TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

D I P L O M A R B E I T

On-line Monitoring of mammalian Cell Cultures for the real-time Extraction of physiological Information

Ausgeführt am Institut für

Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften
der Technischen Universität Wien

unter der Anleitung von Univ.Prof. Dipl.-Ing. Dr.techn. Christoph Herwig und Dipl.-Ing. Dr.techn. Christian Dietzsch

durch

Melanie Feichter, BSc

Mohsgasse 27/ 37, 1030 Wien

07.04.2014 _____

ABSTRACT

Within the last years, more and more attention has been paid to the development of on-line monitoring process devices. Particularly, taking into account that the Food and Drug Administration (FDA) launched the Process Analytical Technology (PAT) guidance, which encouraged biopharmaceutical companies to ensure a pre-defined final product quality. On-line sensors are a great opportunity to gather process understanding and to enhance process development. In course of this thesis the usefulness of on-line and in-line measurements to deduct physiological key parameters and accelerate bioprocess development is evaluated using different human embryonic kidney (HEK) cell clones.

On the one hand an on-line photometric measurement system for the quantification of amino acids and carbohydrates was used. In order to make it useable for cell culture systems it was necessary to meet the high sterility requirements and to adopt the technique to a completely different physiological system. To characterise the system the accuracy and precisions was proven in repeated measurements of standard solutions. The robustness was tested by applying the approach on different cell clones. Furthermore, analytical parameters such as the Limit of Detection, the Limit of Quantitation, the linear working range and the precision and accuracy were determined in the run- up using the device in off-line mode. On the other hand an in-line capacitance probe for the calculation of the viable cell count was implemented. To find a suitable calibration for the in-line device a novel approach based on Multiple Linear Regression was used. These two individual measurements were tied together in order to get a full picture of the bioprocess. The on- line data were transferred into rates and yields, which is the scale independent and time resolved form of information.

In other words, two on-line monitoring devices were coupled and a novel device was created to gather real-time information of mammalian cell cultures. The high frequent determination of physiological data allows to image the dynamic of the culture precisely. Process events, like accumulations and limitation can be detected in real-time and moreover physiological considerations could be drawn using specific rates and yields, which allowed a deeper insight in the metabolism of different cell clones and enhances the process of strain selection and therefore supports and fastens bioprocess development.

Prospectively, this approach may be used not only to detected limitations in- time but furthermore it will provide the basis for intelligent controlling strategies.

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ABBREVIATIONS

4-AA	4-aminoantipyrine
4-AAP	4-amniophenazone
Ad5	human adenovirus 5
ATP	adenosintriphoshate
EHSPT	N-ethyl-N-(2(hydroxyl-3-sulfo-propyl)-3-methyaniline
FDA	Food and Drug Administration
FFU	Florescence Forming Unit
FIA	flow injection analysis
GC-MS	gas chromatography- mass spectrometry
GP	glycoprotein
HBA	4-hydroxybenzoic acid
HEK cells	human embryonic kidney cells
HPLC	high pressure liquid chromatography
IgG	Immunoglobulin G
LCMV	lymphocytic choriomeningitis
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantitation
MFA	metabolic flux analysis
MOI	Multiplicity of Infection
PAT	process analytical technology
PCR	polymerase chain reaction
PID	Proportional-Integral-Differential
POD	peroxidase
rpm	rounds per minute
SNR	signal-to-noise ratio
SOP	Standard Operating Procedure
STR	Stirrer; in this context used to number the cultivations
TCA	tricarboxylic acid cycle
TOOS	N-ethyl-N-(2hydroxy-3-sulphopropyl)-m-toluidine
VIF	Variance Inflation Factor

Symbols

C_m	specific membrane capacitance	$[F \cdot m^{-2}]$
f_c	characteristic frequency	[Hz]
P	viable cell volume fraction (biovolume)	dimensionless
q_{ci}	conversion rate per cells	[g/cells/h]
r	variable cell radius	[m]
r_{ci}	conversion rate	[g/h]

r_k^2	the goodness of fit of the linear model	
s	standard deviation	
$\bar{x}$	arithmetic mean	

Greek Symbols

Δε	maximal capacitance difference	[F·m^{-1}]
σ_c	conductivity of the cytoplasm/intracellular conductivity	[mS cm^{-1}]
σ_m	conductivity of the medium	[mS cm^{-1}]

1. INTRODUCTION

Starting in the mid- 50s with the work of Earle *et al.* (1954) mammalian cell culture established itself as most important and most promising source for new biopharmaceuticals, as today the majority of recombinant proteins (~70 %) is produced by mammalian cell culture and this proportion is still increasing [O'Callaghan *et al.* 2008]. The root of success of mammalians is based on the progress which has been made in gene manipulation and cell fusion leading to the possibility to produce large amounts of physiologically active proteins. Due to their easy and cheap cultivation *Escherichia Coli* cells were first used as hosts but they fail to carry out posttranslational modifications and therefore the products lack biological activity. Yeasts may be able to perform such posttranslational modifications, but they, however, differ significantly from human ones. In contrast to that animal cell hosts are able to perform human post-translational modification as well as protein folding in an authentic manner. There are three cell types which are predominately used. Most important the Chinese hamster ovary (CHO) cell line. Furthermore baby hamster kidney (BHK) cells and human embryonic kidney 293 (HEK293) cells, which were also used in course of this work. The selection of the expression system is determined by its ability to deliver high productivity with acceptable product quality attributes and the preferences of individual companies, which is often influenced by their historical experiences [Li *et al.* 2010]. But with increasing demands on product quality, cost and time efficiency as well as product yields and manufacturing capacity there is less and less space for historical experiences. Today bioprocess development (bpd) pursuing goals like transferability, scalability and process optimization is a key aspect of bioproduction. Intensive development work in cell line, media and bioreactor condition optimization has already led to cell specific productivities of over 20 pg/cell/day [Wurm 2004]. A crucial role in the enhancement of specific productivity per cell represents the selection of highly productive clones. Today, high titers and large capacity has shifted the focus of bioprocess development from achieving even higher titers to controlling product quality and reaching process consistency at all development stages and production scales [Kelley 2009]. In other words in bioprocess development it is important to understand the main reactions that are occurring at various stages throughout the course of a batch and it is necessary to determine the time points at which reaction pathways are affected by certain limitations or excessive accumulation of nutrients. The following approach uses in-line and on-line sensors to gather the required information and therefore applies itself as an essential tool in bioprocess development.

1.1. Human embryonic kidney cells and viral vector

Since their generation more than 30 years ago, human embryonic kidney (HEK) cells are intensively used in the development of viral vaccines, anticancer agents and the production of recombinant adenoviral vectors. In this thesis the cell line HEK293 was used. Exposure to sheared fragments of human adenovirus type 5 (Ad5) DNA generated this expression system. In course of this transformation an Ad5 was incorporated into chromosome 19 of the host genome, a modification used subsequently for the generation of recombinant human adenoviral vectors [Thomas *et al*. 2005]. HEK293F-GP additionally expresses the surface glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) to complement the packing of the LCMV vectors produced in this cell line.

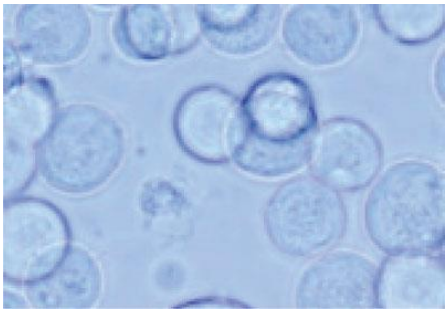


Figure 1 HEK cells [sigmaaldrich.com 2014]

Advantages of HEK293 cells are in general high transfection yields with most gene transfer vehicles, simple adaptation to growth in suspension culture, and adaptation to serum free-media [Graham 1987].

In human embryonic kidney cultures amino acids and carbohydrates are the major substrates for cell metabolism.

Metabolism of HEK cells in cell culture is characterized by high glucose and glutamine uptake rates combined with high rates of ammonium and lactate production [Neermann *et al*. 1996; Schneider *et al.* 1996]. Glucose and glutamine occupy a special position as the cells rely on them to obtaining energy necessary for growth. Nevertheless, the two metabolic by-products, ammonia and lactate, are well-known inhibitors of cell growth and protein production, and can negatively affect the glycosylation pattern of protein products [Yang 2000].

For the utilization of glucose mammalian cells use glycolysis, TCA cycle and oxidative phosphorylation to generate energy, yielding up to 36 mol ATP per mol glucose.

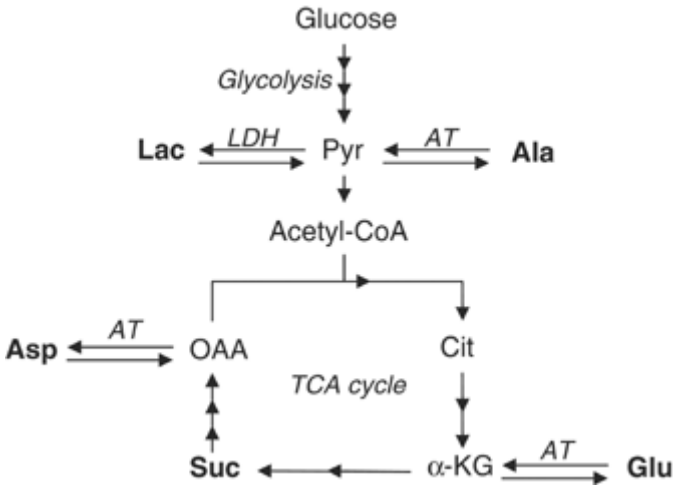


***Figure 2 Scheme of the glucose metabolism**.*
Ala, alanine; Asp, aspartate; AT, aminotransferase; Cit, citrate; Glu, glutamate; α-KG, α-ketogluterate; Lac, lactate; LDH, lactate dehydrogenase; OAA, oxaloacetate; Pyr, pyruvate; Suc, succinate; TCA, tricarboxylic acid [Bak et al. 2006]

The glycolytic pathway is extremely ancient in evolution, and is common to essentially all living organisms. Glucose which is a six-carbon sugar is oxidized and split in two halves to create two molecules of pyruvate (3 carbons each). Doing so the cell obtains energy in the form of ATP. 2 ATP molecules are extracted from each glucose molecule. Pyruvate, which is produced, can now be either metabolised to acetyl-CoA or lactate. If oxygen is available, pyruvate may be converted to acetyl-CoA and enter the Krebs Cycle. Within oxidative phosphorylation acetyl-CoA is completely oxidized generating ATP [biocarta.com 2014]. But at oxygen absence the carbon flow at the pyruvate-branch point can also be diverted to lactate production, since the conversion of glucose to lactate is redox neutral, i.e., there is no net production or consumption of NAD(P)H. Nevertheless, fermentation is less efficient than oxidative phosphorylation in producing ATP. Most mammalian production cells lines walk along the lactate pathway regardless of the level of oxygen supply. This effect is known as "Warburg Effect" [Warburg 1956], or aerobic glycolysis. In addition to a loss of energy, aerobic glycolysis has also another disadvantage, as it is reported that high levels of accumulated lactate can inhibit cell growth and product formation [Lao 1997]. There exist numerous studies which explain this lactate inhibition with a related acidification of the medium and elevated osmolality [Ozturk 1992]. To reduce lactate formation, glucose-limiting feeding strategies have been employed [Ljunggren *et al*. 1994; Europa *et al*. 2000]. To control of the glucose concentration reliable on-line sensors are a necessary prerequisite.

Besides glucose glutamine plays a major role in the metabolism of mammalian cell cultures. Unlike glycolysis, glutaminolysis is not a single, complete metabolic pathway, but forms a network of eight, partly interconnected, alternative metabolic pathways at which glutamine can be oxidised to different degrees and which therefore result in different energy yields and product combinations (**Figure 3**). Glutamine contains two nitrogen atoms, whereby one is present in the form of an amide and one as an amine. Therefore glutamine is crucial for the nitrogen supply and moreover for the synthesis of proteins, amino acids, purine and pyrimidine and thus nucleic acids and nucleotides. As it could be seen in the metabolic scheme in **Figure 3** glutamine is a precursor of glutamate by the action of the enzyme glutaminase. Glutamate is a key amino acid used for the transamination of α-ketogluterate to form other α-amino acids or it can lose the amino group, as NH_4^+, via deamination to 2-oxoglutarat. If glucose levels are low, the cells start to metabolize amino acids to gain energy. Glutamine, as one of the easiest available ones, is especially used by rapidly dividing cell types.

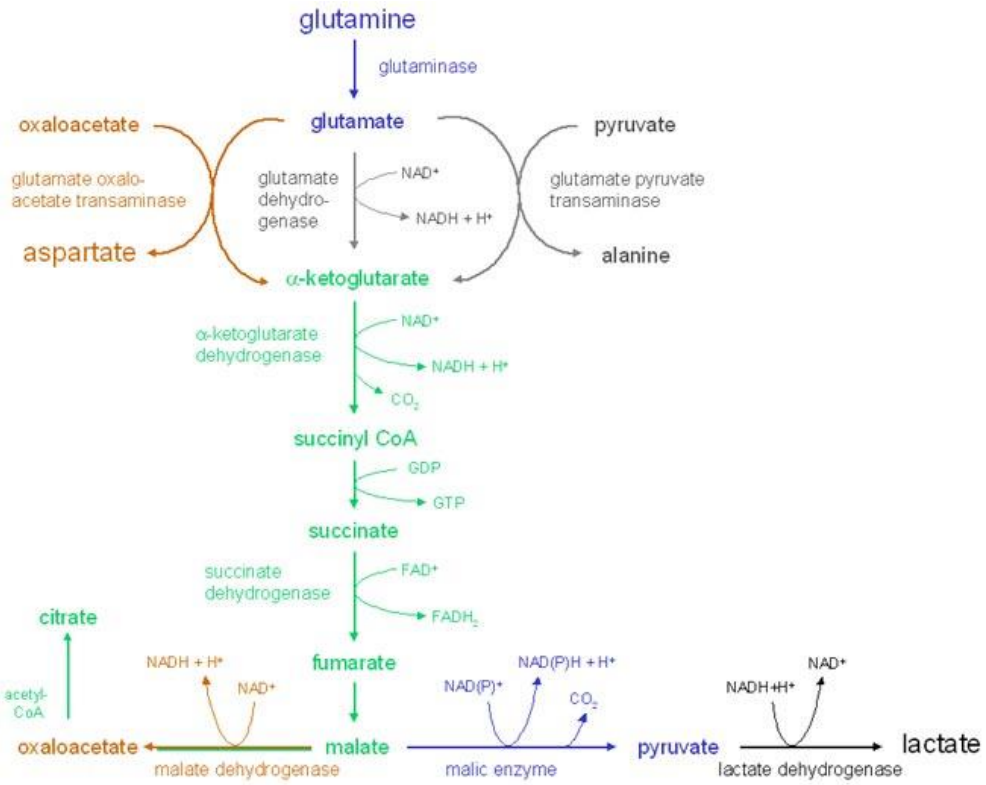


Figure 3 A metabolic scheme of the glutaminolysis.

green, reaction steps of the citrate cycle; brown, reaction steps of the malate aspartate shuttle; blue, enzymes overexpressed in tumor cells; [metabolic-database.com 2014]

Nevertheless, glutamine is unstable and by the chemical decomposition ammonia is released which acts as an inhibitor of cells growth and protein production. Furthermore ammonia is also a produced by deamination of glutamine to glutamate and by the conversion of glutamate to α-ketoglutarate.

Sun *et al.* (2001) showed that the metabolic pathways were altered in cultures with higher ammonia concentrations. Furthermore glucose was more likely converted to lactate by anaerobic metabolism and the reaction of glutamate to α-ketoglutarate was inhibited.

All these facts indicate that a glutamine, glutamate and ammonia have to be monitored and ideally controlled in real time.

The viral vector used in this study was a genetically engineered LCMV vector in which the GP surface protein coding region could be replaced by any gene of interest. Due to deletion of the GP gene, these vectors are replication deficient in any cell substrate except the complementing cell line provided by the sponsor.

1.2. On-line monitoring of the cultivation process

The robust implementation of mammalian cell culture requires not only the genetic and molecular biological optimization of cell lines, culture media and bioreactor culture conditions but also a good understanding of culture performance at different scales. Unexpected variations have a huge impact on the process efficiency and should therefore be monitored and controlled tightly to decrease the costs and increase the product yield but most important to guarantee that the final product meets certain quality attributes. These requirements are not easily fulfilled due to the non-linear behaviour of the cells and a complex metabolic network. Therefore appropriate on-line and off-line sensors capable of providing information in order to enhance process control as well as process characterisation are needed for successful operation [Li *et al.* 2010].

1.2.1 Metabolic compounds

Glucose, lactate, glutamine, glutamate as well as ammonia are key compounds in the metabolism of mammalian cells and therefore real time monitoring of these substances is crucial not only for process understanding but moreover to implement adequate controlling strategies and to obtain a defined and stable process. Hence, different on-line and off-line sensors have successfully been implemented.

One example for such an off-line device is high pressure liquid chromatography (HPLC), using an anion exchange column [Niklas 2010]. Another possibility for off-line determination of glucose and amino acids is gas chromatograph coupled with mass spectrometry (Suk 2010). Ahn *et al.* (2011) used isotopic tracers and gas chromatography-mass spectrometry (GC-MS) for the measurement of labelled glucose to study intracellular fluxes. The aim of not only Ahn *et al.* but most off-line approaches is, first and foremost, to carry out metabolic flux analysis (MFA), considering dynamic changes in growth and metabolism during cultivation [Niklas 2010]. Although MFA works well for microorganisms, it is quite challenging to realise it for mammalian cells, because of compartmentalisation, varying utilisation of multiple substrates, reversibility of metabolite uptake and production, unbalanced growth behaviour and adaptation of cells to changing environment during cultivation [Niklas 2012].

Even though off-line measurements have their justification, today it becomes more and more important for biopharmaceutical companies to ensure a pre-defined final product quality, which can only be reached with the implementation of suitable on-line monitoring devices. In animal cell cultures, metabolite concentration (and also other variables e.g. viable cell count) are normally measured once or twice a day. Derived quantities like substrate consumption and metabolite production rate can only be calculated when a sample is taken. Hence, the informative output of such off-line state variables is rather low during cultivation and as a consequence, off-line sensors may miss variations in the process which are time and cost effective. Advanced on-line monitoring can help to assure process performance and quality during the running process. It can support the operator to take decisions to keep the process within specifications with respect to the design space, because it enables real-time interference which is crucial for optimal and consistent production of biologicals. Robust methods allow faster process characterisation, enhanced quality control and higher productivity.

In-line monitoring devices based on spectroscopic methods, e.g. near infrared, mid- infrared and RAMAN, offer the advantage of simultaneous and high frequency measurement of multiple components without constant withdrawal of on-line samples. Especially small scale reactor benefit from these techniques and furthermore a potential sterility hazard can be avoid. However, the use of spectroscopic methods typically requires the use of chemometric tools for the establishment of robust calibration models and infrared spectroscopy suffers significantly from possible interferences of background compounds [Boudreau and Benton 2008; Vojinovic *et al.* 2006].

On-line analytical devices such as HPLC [Larson 2002] or biosensors, e.g. via Flow Injection Analysis (FIA) [Mulchandani 1996] were successfully applied to cell culture systems, they offer also a few drawbacks. First and foremost HPLC methods are difficult to establish, because in in mammalian cell culture complex media are used which lead to various matrix effects. Furthermore buffer solutions have to be used for maintenance of the sensor units of membrane-based bioanalysers, which causes

not only high costs but also quite laborious handling. Furthermore the limited long term stability of the sensor unit itself presents a major disadvantage [Dietzsch *et al*. 2013]. For the measurement of metabolites and substrates Ozturk *et al*. (1997) reported the on-line implementation of a membrane dependent biochemistry analyser. The disadvantage of membrane processes, in particular when operated in real time, is obviously that they can be saddled with major problems of membrane fouling. Ozturk *et al.* monitored glucose and lactate on-line in a perfusion reactor and the generated data were used to adjust feed rates to maintain their concentrations at desired set points. This approach offers a huge optimization potential regarding to the usage of a membrane- free biochemistry analyser and moreover the number of measured molecules. Dietzsch *et al*. (2013) implemented such a membrane-free, fully automated robot (CuBiAn XC, Innovatis), based on a photometric measurement principle. This on-line device was connected to a bioreactor measuring six compounds (glucose, ethanol, glycerol, acetate, phosphate and ammonium) during different batch cultivations of *Pichia Pastoris*. Within their study Dietzsch *et al*. were able to achieve a good correlation between on-line and off-line concentrations and based on off-line determined biomass data specific rates and yields were calculated giving an inside in the physiological state of the cells throughout the whole cultivation. In course of this thesis the approach is applied on different human embryonic kidney cell clones and coupled with an in-line probe for the determination of the viable cell count.

1.2.2 Viable cell count

On-line measurement can be applied to generated information about metabolic compounds in real time. Therefore the on-line determination of the viable cell density is an essential prerequisite, as it is necessary to evaluate specific rates and yields, which are crucial to extract process knowledge and information from experimental data to reach process understanding. Consequently, biomass measurement is one of the most critical measurements in biotechnological processes. The probes should not only be reliable, precise at high and low cell densities, should show a linear dependency and the possibility of calibration, but they should also be sterilizable, endure temperature and pressure, be corrosion stable and biological inactive [Kiviharju 2008]. Currently available on-line measurement instruments are based on turbidity or optical density, furthermore in-situ microscopy and soft sensors have already been successfully applied. In-situ microscopy, which developed from an off-line into an on-line technology, has already been termed by Bittner *et al.* (1998) and Suhr *et al.* (1995 and 1997). Moreover, Joeris *et al.* (2002) described the potential of this technique to be established as routine tool for the first time. A high-sensitivity laser turbidity probe was used by Konstantinov *et al*. (1992) for on-line monitoring of the cell concentration in batch hybridoma

cultivations. However, all these light-based probes did not distinguish between viable and nonviable cells.

Dorresteijn *et al.* (1996) determined the biomass activity in animal cell cultures from calculated ATP-production rates which was based on on-line measured oxygen uptake and lactic acid production rates. Naciri *et al.* (2008) established a monitoring strategy based on direct measurements of the two major operational parameters, dissolved oxygen and pH, which provides information on both growth and cellular metabolism. Oxygen is necessary for animal cell respiration and would decrease with increasing biomass while lactic acid is given off as a by-product of cell metabolism, lowering the pH of the media [Naciri 2008]. However, mathematical models, are difficult and carry the risk of false results. In course of this thesis dielectric spectroscopy (Fogale BIOMASS SYSTEM®, Fogale nanotech, France), which utilizes the capacitance technology to monitor the viable biovolume that can be linked with the cell density was used (Harris *et al.* 1987). Since only intact membranes should store charge, viable biomass is selectively measured, which offers a big advantage over all light based approaches. This method has already been successfully applied to microbial, animal and plant cells [Markx *et al.* 1991; Fehrenbach 1992; Cerckel *et al.* 1993; Konstantinov *et al.* 1994]. It has also been used cultivating HEK cells [Petiot *et al.* 2011]. Moreover, in 2003 Dowd *et al.* used an automatic perfusion feed rate control based on the estimation of the viable cell concentration of CHO cells using on-line dielectric spectroscopy. They analysed the dependencies of the substrate, metabolite and protein concentrations, along with uptake and production rates as a function of cell specific perfusion rates and consequently they defined those rates which lead to a maximum in recombinant protein concentrations or volumetric productivity.

Obviously, dielectric spectroscopy is a proven technique which has not only been successfully applied to most host systems but has also been used for process monitoring and control.

Nevertheless, to exploit information from dielectric spectra a reliable calibration is needed. In literature, it can be mainly read that two frequencies are used randomly. A low frequency (below 0.1 MHz; for mammalians mostly 1000 Hz), which lies in the critical frequency region and a high frequency (mostly 10 MHz), at which the permittivity corresponds essentially to the permittivity of the culture medium. By subtracting the two permittivities the permittivity of the cell suspension can be determined [Ansorge 2007; Petiot 2012]. The frequency at which the capacitance is measured depends on the organism. Typically, excitation frequencies of around 1,000 kHz are used for bacteria cells and between 500 and 600 kHz for yeasts and mammalian cells [Ducommon 2002]. These linear model is the easiest opportunity to set up a calibration but the frequencies, which are used are not adjusted to the specific cultivation system, e.g. the medium or the organism. To enhance the specificity efforts have been made towards two different methods finding a relvant calibration model between permittivity and viable cell count. First of all, such a model could be found using Cole–Cole equation

on measured spectroscopy spectra, as described by Dabros *et al.* But a disadvantage of this approach may be that that assumptions are made, e.g., the constant value of membrane capacitance (C_m) and intracellular conductivity (r_i), which may not be valid throughout the whole process, leading to deviations between the model and measured values [Parta 2014].

Secondly multidimensional data analysis and modelling, e.g. principal component analysis (PCA) and PLS modeling, is commonly used. Today this may be the preferred method as it seems to be the most accurate one in comparison to linear and Cole-Cole modelling [Dabros 2009; Opel 2010]. In course of this work another multivariate statistical technique was used to find a suitable calibration: Multiple Linear Regression (MLR) based on off-line determined data (manual cell count with a haemocytometer using the Trypan Blue exclusion method as well as automatic cell count with a Cedex HiRes (Roche, Germany)).

1.3. Physiological parameters

Cell culture systems show an inherent complexity, which makes process development more difficult and time consuming compared to microbials. Especially during batch cultivations overflow metabolism may result in a massive overproduction of metabolites which prove to be toxic and product inhibiting at high concentrations (e.g. lactate and ammonia). For the production of biopharmaceuticals and therapeutic proteins variable productivity and product quality should clearly be avoided. Moreover, product quality is a main factor influencing downstream process development. In turn, it is crucial to already aspire optimized product quality in upstream process development. Product quality attributes are inherently connected to the physiological state of the culture and consequently the reliable quantification of physiological parameters is fundamental for clone selection and the definition of optimal operating conditions. This as a first and major step towards the improvement of the final product yields. Hence, it needs distinct variables, which describe the physiological state of the culture. These variables are specific rates and yields, as they can be used to condense the available on- and in-line data into scale independent, transferable and time resolved information. Because specific rates cannot be measured directly, they have to be calculated by other means, e.g., from concentrations at discrete time points. Furthermore, these rates are used to calculate yields. A shift in yields and specific rates hints to physiological state change in the culture and provides important information on the process [Wechselberger and Herwig 2012]. The determination of those variables in real-time would even allow to draw such conclusions immediately, which does not only lead to a faster information flow but is also a first step towards intelligent process control.

1.4. Error propagation and signal-to-noise ratio

The calculation of specific rates and yields for the determination of process information is straightforward and can be carried out in real-time. But as already mentioned for their calculation multiple measurements are needed, each of them prone to random errors, drifts or even gross errors, such as sensor miscalibration and drifts. To evaluate the quality of such sensor signal the signal- to-noise ratio (SNR) is used. The SNR is a commonly used device to decide whether variations in the observed signals are physiological information or random noise. To average over a window of time, which is called moving average, is one possibility to reduce random noise and to increase the SNR. If the expected SNR is defined, it may be used to estimate the maximum temporal resolution for the detectability of dynamic changes and consequently sampling intervals could be scheduled based on this information [Wechselberger *et al.* 2013].

2. AIM OF THE THESIS

Within the last decades, more and more attention has been paid to the development of on-line monitoring process devices, not only because the Food and Drug Administration (FDA) launched the Process Analytical Technology (PAT) guidance, which encouraged biopharmaceutical companies to ensure a pre-defined final product quality, but also because of economic considerations. In course of this thesis the usefulness of on-line and inline measurements to deduct physiological key parameters and accelerate bioprocess development is evaluated using different HEK cell clones.

On the one hand an on-line monitoring device for the real time measurement of the major process parameters a fully- automated, membrane- free biochemistry analyser, based on photometric principles (CuBiAn XC, Optocell, Germany) was connected to the bioreactor set up, which has already been established for microbial processes [Dietzsch *et al.* 2013]. In order to make it useable for cell culture systems it was necessary to meet the high sterility requirements and to adopt the technique to a completely different physiological system. On the other hand an in-line capacitance probe for the calculation of the viable cell count, that has already been successfully applied to microbial, animal, plant and also HEK cells [Markx *et al.* 1991; Fehrenbach 1992; Cerckel *et al.* 1993; Konstantinov *et al.* 1994; Petiot *et al.* 2011] was implemented. To find a suitable calibration for the in-line device a novel approach based on Multiple Linear Regression was used. These two individual measurements were tied together in order to get a full picture of the bioprocess. The on- line data was transferred into rates and yields, which is the scale independent and time resolved form of information. In other words, two on-line monitoring devices were coupled and a novel device was created to gather real-time information of mammalian cell cultures.

Five different substances were measured (glucose, lactate, glutamine, glutamate and ammonia).

In repeated measurements of standard solutions the accuracy and precisions was proven. The robustness was tested by applying the approach on different cell clones, cultivated in 2 L Labfors Infors bioreactors and at the same time it has been used to study physiological variability between them to prove the usefulness of the system regarding clone selection and furthermore bioprocess development. Prospectively, the on-line monitoring device may be used to detected limitations in time and furthermore it will provide the basis for intelligent controlling strategies.

3. MATERIAL AND METHODS

3.1. Cells

Different clones of human embryonic kidney cells HEK293 were used in course of this work (**Table 1**)[1]. As already described in the theoretical part, the cells were exposed to sheared fragments of human adenovirus type 5. HEK293F-GP expresses the surface glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV).

The cell lines were grown in suspension. Thawing was performed according to standard procedures and the cells were split in a 3 to 4 days interval. They were cultured under serum-free conditions in CDM4HEK293 medium supplemented with 4 mM stable L- glutamine or 6 mM GlutaMax. Cultivation in serum-free conditions is an important factor for the production of therapeutics intended for the use in humans. The cells were grown at 36.5°C in a CO_2 incubator at 5% CO_2 and 120rpm.

Table 1 HEK cell clones used in course of the thesis.

cell clone	run	glutamine source	resistance	antibiotic
HEK293-α	STR8	4 mM GlutaMax	Puromycin	0.2 µg/ml Puromycin
HEK293-β	STR13 and 14	6 mM Glutamine	Geneticin	0.2 µg/ml Geneticin
HEK293-γ	STR16	4 mM GlutaMax	Puromycin	0.2 µg/ml Puromycin
HEK293-β	STR17	6 mM GlutaMax	Geneticin	0.2 µg/ml Geneticin

3.2. Virus

Genetically engineered LCMV vector in which the GP surface protein coding region is replaced by any gene of interest. Due to deletion of the GP gene, these vectors are replication deficient in any cell substrate except the complementing cell line. Virus stocks, with a multiplicity of infection (MOI) of 0.001, were stored at -80°C in 1 ml aliquots which were used only once upon thawing.

[1] Author's note: The names of the clones were changed, for reasons of data protection.

3.3. Media and reagents

3.3.1 Medium

In course of the cell-free runs FreeStyle™ 293 Expression Medium was used. This is an animal origin-free, chemically-defined, protein-free medium specifically developed for the ability to support the growth and transfection of 293-F (Invitrogen) cells in suspension.

For all other fermentations CDM4HEK293 (HyClone) was used. HyClone CDM4HEK293 is a chemically defined, animal component-free and protein-free cell culture medium designed to support the growth of HEK 293 cultures, and promote adenovirus and recombinant protein production.
HyClone CDM4HEK293 is a regulatory-friendly medium developed through the metabolic pathway design to support the extremely high productivity and cell density in suspension cultures.
It was either supplemented with 6mM stable L-glutamine (gibco) or with 4mM GlutaMax™ (pAA Laboratories GmbH), which is a stabilized dipeptide of L-Alanyl-L- Glutamine.

3.3.2 Trypan blue solution

Trypan blue stain (Gibco 15250-061) in 0.2 phosphorous buffered solution (PAA H12-002) was used to determine the number of living and dead cells. Living cells appear bright yellow whereas dead cells have a damaged cell membrane taking up the trypan blue stain and appear therefore blue. The procedure was performed manually using a haemocytometer or automated with Cedex Hires.

3.3.3 Cedex solutions

For automated cell count Cedex Hires was loaded with Cedex solutions by Roche. These included Cedex Detergent Solution, Cedex Trypan Blue Solution, Cedex Cleaning Solution and ultrapure water.

3.4. Methods

3.4.1 Bioreactor setup

Batch cultivations were carried out in a 3.6 L volume glass bioreactor (Infors, Switzerland) with a working volume of 2 L and multiple ports to conduced on-line, at-line and in-line measurements. For stirring a 3-blade (45°) impeller was used, powered with an engine on the top of the vessel. Gassing into the medium was realized with a sterile filter and dispensed by an L-shaped macrosparger. To minimize water stripping the outlet air was cooled by an exit gas cooler. For temperature control a heating jacket was applied. To allow sampling and harvesting the reactor was equipped with a super safe sampling valve (Infors). The Infors bioreactor allows direct control of cultivation parameters such as pH, gas inlet flow, temperature and agitation speed via a touch screen control panel.

3.4.2 Bioreactor instrumentation

The bioreactor was placed on a balance to recorded volume gain and loss. Moreover base consumption was logged gravimetrically. The ports on the top plate of the reactor were used for a dissolved oxygen sensor (Hamilton, Switzerland), pH probe (Hamilton, Switzerland) and a carbon dioxide partial pressure (pCO_2) electrode (Mettler Toledo, Switzerland). CO_2 and O_2 in the off-gas was measured on-line with an off-gas monitoring system (DASGIP, Germany). All data were logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

3.4.3 Batch cultivation

After sterilization the medium and the cells were transferred into the reactor aseptically with a seeding density of $3x10^5$ cells/ml. Prior to the seeding of the cells they were infected in a Schott bottle with the virus. For the calculation of the volume of the virus seed for infection following formula (Equation 1) was used:

$$inoculum\ [ml] = \frac{cell\ densitiy\ (\frac{cells}{ml})\ x\ working\ volume\ (ml)\ x\ (MOI)}{Titer\ Vector\ Seed\ (\frac{FFU}{ml})}$$

Equation 1

The pH was maintained constant at 7.2 using a PID controller by addition of 0.5 M Sodiumhydroxide solution. Cultivation temperature was set to 37°C and agitation was fixed to 150 rpm. Dissolved oxygen (pO_2) was set to 40% and maintained constant using a PID controller by cascade aeration via a sterile filter until a flow of 25 mL/min was reached. From this point on pure oxygen was added pulse by pulse. The fermentation parameters are listed in **Table 2**.

Table 2 Fermentation parameters, which were applied on all cultivations.

parameter	
temperature [°C]	37
stirring [rpm]	150
pH	7.2
pO_2 [%]	40

3.4.4 Cell-free runs

Batch cultivations were carried out in a 3.6 l volume glass bioreactor (Infors, Switzerland) with a working volume of 1 l and multiple ports to conduced on-line, at-line and in-line measurements. After sterilization 1 l of the medium was transferred aseptically in the reactor. The bioreactor was equipped with a 0.2 µm in-situ tubular membrane filter (see *Installation, Maintenance and Usage of Membrane Filters for on-line Sampling Device*) which was connected to a peristaltic pump using a PFTE tube with an inner diameter of 0.80 mm. For the pump and to realise the connection between filter probe and PFTE tube a pump tube with an inner diameter of 0.89 mm (PharMed® BPT 070540-80) was used. The bioreactor was equipped with a pO_2 probe. All other ports were closed and process conditions were applied (37°C, 150 rpm). A mixture of oxygen, carbon dioxide and nitrogen was supplied via a sterile gas filter.

At least three samples were taken within one day using a pump speed of 1 ml /min. The pumping time for each sample was set to ten minutes. At the second day the membrane was also back-flushed for another five minutes.

The third cell-free run was conducted using a pump speed of 0.2 ml/min.

3.4.5 Off-line sampling

To validate on-line data, frequent off-line samples were taken to determine the viable cell count, the virus titer and to measure the concentration of different metabolic components in the supernatant. Viable cell count was analysed in triplets by Cedex Hires (Roche, Switzerland). The off-line calculated cell counts were entered into the PIMS and a continuous signal was generated by linear interpolation for the subsequent calculation of specific rates. Alternatively the cells were counted using a Haemocytometer. Cells were diluted with trypan blue stain. A Neubauer improved counting chamber was used for cell count. A cover glass was placed tightly on the counting chamber and the counting dilution was filled in the gap between the chamber and the cover glass. The main divisions separate the grid into 9 large squares. The cells in the four corner squares were counted. For a representative result, an average of 8 squares on the haemocytometer was used. The SOP for the manual cell count using a haemocytometer is annexed in the appendix.

The metabolic compounds were determined by centrifugation of approximately 10 ml of the culture broth (100 g, 4°C, 5 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156). The supernatant was stored at -80°C and at the end of the cultivation all samples were measured using the automatic photometric robot CuBiAn XC (Innovatis).

Furthermore 1 ml aliquots of the cell suspension and the supernatant were stored at -80°C for virus titer analysis performed by the project partner. Fluorescence forming unit (FFU) analysis was conducted as well as a PCR, to determine the vector titer.

3.4.6 On-line analysis of metabolic compounds

Measurements of metabolites (glucose, lactate, glutamine, glutamate and ammonia) were performed on-line with an automated photometric robot (CuBiAn XC, Innovatis). All photometric assays were carried out at 37°C and are listed in **Table 3**.

They are commercial available (Randox, OptoCell GmbH & Co. KG, Germany) and calibration was carried out using four standard points in the respective assay range. Due to higher concentrations of the components under culture conditions automated dilution was conducted by the system. The reagents showed a stability of up to four weeks without recalibration.

Table 3 Commercial available assays for the on-line and off-line determination using CuBiAn XC.

assay	technical range [g/l]
lactate	0,080 – 10.000
glutamine	0,018 – 1.471
glutamate	0,020 – 1.462
ammonia	0,090 – 6.300
glucose	0,000 – 0.176

CuBiAn® XC is a fully- automated, membrane- free biochemistry analyser, based on a photometric measurement principle. An automatic wavelength selection from 340 to 800 nm is possible and furthermore endpoint as well as rate analysis can be conducted from 0.1 to 3.0 absorbance units. A broad range of process relevant substances can be detected such as substrates, metabolites and products (Innovatis).

The key parameters for mammalian cell culture, for which commercially available assays exist are among others IgG (human, humanized), glucose, lactate, ammonia, glutamine, glutamate, inorganic phosphate, LDH and total protein. As already mentioned, all these molecules are measured photometrically, with one exception; IgG is detected by immuno-turbidimetry (bioreaserchonline.com 2014).

In course of this thesis five different molecules were determined using CuBiAn® XC, namely glucose, lactate, ammonia, glutamine and glutamate. In the following, the principals of the used assays will be shortly described.

Glucose, which is frequently used as a substrate, is oxidised enzymatically in the presence of glucose oxidase producing hydrogen peroxide. In a second reaction, called Trinder reaction, H_20_2 reacts with 4-hydroxybenzoic acid (HBA) and 4-amniophenazone (4-AAP) to form a red- violet quinoneimine dye as indicator. The intensity of the final colour is directly proportional to the glucose concentration and is measured at 505 nm (fogalebiotech.com 2014).

$$1.\ Glucose + O_2 + H_2O \xrightarrow{Glucose\ Oxidase} Gluconic\ Acid + H_2O_2$$

Equation 2

$$2.\ H_2O_2 + HBA + 4 - AAP \xrightarrow{Peroxidase} Quinoneimine\ dye + H_2O$$

Equation 3

Lactate production is monitored in industrial processes as a crucial metabolite for cultured mammalian cells. In course of the aerobic glycolysis glucose can be converted into lactate, instead of being completely oxidized to CO_2 and H_2, even if enough oxygen is available. Typically lactate is produced during the exponential growth phase [Zagari 2013].

The concentration of L-Lactate in the sample is determined in the presence of lactate oxidase and oxygen, converting it to pyruvate and hydrogen peroxide. In a second reaction step a peroxidase catalyses the reaction of the peroxide + 4-aminoantipyrine (4-AA) and the hydrogen donor TOOS (= N-ethyl-N-(2hydroxy-3-sulphopropyl)-m-toluidine) to a stable purple dye complex, which can be measured at 700 nm (fogalebiotech.com 2014).

$$1.\ L - Lactate + O_2 \xrightarrow{Lactate\ Oxidase} Pyruvate + H_2O_2$$

Equation 4

$$2.\ H_2O_2 + 4 - AA + TOOS \xrightarrow{Peroxidase} Chromogen + 2\,H_2O$$

Equation 5

Furthermore, glutamine, glutamate and ammonia are important metabolites in cell culture media, as glutamine is a major nitrogen and energy source for many cell lines and should be present at 10 to 100- fold in excess to other amino acids. It is a precursor for peptide and protein synthesis, amino sugar synthesis, purine and pyrimidine and thus nucleic acid and nucleotide synthesis. Furthermore it provides a source of carbons for oxidation in some cells. L-glutamate is in most cells an intermediate product of the glutamine metabolism, produced by the enzyme glutaminase and is the most abundant intracellular amino acid [Newsholme 2003].

The primary waste product of glutamine metabolism is ammonia which is known to inhibit cell growth [Ozturk 1989].

The L-glutamine detection reaction is carried out in two stages. L-glutamate generated from deamination of L-glutamine is oxidized by L-glutamate oxidase to release hydrogen peroxide. Furthermore a peroxidase (POD) catalyses the reaction of the produced H_2O_2 with N-ethyl-N-(2(hydroxyl-3-sulfo-propyl)-3-methyaniline (EHSPT) and 4-aminoantipyrine (4-AA) to generate a quinine dye which is at 700 nm proportional to the amount of L-glutamine in the samples.

The L-glutamate assay works accordingly to the L-glutamine assay [fogalebiotech.com 2014].

$$L-Glutamine+\ H_2O \xrightarrow{glutaminase} L-Glutamate+NH_4^-$$

Equation 6

$$L-Glutamate+\ O_2+\ H_2O \xrightarrow{glutamate\ oxidase} 2-Oxoglutarate+NH_3+\ H_2O_2$$

Equation 7

$$H_2O_2+EHSPT+4-AA \xrightarrow{peroxidase} Quinine\ dye+4\ H_2O$$

Equation 8

Ammonia reacts with α-ketoglutarate and NADPH in the presence of glutamate dehydrogenase (GLDH) to form glutamate and $NADP^+$. The corresponding decrease in absorbance at 340 nm is proportional to the ammonia concentration in the sample [folgaebiotech.com 2014].

$$NH_4^+ +\ \alpha-Ketoglutarate+NADPH \xrightarrow{glutamate\ gedhydrogenase} L-Glutamate+NADP^+ +\ H_2O$$

Equation 9

Set up of on-line connected fully- automated photometric robot

To establish an on-line monitoring device for real time measurement of the major process parameters a physical connection between the photometric robot and the bioreactor was established and moreover an appropriate software interface to create an integration in the process management system was realised. According to Dietzsch *et al.* (2013) a flow- through cuvette was constructed and placed in the on-line port of the photometric robot. Therefore a commercially available half-micro cuvette, which was drilled on both sides and held a volume of 1 ml, was used. To enhance the volume exchange in the cuvette and shorten the required pumping time glass beads were filled in the cuvette, as shown in **Figure 4A**.

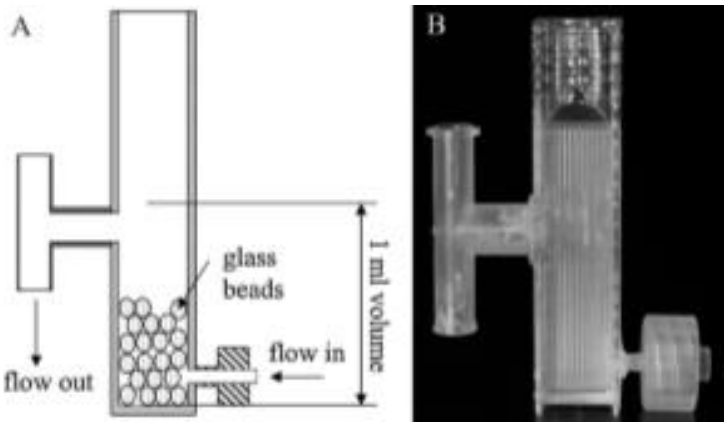


Figure 4 Construction of the flow- through cuvette: (A) schematic illustration; (B) picture of the cuvette (Dietzsch 2013).

This cuvette was connected via a PFTE tube with an inner diameter of 0.80 mm to a peristaltic pump and the pump to an in-situ microfiltration tubular membrane filter (0.2 µm). This connection was realized using an approximately 2 cm long pump tube with an inner diameter of 0.89 mm (PharMed® BPT 070540-80), which was linked to the hose connection. To connect the filter probe respectively the cuvette with the transport line, Luer-Lock connections were used. The length of the whole transport line added up to approximately 1.5m.

Taking a representative sample

As described by Dietzsch *et al.* it needs at least three volume exchanges in the cuvette to take a representative sample. As the volume of the cuvette, the sampling line and the filter device added up to 3 ml and a pump rate of 1ml/min was applied, a pump time of at least 9 minutes should be applied. In this case it was pumped for 10 minutes, which means a sample volume of 10 ml per sample was needed.

As the pump speed was lowered to 0.2 ml/min the pump time was extended to 50 minutes.

3.4.7 Installation, maintenance and usage of membrane filters for on-line sampling device

In-situ microfiltration tubular membrane filters (IBA Heiligenstadt, Germany) were used for the extraction of cell free media out of bioreactors under aseptic conditions. A ceramic filter, made of Aluminiumoxide (Al_2O_3), acted as sterile barrier and filter device. O-rings between the membrane and the filter carrier were used to seal it air-tight. The outermost layer of the membrane offered a porosity of 0.2 µm.

Before reassembling the filter probe, all sealing rings had to be tested visually. It was important to tighten the screw cap hand-tight and to take into account that all O-rings have to be installed centric.

Before installing the filter probe in the fermenter, the membrane integrity had to be tested. As basic test the whole filter probe should be placed in water, in a way that the liquid level was high enough to cover the whole membrane. Back flushing with air should not lead to any visible air bubbles appearing at a distinct point at the surface of the membrane (**Figure 5**).

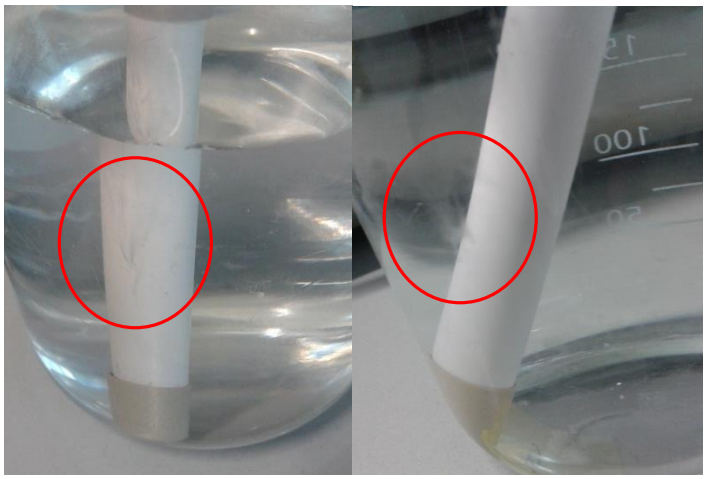


Figure 5 Leakage of the membrane.

Nevertheless, further testing should be carried out to proof membrane integrity. Therefore a suspension of diatomaceous earth in water is filtered through the membrane filter and the optical density of the filtrate is monitored with a spectrophotometer. In case of an increasing turbidity a leakage is considered to be present. This method was applied but turned out not to work for this kind of membranes, as it got block immediately.

Membrane integrity testing was done before and after use. On the one hand to prevent use of a non-integral filter and on the other hand it can detect if the integrity of the filter has been compromised during the process.

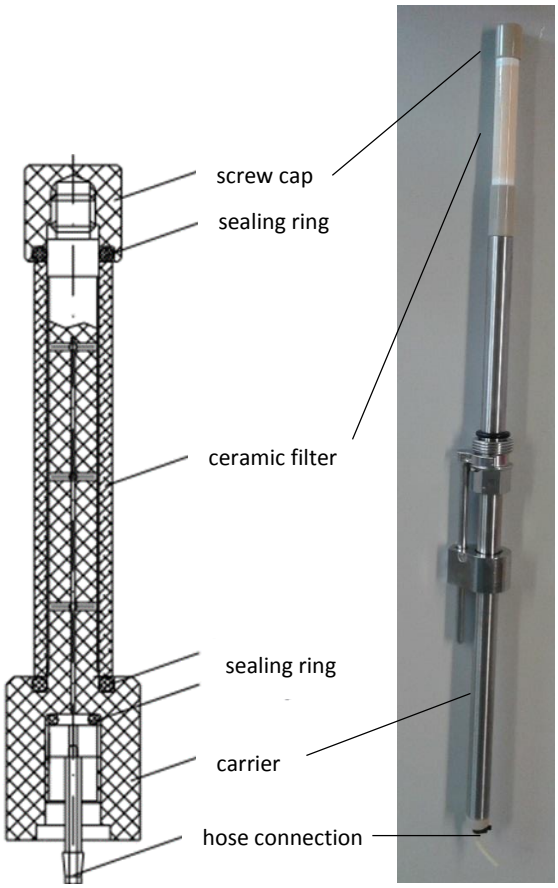


Figure 6 Schematic scheme and picture of a filter membrane.

An internal in-situ membrane filter was placed in a fully equipped bioreactor via a port in the fermenter top using a proper probe holder.

The membrane filter was installed as described in chapter *Set up of on-line connected automatic photometric robot.*

For sterilisation the Luer- Lock connection was detached and the screw connection was closed using a proper Luer-Lock closer. The filter probe was autoclaved with the whole bioreactor. The transport line was not sterilised at all.

After sterilisation the filter probe was reconnected to the transport line and an initial sample was taken. The pump speed was regulated to 1 ml/min according to the specification of the manufacturer. Nevertheless, depending on the own requirements this flow rate may also be lowered. As described in the chapter "*Taking a representative sample"* a pump time of 10 minutes was applied to take a

representative sample. A stop- flow strategy was used and the time between two consecutive samples amounted for six hours, within this time no sample was taken from the bioreactor.

It is recommend to check at least ones a day if the initial flow rate of 1 ml/min is still achieved, as a blockage of the membrane and consequently a lower flow rate may be directly linked to a possible contamination.

This offered a first basic test to review the membrane integrity.

It is important that the membranes should never be allowed to run dry between the usage and the cleaning routine.

The cleaning procedure involved rinsing the system with clear water and afterwards flushing the membrane forward and backward with 1M NaOH for 20-30 minutes.

To avoid uncontrolled precipitations, the system was rinsed with water after each cleaning step until the pH of the filtrate was neutral, which was checked with a pH electrode.

3.4.8 In-line capacitance probe

The viable cell count was determined in-real time using an in-line capacitance probe (Fogale BIOMASS SYSTEM, Fogale nanotech, France).

Principle of dielectric spectroscopy

The Fogale BIOMASS SYSTEM utilizes the capacitance technology for the in-line monitoring of the viable cell density to control cell growth. It uses the dielectric properties of living cells, based on the ability of biological cells to accumulate charges when exposed to an electrical field. Therefore cells with intact plasma membranes behave as tiny capacitors. Dead cells, whose plasma membrane is permeable, are not polarizable and therefore not detected. Furthermore cell debris, gas bubbles, microcarriers and other suspensions are not influencing the signal [fogalebiotech.com 2014].
While the electric field is released, a capacitance is induced caused by the relaxation of the ions. This capacitance increases in correlation to the cell concentration, because the higher the concentration the more plasma membranes get polarised, resulting in a higher capacitance.
Capacitance measurement takes commonly place in alternating fields. The permittivity ε of the cells depends on the electrical-field frequency. At low frequencies (below 0.1 MHz), a lot of ions have time to reach the plasma membranes and to polarise them, before the field changes direction and moves

the ions the opposite way. Accordingly, the measured permittivity is maximal. At high frequencies (above 20 MHz), the cells have not enough time to get polarized. The residual permittivity ε∞ is minimal and corresponds essentially to the permittivity of the culture medium. By subtracting the two permittivities the permittivity of the cell suspension can be determined.
In other words with increasing frequency the permittivity falls from a low- frequency plateau to a high-frequency plateau. This frequency- depending phenomenon is called β-dispersion and is described by the equation of Schwan (Equation 10) including three parameters: the maximal capacitance difference Δε, the critical frequency f_c and the empirical cole-cole parameter α.

$$\Delta\varepsilon = \frac{9}{4} P \cdot r \cdot Cm$$

Equation 10

P, viable cell volume fraction [dimensionless]; r, viable cell radius [m]; C_m, specific membrane capacitance [$F \cdot m^{-2}$];

The above mentioned cole-cole parameter α is an empirical parameter, which reflects heterogeneity of dielectrical properties in cells and has therefore no biological relevance. It should vary between 0 and 1. Nevertheless, in cell cultures α mostly accepts values between 0.1 and 0.2 [Marks 1999]. It is only affected by cell size distribution and whether the cells are spherical or not. Therefore, this parameter can also be used to generate morphological information.

The β-dispersion itself can be described by various parameters and one of them is the characteristic frequency fc (Equation 11).

$$fc = \frac{1}{2\pi r \cdot Cm \left(\frac{1}{\sigma_{in}} + \frac{1}{2\sigma_{out}}\right)}$$

Equation 11

σ_{in}, intracellular conductivity [$S \cdot m^{-1}$]; σ_{out}, medium conductivity [$S \cdot m^{-1}$]

In **Figure 7** a β-dispersion spectra, which is determined by scanning the entire frequency range measuring the permittivity, is shown.

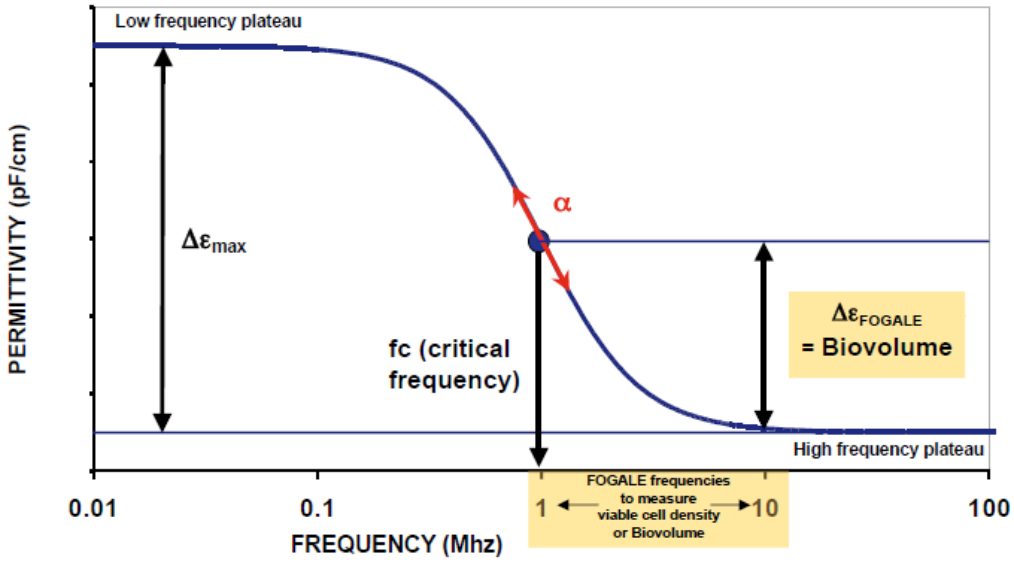


Figure 7 β-dispersion spectra (Esteban 2007)

If the critical frequency f_c, the dispersion coefficient α and the maximal capacitance difference Δε are determined using the β-dispersion spectrum and if it is assumed that specific membrane capacitance as well as the intracellular and the medium conductivity are constant the viable cell radius can be calculated from f_c. The viable cell density can furthermore be calculated using this radius and the Δε [Ansorge 2007; Tiyarenc 2011].

Set up of dielectric spectroscopy probe

The probe was installed using a port in the top of the vessel. The probe position in relation to vessel walls, base of vessel, pH, pO_2 and pCO_2 probe, stirring and presence of aeration is reported in literature to cause interference with the capacitance signal [Dabros *et al.* 2009; Davey and Kell 1998]. Therefore, care has been taken to ensure an equivalent setup for all cultivations. Capacitance measurements were carried out at 20 different frequencies from 0.3 to 10 MHz (so called frequency scan). These capacities were used to develop an adequate model describing the viable cell count.

Furthermore conventional 2 frequency settings, as described before, were used at 1 and 10 MHz respectively 0.3 and 10 MHz to record a permittivity signal. This signal was not used for further calculations.

3.5. Rate and yield calculation

In order to use the on-line determined data for bioprocess quantification, incremental volumetric rates were calculated, accordingly to Equation 12,

$$r_{c_i}\left(\frac{t_n - t_{n-1}}{2}\right) = \frac{c_i(t_n) - c_i(t_{n-1})}{t_n - t_{n-1}}$$

Equation 12

whereby t_n and t_{n-1} describe two consecutive measurement time points for the concentration compound c_i.

Furthermore specific rates were evaluated using the respective in-line determined viable cell count (c_x) (Equation 13).

$$q_{c_i} = \frac{r_{c_i}(t_n)}{c_x(t_n)}$$

Equation 13

Rates were calculated for all components with the respective algebraic sign indicating the flux into or out of the cell (*i.e.* +, release; -, uptake).

Volumetric rates are the basis for the computation of yields (Equation 14).

$$Y_{i,j} = \frac{r_{c_i}}{r_{c_j}}$$

Equation 14

3.6. Calculation of statistical parameters

As average the arithmetic mean $\bar{x}$ was used, which was calculated by adding all the values x_i and dividing the sum by the number of values n (Equation 15).

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

Equation 15

The standard deviation s was evaluated using Equation 16.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

Equation 16

A higher number of replicates result therefore in a smaller standard deviation.

The SNR (Equation 17) compares the arithmetic mean ($\bar{x}$) of a signal to the level of the background noise or the standard deviation of the signal (s).

$$SNR = \frac{\bar{x}}{s}$$

Equation 17

The limit of detection and quantification (Equation 18 and Equation 19) can be used as thresholds for SNR to detected or quantify a component or, in this contribution, variations of specific rates, and yields. Typically, the limit of detection (LOD) is defined as the minimum detectable signals for which the standard deviation (s) is 3. For the limit of quantitation (LOQ) a standard deviation of 12 was supposed.

LOD = 3 * s

Equation 18

LOQ = 12 * s

Equation 19

Concerning the validation of analytical devices robustness of the method is of great importance. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [ICH guideline, 1994]. To proof the robustness of this method it was applied on different HEK cell clones, which showed a completely different growth behaviour.

Furthermore the accuracy and precision of the metabolite measurement device in off-line mode and the precision of the Cedex Hires were determined. Accuracy is defined as the closeness of agreement between a test result and the accepted reference value [McPolin 2009]. To proof the accuracy of the method the mean recovery was determined by eightfold measurement of standard solutions which contain the substrate of interest. The concentration lies in the middle of the working range of each method. Based on McPolin (2009) an acceptance criteria a mean recovery of 90-110% was applied. The precision of a method is the closeness of agreement between independent test results obtained from homogenous test material under stipulated conditions of use [VICH Steering Committee 2009]. Again calculated by eightfold measurement of standard solutions.

The datasets generated in off-line mode were compared with those in on-line mode. That means an original validated bioanalytical method serves as the reference in course of a cross-validation [U.S. Department of Health and Human Services Guidance for Industry 2001].

3.7. Data analysis

Measurements of the viable cell count were executed in triplicates. In the run-up of the first cultivation defined solutions of glucose, L-glutamine, glutamic acid, a calcium salt of lactic acid and ammonium chloride were measured eightfold with CuBiAn XC (**Table 4**). Along the observed standard deviation for the single measurement, the error was propagated to the specific rates as well as to the yield coefficients. The error of determination of the metabolic concentration varied, depending on the method, between 0.9% and 1.3%. Cedex Hires showed a measurement error of 5%. The error of determination of the specific rates and yields was therefore set to 6.5% and 3%, respectively.

4. RESULTS AND DISCUSSION

4.1. Characterization of the method

4.1.1 Data analysis

If data should be compared, discussed and interpreted it is necessary to be conscious about the errors of the single values. The errors which were determined for the single CuBiAn XC methods by eightfold measurements of standard solutions are listed in **Table 4**. The glutamine assay shows with 1.3% the highest and the ammonia assay with 0.9% the lowest percental error.

Table 4 Error in percent of the different methods installed on CuBiAn XC determined in eightfold measurements of defined solutions of the various substrates.

method	error [%]
lactate	1.1
glutamate	1.0
glutamine	1.3
ammonia	0.9
glucose	1.2

4.1.2 Minimizing the risk of contaminations

As already mentioned in *"Material and Methods"* an accurate assembly of the filter probe, integrity tests performed before and after use and careful cleaning of the membranes help reducing the risk of contaminations.

To investigate the influence of the pump rate 3 cell-free runs under process conditions were carried out.

Those cultivations produced concrete results as it could not only be found out that back flushing of the membrane always leads to contaminations but moreover that a lower pump rate of 0.2 ml/min and consequently a longer pumping time of 50 minutes per sample reduces the risk of a contamination.

4.1.3 Analytical parameters describing the on-line monitoring system

After setting up the system, it had to be characterised by the determination of analytical parameters for instance the calibration range, the limit of detection (LOD) or the limit of quantitation (LOQ) for five different components, namely glucose, lactate, ammonia, glutamine and glutamate. Furthermore the method should be proven to be accurate and precise.

Linear working range

A visual determination of the linear working range was conducted. This operation range should preferably cover an area as large as possible and moreover its centre should also coincide with the most abundant range of concentration of the substrate. As example the linear range of lactate is shown in **Figure 8**. The functions for the all analytes are listed in **Table 5**.

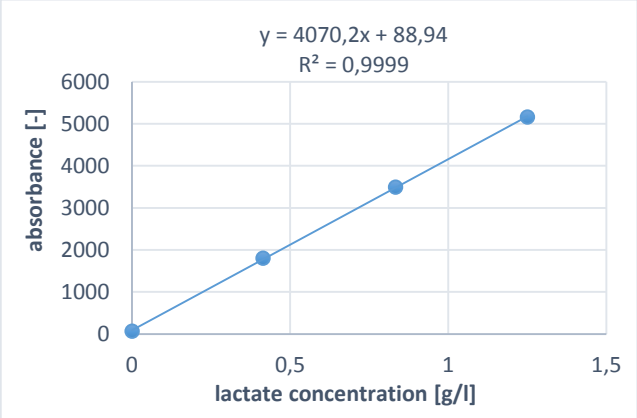


Figure 8 Linear calibration range of lactate

Table 5 Calibration functions and coefficients of determination of the various methods.

analyte	function	coefficient of determination
lactate	y=4070.2x+88.94	0.9999
glucose	y=2301.1x+416,35	0.9995
glutamine	y=9664.7x+416.35	0.9995
glutamate	Y=8967.2x+85.1	0.9997
ammonia	y=-53917x-326.08	0.9994

Precision

According to Janke (1996) the method precision for bioanalytical and pharmacokinetic techniques should not be more than 2.0% relative standard deviation. Comparing **Table 4** it can be proven that this acceptance criteria is fulfilled by all five methods.

Accuracy

To proof the accuracy of the method the mean recovery was determined by eightfold measurement of standard solutions which contain the substrate of interest. The concentration lies in the middle of the working range of each method. The accuracy of the method the values shown in Table 5 could be achieved. Based on McPolin (2009) an acceptance criteria a mean recovery of 90-110% was applied. Again the acceptance criteria was fulfilled by all methods.

Table 6 Mean recovery in percent of the different methods installed on CuBiAn XC determined in eightfold measurements of defined solutions of the various substrates.

method	concentration of the substrate [g/l]	mean recovery [%]
lactate	0.80	90
glutamate	0.60	100
glutamine	0.60	96
ammonia	0.08	100
glucose	3.00	93

Limit of detection and limit of quantitation

In Order to detect the lower band of the working range, the identification of so called limit of detection (LOD) is of high importance. It is defined as the smallest detectable concentration of a substance. In accordance to the Guidelines for the validation and verification of quantitative and qualitative test methods [National Association of Testing Authorities NATA 2013], at this limit, the question if the substrate is present or not, has an error probability of 50%. Those 50% of the cases with false positive detection are due to the signal distribution of the blank value.

The smallest quantifiable concentration is called the limit of quantitation. This is the amount which can be quantitated with a given level of measurement uncertainty (typically 33%) [NATA 2013]. As

simplification it is often assumed that the LOQ is three times the LOD. In case of this study it is supposed that it is even four times the LOD.

Based on eightfold measurements the standard error of the arithmetic mean and furthermore the LOD and LOQ were determined (**Table 7**).

Table 7 Determination of the standard deviation, LOD and LOQ for all substrates.

	lactate [g/l]	glutamate [g/l]	glutamine [g/l]	ammonia [g/l]	glucose [g/l]
standard deviation	0.009	0.006	0.007	0.0004	0.046
LOD	0.027	0.019	0.020	0.001	0.139
LOQ	0.106	0.075	0.079	0.004	0.557

Concerning the LOD and LOQ the assay for the quantification of ammonia is the most and that for glucose the least sensitive one. This is a satisfying situation as the glucose concentration in the media is several times higher than the ammonia concentration.

Sampling frequency

Hence, if data is generated for process quantification, it is important to take into account that it is prone to errors, as these errors have a huge impact on the signal quality and are therefore crucial for the consistency of the calculated elemental balances. Moreover, the signal- to- noise ratio (SNR), the measurement window and the sampling frequency influence the error propagation [Wechselberger and Herwig 2012]. Consequently, the measurement frequency has to be scheduled carefully, not only because of the error calculation but also with regard to process parameters such as the volume.

Within three batch cultivations (STR12, which one was performed in the run-up of this work and STTR13 and STR14 which were part of it) the substrate uptake rates q_s for all five substances were determined and based on that, the sampling frequencies were defined. As q_s is not constant during the fermentation, this leads also to changes in the recommended sampling frequency. To define a general sample frequency an average was calculated. **Table 8** gives and overview of the results. Generally, one must find a compromise between a high sampling frequency, which helps to detect process events and a low one to minimize noise effects.

Table 8 Recommended sampling frequency for all substrates.

	Lactate	Glutamate	Glutamine	Ammonia	Glucose
Sampling Frequency [h]	6	-	9	13	12

Lactate shows the highest sampling frequency with every 6 hours. For ammonia and glucose it is recommended to take a sample every 13 respectively 12 hours. In contrast to that glutamine does not need such an intensive monitoring, taking a sample every 9 hours is sufficient. Furthermore it could be found out that this method is not sensitive enough to measure changes in the glutamate concentration, as it is not possible to distinguish between signal and random noise.

In course of this thesis a measurement frequency of 6 hours was applied for all five substances, based on the lactate assay which needs the most intensive observation. This strategy was also used because the predetermined sampling frequencies (STR12) had to be validated (STR13 and STR14) and therefore the maximum degree of automation could not be reached yet. Furthermore, a higher sampling frequency helps to monitor the development of the different substances and to detect limitations at an early stage. Nevertheless, for further applications the sampling frequency may be adjusted to the recommended values in **Table 8**.

A sampling frequency of only six hours is one big difference to monitoring of microbial cultivations, where it is much higher with up to 0.5 hours [Dietzsch *et al.* 2013].

4.1.4 Implementation of in-situ capacitance probe for the in-line determination of the biovolume

For the determination of the viable cell count the Fogale BIOMASS SYSTEM® was applied. This device uses the β-dispersion phenomenon to measure the viable cell density or biovolume. For the in-line determination of the viable cell count a correlation between the permittivity and the viable cell count has to be found. Therefore frequency scans have been performed over the β-dispersion range. A total of 20 frequencies from 0.3 to 10 MHz were applied by the Fogale BIOMASS SYSTEM® and the corresponding permittivity was measured to establish a complete spectrum of the cell suspension. The spectra were recorded every 12 min. For each spectrum the software calculated the three parameters $\Delta\varepsilon$, f_c, and α in accordance to the β-dispersion model described in the theoretical part.

To find an adequate correlation the viable cell count was determined off-line using Cedex Hires. The precision of this off-line device was proven by numerous triplicate measurements of cell suspensions. The method precision was not be more than 5.0% relative standard deviation. For the calibration a batch cultivation of HEK cells performed by Andrea Egger, MSc was used and data evaluation was carried out with Datalab.

The data of the frequency scan were uploaded to Datalab and a Multiple Linear Regression (MLR) was performed to find a calibration function for the Fogale BIOMASS SYSTEM®. Before carrying out a MLR some prerequisites have to be fulfilled. A major problem of MLR are collinear variables. Two variables are said to be collinear if they are linearly dependent, which means there is a high correlation between them. A model based on highly correlated variables becomes unstable and the coefficients useless for causal interpretation.

The Variance Inflation Factor (VIF) is a common possibility to detect multicollinearities between the independent variables of a model. A particular variable x_k is expressed by a linear model based on all other independent variables. If the calculated model shows a high reliability (i.e. the goodness of fit is high) the tested variable x_k is likely to be (multi)collinear to one or more of the other variables. The VIF of the k^{th} variable is defined by Equation 20.

$$VIF_k = \frac{1}{1 - r_k^2}$$

Equation 20

r_k^2 is the goodness of fit of the linear model for x_k based on all other variables.

The VIF is calculated for all independent variables of a model and the variable which shows the highest value is removed. This step is repeated until the VIF of every variables is smaller than 10 [Lohninger 2010].

In **Table 9** the VIF values for all frequencies are shown.

Table 9 Detection of multicollinearities.

Frequency [kHz]	VIF
300	79,301
373	25234.316
465	126157.53
578	215140.05
720	229587.31
897	262749.31
1117	230974.56
1391	192729.63
1732	125945.50
2156	131117.73
2684	60625.470
3342	43075.145
4161	1.477
5181	21862.479
6451	19332.834
8031	14442.386
10000	9384.327

After eliminating one after another the frequencies listed in **Table 10** remained and were used for further analyses.

Table 10 Variables used for further variable selection.

Frequency [kHz]	VIF
300	1.057
373	1.063
4161	1.006

300, 373 and 4161 kHz were those frequencies which were used for further variable selection. Forward selection, backward elimination and stepwise regression were performed and all three methods showed one common result. The frequencies **300 kHz and 373 kHz** were used to build up a MLR model. This results correlates with the findings in the literature. Justice *et al.* 2011 used this device for the on-line monitoring of stem cell expansion and also found 300 kHz to be the most specific frequency.

The following model could be received and was used for all real-time calculations of the viable cell count:

viable cell count [cells/ml] $=-2{,}158*10^4*\text{permittivity}_{300kHz}+1{,}881*10^5*\text{permittivity}_{373kHz}+4{,}813*10^5$

In **Figure 9** the correlation between the off-line measured cell count using the Cedex Hires and the in-line data gathered permittivity data converted into viable cell counts by the presented model is shown. There is a good correlation for the exponential growth phase of the cells as a straight line with a slope of 1.05 ±0.05 and a correlation coefficient of 0.992 was reached. Nevertheless, the model is not valid for the cell death phase. With the induction of cell death a separate model has to be found. This also applies to very small cell concentrations at the beginning of the cultivation. As this work only pays attention to the exponential growth phase, these deviations are neglected.

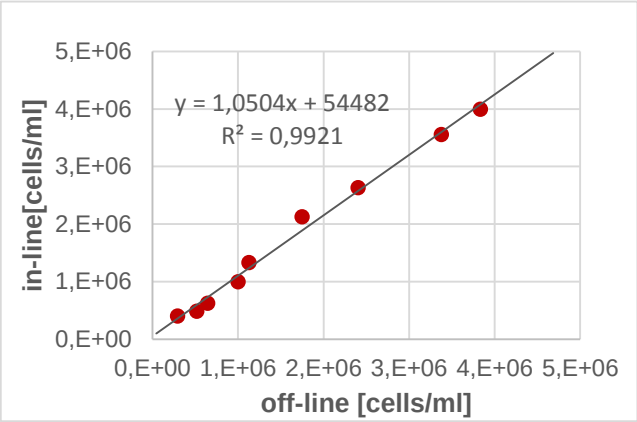


Figure 9 Correlation between off-line viable cell counts and In-line permittivity data converted by the MLR model.

The presented model was applied on every cultivation in which the Fogale BIOMASS SYSTEM was used. In **Figure *10***A and ***Figure 10***B the correlation with the off-line determined data are shown for STR 13 and STR14. The off-line data plotted against the in-line data resulted in a straight line with a slope of 1.07 ± 0.05 with a correlation coefficient of 0.990 and 1.04 ± 0.05 with a correlation coefficient of 0.999.

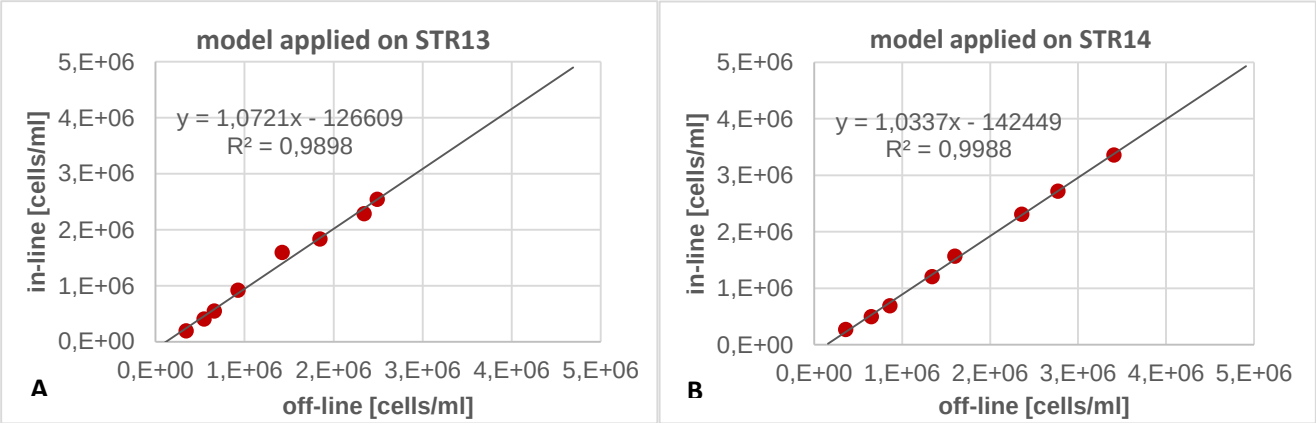


Figure 10 MLR model applied on STR13 and STR14: (A) STR13; (B) STR14.

Therefore it could be concluded, that although a different cell clone was used to produce the calibration line, the model fits almost perfect and a virtually identical results were achieved. This fact is due to variable selection enabling the finding of the most specific frequencies. In literature, it can be mainly read that two frequencies are used randomly. A low frequency (below 0.1 MHz; for mammalians mostly 1000 Hz), which lies in the critical frequency region and a high frequency (mostly 10 MHz), at which the permittivity corresponds essentially to the permittivity of the culture medium. By subtracting the two permittivities the permittivity of the cell suspension can be determined [Ansorge 2007; Petiot 2012]. The frequency at which the capacitance is measured depends on the organism. Typically, excitation frequencies of around 1,000 kHz are used for bacteria cells and between 500 and 600 kHz for yeasts and mammalian cells [Ducommon 2002]. These linear model is the easiest opportunity to set up a calibration.

In course of this work a calibration using MLR based on off-line determined data was conducted after variable selection was performed by forward selection, backward elimination and stepwise regression and therefore a model was found, which perfectly describes the present system. Nevertheless, during the decline phases of the cultures, the model decreased in accuracy, which indicated that physiological changes affecting permittivity were occurring. The same results were observed by Cole *et al.* (2010). They used three methods of analysis (linear modeling, Cole-Cole modeling, and partial least squares regression (PLS)), to correlate the dielectric spectroscopic data with routine biomass measurements in batch and fed-batch CHO cultivations. In contrast to that Parta *et al.* (2014) used the same three approaches and found that only the linear model showed deviations in the decline cultivation phase. Nevertheless, MLR is a fast and easy to use opportunity, with only a single requirement (mulitcolinear variables have to be excluded), which can be perfectly applied on different cell clones, if the exponential growth phase is of major interest. It can keep up with other multivariate

4.2. Physiological characterization of different HEK cell clones

In course of this thesis five batch fermentations were carried out (**Table 11**) using three different HEK293 cell clones and two different glutamine sources. In course of STR8 CuBiAn XC was connected to the bioreactor for the first time. Unfortunately, only a physical connection could be realized and the application to the process management system failed in this early stage of the study. Therefore, one cannot yet speak of on-line but at-line monitoring. Within all other cultivations on-line monitoring of the metabolic compounds lactate, glutamate, glutamine, ammonia and glucose was performed with a sampling interval of six hours. Moreover an off-line determination of the viable cell count was performed for all cultivations and an in-line determination in course of STR13, STR14 and STR16.

Table 11 Batch cultivations carried out in course of this thesis.

run	cell clone	glutamine source	CuBiAn	dielectric spectroscopy probe
STR8	HEK293-A	4 mM GlutaMax	—	—
STR13 & STR14	HEK293-B	6 mM Glutamine	✓	✓
STR16	HEK293-C	4 mM GlutaMax	✓	✓
STR17	HEK293-B	6 mM GlutaMax	✓	—

4.2.1 Determination of physiological parameters

The calculation of specific rates enables the quantification of the main metabolites on a cellular basis. In this case on-line determined metabolite concentration were used to calculate specific rates from one sample to another. The required cell counts were evaluated using in-line determined viable cell counts via Fogale BIOMASS SYSTEM. As described by Wlaschin (20006) most changes are reflected in the metabolic rates of glucose, lactate and glutamine, and are easily detected by tracking their stoichiometric ratios throughout the course of a culture.

4.2.2 Detection of process events

Carrying out 4 batch cultivations using 2 different HEK293 clones lactate, glutamine, glutamate, ammonia and glucose were measured on-line at a frequency of every 6 hours by the automatic controlled on-line device. In parallel, the according off-line data were collected. As an example the datasets of STR14 are shown in the following.

Before discussing the opportunity to detect process events using the on-line monitoring device the accordance between the off-line and on-line data should be examined, this is especially important to prove the accuracy and precision of the on-line monitoring device. That means an original validated bioanalytical method serves as the reference in course of a cross-validation [U.S. Department of Health and Human Services Guidance for Industry 2001]. The accordance of off-line and on-line data will be discussed again in Chapter *"On-line Applicability"*.

The quantitative comparison of the on-line and off-line determined concentrations shows a good correlation, demonstrated by plotting on-line data over off-line date in ***Figure 12***A and ***Figure 12***B.

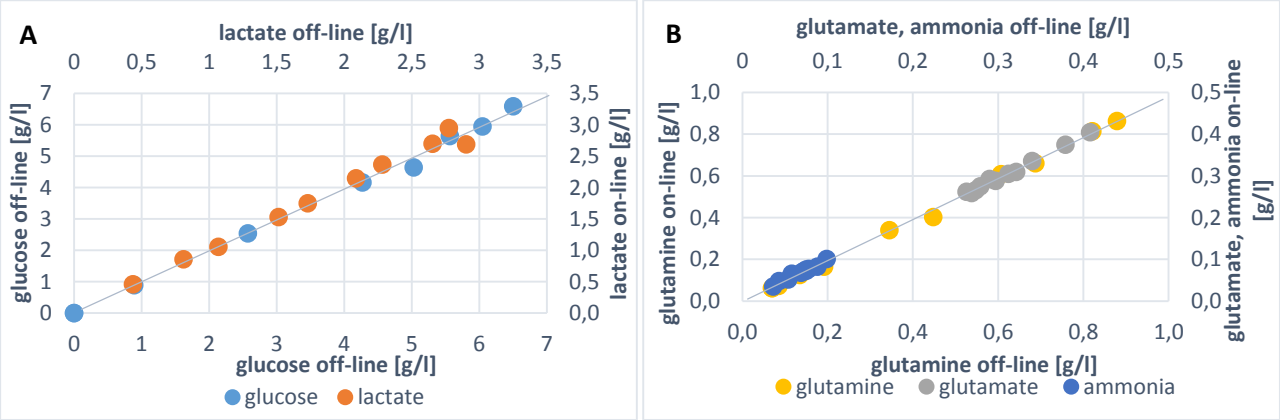


Figure 11 Off-line measured metabolite concentrations of STR14 plotted over on-line measured one: (A) glucose and lactate; (B) glutamine, glutamate and ammonia.

orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia

The maximal percentage settings of the deviations between the two datasets are illustrated in ***Table 12***.

Table 12 Maximal deviation in percent between the off-line and on-line determined concentrations.

method	deviation [%]
lactate	>5
glutamate	>3
glutamine	>10
ammonia	>10
glucose	>5

A maximal deviation of 10% between the off-line and on-line proofs a good correlation. That means the same quantitative information can be drawn using a less working intensive approach and moreover a much higher sampling frequency can be established, which offers the advantage of process monitoring in real-time.

Using the enzymatic robot CuBiAn XC to measure the concentration of metabolites offers many advantages compared to other methods. First and foremost it is simple and easy to conduct. HPLC, which is a well-established technique, does not only need a careful selection of the mobile phase and intensive optimization but also internal standards and calibrators which are expensive to buy and show high maintenance costs. Furthermore for five metabolites (glucose, ammonia, glutamate, glutamine and lactate) at least three different methods have to be applied. The CuBiAn XC is able to measure multiple metabolites using commercially available assay kits and only a simple calibration has to be performed and no recalibration or further maintenance is needed.
Another frequently used method is off-gas analysis. Although this technology offers the advantage of being non-invasive it cannot be used for quantitative considerations, as it lacks specificity. In turn, high specificity is a major advantage of the enzymatic robot.

In ***Figure 12***A the off-line data set of STR14 is shown and in ***Figure 12***B the on-line measured data of STR14 are illustrated. The complete data sets for all other cultivations are annexed in the appendix.

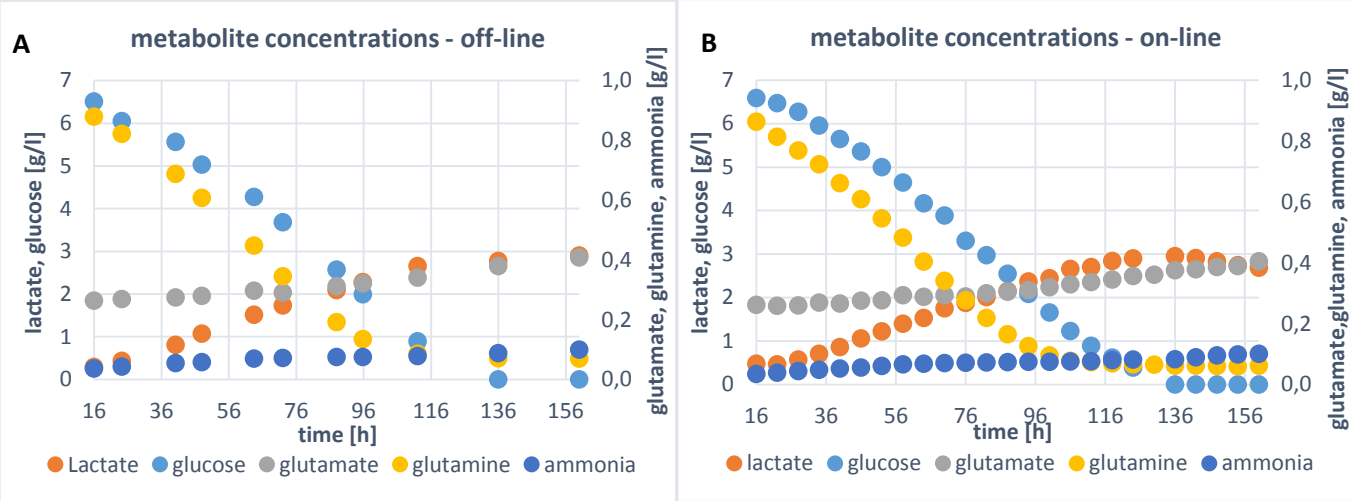


Figure 12 Metabolite concentrations of STR14 measured using CuBiAn XC in off-line and on-line mode: (A) off-line; (B) on-line.

orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia.

Comparing ***Figure 12***A and ***Figure 12***B it is immediately striking that the dynamic of the culture can be precisely imaged using the on-line monitoring device, because a high resolution can be achieved. This is a major advantage concerning the detection of various process events, such as limitations of substrates or accumulations of nutrients.

As an example for the detection of process events the various phases of the glutamine metabolism are discussed in the following. In

Figure 13 the specific glutamine uptakes rates of STR17 which were calculated using on-line and off-line devices are plotted over time. GlutaMax was used as glutamine source, which is a stabilized dipeptide of L-Alanyl-L-Glutamine.

In case of the off-line determined data, it can be seen that the dipeptide was hydrolysed by gradually released peptidases, which leaded to increasing glutamine concentrations in the first 50 hours. This cleavage phase is followed by glutamine uptake and finally, although there is still glutamine present, it is not metabolised anymore, the concentration is maintained. Therefore 3 different phases can be clearly distinguished.

Regarding the on-line data set the same time point (~50 hours) can be detected for the end of the cleavage of the dipeptide and the beginning of the glutamine uptake. These two phases are also followed by a phase at which the glutamine concentration is maintained constant. But the high sampling frequency helps to detect a fourth phase at the end of the cultivation: a glutamine production

period. This increase in glutamine is further supported by the apparent consumption of ammonium beginning 150 hours after induction (data not shown). Ammonia is used in glutamine anabolism via the enzyme glutamine synthetase. Therefore 4 distinct process phases can be differentiated. This last production phase could not be detected using off-line sampling.

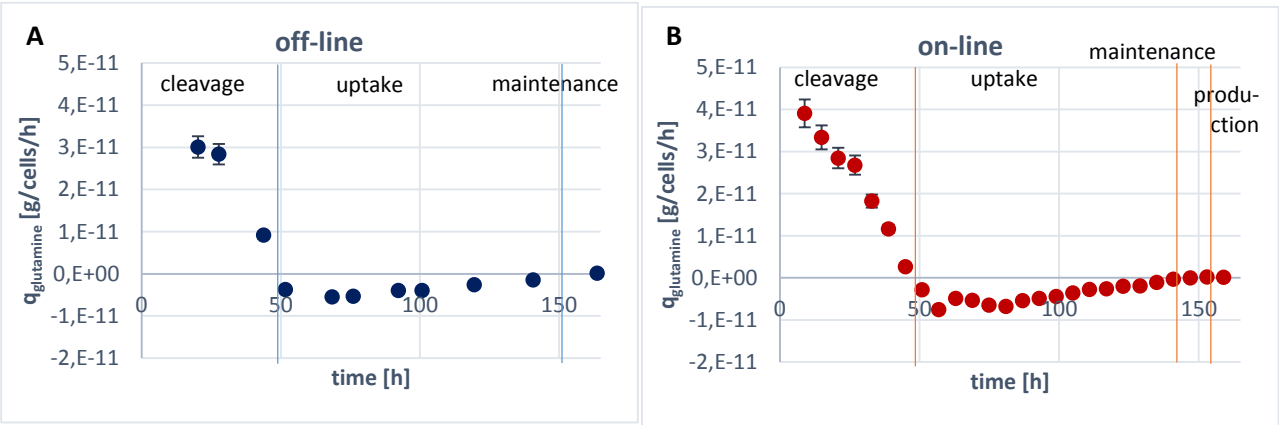


Figure 13 Three different process phase can be distinguished concerning the glutamine metabolism: (A) off-line determined specific glutamine uptake rates; (B) on-line determined specific glutamine uptake rates.

A higher measurement frequency helps to detect the time point at which such process events occur, which is illustrated by comparison of

Figure 13 A and B. Regarding off-line sampling it is hardly or not possible at all to reach a measurement frequency which is as high as those of the on-line monitoring device because too much manpower would be needed.

Furthermore, as visualised in ***Figure 13***A and B, this device is a novel tool for the real-time determination of specific rates. As described by Wlaschin (20006) most changes are reflected in the metabolic rates of glucose, lactate and glutamine, and are easily detected by tracking their stoichiometric ratios throughout the course of a culture. Another advantage of on-line monitoring is the possibility to intervene directly with the process, because the data are available in real-time, which offers the basis for intelligent controlling strategies.

Glucose represents the major substrate of mammalian cells, therefore sufficient supply should be guaranteed and limitations strictly avoided. Such limitations or even a drop under a certain concentration can be easily detected using on-line monitoring. In

Figure 14 A and B once again the off-line and on-line date, in this case the specific glucose uptake rates, are illustrated. In course of the on-line determined data it is easy to follow the progress of $q_{glucose}$. Glucose uptake decreases over time until it is depleted and as a consequence $q_{glucose}$ becomes zero.

Regarding the off-line determined data the limitation occurs sometimes between 130 and 150 hours after the induction (

*Figure 14*A). Using the on-line monitoring the time span can be narrowed down to 138 to 144 hours after induction (

*Figure 14*B).

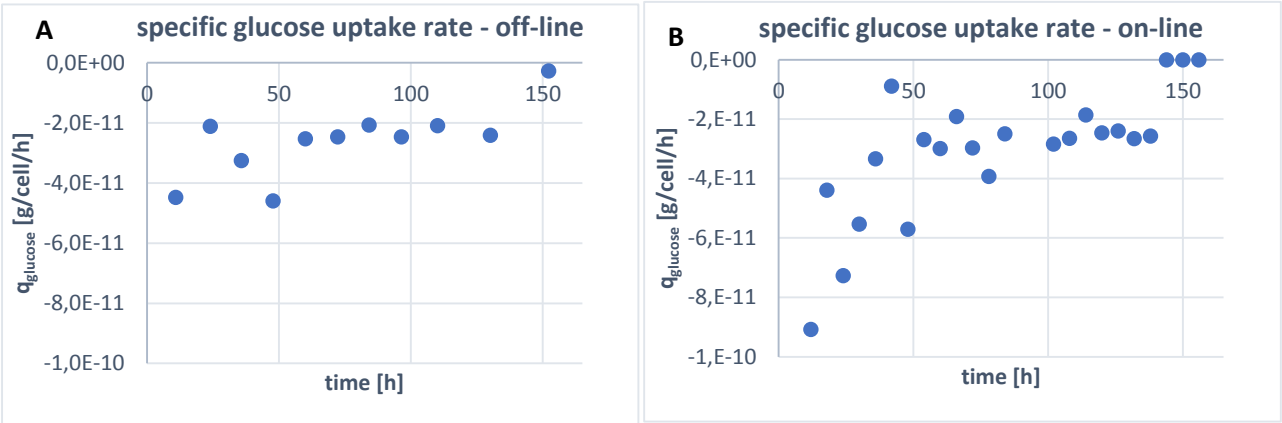


Figure 14 Detection of glucose limitation using on-line and off-line glucose uptake rates: (A) off-line determined specific glucose uptake rate; (B) on-line determined specific substrate uptake rates.

Although a high sampling frequency helps to precisely detect a process event, in course of the specific glucose uptake rate it also leads to very noisy data. As mentioned in the chapter *Characterization of the Method* it is sufficient to measure glucose every 12 hours. Although, the sample number is now quite similar to the off-line data set, the progress of $q_{glucose}$ can be traced better (***Figure 15***) and the complete depletion of glucose can be localised sometimes between 138 and 150 hours after the induction.

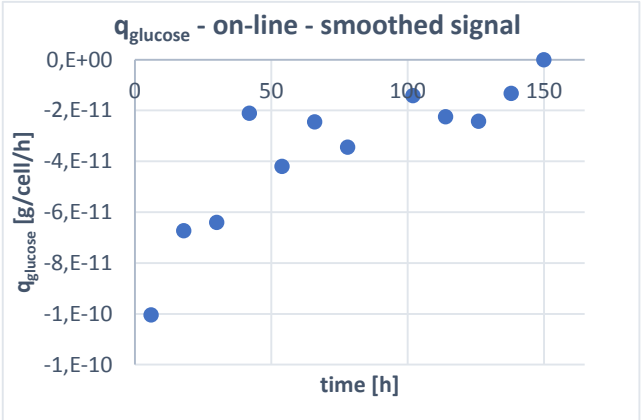


Figure 15 Detection of glucose limitation using a smoothed on-line signal.

In a similar way other metabolites can be monitored. For example it can be of great interest to avoid a critical accumulation of lactate in the media.

Based on that approach feeding strategies can be developed. Therefore, a critical limit for one or more substrates is defined an as soon as that limit is reached the corresponding feed is started automatically.

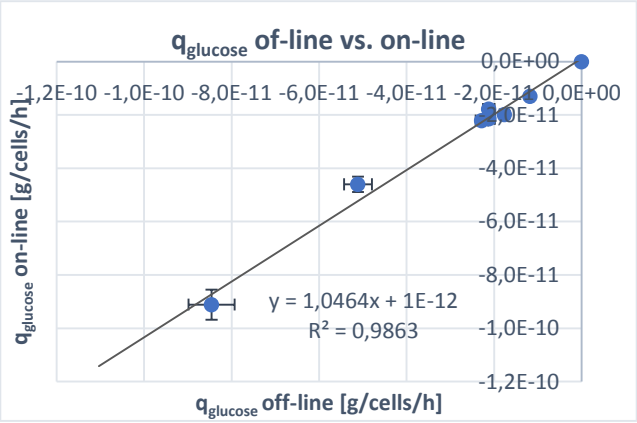


Figure 16 Comparison of on-line and off-line determined specific glucose uptake rates.

A direct comparison of off-line and on-line determined specific glucose uptake rates is shown in ***Figure 16***. The two data-sets show a satisfying correlation. The deviations account for maximal for 7-8% (accept of one outlier with 12%). This error lies within the range of the combined errors of the two coupled methods, which sum up to 7%. Furthermore plotting the on-line over the off-line determined data a straight line could be achieved with a slope of 1.05 ± 0.07 and a correlation coefficient of 0.986, proving the accordance of the two datasets.

4.2.3 Clone selection based the on-line approach

The approach offers the possibility to measure the concentration of metabolites in the supernatant in real-time. For the reliable extraction of information incremental rates and yields were calculated from on-line sample to on-line sample. Such a rate calculation enables to draw conclusions on different physiological cell states as well as the linkage of cell physiology to productivity, which enables profound and fast clone selection.

Two different cell clones (HEK293β and HEK293γ) were cultivated under the same process conditions (STR16 and STR17) and in the following the two strains will be compared considering physiological parameters.

Comparing the off-line determined cell growth curves of both clones it becomes obvious that HEK293γ (STR16) grows significantly slower than HEK293β (***Figure 17***) and does not offer a distinct cell growth peak. Within STR17 a maximum of $3.35*10^6$ viable cells/ml could be reached, compared to $1.13*10^6$ viable cells/ml in course of STR16.

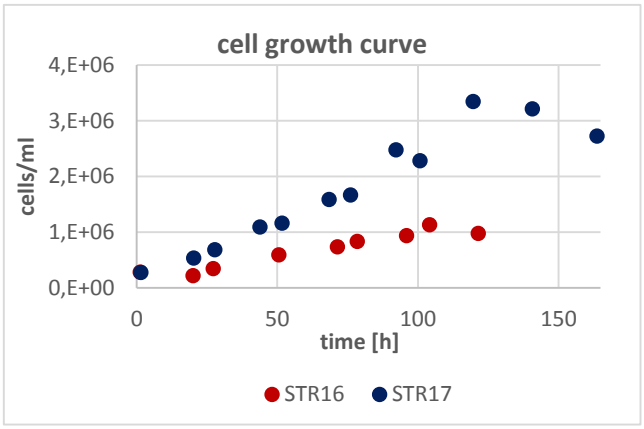


Figure 17 Cell growth curves of HEK293β und HEK293γ.

red, HEK293γ cultivated in STR16; blue, HEK293β cultivated in STR17;

First of all, the possibility to use this on-line monitoring device for two cell clones, showing such a different growth behaviour illustrates the robustness of the method. Accordingly to the ICH the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [ICH guideline 1994]. In this course the variation is caused by the usage of two different cell clones. For both cell clones meaningful specific rates and yields could be determined and therefore the robustness of the method was proven.

Glucose uptake and lactate production

The high frequent on-line determination of carbon sources and metabolites was used to study the metabolic activities of the cells in more detail. As shown in ***Figure 18***A, in which the glucose concentration of STR16 and STR17 are plotted over time, glucose was taken up exponentially until it was depleted 140 hours after inoculation. In course of STR16 no depletion of glucose occurred, as cell death was induced much earlier (approximately 110 hours after inoculation). Nevertheless, a clear trend is shown in ***Figure 18***A: the glucose concentration in the media is not significantly different for the first 100 hours, showing deviations of less than 5%. From that time on the glucose concentration was higher regarding STR16, in which the slower growing strain HEK293γ was used. 110 hours after inoculation the difference was even more than 30%. In ***Figure 18***B the lactate concentration is plotted

over time for both cultivations. As long as glucose was metabolised lactate was produced. After the complete consumption of glucose, lactate was used as a carbon source, a fact underlined by decrease of the lactate concentration in the suspension. In literature comparable results may be found for different cell line, for example Burky (2007) *et al.* cultivated NS0-derived cell lines and published that lactate rise relates to glucose consumption during early culture. As soon as glucose becomes depleted, cells are forced to use lactate as an energy source.

Again, no clear difference can be detected for the first 87 hours, from this time point on up to 17% less lactate is produced by clone HEK293β.

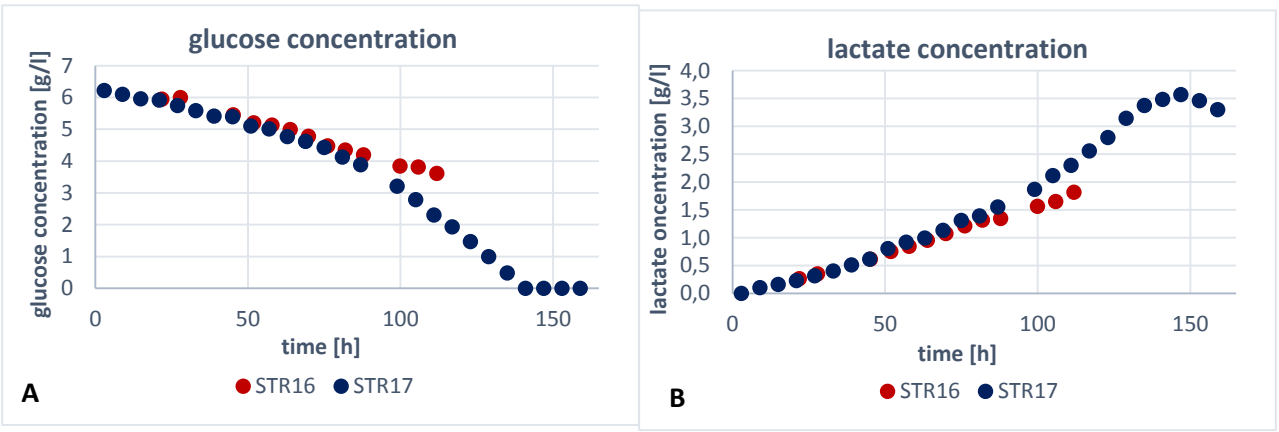


Figure 18 On-line measured glucose and lactate concentrations in the media: (A) glucose concentration; (B) lactate concentration;

red, HEK293γ cultivated in course of STR16; blue, HEK293δ cultivated in course of STR17;

Based on on-line measured metabolite concentrations and in-line determined viable cell counts physiological parameters were evaluated in real-time (***Figure 19***A-D).

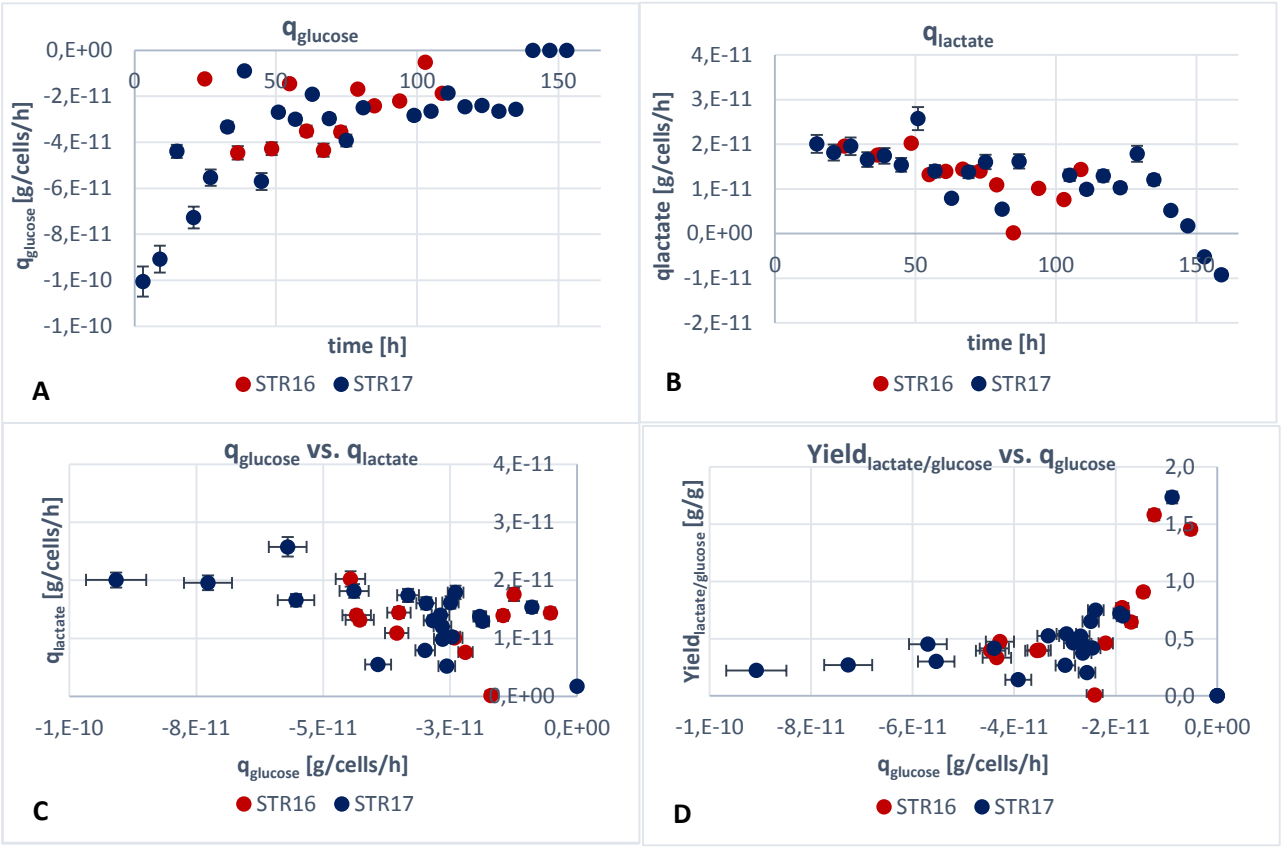


Figure 19 Specific rates and yields for glucose and lactate comparing STR16 and STR17: (A) specific glucose uptake rate over time; (B) specific lactate production rate over time; (C) specific lactate production rate over specific glucose uptake rate; (D) Yield lactate/glucose plotted over the specific glucose production rate;

red, HEK293γ cultivated in course of STR16; blue, HEK293ϐ cultivated in course of STR17;

Considering the specific rates, $q_{glucose}$ decreased continuously until it was fully consumed after 140 hours (***Figure 19*****A**). That decrease concomitant to decreasing lactate production rates (***Figure 19*****B**). This indicates that both rates are strongly related. Therefore this correlation is more closely investigated by plotting both specific rates against each other (***Figure 19***C), which again shows that an increased glucose consumption rate is associated with a higher lactate production rate. Considering process optimization and strain selection it should be kept in mind, that for a reduced release of potential harmful lactate into the supernatant, a lower specific glucose consumption rate should be aspired.

Comparing STR16 and STR17 there is no significant visual difference concerning the specific glucose uptake and specific lactate production rate. Therefore it can be concluded that the divergence in the glucose and lactate concentration in the media could only be related to different viable cell counts.
Moreover, the specific rates were used to calculate yields in real-time. In ***Figure 19***D the yields for STR16 and STR17 are plotted over the specific glucose uptake rate. Obviously, a lower glucose uptake rate leads to a lower $Yield_{lactate/glucose}$. Again, there is no significant difference between STR16 and STR17.

Glutamine uptake and glutamate and ammonia production

Similar to the glucose and lactate metabolism, the uptake of glutamine and production of glutamate and ammonia were compared for clone HEK293β and HEK293γ.
In ***Figure 20***A-C the concentrations of the metabolites in the media are plotted over time. Glutamate uptake was already discussed in the previous chapter "*Detection of Process Events".* GlutaMax was used as glutamine source, which is a stabilized dipeptide of L-Alanyl-L-Glutamine. The dipeptide was hydrolysed by gradually released peptidases, which leaded to increasing glutamine concentrations in the first 50 hours. A production phase is followed by glutamine uptake and finally, although there is still glutamine present, it is not metabolised any more, the concentration is maintained or even increased (in case of STR17). This increase in glutamine is further supported by the apparent consumption of ammonium beginning 150 hours after induction. Ammonia is used in glutamine anabolism via the enzyme glutamine synthetase. Finally, 4 different phases in glutamine metabolism can be clearly distinguished.
Concerning HEK293γ cultivated in STR16 less glutamine was produced from L-Alanyl-Glutamine and furthermore less glutamine was taken up resulting in a higher concentration in the media. Those differences in the glutamine concentrations were temporally 26%.
Glutamate production is directly related to the glutamine metabolism, as glutamine is converted to glutamate by the enzyme glutamase. Glutamate was not produced during cell growth phases and showed only increasing concentrations at cell death phase (***Figure 20***B). As the glutamate concentration changed only slightly over time, a reliable measurement is difficult and almost not possible using this approach, as already discussed in "*Analytical parameters describing the on-line monitoring system"* and therefore this thesis cannot enter this point in a detailed manner. Nonetheless, the general statement that the concentration of glutamate was lower within STR16 might be made.
The ammonia production is also coupled with glutamine metabolism and therefore the concentration of potentially harmful ammonia increases over time. In course of STR17 the concentration nearly

quadrupled from 0.03 g/L to 0.12 g/L (***Figure 20***C). Within STR 16 the concentrations are quite similar the first 70 hours but then started to develop differently leading to 30% lower concentrations. The consumption of ammonia beginning 150 hours after induction is difficult to detect on basis of this raw data.

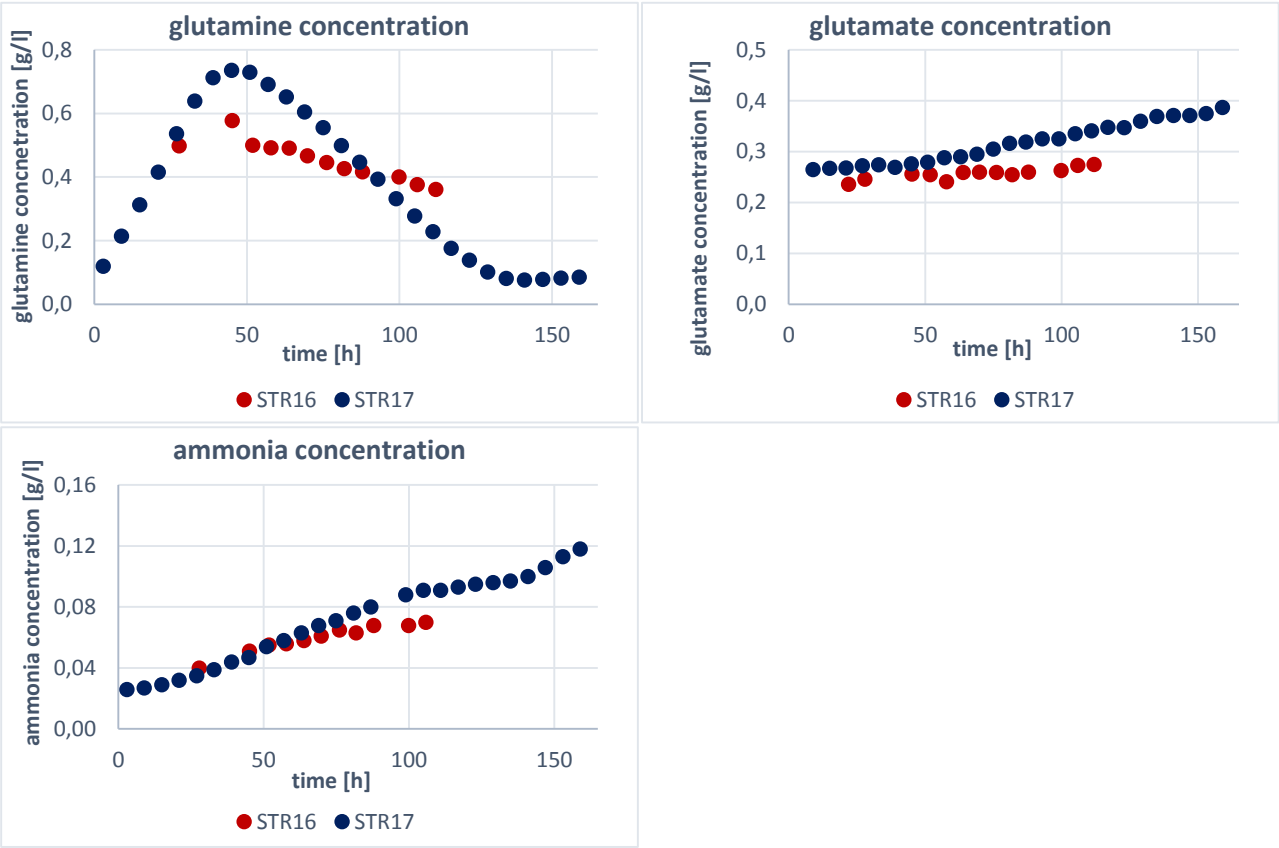


Figure 20 On-line measured glutamine, glutamate and ammonia concentrations in the media: (A) glutamine concentration; (B) glutamate concentration; (C) ammonia concentration

red, HEK293γ cultivated in course of STR16; blue, HEK293β cultivated in course of STR17;

As soon as the viable cell count is included in the considerations, it can be seen that there is no significant physiological difference between the two cell clones (***Figure 21***), that again illustrates the importance of specific rates and moreover the importance of a reliable tool for their quantification.

In case of the specific glutamine uptake rate the dipeptide of L-Alanyl-L- Glutamine was first cleaved and glutamine was released, which is demonstrated by a positive rate. Further glutamine uptake by the cells led to a negative $q_{glutamine}$ (***Figure 21***A). At the end of the cultivation again a slightly positive specific glutamine uptake rate was monitored as it was produced from ammonia.

The specific ammonia production rate assumed relatively high values at the beginning of the cultivation (***Figure 21***B). Furthermore another strong increase can be detected starting approximately 120 hours after the induction. A final decrease results only until the end of the cultivation.

If both specific rates are plotted against each other it could be seen that ammonia is strongly produced within the glutamine production phase and generally at higher glutamine concentrations. Similar off-line determined data can be found in the literature for mammalian cell cultures. Slivac *et al.* (2010) cultivated channel catfish ovary cell lines and found that ammonia production was most abundant in cultures with high glutamine concentrations, which may be due to cell metabolic activity or spontaneous glutamine degradation.

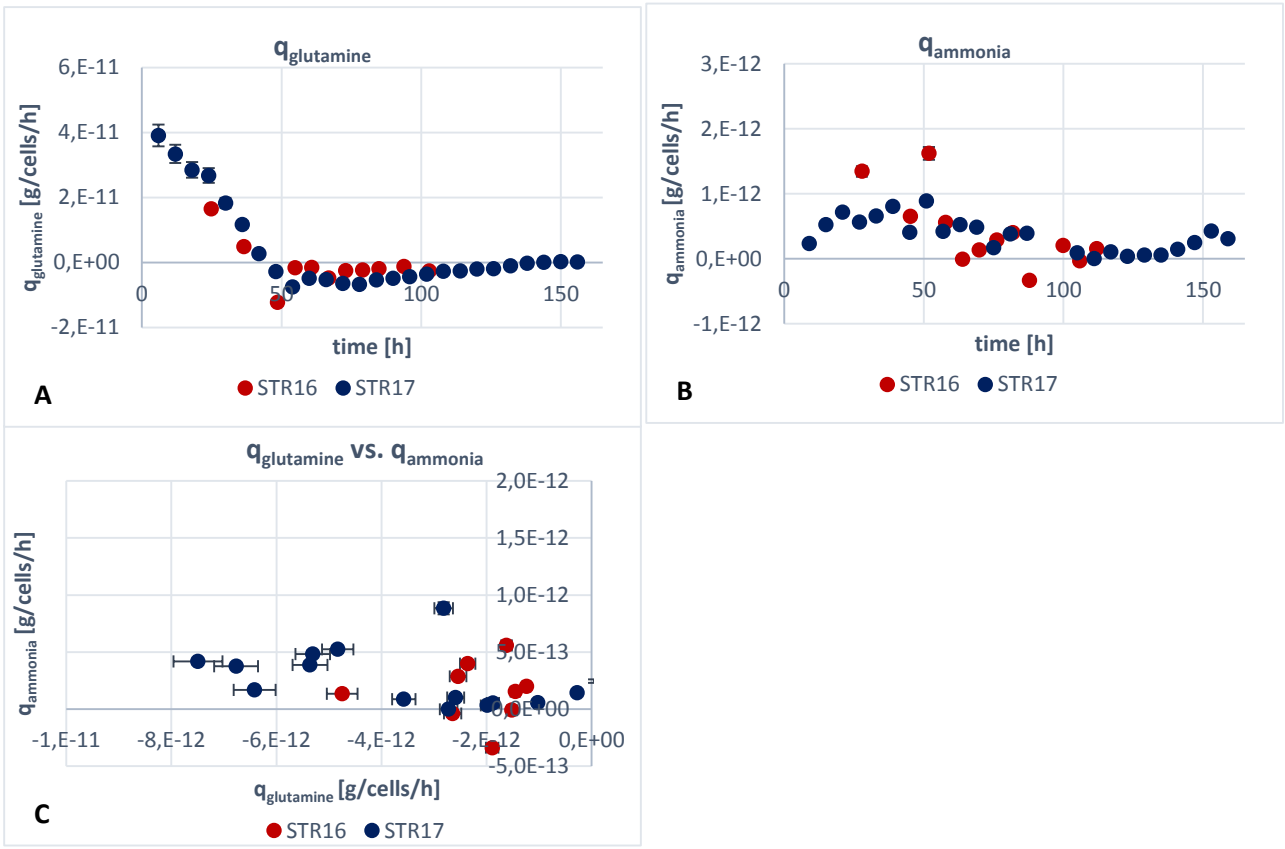


Figure 21 Specific rates and yields for glutamine and ammonia comparing STR16 and STR17: (A) specific glutamine uptake rate over time; (B) specific ammonia production rate over time; (C) specific ammonia production rate over specific glutamine uptake rate;

red, HEK293γ cultivated in course of STR16; blue, HEK293ϐ cultivated in course of STR17;

Conclusions on clone selection

Using the on-line monitoring device, glucose and glutamine uptake as well as the production of waste products (lactate and ammonia) can be quantified and physiological parameters determined. Regarding the concentrations of metabolites in the media, differences between both cultivations can be detected. Taking the viable cell count into considerations and calculating specific rates and yields, it becomes obvious that this differences only occur because of the variable biomass. The two cell clones did not show any physiological differences regarding the glucose and glutamine uptake or ammonia and lactate production.

A possible goal of clone selection may be finding a cell clone which produces only low amounts of ammonia and lactate, as this waste products inhibit cell growth [Hassel *et al*. 1991; Lao 1997] and have also a negative effect on product formation [Lao 1997]. The two cell clones which were discussed in this thesis show no significant differences and therefore further decision criteria such as the product yield may be included in the selection process.

Nevertheless, this robust on-line tool can be used determine specific rates of metabolites and carbohydrates in real-time and can therefore be used for a fast comparison of different cell clones.

4.3. On-line applicability

In the previous chapters different examples for the application of the on-line monitoring approach were shown. Thereby the advantages of the system such as high resolution or low amount of work, which is involved, were highlighted and the accuracy and precision of the off-line methods were proven. Nevertheless, the question rises whether on-line provided data are comparable to conventional off-line records. To find an answer to this question specific rates were calculated using four different approaches. First of all q_s was determined using off-line systems. These data was compared to q_s determined using the CuBiAn in off-line mode and the in-line cell count device and vice versa. And last but not least completely on-line measured records were used for the calculations of q_s. ***Table 13*** summarizes the approaches just mentioned.

Table 13 Different approaches for the determination of specific rates

name	CuBiAn	cell count
off-line+off-line	off-line	off-line
off-line+on-line	off-line	in-line
on-line+off-line	on-line	off-line
on-line+on-line	on-line	in-line

The specific substrate uptake rates for glutamine were determined off-line+on-line, on-line+off-line and on-line+online and were plotted over off-line+off-line calculated q_s (***Figure 22***).

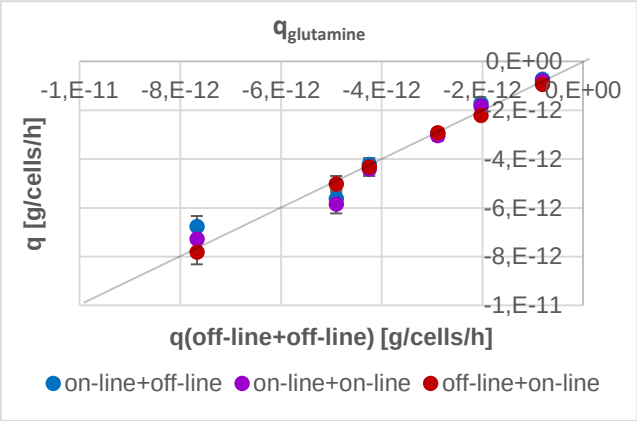


Figure 22 Specific glutamine uptake rates determined using four different approaches to proof the on-line applicability.

blue, on-line+off-line; purple, on-line+on-line; red, off-line+on-line;

The completely off-line determined data show a perfect correlation to those glutamine uptake rates calculated using off-line measurement of the metabolite concentration and an in-line capacitance probe (***Figure 22***). This accordance of almost 100% is a result of the calibration model of the dielectric spectroscopy probe.

The two data-sets which use the CuBiAn XC in on-line mode do not show such a good but also a satisfying correlation. Because of this good accordance and the general advantages of the on-line monitoring system (high resolution and less work intensive) the usage can be recommended.

These results correspond to the findings of Dietzsch *et al.* 2013. In course of their study they could also show that the on-line and off-line determined data described the same values.

Summing up, it can be stated that a novel tool for the determination of specific rates in real-time was found, which can be used for monitoring of metabolites, for media and feed development and feed control. Furthermore it enhances clone selection. All these points underline not only its field of application but also its importance regarding bioprocess development.

5. CONCLUSION

Human embryonic kidney (HEK) cells, and mammalian cell cultures in general, are intensively used in the development of viral vaccines, anticancer agents and the production of recombinant adenoviral vectors. However, their metabolism is far more complex than those of microbials, which makes the achievement of high cell and, more important, product titers extremely difficult. Today, the aspects of product quality as well as cost and time efficiency become more and more important and therefore gaining scientific knowledge and demonstrating process understanding is a big issue not only in accordance to the FDA. Bioprocess development (bpd) pursuing goals like transferability, scalability and process optimization is a key aspect of bioproduction. Furthermore, it also emphasises the achievement of higher yields by selection of better expression systems and gaining scientific understanding to minimize fail batches and avoiding limitations and deviations throughout the cultivations. Basically, this work addressed the last two points. The usefulness and added value of on-line and in-line measurements were evaluated to deduct physiological key parameters and accelerate bioprocess development and characterization of HEK cells.

5.1. Characterization of the method

To establish an on-line monitoring device for the real time measurement of metabolites a fully-automated, membrane- free biochemistry analyser, based on photometric principles (CuBiAn XC, Optocell, Germany) was connected to the bioreactor set up. Hence, a physical connection and moreover an appropriate software interface to create an integration in the process management system were realised. This system had already been applied for microbials [Dietzsch 2012] and was now adapted to mammalian cell cultures. In order to make it useable for cell culture systems it was not only necessary to measure different substances but also to meet the high sterility requirements and to adopt the technique to a completely different physiological system. Therefore, analytical parameters such as the Limit of Detection, the Limit of Quantitation, the linear working range and the precision and accuracy were determined in the run- up using the device in off-line mode. The acceptance criteria for accuracy and precision (90-110% recovery; 2% for the photometric robot and 10% for cell count) were fulfilled for all methods. Based on the calculated LOQ a measurement

frequency of every six hours was fixed. This is another difference to microbial systems were a frequency of 0.5 hours or even higher is used.

This method was applied five batch cultivations of three different HEK cell clones and two different glutamine sources. In course of these fermentations and three cell-free runs, the handling of the membranes, which turned out to be possible subjects of contaminations, was studied and a recommended approach was developed. These advices suggest not only a cleaning routine involving several washing cycles with 1 M NaOH and water after each cultivation and simple optical test before and after usage but also more elaborating bacterial retention tests.

The viable cell count was determined in-real time using an in-line capacitance probe (Fogale BIOMASS SYSTEM, Fogale nanotech, France). This system had been already established for several microbial, plant and also mammalian hosts and also the application on HEK cell cultures was done before [Markx *et al.* 1991; Fehrenbach 1992; Cerckel *et al.* 1993; Konstantinov *et al.* 1994; Petiot *et al.* 2011].

A calibration model for the system was developed based on off-line determined viable cell counts, using an image based analyser (Cedes HiRes). In contrast to the literature, where mainly a dual frequency system based on two more or less randomly chosen frequencies, is applied, [Ansorge 2007; Petiot 2012], MLR was used after variable selection. MLR is easy and fast to use and needs only the exclusion of mulitcolinear variables, which can be achieved by VIF. Therefore the device can be perfectly adjusted to the specific cultivation system, e.g. the medium or the organism. The application of the calibration on several cultivations showed an excellent correlation for the exponential growth phase of the cells, even for different cell clones with a highly different growth profile.

5.2. Physiological characterization of different HEK cell clones

Five components (i.e. glucose, lactate, glutamine, glutamate and ammonia) were analysed with this on-line monitoring system, whereat the device was not sensitive enough to quantify the change in the glutamate signal. A good correlation between on-line and off-line determined concentrations was found for all methods, as the deviations accounted for maximal 10%.

Because a high sampling frequency can be applied in course of the on-line measurements, high resolutions can be achieved and consequently the dynamic of the culture can be imaged perfectly. Based on that, process events, such as limitations of nutrients or accumulations of substances can be

easily identified in real-time, providing a first basis to take counteractions. Especially glucose and glutamine turned out to be critical substrates and were examined in more detail. Concerning the glutamine uptake, using GlutaMax as glutamine source, it can be distinguished between three distinct phases as long as off-line determined data is discussed. First the GlutaMax dipeptide was hydrolysed by gradually released peptidases, which leaded to increasing glutamine concentrations in the first 50 hours. This cleavage phase is followed by glutamine uptake and finally, although there is still glutamine present, it is not metabolised anymore, the concentration is maintained.

Regarding the on-line data set the cleavage of the dipeptide and uptake of glutamine are also followed by a phase at which the glutamine concentration is maintained constant. But the high sampling frequency helps to detect a fourth phase at the end of the cultivation: a glutamine production phase. This increase in glutamine is further supported by the apparent consumption of ammonium beginning 150 hours after induction (data not shown). Ammonia is used in glutamine anabolism via the enzyme glutamine synthetase. Therefore 4 distinct process phases can be differentiated. This last production phase could not be detected using off-line sampling.

In course of the glucose concentration in the media a limitation should be strictly avoided as it is the main substrate. A high frequent on-line monitoring device is the main requirement in developing intelligent controlling strategies, because it can be detected in real-time if the concentrations falls under a certain limit.

Using the determined on-line and in-line values incremental rates and yields could be generated. That has been done based on the metabolite concentrations measured with the on-line coupled CuBiAn XC and the in-line determined viable cell counts using Fogale BIOMASS SYSTEM. Physiological considerations could be drawn using these specific rates and yields, which allowed a deeper insight in the metabolism of different cell clones. The generation of real-time information enhances the process of strains selection and therefore supports and fastens bioprocess development.

It could, for instance, be shown that if two cell clones perform very differently concerning their growth profiles, they may, nonetheless, be physiologically identical. Apparent differences in metabolite concentrations may be traced back to just different viable cell concentrations.

5.3. On-line applicability

To show that on-line provided data are comparable to conventional off-line records, specific rates were determined using four different approaches. First of all q_s was determined using off-line systems. These data was compared to q_s determined using the CuBiAn in off-line mode and the in-line cell count device and vice versa. And last but least, completely on-line measured records were used for the calculations of q_s. These rates showed not only a good correlation to each other but also to completely off-line determined rates. As no significant difference between the off-line and on-line determined data could be detected and the system shows major advantages, its usage can be recommended.

Summing up, it can be stated that a novel tool for the determination of specific rates in real-time was found, which can be used for monitoring of metabolites, for media and feed development and feed control. Furthermore it enhances clone selection. All these points underline not only its field of application but also its importance regarding bioprocess development.

Conclusions at a glance

- The CuBiAn XC is an accurate and precise on-line monitoring device for the determination of metabolite concentrations applied HEK cell clones.

- The system had already been applied to microbials [Dietzsch 2012] and the implementation on mammalian cell cultures illustrates the flexibility and adaptability of this device.

- For the estimation of the viable cell count via an in-line dielectric spectroscopy probe, a calibration based on off-line cell counts has to be done. MLR is a fast and easy opportunity and provides satisfying data in the growth and production phase of the cells.

- The off-line and on-line determined metabolite concentrations differ by maximal 10%.

- The combination of the off-line metabolite measurement device and the in-line impedance sensor can be used to calculate reliable specific rates and yields, thus generating process information in real-time.

- The application of the on-line monitoring device on different cell clones, showing a different growth behaviours proves the robustness of the method.

- The high frequent determination of physiological data allows to image the dynamic of the culture precisely.

- Process events, like accumulations and limitation can be detected in real-time.

- Supports and fastens bioprocess development.

- Physiological considerations could be drawn using specific rates and yields, which allowed a deeper insight in the metabolism of different cell clones and enhances the process of strains selection.

- It is a useful tool for media and feed optimization, as well as adjustment of the feed rate to match changing nutrient demands.

6. OUTLOOK

Further developments may be carried out concerning the integrity test of the membrane filters. A possibility might be bacterial retention test. The membranes are challenged with a solution of culture medium containing bacteria (recombinant Escherichia coli strain, with a resistance for e.g. ampicillin). The effluent is spread onto an ampicillin- agar plates and incubated to allow growth of E. coli cells that passed through the filter membrane and carry the resistance gene.
This test might help to reduce the contamination risk and to enhance the robustness of the system.

Another approach for a possible improvement is the measurement frequency. As mentioned in *"Analytical parameters describing the on-line monitoring system"* on-line samples were taken every six hours and the concentration of all five metabolites was determined, although it is not reasonable for most of them to apply such a high sampling frequency. In **Table 8** the recommended frequencies were listed. It illustrates that based on the signal-to-noise ratio an individual sampling plan for all methods should be developed.
Furthermore, an average specific substrate uptake rate is currently used to calculate an average sampling frequency. But as q_S changes throughout a cultivation it would be beneficial to adjust the frequency correctly. Hence, more samples would be taken in phases which show higher cellular activity and which need therefore more intense observations. For the calculation of q_S off-gas data might be used.

Lessons learned

If this thesis should be carried out again, the experiments would be designed more carefully and forward-looking. More attention would be paid to a correct validation of the analytical methods, especially regarding reproducibility and reliability.

7. TABLE OF FIGURES

8. TABLE OF TABLES

9. APPENDIX

9.1. Standard operating procedures

9.1.1 Manual cell count in suspension/attached cell culture

TECHNISCHE UNIVERSITÄT WIEN Vienna University of Technology	**Standard Operating Procedure**	Bioprocess Technology
	Manual cell count in suspension/ attached cell culture	
Research Division ***Biochemical Engineering***	SOP Number: SOP37 1.0 Status: Effective	Date: 05.04.2013

Version	Number 1.0
Replaced version	None
Author	Feichter Melanie
Date	05.04.2013
Signature	
Authorized by	
Date of authorization	
Signature	

Summary	Biomass measurement in suspension/attached cell culture using Trypan Blue/Crystal Violet assay

Materials

- Haemocytometer Neubauer improved
- Crystal Violet 0.1% (Fluka 61135-25G) in 0.1M citric acid

- Trypan Blue (gibco 15250-061) 0.2% in phosphorous buffered solution (PAA H12-002)
- Diluent (tap water)
- Sample

Equipment

- Microscope with a 100x and 200x magnification

1. Sample preparation – Suspension Cells

a) Transfer at least 1 ml of a representative sample in an Eppendorf tube.
b) Prepare a 1:2 dilution (for Example 200 µL 0.2 % Trypan Blue + 200 µL cell suspension).
c) Vortex the tube.
d) Start cell counting immediately. Proceed with section 3.

2. Sample preparation – Attached cells

a) Transfer 1 ml of a representative sample in an Eppendorf tube (cells with microcarriers).
b) Wait until microcarriers have settled down.
c) Remove 800 µl of the supernatant.
d) Pipet 800 µl of Crystal Violet solution onto the remaining microcarrier pellet and supernatant, resuspend it by vortexing slightly or by pipetting up and down and incubate for 30 minutes at 37°C.
e) The cells are subjected to a slightly hypotonic solution and the nuclei are stained. Proceed with section 3.

3. Haemacytometer

a) Turn on the light of the microscope and select the 20x lens.
b) While the lamp is setting a constant lumination, set up the haemocytometer.
c) Clean the cover glass piece and the chamber with tap water. Pat it dry to prevent scratches on the surface.
d) Breathe onto the chamber and then put the glass cover on it.
e) Pipet up to 15 µl of your prepared sample into the edge of the coverslip of the first and then of the second chamber. Do not overfill the chamber.
f) Use an appropriate dilution of your sample. You should be able to count between 10 and 100 cells.

4. Cell counting

a) The main divisions separate the grid into 9 large squares. Count the cell in the four corner squares (named A, B, C and D in Figure 1).

b) There are three fine lines on the borders of a big square. In every square the cells positioned at the left and upper site should be excluded from counting, and only on the right and the lower site should be taken into account, as indicated in Figure 1. Furthermore, only those cells, which are touching the first line should be counted. The cells on the second or third line should not be regarded.

c) For a representative result, an average of all 8 squares on the haemocytometer is used.

d) Reference pictures attached (what is a living cell, what is a dead cell, what is a particle/cell - Figure 242 and 3).

e) Crystal violet is also staining cytoplasm and dirty particles, so be careful to only count objects with the right size and shape. Nuclei can be also flat (Figure 242 d and h).

f) Calculate cells using the following equation:

$$\frac{\sum squares}{n\ squares} \times dilution \times 10^4 = \frac{cells}{ml}$$

Example: 35 cells in an undiluted sample are 350 000 cells/ml.

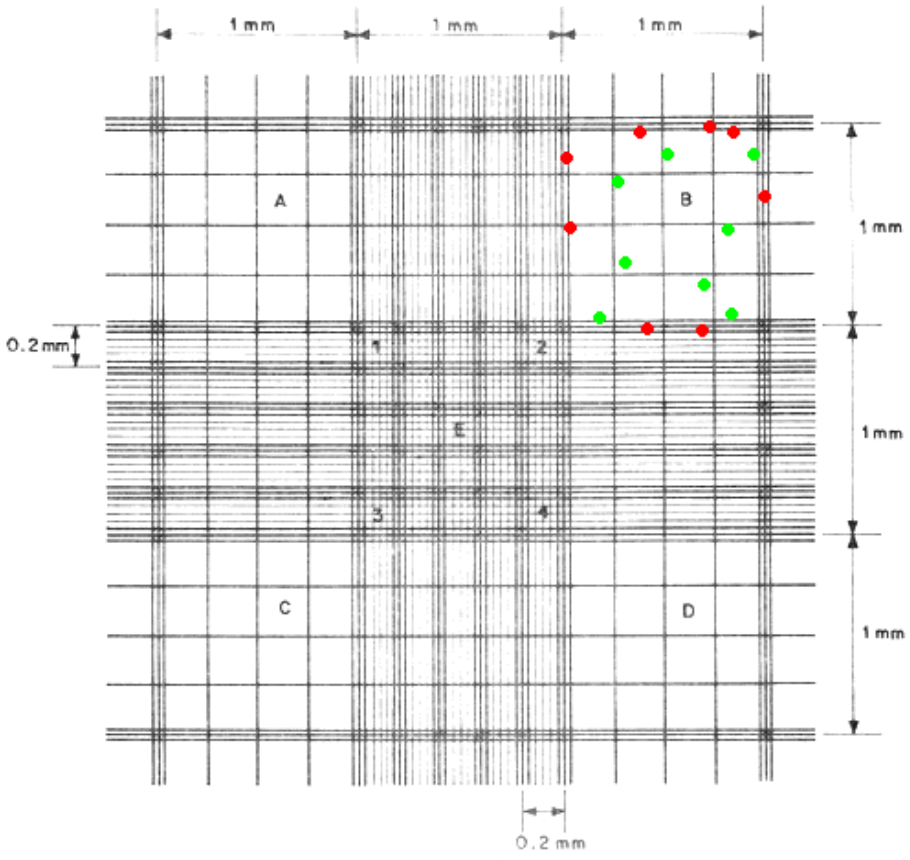


Figure 23: Improved Neubauer Haemocytometer
Green cells, should be counted; red cells should be excluded from counting

Cleaning

1. After completing the count, remove the cover glass and clean the counting chamber with water.
2. Dry the counting chamber with a soft cloth or wipe.

Reference Pictures

Cristal Violet Method

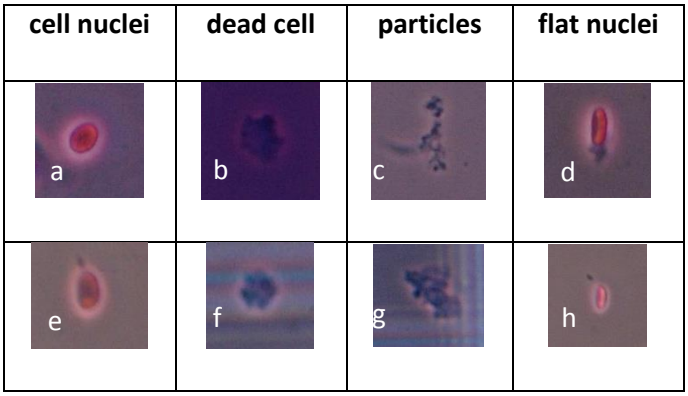


Figure 24: Reference pictures of cells stained with Crystal Violet solution

Trypan Blue Method

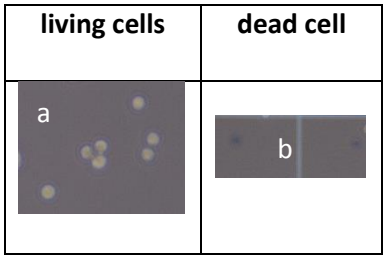


Figure 3 Reference picture of cells stained with Crystal Violet solution

Literature

- ATTC: **Animal Cell Culture Basics. Tips and Techniques for Continuous Cell Lines.** January 2012. 10801 University Blvd. Manassas, VA 20110

End

9.1.2 Cell number measurement of suspension/attached cell culture by Cedex HiRes

Version	1.0
Replaced version	None
Author	Andrea Egger
Date	22.10.2013
Signature	Andrea Egger
Authorized by	Christoph Herwig
Date of authorization	28.10.2013
Signature	

Summary	Cell number measurement of suspension/attached cell culture by Cedex HiRes
Remarks	- For cells which tend to adhere to the surface of the sample cups only one sample should be measured at a time, otherwise you would falsify your measurement results (see reference data and procedure below). - For cells which tend to form big aggregates ACCUMAX™ should be used for cell detachment (see reference data and procedure below).

Materials

- Cedex Reagent Kit (Roche): Detergent Solution (Product Number: 05650658001), Trypan Blue (Product Number: 05650640001), Cleaning Solution (Product Number: 05650666001), MQ water
- Cedex Sample Cup (Roche), Product Number: 05650623001
- Diluent (cell culture medium or PBS)
- Sample
- Pipette tips for a 1000 µl pipette
- ACCUMAX™ (Millipore) if required, Product Number: SCR006

Equipment

- Cedex HiRes
- Pipette 1000 µl

Sample preparation

1. Transfer at least 500 µL of a representative sample in an Eppendorf tube.
2. Mix the sample gently by moving it up and down in the hand.
3. Transfer 300 µl of representative sample into a sample cup.
4. Start cell counting immediately.
5. If your cells tend to adhere to the surface of the sample cup measure only one sample a time and wait till the measurement is finished before placing a new sample in the machine.

Sample preparation for cells which tend to form aggregates

6. Bring ACCUMAX™ to room temperature.
7. Harvest a representative sample of clumped cells, 0.5 or 1.0 ml, and transfer it into an Eppendorf tube.
8. Add the same volume of ACCUMAX™ to the sample and incubate for 5 to 10 minutes at 37° C.
9. Transfer 300 µl of the sample-ACCUMAX™ mix into a sample cup
10. Start cell counting immediately.
11. If your cells tend to adhere to the surface of the sample cup measure only one sample a time and wait till the measurement is finished before placing a new sample in the machine.

Preparing the analyser for measurement

12. Turn on the PC and the Cedex Hires and login as Win-Admin, password Win-Admin.
13. Check that the Cedex server software has already automatically started. If not, start the server by double-clicking on the **Cedex Server** icon on the desktop.
14. Start the Cedex HiRes Software 2.2 by double clicking the **Cedex Client** icon on the desktop (the Cedex server must be started before the Cedex Client can be started). The log-in window will open.
15. Enter the username: Superuser and the password: Spass.
16. Position an empty and clean sample cup on the first position of the sample tray. Leave this sample cup in its position during the entire measurement and do not remove it. Eject the multi sample tray by gently pushing on the tray only when the system is idle. The tray cannot be ejected if the system is moving or the needle is inserted in a cup. This cup is used for prime, LM

Shutdown, calibration and cleaning routines and is called the '*default cup*'.

17. Click on the **HWM** button in the Cedex **Control Center** or else select **Hardware Management** under the **Functions** menu. The **Hardware Management** window will open.
18. The hardware startup will be carried out automatically.
19. If the hardware management has not been started, the current status field will display the message "*HW not started and LM not primed*".
20. Click on **HW Startup** if required. The hardware management will start and the current status field will display the message "*HW started and LM not primed*".
21. Click on **Prime**. The message "*HW started and LM primed*" appears in the current status field when the **Priming** has been completed.
22. Click on × to close the window.
23. Ensure that the reagent kit is not empty. Check the number of remaining runs on top of the Cedex **Control Center**. If the reagent kit is empty, follow the change-procedure in the next chapter.

Changing the Reagent Kit

24. Open the reagent kit chamber by pushing the front upwards until it clicks into place.
25. Remove the tray and replace the trypan blue reagent with a new one. Fill up the cleaning solution reagent to the required level (see label) and the detergent reagent. Fill up the water container with MQ water to the required level (see label) and dispose the waste container.
26. Place all of the reagent containers, as well as the emptied waste container, in the appropriate positions in the reagent tray.
27. Close the front cover by pulling it all the way down. When the front cover is closed, the capillaries are automatically positioned correctly in the individual containers.
28. Select the **Reagent Kit Status** option in the **Functions** menu of the **Control Center**. The **Reagent Kit Status** window will open.
29. Click on the **Replace all containers** button in right-hand corner of the **Reagent Kit Status** window to confirm the changing of the reagents. The reagent control display at the bottom right hand corner of the **Control Center** will turn from red to green and indicate the number of runs left.

Specifying the measurement mode

Either a single measurement or a multiple measurement with the multi sampler can be performed. Note that the multiple measurement mode should only be used when your CELLS DO NOT TEND TO ATTACH to the surface of the sample cup.

For a single measurement proceed with the following instructions:

1. Open the **Measurement** window by clicking on the **Measure** button in the shortcuts area or by selecting **Single Measurement** in the **Measurements** menu.
2. Enter a clearly defined Reactor ID to describe the sample (e.g., the name of the cell line) in the **Reactor ID** area.
3. Enter a clearly defined Sample ID in the **Sample ID** area (e.g., *P001*). The Sample ID can be freely defined. If the intended measurement is not the first in a row of measurements, i.e. if it has the same Reactor ID as a previous measurement, then the software will automatically enter the consecutive Sample ID into the **Sample ID** area (e.g. *P001, P002,...*).
4. The dilution is set by default to 1:1. Never change the dilution in the dilution drop down list. If you have to dilute the cell suspension recalculate the cell number after measurement. The cell concentrations can be determined in the range between 5×10^4 – 1×10^7 cells/ml. Any higher concentration requires a manual dilution.
5. Select the sample volume of 300 µl in the **Volume** drop-down menu. Only the 300 µl option is available.
6. Select the cell type that optimally represents your cells from the **Cell Type** drop-down list. Some cell types are set by default. It is also possible to create individual cell types (see User Manual).
7. Select the desired precision level in the **Precision** drop-down list. The precision level determines the volume to be analysed. A higher analyzed volume will require a somewhat longer measurement time but more cells will be counted, which will lead to a more precise calculation.

For a measurement with the multisampler proceed with the following instruction:

1. The **Multi Run** window automatically displays all 20 possible sample cup positions.

2. Transfer 300 µl of a cell suspension sample into sample cups and place the cups in the desired locations on the multi sample tray.
3. Click anywhere in the row of the **MultiRun Measurements Preparation** area that corresponds to the location of a sample cup containing a sample. The row will be converted to edit mode, and the sample and process parameters for the measurement can be defined as described before.
4. The rows in which entries are in the process of being filled out will remain in edit mode. To clear the entire list of entries and start with a new list, click on the **Clear List** button on the right-hand side of the **Multi Run** window.
5. Activate the status of each sample row by double clicking in the **Status** field of the row to be activated. The row appears blue, and further modification of the sample information is not possible. Return to the **Edit** status by double clicking on the **Status** area of the sample row to allow for further changes if required.
6. Alternatively, all samples can be activated or deactivated simultaneously by double clicking on the word **Status** in the column heading at the top of the **Multi Run preparation** area. Confirm the control query with **Yes** to accept the modification.
7. After some or all rows have been activated, click on the **Start** button on the right-hand side of the multi run window to start the analysis of the activated measurements.

Switching off the Cedex HiRes Analyzer

1. Click on the **HWM** button in the **Shortcuts** area of **the Control Center** to open the **Hardware Management** window. Alternatively, the **Hardware Management** window can be opened by selecting **Hardware Management** in the **Functions** menu at the top of the **Control Center**.
2. Click on the **LM Shutdown** button in the lower right-hand corner of the window to shut down the liquid management.
3. ***For any further maintenance procedures please refer to the User Manual.***

Reference Pictures

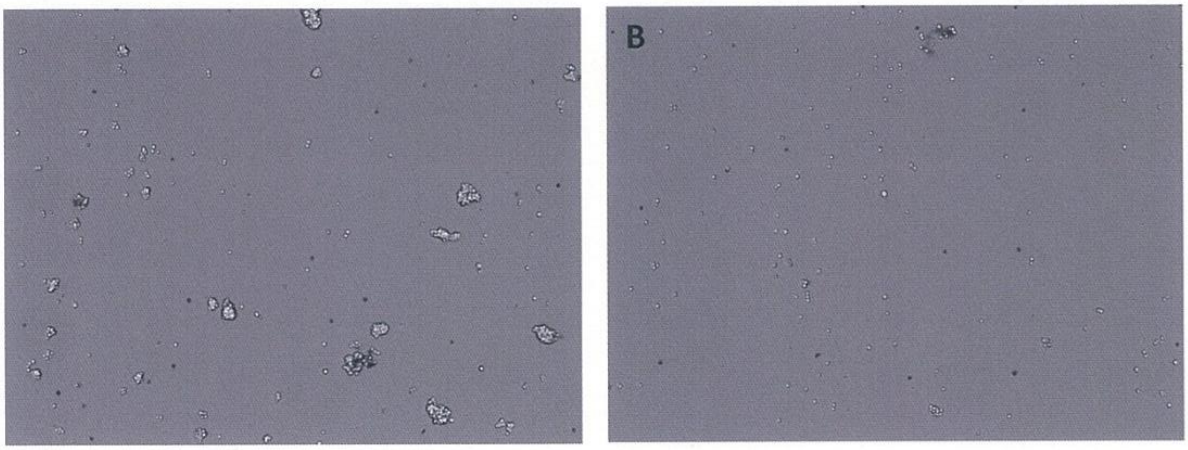


Figure 1: Single measurement of A) untreated cell suspension and B) ACCUMAX™ treated cell suspension. Cells treated with ACCUMAX™ do not form large aggregates compared to untreated cells.

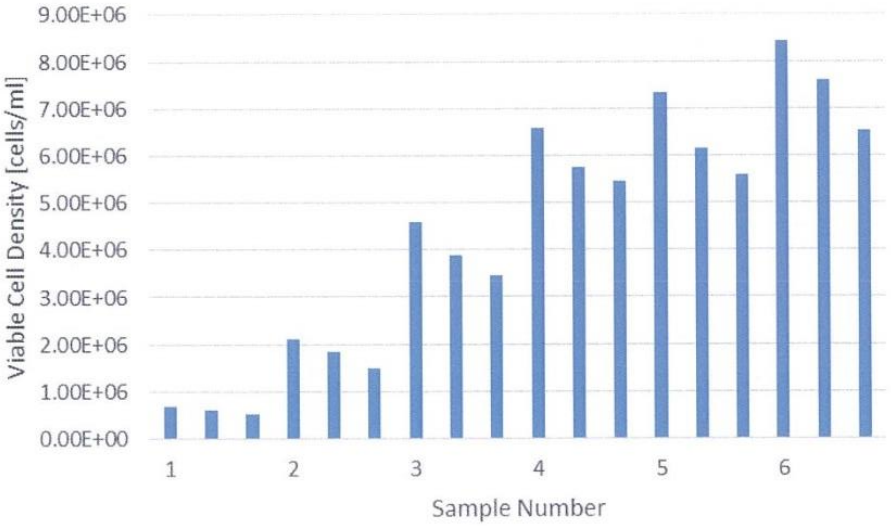


Figure 2: Cedex cell count in triplicates per time point. Cell number decreases per measurement point as cells adhere to the surface of the Cedex sample cups.

Literature

- Cedex HiRes Analyzer Operator's Guide (Software Version 2.2.1)

End

9.2. Applied formulas

calculation inoculum volume [ml]

$$inoculum\ [ml] = \frac{cell\ densitiy\ (\frac{cells}{ml})\ x\ working\ volume\ (ml)\ x\ (MOI)}{Titer\ Vector\ Seed\ (\frac{FFU}{ml})}$$

maximal capacitance difference

$$\Delta\varepsilon = \frac{9}{4}\ P\ \cdot r\ \cdot Cm$$

characteristic frequency for the description of the β-dispersion

$$fc = \frac{1}{2\pi r \cdot Cm\ (\frac{1}{\sigma_{in}} + \frac{1}{2\sigma_{out}})}$$

incremental rate calculation

$$r_{c_i}\left(\frac{t_n - t_{n-1}}{2}\right) = \frac{c_i(t_n) - c_i(t_{n-1})}{t_n - t_{n-1}}$$

specific rate calculation

$$q_{c_i} = \frac{r_{c_i}(t_n)}{c_x(t_n)}$$

calculation of yields

$$Y_{i,j} = \frac{r_{c_i}}{r_{c_j}}$$

arithmetic mean

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

standard deviation

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

signal-to-noise-ratio

$$SNR = \frac{\bar{x}}{s}$$

limit of detection

LOD = 3 * s

limit of quantitation

LOQ = 12 * s

Variance Inflation Factor

$$VIF_k = \frac{1}{1 - r_k^2}$$

9.3. Acquired raw- and processed data

9.3.1 Linear calibration range of the various assays

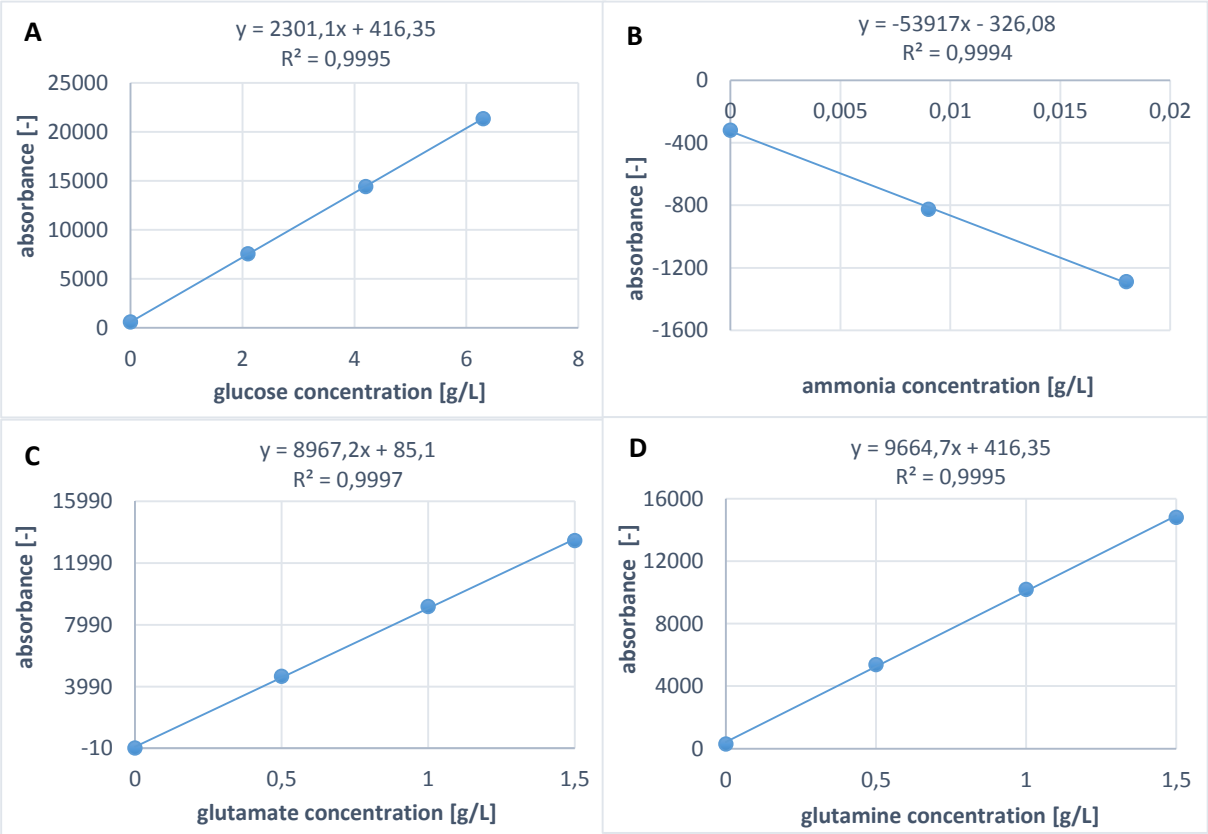


Figure 25 Linear calibration range of the various substrates: (A) glucose; (B) ammonia; (C) glutamate; (D) glutamine;

9.3.2 Acquired data of STR8

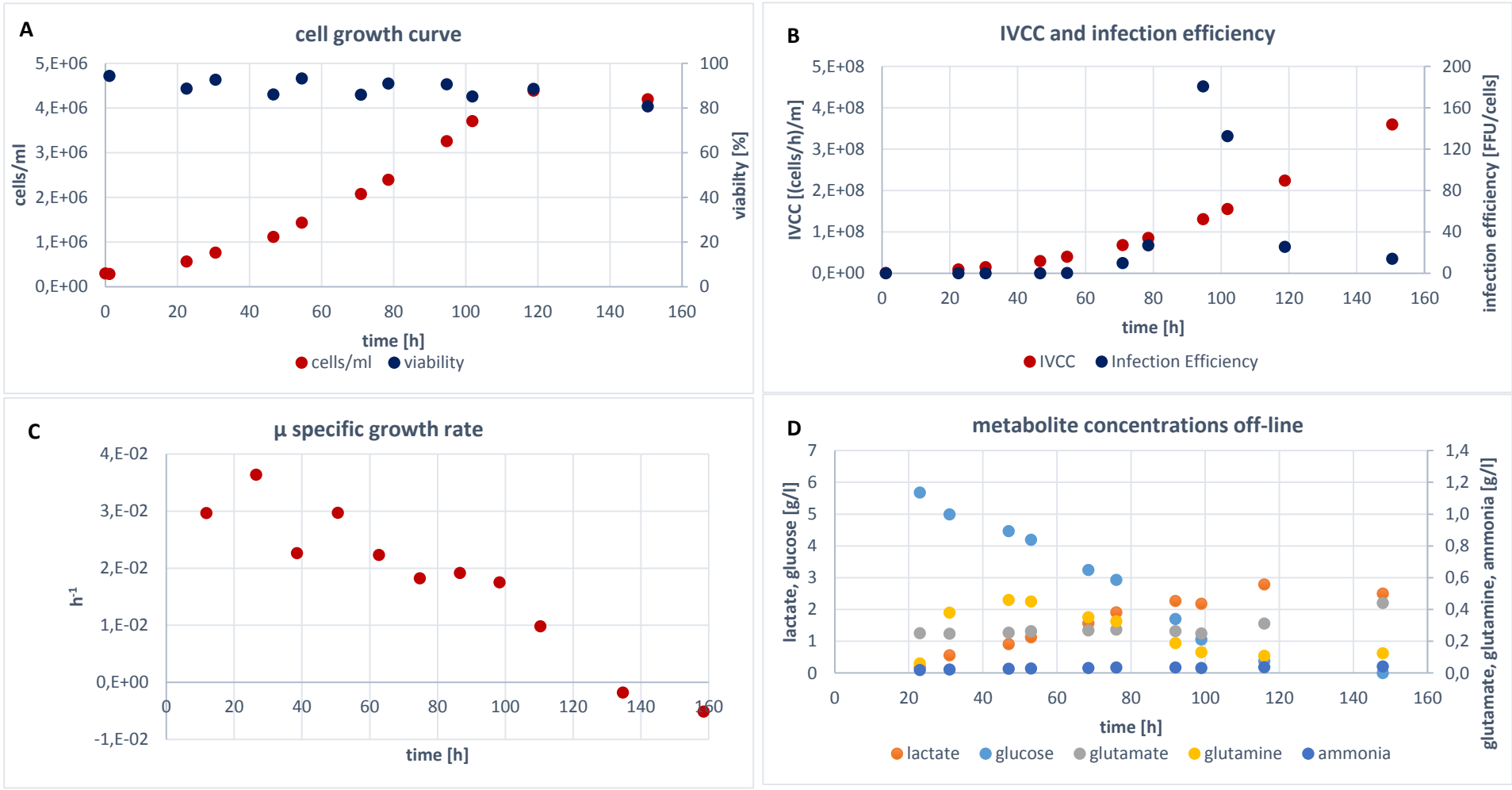


Figure 26 Acquired data of STR8: (A)Viable cell count (red) and viability (dark blue); (B) Integrated viable cell count (red) and Infection Efficiency (dark blue); (C) specific growth rate; (D) off-line measured metabolite concentrations, orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia;

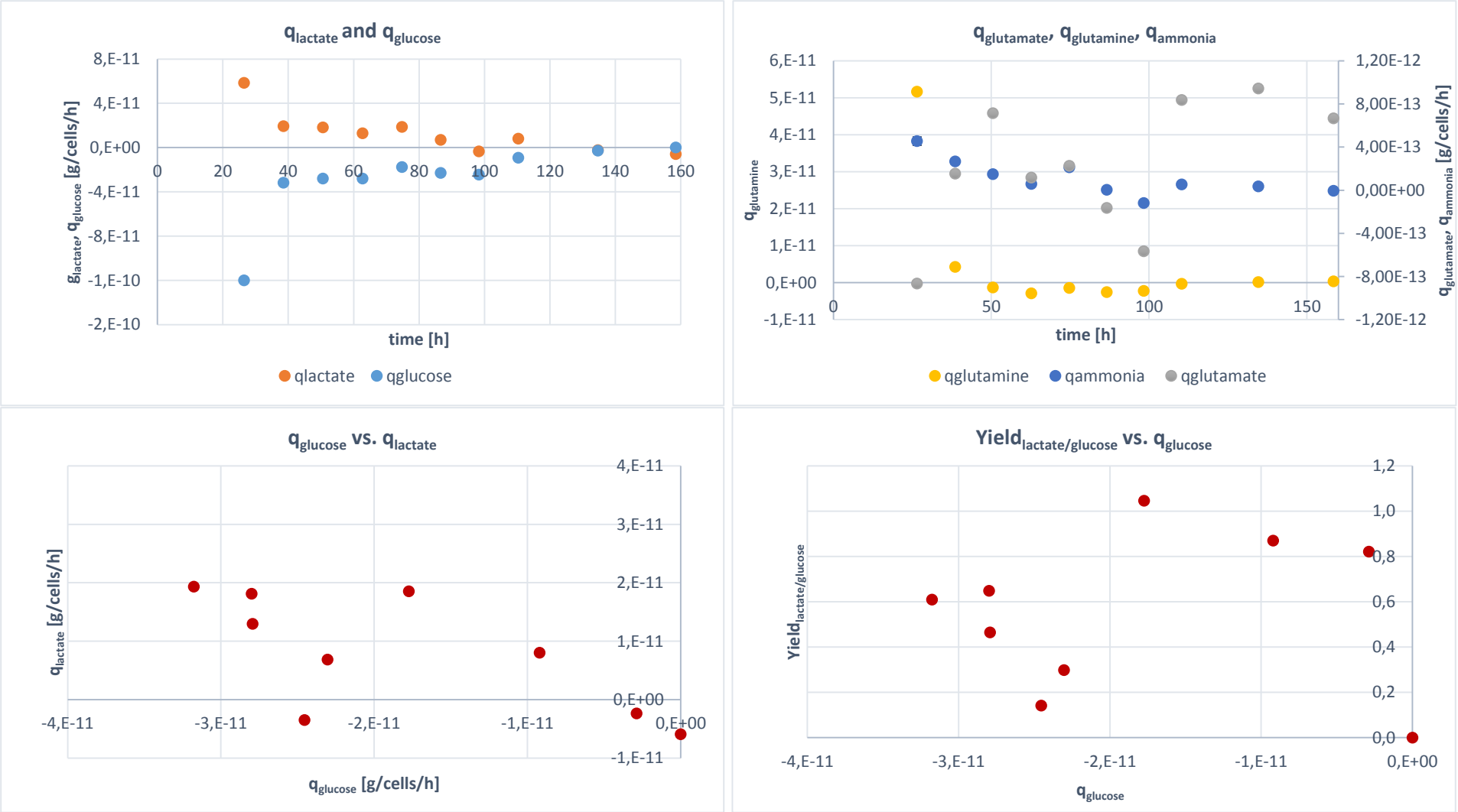


Figure 27 Specific rates and yields of STR8: (A) specific lactate production rate (orange) and specific glucose uptake rate (light blue); (B) specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey); (C) specific lactate production rate plotted over specific glucose uptake rate; (D) $Yield_{lactate/glucose}$ plotted over specific glucose uptake rate;

9.3.3 Acquired data of STR13

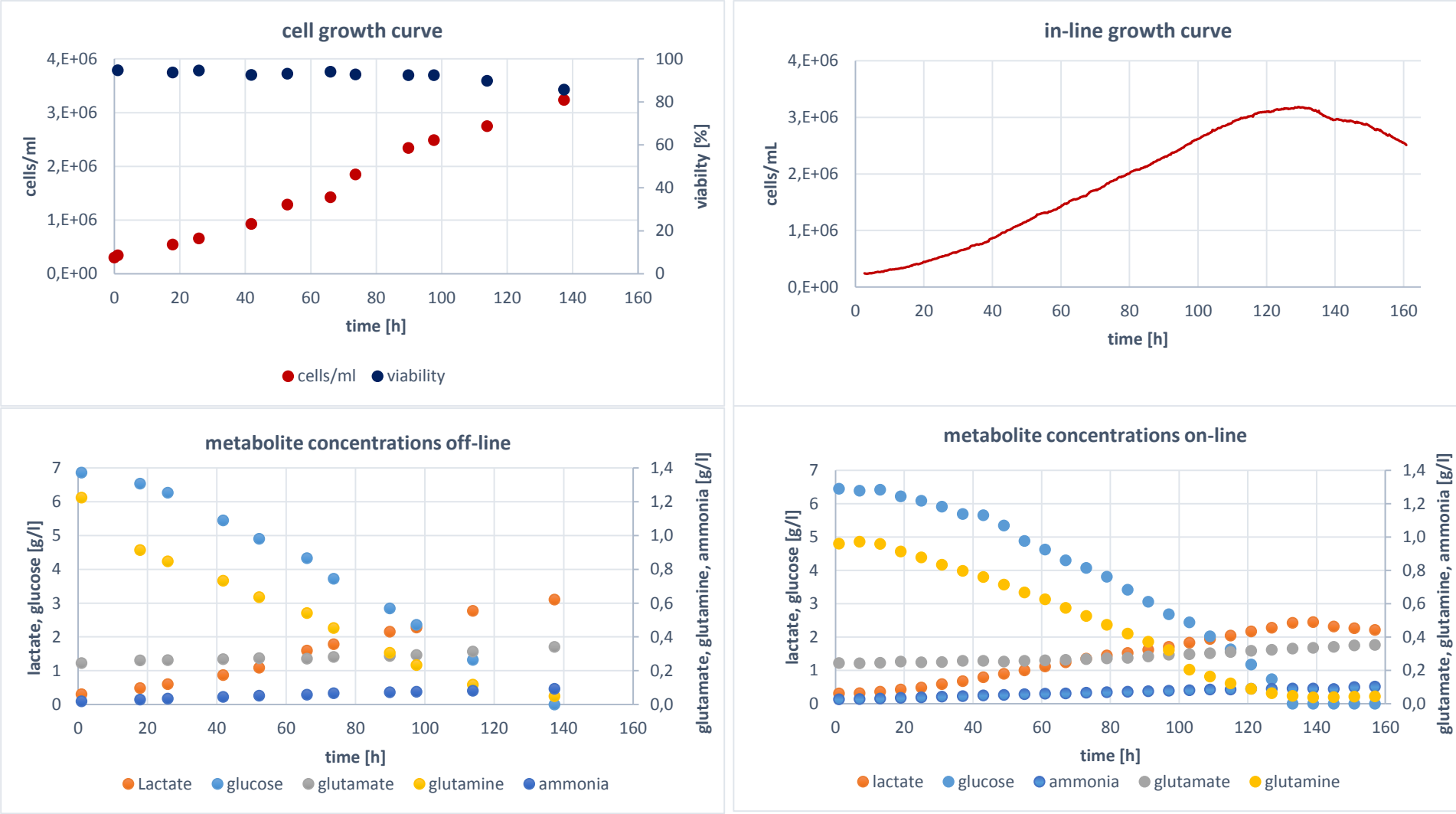


Figure 28 Acquired data of STR13: (A) viable cell count (red) and viability (dark blue); (B) in-line measured viable cell count; (C) off-line measured metabolite concentrations, orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia;

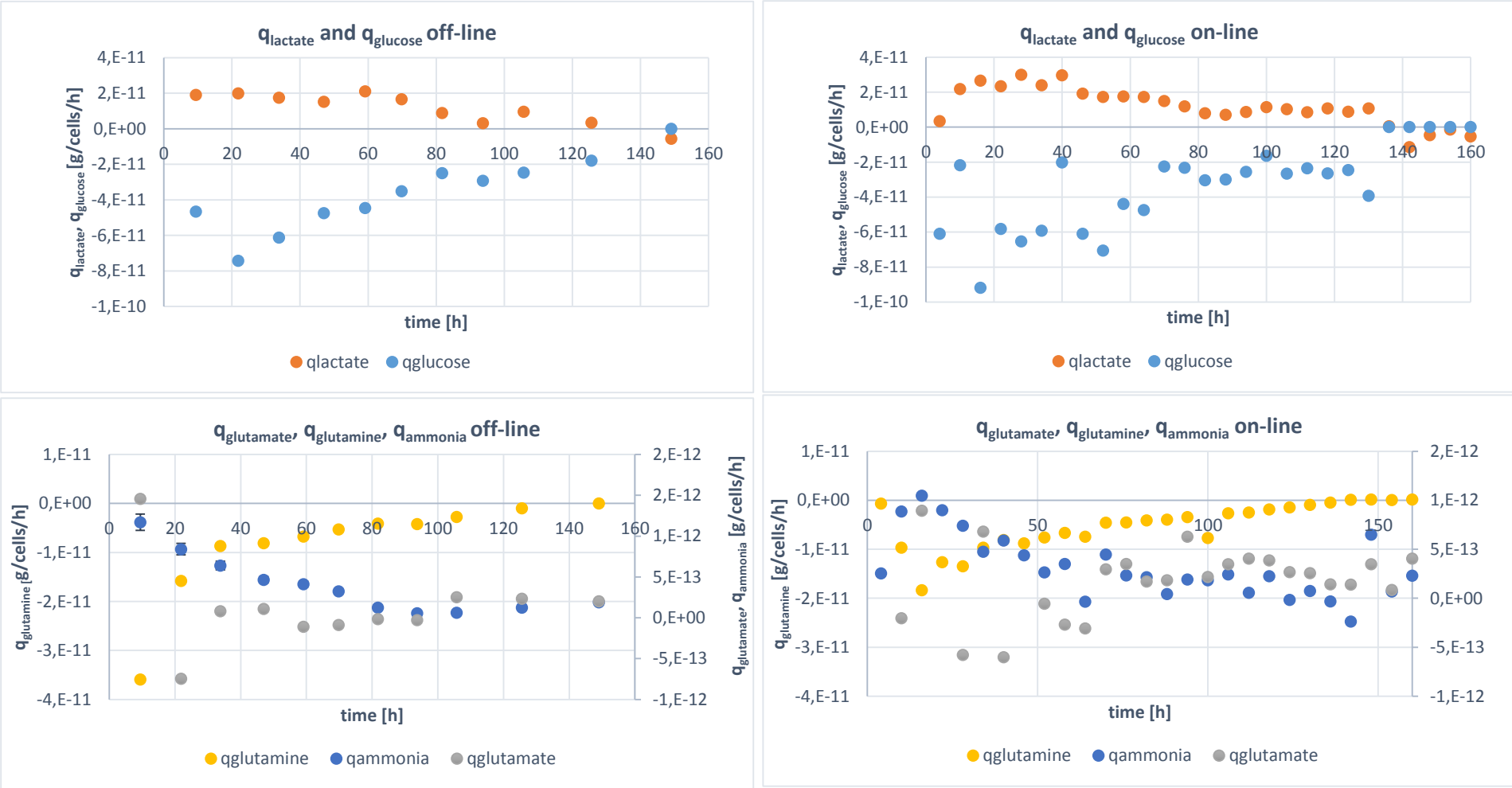


Figure 29 Specific rates plotted over time of STR13: (A) off-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue); (B) on-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue) (C) off-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey); (D) on-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey);

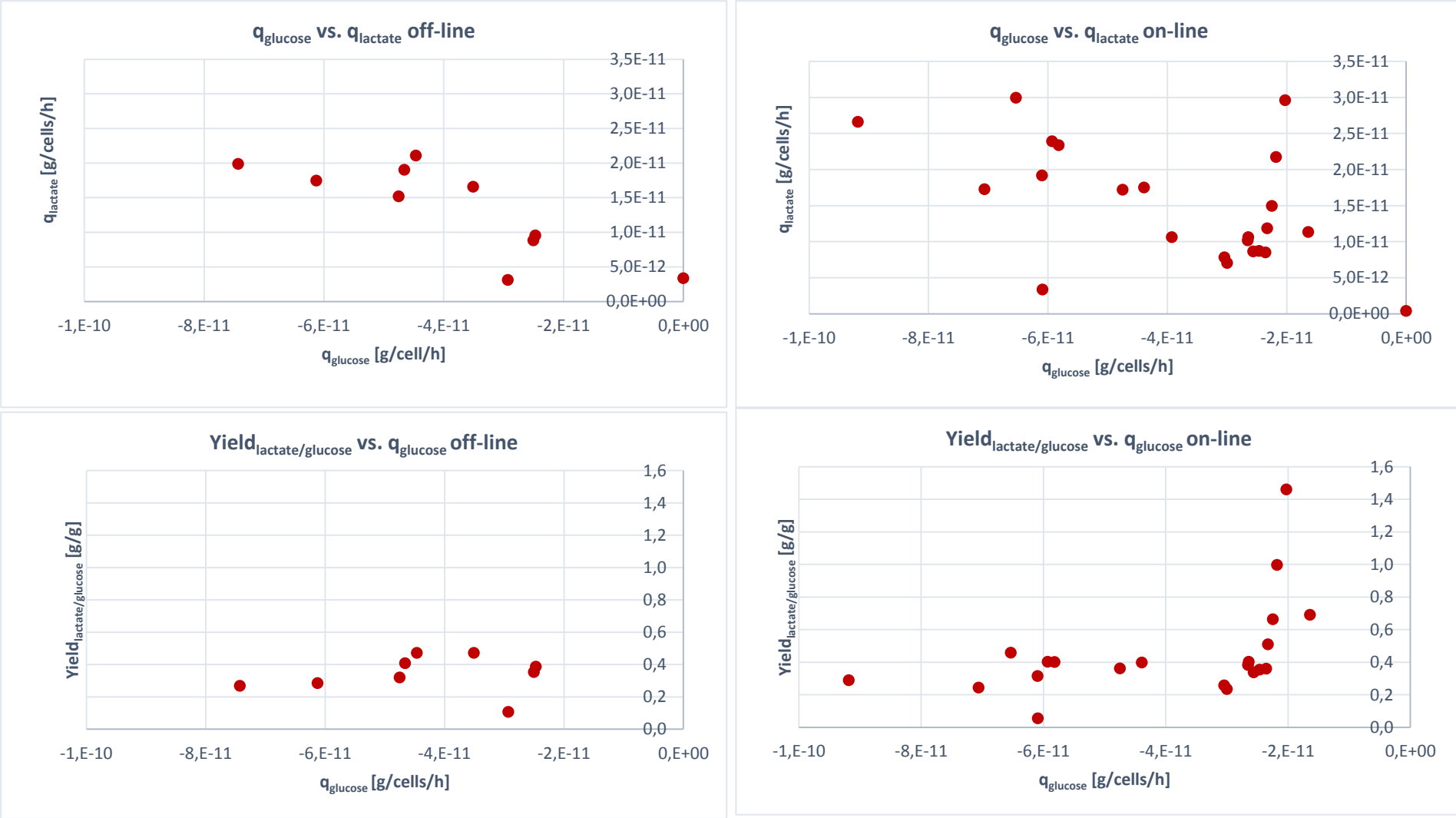


Figure 30 Specific rates and yields of STR13 plotted over the specific glucose uptake rate: (A) on-line determined specific lactate production rate; (B) off-line determined specific lactate production rate; (C) off-line determined $Yield_{lactate/glucose}$; (D) off-line determined $Yield_{lactate/glucose}$;

9.3.4 Acquired data of STR14

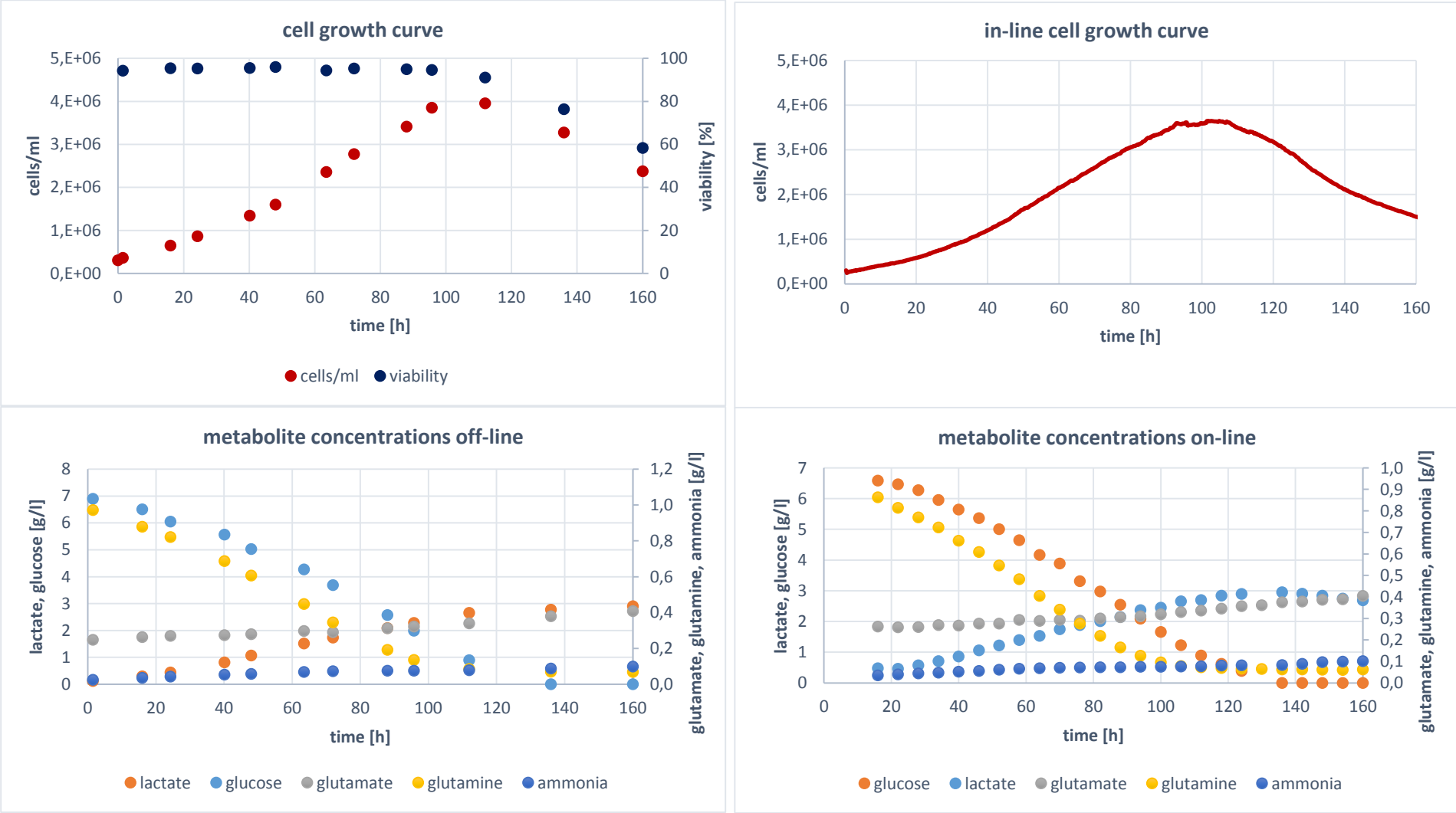


Figure 31 Acquired data of STR14: (A) viable cell count (red) and viability (dark blue); (B) in-line measured viable cell count; (C) off-line measured metabolite concentrations, orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia;

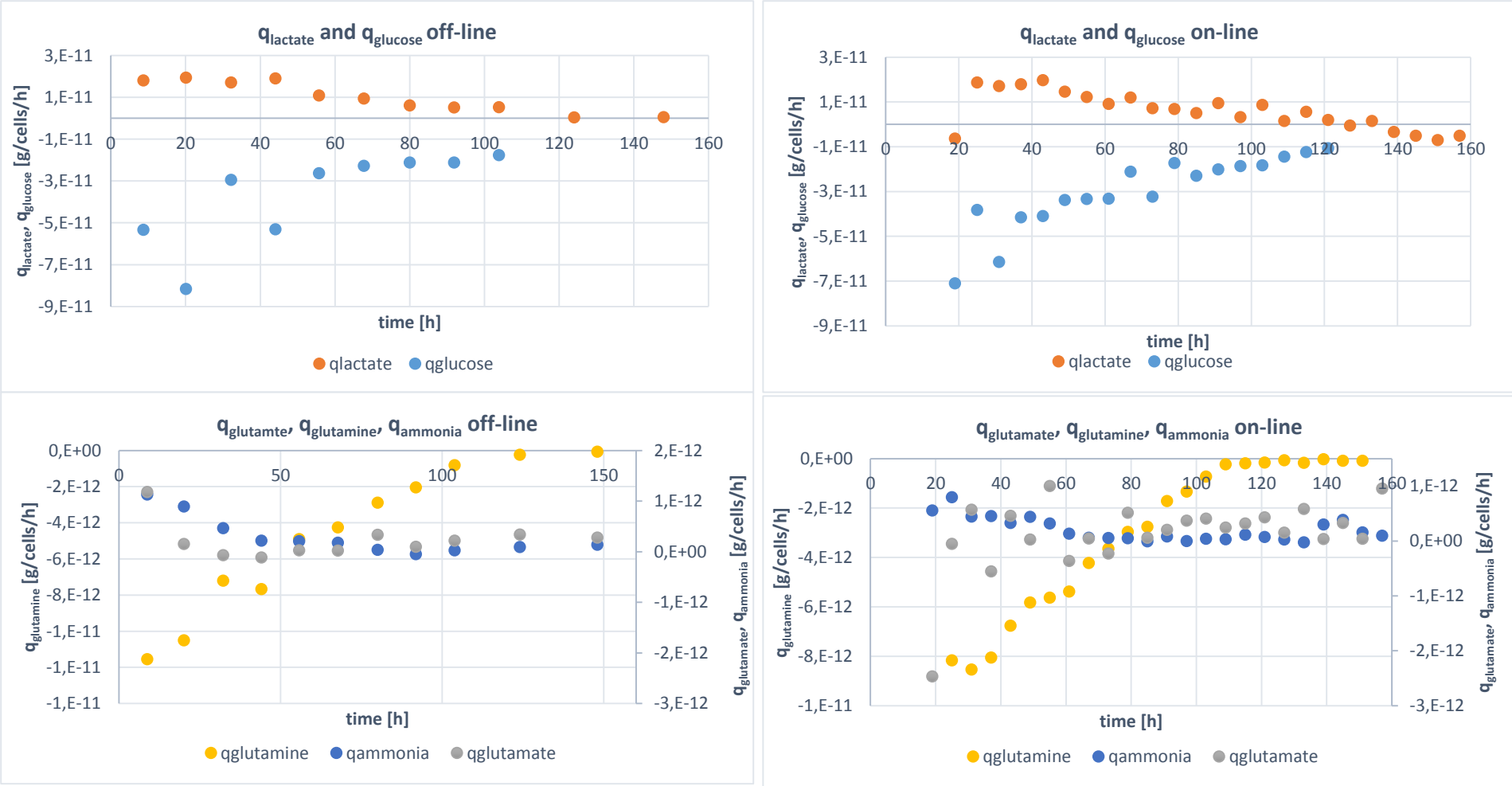


Figure 32 Specific rates plotted over time of STR14: (A) off-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue); (B) on-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue) (C) off-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey); (D) on-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey);

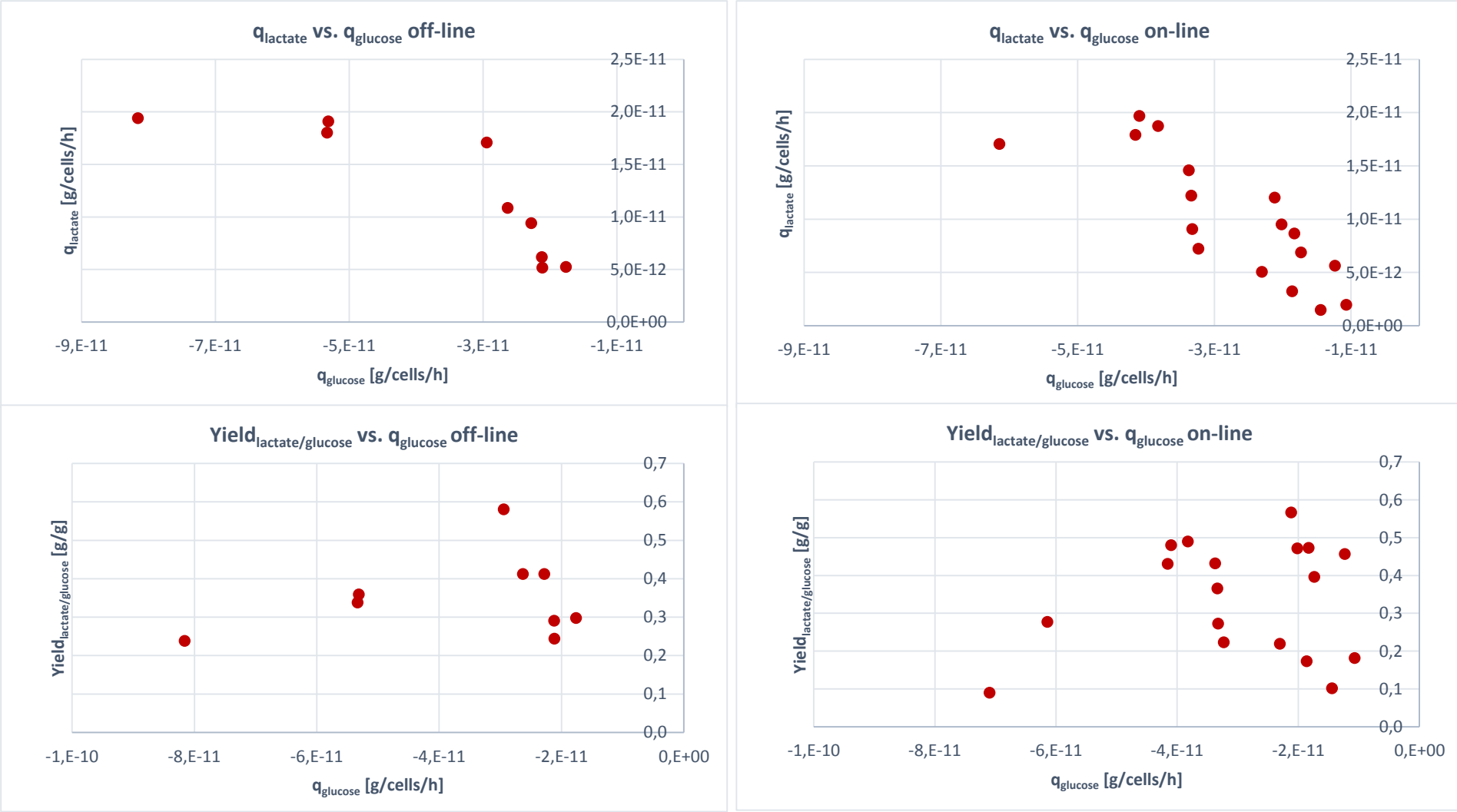
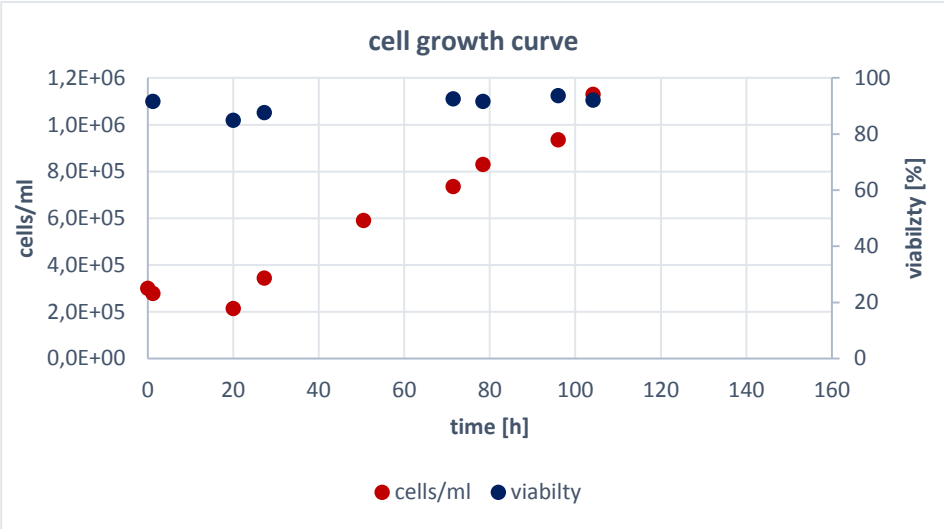
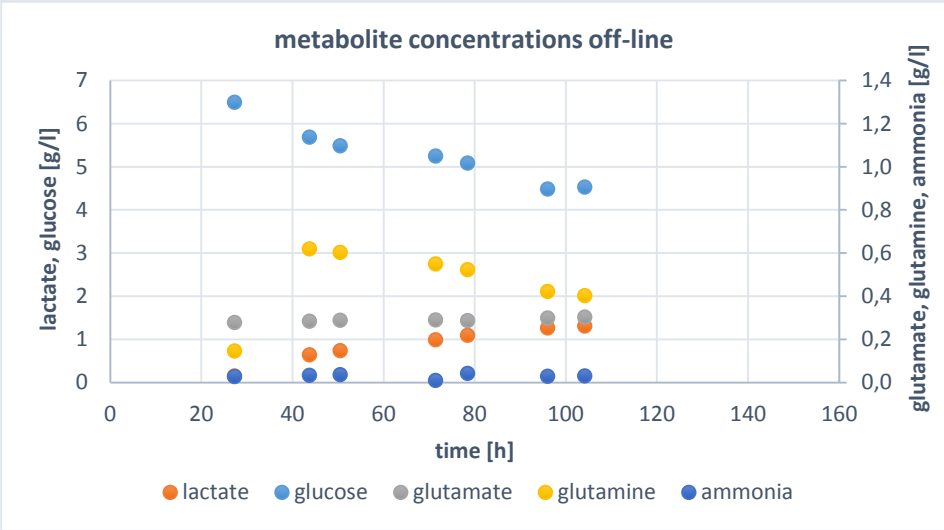


Figure 33 Specific rates and yields of STR14 plotted over the specific glucose uptake rate: (A) on-line determined specific lactate production rate; (B) off-line determined specific lactate production rate; (C) off-line determined $Yield_{lactate/glucose}$; (D) off-line determined $Yield_{lactate/glucose}$;

9.3.5 Acquired data of STR16





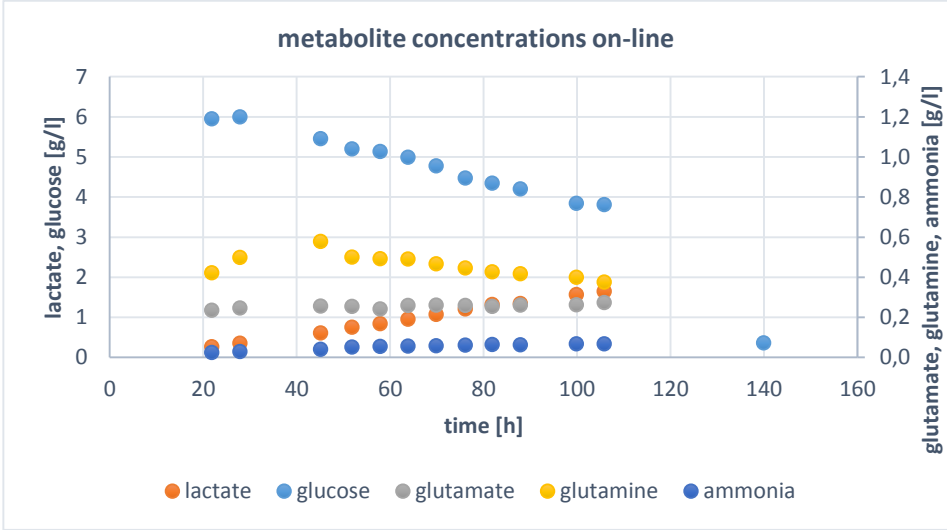


Figure 34 Acquired data of STR16: (A) viable cell count (red) and viability (dark blue); (C) off-line measured metabolite concentrations, orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia;

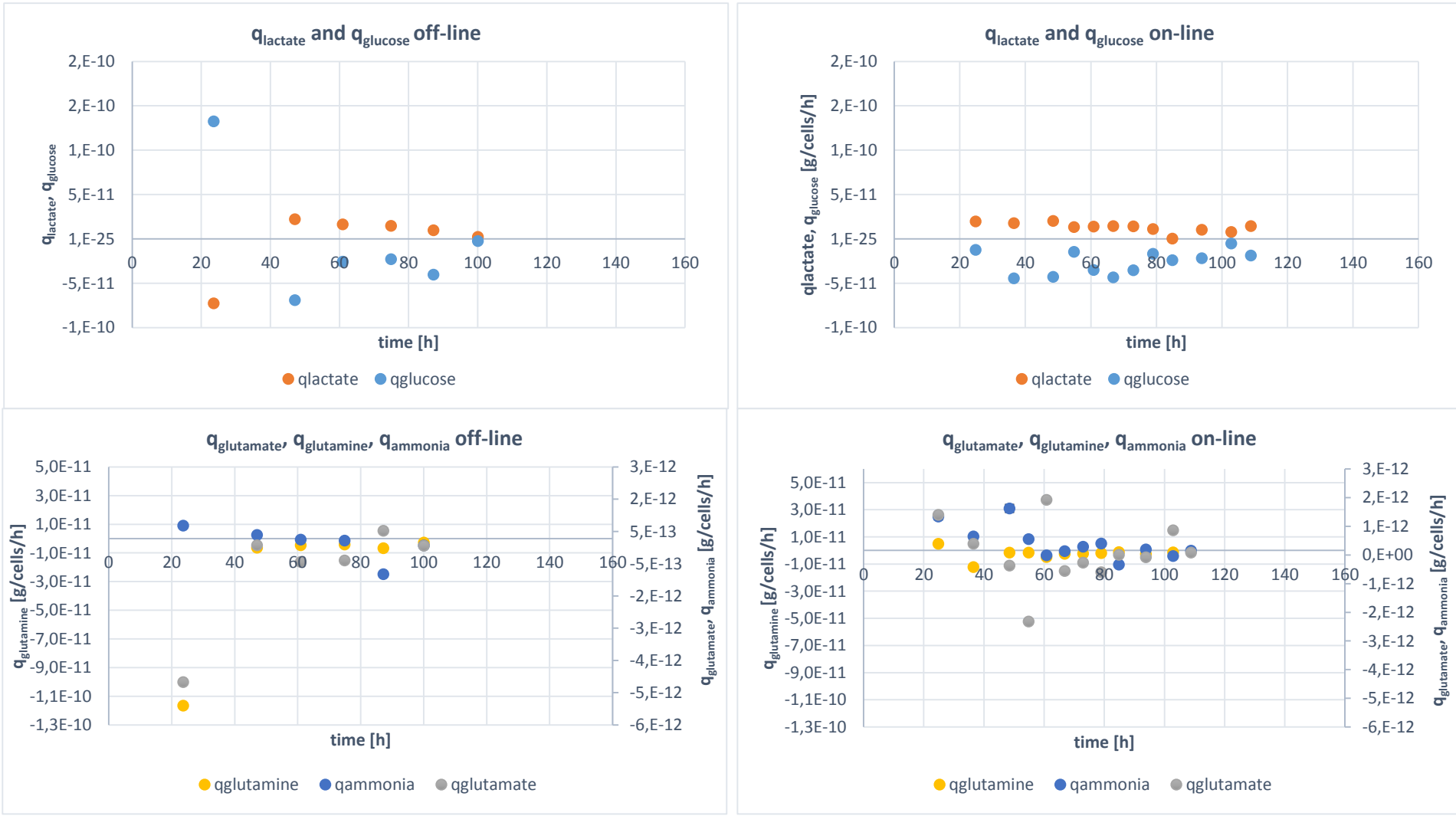


Figure 35 Specific rates plotted over time of STR16: (A) off-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue); (B) on-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue) (C) off-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey); (D) on-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey);

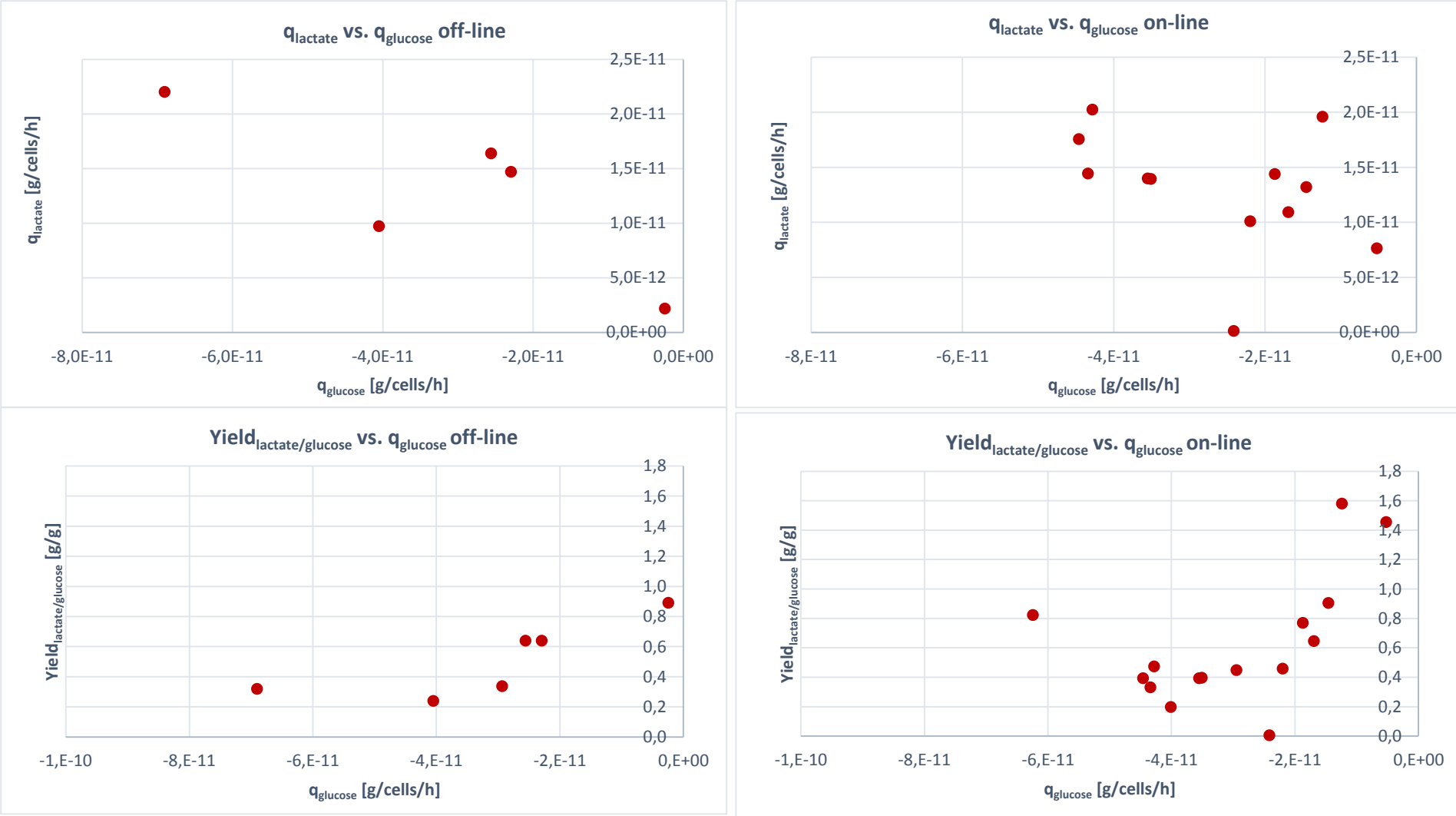
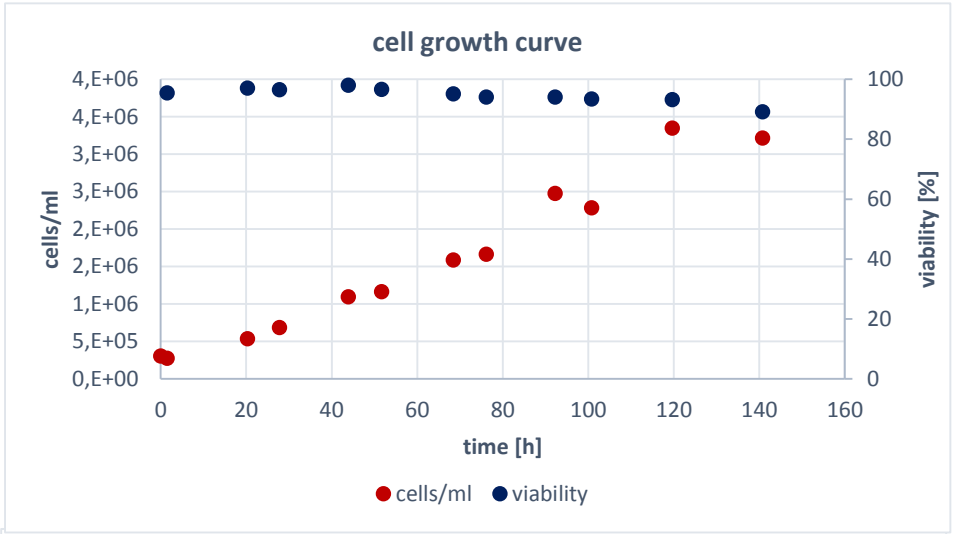
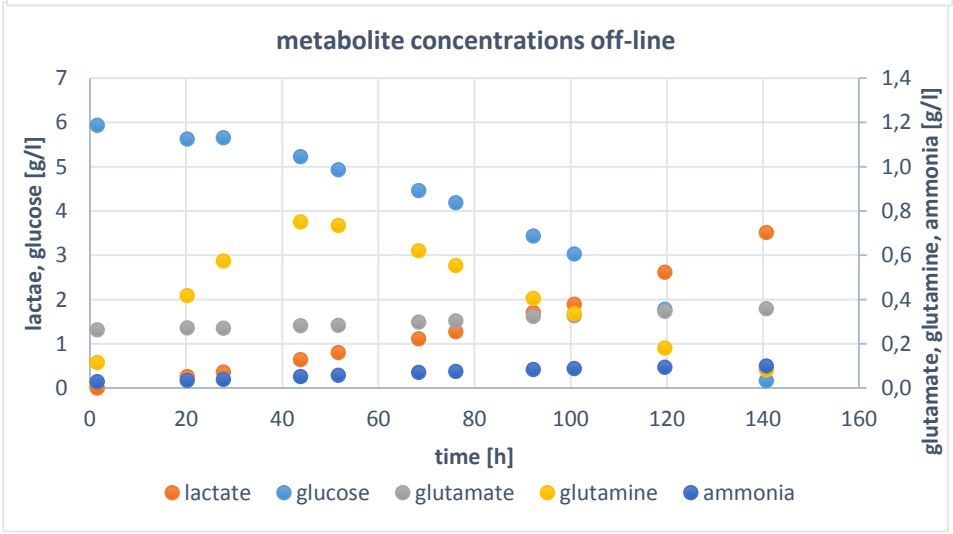


Figure 36 Specific rates and yields of STR16 plotted over the specific glucose uptake rate: (A) on-line determined specific lactate production rate; (B) off-line determined specific lactate production rate; (C) off-line determined $Yield_{lactate/glucose}$; (D) off-line determined $Yield_{lactate/glucose}$;

9.3.6 Acquired data of STR17





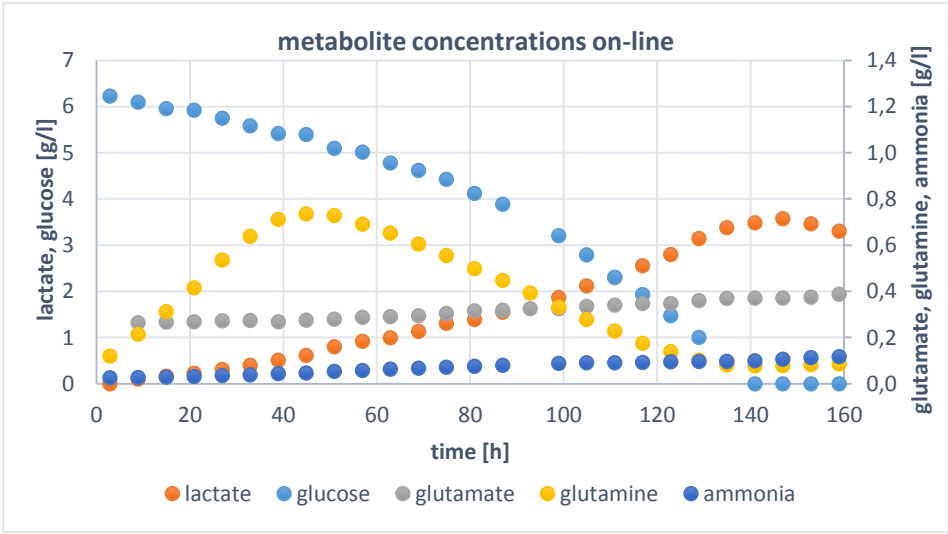


Figure 37 Acquired data of STR17: (A) viable cell count (red) and viability (dark blue); (C) off-line measured metabolite concentrations, orange, lactate; light blue, glucose; grey, glutamate; yellow, glutamine; dark blue, ammonia;

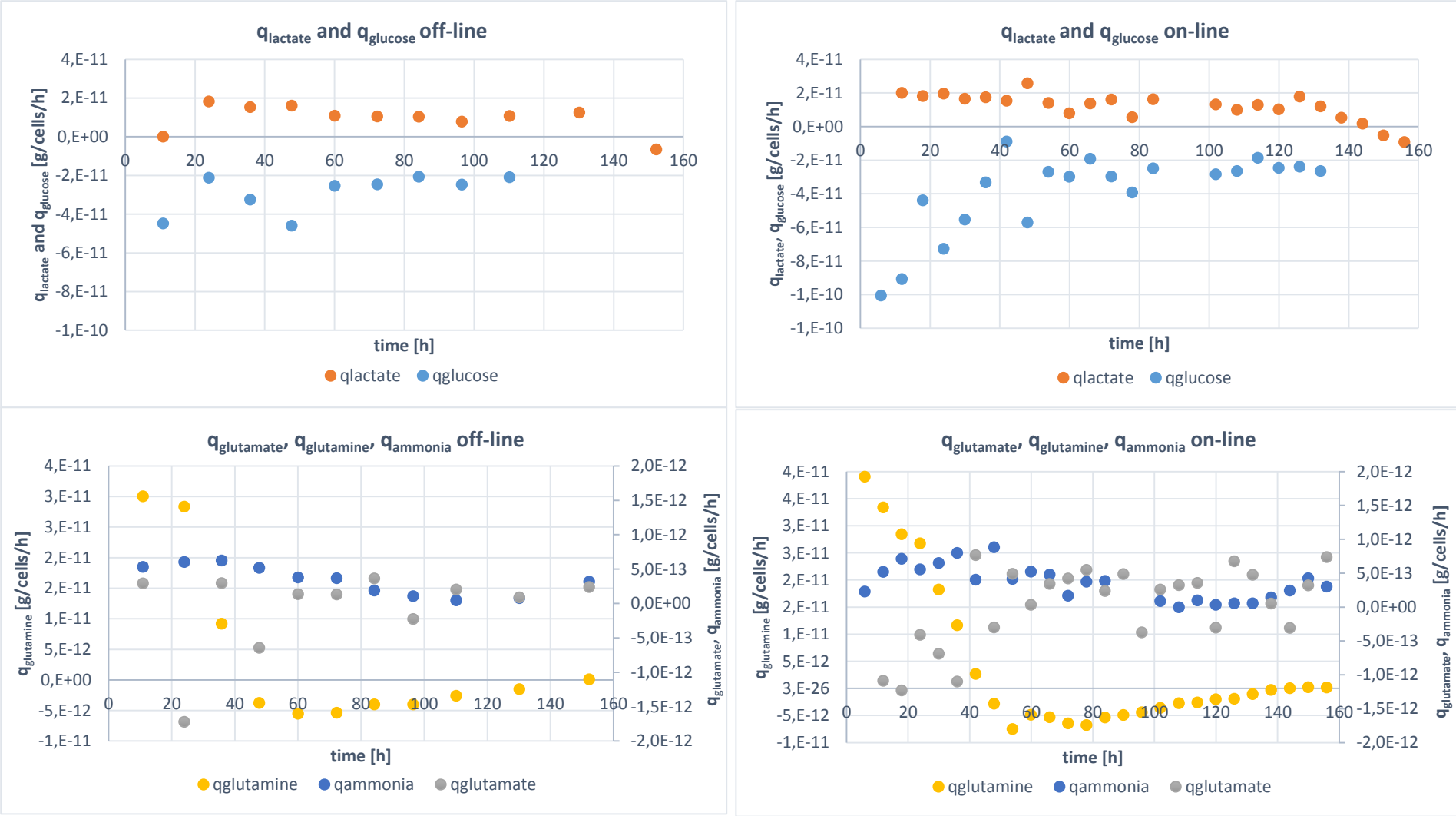


Figure 38 Specific rates plotted over time of STR17: (A) off-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue); (B) on-line determined specific lactate production rate (orange) and specific glucose uptake rate (light blue) (C) off-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey); (D) on-line determined specific glutamine uptake rate (yellow), specific ammonia production rate (blue) and specific glutamate production rate (grey);

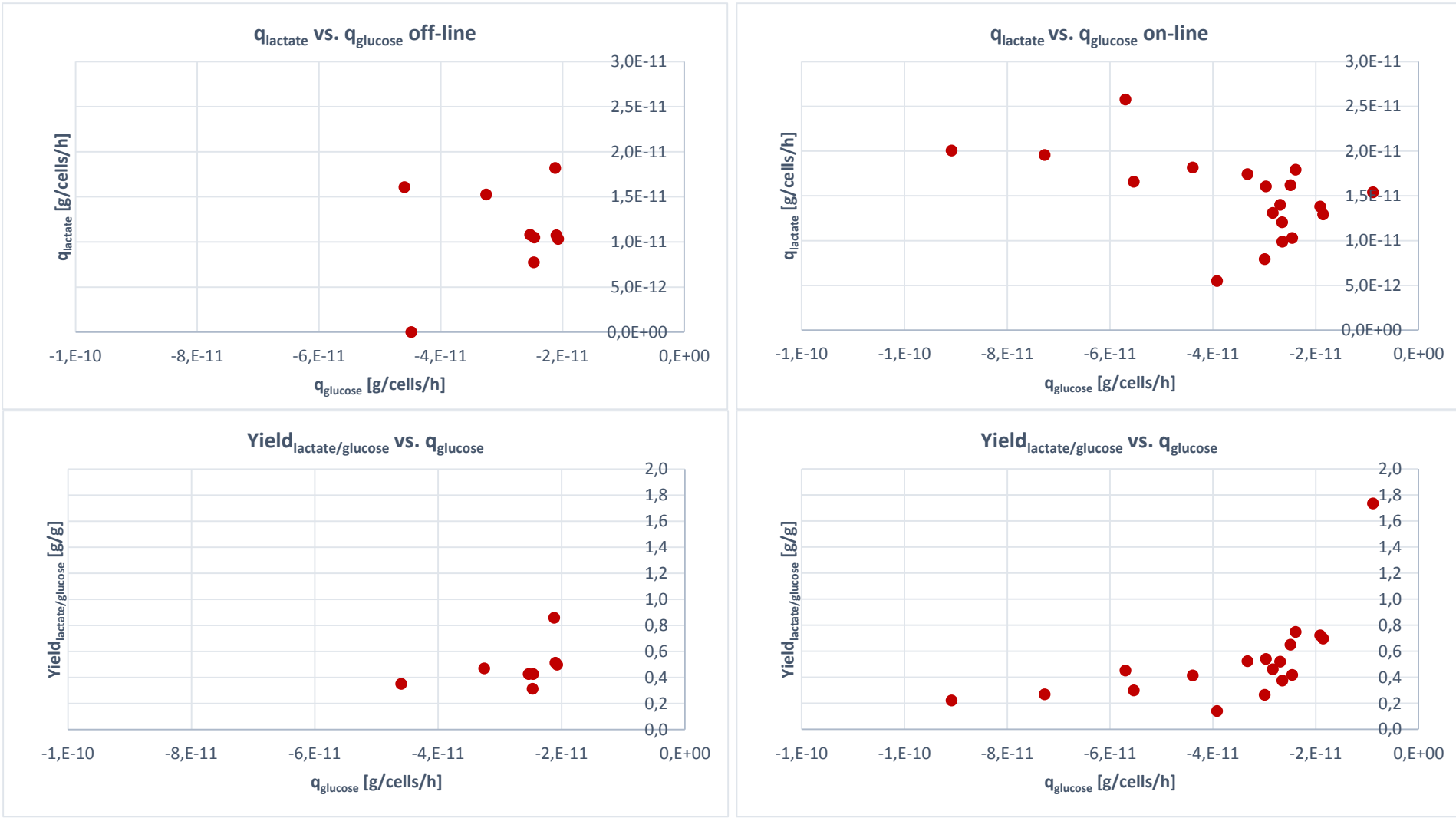


Figure 39 Specific rates and yields of STR17 plotted over the specific glucose uptake rate: (A) on -line determined specific lactate production rate; (B) off-line determined specific lactate production rate; (C) off-line determined $Yield_{lactate/glucose}$; (D) off-line determined $Yield_{lactate/glucose}$;

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