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Sensitivity of Heat-Stressed Yeasts to Essential Oils of Plants

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Eight strains of yeasts (*Candida lipolytica, Debaryomyces hansenii, Hansenula anomala, Kloeckera apiculata, Lodderomyces elongisporus, Rhodotorula rubra, Saccharomyces cerevisiae*, and *Torulopsis glabrata*) were examined for changes in sensitivity to eight essential oils of plants (allspice, cinnamon, clove, garlic, onion, oregano, savory, and thyme) after being sublethally heat stressed. With the exception of garlic oil for all test yeasts, onion oil for *S. cerevisiae*, and oregano oil for *R. rubra*, the essential oils at concentrations of up to 200 ppm in recovery media did not interfere with colony formation by unheated cells. However, some oils, at concentrations as low as 25 ppm in recovery media, reduced populations of sublethally heat-stressed cells compared to populations recovered in media containing no test oils. This demonstrates that the yeasts were either metabolically or structurally damaged as a result of being exposed to elevated temperatures and that essential oils prohibited repair of injury. The size (diameter) of colonies produced on oil-supplemented recovery agar by heat-stressed cells was reduced compared to that observed on unsupplemented agar. Pigment production by heated *R. rubra* was inhibited by oils of oregano, savory, and thyme, but enhanced by garlic and onion oils. The influence of essential oils on survival of yeasts in thermally processed foods and in the enumeration of stressed cells in these foods should not be minimized.

Although sublethal injury and subsequent recovery of bacteria have been recognized for several decades, only within the past decade have food microbiologists recognized that yeasts and molds are susceptible to injury upon being exposed to adverse environmental conditions (18). Fungal cells may be damaged by an array of stress conditions in the environment, but debilitation due to exposure to temperature extremes has been studied most extensively. Heat injury of cells has been demonstrated to result in increased sensitivity to acid pH (11, 13, 16) and sodium chloride (1, 5) and reduced water activity (4).

Heat-stressed fungal cells have been shown to exhibit increased sensitivity to low levels of chemicals used to preserve foods from microbiological and organoleptic deterioration. Sorbic acid (2, 3, 19) and antioxidants such as butylated hydroxyanisole and tertiary butylhydroquinone (7) at levels of less than 0.1% have a lethal effect on damaged cells.

The antimicrobial activity of spices and herbs commonly used to season foods has been documented. Most studies have been directed toward determining the effects of aqueous extracts and essential oils of spices on growth and toxin production by bacteria (6, 9, 12). The study reported here was designed to determine the response of heat-injured cells of food spoilage yeasts to essential oils of plants used in the food industry as seasoning agents.

MATERIALS AND METHODS

Yeast strains. One strain each of *Candida lipolytica, Debaryomyces hansenii* NRRL Y-7268, *Hansenula anomala* 67-455, *Kloeckera apiculata* NRRL Y-1382, *Lodderomyces elongisporus* NRRL YB-4239, *Rhodotorula rubra* C-46A, *Saccharomyces cerevisiae* UGA-102, and *Torulopsis glabrata* were examined. Stock cultures were maintained on potato dextrose agar (pH 5.5) slants at 4°C.

Essential oils. Food-grade quality essential oils were provided by Fritzsche, Dodge and Olcott, Inc., New York, N.Y. Included in the study were oils of allspice, cinnamon, clove, garlic, onion, oregano, savory, and thyme.

Preparation of cells. Yeasts were cultured in a medium consisting of 3.0 g of yeast extract, 5.0 g of peptone, and 10 g of glucose per liter of distilled water (YMPG) (pH 5.5). Cultures (100 ml per 250-ml Erlenmeyer flask) incubated at 30°C for 44 to 48 h under constant agitation served as inocula for all tests.

Recovery medium. YMPG broth (pH 5.5) supplemented with agar (20 g/liter) was used as the basal recovery medium for unheated and heat-stressed cells. Essential oils were diluted in 95% ethanol and added to the molten sterile YMPG agar. After thorough mixing, the oil-supplemented agar was poured into petri dishes and allowed to set at room temperature for 24 h to enable the surface to dry. Levels of 25, 50, 100, and 200 ppm of essential oils were tested; control media consisted of YMPG agar to which neither oils nor ethanol were added and YMPG agar supplemented with 1% ethanol, the highest level added as a carrier in preparing recovery agar containing 25 to 200 ppm of oils.

Procedure for heat stressing. One milliliter of culture was transferred to 100 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a 250-ml Erlenmeyer flask adjusted to a temperature ranging from 44 to 54°C, depending on the relative heat sensitivity of the yeast as determined in a previous study (9). After 20 min of heating with constant agitation in a water bath shaker, a portion of the cell suspension was withdrawn, immediately cooled to 21 to 24°C, serially diluted in phosphate buffer, and surface plated onto recovery media. Nonheated cells from the test cultures were also plated onto recovery media containing various levels of essential oils. The plates were incubated for 5 days at 30°C before colonies were counted. Colonies were also observed for abnormalities in size, color, and morphology. An automatic colony counter (Biotran III; New Brunswick Scientific Co., Inc., Edison, N.J.) was used to determine the total number of colonies exceeding various diameters (≥0.2 mm [total count], ≥0.5, ≥1.0, ≥1.5, and ≥2.0 mm) at the end of the 5-day incubation period. Data presented represent means of

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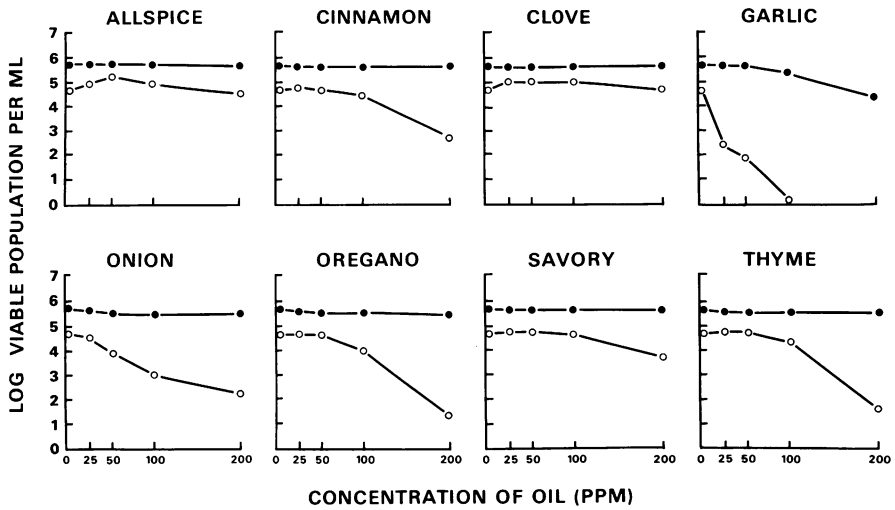

FIG. 1. Effects of various concentrations of essential oils in YMPG recovery agar (pH 5.5) on colony formation by unheated (●) and heated (20 min at 44°C) (○) cells of *C. lipolytica*.

data collected from a minimum of two replicate experiments, each performed in duplicate.

RESULTS AND DISCUSSION

The results of experiments to determine the effects of essential oils on the sensitivity of unheated and heat-stressed *C. lipolytica, H. anomala, L. elongisporus*, and *S. cerevisiae* are illustrated in Fig. 1 to 4, respectively. Data collected from experiments with *D. hansenii, K. apiculata, R. rubra*, and *T. glabrata* are not presented in this series of figures due to space constraints.

With the exception of garlic oil for all test yeasts, onion oil for *S. cerevisiae*, (Fig. 4), and oregano oil for *R. rubra*, the essential oils at concentrations up to 200 ppm in recovery media did not interfere with colony formation by unheated cells. Because of the generally consistent number of colonies formed by unheated cells on recovery agar containing increased levels of essential oils, the presence of heat-injured cells, i.e., cells with increased sensitivity to the oils in agar containing the same concentration range, can be illustrated graphically. Heated cells of *C. lipolytica* showed considerably increased sensitivity to the oils of cinnamon, oregano, and thyme at levels of 100 and 200 ppm and to the oils of garlic and onion at all concentrations tested (Fig. 1). Heated *D. hansenii* cells were considerably more sensitive to all levels of oregano and garlic oils and slightly more sensitive to the other essential oils. The sensitivity of *H. anomala* to all of the essential oils was increased by sublethal heat stress (Fig. 2). The greatest sensitivity was observed with garlic oil, which was lethal to all heated cells at a concentration of 50 ppm.

The heat stress caused only a slight increase in sensitivity of *K. apiculata* to the essential oils. Heated *L. elongisporus* cells showed increased sensitivity to all of the essential oils, even at the 25-ppm level (Fig. 3). The greatest sensitivity was observed with garlic oil; e.g., at the 100-ppm level, no heated cells formed colonies on the recovery medium. *R.*

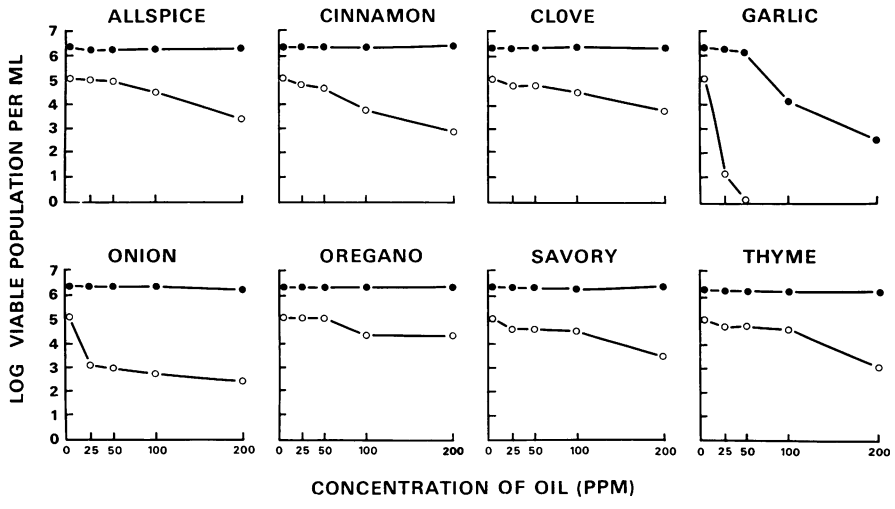

FIG. 2. Effects of various concentrations of essential oils in YMPG recovery agar (pH 5.5) on colony formation by unheated (●) and heated (20 min at 48°C) (○) cells of *H. anomala*.

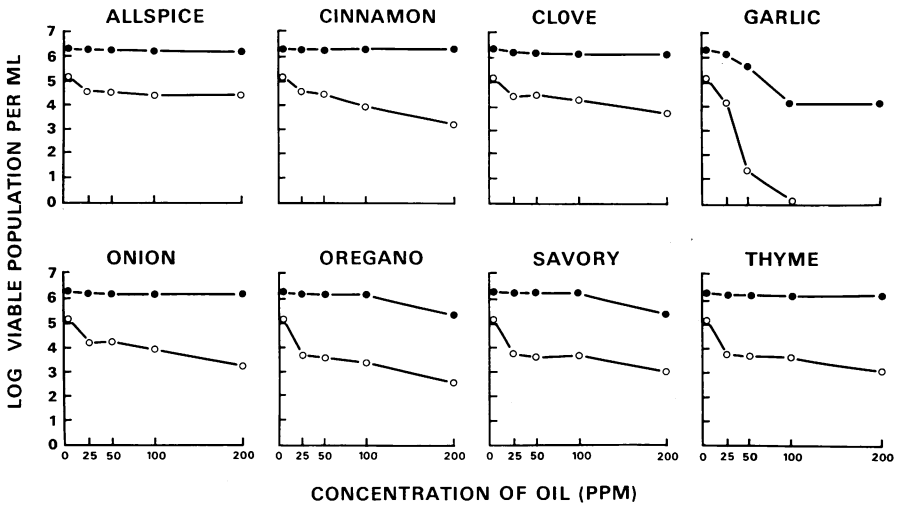

FIG. 3. Effects of various concentrations of essential oils in YMPG recovery agar (pH 5.5) on colony formation by unheated (●) and heated (20 min at 50°C) (○) cells of *L. elongisporus*.

rubra, which was observed to be exceptionally sensitive to several other essential oils tested in our laboratory, showed little increase in sensitivity to many of the oils after heating. Heated *R. rubra* cells did show an increased sensitivity to garlic oil at all concentrations tested, to cinnamon oil at levels equal to or greater than 50 ppm, and to thyme oil at 100 and 200 ppm. Heated *S. cerevisiae* cells showed greater sensitivity to all except the eugenol-containing oils of allspice and clove at all concentrations (Fig. 4). Both heated and unheated cells showed considerable sensitivity to onion oil and especially to garlic oil. Heat stressing of *T. glabrata* had little effect on its sensitivity to the essential oils. A slight increase in sensitivity was observed with 200 ppm of cinnamon oil and 200 ppm of savory oil.

Overall, injury due to heat stress increased the yeasts' sensitivities to the essential oils. Katsui et al. (10) reported that pre- and postincubation temperatures influenced the viability of heat-stressed bacterial and yeast cells. Here, the pre- and postincubation temperature, 30°C, was optimal for growth of most of the test yeasts. Stressed cells require optimum recovery conditions if repair and eventual cell division are to occur.

Leakage of cellular constituents indicates that cellular membrane damage may occur during heat stress (15). Another manifestation of thermal injury may be inactivation of heat-labile enzymes. Grant et al. (8) reported that the yeast *Candida gelida* lost its ability to ferment glucose after it was exposed to temperatures above the optimum, and this loss of fermentative activity was due to the thermal inactivation of pyruvate decarboxylase. Nash and Grant (14) reported that heat stress inactivated ribosomes of this same psychrophilic yeast by impairment of ribosomal function, characterized by a reduced capacity to bind charged tRNA and physical degradation. This effect on ribosomes interferes with the cells' ability to synthesize proteins.

In the studies reported here, it would appear that heat stress may have caused impairment to or lesions in the cytoplasmic membranes, allowing the antiyeast components

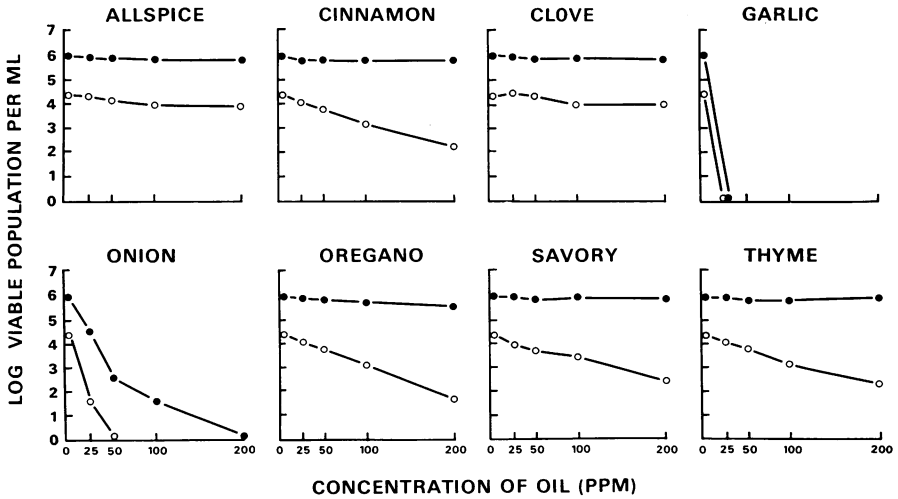

FIG. 4. Effects of various concentrations of essential oils in YMPG recovery agar (pH 5.5) on colony formation by unheated (●) and heated (20 min at 52°C) (○) cells of *S. cerevisiae*.

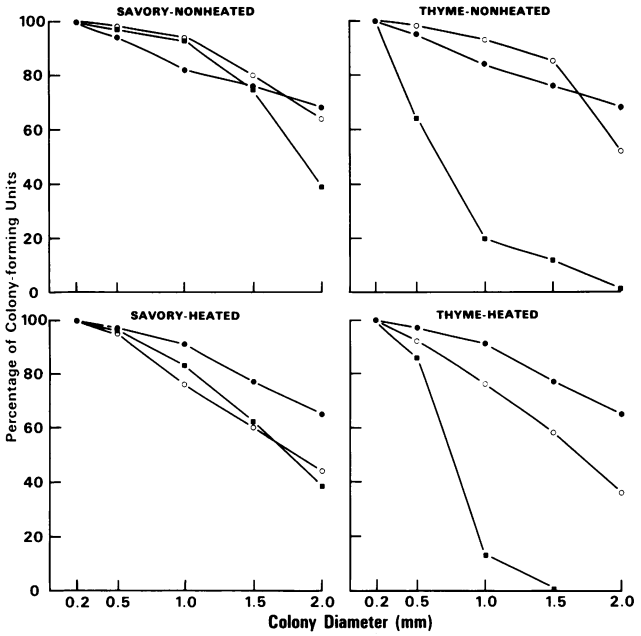

FIG. 5. Effects of 0 (●), 50 (○), and 200 (■) ppm of savory and thyme oils on colony size of unheated and heated (20 min at 44°C) cells of *K. apiculata*.

in the essential oils to move more rapidly into the interior of the yeast cells, where these components apparently impair normal metabolism by their specific modes of action. Another explanation for the observed increase in sensitivity of heated cells is that the essential oils interfered with the recovery (repair) mechanisms required for cell division and consequent colony formation. Schenberg-Frascino (17) reported that active metabolism was required for yeasts to recover from heat-induced damage.

This study also permitted the observation of yeast colonies for changes in morphology, size, and pigment production as affected by heat treatment and essential oils. Colonies exceeding selected diameters (size) were counted and expressed as a percentage of the total colonies. Results for three of the test strains are shown in Fig. 5 to 7. The size of *K. apiculata* colonies was affected by increasing concentrations of savory oil and to a greater extent, thyme oil (Fig. 5). Only 40% of the colonies formed on agar containing 200 ppm of savory oil had diameters of ≥2.0 mm, whereas 64 to 68% of the colonies were ≥2.0 mm in diameter on agar containing 50 and 0 ppm of savory oil. Heat stress did not influence this effect. Thyme oil had a greater effect on the size of *K. apiculata* colonies. With unheated cells growing on agar containing 200 ppm of thyme oil, no colonies exceeded 2.0 mm, whereas only 12% were ≥1.5 mm, and 20% were ≥1.0 mm in diameter. That is, most *K. apiculata* colonies tended to be ≤1.0 mm in diameter when exposed to 200 ppm of thyme oil, whereas most of the colonies were able to grow to 2.0 mm in diameter in the absence of thyme oil or in the presence of 50 ppm of thyme oil. Heat stress slightly enhanced this reduction in final colony size.

Growth of colonies formed from unheated cells of *S. cerevisiae* was not affected by cinnamon oil, but the colonies formed by heat-stressed cells showed some reduction in size (Fig. 6). However, the presence of 50 ppm of cinnamon oil had approximately the same effect as did 200 ppm. Oregano oil, on the other hand, had a pronounced affect on *S. cerevisiae* colony size. Colonies formed on agar not containing oregano oil increased to at least 2.0 mm in diameter, but when exposed to 200 ppm of oregano oil, only 12% of the colonies increased to ≥2.0 mm in diameter. Even at 50 ppm of oregano oil, only 74% of the colonies had diameters of ≥2.0 mm. Heat stress had little influence on this size distribution.

R. rubra colonies were markedly affected by the presence of some essential oils (Fig. 7). Colonies formed by unheated cells of *R. rubra* developed to ≥2.0 mm on control plates (0 ppm), but only 74% of the colonies achieved this size when exposed to 50 ppm of allspice oil. At 200 ppm of allspice oil, no *R. rubra* colonies obtained a diameter of 2.0 mm, and only 22% had diameters of ≥1.5 mm. Heat treatment enhanced the retarding effect of allspice oil on ultimate colony size. Clove oil also caused a reduction in *R. rubra* colony size. The 50-ppm level caused little or no deviation from colony sizes observed on the control plates; however, the presence of 200 ppm of clove oil drastically reduced colony size. Only 50% of the colonies formed from unheated cells were ≥1.0 mm in diameter, whereas only 12% of the colonies formed from heated cells were ≥1.0 mm in diameter in the presence of 200 ppm of clove oil.

In addition to observations on size, *R. rubra* colonies were observed for pigment production. Normal (unheated) *R. rubra* colonies possess a deep coral-orange pigment when grown on YMPG agar. Exposure to some of the essential oils prevented the production of this pigment, Interestingly, garlic and onion oils, which are characteristically inhibitory to *R. rubra* growth, greatly enhanced pigment production at sublethal concentrations. It was noted that *R. rubra* colonies developed heavy pigmentation in the presence of these essential oils 1 to 2 days earlier than did the colonies on the control plate. Conversely, the oils of thyme, savory, and oregano were inhibitory to pigment production. At 25 ppm of these oils, pigment production was only slightly less than that observed in the control; 50 ppm decreased the colony

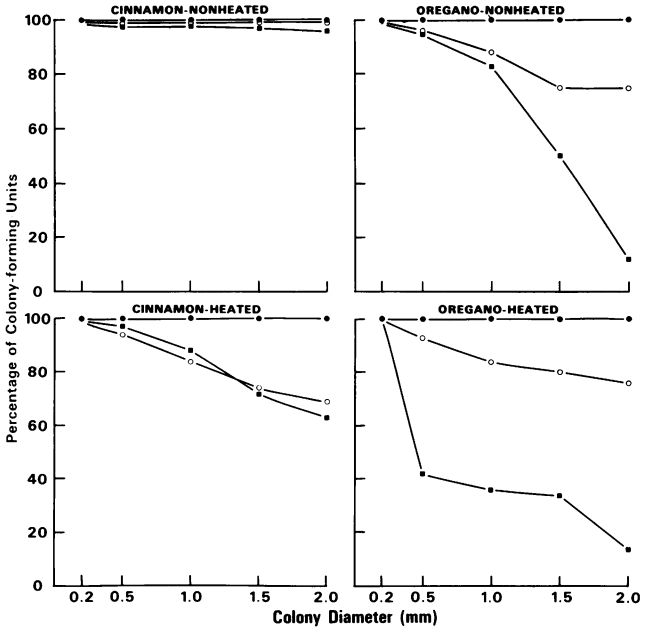

FIG. 6. Effects of 0 (●), 50 (○), and 200 (■) ppm of cinnamon and oregano oils on colony size of unheated and heated (20 min at 52°C) cells of *S. cerevisiae*.

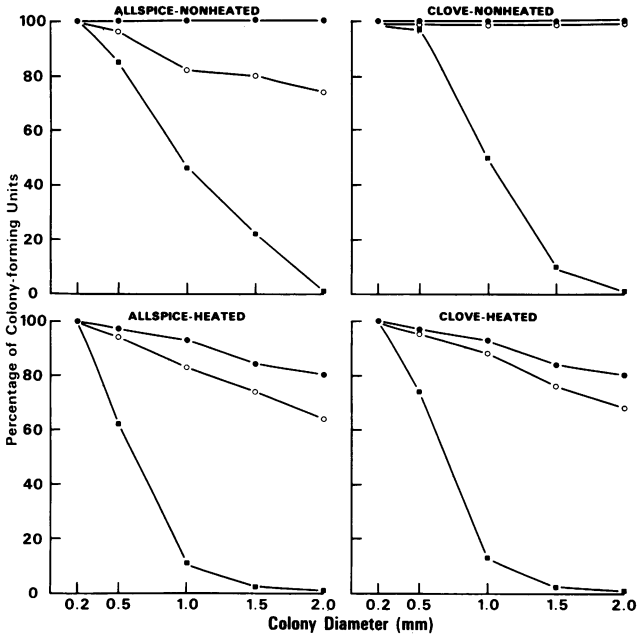

FIG. 7. Effects of 0 (●), 50 (○), and 200 (■) ppm of allspice and clove oils on colony size of unheated and heated (20 min at 54°C) cells of *R. rubra*.

appearance to a very pale coral color; 100 ppm reduced the pigment to a light yellow; and no pigment was observed in colonies formed on agar containing 200 ppm, i.e., colonies were off-white in color.

In summary, eight strains of yeasts which are among those incriminated as spoilage organisms in high-acid foods or used to ferment foods and beverages have been demonstrated to exhibit increased sensitivity to essential oils after being heat stressed. These oils are present in some foods at levels shown to cause lethal effects on heat-injured cells. Thus, the influence of essential oils on reducing populations of yeasts in thermally processed foods should not be minimized. However, further experiments are warranted to determine whether observations from studies reported here using buffer as a heating medium and YMPG agar as a recovery medium also hold true for heating and recovering cells in foods.

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