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ORIGINAL PAPER

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The oxygen transfer rate as key parameter for the characterization of *Hansenula polymorpha* screening cultures

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Abstract Screening cultures are usually non-monitored and non-controlled due to a lack of appropriate measuring techniques. A new device for online measurement of oxygen transfer rate (OTR) in shaking-flask cultures was used for monitoring the screening of *Hansenula polymorpha*. A shaking frequency of 300 rpm and a filling volume of 20 ml in 250-ml flasks ensured a sufficient oxygen transfer capacity of 0.032 mol $(l\ h)^{-1}$ and thus a respiration not limited by oxygen. Medium buffered with 0.01 mol phosphate l^{-1} (pH 6.0) resulted in pH-inhibited respiration, whereas buffering with 0.12 mol phosphate l^{-1} (pH 4.1) resulted in respiration that was not inhibited by pH. The ammonium demand was balanced by establishing fixed relations between oxygen, ammonium, and glycerol consumption with 0.245 ± 0.015 mol ammonium per mol glycerol. Plate precultures with complex glucose medium reduced the specific growth rate coefficient to 0.18 h^{-1} in subsequent cultures with minimal glycerol medium. The specific growth rate coefficient increased to 0.26 h^{-1} when exponentially growing precultures with minimal glycerol medium were used for inoculation. Changes in biomass, glycerol, ammonium, and pH over time were simulated on the basis of oxygen consumption.

Keywords *Hansenula polymorpha* · Material balance
Oxygen transfer rate · Screening condition · Shaking culture

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Introduction

During the last several years, the development of eukaryotes as efficient systems for the production of recombinant pharmaceutical proteins has been the subject of extensive research [1, 2, 3, 4, 5]. Efforts have focused on screening suitable strains for their efficient industrial-scale production capabilities [6]. In these screening processes, shaking bioreactors have been widely used as they are inexpensive and large numbers of reactors can easily be handled in parallel [7]. Indeed, in the field of biotechnology, up to several hundred-thousand individual experiments per company are run every year in shaking bioreactors [8]. Nevertheless, the conditions in these cultures are not well understood. One reason is the lack of appropriate measuring methods for cultures in shaking bioreactors [7, 9]. Consequently the design of screening conditions is frequently based on insufficient empirical results and phenomenological observations. When culture conditions are unsuitable, highly productive strains may remain unidentified, reducing the potential of the developed bioprocess. Therefore, both the characterization and the careful layout of the screening conditions in shaking bioreactors are crucial.

We have recently developed a device for the sterile and non-invasive online measurement of the oxygen transfer rate (OTR) in shaking bioreactors. This device provides an appropriate tool for characterizing screening conditions [10].

In aerobic cultures, almost every physiological activity is coupled to the respiratory uptake of oxygen, making the OTR an excellent indicator of metabolic activity. Physiological responses of aerobic microorganisms to specific culture conditions such as oxygen limitation, nutrient limitation, and inhibiting factors are reflected by the OTR [10].

In the present study a screening project for evaluating recombinant *Hansenula polymorpha* strains was characterized and evaluated by means of the online measurement of the OTR. This yeast is used in the industrial

production of recombinant proteins, such as hepatitis surface antigen vaccines [11]. During screening, suitable production strains were selected from several hundred recombinant clones [12, 13].

Materials and methods

Strains

H. polymorpha wild-type CBS4732, the uracil-auxotrophic host strain RB11 [13], and two RB11-based recombinant strains were studied. The selected recombinant strains produced either human interferon (IFN)-α-2a or human chymotrypsinogen. The physical map of the respective expression vector has been presented elsewhere [14, 15]. Recombinant strains were selected by complementing the uracil-auxotrophy with a plasmid-bound *URA3* gene. The two recombinant strains contained multiple copies of the heterologous expression cassettes, which were integrated into the chromosomes mitotically stable. Heterologous gene expression was controlled by the formate dehydrogenase (*FMD*) promoter in the case of the IFNα-2a-producing strain, and by the methanol oxidase (*MOX*) promoter in the chymotrypsinogen-producing strain. Thus, in both cases elements derived from genes of the methanol utilization pathway and active at glycerol concentrations lower than 4 g l^{-1} were used [16].

General culture conditions

All cultures were shaken on an orbital shaker (LabShaker, Kühner, Basel, Switzerland) with a 25 mm shaking diameter. Cultivations were carried out at 37 °C in unbaffled 250-ml Erlenmeyer flasks with cotton plugs or 250-ml measuring flasks [10]. Cultures were shaken, if not otherwise specified, at a standard shaking frequency of 300 rpm and a standard filling volume of 20 ml. Cultures in standard Erlenmeyer flasks were run parallel to the cultures in measuring flasks and used for off-line analysis of culture parameters during the fermentations.

Preculture

The liquid precultures were inoculated from a yeast extract/peptone dextrose (YPD) plate culture of the respective strain that had been incubated for 2 days at 37 °C. The YPD plate contained (per liter): 20 g glucose monohydrate, 20 g gelatone (Becton Dickinson, Franklin Lakes, N.J., USA), 10 g yeast extract (Becton Dickinson) and 18 g agar. Unless otherwise specified, the strains were precultured in the medium used for the main culture. Exponentially growing precultures were harvested for inoculation at OD_{600} = 8.

Main culture

The main cultures were grown in three different minimal media: SYN 6, a synthetic medium (Rhein Biotech, Duesseldorf, Germany) and two differently buffered yeast nitrogen base (YNB) media. SYN 6 (pH 4.1) contained (per liter): 15 g glycerol, 13.3 g $NH_4H_2PO_4$, 3.3 g KCl, 3 g $MgSO_4{\cdot}7H_2O$, 0.3 g NaCl, 1 g $CaCl_2$, 66.7 mg $(NH_4)_2Fe(SO_4)_2{\cdot}6\ H_2O$, 5.3 mg $CuSO_4{\cdot}5\ H_2O$, 20 mg $ZnSO4{\cdot}7H_2O$, 26.7 mg $MnSO_4{\cdot}H_2O$, 66.7 mg EDTA (Titriplex III, Merck, Darmstadt, Germany), 0.4 mg D-biotin, 133.3 mg thiaminchloride-hydrochloride, 0.7 mg $NiSO_4{\cdot}6\ H_2O$, 0.7 mg $CoCl_2{\cdot}6\ H_2O$, 0.7 mg H_3BO_3, 0.7 mg KJ, 0.7 mg $Na_2MoO_4{\cdot}2\ H_2O$. Ammonium-limited SYN 6 medium was obtained by replacing $NH_4H_2PO_4$ with NaH_2PO_4. YNB 1 medium (pH 6.0, 0.01 mol phosphate l^{-1}) contained (per liter): 15 g glycerol, 5 g $(NH_4)_2SO_4$ and 1.4 g YNB without ammonium sulfate and amino acids (Becton Dickinson). The pH was titrated to 6.0 with sulfuric acid (30%, v/v). YNB 2 medium (pH 4.1, 0.12 mol phosphate l^{-1}) contained (per liter): 15 g glycerol, 13.3 g $NH_4H_2PO_4$ and 1.4 g YNB without ammonium sulfate and amino acids (Becton Dickinson).

If not otherwise specified, SYN 6 main cultures were inoculated with preculture broth using one-tenth of the final culture volume. For inoculation of the YNB main cultures, preculture broth was centrifuged (1000 *g*, 10 min) and the cells were resuspended in one-third of the original preculture broth volume using 9 g NaCl l^{-1}. One-fifteenth of the cell resuspension was used to inoculate the main culture.

Measurement of the OTR

The device for online measurement of the OTR in shaking flasks was described in [10]. The cultivations were conducted in specially designed measuring flasks, ensuring the same hydrodynamics and gas phase conditions as for standard Erlenmeyer flask cultures with cotton plugs. OTR is measured by periodically repeating an automated cycle. During the rinsing phase (20 min), the headspace of the measuring flasks is continuously flushed with air. Subsequently, the headspace is closed air-tight for 10 min (measuring phase). During this phase, the oxygen partial pressure of the gas phase decreases proportionally to the sustained respiration of the liquid culture. The decline of the gaseous oxygen partial pressure is measured and incorporated into the calculation of the OTR [mol $(l\ h)^{-1}$]. Afterwards, a new measuring cycle is initiated by flushing the headspace with fresh air in order to regenerate the gas phase.

Standard analytics

The biomass was determined as cell dry weight by filtering 8 ml culture broth through dried and pre-weighed cellulose acetate filters, pore size 0.2 μm (11107-47-N, Sartorius, Göttingen, Germany). The filter residue was resuspended once in 9 g NaCl l^{-1}, filtered again and dried on the filter at 105 °C until the mass remained constant. The presence of ammonium was determined using an ammonium-sensitive electrode (800-323-4340, Cole-Parmer, Vernon Hills, Ill., USA). Prior to the measurements the electrode was calibrated with several solutions of defined ammonium concentration and ionic strength. The filtered culture broth samples were diluted 1:30 (v/v) in deionized water and adapted to the ionic strength of the calibration solutions with sodium chloride. The IFNα-2a produced was analyzed by HPLC (System Gold, Beckman, Fullerton, Calif., USA). Chromatography conditions were reversed-phase column C18, 250-4 mm (Macherey-Nagel, Düren, Germany), temperature 35 °C and gradient elution with acetonitrile (30–90%, v/v). Protein was detected photometrically at 214 nm (Variable Wavelength Detector 165, Beckman). Glycerol was determined using a standard enzymatic UV test kit (148270, Roche Diagnostics, Mannheim, Germany).

Results and discussion

The influence of oxygen supply and effects of pH and nutrients on microbial cultures is well-known. For this reason, the adjustment of suitable screening conditions is vital. Nevertheless, this is often neglected due to the lack of adequate measuring techniques for shaking cultures. Therefore, the online measurement of OTR in shaking flasks [10] is a valuable tool for qualitative and quantitative analysis of the screening for recombinant *H. polymorpha*.

Oxygen supply

The maximum possible oxygen uptake rate of an aerobic culture is limited by the maximum oxygen transfer capacity (OTR_{max}) [17]. In shaking-flask cultures, OTR_{max} strongly depends on the surface area of the gas-liquid interface of the rotating liquid and its velocity. These parameters are influenced by the operating conditions imposed: shaking frequency, shaking diameter, flask size and shape, and the liquid culture volume [17].

Figure 1 shows the OTR profiles of *H. polymorpha* cultures in SYN 6 medium under different operating conditions, varying the filling volume and the shaking frequencies. A large filling volume of 83 ml and a low shaking frequency of 140 rpm resulted in an extremely low OTR of about 0.002 mol $(l\ h)^{-1}$. The oxygen uptake rate of the culture was limited by the low OTR_{max} at the defined operating conditions. Even after 40 h, cultivation was not complete. By halving the filling volume to 40 ml and by increasing the shaking frequency to 300 rpm, a higher OTR_{max} was observed. Nevertheless, the culture respiration was still limited to an OTR of 0.019 mol $(l\ h)^{-1}$, indicated by the plateau between hours 24 and 34. By reducing the filling volume to 20 ml, an OTR_{max} of about 0.032 mol $(l\ h)^{-1}$ was achieved, deduced from a theoretical correlation of [18]. As the maximum OTR of 0.028 mol $(l\ h)^{-1}$ was below this value, these operating conditions resulted in a non-limiting oxygen supply. The OTR profile was typical for non-limited growth of *H. polymorpha* in SYN 6 medium with glycerol as the sole carbon source. The OTR increased with increasing biomass due to the aerobic turnover of glycerol. At hour 25, glycerol was depleted and the OTR dropped to zero, indicating that the culture had reached stationary. The exhaustion of glycerol was verified by analysis of the culture broth (data not shown). The total oxygen consumption, as calculated by integration of the OTR over the fermentation time, could be correlated with glycerol consumption. The results of the experiments presented in Figs. 1, 2, 3, 4, and 5 demonstrate a constant relation between glycerol and oxygen consumption of $R_{Glyc/O_2} = 0.50 \pm 0.03$mol glycerol per mol oxygen, independent of the varying culture conditions.

The two cultures shown in Fig. 1, shake-incubated at 300 rpm and with filling volumes of 20 and 40 ml, led to similar yields of $Y_{X/Glyc}$ = 33.1 g biomass per mol glycerol and $Y_{X/Glyc}$ = 33.7 g biomass per mol glycerol. No by-products could be detected by HPLC (data not shown) due to the non-fermentability of glycerol. The oxygen limitation of *H. polymorpha* in SYN 6 medium solely reduced the fermentation rate without further influencing metabolism.

The varying OTR_{max} of the cultures resulted in varying fermentation times. This is of significance for the schedule of culture harvesting and product inspection. Heterologous gene expression of *H. polymorpha* usually commences close to the stationary phase; at levels lower than 4 g glycerol l^{-1} until depletion of glycerol the active status of the promoter is maintained [16]. Thus, the pattern of gene expression would vary according to the different culture conditions given in Fig. 1, and a conclusive and reproducible proof of the production capabilities of the examined strains would not be ensured. Therefore an overall equalized oxygen supply is vital as it would allow the cultures to be harvested at a representative end point [7].

As shown in Fig. 1, online measurement of the OTR was an ideal in situ method to achieve suitable oxygen supplies in the screening cultures. The operating conditions shown in Fig. 1 (circles) ensured a non-limited oxygen supply and were therefore chosen for all subsequent investigations (Figs. 2, 3, 4, 5, 6). Otherwise culture phenomena would have been hidden under the "ceiling" of an oxygen limitation.

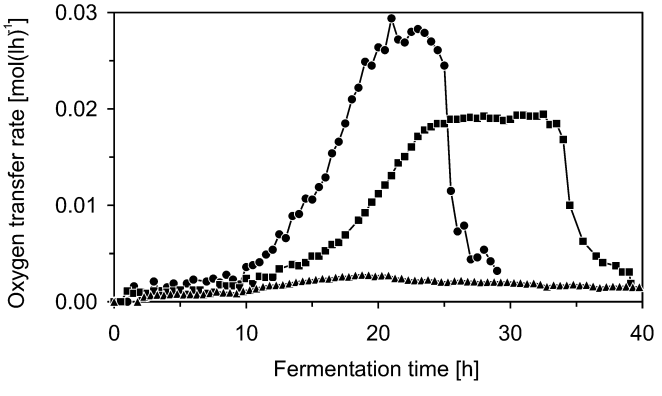

Fig. 1 Oxygen transfer rate over fermentation time for chymotrypsinogen-producing *Hansenula polymorpha* cultures correlated to different filling volumes and shaking frequencies. Cultivation conditions: SYN 6 medium in 250-ml flasks; shaking diameter: 25 mm; filling volumes and shaking frequencies: ● 20 ml, 300 rpm; ■ 40 ml, 300 rpm; ▲ 83 ml, 140 rpm. Oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined for 20 ml, 300 rpm as 0.53 and for 40 ml, 300 rpm as 0.48 mol glycerol per mol oxygen

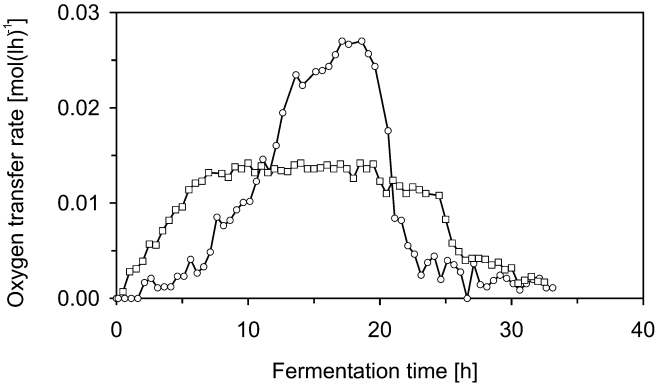

Fig. 2 Oxygen transfer rate over fermentation time for *H. polymorpha* CBS4732 cultures correlated to YNB media with different phosphate buffering. Culture conditions: □ YNB 1: 0.01 mol phosphate l^{-1} (pH 6.0), YNB 2: ○ 0.12 mol phosphate l^{-1} (pH 4.1); standard operating conditions. The oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined for both YNB media as 0.53 mol glycerol per mol oxygen

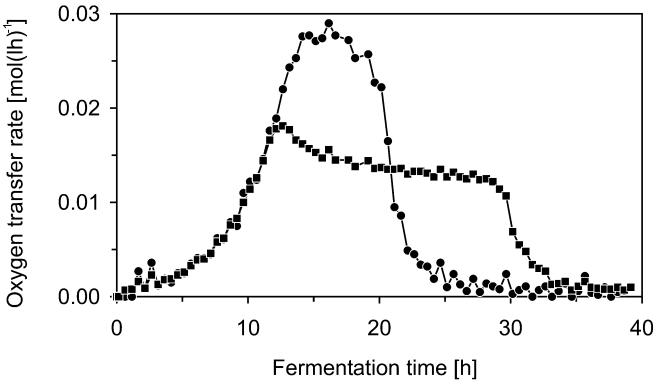

Fig. 3 Oxygen transfer rate over fermentation time for *H. polymorpha* RB11 cultures correlated to different uracil concentrations in SYN 6 medium. Culture conditions: ■ 20 mg uracil l^{-1} ($R_{Uracil/Glyc} = 0.0011$ mol uracil per mol glycerol), ● 40 mg uracil l^{-1} ($R_{Uracil/Glyc} = 0.0022$ mol uracil per mol glycerol); standard operating conditions. The oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined for 20 mg uracil l^{-1} as 0.51 and for 40 mg uracil l^{-1} as 0.48 mol glycerol per mol oxygen, respectively

Screening media

The properties of culture media, such as pH and nutrient supply, affect the growth and metabolism of microbial cultures [7] and have to be assessed for their impact on screening.

pH

The pH value during culturing influences cell metabolism and gene expression [19, 20, 21, 22]. Consequently, it is vital to define those pH ranges that do not impair screening cultures. In our study, YNB 1 and SYN 6

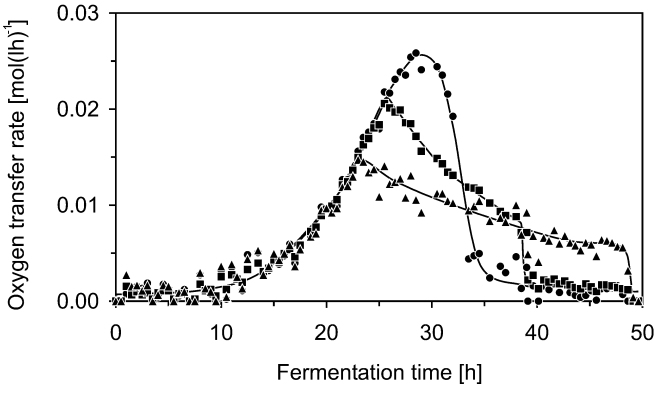

Fig. 4 Oxygen transfer rate over fermentation time for chymotrypsinogen-producing *H. polymorpha* cultures correlated to different ammonium concentrations in SYN 6 medium. Culture conditions: ● 0.12 mol ammonium l^{-1} ($R_{NH_4/Glyc} = 0.712$ mol ammonium per mol glycerol, ■ 0.017 mol ammonium l^{-1} ($R_{NH_4/Glyc} = 0.104$ mol ammonium per mol glycerol), ▲ 0.012 mol ammonium l^{-1} ($R_{NH_4/Glyc} = 0.074$ mol ammonium per mol glycerol); standard operating conditions. The oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined for 0.12, 0.017 and 0.012 mol ammonium l^{-1} as 0.51, 0.51 and 0.53 mol glycerol per mol oxygen, respectively

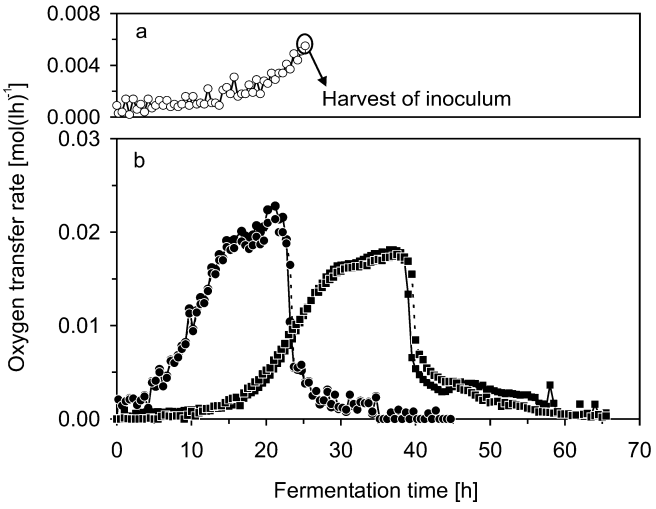

Fig. 5 Oxygen transfer rate over fermentation time for IFNα-2a-producing *H. polymorpha* cultures correlated to different precultivations. **a** Submerged SYN 6 preculture used for inoculation of the cultures of (**b**, *circles*). **b** Main cultures in SYN 6 medium inoculated with ● submerged SYN 6 preculture of **a** or with ■ plate preculture on YPD. The cultures were conducted under standard operating conditions. Oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined for cultures inoculated with submerged SYN 6 preculture and with YPD-plate-derived preculture as 0.52 and 0.48 mol glycerol per mol oxygen, respectively

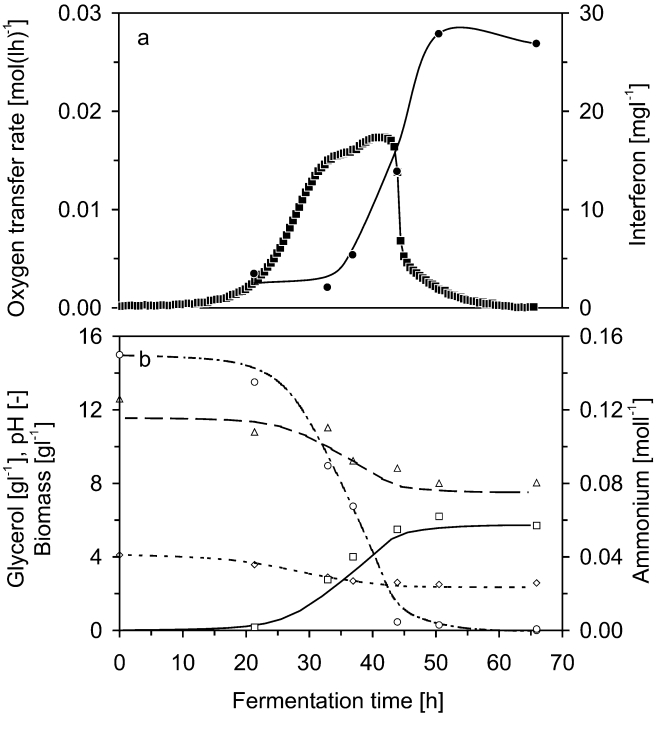

Fig. 6a, b Oxygen transfer rate and culture parameters measured and simulated on the basis of oxygen transfer rate over fermentation time for an IFNα-2a-producing *H. polymorpha* culture. **a** ■ Oxygen transfer rate, ● IFNα-2a. **b** ○ Glycerol, △ ammonium, ◇ pH, □ biomass; *lines*: calculated, *symbols*: measured. The culture was inoculated with cells from a YPD-plate-derived preculture and conducted at standard operating conditions

media were used for screening recombinant *H. polymorpha* production strains. Figure 2 (squares) shows the OTR profile of a cultivation with YNB 1 medium. Although YNB 1 medium provided a fast OTR increase, the OTR remained constant at a level of 0.014 mol $(l\,h)^{-1}$ after 8 h. Plateaus like this usually indicate oxygen limitation [10]; however, the applied operating conditions supported a non-limiting oxygen supply (Fig. 1, circles).

Comparing the ionic strengths and pH values of cultures in SYN 6 and YNB 1 media (Table 1) led to a likely explanation for the OTR plateau observed in Fig. 2 (squares). The low ionic strength of the low phosphate-buffered (0.01 mol phosphate l^{-1}) YNB 1 medium was possibly beneficial for growth, hence, the OTR initially increased rapidly compared to the culture in SYN 6 medium (Fig. 1, circles). But after a short time, the culture in YNB 1 medium was more acidified than that in SYN 6 medium (final pH values 2.4 and 2.8; Table 1). Optimal growth for *H. polymorpha* is at pH 3.0–6.5; beyond this range the specific growth rate coefficients decrease rapidly (unpublished data). Thus, the cultures with low-buffered YNB 1 medium apparently reached pH values leading to reduced growth. This also explains the reduced respiration as indicated by the OTR plateau (Fig. 2, squares). In contrast, in the high-buffered SYN 6 medium (Fig. 1, circles) a higher final pH of 2.8 was maintained (Table 1), and growth and respiration were not influenced. This was confirmed by transferring the buffering (0.12 mol phosphate l^{-1}) and initial pH value (4.1) of the SYN 6 medium to the low-buffered YNB 1 medium. Use of the respectively modified YNB 2 medium (Fig. 2, circles) resulted in a similar non-limited OTR profile as in the case of SYN 6 medium (Fig. 1, circles). The modified YNB 2 medium is thus equivalent to SYN 6 medium with respect to pH.

Uracil supply

The uracil-auxotrophic strain RB11 serves as host for recombinant *H. polymorpha*. During transformation, the uracil-auxotrophy is complemented by *URA3* [23]. The growth characteristics of strain RB11 have been frequently compared with those of recombinant strains in assessing the impact of foreign genes on the vitality of the recombinant strains. The comparison is based on the assumption that external uracil supplementation for strain RB11 results in non-limited uracil availability, comparable to that in recombinant strains complemented with *URA3*.

Table 1 Comparison of *Hansenula polymorpha* cultures in SYN 6 medium and YNB 1 medium

		SYN 6	YNB 1
Ionic strength[a]	[mol l^{-1}]	≈0.9	≈0.2
Phosphate	[mol l^{-1}]	0.12	0.01
Initial pH	[-]	4.1	6.0
Final pH	[-]	2.8	2.4

[a]Assuming total dissociation of the mineral salts

Despite supplementing SYN 6 medium with $R_{Uracil/Glyc}$ = 0.0011 mol uracil per mol glycerol, the OTR profile of the host strain RB11 showed an abrupt decline at hour 12 (Fig. 3, squares). OTR profiles of this kind frequently indicate nutrient limitations at excessive concentrations of carbon source [10]. Obviously, up to hour 12 nutrients were sufficiently available. In this phase the culture grew exponentially, and the OTR increased exponentially as well. At the time of depletion of the essential nutrient, culture growth was limited and thus respiration was reduced, indicated by the decrease of OTR between hours 12 and 30. Thereafter the OTR totally dropped due to the depletion of glycerol.

Various nutrients were added to the SYN 6 cultures in order to compensate for the nutrient limitation (data not shown). In this context, the uracil demand was estimated. [24] specified a uracil demand of 0.011 g uracil per g biomass for *S. cerevisiae*. Taking this number and the theoretical number of a biomass yield of $Y_{X/Glyc}$ = 33.1 g biomass per mol glycerol for a non-limited *H. polymorpha* culture (e.g. Fig. 1, circles), a uracil demand of $R_{Uracil/Glyc}$ = 0.0033 mol uracil per mol glycerol can be deduced. This is three-fold higher than the amount originally applied (Fig. 2, squares). Thus, increased uracil concentrations were tested. The results showed that a demand of $R_{Uracil/Glyc}$ = 0.0022 mol uracil per mol glycerol was sufficient for non-limited cultivations, as seen in the non-limited OTR profile of Fig. 3 (circles). Uracil was identified as the limiting nutrient in strain RB11 cultures.

The non-limiting uracil supply of $R_{Uracil/Glyc}$ = 0.0022 mol uracil per mol glycerol provided a fair basis for comparison of strain RB11 with recombinant strains. The OTR profiles of the chymotrypsinogen-producing strain (Fig. 1, circles) and strain RB11 (Fig. 3, circles) were identical. In this case, the strains were apparently not affected by the transformation step. In contrast, the IFNα-2a-producing strain (Fig. 5b, circles) showed a significant saddle-shaped OTR profile, which will be discussed later.

The use of auxotrophic strains as hosts is common practice although the nutritional compensation of auxotrophies or complementation by marker genes is of complex nature [24]. Low copy numbers of the marker gene may lead to poorly growing strains (gene dosage effect) [25, 26, 27]. Auxotrophic strains, generated by mutagenesis, may have acquired additional deficiencies impairing the performance of the resulting host strains. As shown for the determination of the uracil demand of strain RB11, the online measurement of OTR in shaking flasks provides an accurate qualitative and quantitative approach to defining the specific nutrient requirements of such strains [24].

Ammonium supply

Nitrogen supply has to be well-balanced in order to ensure non-limited synthesis of cellular and recombinant

proteins. Thus, careful determination of the nitrogen demand is crucial. In the case of *H. polymorpha* cultures, ammonium was used as nitrogen source.

At first, the ammonium demand was theoretically estimated by a stoichiometric balance of the cultures [28]. For that purpose, a yield of secreted heterologous protein $Y_{Prot/Glyc} = 0.18$ g protein per mol glycerol (Fig. 6), a biomass yield of $Y_{X/Glyc} = 33.1$ g biomass per g glycerol (Fig. 1, circles), and an average cell composition $C_1H_{1.82}O_{0.47}N_{0.19}$ of *Candida utilis* [28] were assumed. The resulting gross fermentation stoichiometry is depicted in Eq. 1:

$$\begin{array}{lllll} 1\ C_3H_8O_3 & +0.24\ NH_3 & +2.13\ O_2 & & \\ \text{glycerol} & \text{ammonia} & \text{oxygen} & & \\ 1.27\ C_1H_{1.82}O_{0.47}N_{0.19} & +0.0018\ C_{4.51}H_{7.15}O_{1.46}N_{1.28} & \div 1.73\ CO_2 & +3.21\ H_2O & \cdot \\ \text{biomass} & \text{protein} & \text{carbon dioxide} & \text{water} & \end{array} \quad (1)$$

The gross fermentation stoichiometry (Eq. 1) yielded an oxygen-dependent glycerol consumption of $R_{Glyc/O_2} = 0.47$ mol glycerol per mol oxygen. A similar consumption of $R_{Glyc/O_2} = 0.50 \pm 0.03$ mol glycerol per mol oxygen (Figs. 1, 2, 3, 4, 5) was determined experimentally and confirmed the stoichiometry. The material balance resulted in an ammonium demand of $R_{NH_4/Glyc} = 0.24$ mol ammonium per mol glycerol.

In order to specify the ammonium demand of the screening cultures, the estimated demand $R_{NH_4/Glyc}$ was used to prepare SYN 6 medium with excessive and with limiting ammonium concentrations. Figure 4 shows the corresponding OTR profiles of the chymotrypsinogen-producing strain cultivated in those SYN 6 media. The culture with an excessive supply of $R_{NH_4/Glyc} = 0.71$ mol ammonium per mol glycerol showed a non-limited OTR profile as expected (Fig. 4, circles). The ammonium-limited cultures containing $R_{NH4/Glyc} = 0.104$ mol ammonium per mol glycerol or $R_{NH_4/Glyc} = 0.074$ mol ammonium per mol glycerol were characterized by sudden OTR declines (Fig. 4, squares, triangles). These declines indicated the time of ammonium exhaustion as in the uracil-limited case (Fig. 3, squares). Ammonium limitation caused prolonged fermentation times. Biomass yields $Y_{X/Glyc}$ decreased with decreasing $R_{NH_4/Glyc}$ from 33.1 (Fig. 4, circles), over 28.8 (Fig. 4, squares) to 24.5 g biomass per mol glycerol (Fig. 4, triangles).

In the initial phase of the fermentation (prior to ammonium depletion), a linear relationship between ammonium and oxygen consumption could be assumed for *H. polymorpha* [29]. Consequently, the ratios between the initial ammonium amount and the consumed oxygen before ammonium depletion should be constant for different limiting ammonium concentrations. The ammonium consumption was determined as $R_{NH_4/O_2} = 0.12$ mol ammonium per mol oxygen for the cultures supplemented with both limiting 0.074 and 0.104 mol ammonium per mol glycerol (Fig. 4, triangles, squares). Oxygen-dependent glycerol consumption R_{Glyc/O_2} was determined as well (Figs. 1, 2, 3, 4, 5). Hence, the ammonium demand with respect to glycerol could be calculated by division of R_{NH_4/O_2} by R_{Glyc/O_2}. The calculation yielded $R_{NH_4/Glyc} = 0.245 \pm 0.015$ mol ammonium per mol glycerol.This value agrees with the calculated consumption of $R_{NH_4/Glyc} = 0.24$ (Eq. 1). The ammonium content of SYN 6 medium, consisting of monobasic ammonium phosphate, provided a three-fold ammonium excess of $R_{NH_4/Glyc} = 0.72$ mol ammonium per mol glycerol.

A yield of $Y_{X/Glyc} = 33.1$ g biomass per mol glycerol could be determined for cultures not limited by ammonium (Fig. 4, circles). With $R_{NH_4}/Glyc = 0.245 \pm 0.015$ mol ammonium permol glycerol, an ammonium demand of 0.0074 ± 0.00045 mol ammonium per g biomass could be calculated.

The device for the online measurement of OTR could be efficiently employed for developing well-balanced media. Only a few OTR measurements in batch-operated shaking bioreactors facilitated quantitative determination of the demand of the essential nutrient ammonium.

Preculture and Inoculum

Conditions of preculturing can strongly influence the growth and expression characteristics of the subsequent main culture [30, 31, 32, 33, 34]. An active inoculum and an optimal inoculum size (usually 3–10%, v/v) are generally recommended to ensure a main culture with a minimized lag phase and to avoid low reproducibility of production fermentations due to variable biomass concentrations and variable metabolic activity of the preculture [32]. Therefore, assessment of preculturing with respect to a reproducible and efficient main cultivation is important.

Figure 5b shows OTR profiles of an IFNα-2a-producing *H. polymorpha* strain cultivated in SYN 6 medium. The strain was inoculated alternatively from a YPD plate and a submerged preculture with SYN 6 medium. The latter was harvested for inoculation at the early-exponential phase, as indicated by the exponential increase of the OTR in Fig. 5a. Duplicate results are shown in Fig. 5b to demonstrate the reproducibility of the effects.

The densities of the YPD-plate-derived inoculum and the SYN 6 preculture were identical. However, the cultures inoculated by YPD-plate-derived cells exhibited

significantly prolonged lag phases, indicated by a constant OTR near zero for 10 h (Fig. 5b, squares). The plate-derived inocula clearly required time for adapting to the SYN 6 medium of the main culture.

The maximum specific growth rate coefficient of the exponential phase (μ_{max}) can be deduced from the maximum exponential slope of the OTR:

$$\mu_{\max} = \frac{\ln OTR(t) - \ln OTR_0}{t - t_0} \quad (2)$$

The maximum specific growth rate coefficients of the cultures inoculated with YPD plate cells were calculated by Eq. 2. The values were one third lower than those observed for cultures inoculated by a SYN 6 preculture (Fig. 5b, 0.18 h^{-1} compared to 0.26 h^{-1}). Inoculation from a YPD plate irreversibly reduced the specific growth-rate coefficient. The lower specific growth rate coefficient and prolonged lag phase of cultures inoculated from a YPD plate resulted in a cultivation time extended by 15 h over that of a SYN 6 preculture.

The long lag phase of the culture inoculated from YPD plates may have been caused by the shift from YPD complex medium with glucose to SYN 6 minimal medium with glycerol. In contrast to the YPD-plate-derived inocula, the SYN 6-derived preculture resulted in shorter lag phases and higher specific growth rate coefficients (Fig. 5b, circles). This was due to the fact that the cells were taken from the early-exponential phase (Fig. 5a), providing continued exponential growth in the main culture. Moreover, use of the glycerol-supplemented SYN 6 medium avoided adaptive lag phases due to the carbon-source shift described before.

Monitoring was a helpful tool for assessment of precultures. An electronic method for assessment of shaking-flask precultures was developed by [33], and [32] used exhaust-gas analysis to assess fermenter precultures. The devices applied for OTR measurement in this study combine the online analysis of this significant parameter with the simplicity of shaking bioreactors.

Characterization of the IFNα-2a-producing strain by mathematical simulation

The OTR profiles of the IFNα-2a-producing strain exhibited phases with reduced respiration. This could be observed after inoculation with YPD plate-cells (hour 30, Fig. 5b, squares) as well as after inoculation with SYN 6 preculture (hour 15, Fig. 5b, circles). For this reason, the IFNα-2a-producing strain was investigated in detail by mathematical simulation.

Oxygen consumption was correlated to the consumption of glycerol and ammonium and the formation of biomass by Eq. 1. By means of linear balanced equations, we attempted to model several culture parameters on the basis of the OTR profiles. SYN 6 medium was an ideal basis for such balancing since it contains glycerol as a carbon source, which provides a stable metabolism without by-product formation. Moreover, SYN 6 medium lacks undefined complex components, which would have been difficult to balance. The validity of the simulated culture parameters, glycerol, ammonium, biomass, and pH, was checked by direct measurements within the culture broth.

As shown in the investigations before, constant oxygen-dependent glycerol consumption and ammonium consumption could be assumed, on average $R_{Glyc/O_2} = 0.50$ mol glycerol per mol oxygen (Figs. 1, 2, 3, 4, 5) and $R_{NH_4/O_2} = 0.12$ mol ammonium per mol the oxygen (Fig. 4). The ratio of the final biomass to the total oxygen consumption led to an oxygen-dependent biomass yield of $Y_{X/O_2} = 17$ g biomass per mol oxygen, derived from Figs. 1 and 6. The consumed oxygen over fermentation time $c_{O_2}(t)$ was calculated by integration of the OTR according to Eq. 3 and served as the time-dependent guide variable.

$$c_{O_2}(t) = \int_0^t OTR\, dt \quad (3)$$

Oxygen consumption $c_{O_2}(t)$ multiplied with the above ratios yielded the time-dependent courses of the culture parameters (Eqs. 4, 5, 6):

$$c_X(t) = Y_{X/O_2} \cdot c_{O_2}(t) \quad (4)$$

$$c_{Glyc}(t) = c_{Glyc,0} - R_{Glyc/O_2} \cdot c_{O_2}(t) \quad (5)$$

$$c_{NH_4}(t) = c_{NH_4,0} - R_{NH_4/O_2} \cdot c_{O_2}(t) \quad (6)$$

The pH as a function of the fermentation time could be calculated due to the following three constraints:

1. During cultivation, the pH ranged from 4.1 to 2.8. Due to the dissociation constant of ammonium ($pK_{NH_4} = 9.25$ [35]), the protonized ammonium species NH_4^+ was exclusively present in the medium. The assimilation of deprotonized ammonium NH_3 (*assim*) led to the equimolar net release of protons H^+ (*released*) into the medium according to Eq. 7.

$$NH_4^+ \rightarrow NH_3(assim) + H^+(released) \quad (7)$$

2. The resulting acidification was solely buffered by phosphate according to Eq. 8.

$$H_2PO_4^- + H^+(released) \rightleftarrows H_3PO_4 \quad pK_{PO_4} = 2.16\ [35] \quad (8)$$

3. The influence of carbon dioxide produced by the microorganisms on pH was neglected, because the cultures were done at pH values much lower than the relevant pK_{CO_2} of 6.3 of the H_2CO_3/HCO_3^- dissociation pair. Based on these constraints, the course of pH could be calculated by the buffer equation of Henderson-Hasselbach (Eq. 9).

$$pH(t) = pK_{PO_4} - \log \frac{c_{H_3PO_4}(t)}{c_{H_2PO_4^-}(t)} \quad (9)$$

The conversion of buffer species could be described as Eqs. 10, 11, 12and 13:

$$c_{H_3PO_4}(t) = c_{H_3PO_4,0} + c_{H^+(released)}(t) \quad (10)$$

$$c_{H_2PO_4^-}(t) = c_{H_2PO_4^-,0} - c_{H^+(released)}(t) \quad (11)$$

$$\text{with } c_{H^+(released)}(t) = c_{NH_3(assim)} = R_{NH_4/O_2} \cdot c_{O_2}(t) \quad (12)$$

$$\text{and } c_{PO_4(total)} = c_{H_3PO_4}(t) + c_{H_2PO_4^-}(t) \quad (13)$$

Because the initial pH ($t = 0$) and the total phosphate concentration $c_{PO_4(total)}$ of SYN 6 medium were known, the course of pH could be calculated by combining Eq. 3 and Eqs. 9, 10, 11, 12, and 13.

The courses of simulated and measured parameters are presented in Fig. 6a, b for the cultivation of the IFNα-2a-producing strain. Parallel to increasing respiration, glycerol and ammonium were simultaneously consumed and biomass increased. At stationary phase, glycerol was depleted accompanied by a cessation of respiration. Thereafter, biomass and ammonium levels remained constant. Initially, the pH fell from 4.1 to 3.0 due to growth-coupled ammonium consumption. At about hour 35, pH entered the buffer area of the H_3PO_4/$H_2PO_4^-$ dissociation pair and leveled off, finally reaching a pH of 2.7 similar as explained for Fig. 2 (circles). All values (Fig. 6b, symbols) and the independently simulated courses (Fig. 6b, lines) were a close match thus confirming the validity of the linear balance Eq. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and the input values.

The transiently reduced respiration of the IFNα-2a-producing strain at hour 30 (Fig. 6a, squares) seemed to be strain-specific, since the chymotrypsinogen-producing strain, the non-recombinant host strain RB11 and the wild-type strain exhibited standard OTR profiles under identical or similar conditions (circles of Figs. 1, 2, 3, 4). Transiently reduced respiration coincided with the main onset of IFNα-2a formation (Fig. 6a, circles). Possibly, heterologous gene expression affected primary metabolism and thereby transiently reduced respiration. Reduced glycolysis and reduced specific growth rate coefficients were observed when heterologous genes were overexpressed [36]. This was attributed to a burden of primary metabolism imposed by the synthesis of heterologous proteins. A similar effect may have affected the IFNα-2a-producing strain.

Conclusions

Measurement of the OTR in shaking-flask cultures provided valuable data about the culture conditions during screening. The OTR profiles displayed the time-dependent course of the screening cultures and reflected the metabolic activity with respect to different screening conditions. Limiting conditions, such as oxygen limitation, pH inhibition, nutrient limitations and the use of improper precultures, could be identified and eliminated applying defined non-limiting conditions. The use of OTR measurements in shaking flasks was especially beneficial for material balancing of the screening cultures. It enabled the exact determination of nutrient demands and the simulation of several culture parameters. Thereby, defined and reproducible screening conditions, pre-requisite for conclusive strain selection, could be ensured. Moreover, the data generated may serve as a solid basis for the evaluation of strains, the selection of media and operating conditions at a larger scale. The OTR was shown to be a key parameter for characterisation of *H. polymorpha* screening cultures. The OTR was measured online by a device, which is based on easy-to-handle shaking flasks. Thus, there is an efficient small-scale method that alleviates the need for expensive offline sampling.

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Appendix

Symbols

c	Concentration [mol l^{-1}], [g l^{-1}]
H^+ (released)	Released protons [mol]
$H_2PO_4^-$	Dihydrogen phosphate [mol]
H_3PO_4	Phosphoric acid [mol]
NH_3 (assim)	Assimilated ammonium [mol]
NH_4^+	Ammonium [mol]
OTR	Oxygen transfer rate [mol (l h)$^{-1}$]
OTR_{max}	Maximum oxygen transfer capacity [mol (l h)$^{-1}$]
pK_{CO_2}	Dissociation constant of the dissociation pair H_2CO_3/HCO_3^- [–]
pK_{NH_4}	Dissociation constant of the dissociation pair NH_4^+/NH_3 [–]
pK_{PO_4}	Dissociation constant of the dissociation pair $H_3PO_4/H_2PO_4^-$ [–]
R_{Glyc/O_2}	Stoichiometric ratio between glycerol and oxygen [mol mol^{-1}]
$R_{NH_4/Glyc}$	Stoichiometric ratio between ammonium and glycerol [mol mol^{-1}]
R_{NH_4/O_2}	Stoichiometric ratio between ammonium and oxygen [mol mol^{-1}]
$R_{Uracil/Glyc}$	Stoichiometric ratio between uracil and glycerol [mol mol^{-1}]

t_0	Initial time [h]
t	Point of time [h]
Y_{X/O_2}	Oxygen-dependent biomass yield [g mol^{-1}]
$Y_{X/Glyc}$	Glycerol-dependent biomass yield [g mol^{-1}]
$Y_{Prot/Glyc}$	Glycerol-dependent protein yield [g mol^{-1}]
μ_{max}	Maximum specific growth rate coefficient [h^{-1}]

Indices

Glyc	Glycerol
H^+ (released)	Released protons
$H_2PO_4^-$	Dihydrogen phosphate
H_3PO_4	Phosphoric acid
PO_4(total)	Total phosphate
NH_3 (assim)	Assimilated ammonium
NH_4	Ammonium
O_2	Oxygen
X	Biomass
0	Initial point of time

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