Biological and process-engineering characterization of the Minifors 2 laboratory bioreactor

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Application note

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Introduction

Laboratory-scale bioreactors offer a simple, cost-effective tool for developing and optimizing cultivation processes. Familiarity with a variety of process-engineering parameters is crucial for successfully scaling processes up or down.

This application note will first describe a systematic characterization of a stirring reactor, based on the example of the Minifors 2. Using this as a starting point, we will then present examples of microbial cultivation of a methylotrophic yeast *(Komagataella phaffi,* also known as *Pichia pastoris)* that demonstrate system performance at its technical limits.

Technical specifications

The technical specifications of the Minifors 2 (Figure 1) can be summarized as follows:

- Culture vessels: 1.5 L, 3.0 L or 6.0 L total volume with rounded, flat bottoms
- Stirrer speeds range from 150 to 1600 min^{-1} (this application note only examines speeds up to 1200 min^{-1}, which corresponds to a peripheral impeller tip speed of 0.5 to 3.5 m s^{-1})
- Two 6-bladed impellers (Rushton) with diameters of 38, 46 and 54 mm
- Gassing rates of 0 to 2 working vessel volumes min^{-1} (vvm) in L^{-1} min^{-1} by means of two mass-flow regulators
- Replaceable heating/cooling block for temperature control
- Bioprocess platform software (eve®) for recording online parameters

Experimental specifications

a. Process engineering characterization

Process engineering characterization was based on «Recommendations for process engineering characterization of single-use bioreactors and mixing systems by using experimental methods» [1] published by the expert group on single-use technologies within DECHEMA (the Society for Chemical Engineering and Biotechnology). The mixing time was determined using the classic discoloration method, in which an iodine-starch complex (blue) is discolored through the addition of thiosulfate.

The degassing method was used for taking k_La measurements, in which oxygen was first removed by flushing with nitrogen and then reintroduced through the addition of air. Values measured at saturation levels between 10 and 90% were used for the analysis (Eq. 1).

$$k_L a = \frac{\ln (100\text{-}c_t)}{\Delta t} \quad \text{Eq. 1}$$

Power input was recorded using a torque meter. [2] The purpose of the first measurement was to determine torque with respect to air, after which measurements were taken in the appropriate test volume of liquid. Equation 2 was used for analyzing the specific power input (whereby P/V_L is the volumetric power input in W m^{-3}, M is the motor torque in Nm, n is the stirrer speed in s^{-1} and V_L is the volume of liquid in m^3).

$$\frac{P}{V_L} = \frac{M \cdot 2 \cdot \pi \cdot n}{V_L} \quad \text{Eq. 2}$$

Fig. 1: Minifors 2 with 6.0 L glass vessel

b. Cultivation with *Pichia pastoris*

The methylotrophic yeast *Pichia pastoris* was selected as the sample organism for biological characterization of the system. Properties of the yeast include the following:

- High cell-density growth
- High oxygen demand
- Metabolism that becomes more exothermic when methanol is used as a substrate

These features make this yeast suitable for ascertaining the system's potential technical limits. The first step was to use a two-phase batch/pulse method to evaluate how the system responded when switched over to the inducing substrate methanol (MeOH), which places considerable demand on the capacity of the system to dissipate heat. To emphasize the metabolic state and the stress it puts on the system, the biomass was kept within a range that was extreme yet still aerobic (pO_2 0 to 10% at the maximum gassing rate and stirrer speed) (CDW 20 to 30 g L^{-1}).

In a subsequent series of tests, a semi-continuous feed method using the primary substrate (glucose) was employed in order to double the cell density (CDW ~60 g L^{-1}). This was done in order to gauge system performance at the technical limit for oxygen input (pO_2 < 1 %).

To test system stability in relatively long tests, we implemented a continuous cultivation process, setting a defined, specific growth rate and controlled elution. This was followed by the subsequent re-enriching of the biomass. Unlike batch experiments, this method made it possible to compare key physiological parameters (μ_{max} , $Y_{x/s}$) both with and without exclusion of potential limitations associated with the medium.

The use of the online sensors (pO_2, CO_2, and OD_{online}) directly integrated in the system was also evaluated across all of the test series as a way of monitoring culture status in real time and comparing the results to the corresponding offline measurements ($OD_{offline}$, CDW).

Process-engineering characterization

All process-engineering measurements were taken at the maximum working volume, which corresponds to 1.0 L for the 1.5 L reactor, 2.0 L for the 3.0 L reactor, and 4.0 L for the 6.0 L reactor.

a. Mixing time

Measurements were taken at a stirrer speed of 900 min^{-1} (corresponding to a stirrer impellor tip speed of 1.8 m s^{-1} in the 1.5 L, 2.2 m s^{-1} in the 3.0 L and 2.5 m s^{-1} in the 6.0 L culture vessel). Mixing time was less than 2 seconds for all three culture volumes, even at the lowest speeds studied. As stirrer speed increases, mixing time decreases still further, so that thorough mixing can assumed within the range under investigation.

b. k_La value

The k_La value was determined at stirrer speeds ranging from 900 to 1200 min^{-1} and at aeration rates between 1 and 2 vvm for each of the three reactors. Equation 3 (along with the corresponding coefficients in Table 1) can be used for describing the model mathematically. A graphic representation of model-based k_La values can be found in Figure 2. These data clearly demonstrate that oxygen transfer coefficients are similar for all reactors, with k_La values of over 220 h^{-1} achieved in the two smaller reactors and k_La values of over 180 h^{-1} in the 6 L reactor.

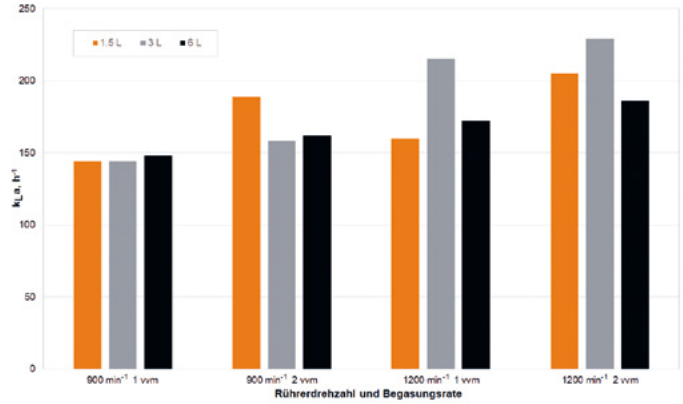

Fig. 2: k_La values for the Minifors 2 at 900 and 1200 min^{-1} and 1 and 2 vvm.

$$k_La = C + n \cdot C_n + B \cdot C_B + n^2 \cdot C_{nn} + B^2 \cdot C_{BB} \quad \text{Eq. 3}$$

Parameter			1.5 L reactor	3.0 L reactor	6.0 L reactor
Constant		C	–1090.05	458.838	841.934
Stirrer speed, min^{-1}	n	C_n	2.25509	–0.8687	–1.4343
Gassing rate, vvm	B	C_B	55.4551	13.8331	13.401
(Stirrer speed)^2	n^2	C_{nn}	–0.00105	0.00053	0.00072
(Aeration rate)^2	B^2	C_{BB}	–3.50337	–	–
Range					
Stirrer speed, min^{-1}				900 – 1200	
Stirrer peripheral speed, ms^{-1}			1.8 – 2.4	2.2 – 2.9	2.5 – 3.4
Aeration, vvm			1 – 2	1 – 2	1 – 2
Coefficient of determination R^2			0.983	0.951	0.915
Predictive quality Q^2			0.952	0.811	0.823
Reproducibility			0.988	0.957	0.882

Table 1: Coefficients for calculating the k_La value for the three Minifors 2 reactors at maximum working volumes.

c. Specific power input

Figure 3 shows the volumetric power input for the three reactors at impeller tip speeds of 0.5 to 3.5 m s^{-1} (corresponding to 250 to 1200 min^{-1}) and fill volumes of 1.0, 2.0 and 4.0 L. As expected, power input was highest at high stirrer speeds. Power input at maximum fill volume and maximum speed (typical cultivation conditions) fell between 2 and 4.5 kW m^{-3}. Comparability between all three reactors is very good.

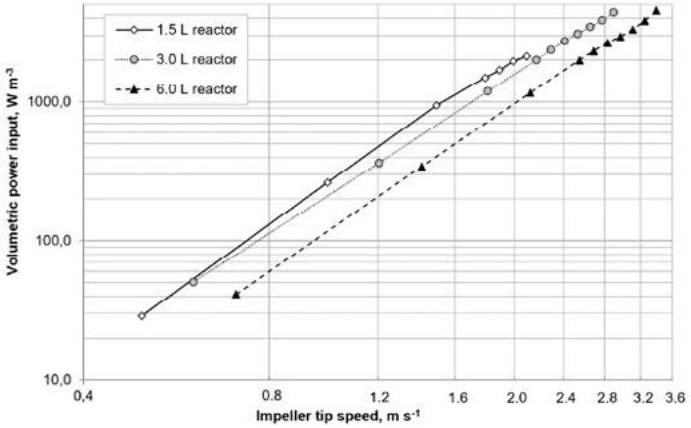

Fig. 3: Volumetric power input (in W m^{-3}) for all three reactors at different impeller tip speeds (in m s^{-1}) and maximum working volume.

Biological characterization

a. Batch/pulse method with MeOH induction

We replicated a classic induction strategy using the AOX promoter and the batch/pulse method (example shown in Fig. 4, process PIP_466). This study showed that the cooling system was capable of sufficiently absorbing the heat that forms suddenly during an MeOH pulse-even under conditions approaching a potential oxygen limit (pO_2 < 10%). The slim design of the reactor combined with maximum stirrer speeds and gassing rates required repeated addition of an antifoaming agent; a similar effect can also be seen in other reactor systems. Under the conditions tested, however, this did not have any noticeable effect on the concentration of dissolved oxygen or on the physiology of the culture.

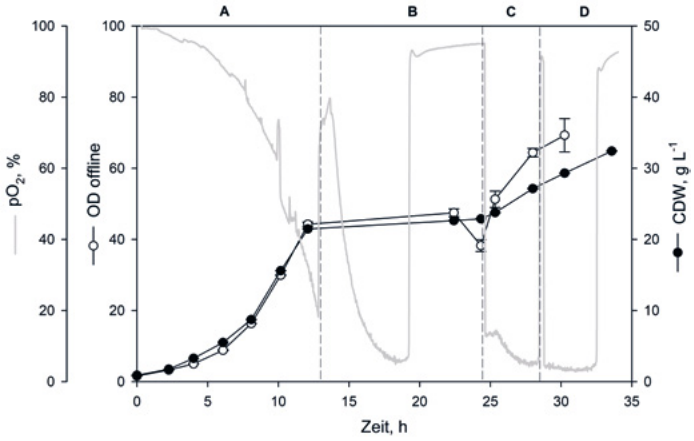

Fig. 4: Process overview of the batch/pulse method showing biomass growth on glucose (phase A, 0 to 13 h) and subsequent induction via MeOH pulses (phases B-D, 1% v/v).

b. Biomass formation with a semi-continuous feed method

A semi-continuous feed method was used for determining the suitability of the Minifors 2 system for cultures in which oxygen demand is high (Fig. 5). A parallel physiological study was also conducted to determine the presence of potential substrate inhibitors. As anticipated, the only way of reaching the technically feasible limit for oxygen input during the gassing phase was through the use of process air. The gradual increase in excess substrate (phase B) resulted in the formation of intracellular storage substances that were not consumed until the available primary substrate had been consumed. Indications in the waste gas composition made different metabolic phases readily identifiable. In applying this process strategy, we were also able to confirm the suitability of the system for studying and describing the physiological characteristics of cultures.

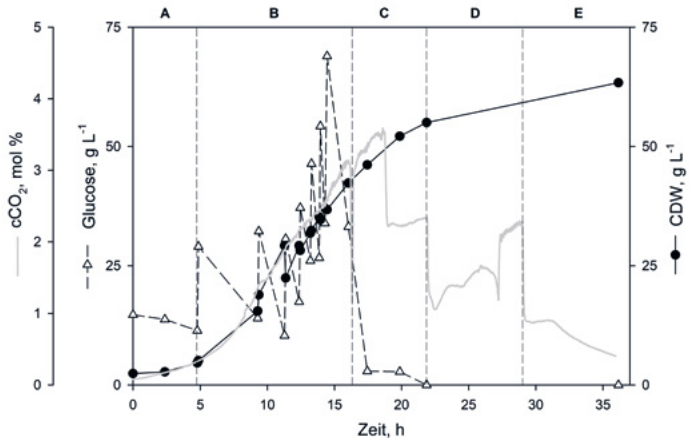

Fig. 5: Process overview of the semi-continuous feed method for biomass formation on 60 g L^{-1}. Repeated glucose pulses (phase B) for testing substrate inhibition. Process phases separated by vertical dotted lines.

c. Continuous method for testing limitations associated with the medium

Continuous cultivation methods represent an efficient tool for measuring key physiological parameters while ruling out the possibilities of nutrient limitations or metabolite inhibition. In tests lasting over ~60 h, cultures passed through various states, matching the states that are typically used in these kinds of studies (initial batch / constant dilution rate / elution / re-enrichment (Figure 6)). The resulting physiological parameters were compared to those from the conventional batch process and found to correspond well (μ_{max}: 0.225/0.251 h^{-1}; $Y_{x/s}$: 0.61/0.65 g g^{-1}; q_s: 0.353/0.382 g g^{-1} h^{-1}).

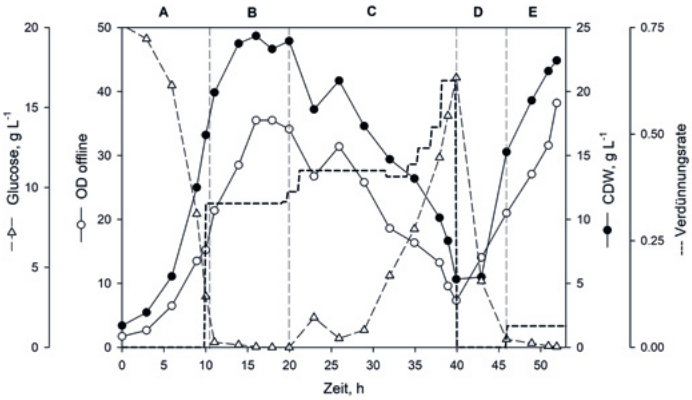

Fig. 6: Process overview of the continuous method. An initial batch phase (A) was followed by a phase with a constant dilution rate (B), a phase with a controlled elution step (C), a biomass re-enrichment phase (D), and a phase focused on product formation (E).

Over the course of the cultivation experiments, we also looked at options for real-time biomass monitoring by means of online sensors that could either be directly integrated in the Minifors 2 or added on separately. The specific goal here was to investigate known problems involving signal saturation or interference caused by vigorous stirring/gassing. [3] This study showed a very strong correlation between online and offline measurements taken with various sensor types (OD, permittivity/dielectric conductivity) and/or offline analytical methods (CDW) (Fig. 7).

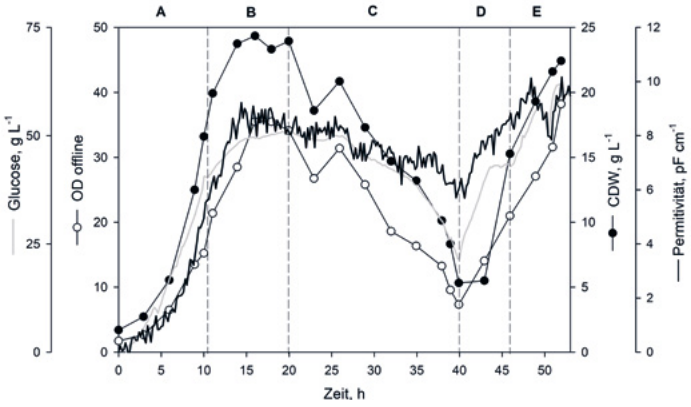

Fig. 7: Comparison between online biomass-monitoring sensors in the Minifors 2 during continuous cultivation (see Fig. 6 for additional process parameters).

Summary

On the basis of process-engineering characterization studies conducted with k_La values up to 220 h^{-1}, blending times less than 2 s (within a cultivation range of 900 to 1200 rpm) and power inputs of up to 4.5 kW m^{-3}, the Minifors 2 was found to be well suited for microbial applications. At the same time, scale-up should not pose any difficulty as the results from the three reactors were found to be highly comparable.

On the whole, the system proved to be very well suited for physiological characterization of bioprocesses involving *Pichia pastoris*. No technical limitations were observed.

Biomass concentrations of up to 66 g L^{-1} (dry cell weight) were readily attainable using the fed-batch method, with no need to introduce additional oxygen. This means that use of the integrated GasMix system, which operates with two mass-flow regulators for adding more oxygen, should further increase achievable biomass concentrations. The modular Minifors 2 also provides a robust, technically simple means of implementing more complex, time-consuming process setups (continuous processes, etc.). Its modular configuration means that, even at bench-scale, the Minifors 2 can make a valuable contribution to the in-depth process understanding embodied by the PAT initiative. [4]

In short, as a baseline for flexible, productive experimental cultures, the Minifors 2 exceeds expectations and meets any challenge.

References

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