Master's thesis report

Lactic acid fermentation aided precipitation of alkaline extracted hemp proteins

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Abstract

Production of Hempseed oil results in large quantities of residual Hempseed Press Cake, which has historically been used for animal feed due its unpalatable high fibre content. Through pH extraction and precipitation, the protein contents can be isolated for use in foodstuffs. This project showcases the possibilities of utilizing lactic acid fermentation to aid the process and potentially improve the protein yield, as well as other properties of the product.

Three precipitation methods were investigated in detail: A control method only using citric acid powder to lower pH, a method utilizing *Lactobacillus plantarum* 299V, and a method utilizing a mix of *Streptococcus thermophilus* och *Lactobacillus delbrueckii* subsp. *Bulgaricus*. It was found that these precipitation methods produced precipitates of similar protein content (dw), and displayed protein precipitation yields of 72.94%, 77.01% and 74.77% respectively.

The protein precipitate samples displayed pasting temperatures of 92.7°C, 95.2°C and 92.0°C, as well as main denaturation temperatures of 90.6°C, 91.4°C and 90.64°C, for the respective precipitation methods. Protein precipitate samples from both of the fermented precipitation methods displayed very clear reductions in concentrations of colony forming units of yeast and Enterobacteriaceae.

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1. Introduction

1.1 Background

The access to safe and nutritious food is a cornerstone of human society, but in recent history an increasing need for ensuring that food production is also sustainable has emerged. Modern technology has enabled food and feed production volumes to rise at an incredible rate, which is not sustainable in the long term. Earth overshoot day of 2024 is estimated to occur on July 25th which, simply put, means that we are currently consuming ecological resources and services twice as fast as the planet is able to recover and regenerate [1]. With this background, there is a great need for sustainably farmed vegetable protein sources as well as minimizing waste streams. This is where the need for extraction of hemp proteins from hempseed press cake (HPC), a byproduct of hempseed oil production, comes into play. Hempseed protein contains all eight essential amino acids, making it a great alternative protein source, but the HPC is unfortunately not very palatable for human consumption and is therefore often used for animal feed. The HPC consists of mainly protein and insoluble fibre, and by separating the protein fraction from the fibre fraction, a protein rich product is gained that can be used in countless foodstuff formulations.

Previous research has already showcased the viability of a pH-dependent separation method, utilizing alkaline extraction and acidic precipitation, of hemp proteins from HPC in both lab scale and pilot scale settings.

1.2 Objectives

This project focuses on investigating fermentation with lactic acid producing bacteria, as a part of the acidic precipitation process, and how this process compares to the chemical precipitation method. This comparison is based on the following criteria:

- The yield of the precipitation process and the protein content of the precipitate.
- The characteristics of the precipitated proteins, such as denaturation temperature and viscosity.
- The microbial flora of the protein precipitate.
- The phytate content of the protein precipitate.

1.3 Scope and limitations

This project was conducted over a 20-week time period, investigating fermentation aided precipitation from two different commercially available bacterial cultures as well as a chemical precipitation method acting as a control.

As part of this study an initial screening process was conducted, which tested the fermentation aided precipitation under the following parameters: temperature, time, choice of microorganism, amount of added microorganism, as well as comparing still vs continuously mixed (shake table) incubation. An in depth optimization of the fermentation process is not the main objective of this study, and is not feasible to properly investigate in the short time span of this study. This means that the results of this study should be used as an indication of how the fermentation process compares to the traditional chemical precipitation process, and should

not be treated as a declaration of the effectiveness of lactic acid fermentation usage for protein precipitation.

1.4 Hypothesis

The precipitation process of the hemp proteins is dependent on pH decrease, meaning that the fermentation precipitation should be able to produce comparable results (in regards to protein yield) to the chemical process, as long as the microorganisms are able to grow and produce organic acids. The chosen microorganisms in this study are well known to significantly decrease pH, as well as being suitable for growth in plant materials, which indicates that a successful pH decrease through fermentation of the HPC protein extract is plausible. Slightly decreased yields, compared to the chemical method, may occur as a because of the lack of control that in the fermentation process or as a result of bacterial metabolism of protein contents for energy purposes. However, the fermentation process may also result in an increased yield as partial proteolytic activity, breaking down the proteins into polypeptides without completely breaking them down, would increase the precipitation of proteins. Furthermore, the protein content of the fermented product may be lower than that of the chemical precipitation due to the increased presence of microorganisms in the final product.

Predicting the rheology and viscosity phenomena of the precipitated proteins is difficult, but it is reasonable to believe that the fermentations process could produce metabolites that alter the properties of the protein precipitates in this regard.

As previously mentioned, fermentation is a known preservative method. Utilizing *Lactobacillus*, which produces an acidic environment as well as using other compounds and mechanisms to prevent competing microorganisms from growing, such as bacteriocins [2], is expected to reduce the presence of pathogens.

There is extensive testing that indicates that fermentation at acidic conditions greatly reduces the phytate content of grains [3, 4], as well as recent results showing clear phytate content decreaseas a result of *Lactobacillus* fermentation in HPC [5]. For this reason, it is to be expected that the fermentation process significantly reduces the phytate content.

To summarize, I hypothesize that the fermentation process will only bring moderate changes to the protein yield, but will significantly improve the microbial profile and decrease the phytate content of the protein precipitate.

2. Background and Theory

2.1 Materials

Hempseed and Hempseed press cake

Hempseeds, a product of the *Cannabis Sativa* plant, are considered a nutritious raw material for foodstuff and feedstuff purposes, and has high contents of both fat and protein. Exact macronutrient contents vary between subspecies of plants and growing conditions, but a general hempseed contains 25-35% (dw) lipids, 20-25% (dw) proteins, 20-30% (dw) carbohydrates (of which 98% are insoluble fibres) and 4-7.6% (dw) ash. [6]

A popular use of hempseeds is the production of hempseed oil, which results in a side stream of Hempseed Press Cake (HPC). The HPC has a high protein content (30-35%, dw) [5], why it is often used for animal feed, but in recent years hemp protein has gained attention as an alternative protein source for food production. Since the high fibre content of the HPC makes it unpalatable for human consumption and unsuitable for certain foodstuff formulations, research in isolating the protein fraction has been conducted. For this purpose, HPC from cold-pressed hempseed oil production is most interesting to use, as a heating procedure would denature the proteins [5].

Hemp proteins

The protein composition of hempseed is dominated by Globulin Edestin (67%-75%), with a large fraction of globular albumin (25%-37%) [7]. Differential scanning calorimetry studies of hemp protein isolate, in the range 60-110°C, have shown a single clear peak in heat flow starting at 86.7°C and peaking at 95.1 °C indicating this is the temperature at which the hemp protein denatures [8]. Studies of Hempseed Protein Isolate (HPI), have displayed an isoelectric point between pH 5.5-5.8 [9].

The amino acid profile of hempseed protein covers all eight essential amino acids, and has notably high amounts of glutamic acid and arginine as well as methionine and cysteine. The lowest occurring amino acid in hempseed is tryptophan. Hemp proteins also have a notably high digestibility score which, along with the amino acid profile, makes hemp protein a great alternative protein source. [10]

Phytic acid and phytase activity

EU regulations state that rapeseed protein isolates can not contain more than 1.5g/100g (dw) of phytic acid. Since there are no regulations yet for hemp protein isolates, this will serve as a benchmark for this report. Previous research on extraction and precipitation of hemp proteins utilizing alkaline extraction and acidic precipitation found that the phytic acid content of the protein isolate ranged between 0.2-1.5g/100g (dw), corresponding to a reduction of 55%-94% respectively. Precipitation at pH 6.00, the highest precipitation pH in the study, yielded the highest phytic acid content reduction while the lowest precipitation pH of 3.00 yielded the lowest phytic acid content reduction. [5]

A study conducted by Reale et. al (2007) investigated the fermentation of cereals utilizing lactic acid-producing bacteria. The study showcased great reductions in phytate content, resulting in a 100% reduction in rye, 95-100% reduction in wheat, and 39-47% reduction in oats. The study

compared 50 strains of lactic acid bacteria and did not find a significant increase in bacterial phytate production, which shows that the decrease of phytate content is not correlated to the strain of lactic acid bacteria used but is rather dependent on the endogenous plant phytase activity. The lactic acid bacteria do, however, produce a favourable environment for phytase activity by lowering the pH, and the highest rate of phytate reduction was seen at pH 5.5. [3]

In another study, focused on the phytase production of different *Lactobacillus* strains, *L. amylovorus* B4552 was found to produce the highest rate of phytase, at a recorded rate of 146 units $ml^{(-1)}$. Following closely were *L. plantarum*, *L. casei*, *L.. pedaococcus*, *L. acidophilus* and *L. delbrueckii*, which produced phytase at rates of 74.4, 28.3, 23.0, 14.6, and 15 units $ml^{(-1)}$ respectively. *L. amylovorus* and *L. plantarum*, found in various plant-derived microbial systems, demonstrated the highest enzyme yields. Conversely, lactic acid-producing bacteria commonly used in milk-based products, such as strains of *L. acidophilus*, *L. casei*, *L. lactis* and *L. delbrueckii* exhibited low phytase production. [4]

2.2 Processing methods

Lactic acid fermentation

Lactic acid bacteria (LAB) are often used in food fermentation applications, and produce large quantities of organic acids, especially lactic acid. Heterofermentative LAB uses carbohydrates to produce lactic acid and other compounds, while homofermentative LAB only produces lactic acid under anaerobic conditions. Most LAB are facultative anaerobic and have an optimal temperature for growth between 20 to 45 °C. *L. plantarum* is an example of a homofermentative LAB and is commonly used in food products such as sauerkraut, kimchi, and sourdough. *L plantarum* also has an extraordinarily high alkali tolerance, with registered growth at pH 8.9. [11]

LAB also produce antimicrobial peptides, called bacteriocins, which can help improve the quality and stability of the product [2]. For example, studies of *L. Amylovorus* and *L. Plantarum* have identified metabolites with antifungal properties [12].

Protein extraction and precipitation

Manipulation of protein solubility can be achieved through pH shifts. By adjusting a mixture of HPC and water to alkaline pH, hemp proteins can be solubilized and therefore separated from remaining solids. Precipitation of proteins from the water phase can be achieved by changing the pH to match the isoelectric point of the protein, which minimizes protein solubility and causes the protein to precipitate. Previous research by Helstad et al. (2022) found the optimal extraction conditions to be constant pH 10.5 for 1h, and the optimal precipitation pH to be 5.5. [5]

2.3 Analysis methods

Ash content

The ash content of a product is defined as the residual inorganic, non-combustible matter and is usually composed of the mineral components of the original sample. The ash content can therefore be a good indicator of the mineral content of foodstuffs, however, a high level of acid-insoluble ash could indicate the presence of sand or other contaminants. [13]

Dumas protein analysis

The Dumas procedure is a combustion method used to determine the total nitrogen content of a sample through complete combustion at high temperature, aided by external oxygen input. The nitrogen in the sample is reduced to nitrogen gas which is measured and quantified using a Thermal Conductivity Detector. To avoid signal disturbances from other combustion products, the gas passes through one or several filters before reaching the detector. [13, 14]

Viscosity analysis

Studies of the apparent viscosity profile (measured in centipoise, cP) as a function of temperature can be measured with equipment such as a Rapid Visco Analyzer (RVA). Analysis in an RVA is conducted by constant stirring of the sample while it is being heated, measuring the resistance experienced by the stirring mechanism. RVA tests are commonly conducted for cereal samples, and can be used to measure gelling properties as well as denaturation of proteins. [15]

Each completed RVA analysis yields information related to several behaviours of the sample. These are displayed in Figure 1. Most important to this project was the temperature at which the viscosity increase starts (pasting temperature, T_{paste}), the temperature at which the viscosity is the highest (peak viscosity temperature, T_{peak}) and the highest recorded viscosity (peak viscosity, $Visc_{peak}$).

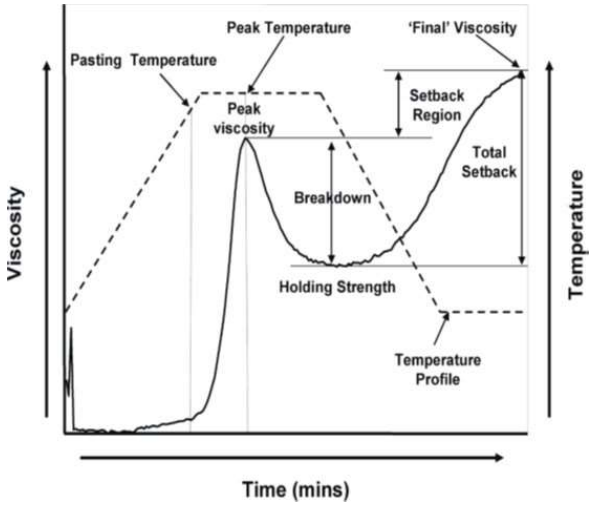

Figure 1: Information gained from RVA analysis [16]

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) measures the difference in the energy input as a function of temperature for a sample and a reference (often an empty container). The sample and the reference are subjected to a temperature range program, which can vary in design depending on the sample. During heating/cooling, endothermic or exothermic phase changes, or other reactions, can occur in the sample. These changes are recorded as positive or negative enthalpy differentials in the results, which are displayed as peaks in the resulting graph. The temperature at which the recorded enthalpy change occurs is measured as the peak temperature, T_{peak}. The enthalpy change, ΔH, of a specific process, in the case of this project being protein denaturation, is gained by integrating enthalpy as a function of temperature over the

temperature values of the chosen peak. Such information can help identify the characteristics of the proteins as well as the relative contents of different proteins with different denaturation temperatures. For an unknown protein mixture, this information can help to identify the protein composition of the sample. [17]

3. Materials and methods

3.1 Materials

The following were the main materials used during this project. Specific chemicals with limited usage, such as for certain analysis methods, have been left out of this list and are instead presented in the respective method descriptions.

The Hempseed Press Cake (HPC) was supplied by the project *Hållbara hampainnovationer – från svinn till vinn* (dnr: 2021-03570) and stored in room temperature. NaOH solution of 2M was created using anhydrous NaOH from VWR chemicals (Leuven, Belgium) and distilled water, and was stored in glass bottles at room temperature. Citric acid powder from VWR chemicals (Leuven, Belgium) was stored in plastic containers in a fume hood. Capsules of concentrated *L. plantarum 299V*, further referred to as 299V, culture from Probi AB (Lund, Sweden) were stored at room temperature. Two commercial cultures, commercial names: "VEGE033" and "VEGE047", from DANISCO (Copenhagen, Denmark) were used in the screening tests and freeze stored. The first DANISCO culture, VEGE033, was also used in the final method and is a mix of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*. The second DANISCO culture, VEGE047, is a mix of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Bifidobacterium lactis* (HN019™) and *Lactobacillus acidophilus* (NCFM®)

3.2 Equipment

For a complete list of equipment, please see Appendix A: Equipment models and producers.

3.3 Sample preparation methods

This section describes the outlines of the laboratory work that conducted to produce all the samples used in this project.

Screening/Experiment design

The extraction method was based on the method developed by Helstad et. al. [2], with minor modifications, i.e. the method was adjusted for lab-scale experiments. The extraction method is further described in the *Extraction* method description.

The major experiment design was conducted in regards to the fermentation. As a first step, 299V culture was used in small batch experiments, testing varying concentrations of added starter culture, incubation temperature and incubation time, as well as inoculation in neutral and alkaline conditions. Samples incubated at 30°C were placed in a *Termaks* heating cabinet from Nordic Labtech (Fjärås, Sweden), and samples incubated at 20°C were placed in a fume hood without further temperature regulation. All pH measurements were conducted using a 914 pH/conductometer from Metrohm (Herisau, Switzerland). Overview of the initial fermentation test parameters can be found in Appendix B: *Screening process overview*.

In the second part of the screening process, in order to follow up on the results of the first part of the screening process, larger volumes (400ml) were fermented at 20°C for 48h, 30°C for 24h and 30°C for 48h respectively. Samples were incubated in the same heating cabinet and at fermentation termination the pH was measured. Samples from these experiments were analysed

to determine protein content. All experiments of the second round of screening were conducted in triplicates.

The third set of screening experiments were incubated for 24 hours at 30°C and 180rpm in a LAB-THERM incubator and LAB-SHAKER shake from Kuhner (Basel, Switzerland).

As a final part of the screening process, experiments were made with the two commercial bacterial cultures VEGE033 and VEGE047. All experiments were incubated at 30°C and 180rpm on a shake table incubator, with incubation times between 24-72h.

The finalized methods for fermentation and precipitation can be found in the *Fermentation and precipitation* method description.

Milling (preparation of HPC sample)

The HPC samples were prepared in a Laboratory mill 3100 and Mill feeder 3170 from Perten instruments (Shelton, CT, USA), fitted with a 0.8mm sieve and operated according to manufacturer's instructions. The milled HPC was stored in airtight plastic containers in room temperature.

Extraction

All extractions were run according to the same method. 50g of HPC was added to 450g of distilled water in a glass beaker and the temperature of the mixture was measured. Each sample was constantly stirred at 450 rpm with a Microstar 7.5 control Stirrer from IKA-Werke (Staufen im Breisgau, Germany) and the pH of each sample was brought up to 10.5 using 2M NaOH solution. The samples were mixed for 1 hour, during which the pH was measured continuously and adjusted within 10.5+/- 0.05 using 2M NaOH solution.

To separate the solubilized proteins from the remaining HPC solids, further referred to as Spent Solids (SS), each sample was put through an Avanti J-15R Centrifuge from Beckman Coulter (Brea, CA, USA) at 7400g for 20 minutes. The resulting supernatant, referred to as the Light Liquid Phase (LLP), was poured off to be used in the precipitation process.

Fermentation and precipitation

In the final study, three different precipitation methods were used, and three process replicates were conducted for each method.

For the control method, referred to as Control, the pH of the LLP was decreased to 4.5 using citric acid powder. For the two fermented methods, referred to as 299V and VEGE033, the pH of the LLP was adjusted to 7 using citric acid powder.

For the 299V and VEGE033 fermentations, the pH-adjusted LLP was transferred to sterilized 500ml Erlenmeyer flasks fitted with rubber corks and air locks, to facilitate a more anaerobic environment, before being inoculated with $3*10^9$ cfu of the respective bacterial culture. Each bacterial culture powder was measured on a precision scale to ensure high reproducibility between process replicates. For each process replicate 250mg and 2.6mg was used of the 299V and VEGE033 cultures respectively. The inoculated samples were fermented in a LAB-THERM incubator and LAB-SHAKER shake table at 30°C and 180rpm for 24 hours. After completed fermentation, the pH of the sample was measured.

Both the control sample (pH 4.5) and the fermented samples were centrifuged in an Avanti J-15R Centrifuge from Beckman Coulter (Brea, CA, USA) at 7400g for 20 minutes. The supernatant was discarded and the resulting precipitate, referred to as the Protein Precipitate (PP), was collected.

To minimize damage and functional changes of the proteins during freezer storage, the pH of the PP samples was adjusted to 7 using 2M NaOH solution and distilled water in a T18 digital Ultra Turrax from IKA-Werke (Staufen im Breisgau, Germany) running at 4-14 krpm before freezing.

At the end of the project one more extraction and precipitation test was conducted using *L. plantarum* 299V according to the methods stated above, with exception of replacing the freezing storage with storage at +4°C overnight. After overnight storage, this sample was tested with RVA and DSC to allow comparison between frozen and non-frozen samples. This process was conducted in one process replicate, and samples from this batch will be referred to as non-frozen 299V samples.

3.4 Analysis methods

This section describes the outlines of the analysis methods utilized in this project.

Dry matter content analysis

To determine the dry matter content, also referred to as DMC, method 44-15a from AACC (American association of Clinical Chemistry), now known as ADLM (Association for Diagnostics & Laboratory Medicine), was used [18]. Analysis was conducted for all samples of SS, PP and pH-adjusted PP. During the screening process all samples were analyzed once, and during the final study all analyses were conducted in triplicates. Dry matter content was determined using equation 1, where the variables m_{sample}, $m_{bowl,empty}$ and $m_{bowl,post}$ denotes the weight of the wet sample used, and the weight of the container before and after the analysis process respectively.

$$Dry\ matter\ content\ [\%] = \frac{m_{bowl,post} - m_{bowl,empty}}{m_{sample,wet}} \quad (1)$$

Protein content analysis and process efficiency

Protein analysis was conducted using a Flash EA 1112 Series from Thermo Electron Corp. (Waltham, MA, USA). The Flash EA 1112 series utilizes gas chromatography to determine the nitrogen content of the sample according to the Dumas process. The setup used Oxygen gas to facilitate combustion and complete oxidation, and Helium was used as carrier gas. Samples of 25mg were enveloped in tin foil discs before being loaded into the machine.

The protein analyzer was calibrated at the start of every run using Aspartic acid, and the calibration was validated to a certainty of ±0.2% protein content. All samples were dried and crushed before being analysed in triplicates. A nitrogen factor of 6.25 was used to determine protein content.

The protein content of the SS and PP of each extraction/precipitation trial was analyzed, as well as a sample from the HPC. These results, along with the dry matter content results, were

used in equations 2 and 3 to calculate the extraction yield, precipitation yield and total process yield respectively. The variables $P_{HPC,db}$, DMC_{HPC} and m_{HPC} represent the protein content (dry basis), the dry matter content and the mass of the HPC (used in a given extraction experiment) respectively. The corresponding variables with different denominators, SS and PP, represent the same values for the Spent Solids (SS) and Protein Precipitate (PP) respectively.

$$Extr.yield\ [\%] = \frac{P_{HPC,db}\, DMC_{HPC}\, m_{HPC} - P_{SS,db}\, DMC_{SS}\, m_{SS}}{P_{HPC,db}\, DMC_{HPC}\, m_{HPC}} \quad (2)$$

$$Prec.yield\ [\%] = \frac{P_{PP,db} DMC_{PP} m_{PP}}{P_{HPC,db}\, DMC_{HPC}\, m_{HPC} - P_{SS,db}\, DMC_{SS}\, m_{SS}} \quad (3)$$

Lipid content analysis

Lipid content analysis was conducted using a SOXTEC AVANTI 2055 Manual Extraction unit and a SOXTEC 2055 Manual control unit from FOSS analytics (Hilleroed, Denmark). The extraction unit was used according to manufacturers instructions, using petroleum ether to extract the lipid content of the sample from a cellulose thimble into an aluminium cup. For each experiment 2-4g of dried sample was used, and the following times and temperatures for each cycle of the process were used. Cooking: 135°C, 20min. Rinsing: 135°C, 40min. Evaporation: 135°C, 15min. Drying: 135°C, 5 min. Lipid content analyses was conducted in triplicates.

The lipid content of the sample was decided using the equation 4, where the variables $m_{sample,dry}$, $m_{bowl,empty}$ and $m_{bowl,post}$ denotes the weight of the sample used, the weight of the container before and after the analysis process respectively.

$$Lipid\ content\ [\%](\mathrm{dw}) = \frac{m_{bowl,post} - m_{bowl,empty}}{m_{sample,dry}} \quad (4)$$

Ash content analysis

To measure the ash content of the HPC and the PP samples, an L14/B150 oven from Nabertherm (Lilienthal, Germany) was used. The weights of empty porcelain containers, as well as the weights of each sample before and after processing in the ash oven, was recorded to determine the ash content of each sample. The peak temperature of 700°C was held for 2 hours during the measurement. Ash content analyses was conducted in triplicates.

To calculate the ash content equation 5 was used. The variables m_{sample}, $m_{bowl,empty}$ and $m_{bowl,post}$ denotes the weight of the sample used, the weight of the container before and after the analysis process respectively.

$$Ash\ content\ [\%](\mathrm{dw}) = \frac{m_{bowl,post} - m_{bowl,empty}}{m_{sample,dry}} \quad (5)$$

Viscosity analysis

A Rapid Visco Analyzer (RVA), specifically the RVA 4800 with a high temperature paddle coupling, from Perten Instruments (Shelton, CT, USA), was used to record apparent viscosity changes in relation to heating of the sample.

The RVA was set to an initial stirring speed of 960rpm for ten seconds, followed by a stirring speed of 160rpm for the rest of the test. The temperature was first increased from 50°C to 140°C at a pace of 15.4°C/min, then held at 140°C for 180sec before being lowered to 50°C at a pace of 15.4°C/min. The TCW3 software was used to record and plot the apparent viscosity changes captured by the RVA. Pasting temperature ($T_{pasting}$), peaking temperature (T_{peak}) and peak viscosity ($Visc_{peak}$) values were gathered from the software, along with plots depicting apparent viscosity, and temperature, as a function of time.

For each process replicate, two RVA tests were conducted, resulting in six tests per finalized precipitation method. All samples were diluted with distilled water to reach a dry matter content of 9% before being analyzed.

Differential Scanning Calorimetry (DSC)

DSC measurements were conducted using a DSC6200 and EXSTAR6000 module from Seiko Instruments (Tokyo, Japan) to record the phase changes of the extracted proteins at varying temperatures. The DSC results were obtained and analyzed using the EXSTAR600 software from Seiko Instruments. Each sample was analyzed in triplicates, and an empty pan was used as reference. The DSC machine was set to heat up the samples from 20°C to 180°C at 10°C/min. Each sample of 6-10mg was weighed up using a C-30 microbalance from CAHN Instruments (Cerritos, CA, USA), with 1 µg precision.

The EXSTAR6000 software allows the user to, from a DSC sample analysis, identify the peak temperature, T_{peak} , of each registered phase change, as well as calculate the enthalpy change, ΔH, produced by each phase change. This information was then compared to the expected characteristics of hemp protein isolate.

For each batch of PP, three DSC tests were conducted, resulting in nine tests per finalized method.

Colony forming units (cfu) count

The live bacterial count was determined as cfu/ml by diluting the samples and growing on selective mediums. The four mediums used were: Tryptic Soy Agar (TSA), Violet Red Bile Dextrose Agar (VRBD), Malt Agar (MA) and Rogosa agar. The chosen agars are selective for: Total microorganism count, Enterobacteriaceae, Yeast/Mould and Lactobacilli respectively. VRBD was incubated for 24h, TSA and Rogosa for 72h, and MA for 120h. All agars were prepared from distilled water and dry substrate from Merck KGaA (Darmstadt, Germany) and then poured into petri dishes in a Laminar Air Flow (LAF) hood before being stored at 4°C. VRBD and Rogosa agar solutions were prepared by boiling for 2 minutes using MR 3001 K Heated magnetic stirrer from Heidolph (Schwabach, Germany). TSA and MA agar solutions were prepared in an autoclave from Certoclave (Leonding, Austria).

PP samples were diluted with a dilution liquid consisting of distilled water mixed with bacteriological peptone from Oxoid (Basingstoke, United Kingdom) and Sodium chloride from VWR (Leuven, Belgium). Dilutions were created in series by transferring 1ml of sample to a tube with 9ml of dilution liquid. The dilutions used for each agar medium/sample combination

are presented in Table 1. After completed dilution, 0.1ml of the sample was transferred to an agar plate and spread with sterilized glass beads. All inoculated agar plates were incubated at 30°C in a *Termaks* heating cabinet from Nordic Labtech (Fjärås, Sweden) before all the colonies were counted. The cfu/ml for each sample was determined using equation 6, where n represents the amount of colonies found on the plate and m is determined by the amount dilution steps made. The 0.1 is present in the denominator as only 0.1ml of sample is transferred to the agar plate, and the 0.5 represents the dilution that happens when the PP is mixed with equal parts distilled water to enable handling the product with a pipette.

$$\frac{cfu}{ml} = \frac{n}{0.5 \cdot 0.1 \cdot 10^{-m}} \tag{6}$$

Table 1: Sample dilutions used for agar inoculation

	Control	299V	VEGE033
TSA (total cell count)	10^{-1}, 10^{-2}, 10^{-3}	10^{-6}, 10^{-7}, 10^{-8}	10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}
Rogosa (Lactobacillus)	10^{-1}, 10^{-2}, 10^{-3}	10^{-6}, 10^{-7}, 10^{-8}	10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}
VRBD (Enterobacteriaceae)	10^{-0}, 10^{-1}, 10^{-2}	10^{-0}, 10^{-1}, 10^{-2}	10^{-0}, 10^{-1}, 10^{-2}
MA (Yeast/Mould)	10^{-0}, 10^{-1}, 10^{-2}	10^{-0}, 10^{-1}	10^{-0}, 10^{-1}, 10^{-2}

Phytate and Iron content analysis

Due to limitations in regards to time and resources, the phytate and iron analysis, planned to be conducted by an external contractor, was not able to be carried out.

3.5 Statistical analysis

Statistical analysis of the data was conducted in Python 3.11.5. One way ANOVA tests were conducted with the “f.oneway”-function from the “scipy.stats” library, Grubbs outliers test was conducted with the “Smirnov_grubbs.test”-function from the “outliers” library and t-tests were conducted with the t.test_ind function from the “scipy.stats” library. All tests were performed with an alpha value of 0.05. If a Grubbs test identified more than half of the data points as outliers, no data points were removed as the variation was likely due to insufficient analysis methods.

ANOVA tests were conducted on analysis data gathered from experiments with Control, 299V and VEGE033 samples from the finalized method. This includes protein content, ash content and lipid content, as well as RVA and DSC results. A Grubbs test was conducted if deemed appropriate in regards to the size, error and expected error of the method for each data set. Each statistical analysis performed is announced in the respective section of the results. T-test results are presented in figures and tables with accompanying lowercase letters in alphabetical order. Duplicate letters for results within the same test indicates they are not significantly different from each other.

3.6 Deviations from stated methods

Due to complications with the RVA machine, two tests for the Control sample group were unable to be performed and the total number of test results were therefore reduced to 4.

After initial analysis of the protein content of the HPC produced uncharacteristically low values, 4 more protein content tests were done on the HPC. The protein content value used for HPC in the report is the average value of all (n=7) HPC protein content results.

The non-frozen 299V test resulted in a diluted PP only containing 7.59% DMC. This made it impossible to use samples with 9% DMC when conducting the RVA measurements (as stated in the method description), and the 7.59% DMC samples were used undiluted when performing those RVA measurements.

4. Results and Discussion

4.1 Screening results and experiment design

The initial fermentation trials with the L. plantarum strain 299V tested pH decrease of the solution, with variations in incubation temperature, amounts of added bacterial culture, starting pH and fermentation time. The results are presented in Table 2. Firstly, it is seen that samples inoculated at alkaline pH (10.5) did produce acidic conditions when incubated at 30°C. This result exceeds the expected growth pH limit of 8.9 for *L. plantarum* but did, however, require extensive fermentation time. Inoculation at alkaline pH could potentially eliminate the need for citric acid usage in this process completely but will not be further discussed in this study due to the mentioned shortcomings making it less viable for industrial use.

Table 2: Initial trial fermentation results for L. plantarum 299V

	Volume [ml]	Microorganism	Added bacteria culture [mg]	Fermentation time [h]	Fermentation temperature [°C]	pH, start	pH, end
Screening 01	100	299V	70	24h	20°C	10.5	9.9
Screening 02	100	299V	70	120h	20°C	10.5	9.26
Screening 03	100	299V	70	24h	20°C	7	5.67
Screening 04	100	299V	70	96h	20°C	7	4.56
Screening 05	100	299V	200	24h	20°C	10.5	10.4
Screening 06	100	299V	200	120h	20°C	10.5	9.57
Screening 07	100	299V	200	24h	20°C	7	5.29
Screening 08	100	299V	200	96h	20°C	7	4.59
Screening 09	100	299V	70	24h	30°C	10.5	9.81
Screening 10	100	299V	70	120h	30°C	10.5	5.29
Screening 11	100	299V	70	24h	30°C	7	4.51
Screening 12	100	299V	70	96h	30°C	7	4.36
Screening 13	100	299V	200	24h	30°C	10.5	9.74
Screening 14	100	299V	200	120h	30°C	10.5	5.72
Screening 15	100	299V	200	24h	30°C	7	4.6
Screening 16	100	299V	200	96h	30°C	7	4.50

Looking at the samples in Table 2 that were inoculated at neutral pH, it is seen that the increased amount of added bacteria culture did not have a consistent effect on the pH of the LLP. The samples incubated at 20°C reached a pH of 5.67 and 5.29 after 24 hours, and 4.56 and 4.59 after 96 hours. The samples incubated at 30°C reached pH values of 4.51 and 4.6 after 24 hours, and 4.36 and 4.50 after 96 hours. This indicates that the pH decrease in the 30°C samples mostly occurred in the first 24 hours, whereas incubation at 20°C requires between 24 to 96

hours to reach the same pH levels. This correlation between increased temperature and pH decrease is most likely due to increased growth of the *L. plantarum* at 30°C, which matches the registered preferred growth temperature of *L. plantarum*.

The results from the second set of screening experiments, further investigating and comparing the pH decrease over time for 20°C and 30°C incubation, is presented Table 3. These pH results are significantly higher than their counterparts in the first round of screening experiments, with the only difference being the larger sample volume. This prompted the development of new screening experiments, presented in Table 4, which were incubated on a shake table. These samples showed a pH decrease to 4.49 within 24h, after which the pH values seem to slowly rise again with increased incubation time, indicating a possible stagnation in the growth of the Lactobacilli, or at least the production of lactic acid. With these results, the parameters of the finalized method for fermentation with *L. plantarum* 299V were set to 24h at 30°C, incubated on a shake table.

Table 3: Results from second round of trial fermentations with L. plantarum 299V

	Volume [ml]	Microorganism	Amount of culture added [mg]	Fermentation time [h]	Fermentation temperature [°C]	pH, end
Screening 17*	400	299V	280	48	20	5.12 +/- 0.07
Screening 18*	400	299V	280	24	30	5.05 +/- 0.03
Screening 19*	400	299V	280	48	30	4.83 +/- 0.02

*n=3

Table 4: results from screening experiments incubated in shake table setup

	Volume [ml]	Microorganism	Amount of culture added [mg]	Fermentation time [h]	Fermentation temperature [°C]	pH, end
Screening 20	400	299V	280	24	30	4.49
Screening 21	400	299V	280	48	30	4.67
Screening 22	400	299V	280	120	30	4.95

The results from the last part of the screening process, evaluating fermentation using the cultures VEGE033 and VEGE047, are presented in Table 5. All samples were incubated at 30°C on a shake table to measure the pH decrease capability with varying fermentation times. The results show that both VEGE033 and VEGE047 can reach pH values close to 4.5, the same as 299V, but it takes 72h for VEGE033 and 48h for VEGE047 which is three and two times longer than 299V respectively.

Table 5: Trial fermentations results for VEGE033 and VEGE047

	Volume [ml]	Microorganism	Amount of culture added [mg]	Fermentation time [h]	Fermentation temperature [°C]	Volume [ml]
Screening 23*	400	VEGE033	2.50	24h	30°C	5.06 +/- 0.02
Screening 24*	400	VEGE033	2.50	48h	30°C	5.14 +/- 0.42
Screening 25	400	VEGE033	2.50	72h	30°C	4.51
Screening 26	400	VEGE047	2.50	24h	30°C	5.46
Screening 27	400	VEGE047	2.50	48h	30°C	4.54
Screening 28	400	VEGE047	2.50	72h	30°C	4.51

*n=2

The results of the protein content analysis of the screening experiment samples are compiled in Table 6. All the samples presented, apart from Screening 17-19, were incubated on a shake table. The "Precipitation Yield" results depict how much of the protein available in the LLP was precipitated into the PP and should be seen as the yield of the fermentation step. It is important to note that a precipitation yield of more than 100%, showcased in several screening samples, is not deemed feasible. Such a result could potentially be the result of the fermentation process altering the amino acid composition, which could render the nitrogen conversion factor of 6.25 inaccurate. Using a lower nitrogen conversion factor for all fermented samples would amend such a problem, but the more likely cause of these high yields is simply inaccuracies in the analysis measurements. The screening sample precipitation yields are therefore treated as arbitrary numbers, with the main purpose of providing insight into their number value in relation to each other, to allow the selection of an optimized method for further testing (as opposed to treating the yield values as representative of the actual process yields).

Looking at the precipitation yields for the 299V samples, the samples incubated on the shake table showcase higher precipitation yields than the samples that were incubated under corresponding conditions in the heating cabinet. This is consistent with the lower pH results of Screening 20 and 21 (pH 4.49 and 4.67) compared to Screening 17-19 (pH 5.12, 5.05 and 4.83). The "PP Purity" results, showcasing the protein content (dw) of the Protein Precipitate (PP), do not show the same clear differences between 299V samples as the precipitation yields, which potentially indicates the PP purity being less sensitive to changes in precipitation pH.

The protein purity results for VEGE033 and VEGE047 do not show any major differences, apart from a lower purity in the samples that were fermented for 72h (Screening 25 and 28) which most likely is due to the longer fermentation time and larger amount of dead bacteria in the PP. The precipitation yields, on the other hand, show some very interesting results. For the VEGE033 samples, there is a clear trend of reduced precipitation yield with increased fermentation time. The reduced protein yield over time could indicate a stronger proteolytic activity in the VEGE033 culture during the fermentation compared to 299V and VEGE047. Since the sample size is very small, this result could also be due an anomaly or handling error.

Lastly, the exceptionally high precipitation yield of the 24h incubated VEGE033 sample (Screening 23) prompted the decision to make this screening method (VEGE033 bacteria, 24h, 30°C) the second finalized fermentation method. This also makes the two finalized fermentation methods identical, aside from the choice of added microorganisms, which provides more possibilities in comparing the effectiveness of the two methods.

Table 6: Precipitation yield and PP purity of Screening experiments

	Microorganism	Fermentation Time	Fermentation temperature [°C]	Precipitation Yield [%]	PP Purity [%]
Screening 17	299V	48	20	94.8 +/- 1.5	78.50 +/- 0.39
Screening 18	299V	24	30	95.8 +/- 3.5	77.00 +/- 1.89
Screening 19	299V	48	30	92.2 +/- 7.6	76.81 +/- 0.23
Screening 20	299V	24	30	118.1	76.88
Screening 21	299V	48	30	110.1	76.56
Screening 23	VEGE033	24	30	131.3	79.00
Screening 24	VEGE033	48	30	110.7	79.88
Screening 25	VEGE033	72	30	85.3	75.50
Screening 26	VEGE047	24	30	99.84	78.19
Screening 27	VEGE047	48	30	91.57	78.00
Screening 28	VEGE047	72	30	104.1	74.88

The dry matter content of the PP from the screening methods, presented in Table 7, shows an interesting trend: with increasing fermentation time, the dry matter content decreases. This is most apparent for VEGE033 samples, where the dry matter content is almost halved as the fermentation time is increased from 24h to 72h. These results show a greater water retention capacity of the solids present in the sample after prolonged fermentation. This is most apparent in screening sample 25 and could be the result of the bacteria producing metabolites such as soluble fibres or other hydrocolloids that are able to trap more water in the precipitate than the original solids were (compare to water content of screening sample 23 that underwent a shorter fermentation). Although these results do not directly influence the yield of the method, it is an interesting result that is worth noting and could be relevant when analysing the rheological properties of the protein isolates.

Table 7: Dry matter content of screening samples

	Screening 20	Screening 21	Screening 22	Screening 23	Screening 24	Screening 25	Screening 26	Screening 27	Screening 28
Bacterial culture	299V	299V	299V	VEGE033	VEGE033	VEGE033	VEGE047	VEGE047	VEGE047
Fermentation Time [h]	24	48	72	24	48	72	24	48	72
Dry matter content [%]	28.97	26.72	25.87	27.17	22.66	15.97	21.01	22.23	19.50

4.2 Extraction and precipitation results of finalized methods

The overall efficiency of the protein extraction and protein precipitation, as well as the purity of the protein precipitate, for each of the three precipitation methods are displayed in Figure 2: Variations between process replicates of the same method are presented in Figures 3-5. The extraction yields in Figure 2 are very similar and with very small standard deviations within each population, which is to be expected as the extraction process does not differ between the three methods. However, the precipitation yields differ between the methods, with 299V displaying the highest precipitation yield, and the control method displaying the lowest precipitation yield. Furthermore, the standard deviations for the precipitation yields are much larger than for the extraction yields. Since these larger variations in the precipitation yield are found in both fermented samples and the control samples, it is reasonable to argue that the variation is a product of the precipitation step itself and not the fermentation process. Furthermore, an ANOVA test p-value of 0.0030 indicates a significant difference in the precipitation yields between the 3 sample populations.

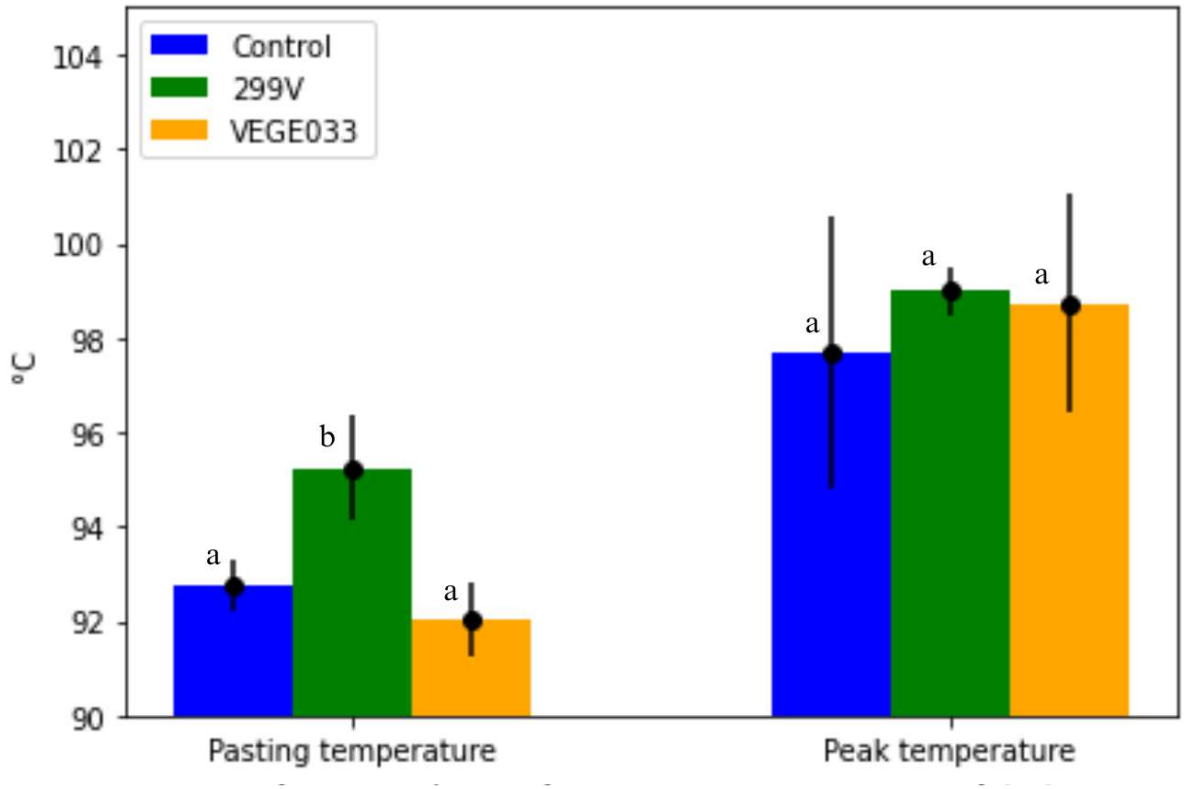

Figure 2: Extraction, precipitation and total yields as well as Protein purity results for Control, 299V and VEGE033 samples

The total yield results presented in Figure 2 follow the same trend as the precipitation yields, which is logical as the total yield is the result of the precipitation and extraction process. Lastly, the PP purity (protein content dw) results presented in Figure 2 are very similar. This strongly suggests that the fermentation process does not significantly affect the purity of the product, which is supported by an ANOVA test p-value of 0.063.

4.3 Ash content results

The results of the ash content analysis, presented in Table 8, show a reduced ash content in the protein precipitates compared to the HPC, indicating that during the extraction and precipitation process, ash contents are removed. This may be due to part of the ash contents never being extracted from the HPC and therefore discarded with the spent solids. It could also be a result of some of the ash contents not precipitating under acidic conditions and therefore being discarded with the residual supernatant, referred to as Spent Liquid (SL). Since the hempseeds are grown in a field, it is reasonable to assume that there is a certain amount of sand and dirt in the HPC. As stated in the background, presence of sand or dirt is a common reason for elevated ash content results, and such a sand fraction would naturally sediment together with the Spent Solids (SS).

Furthermore, a lower average ash content is seen in the fermented samples and compared to the control samples. This is further established by an ANOVA test confirming significant difference between the Control, 299V and VEGE033 sample populations. Since the extraction process is identical for all samples, the ash content of the liquid going into the fermentation/precipitation process is the same as for the control. The difference in remaining ash content after the precipitation step between control samples and fermented samples could be due to the microorganisms metabolizing mineral compounds, creating water soluble ions that do not precipitate during the centrifugation, and are therefore not included in the resulting protein precipitate (PP).

Table 8: Ash contents and Lipid contents of HPC and finalized experiments

	HPC	Control	299V	VEGE033
Ash content (dw) [%]	5.61^{a} +/- 0.11	3.57^{b} +/- 0.20	2.33^{c} +/- 0.15	1.51^{d} +/- 0.46
Lipid content (dw) [%]	9.86^{a} +/- 0.11	6.06^{a} +/- 1.06	6.25^{a} +/- 1.24	6.08^{a} +/- 1.19

4.4 Fat content results

The results of the fat content analysis, presented in Table 8, show a reduced fat content in all three protein precipitate samples compared to the HPC. This is not surprising as there is distinct layer, presumably of a lipid fraction, on top of the supernatant after each centrifugal separation. Parts of this layer adhere the inside of the centrifuge bottle, and therefore is not included in the LLP after the first centrifugation, and the majority of the visible (presumed) lipid layer after the second centrifugal separation is discarded along with the Spent Liquid (SL). Statistical analysis provided no significant difference between the lipid content of the three PP sample populations.

4.5 RVA results

The pasting temperatures, $T_{pasting}$, and peak temperatures, T_{peak}, from the RVA measurements are presented in Figure 3. The pasting temperatures show a clear difference between the methods, whereas the peak temperatures do not. The peak viscosity results, presented in Figure 4, show very large error bars due to the large variation in the results. Grubbs test for the 9 data

sets did not result in the removal of any outliers. ANOVA test values confirm that there is only significant difference between the pasting temperature values, while the peak temperatures and peak viscosities can not be determined to be significantly different.

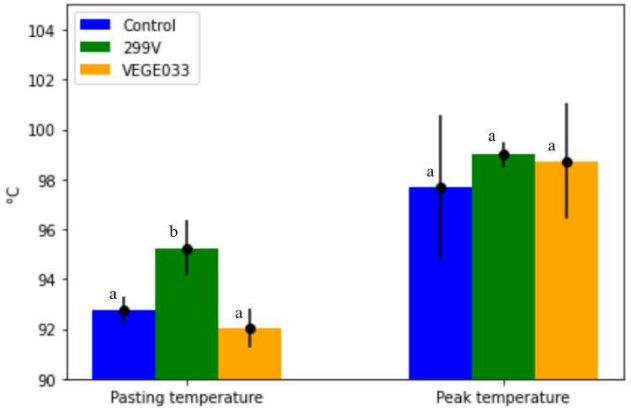

Figure 3: Pasting and peak temeperatures for control, 299V and VEGE033 samples

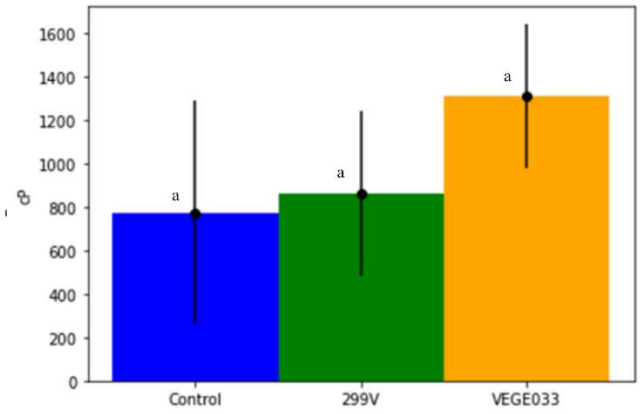

Figure 4: Peak viscosity (cP) for control, 299V and VEGE033 samples

Further understanding of these results is granted by Figures 5-7, showing all RVA curves for each sample group (299V, Control and VEGE033) respectively. In Figure 5, depicting the viscosity curves for 299V, there is a clear grouping of curves that have a viscosity peak around 900cP, and behave very similarly. The two lower curves, with a viscosity peak around 500cP come from the same process replicate, indicating that the difference may be due to a problem with that specific sample. The control sample RVA results, displayed in Figure 6, also showcase peaks at 900cP, as well as 500 and 250cP. Lastly, the RVA viscosity curves of the VEGE033 samples, displayed in Figure 7, do not display any distinguishable pattern, but instead shows viscosity curves with varying shapes and peak intensities.

The viscosity curves at peak viscosity for the non-frozen 299V samples are displayed in Figure 8. The shape of the curves is similar to that of the frozen samples, and the peak viscosities are unfortunately not comparable to the other samples due to insufficient preparation methods. However, the shape and peak values of the 3 non-frozen sample viscosity curves are a lot more consistent than the graphs of the frozen samples, indicating that the freezing process potentially changes the characteristics of the sample and that this change may vary a lot in between samples.

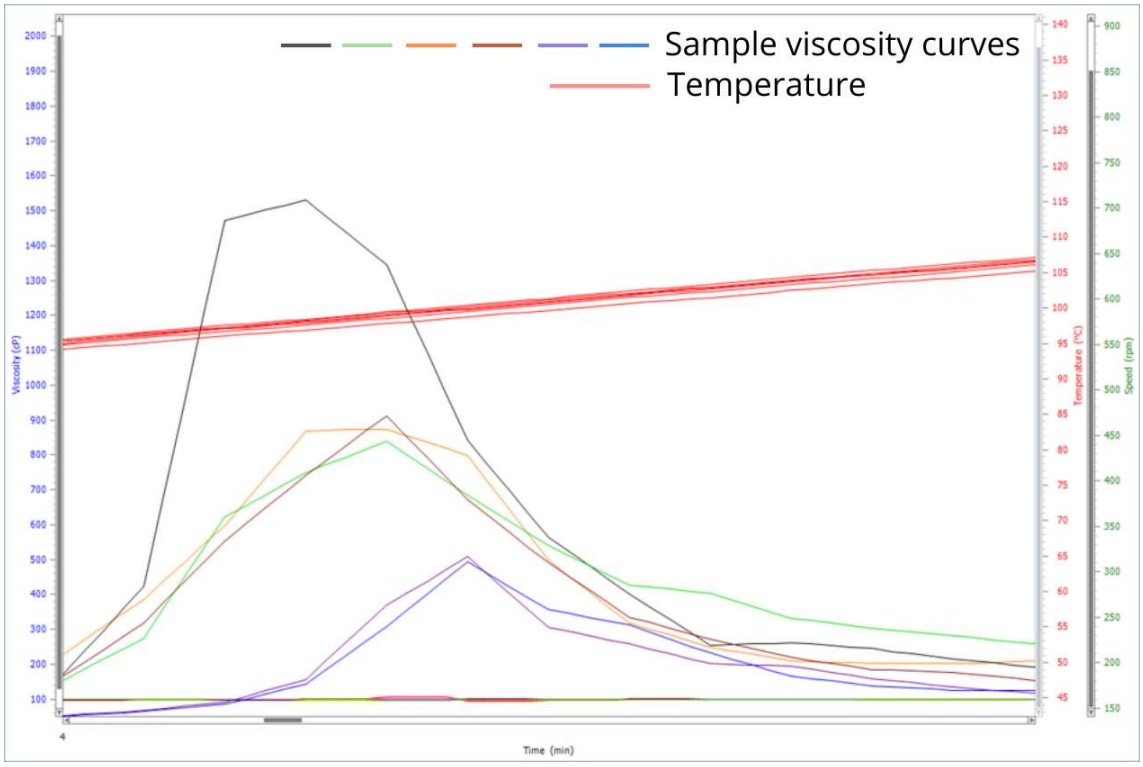

Figure 5: Viscosity peak results of 299V samples

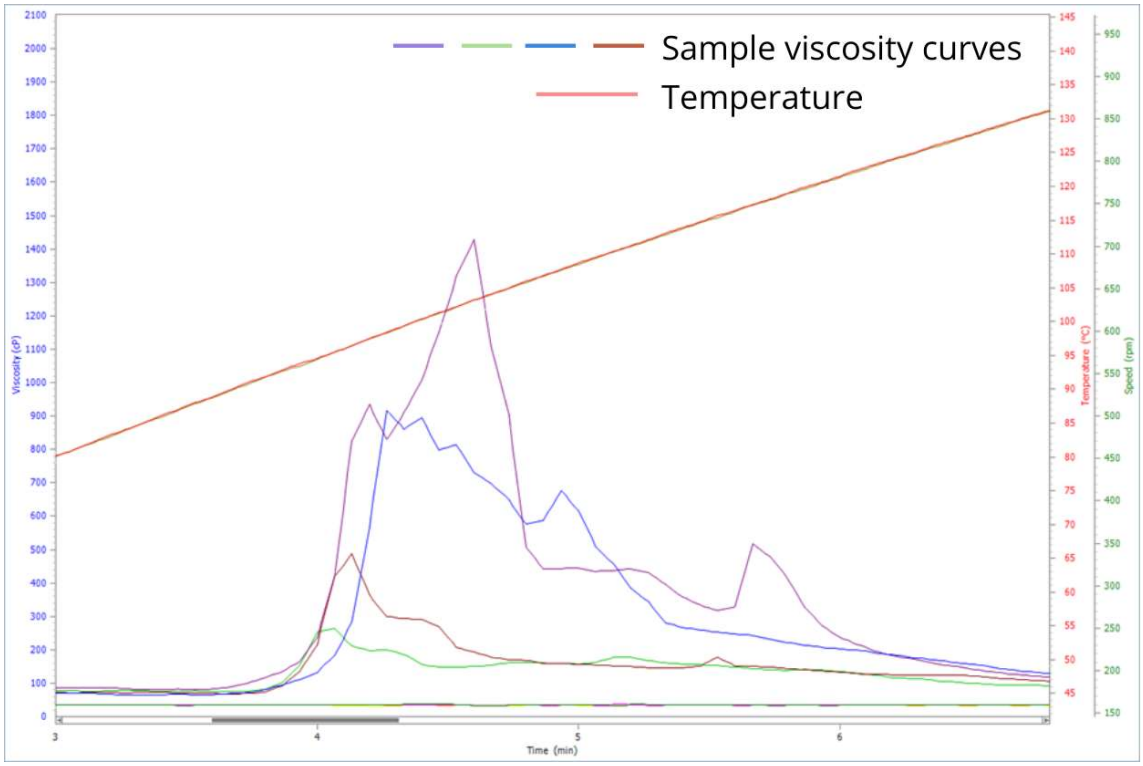

Figure 6: Viscosity peak results of Control samples

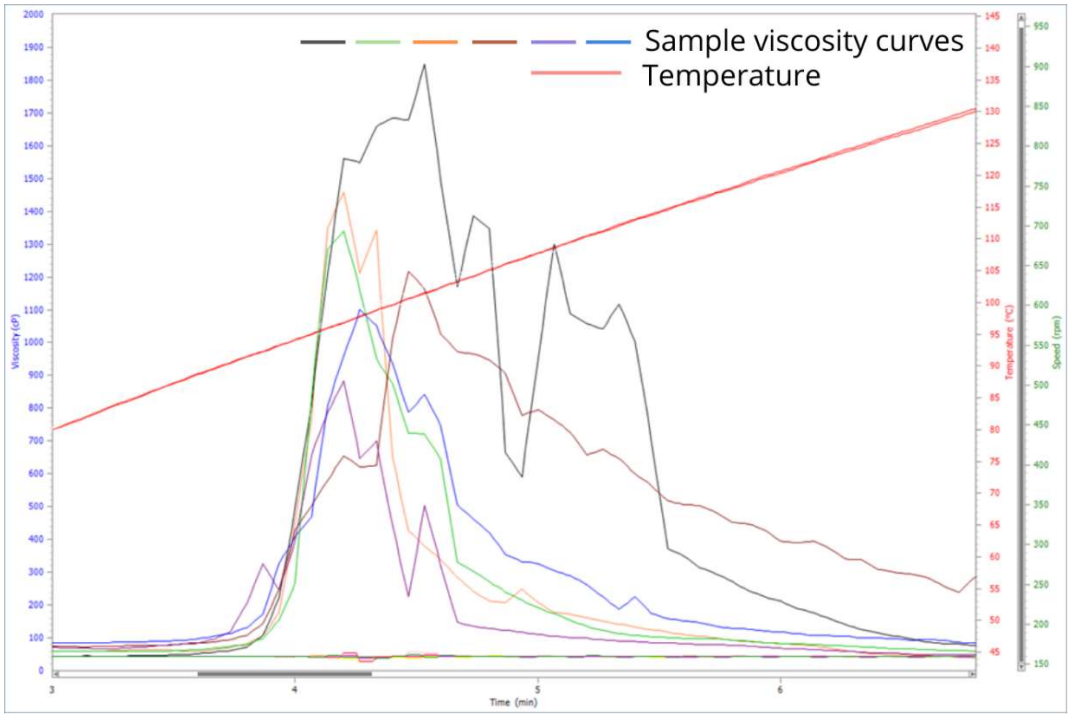

Figure 7: Viscosity peak results of VEGE033 samples

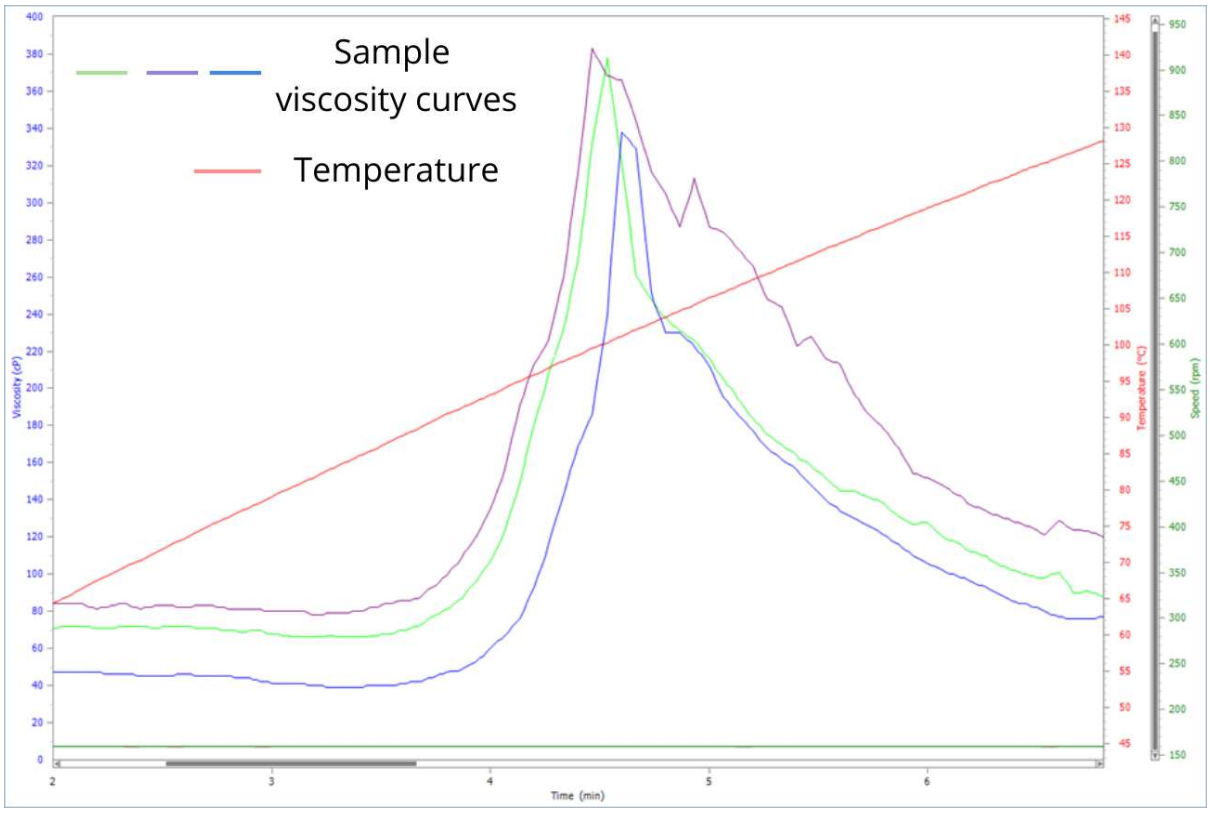

Figure 8: Viscosity peak results of non-frozen 299V samples

The viscosity curve throughout the entire RVA analysis is presented in Figure 9 for the non-frozen 299V samples, and in Figure 10 for one of the control samples. Because all the viscosity curves from the control, 299V and VEGE033 samples were very similar when displayed over

the entire RVA process, the chosen control sample will act as a model graph for all the samples that had undergone freezer storage. The curves of the non-frozen samples showcase several viscosity peaks of comparable intensities throughout the RVA analysis, whereas the frozen sample shows a single viscosity peak after which the viscosity stays low. This would indicate that the only notable viscosity shift in the frozen sample occurs when the observed major protein denaturation occurs. The subsequent viscosity peaks displayed by the non-frozen samples, however, could be the results of other molecular structures activated by the heat. The absence of these peaks in the frozen samples indicate that some heat-activated viscosity-increasing characteristics are lost during the freezing process.

The curds of denatured proteins that were left after each completed RVA analysis looked almost identical throughout all of the samples. The main difference was that the control samples produced curds of a lighter colour than the 299V and VEGE033 samples, indicating that the fermentation process could affect the colour of a potential food formulation using the protein precipitate. The curd from one of the 299V samples is displayed in Figure 11, and the curds from the control and VEGE033 samples are displayed in Figure 12, on the left and right side respectively.

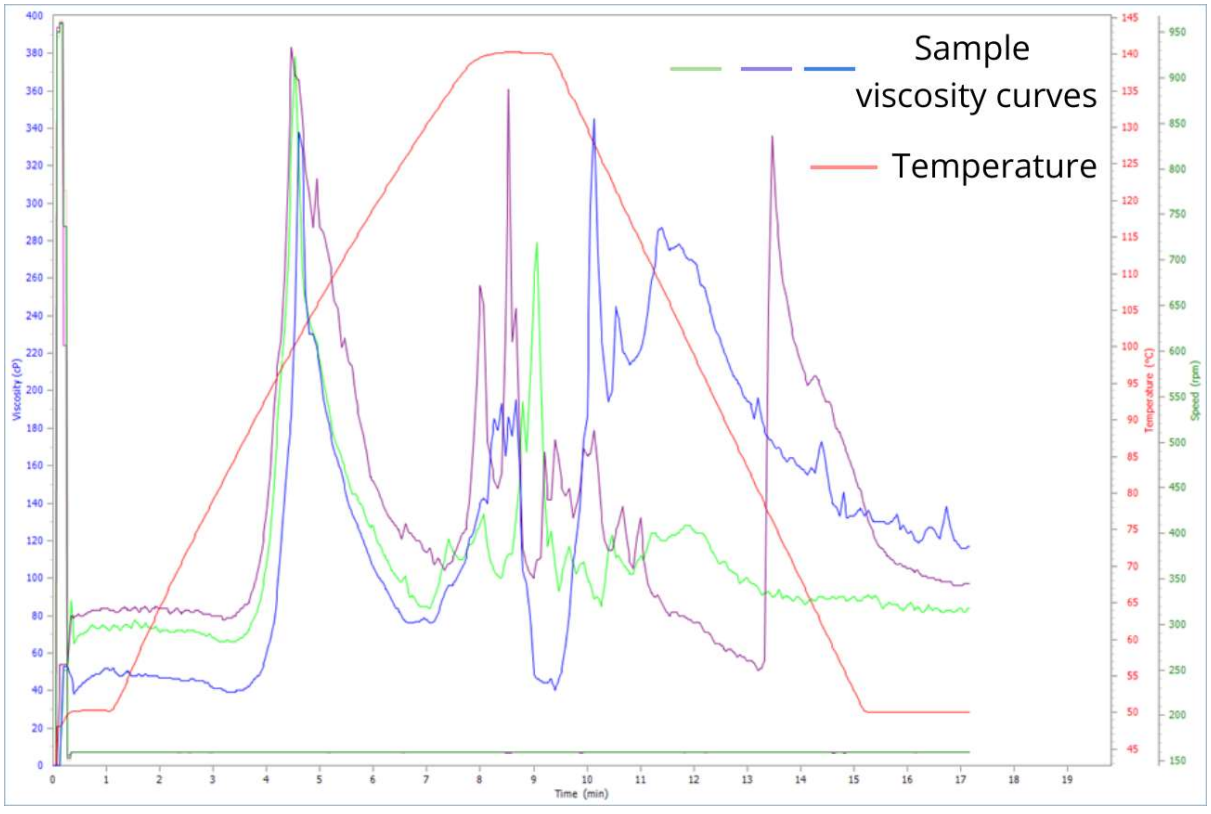

Figure 9: Complete RVA graph of non-frozen 299V samples

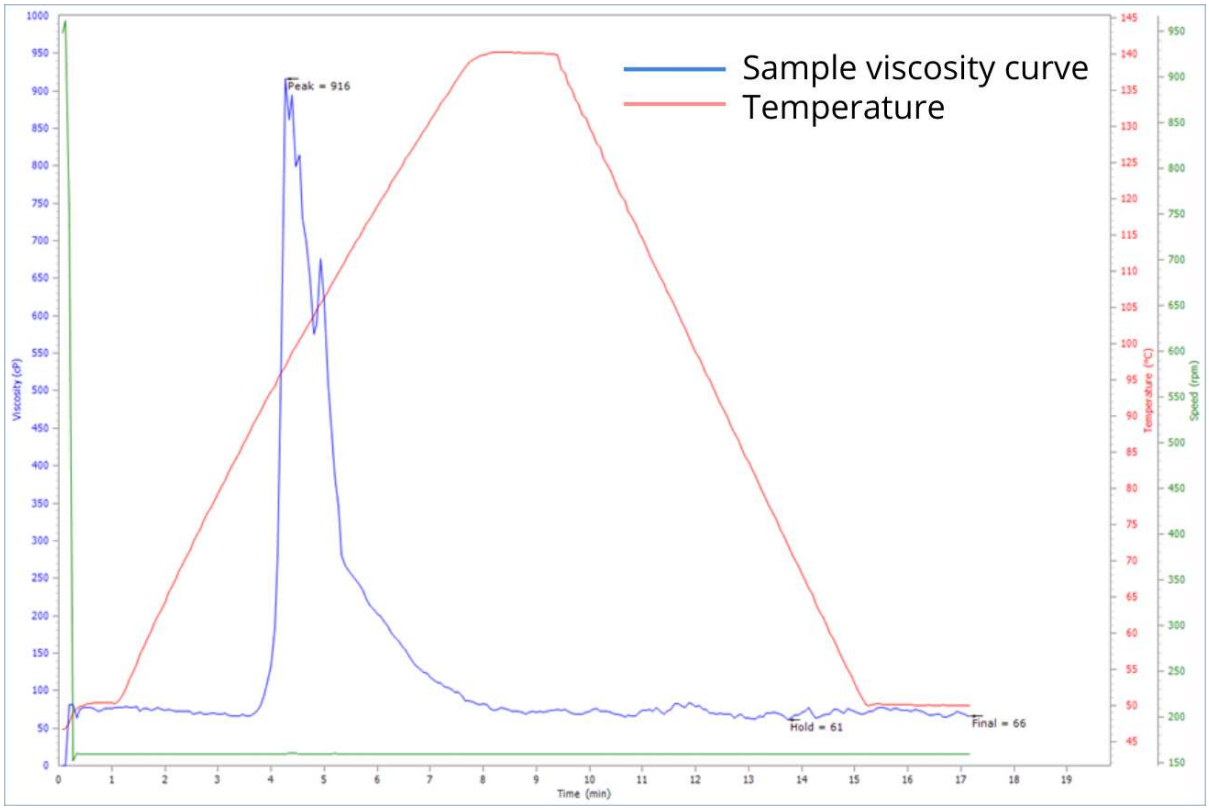

Figure 10: Complete RVA graph of one control sample

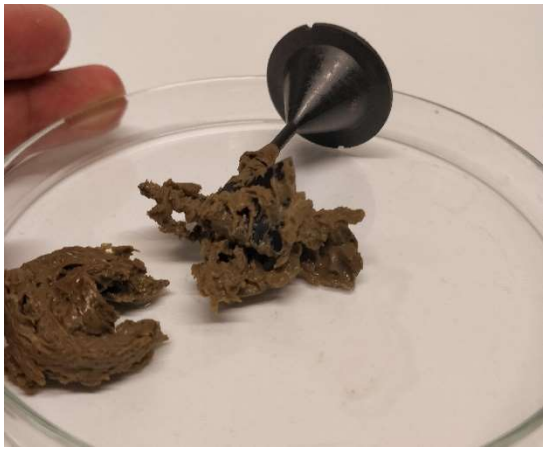

Figure 11: Curds of denatured proteins from 299V samples

Figure 12: Curds of denatured proteins from control (left) and VEGE033 (right) samples

4.6 DSC results

DSC measurements, presented in Figure 13 and Figure 14, show small differences between the three sets of samples, but conclusions are hard to make due to the large standard deviations. A Grubbs test was performed on all 9 data populations, and identified outliers in 5 of them. The test unfortunately identified 5-7 outliers in the respective populations (n_{tot}=9), which means that it is probably not a matter of outliers, but rather a systemic error in the analysis method. ANOVA test p-values of 0.048 and 0.151, for the enthalpy change and the peak temperature respectively, indicate that there is a significant difference in protein denaturation enthalpy

change between the protein precipitates produced by the different precipitation methods, but that no significant difference can be displayed regarding the denaturation temperatures.

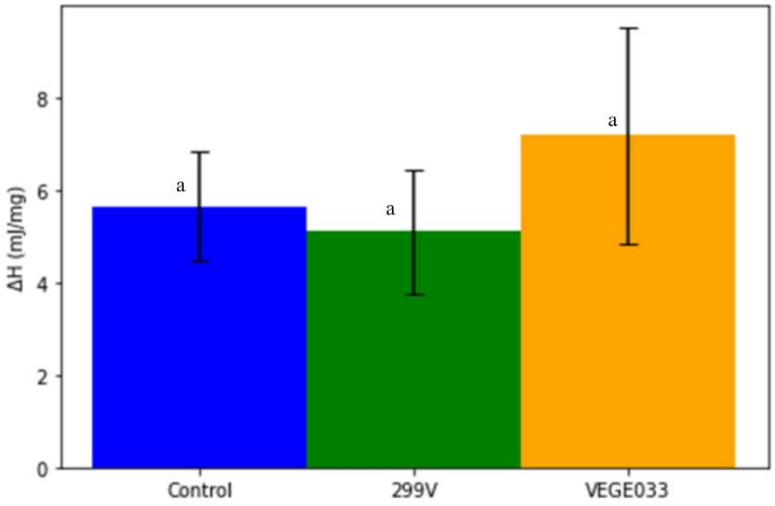

Figure 33: Main enthalpy change observed in DSC tests

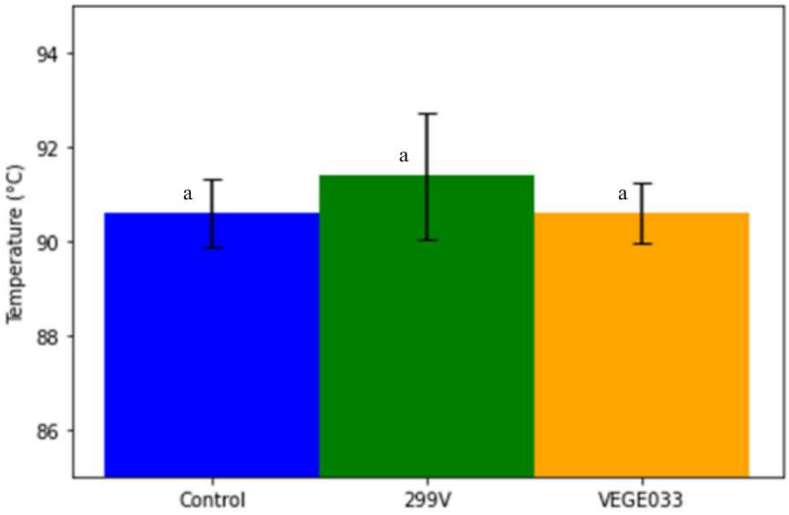

Figure 44: Peak temperature of main anthalpy change from DSC measurements

Compared to the expected viscosity peak temperature of 95°C (displayed by HPI), all three test groups show significantly lower peak temperatures. Furthermore, compared to the reference DSC profile of Hemp Protein Isolate, which exhibits one clear peak with a peak at 95°C, all three sample groups exhibit enthalpy changes at several other different temperatures. This becomes clearer when looking at Figure 15, showcasing the enthalpy as a function of temperature for the Control, 299V, VEGE033 and "fresh" 299V samples. The main peak, present at around 90°C, is consistently the largest enthalpy change but there are smaller enthalpy changes displayed in curves from all four sample populations, making it difficult to draw further conclusions regarding differences in protein composition and behaviour between the precipitation methods or between the frozen and "fresh" samples.

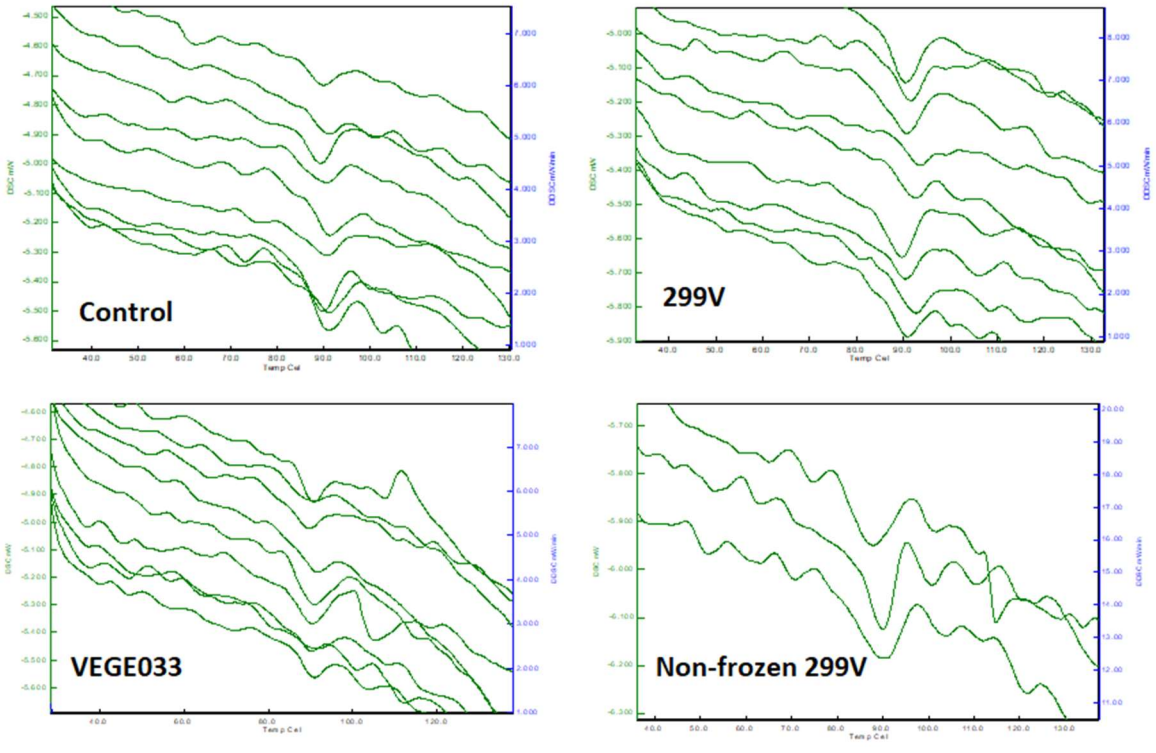

Figure 15: DSC curves for Control, 299V, VEGE033 and non-frozen 299V samples

4.7 Microbial control results

The results from the viable bacterial count, displayed in Table 9, show a significantly lower viable cell count for both Enterobacteriaceae and yeast in the 299V, and VEGE033, samples compared to the Control samples. This indicates that the fermentation process significantly lowers the presence of these pathogens, and potentially improves the shelf life of the PP. Furthermore, the 299V samples display slightly higher viable count of Lactobacillus the total viable cell count, indicating that the majority of live microorganisms present are Lactobacillus, probably 299V. This result, along with the consistent pH reduction and precipitation yield results, strongly indicates that *L. plantarum* 299V is a suitable microorganism for this process.

Table 9: Microbial control results displayed as cfu/ml

	Control	299V	VEGE033
TSA (total cell count) [cfu/ml]	2.59E+04	4.80E+09	8.00E+09
Rogosa (Lactobacillus) [cfu/ml]	0.00E+00	1.30E+10	0.00E+00
VRBD (Enterobacteriaceae) [cfu/ml]	1.14E+03	8.00E+01	0.00E+00
MA (Yeast/Mould) [cfu/ml]	8.80E+03	8.00E+01	3.00E+01

The TSA and Rogosa results for VEGE033, however, look very different. A large total count is seen, on par with 299V, but no growth of Lactobacillus was recorded. This could indicate a failed fermentation, as the VEGE033 culture is a mix of *Streptococcus thermophilus* and

Lactobacillus delbrueckii subsp. *Bulgaricus*, and therefore should have a significant concentration of Lactobacilli. This information, combined with the high concentration of total bacteria, could mean that *L. delbrueckli* simply does not grow on Rogosa. However, it could also mean that the added VEGE033 culture was not able compete with the existing microflora in the HPC, and that the TSA cell count is a wide mix of microorganisms. This theory is further supported by the inconsistent pH values recorded by the VEGE033 fermentations.

5. Conclusion

The results presented in this report clearly indicate that utilizing lactic acid producing bacteria for precipitation of extracted hemp proteins does not compromise the yield or purity of the resulting protein precipitate. Furthermore, the results suggest that utilizing fermentation precipitation could potentially increase the yield of protein, though more extensive research, including optimization of fermentation parameters, needs to be conducted before more definitive conclusions can be made.

The majority of tests related to protein denaturation and rheology changes in this report does not show significant differences between the different precipitation methods tested, apart from small variations in pasting temperature and denaturation temperature, indicating potential variations in cooking temperatures being possible if these differences are further investigated. However, the viscosity analysis results show clear differences between the frozen and non-frozen samples. To understand the cause of this, as well as the role of the fermentation process in these results, more research is needed.

There is clear evidence for the fermentation process greatly reducing the presence of pathogens such as yeast and Enterobacteriaceae, which is likely a result of the low pH and the bacteriocins produced by the LAB. This suggests that fermentation precipitation could be a suitable process choice for increasing the shelf life of the product.

To conclude, the results of this project indicate that utilizing lactic acid bacteria to aid precipitation of hemp proteins could be a valid alternative to chemical precipitation. Before industrial applications are appropriate, more research needs to be conducted to investigate the potential max yield and efficiency of the fermentation process, as well as comparing the effects of the fermentation process to a chemical precipitation conducted over an equal time period.

6. Acknowledgements

First and foremost, I want to thank my supervisors Jeanette Purhagen and Åsa Håkansson for their supervision and mentorship throughout the thesis work. I am so grateful for all the time you have put into this project, and I am honoured to have been a part of it.

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Additionally, I want to highlight the importance of the international academic community who have conducted decades of research, making all the different parts of this project possible. At the time this project is being completed, all 90’000 university students in Gaza have lost access to their university education, and I dedicate this thesis to their right to education and academic freedom.

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