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Bead Structure in Freeze-Drying Processes: Analysis Techniques and Detailed Morphology Studies

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Popular Summary

Freeze drying, or lyophilization, is a vital technology in the biotechnology and pharmaceutical industries, essential for preserving sensitive biological materials. The process involves freezing the material and then placing it in a vacuum, causing the ice to sublimate directly into vapor, thereby bypassing the liquid phase. This method is crucial for maintaining the stability and extending the shelf life of delicate substances such as enzymes.

One of the primary objectives of the study was to investigate the impact of bead size and formulation on the morphology of the pellets. Another key objective was to develop and optimize protocols for pelletization, annealing, and lyophilization, with the goal of improving the structural outcomes of the beads. Additionally, the study employed advanced analytical techniques such as embedding in silicone elastomer, light microscopy, and differential scanning calorimetry (DSC) to examine the morphological and thermal properties of the freeze-dried beads in detail.

The research utilized the quench-freezing method to produce uniform beads of two sizes, 5mm and 2.5mm. Different ratios of sucrose and maltodextrin were used to prepare the formulations. Microscopy methods were employed to inspect the structural integrity and morphology of the freeze-dried beads - light microscopy and stereomicroscopy.

The findings revealed that variations in carbohydrate ratios and bead sizes significantly affect the pore structure and morphology of the beads. Higher concentrations of carbohydrates tended to produce larger pores. Beads with higher sucrose content showed more pronounced voids in their core, indicating potential structural weaknesses. Additionally, a less porous "skin" layer was observed on the surface of all beads, which was more pronounced in higher carbohydrate concentrations due to the rapid freezing process.

However, the study also highlighted some challenges. There were limitations in the resolution and depth of field of the microscopes used, suggesting a need for enhanced visualization techniques. Additionally, improving automated pore annotation using Python scripts and image processing libraries is recommended to handle lower quality images more effectively.

Preface

This degree project was conducted at the Department of Process and Life Science Engineering at the Faculty of Engineering (LTH), Lund University. The project focused on the freeze-drying of beads, aiming to explore the complex relationship between designed formulations and bead structure, by employing bespoke analytical methods and methodologies.

I am profoundly grateful to my supervisors, Anna Fureby and Shuai Bai, for their valuable guidance and insightful feedback throughout the duration of this project. Their expertise and dedicated mentorship were crucial in the formulation and refinement of my research objectives and methods.

Special acknowledgment is due to the department's research engineers, Olexandr Fedkiv and Hans Bolinsson. Their technical support and key practical advice were indispensable during the execution phases of my research.

The collaborative environment at the Department of Process and Life Science Engineering has been tremendously of value. I am thankful for the opportunity to work among peers and professionals who are deeply committed to advancing knowledge and innovation in the field. This project could not have been accomplished without the supportive and inspiring atmosphere provided by the entire department.

Abstract

Freeze drying, also known as lyophilization, is a crucial technology used in biotechnology and pharmaceutical preparation to preserve enzymes and other sensitive biological materials. This thesis investigates how different formulations and pellet dimensions impact the structural integrity of freeze-dried beads, essential for assessing their properties. Specifically, the study focuses on sucrose and maltodextrin formulations and their effects on bead morphology through controlled annealing and freeze-drying processes.

Quench freezing method was designed and then employed to produce uniform beads for analysis of two sizes: 5mm (large) and 2.5mm (small). Several analytical methods, such as Differential Scanning Calorimetry (DSC), Light microscopy, Stereo microscopy were utilized to inspect impacts of the designed formulation on structural integrity and morphology of the freeze-dried pellets.

Key findings indicate that variations in carbohydrate ratios and bead sizes significantly affect the pore structure and morphology of the beads. The study establishes detailed assessment of changes in accordance with the ratio of excipients and concentration of formulation, as well as pellet dimensions, enhancing the understanding and application of this technique in industrial settings.

Keywords: Freeze drying, lyophilization, bead structure, morphology analysis, sucrose, maltodextrin, quench-freezing, Differential Scanning Calorimetry (DSC), light microscopy, carbohydrate formulations, structural integrity, pore structure, pharmaceutical preservation,

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List of Abbreviations

DSC	Differential Scanning Calorimetry
Tc	Collapse Temperature
Tg	Glass Transition Temperature
Tg’	Glass transition Temperature of maximally freeze concentrated solution
Tm	Freezing Line

1. Introduction and Aims of the Study

1.1 Introduction

Background

Freeze drying is a vital process that has been well studied and largely utilized in the pharmaceutical and biotechnology fields. This method is crucial for extending the shelf life and maintaining the stability of sensitive biological materials, such as enzymes, which play a critical role in producing various biomedical and industrial products (1). This process involves removing water from the material by first freezing it and then placing it in a vacuum to sublimate the ice directly into vapor, bypassing the liquid phase. The soft dehydration method preserves structure and bioactivity of materials, making it indispensable for handling delicate materials.

Freeze-drying can be introduced through various formats, including vials and pellets, each offering distinct advantages and challenges. Vial method is commonly used in the pharmaceutical industry due to its ability to produce uniform products and wide versatility when it comes to usage of formulations (20). Filled vials with formulation are slowly cooled - Due to slower freezing processes, this method results in larger ice crystals compared to the pellet method due to the slower freezing process, having an impact on morphology of the cake.

Pellet freeze-drying, on the other hand, involves rapidly quenching drops of the bespoke formulation in liquid nitrogen, resulting in a very rapid freezing rate (4). This leads to the formation of small ice crystals, which creates a different structural matrix (small pores) in comparison to the vial method. Those small ice crystals can be annealed slightly above glass transition temperature of freeze concentrate, where pellets are not in a brittle state - causing small ice crystals to dissolve and redeposit into larger ones. Pellets, having a bigger surface area per volume exposed to vacuum in comparison to vials, exhibit better mass transfer properties - this has implications on better drying efficiency.

There are various applications for freeze-dried beads. One notable example of the application of the beads in the pharmaceutical industry is work executed by Cepheid, a molecular diagnostics company. Cepheid uses freeze-dried pellets in diagnostic tests to ensure stability during long-term storage of reagents and enzymes. The Company has developed Xpert test cartridges that contain freeze-dried beads that are rehydrated during the test process, ensuring reliable results even after extended storage periods (2). Usage of freeze-dried pellets is particularly beneficial in this case, because maintaining the stability of reagents in varying environmental conditions is critical for sensitive test cartridges.

Role of drying aids, such as Sucrose and Maltodextin

Drying aids, such as sucrose and maltodextrin. are well-studied and commonly used in various formulations and thus were strategically used in this study (3). Those carbohydrates yield distinct properties that can be utilized to advantage when planning specific bead structure in different pharmaceutical applications. These carbohydrates are known for their ability to provide bulk and stabilize very fragile biocatalysts, such as enzymes.

Studying the state diagram can be particularly helpful to understand how changes in water content and temperature impact the carbohydrate formulation. It illustrates the phase behavior of the carbohydrate solution during the cooling and drying process.

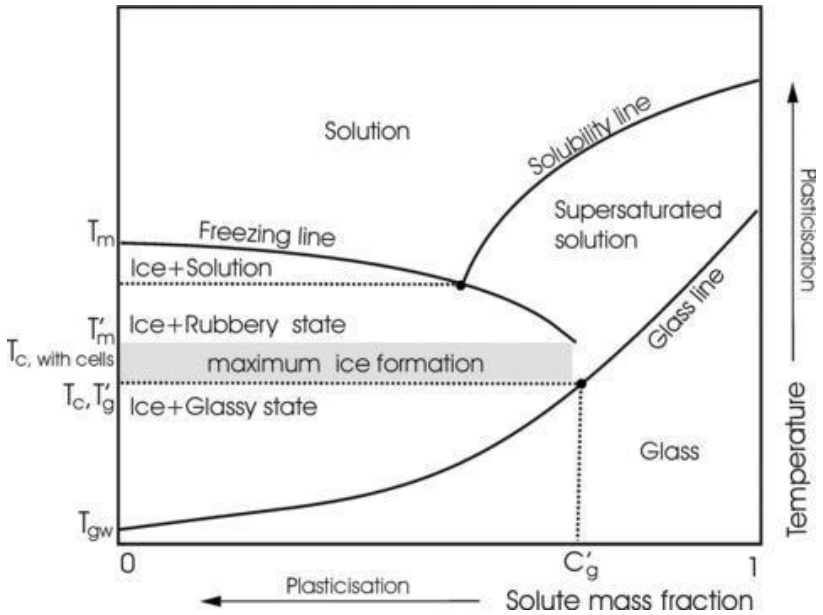


Figure 1: State diagram showing drying matrix and its regions (4)

The Horizontal axis represents the solute mass fraction, ranging from 0 (pure water) to 1 (pure carbohydrate), whereas the vertical axis represents temperature that increases from bottom to top. Freezing line (Tm) marks the boundary where ice starts to form from the carbohydrate solution as the temperature decreases. Above the freezing line, the carbohydrate solution is in a liquid state. Solubility line indicates where the carbohydrate solution becomes supersaturated - above this line solution contains more carbohydrate than it can normally hold at that temperature. Glass line represents transition to a glassy state - below this line material is glassy, which is characterized by a high viscosity and solid-like behavior without a crystalline structure.

Tc, Tg (temperature of collapse, Glass transition temperature) marks the critical temperatures for collapse and glass transition, respectively. Below these temperatures, carbohydrate solution enters a glassy state (5). Knowing the Tg is essential for determining the storage stability of carbohydrate formulations. Below Tg, the carbohydrate solution becomes an amorphous glass, which significantly reduces molecular mobility and chemical reactivity, enhancing stability and shelf life of the product. This is particularly important for preserving the quality of freeze-dried products. When it comes to Tg', which is the glass transition temperature of maximally freeze-concentrated solution, it represents the lowest temperature at which the maximally concentrated solution remains amorphous and glassy (6). Operating below Tg' during freeze-drying ensures that the remaining unfrozen fraction of the solution does not collapse, preserving the structure of the product.

Maltodextrin is a glucose polymer (carbohydrate) that is added to pharmaceutical formulations for crucial properties, such as cryoprotectant ability, as it is reducing ice crystal formation that could otherwise damage the structure and function of sensitive biological material (cells or proteins) (7).

Due to Maltodextrin's very heavy molecular weight, it translates to increase in Tg' of formulation - Tg' means glass transition temperature of maximally freeze-concentrated solution (8). It refers to temperature, at which the solution transitions from a rigid, glassy state to a more flexible, rubbery state in a solution that has been concentrated to its

maximum extent by removing as much water as possible, typically through freezing. This concentration leads to a highly viscous, glass-like state. Implication of higher Tg' is that it helps stabilize the amorphous phase of the product, reducing the likelihood of unwanted changes when environmental temperatures are fluctuating. This helps in stabilizing the amorphous phase of the product, meaning the product is less likely to undergo unwanted changes during storage environment temperature fluctuations (5).

Sucrose on the other hand, acts both as lyoprotectant and cryoprotectant (9). Its role as cryoprotectant centers on preventing damage of fragile biological components (10). As lyoprotectant, it further provides a stabilization effect during a drying phase, helping to preserve structure and function. Lee JC and TimaSheff SN found that sucrose is excluded from the protein domain, increasing the free energy of the system - thermodynamically this leads to protein stabilization, as it is more favorable for the protein to stay folded in this thermodynamic system, rather than unfolded (11). It has similar properties to maltodextrin in the context of increasing Tg' of formulation. This is emphasized by helping the product to be in a glassy state during storage (12).

Those carbohydrate systems are amorphous, which directly impacts various important formulation properties, such as nucleation and Tg' (13). Nucleation refers to the initial process where small clusters of a new phase, such as ice crystals or solid particles, form within the solution. In the context of carbohydrate formulation, this involves nucleation of ice crystals during the freezing process. Proper nucleation thus affects the size and distribution of ice crystals, which in turn influences stability and quality of the final product.

Carbohydrates stabilization mechanisms involve altering the water structure around fragile active ingredients, such as enzymes (14). Carbohydrates like sucrose and maltodextrin increase water-water hydrogen bonds and spatial ordering of water molecules, leading to the dehydration of the protein surface (15),(16). Dehydration makes protein less conformationally flexible, thus more stable. Generally, due to maltodextrin's ability to increase Tg and ability to form protective films, it has a better capacity to provide better long-term stability of freeze-dried products than sucrose (8), (9). A combination of sucrose and maltodextrin is used to leverage immediate protective effects of sucrose and long-term

stability provided by maltodextrin. This formulation can result in a robust protection during both freeze-drying and subsequent storage.

Couple of problems can be described when working with carbohydrate formulations, such as collapse, which is a direct result of increase in temperature during freeze drying, (17). Collapse refers to an occurrence where the structure of the product loses its integrity, shrinks and becomes deformed. This can result in the loss of the desired porous structure of freeze-dried product, leading to dense and compact form (18).

Different conditions and their impact on the pellet morphology

Measuring effects of Formulation composition on freeze-dried pellets is particularly complex because the choice and ratio of excipients directly influences the beads' morphological properties (19). These properties can be assessed in many ways, such as change in pore size, uniformity, mechanical strength - all those properties are critical for beads stability and performance. Additionally, use of different carbohydrates - such as sucrose and maltodextrin, can alter the glass transition temperature - directly having an influence on stability during storage (8). Not only formulation plays a pivotal role there though - when designing experiments, one needs to take into account many other factors, such as freezing rate and annealing time. Rapid freezing for example can promote the formation of small ice crystals, leading to more uniform pore structure and reducing the risk of mechanical damage towards biologically active components. The morphology of freeze-dried beads is influenced by many factors, like freezing rate, annealing time, temperature control, drying pressure and concentration of excipients. All of these factors interplay to determine the structural integrity, stability and performance of freeze-dried material (20).

1.2 Aims of the Study

The overall goal of this thesis is to conduct an analysis on how different approaches in design of freeze-drying formulations and bead sizes impact a bead structure, utilizing a freeze-drying protocol and one-week set annealing time.

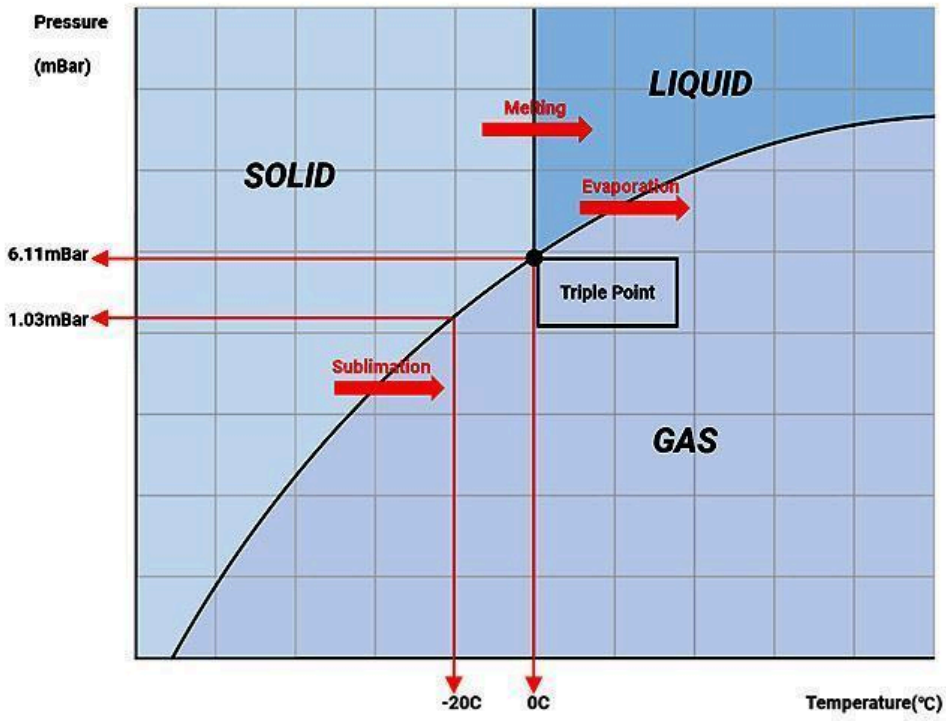
a) To Examine the Impact of Bead Size and Formulation on Structure of Beads:
   This aim concentrates on the hypothesis that differences in bead size dimensions and formulation can significantly affect the physical properties, such as structure and physical stability of beads after the freeze-drying process. This can have implications on pore sizes inside beads, directionality, and occurrence of collapse.
b) To Develop and Optimize Pelletization, Annealing and Lyophilization Protocols:
   By strategizing and designing bespoke pelletization experiments, including choice of syringe pump parameters and correct syringe size, and also optimizing annealing and lyophilization protocols, this research aims to use current knowledge in the field to investigate impact of carbohydrate formulations and dimensions of pellets on morphology of beads. These optimized conditions are expected to mitigate the drawbacks and common problems occurring during several steps of preparing freeze-dried material, such as structural disruptions.
c) To Put to Use Advanced Analytical Techniques for Structural Analysis:
   Employing different techniques such as embedding in silica elastomer, light microscopy, and differential scanning calorimetry (DSC) will allow for a detailed examination of the morphological and thermal properties of freeze-dried beads. This comprehensive analysis will help establish a clearer understanding of the relationship between processing conditions and the structural outcomes of freeze-dried materials.

By achieving these aims, this thesis will contribute valuable insights into the design of more effective freeze-drying processes, ultimately enhancing the preservation practices and extending the viability of sensitive biological materials used in various industries

2. Theoretical Background and Literature Review

2.1 Principles of Freeze Drying

The Freeze drying process can be described by three crucial stages: Freezing, sublimation (primary drying) and desorption (secondary drying). The science behind freeze drying can be better understood with the help of the triple point diagram of water:



F*igure 2: Triple point diagram of water illustrating phase transitions* (21).

The triple point of water occurs at a specific temperature and pressure - 0,01°C and 611,657 Pa, to be exact. This is where water can coexist in three phases: solid, liquid and vapor. The freeze drying process leverages this principle by lowering the temperature and pressure of the system to fall below the triple points, ensuring ice transition directly from solid phase to the vapor phase, without passing through the liquid phase.

Freezing Stage

In the first step, the desired product is cooled down at desired rate, which causes water to solidify. Cooling rate must be optimized based on the nature of the product - rapid freezing leads to smaller ice crystals, which may hinder the sublimation process, while slow freezing favors larger crystals and more porous structure (23). Then, freezing stage is followed by primary drying, resulting in gradually increased temperature under vacuum conditions. Those conditions allow ice to sublime into vapor.

It is important to consider various properties, such as cooling rate, as it is directly impacting freeze-dried products (20). To facilitate better sublimation, it is beneficial to consider cooling rate. Rapid freezing promotes formation of small ice crystals, however these crystals will grow during annealing process through a process called Ostwald ripening. These larger crystals facilitate better sublimation by creating larger pores, which enhance mass transport and improve overall efficiency of the drying process. (24).

Another aspect to take into consideration in that stage of freeze drying is Nucleation. It is a critical phase transition phenomenon and it applies to formation of initial ice crystals from water, then the product is cooled below freezing point. This process is very important, as it directly impacts distribution of ice crystals, which has a direct implication on structural integrity and quality of freeze-dried product. Nucleation can be either Homogeneous (occurs uniformly throughout supercooled liquid) or it can be Heterogeneous (Initiated at specific sites within the liquid) (25).

As the water freezes, solutes are excluded from the ice phase. This increases solute concentration in unfrozen regions. It is important to consider Glass Transition (Tg) when designing outcomes for this phase, as it is the temperature at which the amorphous phase (unfrozen solute) transitions to glassy state (26). This is of utmost importance when one is trying to understand stability of the product during the freezing step and following stages (27).

Annealing Stage

Annealing is a process involving holding a designed formulation at the temperature just below its melting point for a specific period, but above its glass transition temperature (Tg) for a specific, predetermined period, allowing for ice crystal formation (22). Annealing enhances uniformity and size of ice crystals.

Ostwald Ripening is a phenomenon that occurs during the annealing process, where smaller ice crystals dissolve and redeposit onto larger crystals (23). This process results in slow production of larger ice crystals. Larger ice crystals can be beneficial, as they improve

the speed of the freeze-drying process, facilitating a big vapor pathway that is left in the dried area of the product, as ice crystals sublimate (23).

Sublimation Stage (Primary Drying)

Core objective in this step is removal of bulk water in the product by utilizing a sublimation phenomenon. Pressure is decreased to below vapor pressure of ice, causing the sublimation. It transitions from the solid, to gaseous aggregate state (28).

There needs to be a strict temperature controlled environment, as heat is delivered from outside and product temperature must stay below destruction of products skeleton (collapse) temperature. Raising the temperature above the collapse temperature would make the product lose its integrity. It is important to consider drying kinetics in this step. Freeze-drying can be described as a mass exchange process that is relying on heat transport. Mass transfer is crucial - Vaporized water has to be optimally removed from the drying chamber, otherwise It would cause re-condensation (30). This requires a strategically planned vacuum system. Direct removal of water from sublimated material results in highly porous freeze-dried material (31).

Primary drying is considered complete when there is no more ice left in the product. This can be determined through several indicators, such as chamber pressure stabilization - pressure in the drying chamber stabilizes and no longer decreases, indicating that sublimation is complete (30). Another way of determining completion of this step is product temperature plateau - during sublimation the product temperature remains below the shelf temperature due to the cooling effect of sublimation - when sublimation is complete, the product temperature will rise to match shelf temperature.

Desorption Stage (Secondary Drying)

This stage is utilized to remove unfrozen water molecules that were bound to the product. Temperature is usually slowly increased in a low pressure environment to desorb the bound water molecules. It effectively reduces residual moisture for a product, providing it with much better stability during storage (32). In the amorphous syst|em water diffuses to the

surface from a glassy state. It is a long procedure that has a strong impact on the speed of a freeze-drying process.

It is crucial to have in mind the kinetics involved in the desorption process. Exceeding Glass transition (Tg) in this step would lead to unwanted structural changes and collapse of the dried matrix.

For designing freeze-drying processes it is strategically beneficial to use tools like DSC (Differential Scanning Calorimetry) beforehand, pinpointing a glass transition temperature of maximally concentrated solution (Tg'). Those parameters can be used to ensure that freeze-dried products' integrity is still intact.

2.2 Importance of formulations in freeze drying

Excipients Overview

The choice of excipients (bulking agents, cryoprotectants, lyoprotectants, stabilizers) is of utmost importance. Different properties of carbohydrates can be strategically utilized in the freeze drying process for the favor of the wanted product. Especially in the context of biopharmaceuticals on other sensitive materials, choice of Excipients and their concentrations directly impacts the success rate of the freeze drying process (19).

Stabilizers

Stabilizers' role is to protect proteins and other sensitive molecules from physical instability during the freeze-drying process. Stabilizing molecules prevent freeze-dried cake from physical instability (aggregation, structure collapse) and from chemical instability (oxidation prevention, deamidation and isomerization prevention) (34). There are plenty of protective processes involved, one of the most important ones named vitrification; immobilization of proteins in an amorphous matrix and water replacement (hydrogen bond formation with proteins to maintain structure) (35). Stabilizers can also prevent unwanted processes that can occur during lyophilization, such as aggregation. There are many types of stabilizers; the most important ones used are carbohydrates - namely Sucrose and Trehalose (36). Those disaccharides are commonly used due to their high efficiency in stabilizing proteins through vitrification and water replacement phenomenon. Other stabilizers, such as

polymers (Polyethylene Glycol , Polyvinylpyrrolidone) can enhance Tg' and influence phase separation (37), (38).

Bulking Agents

Bulking agents are components in freeze-drying formulations that serve multiple purposes and are very essential to provide enough stability to the structural stability of freeze-dried cake. In the cake formation, bulking agents provide necessary bulk to form porous and structurally sound cake (39). This is essential for mechanical stability, as bulking agents such as Glycine work against collapse or shrinkage of the cake (40). Contribution to the formation of appropriate pore structure facilitates efficient sublimation of the ice during the primary drying phase. Larger and uniform pores are prone to significantly reducing the drying time. Bulking agents can provide necessary support under aggressive conditions in the primary drying step, where temperatures might be higher. Bulking agents, such as dextrans and Ficoll can help to maintain cake structure even above Tg' or collapse temperature (Tc) (41). For synergistic effect, bulking agents can be used together in various ratios and concentrations - their ability to provide structural support, enhance sublimation, prevent collapse makes them a key component in achieving high quality lyophilized cake.

Buffers

Buffers are fundamental Excipients used in the lyophilization process. Their role is to maintain pH of the formulation within a range that ensures activity of active ingredients (42). Buffers also prevent large pH changes that could cause unwanted changes to freeze-dried cakes active ingredients. Popular buffers used for freeze drying are Glycine and Histidine buffers as they provide an exceptional pH control and are compatible with many proteins and carbohydrates. They also do not express a pH shift when buffer salts are crystallized and when the solution freezes. Buffers' ability to maintain a stable ionic environment should also be considered when the active component is an enzyme, as it is important for protein stability (43).

Choice of Maltodextrin and Sucrose for Freeze-drying Formulation in a study

Using a combination of maltodextrin and sucrose in different ratios, it is possible to study the impact of carbohydrate compositions on structure of freeze-dried pellets (44). Each carbohydrate leverages their unique properties to the formulation that can influence morphology and physical characteristics of the final product. In this case,

Maltodextrin serves as an effective bulking agent and is adding volume to the formulation (45). By leveraging its high molecular weight, it also aids with increasing the glass transition temperature (Tg), helping with preventing collapse during sublimation (46).

Sucrose acts as a cryoprotectant and a stabilizing agent (47). It promotes growth of smaller crystals by reducing the amount of freezable water and depressing the freezing points. Sucrose forms hydrogen bonds with proteins and active biomolecules, stabilizing their structure by replacing the water molecules that are removed during drying. Combination of sucrose and maltodextrin can thus serve as an interesting base for research, as each of them can leverage their unique benefits.

Conclusion

When it comes to Excipients, there are different properties that one can take advantage of, depending on the desired outcome. Cryoprotectants like trehalose, sucrose and glycerol help protect biological structures during the first step - freezing (12). Ice crystal formation can be controlled by using those Excipients, as (12). During the drying phase, those agents help form a protective matrix around active pharmaceutical ingredients, such as enzymes - preserving their function.

Carbohydrates in formulations are commonly mixed together to create synergistic effects (48). As an example, Sucrose and Trehalose can be used together as they provide an excellent stabilization by replacing water molecules and forming hydrogen bonds with proteins. Unwanted aggregation and denaturation of protein can thus be completely eliminated.

Maltodextrin is another used carbohydrate in pharmaceutical formulations for freeze drying. This polysaccharide increases both the glass transition temperature of maximally concentrated solution (Tg') and glass transition temperature (Tg) (8). Utilizing different Excipients in formulations can leverage their abilities and cause an improvement of

uniformity and quality of final product, especially for more complex biopharmaceuticals (35). In addition, Maltodextrin is a bulking agent - it provides volume to the formulation and can improve solubility and reconstitution properties of freeze-dried products (49).

2.3 Overview of important techniques for Freeze-dried Cake morphology assessment

Inspection of freeze-dried cakes and beads can be quite a challenging task due to their distinct qualities, such as small pores and brittle structure. There are various analytical methods in place to provide an accurate image of freeze-dried material structure.

Scanning Electron Microscopy (SEM)

The principle of using SEM involves a focused beam of electrons that generates images of the sample surface. Electrons interact with samples to produce a range of secondary electrons, which are then collected to form an image (50).

It is very beneficial to use SEM when inspecting a freeze-dried material, as it is used to visualize its small-scale pore structure, as it is very highly effective for visualizing surface morphology. It can be used to visualize porosity, texture and other structural features both at micro and nanoscale. To better understand rehydration characteristics and stability of freeze-dried cake, SEM uncovers the shape, size and distribution of the pores within it (51). SEM has very high resolution, and allows one to look into Freeze-dried product structural integrity - uncovering issues such as collapse and irregularities that could affect performance.

SEM could be proven indispensable when comparing the effects of different formulations of freeze-dried beads. Different amounts of both cryoprotectants and lyoprotectants can be analyzed to easily determine which formulation provides best structural stability and uniform pore distribution.One of the benefits of using SEM compared to light microscopy is that it provides way greater depth of field compared to optical microscopy (52). On the other hand, when it comes to limitations, SEM equipment is very expensive both to use and maintain and technique requires skilled operators; those factors can deem this method inaccessible and inconvenient to use.

Light Microscopy

Light microscopy uses visible light and lenses to magnify freeze-dried samples, providing both surface and internal structure of cut beads.

Its main application is to initially screen for samples to detect for visible defects and inconsistencies. Depending on freeze-dried cake properties, It could be used to assess overall morphology and structure, but when it comes to pore and surface visualization, it could only be used for this purpose provided pores are big enough or there are staining techniques in place, but its resolution is nowhere near of SEM. Main advantage of this technique is that it is very accessible and fairly cost-effective. It also does not impact samples as much as SEM, so it is more suitable for analyzing sensitive samples. Its main limitations are lower resolution and limited depth of field - which makes it very hard or impossible to view thicker samples in detail.

Equipping a light microscope with polarizing filters could also be very helpful, as it can enhance the contrast of crystalline or fibrous structures within the freeze-dried cake. By rotating the sample, it can allow observers to observe changes in birefringence, which can provide insights into the orientation and distribution of certain components inside the sample.

Benefits of Embedding for Microscopy

It is beneficial to embed freeze-dried cake in silicone or paraffin polymer before inspecting it under the microscope. Freeze-dried cakes are extremely fragile and hygroscopic, meaning they can easily absorb moisture from the air and degrade. Embedding them in a supportive matrix such as silicone elastomer preserves their structure and prevents rapid degradation.

Lyophilized cakes common problem concerns a very fragile, porous material which makes it impossible to make thin slices needed in order to make adequate samples for light microscopy. Handling fragile, non-embedded freeze-dried material poses challenges, primarily because these materials are highly susceptible to morphological changes and environmental factors during handling. This can significantly impact accuracy and reliability of analyses performed using light microscopy. Not thin enough slices under light microscope result in low quality images, making it difficult to observe and photograph pellet

morphology. By embedding the cakes, one can section the material to reveal internal structures and obtain a higher quality image without use of sophisticated material (53). Thus, embedding allows for a detailed cross-sectional imaging that highlights internal structures, providing valuable insights into the lyophilization process and its outcomes (54).

DSC (Differential Scanning Calorimetry)

Differential Scanning Calorimetry is an analytical tool that uncovers thermal properties of different materials. When it comes to freeze-dried biopharmaceutical products, DSC is utilized to analyze phase transitions - crystallization, melting, glass transition. Information derived from phase transitions can be used to better understand how formulation affects efficacy, shelf- life and stability. This method usually requires a small amount of formulation (typically 1-10mg) and an empty pan used as a reference. Data assessment (resulting thermogram) is then analyzed in software to identify thermal transitions and determine Tg, melting, crystallization and sublimation events. Enthalpy changes associated with these transitions are quantified.

Heat flow that is associated with phase transition of formulation is measured during the process of heating or cooling. By comparing it to a known reference, DSC detects heat absorbing (endothermic) or heat releasing (exothermic) events.

DSC can accurately measure glass transition temperature (Tg) of chosen formulation for freeze drying, helping to optimize cycle and storage conditions to ensure that the final product remains in a stable glassy state. This is also true for Tg' - glass transition temperature of maximally freeze-concentrated solution. The graph obtained provides operators an insight of thermal behavior of the sample. During a glass transition, a change in heat capacity can be observed on a graph as a change in DSC heat flow curve. This method can also help understand crystallization and melting behavior or Excipients in formulation, as it measures exothermic crystallization and endothermic melting peaks.

Fluorescent Microscopy

Fluorescent microscopy is founded on fluorescent molecules excitation by specific wavelengths of light. Fluorophores (fluorescent molecules) absorb light at specific wavelengths and then emit it at longer wavelengths. Emitted light can be captured and then used to acquire a highly detailed image of a labeled component.

Fluorescent microscopy can be a viable technique used for inspection of a freeze-dried cake morphology. The principle of the method is that it uses a fluorescent dye or proteins to label a specific morphology component of the inspected sample. It can be particularly proven useful for inspecting internal structures of freeze-dried pellets, where detailed visualization of structural integrity is paramount.

Fluorescent microscopy requires skillful consideration of a marker and then preparation of protocol that allows for a marker to stain a structure one is interested to visualize. Thin sections of freeze-dried cake might be needed for a better penetration of a fluorescent dye and imaging. Freeze-dried cake can be beforehand embedded in silicon elastomer or similar material in order to make preparation of thin slices for microscopy easier.

3. Material and Methods

3.1 Materials

Experiments that took place from January 2024 to May 2024 were conducted at the Department of Process and Life Science Engineering, Division Of Food And Pharma.
Carbohydrates used in formulations that were further used to develop pellets are Sucrose that was obtained from Sigma-Aldrich, Glucidex 12D that was obtained from Roquette / Barentz. Chemicals for embedding were Elastosil Vario 15 and Elastosil Vario 15 Activator bought from Biesterfeld Sweden AB.

3.2 Preparation of formulation

To ensure that formulations are always created with the highest precision, an SOP (Standard Operating Procedure) was created for formulation preparation. Formulations were prepared on 400M precisa Samo Tronic analytical scale. Solutions of different formulations

were achieved by dissolving Sucrose and Maltodextrin with MilliQ water to achieve different concentrations and ratios that were previously predetermined.

To examine the impact of varying carbohydrate ratios on the structural integrity of freeze-dried beads, an in-depth investigation was planned using three different Sucrose - Maltodextrin ratios. These ratios of Sucrose:Maltodextrin (75:25, 65:35, 1:1) were carefully selected to determine how the proportional differences between the two carbohydrates affect the final product's characteristics. To further enhance the scope of the study, each ratio was tested at three different concentrations: 20%, 30%, and 40%. This comprehensive approach allows for a detailed assessment of how both the ratio and concentration of sucrose and maltodextrin influence the structural properties, stability, and overall quality of the freeze-dried beads.

Table 1: Table of Sucrose-Maltodextrin Formulations

Concentration (w/w)	Sucrose:Maltodextrin Ratio	Formulation details
40%	75:25	30% sucrose, 10% maltodextrin
30%	75:25	22.5% sucrose, 7.5% maltodextrin
20%	75:25	15% sucrose, 5% maltodextrin
40%	65:35	26% sucrose, 14% maltodextrin
30%	65:35	19.5% sucrose, 10.5% maltodextrin
20%	65:35	13% sucrose, 7% maltodextrin
40%	50:50	20% sucrose, 20% maltodextrin
30%	50:50	15% sucrose, 15% maltodextrin
20%	50:50	10% sucrose, 10% maltodextrin

Sucrose, a non-reducing disaccharide, was selected for its exceptional ability to stabilize sensitive biomolecules such as enzymes during the freeze-drying process. It functions by replacing water molecules around the enzyme structures, thereby preserving their activity and preventing denaturation during drying. Additionally, sucrose contributes to

the stabilization of protein configurations, preventing aggregation that can occur in the absence of water (55).

Maltodextrin was included as a stabilizer and bulking agent in the formulations to enhance the glass transition temperature (Tg) in the end product. This improves the long-term stability of the freeze-dried product by maintaining it in an amorphous state which is crucial for pharmaceutical applications where consistent dosage and efficacy are paramount (8). The increased Tg ensures that the product remains stable under a range of storage conditions, thereby extending shelf life and maintaining the biological activity of enzymes or pharmaceutical agents. Usage of maltodextrin is important for increasing the Tg of excipient mixture and as a bulking agent, providing necessary volume to the freeze-dried product ends in creation of a porous, structurally sound freeze-dried cake.

Together, the combination of sucrose and maltodextrin in the formulations optimizes both the immediate and long-term stability of bioactive compounds that could be used with this formulation, making it particularly suitable for enzyme storage and pharmaceutical uses where product integrity and efficacy are critical.

3.3 Adjusting syringe pump parameters

To achieve precise control over the droplet size and spacing during the freezing process, the syringe pump settings were optimized through experimentation in a trial and error fashion. Parameters such as flow rate and volume per cycle and size of syringe were finely adjusted to produce uniform droplets aimed at creating consistent droplet sizes across samples. Droplets were measured by using an iPhone 13 Pro Max camera with a slow-mo setting. Two target droplet sizes, large (approximately 5 mm) and small (approximately 2.5 mm), were specifically chosen to explore the influence of droplet size on the structural integrity and drying efficiency of the beads.

Despite optimization of the syringe pump settings to achieve precise control over droplet size and spacing, the initial trials resulted in a mixed distribution of pellet sizes. However, through iterative adjustments to the parameters, It was possible to significantly increase the yield of the desired droplet sizes per adjustment. By tuning the flow rate and volume per cycle, It was possible to minimize variability and produce a higher number of pellets within

the desired size range. This iterative approach enhanced the uniformity and consistency of the final product.

Moreover, with the increased yield of the desired droplet sizes per adjustment, it became notably easier to screen for both small and large pellets, streamlining the quality control process and enhancing the overall efficiency of the experimental procedure.

Figure 3: Droplet measuring technique (aiming for large size, 5mm droplet)

Table 2: Measured pump speed (green highlighted Flow Rates were used for st

L.P Experiment	Solution (30%)	Flow Rate (ml/h)	Approximate Droplets size ±0,1(mm)
1.	1:1 Sucrose Maltodextrin	100	5
2.	1:1 Sucrose Maltodextrin	150	4
3.	1:1 Sucrose Maltodextrin	175	4
4.	1:1 Sucrose Maltodextrin	250	3
5.	75:25 Sucrose Maltodextrin	100	3.5
6.	75:25 Sucrose Maltodextrin	150	3
7.	75:25 Sucrose Maltodextrin	200	3
8.	75:25 Sucrose Maltodextrin	250	2.5

The study used a 50mL sterile Henke-JECT syringe as it is the most convenient size to create the desired amount of material to analyze. Intermediate solution concentration (30%) and ratios were strategically chosen to bring the most accurate results for every formulation used in this experiment. This was critical to understanding the mechanics of fluid dynamics within the context of syringe-based dispensing. It was observed that by increasing the pump speed, the droplets formed were smaller. Conversely, a slower dispensing speed resulted in larger droplets. This phenomenon can be attributed to the shear forces acting on the liquid as it exits the tip of the syringe; at higher speeds, the fluid experiences greater shear, causing it to break into smaller droplets.

Current knowledge in the field supports these findings, indicating that variations in syringe and tip sizes indeed play a pivotal role in droplet size control during syringe-based dispensing processes (56).

Through this experimentation, it became evident that the choice of syringe speed is important in tailoring droplet size.

After those experiments, Syringe Pump flow rates that are highlighted in green color in Table 2. were used to create two bead sizes - small 2.5 and large 5mm.

3.4 Pelletization system

A pelletization system that consists of interchangeable syringe, syringe pump, cryo bucket with liquid nitrogen and container with dry ice and a sieve was specifically designed to produce uniform droplets into a liquid nitrogen bath, creating pellets of two sizes. To control droplet formation and maintain its uniformity, several parameters have to be studied and optimized in order to conduct this process flawlessly. Formulation viscosity of the formulation affects droplet formation and size - too low or high viscosity can result in irregular droplet sizes, and it has to be taken into consideration when designing a pelletization system. The rate at which the droplets are formed by the pelletization system needs to be strictly controlled, to avoid aggregation and uneven freezing. Consistent pressure created by the syringe pump maintains a steady droplet formation rate. Pellets were created by dropping them from a syringe pump located on a table to a cryobucket with liquid nitrogen. Usage of a sieve that fits into a cryobucket allows for efficient collection of frozen pellets, while minimizing exposure to ambient temperatures. It is important to precool aluminum cups on dry ice before transferring the pellets to avoid any partial thawing that would result in pellet morphology changes. Storage on dry ice is temporary and allows pellets to be transported to the annealing freezer as soon as possible.

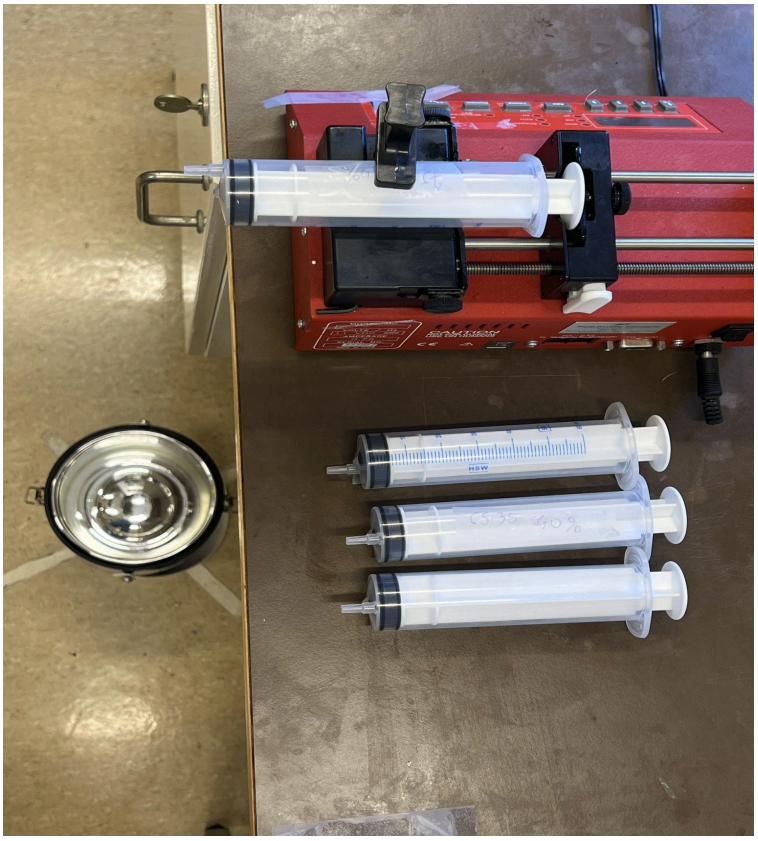


Figure 4: Pelletization system setup

3.5 Production of Beads by Quench-Freezing Methods

Quench-freezing was the method used for production of the beads. This method involved immediate immersion of droplets of the formulation into liquid nitrogen approx temp. $-196\ ^{\circ}C$, resulting in an extremely fast cooling rate and formation of small, uniform ice crystals within pellets. There are several key aspects of this method that need to be considered.

Impact of Rapid Cooling Rate

Rapid cooling results in non-equilibrium freezing, so water from the freeze concentrate does not have time to diffuse to ice crystals before temperature drops below its glass transition temperature (Tg) - This causes formation of small ice crystals and lower Tg compared to a maximally freeze-concentrated solution (20).

Collection Method

Directly after dripping down formulation from the syringe into liquid nitrogen and letting it quench freeze, produced pellets were fished out by a sieve placed in the Cryo Bucket. Fished out pellets were placed in marked cups and put on the tray with dry ice to maintain low temperature. After production of all of the formulations, cups with produced pellets were transported to the freezer at -18°C for the annealing process.

Usage of Needles/ Tubing

Experiments with syringe pumps have shown that usage of tubing or needles is not needed, as achieving target size was harder when trying to implement them. It can be attributed to potential shear stress that liquid was experiencing as it passed through narrow confines of a needle. Using tubing / needles would also introduce risk of blockages, especially when working with viscous and sticky carbohydrates.

3.6 Determination of Tg' for formulation used

Tg' (glass transition of maximally freeze concentrated solution) was analyzed using a Differential Scanning Calorimeter model 6200 from Seiko available at Department of Process and Life Science Engineering. Before the measurements, calibration was performed using Indium. As a reference for both sucrose:maltodextrin measurements an empty aluminum pan was used. The evaluated samples were run in duplicates of 5mg sample solutions that contained 75:25, 65:35, 1:1 10% (w/w). Each carbohydrate ratio is measured separately. Cooling rate of 5°C/min was applied with starting temperature at 20°C and cooled down to -70°C. Using computer and SII EXSTAR 6000 Muse software available at the departament, Tg' was determined and used to decide temperature for the Annealing step.

Temperature for annealing was selected to be above Tg' for all formulations for the process of Ostwald ripening to take place - smaller ice crystals will dissolve and redeposit into larger ones and create larger pores upon sublimation, improving drying efficiency.

Figure 5: Differential Scanning Calorimeter Model 6200 from SEIKO Available at Division of Food and Pharma

3.7 Annealing and Freeze drying of the Beads

The quench-frozen beads were annealed by subjecting them to a controlled temperature, set at -18°C. After one week of annealing time, samples were put on dry ice and transported for the freeze-drying. Martin CHRIST Epsilon freeze-dryer was used for the next stage of processing. In the freeze-drying process, the beads are exposed to carefully controlled conditions of pressure and temperature. The conditions for freeze drying were as follows:

Primary Drying	**Secondary Drying**
Precooled shelf temp: -45°C Shelf Temperature holding: -45°C Pressure: 7,2 Pa Holding: 12 hours	Shelf Temperature 20°C Heating rate: 0.1°C / minute Holding: 12 hours

Figure 6: Martin Christ Epsilon Freeze-dryer available at Division of Food and Pharma

Storage of Dried Product

As lyophilized products are very hygroscopic, obtained pellets were placed in an air-tight desiccator to prevent rehydration from atmospheric humidity. After embedding in Elastosil Vario 15 samples were stored outside the desiccator.

3.8 Embedding Freeze-dried beads in silicone elastomer for further analysis

Freeze-dried cake has been embedded in a hydrophobic polymer in order to immobilize and then make it possible to cut very thin slices of freeze-dried cake. Embedding also allowed freeze-dried cake to be stained and improved the visualization process. (53). Method used involved encapsulation of freeze-dried pellets in silicone elastomer (Elastosil Vario 15) that has been mixed with activator in ratio of 9:1 in order to let the curing process start. Immediately after mixing pellets, Elastosil Vario 15, and Elastosil activator mixture was put into a vacuum chamber to process degassing - removing air bubbles. After all of the air bubbles were removed, samples were left to be cured at room temperature for 24 hours.

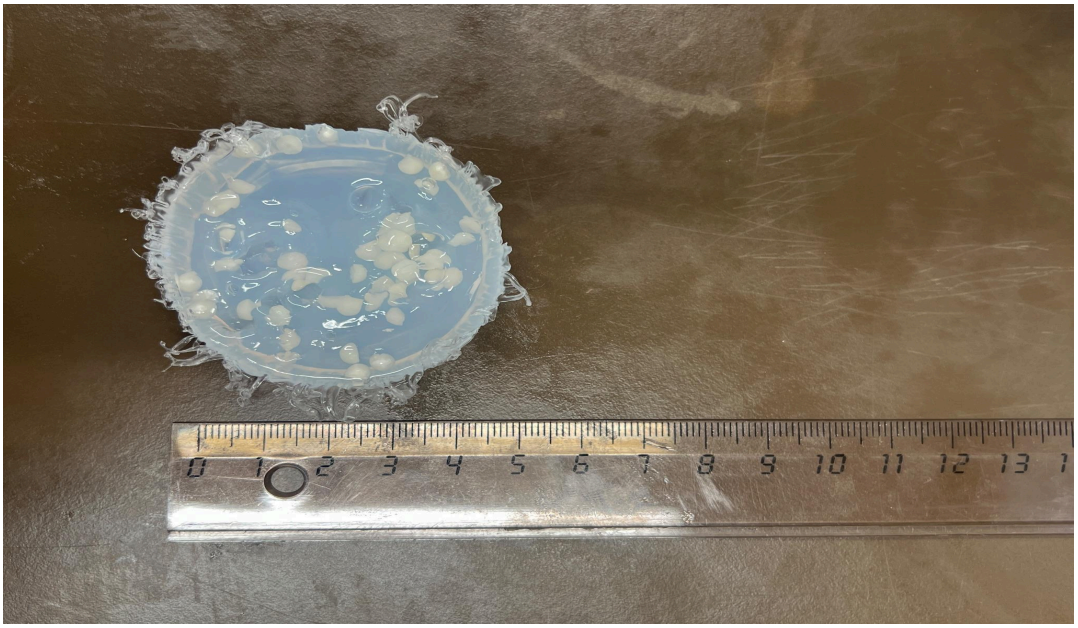


Figure 7: Embedded pellets in de-gassified Silicone Elastomer Vario 15

Samples submerged in Elastosil Vario 15 were placed in Vacuum chamber directly after mixing them with an activator. Degassing in vacuum involves removing trapped bubbles that were introduced by mixing activator and Elastosil. Once the samples were inside the chamber, Vacuum pump was activated, reducing the pressure.

According to Boyle's Law pressure and volume of a gas have an inverse relationship, when temperature is constant (57).

$$\mathbf{P_1V_1=P_2V_2}$$

Following this, the reduction in pressure in Vacuum chamber causes the air bubbles within the mixture to expand. This is because the volume of the gas bubbles increases as the pressure around them decreases. Expanded air bubbles become buoyant and rise to the surface of the mixture and as they reach the surface they burst, releasing trapped air into the vacuum chamber. Released air from the bubbles is removed by the vacuum pump, maintaining a low-pressure environment.

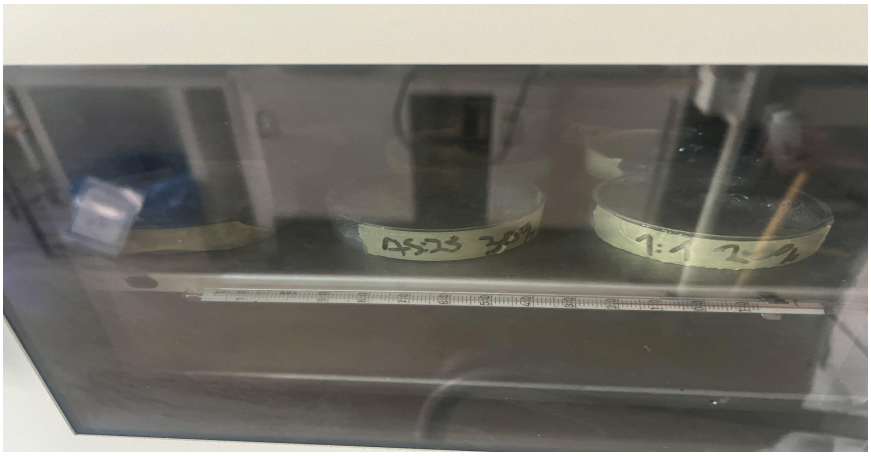


Figure 8: Degassing embedded samples

3.9 Light microscopy and Stereomicroscopy

Light Microscopy

Pellets porosity and morphology was examined by utilization of light microscopy. Light microscope used was Olympus System Microscope BX50, magnification x10. The samples were prepared by cutting a very thin slice by a scalpel, then were stained by Orange Marker sharpie. The scale was superimposed on the images in order to measure structures of the beads.

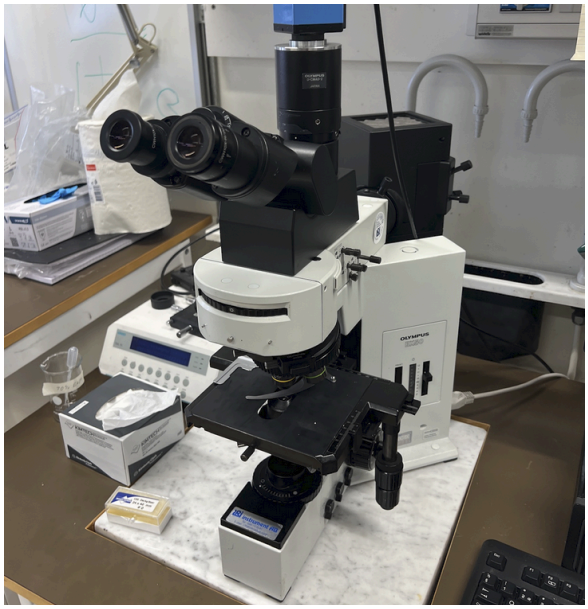


Figure 9: Light microscope available at Division of Food and Pharma

Stereo Microscopy

Pellets were inspected under stereomicroscope SZ-CTV with 60x eyepiece installed. As the light source, SCHOTT KL 1500 Electronic was used. Embedded pellets were cut horizontally and inspected under the microscope.

Figure 10: Stereo microscope available at Division of Food and Pharma with SCHOTT light source

4. Results

4.1 Production of Beads by Quench-Freezing Methods

Quench-freezing was the method used for production of the beads. This method involved immediate immersion of droplets of the formulation into liquid nitrogen approx temp. $-196\ ^{\circ}C$, resulting in an extremely fast cooling rate and formation of small, uniform ice crystals within pellets. There are several key aspects of this method that need to be considered.

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DSC Experiments and Annealing

Table 3: Tg' obtained from DSC experiments

Formulation (30%)	**Tg' (°C)**	**Tg' duplicate (°C)**	**Tg' avg (°C)**
75:25	-28,91	-29,01	-28,96
65:35	-21,91	-22,20	-22,05
1:1	-25,60	Failed attempt	N/A

Temperature obtained by DSC experiments was used to set annealing temperature for the pellets. Annealing was conducted at -18(°C) in the freezer. Directly after quench freezing pellet production, beads were transported on dry ice to the freezer.

Pellets were produced successfully using the Quench-freezing method in liquid nitrogen, one week annealing time and set lyophilization protocol. These pellets were further characterized and compared to each other observing their morphology pre-embedding and after embedding under light and stereomicroscope.

4.2 Morphology of the Beads (pre-embedding)

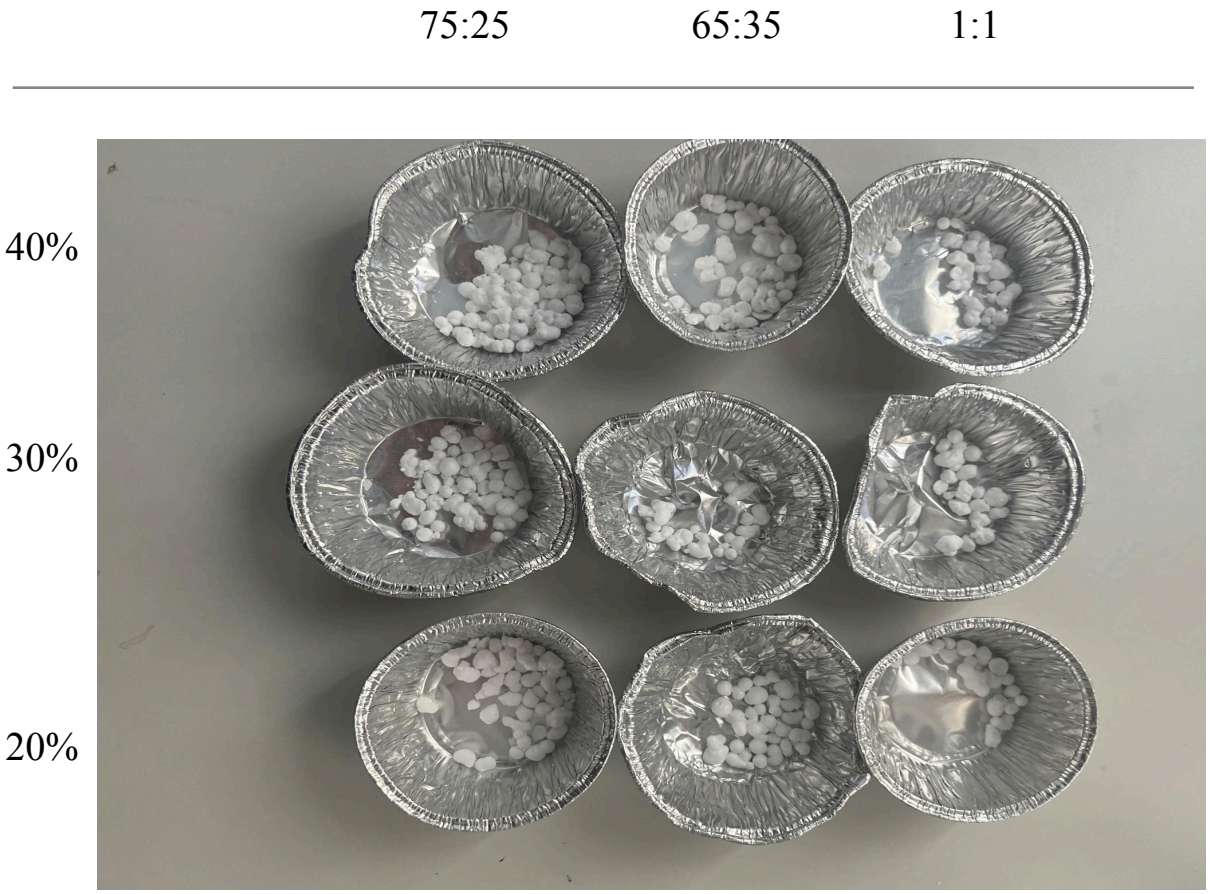


Figure 11: Prepared formulations of beads in aluminum cups (Large) after 2 weeks storage in desiccator

Morphology of Large Bead Size

Large pellets appear to be deformed and aggregated. The most uniform formulation of the Large Pellets is 1:1 Suc:Mal 30%, where the Majority of the pellets (19/30), 63%, were non aggregated and round. Overall, Large pellets produced showed bigger aggregation issues than small pellets during production (especially formulation 65:35 Suc:Mal, 30%) where almost all of the produced large pellets were aggregated, and were thus deformed and not round. During production of the beads, some of the pellets were aggregating into clumps when submerged in liquid nitrogen. It might be explained by the fact that Large pellets were produced first - freshly replenished liquid nitrogen tends to be more turbulent due to the boiling and rapid phase transition from liquid to gas. That was an oversight that was not caught until inspecting pellets post annealing. This turbulence caused the pellets to move

around more and collide with each other, leading to higher aggregation rate than in smaller pellets. Due to lower flow rates used for production of bigger beads and to counteract aggregation, a smaller amount of pellets was produced compared to Small target size. It was observed that some of the beads were observed to be ultra-small, with diameters below 2mm, notably in lower concentration (20%) formulations. This phenomenon can be explained by turbulence caused by freshly replenished liquid nitrogen and rapid boiling could possibly fragment droplets into smaller ones, resulting in beads being much smaller than intended size. Those ultra small beads were screened for and removed from the Large pellet batch.

Figure 12: Prepared formulations of beads in aluminum cups (Small) after 2 weeks storage in desiccator

Morphology of Small Bead size

Smaller beads overall appear to be way more uniform and round, non aggregated into clumps when compared to Large target size. They appear to be intact, with no cracks visibly seen and their very porous nature is not visibly seen by the naked eye. Small sizes were produced when Liquid Nitrogen in cryobucket appeared to be less turbulent, and this reduced turbulence could explain smaller amounts of collisions between the beads, leading to less aggregation. Before Embedding in Silica Elastomer, produced pellets were measured. Clumped and non clumped pellets were screened to match target sizes (2,5 mm and 5mm) and embedded. It is important to note that from the outside, these pellets look pure white and exhibit slightly porous “skin” on the surface.

Embedded Beads

75:25 65:35 1:1

40%

30%

20%

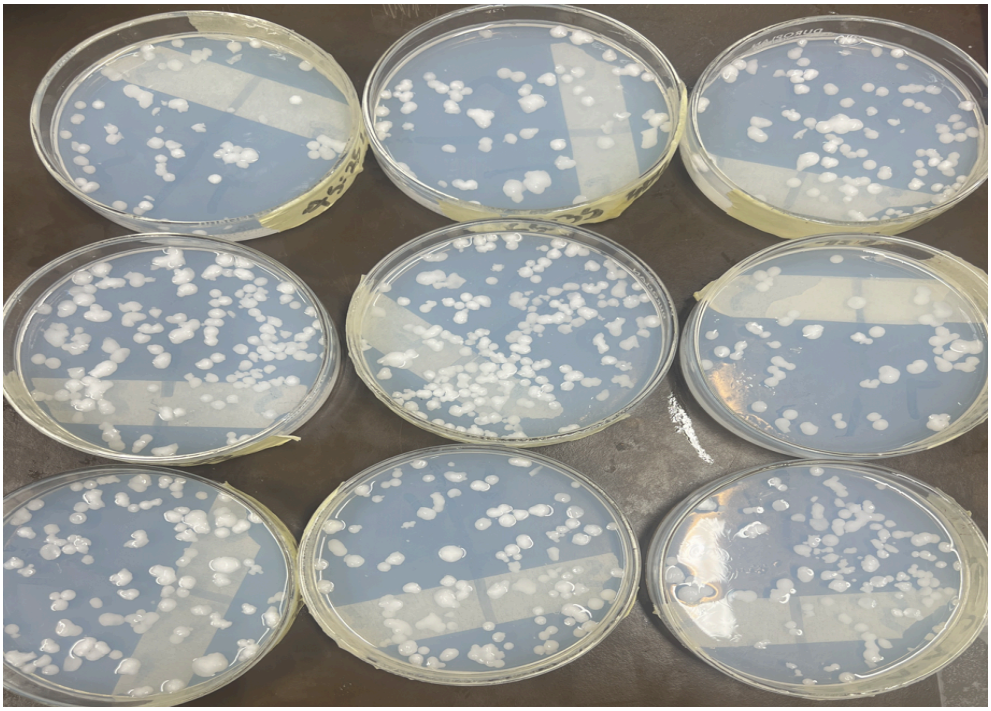


Figure 13: Prepared embedded samples for inspection under light microscope with camera and stereoscopic microscope. Small pellets were always on the left side of the Petri dish, when the tape on the back was aligned horizontally and in the bottom part of the Petri dish.

Impact of Silica Elastomer on Morphology and Experiments (with previous embedding technique)

The use of silica elastomer was successful and did not visibly alter the structure of pellets, which was a good sign after letting it cure for 24 hours. Silica created a hydrophobic, elastic matrix that penetrated the porous structure of the pellets, supporting the pellets without altering their structure. Previous experiments using Optimal Cutting Temperature (OCT compound) and Cryostat to achieve very thin slices for microscopy resulted in the dissolution of the pellets. This was likely due to the OCT compound's properties, which are not compatible with carbohydrates used in a study.

4.3 Stereo Microscope Pellet Analysis

Table 4: 40% Formulations of Small and Large beads

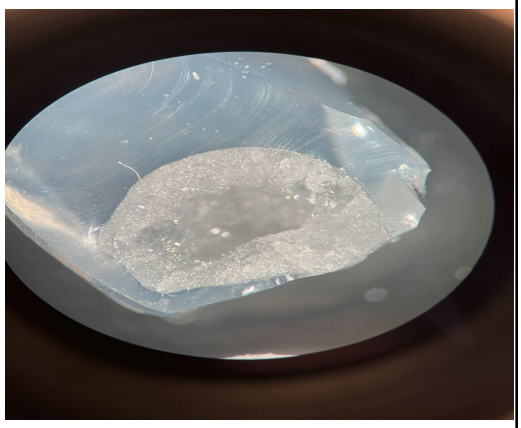
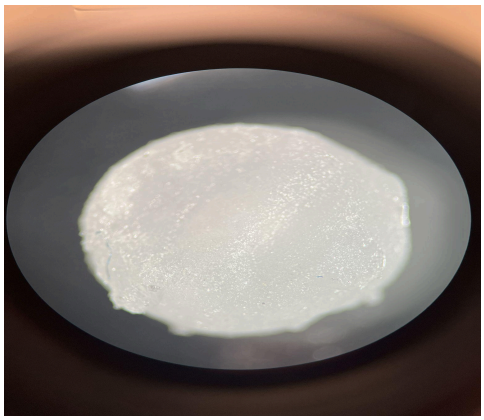
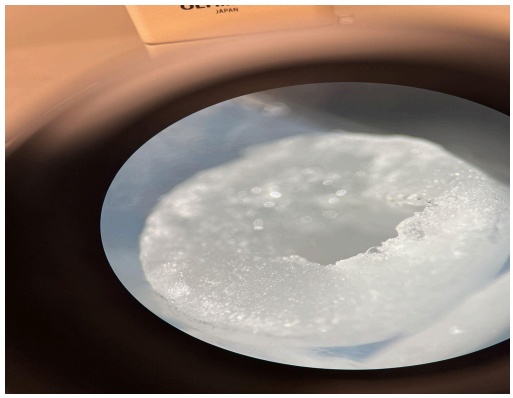
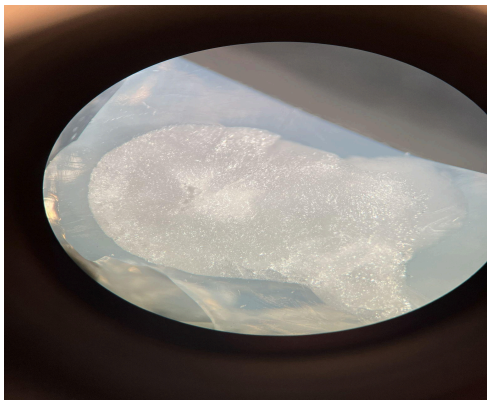
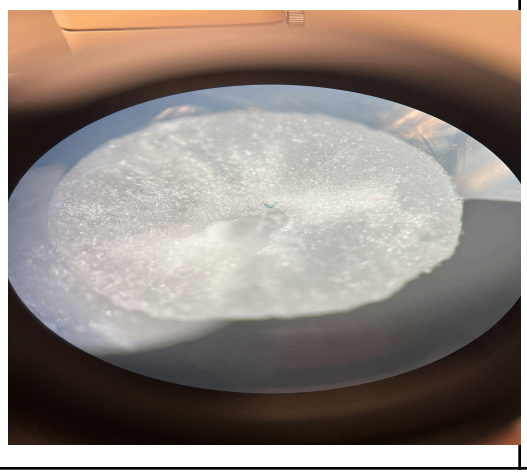
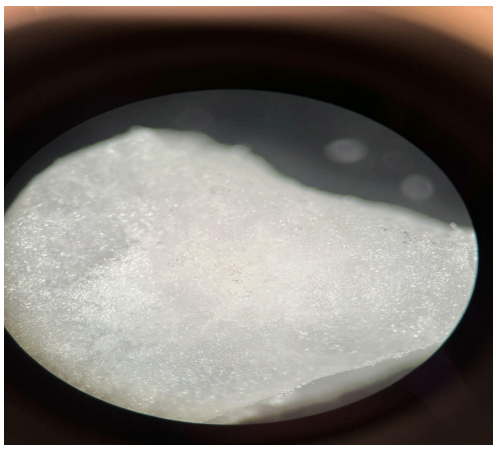
40% formulations	Small	Large
75:25		
65:35		
1:1		

Table 5: 30% Formulations of Small and Large beads

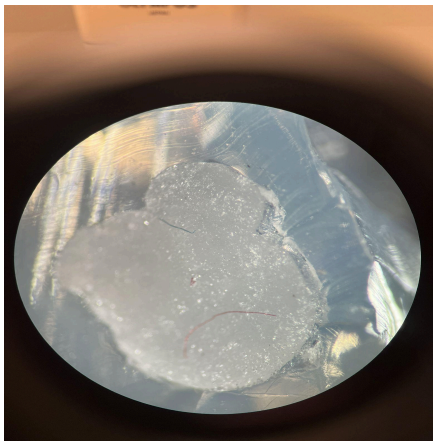
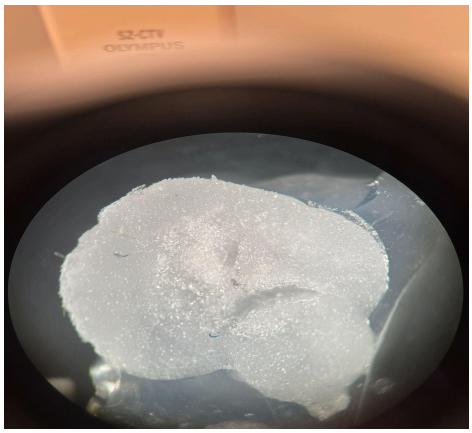
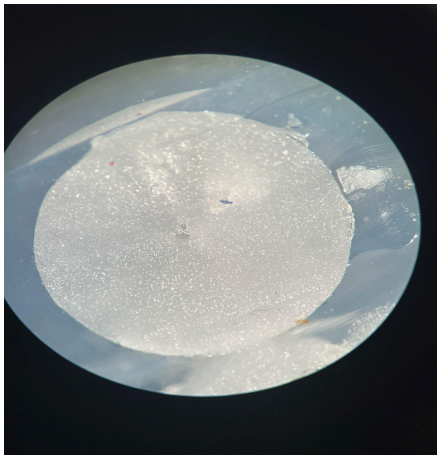
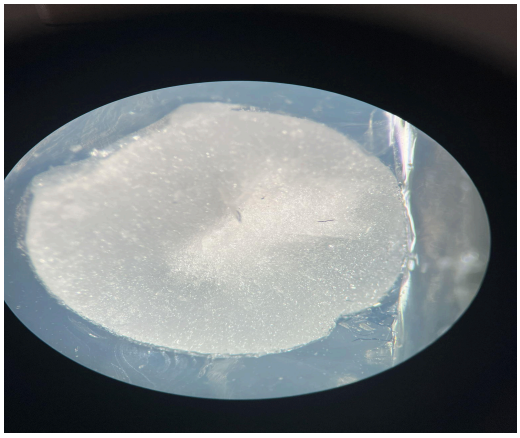
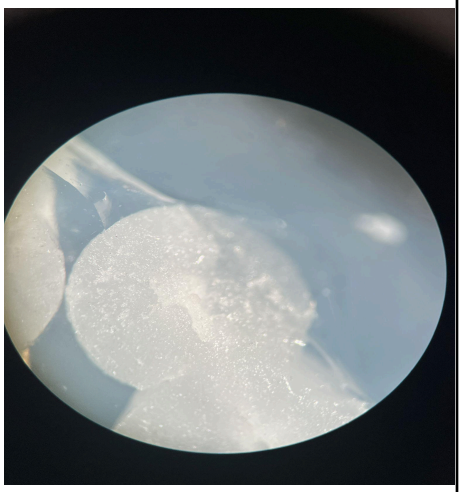
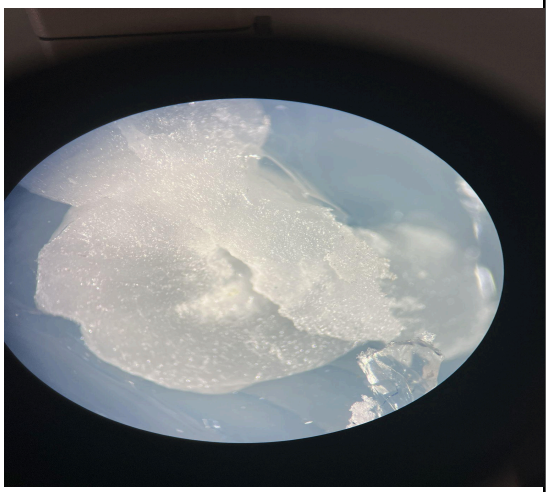
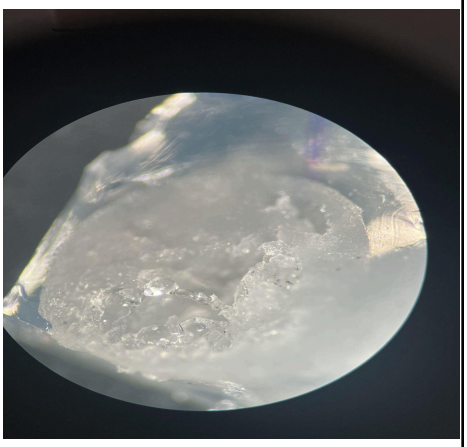
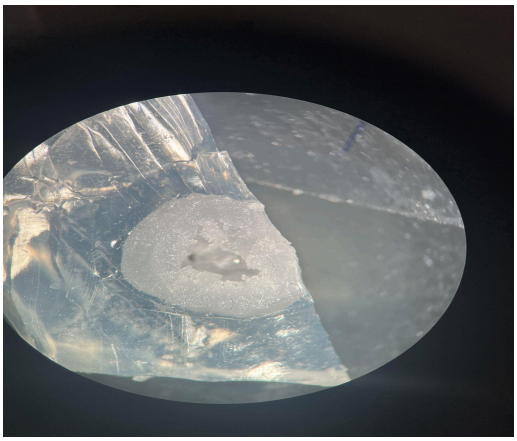
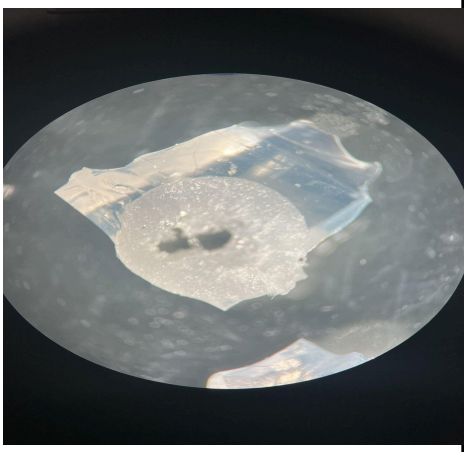
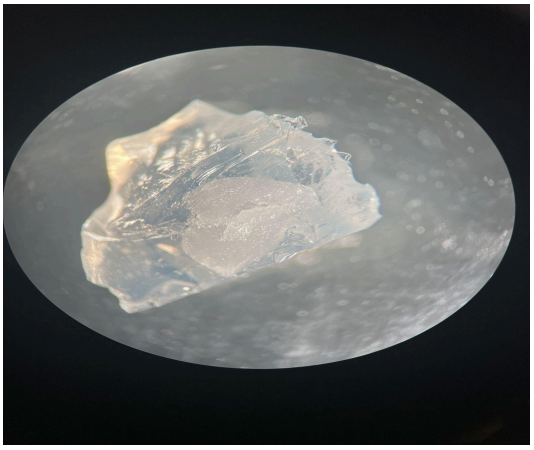
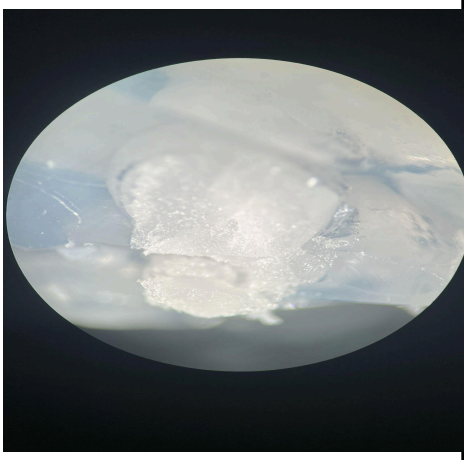
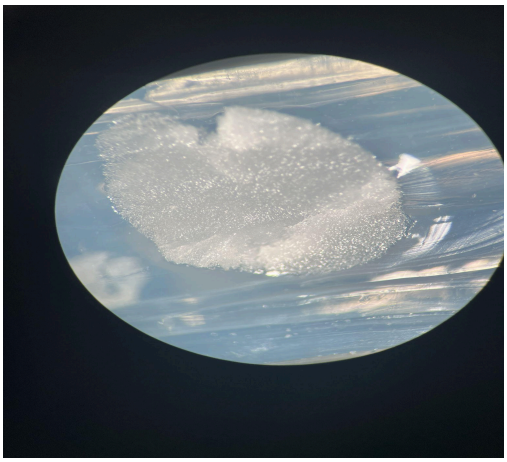
30% Formulations	Small	Large
75:25		
65:35		
1:1		

Table 6: 20% Formulations of Small and Large beads

20% Formulations	Small	Large
75:25		
65:35		
1:1		

Morphology of 40% Formulations

Table 4 showcases morphology of the freeze-dried pellets with a 40% concentration of carbohydrates, analyzed under a stereo microscope. Three formulations were examined: 75:25, 65:35, 1:1 sucrose : maltodextrin ratios. When inspecting morphological characteristics, it is easy to note that small pellets from all three formulations contained noticeable holes in the core. These central holes can be indicators of structural weakness of freeze-dried cake. Similar phenomenon can be noticed in *Table 6.* In 20% 65:35 formulation (Small) and 75:25 formulation (Small and Large), although not as prevalent as in *Table 4.*
These big voids that are mainly present in 40% formulations and could be attributed to higher viscosity, which can affect uniformity. Solutions that are more viscous are prone to not distributing evenly as they freeze, leading to inconsistent ice crystal formation, but viscosity does not explain why some of the less viscous formulation also had voids in the central part of the pellet.

It is also worth noting that voids present in the core are more pronounced in pellets with higher sucrose content. It is suspected that Maltodextrin, with higher molecular weight tends to create a more stable and uniform glassy matrix, reducing the likelihood of producing large voids in the core. Another factor that needs to be taken into account is that Sucrose has a higher affinity for water compared to Maltodextrin (58) - sucrose greater ability to retain water can lead to uneven hydration, causing stress and formation of voids within pellets.

One more explanation of voids in the core of the pellets could be from partial collapse during freeze-drying. When mass transport is restricted, the water vapor generated from sublimation can’t escape efficiently. In consequence, localized temperature increase can occur, surpassing local glass transition temperature (Tg) and leading to structural collapse. At higher solute concentration, the pathways for water vapor are further limited due to reduced pore space, increasing the likelihood of collapse.

Morphology of 30% and 20% Formulations

Table 5 and Table 6. showcase 30% and 20% formulations across three different carbohydrate ratios respectively. What is interesting to note is that the core for the most part seems to be intact in these samples, except for 20% 65:35 Small beads and 20% 75:25 Large beads. There seems to be more void and irregularity in the core of the Samples with higher concentration of sugars. There are a couple of factors that can be in play here, but it is very hard to pinpoint how much they are in play. Higher concentration formulations, such as 40%, have increased viscosity that can hinder the movement of water vapor from the interior to the surface, leading to uneven drying and higher chance of disintegration (26). Dense matrix in 40% concentration poses greater resistance to mass transport - making water vapor difficult to escape during sublimation. The higher resistance to mass transfer increases the risk of local overheating, which can impose stress on the core.

4.4 Stained beads under Light Microscope (40% Formulations)

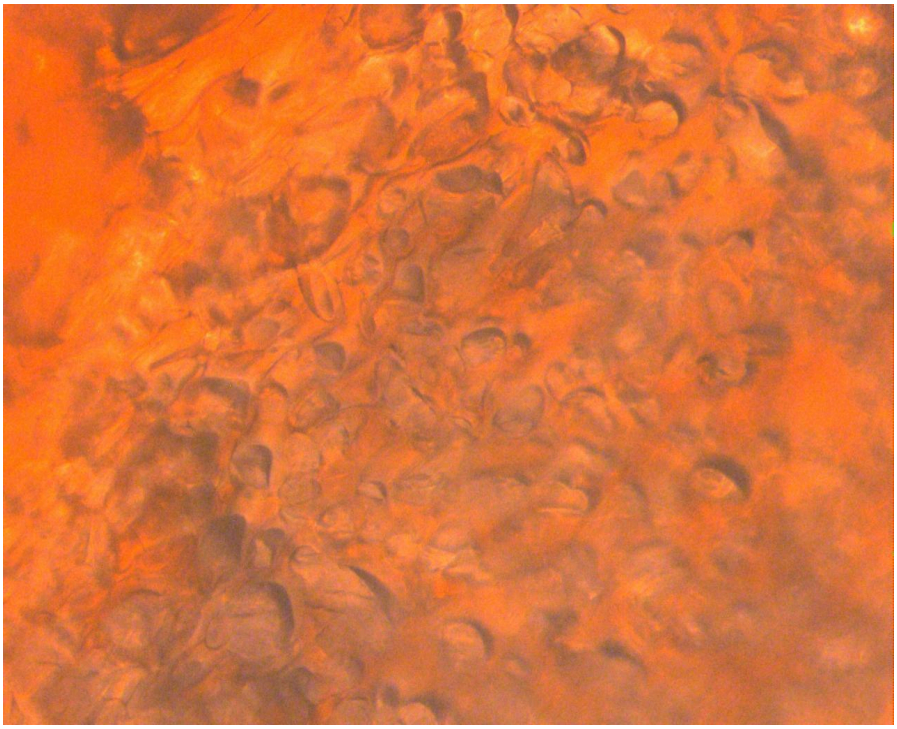


Figure 14: 75:25 40% formulation Large pellet size

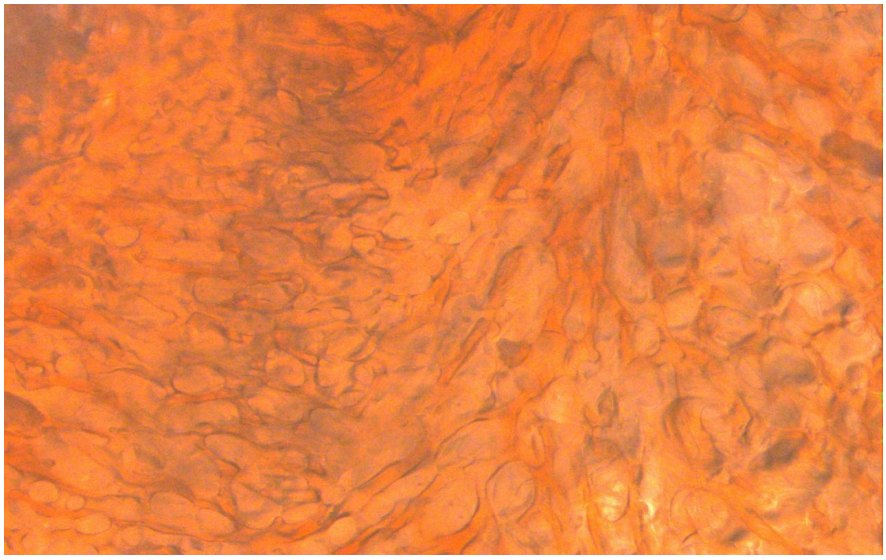


Figure 15: 75:25 40% formulation Small pellet size

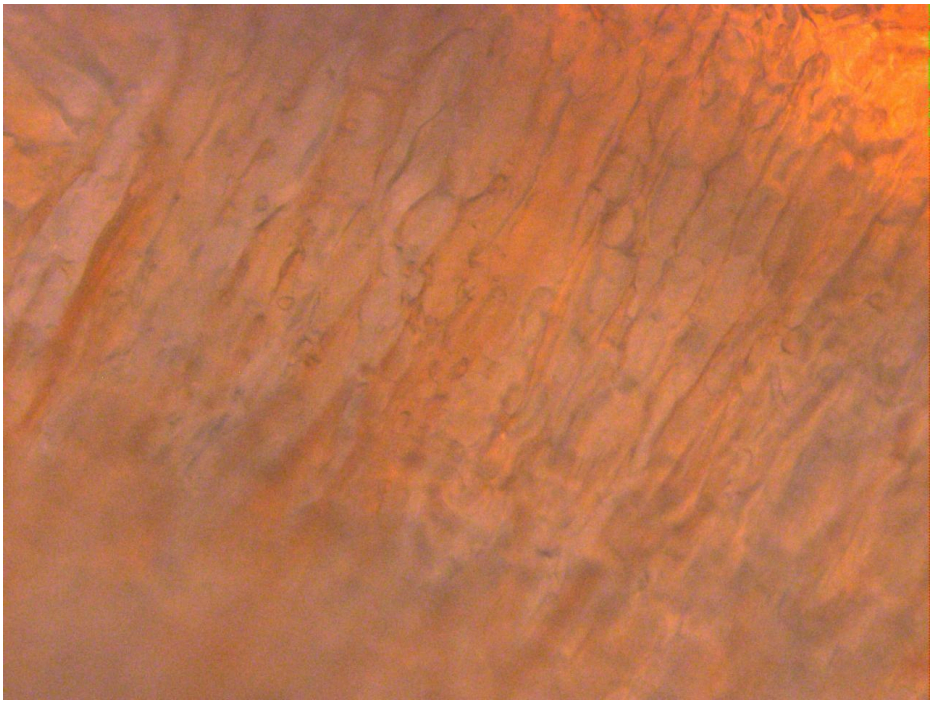


Figure 16: 65:35 40% formulation Large pellet size

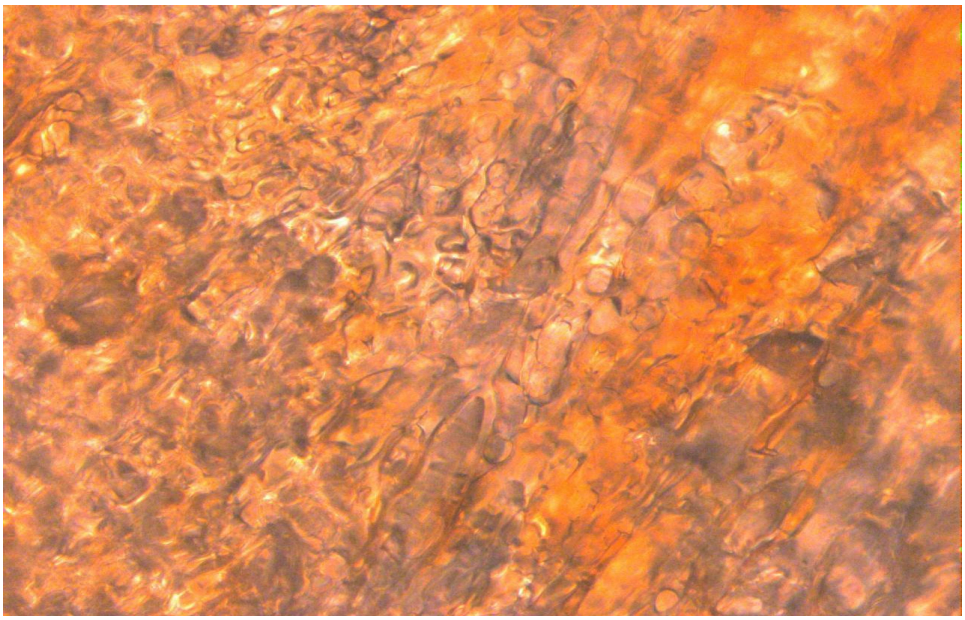


Figure 17: 65:35 40% formulation Small pellet size

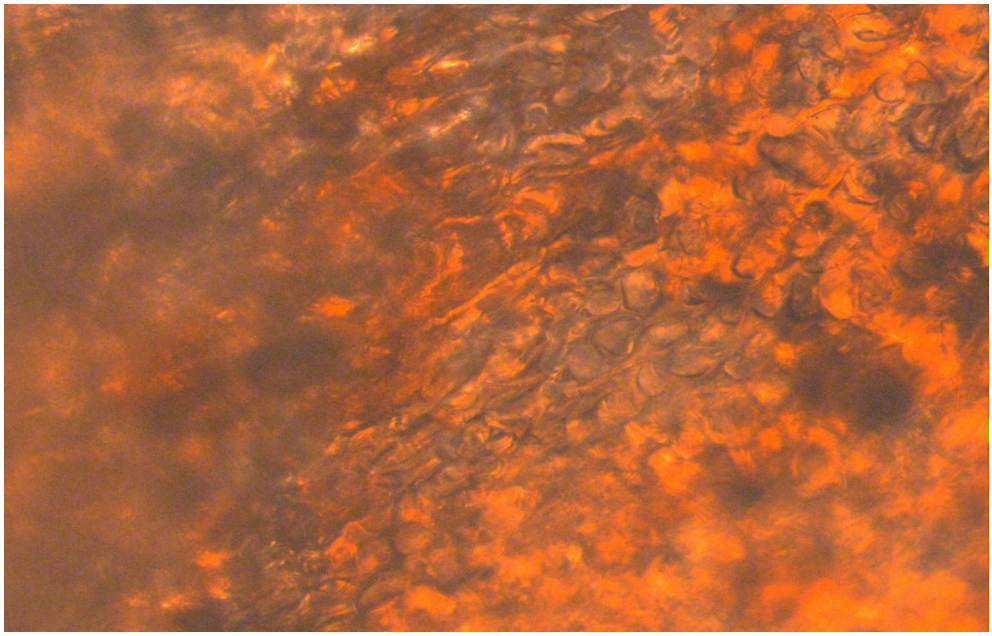


Figure 18: 1:1 40% formulation Large pellet size

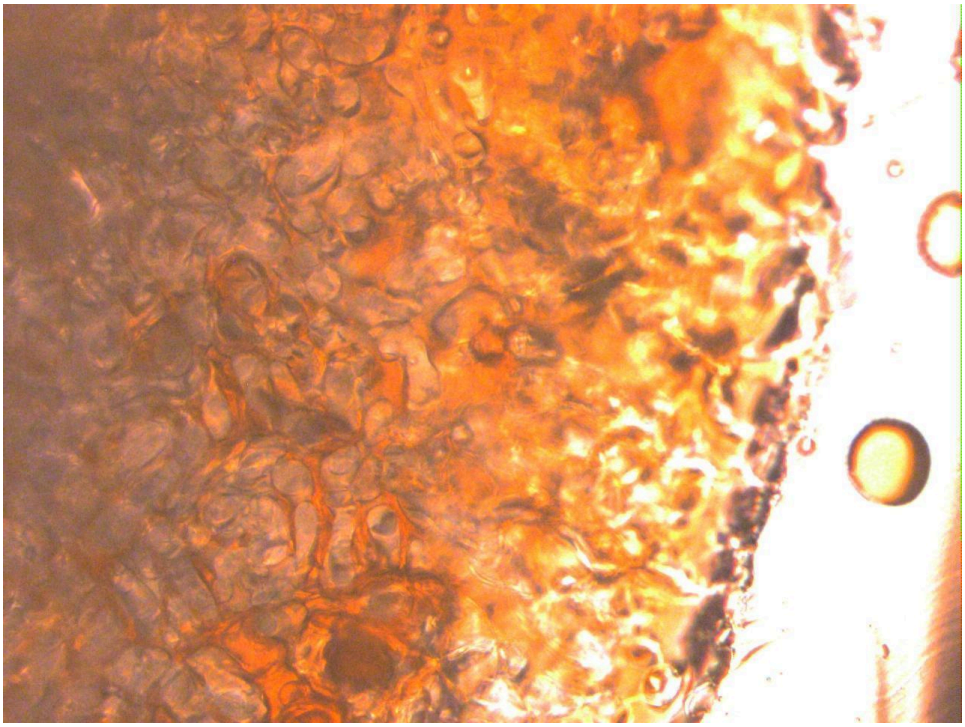


Figure 19: 1:1 40% formulation Small pellet size

4.5 Stained beads under Light Microscope (30% Formulations)

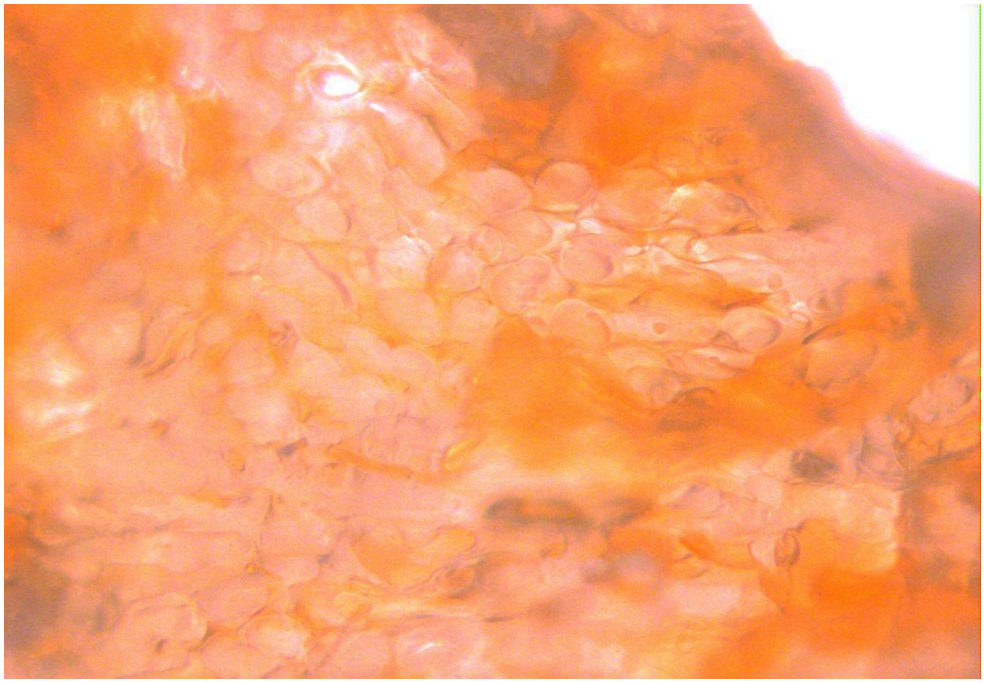


Figure 20: 75:25 30% formulation Large pellet size

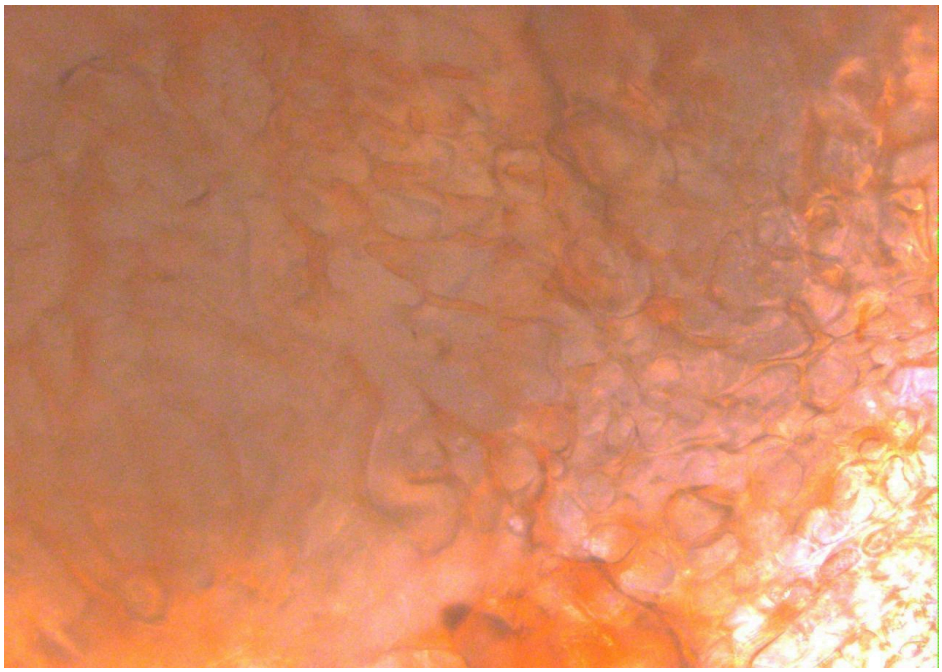


Figure 21: 75:25 30% formulation Small pellet size

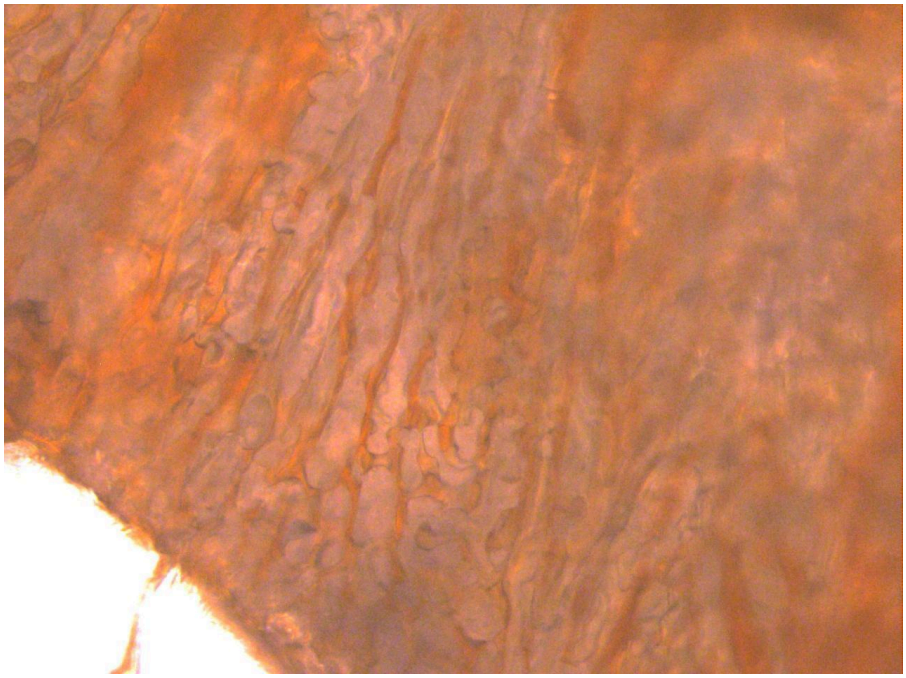


Figure 22: 65:35 30% formulation Large pellet size

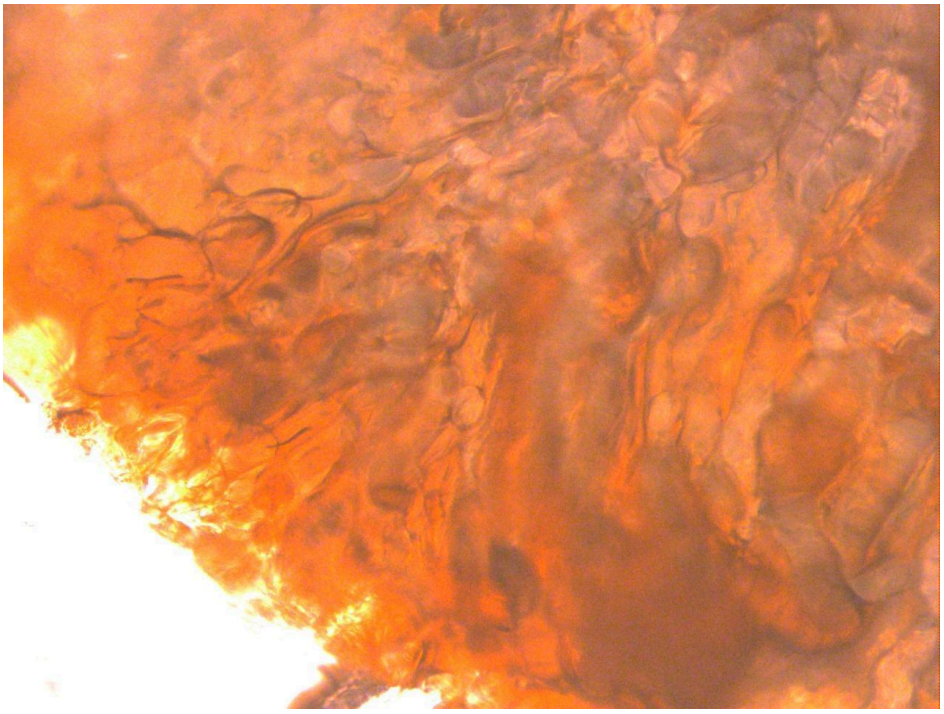


Figure 23: 65:35 30% formulation Small pellet size

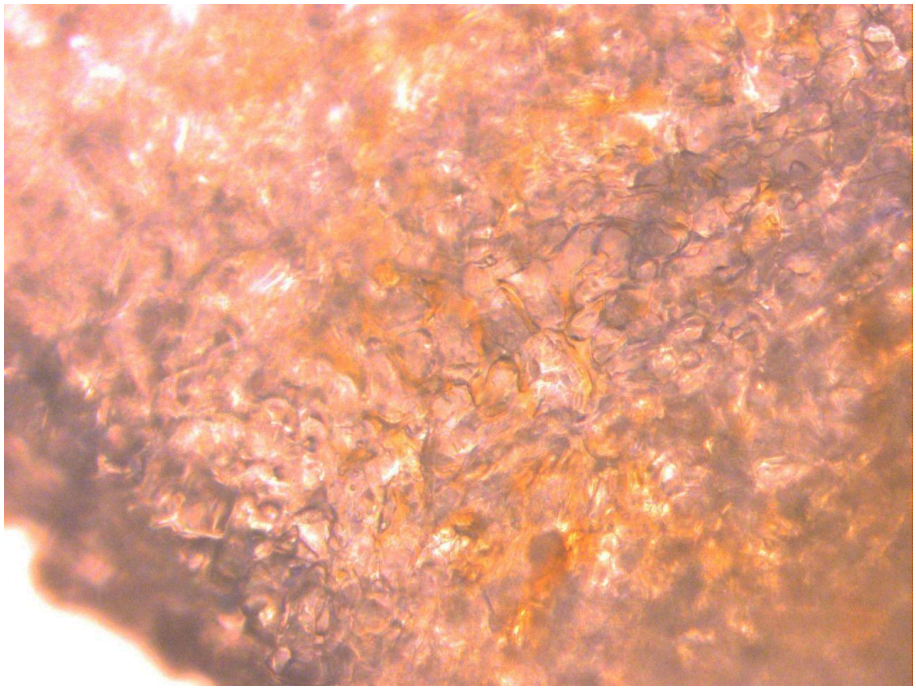


Figure 24: 1:1 30% formulation Large pellet size

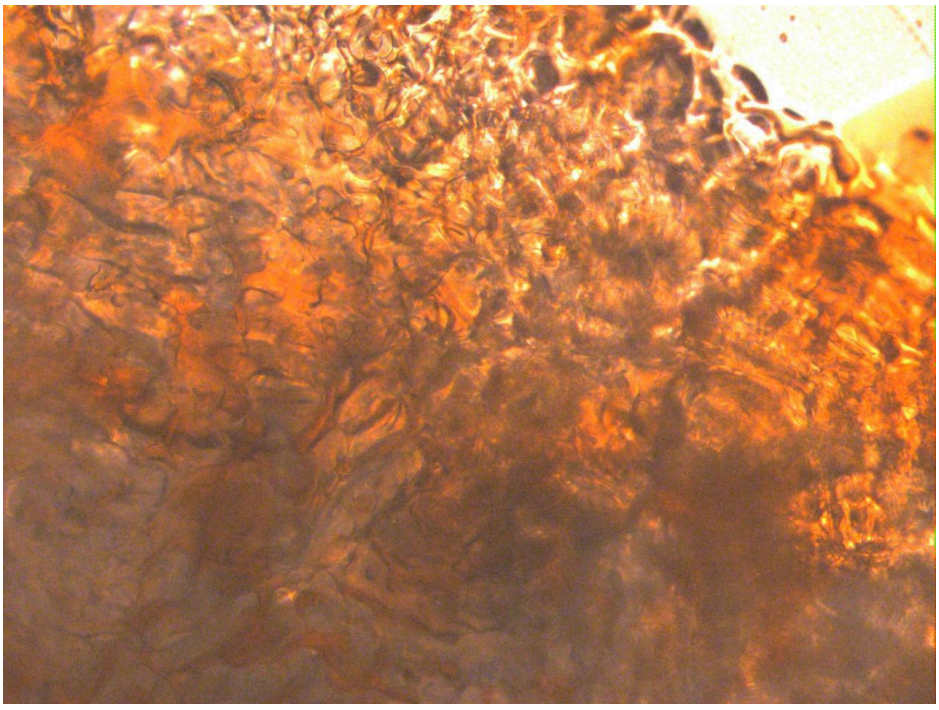


Figure 25: 1:1 30% formulation Small pellet size

4.6 Stained beads under Light Microscope (20% Formulations)

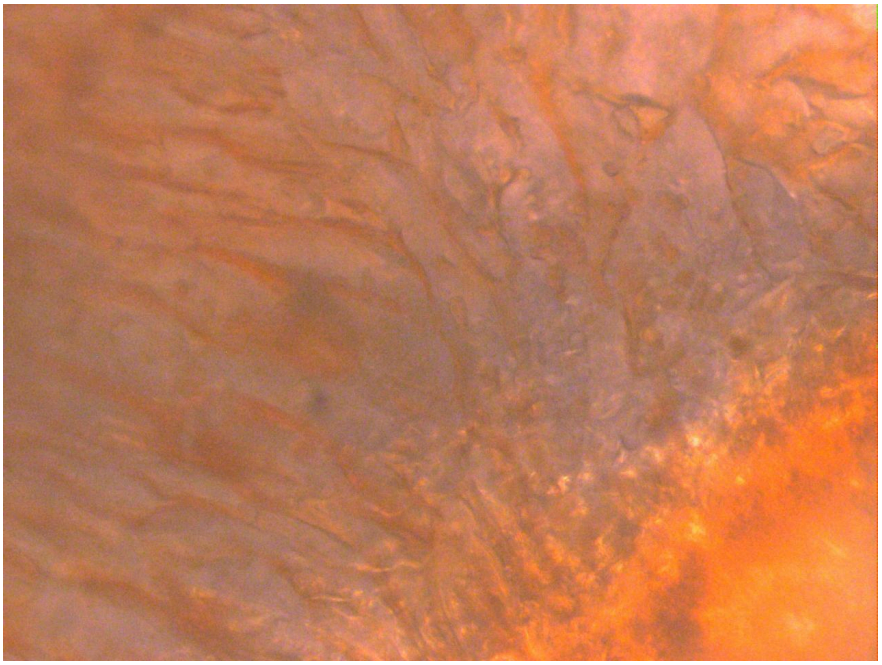


Figure 26: 75:25 20% formulation Large pellet size

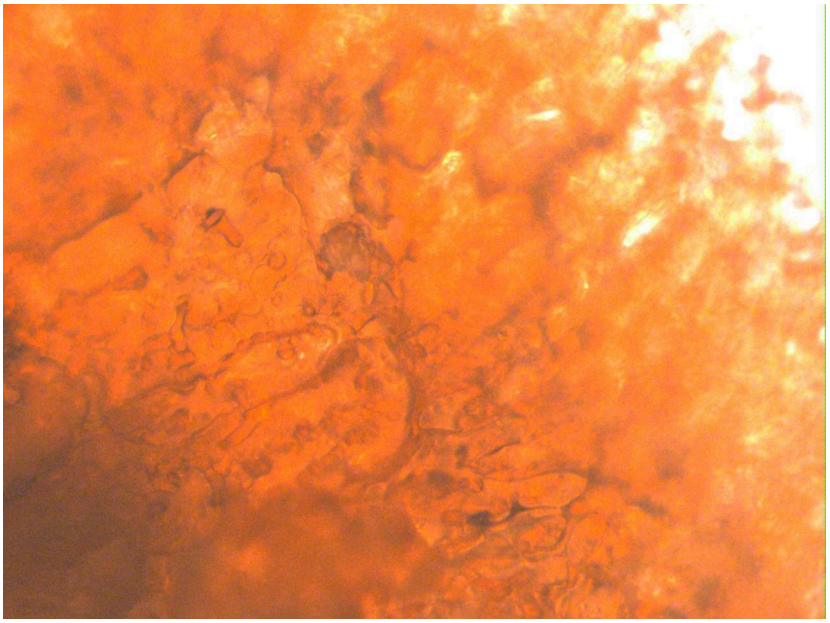


Figure 27: 75:25 20% formulation Small pellet size

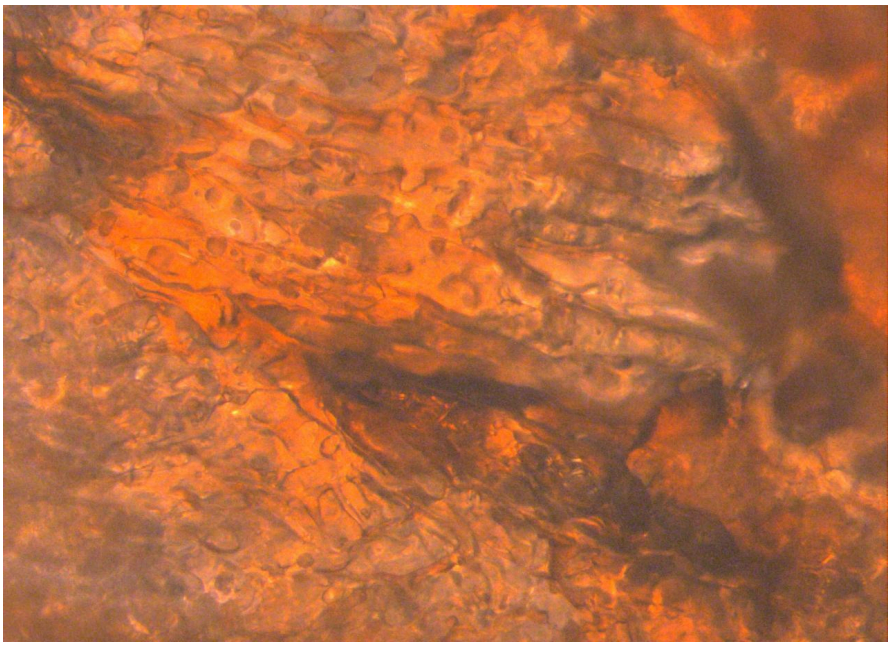


Figure 28: 65:35 20% formulation Large pellet size

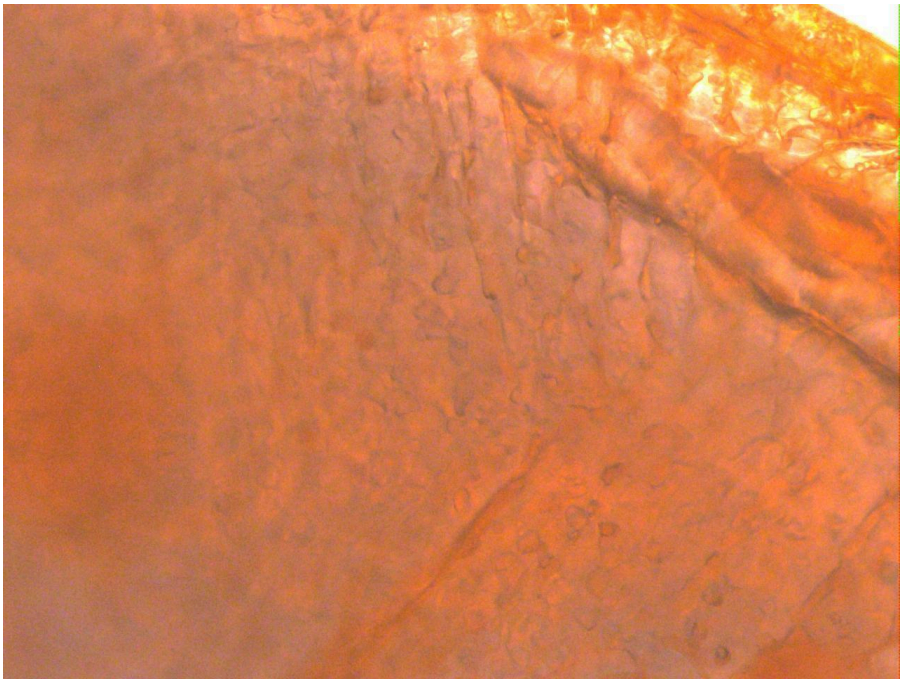


Figure 29: 65:35 20% formulation Small pellet size

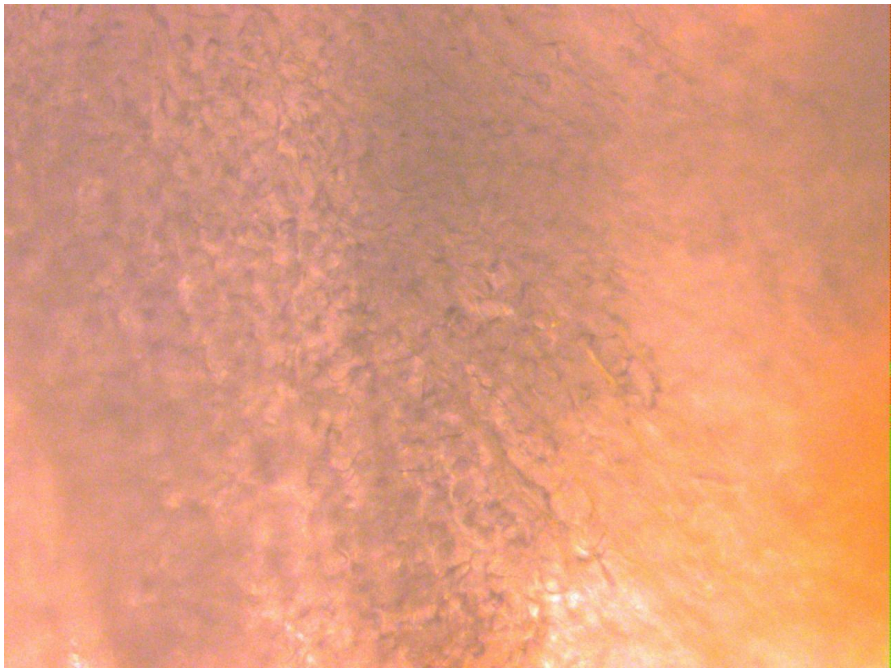


Figure 30: 1:1 20% formulation Large pellet size

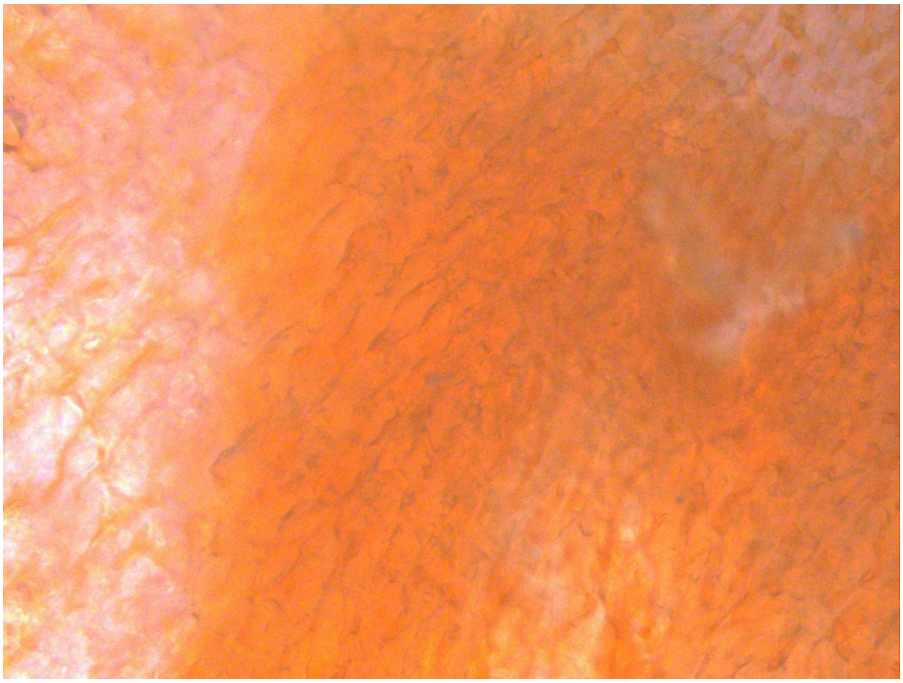


Figure 31: 1:1 20% formulation Small pellet size

All of the samples were cut horizontally by a sharp scalpel into thin slices, and Orange Dye was applied to them from a Faber-CASTELL Orange Sharpie. The water-based

dye from the Sharpie did not adhere to the hydrophobic Elastosil Vario 15 but penetrated into the structure of the beads, allowing for higher contrast and enhanced visualization (53). This staining method significantly improved the ability to observe internal features of the beads under a microscope. However, the field of view of the microscope did not allow for capturing the best pictures. As a result, live inspection by the operator often provided better evaluation than photographs, as the operator could adjust the focus to optimize the view and identify structural details more effectively, in real time.

"Skin on the pellets" (less porous structure on the outside)

Figure 16 shows a 65:35 40% formulation close to the core. One can see in the right top corner the "skin" that was present on the surface of the pellets in all of the formulations, but this picture makes it look even more pronounced. It seems to be less porous than the core of the pellet.

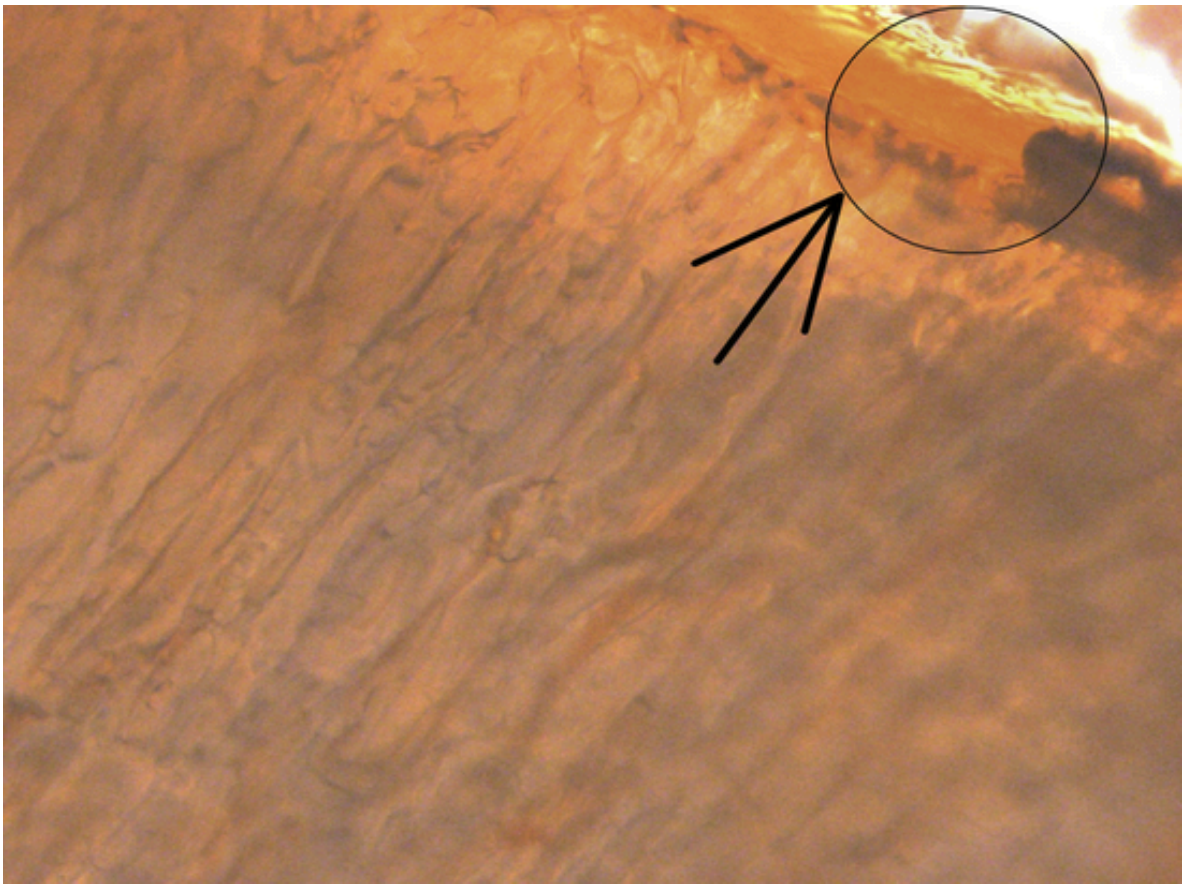


Figure 32: Formulation 65:35 With an arrow pointing to the "skin" part of the pellet

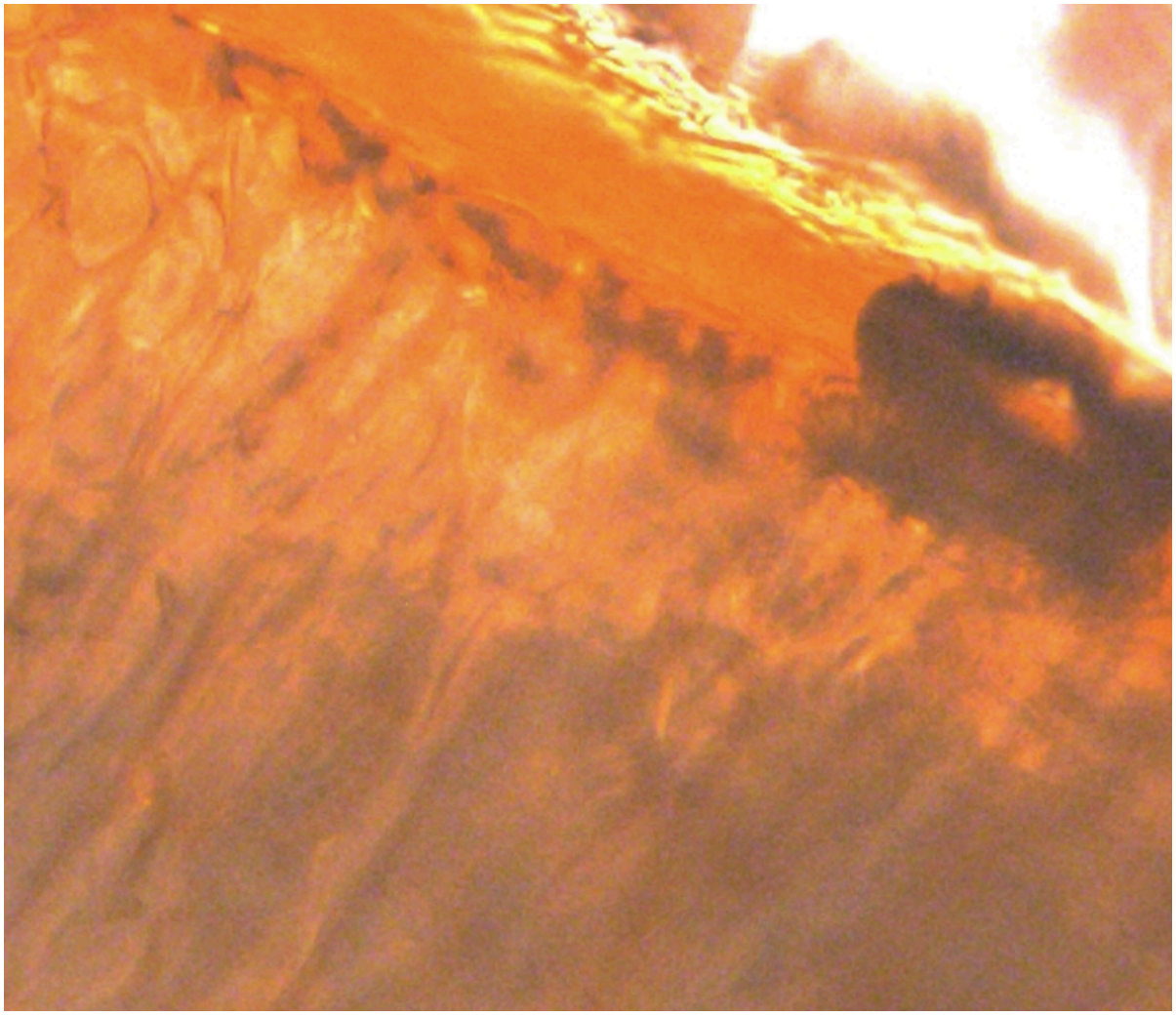


Figure 33: Close up of the "skin" part of the pellet

This phenomenon is apparent in every formulation. It was found that pellets are way more porous inside, especially around the core of the bead, after cutting them. This could be explained by the process of production - quench freezing. During production of pellets, plunging the droplets into liquid nitrogen causes extremely fast freezing, creating a temperature gradient, where outer layers freeze almost instantly, while inner layers take slightly longer. This directional cooling results in radial formation of ice crystals towards the center of the pellets.

Comparison of the "skin" surface between formulations

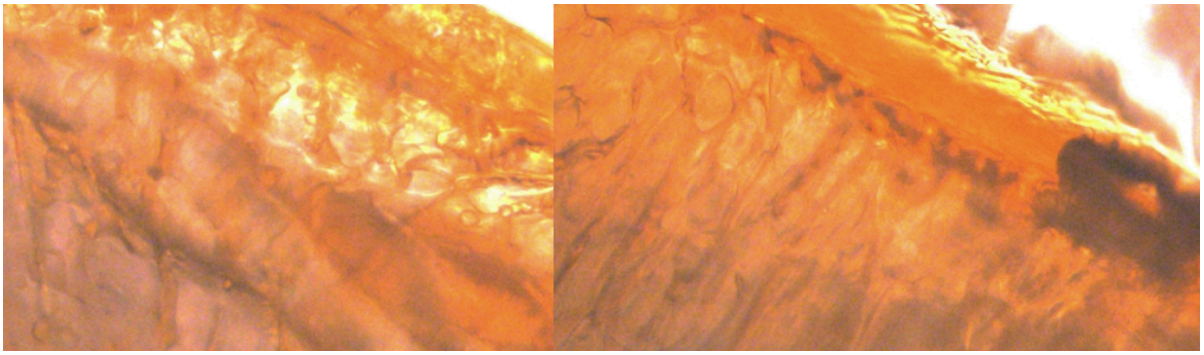


Figure 34: Comparison of 65:35 20% formulation Small pellet size (Left) and 65:35 40% Large pellet size (Right).

It seems that skin on lower carbohydrate concentration (20%) is more porous. Due to the low resolution and depth field of the microscope it is very challenging to draw concrete conclusions. If the skin is really more porous in 20% formulation it could be attributed to lower viscosity and higher number of nucleation sites - more nucleation sites would thus result in more ice crystals and more porous skin surface. Dense structure caused by higher carbohydrate content would provide less space for the movement of water molecules, and since Ostwald ripening relies on the mobility of water molecules to dissolve smaller ice crystals and facilitate individual water molecules to join the crystal lattice of large ice crystals, in higher carbohydrate content pellet it would be expressed as thicker skin.

Pore size comparison

To compare pores, unit measurement rather than micrometers was chosen for the pore size analysis due to technical limitation in superimposing a ruler from departaments computer. This unit measurement provided more practical way to compare pore sizes across different formulations and bead sizes.

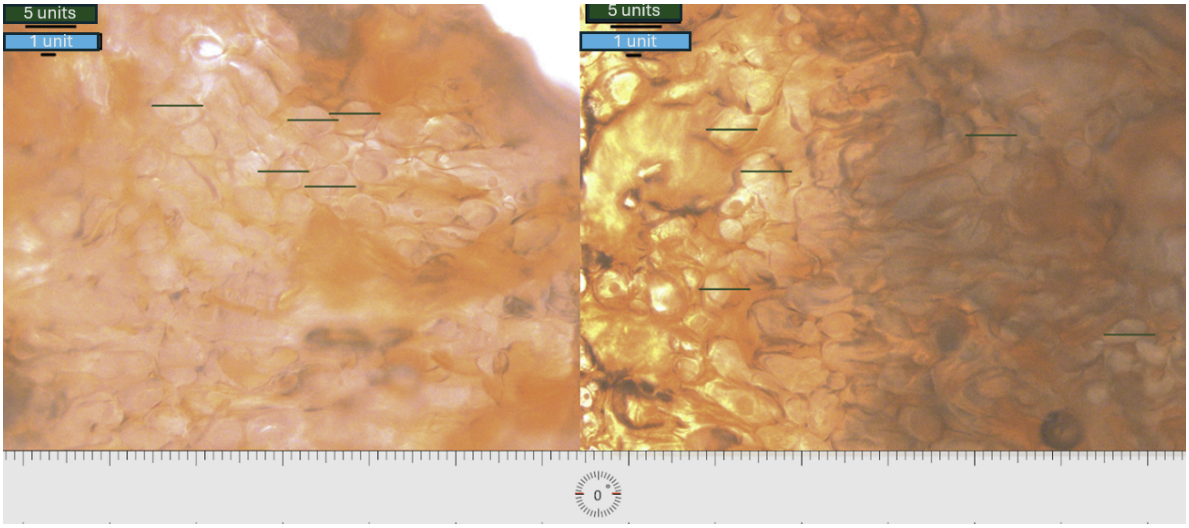


Figure 35: Comparison of 75:25 30% formulation Small (on the left side) and Large (on the right) with 5 unit green markings across the pores showcasing similarities in those pellets.

When comparing Large and Small pellet size formulations of higher concentrations, it became apparent that observed pores have similar size and morphology. 65:35 40% formulation exhibited large, extended long pores that direct towards the core of the bead. 75:25 30% formulation on the other hand exhibits more round shaped pores. This may be the effect of looking at the pores from different angles, depending on the microscope setting. Shape of the pellets changed slightly during live observations depending on how far the microscope condenser focus knob was tweaked. Overall, pellets produced with higher carbohydrate concentrations tended to have pores above 5 units while pellets produced with lower carbohydrate concentrations tended to have pores equal to 1 unit or even smaller. This difference becomes more apparent when inspecting these pellets live under a microscope.

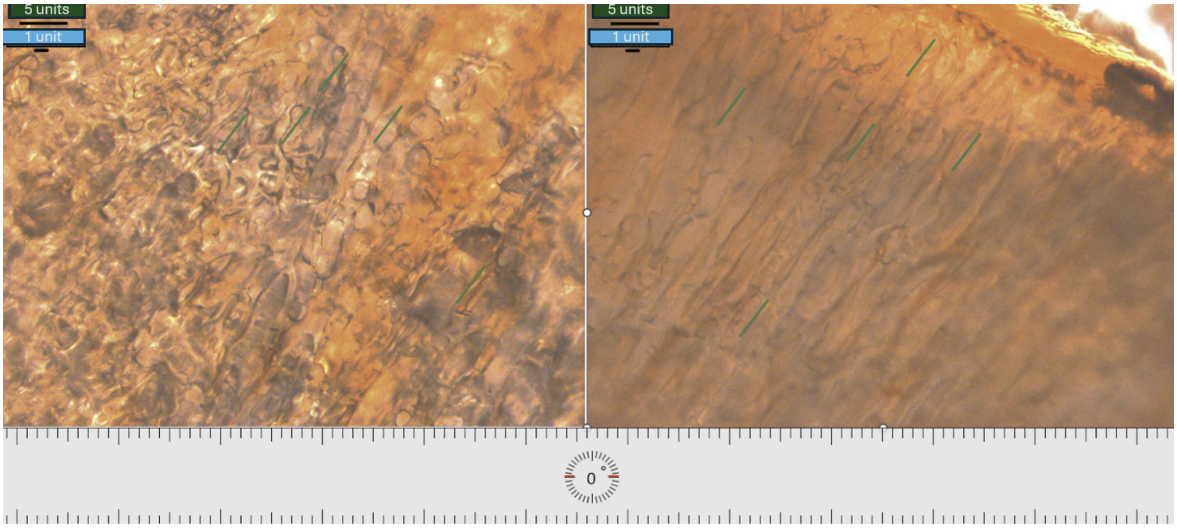


Figure 36: Comparison of 65:35 40% formulation Small (left) and Large pellet (right) size with 5 units green markings across the pores showcasing differences in those pellets.

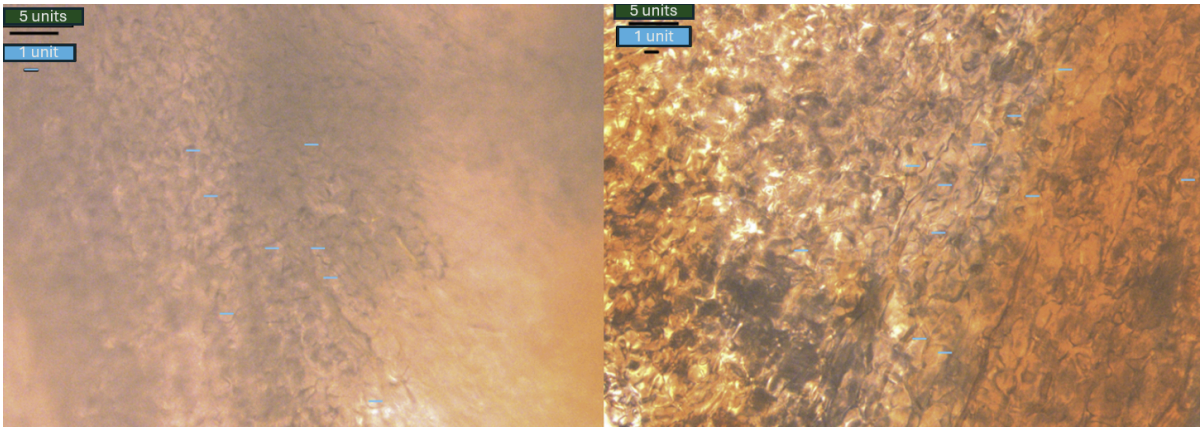


Figure 37: Comparison of 1:1 20% formulation Large (on the left) And Small (on the right) pellet size with 1 unit blue markings across the pores showcasing differences in those pellets.

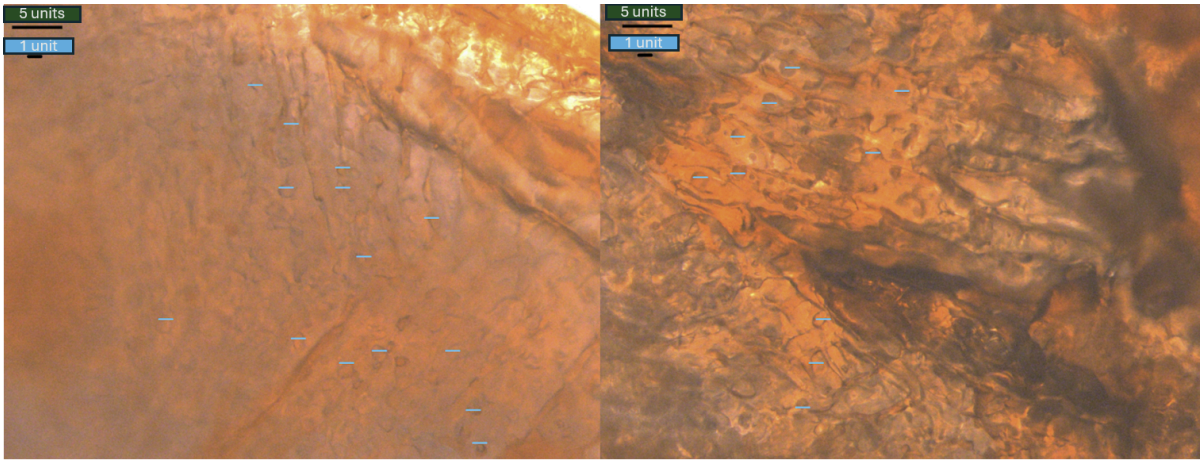


Figure 38: Comparison of 65:35 20% formulation Large (on the left) And Large (on the right) pellet size with 1 unit blue markings across the pores showcasing differences in those pellets.

Comparing images with scale allowed assessing size pores of both formulations with high carbohydrate concentration (40%) and smaller carbohydrate concentration (20%). In formulations with higher carbohydrate concentration, viscosity can slow down the freezing process, resulting in fewer nucleation sites for crystal formation. This leads to fewer but larger ice crystals initially, and during Ostwald ripening their size is further increased.

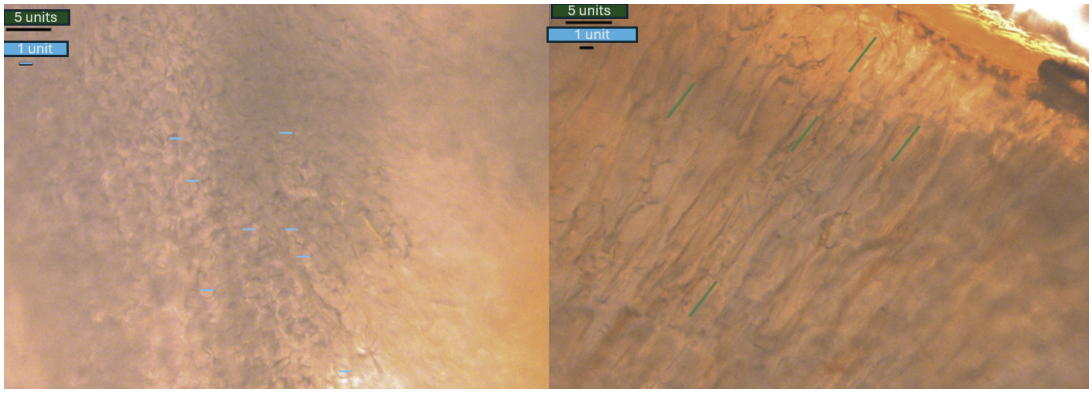


Figure 39: Comparison of 65:35 40% formulation Large pellet size (on the right) and 65:35 20% Large pellet size (on the left) with Blue markings representing 1 unit and green markings representing 5 units

Pore assessment limitation with Python

Automated pore assessment using Python script and utilizing advanced libraries such as OpenCV, NumPy, Matplotlib, Scikit-image, SciPy offers a promising approach. The image

was loaded using OpenCV, then converted into grayscale to simplify analysis. Gaussian blur was applied to reduce noise and improve contour detection. After that, Otsu thresholding method was utilized for binary segmentation and morphological operations were performed to clean up segmented regions. Contours of detected pores were identified and filtered based on area to exclude non porous structures and results were visualized in Matplotlib.

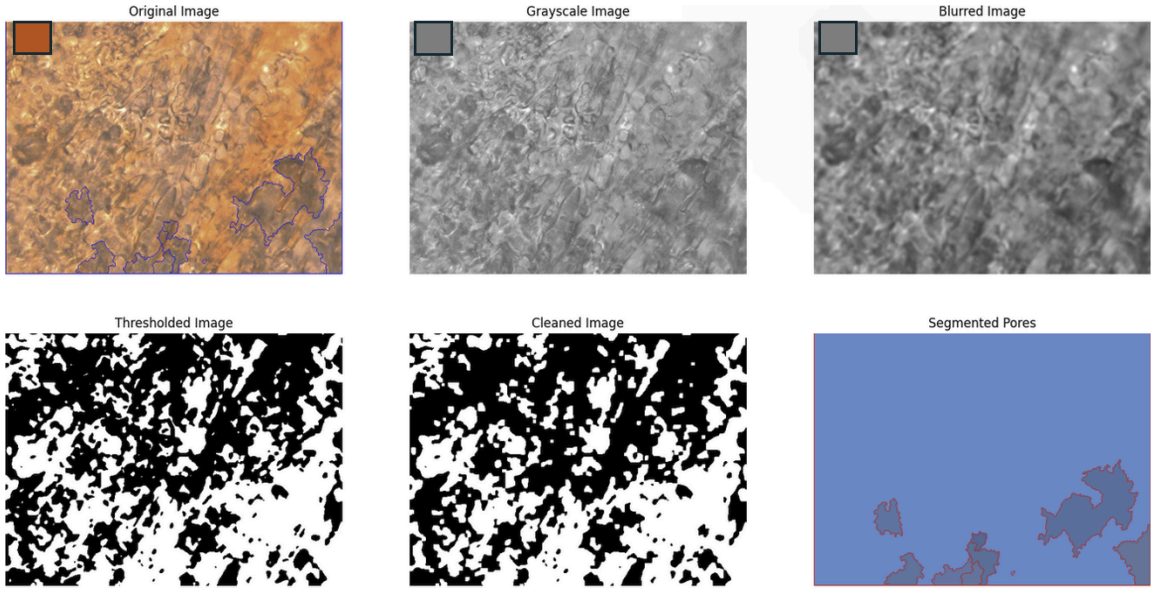


Figure 40: 65:35 40% Small pellet formulation visualized by usage of Matplotlib library

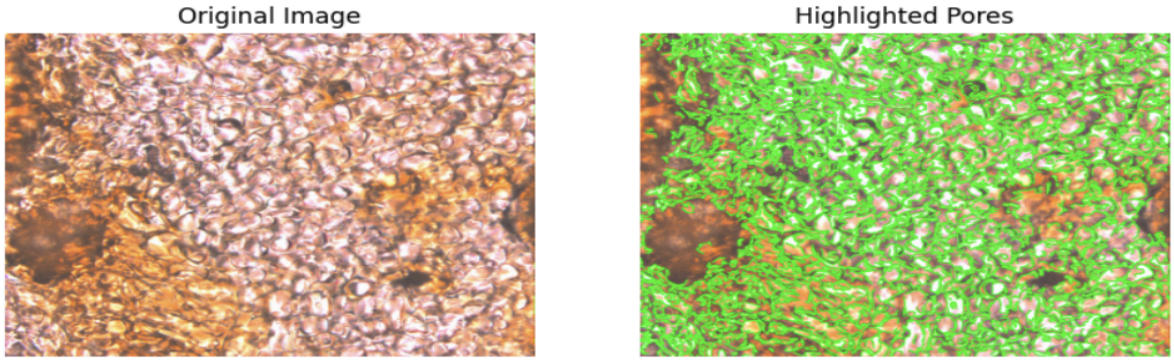


Figure 41: Trial to visualize more pronounced pores by Matplotlib and OpenCV libraries

Initial image quality significantly affected the results. Variations in lighting introduced a lot of artifacts, deeming it impossible to measure the pores. Gaussian blur and Otsu's thresholding were insufficient to measure complexity of the pore structures, leading to poor segmentation. While the script was able to process images and identify potential pore

regions, it failed to provide accurate measurement. Despite the initial setbacks, this method holds a significant promise for future applications. Integration of powerful libraries like OpenCV, Matplotlib gives a framework for detailed image analysis and processing, especially when working with lower-contrast images from light microscopes.

5. Discussion

The research aimed to investigate effects of varying sucrose to maltodextrin ratios, concentrations and bead sizes on the morphology of freeze-dried beads. When comparing large and small pellet size formulations of higher concentrations it became apparent that observed pores have similar size and morphology across high carbohydrate concentrations.

Morphology of 40% formulation

Table 4 demonstrates that the 40% carbohydrate concentration formulations exhibit noticeable voids in their core across all three sucrose to maltodextrin ratios (75:25, 65:35, 1:1). These voids are more pronounced in formulations with higher sucrose content, suggesting structural weakness that could compromise the integrity of the freeze-dried cake. Increased viscosity of 40% concentrated carbohydrate formulations is likely a contributing factor, as this property can hinder uniform distribution of solutes during freezing, leading to inconsistent ice crystal formation. However, the presence of voids in some of the less viscous formulations can indicate that other factors, such as sucrose's higher affinity for water can play a significant role. One more reason could be previously mentioned limited mass transfer - water vapor produced by sublimation cannot escape quickly enough, rising a local temperature to exceed local glass transition temperature and resulting in partial collapse. There could also be some degree of stochastic behavior involved, leading to variations between individual pellets within the same batch and of the same size.

Morphology of 30% and 20% Formulations

Table 5 and 6 show that 30% and 20% formulations generally maintain a more intact core, with fewer voids compared to 40% formulations. The exceptions are two 20% formulations - 65:35 small and 75:25 large pellets, which exhibit an irregular, empty core in

the center. Lower carbohydrate concentrations result in less viscous solutions, facilitating more uniform ice crystal formation, which could further translate in lower frequency of voids in the core. This can also be attributed to improved mass transport during sublimation and a more consistent dehydration process.

Surface Morphology - "Skin Phenomenon"

Research highlighted the presence of a less porous "skin" on the surface of the pellets across all formulations. This skin is likely a result of the quench-freezing method, where the rapid freezing of the outer layers creates a denser structure compared to the more porous interior. This phenomenon is more pronounced in higher carbohydrate concentration due to the suspected lower number of nucleation sites and the influence of Ostwald ripening, which factors the growth of larger ice crystals in a dense matrix and directly impacts porosity.

Pore Size Analysis

Comparison of pore sizes across different formulations *(Figures 35-39)* revealed that higher carbohydrate concentrations tend to yield larger pores, while lower concentrations result in smaller, more numerous pores. There was an attempt on automated pore assessment using Python, and while it is a very promising method it needs further polishing in order to work with lower quality / lower contrast images obtained from light microscopy.

Limitations of the Study

Freeze drying is a very complex process that involves numerous variables like temperature control, pressure regulation, environmental variables, formulation composition. Drawing a correlation between bead size and structural integrity is very challenging due to intricate interplay of these factors. This nature of process makes it difficult to isolate the impact of individual variables on the final product.

This study highlights a significant drawback of the microscope used - the limited resolution and depth of field, which restricted the ability to capture finest details for analysis. Live inspection by the operator often provided better evaluation than photographs taken by

camera attached to microscope, as the operator could adjust the focus and to optimize the view and identify structural details more effectively, in real time.

Embedded pellets needed to be cut in a very thin slice fashion by sharp blade. It was very challenging to get a very thin elastomer slice in repeatable fashion without damaging a very fragile, freeze-dried cake. During experimentation it became clear that cryostat is out of question, due to elastic properties of the Silicone elastomer, so obtaining very thin slices by this method was deemed impossible. There is certainly a promise in this method - provided there is an optimal cutting temperature compound that hardens at low temperature and does not dissolve carbohydrate formulations, then this method would produce thin enough slices to inspect them under light microscope.

Automatic pore assessment couldn't be done due to initial image quality that significantly affected the results of Python script. Variations in lightning introduced many artifacts, and it was not possible to measure the pores accurately. Implementing Gaussian Blur and Otsu's thresholding was proven to be insufficient to the complex pore structure.

6. Conclusions

The findings revealed insights into morphology of the pellets across different carbohydrate formulations and concentrations, as well as sizes. Findings include:

Core Voids that are Predominant at 40% formulations

The 40% carbohydrate concentration formulations exhibited noticeable voids in the core of the pellets across all sucrose to maltodextrin ratios. These voids were more pronounced with higher sucrose content, suggesting that sucrose's higher affinity to water might play a significant role, leading to uneven hydration and stress formation within the pellets, which can result in these voids. Partial collapse due to hindered mass transfer might also be at play - increasing local temperature above glass transition temperature due to poorer water vapor pathways.

More Intact Cores in Lower Concentrations

The 30% and 20% formulations generally maintained a more intact pellet core, with fewer voids compared to 40% formulation. Suspected mechanism for this is that lower carbohydrate concentration results in less viscous solutions, facilitating more uniform ice crystal formation and reducing the likelihood of voids. Improved mass transport during sublimation and more consistent dehydration is also suspected to play a role on the structural integrity of the pellets. More effective mass transport negates

Less Porous "Skin" Surface

All formulations exhibited a less porous outside layer "skin", likely a result of the quench-freezing method. This rapid freezing creates a denser outer structure compared to the more porous interior. This phenomenon is more pronounced in higher carbohydrate concentrations due to suspected lower amounts of nucleation sites and the influence of Ostwald ripening, which facilitates turning small ice crystals into large ice crystals.

Pore Size Variations

Higher carbohydrate concentrations tended to produce larger pores, while lower concentrations resulted in smaller pores. The automated pore assessment using Python for light microscopy shows a lot of promise but requires further refinement to handle lower quality-images effectively.

Microscope Limitations

This study highlights significant drawbacks of the microscope used, including limited resolution and depth of field, which can restrict the ability to capture finest details for analysis. Live inspection by the operator often provided better evaluation than photographs,as the operator could adjust the focus and angle in real-time to optimize the view and identify structural details more effectively.

Perspective on Future Studies

Future studies could address the limitation encountered during this study and further explore the factors that influence freeze-dried pellet morphology. Key areas of improvement include:

1. Enhanced visualization techniques: Improved visualization methods for light microscopy to capture high-resolution images of freeze-dried pellets will provide more detailed insights into their morphology. This might be done by getting thinner slices by developing an optimal cutting compound for carbohydrates and using cryostat for sectioning. This would involve embedding samples in suitable resin that stabilizes the fragile structure of freeze-dried material.
2. Refinement of Automated Image analysis - Improving automated pore annotation using Python scripts and available image processing libraries will enable for accurate and consistent analysis of pore sizes and distributions. This involves addressing variations in lighting and artifacts that affect image quality. Currently image quality might be too low in order to utilize that technique.

7. References

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8. Appendix

8.1 Tables from Syringe pump experiments, considering different Needle Diameters

L.P. Video	Solution	Vol Syringe (ml)	Needle Diameter (G)	Flow Rate (ml/h)	Droplets size (mm)
1	1:1 Sucrose Maltodextrin	50	25	100	3
2	1:1 Sucrose Maltodextrin	50	25	150	2.5
3	1:1 Sucrose Maltodextrin	50	25	175	2.5
x	1:1 Sucrose Maltodextrin	50	25	200	Too high to measure
4	75:25 Sucrose Maltodextrin	50	25	100	3
5	75:25 Sucrose Maltodextrin	50	25	150	2.5-2.7 (hard to estimate)
6	75:25 Sucrose Maltodextrin	50	25	175	2.5
x	75:25 Sucrose Maltodextrin	50	25	200	Too high to measure

L.P. Video	Solution	Vol Syringe (ml)	Needle Diameter (G)	Flow Rate (ml/h)	Droplets size (mm)
7	1:1 Sucrose Maltodextrin	50	18	100	4
8	1:1 Sucrose Maltodextrin	50	18	150	4
9	1:1 Sucrose Maltodextrin	50	18	175	3.5
10	1:1 Sucrose Maltodextrin	50	18	250	3.5
11	75:25 Sucrose Maltodextrin	50	18	100	3

12	75:25 Sucrose Maltodextrin	50	18	150	3
13	75:25 Sucrose Maltodextrin	50	18	200	3
14	75:25 Sucrose Maltodextrin	50	18	250	2.5-3 (hard to estimate)
15	75:25 Sucrose Maltodextrin	50	18	300	

8.2 Standard Operating Procedure for Syringe Pump

Standard Operating Procedure for
Syringe Pump Quenching Freeze Process

Kamil Pietrzak
Friday, 16 Feb 2024
Lund University

Objective: To operate the syringe pump effectively to dispense uniform-sized liquid drops from a solution into a batch of liquid nitrogen for quench freezing.

Equipment:

1. Syringe pump
2. 50 ml syringe
3. Liquid solution to be dispensed (Formulation)
4. Container of liquid nitrogen
5. Droplet collection surface (optional)

Procedure:

1. Setup:
    - Place the syringe pump on a stable surface near the container of liquid nitrogen.
    - Connect the 50 ml (29mm diameter) syringe securely to the syringe pump.
    - Ensure that the other end of the needle is positioned directly over the liquid nitrogen batch where the drops will be dispensed.
2. Setting Parameters:
    - Turn on the syringe pump and access the settings menu.
    - Use the select button to go through the pump menu.
    - Adjust syringe volume to 50 ml.

- Adjust diameter of syringe to 29mm
- Go into the "Vol option menu".
- Set the desired flow rate and desired volume to be dispensed by using arrows and start/stop button. **Note: The flow rate will determine the size of the drops.**

3. Dispensing Drops into Liquid Nitrogen:
    - Position the needle outlet directly over the surface of the liquid nitrogen batch, ensuring a consistent drop trajectory.
    - Start the syringe pump to initiate the dispensing process.
    - Monitor the formation of liquid drops and adjust the pump settings if necessary to ensure uniform size and consistent dispensing.
    - Allow the drops to fall directly into the liquid nitrogen batch for quench freezing.
4. Shutdown:
    - Once the desired number of drops has been dispensed or the process is complete, turn off the syringe pump.
    - Disconnect the tubing from the syringe and clean it thoroughly to prevent clogging.
    - Clean the syringe pump and syringe according to the manufacturer's instructions to maintain equipment integrity.
    - Store the syringe pump and accessories in a clean and dry environment.

Safety Precautions:

- Wear appropriate personal protective equipment (PPE) such as gloves and safety glasses when handling liquid nitrogen.
- Avoid direct contact with liquid nitrogen to prevent frostbite or injury.
- Ensure adequate ventilation in the work area to prevent buildup of nitrogen gas.
- Follow proper disposal procedures for any waste generated during the process.

8.3 Standard Operating Procedure for preparing solutions

Standard Operating Procedure for preparing maltodextrin and sucrose solutions

Kamil Pietrzak
Friday, 8 March 2024
Lund University

Objective:

Prepare 20%, 30%, and 40% w/v solutions of sucrose and maltodextrin in ratios of 65:35, 75:25, and 50:50.

Equipment and Materials:

- Analytical balance
- Beakers or volumetric flasks (100 mL)
- Stirring rod or magnetic stirrer
- Pipettes or graduated cylinders
- Distilled or deionized water
- Sucrose
- Maltodextrin (Glucidex 12D)

Procedure:

1. Preparation

Ensure all equipment is clean and dry. Calibrate the analytical balance as per the manufacturer's instructions.

2. Calculation

Calculate the amounts of sucrose, maltodextrin, and water required for each solution and their ratios. **For a target volume of 60 mL:**

For a 20% w/v solution: Need 12g of solutes in total.
75:25 - > 9g of sucrose, 3g of maltodextrin
65:35 -> 7,8g of sucrose, 4,2g of maltodextrin
50:50 -> 6g of sucrose, 6g of maltodextrin

For a 30% w/v solution: Need 18g of solutes in total.
75:25 - > 13,5g of sucrose, 4,5g of maltodextrin
65:35 -> 11,7g of sucrose, 6,3g of maltodextrin
50:50 -> 9g of sucrose, 9g of maltodextrin

For a 40% w/v solution: Need 24g of solutes in total.
75:25 - > 18g of sucrose, 6g of maltodextrin
65:35 -> 15,6g of sucrose, 8,4g of maltodextrin
50:50 -> 12g of sucrose, 12g of maltodextrin

Based on target volume, values will change.

3. Weighing
Use the analytical balance to weigh out the correct amounts of sucrose and maltodextrin for each solution. Record all weights for accuracy.

4. Dissolving
- For each solution, add the weighed solutes to a beaker or volumetric flask that contains 5-10 mlH of distilled water.
- Use a stirring rod or magnetic stirrer to dissolve the solids completely, ensuring a homogenous mixture.

5. Volume Adjustment
- After the solutes are fully dissolved, transfer the solution to the container - Add distilled water to reach the final volume of 20 mL. This step ensures that the solution is accurately concentrated at 20%, 30%, or 40% w/v.

Notes:

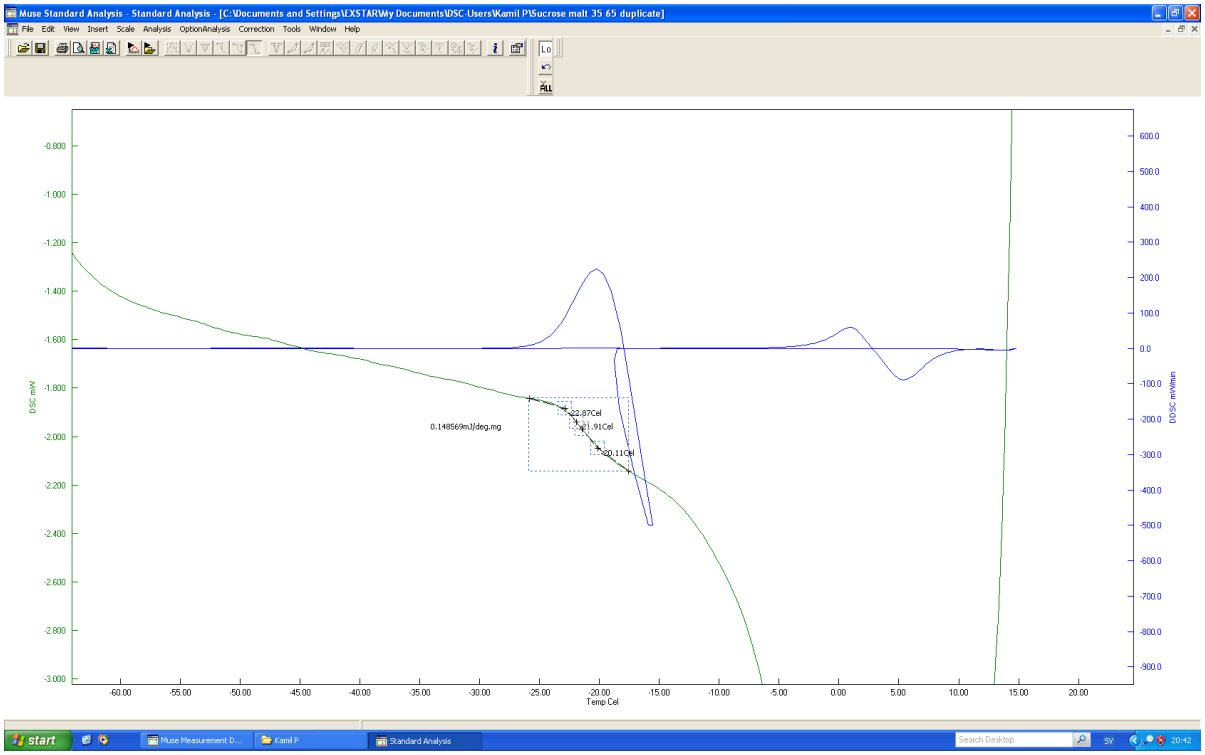
- Verify that the solution is clear and fully mixed.
- Label each solution with its concentration, sucrose:maltodextrin ratio, and the date of preparation.
- Adjustments may be required for solubility issues or specific lab conditions.
- Ensure solutions are stored properly, in labeled containers, and used before the expiry date for the chemicals.
- This SOP assumes room temperature preparation; adjustments may be needed for significant temperature variances.

Safety Precautions

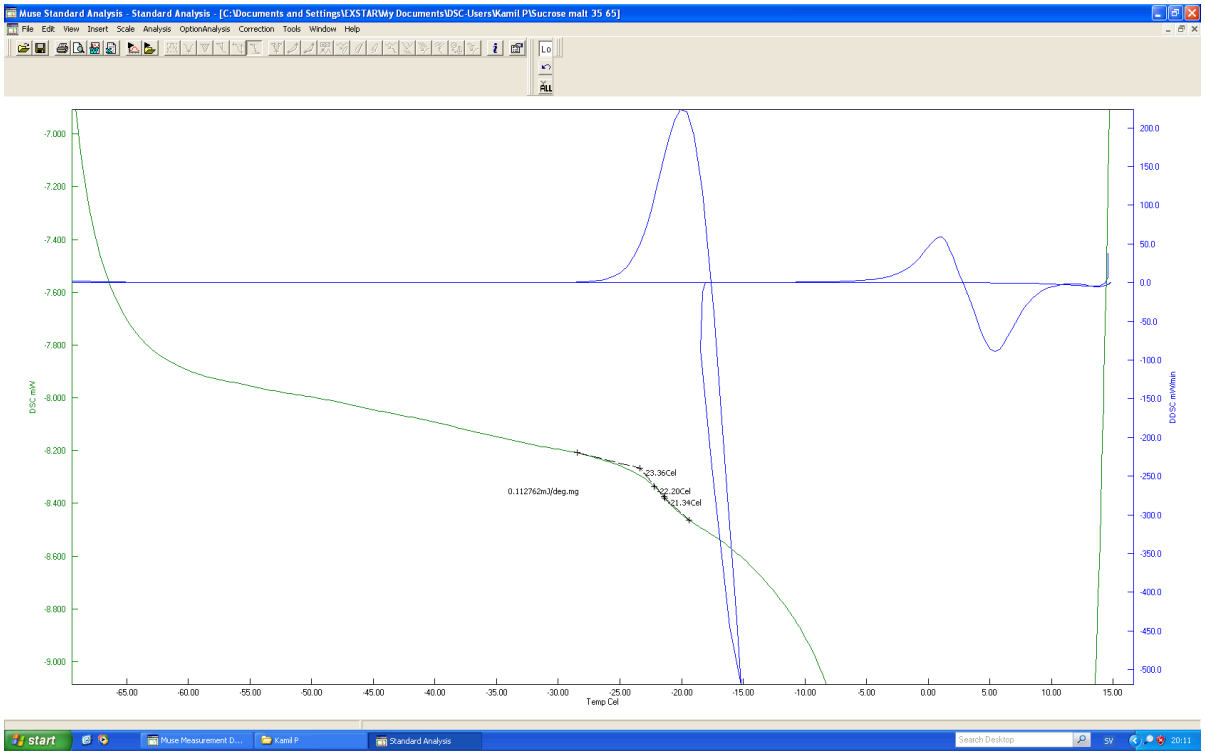
- Always wear appropriate personal protective equipment (PPE), including gloves and safety goggles.
- Handle all chemicals and laboratory equipment according to their safety data sheets and laboratory safety protocols.

8.4 DSC Experiment Graphs

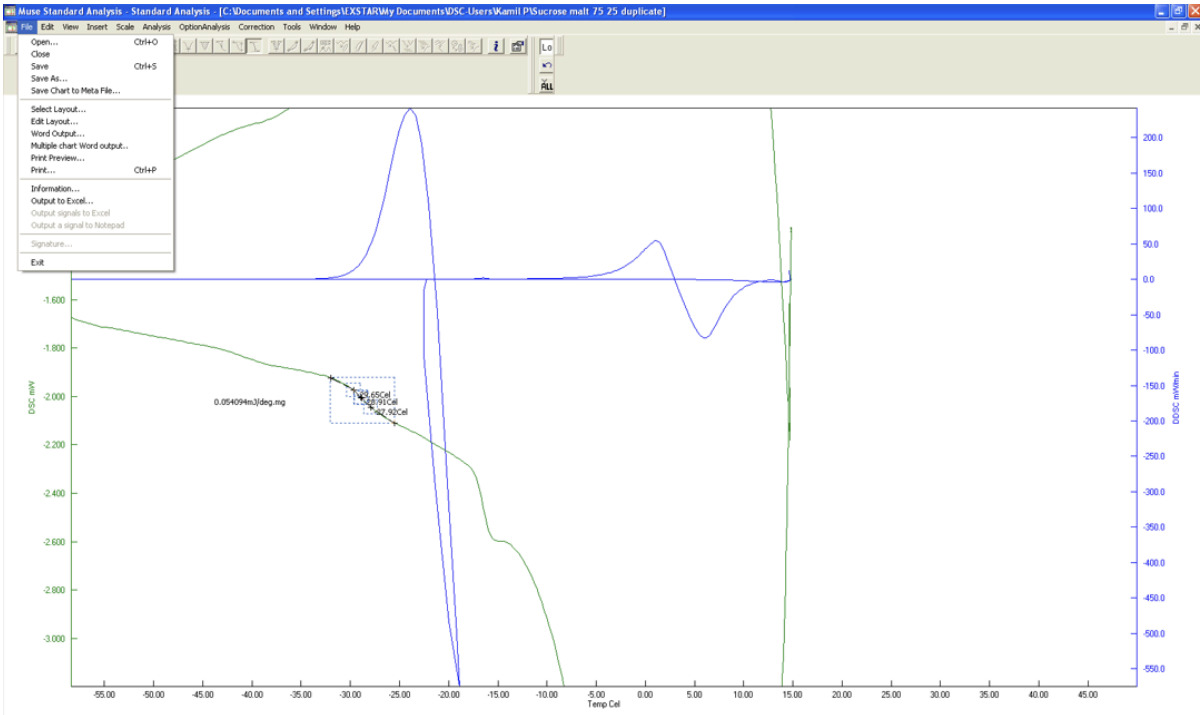
4.1 65:35 Sucrose Maltodextrin 10% (w/w)



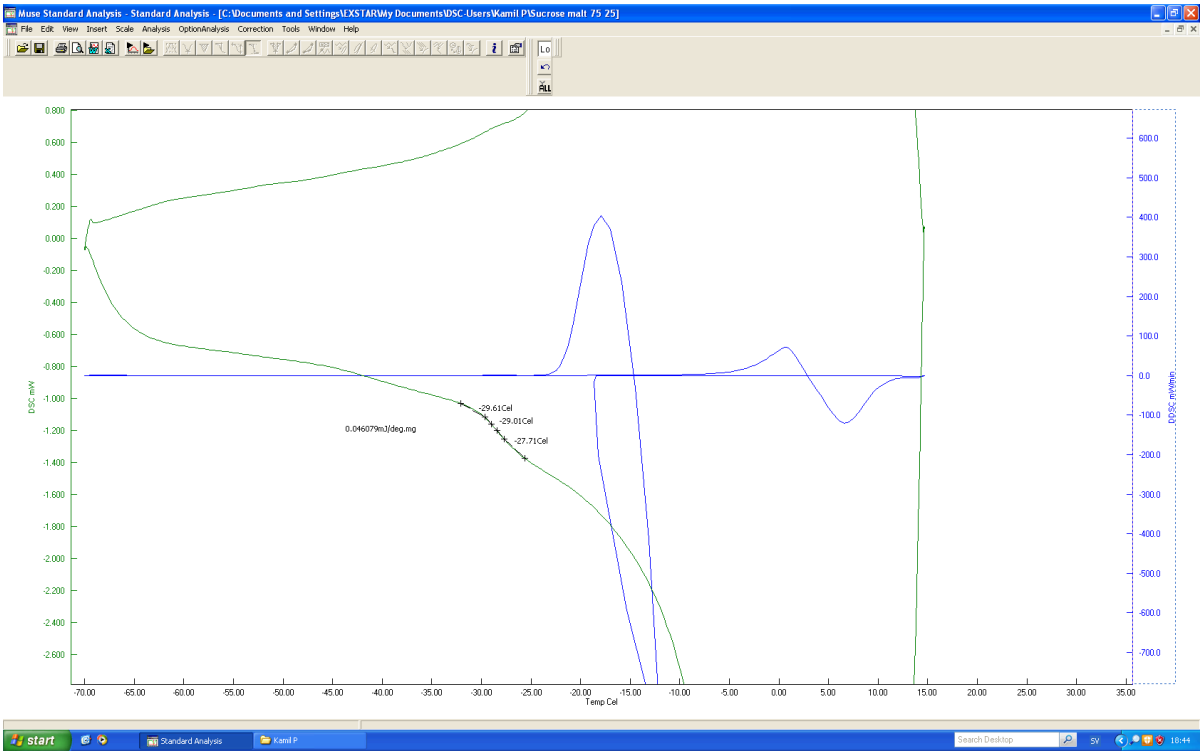
4.1.2 65:35 Sucrose Maltodextrin 10% (w/w) Duplicate



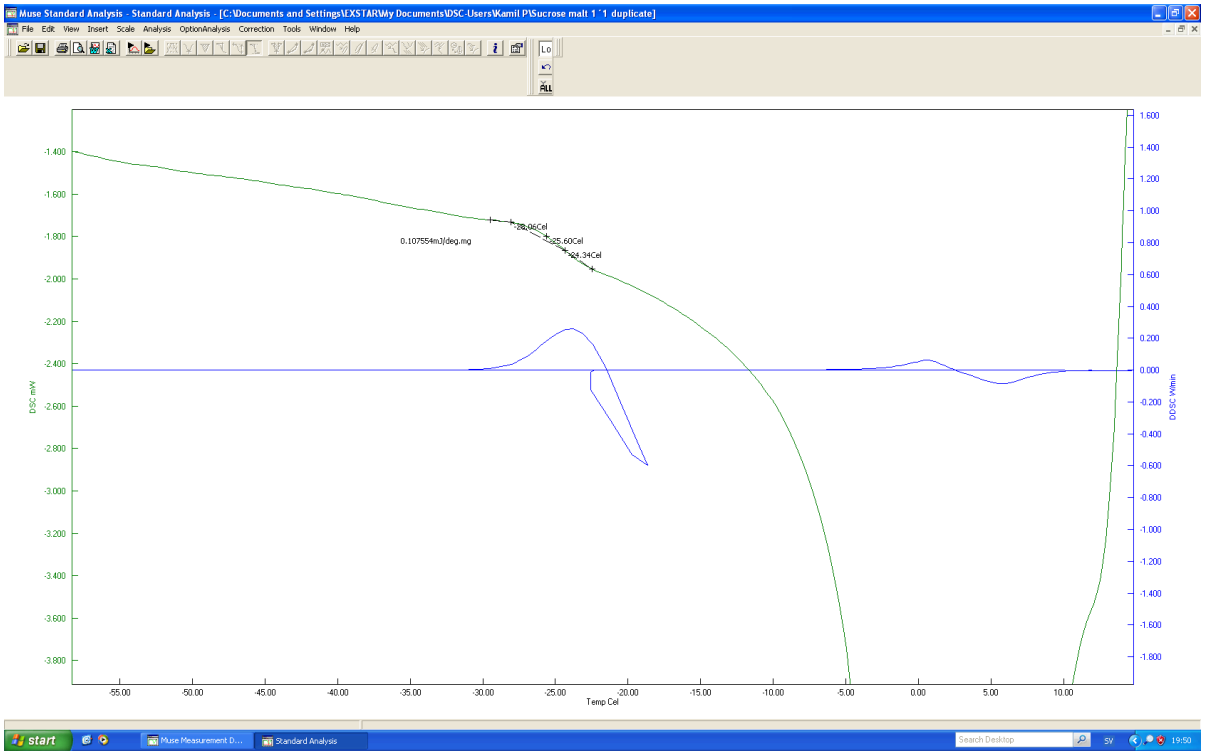
4.2 75:25 Sucrose Maltodextrin 10% (w/w)



4.2.1 4.2 75:25 Sucrose Maltodextrin 10% (w/w) Duplicate



4.3 1:1 Sucrose Maltodextrin



8.5 Tables showcasing light microscopy pictures

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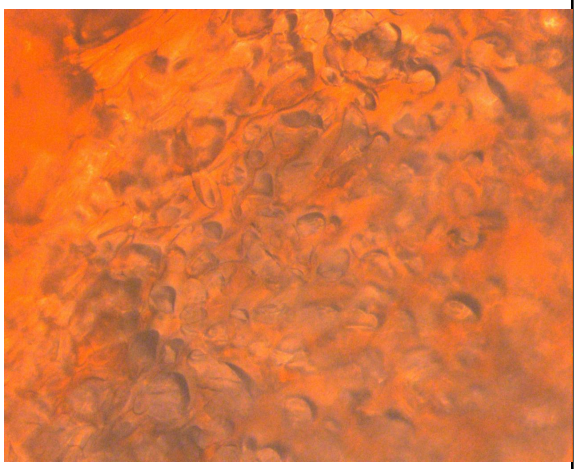
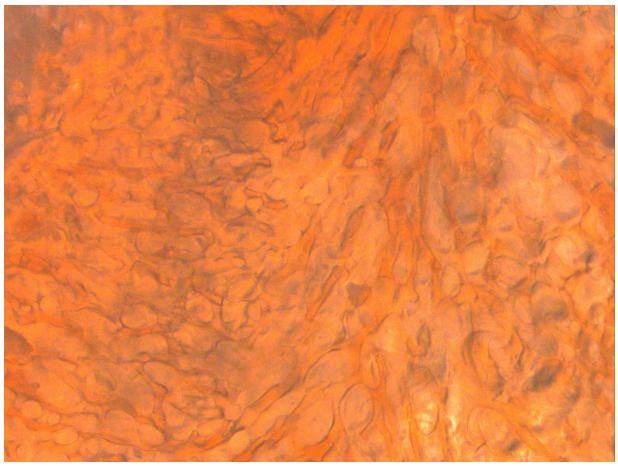
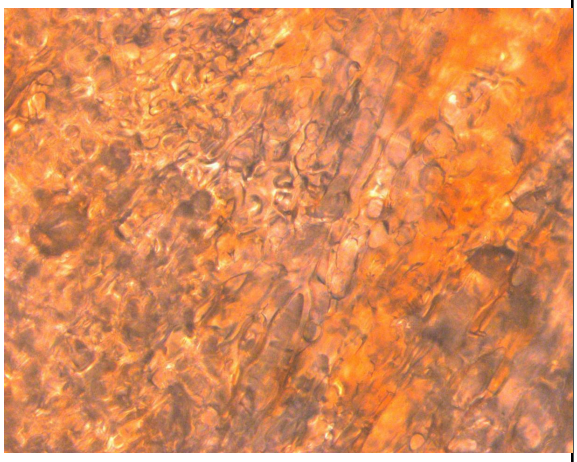
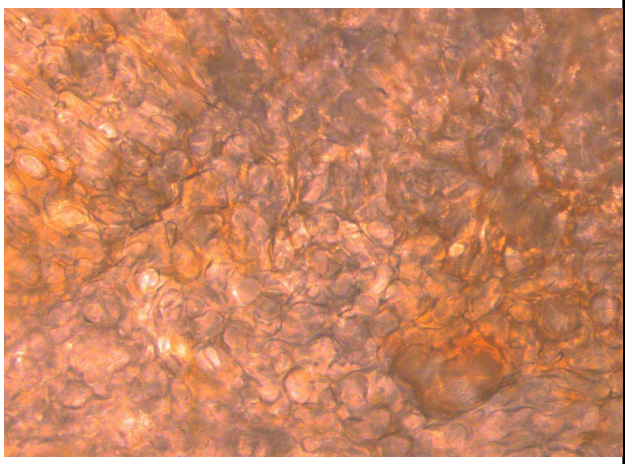
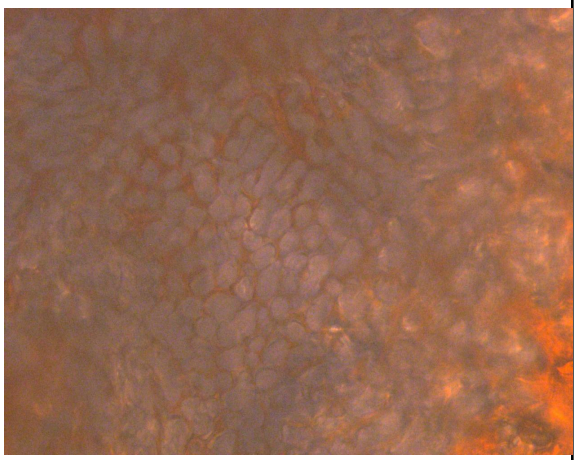
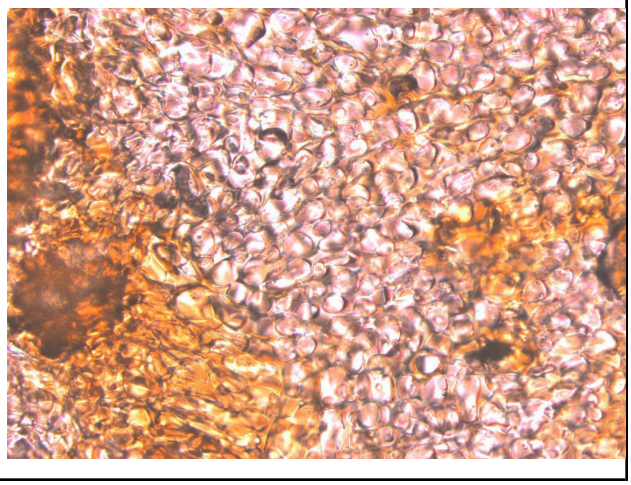
75:25		
65:35		
1:1		

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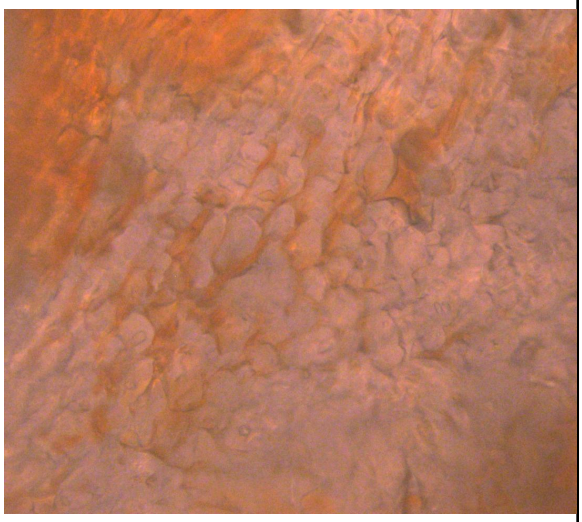
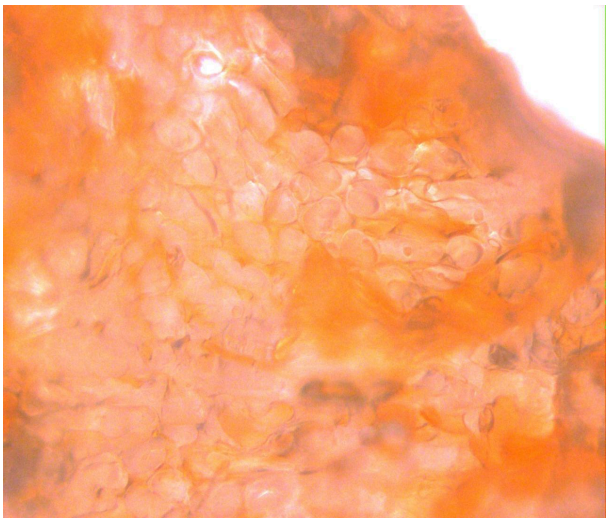
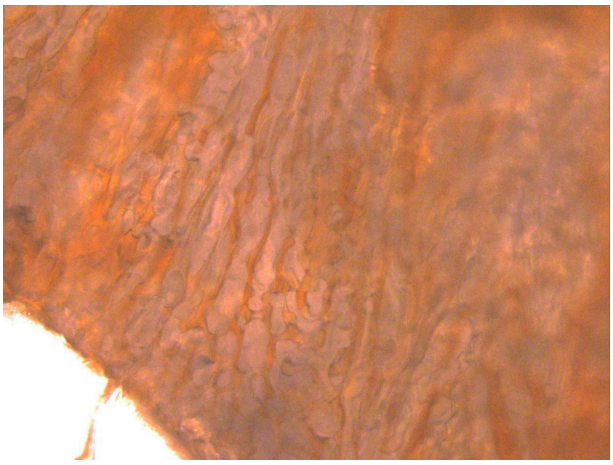
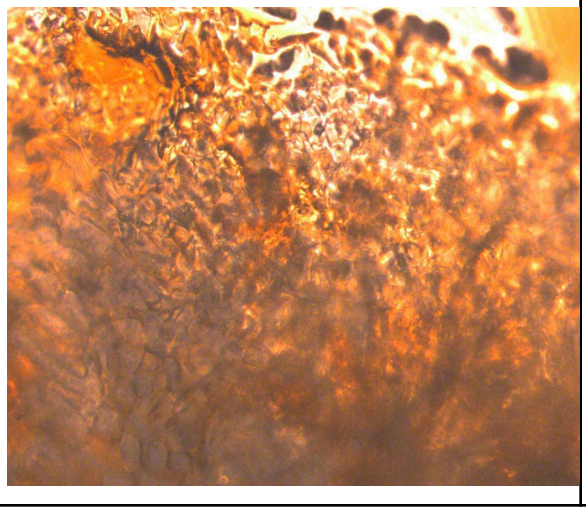
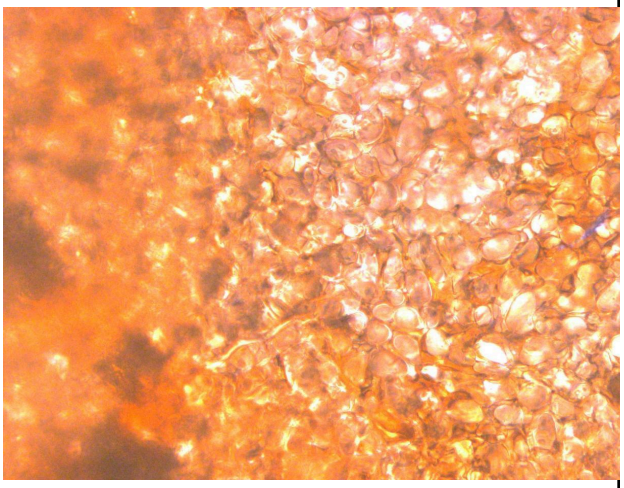
75:25		
65:35		
1:1		

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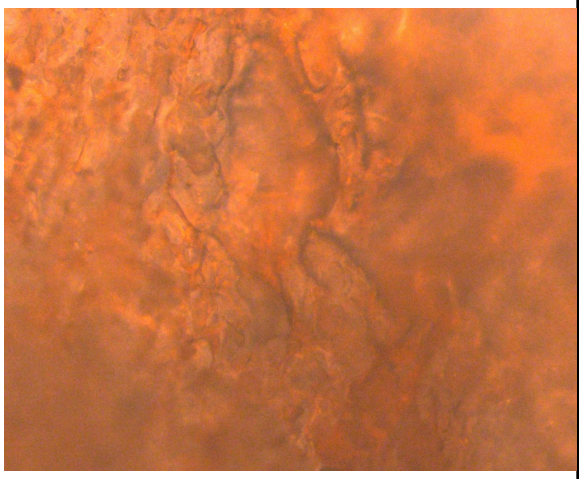
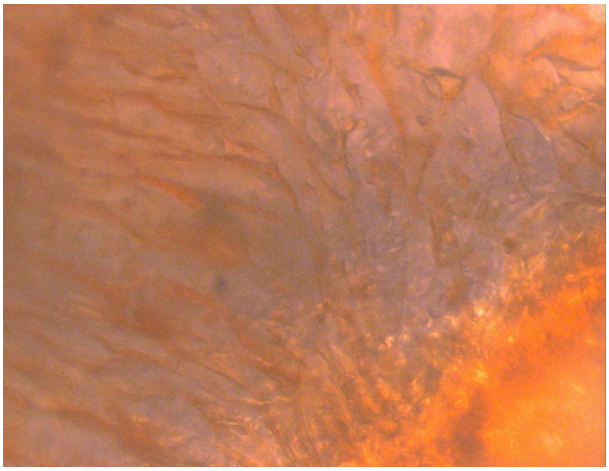
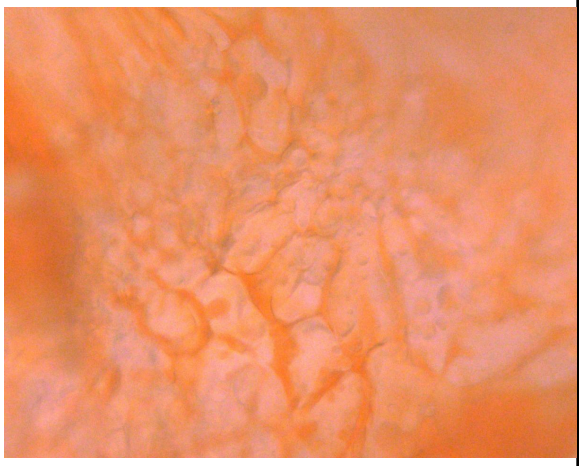
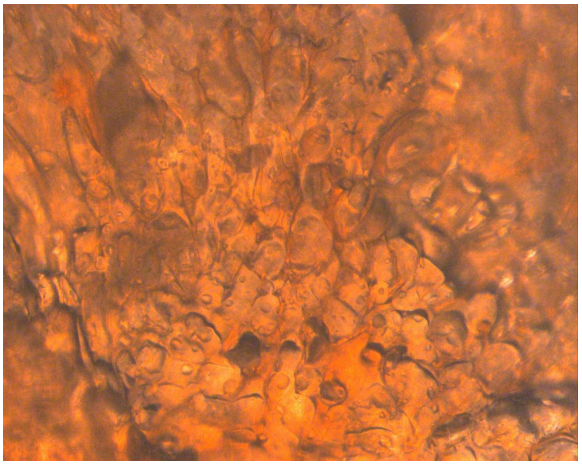
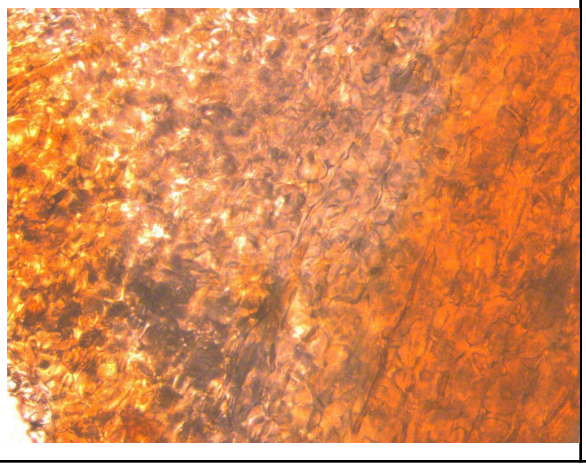
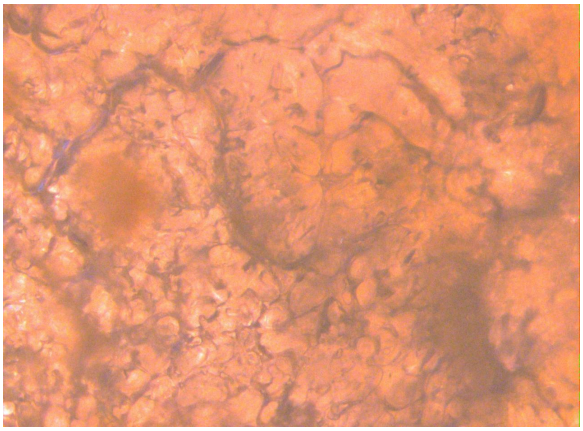
75:25		
65:35		
1:1		

8.6 Code Used to Generate Image by Python Script

```
import cv2
import numpy as np
import matplotlib.pyplot as plt
from skimage import measure, color, morphology
from scipy import ndimage as ndi

# Load the image
image_path = 'C:\\Users\\Kamil\\Desktop\\Directory Python\\65; 35 40% LARGE.p
image = cv2.imread(image_path)

# Check if the image was loaded correctly
if image is None:
    raise FileNotFoundError(f"No such file: '{image_path}'")

# Convert to grayscale
gray_image = cv2.cvtColor(image, cv2.COLOR_BGR2GRAY)

# Apply a Gaussian blur to reduce noise and improve contour detection
blurred_image = cv2.GaussianBlur(gray_image, (11, 11), 0)

# Apply Otsu's thresholding
_, thresholded_image = cv2.threshold(blurred_image, 0, 255, cv2.THRESH_BINARY

# Invert the image
thresholded_image = cv2.bitwise_not(thresholded_image)

# Use morphological operations to clean up the segmented regions
kernel = np.ones((3, 3), np.uint8)
cleaned_image = cv2.morphologyEx(thresholded_image, cv2.MORPH_OPEN, kernel, i
sure_bg = cv2.dilate(cleaned_image, kernel, iterations=3)

# Distance transform and thresholding
dist_transform = cv2.distanceTransform(cleaned_image, cv2.DIST_L2, 5)
_, sure_fg = cv2.threshold(dist_transform, 0.5 * dist_transform.max(), 255, 0
sure_fg = np.uint8(sure_fg)
unknown = cv2.subtract(sure_bg, sure_fg)

# Marker labelling
_, markers = cv2.connectedComponents(sure_fg)

# Add one to all labels so that sure background is not 0, but 1
markers = markers + 1

# Mark the region of unknown with zero
markers[unknown == 255] = 0
```

```
# Apply the watershed algorithm
markers = cv2.watershed(image, markers)
image[markers == -1] = [255, 0, 0]  # Mark boundary with red color

# Color each segment with a unique color
segmented_image = color.label2rgb(markers, image, kind='avg')

# Measure properties of labelled regions
regions = measure.regionprops(markers, intensity_image=gray_image)

# Print equivalent diameters of the pores
for region in regions:
    if region.label == 0:  # skip background
        continue
    print(f'Label: {region.label}, Equivalent Diameter: {region.equivalent_dia

# Display the images
plt.figure(figsize=(20, 10))

plt.subplot(2, 3, 1)
plt.title('Original Image')
plt.imshow(cv2.cvtColor(image, cv2.COLOR_BGR2RGB))
plt.axis('off')

plt.subplot(2, 3, 2)
plt.title('Grayscale Image')
plt.imshow(gray_image, cmap='gray')
plt.axis('off')

plt.subplot(2, 3, 3)
plt.title('Blurred Image')
plt.imshow(blurred_image, cmap='gray')
plt.axis('off')

plt.subplot(2, 3, 4)
plt.title('Thresholded Image')
plt.imshow(thresholded_image, cmap='gray')
plt.axis('off')

plt.subplot(2, 3, 5)
plt.title('Cleaned Image')
plt.imshow(cleaned_image, cmap='gray')
plt.axis('off')

plt.subplot(2, 3, 6)
plt.title('Segmented Pores')

plt.imshow(segmented_image)
plt.axis('off')

plt.show()
```