

Article

Quantifying the Effects of Frequency and Amplitude of Periodic Oxygen-Related Stress on Recombinant Protein Production in *Pichia pastoris*

Mohammadhadi Jazini, Glbahar Cekici and Christoph Herwig *

Research Division Biochemical Engineering, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria;

E-Mails: mohammadhadi.jazini@tuwien.ac.at (M.J.); baharos2@hotmail.com (G.C.)

* Author to whom correspondence should be addressed; E-Mail: christoph.herwig@tuwien.ac.at; Tel.: +43-1-58801-166400; Fax: +43-1-58801-166980.

Received: 24 October 2013; in revised form: 25 November 2013 / Accepted: 2 December 2013 /

Published: 18 December 2013

Abstract: *Pichia pastoris* is an attractive candidate platform for recombinant protein production. Dissolved oxygen is one of the most important factors in the cultivation of *P. pastoris*. However, the effect of oxygen on triggering productivity led to ambivalent results. In our earlier work, a two-compartment system, consisting of a single reactor coupled with a plug flow reactor (PFR), has been proposed as a tool to improve protein quantity and quality. The goal of this work was to investigate the effects of frequency (the residence time of broth in the PFR) and amplitude (the dissolved oxygen level in the reactor) of the stress on productivity, titer and physiology. A recombinant *P. pastoris* strain, which expressed horseradish peroxidase, was used as the model system. Thirteen experiments were performed. Multivariate data analysis was done and the results showed that the residence time did not influence titer, productivity and physiology over the range of residence time studied while dissolved oxygen influenced titer and specific productivity in a quadratic function. In other words, an intermediate level of dissolved oxygen (25%) showed the highest specific productivity and titer, irrespective of the residence time in the PFR. In turn, the variation of the residence time and dissolved oxygen did not influence growth physiology, as quantified in biomass and carbon dioxide yields.

Keywords: oxygen-related stress; two-compartment system; *Pichia pastoris*

1. Introduction

The methylotrophic yeast *Pichia pastoris* is an attractive candidate platform system for recombinant protein production as it can secrete properly folded proteins. It is one of the most effective and versatile systems for expression of heterologous proteins [1] because of these reasons: (1) high growth rate (2) high level of productivity in an almost protein-free medium (3) ease of genetic manipulation (4) and the absence of known human pathogenicity [2].

The AOX1 promoter, which is derived from the alcohol oxidase I gene of *P. pastoris*, is uniquely suited for the controlled expression of foreign genes [3]. The promoter is repressed in the presence of glycerol as well as glucose and induced in the presence of methanol [1]. High cell density is achieved by performing a batch phase followed by a fed batch phase on glycerol or glucose. Subsequently, the culture is fed with a low feed rate of methanol so that the cells adapt themselves to the new carbon source; prior to apply a higher methanol feed rate. After the adaptation period, the methanol feed rate is increased to the designed flow rate and consequently the recombinant product is induced [4].

Operating conditions such as pH, temperature, and dissolved oxygen cause impact on protein production. The dissolved oxygen was reported to be one of the most influencing factors [2]. Production of a single-chain antibody fragment in *P. pastoris* under a low level of dissolved oxygen was done by Trentmann *et al.* [5]. They found that the production was unaffected by oxygen limitation. A similar approach was applied by Hellwig *et al.* to the production of recombinant scFv antibody fragment [6]. In both of these studies, the dissolved oxygen was almost zero during the induction phase because the oxygen uptake rate was close to the maximum oxygen transfer rate. *P. pastoris* was cultivated in a DO-stat culture by Lee *et al.* [7], investigating different dissolved oxygen ranges. The results showed that higher ranges of dissolved oxygen enhanced product titer. Fed-batch cultivation of *P. pastoris* under the control of GAP promoter at hypoxic condition (oxygen-limited condition where dissolved oxygen was kept zero) was reported by Bauman *et al.* [8]. They observed improvement in specific volumetric productivity of the target recombinant protein but this was at the expense of reduction in biomass [9]. Jazini *et al.* [10] investigated the effects of different dissolved oxygen concentrations on protein production during the induction phase. They found out that different dissolved oxygen levels did not change the specific productivity. Hence, they proposed a two-compartment system (a single reactor coupled with a plug flow reactor (PFR)) in which a portion of the broth was circulated continuously between the reactor and the PFR [10]. The dissolved oxygen in the PFR was zero therefore the cells experienced periodic oxygen-limited conditions, hence stress conditions. The cultivation of *P. pastoris* in this system resulted in more than twofold increase in the production of recombinant horseradish peroxidase (HRP). The dissolved oxygen level in the reactor (“dissolved oxygen”) and the residence time of the broth in PFR (“residence time”) were the two main characteristics of the two-compartment system. The former reflects the amplitude of the stress between the two compartments, while the latter represents the frequency of the stress which means how often the cells are exposed to the stress [11]. The residence time was defined as the ratio between the volume of the PFR and the circulation flow. To our knowledge, there is no work investigating the effects of the amplitude and the frequency of the oxygen-related stress (“stress”) in a two-compartment system with the aim of improving protein production.

Hence, the aim of this work was to study the effects of both frequency and amplitude of oxygen-related stress on the production of recombinant HRP in *P. pastoris*. In other words, this work aimed to investigate whether the dissolved oxygen and the residence time are relevant process parameters influencing the product quantity and physiology and a two-compartment system may serve as a basis for a suitable scalable production mode. It is worthwhile to mention that in this work a two-compartment system was used not to mimic the inhomogeneous environment of large bioreactors. For example Amanullah *et al.* [12] investigated the inhomogeneous environment with respect to pH in a two-compartment system using *Bacillus subtilis* as the biological model system. The spatial variation of the dissolved oxygen was studied by Bylund *et al.* [13]. Some other researchers used two interconnected well-mixed reactor as a two-compartment system to mimic the inhomogeneous dissolved oxygen in large bioreactors [14–16].

This is the first report where the two-compartment system was used to apply intended stress to make the cells more productive.

2. Materials and Methods

2.1. Biological Model System

The *P. pastoris* strain KM71 H (aox1:arg4 to aox1::ARG4) was transformed 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, Graz, Austria). The phenotype of the strain corresponded to an AOX1-deficient clone which is characterized as Mut^S (methanol utilization slow) and HRP was secreted into the fermentation broth. The medium, for the batch and fed batch cultivations as well as the detailed composition of the feeds are available elsewhere [4].

2.2. Experimental Setup and Approach

All experiments were conducted in the same way. Every experiment consisted of four phases: (1) the batch phase (2) the fed batch phase (3) the adaptation phase (4) and the induction phase. The first two phases were carried out only to achieve high cell density. The adaptation phase was a period during which the cells adapted themselves to the new feed (methanol). Expression of the target protein was initiated in the induction phase. The feed rate in this phase was adjusted in such a way that specific substrate uptake rate of 1 mmol/L h was set. The detailed description of each phase was fully described before [4].

The experiments were performed by means of a 3 L highly instrumented autoclavable laboratory bioreactor (Infors, Bottmingen, Switzerland) connected to the Process Information Management System (Lucillus, Secure Cell AG, Schlieren, Switzerland) for online process monitoring and control. The bioreactor was equipped with temperature, dissolved oxygen and pH control system which facilitated controlling of process parameters accurately. The cultivation parameters were set to 28 °C and 1,500 rpm of stirrer speed with 2 vvm air flow. In induction phase, the stirrer speed was automatically adjusted to achieve the desired dissolved oxygen level. pH was adjusted by means of a PID controller which added NH₄OH to keep the pH at 5. The off gas passed through the gas analyzer (Servomex, M. Müller AG, Egg, Switzerland) to quantify CO₂ and O₂ content, using infrared and paramagnetic principles, respectively.

A plug flow reactor, which was just a simple silicon tube with a flow-through (Hamilton, Bonaduz, Switzerland) cell at the end, was coupled with the main reactor to build a two-compartment system. The two-compartment system contained a single fermenter (1 L medium) coupled with a plug flow reactor (PFR) of 90 mL. To obtain a RTD of 3.3 min, the circulation flow was adjusted to 27.3 mL/min. The flow-through cell facilitated measurement of pH, DO and temperature. The broth was pumped from the reactor out (ISMATEC, Wertheim, Germany) and then circulated back to the reactor. The PFR was put inside a water bath (LAUDA, Lauda-Königshofen, Germany) to avoid any temperature fluctuations as the cells flowed through the PFR. Detailed specifications and description of the setup as well as the scheme of the plant were presented elsewhere [10] and reproduced in the supplementary materials.

In order to investigate the effects of the amplitude and the frequency of the stress, a design of experiments (DoE) was performed using MODDE (Umetrics, Umeå, Sweden). A full factorial design was carried out. Two factors were defined: (1) the dissolved oxygen in the main bioreactor (2) the residence time in the PFR. The factors were defined as qualitative factors. A qualitative factor is a categorical factor which cannot be measured in numerical terms. Although both dissolved oxygen and residence time can be measured in numerical terms, they defined as qualitative factors because of the reasons described in the following.

A residence time chosen for a small two-compartment system (a system with a small main reactor (for example a 3 L reactor), might not be useful for a larger system because it may be so short that the circulation of the broth has no significant influence on cells. This effect was already observed by Lorantfy *et al.* [11]. Hence, the residence time was defined as a qualitative factor at two levels of high and low. 15 and 3.3 min were assigned to these levels respectively. These values were chosen according to mixing times of large scale bioreactors [17]. Lorantfy *et al.* [11] showed that shorter residence time than 3 min did not influence cell physiology and performance. The dissolved oxygen was also defined as a qualitative factor, because the exact control of the dissolved oxygen was difficult to achieve. For example, when the dissolved oxygen set point was set as 5%, measured values between 3 to 7% were observed. In addition, the calibration of the dissolved oxygen sensor might change in the course of fermentation because of change in pressure and medium composition. Two levels intermediate and high were defined for the dissolved oxygen and 25 and 70% were assigned to them, respectively. The dissolved oxygen in reactor was controlled using stirrer speed as the only manipulated variable. In other words, the dissolved oxygen was regulated independent from feed rate. Hence, the effects of dissolved oxygen level were studied irrespective of the feed rate.

Since the goal of this work was to investigate the effects of frequency and amplitude of the oxygen-related stress on product quantity and physiology, three kinds of responses were defined in the DoE. (I) The specific productivity, derived as the average value of the first 80 h of the induction phase, indicating the rate of the target protein production per biomass (U/g h), (II) the product titer at 80 h after induction, representing the total activity of the target protein in the broth (U) and (III) the biomass and carbon dioxide yields, derived as the average value of the first 80 h of the induction phase, representing global metabolic physiological performance.

A full factorial design of experiments with above mentioned factors (DoE1) resulted in four experiments, while one was executed in duplicate. The list of experiments and their associated dissolved

oxygen levels and residence times are given in Table 1(a). The values of the responses were given in Table A1 in the Appendix.

Table 1. (a) Full factorial design of experiments (DoE1) with the two qualitative factors (dissolved oxygen and residence time) in two levels (high and low levels for residence time, intermediate and high levels for dissolved oxygen). (b) Two additional experiments were performed for further analysis and comparison. (c) Full factorial design of experiments (DoE2) with two quantitative factors (residence time and dissolved oxygen) defined in three levels (0, 3.3 and 15 min for residence time and 5, 25 and 70% for dissolved oxygen).

(a)		
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%)
(b)		
6	Low (3.3 min)	Low (5%)
7	0 min	Low (5%)
(c)		
Exp. No.	Residence time	Dissolved oxygen
1	(3.3 min)	(25%)
5	(3.3 min)	(25%)
6	(3.3 min)	(5%)
7	0	(5%)
8	0	(25%)
9	0	(70%)
10	(3.3 min)	(70%)
11	(15 min)	(5%)
12	(15min)	(25%)
13	(15 min)	(70%)

Two additional experiments were performed for further analysis and comparison as follows (Table 1(b)): (1) An experiment was carried out in the two-compartment system (Exp. 6), in which the dissolved oxygen and the residence time were adjusted at low levels (5% and 3.3 min respectively) and (2). An experiment was performed in one-compartment system (Exp. 7), in which the dissolved oxygen was regulated at a low level (5%). The results of Exp. 6 were compared to those of Exp. 3 and Exp. 1 in order to investigate the effects of the dissolved oxygen level in a univariate manner. Subsequently, Exp. 6 was compared to Exp. 7 in order to study the impact of the circulation of the broth.

In order to confirm the effects observed in DoE1, an additional DoE were performed (DoE2). In this DoE, the factors (dissolved oxygen and residence time) were defined quantitatively. By doing so, it was possible to mathematically demonstrate the quadratic influence of the dissolved oxygen. The residence time and the dissolved oxygen were defined as quantitative factors with three levels (0, 3.3 and 15 min for RT and 5%, 25% and 70% for DO). The zero residence time means no circulation. By these definitions, six more experiments (DoE2) were performed as listed in Table 1(c). The same responses as DoE1 were used. The values of the response were given in Table A2 in the Appendix.

2.3. Analytics

A sample was taken every 12 h from the reactor. They were processed to measure the biomass concentration and the protein activity. The activity of HRP was determined using an enzymatic robot as described elsewhere [4].

Cell mass concentration was quantified gravimetrically. Two milliliters of the culture broth were added to pre-weighted glass tubes, followed by immediate centrifugation at 5,000 rpm for 10 min at 4 °C using a Sigma 3K30 centrifuge with the rotor 11156. The supernatant was removed from the top and used for different extracellular protein measurement. The pellets were re-suspended twice in 2 mL of distilled water using a vortex to wash them and they were centrifuged again like the same conditions mentioned before. The last supernatants were removed carefully and the tubes were dried for 72 h at 105 °C. Tubes were kept in a desiccator before being weighted. During the whole process, the samples were stored on ice to avoid and stop further reactions.

2.4. Data Treatment

All online and offline measurements were pretreated according to the strategy proposed by Herwig *et al.* [18] to assure that they fulfill the carbon balance and degree of reduction balances. Online and offline data were provided in the supplementary documents.

After calculation of responses they were used to build a model which correlated the responses to the factors. The values of responses were provided in the supplementary document. The software MODDE (Umetrics, Sweden) was used for this purpose. For each DoE, the model parameters were modified to get the best precision of prediction and goodness of fit. In addition, the center point of each DoE was performed in duplicate and the model reproducibility and validity were checked to be in the acceptable range proposed by the software. Thereafter, coefficient plots were examined to understand the effects of each factor and their influences on the responses.

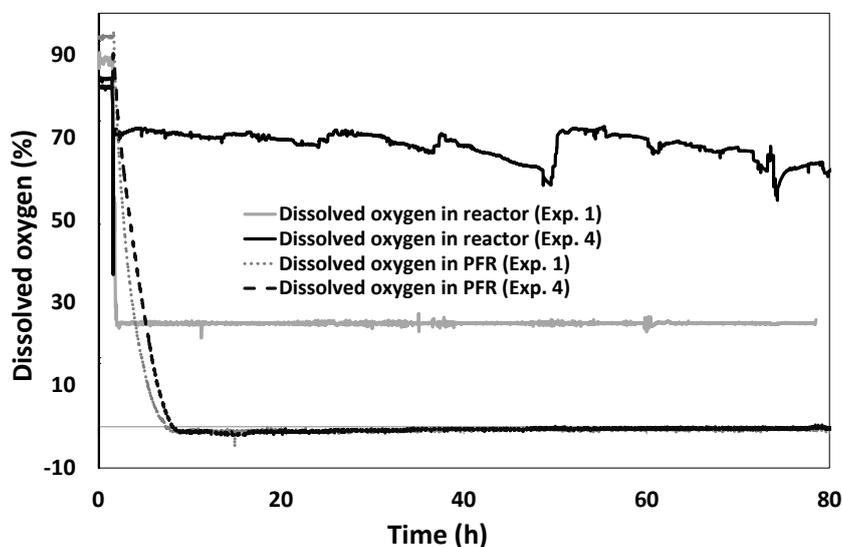
3. Results and Discussion

3.1. Time Course of Dissolved Oxygen Level

Initially, the time course of DO in the two-compartment system is demonstrated with Exp. 1 and Exp. 4 as examples. The dissolved oxygen in the main reactor was well controlled at the level of 25% all over the induction phase in Exp. 1. However, the dissolved oxygen was fluctuating between 60% and 70% in Exp. 4 but it was high enough to be considered as a qualitatively high level of dissolved oxygen. As it is demonstrated in Figure 1, the dissolved oxygen in the PFR could not be differentiated

from zero in both Exp. 1 and Exp. 4. Additional experiment showed that the cells experience oxygen-limitation immediately after they enter the PFR. It took only a few seconds for the cells to take up all oxygen (data not shown). Therefore, oxygen-limited conditions were achieved in the PFR.

Figure 1. Dissolved oxygen in main reactor and in plug flow reactor in experiments 1 and 4 (Exp. 1 and Exp. 4).



3.2. Results DOE1

All experiments showed similar trends in the responses on DOE1 experiments (Figure 2): The specific productivity increased in a linear manner in the beginning and then stayed in a constant level or even decreased (Figure 2(a)). The upward trend in the specific productivity was mainly due to the fact that the cells started to adapt themselves to the new feed rate of methanol and to activate protein synthesis machinery [4]. Such trend has been also observed by Dietzsch *et al.* [4] and Lee *et al.* [7]. The titer, which is the integration of the specific productivity over time and the total biomass, was increasing constantly (Figure 2(a)). However, the rate of increase was different in various experiments. For example, the specific productivity diminished in Exp. 4, its integration over time increased because biomass increased. Figure 2(a) clearly shows the significant difference in the titer between Exp. 1 and Exp. 4: 50,000 and 20,000 U were obtained at 80 h in Exp. 1 and 4, respectively. The specific productivity was 8.5 and 4 U/g h at 70 h in Exp. 1 and 4, respectively. The values of titer which were shown in Figure 2(b) as well as the values used for multivariate studies were all corrected according to the biomass content of the broth in the beginning of the induction phase. In other words, they were brought into the same level at the start point of the induction phase. Doing so, the impact of biomass content was eliminated.

The carbon dioxide yield (Figure 2(b)) was calculated using online signals. The increase in the carbon dioxide yield (about 0.7 C-mol/C-mol in the beginning and 0.8 C-mol/C-mol at the end) was in accordance with the decrease in the biomass yield (about 0.3 in the beginning and around 0.2 at the end) so that the sum of them was unity (Figure 2(b)), hence the carbon balance was conserved. The specific growth rate and doubling time were 0.009 L/h and 77 h during induction phase respectively. These are the typical values for other experiments as well. The reduction in the biomass yield in the

course of the induction phase was also reported by Jahic *et al.* [19] and reasoned by increased cell death under methanol-limited conditions [20,21]. However, as it can be seen in Figure 2(b), the difference in yields between experiment 1 and 4 was not significant.

Figure 2. (A) Specific productivity and titer in experiments 1 and 4 (Exp. 1 and Exp. 4). (B) Biomass and carbon dioxide yield in experiments 1 and 4 (Exp. 1 and Exp. 4).

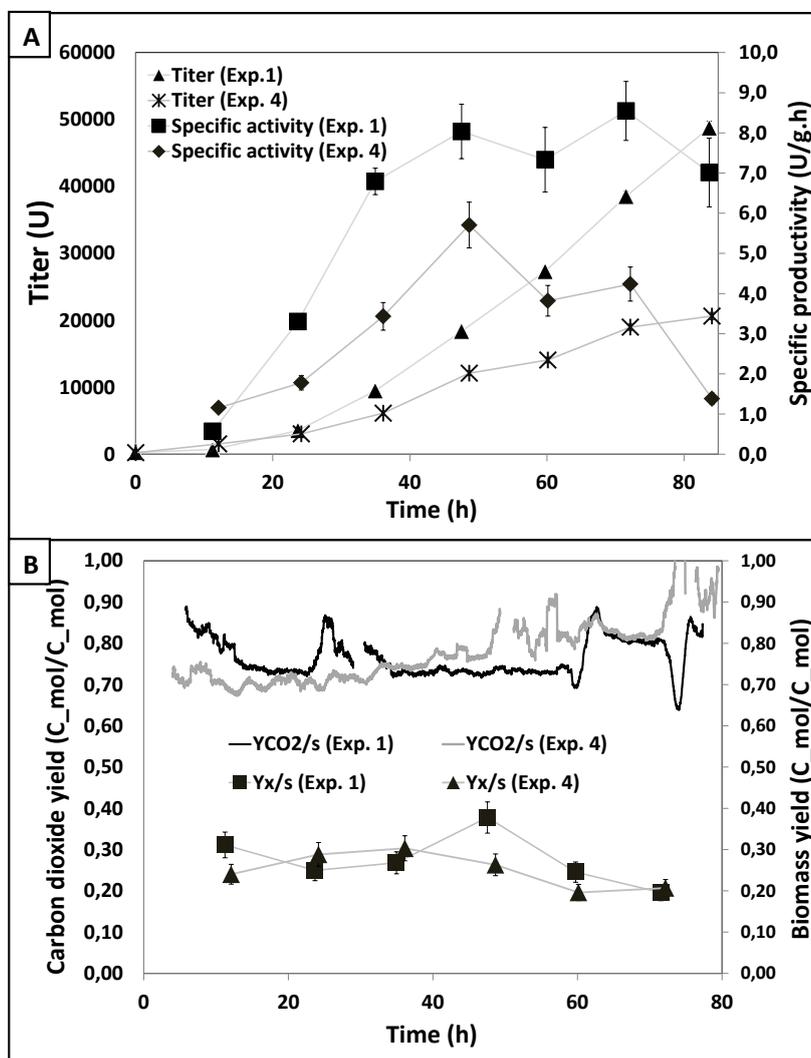


Figure 3 shows coefficient plots resulted from multivariate data analysis performed by MODDE. A coefficient plot represents the level of sensitivity of the factors to regress the responses, which were experimentally determined. The higher the absolute value of the coefficient is, the more significant effect of the factor on the response is concluded. A negative coefficient means that the factor is inversely proportional to the response. In each plot, factors were located in the x-axis and the level of influence to the responses on the y-axis. However, all sensitivities can only be judged for significance, in case the sensitivity exceeds the variability from repeated experiments, indicated by the error bar.

In order to build a model, all individual factors (as listed in Table 1(a)) as well as interaction terms like DO_intermediate*t_low and t_high*DO_intermediate, *etc.*, were taken into account. However, the model coefficients for interaction terms were all zero and they did not have significant contribution to the model (data not shown). Hence, they were eliminated. For each model, the goodness of fit,

the prediction precision and the model validity as well as reproducibility were checked to be higher than 0.99, 0.9, 0.25 and 0.5 respectively (data is not shown here). Hence, the models can be considered as reliable.

Figure 3. Coefficient plots for the models which correlate dissolved oxygen (DO) and residence time (t) as qualitative factors to the (a) specific productivity (b) titer (c) biomass yield (Y_{Xs}) (d) carbon dioxide yield (Y_{CO_2s}).

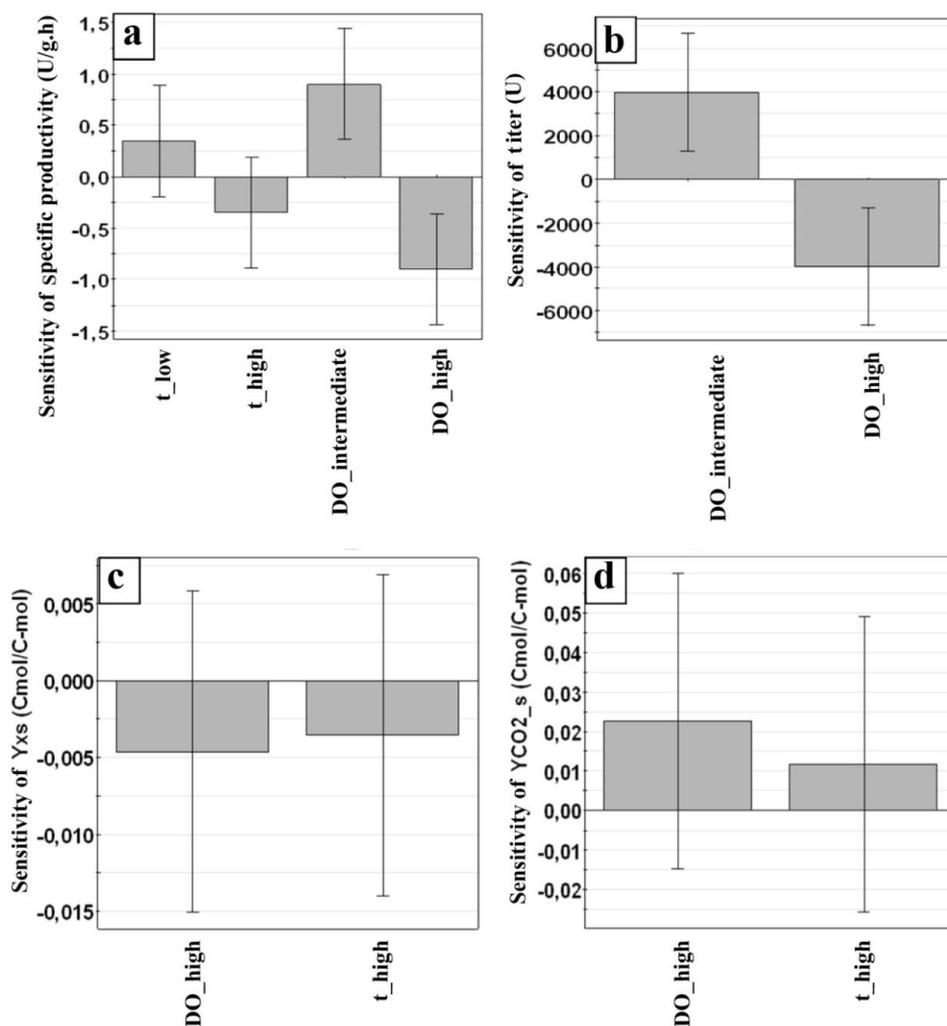


Figure 3(a) shows that the residence time was not significant on specific productivity and on protein quantity. The residence time was set in this work just by adjusting the circulation flow rate. For a longer residence time, a lower circulation flow rate was adjusted. Since the volume of the PFR was constant, the portion of the fermentation broth experiencing the stress did not change. On the other hand, as the PFR volume was recirculated to the main bioreactor, the stress recovery time for the stressed cells in the bioreactor differed with different residence times. Hence the same cell was therefore more frequently exposed to the stress. The results show that it did not matter how often the cells were exposed to the stress. As a biological explanation for this effect, cells react to stress by intracellular responses [22]. This intracellular response which is associated with synthesis of intracellular components reaches an equilibrium state [23]. Our results suggest that it did not matter how long the cells were in this stressed equilibrium condition.

The dissolved oxygen was found to be a significant factor (Figure 3(a,b)). The intermediate level of the dissolved oxygen enhanced specific productivity while the high level of the dissolved oxygen significantly caused reduction in the specific productivity (Figure 3(a,b)). The dependency of the titer on the dissolved oxygen was similar to that of the specific productivity: The more the dissolved oxygen was, the less the titer was obtained (Figure 3(b)).

It has been reported that in the production of recombinant protein in *P. pastoris*, the expression is not the rate limiting step but the secretion is the bottleneck [1]. On the other hand, Hsp70 and Hsp40 chaperone families in the cytoplasm or in endoplasmic reticulum importantly regulate the folding and the secretion of heterologous proteins [24]. As the cells entered the main reactor, they had the opportunity to recover from the stress. It seems that there is a connection between the amount of oxygen available for the cells in the reactor and the expression of Hsp70 and Hsp40 chaperone families. In other words, our results suggest that the high and low levels of dissolved oxygen might reduce the synthesis of chaperones while the intermediate level of dissolved oxygen enhances expression of the chaperones and consequently improves protein secretion. The connection between the expression of chaperon families and the secretion of protein is thought to be just a speculation. However, there may be other reasons, which must be investigated separately. This was out of the focus and aims of this work.

The carbon dioxide and the biomass yields were not significantly influenced by residence time and dissolved oxygen. Also, similar to what was observed for the specific productivity and the titer, no interaction effect of the factors on physiology was found (data not shown). In other words, the imposed stress did not render the global physiology and metabolic performance. This suggests that the stress did not negatively influence cell growth and overall energetic balance. It only influenced the protein expression machinery and secretion.

3.3. Along Which Function is Productivity Coupled to Dissolved Oxygen?

According to the results of above-mentioned data analysis, we found out that the residence time had no impact on the protein quantity. However, the intermediate level of dissolved oxygen resulted in higher specific productivity and higher titer than at high levels of dissolved oxygen. Therefore, this question arose: would protein quantity be higher if the dissolved oxygen is decreased? In order to answer this question, Exp. 6 was performed, in which the same residence time as Exp. 1 (70% dissolved oxygen in the main bioreactor) was set and the dissolved oxygen was lowered to 5%. Hence the two-compartment system resembles a one-compartment system, because the dissolved oxygen level in the reactor is similar to the dissolved oxygen level in the PFR. The results of Exp. 1 and Exp. 6 were compared with each other and no improvement in the specific productivity was observed. The productivity of Exp. 6 was even less than that of Exp. 1 (Figure 4, see Exp. 1 and Exp. 6).

Our results verify therefore, that the two-compartment system with 5% dissolved oxygen in the main reactor behaved similar to the one-compartment system with the same level of DO. In order to proof this hypothesis, it was aimed to execute a one-compartment experiment in which 5% of dissolved oxygen was maintained. Then the results of this experiment (Exp. 7) were compared to those of Exp. 6. The results showed that the productivity was not improved even by maintaining the dissolved oxygen at the level of 5% (Figure 4, Exp. 7 and Exp. 6 in comparison with Exp. 1). Low values of the productivity observed in Exp. 3 and Exp. 6 compared to Exp. 1 (Figure 4, Exp. 3 and

Exp. 6), can therefore be interpreted as a quadratic function of the dissolved oxygen on productivity. In other words, the intermediate level of the dissolved oxygen was suggested as the best level with respect to productivity.

Figure 4. Specific productivity *versus* time of four experiments (Exp. 1, Exp. 3, Exp. 6, Exp. 7).

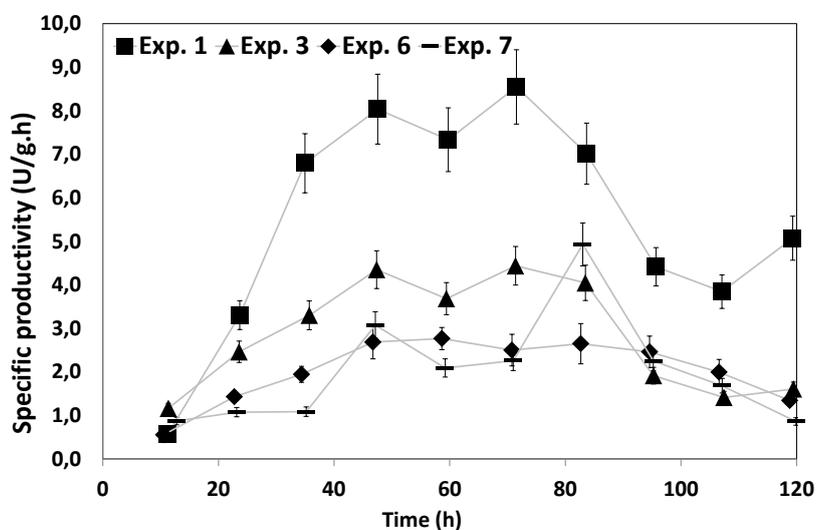
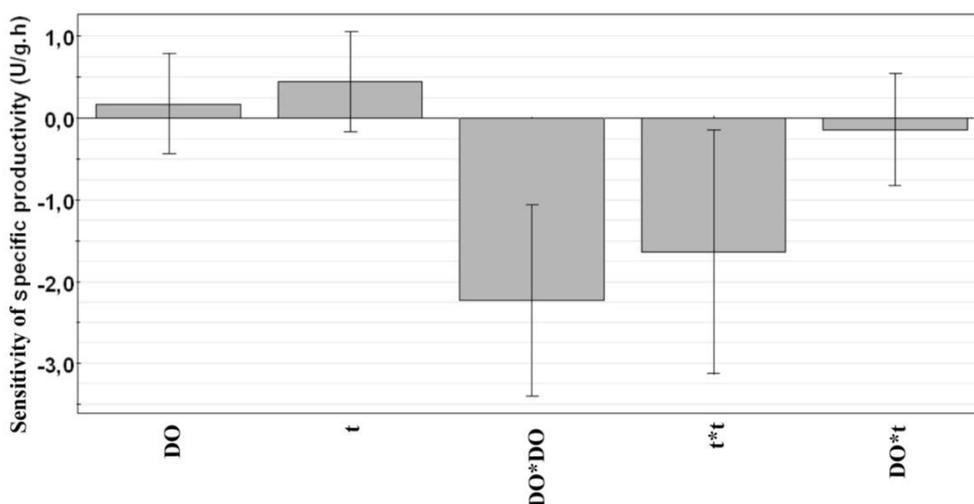


Figure 5. Coefficient plot for the model which correlates dissolved oxygen (DO) and residence time (t) as quantitative factors to the specific productivity.



DoE2 was performed to mathematically demonstrate the fact that the specific productivity was a quadratic function of the dissolved oxygen. The coefficient plot of DoE2 is given in Figure 5. This figure shows that the specific productivity was indeed a quadratic function of the dissolved oxygen. Therefore, in the two-compartment system under study the intermediate level of dissolved oxygen (25%) was recommended to reach the maximum productivity and titer. This quadratic effect may also be interlinked to the expression of Hsp70 and Hsp40 chaperone families at low and high levels of dissolved oxygen. It is assumed that low and high levels of dissolved oxygen repressed the expression of these chaperons. Consequently, the secretion of the synthesized protein was hindered. In Exp. 8, the productivity and titer were nearly the same as those of Exp. 1 (4.95 and 4.6 U/g h, 16,198.2 and

15,980.2 U in Exp. 1 and Exp. 8 respectively). This reflects the observation that the intermediate level resulted in the maximum productivity.

4. Conclusions

Two-compartment processing has been already introduced as a tool to boost the product quantity for the production of HRP using *P. pastoris* as the host [10]. This system benefits from the oxygen-related stress. In this work, the influence of the amplitude (dissolved oxygen level in the main reactor) and the frequency (the residence time of the broth in the PFR) of the stress on specific productivity, titer, biomass and carbon dioxide yield were investigated.

It was concluded that the residence time, in the range that it was studied, did not impact the product titer and specific productivity. We assumed that it was important which fraction of the broth is exposed to the stress and not the length of exposure. In other words, the volume of PFR was determinant.

On the other hand, the dissolved oxygen level in the main reactor was found to be a significant factor on the productivity and titer in a quadratic form. It was concluded that an intermediate level of dissolved oxygen (25%) showed the highest specific productivity and titer, irrespective of the residence time in the PFR. The low and high levels of dissolved oxygen did not enhance productivity. This effect was thought to be somehow interlinked to the fact that the amplitude of the stress might influence the synthesis of Hsp70 and Hsp40 chaperone families differently in various levels of dissolved oxygen, identifying a clear target for future biochemical analysis. This may, in turn, influence the secretion of the target protein.

Finally, a two-compartment processing mode with an intermediate level of dissolved oxygen was suggested as a novel process strategy to boost recombinant protein production. Since the volume of the PFR was concluded to be determinate, it is recommended to choose the ratio between the reactor volume and the PFR volume as a criterion for scale up of the two-compartment system.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Potvin, G.; Ahmad, A.; Zhang, Z. Bioprocess engineering aspects of heterologous protein production in pichia pastoris: A review. *Biochem. Eng. J.* **2012**, *64*, 91–105.
2. Li, P.; Anumanthan, A.; Gao, X.G.; Ilangovan, K.; Suzara, V.V.; Düzgüneş, N.; Renugopalakrishnan, V. Expression of recombinant proteins in pichia pastoris. *Appl. Biochem. Biotech.* **2007**, *142*, 105–124.
3. Cregg, J.; Cereghino, J.; Shi, J.; Higgins, D. Recombinant protein expression in pichia pastoris. *Mol. Biotechnol.* **2000**, *16*, 23–52.
4. Dietzsch, C.; Spadiut, O.; Herwig, C. A fast approach to determine a fed batch feeding profile for recombinant pichia pastoris strains. *Microb. Cell Fact.* **2011**, *10*, doi:10.1186/1475-2859-10-85.
5. Trentmann, O.; Khatri, N.K.; Hoffmann, F. Reduced oxygen supply increases process stability and product yield with recombinant pichia pastoris. *Biotechnol. Progr.* **2004**, *20*, 1766–1775.

6. Hellwig, S.; Emde, F.; Raven, N.P.G.; Henke, M.; van der Logt, P.; Fischer, R. Analysis of single-chain antibody production in *pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. *Biotechnol. Bioeng.* **2001**, *74*, 344–352.
7. Lee, C.Y.; Lee, S.J.; Jung, K.H.; Katoh, S.; Lee, E.K. High dissolved oxygen tension enhances heterologous protein expression by recombinant *pichia pastoris*. *Process Biochem.* **2003**, *38*, 1147–1154.
8. Baumann, K.; Maurer, M.; Dragosits, M.; Cos, O.; Ferrer, P.; Mattanovich, D. Hypoxic fed-batch cultivation of *pichia pastoris* increases specific and volumetric productivity of recombinant proteins. *Biotechnol. Bioeng.* **2008**, *100*, 177–183.
9. Baumann, K.; Carnicer, M.; Dragosits, M.; Graf, A.; Stadlmann, J.; Jouhten, P.; Maaheimo, H.; Gasser, B.; Albiol, J.; Mattanovich, D.; *et al.* A multi-level study of recombinant *pichia pastoris* in different oxygen conditions. *BMC Syst. Biol.* **2011**, *4*, doi:10.1186/1752-0509-4-141.
10. Jazini, M.H.; Herwig, C. Two-compartment processing as a tool to boost recombinant protein production. *Eng. Life Sci.* **2013**, doi:10.1002/elsc.201300038.
11. Lorantfy, B.; Jazini, M.; Herwig, C. Investigation of the physiological response to oxygen limited process conditions of *Pichia pastoris* Mut(+) strain using a two-compartment scale-down system. *J. Biosci. Bioeng.* **2013**, *116*, 371–379.
12. Amanullah, A.; McFarlane, C.M.; Emery, A.N.; Nienow, A.W. Scale-down model to simulate spatial pH variations in large-scale bioreactors. *Biotechnol. Bioeng.* **2001**, *73*, 390–399.
13. Bylund, F.; Castan, A.; Mikkola, R.; Veide, A.; Larsson, G. Influence of scale-up on the quality of recombinant human growth hormone. *Biotechnol. Bioeng.* **2000**, *69*, 119–128.
14. Lara, A.R.; Leal, L.; Flores, N.; Gosset, G.; Bolívar, F.; Ramírez, O.T. Transcriptional and metabolic response of recombinant *Escherichia coli* to spatial dissolved oxygen tension gradients simulated in a scale-down system. *Biotechnol. Bioeng.* **2006**, *93*, 372–385.
15. Lara, A.R.; Vazquez-Limón, C.; Gosset, G.; Bolívar, F.; López-Munguía, A.; Ramírez, O.T. Engineering *Escherichia coli* to improve culture performance and reduce formation of by-products during recombinant protein production under transient intermittent anaerobic conditions. *Biotechnol. Bioeng.* **2006**, *94*, 1164–1175.
16. Sandoval-Basurto, E.A.; Gosset, G.; Bolívar, F.; Ramírez, O.T. Culture of *Escherichia coli* under dissolved oxygen gradients simulated in a two-compartment scale-down system: Metabolic response and production of recombinant protein. *Biotechnol. Bioeng.* **2005**, *89*, 453–463.
17. Lara, A.; Galindo, E.; Ramirez, O.; Palomares, L. Living with heterogeneities in bioreactors. *Mol. Biotechnol.* **2006**, *34*, 355–381.
18. Herwig, C.; Marison, I.; von Stockar, U. On-line stoichiometry and identification of metabolic state under dynamic process conditions. *Biotechnol. Bioeng.* **2001**, *75*, 345–354.
19. Jahic, M.; Wallberg, F.; Bollok, M.; Garcia, P.; Enfors, S.-O. Temperature limited fed-batch technique for control of proteolysis in *pichia pastoris* bioreactor cultures. *Microb. Cell Fact.* **2003**, *2*, doi:10.1186/1475-2859-2-6.
20. Hohenblum, H.; Borth, N.; Mattanovich, D. Assessing viability and cell-associated product of recombinant protein producing *pichia pastoris* with flow cytometry. *J. Biotechnol.* **2003**, *102*, 281–290.

21. Khatri, N.K.; Hoffmann, F. Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant pichia pastoris. *Biotechnol. Bioeng.* **2006**, *93*, 871–879.
22. Mattanovich, D.; Gasser, B.; Hohenblum, H.; Sauer, M. Stress in recombinant protein producing yeasts. *J. Biotechnol.* **2004**, *113*, 121–135.
23. Arsene, F.; Tomoyasu, T.; Bukau, B. The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.* **2000**, *55*, 3–9.
24. Zhang, W.; Zhao, H.-L.; Xue, C.; Xiong, X.-H.; Yao, X.-Q.; Li, X.-Y.; Chen, H.-P.; Liu, Z.-M. Enhanced secretion of heterologous proteins in *Pichia pastoris* following overexpression of *saccharomyces cerevisiae* chaperone proteins. *Biotechnol. Progr.* **2006**, *22*, 1090–1095.

Appendix

Table A1. All responses to be modeled (value and standard deviation) for DoE 1.

Exp. No.	Residence time (min)	Dissolved oxygen (%)	Specific productivity (U/g h)	Titer (U)	Y _{CO₂/s} (C-mol/C-mol)	Y _{x/s} (C-mol/C-mol)
1	Low (3.3)	Intermediate (25)	4.95 ± 0.24	16,198.2 ± 95	0.58 ± 0.021	0.30 ± 0.012
2	High (15)	Intermediate (25)	3.98 ± 0.19	20,665.1 ± 110	0.63 ± 0.022	0.29 ± 0.011
3	Low (3.3)	High (70)	2.87 ± 0.14	10,915.4 ± 50	0.65 ± 0.022	0.28 ± 0.011
4	High (15)	High (70)	2.19 ± 0.10	10,048.3 ± 48	0.64 ± 0.03	0.28 ± 0.011
5	Low (3.3)	Intermediate (25)	4.41 ± 0.22	18,482.8 ± 85	0.59 ± 0.021	0.29 ± 0.011

Table A2. The response to be modeled (value and standard deviation) for DoE 2.

Exp. No	Residence time (min)	Dissolved oxygen (%)	Specific productivity (U/g h)
1	3.3	25	4.95 ± 0.24
5	3.3	25	4.41 ± 0.22
6	3.3	5	2.25 ± 0.22
7	0	5	2.80 ± 0.28
8	0	25	4.60 ± 0.30
9	0	70	2.60 ± 0.31
10	3.3	70	2.20 ± 0.21
11	15	5	2.18 ± 0.20
12	15	25	2.33 ± 0.22
13	15	70	2.08 ± 0.19

Description of the Two-Compartment System

Figure A1 shows a schematic view of the setup. The PFR is just a simple silicon tube with a flow-through cell at the end. The flow-through cell facilitated measurement of pH, DO and temperature. The diameter of the PFR and the circulation flow rate was designed in such a way that turbulent flow was guaranteed. The broth was pumped from the reactor (Pump 1: ISMATEC, Wertheim, Germany). The setup was designed so that the bubbles were separated in a bubble trap and directed to the reactor. The Bubble-free stream was pumped (Pump 2: ISMATEC, Wertheim, Germany) through the flow-through cell (Hamilton, Bonaduz, Switzerland) and then entered back to the reactor. The PFR was put inside a water bath (LAUDA, Lauda-Königshofen, Germany) to avoid any temperature fluctuations when the cells are outside the reactor.

Depending on the volume of the PFR and speed of the circulation, the cells experience oxygen limitation for a certain period of time in the PFR. This period is known as the residence time.

Figure A1. Schematic view of the two-compartment system.

