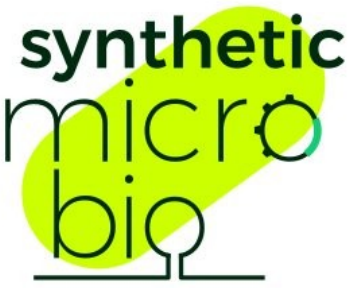

Energetics of Microbial Growth in Parallel Spectrometric Growth Platforms

Bachelorthesis

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Abstract

The bacterium *Escherichia coli* is one of the most studied model organisms. It is of great importance for biotechnology and biomedical science. Over time, more and more complex and sensitive methods have been developed to study biological processes in and around the organism. For example, parallel cultivation in microplate readers allows the collection of many data in parallel instead of single measurements. The BioLector Pro from the company *m2p-labs* is such a microplate reader. It is specialized in measuring metabolic activity. For this purpose optical sensors and fluorescent optodes are used. In contrast to many other measuring instruments, the BioLector Pro does not determine the cell concentration with the conventional OD measuring method, but by back-scattering. The BioLector Pro is particularly distinguished by its factory-calibrated gas exchange parameters. In principle, these make it possible to compare reactor cultures on a large scale. In addition, metabolic rates can be determined with the gas exchange parameters. With the newly generated data, biological processes can be better understood and even predicted. In the process, established relationships are also put in a new light.

The aim of this work is to investigate the growth of *Escherichia coli* with the BioLector Pro using various measurement parameters. The generated data will then be calibrated and tested for their validity. . In addition, the data will be used to calculate quantitative rates and parameters, which could then be plotted in a simple model. With this model the energetic relations between anabolism and catabolism can be calculated. during growth. Despite the simple experimental design, in which the bacteria grew either on glucose or acetate only, the growth curves turned out to be more complicated than expected. Nevertheless, comparisons could be made. It could be shown that back-scatter measurement is suitable for analysing the growth of cell cultures. The average oxygen consumption could also be calculated. The maximum achieved rates correspond to the maximum specific respiration rates of *Escherichia coli* described in the literature. Unfortunately, the graphs were hardly constant and basically very noisy. Nevertheless, interesting biological processes could be identified on the basis of the visualized data. On the one hand, the length growth of *Escherichia coli*, which apparently has an effect on the back-scatter measurements. On the other hand, a second oxygen consumption phase could also be seen on acetate growth, which reminds of diauxia, but is not expected on acetate growth.

The measurement methods used can still be significantly optimized and some errors avoided. Meanwhile, *m2p-labs* offers improved optodes for oxygen measurement, which could also deliver significantly better results. In summary, it can be said that if the experimental conditions and measurement methods are optimized, growth experiments in the BioLector platform could be suitable for the calibration of quantitative models.

Zusammenfassung

Das Bakterium *Escherichia coli* ist eines der am meisten untersuchten Modelorganismen. Es ist für die Biotechnologie und die biomedizinische Wissenschaft von großer Bedeutung. Mit der Zeit wurden immer mehr komplexe und sensitive Methoden, zur Untersuchung von biologischen Vorgängen um und im Organismus entwickelt. Z.b. ermöglicht die parallele Kultivierung in Mikrotiterplattenlesern, das Erheben von vielen Daten parallel anstatt Einzelmessungen. Der BioLector Pro von der Firma *m2p-labs* ist so ein Mikrotiterplattenleser. Er ist spezialisiert darauf metabolische Aktivität zu messen. Dafür werden optische Sensoren und fluoreszierende Optoden verwendet. Im gegensatz zu vielen anderen Messgeräten, bestimmt der BioLector Pro die Zellkonzentration nicht mit dem herkömmlichen OD Messverfahren, sondern über Rückstreuung (back-scatter). Besonders zeichnet sich der BioLector Pro durch seine fabrik-kalibrierten Gasaustauschparameter aus. Diese erlauben prinzipiell, Reaktorkulturen im großen Maßstab vergleichbar zu machen. Zusätzlich können mit den Gasaustauschparameter metabolische Raten bestimmt werden. Mit den neu generierten Daten gelingt es immer besser, biologische Vorgänge zu verstehen und sogar vorherzusagen. Dabei werden auch schon seit langem fest etablierte Zusammenhänge in ein neues Licht gerückt.

Das Ziel dieser Arbeit ist es, das Wachstum von *Escherichia coli* mit dem BioLector Pro durch verschiedene Messparameter zu untersuchen. Die generierten Daten sollen dann kalibriert und auf ihre Aussagekraft geprüft werden. Zusätzlich werden die Daten dazu genutzt quantitative Raten und Parameter zu berechnen, welche dann in einem einfachen Modell aufgetragen werden könnten. Mit diesem Modell lassen sich dann die energetischen Verhältnisse zwischen Anabolismus und Katabolismus während des Wachstums beschreiben. Trotz des einfachen Versuchsaufbaus, in denen die Bakterien entweder nur auf Glukose oder nur auf Acetat gewachsen sind, haben sich die Wachstumskurven komplizierter herausgestellt als erwartet. Dennoch konnten Vergleiche gezogen werden. Es konnte gezeigt werden, dass die back-scatter Messung sich dazu eignet das Wachstum der Zellkulturen zu analysieren. Es konnte auch der durchschnittliche Sauerstoffverbrauch berechnet werden. Die maximal erreichten Raten entsprechen in der Literatur beschriebenen maximalen spezifischen Atmungsraten von *Escherichia coli*. Leider waren die Graphen kaum konstant und grundsätzlich sehr verrauscht. Trotzdem konnte anhand der visualisierten Daten interessante biologische Vorgänge erkannt werden. Zum einen das Längenwachstum von *Escherichia coli*, was scheinbar Auswirkungen auf die back-scatter Messungen hat. Zum anderen war eine zweite Sauerstoffverbrauchsphase auch auf Acetatwachstum zu sehen, welche an Diauxie erinnert, aber auf Acetatwachstum nicht erwartet wird.

Die verwendeten Messmethoden können noch deutlich optimiert und einige Fehler vermieden werden. Mittlerweile werden von *m2p-labs* verbesserte Optoden für die Sauerstoffmessung angeboten, die ebenfalls deutlich bessere Ergebnisse liefern könnten.
Zusammenfassend kann gesagt werden, dass sich bei Optimierung der experimentellen Bedingungen und der Messmethoden, Wachstumsexperimente in der BioLector Plattform zur Kalibrierung quantitativeer Modelle eignen könnten.

List of Abbrevations

a.u.	arbitrary unit
ADP	Adenosine diphosphate
Agar	Agartang, *jap. Fischleim* (Agar-Agar)
ATP	Adenosine-5'-triphosphate
CoA	coenzyme A
CO_2	carbon dioxide
DO	dissolved oxygen
et al.	et alia
g	gram
h	hours
IDE	interactive development environment
L	litre
LB	lysogeny broth
mg	milligram = 10^{-3} gram
mL	millilitre = 10^{-3} litre
mol	base unit of amount of substance
M9	M9 minimal salts solution
OD	optical density
OUR	oxygen uptake rate
O_2	oxygen
rpm	revolutions per minute
TCA	tricarboxylic acid cycle

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1 Introduction

1.1 *Escherichia coli* as an object of investigation

The bacterium *Escherichia coli* (*E. coli*) is one of the best studied model organisms. It is of great importance for biotechnological and biomedical science (Treitz et al. 2016). Therefore, it is important to understand how much energy can be obtained from a fixed amount of substrate and in what ratio the energy is divided between catabolism and anabolism (Russell JB et al. 1995). Catabolism is the phase in *E. coli* in which the degradation of complex molecules takes place (figure 1). Such molecules can be lipids, carbohydrates and proteins. This degradation from complex substances to simple compounds provides energy and basic compounds that are used in the organism (Russell JB et al. 1995)(Schlegel 1992).

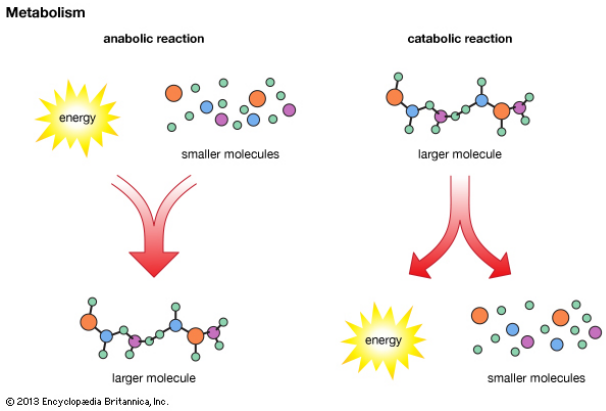

Figure 1: Simple scheme for anabolism and catabolism. Figure source in 6.1 Resources.

Anabolism is the phase in the organism, where the energy and basic compounds obtained from catabolism are metabolized. This produces vital products such as nucleic acids, proteins, lipids and polysaccharides. These products enable the bacterium to grow by cell division (Schlegel 1992)(figure 1).
The growth of a bacterial culture can be divided into several sections. The lag phase, exponential phase, stationary phase and the death phase (Schlegel 1992)(figure 2).

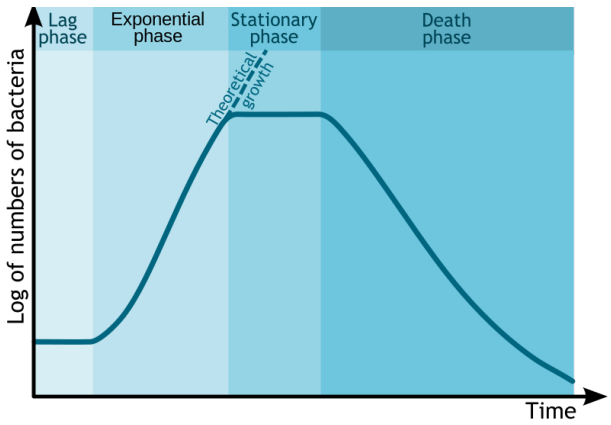

Figure 2: Bacterial growth curve. Figure source in 6.1 Resources .

In the lag phase, the bacterium has receptors to determine which nutrients are available in the medium. Then enzymes are expressed that can metabolize the nutrients. The bacterium distinguishes between energy-rich and energy-weak nutrients and first of all uses the nutrient which provides the most energy through

metabolization. Since the analysis and provision of the enzymes takes time, the lag phase is to be understood as a warm up phase. After the metabolism has been adapted, the exponential phase begins. The exponential phase describes the growth phase of culture, where cell division remains constant over several cycles. Under laboratory conditions, *E. coli* divides every 20 minutes. This doubles the cell count per generation time. The stationary phase occurs when culture approaches the capacity limits of space and/or the availability of the nutrient. There is an equilibrium between cells added by cell division and dying cells. If the majority of nutrients are used up, the death phase occurs. The cells starve gradually until only dead cells remain in the medium.
The growth of *E. coli* on glucose, as the only source of carbon, has been well studied for a long time. With glucose a very fast growth with a high yield can be achieved (Andersen KB. and von Meyenburg K. 1980). Glucose is pre-processed in the cell by glycolysis to pyruvat. This yields 2 ATP per glucose. Pyruvate can then be converted to acetate to maintain the redox balance of glycolysis. The acetat is then secreted to the growth medium. Alternately, pyruvate can be further oxidized to CO_2 by the TCA (tricarboxylic acid cycle) and oxidative phosphorylation, yielding significant higher amounts of ATP per glucose molecule (Schlengel 1992).
However, even when oxygen is present, glucose is glycolytically processed into acetate in the first growth phase. The acetate produced is then taken up again and used in TCA and oxidative phosphorylation. This leads to a second, much slower, growth phase. The described phenomenon was first observed by Jacques Monod, who then referred to it as diauxie (two successive growth phases). Recently, the diauxie has gained attention again through the work of Enjalbert (Enjalbert, Brice et al. 2015). In that work it was shown that *E. coli* did not grow on acetate after glucose exhaustion because acetate anabolism was not completely induced. Growth on glucose can still be continued quickly. Only at high acetate concentrations growth occurs after glucose exhaustion. The basic function of the diauxie is questioned anew with that work.

Nevertheless, this work also investigates the growth of acetate as the only source of carbon (Stéphane et al. 2019)(Min-Kyu Oh et al. 2002). Thereby, the acetate is processed to acetyl-CoA, which is then utilized in the TCA and further in oxidative phosphorylation (Schlengel 1992). As already mentioned at the beginning, that process is not very energy-efficient and leads to slower growth phases. The substrate also influences the yield of the culture. The yield is a coefficient that describes how much "biomass harvest" a culture has gained from the amount of substrate provided. The yield is calculated from the biomass generated by the quantity of substrate used (Shiloach J et al. 2005). Glucose and acetate are fundamentally different in structure and pre-processing. The investigation of the energy distributions at the respective substrate is interesting and could bring new information.

1.2 Measuring methods and BioLector Pro

Over the years, many methods have been developed to investigate cell growth. The most common is to determine the optical density (OD) of cultures at regular intervals (Keiran Stevenson et al. 2016). The optical density measurement is usually measured in a spectrometer or photometer (Daniel C. Harris 2014). The measurement is made with light at a wavelength of 600 nm. The light beam is sent through a cuvette in which the culture to be examined is located. The cells in the culture scatter the light. The remaining light arriving at the sensor is then measured. The more cells there are in the medium, the more light is scattered, resulting in a higher OD. If the OD is higher than the previous measured one, it can be expected that the cells have grown. Despite the simple handling of the OD measurement, it also has disadvantages. The OD measurement is in parabolic dependence to the number of cells (Daniel C. Harris 2014). This makes measurements more unreliable at higher cell densities. Another point is that dead cells are also measured. In other words, the actual death phase of the culture cannot be measured, and in addition, the stationary phase cannot really be distinguished from the death phase. If stationary phases of cell cultures are to be investigated, a measurement in a permanent culture is recommended. For the investigation of the death phase, the colouring of the cells can be used as one method (Georges Knaysi 1935). The dead and living cells can be identified and counted under the microscope. In the work of Stevenson et al. 2007 it was noted that the length growth of *E. coli* had an influence on the OD measurement. An increase in OD was measured although there was no increase in cell count. The increased cell length was also observed in recent analyses in our lab (Bachelorthesis Denise Kaupat, Masterthesis Thomas Rohr). Currently it is not clear why the cells growth.

Filamentation of *E. coli* cells is often observed under nitrogen limitation, in DNA topology and replication or cell division mutants, or late in stationary phase. The effect of such morphological differentiation on optical biomass measurements is only little understood (Stevenson et al. 2016). Also, the influence on measurement techniques is not quite clear.
However, based on the OD measurements, growth curves can be generated. E.g. specific growth rate μ, maximum growth rate μ_{max} and affinity constants to the respective substrate can be calculated (Schlegel 1992). Devices have been developed which not only facilitate and automate the measurement, but also allow a variety of different cultures to be examined simultaneously. One such device is the BioLector Pro from the manufacturer *m2p-labs*. The BioLector Pro makes it possible to examine up to 48 different cultures non-invasively and online, using optical sensors.

The parallel cultivation of different cultures, in microtiter plates, makes it possible to work with small volumes (Keiran Stevenson et al. 2016). As a rule, the range is from $150\mu L$ to 1mL. Unfortunately, cultures often behave differently in lower volumes than in larger ones (Xu et al. 2017). Accordingly, the same experiments cannot always be expected to produce the same results only on a larger scale. For upscalling, a constant k_La is very important. The BioLector Pro has the k_La pre-calibrated for volume and rpm, which can be read in a table (see figure 12). The table allows not only systematic upscalling but also the calculation of metabolic rates from O_2 concentrations, using the k_La.
A further advantage is the possibility to set many different parameters and investigate them at the same time. That avoids potential errors that can occur when each parameter is examined individually in an experiment. On the other hand, it is also possible to examine only a few parameters, but then with several samples per parameter. The experiments are usually designed in that way, since many samples are necessary for statistical tests. It can be disadvantageous, if errors or contaminations occur during the preparation of the experiment, which are not directly noticed. Such a mistake can affect the whole experiment.
The non-invasive online measurement offered by the BioLector Pro also helps to avoid errors in the measurement of the data and disturbances of the cultures. The measured data can be viewed directly without having to be on site. Samples do not have to be taken for every measurement. Taking smples for every measurment often mean stress for the cells, which can lead to falsification of the measurement or strong noise in the data. Stress factors are often temperature and light intensity fluctuations, reduction of oxygen supply but also possible contaminations due to sampling.

The four main parameters to be measured are back-scatter, fluorescence, dissolved oxygen and pH (m2p-labs). The scatter measurement works in a similar way to the OD measurement. However, the light arriving after the scattering is not measured. The back-scatter method measures the light that returns to the starting point at an angle of 180° due to the scattering. The advantage of the back-scatter measurement is that it remains accurate even at high cell densities. This is particularly important for long-term measurements. However, the back-scatter measurement is inaccurate at very low concentrations (m2p-labs). Depending on the wavelength set, the fluorescence sensor can be used to measure different fluorescent substances in the culture. The dissolved oxygen in the medium and the pH value are measured using optodes attached to the measuring plates. A total of up to 6 sensors can be freely installed and replaced. Many different sensors are available. In addition, experiments can be carried out in which the pH and substrate availability can be controlled.

1.3 Quantitative methods

The technological progress and the new, data-intensive methods associated with it place completely new demands on researchers (hhu.de). The large amounts of data, most of which are only digitally accessible, require skills in mathematical modelling, statistics and machine learning in order to make reliable predictions of biological processes. To master this, not only biological background knowledge is necessary, but also a basic understanding of mathematics and informatics. The new methods are executed using programming languages such as *Python* and *R*. Usually most methods are available via *packages*. Nevertheless, the data sets often have to be adapted for the corresponding *package* or, if necessary, you have to write your own *package*. These serve to process the data so far that at the end certain questions can be answered and the results presented visually. Furthermore, whole biological processes can be described as models.

Microbial growth curve were described by quantitative mathematical modelling in the early days of modern microbiology (Monod 1949). But this quantitative experimentation has later given way to more qualitative experiments that sufficed to investigate the genetic make-up of cells by the newly discovered moelcular biology tools. However, recently the quantitative modelling of simple microbial growth processes has regained a certain attention in literature, due to the observed correlations of genome-wide gene regulatory processes with growth rate (Scott et al. 2010, Weise et al. 2015). These models postulate that regulation of gene expression can optimally allocate available nutrient ressources between anabolic and catabolic processes of cell growth to ensure that the maximal possible growth rate is achieved. A proper quantitative calibration of such models is required to rigorously test their predictions and potentially use them to optimize biotechnological processes.

1.4 Aim of this work

The aim of this work is to investigate the growth of *E. coli* cultures with acetate or glucose as the only carbon source using the BioLector Pro. In order to achieve better comparability, in the media the amount of glucose should be added as weight per volume and an equivalent amount carbon mol in form of acetat, respectively. Since acetate has only two C atoms and glucose has six, basically three times the amount of acetate should be provided as a nutrient to achieve the same amount of C atoms. The resulting data should then be further investigated. The scatter measurement and the dissolved oxygen measurement are of particular interest. The scatter measurement should be checked for its credibility, as it is an alternative to the OD measurement. Therefore, the dry weight and the cell count of the cultures should be determined in order to generate correlation graphs. Furthermore, calibrations with the dissolved oxygen data should be carried out. With certain conversions, the average oxygen consumption of the cells could be calculated from them. After the calculation, the generated energy in the cells could be derived. The measured and calculated data could then be further investigated in a simple model to possibly find the distribution of energy between catabolism and anabolism. In addition, possible biologically interesting results should be discussed.

2 Materials

2.1 Microorganisms

Strain	Genotype	Reference
E. coli W3110	*λ⁻ IN(rrnD-rrnE)1 rph-1*	J. Lederberg
E. coli DH5*α*	*fhuA2 Δ(argF-lacZ) U169 phoA glnV44 ϕ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs

2.2 Growth Media and Recipes

Table 3: Recipe for 5x M9 medium. g.mol = g/mol.

M9_salts	g.mol	stock.5x	final_1xM9	for_1L_5x
Na2HPO4.7H2O	268.07	238.7 mM	47.8 mM	64 g
KH2PO4	136.09	110.2 mM	22 mM	15 g
NH4Cl	53.49	93.5 mM	18.7 mM	5 g
NaCl	58.44	42.7 mM	8.6 mM	2.5 g
H2O (milliQ)				up to 0.8 L
2.5 M NaOH				to pH 7.2
H2O (milliQ)				to 1 L
autoclave				121°C/15min

Table 4: Recipe for 100x trace metals. g.mol = g/mol.

Substance	g.mol	concentration	g_for_1L
NA2-EDTA	372.24	13.4 mM	5 g
FeCl3.6H2O	270.33	3.1 mM	0.84 g
ZnCl2	136.32	0.62 mM	84 mg
CuCl2.2H2O	170.48	76µM	13 mg
CoCl2.6H2O	237.93	42µM	10 mg
H3BO3	61.83	162 µM	10 mg
MnCl2.4H2O	197.91	8.1 µM	1.6 mg

Table 5: Overnight medium with same carbon amount in acetate, as glucose medium for *E. coli W3110*. g.mol = g/mol. For Pre-test I without biotin. For Pre-test II, Experiment I and II with biotin.

Ingredients	g.mol	stock	final_M9	for_50mL
M9 salts		5x	1x	10ml
MgSO4	120.37	1M	2mM	100µL
CaCl2	110.98	1M	0.1mM	5µL
uracil		10g/L	178µM	50µL
thiamine	265.35	10g/L	30µM	100µL
trace metals		100x		500µL
biotin		10g/L		50µL
Glucose	198.17	1M	22.2mM (0.4%)	1.11mL
milliQ H2O				38.135mL

Table 6: Overnight medium with same carbon amount in acetate, as glucose medium for *E.coli W3110*. g.mol = g/mol. For Pre-test I without biotin. For Pre-test II, Experiment I and II with biotin.

Ingredients	g.mol	stock	final_M9	for_50mL
M9 salts		5x	1x	10ml
MgSO4	120.37	1M	2mM	100µL
CaCl2	110.98	1M	0.1mM	5µL
uracil		10g/L	178µM	50µL
thiamine	265.35	10g/L	30µM	100µL
trace metals		100x		500µL
biotin		10g/L		50µL
Acetate	136.08	1M	66.6mM (0.4%)	3.33mL
milliQ H2O				35.915mL

Table 7: Overnight medium with same carbon amount in acetate, as glucose medium for *E.coli DH5alpha*. g.mol = g/mol.

Ingredients	g.mol	stock	final_M9	for_50mL
M9 salts		5x	1x	10ml
MgSO4	120.37	1M	2mM	100µL
CaCl2	110.98	1M	0.1mM	5µL
proline		10g/L	2.18mM	1.25mL
trace metals		100x		500µL
Acetate		1M	66.6mM (0.4%)	3.33mL
milliQ H2O				34.815mL

Table 8: Resuspension and final growth media of the Pre-test I, *E. coli W3110* glucose culture. amount_M9 = from 1x M9 Mastermix medium (same as in table 4 but without glucose/acetate.)

conc_glc	amount_M9	from_glc_stock
1% (55.5mM)	3.778mL	222.0µL
0.8% (44.4mM)	3.822mL	177.6µL
0.6% (33.3mM)	3.867mL	133.2µL
0.4% (22.2mM)	3.911mL	88.8µL
0.2% (11.1mM)	3.956mL	44.0µL

Table 9: Resuspension and final growth media of the Pre-test I, *E. coli W3110* acetate culture. conc_ace = same carbon content as the glucose kulture for 1%, 0.6% and 0.2%. amount_M9 = from 1x M9 Mastermix medium (same as in table 5 but without acetate.)

conc_ace	amount_M9	from_ace_stock
166.5mM	2.500mL	500.0µL
99.9mM	2.700mL	300.0µL
33.3mM	2.900mL	100.0µL

Table 10: Resuspension and final growth media of the Pre-test I, *E. coli DH5alpha* acetate culture. conc_ace = same carbon content as the glucose kulture for 1%, 0.8%, 0.6%, 0.4% and 0.2%. amount_M9 = from 1x M9 Mastermix medium (same as in table 6 but without acetate.)

conc_glc	amount_M9	from_glc_stock
1% (55.5mM)	3.778mL	222.0µL
0.8% (44.4mM)	3.822mL	177.6µL
0.6% (33.3mM)	3.867mL	133.2µL
0.4% (22.2mM)	3.911mL	88.8µL
0.2% (11.1mM)	3.956mL	44.0µL

Table 11: Mastermixes for resuspension and final growth media for Pre-test II, Experiment I and II. The mastermixes are for *E. coli W3110* with 0.8% glucose and the same amount of carbon in acetate. The concentration for the final growth medium was adjusted by dilution. ml.1 = ml

Solutions_ace	ml	Solutions_glc	ml.1
M9 salts 5x	28.000	M9 salts 5x	28.000
MgSO4	0.280	MgSO4	0.280
CaCl2	0.014	CaCl2	0.014
trace metals	1.400	trace metals	1.400
biotin	0.140	biotin	0.140
uracil	0.140	uracil	0.140
thiamine	0.280	thiamine	0.280
ace (1M)	4.662	glc (1M)	1.554
milli Q	0.084	milli Q	3.192
final	35.000	final	35.000

2.3 Hardware

Device	Manufacturer
NanoDrop 2000c Spectrophotometer	Thermo Fisher Scientific Inc.
Concentrator plus	Eppendorf AG
XS105	Mettler Toledo
INNOVA®42 Incubator Shaker Series	New Brunswick™
Incubator	BINDER
BioLector Pro	m2p-labs GmbH
FlowerPlate with optodes for pH and DO Lot No.: 1845	m2p-labs GmbH
Gas-permeable sealing foil evaporation reducing layer, Lot No.: 1818	m2p-labs
Centrifuge 5427R	Eppendorf AG
Heraeus Multifuge X3R Centrifuge	Thermo Fisher Scientific Inc.
Safe 2020	Thermo Scientific™
Microscope Axio scope.A1	Zeiss
Axio Cam Km1	Zeiss
Cell counting chamber Neubauer Improved 0.020mm Depth	

3 Methods

3.1 Microbiological Methods

3.1.1 Cultivation of *E. coli*

E. coli plate cultures were grown overnight on LB-agar at 37 °C in a *Binder* at 37 °C. *E. coli* cells were cultivated overnight in 10 mL or 20 mL M9-medium in an *INNOVA®42 Incubator Shaker Series* at 250 rpm and 37 °C. Compostion of M9-medium shown in table 3.

3.1.2 Preparation of the cultures for measuring in BioLector Pro

The OD of the grown cultures was measured. The glucose cultures were diluted to 0.1 OD and the acetate cultures to 0.2 OD, with a M9 1x mastermix without glucose/acetate. Per concentration 6 mL (4 mL in the Pre-test I) of the respective diluted culture was pipetted into a tube. The tubes were then centrifuged at 4500 rpm, 10min. The supernatant was discarded. Thus a cell pellet is obtained, which is resuspended with the corresponding 6 mL (4 mL in Pre-test I) growth medium. For Pre-test I the respective growth medium was mixed together (table [8] [9] [10]). The growth medium for Pre-test II, Experiment I and II was prepared from a main stock (table 11) by a dilution series for the respective concentration. Depending on the replicates, 1 mL was pipetted from the tube per well (in Experiment I 0.875 mL per well, due to an error). The respective 1 mL blanks were mixed directly into a well.

3.1.3 BioLector Pro settings

FlowerPlates with optodes for pH and DO Lot No.: 1845 were used. Used sensors: Biomass sensor ID: 201, Riboflavine sensor ID: 227, pH(HP8) sensor ID: 202, DO(PSt3) sensor ID: 203. An rpm of 1000 and a temperature of 37°C were set. The experiment was started without time limit. Measurements are made every 15 minutes.

3.1.4 Microscopy images

In Experiment I and II samples were taken for microscopy images. 3 µL each for lag phase, exponential phase, peak phase and stationary phase. The phase was determined by observations on live graphs generated by the BioLector Pro software. The samples for the lag phase were taken for all concentrations after 2 hours. For the samples of the exponential phase we waited until the slope did not increase anymore. The peak phase could only be detected when the graph dropped after a constant slope. Thus 15 min after the actual highest peak value. Stationary phase after a constant value had been attained. Note that there are two peaks in the glucose culture. If this was detected, samples were taken too. Per concentration, samples were only taken from one well. In the lag phase the 3 µL were pipetted directly onto the cell counting chamber Neubauer Improved and microscopied. For the exponential, peak and stationary phase, each sample was diluted 1/10. From the dilution 3 µL were taken and pipetted onto the cell counting chamber. The microscope was adjusted so that 12 of the smallest squares were completely visible in the image. 3 images were taken per sample, taking care that not the same 12 squares were seen again. Only the cells in the 12 squares were counted. From the 3 images an average was formed and with this average the cell/µL was calculated with this formula:

$$\frac{cellnumber}{area * chamberdepth * dilution}$$

. With average cellnumber = 126.666, area for 12 squares = 0.03 [mm^2], chamber depth = 0.02 [mm] and e.g dilution = 1/10:

$$\frac{126.66 * 10}{0.03 * 0.02 * 1} = 2111111 cellcount/\mu L$$

.

3.1.5 Determination of the dry cell weight

The determination of dry cell weight was done in Pre-test II, Experiment I and II. Therefore 5 mL eppis were labelled with the respective concentration and pre-weighed (with the XS105 from *Mettler Toldedo*). In Pre-test II, 900 µL culture volume was taken from each replicate of a concentration at the end of the experiment and pipetted into the respective eppi. For this, the sealing foil had to be pierced with the pipette tip. With five replicates, the total volume per concentration is 4.5 mL. Then the cells were centrifuged down at 12000 rpm for 2 min, the supernatant was discarded. The pellet was then resuspended in 3 mL milliQ. Then the cells were pelleted again and the supernatant was discarded. The eppis with the pellet were dried in the concentrator plus of *Eppendorf AG* under vacuum at 60°C for 8 hours (leave eppi lid open). The eppis were then weighed again. The difference gives the biomass in [mg]. For experiments I and II, the steps were carried out as described above. Except that the samples were taken directly as soon as a culture reached the stationary phase. Therefore the experiment had to be stopped for a short time. And the resuspension step, between the two pelleting steps, was skipped.

3.2 Data analysis

Analysis of the collected data was performed using the programming language *R 3.6.0* in the *Rstudio* IDE (interactive development environment). Installed packages: *knitr 1.23, devtools 2.1.0, platexpress 0.1* (from https://github.com/raim/platexpress last updated july 2019) and *pspline 1.0-18*). Also *Rstudio* was used for graph plotting and statistical analysis.

3.2.1 Raw data processing with platexpress

With the function *read.Experiment()* from *platexpress* the raw data from the BioLector Pro and the platelayout were imported. With this function the blank correction is done at the same time. Additionally it is possible to remove wells that were not grown or contaminated. With the function *viewGroups()* from *platexpress* the imported data was visualized. Therefore the mean and the 95% confidence interval over replicates are calculated with this function.

3.2.2 Calculation of saturated liquid concentration of oxygen

(The complete section was taken from the qatp.pdf by Rainer Machne with his agreement.)

Biorector sensors for dissolved oxygen provide values in % of the saturated concentration. The following text outlines how you can calculate actual molar concentrations and gas exchange rates. Mass exchange between gas and liquid is determined by an equilibrium between the gas' partial pressure in the input gas and liquid concentration, known as Henry's law. The saturated liquid concentration C^* can be calculated from the pressure P of the gas, usually as a "partial pressure" of the full e.g. atmospheric pressure, as:

$$C^{=} P \cdot H(T)$$

via the solubility H ("Henry coefficient") of the gas, and solubilities are provided for standard conditions ($T^0 = 298.15K$) with their temperature dependence (van't Hoff equation) in a compilation of experimental values (Sander 2015), and can be calculated for the given liquid temperature T as:

$$H(T) = H^0 e^{dH \cdot (1/T - 1/T^0)}$$

The Henry solubility is usually given in mol m^{-3} Pa^{-1} and this form requires the partial pressure of the gas in Pa, and a conversion from m^{-3} to L^{-1} to come to useful values. The atmospheric pressure is P_0 = 1 atm = 101 325 Pa, and 20.95% of this is the pressure of O_2. To get the saturated concentration, e.g. when bubbling culture medium (before inoculation) with air, we would use:

$$C^*_{O_2} = 0.295 P_O H^0 e^{dH \cdot (1/T - 1/T^0)})$$

,

and the O_2 the compilation provides $H^0_{O_2} = 1.283 * 10^{-5}$mol $m^{-3}Pa^{-1}$, and $dH_{O_2} = 1500K$. At culture temperatures T = 37°C = 310.15K (*E. coli*), we get a liquid concentration of $C^*_{O_2,37°C} = 224.2\mu M$. This is the 100% of the value reported by your dissolved oxygen measurment device, provided you calibrated it properly in the culture medium at the correct temperature.

3.2.3 Calculation of dissolved oxygen [%] into oxygen concentration [mmol/L]

To calculate the dissolved oxygen [%] in the medium into oxygen concentration [mmol/L] in medium we used the following equation:

$$\frac{smoothedDOvalues}{100 * 224.2\mu M}/1000$$

We used the smoothed and normalized DO data for this calculation. 224.2 µM was calculated with the Henry's law.

3.2.4 Calculation of carbon content in measured dry cell biomass

After determining the dry weight of the cells and normalizing to the volume, the carbon content in Cmol/L was calculated:

$$\frac{dcw * 0.48}{mwc}$$

where dcw = dry cell weight in [g/L] mwc = molecular weight of carbon = 12.0107 g/mol. The average carbon content of a cell is between 0.45 and 0.5, we have taken a value of 0.48.

3.2.5 Calculation the oxygen uptake rate (OUR)

In this the section we show how to calculate the $q_{cells}(t)$:

$$q_{cells}(t) = \left(\frac{dC}{dt} - k_L a \cdot (C^* - C(t)) \right) V_l$$

where the change of oxygen over time $\frac{dC}{dt}$ can be calculated from the (smoothed) oxygen concentration timecourse $C(t)$ and the specific mass transfer coefficient $k_L a$. This coefficient is depending from the mixing speed (rpm) and the culture volume and can be read from calibration tables provided by the BioLector Pro manufacturer *m2p-labs* (see figure 12). With 1 mL culture and 1000rpm the $k_L a$ is 260 h^{-1}. C^* is set as 100% and $C(t)$ is the calculated saturation concentration of oxygen. V_l is 0.001 L corresponding to the culture volume. For oxygen $q_{cells}(t)$ is negative, but can also be given as a positive value. Then we speak of an oxygen uptake rate (OUR). To make the data comparable, the $q_{cells}(t)$ is normalized by the biomass data, where we used the scatter values calibrated to biomass from the fits in figure 6. The results for the respective experiments can be seen in figure 10.

4 Results

4.1 Experiments

4.1.1 Pre-test I: quantification of bacterial growth on different nutrients: glucose versus acetate

As a preliminary scan of growth conditions we tested growth of two different strains on minimal growth medium M9 (table 3), supplemented with uracil, thiamine and trace metal (table [5] [6] [7]) and either glucose or acetate as carbon source. To keep experiments quantitatively comparable, glucose concentration where chosen conventionally in weight per volume % and acetate concentrations were adjusted to contain the same amount of carbon atoms (Carbon-mol). Since glucose contains 6-C atoms, therefore 3 times more acetate was added as substrate, because acetate has only 2-C atoms. This leads to a better determination of how much biomass is produced from glucose and how much from acetate.

The *Escherichia coli W3110* cultures were grown over night in 1x M9 medium (table 3) with 0.4% (weight per volume) of glucose or an equivalent amount Carbon-mol, but in the form of acetat. The strain *Escherichia coli DH5α* was cultured in 1x M9 medium, with equivalent amount of Carbon-mol in form of acetate to the glucose kulture (table 7), over night. The cells were then transformed to fresh 1x M9 medium with concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1% glucose (table 5) or acetate (table 6, table 7) as equivalent amount of Carbon-mol in form of acetate. *E.coli W3110* has shown only little growth on acetat medium. After two days of growth, the culture only reached an OD of 0.128. In comparison, the *E. coli W3110* culture from glucose growth achieved after one day an OD of 4.68. The *E. coli DH5α* culture reached after two days an OD of 1.838 on acetate. Therefore not enough cells were available of the *E.coli W3110* acetat culture, to try out all concentrations. For this strain only the acetate concentrations, equivalent to 0.2%, 0.6% and 1% glucose concentration, were tested and only two wells per concentration were used instead of three. For *E. coli W3110* glucose culture and *E. coli DH5α* all concentrations were applied to the plate as triplicates. The plate was a *Flowerplate with optodes for pH and DO* from the company *m2plabs GmbH*. Per well 1 mL culture was pipetted. The measurement was done in the *BioLector Pro* of *m2plabs GmbH* at 1000rpm and 37°C. The visualization and analysis of the measured data was performed in *Rstudio*, an IDE (integrated development environment) of the programming language *R*, with the functions *read.Experiment()* and *viewGroups()* from the package *platexpress*.

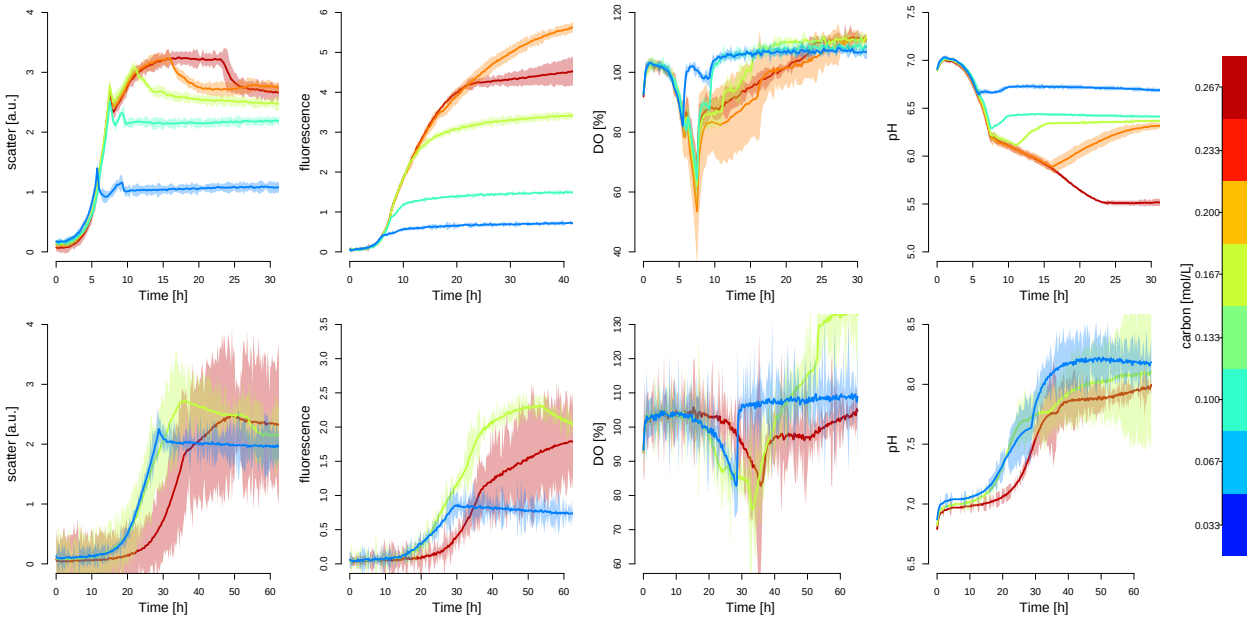

Figure 3: *Comparison of E .coli growth with glucose or acetate as sole substrate. The upper row shows the data of glucose growth. The lower row shows the data of acetate growth. Solid lines are the mean of replicates and the shaded areas show the 95% confidence interval. Three replicates were used for growth on glucose and only duplicates were avaible for growth on acetate. The growth took place in a FlowerPlate with optodes for pH and DO at 1000rpm and 37°C with 1 mL culture per well. Scatter [a.u.] is a measure for the biomass. fluorescence is measured at excitation/emission wavelengths where E. coli shows autofluorescence, most likely steeming from cellular riboflavin pools. DO[%] is the dissolved oxygen concentration of medium, measured with fluorescent optodes. 100% corresponds to the saturated concentration in the absence of oxygen consumption. pH is the pH of the growth medium as measured by fluorecent optodes.*

Strain *DH5α* did not show any growth in this preliminary experiment, therefore the following experiments were restricted to strain *W3110*. The upper row shows the glucose growth data, the lower row the acetate growth data. The scatter values show that acetate growth is not proportional to substrate concentration. With glucose growth a proportionality can be seen, whereas at higher concentrations this proportionality is less and less distant from each other. In addition, peaks can be seen in the scatter graphs. In the case of glucose, two peaks also occur at low substrate concentrations. The scatter values between acetate and glucose growth cannot be compared, since these vary from each other depending on the carbon concentration. A similar ratio can be seen in the fluorescence measurement. The acetate graphs are very chaotic and do not show any proportionality to the substrate concentration. At the same time, the values of fluorescence, on acetate growth, are more than half smaller than on glucose growth. A certain proportionality of the fluorescence, with glucose growth, can be seen, but also here the distances between the graphs differ. The DO [%] rises above 100% in stationary phase, which is due to a known artefact, where the excitation/emission wavelengths used for the DO optodes, overlap with auto-fluorescence of *E. coli* in minimal medium. Nevertheless, it can be seen from the decreasing values that oxygen is consumed for a while and then rises to its original level. The pH values show clear differences between growth on glucose or acetate. The pH value of the glucose culture decreases and the pH value of the acetate culture increases. However, both plots are barely proportional to the carbon concentration. Cultures on acetate show a very long lag phase (see different x-axis ranges in figure 3) and growth startet when the glucose cultures had already reached stationary phase.

4.1.2 Pre-test II: quantification of bacterial growth on different nutrients and M9 concentrations: glucose versus acetate

For the Pre-test II (figure 4) the culture medium was adepted (table [5][6]). Biotin (10g/L) was added and a new thiamine stock was used because of a miscalculation in the old stock. In order to get proportional growth, M9 concentration was increased proportionally to substrate concentration. At a substrate concentration of 0.8% there is a 4x M9 and medium component concentration, at 0.6% substrate concentration there is a 3x M9 medium component concetration and so on. The cells were grown over the weekend in 1x M9 medium with 0.4% glucose or 0.4% acetate. This allows a better habituation time of the cells to the medium, especially the cells in acetate need longer for growth. Then the cells were transfered into fresh medium for another 24h incubation. On the next day the cells were transferred into new medium with either 0.2%, 0.4%, 0.6% and 0.8% glucose or equivalent Carbon-mol amount in acetate. This time five wells were used per concentration instead of three. This experiment was interrupted by mistake for a short time and then had to be continued as a "new" experiment. Therefore the dataset was divided and could not be merged. Therefore the lower row is to be regarded as a continuation of the upper one. At the end of each experiment, 900µL of medium were taken from each well to determine the biomass by weighing. In the glucose graphs this can not be seen. In the acetate graphs this can be seen by the strong decrease of the lines. For the sampling of the strongest acetate concentrations we waited until the end of the experiment. Therefore there is no decreasing line for the maximal concentration.

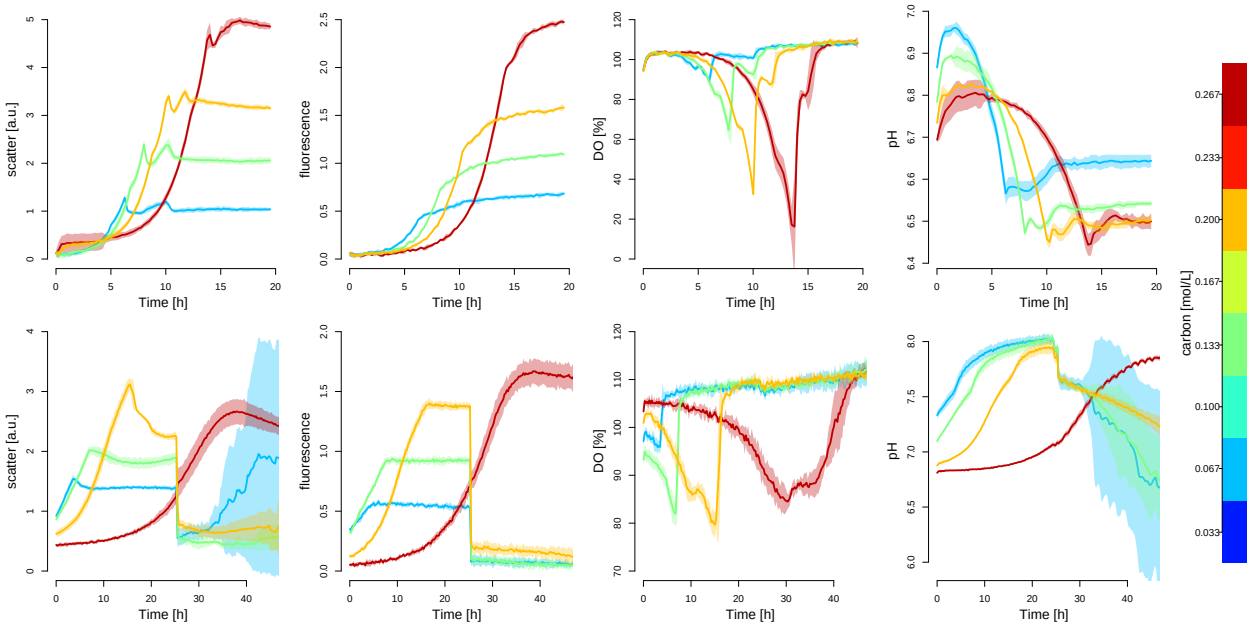

Figure 4: *Comparison of E. coli growth with glucose or acetate as sole substrate. The upper row shows the data of glucose growth. The lower row shows the data of acetate growth. Solid lines are the mean of replicates and the shaded areas show the 95% confidence interval. For the calculation five triplicates were used. The growth took place in a FlowerPlate with optodes for pH and DO at 1000rpm and 37°C with 1 mL culture per well. Scatter [a.u.] is a measure for the biomass. fluorescence is measured at excitation/emission wavelengths where E. coli shows autofluorescence, most likely steeming from cellular riboflavin pools. DO[%] is the dissolved oxygen concentration of medium, measured with fluorescent optodes. 100% corresponds to the saturated concentration in the absence of oxygen consumption. pH is the pH of the growth medium as measured by fluorecent optodes.*

The Pre-test II (figure 4) shows much more proportionality of the scatter graphs to the substrate concentration. This can be seen particularly well in glucose culture (upper row). The acetate culture (lower row) shows a deviation from the proportionality only at the highest substrate concentration. Generally it can be seen that with a higher substrate concentration and thus higher M9 concentration the lag phase of the growth curves is extended. See the scatter and fluorescence graphs. The scatter graphs again show a peak at acetate growth and two peaks at glucose growth. An advantage in the extended lag phase can be seen in the DO graphs.

These have again measured values over 100%, but the oxygen consumption phases are better separated from each other and can be better analyzed. It can be seen, in the DO graphs, that glucose growth is followed by another, but much shorter oxygen consumption after a strong oxygen consumption. With acetate growth, a second oxygen consumption phase also occurs. Unfortunately, this is not well visible for the smaller concentrations, since the data set was split and could not be combined. The pH values of the glucose culture generally decrease. Further down the graphs show a small increase. The pH values of the acetate culture increase without any further specificity.

4.1.3 Experiments I and II: quantification of bacterial growth on different nutrients and M9 concentrations in higher resolutions: glucose versus acetate

The Experiments I and II were made separate on two different plates. Experiment I is only with cultures growing in glucose. Experiment II only with cultures growing in ancetat. The substrate concentrations go from 0.1% to 0.8% in 0.1% steps for glucose. The carbon quantity in the concentrations is the same for glucose and acetate. The M9 concentrations also increase with the Carbon-mol amount. Since no parallel measurements are possible, the acetate growth was performed a few days later. In order to have more calibration data, small samples were taken during glucose growth in the lag phase, exponential phase, peak phase and stationary phase to make microscopy images for cell counts. When the graphs entered the stationary phase, samples were taken again to determine the biomass. The point in time can be recognized by the strongly decreasing graphs. Microscopy samples were also taken from the acetate culture. Due to the long growth time of the aceatate cultures, samples could not be taken for each phase. For biomass determination, samples were normally taken in the stationary phase. For better visualization, after sampling, the scatter and pH data of the acetate cultures (lower row) were removed because they caused strong noises. Therefore the lines in the two plots stop suddenly.

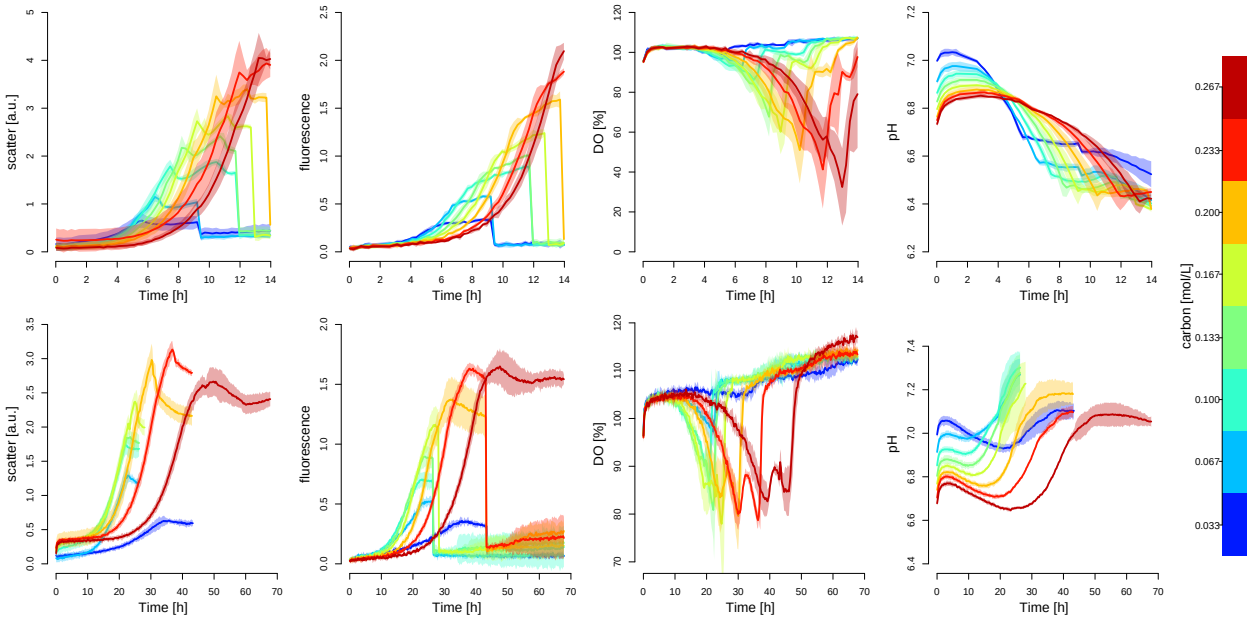

Figure 5: *Comparison of E. coli growth with glucose or acetate as substrate. The upper row shows the data of glucose growth. The lower row shows the data of acetate growth. Solid lines are the mean of replicates and the shaded areas show the 95% confidence interval. For the calculation five triplicates were used. The growth took place in a FlowerPlate with optodes for pH and DO at 1000rpm and 37°C. Due to an error only 0.875mL of the glucose culture was pipetted per well. From the acetate culture 1mL was pipetted per well. Scatter [a.u.] is a measure for the biomass. fluorescence is measured at excitation/emission wavelengths where E. coli shows autofluorescence, most likely steeming from cellular riboflavin pools. DO[%] is the dissolved oxygen concentration of medium, measured with fluorescent optodes. 100% corresponds to the saturated concentration in the absence of oxygen consumption. pH is the pH of the growth medium as measured by fluorecent optodes.*

In figure 5 the scatter graphs show proportional growth in glucose (upper row) and acetate (lower row). Except for the lowest and highest acetate concentration. The graph with the lowest concentration took a long time to reach the stationary phase. The graph with the highest concentration did not reach the highest scatter value. Again a prolonged lag phase can be seen, with increase of substrate and M9 concentration. Also the two peaks in glucose and the peak in acetate can be seen. During the fluorescence measurement of the acetate culture, peaks are also visible at the higher substrate concentrations. DO values higher than 100% were also measured in these experiments. The second oxygen consumption phases can also be seen. Interestingly, the samples with a higher substrate concentration start at a lower pH value. Also in this glucose culture the pH decreases, whereas at the lower end of the graphs a small increase of the pH values can be seen. With the acetate culture the pH first sinks, then rises again.

4.2 Calibrations

The calibration part shows how the generated data was used. On the one hand it should be checked if this data is coherent, on the other hand how it can be used in the model. Furthermore it will be described how to get from the Dissolved Oxygen (DO) values to the average oxygen consumption per cell.

4.2.1 Calibration of scatter measurment with biomass and cell number

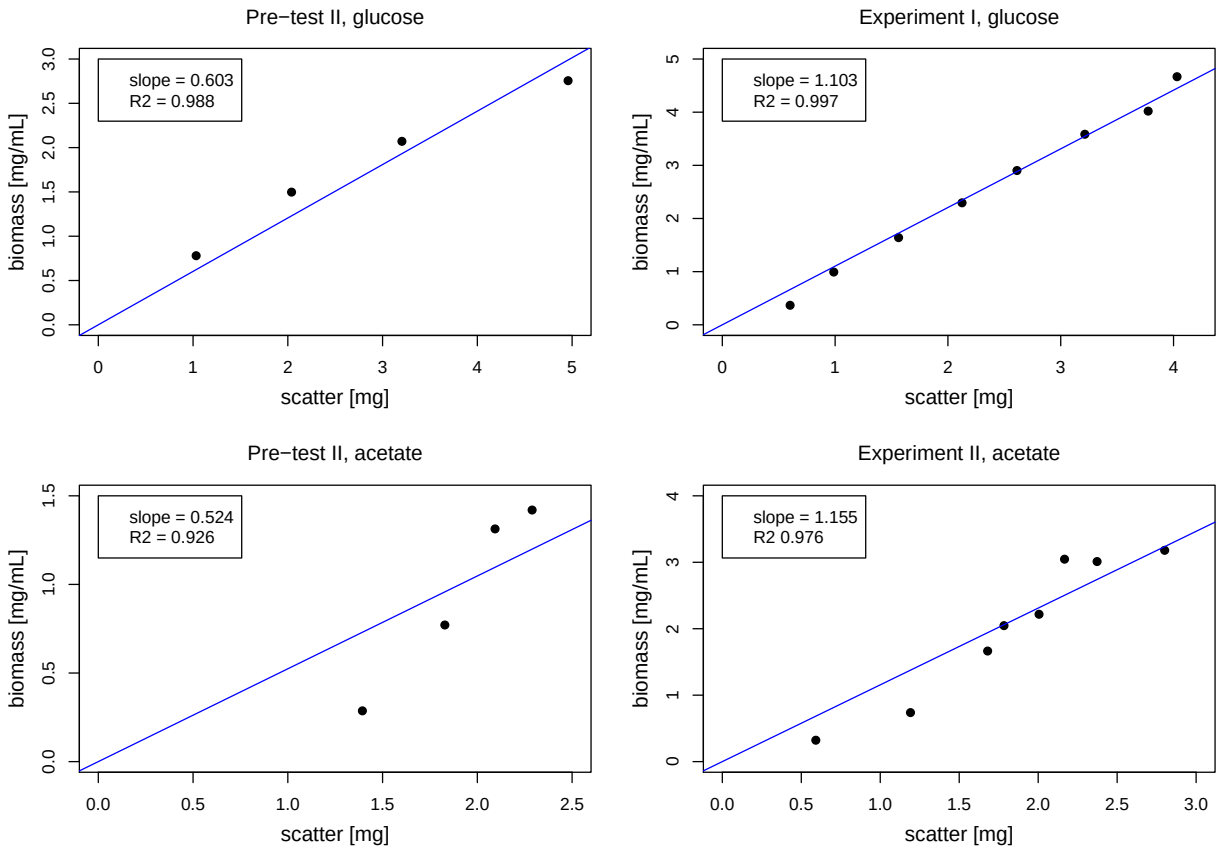

Figure 6: *Biomass against scatter plots from Pre-test II, Experiment I and II. On the y-axis is the biomass [mg/mL]. On the x-axis are the scatter [mg] values. The top row shows the data from the glucose experiments. On the left side from Pre-test II, on the right side from Experiment I. In the bottom row shows the data from the acetate experiments. On the left side from Pre-test II, on the right side from Experiment II. The blue lines are the linear regression lines. Additionally the slope of the respective linear regression and the R^2 = (R2) are given.*

The figure 6 shows the correlation between the biomass and the scatter values (calibrated to [mg] by the blue lines (fits)). The scatter values from the time of sampling were used because the biomass was determined at that time. The samples for biomass determination were always taken when a culture had reached the stationary phase. Therefore the whole experiment was stopped for a short time. From each well 900μL (800μL at experiment I) was taken from the respective culture. The pipette tip had to be pierced through the sealing foil. The foil cannot be removed temporarily. Possibly this would affect the still growing cultures. The experiment was then continued as normal. The respective samples were pipetted together in a tube. The cells were then centrifuged and the supernatant discarded. Then the tube was dried in a vacuum at 60°C for up to 8 hours and then weighed. The difference in weight of the tube (weighed before and after sampling) gives the biomass in mg. In order to be able to compare these values, the samples were normalized to their volume. The linear regression was forced through the origin, since zero scatter is expected for blank-corrected

scatter values. The corresponding biomass values are in the tables [13][14][15][16]. You can see from the slopes that the cultures from Pre-test II are comparable with each other. Also the Experiments I and II are comparable with each other. Between Pre-test II and Experiment I and II no comparison is possible, while the conditions in culture growth and measurement parameters are the same. Experiments I and II have about twice the slopes as Pre-test II. They also reach a higher biomass. On the other hand Experiment I does not reach a higher scatter value than Pre-test II glucose. The scatter value of Experiment II in the other hand has a higher scatter value than Pre-test II acetate. This raises the question in how far the scatter value gives information about the biomass. The R^2 values show that Pre-test II acetate is very different from the other experiments. Experiment I has the best R^2.

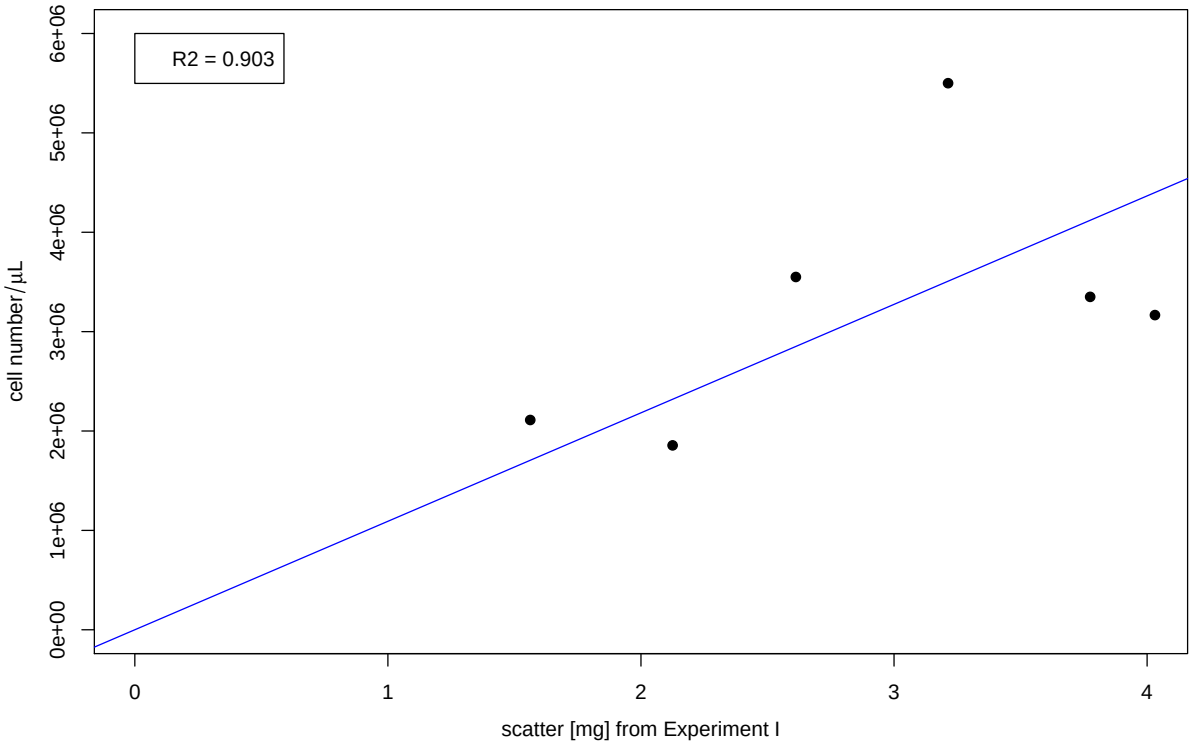

Figure 7: *Cell number/µL (y-axsis) against scatter [mg] from Experiment I (x-axsis). The blue line is the linear regression. Additionally the R^2 is given.*

In addition to figure 6 the figure 7 shows the counted cell number against the scatter. The cell number was determined by counting microscopic images. The Microscopy images were taken at the time of lag phase, exponential phase, peak phase and stationary phase. Therefore the experiment was stopped as soon as one of the cultures reached one of the phase. From this culture 3 µL were taken. For samples from the exponential phase onwards, the sample was diluted 1/10 and applied to the counting plate. The cells were counted in a certain area on the plate and the cell count was recalculated to µL. For the comparison with the scatter values, only the cell count in the stationary phase was used. The points correspond in increasing order to the substrate concentrations of 0.3% - 0.8% in 0.1% steps. For the concentrations 0.1% and 0.2% the images are no longer available and therefore not plotted. A general increase of the cell count to the scatter can be seen, but there is no real correlation. This is also shown by the low R^2 (= R2). The linear regression was forced through the origin, since zero scatter is expected for blank-corrected scatter values.

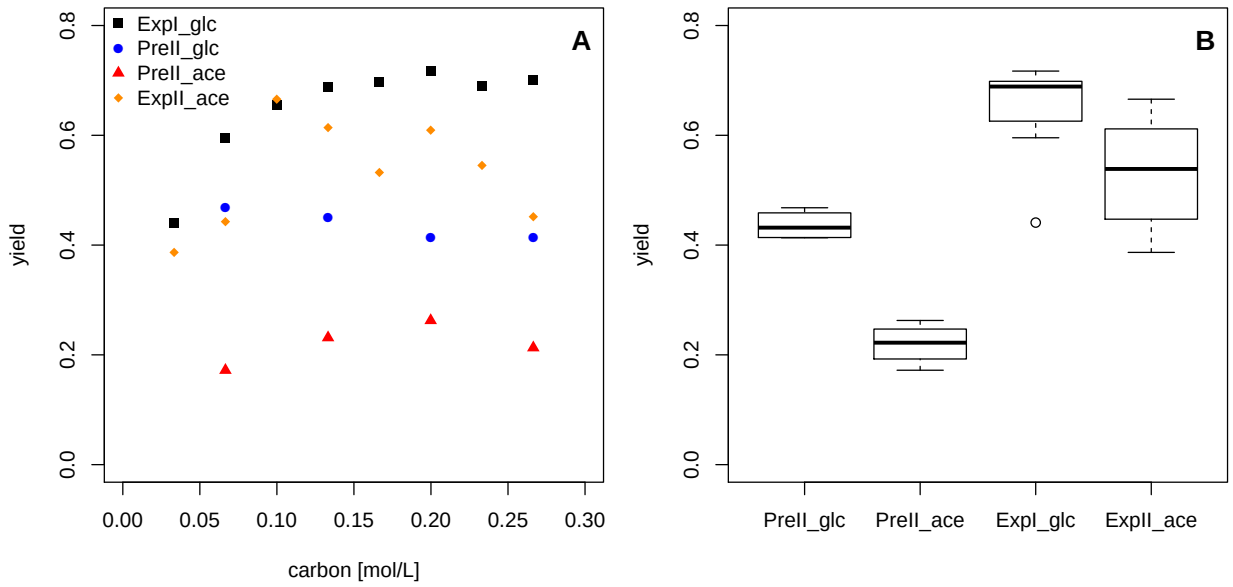

Figure 8: *Plot A shows the yield values of the Pre-test II, Experiment I and II against the carbon amount [mol/L] in the medium. Plot B shows the yield values of the respective experiments in boxplots. ExpI_glc = Experiment I, ExpII_ace = Experiment II, PreII_glc = Pre-test II glucose, PreII_ace = Pre-testII acetate.*

The figure 8 shows the yield values of the respective experiments in two different plots. In plot A the yield value is plotted against the carbon content of the medium. In plot B the yield is plotted in boxplots to have a better comparison between the experiments. The respective values are listed in the tables [13][14][15][16]. The yield values of ExpI_glc and ExpII_ace are clearly higher than the corresponding values of PreII_glc and PreII_ace. Basically, the values of cultures grown with glucose are higher than those grown with acetate. The values of PreII_glc and PreII2_ace do not show a large distribution of the data, also visible from the boxplots in B. ExpI_glc also shows little distribution, but seems to approach a plateau value. ExpII_ace shows a strong scattering of yield values, also seen from the large box plot in B. In plot B it is easy to see that the glucose cultures show a higher yield than the acetate cultures. Nevertheless, the yields of PreII_glc/_ace cannot be compared with those of ExpI_glc and ExpII_ace, although the culture conditions and measurement parameters are identical.

4.2.2 Calculation of metabolic rates: O_2 consumption

This part shows how the average O_2 consumption can be calculated from the DO data. For visualization the data from Pre-test II, glucose are used. The detailed calculations can be found in the methods section.

100% dissolved oxygen represent the concentration, where growth medium is fully saturated with oxygen. In all experiments values higher than 100% were measured. In order to be able to use the data for further calculations, they were normalized. For this the local maximum, before the onset of oxygen consumption by growing cells, were used. Then, with the package *pspline*, a smoothing of the data is done to reduce the noise. The saturation concentration can be calculated via Henry's law from the solubility coefficienct of oxygen (Henry coefficient) and the ambient atmospheric concentration (see Methods). For our conditions we obtain $C^*_{O_2,37°C} = 224.2\mu M$. This value is calculated by the equation 1, which is described in the method part. The resulting dataset can be seen in the figure 9. This differs from the DO plot from Pre-test II glucose (figure 4 only in the y-axis), where now the oxygen content is given in mmol/L instead of a percentage.

$$C^* = P \cdot H(T) \tag{1}$$

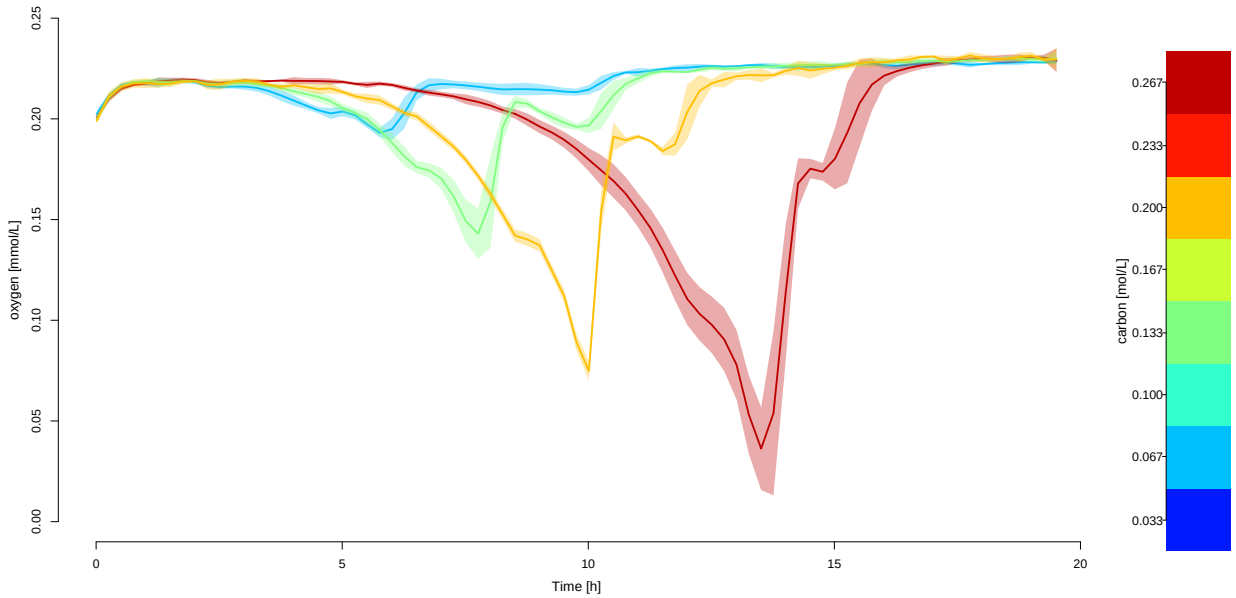

Figure 9: *Plot of the DO values normalized, smoothed and calculated from [%] into [mmol/L]*

With the formula 2 (Müller, S. , Murray, D. B. and Machne, R. 2012) we can now calculate the oxygen uptake rate (OUR).

$$q_{cells}(t) = \left(\frac{dC}{dt} - k_L a \cdot (C^* - C(t)) \right) V_l \tag{2}$$

Where the change of oxygen over time $\frac{dC}{dt}$ can be calculated from the (smoothed) oxygen concentration timecourse $C(t)$ and the specific mass transfer coefficient $k_L a$. This coefficient is depending from the mixing speed (rpm) and the culture volume and can be read from calibration tables provided by the BioLector Pro manufacturer *m2p-labs* (see figure 12). With 1 mL culture and 1000rpm the $k_L a$ is 260 h^{-1}. C^* is set as 100% and $C(t)$ is the calculated saturation concentration of oxygen. V_l is 0.001 L corresponding to the culture volume. For oxygen $q_{cells}(t)$ is negative, but can also be given as a positive value. Then we speak of an oxygen uptake rate (OUR). To make the data comparable, the $q_{cells}(t)$ is normalized by the biomass data,

where we used the scatter values calibrated to biomass from the fits in figure 6. The results for the respective experiments can be seen in figure 10.

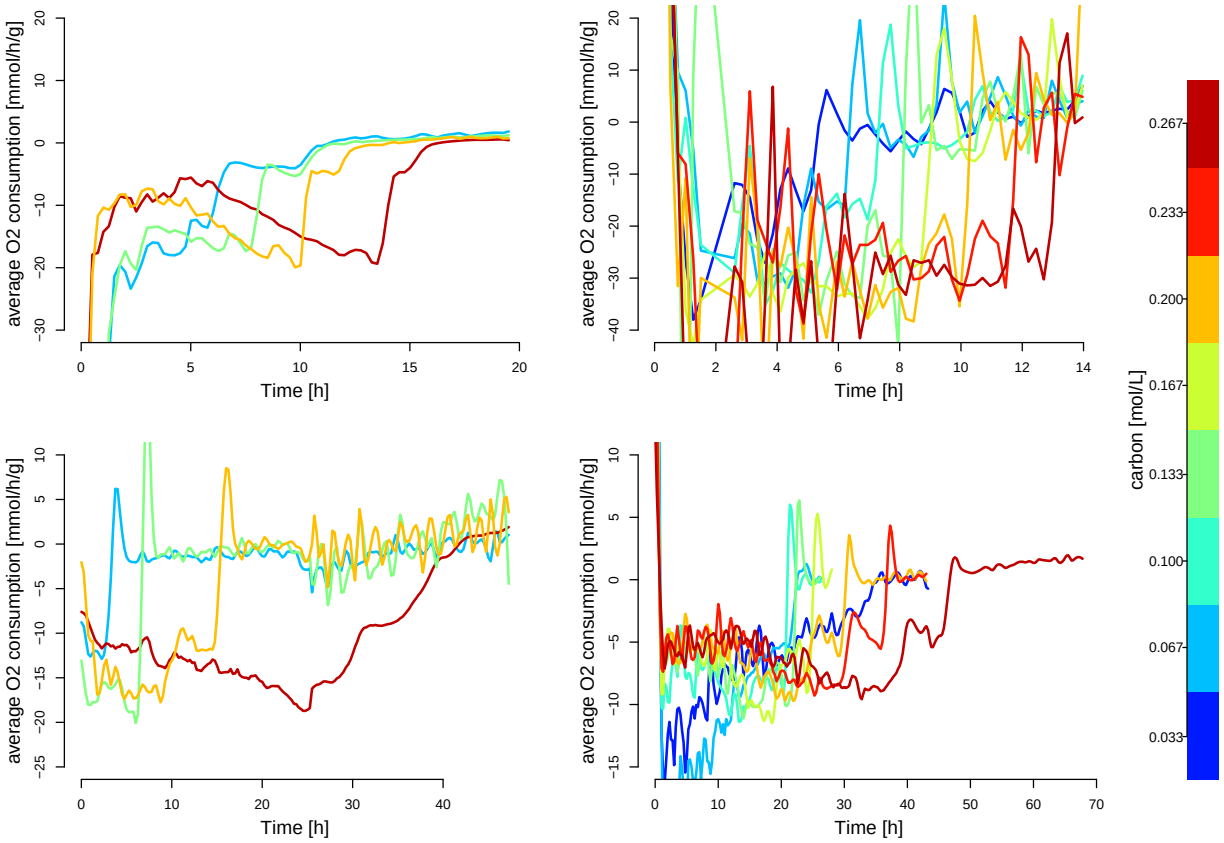

Figure 10: *Plot with the calculated average oxygen consumption [mmol/h/g]. Solid lines are the mean of the respective replicates. In the top row are the glucose experiments. On the left Pre-test II and on the right Experiment I. In the bottom row are the acetate experiments. On the left Pre-test II and on the right Experiment II.*

In figure 10 the oxygen consumption in mmol/h/g is plotted for Pre-test II, Experiment I and II. The graphs correspond to the calculated mean of the respective samples. Each experiment has a different average oxygen consumption. This makes a comparison only conditionally possible. Within the experiments are also differences between the different concentrations. In Experiment II (bottom right) it can be seen that the graphs of the lower concentrations simply rise to zero instead of having a constant value over a certain time. Interesting is the second drop of some graphs just before zero is reached. The maximum value that is reached before the stationary phase is -9 mmol/h/g. The graphs in Pre-test II glucose (top left) show a constant phase at the beginning, but then change to a phase where more and more oxygen is consumed. The maximum value reached is -20 mmol/h/g. Such values are also known from literature (Andersen et al. 1980)(Slavov et al. 2014). Unfortunately the results are generally very noisy and never really constant. It is a permanent change between metabolic phases.

5 Discussion

5.1 Calibration of BioLector Pro Measurments

The BioLector Pro allows to measure multiple parameters simultaneously and non-invasively for a variety of cultures. Up to 48 cultures can be measured simultaneously. With the special microfluidic plates, 32 cultures can be examined simultaneously, whereby it is possible to control the pH or to initiate a nutrient supply. The different parameters are measured by built-in filters or sensitive polymers, which are installed under the wells. Since the filters can be exchanged and different plates with polymers are available, a wide range of measurement data can be generated. In the experiments, four parameters were measured.
The scatter measurements are used to identify the biomass of cell culture. Light with a wavelength of 620 nm is projected onto the culture. The light, which is scattered back at an angle of 180°, is detected. The more light is backscattered from the cells, the more biomass (cells) the culture contains. An increasing scatter value is an indicator for a growing cell culture. The fluorescence data was measured using a riboflavin filter (λ_{Ex}:436 nm / λ_{Em}:540 nm). These describe a crude fluorescence of riboflavin in the cells. The DO (dissolved oxygen) and the pH data were measured using optodes attached to the bottom of the plates. The DO optodes are excited by light (λ_{Ex}:520 nm) and emit a fluorescence signal (λ_{Em}:600 nm). This signal is quenched by oxygen in the medium. The amount of dissolved oxygen correlates accordingly with the degree of quenching. The optode for pH measurement contains a hydronium ions sensitive staining and a reference staining. For example, if the pH decreases, the fluorescence intensity of the hydroniom ions sensitive optode also decreases. The pH value is determined by the resulting phase shift. The measurement is made at the wavelengths λ_{Ex}:470 nm / λ_{Em}:525 nm, at a range from 4.5 pH to 7.5 pH.

In order to be able to use the generated data to answer biological questions or to investigate cell growth dynamics using quantitative models, further calibrations were made.
An important point was to find out, that the measured scatter data are a safe indicator for the biomass. Therefore, in Experiments I and II, samples were taken to determine the biomass of the cultures. The calculated biomass was then plotted against the respective scatter value. In figure 6 the plots can be seen. Except Pre-test II with acetate, all experiments have a good R^2. Basically it can be seen that an increasing scatter is an indicator for increasing biomass. Despite the same conditions under which the cultures grew, Pre-test II cannot be compared with Experiments I and II. The slopes of Experiment I and II are almost twice as large as those of Pre-test II. At the same time the scatter values are similarly large. For glucose growth they are between zero and five. For acetate growth between zero and three. One possible reason, for the gradient differences, could be the time at which the sample was taken. The samples from Experiment I and II were taken shortly after the start of the stationary growth phase. In Pre-test II the cultures were partly in the stationary phase for several hours until the samples were taken.
In addition, the reliability of the scatter values should be investigated by the cell number. In the third and fourth experiment samples were taken during growth to create microscopic images where the cells were counted. For Experiment I, images could be taken for all concentrations and almost all growth phases. For acetate this was not possible because the growth is over 3 days long. The counted cells from Experiment I and the respective scatter values can be seen in figure 7. For the concentrations 0.1% and 0.2% the cell numbers are not available and were therefore not plotted. A general increase of the cell count according to the scatter can be seen. Nevertheless, the increase is very random and is not based on the substrate concentrations. Therefore these data could not be used for further calibrations. These strong deviations in the cell count could have been caused by the dilution. 3µL medium was diluted in 27µL water. Then 3µL was taken for microscopy. With these small volumes, deviations in the distribution of the cells can occur. A solution would be to perform the counting without dilution. Depending on the growth time of the culture, this can become very tedious. A program which makes it possible to count cells automatically would help.

With the determination of the biomass it is additionally possible to determine the yield of the cultures. The yield value ranges from 0 to 1 (0% - 100%) and indicates how much of the substrate of the medium has been converted into biomass. For this, the proportion of carbon in the biomass must be calculated. If the biomass carbon content is then divided by the carbon content of the medium, the yield is obtained. The calculated yield values for the experiments can be seen in the figure 8. In plot A the yield is plotted

against the carbon content in the medium. Plot B shows the boxplots of the yield values of the experiments. Except for ExpII_ace the yield values for the respective experiment are nearly the same. The yield difference between glucose growth and acetate growth is also clearly visible. On glucose growth an average yield of 0.45 can be expected (??). For PreII_glc the yield is slightly lower. The yield of ExpI_glc is much higher than the average. Similar deviations can also be seen in acetate cultures. In general, the acetate yield value is lower than that of glucose. But ExpII_ace has a yield value almost three times higher than exp2_ace. A biological reason, for the higher yield values from exp3_glc and ExpII_ace, is not recognizable. Especially the conditions and the solutions were the same. A mistake in the preparation of the samples could be an important reason for the high yield. The samples from Pre-test II were washed after the first centrifugation and then centrifuged again. The samples from Experiment I and II were not washed in an intermediate step, thus the increasing "biomass" values could also reflect increasing salt concentration from the M9 medium, which was upscaled together with carbon concentrations. In addition, it could be observed that during the preparation of the growth solutions flakes were formed. A possible reason for a higher weight of the unwashed samples and thus a higher yield.

In addition to the verification of the scatter data, the reliability of the oxygen measurement was also examined. The BioLector Pro measures a percentage value that describes how much oxygen is dissolved in the medium. This percentage value is proportional to the maximum value, which differs depending on the conditions and can be calculated with the help of the Henry coefficient, H(t) in equation 1. With the measured data, it is particularly noticeable that values above 100% are measured. Especially after the growth phase the dissolved oxygen concentration seems to increase. Since *E. coli* does not produce oxygen, this seems to be an error in the measuring method. The measurement is made via fluorescence, therefore there is the possibility that the fluorescence signal may combine with the signal of fluorescent substances from *E. coli* overlaps. This would explain the higher measured value. The manufacturers are also aware of this problem and new plates with optimized optodes have already been produced. Since the delivery of the new plates takes some time, the error was corrected by normalization to the local maximum before the growth phase.
Then the data was used to calculate the specific oxygen consumption of the cells (figure 10) by calculating oxygen concentration in mmol/L via Henry's law, then taking the factory-calibrated specific mass transfer coefficient k_La for our conditions to estimate the total oxygen consumption, and finally normalizing by the 'scatter' values, calibrated to biomass to obtain the specific oxygen consumption rate in mmol/h/(g dry cell weight). This procedure combines all calibrations and thus all measurement errors outlined above and interpretation is therefore highly preliminary. It yieled good results for the Pre-test II experiments, but produced very noisy and inconsistent graphs for Experiments I and II. This may again be due to the missing washing step in biomass determination in Experiments I and II or due to frequent microscopy sampling from wells especially in Experiment I. In all experiments a steady state of respiratory activity was apparently never reached. Similar dynamic variation of respiratory vs. fermentative activity throughout constant growth phases had recently been observed in the budding yeast *Saccharomyces cerevisiae* (Slavov et al. 2014). In Pre-test II, maximal respiratory activity was slowly approached and reached maximal values of ca. 20 mmol/h/g only at the end of the main exponential growth phases. Notably, this reflects a value that had been suggested previously as the upper limit of *E. coli*'s "respiratory capacity" (Andersen and von Meyenburg 1981). Similar values were reached on both glucose and acetate as carbon sources, at least in Pre-test II. However, a caveat here is that, as discussed above, the peak in scatter valus at the end of exponential growth phases may likely came from length growth of cells and may thus not reflect actual biomass increase. The maximal respiratory activities were reached close to these time points and thus are also affected by such artefacts of the scatter measurement. Further characterization of the scatter value's dependence on cell morphology and the improved oxygen measurement in novel BioLector plates will be required to allow a more accurate quantification of oxygen consumption rates. The analysis could also benefit strongly from more sophisticated data smoothing approaches.

5.2 Biological results

The calibrations showed that the scatter indicates the growth of the culture. The plots in figure [3][4][5] can now be used to draw further biological conclusions. In Pre-test II, Experiment I and II, a extended lag phase can be seen in the cultures. In these experiments, in addition to the increase in substrate concentration, the quantity of components of the growth medium was also increased. It can be assumed that the increased concentration of the components inhibits cell growth at the beginning. Especially, because in the first experiment, where only 1x M9 was used, no extended lag-phase is visible. The cells were grown in 1x M9 medium and then transferred to the higher concentrated medium. Possibly, the cells first provide active transporters to remove the excess of salts and ions. This requires a certain amount of time and energy, which is then missing during growth. It can also be seen that the acetate cultures have longer lag-phases than the glucose cultures. This could be due to the fact that less energy is gained from acetate and therefore even more time passes for the provision of active transporters. As soon as sufficient transporters are established, more energy will be invested in growth.
Another special feature of the scatter graphs are the peaks. The glucose growth scatter has two peaks and the acetate growth scatter has one peak. It is assumed that at this time the cells show a growth in length and thus influence the scatter measurement. Stevenson et al. 2007 showed in their work that this length increase has an effect on the OD measurement. With a scatter measurement, this behavior could have an even stronger effect. In order to investigate this effect visually, microscopy images were taken at the time of the peaks (figure 13). The pictures show that the cells are longer at the time of the peak than in the other growth phases. In addition, it can be seen that some cells are in the process of division. The second peak in the glucose graphs is a sign of a diauxic shift. Where the glucose culture metabolizes the previously excreted acetate. To what extent the extension of the cells has biological effects cannot be explained by the scatter values and microscopic images alone. Further information can be obtained from the DO measurements. The DO measurements show the period over which the cell generated energy through oxygen consumption. In glucose cultures, two oxygen consumption phases (reversed peaks) can be seen in the DO graphs. First a strong consumption and then a short consumption phase. The acetate cultures also show two oxygen consumption phases, whereby the strength of these differs depending on the experiment. In Pre-test II (figure 4) the first phase is rather short and then there is a strong consumption. In contrast, the two consumption phases of Experiment II (figure 5) are almost equally strong. By plotting the scatter and the respective DO graphs on top of each other, the energy consumption of the cell can be compared at the time of the scatter peaks. See figure 11, where the data from Experiment I and II are shown with glucose (plot A) and acetate (plot B). All other comparison plots are in the appendix, figure [14][15].

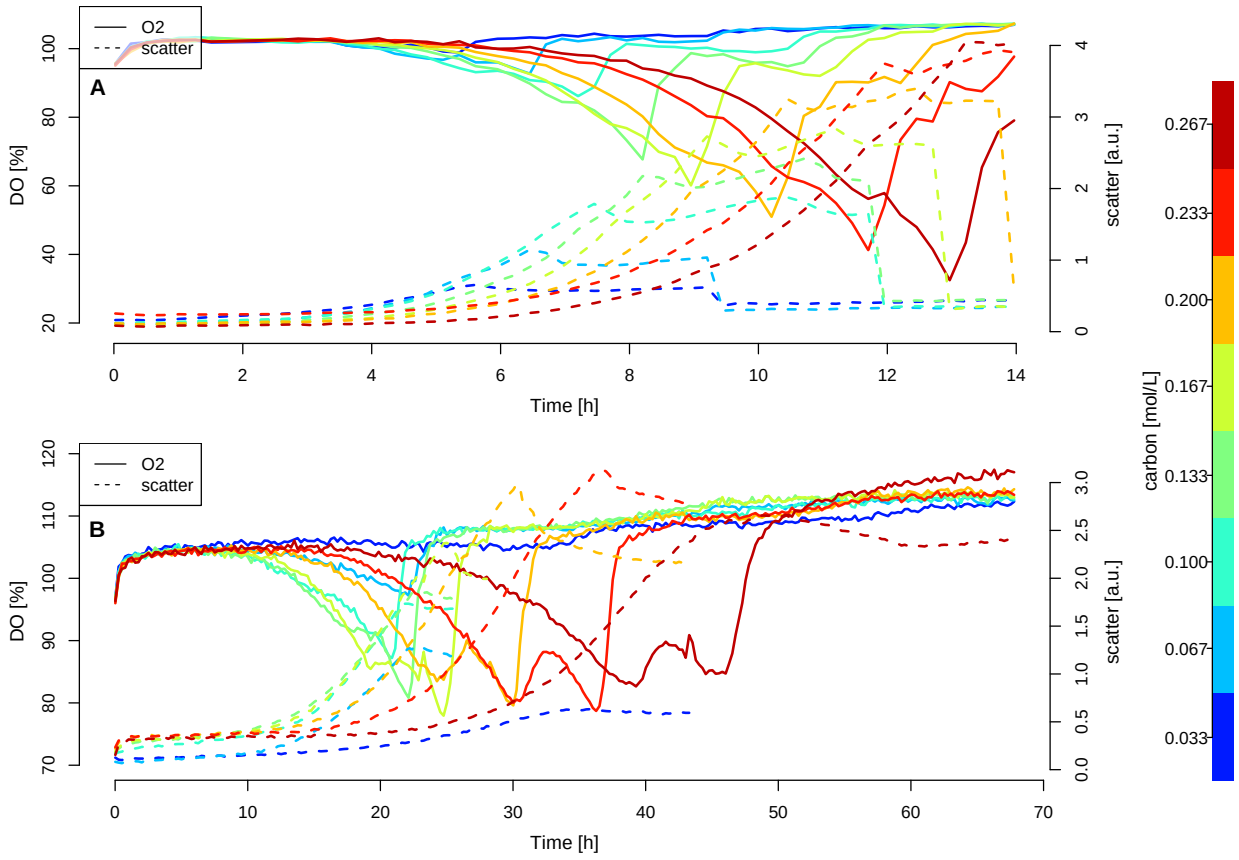

Figure 11: *Overlapping plots of scatter and DO values for Experiment I (A) and II (B)*

Plot A shows that at the time of the first peak most oxygen is consumed. The second peak also matches the second oxygen consumption. This strengthens the assumption on the diauxic shift, where again energy is generated by acetate consumption. With acetate data, the situation is somewhat different. The first smaller oxygen consumption peak is about the time when the cells are in the exponential growth phase. This cannot be seen from the scatter graphs. This consumption peak has similarities to the peak of the glucose culture, which possibly leads back to a diauxie. With acetate, however, no diauxia is to be expected. The second consumption peak coincides with the scatter peak and shows a strong energy canalisation. It could be that the first peak shows the time at which the substrate is used up, both for glucose growth and acetate growth. The second peak is then created by channeling additional resources. In glucose cultures, this is the acetate still present in the medium. In acetate cultures, this could be internal sugar storage or fats/amino acids from dead cells. This last channeling of energy leads to lengthy growth and then to the last cell division.

How much information can be obtained from the fluorescence and pH measurements is still open. These have not been further investigated as this would have been beyond the limits of this work. Furthermore, for the calibration of the data, a deeper understanding of the metabolism and biochemistry of *E. coli* is necessary. The fluorescence graphs are interesting in this context, because they have a similar graph curve as the scatter graphs. They have the extended lag-phase and a growth proportional to the substrate concentration. But they show no peaks. This can be advantageous if the fluorescence data can be used to establish a reference to the biomass. Alternatively, the biomass can then be determined from this without the peak distorting the values. Out of interest, the fluorescence data were calibrated using the same method as the scatter data. But due to the lack of background knowledge, not discussed further.
With the necessary knowledge and mathematical methods, the average H^+ ion flow can be determined from the pH values, which can give information about metabolic activity. Similar to the DO data and the specific oxygen consumption. The graphs also show differences depending on the substrate. Especially at

the beginning a short increase of the pH values can be seen in the experiments. The manufacturer justifies this with the adjustment of the temperature. This could possibly be avoided by adjusting the BioLector Pro and the cultures to the desired growth temperature before measuring. In addition, it is possible to carry out experiments with the microfluidic plates that allow pH control. The planning of these experiments is much more complicated than the experiments with normal plates. In addition, these plates are not really cheap.

5.3 Summary and Outlook

In this work, the reliability of the data generated by BioLector Pro was examined. In addition, it was examined to what extent the energy consumption of *E. coli* can be determined from this data.
In order to examine calibrations and calculations for their reliability, repeatable experiments are essential. Therefore, the experiments have been kept as simple as possible to ensure this. This goal was only partially achieved. Especially after the calculation of the specific oxygen consumption, it was shown that many differences exist between the experiments (figure 10). This made the calculation of the average ATP production redundant, as the values would not have been based on reliable data. To make this possible some improvements can be made. Among other things, further experiments can be done with the improved plates. Furthermore, individual steps can be optimized, e.g. the determination of the biomass could also be done by elemental analysis. In this way, the carbon content could be specified more precisely. This would make the calibration more accurate in general. Furthermore, the growth medium could be optimized, e.g. to avoid prolonged lag phases.
Nevertheless, it could be shown that the scatter is a good indicator for the biomass (figure 6). It was also possible to draw biological conclusions from the graphs. Particularly interesting was the energy consumption during the scatter peaks. Surprisingly, a secound oxygen consumption phase was also seen on acetate medium, which is not shown in the scatter plots. Further information can be obtained from the fluorescence and pH data. Especially the fluorescence measurement can give a lot of information about cell growth and cell metabolism.
In general, it can be said that the BioLector Pro offers a very large potential to observe biological processes from several points of view at the same time. However, some steps still need to be taken to establish the BioLector Pro.

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6.1 Figure references

Figure 1: https://kids.britannica.com/kids/assembly/view/180459

Figure 2: By M • Komorniczak -talk-Illustration by : Michał Komorniczak This file has been released into the Creative Commons 3.0. Attribution-ShareAlike (CC BY-SA 3.0)If you use on your website or in your publication my images (either original or modified), you are requested to give me details: Michał Komorniczak (Poland) or Michal Komorniczak (Poland).For more information, write to my e-mail address: m.komorniczak.pl@gmail.com - Own work by uploader, based in the information and diagrams found in:Rice University, Institute of Biosciences an Bioengineering [1]Alaska Statewide, High School Science Symposium [2]Unit 3 Biology, Further Biology [3]University of Arizona (Course) [4]This W3C-unspecified vector image was created with Inkscape., CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=7731719

Appendix

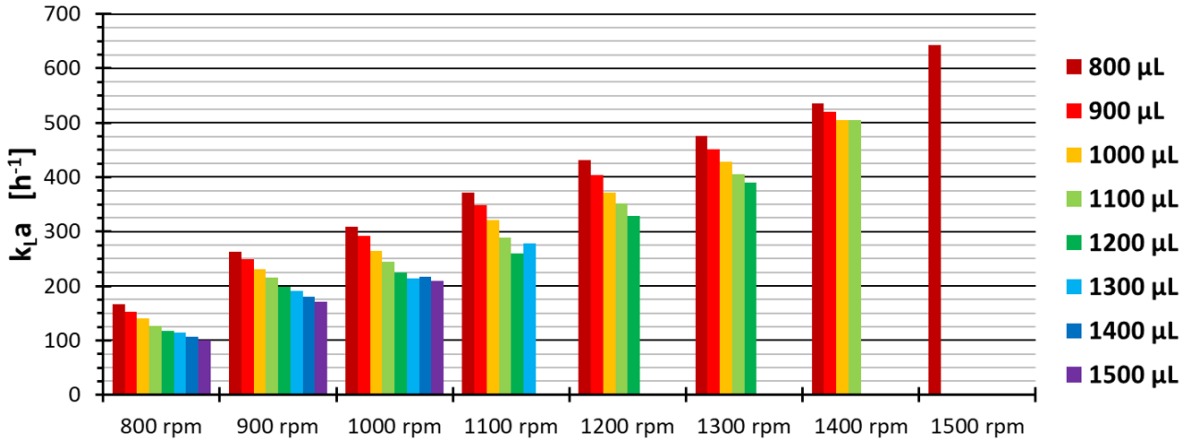

Figure 12: *Plot of the k L a values for specific rpm and culture volume, provided by m2p-labs*.

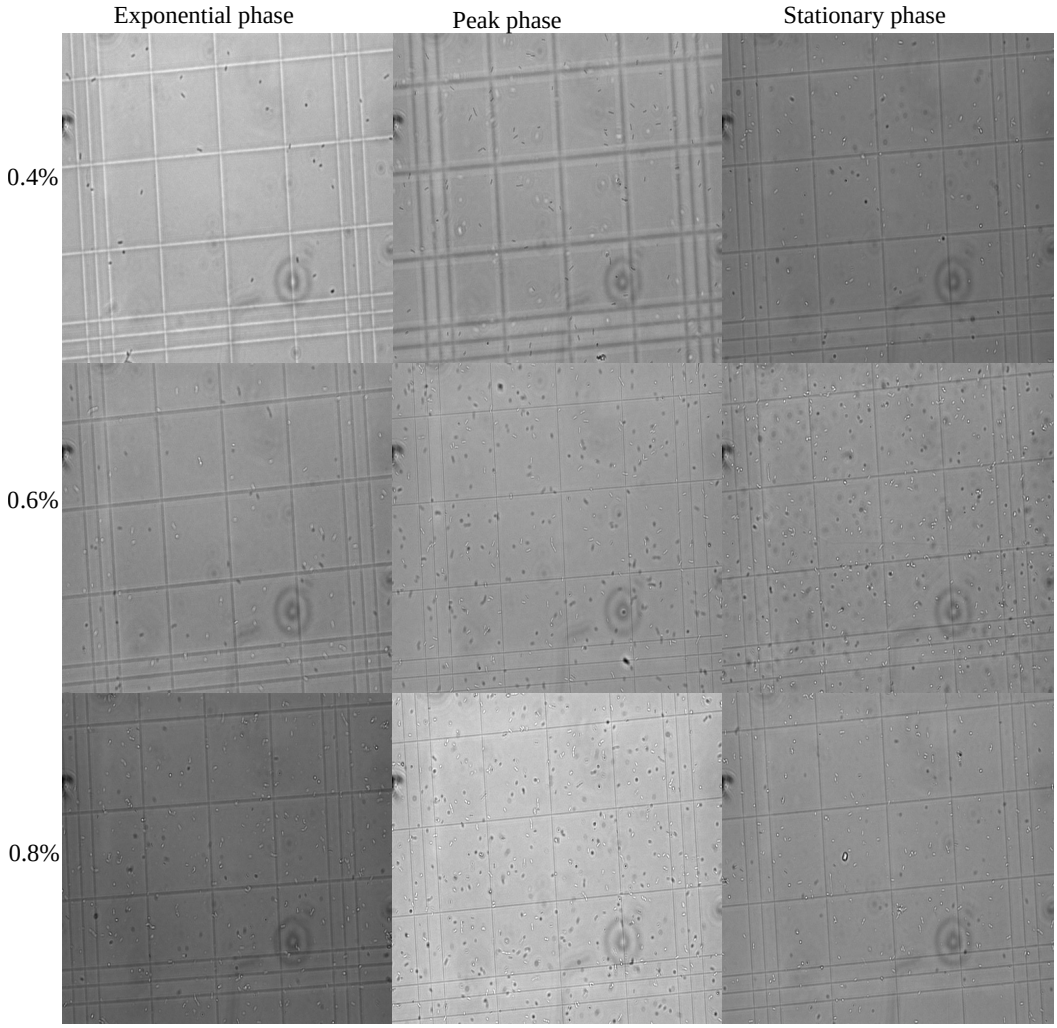

Figure 13: *Mikroskopic pictures of the Experiment I. Only for the concentrations 0.4%, 0.6%, 0.8%. Without the lag phase pictures. From left to right = exponential phase, peak phase, stationary phase. From top to bottom = 0.4% glc, 0.6% glc, 0.8% glc*

Table 13: Table from dry cell weight measurment. Data from Pre-test II, glucose. conc.glc = weight per volume of glucose in medium, eppi_empty = weigth of the empty eppi [mg], eppi_dcw = weight of an eppi with cells after drying [mg], mg_diff. = difference of eppi_dcw/eppi_empty in [mg], mg.mL = normalized to volume (4.5 mL) in [mg/mL], biomasscarbon = carbon content in the dry cells [Cmol/L], mediumcarbon = carbon content in the medium [Cmol/L], yield = yield of the culture (biomasscarbon/mediumcarbon).

conc.glc	eppi_empty	eppi_dcw	mg_diff.	mg.mL	biomasscarbon	mediumcarbon	yield
0.2%	3148.22	3151.73	3.51	0.7800	0.0312	0.0666	0.4680
0.4%	3146.88	3153.62	6.74	1.4978	0.0599	0.1332	0.4493
0.6%	3147.47	3156.79	9.32	2.0711	0.0828	0.1998	0.4142
0.8%	3150.53	3162.93	12.40	2.7556	0.1101	0.2664	0.4133

Table 14: Table from dry cell weigth measurments. Data from Pre-test II, acetate. conc.ace = , eppi_empty = weigth of the empty eppi [mg], eppi_dcw = weight of an eppi with cells after drying [mg], mg_diff. = difference of eppi_dcw/eppi_empty in [mg], mg.mL = normalized to volume (4.5 mL) in [mg/mL], biomasscarbon = carbon content in the dry cells [Cmol/L], mediumcarbon = carbon content in the medium [Cmol/L], yield = yield of the culture (biomasscarbon/mediumcarbon).

conc.ace	eppi_empty	eppi_dcw	mg_diff.	mg.mL	biomasscarbon	mediumcarbon	yield
0.2%	3155.52	3156.81	1.29	0.2866	0.0114	0.0666	0.1720
0.4%	3147.43	3150.90	3.47	0.7711	0.0308	0.1332	0.2313
0.6%	3148.73	3154.64	5.91	1.3133	0.0524	0.1998	0.2627
0.8%	3148.76	3155.15	6.39	1.4200	0.0567	0.2664	0.2130

Table 15: Table from dry cell weigth measurments. Data from Experiment I. conc.glc = weight per volume of glucose in medium, eppi_empty = weigth of the empty eppi [mg], eppi_dcw = weight of an eppi with cells after drying [mg], mg_diff. = difference of eppi_dcw/eppi_empty in [mg], mg.mL = normalized to volume (4 mL) in [mg/mL], biomasscarbon = carbon content in the dry cells [Cmol/L], mediumcarbon = carbon content in the medium [Cmol/L], yield = yield of the culture (biomasscarbon/mediumcarbon), cell_number = cellnumber/µL.

conc.glc	eppi_empty	eppi_dcw	mg_diff.	mg.mL	biomasscarbon	mediumcarbon	yield	cell_number
0.1%	3147.88	3149.35	1.47	0.3675	0.0147	0.0333	0.4410	
0.2%	3157.61	3161.58	3.97	0.9925	0.0397	0.0666	0.5955	
0.3%	3153.17	3159.73	6.56	1.6400	0.0655	0.0999	0.6560	2111111
0.4%	3147.22	3156.40	9.18	2.2950	0.0917	0.1332	0.6885	1855556
0.5%	3157.71	3169.32	11.61	2.9025	0.1160	0.1665	0.6966	3550000
0.6%	3149.15	3163.49	14.34	3.5850	0.1432	0.1998	0.7170	5500000
0.7%	3154.45	3170.53	16.08	4.0200	0.1607	0.2331	0.6891	3350000
0.8%	3147.14	3165.81	18.67	4.6675	0.1865	0.2664	0.7001	3166667

Table 16: Table from dry cell weigth measurments. Data from Experiment II. conc.ace = weight per volume of acetate in medium, eppi_empty = weigth of the empty eppi [mg], eppi_dcw = weight of an eppi with cells after drying [mg], mg_diff. = difference of eppi_dcw/eppi_empty in [mg], mg.mL = normalized to volume (4.5 mL) in [mg/mL], biomasscarbon = carbon content in the dry cells [Cmol/L], mediumcarbon = carbon content in the medium [Cmol/L], yield = yield of the culture (biomasscarbon/mediumcarbon).

conc.ace	eppi_empty	eppi_dcw	mg_diff.	mg.mL	biomasscarbon	mediumcarbon	yield
0.1%	3157.92	3159.37	1.45	0.3222	0.0129	0.0333	0.3867

conc.ace	eppi_empty	eppi_dcw	mg_diff.	mg.mL	biomasscarbon	mediumcarbon	yield
0.2%	3150.72	3154.04	3.32	0.7378	0.0295	0.0666	0.4427
0.3%	3148.41	3155.90	7.49	1.6644	0.0665	0.0999	0.6658
0.4%	3151.85	3161.06	9.21	2.0467	0.0818	0.1332	0.6140
0.5%	3148.42	3158.40	9.98	2.2178	0.0886	0.1665	0.5322
0.6%	3150.96	3164.67	13.71	3.0467	0.1218	0.1998	0.6093
0.7%	3147.23	3161.54	14.31	3.1800	0.1271	0.2331	0.5451
0.8%	3157.06	3170.61	13.55	3.0111	0.1203	0.2664	0.4517

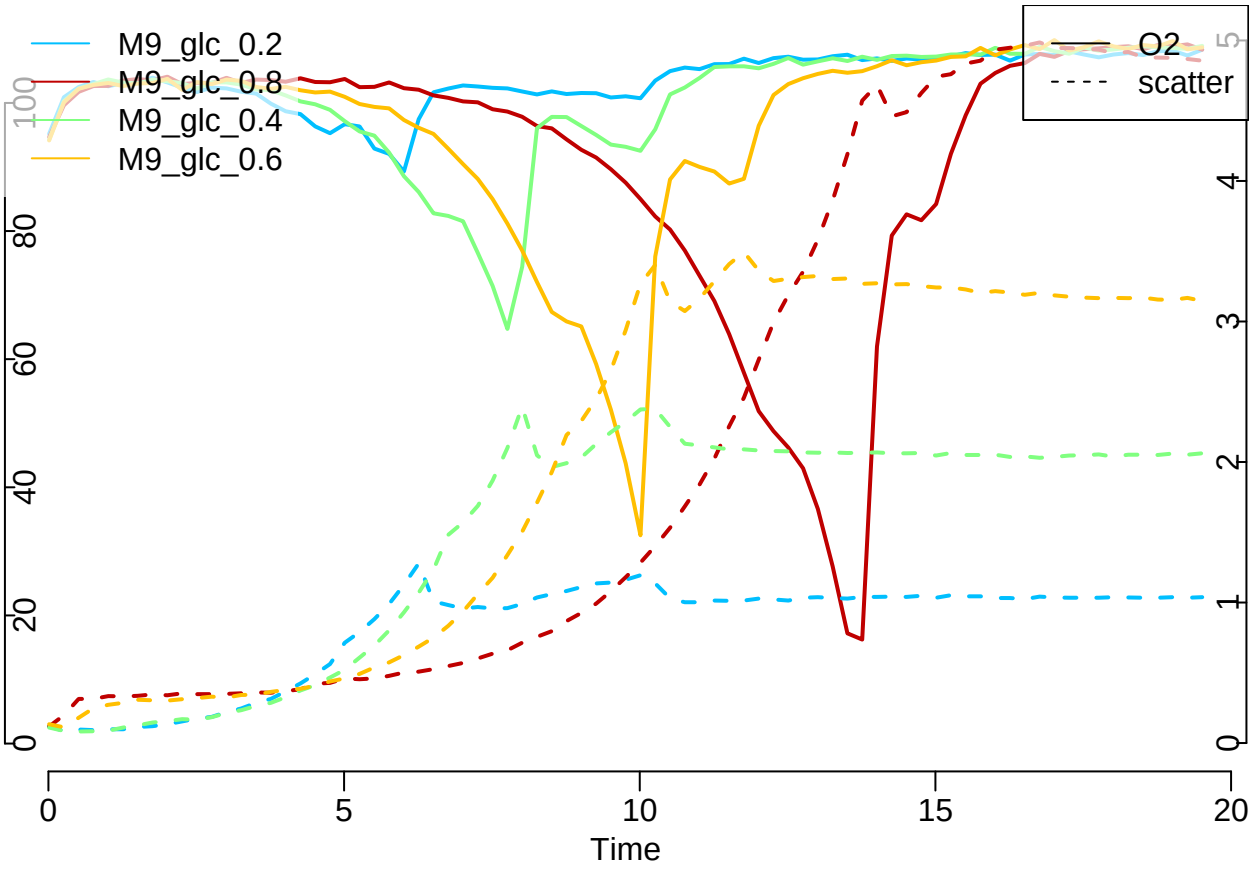

Figure 14: *Overlapping plot of scatter and DO values from Pre-test II glucose*

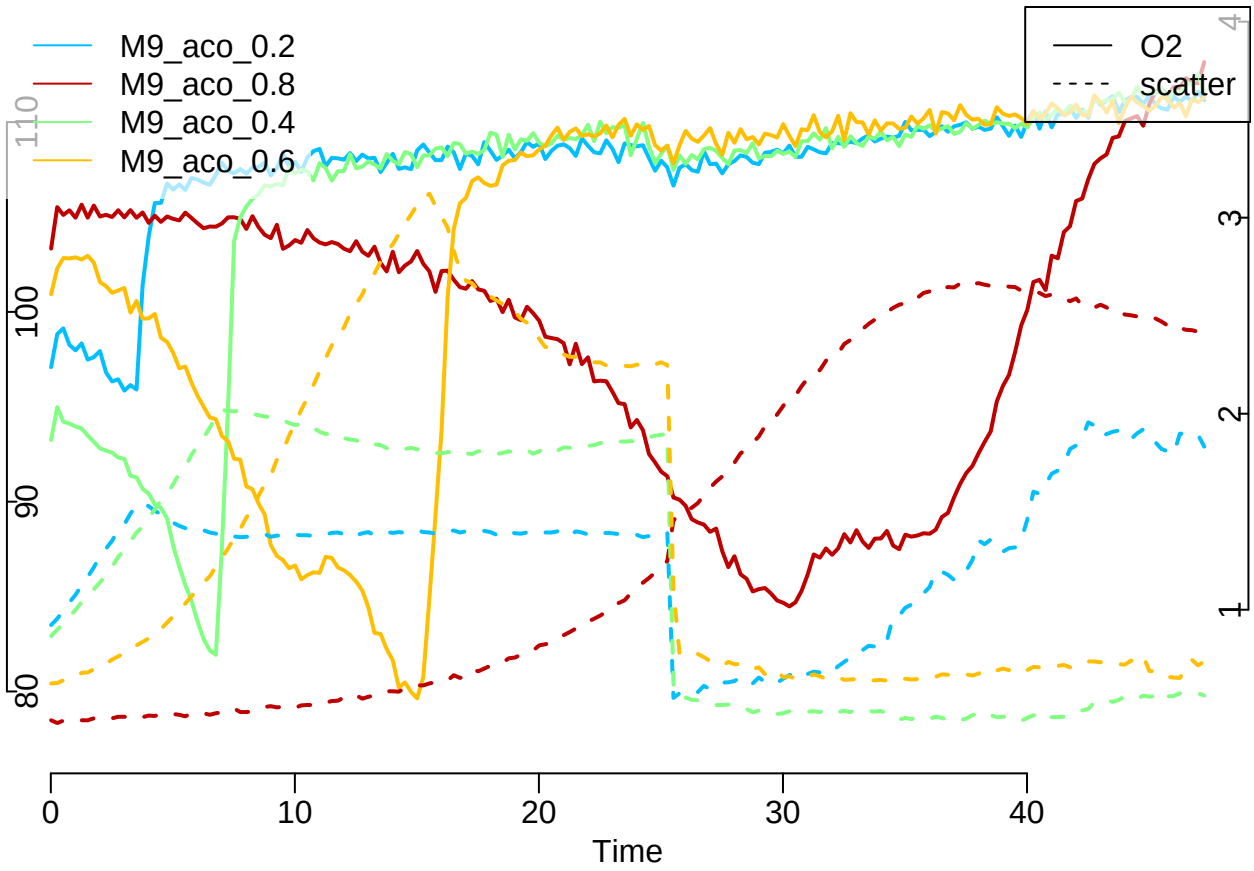

Figure 15: *Overlapping plot of scatter and DO values from Pre-test II acetate*

Declaration of academic honesty | Selbstständigkeitserklärung

I hereby declare that this Bachelor thesis was generated by myself and that all other resources and references used were cited and listed. I also declare that this work was not submitted to an examination committee in any other context.

Hiermit erkläre ich, dass ich die vorlegende Bachelorarbeit selbstständig angefertigt habe und alle genutzten Quellen zitiert und gekennzeichnet habe. Ich erkläre außerdem hiermit, dass die vorliegende Masterarbeit noch nicht in gleicher oder anderer Form einer Prüfungsbehörde vorgelegen hat.

Sergej Britner

Düsseldorf, der 12.08.2019

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