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# PID controlled Constant RQ Fermentation of *Pichia pastoris* in the DASbox® Mini Bioreactor System

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## Abstract

To demonstrate the feasibility of constant respiratory quotient (RQ) based feeding via a script integrated to the DASware® control 5 bioprocess control software, we grew the respiro-fermentative yeast *Pichia pastoris* in the DASbox® Mini Bioreactor System. Further, we compared the results to a standard DO-spike triggered feeding done in the same set up.

The objectives of this study are (1) to demonstrate the feasibility of a constant RQ based feeding using the DASware control 5 professional software and the DASbox Mini Bioreactor System; (2) to show the benefits of constant RQ control in *P. pastoris* fermentation as well as its effect on the amount of feed used and the microbial growth; (3) to explain the measurements necessary for RQ determination as well as the corresponding calculations automatically done by the Eppendorf GA4 gas analyzer module; and (4) to give background information of the RQ, showcasing its potential for usage.

**Abbreviations:**

| Parameter | Unit | Description |
|---|---|---|
| C | mmol/sL | Standard molar volume |
| Fin | sL/h | Gas flow to bioreactor |
| Fout | sL/h | Exhaust gas flow rate |
| vvm | L/L*min | Relative gas supply vvm = Fin/V |
| $XO_2^{in}$ | % | Oxygen concentration in supply gas to bioreactor |
| $XO_2^{out}$ | % | Oxygen concentration in exhaust gas |
| $XCO_2^{in}$ | % | Carbon dioxide concentration in supply gas to bioreactor |
| $XCO_2^{out}$ | % | Carbon dioxide concentration in exhaust gas |
| OTR | mmol/L*h | Oxygen transfer rate |
| CTR | mmol/L*h | Carbon dioxide transfer rate |


**Fig. 1:** DASGIP GA4 Gas Analyzer

## Introduction

*Pichia pastoris* is a methylotrophic yeast largely used for heterologous protein production in biotechnology. As an expression system, the main advantages of *P. pastoris* over *E. coli* is the ability to carry out post-translational modifications such as glycosylation. Compared to mammalian cell culture, *P. pastoris* displays a much faster growth and requires less expensive growth medium, making it ideal for protein production in bioreactors. For optimizing the production yield of a *P. pastoris* culture, one of the most important factors is the feeding strategy.

A convenient and well-established approach for feeding is a strategy based on the amount of dissolved oxygen (DO) in the medium. In this approach the feeding start is triggered by a DO-spike. After that, the DO set point is generally set to a fixed value of e.g. 30 %. However, it is not guaranteed that these conditions lead to full metabolization of the carbon source, and a further optimization of this feeding strategy would be necessary (Hang et al., 2009).

One of the leading strategies for protein production-oriented feeding is based on respiratory quotient (RQ). Constant RQ based feeding ensures that the respiratory metabolism of glucose/glycerol are optimized for protein production purposes and the formation of by-products are limited (Xiong et al., 2010).

In a recent publication, a strategy was introduced which managed to fully convert glucose and additionally, avoid ethanol formation by controlling RQ ≥ 0.9 (Tippmann et al., 2016). The publication has shown additionally a clear improvement in the productivity of the cells due to this feeding strategy.

The intention of this study is to demonstrate the feasibility of constant RQ based feeding for protein production-oriented yeast fermentation using the DASbox Mini Bioreactor System.

### The Respiratory quotient (RQ)

The respiratory quotient is the quotient of Carbon dioxide produced and Oxygen consumed by a culture, expressed with the Carbon dioxide transfer rate (CTR) and the Oxygen uptake rate (OUR). Under conditions of a constant dissolved oxygen concentration the OUR equals the Oxygen transfer rate (OTR) (Clarke, 2013). Therefore, the following calculations were done to determine the RQ:

1) Oxygen transfer rate (OTR):

$$OTR = c \times vvm\left(X_{O_2,in} - \frac{F_{out}}{F_{in}} \times X_{O_2,out}\right)$$

2) Carbon dioxide transfer rate (CTR):

$$CTR = c \times vvm\left(\frac{F_{out}}{F_{in}} \times X_{CO_2,out} - \times X_{CO_2,in}\right)$$

3) Respiratory quotient (RQ):

$$RQ = \frac{CTR}{OTR}$$

The RQ for the metabolization of glucose is 1. This can be explained by the fact that per mol glucose, 6 mol $O_2$ are needed and 6 mol $CO_2$ are produced. Which means that the $O_2$ uptake equals the $CO_2$ transmission and thus their quotient is 1.

Once glucose is fully metabolized the culture starts to consume fermentation by-products. These by-products mainly consist of ethanol which is produced under aerobic conditions in respiro-fermentative yeasts once the tricarboxylic acid (TCA) cycle is overflown by glucose.

As ethanol is more reduced than glucose, the use of ethanol as substrate results in a RQ value below 1. Thus, the RQ value can serve as an inline parameter indicating which substrate is consumed by the culture, and a constant RQ process can be used to optimize fermentation based on specific carbon source.

## Materials and Methods

### Yeast strain and glycerol stocks

*P. pastoris* has been taxonomically reclassified as *Komagataella pastoris* (Peña et al., 2018). To simplify the matter, we will continue to refer to it as *Pichia pastoris* further on.

In this study we used the *Pichia pastoris* strain DSMZ 70382 obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH.

We reactivated the freeze-dried cells in 10 mL YPD medium and incubated them in a 500 mL Erlenmeyer flask with baffles over night at 30 °C, 200 rpm using an New Brunswick™ Innova® 42 incubator. The next morning, we transferred the culture into a 1000 mL Erlenmeyer flask with baffles and added 100 mL sterile YPD-medium. After an incubation period of 6 h at the previous mentioned settings, we checked the culture to be contamination-free using a Zeiss® Primo star microscope.

Next, we measured the $OD_{600}$ of the culture and mixed 800 µL broth with 200 µL 70 % (w/v) glycerol in previously sterilized cryovials to create a *Pichia* working cell bank with $OD_{600}$ of 0.3.

We stored the cryovials at -20 °C and individually thawed at room temperature before the run.

### Fermentation system

For this study, we used the DASbox Mini Bioreactor System for microbial fermentation applications by Eppendorf. Additionally, we connected a GA4 exhaust gas analyzer unit including humidity sensors.

The GA4 measures the two off-gas components oxygen ($O_2$) and carbon dioxide ($CO_2$).

The used GA4 version measures the $O_2$ concentration between 1 % and 50 % using a zirconium dioxide sensor and $CO_2$ is measured by a two-beam infrared sensor. Together with the installed mass flow sensor and humidity sensor, they allow for the online calculation and tracking of the metabolic data of the fermentation (OTR, CTR and RQ). For more detailed information please refer to the GA4 manual, and the Eppendorf Short Protocol 48. Several compensation methods are available for humidity, volume, and pressure compensation.

To prevent liquid or foam from entering the GA4 module and harming the sensitive sensors we installed additional 250 mL Schott bottles as foam traps between the gas outlet of each DASbox mini bioreactor and the GA4.

### Medium Preparation

We prepared three types of media for different applications in this study (Mattanovich et al., 2012):

> One complex medium for inoculum preparation in shake flasks (Table 1)
> One defined minimal medium for the batch stage in the DASbox Mini Bioreactor System (Table 2 and 3)
> One defined feeding medium for the fed-batch stage (Table 4)

### Medium for inoculum preparation

YNB-medium is prepared by fully dissolving the components per liter DI water (Table 1) and sterile filtration using a Ø 0.22 µm sterile filter.

**Table 1: YNB-medium used for inoculum preparation.**

| YNB-medium (per liter) | |
|---|---|
| 1M potassium phosphate buffer | 100.0 mL |
| YNB (without amino acids) | 3.4 g |
| $(NH_4)_2SO_4$ | 10.0 g |
| Biotin | 0.4 g |
| Glucose monohydrate | 20.0 g |

### Media for batch fermentation

Basal salts medium (BSM) containing per liter (Table 2) and PTM1 trace elements solution containing per liter (Table 3):

**Table 2: BSM-medium for batch phase of the fermentation.**

| YNB-medium (per liter) | |
|---|---|
| Basal salts medium (per liter) | |
| 85 % phosphoric acid (v/v) | 26.7 mL |
| $CaSO_4 * 2\ H_2O$ | 1.17 g |
| $K_2SO_4$ | 18.2 g |
| $MgSO_4 * 7\ H_2O$ | 14.9 g |
| KOH | 4.13 g |
| Glucose monohydrate | 44 g |
| Antifoam 204 | 0.3 mL (added separately per vessel) |
| PTM1 | 4.5 mL (added sterily after autoclavation) |

**Table 3: PTM1 trace element solution to complement the BSM medium and the feed.**

| PTM1 trace elements solution (per liter) | |
|---|---|
| $CuSO_4 * 5\ H_2O$ | 6.0 g |
| NaI | 0.08 g |
| $MnSO_4 * H_2O$ | 3.0 g |
| $Na_2MoO_4 * 2\ H_2O$ | 0.2 g |
| $H_3BO_3$ | 0.02 g |
| $CoCl_2$ | 0.5 g |
| $ZnCl_2$ | 20.0 g |
| $FeSO_4 * 7\ H_2O$ | 65.0 g |
| Biotin | 0.2 g |
| $H_2SO_4$ | 5.0 mL |

PTM1 trace elements solution was filter sterilized using a 0.22 µm filter.

We transferred 95 mL BSM medium to the DASbox mini bioreactors and individually added 0.03 mL Antifoam 204 (Sigma-Aldrich®, A6426) to each vessel, which corresponds to an amount of 0.03 % (v/v).

Next, we autoclaved the vessels using a Systec VE-95 autoclave. After cooling to room temperature, the pH of the growth medium was adjusted to 5.0 with 10 % $NH_3$ which additionally serves as nitrogen source during fermentation. We then added 0.45 mL PTM1 solution per vessel which equals an amount of 4.5 mL per liter of basal salts medium. After we did all additions to the medium, the total volume reached ca. 110 mL.

### Feed medium

As feed medium a 33 % (w/v) glucose solution was used (Table 4).

**Table 4: Glucose feed medium.**

| Feed medium (per liter) | |
|---|---|
| Glucose monohydrate | 330 g |
| PTM1 | 12 mL (added after autoclaving) |

### Vessel setup and sterilization

In this study, we did all liquid additions to the bioreactor submersed and separate from each other via an additionally installed triple port.

We installed pH and DO sensors in the Pg13.5 threads of the head plate and used the sample port of the bioreactor for inoculation.

After we did these installations, we filled the bioreactor with medium and autoclaved it for 20 min, using 121 °C liquid cycle setting.

### Inoculum preparation

Before inoculum preparation, we took one cryovial of the previous prepared glycerol stocks to thaw at room temperature.

We allocated 10 mL sterile YNB-medium to two sterile 125 mL Corning® Erlenmeyer flasks with baffles. We inoculated one of the Erlenmeyer flasks using the 1 mL glycerol stock to an $OD_{600}$ of 0.03. Whereas we used the other Erlenmeyer flask, containing only sterile YNB-medium, as sterility control.

We incubated both flasks in an Innova 42 shaker for 17 h at 30 °C, 200 rpm. After that, we started a second pre-culture by transferring 10 mL of the grown culture to 40 mL fresh YNB-medium in a sterile 125 mL Corning Erlenmeyer flask. We grew the second pre-culture for another 7 h at 30 °C and 200 rpm to archive a threshold growth of 15-20 $OD_{600}$.

We inoculated each of the fermenters to reach an initial $OD_{600}$ of 1.

### Sensor calibration

We calibrated the pH sensors in parallel outside of the vessel, before sterilization, following the 2-point calibration procedure of DASware control 5 professional software. We used a pH buffer 7.0 to set the sensors' offset and a pH buffer 4.01 to set the slope.

Before we calibrated the DO sensors and before autoclaving the bioreactors, we filled the sensor cap with fresh electrolyte solution.

We calibrated the DO sensors in parallel inside the vessel after sterilization and addition of the medium supplements. A period of 6 hours is needed to polarize the sensors by connecting them to the control system. The control system provides a polarization voltage to establish an anode and a cathode within the sensor.

It is recommended to set the reactor conditions during DO calibration to the initial set points of the fermentation. In our case, that equals a stirring rate of 400 rpm, a gas flow of 3 sL/h and a temperature of 30 °C. Additionally, we added all het sensitive media after autocaving and before the pH was set to 5.0.

We did the 2-point calibration by sparging in pure nitrogen first to set the offset (0 % DO) followed by sparging in air to calibrate the slope (100 % DO).

### Pump calibration

Before connecting the vessels to the fermentation system, we performed the pump calibration following the procedure of the DASware control 5 professional software. We calibrated the feed pumps in parallel by using the feed medium and setting the density to 1.12 g/mL. We calibrated the base addition-pumps in parallel using water as calibration solution.

### Feed start

*Feed process triggered by DO-spike*

The end of the batch phase of a fed-batch culture is often indicated by a DO-spike. At this point the carbon source within the initial culture medium is consumed, the metabolic activity of the culture and therefore, its oxygen demand drops rapidly, resulting in a sharp increase of DO in the medium.

We took advantage of this DO-spike to trigger an automatic start of the feed pump using an automatic programming script.

Therefore, we implemented a so-called reactor script, based on visual basics, which starts with the inoculation of the fermenter. After a delay time of 12 hours, a low threshold of 30 % DO is anticipated followed by a high DO threshold of 38 % which starts the feed pump, following a step wise exponential-like feed (Table 5). We established this feed profile based on trial and error during the previous runs with *P. pastoris*.

**Table 5: Stepwise feed profile used for the reactors with DO-spike triggered feed start.**

| Step wise feeding profile | |
|---|---|
| Hours since feed start [hh:mm:ss] | Feed rate [mL/h] |
| 00:00:00 | 0.00 |
| 00:00:01 | 0.4 |
| 08:00:00 | 0.4 |
| 08:00:01 | 1.6 |
| 16:00:00 | 1.6 |
| 16:00:01 | 3.2 |
| 32:00:00 | 3.2 |
| 32:00:01 | 0 |

After a total feeding time of 32 h, the pump is automatically turned off. Additionally, we activated the overflow protection

of the reactor which is a safety feature of the DASware control 5 professional software. The overflow protection works as follows: During the course of the experiment, the volume which is added by the pumps is accumulated to the reactor's initial volume. Once the maximal working volume of the reactor is reached, the pumps stop automatically to prevent the reactor from overflowing.

*Automatic RQ control by programmed feeding*

We implemented a reactor script which starts automatically after inoculation (see appendix). After a delay time of 12 hours, the RQ value of 1 is used to start the feeding pump, and further feeding is automatically controlled based on the RQ value.

As stated before, the RQ value can serve as indicator for which substrate is used by the culture. An RQ of 1 indicates the consumption of glucose. A lower RQ indicates the consumption of reduced fermentation by-products.

For creation of a constant RQ based feeding, we programmed a script with built-in PID function to start the feed pump once the RQ value drops below 1, indicating that the glucose has been consumed. We used a script to set the feeding pump to its maximum speed (40 mL/h), when RQ drops to 0.8. Once the RQ value rose to 1 again, the script pauses the feeding automatically.

To reduce the oscillation of the RQ value, we implemented a PID controller within the script with the following PI values ($p = 3.33$, $Ti = 360$ s, $I = 0.0027$ $s^{-1}$).

## Process parameter setup

We kept the *P. pastoris* cultures at 30 °C, pH 5 and 30 % DO.

**Table 6: Process parameters used during fermentation**

| Parameter | Configuration |
|---|---|
| Vessel | DASbox Mini Bioreactor, autoclavable, microbiology |
| Inoculation density | $OD_{600} = 1$ |
| Dissolved oxygen (DO) | 30 %, maintained by DO cascade |
| Agitation | Overhead drive, maximum 1600 rpm, controlled by DO cascade |
| Gassing | Automatic gas flow and mix, controlled by DO cascade |
| Temperature | 30 °C, heating and cooling done by Peltier elements in the bioreactor's positions |
| pH | 5.0, one sided control with 10 % (v/v) sterile ammonium hydroxide solution |
| Sparger | L-Sparger |
| Feeding | Automatically triggered via reactor script |

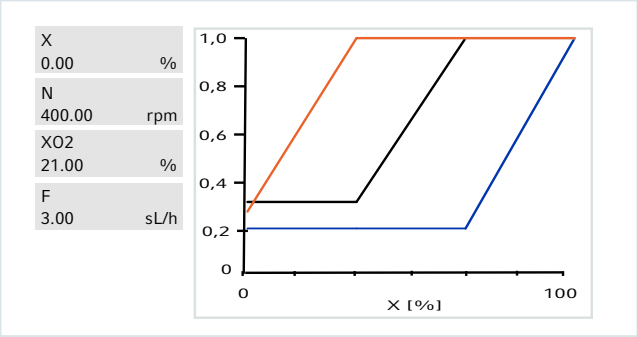


**Figure 2: Scheme of the DO-cascade built in DASware Control 5 professional.**
**First, the stirrer speed is increased (red curve) followed by the gas flow (black curve) and finally oxygen is enriched (blue curve).**

Both setups used the process parameters listed in Table 6.

We designed the DO cascade to maintain the dissolved oxygen at 30 % by first accelerating the agitation from 400 to 1600 rpm, then increasing the air flow rate from 3 sL/h to 9 sL/h (0.5 – 1.5 vvm for 100 mL initial volume), and finally enriching the oxygen concentration in the gas flow from 21 % to 100 %. These three steps corresponded to the DO output of 0 – 40 %, 40 – 80 % and 80 – 100 %.

We lead the exhaust gases into 250 mL Schott bottles which served as foam traps, and then further to the respective channels of the GA4 gas analyzer. The GA4 gas analyzer has a built-in function to calculate RQ using the equations shown in the introduction section of this work.

## Optical density measurements

We measured optical density using an Eppendorf BioPhotometer D30 at a wavelength of 600 nm. Samples were taken regularly during the fermentation.
To achieve an appropriate measurement in the linear range between 0.2 and 0.8 the samples were diluted appropriately.

**Table 7: DO-cascade build in DASware Control 5 professional. First, the stirrer speed is increased followed by the gas flow and finally the oxygen is enriched.**

| | Controller output [%] | | | |
|---|---|---|---|---|
| Parameter | 0 | 40 | 80 | 100 |
| Agitation [rpm] | 40 | 1600 | 0 | 0 |
| Gas flow [sL/h] | 0 | 3 | 9 | 0 |
| Oxygen concentration [%] | | | 21 | 100 |

## Results

We ran *P. pastoris* fed-batch fermentation two times at 30 °C, pH 5.0 and a DO level of 30 % using the DASbox Mini Bioreactor fermentation system for microbial applications.

In one set up, we started the feed automatically by implementing a DO-spike triggered feed started by a script. In the other set up, we successfully implemented a constant RQ-controlled feeding strategy. Throughout the fermentation, we took intermitted samples for optical density measurements to evaluate the growth of the cultures.

### DO-Spike based feeding

As described earlier the DO-spike based feeding is started once the value of dissolved oxygen raises from 30 % to above 38 % which is the case after 39 h inoculation time.
Before the DO spike, we observed a sharp drop of RQ value from 1.3 to 0.6 at 25 h inoculation time, followed by a stabilized RQ value at around 0.8 (Figure 3).

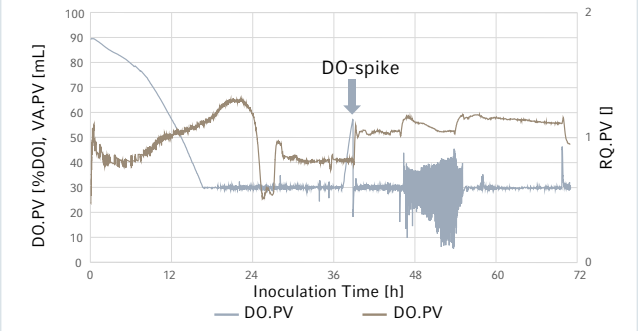


**Fig. 3:** For the DO-spike triggered feeding the course of DO% and RQ is shown over the inoculation time [h].
After 39 h a sharp DO-spike is visible.

We set the flow rate of the feed pump in three steps, where the first step is pulsating and the latter two are constant.

The different stages of the feed are also reflected in the course of the respiratory quotient (see Figure 4). One can see that for a pump flow rate of 0.4 mL/h the RQ is 1, for the next flow rate step (1.6 mL/h) an overshoot of RQ can be observed resulting in a RQ mean value of 1.1. In the final feed step of 3.2 mL/h the RQ raises above 1.1. This is caused by an overflow of the tricarboxylic acid cycle (TCA cycle) where a major part of the carbon source is oxidized to $CO_2$ and water along with by products (Xiong et al., 2010).

After 32 h of feeding (71 h total run time) we turned the pump off. Following the shutdown of the feed the RQ dropped below 1.

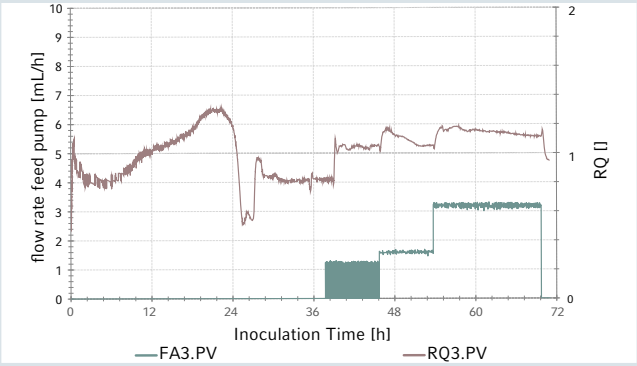


**Fig. 4:** Fermentation graphs of the flow of the feed pump and the respiratory quotient shown during *Pichia pastoris* fermentation with DO-spike triggered feed start.

### RQ controlled feeding

After a delay time of 12 h, the RQ is controlled towards a value of 1 as described above by turning the feed pump on and off.

One can see that after an inoculation time of 36 h, the RQ starts dropping sharply (Figure 5). When it hits 1, the pump starts feeding glucose thus a drop of RQ as low as for the DO-based feeding does not occur. In fact, the RQ settles to 1 with an under shooting to 0.9. Dependent on the RQ controller's output, the pump increases its flow which can be observed at around t=48 h.

During the duration of the feed, the RQ oscillates between 0.9 and 1.1. This is caused by a delay in measurement of the exhaust gas components. As the bioreactor's exhaust gas needs to pass the additional tubing between the reactor and the GA4 before being measured. This can be further minimized by optimization of the controller's PID settings.

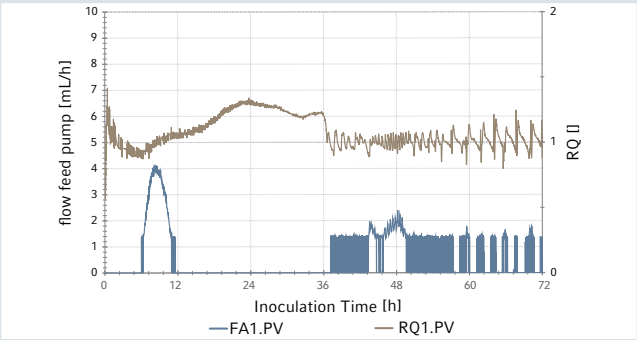


**Fig 5:** Fermentation graphs of the flow of the feed pump (blue) and the respiratory quotient (red) shown during Pichia pastoris fermentation with RQ controlled feeding.

### Growth curves

Based on the $OD_{600}$ data we collected, we drew a growth curve for both fermentations (Fig. 6). Growth curve data are shown and analyzed for the first 56h.

During the batch phase, before feed start, the culture's growth is comparable. After the batch growth phase of the culture, the RQ controlled feeding resulted in a much earlier exponential growth than the DO-spike triggered feeding.

After t=56 h, the DO-spike based fed-batch culture reached an $OD_{600}$ of 245, while the RQ controlled culture reached an $OD_{600}$ of 280.

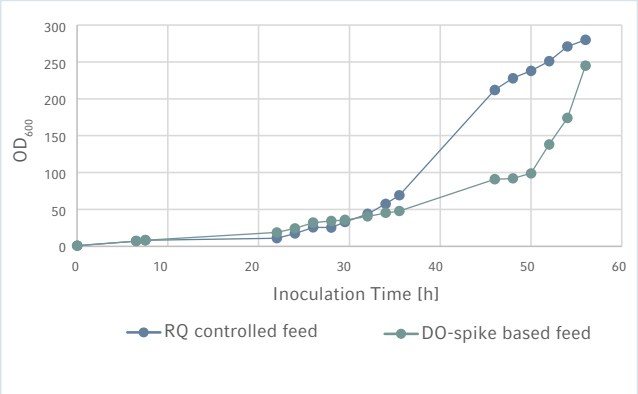


**Fig. 6:** Growth curves of Pichia pastoris with RQ controlled feed and DO- spike based feed.

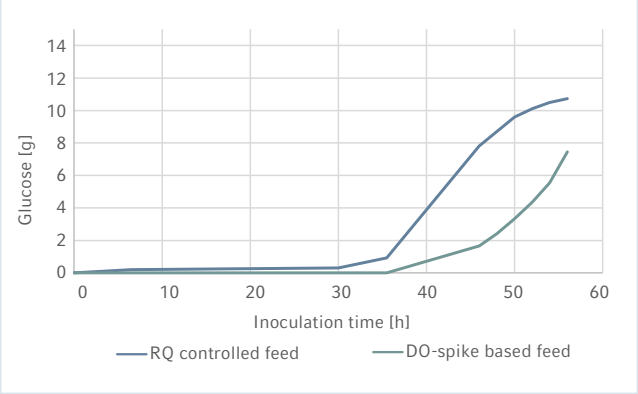


**Fig. 7:** Amount of glucose fed to the reactors. Feeding done by a stepwise feed or as a direct demand of the cells metabolism.

The DO-spike triggered culture reached an $OD_{600}$ higher than 200 after 56 hours inoculation time. We observed an increasing growth rate between hour 50 and 56 of the fermentation.

The RQ controlled culture on the other hand reached an $OD_{600}$ higher than 200 earlier in the fermentation, after 46 h. The growth rate after this point remained nearly static. The amount of glucose fed to the bioreactors in the course of the experiment is shown in Figure 7.

## Conclusion

We successfully demonstrated the implementation of a constant RQ controlled feeding strategy using DASware Control 5 and a DASbox fermentation system.

If the culture's growth profile and biomass accumulation is an indication of a successful fermentation, it can be concluded that the feeding based on constant RQ has multiple benefits.

We observed an $OD_{600}$ of 200 much earlier with the RQ-based feeding strategy compared to the DO-spike triggered feeding strategy. This can be explained by insufficient feeding in the DO-spike triggered set up. The feed steps would need to be adapted, which is not the case for the RQ-based feeding. This makes the RQ-based feeding strategy not only more time efficient, but also more cost effective.

Furthermore, another strong benefit of RQ-based feeding is that the feeding optimization is automatically self-achieved as the culture creates its own demand for feeding based on the drop of RQ value. In contrast to the DO-spike triggered feeding method where usually multiple runs are needed to fully optimize the feeding profile. Thus, less optimization of the feeding profile is necessary, in contrast to the DO-spike triggered feeding method.

This will save additional experiments and therefore material costs, labor costs, system occupancy and time. Thus, constant RQ feeding is one of the most optimal strategies for *Pichia pastoris* based protein production, and it can be easily achieved using Eppendorf small scale fermentation systems equipped with GA4 gas analyzer.

To investigate the hypothesis that the RQ > 1.1 during the last step of DO-spike based feeding results in a TCA cycle overflow the culture broth needs to be further analyzed. This would also give an insight on the theory that RQ-based feeding results in less by-products.

This application note represents a feasibility study towards using constant RQ to control yeast fermentation without optimization of the process. We believe with further optimization and adaption of the RQ based PID feeding script, we will be able to further reduce the RQ value oscillation around the target of 1 in the future.

## Appendix

```
'Constant RQ based feeding programming script with built-in PID control
'(c) 2020, Eppendorf AG
'History:
'V.1.0 msr

'=======> copy to script at least starting here!
'RQ based feeding PI-controller
'Version 1.0, 2020-07-03, (c) Eppendorf AG

'Script parameters
Dim StartDelay_h As Double = 12 'delay after inoculation [h]
Dim RQBasedFeedingSP As Double = 1.0 ' []
Dim MinFeedRate as Double = 0 ' [ml/h]
Dim MaxFeedRate as Double = 40.0 ' [ml/h

Dim tk_h as Double 't(k) runtime  [h]
Dim tk_1_h as Double 't(k-1) [j]

'PI controller with Anti Reset Windup and preset/reset
dim active as boolean
dim reset as boolean = false
dim dt as double
dim sp as double        'set-point
dim pv as double        'process value
dim ti as double = 360 'Reset Time [s]
dim kp as double = 3.33    'Proportional Factor
dim yp as double        'Proportional fraction of controller output
dim yi as double        'Integral fraction of controller output
dim yi_k_1 as double    'yi(k-1)
dim ypi as double       'Controller output (output y)
dim yMax as double = MaxFeedRate 'max output
dim yMin as double = MinFeedRate  'min output
dim preset as double = 0.0 'controller preset

Dim RQ As Double      ' RQ process value []
Dim Feed As Double


if P isNot Nothing Then
  with P
    tk_h = .runtime_h

    RQ = .RQ
    Feed = 0.0

    'Init Storage
    if s is Nothing then
      dim a(3) As Double
```

```
  'a(1) tk-1 [h]
  'a(3) PI controller yi(k-1)
  'a(4) active
  a(1) = 0
  a(2) = preset
  a(3) = false
  s = a
end if

'read from storage
tk_1_h = s(1)
yi_k_1 = s(2)
active = s(3)

' PI controller with Anti Reset Windup and preset/reset
dt = tk_h - tk_1_h
pv = RQ
sp = RQBasedFeedingSP
if active then
  'PI controller
  yp = (sp-pv)*kp
  if reset then yi_k_1 = preset
  yi = yi_k_1+(sp-pv)*kp/ti*dt*3600
  'Anti Reset Windup
  if yi > yMax then yi = yMax
  if yi < yMin then yi = yMin
  ypi = yp+yi
  if ypi>yMax then ypi = yMax
  if ypi<yMin then ypi = yMin
else
  ypi = 0
end if

'debug
'.IntA = ypi

select case .phase
case 0
  .phase = .phase + 1
  .LogMessage("Entering phase " & .phase & ": Waiting for InoculationTime > " & StartDelay_h & " [h]")
case 1
  if .InoculationTime_H > StartDelay_h then
    .phase = .phase + 1
    .LogMessage("Entering phase " & .phase & ": Waiting for RQ < " & RQBasedFeedingSP)
  end if
case 2
  if RQ < RQBasedFeedingSP then
    .phase = p.phase + 1
```

```
      active = true 'start PI controller
      .LogMessage("Entering phase " & .phase & ": start RQ based feeding")
    end if
  case 3
    'RQ based Feeding
    active = true
    Feed = ypi
  end select

  ' select feed pump
  .FASP = Feed

  'Write Storage
  s(1) = tk_h
  s(2) = yi
  s(3) = active

 end with
end if
```

## Literature

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