Food Structure

Volume 9 | Number 1 Article 8

1990

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FOOD STRUCTURE, Vol. 9 (1990), pp. 61-67
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

1046-705X/90$3.00+.00

AN ENZYME/SURFACTANT TREATMENT AND FILTRATION TECHNIQUE FOR THE RETRIEVAL OF *Listeria monocytogenes* FROM ICE CREAM MIX

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Abstract

This study combines an enzyme/surfactant treatment with centrifugation and prefiltration to solubilize food constituents in a dairy product containing *Listeria monocytogenes*, remove the constituents by a second filtration and examine the isolated bacteria under the scanning electron microscope. Treatment of an ice cream mix with a combined 2% (w/w) trypsin and 2% (w/w) Tween 80 solution for 20 minutes at 35°C resulted in proteolysis of the dairy mix without lysing the bacteria. Centrifugation at 4300 x g for 20 minutes at 5°C concentrated the bacteria in the form of a pellet which was subsequently purified through a prefilter system prior to a final filtration through a polycarbonate filter with 0.4 μm pores. The bacteria on the filter were fixed in a glutaraldehyde solution, postfixed with osmium tetroxide, dehydrated in an ethanol series, impregnated with hexamethyldisilizane and air-dried. This procedure made it possible to retrieve sufficient concentrations of purified bacteria on the filter for examination in the SEM.

Initial paper received March 1, 1990
Manuscript received March 29, 1990
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KEY WORDS: *Listeria monocytogenes*, trypsin, Tween 80, ice cream, polycarbonate filters, hexamethyldisilizane

Introduction

In the past, filtration techniques for the examination of dairy products have not been feasible for a variety of reasons. Primarily, dairy products and in particular, ice cream, are inherently viscous because of the presence of fat and casein micelles making filtration virtually impossible. Nutting et al. (1959) investigated the use of membrane filtration to select coliforms and pioneered the use of warmed surfactant (Triton X-100) added to ice cream to maximize its filterable volume. Sharpe et al. (1979) investigated a number of foods and the effects of dilution, temperature, pressure, enzymes and surfactants on their ability to be successfully filtered using hydrophobic grid membrane filter (HGMF) and the membrane filter (MF) techniques. Peterkin and Sharpe (1980) demonstrated the increased recovery of five common bacteria from ice cream using membrane filtration and incubation with a protease and surfactant. Entis et al. (1982) developed a prefiltration, enzyme (trypsin) and surfactant (Tween 80) treatment prior to application of the HGMF technique for the enumeration of coliform bacteria from dairy products. They showed that the use of an enzyme/surfactant technique was not deleterious to the cells as measured by the aerobic plate count.

The above enzyme/surfactant methods have been applied to dairy products to remove interfering substances, allowing bacterial enumeration. Previous efforts by Amelang (1988, unpublished) demonstrated the need to apply this procedure to scanning electron microscopy to obtain "clean" images of bacteria growing in the ice cream mix.

Pasteurized dairy products other than fluid milk have relatively low numbers of microorganisms, < 50,000/g (Anon., 1985) and no foodborne pathogens should be present in the finished product. *Listeria monocytogenes* has been isolated from milk and dairy products and is becoming increasingly well known as a dangerous food pathogen. Over 33 ready-to-eat dairy products, primarily ice cream and novelty items, have been recalled since 1985 because of contamination by *L. monocytogenes*. This organism can proliferate at refrigeration

temperatures and survive at freezer temperatures; hence the reason for concern within the food industry.

This study utilizes the enzyme/surfactant treatment (Nutting et al. 1959; Sharpe et al., 1979; Peterkin and Sharpe 1980; and Entis et al. 1982) prior to the filtration and fixation necessary for observation of L. monocytogenes cells with the scanning electron microscope. Chemical toxicity was performed to ensure that the enzyme/surfactant treatment did not have a deleterious effect on the size or morphology of the cells. Three drying techniques: hexamethyldisilizane-HMDS (Nation, 1983), critical point drying (CPD), and freeze drying were compared to obtain the method which would yield clear micrographs. Morphological size differences, which could occur when L. monocytogenes is grown under less than optimum conditions could lead to misidentification and underreporting of this organism and therefore were investigated.

Material and Methods

Preparation of Inoculum

L. monocytogenes strain Scott A was obtained from the culture collection of M. Doyle at the University of Wisconsin. The culture was maintained on trypticase soy agar supplemented with 0.6% yeast extract (TSYE agar, Baltimore Biological Laboratory-BBL, Cockeysville, MD) slants at 4°C. Prior to use, a 10 ml tube containing trypticase soy broth supplemented with 0.6% yeast extract (TSYE broth, BBL) was inoculated by the loop method and allowed to incubate for 18 h at 35°C.

From an actively growing 18 h culture serial dilutions were made in potassium phosphate buffer (0.1% stock, pH=7.2, Bell et al., 1985) to obtain a concentration of approximately 10^7 cells/ml. Ten milliliters of the culture were added to 500 ml of ice cream mix and incubated for 10 h at 35°C to achieve logarithmic phase of growth. This suspension was then used for the enzyme/surfactant studies.

The ultra-high temperature-treated, unflavored ice cream mix was obtained from HP Hood (Boston, MA) and contained the following ingredients and concentrations (w/v): skim milk, 43.12%; cream 43.93%; condensed skim milk, 5.35%; granulated sugar, 16%; stabilizer, 0.5% and emulsifier, 0.1%. It contained 14% milk fat and was stored frozen at -20°C prior to use.

Enzyme/Surfactant Treatment

The ice cream mix was treated with an enzyme, 2.0% w/w trypsin (Sigma Chemical Co., St. Louis, MO) and a surfactant, either 1.0% w/w Triton X-100 (Alkyaryl polyether alcohol, J.T. Baker Chemical Co., Phillipsburg, NJ) or 2% w/w Tween 80 (Polyoxyethylene sorbitan monooleate, Difco Laboratories, Detroit, MI) prior to filtration. Each 10 ml-treated sample was incubated for 20 min at 35°C and centrifuged at 4300 x g (Sorvall Superspeed RC2-B, Ivan Sorvall Inc., Norwalk, CT) for 20 min at 5°C. The pellet was resuspended in 10 ml potassium phosphate buffer solution. A sample of the suspension was viewed under phase contrast microscopy (Zeiss Axioskop, Carl Zeiss, Inc., West Germany) to ensure the presence of the microorganism. Viable cells exhibiting the tumbling motility characteristic of this organism were evident. The sample was prefiltered using a 13 mm Prefilter Type AP25 (Millipore Corp., Bedford, MA) and the filtrate was subsequently passed through a 13 mm polycarbonate filter 0.4 μm pore (Nucleopore Corp., Pleasanton, CA).

Preparation of Filters for SEM

After final filtration, the filters were transferred from the Swinney holders (Gelman Sciences Inc., Ann Arbor, MI) to CPD specimen-processing capsules (BioRad Microscience, Cambridge, MA) and immersed in 3% glutaraldehyde (GA) in 0.15M sodium cacodylate buffer, pH=7.1 (Electron Microscopy Sciences-EMS, Ft. Washington, PA) for 1 h at room temperature. After removal of the glutaraldehyde and three washes in the same buffer concentration, the samples were postfixed in vapor saturated with 1% osmium tetroxide in 0.15M sodium cacodylate buffer, pH=7.1 (EMS) for 1 h at room temperature under a hood. The samples were then washed in distilled water, followed by dehydration in a gradient series of ethanol baths for 3 min each. Two methods of drying were used. One set of capsules was capped and transferred to a Polaron E3000 Critical Point Dryer (BioRad) and CO_2 (CP=31.5°C and 7.5×10^6 Pa)was used as the final transitional fluid. The second set of capsules was treated with two 3 min baths of HMDS (EMS) and air-dried (AD) for 1 h. All filters were mounted onto aluminum stubs with double sticky tabs and a spot of colloidal silver and sputter-coated with 28 nm of gold in a PS-2 Sputter Coater (International Scientific Instruments-ISI, Pleasanton, CA). The prepared stubs were viewed using an ISI-60 Scanning Electron Microscope operated at an accelerating voltage of 10 kV and photographed using Polaroid 52 film (ISO-400).

Freeze Drying (FD) Method

A 10 ml ice cream mix sample was subjected to the enzyme/surfactant treatment, filtered and the filter was mounted onto an aluminum stub. The stub was initially placed onto a polished copper block cooled in liquid nitrogen and then transferred to a Virtis 15 SRC Sublimator (Virtis Company Inc., Gardiner, NY) and dried according to the manufacturer's instructions. Following sublimation, the stubs were gold coated and viewed in the SEM.

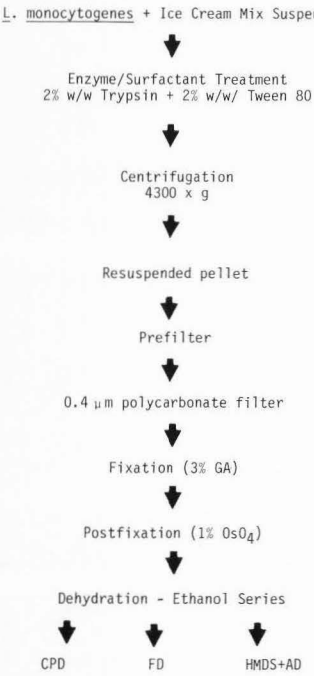
Chemical Toxicity Study

The three chemicals (trypsin, Triton X-100, and Tween 80) were tested for detrimental effects on the growth or colony size of L. monocytogenes prior to use in any treatments. Samples of the culture grown in TSYE broth were incubated for 20 min at 35°C with two concentrations of each chemical (0.5 and 1.0% for Triton X-100, or 1.0 and 2.0% for trypsin and Tween 80). The samples were subsequently serially diluted and plated onto the surface of TSYE broth containing 1.5% agar. After incubation for 48 h at 35°C, these plates were enumerated and colony sizes were measured. These experiments were performed in

duplicate.

Size and Morphology Study of Cells at Different Temperatures

Ice cream mix samples were inoculated with actively growing cultures of L. monocytogenes and incubated at three temperatures: 4°C, 21°C, 35°C for 5 d, 14 h and 10 h, respectively, to obtain cells in logarithmic phase of growth. These time/temperature combinations were determined through preliminary growth curve observations. Following incubation, these samples were treated according to the previously outlined enzyme/surfactant procedure using 2.0% trypsin and 2.0% Tween 80. For examination with the SEM the filters were fixed, dehydrated, and critical point dried using CO_2 as the transitional fluid. Measurements of the bacterial cells in the photomicrographs were taken using the MacIntizer System (GTCO Corp., Rockville, MD) with the MacMeasure Program (Hook and Rasband, 1987) and analyzed using the Dunnett's test procedure (Steel and Torrie, 1980). The following flow chart clarifies the sequence of pretreatments and SEM preparations:



Results and Discussion

Enzyme and Surfactant Treatment

Trypsin combinations with either Triton X-100 or Tween 80 were compared to determine the more suitable surfactant for this treatment. After initial screening, we concluded that the combination of 2.0% trypsin and 2.0% Tween 80 produced images with substantial breakdown of the unfilterable portions of the ice cream mix (i.e., fats and proteins). Figure 1 shows that this combination enhances the breakdown and filtration and allows the recovery of a population of cells. Figure 2, a micrograph of diluted but untreated suspension from preliminary work in this lab by J. Amelang, is evidence that without such treatment, isolation of the bacteria from the ice cream mix is impossible.

The enzyme/surfactant treatments supplement the work reported by Sharpe et al. (1979), Peterkin and Sharpe (1980), and Entis et al. (1982). Previous reports also use enzymes and surfactants prior to the filtering of foods to obtain bacterial cell counts. Our objective, however, was to solubilize corpuscular food constituents without damaging the bacteria, filter off the solution and examine the remaining bacteria by SEM. This method was performed more easily than previous procedures mentioned above because enzyme and surfactant were added directly to the ice cream sample avoiding the need for stock solutions and sample dilution. Using our method, applications for determining microbial counts by the plate count method or observations of cells by bright field could be performed. It is evident from the micrographs that the treatment is effective in breaking down the components of the ice cream mix while not affecting the quantity or quality of L. monocytogenes cells.

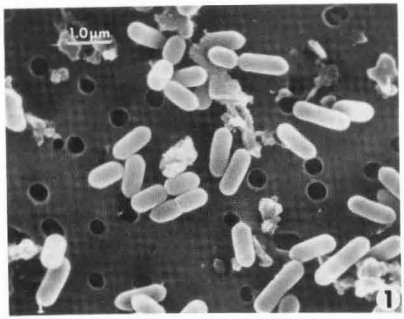
Freeze-Drying Method

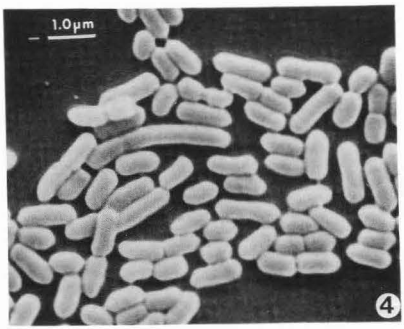
We hoped that a freeze-drying method would circumvent the need for the conventional and time consuming fixing and drying procedures. Examination of Figure 3 showing a filter of a treated sample demonstrates the amount of unsolubilized components obscuring the bacteria and indicates that the additional baths of ethanol in the CPD or HMDS methods of drying actually aid in the further removal of those components.

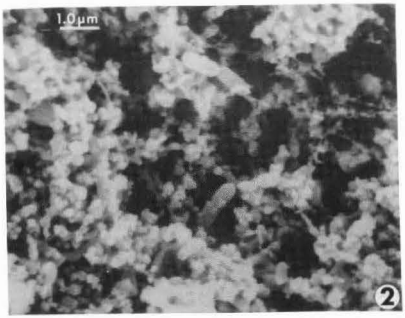
Chemical Toxicity Study

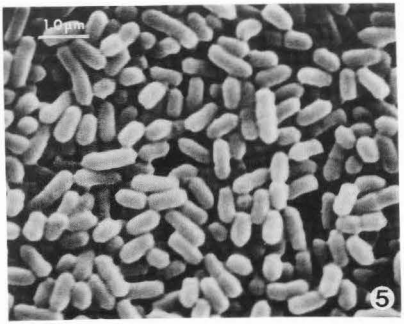
Preliminary chemical toxicity experiments verified that trypsin, Triton X-100, and Tween 80 did not have a deleterious effect on the survival, colony size, or morphology of L. monocytogenes. It is evident from the data obtained in replicated experiments (Table 1) that there is no difference in total numbers and very little difference in colony sizes after exposure to these chemicals when compared with the untreated sample.

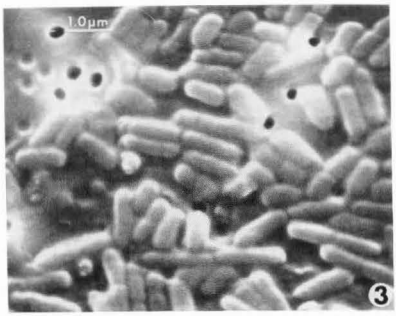
This finding correlates with the work performed by Entis et al. (1982) in which total bacterial cell counts from enzyme-treated and filtered foods were compared with filtered untreated foods. No decrease in numbers was obtained in the treated samples. Further support for survival of bacterial cells is illustrated

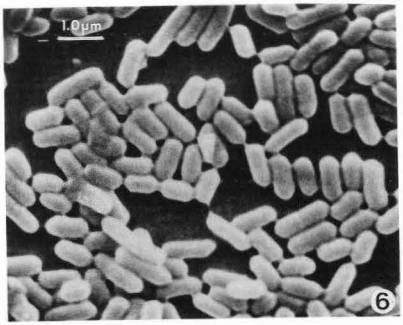












in Figures 4-6 taken from plates incubated for 48 h at 35°C. Upon visual analysis, bacterial cell sizes and morphology appeared to be similar.

Size and Morphology Study at Different Temperatures

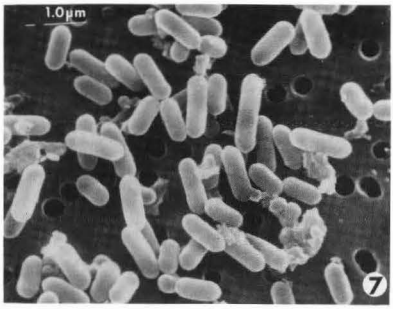
L. monocytogenes grows over a wide range of temperature. We were curious to see if there were vast morphological size differences in the cells when grown in the ice cream mix at different temperatures. Christophersen (1973) raised questions about morphological changes that may or may not be evident in the bacterial cell

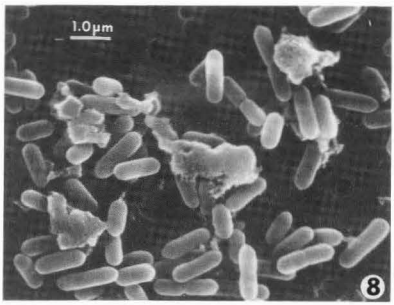
Table 1. Total numbers and size of L. monocytogenes colonies when grown in TSYE broth and treated with varying concentrations of trypsin, Triton X-100, or Tween 80 for 20 min, at 35°C.

Chemical Treatment	Concentration %	Total Number[1] (CFU/ml)	Size[2] (mm)
Untreated		3.6×10^8	3.25
Trypsin	1.0	2.0×10^8	2.85
Trypsin	2.0	1.5×10^8	2.65
Triton X-100	0.5	2.5×10^8	3.00
Triton X-100	1.0	1.5×10^8	2.95
Tween 80	1.0	2.6×10^8	3.25
Tween 80	2.0	3.1×10^8	3.25

[1]Total numbers are estimated from duplicate plates; CFU=colony forming units

[2]Size is represented as the mean of seven colony diameters.





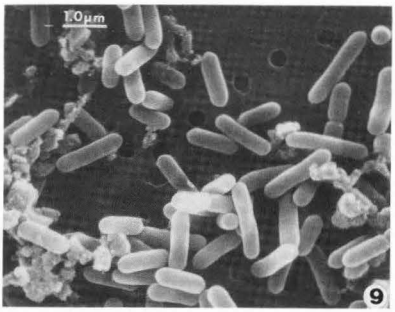


Fig. 1. Ice cream mix containing L. monocytogenes grown for 12 h at 35°C. Sample treated with 2% trypsin and 2% Tween 80 for 20 min, passed through a prefilter and 0.4 µm polycarbonate filter. HMDS + AD. Bar=1 µm.
Figure 2. Ice cream mix containing L. monocytogenes grown for 48 h at 21°C. Treatment included centrifugation and successive filterings (Whatman 49 filter paper and then, 5 µm and 0.45 µm polycarbonate filters). CPD. Bar=1 µm.
Fig. 3. Filter with treated sample of L. monocytogenes in ice cream mix grown for 7 d at 4°C and treated wtih 2% trypsin and 2% Tween 80. FD. Bar=1 µm.

Fig. 4. Untreated sample of L. monocytogenes grown in TSYE broth for 48 h at 35°C. Bacteria fixed on agar. HMDS + air-dried. Bar=1 µm.

Fig. 5. L. monocytogenes grown in TSYE broth for 48 h at 35°C and treated with 2% trypsin for 20 min. HMDS + AD. Bar=1 µm.

Fig 6. L. monocytogenes grown in TYSE broth for 48 h at 35°C and treated with 2% Tween 80 for 20 min. HMDS + AD. Bar=1 µm.

Figs. 7,8,9: L. monocytogenes grown in ice cream mix, treated with 2.0% trypsin, and 2.0% Tween 80, pre-filtered and filtered through a 0.4 µm polycarbonate filter. CPD. Bar=1 µm.

Fig. 7. Culture conditions 7 d at 4°C.
Fig. 8. Culture conditions 12 h at 35°C.
Fig. 9. Culture conditions 15 h at 21°C.

grown under suboptimum temperature conditions. The observance of size differences or significant morphological changes from rods to cocci could indicate inadequacies in the testing of ice cream mix for the presence of pathogens, leading to possible misidentification, especially of L. monocytogenes. Because this is a major concern to the dairy industry as a whole, the samples were inoculated and kept at either 4°C, 21°C, or 35°C for the length of time needed for the cells to achieve logarithmic growth. The samples then underwent the previously described enzyme/surfactant treatment, centrifugation, prefiltration and fixation. All filters were then critical point-dried, gold coated and viewed in the SEM. Figures 7, 8, 9 show samples at all three temperatures. Measurements of the cells were taken from each figure and were compared using statistical analysis. The results indicate no significant size differences among the three temperatures although the samples grown at 35°C did appear smaller than those grown at the lower temperature.

Summary

The presence of L. monocytogenes in ice cream mix has been previously enumerated by plating techniques but because of the density of the mix constituents, microscopic viewing of the sample has been difficult. An enzyme/surfactant treatment with 2.0% trypsin and 2.0% Tween 80 when coupled with centrifugation and filtration yields micrographs of acceptable quality. While the quicker drying method using HMDS can be used to indicate recovery of cells, CPD remains the method of choice where size or morphological changes are concerned. These pretreatment and SEM preparative techniques can be easily used and do not have deleterious effects on the bacteria. The possibility of using SEM to answer microbiological questions in ice cream is now feasible.

Acknowledgement

We are very grateful to J. Amelang for his preliminary work with ice cream mix filtration. This article is authorized for publication as Paper No. 8228 in the series of the Pennsylvania Agricultural Experiment Station.

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Discussion with Reviewers

C.W. Donnelly: Are the methods developed applicable to retrieval of Listeria from other food systems, such as meats? What about tissue samples? If so, the methods could be extremely useful for human and animal epidemiological studies.

Authors: Although experiments addressing other food systems have not been investigated, it can be surmised from the proteolytic and surfactant nature of the treatment that application is feasible.

C.W. Donnelly: Will the developed methods be useful in improving our abilities to isolate and detect Listeria? If so, could the authors define such uses?

Authors: This enzyme/surfactant treatment would be applicable to situations in which interferring food substance could be removed leaving intact cells of L. monocytogenes. This concentrated form of cells could then be added to detection media resulting in a higher chance of recovery. This method would be particularly valuable when cell numbers of L. monocytogenes are low.

R.E. Brackett: Table 1 and Figure 4-6 are used to support the conclusion that trypsin, Triton X-100, and Tween 80 do not have an adverse effect on L. monocytogenes cells. However, data in Table 1 indicate that colony size was reduced by up to 23% (2% trypsin). Also it appears that cells shown in Figure 5 (trypsin-treated) are somewhat deformed compared to those in Figure 6. How do you explain this?

Authors: Reduction in colony size does not necessarily indicate cell size reduction; therefore, colony size was not a major factor in determining the toxicity of the chemicals.

The cells in both of these micrographs were fixed directly on agar, dehydrated, impregnated with HMDS and air-dried. Comparison of cells grown at 35°C in Figures 4 (untreated), 5 (2% trypsin) and 6 (2% Tween 80) all of which were treated with HMDS, and air-dried with those in Figure 8 (2% trypsin + 2% Tween 80) which were also grown at 35°C and critical point dried suggest that size and shape variations may be due to the temperature at which the cells were cultured or to the SEM preparation. The non-deleterious effect of the trypsin treatment is supported by the Total number data illustrated in Table 1.

R.E. Brackett: The materials and methods describe a software program for measuring bacterial cell length and a statistical procedure of analyses. Are results of these analyses not presented because no significant differences were found? Also, were similar measurements done on cells shown in Figures 4-6?
Authors: The results were not presented because in actuality no significant differences were discovered. Similar statistical procedures were not performed on Figures 4-6 because visual analysis of the cells which were critical point dried (Figures 1, 7, 8, 9) in combination with the data collected in Table 1 seemed adequate to ensure no adverse effects upon the cells. Measurements were made on Figures 7-9 to investigate the possibility of decreased size or morphological changes in relation to varied temperatures and times of incubation.

J. Humber: What was the final concentration of L. monocytogenes in the ice cream mix prior to enzyme and surfactant treatment? Would the procedure have been successful when lower cell numbers are present in the ice cream mix - 10 cells per g, for example?
Authors: The final concentration of L. monocytogenes in the ice cream mix prior to treatment was 10^8 cells/ml. It would be possible to recover cells from samples inoculated or contaminated with low numbers of bacteria because the centrifugation and filtration following the treatment not only remove the solubilized food components but concentrate the cells as well.