DECHEMA
Biotechnologie

EXPERT GROUP SINGLE-USE TECHNOLOGY

Recommendation for biological evaluation of bioreactor performance for microbial processes

Authors
Cedric Schirmer[1]*, Thomas Dreher[2], Marco Leupold[2], Robert Glaser[3], Andreas Castan[4], Jason Brown[5], Dieter Eibl[1], Rainer Glöckler[6]

[1] Zurich University of Applied Sciences, Institute of Chemistry and Biotechnology, Wädenswil, Switzerland
[2] Sartorius Stedim Biotech GmbH, Göttingen, Germany
[3] Eppendorf AG, Jülich, Germany
[4] GE Healthcare Bio-Sciences AB, Uppsala, Sweden
[5] Thermo Fisher Scientific, Logan, Utah/USA
[6] swissfillon AG, Visp, Switzerland
* Corresponding author: Cedric Schirmer, cedric.schirmer@zhaw.ch

The authors are active in the working group "Single-Use Microbial" of the DECHEMA expert group "Single-Use-Technology in Biopharmaceutical Manufacturing".

IMPRINT

Editor

DECHEMA

Gesellschaft für Chemische Technik
und Biotechnologie e.V.
Theodor-Heuss-Allee 25
60486 Frankfurt am Main
Tel.: +49 69 7564-0
Fax: +49 69 7564-201
E-Mail: info@dechema.de

Responsible according to the German Press Law
Prof. Dr. Kurt Wagemann
Dr. Kathrin Rübberdt

Layout
Peter Mück, PM-GrafikDesign, Wächtersbach

Print
Seltersdruck & Verlag Lehn GmbH & Co. KG, Selters

2. Revised edition
(corrections on page 8: value given for H^o ; Equation 9; on page 32: Equation A3)

Publication date: September 2019

ISBN: 978-3-89746-222-9

CONTENTS

1 Background

1.1 Introduction

Bioreactors are presently available in various sizes ranging from mL to m^3 scale as steel, glass or single-use systems. Regardless of the choice of bioreactor system, it must meet the requirements of the desired fermentation process. Since the different systems can also differ in their drive type, design and equipment, the comparison or selection of a system is not easy. In order to counteract this problem, DECHEMA issued guidelines in 2016 with standardised methods to obtain process engineering data in various bioreactor systems, data which also form the basis of this guideline. These include, in particular, the power input, the mixing time and the volumetric mass transfer coefficient (k_La) [1]. The working group "Single-Use Microbial" of the DECHEMA expert group "Single-Use Technology in Biopharmaceutical Manufacturing" has made it its task to supplement these methods with a standardised biological microbial characterisation method.

1.2 Characterisation with biological approaches

The biological microbial characterisation of bioreactors is concerned with the evaluation and comparison of the biological performance of a system. Process engineering characterisation, however, often only provides information on the ideal bioreactor design or scale-up. In addition, a transfer of a derived process model to a cultivation process at a later stage is often difficult, due to the use of different cultivation media. For this reason, the use of a model organism in combination with a standardised cultivation strategy enables the evaluation of a bioreactor for a desired process. This idea is by no means new. Adler & Fiechter and Wagner developed model processes with respiratory yeast and a mycelium-forming fungus back in the 1980's [2, 3]. This is precisely where the standardised model process of the working group "Single-Use Microbial" comes in, with the aim of supplementing the process engineering characterisation, which must be completed in the run-up to the investigations on biological performance. *Escherichia coli* is used as a model organism, as it is known for its ready availability, short generation time and great relevance in the biopharmaceutical industry. Additionally, microbial processes place high demands on the bioreactor system regarding oxygen transfer and heat removal.

This enables a global comparison of single-use and reusable bioreactors under real process conditions, thus supporting the investigation and evaluation of various growth-related parameters.

2 Guideline – Experimental determination of biological bioreactor performance with an *Escherichia coli* model process

2.1 Introduction

The *Escherichia coli* model process, from the DECHEMA working group "Single Use Microbial", was developed to determine the performance of bioreactors, especially single-use bioreactors, and evaluate their suitability for microbial applications. The use of a classical medium and a defined process under real and standardised conditions with substrate concentrations changing over time also enables the dynamic investigation of the driving force of oxygen. The classical k_La value measurement method according to the process engineering characterisation (see DECHEMA guidelines, Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods) can lead to problems at high oxygen inputs. The reason is the often slow response time of the pO_2 probe, which can strongly falsify the measurement results and thus limit the measuring range. Since microbial processes in particular place high demands on the bioreactor system with regard to oxygen transfer, this has a special significance. Thus, the *E. coli* model process makes it possible to overcome such problems by determining the k_La value during the cultivation. Moreover, heat removal can also be investigated in paralell.

The *E. coli* model process (Strain: W3110 thyA36 supO λ-; ordering number at the Leibnitz Institute DSMZ-German Collection of Mircoorganisms and Cell Cultures (DSM): 5911) described below is a simple batch process which can be conducted within one working day (7-10 hours) (Figure 1, page 6). It is essential to follow the media preparation, the pre-culture and main culture instructions as well as the data evaluation in the exact order prescribed to obtain reproducible and comparable results. Below, an overview of the whole model process is shown. Detailed instructions for the preparation of the various media components can be found in the appendix. An Excel tool for the standardised data evaluation is also available (download at www.dechema.de or contact the corresponding author).

2.2 Using experimental data for the evaluation of the biological bioreactor performance

The evaluation of biological bioreactor performance is based on online and offline parameters. The optical density at 600 nm (OD_{600}), the dry cell weight (*DCW*), the wet cell weight (*WCW*), the metabolites glucose and acetate, the pO_2 value, the pH value, the temperature, the concentrations of oxygen and carbon dioxide in the exhaust gas, the base consumption and the required volume of antifoam are taken into account. The oxygen uptake rate (*OUR*) as well as the carbon dioxide formation rate (*CER*) can then be calculated from the results of the exhaust gas analysis. The k_La value during the cultivation can also

2 GUIDELINE – EXPERIMENTAL DETERMINATION OF BIOLOGICAL BIOREACTOR PERFORMANCE WITH AN ESCHERICHIA COLI MODEL PROCESS

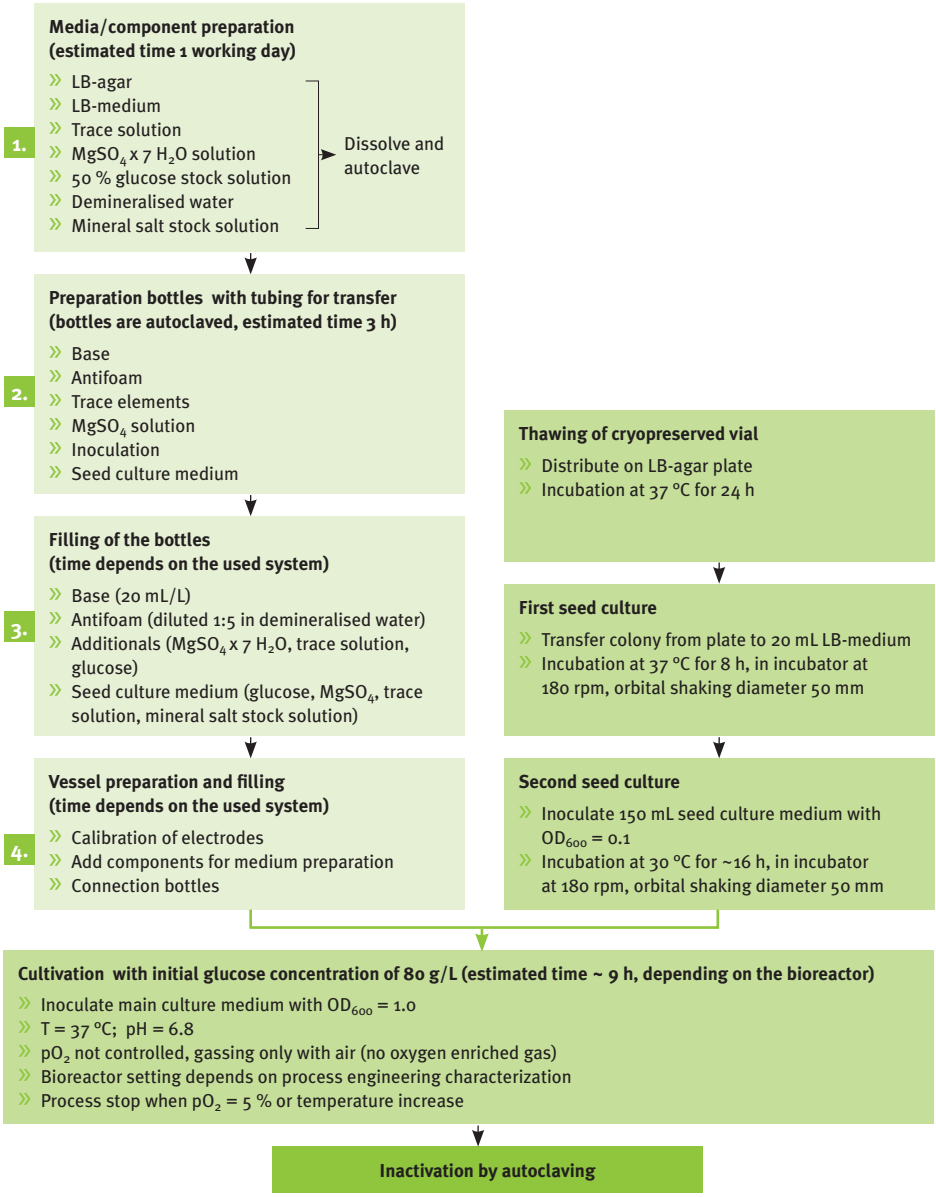


Figure 1: Flow chart of the *Escherichia coli* model process; OD_{600}, optical density at 600 nm.

be calculated from the recorded pO_2 profile and compared with the data from the process engineering characterisation. By taking into account the oxygen transfer rate (*OTR*, Equation 1) and the *OUR* of the microorganisms (Equation 2 and 3), the oxygen gas balance for the liquid phase results as shown in Equations 4 and 5.

$$OTR(t) = k_L a \cdot (c_L^*(t) - c_L(t)) \tag{1}$$

$$OUR(t) = q_{O_2}(t) \cdot X_V(t) \tag{2}$$

The calculation of the *OUR* is normally based on the viable cell counts. Here, the whole cell population is assumed to be viable due to the short process time. The determination of just the colony forming units, in order to calculate $X_V(t)$, would result in a higher margin of error than using the total cell dry weight directly for this study (Equation 3).

$$OUR(t) = q_{O_2}(t) \cdot X(t) \tag{3}$$

$$\frac{dc_L}{dt} = OTR(t) - OUR(t) \tag{4}$$

$$\frac{dc_L}{dt} = k_L a(t) \cdot \left(c_L^*(t) - c_L(t)\right) - q_{O_2}(t) \cdot X(t) \tag{5}$$

If small interval boundaries are used in which the k_La value should be calculated, Equation 7 results after conversion of Equation 6. It becomes apparent that for each pO_2 value recorded online, an offline determined biomass value is necessary. In order to minimise the calculation error when using the difference quotient, the online recording of the pO_2 values should take place in a time interval of less than 1 minute. Since the biomass cannot be determined offline in a similarly short interval, it must be calculated at each of the timepoints from the online recorded values using the calculations listed later on (Equation 11 and 12).

$$\frac{c_L(t_i) - c_L(t_{i-1})}{t_i - t_{i-1}} = k_L a(t_i) \cdot \left(c_L^*(t_i) - c_L(t_i)\right) - q_{O_2}(t_i) \cdot X(t_i) \tag{6}$$

$$k_L a(t_i) = \frac{\frac{c_L(t_i) - c_L(t_{i-1})}{t_i - t_{i-1}} + q_{O_2}(t_i) \cdot X(t_i)}{\left(c_L^*(t_i) - c_L(t_i)\right)} \tag{7}$$

The O_2 equilibrium concentration $c_L^*(t)$ is considered to be constant throughout the entire cultivation period for simplicity. For the calculation, the O_2 solubility in water is assumed and the temperature dependence is taken into account with the aid of the Henry coefficient. Using the Henry coefficient H^θ at standard conditions (25 °C ≙ 298.15 K), a temperature constant C and a cultivation temperature of 37 °C, according to [4, 5], Equation 8 and the following values a suitable Henry coefficient of $H(T) = 9.6244 \cdot 10^{-6}$ mol·m^{-3}·Pa^{-1} can be obtained.

2 GUIDELINE – EXPERIMENTAL DETERMINATION OF BIOLOGICAL BIOREACTOR PERFORMANCE WITH AN ESCHERICHIA COLI MODEL PROCESS

$$H(T) = H^{\Theta} \cdot \exp\left(C \cdot \left(\frac{1}{T} - \frac{1}{T^{\Theta}}\right)\right) \quad (8)$$

H^{θ} = 1.2·10^{-5} mol·m^{-3}·Pa^{-1}

C = 1700 K

T^{θ} = 298.15 K

T = 310.15 K

Finally, the O_2 equilibrium concentration (in mmol·L^{-1}) in the medium can be calculated using the air pressure prevailing on site ($p_{on\ site}$ in Pa) and the molar fraction of O_2 in the air (x_{O_2} = 0.2096) according to Equation 9 [6].

$$c_L^*(t) = const. = c_{L,on\ site}^* = p_{on\ site} \cdot H(T) \cdot x_{O_2} \quad (9)$$

The actual O_2 concentration in the liquid phase $c_L(t)$ can be calculated with Equation 10, depending on the pO_2 value measured.

$$c_L(t) = \frac{pO_2(t) \cdot c_{L,on\ site}^*}{100\%} \quad (10)$$

Furthermore, the biomass concentration in the liquid phase *X(t)* is required. Since the measurement of the *DCW* can be very prone to error, an estimated OD_{600} is used for the determination. This can be calculated with Equation 11 at any time during cultivation. The required values for $OD_{600,\,t=0}$ and μ result from the exponential regression of the OD_{600} values measured hourly offline.

$$OD_{600,estimated} = OD_{600,t=0} \cdot \exp(\mu \cdot t) \quad (11)$$

Using the evaluated assumption that 0.333 g·L^{-1} *DCW* is equivalent to an OD_{600} of 1, the corresponding biomass concentration is obtained with Equation 12.

$$X(t) = 0.333 \cdot OD_{600,estimated}(t) \quad (12)$$

With these values, assuming a constant cell specific oxygen uptake rate during the entire cultivation time of

$$q_{O_2}(t) = const. = q_{O_2} = 16.66\ mmol \cdot g^{-1} \cdot h^{-1} \quad (13)$$

and after rearranging elements in Equation 4 and replacing variables, the current k_La value can be calculated according to Equation 14. In order to ensure comparability with the gassing-out method according to DECHEMA's recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods, the determination of the k_La value according to Equation 14 should only be performed with pO_2 values between 10 % and 90 % [1].

$$k_L a(t_i) = \frac{\frac{c_{L,t_i} - c_{L,t_{i-1}}}{t_i - t_{i-1}} + X_{t_i} \cdot q_{O_2}}{c^*_{L,on\ site} - c_{L,t_i}} \tag{14}$$

To reduce the complexity of the method described, the biomass concentration *X(t)* is assumed as a constant equivalent of the OD_{600} (Equation 12) and the cell-specific oxygen uptake rate is also assumed to be constant (Equation 13) as described above. To minimise the device error for measuring OD_{600}, Eppendorf® provided the BioPhotometer D30 for use during this project. However, those assumptions can also be made dynamically by measuring the *DCW* directly or by using an exhaust gas analyser to ascertain the values. A further assumption concerns the use of chemical antifoaming agents and their influence on the k_La value and the biology of the system. It is clear that the k_La may be affected by changes in a (the interfacial area) and/or changes in k_L (the mass transfer coefficient) through different possible effects [7]. Al-Masry described silicone-based antifoams as negatively affecting the mass transfer in air-lift reactors, due to enhanced bubble coalescence [8], while Routledge et al. described an increase of the k_La between 0.4 % to 0.6 % in shaking flasks using Antifoam A. Koch et al. found that antifoams without silicone oil only slightly affect the oxygen transfer [9], which is also described by Routledge et al. [7]. The proposed Antifoam 204 for this test is a mixture of non-silicone organic defoamers in a polyether dispersion, therefore we do not expect the oxygen transfer to be significantly influenced. Additionally, all participants in the interlaboratory tests operated under the same conditions, thus the influence of the antifoam agent or other influences such as the *CDW* calculated or oxygen solubility determined from literature were comparable amongst experiments, demonstrating that their effect is negligible.

Note: *All calculations are included in the available Excel tool.*

2.3 Verification of the biological performance evaluation method

The standardised *E. coli* model process for evaluating the biological bioreactor performance was extensively tested by the working group "Single-Use Microbial" of the DECHEMA expert group "Single-Use Technology in Biopharmaceutical Manufacturing" in interlaboratory tests to confirm the suitability of the method under real conditions. Single-use and reusable bioreactor systems from different manufacturers with different volumes, from laboratory scale to pilot scale, were investigated. The aim of the investigations was to obtain qualitative information on the reliability of the method using multiple determinations.

For this purpose, the various bioreactor systems were previously characterised according to DECHEMA's recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods [1]. Subsequently, the biological performance evaluation method was carried out at the gassing rates and stirrer speeds which led to the highest k_La value during the process engineering characterisation, since this ensures the highest *OTR* and thus the highest *OUR*.

2 GUIDELINE – EXPERIMENTAL DETERMINATION OF BIOLOGICAL BIOREACTOR PERFORMANCE WITH AN ESCHERICHIA COLI MODEL PROCESS

The investigations were carried out by the respective bioreactor manufacturers. The results obtained show meaningful and reproducible results (Figure 2). Maximum optical densities of ›2 – ›57 were achieved. The resulting k_La values from the biological evaluation method showed values from 32 h^{-1} to 1072 h^{-1} with, partially, strong deviations compared to the values from the process engineering characterisation. This can be explained by the response time of the pO_2 probe, which may not be sufficient when it comes to a rapid signal increase during the gassing-out method, among other reasons. Thus, the biologically determined k_La value might (sometimes) be considerably higher, since the cultivation process lasting several hours results in a slower pO_2 signal change. The position at which the pO_2 probe is installed also has a decisive effect on the k_La. As the biological determination of the k_La value depends on the biomass present, the resulting value may also be lower than the k_La value determined by the process engineering characterisation. The fact that there are zones in the bioreactor that may lead to poorer mixing and oxygen supply, and thus to reduced biomass growth, are also taken into account. These zones often cannot be taken into consideration in a single-point localised k_La measurement and are therefore difficult to detect, thus the resulting k_La value represents only a local approximation, often determined

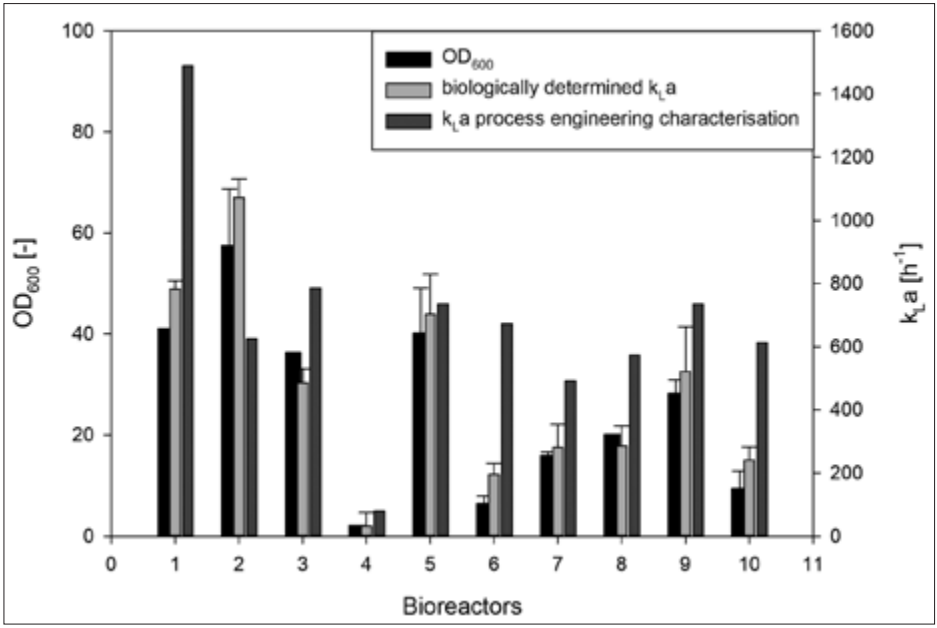


Figure 2: Optical cell densities measured at 600 nm and k_La values from biological characterisation as well as process engineering characterisation in various tested stirred and wave-mixed disposable and reusable bioreactors with working volumes ranging from 250 mL up to 300 L. The data was obtained from different bioreactor manufacturers using this guideline and the operating parameters leading to the highest k_La value. OD_{600}, optical density at 600 nm; k_La, volumetric mass transfer coefficient.

close to the stirrer. An overestimation can therefore be avoided by using a biological method. In addition, the biological characterisation enables the estimation of potentially required adjustments of the process strategy for a process transfer from one bioreactor system to another. This is particularly important for single-use bioreactors, as these systems must not be operated under high pressure, unlike many reusable systems, and require the use of pure oxygen. Furthermore, the assumptions made in this guideline, such as the calculation of the OD_{600} and *DCW*, the oxygen solubility in the medium, the specific oxygen uptake rate and mathematical inaccuracies (see section 2.2), which are all necessary for the determination of the k_La value, also have a direct influence on the results. However, the standardisation of the methods recommended here enables a cross-system comparison and an indication of a suitable field of application for a system. As a result, stirred bioreactors performed better than the wave-mixed systems investigated. Nevertheless, the latter systems are extremely well-suited for inoculum production.

Based on the results of the interlaboratory tests, the applicability of the standardised *E. coli* model process can be confirmed independently of the bioreactor system and cultivation volume.

2.4 Materials

Please be sure to have the following equipment on hand. The overview of chemicals (Table 1) and other equipment (Table 2) needed to ensure comparability is listed below. The formulation of the solutions and medium compositions needed for the experiment are described in the appendix.

2.4.1 Overview of needed general equipment

- Air supply
- Scale
- Bioreactor control system
- Bioreactor system
- Bunsen burner or comparable heat source to open the glass vial with the *E. coli* strain
- Computer-aided data acquisition
- Cryo preserved *E. coli* (Strain: W3110 thyA36 supO λ-; DSM: 5911)
- Cuvettes for the photometer
- Demineralised water
- Laminar flow bench
- Magnetic mixer
- Measuring cup/ volumetric flask
- Nitrogen supply
- Orbital incubator shaker (50 mm orbital diameter)
- Petri dish
- pH probe and control
- Photometer
- Pipettes
- pO_2 probe
- Preparation bottles (minimum 10 x 1 L Schott bottle)
- Tubings for transfer
- Weighing dish

2.4.2 Overview of needed chemicals and materials

The products given were used for the experiments. Products of same quality provided by other suppliers could be used as well.

Table 1: List of the chemicals required for the experiment

Chemical	Vendor / Item no.
$(NH_4)_2$H-citrat	Merck / 1154
$(NH_4)_2SO_4$	Roth / 9218.2
Agar	Sigma-Aldrich / A1296
Antifoam 204	Sigma-Aldrich / A6426
$CaCl_2$ x 2 H_2O	Roth / 5239.2
$CoCl_2$ x 6 H_2O	Merck / 1.02539
$CuSO_4$ x 5 H_2O	Roth / P024.1
D(+)-Glucose, water free	Merck / 1.08337.1000
$FeCl_3$ x 6 H_2O	Merck / 103943
K_2HPO_4	Roth / P749.3
LB-medium powder (Lennox)	Roth / X964.2
$MgSO_4$ x 7 H_2O	Riedel-de Haën / 13143
$MnSO_4$ x H_2O	Roth / 4487.1
Na_2-EDTA x 2 H_2O (Titriplex III)	Merck / 8421
Na_2SO_4	Fluka / 71962
NaCl	Sigma-Aldrich / 71383
NaH_2PO_4 x H_2O	Merck / 1.6346.1000
NH_4Cl	Merck / 1.01145.0500
NH_4OH, 28-30 % NH_3 basis	Sigma-Aldrich / 221228
$ZnSO_4$ x 7 H_2O	Roth / K301.1

Table 2: List of proposed materials

Material	Vendor / Item no.
125 mL disposable baffled shake flask	Corning / 431405
1000 mL disposable baffled shake flask	Corning / 431403

2.5 Experimental setup

2.5.1 Media preparation

Three different media are prepared, one for seed culture 1 (lysogeny broth-medium [LB-medium]), one for seed culture 2 and one for the main cultivation. The latter two are chemically defined media according to Biener et al. and have differing mineral salt and trace element concentrations [10]. Furthermore, a significantly higher glucose concentration is used for the main culture. Figure 3 illustrates the stock solution volumes used. A detailed explanation of the preparation is given in the appendix.

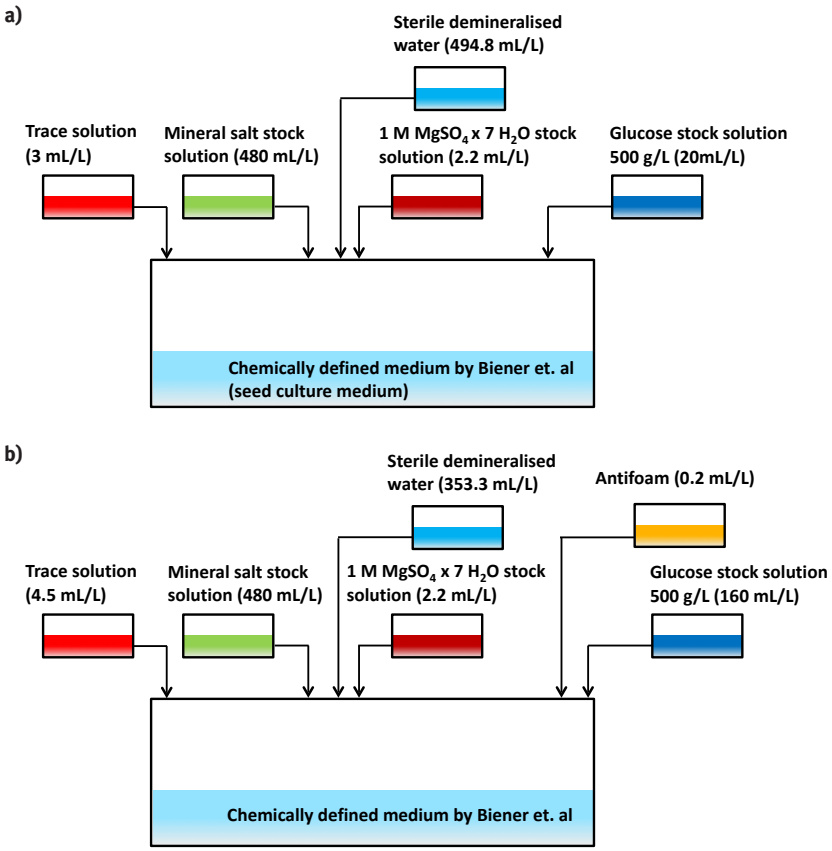


Figure 3: Concentrations and volumes needed for the seed and main culture preparation; (a) seed culture 2 medium, (b) medium used for the main culture.

2.6 Cultivation procedure

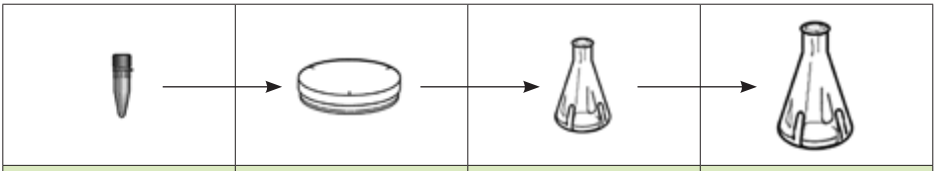
2.6.1 Seed culture

The seed culture preparation comprises several steps (Figure 4), starting from the cryopreserved culture of *E. coli* W3110 (glycerol-tryptic soy broth [glycerol-TSB] culture stored at -80 °C). The required cultivation media are prepared according to the instructions below. Ordering number of the strain is: DSM 5911. Due to mutation risk, please only make use of a freshly ordered strain.

In this document, the recommended procedure for seed culture preparation using baffled shake flasks with a total volume of 1000 mL and a shaking incubator with an orbital diamater of 50 mm is described. It is important to obtain a final optical density in seed culture 2 of $OD_{600} \approx 14$ and that the culture is in the exponential growth phase.

1. **Cryopreserved culture:**
   a) Thaw one cryovial in a waterbath at 37 °C.
2. **Agar plate:**
   a) Smear thawed culture on a LB-Agar plate using a sterile inoculation loop under the laminar flow.
   b) Incubate LB-Agar plate at 37 °C for 24 h.
3. **Seed culture 1:**
   a) Fill 20 mL of LB-medium into a 125 mL disposable baffled shake flask (Corning, 431405) under the laminar flow.
   b) Prewarm shake flask to 37 °C in a shaking incubator (alternatively prewarm medium in the water bath and transfer prewarmed medium into the shake flask).
   c) Transfer 1 colony from the LB-Agar plate into the LB-medium using a sterile inoculation loop under the laminar flow.
   d) Incubate shake flask at 37 °C in a shaking incubator with a 50 mm orbital diameter at 180 rpm for 8 h.
4. **Seed culture 2:**
   a) Fill 150 mL of Biener seed culture medium into a 1000 mL disposable baffled shake flask (Corning, 431403) under the laminar flow.
   b) Prewarm shake flask to 30 °C in a shaking incubator (alternatively prewarm medium in the water bath and transfer prewarmed medium into the shake flask).
   c) Transfer 3 mL of seed culture 1 into the Biener medium using a sterile pipette under the laminar flow. This results in an initial optical density of $OD_{600} = 0.1$ in the 1000 mL shake flask.
   d) Incubate shake flask at 30 °C in a shaking incubator with a 50 mm orbital diameter at 180 rpm for 16 h.

The optical density of seed culture 2 required for the transfer of the inoculum into the production bioreactor is $OD_{600} \approx 14$.



Cryogenic culture	LB-Agar Plate	LB-medium	Chemically defined seed culture medium
» Cryogenic culture of ***Escherichia coli* W3110** (DSM: 5911) used » Thawed to room temperature	» Single loop distributed on the LB-agar plate » Incubated at 37 °C for 24 hours	» First seed culture » 20 mL of LB-medium in 100 mL disposable baffled shake flask (Corning 431405) » Inoculated with a single colony » Incubated in shaking flask incubator at 37 °C and 180 rpm (50 mm orbital diameter) for nearly 8 hours	» Second seed culture » 150 mL of chemically defined Biener seed culture media in 1000 mL disposable baffled shake flask (Corning 431403) » Inoculated with $OD_{600} = 0.1$ » Incubated in shaking flask incubator at 30 °C and 180 rpm (50 mm orbital diameter) for 16 hours

Figure 4: Seed culture preparation. LB, lysogeny broth; OD_{600}, optical density at 600 nm.

Note: *To run the model process in production bioreactors with higher working volumes (e.g. 500 L bags), modified seed culture steps might be necessary, whereby the final optical density of the last seed culture should still be $OD_{600} \approx 14$. This could either mean an increased number of shake flasks in seed culture 2 or, alternatively, replacing the shake flasks in seed culture 2 by a (single-use) bioreactor (operated at 30 °C and a pO_2 setpoint of 35 %), to obtain pre-culture volume required.*

Note: *It is known that the also available American Type Culture Collection (ATCC, 27325) strain might present lower growth performace, so $OD_{600} \approx 14$ might be unattainable with the strain. Here, we recommend using the DSM (5911) strain.*

Note: *If your shaking incubator has an orbital diameter other than 50 mm, the ideal shaking frequency must be verified to obtain an* $OD_{600} \approx 14$ *in the seed culture 2 before starting the main culture. To scale the proposed orbital shaking diameter of 50 mm to your specific shaker, please consult the publications by Klöckner and Büchs [11, 12].*

2.6.2 Main culture

The model process is used to detect limitations of bioreactors. To achieve this, a challenging *E. coli* batch process with a high heat generation and oxygen demand is performed. For the cultivation, the setting should be used which achieved the highest k_La value during the process engineering characterisation (see DECHEMA guidelines, Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods). This setting should be kept constant throughout the entire cultivation. The inoculation volume (corresponding to an inoculation density of $OD_{600} = 1.0$) can be calculated with the Excel tool available. The temperature should be kept at 37 °C and the pH at 6.8 (by using 20 % ammonium hydroxide solution).

During the process, gassing is only performed with air (no extra addition of pure oxygen is allowed). The cultivation is stopped when the pO_2 is at 5 %, as a metabolic shift occurs due to oxygen-limiting conditions or when temperature increases above 37 °C. Based on the pO_2 decrease, cell density reached and temperature trend, assumptions about the bioreactor performance can be made.

Antifoam 204 (Sigma-Aldrich, Art. No.: A6426) is not part of the medium composition mentioned in the preparation instructions in the appendix and is added with a concentration of 0.2 mL/L to the bioreactor prior to the start of the cultivation. All additions during the cultivation (1:5 diluted) must be marked up and, finally, the total amount has to be transferred and added to the Excel sheet.

If the bioreactor has a pressure control (or over pressure mode), please highlight that in the results and run the process without pressure as a control.

Note: *Before starting the model process, make sure that all probes have been calibrated according to the manufacturer's instructions. A two-point calibration is required. For the* pO_2 *probe, calibration must be performed at zero-point (0 %) and 100 %.*

2.6.3 Process monitoring

Mandatory online parameters should be monitored and offline samples should be taken every hour during the process (Table 3) in order to ensure an adequate evaluation of the cultivations.

Table 3: Mandatory and optional analytical parameters have to be determined during the cultivation. *OD_{600}*, optical density at 600 nm.

OD_{600}	Offline	Mandatory
Dry cell weight	Offline	Mandatory
Wet cell weight	Offline	Optional
Glucose concentration	Offline	Mandatory
Acetate concentration	Offline	Mandatory
pO_2	Online	Mandatory
pH	Online	Mandatory
Temperature	Online	Mandatory
Exhaust gas analysis	Online	Optional
Volume Base (ammonium hydroxide)	Online	Mandatory
Volume Antifoam	Online	Mandatory

Note: *The optical density at 600 nm is measured using a photometer. The cultivation medium used serves as the blank value. If an OD_{600} value greater than 1.0 is reached, the sample must be diluted with 0.9 % sodium chloride solution 1:10 (100* µL *of the cell suspension with 900* µL *of the 0.9 % sodium chloride solution) or higher, depending on the expected value. For this purpose, however, the blank value must first be determined with 0.9 % sodium chloride solution on the photometer.*

Note: *The analysis of dry cell weight, glucose concentration and acetate concentration must be performed depending on the method available.*

Note: *When taking a sample, discard the first few milliliters to rinse the transfer line. Afterwards, the actual sample can be taken. In addition, an aseptic sampling must be ensured in order to not contaminate the running process.*

Note: *It is recommended to perform all offline analyses in duplicate.*

2.7 Evaluation

An Excel tool is available for the data evaluation of the model process (download at www.dechema.de or contact the corresponding author). It is recommended that all major results of the model process are summarised in a table and that the bioreactor performance based on all these values (Table 4), and not only a single value, are discussed. An example of a small scale cultivation in a single-use bioreactor is described below. Here, an OD_{600} of 30.3 (dry cell weight = 11.1 g/L) was achieved before the pO_2 dropped to 5 % (Figure 5). The other process relevant parameters, such as pH and temperature, remained constant for the entire process. A k_La of 795 ± 27 h^{-1} was determined based on the pO_2 decrease during the fermentation. Exemplary data evaluation for an *E. coli* process in a small scale single-use bioreactor are shown in Figure 6.

Table 4: Example of an evaluation table for the major results of the model process. OD_{600}, optical density at 600 nm; k_La, volumetric mass transfer coefficient.

Parameter	k_La process engineering [h^{-1}]	k_La model process [h^{-1}]	Final OD_{600} [-]	Process duration [h]	Additional comments - Temperature increase? - Acetate? - Offgas data?	Comments
Bioreactor 1						
Bioreactor 2						

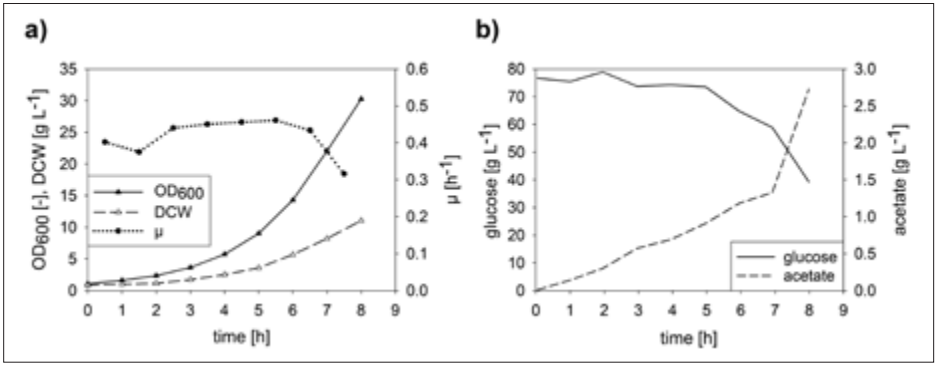


Figure 5: Characteristics of OD_{600}, the *DCW* and the specific growth rate (a) as well as of the metabolites glucose and acetate (b). OD_{600}, optical density at 600 nm; μ, specific growth rate.

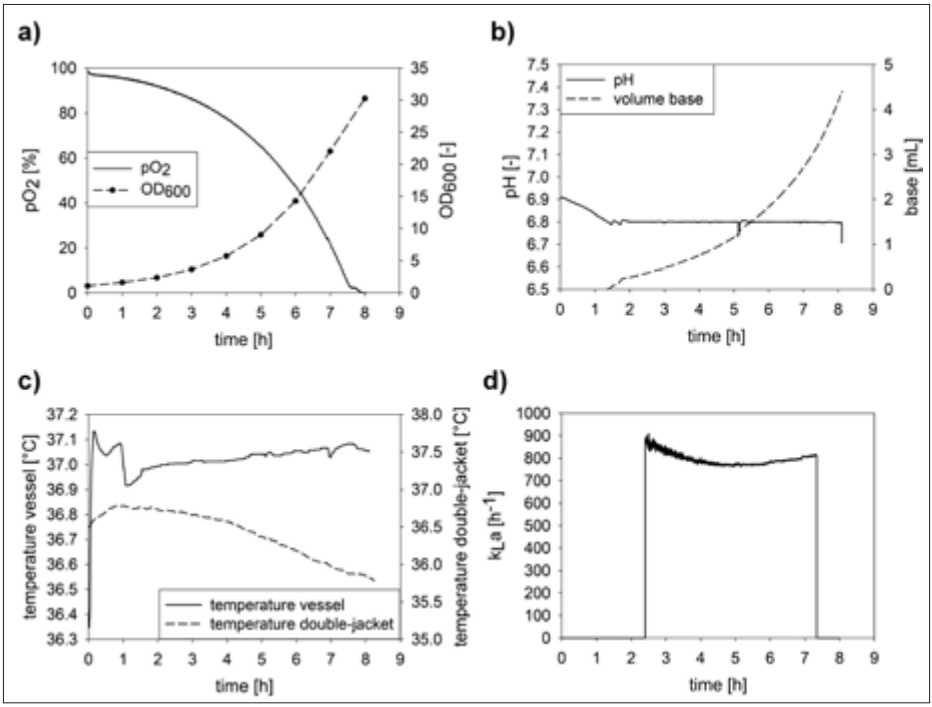


Figure 6: Characteristics of the pO_2 (a), pH characteristics and volume of the added base (b), temperature control (c) and the k_La value determined from the pO_2 decrease during the fermentation (d). OD_{600}, optical density at 600 nm; k_La, volumetric mass transfer coefficient.

3 Acknowledments

The authors would like to thank all participating companies and, in particular, Eppendorf for making the photometers available for all working groups. Special thanks go to Katharina Blaschczok (Roche), Sebastian Rothe (GE Healthcare Europe GmbH), Sebastian Kleebank (Eppendorf AG) and Nephi Jones (Thermo Fisher Scientific) for their technical support.

4 References

[1] Meusel W, Löffelholz C, Husemann U, Dreher T, Greller G, Kauling J, et al. Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods. Frankfurt am Main: DECHEMA; 2016. 31 p. ISBN: 978-3-89746-171-0

[2] Adler I, Fiechter A. Charakterisierung von Bioreaktoren mit biologischen Testsystemen. Chemie Ing Tech. 1983;55(4):322–3. DOI: 10.1002/cite.330550422

[3] Wagner B. Leistungsvergleich von Bioreaktoren für mycelförmiges Wachstum. ETH Zürich; 1987. DOI: 10.3929/ethz-a-000412561

[4] Sander R. Compilation of Henry's law constants (version 4.0) for water as solvent. Atmos Chem Phys [Internet]. 2015 Apr 30;15(8):4399–981. DOI: 10.5194/acp-15-4399-2015

[5] Warneck P, Williams J. The Atmospheric Chemist's Companion. Dordrecht: Springer Netherlands; 2012. DOI: 10.1007/978-94-007-2275-0

[6] VDI-Gesellschaft. VDI-Wärmeatlas. 10. Auflag. Springer Berlin Heidelberg; 2006. 1512 p.

[7] Routledge SJ, Hewitt CJ, Bora N, Bill RM. Antifoam addition to shake flask cultures of recombinant *Pichia pastoris* increases yield. Microb Cell Fact [Internet]. 2011;10(17):1–11. DOI: 10.1186/1475-2859-10-17

[8] Al-Masry WA. Effects of antifoam and scale-up on operation of bioreactors. Chem Eng Process Process Intensif [Internet]. 1999 May;38(3):197–201. DOI: 10.1016/S0255-2701(99)00014-8

[9] Koch V, Rüffer H-M, Schügerl K, Innertsberger E, Menzel H, Weis J. Effect of antifoam agents on the medium and microbial cell properties and process performance in small and large reactors. Process Biochem [Internet]. 1995 Jan;30(5):435–46. DOI: 10.1016/0032-9592(94)00029-8

[10] Biener R, Steinkämper A, Hofmann J. Calorimetric control for high cell density cultivation of a recombinant *Escherichia coli* strain. J Biotechnol. 2010 Mar;146(1–2):45–53. DOI: 10.1016/j.jbiotec.2010.01.004

[11] Klöckner W, Büchs J. Advances in shaking technologies. Trends Biotechnol [Internet]. 2012 Jun;30(6):307–14. DOI: 10.1016/j.tibtech.2012.03.001

[12] Klöckner W, Gacem R, Anderlei T, Raven N, Schillberg S, Lattermann C, et al. Correlation between mass transfer coefficient kLa and relevant operating parameters in cylindrical disposable shaken bioreactors on a bench-to-pilot scale. J Biol Eng [Internet]. 2013;7(28):14. DOI: 10.1186/1754-1611-7-28

5 Abbreviations

ATCC	American Type Culture Collection
DSM	Leibnitz Institute DSMZ-German Collection of Mircoorganisms and Cell Cultures
LB	Lysogeny broth
TSB	Tryptic soy broth

6 Symbols used

a	Interfacial area
c_L	Actual O_2 concentration in the liquid phase
c_L^*	O_2 equilibrium concentration in the liquid phase
C	Temperature constant
CER	Carbon dioxide formation rate
DCW	Dry cell weight
H	Henry coefficient
H^Θ	Henry coefficient at standard conditions
k_L	Mass transfer coefficient
k_La	Volumetric mass transfer coefficient
OD_{600}	Optical density at 600 nm
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
$p_{on\ site}$	Air pressure on site
q_{O_2}	Cell specific oxygen uptake rate
T	Temperature
T^Θ	Temperature at standard conditions
WCW	Wet cell weight
x_{O_2}	Molar fraction of O_2 in air
X	Total cell mass
X_V	Viable cell mass
μ	Specific growth rate

7 Appendix

Please take the necessary and relevant precautions when working with chemical and biological substances. The chemicals given were used for the experiments. Chemicals of same quality provided by other suppliers could be used as well.

7.1 Preparation of LB-medium (Lennox) (1 L)

Chemicals required

20 g	LB-medium powder (Lennox)	Roth, Item no.: X964.2

Preparation

1. Weigh out 20 g LB-medium powder and transfer it into a Schott bottle.
2. Add a magnetic stirrer bar.
3. Fill the bottle to the 1 L mark with demineralised water and homognise by stirring.
4. Autoclave the solution at 121 °C for 20 minutes.
5. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The LB-medium can be stored at 4 °C.
Shelf life: 3 months.

7.2 Preparation of LB-agar (Lennox) (1 L)

Chemicals required

20 g	LB-medium powder (Lennox)	Roth, Item no.: X964.2
15 g	Agar	Sigma-Aldrich, Item no.: A1296

Preparation

1. Weigh out 20 g LB-medium powder and transfer it into a Schott bottle.
2. Weigh out 15 g Agar powder and transfer it into the same Schott bottle.
3. Add a magnetic stirrer bar.
4. Fill the bottle to the 1 L mark with demineralised water and homognise by stirring.
5. Autoclave the solution at 121 °C for 20 minutes.
6. Let the solution cool just enough to be able to handle the flask.
7. In a laminar flow chamber, pour approximately 25-30 mL of the LB-agar per sterile petri dish.
8. Allow the plates to set in the laminar flow chamber with lids slightly opened.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The LB-agar plates can be stored inverted at 4 °C.
Shelf life: 3 months.

7.3 Preparation of a 50 % glucose stock solution (1 L)

Chemicals required

500 g	D(+)-Glucose, water free	Merck, Item no.: 1.08337.1000

Preparation

1. Heat up 300 mL demineralised water to 90 °C in a beaker containing a magnetic stirrer bar.
2. Weigh out 500 g anhydrous D(+)-Glucose.
3. Transfer the glucose slowly into the heated water.
4. Rinse the vessel which contained the glucose with demineralised water.
5. Transfer the demineralised water used for rinsing into the glucose solution.
6. Heat and mix the solution until the whole glucose is dissolved.
7. Transfer the solution into a 1 L volumetric flask and fill it up to the 1000 mL mark using demineralised water.
8. Transfer the solution into a 1 L Schott bottle.
9. Autoclave the solution (121 °C, 20 min).
10. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The glucose solution can be stored at 4 °C.

Shelf life: 1 year.

7.4 Preparation of mineral salt stock solution by Biener et. al (1 L)

Chemicals required

2.1 g	$(NH_4)_2$H-citrat	Merck, Item no.: 1154
4.2 g	Na_2SO_4	Fluka, Item no.: 71962
8.4 g	$(NH_4)_2SO_4$	Roth, Item no.: 9218.2
1.06 g	NH_4Cl	Merck, Item no.: 1.01145.0500
31.6 g	K_2HPO_4	Roth, Item no.: P749.3
7.4 g	NaH_2PO_4 x H_2O	Merck, Item no.: 1.6346.1000

Preparation

1. Fill approximately 600 mL demineralised water into a beaker with a magnetic stirrer bar.
2. Weigh chemicals (analytical balance) and transfer them into the beaker.
3. Solve the chemicals by mixing (magnetic stirrer).
4. Transfer the solution into a 1 L volumetric flask and fill it up to the 1000 mL mark using demineralised water.
5. Transfer the solution to a 1 L Schott bottle and autoclave the solution (121°C, 20 min).
6. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The trace solution can be stored at 4 °C.
Shelf life: 1 year.

7.5 Preparation of trace solution by Biener et. al (1 L)

Chemicals required

0.18 g	$CoCl_2$ x 6 H_2O	Merck, Item no.: 1.02539
0.50 g	$CaCl_2$ x 2 H_2O	Roth, Item no.: 5239.2
0.18 g	$ZnSO_4$ x 7 H_2O	Roth, Item no.: K301.1
0.10 g	$MnSO_4$ x H_2O	Roth, Item no.: 4487.1
10.05 g	Na_2-EDTA x 2 H_2O (Titriplex III)	Merck, Item no.: 8421
8.35 g	$FeCl_3$ x 6 H_2O	Merck, Item no.: 103943
0.16 g	$CuSO_4$ x 5 H_2O	Roth, Item no.: P024.1

Preparation

Caution: The weighing of $CoCl_2$ x 6 H_2O should only performed by trained staff underneath the laboratory exhaust hood.

1. Fill approximately 700 mL demineralised water into a beaker and add a magnetic stirrer bar.
2. Weigh $CoCl_2$ x 6 H_2O (by trained staff under a laboratory exhaust hood) using the special accuracy balance.
3. Weigh $CaCl_2$ x 2 H_2O, $ZnSO_4$ x 7 H_2O, $MnSO_4$ x H_2O and $CuSO_4$ x 5 H_2O by using the special accuracy balance.
4. Weigh Na_2-EDTA x 2 H_2O and $FeCl_3$ x 6 H_2O using the analytical balance.
5. Transfer all substances into the flask and dissolve them by mixing (magnetic stirrer).
6. Transfer the solution into a 1 L volumetric flask and fill it up to the 1000 mL mark with demineralised water.
7. Transfer the solution into a 1 L Schott bottle and autoclave the solution (121 °C, 20 min)
8. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The trace solution can be stored at 4 °C.
Shelf life: 1 year.

7.6 Preparation of 1 M $MgSO_4$ x 7 H_2O stock solution (1 L)

For the preparation of the media by Biener et al., a $MgSO_4$ x 7 H_2O stock solution is used. It is a component of the seed culture medium, the defined medium and feed solution.

Chemicals required

246.5 g	$MgSO_4$ x 7 H_2O	Riedel-de Haën, Item no.: 13143

Preparation

1. Fill 500 mL demineralised water into a beaker and add a magnetic stirrer bar.
2. Weigh $MgSO_4$ x 7 H_2O (analytical balance), transfer it into the beaker and dissolve by mixing (magnetic stirrer).
3. Transfer the solution into a 1 L volumetric flask and fill it up to the 1000 mL mark using demineralised water.
4. Transfer the solution to a 1 L Schott bottle and autoclave the solution (121 °C, 20 min).
5. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The $MgSO_4$ x 7 H_2O stock solution can be stored at 4 °C.
Shelf life: 1 year.

7 APPENDIX

7.7 Preparation of defined main culture medium (80 g/L glucose) by Biener et. al (1 L)

For high cell density fermentations, a minimal medium by Biener et al. 2010 is used.

Chemicals required

480 mL	Mineral salt stock solution by Biener et. al
4.5 mL	Trace solution by Biener et. al
2.2 mL	1 M $MgSO_4$ x 7 H_2O stock solution
160 mL	Glucose stock solution (500 g/L)

Preparation

1. Fill the following sterile solutions into an autoclaved 1 L Schott bottle which contains a magnetic stirrer bar under sterile conditions.
   a) 480 mL sterile mineral salt stock solution
   b) 4.5 mL sterile trace solution
   c) 2.2 mL sterile $MgSO_4$ x 7 H_2O stock solution
   d) 160 mL sterile 50 % glucose stock solution
2. Fill up to the 1 L mark using sterile demineralised water.
3. Mix the solution carefully with the magnetic stirrer.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The minimal medium can be stored at 4 °C.
Shelf life: 1 month.

7.8 Preparation of defined seed culture 2 medium (10 g/L glucose) by Biener et. al (1 L)

For high cell density fermentations, a minimal medium by Biener et al. 2010 is used.

Chemicals required

480 mL	Mineral salt stock solution by Biener et. al
3 mL	Trace solution by Biener et. al
2.2 mL	1 M $MgSO_4$ x 7 H_2O stock solution
20 mL	Glucose stock solution (500 g/L)

Preparation

1. Fill the following sterile solutions into an autoclaved 1 L Schott bottle which contains a magnetic stirrer bar under sterile conditions.
   a) 480 mL sterile mineral salt stock solution
   b) 3 mL sterile trace solution
   c) 2.2 mL sterile $MgSO_4$ x 7 H_2O stock solution
   d) 20 mL sterile 50 % glucose stock solution
2. Fill up to the 1 L mark using sterile demineralised water
3. Mix the solution carefully with the magnetic stirrer.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The minimal medium can be stored at 4 °C.
Shelf life: 1 month.

7.9 Preparation of 20 % ammonium hydroxide solution (1 L)

Chemicals required

670 mL	NH_4OH, 28-30 % NH_3 basis	Sigma-Aldrich, Item no.: 221228

Preparation

1. Fill approximately 670 mL NH_4OH into an autoclaved 1 L Schott bottle which already contains 330 mL sterile demineralised water.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The 20 % ammonium hydroxide solution can be stored at 4 °C.
Shelf life: 1 year.

7 APPENDIX

7.10 Preparation of 0.9 % sodium chloride solution (1 L)

Chemicals required

9 g	NaCl	Sigma-Aldrich, Item no.: 71383

Preparation

1. Weigh out 9 g NaCl and transfer it into a 1 L Schott bottle.
2. Add a magnetic stirrer bar.
3. Fill up to 1 L with demineralised water and solve by stirring.
4. Autoclave the solution at 121 °C for 20 minutes.
5. Let the solution cool down to room temperature and check the filling volume after autoclaving; if necessary, fill up with sterile demineralised water under the laminar flow.

Please note: *The Schott bottle has to be labeled with the content, date of preparation, shelf life and signature!*

The 0.9 % sodium chloride solution can be stored at room temperature.
Shelf life: 3 months.

7.11 Calculations for the evaluation of exhaust gas analysis (optional)

Changes in the metabolic processes in a culture are usually very sensitively reflected by the respiratory quotient (RQ). The RQ can be calculated from the data of the exhaust gas analysis (O_2 and CO_2 content), the gassing rate and the bioreactor volume. The following calculations are also integrated in the Excel tool.

From the mass balance for oxygen in the gas phase, results on the assumption of a quasi-stationary state:

$$\left(\frac{\dot{V}_G}{V_M}\right)^{In} x_{O_2}{}^{In} - \left(\frac{\dot{V}_G}{V_M}\right)^{Out} x_{O_2}{}^{Out} = OUR \cdot V_F \qquad \text{(A1)}$$

Oxygen amount in the inlet air	–	Oxygen amount in the exhaust air	=	Oxygen consumption in the medium

Analogue to the oxygen mass balance, the equation for carbon dioxide can be derived:

$$\left(\frac{\dot{V}_G}{V_M}\right)^{Out} x_{CO_2}{}^{Out} - \left(\frac{\dot{V}_G}{V_M}\right)^{In} x_{CO_2}{}^{In} = CER \cdot V_F \qquad \text{(A2)}$$

CO_2 amount in the exhaust air	–	CO_2 amount in the inlet air	=	Carbon dioxide formation in the medium

Thereby the following definition applies to the molar volume of the gases (V_M).

$$V_M = \frac{R \cdot T_{gas,in}}{p_{bioreactor}} \qquad \text{(A3)}$$

The exhaust air volume flow, which does not have to be equal to the inlet gassing rate due to oxygen consumption and carbon dioxide formation, can be eliminated with the help of the nitrogen balance since nitrogen is not formed or consumed.

$$\left(\frac{\dot{V}_G{}^{In}}{V_M}\right) x_{N_2}{}^{In} = \left(\frac{\dot{V}_G{}^{Out}}{V_M}\right) x_{N_2}{}^{Out} \quad (A4)$$

Nitrogen amount in the inlet air = Nitrogen amount in the exhaust air

Since the mole fractions in a gas are equal to one, the following equations apply to the inlet and exhaust air:

$$x_{N_2}{}^{In} = 1 - x_{O_2}{}^{In} - x_{CO_2}{}^{In} \quad (A5)$$

$$x_{N_2}{}^{Out} = 1 - x_{O_2}{}^{Out} - x_{CO_2}{}^{Out} \quad (A6)$$

By inserting equations A5 and A6 into equation A4, transforming and inserting into equation A1 the OUR can be obtained.

$$OUR = \frac{\dot{V}_G{}^{In}}{V_F V_M}\left(x_{O_2}{}^{In} - \frac{1 - x_{O_2}{}^{In} - x_{CO_2}{}^{In}}{1 - x_{O_2}{}^{Out} - x_{CO_2}{}^{Out}} x_{O_2}{}^{Out}\right) \quad (A7)$$

Analogous to equation A7 the CER can be obtained:

$$CER = \frac{\dot{V}_G{}^{In}}{V_F V_M}\left(x_{CO_2}{}^{Out}\frac{1 - x_{O_2}{}^{In} - x_{CO_2}{}^{In}}{1 - x_{O_2}{}^{Out} - x_{CO_2}{}^{Out}} - x_{CO_2}{}^{In}\right) \quad (A8)$$

The RQ is the ratio of CER and OUR.

$$RQ = \frac{CER}{OUR} \quad (A9)$$

Symbols used:

OUR	Oxygen uptake rate
CER	Carbon dioxide formation rate
x	Mole fraction
V_F	Fluid volume
V_M	Molar volume of gases
R	Gas constant (8.314 J·mol^{-1}·K^{-1})
$T_{gas,\ in}$	Gas inlet temperature
$P_{bioreactor}$	Pressure in bioreactor (mostly ambient pressure)

Indices used:

CO_2	Carbon dioxide
G	Gas
N_2	Nitrogen
O_2	Oxygen

Superscripts used:

In	Inlet
Out	Outlet

Gesellschaft für Chemische Technik
und Biotechnologie e.V.
Theodor-Heuss-Allee 25
60486 Frankfurt am Main

Phone: +49 69 7564-0
Fax: +49 69 7564-201
E-mail: info@dechema.de

www.dechema.de

ISBN: 978-3-89746-222-9