sartorius stedim biotech

Hochschule für Angewandte Wissenschaften Hamburg
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Fakultät Life Sciences
Studiendepartment Biotechnologie

Evaluation of a chemically defined medium for *Pichia pastoris* high cell density fermentation process

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Abstract

Microbial processes are important in modern biotechnology, due to the low requirements of the organisms in comparison to cell culture. Special relevance has *Pichia pastoris* in the production of biopharmaceuticals. It is a eukaryotic protein expression system and able to perform many protein modifications common in mammalian cells. For a successful protein expression an optimised process is important.

Therefore, a fed batch process was evaluated in an established stainless steel system for the comparison of the medium to other media. The process was optimised for maximal cell density. Afterwards, a process transfer to a single use rocking motion type bioreactor was accomplished. Single use systems have the advantages of high flexibility and a decreased effort for cleaning and preparation. The system was originally designed for cell culture applications. For microbial processes the oxygen transfer rate was the limiting parameter in this system. The development of processes for microbial high cell density cultivations are challenging in this system. An important point was the optimisation of the feeding strategy in reference to the oxygen uptake rate. In batch processes the limit of the oxygen transfer rate was reviewed to calculated values. Fed batch processes conducted to increase the maximal cell density. Finally protein expression of human serum albumin with a methanol feed phase was performed.

In the reusable system a maximal cell density of 387 g_{WCW}/L (113 g_{DCW}/L) was reached in a glycerol fed batch process. The results were comparable to industrial high cell density cultivations. In the single use rocking motion type bioreactor the cell density increased up to 292 g_{WCW}/L (82 g_{DCW}/L) in a glycerol fed batch process and up to 315 g_{WCW}/L (92 g_{DCW}/L) in a fed batch process with methanol induction phase. Limitations in the oxygen transfer rate avoid a further increase of the cell density.

The present work shows the possibility of high cell density cultivations of *Pichia pastoris* in single use systems. Furthermore, the expression of the human serum albumin was possible in the single use system.

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Content

I. Abbreviations

ATP	Adenosine triphosphate
AOX	Alcohol oxidase
BSA	Bovine serum albumin
CIP	Cleaning in place
DCU	Digital control unit
DCW	Dry cell weight
HCDC	High cell density cultivation
HSA	Human serum albumin
MFCS	Multi fermenter control system
NADH	Nicotinamide adenine dinucleotide
PES	Poly ether sulfone
pO_2	Oxygen partial pressure
RM	Rocking motion
RO	Reverse osmosis
RPM	Rocks per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIP	Sterilisation in place
STR	Stirred tank reactor
SU	Single use
WCW	Wet cell weight

II. Symbols

$c_{Glycerol}$	Concentration of glycerol	g/L
C_L	Dissolved oxygen concentration	mmol/L
C_L^*	Oxygen saturation concentration	mmol/L
F	Flow rate	L/h
D	Dilution factor	-
k	Correcting factor	-
k_La	volumetric mass transfer coefficient	h^{-1}
OD_{600}	Optical density at 600 nm	-
$OD_{600,Seed}$	Optical density of the seed culture	-
$OD_{600,Start}$	Initial optical density	-
OTR	Oxygen transfer rate	mmol/(L·h)
OUR	Oxygen uptake rate	mmol/(L·h)
q_{O_2}	Specific oxygen uptake rate	$mmol/(g_{WCW}·h)$
r_s	Substrate uptake rate	$g_{substrate}/(L·h)$
r_x	Growth rate	$g_{WCW}/(L·h)$
S	Substrate concentration	$g_{substrate}/L$
S_{feed}	Substrate concentration in the feed solution	$g_{substrate}/L$
V	Volume	L
V_0	Initial volume of the cultivation system	L
V_{Seed}	Transferred volume of the seed culture	L
V_{System}	Volume of the cultivation system	L
$V(t)$	Volume at the feed time t	L
X	Cell density (wet cell weight)	g_{WCW}/L

X_0	Cell density at feed start	g_{WCW}/L
Y_{X/O_2}	Oxygen yield coefficient	$g_{WCW}/mmol$
$Y_{X/S}$	Yield coefficient for substrate	$g_{WCW}/g_{Substrate}$
ΔA	Absorbance difference ($\Delta A = A_1 - A_2$)	-
ΔX	Differential change of the cell density	g_{WCW}/L
ΔS	Differential change of the substrate uptake	$g_{substrate}/L$
μ	Specific growth rate	h^{-1}
μ_{max}	Maximal specific growth rate	h^{-1}
μ_{set}	Fixed value of the specific growth rate	h^{-1}

1 Introduction

Pichia pastoris is nowadays an important organism for the expression of proteins. These proteins are used as biopharmaceuticals for therapeutic use. Common examples are recombinant proteins or monoclonal antibodies (Cregg, 2007) (Hamilton et al., 2007). The microorganism *P. pastoris* is a eukaryote (Suh et al., 2006) and able to perform many post translational modifications like protein folding, disulphide bond formation and glycosylation (Macauley-Patrick et al., 2005). This and the ability to grow on different carbon sources, including methanol, in chemically defined media (Macauley-Patrick et al., 2005) is the reason for the common use of *P. pastoris* as an expression system. The ability to grow on methanol is useful for the expression of proteins. *P. pastoris* is suitable for high cell density cultivations (Cregg, 2007). Cultivation with cell densities above 100 g/L dry cell mass have been reported in reusable stainless steel systems. (Guramkonda et al., 2009)

Reusable stirred tank reactors are established for biopharmaceutical processes. They offer high oxygen transfer rates and mixing rates. The used materials for the cultivation chamber are stainless steel and glass (Chmiel, 2006).

The advantages of a chemically defined medium are the reproducibility of the results. The composition of the medium is identical in every process in contrast to complex media. Therefore, the downstream process is simplified, because only a few substances have to be removed (Hensing et al., 1995). This issue is important in the manufacture of pharmaceuticals.

In the last years single use bioreactors became attractive for the biopharmaceutical industry. Originally designed for the cultivation of mammalian cells, nowadays single use systems are also used for microbial cultivations (Glazyrina et al., 2010). Microbial cultivations are a new challenge for single use systems. The yeast *Saccharomyces cerevisiae* was cultivated successful in a rocking motion single use reactor up to 9.1 g/L dry cell weight (Mikola et al., 2007). Furthermore, *Escherichia coli* was cultivated in single use systems

The aim of this work was the evaluation of a chemical defined medium for *P. pastoris* HCDC in a stainless steel system. After the evaluation was successful a process transfer was carried out to a single use system. Different pH set points and cultivation strategies were tested in the single use system. In a second step after the process transfer the ability of the expression of human serum albumin (HSA) was evaluated.

2 Theory

2.1 Fermentation of *Pichia pastoris*

2.1.1 *Pichia pastoris*

Pichia pastoris is a yeast belonging to the group of Ascomycota (Suh et al., 2006). The organism is facultative anaerobe. It is one of the most attractive eukaryotic expression systems. More than 500 proteins have been produced in *P. pastoris* (Cos et al., 2006). Several reasons made *P. pastoris* popular in the last 25 years. It has only low requirements on the medium compared to mammalian cells. Figure 1 shows *P. pastoris* cells in a phase contrast image.

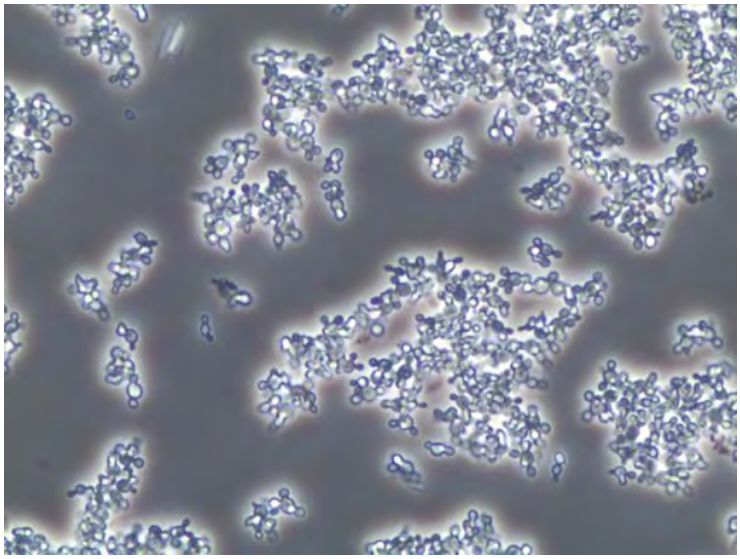


Figure 1: *Pichia pastoris* cells, image was taken by a phase contrast microscope at a magnification of 400x (Sartorius Stedim Biotech)

As a eukaryote, *P. pastoris* is able to perform higher eukaryotic protein modifications, such as glycosylation, disulphide bond formation and proteolytic processing. The produced eukaryotic proteins are potentially in their biological native form and can be secreted extracellular (Macauley-Patrick et al., 2005). Due to this ability the complexity of the downstream process is reduced. Furthermore, the techniques for molecular genetic manipulation are simple and expression systems are commercially available (Cereghino et al., 2000). Furthermore, *P. pastoris* is well suited for high cell density cultivations (HCDC) (Cregg, 2007). It grows at a wide pH range of 3 to 7 in chemical defined media (Macauley-Patrick et al., 2005). The optimal cultivation temperature is 30 °C (Cos et al., 2006). *P. pastoris* is a methylotrophic yeast and therefore able to metabolize methanol as the sole carbon source. This ability was of substantial interest during the 70s of the last century. Single cell protein should be produced by *P. pastoris* as animal food. The oil crisis in the end of the 70s increased the

price for methanol, making the production of single cell proteins commercially unattractive (Cos et al., 2006). Today the ability of methanol metabolism is used for the protein expression in *P. pastoris*.

2.1.2 Expression system and genetics

The phenotypes Mut^+, Mut^S and Mut^- of *P. pastoris* are known, which differ in the utilisation of methanol. In the present work a Mut^S phenotype of *P. pastoris* was used. The "S" in the name of the phenotype implies a slow methanol utilisation. In general, two genes for alcohol oxidase (AOX), *AOX1* and *AOX2*, are present in the genome. The difference between both is the activity of the resulting alcohol oxidase. The activity of the enzyme expressed by *AOX1* is 10 to 20 times higher than the activity of the enzyme expressed by *AOX2*. In the Mut^S strain the *AOX1* gene is genetically manipulated in such a way that no enzyme is expressed by the gene and therefore methanol is metabolised slower compared to strains with both *AOX* genes (Macauley-Patrick et al., 2005).

Protein expression in *P. pastoris* is based on the fact that enzymes for the metabolism of methanol are only expressed when the cells are growing on methanol. For the expression of a target protein, the *AOX1* promoter is commonly used. In the present work, human serum albumin (HSA) is the expressed protein. The gene of HSA is inserted in the genome behind the *AOX1* promoter. This is a tight regulated promoter in the absence of methanol, which is induced 1000-fold when methanol is supplied to the medium (Cereghino et al., 2002). Therefore, HSA is expressed when methanol is added to the medium.

For a short downstream process it is beneficial to secrete proteins into the culture medium. Cell disruption is than unnecessary and the cells can be separated from the medium by centrifugation. For the secretion of an expressed protein a secretion signal is needed. Expression cassettes with secretion signals are commercially available. Commonly used secretion signals are the *S. cerevisiae* α-factor prepro peptide or the *P. pastoris* acid phosphatase (PHO1). The target gene is transferred into the frame of the cassette. As a consequence, the protein is expressed with the secretion signal. In most cases the secretion signal is removed before the protein is secreted. *P. pastoris* secretes only low amounts of endogenous proteins (Cereghino et al., 2000). Therefore, a high percentage of the total protein in the cultivation broth is the secreted target protein, which ensures an economic downstream process.

2.1.3 Metabolism

P. pastoris is able to grow on different carbon sources, such as glucose, glycerol and methanol. In this work glycerol and methanol are used as carbon source. In general *P. pastoris* prefers the respiratory metabolism of the carbon source. In contrast to other yeast the fermentative pathway of the metabolism is decreased in *P. pastoris.* Therefore, only low amounts of ethanol, which is a toxic metabolite, are produced at high concentrations of the carbon source. This special feature makes *P. pastoris* more suitable for HCDC than for instance *Saccharomyces cerevisiae* (Cregg, 2007).

The metabolism of glycerol uses parts of the glycolysis (see Figure 2). Glycerol is phosphorylated by glycerol kinase. The product of this reaction is L-glycerol 3-phosphate. In a next step L-glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate, which can enters the glycolytic pathway. During the glycolytic pathway adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) is formed (Nevoigt et al., 1997). During the metabolism of the carbon source carbon dioxide and water are the main metabolic waste products (Schlegel et al., 1992). The carbon dioxide dissolves to carbonic acid in the cultivation broth and lowers therefore the pH during the cultivation.

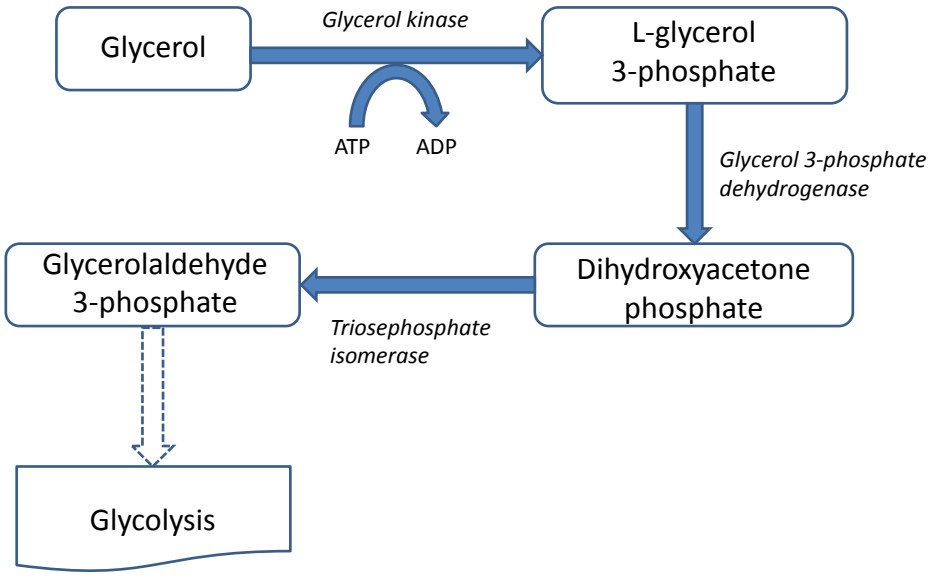


Figure 2: Metabolism of glycerol in *P. pastoris* (Nevoigt et al., 1997)

The metabolism of methanol is shown Figure 3 and requires special enzymes. Methanol is oxidized in the peroxisome by the enzyme alcohol oxidase. The reaction produces hydrogen peroxide and formaldehyde. The hydrogen peroxide is degraded to oxygen and water in the peroxisome by the enzyme catalase. Two molecules of formaldehyde are produced. One molecule leaves the peroxisome and is finally oxidised to formic acid and carbon dioxide. These reactions are the source of redox equivalents, which represents a source of energy for the cells.

The other molecule of formaldehyde is used to form cellular constituents in a cyclic pathway. The reaction is catalysed by dihydroxyacetone synthase (Cereghino et al., 2000).

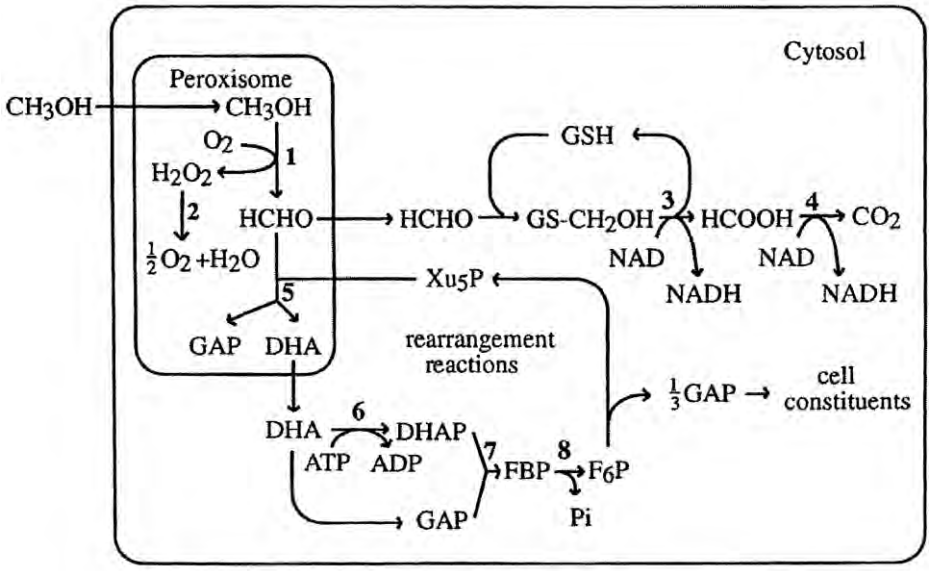


Figure 3: Reaction steps and used enzymes for the metabolism of methanol. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase (Cereghino et al., 2000)

2.2 Bioreactors

2.2.1 Reusable bioreactors

A bioreactor is an enclosed chamber for the cultivation of cells and the expression of the product. It ensures controlled conditions and provides specific requirements in an aseptic environment (Chmiel, 2006). In this chapter bioreactors for microbial cultivations are discussed.

The classical design of bioreactors is the stirred tank reactor (STR). Over 95 % of the today used bioreactors belong to this category of systems. Experiences in the chemical industry result in the multiple use of STRs in the modern biotechnology. Common used materials are glass and stainless steel for reusable bioreactors (Chmiel, 2006). Inside the cultivation chamber a smooth surface for easy cleaning is needed. Stainless steel is therefore electro polished to meet this requirement. The homogenisation of the cultivation broth is important to avoid local concentration gradients during the cultivation. Therefore, agitation is an important function for every bioreactor. This agitation is realised by a stirrers, fixed on a rotating shaft. The shaft can be mounted on the reactor lid or the bottom. A mechanical seal isolates the cultivation chamber from the environment at the shaft feedthrough. Furthermore, magnetic couplings are used for the aseptic power transmission to the stirrer. Different stirrer types e.g. 6-blade-disk impellers are common. Baffles inside the cultivation chamber can

improve the homogenisation in the cultivation chamber by preventing the development of a vortex around the stirrer (Dreher et al., 2013).

The oxygen transfer into the cultivation chamber is realised by pressurized gases, which are supplemented by a sparger at the bottom of the cultivation vessel. The gas bubbles are ascending in the cultivation broth and are dispersed by the stirrer. A longer residence time of the gas bubbles increases the oxygen transfer. Therefore, a high height to diameter ratio is preferable for processes with high oxygen demands like microbial cultivations. A very common height to diameter ratio is 3:1 in Europe and 2:1 in the USA. This ratio increases also the possible heat transfer area of the reactor. The heat transfer is generally implemented by a double jacket supported by warm or cold water.

To provide aseptic conditions in the cultivation chamber a sterilisation method has to be implemented. Stainless steel reactors can be sterilised in place (SIP). The cultivation chamber, the double jacket and further equipment are supplied with steam to reach a sterilisation temperature between 121 °C and 130 °C. Small lab scale glass bioreactors can be sterilised in an autoclave. The inlet and outlet gases are sterilised by filters. To reduce liquid loss and the blocking of the exhaust filters, exhaust gas coolers are used in stainless steel and glass bioreactors. These filters reduce the humidity of the exhaust gas and condensing humidity flow back to the cultivation chamber (Dreher et al., 2013).

The cultivation conditions have to be controlled in their ranges. Therefore, measurement and control systems are important for a reliable process. Online parameters are determined directly inside the cultivation chamber. Often sterilisable electrodes are used. Digital control loops to control the process parameter reliable in the desired ranges.

After cultivation an easy cleaning with a low amount of work is desirable. Many stainless steel STRs are able to clean in place (CIP). This is often realised by diluted base, which is injected to the cultivation chamber by a spray valve (Sartorius, 2011 b).

The advantage of the STRs is the high oxygen transfer rate in comparison to other systems. Processes with a high oxygen demand can be realised in the STR. On the other hand, cleaning and maintenance is time consuming and the time-product yield is therefore decreasing.

2.2.2 Single use bioreactors

During the last years single use (SU) bioreactors becomes widely accepted in all bioprocess production stages (Eibl et al., 2009). The complete cultivation system consists of different parts. Many of these parts e.g. the DCU and the supply system are reusable. Only parts which are exposed to biological agents are designed for single use application. Commonly polymers are used to produce SU parts e.g. the cultivation chamber. After the cultivation no cleaning procedure is needed since the cultivation chamber is discarded. Hence, time and costs for cleaning and sterilisation is decreased and the system availability is increased.

The cultivation chamber can be designed as a rigid vessel or a flexible bag with a two or three dimensional shape (Löffelholz et al., 2013). These bags are made of films, which consist of different

layers. The contact layer of the polymeric film should be chemically inert. In the middle layer a gas barrier is located and the outer layer serves as structural backbone (Brecht, 2009). Similar to the reusable systems the agitation is an important function to homogenise the cultivation broth and eliminate limitations during the cultivation. The two main principles for agitation are mechanically and pneumatically mixed systems. Mechanically systems can be further divided into system with rotating stirrer, tumbling stirrer, oscillating movement or orbital agitation (Löffelholz et al., 2013). A very common agitation principle is the wave mixed system. It belongs to the group of oscillating moved bioreactors. Waves are induced in a two dimensional bag, which is partially filled with cultivation broth. These waves caused a permanent renewal of the fluid surface. Gasses are supplied to the headspace of the bag and therefore surface aeration is possible. The system is originally designed for cell culture applications (Eibl et al., 2009).

Sterile conditions in SU cultivation chambers are usually guaranteed by gamma irradiation. The bag is delivered presterilised with the SU sensors for pH and pO_2, sterile filters and the needed tubing. The exhaust gas filter has a filter heating to avoid filter blockages, caused by the humidity of the exhaust gas.

For the control of the process parameters precise measurement devices are needed. The sterile connection of reusable electrodes is difficult. Therefore, optical chemosensors (optodes) are used in small scale SU bioreactors. A chemical active dye is applied on a transparent observation window. An optic fibre, connected to a light source and an optic detector, is attached to this window. The fluorescence of the dye depends on the pH- or pO_2 and therefore a determination of these process parameters is possible (Glindkamp et al., 2009). A limitation of the optodes is the small measurement range of the pH between 6.0 and 8.5.Wave-mixed systems were used for small scale microbial cultivations and seed cultures (Glazyrina et al., 2010). The system showed limitations for microbial cultivations. The oxygen transfer rate is lower in comparison to STRs. Therefore, special cultivation strategies have to be developed to achieve high cell densities.

2.3 Cultivation of microorganisms

Microbial growth is characterised by cell division. Therefore, an exponential growth characteristic exists. Yeasts as *P. pastoris* are proliferating by sprouting (Schlegel et al., 1992). The proliferation can be described by the growth rate (r_x) (see equation 2.1). Between the growth rate and the cell density is a direct proportionality. The proportionality factor is called the specific growth rate (μ) (see equation 2.2).

$$r_x = \frac{dx}{dt} = \mu \cdot X \tag{2.1}$$

r_x = growth rate in $g_{WCW}/(L \cdot h)$

μ = specific growth rate in h^{-1}

X = cell density in g_{WCW}/L

Rearranged to the specific growth rate:

$$\mu = \frac{r_x}{X} \tag{2.2}$$

For the determination of the cell density at a specific time during exponential growth equation 2.1 can be integrated and rearranged to equation 2.3.

$$X(t) = X_0 \cdot e^{\mu_{max} \cdot (t - t_0)} \tag{2.3}$$

$X(t)$ = cell density at time t in g_{WCW}/L

X_0 = cell density at time t_0 in g_{WCW}/L

The shown equations are suitable for all media, which contain all nutrients in abundance and not inhibiting concentrations. The Monod kinetics describes the relation between the concentration of the limiting substrate and the μ (Chmiel, 2006). It is analogue to the enzyme kinetics by Michaelis and Menten (see equation 2.4). The μ is therefore dependent on the substrate concentration (s) of the limiting substrate, the maximal specific growth rate (μ_{max}) in the medium and the half saturation constant (K_s). The half saturation constant is specific for the organism and the substrate. K_s is the concentration of substrate, if one half of μ_{max} is reached. The value can be determined if μ_{max} is known.

$$\mu = \mu_{max} \frac{S}{K_S + S} \tag{2.4}$$

μ_{max} = maximal specific growth rate in h^{-1}

S = substrate concentration in g/L

A substrate concentration four times higher than the K_s will result in a μ of 80 % of the μ_{max}. Therefore, a high substrate concentration will cause a constant specific growth rate equal to the μ_{max}. Hence, the specific growth rate is an indication for substrate limitations. This relation is shown in Figure 4.

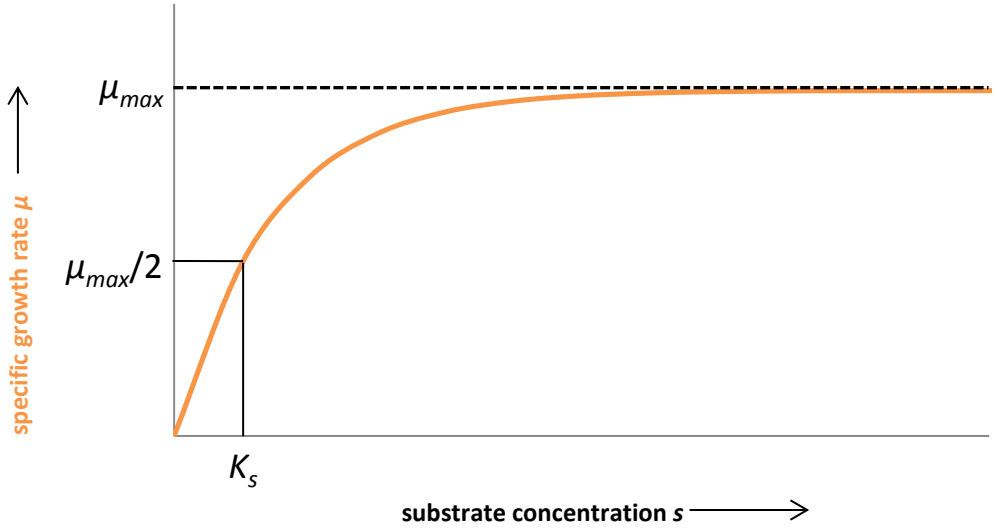


Figure 4: Plot of the Monod kinetics. The plot shows the dependence between the specific growth rate and the substrate concentration.

For economic reasons a medium should contain all components in a small excess and one component in a limiting concentration. Often the limiting component is the carbon source. To optimise the process it is important to know the ratio of the limiting component and the cell density to reach a defined cell density. This is possible by the yield coefficient for substrate ($Y_{X/S}$). It can be calculated by the growth rate (r_x) and the substrate uptake rate (r_s), shown in equation 2.5 (Chmiel, 2006). The substrate uptake rate is the reduction of the substrate concentration over the time.

$$Y_{X/S} = \left|\frac{r_x}{r_s}\right| \tag{2.5}$$

$Y_{X/S}$ = yield coefficient for substrate in $g_{WCW}/g_{Substrate}$
r_s = substrate uptake rate in $g_{Substrate}/(L \cdot h)$

This equation can be simplified to:

$$Y_{X/S} = \frac{cell\ growth}{substrate\ uptake} = \frac{\Delta X}{\Delta S} \tag{2.6}$$

ΔX = differential change of the cell density in g/L
ΔS = differential change of the substrate concentration in g/L

2.3.1 Batch cultivations

During a batch cultivation no compounds are added and no cultivation broth is removed (Chmiel, 2006). The volume is equal (see Figure 5). Only correction fluids like acid, base or antifoam are added

during the process. Therefore, a simple and reliable cultivation strategy is achieved. The medium contains all necessary nutrients at the inoculation.

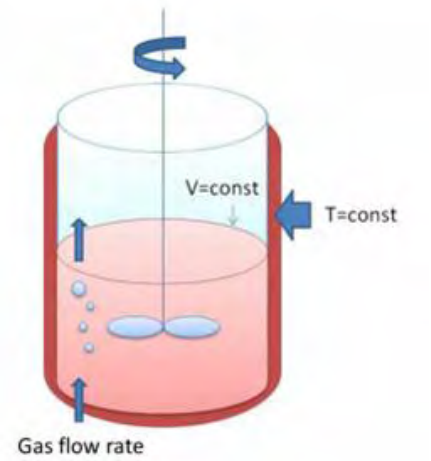


Figure 5: Schematic process description of a batch process (Sartorius Stedim Biotech)

Figure 6 shows the characteristics of the cell density and substrate concentration in a batch cultivation. The cell growth can be separated into four phases. The lag phase is characterised by the absence of cell growth. The cultivation conditions and nutrient concentrations for the cells are changing after the inoculation and the cells have to adopt and express new enzymes for the metabolism of the nutrients. The lag phase is followed by the log or exponential growth phase. In this phase the maximal increase of the cell density in the culture takes place and the cells are growing at μ_{max}. The characteristics of the cell density can be described with equation 2.4. A control of the μ during a batch cultivation is not possible. After this phase, the growth stops in the stationary phase, because a medium component becomes limiting. The cells use their energy reserve. If the reserves are depleted, the cells die and no cell growth occurred. This phase is called death phase.

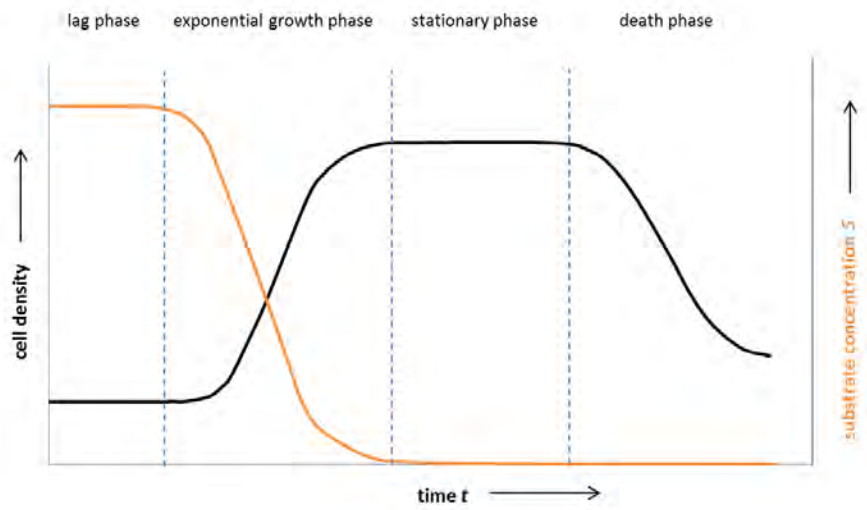


Figure 6: Characteristics of the cell density (black) and substrate concentration (orange) during a batch cultivation. The growth can be separated into four phases: Lag phase, exponential growth phase, stationary phase and death phase.

The cell density at the end of the exponential growth can be calculated with the substrate concentration (equation 2.6). The maximal cell density is limited, because substrate concentrations cannot be increased unlimited. If the substrate concentration exceeds a value, the substrate is inhibiting the cell growth. This is called substrate inhibition (Chmiel, 2006). Furthermore, toxic by-products can accumulate in the medium and inhibiting growth. Therefore, the achievable cell densities in batch cultivations are limited.

2.3.2 Fed batch cultivations

In fed batch cultivations substrates are added during the cultivation and no media is removed. Therefore, the volume increases during the cultivation (see Figure 7). Due to the addition of substrates, it is possible to control the growth rate of the culture in a fed batch process (Chmiel, 2006). This feature is important to extent the growth phase and increase the cell density. Furthermore, oxygen limitations at high cell densities can be avoided, due to lower specific growth rates.

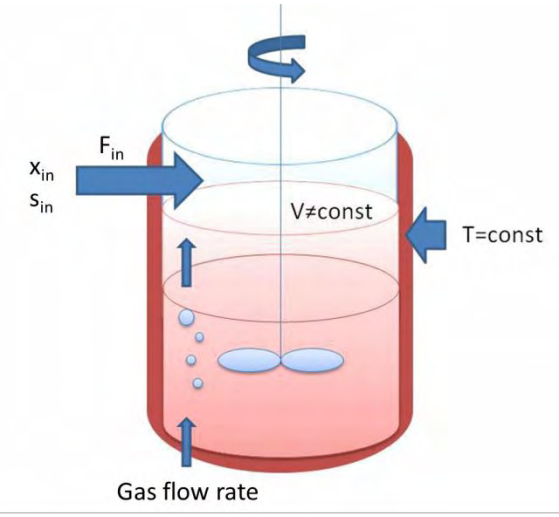


Figure 7: Schematic process description of a fed batch process (Sartorius Stedim Biotech)

The specific growth rate is dependent on the flow rate, due to the substrate supplementation. The flow rate (*F*) is the change of the volume over the cultivation time (see equation 2.7).

$$F = \frac{dV}{dt} \tag{2.7}$$

F = flow rate in L/h
V = Volume in L

For the determination of the volume at a specific cultivation time *t*, the flow rate *F* is integrated over the cultivation time and added to the initial volume (see equation 2.8).

$$V(t) = V_0 + \int_{t=0}^{t} F \cdot dt \tag{2.8}$$

V(t) = Volume at the feed time *t* in L

The fed batch processes in this work were carried out with an exponential feeding strategy. Equation 2.9 shows the calculation of the exponential increasing flow rate at a specific cultivation time (Lee, 1996). With the exponential increase of the flow rate a fixed specific growth rate (μ_{set}) is possible.

$$F(t) = \left(\frac{\mu_{set}}{Y_{X/S}}\right) \cdot \frac{X_0 V_0}{S_{feed}} \cdot e^{\mu_{set} \cdot t} \tag{2.9}$$

μ_{set} = fixed value of the specific growth rate in h^{-1}
X_0 = cell density at feed start in g/L
V_0 = initial filling volume in L
S_{feed} = substrate concentration in the feed solution in g/L

Analogue to equation 2.8, equation 2.9 can be integrated. Therefore, the volume at a specific cultivation time can be determined in equation 2.10.

$$V = V_0 + \int_{t=0}^{t} \left[\left(\frac{\mu_{set}}{Y_{X/S}} \right) \cdot \frac{X_0 V_0}{S_{feed}} \cdot e^{\mu_{set} \cdot t} \right] dt = V_0 + \left[\frac{X_0 \cdot V_0}{Y_{X/S} \cdot S_{Feed}} (e^{\mu_{set} \cdot t} - 1) \right] \quad (2.10)$$

Equation 2.6 shows the linear correlation between the uptake of the substrate and the cell density. Therefore, the cell density has to be increase exponentially if the substrate supplementation increases exponentially. The parameters cultivation volume, flow rate, cell density and specific growth rate in the fed batch phase are shown in Figure 8. The volume is increasing exponentially, due to the exponential increase of the flow rate. The specific growth rate is constantly at μ_{set}, while the cell density increases exponentially.

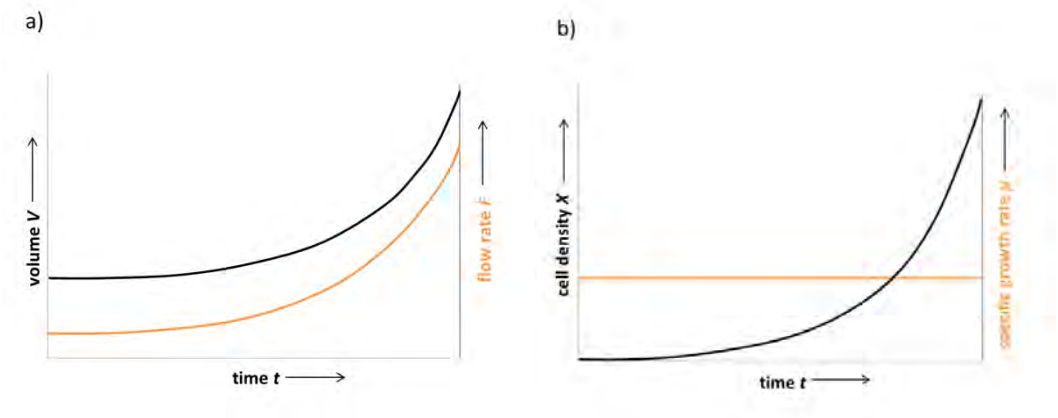


Figure 8: Characteristics for the continuous supplementation of substrate with an exponential feed profile, a) dependency between flow rate (orange) and volume (black), b) dependency between cell density (black) and specific growth rate (orange).

2.4 Oxygen transfer rate and oxygen uptake rate

Many bioprocesses are carried out aerobically. Oxygen is consumed for the cell growth. Therefore, the oxygen concentration in the cultivation broth is an important parameter. Oxygen has only a low solubility in water (Chmiel, 2006). A continuous supplementation of air or pure oxygen is necessary during the cultivation process. Several resistances have to overcome from the gas flow to the oxygen reaction side in the cell. They are shown in Figure 9.

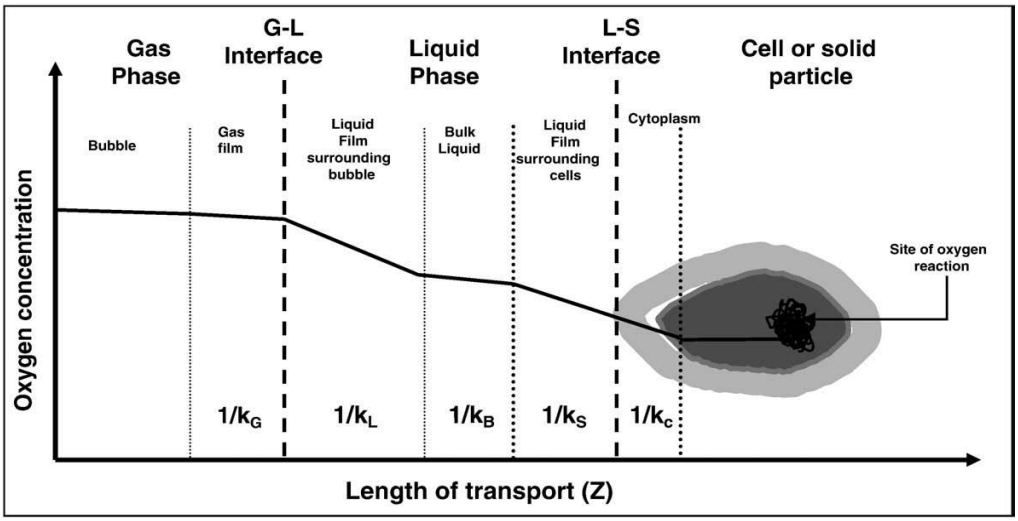


Figure 9: Steps and resistances for oxygen transfer from gas bubble to cell. $1/k_G$: Resistance from the gas bubble to the gas-liquid interface. $1/k_L$: Resistance from the gas/liquid interface to the liquid. $1/k_B$, $1/k_S$, $1/k_C$: Resistances during the oxygen transfer from the bulk liquid into the cell (Garcia-Ochoa et al., 2009).

The first and limiting resistance is the interface between the gas phase and the cultivation broth, which can be described by the two film model (Garcia-Ochoa et al., 2009). This model assumes that two films are present at each side of the gas-liquid interface. These films allow mass transfer by diffusion. The oxygen concentration is in equilibrium to the correspondent phase (Chmiel, 2006). Hence, a concentration difference between both films is present. At the interface, between the two films, equilibrium is present and the oxygen concentration in the liquid can be described by the Henry law (see equation 2.11). This concentration is dependent on the pressure and the oxygen mole fraction.

$$C_L{}^* = \frac{x_i \cdot P}{H} = \frac{p_i}{H} \tag{2.11}$$

C_L* = oxygen saturation concentration in mmol/L
x_i = oxygen mole fraction
P = pressure in Pa
H = Henry coefficient in (Pa·L)/mmol
p_i = partial pressure in Pa

The Henry coefficient describes the saturation concentration of a gas in a liquid phase. It depends on the temperature and the medium composition. An increase of the temperature increases the Henry coefficient and decreases therefore the solubility of oxygen in the cultivation broth (Chmiel, 2006). The cultivation temperature in a cultivation process depends on the optimum of the cultivated organism or the protein expression. Hence, the cultivation temperature can be seldom optimised for the oxygen solubility. Two constructive strategies for the increase of the oxygen saturation concentration are possible. According to the Henry's law an increase of the pressure will increase the

oxygen saturation concentration. This strategy is suitable for reusable stainless steel reactors, which can be pressurised during the cultivation. Polymer films used in single use systems are pressure sensitive and cannot pressurised during the cultivation. The second strategy, which is used for single use systems is the increase of the oxygen mole fraction in the aeration gas by supplementation of pure oxygen.

The oxygen transfer into the cultivation broth is described by the oxygen transfer rate (*OTR*). It depends on the volumetric mass transfer coefficient (k_La) and the oxygen concentration difference (C_L*-C_L) between the aeration gas and the cultivation broth (see equation 2.12).

$$OTR = \frac{dC}{dt} = k_L a \cdot (C_L{}^* - C_L) \tag{2.12}$$

OTR = oxygen transfer rate in mmol/(L·h)

k_La = volumetric mass transfer coefficient in h^{-1}

C_L* = oxygen saturation concentration in mmol/L

C_L = dissolved oxygen concentration in mmol/L

The volumetric mass transfer coefficient is a product of the mass transfer coefficient k_L and the transfer area *a*. Due to the difficulty to determine both values separately, the product k_La is measured (Garcia-Ochoa et al., 2009). The k_La is influenced by the residence time of the gas in the cultivation broth, which is influenced by the gas flow rate and the stirrer configuration. The transfer area *a* is affected by the bubble diameter and the height to diameter ratio in stirred systems or the surface area in rocking motion systems. The dissipated energy affects both values k_L and *a* (Garcia-Ochoa et al., 2009). Typically the dissipated energy is higher in reusable systems, due to the higher agitation rate.

The oxygen concentration difference (C_L*-C_L) is the driving force. An increase of the oxygen saturation concentration increases the driving force and enhances therefore the *OTR*.

Microorganisms metabolise oxygen for aerobe growth. Therefore, a yield coefficient for oxygen can be calculated. This yield coefficient for oxygen is defined analogue to the yield coefficient of substrate (see equation 2.13) (Chmiel, 2006).

$$Y_{X/O_2} = \left|\frac{r_x}{r_{O_2}}\right| \tag{2.13}$$

Y_{X/O_2} = yield coefficient for oxygen in g_{WCW}/mmol

r_{O_2} = oxygen uptake in mmol/(L·h)

If the specific growth rate is divided by the yield coefficient for oxygen the specific oxygen uptake rate is determined. It describes the oxygen demand of one gram wet cell weight (WCW) per hour (see equation 2.14).

$$q_{O_2} = \frac{\mu}{Y_{X/O_2}} \quad (2.14)$$

q_{O_2} = specific oxygen uptake rate in mmol/(g_{WCW}·h)

The oxygen uptake rate (*OUR*) of a culture can be determined if the cell density is known (see equation 2.15). At a static pO_2 during the cultivation the *OTR* and the *OUR* are equal.

$$OUR = q_{O_2} \cdot X \quad (2.15)$$

OUR = oxygen uptake rate in mmol/(L·h)

$$OTR = OUR \quad (2.16)$$

Aerobe cultivation conditions should be ensured during the whole cultivation. Therefore, the *OTR* of a system has to be higher than the *OUR* of the cells for growth without oxygen limitations. For the process optimisation the determination of a maximal possible cell density for aerobe growth is important. With the *OTR* of a system the maximal cell density at a specific growth rate can be determined. Generally a higher μ increases the *OUR*. Therefore, higher cell densities at a lower μ are possible.

2.5 Cryo cultures

The process of freezing and thawing is a way to preserve living cells over long time. Temperatures of -80 °C are reached in freezers for lab use. Furthermore, liquid nitrogen can be used for freezing. Often small volumes of approximately 1 mL are stored for the inoculation of seed cultures.
During the freeze process different freezing rates can occur in the cultivation broth. The cultivation broth is a suspension of medium and microorganisms. Between the cells and the medium different ionic strengths were present. The cytoplasm of the cells has a higher concentration of ions and other substances. Therefore, the medium around the cells is freezing first at low cooling rates (5 °C/min), which are achieved in lab freezers. In consequence, the concentration of substances will increase in the unfrozen parts of the medium, inducing an osmotic pressure to the cell. The cell loses water to the surrounding medium and dehydrates. Furthermore, intracellular crystallisation can cause cell death (Dumont et al., 2004). This issue normally occurs at higher freezing rates as reached in lab freezers
Crystallisation of the medium and intracellular crystallisation can be reduced by the use of cryoprotective additives. Glycerol is often used as cryoprotective agent and reduces the freezing point of water and biological fluids. Therefore, the osmotic pressure is decreased, due to the reduced salt concentration in the unfrozen medium. Furthermore, glycerol is able to enter the cell and prevent the cells from negative crystallisation effects (Hubálek, 2003)

3 Material and methods

3.1 BIOSTAT® D-DCU 10-3

For the evaluation of the chemically defined medium fermentations in a stainless steel stirred tank reactor were carried out. A BIOSTAT® D-DCU 10-3, which is in-situ sterilisable was used for the cultivations. Due to the cylindrical shape with a height to diameter ratio of 3:1, the reactor is suitable for microbial cultivations (Sartorius, 2011 a). For the temperature control a double wall was used, which was supplied with cooling water. The system belongs to a reactor family with filling volumes between 10 L and 200 L. A supply system, a vessel and a digital control unit (DCU) is part of the system. The maximal filling volume is 10 L, the total volume is 1.5 times of the filling volume. At the bottom of the reactor vessel the stirrer device is located Figure 10 shows the reactor vessel and the DCU.

Figure 10: BIOSTAT® D-DCU 10-3, reactor vessel and DCU

Table 1 and Figure 11 show the detailed configuration with 2 x 6-blade disk impellers, which were utilized for the cultivation. The stirrer shaft is sealed by a double mechanical seal. Hence, contaminations of the cultivation broth or the environment should be avoided. Four baffles were installed inside the cultivation chamber. Aeration was realised by a ring sparger with 11 holes and a hole-diameter of 1.3 mm. Supplementation of pressurized air and pure oxygen is possible. For this configuration and an aeration rate of 1 vvm air without overpressure, a k_La value of 308 h^{-1} was determined with the gassing out method (Sartorius internal data). Inlet and exhaust gas were sterile

filtrated by filter cartridges. The reactor is equipped with classical electrodes for the measurement of pH and pO_2. The level of dissolved oxygen in the cultivation broth is controlled by the multistage cascade of the system. Stirrer speed, air gas flow rate and oxygen gas flow rate were the modifiable parameter for the control of the dissolved oxygen level. The temperature was controlled at the set point by the DCU. It is possible to detect foam during cultivations by an antifoam probe. If foam is generated, antifoam solution can be added automatically. The DCU is equipped with peristaltic pumps for base, acid and antifoam and has a graphical interface via touchscreen. The DCU is implemented in the multi fermenter control system (MFCS) for control and data record purposes.

Table 1: Reactor and impeller dimensions of the BIOSTAT® D-DCU II 10-3 (Zahnow, 2011)

Dimension	**Value**
Total volume	15 L
Maximal filling volume	10 L
Reactor height (h)	561 mm
Reactor diameter (d_1)	187.7 mm
Height to diameter ratio (h/d_1)	3:1
Filling height (h_{10L})	363 mm
Height from the bottom to first impeller (h_3)	92 mm
Height between impellers (Δz)	124 mm
Impeller diameter (d_2)	75 mm
Impeller diameter to reactor diameter (d_2/d_1)	0.4
Filling volume upside of impellers (V_1)	6.4 L

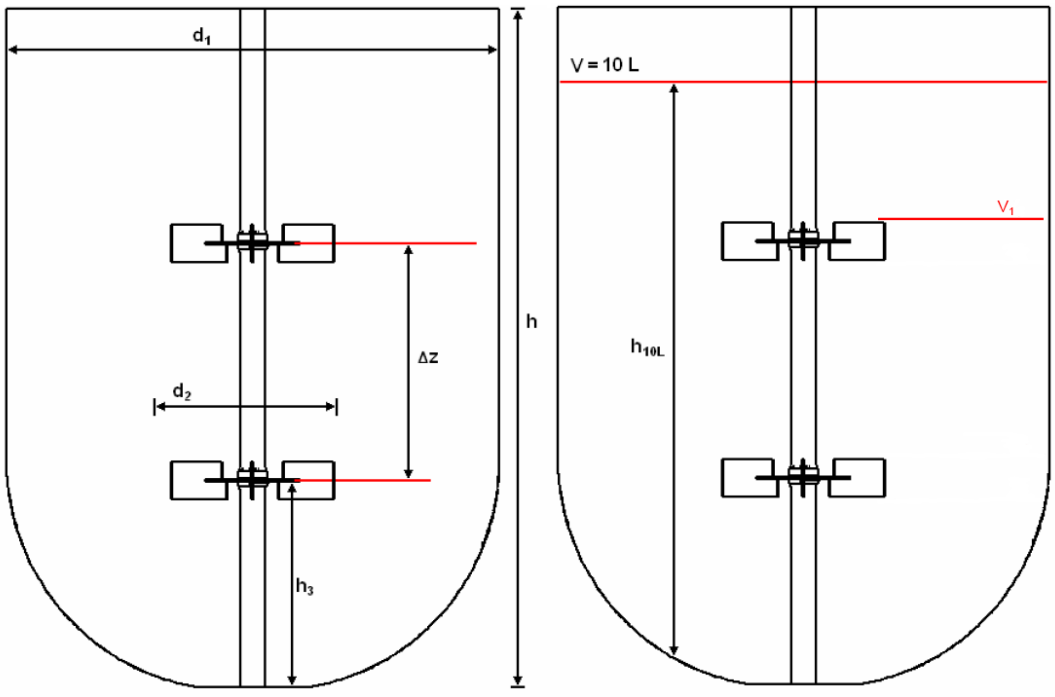


Figure 11: Drawing of the reactor and impeller dimensions of the BIOSTAT® D-DCU 10-3 (Zahnow, 2011)

3.2 BIOSTAT® RM 20 optical

Single use systems become attractive in the last decades (Eibl et al., 2009). The advantages of single use systems are the reduction of labour and time demand. Therefore, in the second part of the experiments a process transfer the single use system BIOSTAT® RM 20 optical was carried out. The agitation principle is based on an induced wave mixing process, resulting from an oscillating movement (Eibl et al., 2010). Therefore, the fluid surface is permanently renewed and bubble free aeration from the headspace is possible. This agitation principle ensures an oxygen transfer with low shear stress (Eibl et al., 2009). Hence, wave agitated mixing is suitable for plant and cell culture (Eibl et al., 2009). Furthermore, microbial cultivations (Glazyrina et al., 2010) were carried out.

The complete system consists of a DCU, a rocker unit and a presterilised two dimensional bag. A working volume between 1 and 300 L is possible in this system. The working volume is one half of the total bag volume. Different versions of the system are available: A basic system for processes with low requirements of the parameter control, a system with optical sensors and additionally perfusion systems for continuous cultivations. The used system has optical sensors and is shown in Figure 12. In this work the second version of the system was used.

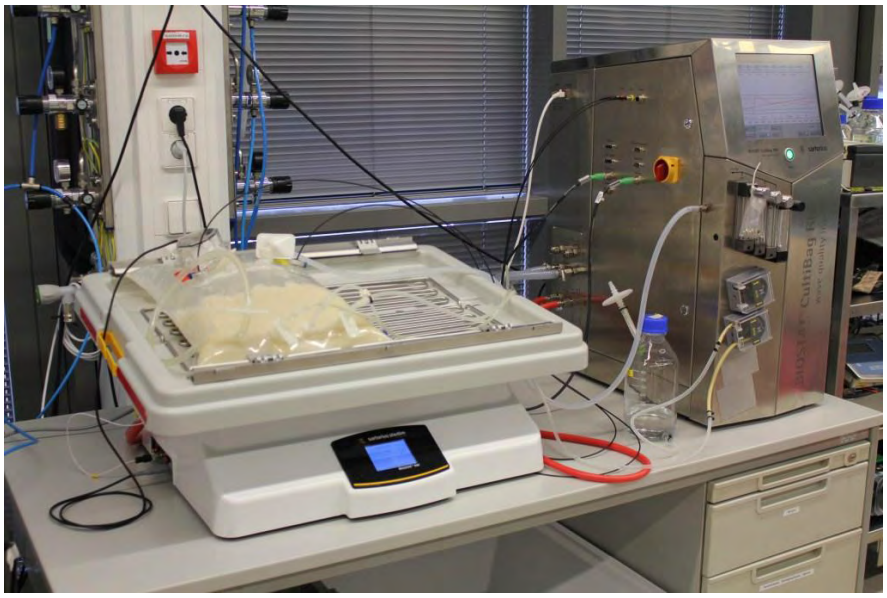


Figure 12: BIOSTAT® RM 20 optical during a *Pichia pastoris* cultivation

The rocker provides the agitation by rocking motion. This rocking motion is generated by a servomotor, which provides an electronic angle setting. The angle is adjustable between 4 and 10 °.The bag is the only part in contact of cultivation broth and is composed of different polymeric layers. It is sterilized by gamma irradiation and fully equipped with ports, tubes and sensors. Inlet and exhaust air flows are passing through sterile filters. Filter heaters can prevent filter blockage, by removing humidity from the filter membrane. The optical sensors are preinstalled and have to be connected with the fibre optic. In addition there are connectors for supplementation of media or feed, taking sample and addition of base or acid. The bag is shown on the rocker tray in Figure 13.

The DCU provides mass flow controllers for air, oxygen, nitrogen and carbon dioxide. Parameters like the angle of the tray, the rocking rate and the aeration rate are controlling the oxygen transfer rate and can be modified by the DCU. Furthermore, pumps for base and acid and digital control loops for the process parameters are implemented in the system. The communication between the DCU and the rocker works in both directions. This enhances the process safety. The DCU has a full graphical interface via touch screen. The system is integrated in the MFCS.

Air and pure oxygen can by supplied to the bag for headspace aeration. For the cultivations a CultiBag® RM 10 L was used. The recommended maximal aeration rate is one half of the total bag volume per minute. Therefore, a gas flow rate of 5 Lpm was applied to the bag. The used configuration with a working volume of 5 L results in a maximal k_La of 45 h^{-1}, determined with the gassing out method (Sartorius internal data). Optical sensors are used for the determination of pO_2 and pH. A full automated feedback control ensures a precise control of pO_2, pH and temperature. The temperature in the cultivation chamber is determined by a Pt100 sensor and adjusted to the set point by a heating/cooling coil in microbial cultivations. Additionally, a heating mat is available for cell culture applications. A reliable temperature control is important at high cell densities and therefore a cooling

unit is used, to tempering the water in the cooling coil. 10 °C is the minimal adjustable temperature in the cooling circuit.

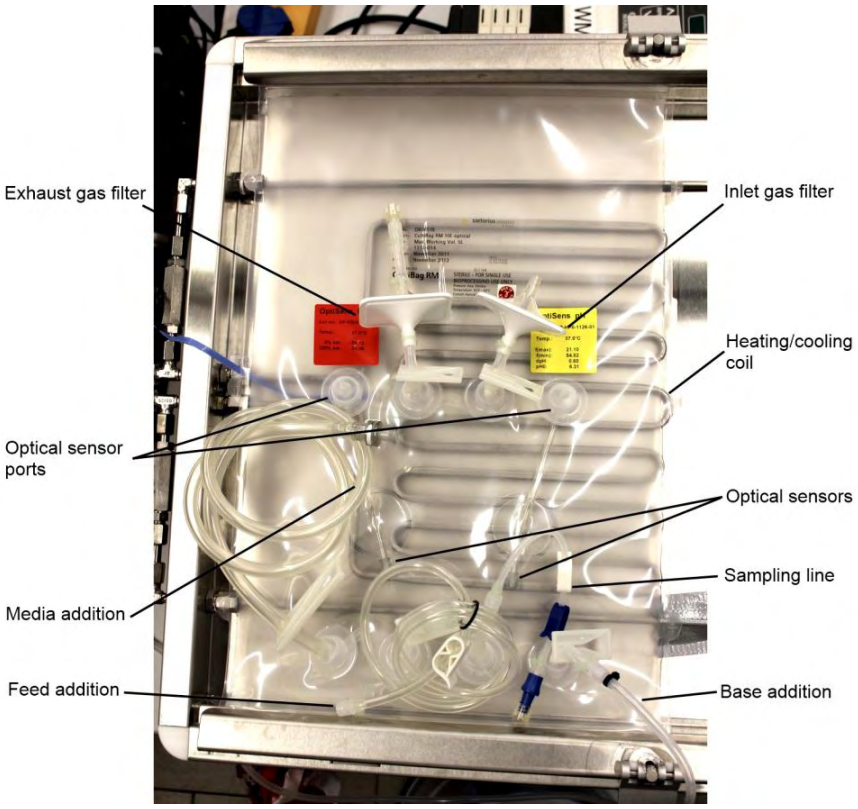


Figure 13: CultiBag® RM 10 L optical, tray of the rocker.

3.3 Preparation of media and solutions

3.3.1 Preparation of 40 % glucose stock solution

The 40 % glucose stock solution was used for the preparation of the YPD agar and medium. It serves as the main carbon source. The composition is listed in Table 2.

Reverse osmosis water (RO-water) was heated in a glass beaker on a magnetic stirrer and glucose was transferred. The solution was mixed until the glucose is completely solved. A Schott bottle was filled with the solution and autoclaved at 121 °C for 20 min (1). Afterwards, it was cooled down to room temperature and the solution was filled up to the corresponding volume with sterile RO-water under sterile conditions (13). The solution was stored at 4 °C.

Table 2: Composition of 40 % glucose stock solution

Chemical	Amount in g/L	Vendor/Article number
D (+) – Glucose (water free)	400	Merck, 1.08337.1000

3.3.2 Preparation of YPD agar

Single colonies were needed for the inoculation of the first step of the seed train. The agar served as medium for a cryo culture of *P. pastoris* and the growth of these colonies. The compounds of the agar are listed in Table 3.

Table 3: Composition of YPD-agar

Chemical	Amount	Vendor/Article number
Bacto peptone	20 g/L	Becton Dickinson (BD), 211677
Yeast extract	10 g/L	Roth, 2363.2
Agar-agar	20 g/L	Roth, 2266.2
40 % glucose stock solution	50 mL/L	See Table 2

Agar-agar, bacto peptone and yeast extract were weighed (5) into a Schott bottle. RO-water was transferred into the bottle and the compounds were solved. Afterwards, the solution was autoclaved at 121 °C for 20 min (1) and cooled down to approximately 60 °C in a water bath (39). 40 % glucose stock solution was added under sterile conditions (13). Afterwards, the plates were prepared. After solidification for approximately 12 h the plates were stored at 4 °C.

3.3.3 Preparation of YPD medium

The YPD medium was used for the first seed culture of the seed train. It was inoculated from the agar plate and served as inoculum for the second seed culture. The composition is shown in Table 4.

Table 4: Composition of YPD-medium

Chemical	Amount	Vendor/Article number
Bacto peptone	20 g/L	Becton Dickinson (BD), 211677
Yeast extract	10 g/L	Roth, 2363.2
40 % glucose stock solution	50 mL/L	See Table 2

For the preparation bacto peptone and yeast extract were weighed (5) into a 1 L Schott bottle. RO-water was transferred into the bottle and the compounds were solved. Afterwards, the solution was autoclaved at 121 °C for 20 min (1) and cooled down to room temperature. Sterile 40 % glucose stock solution was added under sterile conditions (13). The medium was stored at 4 °C.

3.3.4 Preparation of biotin stock solution

The supplementation of biotin to the chemical defined medium is necessary for the cultivation of *P. pastoris*. The components are listed in Table 5.

Table 5: Composition of biotin stock solution

Chemical	Amount in g/L	Vendor/Article number
D(+)-Biotin	0.2	Roth, 3822.1

For the biotin stock solution D(+)-biotin was weighed (3) and solved in RO-water. The dissolving needs approximately 1 h. Afterwards, the solution is sterile filtrated by a 0.2 µm poly ether sulfone (PES) membrane bottle top filter into a sterile vessel. The solution was stored at 4 °C.

3.3.5 Preparation of PTM solution

As trace salt solution in the chemically defined medium (see Table 7), PTM solution was used. It was added sterile after the sterilisation of the medium. Reactions during the autoclaving or losses of substances during the sterile filtration should be avoided by this procedure. The composition of the PTM solution is shown in Table 6.

Table 6: Composition of yeast trace metal (PTM) solution (Zhang et al., 2007)

Chemical	Amount in g/L	Vendor/Article number
$CuSO_4 \cdot 5\ H_2O$	6.0	Roth, P024.1
NaI	0.08	Roth, A134.3
$MnSO_4 \cdot 2\ H_2O$	3.0	Roth, 4487.1
$Na_2MoO_4 \cdot 2\ H_2O$	0.2	Merck, 1.06521.0100
H_3BO_3	0.02	Roth, 6943.2
$CoCl_2 \cdot 6\ H_2O$	0.916	Merck, 1.02539.0100
$ZnCl_2$	20.0	Roth T887.1
$FeSO_4 \cdot 7H_2O$	65.0	Roth, P015.1
H_2SO_4	5.0	Merck, 1.00732.2500

For the PTM solution the components were weighed (3, 5) and transferred into a Schott bottle with RO-water in the order of the table. The solution was mixed by using a magnetic stir bar. The

components were not completely soluble until the sulphuric acid was added. The solution was filled up with RO-water to the desired volume. Afterwards, the solution was autoclaved at 121 °C for 20 min (1), cools down and was stored at 4 °C.

3.3.6 Preparation of chemically defined medium

The chemically defined medium was used for the main cultivations in the BIOSTAT® D-DCU 10-3, the BIOSTAT® RM 20 optical and the second seed culture of the seed train (with reduced glycerol concentration). The medium provides a high concentration of basic elements (Cos et al., 2006). For the cultivation in the RM system the magnesium sulphate concentration was reduced to avoid crystallisation in the medium. The composition of the medium is shown in Table 7.

Table 7: Composition of chemically defined medium (Zhang et al., 2007)

Chemical	**Amount**	**Vendor/Article number**
$C_3H_8O_3$	Seed culture: 20 g/L Batch culture: 60 g/L	Roth, 7530.4
KH_2PO_4	42.9 g/L	Roth, 3904.1
$(NH_4)_2SO_4$	5.0 g/L	Roth, 9218.2
$CaSO_4 \cdot 2H_2O$	1.0 g/L	Roth, P714.1
K_2SO_4	14.3 g/L	Roth, X889.3
$MgSO_4$ (water free)	D-DCU: 11.2 g/L RM: 5.7 g/L	Riedel-de Haën, 13143
PTM solution	4.56 mL/L	See Table 6
Biotin stock solution	4.56 mL/L	See Table 5

For the preparation of the medium glycerol, monopotassium phosphate, ammonium sulphate, calcium sulphate dihydrate, potassium sulphate and magnesium sulphate were weighed (5). All components were dissolved in RO-water. Medium for seed culture was autoclaved at 121 °C for 20 min (1). The medium for the main culture was sterile filtrated by a Sartopore® Platinum BH4 (0.2 µm + 0.45 µm) directly into the cultivation chamber. After the sterilisation PTM solution and biotin stock solution were added under sterile conditions (13). The medium have a high turbidity after the addition of the PTM solution.

3.3.7 Preparation of glycerol feed solution

The glycerol feed solution was used in the fed batch phase as a source of substrate. It was supplemented continuously to the cultivation chamber. The composition of the glycerol feed solution is shown in Table 8.

Table 8: Composition of the glycerol feed solution

Chemical	Amount	Vendor/Article number
Glycerol	630 g/L	Roth, 3822.1
Biotin stock solution	12 mL/L	See Table 5
PTM solution	12 mL/L	See Table 6

Glycerol was weighed and diluted in RO-water. Afterwards, it was autoclaved (1) at 121 °C for 20 min in a Schott bottle. The biotin stock solution, the PTM solution and sterile RO-water were added up to the required volume under sterile conditions (13). The solution was stored at 4 °C.

3.3.8 Preparation of methanol feed

The methanol feed solution was used for the induction of the protein expression. It was supplemented continuously. The compounds of the methanol feed are listed in Table 9.

Table 9: Composition of the methanol feed solution

Chemical	Amount in mL/L	Vendor/Article number
Methanol	976	Roth, 8388.3
Biotin stock sol.	12	See Table 5
PTM solution	12	See Table 6

Due to the high methanol concentration the solution is regarded as auto sterile. Methanol was filled into a Schott bottle. Biotin stock solution and PTM solution were added. The methanol feed was stored in a solvent cabinet at room temperature.

3.3.9 Preparation of antifoam solution

Antifoam solution was automatically added to reduce the foaming in the stainless steel system BIOSTAT® D-DCU 10-3. The composition of the antifoam solution is shown in Table 10.

Table 10: Composition of antifoam solution

Chemical	Amount in g/L	Vendor/Article number
Antifoam 204	100	Sigma, A6426-500G

Antifoam 204 was autoclaved at 121 °C for 20 min (1) and cooled down to room temperature Antifoam 204 was 1 to 10 diluted with sterile RO-water under sterile conditions. The solution was stored at 4 °C.

3.3.10 Preparation of Laemmli buffer

Laemmli buffer was used as sample buffer for SDS-PAGE. It ensures optimal band resolution. The composition of the buffer is shown in Table 11.

Table 11: Composition of Laemmli buffer

Chemical	Amount	Vendor/Article number
Tris-HCl, pH 6.8	62.5 mM	Bio Rad, 161-0737
Glycerol	250 mg/mL	
SDS	20 mg/mL	
Bromophenol blue	0.2 mg/mL	
β-mercaptoethanol	50 mg/mL	Roth, 4227.3

Laemmli buffer prepared by Bio Rad was used. The solution was pre-mixed. Only β-mercaptoethanol was added before the sample preparation.

3.3.11 Preparation of 0.9 % sodium chloride solution

The sodium chloride solution was used for the dilution of the OD_{600} samples.

Table 12: Composition of 0.9 % sodium chloride solution

Chemical	Amount in g/L	Vendor/Article number
NaCl	9.0	Roth, 9265.1

Sodium chloride was weighed (5) and solved in RO-water. Afterwards it was autoclaved at 121 °C for 20 min (1). The solution was stored at room temperature.

3.4 Seed culture preparation

3.4.1 Seed train

Multiple steps are necessary to produce enough seed culture volume for the inoculation of the main culture. Every further step, which is shown in Figure 14, has an increased cultivation volume. For the first step of the seed train, a cryo preserved vial was thawed at room temperature. Parts of the suspension were distributed on an YPD agar plate under sterile conditions with an inoculating loop. The agar-plate was incubated at 30 °C for 48 h (19).

For the first seed culture, 20 mL of YPD-medium were transferred into an 100 mL Erlenmeyer flask with baffles under sterile conditions. The culture was inoculated with a cell colony of the YPD agar plate and incubated in the shaking incubator (11) at a shaking rate of 150 rpm and an orbit diameter of 50 mm at 30 °C for 24 h.

200 mL of chemically defined medium with a glycerol concentration of 20 g/L were transferred into a 1 L Erlenmeyer flask with baffles under sterile conditions for the second seed culture. This culture was inoculated with the first seed culture with an initial OD_{600} of 0.4 (see equation 3.1). The incubation was performed in the shaking incubator (11) at a shaking rate of 150 rpm at 30 °C for 24 h. The orbit diameter was 50 mm. Parts of this second seed culture were used to inoculate the main culture.

The main culture was inoculated with an OD_{600} of 0.5. Equation 3.1 determined the corresponding volume for the seed culture. The seed culture was transferred under sterile conditions to the cultivation chamber.

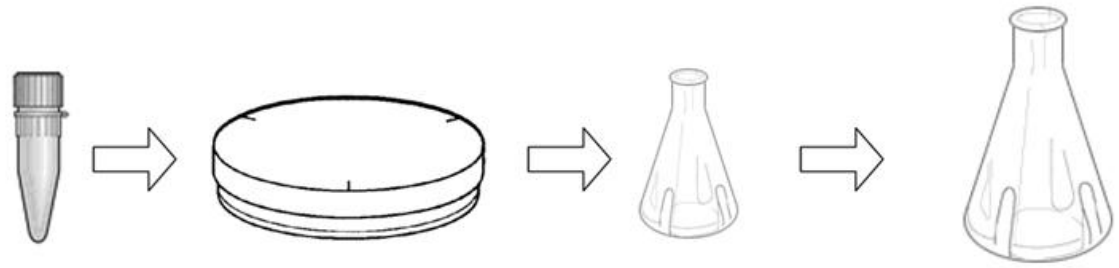
$$V_{Seed} = \frac{OD_{600,Start}}{OD_{600,Seed}} V_0 \tag{3.1}$$

V_{Seed} = transferred volume of the seed culture in L

V_0 = start volume of the cultivation system in L

$OD_{600,Start}$ = initial optical density

$OD_{600,Seed}$ = optical density of the seed culture



Cryo preserved vial | YPD-Agar-plate | First seed culture | Second seed culture

Figure 14: Steps for the preparation of the seed train (Sartorius Stedim Biotech)

3.4.2 Cryo cultures (-80 °C)

The preparation of the seed train is very time-consuming. Therefore, a new method for seed culture preparation should be established. *P. pastoris* was cultivated in a batch culture. The cultivation broth was transferred in a sterile Schott bottle under sterile conditions (13). As cryo protective agent 5 % glycerol was added to the cultivation broth to protect the cells from ice crystals and osmotic stress during the freeze process. The OD_{600} of the culture was determined after the addition of glycerol (30). 40 mL of the cultivation broth with glycerol was converted to 50 mL falcon tubes and freezed at -80 ° C (17). For inoculation the falcon tubes were thawed in a water bath (39) and parts were used to inoculate the main culture. Equation (3.1) was used for the volume determination.

3.5 Cultivation parameters

3.5.1 Cultivation parameters for the BIOSTAT® D-DCU 10-3

For the evaluation of the medium for *P. pastoris* HCDC a glycerol fed batch cultivations was carried out in the BIOSTAT® D-DCU 10-3. An exponential glycerol feed profile was used for the fed batch phase. Cultivations at comparable parameters achieved cell densities up to 381 g_{WCW}/L (Dreher et al., 2013). A feeding strategy was used, which avoids oxygen limitations during the fed batch phase. Furthermore, overflow metabolism should be avoided. Additionally a lower μ_{set} causes a lower demand of pure oxygen during the process. Therefore, a μ_{set} of 0.15 h^{-1} was used in the fed batch phase. Oxygen limitations are improbably at this μ_{set} in the D-DCU 10-3, due to the high k_La of the system. The start volume of the cultivation system (V_0) was 7.0 L and the medium had a pH of 5.0. The pO_2 was controlled at the set point of 30 % by a multistage cascade control shown in Figure 15

The pO_2 was controlled by three parameters: The stirrer speed, the air gas flow rate and the oxygen gas flow rate. In a first phase the stirrer speed increases. In a second phase the gas flow rate for air is increased up to 10 Lpm parallel to the increase of the stirrer speed. Afterwards, the air gas flow is replaced by pure oxygen.

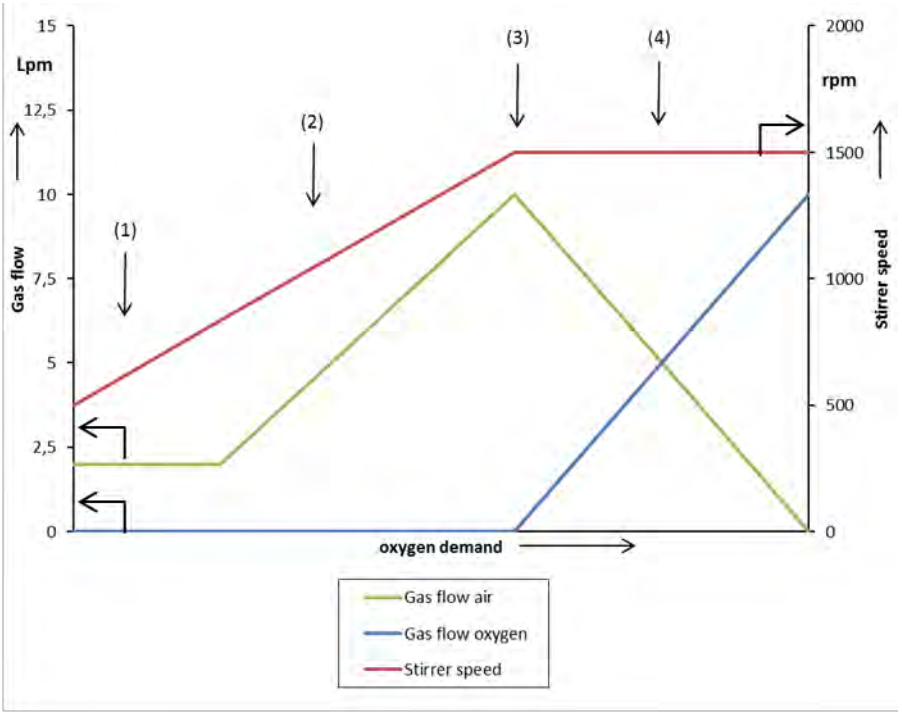


Figure 15: Multistage pO_2-control for the fed batch cultivation in the BIOSTAT® D-DCU 10-3. The cascade can be separated into 4 phases. (1) The initial stirrer speed was 500 rpm and the initial gas flow rate was 2 Lpm. With increasing oxygen demand the stirrer speed was increased. (2) The gas flow rate was increased. (3) The maximal gas flow rate was 10 Lpm and the maximum stirrer speed was 1500 rpm. (4) If necessary pure oxygen was supplied.

3.5.2 Cultivation parameters for the BIOSTAT® RM 20 optical

For the process transfer to a single use system batch cultivations were carried out in the BIOSTAT® RM 20 optical. The process was stepwise developed. The chemically defined medium and the RM-system were first tested in batch cultivations. For the batch cultivations the maximal possible cell density under aerobe conditions in the system was calculated using equation 2.12.

$$OTR_{max} = k_L a(C_L{}^* - C_L)$$

It was assumed that the maximal oxygen transfer rate (OTR_{max}) is equal to the maximal oxygen uptake rate (OUR_{max}), if the pO_2 value is controlled at the set point of 30 %. The OTR_{max} value is the limiting value in the RM system. Therefore, the glycerol concentration is adjusted to this value. The oxygen saturation concentration and the dissolved oxygen concentration can be calculated with by a curve fitting (Truesdale et al., 1955).

$$C_L^* = \frac{p_i}{P \cdot M} \cdot (67.75 - 1.887 \cdot T + 0.0369 \cdot T^2 - 0.000309 \cdot T^3)$$

The oxygen saturation concentration C_L* with pure oxygen is 1.13 mmol/L at 30 °C. The dissolved oxygen concentration in the medium is depending on the pO_2 set point and the oxygen mole fraction in the air. A dissolved oxygen concentration of 0.069 mmol/L is resulting.
The difference between these concentrations is the driving force of the oxygen transfer. Therefore, the OUR can be calculated with these values and the k_La given in chapter 3.2.

$$OTR_{max} = 45\mathrm{h}^{-1}\left(1.13\,\frac{\mathrm{mmol}}{\mathrm{L}} - 0.069\,\frac{\mathrm{mmol}}{\mathrm{L}}\right) = 47.75\,\frac{\mathrm{mmol}}{\mathrm{L{\cdot}h}}$$

OTR_{max} is equal to the OUR_{max}, if the pO_2 can be controlled reliable at the set point by the system.

$$OTR_{max} = OUR_{max}$$

The maximum possible cell density can be determined with the following equation 2.14 and 2.15.

$$X = \frac{OUR \cdot Y_{X/O_2}}{\mu}$$

Y_{x/O_2} was known from former cultivations. The used value is 0.43 g/mmol for wet cell weight. The specific growth rate in these cultivations was 0.24 h^{-1}. The values were determined in batch cultivations in a reusable stirred tank reactor.

$$X = \frac{47.75\frac{\mathrm{mmol}}{\mathrm{L{\cdot}h}} \cdot 0.43\frac{\mathrm{g}}{\mathrm{mmol}}}{0.24\mathrm{h}^{-1}} = 84.65\,\frac{\mathrm{g}}{\mathrm{L}}$$

For the reliability of the process the correcting factor k = 0.9 was used.

$$X_{correcture} = X_{WCW} \cdot k = 84.65\,\frac{\mathrm{g}}{\mathrm{L}} \cdot 0.9 = 76.19\,\frac{\mathrm{g}}{\mathrm{L}}$$

$X_{correcture}$ = cell density in wet cell weight corrected for process reliability in g_{WCW}/L

k = correcting factor

With the yield coefficient for glycerol, known from former cultivations, the maximum reasonable glycerol concentration was determined (see equation 2.6). The value is 2.5 $g_{WCW}/g_{substrate}$.

$$Y_{X/S} = \frac{\Delta X}{\Delta S}$$

Rearranged to the substrate concentration:

$$\Delta S = \frac{\Delta X_{WCW}}{Y_{X/S}} = \frac{76.19\frac{\mathrm{g_{WCW}}}{\mathrm{L}}}{2.5\frac{\mathrm{g_{WCW}}}{\mathrm{g_{substrate}}}} = 30.48\,\frac{\mathrm{g_{substrate}}}{\mathrm{L}}$$

Therefore, the suitable glycerol concentration in a batch process was approximately 30 g/L. This concentration was used for the batch cultivation.

In a next step the feasibility of the exponential feed was tested. For the reliability of the process the glycerol concentration was decreased to 25 g/L. The cell density at the end of the batch phase was determined with the following equation:

$$\Delta X_{WCW} = \Delta S \cdot Y_{X/S} = 25\,\frac{\mathrm{g}}{\mathrm{L}} \cdot 2.5\,\frac{\mathrm{g_{DCW}}}{\mathrm{g_{substrate}}} = 65.00\,\frac{\mathrm{g_{WCW}}}{\mathrm{L}}$$

The μ_{set} was 0.07 h^{-1} in all glycerol fed batch phases in the RM. The possible cell density maximizes, if the fixed specific growth rate is low. A μ_{set} of 0.07 h^{-1} is the lowest possible value, due to limitations of the peristaltic pump. The maximal cell density was calculated with the same equation like in the batch calculation.

$$X_{WCW} = \frac{OUR \cdot Y_{X/O_2}}{\mu} = \frac{47.75\frac{\mathrm{mmol}}{\mathrm{L \cdot h}} \cdot 0.43\frac{\mathrm{g}}{\mathrm{mmol}}}{0.07} = 293.30\,\frac{\mathrm{g_{WCW}}}{\mathrm{L}}$$

The maximum reachable cell density is therefore 293.30 g_{WCW}/L during a fed batch cultivation in the BIOSTAT® RM 20 optical.

At last two induced fed batch cultivations were carried out to determine the ability of protein expression. A constant methanol feed with a transition phase for the adaption of the cells was used in the induction phase. In the transition phase the methanol flow rate was 1.5 mL/(L·h) calculated with the start volume of the cultivation system (V_0). The transition phase lasted for 5 h. Afterwards the methanol flow rate was increased to 4 mL/(L·h) for the induction. The set point for the cultivation temperature was 30 °C in all cultivations. The start volume was different, due to the feed supplementation in the fed batch and induction phase. For optimal oxygen supply the maximal filling volume should not exceed 5 L. An overview of the carried out cultivations is given in Table 13. The used cascades are shown in Figure 16 and Figure 17.

Table 13: Cultivation parameters and set points for the cultivations in the RM-system.

	Cascade	**pH set point**	**Initial filling volume / L**
Batch cultivation	See Figure 16	5.0	5.0
Fed batch cultivation	See Figure 16	5.0	4.0
Induced fed batch cultivation	See Figure 16	5.0	3.6
Induced fed batch cultivation	See Figure 16 and Figure 17	6.0	3.7
Batch cultivation inoculated with cryo culture	See Figure 16	5.0	5.0

For all cultivations except the last induced fed batch cultivation Cascade 1 (see: Figure 16) was used. The initial air gas flow rate was changed to pure oxygen. The rocking rate was the final parameter und

was increased from 35 RPM to 42 RPM. If the rocking rate is increased at low cell densities, the cultivation broth can enter the gas filters, due to the low viscosity of the broth.

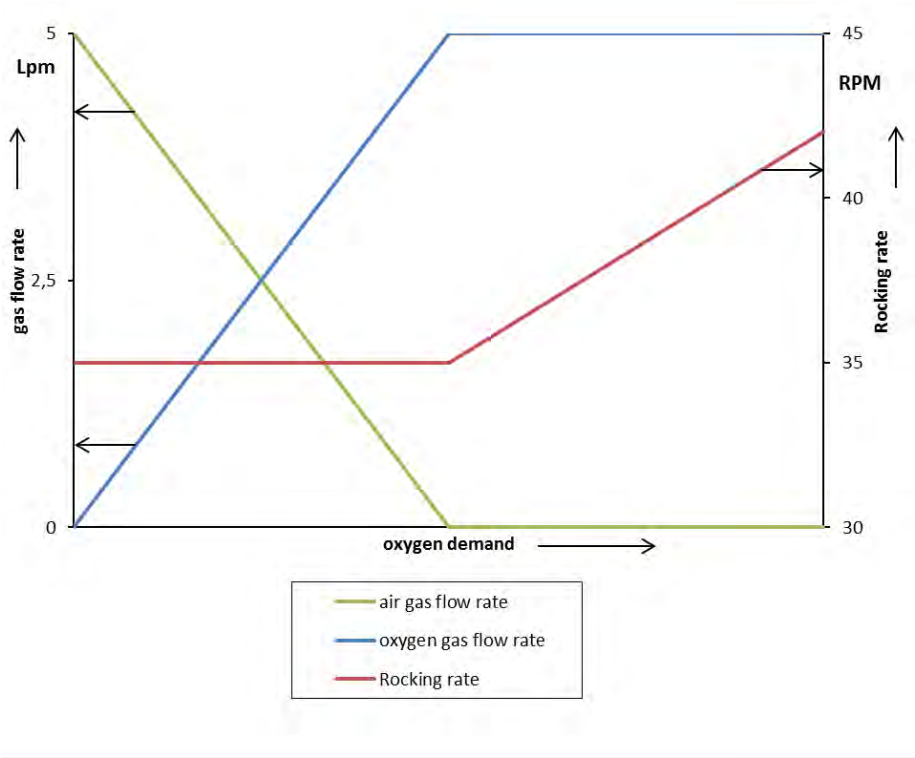


Figure 16: Cascade 1, used in the BIOSTAT® RM 20 optical. In a first step the gas flow is enriched with pure oxygen. In a second step the rocking rate is increased from 35 to 42 RPM.

A simplified cascade was used in the second induced fed batch cultivation (see Figure 16) after the batch phase. The gas flow rate was supplemented with pure oxygen identical to cascade 1. The rocking rate was fixed at a value of 42 RPM. The pO_2 was therefore only influenced by the oxygen concentration in the gas flow rate.

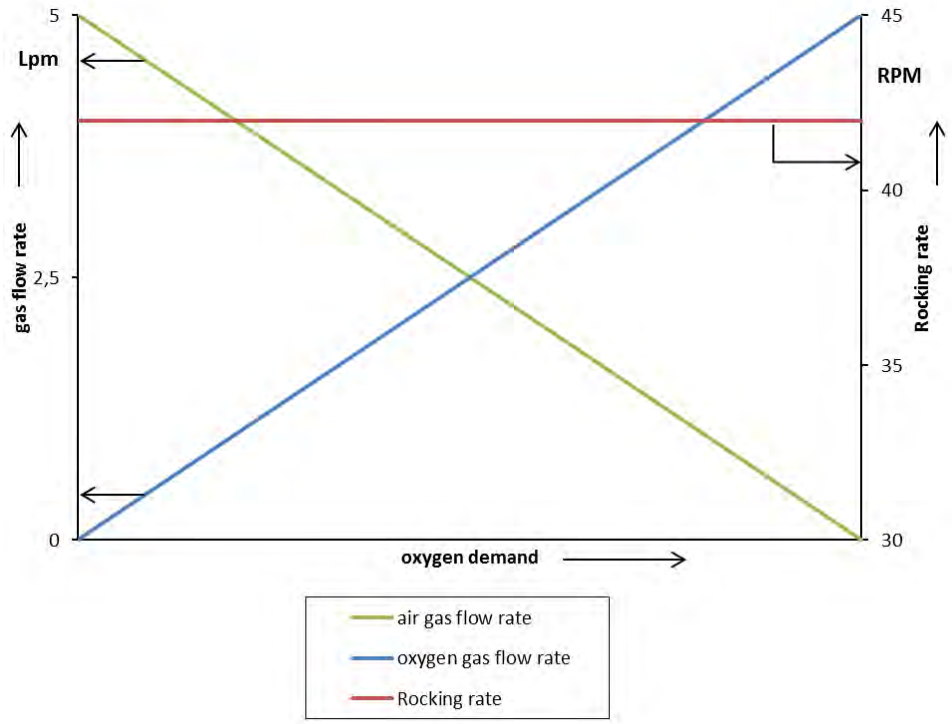


Figure 17: Cascade 2, used in the BIOSTAT® RM 20 optical at high cell densities. In contrast to cascade 1, the rocking rate is fixed to 42 RPM. The gas flow is enriched with pure oxygen.

3.6 Analytical methods

3.6.1 Determination of the optical density

The measurement of the optical density (OD_{600}) gives an indirect statement about the cell density of a cell suspension. The optical density correlates with the dry cell weight and the wet cell weight.
For its measurement single use polystyrene cuvettes with 10 mm thickness were used at a wavelength of 600 nm (30). The medium showed a high self-absorption. Therefore, the OD_{600} samples were blanked against medium. If the measured value was greater than 0.6, the sample was diluted with 0.9 % sodium chloride solution.

3.6.2 Determination of the wet cell weight

To quantify the cell density of a cell suspension the wet cell weight (WCW) was determined. 2 mL of the cultivation broth were centrifuged at 13.000 rpm for 5 min in weighed (3) 2 mL micro tubes. If high cell densities were expected, 1 mL cultivation broth was taken. The supernatant was discarded and the weight of the pellet was determined (3). The WCW measurement was carried out in duplicates.

3.6.3 Determination of the dry cell weight

Like the WCW the dry cell weight (DCW) can be used to get a quantitative statement about the cell density. The DCW was determined by using the Moisture meter (25), a balance with a built in drying function. The glass fibre fleece in the Moisture meter was tared to zero. 2 mL of the cultivation broth were centrifuged at 13.000 rpm for 5 min (10). The supernatant was discarded and the pellet was resuspended with RO-water and pipetted to the filter of the Moisture meter. The sample was dried using infrared radiation. After drying, the determined weight is the dry cell weight. The DCW measurement was carried out in duplicates.

3.6.4 Glycerol analytics

The glycerol concentration in the media was determined photometric with an enzymatic assay (r-biopharm, Cat. No. 10 148 270 035). Glycerol is phosphorylated by enzymes. As a result of this reaction, reduced nicotineamide adenine dinucleotide (NADH) is used and converted to oxidized nicotineamide adenine dinucleotide (NAD). The assay solution contains a defined amount of NADH. Only the reduced NADH form, not the NAD form, shows a light absorption at 340 nm. Therefore, the reduction of the absorption correlates to the glycerol concentration in the solution.
For the glycerol samples, the fermentation broth was filtrated with a 0.2 µm Minisart to remove cells and other particles. The sample volume of 50 µL was mixed with 500 µL solution 1 and diluted with 950 µL RO-water in a single use cuvette. After the addition of 5 µL suspension 2 and resuspending the pre-reaction takes place for approximately 5 to 7 min. Afterwards, the absorbance A_1 was determined (31) and the main reaction was started by addition of 5 µL suspension 3. The absorbance value A_2 was stable after the completion of the main reaction. For the glycerol concentration the absorbance difference ($\Delta A = A_1 - A_2$) is used in the following equation.

$$C_{Glycerol} = \Delta A \cdot D \cdot \frac{2.781\text{g}}{6.3\text{L}}$$

$C_{Glycerol}$ = Concentration of glycerol in g/L
ΔA = Absorbance difference
D = Dilution factor

The measurement range was between 0.04 and 0.4 g/L. If samples exceed this value they have to be adequate diluted.

3.6.5 Protein analysis

During the induction phase human serum albumin should be expressed. To determine the success of the induction, frozen cell free samples were analysed. This was done, using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The samples were thawed and 10 µL were mixed with the same amount of Laemmli buffer. Afterwards, the samples were heated to 95 °C for 5 min. The used gel was an Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Cat. No. 456-9036) by BIO-RAD and 5 µL of processed sample was applied to each lane. In the first and the last lane 4 µL of molecular weight standard was applied. Bovine serum albumin (BSA) was used as a standard. It shows the same characteristics than HSA in the SDS-PAGE. The gel ran for 1 h at 150 V (23) and was stained for 3 h in Coomassie brilliant blue solution. Afterwards, it was decolorized in RO-water over night.

4 Results

4.1 BIOSTAT® D-DCU 10-3

4.1.1 High cell density cultivations with exponential glycerol feed

Initially the feasibility of high cell density cultivations with the chemically defined medium was tested. Therefore, cultivations in an established stainless steel system were carried out. A fed batch cultivation with an exponential glycerol feed was accomplished with a fixed specific growth rate (μ_{set}) of 0.15 h^{-1}. Filling volume was 7 L. The cultivation time was 24.5 h in the batch phase and 9.5 h in the fed batch phase. Cultivation parameters like WCW, specific growth rate (μ) and the online pH value are important to get a statement about the cell growth (see Figure 18).

The WCW increased up to 387 g/L after 34 h. Samples were taken at the second day of the cultivation, due to limitations of the determination of the WCW at low cell densities. Therefore, no data of the batch phase is present. The WCW was 150.6 g/L in the first sample of the fed batch phase. The trend of the WCW shows a strong increase in the fed batch phase. A maximum μ of 0.18 h^{-1} was measured at the beginning of the fed batch phase. The trend has a decreasing character. An average μ of 0.11 h^{-1} was achieved during the fed batch phase. A minimum μ of 0.08 h^{-1} was determined after 34 h at the end of the fed batch phase.

The pH was controlled reliable at the set point over the whole cultivation time.

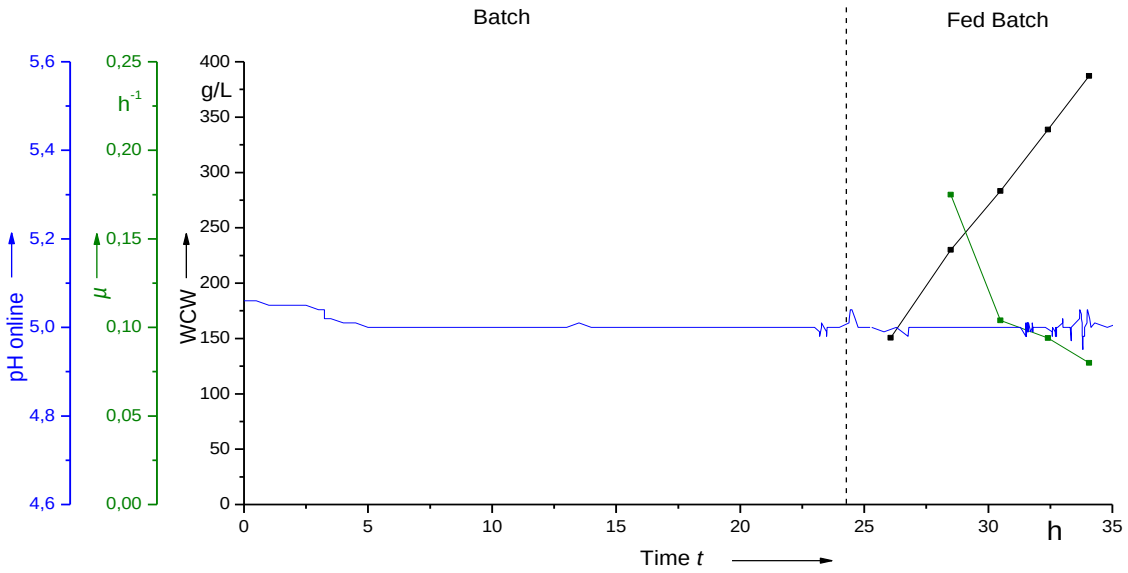


Figure 18: Cultivation parameters of the fed batch cultivation in the BIOSTAT® D-DCU 10-3. The diagram shows the characteristics of the pH (blue), the WCW (black) and the μ (green).

The concentration of glycerol in the cultivation broth of the fed batch is shown in Figure 19. At the beginning of the batch phase the glycerol concentration was 60.8 g/L. This value decreased to zero in

the first determination during the fed batch phase. No glycerol was accumulated during the fed batch phase.

A yield coefficient of 2.5 $g_{WCW}/g_{glycerol}$ was determined with the first WCW value in the fed batch phase. This value is not completely reliable, due to the supplementation of glycerol at the beginning of the fed batch phase.

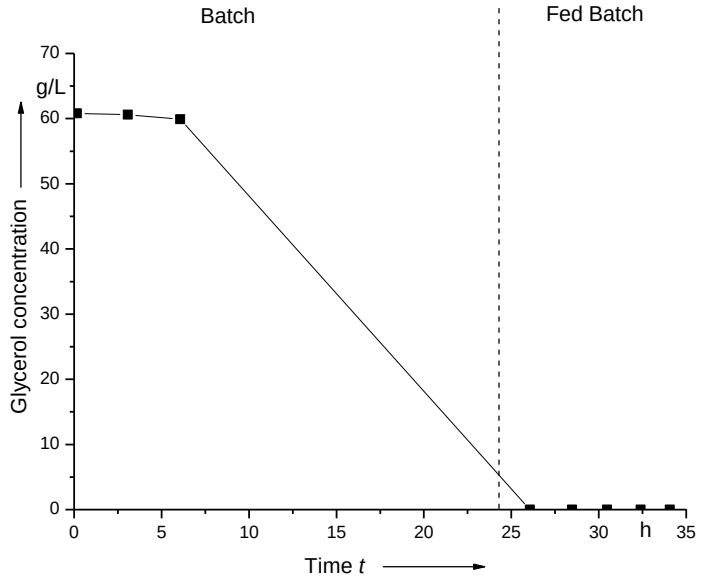


Figure 19: Metabolism of glycerol during the fed batch cultivation in the BIOSTAT® D-DCU 10-3.

For the pO_2 control the parameters stirrer speed, gas flow rate and the supplementation of pure oxygen were used (see chapter 3.5.1). After the point of inoculation the pO_2-decreases exponential and reached the set point of 30 % after 16.0 h. Only slight fluctuations were noticeable. At 19.3 h the pO_2 value showed a strong decrease, due to the addition of antifoam. A second addition of antifoam was necessary at 23.0 h. The batch phase was ending after 24.5 h. A sharp increase of the pO_2 value was noticeable. At 25.0 h the pO_2 value reached the set point again. Fluctuations occurred in the end of the fed batch phase. The fed batch phase was finished after 34.0 h

For the control at the set point the stirrer speed increased at 16.3 h followed by the gas flow at 19.3 h. Both parameters increased exponentially in the batch phases, showing an exponential increase of the *OUR*. The maximum stirrer speed was 1429 rpm at a gas flow rate of 9.2 Lpm. After the end of the batch phase both parameters decreased.

During the further process the stirrer speed and gas flow rate increased exponentially again after 25.0 h. The slope was lower in comparison to the batch phase. This was caused by the reduced μ and therefore a lower *OUR* in the fed batch phase. After 28.9 h stirrer speed and gas flow rate reached the maximal values of 1500 rpm an 10 Lpm and pure oxygen was needed.

No oxygen limitations occurred during the whole cultivation. Therefore, aerobe cultivation conditions could be ensured for the entire cultivation.

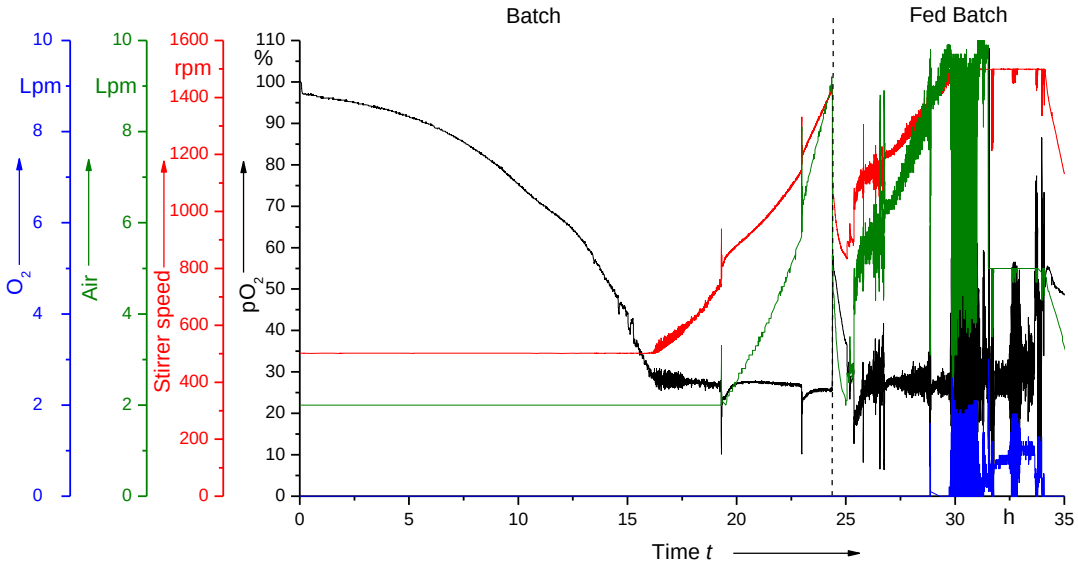


Figure 20: Control parameters of the fed batch cultivation in the BIOSTAT® D-DCU 10-3. The diagram shows the pO_2 value (black), the stirrer speed (red), air gas flow (green) and supplementation of oxygen (blue).

Figure 21 shows the cultivation and jacket temperature during the fed batch cultivation. The jacket temperature decreased at the end of the batch phase. The minimal temperature in the batch phase was 27.0 °C. In the beginning of the fed batch phase the jacket temperature was increasing. After the sharp increase the jacket temperature decreased in the further development of the fed batch phase up to 26.3 °C. This indicates that the cultivation temperature was controlled reliable at the set point for the whole cultivation.

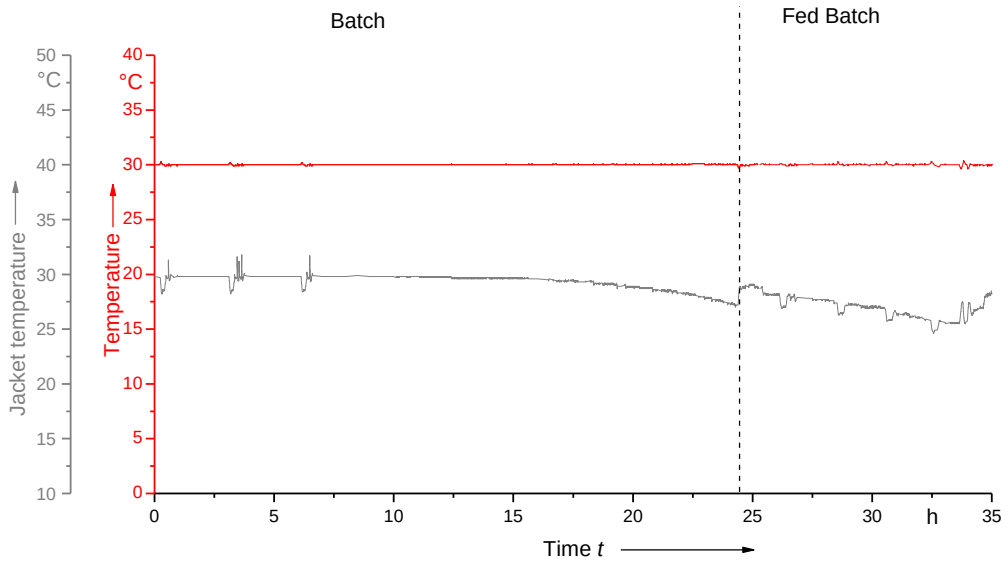


Figure 21: Cultivation temperature (red), and jacket temperature (grey) during the fed batch cultivation in the BIOSTAT® D-DCU 10-3.

4.2 BIOSTAT® RM 20 optical

Single uses systems become attractive in the last decades, due to their easy handling and therefore a reduced time and labour demand for the cultivation preparation. After the experiments in the reusable stainless steel system the suitability of the chemically defined medium in a single use system was evaluated. Therefore, the glycerol concentration in the medium was reduced as calculated in chapter 3.5.2. Batch, fed batch and induced fed batch cultivations were carried out in the BIOSTAT® RM 20 optical.

4.2.1 *P. pastoris* batch cultivation in the BIOSTAT® RM 20 optical

In this first experiment the feasibility of microbial batch cultivation in the CultiBag® RM 10 L optical was tested. Due to the lower k_La of the system the glycerol concentration in the medium was reduced. Therefore, the maximum cell density and the oxygen demand were decreased. The used glycerol concentration was 30 g/L. The duration of the batch cultivation was 22.2 h. The cultivation parameters of this batch cultivation are shown in Figure 22.

For the determination of the cell growth the WCW was determined in the second day of the cultivation. The determination of the WCW is imprecise at low cell densities, like in the first cultivation day. The slope of the WCW showed an exponential increase up to 21.1 h. The maximum WCW was 94.1 g/L

after 22.2 h. The μ was stable up to 21.1 h and showed a strong decrease after that point. The maximum value was 0.21 h^{-1} reached between 19 and 21 h.

The initial pH was 5.10. It decreased to 4.60 overnight, due to a failure in the pH control. The peristaltic pump was not switched to automatic mode. After that point the pH increased up to 5.26 and decreased slowly during the cultivation to 4.98. In conclusion the pH was not controlled reliable at the set point, although the pH value never exceeded growth limiting values of *P. pastoris.*

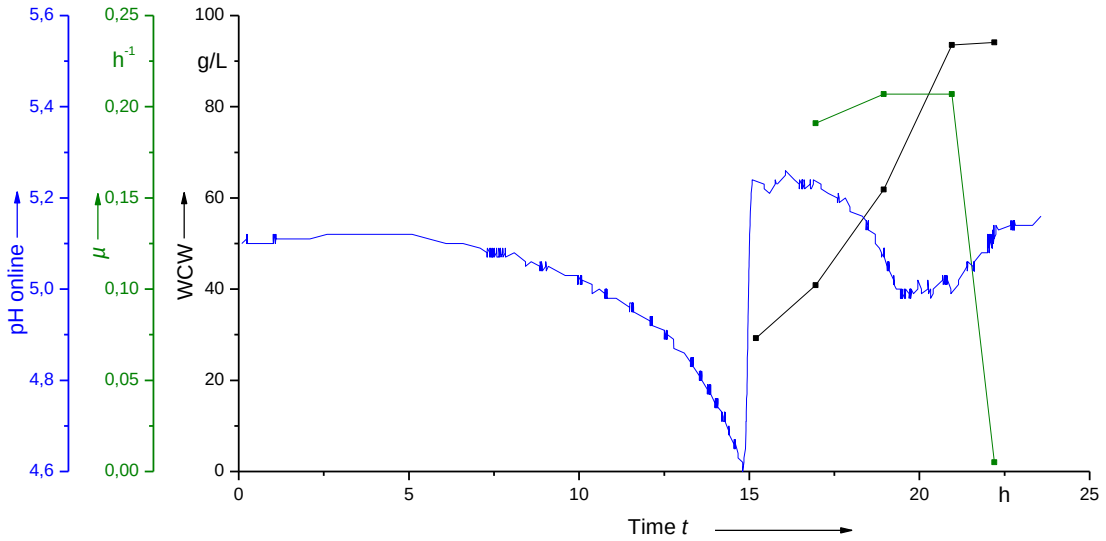


Figure 22: Cultivation parameters of the batch cultivation in the BIOSTAT® RM 20 optical. WCW (black), μ (green) and pH (blue) are shown.

In Figure 23 the characteristics of the glycerol concentration during the batch cultivation is presented. The initial determined glycerol concentration was 31.5 g/L and decreased to 0.1 g/L after 19 h. At the end of the batch cultivation glycerol was completely depleted. Therefore, glycerol was a limiting component in the medium.

The WCW was 94.1 g/L at the end of the cultivation. The resulting yield coefficient is 3.0 $g_{WCW}/g_{glycerol}$.

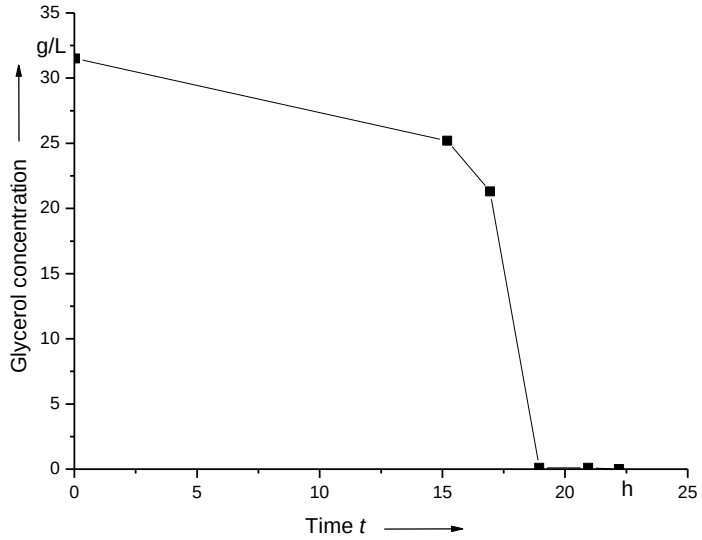


Figure 23: Metabolism of glycerol during the batch cultivation in RM-system

The control parameters for the pO_2 are shown in Figure 24. The pO_2 decreased to 88 % directly after the inoculation. After exponential decrease the pO_2 reached the set point after 8.8 h and was controlled at the set point. In the last two hours of the cultivation fluctuations of the pO_2 occurred and the pO_2 fell partly below the set point. After 22.2 h the pO_2 increased showing the end of the cultivation.

The oxygen gas flow rate increased exponentially to keep the pO_2 at the set point of 30 %. The supplementation of 100 % pure oxygen was reached after 17.8 h and persisted for the rest of the cultivation.

In the next step the rocking rate was increased up to 42 rocks per minute (RPM). This value was reached after 20.6 h. For a half hour the maximal rocking rate was utilized.

Aerobe cultivation conditions could be ensured for the whole cultivation time. Only in the last half hour of the exponential phase the pO_2 decrease below the set point. This was caused by the high oxygen demand of the culture.

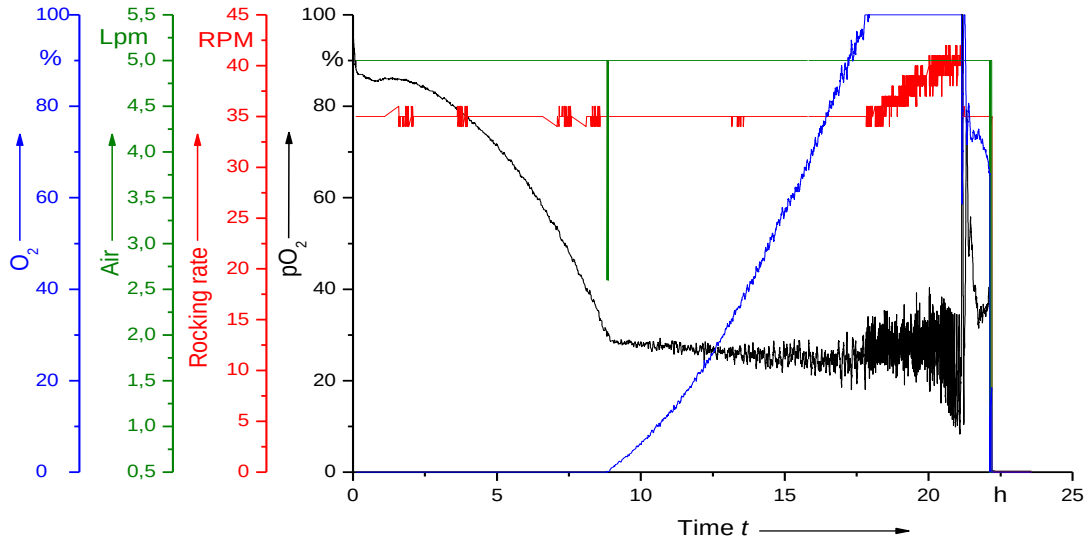


Figure 24: Control parameters during the batch cultivation in the RM-system. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue)

The control of the fermentation temperature was carried out by a heating and cooling coil in the RM-system. The contact area is smaller in comparison to the D-DCU 10-3 stainless steel system. Therefore, the fermentation temperature can be a critical point for HCDC in the RM-system. The temperature development is shown in Figure 25.

During the process the cultivation temperature was kept at the set point of 30 °C. The minimal jacket temperature during the batch process was 17.6 °C.

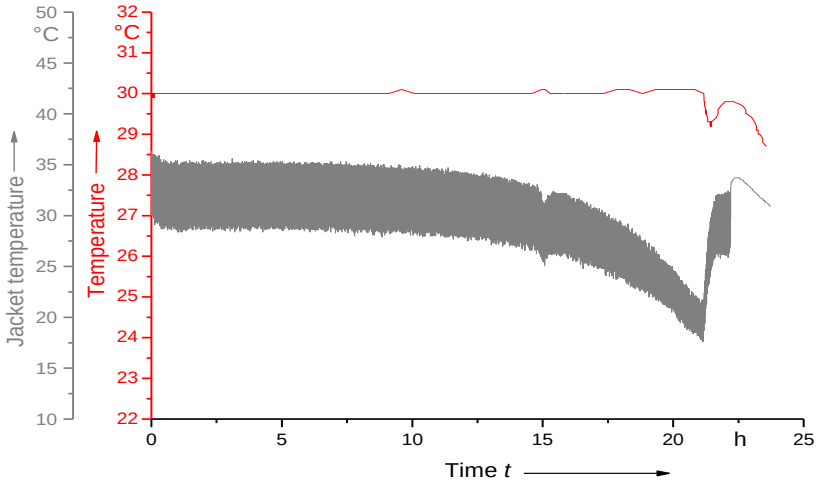


Figure 25: Cultivation temperature (red) and jacket temperature (grey) in the batch cultivation in the RM-system.

4.2.2 High cell density cultivation in the BIOSTAT® RM 20 optical

After the successful batch cultivation in the BIOSTAT® RM 20 optical the cell density should be further increased. Therefore, a fed batch cultivation with an exponential glycerol feed was carried out. In the glycerol fed batch phase the μ_{set} was 0.07 h^{-1}. In Figure 26 the WCW, μ and the pH values were presented. The overall cultivation time was 43.4 h and can be separated in a batch phase for the first 19.8 h followed by the fed batch up to 43.4 h.

During the cultivation an increase of the WCW was determined. In the batch phase a WCW of 67.8 g/L was reached after 18.9 h. In the fed batch phase the WCW was increasing exponentially. The maximal WCW was 292.0 g/L after 43.4 h at the end of the cultivation. The highest determined specific growth rate was 0.19 h^{-1} after 16.9 h in the batch phase. During the batch phase the average specific growth rate was 0.19 h^{-1}. Whereas, this value decreased to 0.067 h^{-1} in the fed batch phase.

In the first 4.6 h after inoculation the online pH value increased to 5.36. After this point the pH value decreased up to 4.98. At 37.7 h the offline pH value was 2.33. The optode was not able to determine the pH value, because the acidic value was not in the measurement range. Therefore, the pH value was corrected manually. In the course of correction more base than necessary was added. This caused a pH increase up to 6.53. In conclusion the pH could not controlled reliable during the fed batch phase.

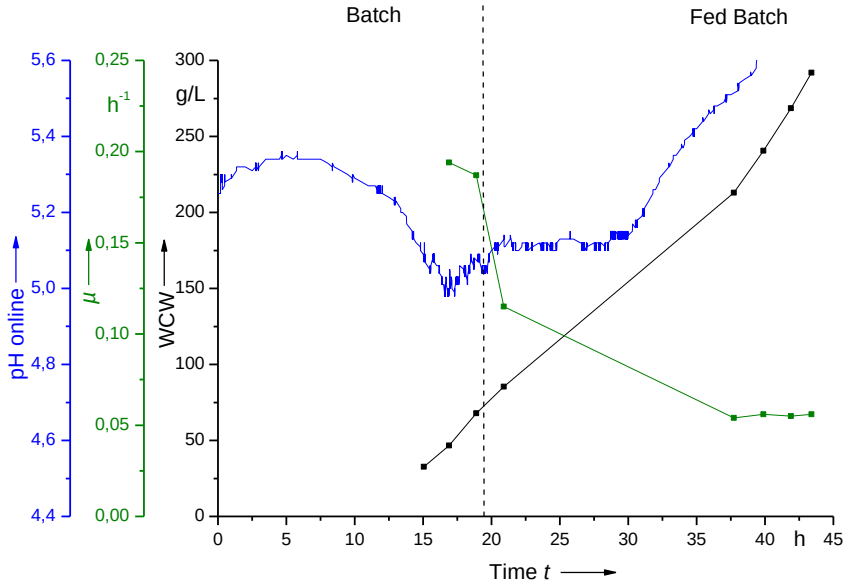


Figure 26: Cultivation parameters of a fed batch cultivation in the BIOSTAT® RM 20 optical. WCW (black), μ (green) and pH (blue) are shown.

The medium for the fed batch process was prepared with a glycerol concentration of 25 g/L. In the batch experiment the pO_2 was decreasing under the set point at the end of the cultivation, due to the high cell density, Therefore, the glycerol concentration was decreased. In the first sample a glycerol

concentration of 25.9 g/L was determined. In the last sample during the batch phase after 18.9 h the glycerol concentration was at 4.7 g/L. This results in a yield coefficient of 3.2 $g_{WCW}/g_{glycerol}$. This value is high in comparison to the yield coefficient in the stainless steel system (2.5 $g_{WCW}/g_{glycerol}$). In the beginning of the fed batch phase the glycerol concentration decreased to 0.1 g/L after 20.9 h. In all following samples the glycerol concentration was lower than the detection limit. Therefore, no glycerol was accumulated in the medium during the fed batch phase.

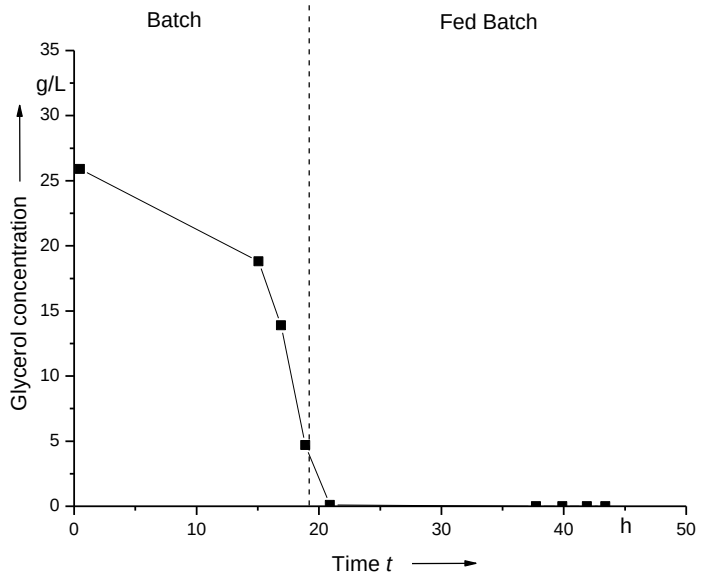


Figure 27: Concentration of glycerol in the cultivation broth during the fed batch cultivation in the BIOSTAT® RM 20 optical

Based on the k_La the *OTR* and consequently the pO_2 control is a critical factor for the BIOSTAT® RM 20 optical. Therefore, the pO_2-trend and the parameters for the control of the pO_2 are shown in Figure 28.

After the inoculation the pO_2 decreased exponentially and reached the set point after 9.9 h cultivation time. It was controlled at the set point. Some fluctuations in the end of the batch phase were noticeable at 18.0 h. In the fed batch phase the fluctuations stop until they occurred again at a cultivation time of 32.5 h. At the end of the cultivation the pO_2 decreased up to 4.0 %.

At 9.9 h the supplementation of oxygen started. The oxygen gas flow reached 100 % after 18.0 h. In the first hours of the fed batch phase the oxygen gas flow decreased up to 69.8 %. From 22.4 h the oxygen supplementation increased again and reached the maximal value after 31.5 h for the rest of the cultivation.

The rocking rate was increased to a maximal value of 38 RPM at the end of the batch phase. In the beginning of the fed batch phase the rocking rate was at the initial set point of 35 RPM and increased

from 35 to 42 RPM between 32.5 and 42.5 h. The cascade was fully exhausted. The system incurred oxygen limitations, due to the high cell density.

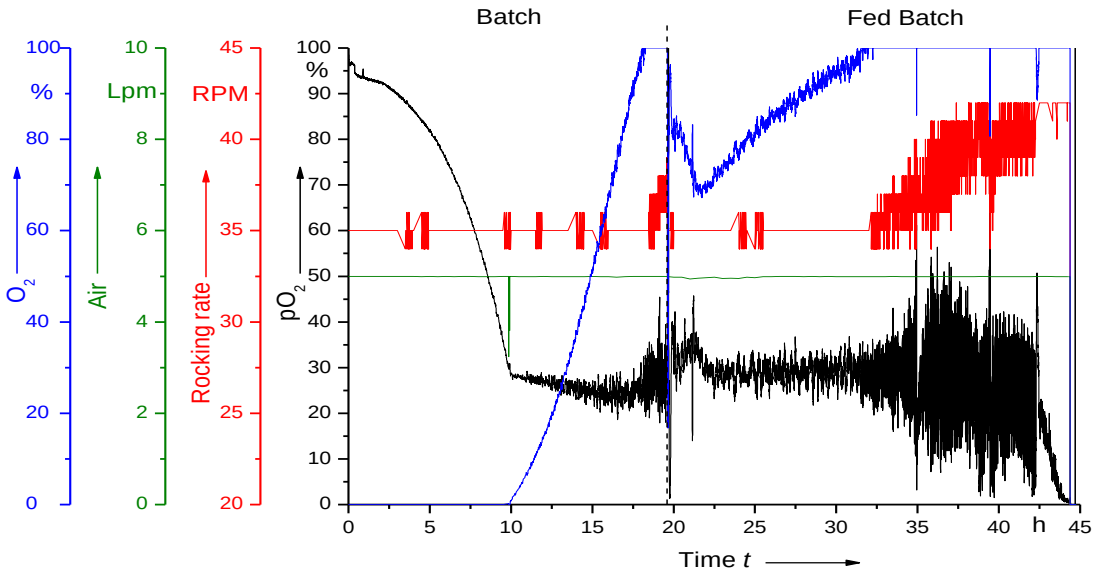


Figure 28: Control parameters in the fed batch cultivation in the BIOSTAT® RM 20 optical. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue) are shown in the diagram.

A reliable temperature control is important, especially in high cell density fed batch cultivations. Therefore, the cultivation temperature and the jacket temperature are shown in Figure 29. The cultivation temperature was kept at the set point, except from a short increase up to 31.6 °C in the fed batch phase after 39.5 h. The reason for this increase is not clarified.

During the cultivation a minimal jacket temperature of 21.3 °C in the batch phase and 16.7 °C in the fed batch phase occurred. The minimal cooling water temperature was limited to 10 °C. A difference of 6.7 °C between the minimal reached and the minimal possible jacket temperature showed a sufficient capacity of the temperature controlling system. Therefore, temperature control was possible in the complete process.

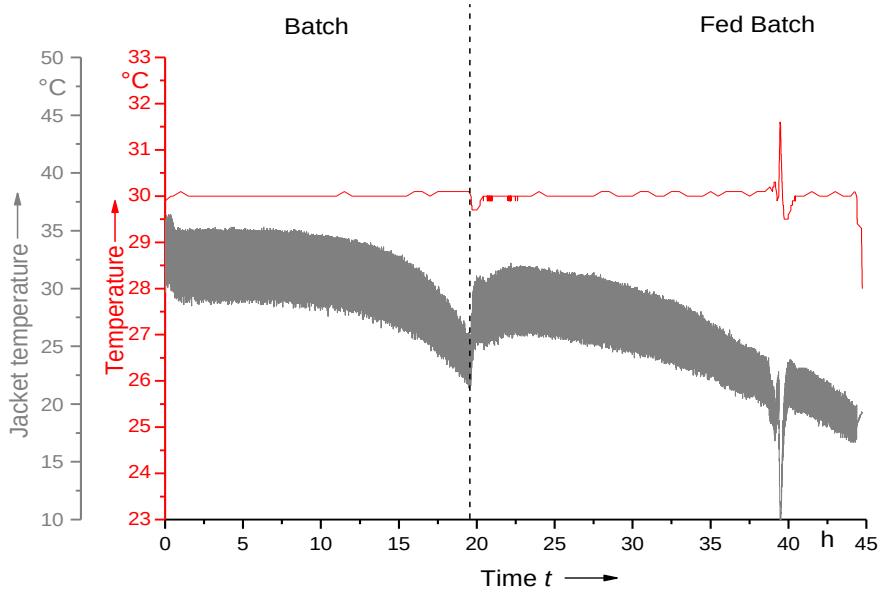


Figure 29: Cultivation temperature (red) and jacket temperature (grey) in the fed batch cultivation in the BIOSTAT® RM II 20 optical.

4.2.3 Protein expression in the BIOSTAT® RM 20 optical

The following cultivation was an induced fed batch process. After the fed batch phase with an exponential glycerol feed an induction phase with a constant methanol feed was carried out (for details, see chapter 3.5.2). The cultivation parameter for all three process phases are shown in Figure 30. The complete cultivation time was 95.6 h and can be separated in a batch phase for 19.8 h, a fed batch phase between 19.8 and 40.0 h and an induction phase until the end of the cultivation.

The WCW showed a significant increase up to the end of the glycerol fed batch phase. In the batch phase a maximal WCW of 88.1 g/L was reached. The value increased up to 266.5 g/L. The maximal WCW was reached after 70.0 h at a value of 315.2 g/L in the induction phase. The specific growth rate was 0.17 h^{-1} directly after the beginning of the fed batch phase at 20.5 h. It decreased to 0.06 h^{-1} in the fed batch phase at 39.5 h. The value is comparable to the calculated value (μ_{set} = 0.07 h^{-1}). In the induction phase the μ were low as expected.

The online determined pH characteristics showed fluctuations. At 39.5 h the pH-valu decreased to 2.40 in the offline determination, due to the limited measurement range of the optode. The pH was corrected manually to a pH of 4.57. In the induction phase the optode determined correctly the pH. In conclusion the pH was not controlled reliable at the set point during the fed batch phase. This result complies with the results in the fed batch cultivation carried out earlier.

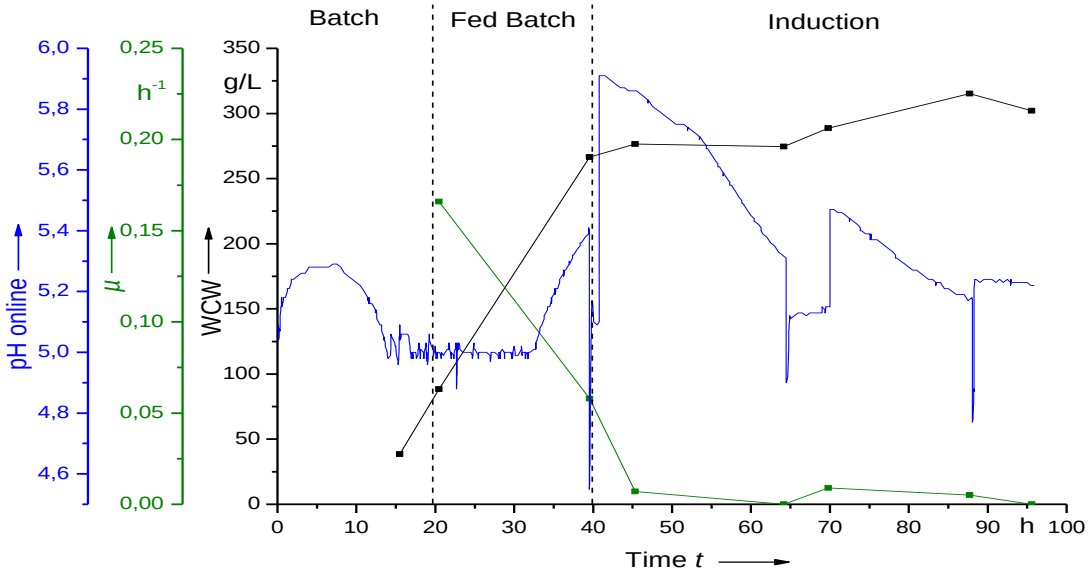


Figure 30: Cultivation parameters of an induced fed batch cultivation in the BIOSTAT® RM 20 optical. WCW (black), μ (green) and pH (blue) are shown.

In the batch and fed batch phase of the cultivation samples for the glycerol determination were taken. The results are shown in Figure 31.

The initial glycerol concentration was 26.8 g/L. After 20.5 h the value decreased to 0.1 g/L. The yield coefficient in the batch phase was 3.3 $g_{WCW}/g_{glycerol}$ determined for the sample after 20.5 h. This value is higher than in the batch and fed batch process in the BIOSTAT® RM 20 optical. During the fed batch phase no glycerol was determined in the cultivation broth. Therefore, glycerol was not accumulating in the cultivation broth. In the induction phase the glycerol concentration was not determined, since no glycerol was added in this phase.

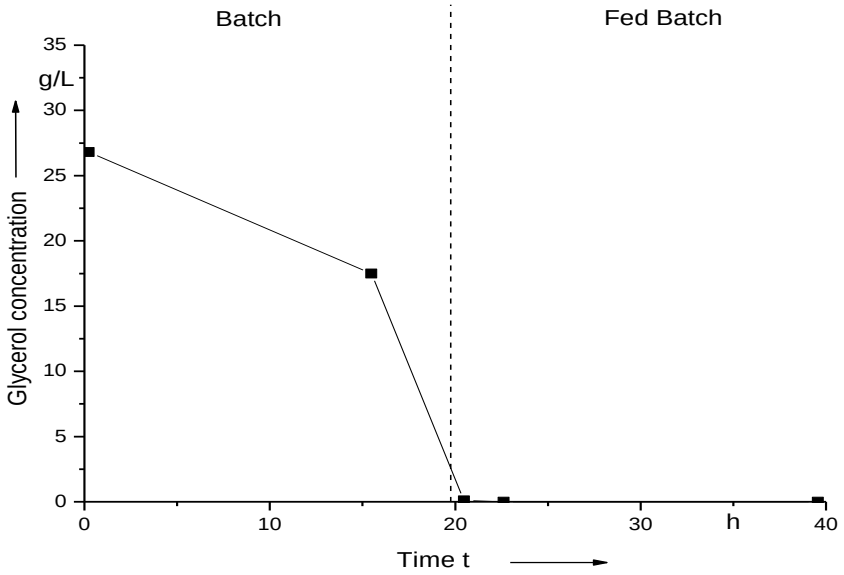


Figure 31: Concentration of glycerol in the cultivation broth during the induced fed batch cultivation in the BIOSTAT® RM 20 optical

For the pO_2-control the same multistage cascade was used as in the fed batch cultivation. The control parameters of the induced fed batch are shown in Figure 32.

Directly after the inoculation the pO_2 decreased exponentially decrease and the set point was reached after 10.0 h. The pO_2 showed strong fluctuations in the fed batch phase and fall slightly below the set point. During the induction phase the pO_2 was kept at the set point.

The oxygen gas flow rate increased exponentially and reached 100 % after 17.9 h. After the end of the batch phase the oxygen gas flow rate decreased for a short period. At 28.0 h the oxygen gas flow reached 100 % in the fed batch phase. In the induction phase the oxygen gas flow was at 100 % for the entire time.

The rocking rate also increased in the end of the batch phase. The maximal value in the batch phase was 41 RPM at 19.8 h. After the end of the batch phase the rocking rate decreased for a short period. After decreasing to 35 RPM in the beginning of the fed batch phase the rocking rate reached the maximal value of 42 RPM in the end of the fed batch phase and the cascade was therefore exhausted in this time.

In conclusion the control of the pO_2 showed strong fluctuations. Therefore, the cascade was changed in the next experiment. The oxygen gas flow rate was the last increased parameter (see Figure 17) Aerobe conditions could be ensured over the whole cultivation, except from short phases at the end of the fed batch phase.

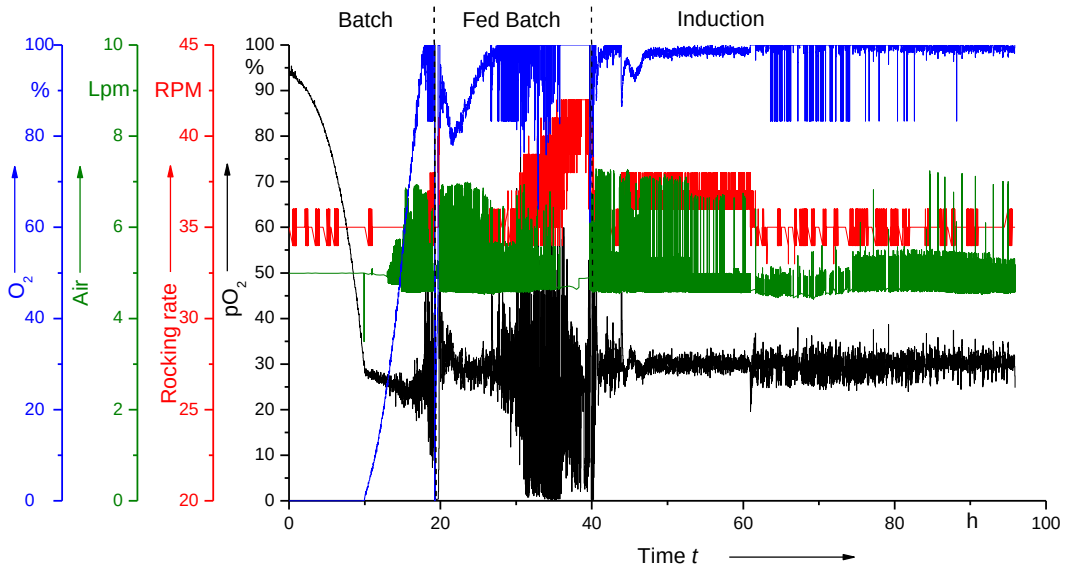


Figure 32: Control parameters in the induced fed batch cultivation in the BIOSTAT® RM 20 optical. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue) are shown.

The set point for the cultivation temperature in the induced fed batch cultivation was 30 °C over the entire process. Cultivation temperature and jacket temperature are shown in Figure 33.

It was possible to control the cultivation temperature close to the set point in all phases of the cultivation. The maximal deviation from the set point was at the change between fed batch and induction phase. A maximal temperature of 30.5 °C and a minimal temperature of 29.1 °C were determined in an overshoot of the temperature control at approximately 40 h.

At the end of the fed batch phase a minimal jacket temperature of 14.8 °C was determined. Similar to the fed batch process earlier a sufficient reserve of cooling capacity was observed.

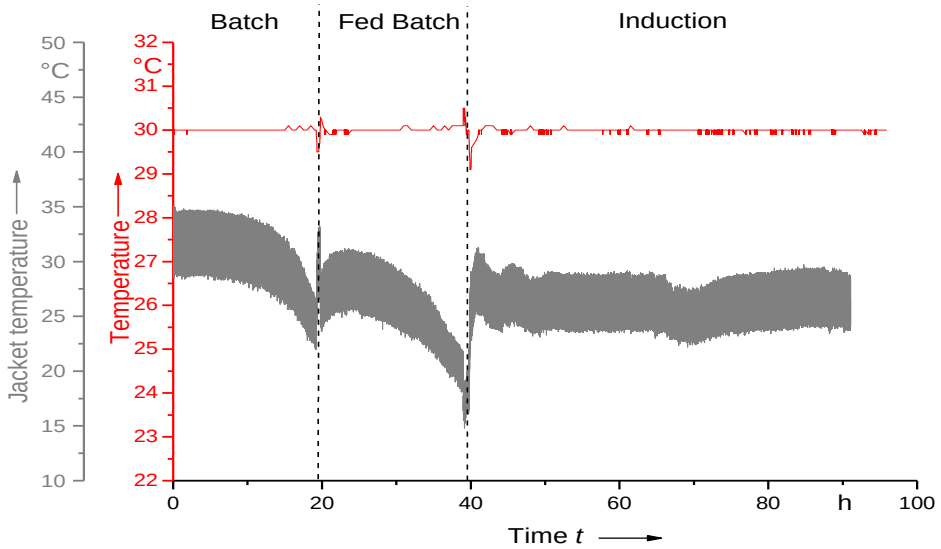


Figure 33: Cultivation temperature (red) and jacket temperature (grey) for the induced fed batch cultivation in the BIOSTAT® RM 20 optical.

The protein expression was proven by SDS-PAGE. The result of the protein expression in the induction phase is shown in Figure 34. The samples are titled with the numbers 1 to 6. On the left side a reference sample was applied to the gel. The standard was BSA instead of HSA.

No rHSA was detectable before the induction was started. After the induction the band intensity increased with further methanol addition. HSA was secreted by the cells into the cultivation broth. The last lane after 56 h has a lower intensity as the lane with 0.2 g/L BSA, which was used as a standard. Therefore, it is supposable that the HSA concentration in the cultivation broth was below 0.2 g/L.

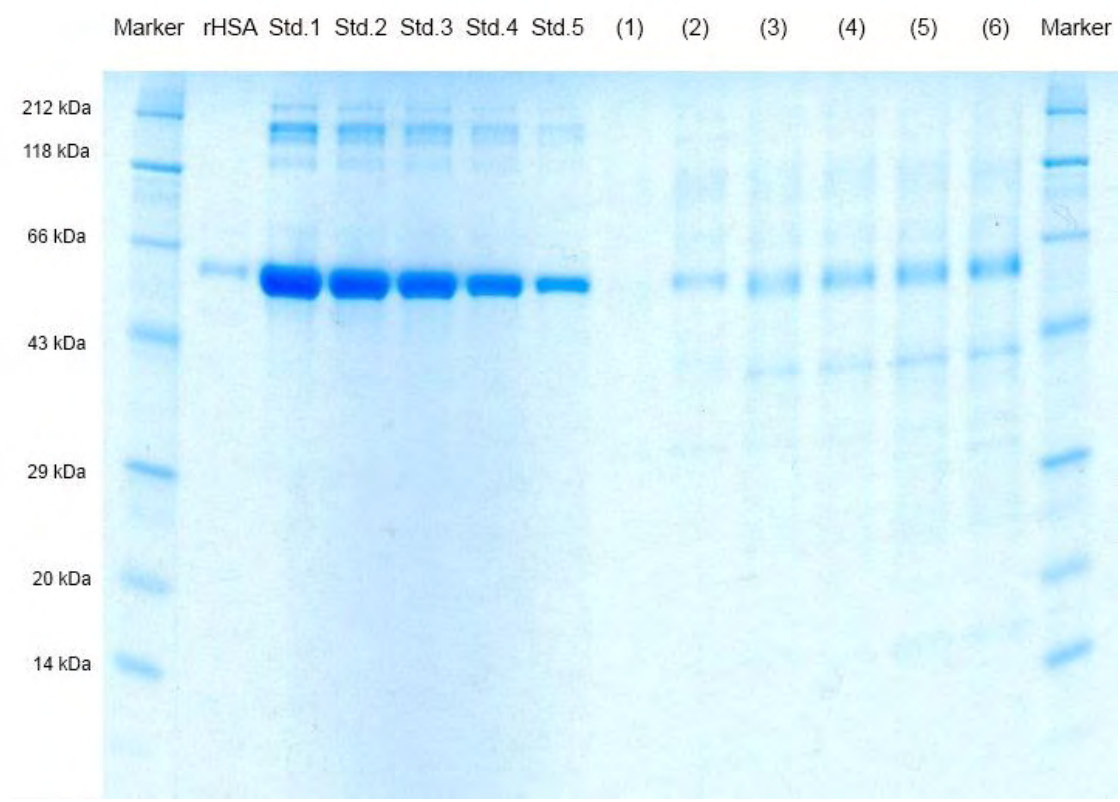


Figure 34: Coomassie brilliant blue stained SDS-PAGE gel of the induced fed batch cultivation. Std.1 = 0.2 g/L BSA, Std.2 = 0.4 g/L, Std.3 = 0.6 g/L, Std.4 = 0.8 g/L, Std.5 = 1.0 g/L. HSA is the target protein. Lane (1) prior to induction, (2) 5 hours, (3) 24 hours, (4) 30 hours, (5) 47 hours, (6) 56 hours after induction.

4.2.4 Influence of the pH on the protein expression in the BIOSTAT® RM 20 optical

The previous experiment (see chapter 4.2.3) indicated that a pH control was difficult, due to the limitations (measurement range between pH 6 and 8.5) of the optodes. *P. pastoris* produce carbon dioxide and other acidic metabolites during growth. The optodes were not able to determine these fast changes in low pH-ranges. Therefore, the pH set point was increased to 6.0 in the following induced fed batch cultivation (see Table 13 in chapter 3.5.2). In the experiment the influence of the changed pH on the cell growth and the protein expression should be determined.

The cultivation parameters WCW, μ and pH are shown in Figure 35. In the batch phase a maximal WCW of 105.6 g/L was determined. The maximal WCW in the fed batch phase was 260.6 g/L. An increase of the WCW up to 284.0 g/L was determined at the end of the cultivation in the induction phase. The WCW was increasing strong in the batch and fed batch phase. In the induction phase the cell density showed an increase of 23.4 g/L. The process time was 88.3 h. During the first 19.8 h the batch phase occurred, followed by the fed batch phase up to 39.3 h. The last phase was the induction phase up to the end of the cultivation. In the batch phase a maximal μ of 0.22 h was measured. During the fed batch phase an average µ of 0.08 h^{-1} occurred. The value is comparable with the calculated μ_{set}.

The μ in the induction phase was approximately zero. The pH was controlled at the set point of 6.0. During the cultivation the optodes had to be recalibrated. No strong decrease below the set point was noticeable like in the cultivations before. The highest deviation was determined after 63.8 h at a pH of 6.17.

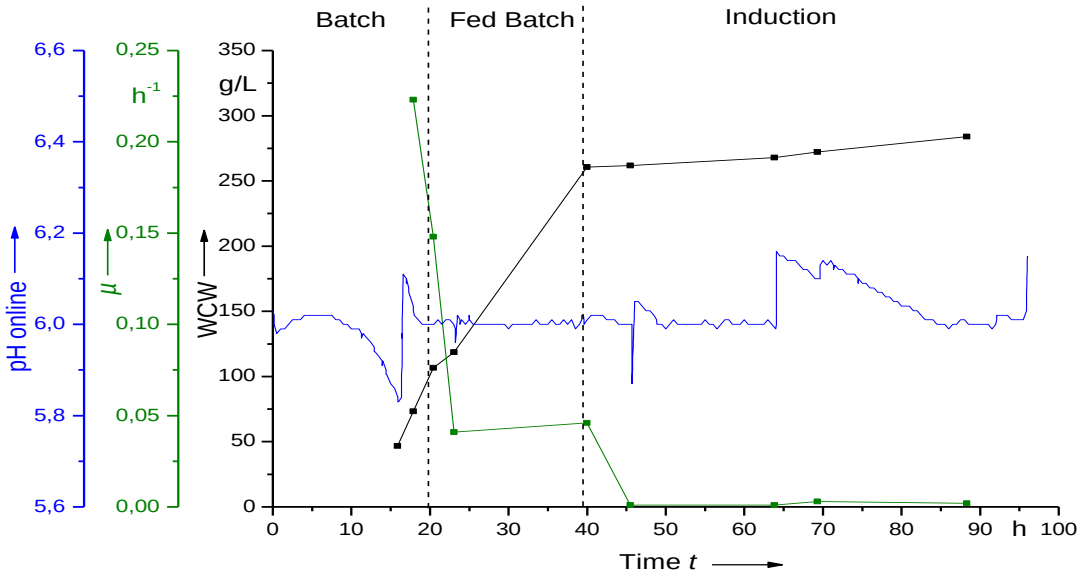


Figure 35: Control parameters in the induced fed batch cultivation in the BIOSTAT® RM 20 optical at pH 6.0. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue) are shown in the diagram.

In Figure 36 the glycerol concentration in the induced fed batch at pH 6.0 is shown. The initial glycerol concentration was 26.5 g/L. In the first sample of the fed batch phase the concentration decreased to 0.1 g/L after 20.4 h. The yield coefficient is 4.0 $g_{WCW}/g_{glycerol}$. This value is high compared to the previous cultivations. During the fed batch phase the glycerol concentration was below the detection limit. The amount of used glycerol feed was weighed. With the information of the feed concentration and the density it was possible to calculate a yield coefficient for the fed batch phase. This yield coefficient was at 1.73 g_{WCW}/L.

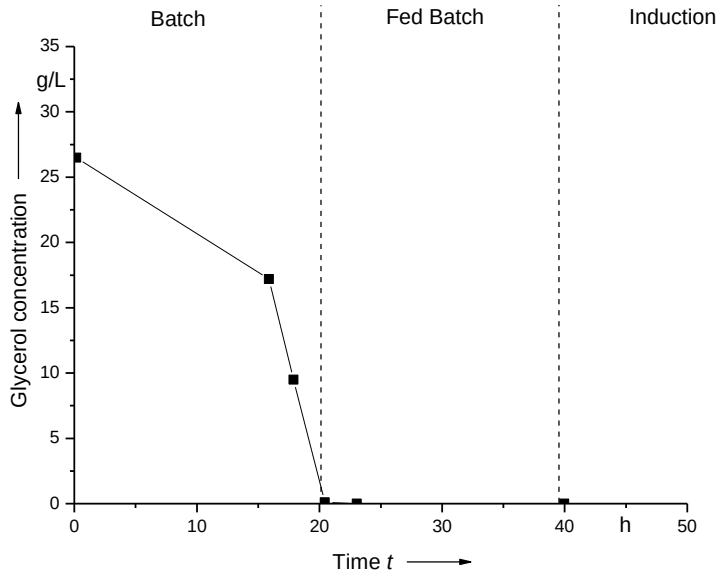


Figure 36: Concentration of glycerol in the cultivation broth during the induced fed batch cultivation in the BIOSTAT® RM 20 optical at pH 6.0.

The control parameters in the induced fed batch at pH 6.0 are shown in Figure 37. The pO_2 was controlled by two different cascades. In the batch phase cascade 1 was used. The oxygen gas flow rate was increased first, followed after the rocking rate. After the feed start the rocking rate was fixed at the maximal value of 42 rpm and the pO_2 was controlled by the oxygen gas flow rate.

After the inoculation the pO_2 decreased exponentially to the set point in 9.6 h. The pO_2 was controlled at the set point for the rest of the cultivation.

The oxygen gas flow rate was increasing exponentially and reached the maximum after 19.0 h, decreased in the beginning of the fed batch phase. In the end of the fed batch phase the oxygen gas flow rate reached 100 % after 38.5 h. During the induction phase the oxygen gas flow rate was at medium level of 70 %.

The rocking rate was not increasing during the batch phase. In the fed batch phase the rocking rate was not part of the cascade and fixed at 42 RPM

The control of the pO_2 was very reliable in the whole cultivation and fluctuations were only existent during the change of the cultivation phases.

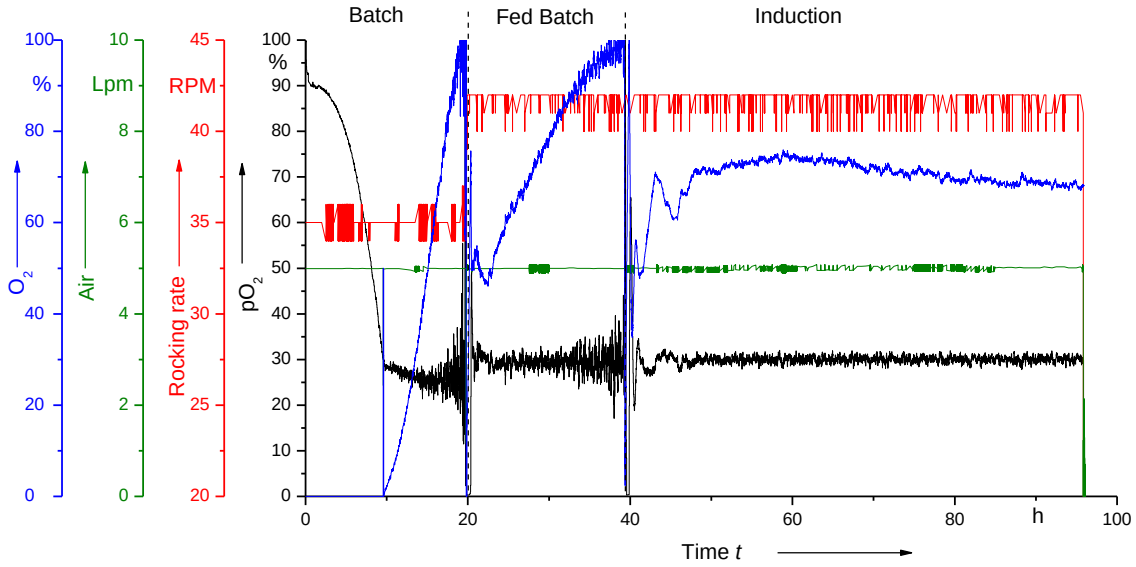


Figure 37: Control parameters in the induced fed batch cultivation in the BIOSTAT® RM 20 optical at pH 6.0. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue) are shown in the diagram.

The cultivation temperature and the jacket temperature are shown in Figure 38. The cultivation temperature was controlled reliable at the set point of 30 °C during the whole cultivation. The minimal measured temperature was 29.2 °C, the maximal temperature was 30.1 °C. At the end of the fed batch phase a minimal jacket temperature of 19.2 °C was reached.

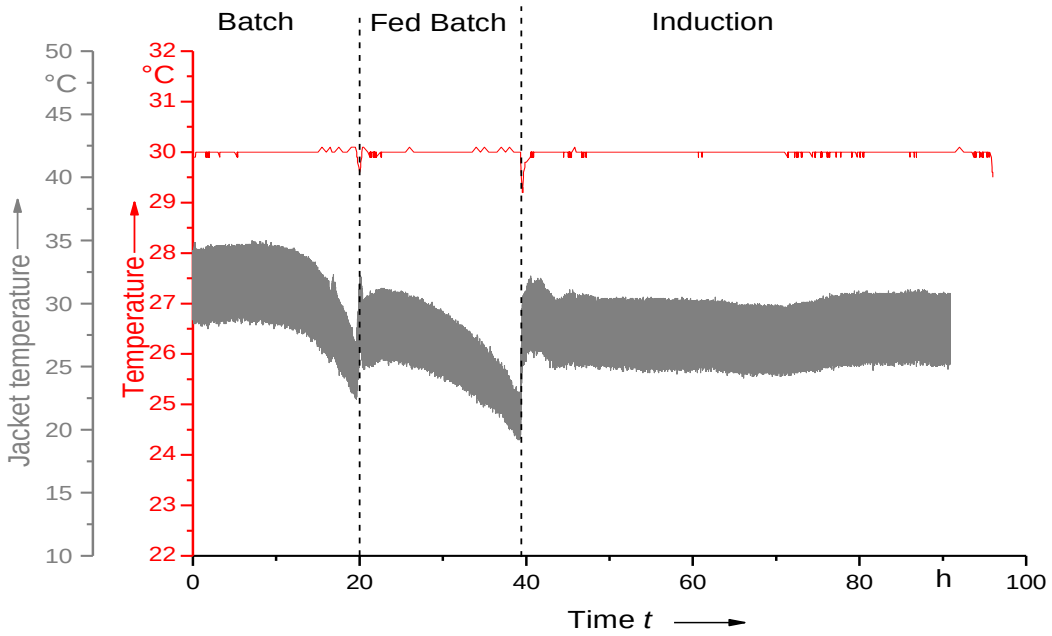


Figure 38: Cultivation temperature (red) and jacket temperature (grey) in the induced fed batch cultivation in the BIOSTAT® RM 20 optical at pH 6.0.

The results of the SDS-PAGE are shown in Figure 39.

The first sample was taken before the start of the methanol feed. A slight band is visible. The band intensity is increasing with further progress of the induction phase. The last sample after 57 h has a comparable intensity as the 0.2 g/L standard of BSA. Therefore, the concentration of HSA in the cultivation broth is approximately 0.2 g/L.

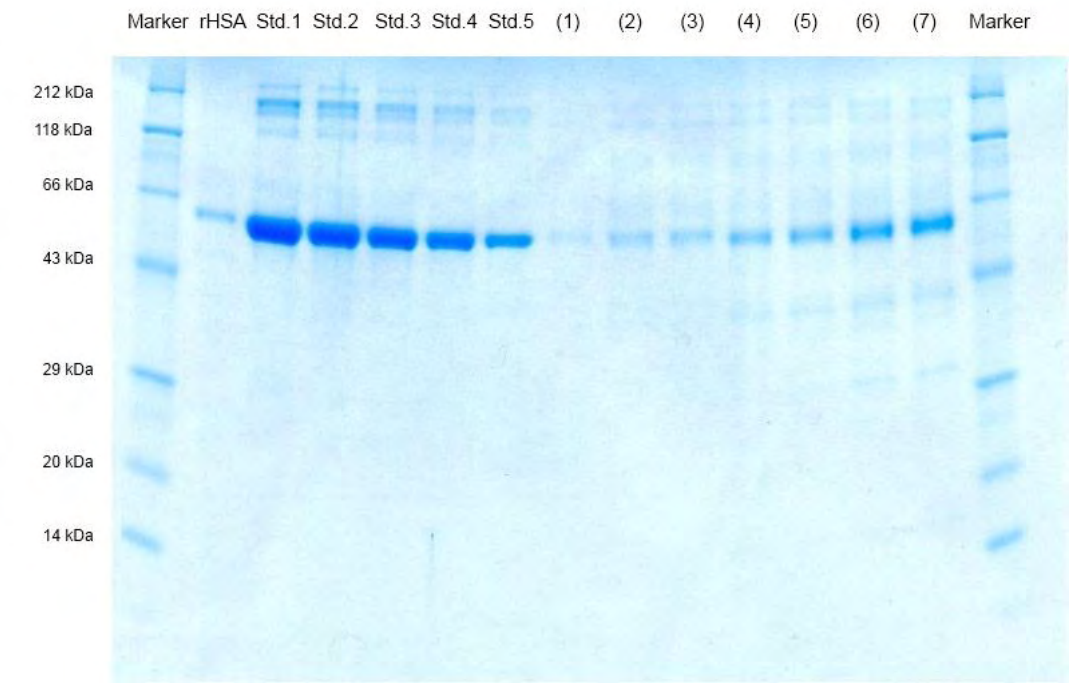


Figure 39: Coomassie brilliant blue stained SDS-PAGE gel of the induced fed batch cultivation at pH 6. Std.1 = 0.2 g/L, Std.2 = 0.4 g/L, Std.3 = 0.6 g/L, Std.4 = 0.8 g/L, Std.5 = 1.0 g/L. rHSA is the target protein. Lane (1) prior to induction, (2) 1 hour, (3) 5.5 hours, (4) 24 hours, (5) 30 hours, (6) 49 hours, (7) 57 hours after induction.

4.2.5 Batch cultivation inoculated with prepared cryo cultures

In this batch cultivation the new seed culture method with cryo cultures was tested. The inoculation cell density was identical to the other cultivations. The cultivation parameters are shown in Figure 40. The WCW was increasing up to 101.2 g/L after 21.0 h at the end of the batch. The maximal μ was 0.20 h^{-1} at the same time. During the batch an average μ of 0.18 h^{-1} was reached.
The pH was controlled at the set point of 5.0 over the whole cultivation.

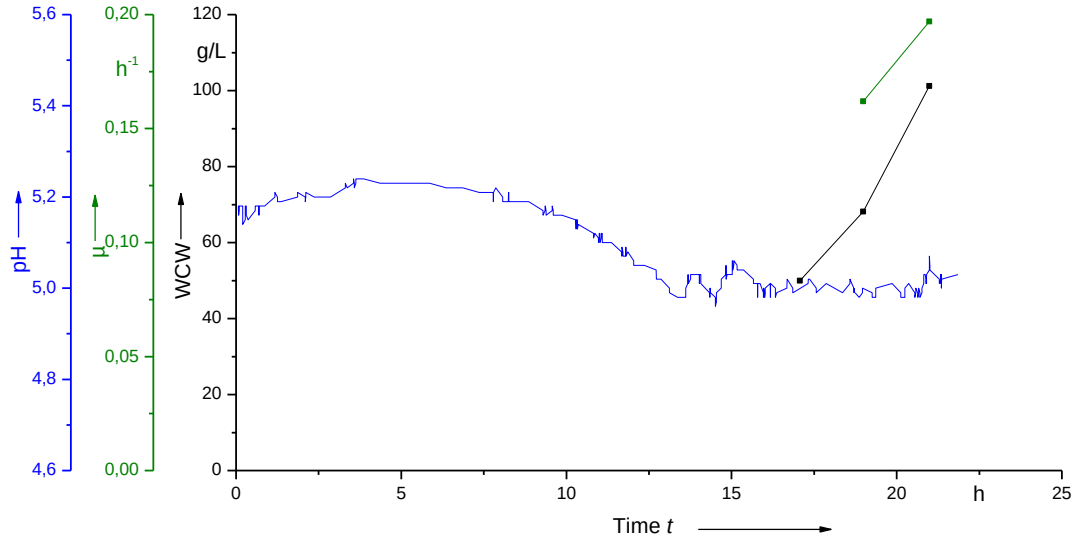


Figure 40: Cultivation parameters of the batch experiment, inoculated with cryo cultures. WCW (black), μ (green) and pH (blue) are shown.

The glycerol concentration in the cultivation broth during the batch process is shown in Figure 41. The initial glycerol concentration was 33.5 g/L. It decreased to 0.1 g/L after 21.0 h cultivation time. The yield coefficient was 3.0 $g_{WCW}/g_{glycerol}$.

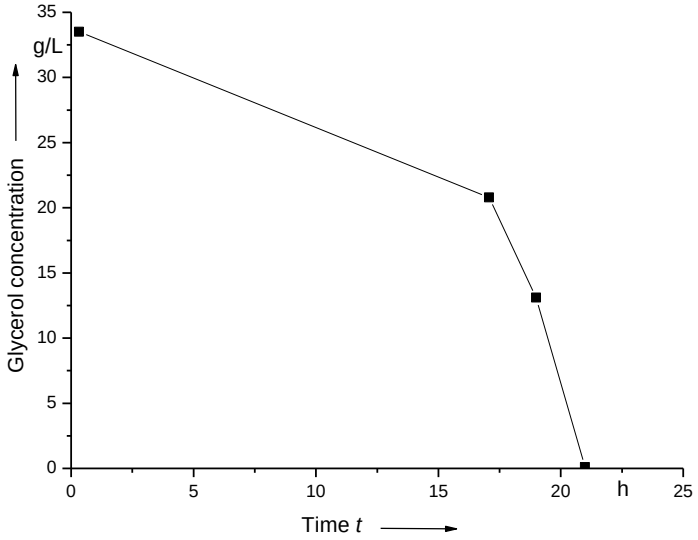


Figure 41: Concentration of glycerol in the cultivation broth during the batch cultivation in the BIOSTAT® RM 20 optical inoculated with cryo cultures.

The control parameters of the batch cultivation, inoculated with cryo cultures is shown in Figure 42. The pO_2 decreased to 90 % after the inoculation. After an exponential decrease the set point was

reched after 8.2 h. Hence, the oxygen gas flow rate was increasing and reached the maximal value of 100 % after 16.9 h. After this point the rocking rate was increased up to 42 rpm. This caused fluctuations in the pO_2 control. In conclusion no oxygen limitations occurred and aerobe conditions were ensured in the whole process.

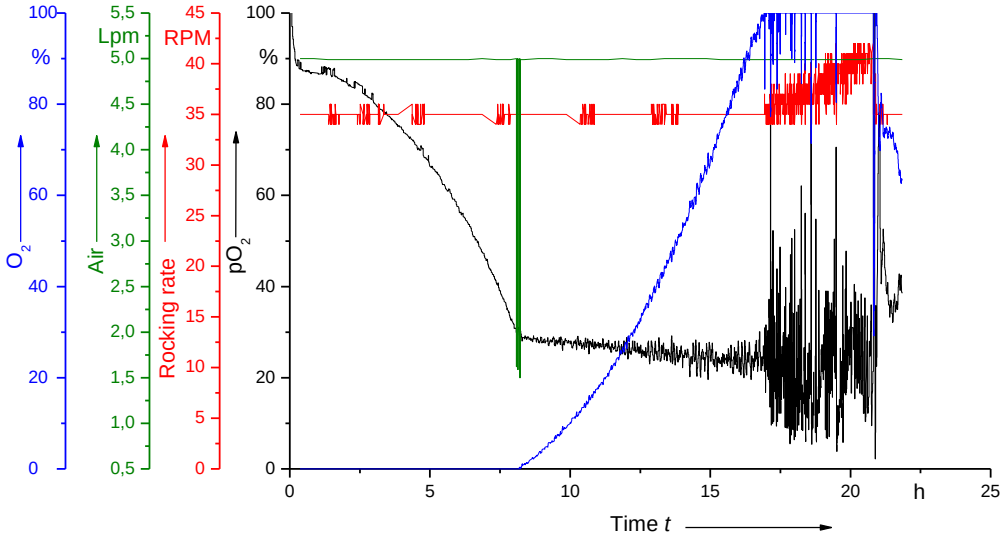


Figure 42: Control parameters in the batch cultivation in the BIOSTAT® RM 20 optical, inoculated with cryo cultures. The pO_2 (black), the rocking rate (red), the air gas flow rate (green) and the O_2 gas flow rate (blue) are shown in the diagram.

Figure 43 shows the temperature development during the batch phase in the BIOSTAT® RM 20 optical, inoculated with cryo cultures. The cultivation temperature was controlled reliable at the set point of 30 °C. The minimal reached cultivation temperature was 16.4 °C

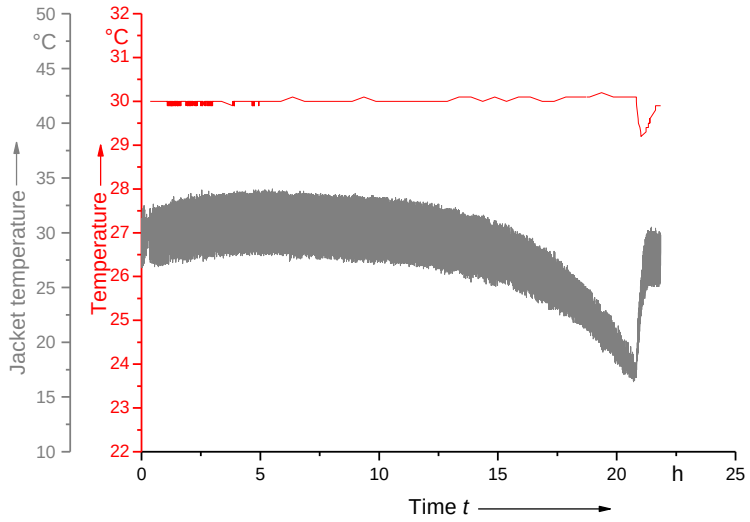


Figure 43: Cultivation temperature (red) and jacket temperature (grey) in the batch cultivation in the BIOSTAT® RM 20 optical, inoculated with cryo cultures.

5 Discussion

The suitability of the chemical defined medium for *P. pastoris* HCDC was evaluated in a stainless steel BIOSTAT® D-DCU 10-3 and a BIOSTAT® RM 20 optical system. Different cultivation processes were carried out. Furthermore, in the process transfer to the SU system the compatibility of the medium and SU systems in general was reviewed.
A new seed culture preparation was tested to decrease the time demand of the seed culture preparation.

5.1 Fed batch cultivations in the BIOSTAT® D-DCU 10-3

For the evaluation of the chemical defined medium a fed batch cultivation in stainless steel STR was carried out. An established stainless steel system was used to avoid limitations of the oxygen transfer rate. Therefore, the suitability of the medium for microbial cultivations was evaluated. During the cultivation no limitations of the pO_2 occur. The fed batch cultivation had an exponentially feed with a fixed specific growth rate of 0.15 h^{-1}. The fixed specific growth rate was chosen to avoid overflow metabolism. Higher growth rate similar to μ_{max} = 0.24 h^{-1} can cause overflow metabolism. A final cell density of 387 g/L WCW was achieved. In a comparable process a WCW of 381 g/L was reached (Dreher et al., 2013). During the batch phase a yield coefficient of 2.5 $g_{WCW}/g_{substrate}$ (0.66 $g_{DCW}/g_{substrate}$) was reached. A yield coefficient of 2.6 $g_{WCW}/g_{substrate}$ (0.71 $g_{DCW}/g_{substrate}$) was reported in the literature (Zhang et al., 2007). Both values were comparable. The specific growth rate (μ) during the fed batch phase was lower than calculated. The average μ during the fed batch phase was 0.11 h^{-1}. In consequence the fed batch phase for reaching the desired cell density was longer than calculated. This was probably caused by neglecting the maintenance metabolism in the calculations. More carbon source was required to reach the cell density and therefore more feed solution was transferred to the cultivation chamber. The cultivation was terminated, because the maximal filling volume of the reactor was reached.
Aerobe conditions were ensured over the whole cultivation time. The multistage cascade for the pO_2 control was not exhausted. The supplementation of pure oxygen caused strong fluctuations of the pO_2. Changed control parameters can improve the pO_2 control. The other controlled parameters, like pH and temperature were controlled reliable at the set points. A higher oxygen transfer rate is possible and therefore an increase of the cell densities is possible by the reduction of the initial filling volume up to 6.4 L or the increase of the glycerol concentration in the feed solution.
In summary the chemical defined medium is suitable for high cell density fed batch cultivations. No limitations of the medium occurred during the cultivation.

5.2 Batch cultivation in the BIOSTAT® RM 20 optical

After the evaluation of the medium in the stainless steel STR a process transfer to a single use system was carried out. A BIOSTAT® RM 20 optical was used for the batch process. In comparison to reusable systems, single use systems have a lower oxygen transfer rate. Due to the lower *OTR* of the BIOSTAT® RM 20 optical the batch cultivation was carried out with a glycerol concentration of 30 g/L (see chapter 3.5.2). This reduces the maximal cell density at the end of the batch phase. Therefore, the cell densities are not directly comparable to the cultivations in the D-DCU. The maximal cell density of 94.1 g_{WCW}/L or 23.5 g_{DCW}/L is higher than expected in the calculations (see chapter 3.5.2) and results in a yield of 3.0 $g_{WCW}/g_{substrate}$ or 0.75 $g_{DCW}/g_{substrate}$. The yield coefficient is therefore higher than in the D-DCU (see chapter 4.1.1). Despite of the higher cell growth, aerobe conditions were ensured over the cultivation time until the glycerol was depleted.

Only a few experiments with yeasts in the RM system were carried out before. *Saccharomyces cerevisiae* was cultivated in a BIOSTAT® RM CultiBag optical 10 L (former: Wave Bioreactor®) up to a dry cell weight of 9.1 g/L (Mikola et al., 2007). This value was exceeded by more than factor two.

The optode was able to determine the pH, although the set point was outside the measurement range. The batch cultivation showed satisfying cell densities and the BIOSTAT® RM II 20 is suitable for *P. pastoris* cultivations.

5.3 Fed batch cultivations in the BIOSTAT® RM 20 optical

After the batch cultivation was satisfying, the possibilities for high cell density cultivations (HCDC) were examined in the BIOSTAT® RM 20 optical. The μ was reduced to the minimal possible value of 0.07 h^1 for optimal growth.

A yield coefficient of 3.2 $g_{WCW}/g_{substrate}$ (0.83 $g_{DCW}/g_{substrate}$) was determined for the batch phase. The cell density increased up to 292.0 g/L and achieved the calculated value (see chapter 3.5.2). A further increase of the cell density was limited by the oxygen transfer rate. Hence, the cell density is lower in comparison to the stainless steel system.

The fed batch phase was longer than expected. The reason is probably the maintenance metabolism like mentioned in chapter 5.1. The μ was nearly the calculated μ_{set} which is not possible, due to the longer fed batch phase. Therefore, measuring inaccuracy influenced the determination of μ.

During the fed batch phase the pH decreases strongly under the set point. The reasons are a deterioration of the chemical dye in the optodes. The chemo sensitive dye has a lifetime of 10 000 measurements (Sartorius, 2011 c).

Reliable temperature control was possible during the cultivation. In this work the first HCDC of *P. pastoris* in a RM system was carried out. Therefore, no comparable data is available. The cultivation

in the BIOSTAT® RM 20 optical was successful and showed the microbial HCDC ability of the system.

5.4 Induced fed batch cultivations in the BIOSTAT® RM 20 optical

The aim of these experiments was protein production in the RM system, after the success of the fed batch cultivation. A fed batch cultivation followed by a constant methanol feed for protein expression was established.

The maximal cell density of 266.5 g_{WCW}/L was achieved in the fed batch phase an increased up to 315.2 g_{WCW}/L during the induction. The yield coefficient showed high values, in comparison to the stainless steel system. The yield coefficient was 3.3 g_{WCW}/ $g_{substrate}$ (0.84 g_{DCW}/ $g_{substrate}$) in the batch phase.

The pO_2 cascade was exhausted to the maximal control parameter similar to the fed batch experiment. Compared to the fed batch cultivation in chapter 4.2.2 the pO_2 value was higher at the end of the fed batch phase. The pO_2 control was not optimal and showed strong fluctuations at the end of the fed batch phase. This was caused by the slow control of the rocking rate, which was the final parameter in the cascade. Aerobe conditions were ensured.

The optode was not suitable for the control of the pH set point at 5.0, because the chemical composition of the patch was designed for the measurement range of 6.0 to 8.5. Hence, a pH set point of 5.0 is not suitable for chemosensors. In the induction phase the pH control was working correct

The target protein HSA was expressed successfully into the medium and was determinable by SDS-PAGE.

The occurred problems with the pH and pO_2 control lead to a repetition of the induced fed batch cultivation with a pH set point at 6.0. *P. pastoris* is able to grow in a pH range between 3 to 7. Additionally the pO_2 cascade was changed. The rocks were set to a fixed value after the batch time and the pO_2 was controlled by the supplementation of oxygen. The pO_2 was controlled reliable at the set point without fluctuations as well as the pH. The optode work reliable at the set point of 6.0 over the whole cultivation time.

The cell density reached 260.6 g_{WCW}/L in the fed batch phase and 284.0 g_{WCW}/L in the induction phase. The yield coefficient showed a further increase in comparison to other cultivations in the RM system. It reached 4.0 $g_{WCW}/g_{substrate}$ (0.88 $g_{DCW}/g_{substrate}$) in the batch phase. All yield coefficients in the RM system were significantly higher than in the D-DCU. A possible explanation is the reduced magnesium sulphate concentration in the medium for the RM cultivation. The higher concentration in the medium for the D-DCU has probably an inhibitory effect on the cell growth. In the second fed batch with induction phase a yield coefficient in the fed batch phase was calculated. It was 1.7 g_{WCW}/L or 0.57 g_{DCW}/L. This value is lower than the yield coefficient in the batch phase, which can be explained with the reduced μ. The energy demand for maintenance is independent of the μ and has therefore a higher percentage on the overall energy demand at low μ.

During the induction phase samples were taken to determine the protein expression. The results were similar to the first induced experiment. Both SDS-PAGE showed concentrations of approximately 0.2 g/L.

The cell densities were comparable to other *P. pastoris* high cell density cultivations. 381 g/L WCW was achieved in a single use stirred tank reactor (Dreher et al., 2013). In the BIOSTAT® RM 20 optical a WCW of 315.2 g/L was reached. The values were comparable and therefore high cell density cultivations are possible In conclusion the protein expression in the RM system was possible. It was possible to establish a protein production step in the BIOSTAT® RM 20 optical. The chemical defined medium and the cultivation system showed the ability for HCDC with following induction phase.

5.5 Batch cultivation inoculated with prepared cryo cultures

The seed train preparation is very time consuming. For the reduction of the process time, cryo cultures were evaluated in a batch cultivation. Cultivation broth was frozen at -80 °C in falcon tubes with glycerol as cryo protective agent.

A cultivation was inoculated with a cryo culture. The cultivation conditions were identical to the other cultivations in the RM system.

The maximal cell density was 101.2 g_{WCW}/L after 21.0 h and is comparable to the batch cultivation in the RM system before (see chapter 4.2.1). An average specific growth rate of 0.18 h^{-1} with a maximal value of 0.20 h^{-1} was determined. Also the specific growth rate is comparable to the batch cultivation. Glycerol was depleted completely resulting in a yield coefficient of 3.0 $g_{WCW}/g_{substrate}$.

The batch cultivation time was 1.2 h shorter in comparison to the batch with the classical seed train. Therefore, cryo cultures were suitable for the process time reduction and can serve as inoculum culture.

6 Outlook

On the basis of the previous experiments the strategy for following experiments can be worked out. The process and the medium were successful evaluated in small scale cultivations. The next step is a scale up of the process to the BIOSTAT® STR 50. This system is a single use stirred tank reactor. For this process transfer the feeding rate and the fixed specific growth rate have to be adapted to the specifications of the STR 50. This system has a k_La, which is between of the two used systems BIOSTAT® D-DCU 10-3 and BIOSTAT® RM 20 optical.

A mathematic model for the growth kinetics of *P. pastoris* can be established to optimise the growth and enhance the process comprehension. A requirement for the modelling is an efficient determination of the target protein concentration. Therefore, a quantitative measurement method has to be evaluated. A chromatographic method or a gel electrophorese with digital band intensity measurement are possible solutions.

If the quantification is established an optimisation of the pH is possible. The optimal pH set point for protein production has to be determined in context to the measurement limitations of the optodes. The organism has a wide pH range for the cell growth. Therefore, this parameter can be easy adapted to the optimum of the system or the protein expression.

Another approach for a higher protein expression can be a mixed feed profile with glycerol and methanol in the induction phase. Especially the used Mut^s strain is suitable for mixed feed profiles (Zhang et al., 2007) (Cereghino et al., 2002).

7 References

R. Brecht: Disposable Bioreactors: Maturation into Pharmaceutical Glycoprotein Manufacturing. book author Regine: Eibl and Dieter Eibl. Disposable Biorectors. Berlin : Springer-Verlag, **2009**.

G. P. L. Cereghino, J. L. Cereghino, C. Ilgen, J. M. Cregg: Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Current Opinion in Biotechnology. **2002**, 13, pp. 329–332.

J. L. Cereghino, J. M. Cregg: Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiology Reviews. **2000**, 24, pp. 45-66.

Chmiel, Horst: Bioprozesstechnik. München : Spektrum akademischer Verlag, Elsevier GmbH, **2006**.

O. Cos, R. Ramón, J. L. Montesinos, F. Valero: Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microbial Cell Factories. **2006**, 5:17.

Cregg, J.M. 2007: *Pichia* Protocols Second Edition. Totowa : Humana Press, **2007**.

T. Dreher, B. Walcarius, U. Husemann, F. Klingenberg, C. Zahnow, T. Adams, D. de Wilde, P. Casteels, G. Greller: Microbial high cell density fermentations in a stirred single use bioreactor. book author: Regine Eibl and Dieter Eibl. Disposable Bioreactors: Second Edition. Berlin : Springer-Verlag, **2013**

F. Dumont, P. Marechal, P. Gervais: Cell Size and Water Permeability as Determining Factors for Cell Viability after Freezing at Different Cooling Rates. Applied And Enviromental Microbilogy. **2004**, January, pp. 268–272.

Eibl, Regine and Eibl, Dieter: Disposable Bioreactors. Berlin : Springer-Verlag, **2009**.

R. Eibl, S. Kaiser, R. Lombriser, D. Eibl: Disposable bioreactors: the current state-of-the-art and recommended applications in biotechnology. Appl Microbiol Biotechnol. **2010**, pp. 86:41–49.

R. Eibl, S. Werner, D. Eibl: Bag Bioreactor Based on Wave-Induced Motion: Characteristics and Applications. book author: Regine Eibl and Dieter Eibl. Disposable Bioreactors. Berlin : Springer-Verlag, **2009**.

F. Garcia-Ochoa, E. Gomez: Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. Biotechnology Advances. **2009**, 27, pp. 153–176.

J. Glazyrina, E. Materne, T. Dreher, D. Storm, S. Junne, T. Adams, G. Greller, P. Neubauer: High cell density cultivation and recombinant protein production with *Escherichia coli* in a rocking-motion-type bioreactor. Microbial Cell Factories. **2010**, 9:42.

A. Glindkamp, D. Riechers, C. Rehbock, B. Hitzmann, T. Scheper, K. F. Reardon: Sensors in Disposable Bioreactors Status and Trends. book author: Regina Eibl and Dieter Eibl. Disposable Bioreactors. Berlin : Springer-Verlag, **2009**.

C. Guramkonda, A. Adnan, T. Gäbel, H. Lünsdorf, A. Ross, S. K. Nemani, S. Swaminathan, N. Khanna, U. Rinas: Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: Application to intracellular production of Hepatitis B surface antigen. Microbial Cell Factories. **2009**, 8:13.

S. R. Hamilton, T. U. Gerngross: Glycosylation engineering in yeast: the advent of fully humanized yeast. Current Opinion in Biotechnology. **2007**, 18, pp. 387-392.

M. C. M. Hensing, R. J. Rouwenhorst, J. J. Heijnen, J. P. van Dijken, J. T. Pronk: Physiological and technological aspects of large-scale heterologous- protein production with yeasts. Antonie van Leeuwenhoek. **1995**, pp. 261-279.

Z. Hubálek: Protectants used in the cryopreservation of microorganisms. Cryobiology. **2003**, 46, pp. 205–229.

S. Y. Lee: High cell-density culture of *Escherichia coli*. Trends in Biotechnology. **1996**, 3, pp. 98–105.

C. Löffelholz, U. Husemann, G. Greller, W. Meusel, J. Kauling, P. Ay, M. Kraume, R. Eibl, D. Eibl: Bioengineering Parameters for Single-Use Bioreactors: Overview and Evaluation of Suitable Methods. Chemie Ingenieur Technik. **2013**, Vol. 85, 1-2.

S. Macauley-Patrick, M. L. Fazenda, B. McNeil, L. M. Harvey: Heterologous protein production using the *Pichia pastoris* expression system. Wiley InterScience. **2005**, 22, pp. 249-270.

M. Mikola, J. Seto, A. Amanullah: Evaluation of a novel Wave Bioreactor® cellbag for aerobic yeast cultivation. Bioproces Biosyst Eng. **2007**, 30, pp. 231-241.

E. Nevoigt, U. Stahl, Ulf: Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae.* FEMS Microbiology. **1997**, 21, pp. 231-241.

Sartorius a: BIOSTAT® D-DCU Your “Fast Lane” to Production. **2011** a.

Sartorius b**:** Manual BIOSTAT® D-DCU and DCU-System. **2011** b.

Sartorius c: Optical pH sensor handling and troubleshooting guide BIOSTAT® Cultibag STR and RM. **2011** c.

H. G. Schlegel, C. Zaborosch: Allgemeine Mikrobiologie. Stuttgart : Gerog Thieme Verlag, **1992**.

S. Suh, M. Blackwell, C. Kurtzman, M. Lachance: Phylogenetics of Saccharomycetales, the ascomycete yeasts. Mycologica. **2006**, 6, pp. 1006-1017.

G. A. Truesdale, A. L. Downing, G. F. Lowden: The Solubility of Oxygen in Pure Water and Sea-Water. Journal of applied chemical. **1955**.

C. Zahnow: Characterization of the BIOSTAT® D-DCU. Göttingen : Sartorius, **2011**.

W. Zhang, M. Inan, M. M. Meagher: Rational Design and Optimization of Fed-Batch and Continous Fermentations. book author: James M. Cregg. Pichia Protocols Second Edition . Totowa : Humana Press Inc., **2007**.

7.1 Figure Index

7.2 Table Index

8 Supplement

8.1 Material

Number	Equipment	Producer
1	Autoclave	Fedegari
2	Balance EA60EDE-I	Sartorius
3	Balance Genius ME215S	Sartorius
4	Balance LA5200 P	Sartorius
5	Balance LA620 S	Sartorius
6	BioSealer (Parameter version P3)	Sartorius
7	BIOSTAT® D-DCU 10-3	Sartorius
8	BIOSTAT® RM 20 optical	Sartorius
9	BioWelder	Sartorius
10	Centrifuge Biofuge Pico	Heraeus
11	Certomat T plus	Sartorius
12	C-Flex tube	Saint Gobain
13	Cleanbench Herasafe KS18	Thermo Scientific
14	CultiBag RM 10L optical DBO010L	Sartorius
15	Enzyme kit glycerol	Biopharm
16	Erlenmeyer flasks with baffles	Schott
17	Freezer (-80 °C) Tus 80-100	Fryka
18	Frigomix 2000	Sartorius
19	Incubator	Köttermann
20	Inoculation bottles	Schott

21	Magnetic stirrer	IKA Labortechnik
22	Marprene tube	Watson Marlow
23	Mini-PROTEAN System + PowerPac 300	Bio Rad
24	Minisart 0.2 µm	Sartorius
25	Moisture meter MA100	Sartorius
26	Peristaltic pump Watson Marlow 205U	Watson Marlow
27	Peristaltic pump Watson Marlow 520U	Watson Marlow
28	Petri dishes	Sarstedt
29	pH and pO_2 probes	Hamilton
30	pH-Meter PB-11	Sartorius
31	Photometer Ultrospec 1000	Pharmacia Biotech
32	Pipet	Eppendorf
33	Polystyrene cuvette	Roth
34	Sartopore Platinum BH4 0.45 µm + 0.2 µm	Sartorius
35	Schott bottles	Schott
36	Silicon tube	Watson Marlow
37	Single use inoculation loop	Roth
38	Single use syringe	Braun
39	Water bath	Memmert