

Influence of Growth Medium on Thermal Resistance of *Pediococcus* sp. NRRL B-2354 (Formerly *Micrococcus freudenreichii*) in Liquid Foods

ABSTRACT

Pediococcus sp. is a nonpathogenic heat-resistant spoilage organism that has been used as a test organism in milk pasteurization studies. These characteristics make this bacterium an attractive test organism to study the mode of bacterial thermal inactivation in a food pilot plant. We report here the effect of growth medium on the thermal D value of this organism in skim milk, whole liquid egg, 10% glucose solution, pineapple juice, apple juice, tomato juice, and water at 60°C. Thermal inactivation was done in a submerged coil; D values were calculated from the linear portion of the survival curves by linear regression analysis. The range of D values of stationary-phase cells grown at 28°C in tryptone glucose yeast extract (TGY) or tryptic soy broth (TSB) was 0.14 to 12.05 min in all heating menstrua tested. The TSB-grown cells exhibited the highest thermal resistance with skim milk and 10% glucose solution as the heating menstrua. Survival curves of the TGY-grown cells indicated the presence of a cell population heterogeneous in thermal resistance. The TSB-grown cells exhibited a cell population uniform in thermal resistance and with a lag time for thermal inactivation. When compared to TGY-grown cells, *Pediococcus* sp. grown in TSB showed a significant ($P < 0.05$) increase in D values by up to eightfold in all heating menstrua. Results from this study suggested that thermal inactivation of *Pediococcus* sp. was dependent on the growth medium and on the heating menstruum with respect to both pH and composition.

Pediococcus sp. (formerly *Micrococcus freudenreichii*) is a gram-positive spherical nonmotile non-spore-forming facultative anaerobe. This bacterium, originally isolated from milk and dairy utensils (15), is a heat-resistant spoilage nonpathogenic organism that has been used as test organism in milk and milk by-products pasteurization studies (7, 15, 16). These characteristics, nonpathogenicity and thermal resistance, made this bacterium an attractive test organism for studying the destruction of bacteria by microwave energy in a food pilot plant (8, 9). While this bacterium is considered to be a test organism in milk pasteurization studies, little is known about the influence of growth medium and heating menstruum on its thermal inactivation kinetics.

This study is a part of ongoing research to develop a cold-pasteurization process utilizing electromagnetic energy. Our data on a nonthermal semicontinuous pilot plant process utilizing microwave energy suggested that the lethal effect of electromagnetic energy on *Pediococcus* sp. was dependent on the growth medium and the heating menstruum (8). To understand and interpret the effects of the process on this bacterium, an understanding of the effects of growth medium and heating menstruum on thermal inactivation

of *Pediococcus* sp. is needed. Thus, the specific objectives of this investigation were to elucidate the effect of growth medium on D values of *Pediococcus* sp. in different heating menstrua.

MATERIALS AND METHODS

Microorganism, culture maintenance, and growth media. *Pediococcus* sp. NRRL B-2354 was supplied by L. K. Nakamura (U.S. Department of Agriculture, Peoria, IL, USA). The culture was maintained on tryptose agar (TA; Difco Laboratories, Detroit, MI, USA) plates at 4°C with biweekly transfers to maintain the strain viability. The two growth media used were tryptic soy broth (TSB; Difco) prepared in distilled water according to the manufacturer's guidelines and supplemented with 0.25% (wt/vol) glucose, and tryptone glucose yeast extract (TGY) broth which was formulated in our laboratory (tryptone, 5 g; yeast extract, 5 g; glucose, 1 g; potassium phosphate dibasic, 1 g; double-distilled de-ionized water, 1 liter; pH 7.00). All ingredients were mixed prior to autoclaving and the media pH did not change following autoclaving.

Inoculum development and general growth procedures. Unless otherwise indicated, the growth temperature was 28°C. A late exponential-phase culture grown in the appropriate medium was used at 1% (vol/vol) to inoculate 50 ml of the same medium. Growth was monitored by measuring optical density at 600 nm with a Shimadzu UV-160 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Cultures were grown to stationary phase ($A_{600 \text{ nm}} = 1.0$), harvested by centrifugation at $16,000 \times g$ for 10 min at 4°C, and washed once with cold sterile distilled water.

Heating menstrua, sample preparation and thermal inactivation. National brands of pasteurized apple juice, pineapple juice, whole tomato juice, and skim milk were obtained from a local source. Pasteurized whole liquid egg was obtained from a local supermarket. Glucose solution (10%, wt/vol) was prepared in tap water. The cell pellet was suspended in the appropriate heating menstruum to a target level of $8 \log \text{CFU/ml}$. Culture samples (9.5 ml) were loaded on a Techne submerged-coil heating apparatus (model tempette TE-8D, Protocol Instruments Limited, West Byfleet, UK) and held at 60°C . Heating time and sampling frequency were based on the heating menstruum. After heating, samples were quickly stored on ice.

Assessment of bacterial viability. The bacterial suspensions were serially diluted in 0.1% peptone (Difco) and surface plated on TA plates with the spiral plating system (Spiral Systems Instruments, Inc., Bethesda, MD, USA). The plates were then incubated at 37°C for 18 to 24 h and the survivors were enumerated using a laser bacterial colony counter, model 500A (Spiral Systems Instruments, Inc.). Cell densities were reported as CFU per milliliter of sample.

D values. *D* values (time to inactivate 90% of the population) were calculated as the negative inverse slope of the linear portion of survivor curves (obtained by plotting logarithms of survival counts versus their corresponding heating times). Linear regression lines were fitted to the linear portion of two sets of independent data. Only survivor curves with more than four values in the linear portion and descending more than two log cycles were used.

Processing time. The processing time to accomplish a 99.99% kill of *Pediococcus* sp. cells was calculated by using the formula $4D$ plus lag time. *D* and lag time represented the *D* value and the time period during which the cell population remained at the inoculation level, respectively.

Statistical analysis. Standard errors were calculated from the regression analysis using SAS software (SAS Institute Inc., Cary, NC). The *t* test was used to determine the significant differences ($P < 0.05$) among the *D* values of *Pediococcus* sp. cells grown in either TGY or TSB.

All chemicals were analytical grade reagents. Glassware was cleaned with Nochromix solution (Godax Laboratories, Inc., Tekoma Park, MD, USA) and rinsed repeatedly with distilled water before use.

RESULTS AND DISCUSSION

We reported on the development of a pilot-plant nonthermal semicontinuous process to inactivate microorganisms with microwave energy (8). The data from our pilot-plant microwave process indicated that TGY-grown cells of *Pediococcus* sp. were more sensitive to microwave energy than cells grown in TSB, and that bacterial inactivation by microwave energy was dependent on the test fluid used. Here we report on the influence of growth medium as well as heating menstruum on the thermal inactivation of *Pediococcus* sp. cells.

Cells were grown to stationary phase in TSB or TGY at 28°C , and their thermal resistance at 60°C in different liquid menstrua was determined. Logarithms of surviving *Pediococcus* sp. CFU per milliliter of heating menstruum were plotted against heating time; the *D* value was obtained by linear regression from the linear portion of the survivor curves (Figure 1). The coefficient of correlation (r^2) range in all heating menstrua tested was 0.855 to 0.996.

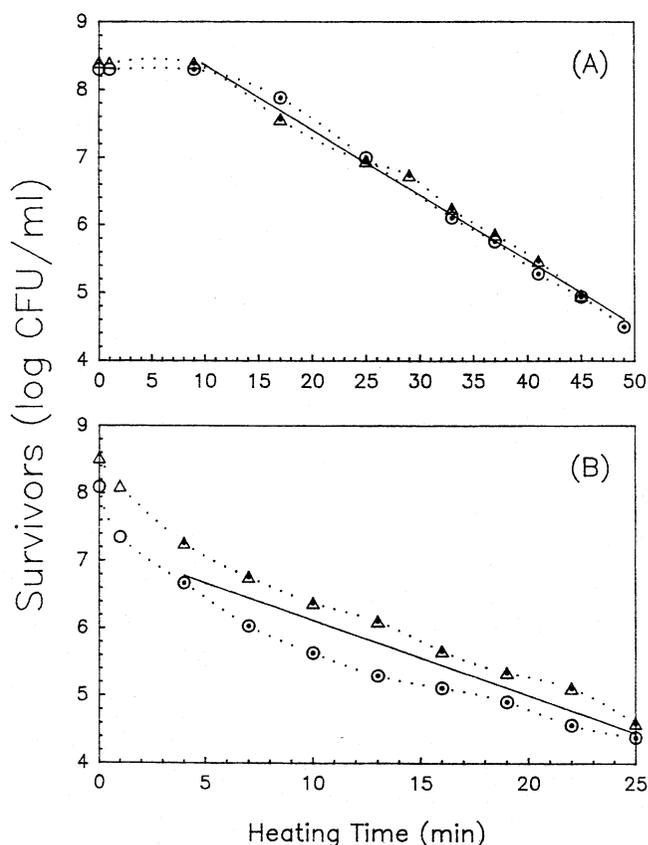


FIGURE 1. Effect of TSB (A) and TGY (B) growth media on heat resistance of *Pediococcus* sp. NRRL B-2354 in skim milk at 60°C . Circles and triangles represent the data of two independent studies. The solid straight line is the average regression plot (filled circles) of the straight portion of the two survivor curves. Cells were grown to stationary phase in the appropriate medium at 28°C .

The representative example of the effect of growth medium on survivor curves at 60°C shown in Figure 1, demonstrated a biphasic inactivation characterized by a shoulder and/or tailing. The TSB-grown cells of *Pediococcus* sp. exhibited a lag time for thermal inactivation (Fig. 1A) in all heating menstrua (Table 1), and a linear decrease in cell concentration during thermal inactivation (Fig. 1A). These data suggested that the cell population was uniform in thermal resistance (4). On the other hand, cells grown in TGY exhibited an initial period of higher thermal sensitivity (Fig. 1B), suggesting the presence of a cell population heterogeneous in heat resistance (4). This initial period of higher thermal sensitivity of the TGY-grown cells was seen in all heating menstrua tested (data not shown).

The effect of growth medium on the *D* values of *Pediococcus* sp. in different heating menstrua is shown in Table 2. The *D* values of TSB-grown cells of *Pediococcus* sp. were significantly ($P < 0.05$) higher (1.2- to 8-fold) than the TGY-grown cells in all heating menstrua tested (Table 2), indicating that the TSB-grown cells were more resistant to thermal inactivation than cells grown in TGY. This growth medium effect was also seen when microwave energy was used to inactivate *Pediococcus* sp. cells at reduced temperatures (8). This variation in *D* values (Table 2), resistance to microwave energy inactivation (8), and initial thermal

TABLE 1. The effect of growth medium^a on the processing time to accomplish a 99.99% kill of *Pediococcus* sp. NRRL B-2354 at 60°C in various heating menstrua

Heating menstruum	Lag time ^b (min)		Processing time (min) ^c	
	TGY	TSB	TGY	TSB
Pineapple juice	ND ^d	0.25	0.56	3.05
Apple juice	ND	0.50	1.32	4.70
Tomato juice	ND	1.50	2.60	6.94
Water	ND	1.50	4.36	19.34
Pasteurized whole liquid egg	ND	1.00	4.76	39.12
10% Glucose solution	ND	3.50	9.84	51.70
Skim milk	ND	10.00	36.28	52.00

^a Cells were grown at 28°C in TGY (tryptone glucose yeast extract) or TSB (tryptic soy broth) to stationary phase, centrifuged, washed once with sterile distilled water and resuspended in the appropriate heating menstruum to a final cell concentration of ca. 8 log CFU/ml.

^b Initial period of thermal resistance.

^c Calculated using the formula $4D + \text{lag time}$.

^d ND, not detected.

protection or sensitivity (Fig. 1) of this bacterium seemed to be an effect of the growth medium regardless of the heating menstruum used. This effect could be due to the higher concentrations of glucose and/or nitrogen source in TSB medium than in TGY. The carbohydrate and/or nitrogen sources have been known to influence the fluidity of the bacterial membrane (6, 10, 11); a decrease in membrane fluidity would increase the thermal resistance of the bacterial cell (2, 3).

The pH and composition of heating menstrua are known to have a strong influence on thermal resistance in bacteria whereby the rate of thermal inactivation increases with a decreasing pH and a decreasing solids content (4, 13, 14). The *D* values of *Pediococcus* sp. decreased with decreasing pH of the heating menstruum (Table 2) as previously reported for other non-spore-forming bacteria (4, 15, 17).

TABLE 2. The effect of growth medium^a on *D* value of *Pediococcus* sp. NRRL B-2354 at 60°C in various heating menstrua

Heating menstruum	pH	<i>D</i> value (min)		Fold increase ^b
		TGY	TSB	
Pineapple juice	3.70	0.14 ± 0.01	0.70 ± 0.04	5.0
Apple juice	3.92	0.33 ± 0.01	1.05 ± 0.03	3.2
Tomato juice	4.45	0.65 ± 0.02	1.36 ± 0.06	2.1
Water	7.30	1.09 ± 0.02	4.46 ± 0.18	4.1
Pasteurized whole liquid egg	7.31	1.19 ± 0.03	9.53 ± 0.41	8.0
10% Glucose solution	7.22	2.46 ± 0.12	12.05 ± 0.71	4.9
Skim milk	6.80	9.07 ± 0.35	10.50 ± 0.61	1.2

^a Cells were grown at 28°C in TGY or TSB to stationary phase, centrifuged, washed once with sterile distilled water and resuspended in the appropriate heating menstruum to a final cell concentration of ca. 8 log CFU/ml.

^b Relative increase in *D* value of cells grown in TSB as compared to those cells grown in TGY.

Unlike thermal inactivation (Table 2), microwave energy had little or no effect on inactivation of *Pediococcus* sp. in pineapple juice, tomato juice, or milk as compared to water, apple juice, or 10% glucose (8). This suggested that the microwave energy was selectively absorbed by the suspended solids in the menstruum instead of the microbial cells. Therefore, microbial inactivation using microwave energy was dependent on the suspended solids in the heating menstruum rather than on the pH (8).

The maximum heat resistance of microorganisms is usually at pH values close to neutrality (1, 5). Although the pH values of water, liquid egg, 10% glucose, and skim milk were close to neutrality, among these four heating menstrua the lowest *D* values were obtained with water and liquid egg (Table 2). While the decrease in *D* value in water may be attributed to the low solids content of this heating menstruum (13, 14), it is not the case for liquid egg, which has a solids content of 25% (12). While the solids content was 10% in skim milk (18) and 10% in glucose solution, the *D* value of *Pediococcus* sp. cells in liquid egg was 13 to 91% of the *D* value in 10% glucose and skim milk (Table 2). The *D* value of *Salmonella senftenberg* 775W in liquid egg at pH 7.4 was reported to be 48% of the *D* value obtained at pH 5.5, suggesting that bacteria were more resistant to thermal inactivation in liquid egg with pH values closer to 5.5 rather than neutrality (5). Thus the lower *D* values obtained in this study for cells in liquid egg compared to those obtained for cells in 10% glucose solution or skim milk could be due to the high pH value of liquid egg.

Although the highest *D* value was obtained with TSB-grown cells heated in 10% glucose (Table 2), skim milk seemed to offer the highest protection from thermal inactivation regardless of the growth medium. The TSB-grown culture exhibited a 10-min lag time for thermal inactivation in skim milk compared to 3.5 min in 10% glucose (Table 1). Also, the calculated processing times to accomplish a 99.99% (4*D* values) kill of *Pediococcus* sp. cells grown in TGY or TSB were the highest when skim milk was the heating menstruum, compared to the other heating menstrua (Table 1). Thermal inactivation of *M. freudenreichii* MS66 in milk and milk by-products was reported to be influenced more by the content of low-molecular-weight solutes rather than by total solids (7, 16). This protective effect could be due to the influence of lactose and milk salts on osmolality, which in turn offer protection from thermal inactivation. Also, the 10% glucose solution seemed to offer good protection from thermal inactivation for cells grown in TSB, where the processing time was similar to that of skim milk (Table 1). Apparently glucose, like lactose, can offer protection from thermal inactivation (14).

This research draws attention to the potentially critical role of *Pediococcus* sp. growth conditions for the interpretation of its thermal resistance (*D* value). Changes in *D* value due to a change in growth medium provide the supporting evidence for this idea. Also, we have shown that thermal inactivation of this bacterium was dependent on the heating menstruum pH and solids content. Therefore the prediction of bacterial thermal inactivation in foods from data obtained

by using one growth medium or one heating menstruum is not advisable. Also, the influence of heating menstruum on microbial inactivation by means of emerging new technology (such as electromagnetic energy, ohmic heating, etc.) may not be the same as with thermal energy.

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REFERENCES

1. Condon, S., and F. J. Sala. 1992. Heat resistance of *Bacillus subtilis* in buffer and foods of different pH. *J. Food Prot.* 55:605–608.
2. Dennis, W. H., and K. B. Yatvin. 1981. Correlation of hyperthermic sensitivity and membrane microviscosity in *E. coli* K1060. *Int. J. Radiat. Biol.* 39:265–271.
3. Hansen, E. W. 1971. Correlation of fatty acid composition with thermal resistance of *E. coli*. *Dansk Tidsskr. Farm.* 45:339–344.
4. Hansen, N.-H., and H. Riemann. 1963. Factors affecting the heat resistance of nonsporing organisms. *J. Appl. Bact.* 26:314–333.
5. Jay, J. M. 1996. High-temperature food preservation and characteristics of thermophilic microorganisms, p. 347–369. *In* J. M. Jay (ed.), *Modern food microbiology*, 5th ed. Chapman and Hall, New York.
6. Julák, J., and M. Mára. 1973. Effect of glucose or glycerin in cultivation media on the fatty acid composition of *Listeria monocytogenes*. *J. Hyg. Epidemiol. Microbiol. Immunol.* 17:329–338.
7. Kornacki, J. L., and E. H. Marth. 1993. Thermal inactivation of *Salmonella senftenberg* and *Micrococcus freudenreichii* in retentates from ultrafiltered milks. *Lebensm. Wiss. Technol.* 26:21–27.
8. Kozempel, M. F., B. A. Annous, R. D. Cook, O. J. Scullen, and R. C. Whiting. 1998. Inactivation of microorganisms with microwaves at reduced temperatures. *J. Food Prot.* 61:582–585.
9. Kozempel, M., O. J. Scullen, R. Cook, and R. Whiting. 1997. Preliminary investigation using a batch flow process to determine bacteria destruction by microwave energy at low temperature. *Lebensm. Wiss. Technol.* 30:691–696.
10. Lechevalier, M. P., and C. W. Moss. 1977. Lipids in bacterial taxonomy—a taxonomist's view. *Crit. Rev. Microbiol.* 6:109–210.
11. McGarrity, J. T., and J. B. Armstrong. 1981. The effect of temperature and other growth conditions on the fatty acid composition of *Escherichia coli*. *Can. J. Microbiol.* 27:835–840.
12. Powrie, W. O., and S. Nakai. 1985. Characteristics of edible fluids of animal origin: eggs, p. 829–855. *In* O. R. Fennema (ed.), *Food chemistry*, 2nd ed. Marcel Dekker, Inc., New York.
13. Reichart, O., and C. Mohácsi-Farkas. 1994. Mathematical modeling of the combined effect of water activity, pH and redox potential on the heat destruction. *Int. J. Food Microbiol.* 24:103–112.
14. Smith, J. L., R. C. Benedict, and S. A. Palumbo. 1983. Relationship of water activity to prevention of heat injury in *Staphylococcus aureus*. *Lebensm. Wiss. Technol.* 16:195–197.
15. Speck, M. L. 1947. The resistance of *Micrococcus freudenreichii* in laboratory high-temperature-short-time pasteurization of milk and ice cream mix. *J. Dairy Sci.* 30:975–981.
16. Speck, M. L., and H. L. Lucas. 1951. Some observations on the high-temperature-short-time pasteurization of chocolate milk. *J. Dairy Sci.* 34:333–341.
17. Splittstoesser, D. F., M. R. McLellan, and J. J. Churey. 1996. Heat resistance of *Escherichia coli* O157:H7 in apple juice. *J. Food Prot.* 59:226–229.
18. Swaisgood, H. E. 1985. Characteristics of edible fluids of animal origin: milk, p. 791–828. *In* O. R. Fennema (ed.), *Food chemistry*, 2nd ed. Marcel Dekker, Inc., New York.