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Effect of Storage Temperature on the Stability of Spray Dried Bacteriophage Powders

Sharon S.Y. Leung[1], **Thaigarajan Parumasivam**[1,2], **An Nguyen**[1], **Thomas Gengenbach**[3], **Elizabeth A. Carter**[4], **Nicholas B. Carrigy**[5], **Hui Wang**[5], **Reinhard Vehring**[5], **Warren H. Finlay**[5], **Sandra Morales**[6], **Warwick J. Britton**[7], **Elizabeth Kutter**[8], and **Hak-Kim Chan**[1]

[1]Faculty of Pharmacy, University of Sydney, Sydney, NSW, Australia

[2]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia

[3]CSIRO Manufacturing, Bayview Avenue, Clayton, Melbourne, Victoria, Australia

[4]Vibrational Spectroscopy Core Facility & The School of Chemistry, University of Sydney, Sydney, NSW, Australia

[5]Department of Mechanical Engineering, University of Alberta, Edmonton, Alberta, Canada

[6]AmpliPhi Biosciences AU, 7/27 Dale Street, Brookvale, Sydney, NSW, Australia

[7]Tuberculosis Research Program, Centenary Institute, and Sydney Medical School, University of Sydney, Sydney, NSW, Australia

[8]The Evergreen State College, Olympia, WA, USA

Abstract

This study aimed to assess the robustness of using a spray drying approach and formulation design in producing inhalable phage powders. Two types of *Pseudomonas* phages, PEV2 (*Podovirus*) and PEV40 (*Myovirus*) in two formulations containing different amounts of trehalose (70% and 60%) and leucine (30% and 40%) were studied. Most of the surface of the produced powders was found to be covered in crystalline leucine. The powders were stored at 4 °C and 20 °C under vacuum. The phage stability and *in vitro* aerosol performance of the phage powders were examined on the day of production and after 1, 3 and 12 months of storage. A minor titer loss during production was observed for both phages (0.2 – 0.8 $\log_{10}$ pfu/ml). The storage stability of the produced phage powders was found to be phage and formulation dependent. No further reduction in titer occurred for PEV2 powders stored at 4 °C across the study. The formulation containing 30% leucine maintained the viability of PEV2 at 20 °C, while the formulation containing 40% leucine gradually lost titer over time with a storage reduction of ~0.9 $\log_{10}$ pfu/ml measured after 12 months. In comparison, the PEV40 phage powders generally had a ~ 0.5 $\log_{10}$ pfu/ml loss upon storage regardless of temperature. When aerosolized, the total *in vitro* lung doses of PEV2 were of the order of 10^7 pfu, except the formulation containing 40% leucine stored at 20 °C which had a lower lung dose. The PEV40 powders also had lung doses of 10^6 – 10^7 pfu. The results

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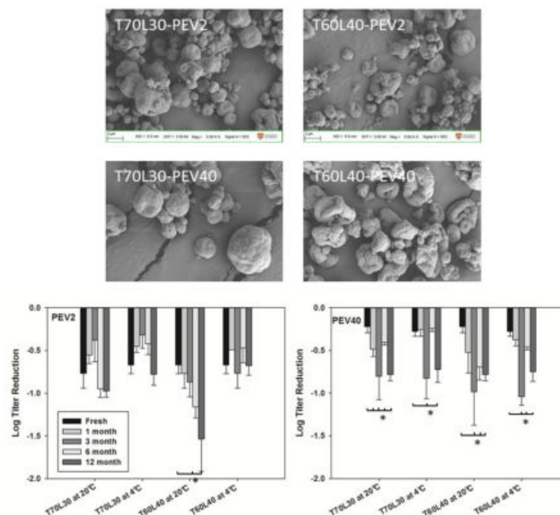
demonstrate that spray dried *Myoviridae* and *Podoviridae* phage in a simple formulation of leucine and trehalose can be successfully stored for one year at 4 °C and 20 °C with vacuum packaging.

Graphical abstract



Keywords

Phage; PEV2; PEV40; pulmonary infections; phage dry powder; antibiotic resistance

1 Introduction

Phage therapy has recently been demonstrated as a promising alternative to conventional antibiotics to treat and prevent pulmonary infections caused by multidrug resistance (MDR) bacterial strains [1–7]. Early research on pulmonary delivery of phage was confined to liquid sprays using intranasal instillation and nebulization as minimum formulation development was required [8]. Nebulization has been a popular choice because of its high efficiency in delivering phage to the lung at a low inhalation flow rate and minimum requirement in inhalation coordination [3, 9]. Recently, increasing efforts have been devoted into developing respirable phage powder formulations for improved storage stability, easy transport and administration. Various dry powder formulation techniques, including freeze drying followed by deagglomeration in a mixer mill [10], spray drying [11–14] and spray freeze drying [15], have been reported to be capable of producing inhalable phage dry powders. Among them, the spray drying technique is favorable because it is a single-step method to produce fine phage powders with the potential of causing only moderate titer loss (1 $\log_{10}$ pfu/ml) upon processing.

As phage are essentially composed of a stable protein capsule enclosing genomic materials, current solid-state phage formulation strategies have largely adapted the knowledge obtained in the development of protein-based pharmaceuticals and viral vaccines [16, 17]. While a 1 – 10 $\log_{10}$ pfu titer reduction was noted when phage was dried with buffer only [18, 19], addition of sugars, such as sucrose [18, 20], lactose [10, 18] and trehalose [11, 14, 20, 21],

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provided excellent protection for phages during the drying process and storage. It is believed that the high transition temperatures (Tg) of these sugars (60 °C for sucrose, 108 °C for lactose and 115 °C for trehalose) and/or their capability as a water substitute play important roles in stabilizing protein/phage in the solid-state [22]. Upon drying, the hydrogen bonding between water molecules and the phage protein coat could be replaced by bonding with the excipients to prevent protein unfolding and immobilize phages within the amorphous glassy matrix [22]. However, crystallization of trehalose kills phage [23]. Therefore, it is crucial for amorphous sugar components to remain well below the glass transition temperature during storage.

In addition to the sugar, L-leucine has been included as a second excipient to improve the powder dispersibility for pulmonary delivery [11, 14, 21]. L-leucine also has surfactant-like properties, in that it is surface active and crystallizes early due to low solubility, thus forming the outer shell of the particles, which could encapsulate the phage and other excipients. The *in vitro* aerosol performance of a trehalose-leucine formulation produced by Matinkhoo et al. [11] was not assessed. However, they showed formulations with a third excipient, either a surfactant or casein sodium salt could achieve an *in vitro* lung mass up to 82.7% of the loaded mass, with a lung dose of the order of 10^6 pfu/mg. These spray dried phage powders were stable with less than 0.15 titer loss under refrigeration for a period of three months. In our recent study [14, 21], we showed that sugar (trehalose and/or mannitol) powder matrix containing 20% leucine could achieve reasonably good aerosol performance (~40% fine particle fraction based on the recovered phage) with satisfactory stability such that no further titer loss was noted after 12 months storage at RH 22% and 4 °C (1 $\log_{10}$ pfu/ml loss upon storage). Vandenheuvel et al. [23] also showed that phages embedded in trehalose powders were most stable when stored at 4 °C and 0% RH. A 54% RH storage condition caused powder crystallization and significant phage inactivation, with the impact being more pronounced for large phage virions (*Myovirus romulus*) than smaller one (*Podovirus* LUZ19). Thermal instability was also reported for the phage powders stored at 25 °C [23]. Since dry powder inhaler products are usually administrated at room temperature and cold-chain storage is limited in most developing countries, more information on the stability of phage powders at higher temperatures is important for future development of phage powder formulations.

Although leucine is a well-studied aerosolization enhancer, its capability of protecting spray dried powders against moisture was recently reported [24, 25]. Li et al. showed an addition of 10 – 20 wt% of leucine could minimize the moisture induced deterioration of the aerosol performance of spray dried disodium cromoglycate powders, which absorbed a significant amount of water without recrystallization under higher relative humidity (60%). In their follow-up work on spray dried salbutamol sulfate, which recrystallized at high RH condition (60%), a higher mass fraction (40%) of leucine was required to eliminate the effect of moisture on the aerosolization performance. Based on a mechanistic model and experimental results, Feng et al. [26] suggested that a minimum leucine threshold must be exceeded to obtain crystalline leucine to form low-density and well-dispersing particles. For trehalose-leucine powders produced with a Büchi B-90 Nano Spray Dryer, a mass fraction 25% of leucine at a total feed concentration of 28.9 mg/mL was required to ensure 100% leucine crystallinity. Our previous studies [14, 15] suggested that the amount of leucine (20%) may

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not be sufficient to form a crystalline shell to protect the amorphous trehalose. In the present study, we extended our work to investigate the effects of leucine content and storage temperature on the long term stability of spray dried phage powders. In addition, two types of *Pseudomonas* phages, PEV2 (*Podovirus*) and PEV40 (*Myovirus*), were studied to elucidate the robustness of the spray drying approach and formulation design in producing inhalable phage powders. The phage stability and *in vitro* aerosol performance of the powders were assessed after 0, 1, 3 and 12 months storage at 4 and 20 °C with vacuum packaging. This is the first successful study demonstrating the long-term room temperature stability of spray dried, inhalable phage powders with less than 1 log_{10} pfu/ml loss and no deterioration in the i*n vitro* aerosol performance after one year.

2 Methods and materials

2.1 Materials

Two *Pseudomonas* lytic phages of different morphologies, a N4-type P*odovirus* (PEV2, 1.4 $\times 10^{11}$ pfu/ml stock titer) and a PB1-like *Myovirus* (PEV40, 2.2 $\times 10^{10}$ pfu/ml stock titer), were used. The phages were isolated from the sewage treatment plant in Olympia, WA, USA by students in the Evergreen State College Phage Laboratory. Phage stocks stored in salt-magnesium buffer (SMB, 5.2 g/l sodium chloride, 2 g/l magnesium sulfate, 6.35 g/l Tris-HCL, 1.18 g/l Tris base and 0.01% gelatin) were supplied via AmpliPhi Biosciences (AmpliPhi Biosciences AU, NSW Australia) and used without further purification. D-(+)-trehalose dihydrate and L-leucine (Sigma – Aldrich, NSW, Australia) were co-spray dried with phage to form a powder matrix to protect the phage particles. Adapting the mathematical model developed by Feng et al. [26] for a Büchi 290 spray dryer, it was estimated that a leucine content of 30% was required to form a fully crystalline leucine shell under the spray drying conditions used in our previous studies [14, 15] to produce the phage powders. Table 1 shows the composition of the four formulations prepared in the present study.

2.2 Powder preparation

The powder preparation was the same as that documented in our previous study [14]. Briefly, an aliquot of 500 μl of the phage stock was added to 50 ml excipient solution of trehalose and leucine at a total solid content of 20 mg/ml prior to spray drying. The mixtures were spray dried using a Büchi 290 spray dryer (Buchi Labortechnik AG, Flawil, Switzerland) using an open-loop setting at a drying gas flow rate of 35 m^3/hr, atomizing air flow rate of 0.742 m^3/hr, and inlet temperature of 60 °C with a liquid feed rate of 1.8 ml/min. The outlet temperature was between 40 and 45 °C. The produced powders were aliquoted into scintillation vials and packed inside a vacuum sealed bag using a Westinghouse vacuum food sealer inside a relative humidity controlled chamber (RH $<$ 20%). The vacuum packed vials were then stored at 4 and 20 °C before use.

2.3 Powder characterization

2.3.1 Particle morphology—Morphologies of the spray dried powders were examined using a field emission scanning electron microscope (SEM) (Zeiss Ultra Plus HD, Oberkochen, Germany) at 3 kV beam accelerating voltage. The samples were scattered on a

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carbon tape and sputter coated with 15 nm of gold using a K550X sputter coater (Quorum Emitech, Kent, UK) before imaging.

2.3.2 Particle size distribution—Optical particle size distributions of the powder formulations were measured using a Malvern Mastersizer 2000 (Malvern Instruments, UK). The powders were dispersed through the measurement window with compressed air at 350 kPa using a Scirocco 2000 dry powder module (Malvern Instruments, UK). The real, imaginary and dispersant (air) refractive indices were set at 1.52, 0.1 and 1.0, respectively. All measurements were done in triplicate. The size distribution was expressed by the volume median diameter (VMD), and span defined as the difference in the particle diameters at 10% and 90% cumulative volume divided by the VMD.

2.3.3 Particle crystallinity—Powder crystallinity was evaluated using an X-ray diffractometer (Model D5000; Siemens, Munich, Germany) under ambient condition. Samples were subjected to Cu Ka radiation at 30 mA and 40 kV, and the scattered intensity was detected with an angular increment rate of 0.04° 2θ/s from 5° to 40°.

The crystalline and amorphous components of the spray dried powders were also determined using a custom built dispersive Raman system with the methodology detailed in Vehring et al. [27] The Raman system utilized a 671 nm diode-pumped solid-state laser (Ventus 671, Laser Quantum, UK) with maximum output power of 500 mW. Briefly, the powder samples were first loaded into a 0.2 μL conical sample holder and kept under a nitrogen atmosphere during the measurement. All spectra were acquired at a temperature of 21±1 °C and a relative humidity of less than 3%. Reference spectra of crystalline L-leucine and trehalose dihydrate were collected from raw material. Amorphous trehalose was obtained by spray drying. The amorphous leucine reference spectrum was approximated by measuring the saturated aqueous solution of leucine and then subtracting the spectrum of water. Characteristic peaks of the reference spectra were used to determine the solid phase of each component.

2.3.4 Thermal analysis—The thermal properties of the powders were analyzed using a differential scanning calorimeter and thermogravimetric analysis (Mettler Toledo, Greifensee, Switzerland). For DSC measurement, each sample (5±1 mg) was weighed in an aluminium crucible which was then crimped to a perforated lid and heated from 30 to 300 °C at a rate of 10°C/min under 250 cm^3/min nitrogen purge. For TGA measurements each sample (5±1 mg) was weighed in an alumina crucible and heated from 30 to 400 °C at a rate of 10 °C/min with dynamic nitrogen flow.

2.3.5 Moisture sorption—The moisture sorption profiles of the powders were analyzed using a DVS instrument (DVS-Intrinsic, Surface Measurement Systems, London, UK). Each sample (~5 mg) was subjected to a dual moisture ramping cycle of 0–90% RH at a step increase of 10%. The moisture content at each relative humidity was determined when it changed by less than 0.02% per minute.

2.3.6 Particle surface components—X-ray photoelectron spectroscopy (XPS) was employed to study the chemical composition of the particle surface. XPS analysis was

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performed with an AXIS Nova Spectrometer (Kratos Analytical Ltd., Manchester, UK), equipped with a monochromated Al Ka source at a power of 180W (12 mA, 15 kV). Powders were filled into shallow wells of custom-made sample holders, and an approximate area of 0.3 mm × 0.7 mm was analyzed. An electron flood gun in combination with a magnetic immersion lens was used to compensate for charging of the samples during analysis. The remaining offsets due to charge neutralization were corrected using a reference binding energy value of 285.0 eV for the C 1 s peak of aliphatic hydrocarbon. The pressure in the main vacuum chamber during analysis was approx. 10^{-6} Pa. Spectra were recorded at a nominal photoelectron emission angle of 0° w.r.t. the surface normal. The sampling depth is in the range of 0–10 nm as the microscopic emission angle is ill-defined in the case of particles (ranging from 0° to 90°). The elemental compositions of powder samples were determined based on survey spectra (160 eV pass energy) using sensitivity factors provided by the manufacturers. High-resolution spectra of individual peaks were recorded at 20 eV pass energy which resulted in a peak width of typically 0.8 – 1.0 eV. In order to calculate relative molar fraction of trehalose and leucine on the surface of the composite particles, reference data were firstly obtained from two pure compounds. The data based on the composite particles were then compared with the reference data, and molar percent of each composition were estimated as follows: The atomic concentration of each element (i.e. C, O, N and S) in the composite particles was assumed to be a linear combination of the corresponding concentration in the pure compounds, appropriately scaled and normalized using the number of respective atoms in one molecule of trehalose or leucine. Element nitrogen (N) is unique in leucine molecules. Thus, based on the experimentally determined N atomic concentration, the molar percent of trehalose and leucine on the surface of particles was estimated. The accuracy associated with quantative XPS is ca. 10% 15%. Precision (i.e. reproducibility) depends on the signal to noise ratio but is usually much better than 5%. The latter is relevant when comparing similar samples as in the case of this study.

2.4 Powder stability

Freshly spray dried phage powder was transferred in a Perspex box at a RH 17% into two 30 ml glass vials which were loosely capped and placed in a vacuum sealed bag. The samples were then stored at 4 and 20 °C for up to a year. The phage viability and powder were examined after 0, 1, 3 and 12 months storage. An amount of 15 mg of powder stored at each condition was dissolved in 300 μl SMB to give a concentration of 50 mg/ml. The number of viable phages in the powder samples was determined by the Miles-Misra surface droplet technique [28]. Serial dilutions, 1:10, were performed by adding 20 μl samples to 180 μl SMB. A volume of 200 μl host bacteria containing ~2×10^9 colony forming units (cfu) was mixed with 5 ml molten soft agar (0.4% Amyl agar, 48 °C). The mixture was then overlayed onto a solidified nutrient agar made of 1.5% Amyl agar and nutrient broth. A volume of 10 ml diluted phage samples were dropped onto the agar lawn in triplicate, air dried and incubated at 37 °C overnight. Samples giving rise to 3–30 plaques were used for phage viability calculation.

2.5 Powder dispersibility

In vitro aerosol performance of the phage powders was assessed at 0, 1, 3 and 12 months storage. An Osmohaler^{TM} coupled to a USP stainless steel throat and a multi-stage liquid

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impinger (MSLI) was operated at 100 l/min for 2.4 s. A sample mass of 20 mg was weighed into a size 3 hydroxypropyl methylcellulose capsule (Capsugel, NSW, Australia). The capsule filling was carried out in a humidity controlled box (17% RH) while the dispersion was conducted at RH of 50 ± 5% and 20 ± 5 °C. SMB was used as the rinsing solvent to determine the viable phage deposition profiles. Experiments were performed in triplicate. The lower cutoff diameters of the MSLI stages 1–4 at 100 l/min are 10.1, 5.3, 2.4 and 1.32 mm, calculated with the adjustment equations given in Appendix XII C of the British Pharmacopoeia. The fine particle fraction (FPF) was defined as the mass fraction of particles with an aerodynamic diameter   5.0 mm with respect to the total recovered dose.

2.6 Statistical analysis

One-way analysis of variance (ANOVA) and unpaired two-sample t test at a confidence level of 95% were employed to identify any statistical significance in particle size and phage deposition. A p value of <0.05 was considered statistically significant.

3 Result and discussion

3.1 Powder characterization

3.1.1 Phage and particle morphology—Figure 1 shows the SEM images of the spray dried powders. The particles are generally spherical with wrinkled surfaces. In our previous study, significant particle fusion was noted for formulations containing 80% trehalose and 20% leucine. By increasing the amount of leucine to 30% and 40%, no particle fusion was noted in the present study, indicating that a higher content of leucine resulted in better coating of the particles. There was no visual difference between powders containing different phages and different amounts of leucine (30% and 40%). The morphologies of the phage particles in the powders stored under desiccation were also examined and no significant changes were found (data not shown).

3.2 Particle size

The volume median diameters (VMD) and span of the particles are presented in Table 2. The results were in accordance with the SEM images, with most particles falling within the inhalable size fraction of less than 5 μm. No significant difference was noted in the particle size between the two formulations for the same phage, while the same formulation composition containing PEV40 was significantly larger than that containing PEV2 ($p < 0.01$). Such differences could be due to batch to batch variation, such as small variations in the buffer and excipient compositions, setting of the atomizer during the spray drying process, and small differences in the ambient temperature. It is also noteworthy that the size obtained by laser diffraction is an optical diameter which is susceptible to changes in particle morphology.

3.3 Powder crystallinity

The XRD profiles of spray dried leucine and matrix containing 30% and 40% leucine are shown in Figure 2a. The XRD traces for powders stored after 12 month were similar to those obtained for the freshly produced powders (data not shown). For the same excipient compositions, similar results were obtained for the two phages. Therefore, only results for

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the PEV2 (F1 and F3) were reported. The crystalline nature of spray dried pure leucine was confirmed in the XRD measurements. For powder matrices containing trehalose and leucine, the appearance of both crystalline peak and broad halo pattern indicated the presence of crystalline and amorphous materials. Most of the peaks noted in the formulations were aligned with the crystalline peaks of spray dried leucine alone, suggesting that the crystallinity of the powder matrices was due to the leucine component. This is confirmed in the Raman measurement (Figure 2b) that only crystalline leucine and amorphous trehalose were detected in all four formulations. The results agree with that reported in Feng et al. [26] that the leucine was purely crystalline for formulations with a leucine mass fraction larger than 25%.

3.4 Thermal analysis

The thermal behavior of phage powders obtained by DSC and TGA analysis is shown in Figure 3. Both formulations showed glass transition temperature (Tg) of trehalose at ~100 °C (Figure 3a). as discussed in our previous work [15], the large endothermic peak at 210 – 260 °C was attributed to sublimation of leucine with the peak height/area increase with increasing leucine content. This peak also corresponded to the weight loss in the TGA results (Figure 3b). The small endothermic peaks at 260 – 280 °C in the DSC traces, accompanying by a further weight loss TGA data, were due to the decomposition or oxidation of trehalose.

3.5 Moisture sorption

An aim of the project is to evaluate whether a crystalline leucine shell could reduce the moisture uptake by amorphous trehalose in the spray dried phage powder formulations, as crystallization of the trehalose matrix has been shown to deactivate phage [23]. The moisture absorption profiles of formulations containing 30% and 40% leucine are shown in Figure 4a. The onset of recrystallization was noted at 50% RH for both formulations, indicating the increase of leucine content up to 40% was not sufficient to form an impermeable crystalline leucine layer to mitigate moisture uptake by the amorphous trehalose. The moisture uptake kinetics of the two formulations at 60% RH (Figure 4b) revealed that trehalose recrystallization took place within 30 minutes exposure, with little difference between the two formulations. The results suggested that the handling and storage of the phage powders below 20% RH (ideally below 5%) would be necessary. Similar moisture sorption profiles were obtained for powders stored after one year (data not shown).

3.6 Surface composition

Table 3 shows the surface atomic concentrations of elements in pure trehalose, pure leucine and the composite phage powders, as well as the estimated molar fractions of trehalose and leucine at the particle surface. The latter values are estimates and should only be interpreted semi-quantitatively. Generally, more leucine in the formulation increased the presence of leucine on the particle surface, with no significant difference noted between the two phages. For formulations containing 30 wt% (55 mol%) leucine, the surface coverage of leucine was 67–70 mol%. When the leucine content increased to 40 wt% (66 mol%), the surface coverage increased to 82 – 89%. This high leucine coverage on the surface could explain the surface corrugation as seen in Figure 1. However, the leucine shell may not have been

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continuous. This could leave channels for the amorphous trehalose to uptake moisture uptake and recrystallize when exposed to high humidity conditions as observed in the DVS results.

3.7 Phage stability

After the spray drying process, a total of ~0.7 and ~0.2 $\log_{10}$ pfu/ml titer reduction was observed in the PEV2 and PEV40 powders, respectively, with no significant difference noted between the two excipient combinations (Figure 5). Since dry powder inhaler medications are generally used at room conditions, adequate stability of the phage powder formulations at room temperature is essential to avoid product degradation prior to use by the patient. In addition, one of the major drivers for the development of phage dry powder formulation is to achieve easier storage, transport and administration. Formulations that are stable at room temperature would be favorable as cold-chain storage could be difficult to achieve in the developing world and during transportation. Therefore, the spray dried powders were transferred into two loosely capped glass vials, then placed inside vacuum sealed package and stored at two temperatures, 4 °C and 20 °C, to assess the effect of temperature on the stability of phage powders.

The titer reduction was determined relative to the titer measured in the liquid feedstock for spray drying. There was no further titer reduction noted for PEV2 phages containing 30% leucine up to a year at both storage temperatures ($p > 0.05$). On the other hand, formulations containing 40% leucine were found to be temperature dependent (p 0.05). While phage powders stored at 4 °C had no further loss across the study, the titer reduction was gradually increased from 0.67 ± 0.10 $\log_{10}$ pfu/ml to 1.54 ± 0.38 $\log_{10}$ pfu/ml after 1 year storage at 20 °C. The gradual loss of phage could be due to further dehydration of powders upon storage. The formulation compositions had limited impact on the storage stability of PEV40 in the powder form. A further loss of ~0.7 $\log_{10}$ pfu was noted after three months. The titer reduction dropped to 0.5 $\log_{10}$ pfu/ml at the 6 month time point, which is within the uncertainties in the plaque assay used to determine the phage titer (± 0.5 $\log_{10}$ pfu/ml). After 12 month storage, the titer reduction of PEV40 was comparable to that obtained at the 3 month time, indicating no further phage inactivation in these powders. Overall, the titer reduction of PEV40 was only ~1 $\log_{10}$ pfu/ml at both storage temperatures. As powders were stored under vacuum and handled at RH < 20%, re-crystallization of trehalose, which has been identified as the major mechanisms responsible for phage inactivation [23], was prevented. This was supported by the similarity in the powder morphologies, crystallinity and DVS profiles between the fresh and stored samples.

Our results show reasonable stability of both the PEV2 (*podovirus*) and PEV40 (*myovirus*), with less than 1 $\log_{10}$ pfu/ml storage loss at the studied storage temperature. This is in contrast to the results reported in Vandenheuvel et al. [23] who showed a higher storage temperature (25 °C) could cause thermal instability of the embedded phages, with the impact being more profound for the larger *myovirus* (*Romulus*) than the smaller *podovirus* (LUZ19). However, Vandenheuvel et al. [23] also highlighted a possibility of leakage of the desiccator in which the *Romulus* phage powder was stored, causing the larger titer reduction

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of the *myovirus*. Nonetheless, the discrepancies in storage stability between our study and Vandenheuvel et al. [23] could also be phage dependent.

3.8 *In vitro* Aerosol Performance

The deposition profiles, total recovery and viable respirable phages (FPF < 5 μm) of F1-F4 after dispersing with an Osmohaler™ at 100 L/min for 2.4 s of the 0, 3 and 12 month old samples are depicted in Figures 6 (PEV2) and 7 (PEV40). While the recovery of trehalose was in the range of 85 – 110% (data not shown), the recovery of viable phage ranged from 12 – 117% with the majority of the dispersion experiments falling at the low end (< 50%). The low recovery may be due to phage inactivation upon impaction with the interior wall of the capsule, inhaler and MSLI during the dispersion process. Dispersion experiments of phage powders at a lower flow rate, 60 L/min, were performed to test this hypothesis. The recovery was found to be in the range of 30–60% (data not shown), indicating either the low flow rate dispersion caused phage inactivation or phage inactivation upon dispersion was not the main cause of the low recovery. Known titer phage suspension (10^5 pfu/ml) of similar order to the phage titer detected in the dispersion tests was used to rinse in the capsule, device, adaptor, throat, all stages of the MSLI and filters as for the real dispersion tests. Then plaque assay was performed on the collected phage suspensions to confirm the impact on phages when they come into contact with the parts in the dispersion tests. Negligible differences were observed between the known phage suspension and those after rinsing for all parts, indicating that phages were not adhered or deactivated during the handling process. Nonetheless, the plaque assay generally has an accuracy of ± 0.5 $\log_{10}$ pfu/ml, which could cause variability in the recovery of phage.

Compared with our previous results which contained 20% leucine in the powder formulations [14], the deposition of viable phage in the capsule and device were significantly lower in the present study (generally ~10%, with a few cases ~20%). As leucine is a surface active compound and the surface coverage of leucine was > 67%, the amount of phage residing on the surface might be reduced, resulting in fewer phage detaching from the powders upon impaction inside the capsule and device. Comparing the two studied phages, the PEV2 phages had a higher capsule and device deposition than the PEV40 phages. This could be because more PEV2 phages were on the surface than the PEV40 phages. As phage particles are essentially composed of a stable protein capsule enclosing the genomic materials, they tend to reside at the surface of the particle matrices during the drying process due to their amphiphilic properties [29, 30]. However, the rate of diffusion of phage from the surface to the center would depend on the proportion of hydrophobic to hydrophilic amino acids of their protein coat. Only a small portion of phages was deposited on the adaptor, throat and Stage 1 of the MSLI. In most cases, the majority of the viable phage deposited on Stage 3 and 4 and gave high FPF values > 40% for both formulations and both PEV2 and PEV40 phages. No specific trends were observed in the deposition profile with the storage time for both formulation compositions and phages.

The corresponding *in vitro* fine particle dose (FPD) of phage quantified as the number of viable phages embedded in particles smaller than aerodynamic diameter 5 μm, is shown in Figure 8. The total fine particle dose of PEV2 phages was of the order of 10^7 pfu, which was

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100 times higher than our previous study [14] caused mainly by the increased titer of viral stocks (from 10^9 to 10^{11} pfu/ml). This indicates that the more concentrated samples examined here did not result in more damage in the production and storage process. In fact, there have been studies with viruses indicating that a higher starting titer leads to greater stability [31]. There were no significant differences in the fine particle dose of viable phage noted for F1 up to 12 month storage, except significantly lower values were obtained at 6 month for powder stored at 20 °C. Significantly lower fine particle dose of viable phage was noted for F2 stored at 20 °C, with the dose decreasing gradually from 7.5 log_{10} pfu to 6.4 log_{10} pfu in one year. Despite a significantly lower fine particle dose being also obtained for F2 stored at 4 °C at certain time points, the difference was within 0.5 log_{10} pfu which lies within the uncertainties of the plaque assay.

The freshly produced PEV40 powder had a fine particle dose of 10^7 pfu. Both formulations had a reduced fine particle dose of phage at both storage temperatures. The reduction was more profound for formulations containing 30% leucine (F3), decreasing gradually from 7.4 log_{10} pfu to 6.3 log_{10} pfu and 6.1 log_{10} pfu for powders stored at 20 °C and 4 °C, respectively. For formulations containing 40% of leucine stored at 20 °C, the fine particle dose of PEV40 had a ~0.5 log_{10} pfu drop after one month storage and plateaued, except the 6 month data which had a much higher phage recovery than other time points as seen in Figure 7. A ~0.5 drop was also noted for F4 stored at 4 °C, but the reduction was gradual, with the 6 month data being exceptional. The reduced fine particle doses of PEV40 phage were aligned with the titer loss of the phage in the powder form (Figure 5), which was ~ 1 log_{10} pfu.

The impacts of storage temperature on the phage stability and fine particle dose were found to be phage specific. Although a high production loss was noted for the PEV2 phages (0.7 log_{10} pfu/ml for F1 and 0.8 log_{10} pfu/ml for F2), they were generally more stable than the formulations containing PEV40 phages upon storage at both temperatures. Such specificity could arise from the distribution of the phages within the particles or interactions between the phages and the excipients. While studying the mechanisms responsible for the stabilization effect of the powder compositions would certainly be valuable for future development of stable phage powder formulations, the stabilizing mechanisms are likely highly complex and beyond the scope of the present study. Nonetheless, we have demonstrated that our proposed powder matrix was suitable to incorporate phages of two different morphologies (*Podovirus* and *Myovirus*) with acceptable stability and a combined loss of <1 log_{10} pfu/ml during production and storage for a period of 12 months.

4 Conclusion

The influences of leucine content and storage temperature on the long term stability of spray dried trehalose powders containing two different phages (*Podovirus* PEV2 and *Myovirus* PEV40) were studied. A low production loss was noted for both the PEV2 (0.7 – 0.8 log_{10} pfu/ml) and PEV40 (0.2 – 0.3 log_{10} pfu/ml) in phage numbers. Although leucine did not form a continuous crystalline shell to protect the powders from moisture sorption, storing the powders under vacuum condition demonstrated acceptable stability (1 log_{10} pfu/ml) at both 4 °C and 20 °C for a period of 12 months. PEV2 phage powders stored at 4 °C were

stable for both formulations studied. While the formulation containing a lower amount of leucine (30%) preserved the viability of PEV2 phages at 20 °C, a ~0.9 $\log_{10}$ pfu/ml storage loss was obtained for the formulation containing 40% leucine after 1 year. The PEV40 phage formulations had inferior storage stability relative to the PEV2 phage ones. In general, a ~ 0.5 $\log_{10}$ pfu/ml storage loss was noted for both formulations and storage temperatures after 1 year. The observations for the fine particle dose of viable phages were consistent with the phage stability in the powders. Despite the titer loss upon storage, the total fine particle dose of PEV2 and PEV40 phages was of the order of 10^6–10^7 pfu. In summary, the proposed simple formulations containing only leucine and trehalose could preserve the PEV2 and PEV40 phages in the powder form and achieve reasonable aerosol performance during a long term storage period of twelve months at both 4 °C and 20 °C. This is the first study to demonstrate long term room temperature storage stability of inhalable phage powders.

Acknowledgments

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ABBREVIATIONS

cfu	Colony formation unit
DSC	Differential scanning calorimetry
DVS	Dynamic vapor sorption
FPF	Fine particle fraction
HPMC	Hydroxypropyl methylcellulose
MDR	Multidrug-resistant
MSLI	Multi-stage liquid impinger
NB	Nutrient broth
pfu	Plaque formation unit
RH	Relative humidity
SEM	Scanning electron microscope
SMB	Salt-magnesium buffer
Tg	Glass transition temperature
TGA	Thermogravimetric analysis
XRD	X-ray diffraction

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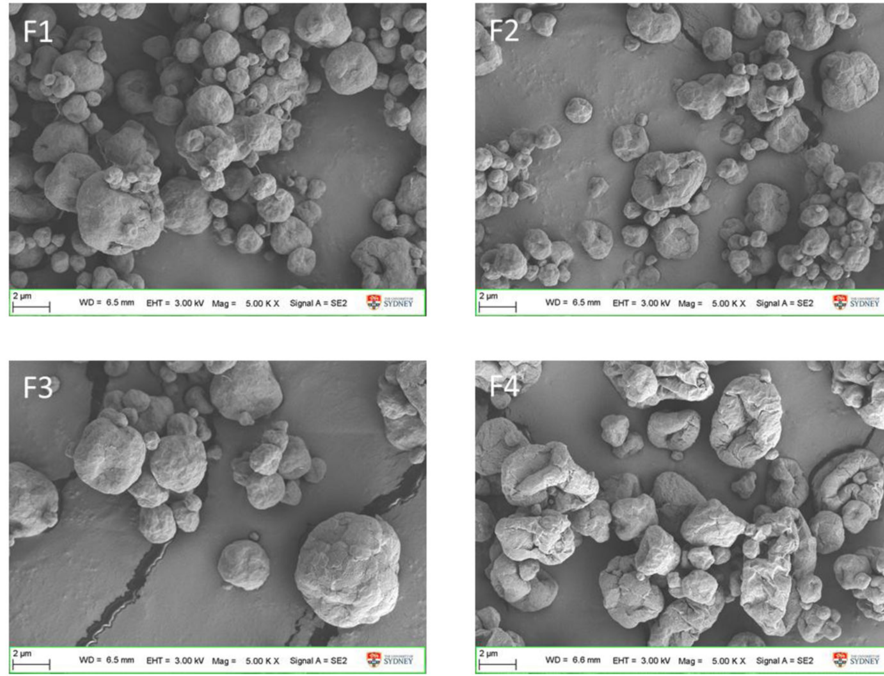

Figure 1.
SEM images of F1 – F4.

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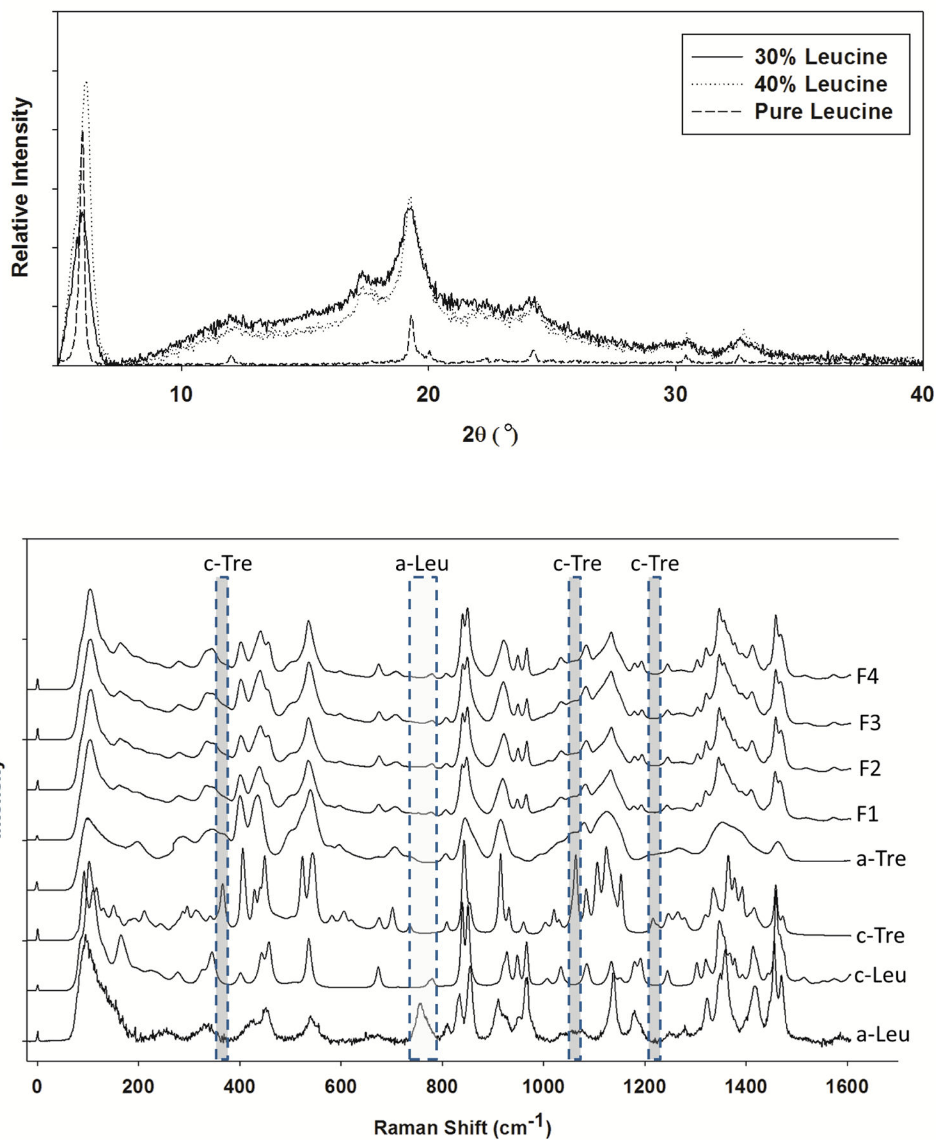

Figure 2.

(a) XRD profiles of the spray dried pure leucine and formulations containing 30% (F1, F3) and 40% (F2 and F4) leucine. (b) Raman spectra of reference materials, crystalline trehalose dihydrate (c-Tre), spray dried amorphous trehalose (a-Tre), crystalline l-leucine (c-leu) and amorphous leucine (a-leu), and spray dried phage powders.

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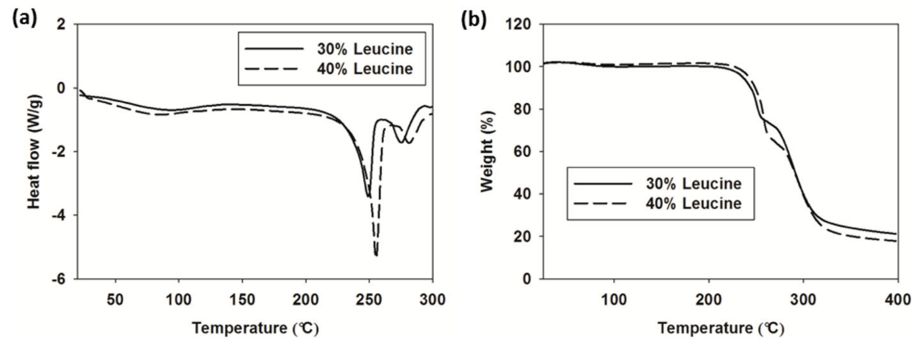

Figure 3.
(a) DSC and (b) TGA curves for formulations containing 30% (solid lines) and 40% (broken lines) leucine.

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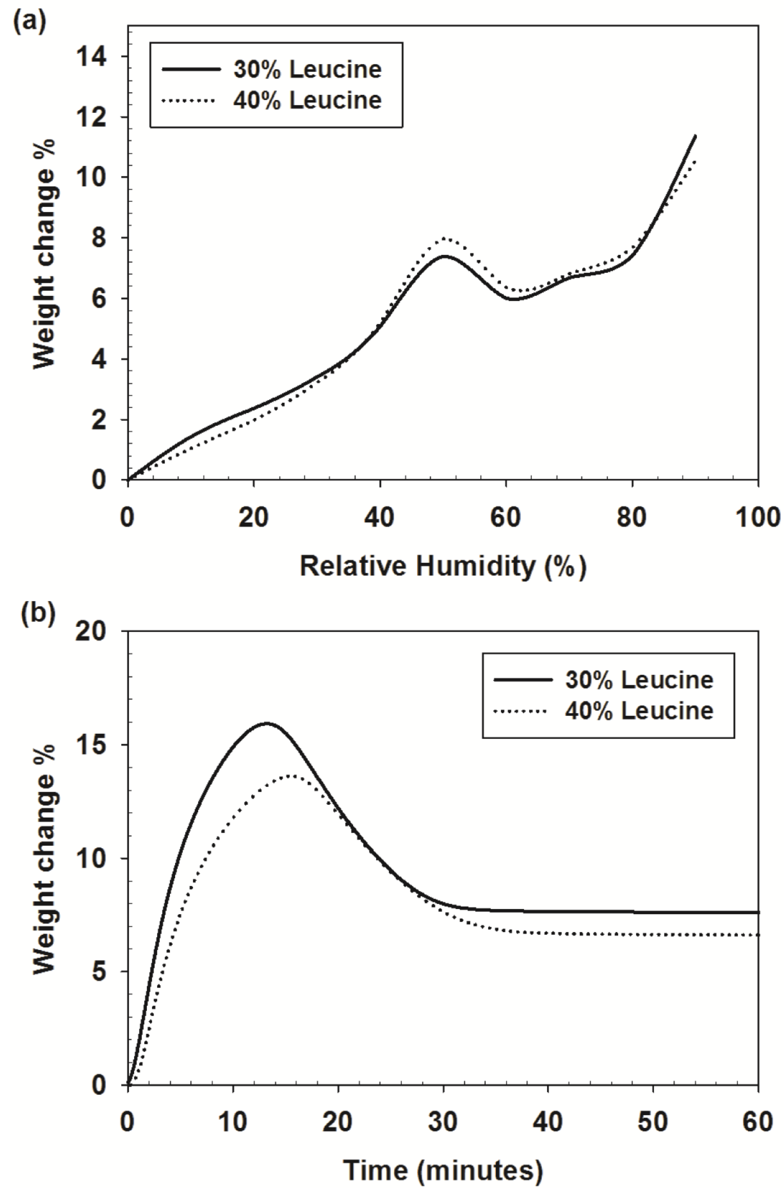

Figure 4.
(a) Vapor sorption profiles and (b) kinetic of moisture uptake at 60% RH for formulations containing 30% (solid lines) and 40% (broken lines) leucine.

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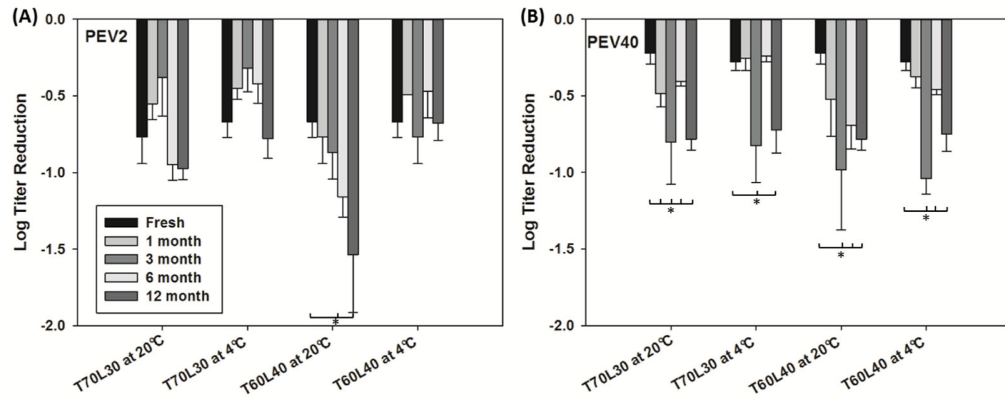

Figure 5.

Phage stability in F1 – F4 formulations of phages PEV2 (A) and PEV40 (B) after storage under vacuum at 4 and 20 °C. Titer reductions at 0 (Fresh), 1, 3 6 and 12 months are relative to the titer measured in the spray dryer liquid feedstock. Asterisk (*) denotes a statistically significantly higher ($p < 0.05$) titer reduction compared with the freshly prepared powders.

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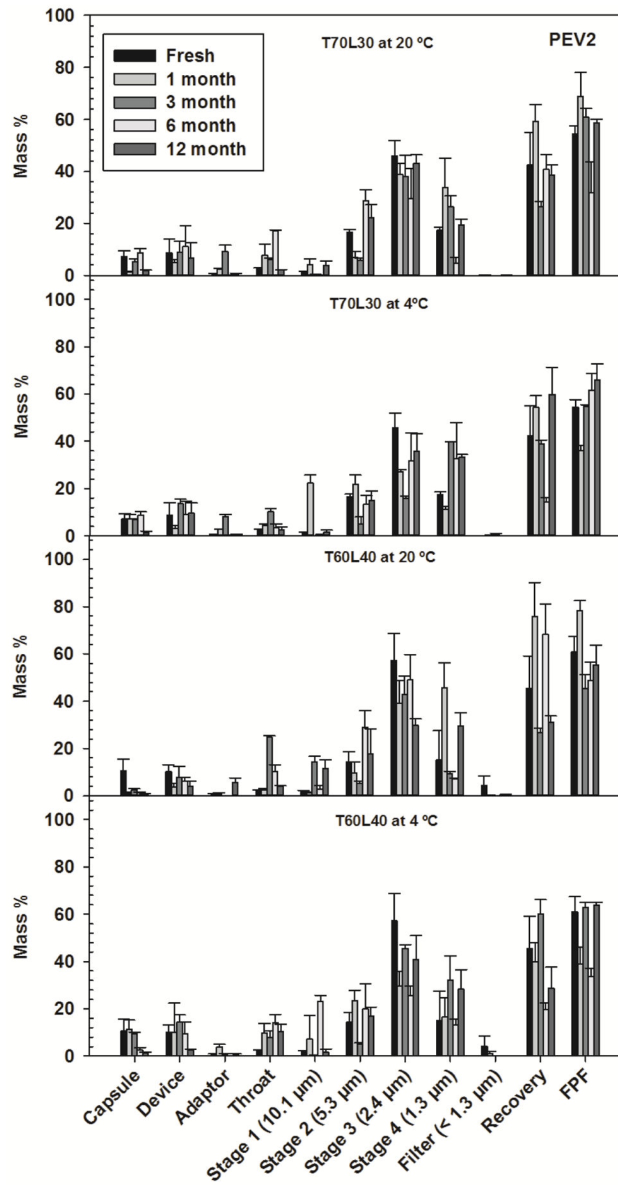

Figure 6.

The distribution profiles, total mass recovery and FPF of viable PEV2 phage. Data presented as mean ± one standard deviation ($n = 3$). All formulations were dispersed at 100 L/min for 2.4 s using the Osmohaler™. The aerodynamic cutoff diameter of each stage is quoted in parentheses.

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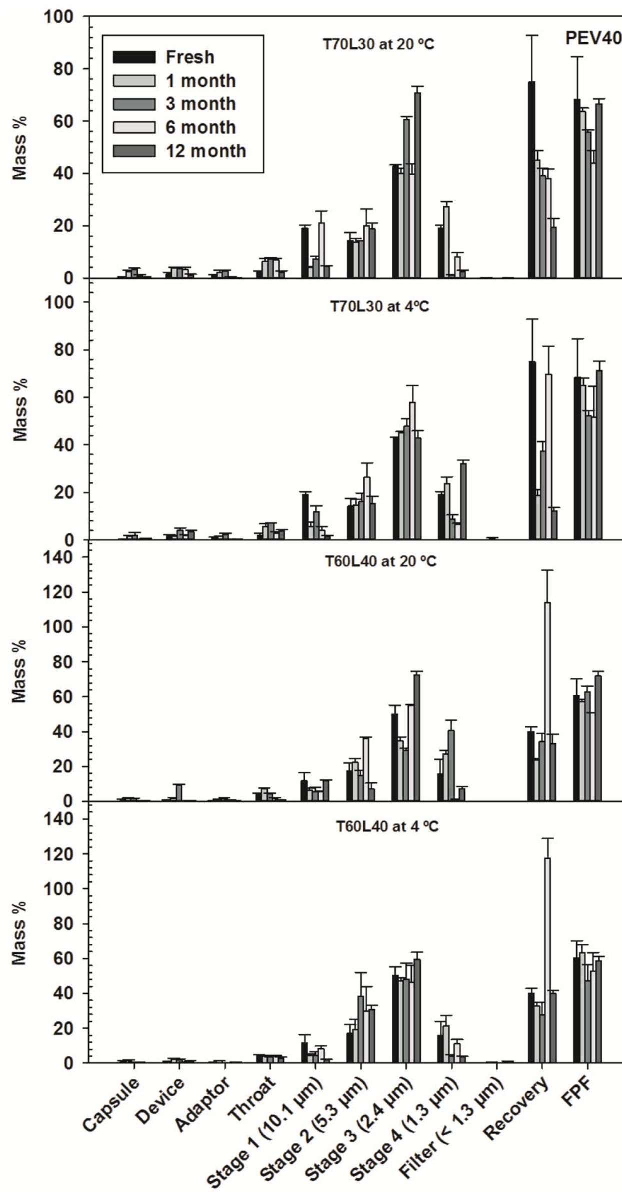

Figure 7.

The distribution profiles, total mass recovery and FPF of viable PEV40 phage. Data presented as mean ± one standard deviation ($n = 3$). All formulations were dispersed at 100 L/min for 2.4 s using the Osmohaler^{TM}. The aerodynamic cutoff diameter of each stage is quoted in parentheses.

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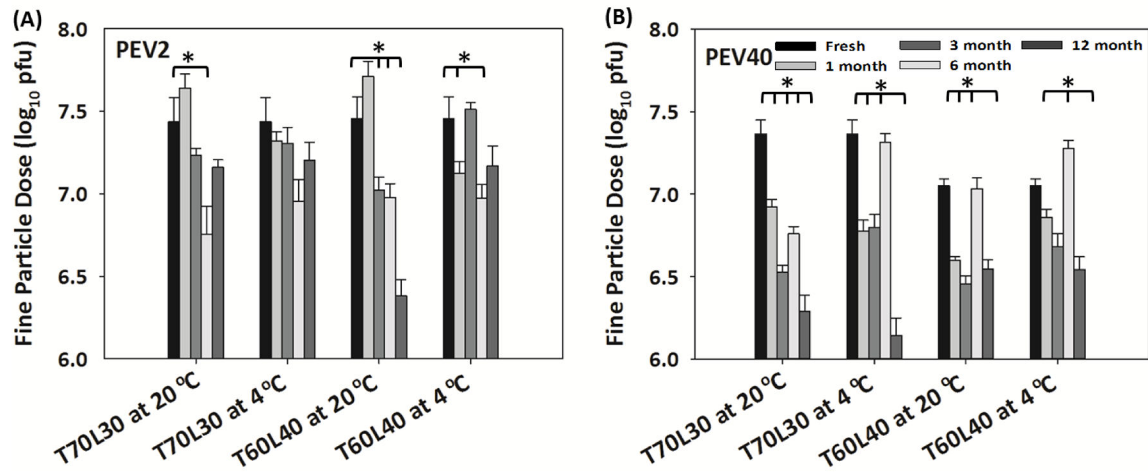

Figure 8.

Total fine particle dose of PEV2 (A) and PEV40 (B) phage of powders stored up to 12 months at 4 and 20 °C. Data presented as mean ± one standard deviation ($n = 3$). Asterisk (*) denotes a statistically significantly higher ($p < 0.05$) titer reduction compared with the freshly prepared powders.

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Table 1

Formulation compositions.

Formulation #	Phage	Starting titer (pfu/ml)	Contents (w/w %)	
			Trehalose	Leucine
F1	PEV2	1.4×10^9	70	30
F2	PEV2	1.4×10^9	60	40
F3	PEV40	2.2×10^8	70	30
F4	PEV40	2.2×10^8	60	40

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Table 2

Optical particle size distribution and titer loss of phage in the solution and freshly produced powders. Plus/minus error is the standard deviation (n= 3).

Formulation #	VMD (μm)	span	Titer loss in solution (log_{10} pfu/ml)	Titer loss in freshly produced powders (log_{10} pfu/ml)
F1 (T70L30)	2.09 ± 0.11	1.73 ± 0.13	0.19 ± 0.16	0.77 ± 0.17
F2 (T60L40)	2.01 ± 0.03	1.57 ± 0.03	0.61 ± 0.10	0.67 ± 0.10
F3 (T70L30)	2.88 ± 0.12	1.93 ± 0.31	0.22 ± 0.11	0.18 ± 0.09
F4 (T60L40)	3.11± 0.09	1.93 ± 0.18	0.22 ± 0.07	0.28 ± 0.06

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Table 3

Atomic concentrations of elements in pure trehalose, pure leucine and the composite phage powders, and the elemental composition at the particle surface.

Compound/ Formulation #	Elemental composition (Atomic concentration, %)					Mass Fraction at the surface (mol%)	
	C	O	N	S	Cl	Trehalose	Leucine
Pure trehalose	55.82	44.08	0.00	0.08	0.04		
Pure leucine	68.16	20.42	11.19	0.11	0.13		
F1	59.96	33.77	4.97	0.48	0.22	32.9	67.1
F2	63.12	28.71	7.12	0.40	0.16	18.3	81.7
F3	60.60	33.03	5.40	0.43	0.21	29.6	70.4
F4	65.20	25.80	8.39	0.29	0.14	11.5	88.5