Epifluorescent Method for Detection of Nonviable Yeast[1]

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ABSTRACT

An epifluorescent method for assessing yeast viability utilizing a protein-staining fluorochrome, the magnesium salt of 1-anilino-8-naphthalene sulfonic acid (Mg-ANS), is described. This method was evaluated against two standard brewery methods for determining yeast viability, specifically the brightfield dye method utilizing methylene blue, and the slide culture method. Results indicate that the Mg-ANS method is simpler, quicker, and as accurate as the slide culture technique and more accurate than the methylene blue method when assessing yeast cultures of low viability. An alternate viability method using acridine orange and nalidixic acid was also attempted, but was less consistent.

Key words: *Fluorescence, Methylene blue, Mg-ANS, Viability, Yeast*

To be able to accurately assess yeast viability is important in order to monitor the relative vigor of pitching yeast and to check yeast after acid or alkaline washing. The ability to rapidly determine the viability of low numbers of wild or culture yeast from the pasteurizer or the packaged product is also desirable. Several methods have been developed over the years for determining yeast viability, including plating, slide culture, and brightfield dye or fluorochrome microchemical cell staining (2,5,8). Most of these methods have disadvantages in their application, especially the classical plate method, which is slow and prone to error (5). Culturing conditions on a solid medium are usually not as ideal as the in situ condition, and often result in an underestimation of yeast viability (12). Although neither is ideal for assessing yeast viability, the slide culture technique and the brightfield dye technique utilizing methylene blue were recommended for use by the European Brewery Convention Yeast Group in 1961 and the Institute of Brewing Analysis Committee in 1970 (5,8). The American Society of Brewing Chemists recommended methylene blue for viability staining in 1976 (1). The slide culture technique assesses viability according to the yeast's ability to bud and form microcolonies on a wort/agar substrate (2,4,5,7,8). While reproducing conditions similar to those in fermenting wort, this method takes up to 18 hr. The methylene blue staining technique is not dependent on cell division to indicate viability, but rather on the permeability of the cell membrane. The membrane of dead or dying cells is permeable to methylene blue, which penetrates and stains the cell (15). Although methylene blue has been the traditional dye used to assess yeast viability in the brewing industry (1), other brightfield dyes also have been successfully employed, such as rhodamine B and eosin (5). Methylene blue provides quick results; however, falsely high viabilities were indicated when this method was used on cultures of low viability (4).

In recent years, yeast viability staining methods have been developed that utilize fluorochromes instead of the traditional brightfield dyes. Many fluorochromes stain viable and nonviable yeast differentially; examples include acridine orange, acriflavine, and primuline O (5). One of the fluorochromes used most frequently for staining viable yeast is fluorescein diacetate (13,14,16).

Fluorochrome cell staining techniques appear to hold promise in improving the accuracy of estimating yeast viability compared with the methods currently recommended for industry. This work, therefore, investigated two new fluorochrome applications for assessing yeast viability. The first procedure, which was originally developed to assess bacterial viability, utilizes acridine orange and nalidixic acid. Theoretically, when incubated in nutrient solution containing nalidixic acid, cell division is blocked without interfering with cell growth. When stained with acridine orange, viable and nonviable cells are differentiated by size and color (9,11). The second method utilizes the protein-staining fluorochrome magnesium salt of 1-anilino-8-naphthalene sulfonic acid (Mg-ANS). Nonviable cells will fluoresce while viable cells will not stain.

EXPERIMENTAL

Epifluorescent Microscope

An American Optical Series 110 Fluorestar microscope with a mercury vapor vertical illuminator was used for all fluorescence work. The fluor cluster used was that designated for use with fluorescein isothiocyanate and acridine orange, which provides excitation in the 500-nm range.

Microscope Staining Methods

Acridine Orange/Nalidixic Acid Staining. Lager yeast was obtained from a production holding tub, and a portion was autoclaved at 121°C for 15 min to render the population nonviable. Both the viable and nonviable yeast slurries were diluted 1:10 in pH 7.2 phosphate buffer. The 0.04% nalidixic acid stock solution was prepared by dissolving nalidixic acid (Sigma Chemical Co.) in 0.05

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NaOH and filtering through a 0.45-μ membrane (9).

To achieve a final nalidixic acid concentration of 0.004%, 0.5 ml of each of the yeast dilutions was mixed in test tubes containing 1 ml of the nalidixic acid stock solution and 7 ml of 0.3% yeast extract, as well as in test tubes containing 1 ml of nalidixic acid stock solution and 7 ml of yeast nitrogen base with 1% glucose. A portion of the cultures was incubated at 20° C and a portion at 28° C for 24 hr before staining with 1 ml of 0.1% acridine orange. The tubes were agitated and, after 3-min staining time, were examined under epifluorescence. Percent viability was calculated by enumerating cells in five fields. Enlarged, greenish-white cells were considered viable and normal-sized reddish orange cells as nonviable.

Results were compared with the calculated percent viability, which was obtained by enumerating the diluted viable and nonviable yeast slurries with a hemacytometer before inoculation. The viability of the viable yeast slurry was determined to be 98.2% by the methylene blue method before mixing, and the calculated percent of viable to nonviable cells in the final dilutions was determined by using that value.

Mg-ANS Staining. Mg-ANS (Nutritional Biochemicals, Cleveland, OH) was dissolved in sterile distilled water at a concentration of 0.3% (10). When stored in a dark bottle at 4° C, the stock solution can be kept for up to six months. Mg-ANS stock solution was mixed with an equal volume of the yeast sample diluted in sterile saline to a concentration of approximately 1×10^7 cells per milliliter, mixed thoroughly, and incubated at ambient temperature for 5 min before microscopic examination at 400 power. Nonviable yeast cells fluoresce green; viable yeast cells do not stain. The ratio of nonviable to viable cells in a microscopic field was determined by first enumerating the fluorescing cells under epifluorescence, then the total field in the brightfield mode. For each test sample, a minimum of 1,000 cells were counted in randomly selected microscope fields.

Methylene Blue Staining. The Fink-Kühles buffered (pH 4.6) methylene blue staining procedure (1) is identical to that of the Mg-ANS method, except that it is performed in the brightfield mode. Cells that stained blue were counted as nonviable while those that remained unstained were counted as viable. At least 1,000 cells were counted in random microscope fields for each test sample.

Slide Culture Method. This method was adapted from the 1979-80 study of the ASBC Subcommittee on Microbiology (2). Brewery wort adjusted to 15.5° P was combined with 1.5 g of agar per 100 ml and autoclaved. The test yeast was suspended in saline to a concentration of approximately 1×10^6 cells per milliliter. A microscope slide was flamed and 1 ml of the molten wort/agar mixture was pipetted onto the surface. After solidification, a drop of yeast suspension was placed at both ends of the slide. The drops were spaced so that a cover slip could be placed lightly on each. The slide was placed in a petri dish, covered, and incubated at 18° C for 17 hr. After incubation, the slide cultures were examined under 400 power. Microcolonies of three or more cells were counted as viable and those of 1–2 cells as nonviable. For each sample, at least 500 microcolonies were counted.

Yeast Viability Measurements from Laboratory Fermentations

Lager culture yeast was inoculated into sterile filtered wort adjusted to 15.5° P at a concentration of 1.5×10^7 cells per milliliter. The culture was incubated on a shaker bath at 18° C for 24 hr and then transferred to a second incubator at 25° C. Samples were withdrawn at intervals, and viability was determined by the Fink-Kühles methylene blue, Mg-ANS, and slide culture methods.

Yeast Viability Measurements from Brewery Samples

Samples of yeast from a pitching tub, at 72 hr of fermentation, at aging fill, and at the end of the aging period were aseptically obtained during a normal brewing operation. Viability was measured using the methylene blue, Mg-ANS, and slide culture methods.

RESULTS AND DISCUSSION

Acridine Orange/Nalidixic Acid Viability Staining

The calculated viability of the mixture of autoclaved/viable cells was calculated to be 33.4%. The actual staining results indicated much higher viabilities, however. For the yeast culture incubated in 0.3% yeast extract, the viability as determined by acridine orange/nalidixic acid staining was 41.0 and 47.0% viability for 20 and 28° C incubation temperatures, respectively. For the culture incubated in yeast nitrogen base plus 1% glucose, some cell replication occurred at the 20° C incubation temperature despite the presence of nalidixic acid, possibly because of the rich nutrient medium coupled with an ideal incubation temperature. Incubation at 28° C resulted in a viability of 66.9%.

Objective viability assessment was difficult because cell enlargement and color were inconsistent. Some cells contained areas of both green and red. These problems may have contributed to the erroneously high viability results that were obtained.

In addition, this procedure was originally developed using procaryotic cells, and several alterations of the method had to be made to use it for yeast viability assessment, such as lengthening the incubation time for cell enlargement from 6 to 24 hr. Results indicated that this procedure did not adapt well to use with eucaryotic cells, because a lengthy incubation period was required and cell staining was inconsistent; therefore, further assessment of this procedure as a yeast viability method was discontinued.

Mg-ANS Viability Staining

Composite data from several yeast fermentations are presented

TABLE I
Percent Yeast Viability as Determined by Mg-ANS,[a] Methylene Blue, and Slide Culture

Slide Culture	Mg-ANS	Methylene Blue
97.7	97.6	96.6
97.5	98.7	97.8
73.2	75.1	86.9
68.3	59.3	81.6
48.1	34.6	55.8
21.3	19.3	63.4
6.1	4.8	29.5

[a] 1-anilino-8-naphthalene sulfonic acid.

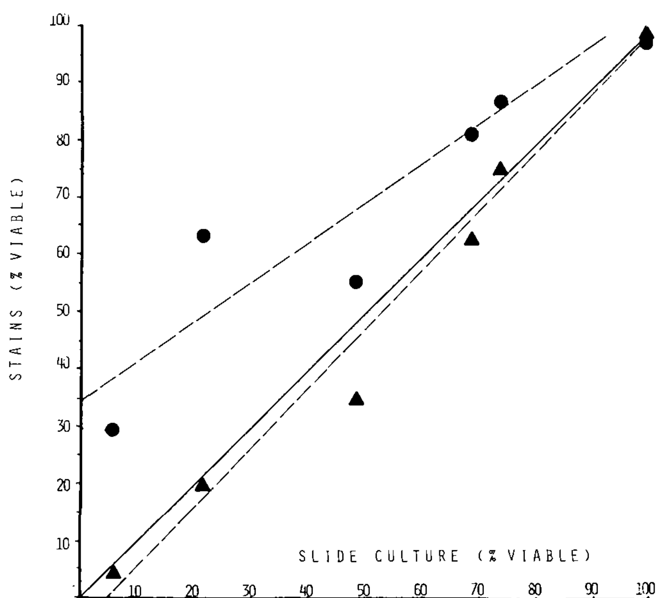

Fig. 1. Viability results of stain methods vs slide culture. ● = methylene blue stain, ▲ = Mg-ANS stain.

in Table I. Since the slide culture results are considered to be the most accurate over a broad viability range, they are the bases of comparison for the methylene blue and Mg-ANS results. At relatively high viability (greater than 90%), both the methylene blue and Mg-ANS results agree closely with the slide culture results. As viability declined, however, the methylene blue results began to diverge, producing higher yeast viabilities than those of the slide cultures, whereas the Mg-ANS results compared favorably with those of the slide cultures even at low percent yeast viabilities.

These data are illustrated in Fig. 1, in which results from the two staining methods were plotted against the slide culture results used as the control. The methylene blue method is quite accurate at higher viabilities, but as viability declines, so does its accuracy. This is illustrated by the slopes (dashed lines) in Fig. 1 for both methylene blue and Mg-ANS. A paired t test was performed with the data from Table I comparing the slide culture against Mg-ANS results, slide culture against methylene blue results, and Mg-ANS against methylene blue results. The data were transformed to be normally distributed using an arc sine transformation. The slide culture and Mg-ANS data were not significantly different at the 95% confidence level, whereas the other two comparisons were significantly different at the 95% confidence level.

The two sets of data that were significantly different, slide culture against methylene blue and Mg-ANS against methylene blue, were analyzed for the 95% confidence interval of the average differences observed. Results showed that the average difference observed could have been 0 to 10% based on the sample size of slide culture against methylene blue, and that 0–13% average difference could have been expected based on the sample size of Mg-ANS against methylene blue.

Although the statistical evaluation is not a definitive indicator, the data show a strong correlation between the standard viability method, slide culture, and Mg-ANS viability values. Conversely, the methylene blue method statistically appears to be dissimilar to both slide culture and Mg-ANS methods. This becomes evident, particularly at low viability values (Fig. 1). These data support previous studies in which the methylene blue method produced inconsistent and falsely high viabilities as yeast culture viability declined (4,5,8,15).

Results from the comparison of the slide culture, methylene blue, and Mg-ANS methods on brewery yeast samples are included in Table II. The culture yeast, yeast from fermenting beer, and yeast from a newly filled aging tank all had high viabilities as indicated by the three methods. Viability measurements varied approximately 2–3% between the three methods. The aging drop yeast viability was 8.5% as determined by slide culture, and the Mg-ANS method gave results in close agreement; however, the methylene blue method again indicated viability to be over twice that found by the slide culture method.

Hypothetical Mechanism of Mg-ANS Staining

The methylene blue stain method reportedly works on the principle of active exclusion from the plasma membrane of a viable yeast cell. Those cells that are weakened or nonviable possess a de-energized membrane that allows entry of the dye into the cell (5). Although this study did not investigate the mechanism of Mg-ANS staining, it may be similar or identical to that of methylene blue.

TABLE II
Percent Yeast Viability of Brewery Yeast Samples

Yeast Sample	Slide Culture	Mg-ANS[a]	Methylene Blue
Pitching yeast	96.1	98.6	98.2
Fermenting beer (72 hr)	96.5	99.4	99.0
Aging fill beer	95.5	97.7	95.6
Aging drop beer	8.5	8.4	19.7

[a] 1-anilino-8-naphthalene sulfonic acid.

The outer cell wall of *Saccharomyces* consists mainly of the complex carbohydrates glucan and mannan (17). Therefore, the fact that the protein stain Mg-ANS will not stain the cell wall is not surprising. If the plasma membrane is de-energized, however, the fluorochrome will likely pass into the cell and stain the protein of the plasma membrane and cytoplasm (6).

Advantages of Fluorescence Microscopy

Fluoroscence microscopy has been frequently employed in recent years to replace more traditional brightfield histochemical techniques. One of the main reasons for the wide acceptance of fluorescence microscopy is its superior sensitivity as compared to brightfield dyes. Usually, 100–1,000 times more dye is required in brightfield systems to yield the same visual qualities as those of fluorochromes (3).

This study found that viability interpretation with Mg-ANS was much less subjective than methylene blue staining when yeast viabilities were below 90%. When viability was below 90%, determination of whether a cell was actually stained with methylene blue was often difficult. With Mg-ANS, little or no question arose as to the cell staining characteristic. An additional advantage of the Mg-ANS stain, as opposed to other fluorochrome stains such as fluorescein diacetate, is that "quenching" or fading of the fluorescence does not occur for several minutes. Thus, the higher interpretative accuracy afforded by the Mg-ANS stain is probably the reason that more consistent yeast viability measurements were obtained with the fluorochrome than with methylene blue for cultures in relatively poor condition.

SUMMARY

The Mg-ANS nonviable yeast stain is recommended as a yeast viability assessment procedure. The Mg-ANS procedure eliminates the need to use two methods of determining yeast viability, combining the best features of two traditional methods while minimizing their disadvantages.

The Mg-ANS method is quick, simple to employ, and accurate over the entire range of yeast viability. The methylene blue stain remains an accurate procedure for assessing viability of cultures over 90% viability and is inexpensive and simple. The Mg-ANS stain, however, provides laboratories with quick, accurate yeast viability results over all ranges of yeast viability.

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