

# LIMITATIONS IN THE USE OF OZONE TO DISINFECT MAPLE SAP

By R.G. Labbe,\* M. Kinsley,  
and J. Wu

Food Microbiology Laboratory, Department of Food Science, University of  
Massachusetts, Amherst, Massachusetts 01003

## ABSTRACT

The sap of the maple sugar tree (*Acer saccharum*) contains 2 to 3% sucrose and is traditionally collected early in the year and concentrated by boiling to produce maple syrup. High levels of microorganisms in the sap occur during holding, leading to a darker syrup with lower economic value. We investigated the use of dissolved ozone as a method to reduce the microbial population in sap. After 40 min of ozone treatment, concentrations of up to 0.30 mg/liter were achieved but were ineffective in reducing the aerobic plate count. Three predominant colonies on nutrient agar were selected for isolation and identification from sap. These included one mucoid and one nonmucoid yeast, both identified as *Candida*, and *Pseudomonas fluorescens*. When suspended in buffer, each was readily inactivated by ozone. Addition of 3% sucrose to the buffer markedly reduced the effectiveness of ozone. With the use of an ozone generator with a larger ozone output, saturating ozone concentrations (1 mg/liter) were achieved within 5 min but were accompanied by only a 1-log reduction in aerobic plate count of maple sap. After 40 min of ozone treatment, a less than 3-log reduction occurred. The results indicate that, because of the presence of sucrose, ozone may be of limited use in reducing the microbial population in sap.

Maple sugar processing is an important seasonal activity in northern climates in North America. Its products, maple sugar and syrup, together with associated tourism activities, represent an important economic benefit to producers and the region. For example, for 1998 the production value of maple products alone was approximately US \$31 million and CAN \$126 million for the United States and Canada, respectively (2,3).

Maple products result from the boiling of sap from the sugar maple tree (*Acer saccharum*). Sucrose comprises 95% of the dry matter of sap, the balance consisting of amino acids, minerals, salts, and other compounds (11). Microbial growth during the collection and holding of sap can adversely affect the quality of the final product, especially with an increase in ambient temperature associated with the later part of the collection season. The quality of maple is based on its color and flavor, the lighter variety being of greater economic value. The enzymatic action of microorganisms on sap sucrose results in darker syrup and development of caramel flavor during the heat processing of sap (14), presumably due to, at least in part, nonenzymatic browning. Aseptic tapping of sugar maple trees results in higher-quality syrup (13) but is not a practical approach.

The documented effectiveness of aqueous ozone against various microorganisms (15) suggested its potential usefulness as a method of reducing microbial loads during the holding of sap. Ozone has been used for decades as a sanitizing agent of drinking water. Its recent affirmation in the United States as a generally recognized

\*Author for correspondence. Tel: 413-545-1021; Fax: 413-545-1262; E-mail:  
rlabbe@foodsci.umass.edu.

safe substance will likely trigger interest in its application to food processing (8). Indeed, its effectiveness in inactivating foodborne pathogenic bacteria has been demonstrated (4, 15). A recent review by Kim et al. (10) identified applications of ozone in the processing of various commodities. Herein, we report the usefulness of ozone as a method of reducing the microbial levels in maple sap.

## MATERIALS AND METHODS

**Mircoorganisms.** Microbial isolates used in this study were obtained from fresh maple sap. One-tenth milliliter was spread plated onto nutrient agar. Three predominant colonies were selected for isolation and identification. Two were yeasts and the third was a gram-negative, motile rod, which was identified by a commercial consulting laboratory as *Pseudomonas fluorescens* using fatty acid profiling techniques. The yeasts, one mucoid and the other nonmucoid, were each identified as *Candida* spp. by similar techniques by the same laboratory. Identification at the species level was unsuccessful by fatty acid profiling and biochemical and morphological characterization. Each isolate was maintained by periodic transfer to nutrient agar slants, incubated at 20 (yeasts) or 32<sup>o</sup> (*P. fluorescens*) for 2 days, and held at 4<sup>o</sup>C.

**Electron microscopy.** Isolates were streaked onto nitrocellulose filters placed on top of Sabouraud dextrose agar (yeasts) or nutrient agar (bacterium). After growth at 20<sup>o</sup>C, the filters were examined using a JSM-5400 scanning electron microscope as previously described (7).

**Ozone treatment.** Initial experiments involved the use of thawed maple sap

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obtained from a local producer of maple products. Experiments were designed to mimic procedures that might be used on a larger scale in field operations. To this end, a stream of ozone was introduced at time zero to samples containing microorganisms (sap or isolated cultures suspended in buffer) rather than the addition of microorganisms to reservoirs containing established ozone concentrations.

Eight hundred milliliters of sap was placed in a 1-liter glass jar and ozone introduced into the bottom of the jar via Tygon tubing fitted to the outlet of an ozone generator. A diffuser stone was attached to the tubing outlet, and the contents of the jar were stirred. Ozone was generated using a Clearwater Tech (San Luis Obispo, Calif.) model UV-275 or UV-2800 with air as the feed gas. Each had a rated output of 0.1 and 1.0 g of ozone per h, respectively. Ozonation was conducted in an exhaust hood.

Each microbial isolate was also exposed to ozone. For this purpose, 500-ml cultures of yeasts and *P. fluorescens* were grown in yeast and mold broth (Difco Laboratories, Detroit, Mich.) or tryptic soy broth (Difco) at 20 or 32°C, respectively, with shaking. Cells (10 ml) were collected at late log phase using a previously determined standard curve (600 nm). Cells were washed twice with 1 mM phosphate buffer, pH 6.0, and resuspended in 10 ml of the same buffer. Eight milliliters were added to 792 ml of the same buffer, yielding initial levels of between 10<sup>5</sup> and 10<sup>6</sup> of viable cells per ml. Following exposure to ozone for various times, decimal dilutions were made in 1.5% peptone and surface plated on Sabouraud dextrose agar (yeasts) or nutrient agar (*P. fluorescens*) and incubated at 20°C for 3 days (yeasts) or 32°C for 2 days (*P. fluorescens*). No increase in survivors was obtained if plates were held for 5 days instead of 3. In certain experiments, 3% sucrose (ACS grade, Sigma Chemical Co., St. Louis, Mo.) was

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added to the buffer into which ozone was generated. All experiments were done in duplicate.

**Measurement of ozone.** The amount of ozone generated was measured by the iodometric method (1).

## RESULTS

Use of the smaller of two ozonators failed to result in any reduction in the aerobic plate count (APC) of maple sap after 40 min, despite oxidizing activity of approximately 0.30 mg/liter (Fig. 1).

We then wished to determine if the microbial population was inherently resistant to ozone treatment. Three predominant microorganisms from sap, two yeasts and one bacterium, were isolated as described in the "Materials and Methods" section. The two yeasts, one mucoid and one nonmucoid, were identified as *Candida* and the bacterium as *P. fluorescens*. Each yeast produced pseudomycelia (Fig. 2A) with characteristic budding (Fig. 2B). Sheneman and Costilow (17) had identified *Candida* and *Pseudomonas* as among the predominant genera isolated from maple tree tap holes.

When yeasts were suspended in 1 mM phosphate buffer, pH 6.0, ozone was readily effective in reducing the viability of the two yeast isolates. A 2.5- to 3-log inactivation of the mucoid yeast was observed within 15 min at an ozone concentration of approximately 0.2 mg/liter (Fig. 3). In the case of the nonmucoid yeast, more than a 4-log reduction occurred within 5 min at an ozone concentration of less than 0.1 mg/liter (Fig. 4). Addition of 3% sucrose to the buffer dra-

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matically reduced ozone effectiveness in each case (Figs. 3 and 4). This is the approximate concentration of sucrose found in maple sap. A similar quenching of ozone effectiveness by sucrose was also observed in the case of *P. fluorescens* (non shown). In that case, a 6-min exposure to ozone was required to obtain a 3-log reduction in viable count, whereas 20 min was required in the presence of sucrose. Interestingly, high oxidizing activity was measured in the presence of sucrose (Figs. 3 and 4). The action of ozone on sucrose apparently created unknown reactive species. An ozone generator with a larger output was used in an attempt to reduce microbial counts in sap (Fig. 5). This unit had a rated output 10-fold greater than the smaller unit, although the dissolved ozone concentration differed by only threefold. With this unit, saturating levels of ozone concentration were reached within 5 min. Nevertheless, after 20 min only a 1-log reduction in the microbial population was achieved; even after 40 min, a less than 3-log reduction in APC was obtained.

## DISCUSSION

The well-known effectiveness of ozone in inactivating microbial cells suggested its use in the processing of maple sap in which high levels of microorganisms can occur during holding at ambient outdoor temperatures. It was, surprisingly,

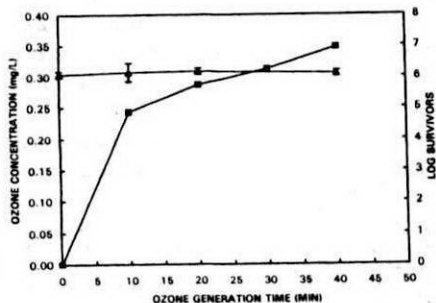


FIGURE 1. Effect of ozone on APC of maple sap. Triangles, log survivors; squares, ozone concentration.

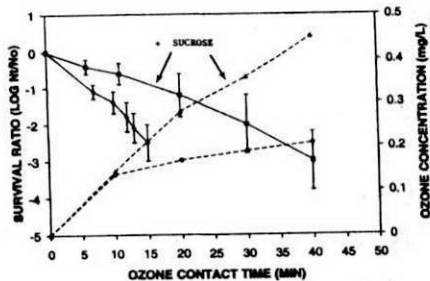


FIGURE 3. Inactivation by ozone of a mucoid yeast isolated from maple sap in the presence or absence of 3% sucrose. Yeasts were suspended in 1 mM phosphate buffer, pH 6.0. Solid lines, yeast survivors; dotted line, ozone concentration.

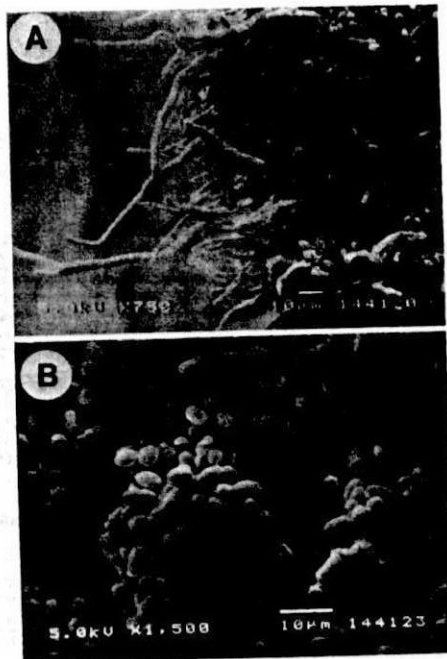


FIGURE 2. Scanning electron micrograph of yeasts isolated from maple sap showing pseudomycelia (A) and budding (B).

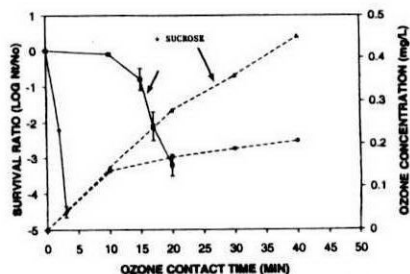


FIGURE 4. Inactivation by ozone of a non-mucoid yeast isolated from maple sap by ozone in the presence or absence of 3% sucrose. Yeasts were suspended in 1 mM phosphate buffer, pH 6.0. Solid line, yeast survivors; dotted line, ozone concentration.

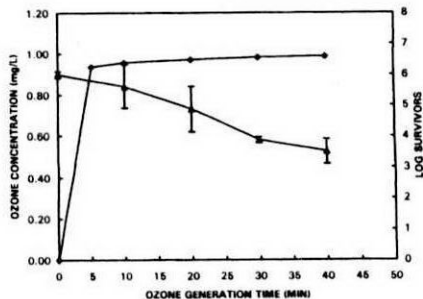


FIGURE 5. Reduction in APC of maple sap by saturating concentrations of ozone. Triangles, log survivors; diamonds, ozone concentrations.

only moderately effective, with less than a 3-log reduction in APC after 40 min even under saturating concentration of ozone, approximately 1 mg/liter.

By contrast, individual yeasts and one bacterium isolated from sap were readily inactivated by ozone when suspended in buffer at ozone concentrations of less than 0.15 mg/liter. Inactivation by ozone was markedly affected by the presence of 3% sucrose. We conclude that sucrose quenched the lethal activity of ozone. It has been previously shown that organic matter can affect the antimicrobial activity of ozone (5, 15, 16). This appears to be dependent on the type of organic material and microbial species. For example, Restaino et al. (15) reported that the death rates of *Escherichia coli* and *Salmonella Typhimurium* were significantly reduced by bovine serum albumin but not by soluble starch.

The antimicrobial activity of ozone has long been known. Less clear is its mode of action. Suggestions for primary targets include unsaturated lipids in the cell surface, enzyme sulfhydryl groups, nucleic acids, and others. Proposed mechanisms for inactivation have been recently summarized (10). The reactivity of ozone is believed to be due to the oxidizing power of free radicals formed in a chain reaction during its decomposition. Indeed, ozone molecules themselves may be relatively nontoxic to microorganisms (6). Organic matter may inhibit this chain reaction (9), although, to our knowledge, the specific interaction of sucrose and ozone has not been investigated. Sucrose may not be the only organic material present and affecting ozone activity. Exopolysaccharides may also be present in sap. Indeed, low-grade sap can contain substantial amounts of such polysaccharides and results in ropy or stringy maple syrup (12). In fact, one of the yeasts isolated in the present work produced mucoid colonies.

To demonstrate any reduction in APC using sap, it was necessary to use an ozone generator with an output greater than the unit that was effective in inactivating yeasts and bacteria suspended in buffer. Associated equipment costs and electrical consumption were each approximately three-fold greater for a volume of 800 ml of sap. This leads to another issue, that of capital costs and operating expenses. The ozone generation systems used in this work are based on exposure of air to UV light. For volumes of maple sap greater than those used herein, alternative methods for ozone generation, such as the corona discharge method, would be required to achieve significant microbial inactivation. Given the results

reported herein, such proposed procedures must be demonstrated by pilot studies using sap collected at various stages of the collection period. Finally, empirical and experimental evidence has long shown that cold storage of sap before quick processing yields lighter-grade syrup. Although aseptic tapping is not practical, disinfection of drill bits, spouts, and collection equipment is a reasonable protocol (13).

### ACKNOWLEDGMENTS

This work was supported in part by a University of Massachusetts Public Service Endowment Grant and by a grant from the North American Maple Syrup Council. We are grateful to South Face Farms, Ashfield, Massachusetts for providing maple sap.

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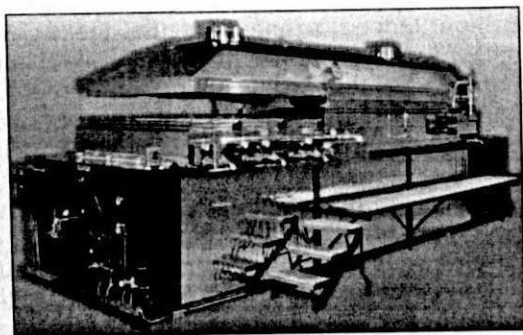
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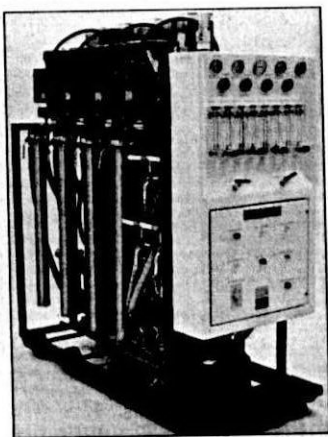
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