Abstract:

The idea behind this project was to realize a major unit operation such as bulk-protein freezing with Quality by Design (QbD)-principles. Freezing is a common method for storage of therapeutic proteins at industrial scale. Identification of Critical Process Parameters (CPP) is important to gain process understanding and improve the controllability. Freezing inside a 700 mL pilot freeze container by Zeta Biopharma was investigated using a test panel generated with Design of Experiments (DoE). Lactic Dehydrogenase (LDH) was chosen as model protein due to its sensitivity towards freezing/thaw stress. 31 experiments, each lasting 24 hours, were performed and the impact of the CPPs on Critical Quality Attributes (CQA) were evaluated. This allowed DoE-based process optimizations. The results indicated that temperature is the main reason for extensive LDH-inactivation during the freezing process. pH-measurements during freezing and thawing suggested that substantial pH drops resulting in pH-values as low as 3.2 are the reason for partial inactivation of the enzyme, observed particularly at low storage temperatures. The change of buffer systems and the addition of surface active substances (Tween 80) to new buffer systems showed significant improvements. The pH drops were not identified after exchanging NAP buffer with Tris-HCl buffers. The determination of the critical process parameters according to QbD principles was successful for LDH and we envisage that the procedure will be applicable to identify optimal freezing conditions for a wide range of other pharmaceutical proteins using this method.

Content

Prologue:

The reason for me to choose this topic for my diploma thesis was my strong interest in the technical subjects during my biotechnology course. As soon as I have heard there was the possibility of a process optimization project I decided to take the chance and to apply for it. After getting introduced into the subject and getting a description of the idea behind this thesis my decision was fixed. Through this project I was able to gain knowledge about the inactivation mechanism that appears during freezing and storing of proteins at temperatures below the freezing point. I think this thesis helped me to improve my existing skills in many different ways. I am really glad that I had the opportunity to work for the RCPE and the Technical University of Graz during this project. Thanks to these two institutions I was able to concentrate completely on the work I had to do without worrying about financial issues. I am also very grateful for all the people who helped me during this journey and were able to motivate me all this time. Especially I want to thank Ulrich Rössl who supervised me and helped me in time of need and Professor Bernd Nidetzky for additional input and ideas for the project.

1. Introduction:

The main aim of this project was to assess a major unit operation like bulk-protein freezing with QbD principles such as CQAs (critical quality attributes) and CPPs (critical process parameters). QbD is used to make objective decisions only on the basis of data. It is an already proven science method and can be used for risk identification. 6 parameters were defined as potential CPPs (most of the CPPs are defined by the FDA) for a freezing process using a 700 mL freeze container provided by Zeta Biopharma and shall be evaluated in a test panel generated with respect to Design of Experiments (DoE)-principles using the software Modde. Operating with Modde makes it possible to screen for relevant parameters within the design space by sampling of only a fractional amount of data points (1).

For the design of the experimental test set up DoE and QbD principles were followed. Both methods are strongly discussed by the FDA (Food and Drug Administration) and the pharmaceutical industry (2-7).

Why is Design of Experiments (DoE) used?

- Development of products and design of new processes
- Modification of existing processes or products
- Optimization of the quality and performance of already existing products
- Optimization of manufacturing processes that are available
- Screening for important process factors
- Cost optimization and reduction of pollution
- Testing of the robustness of products and processes

Areas where DoE is used:

- Chemical industry
- Food industry
- Biotechnology industry
- Pharmaceutical industry

DoE is an important method used for experiments in real life systems but also for deterministic or random simulation models. There are three main problems to which DoE can be applied. The first important objective is screening. The screening process is used for identification of the most influential factors and for determination of the ranges where these factors should be investigated. Usually, the screening procedure only contains a few experiments when compared to the amount of factors that are screened. The second important objective is the optimization procedure. The main idea behind this objective is to define the combination of the important factors that will lead to optimal operating conditions. Since optimization is a more complex process than the screening itself usually one will need more experiments per factor compared to the screening set up. The third experimental objective is the robustness testing. The main idea is to identify the sensitivity of the product or the production set up when small changes in the setting occur. These changes normally are based on fluctuations in the factors occurring when a „bad day“ for productions appears (8).

DoE started with agricultural experiments by Sir Ronald Fisher in the 1920s and continued with chemical experiments by George Box in the 1950s. Design of experiments got more popular because of the increased use of computer codes important for designing chemical processes. For real life experiments it is not practical to investigate too many factors, ten are a maximum. Also the amount of levels for each factor are limited. Five levels per factor are the limit. For experiments following DoE principles a full factorial design is often used, for example a 2^{k-p} design. For this design every k value only contains two different levels/values from all the possible 2^{k} combinations only 2^{-p} have to be performed and observed. For an example a 2^{6-3} design means that from the $2^{6} = 64$ possible combinations only $2^{-3} = 1/8$ have to be performed. So only 8 experiments must be performed and observed. This type of design is allowed when it is assumed that a first order polynomial is an adequate approximation. (9-15).

Quality by Design is a method used for pharmaceutical developments and has a focus on product and process understanding and the control of the process. That means processes are designed and developed with the aim of ensuring a high already defined product quality (1; 16). Therefore, QbD is based on knowledge of the impact of formulations and different process variables on the quality of the product. In the last few years scientists have defined several other points for QbD especially concerning the development of new drugs (3; 5).

For monitoring the product quality CQAs (critical quality attributes) had to be defined. These CQAs are defined by the ISPE PQLI (International Society for Pharmaceutical Engineering Product Quality Lifecycle Implementation) (5). CQAs are biological, microbiological, physical or chemical properties or characteristics that must be monitored to make a statement about the quality of the product. For the test set up that was generated at the beginning of the experiments CQAs were predefined (soluble protein concentration (recovery in %); specific activity (recovery in %); aggregate number (/mL); mean aggregate size (µm equivalent circular diameter)). These CQAs had to be in a specific range to be able to ensure the quality of the results that were achieved. CQAs must not only be attributes of the final products even attributes of raw materials or intermediates can be used as CQAs.

Furthermore, CPPs had to be chosen for designing the very first test set up. There are different opinions of what a critical process parameter is. For some scientists it is any input or output factor that has to be measured and controlled to reach a predefined process quality and consistency (1). Usually, a parameter is said to be critical when a change of this factor

can lead to a different quality of the process or the CQAs. So the importance of a parameter is dependent on the size of the change one is willing to consider, as an example an impeller speed of zero can always be considered as a fail (1).

The interactions between the parameters and their dependence from each other were studied by performing freezing experiments with a variation of the values according to a D-optimal screening design. The defined parameters are freezing time (1-12 h), thawing time (1-12 h), holding time (0-11 h; decrease of the temperature down to -2 °C and keeping it stable at this point, it is a part of the freezing time), set temperature (-10°C,-24°C,-38°C), volume (250 mL, 475 mL, 700 mL) and recirculation of the protein fluid after thawing (Yes or No). The protein that is used for the experiments is Lactic Dehydrogenase (LDH). The sensitivity of this protein towards freezing/thawing (F/T) stresses enables efficient and reliable testing.

The impact of the F/T experiments on LDH stability shall be determined by 4 CQAs.

Enzyme activity was chosen as an overall protein stability parameter. Aggregate number and aggregate size must be regulated as well, because only a certain number of particles of certain sizes are allowed in biopharmaceutical products. For particles with a diameter ≥ 10 µm only 25 particles per ml are allowed. For particles with a diameter ≥ 25 µm 3 particles per ml are allowed (17). Soluble protein concentration was chosen as a complementary aggregation/precipitation parameter.

31 experiments, each lasting 24 hours were performed. After completion regression analysis was employed to fit the results to an interaction model and the impact of the stated CPPs on the CQAs were predicted from the results. This should allow further DoE-based process optimizations.

Conclusions for the general impact of different process factors on protein stability during freezing cannot be drawn from these experiments. Also, LDH is not applied as therapeutic protein in the present form. Nevertheless, the proposed procedure will be valid for identification of CPPs for freezing of various therapeutic proteins such as monoclonal antibodies, cytokines or vaccine components. Knowledge about CPPs will furthermore aid scaling of the freezing process. Even the screening for effective cryoprotectants during formulation development will be possible, making our method a valuable tool for efficient drug product development.

2. Materials and Methods:

Materials:

L-lactic dehydrogenase that origins from rabbit muscle that is stored in ammonium sulfate suspension and lyophilized bovine serum albumin (BSA) were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals and reagents used were from Carl Roth GmbH + Co. KG, Karlsruhe, Germany. The desalting columns PD-10 from GE Healthcare, Little Chalfont, UK, were used for buffer exchange.

Sample preparation:

At the beginning of every experiment the LDH solution, purchased from Sigma-Aldrich (Product No: L2500, 14.3 mg/mL), was desalted using gel filtration columns (PD-10 desalting columns provided by GE-Healthcare) and transferred into 50 mM sodium phosphate buffer with a pH of 7.5. 1620 µg of LDH for 700 mL experiments, 570 µg for 250 mL experiments and 1050 µg for 475 mL experiments were dissolved in sodium phosphate buffer with an end volume of 2.5 mL, before loading on the column. Afterwards, 3-4 mL of buffer were used for the elution of the protein.

The protein concentration was determined by Bradford assay, using Roti-Nanoquant liquid (Carl Roth). This method is based on an absorbance shift after binding of the dye Coomassie Brilliant Blue G-250 to the protein. After the binding reaction an absorbance shift from 465 nm to 595 nm appears. The red colour of the solution that adsorbs at 465 nm shifts through the formation of a dye-protein complex under acidic conditions to a blue colour that adsorbs at 595 nm. With an increase of the protein concentration an increase of light adsorption at 595 nm can be measured linearly. The increase can be determined using a spectrophotometer (18).

The calibration curve was generated using bovine serum albumin in a range of 1-150 µg/ml.

Tab.1 Calibration curve with bovine serum albumin (1-150 µg/mL)

Sample	**BSA stock (0.5 mg/mL) µl**	**Buffer µl**
BSA Standard – 0 µg/ml	0	1000
BSA Standard – 5 µg/ml	10	990
BSA Standard – 10 µg/ml	20	980
BSA Standard – 50 µg/ml	100	900
BSA Standard - 100 µg/ml	200	800
BSA Standard - 150 µg/ml	300	700

The concentration of the LDH in the solution filled into the freezing reactor was 10.75 µg/mL (± 1.37 µg/mL) for each experiment. The filling volumes were 250 mL, 475 mL or 700 ml of protein-sodium phosphate solution.

Design of Experiments:

Modde™ by Umetrics, Umeå, Sweden was used for designing the test panel. It was also used for analyzing and evaluating the results. The interactions between the parameters and their dependence from each other were studied by performing freezing experiments with a variation of the values according to a D-optimal screening design. The defined parameters are freezing time (t_F, 1-12 h), thawing time (t_T, 1-12 h), holding time (t_H, 0-11 h), set temperature (T_S, -10°C,-24°C,-38°C), volume (V, 250 mL, 475 mL, 700 mL) and recirculation of the protein fluid after thawing (Yes or No). The protein that is used for the experiments is Lactic Dehydrogenase (LDH). The sensitivity of this protein towards freezing/thawing (F/T) stresses enables efficient and reliable testing. Specific activity (recovery in %), soluble protein concentration (recovery in %), aggregate number(particlrs/mL) and mean equivalent circular diameter of aggregates were defined as critical quality attributes (CQA). Contour plots and coefficient plots were also generated with Modde™.

Freezing and Thawing:

Every F/T experiment had a duration of 24h. t_F and t_T were defined as the time for reaching the respective target temperature. Freezing of the fluid was done by decreasing the thermofluid temperature linearly from the starting tempreature 20°C to the defined set temperature within the set t_F period. Thawing started 24 hours after experiment start, less the chosen t_T. Therefore the thermofluid temperature had to be increased linearly until 20°C were reached. The final temperature of 20°C had to be reached 23 hours after the experiment start to assure the complete thawing of the liquid within the last hour. t_H was defined as a pre-cooling phase – a part of the t_F – with a thermofluid target temperature of -2°C. When a t_H appeared, the thermofluid had to be cooled down to -2°C as soon as the experiment started. Afterward, the temperature was decreased linearly to the T_S during the rest of the t_F period. Recirculation during thawing was accomplished using a peristaltic pump at a speed of 45 mL/min. The monitoring of parameters like (set temperature, pressure, etc. can be done with the Spylight software. The temperature of the frozen solution can be determined at seven different position's using the PCE-T 800 Multi-Input thermologger (19).

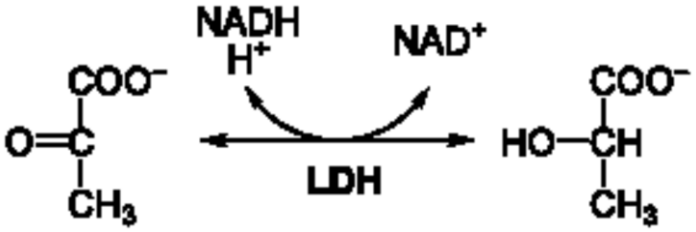
Response Analytics:

After the F/T cycle the solution was withdrawn from the freezer and the analytics were done. Specific activity and soluble protein concentration were measured after removal of aggregates by centrifugation (10 min/ 16.1 x g /4 °C). All parameters were measured before and after every freezing experiment. Values were presented as percentage of initial activity/concentration or as particle concentration (less the initial concentration) and particle diameter.

The counting and sizing of formed protein aggregates was done using an MFI 5100 (Protein Simple, Santa Clara, California). Sample duplicates were measured and averaged. To prevent counting of formed air bubbles circularity- and intensity filters were applied. The MFI is an instrument that enables automatic particle analysis. It combines micro fluidics, image processing and digital microscopy. An image of every particle passing the flow cell is taken. A database containing information about particle count, size, transparency, and morphology and shape is generated. These images can be watched at the monitor in real time (19).

The samples were centrifuged (10 min/ 16.1 x g /4 °C) and the protein concentration was measured as mentioned above using a Bradford assay.

The conversion of L-lactate and NAD+ into pyruvate and NADH was used to determine the specific LDH activity. The lactic dehydrogenase is able to catalyze this reaction.



Pyruvate Lactate

NADH absorbs at 340 nm, NAD+ at 260 nm (20). The amount of transformed NADH can be determined and the specific activity can be calculated. In the centrifuged samples the conversion of 89 mM L-lactate and 4.5 mM NAD^+ to pyruvate and NADH (TRIS buffer, 50 mM, pH 8) was monitored with a spectrophotemeter at 340 nm and 37°C (Beckman Coulter DU 800 spectrophotometer).

The Glass transition temperature (T_g') was identified with a DSC 204 F1 Phoenix™, equipped with a μ-Sensor and an intracooler (Netzsch, Selb, Germany). The heating rate for the DSC measurements was set with 40 K/min. DSC (Differential Scanning Calorimetry) is an important analytical technique that is used to measure the amount of heat that is needed to increase the temperature of a sample. The difference between the heat that is needed to increase or decrease the sample temperature to reach the reference sample temperature is shown in a function of the temperature. The reference probe and the sample are both kept at the same temperature during the whole experimental set up. Usually the sample holding system increases the temperature linear over time so that the generation of a function for the temperature over time is possible. This technique was developed by E.S Watson and O.J. Oneil in 1962 (20).

The idea behind this method is that when the sample undergoes a change in its physical properties like for example a phase transition. The amount of heat that flows to the sample

strongly depends on whether the process itself is an endothermic or an exothermic process. A good example is the phase transition of a sample from a solid state to a liquid state. This type of phase transition will lead to a strong flow of heat to the sample to increase the sample temperature at the same rate as the reference. This is caused by strong adsorption of heat by the sample due to the endothermic phase transition process from the solid to the liquid phase. The results of a DSC experiment are expressed as a curve of the heat flux versus the overall time. This method can be used for the identification of the so called glass transition point (20-22)

DSC measurements and the interpretation were performed at the RCPE by Marko Hainnlütz.

Beside the stability experiments also conformational changes through the experiments should be monitored. Therefore, Circular Dichroism (CD) spectroscopy (Jasco J715 spectropolarimeter, Jasco, Groos-Umstadt, Germany) and fluorescence spectroscopy were used.

Fluorescence spectroscopy is a kind of electromagnetic spectroscopy that is used to analyze fluorescence from samples. It uses light in most cases ultraviolet light that excites electrons in the molecule and brings them to emit light, in most cases visible light. The protein conformation-sensitive fluorophore ANS (1-anilinonaphthalene-8-sulfonate) was used for our fluorescence studies. ANS is a sulphonated naphthalene with an anilin group. The backbone and the anilin ring are hydrophobic and the sulfonate group has negative charge. ANS is able to interact with positive charged amino acids like lysine, histidine and arginine. The aromatic rings of ANS can bin to apolar sides of the protein. This is important because natively folded proteins usually bury the hydrophobic elements inside them. Only under denaturating conditions the hydrophobic elements can get exposed on the surface. Only when the protein is not fully denaturated ANS can bind in a stable way to the protein because it needs the interaction of the sulfonate part with positively charged amino acids and the interaction of the aromatic ring with hydrophobic residues. This is only provided in not fully denaturated proteins. The excitation wavelength of ANS is usually around 350-380 nm. When it bind to proteins it is usually shifted to the blue wavelength range. (23-25).

Optimization:

For further optimization experiments the data of the contour plots were used to reach optimized values for the CQAs by regulating temperature, fill volume, freezing time and holding time as well as recirculation.

In the next step a first series of optimization runs were designed using Modde™. Modde™ suggests specific runs with defined parameters to check if the further generated data allows a prediction of the output factors.

pH Monitoring:

During the F/T experiments the pH value was measured using the InPro 3251electrode attached to an M400 transmitter by Mettler-Toledo (Greifensee, Switzerland). Using the LabView™ software (National Instruments, Austin, Texas) and an NI 9203 current input module the values were recored throughout the run. The calibration of the pH electrode was done at room temperature.

Freezer design:

Using the Zeta pilot freezer by Zeta Biopharma GmbH, Lieboch, Austria makes investigations of bulk freezing effects in volumes up to 700 mL possible. It allows online monitoring of the bulk temperature at up to four different positions. The whole system is shown in picture 1. The vessel is made out of stainless steel. A circulating thermofluid is used for cooling the jacket and the cooling coils inside the vessel. The cooling is done by an external freeze controller (Tango Nuevo thermostat by Peter Huber Kaeltemaschinenbau GmbH, Offenburg, Germany). As thermofluid silicone oil (M40.165.10 by Huber) was used. The temperature measurement during the process was done with an 8-channel PCE-T 800 Multi-Input thermometer. At the beginning of the experiment the temperature of the sample was around 20°C ± 1°C. The thermofluid was equilibrated for 10 min to 20°C. To keep the conditions process-near no seeding was performed. (26)

Pic.1 Zeta Pilot Freeze container without external freeze controller

3. Generation of experimental set up:

For the design of the experimental test set up DoE and QbD principles were followed. Both methods are strongly discussed by the FDA (Food and Drug Administration) and the pharmaceutical industry (2-7).

Therefore six CPPs were defined (holding time (h); freezing time (h), thawing time (h), set temperature (°C), recirculation (Yes/No), fill volume (mL)).

A design space was generated and only a reduced amount of points was tested to get valid models that make a prediction of every other point in between the space possible. The software Modde™ was used to generate the test panel based on DoE-principles (see Tab.2). Modde™ uses response surface modeling to identify the impact of the input parameters on the CQAs. Therefore, the impact of freezing time, holding time, thawing time, fill volume, recirculation and freezing temperature on the output factors protein concentration, activity, aggregate number and aggregate size was determined.

Using DoE a test panel containing 31 experiments that includes three center point experiments was generated. A broad range for activity recovery with values between 0 % and 93.8 % was observed (for details see Tab.3). Also a strong variation over one order of magnitude concerning the aggregate number was monitored. The aggregate size only showed a range between 2.7 and 5.1 $\mu m_{ECD.}$ Experiment number 22 with the highest set temperature and the longest freezing time had to be excluded from further analysis. The bulk temperature profile did not show a complete plateau after freezing time which indicates a not totally frozen solution. Therefore, for model generation only the results of the other 30 runs were used. For each response multiple linear regression (MLR) was applied in Modde™. Insignificant parameters and interactive effects were excluded from the Modde™ – generated coefficient plot to improve model quality.

Tab. 2: Experimental design of the screening project

Run Order	Freezing Time(h)	Thawing Time(h)	Holding Time(h)	Fill Volume (ml)	Set Temper-ature(°C)	Recirculation(Yes/No)
1	12	12	10	700	-10	No
2	12	1	10	250	-38	Yes
3	12	12	0	250	-10	No
4	1	12	0	700	-38	No
5	1	12	0	250	-38	Yes
6	12	1	10	700	-10	Yes
7	12	1	0	250	-10	No
8	12	12	10	250	-38	No
9	12	12	0	700	-38	Yes
10	12	12	10	250	-10	Yes
11	1	1	0	700	-38	Yes
12	12	1	0	700	-10	Yes
13	12	1	0	250	-38	Yes
14	1	1	0	700	-10	No
15	6.5	6.5	5	475	-24	Yes
16	1	1	0	250	-38	No
17	12	1	10	250	-10	No
18	1	12	0	700	-10	Yes
19	12	1	0	700	-38	No
20	12	12	0	250	-38	No
21	1	1	0	250	-10	Yes
22	12	12	0	700	-10	No
23	6.5	6.5	0	475	-24	No
24	12	1	10	700	-38	No
25	1	12	0	250	-10	No
26	12	12	10	700	-38	Yes
27	8.3	6.5	3.3	475	-24	No
28	6.5	6.5	5	475	-24	No
29	12	6.5	5	475	-24	Yes
30	8.3	6.5	3.3	475	-24	No
31	8.3	6.5	3.3	475	-24	No

4. Results and Discussion:

4.1. First experimental set up:

After performing all 31 runs following data were generated:

Tab.3 Experimental design of the screening project with response results

Run Order	Freezing Time(h)	Thawing Time(h)	Holding Time(h)	Fill Volume (ml)	Set Temper-ature(°C)	Recirculation(Yes/No)	Soluble Protein Concen-tration(%)	Specific Activity (%)	Aggregate Number	Mean Aggregate Size(µm)
1	12	12	10	700	-10	No	65.8	55.3	$2.4*10^4$	3.5
2	12	1	10	250	-38	Yes	48.3	13.2	$4.3*10^4$	3.2
3	12	12	0	250	-10	No	67.9	62.6	$1.0*10^4$	3.3
4	1	12	0	700	-38	No	35.1	0	$8.3*10^4$	4.6
5	1	12	0	250	-38	Yes	22.4	24	$3.2*10^4$	2.7
6	12	1	10	700	-10	Yes	43.8	69.9	$4.4*10^4$	4.1
7	12	1	0	250	-10	No	65.5	64	$2.9*10^4$	3.6
8	12	12	10	250	-38	No	42.9	17.8	$5.2*10^4$	4.3
9	12	12	0	700	-38	Yes	44.4	47	$5.7*10^4$	3.5
10	12	12	10	250	-10	Yes	23.3	75.6	$1.4*10^4$	3.8
11	1	1	0	700	-38	Yes	21.2	44.8	$6.8*10^4$	3.7
12	12	1	0	700	-10	Yes	32.5	29.6	$6.6*10^4$	4.3
13	12	1	0	250	-38	Yes	40.4	26.1	$4.5*10^4$	3.4
14	1	1	0	700	-10	No	62.7	76.9	$2.7*10^4$	3.9
15	6.5	6.5	5	475	-24	Yes	45.5	1.8	$3.3*10^4$	3.7
16	1	1	0	250	-38	No	27.4	0	$5.0*10^4$	3.5
17	12	1	10	250	-10	No	55.6	93.8	$3.0*10^4$	3.6
18	1	12	0	700	-10	Yes	61.2	58.3	$3.0*10^4$	4.6
19	12	1	0	700	-38	No	55.7	28.4	$3.3*10^4$	4
20	12	12	0	250	-38	No	40.8	12.4	$7.4*10^4$	3.9
21	1	1	0	250	-10	Yes	59.4	68.9	$2.7*10^4$	3.6
22	12	12	0	700	-10	No	87.0	76.5	$2.5*10^4$	-
23	6.5	6.5	0	475	-24	No	39.5	0	$4.7*10^4$	3.5
24	12	1	10	700	-38	No	35.3	0	$5.2*10^4$	4.5
25	1	12	0	250	-10	No	52.9	71.6	$3.3*10^4$	3.6
26	12	12	10	700	-38	Yes	57.4	29.5	$5.7*10^4$	5.1

27	8.3	6.5	3.3	475	-24	No	33.7	0	$7.3*10^4$	4.1
28	6.5	6.5	5	475	-24	No	43.9	0	$4.9*10^4$	3.9
29	12	6.5	5	475	-24	Yes	37.5	14.3	$1.1*10^5$	4.2
30	8.3	6.5	3.3	475	-24	No	48.8	26.1	$2.7*10^4$	4.2
31	8.3	6.5	3.3	475	-24	No	36.5	0	$5.1*10^4$	3.8

Almost all runs were designed with different freezing parameters except for three runs (27, 30 and 31). Those runs were introduced into the model to make a prediction of the reproducibility of the experiments. Those three runs show similar results which can be seen in a high reproducibility value except for the aggregate number (see below Fig.1). After all runs were performed Modde was used for response surface modeling and analysis of the results. The results of the data set can be seen in Fig.1.

The R2 value gives information about the quality of the model; it shows how good the model fits the experimental data. A high R2 value usually is important for a good model. A model with 0.5 or less only has a low significance. Only the R2 values for the activity, the soluble protein concentration and the particle size were significant.

The Q2 value in dark blue (Fig.1) gives information about how well the model predicts new data. A useable model should therefore have a high Q2 value. Only models with a Q2 value above 0.5 can be described as good models. Q2 values over 0.1 are described as significant models. For protein concentration, activity, aggregate formation and particle size we had a significant model because the Q2 value was higher than 0.1. For the activity even a value over 0.5 (0.67) was reached. Therefore the model for the activity could be described as a good model.

The third column in yellow shows the model validity. When the value is larger than 0.25 the fit of the model to the measured data is agreeable. For all parameters the model validity was higher than 0.25 so there is no lack of fit of the model (Values are set by Modde).

The reproducibility of the models is shown in light blue (Fig. 1). This is the variation of the response under the same conditions (pure error), at the center points, compared to the total variation of the response. The reproducibility results are good except for the aggregate number.

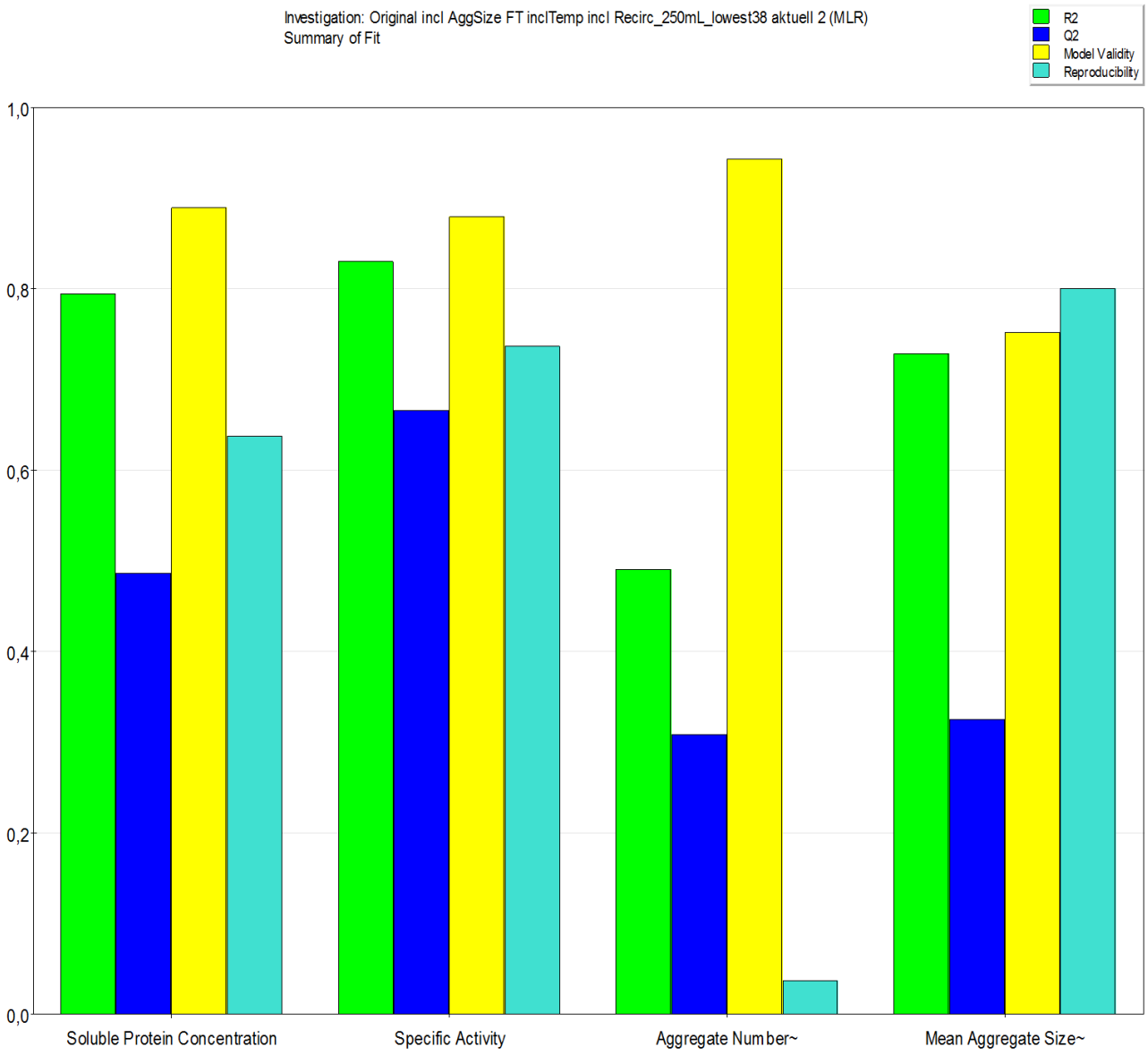


Figure 1 Values R2/Q2/Reproducibility/Model validity for each response see page 19

Tab. 4: Model properties

Responses	R2 value	Q2 value	Reproducibility	Model validity
Protein concentration	0.795	0.487	0.64	0.89
Specific activity	0.830	0.666	0.73	0.88
Aggregate Number	0.491	0.308	0.04	0.94
Mean aggregate size	0.728	0.325	0.80	0.75

Fig.1 clearly shows good Q2 values for the soluble protein concentration and the specific activity; especially for the activity a Q2 value over 0.5 could be reached which means a good prediction quality. However, the reproducibility for the aggregate number is very low which might indicate that it is very difficult to regulate this factor with the given input factors.

In the next step the parameters that have the strongest effect on the experiments should be determined. Therefore coefficient plots for the protein concentration, the activity, the aggregate formation and the particle size were generated using Modde. For those interactions that show a significant impact contour plots could be designed. Contour plots allow the visualization of interactions between process parameters. Optimal process conditions can be identified quickly and act as basis for further optimization

4.2. Soluble Protein concentration:

Following abbreviations are used:

Fre= Freezing time (h)

Tha= Thawing time (h)

Hol= Holding time (h)

Fil= Fill volume (mL)

Temp = Temperature (thermofluid set temperature) (°C)

Reci= Recirculation (Yes/No)

4.2.1. Coefficient plot soluble protein concentration:

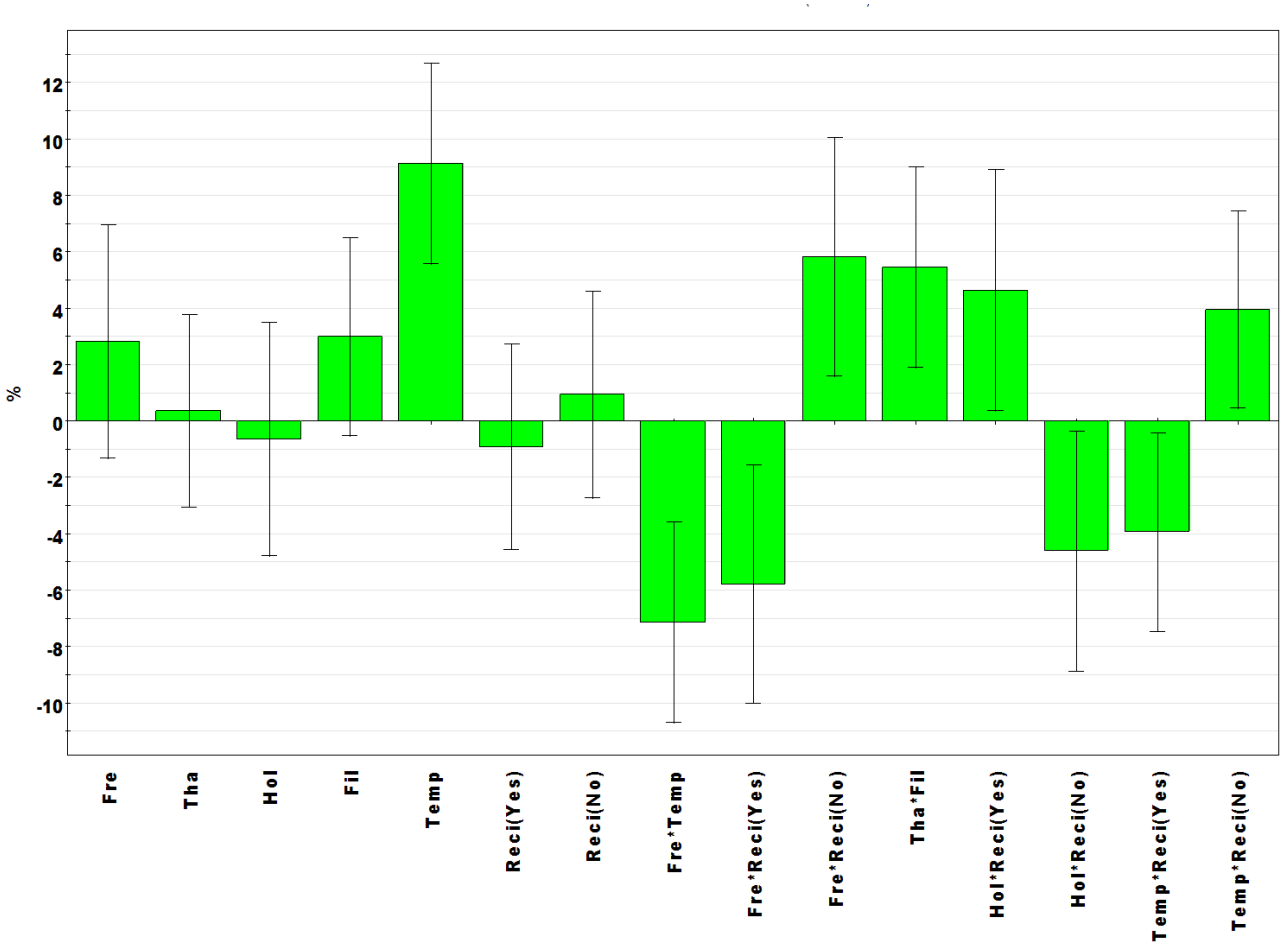


Figure 2 Coefficient plot Protein conc.

Fig.2 shows very clearly that the only significant single parameter that has an effect on the protein concentration is the temperature. It shows that lower freezing temperature leads to lower protein concentration. Higher freezing temperatures around -10°C seem to be beneficial for the protein concentration which can be visualized with contour plots. Although other factors don`t show an effect by themselves they show interactive impact on the protein concentration.

E.g. freezing time + temperature; freezing time and recirculation; thawing time combined with fill volume; holding time and recirculation and temperature combined with recirculation.

4.2.2. Contour plot soluble protein concentration

For the strongest interactive effect a contour plot was designed to see how those two factors affect the protein concentration. In this case the contour plot was designed using freezing temperature and freezing time as factors.

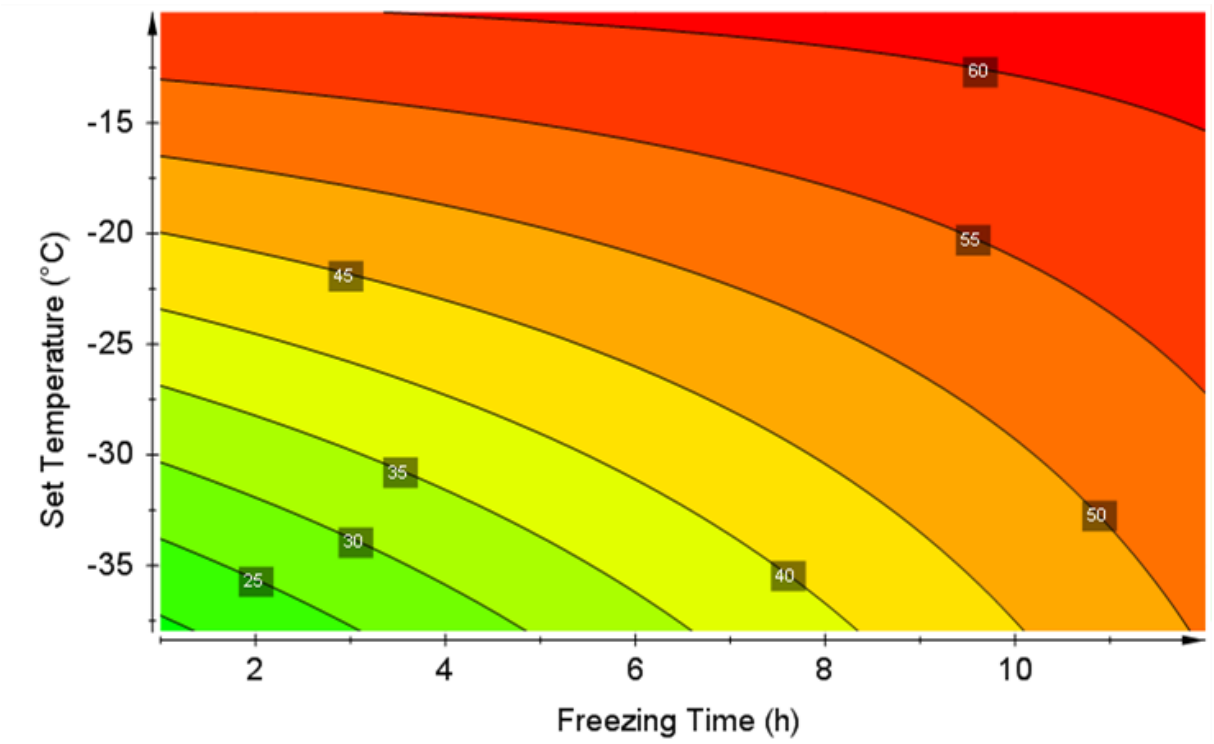


Figure 3 Contour Plot Protein concentration [%]; fill volume: 700 ml, thawing time: 12h, holding time: 10h, recirculation: off.

Fig. 3 shows that for a high protein concentration a high freezing temperature combined with a high freezing time is beneficial for achieving a high protein concentration. The fill volume is set to 700 ml, the thawing time is set to 12h, the holding time is set to 10h and the recirculation is turned off.

4.3 Specific Activity:

4.3.1 Coefficient plot Specific activity

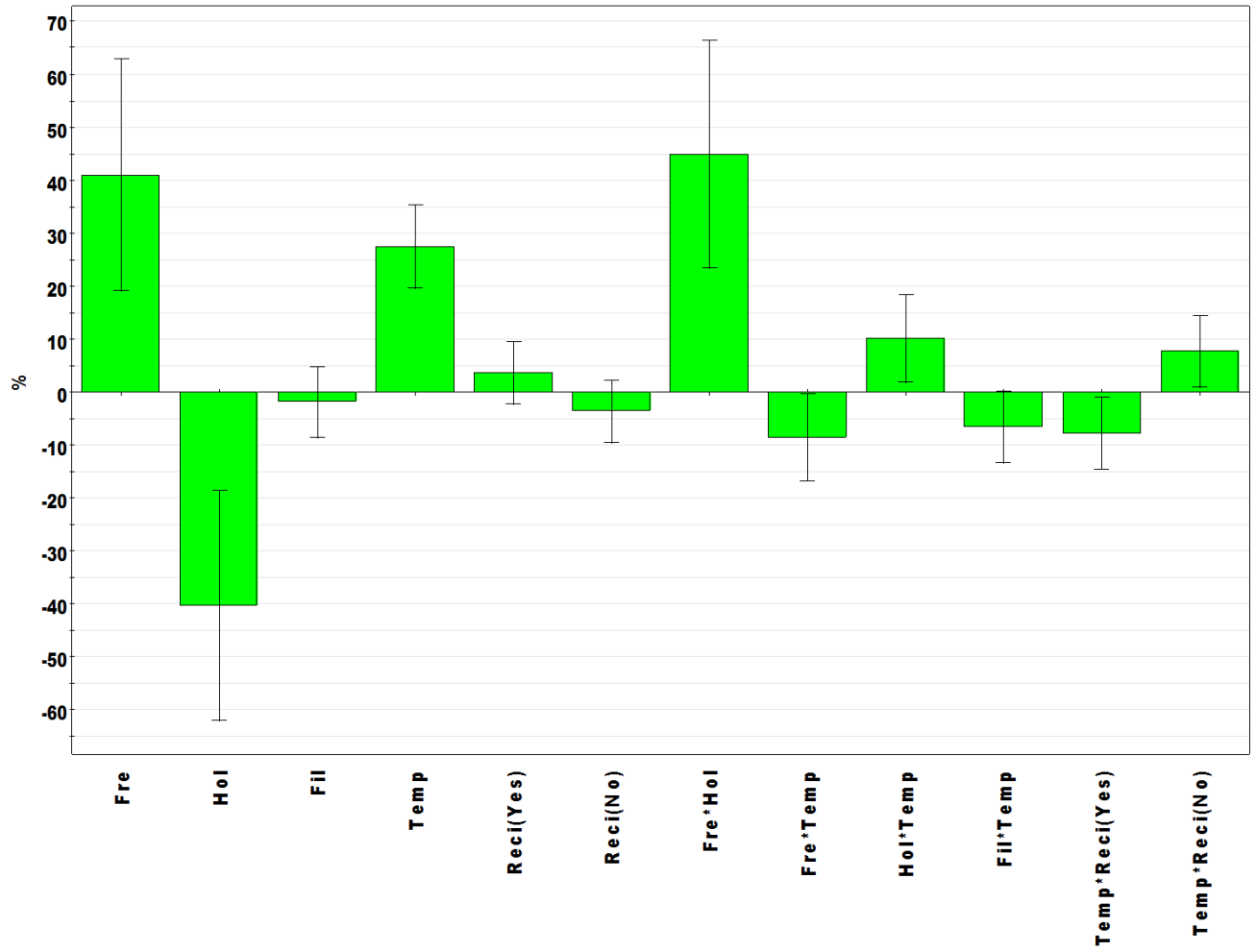


Figure 4 Coefficient plot activity [%]

Fig.4 shows that freezing time, holding time and freezing temperature have a significant impact on the activity. Significant interactive influences can be detected for the freezing time combined with the holding time, for the temperature and the holding time and for the temperature combined with recirculation.

4.3.2. Contour plot specific activity

The contour plot in Fig.5 was designed comparing the two factors freezing time and freezing temperature.

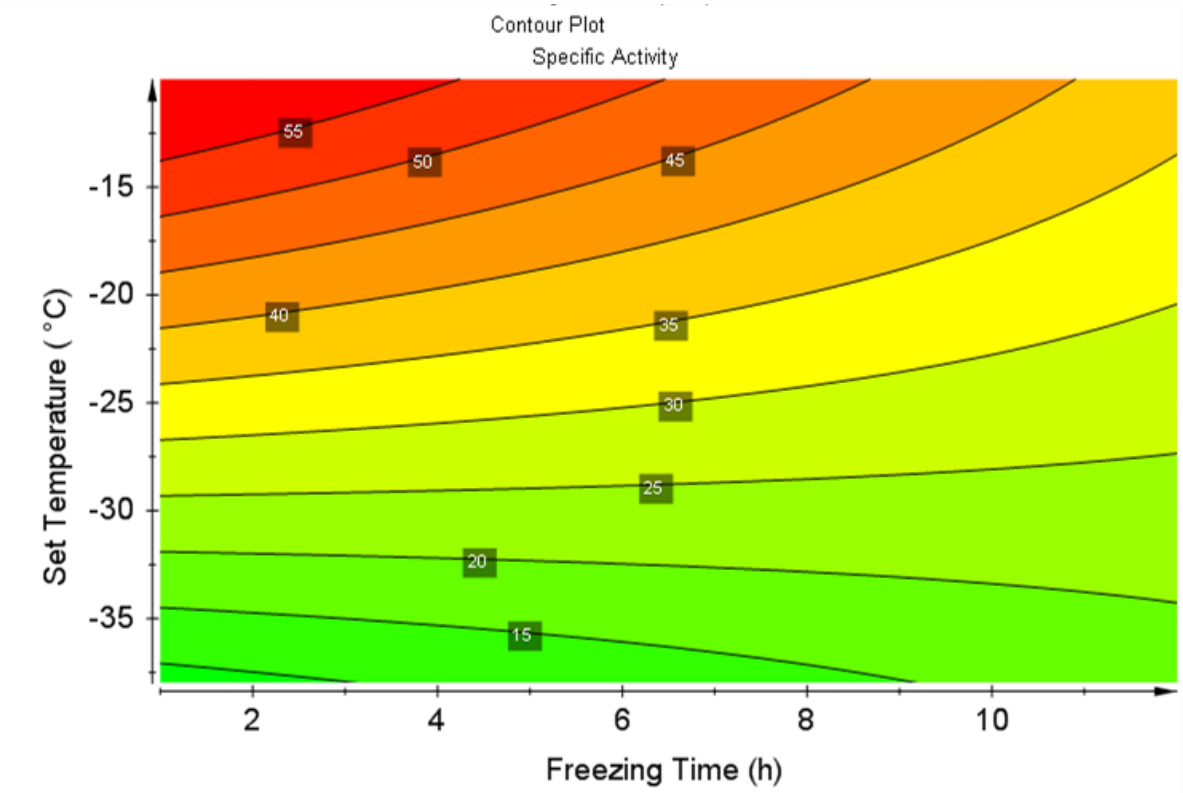


Figure 5 Contour plot specific activity [%]; fill volume: 700 ml, thawing time: 12h, holding time: 0h, recirculation: off.

Fig.5 shows that a high freezing temperature combined with a short Freezing time leads to high enzyme activity. Fill volume is again 700 ml, the thawing time is set to 12h and recirculation and holding time are both not used.

4.4. Aggregate number:

4.4.1. Coefficient plot aggregate number

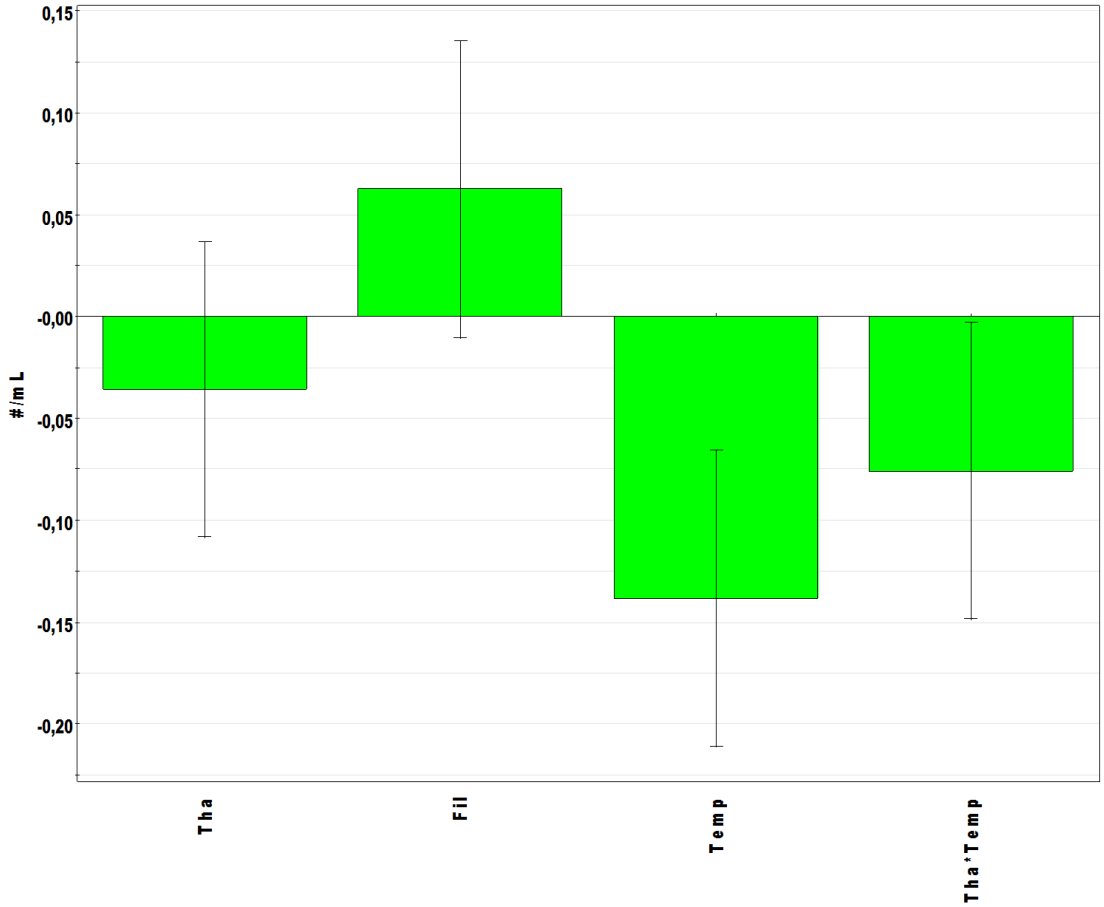


Figure 6 Coefficient plot aggregate number [/mL]

In Fig. 6 the significant factors here are the temperature and the interaction between thawing time and freezing temperature. A contour plot with those two factors can be generated to visualize how aggregate concentration is affected by these two parameters.

4.4.2. Contour plot aggregate number

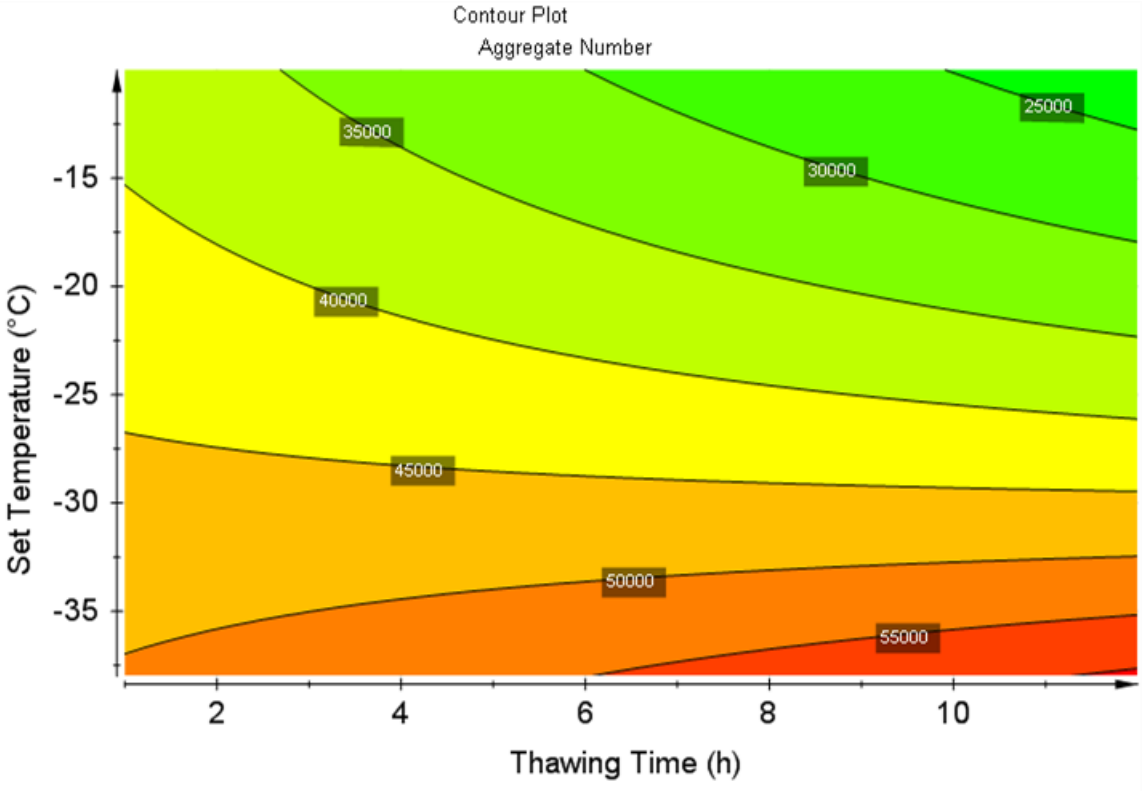


Figure 7 Contour plot aggregate number [/mL]; fill volume: 475ml, thawing time: 6.5h, holding time: 5h, recirculation: off.

To reach a low level of particles a high freezing temperature combined with a long thawing time is preferred. All other parameters are centered thus the thawing time is set to 6.5h, the holding time to 5h the fill volume to 475 ml and recirculation is turned off.

4.5. Mean size of aggregates:

4.5.1. Coefficient plot Mean size of aggregates

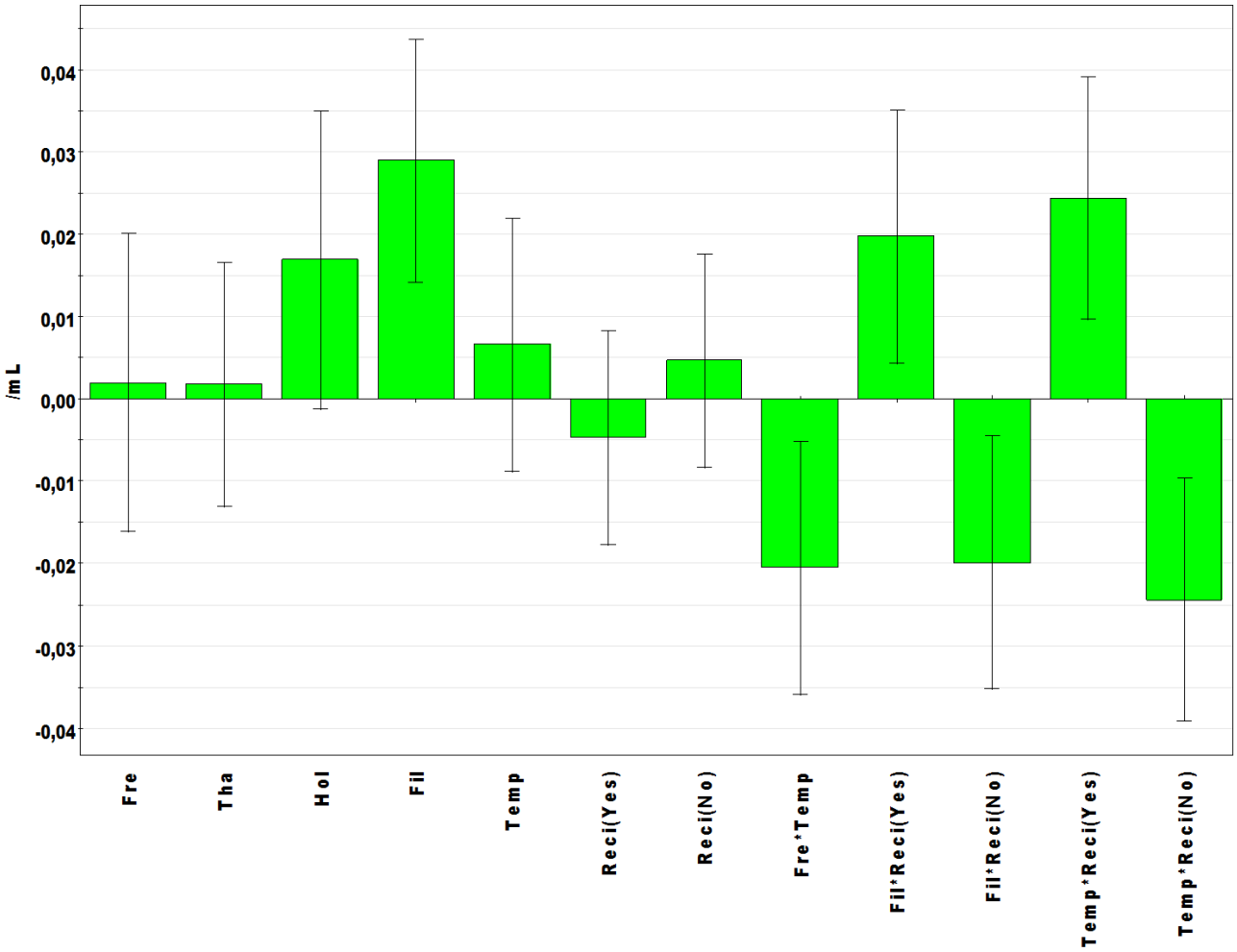


Fig.8 Coefficient plot mean aggregate size [µm]

For mean size of aggregates a high reproducibility was observed. The Q2 value shows significance as well and allows predictions for this response. The main single factor that has an effect on the size of the aggregates is the fill volume. Even five interactions between parameters seem to have an impact. Interactive parameters of importance are: freezing time coupled with freezing temperature, freezing time combined with recirculation and the fill volume combined with recirculation.

4.5.2. Contour plot Mean size of aggregates

In this case two contour plots of the interaction fill volume and freezing temperature were generated to show the strong effect of the recirculation that can be seen in Fig. 9 and 10. The first contour plot Fig. 9 shows the effect witout recirculation, the second one Fig. 10 shows the effect of recirculation.

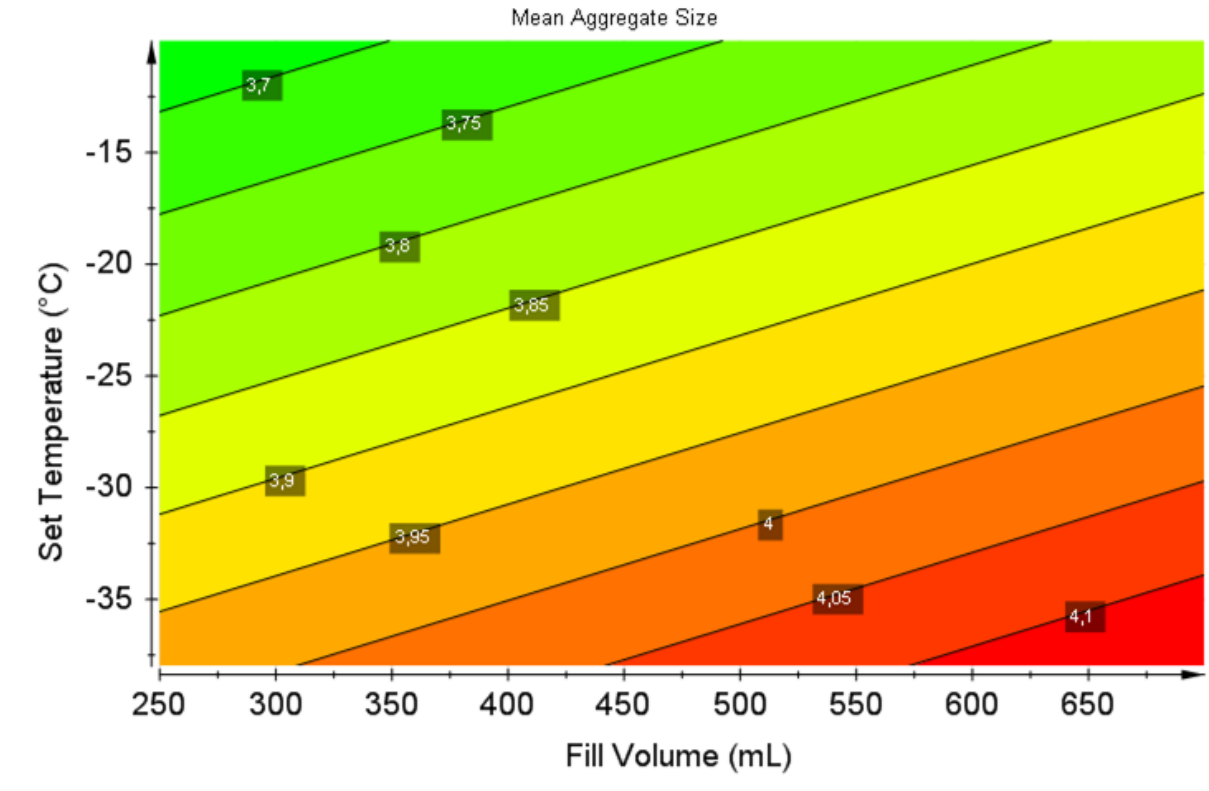


Fig.9 Contour plot mean aggregate size (µm) without recirculation holding time= 6.5 thawing time= 6.5

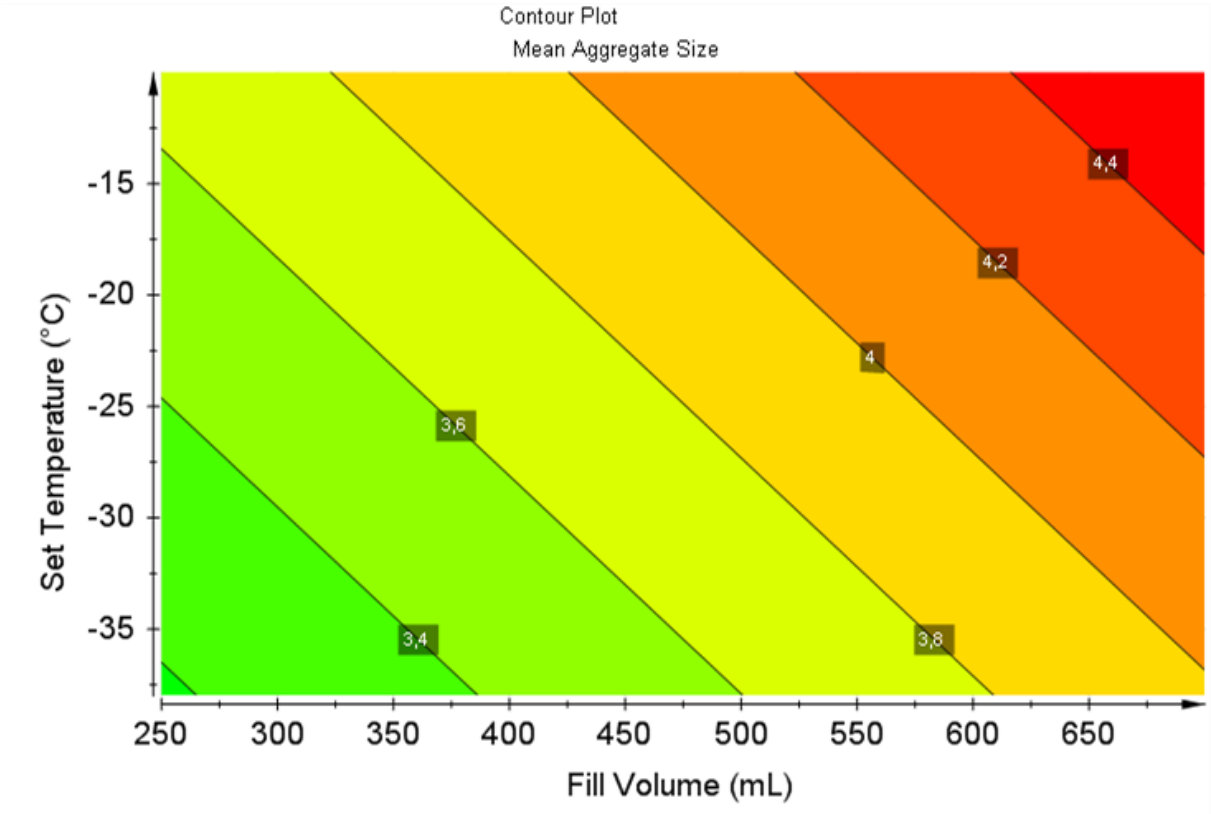


Fig.10 Contour plot aggregate size (µm) with recirculation holding time= 6.5 thawing time = 6.5

Fig. 9 clearly shows that for a small particle size a high freezing temperature and a small fill volume should be combined. As long as the recirculation is turned off. So a high freezing temperature and a low fill volume affect the particle size in a positive way. In Fig. 10 a low freezing temperature combined with a low fill volume and with recirculation is good for achieving small particles.

4.6. Optimizations:

In addition to the Modde™ -generated experiments (runs 1-13), manually designed runs were performed as well (runs 14-17). They were designed to see how good a prediction of the output factors can be done when using bad experimental conditions.

For a preliminary/manually optimized freezing process the following parameters could be chosen (just as an example). These settings should lead to good results:

1. Set Temperature: -10 °C
2. Fill Volume: 700 ml
3. Freezing Time: 2-4 h
4. Holding Time: 0 h
5. Recirculation: No
6. Thawing Time: 12 h

The high freezing temperature should lead to a high protein concentrations and high enzyme activities. Contour plots show that short freezing times combined with short holding times lead to good results for the enzyme activity, protein concentration and aggregate size. Other runs were designed using the optimizer function of Modde™ (see Tab. 5).

Those runs designed with the help of the Modde™ optimizer were performed one after another. The optimizer predicts for every run values for the soluble protein concentration, the activity, the aggregate number and the mean aggregate size. Additionally, an upper and a lower limit were suggested by the software. After finishing up all experiments the results of the experiments were compared to the predicted results by Modde. This should allow to control if the identified influences of the input parameters on the output factors are correct. Three factors were predefined, the fill volume was set with 700 mL, the thawing time was set with 1 hour and the recirculation was turned off in all experiments. This time the pH value was measured during the whole experiment with an electrode that can be used below 0°C (Mettler Toledo). It can measure the pH value within a temperature range of +70°C to -25 °C. The pH changes over time should be monitored to see if the pH value might be affecting the protein stability.

All the values in table 6-9 labeled in green are slightly higher or lower than the limitations sets by Modde. The red values are much higher or much lower than the limited regions allowed by Modde.

During the experiments especially during the activity measurements before the 24 hour experiments, the Lactate dehydrogenase showed higher activities. A reason for that might be the new batch that was used for these follow up experiments. In case of the activity a different batch was used which showed constantly higher activities during the runs:

Activity 1st run series: 112.4 U/mg ± 23.6 U/mg

Activity 2nd run series: 169.9 U/mg ± 27.4 U/mg

Tab.5 Optimization runs designed with the Modde optimizer and manually designed runs

Run Order	Freezing Time(h)	Thawing Time(h)	Holding Time(h)	Fill Volume(mL)	Set Temperature(°C)	Recirculation(Yes/No)
1	2.3	1	0.2	700	-10.9	No
2	1	1	0	700	-10	No
3	4	1	0	700	-11.7	No
4	12	1	8	700	-10	No
5	12	1	6.3	700	-14.9	No
6	4.3	1	0.2	700	-16.4	No
7	1	1	0	700	-15	No
8	10.9	1	1.1	700	-15.5	No
9	12	1	10	700	-15	No
10	12	1	6.1	700	-18.6	No
11	12	1	10	700	-21.9	No
12	6.5	1	0	700	-19.4	No
13	12	1	6	700	-19.4	No
14	12	1	0	700	-24	No
15	6,5	1	5	700	-24	No
16	12	1	0	700	-30	No
17	12	1	0	700	-34	No

4.6.1. Results for soluble protein concentration optimized set up:

As table 6 and Figure 11 show the protein concentration values of some experiments (1;4;5;7;10;11;14;15) are higher than the upper limit values predicted by the optimizer. The variety for the measured concentrations are in between 42-71 %. The green values were only slightly higher than the predicted upper limit which might be caused by a higher stability of the new LDH batch. Still a complete predictability of the concentration was not possible.

Tab6: Results measured for the soluble protein concentration during optimization set up (%)

Run Order	Soluble Proteins concentration predicted (%)	Lower	Upper	Measured
1	57	46	68	69
2	58	46	70	71
3	57	47	67	67
4	53	42	63	70
5	52	43	61	68
6	52	43	60	55
7	51	40	62	66
8	57	48	66	53
9	49	38	59	70
10	51	43	60	61
11	46	36	55	59
12	51	43	59	63
13	51	43	59	55
14	55	45	65	59
15	41	34	49	62
16	53	43	63	42
17	51	41	61	62

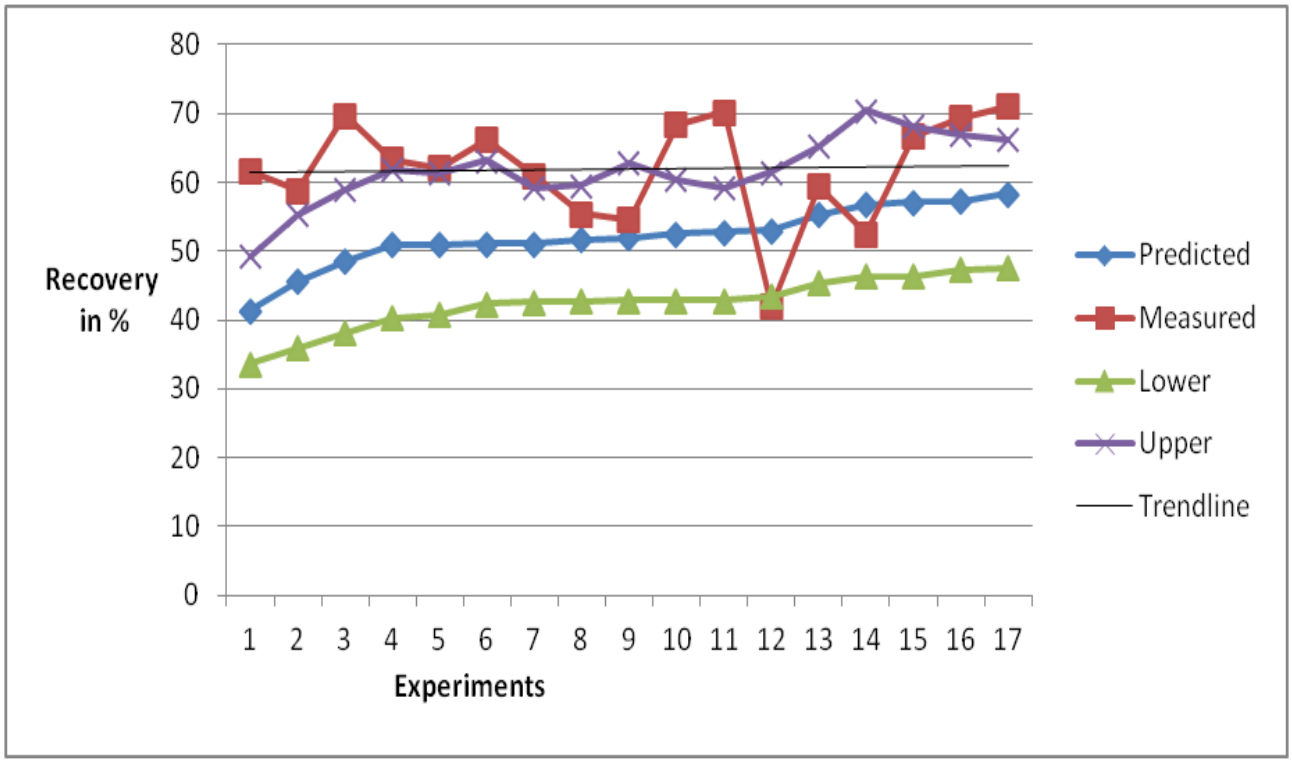


Fig 11: Comparison of Measured values and predicted values for the soluble protein concentration

Experiments were sorted by predicted protein concentration.

4.6.2. Results for the specific activity optimized test set up

As shown in Table 7 and Figure 12 some of the specific activity values that were measured are slightly higher than the predicted upper limits (1;4;5;7;10;11;14;15). Experiment 6 even shows an activity much higher than the predicted upper limit. As mentioned before, the experiments for this optimization part were performed with a LDH batch that showed higher starting activities. This might be a reason for the higher stability of the LDH protein and therefore lead to higher activity recoveries. Still the experiments show that lower freezing temperatures lead to lower post run activities. With recovery rates of 19 % to 25 %.

Tab 7: Results for the specific activity during optimization set up (%)

Run Order	Specific activity predicted (%)	Lower	Upper	Measured
1	55	36	75	83
2	62	41	84	58
3	53	35	71	57
4	61	42	81	83
5	48	32	64	68
6	42	28	57	86
7	52	35	70	62
8	36	19	52	62
9	57	38	75	78
10	42	28	56	56
11	42	27	56	53
12	37	24	51	34
13	41	27	54	77
14	40	29	52	56
15	3	0	22	25
16	30	18	42	19
17	22	10	35	22

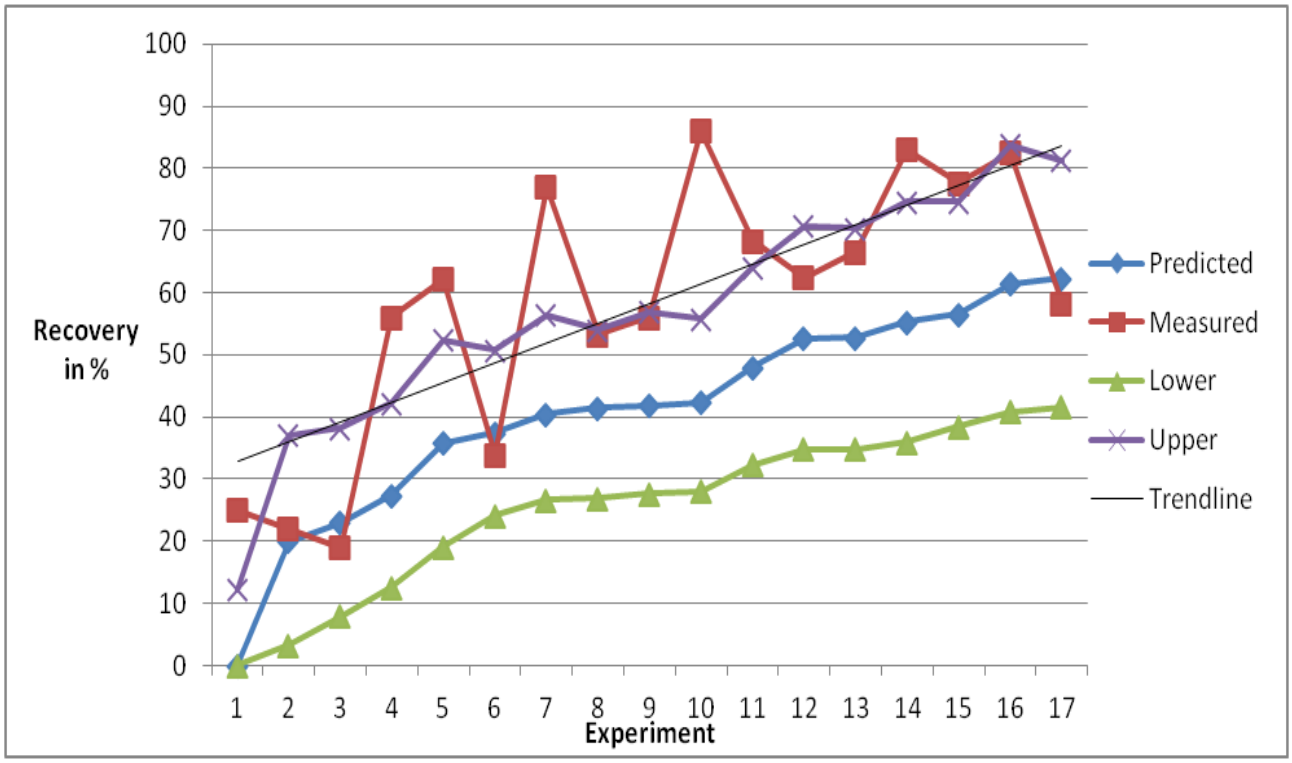


Fig. 12 Comparison of measured activity values with predicted values

Experiments were sorted by predicted activity values.

4.6.3. Results for the aggregate number:

As displayed in Table 8 and Figure 13 the number of aggregates formed during the experiments showed a good predictability. Only one value was outside of the range and showed fewer aggregates than predicted (run 17).

Tab 8: Measured results for the number of aggregates during optimization set up (/mL)

Run Order	Aggregate number predicted (/mL)	Lower	Upper	Measured
1	44200	31200	62600	-
2	43800	30600	62600	45700
3	44500	31700	62600	45700
4	43800	30600	62600	45700
5	46000	33700	62900	60200
6	46700	34600	63100	52600
7	46100	33700	62900	37100
8	46300	34000	63000	46100
9	46100	33700	62900	45000
10	47800	35900	63700	45100
11	49500	37500	65200	54200
12	48200	36300	64000	52800
13	48200	36300	64000	48000
14	51500	44500	59600	48100
15	51500	44500	59600	54500
16	54600	45300	65600	42600
17	56700	45500	70600	35200

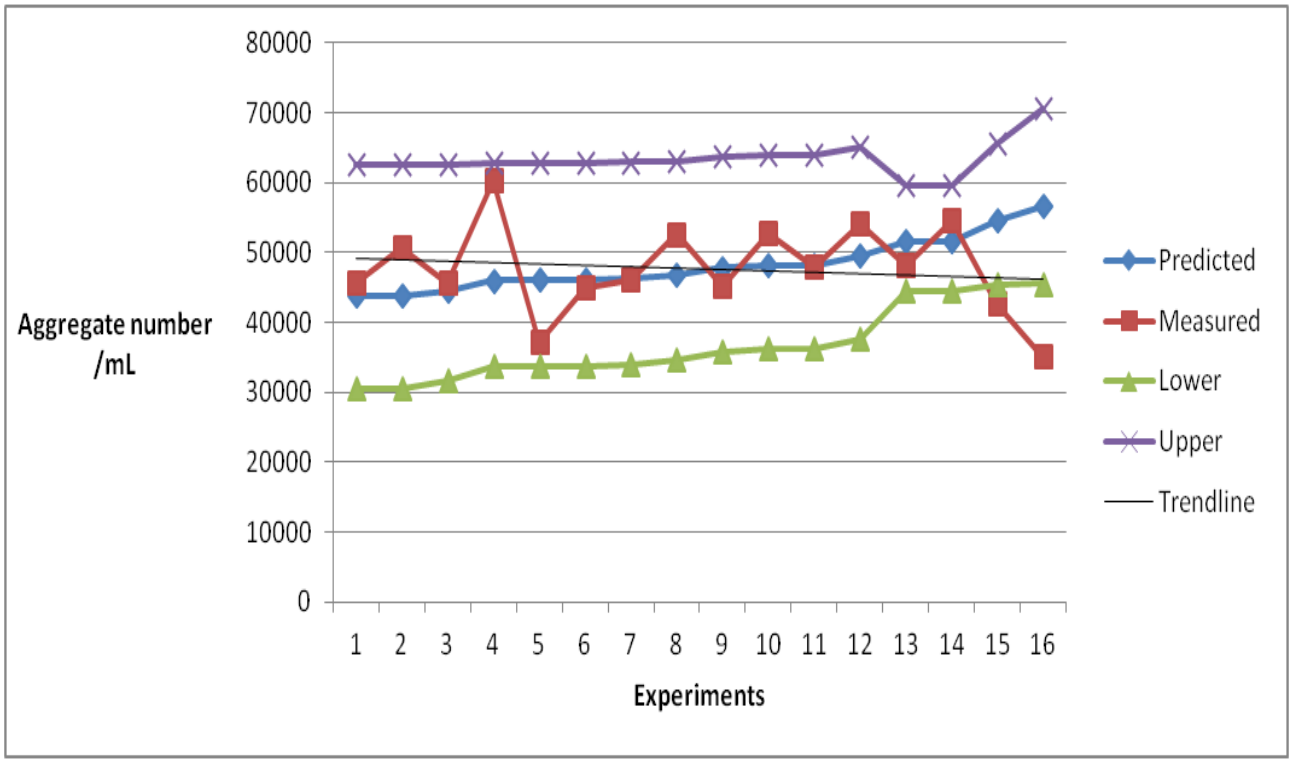


Fig.13: Comparison of measured aggregate number values with predicted values

Experiments were sorted by predicted aggregate number values.

4.6.4 Results for the mean size of aggregates

Tab. 9 and figure 14 show the measured values for the mean size of aggregates compared to the predicted values including the lower and upper limits. The results show a very good predictability almost all values are in in between the range. Only one experiment (run 13) showed a value that is outside the range.

Tab 9: Measured results for the mean size of aggregates during optimization set up (µm)

Run Order	Mean Aggregate Size predicted (µm)	Lower	Upper	Measured
1	3.8	3.4	4.2	-
2	3.8	3.4	4.2	4.0
3	3.7	3.4	4.0	3.8
4	3.7	3.4	4.1	3.9
5	3.8	3.5	4.1	4.2
6	3.8	3.5	4.1	3.8
7	3.8	3.5	4.2	3.4
8	3.7	3.3	4.0	4.0
9	3.9	3.6	4.3	4.3
10	3.9	3.6	4.2	3.7
11	4.0	3.7	4.4	3.7
12	3.7	3.5	4.1	4.0
13	3.9	3.6	4.2	4.5
14	4.0	3.7	4.2	4.1
15	4.0	3.8	4.2	3.8
16	4.1	3.8	4.4	3.5
17	4.2	3.9	4.5	4.1

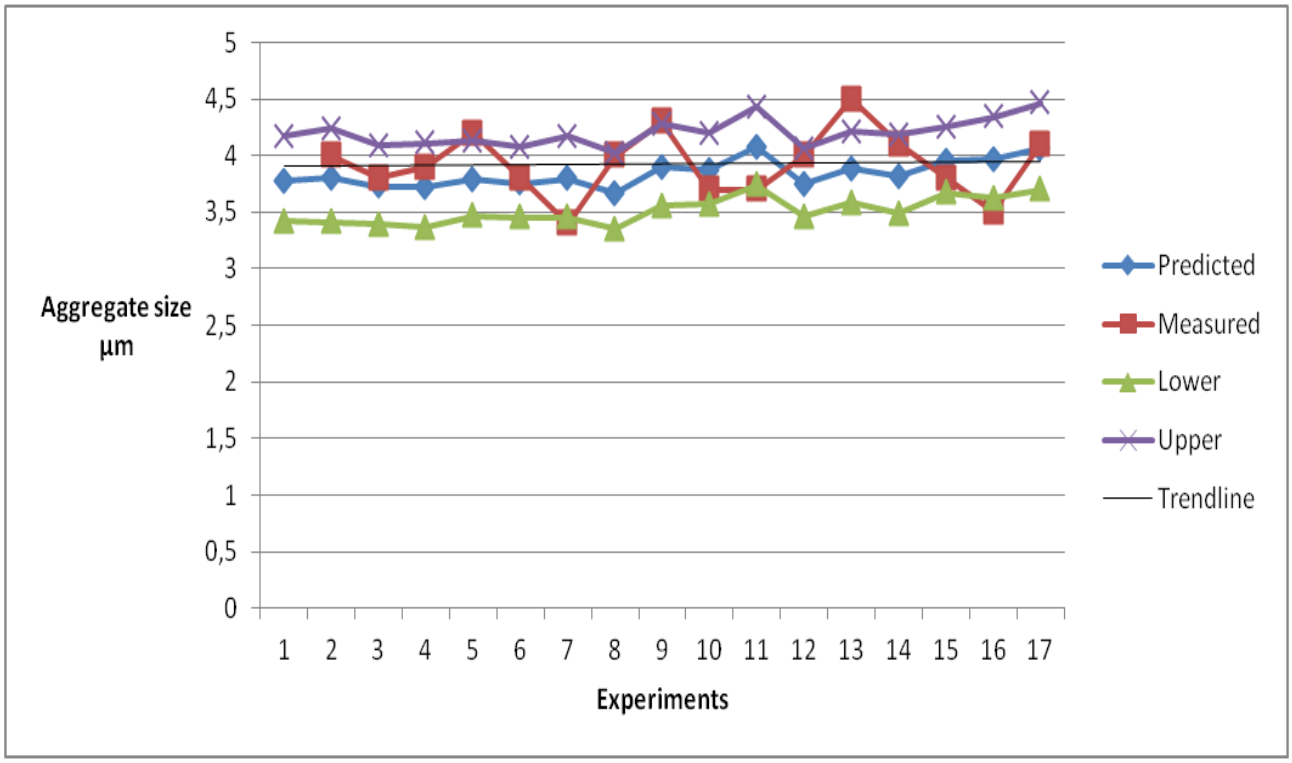


Figure 14: Comparison of measured values for aggregate size and predicted values

Experiments were sorted by predicted aggregate size.

4.6.5 Measurement of the pH value and temperature over time

During most of the experiments of this optimized test set up a pH electrode (Mettler Toledo InPro 3251 (27)) was used to measure the value over time. Figure 15 and 16 show two of these measurements, 15 is with no holding time and 16 is with a holding time. During all the experiments a drop of the pH value during the freezing time of the solution was monitored. As soon as the liquid was frozen the value rises again and the pH was stable during the rest of the run. This drop can be described by different solubilitys of the two compounds of the sodium phosphate buffer (Di- Sodiumhydrogenphosphate, Sodiumdihydrogenphosphate). During the freezing process sequential precipitation of the components lead to a strong pH shift that normalizes after the solution is frozen.

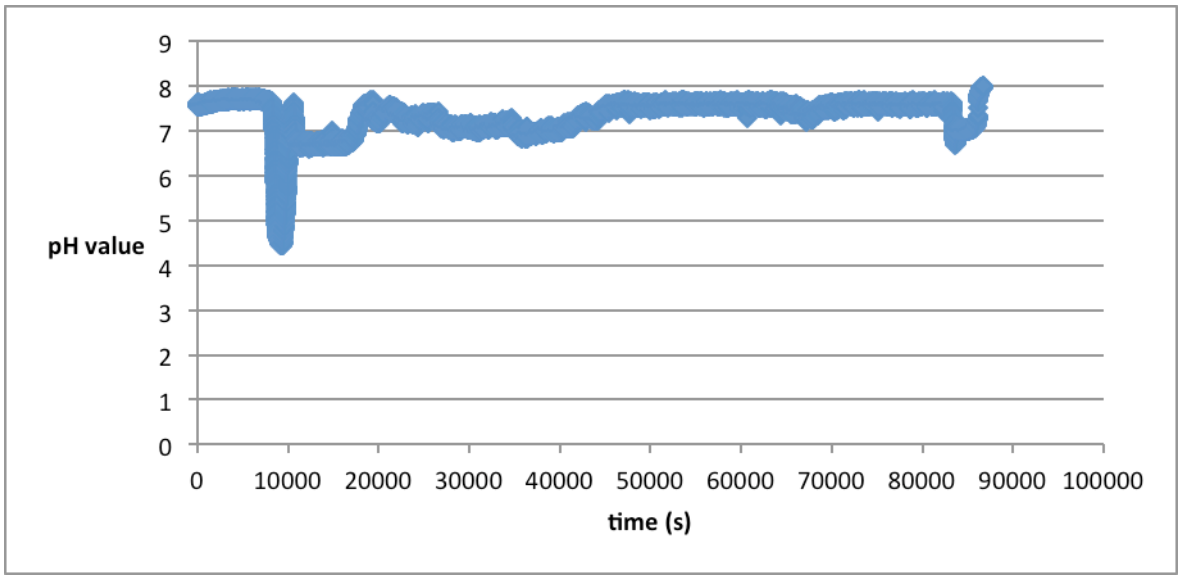


Figure 15: pH curve during freezing experiment without holding time experiment 7

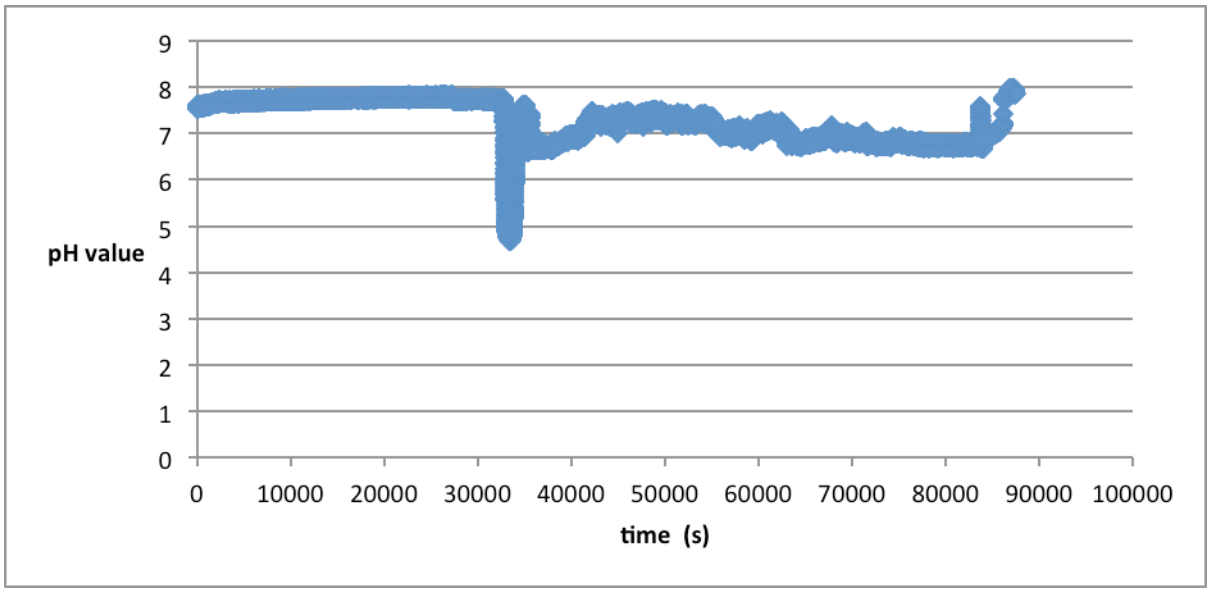


Figure 16: pH curve during freezing experiment with holding time experiment 8

4.7. Second run series for process optimizations:

For this last experimental set six new runs were manually designed, including some changes. For these experiments four temperature settings were chosen: -20°C; -25°C; -30°C; -35°C. In all six experiments the solution is frozen to a set temperature of -20°C before decreasing to final temperature. The freezing of the solution at -20°C before reducing to end temperature should help to generate an equal background for all runs. The low freezing temperatures were chosen to determine effects of cold denaturation. Additionally, in one experiment *Tween 80* (28) was added as a surfactant. *Tween 80* is used to determine if there are positive effects on the preservation of activity and protein concentration, through interactions with the surface. The concentration of *Tween 80* that was chosen was 0.02 %. Therefore, 140mg of the surfactant were added to 700mL of the buffer solution containing the LDH. In one experiment the sodium phosphate buffer was replaced by a 50 mM tris buffer with a pH of 7.5. Tris buffer is used to determine if pH shifts during the phase transition can be inhibited. Those shifts should not occur using Tris buffer.

Some of the parameters were set during all runs:

- Fill volume constant: 700 mL
- Thawing time constant: 1 h
- Freezing time constant: 2 h
- Holding time constant: 0 h
- Recirculation turned off in all experiments

Tab. 10: Manually defined test setup for further optimizations

Run number	Freezing time(h)	Buffer	Tween 80	End temperature(°C)
1	2	Sodium phosphate	No	-30
2	2	Sodium phosphate	Yes	-30
3	2	Tris	No	-30
4	2	Sodium phosphate	No	-35
5	2	Tris	No	-25
6	2	Sodium phosphate	No	-20

After all runs were performed the analysis of the data was done. Table 11 shows that the temperature reduction starting with -25 °C leads to a strong activity loss. The activity loss at -25°C is stronger than the loss at -30°C. That means that there might be a breaking point starting around -24 °C as the experiments at the beginning of the work show. Another unexpected finding was the complete activity loss within the experiment containing *Tween 80* and the strong aggregate formation in the follow up runs (Tab. 13: 2;4;6;7). The tris experiment showed as believed before no pH breakdown. The pH value increased slightly during the freezing process as shown in figure 18 and kept that level for the rest of the run. Interesting was the pH curve of the -30°C and -35 °C experiments as seen in Fig. 17, they showed a strong pH drop to 3.2 and the value stabilized during the whole run and stayed that low. Even the *Tween 80* experiment (Fig.19) showed that pH drop. These pH drops monitored when using sodium phosphate buffer were described by other groups as well for example by Katherine A. et al (29). For better statistical analysis the -30°C experiments with sodium phosphate buffer, the tris experiments and the *Tween 80* experiments were repeated two times as displayed in Tab. 12. After performing another *Tween 80* experiment the values for the aggregate size increased strongly, therefore this experiment was repeated three times instead of two times. The pH shift down to a value of around 3 was monitored during the added *Tween 80* experiments as well (see figure 19). Therefore, a run was performed in which sodium phosphate buffer was replaced by tris buffer containing *Tween 80* to check if this combination shows no pH drop like like the other tris experiments. During this combined tris- *Tween 80* experiment only a slight drop in the pH value was monitored (see Fig.20) and the results for the activity and the aggregate number were much better than the results for the sodium phosphate + *Tween 80* experiment (see table 13). An activity recovery of 83 % as well as almost no aggregate formation was observed.

Tab 11: Results for the first 6 manually designed runs (red values=Tris; green= Tween80)

Run number	Protein concentration(%)	Specific activity(%)	Aggregate number(/mL)	Aggregate size(µm)	Temperature(°C)
1	58	68	39755	3.5	-20
2	46	19	55970	4.0	-25
3	53	25	117113	3.6	-30
4	69	15	52939	3.9	-35
5	77	5	56375	4.1	-30
6	100	-	16669	3.9	-30

Tab.12: Additional runs for better statistical output

Run number	Freezing time (h)	Buffer	Tween 80	End temperature (°C)
7	2	Tris	No	-30
8	2	Sodium phosphate	Yes	-30
9	2	Tris	No	-30
10	2	Sodium Phosphate	Yes	-30
11	2	Sodium phosphate	No	-30
12	2	Sodium Phosphate	No	-30
13	2	Sodium phosphate	Yes	-30
14	2	Tris	Yes	-30
15	2	Tris	No	-30
16	2	Tris	Yes	-30
17	2	Tris	Yes	-30

Tab. 13: Results of additional runs for improved statistical output

Run number	Soluble protein concentration (%)	Specific activity (%)	Aggregate number (/mL)	Mean aggregate size (µm)
7	95	38	31918	4.6
8	92	not detectable	121895	4.1
9	97	44	37430	4.2
10	95	not detectable	164444	4.1
11	90	14	90250	3.5
12	83	17	121895	3.5
13	100	not detectable	142606	4.4
14	83	86	7866	4.3
15	61	41	26401	5.0
16	81	78	5659	3.8
17	88	88	4028	3.8

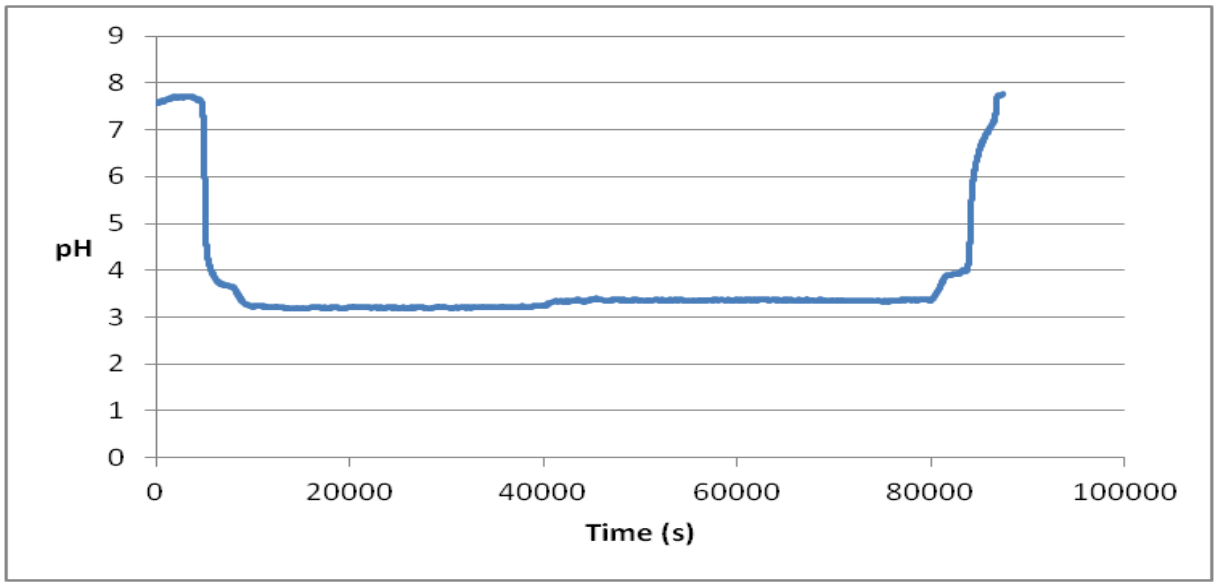


Fig. 17: pH shift during -30°C experiment using NaP buffer

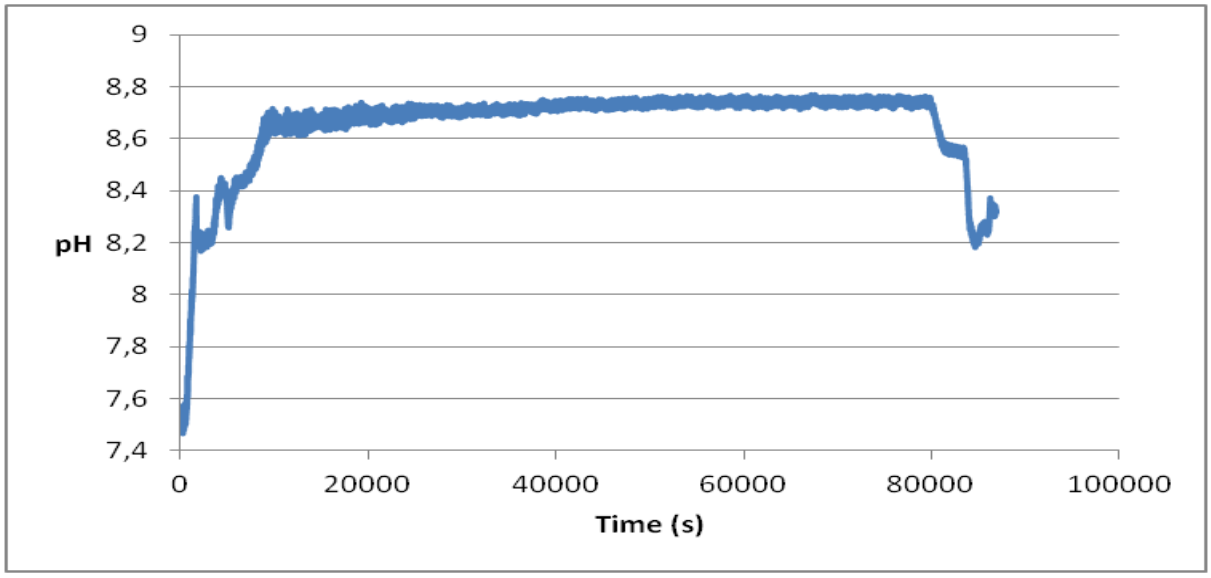


Fig. 18: pH value over time during Tris experiment without Tween 80

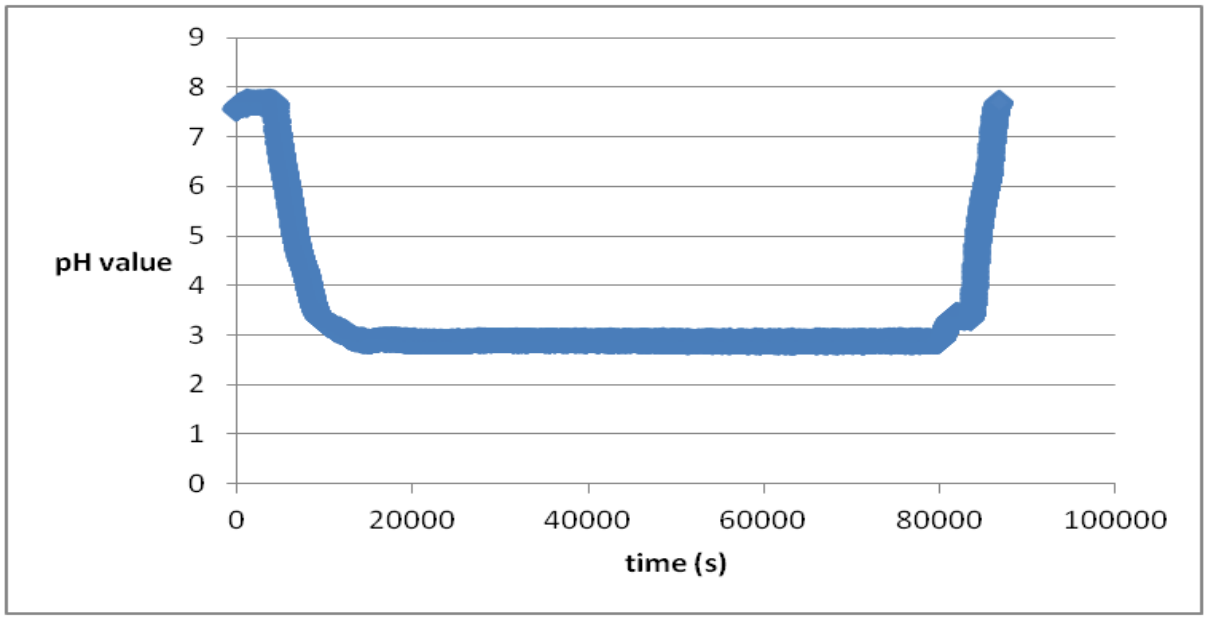


Fig. 19: -30°C experiment NAP buffer with additional 0.02% *Tween 80*

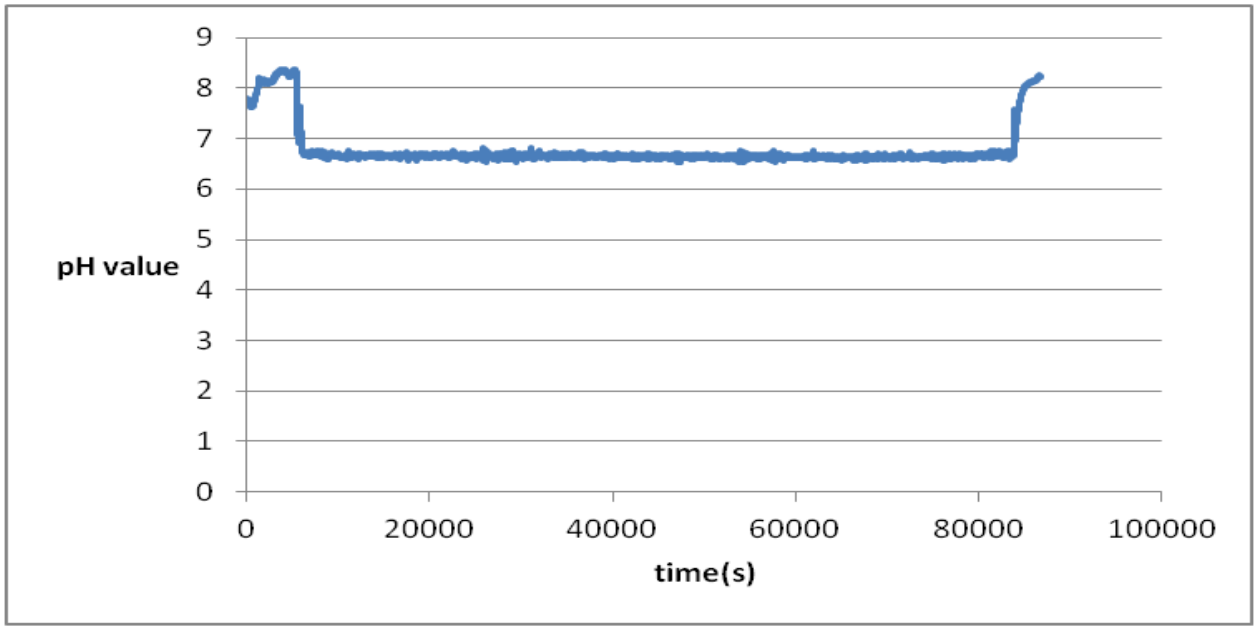


Fig. 20: -30°C experiment containing tris and 0.02% Tween 80

A summary of the results for the experiments that were performed at -30 °C can be seen in Tab.14. It allows an overview of the results from the evaluation of the impact of the pH value and the surface stresses on the LDH performance.

Tab.14 shows the mean values ± SD for the different experiments that were performed at -30°C.

Run number	Buffer	Tween 80 (0.02%)	pH value (-30°C)	Protein Concentration (%)	Specific activity (%)	Aggregate number (/mL)	Mean aggregate size (µm)
3,11,12	NaP	-	3.6±0.1	75.3±19.7	18.7±5.7	109,753±17,053	3.5±0.1
6,8,10,13	NaP	+	3.6±0.7	96.8±3.9	0.0±0.0	148,909±21,032	4.1±0.2
5,7,9	Tris	-	8.7±0.1	89.7±11.0	29.0±21.0	41,908±12,829	4.3±0.3
14	Tris	+	6.7	83.0	86	7,866	4.3

4.8. Analysis of conformational changes:

CD is based on the different absorption of left and right circularly polarized light. Especially UV-CD spectroscopy is used for the identification of the secondary structure of proteins. The spectra of this method can be used to identify the fraction of the protein structure that is in the alpha helix-, beta sheet or other conformation (20;21).

For the first try with lower concentrations a CD-cuvette with a light path of 1 cm was used in order to increase sensitivity. This was necessary due to the low LDH concentration used in our freezing experiments. The first CD-measurements were not successful. The signal intensities were too low to allow for a structural interpretation of the LDH signal. Therefore, spin columns (Vivaspin 20 by Sartorius Stedim) were used to increase the LDH-concentration to achieve better results combined with thinner cuvettes with 0.1 mm light path. The thinner cuvettes should reduce the solvent-related absorption to get better signals for the LDH. However, sufficiently high LDH-concentrations could not be reached using spin columns, most probably due to membrane blocking by aggregates. The highest concentration that was achieved was 454 µg/ml, which was still too low to obtain evaluable CD-spectra.

Also fort he fluorescence spectroscopy the protein concentrations were too low to obtain significant results.

Fig.20-22 show the attempt to determine the LDH folding state using CD spectroscopy. The concentration of the LDH sample was 10 µg/mL. A cuvette with a path length of 1 cm was used because of the low LDH concentration. Fig.20 shows the signal for water, Fig.21 the signal for the buffer and Fig.22 the signal for LDH. The signal for LDH was not good enough to get valuable results.

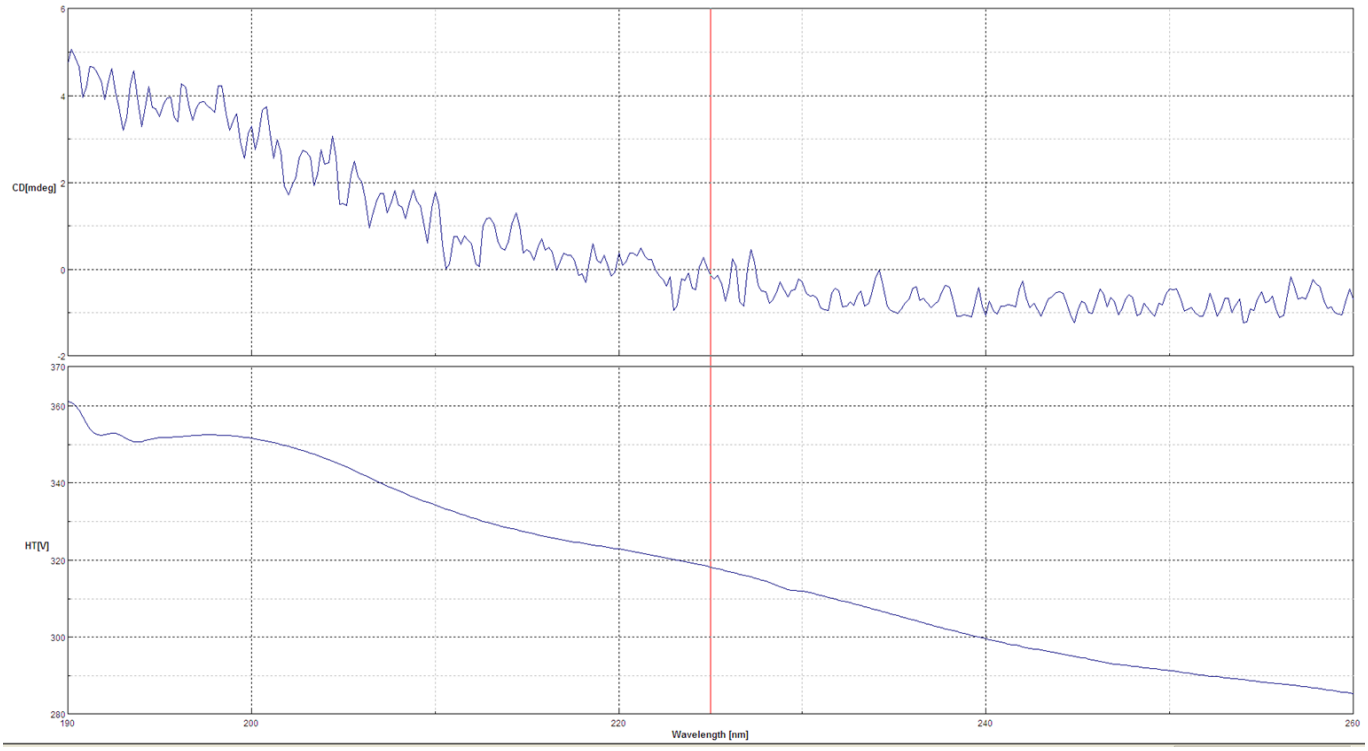


Fig.20 CD signal water

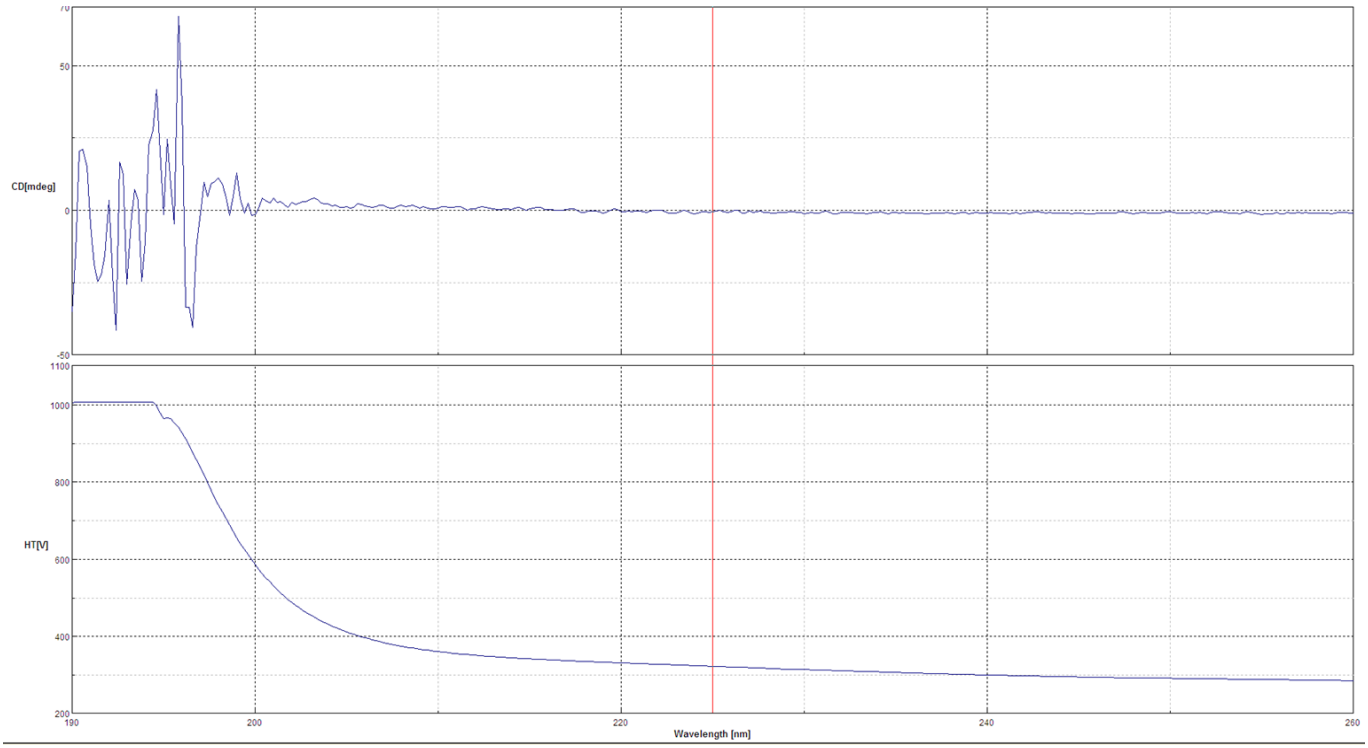


Fig.21 CD signal for buffer

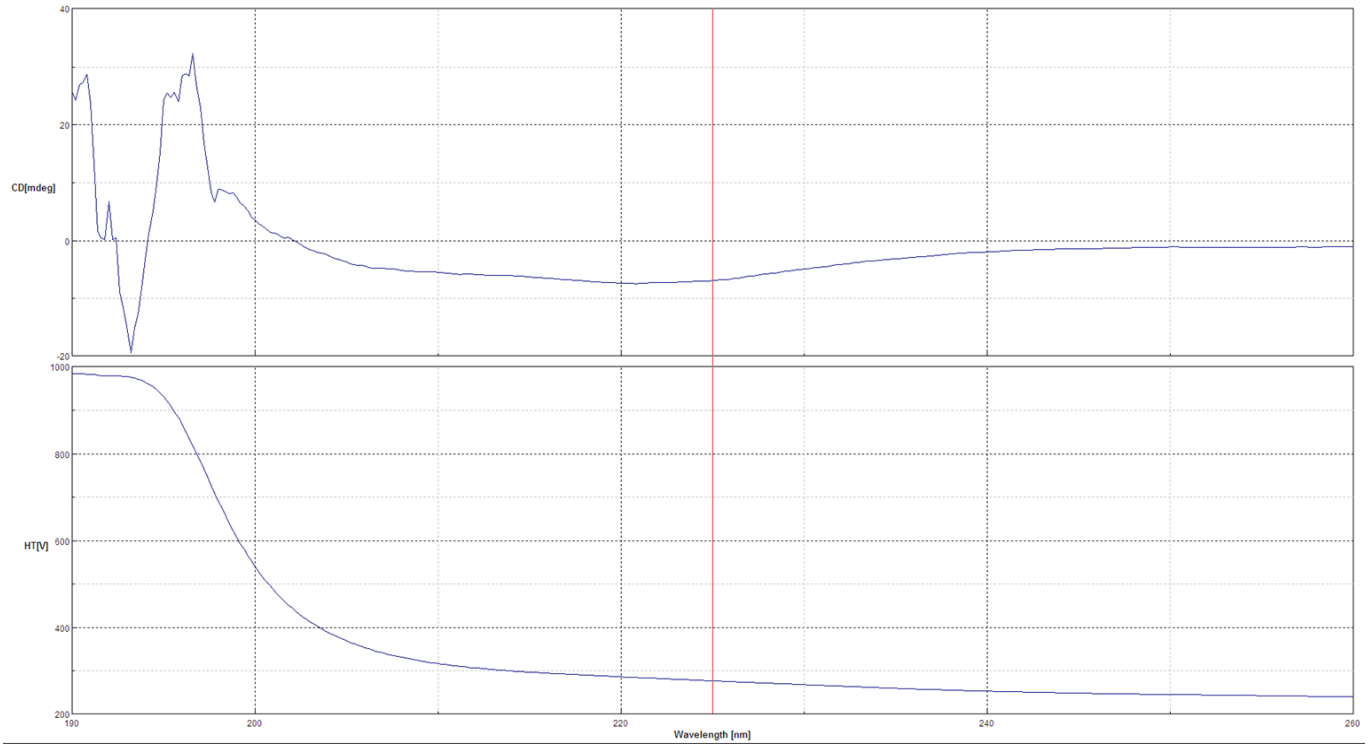


Fig.22 CD signal for LDH

4.9. Glass Transition Point:

The liquid glass transition of amorphous materials is a reversible transition that shows a swift from a hard state into a rubber- like or a molten state. When a viscous liquid is turned into a glass state the change is called vitrification. This liquid glass transition is not a typical form of a phase transition. It does not show a specific transition temperature. There is a specific glass transformation range that is monitored to extend over a bigger temperature range. So the glass transition temperature can be described through convention. This glassy state of the matter usually appears through rapid cooling a solidification from the molten/liquid state. The glass transition temperature that was identified for LDH in Sodium Phosphate buffer was around -27.3 ± 0.7 °C. (31, 32)

To prevent protein aggregation storage beyond glass transition temperature (Tg`) is preferred. The William- Landel-Ferry type diffusive processes that are observed above Tg` show a higher sensitivity towards temperature than Arrhenius type. There is a significantly higher viscosity below Tg`. That means that, even when unfolding appears to some proteins they would not appear as nuclei for further denaturating processes or aggregation due to hindered diffusion. (33)

5. Discussion:

After performing all experiments from the first test set up, the most important single factor for freezing LDH was found to be the temperature. The temperature affects all CQAs except for the particle size and a higher freezing temperature is found to be positive in any case. Only the CQA mean aggregate size shows a dependence on the fill volume. A significant impact of holding time and freezing time was found for enzyme activity.

After analyzing the results many interactive interactions with an impact on the CQAs were discovered. Contour plots were designed that allow later optimizations and improve all responses. The most important finding seems to be that a higher freezing temperature around -10°C is favored. Another parameter (phase transition time) was introduced as potential CPP after finding so many interactive effects. The parameter phase transition time was defined as the time from beginning to the end of the water crystallization that is detectable from the recorded temperature profiles. It could be shown that the phase transition time is affected by the freezing temperature, the freezing time, the fill volume and the holding time. Therefore, it might be difficult to use this as a regulative factor. One thing that could be monitored is that a long phase transition time seems to have a positive effect on enzyme activity and soluble protein concentration. A controlled freezing process using seeding might be a possibility to regulate the phase transition time.

The thawing and holding time only affect the enzyme activity.

One interesting finding is that with long freezing times long holding times are preferred while for short freezing times no holding time at all is favored.

So the temperature seems to have the highest impact on our CQAs. High freezing temperatures, in our case -10°C, lead to higher protein concentrations, enzyme activity and fewer aggregates. Lower temperatures such as -38°C lead to the opposite effect.

The number of aggregates formed seems to be affected by other process parameters that are not monitored in this model.

It seems that only the very low freezing temperature seems to be the reason for all those perturbations. These denaturating effects are described in older research as well. For example Ross Hatley and Felix Franks describe cold denaturation of LDH which is caused only by decreased temperature. (34)

A specific interaction between proteins and ice is described in some papers like in the seminar paper of Strambini and Gabellieri. They were able to show that proteins interact with the surface of ice. This interaction leads to the weakening of the hydrophobic forces and loosening of the native structure which was shown by an increase in tryptophan phosphorescence. The interaction with ice results in a perturbation from both the secondary and the tertiary structure of the protein. Through thawing this effect is reversible although a small part of the protein is irreversibly damaged. They also successfully showed that slow freezing with seeding nucleation leads to lower structural perturbations than without seeding nucleation. So maybe controlled freezing using seeding should be done to retain the protein structure. The CPPs that must be regulated in this case are freezing time, holding time and fill volume to control the time for the phase transition. (35)

It has to be noted that it is not possible to make valuable predictions for the aggregate number on basis of the generated data model due to the bad model quality. A reason for that could be the possibility of aggregate dissolvation during the different thawing programs. This needs to be evaluated in further investigations.

The addition of other protein stabilizators might lead to better results and higher stability. One additional possibility besides *Tween 80* is Ectoine. A natural compound that can be found in several bacterial species. It is a so called compatible solute that helps bacterial cells to survive osmolytic stress. In our case it could be used to protect the enzyme from temperature stress and keep it stable this way. Another possibility is the addition of glycerol with a concentration of 10-50 % to keep the protein stable during freezing and thawing cycles. Since we are not using any chromatographic methods it would be a good possibility to increase protein stability. (36)(37)

The last set of experiments for optimizations and formulations helped to control the predicted impact of the critical process parameters on the responses.

Six additionally runs were manually designed to look for improvements that can be done to get a better protein performance after freezing.

The first run series containing the experiments that were designed by Modde clearly showed that the most significant factor that has a negative impact on the activity is the temperature. Although the prediction of the values by the software were not completely possible for the protein concentration and the activity, still an effect of the temperature was determined. The experiments performed with a low freezing temperature starting with -25°C showed a strong activity loss compared to the runs performed in a temperature range of -10°C to -20°C. The bad predictability might be caused as mentioned before by a higher stability of the LDH. Through the activity measurements better performances were seen in the second part of the work than in the first 31 runs that were performed. Still, a very good prediction was possible for the aggregate number and the mean size of aggregates. Both showed results that were almost in all cases in the predicted range. For both parameters only one value was outside the defined limits.

The second part of the optimization experiments was done to determine the reason for the negative impact on the protein. The temperatures were chosen to see if cold denaturation leads to the decrease in the LDH performance. The experiment with tris buffer instead of sodium phosphate buffer was done to see if the pH drop that was identified has negative a effect on the protein. The additional *Tween 80* experiment was performed to see if the surfactant can lead to improvements in the protein performance after thawing. It was shown that those runs performed with tris buffer show way better results than the ones done with sodium phosphate buffer. Even the *Tween 80* experiment performed with tris buffer leads to better post freezing performances than the one with sodium phosphate buffer. One significant reason for that might be the strong drop in the pH value down to 3 during the -30°C experiments done with Nap buffer. These pH shifts don't occur in the tris experiments. Even the additional *Tween 80*- tris experiment only shows a slight pH shift down to 6 what might be the reason for the strong activity recovery.

In addition to the pH measurements DSC was used to identify the glass transition point. Using this technique a glass transition temperature was identified around -27.3 ± 0.7 °C for

the Lactic Dehydrogenase in sodium phosphate buffer. These temperatures were not reached in the freeze container, so glass transition did not occur. When studying the inner bulk temperature with the data from the pH monitoring it was clear that the pH drop down to 3.6 was persistent when the bulk temperature was below -14 °C - -17°C. Above this temperature range the pH drop only appeared during the phase transition. When the storing phase was reached a neutral pH was monitored.

One conclusion that was made is that a storage at temperatures of lower than -17 °C leads to diffusive mobility in freeze concentrated regions and prevents dissolution of $Na_2HPO_4 \cdot 12H_2O$ precipitate and pH recovery. At temperatures above this range annealing effects are possible that allow $Na_2HPO_4 \cdot 12H_2O$ to dissolve and the pH value to neutralize because of high mobility. So when choosing a temperature range below this critical point the used LDH is exposed to low pH values and precipitated $Na_2HPO_4 \cdot 12H_2O$ throughout the whole storage period. The effect of cold denaturation that leads to LDH inactivation behavior could not be confirmed using this experimental test set up. The negative impact of cold denaturation on the performance of LDH was determined at temperatures way below the investigated critical temperature threshold (33). The possibility of cold denaturation affecting our LDH sample during the setup is very unlikely but still it can’t be ignored since the effect was identified in other case studies differing from this one.

6. Conclusions:

We were able to show that it is possible to identify relevant parameters concerning the freezing process of a protein using QbD principles. Although LDH was used, which is considered as an F/T sensitive model protein, the used method can be assigned also for freezing of many other pharmaceutical proteins at low volume keeping the costs at an attractive level.

The most important findings that were identified are:

- Almost all CQA except for the aggregate size prefer higher freezing temperatures around -10°C.
- Turning on recirculation leads to a strong negative impact on the output factors in most experimental runs.
- The thawing time as a parameter has no significant effect on the CQAs except for the number of aggregates that are formed during the process. The low quality of the model that was generated for the aggregate number must be taken into account for following considerations of the results.

The performed optimization runs cleary show that the critical process parameters that were identified as negative for the protein performance lead to the predicted bad performance. Additional experiments might be needed to improve the prediction quality of this set up.

Further manually designed experiments for studying the LDH inactivation mechanisms clearly showed that the lower set temperature is responsible for the reduction in the LDH stability and performance. We also found that the addition of Tween 80 that should reduce the interfacial stresses lead to no improvements when using NAP buffer. The replacement of sodium phosphate buffer by Tris-HCl buffer lead to a reduction of the number of aggregates formed but did not lead to an improved specific activity retention. The use of Tris-HCl buffer combined with Tween 80 with a concentration of 0.02% showed a significant improvement in the LDH performance after F/T at -30°C.

For better understanding of the inactivation mechanism of the LDH inactivation, when using the 700 mL Zeta Freeze Container for F/T experiments further testing would be needed. Especially the influence of different Tween 80 concentrations and the impact of different buffer systems must be taken into account for a more detailed study of the inactivation process. The use of spectroscopic methods like CD and extrinsic/intrinsic fluorescence was not possible for the determination of the protein structure after the F/T experiments. The reason was the low concentration of the proteins that was set in our tests with 10 µg/mL, the minimum concentration needed for CD couldn't be reached. The fluorescence methods showed no valid signal either.

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8. Tables of Figures:

Figures 1-10 were generated using the software Modde™.

Figure 22: CD signal for LDH