Sysmex UK Ltd.

Evaluation of the CyFlow® Cube 6 flow cytometer unit for the assessment of brewing yeast viability and vitality

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Executive summary

Brewers require their yeast strains to be alive and fit enough to complete fermentation. Exhausted, stressed or damaged yeast cells are not able to ferment as effectively as freshly propagated yeast, and cell death and lysis may result in the formation of off-flavours in the final beer. To ensure the yeast used for pitching is viable and shows good vitality, brewers can use several manual methods such as methylene blue live/dead staining and vitality methods, such as the acidification power test. Flow cytometry offers the potential to measure viability and vitality simultaneously, thereby rapidly providing information on the health of a brewer's yeast population during propagation and prior to pitching. This study compared the methylene blue live/dead staining and acidification power test for vitality with a flow cytometry system developed by Sysmex Ltd. – the Cube 6 – to determine the viability and vitality of a brewing yeast strain during storage for 3 weeks under chilled conditions. Both the manual methods and the Cube 6 unit identified a decreasing yeast viability and vitality over time with the flow cytometry method identifying a higher viability than the methylene blue method, but a lower vitality than the acidification power test. The Cube 6 unit was found to be a faster method for measuring both viability and vitality, while removing much of the user-to-user variability seen with the manual methods.

Background

Yeast is one of the most important raw materials for the production of beer. Thus, brewers require their production yeast to be of high quality to be able to manufacture premium beer. Ideally, the yeast culture cells need to be viable and highly active so that fermentation progresses efficiently and reliably. Traditionally, brewers assess the viability of yeast on a routine basis using the industry standard method of haemocytometer + methylene blue staining; however this method has been criticised as being time-consuming and subject to user-to-user variation. Live yeast cells can show differing fermenting activities according to their age and history (environmental stresses); a measure of a cell's physiological condition is referred to as its 'vitality'. There are a number of tests for yeast vitality, one of which is the acidification power test (APT) that measures the cells' plasma membrane proton efflux in the absence/presence of glucose. Although, the results from this test are considered reliable, the method is time-consuming and only provides an indication of the overall health of a yeast population.

Sysmex has developed a compact bench top flow cytometer for analysis of single cells and microscopic particles (CyFlow® Cube 6) under the control of the CyFlow™ software. The client would like to evaluate the usefulness of the Cube 6 unit to determine the viability and vitality of brewing yeast under industry-relevant stress conditions.

Scope of Work

This assessment evaluated whether the Cube 6 unit was able to determine the cell count, viability and vitality of a strain of brewing ale yeast, stored under refrigerated conditions over three weeks. The Sysmex flow cytometry method was compared with standard manual methods for assessing yeast viability (methylene blue staining) and vitality (acidification power test).

Experimental

The assessment was carried out using a single strain of brewing ale yeast (*Saccharomyces cerevisiae*). The yeast was propagated in wort in stages to produce a yeast slurry in 1 L wort

(approximate cell concentration 5×10^8 cells/mL). The slurry was covered by a layer of wort (~100 mL) to prevent drying-out, and stored at 4°C over a 3 week period. Viability and vitality of yeast cells was performed weekly using the Cube 6 flow cytometry unit and a manual method.

Cube 6 flow cytometry

The flow cytometry viability and vitality stains used in this evaluation were manufactured and provided by Sysmex.

To prepare the sample for measurement of viability and vitality, the yeast slurry was serially-diluted in Ringers solution to produce a yeast suspension at a concentration of ~5×10^6 cells/mL. To assess the viability and vitality of the yeast cells, 10 µL of Solution A (stain for live cells) was added to a 1 mL aliquot of yeast suspension. The sample was then mixed and incubated in the dark at room temperature. After 10 minutes, 10 µL of Solution B (stain for dead cells) was added to the yeast suspension and, after briefly mixing, the sample was run immediately on the Cube 6. The Cube 6 unit used a blue laser (488 nm) to excite the stained cells. The software recorded the forward and side scatter as well as green fluorescence from each cell ('live') and red fluorescence ('dead'). A fluorescent signal from both stains was an indication that the cell was 'vital', defined as structurally-damaged, but metabolically-active.

To set the initial gating for the flow cytometry interpretation, a negative control sample was run as described, without the addition of the biological stains. Each sample (~1 ml volume) was run through the Cube 6 unit at a constant flow rate of 0.6 µL per second. To calculate the total cell concentration of all samples, the true volumetric absolute count (TVAC) feature measured the number of particles in the final 200 µL of sample.

Manual methods

Two manual comparative methods were used for the measurement of yeast viability and vitality. For viability, the yeast slurry was serially-diluted in Ringers solution to produce a yeast suspension with a concentration of ~5×10^6 cells/mL. This suspension was mixed with an equal volume of methylene blue reagent and 10 µL pipetted into a haemocytometer chamber (Neubauer); stained/non-stained cells were counted manually using a microscope at 400x magnification. Methylene blue reagent is reduced by metabolising yeast cells to a colourless form, therefore blue-stained cells were an indication of dead (non-metabolising) yeast cells.

The acidification power test is a manual method to determine yeast vitality, which has been in use in the brewing industry for more than 30 years. The method is based on the biochemical process of healthy yeast to metabolise glucose to produce ATP, whilst excreting protons (H^+) across the cell membrane. The effectiveness of the glucose metabolism can be determined indirectly by a measureable decrease in the pH of the surrounding medium after glucose has been added. The method is relatively time-consuming, involving measuring out 9 grams of wet weight yeast, repeated washing in de-ionised water to remove residual traces of wort and accurate measurement of pH at one minute intervals over a 20 minute period.

Results

Viability

The samples were run through the Cube 6 unit and each detected particle was arranged into a scatter-plot, based on the amount of each biological stain was incorporated into the cell (Figure 1). The presence of the live stain indicated intracellular metabolic activity, whereas the presence of the second stain indicated a compromised cell structure. A high presence of both stains was indicative of structurally-damaged cells that were still able to undergo normal metabolism ('vital' cells).

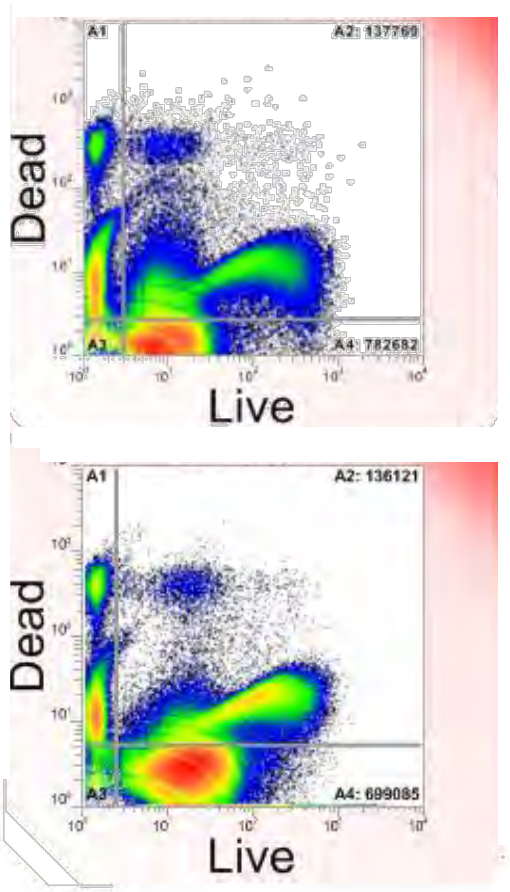

Figure 1: Flow cytometry output from the Cube 6 unit showing Live, Dead and Vital cells for two yeast replicates, analysed after 3 weeks chilled storage. Each point represents a single particle (cell) detected by the unit. Gated quadrants were set manually by the user, based on data from the negative control (unstained cells) and are designated as follows: A1, Dead cells; A2, Vital cells; A3, Unstained cells; A4, Live cells.

When the 'vital' cells were removed from the flow cytometry analysis, both the Cube 6 and manual method indicated that yeast viability decreased over the three weeks cold storage (Figure 2). The yeast were analysed from the same aliquot and the results were very similar. Overall, the Cube 6 analysis indicated higher yeast viability at each week than the manual methylene blue method.

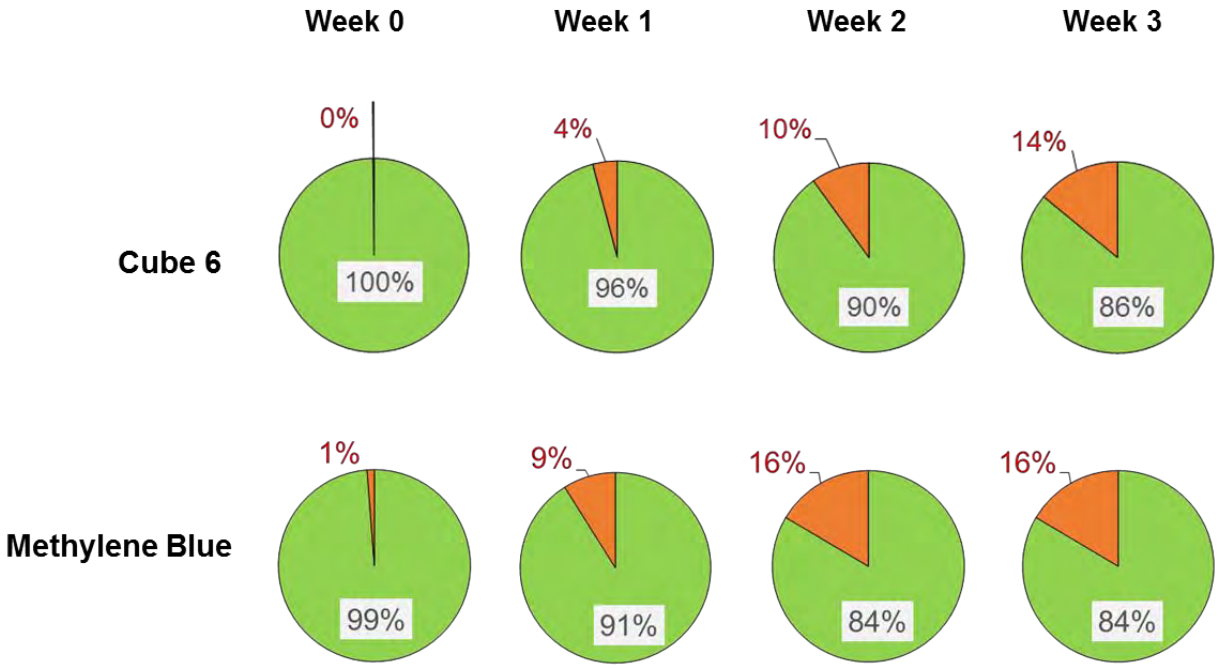

Figure 2: Proportion of live (green) or dead (orange) cells in the yeast population, stored in chilled conditions for 3 weeks using the Cube 6 flow cytometry system and the methylene blue manual counting method. Data represent the average of two independent replicates.

Vitality

As part of the analysis, the Cube 6 unit gives an absolute number of cells, which are stained by both dyes. This fraction of cells are termed 'vital' as they are structurally-compromised but are still metabolically-active. In comparison, the acidification power test produces an overall 'APT score' for a population of yeast, based on changes in extracellular pH. An APT score of 2.7 is considered as an acceptable vitality score for freshly-cropped yeast and a decreasing score indicative of a less vital yeast population. The absolute values from both methods are shown in Tables 1 and 2; however, due to the different outputs it is difficult to compare the two methods directly. The Cube 6 method measures the per-cell vitality, whereas the standard acidification power test gives an overall health of the yeast population. Therefore, when assessing the changing vitality, as measured by the Cube 6, the number of the metabolising cells ('Live' and 'Vital') as a proportion of the total cell number is shown.

As expected, the freshly-cropped yeast showed an excellent vitality by both methods however, during the cold storage, this vitality decreases over time.

Table 1: Yeast vitality of the stored yeast over 3 weeks using the Cube 6 flow cytometry system

	'Live' (cells/mL)	'Vital' (cells/mL)	'Dead' (cells/mL)	Total (cells/mL)	'Vital' + 'Live' (as % of total)
0	4.35×10^{8}	1.83×10^{6}	7.94×10^{5}	4.37×10^{8}	99.8
1	3.89×10^{8}	6.43×10^{7}	1.67×10^{7}	4.70×10^{8}	96.5
2	4.48×10^{8}	8.67×10^{7}	4.94×10^{7}	5.84×10^{8}	91.5
3	2.27×10^{8}	4.19×10^{7}	3.71×10^{7}	3.06×10^{8}	87.9

Table 2: Yeast vitality of the stored yeast over 3 weeks using the acidification power test (APT) method. Data represent the average of two independent replicates. As a guide, an APT score of 2.7 is considered an acceptable value for freshly-cropped yeast.

	APT score	APT vs. week 0 (%)
0	2.68	100
1	2.66	99.4
2	2.63	98.3
3	2.59	96.8

Conclusions

In this study the CyFlow® Cube 6 unit was evaluated for its ability to determine the viability and vitality of a brewing strain of yeast stored under chilled conditions over 3 weeks. The flow cytometry method was compared with widely-used manual methods for the assessment of yeast viability and vitality.

It was expected that the yeast viability and vitality would decrease over time, and this hypothesis was confirmed by both the Cube 6 and manual methods. Viability measurements were very similar, using both methods with the methylene blue method showing lower yeast viability than the Cube 6 unit. However, for the vitality measurement, the manual method (acidification power test; APT) showed a much higher vitality score than obtained by the flow cytometry method. I should be noted that the manual method is based on the overall metabolic activity and health of the yeast population, whereas the flow cytometry method counts the number of individual 'vital' cells. It is possible that the metabolic activity of live and fit cells in a population is sufficient to compensate for less metabolically-active cells, resulting in a high APT score. When interpreting these results, it should be borne in mind that the APT manual method is not a 'gold standard' for measuring yeast vitality, it is one of several existing manual methods used in the brewing industry.

In summary, the Cube 6 unit was able to rapidly analyse a yeast suspension and give absolute cells number for cell count, viability and vitality in around 25 minutes. There was minimal sample preparation time for the Cube 6 method, making the method considerably quicker than the standard manual methods for measuring yeast viability and vitality.

Ease-of-use

Overall the CyFlow® Cube 6 unit was easy to use. The machine is compact, with the unit controlled offline via the pre-loaded CyFlow™ software within the Microsoft Windows 7 operating system. The software was intuitive during the setup stage with regular user prompting of the procedures during start-up, sample priming and shutdown. For the purposes of this assessment, Sysmex provided a detailed user manual for the Cube 6 unit and a Quick Guide, which was sufficient to perform the work outlined here. However, there is a degree of user input and interpretation of the results and a user would benefit from detailed training from Sysmex staff prior to use.

Preparing and running the samples was much faster with the Cube 6 than the manual methods and, by measuring the viability and vitality in one analysis, the Cube 6 unit rapidly provides brewers with data as to the health of their yeast strains. Although there is an initial cost associated with setting up the flow cytometry equipment in a brewing laboratory, the CyFlow® Cube 6 unit does not require additional pieces of equipment such as a microscope or centrifuge that are required for the manual methods. Overall, the method could represent a potential monetary saving to brewers who regularly check the viability and vitality of their yeast strains.