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Removal of Heat-Resistant Bacterial Spores by Filters: Implications for Filter Use in Soft Drink Production

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Removal of Heat-Resistant Bacterial Spores by Filters: Implications for Filter Use in Soft Drink Production

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Abstract

Soft drinks are subject to spoilage induced by bacterial spores resistant to high-temperature. To date, no effective sterilization method that also does not harm the taste profile of the final product has been developed. Filters with pore sizes slightly larger than conventional 0.2µm sterilizing-grade filters were evaluated for their ability to remove heat-resistant bacterial spores. In addition, these filters were evaluated with an eye on improving process parameters such as minimization of heat treatment on final products, limited alteration of flavor and ingredients, and energy saving and cost reduction in the soft drink manufacturing process. Spores from six bacterial species (*Bacillus subtilis, Bacillus stearothermophilus, Bacillus coagulans, Clostridium thermaceticum, Clostridium thermosaccharolyticum* and *Alicyclobacillus acidoterrestris*) were selected as test organisms because they are often isolated as spoilage bacteria in soft drinks. Spores, from each bacterial species, were suspended in 0.067M phosphate buffer solution (pH 7.0) at concentration estimated to be approximately $1x10^5$ cfu/mL. Each 1 L suspension was filtered through a 47 mm test disk. The filtrate was assayed for the presence of viable spores. For filter membranes with absolute pore size ratings of 0.45µm, no spores were detected in the filtrate. For filters with pore size ratings of 0.65µm and 0.8µm, however, spores were detected. The study has shown that Pall filters with pore size ratings of 0.45µm are effective in removing heat-resistant bacterial spores, which suggests application of these filters for bacterial control in the soft drink manufacturing process.

Introduction

Non-carbonated soft drinks delivered in sealed containers such as PET or glass bottles or cans are usually treated by heat sterilization in the manufacturing process to remove spoilage microbes. Conditions of the heat sterilization are strict in some cases and mild in other cases, depending on the type and number of microbes in the soft drink. [1]

Some types of microbes, that adversely impact quality of soft drinks, form spores when nutrients in the surrounding environment are depleted. The formed spores remain dormant until the surrounding environmental conditions are favorable for vegetative growth. Once spores are exposed to nutrient-rich environment, they germinate into vegetative cells and resume propagation (Figure 1).

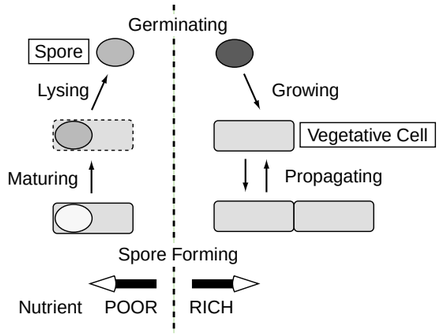

Figure 1 (far right) Life cycle of a spore forming microbe.

Spores are highly resistant to physical and chemical environmental factors such as heat, UV radiation, and pH extremes (acid and alkali)[2]. It is difficult, therefore, to completely kill them with conventional heat treatment techniques.

For example, heat treatment at 121°C is effective, within several seconds, to lower survival rate of vegetative cells of *B. stearothermophilus* to ≤1/10[5]. For spores of *B. stearothermophilus*, the duration of heat treatment needs to be increased to >10-20 minutes to have the same effect.[3]

Extended heat treatment is required to kill spores as discussed above. Such a severe heat treatment, however, alters taste, color, flavor and texture of soft drinks, which negatively affects their commercial value.[4]

A new type of spoilage was reported recently in several cases, which was induced by the presence of heat-resistant acidophil spores in acid soft drinks such as apple juice.[5] Acid soft drinks are thermally treated under relatively mild conditions in order to maintain their commercial value. Such mild heat treatment, however, is not effective in killing heat-resistant acidophil spores.[6] The final product provides a nutrient rich environment, thus, spores unaffected by the heat treatment germinate and propagate.

As described above, soft drinks are often subject to spoilage induced by high-temperature resistant bacterial spores, but no effective sterilization method that does not also degrade the flavor or quality of the product has been developed.

Filters have been used for many years in various process steps of food processing plants. Microbe control in the food processing industry by means of filtration, however, is not yet commonly adopted outside the brewery and wine industries and in mineral water production. This is because sterilizing-grade filters with pore size of 0.2/0.22µm, that have been used extensively in pharmaceutical processes for microbe removal, remove not only microbes but may also remove essential ingredients of soft drinks.

Filters that have pore sizes slightly larger than sterilizing-grade filters were investigated for their heat-resistant bacterial spore removal efficiency. Filtration offers multiple advantages including: minimization of heat treatment on final products; limited alteration of flavor and ingredients; and, energy saving and cost reduction in the soft drink manufacturing process.

Materials and Methods

Filter membranes

Supor® EB (EB), Fluorodyne® DB (DB), Supor® EK (EK) and Supor® 800 (SCW800) membranes (Pall Corporation, East Hills, NY) were used as test membranes in this study. A 0.2µm membrane, Fluorodyne® DFL (DFL), was used as recovery membrane to measure filtrate spore count when test membranes were challenged with spores. All membranes used in this study, were 47 mm disks.

Challenge spores

B. subtilis (IAM1026), *B. stearothermophilus* (IAM1035), *B. coagulans* (IAM1115), *C. thermosaccharolyticum* (isolated from spoiled canned chicken rice), *C. thermaceticum* (isolated from deteriorated canned milk coffee) and *A. acidoterrestris* (DSM2498) were tested in this study. All strains used in this study were provided by the Japan Canners Association.

Spore purification was performed at the Japan Cannners Association. First, bacterial strains were cultured on sporulation media. The media surface was scraped with a swab to collect the bacterial strains. Bacterial strains were stored in cooled deionized water of 10 mL to 15 mL. Fifteen-second-long ultrasonic treatment was repeated on each spore solution at low temperature for four to six cycles. After being centrifuged, pellets were suspended in 5 mM phosphate buffer solution (pH : 8.0). Then 100µg of lysozyme was added to each spore suspension of 1 mL and incubated for 15 to 30 minutes at 20°C. Each suspension was examined by phase contrast microscopy to determine if the spore suspension was contaminated and if the suspensions were monodispersed. The suspensions were frozen and sent to Pall Corporation's Scientific and Laboratory Services Department laboratory at Nihon Pall Ltd.

In the laboratory, 0.067M phosphate buffer (pH : 7.0) was filtrated with a DFL 0.2 µm membrane. To prepare the test solution, each purified spore suspension was suspended in 0.067M phosphate buffer to a volume of 1000 mL at concentration of about 10^5 cfu/mL.

Challenge Procedure

All the tests were conducted in a laminar flow hood. The test membranes (EB, DB, EK and SCW800), recovery membrane (DFL), 47 mm disk holder, silicone tube and glassware were sterilized by autoclaving at 121°C for 30 minutes.

A test membrane and a recovery membrane were mounted in separate disk holders, and moistened with sterile water before performing an integrity test. Only those discs that passed the integrity test (described below) were used in subsequent test steps.

A 1000 mL aliquot of the test solution was introduced to each test membrane at a constant flow rate of 33 mL/minute by means of peristaltic pump, and the effluent (filtrate) was collected in sterile glassware. Each effluent was then heated to optimum temperature (80-100°C) for the induction of spore germination[7]. After spore germination was induced, the temperature was cooled to room temperature. The entire filtrate was then filtered through the recovery membrane at flow rate of 33 mL/minute. When the filtration was completed, the recovery membrane was taken off from the disk holder, was placed on the growth medium plate and incubated at the optimum temperature (50-60°C) for growth. Colony counts were performed to determine the spore count in the filtrate.[8]

The spore count in the challenge suspension was determined in the following way. Ten mL of test solution was diluted to 10^4-10^6 fold. These dilutions were heated to optimum temperature for each spore to germinate.[9] Then all of each solution was filtered with a recovery membrane, and spore count was measured after cultivation.

After the challenge test was completed, an integrity test was conducted again for every test membrane.

Integrity test

The integrity test was performed in accordance with a Manual Bubble Point Test procedure.[10]

A silicone tube was connected to the outlet of disc holders on which test membranes and recovery membranes were mounted, and the other end of the tube was dipped into a beaker filled with water. Compressed air was introduced gradually from inlet of the disc holder, and

pressure of compressed air was recorded as a bubble point (BP) value when bubbles started coming out from the end of silicon tube in the beaker.

A filter was considered acceptable when its BP value is higher than minimum bubble point value shown in Table 1.

Table 1 (far right) Minimum bubble point value for each filter used in this study.

Filter membrane	Minimum BP (mbar)	Minimum BP (psi)
DFL	3180	46
DB	1240	18
EB	1240*	18*
EK	1100*	16*
SCW800	1100*	16*

*preliminary values

Results

Table 2 shows the concentration of spores for each test membrane challenged. Table 3 shows results of the challenge tests. The results are representative of multiple challenge tests on one to two lots of media. No spores of the six types of bacteria were detected in filtrates from EB and DB membranes, both of which have pore size ratings of 0.45µm. *B.subtilis* spores to penetrated the EK (pore size rating of 0.65µm) test membrane. Spores of all six bacterial species tested penetrated the SCW800 (pore size rating of 0.8µm) filters tested.

Table 2 (far right) Concentration of spores present in each challenge

Spore type	Concentration (cfu/mL)
B.subtilis	$3.8x10^4$-$2.5x10^5$
B.stearothermophilus	$1.4x10^4$-$1.0x10^5$
B.coagulans	$3.9\ x10^4$-$1.2x10^5$
C.thermosaccharolyticum	$3.0x10^4$
C.thermaceticum	$3.3x10^4$
A.acidoterrestris	$6.8x10^4$-$4.0x10^5$

Table 3 Spore Challenge Test Results

Test Membrane	Commercial Sterility‡					
	B. subtilis	*B. stearothermophilus*	*B. coagulans*	*C. thermosaccharolyticum*	*C. thermaceticum*	*A. acidoterrestris*
DB	Yes	Yes	NT*	Yes	Yes	Yes
EB	Yes	Yes	Yes	Yes	Yes	Yes
EK	No (LTR^)	NT	Yes	NT	NT	Yes
SCW800	No (LTR)	No (LTR)	No (TR†)	No (TR)	No (TR)	No (LTR)

‡ no challenge spores recovered in the filtrate.

* not tested.

^ limited titer reduction, approximately 1-2 logs.

† titer reduction, approximately 4 logs.

Discussion

Heat-resistant bacterial spores cause a major problem in the soft drink industry as they are not killed effectively in the conventional heat pasteurization process. One potential solution to this problem may be the application of microbial-control filters with pore size ratings of 0.45µm. This study shows that Pall 0.45µm rated filters are effective in completely removing heat-resistant bacterial spores.

A number of soft drink manufacturers currently set a goal to reduce microbe counts by $\geq 10^5$ cfu/mL.[11] The bio-burden level in soft drink manufacturing processes rarely exceeds 10^5 cfu/mL.[12] In Pall's tests, spores were suspended in test solution at concentration of 10^5 cfu/mL corresponding to 10^7 cfu per cm^2 of effective surface area. In this sense, the results of the study simulate bioburden control applications in the soft drink industry.

Bacteria-removal filters have been already introduced in breweries that do not use a heat treatment process.[13] These filters are mounted upstream of the filling process to remove yeast and *Lactobacillus* species. Mineral water that does not go through heat treatment is also treated with sterilizing-grade filters before being bottled.[14]

Few soft drink manufacturing processes, however, have adopted bacterial-control filters. This study, therefore, presents a novel solution to the soft drink industry. Pall filters with pore size ratings of 0.45µm generally do not deteriorate flavor of filtrated soft drinks, and can effectively control spoilage bioburden microorganisms.

When applying microbe-control filters to soft drink manufacturing process, two options are available: one is to combine heat sterilization and bacterial-removal filtration, and the other is to solely rely on bacterial-removal filters.

For filtration applications, there are three options:

1) **Raw material filtration**
   Filter delivered raw materials (*e.g.* sugar solutions and juice) to reduce initial bacteria count in manufacturing process;

2) **Filtration of process intermediates**
   Filter intermediates downstream of critical process steps that may allow microbes to penetrate and propagate. For example, filters may be considered in the extraction, preparation or aging steps, in order to suppress bacterial inclusion into the final product;

3) **Final product filtration**
   Filter final products immediately upstream of the filling step.

Filtration to 0.45µm using the Pall rated filters noted in this study, improves biological shelf stability without exposing the soft drink flavor components to heat degradation. Visual clarity on a consistent basis is an ancillary benefit as these filters are highly effective in removing suspended particulate matter inherent in the product subcomponents, or which are a result of upstream process additions or reactions that result in haze formation during the process. This method is limited to drinks that are not intended to be cloudy or turbid by design.

This filtration process is a means to control quality, assure safety, and produce a product with full flavor character while reducing costs in processing with thermal bio-burden control methods.

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