

# DASGIP Application Note on High Density E.Coli Fermentation

## 1. Introduction

The intention of the experiment was to determine the capability of the “stirrer-pro”-system (DASGIP AG, Juelich, D) to support growth of *Escherichia coli* K12 to high cell densities. Therefore, a fed-batch fermentation in four parallel cultivation vessels was performed. The target of the fermentation was to reach an optical density (OD<sub>600</sub>) of > 100.

## 2. Materials and methods

### 2.1. Fermentation media

All media components are dissolved in deionized water and autoclaved (20 min · 121 °C). Components marked with an asterisk \* are sterilized separately by filtration (0.2 µm). pH-adjustments as indicated are performed prior to sterilization.

#### 2.1.1. TB-glycerol medium (1. preculture)

yeast extract	24.0 g·L <sup>-1</sup>
peptone from casein	12.0 g·L <sup>-1</sup>
glycerol	5.0 g·L <sup>-1</sup>
K <sub>2</sub> HPO <sub>4</sub>	12.54 g·L <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	2.31 g·L <sup>-1</sup>
pH ad 7.0	

#### 2.1.2. PAN-medium for shake-flask cultivation (2. preculture)

glycerol, water-free	10.0 g·L <sup>-1</sup>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.3 g·L <sup>-1</sup>
CaCl <sub>2</sub> ·H <sub>2</sub> O	0.015 g·L <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	3.0 g·L <sup>-1</sup>
K <sub>2</sub> HPO <sub>4</sub>	12.0 g·L <sup>-1</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.0 g·L <sup>-1</sup>
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.075 g·L <sup>-1</sup>
tri-Na-Citrate·2H <sub>2</sub> O	1.0 g·L <sup>-1</sup>
* thiamine solution	1.0 mL·L <sup>-1</sup>
* trace elements solution	1.0 mL·L <sup>-1</sup>

### 2.1.3. PAN-Medium for stirrer-pro cultivation

glycerol, water-free	20.0 g·L <sup>-1</sup>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	3.0 g·L <sup>-1</sup>
CaCl <sub>2</sub> ·H <sub>2</sub> O	0.015 g·L <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	15.0 g·L <sup>-1</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.0 g·L <sup>-1</sup>
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.15g·L <sup>-1</sup>
ZnCl <sub>2</sub>	0.1 g·L <sup>-1</sup>
tri-Na-Citrate·2H <sub>2</sub> O	1.0 g·L <sup>-1</sup>
* thiamine solution	1.5 mL·L <sup>-1</sup>
* trace elements solution	1.5 mL·L <sup>-1</sup>

### 2.1.4. glucose feeding solution

glucose·H <sub>2</sub> O	544 g·L <sup>-1</sup>	(= 500 g·L <sup>-1</sup> glucose)
* thiamine solution	2.0 mL·L <sup>-1</sup>	
* trace elements solution	2.0 mL·L <sup>-1</sup>	

### 2.1.5. trace elements solution

Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·18H <sub>2</sub> O	2.0 g·L <sup>-1</sup>
CoSO <sub>4</sub> ·6H <sub>2</sub> O	0.75 g·L <sup>-1</sup>
CuSO <sub>4</sub> ·5H <sub>2</sub> O	2.5 g·L <sup>-1</sup>
H <sub>3</sub> BO <sub>4</sub>	0.5 g·L <sup>-1</sup>
MnSO <sub>4</sub> ·H <sub>2</sub> O	24.0 g·L <sup>-1</sup>
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	3.0 g·L <sup>-1</sup>
NiSO <sub>4</sub> ·3H <sub>2</sub> O	25.0 g·L <sup>-1</sup>
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	15.0 g·L <sup>-1</sup>
H <sub>2</sub> SO <sub>4</sub> , 30 %	2.0 mL·L <sup>-1</sup>

### 2.1.6. Thiamine-solution

Thiamine-HCl	5.0 g·L <sup>-1</sup>
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sterilized by filtration 0.2 μm

### 2.1.7. pH-correction

An aqueous solution of 10% NH<sub>4</sub>OH was used for correction of the pH-Value. The solution can be assumed to be auto-sterile.

## 2.2. **Strain**

For the experiment, the microbial strain *Escherichia coli* ATCC 23716 (*E.coli* K12) was used.

## 2.3. **Experimental setup**

### 2.3.1. strain conservation

The strain *Escherichia coli* ATCC 23716 was kept as a cryoconserved culture containing 33 % glycerol at -80 °C.

### 2.3.2. first preculture

The first preculture was grown in TB-glycerol medium at 37 °C for 10 h. The cultivation was performed in 250 mL unbaffled Erlenmeyer-flasks with an initial liquid volume of 10 mL. The flasks were shaken at a frequency of 300 min<sup>-1</sup> and an amplitude of 5.0 cm. Wide-neck flasks with cellulose stoppers as sterile closure were used. The culture was inoculated with 4 % of the initial liquid volume of cryoconserved culture.

### 2.3.3. second preculture

The second preculture was grown in medium defined above at 37 °C for 13,75 h. The cultivation was performed in 1000 mL unbaffled Erlenmeyer-flasks with an initial liquid volume of 40 mL. The flasks were shaken at a frequency of 300 min<sup>-1</sup> and an amplitude of 5.0 cm. Wide-neck flasks with cellulose stoppers as sterile closure were used. The culture was inoculated with 10 % of first preculture of the initial liquid volume.

### 2.3.4. main culture

For the main cultivation a stirrer-pro system (DASGIP, D) consisting of 4 x 300 mL working volume stirred vessels was used. Each vessel was completely assembled, filled with 150 mL of the medium and autoclaved. After connecting the vessel and setting pH and temperature to the desired values, the fermentation was inoculated with 15 mL of second preculture. Thus the starting volume of the process was 165 mL in each vessel. The fermentation was started in batch-mode, upon depletion of the supplied substrate the substrate feed was initiated.

Throughout the fermentation all parameters were maintained as given in the list below.

- pH-control

Each vessel was equipped with a pH-probe. The pH-value was kept constant at 6.8 by means of an integrated PID-controller using one channel of an MP8 multi-pump for each vessel dosing 10 % ammonia solution into the fermentation.

- agitation

Each vessel was equipped with a magnetic stirrer bar and agitated at a constant speed of 1000 rpm.

- aeration

Each vessel was aerated at a constant rate of 10.5 L·h<sup>-1</sup>. The gas was sparged into the liquid through a tube-shaped sintered glass frit.

In the final period of the fermentation, the aeration rate was significantly reduced to avoid excessive foaming; in order to maintain oxygen saturation of the broth, pure oxygen was then used for aeration.

- temperature control

All vessels were placed in a thermostat bath with a constant temperature of 36.5 °C.

- DO-control

The dissolved oxygen concentration was kept above 40 % saturation (set point) by means of a gas-mixing station MX4/4.

- substrate dosage

During the fed-batch phase of the fermentation, the substrate solution was dispensed into the vessels by means of a MP8 multi-pump module with one channel configured for each vessel. Substrate dosage was controlled by a time-dependent profile defined by the following equation:

$$f(x) = P_1 + \frac{P_2}{1 + e^{-P_3(t-P_4)}} + \frac{P_5}{1 + e^{-P_6(t-P_7)}} \text{ with } P_1 = 0,4 / P_2 = 2,5 / P_3 = 0,27 / P_4 = 8 / P_5 = P_6 = P_7 = 0.$$

### 3. Results and discussion

#### 3.1. precultures

The first preculture, run under the described conditions reached an optical density of  $OD_{600}=13.3$  after 10 h of incubation. 10 mL of this culture were used for inoculation of the second stage. The second preculture grew from a starting value of  $OD_{600}=1.5$  to  $OD_{600}=10.5$  within 13.75 h following the protocol described above.

#### 3.2. Main cultivation

The four cultivation vessels were inoculated with 15 mL of second preculture. After a fermentation time of 7 h the glycerol supplied initially was exhausted and the fed-batch phase of the fermentation was subsequently initiated. A solution of  $500\text{g}\cdot\text{L}^{-1}$  glucose was dispensed into the fermenters following the profile described above. In figure 1 the dosing profile and the complete dosage is displayed. This profile assures a growth-limiting supply of carbon source for this strain of *E. coli*. Thus, glucose is not detectable in the culture broth throughout the fermentation.

Within the total fermentation time of 46.8 h, the optical density ( $OD_{600}$ ) of the fermentation broth could be increased to a mean value of 169.5. This corresponds to a cell concentration (cell dry weight, cdw) of  $65.5\text{g}\cdot\text{L}^{-1}$ . The courses of  $OD_{600}$  and cdw are displayed in figure 2a and figure 2b, respectively.

One problem occurring during the fermentation was an excessive foam formation. At biomass concentrations of  $OD_{600} > 100$  this foam could not be anymore controlled by addition of anti-foam agent (Sigma 204). Though, the problem could be solved by a simple procedure: After disconnecting the supply of pressurized air to the system, the fermentation was left only with a supply of pure oxygen. The DO-saturation was then controlled by the amount of oxygen supplied to each vessel. As this resulted in a strong decrease of the total aeration rate from  $10.5\text{L}\cdot\text{h}^{-1}$  to less than  $1\text{L}\cdot\text{h}^{-1}$ , foam formation was diminished and the addition of antifoam agent no longer required.

Throughout the fermentation, pH was controlled with a set point of 6.8; the time course of the pH-value is displayed in figure 3.

An MX4/4 gas mixing device was used to maintain dissolved oxygen saturation at a desired value of 40 %. The time course of DO is displayed in figure 4a. The frequent sharp drops in dissolved oxygen saturation each indicate the addition of antifoam agent into the individual

fermentation vessel. DO was maintained by addition of pure oxygen to the gas supply. In the later fermentation phase (33 h post inoculation) the aeration rate had to be decreased significantly by disconnecting the pressurized air supply leaving a pure oxygen supply. Figure 4b displays the amount of oxygen added and the supply with pure oxygen in the late fermentation, respectively. For technical reasons, DO could not be controlled automatically in vessel I. Thus, the oxygen partial pressure in the gas supply was fixed to 40 %.

With the exception of single spikes in oxygen supply caused by reduced mass transfer due to antifoam addition, the consumption of oxygen remains on moderate levels demanding partial pressures of no more than 40 % in the gas supply. In the later fermentation phase, the demand for pure oxygen is less than  $1 \text{ L}\cdot\text{h}^{-1}$ . These results clearly demonstrate, that much higher growth rates and even higher biomass concentrations could have been achieved using the stirrer-pro system.

The stirrer-pro system, equipped with submerged aeration and a gas-mixing device, is thus clearly able to bear high-cell-density fermentations without restrictions compared to a standard-type stirred-tank reactor.

#### **4. Summary**

The stirrer-pro system (DASGIP AG, D) was used to demonstrate the capability of the system to sustain a high-cell-density fermentation using *Escherichia coli*. Within a total fermentation time of 46.8 h an optical density of  $\text{OD}_{600}=170$  was achieved corresponding to a cell-dry-weight concentration of  $65.5 \text{ g}\cdot\text{L}^{-1}$ . Regarding the actual consumption of oxygen during the fermentation run, higher growth rates and even higher cell densities could have been achieved.

Foam formation, occurring frequently in high-cell-density fermentation, could be overcome by switching to a pure oxygen supply at a strongly reduced aeration rate.

It could be demonstrated, that the stirrer-pro system is capable of sustaining very high cell densities of fast growing of *Escherichia coli* without restrictions. High-cell-density fermentation, though, requires a system equipped with a submerged gassing device and a gas mixing device.

Fig. 1: substrate dosage

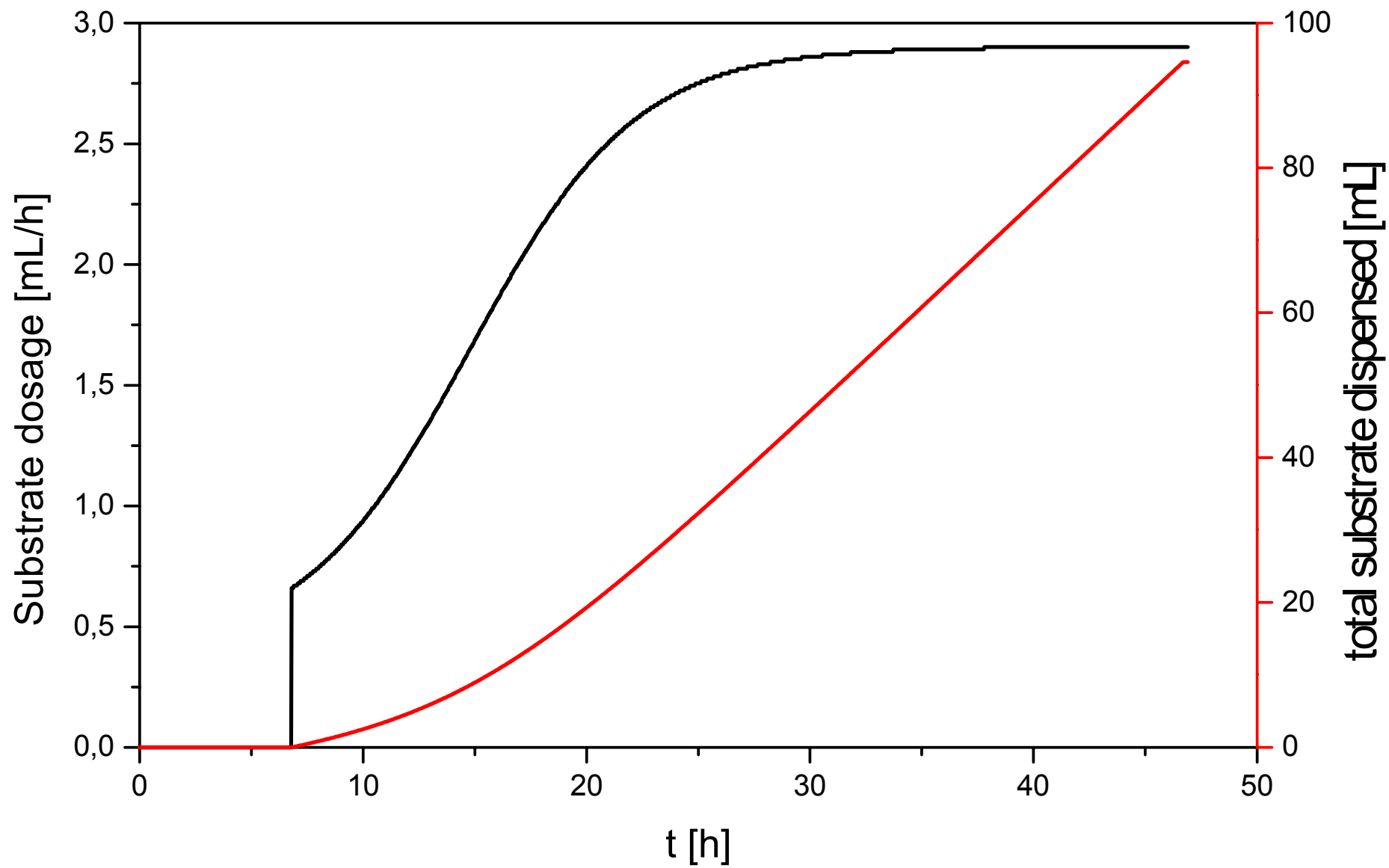


Fig. 2a: Optical density

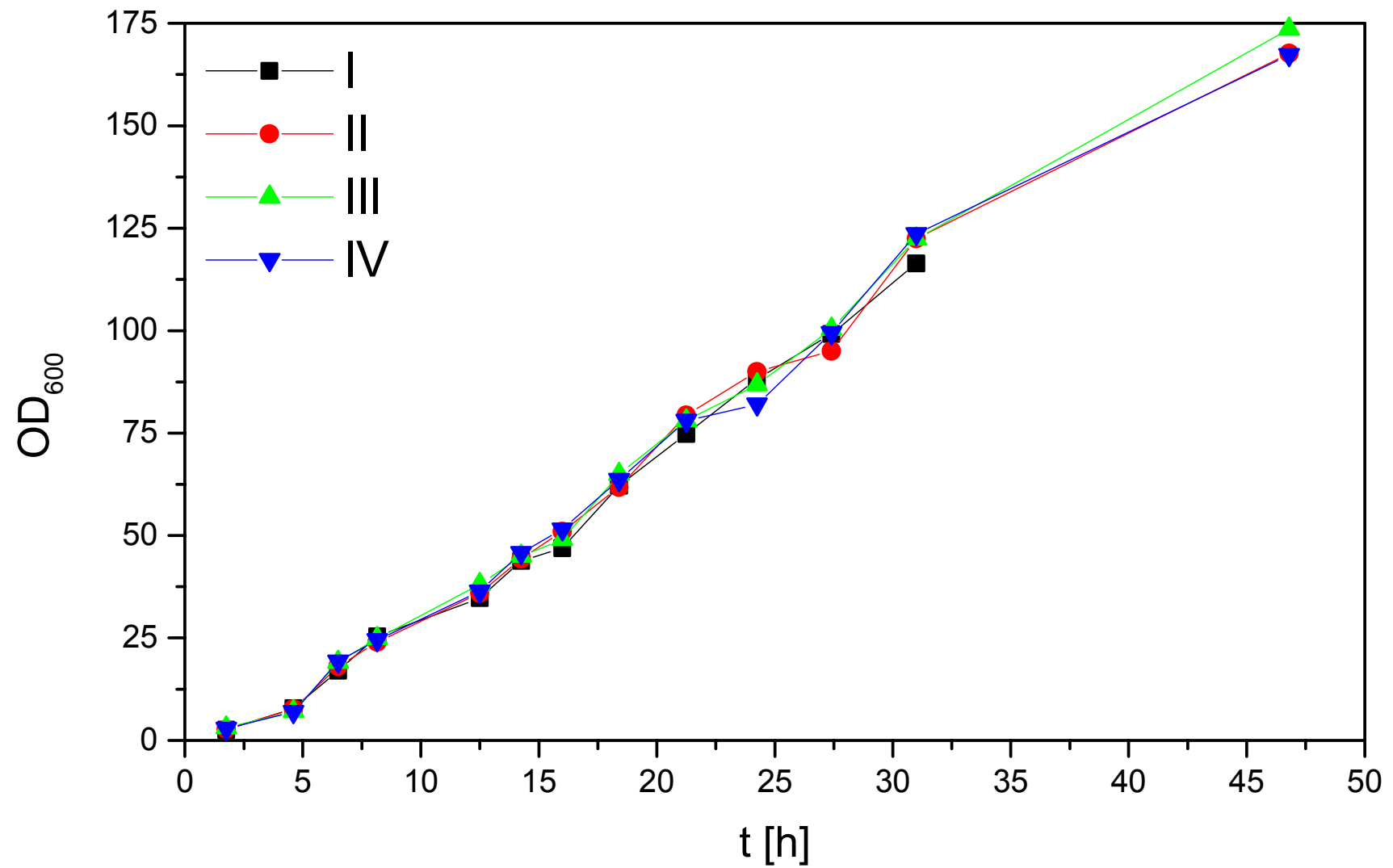


Fig. 2b: cell dry weight

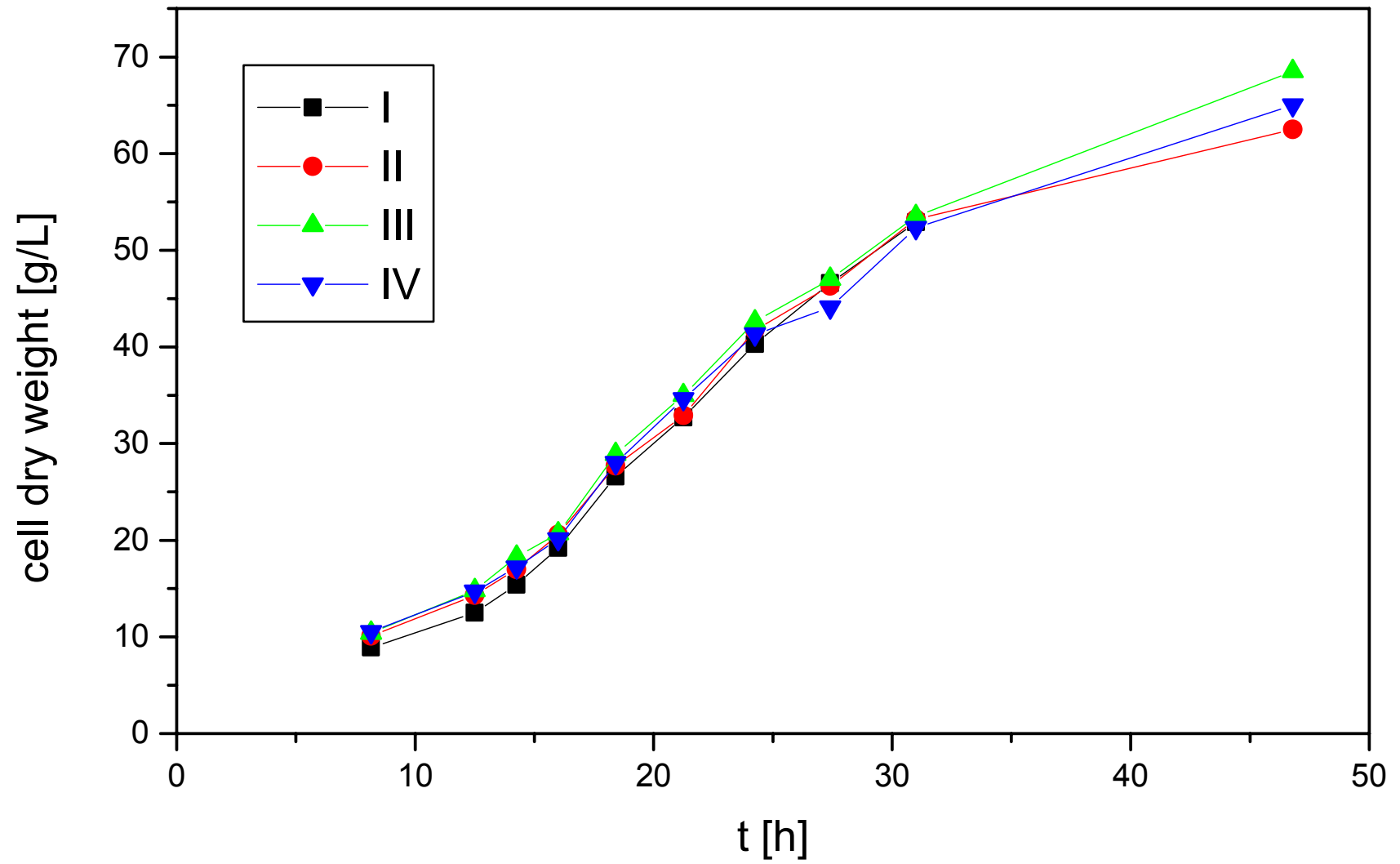


Fig. 3: pH-value

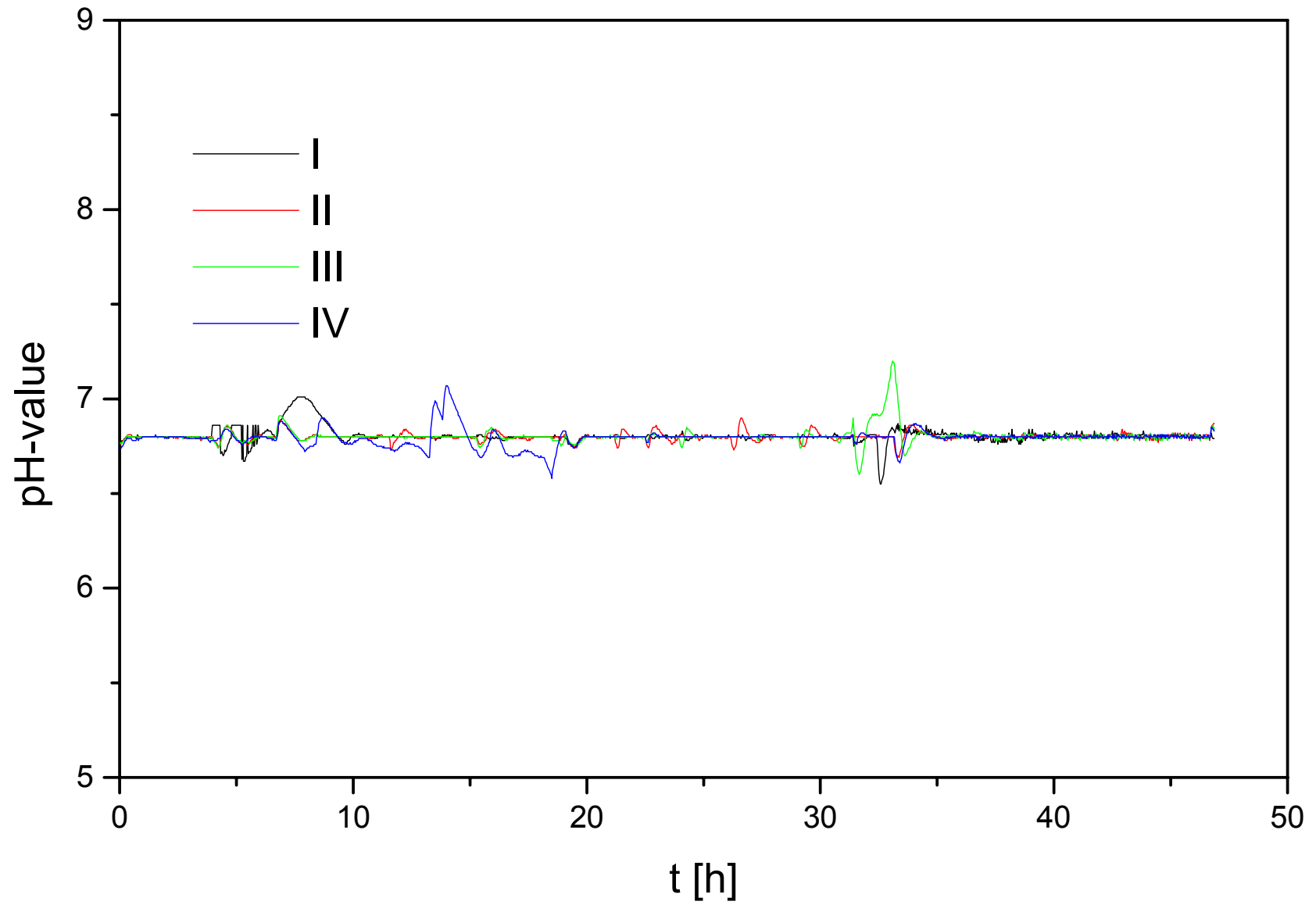


Fig. 4a: DO-saturation

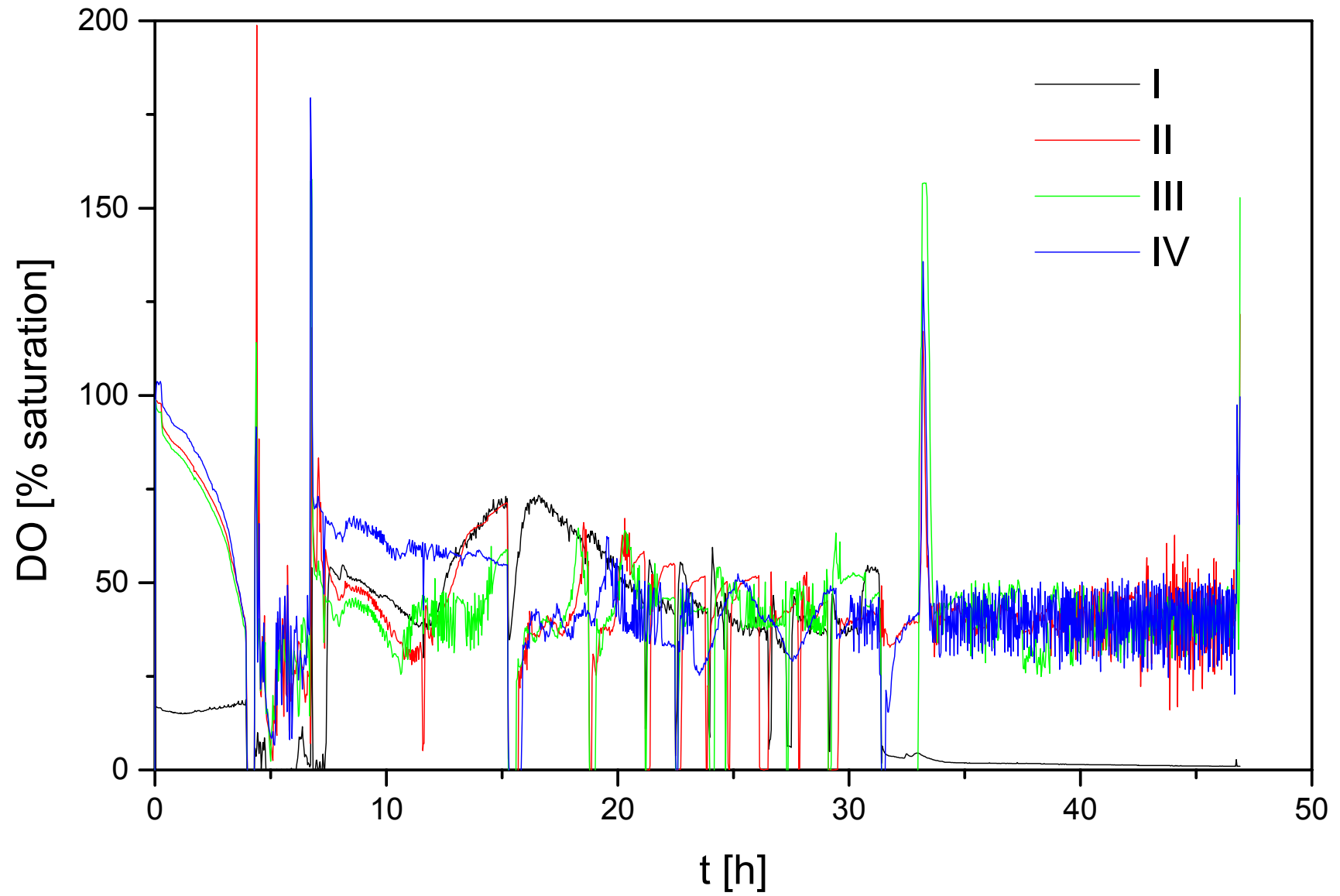


Fig. 4b: oxygen supply

