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Scale-down of vinegar production into microtiter plates using a custom-made lid

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As an important food preservative and condiment, vinegar is widely produced in industry by submerged acetic acid bacteria cultures. Although vinegar production is established on the large scale, up to now suitable microscale cultivation methods, e.g. using microtiter plates, are missing to enable high-throughput cultivation and to optimize fermentation conditions. In order to minimize evaporation losses of ethanol and acetic acid in a 48-well microtiter plate during vinegar production a new custom-made lid was developed. A diffusion model was used to calculate the dimensions of a hole in the lid to guarantee a suitable oxygen supply and level of ventilation. Reference fermentation was conducted in a 9-L bioreactor to enable the calculation of the proper cultivation conditions in the microtiter plate. The minimum dissolved oxygen tensions in the microtiter plate were between 7.5% and 23% of air saturation and in the same range as in the 9-L bioreactor. Evaporation losses of ethanol and acetic acid were less than 5% after 47 h and considerably reduced compared to those of microtiter plate fermentations with a conventional gas-permeable seal. Furthermore, cultivation times in the microtiter plate were with about 40 h as long as in the 9-L bioreactor. In conclusion, microtiter plate cultivations with the new custom-made lid provide a platform for high-throughput studies on vinegar production. Results are comparable to those in the 9-L bioreactor.

[**Key words:** High-throughput vinegar production; Acetic acid bacteria; Scale-down; Microtiter plate lid; Reduction of evaporation losses; BioLector; Flowerplate]

Vinegar is very important as a food preservative or condiment (1–3). In vinegar production, acetic acid bacteria stoichiometrically oxidize ethanol with oxygen to acetic acid and water (4). This oxidation reaction is catalyzed by membrane bound alcohol and aldehyde dehydrogenases that are linked to the respiratory chain (5). There are several fermentation methods to produce vinegar such as traditional methods or solid-state fermentation (6). Yet today, vinegar is predominantly manufactured by submerged fermentation first described in 1949 (7). Since the acetic acid bacteria are obligatory aerobic in vinegar production (7–13), a sufficient oxygen supply is essential for submerged fermentation. The substrate ethanol and the product acetic acid are relatively volatile. Thus, to avoid excessive evaporation and product losses, it is favorable to disperse the air for oxygen supply into fine air bubbles instead of using high aeration rates (9). In bioreactors for vinegar production, special turbines generate these fine air bubbles and, at the same time, homogeneously mix the air and liquid in the reactor (4). Consequently, industrial aeration rates are as low as 2 vvh (volumes air/volumes culture medium/hour) to 5 vvh.

The bacterial culture in vinegar production is a mixture of different strains of acetic acid bacteria for various reasons: First, industrial submerged fermentations are inoculated with microbiologically undefined bacterial cultures taken from other vinegar productions (4). Second, vinegar is produced in unsterile bioreactors where the best adapted acetic acid bacteria strains thrive due to higher tolerance to high ethanol and acetic acid concentrations. Third, strains of acetic acid bacteria show strong genetic variations that lead to a heterogeneous population. These genetic variations are mainly caused by insertion sequence elements or by plasmid rearrangements (14,15).

At the high concentrations of acetic acid and ethanol in vinegar production, the acetic acid bacteria are obligatory aerobic. They rapidly die as soon as the aeration is interrupted. Thus, they are difficult to isolate and typical, microbiological inoculation cascades are not appropriate for vinegar production (16). Therefore, it is reasonable that vinegar is produced industrially in repeated batch, repeated fed batch or cascaded repeated fed batch fermentations (4,17,18). Such kind of repeated batch processes show good productivity, since high ethanol and acetic acid concentrations ensure the selectivity of the particular bacterial strains.

The smallest scale used for submerged production of vinegar under industrially high ethanol and acetic acid concentrations is typically in the magnitude of liters in laboratory bioreactors such as the 9-L Frings-Acetator (4,18). Only recently, reproducible batch fermentations for vinegar production were achieved on the milliliter scale in shake flasks by ensuring a constant oxygen supply during inoculation (16). Furthermore, a shake flask system could be developed even for extended repeated batch vinegar production (19). In general, submerged small-scale cultivation is advantageous to develop or optimize bioprocesses, particularly with respect to biocatalyst and medium screening. Small-scale cultivation enables

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parallel and automated experimental setups. This facilitates a higher throughput and, at the same time, saves media, space, workload and, thus, costs (20–26). Shaken vessels comprising shake flasks, test tubes and microtiter plates are preferred in small scale cultivation (22). Substantial progress has been made with the invention of the so called BioLector (27–30), a microtiter plate fermentation system with scattered light and fluorescence optic measurement techniques. Furthermore, different well geometries for microtiter plates have been studied to assure high oxygen transfer capacities and sufficient oxygen supply for microorganisms during cultivation (31). The 6-petal flower shape with an edge diameter of 5 mm proved to be best and is marketed as Flowerplate by m2p-labs GmbH (Baesweiler, Germany).

Regarding to microtiter plates, evaporation losses of fermentation medium are a critical issue. Evaporation can be reduced with suitable seal membranes that preferably minimize the evaporation of water vapor while having no relevant impact on gas exchange (23). These seal membranes are typically made from polymer materials such as copolymers of ethylene and vinyl acetate as well as polyethylene, silicone or rayon (23). Zimmermann et al. (32) classified various seal membranes, including also wound dressings, into two groups. In the first group the oxygen transfer is high, but water vapor retention is relatively low. On the contrary, in the second group, water retention capability is comparably high but oxygen transfer is low. All the wound dressings they examined fall into the second group except for one. This wound dressing, Tendra Mefilm, exhibits both good oxygen permeability and water retention capability.

In addition to different seal foils, Arain et al. (33) also examined oxygen diffusion through a layer of paraffin oil or paraffin wax as well as the plate material. Paraffin oil is commonly used to prevent evaporation of samples in microtiter plate wells and can reduce gas exchange with the ambient air. Oxygen flux through paraffin oil into the sample was considerably increased as soon as the microtiter plate was shaken. Whereas different paraffin wax layers showed lower oxygen permeability than cellulose acetate foils, these paraffin wax layers showed higher oxygen permeability than polyester foils. Furthermore, aluminum foils were impermeable to oxygen (33).

Seal membranes may impair to some extent quantitative work, since droplets may cover parts of the membrane, especially when vigorous shaking is involved. Due to such droplet formation, the conditions in the various wells may differ from each other (25,34). Well-to-well heterogeneity also occurs in microtiter plates covered by conventional hard plastic lids. These lids allow rapid gas exchange around their perimeters but a lesser rate of gas exchange toward the center of the microtiter plate (Perlman D., US patent 5858770, 1999).

Another approach to reduce evaporation losses and cross-contamination between adjacent wells is to use a sandwich lid for 96 well plates (34,35) commercialized by Enzyscreen B.V. (ER Haarlem, The Netherlands). This lid is composed of a perforated spongy silicone base layer, a perforated top plate and a thin 2 mm cotton wool layer between the base layer and the top plate. Each of the 96 holes in the base layer and top plate is positioned over the center of each well and has a diameter of 1.5 mm and 6 mm, respectively. The top plate is made either of rigid polypropylene or stainless steel. The microtiter plate and the lid are then tightly clamped together onto the shaking platform. In cultivation experiments with the bacterium *Pseudomonas putida*, evaporation losses were less than 2% per well and day for filling volumes of 500 μL–1000 μL. Typically evaporation is also minimized by placing the microtiter plate in humidified chambers (23).

Recent studies have already demonstrated that the scale-down of vinegar production to the milliliter shake flask scale is possible by specific techniques (16,19). However, there are no microscale cultivation methods available for vinegar production that would allow for bacterial strain selection or fermentation media investigation. Thus, the aim of this current study is to present a new method to scale-down vinegar production to the microliter scale for high-throughput cultivation. A new custom-made lid is introduced specifically for vinegar production to minimize evaporation losses of ethanol and acetic acid while allowing sufficient oxygen transfer. This paper will first present the theory on oxygen supply in the microtiter plate with the custom-made lid and the estimation of appropriate shaking conditions based on a reference fermentation in a 9-L bioreactor in the Materials and Methods section. Then, vinegar production in a microtiter plate with the custom-made lid is empirically evaluated.

MATERIALS AND METHODS

Oxygen balance in microtiter plate with custom-made lid The envisaged custom-made lid has one borehole per well through that gas exchange with the ambient air takes place during fermentation. Fig. 1 depicts the oxygen supply in a microtiter plate well with such a lid. Oxygen that is consumed by the bacteria in the medium is replenished from the headspace of the well. Oxygen from the ambient air, in turn, diffuses through the borehole into the headspace under the microtiter plate lid. Analogous to the diffusion through a cotton plug in shake flasks (36), the oxygen transfer rate OTR_{lid} through a borehole in the microtiter plate lid is estimated as follows (Eq. 1). The nomenclature of all equation symbols is additionally listed before the References section.

$$OTR_{lid} = k_{lid,O_2} \cdot \frac{p_{O_2,air} - p_{O_2,g}}{V_l \cdot p_{abs}} \quad (1)$$

The terms p_{abs}, $p_{O_2,air}$ and $p_{O_2,g}$ denote the absolute pressure, oxygen partial pressure of the ambient air and oxygen partial pressure in the gas phase of the microtiter plate well, respectively. The term V_l is the filling volume of the well. The mass transfer coefficient of oxygen k_{lid,O_2} can be approximated considering Fick's diffusion law (36,37):

$$k_{lid,O_2} = \frac{D_{e,O_2} \cdot A}{V_m \cdot H} = \frac{D_{e,O_2} \cdot p_{abs} \cdot \pi \cdot D^2}{4 \cdot R \cdot T \cdot H} \quad (2)$$

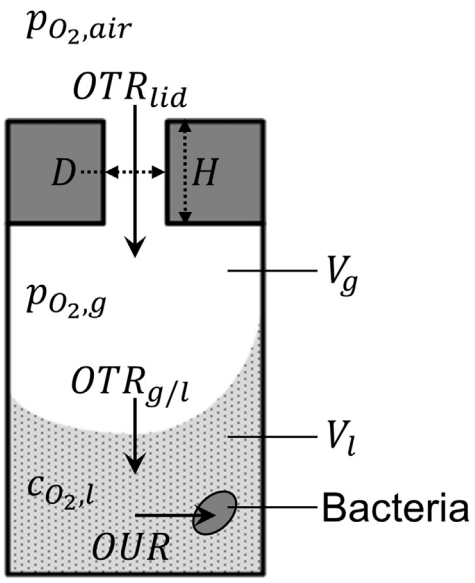

FIG. 1. Oxygen supply in microtiter plate well during fermentation. Microorganisms take up dissolved oxygen for metabolism. The depleting dissolved oxygen in the medium is replenished with oxygen from the gas phase in the well headspace passing the gas-liquid interface. Driven by the arising concentration gradient, oxygen diffuses through a borehole in the microtiter plate lid from the ambient air to the well headspace. $c_{O_2,l}$: oxygen concentration of the medium at the gas-liquid interface; D: diameter of the borehole in the microtiter plate lid; H: height of the microtiter plate lid; $OTR_{g/l}$: oxygen transfer rate at the gas-liquid interface; OTR_{lid}: oxygen transfer rate through the borehole in the microtiter plate lid; OUR: oxygen uptake rate of the bacteria; $p_{O_2,air}$: oxygen partial pressure of air; $p_{O_2,g}$: oxygen partial pressure in gas phase of microtiter plate well; V_g: gas volume of the well headspace; V_l: filling volume of the well.