Report on the evaluation of the Oculyze yeast cell-counting system

The Start-up Oculyze has developed a system for the determination of yeast cell concentration. It consists of an App and a smartphone microscope with an automated image recognition system. The Oculyze-App is used to record microscopic images which are forwarded to an image recognition server. The corresponding software enables an automated determination of the cell concentration and the viability (live/dead ratio) of the respective yeast population. For the determination of the viability the yeast cells have to be stained with methylene blue previously. Both parameters (cell concentration and viability) can be measured simultaneously. The user receives the result within seconds on the app.

The VLB-Berlin was commissioned as an external and independent institute for a system validation to demonstrate the correct functionality of the Oculyze counting system. The following analysis were performed:

- Determination of cell concentration and viability of different brewer's yeast strains using the following methods:
  - Oculyze System
  - Manual evaluation with the Thoma chamber at a microscope by staff with appropriate expertise
  - Another automated system, that is designed for the determination of the cell concentration and the live/dead ratio of brewer's yeast → NucleoCounter YC-100
- Analysis of at least three different brewer's yeast strains; each in three different batches
- Analysis of all yeast strains and batches in watery solution and wort. Measurements within wort where done to evaluate the influence of turbid particles on the analysis of cell concentration.
- Each batch of the yeast populations was analysed with the respective systems three times.

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Three different bottom fermenting yeast strains, coded as ug1, ug2 and ug3, were used. They were cultured in YEPD medium. This medium has the following composition:

- 1% yeast extract
- 1% peptone
- 5% glucose

For each yeast strain two different main cultures were prepared from a 24 h pre-culture (static culture at 26 °C). Therefore 100 ml of YEPD medium was inoculated with 1.2 ml of the pre-culture in a 250 ml Erlenmeyer flask. The batches were incubated at 26 °C under continuous shaking at 80 rpm. To allow for distinct values for the viability measurement, the yeast cultures were exposed to different conditions in order to reduce viability. The analysed yeast populations were treated as followed:

1. Batch: Main culture 2 d, 26 °C, continuous shaking at 80 rpm
2. Batch: Main culture 9 d, 26 °C, continuous shaking at 80 rpm
3. Batch: One half of the second batch was incubated for another 24 h at 37°C as static culture. A part of the yeast suspension of ug2 and ug3 was killed after the incubation at 37 °C via heat treatment and mixed with the respective remaining other half of the (living) yeast suspension.

The cells were washed with tap water before the measurements. A volume of 45 ml of yeast suspension was centrifuged for 5 min. at 1800 g. The pellet was resuspended in tap water and centrifuged again. The resuspended pellet was then used for the analysis. Furthermore, the yeast cells were also analysed in wort. For this purpose a 11.5 °P wort from the VLB-Berlin pilot brewery was inoculated with the washed yeast cells. The concentration should be at $2x10^7$ cells/ml. To achieve this concentration, the inoculation volume was calculated by the formula [1]. The used cell concentration c_1 was the result of the Thoma chamber counting.

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[1] $c_1 \times V_1 = c_2 \times V_2$

c_1: concentration of the yeast cells in watery solution
V_1: volume to be calculated (addition to the wort)
c_2: desired volume in the wort solution
V_2: volume of the wort solution with the desired yeast concentration

The yeast viability indicates the percentage of living cells in a cell population. It is calculated according to formula [2]. Therefore, in addition to the measurements of the total cell concentration, the amount of dead cells has to be determined.

[2] $\text{viability}[\%] = 100 - \left(\frac{\text{amount or concentration of dead cells} * 100}{\text{amount or concentration of total cells}}\right)$

amount or concentration of total cells: living + dead cells

The methods used for the measurements are briefly described below.

NucleoCounter:

This method is based on the incorporation of the fluorescent dye propidium iodide into the double stranded DNA in the nucleus of the cells. The NucleoCounter system determines the amount of fluorescent cells via a fluorescence microscope. For the measurement of the total cell concentration, the yeast cells have to be treated with a lysis buffer. This step makes the cell wall permeable for the dye. This buffer was also used to dilute the yeast suspension, in order to reach the linear measuring range of the system. The analysis is carried out by a so-called NucleoCassette, which acts as a pipette and already contains the immobilized fluorescent dye. Using the fluorescence microscope within the device, fluorescent cells can be identified.

For the determination of the viability, it is necessary to determine the amount or the concentration of only dead cells in the yeast population. For this purpose a second measurement has to be carried out. The yeast population is not treated with the lysis buffer, so that only dead cells of the yeast population are identified by the system. If the amount of dead cells is very high, a dilution using tap water has to be performed previously. The amount of dead cells of the batches 1 and 2 were determined without

any dilution. The cell suspension of ug2 and ug3 in batch 3 were diluted 1:10 in tap water. The viability was calculated after both measurements according to formula [2]. The determination of the total cell concentration and the viability of a yeast population via a NucleoCounter require two measurements and therefore two NucleoCassettes. Used Cassettes can't be reused.

Thoma chamber:

The Thoma chamber or haemocytometer is a counting chamber, which is used to determine the number of cells by direct counting under the light microscope. The Thoma chamber is a glass plate (comparable to a thicker microscope slide) with four longitudinal grooves cut into the middle, so that three bars are formed in the middle. The bars on the outside are slightly higher than the middle one. On the middle bar is an etched grid of vertical lines. This grid consists of 16 large squares. Each large square is divided into 16 small squares. All squares have a defined area and depth. Due to the specific placement of a cover slip over the three bars, a cavity with a defined volume is created between the cover glass and the middle bar. By filling this cavity with a yeast suspension, the yeast cells can be counted under the microscope in the respective squares. Based on the number of counted squares, the cell concentration can be determined by a specific formula.

To determine the viability of a yeast population, the yeast cells are treated with methylene blue. The sample is mixed in a ratio of 1:2 with the methylene blue solution. This dye penetrates into the cells. Living cells can neutralize the dye and appear colourless under the light microscope. Dead cells appear blue because they cannot neutralize the dye. By counting the total cells and the blue (dead) cells, the viability of the yeast population can be calculated according to formula [2].

The data generated with the Thoma chamber during the validation is based on cell counts in 100 small squares. The calculation of the cell concentration was performed according to formula [3].

$$[3] \quad \text{cell concentration}[\text{cells/ml}] = \frac{\text{amount of counted cells}}{100} * 4 * 10^6 * \text{DF}$$

DF: dilution factor

The methylene blue treated cells can be used directly for the determination of the cell concentration and viability. In order to have a countable range for the cell counting, the yeast cells in the watery solution were diluted 1:5 with tap water. Subsequent treatment with methylene blue resulted in an additional dilution of 1:2. The resulting final dilution factor for counting was therefore 1:10. The yeast cells in wort were mixed directly with the methylene blue solution. The dilution factor was therefore 1:2.

Oculyze system:

The principle of the Oculyze system has already been described at the beginning of this report. Basically it automates the Thoma chamber method. Microscopic images of the yeast cells are captured and evaluated automatically for the cell concentration. Cells stained with methylene blue can also be differentiated by the used image recognition. This enables an automated determination of the amount of dead cells and therefore the viability calculation of a yeast population. Both values (total count and viability) can be determined simultaneously.

The respective samples, which were prepared for the use in the Thoma chamber, were also used for counting with the Oculyze system.

The results of the measurements with the different method are shown in Table 1 (measurements of the cells in water) and Table 2 (measurements of the cells in wort). The respective mean values and standard deviations are represented graphically in Fig. 1.

Table 1: Cell concentration and viability in three different yeast strain populations in different batches. Measurements were carried out using three different yeast cell counting systems (yeast cells in water).

Batches*	NucleoCounter		Thoma-Chamber		Oculyze	
	Cell Concentration [cells/ml]	Viability [%]	Cell Concentration [cells/ml]	Viability [%]	Cell Concentration [cells/ml]	Viability [%]
ug1						
1	1,15E+08	98,46	1,46E+08	99,45	1,36E+08	98,95
	1,22E+08	98,58	1,30E+08	96,62	1,70E+08	99,58
	1,18E+08	98,59	1,27E+08	97,17	1,77E+08	99,60
2	1,45E+08	98,25	2,12E+08	99,43	1,87E+08	99,64
	1,57E+08	98,34	2,19E+08	98,36	1,91E+08	99,25
	1,61E+08	98,33	2,07E+08	98,65	2,19E+08	100,00
3	1,57E+08	98,93	1,98E+08	98,99	2,00E+08	99,29
	1,57E+08	98,82	2,00E+08	98,40	2,19E+08	99,57
	1,51E+08	98,73	1,77E+08	98,42	1,54E+08	99,53
ug2						
1	1,77E+08	99,33	1,66E+08	99,04	2,31E+08	99,07
	1,83E+08	99,37	1,88E+08	97,66	2,01E+08	100,00
	1,83E+08	99,41	1,73E+08	99,54	2,14E+08	99,33
2	1,82E+08	99,38	2,11E+08	98,86	2,39E+08	99,70
	1,69E+08	99,31	2,24E+08	99,64	2,14E+08	99,33
	1,77E+08	99,40	2,51E+08	100,00	2,22E+08	99,68
3	1,84E+08	80,75	2,73E+08	64,10	2,54E+08	70,14
	1,89E+08	80,84	2,40E+08	59,50	2,35E+08	71,12
	1,86E+08	78,12	2,34E+08	60,68	2,53E+08	70,34
ug3						
1	1,42E+08	96,36	1,56E+08	97,20	1,85E+08	99,61
	1,50E+08	96,50	1,86E+08	96,34	1,75E+08	95,51
	1,48E+08	96,42	1,84E+08	97,39	1,81E+08	98,03
2	1,43E+08	97,08	2,11E+08	97,91	1,97E+08	98,55
	1,46E+08	97,02	2,00E+08	98,20	2,04E+08	97,89
	1,38E+08	96,92	2,12E+08	96,79	2,06E+08	100,00
3	1,45E+08	74,30	2,22E+08	59,64	1,99E+08	68,10
	1,39E+08	75,05	2,17E+08	62,58	1,97E+08	68,84
	1,49E+08	74,28	2,05E+08	60,59	2,00E+08	71,79

*Batches:

1: yeast main culture: Incubation: 2 d, 26 °C, aerobic, 80 rpm

2: yeast main culture: Incubation: 9 d, 26 °C, aerobic, 80 rpm

3: yeast main culture from batch 2: further incubation 1 d aerobic at 37 °C (static culture); for the yeast strains ug2 and ug3 a part of the yeast suspension was additionally killed by heat treatment and mixed with the remaining (live) cells.

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Table 2: Cell concentration and viability in three different yeast strain populations in different batches. Measurements were carried out using three different yeast cell counting systems (yeast cells in wort).

Batches*	NucleoCounter		Thoma-Chamber		Oculyze	
	Cell concentration [cells/ml]	Viability [%]	Cell concentration [cells/ml]	Viability [%]	Cell concentration [cells/ml]	Viability [%]
ug1						
1	1,95E+07	98,63	2,54E+07	96,85	2,21E+07	99,43
	1,90E+07	98,45	2,73E+07	98,83	2,47E+07	98,84
	1,96E+07	98,44	2,23E+07	99,28	2,27E+07	99,37
2	1,23E+07	98,55	2,38E+07	97,98	2,17E+07	96,71
	1,51E+07	98,81	2,15E+07	98,88	2,30E+07	98,76
	1,67E+07	98,72	2,48E+07	98,84	2,05E+07	100,00
3	1,54E+07	99,04	2,02E+07	99,21	2,14E+07	98,67
	1,60E+07	99,10	2,50E+07	99,36	2,24E+07	100,00
	1,56E+07	98,91	2,18E+07	97,06	2,34E+07	98,17
ug2						
1	1,86E+07	98,89	2,52E+07	99,05	2,33E+07	94,50
	2,05E+07	99,12	2,44E+07	99,77	2,21E+07	98,71
	1,86E+07	98,83	2,39E+07	99,00	2,56E+07	98,32
2	1,39E+07	99,11	2,63E+07	99,39	2,40E+07	98,21
	1,86E+07	99,22	2,49E+07	99,36	2,90E+07	98,56
	1,57E+07	99,00	2,49E+07	99,36	2,56E+07	98,88
3	1,48E+07	86,72	1,24E+07	61,29	2,14E+07	62,00
	1,44E+07	86,37	2,55E+07	63,29	2,39E+07	71,86
	1,46E+07	81,33	2,22E+07	68,29	2,23E+07	71,15
ug3						
1	1,64E+07	96,06	2,39E+07	99,33	1,83E+07	99,22
	1,63E+07	96,22	2,38E+07	96,97	2,30E+07	96,35
	1,73E+07	95,96	2,61E+07	97,85	1,96E+07	96,89
2	1,57E+07	97,43	2,36E+07	98,64	2,27E+07	97,48
	1,48E+07	96,82	2,53E+07	96,21	2,27E+07	94,97
	1,49E+07	97,29	2,46E+07	95,77	2,24E+07	92,36
3	1,27E+07	89,27	2,10E+07	70,95	2,20E+07	64,29
	1,29E+07	91,63	1,85E+07	68,00	2,14E+07	66,00
	1,32E+07	89,38	1,80E+07	66,22	1,76E+07	72,36

*Batches:
1: yeast main culture: Incubation: 2 d, 26 °C, aerobic, 80 rpm
2: yeast main culture: Incubation: 9 d, 26 °C, aerobic, 80 rpm
3: yeast main culture from batch 2: further incubation 1 d aerobic at 37 °C (static culture); for the yeast strains ug2 and ug3 a part of the yeast suspension was additionally killed by heat treatment and mixed with the remaining (live) cells.

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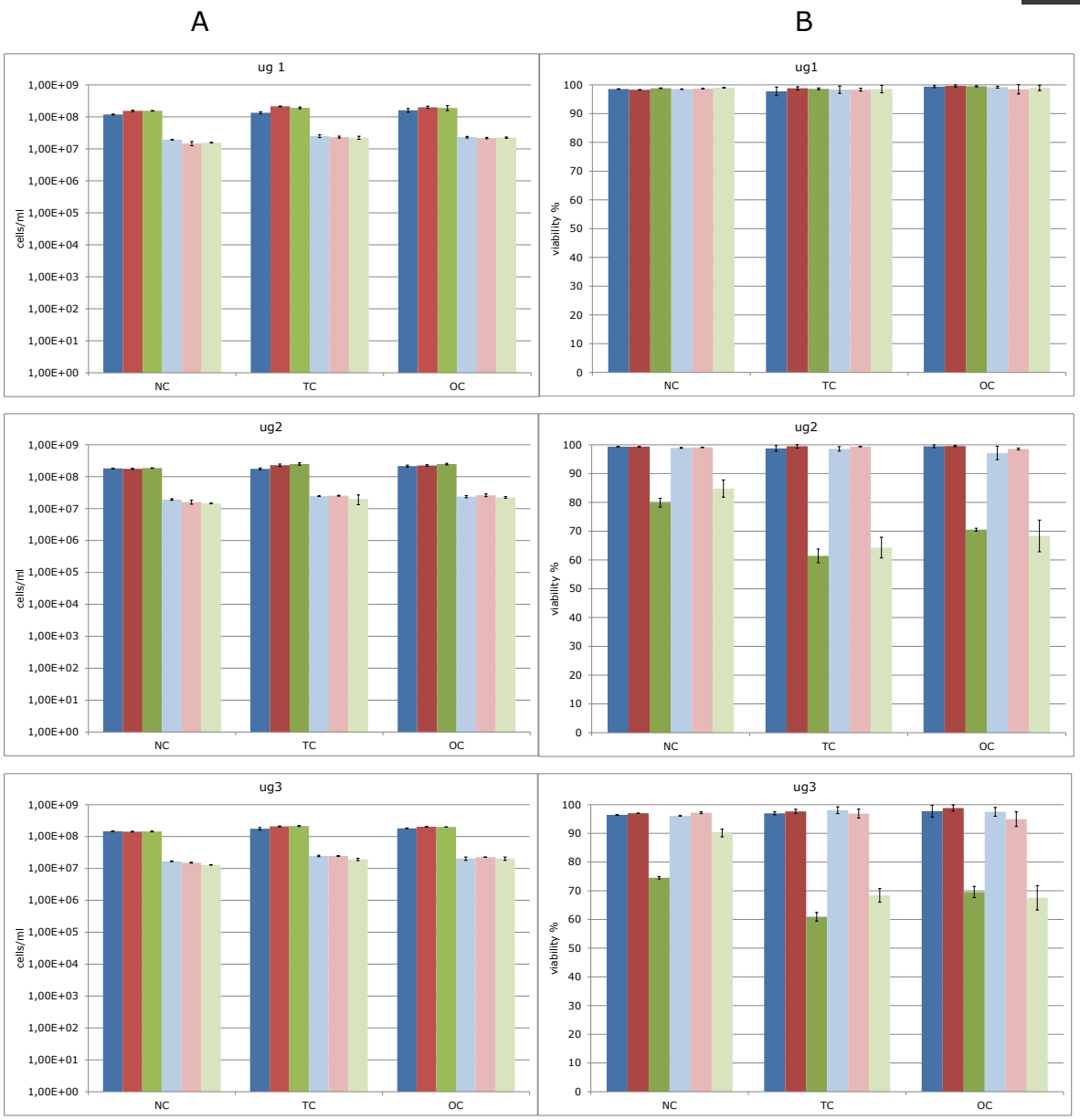

Figure 1: Graphical representation of the mean values and standard deviations of the cell concentration (vertical line A) and viability (vertical line B); three different yeast strain populations (ug1, ug2, ug3) in different batches. The measurements were carried out using different measuring systems
NC: NucleoCounter; TC: Thoma chamber; OC: Oculyze system
The mean values and standard deviations were calculated with the respective values of Table 1 and 2

Batch1 in water Batch2 in water Batch3 in water
Batch1 in wort Batch2 in wort Batch3 in wort

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Concerning the determination of the cell concentration, the results gained with the Oculyze system are comparable to the counts and calculations of the Thoma chamber method. The results gained from the NucleoCounter were partly lower compared to the other two systems. Concerning the viability, the different measurement methods barely showed any differences for high viabilities. The standard deviation is minor.
In the case of yeast cultures with low viability, as shown for the yeast strains ug2 and ug3 in batch 3, differences in the different measurement methods were noticeable. The results from the Thoma chamber and the Ocuylze system, where methylene blue was used for the staining of dead cells, are closer to each other, compared to the NucleoCounter system. Furthermore it can be seen that for the methylene blue method the standard deviation of the viability measurement increases with the decrease of the viability (i.e. the proportion of the dead (blue) cells increases). This may be due to the different intensity of the blue staining when a high ratio of dead cells is present.

Due to the different measuring method of the NucleoCounter, the results may differ partially compared to those of the Thoma chamber method and Oculyze system. Due to the direct counting with the Thoma chamber or Oculyze system every single cell (also connected cells) can considered for counting.

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Conclusion:

The results of the Oculyze system are closely comparable with those obtained using the microscopic Thoma chamber method, as both measurements are based on the same principle. However, using the Oculyze system does not require laboratory equipment such as a microscope and does not require trained staff for the microscopic examination. Using the Thoma chamber everything has to be done manually (counting the cells and calculation of the results), while the Oculyze system automates the counting and calculation of the results. With the Oculyze system the cell concentration and viability can be analysed within one fast measurement. The measurements of the Oculyze system or the Thoma chamber are not influenced by particles within the wort.

With the NucleoCounter, two independent measurements are required to obtain cell concentration and viability results. This also requires two separate NucleoCounter-cassettes, which are not reusable. The Oculyze chamber can be reused several times after careful cleaning and drying.

As with all analysis methods, measurements in triplicate are recommended.

Finally we can say, that the Oculyze system offers a very good, fast and cost efficient alternative for the determination of the cell concentration and viability of bottom-fermenting yeast strains without the need of complex laboratory equipment.

We thank the Oculyze team for their trust and the good cooperation during the evaluation project.

If you have any further questions do not hesitate to contact us and we are looking forward to future cooperation’s.

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