

# Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates

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## Abstract

Growth of heterogeneous culture collections in microtiter plates is advantageous for logistic reasons and also in enabling significant savings in medium costs, labor input and use of equipment during large screening projects. The main hurdles to overcome for aerobic microbial strains are the prevention of cross-contamination and excessive evaporation while assuring sufficient aeration rates. For this purpose we developed a sandwich spongy silicone/cotton wool cover to close the wells of square-deepwell microtiter plates. Oxygen transfer rates were derived from growth curves of *Pseudomonas putida* and were shown to be threefold higher during orbital shaking at a shaking diameter of 5 cm at 300 rpm ( $24 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$  at a culture volume of 0.75 ml) in comparison to a shaking diameter of 2.5 cm. Photographic analysis showed a clear influence of the shaking diameter on the hydrodynamic behavior in the wells; during shaking at a 2.5 cm amplitude, out-of-phase conditions occurred resulting in poor vertical mixing, while a 5 cm shaking amplitude led to an optimal surface to volume ratio and a turbulent flow. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Heterogeneous culture; Microtiter plate; Oxygen transfer rate

## 1. Introduction

In the last decades, technological improvements in analytical systems have continuously led to lower detection limits for most organic compounds. At the same time, the minimum amount of sample volume required has decreased substantially as well. Recent important developments in this respect include the introduction of miniature HPLC columns, and the development of sensitive HPLC–MS systems which are also applicable for low molecular weight compounds. A logical consequence of these developments is that the minimum amount of microbial biomass needed for the purpose of screening for new biocatalysts or secondary metabolites is decreasing accordingly.

These smaller requirements for biomass levels open up the way for the use of miniaturized growth systems for screening heterogeneous microbial culture collections. The use of 96-well or 384-well microtiter plates is attractive in this respect in the light of their wide availability in various shapes and volumes, and the large choice in equipment for handling microtiter plates (dispensers, centrifuges, multipipettes etc.). Until now, use of microtiter plates for the growth and maintenance of microbial strains has been mainly limited to

clonal libraries in *Escherichia coli* [1–3] and yeasts [4,5]. Major bottlenecks in the use of microtiter plates for the maintenance, handling and growth of aerobic heterogeneous culture collections are the risks of cross-contamination and poor aeration rates.

We have addressed these problems with (a) the development of a suitable cover system for square-deepwell microtiter plates, and (b) the comparison of various shaking parameters with respect to mixing properties and oxygen transfer rates (OTRs).

## 2. Development of a suitable cover system for square-deepwell microtiter plates

A cover system to be used for deepwell square microtiter plates during orbital shaking should meet the following requirements: (i) the cover should prevent direct well-to-well spilling of cell suspension by small splashes or aerosols, (ii) the cover should prevent airborne contaminants to enter the wells, (iii) the cover should prevent excessive evaporation of water from the culture (no more than 5% volume loss per day), (iv) the cover should allow a sufficient degree of replacement of the headspace air (venting) to maintain an oxygen level in the headspace of more than 75% of air saturation.

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### 2.1. Sandwich cover

The above criteria are met when applying the following sandwich cover: a deepwell microtiter plate is covered with a spongy silicone plate (thickness 8 mm, non-permeable film at both sides, quality 2660 shore, Maag Technik AG, Dübendorf, Switzerland), perforated with 96 holes of 1.5 mm diameter (holes positioned exactly above the centers of the wells). This spongy silicone plate in its turn is covered, respectively, with a thin layer (2 mm) of cotton wool and a stainless steel lid of 1.5 mm thickness, perforated with 96 holes of 6 mm diameter. The microtiter plate and the sandwich cover are clamped together with a force of approximately 500 N using an appropriate clamp, causing the silicone plate to become uniformly compressed by a factor 2 (from a thickness of 8 to 4 mm).

### 2.2. Headspace air refreshment rates

It was calculated that at a hole diameter of 1.5 mm, the rate of refreshment of the headspace air is sufficient to maintain an oxygen concentration in the headspace of more than 15% even at oxygen uptake rates of  $20 \text{ mmol l}^{-1} \text{ h}^{-1}$  at working volumes of 1 ml.

### 2.3. Evaporation

With working volumes of 0.75 ml, the evaporation rate during orbital shaking at 300 rpm was measured to be  $10 \mu\text{l}$  per day at an environmental relative air humidity of 40–60%. This rate of evaporation is sufficiently low if the strains can be harvested within 10–20 days (dependent on the growth rate of the strains). For extremely slow growing strains it might be considered to increase the environmental air humidity in order to decrease the evaporation rate. It should be noted, however, that air humidification almost inevitably leads to colonization of the incubation chamber with fungi. Therefore, a better solution for slow growing strains consists of the use of spongy silicone plates in which the 96 holes are filled with short pieces of teflon or silicone tubing with an internal diameter of 0.5 or 1 mm.

## 3. Effect of shaking parameters on mixing pattern and oxygen transfer rates

To improve the aeration rates in microtiter plates the following approaches can be considered: (i) use of pure oxygen, (ii) use of oxygen enriched air, (iii) use of high pressures, (iv) magnetic stirring of all wells, (v) mild sonication methods. Each of these methods has disadvantages ranging from safety problems (spontaneous combustion of plastics at high oxygen concentrations, explosions at high pressures) to toxic effects of high oxygen concentrations for a variety of strains, high costs associated with individual magnetic stirring of wells, etc. For these reasons, we concentrated

our study mainly on conventional aeration methods, especially horizontal orbital shaking at various shaking diameters.

### 3.1. Quantification of oxygen transfer rates

Square-deepwell microtiter plates containing cell suspensions of *Pseudomonas putida* (0.75 ml per well) growing on a mineral medium with glucose as the sole carbon and energy source were incubated at  $25^\circ\text{C}$  on an orbital shaker with a shaking diameter of either 2.5 or 5 cm (Kühner AG, Basel, Switzerland). At regular time intervals, small samples (10–30  $\mu\text{l}$ ) were taken from the cultures using a 50  $\mu\text{l}$  syringe (Hamilton, USA). After dilution in a physiological salt solution, the  $\text{OD}_{540}$  was measured in glass microcuvettes. The growth curves were translated into OTRs in the following way. The average yield on glucose was measured to be  $0.363 \text{ g dry wt. (g glucose)}^{-1}$ , corresponding to  $2.88 \text{ mol C (mol glucose)}^{-1}$  if it is assumed that the carbon content of bacterial biomass is 53%. Assuming an elemental ratio in biomass of C:H:N:O of 1:1.666:0.2:0.27 and given that ammonia is the sole nitrogen source, the following overall equation for biomass formation can be derived:  $\text{C}_6\text{H}_{12}\text{O}_6 + 0.576\text{NH}_3 + 2.91\text{O}_2 \rightarrow 2.88\text{CH}_{1.666}\text{N}_{0.2}\text{O}_{0.27} + 3.12\text{CO}_2 + 4.47\text{H}_2\text{O}$ . The corresponding oxygen consumption rate of  $42.1 \text{ mmol (g of dry wt. formed)}^{-1}$  was used to calculate OTRs directly from the rate of increase in biomass in the linear (oxygen limited) part of the growth curve using linear regression.

The OTRs determined in this way appeared to be strongly influenced by the shaking diameter at 300 rpm and a working volume of 0.75 ml. A shaking diameter of 5 cm resulted in a threefold higher OTR ( $24 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$ ) in comparison to a shaking diameter of 2.5 cm ( $8 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$ ). The OTR at a shaking diameter of 5 cm ( $24 \text{ mmol O}_2 \text{ l}^{-1} \text{ h}^{-1}$ ) is similar to that of a 30 ml culture in a 300 ml Erlenmeyer flask shaken at 300 rpm [6]. The OTRs at a shaking diameter of 5 cm allowed *P. putida* to reach a cell density of almost  $10 \text{ g dry wt. l}^{-1}$  during growth on a glucose mineral medium at a culture volume of 0.75 ml.

### 3.2. Hydrodynamic behavior during shaking

The hydrodynamic behavior of 0.75 ml of an aqueous solution of bromocresol blue (1%, w/v) in a square microtiter plate well was studied under various shaking conditions, using a Powershot 70 digital camera (Canon, Japan). In correspondence with the results for the OTRs in the previous section, it was observed that the degree of mixing at a shaking diameter of 2.5 cm was poor in comparison to a shaking diameter of 5 cm (see Figs. 1 and 2).

The figures also show that the angle of the aqueous surface with the horizontal plane was lower at a shaking diameter of 2.5 cm, which can be attributed to the lower centrifugal force. The higher OTRs at a shaking diameter of 5 cm are probably not only due to the larger surface area but also to

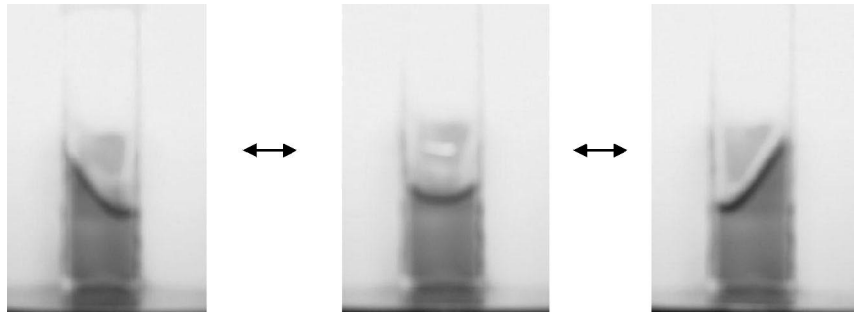


Fig. 1. Shaking pattern during orbital shaking at 300 rpm in a square microwell of 2 ml at a shaking amplitude of 2.5 cm and a working volume of 0.75 ml ( $\text{OTR} = 8 \text{ mmol l}^{-1} \text{ h}^{-1}$ ).

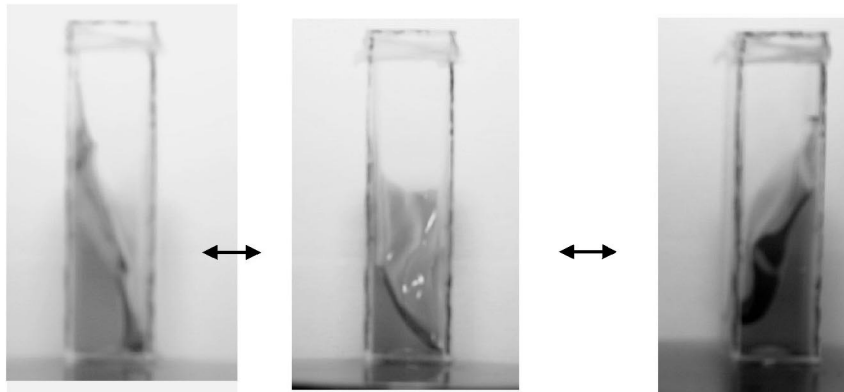


Fig. 2. Shaking pattern during orbital shaking at 300 rpm in a square microwell of 2 ml at a shaking amplitude of 5 cm and a working volume of 0.75 ml ( $\text{OTR} = 24 \text{ mmol l}^{-1} \text{ h}^{-1}$ ).

a better degree of vertical mixing. The photos taken at a shaking diameter of 5 cm show small waves occurring at the surface.

It can be concluded that the combination of (i) the application of the described sandwich covers and (ii) the use of orbital shakers with a shaking diameter of 5 cm have solved the major problems previously associated with the use of microtiter plates for the growth of aerobic strains.

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