TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Establishing of an equivalent process platform for *P. pastoris* fermentation in different scales

Master Thesis

by

Bernhard Kreppel

To obtain the academic degree of a

Master of Science (M. Sc.)

Institute:
Technische Universität Wien –
Institut für Verfahrenstechnik, Umwelttechnik und Techn.Biowissenschaften

Supervisor:
Dr. rer. nat, MSc, Dipl.-Ing. Jens Fricke

Supervising Professor:
Univ.Prof. Dipl.-Ing. Dr.techn. Christoph Herwig

_____ _____

Date Signature (Student)

Statutory Declaration

"I declare in lieu of an oath that I have written this master thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This master thesis has not been submitted elsewhere for examination purposes."

Date: 28.02.2018

Bernhard Kreppel

Abstract

Scale-up problems of processes have been known in all producing sectors and technologies. However biotechnology with the extraordinary complexity of working with living organisms puts itself in an exceptionally challenging position. Scale-up issues can become very time consuming and costly since it delays progress to full-scale production and can even lead to a stop of development.

This thesis focused on scaling-up a *P. pastoris* fermentation from a 5L lab scale fermenter to a 60L pilot plant fermenter. Prior to this, CFD simulations of the 60L fermenter with different fermenter setups were performed. The result was an identification of the most suitable setting of agitator and feed position as well as agitation to ensure homogeneity in the reactor's liquid phase. These calculations were verified by several *E. coli* fermentations with the output that the simulated highest homogeneity results in the highest substrate to biomass yield.

A scale-up of a *P. pastoris* fermentation in lab scale reactor, whose liquid phase is considered to be highly homogeneous, was chosen to consolidate the findings for parameters by the CFD simulations. The process started with a batch phase and continued with a fed batch phase, both with glycerol as the C-source to gain biomass. A pulse with methanol was conducted afterwards so the organism adapted to the new C-source methanol and changed its metabolic state, in which *P. pastoris* metabolized methanol to biomass as well as the product horseradish peroxidase (HRP), an extensively used enzyme in biotechnological and medical applications. After the complete consumption of methanol, a fed batch phase followed with a step-wise increase of cell specific substrate uptake rate. An intended accumulation of methanol identified the maximum cell specific uptake rate of methanol of 0.12 $g_S/(g_X*h)$.

The fermentation process was successfully executed in both fermenters. Metabolic rates and quotients were calculated to quantify the comparability of the fermentation in different scales. Calculated values for all other phases were equivalent and do not differ significantly. For example, for the batch phase, the cell specific growth rate was found to be 0.23 h^{-1} in 5L bioreactor and 0.21 h^{-1} in the 60L fermenter, substrate to biomass yield 0.72 g_S/g_X (5L) and 0.63 g_S/g_X (60L). Those values resulted in a cell specific substrate uptake rate of 0.32 $g_S/(g_X*h)$ in the small scale and 0.33 $g_S/(g_X*h)$ in the pilot plant scale.

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Symbol	Unit	Description
μ	h^{-1}	Cell specific growth rate
μ_L	kg/(m*s)	Dynamic viscosity
c_A	g_A/L or mol_A/L	Concentration of substance A
CER	mol_{CO2}/h	Carbondioxid evolution rate
K_S	g/L	Half-velocity constant – the value of S when $q_s/q_{s,max}$ = 0.5
m	g	Mass
n	mol	Amount of substance
$n_{agitator}$	min^{-1}	Rounds per minute by agitator
Ne	-	Newton number
OUR	mol_{O2}/h	Oxygen uptake rate
P	W	Power input
p	Pa	Pressure
$q_{S/X}$	$g_S/(g_X*h)$	Cell specific substrate uptake rate
r_A	mol_A/h or g_A/h	reaction rate of substance A
Re	-	Reynolds number
RQ	mol/mol or g/g	Respiratory quotient
S	g/L	Concentration of limiting substrate
t	h	Time
T	°C	Temperature
V	L	Volume
V_M	m^3/mol	Molar volume
vvm	Q_{air}/VL	Volumetric gas flow rate per volume of liquid phase
X	g_X	Biomass
$Y_{P/S}$	g_P/g_S or mol_P/mol_S or c-mol_P/c-mol_S	Substrate to product yield (e.g. glycerol to biomass)
ρ	g/L	Density

1. Introduction

Scaleability has major impact on process development, e.g. technical feasibility in different scales and economic reasonability. Especially inhomogeneities of substrates or physical bioprocess parameters can greatly vary in scale. This can impact the performance of the process in larger scales which makes economical unreasonable to pursue development of such processes. Hence, predictions of the expected outcome of fermentations are desired to avoid waste of resources and time. Scale independent conversion rates and yield coefficients are used to compare fermentations in different scales. To maintain homogeneity in larger scales mixing power input can be increased which may result in damaging shear stress for organisms; especially mammalian cell cultures are sensitive (F. Bylund, 2000). Different approaches are established to face these issues. Geometric similarity is a widely used in biotechnology (L.-K. Ju, 1992). In this strategy all characteristic proportions, e.g. ratio of agitator diameter and tank diameter, are constant over all scales and scale independent numbers can be determined.

Literature (L.-K. Ju, 1992) suggests following significant and critical parameters to be kept constant to successfully scale up bio reactors:

1. Reactor geometry;
2. Volumetric oxygen transfer coefficient k_La;
3. Maximum shear;
4. Power input per unit volume of liquid phase P_L/V
5. Volumetric gas flow rate per unit volume of liquid phase (Q_g/V_L);
6. Superficial gas velocity v_s;
7. Mixing time;
8. Impeller Reynolds number;
9. Momentum factor;

Depending on the application, type and aim of the fermentation different criteria should be targeted. The first criterion is based on empirical correlations in reactors with the same or very similar geometries (e.g. specific power input, volumetric gas flow rate and k_La value). Height to diameter of the vessel ratio, distances between stirrers to diameter of stirrer value ratio and impeller to vessel diameter ratio comprise the geometry. Criterion two often applies as the aerobic fermentations are usually oxygen limited. A constant k_La value ensures enough oxygen solved in the liquid phase and therefore available for organisms. Mammalian cell cultures are very sensitive to shear stress (G. Kretzmer, 1990). Exposing cells to lethal shear force causes

unrepairable damage in cell morphology. The forth criterion is likewise the previous one and aims to prevent damaging shear forces for the organisms. Also keeping the power input per liquid volume constant is often applied in penicillin production (Chopra, 2004) as the penicillin titer is a function of the specific power input. Criterion 5 and 6 address like number 2 aerobic fermentations but are easy to realise without any further experiments. The seventh criterion is aiming for ensuring homogeneity whereas 8 and 9 more relate to shear stress (N. Blakebrough, 1966).

Problems with geometric similarity can occur since mostly the heat transfer is just possible using the surface of the bioreactor. While in small scales required heat transfer capacity is given, in large-scale fermenters might not be the case. This is due to the fact that the volume (and therefore heat build-up due to cell growth and mixing power input) increases to the power of three whereas the surface for cooling and heating just to the power of two (lower surface-to-volume ratio). Generally a constant specific power input P_I/V is recommended. These issues can be solved by consider this relation beforehand in process development, cooling strategies (e.g. suitable cooling medium) and bio reactor design.

As undesired inhomogeneities in large bioreactors are to be prevented, CFD (Computational Fluid Dynamics) software is often used to simulate fluid dynamics in bioreactors (F. Bezzo, 2003). Within the diploma thesis of D. Pavlicek (Pavlicek, 2013) several CFD simulations with different bioreactor setups as well as different operation modes were executed. The variating parameters were impeller distance, feed position and stirrer speed.

As significant values for homogeneity UI scalar and UI velocity were chosen. The UI scalar is the uniformity index which shows how uniform the flow is through a given section. So the higher the UI scalar value, the higher homogeneity is to be expected. Simulations have been executed to identify the bioreactor setup with the highest homogeneity. An example is shown in Figure 1 and results of the study are shown in Table 1.

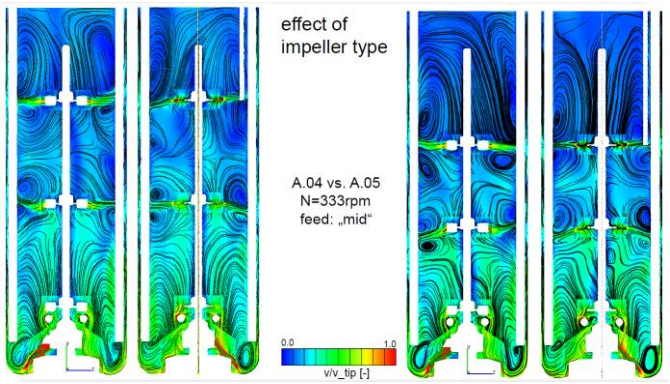

Figure 1 Relative velocity of A.04 and A.05 operation mode (Wechselberger, 2014)

	impeller	feed	N [rpm]	vtip [m/s]	UI_velocity	UI_scalar
A.1	2.5xD	top	333	1,74	0,748	0,610
A.2	2.0xD	top	333	1,74	0,722	0,625
A.4	2.5xD	mid-N12	333	1,74	0,747	0,641
A.5	2.0xD	mid-N12	333	1,74	0,721	0,597
A.7	2.5xD	down-N14	333	1,74	0,748	0,609
A.8	2.0xD	down-N14	333	1,74	0,722	0,649
A.10	2.5xD	top	1000	5,23	0,749	0,609
A.11	2.0xD	top	1000	5,23	0,723	0,628
A.13	2.5xD	mid-N12	1000	5,23	0,749	0,669
A.14	2.0xD	mid-N12	1000	5,23	0,723	0,580
A.16	2.5xD	down-N14	1000	5,23	0,749	0,611
A.17	2.0xD	down-N14	1000	5,23	0,723	0,646
A.19	2.5xD	top	666	3,49	0,749	0,609
A.20	2.5xD	mid-N12	666	3,49	0,748	0,647
A.21	2.5xD	down-N14	666	3,49	0,748	0,614

Table 1 Results of CFD simulations for ZETA BIRE with different setups (Wechselberger, 2014)

Calculating the UI scalar for certain setups within the design space result in the preferred A.13 setup. The lightened A.13 setup includes an impeller distance of 2.5 times the diameter of the impeller and less surprisingly a mid-feeding position as well as the maximum stirrer speed.

The validation of the CFD simulated mixing times has been performed with conductivity experiments as suggested by literature and shown in Figure 2 (Qinghua Zhang, 2009).

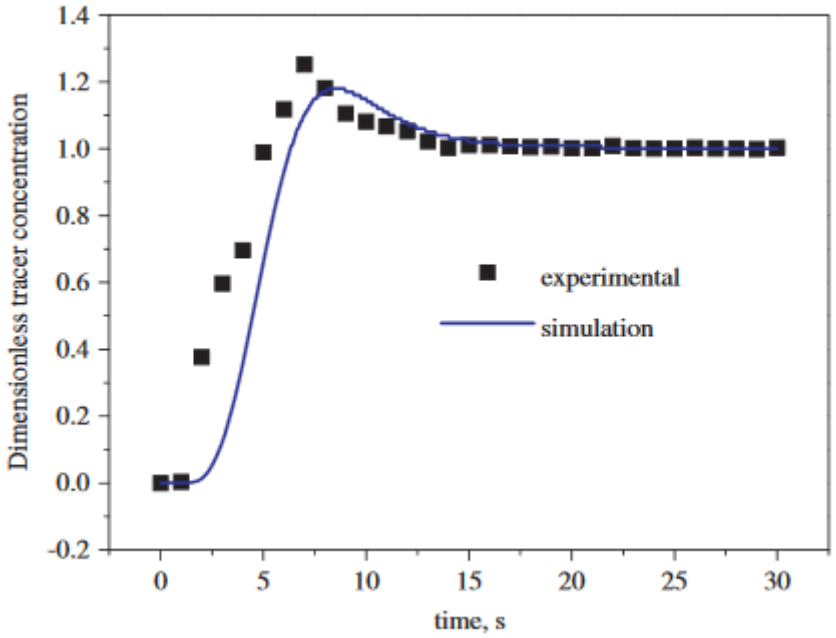

Figure 2 comparison between experimental and predicted tracer response as a function of time (Qinghua Zhang, 2009)

Since the CFD simulations are quite computationally intensive the following process parameters were chosen for the validation study:

	impeller	feed	stirrer speed [rpm]		impeller	feed	stirrer speed [rpm]
M.01	2.0xD	top-mid-mt	50	**M.04**	2.0xD	top-mid-mt	900
M.02	2.0xD	top-mid-mt	1000	**M.05**	2.0xD	top-mid-mt	200
M.02_D2	2.5xD	top-mid-mt	1000	**M.06**	2.0xD	top-mid-mt	300
M.03	2.0xD	top-mid-mt	525				

Therefore mixing times were investigated to quantify the homogeneity of the operation modes.

To increase the reliability of the results two probes were in installed and compared as shown in Figure 3.

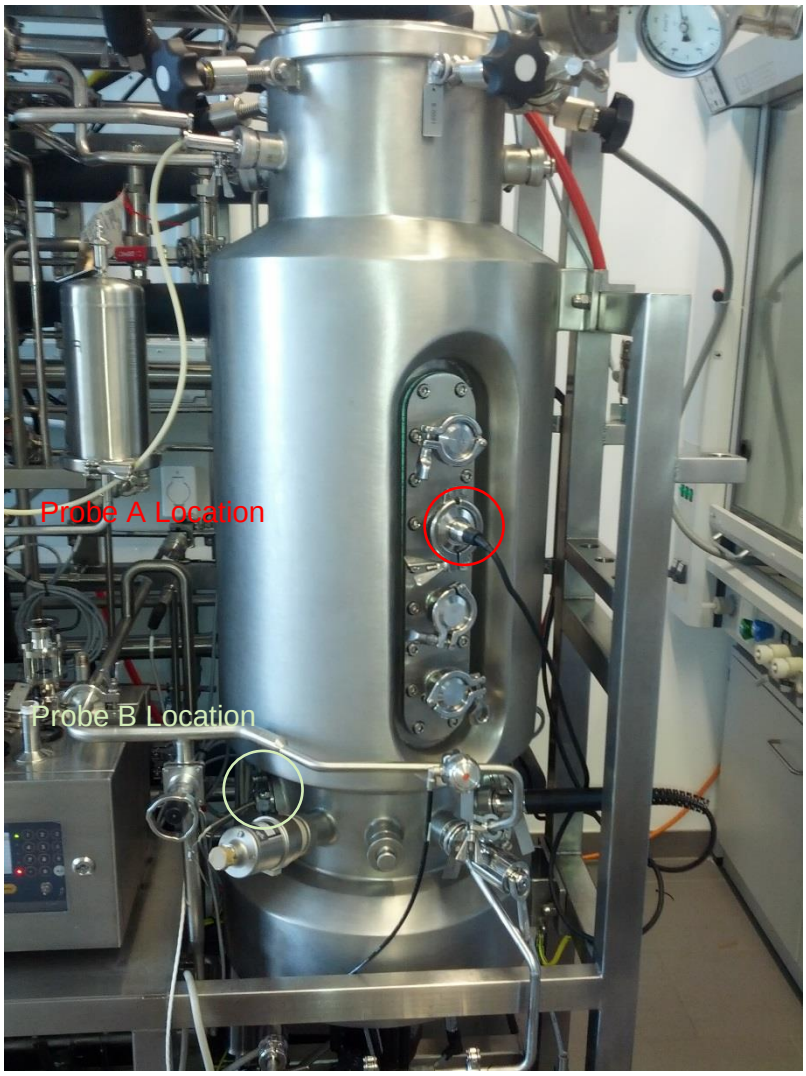

Figure 3 locations of probes for mixing time experiments (Wechselberger, 2014)

For each bioreactor setup six shots of the tracer were injected over the defined feed slot. The mixing time is defined as the average time to reach the final concentration (stable; +/- 5%) after each tracer addition. The different locations of the probes resulted naturally in different conductivity profiles over time as shown in Figure 4 and Figure 5. The experimental parameters were:

Parameter	Setting
Agitation	50 rpm
Aeration	0
Temperature	30°C
Impeller distance	2 times impeller diameter

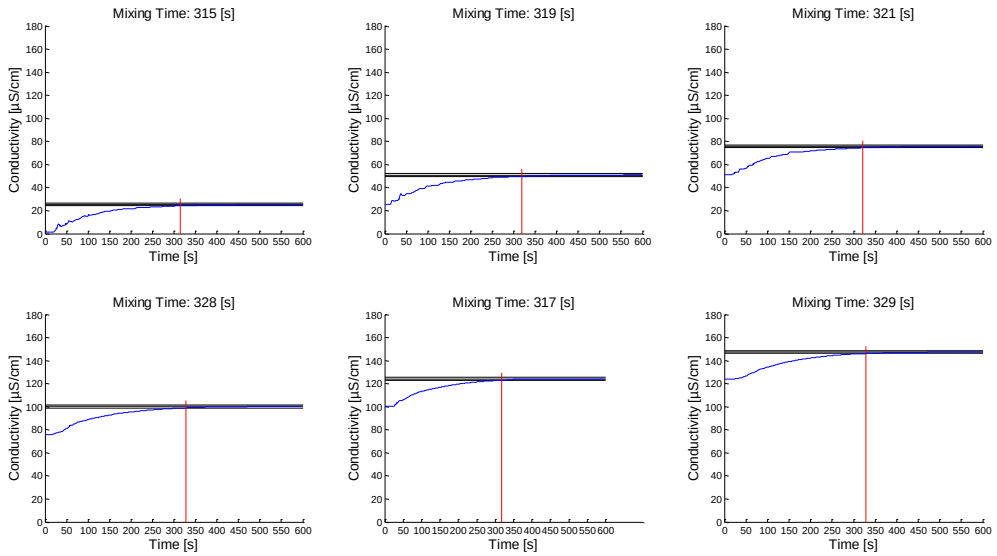

Figure 4 results for probe A (Wechselberger, 2014)

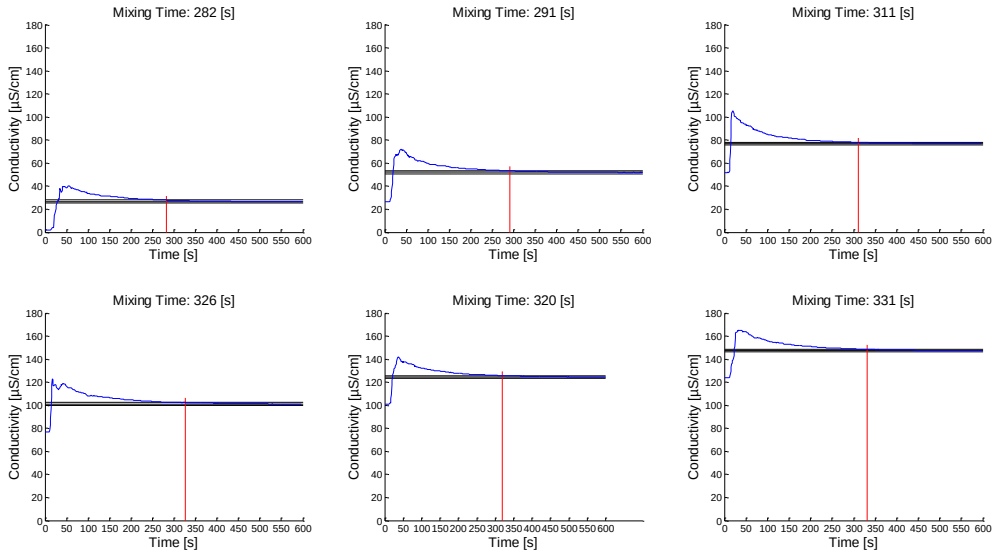

Figure 5 results for probe B (Wechselberger, 2014)

The difference between the mean mixing time of 310s and 320s was insignificant so the results can be trusted. After the CFD model had been experimentally validated, an impact on physiological parameters had to be investigated.

Quantitative understanding of fermentations is important to successfully plan and execute efficient bioprocesses (Sagmeister, 2012). Both approaches just quantify rheological phenomena, so physiological impact on fermentations was investigated. Inhomogeneities can result in damaging organisms (e.g. high concentration of acid, base, inhibition (Aiba S., 1968)) as

well as in shortage of limiting substrate for organisms. Figure 6 and Formula 1.1 showing the monod equation (Monod, 1949), which describes the impact of limited availability of substrate on cell specific substrate rates.

$$q_S = q_{s,max} * \frac{S}{S + K_S}$$

Formula 1.1 Monod equation for cell specific substrate rate

$\mathbf{q_S}$	cell specific substrate rate [$g_s\ g_x^{-1}\ h^{-1}$]
$\mathbf{q_{S,max}}$	maximum of cell specific substrate rate [$g_s\ g_x^{-1}\ h^{-1}$]
S	concentration of limiting substrate [$g_s\ L^{-1}$]
K_S	half-velocity constant – the value of S when $q_s/q_{s,max} = 0.5$ [$g_s\ L^{-1}$]

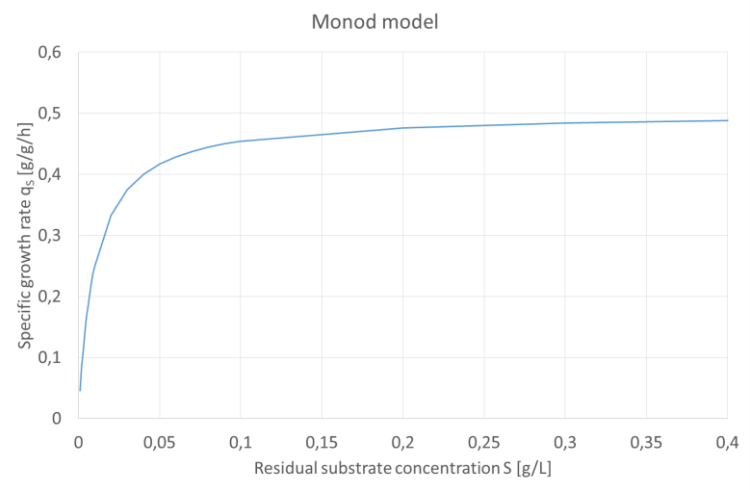

Figure 6 Monod kinetics showing the influence on specific growth rate over residual substrate concentration for K_s=0.01 g/L and $q_{s,max}$=0.5 [$g_S/g_x/h$]

Obviously it is important to avoid unintentional shortage of substrate at any moment of the fermentation going. Although the monod equation is well established ((Chmiel, 2006), (Storhas, 2003)) an impact of investigated inhomogeneities have to be carried out with experiments.

Patrick Wechselberger et al. performed several fermentations with *Escherichia coli* bacteria. As physiological parameter the substrate to biomass yield $Y_{X/S}$ is chosen as inhomogeneities in large scales lead to an increase in this yield (Enfors et al., 2001). The results are shown in following figure:

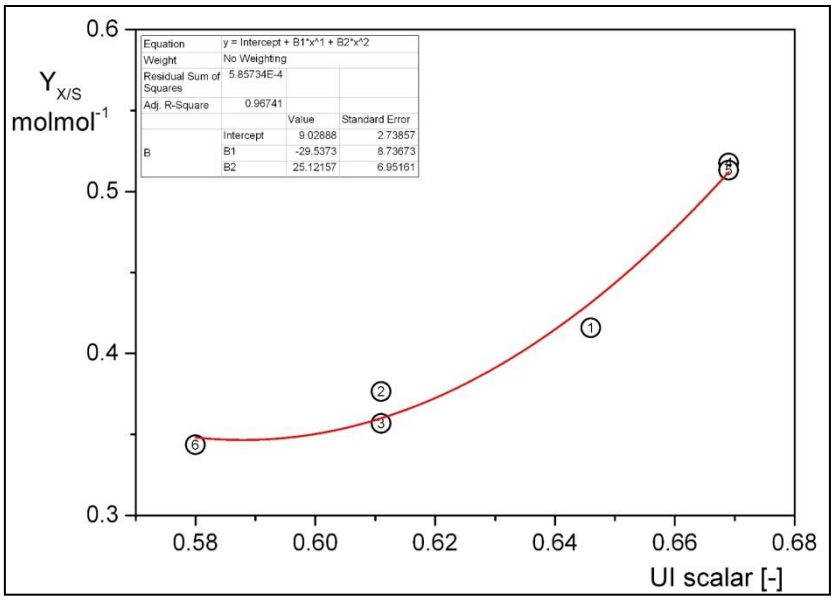

Figure 7 Correlation between UI scalar value of CFD simulations and substrate to biomass yield [g_X/g_S] in 60L BIRE reactor (J. Fricke, 2015)

Figure 7 visually displays the correlation that leads to the results shown in Table 1. As stated this is to be belived the best set up to avoid inhomogenities, which has to be varified by further experiments.

High homogeneity has a significant impact on the process, e.g. distribution of substrates and temperature control, and is commonly obtained by mixing via stirrer. To characterize the fluid dynamic state of a liquid phase the Reynolds number is often used which is defined as the ratio of momentum forces to viscous forces ("Mischvorgänge", 1998). For stirred vessels the following equation is commonly used (Chmiel, 2006)

$$Re = \frac{n \cdot d_{agitator}^2 \cdot \rho_L}{\mu_L}$$

Formula 1.2 equation for Reynolds number in stirred vessels

Re	Reynolds number [-]
$\boldsymbol{\rho_L}$	density of the fluid [kg m^{-3}]
$d_{agitator}$	diameter of the agitator [m]
μ_L	dynamic viscosity [kg m^{-1} s^{-1}]

The turbulence in stirred vessels is partitioned in three main areas. In the range of Re=0…2300 the flow patterns in the liquid phase are considered as laminar and gradients of concentrations and temperatures common. In the following transition area (Re=230…10000) laminar and

turbulent flow patterns occur in the phase. Reynold numbers above 10000 the system is fully turbulent ("Mischvorgänge", 1998).

$$P = \mathrm{Ne} \cdot \rho_L \cdot n_{agitator}^3 \cdot d_{agitator}^5$$

Formula 1.3 equation for power input into the liquid phase by stirrer

P	Power input [W]
Ne	Newton number [-]
$\boldsymbol{\rho_L}$	density of the fluid [kg m^{-3}]
$d_{agitator}$	diameter of the agitator [m]
$n_{agitator}$	round per minute by agitator [min^{-1}]

In the equation the Newton number (also known as Power number) characterizes the bio reactor system with within a certain setup (stirrer, baffles) and range of agitation, gaseous input, volume. Is the liquid phase of the reactor in a turbulent state (Re > 10^3), the Newton number is considered as constant.

1.1. Balances

Microorganisms need a C-source for producing biomass. Depending on the organism, the quantity and type of available C-sources, the fermentation mode (e.g. anaerobe, aerobe), the reaction of substrate to products differ.

Fermentation runs in fully instrumented bioreactors provide immense amounts of online- and offline data. Closing balances proves the reliability of measured and calculated values.

Mass balances and C-balances are most commonly used in bioprocess engineering (McNeil, et al., 2008).

1.1.1. Mass balance

The mass balance is used to calculate values not directly measured, e.g. mass of liquid phase in fermenter. It is calculated with the general approach:

$$m_{input} = m_{output} + m_{Accumulation}$$

Formula 1.4 Mass balance

m_{input}	mass transferred in the process [g]
m_{output}	mass transferred out of the process [g]
$m_{Accumulation}$	mass accumulated in the process [g]

If the outlet of a bioreactor is combined with an chiller, the condensate is led back in the reactor and the mass balance is easier to close.

1.1.2. C-balance

The C-balance (carbon balance) is easier to close, since not all in- and outputs of the process contain carbon. All masses of carbon containing substances are calculated in the unit Mol as following formula suggests:

$$n_{C.input} = n_{C,output} + n_{C,Accumulation}$$

Formula 1.5 Carbon balance

$m_{C,input}$	carbon transferred in the process [mol]
$m_{C,output}$	carbon transferred out of the process [mol]
$m_{C,Accumulation}$	carbon accumulated in the process [mol]

1.2. Physiological parameter

In chapter 1 scale-up strategies on are described. While theoretically convincing, the applicability to be verified experimentally. To quantify biologically the strategy, physiological parameters are used to provide further information.

Quantitative information on microbial growth is needed in many fermentation and biological waste treatment processes. Therefore significant physiological parameters are used, e.g. substrate to biomass or substrate to product ratios called yields. Depended on organism and strain maxima of certain yields can be observed. Process design has a huge impact on determined yields, e.g. concentrations of substrate or shear stress.

1.2.1. Biomass growth rate

One of the most common and important parameters (Chmiel, 2006) in fermentations is the specific growth rate μ, which is the reciprocal of the time the organism in a certain metabolic state takes to double the biomass within the process. It is part of the Monod equation which is described following:

$$\mu = \mu_{max} * \frac{s}{s + K_s}$$

Formula 1.6 Cell specific growth rate

μ	specific growth rate [gx/(gx*h)]
s	substrate concentration in liquid phase [g/L]

$X_{total,i}$ total bio mass [g]

k_s saturation constant [g_s/L]

The formula for summing up the produced biomass within the process is calculated as following

The cell specific growth rate can also be described as

$$\mu = \frac{\ln\left(\frac{X_{total,i}}{X_{total,i-1}}\right)}{t_i - t_{i-1}}$$

Formula 1.7 Cell specific growth rate

μ specific growth rate [g_x/(g_x*h)]

$X_{total,i}$ total bio mass [g]

t_i time [h]

1.2.2. Yields

Yields are the quotient of up taken substrate (e.g. glycerol, oxygen) and the product (e.g. biomass, protein, enzyme). A substrate to biomass yield is an important physiological parameter for fermentation parameters as well as experimental design. It is calculated as the following formula:

$$Y_{X/S} = \frac{m_{X,produced}}{m_{S,converted}}$$

Formula 1.8 Substrate to bio mass yield

m_x mass of bio mass [g]

m_s mass of substrate [g]

1.2.3. Specific substrate rates

Is a certain substrate limited in fermentations, another metabolic parameter is important. The specific substrate uptake rate $q_{S/X}$ or q_S describes the uptake of substrate per gram biomass and hour [g_s/(g_x*h)] as following:

$$q_{S/X} = \frac{m_{s,converted}}{\frac{X_{BR,i} + X_{BR,i-1}}{2} * (t_i - t_{i-1})}$$

Formula 1.9 Substrate to bio mass yield

$q_{S/X}$	cell specific substrate uptake rate [$g_s/(g_x*h)$]
X_{BR}	biomass in liquid phase of bioreactor [g]
m_s	mass of substrate [g]
t	time [h]

The specific substrate uptake rate q_s can also be calculated via following ratio:

$$q_s = \frac{\mu}{Y_{\frac{X}{S}}}$$

Formula 1.10 Substrate to bio mass yield

q_s	specific substrate uptake rate [$g_s/(g_x*h)$]
μ	specific growth rate [1/h]
$Y_{X/S}$	yield of substrate to biomass [g_x/g_S]

As the yield is defined by the metabolic state of the organism, a certain growth rate can be targeted in planning fermentations by adjusting the substrate feed to the measured yield. This strategy for controlling fermentations applies only if the aimed substrate is the only limiting factor.

Calculations of metabolic rates, yield coefficients were conducted with Matlab r2013 b

1.2.4. Off-gas analytic

The off-gas of the fermentation has several benefits for the operator of the fermentation. The online signal of oxygen and carbon dioxide offers information about the metabolic state of the organism. In this chapter important rates and quotients of off-gas analysis is described

Respiratory quotient

The respiratory quotient (RQ) is the ratio of produced CO_2 divided by the converted O_2 on a molecular basis while substrate is metabolized. Although it can be calculated by balances, it varies depending on the process parameters.

The RQ (Formula 1.13) is calculated via formula as a quotient of the converted oxygen (Formula 1.11). It can also be described as a yield $Y_{CO2/O2}$.

and the produced carbondioxid (Formula 1.12)

$$r_{O2} = \left[-\left(\dot{V_{Air}} * c_{O2,Air} + \dot{V_{O2}} * c_{O2,pureO2}\right) + \dot{V_{out}} * c_{O2,out}\right] * V_M$$

Formula 1.11 oxygen reaction rate O_2

r_{O2}	reaction rate of O_2 [mol/h]
$\mathbf{V_M}$	molar volume [m³/mol]
$\dot{\mathbf{V}}_{\boldsymbol{air}}$	Air flow in [NL/min]
$c_{O2,x}$	concentration of oxygen [%]

$$r_{CO2} = -\dot{V_{Air}} * c_{CO2,Air} + \dot{V_{out}} * c_{O2,out}$$

Formula 1.12 carbon dioxide reaction rate CO_2

r_{CO2}	reaction rate of CO_2 [mol/h]
$\mathbf{V_M}$	molar volume [m³/mol]
$\dot{\mathbf{V}}_{\boldsymbol{air}}$	Air flow in [NL/min]
$c_{CO2,x}$	concentration of CO_2 [%]

$$RQ = \frac{r_{CO2}}{r_{O2}}$$

Formula 1.13 respiratory quotient RQ

r_{CO2}	reaction rate of CO_2 [mol/h]
r_{CO2}	reaction rate of CO_2 [mol/h]
RQ	respiratory quotient [-]

Oxygen Uptake Rate (OUR)

The oxygen uptake rate (OUR) is more common than the oxygen reaction rate r_{o2} mentioned above since it is volume independent. It is one of the most important physiological parameters in fermentations. Unlike q_{O2} it can be calculated via the online signal in realtime, since it is not dependant on the cell concentration (which can only be estimated between two samplings or via modelling e.g. correlations using turbidity probes or biomass estimation with balancing off-gas).

The following formula for OUR is applied:

$$OUR = \frac{r_{O2}}{V_L}$$

Formula 1.14 Equation oxygen uptake rate

OUR	Oxygen uptake rate [mol/(L*H)]
r_{O2}	reaction rate of O_2 [mol/h]
V_L	Volume of liquid phase in bioreactor [L]

Is the oxygen available in the liquid phase not limited, OUR = OTR (oxygen transfer rate), which is the transferred oxygen from the gas into the liquid phase. The OTR also characterizes the setup of the bioreactor and the chosen set of parameters. Combined with the q_{O2}, a maximum cell concentration of an organism (in certain metabolic state) can determined, without limiting available oxygen in the liquid phase.

design space of fermentations.

Carbon dioxide evolution rate (CER)

Similar to the OUR, the carbon dioxide evolution rate (CER) is the equivalent to the carbon dioxide reaction rate r_{CO2}. It is also more common for the similar reasons. It is calculated with the following formula:

$$CER = \frac{r_{CO2}}{V_L}$$

Formula 1.15 equation reaction rate CO_2

CER	Carbon dioxide evolution rate [mol/(L*H)]
r_{CO2}	reaction rate of CO_2 [mol/h]
V_L	Volume of liquid phase in bioreactor [L]

1.3. Power Input

Agitation via impellers in stirred tank reactors (STR) has several purposes as following

- Ensure maximal homogeneity to avoid temperature, concentration, etc. gradients
- Increase gas hold-up for higher oxygen transfer from gas to liquid phase

Nevertheless power input can also be limited to other factors like shear stress (especially mammalian cultures are shear-sensitive), dissipation in the liquid phase, which can cause cooling problems, unwanted vortexing and economic reasons (Storhas, 2003).

2. Material and methods

2.1. Strain

P. pastoris is a well-established protein expression host with an wide application in production of industrial enzymes and biopharmaceuticals (Mudassar Ahmad, 2014). Advantages are achievable high cell densities, well established promoters and high titers.

In the fermentations *Pichia pastoris* yeast was used, which is to date the most promising recombinant production platform (Capone S, 2015). It was modified to produce the enzyme horseradish peroxidase (HRP), which is naturally found in roots of horseradish. Conjugating to antibodies and lectins, HRP is widely used in medical diagnostics (Capone S, 2015). The strain used is a Wild type 4/8, which means that four out of eight superficial N-glycosylation sites of the product HRP are removed (Capone S, 2015). This vector is driven by the alcohol oxidase promoter (*AOX1*). Hemin is used, which is a cofactor to provide a better yield and product rate. As described in 1.1, the reaction of the substrate to product (e.g. biomass, inter and extracellular proteins) conversion is dependent on the fermentation situation. The expected reaction can be calculated beforehand to design the experiment and determine the parameters (e.g. substrate concentrations, aeration). Usually the ASTM (American Society for Testing and Materials) D3172 method is applied to determine the composition of biomass (ASTM International, 2013). The analysis of the strain results in following composition:

Table 2 Chemical composition of *P. pastoris* strain WT 4/8

Substance	Symbol	Amount	Unit
Carbon	**C**	**1**	**mol/C-mol**
Hydrogen	**H**	**1,691**	**mol/C-mol**
Nitrogen	**N**	**0,176**	**mol/C-mol**
Oxygen	**O**	**0,502**	**mol/C-mol**
Ash	**ash**	**0,0603**	**mol/C-mol**
Molecular weight	**M**	**25,74**	**g/C-mol**

With the composition of the biomass (Table 2), the designed determined media as nitrogen and carbon sources and the expected metabolism of the organism the following reaction equation can be arranged

$$C_3H_8O_3 + a\ NH_3 + b\ O_2 \rightarrow c\ CH_{1,69}N_{0,18}O_{0,5} + \ d\ H_2O + e\ CO_2$$

As seen, the equation is undetermined and cannot be solved without further experiments to observe conversion rates and determine the equation.

2.2. *P. pastoris* fermentation setup

Microbial metabolism describes the ability of microbes to metabolize substances to obtain nutrients and energy. The quality and quantity of the availability of substrates result in different metabolism modes and is described in metabolic pathways (Ron Caspi, 2012). Through experiments the pathways can be described and quantified.

Cell banks are facilities which store specific genome with cryopreservation for the purpose of future use in research or industrial processes or analytics. The cell broth is transferred into cyro vials (1-2mL) and deep-frozen fast enough to avoid damages of the cells, e.g. with with liquid nitrogen (Harel, 2013). Variations of cell banks such as SCBs (Safety Cell Banks), MCBs (Master Cell Banks), WCBs (Working Cell Banks) and PPCBs (Post-Production Cell Banks) meet demands, depending on the purpose and amount of frozen vials required (Chmiel, 2006).

SCBs store several different clones of a cell line with promising growth rates, product titres and quality. Has a candidate asserted itself to be chosen for the MCB, it is cultivated several times to an amount of 100 to 300 vials. A few of them will be chosen for a WCB from which, as the name implies, the vials are used for cultivations for production or research purposes. For reliable results in experiments and industrial production, a well-established cell bank system is essential (Chmiel, 2006).

The available vials with desired clone of the defined cell line further processing is to be targeted. The process diagrams below lead through the following process steps conducted.

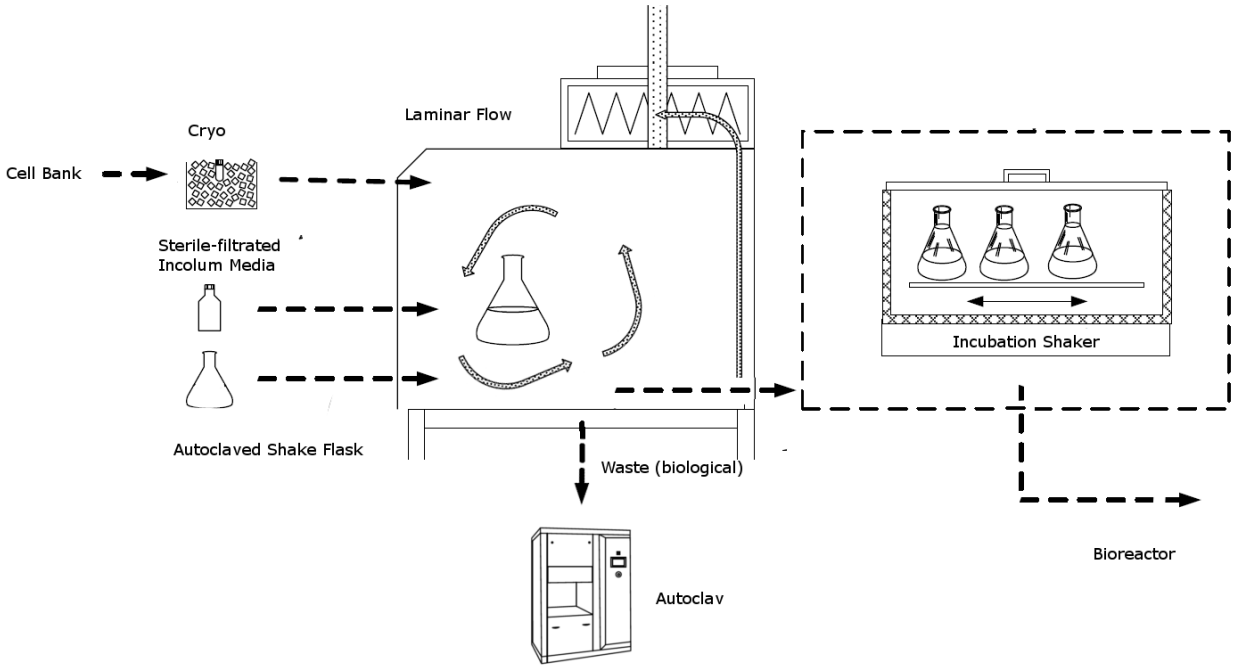

Figure 8 Process flow diagram of incolum preperation

For fermentations executed in bioreactors, 10% of the starting volume should be the final broth (with desired cell density) of the previous fermentation step in a smaller scale us advised. However inoculum broth can be cultivated directly from cryo vials described in the text above (Chmiel, 2006). The inoculum preparation is shown above in Figure 8.

After taking the cryo vial of the WCB, the thawed cell line and sterile-filtrated inoculum media (see also 3.2.1) are set into a laminar flow for further processing. After transferring both into an autoclaved shake flask, it is put into an incubation shaker for 24h. The carbon source in the preculture media should be metabolised into bio mass after this period. However the bio mass is to be measured (see also 3.3.1) at-line to ensure process control and vary the process parameters if necessary. The inoculum is then transferred into the bioreactor, as shown in the following process flow diagram.

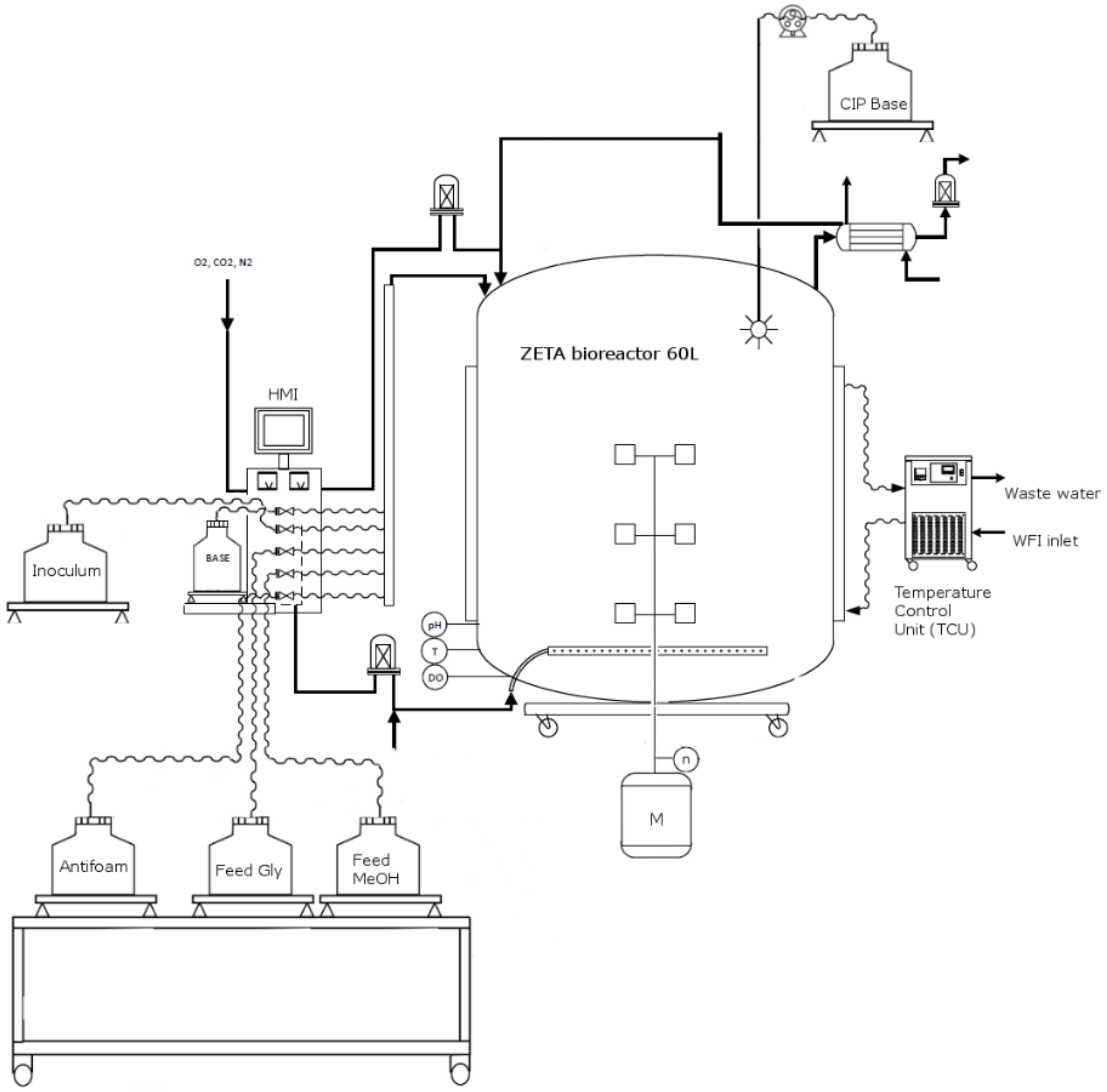

Figure 9 Process flow diagram of ZETA bioreactor

In Figure 9 the process flow diagram is shown. The transferred inoculum as well as the batch media is set on a scale and pumped into the reactor to balance the volume of the bioreactor (Formula 1.4). As seen in the diagram, the process requires two feeds, glycerol feed (growth media) and methanol feed (product media). The reacot is equipped with the commonly used pH, Temperature and desolved oxygen measurement instruments (McNeil, et al., 2008). A Temperature Control Unit (TCU) for SIP and temperature control within the process responsible. CIP base is also set up for the cleaning in place step after the process. As described in 3.4.1, all mass flows in the reactor are controlled and recorded over the SCADA system and can be monitored on the HMI next to the reactor. This enables the operator to take action if necessary. Process flow diagrams are an overview of the process and are not supposed to cover all details.

Nevertheless they are an important tool to set up process plants. Additionally they are the basis for Piping and Instrumentation Diagrams (P&ID).

The volume of the preculture is dependent on the final batch volume, since commonly 10% (also depending on the final cell density) of the starting batch volume is the preculture..

3.1.1. Fermentation strategy

The goal of the process is to analyses if the chosen setup of the ZETA bioreactor is suitable for avoidance of scalability problems. Therefore the fermentation has to be characterized in a scale independent way. The overall strategy is shown in Figure 10. It is determined by values that can be controlled easily, e.g. temperature, pH, but also calculated values that are expected by knowledge of the organism, e.g. specific growth rate, cell concentration. With 0.5% v/v ratio of the methanol pulse, the experimental design follows scientific findings (Christian Dietzsch, 2011).

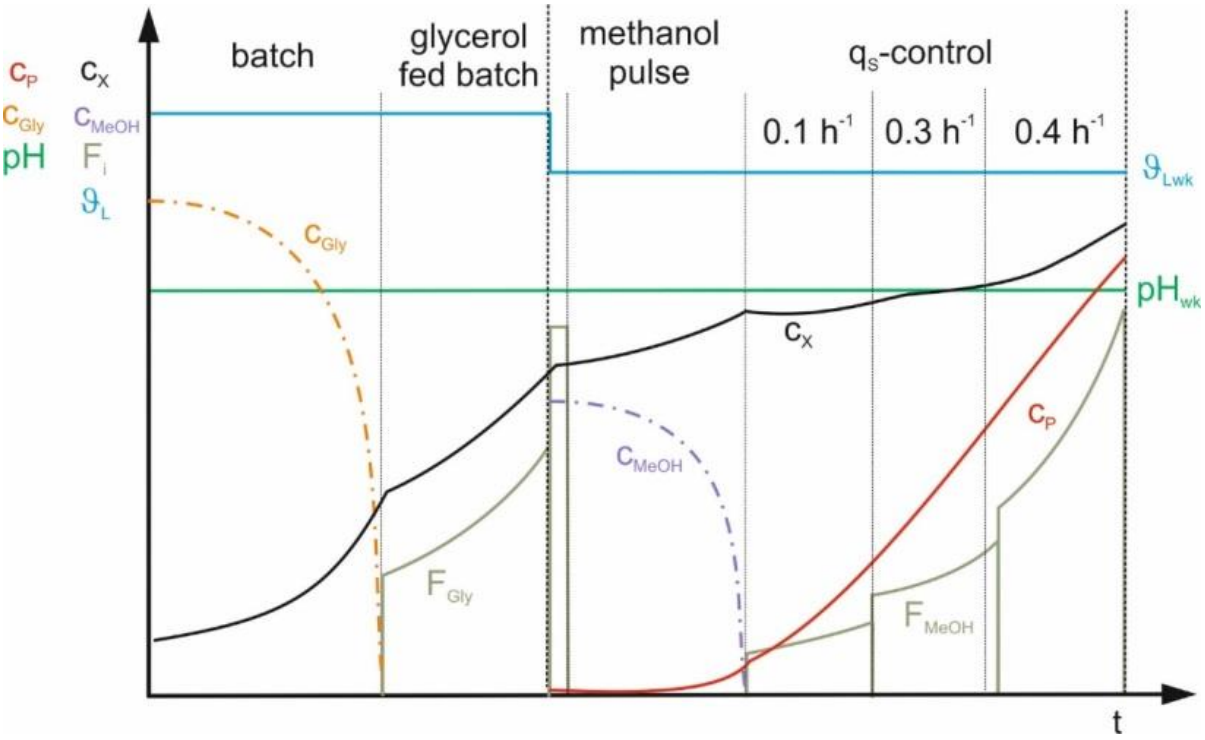

Figure 10 Experimental plan for fermentation

With calculated concentration of methanol in the feed of 719 g/L and a specified pulse resulting in 0.5% volume methanol / volume liquid phase, the mass to be added is calculated within the process as following:

$$m_F = \rho_F * V_F = \rho_F * V_{MeOH} * \left(\frac{\rho_{MeOH}}{\rho_F}\right) = \rho_F * 0{,}05\, V_{BR} * \left(\frac{\rho_{MeOH}}{\rho_F}\right)$$

For the targeted volume at the beginning of the pulse phase an estimated volume of 50L for the ZETA bioreactor and 2.7L for the small scale reactor, which was calculated with batch volume with added glycerol fed batch feed (added base and antifoam counterbalance removed sample volume).

As there are just base components expected to be produces, only acid is needed to adjust the pH level while the fermentation is running. Volumetric gas flow rate per volume liquid phase is set to 2 $\dot{V_{air}}/(V_L * min)$ also written as 2 vvm. A temperature of 37°C in the growth phase on glycerol and 20°C in the production phase on methanol is targeted. The pH level is constant set to 5.

2.2.1. Fermenter setup

As described in chapter 1 issues can be encountered in scaling up processes. To avoid inhomogeneity within the broth, a CFD simulation was performed and validated to assure a maximum in homogeneity. Different setups were investigated with various parameter settings:

- Position of feed addition
- Stirrer type, e.g. Rushton
- Agitation in rounds per minute
- Biomass growth rate (impact on heat production and feed rate)

Following figure is showing the performed CFD simulation.

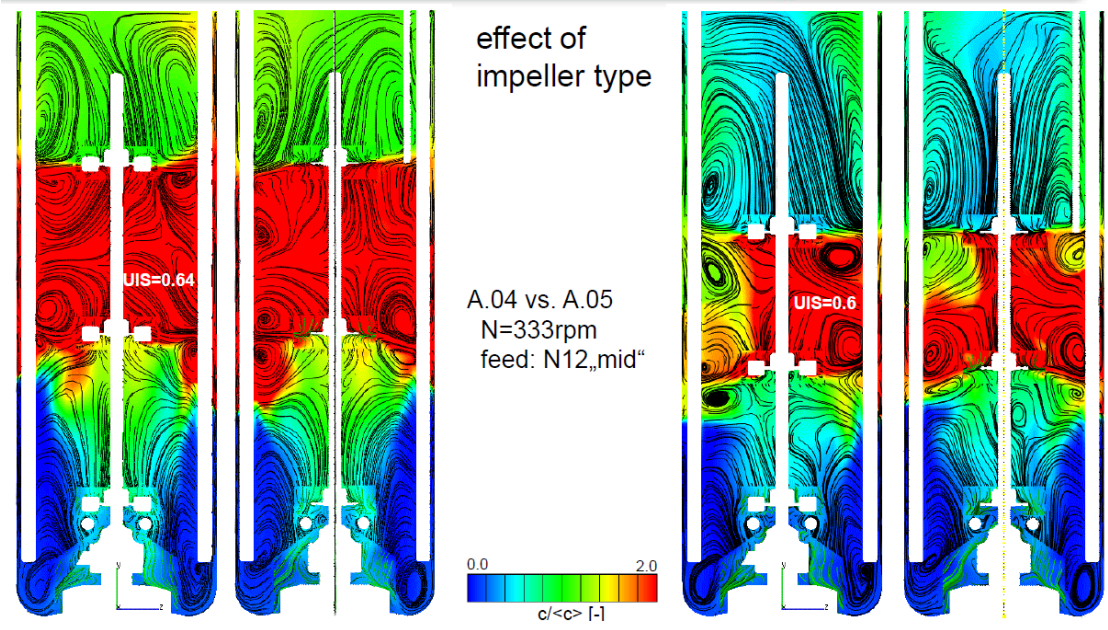

Figure 11 CFD simulation of the 60L ZETA bioreactor with different impeller distances

As shown in the legend the colors show concentrations of substrate after feeding.

To quantify homogeneity an UI scalar was established.

No. CFD	No.	$Y_{x/s}$ [molmol^{-1}]	UI Skalar [-]	Cultivation settings Impeller Distance, Feed position, Stirrer speed
A.17	1	0.416	0.646	2.0 D, low, 1000 rpm
A.16	2	0.377	0.611	2.5 D, low, 1000 rpm
A.16	3	0.357	0.611	2.5 D, low, 1000 rpm
A.13	4	0.518	0.669	2.5 D, mid, 1000 rpm
A.13	5	0.513	0.669	2.5 D, mid, 1000 rpm
A.14	6	0.344	0.580	2.0 D, mid, 1000 rpm

2.2.2. *P. pastoris* fermentation

As stated in chapter 2.1, *P. pastoris* is an expression system with an broad application range. Despite generic process steps within fermentations, specific variations may apply and will be discussed following.

3.2. Media

3.2.1. Preculture

For bioreactor cultivations a certain amount of initial cell density is necessary to match the supposed fermentation running time. Therefore a preculture/inoculum is cultivated in shake flasks using a Yeast Nitrogen Base (YNB) medium with added glycerol as C-source as shown in Table 3.

Table 3: Preculture (YNB)

Preculture in Yeast Nitrogen Base (YNB)		
Components	%Vol	Remarks
Phosphate Buffer 1M pH6	10	autoclave separately
YNB 10 X	10	filter sterilze
Biotin 500X	0,2	filter sterilze

Glucose 20 % w/v	10	autoclave separately
Zeocin	0,02	after autoclave
Water	69,78	autocalve in shake flask

The final volume of the preculture is defined by the initial biomass concentration in the bioreactor.

3.2.2. Batch media

The media for the batch phase mainly contains C-sources for the organism to grow and additionally metabolite supplements, e.g. trace salts and P-sources. Basel salts medium (BSM) was used with a certain amount of glycerol. The glycerol concentration is determined by the desired cell density.

As in the preculture media the ratio is given in Table 4 and the final volume depends on the fermentation strategy and desired cell concentration at the end of the batch phase.

Table 4: Batch media (BSM)

Basel salts medium (BSM)		
Components	quantity per L	Unit
H3PO4, 85 %	10.79	ml
CaSO4.2H2O	0.18	g
K2SO4	13.62	g
MgSO4.7H2O	2.24	g
KOH	4.13	g
Antifoam Structol	300	µl
PTM_1 Trace Salts	4.35	ml

The media also contains a specific mixture of trace salts. The recipe is listed in Table 5.

Table 5: PTM_1 Trace Salts

PTM_1 Trace Salts		
Components	quantity per L	Unit
Cupric sulfate.5H2O	6	g
Sodium iodide	0,08	g
Manganese sulfate.H2O	3	g
Sodium molybdate.2H2O	0,2	g
Boric acid	0,02	g
Cobalt chloride.6H2O	0,91	g
Zinc chloride	20	g
Ferrous sulfate.7H2O	65	g

Biotin	0,2	g
Sulfuric acid	5	ml
Water	995	ml

PTM_1 has to be sterile filtrated for storage.

3.2.3. Glycerol feed

An additional feed containing glycerol is used to counteract undesired metabolism (by-products) while achieving the cell density. Another aspect is the possibility to have a higher level of process control as the C-source input can be adjusted suitable to the situation. To avoid shortage of trace salts, PTM_1 is also added to the fed batch medium. The fed batch media has to be sterilized beforehand since the media is added without a sterile filter.

3.2.4. Methanol feed

As described in chapter 2.1, methanol is fed to product HRP. As pure methanol is sterile, just autoclaved water is added under the laminar flow bench to dilute the methanol feed for the desired concentration. Hemin as an cofactor to provide higher yields and product rates is added in a concentration of 1mM to the methanol feed.

3.2.5. Acid

For controlling the pH level of the broth, a 12.5% v/v Ammonium acid is used. Base media for regulating is not required since the expected products are basic.

3.3. Analysis

For the determination of important parameters and values, the applied analytical methods are discribed in the following chapter.

3.3.1. Biomass concentration

To quantify the cell growth rate offline, the biomass dry weight was determined. A certain volume of cell suspension was centrifuged (Sigma 4k15, rotor 11156) RZB 5000, 10 min, 4°C) in pre-weighted glass tubes, washed with 5 mL of deionized water and centrifuged with same conditions again. The supernatant was stored (-20°C) for further analysis while the solid compounds were washed with distilled water using a Vortex. After a second centrifugation (RZB 5000, 10 min, 4°C) and discarding the supernatant, the tubes were stored in an incubator at 95°C for 72 hours. By weighting the tried tubes the biomass concentration can be calculated using the following formula (2.1).

$$Biomass\ \left[\frac{g}{l}\right] = \frac{\left(m_{dried\ tube} - m_{empty\ tube}\right)}{V_{sample}} \qquad (2.1)$$

Formula 2.2 Biomass dry weight

Biomass	Biomass concentration in liquid phase of bioreactor [g/L]
$m_{dried\ tube}$	total weight of the tube with biomass and tube [g]
$m_{empty\ tube}$	weight of the tube without biomass [g]
V_{sample}	Volume of the sample taken

The uncertainty of the method is calculated to be 0.73 g_x for the measurements since percentage deviation depends on the biomass in the measured vial. To describe the correct amount of biomass produced in the process, the biomass removed by sampling has o be considered in the total biomass calculation as following:

$$X_{total} = c_{X,BR} * V_L + m_{sampling}$$

Formula 2.3 Total biomass

X_{total}	biomass inserted via preculture and produced in the process [g_x]
$c_{x,BR}$	concentration of biomass in the bioreactor [g_x/L]
V_L	Volume of liquid phase in the bioreactor [L]
$m_{sampling}$	biomass removed from the bioreactor for sampling [g]

The formula integrates all biomass since also contained biomass in removed sample volume.

3.3.2. Cubian (HRP, enzyme assays)

The protein activity in the supernatant was determined by using an ABTS assay in an enzymatic analyzer (CuBiAn© XC, Innovatis). 10 µL of sample were mixed with 140 µL 1 mM ABTS solution (pH 6.5, 50 mM potassium phosphate buffer) and after 5 min at 37°C of incubation, 20 µL 0.078% (w/w) H_2O_2 were added to start the reaction. Changes in absorption at a wavelength of 415 nm were measured for 80 s and rates calculated. Standard curves were prepared with commercially available HRP preparation (Type VI, Sigma-Aldrich) within the range of the concentrations of the samples.

3.3.3. Optical density

Dry weight measurements for quantification of the cell density are accurate and the state of the art (McNeil, et al., 2008), however it is not practical to determine the concentration at-line since the procedure is time-consuming. Correlations between optical absorption and dry weight provide remedy in this case. The optical densities of the samples are determined at 600 nm. With this correlation it is possible to monitor the process at-line and take actions if necessary.

3.3.4. HPLC (Glycerol, MeOH)

While HPLC can be used for purification and analytical purposes, here it is just used for quantifying the substrate concentration in the feeds as well as in the broth samples taken within the fermentation running.. Ion chromatography uses affinity of ions and polar molecules to separate them. While it is often used for protein purification and amino acids, here it quantifies glycerol and methanol.

Since the substance rather elute within a certain time range than a point, the detected signal is integrated over time. Standards within the expected range of the samples taken are used calculate a correlation between concentration and measured signal by the detectors (Storhas, 2003). The detector used is a refractive index detector (RID) which is found to be very useful to determine methanol in pharmaceutical products (K. A. Shaikh, 2010).

Combing the results with the measured biomass in 3.3.1 a metabolic rate can be calculated as described in 1.2.3.

The used HPLC analys method is shown in Table 6.

Table 6: HPLC equipment and methode

Equipment	
System	Agilent Technolgies Series 1100 HPLC
Column	Supelcogel C-610H (Sigma-Aldrich)
Precolumn	Supelcogel h Guard Column (Sigma-Aldrich)
Detectors	DAD and RID
Methode	
Principle of separation	ion exchange
Temperature	30°C
Mobile phase	0.1% H_3PO_4 containing traces of Na_3N
Flow rate	0.5 ml/min
Sample Injection Volume	10 µl
Detection	Refractive Index Detector (RID)

Standard solutions of methanol and glycerol are measured for correlating signal to concentration. Figure 12 shows the correlation for the methanol standards while Figure 13 shows the correlation for the glycerol standards.

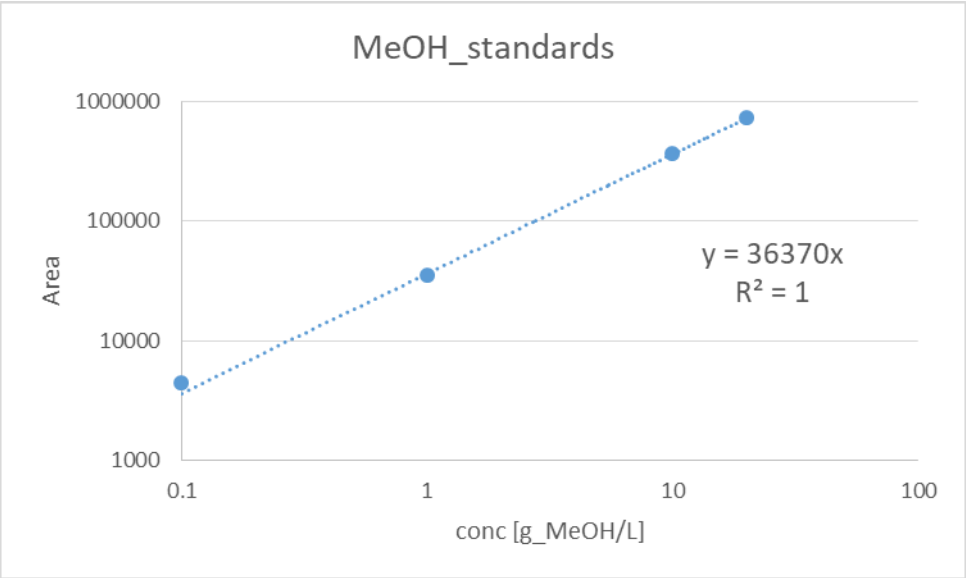

Figure 12 Correlation of signal to methanol concentration for HPLC measurements

The retention time of 23.8min is identified by the standards and an uncertainty of 0.14 g_{Gly}/L was calculated for methanol, since percentage deviation is depended on the methanol concentration.

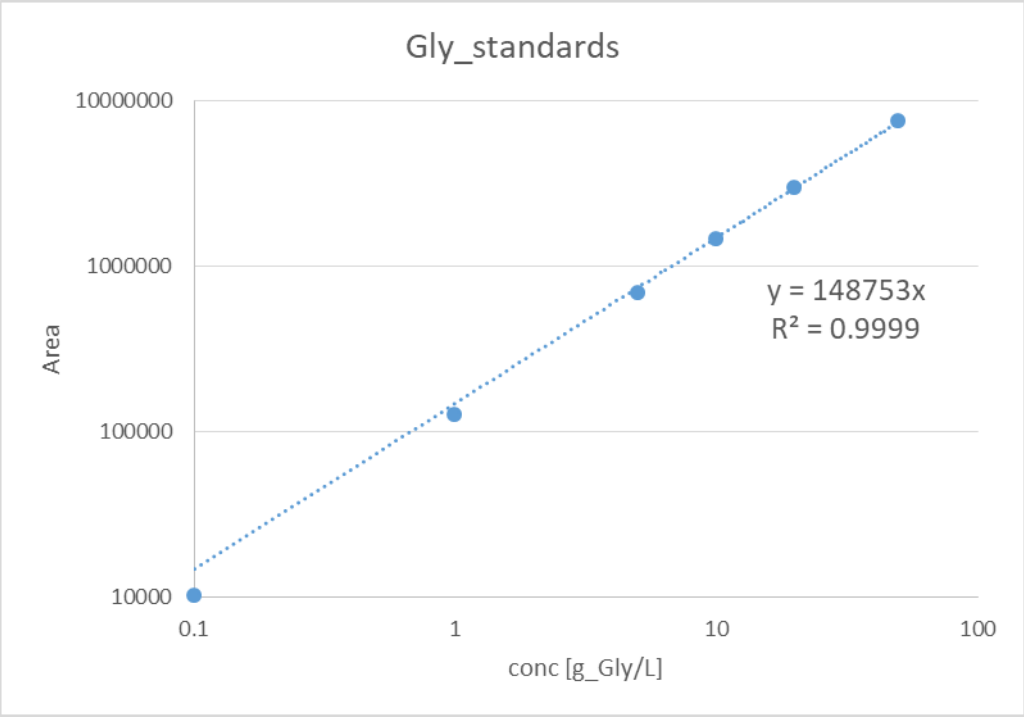

Figure 13 Correlation of signal to glycerol concentration of HPLC method to determine concentrations in the fermenter's liquid phase

The retention time of 17.8min is identified with the standards measurement and an uncertainty of 0.016 g_{MeOH}/L is calculated. Also percentage deviation would not be satisfying the results.

3.4. 60L ZETA pilot plant

For conducted fermentations a bioreactor of the industry partner ZETA Bipharma GmbH, Graz, was used. It is considered being suitable to follow the enforced Process Analytical Technology (PAT; (FDA, 2004) and Quality by Design (QbD; (e.V., 2007). As the regulatory bodies demand

3.4.1. Automation structure

The ZETA bioreactor is equipped with SCADA software. SCADA is an acronym for "Supervisory Control and Data Acquisition" and enables the operator to plan, monitor, analyse and store data of executed processes in the system. This ability is necessary to gain large process knowledge and understanding, to optimize and analyse experiments. SCADA is a control system architecture for high-level process supervisory management. It interfaces programmable logic controllers and PID controllers to transfer desired processes (e.g. experimental plans Figure 10) into a sequence of operation sections. SCADA offers the ability to monitor the process as well as issue process commands e.g. modifying parameters while the system is operating (McNeil, et al., 2008). Logic controllers and calculations are performed in real-time by interacting permanently with sensors and actuators to facilitate closed-loop rather than open-loop controls (Boyer, 2010).

Following levels of manufacturing control operation is shown:

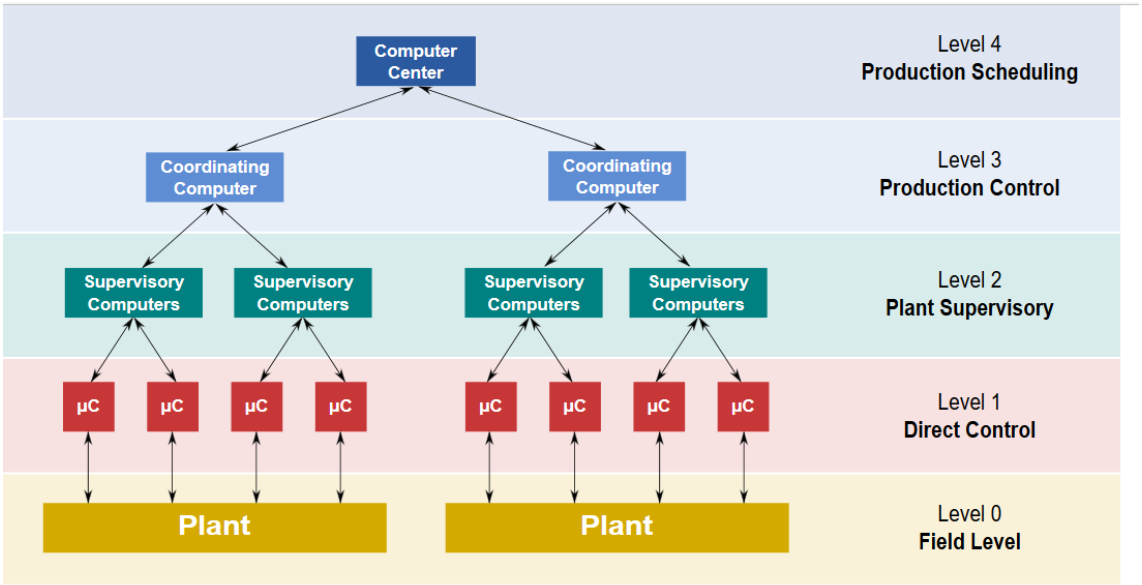

Figure 14 Levels of manufacturing control operation (Pugliesi, 2017).

All levels are interacting with the previous and the following levels.On the level 0 (Field Level) sensors (e.g. temperature, pressure, flow, etc.) and control elements (e.g. control valves, brakes, motors) are set up on process relevant positions. Level 1 (Direct Control) contains I/O (input/output) programmable logic controllers (PLCs) and remote terminal units (RTUs). Signals and data of level 2 I/O modules converge in level 2 (Plant Supervisory), where the SCADA software and computing platform resides. Since PLCs and RTUs carry out control actions (e.g. adjusting power level of pump if measured flow rate differs from flow set point), the SCADA system is primary a supervisory tool, however interventions (e.g. manually changing power level of pump) are possible (overwriting level 1 set points). To perform the process as desired, alarm functions are also enabled to be displayed and recorded, such as unwanted high temperature or pressure. Since the ZETA bioreactor system is not embedded in higher levels of manufacturing architecture, level 3 and 4 will not be discussed.

Monitoring processes in real-time requires data acquisition of level 0 devices and sensors data. These data are compiled and formatted to enable the operator to supervisor the process and take action if needed. The formatted data is commonly displayed in an R&I alike graphic to facilitate an easy orientation and overview of the running process. It is displayed on a Human-Machine-Interface (HMI) as shown below.

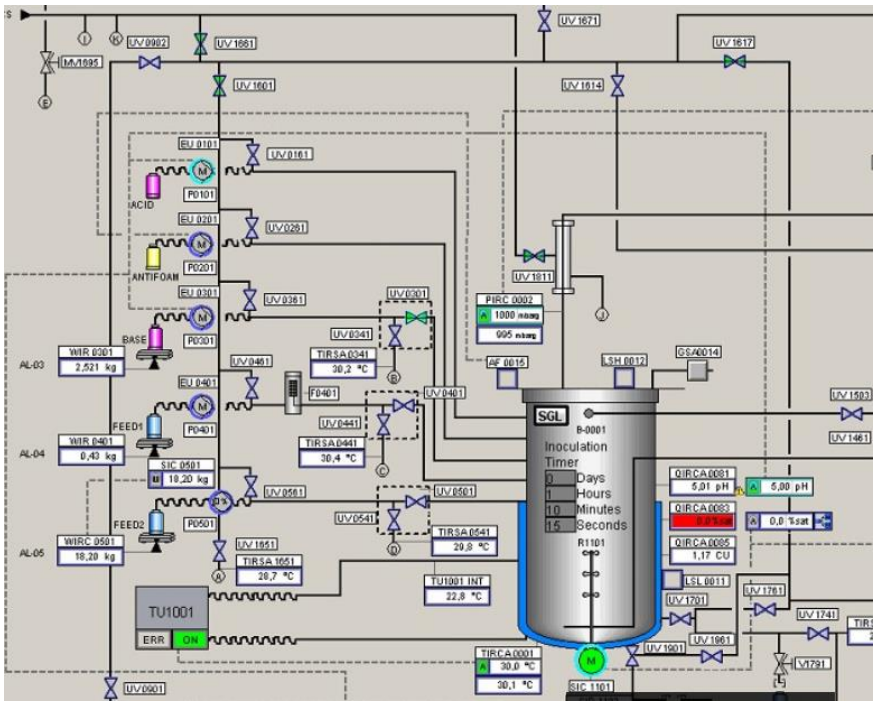

Figure 15 Overview of bioreactor on HMI

The interface should be adjusted to every process plant with showing all significant signals, e.g. dissolved oxygen, pressure, temperature in the vessel, piping, off-gas analyzer. However general graphical standards should be followed.

Processes analyses are usually performed with various tools such as MS Excel and Matlab, thus an export function of the data acquisition is a basic function of SCADA systems. However it is possible to calculate values and show historian data to support the operator's ability to direct the process by displaying on HMI.

As many sensors and actuators are included in a process plant, it is necessary to have clearness in naming and identifying all devices, usually organized in tag databases (Mehra, 2011). As seen in Figure 15, sensors and actuators have tag names on the shown P&ID on the HMI.

Following list contains functions a SCADA system should at least fulfil (McNeil, et al., 2008):

- online data collection;
- off-line data collection;
- online data storage;
- off-line data storage;
- data presentation;
- calculations on measured data;
- data import and export;
- time and event based actions;
- recipe definition, management and execution.

3.4.2. Instrumentation

The instrumentation of a bioreactor has to meet the demands of intended processes performed in the bioreactor. Depending on the process parameters and feeding strategies, the ranges, measurement uncertainties and amount of pumps, scales, piping, etc. have to be adjusted. The minimum requirements of the intended processes on the bioreactor would result in certain inflexibility, so a higher degree of equipment is recommended (Storhas, 2003). The instrumentation of the Zeta BIRE is listed below:

Table 7 Instrumentation of Zeta Bioreactor (Zeta, 2018)

Bioreactor Main System Equipment	Mobile Package (Rack), integrated drip pan and plates for scale and feed cans 1 Reactor with 2 types of agitator 1 can 40L for Feed 1

	1 can 2L for Feed 2
	2 cans 1,5L for acid and base
	2 cans 400 mL for Antifoam and other media
	3 peristaltic pumps digital controlled
	2 peristaltic pumps continuously controlled
Functional and Hardware Description	
General	Cultivation of microbial and cell cultures
	Sterile Feeding, inoculation and addition of acid, base and antifoam while process operation
	in a closed system.
	Sterile sampling anytime, thermal Inactivation
CIP	Integrated CIP-System with pump, cleaning of bioreactor system incl. connected pipework.
	Cleaning media prepared in bioreactor.
SIP	Fully automated SIP bioreactor full, empty, feed lines (parallel / single) harvest line, CIP line
Processes	Standby (pressure controlled), pO2 cascade control, tightness testing, fed-batch, batch processing, tempering 4°c to 40°C, transfer of Media /inoculum /additives /harvest
Technical Details Bioreactor	
Working volume	20 - 40 L cell culture
	20 - 60L microbial culture
Fermentation temperature	15 – 40°C (± 0,2°C)
Operation Pressure	-1 / 6 bar
Operation temperature	0 – 135 °C
Sterilization temperature	122 – 135°C (± 1°C)
Fermentation pressure	0 – 2 bar (± 0,1 bar)
Agitator for cell culture	Segmental stirrer, (d/D = 0,4; Dip-speed app. 2 m/s, 3-blades) 2 elements, variable movable on shaft
Agitator for cell microbial	Rushton Impeller (power input 10 W/L; d/D = 0,3-0,33; Dip-speed < 10 m/s, 6-blades) 3 elements, variable movable on shaft
Gasification Process Air Head	0,1 – 2 vvm
space	0,1 – 2 vvm
Gasification Process Air Sparger	0,1 – 1 vvm
Gasification Oxygen Head space	0,1 – 1 vvm
Gasification Oxygen Sparger	0,1 vvm
Gasification Nitrogen Sparger	0,1 vvm
Gasification CO2 Sparger	2 – 12 (± 0,05)
pH value control	0 – 100 % (± 1%)
P O2 control	10 L - 50 L (100 L/h), incl. Filtration, weigh controlled
Feed 1/ Media addition	200 - 6.000 mL/h, weigh controlled
Feed 2/ Inoculum addition	Each 1,5 L (5 L/h) , weigh for base
Acid / Base addition / Antifoam addition	400 mL (1,5 L/h)
Bioreactor Vessel Data	
Vessel Material	1.4435

Double Jacket	316L
Isolation cover	1.4301
Screws	A4 quality
Sealings / O-rings	EPDM (FDA approved)
Surface Quality	inner: Ra ≤ 0,6 µm, e- polished, outer: Ra ≤ 1,6 µm
Ports	
Vessel Lid	1 Nozzle for rupture disc
	1 Nozzle for exhaust air
	1 Nozzle for pressure gauge
	1 Nozzle for pressure transmitter (in exhaust air line)
	1 Nozzle for light
	1 Nozzle for Foam detection
	1 Nozzle NA-Connect spare
	1 Nozzle Ingold spare
Vessel Collar	5 Nozzles Media addition
	2 NA-Connect with sight glasses, opposite 1 longitudinal sight glass,
	1 Nozzle for pH- Probe
Lateral	1 Nozzle for pO2- Probe
Low Nozzle Belt	1 Nozzle for OD- Probe
	1 Nozzle for dry run protection
	1 Nozzle for sampling valve, steamed
	1 Nozzle In-gold spare,
	1 Nozzle NA-Connect spare
Vessel bottom	1 welded nozzle for temperature probe
Sparger	Ring-Sparger, dismountable
Heating / Cooling Cycle	Sanitizing empty and full. Heating from 20°C to 122°C in 60 min. Cooling from 20°C to 4°C in 25 min-
Gas module	Pressure reducing, self degassing each for CO2, N2, O2, Process Air for Sparger and O2 for head space
Submerse and Head space	1xMFC; control valve and non-return valve for process air and O2
Submerse	1xMFC; control valve and non-return valve for N2 and CO2 for Sparger
Gas Filter	2 x sterile filter, WIT ready 0,2 µm
Exhaust Air	1 Exhaust air cooler (chilled water 6/12°C).
	1 sight glass
	Gas analyzer (CO2 and O2)
	1 Sterile filter, WIT ready
Automation	1SCADA control with long-term data storage, FDA 21 CFR Part 11 compliant, OPC interface, Integration control relevant parameter from external data sources, data transfer to external devices
HMI	PID scheme visualization
	Color display, TFT, 19“

	Password protection in 4 levels (free configuration), Admission acc. FDA 21 CFR Part 11
Essential control loops	pO2-control, freely combined with gas module; rotation speed; pressure; Feed: as ramps, exponential, time controlled, set-point controlled, feed profiles; incl. weigh data, pO2 Signal and off-gas analyzer

3.4.3. Recipe structure

Cultivating successfully any kind of organism in a bioreactor requires various process phases. Although the process parameters may vary and depend on the organism, cell line and scope of the fermentation, there are either mandatory or very common sections which usually building the frame of the overall process. The process described as a Process flow diagram (PFD) in 2.1 is now to be transferred into different phases and steps possible in the used fermenter systems. Not only the fermentation itself has to be considered but also important not directly to the specific process dedicated work packages.

As it is mandatory in biotechnology to avoid contamination, a SIP (sterilise in place) step is necessary to meet cell free requirements. Clean-in-place (CIP) is a method of cleaning the interior surfaces of pipes, vessels, process equipment, filters and associated fittings, without disassemble the peripherie.

The recipe for the CIP is usually obligate by regulatory bodies. Mostly acids as well as bases are used for intended cleaning results but variations due to expected level of impurities are possible, especially in considering economic and ecological.

3.4.3.1. Operations

The SCADA system enables the operator to choose between different pre-assembled operation blocks, covering most commonly used in fermentations. Along the process flow a sequential arrangement of the blocks is to be built as seen in Figure 16.

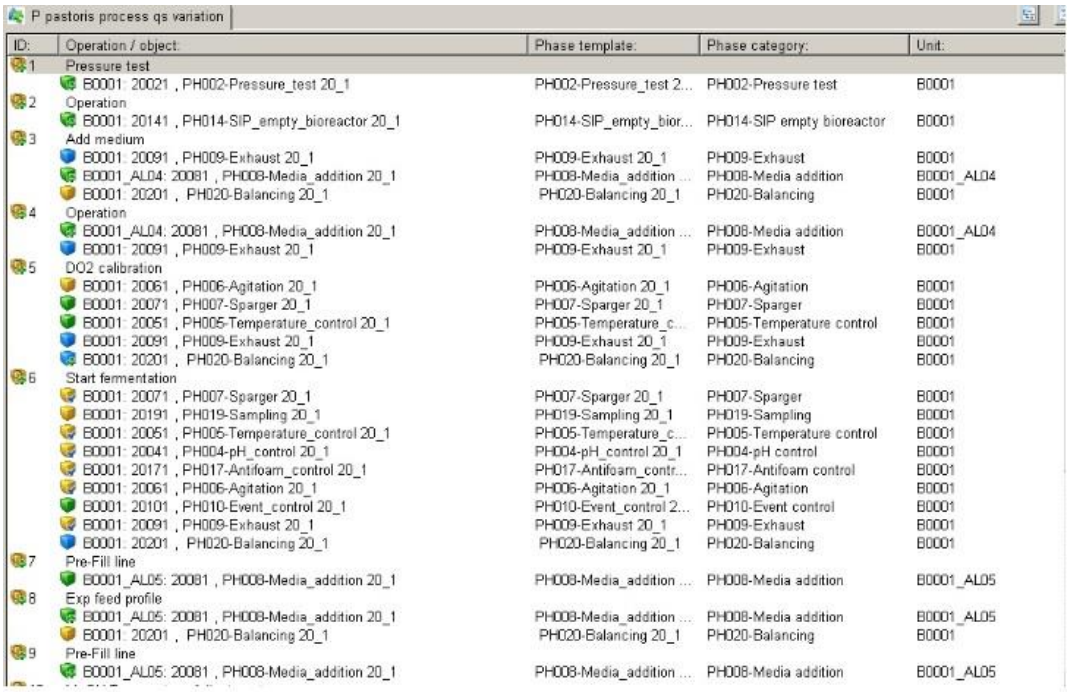

Figure 16 Recipe of a *P. pastoris* fermentation with an overview of sequential arrangment of operations

3.4.3.2. Phases

The process is divided into different process operations with a defined purpose and target, as stated in 3.4.3.1. To achieve desired outcome under controlled parameters, operation contain so called phases which are dedicated to certain level 1 PLCs and RTUs. Phases are specified in functional specifications (FS), including a various set of informations and requirements explained on the following example table:

Table 8 Example of phase: Temperature control

Topic	Content	Example
General information	Description of purpose and functionality of phase	Max. Temp reached -> cooling Min. Temp- reached -> heating
Parameters	Input parameters, engineering parameters, output parameters	Input: temperature control range +/- eng.: jacket temperature output: phase timer
Alarms	phase specific alarms, alarms of test points	Sensor is not ready Bioreactor temp. exceeds control range limits
Sequence	Phase divided in a sequence of steps	Start -> Heating -> Stabilization -> Maintaining temp. -> End

Controller	Lool logic for control unit described	PID values

Phases can be arranged in new operations to match requirements of novel processes if existing pre-built operations are not capable to do so.

4. Results

4.1. Automation

Temperature, pH and substrate feed is controlled by PID controller. In fermentation processes the feed addition is controlled gravimetrically given the higher accuracy over volumetric feeding.

Since default values for the controlling parameters were considered to be insufficient for the purposes of the fermentation, the controller has been optimized.

4.1.1. Optimization of pump controller

Fermentation systems are used to provide an optimal growth environment for many different types of cell cultures. Therefore fermenters are required to ensure the ability to carefully control temperature, pH, dissolved oxygen and available substrates. Especially pH and added substrate are essential for reproducibility and controlling the process. As creating a correlation of pump power output and volume flow lacks of accuracy, gravimetric feeding is prefered. As written in the function specification for media addition (GmbH, 2012) ZETA BIRE uses a PID controller for media addition. Set points for the media weight are permanently given by following:

$$m_{i,SP} = m_{i-1,SP} - \dot{F}_0 * e^{\mu} * t$$

Formula 4.1 Exponential function for media addition according to function specification

m	mass of feed flask on scale [kg]
$\dot{F}_0$	intial feed [kg/h]
μ	exponential growth rate [h-1]
t	time [h]

While most of the control strategies were found to be suitable for fermentation purposes, pump control had to be adjusted to the required needs. Therefore a fitting method by Ziegler and Nichols (Ziegler, et al., 1942) as shown in Table 3 was chosen and implemented.

	Typ of contol	Set value		
		K_R	T_I	T_D
Method 1	P	0.5 K_{Rkrit}	-	-
	PI	0.45 K_{Rkrit}	0.85 T_{Pkrit}	-
	PID	0.6 K_{Rkrit}	0.5 T_{Pkrit}	0.12 T_{Pkrit}

Method 2	P	$\frac{1}{K_S}\ \frac{T_a}{t_u}$	-	-
	PI	$\frac{0.9}{K_S}\ \frac{T_a}{t_u}$	3.33 T_u	-
	PID	$\frac{1.2}{K_S}\ \frac{T_a}{t_u}$	2 T_u	0.5 T_u

Table 9 Control set values according to Ziegler and Nichols

According to the method the feed was set to similar levels as used in the following fermentations. The results are shown in Figure 17

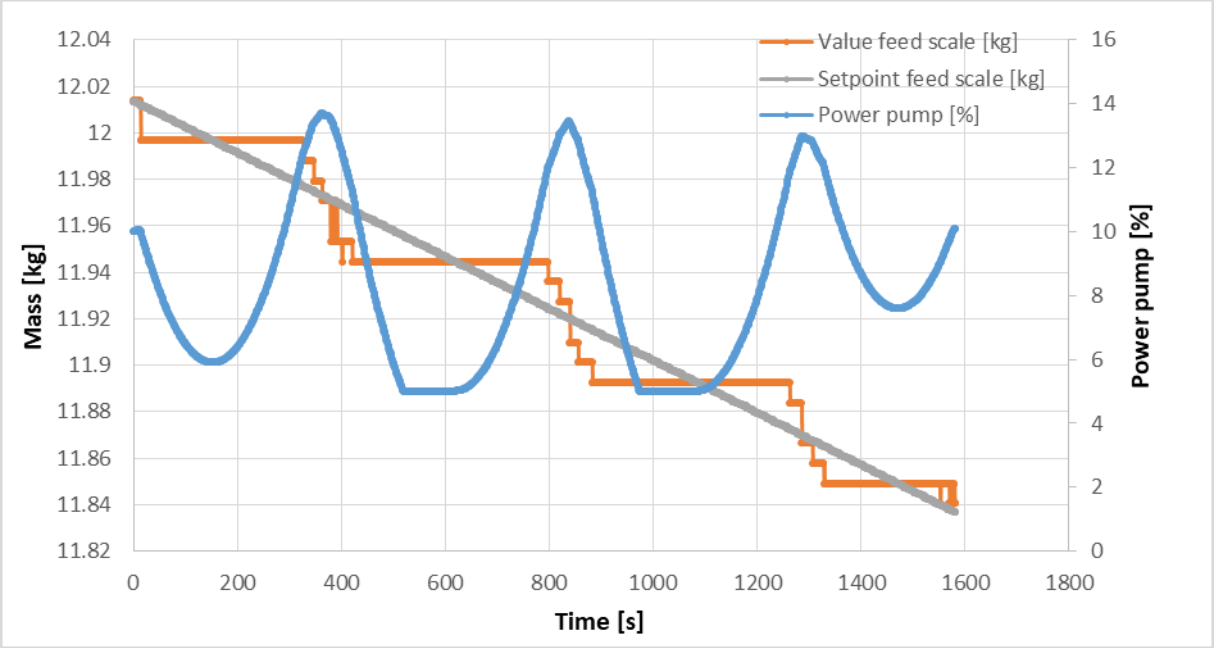

Figure 17 feed for determination of values for PID controller. Set points were calculated following Formula 4.1. The actual values of the scale were measured. The power of the pump was oscillating around the correct power input level correlating with the set mass

As shown in the figure above the set values for the P-, I- and D-components are not suitable chosen for the intended feed rate. Additionally disadvantageous circumstances occurred since the pump switches off below a power of 5%. This also required adjustments in substrate concentrations in the fermentation strategy.

As suggested by Ziegler & Nichols, necessary values were calculated and optimized parameters for the controller were set.

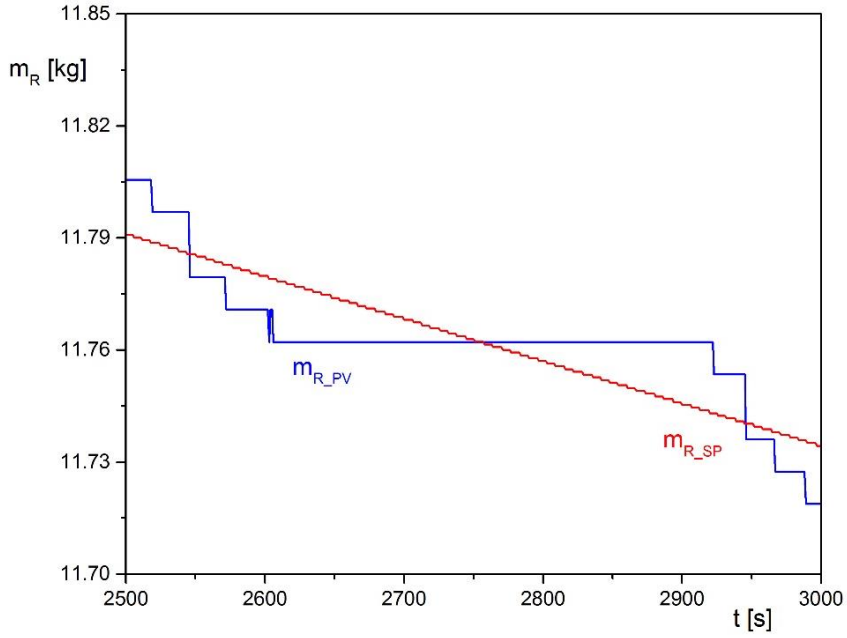

Figure 18 Setpoint and actual value of the scale feed Zeta BIRE

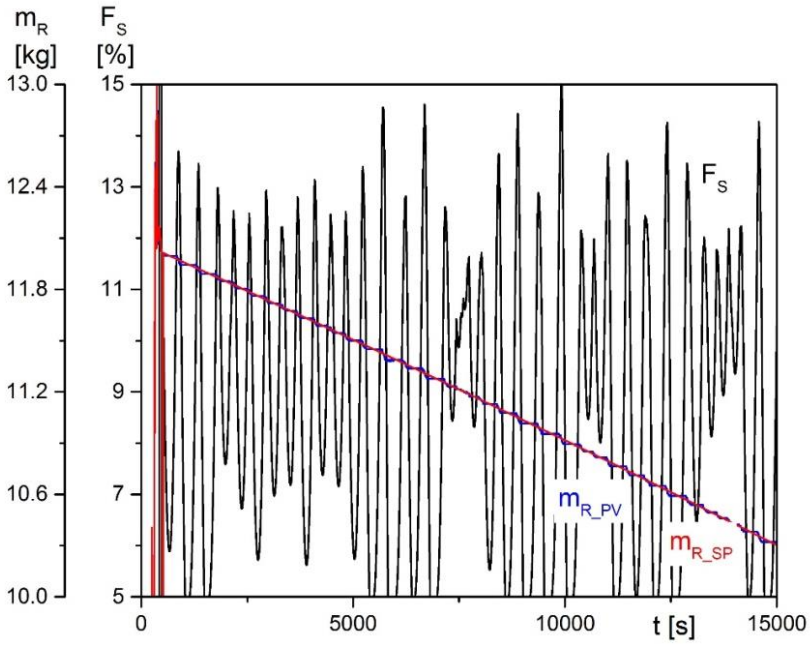

Figure 19 Pump signal shown over 4h black line shows the power of the pump [%], red the setpoint, blue the actual value of the scale. Before the optimization, the power of the pump Is very unsteady which results in uneven feed input as shown in Figure 18

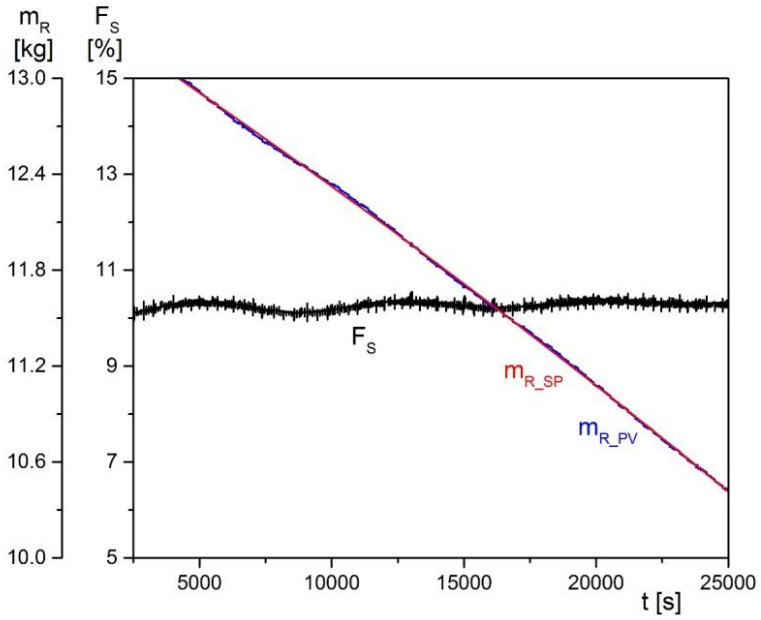

Figure 20 Pump power [%], setpoint and actual vale of scale of Zeta BIRE after optimization

Eventually the value of the scale adjusted to the set value given by the exponential function. Therefore not only an improvement of constant substrate uptake was achieved but also inhibition by high concentrations of methanol was prevented.

4.2. Glycerol phase of *P. pastoris* fermentation

The fermentation is based on two different C-sources, glycerol for biomass production, methanol to produce HRP as a product (see also 2.2). In the following chapter the focus is focused on the glycerol phase, namely the batch and fed batch.

Comparing both fermentations of different scales to show the comparability of the fermenters set up

4.2.1. C-balance

The reliability of the results are ensured by closing mass balances. Since the off-gas data for the batch phase in the 60L reactor are lost, only the fed batch phase on glycerol is to be examined. Glycerol as the only C-source and CO2 in off-gas as well as the produced biomass is to be analyzed.

The produced CO2 in this phase is calculated as following:

$$C - mol_{CO2,produced} = \sum_{t=fed\ batch,end}^{t=fed\ batch,end} r_{CO2} * \Delta t$$

The produced biomass as well as the added substrate has already been calculated in chapter 1.1.2.

Resulting in following balance:

$$Balance = \frac{mol_{C,CO2,produced} + mol_{x,produced}}{mol_{C,substrate,metabolised}} =$$

$$Balance = \frac{18{,}4mol_{CO2} + 86{,}16mol_X}{116mol_C} = 90\%$$

Fermentations, as all processes, can be quantified in various ways, depending on the scope and strategies. In the following chapter the most common ones are in focus as well as specifically reasonable for this experiment.

4.2.2. Glycerol and biomass concentration in 5L and 60L scale

Since the conditions (pH, temperature and substrate availability) are constant within the phases, the organism is found to be in a certain metabolic state in the phases.

The C-source (glycerol and methanol) represents the limiting as well as the nutrient with the highest conversion rate in this fermentation. For this reason, most interpretations and quantifications of the process is related to the C-sources. Following the glycerol and biomass concentration is shown over time.

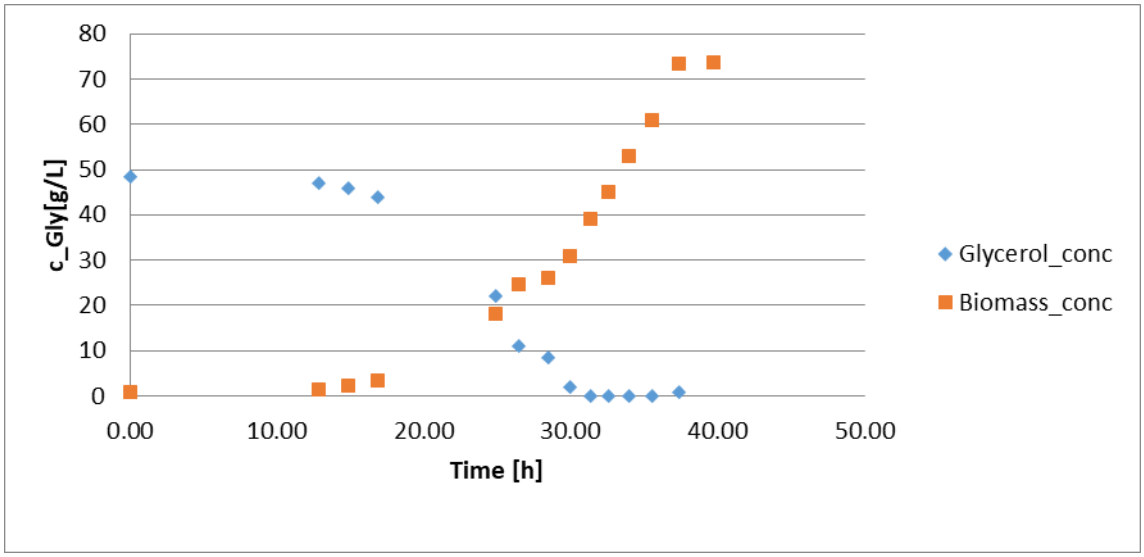

Figure 21 Fermentation with glycerol and biomass concentration over time [h] in 60L scale

In Figure 21 the concentration of glycerol and biomass is plotted over time. As expected due to conversion the glycerol concentration is declining while the biomass is increasing. From process time 0h until 30h the fermentation is a batch process hence no C-source is fed. The glycerol is

converted entirely into biomass. Following a fed batch is performed with a mass flow of glycerol which is lower than the maximum specific conversion rate $q_{x/s}$ ($g_s/(g_x*h)$) of *P. pastoris* hence the fed glycerol is not accumulating and the concentration is not detectable.

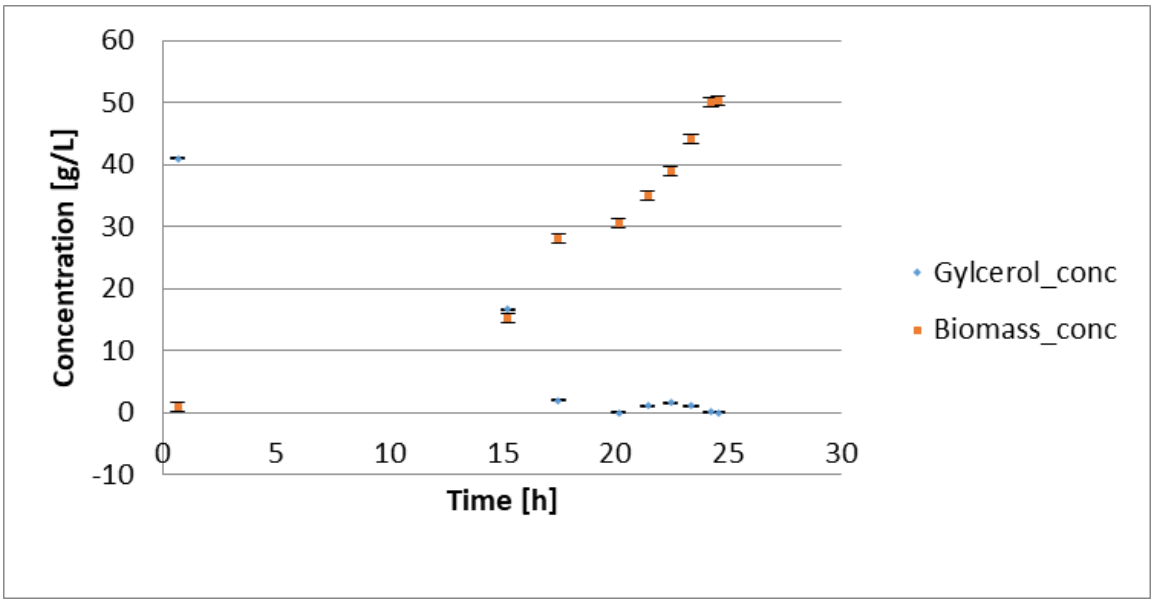

Figure 22 Fermentation with glycerol and biomass concentration over time [h] in 5L scale

As previous, Figure 22 shows the concentration of glycerol and biomass over time.

4.2.3. Substrate to biomass yield in 5L and 60L scale

The yield as described in 1.2.2 shows the ratio of produced biomass per substrate. In batches without feed of anti-foam or pH-regulating agents and the ratio of c-source nutrient and biomass concentration equals the yield $Y_{x/s}$. To meet demands of the fermentation performed, the Formula 1.8 is modified and result in following formula:

$$Y_{\frac{X}{S}} = \frac{m_{X,produced}}{m_{S,converted}} = \frac{V_{BR,n} * c_{x,n} - V_{BR,n-1} * c_{x,n-1}}{V_{BR,n} * c_{s,n} - V_{BR,n-1} * c_{s,n-1} + V_{Feed} * c_{s,Feed}}$$

Formula 4.2 Modified substrate to bio mass yield calculation

m_x	mass of bio mass [g]
m_s	mass of substrate [g]
V_{BR}	Volume of bioreactor [L]
c_x	concentration of biomass [g/L]
cs	concentration of substrate [g/L]

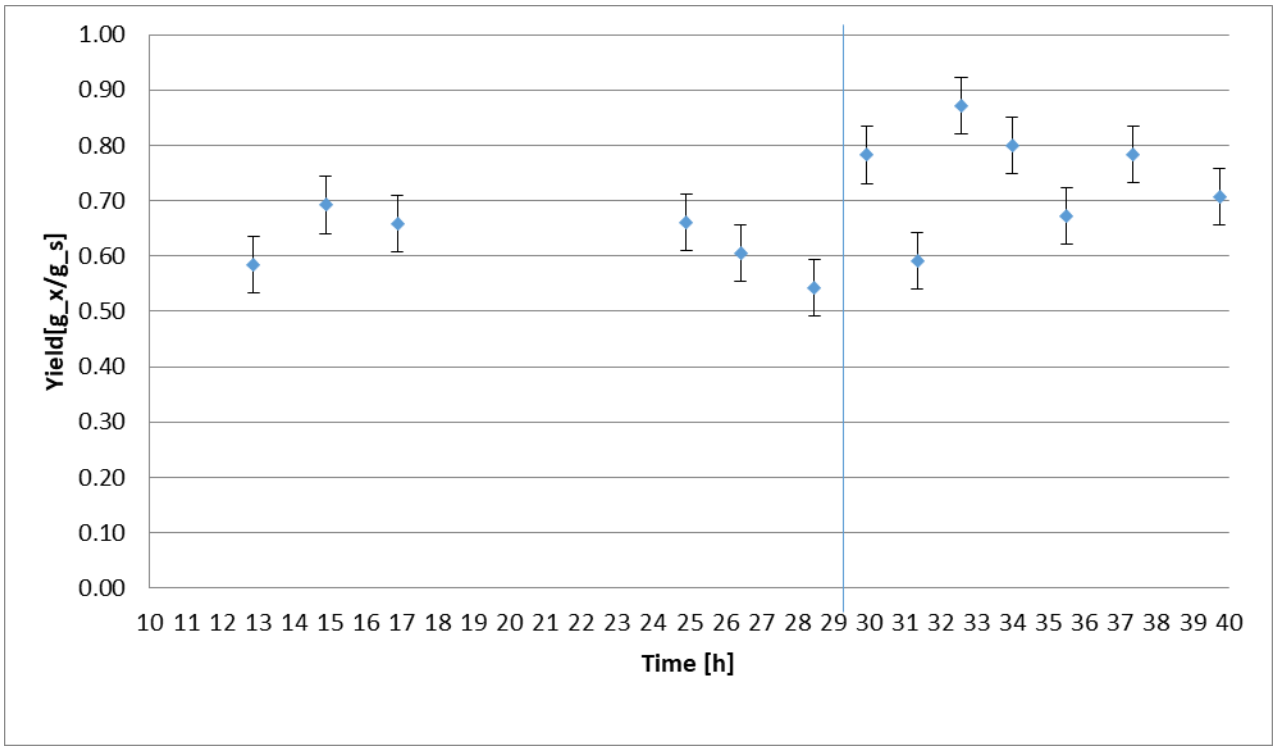

Figure 23 Yield of converted substrate [gs] into biomass [gx] over time [h] in 60L Scale

The first yield can be determined after a process time of 12,9h since is calculated with the previous state of the fermentation (see also Formula **4.2**). Until process time 29.5h (blue line) the batch phase, the fed batch started just after that. The average yield in the batch phase is 0.63 [g_x/g_s], in the fed batch phase 0.75 [g_x/g_s]. The yield should not differ.

The pathway of *P. pastoris* with the substrate glycerol shows no significant production of other products than biomass. Therefore all substrate will be converted into biomass.

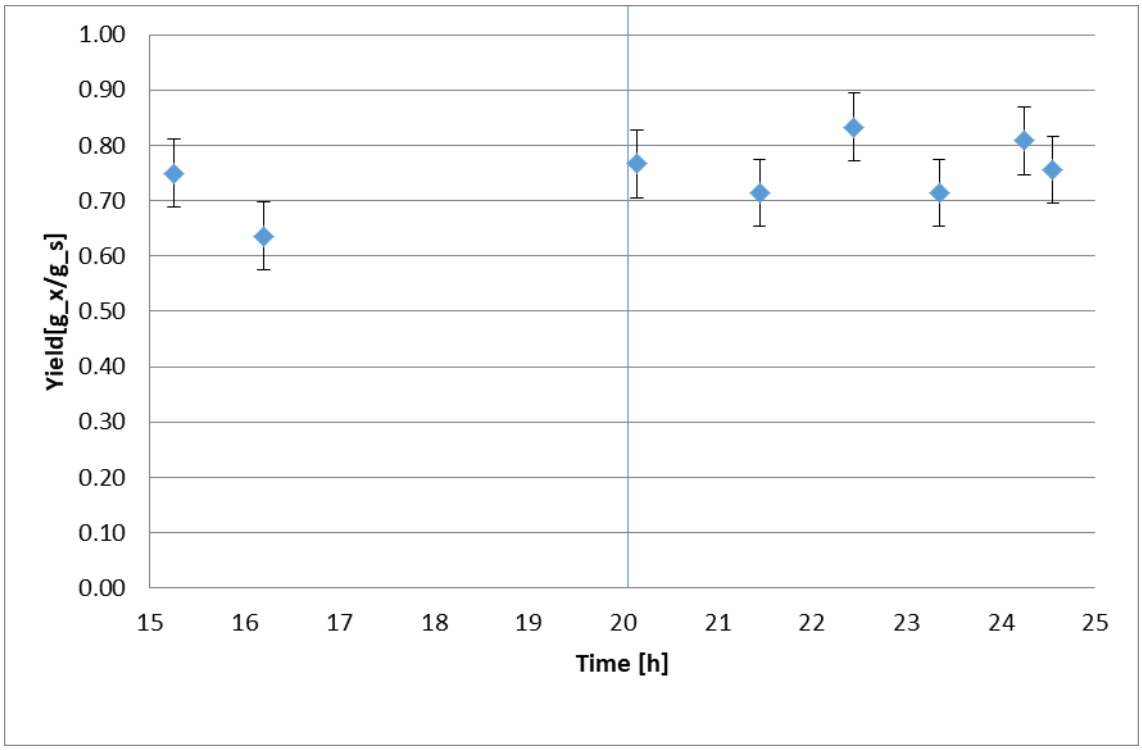

Figure 24 Yield of substrate [g_s] and biomass [g_x] over time [h] in 5L scale

The mean yield in 5L scale is 0.72 [g_x/g_s] in batch and 0.77 [g_x/g_s]in fed batch (beginning 20,15h process time, blue line). As in the 60L scale glycerol phase, the yield should not differ since it the organism is in the same physiological state. Nevertheless the yield varies also due to close sampling times.

4.2.4. Specific growth rate µ in 5L and 60L scale

One of the most important physiological parameters is the cell specific growth rate as described in 1.2.1. In the following chapter the results of both the 5L and 60L scale fermentations is shown and discussed.

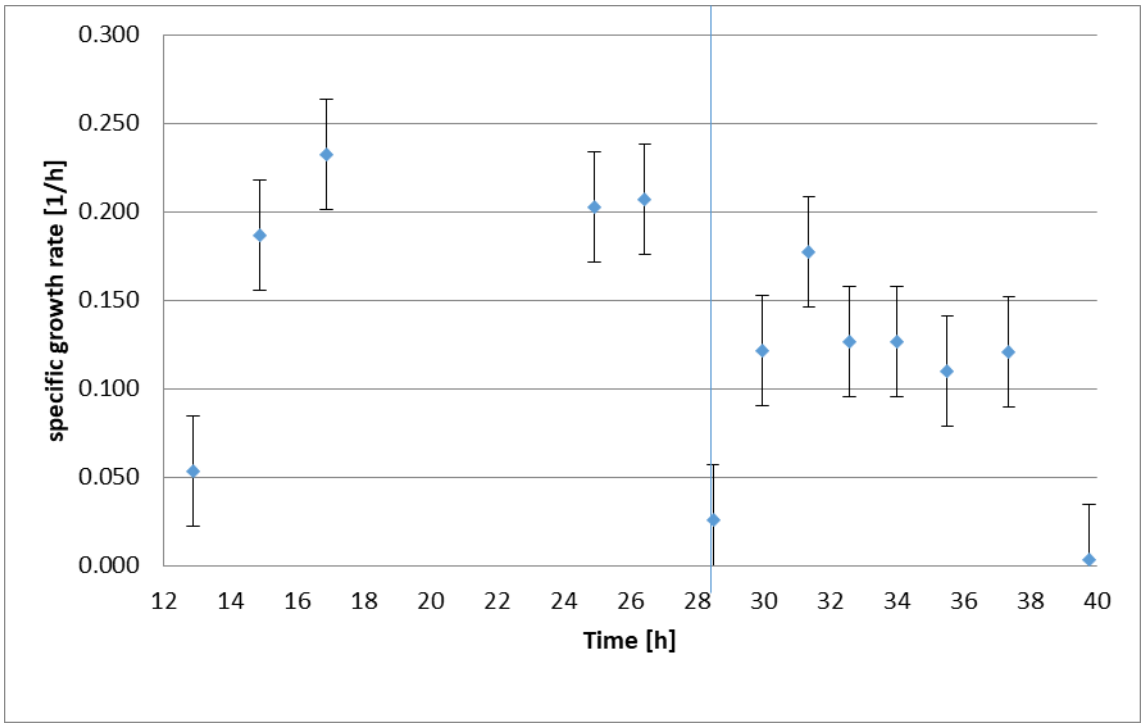

Figure 25 Specific growth rate [1/h] in 60L scale

Figure 25 shows the specific growth rate of the fermentation in 60L scale. Differently to the yield, the lack phase and the end of batch phase should not be considered for the specific growth rate μ since it is time depended. The maximum growth rate is stated in literature (Capone S, 2015) with the value of μ_{max}=0.2 h^{-1}. The single rates vary heavily, so the overall phase cell specific growth rate is considered to be more significant to make a statement. It has an value of μ_{max}=0.21h^{-1} for the overall batch phase (excluding the first measurement, which includes the lag phase). The blue line indicates the beginning of the fed batch phase. As the batch phase, the individual rates vary heavily due to short sampling times. Since the physiological state of the organism is identical over the fed batch phase, a cell specific growth rate over the phase is more substantiated statement. The overall phase specific growth rate is 0.14h^{-1}.

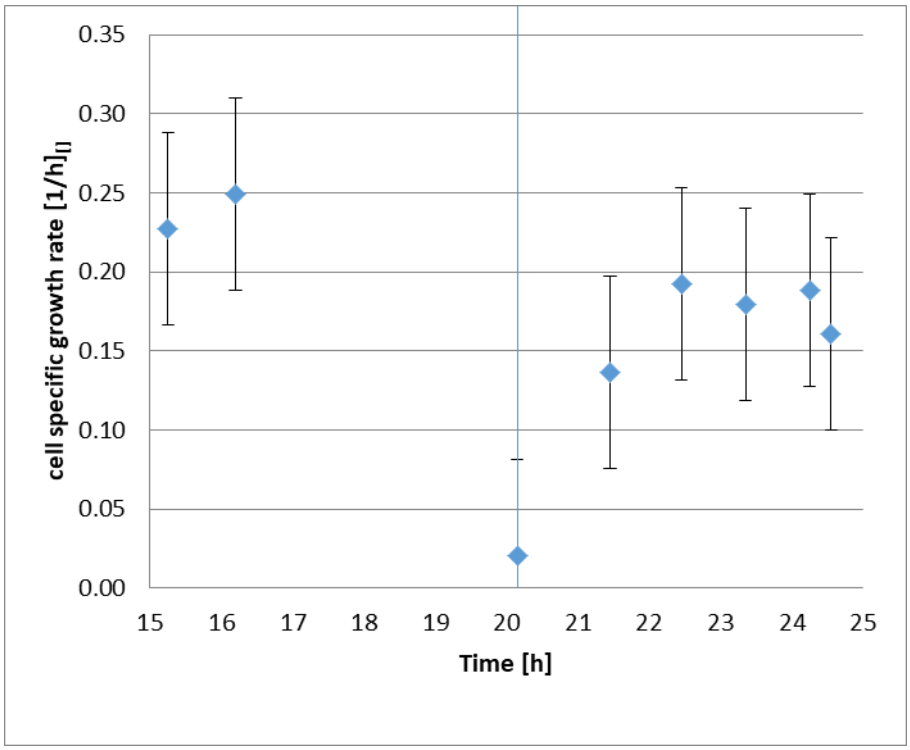

Figure 26 Cell specific growth rate [1/h] in 5L scale

In Figure 26 the specific growth rate in the batch phase is in average μ_{max}=0.23 h^{-1}, which is higher than the literally stated μ_{max}=0.2 h^{-1}. The first point at 15.25h can be excluded due to the expected lag phase occurring in most fermentation. The blue line again indicates the beginning of the fed batch phase.

The average in the fed batch phase is μ=0.16 h^{-1}, where only the five last measurements where included due to the lack phase. The archived growth rate fits quite well to aimed μ=0.15 h^{-1} in the designed feed.

4.2.5. Specific substrate uptake rate in 5L and 60L scale

Stated in the fermentation strategy the $q_{s/x,max}$ in the experiment design was to be determined with 0.338 $g_s/(g_x*h)$. Differently to the yield, the lack phase and the end of batch phase should not be considered for the $q_{s/x}$ value since it is a rate.

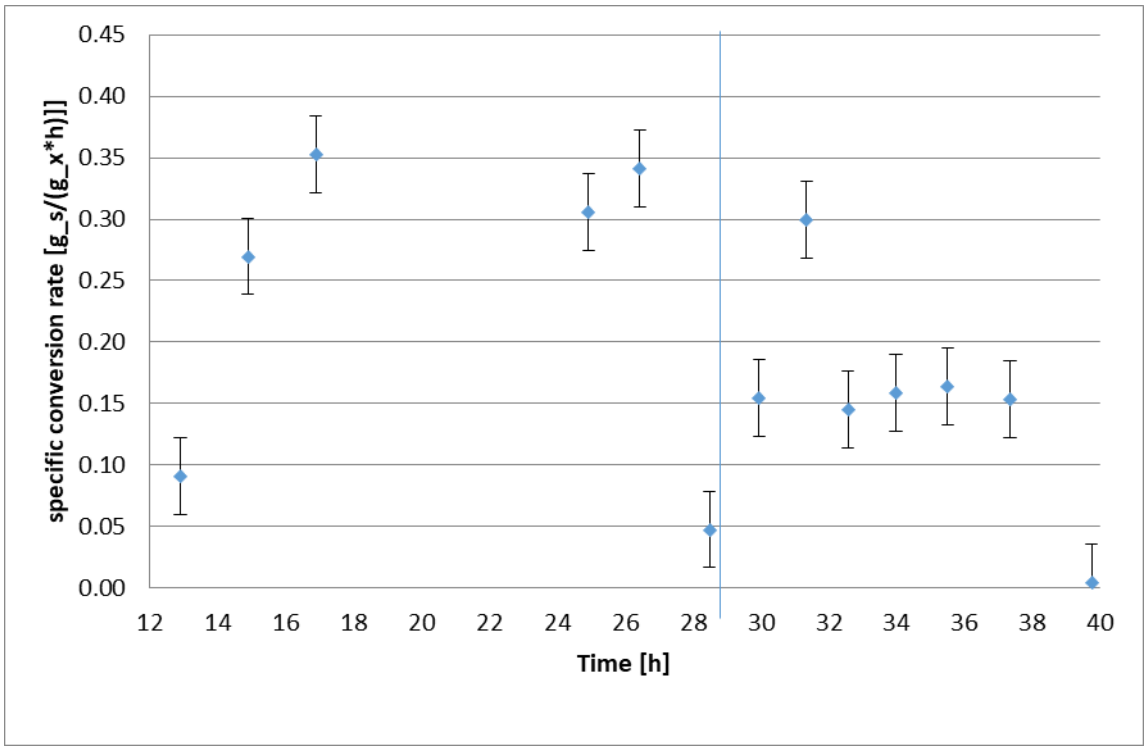

Figure 27 Specific substrate uptake rate q_s/x over time in 60L scale

Figure 27 shows the results of the 60L scale fermentation with an average specific substrate uptake rete $q_{s/x,max}$ of 0.30 $g_s/(g_x*h)$ for the batch phase. In planning of the experiment, the fed batch was designed with the value of $q_{s/x}$ =0.28 $g_s/(g_x*h)$. Similar to the cell specific growth rate, q_s is time dependent and is reasonably calculated for the phases the organism is in a steady physiological state.

The average of the specific conversion rate is just $q_{s/x}$=0.17 $g_s/(g_x*h)$ for the second part of the fermentation.

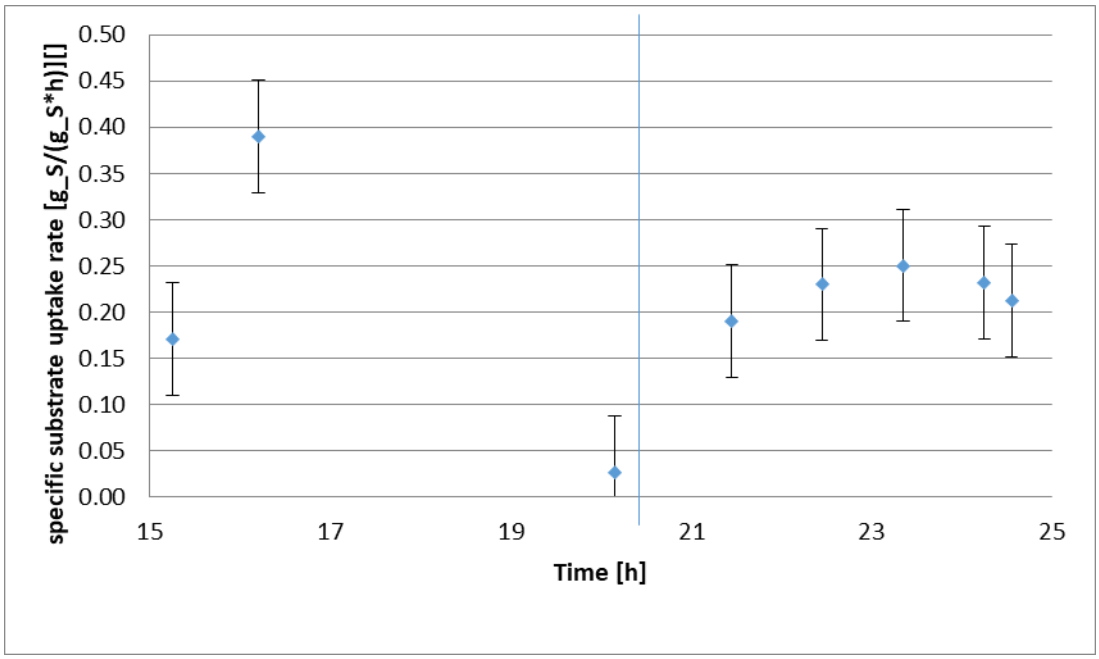

Figure 28 Specific substrate uptake rate q_s/x over time in 5L scale

Figure 28 shows the specific conversion rate. The first value includes the lack phase and does not show the $q_{s/x,max}$ value for the batch phase. The second value $q_{s/x,max}$=0.32 $g_s/(g_x*h)$ just shows little difference to the maximum of 0.34 $g_s/(g_x*h)$ calculated in previous fermentations and anticipated in the experimental design. The fed batch fermentation result in an average of $q_{s/x}$=0.28 $g_s/(g_x*h)$ which differs to desired value $q_{s/x}$=0.21 $g_s/(g_x*h)$. However it should be compared to the values resulting in the 5L scale fermentation.

4.2.6. Off-gas analysis

In chapter 1.2.4 important rates and quotients of off-gas analysis are described. For the 60L fermentation the following results are discussed.

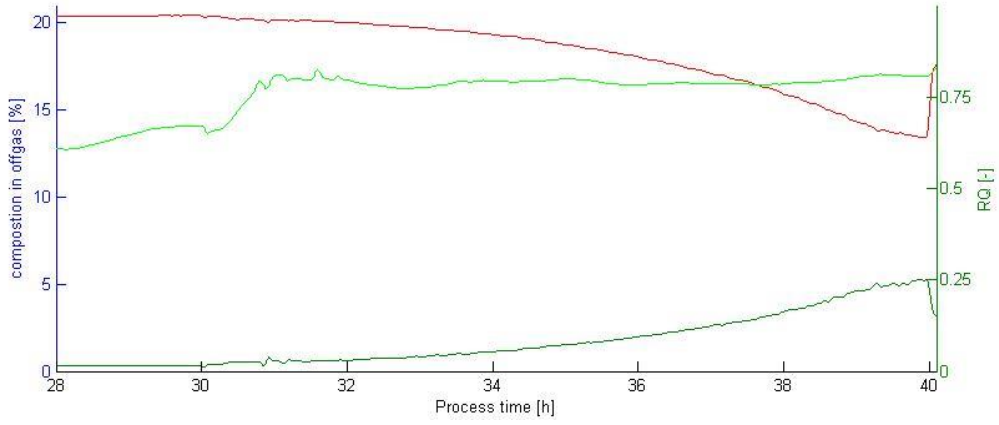

Figure 29 Off-gas and respiratory quotient of fed batch phase in 60L scale

Figure 29 shows the percentage of oxygen and carbondioxid in the off-gas of the bioreactor 60L scale. As expected the O2 content in the off-gas declines with higher conversion rate of substrate.

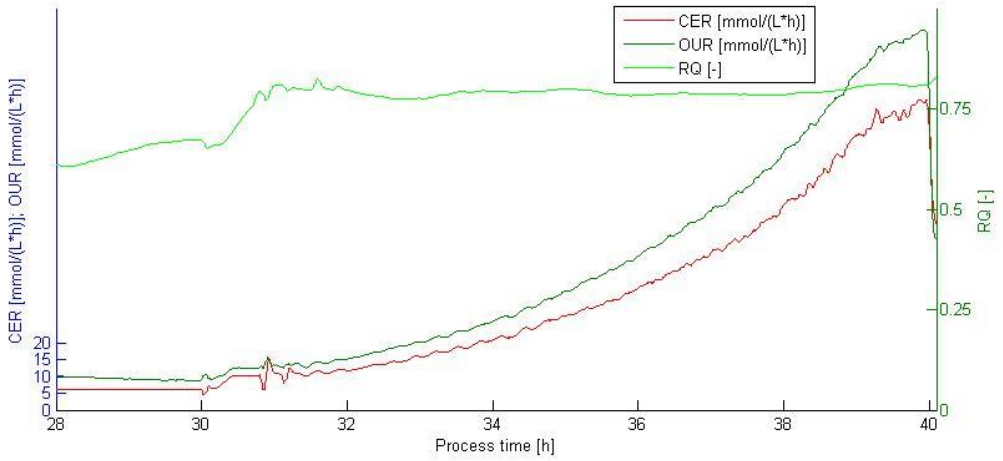

Figure 30 CER, OUR and RQ of fed batch on gylcerol in 60L scale

In Figure 30 the CER, OUR and RQ are shown. As expected the CER and OUR increase by the time (and amount of glycerol metabolized), while the RQ (as a quotient of OUR and CER) stays constant as the metabolic steady state of the organism

4.2.7. Activity HRP

The activity of the target enzyme was measured as stated in 3.3.2. For the 5L fermentation the following figure shows the activity of HRP over time.

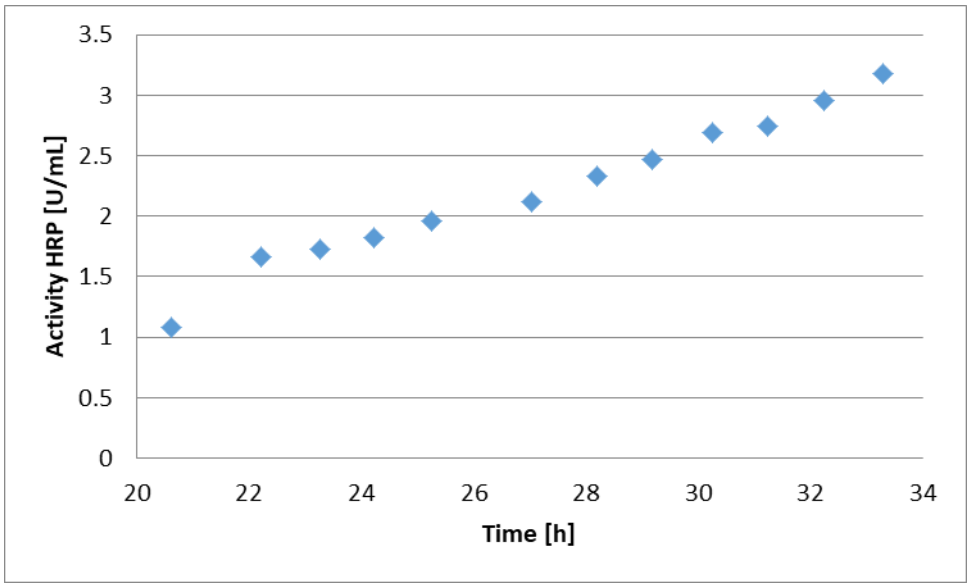

Figure 31 Acivitiy [U/mL] of horseradish peroxidase (HRP) over time [h] 5L scale

An increase of activity is to be noticed over time starting with the pulse at process time 20.6h. The activity of HRP in the 60L fermentation cannot be measured. The reason is unclear, since the same strain with the same metabolic rates and data have been cultivated.

4.2.8. Direct comparison of metabolic rates and yields in 5L and 60L scale

After discussing the results of the metabolic rates and yields separately in the chapters before, a direct comparison of the results is shown following in Table 10.

Table 10 Comparison of metabolic rates and yields of 5L and 60L scales

Phase	Metabolic rates and yields	Scale 5L	Scale 60L
Batch	Substrate to Biomass Yield $Y_{X/S}$ [g_x/g_S]	0.72	0.63
	Specific Growth Rate μ [h^{-1}]	0.23	0.21
	Specific Substrate Uptake Rate $q_{s/x}$ [$g_s/(g_x*h)$]	0.32	0.33
Fed batch on Glycerol	Substrate to Biomass Yield $Y_{X/S}$ [g_x/g_S]	0.77	0.73
	Specific Growth Rate μ [h^{-1}]	0.16	0.13
	Specific Substrate Uptake Rate $q_{s/x}$ [$g_s/(g_x*h)$]	0.21	0.18
Fed batch q_s=0.1 $g_s/(g_x*h)$	Substrate to Biomass Yield $Y_{X/S}$ [g_x/g_S]	0.22	0.32
	Specific Growth Rate μ [h^{-1}]	0.02	0.02
	Specific Substrate Uptake Rate $q_{s/x}$ [$g_s/(g_x*h)$]	0.06	0.07
Fed batch q_s=0.3 $g_s/(g_x*h)$	Substrate to Biomass Yield $Y_{X/S}$ [g_x/g_S]	0.36	0.25
	Specific Growth Rate μ [h^{-1}]	0.04	0.03
	Specific Substrate Uptake Rate $q_{s/x}$ [$g_s/(g_x*h)$]	0.11	0.10

Fed batch q_s=0.4 $g_s/(g_x*h)$	Substrate to Biomass Yield $Y_{X/S}$ [g_x/g_S]	0.34	0.32
	Specific Growth Rate μ [h^{-1}]	0.04	0.03
	Specific Substrate Uptake Rate $q_{s/x}$ [$g_s/(g_x*h)$]	0.12	0.10

In the batch and fed batch phase three main values are shown, substrate to biomass yield, specific growth rate and specific substrate uptake rate.

Comparing the values directly against each other, the batch and the fedbatch phase on glycerol, the results differ minimal. Matching the experimental design is desired while the differences between the 5L and 60l scale are more likely to have a stronger statement in comparability of fermentation. The methanol phase Is divided into four main parts. The pulse and three exponential feeds with different assumptions on q_s. The first and second exponential feed is almost identical. The third feed (as described in 3.1.1) is above the level of maximum capability of q_s and differs quite a lot.

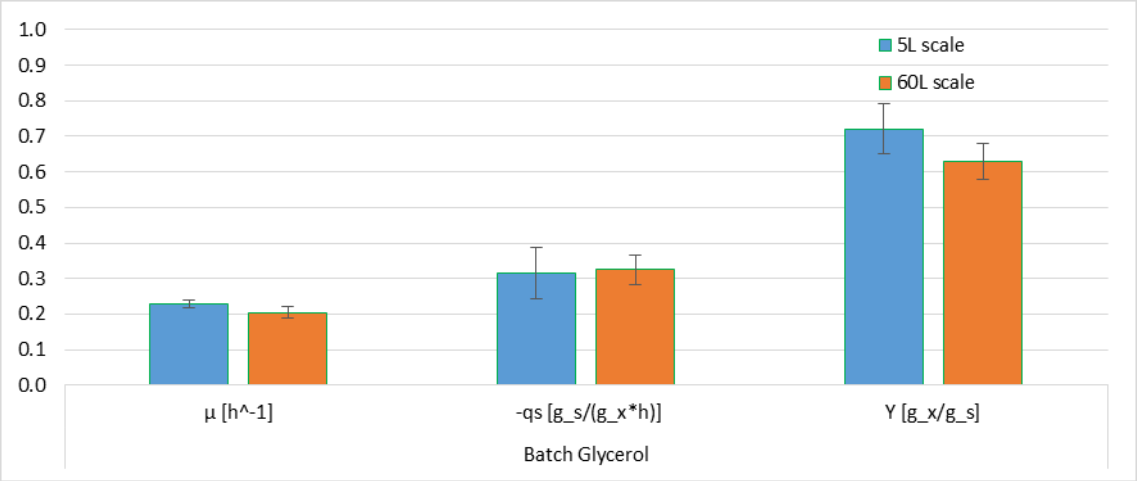

Figure 32 Comparison of physiological paramters of batch phase on glycerol of 5L and 60L scale fermentation

Figure 32 shows the physiological parameters cell specific growth rate μ [h^{-1}], specific substrate uptake rate q_s [$g_S/(g_X*h)$] and substrate to biomass yield $Y_{X/S}$ [g_X/g_S]. As shown, there are no significant differences in the physiological parameters to be seen.

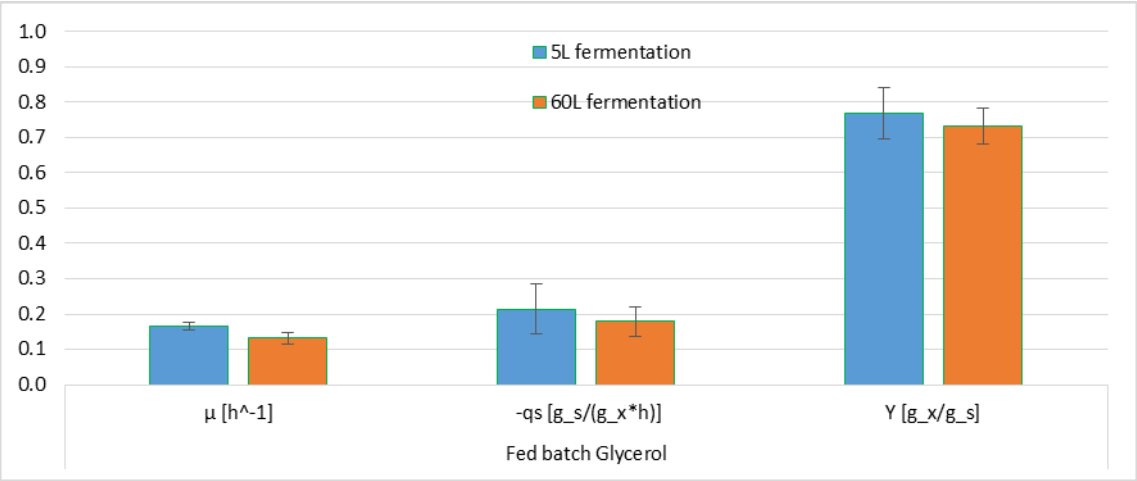

Figure 33 Comparison of physiological parameters of fed batch phase on glycerol of 5L and 60L scale fermentation

As the batch phase in Figure 32 also the fed batch phase shown in Figure 33, no significant difference between the scales is to be seen. The yield does not differ significantly between batch and fed batch phase, which indicates a metabolic steady state. The cell specific growth rate µ and cell specific substrate uptake rate q_S is dependent on the available substrate and is therefore different.

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4.3. Range of specific power

As described in 1.3, the power input has an impact on the fermentation circumstances. The higher the power input, the higher the degree of homogeneity in the liquid phase. The resulting shear stress can impact the physiological conditions of mammalian cultures and, in worst case, can actually kill them. *P. pastoris* is robust and likely withstands shear stress (Julien, 2006).

In 1.3 the specific power input is calculated. The following table shows the parameters used in **Formula 1.3**.

Table 11 Parameter set for specific power input calculation for 5L labscale reactor und 60L pilot plant reactor

	60L Zeta BIRE	5L lab BIRE
Ne [-]	4.5	4.5
ρ_L [kg/m³]	1100	1100
$d_{stirrer}$ [cm]	10	5.5
V_L [L]	30…60	3…5

For the two different bioreactors the specific power input over the rotation is shown in following figure.

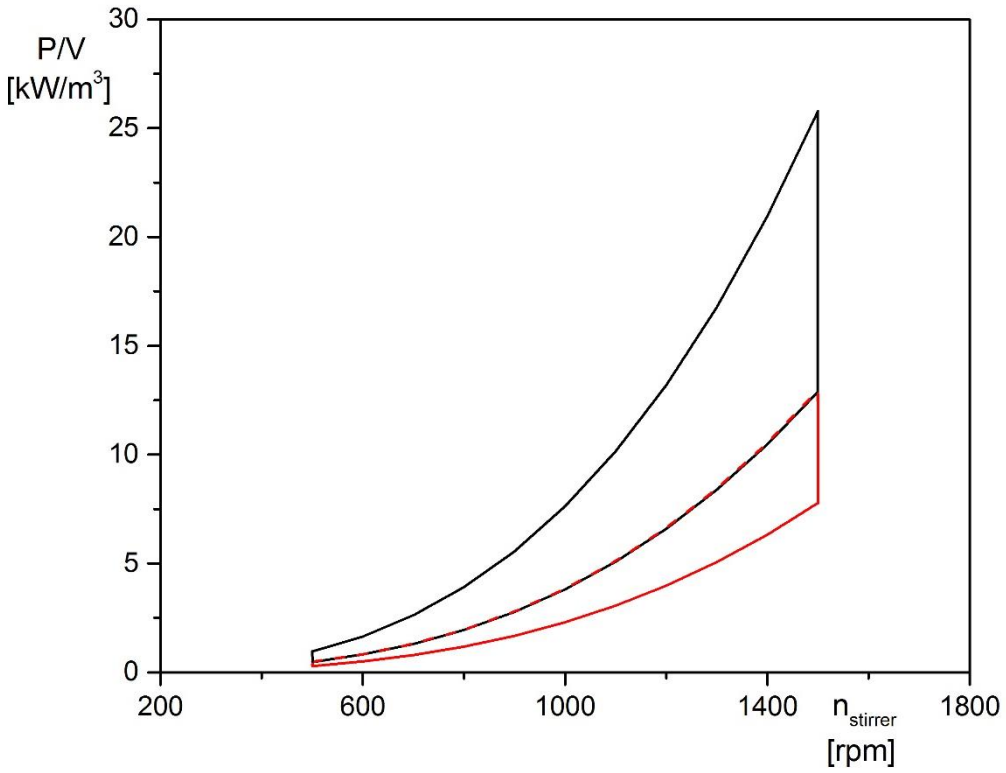

Figure 34 Specific power input of the two bioreactors. The red area is the lab scale 5L system, the black one is for the 60L pilot plant system

In Figure 34 the specific power input is shown. The black area displays the specific power input in the 60L pilot plant, the red area the 5L lab scale bioreactor. Since the agitation varies for the

oxygen control in the liquid phase and the volume (sampling, feed, pH control), an area is calculated for the specific power input. The working areas are shown in the figure below.

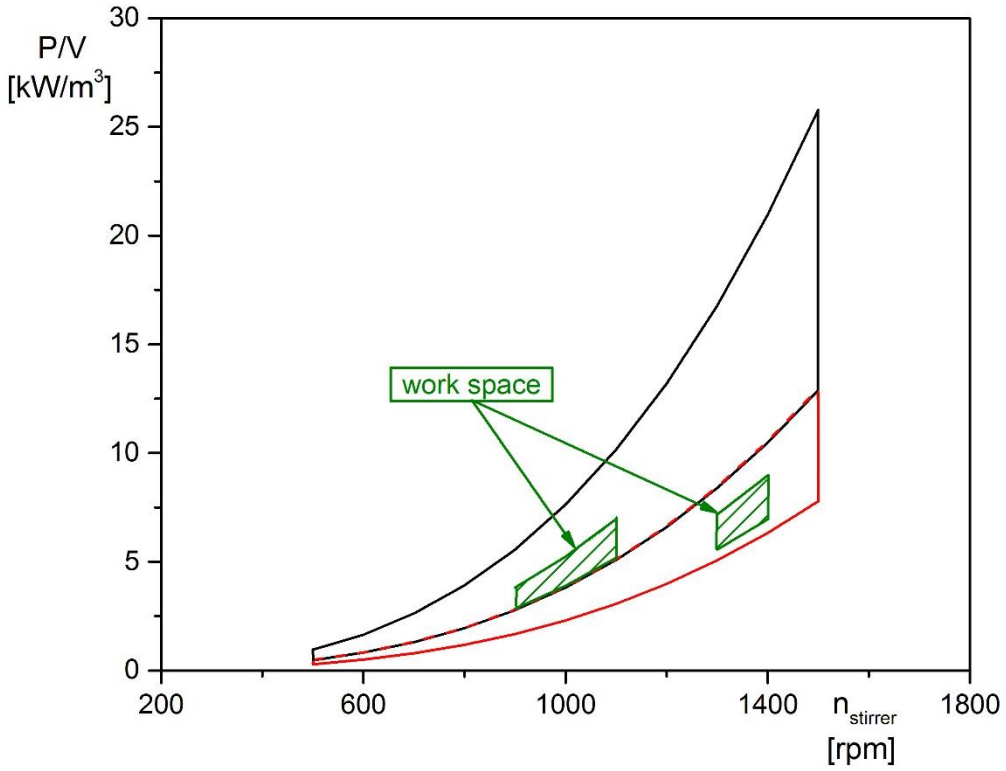

Figure 35 Specific power input work space for the fermentations conducted in the two bioreactors

An important scale up criteria is to keep same conditions in different scales (see also chapter 1). For the fermentation in the 5L scale (red area), the specific power input was in range of 5.5…9.2 kW/m³, in the 60L scale (black area) in range of 2.9 … 7.0 kW/m³. The ranges are even close and the specific power input can be assumed.

5. Conclusion and outlook

Scalability is a big issue in process technology in general, where bioprocesses are no exceptions. The main causes were discussed in the thesis and a scale-up strategy was chosen for this specific *P. pastoris* fermentation where the common yeast strategy was applied. Volumetric gas flow rate per volume of liquid phase, specific power input and concentrations of substrates were to be set identical in the fermentation in 5L and 60L. The process equipment understanding and knowledge was deepened and the process sequences were optimized (e.g. pump control optimization for smooth feeding).

The fermentation was quantified with the most common parameters for fermentations, namely specific growth rate (μ), substrate to biomass yield ($Y_{S/X}$) and specific substrate uptake rate ($q_{S/X}$). Off-gas analytic was used to calculate oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ).

The specific growth rate μ on glycerol over the batch phase of *P. pastoris* was 0.23 h^{-1} in the 5L lab scale reactor and 0.21 h^{-1}, which is close to the assumed 0.2 h^{-1}. The value for specific growth rate μ in the experimental design was 0.15 h^{-1} for the fed batch phase, which were 0.16 h^{-1} in the 5L and 0.13 h^{-1} in the 60L reactor. With an uncertainty of 0.02 h^{-1}, the difference was not significant.

Substrate to biomass yield as an important physiological parameter was also calculated and compared. The yield in the batch phase in the 5L fermentation was calculated to be 0.72 g_S/g_X, whereas 0.63 g_S/g_X was calculated in the 60L pilot scale reactor. In the 5L small scale reactor the substrate to biomass yield was 0.77 g_S/g_X, compared to 0.73 g_S/g_X in the 60L scale. The yield should not differ in different phases as long as the metabolic state (e.g. different C-source substrate) was steady. Since the uncertainty is between 0.05 to 0.09 g_S/g_X, the difference is not significant.

The cell specific substrate uptake rate q_S was also determined for the different phases. For the batch phase on glycerol in the 5L lab scale fermenter the specific substrate uptake rate q_S for glycerol was 0.32 $g_S/(g_X*h)$, the 60L pilot plant scale reactor value was 0.33 $g_S/(g_X*h)$. In the fed batch phase it was 0.21 $g_S/(g_X*h)$ in the 5L reactor, 0.18 $g_S/(g_X*h)$ in the 60L reactor. Since glycerol was not limited in the batch phase, a lower cell specific growth rate μ and cell specific substrate uptake rate was to be expected. After an adaption pulse with methanol, a stepwise increase of intended cell specific substrate uptake rates q_S were executed. For the first phase, a specific growth rate of 0.02 h^{-1} were calculated in the first phase. A substrate to biomass yield of

0.22 g_X/g_S in 5L and 0.32 g_X/g_S in 60L resulting in a specific uptake rate of 0.06 $g_S/(g_X*h)$ in 5L and 0.07 $g_S/(g_X*h)$ in 60L. After an increase of methanol feed, the q_S was calculated to be 0.11 $g_S/(g_X*h)$ in the 5L, 0.12 $g_S/(g_X*h)$ in the 60L bioreactor. During the third part the specific uptake rate of methanol did not increase and methanol accumulated as intended.

The major achievement of this thesis was the establishment of a scale-up platform of *P. pastoris* fermentations with HRP production with equivalent metabolic rates, yields and performance in both lab scale 5L and pilot plant scale 60L.

The fermentation of *P. pastoris* was successfully transferred from 5L to 60L scale due to optimization and deepened equipment understanding. Nevertheless further investigations to verify the CFD simulations should be conducted. The sensitivity of the *P. pastoris* strain and chosen fermentation is to be challenged in different fermenter set ups, to wit results of worst and best position should be compared. While conducting these experiments, the amount of sampling and sampling time intervals should be adjusted to the period a significant progress within the fermentation is to be expected. Also other established process strategies should be considered (e.g. multiple pulses, variations on concentrations) as well as other organisms (e.g. shear-sensitive organisms).

A further characterization of the bioreactor (e.g. k_La values, shear stress, measured power input) could also support the results of the CFD simulations and provide information for tailored fermenter set ups to take specific requirements of organisms into account (e.g. shear-sensitive).

Since the fluid flows in CFD were simulated for the 60L volume, the set ups should be calculated for other volumes to challenge the rating of homogeneity for the parameter settings investigated.

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