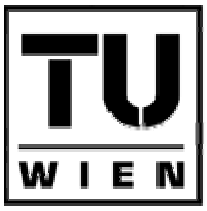


TECHNISCHE UNIVERSITÄT WIEN

VIENNA UNIVERSITY OF TECHNOLOGY

# D I P L O M A R B E I T

Bio-suspensions in the ultrasonic h-shape filter

Ausgeführt am Institut für

Allgemeine Physik
der Technischen Universität Wien

unter der Anleitung von Prof.Dr. Ewald Benes
und
Dr. Stefan Radel

durch

Cosima Koch

Fasangasse 4
2283 Obersiebenbrunn

Wien, am 15.9.2009

# Contents

**Abstract**

The ultrasonic h-shape filter was used for the separation of suspensions of the yeast *Saccharomyces cerevisiae*. The separation efficiency of the filter, i.e. the ability to retain suspended yeast cells, was assessed. To examine the influence of the separation process on the cells, cell viability by methylene blue counts, possible cell rupture assessed by the protein concentration of the supernatant and growth were investigated before and after sonication.

The filter was operated at a frequency of 2.1MHz at a power input of 3W true electrical power input. The filter was shown to work well for cultured yeast suspended in Malt Extract Broth, for which a separation efficiency of 89+/-6% was found. Wet yeast suspended in phosphate buffered saline (PBS, 0.9% NaCl), PBS 2x (1.8% NaCl) and $H_2O$ tap was not retained as efficiently.

For all the suspensions cell viability was not affected by sonication, remaining at high levels. However, in two cases when cultured cells suspended in Malt Extract Broth were driven through the resonator, a significant decrease of cell viability of the sonicated cells was measured. Cell concentration of retentate samples of cultured yeast/Malt Extract Broth-suspensions was 20% higher for counts 18 hours after sonication than that of counts immediately after sonication (99% significance, 2-sided t-test). For control groups and the filtrate samples no such increase was detected. Cell leakage caused by the separation process was detected by a significant increase in UV O.D. for cells suspended in PBS. For PBS 2x- and $H_2O$ tap-suspensions no significant difference in UV O.D. between control and sonicated groups, respectively, was found.

## Kurzfassung

Der h-Separator, ein spezieller Ultraschallfilter, wurde für die Separation von *Saccharomyces cerevisiae* getestet. Die Separationseffizienz des Filters, also die Fähigkeit suspendierte Hefezellen zurückzuhalten, wurde gemessen. Der mögliche Einfluss des Separationsprozesses auf die Hefezellen wurde durch die Messung der Viabilität der Zellen mit Methylen Blau sowie der Proteinkonzentration des Überstandes (Messung der optische Dichte für UV-Licht) zur Detektion möglicher Zerstörung von Zellenmembranen und Messung des Zellwachstums vor und nach der Separation untersucht.

Der Filter wurde mit 3W true electrical power input bei einer Frequenz von 2.1MHz betrieben. Für Suspensionen von gezüchteten Hefezellen in Malt Extract Broth konnte eine hohe Separationseffizienz von 89+/-6% gefunden werden. Für Presshefe, die in phosphatgepufferter Salzlösung (PBS, 0.9% NaCl), PBS 2x (1.8% NaCl) und Leitungswasser suspendiert war, konnten nicht so hohe Separationseffizienzen erreicht werden.

Die Viabilität der Zellen wurde in keiner der Suspensionen durch Separation beeinträchtigt. In zwei Fällen kam es jedoch bei der Separation von gezüchteter Hefe in Malt Extract Broth zu Turbulenzen, die zu einer signifikanten Verminderung der Viabilität der beschallten Zellen führten. Die gemittelte Zellkonzentration der Retentat-Proben von gezüchteten Hefezellen/Malt Extract Broth-Suspensionen war 18 Stunden nach der Beschallung um 20% höher als gleich nach der Beschallung (99% Signifikanz, 2-seitiger t-test). Bei Kontrollgruppen und den Filtrat-Proben konnte keine signifikante Steigerung festgestellt werden. Für Hefezellen die in PBS suspendiert waren, wurde eine signifikante Steigerung der optischen Dichte für UV-Licht gemessen welche auf ein Auslaufen der Zellen zurückzuführen ist. Für die Suspensionen in PBS 2x und Leitungswasser konnte kein signifikanter Unterschied zwischen der optischen Dichte für UV-Licht der Kontrollgruppen und der beschallten Zellen gefunden werden.

# 1. Introduction

The first observation of particle manipulation by ultrasound was made by Kundt and Lehmann over a century ago [1]. They observed the aggregation of particles previously homogeneously suspended in a fluid in specific regions when a standing ultrasonic standing wave was present. This concentration is due to forces exerted on the particles in dispersion when irradiated with ultrasound. These forces are called acoustic radiation forces and are caused by the spatial gradient of the acoustic pressure of the sound wave. The forces are size dependent, i.e. they depend on the relation between the particle size and the wavelength of the acoustic wave, and they act in the direction of sound propagation. The so called acoustic contrast factor, a coefficient of the said forces which depends on the ratio of mass densities and the ratio of speeds of sound of the particle and the fluid, respectively, gives rise to the direction of the forces and their strength. When a suspension of particles in a liquid is irradiated by an ultrasonic plane standing wave field and the particles are denser and their speed of sound is larger (than that of the liquid), the particles will be driven into to pressure nodal planes. An example for this would be particles like polysterene beads or biological cells suspended in water or saline (water, adjusted to the physiological salt concentration inside the cells). If gas bubbles suspended in a liquid like water are subject to ultrasonic standing waves, these bubbles will be driven into the pressure antinodal planes, which are the displacement nodal planes. A more detailed explanation will be given later.

The effect has been shown to work for the retention (or filtering), sorting and handling of micron sized particles [2-5] and such delicate things as biological material [6]. Possible fields of application are cell retention [7-9], specific concentration of particles like DNA or antibodies/antigens for detection and further analysis [10-13]. Large scale industrial applications have emerged, e.g. the 250L BioSep AppliSens by Applikon Dependable Instruments bv (Schiedam, The Netherlands) [14]. Also, in the last years, microfluidic devices have been developed and successfully applied for the manipulation of microparticles and cells [15, 16].

Cell viability and proliferation, respectively, is not affected by the handling with ultrasonic standing plane wave fields at low energy densities, as has been reported for numerous cell lines [9, 17-21]. This holds true as long as the cells remain in the protective area of the pressure nodal planes. When they are subject to propagating waves, cell viability was found to be significantly reduced [22, 23]. Furthermore, morphology changes in yeast cells that were subject to ultrasonic plane wave fields have been reported [22]. There have

also been reports of increased cellular uptake when cells are subject to ultrasonic standing waves in the presence of acoustic contrast agents [24, 25].

The h-shape filter is an ultrasonic flow-through particle filter that relies only on the acoustic radiation forces. It has been developed to concentrate particles in suspension, and thus separate a phase with high particle concentration (enriched phase or retentate) from a phase with a low concentration of particles (cleared phase or filtrate) without depending on gravitational forces. It can thus be used in low and zero-gravity environments, e.g. space stations, and to split up suspensions in which the medium and the particles have the same mass density. This has been shown to work in zero-gravity flights with polysterene beads and the algae *Spirulina platensis* suspended in water and salt water, respectively [26]. Under normal gravitational conditions on earth, experiments with polysterene beads suspended in water in which the salt content was adjusted so that its mass density corresponded to that of the polysterene beads were successfully conducted by Hauser (data not published).

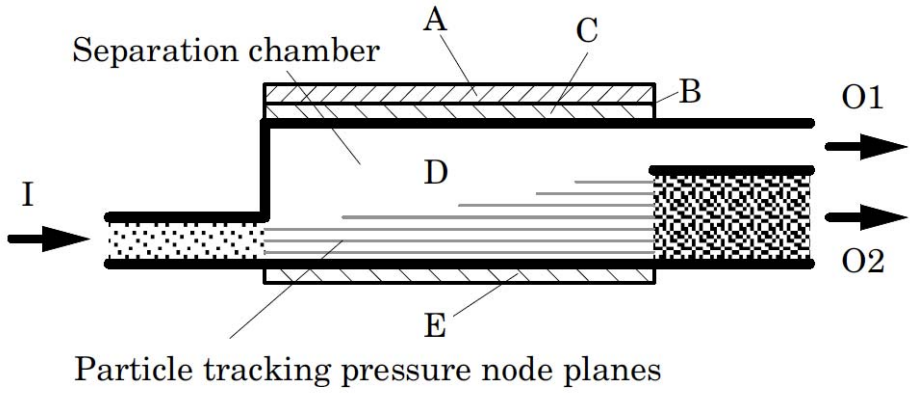


**Figure 1: Schematic sketch of the h-shape filter. The suspension enters the separation chamber through the inlet (I), in which an ultrasonic plane wave is induced by a PZT transducer (A) glued (B) to a glass carrier (C). The wave is reflected from the glass carrier (E) on the other side of the chamber, thus a standing wave field is built up. The cells entering the separation chamber are forced into the pressure nodal planes and travel to the retentate outlet (O2) as if guided by rails. Only the cleared liquid leaves the filtrate outlet (O1).**

Figure 1 [27] shows a sketch of the h-shape filter. The cell or particle suspension enters the separation chamber through the inlet (I). An ultrasonic standing wave is present in the separation chamber, induced by a PZT transducer (A) glued (B) to the top glass wall (C) of the chamber; the bottom glass wall acts as a reflector (E). The cells or particles are held in the pressure nodal planes situated in the lower part of the chamber due to the acoustic radiation forces. Drag force moves them towards the outlets within these planes. With the correct choice of energy density of the acoustic field and the flow velocity of the suspension, the

pressure nodal planes act as rails that guide the cells to the retentate (or enriched) outlet (O2). Only the cleared liquid leaves the chamber through the filtrate (or cleared) outlet (O1).

The aim of this work was to test the ultrasonic h-shape filter with suspensions of yeast cells and to observe the effects of the ultrasonic irradiation on the cells' viability, integrity and growth. The following hypothesises were tested for:

- As cells would have to pass through several pressure antinodal planes before being collected from the filtrate outlet, one would expect decreased cell viability in the samples taken from this outlet.
- Cells could be ruptured when passing through the antinodal planes which would lead to an increase in the protein content of the sonicated samples compared to control groups.
- Viability specific retention, i.e. non-viable cells are not retained as well as viable cells due to different acoustic properties, by analysing the connection between cell viability in the filtrate and retentate samples, respectively, and separation efficiency.

Furthermore, the influence of sonication on cell growth was investigated.

*Introduction to the theory of ultrasonic particle manipulation*

Gröschl [28] gave an exceptionally good overview of the theoretical background of ultrasonic particle manipulation and the introduction is guided by this.

The explanation for the observed effects was 1934 delivered by King [29]. He integrated the radiation pressure exerted by a plane standing acoustic wave over the surface of a rigid sphere in an ideal, i.e. non-viscous fluid. This derivation taking second order effects from the scattered sound field into consideration led to a nonvanishing time-averaged force displacing the particle. This effect is called the *axial primary radiation force* to express that it is originating from direct (primary) interactions of the particle and the initial sound field in direction of sound propagation (axial). Yosioka and Kawasima [30] delivered a description of the axial primary radiation force of a plane standing wave as

$$\langle F_s \rangle = 4\pi \cdot \rho \cdot \widehat{\Phi}_s (ka)^3 K_s(\lambda, \sigma) \sin(2kx). \quad (1)$$

The angle brackets in Equation (1) refer to the time averaging over one period of the cycle. Equation (1) was derived by replacing King's rigid particle by a compressible sphere in a host liquid of the mass density $\rho$. Furthermore the axial primary radiation force $F_s$ depends

on the wave number $k$ (the angular frequency divided by the speed of sound – $k = \frac{w}{v}$), the particle's radius $a$, and the amplitude $\widehat{\Phi}$ of the standing wave field's velocity potential

$$\widetilde{\Phi}_s = \widehat{\Phi}\left\{e^{i(wt-kx)} + e^{i(wt+kx)}\right\}. \quad (2)$$

$K_s(\lambda, \sigma)$ is the acoustic contrast factor of the standing wave which describes the strength and the direction of the axial primary radiation force. It is a function of the material properties of the particle and the host liquid and is given by

$$K_s(\lambda, \sigma) = \frac{1}{3}\left(\frac{5\lambda-2}{2\lambda+1} - \frac{1}{\lambda\sigma^2}\right), \quad (3)$$

where $\lambda = \rho_0/\rho$ is the ratio of the mass densities and $\sigma = v_0/v = k/k_0$ the ratio of speeds of sound of the particle (denoted by the subscript 0) and the host liquid, respectively.

This description of the axial primary radiation force is valid in the limiting case $ka \ll 1$ and $k_o a \ll 1$, thus the particle has to be small compared to the wavelength, and the mass densities of the particle and the liquid must be of the same order of magnitude.

The *transverse primary radiation force* emerges from uneven distributions of the amplitude $u_s(y)$ over the surface of a transducer[1], i.e. the sound source. These deviations arise from the boundaries of real resonators and lead to a force exerted perpendicular (transverse) to the direction of sound propagation. The force leads to a further concentration of the particles within the pressure nodal planes.

[1] It has to be emphasized that nevertheless one deals with a plane wave, i.e. the phase of the wave is unaffected and therefore the surface of equal phase is flat!

# 2. Materials and Methods

## 2.1. Statistical definitions

The standard deviation $s$ is defined by

$$s_x = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2} \quad \text{with } \bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i \,, \tag{4}$$

where $x_i$ are the measured values, $N$ is the number of trials and $\bar{x}$ denotes the mean (average) of the measured readings.

To compare the means of sets of trails to each other Student's t-test was used. This common statistical test tests for a null hypothesis $H_0$ that two means of sets of data are equal and returns the probability for this null hypothesis being true. A probability of $P \leq 0.05$ therefore means that the null hypothesis is false, i.e. the hypothesis H that the means are different is accepted at a significance level of 95%.

A two-sided t-test is performed when no indication exists, if one of the groups has a higher or lower average than the other group, e.g. when arbitrarily choosing one hundred people, splitting them into two groups and comparing the average IQ of the two groups. Thus a two-sided t-test was performed for the separation efficiencies observed in the different media, as there was no indication which one of the media would result in a higher efficiency, and for comparison of average cell concentrations found in the respective samples for the cultured cells sonicated in Malt Extract Broth immediately and 18 hours post-sonication. When one of the groups has undergone treatment that influences the measured variable, a one-sided t-test can be performed. Picking up the example for the two-sided t-test, this would mean that one of the groups was specially trained before performing the IQ test. One would clearly expect that this group has higher average IQ than the unprepared and untrained group. One thing has to be noted: When performing a one-sided t-test one has to define the hypothesis H beforehand, i.e. which of the groups is expected to have the higher or the lower average, respectively. If the averages turn out the other way, i.e. the sample one had expected to show the higher average turns out to have a lower average than the other an vice versa, the difference in averages can be significant (thus the null hypothesis would be rejected), but the hypothesis still has to be rejected. For comparing means of control groups to the sonicated groups a one-tailed t-test was used, as the separation process was believed to influence the measured parameters, i.e. cell viability would be reduced and the UV O.D. would be increased by sonication, as especially the cells found in the filtrate would have to cross the pressure antinodes which has been shown to cause damage to yeast cells [31, 32]. Therefore,

the respective hypothesises H chosen were that the average cell viability found in the sonicated samples would be lower than that of control groups and that the average and UV O.D. of sonicated samples would be higher than that of the control groups.

The test statistic is calculated by

$$t = \frac{|\overline{x_1} - \overline{x_2}|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \quad (5)$$

where $n_1$ and $n_2$ are the sample sizes of the groups $x_1$ and $x_2$, respectively. The degrees of freedom are given as $df = \frac{n_1 + n_2 - 2}{2}$ [33].

As sampling and pipetting errors are almost unavoidable and the methods used to obtain data are very sensitive to these, data points that were noticeably higher or lower than the others were tested by Grubbs' test for outliers (also known as extreme studentized deviate). The test-statistic G is defined as

$$G = \frac{\max_{i=1,\ldots,N} |x_i - \bar{x}|}{s} \quad (6)$$

and the null hypothesis that the data contains no outliers is rejected with a significance level $\alpha$ if

$$G > \frac{N-1}{\sqrt{N}} \sqrt{\frac{t^2_{\alpha/(2N),N-2}}{N-2+t^2_{\alpha/(2N),N-2}}} \quad (7)$$

where $t_{\alpha/(2N),N-2}$ is the critical value of the t-distribution with *N-2* degrees of freedom at a level of significance of $\frac{\alpha}{2N}$. All experiments were carried out in triplets therefore *N-2* was 1 and $\alpha$ was chosen to be 0.01, hence the level of significance 99%. If an outlier was detected it was not used for further analysis of the sample. The outliers will be indicated by striking through in the results section.

## 2.2. Cell culture and preparation of yeast suspensions

*Saccharomyces cerevisiae* (baker's yeast), which is spherical with a diameter of 5-10µm, was bought from the supermarket as block yeast.

For the experiments with wet yeast suspended in different media, about 0.2g of wet yeast were suspended in 300mL PBS (phosphate buffered saline, 0.9% NaCl, adjusted to pH 7), $H_2O$ tap or PBS 2x (PBS with 1.8% NaCl) which resulted in a final concentration of $4*10^6$ cells/mL.

For the experiments using cultured yeast, the following cultivation protocol was applied: A streak was made on a Malt Extract Agar (MEA, Fluka) plate and incubated at 30°C for 48 hours. A single colony was obtained from the streak plate with a loop and inoculated in Malt Extract Broth (0.4g in 40mL $H_2O$ deionized, MEB, Fluka) in an Erlenmeyer flask. The inoculum was left in an incubator equipped with a magnetic stirrer at 30°C overnight. Then, 10mL of the inoculum were transferred to 190mL of malt extract broth (4g in 200mL $H_2O$ deionized) and left in the incubator for about 55 hours at 30°C.

### 2.3. Haemocytometer counts and viability

To determine the cell concentration in a suspension, i.e. cells per mL, a "Neubauer improved" haemocytometer was used. This microscope glass slide consists of 2 chambers of defined height, which have a grid of perpendicular lines etched into their bottom. A microscope cover slip is placed on top of the haemocytometer, held at a distance of 0.1mm over the counting chambers by raised edges. The lines have a well defined distance, so the squares formed by these lines are of known area. As the exact height of the chamber is know, one can calculate the aliquot above them. For yeast cells, the number of cells in ten of the smallest squares, which have an area of 0.04mm$^2$, is counted in each chamber and the average between the two counts is taken. The volume over one square corresponds to 4nL, therefore each cell stands for 250000 cells in one mL. To calculate the number of cells per mL, i.e. cell concentration, the averaged number counted in the two chambers is multiplied by 25000 (as one counts 10 squares per chamber which corresponds to a volume of 40nL of the solution).

The viability of the cells was assessed by methylene blue staining (m.b.) as a standard method for detection of non-viable yeast cells as described by Jorgensen and Hansen [34]. This stain dyes non-viable cells blue, while viable cells remain uncoloured as they reduce the dye. The dye is mixed with the cell solution 1 in 2 before cell concentration is assessed by haemocytometer counts. To calculate the cell concentration of the diluted solution, the number of cells counted has to be multiplied by two, i.e. the average number of cells in the two chambers has to be calculated by multiplying by 50000 if ten squared per chamber were counted. The viability is calculated as:

$$cell\ viability = \left(1 - \frac{c_s}{c_t}\right) * 100\% ,$$

where $c_s$ is the concentration of stained cells (cells/mL) and $c_t$ is the total concentration of cells in the solution, i.e. stained and non-stained cells.

It has to be emphasized that the cell viability only gives the fraction of the cells found in the sample that are viable. When the cell viabilities will be presented in the Results section, this has to be considered, as the total concentration found in the respective samples can be different, e.g. the concentration in the filtrate samples is expected to be lower than that of the retentate, but the cell viability, i.e. the fraction of cells that are not stained blue, can be the same. Moreover, cells that are possibly ruptured during sonication would not be found in the sonicated samples.

### 2.4. Cell damage assessment

To see if cells were ruptured by sonication, the protein content of the suspension was assessed by measuring the UV optical density (UV O.D.) at 280nm wavelength.

The absorbance A of a liquid is defined as:

$$A = -\log_{10}\left(\frac{I}{I_0}\right)$$

where $I_0$ and I are the intensities of the light before and after the light has passed the liquid, respectively. Protein, to be more precise specific amino acids contained in the protein, absorbs light at 280nm resulting in a higher absorbance A, i.e. increased optical density if more protein is present in the liquid [35]. According to the Lambert-Beer law the relationship between absorbency and concentration of the absorbing substance is linear and thus the concentration of protein can be calculated if one knows the absorbance coefficient $\varepsilon$.

Before measurement, the samples were spun down in a centrifuge (Hettich) at 6000 rpm for 10 minutes. Subsequently, the supernatant was collected with a pipette and transferred into another Eppendorf and kept in a refrigerator until the measurements were conducted. The measurements were made with a Hewlett Packard 8452A diode array spectrometer. As one can see in the equation it is necessary to determine $I_0$, the intensity of the incident light; for this purpose a “blank“ measurement was taken of the quartz cuvette (10 mm path length) filled with the respective medium the samples were suspended in, i.e. the spectrometer was blanked with PBS for samples of yeast suspended in PBS, $H_2O$ for yeast suspended in $H_2O$, etc. The samples were vortexed before being carefully pipetted into the quartz cuvette to avoid bubble formation. Three measurements were made of each sample and the averaged value was used for further analysis.

## 2.5. Separation efficiency

To evaluate the quality of the filtration process of the h-shape filter the separation efficiency was determined. The separation efficiency is a measure of how well cells are kept from exiting through the filtrate outlet. It is calculated as

$$separation\ efficiency = \left(1 - \frac{c_f}{c_i}\right) * 100\%$$

where $c_f$ is the concentration of the cells found in the filtrate outlet and $c_i$ is the concentration of cells entering the filter, i.e. the average concentration of the control and sham groups.

## 2.6. Experimental set-up and procedure

After preparation of the respective suspension it was put in a reservoir that was equipped with a magnetic stirrer to ensure good mixing of cells and the medium.

A control sample was taken (two 1.5mL Eppendorfs for the experiments with wet yeast suspended in different media and about 15mL in sterilized test tubes when experimenting with cultured yeast). The h-shape filter was fixed at an angle of -45° to minimize the effect of gravity with the inlet at the highest point and the retentate outlet at the lowest, i.e. a downward pointing flow velocity. Böhm et al. [26] present a detailed analysis of the influence of different angles. In Figure 2 a schematic overview of the experimental set-up is given.

A peristaltic pump (Abimed Mini Plus) equipped with a pump head allowing up to four tubes to be operated at the same time was used to feed the suspension into the filter. For reasons of flow uniformity and avoidance of turbulence, the two tubes attached to the retentate and filtrate outlet were used (see Figure 2). (At first, two peristaltic pumps were used; one to feed into the inlet and one to pump from one of the outlets, respectively. This proved to be very instable, as there is a short moment of stalling when one of the cylinders that squeeze the tube stops squeezing it or starts to squeeze it again. The stalling lead to unstable flow conditions in the separator and subsequent difficulties in keeping the separation working. Synchronicity was hard to achieve or impossible, as the pumps were started manually and often driven at different speeds. A slight improvement could be made by pumping from both of the outlets, but the most stable conditions resulted from using just one pump.) The cleared flow-through was set to about 14L/day, corresponding to 4.13 rounds per minute for this pump head and the chosen tube diameter of 0.8mm. As the volume flow through the two outlets was set to be the same, this corresponded to a total flow-through of around 28L/day. The filter was filled inversely, i.e. through the outlets, to facilitate the

elimination of air in the tubes and the filter. Great attention was being paid to make sure that no air bubbles were present during sonication, as they could change the properties of the sound field and act as obstacles for the cells. When the system was filled, a sham-exposed (or sham, for short) sample was taken. Then the direction of pumping was reversed and the ultrasound was switched on.

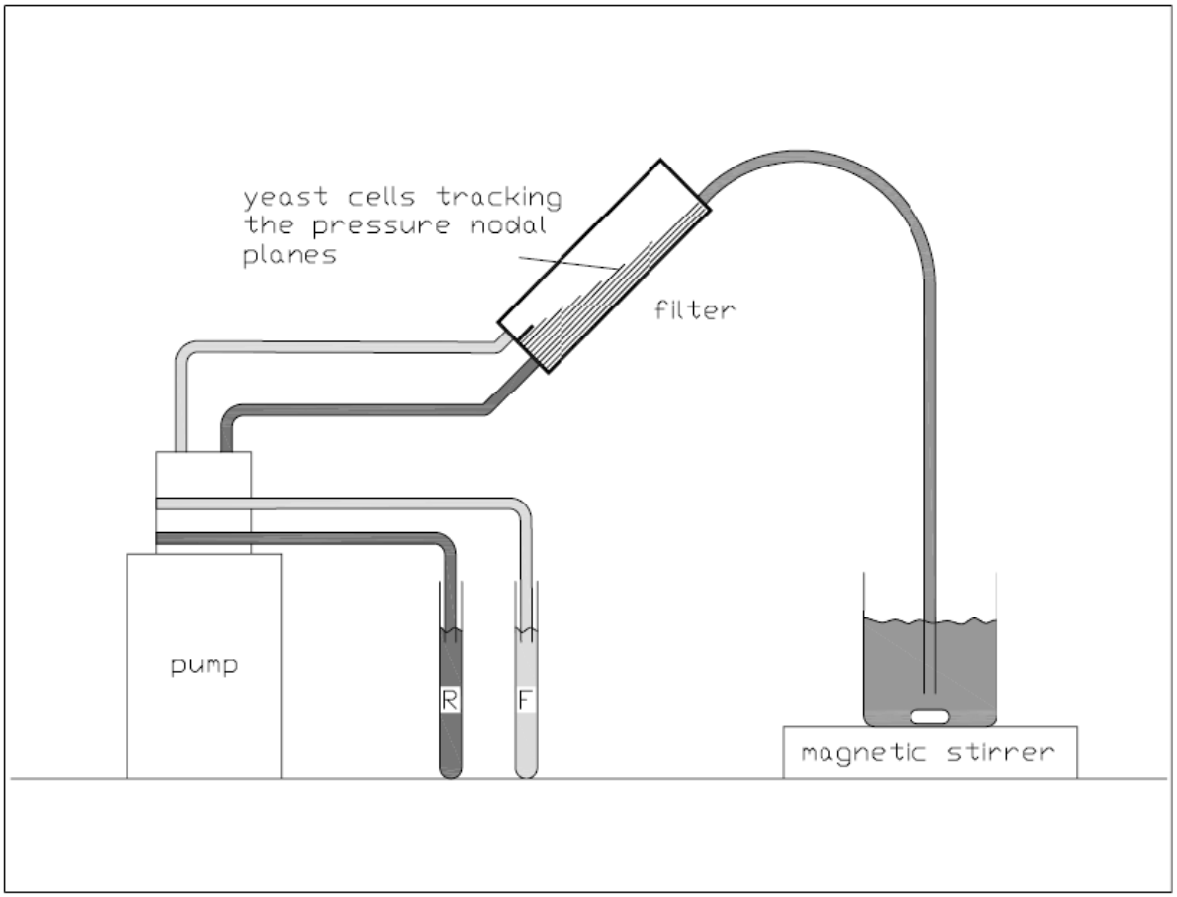


**Figure 2: Schematic view of the experimental set-up. The reservoir was equipped with a magnetic stirrer to ensure good mixing of the yeast suspension. The yeast suspension was pumped into the h-shape filter, which was inclined by -45°, and the yeast cells were driven into the pressure nodal planes. These acted like rails and guided the cells to the lower of the two outlets, the retentate outlet. The enriched suspension is represented by dark grey colour. Cleared host liquid (represented by light grey) left the filter through the filtrate outlet (upper). Samples from the two outlets were collected after the peristaltic pump.**

The frequency was set to approximately 2.1MHz at 3W true electrical power input (t.e.p.i.). The frequency was adjusted visually, i.e. set to a frequency at which a stable alignment of the yeast cells in the pressure nodal planes was observed. In all the experiments the chosen frequency was also a good electrical resonance (cosine of the ratio of admittance and susceptance equal to 1 or 0.9 as a measure for the resonance).

Two different procedures were followed for sample collection at the outlets:

In the experiments using suspensions of wet yeast, two 1.5mL samples were collected from each outlet (retentate and filtrate, respectively) in Eppendorf micro-centrifuge tubes every two minutes after a stable alignment had been established in the resonator. Thus five samples could be obtained from each outlet.

When cultured yeast was sonicated in the culture medium, sterilized test tubes were used for sample collection. The samples were taken from the respective outlets starting at about 4 minutes after the establishment of the stable alignment and about 15mL were collected in each test tube. This took about 2 minutes.

In Figure 2 the test tubes in which the respective samples were collected are named R for the sample collected from the retentate outlet and F for the sample collected from the filtrate outlet.

Due to the relatively small amount of suspension, i.e. 300mL and 200mL for re-suspended wet yeast and yeast in Malt Extract Broth, respectively, the total duration of one run was about 10 to 12 minutes.

Haemocytometer counts were performed right after the experiment. Then, the samples were spun down in a centrifuge (Hettich) at 6000 rpm for 10 minutes and the supernatant was transferred to a fresh micro-centrifuge tube for UV O.D. measurements.

To investigate if the ultrasonic filtration process had an influence on the cell growth rate, the cultured cells sonicated in Malt Extract Broth were not only counted right after the experiment, but also 18 hours post-sonication. After the first analysis (haemocytometer counts, viability assessment by m.b. staining and preparation of the samples for UV OD measurements), the remainder of the samples were left under the laminar flow at room temperature.

# 3. Results

The results will be presented in different sections for each experimental configuration, i.e. for the wet yeast suspended in the respective media and the cultured yeast sonicated in Malt Extract Broth. Immediately after the respective results, these will be discussed.

## 3.1. Suspensions of wet yeast

Separation efficiency, viability and UV O.D. data for suspensions of wet yeast in PBS, $H_2O$ tap and PBS 2x, respectively, will be given in the following chapters.

### 3.1.1. Separation efficiency for different host liquids

The following observation was made for all the experimental runs with wet yeast suspended in the different host liquids: When the ultrasonic field was switched on, the cells were almost instantaneously driven into the pressure nodal planes and a stable alignment developed. Over time, the pressure nodal planes in the lower part of the acoustic filter, i.e. those that would "guide" the cells to the retentate outlet, became more and more populated with yeast cells, which seemed to be held back in the resonator. Especially close to outlets cells would also start filling up the pressure nodal planes in the upper part of the filter. A kind of "plug" of cells appeared to be formed in close proximity to the outlets. This area would be steadily filled up, until the "plug" would quickly break up and the cells would be dragged into both outlets.

*PBS-suspensions*

In Figure 3 the separation efficiency for wet yeast/PBS suspensions is shown at different times after the ultrasound (US) had been switched on and a stable alignment was achieved. Run 1 through 3 show a similar change of separation efficiency over time, increasing to a maximum at 4:00 [min:sec] after the US had been switched on and then gradually decreasing over time. Only run 4 showed a different progression over time, with a slight decrease in separation efficiency from 2:00 to 6:00 and a strong increase towards the end of the run. The average separation efficiency over all times of the four runs was found to be 54.5+/-17.9%. In Table 2 the exact values of the separation efficiency found at the respective times during the different runs are listed.

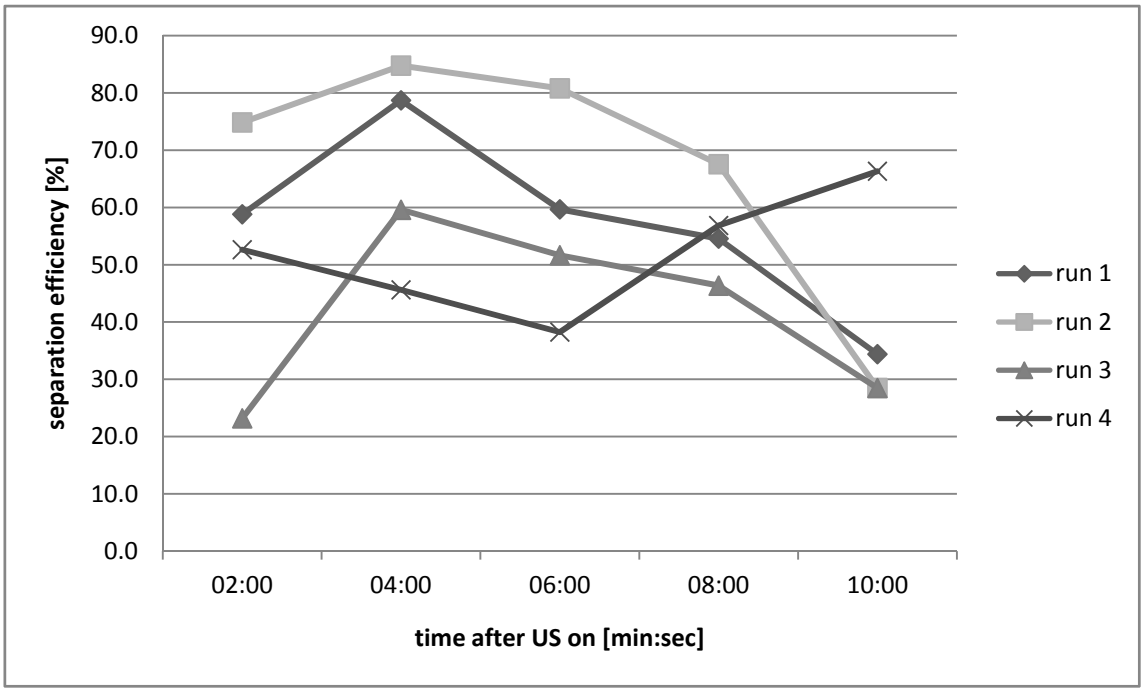


**Figure 3: Separation efficiency found for wet yeast suspended in PBS at different times after the US had been switched on.**

*PBS 2x-suspensions*

Figure 4 shows the separation efficiencies found in the runs at different times after the US had been switched on. The highest separation efficiency was achieved in run 3 with 69.9% however this was also the run with the highest fluctuation of separation efficiencies at the different times. In run 2 the separation efficiency seems to reach a somewhat stable state at around 60% after starting at a very low level. This was probably due to difficulties in establishing a stable alignment of the cells in the pressure nodal planes in the beginning of the run. The averaged separation efficiency for the three runs was found to be 49.2+/-16.9%

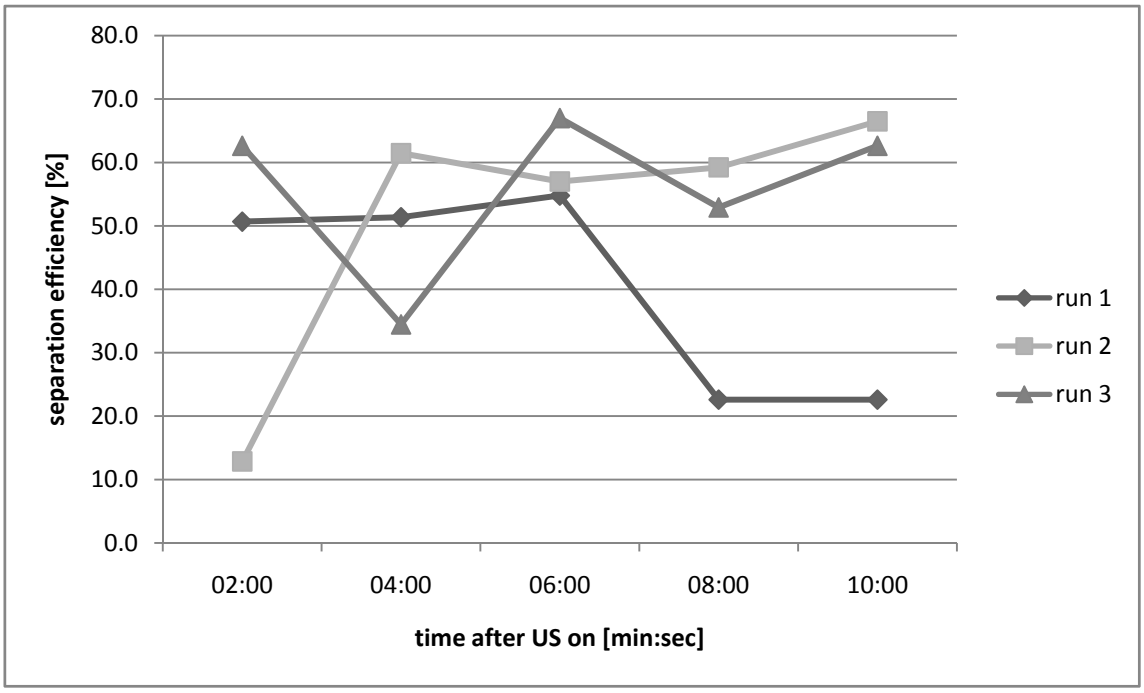


**Figure 4: Separation efficiency for wet yeast suspended in two-fold concentrated PBS (PBS 2x) measured at different times after the US had been switchen on.**

*$H_2O$ tap-suspensions*

The separation efficiency for wet yeast suspended in $H_2O$ tap was very unreliable and varied greatly between and within run 1 and run 2 as one can see in Figure 5. In Run 3, however, separation efficiency steadily increased from 18.8% found at time t = 2:00 to 61.7% at time t = 10:00. A similar development, however with higher fluctuations, was observed in run 1, separation efficiency was low from the very beginning and increased towards the end of the experimental run. Run 2 showed a different development, after starting off at the same low level as run 1 and 3, separation efficiency decreased to values even below 0%, i.e. more than half of the cells that were entering the separator left it through the cleared outlet. The average separation efficiency for wet yeast/$H_2O$ suspensions was 27.0+/-27.7 % in the three runs.

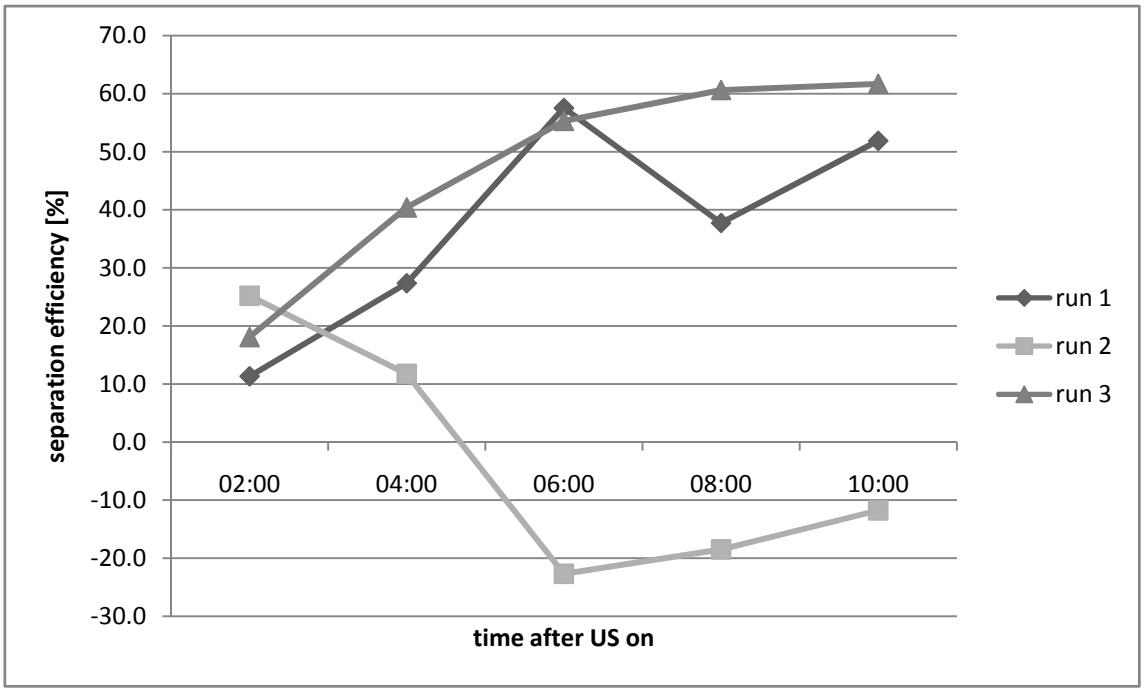


**Figure 5: Separation efficiency for wet yeast suspended in $H_2O$ tap at different times throughout the runs. Notice how the separation efficiency drops below 0% for run 2 – this means that more cells per mL were leaving the resonator through the filtrate outlet than were present in the original suspension.**

*Discussion*

The highest average separation efficiency was found for wet yeast/PBS suspensions. Wet yeast/PBS 2x suspensions showed slightly lower average separation efficiency, but the difference between the averages is not significant. The maximum separation efficiency found for PBS 2x-suspensions was lower than that for PBS-suspensions, as was the minimum separation efficiency. For $H_2O$-suspensions separation efficiency varied a lot more than for PBS- and PBS 2x-suspensions. Furthermore the separation efficiencies found were somewhat lower than those found for PBS- and PBS 2x-suspensions.

The disappointingly low separation efficiency for yeast suspended in PBS, PBS 2x and $H_2O$ tap, respectively obtained by the h-shape filter came as a surprise after the good results reported by Böhm et. al. for *Spirulina platensis* [26]. Apparently, the acoustic contrast factor between *Saccharomyces cerevisiae* and the media used is not large enough for the axial primary radiation force to completely overcome the drag force of the liquid. Even though the salt content does have an influence on the sound velocity and the mass density of a liquid, and thus on the acoustic contrast factor (as can be seen in Equation (3)), the change in salinity from 1 ppt (parts per thousand) for $H_2O$ tap (acoustic contrast factor = 0.112) to 9 ppt for PBS (acoustic contrast factor = 0.114) and 18 ppt for PBS 2x (acoustic contrast factor = 0.106),

respectively, only reduces the acoustic contrast factor by 4% and 11%, respectively. For these calculations the speed of sound in PBS and PBS 2x was calculated to be $v_{PBS} = 1506\frac{m}{s}$ and $v_{PBS2x} = 1516\frac{m}{s}$, respectively, using Coppens equation at 25°C and 0m water depth [36]. Mass density was interpolated between fresh water (mass density $\rho_{fresh\,water} = 1000\frac{kg}{m^3}$) and of sea water with a salt content of 3.5% and a mass density of $\rho_{sea\,water} = 1025\ \frac{kg}{m^3}$. Thus mass density of PBS was estimated to be $\rho_{PBS} = 1006\frac{kg}{m^3}$ and that of PBS 2x to be $\rho_{PBS2x} = 1013\frac{kg}{m^3}$. For the mass density of yeast cells $\rho_{yeast} = 1114\frac{kg}{m^3}$ was used [37] and the speed of sound was calculated using the compressibility of erythrocytes to be $v_{yeast} = 1642\frac{m}{s}$ [38].

Moreover, the difference in salt concentration changes the osmolarity, i.e. the number for solute concentration, of the medium. The ion content of PBS corresponds to the ion concentration normally found inside yeast cells, this means the two are isoosmotic and isotonic. A good overview of the effect of hypo- and hyperosmotic stress is given by I. Dawes in the book "The metabolism and molecular physiology of *Saccharomyces cerevisiae*" [39]. Morris et al [40] reported the instantaneous loss of intracellular water when *Saccharomyces cerevisiae* were placed in a 5Osm NaCl suspension. Consequently, the cell volume was reduced by an average of 35%. Stelios et al. reported that cell viability was not affected by a salt content of 4%, 6% and 10%($^{w}/_{v}$) in the medium during fermentation for ethanol production [41]. As the salt content of PBS 2x is higher than the intracellular salt content, i.e. it is hyperosmotic, it is likely that the cells' content would be reduced. Thus the acoustic radiation forces, which depend on the volume of the particles, would be smaller for the cells suspended in PBS 2x than for those suspended in PBS. The result would be lower separation efficiency for the PBS 2x-suspensions than for PBS-suspensions. However, no significant difference in separation efficiency was found for the two media. The opposite holds true for cells suspended in $H_2O$ tap, in which the ion concentration is below the intracellular level, i.e. $H_2O$ is hypo-osmotic. Thus, a net flow of water into the cell would lead to swelling, i.e. an increase in cell diameter, and consequently to a larger axial primary acoustic radiation force. Moreover, there is a physiological difference between the cells suspended in the different media, which could result in different compressibilities of the yeast cells. However, this is not reflected by the separation efficiency data found. There are no significant differences in the average separation efficiency in the different media. However, the changes of separation efficiency during the individual experimental runs is higher for PBS 2x- and $H_2O$ tap-

suspensions, respectively, than for cells suspended in PBS. This could be an effect of the small difference in acoustic contrast.

The cells were held back in the acoustic filter by the transverse primary acoustic radiation force. This force arises from the fact that the resonator has a limited area and the energy density is highest towards its centre. It acts perpendicular to the direction of speed of sound and results in a concentration of the cells in regions with maximal amplitude within the pressure nodal planes. Apparently this force is very strong in this set-up and led to the formation of the "plug" of cells which could be a reason for the low separation efficiencies observed.

### 3.1.2. Cell viability (m.b.)

In Table 1 the average cell viabilities of the respective samples for the three different media are given. As sham treatment did not change cell viability (two-sided t-test) for any of the media used, data from both groups was used to check for statistical significance of differences in cell viability of non-sonicated and sonicated cells, i.e. cells found in the filtrate and the retentate outlet. When cell viabilities for filtrate and retentate samples, respectively, are given for single runs, these were calculated as averages over the five samples taken in the respective run. For the calculation of the t-test, data of all the single samples were used.

For wet yeast suspended in PBS and PBS 2x the viability was found to be around 99% for control groups and sham exposed cells, remaining at that level for retentate and filtrate samples. $H_2O$ tap proved to be of negative influence on the viability of the cells, however, no difference between sonicated cells and the non-sonicated groups (control and sham) was found. A more detailed analysis of the data as well as possible correlations between separation efficiency and cell viability will be given for the different media.

Table 1: Averaged and rounded cell viabilities for control, sham, retentate and filtrate samples, respectively, in the different media.

| cell viability [%] | control | sham | retentate | filtrate |
|---|---|---|---|---|
| PBS | 99 | 99 | 99 | 99 |
| PBS 2x | 99 | 99 | 99 | 99 |
| $H_2O$ tap | 89 | 89 | 88 | 91 |

*PBS-suspensions*

In Figure 6 the averaged cell viability for yeast/PBS suspensions is shown. The control samples had the highest average cell viability at 99.5+/-0.6%; sham treatment did not influence the cells' viability, which remained at 99.3+/-0.6%. For the retentate samples the cell viability was found to be 99.0+/-0.1%, omitting one outlier (indicated by striking out in Table 2) according to Grubb's test (99% significance level). The filter process led to a very slight decrease of cell viability for the filtrate samples to 98.5+/-1.1%, however, this was not significant (t-test). Again, this average was calculated omitting one outlier (also indicated in Table 2) according to Grubb's test (99% significance level).

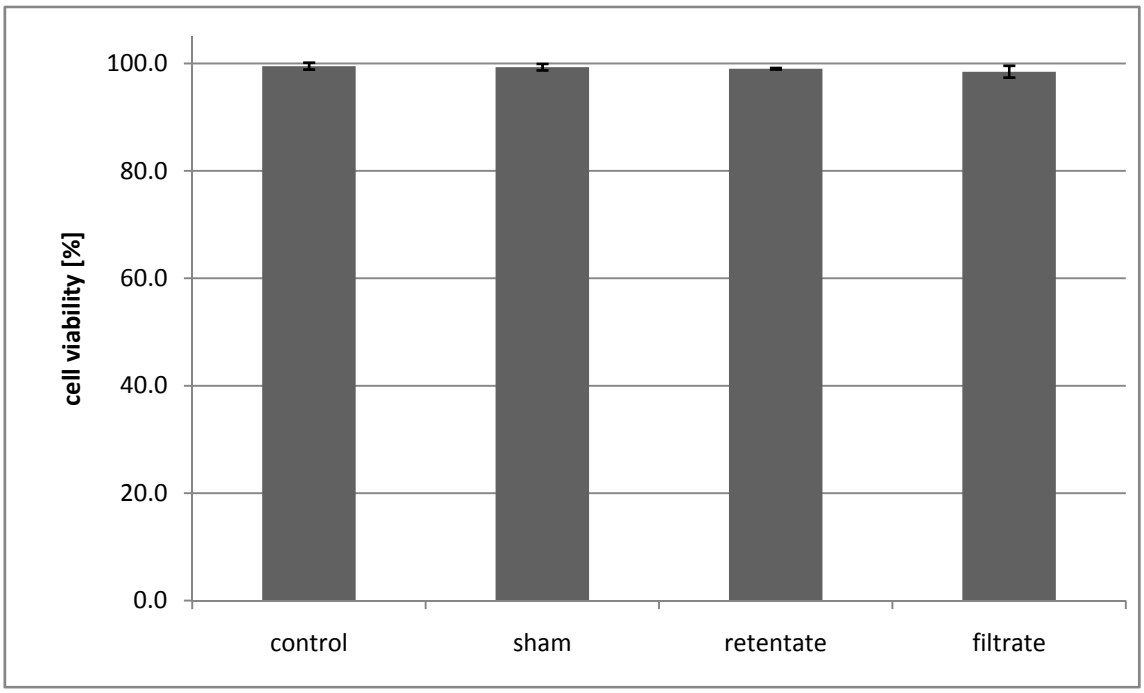


**Figure 6: Average cell viability for wet yeast suspended in PBS. For the retentate and filtrate sample the average over all times and runs was calculated. Note the relatively small error, i.e. standard deviation.**

In Figure 7 the cell viabilities for the two outlets are plotted over the respective separation efficiencies. There seems to be no monotonic relationship between the cell viability found in either of the outlets and the separation efficiency. The most obvious thing speaking against monotonicity is that there are a few filtrate samples (dark grey diamonds) with a viability of 100.0%, however the separation efficiencies found at the respective times varied from 23.2% to 80.0%. The retentate cell viabilities scatter around a "plateau" at the average of 99.3% for the different separation efficiencies.

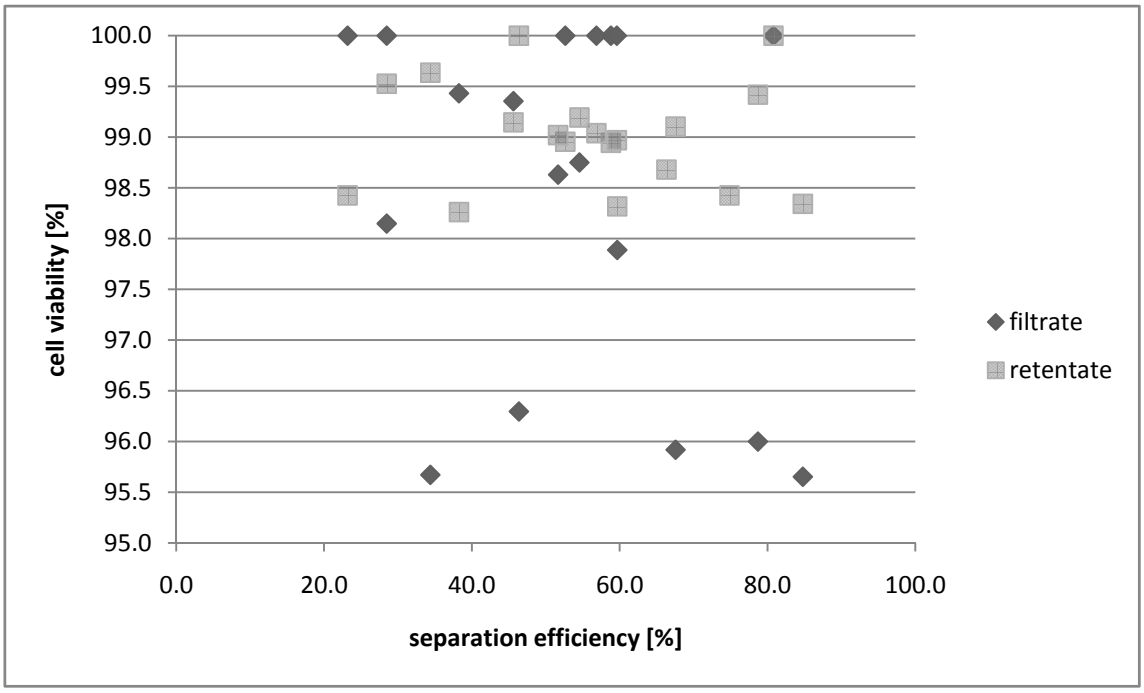


**Figure 7: The cell viabilities of the retentate and filtrate samples of wet yeast/PBS suspensions, respectively, plotted over the corresponding values of separation efficiency at the time of sample taking.**

Table 2: Separation efficiency and the respective cell viabilities of retentate and filtrate samples of the four experimental runs at the different times – the outliers according to Grubb's test for outliers are crossed out.

| run | time [min:sec] | separation efficiency [%] | cell viability [%] | |
|---|---|---|---|---|
| | | | filtrate | retentate |
| 1 | 2:00 | 58.8 | 100 | 98.9 |
| | 4:00 | 78.7 | 96.0 | 99.4 |
| | 6:00 | 60.0 | 97.9 | 98.3 |
| | 8:00 | 54.5 | 98.8 | 99.2 |
| | 10:00 | 34.4 | 95.7 | 99.6 |
| 2 | 2:00 | 74.8 | ~~86.8~~ | 98.4 |
| | 4:00 | 84.8 | 95.7 | 98.3 |
| | 6:00 | 80.8 | 100 | 100 |
| | 8:00 | 67.5 | 96.0 | 99.1 |
| | 10:00 | 28.5 | 98.1 | 99.5 |
| 3 | 2:00 | 23.2 | 100 | 98.4 |
| | 4:00 | 59.6 | 100 | 99.0 |
| | 6:00 | 51.7 | 98.6 | 99.0 |
| | 8:00 | 46.4 | 96.3 | 100 |
| | 10:00 | 28.5 | 98.1 | ~~91.9~~ |
| 4 | 2:00 | 52.6 | 100 | 99.0 |
| | 4:00 | 45.6 | 99.4 | 99.1 |
| | 6:00 | 38.2 | 99.4 | 98.3 |
| | 8:00 | 56.9 | 100 | 99.0 |
| | 10:00 | 66.3 | 100 | 98.7 |

*PBS 2x-suspensions*

Wet yeast/PBS 2x suspensions were found to have unaffected cell viability as one can see in Figure 8. Average cell viabilities for controls and shams were 99.5+/-0.8% and 99.0+/-1.1%, respectively, and remained at that level for retentates and filtrates with 99.7+/-0.2% and 99.2+/-0.5%, respectively.

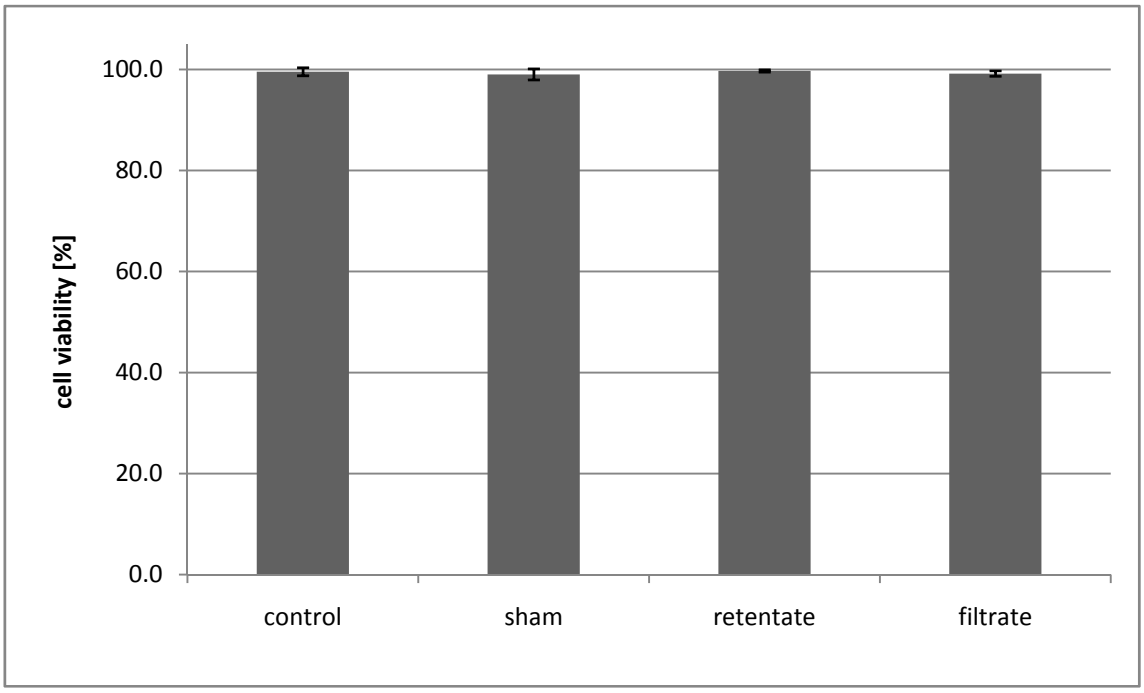


**Figure 8: Average cell viability for yeast/PBS 2x suspensions. As for yeast suspended in PBS, cell viability is high and is not significantly altered by the filtration process.**

In Figure 9 the cell viability is plotted over the respective separation efficiency found for wet yeast/PBS 2xsuspensions. The rather low cell viabilities found for four of the filtrate samples (denoted by a circle) are not outliers according to Grubb's test for outliers. Even though these values would speak for a monotonic relationship between the two measurands, it is very unlikely, as cell viabilities of 100.0% were found for several different separation efficiencies. When omitting these data, the dependence of filtrate and retentate cell viability, respectively, is similar.

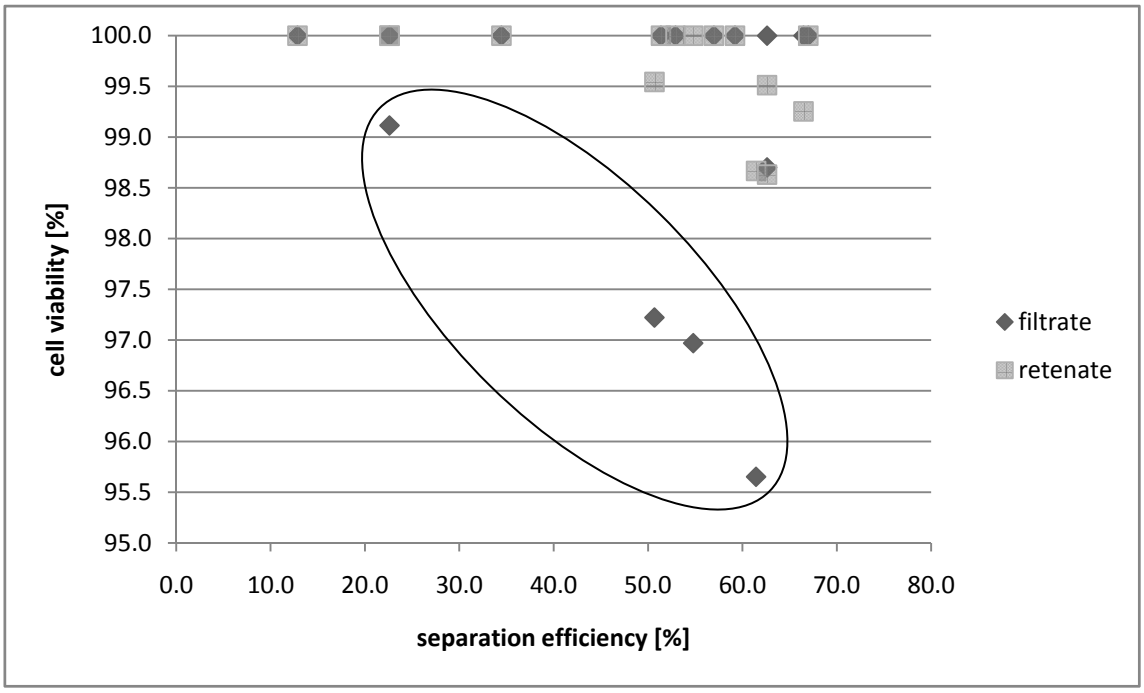


**Figure 9: Cell viability vs. separation efficiency for wet yeast/PBS 2x suspensions. The relatively low cell viability of four of the filtrate samples (circled) are not outliers according to Grubb's test for outliers. Concerning the relationship between filtrate separation efficiency and cell viability it has to be emphasized that most of the samples have a cell viability of 100% - they are "hidden" behind the semi-transparent retentate data points. The circled data points conceal the fact that the relationship is basically non-existent.**

*$H_2O$ tap-suspensions*

For cells suspended in $H_2O$ tap, Figure 10 shows that the average cell viability was relatively low and standard deviation was high. For control and sham groups the average cell viability was found to be 89.5+/-10.0% and 89.2+/-11.9%, respectively. Sonication did not significantly affect the cells' viability, as an average of 87.9+/-15.1% of the cells found in the retentate samples and 91.3+/-9.8% of the cells found in the filtrate samples were viable. Especially the samples taken in run 2 displayed very low cell viability, as one can see in Table 3. These values give rise to the high standard deviations found. As for the other runs, sonication and the separation process did not significantly alter cell viability in run 2. Retentate and filtrate cell viability were not significantly different from those found in the control and sham sample. However, average cell viability was found to be significantly higher for filtrate samples than for retentate samples (99% significance, 2-sided t-test).

Table 3: Cell viabilities for control, sham, retentate and filtrate samples, respectively, for each of the three runs with yeast/$H_2O$ tap suspensions. For the retentates and filtrates the cell viability is given as an average over all the samples in one run.

| cell viabilities [%] | control | sham | retentate | filtrate |
|---|---|---|---|---|
| run 1 | 99.0 | 99.5 | 99.2 | 99.1 |
| run 2 | 79.0 | 76.2 | 70.8 | 81.8 |
| run 3 | 90.5 | 92.0 | 93.7 | 92.8 |

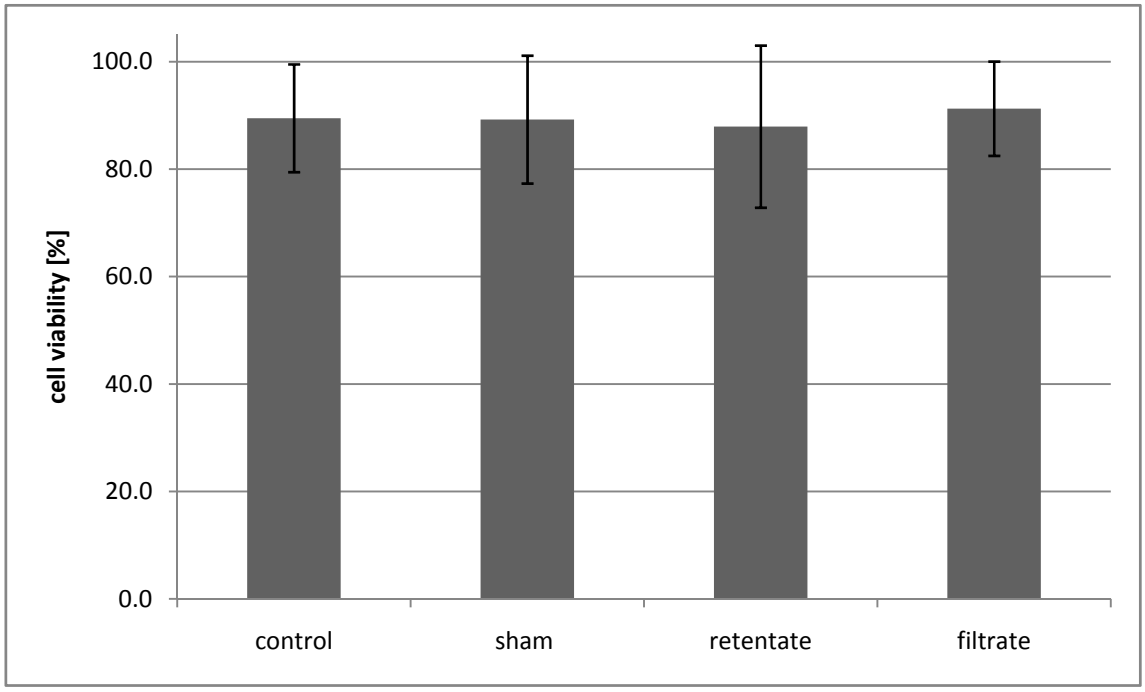


**Figure 10: Average cell viability for yeast/$H_2O$ tap suspensions. It is obvious that $H_2O$ is not an ideal medium for the yeast cells, as even the control groups show a lower viability compared to the controls of cells suspended in PBS or PBS 2x.**

The data that represent the samples of the second run are circled in black in Figure 11. These data are not outliers according to Grubb's test for outliers which is due to the way the test is works: the 5 values make the standard deviation $s$ very large, so that $G$ is a small number which makes the significance condition given in the Equation (7) is unlikely to be fulfilled. Figure 11 shows the cell viabilities of the samples taken from the respective outlets over the separation efficiencies found at the corresponding times. The circled data points are those found in run 2. When omitting these, the connection between filtrate cell viability and separation efficiency is clearly not monotonic. The same is true for dependence of the retentate cell viability on the separation efficiency.

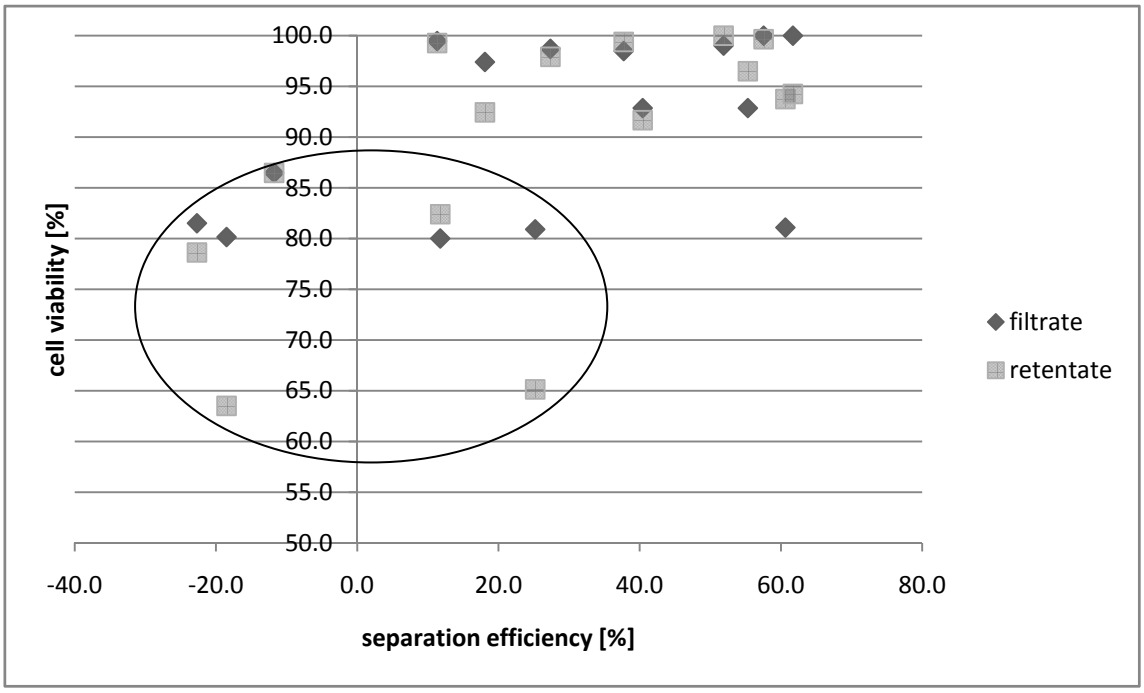


**Figure 11: Cell viability for the filtrate and retentate samples, respectively, over the corresponding separation efficiencies for wet yeast/$H_2O$ tap suspensions. The data points of experimental run 2 are encircled by the black ellipse.**

*Discussion*

As already mentioned, sonication did not influence cell viability in any of the media used. This is in accordance with a previous report about yeast cells subject to ultrasonic plane wave fields by Radel et al. [22].

There seems to be no monotonic relationship between the viability of the yeast cells found in the filtrate and the separation efficiency for any of the three media used, i.e. no viability specific retention could be detected. In run 2 of the $H_2O$ tap-suspensions cell viability was already low for the control and sham-exposed samples and it seems likely that the low cell viability gives rise to the extremely low separation efficiencies found[2]. This would speak for viability specific retention, however not in the sense it was expected to work, i.e. viable cells are retained better than non-viable cells. An interesting fact is that the average cell viability is significantly larger for the filtrate samples than for the retentate samples (two-sided t-test, 95% significance level) (see Table 3 – the standard deviation for filtrate samples is $s = 2.7\%$, while for retentate samples it is $s = 9.0\%$) – that would suggest that the non-viable cells are ruptured when moving through the pressure anti-nodes to reach the filtrate outlet and hence are not found there anymore. The larger fraction of non-viable cells present

[2] It has to be emphasised that a new package of fresh yeast, i.e. freshly bought from the supermarket with a best before date at least two weeks after the experiment, was used for all the experimental run and that a nice and stable alignment was observed during experimental run 2.

could have an influence on the acoustic radiation forces acting on all the cells, thus cell separation efficiency could be reduced.

### 3.1.3. Optical density (UV O.D.)

Possible cell leakage and rupture, which would lead to an increase in protein content of the supernatant, were assessed by comparing the UV O.D. of control and sonicated samples, respectively.

When looking at averaged UV O.D. values found in the different media before and after sonication in Figure 12, a few things become apparent. Firstly, the wet yeast/$H_2O$ tap suspension shows the smallest UV O.D. averaging to 0.05+/-0.03 for control and sham samples and 0.04+/-0.02 for the sonicated samples. Secondly, standard deviations are quite large; again especially for the suspensions in $H_2O$ tap. Furthermore, the average UV O.D. for wet yeast/$H_2O$ tap and wet yeast/ PBS 2x suspensions, respectively, is basically the same for control and sonicated samples. On the contrary, for the wet yeast/PBS suspensions, the average control UV O.D. is 0.05+/-0.01 while the average sonicated UV O.D. is higher, being 0.08+/-0.02. According to Student's t-test, this difference of averages is significant at a level of 99% (one sided).

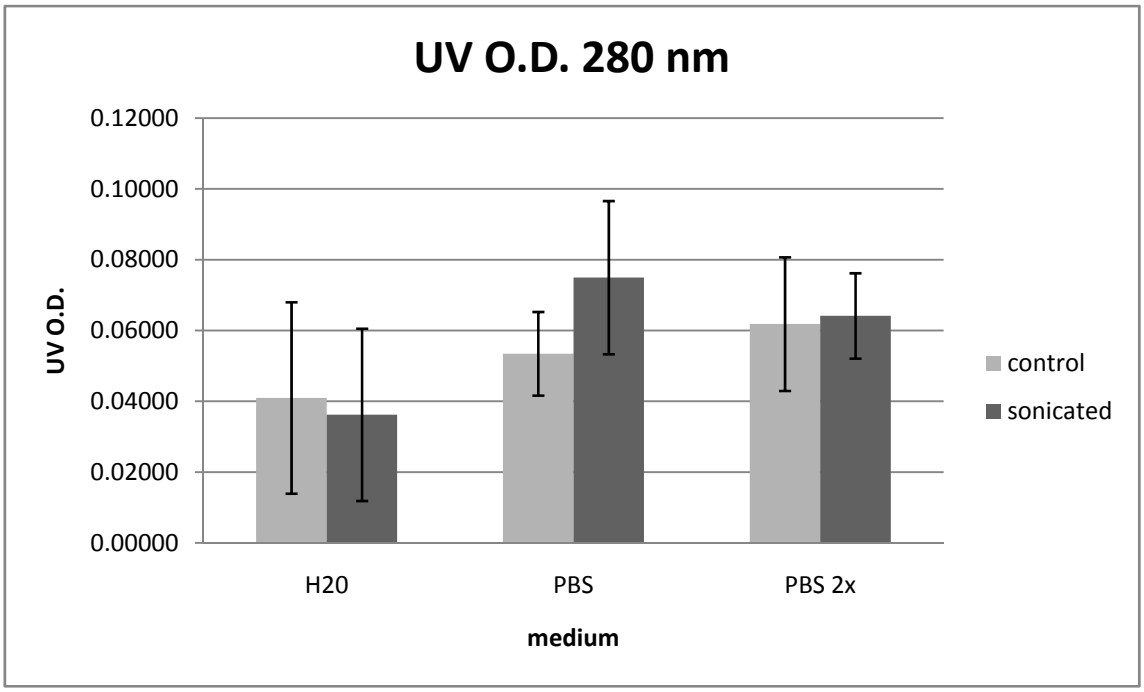


**Figure 12: UV O.D. for the three different media. As control the average of control and sham samples of all the runs with a specific medium was taken; error bars indicate the standard deviation. The sonicated value was calculated by averaging over all the filtrate and retentate samples of the runs with the respective media. Again, the error bars correspond to the standard deviations.**

The UV O.D. values for the different media and possible connections with separation efficiency will be presented in the following three chapters.

*PBS-suspensions*

The UV O.D. of control, filtrate and retentate samples taken at the indicated times averaged over three experimental runs (UV O.D. was not assessed for run 1 as the UV-vis photo-spectrometer was not available at the time of the experimental run) did not vary greatly over time (Figure 13). Filtrate and retentate UV O.D. were not significantly different from each other when comparing them at specific times after the US had been switched on or when averaging over all times for filtrate and retentate, respectively (two-sided t-test). Thus, for the analysis of relationship with separation efficiency the average of both outlets was taken. As mentioned before, the control UV O.D. is significantly lower than the average UV O.D. of the two outlets.

In Figure 14 the separation efficiency at the different times of sample taking is plotted along with the respective UV O.D. values of the sonicated samples, i.e. the average UV O.D. of filtrate and retentate samples. Even when taking into account the different scales of the respective y-axis, separation efficiency shows a much greater variance than UV O.D. This becomes more obvious when looking at the averages and relative standard deviations (RSD) listed in Table 4: while the RSD for the separation efficiency varies from 18.6% to as high as 53.2% while for UV O.D. it varies between 14.9% and 36.7%. This is already one indication that a strong relationship between UV O.D. and separation efficiency did not exist.

Table 4: Average separation efficiency and UV O.D., respectively, and the relative standard deviations of those for the samples taken at the indicated times.

| time [min:sec] | separation efficiency [%] | RSD [%] | UV O.D. | RSD [%] |
|---|---|---|---|---|
| 2:00 | 50.2 | 51.6 | 0.09131 | 34.7 |
| 4:00 | 63.3 | 31.3 | 0.06789 | 24.5 |
| 6:00 | 56.9 | 38.2 | 0.06457 | 28.0 |
| 8:00 | 57.0 | 18.6 | 0.07724 | 36.7 |
| 10:00 | 41.1 | 53.2 | 0.07198 | 14.9 |

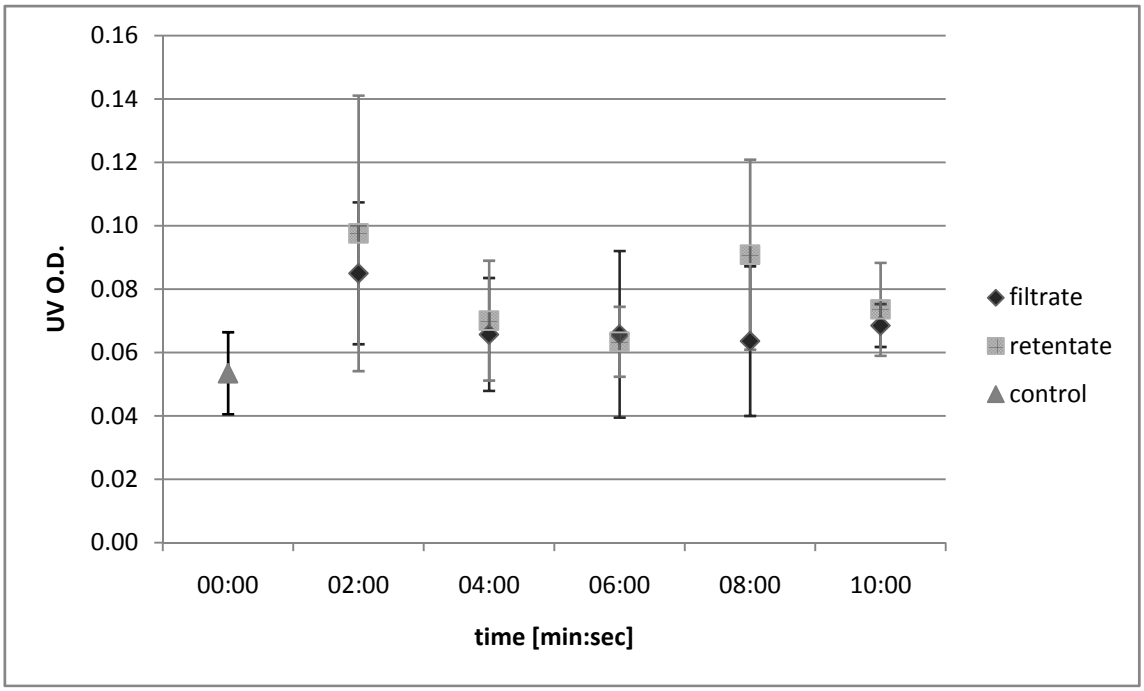


**Figure 13: Averaged UV O.D. for control, filtrate and retentate samples, respectively, for wet yeast/PBS suspensions taken at the times indicated on the x-axis after the US had been switched on.**

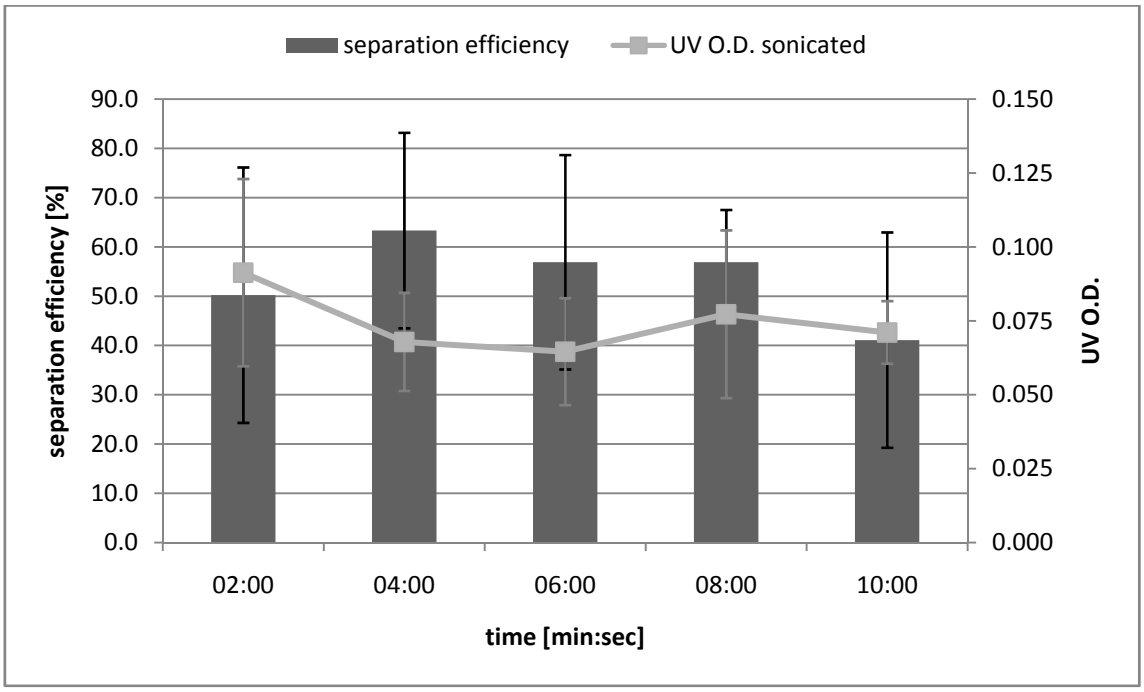


**Figure 14: Comparison of the average separation efficiency and average O.D. (of all sonicated samples) for wet yeast/PBS suspensions.**

*PBS 2x-suspensions*

For wet yeast/PBS 2x suspensions, UV O.D. values were relatively stable over time, not changing a lot between the different times at which samples were taken within one run and

when comparing the samples taken at the same time in the different runs, respectively (see Figure 15). This lead to small standard deviations and only small differences between the average UV O.D. values found at the different times for filtrate and retentate samples, respectively. One sample (filtrate @ 10:00 after US on in run 1) was neglected as an outlier according to Grubb's test (99% significance). Again, the UV O.D. values of filtrate and retentate samples were not significantly different from each other, so their average was used for further analysis (marked as "sonicated"). Also, no significant difference between control samples and sonicated samples could be detected.

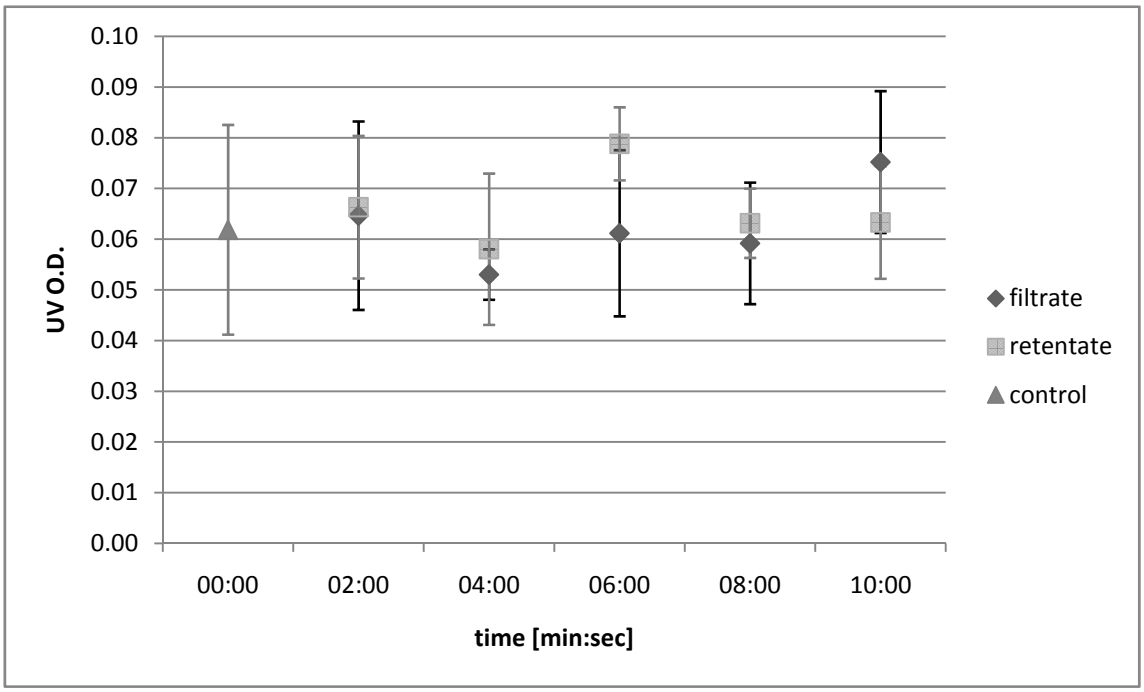


**Figure 15: UV O.D. of the samples of PBS 2x-suspensions taken at the time indicated. The control is an average of control and sham samples taken before the experimental set-up was filled and the US had been switched on, respectively.**

In Figure 16 average separation efficiency and the corresponding average sonicated UV O.D. for the different times are shown. Due to the large standard deviations of the separation efficiencies, no connection between separation efficiency and UV O.D. could be found. This becomes even more apparent when analysing a scatter plot of the two measurands (not shown).

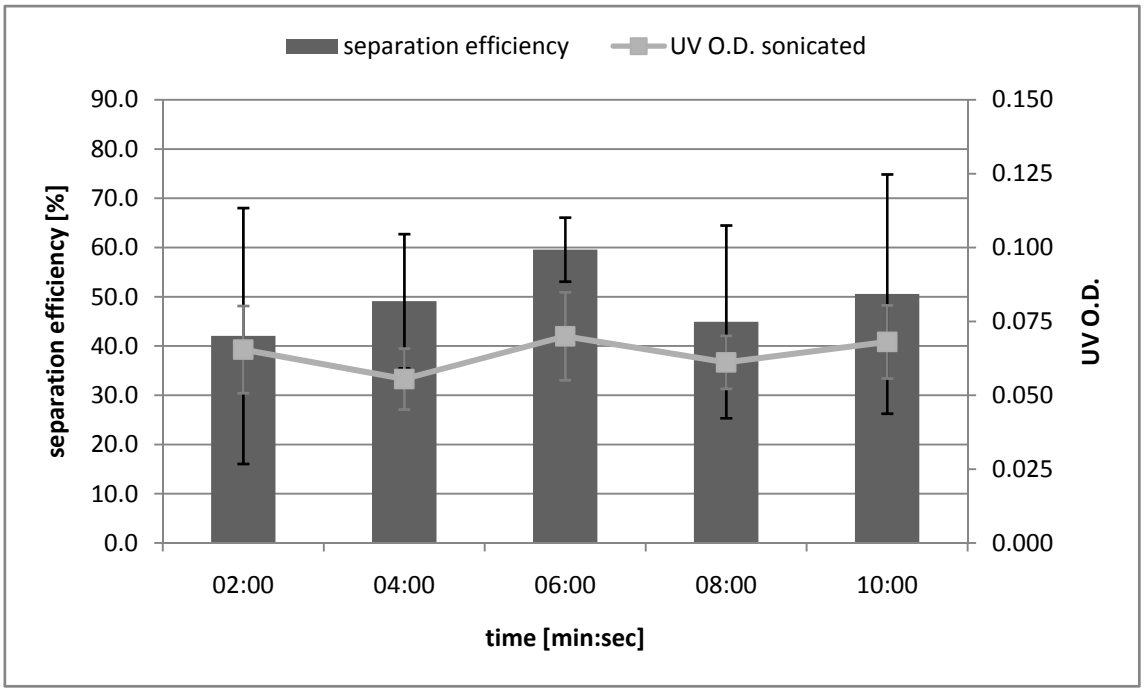


**Figure 16: The separation efficiency (bars) and the UV O.D. (squares) measured when filtering wet yeast/PBS 2x suspensions plotted over time.**

*$H_2O$ tap-suspensions*

The UV O.D. values of wet yeast/$H_2O$ tap-suspensions were rather low and showed large variance. The average retentate UV O.D. was found to be at all times larger than the average filtrate UV O.D. (Figure 17), nevertheless the difference between the retentate and filtrate UV O.D.s, respectively, was not significant. The standard deviations were also larger for the retentate samples than for the filtrate samples. For filtrate and retentate samples the average UV O.D.s were not significantly different from those found in the sham and control groups.

Figure 18 shows the average UV O.D. of the sonicated samples at the indicated times and the corresponding average separation efficiency. The development over time looks quite parallel for the two measurands, except for the samples taken at $t = 2{:}00$. For these, the separation efficiency is very low with a relatively small standard deviation (18.2% with a relative standard deviation (RSD) of 38.2%, which is low compared to the RSDs of separation efficiencies at the other times of assessment), while average UV O.D. was comparatively high (compared to the average UV O.D.s found for the sonicated wet yeast/$H_2O$ tap samples). Plotting separation efficiency over UV O.D. revealed that there was not monotonic relationship between the measurands (not shown).

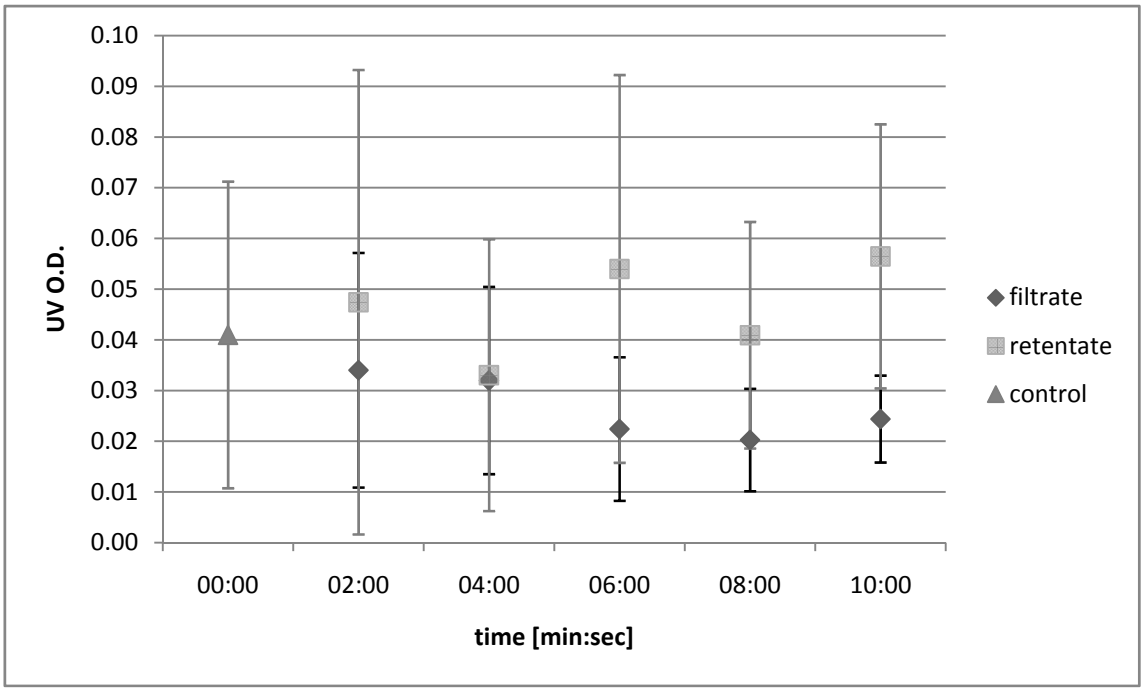


**Figure 17: The change of average filtrate and retentate UV O.D., respectively, of wet yeast suspended in $H_2O$ tap over time. Especially the retentate standard deviation is quite large.**

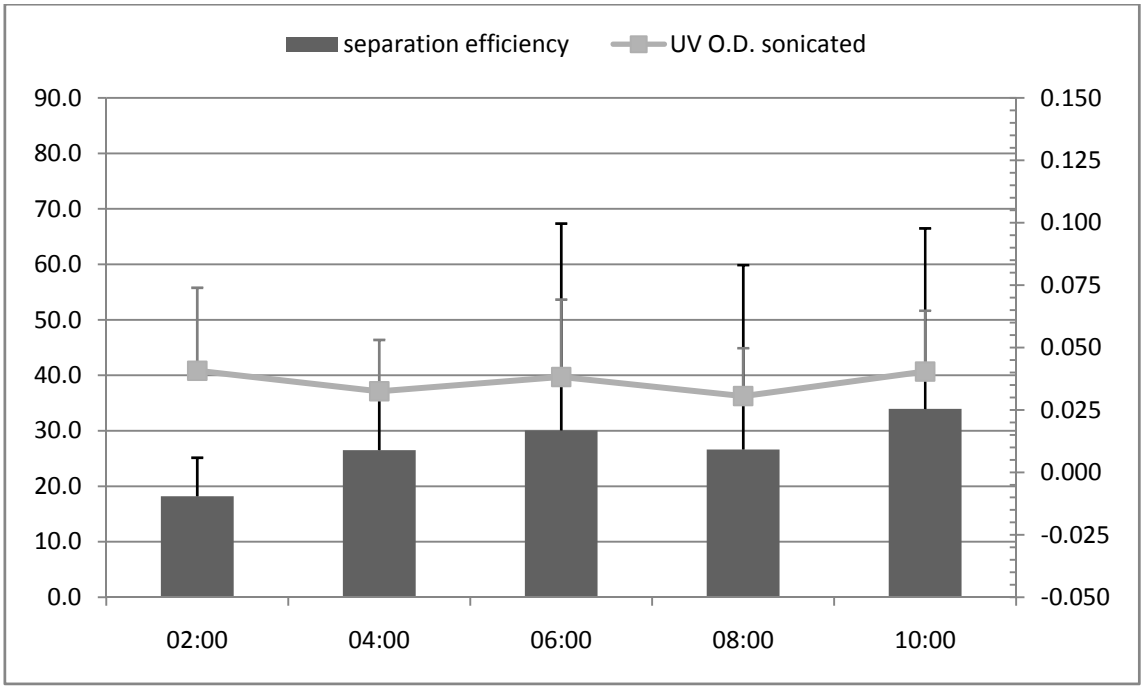


**Figure 18: Average UV O.D.s and separation efficiencies found at the indicated times after the US had been switched on for wet yeast/$H_2O$ suspensions**

*Discussion*

As UV O.D. is a measure for protein in the supernatant of the samples, a rise in UV O.D. after sonication would correspond to cell leakage induced by the separation process. The

reason for such a leakage could be the large pressure changes that the yeast cells undergo when moving from one pressure nodal plane to another [8].

In the h-shape filter, however, only PBS-suspensions show a significant increase in UV O.D. after sonication compared to control and sham-exposed groups. Even though the difference of average UV O.D. before and after sonication is very small, especially in comparison to data reported by Radel [8] in a batch set-up that showed turbulent behaviour in a lager sonication chamber for longer periods of time, this increase can be interpreted as a result of cell leakage. The fact that the PBS 2x- and $H_2O$ tap-suspensions don't show an increase in UV O.D. after sonication could have physiological reasons: a possible explanation for the absence of cell leakage could be that the yeast cells are subject to stress due to the osmolarity of $H_2O$ tap and PBS 2x, respectively. This stress could lead to a strengthening of the cells and subsequently fewer cells would be ruptured by the additional stress induced by the acoustic radiation forces (and the associated pressure differences in the internodal space).

As mentioned before, all the UV O.D. values detected in run 2 of the wet yeast/$H_2O$ tap-suspensions were lower than those found in the other runs (also those of control and sham samples). The cell viability was lower than that of the other runs of $H_2O$-tap suspensions as well; again, at all times and control and sham samples, respectively, too. Maybe the cells used for the suspension were more robust than the ones used in the others, thus, instead of breaking up when non-viable, their cell membrane remained intact. This would explain both the low UV O.D. values and the low cell viability.

### 3.2. Suspensions of cultured yeast sonicated in Malt Extract Broth

The second set of experiments was conducted with cultured yeast suspended in the Malt Extract Broth it had been cultured in. Findings of separation efficiency, cell viability and cell growth, respectively, following sonication will be given. UV O.D. measurements were conducted for all the experimental runs, the data however, is of no use as Malt Extract Broth contains such a lot of protein that the photo-spectrometer is basically blind.

In these experiments, only one big (10-15mL) sample was taken from retentate and filtrate outlet, respectively, due to the larger amount of suspension needed to monitor growth after sonication. In contrast to the results given before, where samples were taken every two minutes after US had been switched on, thus giving a development of the respective measurands over time, the results given in this section can be considered as time-averages.

One experimental run did not show stable alignment during sonication (run 2), but the cells were turbulently driven through the resonator. In another run (run 4), turbulent behaviour

could be stopped by switching the US off and on again. The averages given in the following chapters will always be taken over the runs that displayed a stable alignment unless indicated otherwise.

For experimental run 4 through run 7 additional cell counts were performed 18 hours after the US had been switched on for growth assessment. A comparison between the data found right after sonication and the data found 18 hours post-sonication will be given in the respective chapters.

### 3.2.1. Separation efficiency

Rather high separation efficiencies were found for cultured yeast suspended in Malt Extract Broth. The average separation efficiency found was 87.3+/-5.8%.

Table 5 shows the separation efficiencies found in the single experimental runs. As mentioned before during run 2 a stable alignment was not observed. The cells were driven through the resonator. Turning the US off and on again and changing the driving frequency did not lead to an alignment of the cells. The turbulent behaviour lead to a separation efficiency of -63.7%, i.e. the set-up was not working as a filter. The cell concentration found in the retentate sample was $3.55*10^7$cells/mL and in the filtrate samples it was $4.18*10^7$cells/mL. Run 4 showed turbulent behaviour in the beginning, but a stable alignment could be obtained by switching the US off and on again. Samples were taken shortly after the stable alignment was visible and about 4 minutes later. Thus, there are two values given for run 4, one for stable alignment and one for turbulent behaviour, respectively. Even though separation efficiency increased from 0.2% to 79.4% for this run, it remains the lowest value found for non-turbulent runs.

Table 5: Separation efficiencies found in the respective runs for cultured yeast suspended in PBS.

| separation efficiency [%] | run 1 | run 2 | run 3 | run 4 | run 5 | run 6 | run 7 |
|---|---|---|---|---|---|---|---|
| stable alignment | 80.9 | | 88.2 | 79.4 | 93.1 | 92.1 | 89.7 |
| turbulent | | -63.7 | | 0.2 | | | |

*Discussion*

The separation efficiencies found for cultured yeast sonicated in Malt Extract Broth are high compared to those found for wet yeast suspended in PBS, PBS 2x and $H_2O$ tap, respectively (see chapter 3.1.1). The parameters of the ultrasonic field, i.e. frequency and energy density, were not changed. There are three possible reasons for this:

1. The acoustic contrast factor of Malt Extract Broth and cultured yeast is higher than that of the other media and wet yeast.
2. The size distribution of the cultured yeast cells shifts towards larger cells compared to that of wet yeast, which would increase the primary acoustic radiation force.
3. Not being in stationary phase as long as wet yeast from the supermarket could give rise to different acoustic properties of cultured yeast.

Of course the higher separation efficiency could also be due to a combination of any of these possible factors.

As a possible field of application for the ultrasonic h-shaped filter is in harvesting cells from perfusion-bioreactors, the separation efficiency of yeast cells from Malt Extract Broth is more important than that of yeast cells from PBS, $H_2O$ tap and PBS 2x, respectively. However, separation efficiencies around 90% are rather low for terrestrial commercial applications, as separation efficiencies of around 99% can be reached by systems that employ Ultrasonically Enhanced Settling [8].

As one would expect, the separation efficiency was greatly reduced when cells were not stably aligned in the pressure nodal planes of the ultrasonic standing wave field. The Stokes' (drag) force then plays a major role in the separation of the cells into the two outlets. As the volume flux through both outlets was set to be the same, the suspension would be divided equally between the two outlets. However, the diameter of the filtrate outlet is only half that of the retentate outlet, so the flow velocity through the filtrate outlet is two times bigger than that through the retentate outlet. The higher cell concentration would be expected in the filtrate outlet, as the drag force is a function of velocity. This is reflected by the fact that in run 2, in which stable alignment could not be established, the cell concentration found in the filtrate outlet is higher than that found in the retentate outlet.

### 3.2.2. Cell viability (m.b.)

The cell viabilities found right after sonication in the different runs are shown in Table 6. Average control cell viability was high at 98.5+/-0.7% and was not significantly changed by sham exposure (97.7+/-2.7%). Cell viability remained high for the retentate samples, averaging to 97.9+/-2.4%. For the filtrate samples the average cell viability was found to be 93.6+/-5.5% which is lower than the average cell viability of control and sham, however, insignificantly. An outlier was detected using Grubb's test with a level of significance of 99% (control run 1); it was omitted in further calculations and is indicated by crossing out in Table 6.

When stable alignment of the cells was not possible, cell viabilities of the sonicated cells, i.e. those found in the filtrate and retentate samples, decreased drastically. The average cell viability found for the "turbulent" retentate samples is significantly lower than that for the retentate samples collected when stable alignment was present (one sided t-test, significance level of 99%). The same is true for the "turbulent" filtrate samples; again average cell viability is significantly lower than that found for filtrate samples of runs with a stable alignment of the cells.

Table 6: Cell viabilities found immediately after the US had been switched for the different runs in the respective samples. According to Grubb's test for outliers, the cell viability found for the control sample in run 1 is an outlier at a significance level of 99%.

| cell viability [%] | run 1 | run 2 | run 3 | run 4 | run 5 | run 6 | run 7 |
|---|---|---|---|---|---|---|---|
| control | ~~88.5~~ | 98.0 | 98.3 | 99.4 | 99.4 | 97.8 | 98.2 |
| sham | 94.8 | 98.4 | 100.0 | 100.0 | 100.0 | 97.6 | 93.3 |
| retentate | 93.6 | | 99.3 | 99.6 | 99.6 | 98.4 | 96.9 |
| filtrate | 86.7 | | 88.2 | 94.6 | 93.1 | 100.0 | 99.1 |
| ret turbulent | | 65.5 | | 85.8 | | | |
| fil turbulent | | 67.7 | | 81.8 | | | |

Figure 19 shows the average cell viabilities found for these four runs immediately and 18 hours after sonication are shown. The first thing one notices is that the average cell viabilities for all the respective groups are slightly higher 18 hours post-sonication than immediately post-sonication. The differences are not significant according to Student's t-test, however. When comparing the cell viabilities of the corresponding samples immediately after sonication and 18 hours after sonication one finds that only for the retentate samples' cell viabilities were (slightly) higher in the later count for all the runs. The cell viabilities found in the filtrate samples basically didn't change during the 17 hours after sonication for run 4 and run 6, while for run 5 cell viability increased from 93.1% to 100.0% and in run 7 it decreased from 99.1% to 95.5%. For the control and sham samples the development is similar to that found for filtrate samples: The changes are slight and are both positive and negative. Furthermore, the standard deviations of sham, retentate and filtrate samples, respectively, are lower 18 hours after sonication than immediately after sonication. The average cell viability 18 hours after sonication of retentate samples is significantly higher than that of control and sham samples counted 18 hours after sonication. For filtrate samples the average cell viability is not significantly different from that of control groups at both times.

For the cells that were driven through the resonator in run 4, cell viability decreased from 85.8% to 81.2% for the retentate sample and from 81.8% to 70.8% for the filtrate sample, respectively.

Table 7: Cell viabilities found 18 hours after the US had been switched on, i.e. after sonication, for the different runs in the respective samples.

| cell viability [%] | run 4 | run 5 | run 6 | run 7 |
|---|---|---|---|---|
| control | - | 98.0 | 99.4 | 99.4 |
| sham | 100.0 | 99.0 | 98.9 | 98.9 |
| retentate | 100.0 | 100.0 | 99.4 | 99.4 |
| filtrate | 94.2 | 100.0 | 100.0 | 95.5 |
| ret turbulent | 81.2 | | | |
| fil turbulent | 70.8 | | | |

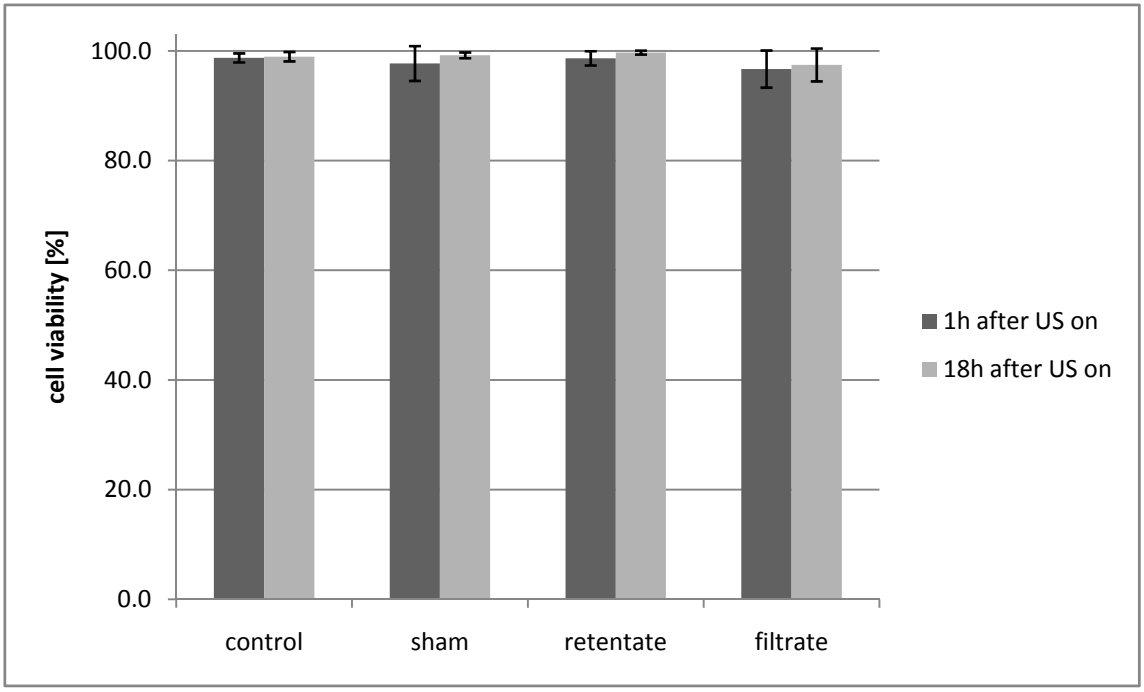


**Figure 19: The average cell viabilities found for run 4 through run 7 (cultured yeast sonicated in Malt Extract Broth). The dark grey bar shows the cell viabilities found immediately after the US had been switched on while the light grey bar shows the averages found 18 hours after sonication.**

Looking at cell viability and separation efficiency of all seven experimental runs, including the runs that did not show stable alignment, one finds rather good correlation between the two measurands. The turbulence which led to low separation efficiency and cell viability, respectively, makes a linear dependence of the two values likely. When omitting these values however, the dependence of the two measurands is clearly not monotonic anymore.

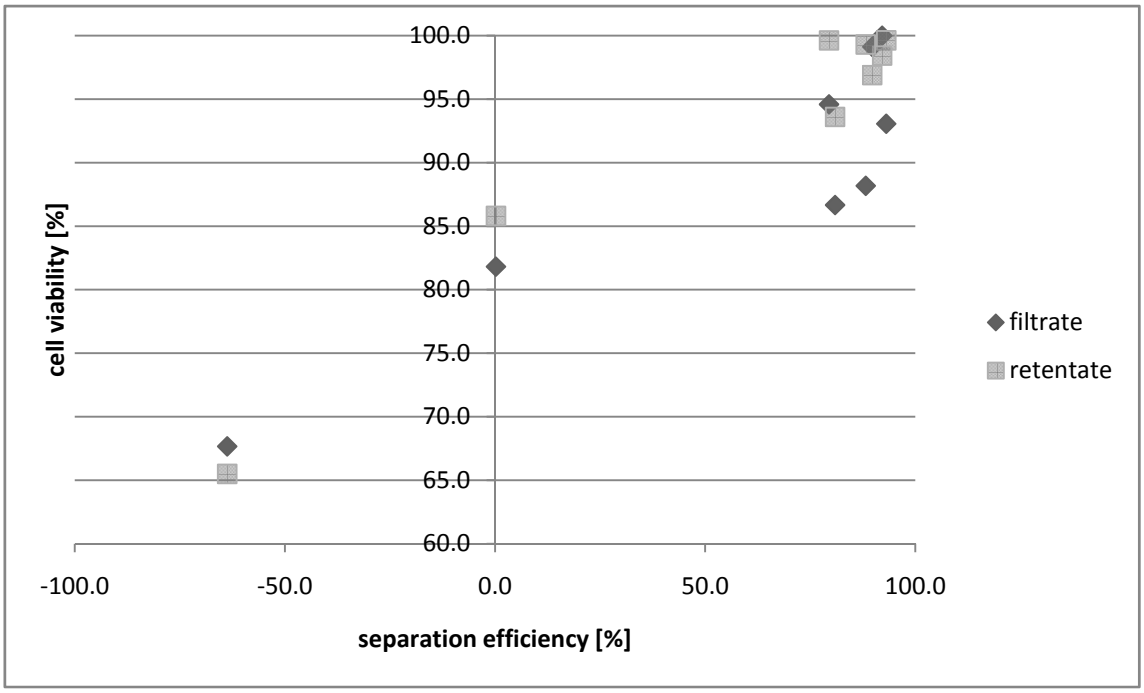


**Figure 20: Cell viability of filtrate and retentate samples plotted vs. the respective separation efficiency found for cultured yeast suspended in Malt Extract Broth.**

*Discussion*

Depending on which data one takes into account two different results were found: When only analysing the runs that were counted immediately and 18 hours post-sonication, i.e. run 4 through 7, cell viability assessed immediately after sonication remains unaltered by sonication. No significant difference in cell viability between control groups and sonicated groups was detected. This is also true when looking at all the runs that showed stable alignment at least temporarily (run 1 and run 3 through run 7).

The most interesting phenomenon observed were the runs that showed turbulent behaviour. The reason for the turbulence is not clear, but a similar behaviour was reported by Radel et al. when yeast cells were suspended in water rich ethanol mixtures [42]. There, a stable alignment of the cells was not possible due to the addition of 12% $^{v}/_{v}$ of ethanol to the water the cells were suspended in. This caused the cells to be turbulently driven through the resonator. As ethanol was not deliberately added in the experiments conducted here, it is possible that the surface of the culture medium in contact with air was not large enough to ensure sufficient oxygen supply of the yeast cells[3]. This would be an explanation why ethanol could have been produced during cultivation. In another work Radel [8] showed that stable

[3] Thus the cells were anaerobically fermenting the Maltose contained in Malt Extract Broth to ethanol and carbon dioxide.

alignment was present as long as the ethanol concentration in $H_2O$ stayed below 8% ($^{v}/_{v}$) and in saline (0.9% NaCl) below 15% ($^{v}/_{v}$), respectively. As the natural limit of ethanol production of *Saccharomyces cerevisiae* is around 12% ($^{v}/_{v}$) ethanol, it is possible that the threshold of 8% ($^{v}/_{v}$) was reached. The only thing that remains without explanation is why only two out of seven runs showed turbulent behaviour if indeed the lack of oxygen supply was the reason for the turbulences. Possibly the cotton plugs used to close the Erlenmeyer during cultivation were too dense in the runs that showed turbulent behaviour, hence inhibited the oxygen delivery.

Also, cell viability was greatly reduced in the samples collected from the outlets when stable alignment was not present. Again, this is in accordance with the results found by Radel [8, 32] and it is another indication that yeast cells that leave the protective area of the pressure nodal planes are subject to damage. In this case it is clear that the reduced cell viability is a consequence of the cells' being turbulently driven through the ultrasonic resonator.

Cell viability did not change significantly when comparing counts made immediately after sonication and 18 hours after sonication. It could even be the case that the fraction of viable cells becomes larger, as non-viable cells might degrade over time (that would explain the mild increase of average cell viability when comparing the counts immediately and 18 hours after sonication). An indication for this is the fact that average retentate cell viability 18 hours post-sonication is significantly higher than that of control groups counted 18 hours after sonication. The hypothesis made in the Introduction, i.e. that cell viability would be reduced by sonication, is thus could not be confirmed when taking into account the development over time.

The correlation between cell viability and separation efficiency is very high for this experiment, especially for the retentate cell viability and separation efficiency. Due to the fact that cell viability decreased when the cells were driven through the resonator and the separation efficiency was consequently greatly reduced as well, a great range of values could be considered. As this is represents a very special case of the interaction between ultrasonic plane wave field and the yeast cells, it shouldn't be taken into consideration when calculating the correlation of separation efficiency and yeast cell viability. When omitting these data, the relationship becomes non-monotonic.

### 3.2.3. Growth stimulus

When comparing the cell concentration of the respective outlets immediately after sonication and 18 hours after sonication for the runs that showed stable alignment, an increase

of averaged concentrations 18 post-sonication can be seen (see Figure 21). The difference between the averages is significant for the retentate samples, for which an average of $6.35*10^7$+/-$6.56*10^6$cells/mL was counted immediately after sonication and $7.88*10^7$+/-$3.4*10^6$cells/mL 18 hours after sonication, respectively. That means that in the second count the cell concentration had increased by an average of 24% compared to the first count. This is only the second largest increase of cell concentration, as the number of cell/mL in the filtrate outlet increased by an average of 100%. Due to the very small total number of cells found in all the filtrate samples and its great variance, this increase is, however, not significant. For the control and the sham groups, cell concentration increased by 13% and 6% on average, respectively. Again, these increases are not significant according to Student's t-test.

The cell concentrations found for the samples that were turbulently driven through the resonator in run 4 were as follows: $3.88*10^7$cells/mL and $3.45*10^7$ cells/mL for the retentate immediately and 18 hours after sonication, respectively, and $3.58*10^7$cells/mL and $4.20*10^7$cells/mL for the filtrate immediately and 18 hours after sonication, respectively. As only one of the runs in which the cells were driven through the resonator was counted 18 hours after sonication, no reliable statistics can be given for these.

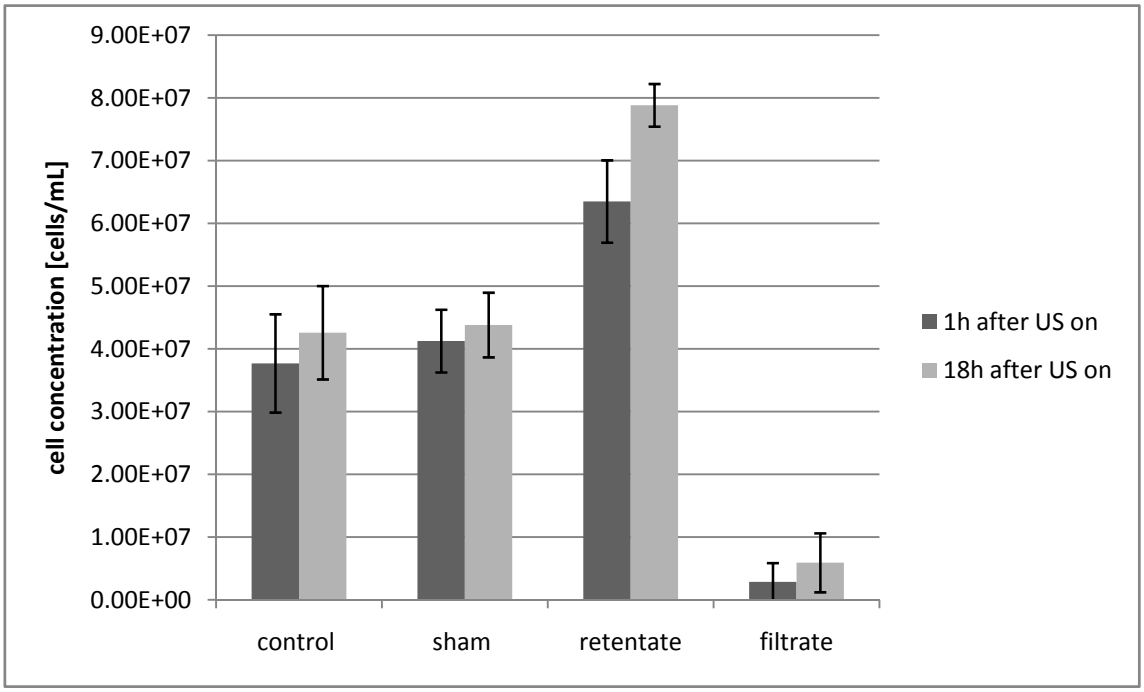


**Figure 21: Average cell concentration found in the respective samples immediately post-sonication (dark grey bars) and 18 hours post-sonication (light grey bars) of cultured yeast suspended in Malt Extract Broth. The data found when the cells were driven through the resonator were omitted. The error bars indicate the standard deviation.**

*Discussion*

The fact that the cell concentration in the retentate sample had increased by an average of 20% during the 17 hours between the respective counts, indicates that the cells are still able to reproduce after sonication. It seems that they even do so more productively than the un-sonicated cells of control and sham groups. Furthermore, average cell viability of the retentate sample 18 hours after sonication is significantly higher than that of control groups determined at the same time. This could be an indication that the non-viable cells lysed within the 17 hours between the counts. They could thus provide nutrition for the viable cells which the control and sham groups lack.

Also, the stress induced on the cells that undergo filtration by sonication could stimulate the cells to grow. However, as the cultured yeast had gone into stationary phase before sonication, it is very unlikely that there would be enough nutrition for the cells to replicate or too many end products that would limit cell reproduction.

An increase of microbial productivity by ultrasonic irradiation has been reported before for *Saccharomyces cerevisiae* and other organisms [43, 44].

As only one run that showed turbulent behaviour was counted immediately and 18 hours after sonication, no reliable conclusions can be drawn for the long term effects of the turbulence on cell viability.

# 4. Conclusions

The ultrasonic h-shape filter was found to be suitable for the separation of cultured yeast cells from Malt Extract Broth. An average separation efficiency of 87.3+/-5.8% was found. For the filtration of wet yeast suspended in PBS, PBS 2x and $H_2O$ tap, respectively, the acoustic and flow parameters need further optimization. With settings used in this report, separation efficiencies were found to be rather low and unstable over time.

Cell viability was not affected by sonication for wet yeast in the respective host liquids and for cultured yeast in Malt Extract Broth, respectively. The hypothesis that the cells found in the filtrate outlet would show damage, i.e. lower cell viability, because they would have to cross regions with high pressure amplitudes to reach this outlet could not be verified experimentally. The filtrate cell viabilities found in all the experiments were not significantly different from the control and sham cell viabilities. Furthermore, retentate and filtrate cell viabilities were found to be the same, thus cell viability specific separation as reported for mammalian cells by Gaida et al. [45] could not be verified. Cell viability remained constant for long periods after sonication for cultured yeast sonicated in Malt Extract Broth, thus it can be concluded that the ultrasonic filtration process has no negative long-term effects on cell viability. The negative effect of the turbulence which was observed in two runs of cultured yeast suspended in Malt Extract Broth on cell viability were apparent, however, long term effects were not investigated.

Rupturing of cells, corresponding to an increase in UV O.D., was not detected for PBS 2x- and $H_2O$ tap suspensions. However, a significant increase in UV O.D. was found for the cells sonicated in PBS. As the increase was very small, one can conclude that only a relatively small number of cells were ruptured during sonication. It seems the stress induced on the cells by being suspended in a hypo- or hyperosmotic medium, respectively, i.e. $H_2O$ tap and PBS 2x, lead to a strengthening and thus fewer cells were ruptured when sonicated in these media.

For the cultured yeast sonicated in Malt Extract Broth a significant increase in cell concentration was found in the retentate samples when comparing the counts immediately after sonication and 18 hours after sonication. The cell concentration had increased by an average of 24% during the 17 hours. For control, sham and filtrate groups, respectively, the increase was smaller and not significant. It seems that the ultrasonic irradiation stimulates cells growth either by breaking up dead cells, hence providing nutrition, or by inducing physiological stress on the cells that triggers cell growth.

# 5. Outlook

The h-shape filter proved to be a good tool for the separation of cultured yeast from Malt Extract Broth, however, at the used settings, separation of wet yeast from PBS; PBS 2x and $H_2O$ tap was not very satisfactory. This could surely be improved by optimising the acoustic parameters and the ratio of flow through the two outlets. Also, it would be interesting to further investigate the reasons for the difference in separation efficiency of cultured yeast and wet yeast.

The reason for the turbulent behaviour which was observed on two occasions for cultured yeast suspended in Malt Extract Broth would definitely be worth further investigation. The alcohol content of the solution could be measured before starting sonication, to if was indeed that reason for turbulence. Also, the long term effect of being turbulently driven through the sound field would be worth further research.

Further examination of the growth stimulus induced by ultrasonic irradiation should be performed to assess the effect on the cells found in the filtrate.

**Acknowledgements**

Prof.Dr. Ewald Benes and Dr. Stefan Radel shall be especially thanked for their supervision and guidance. They were always supportive, ready to answer questions and for discussions. Both have encouraged me and my work and have given me the opportunity to participate in international conferences to present it. Dr. Stefan Radel shall be especially thanked for the vivid discussions, the proof-reading and his knowledge about and inspiring fascination with the interaction of ultrasound and cells.

My colleagues at the Sensors and Ultrasonics group, who were always helpful, ready for discussions and great to work with, are thankfully acknowledged. Special thanks to Thomas Zika for his artful soldering.

My parents Elisabeth and Meinhard who have always supported me in every possible way from close by and far away are most thankfully acknowledged.

Thomas Hübner shall be thanked for his patience and his help with CAD. The biggest Thank you goes to him for this loving support – without you, this work wouldn't have come into being!

## References


[1] A. Kundt and O. Lehmann, *Longitudinal vibrations and acoustic figures in cylindrical columns of liquids.* Annalen der Physik und Chemie (Poggendorff's Annalen), 1874. **153**: p. 1-12.

[2] R.E. Apfel, *Acoustic radiation pressure - principles and application to separation science*, in *Fortschritte der Akustik - DAGA '90*. 1990, DPG-GmbH: Bad Honnef, Germany. p. 19-36.

[3] C.J. Schram, *Manipulation of particles in an acoustic field*, in *Advances in Sonochemistry*, T.J. Mason, Editor. 1991, JAI Press Ltd.: London. p. 293-322.

[4] T.L. Tolt and D.L. Feke, *Separation of dispersed phases from liquids in acoustically driven chambers.* Chem. Eng. Sci., 1993. **48**(3): p. 527-540.

[5] Z. Mandralis, W. Bolek, W. Burger, E. Benes, and D.L. Feke, *Enhanced synchronized ultrasonic and flow-field fractionation of suspensions.* Ultrasonics, 1994. **32**(2): p. 113-121.

[6] W.T. Coakley, G. Whitworth, M.A. Grundy, R.K. Gould, and R. Allman, *Ultrasonic manipulation of particles and cells.* Bioseparation, 1994. **4**: p. 73-83.

[7] M. Gröschl, W. Burger, B. Handl, O. Doblhoff-Dier, T. Gaida, and C. Schmatz, *Ultrasonic separation of suspended particles - Part III: Application in biotechnology.* Acustica - acta acustica, 1998. **84**(5): p. 815-822.

[8] S. Radel, *Ultrasonically Enhanced Settling: The effects of ultrasonic plane wave fields on suspensions of the yeast Saccharomyces cerevisiae*, 2002, Vienna University of Technology.

[9] F. Trampler, S.A. Sonderhoff, P.W.S. Pui, D.G. Kilburn, and J.M. Piret, *Acoustic cell filter for high density perfusion culture of hybridoma cells.* Bio/Technology, 1994. **12**(3): p. 281-284.

[10] R.A. Barnes, P. Jenkins, and W.T. Coakley, *Preliminary clinical evaluation of meningococcal disease and bacterial meningitis by ultrasonic enhancement.* Arch Disease Child, 1998. **78**: p. 58.

[11] S.J. Gray, M.A. Sobanski, M. Guiver, W.J. Marsh, R. Borrow, E.B. Kaczmarski, R.A. Barnes, and W.T. Coakley, *PCR-based diagnosis of meningococcal infection can be complemented by ultrasound enhanced latex immuno-assay.* J Clin Microbiol, 1999. **37**: p. 1797-1801.

[12] M. Wiklund, J. Toivonen, M. Tirri, P. Hänninen, and H.M. Hertz, *Ultrasonic enrichment of microspheres for ultrasensitive biomedical analysis in confocal laser-scanning fluorescence detection.* J Appl Phys, 2004. **96**: p. 1242.

[13] M. Wiklund, S. Nilsson, and H. Hertz, *Ultrasonic trapping in capillaries for trace-amount biomedical analysis.* Journal of Applied Physics, 2001. **90**: p. 421.

[14] T. Gaida, O. Doblhoff-Dier, H. Katinger, M. Gröschl, and E. Benes, *Scale-up of ultrasonic resonance field cell separation devices used in animal cell technology*, in *Animal Cell Technology: Developments towards the 21st Century*, E.C. Beuvery, J.B. Griffiths, and W.P. Zeijlemaker, Editors. 1995, Kluwer: Dordrecht. p. 699-703.

[15] F. Petersson, A. Nilsson, C. Holm, H. Jönsson, and T. Laurell, *Separation of lipids from blood utilizing ultrasonic standing waves in microfluidic channels.* The Analyst, 2004. **129**: p. 938-943.

[16] T. Lilliehorn, M. Nilsson, U. Simu, S. Johansson, M. Almqvist, J. Nilsson, and T. Laurell, *Dynamic arraying of microbeads for bioassays in microfluidic channels.* Sensors and Actuators B, 2004(in press).

[17] J. Hultström, O. Manneberg, K. Dopf, H. Hertz, H. Brismar, and M. Wiklund, *Proliferation and viability of adherent cells manipulated by standing-wave ultrasound in a microfluidic chip.* Ultrasound in Medicine & Biology, 2007. **33**(1): p. 145-151.

[18] O. Doblhoff-Dier, T. Gaida, H. Katinger, W. Burger, M. Gröschl, and E. Benes, *A novel ultrasonic resonance field device for the retention of animal cells.* Biotechnology Progress, 1994. **10**(4): p. 428-432.

[19] D.G. Kilburn, D.J. Clarke, W.T. Coakley, and D.W. Bardsley, *Enhanced sedimentation of mammalian cells following acoustic aggregation.* Biotechnol. Bioeng., 1989. **34**(4): p. 559-562.

[20] T. Bjorling, U. Dudel, and C. Fenge, *Evaluation of a cell separator in large scale perfusion culture*, in *Animal Cell Technology: Developments towards the 20th Century*, E.C. Beuvery, J.B. Griffiths, and W.P. Zeijlemaker, Editors. 1995, Kluwer Academic Publishers Inc.: Dordrecht, NL. p. 671-675.

[21] O. Doblhoff-Dier, T. Gaida, W. Burger, F. Trampler, M. Gröschl, E. Benes, J.M. Piret, and S.M. Woodside, *Review of the viability of mammalian cells in ultrasonic standing wave fields.* Acustica - acta acustica, 1999. **85**(Suppl. 1): p. S399-400.

[22] S. Radel, A.J. McLoughlin, L. Gherardini, O. Doblhoff-Dier, and E. Benes, *Viability of yeast cells in well controlled propagating and standing ultrasonic plane waves.* Ultrasonics, 2000. **38**(1-8): p. 633-637.

[23] H. Böhm, P. Anthony, M.R. Davey, L.G. Briarty, J.B. Power, K.C. Lowe, E. Benes, and M. Gröschl, *Viability of plant cell suspensions exposed to homogeneous ultrasonic fields of different energy density and wave type.* Ultrasonics, 2000. **38**(1-8): p. 629-632.

[24] S. Khanna, B. Hudson, C. Pepper, N. Amso, and W.T. Coakley, *Fluorescein isothiocynate-dextran uptake by chinese hamster ovary cells in a 1.5 mhz ultrasonic standing wave in the presence of contrast agent.* Ultrasound in Medicine & Biology, 2006. **32**(2): p. 289-295.

[25] M. Postema and O.H. Gilja, *Ultrasound-Directed Drug Delivery.* Current Pharmaceutical Biotechnology, 2007.

[26] H. Bohm, L. Briarty, K. Lowe, J. Power, E. Benes, and M. Davey, *Quantification of a novel h-shaped ultrasonic resonator for separation of biomaterials under terrestrial gravity and microgravity conditions.* Biotechnology and Bioengineering, 2003. **82**(1).

[27] H. Böhm, L.G. Briarty, K.C. Lowe, J.B. Power, E. Benes, and M.R. Davey, *Quantification of a novel h-shaped ultrasonic resonator for separation of biomaterials under terrestrial gravity and microgravity conditions.* Biotechnol. Bioeng., 2003. **82**(1): p. 74-85.

[28] M. Gröschl, *Ultrasonic separation of suspended particles - Part I: Fundamentals.* Acustica - acta acustica, 1998. **84**(3): p. 432-447.

[29] L.V. King, *On the acoustic radiation pressure on spheres.* Proc. R. Soc. London, 1934. **A147**: p. 212-240.

[30] K. Yosioka and Y. Kawasima, *Acoustic radiation pressure on a compressible sphere.* Acustica, 1955. **5**: p. 167-173.

[31] S. Radel, A.J. McLoughlin, L. Gherardini, O. Doblhoff-Dier, and E. Benes, *Breakdown of immobilisation/separation and morphology changes of yeast suspended in water-rich ethanol mixtures exposed to ultrasonic plane standing waves.* Bioseparation, 2000. **9**(6): p. 369-377.

[32] S. Radel, A.J. McLoughlin, and E. Benes, *Viability of yeast cells in propagating ultrasonic waves (A).* J. Acoust. Soc. Am., 1999. **105**(2): p. 1076.

[33] P. Zöfel, *Statistik in der Praxis*. 2 ed. 1988, Stuttgart: Fischer

[34] A. Jorgensen and A. Hansen, *Mikroorganismen in der Gärungsindustrie*. 1956, Nürnberg. p. 64,163

[35] H. Mach, D.B. Volkin, C.J. Burke, and C.R. Middaugh, *Ultraviolet Absorption Spectroscopy*, in *Protein Stability and Folding - Theory and Practice*, B.A. Shirley, Editor. 1995, Humana Press Inc.: Totowa, NJ. p. 91-114.

[36] A.B. Coppens, *Simple equations for the speed of sound in Neptunian waters.* Journal of the Acoustic Society of America, 1981. **69**(3): p. 862-863.

[37] J.J. Hawkes and W.T. Coakley, *A continuous flow ultrasonic cell-filtering method.* Enzyme and Microbial Technology, 1996. **19**: p. 57-62.

[38] M.A.H. Weiser and R.E. Apfel, *Extension of acoustic levitation to include the study of micron sized particles in a more compressible host liquid.* J. Acoust. Soc. Am., 1982. **71**: p. 1261-1268.

[39] I.W. Dawes, *Osmotic stress (hypo- and hyperosmotic stress) and salt stress*, in *The metabolism and molecular physiology and Saccharomyces cerevisiae*, J.R. Dickinson and M. Schweizer, Editors. 2004, CRC Press. p. 402-405.

[40] G.J. Morris, L. Winters, G.E. Coulson, and K.J. Clarke, *Effect of Osmotic Stress on the Ultrastructure and Viability of the Yeast Saccharmomyces cerevisiae.* Journal of General Microbiology, 1986. **132**: p. 2023-2034.

[41] L. Stelios, W. Graeme, and N.E. T., *Effect of salt hyperosmotic stress on yeast cell viability.* Proc Nat Sci, Matica Srpska Novi Sad, 2007(113): p. 271-284.

[42] S. Radel, L. Gherardini, A.J. McLoughlin, O. Doblhoff-Dier, and E. Benes, *Breakdown of immobilisation/separation and morphology changes of yeast suspended in water-rich ethanol mixtures exposed to ultrasonic plane standing waves*, in *Bioseparation*. 2000. p. 369-377.

[43] Y. Chisti, *Sonobioreactors: using ultrasound for enhanced microbial productivity.* Trends in Biotechnology, 2003.

[44] K. Matsuura, M. Hirotsune, Y. Nunokawa, M. Satho, and K. Honda, *Acceleration of cell growth and ester formation by ultrasonic wave irradiation.* 1994. **77**(1): p. 36-44.

[45] T. Gaida, O. Doblhoff-Dier, K. Strutzenberger, H. Katinger, W. Burger, M. Gröschl, B. Handl, and E. Benes, *Selective retention of viable cells in ultrasonic resonance field devices.* Biotechnology Progress, 1996. **12**(1): p. 73-76.

## Curriculum Vitae

Cosima Koch

Date of birth: 07.09.1982

### Education

| | |
|---|---|
| 2000 – present | Vienna University of Technology, curriculum: technical physics |
| 8/2008 | Summer School "Electronics for Physiologists" held by the Medical University of Vienna, Institute for Pharmacology |
| 09/2005 – 05/2006 | TASSEP (TransAtlantic Science Students Exchange Programme) scholarship : exchange year at Université de Montréal, Canada |
| 10/2004 – 07/2005 | ERASMUS scholarship : exchange year at University of Leipzig, Germany |
| 09/1998 – 06/2000 | Secondary school in Gänserndorf, Austria<br>Matura (school leaving exam): 06/2000 at Bundesgymnasium Gänserndorf |
| 01/1998 – 06/1998 | Burley High School, Burley ID, USA<br>Academic Year in America |
| 10/1992 – 12/1997 | Secondary school in Gänserndorf, Austria |