Microbiology

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Key words: *Methylene blue staining, EPI-fluorescent staining, Slide culture method.*

CONCLUSIONS

1. The methylene blue (1,4) and EPI-fluorescence (5) methods for measuring yeast viability gave comparable results and showed similar variabilities for both the ASBC test yeast and the collaborator's own yeast.
2. The slide culture method (2,3,6) gave lower viabilities for both yeasts and also showed greater variability than the other two methods.

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RECOMMENDATIONS

1. Accept the EPI-fluorescence method (Appendix A) as an alternate procedure to methylene blue staining for measuring yeast cell viability.
2. Accept the MYPG slide culture procedure (Appendix B) for measuring yeast cell viability.

PROCEDURE

Yeast Culture Preparation

Each collaborator was supplied with a brewer's yeast culture. In addition, it was suggested that a yeast of the collaborator's choice be tested. The culture medium (MYPG broth + 6% maltose) was also supplied by the chairman. The broth medium was prepared by dissolving 8.1 g of medium in 100 ml of distilled water in 250-ml flasks and autoclaving for 15 min at 15 psi.

The yeast culture was revived by growing in this medium at 50° F

TABLE I
Indicated Yeast Viability Through a Three-Week Storage Period, Comparing Methylene Blue and EPI-Fluorescence Staining with Slide Cultures

Method	Culture	Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	N	Av.	Std. Dev.	c.v.
Methylene blue	ASBC	1	83.0	96.2	93.7	95.9	94.5	97.6	95.8	88.6	96.0	98.8	87.1	95.6	99.0	96.0	96.0	96.3	96.8	93.6	18	94.3	4.23	4.48
		7	78.0	90.3	77.9	81.2	81.2	82.9	73.9	78.4	93.5	68.2	73.1	83.3	76.4	95.8	83.0	72.6	78.9	86.0	18	79.9	6.43	8.05
		14	74.2	86.9	76.3	75.9	75.9	66.7	72.1	74.4	77.0	67.2	79.6	76.6	81.4	80.9	82.6	69.6	72.4	78.8	17	76.0	5.77	7.59
		21	74.2	86.1	75.7	76.4	76.4	77.4	70.2	82.4	56.0	64.9	81.7	79.8	81.8	50.0	72.8	79.8	70.5	80.5	17	75.5	7.65	10.13
Slide culture	ASBC	1	83.6	91.8	94.8	92.3	94.8	96.5	95.5	68.8	95.5	98.2	78.4	92.7	96.0	...	89.0	94.7	97.0	90.9	18	91.2	7.67	8.41
		7	77.0	57.5	74.8	83.9	79.4	74.3	80.3	73.8	89.0	68.0	63.3	71.2	82.0	87.0	89.0	69.1	88.4	70.2	18	75.9	9.02	11.88
		14	61.6	78.5	75.3	84.2	79.9	68.7	75.7	65.8	72.5	61.5	45.6	78.5	64.5	82.0	72.6	67.4	90.3	60.7	17	71.4	10.62	14.87
		21	49.3	73.0	71.5	79.2	78.4	69.5	75.6	42.2	49.5	62.9	11.5	76.2	82.5	80.0	76.0	59.4	81.8	56.9	17	64.9	18.90	29.12
EPI-fluorescence	ASBC	1	...	...	...	...	92.4	98.1	...	72.2	...	98.6	85.8	92.1	97.2	98.0	95.2	90.4	97.9	91.2	12	92.0	7.65	8.32
		7	...	...	...	...	80.3	72.2	...	70.8	...	71.3	72.8	77.9	72.6	82.0	75.0	73.7	80.1	87.5	12	75.8	5.12	6.67
		14	...	...	...	...	80.8	68.9	...	80.2	...	66.0	74.9	74.7	82.1	77.6	80.5	...	67.9	79.5	10	75.1	6.22	8.28
		21	...	...	...	...	76.9	69.2	...	69.6	...	68.3	70.6	74.5	71.3	73.0	71.2	67.0	68.1	83.2	11	70.7	3.04	4.32
Methylene blue	Collab.	1	87.6	89.0	97.1	96.3	96.5	99.3	95.0	97.6	...	99.6	93.2	96.0	100.0	95.0	100.0	93.7	95.6	92.7	17	95.6	3.75	3.92
		7	82.4	87.7	92.8	88.1	97.8	90.7	86.3	74.4	...	95.6	88.1	97.7	90.0	97.0	95.0	49.6	87.3	88.5	17	87.1	11.63	13.35
		14	78.0	86.2	91.1	90.5	96.8	87.0	86.6	92.0	...	92.2	87.5	96.2	92.4	88.2	89.0	46.0	45.8	85.7	16	83.5	15.91	19.05
		21	70.5	83.4	91.3	87.4	96.2	87.1	83.1	93.2	...	95.9	85.2	93.9	86.4	75.0	87.9	77.9	49.5	85.5	16	84.9	11.97	14.10
Slide culture	Collab.	1	88.4	91.8	95.9	94.3	73.1	97.3	95.9	90.8	...	98.8	67.5	70.6	90.4	...	87.0	93.2	97.6	93.0	17	83.1	23.43	28.19
		7	87.2	66.0	85.1	84.4	45.9	79.1	84.3	69.2	...	89.9	73.4	47.0	89.0	90.0	94.0	64.0	89.6	84.9	17	77.0	14.89	19.34
		14	71.8	73.7	76.9	85.5	52.8	71.7	87.3	56.8	...	88.7	37.6	56.6	88.7	86.0	86.0	80.8	72.2	75.7	16	72.2	15.10	20.91
		21	57.3	64.6	79.3	87.0	53.1	64.3	83.7	42.0	...	79.3	5.0	58.5	85.3	85.0	85.0	85.6	56.6	78.3	16	66.2	22.70	34.29
EPI-fluorescence	Collab.	1	...	...	...	...	96.4	99.0	...	94.0	...	99.5	84.7	96.7	92.0	98.4	96.0	87.5	98.1	90.5	12	93.1	6.05	6.50
		7	...	...	...	...	97.0	88.6	...	88.0	...	95.4	87.4	95.9	82.0	90.6	52.0	52.6	84.0	88.9	12	83.3	15.89	19.08
		14	...	...	...	...	95.7	84.3	...	90.2	...	92.6	85.2	93.5	86.9	87.1	85.1	...	37.6	89.6	10	84.0	17.86	21.26
		21	...	...	...	...	94.9	64.7	...	92.6	...	94.2	80.4	92.2	91.6	86.6	84.8	81.5	39.9	89.7	11	82.1	17.67	21.63

for 5–7 days. This provided vigorous cells for the test. Fresh cells from the initial fermentations were used to inoculate the test flasks at the rate of 10^7/ml. The flasks were incubated at 50° F.

Sampling and Viability Estimation

Each yeast fermentation was sampled 1, 7, 14, and 21 days after inoculation. A sterile sample was withdrawn with a 5-ml pipet to provide sufficient cells for a hemocytometer count and subsequent viability estimation. All dyes and reagents for the following procedures were provided by the subcommittee chairman. Details for each test procedure are outlined in Appendixes A and B.

RESULTS AND DISCUSSION

The data are presented in Table I. Examination of the average percent viable cells across days indicates a general decrease in viability with storage time. It is also seen that the variability, as measured by standard deviation, is generally larger for the slide culture method in relation to both the methylene blue and EPI methods. Greater variability is seen for the collaborator's yeast data as opposed to the ASBC yeast data.

Statistical analysis of these data is not amenable to a straightforward treatment, since differences in viability across time are affected by individual laboratory holding procedures. Thus, comparisons across laboratories measure the effect of these holding procedures combined with any between-laboratory method variation and confound the estimation of method variation only. To eliminate this problem, analysis of variance techniques can be used with difference data, ie, the value of the difference between viable percentages using a given method and a base method. This approach allows the estimation of storage time and collaborator effects on the consistency of the given method with respect to the base method. Since the methylene blue method was most familiar to the majority of collaborators, it was chosen as the base method.

Table II contains the analysis of variance for the methylene blue vs slide culture methods for ASBC supplied yeast and collaborator's yeast separately. No evidence for inconsistency between methods was found for either ASBC or collaborator's yeast with respect to storage days; however, collaborators were not consistent. Standard deviations of the differences were quite high, being 10.96 and 11.59% for ASBC and collaborator's yeast, respectively.

Table III contains the analysis of variance for the methylene blue vs EPI-fluorescence methods for ASBC supplied yeast and collaborator's yeast separately. In both cases, no inconsistencies were shown for days of storage or collaborators. Standard deviations of the differences were relatively high at 6.67 and 8.19% for ASBC and collaborator's yeast, respectively. An F-test of these pooled values against the methylene blue vs slide culture pooled standard deviations shows them to be significantly smaller in magnitude.

Correlation analysis, as shown in Table IV, shows significant positive correlation for all but the methylene blue vs slide culture method using collaborator's yeast. In this instance, no significant correlation exists.

From the statistical analyses conducted, it may be stated that the methylene blue and EPI-fluorescence methods tend to yield equivalent information with better precision since storage collaborator effects are not significant, even with disparate yeasts. The slide culture method, at least in comparison to the methylene blue method, does not yield equivalent results, is inconsistent between collaborators, and has less overall precision.

TABLE II
Analysis of Variance Table—Viable Yeast Percentage: Difference Between Methylene Blue and Slide Culture Methods

Source of Variation	Degrees of Freedom	Mean Square	F-Value
	ASBC Yeast		
Days	3	137.65	1.15 (NS)[a]
Collaborator	17	376.69	3.14 **[b]
Error estimate	50	120.5	
Estimated std. deviation of difference = 10.96%			
	Collaborator's Yeast		
Days	3	323.03	2.41 (NS)
Collaborator	16	1,087.00	8.10 **
Error Estimate	47	134.25	
Estimated std. deviation of difference = 11.59%			

[a](NS) = Not significant at $P \leq 0.05$.
[b]** = Significant at $P \leq 0.01$.

TABLE III
Analysis of Variance Table—Viable Yeast Percentage: Difference Between Methylene Blue and EPI-Fluorescence Methods

Source of Variation	Degrees of Freedom	Mean Square	F-Value
	ASBC Yeast		
Days	3	19.21	0.43 (NS)[a]
Collaborator	11	35.89	0.81 (NS)
Error estimate	32	44.56	
Estimated std. deviation of difference = 6.67%			
	Collatorator's Yeast		
Days	3	13.98	0.21 (NS)
Collaborator	11	79.63	1.19 (NS)
Error estimate	32	67.07	
Estimated std. deviation of difference = 8.19%			

[a](NS) = Not significant at $P \leq 0.05$.

TABLE IV
Viable Yeast Percentage: Correlation Analyses

Method Pairing	No. of Obs.	Correlation Coefficient
	ASBC Yeast	
Methylene blue vs slide culture	71	0.47**[a]
Methylene blue vs EPI	47	0.79**
	Collaborator's Yeast	
Methylene blue vs slide culture	67	0.17 (NS)[b]
Methylene blue vs EPI	47	0.82 **

[a]** = Significant at $P \leq 0.01$.
[b]Not significant at $P \leq 0.05$.

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APPENDIX A

SLIDE CULTURE METHOD FOR YEAST VIABILITY

Culture Medium

Ingredients	*Concentration (g/100 ml)*
Malt extract (Difco 01867)	0.30
Yeast extract (Difco 0127)	0.30

Peptone (Difco)	0.50
Glucose	1.00
Agar	1.50

Supplements

Maltose (Sigma), 6%

Zinc sulfate. $7H_2O$, 1 ml of 1.5% solution per 100 ml of medium if production yeasts are used (7).

Procedure

With forceps, hold a 3 × 1-in. slide, and flame over burner. Pipet approximately 1 ml of molten MYPG agar over slide.

After agar has solidified, pipet two drops (from Pasteur pipet) of test suspension (approximately 1×10^6 cells/ml) on agar. Space drops so that cover slip can be placed on each easily. Do not apply pressure to cover slip. Check preparation at 250× to make sure that overcrowding of cells is not a problem.

Place prepared slide in petri dish, cover, and mark. Incubate test slide at room temperature (20–22°C) for 18 hr.

After incubation, examine slide cultures at 250× magnification. Cells that give rise to micro-colonies are counted as viable. Single cells not giving rise to a colony are considered dead. Count at least 500 micro-colonies and cells.

Calculation

Calculate % viable as follows:

$$\frac{\text{No. micro-colonies}}{\text{Total no. cells and colonies}} \times 100 = \%\ \text{viable cells.}$$

Note

Usually the area near the edge of the cover slip is not suitable for counting. Micro-colonies near the edge tend to be confluent. Use center of slide where distribution is better for counting.

APPENDIX B

EPI-FLUORESCENCE STAINING FOR YEAST VIABILITY

Reagents

(a) *8-Anilino-1-naphthalone sulfonic acid-magnesium salt* (Mg-ANS). Make up 0.3% solution of Mg-ANS (ICN Pharmaceuticals, Inc., Life Science Group, Cleveland, OH) in sterile water. This solution can be stored in dark bottles at 4°C for 7 days.

(b) *Saline blank*. Dissolve 0.85% NaCl in distilled water; dispense 9 ml into each 150 × 20-mm test tube, cap, and sterilize by autoclaving at 15 psi for 15 min.

Procedure

Add 2 ml yeast suspension ($1-2 \times 10^6$ cells/ml) to test tube, then mix with equal volume of Mg-ANS dye solution. Let mixture stand 1–5 min.

Place 1–2 drops of above on clean slide and cover with cover slip. Examine slide microscopically at 250× magnification of fluorescence microscope.

Cells fluorescing (cytoplasm as well as periphery) are considered dead. With double tally register, count total number of cells and number of dead cells (fluorescing). Observe at least 500 cells (see Note).

Calculation

Calculate % viable as follows:

$$\frac{\text{No. nonfluorescing cells}}{\text{Total no. fluorescing and nonfluorescing cells}} \times 100 = \%\ \text{viable.}$$

Note

Count mother cells with attached buds as one cell.