

A Novel Method For Quantitation of Active Yeast Cells

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Abstract

Yeast performance is critical to the development of quality beer. For this reason, methods of yeast analysis are an important element of the brewing process. Traditional methods including hemacytometer counting and methylene blue staining are rapid, but inaccurate and unreliable. Slide culture is an accurate measure of yeast viability, but requires a lengthy incubation period of 18 to 24 hours. As an alternative, we have developed a fluorometric assay, the Easy Count, based on the metabolic activity of the yeast culture to provide brewers with a rapid and accurate estimation of active cell number. This method was compared to the hemacytometer counting technique as an estimation of cell number, and to both methylene blue staining and slide culture as measures of vitality and prediction of fermentation performance. The Easy Count method correlated to the hemacytometer, methylene blue, and slide culture with R^2 values of .985, .987, and .962 respectively, $P < .0001$. An error analysis was carried out on the Easy Count, hemacytometer and methylene blue staining techniques for multiple operators performing the tests. There was less error associated with the Easy Count than with the microscopic methods. The Easy Count was also used to track the activity of a culture during fermentation. These results suggest that this novel method can be used to determine the active number of cells in a commercial brewery. Thus, the Easy Count could be used to determine correct pitching rates, monitor fermentation and propagation, and for other applications involving cell quantitation.

Key Words : yeast, brewing, hemacytometer, fermentation, fluorometry, quantitation

Introduction

Consistent yeast performance during fermentation requires accurate cell counts to ensure correct pitching, monitor growth during fermentation, and assess viability. Traditional methods of cell enumeration and viability determination include hemacytometer counting, methylene blue staining, and slide culture. A high degree of experimental error is associated with both the hemacytometer and methylene blue staining methods (1,2,3,7). The hemacytometer requires cumbersome sample preparation and relies on the skill of the operator for accurate counts. Methylene blue has been reported to be unreliable when viabilities fall below 90% (1,2,3,4). In addition, neither method gives a reliable indication of yeast performance during fermentation (4). Slide culture, while accurate for determining yeast viability, requires an incubation time of 18 to 24 hours, preventing the brewer from obtaining results in a reasonable

time (5). As an alternative, we have developed the Easy Count, a simple, rapid, accurate method for active yeast quantitation. This novel technique, which is based on a culture's metabolic activity, not only provides the brewer with an accurate estimation of cell number, but also an indication of culture health. The Easy Count test should allow brewers to rapidly and accurately determine the total number of active cells in yeast slurry. The new method could be employed to determine pitching ratios in the brewery, and to monitor culture activity during fermentation.

Materials and Methods

All chemicals were reagent grade and obtained from Sigma Chemical Co., St. Louis, MO unless otherwise noted.

Yeast

All yeast cultures were obtained from Wyeast Laboratories, Mt. Hood, OR. Yeast samples for the experiments comparing the hemacytometer, methylene blue staining, and slide culture to the Easy Count method were a 1084 strain of *Saccharomyces cerevisiae*. Yeast cultures tested during laboratory scale fermentations were strain 1968. Brewery scale fermentations were performed using yeast strain 1056.

Hemacytometer Counts

Hemacytometer counts were performed according to the ASBC method (6,8). Samples were removed from a slurry with an initial concentration of 198 million cells per ml, as determined by hemacytometer count, and diluted in spent wort to maintain cell integrity. Each dilution was counted in the hemacytometer as well as measured using the Easy Count method. Experiments were carried out in triplicate.

Methylene Blue Staining

Methylene blue staining was performed according to the ASBC method (6,8). Samples were removed from a slurry with an initial concentration of 198 million cells per ml, as determined by hemacytometer count, and diluted in spent wort to maintain cell integrity. Each dilution was stained and counted in a hemacytometer, as well as measured using the Easy Count method. Hemacytometer counts were corrected for viability according to the staining results. Experiments were carried out in triplicate.

Slide Culture

Slide culture was performed according to a modified version of the protocol for preparation of slide cultures for the examination of yeast and mold (5). Ten ml of yeast strain 1028 at a concentration of 433 million cells per milliliter, as determined by a hemacytometer count, were placed into a 43 degree Celsius water bath. Aliquots were removed at time intervals 0, 2, 4, 6, 8, 10, 15, 20, 30, and 40 minutes. Each was tested using the Easy Count. Measurements were taken in triplicate and averaged. Slide culture samples were diluted 1:100 into wort containing 6% gelatin. 10 μ l of the sample were then placed on a micro slide, covered and sealed with petroleum jelly. Each slide was incubated for 20 hours at 18 degrees Celsius before microscopic examination (Microscope model, Leica DMLB). Viability was determined with the assumption

that living cells had formed micro colonies, while nonviable cells remained single.

Determination of Active Cell Number Using the Easy Count

The Easy Count method for determining total active cell number is based on the metabolic activity of the yeast culture. The technology involves exposing cells to proprietary chemicals that enter cells through diffusion. These molecules are converted to a fluorescent form by metabolically active cells. This fluorescent signal is quantified in a handheld battery operated fluorometer model GP320 GenPrime Inc, Spokane, WA. The protocol is as follows; 50 μ l of yeast sample was added to 500 μ l of cell prep solution in a 1ml glass test cuvette. 50 μ l of dye solution was added; the cuvette was capped, and incubated for 5 minutes. After incubation, the cuvette was shaken, and the fluorescent signal quantitated in the GP320. These values were compared to the hemacytometer and methylene blue staining methods by performing Easy Count tests on the diluted samples from these experiments. Readings were taken in triplicate and averaged. These relationships were analyzed by linear regression using Statview, SAS institute, Cary NC.

Fermentation Tracking

Laboratory Scale: Laboratory scale fermentation tracking was carried out in a 300ml flask by inoculating 150ml wort with 5ml yeast strain 1968, with an initial concentration of 420 million cells per ml, and monitoring growth using a hemacytometer and the Easy Count. Cells were grown at room temperature (21°C). Samples were taken every 45 minutes for 5.25 hours and then periodically over the next 48 hours.

Brewery Scale: Brewery Scale fermentation tracking was carried out during a typical fermentation cycle, at the Steam Plant Grill, Spokane, WA 99201. Hemacytometer counts and corresponding readings using the Easy Count were made daily for 13 days beginning immediately following pitching.

Error

Percent error between operators was determined for the Easy Count method, hemacytometer counts, and the methylene blue staining method. Error analysis was performed using Microsoft Excel.

Hemacytometer: Three operators performed hemacytometer analysis of a yeast strain 1028 slurry according to the ASBC method. Each

operator prepared and measured 15 samples. Results were averaged for each operator, and error between operators was calculated.

Methylene Blue: The 15 hemacytometer samples from above were stained with methylene blue according to the ASBC method. Each of the three operators counted stained cells for each sample. Results were averaged for each operator, and error between operators was calculated.

Easy Count: Easy Count tests were performed on 15 replicate samples by each of the three operators. Results were averaged for each operator, and error between operators was calculated.

Results and Discussion

Hemacytometer Counts

Figure 1 shows the correlation between the Easy Count values and the cells/ml results of the hemacytometer. A statistically linear relationship was found between cell counts obtained by the ASBC standard method of microscopic examination using a hemacytometer and values obtained using the Easy Count, $R^2=0.985$.

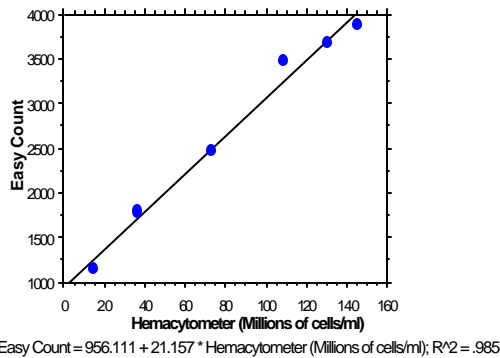


Figure 1. Linear correlation plot of hemacytometer counts vs. Easy Count Readings. Data points are the mean of three samples. The linear relationship is significant $P<.0001$.

Methylene Blue Staining

Figure 2 illustrates the linear correlation found between the Easy Count method and the ASBC method for methylene blue staining. A statistically linear relationship was found between the Easy Count, and hemacytometer counts corrected for viability, $R^2=0.987$

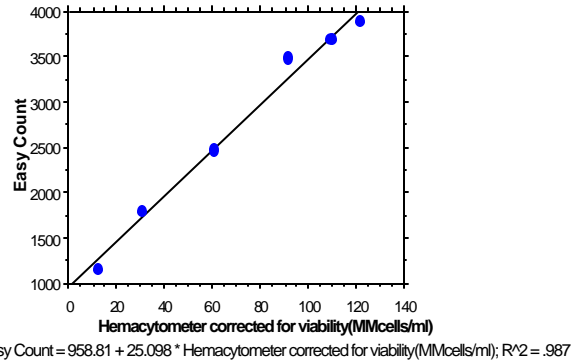


Figure 2. Linear correlation plot of hemacytometer counts that have been corrected for viability using methylene blue stain vs. Easy Count Readings. Data points are the mean of three samples. The linear relationship is significant $P<.0001$.

These results suggest that the Easy Count can be used to accurately predict active cell number. Using the results of the correlation, it is possible for the brewer to accurately determine the correct pitching rate using the Easy Count method based on 1 million active cells per ml per degree plato of wort. Additionally, the method can be used to monitor fermentation, propagation, and for other applications involving the quantitation of cells.

Slide Culture

A linear relationship was found between the Easy Count and slide culture for yeast viability, as shown in Figure 3.

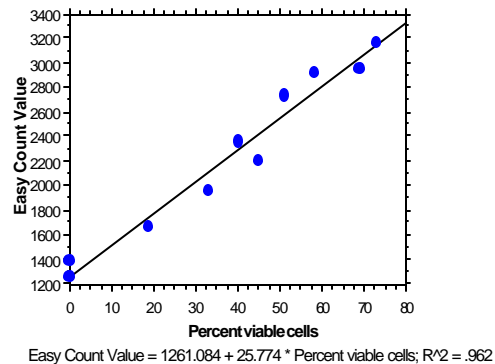


Figure 3. Linear correlation between percent viability as measured by slide culture, and Easy Count values. Data points are the mean of three samples. The linear relationship is significant $P<.0001$

The correlation to slide culture confirms that the Easy Count only measures active cells, since the total number of cells in this experiment remains constant.

Fermentation Tracking

Cell growth was measured during laboratory and brewery scale fermentations using both the ASBC method for hemacytometer counts, and the Easy Count method. Figure 4 shows cell growth tracked by both methods during laboratory scale fermentation. Figure 5 is an example of a brewery scale fermentation tracked by both methods.

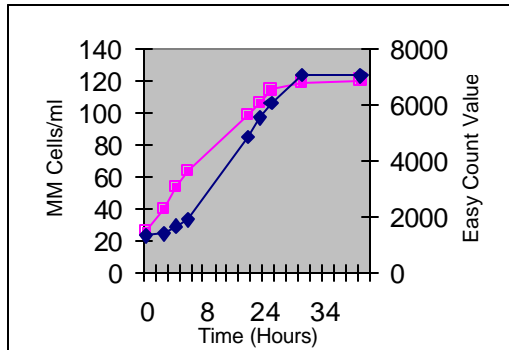


Figure 4. Fermentation tracking in a laboratory fermentation using both the Easy Count and hemacytometer methods. Time 0 is the time that the cells were pitched into fresh wort. The Y-axes represent cell counts using a hemacytometer (squares) and active cells using the Easy Count (diamonds). Data points are the mean of three samples.

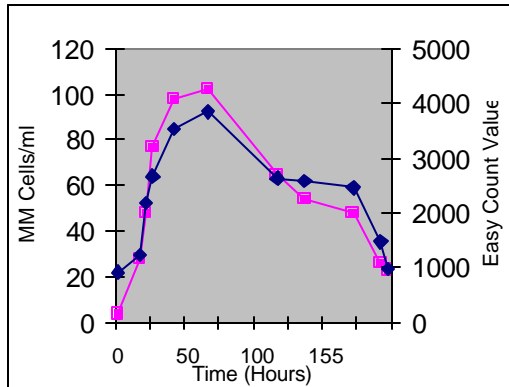


Figure 5. Fermentation tracking during brewery scale fermentation. The Y-axes represent cell counts using a hemacytometer (squares) and the Easy Count (diamonds). Data points are the mean of three samples.

Error

Results from the experiments were averaged for each operator as shown in Table 1. Percent error between operators was calculated by dividing the standard deviation of the mean by the mean, and multiplying the result by 100. Easy Count reported significantly lower error between operators than the other methods. These results are graphed in Figure 6.

Table 1
Percent Error Between Operators

Data in Millions of cells/ml	Easy Count mean	Methylene Blue live mean	Methylene Blue dead mean
Operator 1	195.9	158.6	27.9
Operator 2	197	122.3	23.6
Operator 3	187.7	187.7	40.3
Mean	193.5	156.2	30.6
Std. Dev.	5.1	32.8	8.7
%Error	2.6	21.0	28.3

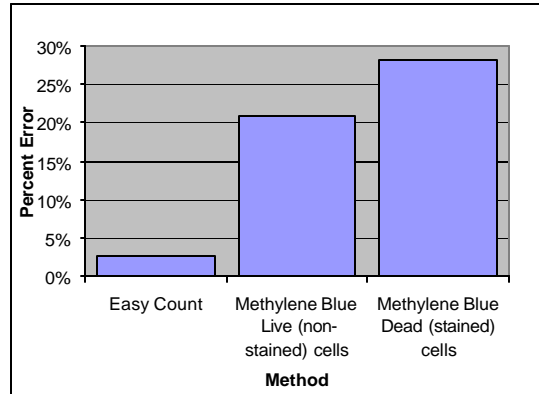


Figure 6. Percent error between operators for the three methods is shown. Methylene Blue dead (stained) cells reported differences between operators of 28.3%, while Methylene Blue live (non-stained) cells was 21.0%. The error between operators for the Easy Count was significantly lower, at only 2.6%.

Results of the error experiments confirm previous research reporting the inaccuracies of hemacytometer counts and methylene blue staining (1,2,3,4,7). The low error associated with the Easy Count method is an improvement on these traditional techniques. Percent error is of particular importance to the brewer due to the exacerbation of inaccuracies in the calculation of cells/ml. For example, when calculating cells/ml from a hemacytometer count of 180 live cells, and 15 dead cells, (counting all 25 fields and using a 1:100 dilution) the result would be 180 million live cells/ml (180*100*10000) and 15 million dead cells/ml (15*100*10000). If the error between operators when performing the live cell test is 21%, then the live cell result could be between 142-218 million cells/ml, a difference of 76 million

cells/ml. With a percent error of 28% between operators, the dead cell result could be between 11-19 million cells/ml. This could result in reported viabilities between 87% and 96% for the same sample. The Easy Count has much less error associated with its performance. A reading of 6000 in the Easy Count would be 197 million active cells/ml (see equation generated in Figure 2.). A percent error of 3% between operators gives a range between 191-203 million cells/ml, a difference of only 12 million cells/ml. The very low error associated with the performance of the Easy Count provides much more reliable information to the brewer.

Conclusion

The Easy Count has been developed to provide a method of analysis for the brewing industry that is rapid, accurate, and easy to use. The test has better precision and accuracy than traditional hemacytometer and methylene blue staining techniques, and can be completed in less than 10 minutes. The novel technology not only correlates significantly to conventional methods of cell enumeration, but improves on these procedures by providing information on the overall health of the culture. The Easy Count technology can be used to monitor growth during fermentation and propagation, to determine correct pitching rates, and for other applications that involve cell quantitation.

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