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Oxygen transfer by orbital shaking of square vessels and deepwell microtiter plates of various dimensions

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Abstract

Oxygen transfer rates (OTRs) during orbital shaking were measured for differently sized polypropylene vessels that were either square or round in the horizontal plane, using an enzymatic method involving glucose oxidase, horseradish peroxidase (HRP), and 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS). In comparison to non-shaking conditions (3.2 mmol O_2 l^{-1} h^{-1}), OTRs in 4 mm × 4 mm vessels (corresponding to wells in 384-square deepwell microtiter plates) at a working volume of 0.125 ml could only be significantly enhanced at 300 rpm if a shaking diameter of 50 mm was applied (25 mmol O_2 l^{-1} h^{-1}) instead of 25 mm (6.7 mmol O_2 l^{-1} h^{-1}). Larger square vessels (18 mm × 18 mm and 50 mm × 50 mm) yielded high oxygen transfer rates and a regular shaking pattern, demonstrating that vessels in this size range could be a viable, space-efficient, alternative to baffled or unbaffled Erlenmeyer shaking flasks. Round vessels (internal diameter 6.6 mm) resulted in OTRs that were approximately 50% of those measured for square vessels in the same size range.

Keywords: Oxygen transfer rates; Microbial; Aerosols; Aeration; Miniaturised growth vessels

1. Introduction

In the early days of microbiology, Erlenmeyer flasks were a logical choice for the cultivation of axenic microbial strains: (i) such flasks were readily available as they were already broadly applied in chemical laboratories, (ii) the conical shape allowed vigorous shaking while avoiding the spillage of culture from the opening, (iii) the relatively small opening at the top limited the rate of evaporation, allowing the cultivation of slow-growing strains without moisturizing the ambient air, (iv) the relatively large surface of the bottom allowed a high surface-to-volume ratio, which is a main determinant of the oxygen transfer rate (OTR). These combined features have helped Erlenmeyer flasks to survive the complete 20th century as the method of choice for the generation of cell mass in the range of 0.01–5 g dry weight.

Alternative growth vessels tested so far proved inferior to Erlenmeyers in one or more of the above-mentioned aspects; growth in tubes usually suffers from a lower specific OTR due to a lower surface-to-volume ratio; baffled flasks tend to yield inconsistent results (flask-to-flask variations), and lead to more aerosols, wall growth, and hydromechanical stress [1–3].

For the handling and growing of large numbers of aerobic strains (as, e.g. required during screenings for new enzymes or secondary metabolites), however, the Erlenmeyer flask is markedly inconvenient. Drawbacks include difficulties with robotic handling and the limited possibilities for miniaturisation/parallelisation. At the same time, improved analytics and more sensitive bioassays have reduced the minimal amount of biomass required during screenings to the range of 0.1–1 mg dry wt. This is compatible with culture volumes of 0.1–0.5 ml. Previous work [4,5] proved the applicability of 96-square deepwell plates for the generation of cell mass in the low milligram range, using 0.5 ml cultures of a strictly aerobic *Pseudomonas putida* strain. Under appropriate orbital shaking conditions, OTRs similar to those seen for shake flasks were attained (up to 38 mmol O_2 l^{-1} h^{-1} at a working volume of 0.5 ml). Cell densities of 9 g dry wt. l^{-1} were readily achieved under these conditions.

The feasibility of further miniaturization of growth vessels for aerobic strains (to, e.g. 384-well plates) will depend on the extent at which similarly high OTRs can be achieved by simple "non-invasive" aeration techniques, such as orbital shaking. At smaller dimensions, however, the increased importance of adhesive and capillary forces may be expected to reduce the degree in which centrifugal forces are able to

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increase the surface-to-volume ratio during orbital shaking. The goal of the present paper was to study the effect of the shape and size of polypropylene (microtiter) wells on the OTR. Also larger square vessels were tested.

2. Material and methods

2.1. Measurements of OTRs

A 120 ml bottle was filled with 100 ml of an aqueous solution (pH 7.0) containing 100 mM K_2HPO_4/KH_2PO_4 buffer, 1.5 U ml^{-1} (measured at 25 °C) glucose oxidase (Aldrich), 5 U ml^{-1} horseradish peroxidase (HRP, Aldrich), and 1 mM 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS, Fluka). The bottle was closed with a screw cap with two holes of 1 mm diameter and was flushed with nitrogen (100 ml min^{-1}) using a Teflon tube for 30 min. Subsequently, a nitrogen-flushed glass syringe (Hamilton) was used to add 1 ml of a (nitrogen-flushed) glucose solution (1 M). The (empty) vessels to be tested were mounted on an orbital shaker, which was then allowed to reach a desired frequency. Subsequently, appropriate amounts of the anoxic mixture (pre-warmed at 25 °C) were added rapidly (within 1 s) to the (shaking) vessel using a nitrogen-flushed glass syringe via a 20 cm long Teflon tube of 0.8 mm internal diameter, which was taped to the top of the vessel. After a defined time period (between 15 and 60 s), the reaction was stopped by the rapid addition of an equal volume of HCl (1.0 M) to the (still shaking) vessel using the same Teflon tube and a syringe. The time period after which the reaction was stopped was chosen to result in an absorbance at 725 nm (A_{725}) between 1 and 3. In practice, this implicated that under low oxygen transfer conditions (below 20 mmol O_2 l^{-1} h^{-1}) the reaction was stopped after 30, 45 or 60 s, while under high oxygen transfer conditions (above 20 mmol O_2 l^{-1} h^{-1}) the reaction was stopped after 15 or 30 s. The A_{725} was measured using a glass microcuvette and a Spectramax plus spectrophotometer (Molecular Devices, USA). A scheme of the coupled reaction is shown in Fig. 1.

2.2. Calculation of OTRs from the A_{725}

The A_{725} as measured above was used to calculate the amount of oxygen consumed. It was assumed that 1 mmol l^{-1} of oxygen consumed correlates with an increase in A_{725} of 19.1 (value before correction for the dilution due to the addition of HCl). This value was determined by supplying the enzyme/ABTS mixture described above with a limited concentration of glucose (100 μM), and subsequent measurement of the A_{725} (after all glucose was consumed, and after acidification). For unknown reasons, the value of 19.1 is lower than the theoretical value of 28.0 that would result from a stoichiometry of 2 mol of $ABTS^+$ formed for every mole of O_2 consumed, using a (measured) molar extinction coefficient at 725 nm of $ABTS^+$ of 14.0 at pH 0 (determined empirically using a 100 μM solution of ABTS supplied with horseradish peroxidase and a surplus amount of hydrogen peroxide).

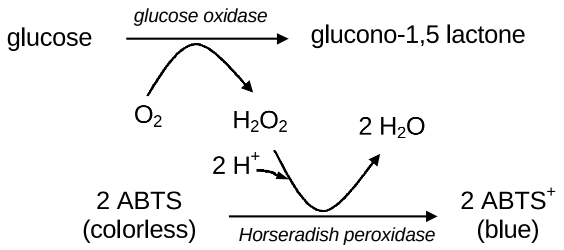

Fig. 1. The coupled enzyme reaction used for the determination of OTRs.

2.3. Orbital shaking conditions and vessels

Orbital shakers with a 50 mm shaking diameter (Kühner AG, Basel, Switzerland) or 25 mm shaking diameter (Unimax 2010, Heidolph, Nürnberg, Germany) were used. The shaking frequency was varied from 0 to 400 rpm. All experiments were performed at 25 °C.

Five rectangular vessels (all square in the horizontal plane) with the following dimensions were custom-made using high density polypropylene plates of 5–8 mm thickness and silicone paste (width × height): 4 mm × 23 mm, 8 mm × 45 mm, 18 mm × 93 mm, 50 mm × 260 mm, and 100 mm × 500 mm. In addition, a deepwell microtiter plate with round polypropylene wells of 7 mm internal diameter (Greiner) was obtained from Polylabo (Geneva, Switzerland).

3. Results

3.1. Validation of the glucose oxidase/HRP method

In order to determine whether the oxidation of ABTS follows zero order kinetics, various reaction times (time intervals between the addition of the anoxic reaction mix to the shaking vessel, and the termination of the reaction by the addition of HCl) were tested using a 8 mm × 8 mm square well shaken at a frequency of 300 rpm at a shaking diameter of 50 mm and a volume of 500 μl. The results (Fig. 2) indicate that the increase in A_{725} in the first 30 s reaction (between an A_{725} of 0.2 and 3) was undistinguishable from zero-order. The data point at time 0 represents the A_{725} when the HCl was added to the well prior to the addition of the anoxic reaction mix. From three independent measurements using an incubation period of 15 s, an OTR of 33.6 ± 4.4 mmol O_2 l^{-1} h^{-1} was calculated.

3.2. Effect of the size of square vessels on the OTR

Differently sized square vessels (five different dimensions in the horizontal plane) were tested at a frequency of 300 rpm and a shaking diameter of 25 or 50 mm. Working

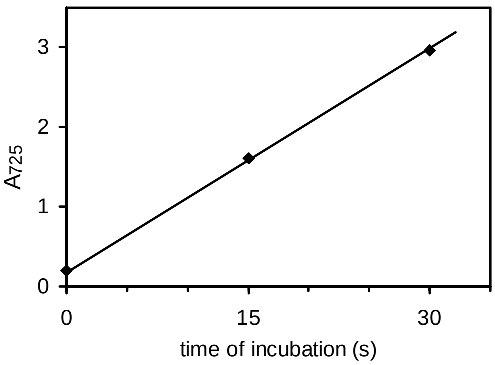

Fig. 2. Demonstration of zero-order kinetics of ABTS oxidation using the enzymatic method shown in Fig. 1; increase of the A_{725} in time during shaking of a 8 mm × 8 mm vessel with a working volume of 0.5 ml at 300 rpm and a shaking diameter of 50 mm (see text).

volumes were chosen to result in the same filling height of 7.8 mm (corresponding to the filling height in a well of a regular 96-square deepwell plate filled with 0.5 ml, for which reference data are available). The results (Table 1) show that larger vessels result in a relatively high specific OTR. This effect of the size is more pronounced at the low shaking diameter of 25 mm. At a shaking diameter of 50 mm, the three intermediately sized vessels (8 mm × 8 mm, 18 mm × 18 mm, and 50 mm × 50 mm) gave rise to a visually regular shaking pattern: the bulk of the liquid easily followed the movement of the shaker (although there was some splashing in the 50 mm × 50 mm vessel). The largest vessel (100 mm × 100 mm) gave rise to extensive splashing and a chaotic shaking pattern (the bulk of the liquid failed to follow the movement of the shaker). Visual assessment of the shaking pattern in the smallest vessel (4 mm × 4 mm) was not readily feasible. The shaking patterns at a shaking diameter of 25 mm were similar to those at a 50 mm shaking diameter, except that not only the 100 mm × 100 mm vessel but also the 50 mm × 50 mm vessel gave rise to extensive splashing and a chaotic shaking pattern.

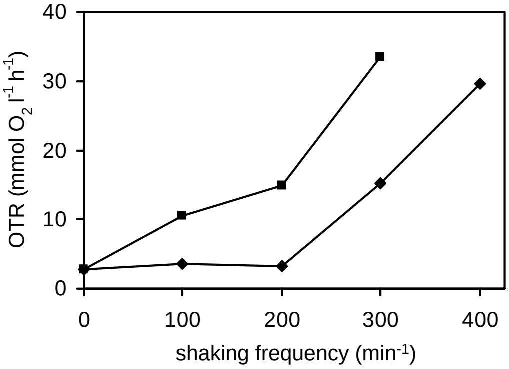

Fig. 3. Influence on the OTR of the frequency of orbital shaking of a 8 mm × 8 mm vessel at shaking diameters of 25 mm (◆), and 50 mm (■).

3.3. Effect of the shaking frequency on the OTR

The shaking frequency appeared to have a strong effect on the OTR in 8 mm × 8 mm wells at both shaking diameters tested (25 and 50 mm). The effect was most pronounced at a shaking diameter of 25 mm; shaking frequencies of 100 and 200 rpm did not yield any significant increase in OTR in comparison to the complete absence of shaking (Fig. 3).

3.4. OTRs in round deepwells

The OTR in polypropylene round deepwells (internal diameter 6.6 mm) was measured at a working volume of 0.26 ml (corresponding to the same height of 7.8 mm as used for the square wells described in the previous sections) at shaking diameters of 25 and 50 mm. At a shaking diameter of 50 mm and a shaking frequency of 300 rpm, the OTR was 16.4 mmol O_2 l^{-1} h^{-1}, which is about 50% of the OTR found for square wells of a similar dimension (8 mm × 8 mm, 33.6 mmol O_2 l^{-1} h^{-1}) filled to the same height. At the lower shaking diameter of 25 mm, the OTR was 76% lower at 4.0 mmol O_2 l^{-1} h^{-1}, which is only

Table 1
Effect of the shaking diameter on OTRs in growth vessels of various dimensions during orbital shaking at 25 °C and 300 rpm

Size[a]	Volume (ml)	Oxygen transfer rates (mmol O_2 l^{-1} h^{-1})		Theoretical surface-to-volume ratio[b] (mm^{-1})	
		25 mm[c]	50 mm[c]	25 mm[c]	50 mm[c]
Square wells					
4 mm × 4 mm	0.125	6.7	25.4 ± 5.9	0.21	0.34
8 mm × 8 mm	0.5	15.2 ± 2.4	33.6 ± 4.4	0.21	0.31
18 mm × 18 mm	2.5	39.3	50.5 ± 3.5	0.17	0.20
50 mm × 50 mm	19.5	47.7[d]	58.1	0.10	0.12
Round wells					
6.6 mm[e]	0.26	5.0	16.4	0.21	0.31

[a] Dimensions in the horizontal plane.

[b] Assuming that the surface of the liquid is flat and its angle with the horizontal plane is a function of the centrifugal and gravitational acceleration only.

[c] Shaking diameter.

[d] Irregular shaking pattern (see text).

[e] Internal diameter.

slightly higher than the OTR measured in the absence of shaking (2.7 mmol $O_2\,l^{-1}\,h^{-1}$). An increase of the shaking frequency to 400 rpm (25 mm shaking amplitude) yielded only a slight increase in the OTR to 5.0 mmol $O_2\,l^{-1}\,h^{-1}$.

4. Discussion

For the quantification of OTRs in square and round mini-vessels, we used an enzymatic method involving glucose oxidase, horseradish peroxidase and ABTS (see Fig. 1). Glucose oxidase has been used previously for the quantification of OTRs in bioreactors (reviewed in [6]). In these earlier studies, however, the reaction was not coupled to the oxidation of ABTS but to the lactonase-catalyzed conversion of glucono–lactone to gluconic acid that was then quantified by acidometric titration. The enzymatic method we used is more convenient than the more widely applied method involving the cobalt-catalyzed oxidation of sulfite and the measurement of the oxygen consumption from the headspace, which becomes increasingly tedious and inaccurate at lower working volumes (note: this disadvantage has been recently partially alleviated by the development of an optical method to follow the oxidation of sulfite, [7]). Another alternative method, involving the measurement of the increase in biomass of a culture of *P. putida* in time, as used in a previous study [4] also becomes less workable at smaller working volumes. Besides convenience and easy down-scaling, the enzymatic method offers the additional advantage of a low ionic strength of the buffer system (100 mM phosphate buffer), which is in the same range of commonly used microbial growth media. High salt concentrations as required for the chemical method involving the cobalt-catalyzed oxidation of sulfite (0.5 M sodium sulfite) are known to lead to a lower solubility of oxygen, and so to a systematic underestimation of the maximal OTRs attainable in relevant microbiological media. In this light, it was not unexpected that the OTRs measured in this study for 8 mm × 8 mm square wells at 300 rpm and a shaking diameter of 50 mm (33.6 mmol $O_2\,l^{-1}\,h^{-1}$) are closer to the corresponding value derived from the oxygen limited growth rate of *P. putida* CA-3 (37.8 ± 2.6 mmol $O_2\,l^{-1}\,h^{-1}$, [4]), than the value derived from the cobalt-catalyzed oxidation of sulfite (27.3 ± 0.4 mmol $O_2\,l^{-1}\,h^{-1}$ [4]). Since the glucose oxidase is present in excess, it may be expected that most oxygen that diffuses into the bulk liquid is consumed immediately. The method was validated by stopping the reaction after various time intervals and using different concentrations of glucose oxidase: at higher levels than the glucose oxidase activity used (1.5 U ml^{-1}), the concentration of oxidized ABTS was not affected, from which it can be concluded that practically all oxygen taken up in the bulk liquid was consumed immediately giving rise to H_2O_2 (results not shown).

A main goal of the present study was to determine to what extent the OTR is affected by further miniaturization of square growth vessels. The OTRs in square vessels of 4 mm × 4 mm (corresponding to wells in 384 well plates) at a shaking diameter of 50 mm at 300 rpm was found to be 25% lower than in 8 mm × 8 mm vessels under the same shaking conditions. This difference was more pronounced (56% lower) when a shaking diameter of 25 mm was applied (Table 1). The strong influence of the shaking diameter in small vessels (a factor 5 for the 4 mm × 4 mm vessel) in comparison to larger vessels (a factor 1.2 for the 18 mm × 18 mm vessel) may be explained by the relatively strong adhesive and capillary forces in comparison to the gravitational forces in small vessels. Previous photographic studies of 8 mm × 8 mm wells [4,5] showed that the gravitational force at 300 rpm was sufficient to generate an angle of the aqueous surface with the horizontal plane in accordance with the Froude number (the ratio between centrifugal and gravitational acceleration) at a shaking diameter of 50 mm, but not at 25 mm. During the latter conditions (shaking diameter 25 mm), we assumed that the centrifugal force is not sufficient to overcome the adhesive and capillary forces, leading to so-called out-of-phase conditions [8]. The present observation that the OTR in 4 mm × 4 mm vessels is lower than in 8 mm × 8 mm vessels in despite of a slightly higher theoretical surface-to-volume ratio (Table 1) suggests that in the 4 mm × 4 mm vessel such out-of-phase conditions not only (strongly) occur at a shaking diameter of 25 mm but also, albeit rather mildly, at a 50 mm shaking diameter. On the basis of these results, we predict that an increase of the shaking diameter to 75 or 100 mm could result in significantly higher OTRs for 4 mm × 4 mm vessels.

A second goal of this study was to determine the OTRs in square vessels larger than those present in 96 well microtiter plates. An interesting observation is the increasing OTR with an increasing vessel size, even though the theoretical surface-to-volume ratio (assuming that the surface of the liquid is flat and its angle with the horizontal plane is a function of the centrifugal and gravitational acceleration only) is decreasing with an increasing vessel size (Table 1). We assume that this is mainly due to the smaller importance of adhesive and capillary forces at increasing vessel sizes, as discussed in the previous paragraph. Interestingly, only the 18 mm × 18 mm vessel showed no significant influence of the shaking diameter; the visual shaking pattern was regular, and the OTRs were similar. For the 50 mm × 50 mm vessel the shaking pattern was only regular at a shaking diameter of 50 mm. At a shaking diameter of 25 mm, out-of-phase conditions characterized by splashing and a chaotic shaking pattern occurred. In absolute terms, the OTRs measured for the 18 mm × 18 mm and 50 mm × 50 mm vessels at a 50 mm shaking diameter were high (50–60 mmol of $O_2\,l^{-1}\,h^{-1}$) in comparison to Erlenmeyer flasks during orbital shaking under similar conditions [3]. Therefore, square vessels could be a viable alternative to Erlenmeyer flasks for culture volumes in the range of 2–20 ml. Square vessels in this size range can be considered as a compromise between regular Erlenmeyer flasks and baffled flasks, without the disadvantages associated with baffled flasks (flask-to-flask variation and splashing [1]). Taking into account that the shaking platform can be used in a more space-efficient way, it can be readily estimated that the use of square vessels could increase the OTR expressed per unit of shaking platform area by a factor of 3–4 in comparison to regular shake flasks.

The round deepwells (6.6 mm in diameter) in 96-well microtiter plates behaved similarly to 4 mm × 4 mm square wells with respect to the strong influence of the shaking diameter on the OTR (more than a factor 3 difference between 25 and 50 mm shaking diameter). The OTR in such 96 round deepwell plates was more than two-fold lower than in wells of its square well counterpart, leading to the conclusion that round deepwell plates are inferior both with respect to OTRs and attainable working volumes.

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