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ORIGINAL PAPER

Application of an improved continuous parallel shaken bioreactor system for three microbial model systems

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Abstract A continuous parallel shaken bioreactor system, combining the advantages of shaken bioreactors with the advantages of continuous fermentation, was specifically manufactured from quartz glass and provides a geometric accuracy of <1 mm. Two different model systems (facultative anaerobic bacterium *C. glutamicum*, and Crabtree-negative yeast *P. stipitis*), whose growth behaviour and metabolite formation are affected by dilution rate and oxygen availability, were studied. The transition from non-oxygen to limited conditions as function of the dilution rate could precisely be predicted applying the approach described by Maier et al. (Biochem Eng J 17:155–167, 2004). In addition, the Crabtree-positive yeast *S. cerevisiae* was simultaneously studied in the continuous parallel shaken bioreactor system and in a conventional 1-L bioreactor, for comparison. Essentially the same results were obtained in both types of bioreactors. However, many more reading points were obtained with the parallel shaken bioreactor system in the same time at much lower consumption of culture media.

Keywords Continuous fermentation · Parallel operation · Medium consumption · Oxygen transfer rate · Crabtree-effect · Shaking parameters

List of symbols

D	dilution rate (1/h)
D_{crit}	critical dilution rate (1/h)
d_o	orbital shaking diameter (mm)
Fr	froude number (–)
g	gravitational force (m/s^2)
n	shaking frequency (rpm)
OTR	oxygen transfer rate [mol/(L h)]
OTR_{max}	maximum oxygen transfer capacity [mol/(L h)]
OTR_{demand}	maximum oxygen transfer rate required at a specific dilution rate [mol/(L h)]
T	temperature (°C)
V_L	filling volume (mL)
$Y_{Oxygen/Substrate}$	oxygen consumption per mol glucose (mol/mol)
$Y_{X/Glu}$	glucose-dependent biomasss yield (g/g)
μ_{max}	maximum specific growth rate (1/h)
$Ø_i$	inner diameter of hoses (mm)

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Introduction

Microbial processes are based on cultivation of microorganisms and are mostly carried out in a liquid culture broth. A number of factors including nutrient contents, product and by-product concentration, osmotic pressure, pH, dissolved oxygen, viscosity and antimicrobial constituents affect the growth and the survival of microorganisms in a culture [19]. Continuous cultivation of microorganisms, compared to batch operation, offers the advantages of constant environmental conditions for biological systems, which can be highly sensitive to process variations [9]. Therefore, continuous cultures are very suitable to study and characterize the specific properties of a microbial strain. During the last 6 decades, many types of continuous reactor systems have been introduced [4, 15, 21–23, 29, 36, 38–40]. The conventional continuous culture systems are

mostly based on stirred tanks. In spite of the numerous advantages, the complex set up and large filling volume of these conventional systems represent a significant barrier for the broad application of continuous culture of microorganisms [33, 40]. Recently a number of miniaturized, mostly stirred parallel bioreactor systems were introduced which partly allow a quasi-continuous operation. These systems are reviewed e.g. by Betts and Baganz [6].

As an alternative to the existing systems we have introduced and fully characterized in our previous study a novel continuous parallel shaken bioreactor system, combining the advantages of shaken culture technology with the advantages of continuous operation [1]. With this new reactor system continuous fermentation experiments can be performed in a cost- and time-saving manner. One of the significant advantages of the new technique is the possibility to simultaneously operate many of the bioreactors in parallel on a single shaker. The geometric reproducibility of these reactor vessels, therefore, plays an important role. The first generation of the continuous parallel shaken bioreactor system consisted of four special reactor vessels modified from the 250-mL Erlenmeyer flasks. An outlet for the overflow of fermentation broth and exhausted gas was melted onto the side face of the flask [1]. However, due to the flow property of molten borosilicate glass during the manufacturing of the modifications of these flasks, it was not possible to achieve a geometric accuracy of <1 mm. The variation in the geometry and the position of the outlets strongly influences the filling volume of each shaken bioreactor. Therefore, it was necessary to determine the specific filling volume of each individual reactor. Besides operational difficulties, this problem represented a barrier for the operation of many more reactors on a single shaker.

Although the experiments carried out in the last study [1] proved the general usefulness of the new technique, the system required improvement with respect to geometric uniformness and handling. It is the aim of this paper to introduce a continuous parallel bioreactor system with well-defined reactor geometry and to demonstrate the applicability of this system for the cultivation of three microbial model systems. Special emphasis is put on ensuring sufficient oxygen supply of the cultures.

Improved vessels and principle of the new continuously operated shaken bioreactor system

New reactor vessels were manufactured from cylindrical quartz glass (Fig. 1) with a diameter and height of 85 mm. In contrast to borosilicate glass used in Erlenmeyer flasks, quartz glass has an extremely low coefficient of thermal expansion and a low refractive index. It is particularly suitable for the required modifications with accurate geometric reproducibility. The quartz outlet tubes with an inner diameter of 2 mm could be melted onto the sides of the quartz glass cylinders at a height of 70 mm applying a jig, resulting in a geometric accuracy of <1 mm (Fig. 1). This cylindrical quartz glass body was clamped between two metal parts (base and cover) and was held together by lathed rods. The sealing of the vessel at the top and the bottom is ensured by silicon rings. The vessel is closed at the top by a borosilicate glass lid carrying two glass screw connectors. The lid is fixed to the upper metal ring by a screw cap made of PEEK. The gas volume of an empty reactor is about 520 mL.

The new system was designed for six parallel bioreactors. It will be termed CosBios system in the following and is schematically shown in Fig. 2. In operation the substrate is continuously delivered from a common feed stock bottle to all individual culture vessels by a single multi-channel peristaltic pump. The aeration rate is adjusted by a gas flow controller for each reactor, and the air is pre-humidified. When the filling volume inside the shaken reactor is increased by feeding and the rotating liquid reaches the lower edge of the outlet, the fermentation broth is accelerated into the outlet by centrifugal force. The excess fermentation broth and exhaust gas leave the culture vessel through the same overflow tube. Thus, the overflow of excess fermentation broth is continuously driven by the exhaust gas. This action keeps the culture volume in the reactor at a constant level. The circulating motion of the fermentation broth induces sufficient mixing and aeration similar to that of a conventional shake flask. Using hoses with different inner diameters on a single multi-channel peristaltic pump, various dilution rates can be simultaneously adjusted in the different vessels in a parallel operation. The combination of hoses with different inner diameters, the rotation rates of the feeding pump, the height of the outlet, the diameter of the cylindrical reactor, the shaking diameter and the shaking frequency of the shaker determines the dilution rates.

In conclusion, the introduced system allows for six parallel continuous fermentations employing only one drive, one feed stock bottle and one multi-channel feeding pump and no outlet pump. Oxygen supply is provided by surface aeration. Therefore, no bubbles are introduced into the fermentation broth and no changing gas hold-up influences the filling volume in the reactors. In contrast, a fermentation system with conventional stirred tank reactors would have required a drive, a feeding pump and additionally an outlet pump for each of the vessels. If an overflow is used for controlling the filling height, changing gas hold-up may influence the filling volume in the reactors.

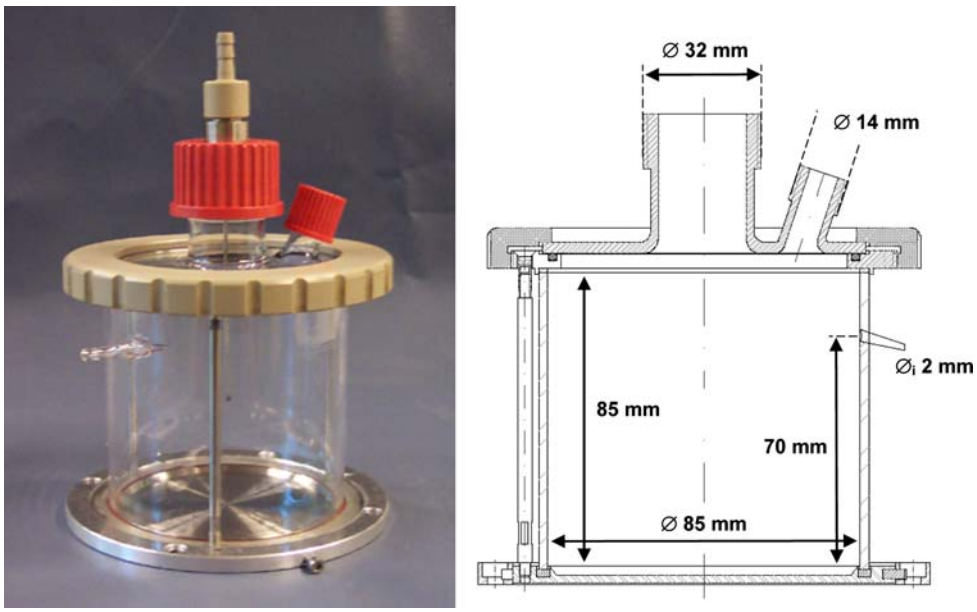

Fig. 1 Picture and mechanical drawing and of a single CosBios reactor with a total volume of 520 mL

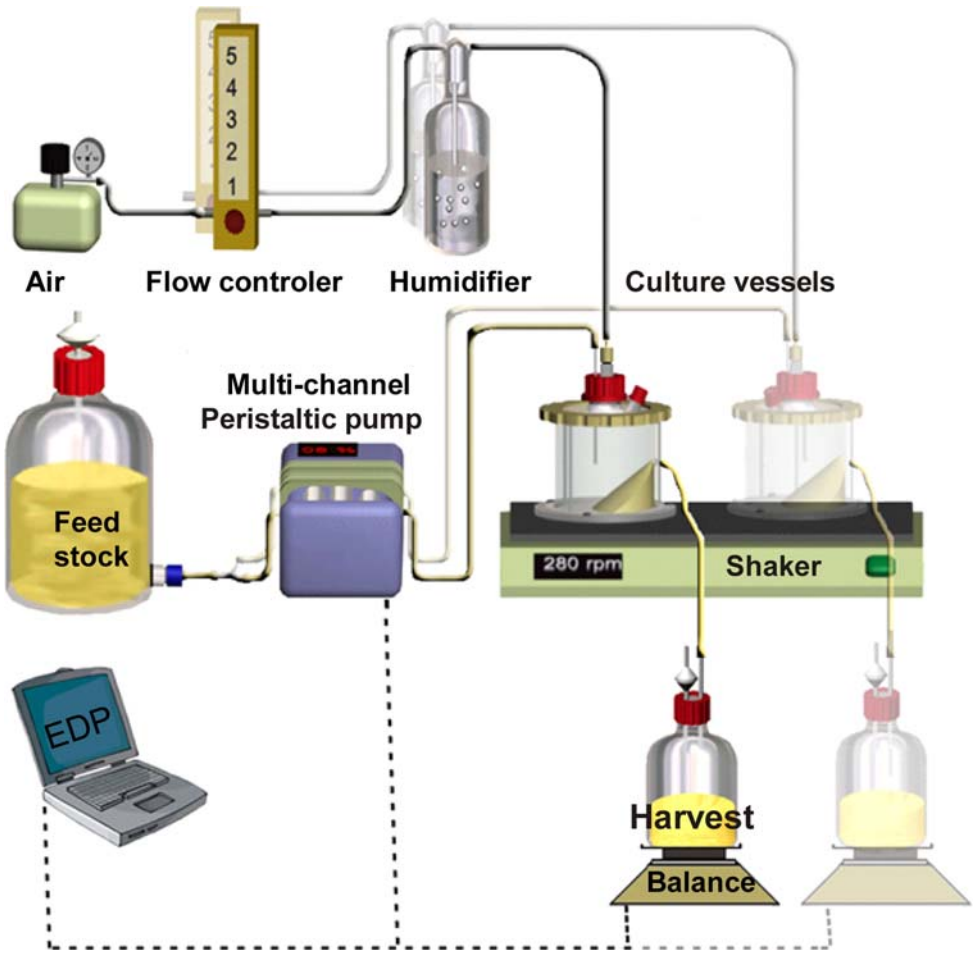

Fig. 2 Set up of the CosBios system

Significance of oxygen supply

Many valuable fine chemicals, food, beverage and pharmaceutical products are produced by aerobic fermentation. In aerobic fermentation processes oxygen is the key substrate. Numerous effects of the extent of oxygen supply on microbial systems are reported [10, 13, 20, 25, 26, 32, 34, 35].

The maximum oxygen transfer capacity (OTR_{max}) in shaken bioreactors depends on the surface-to-volume ratio of the rotating liquid including the wetted reactor wall [27]. It is affected by many factors such as geometrical and operational characteristics of the reactors, type, concentration and viscosity of the culture media. Therefore, OTR_{max} represents the most important parameter controlling the design and operation of a new shaken culture technique and may limit its usage in biological experiments. Thus, it was essential to investigate OTR_{max} of the novel culture vessels.

Materials and methods

Strains

Three strains, whose growth behaviour and metabolite formation are obviously affected by oxygen supply, were selected. The wild type-strains of *Corynebacterium glutamicum* (*ATCC 13032*) and *Pichia stipitis* (*CBS 5774*) were used to monitor oxygen limitation in continuous culture. To demonstrate the performance of the CosBios system, fermentations of *Saccharomyces cerevisiae* (*ATCC 32167*) were carried out in comparison to a 1-L continuous stirred tank fermentor.

Corynebacterium glutamicum is a facultative anaerobic bacterium and can utilize glucose, and organic acids (lactate, acetate) as carbon sources. Oxygen deprivation leads to anaerobic growth, resulting in the formation of organic acids, such as lactate or succinate, from glucose [30]. The two yeasts *P. stipitis* and *S. cerevisiae*, reveal fundamentally different modes of metabolic regulation in glucose-containing media. In the Crabtree-positive yeast *S. cerevisiae*, elevated glucose concentration induces a catabolite repression, resulting in low levels of transcription of genes involved in respiration. When the dilution rate in a continuous culture is increased above a certain value, the critical dilution rate (D_{crit}), ethanol formation occurs and the biomass yield decreases. In contrast to the Crabtree-positive yeast *S. cerevisiae*, the enzymes pyruvate decarboxlase and alcohol dehydrogenase of the Crabtree-negative *P. stipitis* are regulated independently of the glycolytic flux [31]. This yeast exhibits predominantly respirative metabolism even at high glucose concentrations due to relatively high activities of acetaldehyde dehydrogenase and acetyl coenzyme-A synthetase. In case of oxygen deprivation ethanol formation occurs [37].

Media

Two different media were used in the experiments of *C. glutamicum*. A simple complex medium for pre-culture (20 g/L glucose, 10 g/L yeast extract (Roth, Germany), 10 g/L casein peptone (Roth, Germany), 2.5 g/L NaCl and 0.25 g/L $MgSO_4 \cdot 7H_2O$) [8] and a chemically defined medium for the main cultures (20 g/L, respectively 10 g/L glucose, 20 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 2 g/L K_2HPO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2$, 0.03 g/L protocatechuic acid, 0.2 mg/L D-biotin, 10 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $MnSO_4 \cdot H_2O$, 1 mg/L $ZnSO_2 \cdot 7H_2O$, 0.2 mg/L $CuSO_4$, 0.02 mg/L $NiCl_2 \cdot 6H_2O$, pH 7.0) [14]. Agar plates were prepared by the addition of 15 g/L Bacto-Agar (Difco, Germany) into complex medium. The pre-culture and main culture of *P. stipitis* were carried out in a chemically defined PMM-YE medium [11] prepared by combining the following separate solutions for 1 L; solution A (30, 15 or 5 g glucose depending on experimental set-up was dissolved in 150 mL of deionised water and autoclaved), solution B (10 g granulated yeast extract (Merck, Germany) were dissolved in 100 mL deionised water and autoclaved), solution C (18.75 g KH_2PO_4, 6 g $(NH_4)_2HPO_4$, 1.13 g $MgSO_4 \cdot 7H_2O$ and 5 g $(NH_4)_2SO_4$ were dissolved in 700 mL deionised water and pH was adjusted to 5.0 by addition of HCl before autoclavation), and solution D (1.7 g YNB without amino acids (Difco, Germany) and ammonium sulphate was dissolved in 50 mL deionised water and sterile filtered). The pH of the final solution was adjusted to pH 5.2. Agar plates were composed of 20 g/L glucose, 20 g/L peptone (Roth, Germany), 10 g/L yeast extract (Roth, Germany), 20 g/L Bacto-Agar (Difco, Germany). Cultivations of *S. cerevisiae* were carried out in a complex growth medium with 5 g/L or 10 g/L glucose, 5 g/L yeast extract (Roth, Germany), 5 g/L casein peptone (Roth, Germany), and 1 drop of antifoam agent per litre (Plurafac LF 1300, BASF, Ludwigshafen, Germany). Antifoam agent was not required in shake flask but was used to provide a similar chemical environment to that of conventional stirred tank fermenter. All chemicals were delivered by Fluka, Germany unless specified otherwise. The filling volume of each parallel shaken bioreactor was 27 mL. The corresponding requirement of fresh medium is, for a main culture running 10 days at dilution rates varying between 0.1 and 0.7, approx. 10 L.

Cultivation and modes of operation

Agar plates of *C. glutamicum* and *S. cerevisiae* were weekly inoculated and stored at 4 °C, while the *P. stipitis*

plates used for inoculation were always 24-h-old. All precultivations were carried out by transferring a loop of cells from agar plates into a 250-mL Erlenmeyer flask containing 27 mL preculture medium, followed by incubation at 30 °C for 12 h. The shaker (Adolf Kühner AG, Birsfelden, Switzerland) adjusted to a shaking diameter of 50 mm was operated at a shaking frequency of 275 rpm. After precultivation, the culture broths were centrifuged for 20 min at 4,000*g*. The pellet was washed two times with 0.9% NaCl and centrifuged again. To prepare inocula, the biomass was resuspended in the media used for the main culture to obtain an optical density of 1.5. The bioreactors containing 26 mL medium for main cultivation were inoculated with 1 mL inocula and were operated in batch mode at 30 °C for 12 h before continuous operation was started. The shaker (shaking diameter 50 mm) was operated in all experiments at a shaking frequency of 275 rpm, except for an experiment with *C. glutamicum*, where it was operated at 300 rpm in order to eliminate oxygen limitation. The continuous cultures were assumed to be in steady state when five retention times have been passed.

The volumetric aeration rate of each shaken reactor was 2 vvm and was adjusted by a precision pressure controller (FDR. 02 B2, Wika Alexander Wiegend GmbH & Co., Klingenberg, Germany) and a thermal mass flow controller (F-210C-FB, Bronkhorst High Tech B.V., Ruurlo, The Netherlands). The substrate feed was provided by a multichannel peristaltic pump (IPC-N-8, Ismatech, Glattbrug, Switzerland). To attain different dilution rates for each reactor, pump hoses (Ismaprene, Ismatech Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany) with six different inner diameters of 1.02, 1.14, 1.30, 1.42, 1.52, and 1.65 mm were used. In addition to the variation of the hose diameter, different rotation rates of the feeding pump between 0.34 and 1 rpm were utilized to vary the dilution rates in a wide range. Table 1 shows that the range of dilution rates at the different rotation rates of the feed pump overlaps. This was useful to prove the reproducibility of results obtained before at different rotation rates of the feed pump, as explained in more detail later (Fig. 8). The reactors, including all fittings and tube connections, were sterilized in an autoclave according to standard procedures for 20 min. at 121 °C and 1.2 bars.

Table 1 Dilution rates of CosBios reactors (shaken at 275 rpm with 27 mL filling volume) resulting from different combinations of the inner diameter of the hoses ($Ø_i$) and the rotation rates of the feeding pump

$Ø_i$ (mm)	Dilution rate (1/h)			
	At rotation rates of the feeding pump (rpm)			
	0.34	0.56	0.78	1
1.02	0.10	0.16	0.23	0.29
1.14	0.12	0.20	0.28	0.36
1.30	0.15	0.26	0.36	0.46
1.42	0.18	0.30	0.42	0.54
1.52	0.21	0.34	0.48	0.62
1.65	0.24	0.40	0.56	0.72

Sampling and analytical methods

For sampling of the CosBios system, the harvest bottles were replaced by sample tubes placed in an icebox. The shaking frequency was increased from 275 to 300 rpm for about 1 min, resulting in an increased outflow of fermentation broth. In this way, about 7 mL of culture broth could be harvested from the reactors. After completion of this procedure, the standard settings were readjusted again. For the determination of cell dry weight, a 2-mL sample was centrifuged for 20 min at 4,000*g*. The pellet was washed two times, and the biomass was dried for 24 h at 105 °C and weighed. Cell-free extracts were frozen and kept at −20 °C for later quantification of the glucose and ethanol concentration by HPLC.

Measurement of the maximum oxygen transfer capacity (OTR_{max})

The maximum oxygen transfer capacity (OTR_{max}) in the shaken bioreactors was measured by using the cobalt-catalyzed sulphite oxidation method described in Hermann et al. [24]. The vessels were filled with defined liquid volumes of a standard solution of 0.5 M sulphite. Depletion of sulphite is accompanied by a drop of the pH value, which can be followed by the colour change of a pH indicator. The colour change was recorded by a camera (DCR-VX700E, Sony, Germany). The OTR_{max} is proportional to the length of the sulphite oxidation time until sulphite depletion.

On-line measurement of the OTR_{max} in shaken flasks was carried out in the Respiratory Activity Monitoring System (RAMOS) described in Anderlei and Büchs [2] and Anderlei et al. [3]. In this device, the cultivations were conducted in specially designed measuring flasks, ensuring hydrodynamics and gas phase conditions identical to those in normal cultures in Erlenmeyer flasks with cotton plugs. This method is based on the measurement of the decline of the gaseous oxygen partial pressure in culture vessels [2].

Results and discussion

Bioreactor set up and operating characteristics

All experiments carried out for the characterization of the first generation of continuous shaken bioreactor system [1] were repeated for the improved design of the CosBios system. The steadiness of liquid overflow and influence of aeration rate, substrate feeding rate, shaking frequency (data not shown) and shaking diameter on filling volume proved reproducible results.

Influence of shaking frequency (n) and shaking diameter (d_o) on filling volume (V_L)

The filling volume in the reactors, determined by the reactor geometry, the shaking frequency and the shaking diameter, is the key parameter controlling the dilution rate. Thus the filling volume of the reactors was investigated by changing the shaking frequency (150–325 rpm) and the shaking diameter (25, 50, and 70 mm). Experiments were carried out with six parallel shaken reactors. Complex growth medium for *S. cerevisiae* specified above was used as test solution. The airflow rate was kept constant at 45 mL/min. As shown in Fig. 3, the filling volume decreases with increasing shaking frequency as well as with increasing shaking diameter. At a shaking frequency of 275 rpm the filling volume in the reactors amounts to 55, 27 and 20 mL at shaking diameters of 25, 50 and 70 mm, respectively, while 39, 20 and 16.5 mL were obtained at 300 rpm. The fluctuations of the filling volumes in six reactors for each set point were very small for shaking frequencies from 200 to 325 rpm. This result demonstrates that the improved reactor design ensured the same filling volumes in all culture vessels at shaking frequencies higher than 200 rpm.

In order to be able to design experiments, a mechanistic model is desirable for the prediction of the filling volume and the maximum oxygen transfer capacity as function of the operating conditions. Therefore, the approach of Maier et al. [28], based on the liquid distribution inside a shaken bioreactor [7] was modified and adapted to the cylindrical shape of the improved continuous reactors. The calculated values are shown in Fig. 3 as solid curves. The dependency of the filling volume on shaking frequency and on shaking diameter is very well represented by this model.

Effect of shaking parameters and vessel shape on the maximum oxygen transfer capacity (OTR_{max})

As shown above, the shaking frequency and shaking diameter have a strong effect on the filling volume and, hence, on the surface-to-volume ratio of the rotating liquid. This influences the maximum oxygen transfer capacity (OTR_{max}). Measurements for the determination of the OTR_{max} in the CosBios reactors were carried out by using the sulphite oxidation method. The OTR_{max} values were determined at different shaking frequencies (150–300 rpm) on a shaker with a shaking diameter of 50 mm. For comparison conventional Erlenmeyer flasks and a cylindrical vessel of the same diameter as the continuous reactors but without outlet were included in the study. The latter two vessels were filled with 20 mL sulfite solution. This volume certainly does not change with shaking frequency, in contrast to the continuous reactors, where the filling volume changes from 130 to 20 mL for shaking frequencies between 150 and 300 rpm. The volumetric aeration rate for the continuous reactors was kept constant at 2 vvm according to the filling volumes represented in Fig. 3.

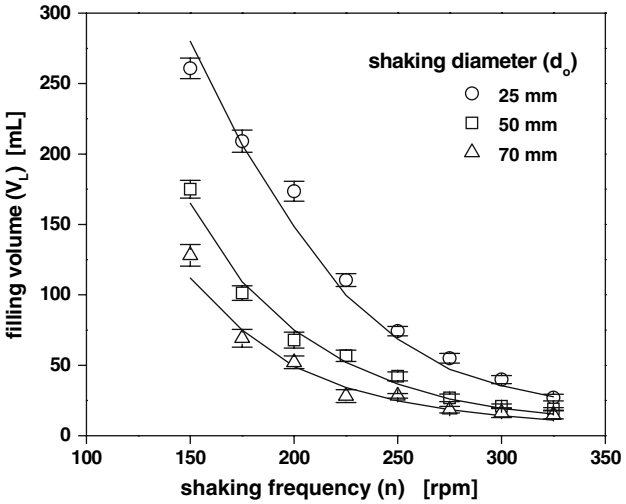

Fig. 3 Operational filling volumes in the CosBios bioreactors versus shaking frequencies at different shaking diameters. Comparison of model calculations (*solid curves*) and measured values (*points*). Air flow rate = 45 mL/min, substrate feed rate = 0.1 mL/min, temperature = 30 °C. *Vertical bars* represent the standard deviation of six parallel reactors

The results represented as open symbols in Fig. 4 show that the OTR_{max} in the CosBios bioreactors is very low at low shaking frequencies due to high filling volumes and low surface-to-volume ratio of the rotating liquid. However, it increases exponentially with increasing shaking frequency and respectively decreasing filling volume. It is also obvious that the OTR_{max} in a cylindrical shaken vessel at a constant filling volume of 20 mL is significantly higher than in an Erlenmeyer flask and increases substantially at shaking frequencies $\geq$150 rpm. The OTR_{max} of the cylindrical vessel with the filling volume of 20 mL at 300 rpm reaches 0.05 mol/(L h), which is 1.6-fold higher than that of an Erlenmeyer flask operating at the same condition. This is due to the fact that the liquid is spread over a much

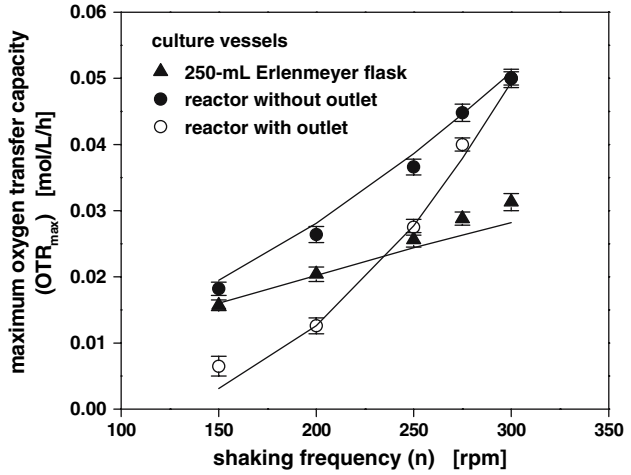

Fig. 4 Comparison of the maximum oxygen transfer capacity (OTR_{max}) in a 250-mL Erlenmeyer flask and in the CosBios bioreactors with- and without outlets versus shaking frequency at a shaking diameter of 50 mm, temperature = 30 °C, aeration rate in reactors with outlet = 2 vvm. The Erlenmeyer flask and the cylindrical CosBios reactor without outlet contained a constant filling volume of 20 mL. Model calculations are shown as *solid curves* and the measured values as points. *Vertical bars* represent the standard deviation of three parallel vessels.

larger wall area by the centrifugal force in a cylindrical vessel than in a conical one. At 250 rpm the continuous reactor with outlet shows about the same OTR_{max} as the Erlenmeyer flask, although the filling volume in the continuous reactor (44 mL) is larger than in the Erlenmeyer flask (20 mL). At 300 rpm the filling volume of the continuous reactor with outlet is similar (20 mL) to that of the cylindrical vessel without outlet and has the same OTR_{max}.

It is noteworthy that all measurements agree quite well with the calculations by the modified model according to Maier et al. [28], which does not contain any fitting parameter. It also proves that this tool can be used to design a continuous shaken bioreactor system and to choose operating conditions ensuring a sufficient oxygen supply to the cultured cells. It should also be pointed out that the OTR_{max} values discussed above only apply to the sulfite system. If culture media are used, which usually contain smaller amounts of dissolved compounds, the ionic strength is lower and, therefore, the oxygen solubility and diffusion coefficient, i.e. the k_L value is higher [28]. This results in a higher OTR_{max} for culture media. It has been shown for several culture systems that there is a linear relationship between the OTR_{max} of the respective culture media and the sulfite system. Details for the specific culture media investigated in this work will be specified later.

In order to design the optimal shaking parameters for biological experiments, Fig. 5 provides the shaking frequency and filling volume on shakers with three different shaking diameters (25, 50 and 70 mm) as function of the required OTR_{max}. For higher oxygen demand of a microbial culture higher shaking frequencies and, as a result, lower filling volumes have to be chosen. The shaking diameter does not have a strong influence on the resulting filling volumes, whereas higher shaking frequencies are required for small shaking diameters at the same OTR_{max}. If it is considered that bacterial and fungal microorganisms require oxygen transfer rates typically in the range of 0.05–0.25 mol/(L h) [41], the OTR_{max} may be insufficient on a shaker with a shaking diameter of 25 mm. Shakers with higher shaking diameters (50 and 70 mm) provide sufficient oxygen at shaking frequencies ≥275 rpm.

The acceleration of the excessive culture broth into the outlet is also an important operational parameter for the continuous operation. The centrifugal force is induced in a shaken bioreactor in radial direction by the orbital motion of the shaker table. This force is represented in Fig. 5 by the Froude number (Fr), which is defined as the ratio of inertial forces to gravitational forces according to Eq. (1).

$$Fr = \frac{(2\pi \cdot n)^2 \cdot d_o}{2g} \quad (1)$$

As shown in Fig. 5 the Froude number (Fr) is increased by increasing the shaking diameter. Therefore, a large shaking diameter provides a higher acceleration of the excessive culture liquid into the outlet compared at a fixed OTR_{max}.

Although a shaking diameter of 70 mm seems to be advantageous in terms of more oxygen transfer at the same shaking frequency, a shaker adjusted to a shaker diameter of 50 mm was chosen for further experiments. The reason for this decision was of practical nature. During the sampling procedure, described in the "Materials and methods" part, increasing the shaking frequency does not provide

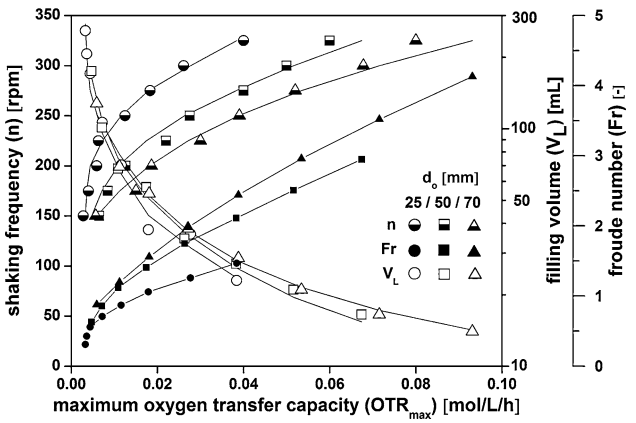

Fig. 5 Shaking frequency (n), resulting filling volume (V_L) and calculated Froude number on shakers with three different shaking diameters (d_o) of 25, 50 and 70 mm as function of the required maximum oxygen transfer capacity (OTR_{max}) of the culture. Model calculations are shown as *solid curves* and the measured values as *points*

enough sample for analytics in case of a shaking diameter of 70 mm. Increasing the shaking frequency from 275 to 300 rpm for a shaking diameter of 50 mm provides 7 mL overflow of culture broth and a reduction of filling volume from 27 to 20 mL, whereas the amount of overflow at a shaking diameter of 70 mm results just in 3.5 mL and the filling volume is reduced from 21 to 16.5 mL.

Biological validation

Examination of oxygen limitation in a continuous culture of C. glutamicum

C. glutamicum produces lactate, when oxygen is limited during aerobic growth [12]. To examine the effect of oxygen limitation in a continuous fermentation, the concentrations of biomass, glucose and lactate versus dilution rate were investigated in the CosBios system (Fig. 6). A continuous fermentation of *C. glutamicum* was carried out using a chemically defined medium containing 20 g/L glucose as C-source (Fig. 6a). The biomass decreases significantly with dilution rate. Already at a dilutions rate of 0.2 1/h a significant glucose concentration well above the limiting level was detected. Concurrently lactate appears. This is a clear indication for oxygen limitation.

Figure 6b shows another continuous culture experiment, which was conducted at lower glucose concentration in the feed of 10 g/L. In this experiment a constant biomass yield of about 0.49 g/g was observed for dilution rates $D < 0.4$ 1/h. However, the biomass yields decreased at the dilution rates >0.4 1/h. The appearing lactate formation and a high residual glucose concentration in this range also indicates the onset of an oxygen limitation.

Figure 6c shows a continuous culture experiment with 10 g/L glucose in the feed at a shaking frequency increased from 275 to 300 rpm, leading to a reduction of the filling volume from 27 to 20 mL. As explained above, this results in a twofold effect in favour of increasing the capacity of oxygen transfer. Figure 6c shows a constant biomass yield of 0.47 g/g for all dilutions rates $D < 0.6$ 1/h. The complete absence of lactate formation and a sharp increase of glucose concentration at the wash out point indicates that no oxygen limitation is experienced under these conditions. Therefore, the maximum specific growth rate (μ_{max}) of *C. glutamicum* could be determined as 0.56 1/h under given non-oxygen limited cultivation conditions.

Estimation of the oxygen demand of a culture to avoid oxygen limited conditions

As demonstrated by the three independent continuous culture experiments in Fig. 6, it is a drawback of the current stage of development of the CosBios system that the dissolved oxygen concentration cannot yet be measured. Therefore, an oxygen limitation should be excluded employing another approach.

The oxygen demand of a microorganism as function of the dilution rate can be determined using Eq. (2).

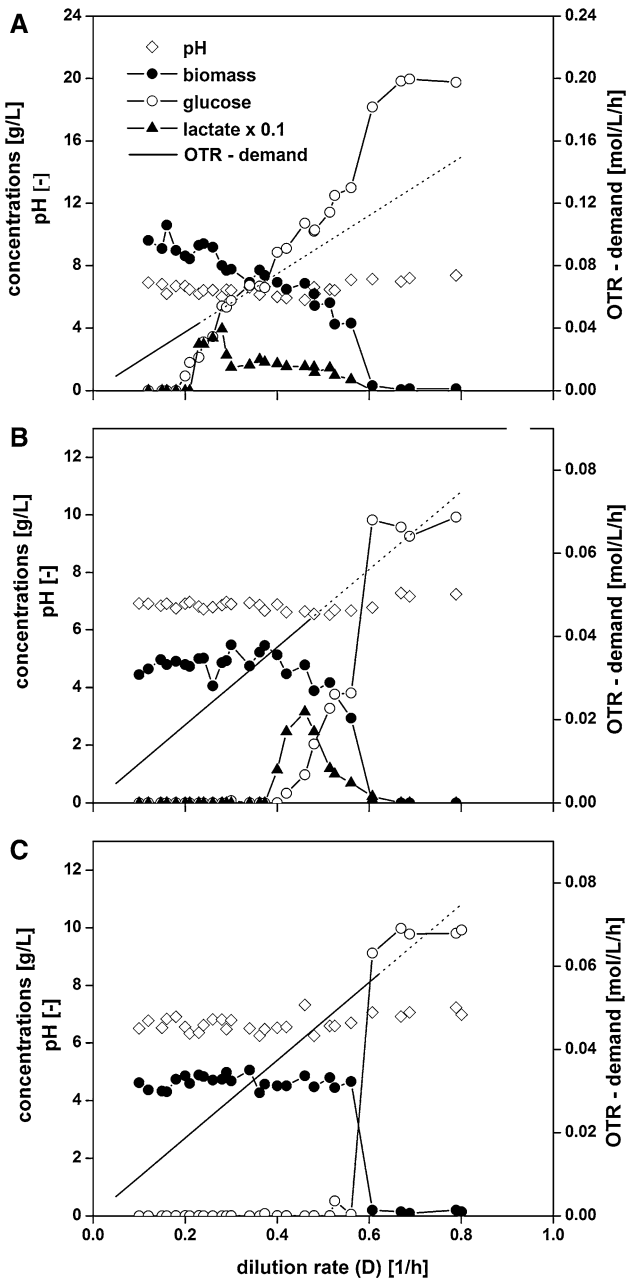

Fig. 6 Fermentation of *C. glutamicum* in the CosBios system; **a** 20 g/L and **b** 10 g/L glucose in the feed stock and a shaking frequency of 275 rpm, resulting in a filling volume of 27 mL; **c** 10 g/L glucose in the feed stock and a shaking frequency of 300 rpm, resulting in a filling volume of 20 mL, shaking diameter = 50 mm, aeration rate = 2 vvm, 30 °C. The OTR-demand, calculated with Eq. (2), is represented as a *continuous line* for non-oxygen limited conditions and as *dashed line* for oxygen limited conditions

$$OTR_{demand} = D \cdot C_S \cdot Y_{Oxygen/Substrate} \quad (2)$$

In this equation the oxygen consumption per mol glucose ($Y_{Oxygen/Substrate}$) can be estimated either theoretically, using a stoichiometric balance of a culture [5], or practically, using measurements of the oxygen transfer rate (OTR) over time. The integral of an OTR profile will provide an exact experimental value of $Y_{Oxygen/Substrate}$. Such OTR profiles can be obtained from exhaust gas measurements of a stirred tank fermentor or, much more conveniently, using a device for online measurement of the oxygen transfer rate (RAMOS), as described in the "Materials and methods".

Therefore, the maximum oxygen transfer capacity of the *C. glutamicum* culture was investigated as function of the operating conditions with a RAMOS device (Fig. 7). All three cultures show the same kinetics in the first part of the fermentation. The culture with only 10 g/L glucose is terminated after about 7 h, indicated by the sharp decrease of the oxygen transfer rate. The cultures with 10 mL filling volume and 10 and 20 g/L glucose show a horizontal plateau at a OTR level of 0.054 and 0.032 mol/(L h), respectively, which is a clear indication of an oxygen limitation during this phase of the fermentations [2]. Calculating the integral of the OTR curves, the oxygen consumption of *C. glutamicum* per mol glucose ($Y_{Oxygen/Substrate}$) was determined as 1.7 mol oxygen/mol glucose consumed. With this value a linear relationship between dilution rate and the relevant OTR-demand was calculated using Eq 2. The results are depicted in Fig. 6 as continuous lines for the range of dilution rates where the OTR demand is lower than the maximum oxygen transfer capacity (OTR_{max}) of the CosBios system, whereas the dashed part of the lines indicates the range of dilution rates where the culture suffer from the oxygen limitation due to higher oxygen demand than the maximum oxygen transfer capacity of the culture system.

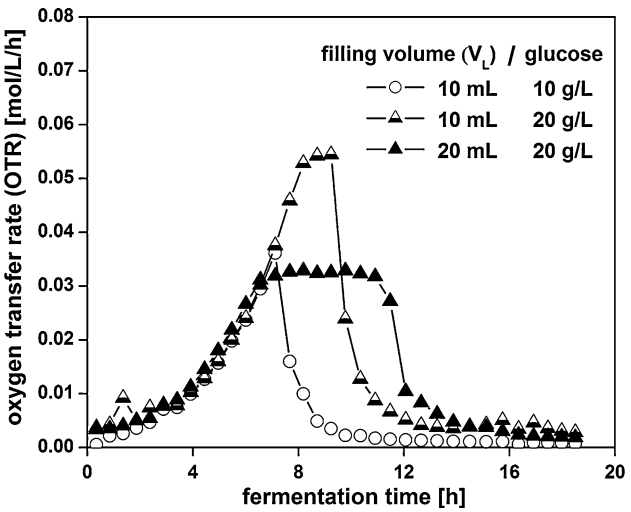

Fig. 7 Online measurement of oxygen transfer rate during a cultivation of *C. glutamicum* in the RAMOS device ($n = 275$ rpm, $d_o = 50$ mm, $T = 30$ °C) with variation in filling volume (20 and 10 mL) and glucose concentration (10 and 20 g/L)

For the estimation of the point of transition from unlimited to limited conditions, the maximum oxygen transfer rate capacity (OTR_{max}) of the reactors of the CosBios system is required for the culture medium used for *C. glutamicum*. It was determined through comparison of the results obtained in the RAMOS device for the sulfite system and the culture medium for the given operating conditions. The OTR_{max} of the 0.5 M sulfite system at 275 rpm (20 mL filling volume) were taken as 0.029 mol/(L h) from Fig. 4. The corresponding value for the culture medium was obtained from the horizontal plateau of the culture with 20 g/L glucose in Fig. 7 as 0.032 mol/(L h). Though, the proportionality factor between the OTR_{max} values of a culture of *C. glutamicum* and a 0.5 M sulfite solution is calculated as 1.11, multiplying this factor with the OTR_{max} of 0.040 mol/(L h) obtained in the CosBios reactors via sulphite method operating at 275 rpm with a shaking diameter of 50 mm (Fig. 4), the OTR_{max} of a *C. glutamicum* culture is estimated as 0.044 mol/(L h). In case of any OTR-demand higher than 0.044 mol/(L h), the continuous culture of *C. glutamicum* in the CosBios reactors will suffer from oxygen deprivation under these shaking conditions.

The calculated transition from non-limited conditions, indicated by the change from a continuous to a dashed line, agree in Fig. 6a and b well with the dilution rate above which lactate as anaerobic metabolite appears in the culture broth and glucose concentration increases. In contrast, Fig. 6c does not show any experimental evidence for oxygen limited conditions, which is also proven by the calculated maximum oxygen transfer capacity of 0.058 mol/(L h) at 300 rpm. This value is obtained by multiplying the corresponding sulfite value (0.05 mol/(L h)) from Fig. 4 by 1.11. Therefore, the described method can be used to design experiments such that oxygen limited conditions can be excluded. In this case, online measurement of the dissolved oxygen concentration in the fermentation broth is not really required.

Examination of oxygen limitation in the continuous culture of crabtree-negative P. stipitis

In addition to *C. glutamicum*, continuous cultivations of *Pichia stipitis* were carried out to investigate the performance of the CosBios system. As it is described in literature [16, 31], *P. stipitis* is a Crabtree-negative yeast and produces ethanol under oxygen-limited conditions. Referring to Fig. 8 not only the obtained specific results are

presented and discussed, but also the experimental procedure is briefly described to illustrate the handling of the CosBios system.

In this study, a continuous culture of *P. stipitis* has been conducted at first with high concentration of glucose (30 g/L) in the feedstock. As described in "Materials and methods", six set points of dilution rate can be adjusted in parallel depending on the rotation rate of the substrate-feeding pump. The rotation rate of the feeding pump was initially 0.34 rpm, resulting in dilution rates between 0.1 and 0.24 (Table 1), as also depicted at the top of Fig. 8. After five retention times at 0.34 rpm, samples from each reactor were harvested and analyzed for biomass, glucose and ethanol concentration. As shown in Fig. 8, the biomass concentration dropped from 10.1 to 5.6 g/L and the glucose-dependent biomass yield ($Y_{X/Glu}$) from 0.33 to 0.19 g/g. The fact that ethanol could be detected in four reactors pointed to an oxygen limitation. Without interrupting of culture system, the used feed stock bottle containing 30 g/L glucose was replaced by a new feed stock bottle containing 15 g/L glucose. The system was perpetually operated for the next five retention times at the same rotation rate of the feeding pump (Fig. 8). After analysis of the samples, the rotation rate of the feeding pump was increased to 0.56 rpm, in order to enlarge the range of dilution rates. As mentioned earlier, the two lowest dilution rates at a rotation rate of 0.56 rpm lie in the range of the dilution rates adjusted at 0.34 rpm (see top of Fig. 8). For the lowest six dilution rates a similar biomass concentrations of 6.5 g/L with a biomass yield ($Y_{X/Glu}$) of about 0.43 g/g and no ethanol formation was obtained. For the higher dilution rates a decrease of the biomass concentration and significant ethanol formation was found, which again indicates an oxygen limitation. In the following step, the operation was continued at the same rotation rate of the feeding pump (0.56 rpm) by replacing the feed stock bottle with one with a glucose concentration of 5 g//L (Fig. 8). Finally this experiment was completed by operating the feeding pump at a rotation frequency of 0.78 rpm. In all reactors, $Y_{X/Glu}$ was obtained as 0.46 g/g and no ethanol was found.

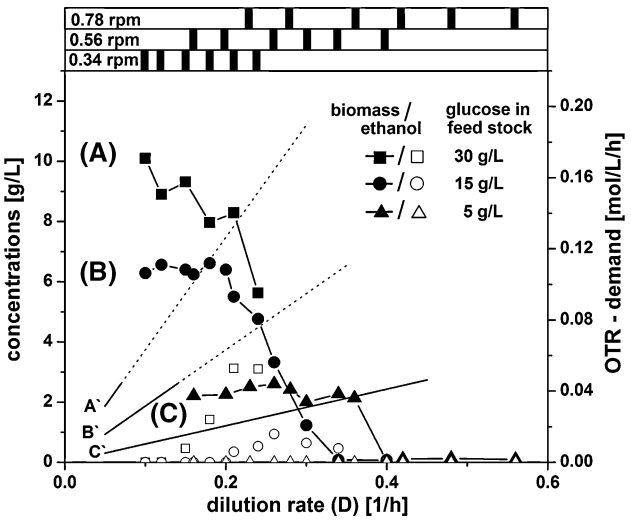

Fig 8 Fermentation of *P. stipitis* in the CosBios system with the variation of glucose concentration in feeding stock; **a** 30 g/L glucose in the feed stock and at a rotation rate of the feeding pump of 0.34 rpm; **b** 15 g/L glucose and a rotation rate of the feeding pump of 0.34 and 0.56 rpm; **c** 5 g/L glucose at a rotation rate of 0.56 and 0.78 rpm. ($n = 275$ rpm, $V_L = 27$ mL, $d_o = 50$ mm, aeration rate = 2 vvm, $T = 30$ °C). The sketch above the diagram indicates the dilution rates corresponding to the rotation rates of the feeding pump. Oxygen transfer rate (OTR) demand is represented with *continuous lines* for oxygen unlimited conditions and with *dashed lines* for oxygen limited conditions

As already explained in detail above for *C. glutamicum*, the maximum oxygen transfer capacity for the culture medium of *P. stipidis* and $Y_{Oxygen/Substrate}$ (3.81 mol/mol) was determined in the RAMOS system. From these values the relationship between the oxygen demand of the culture and the dilution rate was calculated according to Eq. (2). In Fig. 8 non-oxygen limited conditions are indicated by continuous lines, whereas dashed lines represent oxygen-limited conditions. Absolutely consistent results are obtained. The continuous culture with 30 g/L ethanol is oxygen limited at all dilution rates investigated and only relatively low $Y_{X/Glu}$ values smaller than 0.33 g/g are obtained. The continuous culture with 15 g/L glucose shows constant $Y_{X/Glu}$ values 0.43 g/g in the range of dilution rates without oxygen limitation. The culture at a glucose concentration of 5 g/L is non-oxygen limited at all dilution rates, supported by the lack of ethanol formation and the calculated oxygen demand and OTR_{max}. The biomass yield $Y_{X/Glu}$ is similar (0.46 g/g) as for non-oxygen limited conditions in a medium with 15 g/L glucose. The maximum specific growth rate μ_{max} of *P. stipitis* in continuous culture was determined as 0.36 1/h. The whole experiment providing 30 steady state points was carried out within only 15 days with a total consumption of 10 L medium.

Comparative study on continuous culture of S. cerevisiae *in the CosBios system and in a 1-L stirred tank*

The availability of glucose has a strong influence on the respiration of the Crabtree-positive yeast *S. cerevisiae*, in contrast to the Crabtree-negative yeast *P. stipitis*, which responds only to the oxygen availability rather than to glucose concentration [17, 18]. This phenomenon known as Crabtree-effect is characterized in continuous culture of *S. cerevisiae* by a critical dilution rate above which ethanol formation occurs, while the biomass yield decreases. In this

study, *S. cerevisiae* was cultivated in two different media, (a) including 5 g/L glucose to investigate the regulation of respiratory and fermentative metabolism and (b) including glucose of 10 g/L to examine the continuous culture of *S. cerevisiae* under oxygen limitation. All experiments were simultaneously carried out in the CosBios system and in a 1-L stirred tank fermentor.

Figure 9a shows the results of continuous cultivations in simple complex medium containing 5 g/L glucose. There is a perfect agreement between the results from the CosBios system and the 1-L stirred tank fermentor. The biomass concentration drops from 2.4 to 1 g/L at dilution rates $D > 0.26$ 1/h. This correlates with the formation of up to 1.3 g/L ethanol at $D = 0.4$ 1/h. More set-points and smaller intervals between the set-points in the range of dilution rate, where the Crabtree-effect is emerging, could be adjusted with the CosBios system. Simultaneous operation of six bioreactors clearly shortens the total duration of experiments to create an *X–D* diagram like Fig. 9a. Just

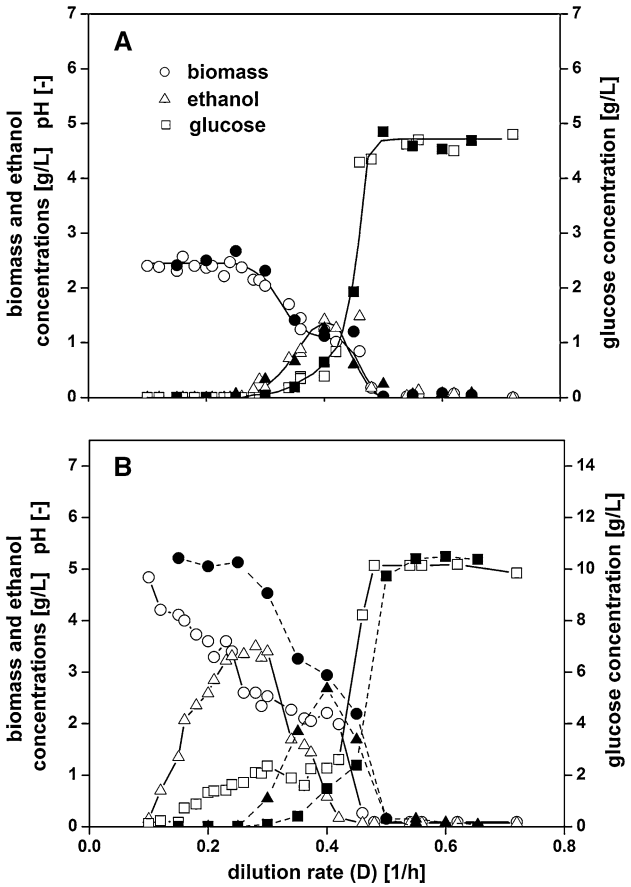

Fig. 9 Fermentation of *S. cerevisiae* in the CosBios system represented by open symbols ($n = 275$ rpm, $V_L = 27$ mL, $d_o = 50$ mm, aeration rate = 2 vvm, $T = 30$ °C) and in a conventional 1-L stirred tank fermenter represented by *closed symbols* (aeration rate = 1 vvm, stirring speed = 900 rpm). **a** 5 g/L and **b** 10 g/L glucose in the feeding medium

9 days were required to create the *X–D* diagram with 24 steady state dilution rates, consuming only 10 L of medium, whereas 14 days were required to generate a similar diagram with only 12 steady states, consuming over 80 L of medium in the conventional 1-L stirred tank fermenter. To generate also 24 steady states in the 1-L stirred tank, 212 L of medium and 26 days would have been required.

A second comparison was performed by feeding the culture with a stock solution containing 10 g/L glucose (Fig. 9b). The dissolved oxygen concentration in the stirred tank fermentor was monitored by an electrode and never reached oxygen-limiting levels. Therefore, the results are qualitatively very similar to that in Fig. 9a. The biomass concentration was more or less constant up to a critical dilution rate of $D = 0.3$ 1/h. At higher dilution rates, a decline in biomass yield followed by ethanol formation was determined. At dilution rates, $D \geq 0.35$ 1/h glucose concentration starts to rise and consequently biomass and ethanol concentration decreased.

The fermentation in the CosBios system differs from that in the 1-L stirred tank. The biomass concentration decreased from 4.8 to 3.4 g/L at dilution rate from 0.1 to 0.26 1/h. It is also detected that glucose is not completely consumed. Furthermore, a strong ethanol formation was obtained. It has to be concluded that the oxygen supply in the CosBios system was not sufficient to compensate the oxygen demand of the culture. The decline in biomass is directly related to the amount of oxygen supplied, leading to an oxido-reductive fermentation.

Conclusion

In the present study we report on an improved design of a continuous parallel shaken bioreactor (CosBios) system. The improved reactor vessels manufactured from quartz glass provided a geometric accuracy of <1 mm and an exact positioning and dimensions of the outlets. Therefore, a defined and reproducible filling volume as function of the shaking parameters was ensured for different parallel shaken reactors. The influence of the shaking frequency and the shaking diameter on the filling volume, the centrifugal acceleration and the oxygen transfer rate (OTR) were examined and the optimal operating conditions for the CosBios system were determined. Depending on the combination of the inner diameter of the feeding hose, the rotation rate of the feeding pump, shaking diameter and shaking frequency of the shaker, a broad range of set points for the dilution rate can be adjusted in parallel shaken vessels. This is especially attractive for rapid generation of an *X-D* diagram.

The maximum oxygen transfer capacity (OTR_{max}) of the CosBios system was investigated by using the sulfite

oxidation method and the results were compared with that of conventional 250-mL Erlenmeyer flask. The results led to the conclusion that the OTR_{max} is substantially higher in cylindrical vessels than in conical Erlenmeyer flasks (1.6-fold higher OTR_{max} at a shaking frequency of 275 rpm and a shaking diameter of 50 mm).

The performance of the CosBios system was investigated by examining the facultative anaerobic bacterium *C. glutamicum*, and the Crabtree-negative yeast *P. stipitis.* In addition, the Crabtree-positive yeast *S. cerevisiae* was studied, in comparison to a conventional stirred tank 1-L bioreactor. The comparative study has proven that the CosBios system accurately mimicked the conventional 1-L bioreactor. The new technique can be used to carry out a continuous fermentation in a cost- and time-saving manner and to create an *X-D* diagram more accurately in comparison to continuous culture in conventional 1-L stirred tank reactors. Many more steady state points can be obtained with the CosBios system and smaller intervals between the dilution rates allows an accurate investigation of cultures exhibiting transitions between the metabolic regimes, as a Crabtree-effect in yeasts. Simultaneous operation of 6 shaken bioreactor drastically shortens the total duration of experiments and the total medium consumption. Shortening the fermentation time to create a complete *X-D* diagram is also advantageous to minimize problems with wall growth.

The experiments with all three microorganisms also clearly demonstrated that the oxygen supply has to be carefully controlled in the CosBios system in order to avoid oxygen-limited conditions. This task is not easily accomplished as the dissolved oxygen concentration cannot yet be measured. However, a method was illustrated to a priori evaluate the oxygen demand of the culture as function of the dilution rate and to estimate the critical dilution rate leading to a transition from non-oxygen limited to limited conditions. The operating conditions of the CosBios system have to be designed such that this critical dilution rate is always larger than the maximum specific growth rate of the investigated microorganisms.

The CosBios system is particularly useful to investigate the genetic stability and behaviour of genetically modified microorganisms, respectively, the analysis of their metabolic fluxes, along the generations. In addition, different strains (mutagenised or recombinant clones) or different media can be compared in parallel cultures at equivalent conditions (same dilution rates in all vessels). Specific requirements for growth and product formation can be determined by adding or eliminating chemical components systematically from the media formulation. This culture system could be also applied for evolutionary selection procedures of strains.

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