Stabilization of HSV-2 Viral Vaccine Candidate by Spray Drying

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ABSTRACT

This work demonstrates that an HSV-2 candidate vaccine can be thermostabilized by spray drying to reduce cold chain demands. This work is also to optimize the process responses by varying spray dry parameters for pre-screened suitable excipients; and to determine the validity of current prescreening techniques. Vaccine activity losses were measured by *in vitro* plaque forming assay with Vero cell line. An accelerated storage condition of 45°C for 10 days was used to determine spray dried sample stability. Prescreening studies demonstrated that trehalose and sucrose were superior to other tested excipients spray dry thermal stabilization of HSV-2. Subsequent optimization by design of experiments (DOE) of activity responses to spray dry parameter changes demonstrated significant differences between trehalose and sucrose for stability of the viral vaccine. Model parameters included the drying conditions inlet temperature, spray gas flow rate, and solids concentration for the model responses of vaccine stabilization. Trehalose was an effective and robust stabilizing excipient for spray drying HSV-2 vaccine. In contrast, stabilization by sucrose was greatly dependent on the spray dry process parameters. These DOE differences indicated inadequate excipient selection by prescreening methods and the variability demonstrated current prescreening techniques may not be adequate for determining optimal excipients.

KEYWORDS: vaccine, HSV-2, stabilization, spray dry, formulation, optimization, DOE

1. INTRODUCTION

Global vaccination interests will be achieved by improving thermal stability of vaccine products (Brandau et al., 2003; Ford et al., 2016). Enhanced thermal stability may alleviate many of the costs associated with the cold-chain transport and storage currently necessary to ensure vaccine efficacy in limited resource settings, particularly in developing countries. Stability at more relevant ambient temperatures for weeks or months would reduce challenges of transport to remote areas and maintenance of vaccine supplies (Brandau et al., 2003). Thus, strategies for increasing thermal stability of vaccine components are viewed as addressing a large challenge to improve global vaccine deployment.

Drying of active biologics has been shown to improve product shelf-life by molecular immobilization and protection from harsh environmental conditions, such as temperature, oxygen and water in general (Kanojia et al., 2018; Lovalenti et al., 2016). Spray drying has recently been proposed as an effective drying process for biologicals because it can remove water in a controllable and scalable manner with shorter process times and lower cost (Sosnik and Seremeta, 2015). For spray drying, solution feeds containing stabilizing excipients and a biological of interest are sprayed by a pressurized gas through a nozzle into a drying chamber of controlled inlet temperature. Evaporation of the liquid phase from the small, sprayed droplets leads to dry particle formation (Vehring et al., 2007). Once dried, the sugar, amino acid, or polymer excipients act as a restrictive, stabilizing matrix for the active biological (Kanojia et al., 2017). Several excipients have gained favor as effective molecules for stabilization, though several reports in the literature have demonstrated that excipient formulation and spray drying conditions are unique to the biological agent being investigated (Kanojia et al., 2017; Toniolo et al., 2019).

Genital herpes is one of the most prevalent sexually transmitted diseases, with herpes simplex virus type 2 (HSV-2) infection representing a global health burden estimated at over $400 million in 2012 (Looker et al., 2015). An effective HSV-2 vaccine has become more important in recent years due to the increased risk of human immunodeficiency virus (HIV) infection following HSV-2 acquisition (Looker et al., 2015). The development and delivery of a thermostable HSV-2 vaccine is still an unmet global medical need and thus would also reduce the burden of HSV-2 and HIV globally. We used the HSV-2 candidate developed by Sanofi Pasteur [8] to investigate and demonstrate the feasibility of spray drying as a potential means to stabilize the viral component, which is known to very rapidly lose activity at elevated temperatures (Plummer and Lewis, 1965). We have tested the spray dried HSV-2 candidate stability by storage at 45°C for 10 days to demonstrate plausibility of the vaccine use in remote areas such as sub-Saharan Africa.

The development of a dry powder vaccine requires prescreening of excipients to determine appropriate selection and suitability of each material for use with the unique biological. Numerous studies in the past have performed base case prescreening efforts to decide upon excipient selection (LeClair et al., 2016a; Ohtake et al., 2010; Smith et al., 2003; Sou et al., 2013). In some cases, top performing formulations were investigated further to optimize with the spray drying process for responses such as activity, particle size and yield (Kanojia et al., 2016; LeClair et al., 2016b; Saboo et al., 2016). This base case prescreening of excipients operates under the presumption that all formulations are affected similarly under changing spray dry conditions. Previous reports have shown different activity responses according to spray dry condition changes depending on the biological in use (LeClair et al., 2016b; Mohajel et al., 2012). However, a direct comparison of differing activity responses according to different

92 formulations for the same biological has not been investigated. This type of effect would
93 demonstrate that selection of an excipient formulation is more complicated than current work
94 dictates because the performance for a set of excipients is largely dependent on spray drying
95 conditions.

96 In the present work, we conducted a preliminary screening investigation to determine the
97 best stabilizing spray dry formulations for HSV-2 activity losses, occurring in the feed solution
98 during spray drying, and after 10 day storage at an elevated temperature of 45°C. The goal of the
99 studies was to achieve minimum activity losses during the drying process, i.e. below 0.5 $\log_{10}$
100 pfu/mL. We further investigated the two top performing excipients (trehalose and sucrose) by
101 DOE analysis to model activity losses as affected by spray drying parameters, namely inlet
102 temperature, spray gas flow rate and solids concentration, as well as the significant interaction
103 factors. The choice of two DOE setups for top performing excipients was undertaken to compare
104 each model to determine validity of the prescreening method by performance for each
105 formulation over a range of processing parameters. Furthermore, we conducted a technology
106 transfer study by using a 10x larger batch size in a different facility to verify the prediction
107 model and the robustness of the optimized formulation and process. The current study not only
108 demonstrated that spray drying improved HSV-2 viral vaccine thermal stability, but also
109 provided a methodology of creating prediction models of spray drying parameters with process
110 losses. In addition, it validated the prediction models based on another study at a different
111 facility by means of technology transfer. Additionally, the DOE model of sucrose process losses
112 and further examination of storage losses demonstrated a lack of predictive power by the
113 prescreening trials. The result of this series of exercises suggested that the interactions between

the excipient matrix and the process parameters are important factors to consider in the excipient selection phase.

2. MATERIALS AND METHODS

2.1. Materials

Trehalose dihydrate, sucrose, lactose, mannitol, dextran (Mr. 40000 kDa), sodium chloride (NaCl), potassium glutamate and L-histidine were all purchased as USP grade excipients from Sigma-Aldrich (Ontario, Canada). HSV-2 model vaccine was produced in the Bio-process R&D Department facility at Sanofi Pasteur Ltd. Canada, both process and compositions as previously described (Da Costa et al., 2000). It was shipped to McMaster University for subsequent spray drying at a viral concentration of 7.5 $\log_{10}$ pfu/mL within a composition of: 10% sucrose (w/v), 160 mM NaCl, 50 mM potassium glutamate and 10 mM histidine.

2.2. Spray Drying of HSV-2

Powders were produced by spray drying with a Mini Spray Dryer B-290 (Büchi; Switzerland). The spray dryer was equipped with a 0.7 mm spray nozzle and high performance cyclone. The pressurized spray gas was dried and filtered using an in-line silica gel desiccant air dryer (McMaster-Carr; Elmhurst, IL) and Aervent® 0.2 μm filter (EMD Millipore; Billerica, MA). The spray dried liquid feed formulations were composed of dissolved excipient with 160 mM NaCl, 50 mM potassium glutamate, 10 mM histidine, 0.78% sucrose (w/v, due to bulk HSV-2 formulation) and final HSV-2 viral concentration of 6.4 $\log_{10}$ pfu/mL. The dissolved excipient concentration was 4% (w/v) for prescreening measurement and varied between 1.6% and 18.4% (w/v) in the DOE runs. 500 μL of liquid formulation was pipetted into an autoclaved

3 mL Type I clear glass vial and hemetically sealed by crimping for measurement of activity in the prepared feed solutions (prior to spray drying); losses in the feed solution are referred to as 'prespray dry losses' for monitoring the stability of the formulation process, not for spray drying process. The spray dryer inlet temperature was set at 110°C for prescreening measurement, and varied between 93°C and 127°C in the DOE. The pressurized spray gas flow rate was set at 359.5 L/h for prescreening measurement and varied between 206 L/h and 513 L/h in the DOE. Spray dried powders were collected after processing and weighed into hemetically sealed autoclaved 3 mL Type I clear glass vials. Samples were then analyzed as a process loss measurement, or stored at 45°C for 10 days for future storage loss analysis within the hemetically sealed vials.

2.3. DOE Modeling for Excipients

Modeling of HSV-2 spray drying in trehalose and sucrose formulations was accomplished by Box-Wilson Central Composite Design (Table 1). The experimental design was set up in Design-Expert 7.0 (Stat-Ease, Minneapolis, MN). Three separate spray dry parameters were examined within this DOE study per excipient formulation (trehalose or sucrose): inlet temperature, spray gas flow rate and concentration of the excipient, which was simply referred to as excipient concentration. These parameters were chosen due to their significant effects on spray dry biological stability, and their familiarity within the literature on spray drying pharmaceutics (Leclair et al., 2016; Saboo et al., 2016). Recently, work has shown that the liquid feed rate may have an effect on processing spray dry stability (Grasmeijer et al., 2019). This parameter may be further investigated in future work. Low (-1) and high (+1) settings were respectively: 100°C and 120°C for inlet temperature (*T*), 268 L/h and 451 L/h for spray gas flow rate (*S*), and 5% (w/v) and 15% (w/v) for excipient concentration (*C*). A circumscribed central

composite design with α = 1.682 was applied to enhance second order effects, thus the DOE model included runs at ±1.682 for each factor along the baseline. For example, inlet temperature second order effects were enhanced by inclusion of the points (T = 127°C, S = 359.5 L/h, C = 10% (w/v)) and (T = 93°C, S = 359.5 L/h, C = 10% w/v), as modeled by (±1.682, 0, 0). All formulations included the 160 mM NaCl, 50 mM potassium glutamate, 10 mM histidine, 0.78% sucrose (w/v, due to bulk HSV-2 formulation) and final HSV-2 viral concentration of 6.4 $\log_{10}$ pfu/mL. Three replicates were completed at the (0, 0, 0) model midpoints (T = 110°C, S = 359.5 L/h, C = 10 (% w/v)) for error analysis within the DOE.

2.4. Glass Transition Temperature

Thermograms of the spray dried powders were measured by modulated differential scanning calorimetry (MDSC) with a Q200 Differential Scanning Calorimeter (TA Instruments; DE, USA). The samples (5- 10 mg) were measured into a hermetically sealed aluminum pan. Pans were equilibrated at 4°C for five minutes. The sample temperature was modulated by ±0.60°C every 40 s and increased at a ramp rate of 3.00°C/min up to a maximum temperature of 200°C. Samples were purged with a nitrogen flow gas at a flow rate of 50 mL/min during the measurement. Thermograms were evaluated for glass transition temperatures (Tg, °C) using TA Universal Analysis Software (TA Instruments; DE, USA).

2.5. Residual Moisture

Approximately 150 mg of spray dried powder was measured into a Mettler Toledo HG63 Moisture Analyzer (Mettler Toledo, Switzerland). The sample was heated linearly to 140°C and held for 10 minutes to evaporate all bound water. Residual moisture was then reported by the moisture analyzer as a mass percentage (% w/w).

2.6. *In Vitro* HSV-2 Activity Testing

Activity loss of HSV-2 vaccine was determined by plaque forming assay with Vero cell lines (obtained from Sanofi Pasteur Ltd., Ontario, Canada). Measured activity was reported as $\log_{10}$ pfu/mL.

2.6.1. Culturing of Vero Cells

Vero growth media was prepared as Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Sigma Aldrich, Ontario, Canada) with 50 mM sucrose, 10% fetal bovine serum (FBS; Invitrogen, Ontario, Canada), 10 mL/L Geneticin (Sigma Aldrich, Ontario, Canada), 2 mL/L HyClone™ LS250 (Sigma Aldrich, Ontario, Canada) and 4 mM glutamate (Sigma Aldrich, Ontario, Canada). At 80-90% confluency of Vero cells, cultures were split for plating. Cell monolayers were washed with Dulbecco's Phosphate-Buffered Saline (Invitrogen, Ontario, Canada) before trypsinizing with TrypLE (Thermo Fisher Scientific; ON, Canada). The cell suspension was counted and then diluted to a concentration of 6 x 10^5 cells/mL for use within the plaque forming assay.

2.6.2. Determination of HSV-2 by Plaque Forming Assay

Using a 48-well plate (Corning Cooperated; Durham, USA), wells were seeded with 250 µL of the cell suspension and then incubated overnight at 36.0°C/5% CO_2 for complete surface coverage. Spray dried samples were reconstituted in culture media (DMEM/F12 + 1% FBS) to a HSV-2 input concentration (i.e. activity assuming zero losses) of 6.4 $\log_{10}$ pfu/mL after accounting for bound water in the powders. Serial dilutions were prepared and plated in triplicate by 300 µL of solution into each well within the 48-well plate. Negative controls (containing no HSV-2) were prepared and plated for quality measures. Viral dilutions were then incubated at 34°C/5%CO_2 for 48 hours. Afterward, culture media was discarded and the cell monolayer was covered with a solution of 57% Crystal Violet Stain (Sigma-Aldrich; ON, Canada) and 43%

methanol (EMD Millipore; ON, Canada). The cell monolayer was fixed and stained for 60 minutes before discarding of the staining solution. Residual stain was rinsed with water until water ran clear. 48-well plates were then inverted and dried. Following the staining procedure, viral plaques were visible as holes within the cell monolayer. Plaques were counted by a Viruscope (MicroVision Instruments; Evry, France) to determine the resulting titre of the sample tested. Activity loss was then calculated as the difference between HSV-2 input and final measured titres.

2.7. Data Analysis

DOE measurements were analyzed with Design-Expert 7.0 and MATLAB 9.3 (MathWorks; MA, USA) using analysis of variance (ANOVA) to model parameter effects on the response. Each model was found to be statistically significant ($p \leq 0.05$), indicating that variations in activity response were not due to random noise. Investigated parameters were determined to be statistically significant ($p \leq 0.05$) in effect on activity within the models.

3. RESULTS

3.1. Excipient Screening

Numerous excipients were tested for spray drying to determine acceptable formulations for HSV-2 stability (five examples are shown in Fig. 1 and Table 2). These excipients were prescreened for spray dry suitability with HSV-2 by the method similar to previous works (Lovalenti et al., 2016; Ohtake et al., 2010). Samples were dried under fixed process parameter settings of spray drying as described in the methods, determined as reasonable conditions in previous work with other vaccine platforms [11, 15]. The stability of each formulation was determined by measurement of prespray dry loss (activity losses during formulation mixing

manipulation), process loss (activity losses from spray dry processing), and storage loss (activity losses from storage at 45°C for 10 days). These combined losses for a selection of tested excipient formulations, including the two top performing cases (i.e. trehalose and sucrose), are shown in Figure 1 and Table 2. Prescreening was based on performance of the formulations by the measured activity losses. Trehalose and sucrose demonstrated the lowest total activity losses at 1.40 $\log_{10}$ pfu/mL and 1.76 $\log_{10}$ pfu/mL, respectively (Table 2). The greatest combined activity losses were observed for the excipient formulations of lactose and a lactose/trehalose blend where viral activity was below the detection limit (<3.5 $\log_{10}$ pfu/mL, Table 2).

The restrictive and stabilizing matrices of sucrose and trehalose were further characterized for their glass transition temperature (T_g) due to the relevance of this transition on imparting biological thermal stability (Ameri and Maa, 2006). The T_g was 116.2°C for the trehalose formulation and 58.4°C for the sucrose formulation (Table 2), indicating immobility of the biological should exist far above room temperature for trehalose. Table 2 shows that all tested excipients in the screening study exhibited sufficiently high T_g for the purpose of thermal stability but in comparison to the results in Fig 1, it is evident that this characteristic of the matrix is insufficient to ensure their suitability. Design of Experiments (DOE) trials were then prepared to better quantify and evaluate vaccine activity losses within the two top formulations.

3.2. Optimization of Trehalose and Sucrose Matrix by DOE

A Box-Wilson Central Composite Design and data analysis was described in the methods section. The randomized DOE run tables and raw data of the responses are reported in the Supplemental Information. The main responses of the DOE used for the analysis were the drying process loss and storage loss, as discussed below.

3.2.1. Loss of HSV-2 Activity during Processing ("Process Loss")

252 The loss of HSV-2 activities related to spray dry processing was investigated by DOE
253 studies for the excipient matrices, trehalose and sucrose, separately. Losses were determined
254 from adjustment of the spray dryer inlet temperature (*T*, °C), spray gas flow rate (*S*, L/h) and
255 trehalose or sucrose concentration (*C*, % w/v). The process loss was modeled by ANOVA and
256 resulted from thermal and mechanical stresses imposed by the spray dryer and physiochemical
257 interactions of the biological with the matrix excipient during drying (Ameri and Maa, 2006).
258 The models accounting for the main factors and interactions are given as Equations 1.1 and 2.1
259 for trehalose and sucrose, respectively.

$$Process\ loss\ (\log)\ of\ trehalose\ matrix = 2.66 + 9.56 \times 10^{-3}T - 1.50 \times 10^{-2}S - 5.5 \times 10^{-2}C + 2.00 \times 10^{-5}S^2 \quad \textbf{1.1}$$

$$Process\ loss\ (log)\ of\ sucrose\ matrix = -236.89 + 4.36T + 0.67S - 0.47C - 1.23 \times 10^{-2}TS + 4.17 \times 10^{-3}TC - 1.99 \times 10^{-2}T^2 + 5.56 \times 10^{-5}T^2S \quad \textbf{2.1}$$

260 Process loss surface plots generated by these models are shown in Figures 2 and 3. For
261 Equation 1.1, the significant parameters were inlet temperature, T ($p < 0.1$), second-order spray
262 gas flow rate, S^2 ($p < 0.05$), and sugar excipient concentration, C ($p < 0.05$). The model fit the
263 data (residual lack of fit: $p > 0.1$) and was not due to random noise ($p < 0.05$). For Equation 2.1,
264 the significant parameters were inlet temperature, T ($p < 0.1$), spray gas flow rate, S ($p < 0.05$),
265 inlet temperature and sugar excipient concentration interaction, TC ($p < 0.05$), and second-order
266 inlet temperature and spray gas flow rate interaction, T^2S ($p < 0.05$). This model also fit the data
267 (residual lack of fit: $p > 0.1$) and was not due to random noise ($p < 0.05$). Inlet temperature (*T*),
268 spray gas flow rate (*S*) and their combined effect (*TS*) on HSV-2 vaccine activity are shown in

Fig. 2A and Fig. 2B for the trehalose- and sucrose-based formulations respectively, with solids concentration fixed at 10% (w/v). Inlet temperature (*T*), sugar excipient concentration (*C*) and combined effects (*TC*) on process loss of HSV-2 candidate vaccine are shown in Fig. 3A and Fig. 3B for trehalose- and sucrose-based formulations respectively, with spray gas flow rate fixed at 359.5 L/h.

The trehalose-based formulations demonstrated an increase in process loss with the HSV-2 candidate vaccine for increasing spray dryer inlet temperatures (Fig. 2A). The effect of the spray gas flow rate had a parabolic trend, with lowest process losses at the central condition. There was no significant interactive effect between spray dryer inlet temperature and spray gas flow rate for the trehalose formulation.

The sucrose-based formulations exhibited similar overall trends for HSV-2 process losses. Increasing losses in activity were witnessed for increasing inlet temperature (seen in Fig. 2B). While the trend of increasing HSV-2 activity losses for increasing spray dryer inlet temperatures remained consistent between trehalose and sucrose formulations, the magnitude of these associated losses varied. This is seen clearly by the interaction effect between spray dryer inlet temperature and spray gas flow rate, which was only applicable for the sucrose formulation model. This interaction demonstrated moderate inlet temperatures (approximately 110°C) to be more ideal at high spray gas flow rates (450 L/h) and detrimental at low spray gas flow rates (268 L/h). Minimal process losses were ultimately found at moderate inlet temperature and high spray gas flow rate (i.e. 110°C and 451 L/h) for sucrose, in distinct contrast to the trehalose formulation where minimal losses occurred at an inlet temperature of 100°C and spray gas flow rate of 360 L/h.

291 The effect of excipient concentration on process losses for the trehalose- and sucrose-
292 based formulations is shown in Fig. 3 (A: trehalose, B: sucrose). Increasing concentration of
293 trehalose within the spray dried system reduced activity loss for the HSV-2. The effect was
294 further demonstrated by the negative coefficient for excipient concentration within the trehalose
295 process loss model (Equation 1.1). Minimal process loss was modeled to occur at the greatest
296 trehalose concentration and lowest inlet temperature (approximately 0 $\log_{10}$ pfu/mL, perhaps due
297 to boundary effects, shown Fig. 3A). There was no interaction effect between inlet temperature
298 and excipient concentration within the spray dried trehalose formulation.
299 The effects of sucrose concentration and inlet temperature on process loss are shown in
300 Fig. 3B. These effects were dissimilar in trend to the trehalose-based formulation due to the
301 interaction effect. The negative coefficient for excipient concentration (*C*, Equation 2.1)
302 indicated a decreasing process loss for increasing sucrose concentration, as observed at the low
303 inlet temperatures, but the significant interaction parameter between inlet temperature and
304 excipient concentration (*TC*) indicated that process loss drifted upward in value for the combined
305 effect of increasing inlet temperature with increasing solids concentration (Fig. 3B). This
306 interaction effect was absent from the trehalose-based model, and is greatly noticeable in
307 comparing each model (Fig. 3A, 3B).
308 For the trehalose process loss model, HSV-2 activity is minimized as an activity loss less
309 than 0.50 $\log_{10}$ pfu/mL for maximum solids concentrations (15.00 %w/w) at all combinations of
310 DOE inlet temperature and spray gas flow rate. At a mid-level solids concentration (10.00
311 %w/w), HSV-2 activity loss is below 0.50 $\log_{10}$ pfu/mL at all spray gas flow rates for minimum
312 inlet temperature (100°C), and between the spray gas flow rates of 318.50 – 430.00 L/h. At

minimum solids concentration (5.00 %w/w), the HSV-2 activity loss is greater than 0.50 $\log_{10}$ pfu/mL for all inlet temperature and spray gas flow rate DOE combinations.

For the sucrose process loss model, HSV-2 activity is minimized at less than 0.50 $\log_{10}$ pfu/mL for maximum solids concentrations (15.00 %w/w) at minimum spray gas flow rate (268.00 L/h) and up to inlet temperatures of 101.2°C. In comparison for the same solids concentration, the process loss is below 0.50 $\log_{10}$ pfu/mL at a maximum spray gas flow rate (451.00 L/h) up to inlet temperatures of 116.2°C. At a mid-level solids concentration (10.00 %w/w), HSV-2 loss of activity is greater than 0.50 $\log_{10}$ pfu/mL for all DOE inlet temperatures at minimum spray gas flow rate (268.00 L/h), and between inlet temperatures of 101.9°C and 117.5°C at maximum spray gas flow rate (451.00 L/h). Lastly, at minimum solids concentration (5.00 %w/w), HSV-2 loss of activity is again greater than 0.50 $\log_{10}$ pfu/mL for all DOE inlet temperatures at minimum spray gas flow rate (268.00 L/h). Similarly, activity loss is below 0.50 $\log_{10}$ pfu/mL at DOE inlet temperatures between 104.1°C and 119.2°C for maximum spray gas flow rate (451.00 L/h).

By summarizing the conditions for which HSV-2 activity process loss is less than 0.50 $\log_{10}$ pfu/mL, it is evident that trehalose is more robust of an excipient formulation than sucrose for this particular biological. HSV-2 activity is at satisfactory levels for a greater range of spray drying process parameters when dried with trehalose compared to sucrose.

3.2.3. Loss of HSV-2 Activity during Storage ("Storage Loss")

The loss of HSV-2 activity during storage was measured using the DOE runs to provide further comparison between the two sugar excipient bases. However, neither formulation resulted in a model of any significance. Thus prediction of storage losses by evaluation of the

inlet temperature, spray gas flow rate and excipient concentration was not possible from the current results.

However, the trehalose-based formulation proved to be very robust as the storage loss remained relatively consistent over a wide window of tested spray dryer conditions (Fig. 4). The minimal measured storage loss was approximately 0.0 $\log_{10}$ pfu/mL, while the greatest tested storage loss was approximately 1.3 $\log_{10}$ pfu/mL, indicating similar storage losses between samples despite the wide range of processing conditions. Overall, the dry powder vaccine based on trehalose shows suitable thermal stability.

Conversely, the sucrose-based formulation demonstrated less robustness in terms of storage stability. The minimal tested storage loss was approximately 0.4 $\log_{10}$ pfu/mL while the greatest storage loss was beyond the detection limit (>5 $\log_{10}$ pfu/mL) (Fig. 4). This large variance indicates that HSV-2 storage stability is affected by the spray dry process conditions, although the model used lacks significant power to resolve these differences or the specific interactions leading to storage stability are too complex to be mapped by the model parameters used.

3.3. Extended Formulation Analysis

DOE results were primarily compared above to activity loss as the dominant response for determining the suitability of formulation matrix and spray dryer operation parameters. However, additional responses were evaluated to investigate the differences between the two matrixes on thermal stability. HSV-2 process losses were plotted against outlet temperatures, which is an indirect process variable that correlates with many of the input variables and yet represents the final drying temperature and ambient environment for collected particles until a run has finished (Fig. 5). Spray drying with the trehalose-based formulation showed no

observable trend, as determined by the Pearson correlation coefficient, r (Fig. 5A, r = 0). In comparison, there was a positive linear trend with increasing process loss of HSV-2 for increasing spray dryer outlet temperature with the sucrose-based formulation (Fig. 5B, r = 0.47).

Additionally, residual moisture of the spray dried powder samples from each DOE run were correlated to the spray gas flow rate for the sucrose-based formulation (Fig. 6). A negative linear relationship was determined between residual moisture and the spray gas flow rate (Fig. 6, r = -0.47). Decreasing residual moisture from increasing spray gas flow rates agrees with known theory and previous spray drying studies (Kanojia et al., 2016). The investigation of residual moisture for spray dried sucrose samples was especially important in comparison to that of spray dried trehalose samples due to the effects of moisture on dry powder T_g (Hancock and Zografi, 1994), and sucrose sample T_g near ambient conditions (58.4°C Table 1) that contrasts high trehalose particle T_g (Hoe et al., 2014).

Overall, extra analysis of the sucrose-based spray drying process revealed a positive linear correlation between spray dryer outlet temperature and HSV-2 process loss, and a negative linear correlation between spray gas flow rate and residual moisture.

3.4. Technology Transfer Study

As previously discussed, the minimum process titer loss can be achieved at maximum trehalose concentration for all combinations of DOE inlet temperature and spray gas flow rate, preferably at lower inlet temperature and medium spray gas flow. To further examine and challenge the robustness of the trehalose matrix, a confirmation study was conducted in a different facility to mimic 10x batch size technical transfer. An optimized formulation with low process loss was chosen from the prediction model (Equation. 1.1). The confirmation study

conditions and results are summarized in Table 3 below. The same nozzle design was used, thus the particles formed were expected to be similar. Future work is necessary to confirm this.

Slightly higher process losses were observed in the large-scale batch. The deviation from the prediction model was likely caused by the different type of cyclone used on each of the two spray dryers and producing a larger batch size, which led to a longer residence time in the powder collector at temperature around 50°C. The difference in cyclone usage likely led to different particle size distributions between batches, as particle separation is dependent on the cyclone radius. All other responses i.e. residual moisture and stability were consistent with the predictions.

4. DISCUSSION

4.1. Formulation Screening

Sugars are the most important stabilizers for spray drying vaccines [6]. Our preliminary formulation screening investigated different sugars and its combination. The screening criteria was set as the minimal viral activity loss during overall processes including prespray dry, spray drying and storage. Among different sugars, trehalose and sucrose were more effective at replacing the stabilizing water bonds with the biological by hydrogen bonding; our preliminary work thus has the similar in findings (Table 2 and Fig. 1). It is known that individual excipient-biological interactions are unique and conditional on the biological (HSV-2 in this case) being stabilized effectively as water is replaced by a glassy matrix (Alcock et al., 2010; Ohtake et al., 2010; Saluja et al., 2010; Toniolo et al., 2019). This is why it is necessary to conduct prescreening trials for each new biological being spray dried, at least until models of the stabilizing mechanism are properly derived.

In addition, the T_g of these formulations was measured to be 116.2°C for the spray dried trehalose powder and 58.4°C for the spray dried sucrose powder (Table 2), which was also in agreement with previous literature findings (Ding et al., 1996; Shamblin et al., 1996). However, lactose, mannitol, dextran and/or its combinations did not preserve the viral titer well, even though some formulations demonstrated a higher Tg value. There are many hypotheses that explain how sugars function as stabilizers for drying [6], such as hydrogen bonding, glassy matrix immobilization, specific molecular fit into irregular surfaces of target biological and/or T_g of amorphous matrix; to us it is more likely that there are complex and dynamic factors that influence both the drying process and storage. Thus, the selection of an appropriate excipient with overall titer preservation is very important for stabilizing vaccine antigen during the spray drying process and future storage.

The excipient screening was performed at one set of values for inlet temperature, spray gas flow rate and solids concentration for comparison of titer losses; the values were selected from preliminary trials for their robustness in preparing particles from multiple excipients with acceptable activity. This method of prescreening is commonly used for determining spray dry formulations. Our subsequent analysis showed that sucrose could have been dropped as a formulation excipient for spray drying earlier in the study. Alternatively, another excipient choice may have performed markedly better, however, it was not evaluated further because of poor performance at just the tested prescreen spray dry conditions.

4.2. Comparison of Process Loss between Formulations using DOE studies

In comparison to the widely utilized drying process of lyophilization, spray drying is advantageous due to cheaper processing costs, semi-continuous processing and greater process control due to the various spray dry processing parameters (i.e. inlet temperature, spray gas flow

425 rate, etc.). Spray dry processing imparts thermal and shear stresses on the biological as it is being
426 dehydrated (Ameri and Maa, 2006). Stabilization occurs by proper incorporation of biological
427 within the dry excipient matrix (Yu, 2001). And so, appropriate processing must strike a balance
428 between rapidly immobilizing the viral vector in the sugar matrix to minimize deactivation while
429 accepting the spray dry processing leads to damage as well. Prior work has identified
430 atomization and dehydration to be significant factors for spray dry process loss (Grasmeijer et
431 al., 2019). The modeled process losses of HSV-2 for the trehalose formulation (Fig. 3A)
432 followed the same trends previously witnessed for spray drying of a type 5 human adenoviral
433 vector (AdHu5) within a mannitol/dextran formulation (LeClair et al., 2016b). Increasing inlet
434 temperatures increases the free energy of the system, and thus promoted deactivation of the
435 biological (Fig. 3A) (Dill, 1990; Huang, 1986). The effects of high inlet temperature are offset
436 by faster immobilization via increasing spray gas flow rate to reduce the droplet size, which in
437 turn minimizes drying time and biological-excipient separation by diffusion (as previously
438 explained by Vehring) (Vehring, 2008; Vehring et al., 2007). However, spray gas flow rate
439 exhibits a parabolic trend since significantly high flow rates promote more losses by imposed
440 high shear rates that are detrimental to biological stability and efficacy (Ghandi et al., 2012; Maa
441 and Hsu, 1997).

442 In contrast, the sucrose matrix showed a different behavior resulting in minimal activity
443 losses occurring at a different process point, and caused much greater variation within the
444 modeled HSV-2 process losses (for this operating window, trehalose losses varied between
445 approximately 0.25 $\log_{10}$ pfu/mL – 0.65 $\log_{10}$ pfu/mL whereas sucrose losses varied between
446 approximately 0.20 $\log_{10}$ pfu/mL – 1.20 $\log_{10}$ pfu/mL). The interaction effect between inlet
447 temperature and spray gas flow rate accredited to the differences with sucrose on HSV-2 process

448 loss is largely attributed to the low T_g of the excipient, as seen in Table 1. Low matrix T_g caused
449 instability of HSV-2 throughout processing, and thus losses were heavily dominated by spray
450 dryer temperature and free energy within the system (Amorij et al., 2008). Residual moisture of
451 spray dried sucrose powder increased for decreasing spray gas flow rates (Fig. 7). The sample T_g
452 is further depressed by residual moisture due to the well-established plasticizing effects of water
453 on disaccharides (Roos, 2002). Due to the increased instability within spray dried sucrose
454 powder because of sample T_g, HSV-2 losses from processing were more susceptible to increased
455 free energy and residual moisture. The balancing and effects of these forces on HSV-2 activity
456 are shown in Fig. 3B. Thus, process losses for a sucrose matrix were perhaps more sensitive to
457 spray dry parameters due to its T_g.

458 The effect of sugar concentration has a significant influence on process loss (Fig. 3). The
459 modeled trends for trehalose agreed with previous DOE studies of AdHu5 spray dried within a
460 mannitol/dextran matrix, though the magnitude of effect was much greater in the prior work
461 (LeClair et al., 2016b). Increasing trehalose concentration allowed for a greater availability of
462 sugar-biological stabilization to replace water bonds when dehydrated by spray drying (Patist
463 and Zoerb, 2005). This was demonstrated within the modeled operating window because the
464 greatest processing loss of HSV-2 efficacy occurred at the lowest spray dried trehalose
465 concentration (Fig. 3A). In comparison, spray dried sucrose particles were less effective at
466 stabilizing the HSV-2 at greater solids concentration within the same operating window (Fig.
467 3B). This is indicative of perhaps weaker interactive effects between sucrose and the specific
468 biological used (HSV-2), which was not identified during the standard prescreening methods.
469 Furthermore, the interaction effect of inlet temperature and excipient concentration was unique
470 to the modeled HSV-2 process loss for the sucrose matrix. It has been previously established that

greater solute concentrations within a spray dried feed increases the outlet temperature (Shishir and Chen, 2017). By reducing the amount of free water per drying droplet and increasing the amount of hydrating solute, there is less evaporative cooling due to the decrease in rate of water flow through the system and increase in retained water within the extra solids content. These factors cause an increase in the outlet drying temperature. For a stabilizing matrix with high T_g, like trehalose, the equilibrium drying temperature will have little effect on HSV-2 activity losses associated with processing (Fig. 5A, for trehalose). However, these increases appeared much more detrimental for matrices of lower T_g, such as sucrose (Fig. 5B) (Patist and Zoerb, 2005), where comparison of outlet temperature to the measured HSV-2 process loss showed a positive correlation. Thus, the increase in outlet temperature due to the combined effects of inlet temperature and solids concentration had a greater effect on the sucrose formulation, causing the differences in observed process loss models (Fig. 3). It is again because of these differences in response to spray drying parameters that a prescreening excipient comparison is difficult to achieve at a specific spray dry setting.

Overall, the optimized spray drying parameter settings obtained from the prediction model for trehalose formulation are: low inlet temperature i.e. 105°C; high solid concentration i.e. 18%w/w; and medium gas flow rate i.e. 360 L/h. The predicted process titer loss would be below measurable amounts. The optimized spray drying parameter setting obtained from the prediction model for sucrose formulation is: low inlet temperature i.e. 105°C; medium high solid concentration i.e. 15%w/w; and medium gas flow rate, i.e. 360 L/h. The predicted process titer loss would be near 0.42 $\log_{10}$ pfu/mL.

4.3. Comparison of Storage Loss between Formulations using DOE Studies

493 After storage for 10 days at 45°C, the susceptibility of the sucrose formulation to activity
494 losses was even more apparent. Trehalose proved to be a very robust formulation matrix,
495 demonstrating minor activity losses for HSV-2 after storage over a wide range of tested spray
496 dry conditions (Fig. 4). In contrast, retained HSV-2 activity after storage was very poor for many
497 spray dry conditions of the sucrose formulation matrix. The varying range of storage losses
498 demonstrated that sucrose could be an effective stabilizer at some spray dry conditions, and a
499 much poorer stabilizer at others. This is demonstrated within a mock prescreen spray dry setting
500 shown in Figure 7. When spray dried with an inlet temperature of 120°C, spray gas flow rate of
501 451 L/h and excipient solids concentration of 15% (w/v), trehalose vastly outperforms sucrose as
502 a stabilizer (Fig. 7). Total HSV-2 activity losses from prespray dry, processing and storage
503 amounted to <1.5 $\log_{10}$ pfu/mL for trehalose and were beyond the detection limit for sucrose (>5
504 $\log_{10}$ pfu/mL). It is reasonable to predict that if a prescreening of excipients were performed at
505 this new set of spray dry settings, sucrose would not have been considered for use in the spray
506 drying of the HSV-2 vaccine (in comparison to Fig. 1). This overlooks the fact that sucrose is an
507 effective stabilizing excipient at certain conditions (i.e. previously demonstrated storage losses
508 below 0.5 $\log_{10}$ pfu/mL). The observed differences between trehalose and sucrose at these
509 conditions are demonstrative of the differences in magnitude and response for spray dried HSV-2
510 activity prespray dry, process and storage losses. The cause for this differentiation is largely
511 attributed to the specific molecular interactions between trehalose/sucrose and HSV-2, the T_g of
512 each matrix and the equilibrated state the particle is spray dried into, though further study would
513 be necessary to establish this. The fact is, the spray drying conditions used for prescreening
514 impose a bias in excipient selection that may not be suitable to determine their effectiveness for
515 stabilizing biologicals through spray drying and long term storage. It is then proposed that more

rigorous screening is necessary in early formulation development of spray dried vaccines, perhaps as an example by taking better account of storage loss over other losses.

To overcome the implications of the commonly used trial-and-error method, one potential approach is to adjust spray dry settings depending on predicted formulation T_g as described within a previously published valuable user-friendly model (Grasmeijer et al., 2013). However, this is only applicable for the formulation matrix that is heavily affected by spray dry processing due to low T_g and for T_gs that can be accurately predicted; the implications on T_g will be dependent on the level of residual moisture in each sample. Additionally, it leaves open to interpretation the particular spray dry conditions that would be best for prescreening experiments. Thus, such an approach is beneficial in planning of prescreening experiments, but does not conclusively solve issues altogether. This approach also cannot determine how effectively a chosen excipient interacts with the biological of interest. Alternatively, it is much more appropriate to prescreen potential excipients across more than one set of spray dry conditions. In doing so, researchers are better able to understand the robustness of certain excipients and determine suitable boundaries of processing for prescreen comparison of each tested formulation matrix. This proposal greatly increases the time and resources of prescreening excipient stabilization studies because of the need to repeat across additional spray dry process changes. Thus, our work also highlights a current need for the development of high-throughput tools capable of replicating a spray dry environment in order to alleviate the current costs of prescreening and optimizing a thermally stable vaccine formulation.

4.4. Validate Technology Transfer of the Prediction Model

The confirmation studies at 10x the optimized batch size demonstrated the feasibility and technology transfer of the spray dry process for thermally stable vaccine formulations; and

539 achieved reasonable process titer losses and storage titer losses (Table 3). The deviation from the
540 prediction model was likely due to the larger batch size causing dried powder to experience
541 longer residence time in the powder collector at elevated temperature (about 50°C). In addition, a
542 small high-efficiency cyclone was used in the DOE study, which has a greater tendency to
543 collect fine particles than the larger cyclone used in the confirmation trial. It is likely that the
544 particle size distribution of each collected powder would influence the process loss and
545 especially storage loss of the HSV-2 vaccine. Further work may build on these findings to better
546 highlight this.
547
548 **5. CONCLUSIONS**
549 This manuscript is the first instance of directly comparing excipient matrices for use in
550 the spray drying of an HSV-2 vaccine candidate and examining their effects on process and
551 storage stability profiles. The work demonstrates inefficiencies in prescreening prediction of
552 stabilizing spray dry excipients. A series of evaluation steps were covered in the study,
553 highlighting the optimization of a thermally stable spray dried powder form of an HSV-2 vaccine
554 candidate. The results from the optimization trials for the different formulations demonstrated a
555 differing stabilization profile for the matrix sugars that were selected by prescreening efforts.
556 Trehalose was the most robust and effective stabilizing excipient in the study. The activity loss
557 variation in the DOE models between trehalose and sucrose revealed the impact of spray dry
558 process parameters on measured bio-activity. This work demonstrated the need to consider
559 potential interaction between excipient matrix and process parameters in early stage formulation
560 development for spray drying. Very similar process losses were achieved for the selected
561 trehalose formulation during the confirmation trial which examined the technology transfer of

562 the DOE prediction models by producing larger batches at a different facility. Future work
563 examining stability of spray dried HSV-2 vaccines at other storage conditions (i.e. 5°C,
564 controlled room temperature) is necessary to better define stability profiles and understand any
565 degradation that may occur from storage.
566
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575
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LIST OF FIGURE CAPTIONS

Figure 1. Activity loss (log_{10} pfu/mL) for selected excipient formulations. HSV-2 activity losses were combined by prespray dry loss (solid bar, losses due to dilution and mixing), process loss (striped bar, losses due to spray drying) and storage loss (clear bar, losses due to storage for 10 days at 45°C). * denotes viral activity was below detection limit (>5 log_{10} pfu/mL).

Figure 2. Process loss (log_{10} pfu/mL) of HSV-2 activity after spray drying at a solids concentration of 10% (w/v) with varying inlet temperature (°C) and spray gas flow rate (L/h). Models shown are for trehalose (A) and sucrose (B) formulations.

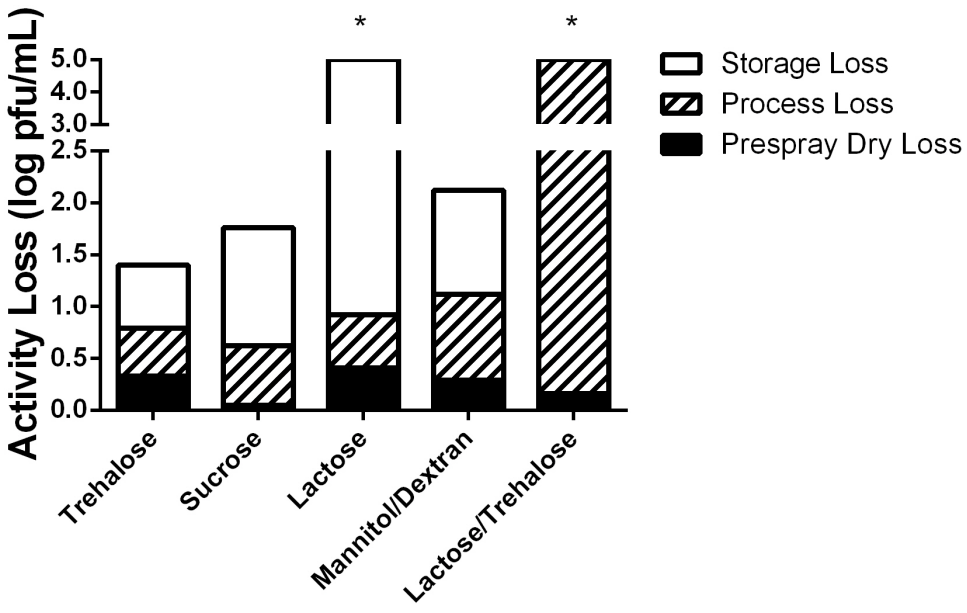
Figure 3. Process loss (log_{10} pfu/mL) of HSV-2 activity after spray drying at a spray gas flow rate of 360 L/h with varying inlet temperature (°C) and base excipient concentration (% w/v). Models shown are for trehalose (A) and sucrose (B) formulations.

Figure 4. Box and whisker plot of HSV-2 activity losses (log_{10} pfu/mL) after storage at 45°C for 10 days. Whiskers represent minimum and maximum responses. The line bisecting the box was the median (>5 log_{10} pfu/mL for sucrose sample)(n=17). * denotes activity losses were below detection limit (>5 log_{10} pfu/mL).

Figure 5. Measured process loss (log_{10} pfu/mL) for each DOE sample with corresponding outlet temperature (°C). Spray dry formulations shown are trehalose (A) and sucrose (B).

Figure 6. Measured residual moisture (% w/w) for DOE samples with specified drying spray gas flow rates (L/h).

Figure 7. Combined HSV-2 activity losses (log_{10} pfu/mL) for a trehalose and sucrose formulation spray dried with inlet temperature of 120°C, spray gas flow rate of 451 L/h and solids concentration of 15% (w/v).



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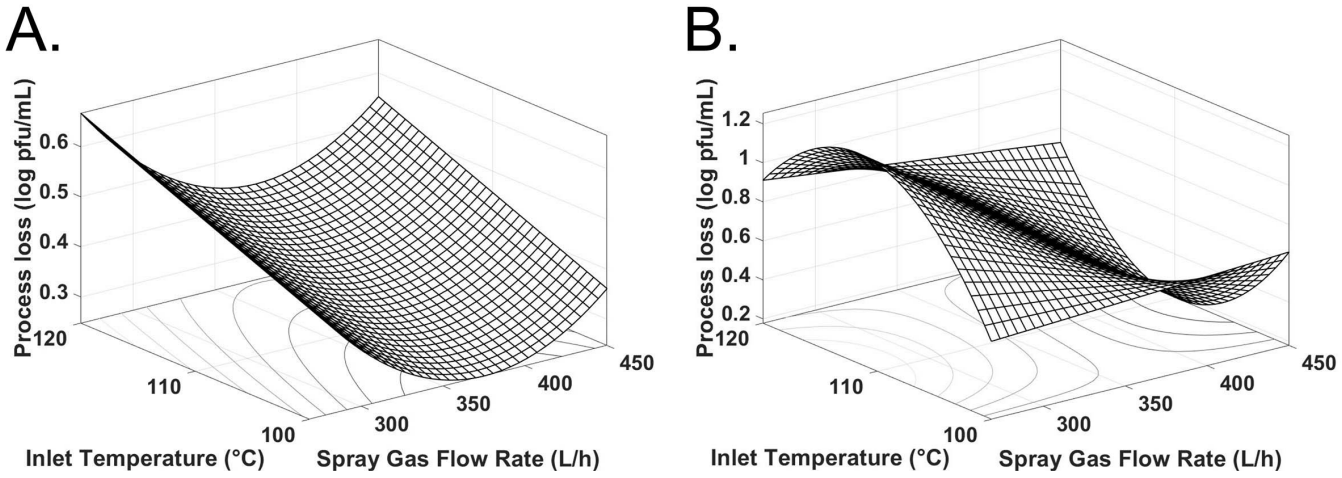

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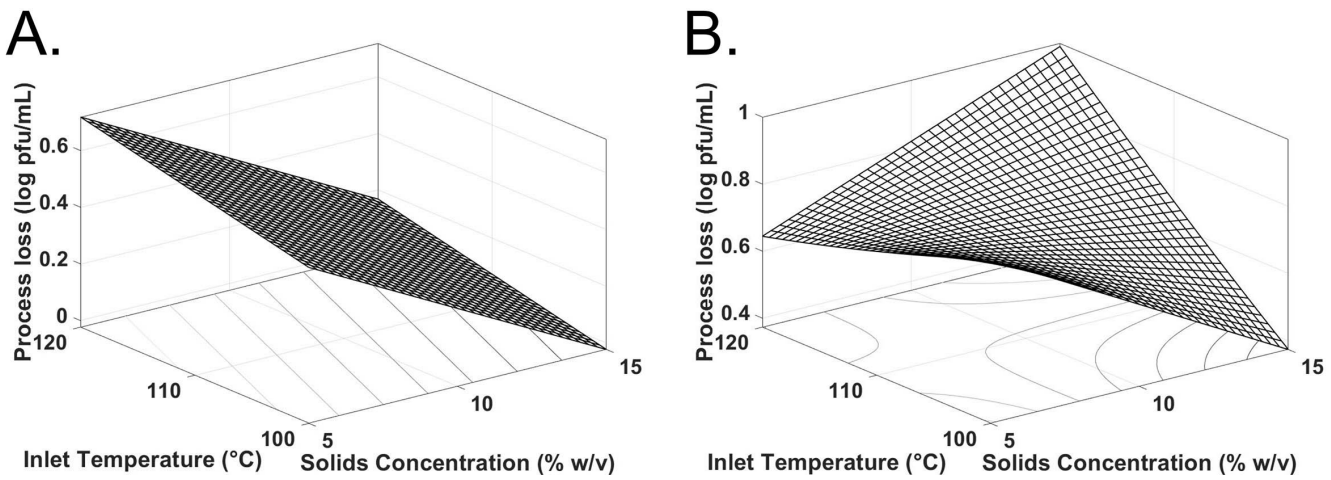

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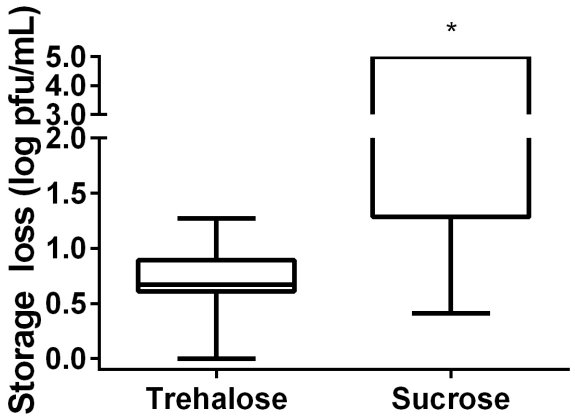

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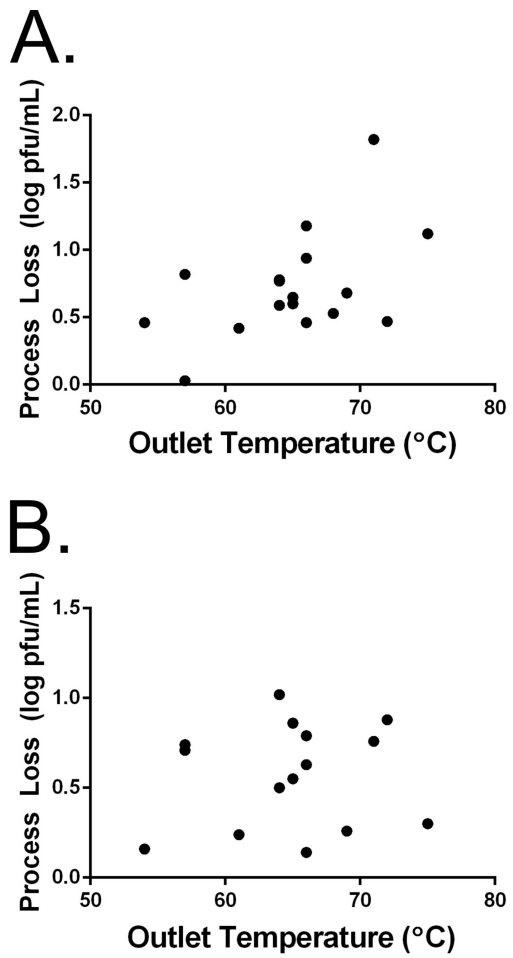

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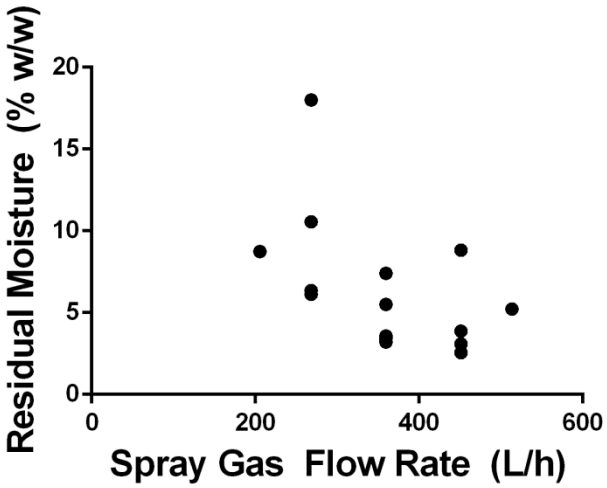

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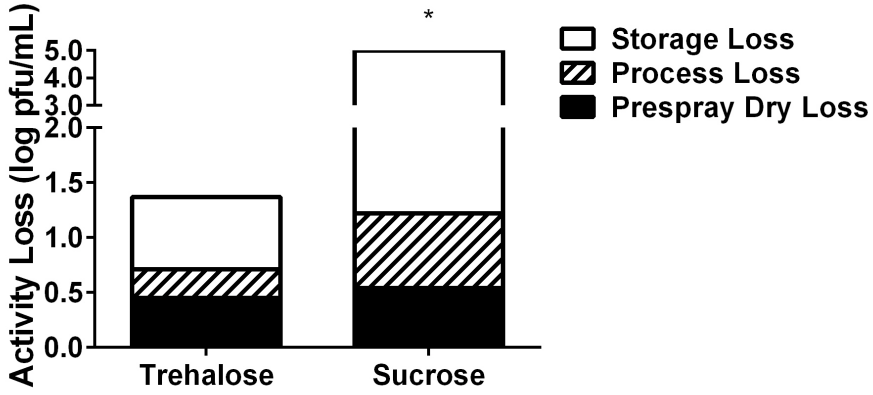
740 **Figure 6.** Measured residual moisture (% w/w) for DOE samples with specified drying spray gas flow rates (L/h).

741



742

743 **Figure 7.** Combined HSV-2 activity losses (log_{10} pfu/mL) for a trehalose and sucrose formulation spray dried with inlet
744 temperature of 120°C, spray gas flow rate of 451 L/h and solids concentration of 15% (w/v).

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