Killer Yeast Identification

Subcommittee Members: I. Russell, *Chairman*; D. Bendiak; R. Berndt; B. K. Blenkinship; D. Fassnidge; S. Fernandez; M. Frati; B. Hjortshoj; S. Holmay; W. M. Ingledew; R. Jones; Y. Lin; T. Lom; M. Moll, European Brewery Convention (EBC); R. B. Saha; H. H. Schopmeyer; D. O. Schisler; G. G. Stewart; P. Swindall; E. L. Van Engel; C. E. Wallin; S. A. Wright; and J. E. Middlekauff (*ex officio*).

Key words: Toxin, Plate assay

CONCLUSION

1. The method tested for assaying whether a yeast strain exhibits killer activity was found to give clear and consistent results.

RECOMMENDATIONS

1. Adopt the killer yeast medium and method as described in the Appendix for inclusion in "Methods of Analysis."

2. Dissolve this subcommittee.

This year, the Subcommittee's task was to evaluate the killer yeast plate assay (1–4) for possible adoption by the Society and inclusion in the "Methods of Analysis."

Killer yeasts are reported as contaminants in both batch and continuous brewery fermentations. Infection of a fermentation by a killer yeast results in the death of the pitching yeast and usually in a beer that is very different from the desired product (e.g., phenolic note, estery off-flavors) (1,4). The majority of brewing strains are sensitive to the action of killer yeasts, and contamination of less than 0.1% of the pitching yeast can result in the total elimination of the brewer's yeast within 24 hr (3).

PROCEDURE

Each collaborator was supplied with three control *Saccharomyces cerevisiae* yeast cultures. Strain A (Labatt strain 1438, S6, obtained from H. Bussey) was the yeast employed as a sensitive lawn, strain B (Labatt strain 1465, killer, A820, obtained from H. Bussey) was the positive control, and strain C (Labatt strain 19, obtained as NCYC 1243) was the negative control. In addition, 10 strains of yeast for which killer character was to be determined were sent to each collaborator.

The cultures were revived on a nutrient medium of the collaborator's choice and inoculated into 10-ml volumes of YEPD at 21° C, for three days. An agar base was prepared by adjusting the pH of PYG medium to 4.5 and, after autoclaving, pouring 20 ml into standard petri dishes.

Overlay tubes were prepared by adjusting the pH of PYG medium to 4.5 and adding 0.003% methylene blue. The medium was stirred on a hot plate until dissolved, and 10-ml aliquots were added to standard size test tubes, which were capped and autoclaved. Before use, the medium was liquefied by stream-steaming for 20 min and placed in a 50° C water bath.

A cell count was carried out on strain A and appropriate dilutions were made to yield 10^5 cells/ml. Each overlay tube was inoculated with 0.1 ml (10^4 cells) of strain A and poured over the agar base. The plates were swirled to ensure that the overlay covered the entire surface of the agar plate and allowed to solidify for 10 min.

Aliquots (0.01 ml) from the YEPD solution of the test strain, the positive control (strain B), and the negative control (strain C) were gently spotted onto the surface of the overlay plate, and the plates were allowed to dry for 30 min. The plates were incubated at 21° C for three days and examined for a zone of clearing of the lawn around the test strains.

RESULTS AND DISCUSSION

The results of the 22 collaborators are presented in Table I. In all cases, duplicate results were in agreement. There was excellent

TABLE I
1986 ASBC Collaborative Study Results

Saccharomyces spp. Strains	Collaborators 1-21	Collaborator 22
1	No clearing[a]	No clearing
2	No clearing	No clearing
3	No clearing	No clearing
4	Clearing	Clearing
5	No clearing	No clearing
6	Clearing	Clearing
7	Clearing	Clearing
8	Clearing	*No clearing*[b]
9	Clearing	*No clearing*[b]
10	No clearing	No clearing

[a] Represents the observation for both replicates.
[b] Italized data are not in agreement with other collaborators.

agreement among the collaborators with the exception of one collaborator who encountered difficulty with two of the strains. This may have been caused by the density of the yeast lawn, which is a critical factor in obtaining clear results, or insufficient growth in the YEPD medium. In summary, the collaborators found the test simple to conduct and encountered no difficulties.

Note Added in Proof:

The three strains employed in this study have been deposited into the ARS Culture Collection and assigned the following numbers:

NRRL Y-17007 (Strain A, sensitive lawn)
NRRL Y-17008 (Strain B, killer positive, control)
NRRL Y-17009 (Strain C, killer negative, control)

They can be obtained by writing to the ARS Culture Collection, United States Department of Agriculture, Agricultural Research Service, Midwest Area Regional Research Center, 1815 North University Street, Peoria, IL 61604.

LITERATURE CITED

1. Hammond, J. R. M., and Eckersley, K. W. *J. Inst. Brew.* 90:167, 1984.
2. Panchal, C. J., Meacher, C., Van Oostrom, J., and Stewart, G. G. *Appl. Environ. Microbiol.* 50:257, 1985.
3. Russell, I., and Stewart, G. G. *J. Am. Soc. Brew. Chem.* 43:84, 1985.
4. Young, T. W. *J. Inst. Brew.*87:292, 1981.

APPENDIX

KILLER YEAST IDENTIFICATION

Killing by a killer yeast is an easily scored and well-defined phenotype (1–4). An agar plate seeded with a sensitive strain and then inoculated with a killer strain will produce a clear zone of inhibition of the seeded sensitive lawn (Fig. 1). This technique is a simple screening assay to determine if a yeast secretes killer toxin.

Media

(a) YEPD Medium

Yeast extract	10.0 g
Peptone	20.0 g
Glucose	20.0 g
Distilled water to	1 L

Sterilize the media for 20 min at 121° C.

(b) PYG Agar base

Peptone	3.5 g
Yeast extract	3.0 g
Potassium phosphate monobasic KH_2PO_4	2.0 g
Magnesium sulfate $MgSO_4 \cdot 7H_2O$	1.0 g
Ammonium sulfate $(NH_4)_2SO_4$	1.0 g
Glucose	10.0 g
(Adjust pH to 4.5 with 1*N* HCl)	
Agar	30.0 g
Distilled water to	1 L

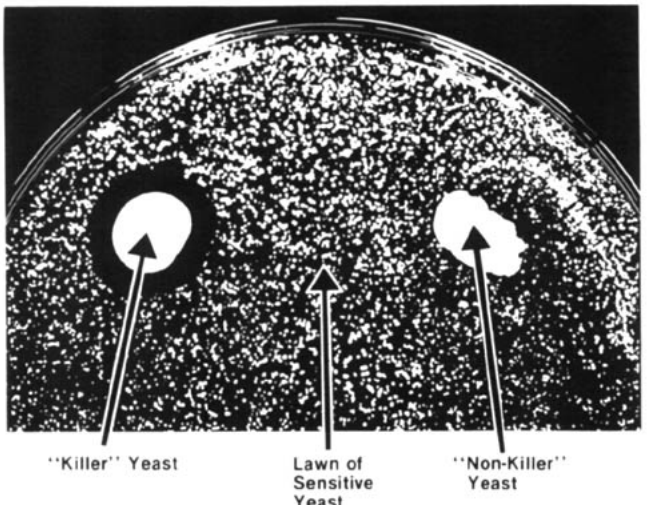

Fig. 1. Killer-sensitive and insensitive phenotypes. An agar plate seeded with a sensitive strain is inoculated with a killer yeast and a nonkiller yeast in addition to the test strain.

Sterilize the media for 20 min at 121° C and, after cooling, pour into petri dishes.

(c) PYG Overlay tubes

Peptone	3.5 g
Yeast extract	3.0 g
Potassium phosphate monobasic KH_2PO_4	2.0 g
Magnesium sulfate $MgSO_4 \cdot 7H_2O$	1.0 g
Ammonium sulfate $(NH_4)_2SO_4$	1.0 g
Glucose	10.0 g
Methylene blue	0.03 g
(Adjust pH to 4.5 with 1*N* HCl)	
Agar	12.0 g
Distilled water to	1 L

Sterilize the media for 20 min at 121° C. To reliquefy, streamsteam for 20 min and place into a 50° C water bath.

Apparatus

(a) *Autoclave*
(b) *Water bath,* 50° C
(c) *Petri dishes and test tubes*
(d) *Cell counting chamber*
(e) *Pipettes*
(f) *Incubator,* 21° C
(g) *Platinum loop*

PROCEDURE

1. Streak a loopful of a pure culture of the yeast to be tested onto nutrient media plates. Also streak out the strain to be used for the killer-sensitive lawn and a positive and negative control.
2. Inoculate bottles containing 10 ml of YEPD medium with a loopful of cells from the nutrient media plates and incubate for 72 hr.
3. Perform a cell count on the strain to be used for the lawn and dilute to 10^5 cells/ml. Inoculate each overlay tube with 0.1 ml (10^4 cells) of the lawn strain and pour this tube over the agar base.

4. Swirl the plates to ensure that the overlay covers the entire surface of the agar base and allow to solidify.
5. Gently spot 0.01 ml of the test strain from the YEPD solution onto the surface of the overlay plate. Allow the plate to dry completely before moving it (30 min).
6. Incubate the plate at 21° C for three days and examine under light for a zone of inhibition. Sensitive cells killed by killer toxin accumulate methylene blue and appear as a ring of blue cells around the clear zone of inhibition.
7. Always ensure that a negative and positive control are present on the same plate as the test strain.

REFERENCES

1. Hammond, J. R. M., and Eckersley, K. W. *J. Inst. Brew.* 90:167, 1984.
2. Panchal, C. J., Meacher, C., Van Oostrom, J., and Stewart, G. G. *Appl. Environ. Microbiol.* 50:257, 1985.
3. Russell, I., and Stewart, G. G. *J. Am. Soc. Brew Chem.* 43:84, 1985.
4. Young, T. W. *J. Inst. Brew.* 87:292, 1981.