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Early bioprocess development using *Komagataella phaffii*: application scenarios for the microTOM mini bioreactor system

David Wollborn^{1*}, Carol J. Hartley¹, Andreas Schulte² and Juan C. P. Correa²

Abstract

Developing novel bioprocesses usually starts with an iterative cycle to engineer genetically modified organisms. This leads to large strain libraries that differ in their genetic makeup, showing different growth and production performance. Finding the optimal production strain requires extensive screening and is arguably one of the most important steps for large-scale productivity. For aerobic processes, it is important to ensure the cultivation conditions avoid oxygen limitation. The oxygen transfer rate (OTR) is a parameter to monitor metabolic activity. The micro-scale Transfer rate Online Measurement (microTOM) system allows online monitoring of the respiration activity in 96-individual wells of a microtiter plate. This novel late-stage beta version prototype, provided by Kuhner Shaker, was trialled with the common microbial host *Komagataella phaffii*. It is demonstrated how the microTOM system can be applied to rapidly determine essential parameters for bioprocess design. Using a 96-well microtiter plate format enabled high-throughput investigation of three strains in parallel. The methanol uptake and growth rate was estimated, which can be used in process models to guide scale-up and decisions regarding feeding rates in fed-batch operation mode. Further, we assessed seven media formulations with two carbon sources, the impact of filling volume on the maximum oxygen transfer capacity, eight different antifoam agents, and growth rates were estimated at five different temperatures. These findings can help select screening conditions and demonstrate how the microTOM system can accelerate bioprocess development. Until now, obtaining high-resolution OTR datasets at high-throughput, suitable for deriving critical process parameters, has not been feasible. Thus, the microTOM supports robust, data-driven bioprocess development.

Keywords Mini bioreactor, microTOM, OTR, *Komagataella phaffii*, *Pichia pastoris*, Small scale cultivation, Early-stage bioprocess development

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Introduction

In early bioprocess development, small-scale cultivation systems such as microtiter plates (MTPs) or shake flasks are often employed to test multiple cultivation conditions and/or strains with reasonable experimental time and throughput.

MTPs offer key advantages in laboratory workflows due to their low cost, reduced culture media consumption, and suitability for high-throughput applications [1, 2]. The 96-deepwell format is a widely adopted standard, making MTPs compatible with automated liquid handlers such as those used in bio-foundries [3]. This standardisation supports seamless integration with robotic liquid handlers and analytical instruments, enabling consistent and scalable automation across various applications.

Hence, various mini-bioreactor systems with integrated online monitoring have been developed. Commercially available systems compatible with MTP formats include: The BioLector® (Beckman Coulter Life Sciences GmbH), the Growth profiler 960 (EnzyScreen B.V.), the Applikon micro-Matrix system (Geringe AB), and the micro-24 MicroReactor system (Pall Corporation). For cell culture applications there is the microtiter system C.NEST® and C.BIRD™ (CYTENA GmbH) offering enhanced mixing, with temperature and CO₂ control, but without online measurement of bioprocess parameters.

These systems focus on non-invasive measurement of parameters such as biomass via backscatter, dissolved oxygen or pH using dye-based optodes and lack the capability to directly measure oxygen transfer rate (OTR), a critical parameter allowing process insight and deeper process understanding. An overview of the correlation of oxygen uptake rate (OUR), dissolved oxygen tension (DOT), OTR and the maximum oxygen transfer capacity (OTR_{max}) is given in the supplementary data (Supplementary Fig. 7 and Equation S1 to S3).

The OTR is an important parameter that directly correlates with metabolic activity in microbial cultures and can reveal biological phenomena such as substrate limitations, diauxic growth, or oxygen limitation [4, 5, 6, 7]. Various application scenarios have shown auxotrophic deficiencies [8], detection of metabolic burden caused by induced protein production [9, 10, 11], nutrient limitation in media development [12, 13, 14], characterisation of yeast extract qualities [15, 16], cellulose digestibility [17], determination of biocompatibility of materials [18, 19] and the impact of mutagenic substances and toxins [20, 21, 22].

The OTR further enables oxygen balancing when using different carbon sources [23, 11, 24]. Knowing the total amount of consumed oxygen further allows to calculate biological heat generation using the oxycaloric equivalent for the respective carbon source used [25, 26].

More importantly, the OTR measurement can be useful for deriving parameters that are helpful in supporting bioprocess scale-up. The OTR_{max} allows the calculation of the volumetric mass transfer coefficient ($k_L a$) [27, 28, 29]. The $k_L a$ is a parameter that is often used as scale-up criterion and maintained constant across scales [30]. A successful translation from MTP to continuously stirred tank reactor (CSTR) was demonstrated by [31, 32] and [33]. Even though successful scale transfer has been reported previously, this study does not include a scale-up component. Instead, the data presented here aim to showcase various application scenarios for the **micro-scale Transfer rate Online Measurement** (microTOM) mini bioreactor system. We demonstrate a robust framework for hypothesis generation and parameter screening to obtain OTR-based estimates as comparative screening metrics for rapidly assessing differences between strains.

In this study, a late-stage beta version prototype for online OTR measurements in 96-well microtiter plates manufactured by Kuhner Shaker GmbH, named microTOM, was used. The measurement principle is described by Dinger et al. [34]. Three different methylotrophic *Komagataella phaffii* (formerly known and still often referred to as *Pichia pastoris*) yeast strains were used as a model organism due to their industrial relevance in biotechnological processes [35, 36]. The strains have different methanol utilisation (Mut) phenotypes. Mut⁺ enables unhindered methanol metabolism as the strain has both alcohol oxidases (AOX1 and AOX2), and Mut^S shows a reduced (slow) methanol metabolism rate due to a deletion of the *AOX1* gene. The remaining AOX2 still results in AOX production, but at a much lower rate [37]. Here, *K. phaffii* BG10 (Mut⁺), *K. phaffii* X-33 (Mut⁺) and *K. phaffii* CS310 (Mut^S) strains were used.

Generally, the use of methanol as inducer or substrate is controversially discussed. On the one hand, there is the argument of toxicity and flammability which makes it challenging to store and apply methanol in an industrial setting [38]. This also leads to suggested methods to reverse engineer existing production strains using the methanol dependent pAOX promoter system [39]. At the same time, there is the argument to push for single carbon feedstocks (such as CO₂, methane, formate and methanol) as a renewable feedstock that is not dependent of sugars [40]. That includes adapting non-methylotrophic yeasts such as *Saccharomyces cerevisiae* to methanol as a substrate [41]. From this debate, it can be concluded that methanol will most likely retain its relevance as a feedstock for certain processes and applications.

The microTOM system was used to investigate different media compositions and carbon sources, the determination of growth rates at different cultivation temperatures, to estimate the methanol uptake rates for

the three different *K. phaffii* strains, and to test the effect of eight different chemical antifoam agents on the OTR.

The selected examples are common parameters chosen for assessment when characterising new strains or a new clone library. Media composition should be addressed as it can directly affect oxygen requirements [42] or requires adjustments to meet the nutrient requirements for genetically modified strains [43]. Further, cultivation temperature needs to be considered and determined for different bioprocesses. Lower cultivation temperatures are often selected to support product formation and protein folding. Hence, the temperatures impact on the growth rate is valuable information for modelling and up-scaling of the bioprocess [10, 44].

Chemical antifoam agents are commonly used on demand in stirred tank bioreactors to efficiently and cost effectively control foam formation. However, they should be selected with care, as there are various types, such as esters, fatty acids, polyglycols, siloxanes, alcohols and sulfonates, that may negatively affect microbial metabolism, mass transfer, the quality of the final product and the downstream operations [45, 46]. Therefore, it is advisable to screen multiple antifoam agents early on to evaluate their specific effects on the bioprocess. The effects of antifoam agents can vary significantly; while some studies report detrimental impacts on yeast cell viability and ethanol production capacity [47], others report enhanced productivity and protein secretion [48]. These contrasting findings highlight the importance of carefully selecting a suitable antifoam agent for each specific bioprocess.

Additionally, the filling volume has a major impact on oxygen mass transfer into the medium and thereby affects cell metabolism affecting cell performance. Oxygen limiting conditions should be avoided to maintain reproducible conditions across cultivation scales [49].

In this study, the microTOM system was employed to investigate these phenomena during early-stage bioprocess development at the MTP scale. A photo of the microTOM system is shown in Fig. 1.

The novelty of the microTOM system lies in its ability to combine high-throughput cultivation in a 96-well MTP format with online, and well-resolved, quantification of oxygen transfer. As demonstrated in earlier studies, OTR measurements at small scale (for example, in shake flasks) can provide highly relevant implications about metabolic activity in aerobic cultivations.

By transferring this measurement capability to the 96-well MTP scale, the microTOM system substantially extends the scope of high-throughput microbial screening. The large number of wells support broad experimental designs with multiple strains, conditions, and replicates, thereby improving statistical robustness and enabling more informative early-stage bioprocess development.

In this study, we investigate several representative questions that commonly arise in early-stage bioprocess development, using them as illustrative application scenarios.

At the same time, it is important to note that conclusions derived from OTR measurements are limited to physiological interpretations that are directly linked to aerobic metabolic activity and do not substitute further analyses. The microTOM system can help to quickly assess a large parameter space and narrow down the number of experiments in the following steps of bioprocess development [50].

Materials and methods

The micro-scale transfer rate Online Measurement system (microTOM)

The foundations for the **micro** scale Transfer rate Online Measurement system from Kuhner Shaker, referred to as microTOM in this manuscript, were originally developed at the chair of biochemical engineering at RWTH University. The underlying prototype is described and characterised in detail in the work of Dinger and colleagues [34]. Building on this foundation, Kuhner Shaker has advanced the system to a late-stage beta version, which is the device used in this study. For a detailed explanation



Fig. 1 Photographs of the microTOM system by Kuhner Shaker

of the measurement principle and a thorough evaluation of measurement accuracy, readers are kindly referred to the publication of Dinger et al. [34]. In particular, Fig. 4 in their work illustrates the measurement principle, and Fig. 8 summarises measurement precision by comparing theoretical and experimentally obtained coefficients of variation. Differences between outer and inner wells (edge effects) are not significant (unpublished data).

The measurement principle and associated performance parameters remain unchanged, as the sensor plate itself was not modified during the development process. The oxygen sensitive sensor comprising a fluorescent dye exhibits a drift of less than 1% per year. Furthermore, calibration with fresh air prior to each experiment, or at monthly intervals, is considered appropriate to ensure accurate measurements. The late-stage beta version has been tested at maximum shaking frequencies of 300 rpm (50 mm shaking diameter), 400 rpm (25 mm shaking diameter), and 1000 rpm (3 mm shaking diameter).

Microorganisms

The *Komagataella phaffii* (formerly known as *Pichia pastoris*) strains BG10, X-33 and CS310 were used. The BG10 strain is a derivative of strain *K. phaffii* NRRL Y-11430/ CBS 7435 strain that was originally deposited into the US Agricultural Research Service (ARS) Culture Collection. It carries both alcohol oxidase genes (*AOX1* and *AOX2*) and has native methanol utilisation (*Mut*⁺) [51]. Therefore, the BG10 strain is regarded as a wildtype strain in this study. BG10 has a mutation in the *HOC1* gene leading to a truncated version of HOC1. The *HOC1* gene is associated with the cell wall and its structural integrity, and its deletion may result in higher transformation efficiency. This truncation can also positively impact protein secretion efficiency [52]. The BG10 strain used in this study is cured of cytoplasmic killer plasmids and was originally sourced from [53].

The X-33 strain is part of a commercially available “*Pichia pastoris* Expression Kit” by the company Invitrogen [54] it can also be regarded as wildtype as it has the *Mut*⁺ and *His*⁺ genotype.

The CS310 strain was engineered by CSIRO, and it is a *Mut*^S version of *K. phaffii* NRRL Y-11430. The *AOX1* gene was deleted, resulting in a slow methanol utilisation (*Mut*^S) genotype. This strain is similar to the BioGramatics BG11 strain.

Media and solutions

All chemicals applied for media preparation were of analytical grade and purchased from Thermo Fisher Scientific or Merck Life Science Pty Ltd, if not stated otherwise. For the experiments, the microorganisms were grown in a defined mineral medium, referred to as Seed medium. It consists of a base medium, a trace element stock

solution, a carbon source stock solution (e.g. 500 g/L glucose or 500 g/L glycerol, autoclaved) and a vitamin stock solution. The base medium composition was as follows: 2 g/L potassium hydroxide (KOH), 1 g/L potassium sulphate (K_2SO_4), 2 g/L magnesium sulphate heptahydrate ($MgSO_4 \times 7 H_2O$), 1 g/L calcium sulphate dihydrate ($CaSO_4 \times 2 H_2O$), 2 mL/L phosphoric acid (H_3PO_4) (85% w/v), 5 g/L ammonium sulphate ($(NH_4)_2SO_4$), and to obtain a 100 mM citric acid buffer (pKa 6.4), 25.7 g/L sodium citrate dihydrate ($C_6H_5Na_3O_7 \times 2 H_2O$) and 2.42 g/L citric acid ($C_6H_8O_7$) were added. The components were dissolved in 800 mL of deionised water, and the pH was adjusted to pH 6.2 using 4 M KOH.

The trace element solution was concentrated 100-fold with the following composition: 5 g/L copper sulphate heptahydrate ($CuSO_4 \times 5 H_2O$), 0.08 g/L sodium iodide (NaI), 3 g/L manganese sulphate monohydrate ($MnSO_4 \times H_2O$), 0.24 g/L disodium molybdate dihydrate ($Na_2MoO_4 \times 2 H_2O$), 0.024 g/L boric acid (H_3BO_3), 0.73 g/L cobalt chloride hexahydrate ($CoCl_2 \times 6 H_2O$), 20 g/L zinc sulphate heptahydrate ($ZnSO_4 \times 7 H_2O$), 40 g/L iron sulphate heptahydrate ($FeSO_4 \times 7 H_2O$), 6 mL/L sulphuric acid (H_2SO_4 98% w/v), and 0.13 g/L nickel sulphate hexahydrate ($NiSO_4 \times 6 H_2O$). The components were solubilised in deionised water and filter-sterilised using a 0.22 µm sterile filter.

The vitamin solution was also prepared as 100-fold concentrated stock, with the following composition: 0.024 g/L biotin was solubilised in 100 mL/L of a 50% v/v isopropanol mixture. Afterwards, 13.35 g/L Thiamine hydrochloride was added to 900 mL deionised water and both mixtures combined, and filter-sterilised using a 0.22 µm sterile filter. The vitamin solution was stored at 4 °C.

An alternative culture medium was used for comparison, referred to as mineral synthetic medium 6, using 200 mM MES buffer (Syn6-MES). It consists of a base medium, a 100x micro element stock solution, a trace element stock solution, a carbon source stock solution (e.g. 500 g/L glucose or 500 g/L glycerol, autoclaved), a 100 g/L calcium chloride solution (autoclaved), and a vitamin stock solution [55]. The base medium composition is as follows: 1.0 g/L potassium phosphate (KH_2PO_4), 7.66 g/L ammonium sulphate ($(NH_4)_2SO_4$), 3.3 g/L potassium chloride (KCl), 3.0 g/L magnesium sulphate heptahydrate ($MgSO_4 \times 7 H_2O$), 0.3 g/L sodium chloride (NaCl), and 27.3 g/L 2-(N-morpholino)-ethanesulfonic acid, 4-morpholineethanesulfonic acid (MES). All medium components were dissolved in 900 mL deionised water, and the pH was adjusted to 6.0 with 1 M NaOH. The basic medium solution was sterilised via autoclaving (121 °C for 30 min). The micro element stock solution consists of: 6.65 g/L EDTA (ethylenediamine tetraacetic acid disodiumsulfate), 6.65 g/L diammonium iron disulphate

hexahydrate $((\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \times 6 \text{ H}_2\text{O})$, 0.55 g/L copper sulphate pentahydrate $(\text{CuSO}_4 \times 5 \text{ H}_2\text{O})$, 2 g/L zinc sulphate heptahydrate $(\text{ZnSO}_4 \times 7 \text{ H}_2\text{O})$ and 2.65 g/L manganese sulphate monohydrate $(\text{MnSO}_4 \times \text{H}_2\text{O})$.

The trace element stock solution consists of: 0.065 g/L nickel sulfate hexahydrate $(\text{NiSO}_4 \times 6 \text{ H}_2\text{O})$, 0.065 g/L cobalt chloride hexahydrate $(\text{CoCl}_2 \times 6 \text{ H}_2\text{O})$, 0.065 g/L boric acid (H_3BO_3) , 0.065 g/L potassium iodide (KI), and 0.065 g/L disodium molybdate dihydrate $(\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O})$. All stock solutions were filter sterilised using a 0.22 μm filter. The same vitamin stock solution was used for both defined mineral mediums.

To obtain 1 L Syn6-MES medium, 940 mL basic medium was supplemented with 10 mL of 100-fold CaCl_2 solution, 10 mL of the micro-elements stock solution, 10 mL of the vitamin stock solution, 10 mL of the trace element solution, and the residual 20 mL was used to add a carbon source stock solution.

Additionally, yeast, peptone, dextrose (YPD) medium was used as reference cultivation medium. 10 g/L of yeast extract and 20 g/L of bacteriological peptone were solubilised in 800 mL deionised water and autoclaved. A dextrose stock solution (500 g/L) was prepared and autoclaved separately. The final medium was combined to a final composition of 20 g/L dextrose (800 mL YE, 40 mL dextrose stock solution and 160 mL sterile deionised water). Different types of yeast extract were used, and they are mentioned in the caption of the respective experiment. The following yeast extracts were used: Flavex Powder Type 401 Savoury Low Salt 1 (provided by Cell BioScience), and Kerry yeast extracts: Hy-Yest 412, Hy-Yest 466 and Hy-Yest 455, provided by Total Lab Systems.

Antifoam agents

In total, 8 different antifoam agents were tested at 2 different concentrations. The following antifoam agents were obtained from Schill+Seilacher “Struktol” GmbH, Germany: Struktol® J 673 A (alkoxylated fatty acid ester on a vegetable basis), Struktol® J 647 (combination of polyglycol ethers of aliphatic alcohols), Struktol® SB 2121 (polyalkylene glycols), Struktol® SB 2228 (polyalkylene glycols). Antifoam 204 (mixture of organic polyether dispersions), Antifoam A Concentrate (active silicone polymer) and polypropylene glycol were obtained from Sigma Aldrich/ Merck KGaA, Germany, and FOAMSTOP™ Antifoam (Dimethylpolysiloxane) from BeerCo Pty, Australia. All antifoams were tested using concentrations of 1 mL/L medium (0.1% v/v) and 5 mL/L (0.5% v/v). These concentrations are regarded as typical usage level, based on the antifoam agent’s cloud point, and are within, but near the upper limit of, commonly added antifoam amounts.

Pre-cultures

Pre-cultures were conducted in defined mineral Seed media, with 10 g/L of glycerol as carbon source. The respective *K. phaffii* strains were picked as single colonies from YPD-AGAR plates and transferred into the medium. 250 mL shake flasks were operated with 10 mL filling volume, 300 rpm and 25 mm shaking diameter at 28 °C. The cultures were grown over night (approximately 18 to 24 h).

MTP cultivations

All cultivations were performed in 96-well deepwell MTP (DeepWell Plate 96/1000 μL by Eppendorf) with a square well geometry and round bottom. The plates were sealed with “Breathe Easier” gas-permeable sealing films by the company Diversified Biotech. The plate was incubated using the late-stage beta version of the microTOM from Kuhner Shaker located inside a LT-X orbital shaker by Kuhner Shaker (see Fig. 1).

The standard operation conditions were a filling volume of 0.6 mL per well, operated at a shaking frequency of 300 rpm, a shaking diameter of 50 mm, at 28 °C and a relative humidity higher than 80%, as the system uses gas-washing bottles for supplying humidified pressurised air for active surface aeration. Deviating experimental conditions are specified in the description of the respective experiment. Initial OD_{600} and number of replicates are stated in the caption of the corresponding experiment.

OTR based growth rate estimation

To obtain OTR based growth rate estimates at different cultivation temperatures, the exponential phase was identified by applying a natural log transformation to the obtained OTR signals. For each selected time interval, the transformed data were fitted using a linear model of the form $y = a \cdot x + b$, where a is the slope (specific growth rate) and b the intercept. Model parameters were estimated by ordinary least squares minimization using OriginPro 2022b (v9.9.5.167). Linear fits and corresponding Pearson’s R^2 values for all conditions and replicates are provided in Supplementary Fig. 8, while error bars in Fig. 3 indicate the standard error of the slope.

Results and discussion

A microTOM late-stage beta version prototype system was applied in various application scenarios using methylotrophic *K. phaffii* strains. The results are described and discussed in the following paragraphs.

Testing strain performance on differently composed media

One of the first steps in bioprocess development is the selection of a culture medium to check compatibility of the ingredients. The selected medium can also affect strains differently, thus, several clones are usually

tested to analyse growth performance. Testing different media and strains quickly leads to a large experimental load with an equally large amount of samples for analysis. Measuring the OTR at 96-well MTP scale with the microTOM system can help to quickly analyse many conditions without the need of offline measurements at reasonable throughput. Thereby, it narrows down the number of cultivations needed at later stages of the bioprocess development.

Three different *K. phaffii* strains (BG10 Mut⁺, X-33 Mut⁺ and CS310 Mut^S) were tested on two carbon sources (glucose and glycerol) in four different YPD media (varying the type of yeast extract), and two defined mineral media. The results are shown in Fig. 2.

The top row of Fig. 2 (panels A to C) illustrates the growth behaviour of the *K. phaffii* strains BG10 Mut⁺, X-33 Mut⁺ and CS310 Mut^S, using 10 g/L glycerol as carbon source in different media. Comparing the strains individually, BG10 (Fig. 2, panel A) shows no OTR differences between the different yeast extracts tested. Further, both mineral media (Syn6-MES and Seed) result in an identical OTR trend. Mineral media causes a slight delay in growth, nonetheless the batch phase for all tested media ends after approximately 20 h.

The growth behaviour of strain X-33 (Fig. 2, panel B), shows a different OTR course for the tested yeast extracts in YPG medium. YP2, YP3 and YP4 show a double peak, between 10 and 19 h, whereas YP1 shows a single peak on an overall higher OTR level (up to 21 mmol/L/h compared to 19.5 mmol/L/h). But a second peak occurs in the shoulder of the OTR for YP1 at 16 h. These findings

demonstrate, as has been shown previously, that measuring the OTR can reveal growth performance differences between yeast extracts from different manufacturers or with varying compositions [16, 56, 57]. Growth performance on mineral Syn6-MES and Seed shows a delay in growth and no additional OTR peaks that may indicate overflow metabolite formation are observed.

K. phaffii strain CS310 shows a slight delay in growth for all tested conditions as it was inoculated with a 10-fold lower starting optical density (OD_{600} = 0.02 instead of OD_{600} = 0.2).

Overall, the CS310 strain performs in a similar manner to the BG10 strain on all tested media, also showing a slightly reduced growth rate on the tested mineral media Syn6-MES and Seed.

Interestingly, using the same strains and media, but with 10 g/L glucose rather than 10 g/L glycerol results in a different growth performance. The BG10 and CS310 perform very similar and there is no visible difference between the four different YPD media tested (Fig. 2, panel D and F). However, using 10 g/L glucose as carbon source results in differences between Syn6-MES and Seed media for both strains. On Syn6-MES medium, there is an additional peak after 16 h, and the maximum OTR achieved is lower than for the tested YPD medium (13 mmol/L/h compared to 22 mmol/L/h). A possible explanation is that complex media often provides better growth as metabolites such as amino acids and peptides do not need to be synthesised by the cells as they are available in the medium [15]. A third peak becomes visible after approximately 30 to 32 h. These two additional

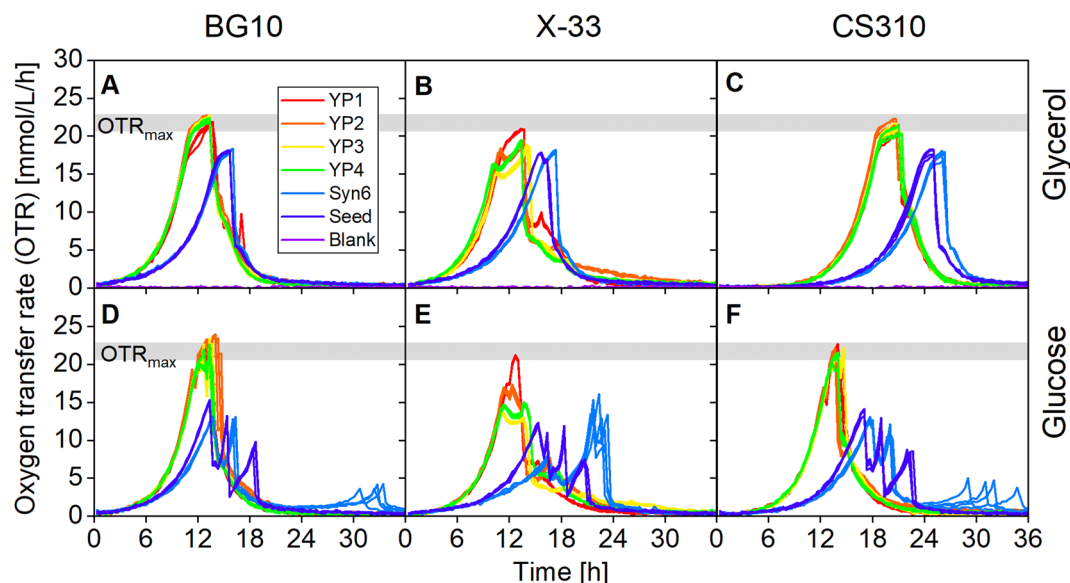


Fig. 2 Cultivation of three different *K. phaffii* strains (BG10 Mut⁺, X-33 Mut⁺ and CS310 Mut^S) in a single 96-square deep well MTP, $N=4$ replicates per condition, in a microTOM system. Cells with an initial optical density (OD_{600}) of 0.2 were cultivated at 28 °C. Strain CS310 on glycerol was inoculated with an initial OD_{600} of 0.02. YP1 = Flavax Powder Type 401, YP2 = Hy-Yeast 412, YP3 = Hy-Yeast 466, and YP4 = Hy-Yeast 455. The theoretically achievable OTR_{max} ranges from 23 to 21 mmol/L/h and is depicted as a grey bar. It was determined experimentally (see Fig. 7)

peaks are consistent with the formation of overflow metabolites. *K. phaffii* is known to produce metabolites from the respiro-fermentative pathway, such as ethanol, acetate and arabitol [58, 59, 60]. It is likely that the peak after 16 h can be attributed to the metabolism of ethanol as shown in previous studies [14, 61]. Offline measurements would be required to experimentally confirm the presence of a metabolite.

Despite a highly similar composition, the mineral Seed medium shows a different OTR profile than the Syn6-MES medium. The third OTR peak becomes apparent much earlier in this medium; after approximately 18 h, compared to 30 h in Syn6-MES medium.

The X-33 strain shows a different growth behaviour (Fig. 2, panel E). Similar to the results on glycerol, there are differences between the different YPD media, when using glucose as carbon source. The double peak formation between 12 and 14 h for YP3 and YP4 becomes even more apparent. YP2 shows a similar pattern at a higher OTR level and YP1 does not show a double peak. The two tested mineral media lead to a more delayed initial growth on glucose, compared to the tested YPD media, and also when compared to their performance with glycerol as carbon source (Fig. 2, panel B).

The Syn6-MES medium results in 3 distinct peaks at 17, 22 and 23 h. As mentioned earlier, it is likely they correspond to the overflow metabolites ethanol, acetate and arabitol. The mineral Seed medium also results in 3 distinct peaks, appearing at 11, 12 and 21 h. The expression of the peak height also varies which suggests a difference in concentration of the individual overflow metabolites. In order to clarify the differences observed, further investigation and offline samples would be necessary.

Nonetheless, the results demonstrate how the growth performance of different strains and using different cultivation media can be quickly assessed using the microTOM system. The number, height and time point of occurrence of different OTR peaks indicate changes in the cell's metabolism and can aid selection of the optimal cultivation conditions.

Temperature screening to identify maximum growth rate

The cultivation temperature is another crucial parameter that needs to be considered early in bioprocess development. Cultivation temperature affects the speed of catalytic reactions and their yields. Importantly, the temperature optimum within a microbial system can vary depending on whether the focus is on growth or product formation. Since temperature correlates directly with the growth rate, it is often lowered in order to allow more time for protein folding which can enhance production of correctly folded proteins [62, 63, 64, 65].

Previous studies have showed that the OTR signal is proportional to the biomass signal (e.g. scattered light)

[66]. Hence, the OTR can be used to estimate the growth rate and compare the impact of varying cultivation conditions (e.g. cultivation temperature) on the cell growth kinetics. It is important to emphasise that this method has its limitations, which is why the resulting specific growth rate coefficients cannot be considered to be as accurate as those obtained from biomass measurements. Nevertheless, the OTR allows for rapid screening of many different conditions to identify those under which maximum growth can be expected. The method presented provides OTR-derived apparent values that are suitable for rapid comparative screening. It is not appropriate for determining precise kinetic parameters of temperature-dependent growth rates; for that purpose, methods that directly measure biomass (e.g., backscatter calibrated against cell dry weight or offline cell dry weight measurements over time) are required. This illustrates how non-invasively measuring the OTR not only gives insight into metabolic stages but also allows to derive values for growth rates to gain deeper bioprocess understanding.

For a precise estimation of the specific growth rate (μ) using the OTR, it is necessary to know the yield coefficient of biomass on oxygen (Y_{X/O_2}). It needs to be assumed that Y_{X/O_2} is constant during the cultivation [67] and that the oxygen demand for cell maintenance can be neglected. This technique has been successfully applied for *Escherichia coli* [66] and *Corynebacterium glutamicum* [68] cultivations.

Here, the OTR serves as an indicator for specific growth rate estimation of *K. phaffii* cultures to elucidate the effect of different cultivation temperatures on growth rate. The Y_{X/O_2} or cell maintenance was not considered, and the obtained growth rates cannot be regarded as precise kinetic constants. Three different *K. phaffii* strains (BG10 Mut⁺, X-33 Mut⁺ and CS310 Mut^S) were cultivated on defined mineral Seed medium using 10 g/L glycerol as carbon source. The strains were cultivated at 20, 25, 28, 32, 34 and 37 °C (see Fig. 3).

The OTR data in Fig. 3, panel A shows the OTR over time for *K. phaffii* strain BG10 at 20, 25, 28, 32, 34 and 37 °C. Please note that only one replicate per condition is shown to emphasize the methodology. All individual measurements are provided in Supplementary Fig. 1. The time point when the OTR signal rapidly drops after reaching the maximum value, indicates when all glycerol is consumed and the batch ends [61, 69]. Depending on the cultivation conditions, the batch end is reached at different timepoints, as the growth rate and thereby the slope of the OTR signal varies. To quantify this effect, the OTR data were linearised by calculating the natural logarithm, and the linear segment representing the exponential growth phase was used to estimate the growth rate (Supplementary Fig. 8).

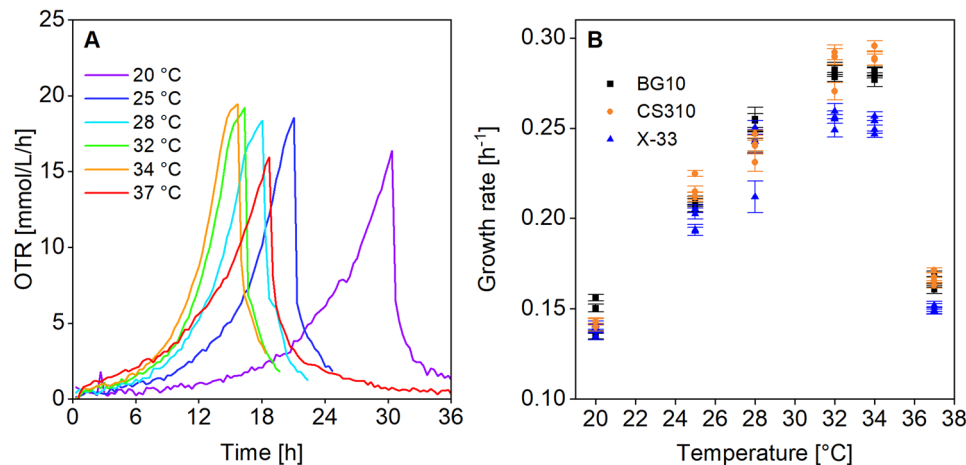


Fig. 3 **A** OTR data for *K. phaffii* strain BG10 at different cultivation temperatures (only 1 of 4 replicates shown, all replicates shown in Supplementary Fig. 1). **B** The growth rate was estimated from the OTR data and plotted against the different cultivation temperatures to obtain a temperature response curve. Cultivations were conducted in defined mineral seed medium at pH 6.2 (100 mM citrate buffer) with 10 g/L glycerol, $N=4$ replicates per condition, in a microTOM system. Error bars indicate the standard error of the slope. Cells with an initial optical density (OD_{600}) of 0.2 were cultivated at various temperatures: 20, 25, 28, 32, 34 and 37 °C

The slope of each linear fit was interpreted as the estimated growth rate and was plotted against its respective temperature, resulting in a bell-shaped curve (Fig. 3, panel B). The 4 replicates together show the distribution of values. While this method does not yield absolute kinetic parameters, it provides a rapid mean to identify growth optima and tolerable temperature ranges for a given strain under specific conditions.

It also is important to note that Y_{X/O_2} may change with cultivation temperature. Therefore, differences in the kinetics of oxygen consumption are not solely correlated with changes in growth rate but may also result from shifts in other metabolic processes. As expected, the temperature response curve shows a decline in growth rate at both, low and high temperatures, with a peak at an optimal temperature between 32 and 34 °C for two of the tested *K. phaffii* strains (BG10 and CS310). Interestingly, *K. phaffii* strain X-33 shows a growth rate plateau from 28 to 34 °C, and an overall slower growth rate than the other two tested strains. This observation matches the strain's reported temperature optimum of 28 to 30 °C [65].

These results demonstrate how the microTOM system can be used to conduct temperature profile screening to obtain growth optima curves. This is particularly useful when screening clone libraries, as product formation can affect growth kinetics through side-effects caused by excess metabolic burden [11, 66, 70].

Estimating methanol uptake rate in *K. phaffii* Mut⁺ and Mut^S strains

K. phaffii is a methylotrophic yeast capable of using methanol as sole carbon source. The yeast is known for its strong and highly regulated methanol inducible

promoters [37]. This makes it an attractive host for heterologous protein production. *K. phaffii* hosts can be differentiated into three genotypes regarding methanol metabolism. Methanol utilisation plus (Mut⁺) describes the wildtype that is capable of utilising methanol at maximal rate, as Alcohol oxidase 1 and 2 are functional in these strains. Mut^S strains can metabolise methanol slowly, as the Alcohol oxidase 1 is deleted or truncated and Mut⁻ strains, which do not have any functional Alcohol oxidases [71]. In the *K. phaffii* CS310 Mut^S strain used in this study the *AOX1* gene was deleted.

A common challenge of designing a *K. phaffii* process is to determine a methanol feeding rate that is sufficient to maintain protein induction but does not lead to an accumulation of methanol in the culture broth. If methanol concentration is too high, it can negatively affect strain performance and have a detrimental effect on the cells [72].

The OTR course of *K. phaffii* cultures growing on glycerol, followed by growth on methanol can be divided into 4 phases (I to IV) [11]: Phase I: growth on glycerol until depletion, phase II: methanol adaption, phase III: methanol consumption phase and phase IV, methanol depletion (Fig. 4). To allow for methanol adaptation, 1% (v/v) methanol was added after 31 h, giving the cells time to adjust their metabolism and enabling more accurate uptake-rate determination during subsequent pulses. Although minimal non-linear consumption may still occur, spiking with different methanol concentrations consistently yielded similar uptake rates, highlighting the practical reliability of the method, within its constraints. The differences between the methanol consumption patterns for the *K. phaffii* BG10 Mut⁺ strain (Fig. 4, panel A) and the *K. phaffii* CS310 Mut^S strain (Fig. 4, panel B)

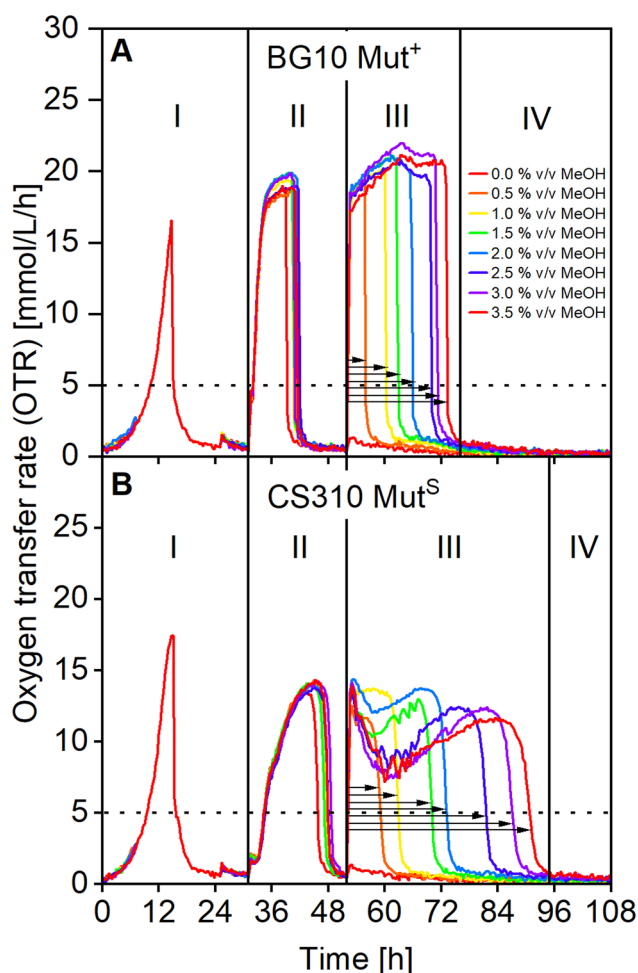


Fig. 4 Cultivations were conducted in defined mineral seed medium at pH 6.2 (100 mM citrate buffer) with 10 g/L glycerol, $N=4$ replicates per condition (Only one replicate is depicted to demonstrate the methodology, replicates are shown in Supplementary Fig. 2), in a microTOM system. Cells with an initial optical density (OD_{600}) of 0.2 were cultivated and 1% v/v methanol was added after 31 h to allow for methanol adaptation (II). Various methanol additions followed at concentrations of 0.5, 1, 1.5, 2, 2.5, 3 and 3.5% v/v after 52 h (III). Vertical arrows show the Δt values that were determined as time for methanol consumption for the respective methanol concentrations. The horizontal dashed line at 5 mmol/L/h marks the boundary condition; once OTR dropped below, methanol consumption phase was considered complete (IV). **A** *K. phaffii* BG10 Mut⁺ and **B** *K. phaffii* CS310 Mut^S

can be clearly identified. The difference is expected as the strains phenotype differentiates their ability to metabolise methanol. Please note that only one replicate per condition is shown to emphasize the methodology. All individual measurements are provided in Supplementary Fig. 2. The *K. phaffii* CS310 Mut^S metabolises methanol more slowly, which becomes apparent in a longer methanol adaptation phase of 17 h (Fig. 4, panel B phase II) compared to only 10 h for the *K. phaffii* BG10 Mut⁺ strain (Fig. 4, panel A, phase II). Here, different methanol concentrations were added after 52 h: 0.5, 1, 1.5, 2, 2.5, 3

and 3.5% v/v, which resulted in an instant onset of methanol consumption and metabolic activity as indicated by a sudden OTR level increase. Since the concentration of added methanol is known, the methanol uptake rate can be estimated by using the time required to completely metabolise the methanol. The Δt value that was required to achieve complete metabolisation for the different concentrations is illustrated by the black horizontal arrows in Fig. 4, panel A and B. The horizontal dashed line at 5 mmol/L/h marks the defined boundary condition. Theoretically, an OTR of 0 mmol/L/h would indicate complete methanol consumption. In practice, the OTR signal never instantly falls directly to zero. Instead, a persistent baseline approximately between 0 and 2 mmol/L/h is typically observed (Fig. 4, panel A and B, phase IV).

A commonly mentioned explanation as to why the OTR does not drop to zero is that residual oxidative metabolic activities (e.g., maintenance respiration or the oxidation of intracellular storage compounds) contribute to the observed measurable oxygen uptake [7]. This putative explanation is further supported by the blank measurements (non-inoculated culture medium) showing a baseline OTR noise between 0 and 0.3 mmol/L/h (Fig. 2, panel A to C). For this reason, a threshold value larger than 2 mmol/L/h was selected. The methanol consumption phase was considered complete when the OTR dropped below the selected threshold. As expected, the *K. phaffii* BG10 Mut⁺ strain consumed the various methanol concentrations faster (highest concentration of 3.5% v/v within approximately 25 h) than the *K. phaffii* CS310 Mut^S strain (approximately 42 h) (Fig. 4, panel A and B respectively). The Δt values for all conditions were determined and plotted against the corresponding methanol concentration. The results are shown in Fig. 5.

Here, seven different methanol concentrations were spiked, and every concentration for itself lead to the same uptake rate. This is verified by a Pearson's r value of 0.977 for *K. phaffii* BG10 Mut⁺ (Fig. 5, panel A) and a Pearson's r value of 0.984 for *K. phaffii* CS310 Mut^S (Fig. 5, panel B). The results clearly show the difference in methanol utilisation rate, as the BG10 Mut⁺ strain shows an uptake rate of $1.17 \text{ g/L/h} \pm 0.03$ and the CS310 Mut^S strain only $0.66 \text{ g/L/h} \pm 0.02$. The *K. phaffii* X-33 Mut⁺ was determined with a methanol uptake rate of $1.13 \text{ g/L/h} \pm 0.02$ (see supplementary data, Supplementary Fig. 3). As the methanol uptake rate is dependent on the cell density at the time of methanol spiking, these values need to be converted to g/g/h (g methanol per g dry cell weight per hour). Here, a cell density of 5.5 g/L cell dry weight (CDW) was achieved *K. phaffii* BG10 Mut⁺, resulting in a methanol uptake rate of 0.21 g/g/h . *K. phaffii* CS310 Mut^S reached 6.25 g/L CDW (respective methanol uptake rate 0.11 g/g/h) and *K. phaffii* X-33 Mut⁺ 5.0 g/L (respective methanol uptake rate 0.23 g/g/h). The

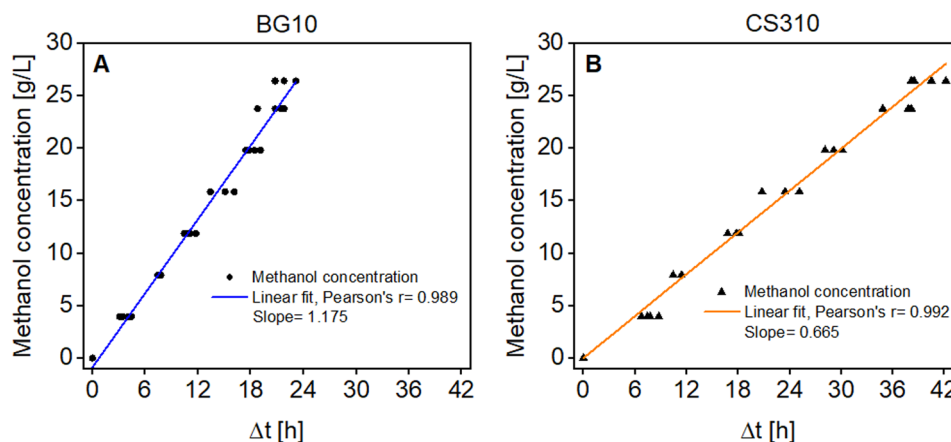


Fig. 5 Estimate of methanol consumption rate for **A** *K. phaffii* BG10 Mut^S strain and **B** *K. phaffii* CS310 Mut^S strain (data for *K. phaffii* strain X-33 in Supplementary Fig. 3). The known methanol concentrations that were added are plotted against the time until the spiked methanol was consumed (Δt) for each individual replicate, $N=4$. The slope of the linear fit results in a methanol uptake rate in g/L/h

formation of additional biomass growth on methanol was neglected. These values are in accordance with literature, as [73] report methanol uptake rates of 7.1 mmol_{methanol}/g_{cell dry weight}/h for Mut⁺ strains and 1.42 mmol_{methanol}/g_{cell dry weight}/h for Mut^S strains. These values can be converted to 0.23 g/g/h and 0.05 g/g/h respectively.

The methanol uptake rate determined for the CS310 Mut^S strain was higher than reference values in literature. A possible explanation is, that the CS310 strain used in this study did not carry a recombinant gene, unlike the Mut^S strain used by [73]. Dietzsch and colleagues [74] observed significant variation among Mut^S strains expressing different target proteins. Their obtained values varied from 0.039 g/g/h to 0.062 g/g/h. This observation is often referred to as metabolic burden [11].

The method presented here is best suited for comparing methanol uptake rates between strains to obtain an initial estimate during early screening. A quick method to roughly estimate methanol consumption for multiple strains is useful and desirable. OTR monitoring also shows if methanol is still present or metabolised and thereby indicates if additional feeding pulses are necessary. Knowing if methanol is still present can also be used to adjust processing time in screening experiments [61]. Thus, this method is intended for the quick estimation of methanol uptake rates for multiple clones without the need to analyse the methanol concentrations via HPLC or other analytical methods. It is also similar to the method suggested by [75].

It is important to note that the method still requires determination of the cell dry weight prior to the methanol pulses to enable the estimation of the uptake rate. The method assumes negligible methanol evaporation and minimal biomass growth on methanol so it is not suitable for examining *K. phaffii* strains that require substantially longer induction phases on methanol as

evaporation effects can become more pronounced under these conditions. Accurate determination of uptake rates requires offline quantification of methanol concentrations because evaporation rate depends on factors such as initial methanol concentration, filling volume, shaking speed, temperature, and gassing rate.

Influence of antifoam agents on OTR_{max}

Foaming events are a common issue for bioprocesses that are actively aerated by air sparging and agitation in bioreactors. Foam is defined as a gas phase dispersed in a liquid phase where the gas bubbles are stabilised by a liquid film forming on the surface of the culture medium. Media composition and the presence of microorganisms influence the degree of foaming and foam stability as both affect surface-active components such as proteins, lipids, fatty acids and others [76].

It is crucial to control foam formation, as it can affect mass transfer of gas into, or out of (pCO₂) the culture medium at the gas-liquid interface, cause culture inhomogeneities and lead to loss of culture broth and cells through the off-gas line, which might also cause blockage of off-gas filters [45, 47, 77, 78].

A cost-effective method to efficiently tackle foam formation is the addition of chemical antifoam agents. These often act as surfactants and can be used to destroy foam or prevent its formation. In shaken small scale cultivation systems (MTPs and shake flasks), foaming is not commonly encountered as air is not provided by air sparging into the culture liquid as it is the case in bioreactors, but via surface aeration. However, it can occur when baffles are used to enhance mixing. It is recommended to screen the impact of antifoam agents on the bioprocess as different antifoams can have different effects, such as being inhibitors or get metabolised thereby affecting growth and process performance [48, 79].

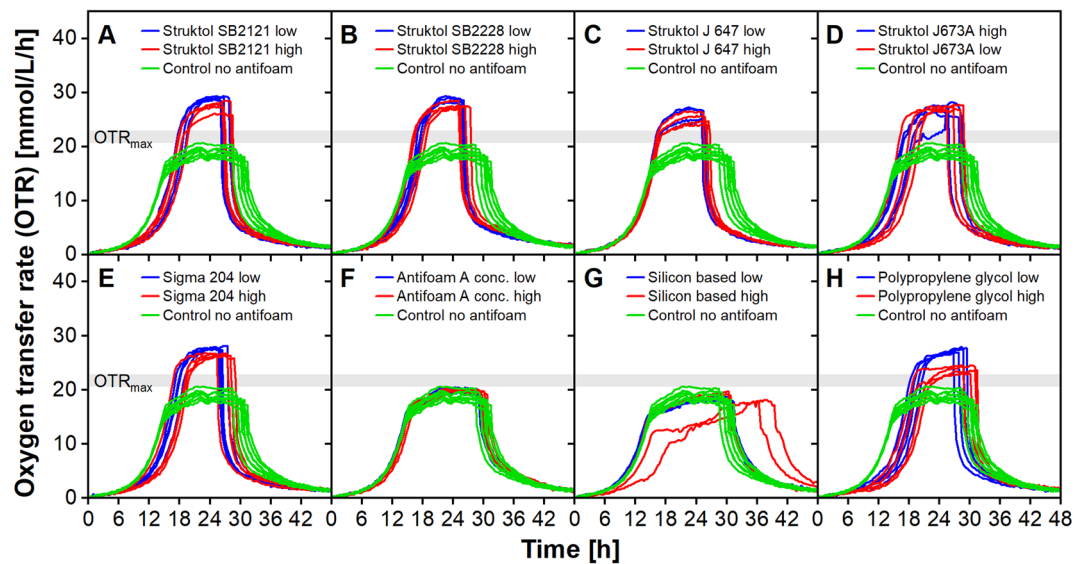


Fig. 6 Cultivation of *K. phaffii* BG10 Mut⁺, with various chemical antifoam agents (stated in graphic), $N=4$ replicates per condition, and $N=8$ for the control without antifoam-addition, in a microTOM system. Cells with an initial optical density (OD_{600}) of 0.2 were cultivated at 28 °C in defined mineral seed medium at pH 6.2 (100 mM citrate buffer) with 40 g/L glycerol. The theoretically achievable OTR_{max} ranges from 23 to 21 mmol/L/h and is depicted as a grey bar (as determined in Fig. 7)

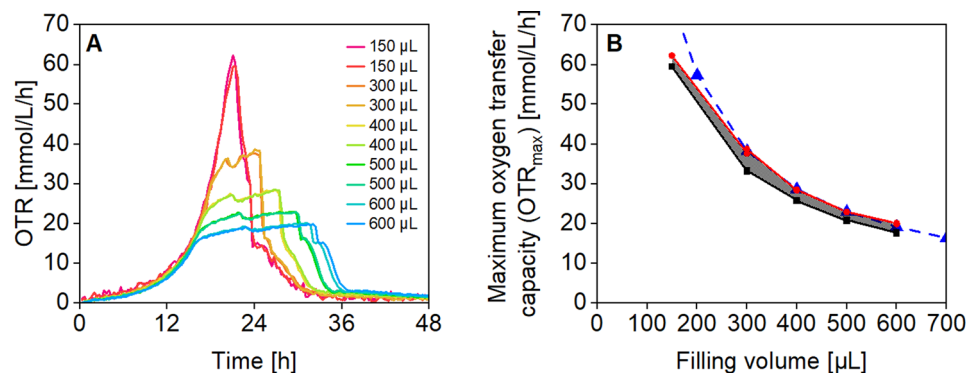


Fig. 7 **A** Cultivation of *K. phaffii* BG10 Mut⁺ at various filling volumes (0.15 to 0.6 mL), $N=2$ replicates per condition, in a microTOM system (data for other strains can be found in Supplementary Fig. 4). Cells with an initial optical density (OD_{600}) of 0.2 were cultivated at 28 °C in defined mineral seed medium at pH 6.2 (100 mM citric acid buffer) with 40 g/L glycerol. **B** The lower (black squares) and upper (red circles) OTR_{max} levels were plotted over the respective filling volume. Blue triangles and dotted line depict the correlation to estimate OTR_{max} in MTPs by Dinger et al. [34]

Here, eight different antifoams were tested at 96-square deepwell MTP scale (Fig. 6).

Every panel in Fig. 6 shows the course of the OTR for one antifoam agent at a high (0.5% v/v) concentration in red, and a low (0.1% v/v) concentration in blue. The results for the positive control, where no antifoam agent was added, is plotted in green.

The antifoams Struktol® SB2121, SB2228, J647, and Sigma 204 can be classified as polyether-based compounds. They are mixtures of polyalkylene glycols (polyether) or polyglycol ether (polyether) with different molecular weights resulting from differences in aliphatic carbon chain length and side groups. They are water soluble and affect surface tension. High molecular weight aliphatic alcohols have a similar effect. Struktol® J647

includes a mix of polyglycol ethers and aliphatic alcohols [76, 77].

The chemical similarity of these antifoam agents explains the highly similar results obtained in the cultivation. All mentioned antifoams (Fig. 6, panel A, B, C, D, E and H) show a similar course of the measured OTR. The addition of antifoam agents at both tested concentrations seems to positively impact the maximum achievable oxygen transfer capacity (OTR_{max}). The OTR_{max} value of the control is approximately 19 mmol/L/h which is in accordance with the previously determined OTR_{max} value under these cultivation conditions of 21 to 23 mmol/L/h, marked with a grey bar (see Fig. 7). Antifoam addition leads to OTR_{max} values of 26.9 to 28.8 mmol/L/h. This is an increase of approximately 40 to 50%. No foam

formation was expected, and this effect was observed in shaken and surface-aerated cultivation systems. In air-sparged and stirred bioreactors, antifoam usually negatively affects mass transfer [80].

The lower concentration of 0.1% v/v of added antifoam also appears to lead to higher OTR_{max} values than the higher concentration of 0.5% v/v. This effect is especially apparent when using polypropylene glycol (Fig. 6, panel H).

The subsequent drop of the OTR to nearly zero indicates complete depletion of the carbon source, in this case glycerol. The exact timing of batch termination varies slightly across replicates.

Interestingly, Antifoam A Concentrate (active silicone polymer) and FOAMSTOP™ Antifoam (Dimethylpolysiloxane) (Fig. 6, panels F, G) do not affect the OTR_{max} value. The addition of FOAMSTOP™ appears to affect the variability between replicates. All tested antifoams did not negatively affect microbial growth, suggesting their compatibility with the tested biological system, as the initial increase in OTR within the first 12 to 16 h was highly comparable to the antifoam agent free control.

The results suggest adding surface active antifoams to MTP cultivations might be beneficial during screening experiments as it increases the OTR_{max} value and thereby contributes to avoiding oxygen limiting conditions. The influence of the hydrophilic/hydrophobic surface properties of a shake flask wall on the OTR is well described for different materials [81]. Surface wettability directly affects the OTR_{max} . A change in surface tension, caused by the addition of antifoam, could increase the surface area available for gas/liquid mass transfer. Especially, as the used MTPs are manufactured from polypropylene which is hydrophobic, reducing surface wettability [81]. However, further experimental testing would be required to identify the underlying mechanism. The results show that different antifoams have different effects on the bioprocess and they should be considered early on, not only at bioreactor scale. Early testing of antifoam agents can identify biological incompatibility (toxicity or inhibition) and potentially positive effects on secretion. It is crucial to distinguish whether any observed benefit arises from the antifoam's properties or from its effect to increase the OTR_{max} in surface aerated small scale cultivation systems, which can prevent oxygen limitation compared to screenings without antifoam agents.

It is worth noting that in non-surface aerated cultivation systems such as CSTRs, the addition of antifoam agents often results in a significant reduction of the OTR. This effect is commonly attributed to interactions of the antifoam agent at the gas-liquid interface, where it can introduce an additional barrier to oxygen mass transfer, thereby reducing the $k_L a$ value [82].

For this reason, one must exercise caution when directly transferring MTP findings to larger scale, especially with regard to mass-transfer effects. However, insights into the toxicological compatibility or the metabolism of the antifoam agents used should remain transferable across scales as these are biological rather than physicochemical effects.

Filling volume testing to determine maximum oxygen transfer capacity

It is often argued that data obtained from MTP cultivations cannot be translated to larger bioreactor systems due to factors such as insufficient mass transfer of oxygen into the liquid phase [83, 49]. The findings from this study highlight the importance of thoroughly selecting the parameters to achieve oxygen unlimited conditions and sufficient mixing, as this can enable the transfer of results from MTP scale to stirred tank reactor scale [84, 85]. The crucial factors to be considered are filling volume per well, shaking speed, well geometry, and shaking diameter [86, 87]. Here, all the *K. phaffii* strains BG10 Mut⁺, X-33 Mut⁺ and CS310 Mut^S were cultivated at various filling volumes (0.15, 0.3, 0.4, 0.5 and 0.6 mL) using 40 g/L glycerol. The high glycerol concentration ensured reaching oxygen limiting conditions, that become apparent in the formation of an OTR plateau that corresponds to the respective OTR_{max} -level [88] (Fig. 7).

Figure 7, panel A shows the OTR signal over cultivation time for *K. phaffii* BG10 at various filling volumes. The lowest filling volume of 150 µL reaches a maximum OTR of approximately 65 mmol/L/h. The occurring peak and thus lack of a signal plateau indicates oxygen limiting conditions were not reached, despite the high initial glycerol concentration of 40 g/L. All other conditions tested, however, show oxygen-limiting conditions, resulting in the formation of a plateau of the OTR signal. It should be noted that the level of this plateau varies. It starts at a low level and then rises slowly until the signal drops abruptly due to the complete metabolism of the glycerol provided. This slow increase of the obtained OTR plateau level can be explained by a change in osmolarity. Medium osmolarity directly affects the OTR_{max} that is achievable in a cultivation medium [29, 61]. For better illustration, the low plateau OTR values at the beginning (black squares) and the higher plateau OTR values towards the end (red circles), for each tested filling volume were extracted as “maximum oxygen transfer capacity” values and plotted against the respective filling volume (Fig. 7, panel B). This representation also allows a comparison with the empirical correlation (formular 1) described by Dinger et al. [34].

$$OTR_{max} = 0.008 \cdot V_L^{-1.00} \cdot d_0^{0.40} \cdot n^{1.00} \quad (1)$$

The empirical correlation for the OTR_{max} depends on filling volume (V_L [mL]), shaking diameter (d_0 [mm]) and shaking speed (n [rpm]). Despite being valid for 96-round deepwell MTPs the data fits the prediction in the observed parameter space very well. The theoretical OTR_{max} as depicted as a blue dashed line with triangles (Fig. 7, panel B). It also nicely illustrates that the OTR_{max} was not reached for the filling volume of 150 μ L.

Filling volumes larger than 600 μ L were also tested (700 μ L, 800 μ L and 1000 μ L) and the data is depicted in Supplementary Fig. 4. At the tested conditions, these filling volumes did not lead to the formation of an oxygen limited plateau phase, but exhibited considerable variability between replicates, suggesting that cultivation conditions were suboptimal or inconsistent. This is in accordance with literature [27] that states that the capillary forces present in the small polystyrol wells cannot be overcome at 300 rpm and 50 mm shaking diameter. In connection with this theory, it is also necessary to have a minimum shaking speed in order to form a liquid crescent. Furthermore, if the filling volume is too high, the liquid crescent may not reach the bottom of the well, resulting in inhomogeneous mixing in the well. This can lead to sedimentation of cells despite shaking and causes inconsistent results. The results are in accordance with literature as [87] describe filling volumes larger than 600 μ L as critical for fully mixed conditions at 300 rpm and 50 mm in 96-square deepwell MTPs. Duetz et al. [89, 90] describe a similar effect and state that there is a “risk of settling cells” under the described conditions when exceeding a filling volume of 600 μ L per well (see Supplementary Fig. 5). Further, a picture of the MTP from the filling volume screening experiment was taken immediately after the shaker was stopped (Supplementary Fig. 6). The image indicates that cells had already sedimented at filling volumes exceeding 600 μ L, despite continuous orbital shaking, suggesting insufficient mixing or suspension efficiency under these conditions.

These findings highlight the importances of thoroughly selecting the applied cultivation conditions to achieve oxygen unlimited conditions and sufficient mixing. Particularly where MTP screening is performed under oxygen-limited conditions (for example due to choosing a too high filling volume).

Conclusion and outlook

In this study, a late-stage beta version prototype of a microTOM system developed by Kuhner Shaker GmbH was tested to determine relevant parameters necessary for bioprocessing, using three strains of the methylotrophic yeast *K. phaffii* as a model organism.

The study demonstrates that measuring the OTR is a suitable parameter to reveal differences in production strain performance related to different yeast

extract products and media composition (complex versus defined mineral medium) as well as the effect of the selected carbon source (glucose versus glycerol). The microTOM system was found to be suitable for quickly and efficiently testing the optimum temperature for growth. A method was also presented to rapidly estimate the methanol uptake rate for *K. phaffii*. The BG10 strain with the Mut⁺ phenotype showed an uptake rate of 0.21 g/g/h, while *K. phaffii* CS310 Mut^S showed a methanol uptake rate 0.11 g/g/h.

Antifoam agents based on polyalkylene glycols increased the OTR_{max} to values approximately 40 to 50% higher than those obtained under non-antifoam containing conditions. This highlights the importance of including antifoam agents during strain screening in small scale cultivation systems. An increase in OTR_{max} can prevent oxygen limitations and therefore promote improved cell growth leading to enhanced productivity.

Filling volume screening experiments also confirmed that 96-square deep well plates should not be operated at filling volumes exceeding 600 μ L, when operating at 50 mm shaking diameter and 300 rpm.

It is often argued that MTPs offer limited process control, resulting in screening results that lack relevance for bioprocess development and scale-up. This is typically attributed to the operation conditions, as experiments are usually conducted in batch mode, where all nutrients are present from the beginning, resulting in microbial growth at maximum rate. Parameters such as dissolved oxygen and pH are also not controlled. Therefore, the cultivation conditions in MTPs usually differ significantly from the nutrient-limited fed-batch mode conditions that are commonly applied at industrial production scale. In bioreactors, growth rate and thereby metabolic activity is tightly controlled to ensure process stability and efficiency [91]. The extent to which the microTOM online OTR measurement can directly support scale-up from MTP to CSTR scale requires further investigation.

The microTOM system addresses this issue, by allowing the user to rapidly screen a clone library and identify bioprocess parameters such as media composition, effect of media components, growth rate, temperature optimum or substrate uptake rate. Even though the dissolved oxygen levels cannot be controlled, monitoring the OTR can help to avoid oxygen limiting conditions. pH buffers can also be used and the OTR can indicate if the buffer capacity is sufficient in order to prevent inhibitory effects due to pH shifts [69]. Additionally, different feeding technologies are available to be deployed at MTP scale, for example FeedPlates®, feeding rings, microfluidic systems, and enzymatic release systems [92, 84].

The microTOM system advances high throughput screening of microbial hosts organisms by enabling online monitoring of the OTR at 96-well MTP scale. The

number of wells facilitates comprehensive screening with sufficient replicates to enhance data robustness.

Abbreviations

CSTR	Continuously stirred tank reactor
DOT	Dissolved oxygen tension
V_L	Filling volume
OTR_{max}	Maximum oxygen transfer capacity
microTOM	Micro-scale Transfer rate Online Measurement system
MTP	Microtiter plate
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
μ	Specific growth rate
k_{La}	Volumetric mass transfer coefficient

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13036-026-00691-4>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

DW designed and executed the experiments, analysed the data, and prepared the manuscript. CJH provided the tested *K. phaffii* strains and critically revised the manuscript. JCPC and AS provided technical support with the provided microTOM prototype, assisted in trouble shooting and data processing and critically revised the manuscript.

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Data availability

All data supporting the findings of this study are available in the article and its Supplementary Information files. Data generated by the microTOM system are provided as .csv files within the Supplementary Information.

Declarations

Generative AI and AI-assisted technologies in the writing process

The authors used Microsoft Co-pilot during the preparation of this work to refine grammar and punctuation, as well as to ensure clarity in writing. The authors reviewed and edited the content after applying this tool and take full responsibility for the content of the publication.

Competing interests

The authors declare no competing interests.

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