Bioprocess Biosyst Eng
DOI 10.1007/s00449-007-0119-y

ORIGINAL PAPER

Evaluation of a novel Wave Bioreactor® cellbag for aerobic yeast cultivation

Mark Mikola · Jennifer Seto · Ashraf Amanullah

Received: 29 January 2007 / Accepted: 6 February 2007

Abstract The Wave Bioreactor® is widely used in cell culture due to the benefits of disposable technology and ease of use. A novel cellbag was developed featuring a frit sparger to increase the system's oxygen transfer. The purpose of this work was to evaluate the sparged cellbag for yeast cultivation. Oxygen mass transfer studies were conducted in simulated culture medium and the sparged system's maximum oxygen mass transfer coefficient (k_La) was 38 h^{-1}. These measurements revealed that the sparger was ineffective in increasing the oxygen transfer capacity. Cultures of Saccharomyces cerevisiae were successfully grown in oxygen-blended sparged and oxygen-blended standard cellbags. Under steady state conditions for both cellbag designs, k_La values as high as 60 h^{-1} were obtained with no difference in growth characteristics. This is the first report of a successful cultivation of a microbe in a Wave Bioreactor® comparing conventional seed expansion in shake flasks and stirred tank bioreactors.

Keywords Wave Bioreactor · Disposable bioreactor · *Saccharomyces cerevisiae* · Oxygen transfer · Seed culture

List of symbols

C^*	saturated dissolved oxygen concentration in the liquid phase (mmol L^{-1})
C_1	dissolved oxygen concentration at time t_1 (mmol L^{-1})
C_2	dissolved oxygen concentration at time t_2 (mmol L^{-1})
CER	carbon dioxide evolution rate (mmol L^{-1} h^{-1})
DO	dissolved oxygen (% air sat)
k_La	volumetric oxygen transfer coefficient (h^{-1})
OD	optical density
OTR	oxygen transfer rate (mmol L^{-1} h^{-1})
OUR	oxygen uptake rate (mmol L^{-1} h^{-1})
PBS	phosphate buffered saline
RPM	rocks per minute (min^{-1})
RQ	respiration quotient (CER/OUR)
s	substrate (g L^{-1})
vvm	volume of gas sparge per volume liquid per minute (L L^{-1} min^{-1})
$Y_{x/s}$	yield coefficient, g biomass/g glucose
x	biomass (g)

M. Mikola (✉) · J. Seto · A. Amanullah
Fermentation and Cell Culture, Merck and Co.,
P.O. Box 4, Mailstop WP26C-1,
West Point, PA 19486, USA
e-mail: mark_mikola@merck.com

Introduction

A recent development in cell cultivation technology is the Wave Bioreactor® introduced by Wave Biotech LCC (Bridgewater, NJ, USA). The bioreactor system consists of a disposable, flexible, sterile gamma-irradiated cellbag that sits on a rocking thermo-platform (Fig. 1). A cellbag is a chamber partially filled with media and inflated with air using the integral sterile inlet filter. The disposable contact material eliminates the need for cleaning and its validation, thereby significantly reducing costs in cGMP operations. In addition, the Wave Bioreactor® can be installed and used rapidly for process development and clinical manufacturing, thus minimizing the time to market for biological products. The rocking motion imparts mixing and promotes oxygen transfer. The combined advantages of a fully closed, disposable system with process monitoring capa-

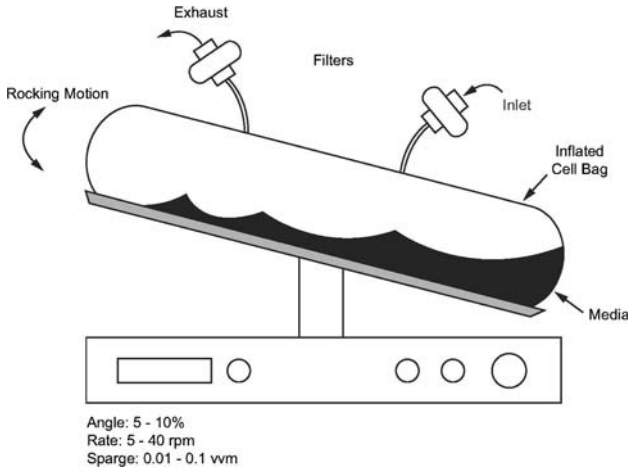

Fig. 1 Schematic diagram of a Wave Bioreactor®

bilities (pH and dissolved oxygen), makes it highly attractive over traditional systems for animal cell culture such as shake flasks and stirred tanks. Batch cultivation of mammalian, insect and plant cells [1–3] have been reported in Wave Bioreactors®.

However, the Wave Bioreactor® has not been evaluated for aerobic microbial growth, since microorganisms typically require higher oxygen transfer rates than those of animal and plant cells (30–100 mmol L^{-1} h^{-1} compared to approximately 1–10 mmol L^{-1} h^{-1}). The only exception is one report of yeast cultivation used for a catalytic bioreduction which gives few details of the cultivation parameters employed [4]. Based on the reported oxygen transfer capacity of the original cellbag system [5, 6], it was assumed that the Wave Bioreactor® could not meet such high oxygen transfer demands, even at moderately low biomass concentrations.

In an attempt to utilize the Wave Bioreactor® system for aerobic microbial growth, a new cellbag has been developed featuring a frit sparger that generates small gas bubbles near the lower center of the cellbag (Fig. 2). Thus, the incoming air passes directly into the liquid phase rather than through the headspace of the cellbag. With more assumed gas–liquid interface, the sparged design was anticipated to increase oxygen transfer. This paper describes the oxygen mass transfer characteristics of the new sparged Wave Bioreactor® design and its application for microbial cultivation.

The Wave Bioreactor® system is envisioned to be used as a seed expansion step. The purposes of the seed stage(s) are to provide the production vessel with an inoculum source of consistent biomass and culture physiology. Typically, a seed expansion does not require the same rigor for growth conditions as the final production step. The seed step final biomass is much lower and therefore has a lower demand for oxygen and media

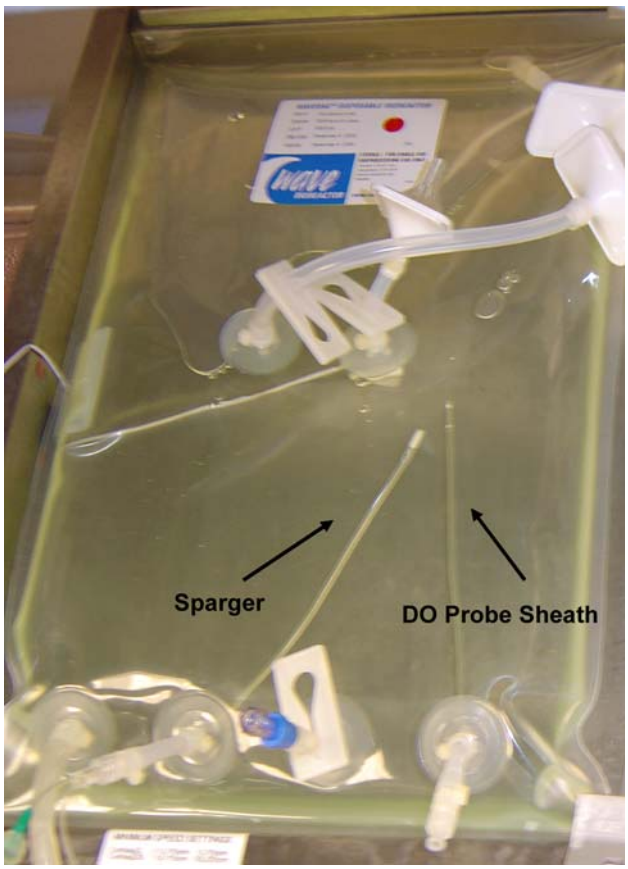

Fig. 2 Picture of new sparged cellbag showing the DO probe and sparger

buffering capacity. Traditionally, expansion steps have occurred first in shake flasks typically under uncontrolled conditions of pH and dissolved oxygen followed by small stirred tank bioreactors operated under controlled conditions. Shake flask cultures of *Saccharomyces cerevisiae* and *Escherichia coli* have been shown to become oxygen limited [7]. The Wave Bioreactor® has the potential to provide increased oxygen transfer compared to shake flasks due to the ability to supply air, via the sparge or the headspace, directly to the culture. Given the attributes of the seed step, the Wave Bioreactor® appears to be well suited if the oxygen demand of the culture can be met. The Wave Bioreactor® has additional advantages over shake flask cultures. Specifically it offers the convenience of using one fully closed cell expansion step that can generate culture volumes at 5–10 L scales that would be equivalent to multiple shake flasks.

The purpose of this work was to evaluate the new sparged Wave Bioreactor® for yeast cultivation. First, oxygen mass transfer studies were conducted with simulated culture medium utilizing the sparger under various combinations of rocking speeds (10, 25 and 40 rpm), rocking angles (5°, 7.5° and 10°), sparging rates (0.02, 0.06

and 0.1 vvm), and temperatures (30 and 37 °C). Secondly, 5 and 10 L cultures of *Saccharomyces cerevisiae* were grown in oxygen-blended Wave Bioreactors®.

The AlcoFree® *Saccharomyces cerevisiae* strain [8] was used for all cultivation studies. This strain is unique in the fact it does not ferment significantly in the presence of excess glucose with sufficient oxygen present as do wild type strains [9]. This strain was metabolically engineered to limit glucose transport into the cell, which results in a reduced amount of pyruvate produced from glycolysis [8]. This lower pyruvate concentration favors respiratory metabolism and promotion of biomass formation over fermentation and ethanol production. This strain undergoes fermentative metabolism only when oxygen is limiting [8] essentially imparting a Crabtree negative [10] phenotype. Thus, by using the AlcoFree® strain, one can assume that biomass yield is highly dependent on the bioreactor's oxygen transfer capacity. While AlcoFree® growth rate is about 70% compared to wild type the oxygen consumption rate is 4.5 times higher than wild type [8, 11]. Based on this relationship of biomass to oxygen and the higher oxygen demand compared to wild type *S. cerevisiae*, the AlcoFree® strain is well suited as a model organism for oxygen transfer studies.

Materials and methods

Wave Bioreactor® system

A 20-L Wave Bioreactor® system was used with both standard cellbags (Fig. 1) and novel sparged cellbags (Fig. 2) which were designed by Wave Biotech LCC. The 20-L system is compatible with 2 × 10-L cellbags, each 5 L working volume, or 1 × 20-L cellbag, 10 L working volume. The three layer bag is composed of low density polyethylene, ethylene vinyl alcohol and ethylene vinyl acetate and meets US Pharmacopoeia Class VI testing for leachables. The frit sparger is made of polyethylene with a pore size range of 70–100 μm. The thermo-platform rocking creates mixing and enhances oxygen transfer [5]. It also provides heating for temperature control. For the standard cellbag, air is introduced into the headspace through a sterilizing 0.2 μm inlet filter and exits through the exhaust filter, which is heated to prevent blockage due to condensation. For the sparged bag, air is supplied through the sparger and exits through the heated exhaust filter. All cellbags are fitted with a backpressure control valve which ensures that the chamber is always fully inflated at any airflow and prevents over inflation leading to potential bursting of the chamber.

Both the standard and sparged configurations of the cellbags have an oxygen permeable sheath for placement of a dissolved oxygen (DO) probe. This sheath is necessary to provide a sterile barrier between contact of the culture media and the non-sterile DO probe. The tip of the DO probe is then located near the bottom and center right of the bag.

The bioreactor system, for both 10 and 20-L cellbags, can be operated with gas flow rates in the range 0.1–1 L min^{-1}, rocking rates up to 40 rpm and at rocking angles between 5° and 15°, as supplied by the manufacturer. However, much higher gas flow rates are possible based on the capacity of the inlet and exhaust filters.

Dissolved oxygen probe

For dissolved oxygen (DO) measurements, Wave Biotech's optical DO instrument (model DOOPT) was used. This instrument is similar to other optical DO instruments in that the measurement theory involves the luminescence decay time of a luminophore [7]. The luminophore exploited is a transition metal complex of ruthenium, which emits light on excitation by incident light. In the presence of oxygen, this excitation is quenched. Thus, a relationship exists between the DO concentration and the light emitted from the luminophore on the tip of the probe. Typically, an optical DO probe uses luminescence intensity to estimate DO concentration; in Wave Biotech's instrument, a phase-modulation technique is utilized to measure the shift in the phase angle between the excited and the emitted light signal. This reduces the impact of photo bleaching, and decay time is not influenced by the optical properties of the sample, such as turbidity, refractive index and color. The phase shift is related to the dissolved oxygen concentration according to the Stern–Volmer relationship [7].

The probe was calibrated every second day of use per the manufacturer's instruction. Dissolved oxygen readings were taken every 30 s and recorded on a computer for subsequent analysis.

Oxygen transfer measurement

The oxygen transfer capabilities of the 10-L sparged bag were characterized by calculation of the volumetric oxygen transfer coefficient (k_La) at various combinations of rocking speeds (10, 25 and 40 rpm), rocking angles (5°, 7.5° and 10°), sparging rates (0.02, 0.06 and 0.1 vvm), and temperatures (30 and 37 °C). Although the gas flow rates chosen were low for microbial cultures, this limitation was due to capacity of the air supply pump rotometer. The classic dynamic method was used for k_La measurement [12]. To simulate fermentation medium, 5 L of phosphate buffered saline (PBS) with 0.3 mL L^{-1} UCON antifoam (LB-625, Union Carbide, Dow, Midland, MI, USA) were added to a 10-L sparged cellbag.

For DO measurement, the optical DO probe was placed in a sheath that is part of the standard bag configuration. The silicone tip of the sheath was removed in order to decrease the response time of the probe system. The sheath increases the response time of the bare probe from 6 s to 3–5 min (DOOPT model, DO probe instrument manual Wave Biotech LCC). The response time with the sheath is too long compared to the characteristic time for oxygen transfer ($1/k_La$) making it unsuitable for such measurements [13].

The simulated culture media was de-oxygenated by supplying nitrogen gas through either the sparger for the new cellbag design or through the inlet filter for the standard cellbag design until the DO concentration was less than 10% of saturation. Following de-oxygenation, compressed air was supplied to the bag at specified flow rates and the resulting increase in DO was recorded as a function of time using an optical DO probe (Wave Biotech, LLC, Bridgewater, NJ, USA). Also note that neither the DO probe nor the sparger is fixed to the inner bag surface and therefore, both move with the rocking motion of the bag.

Experiments were also conducted to study the influence of headspace nitrogen on k_La measurements using the same sparged cellbag. In these experiments nitrogen gas in the headspace was purged with air before initiating the k_La study. To accomplish this, rocking was stopped after de-oxygenation and air was fed through the top inlet filter at 0.5 L min^{-1} for 10–15 min. During this time, a small rise in DO (~5% increase) was typical, due to diffusion. Rocking was resumed at the same time that re-oxygenation and data collection began for the DO measurement.

k_La Measurement

The k_La was calculated from the slope of the following dynamic method mass balance equation:

$$\ln\left(\frac{C*-C_1}{C*-C_2}\right) = k_La\,(t_2 - t_1), \quad (1)$$

where C^* is the saturated dissolved oxygen concentration in the liquid, C_1 is the dissolved oxygen concentration at time t_1, and C_2 is the dissolved oxygen concentration at time t_2 [12]. The left-hand expression of the Eq. 1 was the transform used to linearize the dissolved oxygen versus time data. The linearized transform was expressed as a function of time and a best fit line was used to determine the slope which is the mass transfer coefficient, k_La. The accuracy of these measurements is dependent upon the assumption that the liquid and gas phases are well mixed. Liquid mixing time of a 20-L bag with 10 L of water was determined to be approximately 5–10 s [5] at rocking rates greater than 6 rpm, indicating a well mixed system.

Microbial cultivation

The AlcoFree® *S. cerevisiae* strain [MATa *MAL2-8^c SUC2 hxt17Δ ura3-52 gal2Δ::loxP stl1Δ::loxP agt1Δ::loxP ydl247wΔ::loxP yjr160cΔ::loxP hxt13Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10Δ::loxP hxt8Δ::loxP hxt514Δ::loxP hxt2Δ::loxP hxt367Δ::TM6** (*TM6** is *HXT1* bp 1–741/*HXT7* bp 742–1,713, mutation S279Y)] was grown from a 1 mL vial source (Gothia Yeast Solutions, Gothenburg, Sweden) stored at –70 °C.

The first preculture was grown in 50 mL of medium of the following composition in a 250-mL Tunair® shake flask [14]: 1.7 g L^{-1} Yeast Nitrogen Base without amino acids or ammonium sulfate, 25 g L^{-1} maltose monohydrate, 5 g L^{-1} ammonium sulfate and 2 g L^{-1} soy peptone (Kerry Bio-Science, Hoffman Estates, IL, USA). After 24 h of cultivation in a rotary shaker incubator at 30.0 °C and 250 rpm at 1 in. stroke length, the entire flask contents were transferred to a 2-L flask with 350 mL of medium. This second preculture (400 mL working volume, 2-L unbaffled flask with a foam plug) was of the same composition as the first preculture without the soy peptone component and incubated at the same conditions. The components for all media were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted.

The Wave Bioreactor® and control shake flask medium was based on a 2× concentration of a published medium [15] of the following composition per liter: 10 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 20 g glucose, 0.025 g $CuSO_4{\cdot}5H_2O$, 2 mL Solution 1, 2 mL Solution 2, 10 g succinic acid, and 0.3 mL UCON LB-625. The succinic acid and UCON were additions to the original medium to increase the buffering capacity and provide an antifoam agent, respectively. Solution 1 (''Vitamin Stock, 1000X'') contained the following per liter: 0.05 g biotin, 1 g calcium pantothenate, 1 g nicotinic acid, 25 g inositol, 1 g thiamine HCl, 1 g pyridoxine HCl, 0.2 g para-aminobenzoic acid. Solution 2 (''Trace Element Stock, 1000X'') contained the following per liter: 15 g EDTA, 4.5 g $ZnSO_4{\cdot}7H_2O$, 0.3 g $CoCl_2{\cdot}6H_2O$, 1 g $MnCl_2{\cdot}4H_2O$, 0.3 g $CuSO_4{\cdot}5H_2O$, 4.5 g $CaCl_2{\cdot}2H_2O$, 3.0 g $FeSO_4{\cdot}7H_2O$, 0.4 g $Na_2MoO_4{\cdot}2H_2O$, 1 g H_3BO_3, 0.1 g KI. Before inoculation, the medium was adjusted to a pH of 5.5 using NaOH and filtered through a sterilizing grade 0.2 μm filter directly into a cellbag.

In the Wave Bioreactor®, cultures were grown at a temperature of 30 °C, rocking rate of 40 rpm, angle of 10° and initial sparge rate of 0.1 vvm. These conditions were selected on the basis of providing maximum oxygen transfer capacity as determined by dynamic k_La measurements. The second pre-culture shake flask was used for inoculation of the Wave Bioreactor®. The inoculum was

2% v/v (by final volume) for both 5 and 10 L cultures. To initiate a 2-L control shake flask culture, 400 mL of culture from a cellbag were aseptically removed. Control flasks were cultivated in a rotary shaker incubator at 30 °C, 250 rpm and a 1 in. stroke length. The control 2-L flasks were not baffled and were capped with foam plugs. Vial and culture purity were routinely monitored before and after inoculation and at the end of each cultivation by plating onto Columbia Blood and Tryptic Soy Agars and observing subsequent growth.

Oxygen blending

Oxygen blending was used in yeast cultivations to increase the oxygen transfer of the cellbags in addition to the sparger. Three oxygen sources were utilized: (1) compressed air, (2) a 92% oxygen cylinder, and (3) a 91%+ oxygen generator. The oxygen generator (Wave Biotech LLC, Bridgewater, NJ, USA) was capable of producing 2 L min^{-1} of 91%+ oxygen from room air using automated zeolite adsorption beds, which adsorb nitrogen from the air to produce oxygen-enriched gas. A desired oxygen concentration was achieved by manually blending different gas flows from either the 92% oxygen cylinder gas or oxygen generated gas with the compressed air.

Analysis

A mass spectrometer (PEI1200E, Orbital, Pomna, CA, USA) was used to monitor the exit gas composition of the Wave Bioreactor® culture. Oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiration quotient (RQ) were calculated from the mass spectrometer data. In addition, the previously described optical DO probe was used to measure and record DO in the Wave. To wet the probe sleeve, 1 mL of PBS was injected into the sheath before the insertion of the DO probe. Inclusion of the DO sheath resulted in an increased response time of 3–5 min, but it was required for the yeast cultivation to maintain a sterile barrier between the culture and the DO probe. Optical density (OD) was measured by spectrophotometer (Milton Roy Spectronic 501/601, Rochester, NY, USA) at 600 nm. Dry cell weight was measured in duplicate for each sample by filtration of a 3 mL sample and drying to constant weight in a microwave oven (Sharp R-4065 850W, Mahwah, NJ, USA). Off line pH was measured by a Bioprofile 200 (Nova Biomedical Corporation, Waltham, MA, USA). Glucose and ethanol concentrations were measured by a YSI biochemistry analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA).

Steady state k_La measurement

The following steady state equation, where OUR = OTR (oxygen transfer rate), was used to calculate k_La values during the exponential growth phase:

$$\text{OUR} = k_La \times (C^* - C_1), \quad (2)$$

where C^* is the saturated dissolved oxygen concentration in the liquid and C_1 is the dissolved oxygen concentration at time the OUR measurement was taken.

Results and discussion

Oxygen mass transfer studies in PBS

The oxygen transfer capacity of 10-L sparged Wave Bioreactor® cellbags was evaluated by calculating the oxygen mass transfer coefficient, k_La. Figure 3 shows an example of the raw data transformation and analysis. The experimental conditions were 40 rpm rocking, 0.1 vvm sparge, 10° rocking angle and 30 °C. The dissolved oxygen and linearized transform data are plotted as a function of time. A best fit line was drawn using the linear transform. The slope of this line is the transfer coefficient, k_La, determined at the specific conditions. Under these conditions the transfer coefficient was calculated to be 9.24 h^{-1}.

Figure 4a shows k_La calculations at rocking rates of 10, 25 and 40 rpm at sparge rates of 0.02, 0.06 and 0.1 vvm without headspace exchange. The figure illustrates an increasing dependence of k_La on sparge rate as rocking rate increases with maximum values of k_La in the range 2–9 h^{-1}. Figure 4b k_La measurements at 0.1 vvm, rocking rates of

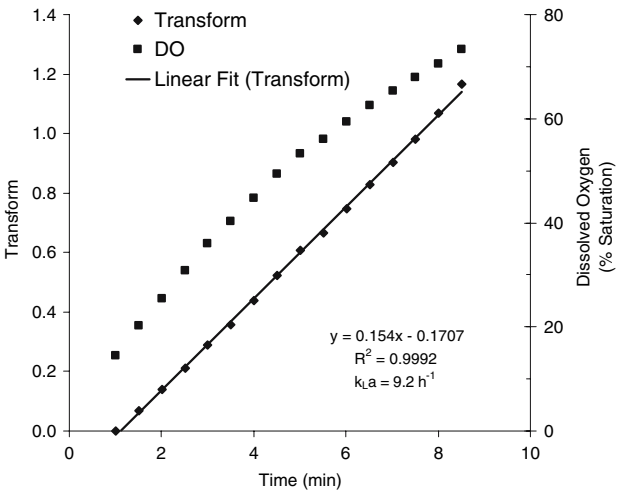

Fig. 3 Data analysis example for determination of k_La, no headspace exchanged: 40 rpm, 10°, 0.1 vvm, 30 °C

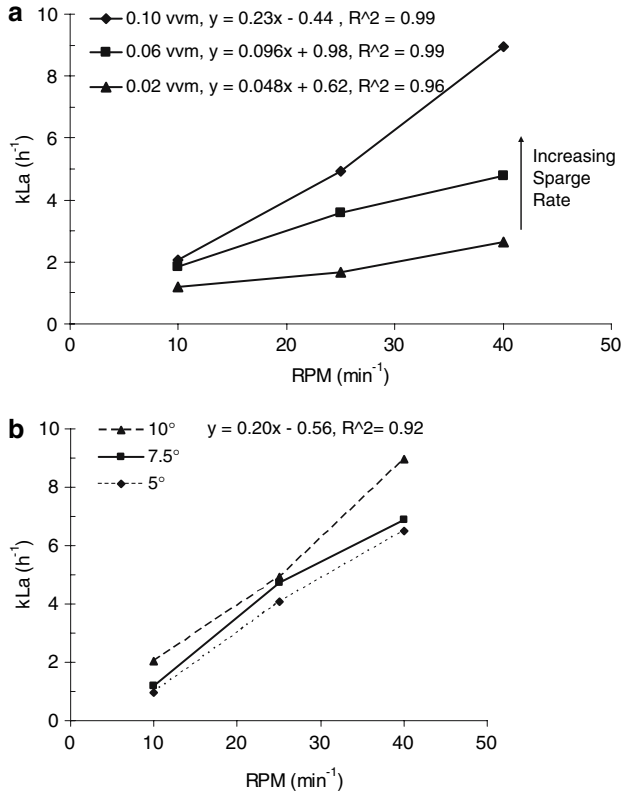

Fig. 4 **a** Effect of sparge rate and rocking rate on oxygen transfer coefficient: temperature 37 °C, angle 10°. **b** Effect of angle and rocking rate on oxygen transfer coefficient: sparge rate 0.1 vvm, temperature 37 °C

10, 25 and 40 rpm and rocking angles of 5°, 7.5° and 10° without headspace exchange. These results demonstrate a lesser dependence of k_La on rocking angle.

The highest k_La achieved using the sparger for aeration without headspace exchange was 9 h^{-1} which is comparable to values previously reported for the standard cellbag configuration [5]. In that study, the author evacuated the cellbag headspace and re-inflated the bag with air prior to initiation of data collection.

Knevelman et al. [6] reports that k_La has a strong dependence on rocking rate and angle but not air flow rate, again for the standard cellbag configuration. These differences may be due to conditions not stated by the authors such as media composition. Under some conditions, Knevelman et al. [6] reported k_La values that are two- to fourfold higher than measured in this study at nearly comparable bioreactor conditions. k_La is known to be sensitive to liquid composition, particularly antifoams which have a tendency to reduce it [16]. The addition of UCON antifoam to PBS in our study resulted in a decrease in k_La from 68 to 38 h^{-1} at 40 rpm, 10° rocking angle, 0.1 vvm and 30 °C. Furthermore, the lower k_La values obtained in our study may be due to the use of a sparger without headspace gas exchange as performed by Singh [5].

Experiments were conducted to investigate the influence on k_La measurements from the presence of a nitrogen headspace at the initiation of data collection since it was thought that the gas entrained during the rocking motion could have a significant contribution to the oxygen transfer rate. k_La measurements were made at 40 rpm with and without headspace exchange in triplicate. This was done to confirm the results and give an indication of the error in measurements. It is evident from Fig. 5 that while headspace exchange has little effect on k_La at the lower rocking rates, it has a significant impact at the higher rocking rate with up to fourfold higher k_La values (38 h^{-1}). This k_La result was similar in magnitude to the value obtained using the steady state method under similar operating conditions. At lower rocking rates of 10 and 25 rpm, the use of the sparger is equivalent to gas exchange only through the headspace [5, 6] given both methods result in similar k_La values. The highest rocking rate, 40 rpm, was chosen for yeast cultivation.

Based on the headspace exchange experiment, the sparger was not an advantageous addition to the Wave Bioreactor®. This result is not surprising, for several reasons: (1) the sparger is not fixed to the bottom of the cellbag, so the tip oscillates from the bag bottom to the liquid–gas interface at high rocking rates. Without a significant depth of liquid above the sparger tip there is minimal gas residence time making the sparger ineffective. (2) The bubbles exiting the sparger only travel in the direction of the wave motion, even at the highest rocking rate, so there is no dispersion in the lateral direction. The lack of lateral mixing also detracts from the sparger's efficacy. (3) The sparger itself is very small, which caused both excessive back pressure and minimal bubble forma-

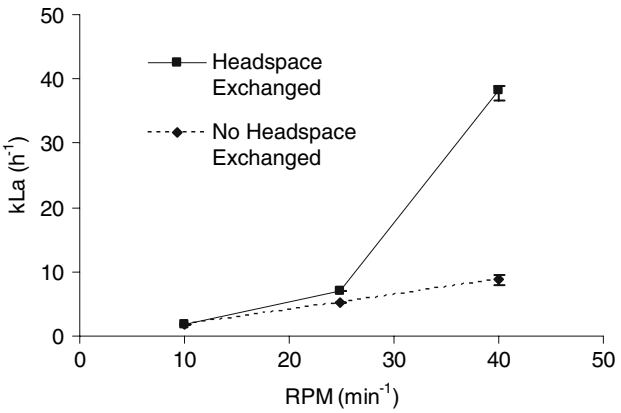

Fig. 5 Effect of headspace exchange on oxygen transfer coefficient in a sparged Wave Bioreactor®; sparge rate 0.1 vvm, temperature 30 °C, angle 10°

tion. Furthermore, this data demonstrates that the entrainment of headspace gas from the rocking motion is the major route of oxygen transfer at the highest rocking rate and is equivalent to sparging at lower rocking rates. As shown in Fig. 5, the higher the rocking rate the more important the headspace gas contribution becomes. When observing the bioreactor it is obvious that much more of the headspace gas is entrained with increasing rocking rate. At the highest rocking rate of 40 rpm, the liquid sweeps over the top of the cellbag with each rock. This indicates there is a critical rocking rate at which the oxygen transfer dramatically increases due to an increase in contact area between the liquid and gas phases.

5 L yeast cultivation

To evaluate the Wave Bioreactor® for microbial cultivation, the AlcoFree® strain of *S. cerevisiae* was used to compare growth in a cellbag to that of a shake flask. The first experiment was performed in a 10-L sparged cellbag with 5 L working volume. The Wave Bioreactor® culture was grown at a temperature of 30 °C, rocking rate of 40 rpm, rocking angle of 10° and initial sparge rate of 0.1 vvm. To overcome apparent oxygen limitations, the sparge oxygen concentration was increased stepwise during the course of the experiment to aerate the culture. During the initial growth period, the cellbag was sparged with 0.1 vvm of 40.9% oxygen. As the culture entered its exponential growth phase, the percent of oxygen was increased stepwise from 40.9 to 91.2% to maintain a DO above 30% (see Fig. 6). Upon depletion of the glucose (and hence lowered oxygen demand) the inlet gas oxygen concentration was lowered to 21% using compressed air.

As shown in Fig. 7a, the 5 L Wave Bioreactor® culture grew to a higher optical density than the control shake flask. The Wave Bioreactor® reached a final OD of 26.0 (9.1 g L^{-1} DCW), whereas the flask reached a final OD of 18.8 (5.6 g L^{-1} DCW). In addition, the maximum specific growth rate was almost twofold higher at 0.24 h^{-1} for the Wave culture. Dry cell weights, maximum specific growth rates and biomass yields for the Wave Bioreactor® and the shake flask cultures are summarized in Table 1.

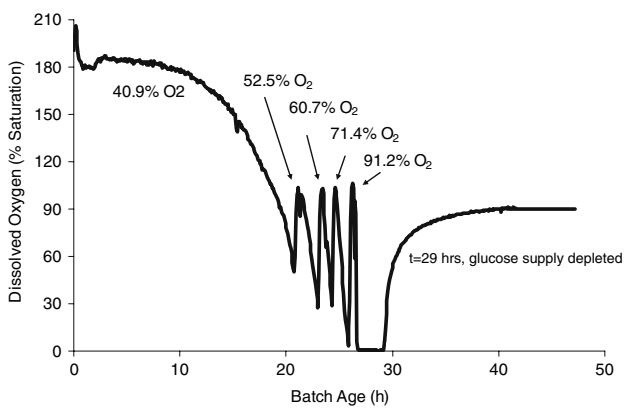

Fig. 6 Sparged wave culture dissolved oxygen profile

Similarly to the specific growth, the yield of biomass from glucose, $Y_{x/s,}$ was higher for the Wave Bioreactor® compared to that of the shake flask culture, 0.44 versus 0.28 g biomass/g glucose, respectively. The biomass yield for the Wave Bioreactor® culture was calculated near the point of glucose exhaustion in order to compare the yield at the same point for the control flask. The bioreactor culture essentially generated the theoretical maximum yield of 0.5 g biomass/g glucose for yeast grown on glucose under non-limiting dissolved oxygen conditions [11]. For the flask culture the final biomass yield was reduced due to increased ethanol production of fermentative metabolism. As shown in Fig. 7b, c, the glucose consumption is the same for both cultures but the maximum ethanol concentration of the bioreactor is nearly fourfold less than that of the flask (1.3 vs. 4.7 g L^{-1}), further illustrating that the bioreactor is well oxygenated compared to the shake flask culture. Since AlcoFree® does not produce ethanol due to carbon overflow metabolism this indicates that the control shake flask culture became oxygen limited before the Wave Bioreactor® culture.

Figure 8 shows the off-gas data for the same 5 L culture. A maximum CER of 41 mmol L^{-1} h^{-1} and a maximum OUR of 44 mmol L^{-1} h^{-1} were achieved. RQ was never greater than 1, which indicates that the AlcoFree® yeast were mostly respiring as expected. Furthermore, steady state k_La values were calculated at various points during the exponential growth phase and are also indicated in Fig. 8. The measured k_La values at 21, 24 and 25 h were 43, 60 and 58 h^{-1}, respectively, and reflect the increasing concentration of oxygen in the inlet gas (Fig. 6). These values compare to 38 h^{-1} as determined by the dynamic method using air only (Fig. 5). This shows that in spite of increasing the inlet oxygen concentration by about 4.5-fold, k_La only increased by a factor of 1.5, further indicating that at the high rocking rate used in this experiment the inclusion of the sparger does not significantly enhance oxygen transfer. This is also evident from the results of the k_La determination following nitrogen headspace exchange at these operating conditions (Fig. 5).

Further comparison between the Wave Bioreactor and shake flask cultures can be made in regards to oxygen transfer and limitation. Oxygen transfer measurements were not made from the control shake flask, however, a k_La value of 24 ± 3 h^{-1} has been reported [17] for similar flask cultivation conditions. Anderlei et al. [18] reported OUR measurements in shake flask again under similar conditions and found the *S. cerevisiae* culture to be oxygen limited

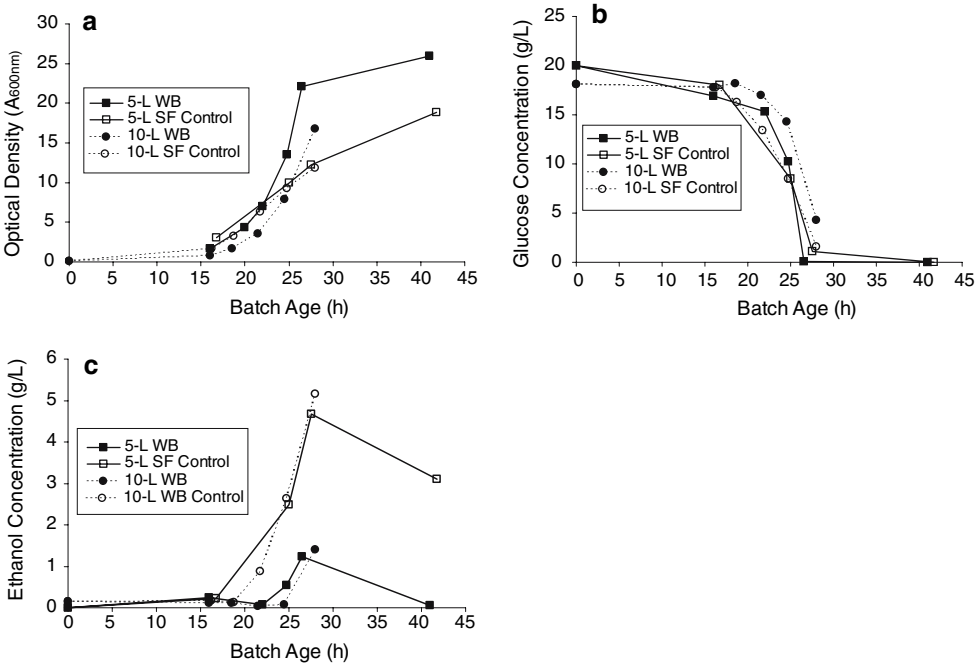

Fig. 7 **a** Sparged wave and shake flask cultures optical density profiles. **b** Sparged wave and shake flask cultures glucose profiles. **c** Sparged wave and shake flask cultures ethanol profiles

Table 1 Wave Bioreactor® and shake flask AlcoFree® cultivation data

Culture	Final dry cell weight (g L^{-1})	Maximum specific growth rate (h^{-1})	Yield (g biomass/g glucose)
5 L Wave Bioreactor®	9.1	0.24	0.44
Shake flask (5 L control)	5.6	0.13	0.28
10 L Wave Bioreactor®	6.9	0.25	0.43[a]
Shake flask (10 L control)	4.2	0.17	0.22

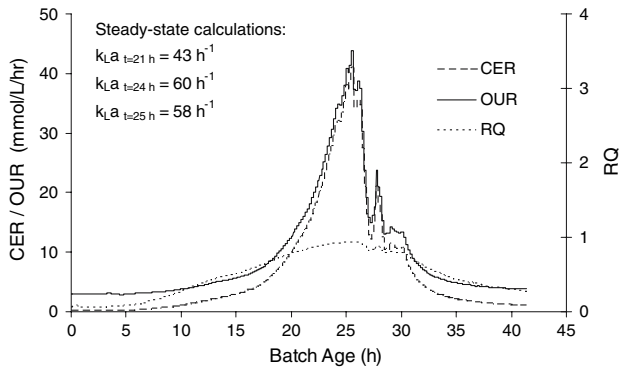

Fig. 8 5 L sparged wave culture off gas profile

from 13 h of cultivation until carbon exhaustion. These references help to support the observation that the control flask cultures are indeed oxygen limited, although the key evidence for this is provided by the ethanol profiles by means of the AlcoFree® apparent phenotype.

Scale-up to 10 L yeast cultivation

Following successful demonstration of growth of a 5 L *S. cerevisiae* culture in a 10-L sparged cellbag, the process was scaled to a 20-L standard cell bag with a 10 L working volume). A standard 20-L cellbag was employed in this experiment since the sparger had proven ineffective in improving oxygen transfer compared to the standard cellbag at the chosen bioreactor operating conditions. If successful, this larger cultivation volume would be useful to inoculate larger production vessels. Oxygen enrichment was used once again to overcome the cellbag's oxygen transfer limitations. During the initial growth period, the culture was aerated with 0.1 vvm of 30% oxygen. As the culture entered its exponential growth phase, both the percent of oxygen and flow rate were increased to maintain the DO. Process interventions were minimized to two events by design in order to reduce operator manipulations since the cultures were not continuously monitored by the operators. The DO was maintained above 30% during most of the experiment. The culture was gassed at 0.1 vvm 30% oxygen until 20 h when the oxygen concentration was changed to 39%. The last adjustment occurred at approximately 24 h and increased the flow rate to 0.2 vvm and the oxygen concentration to 58%. At 28 h this experiment was terminated prior to substrate exhaustion due to a blinded exhaust filter. However, the 10 L Wave culture ended within an hour of glucose exhaustion based on the glucose consumption rate.

As shown in Fig. 7a, the shake flask culture had higher OD than the Wave Bioreactor® culture for the first 25 h of

growth due to a longer lag phase in the latter as evident by a delay in glucose utilization (Fig. 7b). The Wave Bioreactor® culture reached a final OD of 16.8 (6.9 g L^{-1} DCW), whereas the shake flask culture reached a final OD of 11.8 (4.2 g L^{-1} DCW). For this experiment the maximum specific growth rates for the Wave and shake flask cultures were similar. Dry cell weights, maximum specific growth rates and biomass yields of the cultures are shown in Table 1.

The yield, $Y_{x/s}$, of biomass from glucose consumed was higher for the Wave Bioreactor® compared to that of the shake flask culture; 0.43 versus 0.22 g biomass/g glucose, respectively. Again, the bioreactor culture essentially generated the theoretical maximum yield of 0.5 g biomass/g glucose for yeast grown on glucose under non-limiting dissolved oxygen conditions [11]. These calculated yield values are comparable to the 5 L Wave and corresponding control flask cultures since all the yields were calculated near the point of glucose exhaustion. As shown in Fig. 7b, c, the glucose consumption is the same for both cultures but the maximum ethanol concentration of the bioreactor is nearly fourfold less than that of the flask (1.4 vs. 5.2 g L^{-1}), illustrating that under these conditions the bioreactor is well oxygenated at the 10 L scale although unsparged. The data shown in Fig. 7a–c demonstrate the reproducibility and scalability of the 5 and 10 L cultures. This also reinforces the finding that under these operating conditions, the Wave Bioreactor® cultures outperform the shake flask cultures with respect to biomass production and oxygen transfer. The 5 and 10 L cultures can be considered as a replicate (even though the scales are different) given the similarity of the results obtained.

The 10 L culture off-gas data are shown in Fig. 9. A maximum CER of 44 mmol L^{-1} h^{-1} and a maximum OUR of 46 mmol L^{-1} h^{-1} were achieved. These values are nearly identical to those obtained with the 5 L culture (Fig. 8). RQ never exceeded 1, which indicates that the yeast were mostly respiring. Steady state k_La's were calculated at various points during the exponential growth phase (Fig. 9), and were similar to those of the 5 L culture.

Yeast cultivation

Both the 5 L and 10 L Wave Bioreactor® cultures reached higher optical density and dry cell weights than the corresponding shake flask cultures. There was essentially no difference in the performance of the two cultivation experiments. The first used a sparged bag with a 5 L culture and the second used a standard bag with a 10 L culture. The maximum k_La calculated by the steady state method was equivalent for each culture, approximately 60 h^{-1}.

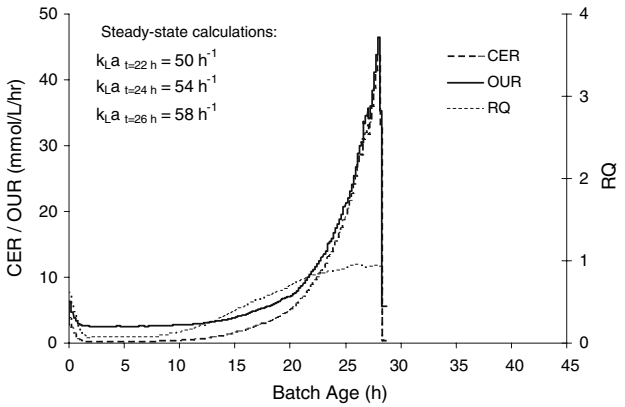

Fig. 9 10 L wave culture off gas profile

In Table 2, OUR values from seed cultures of multiple representative organisms [19, 20] are compared to values obtained in the Wave Bioreactor®. Note that all cultures referenced in the table, other than this study, are seed cultures and typical seed biomass of 5 g L^{-1} was assumed and the corresponding OUR value was used. In comparing the maximum OUR obtained in the 5 and 10 L cultures it is evident that the Wave Bioreactor® can easily meet the required oxygen demand of the representative organisms. For instance this OUR could easily be met by operating a 10 L Wave bioreactor at 40 rpm, rocking angle of 10°, 0.1 vvm and 60% oxygen in the inlet gas. Therefore, the Wave Bioreactor® exhibits great potential for microbial cultivation, particularly for a seed expansion step. However, there may be other limiting factors for different organisms such as pH control that must be addressed for the successful use of the Wave Bioreactor®.

Recommendations for Wave Bioreactor® and sparged cellbag improvements

Several modifications to the current Wave Bioreactor® sparged cellbag can be made in an attempt to improve the oxygen transfer capacity. First a larger sparger or multiple spargers would increase the number of bubbles. A larger sparger can be implemented in the form of a long tube or a mat similar to the floating filter used in a perfusion cellbag

Table 2 OUR comparison of representative seed cultures

Culture	OUR (mmol L^{-1} h^{-1})	References
S. cerevisiae AlcoFree®	44–46	This study
S. cerevisiae	40	[20]
A. niger	22	[19]
C. sorphophila	30	[19]
E. coli	25	[19]

[21] and a larger sparger would help to minimize back pressure. In addition, fixing the sparger(s) to the bottom of the cellbag would take maximize advantage of the liquid height. The current bags were designed to be shallow to maximize oxygen transfer with the headspace. A deeper liquid height would allow for longer bubble residence times, although this would have to be balanced with the oxygen transfer due to the headspace gas entrainment created by the wave motion. Baffles could be added to the bottom of the bag to increase axial mixing, which was noted as poor, especially at lower rocking rates. Lastly, an improvement to the exhaust filter is needed since it easily blinds at high rocking rates due to liquid splashing. This occurred in our study despite using a filter heater and having an extra length of tubing to keep it at a greater distance from the cellbag.

Microbial cultures produce a significant amount of heat due to metabolism. At the end of the growth period in the 10 L culture, it was observed that the temperature of the cellbag had increased to 31.5 °C. However, as the current use is envisioned to be a seed expansion step, the biomass would not be as high as the test cultures and this problem may not be encountered. Alternatively the rocking thermo platform could be modified to provide cooling and operate more like a stirred tank bioreactor jacket.

Further studies should be conducted to evaluate the use of the Wave Bioreactor® for microbial seed expansion and possible replacement of traditional stirred tanks. In this context, as an example, a single 10 L culture could be used to inoculate a 300-L fermentor. In addition, smaller Wave Bioreactors® (1 L working volume) could be inoculated directly from a frozen vial and therefore circumvent the need for an initial shake flask expansion step.

Conclusions

The Wave Bioreactor® shows great promise as a microbial bioreactor, particularly for a seed expansion step and therefore potentially replacing the need for shake flasks or stirred tank bioreactors. Five and 10 L cultures of *S. cerevisiae* were successfully grown in oxygen-blended Wave Bioreactors®. Under steady state conditions, k_La values as high as 60 h^{-1} were obtained. Dynamic k_La measurements were also made and correlated to the operating conditions of the Wave Bioreactor®. It was also shown that the inclusion of the sparger in the cellbag does not effectively increase oxygen mass transfer compared to the standard cellbag. Rather, it is the headspace gas entrainment due to the wave motion of the liquid that is the major contributor to oxygen transfer, particularly at the higher rocking rates required for microbial cultivation.

Acknowledgments The authors would like to acknowledge and thank Dr. Arthur L. Kruckeberg for supplying the AlcoFree® strain and for useful discussions.

References

1. Namdev PK, Lio P (2000) Assessing a disposable bioreactor for attachment-dependant cell cultures. BioPharm 13:44–50
2. Weber W, Weber E, Geisse S, Memmert K (2002) Optimisation of protein expression and establishment for the Wave Bioreactor® for Baculovirus/insect cell culture. Cytotechnology 38:77–85
3. Palazón J, Mallol A, Eibl R, Lettenbauer C, Cusidó R, Pinol MT (2003) Growth and ginsenoside production in hairy root cultures of using a novel bioreactor. Planta Med 69:344–349
4. Jablonski-Lorin C, Melillo V, Hungerbuhler E (2003) Stereoselective bioreductions to a chiral building block on a kilogram scale. Chimia 57:574–576
5. Singh V (1999) Disposable bioreactor for cell culture using wave-induced agitation. Cytotechnology 30:149–158
6. Knevelman C, Hearle DC, Osman JJ, Khan M, Dean M, Smith M, Aiyedebinu A, Cheung K (2002) Characterisation and operation of a disposable bioreactor as a replacement for conventional stem in place inoculum bioreactors for mammalian cell culture processes. In: 224th Meeting of American Chemical Society, August 18–22, Boston
7. Tolosa L, Kostov Y, Harms P, Rao G (2002) Noninvasive measurements of dissolved oxygen in shake flasks. Biotech Bioeng 80:594–597
8. Otterstedt K, Larsson C, Bill RM, Ståhlberg A, Boles E, Hohmann S, Gustafsson L (2004) Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*. Eur Mol Biol Organ Rep 5:532–537
9. Verduyn C, Zomerdijk TPL, Van Dijken JP, Scheffers WA (1984) Continuous measurement of ethanol production by aerobic yeast suspension with an enzyme electrode. Appl Microbiol Biotechnol 19:181–185
10. Elbing K, Larsson C, Bill RM, Albers E, Snoep J, Boles E, Hohmann S, Gustafasson L (2004) Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:5323–5330
11. Diderich JA, Schepper M, van Hoek P, Luttis AH, van Dijken JP, Pronk JY, Klaassen P, Boelens HFM, Teixeira de Mattos MJ, van Dam K, Kruckeberg AL (1999) Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem 274(22):15350–15359
12. Rehm H-J, Reed G, Puhler A, Stadler P (1993). *Biotechnology*. VCH Verlagsgesellschaft mgH. Weinheim Federal Republic of Germany, pp 190–192. ISBN # 1-56081-153-6
13. Merchuk JC, Yona S, Siegel MH, Ben Zvi A (1989). On the first-order approximation to the response of dissolved oxygen electrodes for dynamic k_La estimation. Biotechnol Bioeng 35:1161–1163
14. Tunac JB (1989) High-aeration capacity shake-flask system. J Ferm Bioeng 68:157–159
15. Verduyn C, Postma E, Scheffers WA, Van-Dijken JP (1992) Effect of benzoic acid in metabolic fluxes in yeasts a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8:501–517
16. Zlokarnik M (1978) Advances in biochemical engineering. Springer, Berlin, vol 8, p 134
17. Gupta A, Rao G (2003) A study of oxygen transfer in shake flask using a non-invasive oxygen sensor. Biotechnol Bioeng 84:351–358

18. Anderlei T, Zang W, Papaspyrou M, Buchs J (2004) Online respiration activity measurement *OTR, CTR, RQ) in shake flasks. Biochem Eng J 17:187–194
19. Junker B, Seeley A, Lester M, Kovatch M, Schmitt J, Boryscwicz S, Lynch J, Zhang J, Greasham R (2002) Use of frozen bagged seed inoculum for secondary metabolite and bioconversion processes at the Pilot scale. Biotechnol Bioeng 79:628–640
20. Tan T, Zhang M, Gao H (2003) Esgosterol production by fed-batch fermentation of *Saccharomyces cerevisiae*. Enzyme Microbial Tech 33:366–370
21. Pierce LN, Shabram PW (2004) Scalability of a disposable bioreactor from 25L–500L Run in the perfusion mode with a CHO-based cell line: a tech review. BioProcess J 3:1–6