ_{max}). Acceleration rate is thereafter decreased in reactor one, and the A-stats continue. The process is repeated until two sequential acceleration rates provide the same, true μ_{max}.	Missing

a - For example, the 30 different perturbations could be 30 different IPTG concentrations.; b - Batch phase lasting ten hours preceded each chemostat phase. Chemostat was stabilised for 5 residence times (residence time τ was defined as the time necessary to dilute the culture twofold at constant dilution rate).; c - In the daughter-reactors, the perturbations were always applied at the environmental conditions of the mother-reactor at the time of transfer.; d - For example one IPTG concentration.; e - Acceleration rate a=0.01 h^{-2};f - Acceleration rate 2 $\%\cdot h^{-1}$.

5.2.1 Theoretical Time-Efficiency Analysis of 30 Perturbations in Steady state Chemostat Conditions

Technologically simplest solution in perturbation analysis is to stabilise culture in chemostat and apply the perturbation. It is noted that this is not growth space scanning as the physiological state prior to the perturbation is newer changed, rather it is an example of a detailed analysis of single steady physiological state fixed in growth space. Row 2 in Table 3 explains the cultivation simulations used to analyse MD systems time-efficiency. Figure 10 A further explains the cultivation set-up in the MD system.

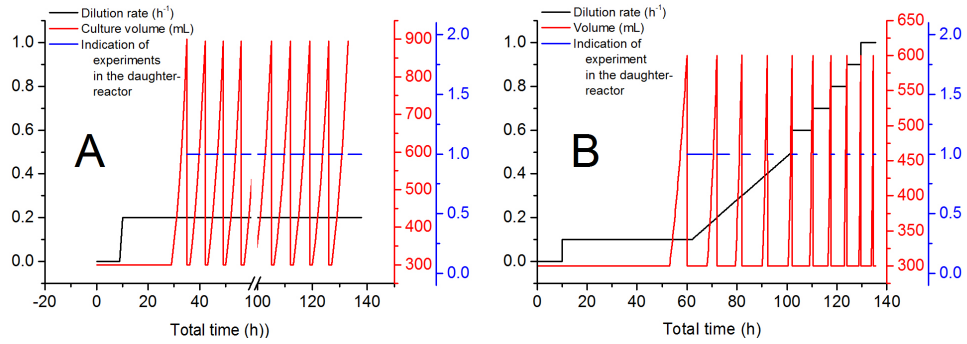

Figure 10. Perturbation analysis in the MD system. The rapid drop in culture volume indicates culture transfer. The blue line indicates experiment in the daughter-reactor(s). A – Analysis of 30 different perturbations at dilution rate 0.2 h^{-1} in chemostat in the MD set-up in the three-reactor system. B – Analysis of one perturbation at 10 different growth rates in the MD set-up in the two-reactor system. When the time to reach next dilution rate in A-stat cultivation exceeds the time necessary to stabilise culture for five residence times in chemostat, the new dilution rate is obtained in chemostat.

The need for a new experiment following each perturbation renders the reference chemostat system inefficient, as the calculations show that the MD system will be ~300 % faster when 30 experiments are simulated. If the number of perturbations is decreased to 12, the time-efficiency of the MD system will be ~200 %, thus substantial gain is achieved at a low number of perturbations. If the number of perturbations analysed is increased, the time-efficiency of the MD system increases asymptotically to near 400 % due to the mathematical nature of the calculus (discussed in section 5.2.5). Theoretically, in the MD system the number of transfers and following experiments is not limited, thus the transfer-analyse sequence can be run infinitely, thereby increasing productivity. In practice, one-time limiting factor in any continuous cultivation is the appearance of mutants (Ferenci, 2008). The occurrence of mutations can be validated by returning the culture to initial growth conditions and checking for altered phenotype or by comparing DNA sequences at the beginning and end of cultivations.

5.2.2 Theoretical Time-Efficiency Analysis of Perturbations at Ten Different Growth Rates

The optimisation of growth rate is critical in bioprocess development, for instance, it is known to have an effect on the recombinant protein productivity (Sandén et al., 2003). It is possible to study the effect of growth rate on perturbations in chemostat or A-stat cultivations, by inducing recombinant protein production at different growth rates. Row 3 in Table 3 explains the cultivation simulations to analyse MD systems time-efficiency when the effect of single perturbation (e.g. single IPTG concentration) was studied at ten different growth rates in both reference chemostat and MD systems. We note that a changestat cultivation provides many more QSS physiological states than chemostat, including the ones detailed in the perturbation simulation analysis in the reference chemostat. Figure 10 B further explains the cultivation set-up in the MD system.

If the experiment time in the MD set-up coupling QSS growth space scanning with perturbation experiments is compared to the same processes run in two traditional bioreactors in chemostat, the MD method provides data 114 % faster. The assumption of increased number of experiments leads to greater efficiency – with five different perturbations analysed at each growth rate studied, the MD method is 174 % faster. When the number of bioreactors is assumed to change to six (one mother-reactor + five daughter-reactors) the MD system is 190 % more efficient. It is emphasised that calculation is valid as long as the increase in volume does not introduce extra cultivation time; this is realised when mL scale bioreactors are used as daughter-reactors (further discussed in paragraph 5.2.5). These results show that the MD system is efficient in coupling growth space scanning with perturbation analysis especially if the daughter-reactors are small-scale bioreactors operating in mL scale.

5.2.3 Theoretical Time-Efficiency Analysis of Perturbations at Ten Growth Rates at Eleven pH values

Recombinant protein production optimisation involves studying many parameter combinations as pre- and post-induction conditions can be modified extensively (e.g. varying growth and feeding rate, IPTG concentration, T, pH, C/N ratio, substrates) (Yang, 1996; Shin et al., 1997). Physiological states at different combinations of environmental parameters can be achieved in chemostat or changestat experiments, and productivity analysed by inducing recombinant protein production in these different conditions. In the calculated example, single IPTG concentration is applied at ten growth rates and 11 pH values (in total 110 combinations) to study the combined effects of growth rate and pH on recombinant protein productivity. This is achieved by assuming that the reactors in the MD system are divided into three layers, between which culture transfer is possible (see row four of Table 3 and Figure 11 for details).

While 110 perturbation experiments are run in the perturbation reactors only a single experiment is required in the mother-reactor (A-stat reactor) and 20 experiments in the primary daughter-reactors (D-stat reactors). This results in considerable time saving, as cultivations in the QSS in MD system provide the data ~166 % faster. In the cultivation simulation, one IPTG concentration is studied, but the time-efficiency will increase if more IPTG concentrations would be explored. Upon increasing the number or IPTG

concentrations analysed in the five-reactor set-up, the efficiency nears ~220 %, due to the mathematics of the calculus. We note that typically recombinant protein production in *E. coli* is realised at lower growth rates to avoid overflow metabolism, thus the MD system should further increase in time-efficiency, as at lower growth rate the stabilisation must last longer in traditional chemostat experiments.

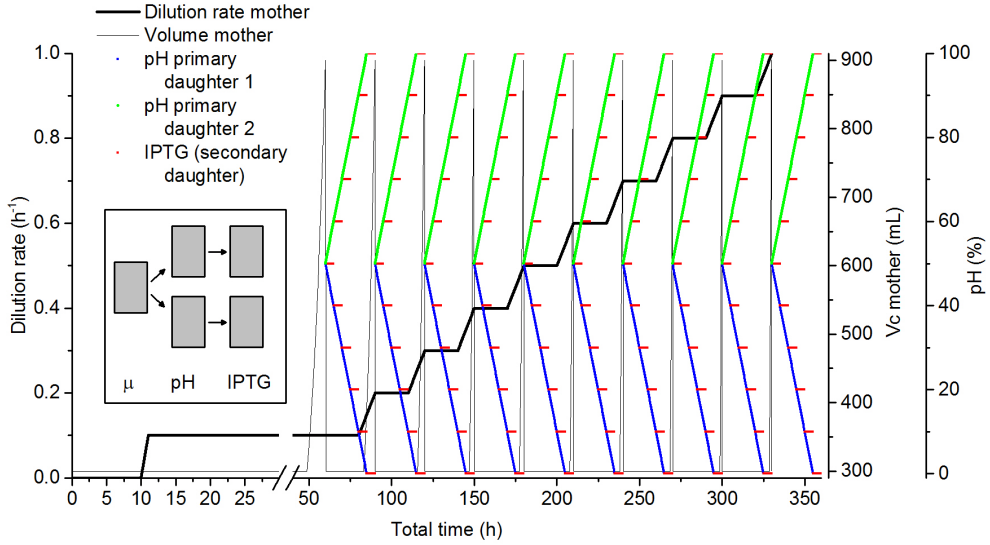

Figure 11. A theoretical example of growth space scanning coupled to perturbation experiments undertaken in five reactor MD system, combining two different changestat cultivations with perturbation analysis. Black marks primary mother (A-stat), green and blue two primary daughter- (D-stat), and red two secondary daughter-reactors (perturbation reactors). Transfers into the two primary daughter-reactors at D interval 0.1 h^{-1} are marked by a rapid decrease of culture volume in A-stat reactor. The culture from D-stat reactors at pH interval 10 % is further transferred to two secondary daughter-reactors for perturbation analysis (marked by the red horizontal lines). After 5 hours of perturbation monitoring in the perturbation reactor, the culture within is discarded and replaced with culture from D-stat reactors (now at new pH). Once the D-stat cultivation covers the whole pH range the culture within the D-stat reactor is discarded, followed by re-fill from the A-stat reactor now operating at increased growth rate (indicated by decreased culture volume). D-stat cultivations at increased growth rate follow, and repetition of the steps enables conducting the 110 perturbation experiments.

5.2.4 Iterative Scheme for the Determination μ_{max}

Previous works show that too high acceleration rates in A-stat cultivations will result in premature washout; generally the higher the acceleration rate will be, the smaller will be the value of the apparent μ_{max} (van der Sluis et al., 2001), (Adamberg et al., 2009). Theoretically, this phenomenon can speed up screening for the maximal growth rate. In this iterative cultivation set-up in the MD system, A-stat cultivations would run sequentially at decreasing acceleration rates. We emphasise that in the example calculations the correlation between acceleration rate and maximal growth rate was pre-given based on Appendix Table 1; in practice, an online probe determining the maximal growth rate should be used. Row five of Table 3 and Figure 12 present the details of the cultivation setup.

The calculations show that the MD system is 133 % more efficient in determining the maximal specific growth rate, compared with A-stat ran in a separate reactor at the lowest acceleration rate of Appendix Table 1. The effect is not huge at the pre-given cultivation settings, and surely more efficient iterative schemes can be devised; still, this shows an interesting application for the MD system. Additionally, a similar set-up can be used to validate if the acceleration rate applied in a changestat cultivation is small enough to keep the culture in quasi-steady state. This validation could be done by transferring culture from the changestat in the mother-reactor into chemostat in the daughter-reactor and monitoring the physiological state. If the culture in the mother-reactor is in QSS, the physiological state in the chemostat in the daughter-reactor would remain constant.

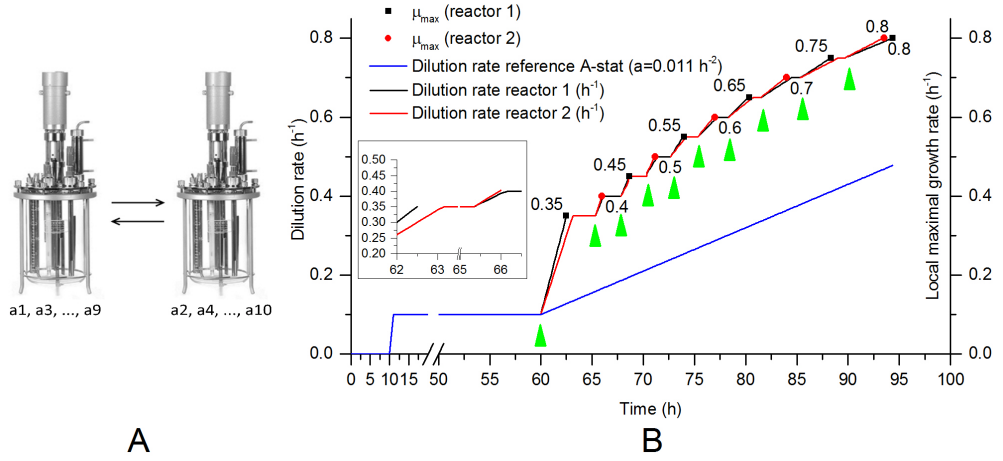

Figure 12. Iterative determination of the maximal growth rate in the MD system. A - Culture transfer is possible between two reactors in both directions; acceleration rates used in both reactors are given. B – Black and red continuous lines indicate dilution rate in the MD system, blue in the reference A-stat operated traditionally with acceleration rate 0.011 h^{-1} (does not reach μ_{max} in the figure). Green arrows indicate points of culture transfer. The different acceleration rates are visible in the different slopes of the dilution rates. The inset shows in detail the dilution rate behaviour at the first two iterations. Reactor 1 (black line) reaches μ_{max} and is cleaned in the first iteration (gap in dilution rate at time 62.5 h). Reactor 1 is re-filled when the black line reappears (65.4 h), in the second iteration. Note that now the acceleration rate in reactor 1 is decreased. In iteration two, reactor 2 (red line) reaches μ_{max} first and is cleaned (gap in dilution rate at time 66 h).

5.2.5 Factors Affecting the Time-Efficiency of the MD System

In addition to the cultivation algorithm set-up, several other parameters influence the time-efficiency of the MD systems, such as the number of reactors used per experiments conducted, the length of an experiment in the daughter-reactors after perturbation, dilution rates used, etc. The possibilities for growth space analysis in the MD system are huge, resulting in different time-efficiencies. Below we analysed the cultivation set-up where perturbations were introduced in chemostat physiology (D=0.2 h^{-1}) in 30 theoretical perturbation experiments, undertaken in both traditional (chemostat in single reactors) and MD systems.

A specific characteristic of the MD method is the added process time from the mandatory volume increase phase in the mother-reactor, necessary to provide the biomass for filling the daughter-reactors. In an ideal case, this phase is completed while the daughter-reactors are in experiment or cleaning phase. Table 4 column nine shows that theoretically increasing volume three-fold will not affect total process time as it can be completed while perturbation analysis is conducted in the daughter units. Therefore, two culture transfers to daughter-reactors of equal size to the mother-reactor can be made without introducing extra experiment time. However, if the daughter-reactors are small-scale minireactors the culture in the mother-reactor can be used to inoculate many more bioreactors.

It is evident that the time efficiency of the MD system in comparison with traditional system is larger when the number of reactors in relation to the number of experiments is small (Table 4). For example, 30 perturbation experiments in a limited number of reactors in the traditional set-up will lead to the need to start stabilisation phase on several occasions, while the stabilisation is conducted only once in the MD system, regardless of the number of perturbation experiments made. As the number of reactors used approaches the number of experiments conducted, the time efficiency of the MD system decreases, and if the number of bioreactors matches the number of perturbations analysed, the traditional system will become more time-efficient. In the MD system, experiment time will be added due to the need to make one extra culture transfer, as only 29 reactors are available for the 30 perturbation experiments (in the mother-reactor a perturbation was never assumed), while in the traditional set-up the 30 experiments can be completed simultaneously). In the example considered the MD system was more time-efficient than the reference system if the number of experiments exceeded the number of reactors.

Table 4. Experiment time comparison of 30 theoretical perturbation experiments started in chemostat at dilution rate D=0.2 h^{-1} in traditional chemostat and MD systems, at increasing number of bioreactors. The MD and traditional systems always contain the same number of bioreactors. The preparatory phases consist of batch (ten hours) and stabilisation (25 h) phases. In the traditional system, culture is stabilised in chemostat at dilution rate D=0.2 h^{-1} after which perturbation is applied, and the effect monitored for five hours. To study next perturbation, a new chemostat experiment is required. In the MD system, perturbation analysis takes place in the daughter-reactors after culture transfer. Culture in the daughter-reactors is discarded once the analysis is complete, and is replaced with QSS culture from the mother-reactor. The culture in the mother-reactor is all the time at dilution rate D=0.2 h^{-1} and is never perturbed.

Nr of reactors used for the 30 perturbation experiments	Total experiment time in traditional system (h)	Total experiment time in MD system (h)	Time-efficiency of the MD system (%)	Length of batch and stabilisation phases in MD system (h)	Length of batch and stabilisation phases in traditional system (h)	Perturbation analysis length in MD system (h)	Perturbation analysis length in traditional system (h)	Variable volume phase length in MD system (h)
2	628	243	258	10 / 25	150 / 375	150	75	3
3	418	138	303	10 / 25	100 / 250	75	50	5
10	124	61	203	10 / 25	30 / 75	20	15	9
20	82	47	174	10 / 25	20 / 50	10	10	15
25	82	47	174	10 / 25	20 / 50	10	10	16
29	82	47	174	10 / 25	20 / 50	10	10	17
30	40	47	85	10 / 25	10 / 25	10	5	17

The perturbation experiment's length has an effect on time-efficiency. The effect was evaluated in the most time-efficient case of Table 4 in the example with three bioreactors (one mother and two daughter-reactors). The efficiency of the MD system decreases as the perturbation experiment's length increases. Figure 13 shows the effect of monitoring perturbation for either one or five residence time. The MD system is ~300 % faster when the perturbation is analysed for one residence time and is ~140 % faster when its effect is analysed for five residence times. The MD system results in time efficiency by decreasing the total time needed for culture stabilisation. Therefore, everything that results in reducing the proportion of the stabilisation phase in comparison with total experiment time (such as increasing the perturbation monitoring time) must reduce the time efficiency of the MD system.

While each experiment should be analysed separately based on the growth rate applied and molecular effects expected, lengthy experiments in the daughter-reactors are typically not needed as the physiological response usually develops within the first two residence times after perturbation. For instance, if the effect of the perturbation totally halts microbial growth, then in chemostat the result will be an exponential decrease of biomass concentration to 63 % and 86 % of the initial value by the end of residence times 1 and 2, respectively. The response on the level of metabolome or transcriptome has been shown to be rapid; even expression, synthesis and insertion of specific transporters has been reported to take place within the first few residence times (Kresnowati et al., 2008; Lara et al., 2009; Soini et al., 2005).

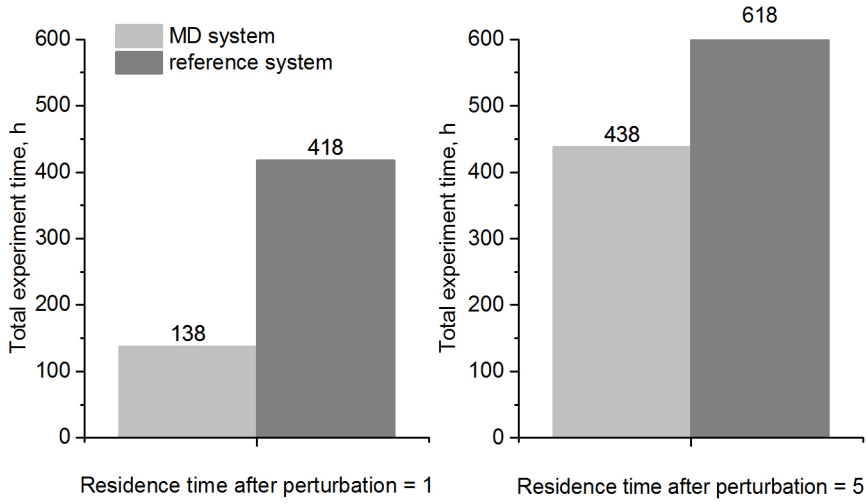

Figure 13. The effect of the perturbation monitoring length on total experiment time in the three reactor example from Table 4, where perturbation is introduced in chemostat at D=0.2 h^{-1}. In the reference system, three reactors are operated in parallel in chemostat, and after perturbation analysis, new experiments are started. In the MD system, the mother-reactor constantly operates in chemostat and is used to fill the two daughter-reactors at regular intervals (after the perturbation analysis within the latter is finished).

The next parameter affecting MD system's time-efficiency is the dilution rate at which perturbations are applied. A low dilution rate will increase the length of stabilisation period, rendering MD method more efficient. This is true when the length of the perturbation experiment was constant, but if that length was correlated with residence time, the effect of dilution rate diminished.

5.3 Technical Analysis of the MD System

The MD system can be viewed to consist of four main components: cultivation algorithms, bioreactor hardware, control software for the MD system specific operations (volume increase and culture transfer), apparatus for culture transfer. Cultivation algorithms for growth space analysis were discussed above. The control software for MD specific phases used in current work was sufficient to provide semi-automated operation, for instance, cultivations in the daughter-reactors started automatically upon culture transfer. However, several manual steps remained, such as opening the valves for culture transfer or cleaning the transfer lines and daughter-reactors after experiments. These manual operations make the system cumbersome to use and should be replaced with automated procedures to undertake more comprehensive growth space analysis. The transfer and cleaning phases should start automatically upon reaching some trigger (time or event). The speed and the accuracy of process control is especially important during the short period of culture transfer, as actuators must respond to the quickly changing environment. Dedicated software allowing unbiased and repeated transfers and following cultivations is therefore needed.

The apparatus for culture transfer would need to be such that culture transfer does not introduce any interference to the cultivation process, regardless of biomass density and growth rate. Overpressure was used as the driving force of culture transfer in current work, with biomass flow rate through the transfer tube correlating with the pressure applied. To avoid substrate depletion in the tube the transfer should be rapid, therefore the overpressure applied should be high. However, we noticed pH changes due to CO_2 concentration changes in experiments with applying too high overpressure. Devising of a proper culture transfer system matching the needs is currently underway.

The bioreactors used as daughter-reactors must be cleanable and sterilisable in place (to be compatible with the repeated culture transfers). The technological set-up will be analysed in detail in future studies. The focus of current paragraph is to analyse different bioreactor platforms (with emphasis on small-scale bioreactors) for the suitability to conduct experiments started from QSS after culture transfer.

In current work, it was shown that maintaining QSS physiological state after culture transfer from one laboratory scale STR to another laboratory scale STR is possible if hard- and software settings are appropriate. In certain cases connecting two laboratory scale STRs is desirable, for example, if back-and-forth culture transfers are expected such as in the iterative A-stat. However, the efficiency calculations show that to achieve better time-efficiency the daughter-reactors of the MD system should be an order of magnitude smaller than the mother-reactor, as then no extra time is introduced due to the culture volume increase phase.

Maintaining the physiological state in the small-scale daughter-reactors after culture transfer is best achieved if the daughter-reactors are scale down versions of laboratory scale STRs providing homogeneous conditions regarding heat and mass transfer, and equipped with fast and accurate sensors. The instrumentation need of the daughter-reactors is dependent on the cultivation procedure applied, for instance in fed-batch cultivation maintaining culture volume constant is not important but is critical in chemostat cultivation. For classical chemostat cultivation in small-scale reactors, typically overflow ports control culture volume. The accuracy of this method is not very good and can affect the physiological state, especially if waves occur in the reactor (due to stirring or gassing). Still, small-scale chemostat devices should be suitable for use in the MD system, such as those described in (Bower et al., 2012; Gebhardt et al., 2011).

For best control of the physiological state, the feeding in daughter-reactor should be continuous such as described by Schmideder (Schmideder et al., 2015). Pulsed feeding, while more economical as one pump can serve many reactors, can result in heterogeneities in substrate concentration in the culture liquid, which can be critical in steady state. However, the pulsed operation described for instance by Takahashi et al is acceptable if the concentration of substrate is high deliberately, such as in auxostat cultivation (Takahashi et al., 2014). For substrate addition, the most accurate option is the use of syringe pumps. In the MD setup the experiment in the daughter-reactor can be designed to be short enough to consume only the contents of a single disposable syringe. In this regards the one reactor with one 3D printed syringe pump set-up, such as that proposed by (Bareither et al., 2013) or (Ali et al., 2012) would be an ideal solution.

The daughter-reactor must not necessarily be equal to laboratory scale STR in terms of mass transfer capabilities but should provide enough mixing power to maintain undisturbed steady state. It has been shown that reactors with different k_La values can result in equal processes, and the difference in maximal mass transfer rate becomes

relevant only at high growth rate and elevated biomass concentration conditions (Betts et al., 2014; Seletzky et al., 2007). In cases where increased mass transfer is needed, the minireactors with k_La value of 1440 h^{-1} should be used, such as those proposed by Schmideder (Schmideder et al., 2015).

The level of instrumentation utilized in the daughter-reactors also depends on the cultivation procedure - there can be a situation where the daughter-reactor does not need measuring and control of certain parameters, while still maintaining the physiological state. As an example, if aeration and mixing grant that the concentration of dissolved oxygen remains above the critical value and its exact value is not of importance the daughter-reactor can operate without an oxygen sensor. However, if pO_2 and pH measurement are needed, the fast and accurate optical sensors should be used, as-long as the somewhat limited operational range allows (Janzen et al., 2015). Preferably, acid and base control for pH would be present. However, in certain cases, if the culture medium contains enough buffer, pH control by actuators can even be absent. If individual temperature control for each reactor is not required it is feasible to place the daughter-reactors into an incubator, as then there will be a minimal error in temperature during the culture transfer phase.

6 CONCLUSIONS

The main conclusion of the thesis was that the MD system can be used for starting (in the daughter-reactors) QSS cultivations with cells in precisely determined physiological state without stabilisation. The system allows decreasing experiment time and combining in the different reactors of the system different changestat cultivation methods for efficient growth space scanning. Specific conclusions of the thesis were:

a. A semi-automated prototype of the MD system was developed and evaluated. Technological limitations and possibilities for the creation of an automated MD system were analysed. The most critical technological limitations that need to be solved in an automated system are transfer valves and in place cleaning.

b. The preparatory phases for starting cultivations in the MD system – the QSS cultivation with increasing culture volume and the biomass transfer – do not alter the physiological state of the culture statistically significantly.

c. Growth space scanning cultivations started in the MD system are possible, as experimentally conducted A-stat cultivation undertaken in the MD system resulted in statistically equal data (specific biomass and product formation rates) to a separately conducted A-stat cultivation. However, for more comprehensive scanning of the growth space, the algorithms developed should be run in an automated system.

d. The experimentally conducted production of GFP from QSS physiology showed that the MD system could be used to combine QSS growth space scanning with perturbation analysis (e.g. useful in testing different environmental conditions for biologics production). Further, this widens the applicability of the MD system into production processes where a mother-reactor can provide production ready culture to the daughter-reactors when required.

e. The theoretical efficiency analysis of cultivations conducted in the MD system shows that perturbation analysis in the system is more time-efficient than single reactor chemostat systems operated in parallel if the number of experiments exceeds the number of bioreactors. To maximise efficiency the volume of the daughter-reactors in the MD system should be about a magnitude smaller than the volume of the mother-reactor.

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CURRICULUM VITAE

Personal data

Name:	Sten Erm
Date & place of Birth:	11. April 1980, Tallinn
Citizenship:	Estonian
Email:	sten.erm@gmail.com
Phone:	+372 55 539 779

Work Experience

2004 – Present	Center of Food and Fermentation Technologies, researcher
2017 – Present	Lallemand Inc., Corporate R&D, scientist
2012 – 2017	Biotecha Estonia, sales manager and service engineer

Education

2007 – present	PhD studies, Tallinn University of Technology
	Thesis: Use of Mother-Daughter Multi-Bioreactor Systems for Studies of Steady State Microbial Growth Space
2004 – 2007	MSc studies, Tallinn University of Technology
	Thesis: Study of Growth Space of Microorganisms
2000 – 2004	BSc studies, Tallinn University of Technology, department of Chemical Engineering

Teaching and MSc Supervising

2010 – 2012 (spring)	University of Helsinki. Hands on course in fermentation technology (laboratory work teacher)
2008 – 2010 (autumn)	Tallinn University of Technology, Chemistry and Material Science for non-chemistry major students (practical calculations and laboratory work teacher)
2016	Sander Pihelgas, thesis: Optimization of GFP Production in *Escherichia coli* BL21 (DE3) in Multi Bioreactor System (co-supervisor)

2010	Dagmar Undrits, thesis: Effect of variable volume cultivation and culture transfer to the physiological state of the organism under study (co-supervisor)
2009	Merli Špitsmeister, thesis: Development of a quantitative method for fatty acid determination in *Lactococcus lactis* and *Escherichia coli* (co-supervisor)

Honors

2015	Winner of Prototron competition

Languages

Estonian	Native
English	Fluent
Finnish	Intermediate
Russian	Intermediate

ELULOOKIRJELDUS

Isikuandmed

Nimi:	Sten Erm
Sünniaeg ja -koht:	11. April 1980, Tallinn
Kodakondsus:	Eesti
Email:	sten.erm@gmail.com
Telefon:	+372 55 539 779

Töökogemus

2004 – tänaseni	Toidu- ja fermentatsioonitehnoloogia arenduskeskus, teadur
2017 – tänaseni	Lallemand Inc., Corporate R&D, teadlane
2012 – 2017	Biotecha Eesti, müügijuht ja hooldusinsener

Haridus

2007 – 2017	Doktoriõpe, Tallinna Tehnikaülikool, keemia- ja geenitehnoloogia
	Väitekiri: Ema-tütar multi-reaktorite süsteemide kasutamine mikroorganismide püsiseisundis kasvuruumi uurimisel
2004 – 2007	Magistriõpe, Tallinna Tehnikaülikool, biotehnoloogia ja rakenduskeemia
	Väitekiri: Mikroorganismide kasvuruumi uurimisest
2000 – 2004	Bakalaureuseõpe, Tallinna Tehnikaülikool, keemitehnika ja keskkonnakaitse

Õpetamine ja magistritööde juhendamine

2010 – 2012 (kevad)	Helsingi Ülikool, Fermentatsioonitehnoloogia (laboratoorse töö õpetaja)
2008 – 2010 (sügis)	Tallinna Tehnikaülikool, Keemia ja materialiõpetus mittekeemikutele (praktikumi ja harjutustunni õpetaja)
2016	Sander Pihelgas, väitekiri: Kasvukiiruse ja substraadi lisamise profiili mõju GFP tootlikkusele poolperioodilisel kultivatsioonil koos efektiivsuse analüüsiga (kaasjuhendaja)

2010	Dagmar Undrits, väitekiri: Rakukultuuride ruumala suurendamise ja ülekandmise mõju nende füsioloogilisele seisundile segureaktoris järjestik-paralleelsel pidevkultiveerimisel (kaasjuhendaja)
2009	Merli Špitsmeister, thesis: Rasvhapete kvantitatiivse määramise metoodika väljatöötamine *Lactococcus lactis*`es ja *Escherichia coli*`s (kaasjuhendaja)

Tunnustused

2015	Prototron võistluse auhind

Keelteoskus

Eesti	emakeel
Inglise	kõrgtase
Soome	kesktase
Vene	kesktase

APPENDICES

Table 1. Pre-given theoretical correlation between acceleration rate and local maximal growth rate.

Iteration	i	i+1	i+2	i+3	i+4	i+5	i+6	i+7	i+8	i+9	i+10
Acceleration index	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8	a_9	a_{10}	a_{11}
Acceleration (h^{-2})	0.1000	0.080	0.064	0.051	0.041	0.033	0.026	0.021	0.017	0.013	0.011
Local maximal specific growth rate (h^{-1})	0.3500	0.4	0.45	u0.5	0.55	0.6000	0.6500	0.7	0.75	0.8	0.8

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Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures

Ranno Nahku[a,b], Kaspar Valgepea[a,b], Petri-Jaan Lahtvee[a,b], Sten Erm[a,b], Kristo Abner[a,b], Kaarel Adamberg[b,c], Raivo Vilu[a,b,*]

[a] Tallinn University of Technology, Department of Chemistry, Ehitajate tee 5, 19086 Tallinn, Estonia
[b] Competence Centre of Food and Fermentation Technologies, Akadeemia tee 15b, 12618 Tallinn, Estonia
[c] Tallinn University of Technology, Department of Food Processing, Ehitajate tee 5, 19086 Tallinn, Estonia

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ABSTRACT

Specific growth rate dependent gene expression changes of *Escherichia coli* K12 MG1655 were studied by microarray and real-time PCR analyses. The bacteria were cultivated on glucose limited minimal medium using the accelerostat method (A-stat) where starting from steady state conditions (chemostat culture) dilution rate is constantly increased. At specific growth rate (μ) 0.47 h^{-1}, *E. coli* had focused its metabolism to glucose utilization by down-regulation of alternative substrate transporters expression compared to $\mu = 0.3\ h^{-1}$. It was found that acetic acid accumulation began at $\mu = 0.34 \pm 0.01\ h^{-1}$ and two acetate synthesis pathways – phosphotransacetylase-acetate kinase (*pta-ackA*) and pyruvate oxidase (*poxB*) – contributed to the synthesis at the beginning of overflow metabolism, *i.e.* onset of acetate excretion. On the other hand, *poxB*, *pta* and *ackA* expression patterns suggest that pyruvate oxidase may be the only enzyme synthesizing acetate at $\mu = 0.47\ h^{-1}$. Loss of glucose and acetate co-utilization represented by down-regulation of *acs-yjcH-actP* operon between specific growth rates 0.3–0.42 h^{-1} and acetic acid accumulation from $\mu = 0.34 \pm 0.01\ h^{-1}$ allows one to surmise that the acetate utilization operon expression might play an important role in overflow metabolism.

1. Introduction

A continuous culture method chemostat was introduced in 1950 by Novick and Szilard (Novick and Szilard, 1950) and since then it has been widely used for studying microorganisms in defined (steady state) conditions at constant dilution rate which is equal to specific growth rate (μ) of cells. The presence of steady state is its main and most important advantage over batch cultivation where dependence of growth characteristics on specific growth rate is hard to determine. Conducting experiments in batch cultures result in complex data patterns reflecting uncontrolled changes of growth conditions, which are often difficult or even impossible to interpret (Hoskisson and Hobbs, 2005). It has been proposed that steady state can be obtained in batch cultures in exponential phase while substrate consumption by cells is maximal, however, within that time, metabolite concentrations and pH are constantly changing and latter is known to affect gene expression (Richard and Foster, 2004, 2007; Rosenthal et al., 2008). For these reasons, chemostat is a better choice for studying bacterial physiology. On the other hand, to obtain steady state in chemostat, five culture volumes of medium are usually needed to be pumped through the reactor in fixed conditions, making cultivation experiments time consuming and thus limiting the number of specific growth rates to be studied. Moreover, chemostat culture is open to random mutations and therefore, it is important to reduce the duration of experiment (Ferenci, 2008).

Accelerostat (A-stat) method was introduced to overcome the problems associated with chemostat (Paalme and Vilu, 1993). A-stat begins as chemostat: batch phase is followed by stabilization of the culture at fixed specific growth rate to obtain steady state. Subsequently, the dilution rate which equals specific growth rate is changed with constant speed until the culture cannot keep up with the rising dilution rate, resulting in wash-out. It has been shown before that transition from acceleration phase to chemostat does not result in a significant change in culture parameters if the chosen changing rate of dilution rate is not too fast (Adamberg et al., 2009). In the latter case, culture is in quasi-steady state which is a physiological state of a microorganism where every point represents the corresponding steady state value. Great advantages of A-stat are the possibilities to monitor bacterial growth in real time to study physiology in a large variety of specific growth rates while reducing the duration of experiment at the same time.

* Corresponding author at: Mailing address: Tallinn University of Technology, Department of Chemistry, Akadeemia tee 15, 12618, Tallinn, Estonia.
Tel.: +372 6204831; fax: +372 6202828.
E-mail address: raivo@kbfi.ee (R. Vilu).

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Producing recombinant proteins and other biotechnologically important substances has significantly increased in recent years. *Escherichia coli* is often used as a production system due to its ability to grow aerobically on glucose and reach high biomass concentrations. In addition to its relatively low cultivation cost, *E. coli* is a Gram-negative bacterium which makes purification of products relatively cheap. Generally, production yields are better at higher specific growth rates, however, this is where the main problem in aerobic *E. coli* cultivation on glucose arises – excretion of acetic acid as the result of overflow. It is believed that acetate production, *i.e.* overflow metabolism is caused by an imbalance between glucose uptake and TCA cycle capacity resulting in pyruvate and acetyl-CoA accumulation (Akesson et al., 1999; Farmer and Liao, 1997; Wolfe, 2005). Acetate is known to reduce maximum growth rate and inhibit protein production, moreover, it diverts carbon from biomass formation (Nakano et al., 1997; Contiero et al., 2000; March et al., 2002). For those reasons, many strategies have been developed to abolish acetate production (De Mey et al., 2007; Eiteman and Altman, 2006; Wolfe, 2005).

Two main pathways are known synthesizing acetate. Acetyl-CoA is converted into acetyl-P by phosphotransacetylase (*pta*) through phosphotransacetylase-acetate kinase (*pta-ackA*) pathway and acetate kinase (*ackA*) catalyzes the reaction from acetyl-P to acetate. Alternatively, acetate can be synthesized directly from pyruvate in the presence of pyruvate oxidase (*poxB*) (Phue and Shiloach, 2004; Phue et al., 2005). Deletion of *pta-ackA* pathway results in a significant reduction of acetate excretion, specific growth rate and elevation in formate and lactate production (Chang and Cronan, 1999; Contiero et al., 2000; Dittrich et al., 2005). *poxB* disruption results in reduced biomass yield (Abdel-Hamid et al., 2001). Despite the considerable amount of studies with genetically engineered *E. coli* strains, there is still no clear understanding about the regulation of acetic acid synthesis pathways, making the matter intriguing. As overflow metabolism is known to be specific growth rate dependent, continuous monitoring of specific growth rate effect on bacterial physiology should be made for gaining more detailed insights into overflow metabolism regulation. The method enabling the latter is A-stat.

The aim of this study was to characterize *E. coli* K12 MG1655 metabolism at various specific growth rates at transcriptional level using A-stat cultivation method which has not been done before. Additionally, accelerostat experiments gave an excellent opportunity to precisely monitor the course of metabolism and directly connect growth phenotype to gene expression data. Special attention was drawn for characterising acetate metabolism.

2. Materials and methods

2.1. Bacterial strain and culture medium

The *E. coli* K12 MG1655 strain used in the current study was obtained from Statens Serum Institute, Denmark. Growth and physiological characteristics were determined using a defined medium with the composition as follows ($g\,l^{-1}$): $FeSO_4{\cdot}7H_2O$ 0.005, $MgSO_4{\cdot}7H_2O$ 0.5, $MnSO_4{\cdot}5H_2O$ 0.002, $CaCl_2{\cdot}2H_2O$ 0.005, $ZnSO_4{\cdot}7H_2O$ 0.002, $CoSO_4{\cdot}7H_2O$ 0.0006, $CuSO_4{\cdot}5H_2O$ 0.0005, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.0026 were dissolved in 50 ml 5 M HCl; C-source – α-(D)-glucose 10, N-source – NH_4Cl 3.5 and buffer – K_2HPO_4 were autoclaved separately and mixed together afterwards.

2.2. Accelerostat cultivation

The A-stat cultivation system consisted of 1.25 l Biobundle bioreactor (Applikon Biotechnology B.V., Schiedam, the Netherlands) controlled by an ADI 1030 biocontroller (Applikon Biotechnology B.V.) and a cultivation control program "BioXpert NT" (Applikon Biotechnology B.V.). The system was equipped with pH, pO_2 and temperature sensors. Two variable speed pumps (feeding and out-flow) were controlled using "BioXpert NT" control software. The bioreactor was set on a balance whose output was used as the control variable to ensure constant culture volume (300 ± 1 ml). Similarly, the inflow was controlled through measuring the mass of the fresh culture medium.

Four cultivation experiments with accelerations – 0.01 h^{-2} or 0.005 h^{-2} – were carried out at 37 °C under aerobic conditions with an agitation speed of 500 rpm. pH of the culture was controlled using 5 M NaOH and temperature with a heating blanket. The growth characteristics of the bacteria in A-stat experiments were calculated on the basis of OD, total volume of medium pumped out from bioreactor (L) and organic acid concentrations in culture medium (mM). Formulas and cultivation system used were as described in previous studies (Kasemets et al., 2003; Adamberg et al., 2009).

2.3. Analytical methods

The concentrations of organic acids (lactate, acetate and formate), ethanol and glucose in the culture medium were analyzed by high pressure liquid chromatography (Alliance; Waters Corp., Milford, MA), using a BioRad HPX-87H column (Hercules, CA), and isocratic elution at a flow rate of 0.6 ml min^{-1} with 0.005 M H_2SO_4 at temperature 35 °C. Refractive index detector (model 2414; Waters Corp.) was used for detection and quantification of the substances. The samples of culture medium (1.5 ml) were centrifuged ($14{,}000 \times g$, 4 min), supernatant was collected and analyzed directly or stored at −20 °C before HPLC analyses. Data processing was performed using Empower software (Waters Corp.).

Biomass concentration was measured gravimetrically: centrifuging 10 ml of culture, washing twice with physiological solution, resuspending in dH_2O, drying on aluminum plates at 100 °C for 24 h and cooling down in a desiccator prior to weighing.

2.4. Gene expression profiling

The microarrays used in the study were obtained from KTH Microarray Center (Stockholm, Sweden). *E. coli* Genome Oligo Set Version 1.0 (Operon Biotechnologies Inc., Huntsville, AL) containing 4289 *E. coli* K12 open reading frames was used in the spotting process. For microarray analyses, steady state chemostat culture of *E. coli* K12 MG1655 was used as a reference ($\mu = 0.3\ h^{-1}$). Quasi-steady state point at specific growth rate $0.47 \pm 0.02\ h^{-1}$ was compared to the reference sample in triplicate biological parallels. Compared samples were obtained from experiments carried out with 10 $g\,l^{-1}$ of glucose in medium.

RNA degradation was halted, total RNA extracted, cDNA synthesized and labeled as described previously (Lahtvee et al., 2009), with minor modifications: less concentrated lysozyme solution, no proteinase K and 25 μg of total RNA was used for cDNA synthesis. Microarrays were incubated for 30 min at 42 °C in prehybridization solution containing (per slide): 0.5 μg BSA, 12.5 ml 20× SSC, 0.5 ml 10% SDS. Slides were rinsed 10 times in MilliQ water, twice in 2-propanol and dried with centrifugation. After purification of the labeled cDNA, the hybridization solution (20× SSC 16.3 μl, 100% formamide 32.5 μl, 10% SDS 0.65 μl, *E. coli* tRNA 1 μl (5 μg)) was added, followed by hybridization for 35 h at 42 °C.

2.5. Microarray data analysis

Microarray slides were scanned using an Affymetrix 428 Array Scanner (Santa Clara, CA). Spot intensities and corresponding

background signals were quantified with Genepix Pro (version 6; Axon Instruments [http://www.moleculardevices.com/pages/software/gn_genepix_pro.html]). Spots which had signal-to-noise ratio less than three were filtered. Further analysis was carried out in R environment (version 2.6.1 [http://www.r-project.org/]) using KTH package (KTH Microarray Center [http://www.biotech.kth.se/molbio/microarray/dataanalysis/index.html]). Flagged spots and background were extracted before "printTipLoess" normalization. Data from the independent experiments were combined and further analyzed with web based tool Marray (G-language Project [http://www.g-language.org/data/marray/software/map2swf.cgi]) that enables gene expression data visualization on KEGG pathways. Input data and instructions for Marray and Supplementary Table S1 for gene expression measurement results can be found at our homepage (http://tftak.eu/?id=68). DNA microarray data is also available at NCBI Gene Expression Omnibus (Reference series: GSE18183).

2.6. Real-time PCR assay

RNA was extracted and cDNA synthesized as described by Lahtvee et al. (2009), except regular nucleotides were used for cDNA synthesis. Real-time PCR reactions were carried out using Roche LightCycler 2.0 (Basel, Switzerland) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Per one reaction 5.25 μL 2× platinum SYBR Green qPCR SuperMix-UDG, 3 μL primer mix (1.67 μM), 0.5 μL BSA and 2 μL cDNA (concentration ca 20 ng μL^{-1}) were used. PCR conditions were set according to the Platinum SYBR Green qPCR SuperMix-UDG manual. Gene expression changes for *acs*, *pta*, *ackA*, *poxB*, *sucC* were derived comparing samples from dilution rates of 0.36 h^{-1}; 0.42 h^{-1}; 0.45 h^{-1}; 0.47 h^{-1} to the reference sample at dilution rate 0.3 h^{-1}. All samples were normalized according to biomass and ribosomal RNA content. The changes in ribosomal RNA content were estimated using gel electrophoresis. Equal volumes of total RNA were loaded to 1% agarose gel and rRNA band intensities were compared. As equal amounts of total RNA (25 μg) were used as a starting material for cDNA synthesis, it was assumed that higher rRNA amount lead to proportional reduction of mRNA pool.

3. Results

3.1. A-stat growth characteristics and reproducibility

Three parallel experiments were carried out (with 10 g L^{-1} glucose) where the culture was stabilized in chemostat at 0.3 h^{-1} and after achieving steady state, dilution rate was smoothly changed (increased) with acceleration of 0.01 h^{-2} (Fig. 1). A-stat enabled precise determination of *E. coli* K12 MG1655 growth parameters – acetate overflow began at $\mu = 0.34 \pm 0.01\ h^{-1}$ and cells had problems keeping up with rising dilution rate at specific growth rate $0.48 \pm 0.02\ h^{-1}$ (Fig. 1). Glucose started to accumulate in the culture medium simultaneously with the latter point. Three characteristic cultivation points were chosen to validate reproducibility: specific growth rate when acetate starts to accumulate ($\mu = 0.34 \pm 0.01\ h^{-1}$), specific growth rate when μ started lagging D ($\mu = 0.48 \pm 0.02\ h^{-1}$) and specific growth rate when glucose started to accumulate ($\mu = 0.48 \pm 0.02\ h^{-1}$). The average relative standard deviation of each of these three points in quasi-steady state cultures was 3.3%. For further characterization of the good reproducibility of A-stat experiments it was shown that the average relative standard deviation of biomass yields (Y_{xs} [g g^{-1}]) was 5.4% in quasi-steady state conditions (data not shown).

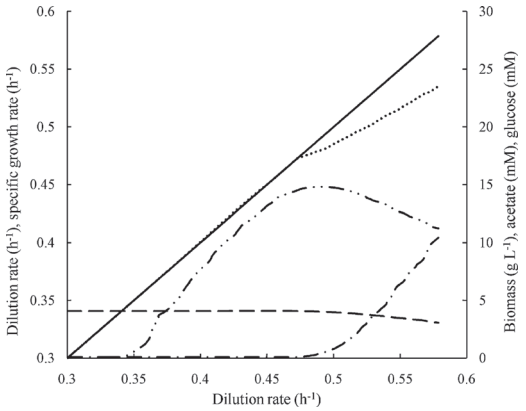

Fig. 1. A-stat cultivation of *E. coli* K12 MG1655 with 10 g L^{-1} of glucose as the limiting substrate. Measurement rate for all parameters was dilution rate 0.01 h^{-1}. Solid line represents dilution rate (h^{-1}), dotted line – specific growth rate (h^{-1}), dashed line – biomass concentration (g l^{-1}), dashed dot line – glucose concentration (mM), dashed dot dot line – acetate concentration (mM).

3.2. Glucose repression in E. coli K12 MG1655

In microarray analysis quasi-steady state points at specific growth rate $0.47 \pm 0.02\ h^{-1}$ and the reference steady state points at 0.30 h^{-1} were compared. According to the data, alternative substrate utilization and transport transcript expression analysis revealed 3–20-fold reduction of genes associated with the metabolism of maltose (*malBEFKM*), β-methylgalactoside (*MglABC*), D-ribose (*rbsABC*), L-arabinose (*araF*), galactitol (*gatABC*), N-acetyl glucosamine (*nagE*), acetate (*actP*, *yjcH*) and C_4-dicarboxylates (*dctA*). Glucose repression was also seen by strong down-regulation of *tnaA*, *cstA*, *aspA*, *dadX* and *ilvBN* (see Supplementary Table S1), observed as well by Oh and colleagues (Oh et al., 2002).

3.3. Struggle against acetate stress

The major sugar transport system in *E. coli* is the phosphotransferase system (PTS) enabling fast glucose transport. Latter causes accumulation of pyruvate that may lead to acetate production (Vemuri et al., 2006). Microarray data showed a times decrease in the expression of glucose specific PTS gene *ptsG* at higher growth rate while glucose consumption per biomass produced was constant. Reduced glucose uptake rate through PTS is likely compensated by increase in alternative non-PTS transport. We found 3- and 5-fold increase in galactose permease (*galP*) and predicted transporter *tsgA* respectively, but a 2.5 times decrease in glucokinase (*glk*) expression. Alternatively, pyruvate pool could have been reduced by converting phosphoenolpyruvate to oxaloacetate by minor up-regulation (1.6 times) of phosphoenolpyruvate carboxylase (*ppc*), 9-fold down-regulation of phosphoenolpyruvate carboxykinase (*pck*) and down-regulation of pyruvate synthesis from malate by NADP-dependent malic enzyme (*maeB*) (Fig. 2).

Acetic acid effect on metabolism was also observed by three fold up-regulation of acid stress genes *hdeAB* at $\mu = 0.47\ h^{-1}$ where extracellular acetate concentration was approximately 15 mM. Similarly, around 2-fold elevation in *gadW* expression, known activator of acid response genes, occurred (Tramonti et al., 2008).

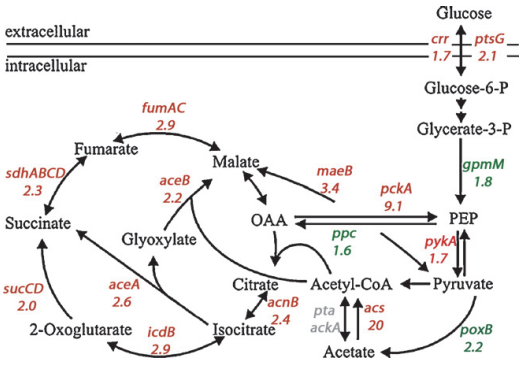

Fig. 2. Central carbon metabolism gene expression changes at $\mu = 0.30\,h^{-1}$ vs. $0.47\,h^{-1}$ in *E. coli* K12 MG1655 A-stat cultivation. Down-regulated genes are indicated in red, up-regulated in green and no significant changes are indicated in grey. Number below gene name shows the expression fold-change (average fold change is shown in case of several genes). Arrowhead indicates the expected reaction direction. The figure is based on Nanchen et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Energy metabolism

Most of the energy for anabolic reactions during aerobic growth of *E. coli* K12 on glucose is derived from NADH, $FADH_2$ generated in Krebs cycle and ATP which is synthesized via oxidative phosphorylation. Microarray data showed that Krebs cycle operon genes *sdhCDAB*, *sucABCD* and *fumAC* were averagely 2-fold down-regulated at specific growth rate $0.47\,h^{-1}$ compared to $0.3\,h^{-1}$ (Fig. 2). Expression of *sucC* (succinyl-CoA synthetase, β subunit) was quantified as described above using real-time PCR assay which confirmed the decrease of *sucC* at mRNA level (Fig. 3). In oxidative phosphorylation, no significant changes in ATP synthase, cytochrome *c* oxidase, cytochrome *bd* complex and NADH dehydrogenase I subunits encoding mRNA levels were monitored. At the same time, a significant increase of *ndh* (NADH dehydrogenase II, 3-fold), *mqo* (malate dehydrogenase, 9-fold), *pyrD* (dihydroorotate oxidase, 2-fold) and *poxB* (pyruvate oxidase, 2-fold) expression

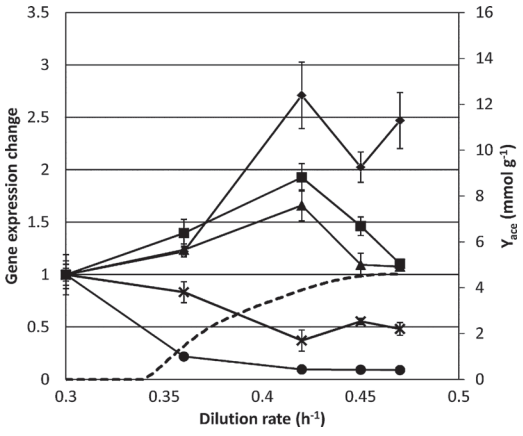

Fig. 3. Gene expression changes in *E. coli* K12 MG1655 A-stat cultivation. *ackA* (■), *pta* (▲), *poxB* (♦), *acs* (●) and *sucC* (×) – relative gene expression of acetate kinase, phosphotransacetylase, pyruvate oxidase and acetyl-CoA synthase, respectively. Dashed line represents specific acetate production per biomass (Y_{ace}: mmol g^{-1}). Error bars represent standard deviations of three to five technical replicates.

levels was observed at rising dilution rates (Supplementary Table S1 and Fig. 2).

3.5. Acetate production

Two main pathways are known to synthesize acetate – phosphotransacetylase-acetate kinase (*pta-ackA*) and pyruvate oxidase (*poxB*). Microarray data revealed that *pta* and *ackA* had no significant changes in mRNA expression levels between compared specific growth rates, however the expression of *poxB* increased around two times at higher specific growth rate ($0.47\,h^{-1}$). Concurrently, acetate utilization and transport operon genes acetyl-CoA synthetase (*acs*), *yjcH* and *actP* were strongly repressed (>20 times) (Fig. 2). To confirm and further characterize the course of acetate metabolism, *pta*, *ackA*, *poxB* and *acs* genes were validated with real-time PCR (Fig. 3). The analysis confirmed microarray data, moreover, it was revealed that *pta-ackA* pathway was slightly induced in parallel with acetate accumulation and down-regulated back to the pre-overflow metabolism level ($\mu = 0.3\,h^{-1}$) after acetate production had reached its near-maximum value ($\mu = 0.42\,h^{-1}$). In contrast, *poxB* expression increased and stayed 2-fold higher compared to chemostat at $0.3\,h^{-1}$ (Fig. 3).

4. Discussion

Modeling cell metabolism and creating strains of microorganisms for production of various biotechnologically important substances is a challenge for the 21st century. These goals can be achieved exclusively if data about microorganism's physiology is acquired from controlled steady state conditions. Besides well-known chemostat, accelerostat offers good quality data with high reproducibility and complements chemostat in two aspects – it enables to study dynamic response of cells, monitor metabolic switch points corresponding to specific growth rate and empowers the possibility to acquire (quasi-steady state) samples from vast number of specific growth rate values during one continuous experiment.

Whereas the glucose yield (Y_{glc}, mmol g^{-1}) was constant throughout the experiments, changes in glucose uptake genes expression were observed. A decrease in *ptsG* expression at higher glucose consumption rates was seen. *ptsG* encodes the glucose specific phosphotransferase system (PTS) transporter, which is a major glucose transporter in *E. coli* K12 (Becker et al., 2006). *ptsG* expression has been studied previously in glucose limited chemostat cultures at different specific growth rates by Seeto et al. (2004). This study is in consonance with our data, showing lower *ptsG* expression at higher specific growth rates. Glucose transport via PTS generates pyruvate directly, since one mole of pyruvate is produced per one mole of glucose taken up. That may lead to accumulation of pyruvate and trigger the beginning of acetate accumulation resulting in stress (Vemuri et al., 2006). Acid stress response was confirmed on transcriptional level (up-regulation of *hdeAB*, *gadW*). Therefore, it is beneficial for the bacteria to reduce glucose transport through PTS, because it provides *E. coli* with an opportunity to bypass some of the carbon "around" pyruvate. It is known that over-expression of phosphoenolpyruvate carboxylase (*ppc*), knockout of phosphoenolpyruvate carboxykinase (*pck*) reduces acetate formation (Farmer and Liao, 1997; Yang et al., 2003). As phosphoenolpyruvate is needed for the uptake of glucose via PTS, conversion of PEP to oxaloacetate by *ppc* reduces the available pool of PEP, resulting in a decrease of pyruvate accumulation. Indeed, we observed up-regulation in *ppc* and a remarkable decrease in *pck* mRNA levels at higher dilution rates. When PTS transport is reduced, it is likely that *E. coli* K12 MG1655 consumes some of glucose through an alternative non-PTS type transporter(s).

One such glucose transport system is encoded by *galP* (galactose permease). It has been shown that over-expression of *galP* and glucokinase (*glk*) in PTS disruption mutant reduced acetate formation and increased recombinant protein production (DeAnda et al., 2006; Wong et al., 2008). Gene expression analysis revealed a more than 3-fold up-regulation of *galP* expression with rising dilution rate. Another candidate for non-PTS glucose uptake could be the putative transporter *tsgA* which expression increased more than five times under catabolite repression conditions. However, expression of glucokinase (*glk*) catalyzing glucose phosphorylation was down-regulated at higher specific growth rates (Supplementary Table S1). Thus, alternative enzymes which are able to catalyze glucose uptake and therewith reduce acetate production should be present.

Considering microarray data about acetate metabolism in the literature, it should be concluded that there is no clear explanation about the synthesis routes from pyruvate to acetate, making the data about acetate metabolism controversial (Ishii et al., 2007; Vemuri et al., 2006). The main pathway for acetate synthesis at higher specific growth rates can be through *pta-ackA* (Ishii et al., 2007) or co-expression of *ackA* and *poxB* (Vemuri et al., 2006). Phue and Shiloach found that in *E. coli* K12 batch culture pyruvate oxidase (*poxB*) is a major route for acetate synthesis (Phue and Shiloach, 2004). Our study showed that at the beginning of overflow metabolism ($D = 0.34 \pm 0.01\ h^{-1}$), *pta-ackA* pathway is induced, but shifted down to $D = 0.3\ h^{-1}$ level before wash-out of the culture ($D = 0.48\ h^{-1}$). Concurrently, *poxB* expression increased approximately 2-fold (Fig. 3). *poxB*, *pta* and *ackA* expression patterns at higher specific growth rates suggest that pyruvate oxidase may be the only enzyme synthesizing acetate which is in concordance with Phue and colleagues (Phue and Shiloach, 2004; Phue et al., 2005). It is possible that *poxB* is favorable to balance NADH/NAD^+ ratio as the maximal NADH throughput in respiratory chain is exhausted, inhibiting additional NADH production via pyruvate dehydrogenase.

It has been shown by Vemuri and colleagues that excess NADH production will eventually down-regulate TCA cycle. Similarly, expression of respiratory chain components (NADH dehydrogenase I subunits, ATP synthase) encoding genes will be shifted down as well (Vemuri et al., 2006). From our data, NADH accumulation seems to be diminished by up-regulation of *ndh* that encodes alternative NADH dehydrogenase (complex II) (Bott and Niebisch, 2003) and by reduction of TCA flux. The latter causes reduction of electron donation to quinone pool, but *E. coli* seems to compensate it by enhancing the gene expressions of the enzymes mediating other routes for electron transfer (*mqo*, *pyrD* and *poxB*).

Many genes that encode proteins responsible for utilization and transport of substrates other than glucose were strongly repressed at higher specific growth rates. This effect is known as carbon catabolite repression (CCR), which is a metabolic response of the bacteria to utilize the substrate that is most abundant and guarantees the fastest growth (comprehensive review by Görke and Stülke, 2008). According to our results and data from the literature, it can be concluded that at relatively low specific growth rates, *E. coli* K12 MG1655 cells are ready for co-utilization of different substrates without the need of gene expression changes (Franchini and Egli, 2006; Ihssen and Egli, 2005; Lendenmann et al., 1996). Ability to rapidly utilize more than one substrate at a time gives *E. coli* an advantage in its natural environment. Since the competition in gastrointestinal tract for substrates is high, the bacteria that consume nutrients the fastest might have a significant advantage.

When looking at co-utilization of growth substrates from a different prospective, it is intriguing that according to real-time PCR data, down-regulation of *acs* occurs between dilution rates $0.3\ h^{-1}$ and $0.42\ h^{-1}$ (Fig. 3). Therefore, it can be expected that the ability to co-utilize glucose and acetate is lost between the mentioned dilution rates. Latter may have a significant influence to the beginning and course of overflow metabolism since acetic acid accumulation starts at dilution rate $0.34 \pm 0.01\ h^{-1}$ according to our data. However, the relationship between glucose repression and acetate-glucose co-utilization is poorly studied. Therefore, we are currently investigating the latter phenomenon using A-stat and dilution rate stat (D-stat) (Lahtvee et al., 2009) with adding acetic acid smoothly to the bioreactor while keeping the limiting substrate concentration constant.

A-stat is a cultivation method that enables real-time monitoring of culture parameters, *e.g.* culture optical density, oxygen consumption and by-product formation during continuous change of specific growth rate. Latter is especially important to characterize metabolic phenomena, *e.g.* overflow metabolism. *E. coli* K12 MG1655 has been studied before at different specific growth rates using chemostat and microarrays (Vemuri et al., 2006). Comparing gene expression changes at similar specific growth rates in the latter chemostat experiments and A-stat revealed that most of the strategies that *E. coli* K12 MG1655 execute when coping with rising dilution rate are essentially the same in both cases. Therefore, when studying bacterial metabolism at various specific growth rates A-stat could be considered as an effective and reproducible method for precise determination of switch points in metabolism regulation, *e.g.* beginning of overflow metabolism, induction and repression of specific genes, etc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.10.007.

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Publication II

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ORIGINAL PAPER

Multiplying steady-state culture in multi-reactor system

Sten Erm · Kaarel Adamberg · Raivo Vilu

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Abstract Cultivation of microorganisms in batch experiments is fast and economical but the conditions therein change constantly, rendering quantitative data interpretation difficult. By using chemostat with controlled environmental conditions the physiological state of microorganisms is fixed; however, the unavoidable stabilization phase makes continuous methods resource consuming. Material can be spared by using micro scale devices, which however have limited analysis and process control capabilities. Described herein are a method and a system combining the high throughput of batch with the controlled environment of continuous cultivations. Microorganisms were prepared in one bioreactor followed by culture distribution into a network of bioreactors and continuation of independent steady state experiments therein. Accelerostat cultivation with statistical analysis of growth parameters demonstrated non-compromised physiological state following distribution, thus the method effectively multiplied steady state culture of microorganisms. The theoretical efficiency of the system was evaluated in inhibitory compound analysis using repeated chemostat to chemostat transfers.

Keywords Bioprocess optimization · Continuous cultivation · Variable volume cultivation · Physiological state · Sequential-parallel cultivation

Introduction

It is generally accepted that systems biology approaches need cultivation tools that allow precise determination of quantitative relationships between environmental and growth/cellular parameters. This is realized in stabilized continuous cultivations (chemostat) where the physicochemical environment of cells is strictly controlled thus defining the physiological state of the cells under study [1, 2]. Physiological state of cells is reflected in their overall physiology—specific growth rate, gene expression, metabolism, etc.; it can be deduced and characterized by measuring time profiles of key metabolites consumed and produced [3]. On the contrast with continuous cultivations the physiological state is seldom truly fixed in batch cultivations due to changing environment, still batch cultivations are preferred in many cases as continuous experiments are preceded by stabilization phase, which renders them time and material consuming. Also, lengthy continuous cultivations are not desirable since mutants can outgrow the initial population [4–6]. Stabilization time per experiment is reduced in change stat cultivations but these methods also require initial stabilization for every experiment [7–9]. Described herein are a method and a system where experiments are started with cells in steady state physiology from the moment they reach the bioreactor. This enables: (1) to start cultivation experiment in each

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S. Erm (✉) · K. Adamberg · R. Vilu
Competence Center of Food and Fermentation Technologies, Tallinn, Estonia
e-mail: sten@tftak.eu

S. Erm · K. Adamberg · R. Vilu
Department of Chemistry, Tallinn University of Technology, Ehitajate tee 5, Tallinn, Estonia

K. Adamberg
Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, Tallinn, Estonia

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reactor from the defined physiological state established in just one reactor, thus reducing culture history related variance; (2) to save cultivation time and consumables due to decreased culture stabilization time; (3) to apply the method (with proper hardware) in an array of miniaturized reactors with multiple laboratory scale main reactors and/or sequential transfers to automatically study wide range of growth space of cells efficiently.

The prototype devised consists of three laboratory scale bioreactors connected with silicon tubing, thus forming a network (the parallel–sequential system). The method applied in the parallel–sequential system comprises the following steps: bacterial culture is stabilized in one bioreactor (termed the mother reactor) using desired continuous cultivation method (e.g., chemostat). After stabilization culture volume is increased while all other environmental and cellular parameters are kept constant. This can be considered a dilutionstat (D-stat) cultivation method in which outflow is turned off and feed flow rate is altered continuously such that dilution rate (D) remains constant. It should be emphasized that throughout this phase the culture will remain identical to the culture in the initial chemostat and the kinetic equations of chemostat apply. After the required volume is obtained, microorganisms are transferred into pre-set empty bioreactors (termed daughter reactors). The volume transferred equals the total operating volume of the daughter reactors and process' conditions are controlled in all bioreactors starting from the moment culture transfer begins, resulting in minimum deviation from pre-set environmental parameters. Following transfer, cultivation experiments are carried out in the daughter reactors, ensued by in situ cleaning of them without disconnecting the network. Thus, the daughter reactors are ready for another round of experiments. In theory the sequence transfer-experiment-cleaning can be continued infinitely; in reality the culture in the mother reactor should be replenished at desired intervals to avoid mutations. The number and roles of the bioreactors in the network are not fixed and the concept can be applied to an array of reactors (several hundreds of microreactors as an example). Also the mother reactor of one experiment can be daughter reactor in the next, thus the operator can maintain and multiply steady states obtained in whatever bioreactor of the system.

With the novel scheme there are two critical steps to be considered—variable volume cultivation in D-stat and culture transfer between bioreactors. Theoretical basis for kinetics of variable volume continuous cultivation are derived from its material balance, as described by Dunn and Mor in 1975 [10]. For instance they modeled balanced biomass growth and residual substrate concentration behavior at fixed dilution rate when cultivation volume was left to change, thus resulting in ever diminishing dilution rate. Variable volume cultivation is common in fed-batch cultivations; one of the simplest control strategies for avoiding overflow metabolism in fed-batch cultivation is keeping the growth rate below critical by using proper exponential substrate flow [11]. In fed-batch setup, however, the culture's characteristics change, by default the concentration of biomass or some other product is anticipated to increase. Differently from that in our non-typical fed-batch experiment all environmental and culture parameters apart from culture volume are maintained unaltered during volume increase phase so that concentration of biomass and substrates or products excreted remains constant. It should be noted that the maintenance of cultures' physiological homogeneity during variable volume phase is best assured in well mixed and properly controlled bioreactors, variable volume cultivation in industrial scale or in micro scale bioreactors can lead to culture heterogeneity due to poor mixing characteristics.

The second critical operation of our method is the culture's transport between reactors. Culture transfer between reactors is a well-known concept, particularly in the field of chemical engineering where it is used for achieving optimal conversion rates [12]. It is also used in biotechnology, a typical example being pre-growing inoculum in small scale and transporting it into the production unit; however, little attention is paid at maintaining a truly unaltered physiological state at culture transfer. In research laboratories the most common case of culture transfer between bioreactors is the repeated batch, where cells from later stages of batch cultivation are introduced into a fresh medium. This approach was successfully used by Lenski et al. [13] to study bacterial evolution through remarkable 44,000 generations. Although fit for purpose the procedure inherently introduces changes in the physiological state of the organism being transported as stationary phase cells are diluted into fresh medium with excess substrate.

Periodic biomass transfer in fed-batch regime was used to produce malaria vaccine when cultivating *Pichia pastoris* by Fricke et al. [14]. In their study a 6 L mother reactor and six 1 L daughter reactors were used for screening the optimal environmental conditions to produce the desired vaccine. In the mother reactor culture volume was increased, followed by transport to the daughter reactors. Similarly to the previous example the aim was to transfer inocula to the daughter reactors as the culture in daughter reactors was diluted twofold after the transfer. Temperature and other environmental conditions were varied in the daughter reactors following distribution, showing that the aim was not to keep physiological state after transfer equal to that in the mother reactor.

Continuous biomass transfer between two connected chemostats was used by Lueders et al. [15] to study heat

shock response in *Escherichia coli*. The effluent from one bioreactor was used as inflow into the second. The aim was to continuously heat shock the cells in the daughter reactor and analyze the adaptation of their proteome to the temperature increase from 37 to 47.5 °C. In addition to altered temperature between reactors an extra inflow into the stress bioreactor was established in order to maintain equal glucose concentration in both bioreactors, showing that dilution rate and hence physiological state was not maintained during the transfer; also differently from our approach, the transfer of biomass was continuous.

Continuous biomass transfer between bioreactors of different kind has been used to mimic culture heterogeneities found in industrial cultivation systems. In a nice example, Lara et al. [16] simulated feeding zones of large bioreactors in a setup consisting of laboratory scale stirred tank reactor (STR) coupled to a plug flow reactor (PFR). The outflow of the PFR was fed back into the STR thus forming a continuous loop. Concentrated glucose solution was introduced into the PFR, response in metabolism was monitored. As anticipated, the different operating conditions in the PFR and STR led to altered physiology of the culture.

In current work we evaluated the possibility of increasing biomass amount in steady state using variable volume D-stat cultivation followed by culture transfer in a network formed by three laboratory scale bioreactors. Throughout the process environmental conditions were kept constant. The maintenance of resulting constant physiology was evaluated by determining key process parameters and metabolites (biomass yield, CO_2 and acetate production). Further, the results from accelerostat (A-stat) cultivation experiments conducted in the novel system were compared to a traditionally conducted control A-stat experiment using statistical methods. Our results show that the cultivation experiment in the parallel–sequential scheme provides identical results when compared with the same cultivation procedure conducted in the traditional way.

Materials and methods

Bacteria and medium

In all experiments *E. coli* K12 MG1655 was used, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Inoculation procedure was as follows: 5 mL of glucose containing mineral medium was added to lyophilized culture, incubated at 37 °C for 24 h. Syringe was used for inoculation of pregrown inocula into the bioreactor. The defined mineral medium+glucose used in all experiments is described elsewhere [17].

Analytical methods used

Throughout experiments optical density, dry cell weight by gravimetry and major metabolites by HPLC were determined. The procedures are described elsewhere [17].

Cultivation procedure and system

Bacteria were inoculated into one of the reactors of the system (termed the mother reactor) and cultivated in batch regime until glucose and acetate were consumed from the medium. This batch cultivation was followed by chemostat cultivation at dilution rate 0.1/h with stabilization at constant settings lasting for at least five residence times. Chemostat phase (culture volume 300 mL) was ensued by variable volume cultivation with no outflow (end volume 900 mL) to increase the amount of steady state biomass. The inflow pump's flow rate was recalculated every second thus taking into account the exponential increase of the culture's volume. After desired volume was reached culture transfer to pre-set daughter reactors was realized by means of $\sim$1 bar overpressure using a silicon hose 0.5 m long with inner diameter 5 mm as transport line (total volume of one line 3 mL) (Fig. 1). Prior to biomass transfer the daughter reactors containing 300 mL of physiological solution (0.9 % solution of NaCl) were brought to experiments conditions and rapidly drained, establishing identical conditions in all reactors already at the time of transfer. The time to complete culture transfer to one reactor was $\sim$30 s, resulting in residence time inside the tube of $\sim$0.3 s. After ascertained of unaltered physiology the effect of growth rate change on the metabolism of *E. coli* was studied in A-stat cultivation with acceleration rate 0.01/h^2.

Possible effects on physiology arising from variable volume cultivation were monitored by analyzing bacterial broth during variable volume cultivation and also for one residence time before and after it. Effects due to culture transfer were screened for within one residence time before and after culture transfer.

The cultivation system consisted of three 1.25 L bioreactors (Applikon). Accessory devices (pumps, heating, etc.) were managed using EZ controller (Applikon) which was in turn controlled by BioXpert XP software (Applikon). Bioreactors were equipped with in situ sensors for pH, T, dO_2 and optical density (Truecell); temperature was kept at 37 °C using a heating blanket and pH at 7 with titration of 2 M NaOH. The culture was aerated via mass flow controller (MFC) at air flow rate 150 mL/min. In the case of variable volume cultivation the dO_2 level was maintained at 40 % by sparging pure oxygen via second MFC into the culture medium according to proportional-integral (PI) algorithm implemented in the software. For in

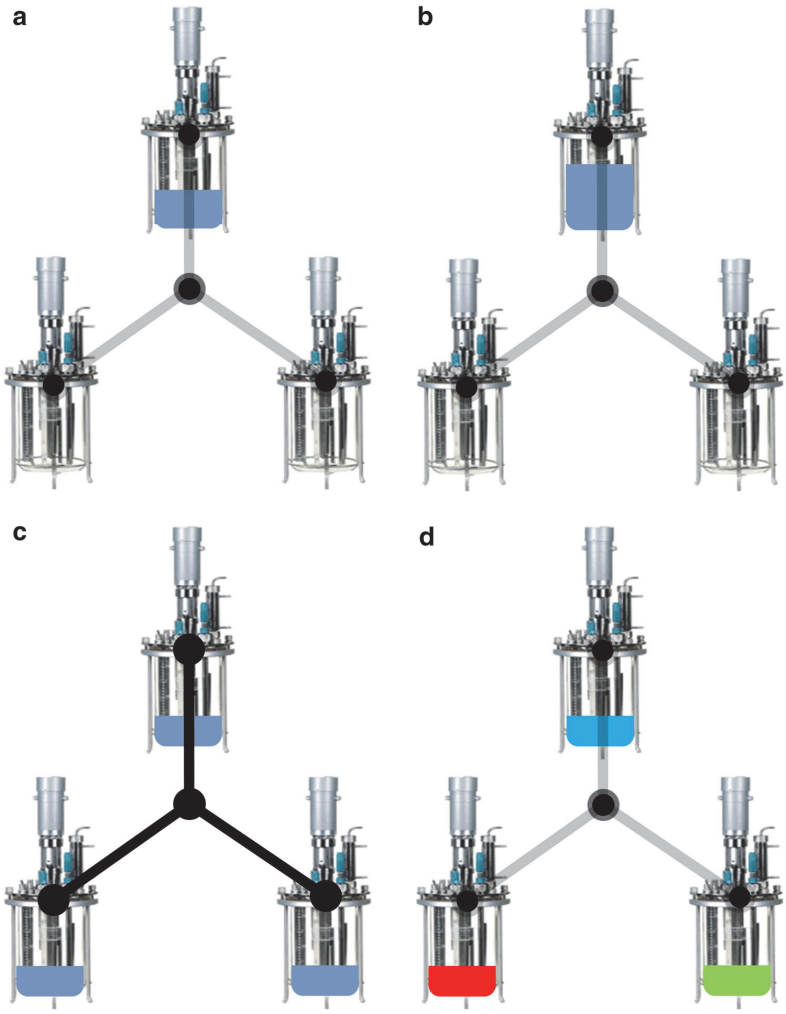

Fig. 1 Technological setup showing different phases in one set-up of parallel–sequential cultivation. During **a** culture is stabilized in the mother reactor in chemostat regime, followed by variable volume cultivation (depicted **b**). This phase is followed by **c** where culture is transferred into several bioreactors. **d** Illustrates growth-space analysis started from identical physiological state. In current study the growth space analysis conducted was an A-stat experiment (color figure online)

line gas analysis (O_2 and CO_2) gas sensors were used (Bluesense). Dilution rate was calculated by online weighting of the inflow and the bioreactor. Rushton turbines were used for mixing at 800 rpm. Data collection interval for the critical online variables (weights, dO_2) was 1 s, for other variables it varied from 10 s to 5 min.

Additional four experiments were conducted at dilution rate exceeding critical growth rate ($D = 0.4$/h), resulting in overflow metabolism and thus acetate concentration in the broth within confidently quantifiable range. In each experiment, after initial stabilization in chemostat at volume 300 mL the culture volume was set to increase to 600 mL in D-stat cultivation, followed by transfer of 300 mL of culture into pre-set empty bioreactor. The specific production rate of acetate during variable volume cultivation and after culture transfer was compared statistically (t test) to the specific production rate of acetate during the initial chemostat at 300 mL.

The built-in functions of Microsoft Excel were used for statistics, figures were created using OriginPro 9.1.

Results and discussion

The successful multiplication of physiological state was validated by conducting an A-stat experiment after culture transfer. Criterion for successful validation was statistically similar physiology of cells in A-stat cultivations

when three experiments in the parallel–sequential system were compared to an A-stat conducted in the traditional way.

The effects of the two procedures preceding any cultivation experiments in the parallel–sequential setup—variable volume cultivation and culture transfer—were analyzed by statistically comparing the physiological state of cells before, during and after applying the novel steps.

Comparison of parallel–sequential and traditional A-stat

The steady state obtained in stabilized chemostat in the mother reactor was verified by determining growth parameters within one residence time; if these were constant, variable volume cultivation and following culture transfer in the bioreactor network were carried out. After transfer, three accelerostat experiments starting from the physiological state of the mother reactor were conducted. Stabilization in just one reactor instead of three resulted in $\sim$2.5 times less medium consumed during stabilization period. Statistical analysis [single factor analysis of variance (ANOVA)] was applied to compare parameters obtained during A-stat cultivation in parallel–sequential system to the corresponding ones obtained in the traditional procedure (control A-stat). Two fluxes that exhaust most of the consumed carbon at aerobic growth of *E. coli* are carbon dioxide and biomass, a parameter considering both is the specific carbon dioxide production rate (q_CO_2). The average relative standard deviation of q_CO_2 in the four A-stat experiments was $\sim$5 %, a value comparable to that of three independent chemostat experiments (inset in Fig. 2), showing that little variation was induced due to the culture transfer. A-stat data from the parallel–sequential experiments was statistically equal to the data from traditional A-stat as the P value at most dilution rates exceeds threshold value 0.05 (supplementary tables S1, S2).

Metabolite profiles of *E. coli* MG1655 change with increasing growth rate in A-stat experiments [18]. Therefore, the profiles of acetic acid (ace), dihydroorotic acid (dho), lactic acid (lact) and acetylaspartic acid (aceasp) were monitored throughout the acceleration phase to compare the results from parallel–sequential cultivation to those obtained in traditional A-stat. Based on measured data the metabolite production per dry biomass (O_aceasp, O_ace, O_dho, all mmol/g) of three A-stat cultivations in parallel–sequential scheme and the control A-stat were statistically similar with $P > 0.05$, excluding lact ($P < 0.05$) (presented in Fig. 2). At most dilution rates the parallel–sequential A-stat data yields statistically equal data to that from the traditional A-stat (supplementary tables S3–S7). The dissimilarity in case of lactate is explained by the fact that the concentrations of minor metabolites were quite small, thus the error introduced by measurement uncertainty was significant (concentration of dho ranged from 0.05 to 0.19 mM, aceasp from 0 to 0.15 mM, lact from 0.05 to 0.11 mM).

Continuous monitoring of cellular metabolism in response to increasing growth rate has an important and practical value during growth space analysis, a classical

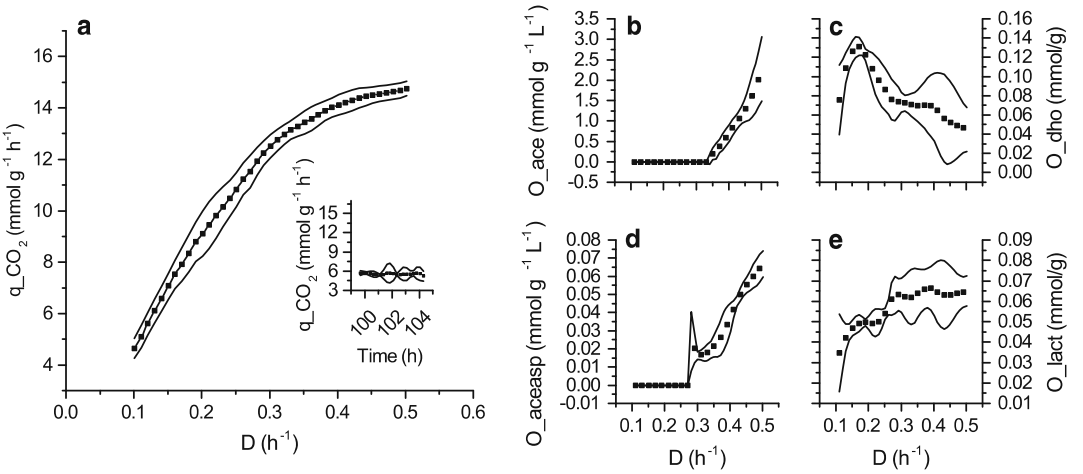

Fig. 2 *Left* **a** depicts average specific carbon dioxide production rate q_CO_2 with experimentally determined standard deviation in four A-stat experiments (three from parallel–sequential and one from traditional A-stat). The average relative standard deviation, presented by continuous line, was $\sim$5 %; a similar value was obtained from three chemostat experiments (inset on **a**). *Right* **b**–**e** present averaged flux values of metabolites per biomass from four A-stat experiments (three parallel–sequential+control A-stat)

Table 1 Values of dilution rate (equals growth rate) at shift points of selected parameters trend lines in A-stat experiments, three from parallel–sequential scheme (identifier MD added) and one traditional (control)

Parameter	Dilution rate in MD A-stat (mother)	Dilution rate in MD A-stat (daughter 1)	Dilution rate in MD A-stat (daughter 2)	Dilution rate in control A-stat	Average dilution rate	RSD (%)
OD (max)	0.53	0.54	0.51	0.52	0.52	2.04
O_dho (max)	0.15	0.16	0.17	0.16	0.16	5.10
O_dho (min)	0.43	0.50	0.47	0.42	0.46	8.12
$\mu_{critical}$ (based on O_ace)	0.36	0.34	0.34	0.33	0.34	3.67
Increase of O_aceasp	0.29	0.28	0.28	0.28	0.28	1.77
q_CO_2 (max)	0.49	0.42	0.47	0.48	0.47	6.69

RSD is relative standard deviation, subscript "max" and "min" denotes maximum and minimum values, $\mu_{critical}$ the onset of overflow metabolism reflected in acetate's flux increase

example with *E. coli* being determining the onset of overflow metabolism of acetate. Therefore, it is crucial that the metabolic shift points obtained in A-stat experiment carried out in parallel–sequential system correspond to those obtained in the traditional A-stat. Results show that relative standard deviation of specific growth rate at metabolic shift points was below 10 % when comparing sequential–parallel and control A-stat. This indicates that the results of parallel–sequential A-stat experiment are close to the ones obtained in a traditional A-stat (Table 1).

Metabolic flux analysis (MFA) was used to compare sequential–parallel and traditional A-stat experiments, procedure described in [18]. The average standard deviation of all fluxes in the four experiments was 32.3 %; however, in case of the major carbon fluxes—glycolytic and Krebs cycle—it was 7.4 and 7.8 %, respectively. The small standard deviations of these fluxes show that parallel–sequential method is applicable in the modeling of growth space in *E. coli* cells. Large variation observed for fluxes of pentose phosphate cycle (74.4 %) can be explained by relationship of this pathway to nucleotide metabolism where excretions of several pyrimidine intermediates (e.g., dho) were observed, the effect of which induced large variations in the MFA on the relative scale.

Variable volume cultivation

E. coli was cultivated and growth parameters analyzed during variable volume D-stat experiments to look for possible effects induced by the method. In one set of experiments culture parameters during the D-stat cultivation and the following chemostat (at volume 900 mL) were compared to the corresponding ones from the preceding chemostat (at 300 mL) using ANOVA. It can be seen that the specific carbon dioxide production rate q_CO_2 remained statistically constant throughout the variable volume cultivation, with *P* value exceeding threshold of 0.05 (Fig. 3a; supplementary tables S8, S9).

During variable volume cultivation the relative standard deviation of minor metabolite concentrations [dho, aceasp, carbamoylaspartate (carbasp), lact] remained in the range of control experiments with value and standard deviation similar to those of the average of three chemostats preceding volume increase (data not shown). The production of metabolites per biomass in the initial chemostat was compared statistically to the succeeding variable volume cultivation. The data shows that in case of lact and aceasp production remained similar in both phases, but with dho and carbasp the *P* value was smaller than 0.05 indicating altered physiology. It should be stated that small concentrations of the metabolites were used to calculate productivities thus the error introduced by analysis is significant. Another explanation for the cause of deviation is hardware related, as control parameter's values differed in different phases of the variable volume cultivation. The average relative standard deviations of dilution rate, pH, T during the whole experiment were 10, 0.12, 0.13 %, respectively; clearly the most fluctuating control parameter is dilution rate and this fluctuation can cause some disturbance to the well-established chemostat physiology. The fluctuation is comparable to the reproducibility of three separate chemostat experiments (respective relative standard deviations for dilution rate, pH, T in chemostat are 14, 0.15, 0.14 %); thus variable volume cultivation did not induce excess variation in control parameters. However, when the average value of dilution rate at the initial chemostat (at 300 mL) was compared to the one from chemostat at (900 mL) the dilution rate was in average $\sim$3 % higher. This increase can induce a physiological response and should be avoided.

To avoid large error due to calculations involving small concentrations, additional experiments were conducted in which the concentration of metabolites reflecting physiology was significantly increased. Acetate was chosen as the

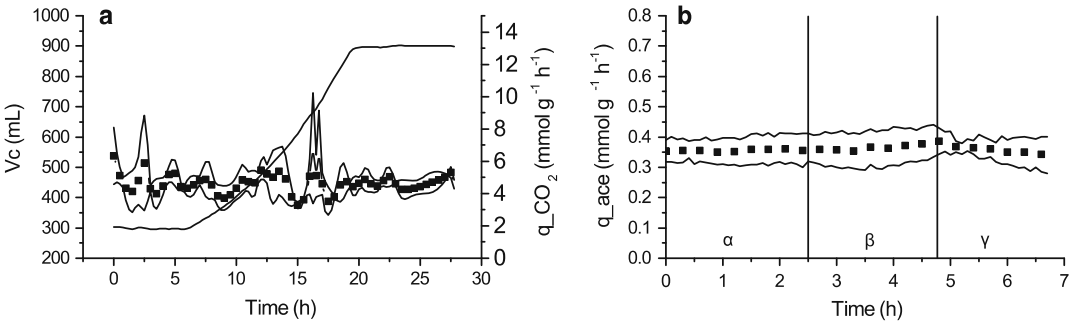

Fig. 3 *Left* **a** depicts average specific carbon dioxide production rate q_CO_2 from three variable volume experiments at dilution rate 0.1/h. Experimentally determined average relative standard deviation was 15 %. Three phases of the cultivation can be distinguished based on culture volume (initial chemostat at volume 300 mL, variable volume cultivation with increasing volume from 300 to 900 mL, chemostat at 900 mL). **b** Depicts average specific acetate production rate of three experiments at dilution rate 0.4/h. Compared are chemostat at 300 mL (denoted α), variable volume cultivation (denoted β) and at chemostat after transfer (denoted γ)

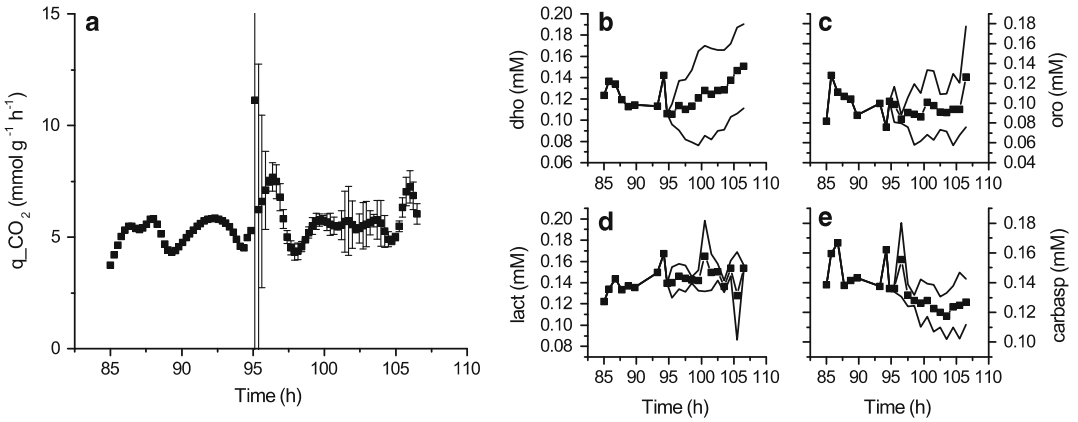

Fig. 4 *Left* **a** represents specific carbon dioxide production rate q_CO_2 values in the mother reactor and in the daughter reactors following culture transfer. The large deviation after culture transfer in time frame ~95–96 h is an artifact due to splining error. *Right* figures **b–e** show concentration profiles of minor metabolites before and after the culture transfer. *Thin continuous line* represents standard deviation. The average relative standard deviations were dho 27 %, oro 27 %, lact 10 %, and carbasp 10 %. The moment of transfer is visible from time point 95 h when the standard deviation lines appear

subject as its concentration is easily monitored; additionally acetate production will change rapidly due to fluctuating environmental conditions. The statistical comparison (*t* test) of initial chemostat at 300 mL with variable volume cultivation demonstrated that according to specific acetate production rate the two phases represented the same population, with P value > 0.05 (Fig. 3b; supplementary tables S10, S11).

Culture transfer

The possible effects induced by culture transfer were analyzed using ANOVA by comparing culture parameters before and after culture transfer. ANOVA showed that during culture transfer the two major carbon fluxes—biomass synthesis and production of CO_2 (considered together as q_CO_2) did not derail statistically as the distributions before and after culture transfer were similar ($P > 0.05$) (Fig. 4; supplementary tables S12, S13). The large error after culture transfer is explained by calculation bias (splining of data) due to fast change of dilution rate.

Minor metabolite concentrations before and after culture transfer were statistically similar ($P > 0.05$) in case of orotate (oro), dho and lact but not in the case of carbasp (Fig. 4); the dissimilarity of carbasp can be explained by measurement uncertainty at small concentrations and hardware related challenges discussed below.

Table 2 Minimal or maximal value of main environmental parameters in all reactors after culture transfer within the period of one residence time

Reactor	D (h^{-1})	D (%)	dO_2 (of saturation %)	dO_2 (deviation %)	pH	pH (%)	T (°C)	T (%)
M	0.09	−10.00	77.70	11.00	7.04	0.57	36.70	−0.81
D1	0.09	−10.00	68.00	−2.86	7.07	1.00	36.40	−1.62
D2	0.11	10.00	72.10	3.00	7.24	3.43	36.40	−1.62

Percentage illustrates the deviation of parameter from its set-point value

M denotes mother reactor D1 and D2 daughter reactors

Set-point for dilution rate was 0.1/h, for dO_2 70 %, for pH 7, for T 37 °C

To avoid error due to small concentration of metabolites, the effect of culture transfer at higher dilution rate ($D = 0.4$/h) was conducted. Statistical comparison (t test) of specific acetate production between the initial chemostats at 300 mL and chemostats in the receiver reactors after the culture transfer indicates prevalence of unaltered physiological state of culture (Fig. 3; supplementary tables S14, S15).

Alike the variable volume cultivation the culture transfer should have minimal effect on cellular physiology. Ideally this is achieved when control parameters in different bioreactors remain equal. In order to compare the behavior of control variables during culture transport the environmental parameters values (dilution rate, T, pH, dO_2) after transport were compared to the desired set-point values. Due to the rapid culture volume change during transport the calculated dilution rate was biased as calculation is based on 3 min average, hence analysis of dilution rate stability between reactors was omitted in the turbulent region (about 5 min after transport). The maximal/minimal value of environmental parameters from all reactors during the chemostat phase after the culture transfer was compared to the corresponding parameters set-point value (Table 2).

The deviation of cultivation parameters from set-point values is one of the reasons for the variation in cultivation parameters in different bioreactors. There will also be some bias induced from the control interval length during culture transfer. As the cells inside transfer tube are not in homogeneous STR conditions, this phase should be completed as fast as possible. However, the desired fast change in culture volume during transfer (10 mL/s) introduces an inherent error in dilution rate, the scale of which is dependent on the control unit's data control capacity. We used 1 s interval for control, a period which results in 3 % deviation in one second in dilution rate during culture transport at 300 mL (1 % at 900 mL) this deviation is corrected at the next second, etc. However, as the following A-stat cultivation clearly showed, the small change induced in physiology by bias of control parameters did not have an effect on the growth characteristics of *E. coli* in A-stat.

The deviation of cultivation parameters can lead to stressed microbial culture, which can be prone to mutate. As culture growth conditions change, the mutations favoring those conditions are amplified within the population. However, in our system the cells are potentially under stressful conditions only during culture transport, which comprises a short period (30 s). Before and after steady state conditions prevail. So it is reasonable to assume that even genetically unstable strains can be used within the devised system successfully. Mutations are also a subject to consider upon all long lasting cultivations. Data from continuous cultivation experiments with *E. coli* K12 MG1655 (strain used also in current work) by Nahku et al. suggest no extraordinary mutation rate during 20 generations in continuous cultivation; however, it has been shown that long continuous cultivation experiments will amplify favorable phenotypes [5, 20]. Thus, the robustness of organism used should be analyzed case by case for the suitability in parallel–sequential cultivation and for continuous cultivation in general.

Efficiency analysis of the sequential–parallel system

The procedure of A-stat carried out in current work can be optimized if validation of constant physiology after variable volume cultivation and culture transfer is not conducted. The procedure is most efficient when the variable volume cultivation forms part of the stabilization phase, bacteria are transferred to the daughter reactors at the moment the desired culture volume is obtained, and cultivation experiment started as soon as transport between bioreactors is completed (Fig. 5, blue lines). When compared to the procedure used in current study the more effective case consumes 44 % less substrate during the preparative phase of parallel–sequential cultivation (initial batch culture and stabilization before A-stat). The method devised is substantially more effective when compared to three A-stat experiments carried out traditionally in three separate bioreactors—the optimized parallel–sequential scheme uses ~61 % less feed in the preparative phase.

The power of the method described herein lies in the fact that experiments from identical physiological condition can be started with no prior stabilization period in the

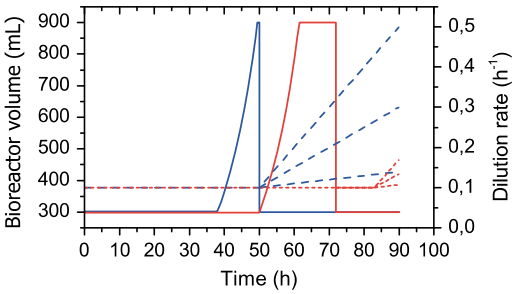

Fig. 5 Comparison of optimized parallel–sequential cultivation scheme (*blue*) with the one used in the study (*red*). *Continuous line* represents culture volume, *dashed line* dilution rate. The stabilization in chemostat regime before and after culture transfer in the actually used scheme was one residence time. Start of A-stat experiment can be seen by the increasing dilution rate (color figure online)

Table 3 Comparison of time spent for 30 inhibitor analysis experiments in parallel–sequential and traditional set-ups of bioreactors

Phase (h)	Parallel–sequential setup	Traditional setup
Initial batch growth	10	300
Stabilization phase	25	750
Inhibitor's effect analysis in chemostat	300	300
Set-up time	58	58
Total per array	214	469.33

The variable volume cultivation was part of the stabilization phase, experiments started at the moment biomass from the mother reactor reached the daughter reactors

daughter reactors. After experiment the daughter reactors can be rinsed and sterilized, thus preparing them for another culture transfer without altering the culture in the mother reactor. As an example the time necessary to analyze the effect of three inhibitors at ten different concentrations in traditional and parallel–sequential system in chemostat cultivation (three reactors in both cases) was analyzed theoretically. It was assumed that the cellular response, e.g., growth rate decrease, is obtained within two residence times after the introduction of inhibitor. In this case the novel method will provide the necessary data in 213 h while the traditional method within 470 h, meaning the novel method is $\sim$2.2 times more effective. The time necessary to complete the theoretical study using the two methods is depicted below in Table 3.

Conclusions

A method and a system for fast transport of microorganisms in steady state from one bioreactor into several was devised. It was shown that experiments conducted in the parallel–sequential system were statistically equivalent to an A-stat executed in traditional manner. The novel phases of cultivation in the parallel–sequential scheme, the variable volume cultivation and the culture transfer between bioreactors, were shown not to compromise the physiological state of the cells. As stabilization is needed only once per many experiments the application of parallel–sequential cultivation results in savings of cultivation time and medium. Although herein the applicability of the method was shown using traditional laboratory STR type bioreactors, the method is very suited to be used in the field of mini- or micro-reactors. Mini reactor systems where tubing lengths can be minimized or totally removed should be developed for scanning the growth space of cells with full quantitative analysis of metabolism and growth regulations. Microreactor systems required for fast screening of different strains and diffusion-based microplate technologies developed for chemostat (as described in [19]) can be an attractive and suitable platform for parallel–sequential experiments in micro scale.

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Efficient Growth Space Scanning and Perturbation Analysis of Microbial Processes using Multi-Bioreactor Systems

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Keywords:	Chemostat, Changestat, Growth space analysis, Physiological state, Quasi-steady state

Efficient Growth Space Scanning and Perturbation Analysis of Microbial Processes using Multi-Bioreactor Systems

Sten Erm[1, 2] (corresponding author), Kaarel Adamberg[1, 3], Raivo Vilu[1, 2]

[1] Center of Food and Fermentation Technologies, Akadeemia 15A, Tallinn, Estonia

sten@tftak.eu, mob. 0037255539779

[2] Department of Chemistry, Tallinn University of Technology, Ehitajate tee 5, Tallinn, Estonia

[3] Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, Tallinn, Estonia

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Abstract

Starting cultivations in steady state conditions with no stabilisation phase can be realized using mother-daughter (MD) systems by maintaining microbial culture in controlled steady state physiology in the mother-reactor and periodically transferring aliquots of the culture without perturbing the established physiological steady state into daughter-reactors. In the daughter-reactors the physiology of the cells can be studied in steady or quasi-steady state experiments or in controlled perturbation experiments. Important element of this system is the use of changestat cultivations in the mother-reactor to obtain different physiological states. It was shown that the specific productivity of GFP during the induced period in the MD and reference chemostat cultivations was coinciding with Pearson correlation coefficient 0.97 – 0.99. Theoretical calculations show that the study of steady state microbial physiology and productivity in perturbation experiments in the MD system is up to 400% less time consuming than experiments run in parallel in separate reactors.

Keywords:

Chemostat;

Changestat;

Growth space analysis;

Physiological state;

Quasi-steady state;

Introduction

The physiological states of microbes depend on their growth environment and culture history (Malek 1975). The continuum of physiological states and environmental parameters can be viewed to form an *n*-dimensional growth space. Comprehensive growth space analysis cannot be considered a popular concept in current scientific research, however, it has been used as a helpful concept in gene regulation analysis, metabolic flux analysis, bioprocess optimisation, etc. (Gerosa et al. 2015; Holms 1996; Warikoo et al. 2012). The most difficult aspect for grasping the growth space concept is that physiological states of cells depend besides values of environmental parameters also on the history of the cells – as cells are highly hysteretic systems. However, there exists a specific subset (sub region) of growth space, which is free from the nearly infinite number of ambiguous relationships – the steady state growth space (SSGS). SSGS has a special role in the continuum of physiological states of microbes as one-to-one relationship between environmental and cellular parameters exists there, which means that in SSGS the values of environmental parameters are fully determining the steady state physiological states of microorganisms (Adamberg, Valgepea, and Vilu 2015). Although the number of parameters affecting physiology (and therefore the number of dimensions of growth space) is large, a few are considered most relevant in practical studies – limiting substrate composition, growth rate, pH and temperature. An illustration of simplified theoretical steady state growth space where a bioprocess descriptor (volumetric productivity) is depicted as a function of pH and substrate availability is presented in Fig. 1 a. Although in case of choosing other parameter combinations more complicated surfaces are expected to describe the relationships between environmental parameters and those determining the steady state of cell physiology, it can be reasonably assumed that there exist productivity etc. optima and other specific points in the SSGS which would be important to understand design principles and constraints of the cell metabolism. The most conventional way of studying steady state growth space of bacteria is using chemostat cultivations run sequentially or in parallel as shown in Fig. 1 a (Bull 2010). Significant increase in efficiency of studying SSGS was achieved in the result of introduction of changestat methods (Paalme et al. 1995) allowing true scanning of the growth space (see Adamberg, Valgepea, and Vilu 2015). Quasi-steady state scanning of SSGS using changestat cultivations in individually run reactors is illustrated in Fig. 1 b and c. In Fig. 1 b the depicted physiological states are reached by altering the growth rate in three separate A-stat cultivations by changing dilution rate continuously. The same physiological states can be reached by moving in “perpendicular” direction to previous in the illustrated growth space by changing pH continuously in three separate D-stat cultivations as shown in Fig. 1 c (Kasemets 2003).

While changestat algorithms offer convenient means to scan the SSGS they are started as chemostats, and thus include lengthy preparatory batch and stabilisation phases. These preparatory phases are not necessary in the MD systems where a quasi-steady state culture in mother-reactor can be transferred into other reactors (daughter-reactors), as described by Erm et al. (Erm, Adamberg, and Vilu 2014). In these multi-reactor systems the physiological states of the cultures are maintained during and after the culture transfer, therefore making possible starting of different changestat or chemostat experiments in the daughter reactors from the same initial physiology. An illustration of quasi-steady state growth space scanning in the MD system is depicted in Fig. 1 d where culture in the physiological state 1, initially developed in the mother-reactor, is transferred to two daughter-reactors, and growth space scanning in all three reactors in three different directions is continued. By repeating the transfer-analyse-discard sequence in principle the whole SSGS can be scanned. Further, the MD system is very suitable for coupling SSGS scanning with perturbation experiments, such as recombinant protein production. In this case culture in particular physiological states illustrated by red dots in Fig. 1 is transferred to the daughter-reactors for perturbation experiments. Perturbation experiments are used widely (reviewed by (Spadiut et al. 2013)) and can result in exit from SSGS. The exit from SSGS necessitates a new stabilisation or experiment in order to study another point in the SSGS. Decoupling growth and perturbation reactors has been used in previous works (Curless et al. 1991; Fricke et al. 2011; Lüders, Fallet, and Franco-Lara 2009; Shin et al. 1997). In these earlier studies chemostat or fed-batch cultures at fixed growth rates have been transferred to perturbation reactors where the physiological states of cells were changed. However, to our knowledge, previous works have not focused on starting cultivations in the daughter-reactors from the physiological state established in the mother-reactor; further, the previous works have not analysed coupling comprehensive growth space scanning in the mother-reactor (possible with changestats) with perturbation experiments in the daughter-reactors.

Fig 1

The time efficiency of analysing SSGS using MD systems was studied in special case of perturbation analysis in current paper. In perturbation analysis the cells in prepared steady states were perturbed in controlled processes – inducing recombinant protein production, changing pH or temperature stepwise, etc. It was shown in a series of experiments in this paper that the results of the perturbation analysis using GFP were practically the same whether obtained in reference chemostat experiments carried out in separate bioreactors or in analogous experiments in MD systems. Further several theoretical SSGS studying scenarios were compared in parallel in independent chemostats and MD systems. The calculations showed that although experiment time is reduced

maximally if the network of bioreactors in the MD system is large, considerable time savings (e. g. 200 %) are obtained also with relatively small number of experiments conducted in a network consisting of a few interconnected bioreactors, if the experiments would be designed and carried out properly.

Materials and Methods

GFP Productivity Experiments

Description of Experiments and Cultivation System

Recombinant protein production experiments were used to compare the results in reference single reactor system and MD system. The recombinant protein production was chosen as an example of perturbation experiment as it is known to introduce significant changes in physiology of cells, and is important for the optimization of industrial processes (Carneiro, Ferreira, and Rocha 2013). Experiments in both cases were conducted in 1 L laboratory scale bioreactors (Applikon) with EZ controllers and BioXpert W7 software (control interval 1 second).

The reference cultivation in the single reactor system was stabilised as chemostat, after which the continuous steady state cultivation was changed to fed-batch and production of GFP induced. The growth rate was the same in chemostat and fed-batch cultivations, and two growth rates were studied (0.1 h^{-1} and 0.5 h^{-1}). The GFP production process was monitored for five hours, samples taken hourly.

The MD system consisted of a mother-reactor and a daughter-reactor (Fig. 2 a). The culture stabilisation was done in the mother-reactor, followed by increasing the culture volume in quasi-steady state μ-stat cultivation (cultivating at constant biomass density at constant growth rate with exponentially increasing culture volume) after which an aliquot of culture corresponding to the working volume of the daughter-reactor was transferred into the daughter-reactor. The biomass transfer via silicon hose (length ~300 mm, inner diameter 5 mm) connecting the bioreactors was achieved by pressurising the mother-reactor (~1 atm). To minimise the effect of culture transfer on cell physiology the daughter-reactor was kept at pH and temperature of the mother-reactor prior the transfer. The buffered solution in the daughter-reactor was drained moments before culture transfer.

Dissolved O_2, gas phase O_2 and CO_2 were monitored in the daughter reactor after the transfer of the culture for one hour to confirm unaltered physiological state. After that the chemostat phase was switched to fed-batch and GFP production initiated. The statistical comparison of physiological states before culture transfer, during the μ-stat and after culture transfer in the MD system were not conducted in current work as this was done previously (Erm, Adamberg, and Vilu 2014). However, the specific productivity of GFP (q_{GFP}, $FU \cdot g^{-1} \cdot h^{-1}$) during the production phase of cultivations carried out in the MD system and in reference single reactor chemostat system

were compared in correlation analysis using OriginPro. The experiments at both growth rates were conducted in the MD set-up in two, and in traditional set-up in one replica.

Strain, media, analysis

GFP production analysis was carried out using kanamycin resistant *Escherichia coli* BL21(DE3) pET42-GFPuv (Clontech). Expression of recombinant protein was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). All chemicals were from Sigma-Aldrich, if not stated otherwise. The feeding medium in continuous cultivation was [all $g \cdot L^{-1}$] α-(D)-glucose 5; NH_4Cl 3.5; casamino acids (LabM) 2.5; K_2HPO_4 (Merck) 2; $FeSO_4 \cdot 7H_2O$ 0.005; $MgSO_4 \cdot 7H_2O$ 0.5; $MnSO_4 \cdot 5H_2O$ 0.002; $CaCl_2 \cdot 2H_2O$ 0.005; $ZnSO_4 \cdot 7H_2O$ 0.002; $CoSO_4 \cdot 7H_2O$ 0.0006; $CuSO_4 \cdot 5H_2O$ 0.0005; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) 0.0026. The fed-batch medium contained the same components, but concentrations were increased 20 times. In both cases the media contained antifoam 15 $\mu l \cdot L^{-1}$ and kanamycin 50 $mg \cdot L^{-1}$. Induction of the GFP production was triggered by adding IPTG 0.5 $mmol \cdot L^{-1}$. For fluorescence analysis biomass was washed twice with ice cold PBS and diluted to biomass concentration 0.35 $g \cdot L^{-1}$, fluorescence was determined by Fluo-Imager M53 (Skalar) (excitation 380-500 nm and emission 420-735 nm). Organic acids and glucose were separated using Alliance HPLC system (Waters) with HPX-87H column (BioRad) and determined with refractive index detector (Waters).

Theoretical Time-Efficiency Analysis of Cultivations in the MD System Coupling Growth Space Scanning and Perturbation Experiments

Three theoretical scenarios of perturbation analysis in the different regions of microbial growth space were compared in reference chemostat and MD systems. The number of perturbation experiments and reactors in the reference chemostat experiments and in the MD experiments was always considered equal. Time efficiency of using MD system was calculated by dividing total experiment time of the reference chemostats with the total experiment time in the MD systems. In the case of reference system all reactors were stabilised as chemostats, after which perturbation was assumed to be applied. A new experiment was assumed necessary in order to study another physiological state as the perturbation was assumed to disrupt the steady state physiological state.

In the MD system the mother-reactor was assumed to be used for quasi-steady state growth space scanning and for periodically filling the working volume of daughter-reactors in which perturbation experiments were assumed to be conducted. In order to study another physiological state the culture in the daughter-reactors was assumed to be discarded after the perturbation experiment and replaced with quasi-steady state culture from the mother-reactor. The overview of the theoretical experiments is given in Table 1.

Table 1. Overview of the theoretical perturbation experiments conducted in reference chemostat and MD systems.

Experiment description	Reactor set-up in the reference system	Description of cultivation process used in the reference system	Reactor set-up in the MD system	Description of cultivation process used in the MD system	Perturbation analysis length
30 different perturbations in chemostat at D=0.2 h^{-1}.	3 reactors operated in parallel in chemostat[a].	In total 30 perturbation experiments. Stabilisation of culture in all reactors as chemostats[a] at D=0.2 h-1. Followed by perturbations in all reactors. By changing the nature of perturbation and repeating the process the 30 perturbation experiments were assumed to be conducted.	3 reactors, one mother-reactor (operated in chemostat[a]) and two daughter-reactors (for carrying out perturbation experiments).	In total 30 perturbation experiments in the daughter reactors, chemostat[a] culture at D=0.2 h^{-1} maintained in the mother-reactor. Stabilisation of culture in chemostat in the mother-reactor at D=0.2 h^{-1}, followed by transfer of culture into daughter-reactors where the perturbations were applied. Following perturbation analysis the culture in the daughter-reactor was assumed to be discarded. Procedure was repeated until 30 perturbation experiments were analysed.	One residence time.
Single perturbation at 10 different growth rates (0.1 h^{-1} to 1 h^{-1}, interval 0.1 h^{-1}).	2 reactors operated in parallel in chemostat[a].	In total 10 perturbation experiments. Stabilisation of culture in chemostat[a] at D=0.1 h-1, followed by perturbation analysis and discarding of culture. Procedure assumed to be repeated in case of all dilution rates indicated.	2 reactors, one mother-reactor (operated in A-stat[a, b] and one daughter-reactor (for carrying out perturbation experiments).	In total 10 perturbation experiments in the daughter-reactor starting at different dilution rates indicated, A-stat[a, b] run from D=0,1 h^{-1} to D=1,0 h^{-1} in the mother-reactor. Stabilisation of culture in chemostat[a] at D=0.1 h^{-1} in the mother-reactor followed by smooth growth rate increase in A-stat cultivation. Transfer of culture to daughter-reactor from the A-stat reactor at indicated D values for perturbation analysis, after which culture in the daughter-reactor was assumed discarded. By repeated transfers the perturbation experiments at 10 growth rates were assumed to be conducted.	One residence time.
Perturbations at 10 different growth rates (0.1 h^{-1} to 1 h^{-1}, step 0.1 h^{-1}), and at each growth rate at 11 different pH values (0 % - 100 %, step 10 %).	5 reactors operated in parallel in chemostat[a].	In total 110 perturbation experiments. Stabilisation of culture in five reactors as chemostats, followed by perturbation analysis in all reactors, after which culture was discarded. The experiments at indicated growth rates and pH combinations were assumed to be carried out – altogether 110 chemostat perturbation experiments.	5 reactors – reactor one for A-stat[a, b], reactors two and three for D-stat[c], reactors four and five for perturbation analysis.	In total 110 perturbation experiments conducted in the two perturbation reactors, A-stat[a, b] and D-stat[c] in the mother-reactors. In the reactor 1 culture was assumed to be stabilised in chemostat[a], followed by smooth growth rate change in A-stat cultivation. Transfer of culture to reactors 2 and 3 followed at indicated D values. In the reactors 2 and 3 pH was changed from 0 % to 100 % in D-stat cultivations (starting from 50 % in both cases). After scanning the pH effect the culture in D-stat was discarded and replaced with culture from A-stat operating now at increased dilution rate. At pH interval 10 % culture was transferred from the D-stat reactors (reactors 2 and 3) to the next two daughter-reactors (reactors 4 and 5) for perturbation analysis. By repeated transfers at 110 combinations of growth rate and pH the 110 perturbation experiments were assumed to be conducted.	Five hours.

a – Theoretical chemostat and A-stat cultivations were started as batch lasting 10 hours followed by stabilisation in chemostat for five residence times at constant conditions.

b - Acceleration rate 0.01 h^{-1} used in the A-stat

c - pH changing rate 2 %·h^{-1} used in the D-stat

In order to gain the biomass necessary to fill the daughter-reactors μ-stat cultivations with increasing culture volume (cultivating at constant biomass density at constant growth rate with exponentially increasing culture volume) were assumed to be carried out in the mother-reactor prior each transfer. The MD system was assumed to be formed either two or three layers of bioreactors with culture transfer between the layers (Fig. 2). In the three-layer system the first two layers were assumed to be used for growth space scanning (A-stat and D-stat cultivations, details in Table 1), and the third layer for perturbation analysis. The bioreactor of the first layer was

always assumed to be laboratory scale reactor (operating in L scale), while the layer two and layer three reactors were either laboratory or small scale (operating in tens of mL scale) reactors. Layer one was assumed to contain one reactor while layers two and three could have more, but in our case the total number of reactors in the MD systems was either three or five as shown in Fig. 2.

Fig 2

Theoretical calculations were run in MS Excel, figures were created with OriginPro 9.1.

Results and Discussion

Experimental Recombinant Protein Production in the chemostat and MD System

In the experimental part of current study the effect of two growth rates (μ_{set}=0.1 h^{-1} and μ_{set} =0.5 h^{-1}) on the physiology of *E. coli* during GFP production was analysed. The recombinant protein production was chosen as this represents a typical perturbation experiment resulting in exit from the SSGS. The GFP specific productivity during the induced phase was compared in reference chemostat cultivations in single reactors and in the MD system. In the reference experiments the culture was stabilised in chemostat after which recombinant protein production was induced and the effect on physiology monitored. In the MD system the culture was stabilised in the mother-reactor followed by transferring the culture in quasi-steady state into the daughter-reactor for the induction of recombinant protein production. The perturbation experiment can be started with the physiological state established in the mother-reactor only if transferring culture between the reactors of the system does not change its physiological state. The unaltered physiology during the specific phases of cultivation in the MD system (the volume increase µ-stat and the culture transfer) was validated statistically for *E. coli* K12 in a previous study, and was thus not detailed herein (Erm, Adamberg, and Vilu 2014).

Prior the induction the bacterial growth followed pre-set profile. However, post induction at higher feeding rate the measured growth rate decreased during the induced period (five hours) practically linearly from 0.5 h^{-1} to 0.035 h^{-1}, with acetate detected in the medium three hours after induction (Fig. 3 a). The high feeding rate combined with decreasing growth rate resulted in increased glucose flux per biomass, leading to the onset of overflow metabolism. The decrease in growth rate is most likely due to the metabolic burden introduced by the recombinant protein production (Carneiro, Ferreira, and Rocha 2013). Initially higher specific and volumetric productivity at higher feeding rate was observed (Fig. 3 b and c), which can be explained by increased translational power, e. g. it has been shown that cells at growth rate 0.5 h^{-1} contained ~500 % the ribosomes of cells at growth rate 0.1 h^{-1} (Sandén et al. 2003). By the last time point post induction the cumulative impact of stress from recombinant protein and acetate resulted in decrease in specific productivity of the GFP (**Error!**

Reference source not found.3 b). The aforementioned effect is seen also in the volumetric productivity of GFP at higher feeding rate, as it decreases 25 % in the last hour post induction[1] (Fig. 3 c). Interestingly GFP production at higher growth and feeding rate resulted in ~25 – 40 % higher fluorescence per gram of biomass rather constantly throughout the experiment (data not shown).

The lower feeding rate did not result in overflow metabolism, and the specific growth rate of bacteria followed the pre-set growth profile (Fig 3). As a result the volumetric productivity of GFP at lower feeding rate increased near linearly throughout the induction phase.

Fig 3

The data showed that the specific productivity of GFP during production phase was well comparable in experiments conducted in the reference and MD systems at both growth rates, with the correlation coefficients being 0.99 and 0.97 for feeding rates 0.5 h^{-1} and 0.1 h^{-1} respectively (Supplementary Table 1 – 2, Supplementary Figure 1 – 2).

Theoretical Time-Efficiency Analysis of Perturbations in Steady-state Chemostat Conditions

30 Different Theoretical Perturbation Experiments at Fixed D=0.2 h^{-1} (see Table 1 for details)

The simplest case in steady-state perturbation analysis is to introduce the perturbation in chemostat after stabilisation; and as the perturbation disrupts the physiological state the culture must be discarded after the results of perturbations have been obtained in order to study another perturbation. This case was analysed by modelling cultivations conducted in reference and MD systems in case of 30 different theoretical perturbation experiments (such as 30 different IPTG concentrations) started from single physiological state obtained in chemostat (D=0.2 h^{-1}) (see Table 1 for details). It is noted that this is not growth space scanning as the physiological state in the mother-reactor is not changed, rather it is an example of detailed analysis of single position of SSGS.

The need for a new experiment following each perturbation renders the reference chemostat system inefficient, as the calculations show that the MD system was ~300 % faster when 30 perturbation experiments were undertaken. At 12 perturbation experiments the time-efficiency of the MD system was ~200 %, thus substantial gain is achieved already at low number of perturbations. As the number of perturbations analysed was increased the time-efficiency of the MD system increased asymptotically to 400 % due to the mathematical nature of the calculus (Fig. 4 a, Supplementary Tables 3 – 8, Supplementary Figure 3).

[1] The decrease in QGFP is from 95949 to 45085 (FU h-1 L-1; FU – fluorescence units).

Theoretically in the MD system the number of transfers and following experiments is not limited, thus the transfer-analyse sequence can be run infinitely thereby increasing productivity. In practice one limiting factor in carrying out any continuous cultivation is the appearance of mutants (Ferenci 2008). The occurrence of mutations can be validated by returning the culture to initial growth conditions and checking for altered phenotype or by comparing DNA sequences in the beginning and end of cultivations but needless to say that these would make experiments much more complicated and more time-consuming. These options were not analysed in the present paper.

Theoretical Time-Efficiency Analysis of Perturbations at Ten Different Growth Rates (see *Table 1* for details)

The effect of growth rate is important in bioprocess optimisation, for instance it is known to have an effect on the recombinant protein productivity (Sandén et al. 2003). The effect of growth rate on productivity can be studied in chemostat or in A-stat cultivations by inducing recombinant protein production at different growth rates. In the calculations the effect of single perturbation (e.g. single IPTG concentration) was analysed in a two reactor system in both reference and MD systems (details in Table 1).

While chemostat cultivations provide discrete results (one stabilisation phase required for one physiological state) changestat cultivations provide many more quasi-steady state physiological states, including the ones, which were assumed to be detailed in the perturbation analysis in the reference chemostat (Fig. 1 a and b). In the calculated example in the MD system two quasi-steady state cultivations were supposed to be taking place simultaneously in the mother-reactor (growth rate and culture volume increased in parallel), and culture was assumed to be transferred into the daughter-reactor for perturbation experiments at interval of 0.1 h^{-1}. At higher dilution rates than 0.6 h^{-1} it was more reasonable to stabilize the culture in chemostat for five volumes instead of increasing dilution rate in A-stat as the acceleration period exceeded the stabilisation period (Fig. 2 a).

When the experiment time in the MD set-up was compared to the experiment time of reference chemostats the MD method provided data 114 % faster[2]. Assumption of increased number of experiments led to increased efficiency – with five different perturbations analysed at each growth rate studied, the MD method was 174 % faster[3]. When the number of bioreactors was assumed to change to six (one mother-reactor + five daughter-reactors) the MD system was 190 % more effective[4] (Supplementary Tables 9 – 13). The calculation is correct as long as μ-stat cultivation to increase culture volume for transfer does not introduce extra cultivation time, this

[2] 135 h / 154 h, experiment time in MD system / experiment time in traditional system, respectively.
[3] 444 h / 773 h, experiment time in MD system / experiment time in traditional system, respectively.
[4] 135 h / 256 h, experiment time in MD system / experiment time in traditional system, respectively.

is realized when mL scale bioreactors are used as daughter-reactors. These results showed that the MD system was efficient in coupling growth space scanning with perturbation analysis especially if small-scale bioreactors operating in mL scale are used as the daughter-reactors.

Fig 4

Theoretical Time-Efficiency Analysis of Perturbations at Ten Growth Rates at Eleven pH values, Altogether 110 Perturbation Experiments (see Table 1 for details)

Optimisation of recombinant protein production involves studying the effect of many parameter combinations on productivity as pre- and post-induction conditions can be modified extensively (e. g. varying growth and feeding rate, IPTG concentration, T, pH, C/N ratio, substrates, etc.) (F.Yang 1996; Shin et al. 1997). The physiological states at different combinations of environmental parameters are best achieved in chemostat or changestat experiments. Productivity can be analysed by inducing recombinant protein production in these different conditions. In the MD system near infinite number of pre-induction physiological states can be obtained by combining changestat cultivations in the mother- and daughter-reactors, as illustrated in Fig. 1 d. In this set-up the productivity analysing perturbation experiments starting from the physiological state established in the mother-reactors would be conducted in the last layer bioreactors (Fig. 2 b). Additionally, as cultivations in the perturbation reactors are conducted autonomously from the changestats, the post-expression conditions can also be varied.

In the theoretical example the combined effects of ten growth rates each at 11 pH values on recombinant protein productivity at fixed IPTG concentration were analysed in reference chemostat and MD system (details in Table 1). It is emphasised that in the MD system both the growth rate and pH were changed continuously and smoothly in quasi-steady state thus providing cultivation data at much higher density than chemostat, as illustrated in Fig. 5.

To cover the analysed growth space only a single experiment was required in the mother-reactor, 20 experiments in primary daughter-reactors and 110 experiments in the secondary daughter-reactors (see Table 1 for details). This results in time saving, as cultivation in the quasi-steady state in MD system provides the data ~166 % faster[5] (calculations in Supplementary Table 14 – 17). Upon increasing the number of IPTG concentrations analysed in the five reactor set-up at each growth rate and pH combination the efficiency nears ~220 %, due to the mathematical nature of the calculus (Supplementary Table 14 - 17, Supplementary Figure 4). Typically recombinant protein production in *E. coli* is realised at lower growth rates to avoid overflow metabolism, thus

[5] 378 h / 629 h, experiment time in MD system / experiment time in traditional system, respectively.

the MD system should further increase in time-efficiency, as at lower growth rate the total length of stabilisation must last longer in traditional chemostat experiments.

Fig 5

The Effect of MD System Specific Parameters on Time-Efficiency

The effect of number of bioreactors on total experiment time was evaluated in 30 theoretical perturbation experiments (details in Table 2, Supplementary Table 18 - 20). It is evident that the time efficiency of the MD system in comparison with traditional system is larger when the number of reactors in comparison with number of experiments conducted is small. The reason is that running 30 perturbation experiments in a small number of reactors in the traditional set-up will lead to the need to start stabilisation phase on several occasions, while the stabilisation is conducted only once in the MD system regardless of the number of perturbation experiments made. As the number of reactors used approaches the number of experiments conducted the time efficiency of the MD system decreases, and if the number of bioreactors matches the number of perturbations analysed, the traditional system will become more time-efficient than the MD system (in the MD system extra experiment time will be added due to the need to make one extra culture transfer, as only 29 reactors are available for the 30 perturbation experiments (in the mother-reactor a perturbation was never assumed), while in the traditional set-up the 30 experiments can be completed simultaneously).

Table 2. Experiment time in case of 30 theoretical perturbation experiments with increasing number of bioreactors, conducted in traditional chemostat and MD systems, both at D=0.2 h^{-1}. The MD and traditional systems always contained the same number of bioreactors. The preparatory phases were supposed to consist of batch (ten hours) and stabilisation (25 h). In the traditional system perturbation was applied after stabilisation, the effect of perurbation was monitored for five hours. In order to study next perturbation a new chemostat experiment was assumed to be required. In the MD system perturbation analysis for five hours was supposed to take place in the daughter-reactors, with culture discarded once the analysis was complete, and replaced with quasi-steady state culture from the mother-reactor. The mother-reactor was reserved for maintaining the physiological state of culture and perturbation was never applied there.

Nr of reactors	Total experiment time in reference system (h)	Total experiment time in MD system (h)	Time-efficiency of the MD system (%)	Batch / stabilisation length in MD system (h)	Batch / stabilisation length in reference system (h)	Perturbation analysis length in MD system (h)	Perturbation analysis length in reference system (h)	Variable volume phase length in MD system (h)
2	628	243	258	10 / 25	150 / 375	150	75	3
3	418	138	303	10 / 25	100 / 250	75	50	5
10	124	61	203	10 / 25	30 / 75	20	15	9
20	82	47	174	10 / 25	20 / 50	10	10	15
25	82	47	174	10 / 25	20 / 50	10	10	16
30	40	47	85	10 / 25	10 / 25	10	5	17

The effect of perturbation experiment's length was evaluated in the most time-efficient case of **Error! Reference source not found.** in the example with three bioreactors (one mother and two daughter-reactors). The efficiency of the MD system decreases as the perturbation experiment's length increases (Fig. 4 a). The MD system results in time efficiency by decreasing the total time needed for culture stabilisation. Therefore, everything that results in reducing the proportion of the stabilisation phase in comparison with total experiment time (such as increasing the perturbation monitoring time) must reduce the time efficiency of the MD system.

While each case should be considered separately based on the growth rate applied and molecular effects expected, lengthy experiments in the daughter-reactors are typically not needed as the physiological response develops usually within the first two residence times after perturbation. For instance, if the effect by the perturbation totally halts microbial growth, then in chemostat the result will be exponential decrease of biomass concentration to 63 % and 86 % of the initial value by the end of residence times 1 and 2, respectively. The response on the level of metabolome or transcriptome has been shown to be rapid; even expression, synthesis and insertion of specific transporters has been reported to take place within the first few residence times (Kresnowati et al., 2008; Lara et al., 2009; Soini et al., 2005).

The next parameter affecting MD system's time-efficiency in comparison to the traditional system is the dilution rate at which perturbations are applied. A low dilution rate will increase the length of stabilisation period, rendering MD method more efficient.

Another specific characteristic of the MD method is the added process time from the mandatory volume increase phase, necessary for providing the biomass to fill the daughter-reactors. In an ideal case this phase is completed during stabilization phase or during perturbation experiment. From Table 2 it is seen that theoretically increasing volume three fold will not affect total process time as it can be completed while perturbation analysis is conducted in the daughter units. Two culture transfers to daughter-reactors of equal size to the mother-reactor can therefore be made without introducing extra experiment time. However, if small scale minireactors would be used as daughter-reactors the number of experiments can be increased without extra time.

Conclusions

Selected applications of a cultivation system (MD system) allowing to start perturbation experiments from quasi-steady state with no stabilisation were described. A proof of concept analysis of GFP production in the MD set-up showed that the method can be used for recombinant protein productivity analysis, as good correlation of specific GFP productivity during the production period between the MD system and reference chemostat in single reactors was observed. Based on theoretical calculations the MD system provides effective means for perturbation analysis, decreasing experiment time several fold even with relatively low number of experiments and a few bioreactors. Furthermore, the MD setup allowed to conveniently combine different quasi-steady state cultivation methods for scanning the growth space of micro-organisms. It is emphasised that the MD system is not limited to perturbation analysis as starting experiments from quasi-steady state allows for instance to conduct tracer analysis or substrate pulse analysis equally well.

The MD system is efficient when the experiments are planned and carried out carefully, in accordance with the QbD initiative. In the theoretical examples of current work the time-efficiency of MD system was up to 400 % higher in comparison with standard chemostat systems, but more effective cultivation scenarios can the devised, for instance by adding a layer of microfluidics reactors. However, it should be kept in mind that the culture transfer logistics will therefore be rendered more complex as-well. Still, as the MD system allows convenient and efficient growth space scanning and productivity analysis, further development should be directed to automatization of the system, which would enable to run automated experiments within several weeks while keeping physiological state of cells according to pre-set state and requiring minimal manual intervention.

Supplementary Material

Supplementary Tables 1-2, Supplementary Figure 1-2

Supplementary Tables 3-5

Supplementary Tables 6-11, Supplementary Figure 3

Supplementary Tables 12

Supplementary Tables 13-17

Supplementary Tables 18-21, Supplementary Figure 4

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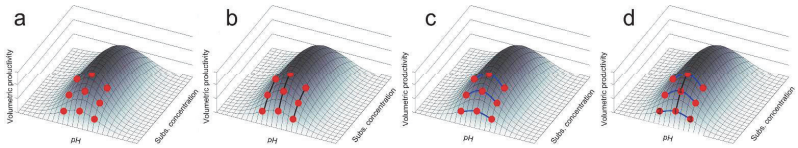

Possible cultivation scenarios to analyse the SSGS of cells. a – Nine chemostat experiments with inherent stabilisation. b – SSGS scanning in quasi-steady state in A-stat cultivation experiments – the same nine states from Fig. 1 a are studied among the others on the trajectories shown. c – SSGS scanning in three quasi-steady state D-stat cultivations with pH change. d – SSGS scanning in two directions in quasi-steady state A-stat and D-stat cultivations in the MD system

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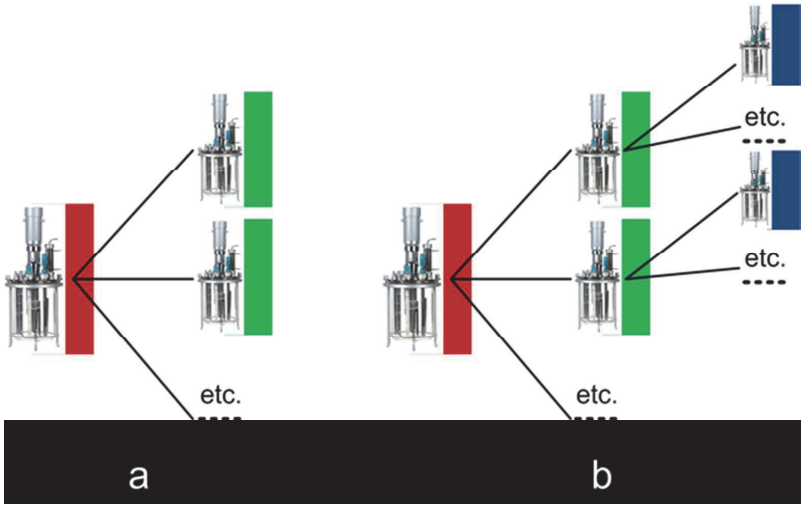

a – MD system operated in two layers of bioreactors, layer one (mother-reactor) is depicted by red, layer two (daughter-reactor) by green. Quasi-steady state culture was assumed periodically transferred from the mother-reactor to the daughter-reactors for the perturbation experiments with no stabilisation period. In the mother-reactor changestat cultivations were used to reach different physiological states (scanning of growth space). Fig. 2 b – The process is similar to the one described in Fig. 2 a, but a changestat cultivation in layer two bioreactor was used to move in quasi-steady state growth space into a different direction than in the layer one mother-reactor. The third layer of bioreactors (blue) was reserved for perturbation analysis. By repeating transfers between layers one and two, and between layers two and three, a comprehensive growth space scanning with perturbation analysis could theoretically be realised

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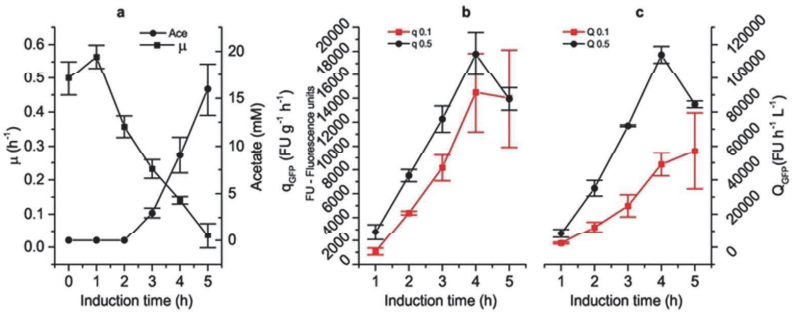
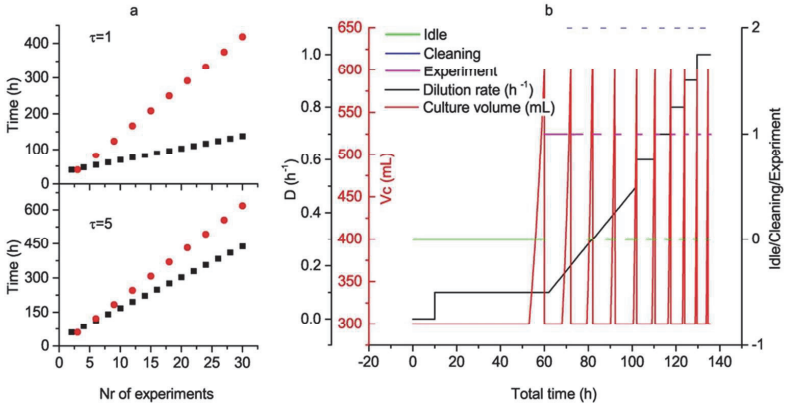


Bioprocess descriptors during the production phase. The error bars represent standard deviation for three experiments, two conducted in the MD system and one started from traditional chemostat. a – Specific growth rate and acetate concentration in higher (D=0.5 h-1) feeding rate experiment. b – GFP specific productivity (determined as fluorescence) at high and low (D=0.1 h-1) feeding rate experiments. c - Volumetric productivity of GFP at high and low feeding rate experiments

60x24mm (300 x 300 DPI)



a – The perturbation time length in the daughter-reactor (denoted as residence time, τ) and the number of perturbation experiments determines the time efficiency of the MD system. Red circles denote theoretical experiments in traditional, black squares in MD system. In both systems the perturbation experiment was supposed to last for either one or five residence times, after which the culture was discarded. In the traditional system a new experiment was needed to study the next perturbation while in the MD system perturbation analysis in the daughter-reactor could be started right after culture transfer from the mother-reactor. b – Coupling growth rate scanning to perturbation analysis in a two reactor system. Dilution rate (equals growth rate) was theoretically varied in A-stat cultivation in the mother-reactor (acceleration rate a=0.01 h-2). Culture transfers, depicted by rapid drop in culture volume (Vc), from the mother-reactor into the daughter-reactor was assumed at ten dilutions rates, from D=0.1 h-1 to 1 h-1, with step 0.1 h-1 (D=0.1 h-1, 0.2 h-1, 0.3 h-1, etc.). Following each transfer, a perturbation was supposed in the daughter-reactor, while in the mother-reactor the dilution rate was increased to the next set-point value in quasi-steady state. The analysis of perturbation lasted one residence time, after which the culture in the daughter-reactor was discarded and the reactor re-set for next transfer. Subsequent transfers and perturbations followed for all analysed growth rates. When the time to reach next dilution rate in A-stat cultivation exceeded the time necessary to stabilize culture for five residence times in chemostat, the new dilution rate was obtained in chemostat. Idle, cleaning or experiment phase in the daughter-reactor is depicted

80x42mm (300 x 300 DPI)

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