Tutorial: Microbial and cell growth in microtiter plates.

Wouter A. Duetz, Markus Kühner, Rene Lohser

Trend towards lower culture volumes. Construct and mutant libraries, as well as collections of bacteria, fungi, streptomycetes, and eukaryotic cell lines are being used in a wide range of research areas. As diverse as these areas may be - ranging from traditional streptomycete screening aimed at the discovery of new drugs to the use of RNAi libraries in recent years - there is one common denominator: ever smaller culture volumes suffice. Receptor binding assays, cell based (inhibition) assays, and other bioassays alike are being miniaturized to an extent that they can be well performed with pg or ng amounts of cell products. The same holds for e.g. LC-MS assays for biocatalyst screenings and medium optimization studies.

The challenge of the last 5 years has been to miniaturize microbial and cell growth systems while maintaining the quality of growth and axenicity as traditionally achieved in shake flasks and bioreactors. The use of the standard microtiter plate format is attractive because of the availability of compatible robots, readers, and other equipment. The present tutorial describes the state of-the art of methods available to turn the individual wells from microtiter plates into high-quality "mini-reactors".

Well closures. The most critical aspect of microbial growth in microtiter plates is the closure system of the individual wells, which has to meet three requirements: i) prevent (cross)-contamination even during vigorous shaking, ii) permit the exchange of headspace air, iii) limit evaporation. Equal physical conditions in all wells is a further requirement: the wells in the corners should have exactly the same characteristics as the wells in the middle of the microtiter plate. This is especially important in the area of medium optimization and mutant screening where productivity improvements as low as 5% should be detectable.

Gas permeable membrane filters (sealing tapes) are available from many suppliers, and are well suitable for qualitative work (e.g. to generate small amounts of plasmid DNA) but have a number of distinct disadvantages for quantitative work. Firstly, during vigorous shaking, droplets may cover smaller or larger parts of the membrane, leading to random well-to-well variations in evaporation and supply of oxygen. Secondly, the partial dissolving of (potentially toxic) glue material may harm growth. An additional drawback of plastic membranes is their relatively low diffusion coefficient for oxygen and high diffusing coefficient for water. The result is substantial water loss by evaporation while the oxygen demand of the culture is often not met.

The most complete solution that overcomes the above-mentioned problems of sealing tapes has been developed by ENZYSCREEN in Holland, is marketed by KüHNER AG in Switzerland and distributed in the US by BIOPRO INTERNATIONAL INC., and consists of a sandwich cover consisting of the following layers: i) a silicone layer with 96 small holes in the middle of each well, ii) a layer of cotton wool, and a rigid (stainless steel) lid with 96 holes (Fig. 1,2). The sandwich cover and the microtiter plate are clamped together tightly in order to prevent spillage of the culture fluid during high G-force orbital shaking, and to ensure that exchange of headspace air occurs solely through the center holes. In this system, the rate of exchange of headspace air (and so also the evaporation) is accurately controlled by the diameter of the holes in the silicone layer; holes of 0.5-1 mm result in water evaporation rates of 10-20 µl per well per day at 50% humidity and 30°C. A thin stainless steel foil with 200 µm holes inserted between the silicone layer and the cotton wool layer results in even lower evaporation rates (4-6 µl per well per day) and is advisable for 100-200 µl cultures in lowwell plates. Such evaporation rates correspond to 2-5% of the culture volume per day, which is acceptable for most purposes. Therefore, no air humidification is required, which avoids the nuisance of fungal wall growth to occur (as is often seen in humidified incubators).

Since the diffusion coefficients of oxygen and water in air are practically equal (0.21 cm^2/s at 20°C), it can be readily calculated that an evaporation rate of 10 µl per day at a relative humidity of 50% correlates with an exchange rate of headspace air of 450 µl (containing 3.6 µmol O_2) per minute, provided a 'mechanical' diffusion barrier such as cotton wool is used). This guarantees a sufficient oxygen concentration (above 18%) in the headspace even at oxygen consumption rates in the culture as high as 40 mmol/l/h. It should be noted that air supply rates in this range correlate well with the general rule of thumb for lab-scale stirred tank bioreactors: one vessel volume of air per minute.

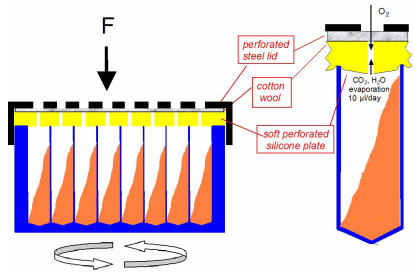

Fig 1. Schematic view of a sandwich cover for deepwell plate cultures, to reduce evaporation, and allow vigorous orbital shaking without cross-contamination.

A sufficiently high and universal exchange rate of headspace air is a prerequisite, but not a guarantee that the cells growing in the wells are supplied with enough oxygen; gas-liquid transfer is another limiting factor. Optimal incubation conditions are described in the next section.

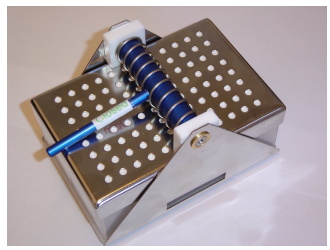

Fig 2. Sandwich covers convert all individual wells of square deepwell plates into 96 'mini-reactors'

Orbital shaking conditions for various microtiter plates

The type of microtiter plate to be used is project-dependent. For culture volumes of ~ 250 µl, good results can be obtained with regular polystyrene 96-lowwell plates as long as one is prepared to settle for moderate oxygen transfer rates.

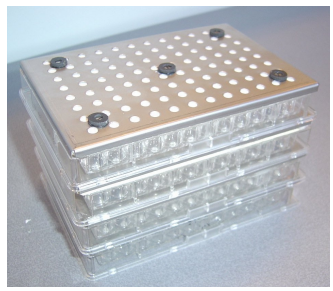

Fig 3. Lowwell microtiter plates can be stacked on an orbital shaker if special sandwich covers allowing a free airflow between the plates are used.

The advantage of such lowwell plates is that they can be stacked, provided special sandwich covers are used that maintain a space between the cover and the

microtiter plate put on top (Fig. 3). Shaking at 300 rpm and a 50 mm shaking amplitude results in oxygen-transfer rates (OTR) of around ~15 mmol O_2/l/h (Hermann *et al.*, 2002, Biotechnol. Bioeng. 81: 178-186). This OTR can be almost doubled by lowering the culture volume to 100 µl or by increasing the orbital shaking frequency to 350 rpm (note: only advisable when using sandwich covers as described above to prevent spillage of culture fluid). The hydrodynamic pattern during shaking is regular and non-turbulent, so also suitable for strains and cell cultures that are susceptible to shear forces.

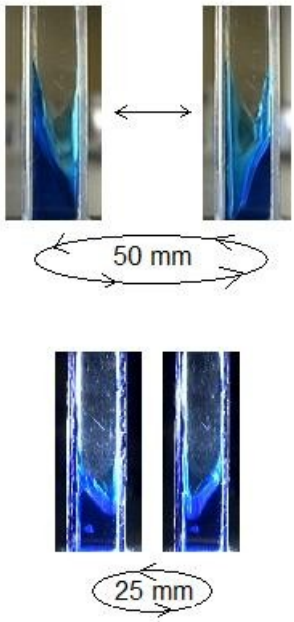

Fig. 4. Hydrodynamic shaking patterns during orbital shaking of an (isolated) 96 square deepwell at 300 rpm at shaking amplitudes of 50 mm and 25 mm. The OTR at 50 mm is three fold higher than at 25 mm

For higher OTRs (up to 40 mmol/l/h) and higher culture volumes (0.5-1 ml), the use of square 96 deepwell plates is advisable (Duetz *et al*., 2000, Appl. Environ. Microbiol. 66:2641-2646). The square shape in the horizontal plane results in a turbulent shaking pattern (Fig. 4) and therefore better mimics the situation in e.g. stirred tank bioreactors than roundwell microtiter plates do. For this reason, square deepwell plates are especially well applicable for e.g. medium optimization studies for large-scale production of e.g. secondary metabolites. A major determinant of good aeration rates is the shaking amplitude. The best results are obtained by relatively high shaking amplitudes (50 mm or more) since the generated G-forces are sufficiently high to induce mixing until the very bottom of the wells, as is illustrated by Fig. 4. Cell densities of almost 10 g dry wt per liter are readily attained at growth rates similar to shake flasks incubated under optimal conditions (Fig. 5)

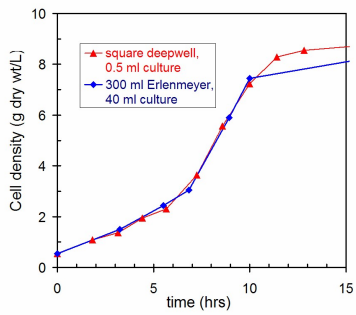

Fig. 5. Growth curves of a *Pseudomonas putida* strain in wells of a deepwell microtiter plate, and an Erlenmeyer shake flask during orbital shaking at 300 rpm and a shaking amplitude of 50 mm.

Square 24-well square deepwell plates at culture volumes of 2-4 ml are favored in some research areas, such as medium optimization for streptomycetes or fungi. A major reason for the use of 24-well plates for pellet-forming microorganisms is to reduce the effect of statistical variations in the number of pellets per well on the concentration of the metabolite. It should be noted here that for 24-square well plates, 300 rpm at an amplitude of 25 mm instead of 50 mm is sufficient to achieve aeration rates of 40-50 mmol /l/h.

Other aeration methods.

The use of orbital shaking as described above is attractive because it is effective, economical and simple. Furthermore, it helps to minimize well-to-well variations since the G-force is exactly identical in each well and therefore also the hydrodynamic patterns and oxygen transfer rates show very little variation. The use of small stirring bars or air-bubbling systems is more elaborate and almost inevitably leads to higher well-to-well variations.

Inoculation of wells.

For some strains such as *E. coli*, it is possible to inoculate microtiter plate cultures from a thawed master plate using a pipetting robot. A distinct disadvantage of this procedure, however, is that for most microorganisms 90-99.9% of the cells remaining in the master plate loose viability with each freeze-thaw cycle. For other (heterogeneous) culture collections, an additional drawback is that if cells from frozen stocks are inoculated directly into liquid cultures, the viability is often quite poor.

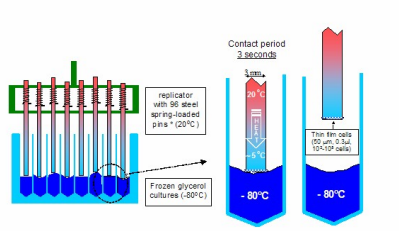

Fig. 6. sampling of 96 frozen microbial stocks without thawing the remaining culture, by using a spring-loaded replicator

For these reasons we advise the following procedure. The strains are stored in the presence of 15% (v/v) glycerol in deepwell microtiter plates at -80 oC. A special, sterile, replicator (developed at the ETH Zürich, and made commercially available by ENZYSCREEN and KüHNER) with 96 spring-loaded pins is pressed onto the 96 frozen glycerol stocks (-80 ºC), and samples 0.3 µl per well by heat transfer, without thawing the remaining glycerol culture (Fig. 6). Since the master plate remains frozen the viability is not affected by repeated sampling. The same master plate can therefore be used for several hundreds of times. The sampled cells are transferred onto an agar medium suspended into a microtiter plate; the resulting colonies can be used after 1-3 days for inoculation of the liquid cultures.

Further miniaturization in future?

For suspended cultures, an intrinsic hurdle for further miniaturization is the surface tension and cohesion of aqueous cell suspensions. In culture vessels smaller than ~3.5 mm in diameter, the surface tension can not be overcome by the centrifugal forces applied by e.g. orbital shaking. The result is a dramatically low OTR and the absence of any significant mixing of the liquid, even if G-forces above 5 are applied. These intrinsic (physico-chemical) limitations have lead us to believe that the scope for high-quality cultivation of liquid cultures at this scale (e.g. in 384 well plates) is minimal.

Further parallelisation (more cultures per square meter) is more likely to be achieved by reducing the height of the cultures, and stacking them.

Wouter Duetz is CEO of Enzyscreen bv, Leiden, the Netherlands (www.enzyscreen.com), a company specialized in miniaturized growth systems. Markus Kühner is CEO of Kühner Ag, Basel, Switzerland, a supplier of orbital shakers (www.kuhner.com). Rene Lohser is CEO of Biopro, Farmingdale New York, a supplier of biotechnology equipment (www.biopro.com).