Some Factors Affecting the Activity of Diethylpyrocarbonate as a Sterilant

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Quantitative data indicated logarithmic death in 5° Brix Concord grape juice when concentrations of cells under $10^7$/ml were exposed to diethylpyrocarbonate (DEPC). Species differed considerably in their resistance; e.g., 50 ppm reduced the viable count of Saccharomyces cerevisiae over nine $\log_{10}$ cycles, whereas 200 ppm reduced the count of Byssoschlamys fulva ascospores by only about 1 log. DEPC lethality was enhanced by higher temperatures; destruction at 40°C was 10- to 100-fold greater than at 20°C. Studies on death rates showed that most yeasts and fungal spores were killed during the first hour of exposure, whereas 24 h or longer was needed for maximal destruction of several lactic acid bacteria. Repair of DEPC-induced damage was believed responsible for the slower death rates of the lactics.

Diethylpyrocarbonate (DEPC) has been used for the preservation of various acid foods such as fruit juices, wine, and beer. An attractive property of the compound is that it is rapidly hydrolyzed to carbon dioxide and ethanol and thus persists for only a short time in aqueous solutions. Although DEPC can no longer be added to foods in the United States, it is likely that applications will be found for its use as a sterilant of other materials.

Numerous studies have dealt with its effectiveness as a preservative of various foods, mostly fruit products (1, 3, 5, 7, 8). In general, these reports have shown the levels of DEPC that either preserved or failed to preserve a food contaminated or inoculated with various organisms. Surprisingly, little quantitative information is available regarding the response of different organisms to the compound when treated under various conditions, and such information was the objective of our research.

MATERIALS AND METHODS

Cultures. Various media and conditions were used for the propagation of the different test organisms. Saccharomyces cerevisiae and a flor sherry strain of yeast were grown in 5° Brix Concord grape juice supplemented with 0.2% yeast extract. The broth cultures, 50 ml in 500-ml Erlenmeyer flasks, were incubated for 24 h at 30°C on a rotary shaker. Conidia of Aspergillus niger were flushed with sterile water from the surface of potato-dextrose-agar slant cultures that had incubated for 3 days at 32°C. The spores were washed eight times in water before being stored as aqueous suspensions at 5°C. The conidia of Byssoschlamys fulva were obtained from a strain that produced large numbers of this spore type and very few ascospores (11). The media and harvest procedures were similar to those used with A. niger. Ascospores of B. fulva were from strain H-36 that was grown as a pellicle over 15° Brix Concord grape juice for 28 days at 32°C. Details regarding our methods of harvesting, freeing of the spores from ascii, and cleaning have been published (11, 12). Leuconostoc mesenteroides and Lactobacillus plantarum were grown in Tryptone-glucose-yeast extract (TGYE)-salts-broth (13) and incubated 48 h at 32°C.

The viable count of the yeasts and molds was made on potato-dextrose-agar, pH 3.5, by pour-plate techniques. The two lactic-acid bacteria were plated on TGYE-salts agar. When very low numbers of survivors were expected, most-probable-number techniques were applied by use of the appropriate broth media.

Sterilization trials. Concord grape juice, pH 3.4, adjusted to a concentration of 5° Brix with water served as the standard treatment menstruum. In a typical trial, juice equilibrated to a certain temperature was inoculated with cells that had been diluted in similar juice to give a desired viable population. A solution of diethylpyrocarbonate (DEPC), freshly diluted in absolute ethanol, was then quickly added and thoroughly mixed. The volume of alcoholic DEPC was such that the final ethanol concentration in the treatment medium never exceeded 2%. Incubation in...
RESULTS AND DISCUSSION

Cell concentration. In a number of trials different cell populations were exposed to a given level of DEPC. As data with two organisms illustrate (Table 1), the percent survival or log₁₀ reduction in viable count was, in general, independent of the initial population. The exception to this was when relatively dense suspensions were treated as can be seen with the flor yeasts where the proportion that was destroyed was less at cell concentrations of 10⁷ per ml or more. The effect of high concentration was most evident with the two yeast cultures, perhaps because their relatively large cells reacted with sufficient DEPC to deplete it from the treatment menstruum.

As a result of these studies, the cell concentration used in most experiments was sufficiently diluted to assure logarithmic destruction and thus permit the data to be presented either as percent survival or log₁₀ reduction.

Species resistance. The six organisms that were studied showed marked differences in resistance (Table 2). S. cerevisiae 223, a strain used in winemaking, was most sensitive, whereas the ascospores of B. fulva were affected least. Our data with S. cerevisiae are in agreement with various reports (2) of yeasts readily killed by DEPC. On the other hand, the much higher survivals manifested by the flor yeast, believed to be a strain of S. oviformis, indicates considerable difference between species. A relationship may exist between resistance to DEPC and to other factors. Thus, the ascospores of B. fulva are able to withstand other adverse effects, such as being heated for several hours at 80 C and being exposed to strong solutions of ethanol, whereas the more DEPC-sensitive conidia of B. fulva and A. niger cannot survive these treatments.

Differences in lethality figures often were observed among experiments conducted under supposedly identical conditions. For example, in 17 trials in which L. plantarum was exposed to 100 ppm of DEPC, the log₁₀ reduction values ranged from 0.72 to 3.6, 2.5 being the average. The reasons for this variation are not well understood, although a difference in the physiological age of cultures is suspected to be a factor. Supporting this idea were the results of one study in which 24-, 31-, 48- and 55-h cultures of L. plantarum yielded log₁₀ reductions of 2.4, 1.3, 0.86, and 0.83, respectively.

The data in Table 2 also show the effect of varying the level of DEPC. As one would expect, increasing the concentration resulted in greater cell destruction. Plots of kill data versus DEPC levels failed to give straight-line responses, therefore it has not been possible to extrapolate accurately the effect of exposing an organism to even higher concentrations.

Treatment temperature. The maintenance of higher temperatures during the incubation of cells with DEPC resulted in significantly lower percent survivals (Table 3). It can be seen that with three of the organisms, the survival figures at 20 C were 10- to 100-fold higher than at 40 C, whereas with the ascospores of B. fulva no lethal

| Table 1. Effect of cell concentration on the lethality of diethylpyrocarbonate |
|---------------------------------|-----------------|-----------------|-----------------|
| Microorganism                   | Viable count/ml × 10⁶ | Survival (%) | Log Reduction (log₁₀ n₀/n)² |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Before          | Post-treatment  |                  |                  |
|                                | Pre-treatment   | (50 ppm DEPC)  |                  |                  |
| Flor yeast                      | 640             | 400             | 62              | 0.21             |
| 320                             | 62              | 19              | 0.71            |
| 160                             | 5.2             | 3.2             | 1.5             |
| 100                             | 0.92            | 0.92            | 2.0             |
| 40                              | 0.042           | 0.10            | 3.0             |
| 2.0                             | 0.0020          | 0.10            | 3.0             |
| 0.20                            | 0.00026         | 0.13            | 2.8             |
| A. niger conidia                | 290             | 170             | 59              | 0.23             |
| 29                              | 15              | 52              | 0.28             |
| 2.9                             | 1.5             | 52              | 0.28             |
| 0.29                            | 0.16            | 55              | 0.29             |

²n₀, Initial viable count; n, survivors after DEPC treatment.
effect was even detected at temperatures below 60 C. In other trials with B. fulva, even higher temperatures (65 and 70 C) did not result in greater ascospore destruction, indicating that there is an optimal treatment temperature that may vary with the microorganism.

Some commercial suppliers of DEPC have recommended treating products at a low temperature, 10 to 12 C, presumably because hydrolysis is retarded, and therefore sterilization will be more effective. Our data indicate that lethal reactions with the cell are inhibited by low temperatures to an even greater extent than hydrolysis, as has been suggested by Shibasaki et al. (10).

**Destruction rates.** Studies on the changes in viable count with exposure time indicated different responses, depending upon the microorganism. The results with S. cerevisiae and the conidia of A. niger and B. fulva were similar to the flour yeast results (Fig. 1) in that most of the cells were killed during the first 30 min of treatment. The highly resistant ascospores of B. fulva required a somewhat longer exposure (4-6 h) to achieve maximal destruction. The greatest difference was with L. plantarum (Fig. 1) and L. mesenteroides in that an incubation of 24 h or longer was needed before DEPC-induced death was completed.

The results with the lactic acid bacteria were puzzling, because at the temperature of the experiment (30 C) hydrolysis of DEPC should have been complete by four h (4), and thus more death would not be expected. It was thought at first that some product formed by the reaction of DEPC with a grape juice constituent was toxic to these organisms. However, in subsequent experiments the survival of L. plantarum incubated for 24 h in DEPC-treated juice was found to be comparable to that of cells centrifuged out after four h and reincubated for 20 h in fresh, DEPC-free juice. More evidence against the hypothesis of a toxic-reaction product was that no decrease in viable count was detected when L. plantarum was inoculated into juice four h after DEPC had been added.

**Injury repair.** The exposure of L. plantarum to DEPC for relatively brief periods before being plated or transferred to fresh grape juice indicated that this organism might be affected as rapidly as others, but that damage could be repaired or reversed if the cells were placed soon

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**Table 2. Resistance of various species to different concentrations of diethylpyrocarbonate (DEPC)**

| Microorganism | Avg reduction in viable count (log10n/n)* in DEPC (ppm) |
|---------------|----------------------------------------------------------|
|               | 50   | 100  | 150  | 200  |
| S. cerevisiae 223 | 9.1  |
| Flor sherry strain yeast | 2.6  |
| A. niger ATCC 1004 conidia | 0.58 | 1.8  | 2.6  | 3.4  |
| B. fulva conidia     | 0.65 | 2.9  | 3.9  | 1.2  |
| B. fulva ascospores  | 2.5  |
| Leuconostoc mesenteroides C-33 | 2.0  | 6.2  |
| L. plantarum B-246   | 2.0  |

*n, Initial viable count; n, survivors after DEPC treatment.

**Table 3. Percent survival after incubation with diethylpyrocarbonate (DEPC) at various temperatures**

| Microorganism          | DEPC (ppm) | Temperature (C) |
|------------------------|------------|-----------------|
|                        | 20  | 30  | 40  | 60  |
| Flor sherry strain yeast | 50  | 16  | 3.0 | 0.18 |
| A. niger conidia       | 50  | 67  | 33  | 6.4 |
| B. fulva ascos-      | 200 | 100 | 100 | 13  |
| B. plantarum          | 100 | 6.4 | 1.2 | 0.048* |

*Survival at 36 C
enough into a suitable medium. The phenomenon of repair has been observed with organisms injured by heat (6), freezing (9), and various other stresses. The effect of exposing *L. plantarum* to DEPC for as short a time as 10 min was quite evident when the cells were subsequently held in fresh grape juice for 24 h before being plated (Fig. 2). This survival curve indicated that most cell damage occurred during the first 1.5 h of incubation with DEPC. The much higher survivals obtained when the DEPC-treated cells were cultured immediately indicated that many could recover when transferred to the TGYE-salts medium.

Additional studies revealed that the post-treatment suspension medium had considerable effect on survival. The death rates in certain menstrua, such as peptone and distilled water, were considerably more rapid than in grape juice (Table 4). This accelerated death, as compared with grape juice, also was observed in solutions of 0.1 M potassium phosphate of several pH values and in various concentrations of glucose. In acid-hydrolyzed casein, on the other hand, no further decrease in the viable count was detected when cells were incubated in this medium for 20 h after a 4-h exposure to 100-ppm DEPC in grape juice (Fig. 3). Varying the strength of the casein hydrolysate solutions indicated that concentrations as low as 0.2% would give maximal recoveries; in fact, this was the level that appeared to be optimal in a number of trials.

One possible explanation for the above results is that certain media are less favorable than others to DEPC-stressed cells, with the result that more rapid death occurs. Osmotic shock is not believed to be the adverse condition, for a significant drop in the viable count occurred in dilute peptone, but not in dilute Casamino Acids solution.

Another possibility is that actual reversal of damage (cellular repair) takes place in some of the suspension medium as well as on the TGYE-salts-agar, and the data would indicate that maximal repair was afforded by the Casamino Acids solution and that some recovery may also have occurred in the grape juice.

**Table 4. Effect of the post-treatment menstruum on the survival of diethylpyrocarbonate (DEPC)-treated *L. plantarum*.**

| Menstruum                      | DEPC (100 ppm) | Viable count/ml × 10⁴ |
|--------------------------------|----------------|-----------------------|
|                                |                | Suspension time        |                         |
|                                |                | (min at 30°C)          |                         |
|                                |                | 30  | 60  | 90  |                         |
| 5º Brix Concord grape juice    | Control*       | 730 | 780 | 820 |                         |
| Peptone (0.1%)                 | Control        | 150 | 130 | 70  |                         |
| Distilled water                | Control        | 750 | 720 | 680 |                         |
|                                |                | 1.6 | 0.085 | <0.01 |                         |
|                                |                | 840 | 810 | 670 |                         |
|                                |                | 2.2 | 0.10 | 0.05 |                         |

*After a 4-h exposure in grape juice to DEPC, the cells were washed once in the test menstruum and incubated as a 10⁻¹ dilution.
* Not treated with DEPC.
ACTIVITY OF DIETHYL PYROCARBONATE

To determine whether a specific amino acid was responsible for the activity of the casein hydrolysate, DEPC-treated cells were incubated in solutions of L-aspartic acid, L-alanine, L-valine, L-phenylalanine, DL-leucine, L-serine, L-arginine, L-cystine, and DL-threonine. None improved the survival of DEPC-stressed *L. plantarum*. It was concluded that either the active compound (amino acid, peptide, etc.) was not tested, that a combination of amino acids was required, or that the concentration used (4 mg/ml) was unsuitable.

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