

Selina Uhlendorff, Lisa Marie Dreyer, Lisa Kemper, Juliette Halli, Jan Schneider, Björn Frahm and Miriam Pein-Hackelbusch

Towards standardised yeast viability measurement: A critical evaluation and systematic comparison of staining parameters

Determining yeast cell viability is critical, as yeast cells serve as both model and production organisms. Viability, defined as the proportion of living cells to the total cell count, is essential for process monitoring as well as optimising processes and product quality. Despite their widespread use, dye-based viability assessments lack standardised protocols, and literature reports inconsistencies and debated mechanistic foundations. To address this gap, we systematically evaluated the effects of staining solution pH (3.0, 7.8 and 10.6), dye concentration, and yeast strain (baker's yeast *Saccharomyces cerevisiae*, and brewer's yeasts W-34/70, and WB-06) on the reliability of image-based methylene blue (MB) and trypan blue (TB) viability assays. Highly alkaline conditions (pH 10.6) produced unreliable results, while higher dye concentrations improved discrimination between viable and non-viable cells without shifting mean viability. MB and TB exhibited broadly similar behaviour, indicating that reported MB inaccuracies are likely method-related rather than dye-specific. Across multiple strains, no single parameter set was universally optimal, but pooled analysis identified MB at pH 3.0 as the most robust and reproducible condition. These findings support acidic MB staining at higher concentrations for automated, cross-strain viability assessments and reinforce models in which dye exclusion, rather than intracellular decolourisation, determines viability-dependent contrast.

Descriptors: Automated cell analysis, brewer's yeast, methylene blue, pH dependent staining, viability assay

1 Introduction

In beer production, product quality depends largely on the physiological performance of the yeast, making consistent yeast quality crucial for industrial production [1, 2]. Consequently, the analysis of both pitching and harvested yeast is particularly important, especially during serial repitching [3]. This typically requires assessing two criteria: viability and vitality. Viability is defined as the proportion of living cells in the total cell population, whereas vitality can be defined as a measure of the physiological state of the living cells [4]. A wide variety of methods have been developed to determine yeast viability, as summarized, among others, by Heggart et al. [5] and Thiele [4]. The methods are based on the determination of

metabolic activity, the measurement of cellular components, or the measurement of the glycolytic flux rate or fermentation capacity. Among the numerous methods available, the determination of intracellular pH, the Acidification Power Test and the measurement of fermentation pressure have gained particular significance in both research and practice. The assessment of viability, which is the focus of this study, is commonly performed using staining methods in combination with cell proliferation assays or by plate counting [1], while alternative approaches include flow cytometry [6] and impedance-based methods [7, 8]. Methylene blue (MB) staining has been used for the assessment of yeast cell viability in brewing for decades and remains included in established standard reference methods, such as those published by the European Brewery Convention [9]. Trypan blue (TB), although developed primarily for mammalian cell assays, has occasionally been adapted to yeast viability assessment using the same dye-exclusion principle [10, 11].

With regard to the mode of action of MB in distinguishing between viable and dead cells, there is a widely accepted theory: MB penetrates both viable and dead cells, but is reduced to a colourless leuco form by viable cells, causing them to appear unstained [12–16]. Other research groups, however, hold a differing view. They argue that MB cannot simply penetrate viable cells due to its positive charge and hydrophilicity. It is rather converted into a colourless, uncharged, lipophilic form by transmembrane reductases on the cell surface and only then gets absorbed by the cells [17–19]. Both theories contrast with the third theory that MB can only penetrate

<https://doi.org/10.23763/BrSc26-10uhlendorff>

Authors

Selina Uhlendorff (ORCID ID: 0000-0002-0502-8032), Lisa Marie Dreyer, Lisa Kemper (ORCID ID: 0009-0004-2306-2535), Juliette Halli, Jan Schneider (ORCID ID: 0000-0001-6401-8873), Miriam Pein-Hackelbusch (ORCID ID: 0000-0002-7920-0595), OWL University of Applied Sciences and Arts, Institute for Life Science Technologies ILT.NRW, Campusallee 12, 32657 Lemgo, Germany; Björn Frahm (ORCID ID: 0000-0003-4046-7804), OWL University of Applied Sciences and Arts, Biotechnology and Bioprocess Engineering, Campusallee 12, 32657 Lemgo, Germany; corresponding author: miriam.pein-hackelbusch@th-owl.de



Table 1 Diversity of methylene blue (MB) solutions used for viability staining of yeast cells. All references used dye concentrations of 0.01 % (w/v). 'Comment' refers to our understanding of the actual pH value of the MB solutions in the referenced works.

Buffer system	pH value	Reference	Comment
glycine	10.6	[1]	–
glycine	10.6	[12]	–
glycine	5.5; 10.6	[23]	pH of glycine solution was adjusted to 5.5 and 10.6.
phosphate-buffered saline	9	[20]	–
potassium dihydrogen phosphate	4.2 – 4.5 ^{a)}	[9]	no information on pH; pH is expected to be around 4.2–4.5 [25]
sodium citrate	7 – 8 ^{a)}	[1]	no information on sodium citrate used, but hint to pH: 'neutral, citrate-buffered methylene blue'
sodium citrate	3 ^{a)}	[12]	upon request: monosodium citrate was used, indicating a pH of around 3–4
sodium citrate	n.a.	[26]	no information on pH value or sodium citrate used
sodium citrate	n.a.	[27]	no information on pH value or sodium citrate used
sodium citrate dihydrate	n.a.	[9]	no information on pH value or sodium citrate used
sodium citrate dihydrate	n.a.	[28]	no information on pH value or sodium citrate used; [1] refer to this reference and describe the citrate-buffered solution as having a neutral pH.
sodium citrate dihydrate	n.a.	[13]	no information on pH value or sodium citrate used
sodium citrate dihydrate	n.a.	[6]	no information on pH value or sodium citrate used
sodium citrate dihydrate	n.a.	[14]	no information on pH value or sodium citrate used
sodium citrate dihydrate	n.a.	[29]	no information on pH value or sodium citrate used
trisodium citrate	8 ^{a)}	[30]	no information on pH, which would be expected to be around pH 8 for trisodium citrate
trisodium citrate dihydrate	8 ^{a)}	[15]	no information on pH, which would be expected to be around pH 8 for trisodium citrate
trisodium citrate dihydrate	8 ^{a)}	[22]	no information on pH, which would be expected to be around pH 8 for trisodium citrate

a) pH values were not provided in the original reference, but were inferred by us (see comment column).

permeable cells with damaged membranes [9, 20, 21]. Evidence for this was provided by experiments conducted by Matsumoto et al. [22], in which the coloured supernatant was measured photometrically after centrifugation of the stained cells instead of examining the cells themselves. It was shown that the selective staining of dead cells with MB is mainly due to the dye penetration into dead cells and not to the decolourisation by living cells.

In addition to these mechanisms, the pH value of the staining solution appears to affect dye uptake. In this context, Sami et al. [23] proposed that the cell membrane normally prevents the positively charged dye MB from entering the cell. However, in an alkaline environment, the membrane loses this barrier function because the difference in hydrogen ions across the membrane is cancelled. These observations suggest that both membrane permeability and environmental pH contribute to the staining patterns.

A survey of published staining protocols, however, reveals a persistent reporting gap. In a substantial proportion of protocols, the pH value of the staining solution is either not stated explicitly or is implicit in the buffer composition in ways that permit a wide range of plausible values (Table 1). Where the pH value is reported, values span approximately the range from pH 3 to pH 10 without clear

consensus on which condition yields optimal performance. Ionic strength, which depends on buffer composition and is known to affect cationic dye behaviour in solution [24], is reported even less consistently. This heterogeneity makes it difficult for practitioners to select a defensible standard protocol, and complicates inter-laboratory comparison of results.

The European Brewery Convention published an article stating that a citrate-buffered MB staining solution results in stronger staining contrasts, which leads to lower measured viabilities compared to a phosphate-buffered MB solution. Here, viability tends to be overestimated due to weaker contrasts (approximately in the viability range of 40–90 %). As an explanation, the authors expressed that 'in the presence of citrate, the cells dispersed more readily and gave more clear-cut staining' [9]. However, these results were discussed only in relation to the buffer system; the pH value itself was not reported. While the pH of a potassium dihydrogen phosphate solution would be expected to be approximately pH 4.2–4.5 [25], the pH of phosphate buffers based on potassium dihydrogen phosphate can range from pH 2 to pH 9 [31]. A similar issue arises for citrate-buffered solutions, since these can be based on monosodium citrate, resulting in pH 3 solutions [32], or based on trisodium citrate, resulting in pH 8 solutions [33].

Smart et al. [1] further reported that the use of acidic phosphate-buffered MB (pH 4.6) did not allow discrimination between viable and dead cells, in contrast to a neutral citrate-buffered solution. When comparing a citric MB solution (upon request: monosodium citrate, pH approx. 3–4) with an alkaline MB solution with a pH of 10.6, White et al. [12] found that the acidic solution delivered higher measured viabilities (in the range of 80–100%). No clear explanation was provided for this observation; it was merely noted that the uptake rate of MB into yeast cells increases at alkaline pH values. Other researchers also found that a pH value in the range of pH 4–6 leads to lower staining contrasts than at an alkaline pH value (pH > 10) [23]. This had the effect that viability tended to be overestimated when acidic MB solutions were used.

In the study by Matsumoto et al. [22], MB staining solutions with and without trisodium citrate dihydrate were compared, without addressing the pH. However, in the case of trisodium citrate, we expect the pH value to be around 8 [33]. In the referred study, the authors measured the supernatant from stained cell suspensions of different viabilities photometrically, after the stained cells had been centrifuged. It was shown that it was not possible to distinguish between viable and dead cells when using MB solutions without trisodium citrate. However, when using the MB solution containing trisodium citrate dihydrate, a clear distinction could be made.

Literature further indicates that the conceptual foundations remain debated and that inconsistencies question the comparability of MB-based viability assessments (see Table 1).

However, to the best of our knowledge, neither a standardised protocol nor a systematic review on this topic has been published yet. For this reason, the objective of the present study is to address this reporting gap by systematically evaluating the influence of pH of the dye solutions, dye concentration, and yeast matrix on the staining performance of MB and TB across three *Saccharomyces* strains representative of brewing applications. This work does not aim to validate the methods against an external biological reference such as colony-forming-unit (CFU) plating, which has been addressed elsewhere in the literature [22], nor to resolve mechanistic questions about dye–cell interactions. Rather, the goal is to provide an evidence-based decision framework that supports the selection of staining parameters in routine yeast quality control. The framework is illustrated with reference to common brewing tasks including pitching control, in-process fermentation monitoring, and post-cropping assessment, while the experimental evaluation itself was performed under controlled laboratory conditions using a defined unhopped wort matrix.

2 Material & Methods

2.1 Yeast suspensions

Three dry yeasts belonging to the genus *Saccharomyces* were examined, including standard baker's yeast *Saccharomyces cerevisiae* (*S. cerevisiae*; commercially available, Dr. August Oetker Nahrungsmittel, Bielefeld, Germany) and two brewing yeasts *Saccharomyces pastorianus* (Saflager™ W-34/70, Fermentis by Lesaffre, Marcq en Baroeul, France) and *Saccharomyces cerevisiae* (SafAle™ WB-06,

Fermentis by Lesaffre, Marcq en Baroeul, France). These three yeasts were selected to include the most well-known and widely used yeast, baker's yeast, along with two brewing yeasts chosen to represent a bottom-fermenting strain (W-34/70) and a top-fermenting strain (WB-06). It should be noted that commercial dry yeast preparations may contain emulsifiers such as sorbitan monostearate, and a possible influence on the staining system cannot be fully excluded.

The yeasts were suspended in tap water in the first experimental plan and in unhopped beer wort (12°Plato) for the second experimental plan. In both cases, the dry yeasts were rehydrated in the respective liquid for 30 min before further use. The wort was prepared from diluted malt extract (Weyermann® Bavarian Pilsner, Weyermann Spezialmalze, Bamberg, Germany). Since the wort was not subjected to a conventional brewing process, it was autoclaved (20 min, 121 °C, 2 bar abs.) to provide a standardised heat treatment. Furthermore, it was centrifuged (10 min, 4612 × g) to reduce the amount of larger particles that could interfere with the viability analysis, thereby approximating the clarification occurring during wort production. Only the supernatant was used for further experiments. The contribution of the ionic strength, especially with regard to the wort matrix, is negligible in the present study. The cells were assayed in a wort matrix, whose ionic strength is already set by its intrinsic inorganic electrolyte load (K⁺, Mg²⁺, Ca²⁺, Na⁺, phosphate, chloride and sulfate) derived from the malt and brewing liquor.

To generate samples with reduced viability, 50 mL of the cell suspensions were microwaved for 90 s (700 W, 2450 MHz), resulting in a stock cell solution with 0 % viability. The effectiveness of the treatment was confirmed microscopically by the absence of viable cells. In the following, reference will be made to theoretical viabilities, meaning that freshly rehydrated dry yeasts relate to a viability of 100 %, while microwave-treated yeasts have a viability of 0 %. These conditions should be interpreted as operational reference states rather than absolute biological endpoints. They were used to establish reproducible lower- and upper-bound conditions within the experimental system for the comparison of staining performance across different parameters. Although it does not impair the findings of the present study, it should be noted that the actual viability of rehydrated dry yeast is often below the theoretical value of 100 %, since a certain proportion of dead cells is typically present as a result of the drying process. For commercial active dry brewing yeast, post-rehydration viabilities of approximately 65–85 % have been reported, with values of 65–75 % for a lager strain and 75–85 % for ale strains [34].

In addition, intermediate viability levels can be generated by mixing defined proportions of viable and non-viable stock suspensions. Such mixtures (25 %, 50 %, 75 %) were prepared for selected conditions to verify the transferability of staining behaviour across the full viability range (Supplementary Information; intermediate viability levels).

2.2 Staining solutions

Staining solutions were prepared according to table 2 and stored in the dark at room temperature for the duration of the experi-

Table 2 Details on the preparation of staining solutions at different pH values and concentrations

Dye (concentration)	Dye solution pH value	Preparation
Methylene blue (MB) solution (0.01 % / 0.1 %)	3	Acidic (acetic acid) MB solution (0.25 % aqueous solution, Th. Geyer GmbH & Co. KG, Renningen, Germany) was diluted to a concentration of 0.01 % / 0.1 % (v/v) with dist. water.
	7.8	2 g tri-sodium citrate dihydrate (Merck KGaA, Darmstadt, Germany) were dissolved in 100 mL dist. water. 1 mg / 10 mg MB (Merck KGaA, Darmstadt, Germany) was added to 10 mL of this solution.
	10.6	0.751 g glycine was dissolved in dist. water, the pH adjusted to 10.6 with 1 M NaOH, and filled up to 100 mL with dist. water. 1 mg / 10 mg MB (Merck KGaA, Darmstadt, Germany) was added to 10 mL of this solution.
Trypan blue (TB) solution (0.02 % / 0.2 %)	3	Dist. water was adjusted to pH 3.0 using 100 % acetic acid. 2 mg / 20 mg TB (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to 10 mL of this solution.
	7.8	TB (0.4 %, VWR International GmbH, Hannover, Germany) was diluted to a concentration of 0.02 % / 0.2 % (v/v) with dist. water.
	10.6	0.751 g glycine was dissolved in dist. water, the pH adjusted to 10.6 with 1 M NaOH, and filled up to 100 mL with dist. water. 2 mg / 20 mg TB (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to 10 mL of this solution.

ment. The evaluated pH values refer to the prepared dye solutions prior to mixing with the yeast suspensions. This approach was deliberately chosen to reflect both common laboratory practice and the procedures described in the existing literature, where staining solutions are typically characterised prior to sample addition. In practical applications, the final pH after mixing is variable and depends on multiple factors, including the composition of the sample matrix, the buffering capacity of the medium, and the physiological state and metabolic activity of the yeast cells. By defining pH at the level of the dye solution, this study aims to capture experimentally controllable input parameters while maintaining relevance to real-world application conditions. Furthermore, the staining solutions contain a defined concentration of buffer ions, so the pH remains constant upon mixing. This was confirmed by measuring the pH before and after addition of the staining solution to the yeast-containing sample, where no significant change occurred. All pH values reported hereafter therefore refer to the pH of the staining solution.

Based on the device-specific requirements of the cell counter, which, according to the manufacturer’s manual, requires a higher dye concentration than the 0.01 % (w/v) typically used in bright-

field microscopy, this study evaluated two dye concentrations for each dye: a ‘high’ concentration (0.1 % for MB; 0.2 % for TB) and a ‘low’ concentration (0.01 % for MB; 0.02 % for TB). Depending on the preparation procedure, dye concentrations were expressed either as weight/volume (w/v) when the dye was weighed as a solid powder or as volume/volume (v/v) when a commercial liquid stock solution was diluted.

2.3 Analysis

To reduce analysis time and avoid errors caused by subjective perception and interpretation by the analyst, viability measurements were performed using a semi-automated image-based cell counter [35]. The analysis was conducted using a Countstar® Mira FL (Alit Biotech Shanghai, P.R. China). According to the user manual, the operating range is $1 \times 10^4 - 3 \times 10^7$ cells mL⁻¹. Image acquisition and analysis typically require <20 s per sample. The instrument uses a single-use plastic slide containing five chambers. Three of these chambers were loaded with 20 µL of each test sample (yeast samples mixed with equal volumes of staining solution) in order to account for potential variability in cell distribution and chamber loading. Because all chambers were filled from the same mixed

suspension, measurements obtained from the three chambers represent technical replicates. After a sedimentation time of 3 min, the samples were imaged and analysed immediately to ensure consistent incubation times across all measurements. The parameter settings followed the manufacturer’s recommendations: ‘Focal length = 1.271’; ‘Consumable = Countstar Slide-8.0X’; ‘Exposure = 160’; ‘Gain = 0’; ‘Prob = 0.25’; ‘Nms = 0.25’; ‘Min-Size = 4.0’; ‘Max-Size = 25.0’; ‘Thresh = 10’; ‘Fit-Min = 5.0’; ‘Fit-Max = 25.0’; ‘Min-Ave-Gray = 0.0’; ‘Max-Ave-Gray = 255.0’; ‘Activity = Close’. All analyses were performed using these fixed manufacturer-defined settings to ensure comparability across all experiments. Consequently, the results of this study refer specifically to measurements

Table 3 Variable and constant factors and their respective levels in experiment 1

	Factor	Level
Variable	Dye type	Methylene blue (MB); Trypan blue (TB)
	Dye solution concentration	High (0.1 % MB; 0.2 % TB); Low (0.01 % MB; 0.02 % TB)
	Dye solution pH value	3.0; 7.8; 10.6
	Theoretical viability	0 %; 100 %
Constant	Yeast strain	Baker’s yeast
	Total Cell Density (TCD) stock cell solution	$1 \cdot 10^7 - 3 \cdot 10^7$ cells mL ⁻¹
	Yeast matrix	Water

obtained with the Countstar® Mira FL under these defined analysis conditions.

The Δ Viability parameter was calculated by subtracting the means of the triplicate measurements (technical replicates) for the 0 % theoretical viability samples from those of the 100 % viability samples; these results were then averaged over three days and three yeasts, and the standard deviation was determined.

Data analysis was performed using JMP (version 18.2.2). Figures were prepared using Python (version 3.12.1) with NumPy (version 1.26.2), Matplotlib (version 3.8.2), and Pandas (version 2.2.3).

2.4 Experimental design

The experiments and selected parameters were designed in a systematic, stepwise manner. In the first experiment, a broad overview was obtained by examining baker’s yeast suspended in water. This simplified system was intentionally used to evaluate the influence of staining parameters under controlled conditions with minimal matrix interference. In the second experiment, two brewing yeast strains were included, and wort was used as the matrix to better reflect real-world conditions in the brewery application field. The use of water in the first experiment was therefore not intended to assume equivalence between water-based and wort-based samples, but rather to provide an initial baseline before investigating the staining performance in a more complex and application-relevant matrix.

The first experiment was designed as a randomised full-factorial combination of the conditions listed in table 3. Two dyes, MB and TB, were used, each tested at two concentrations: high (0.1 % MB; 0.2 % TB) and low (0.01 % MB; 0.02 % TB). Staining was performed with staining solutions of pH level 3.0, 7.8, and 10.6. Samples were prepared using baker’s yeast suspended in water, with a total cell density of $1 \times 10^7 - 3 \times 10^7$ cells mL⁻¹. Theoretical viabilities of 0 % and 100 % were considered to represent dead and live cell populations.

All analyses were performed using a semi-automated cell counting device. The same operator repeated the entire experimental plan on three different days within one week. Experiments performed on different days were treated as independent experimental replicates, and a fresh yeast suspension was prepared each day. In total, this resulted in 72 observations.

The second experiment was based on a randomised full-factorial design combining all the levels shown in table 4. Two dyes were tested: MB (0.1 %) and TB (0.2 %). Each dye was applied at two pH levels (3.0 and 7.8). Yeast suspensions were prepared in wort with a cell concentration of $1 \times 10^7 - 3 \times 10^7$ cells mL⁻¹. Three yeast types were used: baker’s yeast, W-34/70, and WB-06. To examine different viability conditions, samples with theoretical viabilities of 0 % and 100 % were considered. Again, the same operator repeated the entire experimental setup on three separate days within one week. Comparably to experiment 1, also experiment 2 resulted in 72 observations. Each day, a fresh yeast suspension was prepared.

Table 4 Variable and constant factors and their respective levels in experiment 2

	Factor	Level
Variable	Dye type	Methylene blue (MB); Trypan blue (TB)
	Dye solution pH value	3.0; 7.8
	Theoretical viability	0 %; 100 %
	Yeast strain	Baker’s yeast; W-34/70; WB-06
Constant	Dye solution concentration	High (0.1 % MB; 0.2 % TB)
	Total Cell Density (TCD) stock cell solution	$1 \times 10^7 - 3 \times 10^7$ cells mL ⁻¹
	Yeast matrix	Wort

The use of the chosen reference states (0 % and 100 % theoretical viability) provided clearly separated conditions that enabled a robust comparison of staining behaviour across the tested parameter combinations. Within this framework, the analysis focused on relative differences between staining conditions rather than on establishing absolute viability values or validating the staining approach itself. While such boundary conditions do not permit a direct assessment of linearity across intermediate viability levels, additional experiments performed for a representative staining condition (MB, pH 3.0, 0.1 %) demonstrated that mixtures of viable and non-viable cells (25 %, 50 %, and 75 % theoretical viability) resulted in proportional combinations of stained and unstained cells (see Supplementary Information; intermediate viability levels). The observed trends in staining behaviour remained consistent with those obtained for the extreme conditions, indicating that the parameter-dependent effects identified in this study were transferable across the full viability range. Based on this consistency, the systematic analysis was restricted to the two extreme reference states to allow for a controlled and tractable experimental design.

3 Results & Discussion

3.1 First experiment

S. cerevisiae was analysed under varying dye type, varying dye concentration, and different pH value of the staining solution. Figure 1 summarises the results of experiment 1.

Highlighting the results obtained with MB, deviations in the mean values are evident at pH 10.6, compared to the other pH levels. This applies in particular to high dye concentrations for both 0 % and 100 % theoretical viability, while at low concentrations no deviation is observed for 0 % theoretical viability. Using TB at pH 10.6 at high concentration and a theoretical viability of 0 %, however, resulted in a mean viability of 22 %, which is considerably higher than at pH 3.0 and pH 7.8, with mean viabilities of 1 % and 9 %, respectively. At pH 3.0, deviations in the mean values for TB at 0 % viability are noticeable compared to the other pH levels, both at low and high dye concentrations. In detail, for the low dye concentration we found a mean viability of 2 % at pH 3.0 compared to 10 % at pH 7.8 and 11 % at pH 10.6.

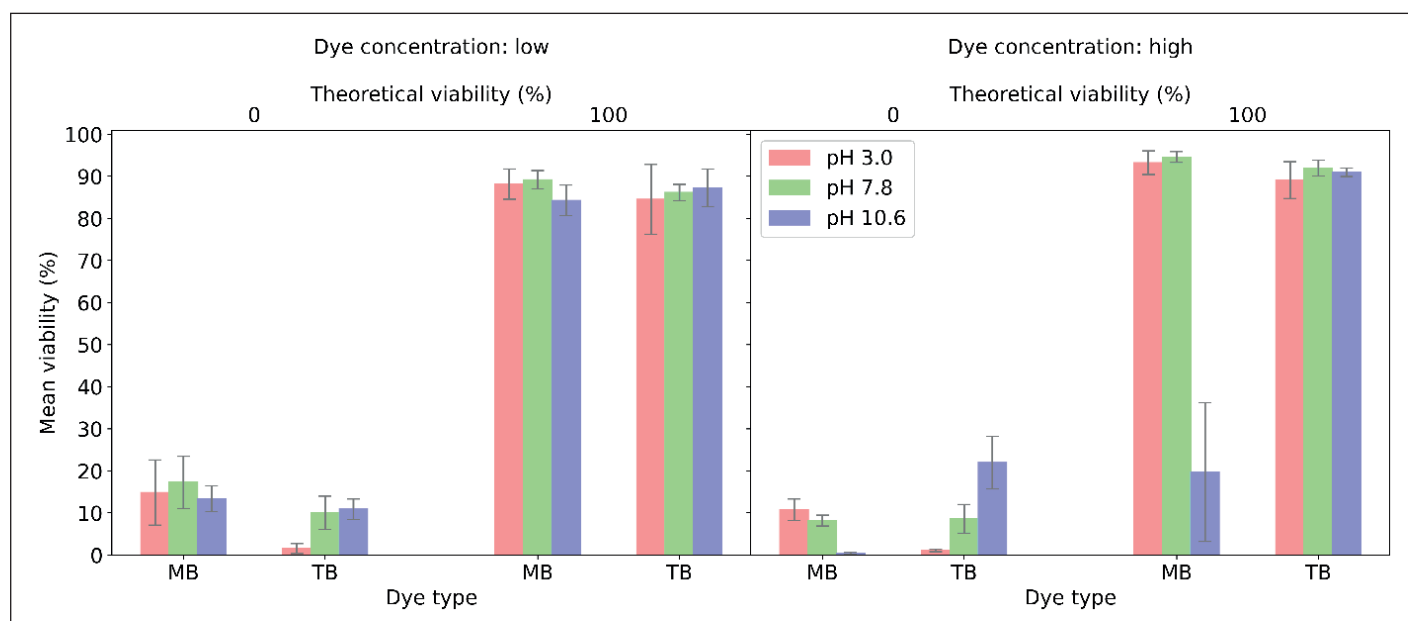


Fig. 1 Mean ($n=9$ [3 technical x 3 experimental replicates]) standard deviations of the measured viabilities of *Saccharomyces cerevisiae* under varying conditions. The factors examined were dye type (methylene blue, MB; trypan blue, TB), dye concentration (low: 0.01 % MB, 0.02 % TB; high: 0.1 % MB, 0.2 % TB), and pH of the staining solution (left to right 3.0, 7.8 and 10.6). Two theoretical viability levels (0 % and 100 %) were used.

To further contextualise the role of pH under the applied experimental conditions, the pH of the staining mixtures after combining dye solutions with yeast suspensions was determined for all conditions. Across all strains, the final pH values closely followed the initial pH of the staining solutions, with only minor shifts after mixing. Differences between yeast suspensions in wort (pH 5.2) and water (pH 7.8) primarily reflected their respective initial pH levels rather than distinct buffering effects, and strain-specific influences were negligible. Overall, these results indicate that, under the conditions applied in this study, the relative pH conditions defined by the staining solutions are largely maintained after mixing and provide a consistent basis for interpreting the observed effects.

Summing up the pH-related investigations (Table 5), our results support the hypothesis that alkaline MB solutions lead to increased staining contrast and underestimated viabilities [12, 22, 23] at high dye concentration and 100 % theoretical viability.

Disregarding the discrepancies described above, the results in figure 1 indicate no substantial differences between MB and TB staining. Both dyes showed the same general trend in differentiating between viable and non-viable cells. This observation suggests that the inaccuracy of MB staining discussed in the literature [36] may either also apply to TB or be less attributable to the dye itself and more dependent on the analytical method. In contrast to the commonly employed bright-field microscopy, our study utilised an automated cell counter, which may have reduced some of the methodological biases.

Indeed, depending on the chosen reference measurement, there is disagreement in the literature regarding whether viability assessed using MB staining and bright-field microscopy is overestimated [1, 28, 36] or underestimated [6], and how this bias depends on the underlying viability of the sample [26]. Importantly, these discrepan-

cies are often not solely attributable to the staining procedure with MB itself, but rather to the analytical method commonly used: bright-field microscopy. Both the inherent limitations of microscopy, such as the substantially smaller sample size compared to other analytical methods [6], and factors specific to MB staining contribute to the variability and lack of reproducibility observed. For example, faintly blue-stained cells may be classified as either viable or non-viable depending on the operator's subjective judgement [1]. This highlights the impact of the analysis method on viability assessment.

Furthermore, the similar staining behaviour observed for MB and TB may indicate a similar mode of action for both dyes. TB can only penetrate and stain cells with compromised membranes [9, 21, 37]. Accordingly, these results appear to support the hypothesis proposed by [9, 20, 21], suggesting that MB only penetrates cells with damaged membranes, and that the reduction to the colourless leuco form can be neglected in viability staining. This interpretation is further supported by recent work [22], which demonstrated that living cells do not significantly take up MB, reinforcing the importance of membrane permeability as the mechanistic key variable. Instead, the strong and reproducible discrimination between viable and non-viable cells achieved at acidic pH supports models in which viable cells largely exclude the dye and staining is primarily governed by membrane permeability modulated by the environmental pH value. Overall, the findings narrow the set of plausible mechanisms and favour permeability-based explanations over those centred on intracellular MB conversion.

The concentration of the dye also does not appear to have a clear effect on the mean viability values within the investigated concentration ranges (Fig. 1). However, upon closer inspection, the standard deviations tend to be lower at higher dye concentrations for pH 3.0 and pH 7.8. Across both dyes and both pH levels, the mean standard deviation at high dye concentration was 4 % for 0 % viability

(vs. 8 % at low concentration) and 3 % for 100 % viability (vs. 5 % at low concentration). Thus, elevated dye concentrations seemed to enhance the distinction between viable and non-viable cell populations for both dyes, suggesting improved staining efficiency and clearer discrimination at higher concentrations (Table 5).

This contrasts with the commonly accepted and used MB concentration of 0.01 % (Table 1), which corresponds to the lower concentration tested in the present study. We explain this discrepancy by the fact that the 0.01 % concentration works best for viability determination using bright-field microscopy. We, however, performed our analyses with an automated cell counter, for which the application of higher dye concentration is beneficial.

3.2 Second experiment

Based on the findings that pH value 10.6 and low staining concentrations lead to less reliable results (experiment 1), a further experimental design was developed. The pH values were now only varied between acidic and neutral pH, and the higher dye concentration of 0.1 % for MB and 0.2 % for TB was selected. Based on the initial findings, the experimental range was now extended to three different yeast strains and the experimental conditions for brewing yeasts were made more realistic by suspending the yeast in beer wort (autoclaved and centrifuged) instead of water (Fig. 2). While wort represents a more realistic process matrix, its complex composition (e.g., turbidity, background particles, and polyphenolic compounds) may potentially influence dye behaviour. Therefore, possible matrix-specific effects cannot be fully excluded and should be investigated in future studies, for example by comparison with established reference staining methods such as Carboxyfluorescein diacetate.

Regarding MB staining, the W-34/70 and WB-06 strains showed higher mean viabilities at pH 3.0 in the 100 % theoretical viability samples, whereas for baker's yeast higher viabilities were observed

at pH 7.8 (87 % vs. 80 % at pH 3.0) (Figure 2). For samples with 0 % theoretical viability, the pH value of the staining solution appeared to have no influence on the measured viability of baker's yeast. However, for W-34/70, pH 7.8 resulted in lower viabilities (28 % vs. 33 % at pH 3.0), while for WB-06, lower viabilities were observed at pH 3.0 (12 % vs. 16 % at pH 7.8). The TB staining data, however, indicate a tendency for measured viabilities to be higher at pH 7.8 than at pH 3.0, at both 0 % and 100 % theoretical viability. These overall results therefore suggest that the measured viability is influenced by the type of dye, the pH of the staining solution, and the yeast strain. Variations in cell wall architecture, membrane permeability, metabolic redox capacity, and the degree of cell aggregation may influence both dye accessibility and intracellular reduction of methylene blue, resulting in different staining.

Based on the dye-specific results, no combination of parameters for reliably assessing yeast cell viability could be recommended as generally suitable. We therefore evaluated whether pooling the results across all yeast strains (Fig. 3) as an overall mean ($n=27$) could be beneficial in terms of general statements. This approach is based on the premise that an ideal method for yeast viability determination should be broadly applicable across different yeast strains, without requiring strain-specific adjustments.

When considering the overall data (Fig. 3), it becomes apparent that the considered pH value between acidic and neutral pH does not affect the outcome of MB staining. In the case of TB staining, higher viabilities of 86 % were observed at pH 7.8 for samples with a theoretical viability of 100 % compared to pH 3.0 with a mean viability of 69 %. However, it is important to note that for samples with a theoretical viability of 0 %, the measurements at pH 7.8 were also higher (mean viability of 21 %) than at pH 3.0 (mean viability of 6 %). Ideally, the measured viability should be as high as possible for samples with a theoretical viability of 100 %, and as low as possible for samples with a theoretical viability of 0 %.

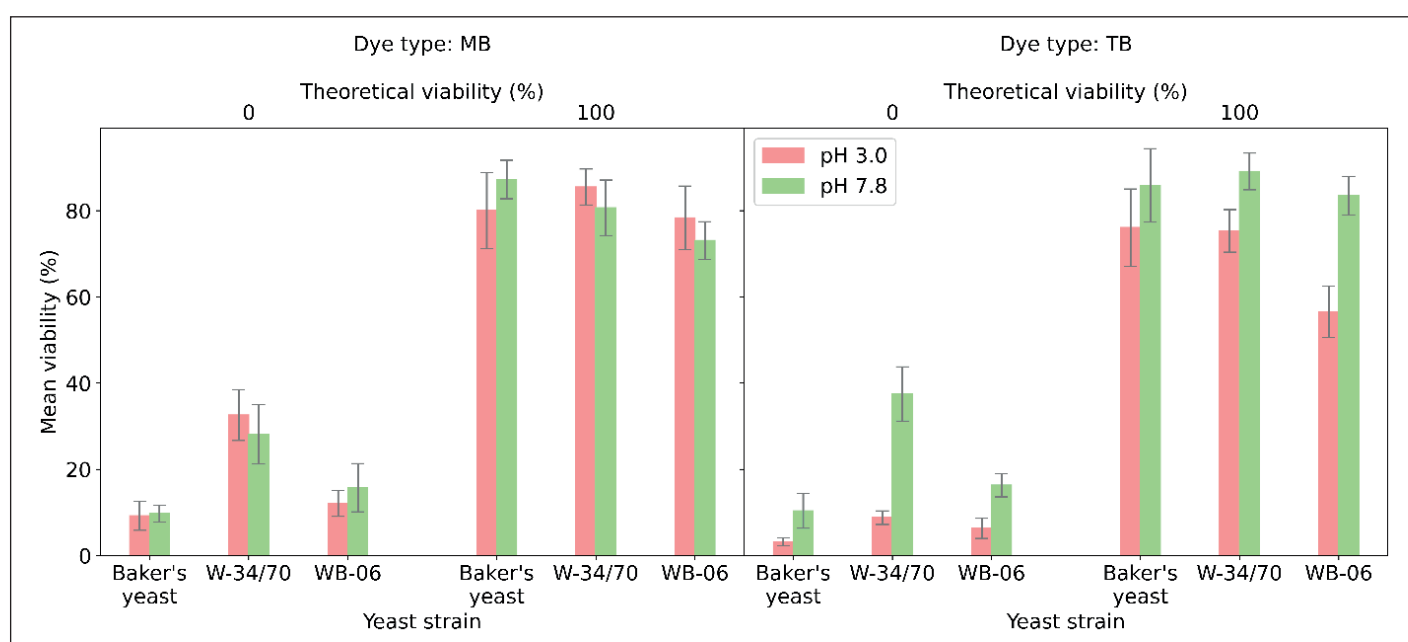


Fig. 2 Mean ($n=9$ [3 technical x 3 experimental replicates]) standard deviations of the measured viabilities of all replicates under varying conditions. The factors examined were dye type (methylene blue, MB; trypan blue, TB), pH of the staining solution (left to right 3.0, 7.8) and yeast strain (baker's yeast; W-34/70; WB-06). Two theoretical viability levels (0 % and 100 %) were used.

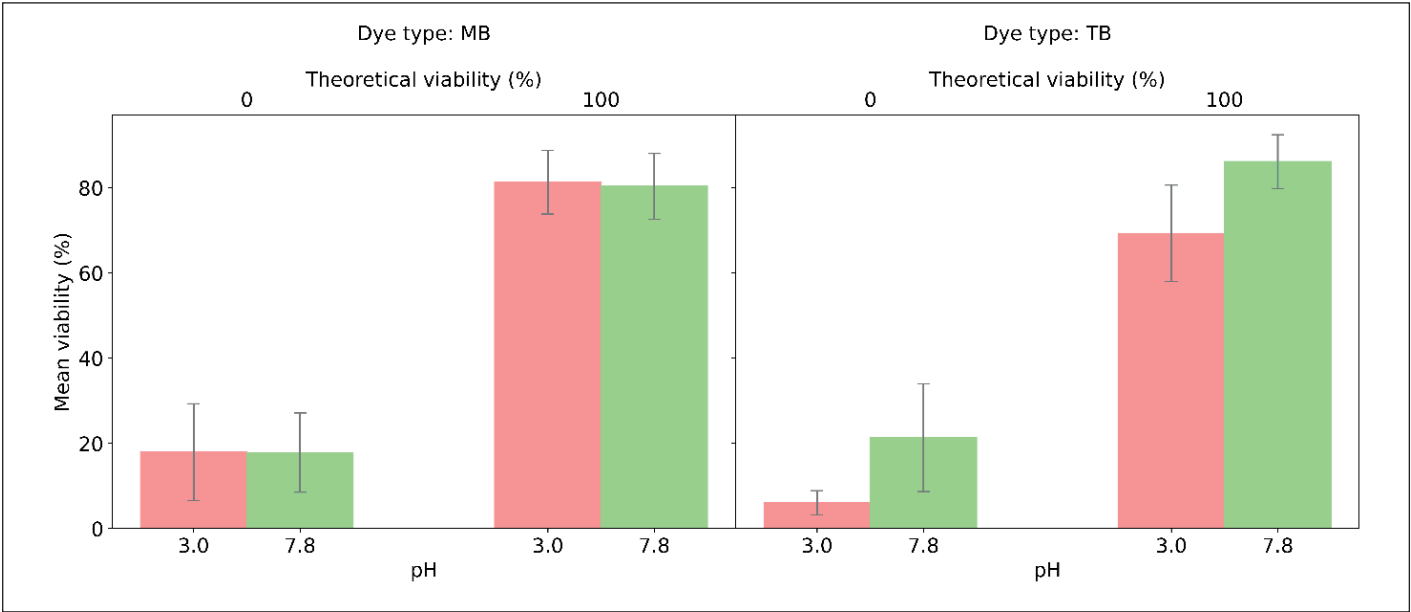


Fig. 3 Mean (n=27 [3 technical x 3 experimental replicates x 3 yeasts]) standard deviations of the measured viabilities over all yeast strains (baker's yeast; W-34/70; WB-06) in wort under varying conditions. The factors examined were dye type (methylene blue, MB; trypan blue, TB) and pH of the staining solution (3.0, 7.8). Two theoretical viability levels (0 % and 100 %) were used.

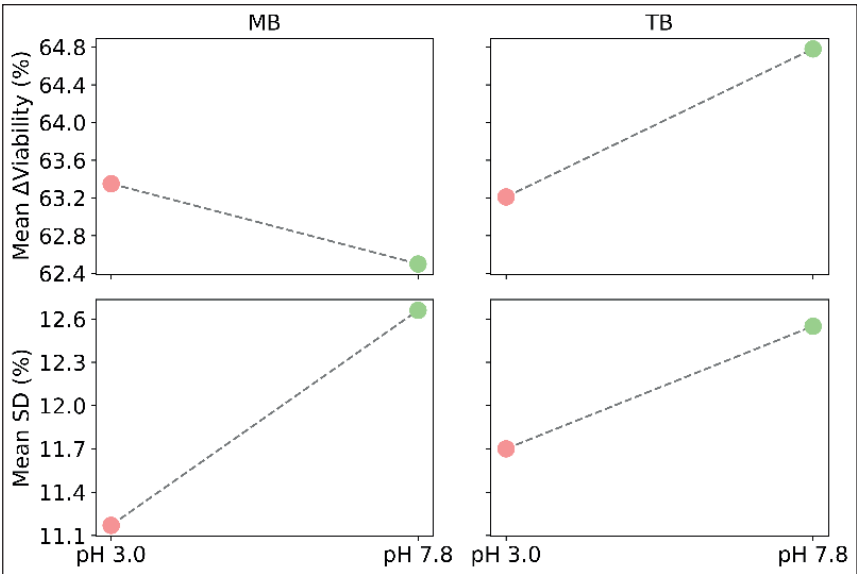


Fig. 4 Interaction analysis. Influence of dye type (methylene blue, MB; trypan blue, TB) and pH of the staining solution (3.0, marked in red; 7.8 marked in green) on the mean ΔViability (n=9 [3 experimental replicates x 3 yeasts]) and the corresponding mean standard deviations (SD) for ΔViability at high dye concentration (0.1 % MB; 0.2 % TB)

To assess the ability of each staining method to discriminate between viable and non-viable cells, we calculated the difference between the mean measured viability of the 100 % and 0 % theoretical viability samples. We defined this parameter as ΔViability, which reflects the method's separation capability. A higher ΔViability indicates a better ability to distinguish between viable and dead cells. At the same time, a low standard deviation of ΔViability is desirable, as it reflects the precision and reproducibility of the method's discriminatory power.

A corresponding interaction analysis (Fig. 4) revealed just small differences between the individual parameter settings. However,

MB staining at pH 3.0 showed the most favourable performance (Table 5), characterised by the highest mean ΔViability and comparatively low variability across all yeast strains. This suggests that MB under acidic conditions provides reliable and reproducible differentiation between viable and non-viable cells. Considering that TB performed slightly less effectively and is furthermore classified as a CMR substance (Carc. 1B) [38], its routine use should be avoided whenever alternative dyes demonstrate comparable or superior performance. In this context, MB at pH 3.0 appears to represent a less hazardous and more effective alternative under the tested conditions in yeast viability assessment.

4 Conclusion

This study systematically evaluated the influence of the pH of the staining solutions, dye concentration, dye type, and yeast strain on the reliability of image-based viability measurements using MB and TB staining. The findings of this study therefore apply to the tested staining parameters within the analytical framework of the Countstar® Mira FL and should not be interpreted as a general validation of automated image-based viability assessment methods. By applying a reproducible experimental design and minimising subjective interpretation through automated image analysis, the work provides a comprehensive and transparent assessment of key methodological factors that have been reported in the literature as sources of inconsistency in viability determination.

Neither dye type nor concentration alone yielded universally superior performance, but trends emerged. First, highly alkaline staining conditions (pH 10.6) yielded implausible and unreliable viability

Table 5 Summary of all methylene blue (MB) and trypan blue (TB) staining variants tested in this study and their qualitative suitability for automated yeast viability determination according to figures 1 – 4. Conditions marked with an asterisk were evaluated across baker's yeast, W-34/70, and WB-06 in wort (experiment 2), all other conditions were evaluated in experiment 1 using baker's yeast in water. 'Limited' indicates a relative methodological assessment within the tested conditions and does not imply that the respective staining variant is generally unsuitable for viability determination. Recommended staining solution: 0.1 % MB solution at pH 3.0 (acidified with acetic acid)

Dye type	Dye conc.	pH 3.0	pH 7.8	pH 10.6
MB	0.01 %	Limited; clear separation of viable and non-viable; variability tended to be higher than at 0.1 % MB	Limited; clear separation of viable and non-viable; variability tended to be higher than at 0.1 % MB	Limited; viable samples deviated from pH 3.0 and 7.8, whereas non-viable samples remained largely unaffected
MB	0.1 %	Recommended*; clear separation with low variability and most favourable overall cross-strain performance	Suitable*; clear separation of viable and non-viable and lower variability than at 0.01 % MB	Not recommended; pronounced underestimation of viable samples and reduced overall reliability
TB	0.02 %	Limited; clear separation and lowest apparent viability of non-viable samples among the tested pH values; variability tended to be higher than at 0.2 % TB	Limited; clear separation of viable and non-viable, but higher apparent viability of non-viable samples than at pH 3.0	Limited; clear separation of viable and non-viable, but higher apparent viability of non-viable samples than at pH 3.0
TB	0.2 %	Suitable*; clear separation of viable and non-viable; low apparent viability of non-viable samples; variability tended to be lower than at 0.02 % TB	Suitable*; higher apparent viability of viable samples, but also of non-viable samples, compared with pH 3.0	Not recommended; increased apparent viability of non-viable samples and consequently reduced discrimination

estimates, particularly for MB at high concentration. Second, MB and TB exhibited broadly similar staining behaviour, suggesting that previously reported MB inaccuracies are likely linked to bright-field microscopy rather than the dye. Automated image analysis mitigated several of these biases.

Dye concentration did not systematically shift mean viability values, but higher concentrations reduced variability and improved the distinction between viable and non-viable cells, enhancing staining efficiency. Across multiple yeast strains, no single parameter configuration consistently outperformed others, highlighting strain-dependent variability and the difficulty of defining universally optimal conditions.

Nevertheless, when data across all strains were pooled – reflecting the requirement for broadly applicable viability methods – interaction analysis identified MB staining at pH 3.0 as the most favourable overall condition across the tested yeast strains. This parameter combination yielded the highest Δ Viability with comparatively low variability, indicating reproducible and reliable discrimination between viable and non-viable cells. Given TB's slightly lower performance and CMR classification, MB at acidic pH is a practical and safer choice for routine yeast viability measurements.

Overall, this study provides a systematic foundation for selecting staining conditions that enhance reproducibility and interpretability in yeast viability assessments. The results support the use of MB at pH 3.0 and higher dye concentrations for automated image-based analysis, applicable across tested strains and viability ranges.

The systematic results obtained in this study also help to evaluate the conflicting staining mechanism models discussed in the literature. The superior reproducibility of staining at pH 3.0, combined with the reduced variability observed at higher dye concentrations, aligns with models in which MB can only penetrate non-viable cells. These findings are consistent with the interpretation that dye

exclusion rather than intracellular decolourisation is the primary factor defining viability-dependent contrast.

Acknowledgement

This study was funded by the German Federal Ministry of Research, Technology and Space through the funding programme 'Forschung an Fachhochschulen' under contract number 13FH045KX2. Selina Uhlendorff gratefully acknowledges the Joachim Herz Foundation for supporting her through the Add-on Fellowship.

Conflict of interest

The authors declare there are no conflicts of interest.

Author contributions

Uhlendorff, S.: Conceptualisation, Methodology, Validation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualisation, Funding acquisition. Dreyer, L. M.: Validation, Formal analysis, Investigation, Writing - Review & Editing. Kemper, L.: Validation, Writing - Review & Editing. Halli, J.: Validation, Writing - Review & Editing. Schneider, J.: Validation, Writing - Review & Editing, Funding acquisition. Frahm, B.: Validation, Resources, Writing - Review & Editing, Funding acquisition. Pein-Hackelbusch, M.: Conceptualisation, Validation, Writing - Review & Editing, Project administration, Funding acquisition.

5 References

1. Smart, K. A.; Chambers, K. M.; Lambert, I.; Jenkins, C. and Smart, C. A.: Use of Methylene Violet Staining Procedures to Determine Yeast Viability and Vitality, *Journal of the American Society of Brewing Chemists*, **57** (1999), no. 1, pp. 18-23.
2. Veloso, A. C. and Ferreira, E. C.: Online Analysis for Industrial

- Bioprocesses, Current Developments in Biotechnology and Bioengineering, Elsevier, 2017, pp. 679-704.
3. Wang, J.; Ding, H.; Zheng, F.; Li, Y.; Liu, C.; Niu, C. et al.: Physiological Changes of Beer Brewer's Yeast During Serial Beer Fermentation, *Journal of the American Society of Brewing Chemists*, **77** (2019), no. 1, pp. 10-20.
 4. Thiele, F.: Einfluss der Hefevitalität und der Gärparameter auf die Stoffwechselprodukte der Hefe und auf die Geschmacksstabilität, Dissertation, Technische Universität München, München, 2006.
 5. Heggart, H.; Margaritis, A.; Stewart, R. J.; Pilkington, M.; Sobezak, J. and Russell, I.: Measurement of brewing yeast viability and vitality: A review of methods, *Technical quarterly-Master Brewers Association of the Americas*, **37** (2000), no. 4, pp. 409-430.
 6. Boyd, A. R.; Gunasekera, T. S.; Attfeld, P. V.; Simic, K.; Vincent, S. F. and Veal, D. A.: A flow-cytometric method for determination of yeast viability and cell number in a brewery, *FEMS yeast research*, **3** (2003), no. 1, pp. 11-16.
 7. Opitz, C.; Schade, G.; Kaufmann, S.; Di Berardino, M.; Ottiger, M. and Grzesiek, S.: Rapid determination of general cell status, cell viability, and optimal harvest time in eukaryotic cell cultures by impedance flow cytometry, *Applied Microbiology and Biotechnology*, **103** (2019), no. 20, pp. 8619-8629.
 8. Tibayrenc, P.; Preziosi-Belloy, L. and Ghommidh, C.: On-line monitoring of dielectrical properties of yeast cells during a stress-model alcoholic fermentation, *Process Biochemistry*, **46** (2011), no. 1, pp. 193-201.
 9. European Brewery Convention - Yeast Group: Estimation of Yeast Viability, *Journal of the Institute of Brewing*, **68** (1962), no. 1, pp. 14-20.
 10. Andrews, P. D. and Stark, M. J.: Type 1 protein phosphatase is required for maintenance of cell wall integrity, morphogenesis and cell cycle progression in *Saccharomyces cerevisiae*, *Journal of Cell Science*, **113** (2000), no. 3, pp. 507-520.
 11. Karpova, T. S.; Lepetit, M. M. and Cooper, J. A.: Mutations that enhance the cap2 null mutant phenotype in *Saccharomyces cerevisiae* affect the actin cytoskeleton, morphogenesis and pattern of growth, *Genetics*, **135** (1993), no. 3, pp. 693-709.
 12. White, L. R.; Richardson, K. E.; Schiewe, A. J. and White, C. E.: Comparison of Yeast Viability/Vitality Methods and Their Relationship to Fermentation Performance, in Smart, K. (Ed.): *Brewing Yeast Fermentation Performance*, Blackwell Science, Oxford, UK, 2003, pp. 138-148.
 13. Painting, K. and Kirsop, B.: A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining, *World Journal of Microbiology & Biotechnology*, **6** (1990), no. 3, pp. 346-347.
 14. Kwolek-Mirek, M. and Zadrag-Tecza, R.: Comparison of methods used for assessing the viability and vitality of yeast cells, *FEMS yeast research*, **14** (2014), no. 7, pp. 1068-1079.
 15. Thomson, K.; Bhat, A. and Carvell, J.: Comparison of a new digital imaging technique for yeast cell counting and viability assessments with traditional methods, *Journal of the Institute of Brewing*, **121** (2015), no. 2, pp. 231-237.
 16. Li, G.; Wei, M.; Sun, J.; Zhang, Y. and Zhang, R.: Low false positive and accurate detection of yeast cell viability and concentration using an automatic staining and lensfree imaging platform, *Biochemical and Biophysical Research Communications*, **525** (2020), no. 3, pp. 793-799.
 17. Bongard, R. D.; Merker, M. P.; Shundo, R.; Okamoto, Y.; Roerig, D. L.; Linehan, J. H. et al.: Reduction of thiazine dyes by bovine pulmonary arterial endothelial cells in culture, *The American Journal of Physiology*, **269** (1995), 1 Pt 1, L78-L84.
 18. May, J. M.; Qu, Z. and Whitesell, R. R.: Generation of oxidant stress in cultured endothelial cells by methylene blue: protective effects of glucose and ascorbic acid, *Biochemical Pharmacology*, **66** (2003), no. 5, pp. 777-784.
 19. Bapat, P.; Nandy, S. K.; Wangikar, P. and Venkatesh, K. V.: Quantification of metabolically active biomass using Methylene Blue dye Reduction Test (MBRT): measurement of CFU in about 200 s, *Journal of Microbiological Methods*, **65** (2006), no. 1, pp. 107-116.
 20. Chilver, M. J.; Harrison, J. and Webb, T. J. B.: Use of Immunofluorescence and Viability Stains in Quality Control, *Journal of the American Society of Brewing Chemists*, **36** (1978), no. 1, pp. 13-18.
 21. Passow, H.; Rothstein, A. and Loewenstein, B.: An all-or-none response in the release of potassium by yeast cells with methylene blue and other basic redox dyes, *The Journal of General Physiology*, **43** (1959), no. 1, pp. 97-107.
 22. Matsumoto, A.; Terashima, I. and Uesono, Y.: A rapid and simple spectroscopic method for the determination of yeast cell viability using methylene blue, *Yeast*, **39** (2022), 11-12, pp. 607-616.
 23. Sami, M.; Ikeda, M. and Yabuuchi, S.: Evaluation of the alkaline methylene blue staining method for yeast activity determination, *Journal of Fermentation and Bioengineering*, **78** (1994), no. 3, pp. 212-216.
 24. Wani, H. A.; Shaikh, V. R.; More, D. H. and Patil, K. J.: Studies related to changes in ionic strength due to addition of sodium chloride to an aqueous solution of cationic dye methylene blue (MB): Electronic spectra analysis for dimerization equilibria, *Journal of Molecular Liquids*, **361** (2022).
 25. Council of Europe (Ed.): *European Pharmacopoeia*, Monograph 0920, Kaliumdihydrogenphosphat, Council of Europe, Strasbourg, France, 2023.
 26. Saldi, S.; Driscoll, D.; Kuksin, D. and Chan, L. L.-Y.: Image-Based Cytometric Analysis of Fluorescent Viability and Vitality Staining Methods for Ale and Lager Fermentation Yeast, *Journal of the American Society of Brewing Chemists*, **72** (2014), no. 4, pp. 253-260.
 27. Eigenfeld, M.; Wittmann, L.; Kerpess, R.; Schwaminger, S. and Becker, T.: Quantification methods of determining brewer's and pharmaceutical yeast cell viability: accuracy and impact of nanoparticles, *Analytical and Bioanalytical Chemistry*, **415** (2023), no. 16, pp. 3201-3213.
 28. Pierce, J. S.: Institute of Brewing: Analysis committee measurement of yeast viability, *Journal of the Institute of Brewing*, **76** (1970), no. 5, pp. 442-443.
 29. Atanasova, M.; Yordanova, G.; Nenkova, R.; Ivanov, Y.; Godjevargova, T. and Dinev, D.: Brewing yeast viability measured using a novel fluorescent dye and image cytometer, *Biotechnology & Biotechnological Equipment*, **33** (2019), no. 1, pp. 548-558.
 30. Back, W.: *Fruchtsaft- und Limonadenbetriebe, Wasser, Betriebshygiene, Milch und Milchprodukte, Begleitorganismen der Getränkeindustrie*, Carl Getränke-Fachverl., Nürnberg, 2000.
 31. Council of Europe (Ed.): *European Pharmacopoeia*, 07/2021:40103, Council of Europe, Strasbourg, France, 2021.
 32. Carl Roth GmbH + Co. KG: Safety data sheet Mono sodium citrate ≥99 %, anhydrous, extra pure, 2024.
 33. Carl Roth GmbH + Co. KG: Safety data sheet tri-Sodium citrate dihydrate CELLPURE® ≥99 %, 2024.

34. Jenkins, D. M.; Powell, C. D.; Fischborn, T. and Smart, K. A.: Rehydration of Active Dry Brewing Yeast and its Effect on Cell Viability, *Journal of the Institute of Brewing*, **117** (2011), no. 3, pp. 377-382.
35. Uhlendorff, S.; Odefey, U.; Frahm, B. and Pein-Hackelbusch, M.: Performance Comparison between Semi-Automated and Manual Cell Counting for Animal Cell Culture, *Chemie Ingenieur Technik*, **98** (2026), 1-2, pp. 57-63.
36. O'Connor-Cox, E.; Mochaba, F. M.; Lodolo, E. J.; Majara, M. and Axccl, B.: Methylene blue staining: Use at your own risk, *Technical Quarterly*, **34** (1997), no. 1, pp. 306-312.
37. Strober, W.: Trypan Blue Exclusion Test of Cell Viability, *Current Protocols in Immunology*, **111** (2015), A3.B.1-A3.B.3.
38. Carl Roth GmbH + Co. KG: Safety data sheet Trypan blue (C.I. 23850) for microscopy, 2024.

Received 16 July 2026, accepted 12 August 2026