

Impact of out-of-phase conditions on screening results in shaking flask experiments

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Abstract

Screening projects dealing with filamentous microorganisms in shaking flasks may generate strains showing a less filamentous morphology with a decreased apparent viscosity of the fermentation broth. The apparent viscosity of the fermentation broths showing pseudo-plastic flow behavior can be calculated by known relations, if the average shear rate is known. A method is presented allowing the determination of the relevant average shear rate, and thus, apparent viscosity of the fermentation broth at given operating conditions of the shaking flask experiment. At elevated apparent viscosity, shaking flask fermentations are subject to the recently discovered out-of-phase conditions. Measurements of the oxygen transfer capacity (OTR_{max}) in a highly viscous fluid have clearly shown reduced mass transfer, and therefore a reduced productivity of the investigated strains, when out-of-phase conditions are present. This leads to a selection pressure preferring a less filamentous morphology accompanied by lower apparent viscosity in screening projects in shaking flasks. In two completely different cases, the apparent broth viscosity of several consecutive strain generations was investigated. The later strain generation showed a lower apparent broth viscosity compared to the predecessor strain. In a third case, it was shown that out-of-phase conditions prevent the development of an improved culture medium.

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1. Introduction

Screening for improved production strains and culture media plays a crucial part in process development and optimization in biotechnological industry. On the search for new or improved strains or media components, a large number of experiments is carried out. In this stage of process development, shaking flasks have shown to be an invaluable tool in R&D laboratories. However, assignment of “increased product titer” to “improved strain or medium” requires suitable and reproducible experimental conditions. Especially oxygen limitation is easily encountered in shaking flasks. An insufficient supply of this vital substrate to most industrially relevant microorganisms can alter metabolism and hence mislead the outcome of the experiment.

In the case of microorganisms showing filamentous morphology, viscosity can pose additional problems in shaking flask cultures. A recently described phenomenon could be

encountered, called “out-of-phase” conditions [1–3]. Under “regular” operating conditions, the liquid rotates “in-phase” with the movement of the shaker table. But, with increasing viscosity, the rotational movement of the liquid is continuously reduced until a complete breakdown of the fluid occurs, resulting in an undefined swashing on the bottom of the flask. This out-of-phase condition is characterized by reduced power input, mixing and mass transfer. Culturing filamentous microorganisms in shaking flasks, the viscosity of the fermentation broth increases with fermentation time, due to formation of biomass. The rotational fluid movement will increasingly tend to out-of-phase conditions, resulting in impeded mass transfer, altered metabolism, and most probably in reduced product titers. As long as the viscosity of all individuals in a large experimental set develops in the equivalent way over time, the detrimental effect of the out-of-phase operating conditions will derogate the performance of all individual experiments in the equivalent way. Under these conditions, improved strains and media can hardly be identified. Even a lower final product titer of an improved strain or medium, compared to its reference, will be obtained, if the improvement is accompanied by higher viscosity resulting in out-of-phase conditions. In practice, this

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Nomenclature

a	fitting parameter (s^{b-1}), Eq. (8)
b	fitting parameter, Eq. (8)
d	largest inner diameter of shaking flask (mm)
d_0	shaking diameter (mm)
g	gravitational acceleration (m/s^2)
K	consistency factor of Ostwald-deWaele law ($mPa s^m$)
m	flow index of Ostwald-deWaele law
n	shaking frequency of shaking machines (rpm)
n_{STR}	stirrer speed in stirred tank reactors (rpm)
P	power (W)
V_L	filling volume of shaking flask (ml)

Dimensionless numbers

Fr_a	axial Froude number, Eq. (4)
Ph	phase number, Eq. (1)
Ph_{crit}	critical phase number for the fluid motion to be in-phase
Re	flask Reynolds number, Eq. (3)
Re_f	liquid film Reynolds number, Eq. (2)

Greek letters

$\dot{\gamma}$	average shear rate (1/s)
η	apparent dynamic fluid viscosity ($mPa s$)
η_{crit}	maximum apparent fluid viscosity for in-phase conditions ($mPa s$), Eq. (5)
ρ	liquid density (kg/m^3)

improved strain or medium would not be identified due to its supposedly lower productivity. Instead, mutants evolving from the strain development procedure, which are characterized by altered morphology (with lower viscosity) would attract attention. Under these circumstances, the hydrodynamic flow of the fermentation broth for these mutants with altered morphology may be in-phase. The lower viscosity would allow a normal rotational movement of the liquid, along with high power input, high mixing intensity, and high oxygen supply. These strains will exhibit relatively high metabolic activity and product titers compared to the reference. As a consequence, strains will mistakenly be selected in the screening procedure, although, under suitable operating conditions, they would not show increased productivity [1].

Also medium development is subject to the risks implied by out-of-phase conditions [1]. Suppose a medium component, which would lead to higher productivity under normal conditions, causes an increased viscosity of the fermentation broth. Its higher viscosity will transfer the fluid movement into out-of-phase conditions, if the operating conditions of the shaking flask experiment are unsuitable. The presumably advantageous effect of this component will not be identified. Screening at or close to out-of-phase conditions will therefore lead to a medium composition with lower viscosity.

A methodological approach to quantify the out-of-phase phenomenon with regard to operating conditions is presented in the work of Büchs et al. [1]. A dimensionless phase number (Ph) is introduced as a mathematical tool for the identification of out-of-phase conditions. In this dimensionless number, the apparent viscosity of the fermentation broth (η) represents a key parameter. For stirred tank reactors, the apparent viscosity of pseudo-plastic fluids is easily accessible by the relation of Metzner and Otto [4]. However, until today no methods are known neither for the evaluation of the apparent viscosity nor the average shear rate in shaking flasks.

This work focuses on the relevance of the out-of-phase conditions for screening projects induced by increasing viscosity. The misleading effect of this phenomenon in screening projects was investigated for two entirely different strain developments (referred to in the text as Cases 1 and 2) and one medium development (Case 3). A method is presented for the evaluation of the average shear rate of pseudo-plastic fluids in shaking flasks.

2. Theoretical approach

The dimensionless phase number, Ph [1] depends on the operating conditions of the shaking flask (shaking frequency (n), shaking diameter (d_0), maximum inside shaking flask diameter (d), and filling volume (V_L)) as well as on the hydrodynamic state of the fluid motion described by the liquid film Reynolds number (Re_f) and the axial Froude number (Fr_a), according to Eqs. (1)–(4):

$$Ph = \frac{d_0}{d} (1 + 3 \log_{10}(Re_f)) \quad (1)$$

with

$$Re_f = Re \frac{\pi}{2} \left(1 - \sqrt{1 - \frac{4}{\pi} \left(\frac{V_L^{1/3}}{d} \right)^2} \right)^2 \quad (2)$$

$$Re = \frac{\rho n d^2}{\eta} \quad (3)$$

and

$$Fr_a = \frac{(2\pi n)^2 d_0}{2g} \quad (4)$$

Although there is a steady transition between the undisturbed rotational fluid motion and its complete breakdown, the condition $Ph > Ph_{crit} = 1.26$, with $Fr_a > 0.4$ as a boundary condition, is introduced as criterion for the in-phase fluid motion by Büchs et al. [1]. Applying Eqs. (1)–(3) to the operating conditions of shaking flask experiments, it is possible to calculate a critical maximum apparent viscosity (η_{crit}) at which the fluid movement inside the flasks will

change from in-phase to out-of-phase conditions:

$$\eta_{\text{crit}} = \rho n d^2 \times 10^{(1-Ph_{\text{crit}}(d/d_0))/3} \times \frac{\pi}{2} \times \left(1 - \sqrt{1 - \frac{4}{\pi} \left(\frac{V_L^{1/3}}{d} \right)^2} \right)^2 \quad (5)$$

for $Ph_{\text{crit}} = 1.26$ within the boundary condition of $Fr_a > 0.4$.

Fermentation broths containing filamentous microorganisms show pseudo-plastic flow behavior, where the viscosity depends on the shear rate. This relationship can be described by the Ostwald-deWaele (or power-) law:

$$\eta = K \dot{\gamma}^{m-1} \quad (6)$$

The determination of the apparent fluid viscosity inside a shaking flask at given operating conditions is not trivial. Either the apparent viscosity or the average shear rate has to be assessed. For stirred tank reactors, the average shear rate is easily calculated using a relation provided by Metzner and Otto [4]. In contrast, no relation between the average shear rate and operating conditions is available for shaking flasks. However, the method presented in this work to quantify the average shear rate in shaking flasks is based on the same approach of Metzner and Otto [4]. They have used the assumption, that two fluids causing the same volumetric power consumption (P/V_L) at the same geometrical and operating conditions in a mixing device must have the same apparent viscosity. Therefore, a set of two experiments is needed (Fig. 1): the specific power consumption of a more or less Newtonian fluid (dotted lines) is compared to the one of the investigated pseudo-plastic fluid (solid lines). At the same

time, the respective viscosity curves (viscosity in function of the shear rate) are measured. In each diagram, there will be one intersection point, indicating the same hydrodynamic state. Correlating the viscosity from the intersection point in the second diagram to the shaking frequency at the intersecting point in the first diagram, the average shear rate (and thus the apparent viscosity) of the pseudo-plastic fluid inside a shaking flask at a certain operating condition can be determined (cf. Fig. 1).

3. Material and methods

3.1. Microorganisms and culture conditions

The investigations were carried out using different mutants of two different fungi showing more or less filamentous morphology (Cases 1 and 2). The two collections of different mutants represent consecutive generations of strain development projects, which both were carried out during a time span of at least 10 years. The mutants were obtained by conventional mutation and selection procedures. All culture broths show pseudo-plastic flow behavior. The culture experiments were carried out in Erlenmeyer flasks according to German industrial standard DIN 12380. Agitation was realized by orbital shaking machines (Kühner AG, Birsfelden, Switzerland) with shaking diameter as indicated in the experiments. In Case 1, four strains were used. The screening experiments were carried out in shaking flasks of 250 ml nominal volume, 25 ml filling volume, 25 mm shaking diameter, at 300 rpm shaking frequency. In Case 2, five strains were used. They were cultivated in flasks of 500 ml nominal volume, 30 ml filling volume, 70 mm shaking diameter, at

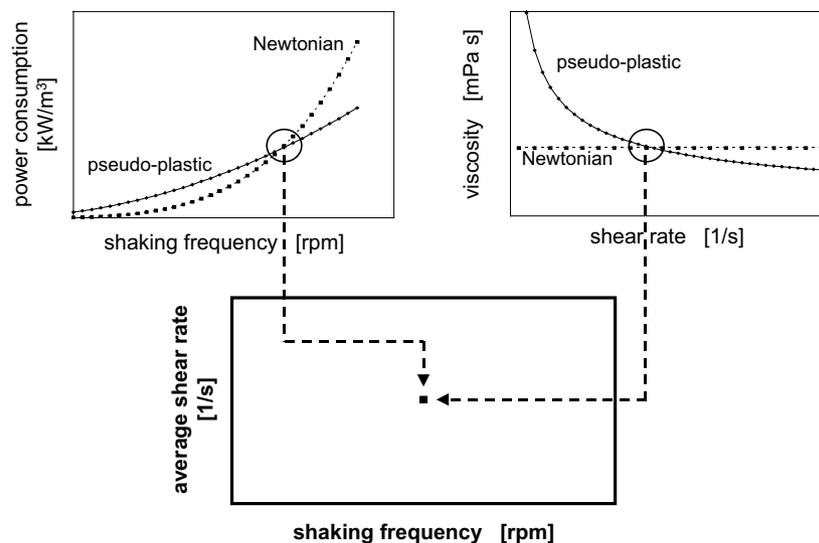


Fig. 1. Methodology for the determination of the average shear rate as function of shaking frequency in shaking flasks modified after Metzner and Otto [4]. With a pseudo-plastic and a Newtonian fluid, the power consumption is measured as function of the shaking frequency (top left) as well as the viscosity as function of the shear rate (top right). The intersection point in each diagram indicates the same hydrodynamical state. This information is then combined yielding the average shear rate in function of the shaking frequency (bottom).

230 rpm shaking frequency. In Case 3, a medium development project was investigated. For one microorganism, the medium composition was varied only with respect to the concentration of a complex carbon source (plant seed flour). The concentration was doubled, halved and in one experiment this component was completely omitted. Shaking conditions were equivalent to those in Case 1.

3.2. Viscosity measurements

The experiments were carried out in several equivalent flasks operated in parallel for one strain or medium. Viscosity measurements of the fermentation broth were made periodically throughout the fermentation. For each viscosity measurement, one or several (to obtain sufficient sample volume) new until then untouched flasks were removed from the shaking machine. The viscosimeter used in Cases 1 and 3 was a cone-plate device (Rotovisco RV100, measuring device CV100, Haake Meßtechnik, Karlsruhe, Germany). In Case 2, viscosity was measured according to the principle of Searle, where the fluid is sheared between two concentric cylinders, the outer one fixed, the inner one rotating at defined speed (Rheomat 115A, Contraves, Switzerland).

3.3. Determination of relevant average shear rate

The average shear rate was determined by measuring the power input and the viscosity of the investigated culture broths after the method described above (see theoretical approach). The power consumption in shaking flasks was measured according to the method proposed by Büchs et al. [5]. In Cases 1 and 3, the culture broth was used directly to establish the average shear rate versus shaking frequency relationship. In Case 2, a sample was taken from a fermentation of the most recent tested mutant at the culture time with the highest viscosity. The viscous flow behavior of this fermentation broth was characterized based on Eq. (6) with $K = 2.57 \text{ mPa s}^m$ and $m = 0.43$. The fermentation broth was substituted by a model liquid expressing the same flow behavior, consisting of 0.7% (w/v) carboxymethylcellulose (Fluka Chemie AG, Buchs, Switzerland), 0.7% (w/v) xanthan gum (Fluka Chemie AG), and 0.5 M sodium sulfate (Merck KGaA, Darmstadt, Germany). This substitution was made in order to simplify and standardize the experiment. As near-Newtonian fluid, aqueous solutions of polyvinylpyrrolidone (PVP, Luviskol K90 powder, BASF AG, Ludwigshafen, Germany) in different concentrations as indicated in the figures were used. As the viscosity of this fluid may slowly change with time, the viscosity was measured shortly before a power consumption measurement was conducted. Additionally, for the quantification of the influence of the filling volume on the hydrodynamic state (in-phase or out-of-phase conditions), experiments were conducted with a flask filling volume of 50 ml instead of 30 ml, while all other operating conditions were kept constant.

3.4. Measurement of the oxygen transfer rate

The investigation of the influence of the out-of-phase phenomenon on the oxygen transfer rate was carried out by the sulfite method proposed by Maier and Büchs [6], using the measuring device described by Anderlei and Büchs [7] and Anderlei et al. [8]. The oxygen transfer rate measured according to this method is regarded as a maximum value (dissolved oxygen concentration being close to zero) at given operating conditions of a shaking flask experiment and thus is called maximum oxygen transfer capacity. For the investigation of the mass transfer into fluids with water-like viscosity, aqueous sodium sulfite 0.5 M (Carl Roth GmbH and Co., Karlsruhe, Germany) solution containing as a catalyst cobalt sulfate (Fluka Chemie AG, Buchs, Switzerland) at a concentration of 10^{-7} M as well as a $0.012 \text{ M HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ buffer (Carl Roth GmbH and Co., Karlsruhe, Germany) was used. For quantifying the oxygen transfer into the viscous model liquid (Case 2) described above, sodium sulfate was substituted by 0.5 M sodium sulfite in order to provide an oxygen consumer. Cobalt sulfate was added accordingly (concentration: 10^{-7} M), but no buffer was added.

4. Results and discussion

4.1. Determination of the relevant shear rate

The investigation of the evolution of viscosity for all three cases requires the determination of the relevant average shear rate beforehand. In order to quantify this value for the rotational movement of a pseudo-plastic fluid inside a shaking flask, the method described above was applied. The whole procedure following this method will now be described in detail for Case 2.

Fig. 2 shows the flow curve of the pseudo-plastic model liquid simulating the fermentation broth of Case 2 (open squares). The flow curves of three near-Newtonian fluids (aqueous PVP solutions in concentration of 80 g/l (filled circles), 70 g/l (filled lozenges), and 60 g/l (filled triangles) are also shown. The intersection points of the pseudo-plastic with the Newtonian fluids are marked by circles, and their respective values of viscosity and shear rate are explicitly denoted in the caption of Fig. 2. Fig. 3 shows the measured specific power consumption as function of the shaking frequency for the mentioned fluids for a filling volume of 30 and 50 ml. In Fig. 3a, an intersecting point can be identified at $n \cong 240 \text{ rpm}$ (model liquid with PVP 70 g/l-solution). The corresponding intersection point of the model liquid with PVP 70 g/l-solution in Fig. 2, indicates an average shear rate of $\dot{\gamma} = 254 \text{ 1/s}$ at these operating conditions. All other intersecting points can be identified in the same way. These points were transferred into Fig. 4, depicting the dependence of the average shear rate on the shaking frequency. The curves of the Newtonian and pseudo-plastic fluids in Fig. 3 intersect at a very flat angle. Small inaccuracies of the power

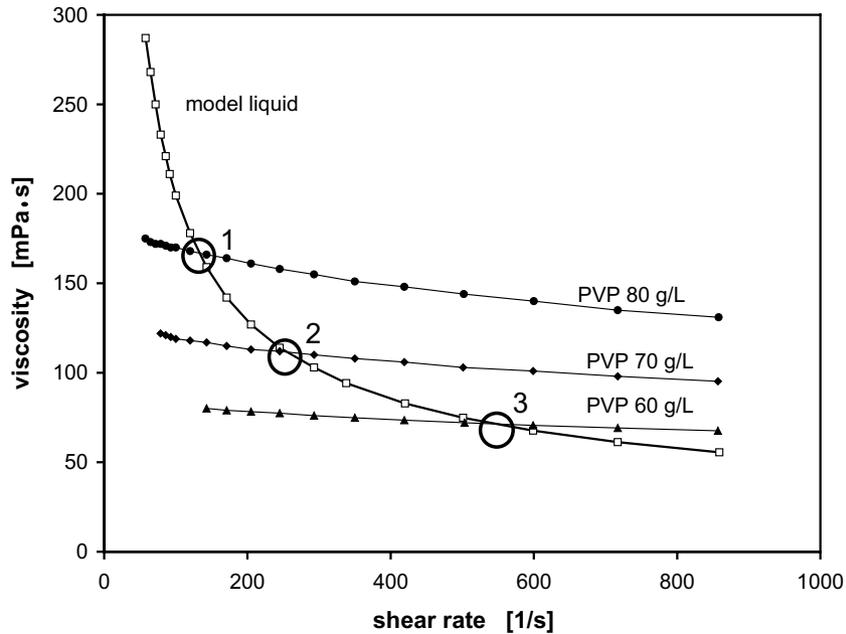


Fig. 2. Viscosity curve of a model liquid (\square) representing an fermentation broth, compared to aqueous solutions of polyvinylpyrrolidone (PVP) in concentration of 80 g/l (\bullet), 70 g/l (\blacklozenge), and 60 g/l (\blacktriangle). Intersection points: no. 1 ($\dot{\gamma} = 116$ l/s; $\eta = 171$ mPa.s), no. 2 ($\dot{\gamma} = 254$ l/s; $\eta = 110$ mPa.s), no. 3 ($\dot{\gamma} = 553$ l/s; $\eta = 70$ mPa.s).

consumption measurements cause relatively large shifts of the relevant shaking frequencies. Therefore, the accuracy of this method to evaluate the average shear rate is limited.

In order to assess the average shear rate at other than the evaluated shaking frequencies, a suitable correlation between $\dot{\gamma}$ and n had to be found. For stirred tank reactors, Metzner and Otto [4] have proposed a linear correlation:

$$\dot{\gamma} = Kn_{\text{STR}} \quad (7)$$

In the case of shaking flasks, no relation has yet been published. The data points in Fig. 4 rather imply a superproportional relationship containing an exponent on the shaking frequency. Therefore, the following equation, containing the fitting parameters a and b , was applied:

$$\dot{\gamma} = an^b \quad (8)$$

The parameters a and b of Eq. (8) were fitted to all data points shown in Fig. 4 by non-linear regression, yielding: $a = 20.9s^{b-1}$ and $b = 1.94$. With these parameters and Eq. (8), it is now possible to determine the average shear rate for other shaking frequencies if all other operating conditions are kept constant. A value for the apparent viscosity at given operating conditions can now be obtained. For the other two examples (Cases 1 and 3), the same procedure was applied (data not shown).

These investigations to establish the correlation between the average shear rate and the operating conditions of shaking bioreactors should be regarded as preliminary work. Much more efforts have to be dedicated to this area to introduce a general model, which is valid for all kinds of

pseudo-plastic fluids and operating conditions. Such work is currently conducted in our laboratory.

4.2. Impact of out-of-phase conditions on strain development, Case 1

Fig. 5 shows the apparent viscosity of the fermentation broths of the different strain generations at the relevant average shear rate ($\dot{\gamma} = 325$ l/s) over fermentation time. The maximum viscosity for the in-phase condition calculated from Eq. (5) is also shown as a horizontal, dashed line.

The apparent viscosity for all four strains increases simultaneously at the beginning of the fermentation. As the apparent viscosity gets closer to the maximum value for in-phase operating conditions, each of the four curves takes a different course. It can be noticed clearly, that with each of the consecutive strain generations, the apparent viscosity of the fermentation broth becomes lower and lower. While the apparent viscosity of the wild type strain (I) and stage II both remain above the maximum value for in-phase operating conditions, stage III and IV fall well beneath it. The most recent strain tested clearly shows the lowest apparent viscosity. This is verified by microscopical observations. The wild type strain unequivocally shows a filamentous morphology. No other growth morphologies are known for this species. The most recent strain tested develops some kind of filaments at the beginning of the fermentation time, later it more or less disintegrates into single cells.

These observations can be explained by the until recently unknown and unintended selection pressure caused by the out-of-phase phenomenon, as explained in the introduction.

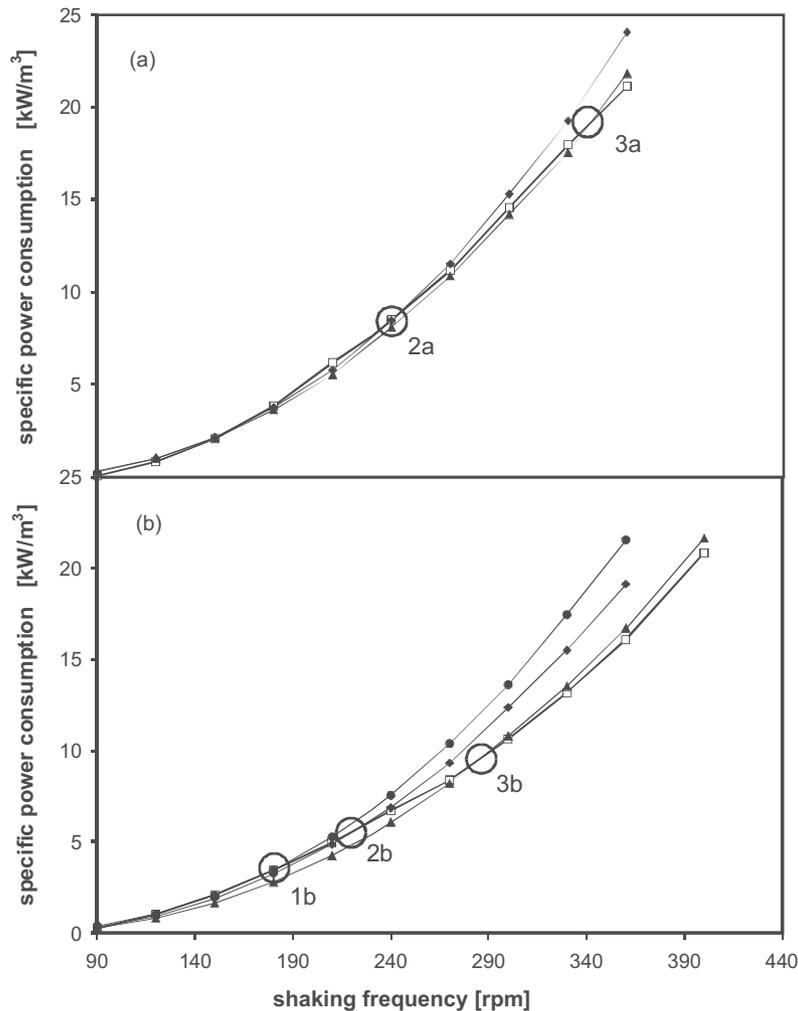


Fig. 3. Power consumption of model liquid (\square) representing an fermentation broth and aqueous solutions of polyvinylpyrrolidone (PVP) in concentrations of 80 g/l (\bullet), 70 g/l (\blacklozenge), and 60 g/l (\blacktriangle) in shaking flasks of 500 ml nominal size, at shaking diameter of 70 mm, and filling volume of (a) 30 ml and (b) 50 ml. Intersection points: no. 2a ($n = 240$ rpm; 8.4 kW/m^3), no. 3a ($n = 340$ rpm; 19.1 kW/m^3), no. 1b ($n = 180$ rpm; 3.4 kW/m^3), no. 2b ($n = 220$ rpm; 5.8 kW/m^3), no. 3b ($n = 285$ rpm; 9.5 kW/m^3). Digits in intersection point numbers refer to Fig. 2.

In the long lasting screening procedure, strains were chosen as “improved” mutants by their product titer. Obviously, the increased product titer was a result of lower apparent viscosity caused by an altered morphology (resulting in in-phase condition and therefore improved mass transfer, mixing, etc.). Strains with a higher specific productivity, but with a more filamentous morphology resulting in higher apparent viscosity could not have been identified due to an undersupplied metabolism at those given operating conditions.

4.3. Impact of out-of-phase conditions on strain development, Case 2

Case 2 represents a completely different filamentous fungus and process. Fig. 6 shows the time course of the apparent viscosity at the relevant shear rate ($\dot{\gamma} = 283 \text{ l/s}$) of five consecutive strain generations. The apparent viscosity for each strain generation is lower than the one of its predecessor.

The apparent viscosity of the wild type strain reaches values clearly above the critical value representing the limit to out-of-phase conditions. Most remarkably, already the second generation falls beneath it. The apparent viscosity virtually seems to align with the boundary line. All of the other strains have a lower apparent viscosity, which is more or less in the chronological order of their generation. Also in this case, there are strong indications that selection pressure preferred a less filamentous morphology leading to lower apparent viscosity.

The comparison of Cases 1 and 2 also reveals the strong influence of the shaking diameter, as already has been pointed out by Büchs et al. [1,2]. In Case 1, a maximum apparent viscosity of about 35 mPa s is observed on a shaking machine with 25 mm shaking diameter (Fig. 5). In Case 2 (Fig. 6), a relatively high maximum apparent viscosity of about 110 mPa s is reached with a shaking machine of 70 mm shaking diameter. Considering Eq. (1), this is not

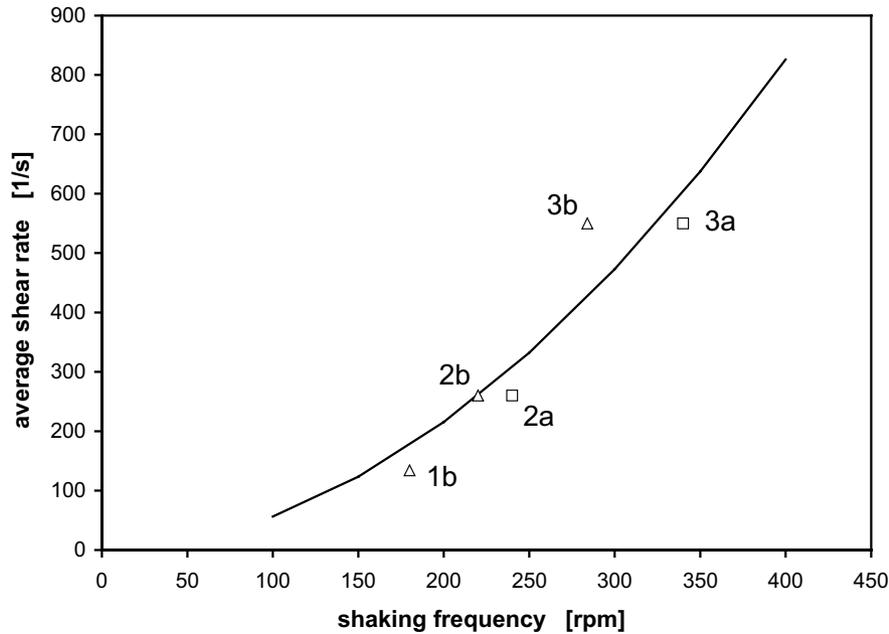


Fig. 4. Dependency of average shear rate on shaking frequency of a pseudo-plastic model liquid representing an fermentation broth in shaking flasks of 500 ml nominal size, at shaking diameter of 70 mm, and filling volume of 50 ml (Δ) and 30 ml (\square). Data points correspond to intersecting points in Figs. 2 and 3. Model curve was obtained by fitting Eq. (8) to the data points.

surprising. The Ph -number relates proportionally to the shaking diameter ($Ph \propto d_0$). If highly viscous liquids are encountered inside the shaking flask, the usage of shaking machines with a large shaking diameter is a practical way to account for the influence of viscosity.

It is also interesting to note that in the German Institute now known as Hans-Knöll-Institut, one of the major antibiotics research centers in the former Warsaw Pact hemisphere, self made shaking machines with a shaking diameter of 100 mm were used. When in 1989 the po-

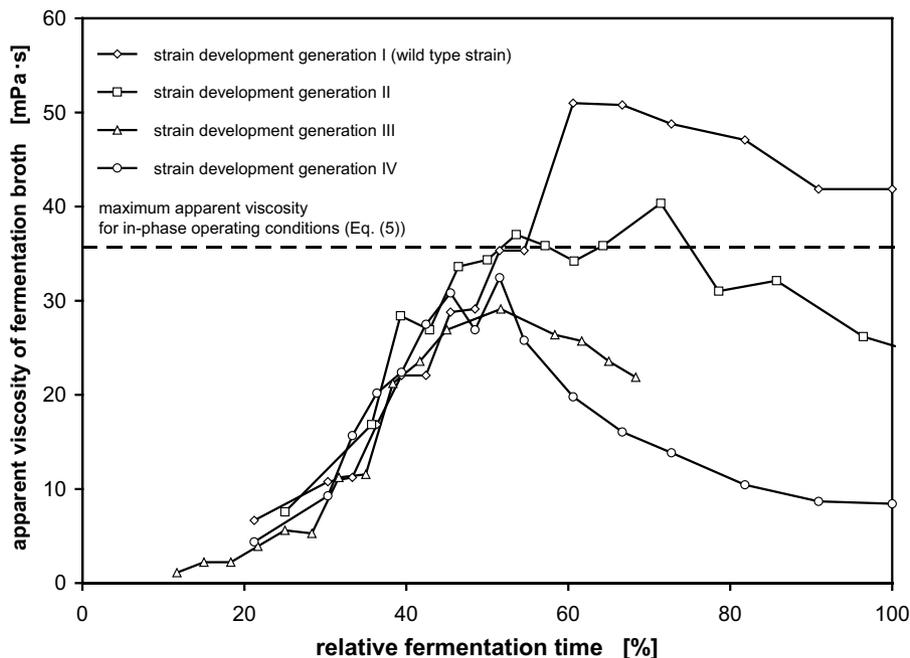


Fig. 5. Apparent viscosity over fermentation time for a filamentous growing microorganism (Case 1). Four consecutive generations in strain development are shown in chronological order (oldest: I, most recent: IV). The apparent viscosity is given at the relevant average shear rate of $\dot{\gamma} = 325$ 1/s. Culture conditions: shaking flasks of 250 ml nominal volume, 25 ml filling volume, 25 mm shaking diameter, 300 rpm shaking frequency. Maximum apparent viscosity for in-phase conditions calculated after Eq. (5).

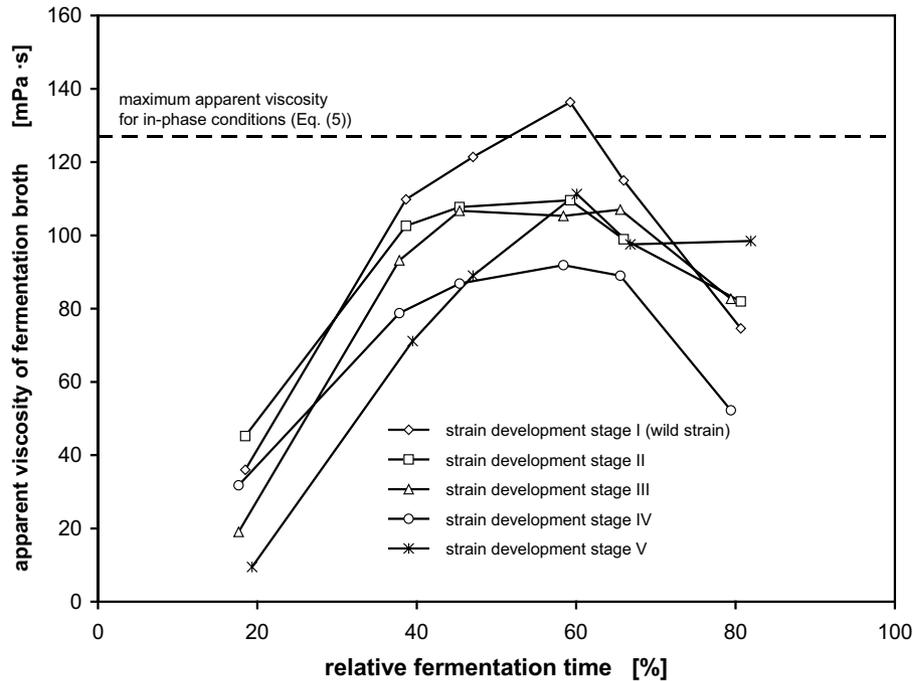


Fig. 6. Apparent viscosity over fermentation time for an filamentous growing microorganism (Case 2). Five consecutive generations in strain development are shown in chronological order (oldest: I, most recent: V). The apparent viscosity is given at the relevant average shear rate of $\dot{\gamma} = 283$ l/s. Culture conditions: shaking flasks of 500 ml nominal volume, 30 ml filling volume, 70 mm shaking diameter, 230 rpm shaking frequency. Maximum apparent viscosity for in-phase conditions calculated after Eq. (5).

litical situation changed, the institute purchased modern Western equipment with a shaking diameter of 25 mm [9]. Interestingly, the culture experiments with filamentous microorganisms at the Hans-Knöll-Institut did not succeed at all on the new shaking machines. As a consequence, new custom-made shaking machines with 100 mm shaking diameter were commissioned. With this equipment, satisfying results were again obtained. With reasonable certainty these problems at the Hans-Knöll-Institut at that time have to be attributed to the out-of-phase phenomenon, illustrating the strong influence of the shaking diameter. Considering this, it is very unfortunate, that in the vast majority of all publications dealing with shaking bioreactors, the shaking diameter is not at all specified.

4.4. Influence of the out-of-phase conditions on oxygen transfer

Fig. 7 shows the oxygen transfer capacity as function of the shaking frequency in 500 ml shaking flasks with filling volumes of 20, 30, and 50 ml. The dotted lines with open symbols represent the results of the aqueous sulfite system. The solid lines with filled symbols show results of the pseudo-plastic model-liquid representing the investigated culture broth of the filamentous fungus (the apparent viscosity at these operating conditions is in the range of 100 mPa s). All oxygen transfer capacities (OTR_{max}) increase with increasing shaking frequency. The oxygen trans-

fer capacities of the highly viscous model liquid are generally lower than those for the aqueous solution. However, the difference in case of 50 ml filling volume (triangles) is relatively small and remains constant for all shaking frequencies. In contrast, with 30 ml filling volume (squares), the difference between the two investigated solutions is relatively large at low shaking frequencies. With increasing shaking frequency, this difference becomes smaller. This effect is even more pronounced when the filling volume is further reduced to 20 ml (circles).

These observations correlate well with the theory of the out-of-phase phenomenon as illustrated in the introduction: The rotational liquid movement in shaking flasks tends to be in-phase with increasing shaking frequency or increasing filling volume [1]. With a filling volume of 50 ml, the liquid volume is large enough to ensure in-phase conditions over the whole range of shaking frequencies. The constant and small difference in OTR_{max} between the model liquid and the aqueous solution probably is caused by the direct effect of the elevated viscosity on the gas-liquid mass transfer. However, reducing the filling volume to 30 ml, out-of-phase conditions occur at low shaking frequencies, but turn into in-phase conditions when the shaking frequency is increased. This explanation holds even more for 20 ml filling volume, where the difference between the OTR_{max} values of the aqueous system and the highly viscous model liquid at low shaking frequencies is considerably larger than at the filling volume of 30 ml. Only at shaking frequencies

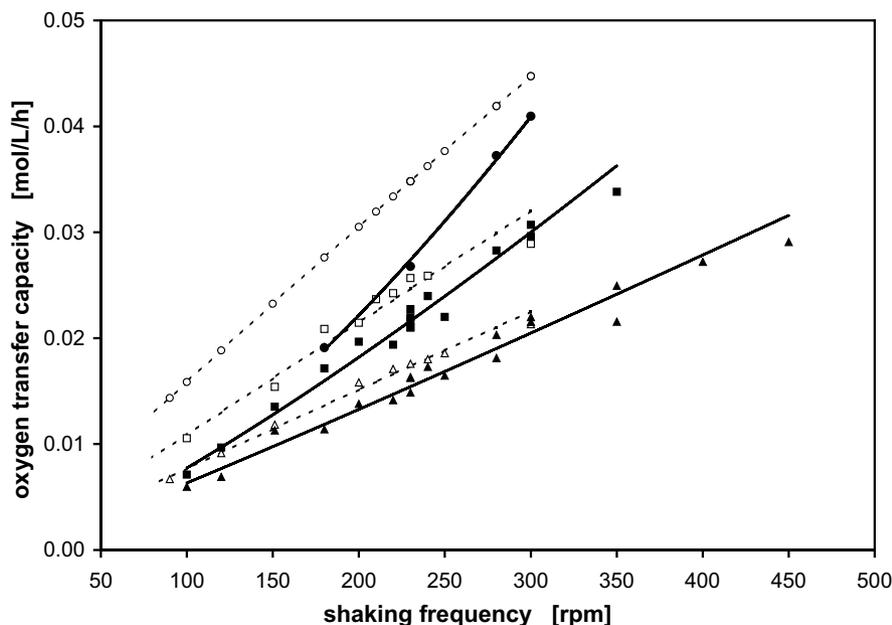


Fig. 7. Oxygen transfer capacity of a pseudo-plastic model liquid representing a fermentation broth and consisting of xanthan gum, carboxymethylcellulose, and 0.5 M sodium sulfite as an oxygen consumer ((▲), (■), (●), solid lines). For comparison, the oxygen transfer capacity of an aqueous 0.5 M sulfite system ((△), (□), (○), dotted lines) is shown. Operating conditions: shaking flasks of 500 ml nominal size, at shaking diameter of 70 mm, and filling volumes of 50 ml (▲, △), 30 ml (■, □), and 20 ml (●, ○).

larger than 300 rpm, the curves for the two liquids approach each other, indicating a transition into the in-phase condition.

These observations clearly support the prediction of reduced gas/liquid mass transfer at out-of-phase conditions in shaking flasks, as illustrated in the introduction. Considering the difference between the curves for the aqueous and the viscous model liquid at the specific operating conditions used in the screening project (30 ml filling volume, shaking frequency of 230 rpm), it has to be concluded that the hydromechanical flow is still in-phase but very close to the transition to the out-of-phase condition. This clearly confirms the conclusion drawn from Fig. 6, where the viscosity of the investigated strain (strain development stage V) remains below the maximum apparent viscosity for in-phase operating conditions.

4.5. Impact of out-of-phase conditions on medium development

Detrimental effects of the out-of-phase conditions are not only observed on the strain development conducted over a long period of time, leading to altered morphologies. The out-of-phase conditions can also effect on a short term base the results of medium development, as will be shown for Case 3.

Fig. 8 depicts the apparent viscosity at the relevant average shear rate ($\dot{\gamma} = 325 \text{ l/s}$) over fermentation time for a strain at varying concentrations of added plant seed flour. This compound is one of the complex nutrients in this

medium. In accordance with common practice, during this process development and optimization, the concentration of the different constituents were readjusted from time to time. It can clearly be recognized from Fig. 8, that the apparent viscosity of the fermentation broth increases with increasing concentration of the plant seed flour. The physiological reason for this correlation between apparent viscosity and the concentration of plant seed flour was not investigated. Interestingly, the medium readjustment, which was periodically carried out, always resulted in an unchanged concentration of plant seed flour (this constant value here is referred to as 100%). Higher concentration did not show any beneficial effect on product titer in shaking flask experiments (data not shown). However, when a medium with a concentration of plant seed flour of 200% was investigated in a stirred tank fermentor, ensuring sufficient mixing and oxygen supply, a surprisingly high product titer was obtained (data not shown). The fact, that the beneficial effect of increased concentration of plant seed flour was not discovered in shaking flasks experiments under the applied operating conditions can again be explained by the out-of-phase phenomenon. The high concentration of plant seed flour (200%) led to an apparent viscosity higher than the critical value that characterizes the transition from suitable in-phase to unsuitable out-of-phase conditions. Therefore, the metabolism of the fungi was limited by insufficient mixing and oxygen supply. The superior properties of the medium with the high concentration of plant seed flour could not be identified in shaking flasks under the applied unsuitable operating conditions.

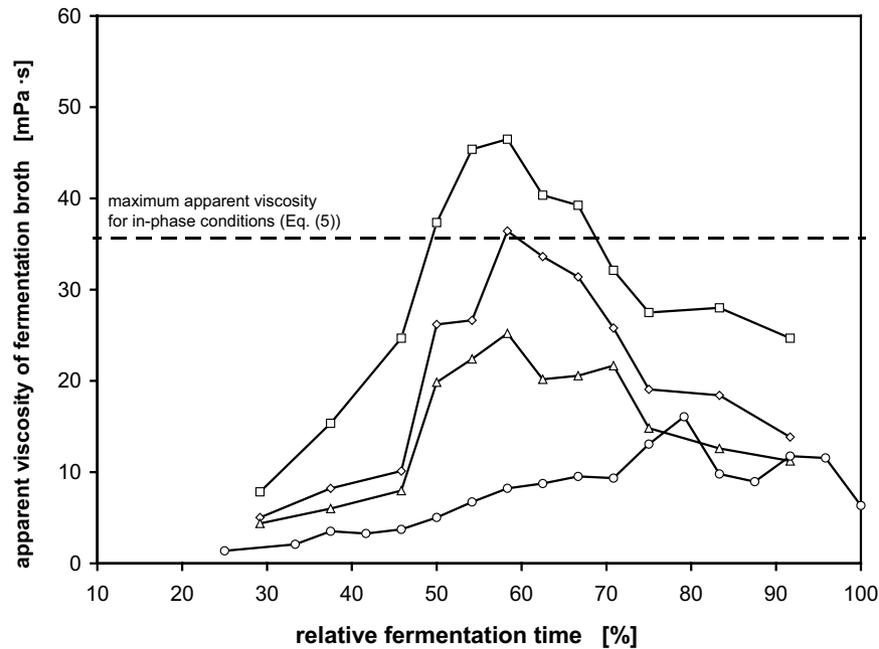


Fig. 8. Apparent viscosity over fermentation time for an filamentous growing microorganism (Case 3) with varying concentration of the complex carbon source (plant seed flour). Original medium (100% plant seed flour) (◇); 200% (□); 50% (△); 0% (○). The apparent viscosity is given at the relevant average shear rate of $\dot{\gamma} = 3251/\text{s}$. Culture conditions: shaking flasks of 250 ml nominal volume, 25 ml filling volume, 25 mm shaking diameter, 300 rpm shaking frequency. Maximum apparent viscosity for in-phase conditions calculated after Eq. (5).

5. Conclusion

The investigation of three screening projects in shaking flasks gave strong indications for the impact of the out-of-phase phenomenon on strain and medium development. As this phenomenon, resulting in reduced mixing and mass transfer, occurs at elevated viscosity, the identification of highly productive but viscously growing strains may not be possible. For two independent cases at entirely different operating conditions, the change of the morphology and the decrease of the apparent viscosity of the culture broth with each consecutive generation of strain development could be demonstrated. It has also been noticed for *Streptomyces* species that the culture broth viscosity of production strains generally tends to be lower compared to the broth viscosity of wild type strains [10]. Though, it is also probable that there is a physiological relation between growth morphology and productivity. In our work completely different microorganisms were considered, synthesizing totally different products. No explanation is available why in all these cases there should be a link between the metabolic pathway responsible for the investigated product and the mechanism triggering the morphological change. Probably during the random mutagenesis and selection cycles, the product metabolism as well as the mechanism responsible for altered morphology were both affected in parallel.

Our results lead to the strong suspicion that a general problem of screening projects, dealing with filamentous microorganisms in shaking bioreactors has been uncovered.

Performing screening with filamentous growing microorganisms at or near the out-of-phase boundary line (maximum apparent viscosity) lead to altered morphology of the mutated and selected strains with lower culture broth viscosity. Though, under the view point of production scale, one may argue that a lower culture broth viscosity is even advantageous with respect to the required agitation power consumption. This may be true, but as illustrated in this work, a lower culture broth viscosity is rather an unconscious result of the common screening practice and not the aim of strain development. There is a high risk of not recognizing the effects of changes in strain metabolism or medium composition when the operating conditions of the shaking flask experiment are unsuitable for highly viscous fermentation broths. Careful experimental planning with respect to out-of-phase conditions is vital in the early screening steps and for process development. If the strain development is once driven in an unfavorable direction, later compensation will hardly be possible.

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References

- [1] J. Büchs, U. Maier, C. Milbradt, B. Zoels, Power consumption in shaking flasks on rotary shaking machines. II. Nondimensional

- description of specific power consumption and flow regimes in unbaffled flasks at elevated liquid viscosity, *Biotechnol. Bioeng.* 68 (2000) 594–601.
- [2] J. Büchs, S. Lotter, C. Milbradt, Out-of-phase operating conditions, a hitherto unknown phenomenon in shaking bioreactors, *Biochem. Eng. J.* 7 (2) (2001) 135–141.
- [3] S. Lotter, J. Büchs, Utilization of power input measurements for optimisation of culture conditions in shaking flasks, *Biochem. Eng. J.*, this issue.
- [4] A.B. Metzner, R.E. Otto, Agitation of non-Newtonian fluids, *AIChE J.* 3 (1) (1957) 3–10.
- [5] J. Büchs, U. Maier, C. Milbradt, B. Zoels, Power consumption in shaking flasks on rotary shaking machines. I. Power consumption measurements in unbaffled flasks at low liquid viscosity, *Biotechnol. Bioeng.* 68 (2000) 589–593.
- [6] U. Maier, J. Büchs, Characterisation of the gas–liquid mass transfer in shaking bioreactors, *Biochem. Eng. J.* 7 (2) (2001) 99–107.
- [7] T. Anderlei, J. Büchs, Device for sterile on-line measurement of the respiration rates in shaking flasks, *Biochem. Eng. J.* 7 (2) (2001) 157–162.
- [8] T. Anderlei, W. Zang, J. Büchs, Online respiration activity measurement (OTR, CTR, RQ) in shake flasks, *Biochem. Eng. J.*, this issue.
- [9] Prof. Dr. Fleck, Hans-Knöll-Institut, Jena, Germany, personal communication.
- [10] Prof. Bushell, University of Surrey, Great Britain, personal communication.