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DIPLOMARBEIT

Studying the effects of frequency and amplitude of periodic oxygen-related stress on recombinant protein production in *Pichia Pastoris*

Ausgeführt am Institut für

Verfahrens-, Umwelttechnik und technische Biowissenschaften

der Technischen Universität Wien

unter der Anleitung von

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This thesis is dedicated to my husband, Ing. Hakan Çekici.

Vienna, June 2014 **Gülbahar ÇEKICI**

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Abstract

Dynamic variations of operating conditions in which microorganisms are living and growing influence the performance of the culture. These variations impose stress on cells and can render positive or negative metabolic actions.

The aim of this work was to apply oxygen-related stress on the cells of *Pichia pastoris* periodically by means of a two-compartment system (*a single reactor coupled with a Plug Flow Reactor)* in order to observe the effects on protein quality and quantity. Specifically we aimed at investigating different amplitude (the dissolved oxygen level in the reactor) and frequency (residence time of broth in Plug Flow Reactor) of stress on cells to understand how the specific productivity, titer and cell physiology change. The results were compared with the reference run that is a production strategy executed in normal single reactor (one-compartment system).

The influence of different dissolved oxygen levels in reactor was going to be explored. There were no major differences in the specific productivity and titer (total activity) observed between the experiments, which were made at different dissolved oxygen (DO) levels in the one-compartment system. The analysis showed, that the DO level in one-compartment system does not play an important role as long as the culture is not oxygen limited. However, the results of the two-compartment experiments demonstrated that the protein production can be increased to several times without influencing the physiology of the cells. In the two-compartment system, the dissolved oxygen in the Plug Flow Reactor (PFR) was zero.

A multivariable data analysis was performed to check the accuracy of the results, and the analysis reflected that the oxygen-related stress increase the protein quality and quantity. The frequency of the stress (residence time), however, has no influence on these variables. It is assumed that it does not matter how long the cells stay in this equilibrium state however it is important to only experience the oxygen-related stress.

Environmental conditions such as temperature, pH, or oxygen content in reactor, play an important role in the cellular production. Therfore, the changes in these parameters can lead to the enormous changes in the cell metabolism. To compare the different setups, such as one-compartment system and two-compartment system, it is necessary to ensure that the cell physiology is the same in both contruction. To that end, there are physiological parameters such as biomass yield and carbon dioxide yield ($Y_{X/s}$ and $Y_{CO2/s}$), which are determined off-line. The results of Multivariate Data Analysis were also demonstrated that the $Y_{X/s}$ and $Y_{CO2/s}$ are the same in all experiments, and thus the physiology of the cells remains unchanged.

In two-compartment system, the circulation of the fermentation broth between two different zones, the main reactor as homogenously aerated zone and the PFR as oxygen-free zone, caused an oxygen-related stress on the cells. The changes in environmental conditions caused by the periodic circulation led to remain the cells in their active state over the process time and thus to more protein production. The increase in the production of horseradish peroxidase (HRP) in *Pichia pastoris* due to the circulation was remarkable, and that meant the two-compartment setup should be further developed. Since the DO in PFR is always kept to zero, the critical factors in the decision of an optimal two-compartment system are the DO level in the main reactor (amplitude) and the residence time, which the cells in the PFR spend (frequency). The analysis showed that the high and low level of DO

(70% and 5%) influences negatively and the intermediate level of DO (25%) results in two-fold increase in the specific productivity and titer (quadratic effect).

The two-compartment system as a novel process technology is a proper tool to apply oxygen-related stress and successfully operates in laboratory-scale bioreactors. Hence, the analysis showed that this new proposed methodology could be implemented in the large-scale systems with less investment costs and more income, which represent crucial factors for biopharmaceuticals.

1 Introduction

The fast and low-cost production of recombinant proteins in both at laboratory scale and also industrial scale requires the proper selection of a suitable host cell. The yeasts are considered as a simple powerful expression tool for the production of many recombinant proteins since the development of modern biotechnology in scientific research.

With regard to fermentation, there are several factors affecting production yield, including culture medium composition, strain type, and non-nutritional factors, such as culture pH, agitation rate, dissolved oxygen, methanol induction, and fermentation strategy (Li et al. 2007).

As described in the numerous reviews, dissolved oxygen (DO) is an important operation parameter for process optimization and has a great influence on cell metabolism in *Pichia* cultivations. Redox processes play a major role in heterologous protein production, both related to the oxidation of the product to form disulfide bonds, and to oxidative stress of the host cell during cultivation (Gasser et al. 2008). If in a process, the dissolved oxygen amount is not sufficiently provided, it may lead to a micro-environmental stress factor, which renders positive or negative metabolic actions. Generally dissolved oxygen should be kept consistently above 25 percent to avoid oxygen limitation but different proteins need different optimal levels that can only be determined by experiments (Anumanthan et al. 2007).

During the recent years, it has become evident that a variety of metabolic and environmental stresses may have a strong impact on recombinant protein production (Gasser et al. 2008). To day, the oxygen-limitation represents an interesting platform to investigate how the cells respond to the oxygen-related stress conditions in terms of their physiology and productivity. However, there are conflicts about the influence of oxygen limitation and consequent stress on the productivity of the cells. Cultivation of *Pichia pastoris* under at a hypoxic condition was reported by Baumann et al. 2007. Baumann et al. 2007 explained that hypoxic conditions in a fed batch fermentation lead to a three- to six fold increase of specific productivity of protein secreting by *P. pastoris* and threefold reduced fed batch times. Although increased specific volumetric productivity of the target recombinant protein was observed, this was at the expense of the reduction in biomass. Charoenrat et al. 2005 also recommends an oxygen-limited fed-batch technique to improve the oxygen transfer rate and productivity.

Lee et al. 2003, reported the effect of high dissolved oxygen tension on the expression system of the model protein elastase inhibiting peptide (EIP) in *Pichia pastoris*. They reported, that higher DO tension simulated the methanol utilization pathway and enhanced the expression titre of EIP about three-fold. However, there was a need to add pure oxygen in the air to maintain a high DO setpoint.

Production of a single-chain antibody fragment in *P. pastoris* under low dissolved oxygen condition, where the methanol concentration was controlled at different levels, was done by Trentmann et al. 2004 and the feed rate was the manipulated variable. They found production of recombinant protein was unaffected by oxygen limitation. Similar approach was applied and the same results were found by Hellwig et al. 2001 for production of recombinant scFv antibody fragment.

However, there is no reported work studying the effects of different DO levels on recombinant protein production. It has been no method proposed, which results in a certain DO in a single reactor

(one-compartment system) an increased amount of recombinant proteins and boost the protein quality. If DO in the one-compartment system plays an important role to the improvement of productivity, it should be experimented and found out how much they should be set in the reactor so that optimum productivity is obtained. Running the process at 0% DO is not recommended due to the risk of methanol accumulation, acting lethal to the cells. For this reason, a two-compartment system is concluded to realize oxygen related stress: A single reactor coupled with a plug flow reactor (PFR) in which a portion of the broth is circulated continuously between the reactor and PFR.

1.1 *Pichia pastoris*

The methylotropic yeast, *Pichia pastoris,* is one of the mostly occurring yeast hosts in practice and industrial biotechnology due to its advantageous properties. *P. pastoris* is often used for the production of recombinant proteins, for example, due to its fast growth rate, where the reactor is being driven in fed-batch operation to ensure exponential growth. Other important characteristics are: the ability to produce foreign proteins at high levels, either intracellularly or extracellularly (Cereghino et al. 1999), ease of genetic manipulation of well-characterized yeast expression vectors, as well as elimination of endotoxin and bacteriophage contamination (Li et al. 2007).

A further characteristic of *P. pastoris* is the availability of an alcohol oxidase 1 gene (AOX1), which plays the main role in the expression of the target protein. AOX1 promoter makes up to more than 90% of the alcohol oxidase enzyme (Cos et al. 2006), which catalyzes the first step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide (Cereghino et al. 1999).

Three different phenotypes of *P. pastoris* strains are well known, regarding their utilization of methanol: The wild type or methanol utilization plus phenotype (Mut^+), and those resulting from deletions in the AOX1 gene (methanol utilisation slow Mut^s) or both AOX genes (methanol utilisation minus Mut^-) (Cos et al. 2006). According to Li et al. 2007, strains with deleted AOX genes are sometimes better producers of foreign proteins than wild type strains and require much less methanol to induce expression.

For the performed experiments the *P. pastoris* phenotyp Mut^s with a plasmid containing the gene for the horseradish peroxidase isoenzyme C1A (HRP) was used.

1.2 HRP (Horseradish Peroxidase)

HRP belongs to the plant peroxidases and is mainly isolated from the roots of horseradish, Amoracia rusticana. There are a number of distinctive peroxidase isoenzymes from horseradish roots of which the C isoenzyme (HRP C) is the most abundant (Veitch et al. 2004).

HRP is a heme-containing enzyme that utilizes hydrogen peroxide to oxidase a wide variety of organic and inorganic compounds, such as aromatic phenols, phenolic acids, indoles, amines and sulfonates (Veitch et al. 2004). The reaction is a three-step cyclic process, in which the enzyme is first

oxidised by H_2O_2 and then reduced back to the native form in two sequential steps involving the formation of two enzyme intermediates, Compounds I and II (Azevedo et al. 2003).

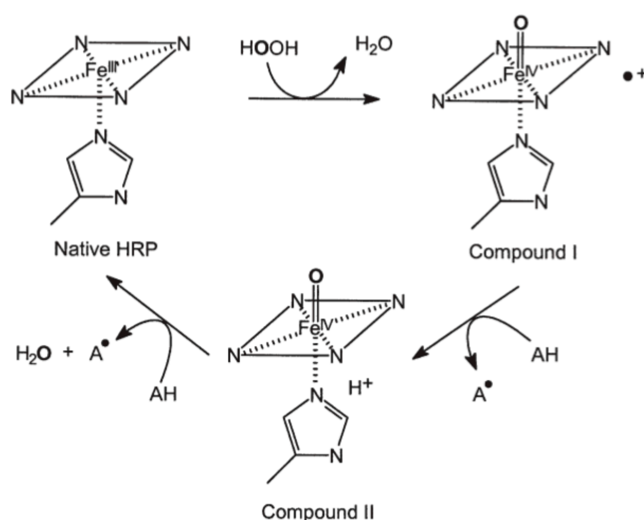


Figure 1: Reaction cycle of HRP, showing the enzyme intermediates, Compounds I and II (Azevedo et al. 2003).

While Compound I is a high oxidation state intermediate comprising an Fe(IV) oxoferryl centre and a porphyrin-based cation radical, Compound II represents an Fe(IV) oxoferryl species that is one oxidising equivalent above the resting state (Veitch et al. 2004).

This cycle in figure 1 can be simplified in the following steps:

$$\text{HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O \qquad (1)$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH\bullet \qquad (2)$$

$$\text{Compound II} + AH_2 \rightarrow \text{HRP} + AH\bullet, \qquad (3)$$

where AH_2 is an electron donor substrate and AH• is the radical reaction product (Zakharova et al. 2011). Further information to the catalytic mechanism of HRP can be found in the literature.

HRP as a multifunctional plant enzyme has been used in many ways. It is used not only analytically, in clinical diagnosis or enzyme assays but also in cancer research. According to Veitch et al. 2004, a combination of horseradish peroxidase and indol-3-acetic acid or its derivatives is currently being evaluated as an agent for use in targeted cancer therapies.

1.3 Process design

Different coupled reactor systems such as stirred tank reactor/stirred tank reactor (STR/STR) and stirred tank reactor/plug flow reactor (STR/PFR) have been described in the literature and used as scale-down tools (Bylund et al. 1999) to study different parameters such as substrate gradients (George et al. 1993) or oxygen transfer (Sandoval-Basurto et al. 2005). For example, by George et al. 1993 a two-compartment system was presented, which consist of a PFR and a STR, between which the medium is circulated to study of substrate concentration gradients on *Saccharamyces cerevisiae.* In this present work of George et al. 1993, the PFR was an aerated zone, where the medium is mixed with the substrate feed and the oxygen, while in our work the air bubbles are completely removed before the culture medium was pumped into the PFR, and thus a completely oxygen-free zone was ensured. Another two-compartment system was proposed by Sandoval-Basurto et al. 2005 to study of dissolved oxygen tension (DOT) gradients on the metabolic response of *E. coli*. Sandoval-Basurto et al. 2005 used two interconnected STRs; one of them maintained at anoxic conditions while the other presented an anerobic zone, between which the cells are circulated at different circulation times.

The different two-compartment systems used by some authors were mainly *Saccharomyces cerevisiae* and *E. coli*. For example, Bylund et al. 1999 taken *E. coli* as a model system and studied such as George et al. 1993, the effect of substrate gradients in a two-compartment system, composed of one STR an aerated PFR to avoid oxygen limitation.

A two-compartment consist of a STR and a PFR with the model system *Pichia pastoris* has been first described by Lorantfy et al. 2012, to investigate the effect of oxygen limitation on the culture performance quantitatively. Lorantfy et al. 2013 has studied with the aid of repetitive batch experiments the impact of dissolved oxygen concentration by different setup configurations and different residence times in the PFR. The conclusion of this work was that the overall performance was not affected by either slight change in residence time or small variation in DO.

Also Lara et al has published several reports in which the effect of dissolved oxygen has been studied. Lara et al. 2006-b has investigated the impact of dissolved oxygen tension (DOT) gradients on the culture performance of recombinant *E. coli* in a two-compartment system, in which the cells continuously circulated between two interconnected STRs, an anerobic (0% DOT) and an aerobic (10% DOT) vessel. The conclusion was that the oscillating DOT fermentation caused an increase between 1.5- to ever 6-fold of transcription levels of studying genes on *E.coli*. Differences in transcription levels between aerobic and anaerobic compartments were also observed, indicating that *E. coli* can respond very fast to intermittent DOT conditions (Lara et al. 2006-b).

So far, two-compartment systems were used only to simulate the inhomogeneities in large-scale bioreactors and therefore the residence time was set constant in such a way that it mimics the micro-environmental changes in large bioreactors (Neubauer et al. 2010). To our knowledge, there is no work investigating the effects of amplitude and frequency of oxygen-related stress in a two-compartment system.

In this work a two-compartment system was proposed to apply stress with the aim of obtaining higher titer. The previous related work done by Jazini et al. 2013-b showed that circulation of broth between two zones of oxygen limited (0% dissolved oxygen in PFR) and non-oxygen limited (25%

dissolved oxygen in bioreactor) in a certain residence time (3.3 min) increases the specific productivity.

The circulation is actually a means of applying periodic stress to the cells, which is characterized with the residence time and amplitude. Residence time represents the frequency of stress imposed to the cells and the difference in DO between reactor and loop represent the amplitude of stress applied to the cultures. Now the question is how the effects of higher amplitude and frequency of oxygen-related stress on the productivity are.

1.4 Goal

In this work, oxygen-related stress was studied to improve the product quality and quantity of a plant derived Horseradish Peroxidase (HRP) by methylotropic yeast *P. pastoris*. There should be proposed a new process technology, which presents the optimal tool with a certain amplitude (strength of the applied dissolved oxygen) and frequency (duration of the stress) in accordance with the two-compartment system to boost the protein quality and quantity.

It should first be clarified, whether dissolved oxygen is a relevant process parameter for production of recombinant protein in a one-compartment system. Due to the lack of information in the literature, the influence of different DO levels on productivity and physiology of cells in one-compartment system should first be carefully experimented and discussed in detail.

A two-compartment technique (a single reactor coupled with a PFR) is used and compared with the conventional one-compartment system (a single reactor) to study if DO level in reactor (amplitude of stress) and residence time of broth in the PRF (frequency of the stress) are relevant and significant process parameters for optimization. The residence time in one-compartment system is irrelevant, since the circulation of the broth with this setup is not possible.

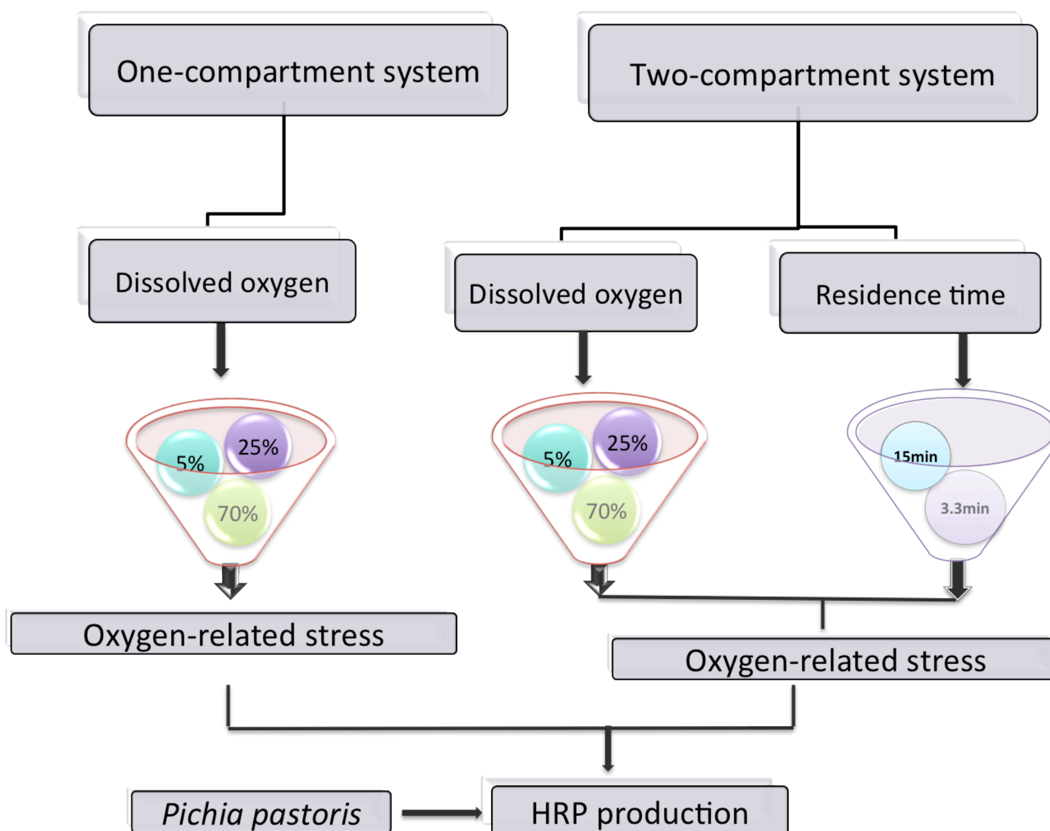


Figure 2: General formulation of the work

In two-compartment system, the circulation of the broth between the main bioreactor as homogeneously aerated zone and the PFR as oxygen-free zone triggers a stress. The amplitude and frequency of this stress are key parameters in two-compartment system, with which the strength

and duration of the oxygen-induced stress can be adjusted so that the productivity of the cells is increased. Another aim of this work was the investigating of the different amplitude and frequency of stress on cell physiology and product quality and quantity of recombinant protein production in *P. pastoris* under control of AOX1 promoter by using a two-compartment system.

Finally, the results from the experiments and the statements should be analyzed and checked for accuracy by application of a design of experiments (DoE).

2 Materials and methods

2.1 Microorganism

In this work, the strain used is *Pichia pastoris* KM71 H (arg4 aox1: arg4)/Mut^S with a plasmid containing the gene for the horseradish peroxidase isoenzyme C1A (HRP) and was gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria), (Dietzsch et al. 2011-a). The Target protein HRP will be secreted into the medium and isolated by centrifugation of the fermentation broth.

The stock cultures were replicated by preparing from 0.5 ml pre-culture and 0.5 ml 50% sterile glycerol and stored in cryo vials deep-frozen at -80°C.

2.2 Culture media

The medium composition for pre-culture:

1M Phosphatbuffer, pH=6	10 ml
10*YNB sol with $(NH_4)_2SO_4$ without amino acids	10 ml
500*Biotin solution	0.20 ml
Glycerol 20 % w/v	10 ml
Water, autoclaved	69.8 ml

Table 1: Medium for pre-culture

Batch medium per liter:

Glycerol (98%)	40.8 g
H_3PO_4 (85%)	26.70 ml
$CaSO_4 \cdot 2H_2O$	1.18 g
K_2SO_4	18.20 g
$MgSO_4 \cdot 7H_2O$	14.90 g
KOH (85%)	4.86 g
Antifoam Structol J673	150 µl
PTM1 (trace elements)	4.35 ml

Table 2: Batch medium

The trace elements solution (PTM1) may not autoclaved and should be filter sterilized before use.

Trace elements solution (PTM1) per liter:

$CuSO_4 \cdot 5H_2O$	6.0 g
NaI	0.08 g
$MnSO_4 \cdot H_2O$	3.0 g
$Na_2MoO_4 \cdot 2H_2O$	0.2 g
H_3BO_3	0.02 g
$CoCl_2$	0.5 g
$ZnCl_2$	20.0 g

$FeSO_4 \cdot 7H_2O$	65.0 g
Biotin	0.2 g
H_2SO_4	5 mL

Table 3: Components of trace element solution (PTM1)

Glycerol feed composition per liter:

Glycerol (95%) Merck	714.29 g
Antifoam Structol J673	300 µl

Table 4: Components of glycerol feed

Methanol feed composition per liter:

Methanol	300 g
PTM1 (trace elements)	12 ml
Antifoam Structol J673	300 µl

Table 5: Components of methanol feed

Base feed:

NH_4OH	5M

Table 6: Component of base feed

Shortly after the start of the induction phase was a 5 ml of 1 mM δ-Aminolevulinic acid (δ-ALA) solution prepared and added into the reactor in a sterile manner. The detailed explanation of medium and feed composition can be found by Dietzsch et al. 2011-a.

To obtain the glycerol concentration of batch medium was using a calibration curve determined by previous experiments using density.

$$c_{glycerol}\,[g/l] = 4.898\, *\, \rho_s\,[g/l]\, -\, 4936.7 \qquad (1)$$

The density measurement of the batch medium was performed before autoclaviation, and that´s of glycerol feed after autoclavation. To this purpose, a 25 ml and a 50 ml volumetric flask were pre-weighted with a balance to determine the dry weight. The flasks were filled with medium as accurately as up to mark and then reweighted. The density is calculated from the difference in weight between the full and empty volumetric flask and its volume. Methanol feed and base solution were not autoclaved.

To determine the concentration of the base solution was a need to dilute this solution to one-tenth of its original concentration. For this aim, 2 ml of ammonium hydroxide solution (NH_4OH) with a few drops of an indicator called Bromothymol blue was titrated with a 0.25M solution of potassium hydrogen phthalate (KHP) in a 20 ml beaker with a magnetic stirrer. When the solution in the beaker turned from blue to green the end point has been reached. At this point the amount of KHP solution taken from the burette is recorded.

The molarity of base solution was estimated as follows:

$$molarity\ base\ [mol/l] = f\ *\ \frac{molarity\ of\ KHP\ [mol/l] * base\ consumption(KHP)\ [ml]}{volume\ base\ (diluted,\ \ before\ titration)\ [ml]} \qquad (2)$$

where f is the dilution factor of base before titration.

2.3 Fermentation conditions

The fermentation process of *P. pastoris* to the production of recombinant protein is carried out in four steps. It is started with a glycerol-batch phase, where the cells become accustomed to the glycerol and achieve a defined cell density after a certain time. Second step is a glycerol-fed-batch phase to achieve a sufficiently high cell density. In the third step, is started with a slow addition of methanol to adapt the cells to the methanol. In the final stage, the cells are fed for a long time in an induction phase with a certain higher flow rate of methanol feeding to produce the target protein (see figure 7: Fermentation strategy).

In this study, two-compartment experiments were carried out in a highly instrumented autoclavable laboratory bioreactor (Infors, Switzerland) with a working volume of 3 liter. The bioreactor was connected to the Process Information Management System (Lucullus, Biospectra AG, Switzerland) in order to monitoring and control the process. For one-compartment experiments was used both 3 liter Infors-bioreactor as well as 2.3 liter DASGIP-bioreactors (see figure 3 and figure 4).

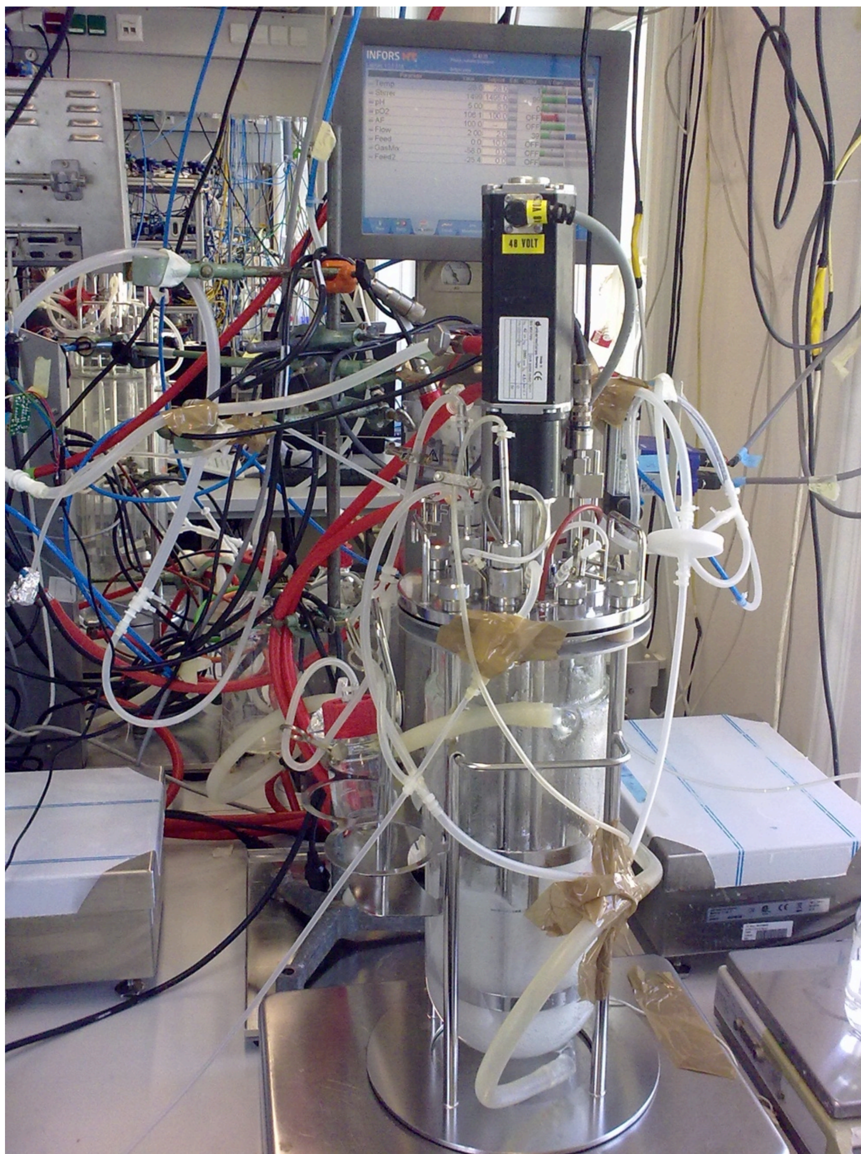


Figure 3: This figure shows a one-compartment experiment using 3 liter Infors-bioreactor during batch phase.

Figure 4: DASGIP-experiments as one-compartment system using 2.3 liter DASGIP-bioreactor. The DASGIP experiments had the advantage that you could run multiple reactors with different parameters such as DO, pH, T, simultaneously: Four one-compartment experiments were conducted at the same time with different DO level (5%, 25%, 70% and uncontrolled but above 25%).

The cultivation temperature was set to 28 °C through the jacket surrounding the bioreactor to avoid temperature limitation. The culture was aerated with 2 vvm air flow through the sparger by using of filtered air and agitated at 1450 rpm (see table 7) to keep the fermentation broth homogeneous. The specific substrate uptake rate is maintained at 0.5 mmol/g.h in adaptation phase and at 1 mmol/g.h during the whole induction phase to control the glycerol and methanol feeding rates. The pH level was kept constant at 5 by means of PID controller by addition of ammonium hydroxide solution to the bioreactor.

Parameter	
Temperature (°C)	28
Agitation (rpm)	1450
pH	5

Table 7: Cultivation parameters

The off gas passed through the gas analyzer (Sevomex, M. Müller AG, Switzerland) to quantify CO_2 and O_2 content, using infrared and paramagnetic principles, respectively

All cultivation parameters, like reactor, feed and base weight, temperature, pH, dissolved oxygen, agitation speed as well as CO_2 and O_2 in the off gas were measured und monitoring in Lucullus Process Information Management System (PIMS, Biospectra AG, Switzerland).

The feed rates, like base, glycerol and methanol, were calculated from the signal of a balance on which the feed solution was placed.

The bioreactor vessel was autoclaved at 121 °C for 20 min. Before autoclaving, a pressure test was carried out to check for air-proof of the bioreactor.

The calibration of pH sensors was performed outside the bioreactor vessel using the pH buffer solutions of pH 7 and 4 before autoclavation.

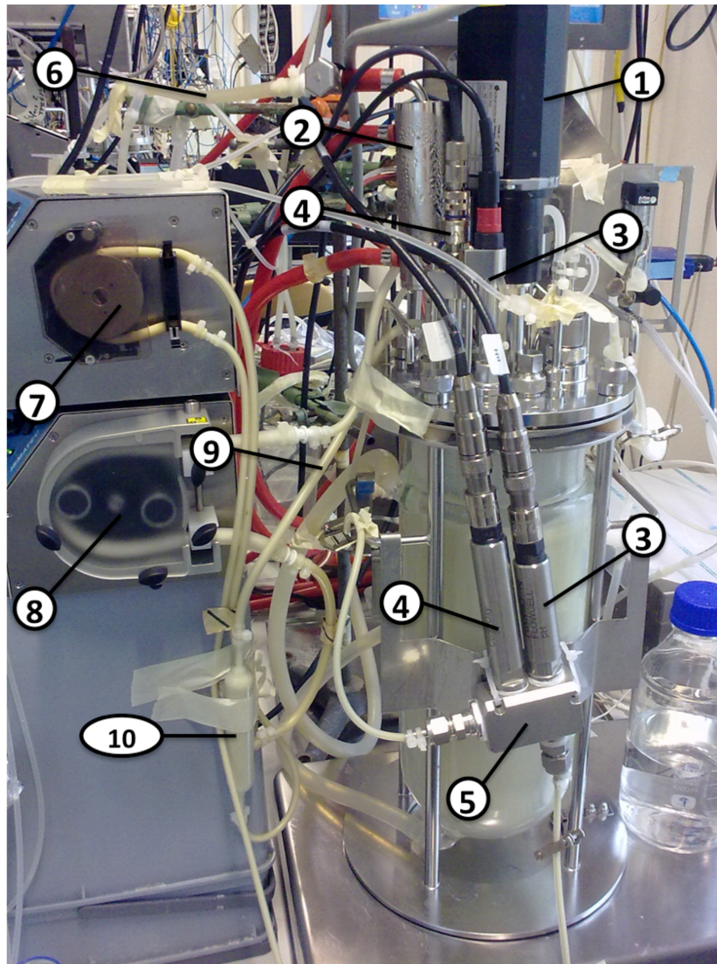
The DO (dissolved oxygen) in bioreactor was calibrated at 0% by using of pure N_2 and at 100% by using of air. After calibration, DO in bioreactor remained at approximately 100% until inoculation. An agitation cascade was selected in the controller to maintain DO at setpoint through automatic adjustment of agitation speed. When the desired dissolved oxygen was no longer possible to create with the agitator, pure oxygen was added into the inlet air controlled by means of a mass flow controller.

Cultivations were conducted with an initial working volume of about 1 Liter. A 1000 ml shake flask with baffles, containing 100 ml pre-culture medium, was inoculated with 1 ml of inoculum (frozen stokes, at -80°C) under a laminar flow. After inoculation, the shake flask was incubated for 24h at 28 °C on Multitron Standard (Infors HT) Incubator at 230 rpm so the cells grow and reproduce. After 24h, twice 5 ml of the preculture was distributed in 2 sterile pre-weighted glas tubes and centrifuges for 10 min at 5000 rpm at 4°C to determine the biomass concentration at the beginning of batch phase. With the inoculation of preculture into the reactor already started the process with a batch phase.

Trace elements solution was filtered and transferred to the reactor through a septum using a 0,2 µm filter and a sterile syringe with a needle.

2.4 Experimental setup of the two-compartment system

As mentioned earlier, there are to find different two-compartment systems in the literature, and the interest in two-compartment systems grows with the increasing number of successful experiments. In our work, it comprises a main reactor, a bubble trap to eliminate the air bubbles, a PFR to realize an oxygen-related stress, and a flow cell to measure of DO and pH of the broth after the PFR (see figure 5).

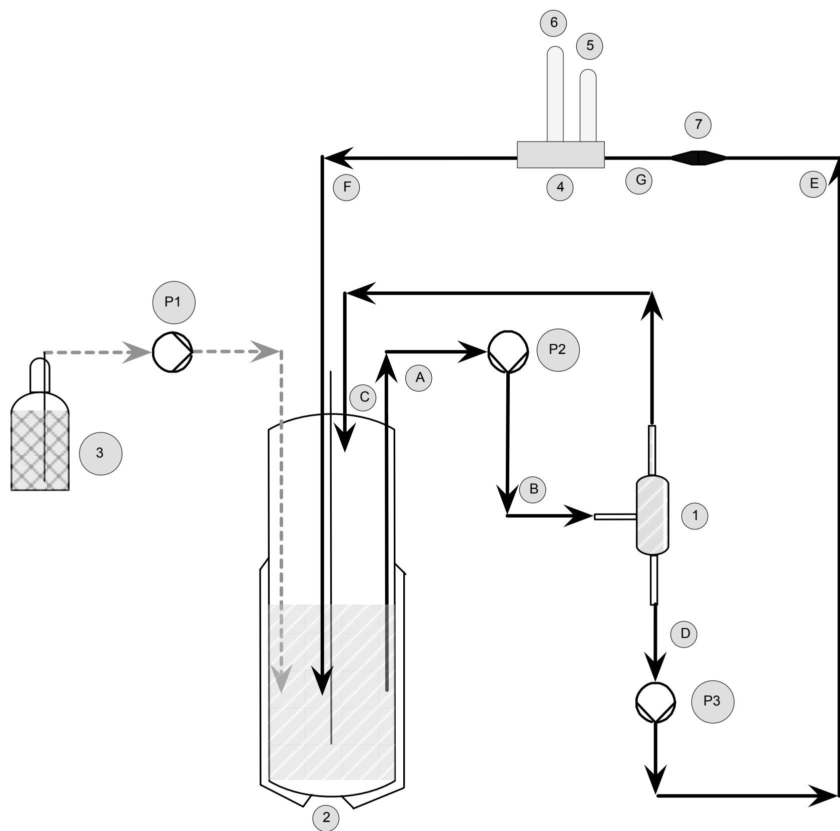


① Stirrer
② Cooler
③ pH sensor
④ DO sensor
⑤ Flow cell
⑥ Off-gas
⑦ Pump2
⑧ Pump1
⑨ Bypass
⑩ Bubble trap

Figure 5: This figure shows a two-compartment experiment using 3 Liter Infors-bioreactor with a detailed marking.

The schematic view of the two-compartment system has been depicted in figure 6 in detail. The two-compartment experiments were conducted only in a 3 Liter Infors-bioreactor.

The fermentation broth was pumped out of the fermenter into the plug flow reactor (PFR), which had a holding volume of 90 mL. PFR is in fact a 3 m long silicone tube with 4.4 mm in diameter. Temperature of the PFR was maintained at 28 °C by using a water bath (LAUDA, Germany) to keep the temperature of the broth in PFR in the same with the in main reactor. Since the fermenter contents are stirred constantly, this leads to the formation of many bubbles in the reactor. To create an efficiently oxygen limitation should be avoided that the air bubbles get into the PFR. A bubble trap was used to allow the entrained gases in fermentation broth to escape so as not to introduce air into the PFR. In order to eliminate bubble has two pumps used. Pump2 was used to flow back the bubble-containing stream into the bioreactor via a bypass line (Jazini et al. 2013-b). Pump3 (a peristaltic pump) was previously carefully calibrated to set the desired residence time exactly, and allows the circulation of bubble-free fermentation broth from the bubble trap to the PFR. The pump setpoint, the flow rate of the pumps through the PFR, the dimensions of the PFR, as well as the diameter and kind of the used tubings were detailed described in figure 6. The circulation was only started after adaptation phase, and performed during entire induction phase until the end of the experiment. The flow cell was connected right at the end of the PFR, and it allows the measurement of the pH and DO of the fermentation broth after the PFR.



Plug Flow Reactor

1 Bubble trap

2 Fermenter

3 Feed bottle

4 Flow cell

5 pH sensor

6 pO2 sensor

7 Connector

P1 LAMDA pump, preciflow, silicon tubing, ID = 2.5 mm, OD = 4 mm

P2 ISMATEC pump, Ecoline VC-280, (10%, 230ml/min, 13 sec res. time in bypass),
Tygon tubing 6.4*11.1*2.35

P3 ISMATEC pump, Ecoline VC-360, (7%, 21.4 ml/min, 3.3 min res. time),
(2%, 4,7ml/min, 15 min res. time) Pharmed, ID = 3.5 mm, OD = 6.5 mm, L = 20.5 cm

A Silicon tube, ID = 4.5 mm, OD = 7mm, L = 29 cm

B Silicon tube, ID = 5.5 mm, OD = 8mm, L = 24 cm

D Silicon tube, ID = 4.5 mm, OD = 7mm, L = 25 cm

E Silicon tube, ID = 3.5 mm, OD = 6mm, L = 7.53 m

F Silicon tube, ID = 3.5 mm, OD = 6mm, L = 46 cm

G Silicon tube, ID = 3.5 mm, OD = 6mm, L = 32 cm

A-B-C 50 ml

D-E-F 70 ml

Figure 6: Schematic view of the two-compartment system: A single fermenter coupled with a Plug Flow Reactor (PFR). Bubble elimination in PFR is done by means of a bubble trap (1). Bubble containing stream flows back to the fermenter via a bypass line (gray dashed line).

2.5 Process strategy

The fermentation strategy that we have in this study performed, consists of four phases (see figure 7): Each experiment started with a batch phase based on glycerol and run about 22 hours such a long time until the submitted glycerol completely consumed and finally the CO_2 signal decreases. Thereafter was the fed-batch phase started with an initial biomass density of about 20 g/l, and as long as the cells were fed with glycerol to reach a high cell density of about 63 g/l. In the third phase, the glycerol feed was stopped and the cells are grown at a slow flow rate of methanol feed. The feed flow rate through the STR was adjusted so that during the adaptation phase a substrate uptake rate (qs) of 0.5 $mmol.g^{-1}.h^{-1}$ is maintained. Methanol-feed should be added slowly at the beginning, so that the cells adapt to the new carbon source, furthermore methanol is lethal at high levels in the reactor to the cells. Finally, after approximately 10 hours adaptation time, the induction time at higher flow rate of methanol feeding with qs of 1 $mmol.g^{-1}.h^{-1}$ begins, whereby the production of the target protein is accelerated.

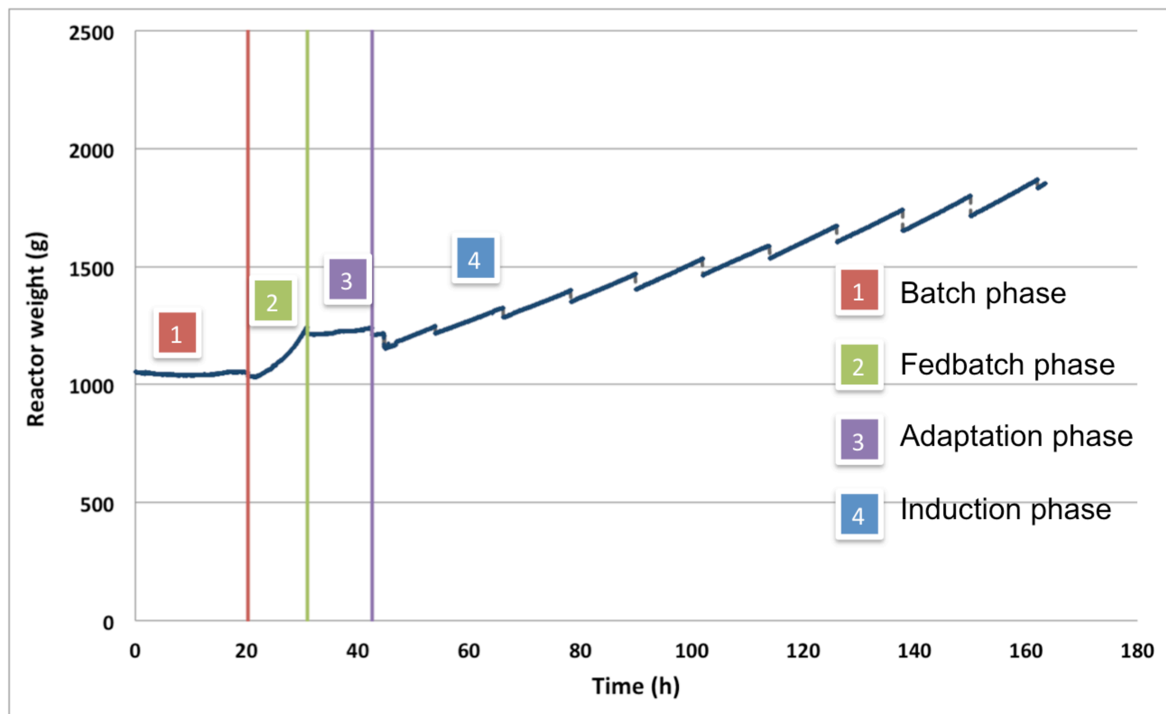


Figure 7: Fermentation strategy

2.6 Sampling

The first sample for biomass determination was taken from the pre-culture. The next one at the end of the batch, then at the end of the fed-batch and adaptation phase and afterwards the sampling was done in 12 h intervals during the induction phase. The stairs during the induction phase in the figure 7 indicate the process times where the samples were taken. Sampling was conducted through an immersion pipe from fermenter into a 100 ml schott bottle. The remaining broth in the sampling line

was flushed back over a bypass by inlet air stream. Each sample was then assayed according to the biomass concentration, protein content, protein activity and substrate concentration.

2.7 Analytical methods

2.7.1 Off-line measurements

2.7.1.1 Biomass determination

Biomass concentration was estimated by measuring the absorbance of the broth at 600 nm (OD600) in a spectrophotometer and by gravimetric analysis by using a microwave as a quick method as well as an oven as a reference method.

The optical density measurement was performed at a Hitachi 1100 UV/VIS spectrometer at a wavelength of 600 nm. In this purpose, the samples were diluted with deionized water to a final OD600 of 0.2-1.0. The correlation between OD600 and biomass concentration had been determined over an established linear regression experimentally before:

$$c\ [g/l]\ = (OD600 \times 0.3507 \times dilution) - 0.0203 \qquad (3)$$

Since the values of OD measurements were sometimes unreliable, the biomass concentration was determined mainly by microwave method.

The determination of the cell dry weight by microwave as a quick method was performed according the SOP (Standard Operation Procedure) by Herwig et al. The samples of culture broth (4x0.5 ml) were added to pre-weighted glass tubes, suspended with 2 ml deionized water and centrifuged at 5000 rpm for 10 minutes using a Sigma 3K30 centrifuge with the rotor 11156. The supernatant was removed carefully from the top. The cell pellets were re-suspended in 5 ml of deionized water using a vortex to remove salts and centrifuged again. The pellets in tube were dried for 10 minutes in a microwave (Philips, Netherland) at 500 Watts and then another 10 minutes at 900 Watts. Afterwards the tubes containing the cell pellets were dried in a desiccator for a short time (5-10 min) until they have cooled, before being weighted.

The oven method as a reference method to determine the cell dry weight is similar to quick method and was carried out simultaneously. 2 ml of culture broth were added to pre-weighted glass tubes and centrifuged at 5000 rpm for 10 minutes using a Sigma 3K30 centrifuge with the rotor 11156. The cell pellets were re-suspended twice with 2 ml deionized water and centrifuged again at the same conditions. The supernatant were discarded and the tubes containing the cell pellets were dried for 72 hours at 105°C oven (Heraeus, Germany) to constant weight. All these steps were performed in duplicate.

2.7.1.2 Protein determination

Proteins were secreted into the fermentation broth and separated from the biomass by centrifugation. To determine the extracellular compounds, 4x1.5 ml of the samples were centrifuged at 14000 rpm for 10 min in Eppendorf tubes using a centrifuge. The resulting supernatant was transferred to 3x Eppendorf tubes and frozen at -20°C for subsequent analysis such as Bicinchoninic Acid (BCA) assay, enzymatic assay.

The extracellular protein concentration in the supernatant was assayed by using the Bicinchoninic Acid Kit for Protein Determination (Sigma, BCA1-1KT) at 562 nm. The reference protein, bovine serum albumin (BSA) was used as the standard and diluted from the initial concentration (1 g/l) to five different concentrations (50, 100, 200, 300 and 400 µg/l). The supernatants diluted so that the samples are present in concentrations in the calibration range. A BCA reagent was prepared, where 50 ml of Bichionic Acid Solution was well mixed with 1 ml of Copper II Solution. To determine the protein content, 50 µl of the prepared BCA reagent was pipetted into 1 ml of the sample in an eppendorf tube and mixed using a vortex. The resulting samples were immediately boiled using water bad at 60°C for 15 min. Thereafter, the samples were placed in an ice bath to stop further reactions. After cooling, blank with BCA reagent and the samples were measured using a spectrometer in disposable plastic cuvettes at 562 nm. The measured values from the BSA standard solutions were used to plot a calibration curve. The extracellular protein concentration [g/l] was calculated due to the equation of the trend line from the calibration curve. To calculate the total protein [g], the extracellular protein concentration [g/l] was multipled with the total volume [l].

2.7.1.3 Protein activity

In addition to the determination of the extracellular protein concentration, the measurement of the protein activity is important to be able to calculate other significant parameters such as titer (total activity) [U] and specific activity [U/$mg_{Protein}$]. The first indicating the amount of the target protein in the fermentation broth, the latter provides information about the quality and cleanliness of the target protein.

To get the total activity [U], the activity was multipled with the total volume. The specific activity of the target protein is commonly referred to in units of activity per milligram protein (Rosenberg et al. 2006) and was calculated by dividing the protein activity [U/ml] through the extracellular protein concentration [g/l].

For this purpose, the activity of HRP was measured in U/ml by using an enzymatic analyzer robot (CuBiAn©XC, Innovatis) after each sampling. For this purpose, two different reagent solutions were prepared and placed in the provided reagent chamber into CUBIAN: 1 mM ABTS (2.2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid) and 0.075% H_2O_2. The cell free samples were appropriately diluted according to the needs. By automatic pipetting begins the first step of the measurement with the addition of 10 µl of the sample to 140 µl of 1 mM ABTS. In the second step, the reaction mixture was reacted by automatically addition of 20 µl of 0.075 % H_2O_2 into a slightly green colored end product with maximum absorbance at 415 nm. Calibration was done using commercially available

horseradish peroxidase (Type VI-A, Sigma-Aldrich, P6782, Lot# 118K76703) as standard at six different concentrations (0.02; 0.05; 0.1; 0.25; 0.5 and 1.0 U/mL) (Dietzsch et al. 2011-a). The supernatants were diluted appropriately and the protein activity [U/ml] was determined on CUBIAN by changing the absorbance at 415 nm.

2.7.1.4 Substrate concentration

Methanol and glycerol concentrations in the feed solutions and the fermentation broth were measured in undiluted samples from supernatants by high-pressure liquid chromatography (HPLC, Agilent Technologies, USA) equipped with a Supelcogel C- 610 H ion-exchange column (Sigma-Aldrich, USA) by isocratic elution at 500 µl/min and 30 min with a solution of 0.1% H_3PO_4 as the mobile phase. In the course of this, seven different concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g/l) of methanol and six different concentrations (0, 2, 5, 10, 15 and 20 g/l) of glycerol were used as standards.

2.7.1.5 Physiological parameters

In the cell running during to growth many chemical reactions, that convert the necessary raw materials to energy and metabolic products.

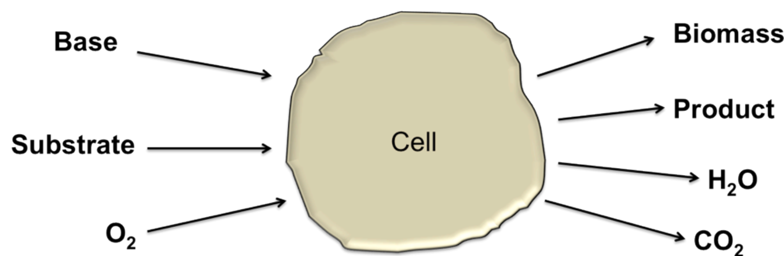
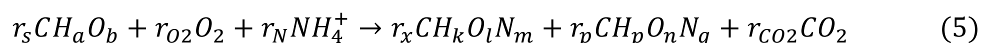


Figure 8: Formation of the metabolic products in the cell

The general formulation of the material balance in a living cell system can be simplified as:

$$input - output + conversion = accumulation \quad (4)$$

and the respective stoichiometric reaction can be written as follows:



$$r_s CH_a O_b + r_{O2} O_2 + r_N NH_4^+ \rightarrow r_x CH_k O_l N_m + r_p CH_p O_n N_q + r_{CO2} CO_2 \quad (5)$$

The volumetric substrate uptake rate, r_s, oxygen uptake rate (OUR), r_{O2}, ammonia uptake rate, r_N, volumetric growth rate, r_X, volumetric product formation rate, r_P, and carbon dioxide evolution rate (CER), r_{CO2} are most important physiological parameters and can be calculated using on-line and off-line data. Since during the batch-phase no substrate-feeding exists, the r_s is only calculted for fed-batch and induction cultures.

The common balance equations for fed-batch and induction phase:

$$r_s \left[\frac{\text{mmol}}{\text{l·h}}\right] = \frac{m_{feed,t} - m_{feed,t=0}}{\rho_{feed}} \cdot x_{feed} \cdot \frac{1}{V_{R,t}} \cdot \frac{1000}{M_X} \cdot \frac{1}{(t-t_0)} \tag{6}$$

$$r_{O_2} = OUR \left[\frac{\text{mmol}}{\text{l·h}}\right] = -\Gamma \cdot 60 \cdot \frac{1}{M_V} \cdot \left(y_{O_2,in} - r_{inert} \cdot y_{O_2,out}\right) \cdot \frac{1000}{V_R} \tag{7}$$

$$r_N \left[\frac{\text{mmol}}{\text{l·h}}\right] = \frac{m_{base,t} - m_{base,t=0}}{\rho_{base}} \cdot M_{base} \cdot \frac{1}{V_{R,t}} \cdot \frac{1000}{(t-t_0)} \tag{8}$$

$$r_X \left[\frac{\text{mmol}}{\text{l·h}}\right] = \frac{X_t \cdot V_{R,t} - X_{t=0} \cdot V_{R,t=0}}{V_{R,t}} \cdot \frac{1000}{M_X} \cdot \frac{1}{(t-t_0)} \tag{9}$$

$$r_P \left[\frac{\text{mmol}}{\text{l·h}}\right] = \frac{c_{P,t} \cdot V_{R,t} - c_{P,t=0} \cdot V_{R,t=0}}{V_{R,t}} \cdot \frac{1000}{M_P} \cdot \frac{1}{(t-t_0)} \tag{9}$$

$$r_{CO_2} = CER \left[\frac{\text{mmol}}{\text{l·h}}\right] = \Gamma \cdot 60 \cdot \frac{1}{M_V} \cdot \left(y_{CO_2,out} \cdot r_{inert} - y_{CO_2,in}\right) \cdot \frac{1000}{V_R} \tag{10}$$

where r_{inert}, called as the inert gas ratio, indicating the relation of total mols of gas in the outlet to the inlet:

$$r_{inert} = \frac{1 - y_{O_2,in} - y_{CO_2,in}}{1 - y_{O_2,out} - y_{CO_2,out} - ex_{H_2O}} \tag{11}$$

with ex_{H2O}, defined as the water content in the off-gas:

$$ex_{H_2O} = 1 - \frac{y_{O_2,wet}}{y_{O_2,in,air}} \tag{12}$$

The biomass yield ($Y_{X/s}$) and the carbondioxide yield ($Y_{CO2/s}$) are other two important physiological parameters and equal to the biomass formation and CO_2 production divided by the substrate consumption:

$$Y_{X/s} = {}^{r_X}\!/_{r_s} \tag{13}$$

$$Y_{CO_2/s} = {}^{r_{CO_2}}\!/_{r_s} \tag{14}$$

The oxygen yield ($Y_{O2/s}$), so the relation of O_2 consumed to substrate consumed, depends on the $y_{O2,wet}$, called as the molar fraction of oxygen in the off-gas without any biological reaction.

$$Y_{O_2/s} = {}^{r_{O_2}}\!/_{r_s} \tag{15}$$

The specific rate of a microbial activity is equal to the volumetric rate for that activity divided by the cell concentration (c_X) performing that activity, and usually defined for growth, product formation and substrate uptake (McNeil et al. 2008). Specific rates could be calculates as follows:

Specific substrate uptake rate:

$$q_S = {}^{r_S}/_{c_X} \qquad (16)$$

Specific product formation rate:

$$q_P = {}^{r_P}/_{c_X} \qquad (17)$$

During batch and fed-batch phase, the growth of culture followed an exponential growth rate μ [h^{-1}] according to the equation 18:

$$X\,[g] = X_0 \cdot e^{\mu \cdot t} \qquad (18)$$

To produce a product quickly and at high levels, high biomass concentration is necessary. To do this, methods must be established which will estimate as precisely as possible the specific growth rate and therefore help to control the cell growth. Equation 19 was used to calculate the specific growth rate as follows:

$$\mu\,[h^{-1}] = ln \frac{X_t \cdot V_{R,t}}{X_{t=0} \cdot V_{R,t=0}} \cdot \frac{1}{(t-t_0)} \qquad (19)$$

2.8 Design of experiments (DoE)

Design of experiments (DoE) provides a toolset for the statistical layout and the subsequent statistical evaluation of experiments in a predefined experimental range (Sagmeister et al. 2011). The aim is to obtain the smallest possible set of experiments that have to be performed in which all of the relevant factors are varied systematically, to identify optimal conditions, the magnitude of influence of a factor as well as the existence of interactions and synergies between factors (Seifert et al. 2010).

To achieve the goals of this study a full factorial design of experiments (DoE) using MODDE (Umetrics, Sweden) were performed. The regression coefficient, the prediction precision and the model validity as well as the reproducibility of the model system were checked. The significance of the effects was judged based on the coefficient plot.

The dissolved oxygen and the residence time were defined both qualitatively and quantitatively as factors for the DoE. The dissolved oxygen was therefore chosen in the levels 5% as low, 25% as intermediate and 70% as high. The residence time was varied between 3.3 and 15 minutes. The center point at 25% of dissolved oxygen and 3.3 min of residence time was performed in dublicate. The dissolved oxygen was regulated independent from feeding rate. All metabolites, product content and activity and biomass were measured every 12 hours.

In order to find the optimum process parameters, the responses were chosen as follows:

- Specific productivity (average of first 50 hours)
- Titer (total activity at 80 hours)
- Biomass and carbon dioxide yield (the average value of the first 80 hours over the induction phase)

The specific productivity (the average value of first 50 hours of induction phase) and titer (the titer at 80 hours after induction) were chosen as representatives of product quantity. The former means the rate of target protein production per biomass [U/g.h] and the latter reflects the total activity of target protein in broth [U]. The biomass and carbon dioxide yield (the average values of the first 80 hours of the induction phase), which are the singe of physiology, were defined as responses as well.

All these factors were first examined by a full factorial design of experiments (DoE1). In this DoE the factors DO and residence time) were defined qualitatively.

The list of experiments for DoE1 is given in Table 8:

Exp.no	residence time	dissolved oxygen
1	low (3.3 min)	intermediate (25%)
2	high (15 min)	intermediate (25%)
3	low (3.3 min)	high (70%)
4	high (15 min)	high (70%)
5	low (3.3 min)	intermediate (25%)

Table 8. List of experiments for DoE1 with two qualitative factors (residence time and dissolved oxygen) defined in two levels for residence time (low (3.3min) and high (15min)) and two levels for dissolved oxygen (intermediate (25%) and high (70%)). The center point at DO=intermediate (25%) and residence time=low (3.3 min) was performed in duplicate and indicated here in gray (see Exp.1 and Exp.5). All these experiments were performed in two-compartment sytem.

The experiments in table 8 were carried out only in a two-compartment system to study the effects of different amplitude and frequency of the stress in this novel setup qualitatively. The corresponding full factorial design of experiments (DoE1) was shown in figure 9.

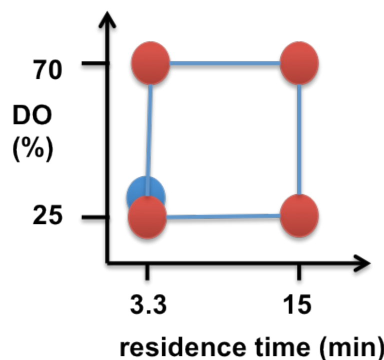


Figure 9. The full factorial design of experiments (DoE). Each circle represents one individual fermentation run. The center point at DO=25% and residence time=3.3 min was performed in duplicate, illustrated as two superimposed circles with different color. All these experiments were performed in two-compartment system.

In two-compartment system, the cells were circulating between two zones, a bioreactor with sufficient oxygen and a PFR without oxygen to realize an effective stress on cells. However, not only in PFR, as well as in bioreactor, the oxygen supply can be maintained as little as possible, and this perhaps may lead to more effective stress in this system. To elucidate this prediction, a further two-compartment experiment with DO of 5% and residence time of 3.3min (Exp.6 see table 9) was conducted. An additional experiment (Exp.7) was carried out in one-compartment system with a dissolved oxygen level at 5% to comparison and to study the impact of circulation of the broth.

To analyze and reinforce of the DoE1-statements in a wider range, a second full factorial design of experiments (DoE2) were performed. DoE2 with corresponding DO and residence time levels is visualized in the following figure:

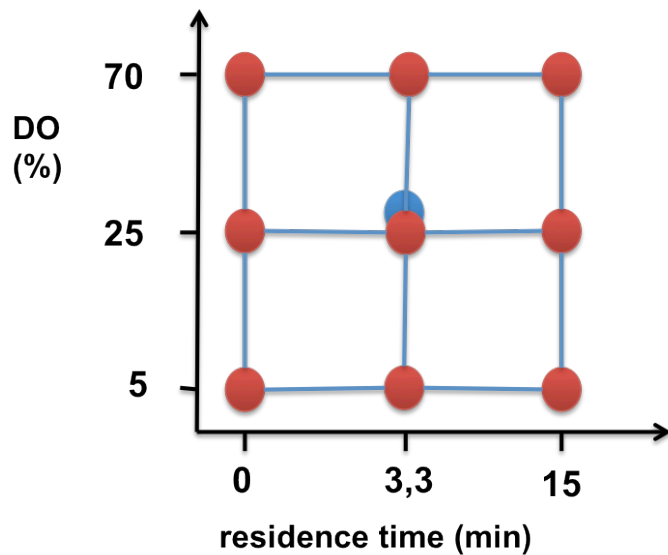


Figure 10. The full factorial design of experiments (DoE2). Each circle represents one individual fermentation run. The center point at DO=25% and residence time=3.3 min was performed in duplicate, illustrated as two superimposed circles with different color. The experiments with zero residence time were carried out in a single reactor and represent only the one-compartment experiments. In this DoE, DO and residence time were defined quantitatively.

The list of all experiments for DoE2 is given in table 9, resulting in a total number of 10 fermentation runs:

Exp.no	Residence time [min]	Dissolved oxygen [%]
1	3.3	25
2	15	25
3	3.3	70
4	15	70
5	3.3	25
6	3.3	5
7	0	5
8	0	25

9	0	70
10	15	5

Table 9. List of all performed experiments. The first five experiments are the same in Tab.7, they were considered here only quantitatively. The center point at DO=25% and residence time=3.3min was performed in duplicate and indicated her in gray (Exp.1 and Exp.5). The experiments with zero residence time (Exp.7, Exp.8 and Exp.9) represent the one-compartment experiments.

In the two-compartment system used in this study, only the overall performance of biological system was investigated. Instantaneous reaction of the cells and their intracellular response to the stress was not of interest. The overall performance was not affected by either slight change in residence time or small variation in DO (Lorantfy et al. 2013) Therefore three levels of dissolved oxygen were defined far from each other to represent only low (5%), middle (25%) and high (70%) levels of dissolved oxygen. As demonstrated by Lorantfy et al. small variations in residence time has not significant effect. Therefore only two levels of residence time (3.3 min and 15 min) were chosen. The lower and higher levels were set according to the different mixing times reported in literature for large scale reactors (Lara et al. 2006-a).

3 Results

This thesis is published:

Jazini, M.; Cekici, G.; Herwig, C.: **Quantifying the Effects of Frequency and Amplitude of Periodic Oxygen-Related Stress on Recombinant Protein Production in *Pichia pastoris***. *Bioengineering* **2014**, *1*, 47-61; doi:10.3390/bioengineering1010047.

3.1 Method development for physiological feeding

3.1.1 Biomass determination

In this study, the biomass concentration was obtained with different analytical methods. The results of gravimetric methods from a two-compartment experiment (Exp.3) based on the total biomass were presented in figure 11.

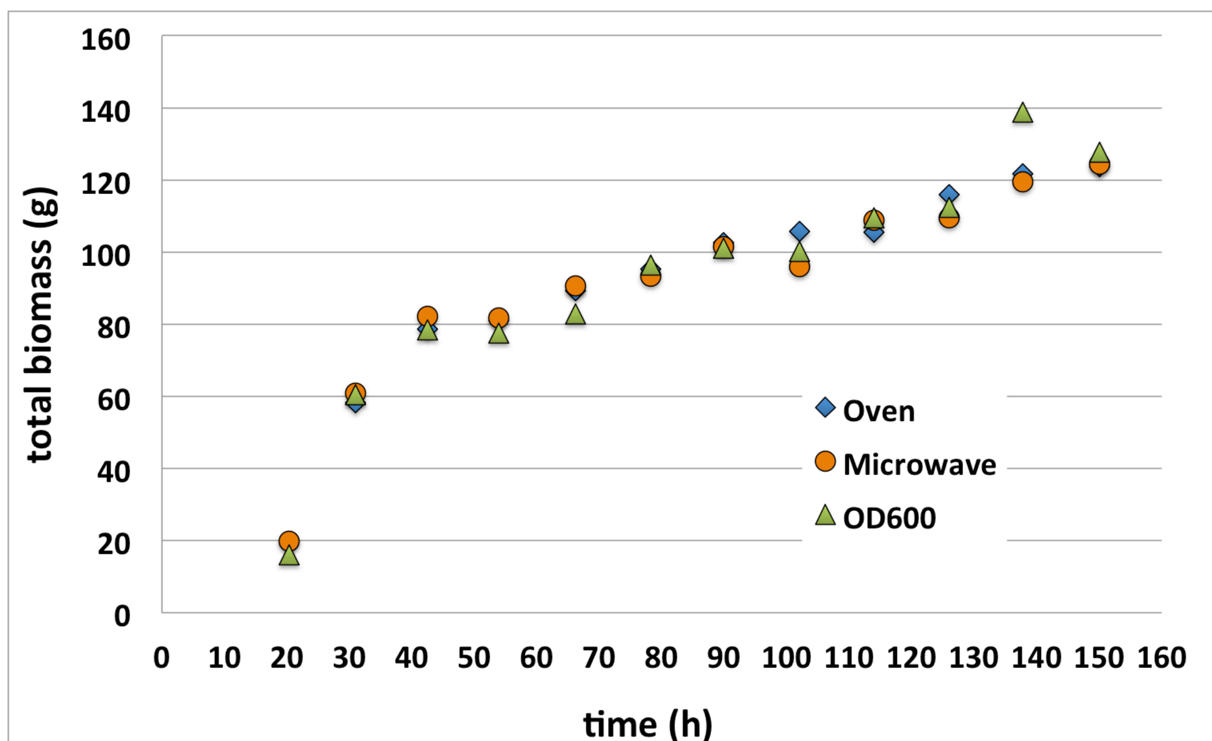


Figure 11: These figure shows for *Experiment 3 (a two-compartment system with DO=70% and residence time=3.3 min)* the variation of total biomass (g) by different methods vs. time

The fermentation took approximately 150 h, wherefrom the batch done the first 20 hours, and the fed-batch the following 10 hours. The adaptation phase lasted about 12 hours and followed by the induction phase at about 42 hours after the start of the process. The figure 11 clearly shows, an increase in total biomass after 20 hours of fermentation with time. This is because of the cell density increases in the fermentation broth as they multiply. After conversion to the methanol, so after

about 30 hours, the biomass concentration remained approximately constant. But it isn´t to be seen here a remarkable difference between OD_{600} method and microwave method as well as oven method. However, at some points, especially by total biomass at 66 and 137 hours, the values obtained by the OD 600 method differ sometimes slightly from the other two methods, and this explains why we have the microwave method necessarily performed as a quick and safe method after each sampling, although the biomass determination by OD600 very fast occurs. Another point, namely, the total biomass in 102 hours differs in all methods from each other, and in such cases we have the biomass concentration estimated according to the previous biomass to the feed flow rate to adjust.

3.1.2 q_s control

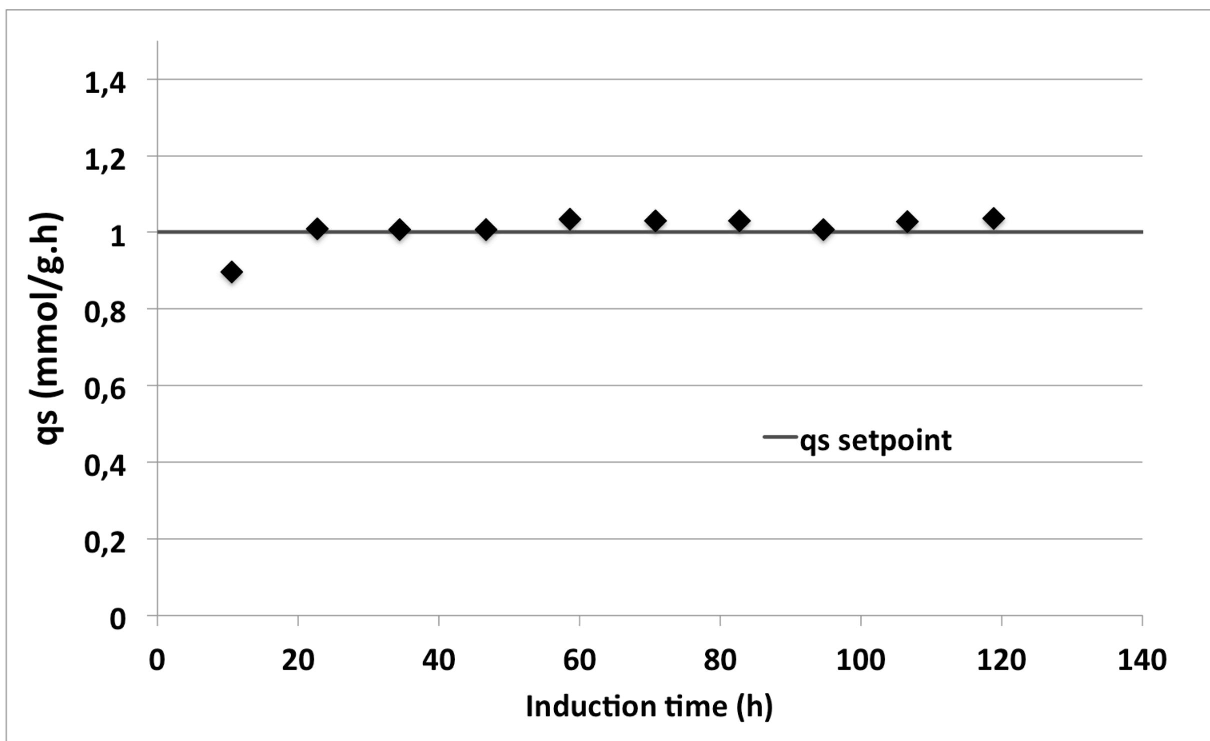


Figure 12: This figure shows for Experiment 6 the substrate uptake rate (q_s) obtained during the whole induction phase

As mentioned, our feeding strategy was based on maintain the specific substrate uptake rate at 1 mmol/g.h depending from methanol feed flow rate during the induction phase. The figure 12 demonstrates how successful this control conducted. However, here is a difference between the first resulting and the subsequent qs pulses remarkable. At the beginning of the induction phase, a q_s of about 0,9 mmol/g.h was obtained, during the next calculated q_s was exactly at 1 mmol/g.h. This behavior of q_s was obtained in all the experiments. To determine this, it is sufficient to compare the cell dry weight after the adaptation phase with which after the beginning of induction phase from each experiment. For example, in Experiment 3 was the cell dry weight after adaptation phase at 69.33 g/l, while after induction a cell dry weight of 64.85 g/l in bioreactor was determined (data not shown). In contrast to the total biomass, it was typical in each experiment that the cell dry weight at the beginning of the induction phase decreases slightly due to the increased methanol feeding (data

not shown). Since the methanol feeding suddenly after the adaptation phase increased to double, leading perhaps to the fact that the some cells are no longer viable. The consequence is then a decline in cell dry weight and thus a low value of qs because the cells do not use the energy source to grow, but to survive and maintain their metabolic state.

3.1.3 Feed flow control

The following equations were used for the exponential feeding strategy to establish the glycerol-feeding rate in fed-batch experiments. The parameter values used to obtain an exponential feeding profile were μ_{set} = 0.15 h^{-1} (set growth rate), $Y_{X/S}$ = 0,45 g g^{-1}.

Initial glycerol-feeding rate:

$$F_0\ [g/h] = \frac{\mu set\ [h^{-1}] * c_{x0}\ [g/l] * V_{R0}\ [l] * \rho_S\ [g/l]}{c_S\ [g/l] * Y_{X/S}\ [(C-mol)/(C-mol)]} \qquad (20)$$

The exponential glycerol-feeding rate as a function of time during the fed-batch phase:

$$F_t\ [g/h] = F_0\ [g/h] * e^{\mu\ [h^{-1}] * t\ [h]} \qquad (21)$$

where c_{x0} is initial biomass concentration [g/l]; V_{R0} is the initial culture volume [l] in the reactor; F_0 is the initial glycerol feeding rate [l/h]; μ is the specific growth rate [h^{-1}]; c_S is the glycerol concentration in the feed medium [g/l]; $Y_{X/S}$ is the biomass yield [C-mol/C-mol]; ρ_s is the density of glycerol feed medium [g/mol] and t is the time [h].

To estimate the feeding rate regardless of the current phase the following equation was used:

$$Pump\ setpoint\ [\%] = F_0\ /\ 1.8648 \qquad (22)$$

This equation is the result of a calibration that was previously carried out experimentally for peristaltic pumps. The creation of a linear calibration curve was performed using of the batch medium.

During the induction phase, an overfeeding of methanol feed can lead to it´s accumulation, which affects adversely the cell growth, and this make the flow control an important process operation in *Pichia* Fermentation. The control of feed flow rate during the induction phase was done with the PID controller of pump setpoint to ensure a substrate uptake rate (qs) of 1mmol.g^{-1}.h^{-1} according to a linear growth of culture (see figure 13).

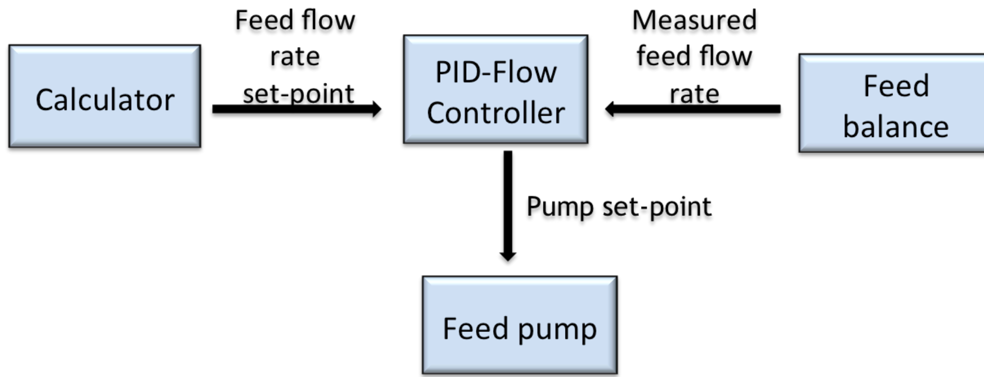


Figure 13: Schematic view of feed flow control by means of PID controller during induction phase

The linear feeding profile is applied according to the following equation:

$$Flow\ rate\ [g/h]\ = \frac{q_s\,[mmol.g^{-1}.h^{-1}] * 10^{-3}\,.X\,[g] * M_s\,[g/mol]\,.\rho_s\,[g/l]}{c_s\,[g/l]} \qquad (23)$$

The feed flow rate through the STR was calculated after each sample according to the Equation 23 during the whole induction phase and controlled by means of a gravimetrically based PID controller to achieve a substrate uptake rate of 1 mmol/g.h.

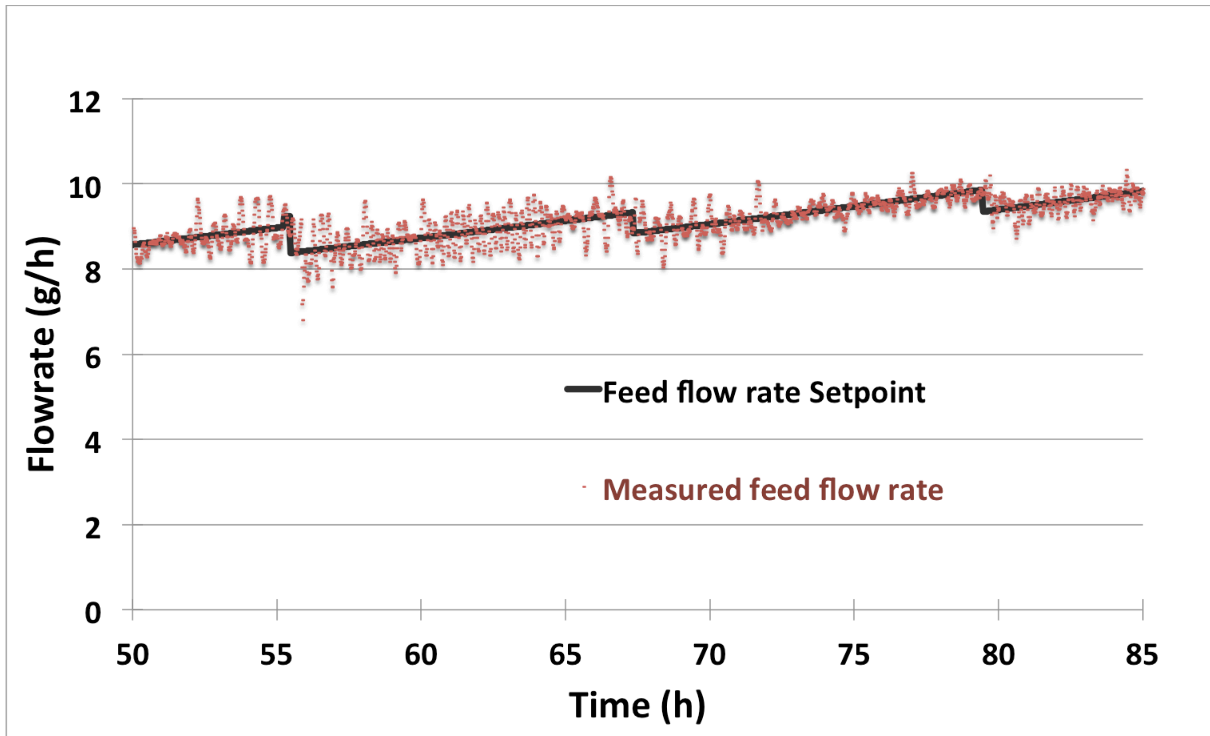


Figure 14: This figure shows for Experiment 3 the feed flow rate (g/h) obtained during induction phase between 50 and 85 hours

Figure 14 shows the online data of feed flow rate setpoint and measured feed flow rate during the induction times between 50 and 85 h. The online data between these time points were selected only to look closer that the flow control with the applied strategy has worked well enough to feed the cells with methanol and thereby to avoid the deadly methanol accumulation.

3.2 One-compartment system

3.2.1 Specific productivity

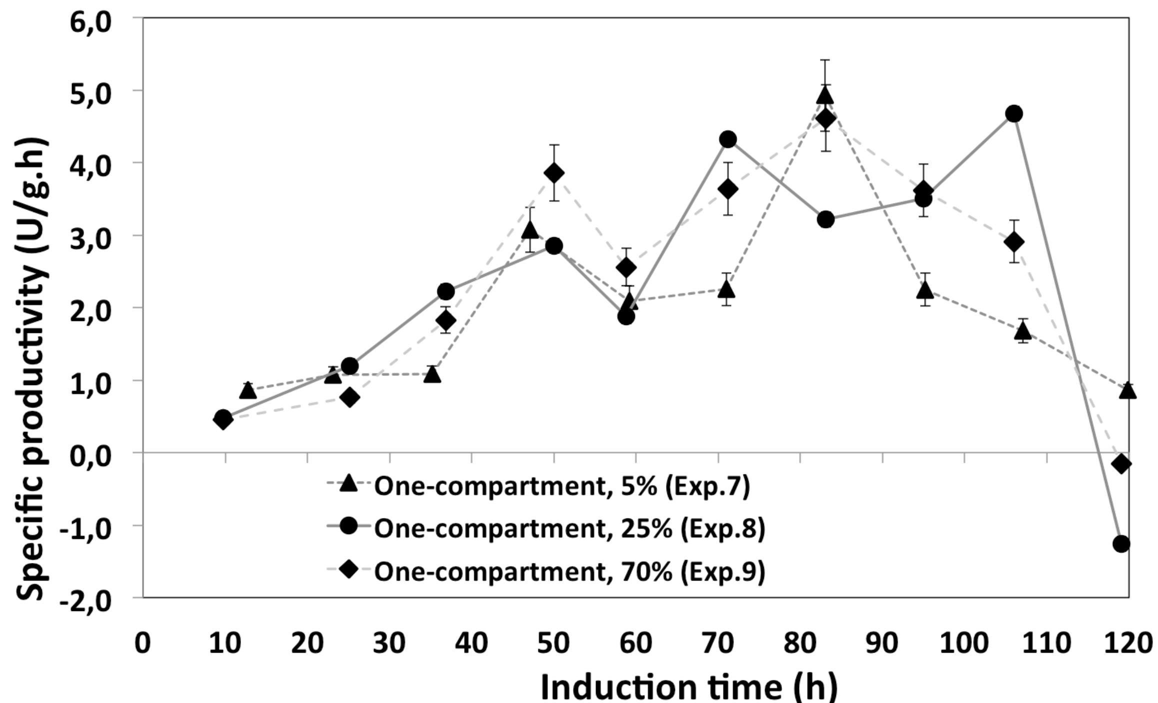


Figure 15: Specific productivities of all one-compartment experiments (Exp.7, Exp.8 and Exp.9) during induction phase. The upward, constant and downward trend in specific productivity is striking in this figure.

The specific productivity is the quantity of protein that was produced per gram of cell and per hour. All one-compartment experiments (Exp.7, Exp.8 and Exp.9) in terms of their specific productivity were indicated in figure 15. It can be seen that the specific productivity has a typical profile in all experiments. It rises in the first 50 hours of near linear, and then it remains approximately constant for a long moment, and from about 100 hours of induction time begins to decline. This diagram is therefore too important because it can determine when and how long to reach a maximum specific productivity and when to stop the experiment. Another statement from this figure is, that there is no difference in the specific productivity between three one-compartment experiments to note. This means that different dissolved oxygen level in one-compartment system does not affect the quantity of protein. And it was also found no differences between the values of specific productivities by statistically studies using one-way ANOVA. The Exp.7 shows a maximal specific productivity of 4.93 U/g.h at 83 hours, and this amounts in Exp.9 4.61 U/g.h at the same time, where Exp.8 reached a maximum specific productivity of 4.68 U/g.h in 106 hours, that is with a time delay of 23 hours.

3.2.2 Total activity (titer)

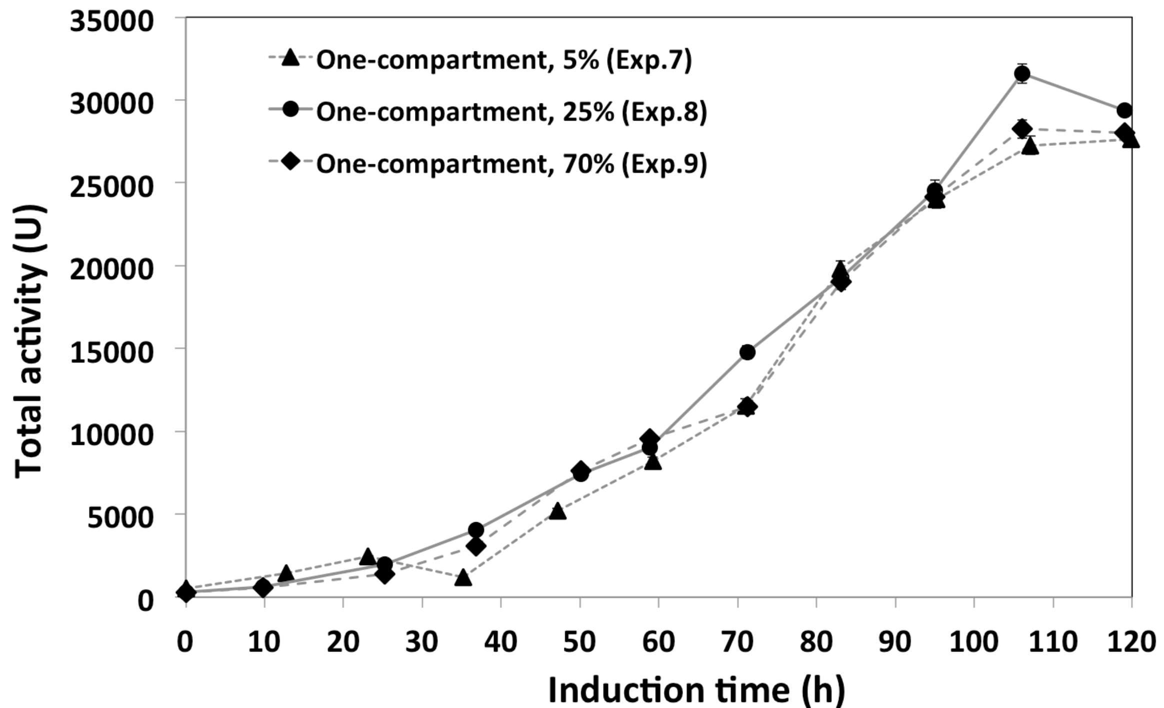


Figure 16: Total protein activities (titer) of all one-compartment experiments (Exp.7, Exp.8 and Exp.9) during induction phase

The titer gives the total number of active proteins in the respective sampling time in bioreactor, and is mathematically the integration of specific productivity over the total biomass and time. The titer shows an increasing trend over the induction time due to the increase of total biomass and time although the specific productivity has remained constant from 50 hours of induction time and decreased afterwards. As it can be seen in figure 16, there is no difference between titers of three one-compartment experiments (Exp.7, Exp.8 and Exp.9). Particularly at 83 and 95 hours, the numbers of total active proteins in bioreactor are almost the same (19807 U, 19257 U and 19045 U at 83 hours and 24018 U, 24553 U and 24163 U at 95 hours in Exp.7, Exp.8 and Exp.9, respectively). The decrease of titer at 120 hours is due to the decline of total biomass.

3.3 Two-compartment system

3.3.1 Dissolved oxygen (DO) control

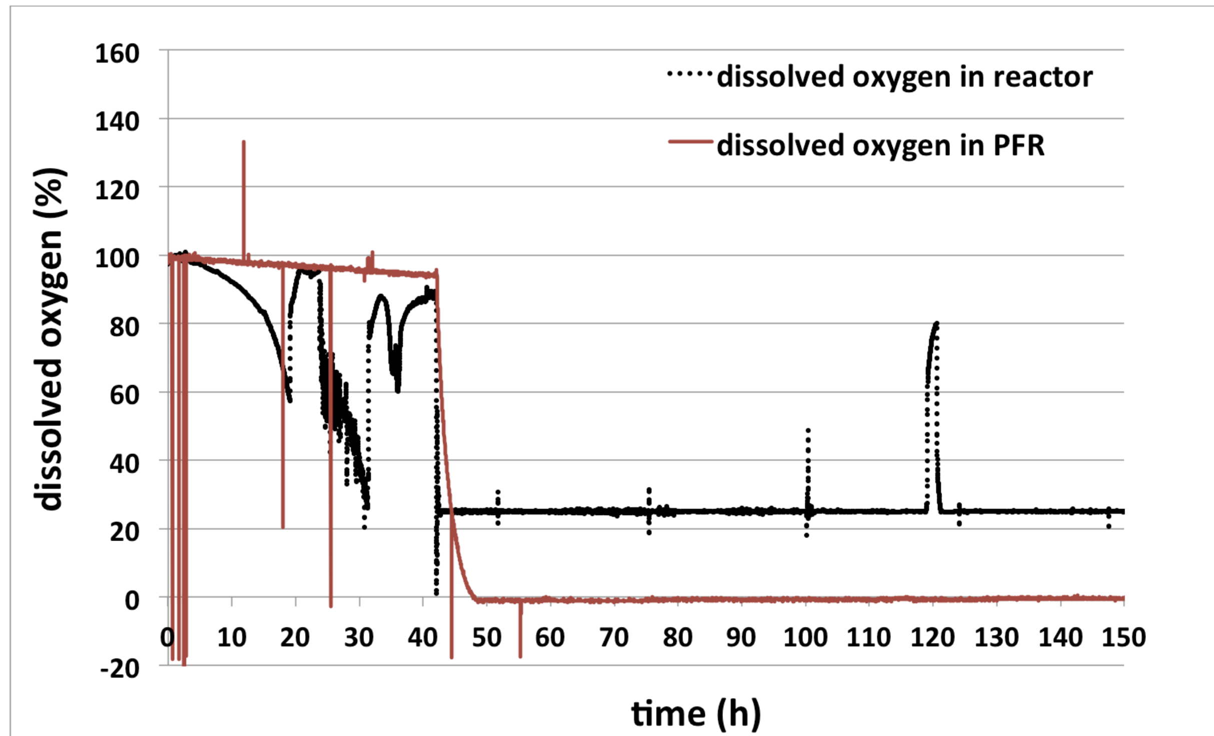


Figure 17: Measured dissolved oxygen (DO) in bioreactor (black line) and in PFR (red line) from Experiment 1 (=center point with DO=25% and residence time=3.3 min).

As mentioned before, the two-compartment system consists of an aerated labor-scale bioreactor and a PFR as an oxygen-free zone, where the air bubbles are completely removed via a bypass line before the culture medium was pumped into the PFR (see figure 6). At the end of the PFR, a flow cell was connected and it allows by means of integrated sensors the measurement of the pH and DO of the fermentation broth after the PFR. The figure 17 gives us clear statements that the bubble-removal in the two-compartment system has worked well with a DO of 0% in the PFR during the whole induction phase (red line, from 47 hours up to end of fermentation). The circulation has started from about 41 hours, and the dissolved oxygen in about 6 hours has decreased to 0%. Why did it take so long until we finally reached an oxygen-free zone? The PFR was empty until the start of the induction phase, and there is air in the PFR was located. The air has been mixed from the start of the induction phase to the broth, and it has taken so much time to get out all the air was out of the PFR. A second reason is due to the dead volume in the flow cell. An efficient oxygen-free zone was achieved from about 47 hours after the start of fermentation in this presented two-compartment system called as Experiment 1, so the center point with DO of 25% in bioreactor und residence time of 3.3 min.

The setting of dissolved oxygen at 25% in the bioreactor was successfully performed. Hence, the DO signal in this experiment was disturbed due to the lack of supply of feed at about 120 hours. The other signal disturbances of DO occur due to poor calibration of DO sensor.

3.3.2 Specific productivity

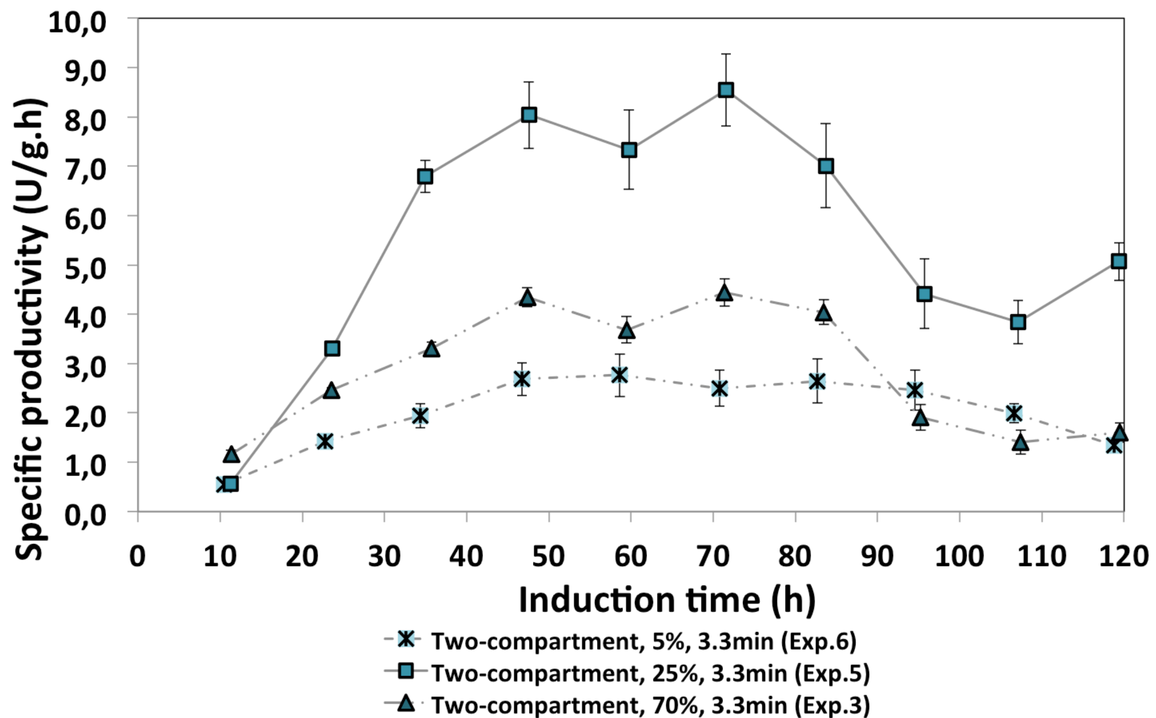


Figure 18: Specific productivity of two-compartment experiments (Exp.3, Exp.5 and Exp.6) at residence time of 3.3 min

As mentined before, the residence time, which the cells spend in the PFR, has no significant effect on the protein quantity. According to this statement, here only the experiments were compared with each other, which were carried out at different DO levels at the same residence time.

The typical behavior of the specific productivity was also observed in two-compartment system: the upward, constant and downward trend over the induction time. The figure 18 clearly shows the significant differences in specific productivity in two-compartment experiments, which has been performed at different DO levels with the residence time of 3.3 min. The Exp.5, which we have identified as the center point in our work, shows a specific productivity of 8.55 U/g.h at 70 hours, altough it was in the other two experiments at the bottom (4.4 and 2.5 U/g.h at 70 hours in Exp.3 and Exp.6, respectively).

An possible declaration on the different HRP secretion of Pichia pastoris at the biochemical level has already been discussed in literature. According to Zhang et al. 2006, the Hsp70 and Hsp40 chaperone families in the cytoplasm or in ER (endoplasmic reticulum) importantly regulate the folding and secretion of heterologous proteins. Molecular chaperones are defined as molecules that prevent protein aggregation and facilitate folding by maintaining polypeptides in productive folding pathways (Gething et al. 1992). It appears, that the oxygen-related stress in two-compartment system enhanced the systhesis of Hsp70 and Hsp40. On the other hand, the secretion of recombinant protein HRP due to Hsp70 and Hsp40 chaperones might be depended on stress condition during the fermentation process. In two-compartment system, the circulation of broth zwischen bioreactor and the PFR triggers a stress, which depends on the dissolved oxygen level in the bioreactor. A two-compartment experiment with 5% dissolved oxygen in the bioreactor and 0% dissolved oxygen in the loop (Exp.6) has revealed less efficiency like a one-compartment experiment, because the dissolved

oxygen in bioreactor is just as little as in the loop. Since the cells should get on the whole process time with little oxygen, they adapt themselves to stressful conditions and produce as little active proteins such as in a one-comparment system. So, the low and high level of dissolved oxygen in bioreactor might cause that the chaperone molecules were less synthesized and less HRP in the culture medium was secreted. It seems, that under stress condition the intermediate level of DO (25%) appears to be optimal to improve the synthesis of chaperone molecules and secretory expression of HRP. In summary, the intermediate level in two compartment system has increased the protein quantity and thereby a better specific productivity was achieved. This statement will be considered and interpreted in DoE1.

3.3.3 Total activity (titer)

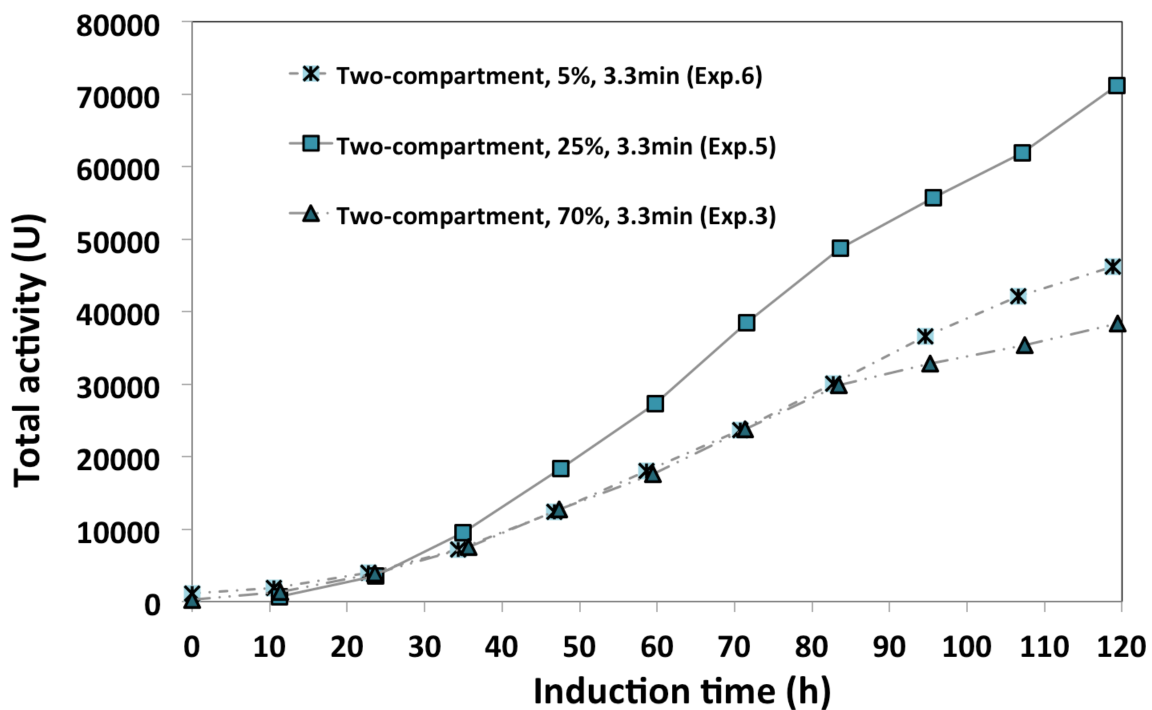


Figure 19: Total activity (titer) of two-compartment experiments (Exp.3, Exp.5 and Exp.6) at residence time of 3.3 min

Figure 19 shows the total activities of two-compartment experiments which were conducted at residence time of 3.3 min. The highest value was observed again in Exp.5, which were performed at DO of 25%. The titer in Exp.5 has increased at the end of induction phase up to 71 243 U. The titer in other two experiments were similar up to 83 hours (about 30 000 U), and then it has risen in Exp.3 slower due to the sudden descent of the specific productivity from this time (see figure 18).

3.3.4 Quadratic effect of DO

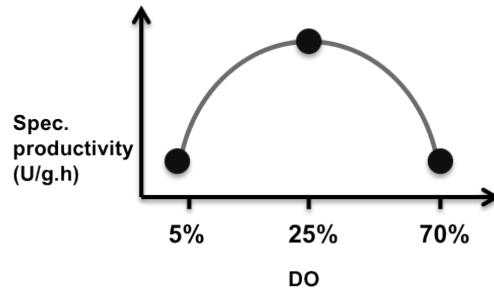


Figure 20: Quadratic effect of dissolved oxygen on specific productivity and titer in two-compartment system

In figure 18 and 19, it was clear to see, that the both the specific productivity and titer in the Exp.5 was the highest over the entire induction time. This two-compartment experiment, referred to as center point in our work, has revealed more than two-fold values both in specific productivity and titer. The two-compartment experiments with DO of 5 and 70% had approximately the same results like in one-compartment experiments. These results have indicated that there was a quadratic effect between the dissolved oxygen and the response variables such as the specific productivity and titer in two-compartment system. Since the factors were defined in DoE1 qualitatively, the mathematical investigation of the quadratic effect of the DO on response variables will be considered and interpreted in DoE2.

3.3.5 Results from DoE1

In the context of DoE1, five experiments were carried out in total (as listed in table 8), where the center point was performed in duplicate (Exp.1 and Exp.5), and the factors (DO and residence time) were defined qualitatively (as low, middle and high). As mentioned before, all of the experiments in the context of DoE1 were conducted in two-compartment system. At the beginning of modeling, all terms like linear, quadratic and interaction term were taken into account in order to investigate their influence on the response variables. Non-significant terms were deleted to modify the regression coefficient (R^2), the prediction precision (Q^2), and the model validity as well as the reproducibility of the model system.

The figure 21 shows the correlation of specific productivity (the average value of first 50 hours of induction phase) to the factors. Hence, no interaction terms (like Res(low)*DO(intermediate) or Res(high)*DO(high)) and quadratic terms (like Res(low)*Res(low) or DO(high)*Do(high)) with the dissolved oxygen and residence time were detected. To obtain a model with higher precision, all interaction and quadratic terms were deleted.

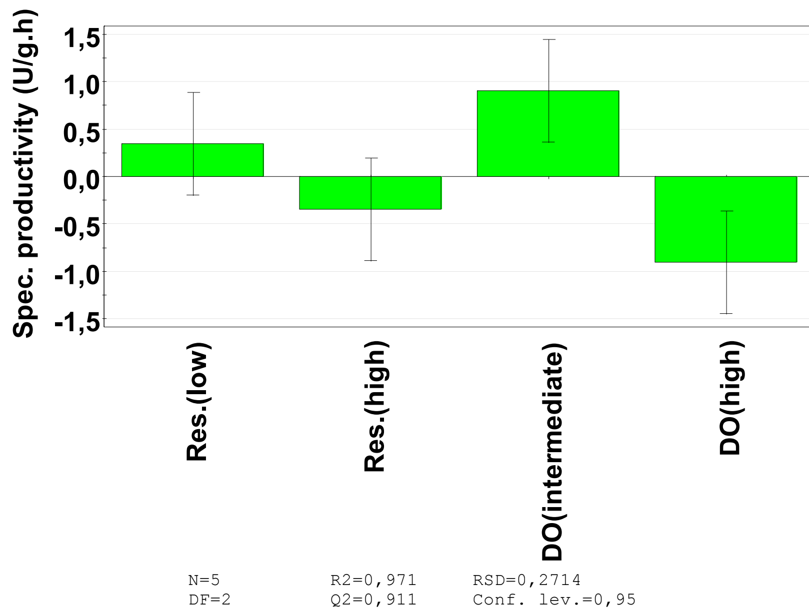


Figure 21: Coefficient plots for DoE1 to the specific productivity.

Dissolved oxygen and residence time were included only as linear terms. No affect of residence time on the specific productivity was dedected. Dissolved oxygen was identified as the sole influential factor: during DO in intermediate level had a positive effect on specific productivity, DO in high level has influenced in a negative manner. In other words, in two-compartment system, the high the DO in bioreactor was, the less the specific productivity was resulted.

The figure 22 shows the basic model statistics of the regression model with a very high regression coefficient (R^2), prediction precision (Q^2), and model validity as well as a fairly high reproducibility.

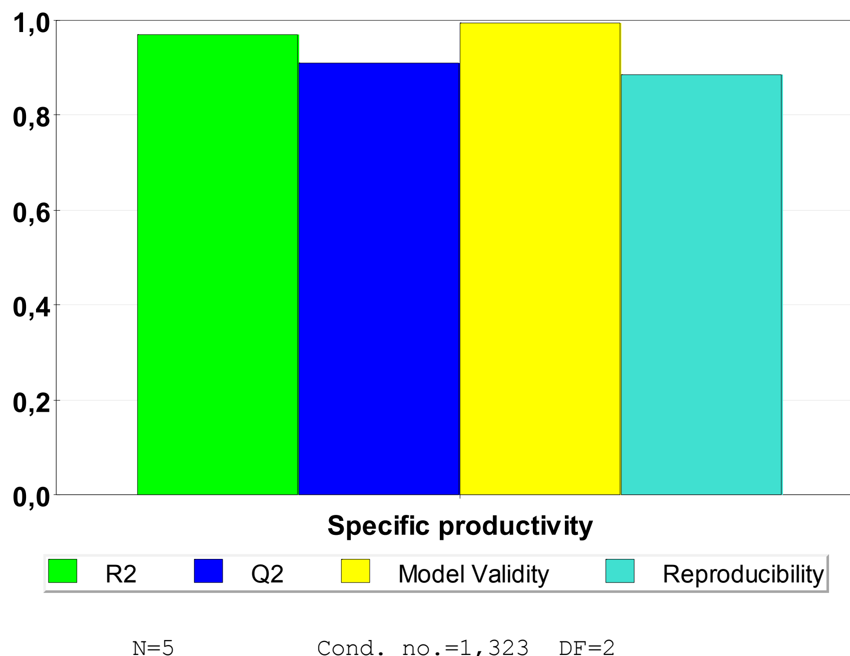


Figure 22: Regression model for DoE1 to the specific productivity with regression coefficient (R^2), prediction precision (Q^2), model validity and reproducibility of the model system

The figure 23 shows the correlation of total activity (titer at 80 hours of induction phase) to the factors. As in the case of specific productivity, there is no interaction and quadratic term with the dissolved oxygen and residence time were detected. To obtain a model with higher precision, all interaction and quadratic terms were deleted.

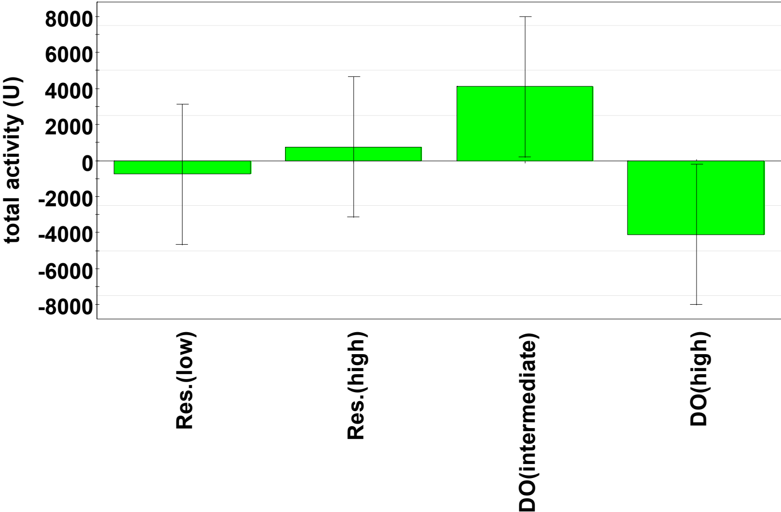


Figure 23: Coefficient plots for DoE1 to the total activity (titer)

Hence no affect of residence time on the total activity was dedected, therefore the corresponding plots from the residence time were deleted to create a better model.

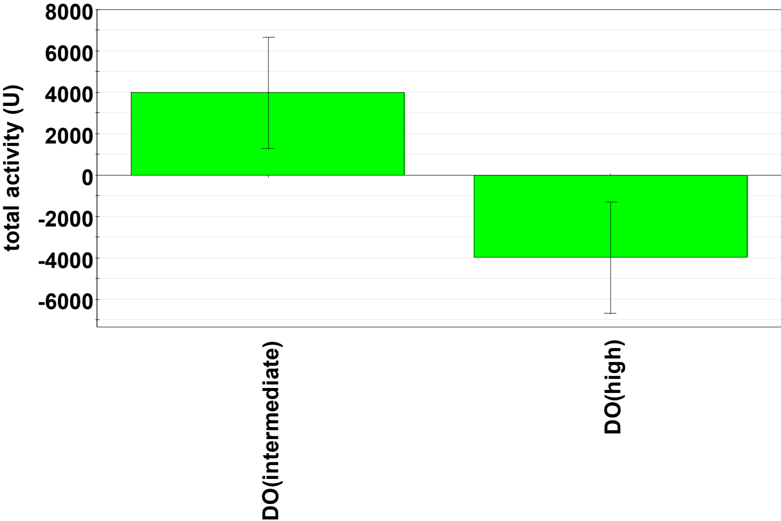


Figure 24: Corrected coefficient plots for DoE1 to the total activity (titer)

As in the case of specific productivity, dissolved oxygen was identified as the sole influential factor to the titer: the intermediate level of DO results has given the maximum titer, and an experiment was terminated with little titer, the more DO was fed into the bioreactor. The residence time can be considered as insignificant. The summary of fit is shown in figure 25

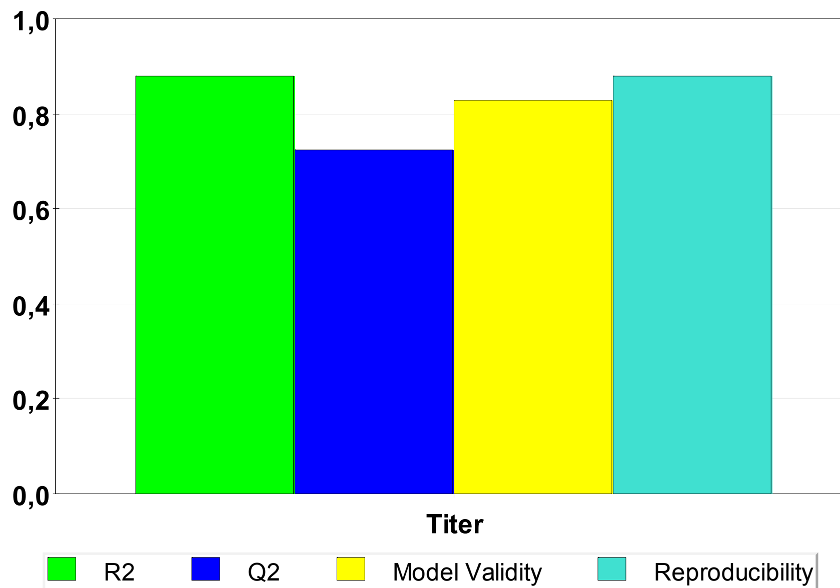


Figure 25: Regression model for DoE1 to the total activity with regression coefficient (R^2), prediction precision (Q^2), model validity and reproducibility of the model system

The physiological parameters, like biomass yield and carbon dioxide yield (the average value of the first 80 hours of the induction phase) were analyzed as responses to study of the influence of the applied stress on the cell physiology and cell metabolism. Since all confidence intervals of responses have touched the zero line (x-axis), these can be regarded as insignificant (see figure 26-27). Hence, it was observed that neither dissolved oxygen nor residence time has an influence on the yields in two-compartment system.

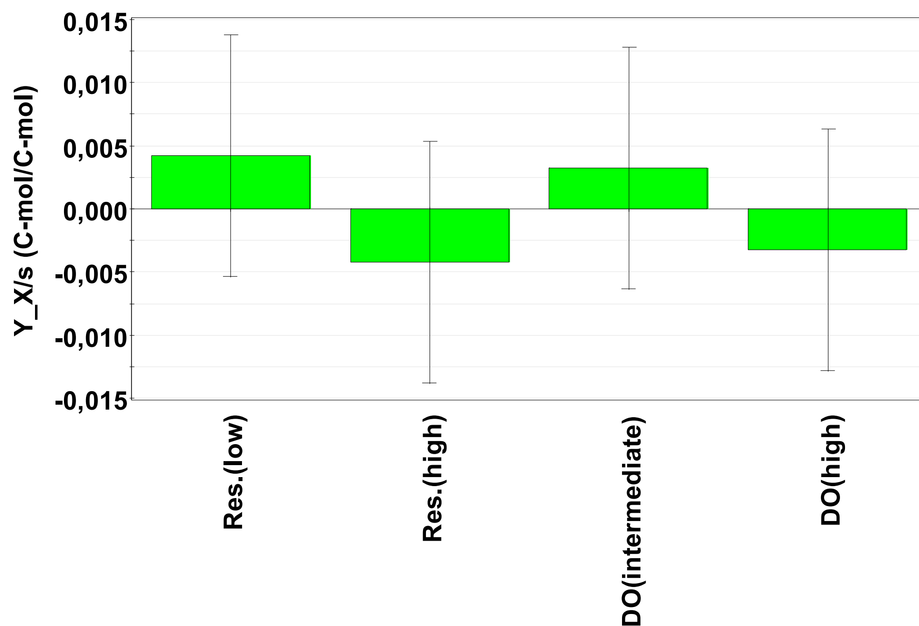


Figure 26: Coefficient plots for DoE1 to the biomass yield ($Y_{X/s}$)

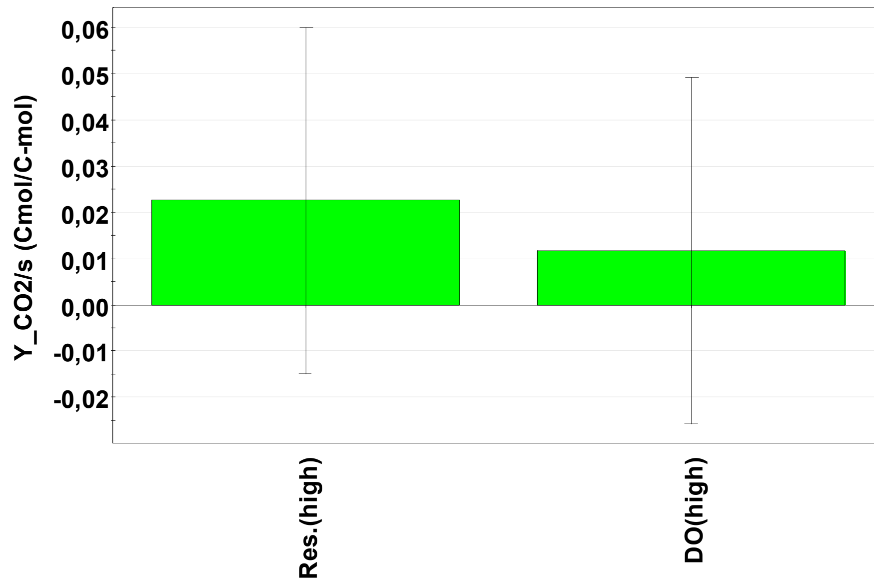


Figure 27: Coefficient plots for DoE1 to the carbondioxide yield ($Y_{CO2/s}$)

3.4 Two-compartment system vs. one-compartment system

To study the impact of imposed stress on the response variables, like the specific productivity and the total activity (titer) as well as yields and to understand the difference between a two-compartment system and a one-compartment system, two experiments were compared in this part: the Exp.8 which was carried out in one-compartment system with 25% of DO and Exp.5, which has illustrated the central point in two-compartment system with 25% of DO and 3.3 minutes of residence time.

3.4.1 Specific productivity

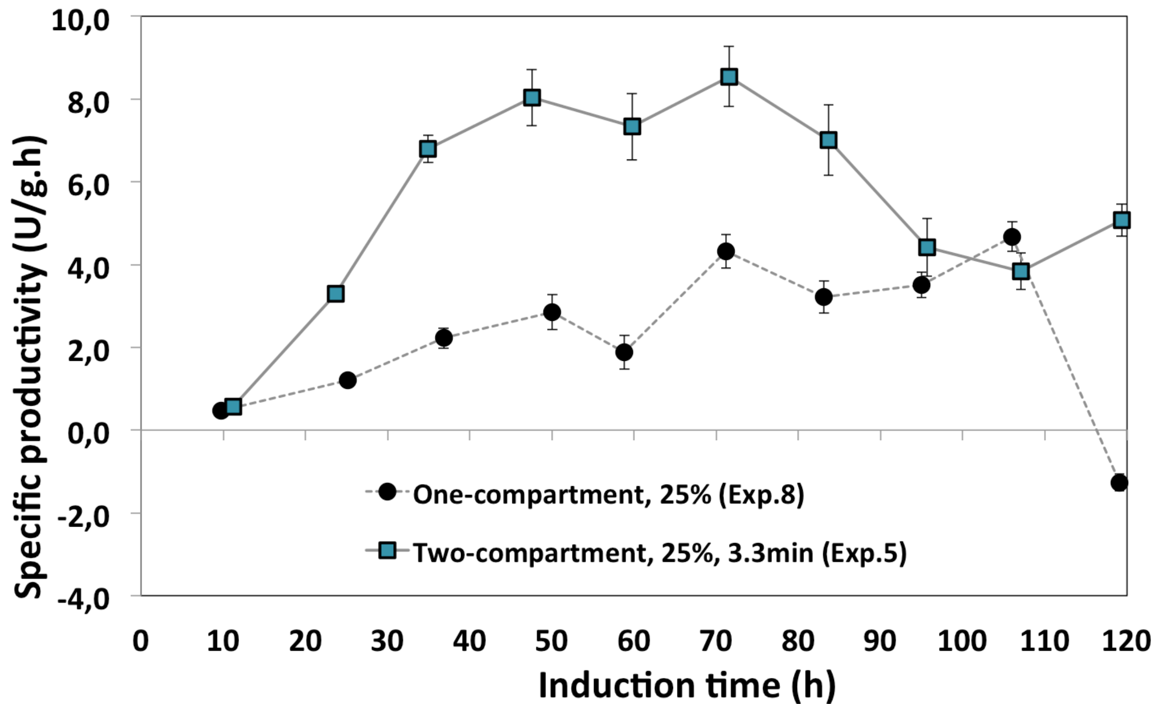


Figure 28: Comparison of the specific productivity of center point (Exp.5) to an one-compartment experiment (Exp.8) with the same value of DO

The figure 28 clearly shows the significant difference in specific productivity in two experiments, which has been performed at the same DO level but in two different setups. The specific productivity in Exp.5, which we have identified as the center point in our work, was determined to be approximately 8.55 U/g.h at 70 hours, as compared to 4.3 U/g.h observed for Exp.8 (one-compartment system). This difference might be due to the effect of oxygen limitation. The circulation of the broth in absence of the oxygen in PFR might have affected the activity of the recombinant proteins positive.

3.4.2 Extracellular protein content

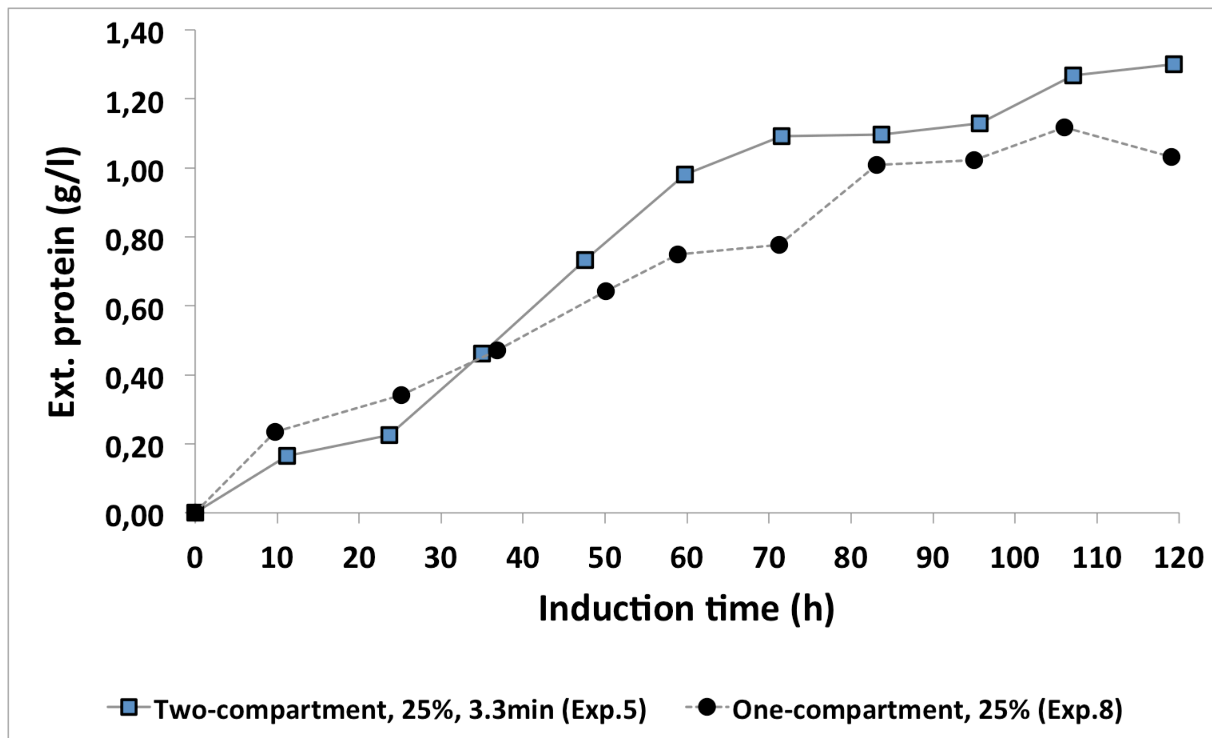


Figure 29: Extracellular protein content during induction phase

A higher protein content is observed as mentioned during the induction phase, what means *P. pastoris* produced HRP at high levels using methanol as carbon source. The results, which we have determined by BCA assay, were here based on the total volume. In addition, the protein content, which we have in adaptation phase assumed to be zero and, therefore, removed from the subsequent values. As is obvious in figure 29, the difference in extracellular protein content in culture supernatants between both experiments is low. The protein amounts for both experiments were initially of the same, but it increases for two compartment system a bit more after 50 hours and reached up to 1.3 g at the end of induction phase, while it was calculated for Exp.8 at the same time 1.03 g. The protein amount was by use of the two-compartment system a little improved.

3.4.3 Total activity (titer)

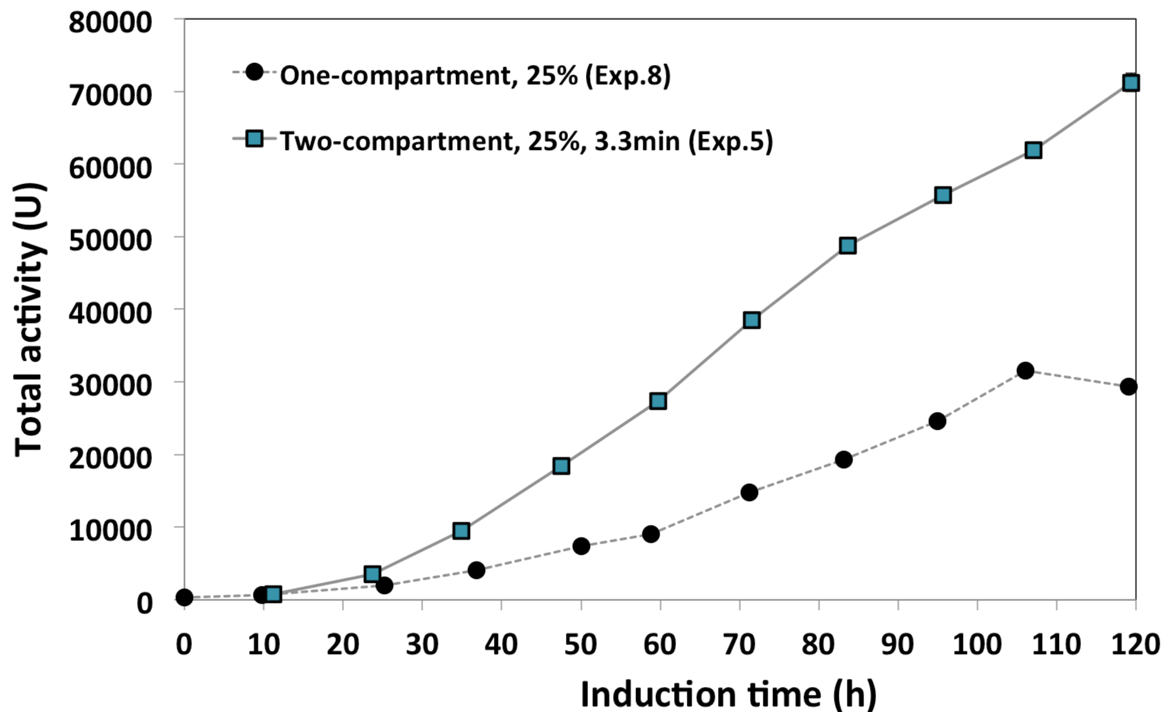


Figure 30: Comparison of the total activity (titer) of center point (Exp.5) to an one-compartment experiment (Exp.8) with the same value of DO

Figure 30 shows the total activities of Exp.5 and Exp.8. The highest value was observed again in Exp.5, which were performed in two-compartment system at 25% of DO. The total activities for both experiments were initially of the same, but it increases for two compartment system more rapidly and reached up to 71 243 U at the end of induction phase, while it was calculated for Exp.8 (one-compartment system) at the same time 29 389 U. The results imply, that the oxygen-limitation cause an apparent increase in total activity. This is a proof of how effectively the stress conditions on the cells act.

3.4.4 Yields

Figure 31 and 32 show how the biomass yield and carbon dioxide yield of Exp.5 and Exp.8 running versus the time. These two yields are important for the carbon balance and for the analysis of physiological states of the cells during the process. As mentioned before, the CO_2 content in the exhaust gas was measured online and these measured values were used for the calculation of carbon dioxide yield. To calculate the biomass yields both online and offline data were needed. For the formulas to calculate both biomass yield and carbon dioxide yield see equation 13 and 14. The biomass yield in both experiments was the same and amounted on the average about 3.5 C-mol/C-mol. The carbon dioxide yield in Exp.5 was up to 70 hours at 6 C-mol/C-mol, afterwards it has remained at about 7 C-mol/C-mol as in Exp.8. Hence it has been proved that the oxygen-related stress has no influence on the yield coefficients, and therefore the oxygen limitation in two-compartment system was no significant factor to change the cell physiology.

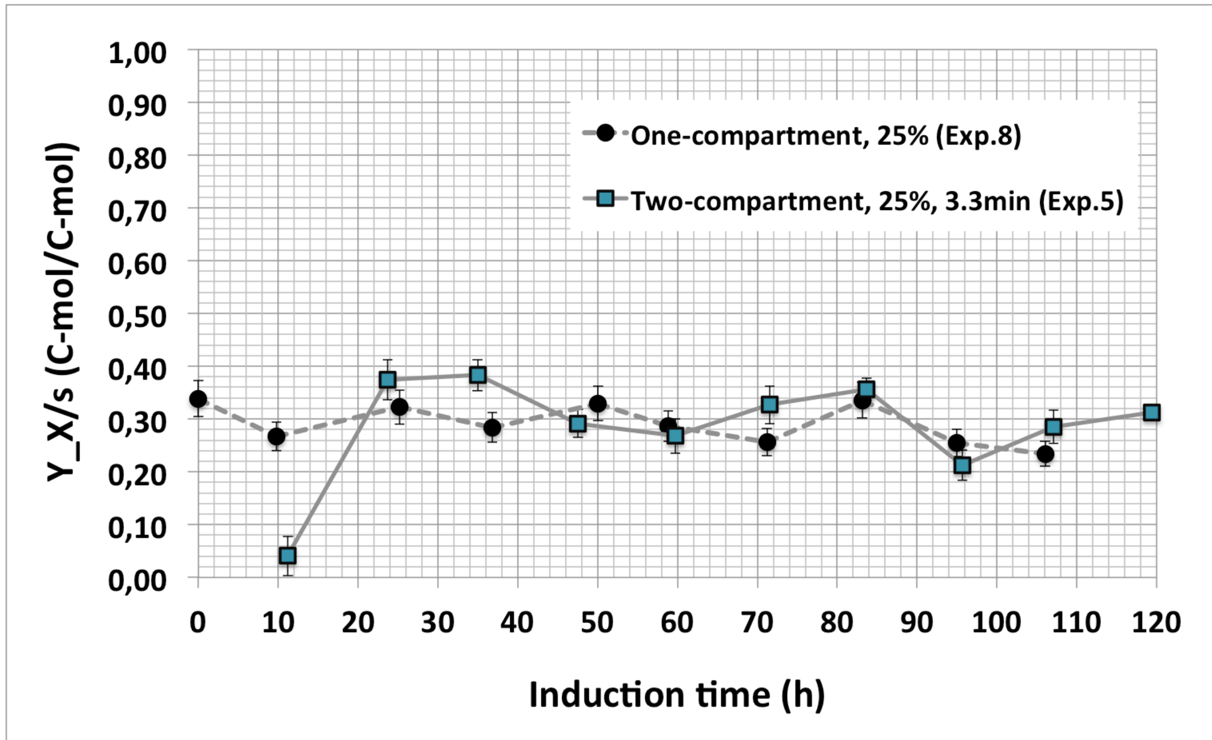


Figure 31: Comparison of the biomass yield of center point (Exp.5) to that of an one-compartment experiment (Exp.8) with the same value of DO

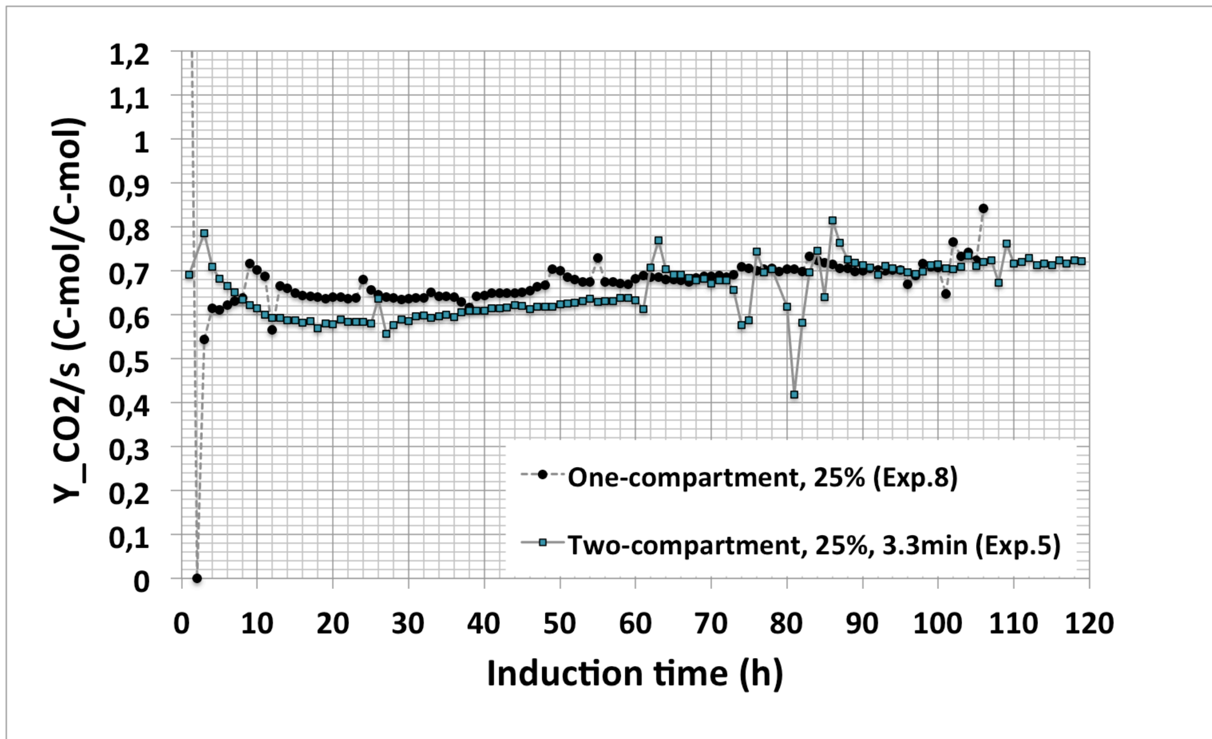


Figure 32: Comparison of the CO_2 yield of center point (Exp.5) to that of an one-compartment experiment (Exp.8) with the same value of DO

3.4.5 Result from DoE2

In the context of DoE1, all of the factors like DO and residence time and the response variables like specific productivity, titer and yields were evaluated qualitatively. To analyze and reinforce of the DoE1-statements mathematically, a second full factorial design of experiments (DoE2) were performed.

In this part, only the result of specific productivity was taken into account to demonstrate the quadratic effect of dissolved oxygen on these response variables. Figure 33 shows the coefficient plots of DoE2 to the specific productivity. The confidence interval of the quadratic term DO*DO was in this figure to take particular, it include not zero and can be regarded as significant. Since the coefficient plot of DO*DO was below the x-axis, it can be concluded that it has influenced the response variable adversely. This means, the higher the DO in the reactor; the less will be the specific productivity. Intermediate level of dissolved oxygen results in better protein quality and quantity.

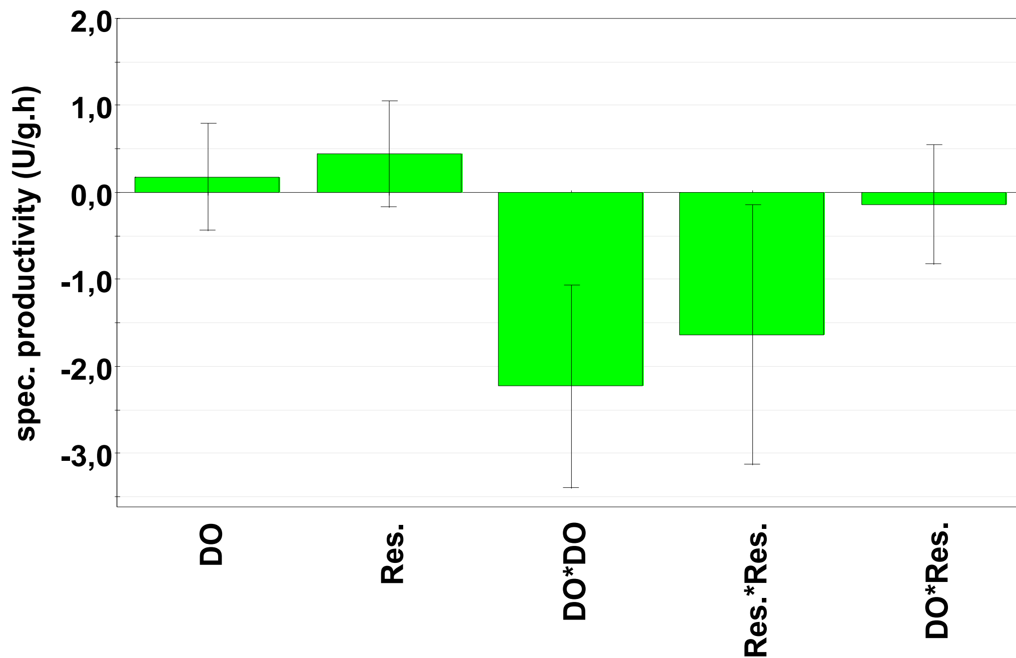


Figure 33: Coefficient plots for DoE2 to the specific productivity

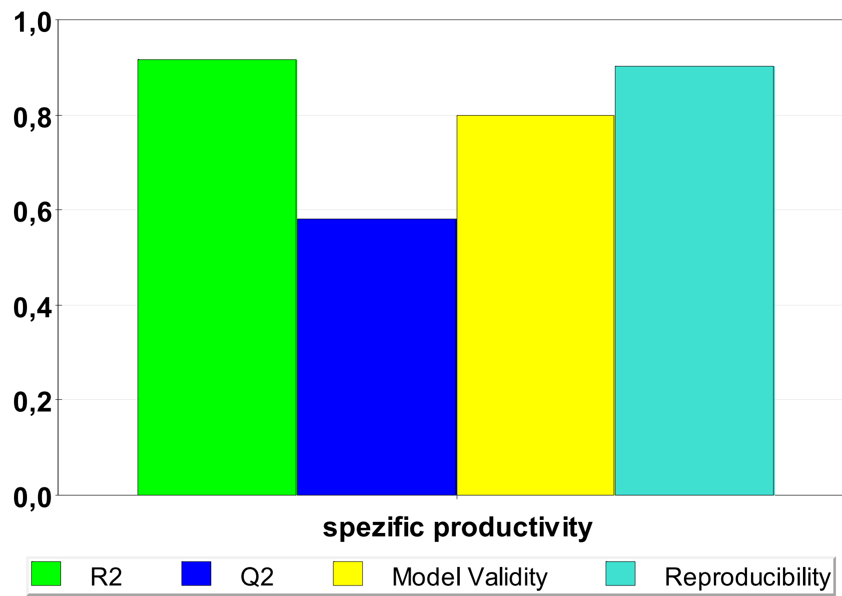


Figure 34: Regression model from DoE2 to the specific productivity with regression coefficient (R^2), prediction precision (Q^2), model validity and reproducibility of the model system

3.5 Discussion

In this study we show that oxygen limitation enhances specific productivity and the titer by using a two-compartment system. As can be seen in figure 15, 18 and 28, the specific productivity has a typical profile in all experiments. The upward, constant and downward trends in specific productivity after specific times were very noticeable. In two-compartment system, after 70 hours in induction phase specific productivity decreases (see figure 18 and 28). From these results we can decide when to stop the process. For example, if we want to increase the specific productivity, then it makes no sense to let the process run for more than 70 hours. In contrast to the specific productivity, the titer shows an increasing trend with the time (see figure 16, 19 and 30). As we arrive at two-compartment system more total activity, we can conclude that the process time is dependent on the goal: if we want to have ever more active proteins, we can make the process run as long as it is possible. But if we get more specific productivity, then the whole process should be a total stop after 70 hours.

Earlier it was mentioned that the DO level by some authors (such as by Anumanthan et al. 2007) to not be kept below 25% in bioreactor in order to avoid the oxygen limitation. Baumann et al. 2007 described the consequences of hypoxic condition in *Pichia pastoris* cultivation with three different protein producing strains by supplying the different oxygen concentration in the inlet air. After successful experiments in chemostat cultures, wherein a 2.5-fold rise in the specific productivity was achieved, the author has tested the experiments in fed-batch, so in a one-compartment system. That found that the fed-batch processes under the hypoxic condition resulted in three to six fold increased specific productivity. However, according to our results of one-compartment experiments, it does not matter in terms of the specific productivity whether DO is at 5%, 25% or 70% (see figure 15). But for operational reasons it is important to adjust in the reactor a DO level, which is easy to create and control, such as DO of 25%. It can be ruled out that in a one-compartment system a low or a higher DO in the reactor is not an effective solution to achieve increased protein quantity.

An oxygen-limited fed-batch technique, which according to our approach represents a one-compartment system, for the production of recombinant Thai Rosewood β-glucosidase in *Pichia pastoris* has been already described in Charoenrat et al. 2005. They found that the oxygen limitation in fed-batch increased the specific activity [$U/mg_{Protein}$] of β-glucose and the specific product concentration in the broth due to the less protein release. Since the specific activity [$U/mg_{Protein}$] is a function of total activity [U] and the extracellular protein amount [mg], it should be the total activity also increases with diminishing protein release. However, in contrast to the work of Charoenrat et al. 2005, we could detect no difference in terms of the total activities between one-compartment experiments with 25% and 5% DO, which was supposed to cause oxygen limitation (see figure 16).

Lee et al. 2003, reported, that higher DO tension simulated the methanol utilization pathway and enhanced the expression titre of the model protein elastase inhibiting peptide (EIP) about three-fold. Contrary to this assumption, we could not find a difference in the experiments that was carried out in one-compartment system at high (70%) and low (5%) DO levels (see figure 15).

Trentmann et al. 2004, studied in a one-compartment system the production of a single-chain antibody fragment in *P. pastoris* under low dissolved oxygen condition. They found, production was unaffected by oxygen limitation. This is in agreement with our results from one-compartment sytem experiments, that DO level in one-compartment system is not a significant factor to influence the protein production in a positive way.

In two-compartment system, the circulation of the fermentation broth between the main reactor as homogenously aerated zone and the PFR as oxygen-free zone caused a stress. The investigation of amplitude (different level of dissolved oxygen) and frequency (residence time in PFR) of the oxygen-related stress on the *Pichia pastoris* cells has revealed different statements about their affect on the response variables. In two-compartment system, the amplitude can be considered as a significant factor to influence the response variables positive. A two-compartment system with low level (5%) and high level (70%) of the dissolved oxygen has resulted like a one-compartment system with less specific productivity, while the intermediate level (25%) of the dissolved oxygen increased the specific productivity more than two-fold (see figure 18). If we look at the figure 29, we can say, that the cells can produce a little more proteins under stress conditions than in normal ambient conditions. This can be because of either production of more protein or the same protein, which are more active. Hence, if we want to achieve with the same protein content more total activity, then oxygen related stress could help us to increase the total activity. Also figure 30 shows this clearly positive impact that we have by two compartment system with 25% of DO more than two-fold more total activity at the end of induction phase than by one-compartment system.

George et al. 1993 has presented an aerated two-compartment sytem, which consists of an aerated PFR and aerated STR, and it has been compared with a one-compartment system to study the aerobic ethanol production of *Saccharamyces cerevisiae*. In contrast to our work, George et al. 1993 mixed the culture medium with the substrate feed and oxygen at the inlet of the PFR to lead the cells to a repeated high sugar concentration. They reported, that the differences in sugar concentration between the two reactors increased the ethanol production and resulted in less biomass yield. However, in our work, the PFR indicating an oxygen-free zone, and the substrate was feed only in the STR.

Another two-compartment system was proposed by Buyland et al. 1999, to study the cell responses of *E.coli* on the oscillating substrate gradients. It consisted of a STR/PFR system with an aerated PFR to avoid the oxygen limitation. Bylund et al. 1999 declared that not only substrates gradients but also oxygen limitation as secundary effect due to high glucose concentration is responsible for reduction of biomass and increased by-product formation. In comparison to the work doing by Bylund et al. 1999, in our approach, the substrate was fed only into the main reactor, thus the substrate gradient was avoided, and therefore, the oxygen limitation due to any substrates gradient was excluded.

As in the literature has already been reported, the Hsp70 and Hsp40 chaperone families regulate the folding and secretion of heterologous proteins (Zhang et al. 2006). According to Potvin et al. 2012, the expression is not the rate-limiting step but the secretion is bottleneck. It seems, the secretion of recombinant protein HRP due to Hsp70 and Hsp40 chaperones might be increased on the oxygen-related stress during the fermentation process. The secretion of more active protein under oxygen-related stress condition can be explained of the more activity of Hsp70 and Hsp40 chaperone families. The circulation of broth between the bioreactor and the PFR probably caused that the cell metabolism under stress conditions remains active. But, we have seen that these statements are only valid in the case of the two-compartment system with 25% dissolved oxygen, so for the central point in DoE1 and DoE2. In two-compartment system, a DO of 5% in the main reactor together with the DO of 0% in PFR has caused no oxygen-related stress on the cells, because of the different leveling between the DO is too low, that the cells during the circulation between the two parts can immediately adapt to the environmental conditions. Otherwise, in two-compartment system, a main reactor with 25% dissolved oxygen along with PFR, as oxygen-free zone can be an optimal setup, to

have more protein quantity. This means that the cells form the proteins faster under stress conditions, and we can therefore conclude that the oxygen-related stress increases the productivity of cells, when it is carried out optimally.

A quadratic effect of dissolved oxygen on the specific productivity and the titer was invented through the comparison of two-compartment experiments at different DO levels (see figure 18 and 19). It has been shown, at the low and the high level of dissolved oxygen was less specific productivity and titer were obtained, and that just as in a one-compartment system. These statements were analyzed mathematically with the aid of a second full factorial design of experiments (DoE2). It was confirmed by the DoE2, that the intermediate level of DO results in better productivity.

As can be seen in figure 31 and 32, the biomass and carbon dioxide yiels are the same in both two-compartment and one-compartment experiments. We could not detect significant differences in $Y_{CO2/s}$ and $Y_{O2/s}$ between both experiments. These results also indicate that the cells are in fermenter on both experiments in the same physiological condition and the oxygen limitation in reactor does not have any influence on physiology of the cells. Therefore, using two-compartment system could not affect the primary metabolic variables.

After sampling, the biomass concentration was determined with three different methods: indirect measurement with optical density, estimation using fixed yield, direct measurement using microwave. Since the values of OD measurement were unreliable, the biomass concentration was determined mainly by microwave method as a cross-check for the biomass estimated with fixed yield. Using this strategy, we were able to control qs well (see figure 12). This demonstrates the capability of this approach for controlling qs.

As mentioned, we have tried using a bubble trap and a bypass to eliminate the entrained gases in broth circulated in loop. The result shows clearly that this is an optimal method to provide a DO of 0% (see figure 17).

Why residence time was not affected the protein production?

The circulation of the fermentation broth between the main reactor as homogeneous mixed zone and the PFR as oxygen-free zone was to be main task of our two-compartment system. Not only the amplitude but also the frequency of the oxygen-related stress was of particular interest, and has been extensively studied in this work. The residence time, which the cells spend in PFR during the circulation, was defined as the frequency of the stress, and was adjusted to the desired value (3.3 min and 15 min) with the aid of circulation flow rate. The circulation caused that the cells metabolize in the bioreactor in their normal state, while the environmental conditions in PFR force the cells more and more to stay active. Since the fermentation broth in the main reactor is completely mixed, and the volume of the broth in the PFR remains constant, each cell has the opportunity to pass as much as possible as the other cells through the PFR. Every time when the cell is out of a zone to another zone, it tries to adapt to new conditions. But, it was found that it plays no significant role, as long as the cells are in PFR under oxygen-limitation, it is important to bring the cells as much as possible under oxygen-related stress.

Sandoval-Basurto et al. 2005, used two interconnected STRs as two-compartment system with different circulation times to study the DOT gradients on the model system *E.coli*. In comparison to our work, they reported, that the circulation between an aerobic and an anaerobic STR even at the short circulation times resulted in reduced recombinant protein productivity.

The impact of different residence times using a two-compartment scale-down system was recently reviewed by Lorantfy et al. 2012. In our work, the two-compartment experiments were performed either with 3.3 or with 15 minutes of residence time. The qualitative analyses by DOE1 have shown that the residence time does affect neither the specific productivity nor the product titer (see figure 21 and 23).

4 Conclusion

In this work, we proposed a two-compartment system consisting of a STR as main reactor and PFR as oxygen-limited zone, to study the effect of the oxygen-related stress on the production of HRP in *Pichia pastoris*. The proposed technique was very easy to set up in labor-scale. The volume of the cell culture, which flows between the main reactor and PFR, has amounted to about 10% of the batch volume.

The oxygen limitation and consequent stress on the cells were carefully examined at different DO levels (amplitude of stess) and residence times (frequency of stress) to understand how the cell physiology and productivity change. Hence, the amplitude, which indicating the amount of the dissolved oxygen in the main reactor, was chosen in three levels: 5, 25 and 70%, respectively. The frequency, which representing the time of the circulation between the main reactor and PFR, was adjusted with the aid of circulation flow rate in two levels: 3.3 and 15 minutes, regarding to the mixing time of the large bioreactors. In two-compartment system, the DO level in the PFR was always kept at 0% to create a completely oxygen-free zone and to quantify the cellular responses between an aerated and anerobic part. Since in one-compartment system the PFR part was not attached, the residence time in this approach was irrelevant.

There were also one-compartment experiments performed as reference runs, where only the STR as normal single reactor was used. The result of the one-compartment experiments at different DO levels (5%, 25% and 70%) has demonstrated that in this approach the DO level does not play an important role to trigger the recombinant protein production of the cells. The specific productivity and the total activity were in all of the experiments the same, as long as the culture is not oxygen limited.

However, in two-compartment system, the results of an experiment with 25% DO in the main reactor and with zero oxygen in the PFR looked quite different. This experiment, what we have called the central point in our multivariable data analysis, showed a 2.5-fold increase in the specific productivity and resulted in more than two-fold in the total activity. The results of DOE1 were demonstrated qualitatively that the two-compartment experiments with low level (5%) and high level (70%) of the dissolved oxygen have resulted like a one-compartment system with less specific productivity. Hence, low and high level of DO influence negatively, during the intermediate levels result in improved protein quantity (quadratic effect). With the aid of a further data analysis, namely DOE2, the quadratic effect of DO in intermediate level (25%) on the response variables such as specific productivity and total activity was dedected quantitatively.

While the DO at intermediate level for the production of recombinant proteins played a major role, it was proved that the residence time is not a relevant process parameter for a two-compartment system. It is assumed that it does not matter how long the cells stay in this equilibrium state however it is important to only experience this stress. Based on the DOE1 and DOE2, no interaction effect between dissolved oxygen and residence time could be determined.

Oxygen-related stress is a tool to enhance protein quality and quantity. Since the physiology parameters, such as biomass yield and carbondioxide yield in all experiments were the same, no effect of oxygen-related stress on physiologie of the Pichia pastoris cells was observed.

The two-compartment system as a novel process technology is a proper tool to apply oxygen-related stress and successfully operates in laboratory-scale bioreactors. The economic analysis of two-compartment and one-compartment processing was reported by Jazini et al. 2013-c. Hence, the analysis showed, that the two-compartment system as a proper process technology could be implemented in the large-scale systems with less investment costs and more income, which represent crucial factors for biopharmaceuticals.

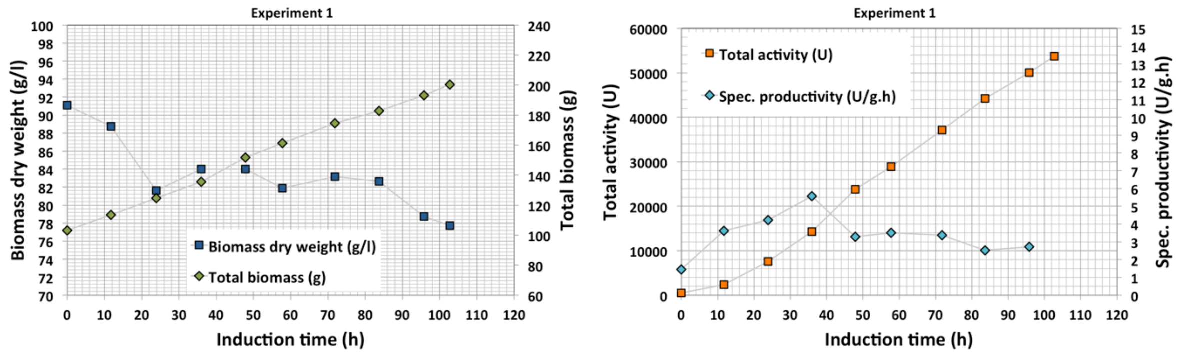
5 Outlook

The oxygen-related stress makes the two-compartment system to a simple useful process technology to improve the protein quantity and quality without affecting the physiology of the cells. According to the results obtained in this work, the periodic oxygen limitation positively affects the productivity of target protein. The proposed two-compartment system as a novel technology offers many advantages and is relatively easy to set up in the laboratory scale. In this work, we have performed the experiments with the strain of *Pichia pastoris* containing the HRP. The two-compartment system can also be used for other strains of *Pichia pastoris*.

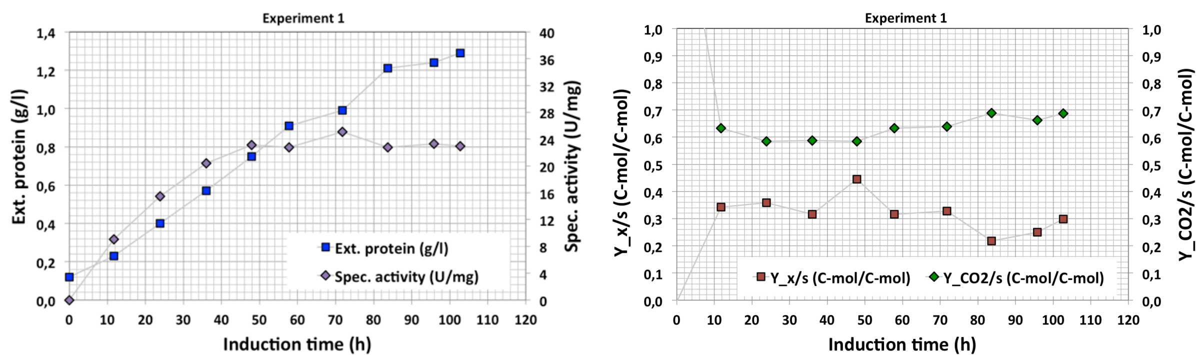
In a single reactor the modification of certain parameters such as dissolved oxygen and temperature is complex, since it requires a lot of time. In two-compartment system, the plug flow reactor allows these parameters during the circulation relatively easy to change and control. Therefore, the two-compartment system allows the application of other kinds of stress to the cells like temperature-related stress.

6 Appendix

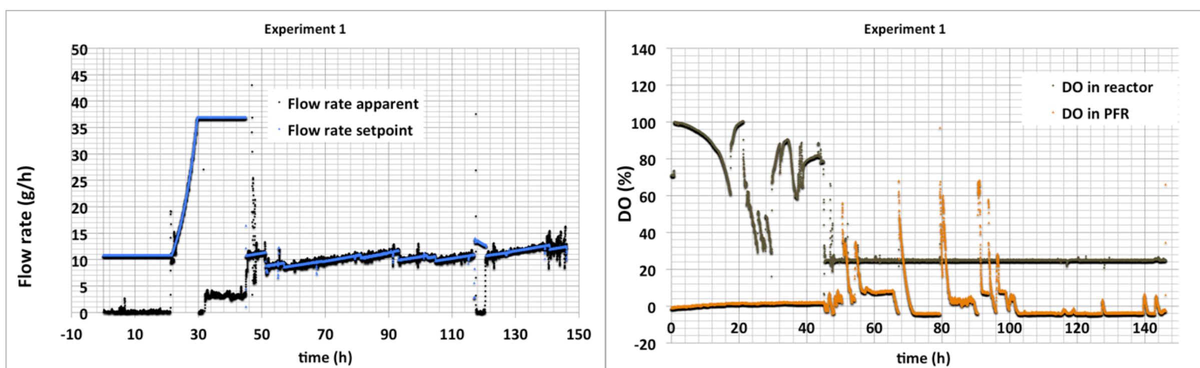
6.1 Acquired data of experiments



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

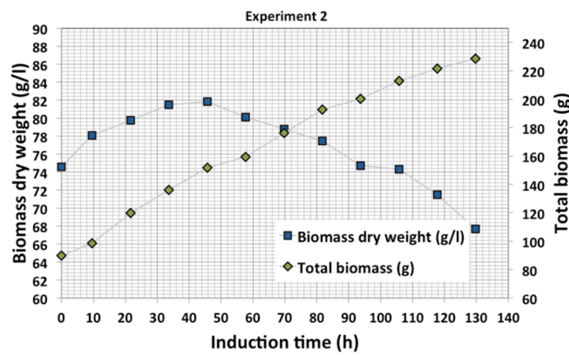


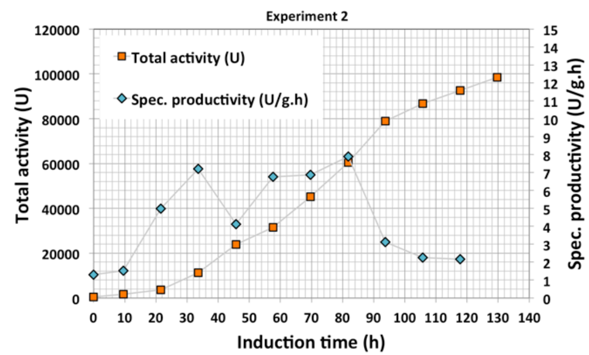
b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.



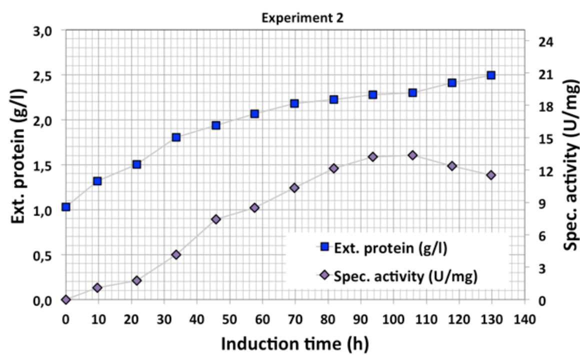
c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

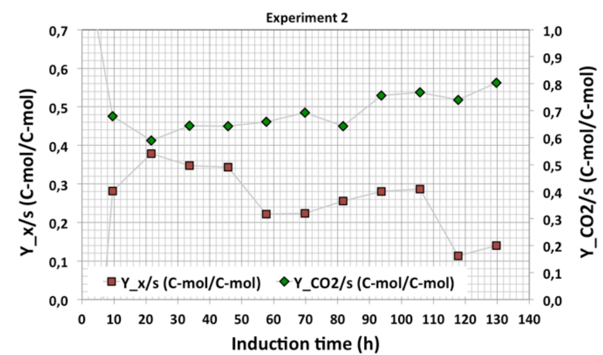
Figure 35: Experiment 1: Fermentation in two-compartment system at 25% of dissolved oxygen and 3.3 min of residence time.



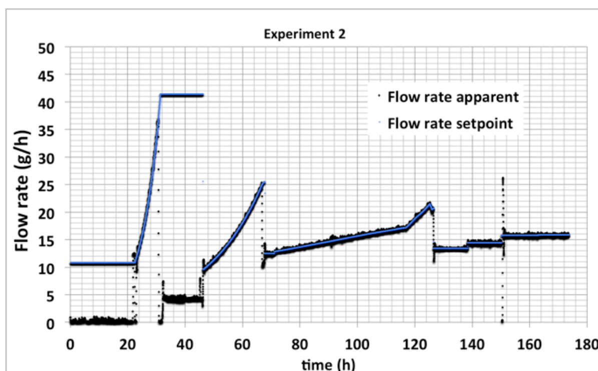


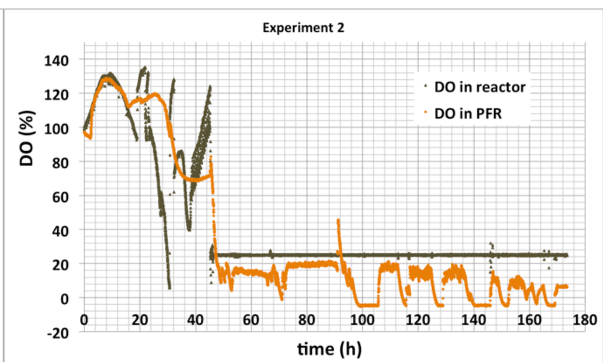
a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.





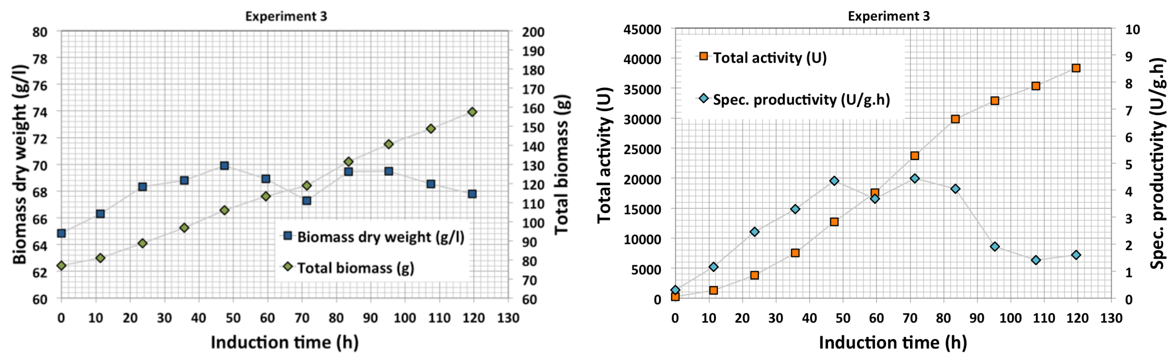
b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.



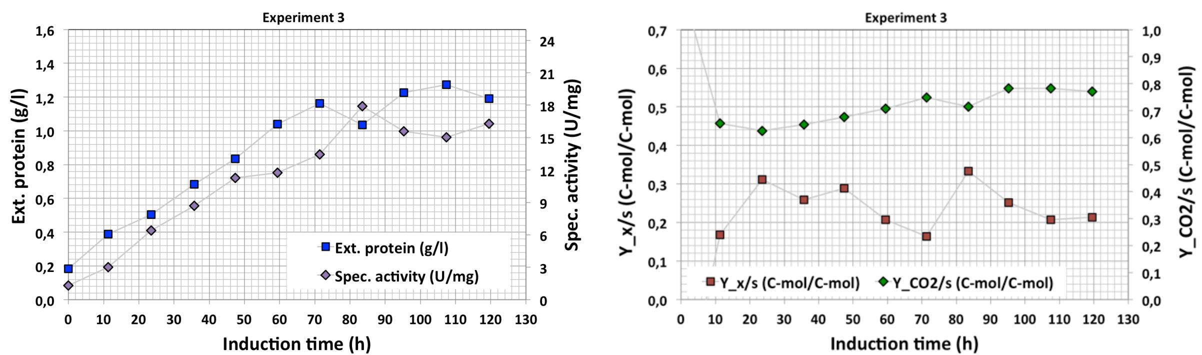


c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

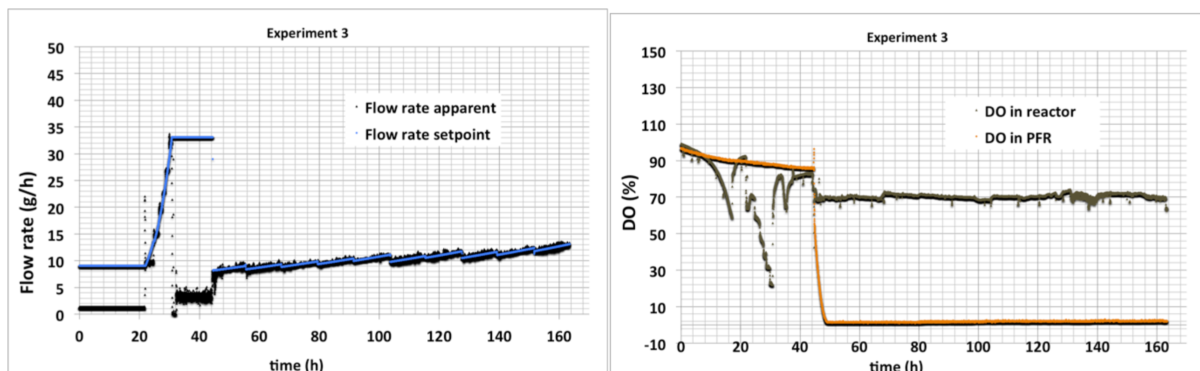
Figure 36: Experiment 2: Fermentation in two-compartment system at 25% of dissolved oxygen and 15 min of residence time.



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

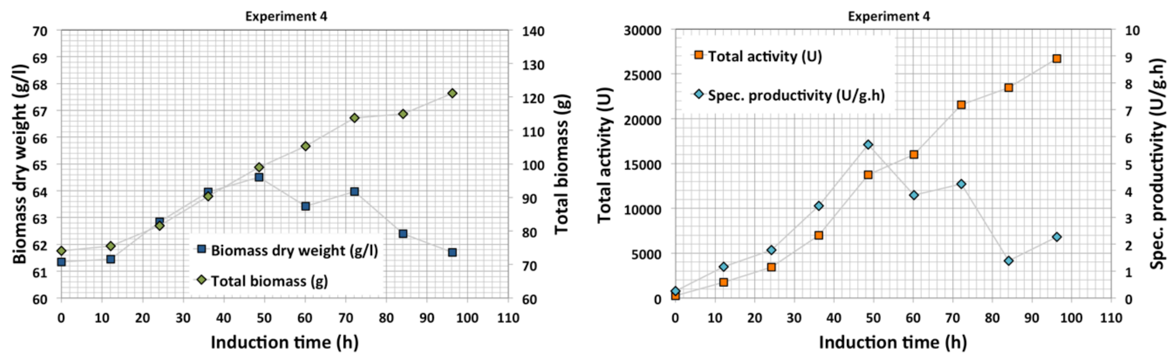


b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.

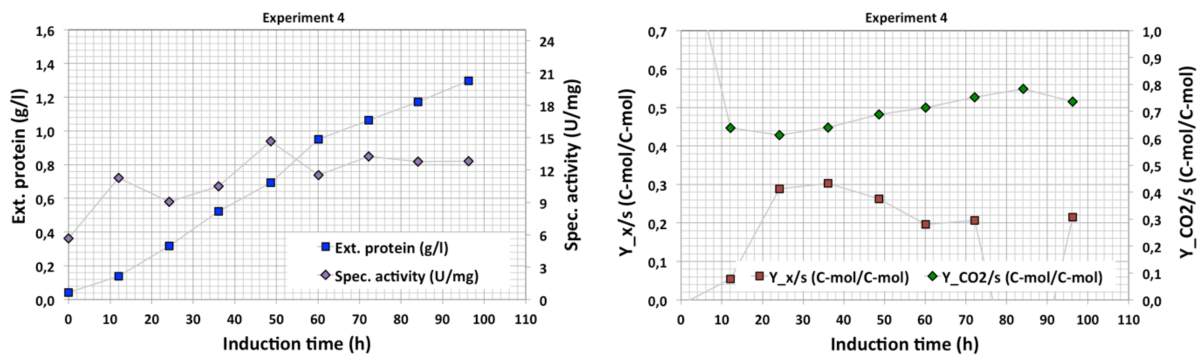


c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

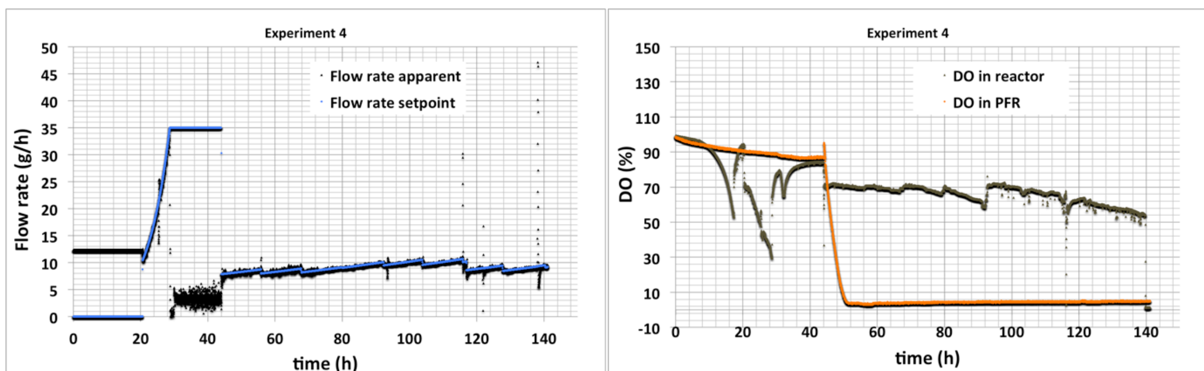
Figure 37: Experiment 3: Fermentation in two-compartment system at 70% of dissolved oxygen and 3.3 min of residence time.



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

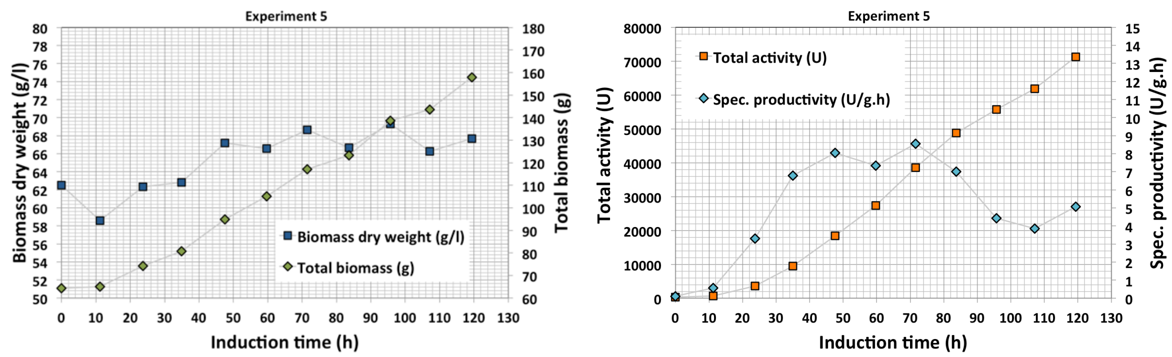


b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.

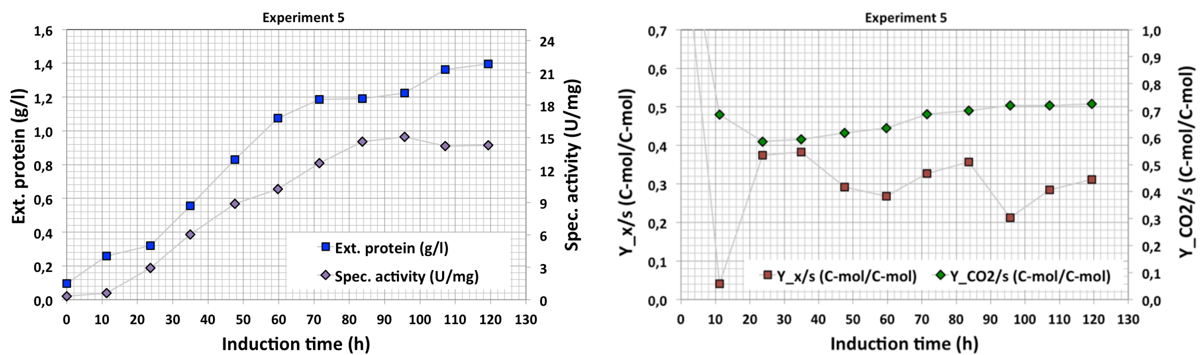


c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

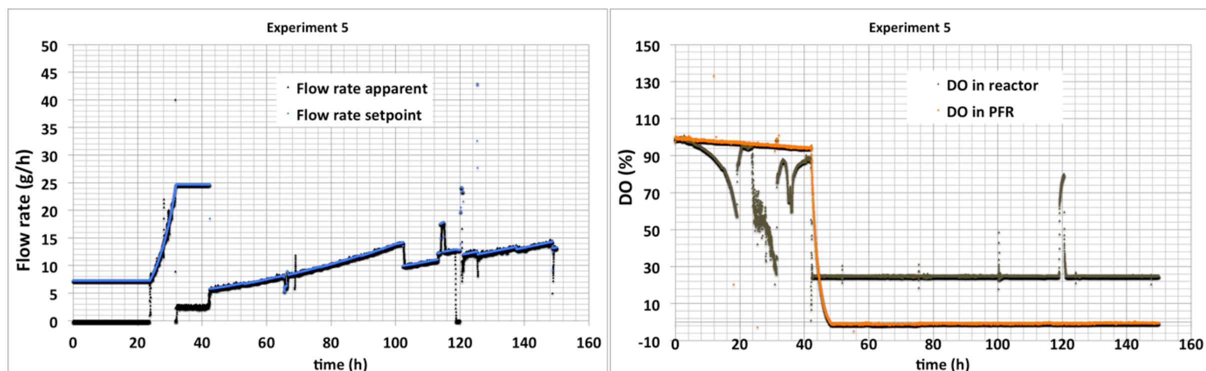
Figure 38: Experiment 4: Fermentation in two-compartment system at 70% of dissolved oxygen and 15 min of residence time.



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

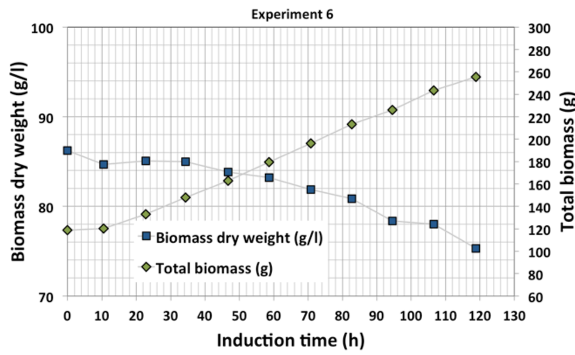


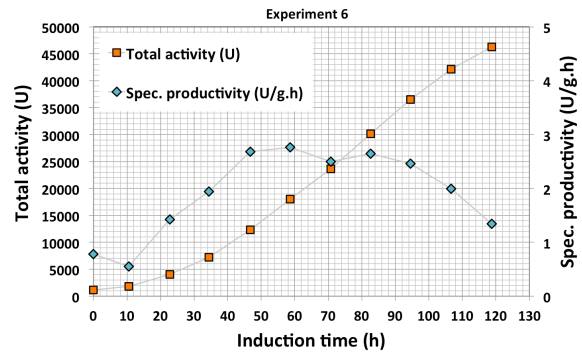
b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.



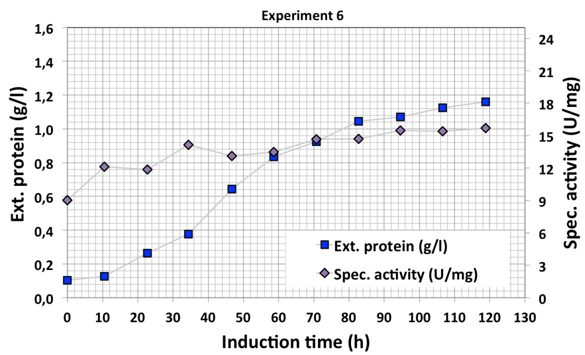
c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

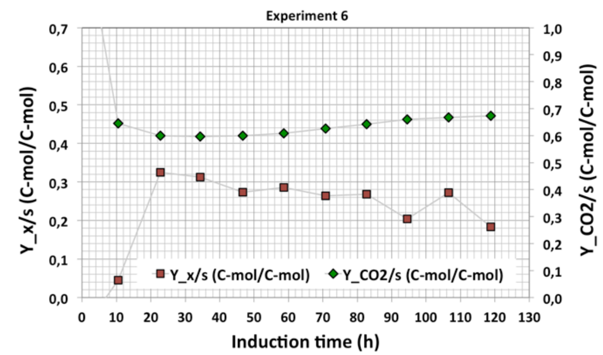
Figure 39: Experiment 5: Fermentation in two-compartment system at 25% of dissolved oxygen and 3.3 min of residence time.



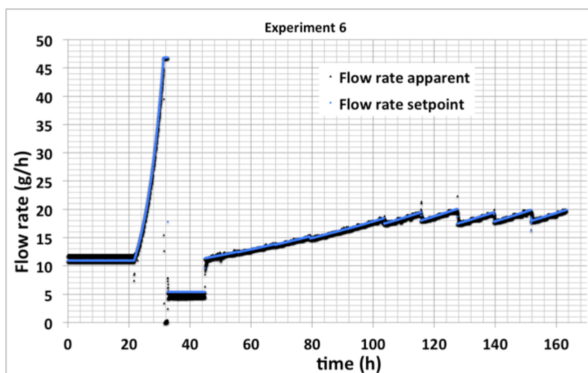


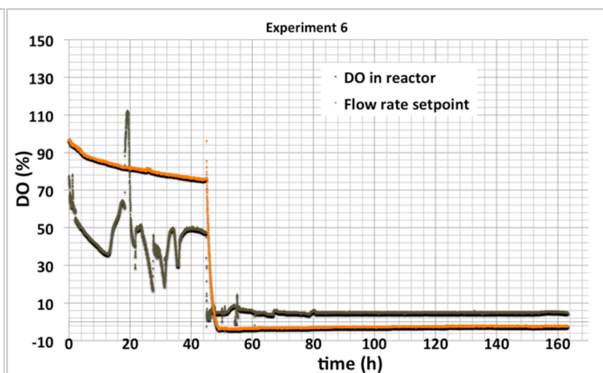
a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.





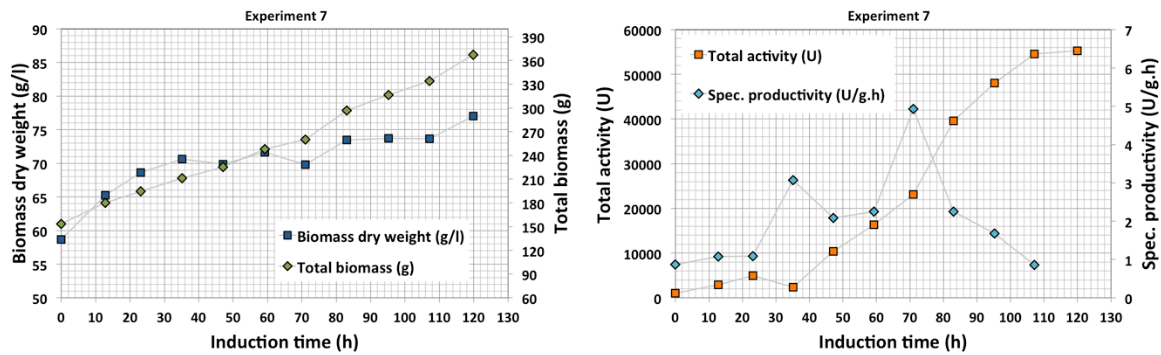
b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.



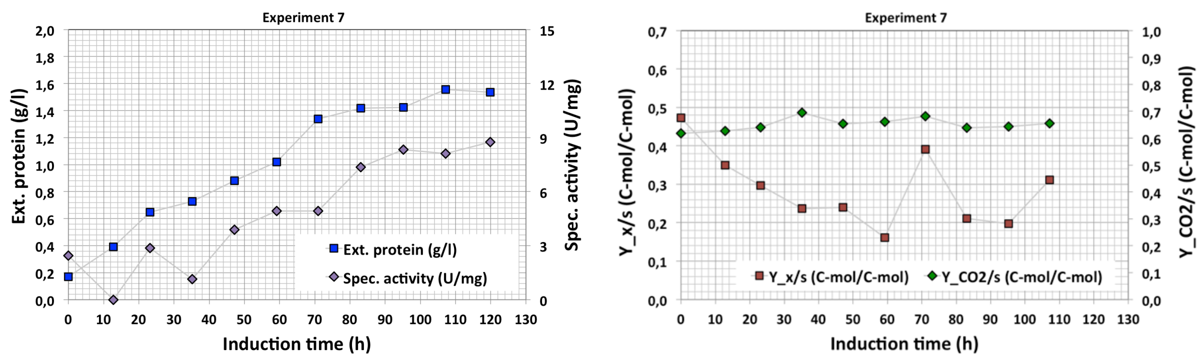


c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line.

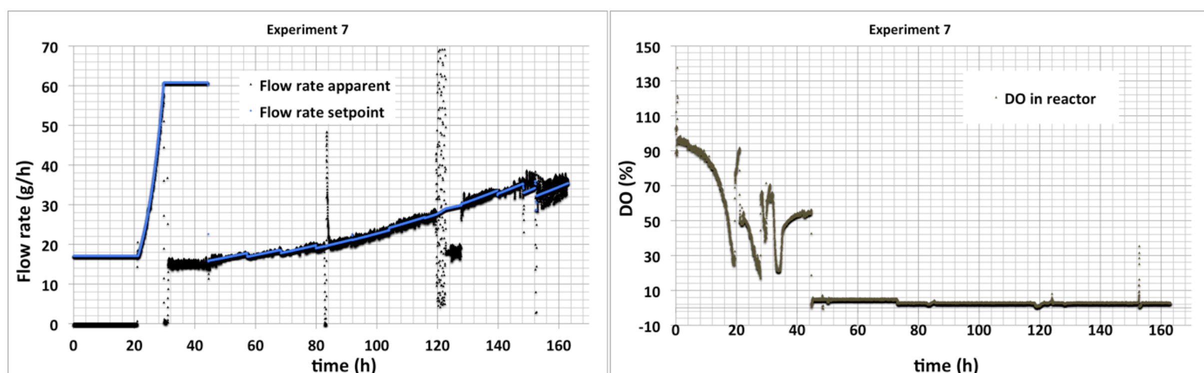
Figure 40: Experiment 6: Fermentation in two-compartment system at 5% of dissolved oxygen and 3.3 min of residence time.



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

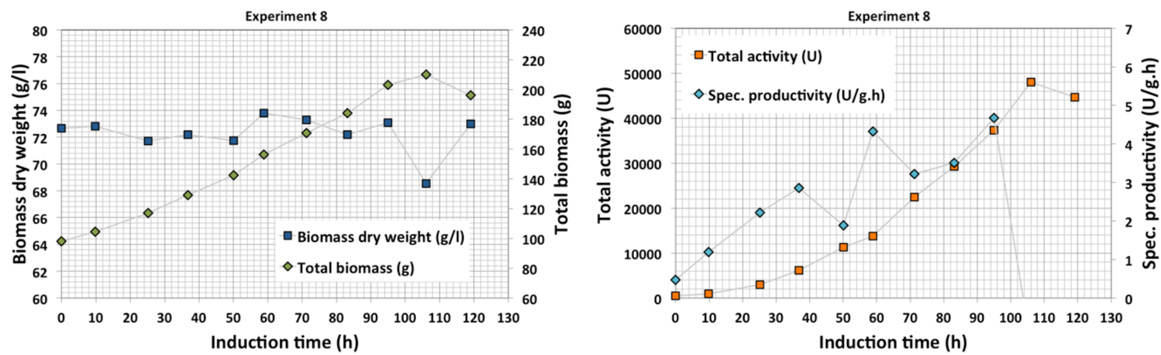


b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.

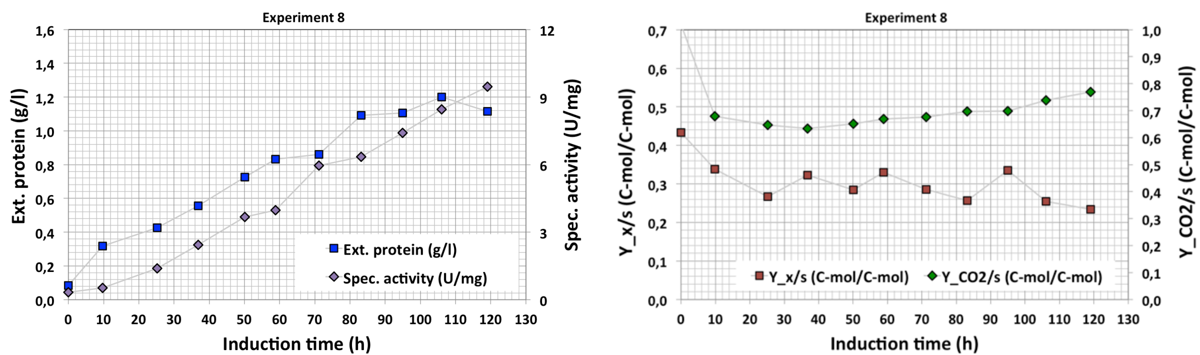


c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor. x-axis: process time. All of these data were determined on-line.

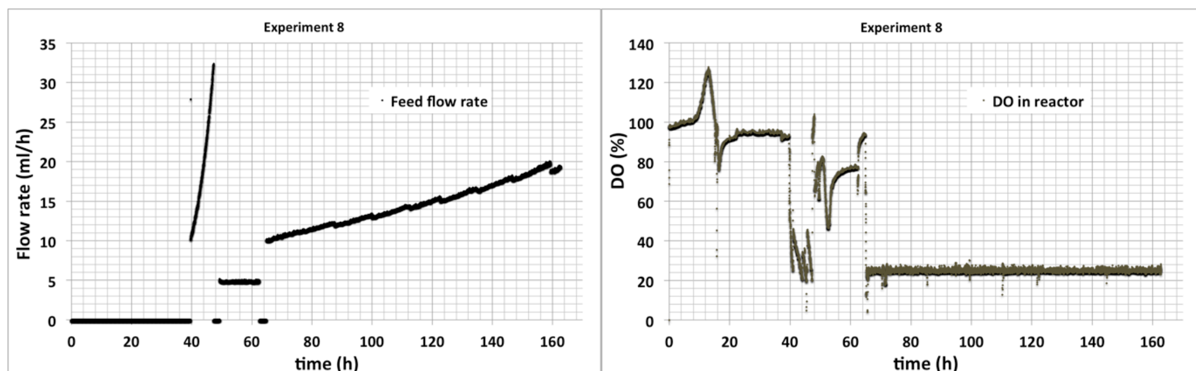
Figure 41: Experiment 7: Fermentation in one-compartment system at 5% of dissolved oxygen.



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

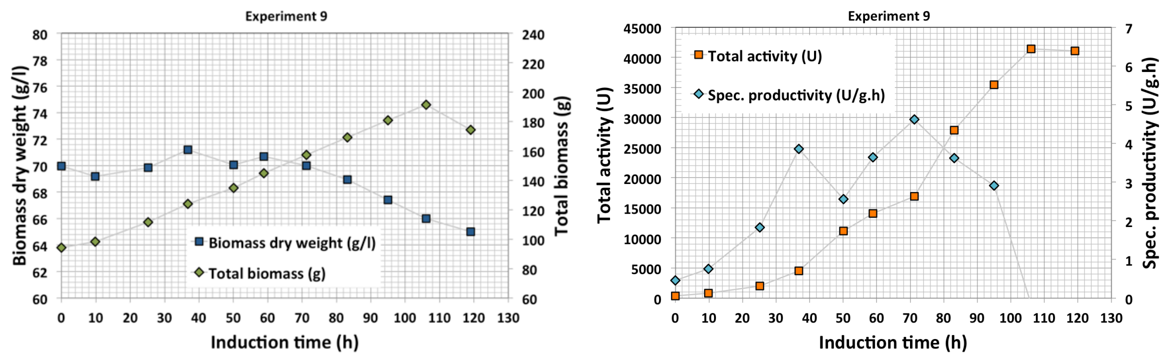


b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.

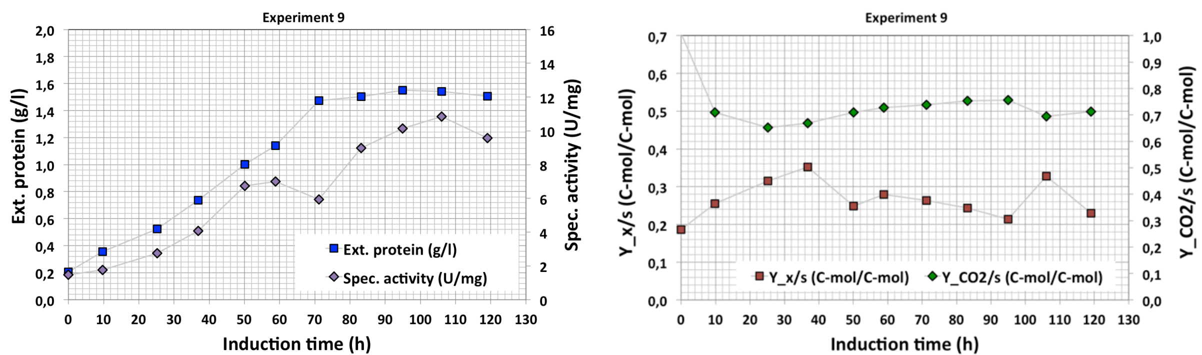


c) Left: feed flow rate apparent(ml/h). Right: DO (%) in reactor. x-axis: process time. All of these data were determined on-line. Flow rate setpoint was not saved.

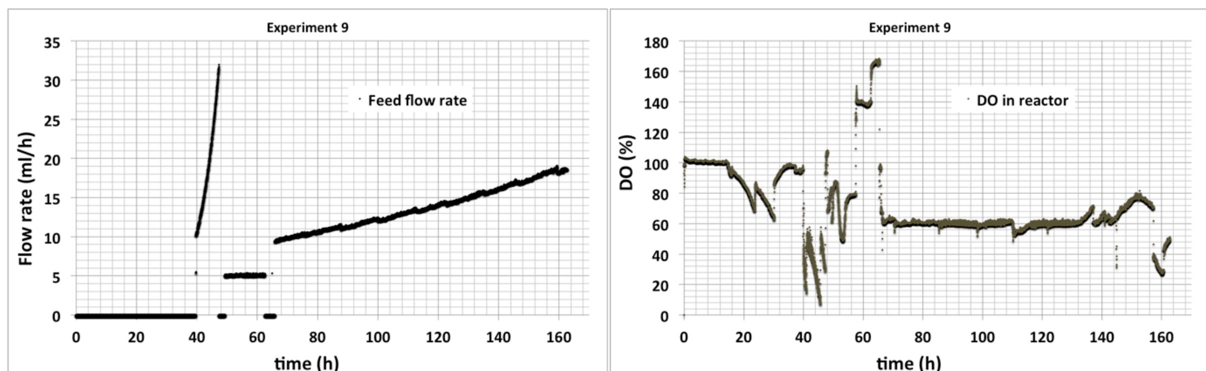
Figure 42: Experiment 8: Fermentation in one-compartment system at 25% of dissolved oxygen and 3.3 min of residence time (DASGIP experiment).



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.

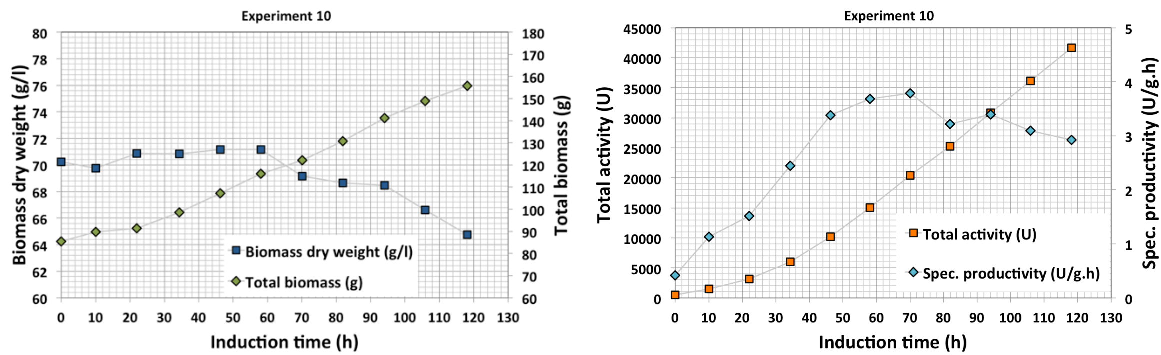


b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line.

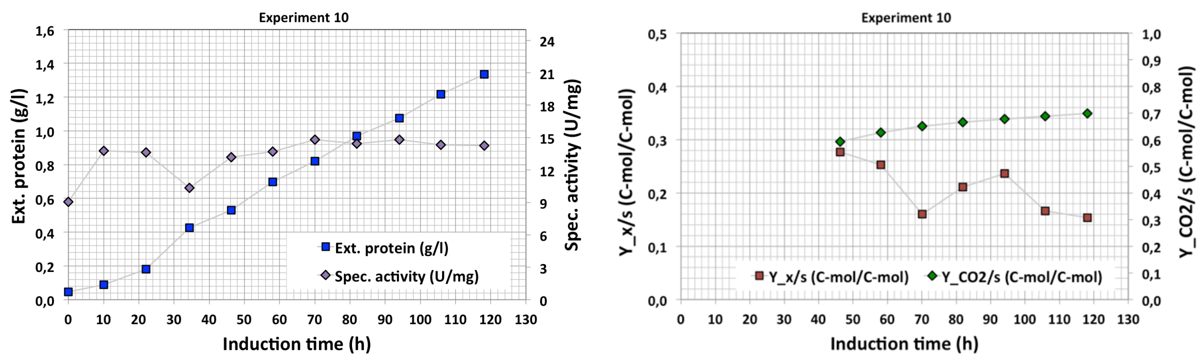


c) Left: feed flow rate apparent(ml/h). Right: DO (%) in reactor. x-axis: process time. All of these data were determined on-line. The flow rate setpoint was not saved.

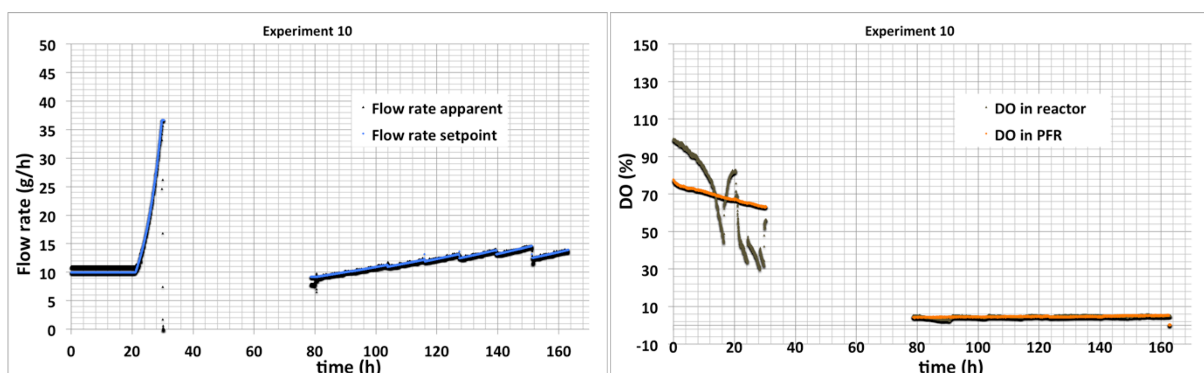
Figure 43: Experiment 9: Fermentation in one-compartment system at 5% of dissolved oxygen and 3.3 min of residence time (DASGIP experiment).



a) Left: biomass dry weight (g/l) and total biomass (g). Right: total activity (U) and specific productivity. x-axis: induction time. All of these data were determined off-line.



b) Left: extracellular protein (g/l) and specific activity (U/mg). Right: biomass yield (C-mol/C-mol) and carbon dioxide yield (C-mol/C-mol). x-axis: induction time. All of these data were determined off-line. By this experiment was a problem with Lucullus between 30 and 78 hours, and the on-line data could not be saved.



c) Left: feed flow rate setpoint (g/h) and feed flow rate apparent (g/h). Right: DO (%) in reactor and in PFR. x-axis: process time. All of these data were determined on-line. By this experiment was a problem with Lucullus between 30 and 78 hours, and the on-line data could not be saved.

Figure 44: Experiment 10: Fermentation in two-compartment system at 5% of dissolved oxygen and 15 min of residence time.

6.2 List of used formulas

Material balance [-]	$\dot{V}_{in} \cdot c_{i,in} - \dot{V}_{out} \cdot c_{i,out} + V_R \cdot r_i = \frac{\delta c_i}{\delta t} \cdot V_R + \frac{\delta V_R}{\delta t} \cdot c_i$
Respiratory quotient (RQ) [-]	$RQ = \frac{CER}{OUR}$
C – balance [-]	$Y_{x/s} + Y_{Protein/s} + Y_{CO_2/s} = 1$
Degree of reduction (DR) balance [-]	$\frac{r_x \cdot \gamma_x + r_{protein} \cdot \gamma_{protein}}{(r_{O_2} \cdot \gamma_{O_2} + r_s \cdot \gamma_s) \cdot (-1)} = 1$
Nitrogen balance [-]	$\frac{r_x \cdot x_n + r_{protein} \cdot x_p}{-r_{NH_4}} = 1$

Symbols

c	concentration	[g/l]
CER	carbon dioxide evolution rate	[C-mmol/l.h]
DO	dissolved oxygen	[-]
PFR	plug flow reactor	[-]
OD600	Optical density at 600 nm	[-]
OUR	oxygen uptake rate	[mmol/l.h]
RQ	respiratory quotient	[-]
r_{inert}	inert gas ratio	[-]
t	time	[h]
V_R	reactor volume	[l]
$\dot{V}$	flow rate	[g/h]
x_{H2O}	water content in off-gas	[-]
Y	yield	[C-mol/C-mol]

Greek symbols

ρ	density	[g/l]
µ	specific growth rate	[1/h]
Γ	gas flow	[l/min]

Indices

CO_2	carbon dioxide
in	In-coming
N	nitrogen
O_2	oxygen
out	Out-going
R	reactor
s	substrate
X	biomass

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