Dichlorofluoromethane Inactivates Saccharomyces cerevisiae

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Saccharomyces cerevisiae was incubated in aerosol cans containing YM broth and dichlorofluoromethane (f-21). The presence and number of viable cells were determined by inoculating (1% vol/vol) YM broth and by the plate count procedure (YM agar). Inactivation of the yeast was greater or more rapid when: (i) the thermodynamic activity (saturation value) of f-21 became greater through increasing the concentration of chemical from 0.5 to 1.5% (wt/wt) in a given volume (20, 40, or 80 ml) of broth, or by holding the concentration of chemical constant but increasing the volume of broth in the test vessel, (ii) the temperature of treatment was increased (7, 22, 37, and 47 C), (iii) samples with 1.5% (wt/wt) f-21 were agitated, (iv) young (8 h) rather than old (36 h or 10 days) cells were treated, and (v) cells were grown in YM broth without, rather than with, glucose. Adjusting the pH (6.3 to 4.0) of broth before treatment, pretreating the substrate with f-21, or distilling the chemical before use had no effect on viability of cells when treated with f-21. Yeast cells inactivated by f-21, chlorine, or heat were more resistant to disruption by sonic treatment than were viable cells.

Dichlorofluoromethane (f-21; CHCl₂F) is a colorless, nearly odorless, nonflammable, and non-explosive gas at ordinary temperatures and is readily liquefied at pressures below its vapor pressure (2, 3, 5). The compound boils at 8.9 C, freezes at −135 C, and is slightly soluble in water (0.95% by weight at 25 C) (3, 5). It is stable under neutral and acidic conditions but undergoes hydrolysis rapidly under alkaline conditions. f-21 forms a type II hydrate consisting of 17 mol of water per mol of chemical and having a melting point of 8.62 C. It is relatively nontoxic, being classed between groups 4 and 5 in the Underwriters’ Laboratories’ classification (1).

Relatively little published information is available concerning the effects of fluorinated hydrocarbons on microorganisms. Lie (M.S. thesis, Univ. of Wisconsin, Madison, 1966) found that trichlorofluoromethane was detrimental to Pseudomonas striata. According to Reed and Dychala (9), dichlorodifluoromethane and 1,2-dichloro-1,2-tetrafluoromethane inhibited growth of Streptococcus agalactiae, Aspergillus niger, and Paeclomyces varioti. Inhibition of methane synthesis by rumen fluid was observed by Bauchop (4) when the fluid was treated with Antifoam. Bauchop attributed the inhibition to dichlorodifluoromethane, which served as a propellant for the silicone Antifoam.

A more extensive study was done by Prior et al. (8), who used propane, dichlorodifluoromethane, and 1,1-difluoro-1-chloroethane. These authors found that dichlorodifluoromethane and 1,1-difluoro-1-chloroethane partially or completely inactivated populations of many different microorganisms, including Saccharomyces cerevisiae. No reports have appeared on how f-21 affects microorganisms.

This study was done to determine if f-21 can inactivate S. cerevisiae and, if so, to define some of the parameters that govern this inactivation. Since f-21 is relatively inert and nontoxic, any antimicrobial property it possesses could be useful in some industrial applications.

MATERIALS AND METHODS

Treating S. cerevisiae. S. cerevisiae NRRL Y-2034 (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, I11.) was propagated (16 h at 22 C) twice in YM broth (Difco) before it was used as a test culture. For some experiments, YM broth prepared to contain all ingredients of the medium except glucose was used to grow the yeast, and for others regular YM broth and incubations of 8, 12, and 36 h, or 10 days were used instead of 16 h.

Sterile YM broth was inoculated to contain approx-
imately 10⁴ yeast cells/ml and then was dispensed (20-, 40-, or 80-ml quantities) into sterile double-
epoxy-lined metal cans (5.5 by 6.0 cm, 97-ml capacity,
Continental Can Company, Chicago, Ill.). Cans
were then weighed and the contents were chilled to
7 C. The headspace contained air of normal composi-
tion.

Liquid f-21 (99.0% purity, Matheson Co.; solubility
in water at 37 C is 0.62 g/100 ml) at 7 C was pipetted
into the precooled samples, and cans were then sealed
(model LC sealer, John R. Nalbach Engineering Co.,
Inc., Chicago, Ill.) with sterile stainless-steel caps
that lacked dip tubes (Avoset Co., Oakland, Calif.).
Sealed cans and their contents were shaken thor-
oughly and then weighed again. All cans that con-
tained the desired weight of f-21 or up to 0.05% more
than the desired weight were used for a given experi-
ment. The calculated fraction of saturation (grams of
dichlorofluoromethane added divided by grams of
chlorofluoromethane needed to saturate the liquid
and vapor phases in the aerosol can) attained under
the various conditions used in this study are listed in
Table 1. “Fraction of saturation” (or thermodynamic
activity) will hereafter be designated by the symbol S.

Cans of inoculated YM broth without added f-21
were sealed and used as controls. When necessary,
treated samples and appropriate controls were placed

Table 1. Calculated* fraction of saturation obtained
when f-21 was added to 97-ml aerosol cans containing
various amounts of broth

| Temp (C) | f-21 added | Can contents | Fraction of saturation obtained |
|---------|------------|--------------|--------------------------------|
| 7       | 0.3        | 1.5          | 20 | 77 | 0.38 |
| 22      | 0.1        | 0.5          | 20 | 77 | 0.11 |
|         | 0.15       | 0.75         | 20 | 77 | 0.17 |
|         | 0.3        | 1.5          | 20 | 77 | 0.33 |
|         | 0.2        | 0.5          | 40 | 57 | 0.18 |
|         | 0.3        | 0.75         | 40 | 57 | 0.27 |
|         | 0.6        | 1.5          | 40 | 57 | 0.53 |
|         | 0.4        | 0.5          | 80 | 17 | 0.25 |
|         | 0.6        | 0.75         | 80 | 17 | 0.58 |
|         | 1.2        | 1.5          | 80 | 17 | 0.76 |
| 37      | 0.3        | 1.5          | 20 | 77 | 0.26 |
|         | 0.6        | 0.75         | 80 | 17 | 0.40 |
| 47      | 0.3        | 1.5          | 20 | 77 | 0.22 |
|         | 0.6        | 0.75         | 80 | 17 | 0.40 |

* Based on data from the Matheson gas data book
(5) and DuPont Technical Report KSS-3560 (7a). It
was assumed that f-21 behaves as an ideal gas and
that the solubility of f-21 in water and in YM broth was
equal.

* Grams of f-21 present/grams of f-21 needed for
saturation of liquid and gas phases.

in a water (50 C) bath and warmed to the desired
incubation temperature. Samples were then incu-
bated quiescently or with agitation (180 cycles/min,
platform shaker, model 6000, Eberbach Co., Ann
Arbor, Mich.) at uncontrolled room temperature or at
7, 22, 37, or 47 C.

Determining viability of treated yeast cells.
Duplicate cans of treated yeast cells in broth were
removed from incubation at various intervals and
weighed to insure that no loss of f-21 had occurred.
The bottom of each acceptible can was swabbed with
alcohol and pierced (pierced by growth of yeast in fresh
or treated broth, always coincided with appearance
of colonies on YM agar. Plates were incubated at 22 C
for 5 days and then colonies, if present, were counted.
Additionally, YM broth was inoculated (1%) with treated
sample, incubated at 22 C for 5 days, and then examined
for growth of S. cerevisiae. Finally, 10 ml (or somewhat
less when necessary) of treated sample was transferred to a
sterile test tube, shaken to facilitate removal of f-21,
and incubated at 22 C for up to 7 days. Viability of
cells, as indicated by growth of yeast in fresh or
treated broth, always coincided with appearance
of colonies on YM agar. Hence, data reported in this
paper are limited to those obtained with the plate
count method.

Resistance of treated cells to sonic treatment.
YM broth was inoculated to contain approximately
10⁴ cells of S. cerevisiae per milliliter and then was
incubated quiescently for 16 h at 22 C. Substrate and
cells were cooled to 7 C and portions were treated with
1.5% f-21 for 2 h, 200 mliters of chlorine (BK powder,
Pennwall Corp., Philadelphia, Pa.) per milliliter of heat
(80 C) for 30 min. All treatments rendered the
substrate free of viable cells. Cells were recovered by
centrifugation, washed twice with sterile distilled
water, and then suspended in distilled water so that
an optical density value of 0.80 to 0.85 at 500 nm
(Spectronic 20, Bausch & Lomb) was obtained. Cells
were then sonically treated at 9 A for 60 min (Sonifier,
model LS-75, Branson Instrument Co., Inc., Stam-
ford, Conn.). Lysis of cells was determined by a
change in optical density of the cell suspension and by
the presence of soluble protein in the cell-free prep-
paration. Samples for protein determination were fil-
tered (0.45 mm pore size, Millipore Corp., Bedford,
Mass.) to remove whole cells and any fragments.
Protein was then measured by the method of Lowry et al. (6).

RESULTS AND DISCUSSION

Concentration of f-21. Three concentrations
(0.5, 0.75, and 1.5% wt/wt) of f-21 were used to
treat 12-h-old cells of S. cerevisiae. As expected,
an increase in concentration of chemical when the
volume of broth was constant resulted in greater
and/or more rapid inactivation of the yeast (Fig. 1).
When the concentration of chemical was held constant but the volume of broth
in cans, and hence headspace, was varied, the
greatest biocidal activity, regardless of concen-
tration of f-21, always occurred in cans with the
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Fig. 1. Survival and inactivation of S. cerevisiae at 22 C in 20, 40, and 80 ml of YM broth that contained 0.5, 0.75, or 1.5% f-21.

The largest volume of liquid. Hence, complete inactivation by 1.5% (wt/wt) of f-21 occurred in <1 h in 80 ml of broth, but 3 and 12 h were required when the same concentration of chemical was added to 40 and 20 ml of broth, respectively.

The reason for this behavior becomes clear when the data on degree of saturation (Table 1) are examined. At the 0.5% concentration of f-21, increasing the volume of broth from 20 to 80 ml caused the S value for f-21 to rise from 0.11 to 0.25. At the 0.75 and 1.5% concentrations of f-21, corresponding increases in the S value were, respectively, 0.17 to 0.38 and 0.33 to 0.76. Interestingly, 0.75% f-21 in 80 ml of broth resulted in the same inactivation time as 1.5% f-21 in 20 ml of broth. This would be expected since f-21 in these two samples was present at approximately the same S value (0.38 for 0.75% in 80 ml, 0.33 for 1.5% in 20 ml).

It is obvious from these data that, as the volume of liquid increased and the headspace decreased, less chemical was lost from the quiescently incubated liquid into the headspace during establishment of equilibrium.

In another experiment, f-21 was added to provide concentrations of 0.50, 0.75, 0.90, 1.0, 1.1, 1.2, 1.3, and 1.5% by weight in 80 ml of YM broth inoculated with a 12-h-old culture of S. cerevisiae, and these samples were then held quiescently at 22 C. The time required for complete inactivation of yeast cells (approximately 107/ml) was determined and expressed as the log of time in minutes (Fig. 2). At a concentration of 0.25% (not shown on the figure), f-21 was not biocidal, but it was biostatic for 48 h. When concentrations of 1.3 and 1.5% were used, f-21 completely inactivated the yeasts in <2 min. This test was done only with the 80-ml volume of broth since the previous experiment (Fig. 1) showed that this condition was optimal over the range tested for inactivation of the yeast.

Age of culture. YM broth was inoculated with yeast cells of different ages (8 h, 36 h, and 10-day-old broth cultures and 10-day-old YM agar slant cultures). Twenty-milliliter quantities of such broth received 1.5% f-21 (S = 0.33) and 80-ml quantities received 0.75% (S = 0.38) of the chemical. Samples were incubated qui-
escently at room temperature for 24 h. Cells (no detectable spores by malachite green procedure of McClung [7]) from the 10-day-old agar slants were most resistant to f-21 (Fig. 3). Ten-day-old cells from broth were somewhat less resistant to the chemical than those from the agar slant. Still less resistance was noted with 36-h-old cells, and 8-h-old cells were most easily inactivated by the chemical.

Once again little difference was apparent between the biocidal effects of 1.5% f-21 in 20 ml of broth and 0.75% f-21 in 80 ml of broth. The similarity of their S values is of obvious importance.

Temperature. Two concentrations of f-21, 0.75% in 80 ml and 1.5% in 20 ml of YM broth containing 12-h-old yeast cells, were used to determine inactivation of S. cerevisiae at 7, 22, 37, and 47 C. Total inactivation of yeast occurred most rapidly (between 1 and 3 h) at 47 C and was slowest (between 16 and 24 h) at 7 C (Fig. 4). Periods of 9 to 12 h at 22 C and 5 to 7 h at 37 C were required to inactivate the yeast (Fig. 4). These data once again indicate that 0.75% f-21 in 80 ml yielded results essentially the same as those obtained with 1.5% f-21 in 20 ml of broth.

This result is not surprising at 7 and 22 C since the two samples had very similar S values at these two temperatures. However, at 37 and 47 C the calculated S values differ considerably (0.40 versus 0.26 at 37 C and 0.40 versus 0.22 at 47 C, with the 0.75% 80-ml sample having the higher S value in both instances).

The insensitivity of S. cerevisiae to f-21 when the S value ranged from 0.2 to 0.4 may be partially accounted for by the trend shown in Fig. 2. From this plot it is evident that the 0.5% (S = 0.25) concentration of f-21 in 80 ml of broth was weakly biocidal at 22 C, and that rapid changes in effectiveness with increasing concentration of f-21 did not occur until 0.75% (S = 0.38) was surpassed. Furthermore, the instability of control cells at 47 C may have tended to obscure differences between the two test samples.

Agitation during incubation. Two concentrations of f-21 (0.5 and 1.5% in 20 ml of YM broth) and two levels of yeast cells (approximately 10^8 and 10^9/ml) were used to determine

![Fig. 3. Inactivation of young and old cells of S. cerevisiae by f-21.](image)

![Fig. 4. Inactivation of S. cerevisiae by f-21 at 7, 22, 37, and 47 C.](image)
whether agitation (180 cycles/min) during incubation at 22 °C influenced inactivation of *S. cerevisiae* by the chemical. The data (Fig. 5) indicate that agitation enhanced the effectiveness of f-21 when the substrate contained 1.5% of the chemical but not when 0.5% was present. Agitation afforded the yeast better access to substrate and O₂, thus apparently overcoming the biostatic effect of 0.5% f-21.

**Presence of glucose in the substrate.** *S. cerevisiae* was propagated in YM broth with and without glucose and then was treated with 1.5% f-21 (S = 0.33) in 20 ml of YM broth with and without glucose. Incubation during treatment was quiescent and at room temperature. The data (Fig. 6) indicate that both growth and treatment of the yeast in broth free from glucose were needed before resistance to f-21 was reduced. In this instance, total inactivation occurred in 5 h rather than 9 h, as was true of the other treatments.

**Factors without effect on inactivation of yeasts.** The pH of YM broth, normally 6.3, was adjusted to 4.0 with 1 N HCl. A 20-ml volume of broth was inoculated to contain approximately 10⁴ yeast cells/ml (12-h-old culture) and then treated with 1.5% of f-21 (S = 0.33). Samples were incubated for 12 h at 7 and 22 °C. This change in pH of the substrate had no apparent effect on inactivation of yeast by f-21 (data not shown).

The f-21 used in some experiments was slightly yellow in color. To help insure that inactivation of yeasts resulted from the chemical and not from an impurity, f-21 was distilled at 9 °C and a clear colorless liquid was obtained. This liquid was then tested (0.75% in 80 ml of YM broth containing approximately 10⁴ yeast cells/ml) at room temperature, and results were not significantly (P = 1%) different from those obtained with commercial f-21 (data not shown). Of course, distillation does not assure complete removal of contaminants, if any were present, but it is a process that is commonly used to purify various substances.

In another experiment, YM broth was treated with 1.5% f-21 for 24 h at room temperature.

![Fig. 5. Behavior of *S. cerevisiae* at 22 °C when treated with 0.5 and 1.5% f-21 in 20 ml of YM broth held with and without agitation. Symbols: ○, control; 10⁴ cells/ml; ●, control, 10⁸ cells/ml; ■, treated, 10⁴ cells/ml; ●, treated, 10⁸ cells/ml.](image)

![Fig. 6. Inactivation of *S. cerevisiae* propagated in YM broth with and without glucose and treated with 1.5% f-21 in 20 ml of YM broth with and without glucose.](image)
The chemical was then removed by warming the broth to 50 C and flushing with air for 30 min. Treated broth was inoculated with S. cerevisiae and incubated at 22 C for 24 h. Growth of yeast in treated and untreated broth was similar (data not shown).

**Resistance of treated yeast cells to lysis by sonic treatment.** Yeast cells were inactivated by f-21 (1.5%), chlorine (200 μliters/ml), or heat (80 C) and then subjected to an ultrasonic treatment. Although this treatment caused marked lysis of viable cells (control), inactivated cells remained intact (data not shown). This suggests that inactivation, regardless of the method used, may have altered the cell wall sufficiently to prevent lysis by the ultrasonic treatment. Microscope observation of inactivated cells after sonic treatment revealed the absence of cell wall fragments.

Data reported in this paper have demonstrated the efficacy of f-21 to inactivate S. cerevisiae under a variety of conditions. Although f-21 is not approved by the Food and Drug Administration for use with foods, use of the compound to control microorganisms in nonfood industrial applications may be feasible. Depending on the potential application, it may be necessary to do additional tests to determine that microorganisms involved are susceptible to the action of f-21.

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