Proof of Concept: Containment Systems that Prevent Freeze-Dryer Contamination When Lyophilizing Escherichia coli (JM 109)

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This study describes the use of containment systems to prevent escape of microorganisms during lyophilization, thereby avoiding contamination of freeze-drying equipment. Cultures of Escherichia coli (JM 109) of an approximate cell concentration of 10^9 cfu/mL were suspended in 0.9% saline, aseptically dispensed into vials, double-wrapped in either medical-grade paper or Tyvek sterilization pouches and freeze-dried. An intentional collapse phenomenon was observed during the freeze-drying process, ejecting debris and aerosols from the vials, thus representing a worst-case challenge for containment. Following freeze drying, the layers of the pouches were tested for microbial contamination using 3 M Clean-Trace surface ATP analyzer swabs, surface swabs, and tryptone soya agar contact plates. The paper and Tyvek pouches were able to contain a maximum cell concentration of 1×10^6 cfu/mL of E. coli recovered from ejected debris. Some penetration through the first paper pouch layer was observed (although not Tyvek); however, this was successfully retained by the second, outer layer preventing contamination of the lyophilization apparatus and laboratory environment.

Keywords Containment; Contamination; Culture; Freeze drying; Lyophilization; Preservation

INTRODUCTION

Freeze drying is the method preferred by Microbial Resource Centres (MRC) and other microbiology laboratories around the world to preserve microbial cultures often used by the biotechnology industry.[1] It has been demonstrated that viabilities of some freeze-dried strains can be maintained for up to 20 years if high initial cell concentrations of between 10^6 and 10^{10} cells=mL are used.[2] However, if cake collapse is observed when freeze drying cell densities of this order, the resultant aerosol generation can contaminate the freeze-drying chamber and condenser.[3] MRCs preserve many different types of microorganisms so that contamination of the freeze dryer and condenser leads to increased risk of cross-contamination and compromised culture purity, thereby extending decontamination procedures, prolonging downtime of the freeze dryer, and increasing costs.[4]

Loss of substrate during the sublimation stage of freeze drying is often termed ablation[5] and is partly considered a consequence of particle entrainment in high-velocity subliming water vapor.[6] Crude in-line filters have been shown to prevent microbial contamination of the freeze-dryer condenser[7,8] and indeed the use of filter boxes have been suggested to contain product, thus preventing contaminating the freeze dryer by ablation.[9] Currently, however, containment systems that are specifically designed for containment of aerosols and particles generated by ablation during freeze drying of microorganisms do not exist.

Recently, medical-grade paper and Tyvek sterilization pouches were used to perform sterile freeze drying of a pharmaceutical protein in a non-sterile environment where these containment barriers were demonstrated to prevent bacterial contamination of nutrient-rich growth media during a freeze-drying process simulation.[10] It was postulated that these barriers may also restrict the movement of aerosolized particles formed during ablation when microbial cultures were freeze dried, therefore providing an inexpensive method to prevent contamination of the freeze dryer. A freeze-drying process previously used to investigate ablation of E. coli from vials[5] was adapted for use with paper and Tyvek pouches. It was understood that resistance to water vapor during sublimation causes an increase in product temperature at constant shelf temperature (T_s) due to a reduction in the latent heat loss brought about by decreased sublimation rates.[10] To compensate for the reduced sublimation rate imposed by barriers, shelf temperature (T_s) was decreased. The aim of this study was to determine whether medical-grade paper and Tyvek pouches could contain bacterial cells during freeze drying when an intentional collapse was induced.

MATERIALS AND METHODS

Schott type I borosilicate 2 mL tubular glass vials (Adelphi Healthcare Packaging, Haywards Heath, West Sussex, UK) and 152-mm-square aluminium trays of 0.7 mm thickness were used for all tests. Chevron-style self-seal sterilization pouches 305 mm × 381 mm were used for all containment experiments. SBW pouches were formed from latex-impregnated medical-grade paper with a laminated polyester base (Marathon Laboratory Supplies, London, UK) and SPS Medical pouches formed from Tyvek 1073B with a laminated polyester base (MET Ltd, Ashford, Kent, UK). All parts were autoclaved in an Astell AMA420 autoclave (Astell Ltd, Siddcup, Kent, UK). An Edwards Super Modulyo (Crawley, West Sussex, UK) pilot-scale freeze dryer (housed in the open laboratory) was used throughout. It was fitted with calibrated RTD temperature probes and calibrated Edwards active Pirani gauges. Data were captured using a Eurotherm Chessell (Worthing, East Sussex, UK) Model 346 chart recorder.

Freeze-Drying Cycle Development Using 0.9% Saline

A freeze-drying method developed for E. coli[5] was adapted using vials containing a 1 mL fill of 0.9% saline suspending medium (without microorganism). The shelves were pre-cooled to −40°C and the trays of unwrapped vials were placed on the shelves and held for 4 h. The T_s was ramped to 4°C in 1 h and then held for 16 h with a chamber pressure (P_c) of 7 Pa (52.5 mTorr). To compensate for the higher product temperature (T_p) caused by barrier resistance,[10] T_s was reduced to 0°C for paper pouches and to −5°C for Tyvek pouches.

Containment Experiments

E. coli (JM 109) (Promega UK, Southampton, UK) was grown up on tryptone soya agar slopes (Oxoid, Basingstoke, Hampshire, UK) by inoculating with one colony from a stock culture plate and incubating at 37°C for 16 hours. The bacterial lawn was re-suspended in 10 mL of sterile 0.9% saline for irrigation USP (Baxter Healthcare, Compton, Berkshire, UK) and the total viable count (TVC) of the suspension was determined by serial decimal dilution in maximum recovery diluent (MRD) (Oxoid, Basingstoke, Hampshire, UK), plating on tryptone soya agar (TSA) plates (Oxoid, Basingstoke, Hampshire, UK) and incubating at 37°C for 48 h. Aliquots of 1 mL of bacterial suspension were then aseptically pipetted into seven autoclaved 2 mL vials. The vials were held in close hexagonal packing in the center of on autoclaved aluminum tray using aluminum strips. Two duplicate trays were prepared and double-wrapped using paper pouches and freeze-dried using $T_s = 0°C$. This process was then repeated, double wrapping trays in Tyvek pouches and freeze drying them using $T_s = -5°C$.

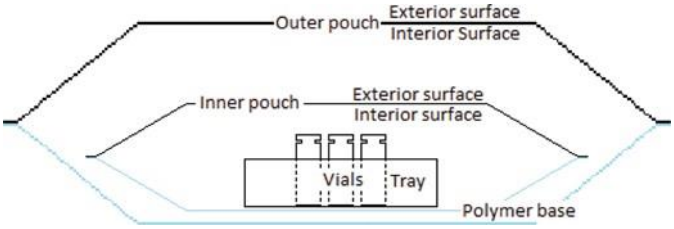

FIG. 1. Diagram to illustrate double-wrapping of trays of vials in sterilization pouches, indicating the interior and exterior surfaces of outer and inner pouches that were tested for E. coli JM 109.

When freeze drying was complete, the trays of vials were removed from the freeze dryer and the interior and exterior surfaces of the inner and outer pouches (Fig. 1) sampled with 3 M Clean-Trace surface ATP analyzer swabs (3 M, St. Paul, MN, USA) and TSA 24 cm^2 contact plates (Oxoid, Basingstoke, Hampshire, UK). Contact plates were applied to the pouches directly above where the vials were situated in the trays and a 100 cm^2 area adjacent to this was sampled with the 3M Clean-Trace surface ATP analyzer swab. The 3M Clean-Trace surface ATP analyzer measures luminescence in relative light units (RLU) that are proportional to the amount of ATP present and, therefore, give an indication of the cleanliness of a surface.[11] In addition, the total surface (870 cm^2) of the interior of the inner pouch was swabbed with a sterile swab (wetted with sterile saline) and plated out on a TSA agar. All plates (contact and swabs) were incubated for 48 h at 37°C. To recover any debris that had ablated from the vials, the trays were rinsed with 1 mL of sterile water for irrigation USP (Baxter Healthcare, Compton, Berkshire, UK) and TVC performed to determine the number of organisms present.

RESULTS

Freeze-Drying Cycle Development Using 0.9% Saline

Freeze-drying trays of unwrapped vials filled with 0.9% saline and utilizing the method originally developed by Adams[5] induced a collapse phenomenon, causing ejection of material from vials that coated the underside of the dryer shelf above the tray. A T_s of 4°C yielded an initial T_p of −28°C that slowly increased over 9 h until completion of drying was observed (taken as $T_p = T_s$). When the cycle was conducted with trays and vials double-wrapped in medical-grade paper using T_s of 0°C, ablated debris was observed on the internal layer of the inner pouch. T_p was −28°C and total drying time was 9 h, indicating that the vials had experienced the same conditions as the unwrapped vials. Similarly, using Tyvek pouches, the T_s was reduced to −5°C, and again debris was observed, T_p was −26°C, and drying time was 9 h. Thus, a consistent freeze-drying process yielding observable blowout with paper ($T_s = 0°C$) and Tyvek ($T_s = -5°C$)

TABLE 1
RLU readings obtained using 3M Clean-Trace surface ATP analyzer swabs of paper and Tyvek pouches

	RLU of exterior surface of the outer pouch	RLU of interior surface of the outer pouch	RLU of exterior surface of the inner pouch	RLU of interior surface of the inner pouch
Paper 1	94	72	7	13
Paper 2	17	25	891	31,231
Tyvek 3	91	15	18	24,275
Tyvek 4	295	56	51	31,189

pouches was obtained. Duplicate trays with vials containing bacteria were used for all subsequent experiments with these conditions representing a worst case for aerosol generation and potential freeze dryer contamination.

Containment of E. coli Using Sterilization Pouches Prepared from Medical-Grade Paper and Tyvek

Two sets of duplicate pouches prepared from medical-grade paper and Tyvek were processed using equivalent freeze-drying protocols previously developed. These sets of pouches were analyzed identically by visual inspection, 3 M Clean-Trace surface ATP analyzer swabs, surface swabs, contact plates, and cell density recovery by determination of material ejected or ablated from the vials. For the purposes of this study and to aid interpretation of these data, the pouches containing trays of vials are termed paper 1, paper 2, Tyvek 3, and Tyvek 4.

Visual Inspection

Inspection of the trays following freeze drying indicated no debris in paper 1; however, debris deposits had collected in paper 2, Tyvek 3, and Tyvek 4. The interior surface of the inner pouch of paper 2 showed some absorption of deposited crystalline material. Further inspection showed some of the crystalline debris had penetrated through the paper layer of the inner pouch. The interior surface of the inner pouches of Tyvek 3 and Tyvek 4 also showed some absorption of deposited salt crystals. However, this had not penetrated through the Tyvek material as observed with paper 2.

3 M Clean-Trace Surface ATP Analyzer

According to the manufacturer's instructions for the 3 M Clean-Trace surface ATP analyzer, a surface can be considered contaminated if a reading of >250 RLU is obtained. Where no debris was observed in paper 1, a reading of <250 RLU was recorded on the interior layer of the inner pouch. Swabbing of the interior layer of the inner pouch of paper 2 indicated an RLU reading of >250 RLU. The exterior of the inner pouch of paper 1 detected a reading of <250 RLU where paper 2 detected a reading of >250 RLU. The interior and exterior of the outer pouches of paper 1 and paper 2 both detected readings of <250 RLU (Table 1).

Results obtained from the Tyvek duplicates (Table 1) indicated readings of >250 RLU on the inner layers of the interior pouches of Tyvek 3 and Tyvek 4. The exterior of the inner pouches and the interior of the outer pouches of Tyvek 3 and Tyvek 4 both recorded readings of <250 RLU. The exterior of the outer pouch of Tyvek 3 recorded a reading of <250 RLU; however, Tyvek 4 recorded a result >250 RLU. These outer layers had been exposed to the environment during transport to the freeze dryer and it would, therefore, be possible for contamination to occur during this procedure.

Analysis of the freeze-dryer shelves detected readings of <250 RLU. Positive controls were taken as a swab of the palm of the hand were detected as readings of >250 RLU, indicating a contaminated surface (Table 2). Swabbing of the hand using the 3M Clean-Trace surface ATP analyzer yielded results several orders of magnitude greater than the contaminated surface indication of >250 RLU. The results of positive controls and contaminated surfaces tested during this study all reflected this large range of detection.

Surface Swabs

Swabs collected using sterile saline from the interior of the inner pouch detected considerable numbers of E. coli for paper 2 where paper 1 showed no growth of contaminating microorganism. The swabs collected using sterile saline from the interior of the inner pouches of Tyvek 3 and Tyvek 4 showed considerable numbers of E. coli for both duplicates (Table 3).

TABLE 2
RLU readings obtained using 3M Clean-Trace surface ATP analyzer swabs of freeze-dryer shelves and positive control (hand) for paper and Tyvek pouches

	Shelf	Upper shelf	Control (hand)
Paper 1 and 2	61	80	15,098
Tyvek 3 and 4	32	57	4,467

TABLE 3
Ablated E. coli following freeze drying in paper and Tyvek pouches determined using surface swabs of the interior surface (870 cm^2) of the inner pouch after plating out and 48 hours incubation at 37°C (data given in cfu/64 cm^2)

Interior surface, inner pouch	Colony-forming units
Paper 1	<1
Paper 2	140
Tyvek 3	TNTC
Tyvek 4	TNTC

TNTC = too numerous to count.

Contact Plates

The interior of the inner pouch of paper 2 (exposed directly to debris) showed large numbers of contaminating microorganism where only 5 cfu was detected for the equivalent surface of paper 1. The external layer of the inner pouch of paper 2 indicated 1 cfu; however, no growth was detected on the same surface for paper 1. The inner layer of the exterior pouches of paper 1 and paper 2 showed no growth of contaminating microorganism. The outer layers of the exterior pouches of paper 1 and paper 2 that had been exposed to the environment indicated low levels of contamination (Table 4).

Contact plates also indicated large numbers of contaminating microorganism on the interior surface of both inner pouches Tyvek 3 and Tyvek 4 (exposed directly to any debris). The external layer of the inner pouch of Tyvek 4 indicated no growth, although 1 cfu was detected on Tyvek 3. The inner layers of the exterior pouches of Tyvek 3 and Tyvek 4 showed no growth of contaminating microorganism. The outer layers of the exterior pouch that had been exposed to the environment of Tyvek 3 and Tyvek 4 indicated some low level contamination (Table 4).

Cell Densities

The initial cell densities of the inocula for the ablation experiments were equivalent to those obtained during a previous study[5] of 2.25×10^9–1.35×10^9. Following freeze drying, it can be seen that, where debris and ablation had been observed, and microorganisms detected using the previous techniques, considerable cell densities were recovered (Table 5).

DISCUSSION

Formulating the E. coli in 0.9% saline and allowing the product temperature (T_p) to rise above the eutectic point (T_{eu}) of sodium chloride during freeze drying caused cake collapse. This collapse phenomenon was intended to eject sodium chloride debris (containing resuspended E. coli) capable of grossly contaminating the freeze dryer. It was found that the paper and Tyvek barriers were able to contain large numbers (3.33×10^3–1.0×10^6 cfu/mL) of E. coli recovered from this debris.

Visual inspection of the inner layer of the interior pouches of paper 2, Tyvek 3, and Tyvek 4 indicated a crystalline deposit thought to be absorbed, aerosolized ejected debris. When this deposit was analyzed with contact plates and surface swabs, it was found to be grossly contaminated with E. coli. Moreover, the equivalent 3M Clean-Trace surface ATP analyzer swabs detected surface contamination higher than luminescence detected as a positive control.

TABLE 4
Ablated E. coli following freeze drying in paper and Tyvek pouches determined using contact plates after 48 hours incubation at 37°C (data given in cfu/24 cm^2)

	cfu Exterior surface of outer pouch	cfu Interior surface of outer pouch	cfu Exterior surface of inner pouch	cfu Interior surface of inner pouch
Paper 1	4	<1	<1	5
Paper 2	3	<1	1	TNTC
Tyvek 3	2	<1	1	TNTC
Tyvek 4	1	<1	<1	TNTC

TNTC = too numerous to count.

TABLE 5
Cell densities of E. coli before and after freeze drying using paper and Tyvek pouches

	Paper 1 (cfu/mL)	Paper 2 (cfu/mL)	Tyvek 3 (cfu/mL)	Tyvek 4 (cfu/mL)
Pre-freeze drying	1.67×10^9	1.17×10^9	1.5×10^9	1.5×10^9
Post-freeze drying	Not detected	1.0×10^6	4.17×10^3	3.33×10^3

Testing the external layer of the interior pouches paper 2, Tyvek 3, and Tyvek 4 using contact plates indicated a considerable reduction in the number of organisms detected and negative 3M Clean-Trace surface ATP analyzer readings (<250 RLU) when compared to the internal layers. However, this cannot be considered absolute retention as 1 cfu=24 cm^2 was detected on the exterior layer of the inner pouch of paper 2 and Tyvek 3 (although this could have been chance contamination as colonies were not formally identified). This was not the case for paper 2, where a positive Clean-Trace surface ATP analyzer reading (>250) was detected. It was observed that this pouch contained the largest amount of debris along with penetration or breakthrough of crystalline material from the inner surface to the outer surface of the pouch. It is thought that, due to the semi-absorbent nature of the paper material, some of the aerosolized collapsed material had potentially soaked through the paper. This breakthrough was not observed using the Tyvek packages where the hydrophobicity of the material would have prevented this.

Importantly, sampling of the inner layer of all of the outer pouches (paper and Tyvek) with contact plates failed to demonstrate bacterial contamination. Furthermore, negative readings were obtained using 3M Clean-Trace surface ATP analyzer swabs. This result indicates that the small amount of contamination detected on the exterior layer of the inner pouches was contained by the exterior pouch and prevented from reaching the freeze dryer and environment.

Analysis of the exterior surface of the outer pouches indicated minor levels of contamination as detected by contact plates and 3M Clean-Trace surface ATP analyzer swabs. This was attributed to chance contamination from environmental exposure during transportation from the aseptic area to the freeze dryer and back again.

The results suggest that the vials contained in paper 1 did not undergo collapse, as only minor contamination and no debris were detected. This was confirmed after inspection of the vials showed an acceptable cake structure was present. When freeze-drying vials in pouches, care must be taken to ensure that the base is in proper contact with the shelf during loading, which can be difficult to confirm visually due to the nature of the pouches. As both trays were treated as duplicates and freeze-dried upon the same shelf, it is postulated that the paper 1 did not have proper contact with the shelf during freeze drying, reducing heat transfer, allowing a lower T_p, and thus avoiding collapse. Although inconsistent with the other pouches, this result does indicate that if freeze drying occurs without collapse, then a double layer of medical-grade paper is adequate to contain any ablation generated by processes other than collapse. This is an important observation, as the usual aim of preservation by freeze drying is the production of an elegant, friable cake avoiding collapse.

The freeze drying of microorganisms can lead to contamination of the freeze dryer. This can be exacerbated if collapse is observed, leading to the formation of aerosols distributing microorganisms around the freeze dryer. Using lower initial cell concentrations is a strategy to limit the amount of contaminating microorganism released during ablation. However, this is likely to affect the long-term viability of preserved cultures by a reduction in cell survival.[3] This study indicates that it is possible to contain high initial concentrations (10^9 cfu=mL) of Escherichia coli when badly formulated in a medium that collapses by using a double layer of either paper or Tyvek pouch containment. Therefore, this method could potentially enable MRCs and other laboratories to preserve high cell density cultures by freeze drying without issues of batch-to-batch or cross-contamination due to ablation or the gross contamination brought about by accidental cake collapse. The use of these inexpensive and disposable containment systems by MRCs or other laboratories could then increase efficiency and throughput and reduce costs for the preservation of cultures.

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