Use of Immunofluorescence and Viability Stains in Quality Control[1]

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ABSTRACT

Immunofluorescence has been evaluated as a rapid quality control technique for the detection of low levels of wild yeasts in culture yeast or other brewery samples. Antisera have been prepared against antigenic groups A to F, pooled and absorbed with culture yeast. Using the indirect staining method and fluorescein isothiocyanate or rhodamine as fluorochrome, levels as low as 10 wild yeasts per million cells can be detected in three hours. Combined immunofluorescence and viability staining allows the differentiation of live and dead, culture and wild yeast cells, on the same slide by alternating the light sources. The preferred method uses fluorescein isothiocyanate, excited by incident blue light, for wild yeast detection, combined with methylene blue, viewed by transmitted light, for viability differentiation. Fluorescein diacetate is a useful viability stain for yeasts although agreement between results comparing it with methylene blue and slide culture viabilities is not exact for heat-stressed cells. Some nonbrewing yeasts require heating for two minutes at 50°C before consistent results are obtained with fluorescein diacetate. An examination of the mechanism of the action of methylene blue as a viability stain has suggested that the action is one of permeability, rather than permeability followed by enzymatic reduction.

Key words: *Fluorescein diacetate, Immunofluorescence, Methylene blue, Viability, Yeast.*

Serological techniques are not widely used in quality control in the brewing industry except, perhaps, in the U.K. However, there are a number of applications where they can be of value. These include the rapid detection of wild yeast cells in the presence of the culture yeasts.

Yeast genera can be classified (6) into one or more of six antigenic groups, A to F. Specific antisera against each of these groups can be prepared (7) and Campbell has studied the serological reactions of brewing yeasts (8), *Saccharomyces* species (4,5), and various other genera (6) in considerable detail. Brenner *et al.* (2) showed a relation between *Saccharomyces* antigenic groups and their growth on Schwarz Differential Medium. More recently Callejas *et al.* (3) have reported that Ouchterlony immunodiffusion patterns are promising in the recognition of wild and culture yeast strains. Cowan (10) has studied yeast antigenic structure using the sensitive technique of micro-immune electrophoresis.

We have been using immunofluorescence for some years as a straight-forward technique for the rapid detection of contaminating yeasts in either ale or lager pitching yeast (9) and we have also used the method to study the antigenic responses of parent and mating strains of brewing yeasts (9). We have chosen to use pooled A to F antisera produced using six strains of yeast, in preference to the Institute of Brewing Analysis Committee recommended method (18) which covers the antigenic groups A to D only, and which is produced using only two yeast strains and gives a lower staining intensity. We have preferred the use of fluorescein isothiocyanate (FITC) as fluorochrome because it is readily available commercially conjugated with anti-rabbit immunoglobulin, thus enabling use of the indirect staining technique (23). Haikara and Enari (15) have also used this conjugate in the detection of wild yeast contaminants by the immunofluorescence technique.

A serious disadvantage in the practical adoption of the system has been the failure to differentiate live and dead cells. We have been studying the combined use of immunofluorescent and viability stains so that live and dead, culture and wild yeasts, can be differentiated on the same slide field. This paper details the results obtained when yeast samples are examined alternately with FITC-exciting blue light wavelengths and rhodamine-exciting green light and when incident fluorescent light is combined with transmitted white light to illuminate the specimen.

The potential use of fluorescein diacetate as a viability stain has been established. Initial work by Rotman and Papermaster (22) was developed by Paton and Jones (21) and its use in brewery quality control has been described by Molzahn and Portno (19). Zeigler *et al.* (24) have also studied viability staining using this compound. Fluorescein diacetate is itself nonfluorescent but is hydrolyzed by esterases in live cells to fluorescein. Dead cells reputedly do not possess active esterases and thus do not release the fluorochrome.

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We have evaluated fluorescein diacetate as a viability stain in our situation and have compared the reactions of normal viable cells and heat-shocked cells with fluorescein diacetate, with methylene blue, and by slide viability culture. It is clearly important to know how these methods agree. Viability, as judged by the methylene blue staining, is known to vary with the state of the yeast and agrees more closely with slide viability results when the cells are "in good condition" (14). The mechanism of methylene blue action has been uncertain, however. This uncertainty has been summed up by Hough (17) in his review, "living cells either prevent the entry of the dye into the cytoplasm or they reduce the dye to the colourless 'leuco' form. Dead or dying cells stain blue". We have attempted to clarify the mechanism.

Although we are primarily concerned here with the detection of yeasts, some bacteria can be detected specifically by immunofluorescence. Dadds *et al.* (11) have shown that all *Zymomonas* strains will react with at least one of two specific *Zymomonas* antisera. Dolezil and Kirsop have studied the serology of some *Lactobacillus* (12), as well as *Pediococcus* and *Micrococcus* species (13), in brewing contexts. These publications do not cover viability staining.

EXPERIMENTAL

Yeast Cultures

Yeast cultures used in these viability studies were brewing strains *Saccharomyces cerevisiae* (AB 1) and *S. carlsbergensis* (AB 140) and several wild yeast strains including *S. diastaticus* (NCYC 447), *Hansenula subpelliculosa* (NCYC 119), and *Kloeckera apiculata* (NCYC 465).

Yeasts were maintained on MYGP agar at 4°C and subcultured in MYGP broth or beer before use.

Media

Phosphate buffered saline (PBS) was per liter,

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$NaH_2PO_4 \cdot H_2O$	0.22 g
Na_2HPO_4	1.2 g
NaCl	8.5 g

The solution was filtered through a 0.22 μm membrane filter to remove particulate matter, which might interfere with subsequent microscopic examination, and was autoclaved at 121°C for 10 min. The nominal pH was 7.4.

Preparation of Antisera

Antisera against yeast strains of antigenic groups A to F (Table I) were prepared in rabbits using standard serological techniques and based on the injection routine developed by Campbell and Allan (7). Antisera titers were determined by standard tube or slide agglutination tests; titers of at least 1:320 were desired. Absorption of antibodies from pooled sera (mixed equiproportionally by volume) was based on the technique of Campbell and Allan (7) except that the absorbing yeasts were grown in ale wort in preference to Sabouraud medium. Routinely, the pooled antiserum was absorbed eight times with the chosen culture yeast. Sera, containing sodium azide as preservative, were stored at 4°C. Diluted sera prepared for use were kept frozen at −20°C until required.

TABLE I
Yeast Antigenicity

Antigenic Group	Yeast
A	*Saccharomyces carlsbergensis* NCYC 1116
B	*Saccharomyces fragilis* NCYC 100
C	*Saccharomyces cerevisiae* var. *ellipsoideus* CBS 1395
D	*Saccharomyces microellipsodes* NCYC 411
E	*Schizosaccharomyces pombe* NCYC 132
F	*Hansenula subpelliculosa* NCYC 119

Immunofluorescence Staining Procedures

The indirect procedure shown in Fig. 1 was used routinely when immunofluorescence staining alone was required. When combined with methylene blue viability staining, the modified tube technique shown in Fig. 2, was adopted. It was important in both procedures that the cells under treatment were not allowed to dry out once staining had commenced.

Uninactivated horse serum[2] was used as a source of complement.

Conjugates

Commercially available sheep anti-rabbit immunoglobulin labelled with FITC[2] was reconstituted from the freeze-dried form in PBS to the recommended dilution. Rhodamine-labelled conjugate was also used but was less readily available commercially.

[2]All of the commercial sera used were obtained from Wellcome Reagents Ltd., Hither Green, London SE13 6TL, England.

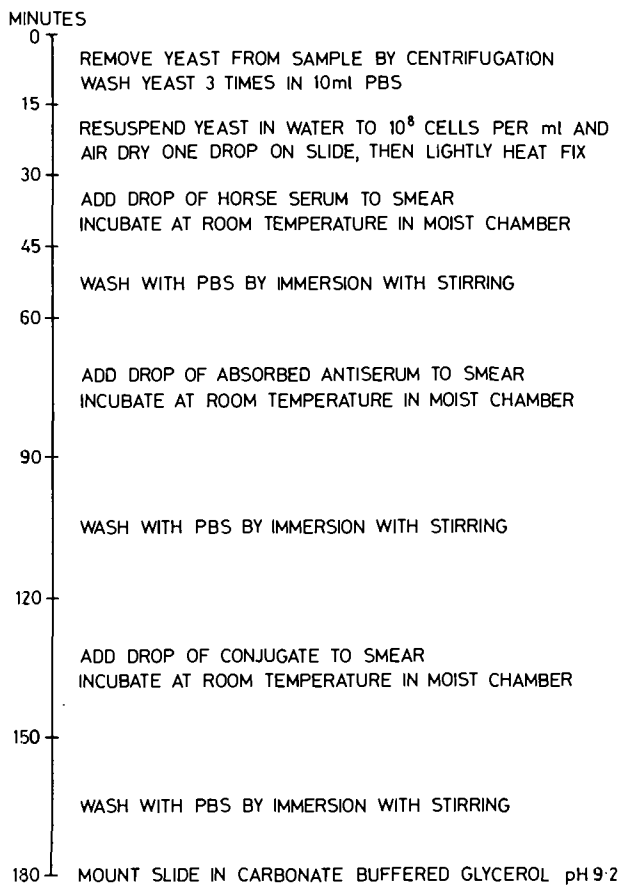

Fig. 1. Indirect immunofluorescence staining procedure.

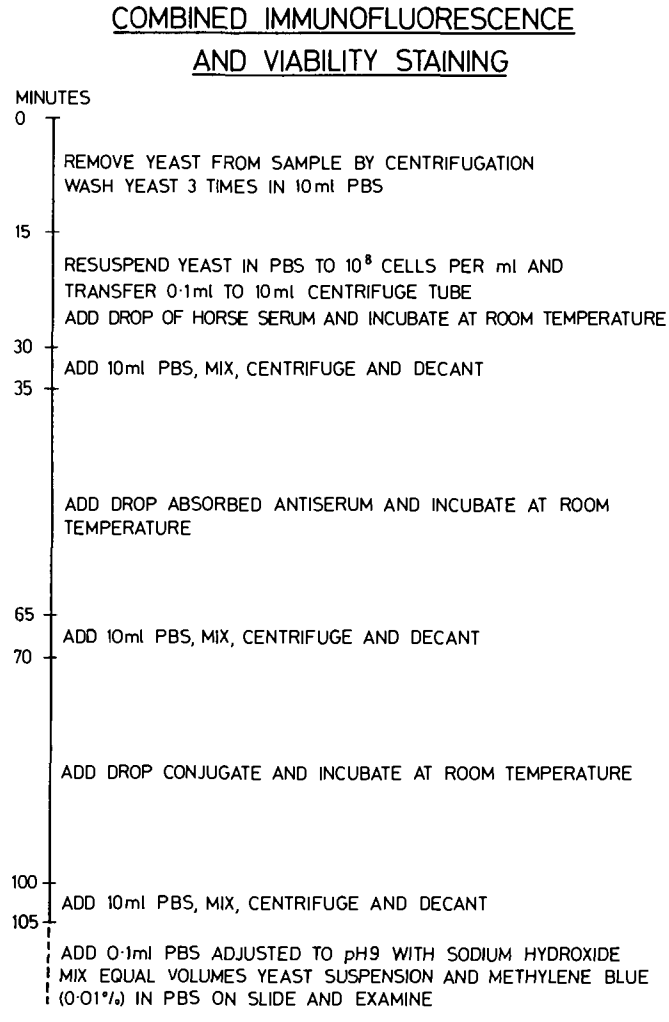

Fig. 2. Combined immunofluorescence and viability staining.

Microscopy

Work was carried out initially using a transmitted light microscope (Wild M 20) having uv and white light sources. FITC was excited under blue light (490 nm) and rhodamine under green light (540 nm) using standard filter systems; for examination appropriate barrier filters were used. Dark-ground illumination was used for each fluorochrome. Yeast cells serologically tagged with FITC fluoresced yellow-green whereas cells tagged with rhodamine fluoresced red. Later work involved the use of a microscope having transmitted white light and incident fluorescence facilities (Reichert Biovar) with the capacity to blend lights onto the specimen. Fluorochromes were excited using similar filters to those mentioned.

Viability Stains

Twenty-nine potential viability stains were evaluated. Those that showed differentiation between live and dead (heated at 60°C for 15 min in saline) yeast cells under blue light (490 nm), green light (540 nm) or white light are listed in Table II. Concentrations of 40 mg/l. in Sorensen's phosphate buffers of pH values 6.5 – 7.5 were tested and contact times between stains and cells were not less than 2 min.

Methylene blue solutions were prepared in PBS and diluted as required to give a final concentration of 50 mg/l. Cells staining blue were considered to be nonviable. A staining time of not less than 2 min was used but was not found to be critical.

Fluorescein diacetate (Sigma, or Koch-Light) stock solution was a 0.5% solution in acetone stored at −20°C. For use 0.05 ml stock solution was added to 10 ml PBS. A staining time of 15 min was used, cells then being washed with PBS. Fluorescent cells were considered to be viable.

TABLE II
Stains Showing Viability Differentiation in Yeasts

Stain	Viability Differentiation Under		
	Blue light FITC[b]	Green light Rhodamine	White light
Acridine orange	Fair	Good	Fair
Acridine yellow	Good	–[a]	–
Acriflavine	Good	Good	Good
Atebrine	Good	Fair	–
Berberine sulphate FS	Good	Good	–
Brilliant dianil green G	Good	Good	–
Congo red	–	Poor	–
Coriphosphine	Good	Good	–
Euchrysine 2GNX	Very Good	Good	–
Evans blue	–	Fair	Fair
Lissamine flavine	Good	Good	–
Lissamine rhodamine RB200	–	Fair	–
Methylene blue	–	–	Good
Neutral red	Fair	Good	–
Pontamine sky blue	–	Good	–
Primuline	Good	Fair	–
Pyronine Y	Fair	Good	–
Rose bengale	Fair	Very Good	Fair
Thioflavine S	Fair	Fair	–
Thionine	–	Fair	–
Titan yellow	Poor	Poor	–
Uranine	Good	Good	–
Xylenol orange	–	Fair	–

[a]– = No differentiation.
[b]FITC = Fluorescein isothiocyanate.

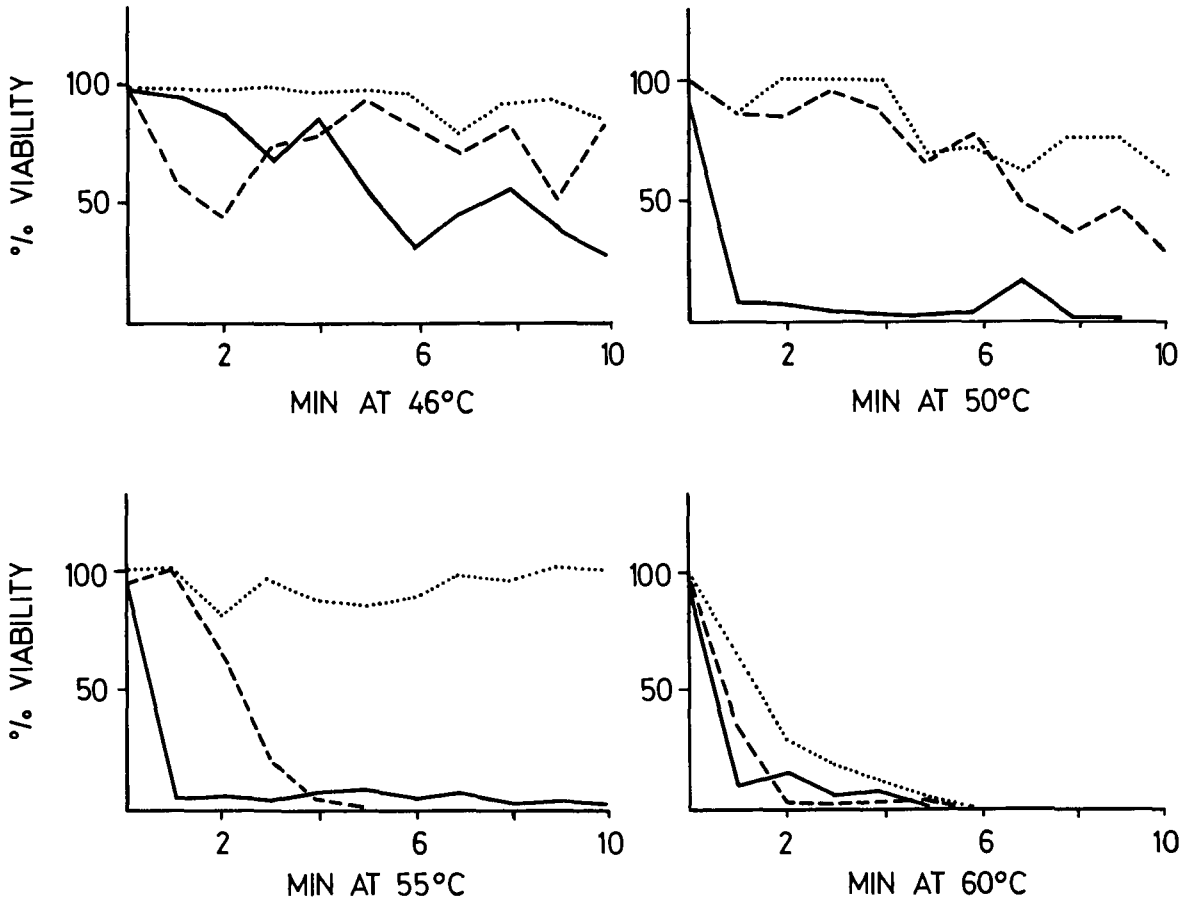

Fig. 3. Viabilities of *Saccharomyces cerevisiae* AB 1 in beer. Dotted lines = methylene blue; dashed lines = fluorescein diacetate; unbroken lines = slide culture.

Slide Culture Viability

Slide culture viabilities were carried out (16) using Wallerstein Laboratory Nutrient (WLN) agar, incubation being aerobically overnight at 28°C.

Heat Shocking of Cells

Yeast cells were heated in either saline (0.25%), PBS, or beer at selected time and temperature conditions. Stressed cells for the comparative work using fluorescein diacetate were heat-shocked in beer at selected temperatures to simulate inadequate brewery pasteurization. Mixtures of live and heat-stressed cells were prepared as required.

RESULTS AND DISCUSSION

Immunofluorescence Staining Procedures

Levels of fluorescing contaminating yeast cells as low as 10 per 10^6 primary yeasts can be detected on slides. However, if this degree of sensitivity is required, it is necessary to ascertain whether any particular primary yeast has a base-level of nonspecific staining. We have encountered the situation with one of our ale yeasts where even 16 absorptions of the pooled antiserum has failed to remove all traces of nonspecific staining, leaving levels of approximately 10 cells per 10^6 that will stain.

One of the principal uses of the technique is in the examination of pitching yeast; however, it also is used to examine concentrated beer deposits, and liquid brewing adjuncts as delivered to the brewery.

The indirect staining technique shown in Fig. 1 is normally trouble-free. The modified version (Fig. 2), in which methylene blue is included, is kept as short as possible to minimize the risk of live cells being killed by the serological steps. For this reason the immunofluorescent tagging is carried out in a tube in preference to on a slide, and the methylene blue is added at the final stage since it will wash out of the cells if added earlier. We have shown that the serological steps do not increase the percentage of cells staining with methylene blue in a yeast suspension if carried out as shown. However, we believe that other combinations of time may be equally successful and that optimum conditions should be established by each worker to suit a particular situation. The precise pH value of the final methylene blue solution is not critical but should be in the range pH 8 – 9 to optimize the FITC fluorescence. Glycerol mountant should not be used in this instance as it increases the percentage of cells that stain blue.

Combined Immunofluorescence and Viability Staining

Table II shows the degree of differentiation obtained between viable and dead cells using a range of potential viability stains under the three light systems. Not unexpectedly, the color of each stain under the FITC system was green-yellow and under the rhodamine system was orange-red. Furthermore, when the viability stain was examined in conjunction with the appropriate conjugate, the stronger fluorescence of the conjugate masked that of the viability stain.

In an attempt to solve this problem identical fields were examined using different filter systems. Some success was obtained; the most satisfactory combination being rhodamine conjugate and

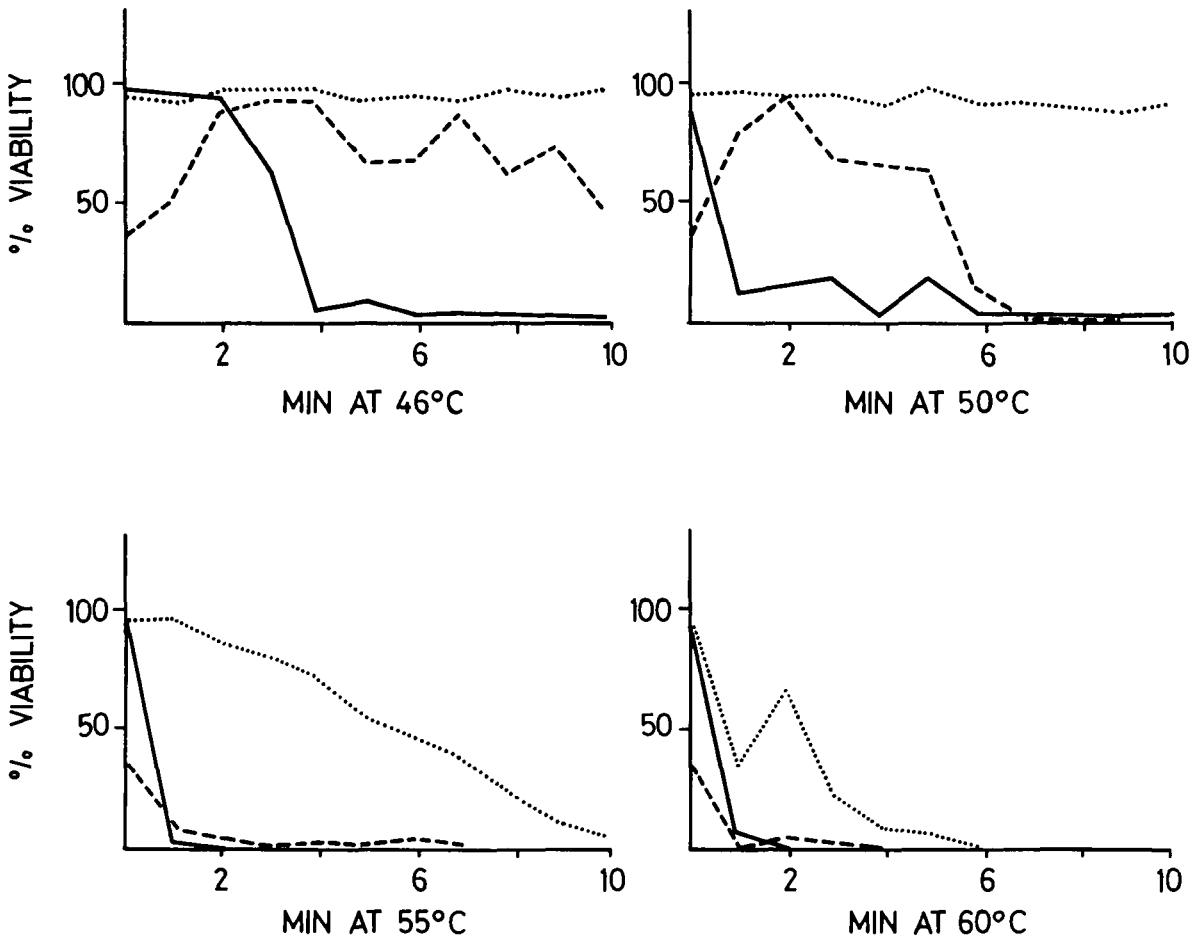

Fig. 4. Viabilities of *Hansenula subpelliculosa* in beer. Dotted lines = methylene blue; dashed lines = fluorescein diacetate; unbroken lines = slide culture.

euchrysine 2GNX for viability. In this case wild yeasts fluoresced red under green light and dead yeast cells showed brighter yellow centers under blue light fluorescence. Thus, all combinations of live and dead, culture and wild yeast cells could be differentiated. The method suffered from the disadvantage that the operator had to remember the results under one light system when looking at the field under the other.

The use of a microscope with facilities for blending incident fluorescent light and transmitted white light allowed the use of nonfluorescent viability stains. We found that the combination of FITC and methylene blue gave good results.

Fluorescein Diacetate as Viability Stain

In a quality control situation where viable organisms, if present, have been collected on a membrane filter, direct microscopic examination of the membrane is potentially a rapid method for detection. The use of a black membrane filter and a fluorescent viability stain (21) gives good contrast for examination using an incident light microscope.

We have compared viabilities of a brewing strain of *S. cerevisiae* (AB 1) and a wild yeast *H. subpelliculosa* by slide culture, and by methylene blue and fluorescein diacetate staining techniques after heat-shocking in beer. With both yeasts (Figs. 3 and 4), viability was lost first by slide culture, then by fluorescein diacetate and finally by methylene blue as heat treatment increased. *Hansenula* (Fig. 4) showed a peculiarity of fluorescein diacetate viability that was found to occur with certain other wild yeasts, including *S. diastaticus* and *Kloeckera apiculata, i.e.*, a short period of about two min at 50°C was necessary before the cells behaved as expected with the viability stain. Unless this pretreatment is given to these cells, viability results obtained are very low and variable. Molzahn and Portno (19) found that preincubation with sodium acetate was necessary to give reliable staining with some yeasts.

It is clear from these results that agreement between these three methods is not good under certain stress conditions and care must be taken in interpreting results. The results are in agreement with the generally accepted view that methylene blue gives misleadingly high viabilities for yeast cells in poor condition.

Limitations of the Combined Staining Techniques

It is apparent that stressed yeast cells can show disagreement between viability as assessed by methylene blue staining and by slide culture. This fact must not be overlooked when results by the combined immunofluorescence-methylene blue staining technique are considered. However, methylene blue results do over estimate the percentage of viable cells in stress conditions and thus err on the safe side in quality control work. Furthermore, despite its limitations, it appears to be an accepted stain for yeast viability determination. It is, of course, possible that organisms heat-stressed in liquid may not show true viability on transfer to a solid growth medium; the whole topic of cell life and death must be treated with caution.

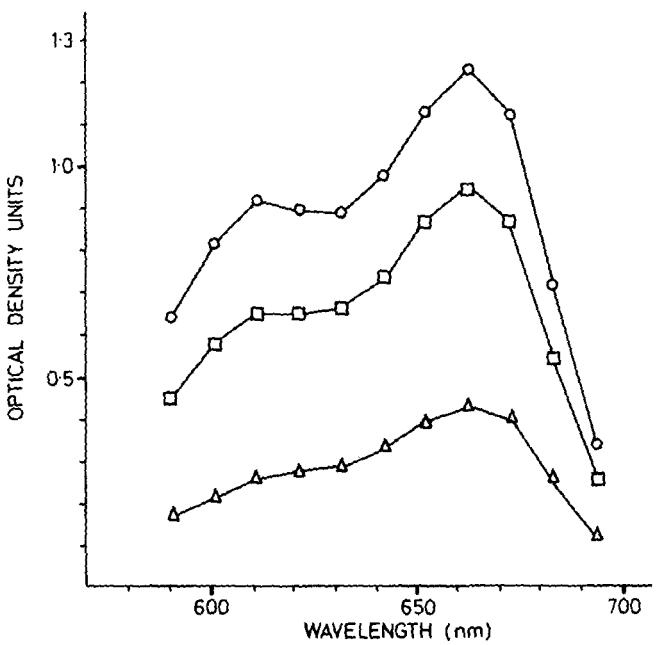

Fig. 5. Absorption spectra of methylene blue (100 mg/l.) after contact with *Saccharomyces carlsbergensis* AB 140. Circles = control, no cells added; squares = 10^6/ml live cells added; triangles = 10^6/ml dead cells added.

Mechanism of Action of Methylene Blue

There is general agreement in the literature that viability staining techniques depend on the permeability of the plasma membrane. This has recently been reiterated by Parkkinen, *et al.* (20) in work on bakers' yeast but uncertainty has existed as to whether methylene blue is subsequently reduced (by a reductase) in live cells (1). We believe that this uncertainty still exists and consequently we carried out studies to attempt to clarify the mechanism. We did not obtain convincing proof to support one mechanism specifically, although evidence points more strongly toward a permeability effect in that the stain does not appear to enter viable cells.

Identical concentrations of live and dead yeast cells suspended in PBS pH 7.4 containing 100 mg/l. methylene blue removed different amounts of the stain from solution (Fig. 5). In addition to removing more stain, the dead cells were visibly blue. The graphs correspond to a contact time of 20 min but the absorbance at about 660 nm for live cells remained unaltered over a period of 2 hr, suggesting that, if enzymatic reduction was occurring: 1. It occurred before measurements were made; and 2. it was to a fixed extent, unlike many bacterial systems where complete decolorization would occur.

We also found that live cells that had been in contact with methylene blue could not be made to turn blue by subsequent killing with heat. Furthermore, alteration of the redox potential of the methylene blue solution by varying the pH from 5–8 gave similar viability results.

We were unable to cause metabolizing brewers' yeast cells to reduce methylene blue in MYGP broth. *Bacillus* species would effect this reduction quite readily although access of atmospheric oxygen would allow redevelopment of the blue oxidized form. Had it been possible to induce metabolizing yeast cells to reduce methylene blue, it would have been possible to study systems affecting this process.

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Literature Cited

1. AMERICAN SOCIETY OF BREWING CHEMISTS. Report of the Subcommittee on Yeast Examination (Biological). Wallerstein Lab. Commun. *6*: 198 (1943).
2. BRENNER, M. W., EGAN, W. P., and HSU, W-P. *Amer. Soc. Brew. Chem., Proc. 1972*, p. 75.
3. CALLEJAS, C. C., RODRÍGUEZ, A. M., and CANALES, A. M. *J. Amer. Soc. Brew. Chem. 35*(1): 12 (1977).
4. CAMPBELL, I. *J. Appl. Bacteriol. 31*: 515 (1968).
5. CAMPBELL, I. *J. Gen. Microbiol. 63*: 189 (1970).
6. CAMPBELL, I. *J. Appl. Bacteriol. 34*(1): 237 (1971).
7. CAMPBELL, I., and ALLAN, A. M. *J. Inst. Brew. 70*: 316 (1964).
8. CAMPBELL, I., and BRUDZYNSKI, A. *J. Inst. Brew. 72*: 556 (1966).
9. CHILVER, M. J., WEBB, T. J. B., and MARTIN, P. A. *Proc. 4th International Symposium on Yeasts (Part 1), Vienna, 1974*, p. 227.
10. COWAN, D. *J. Inst. Brew. 83*: 3 (1977).
11. DADDS, M. J. S., MARTIN, P. A., and CARR, J. G. *J. Appl. Bacteriol. 36*: 531 (1973).
12. DOLEZIL, L., and KIRSOP, B. H. *J. Inst. Brew. 81*: 281 (1975).
13. DOLEZIL, L., and KIRSOP, B. H. *J. Inst. Brew. 82*: 93 (1976).
14. EUROPEAN BREWERY CONVENTION YEAST GROUP. *J. Inst. Brew. 68*: 14 (1962).

15. HAIKARA, A., and ENARI, T-M. *Eur. Brew. Conv., Proc. Congr. 15th, Nice, 1975,* p. 363.
16. HALL, J. F., and WEBB, T. J. B. *J. Inst. Brew. 81*: 471 (1975).
17. HOUGH, J. S. *The Brewer 59:* 69 (1973).
18. INSTITUTE OF BREWING ANALYSIS COMMITTEE. *J. Inst. Brew. 79:* 134 (1973).
19. MOLZAHN, S. W., and PORTNO, A. D. *Eur. Brew. Conv., Proc. Congr. 15th, Nice, 1975,* p. 479.
20. PARKKINEN, E., OURA, E., and SUOMALAINEN, H. *J. Inst. Brew. 82:* 283 (1976).
21. PATON, A. M., and JONES, S. M. *J. Appl. Bacteriol. 38:* 199 (1975).
22. ROTMAN, B., and PAPERMASTER, B. W. *Proc. Natl. Acad. Sci. (U.S.) 55*: 134 (1966).
23. WELLER, T. H., and COONS, A. H. *Proc. Soc. Exptl. Biol. Med. 86:* 789 (1954).
24. ZIEGLER, G. Z., ZIEGLER, E., and WITZENHAUSEN, R. *Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 1. Orig., Reihe A, 230:* 252 (1975).

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