Improving the sensorial properties of pea protein through fermentation

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Abstract

Lactic acid fermentation has been a widely used process for many years, offering nutritional and sensory benefits to plant proteins. In this study, the objective was to produce a fermented pea powder with enhanced sensory properties by combining previous research from FrieslandCampina. A screening was performed on a set of cultures obtained from a contract research organization (CRO), starter Culture A, Culture B and Culture C. The best combination was determined based on the volatiles obtained from GC analysis, pH kinetics, and their effects on physicochemical and sensorial properties, with the Culture B being the most suitable. Pretreatments such as pasteurization and the combination of pasteurization and homogenization were used, and the intensity of stirring during fermentation was found to play a crucial role in the physicochemical properties of the pea protein medium. After that the product was again homogenized and pasteurized, with viscosity measurements taken for each process. SDS-PAGE analysis showed signs of albumin hydrolysis. By comparing the reference with the fermented protein chemically, there is a major drop in the protein level, while glycose was mostly utilized for the growth of microorganisms. Sodium and potassium displayed a huge increase due to the caustics used for neutralization. GC analysis was performed in various stages of the process, to determine the volatile compounds and the effect of each process stage. Overall, fermentation had a huge impact on the volatile compound profile, reducing the off flavors of the pea protein. Spray drying mitigates some compounds and increases the levels of hexanal. The use of a lower inlet temperature during spray-drying resulted in decreasing the levels of benzaldehyde. Comparing the proteins that were developed in this experiment with the protein that was developed in CRO they had enhanced volatile profiles. However, during the sensory analysis, the CRO protein was preferred. This is due to the fact of the caustics' effect that were used during neutralization. From that experiment a process diagram is proposed. Further research needs to be done for the combination of caustics that should be used for neutralization, the optimization of the production process, reduction of the fermentation time

and the increase of the production yield. In Figure 1 a proposed diagram for the fermented pea protein production is presented.

Keywords: Lactic acid fermentation, fermented pea powder, cultures and screening, physicochemical properties, sensory analysis.

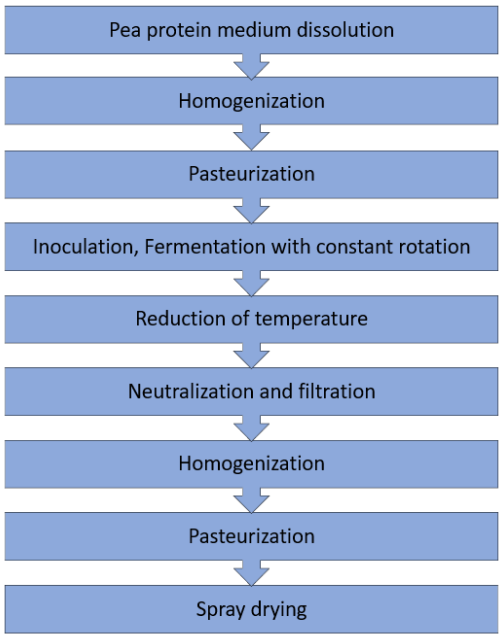

Figure 1 Suggested process diagram for the production of a fermented pea protein.

Acknowledgments

Firstly, I would like to express my heartfelt gratitude to the esteemed Lund University for providing me with an incredibly enriching and memorable semester. I am truly grateful for the opportunity to undertake this internship, which has been an invaluable experience. A special thank you goes to Eric and Jenny for their unwavering support and guidance throughout this journey.

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Lund, June 2023

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List of abbreviations

ANFs	Anti-nutritional factors
CAGR	Compact annual growth rate
CRO	Contract Research Organization
DMDS	dimethyl disulphide
GC	Gas chromatography
HPLC	High performance liquid chromatography
kDa	kilo-Dalton
KOH	Potassium hydroxide
LAB	Lactic acid bacteria
LAF	Lactic acid fermentation
LOX	Lipoxygenase
MS	Mass spectrometry
NaOH	Sodium hydroxide
PET	Polyethylene
rpm	Rotations per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
UHT	Ultra high temperature
USD	United States Dollars

1 Aim & Objectives

FrieslandCampina Innovation Centre has conducted extensive research to determine the optimal conditions required for the development of fermented powdered protein, which include pre-treatments of protein, culture varieties, and fermentation temperature. Building on this previous work, the main objective of this thesis is to develop a powdered protein with improved sensory properties, which involves achieving rapid acidification and a desired aroma, taste, and color of the protein powder, while it could be also easily adapted to the process equipment that the facilities of the FrieslandCampina Innovation possess. The study adopted a scientific approach to evaluate the results, employing analytical techniques such as gas chromatography-mass spectrometry (GC-MS) to identify and quantify the volatile compounds present in the protein samples and the chemical composition of the fermented pea protein will be reviewed, highlighting the differences after fermentation. In the end, an informal sensory analysis will be conducted and compare the fermented pea protein that will be produced in this experiment with another one that was produced by a Contract Research Organization (CRO). Also, the packaging requirements of that product will be further investigated, in order to propose an appropriate packaging for the developed product.

2 Introduction

2.1 Pea protein: market opportunities and challenges

In recent years, pea protein has gained significant attention as a sustainable alternative to animal-based proteins. The pea protein market is growing at a rapid pace, driven by increasing consumer awareness about the benefits of plant-based diets and a growing demand for sustainable protein sources (Grand View Research, 2022). The COVID-19 pandemic has also contributed to the growth of the pea protein market, as consumers have become more health-conscious and are looking for products that support a healthy lifestyle.

Despite the growing demand for pea protein, there are still several challenges that need to be addressed. The low solubility and undesirable aroma of pea protein have been major issues in the food industry, leading to lower demand for pea protein-based products (Schindler, 2012; Pontonio and Rizzello, 2021). To overcome these challenges, researchers are developing new processing techniques and flavor masking technologies to improve the sensory qualities of pea protein (Lan, 2019).

Anti-nutritional factors (ANFs) in pea protein are also a significant challenge that needs to be addressed. Researchers are exploring various strategies to reduce the levels of ANFs in pea protein, including genetic modification, fermentation, and enzymatic treatment (Karlund et al., 2020). In addition, the development of pea varieties with reduced ANF content could help increase the utilization of pea protein in the food industry.

Despite these challenges, the pea protein market is expected to continue to grow in the coming years. According to a report by Grand View Research, the global pea protein market size was valued at USD 547.8 million in 2020 and is expected to reach USD 1.4 billion by 2028, growing at a CAGR of 12.8% from 2021 to 2028 (Grand View Research, 2022). The increasing demand for plant-based proteins, coupled with the growing need for sustainable food sources, is expected to drive the growth of the pea protein market in the coming years.

2.2 Lactic acid fermentation: bringing the past into the future

Lactic acid fermentation (LAF) has been used for centuries to preserve and enhance the flavor of food products. The process involves the conversion of carbohydrates to lactic acid by lactic acid bacteria (LAB), which results in the production of various metabolites, such as organic acids, volatile compounds, and bioactive peptides. The use of LAF has been documented in several traditional food products, including yogurt, cheese, sourdough bread, sauerkraut, and kimchi (Wolfe and Dutton, 2020).

The history of LAF dates back to ancient times, where it was used by humans for preserving and enhancing the nutritional value of food. One of the earliest recorded uses of LAF was by the Babylonians, who used it to ferment milk and produce sour milk (Hutkins, 2019). Similarly, the Greeks and Romans used LAF to make cheese and yogurt, respectively (Wolfe and Dutton, 2020). In Asia, LAF has been used for centuries to produce various fermented foods, such as miso, soy sauce, and tempeh (Tamang et al., 2016).

In recent years, LAF has gained attention as a promising method to address the off-flavors and anti-nutritional factors (ANFs) in legume-based food products, such as pea protein. ANFs, including phytic acid, saponins, and trypsin inhibitors, can hinder nutrient availability and affect the digestibility of proteins (Karlund et al., 2020). LAF has been shown to decrease the presence of ANFs in legumes and increase the bioavailability of macro- and micronutrients (Tangyu et al., 2019; Mehak et al., 2021).

Moreover, LAF can improve the sensory and functional properties of legume-based food products. For instance, LAF has been shown to reduce the presence of off-flavors, such as hexanal, and increase the acceptability of soy-based dairy alternatives (Blagden and Gilliland, 2005). LAF can also produce bioactive peptides with inhibitory activities towards metabolic-syndrome-associated enzymes and decrease various gastrointestinal symptoms related to legume consumption (Karlund et al., 2020).

Recent studies have investigated the use of LAF in improving the properties of pea protein. Pea protein inoculated with *Lactobacillus casei* displayed the best properties among other samples, regarding the aroma and bitterness (Garcia Arteaga et al., 2021). LAF with lactobacillus species was able to reduce the presence of aldehydes like hexanal, heptanal, and pentanal, which are products of oxidation (Ben-Harb et al., 2019). Finally, LAF

decreases the ANFs of pea protein samples and increases their sensorial properties (Pontonio and Rizzello, 2021).
LAF has a long history of use in food preservation and flavor enhancement. It has also shown promise as a method to address the off-flavors and ANFs in legume-based food products, such as pea protein. With further research and development, LAF could potentially improve the nutritional and sensory properties of legume-based food products and increase their acceptability among consumers.

3 Materials & Methods

Pea protein Isolate 85A (also known as Plantaris pea protein), 85B and 80 were provided by AGT Foods and Ingredients (Regina, Canada). Also, syrup SIRODEX 410 (Tereos, Moussy-le-Vieux, France), yeast extract (Sigma-Aldrich, St. Lous, United States), protein hydrolysate and Proyield Pea PCE80B (FrieslandCampina Ingredients North America, Delhi, United States) were to develop a fermentation medium. Set of cultures were given by the CRO. These cultures were named Culture A, Culture B and Culture C.

3.1 Preparation of fermentation and monitoring of the fermentation

Fermentation mediums (7.5% protein) were prepared by adding pea protein isolate gradually to demi water while stirring with an overhead stirrer. Glycose syrup (1%), yeast extract (1g/L) and protein hydrolysate (1g/L) were selectively added and mixed into the mediums according to the experimental design. The medium was then treated with pasteurization (73 °C; 15 s) and it was stirred until, the product was homogenous, using a Gronfa Ferment-water bath 13160 (Gronfa, Zupthen, Netherlands) and an overhead stirrer VWR VOS 40 digital (VWR, digital), to continuously mix the samples. The medium after treatment was then distributed into Schott bottles (250 mL), which were previously autoclaved at 121 °C for 60 min. Each bag of frozen starter cultures (500 DCU) was thawed in 2000 mL ‘warm-up’ solution (1% protein, 1% sugar) to get the starter cultures ready for the fermentation environment. Frozen adjunct cultures (2 g) were diluted and thawed in demi-water (80 mL) before use.
During the fermentation process, various fermentation mediums were inoculated with starter cultures at a volume of 200 µL per 250 mL of medium, and when necessary, adjunct cultures were added at a volume of 100 µL per 250 mL of medium. The fermentation was performed at a

temperature of 42 °C for a duration of 20 hours. To monitor the fermentation progress, the pH of the inoculated samples was continuously measured and recorded every 5 minutes using the Cinac system manufactured by AMS Alliance, Frépillon, France (Appendix A & B). Moreover, The pH of fermented samples was measured manually using a pH meter (Mettler Toledo, Tiel, The Netherlands), in order to check if the pH values are in line with what is displaying in the Cinac system. The variables of protein (7,5%), carbohydrate source (1%) and supplements (1g/L) were determined and kept stable, stepping on the previous findings on this subject.

3.2 Scale up of the experiment

For the scale-up of the experiment, the medium was fermented in the buckets of the water bath. After the pasteurization, a method that was identical to the previous set-up of the experiment, the water bath was set at 42◦C, which was the fermentation temperature, and was left overnight, for approximately 12 hours. Previously, the buckets and stirrers that were used were autoclaved at 121 °C for 60 min.
Similar to the previous conditions of the experiment, the fermentation mediums that were prepared, were inoculated with starter cultures and adjunct cultures. The quantity of the starter and adjunct cultures was depended. on the net weight of the sample that would be fermented. Since in that instance the CINAC system could not be used, wireless pH probes were used instead to measure the pH every 5 mins, by utilizing PASCO system (VOS Instrumenten BV, Zaltbommel, Netherlands). In addition, the pH was also measured, by using the same pH meter that was used in the small-scale experiment (Appendix C&D)

3.3 Neutralization, spray drying and freeze drying and analysis of samples

After the fermentation, samples were neutralized with various caustics: sodium hydroxide, potassium hydroxide and sodium bicarbonate (Sigma-Aldrich Chemie, Schnelldorf, Germany) and also with various pH ranges

(6,7-7,8). The caustics were slowly added to the sample while stirring and the value of the pH was measured, using the PASCO system (VOS Instrumenten BV, Zaltbommel, Netherlands) Samples were then processed with the Janke & Kunkel Ultra-Turrax T50 (Janke & Kunkel, Germany), at 5000 rpm for 4 minutes. After the neutralization, samples were treated with UHT (80°C; 30 s) in combination with downstream homogenization (150bar/50bar). Samples were afterwards collected. Part of the collected samples was spray dried by using a Buchi mini Spray Dryer B-290 (Appendix E) and also freeze dried by a Buchi mini freeze Dryer L-200.

Samples, both in the small scale and the larger scale were gathered before, during and after fermentation during each trial for various analysis that will be explained below.

3.3.1 Microbiological analysis

The microbial composition of fermented samples was analyzed through enumeration of bacterial population, yeast and mold, as well as quantification of specific bacterial species such as *Bacillus cereus* and *Enterobacteriaceae*, using advanced microbiological techniques at the FrieslandCampina LQS Microbiology laboratory.

3.3.2 Chemical analysis

High-performance liquid chromatography (HPLC) was employed to quantify the sugar contents and spot the differences of both the fermented samples and the raw fermentation mediums. Furthermore, the protein contents and elemental compositions of the raw fermentation mediums were determined using the Kjeldahl method and inductively coupled plasma (ICP) spectroscopy, respectively. All of these analytical procedures were conducted at the Chemistry laboratory of FrieslandCampina LQS.

3.3.3 Volatile-compound analysis

The identification of both newly formed volatile compounds during fermentation and original plant-based volatile compounds after fermentation was performed using gas chromatography mass spectrometry (GC-MS). To this end, samples were collected before and after fermentation (4 g/vial) and sent for GC-MS measurements by internal analysts. Raw fermentation mediums collected before fermentation were also analyzed as references. In order to scale up the experiment, the samples were neutralized and pasteurized before being sent for analysis, spray and freeze-dried counterparts were also analyzed to evaluate the effect of spray drying on volatile compounds. These analyses were conducted in accordance with established scientific protocols to ensure accuracy and reproducibility of the results.

3.3.4 Viscosity and Storage-Loss modulus measurement

The rheological behavior of the samples during fermentation was analyzed by measuring their viscosity. A controlled-stress rheometer, Paar Physica MCR 302 (Anton Paar, Graz, Austria), was employed for this purpose. The system consisted of a concentric cylinder CC27 and bob measuring system. The measurements were conducted at a range of shear rates from 1-200 s^{-1} and the temperature was maintained at 20°C.
As for the measurements of the Storage and Loss modulus, it was conducted with the same equipment. The difference is that the temperature was set constant at 42°C and the pea protein matrix was inoculated with cultures before being inserted to the equipment to measure the Storage and Loss Modulus for 5 mins for 15hours. The results were expressed in Pascal (Pa).

3.3.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

In order to determine the molecular weight (Mw) of pea protein subunits after before and after fermentation, SDS-PAGE was performed. The samples were heated to 95◦C for 5 min and then cooled to room temperature. Samples were centrifuged to remove excess liquid, and 10µL

of each sample was loaded into the wells of a 4-15% Criterion™ TGX Stain-Free™ Precast protein gel along with 5 µL of Stain-free ladder to serve as a molecular weight standard. Electrophoresis was run in a 1x TGS buffer at a constant voltage of 100 V for 5 min, which increased to 150 V for 45-50 min until the dye reached ~0.5 cm before the end of the gel. The gel was then analyzed using a ChemiDoc MP+ Imaging System, and the intensity of the bands was quantified using ImageJ software (https://imagej.nih.gov/ij/).

3.4 Sensory evaluation

In view of time constraints, a preliminary sensory assessment was conducted within the organization, involving 16 untrained colleagues as participants. The evaluation comprised three phases. In the first phase, the participants were presented with two samples: a reference solution consisting of 80% AGT protein and 6% protein, and a fermented pea protein solution with a 6% concentration, which was produced at the Contract Research Organization (CRO). In the second phase, a 6% fermented pea protein solution produced using the cultures A and B as compared to the reference solution in a sensory test categorized as "Detailed comparison to reference".
Finally, the participants were asked to indicate which of the three protein solutions was perceived as the most "neutral". To begin with, the samples were prepared and served to the participants in a coded manner. Water was provided to neutralize the palate between different tastings. The responses were gathered using online forms, and the findings were subsequently analyzed.

4 Results & Discussion

Throughout the experiments, various parameters and variables including protein concentration, percentage of glucose added, and the hydrolysate and yeast extract concentrations were set in order to speed up the fermentation process. The protein concentration remained constant at 7.5%, while the carbohydrate level was kept stable at 1% and the fermentation temperature was set at 42◦C.

4.1 Selection of starter, adjunct cultures and pre-treatment methods of the medium

In the previous research conducted at the FrieslandCampina Innovation Center, the variables of protein concentration and carbohydrate level were established for the fermentation process using plant-based proteins and LAF. As seen in Figure 2, set Cultures A, B and C were prepared, with the fermentation mediums undergoing pasteurization and homogenization treatments. The mediums were afterwards inoculated with the different combination of microorganisms that are displayed in Figure 2. The correlation between time and pH was recorded and the time that it took for the samples to reach a value of pH 5 from an average pH of 6,96 was compared among the samples that had different cultures.

By keeping the starter culture stable and varying the adjunct cultures, it was found that all samples containing Culture A displayed a faster acidification rate. However, sensory screening revealed that the taste of these samples was not acceptable due to a sour aftertaste.

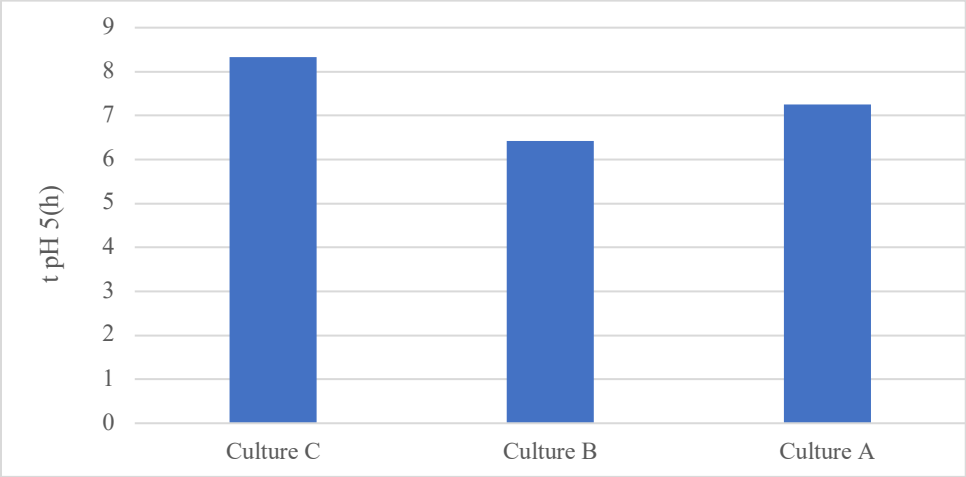

Figure 2 The time taken for the pea matrix samples, inoculated with various combinations of cultures, to reach pH 5.

In contrast, samples containing Culture B were well-liked by members of the FrieslandCampina Ingredients Team during the sensory analysis. One disadvantage of the samples was the formation of a strong gel after fermentation, which made extraction, neutralization, and handling of the samples challenging. Jesse Chen noted in his report that the homogenization treatment gave the samples a yogurt-like texture but made them difficult to process through spray drying (Chen, 2023). In general, pea protein isolates solutions during fermentation exhibit a gel formation. Klost et.al. suggested that the legumin fraction played a significant role in hydrophobic protein-protein interactions during the fermentation of pea protein. Furthermore, the findings suggest that a minor fraction of vicilin was incorporated into the gel due to electrostatic interactions between the basic legumin-β chain and vicilin (Klost et. al., 2020). In addition, it is reported that Homogenization is a technique that involves decreasing the particle size of a liquid by applying high pressure or by passing it through a small opening. When applied to pea protein fermentation, homogenization can improve the uniformity of the mixture by dispersing protein particles more evenly and breaking up any clumps or aggregates. This can enhance

the formation of a stronger gel during fermentation since smaller protein particles interact more efficiently with each other (Levy et. al, 2022, Moreno et al., 2020).
It is also worth mentioning that samples that contained A and B starter cultures exhibited faster acidification rate, both in lag phase and the time needed to reach the pH 5. This is probably due to the complexity and vast variety of microorganisms that these cultures have compared to the Culture C.

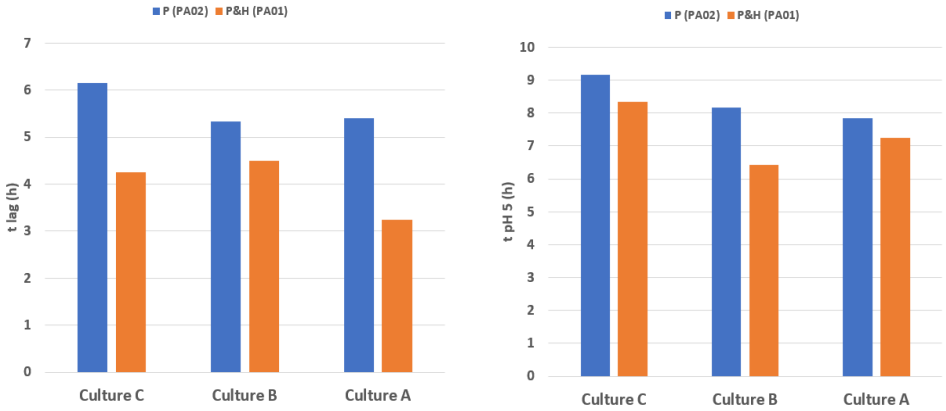

Figure 3 The time of samples to reach pH 5 (graph 1) and the lag phase (graph 2), when various cultures combinations were used. Blue columns depict the samples that their medium was only pasteurized (P), while orange columns depict the samples that their medium

Figure 4 Pea protein samples, after fermentation. Prior to the fermentation, the pea protein matrix was homogenized.

Consequently, the experiment was repeated without the homogenization step as a pre-treatment. The results indicated that while homogenization facilitated faster acidification, omitting this step led to easier handling, extraction, and neutralization of the samples. This comes in terms with Chen's report, in which he reported that the fermentation time was significantly reduced by applying homogenization in the pea protein medium, that is intended for fermentation. As stated by Chen, In terms of pH kinetics over time, the starter cultures A and B showed more similar trends between the pasteurized-only and pasteurized-homogenized mediums (Chen, 2023).

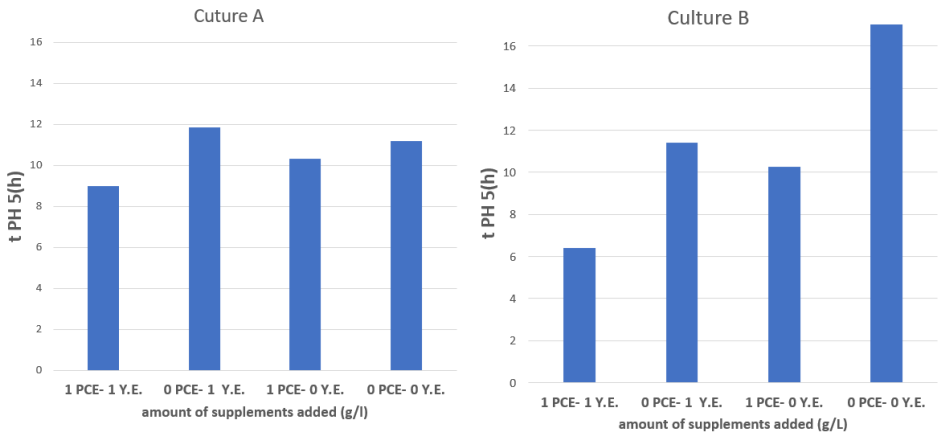

Figure 5 Time needed for samples to reach pH 5 . Samples were inoculated with Cultures A and B, under various combinations of supplements used. PCE stands for the protein hydrolysate used for the experiments, while the Y.E. stands for the yeast extract.

Furthermore, the impact of yeast extract and protein hydrolysate was investigated in this trial. The fermented samples were prepared with three different conditions: a) yeast extract at a concentration of 1g/L, b) protein hydrolysate at a concentration of 1g/L, and c) a combination of both at a concentration of 1g/L for each. By analyzing the pH kinetics over time, it was found that the combination of yeast extract and protein hydrolysate led to a faster acidification than the previous two conditions. The protein hydrolysate employed in this study is abundant in di- and tripeptides, which aid in the acidification process by stimulating the proliferation of bacterial cell cultures. This hydrolysate is sourced from FrieslandCampina Ingredients (2019). Additionally, yeast extract serves as a vital nutrient

source for the cell culture by providing various growth factors, free amino acids, peptides, vitamins, and other necessary nutrients. The use of yeast extract has been found to enhance the biomass and stability of Lactic Acid Bacteria (LAB), as documented by Angel Yeast (n.d.).
In the subsequent trials, the experimenters evaluated two distinct pea protein variants. Although 85A pea protein has outstanding techno-functional characteristics, it is relatively expensive as a raw material. Hence, AGT's 85% and 80% pea proteins were employed to make the project economically feasible. While both pea protein cultures showed a similar trend during fermentation, the economic factor played a significant role in selecting the 80% pea protein.

4.2 Effect of caustics and neutralization on the fermented matrix

In the early experiments, 10% NaOH was used for the neutralization of proteins after fermentation, which was found to cause a salty and caustic aftertaste in the samples. To improve the taste, a mixture of 10% NaOH/KOH (50:50) was used instead, which was found to be more acceptable in an informal sensory test. Sodium bicarbonate was also considered as an alternative, but due to supply chain issues (samples were available end of May), it was not available for use. The end pH values after neutralization were also examined, and the results showed that higher pH values led to thinner viscosity in the samples. However, the use of additional caustics made it less desirable in terms of organoleptic properties. Therefore, it was decided to proceed with a pH of 7 as the end-value after neutralization in the upcoming trials. For the viscosity and the preference of a consumer during the sensory analysis, more will be further discussed later

4.3 Microbiological analysis

The objective of sending the samples to the laboratory quality control section (LQS) was two-fold. Firstly, it was aimed to investigate the potential microbial growth and pH variations within the pea protein matrix,

to prevent any spoilage and the proliferation of pathogenic microorganisms. Secondly, the analysis was conducted to evaluate the effectiveness of the pasteurization process in eliminating all microorganisms present in the samples and to ensure the product's safety for human consumption by detecting any possible pathogenic microbes. The findings revealed a notable growth of bacteria in some of the samples, and some samples were identified to contain *Bacillus cereus*, a pathogenic bacterium. After spray drying, all of the microorganisms that were investigated, were eliminated.

4.4 Chemical analysis

The fermented samples were analyzed by the LQS-Chemistry department to determine their protein content, carbohydrate content, mineral content, and the presence of lactic and citric acid. By reviewing the major differences between the samples (Table 1), the protein levels dropped after fermentation (from approximately 77% to 67%), while carbohydrate levels significantly decreased from 1% to below 0,1%, indicating the metabolism of microorganisms that converted glucose into lactic acid. This was confirmed by the increase in lactic acid detected in all of the samples. The addition of 10% NaOH/KOH for neutralization resulted in an increase in minerals, particularly sodium and potassium.

Table 1 Differences between the AGT 80% (reference) and a fermented pea protein, inoculated by Culture A.

Sample	Protein %	Glucose %	Sodium (Na) %	Potassium (K) %
AGT 80%	77,5	0,8	0,88	0,033
Culture A	67,4	<0,1	2,1	1,7

4.5 Volatile Compound analysis

Aroma is a crucial aspect of the sensory perception of plant-based products, and the formation of aroma is largely influenced by volatile compounds. Among these compounds, hexanal, benzaldehyde, and pentanal have been identified as markers of beany off-flavours (Fischer et al., 2022). The use of LAF has been shown to reduce off-flavour molecules and modify the aroma profile of pea protein (Schindler et al., 2012). LAF can also increase the production of certain volatile compounds that can act as aroma maskers or modulators. For example, the buttery-flavoured diacetyl was found to increase when pea emulsions were subjected to LAF with streptococci (Engels et al., 2022). On the other hand, the volatile sulphur compound dimethyl disulphide (DMDS) is associated with a rotten odour and has a very low odor threshold, which suggests that it plays a crucial role in the aroma of pea protein extract (Schindler et al., 2012). In combination with the previous work that was performed in the facilities of the FrieslandCampina Innovation Center by Jesse Chen, the quantification of five volatile compounds (hexanal, benzaldehyde, pentanal, diacetyl and DMDS) was carried out before and after LAF, and various process steps or addition of antioxidant ingredients were evaluated.
The volatile compound analysis was performed mainly on the combination of Culture A and Culture B. As stated before, the combination of these two displayed a preference on the various informal sensory tests that were performed.

4.5.1 Effect of fermentation

During this experiment, the volatile compounds of Culture A and B fermented pea protein samples of two trials (PA06 and PA07) were analyzed. According to Figure 7, there is a significant effect on all the volatile compounds that are associated with that with the off flavor and taste of a pea protein. There is also a repeatability of the results, excluding the results of benzaldehyde
Absolute values of diacetyl levels, corrected to the reference were also analyzed. As seen in Figure 8, the diacetyl are hugely increased.

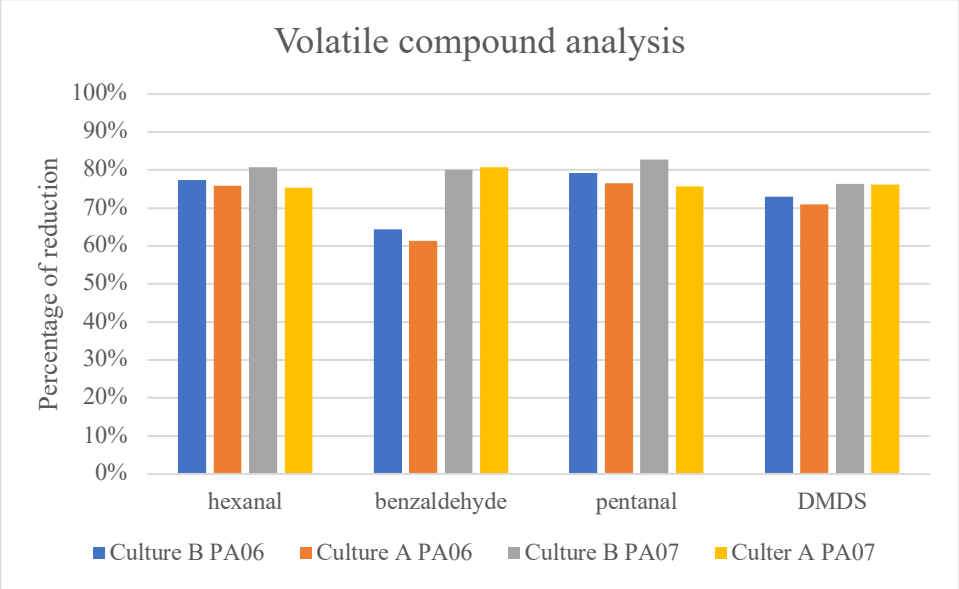

Figure 6 Percentage of reduction of the volatile compounds of fermented pea protein samples. A and B refer to the cultures that inoculated the pea protein sample, while PA06 and PA07 refer to the number of the trial.

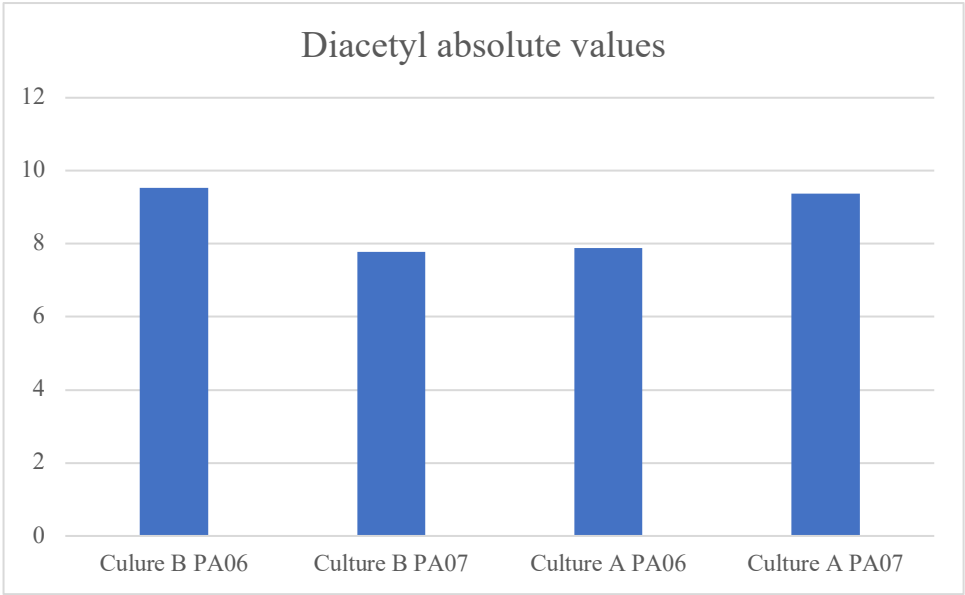

Figure 7 Absolute values of the diacetyl levels. PA06 and PA07 refer to the number of the experimental trial, while A and B refer to the cultures that inoculated the pea protein solution.

4.5.2 Effect of spray drying

In the subsequent trials, the samples obtained from the fermentation process were subjected to spray-drying, with the Buchi spray dryer that was mentioned earlier, with an inlet temperature of 185◦C and an outlet temperature of 95-100◦C. To maintain the set temperatures, the flowability was adjusted. Following the fermentation, an unpleasant odor was detected, during the sensory tasting sessions. Comparison with samples freeze-dried during small-scale experiments suggested that the off-flavor could have arisen due to the Maillard reaction, where remaining glucose in the pea matrix reacted with amino acids.
The GC analysis was conducted on four samples, which included two samples inoculated with A and B Cultures. Additionally, their spray-dried counterparts were analyzed. Reviewing Figure 9, during spray drying some volatile compounds, such as benzaldehyde, were mitigated and others such as hexanal increased. This observation aligns with the findings of previous experiments conducted on pea protein isolates, which indicated that while certain volatile compounds could be reduced, others could be increased as a result of the acceleration of free radical-initiated lipid oxidation (Lan et al., 2018).

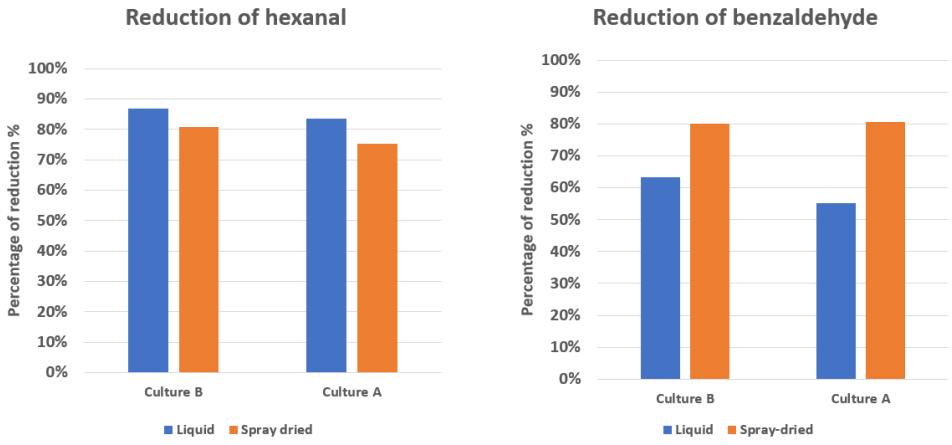

Figure 8 Percentage of reduction in the volatile compounds of hexanal (A) and benzaldehyde (B) in the liquid form and after spray drying in samples that were inoculated with A and B cultures.

Moreover, by reviewing Figure 10, diacetyl levels were mitigated at a high rate during spray drying.
Thus, a series of inlet and outlet temperatures were evaluated to investigate the effect of spray drying temperature on the volatility of compounds. Specifically, inlet temperatures of 165◦C and outlet temperatures ranging from 80-85◦C were tested. The objective was to assess whether reducing the spray drying temperature could have a significant impact on the concentration of volatile compounds. Figure 11 shows that the samples spray dried at 165◦C exhibited a a greater reduction of benzaldehyde compared to those spray dried at 185◦C. Interestingly, other compunds, such the diacetyl levels remained stable throughout both conditions.
Thus, it is safe to assume that by lowering the spray-drying temperature significantly mitigates the off-flavors of the pea protein solution, compared to a higher temperature.

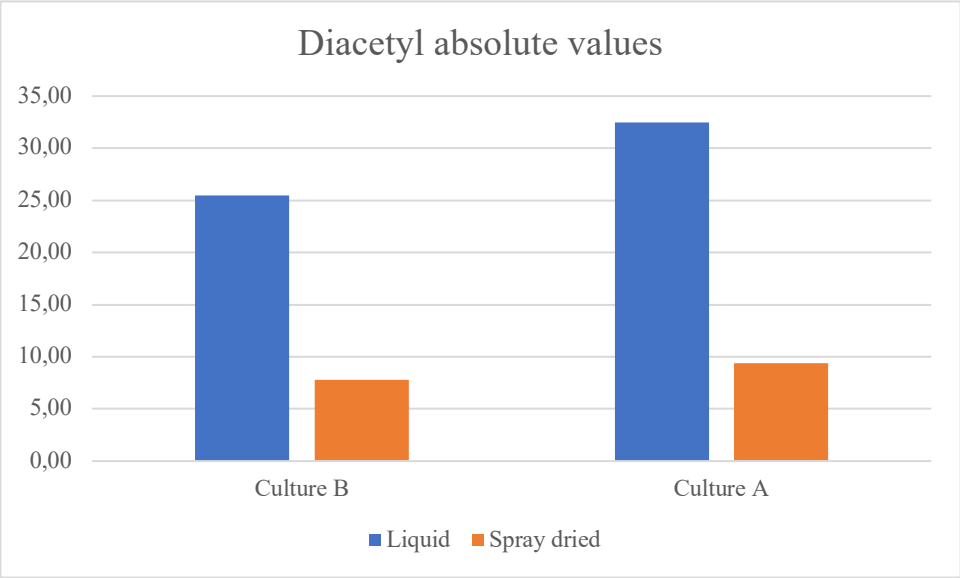

Figure 9 Diacetyl absolute values, corrected with the reference before and after spray drying A and B refer to the cultures that inoculated the pea protein medium.

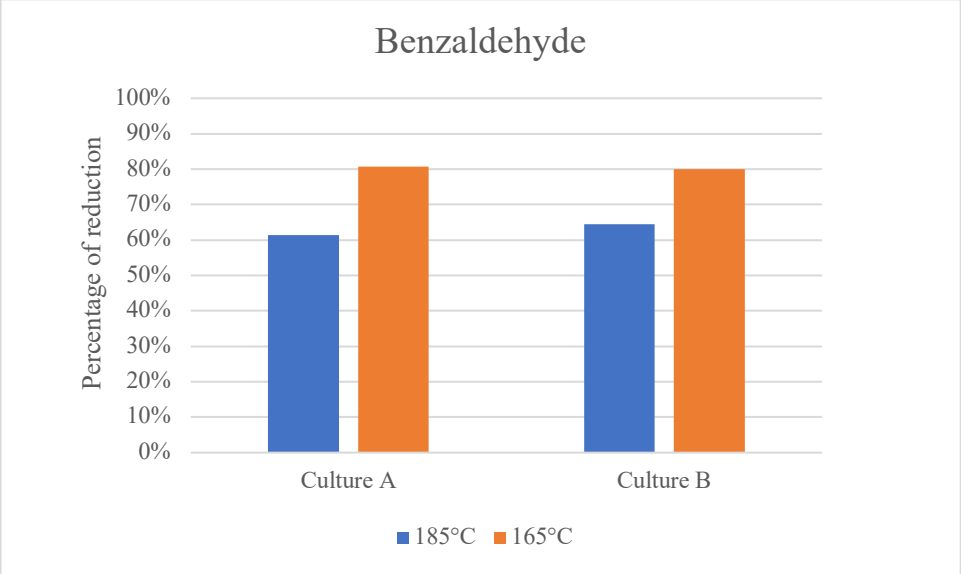

Figure 10 Benzaldehyde percentage of reduction and comparing the differences between 185◦C and 165◦C inlet temperature. A and B refer to the cultures that inoculated the pea protein medium.

4.5.3 Comparison with other fermented pea proteins

A GC analysis to determine the volatile compounds was performed in a pea protein solution that was fermented with Culture A and another sample that was inoculated by Culture B, in comparison with a pea protein that was produced in the facilities of the CRO, which is in collaboration with FrieslandCampina for the development of a fermented pea protein.

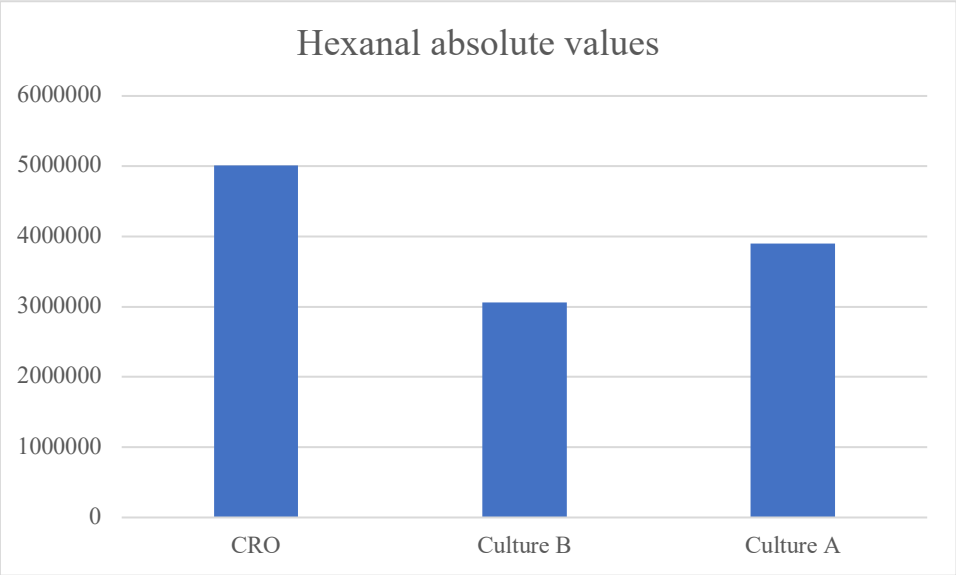

Figure 11 Absolute values of hexanal measured in the CRO protein, the samples that were inoculated by cultures A and B.

Firstly, by observing Figure 12 the Cultures A and B samples had lower absolute values than the CRO regarding the hexanal compound. Moving on to Figure 13, Cultures A and B displayed better performance on the absolute values of the diacetyl levels.

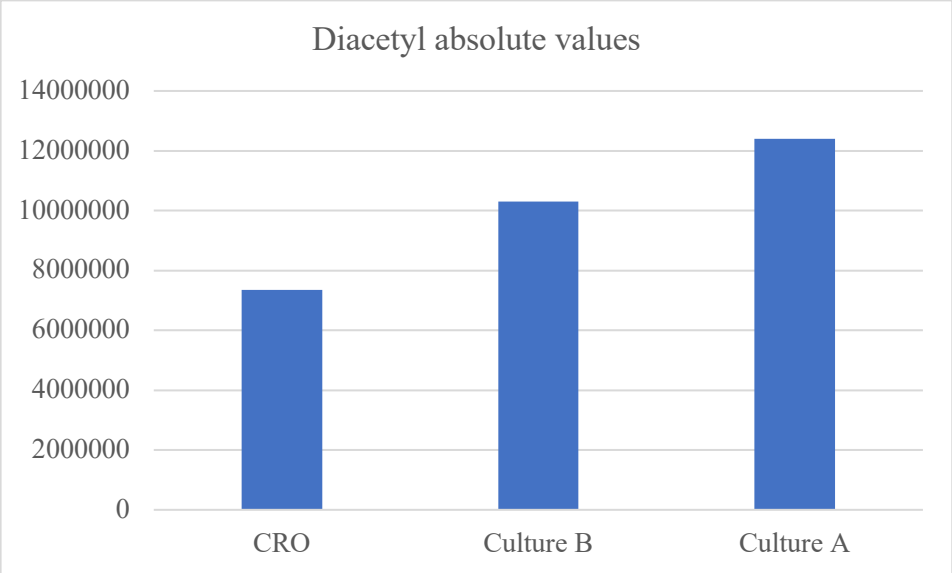

Figure 12 Absolute values of diacetyl values measured in the CRO protein, the samples that were inoculated by cultures A and B

Based on the results, the fermented 80% pea protein exhibited better performance on volatile compounds, compared to the CRO protein. However, it has to be stated that the CRO protein originated from a different pea protein source than the developed samples. Moreover, CRO followed a different process to develop the protein that the one that was suggested in this Master Thesis. However, it was found useful to compare the developed samples to other fermented pea proteins.
The results of the volatile compound analysis were deemed satisfactory. By these results, it was concluded that a sensory analysis should be conducted to further evaluate the organoleptic properties.

4.6 Effect of rotation per minutes during fermentation

The initial trials involved fermenting samples in water baths to assess the feasibility of scaling up the experiment. The pH kinetics of the larger-scale fermentation were identical and followed the same trend as those of the small-scale experiment. During these experiments, the combination of Culture A was preferred. Although A and B Cultures displayed similar trends to their volatile behavior when they were inoculated in a pea protein medium, B was more difficult to process in the equipment that was provided by FrieslandCampina. This may be to the fact that this starter culture is able to produce exopolysaccharides EPS are complex carbohydrates or polysaccharides that are synthesized and secreted by microorganisms into their surrounding environment. The prefix "exo-" in exopolysaccharides refers to their external secretion, meaning they are released outside the cells. which is known to alter the viscosity of the sample. It is known that by the presence of exopolysaccharides, the viscosity of the fermented matrix is increased (Lynch et.al., 2018). Furthermore, the effect of different rpm levels during fermentation was investigated. Samples were subjected to fermentation in water baths with rpms set to 120, 170, and 220. It was observed that a higher rpm resulted in less gel formation after fermentation, without apparent effects on the fermentation process. This could be attributed to the similarity of pH kinetics among the different samples. Moreover, pre-treated pea medium that was homogenized prior to the fermentation resulted in a smooth texture after the fermentation, when it was being constantly stirred at 250rpms during fermentation.

To further evaluate the findings, the viscosity of the fermented pea medium was measured at the different stages of the process. The results indicate that after fermentation, there is a slight increase in the viscosity, which is reduced when the product is neutralized. However, homogenization significantly enlarges the viscosity value, which is kept relevantly stable after the fermentation process.

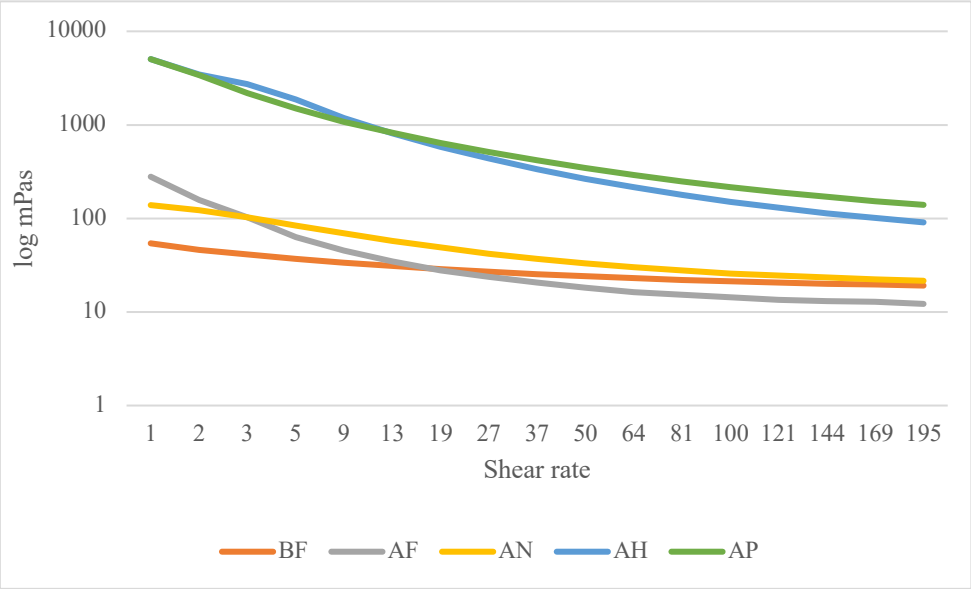

Figure 14 Viscosity measurements, during the production process of a fermented pea protein. The viscosity is depicted in a logarithmic scale (BF: before fermentation, AF: after fermentation, AN: After neutralization, AH: After homogenization, AP: After pasteurization.

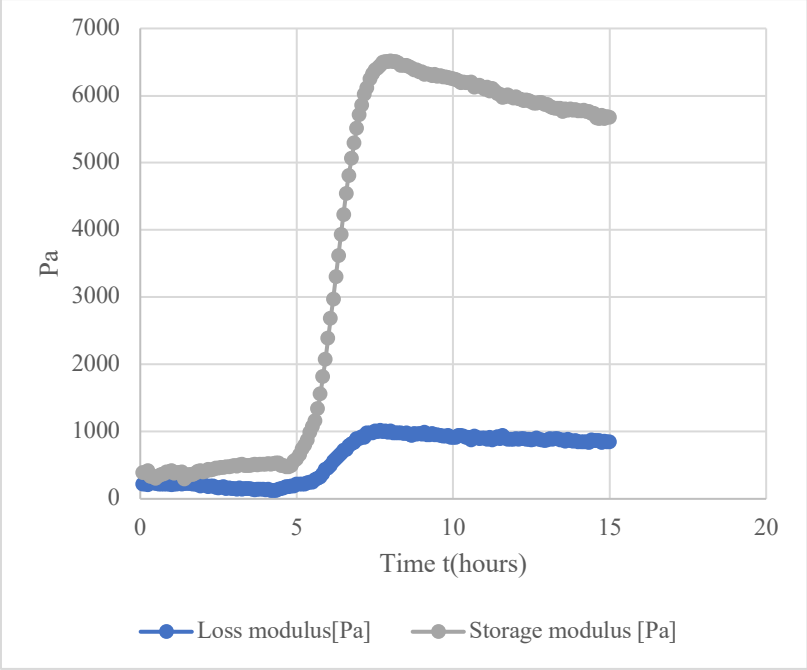

Figure 13 Depiction of Storage and Loss modulus over time during fermentation.

Storage and loss modulus were also measured during the fermentation of a non-stirred sample. The storage (G') and loss modulus (G'') provide insight into the visco-elastic behavior of a fluid under oscillatory shear, with the storage modulus representing the elastic component and the loss modulus representing the viscous component (Zin, 2019). The crossover frequency at which these moduli intersect corresponds to the reciprocal relaxation time of the fluid. As an individual could observe below in Figure 14, the storage modulus during fermentation increases significantly. The increased value validates the observation that was reported earlier in the experiment, where it was stated that with homogenization as a pretreatment, the fermented sample from a very strong gel.
By these two measurements, it is evident that rotation during fermentation significantly alters the physicochemical properties of the pea protein matrix.

4.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

To assess the protein composition of AGT pea protein isolate 80% and a fermented sample that was inoculated with Culture A, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted. Both samples had solid contents of approximately 9.4%. The dominant protein fractions in peas are represented by the 7S and 11S proteins ratios (7S/11S). The amino acid composition may have been affected by fermentation (Skalickova et al., 2022). The protein composition of the samples is crucial for evaluating their functional properties, such as emulsifying, foaming, and gelling, as well as their nutritional properties, including digestibility (Cui et al., 2020; Park et al., 2010). Furthermore, the percentage of albumin (albumin %) and Lipoxygenase (LOX, LOX %), was investigated, in order to check if pea protein fermentation has any significant effect on these subunits.
The electrophoresis patterns of all the samples revealed multiple bands with sizes ranging from 90 kDa to 10 kDa. According to Mession et al. (2015), the polypeptides with a Mw of 85-90 kDa correspond to lipoxygenase isozymes LOX-1 and LOX-2/3. Legumin (11S) is a hexameric protein with acidic (Leg α ~40 kDa) and basic (Leg β ~20 kDa) polypeptides (Gao et al., 2020), so the bands at around 38-40 kDa and ~20 kDa likely represent the α

and β parts of legumin, respectively. Vicilin (7S) is a trimeric protein with subunits ranging from 12-33 kDa, each subunit being 40-50 kDa, and according to Mertens et al. (2012), the Mw of vicilin α+β and β+γ can be 35 and 30 kDa, respectively. Additionally, vicilin α has a Mw of 21.2 kDa, which means the bands between 25-37 kDa correspond to vicilin monomeric subunits. Monomeric subunits of convicilin with a Mw of approximately 70 kDa were also present in the dispersions, while lower Mw bands (~10-15 kDa) were attributed to the albumin fraction.
Based on the observations presented in Figure 16, it can be concluded that the fermented proteins exhibited a lower band intensity in the gel picture when compared to the AGT pea protein isolate 80%. This finding is further substantiated by Peaks analysis conducted using the Image J program, which demonstrated that the peaks in the fermented protein solution were lower than those in the AGT 80% solution. The protein composition of the samples was assessed by determining the 7S/11S ratio and the percentages of LOX and albumin, which are essential for evaluating the functional and nutritional properties of protein ingredients. While the 7S/11S ratio and LOX percentage were comparable between the two samples, the albumin percentage was significantly reduced in the fermented pea protein sample compared to the AGT 80% sample, indicating a noteworthy alteration in the protein composition due to fermentation. Thus, albumin fractions may be hydrolyzed during the fermentation of a pea protein .However, it is noteworthy to mention that during the fermentation of this sample, it was

constantly mixed. This indicates that the precipitates that occur during fermentation may contain albumin fractions.

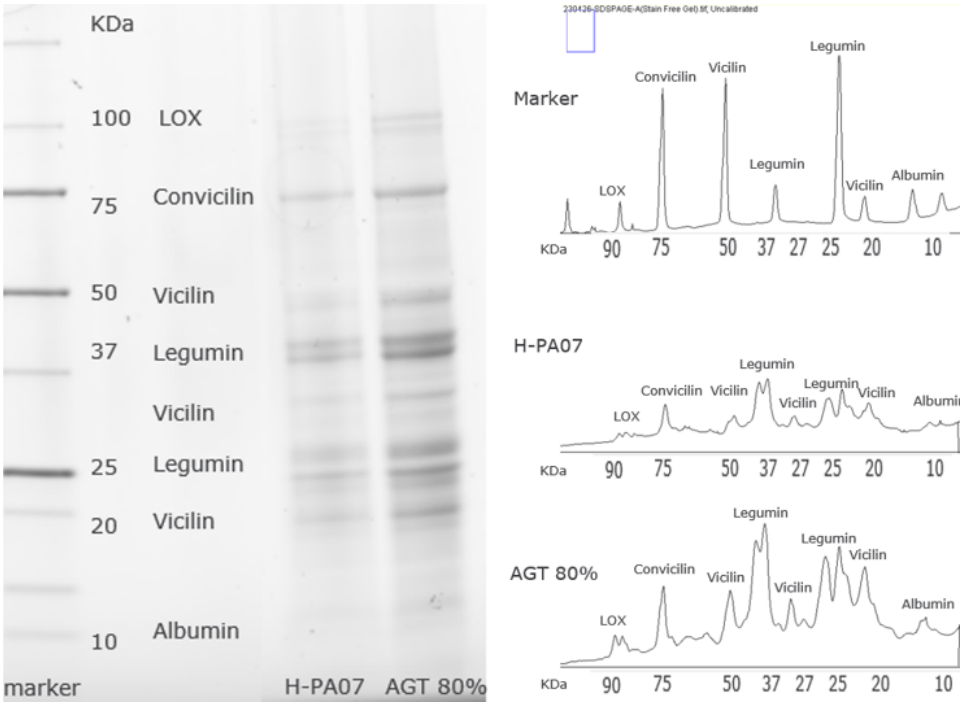

Figure 15 Results of the SDS Page analysis, with bands displayed on the left and peak analysis on the right. Using the ImageJ program, the bands for each sample were analyzed, resulting in peaks that show the molecular weight in kDa for each sample.

Table 2:Approximate 11S legumin/7S vicilin (11S/7S) ratios and LOX and albumin percentages (%) between reference (AGT 80% and culture A sample (H-PA07)

	AGT 80%	H-PA07
LOX %	3,14%	3,08%
Albumin%	4,28%	2,49%
7s/11s	1,57	1,55

4.8 Sensory evaluation

During the course of an informal sensory evaluation, a fermented pea protein sample (Culture A sample) and the CRO protein was compared to a reference solution consisting of 80% AGT pea protein. Both samples exhibited notable distinctions from the reference, with enhanced organoleptic qualities. A subsequent comparison was conducted between the two fermented pea proteins.
The majority of participants expressed a preference for the CRO-produced protein, which was also found to have a neutral odor perception. However, some participants reported experiencing a dry mouthfeel after consuming the CRO protein, while others gave the Culture A sample positive reviews. In contrast, the developed sample elicited a strong bitter and caustic taste, which was deemed unfavorable by participants and detracted from its overall appeal. As such, the taste attribute of this sample discouraged participants from selecting it over the CRO sample as their preferred option.
This taste is probably attributed to the use of caustics for neutralization, as their comments (limey, caustic and bitter) are hugely associated with sodium and potassium hydroxide.

5 Packaging requirements

Moreover, the packaging requirements of the powdered proteins will be examined to determine the ideal materials and atmosphere for storage during its shelf life.

Selecting the right packaging for vegan protein powder can be a complex process. Manufacturers must balance the need for functional packaging that protects the product and maintains its quality, with the desire for sustainable materials that minimize waste and environmental impact (Versino et al. 2023).

One challenge that manufacturers face when packaging vegan protein powder is maintaining the freshness and quality of the product. Protein powder is sensitive to moisture, light, and air, which can cause it to degrade over time. To prevent spoilage, packaging must be designed to create a barrier against these factors (Hadidi et al., 2022).

Another challenge is selecting packaging materials that align with the values of consumers nowadays. Many people are concerned about the environmental impact of packaging and prefer materials that are eco-friendly and sustainable. This can make it challenging for manufacturers to find materials that meet both functional and ethical requirements (Boz et al.).

In the case of a 20kg vegan protein powder, the selected packaging material is a paper bag with a plastic lining made of polyethylene (PE). This type of packaging offers several advantages. Firstly, it is strong and durable, providing adequate protection for the product during shipping and storage. The vacuum-sealed bag will help keep the product fresh for longer (Adibi et al., 2023). Secondly, the combination of paper and plastic makes the packaging more sustainable than using all plastic. Paper is a renewable resource and can be recycled, while the PE lining helps to protect the product from moisture and air. By using a plastic lining with PE, manufacturers can achieve a balance between functionality and sustainability(Kumar et al., 2021, Omnexus n.d.) .

When labeling the packaging, manufacturers must ensure that they comply with relevant European regulations. Under EU regulation, certain mandatory information must be inscribed on the packaging, including the

name and address of the manufacturer or importer, the weight of the product, and a list of ingredients. Additionally, the packaging should include instructions for use, storage, and any necessary warnings or precautions (European commission, n.d.).

In conclusion, selecting the right packaging for vegan protein powder requires careful consideration of functional and ethical factors. The combination of a paper bag and a PE plastic lining provides a sustainable and functional solution for packaging a 20kg fermented pea protein powder. Compliance with European regulations is also crucial to ensure that the product is safe and properly labeled. It is also necessary that FrieslandCampina should conduct various tests like organoleptic, chemical, microbiological etc. to ensure the shelf life of the product with the selected packaging.

6 Conclusions

This study aimed to evaluate the feasibility of producing a fermented pea protein powder by optimizing the selection of starter and adjunct cultures. The experiment was designed based on previous research that established key parameters such as pea protein and carbohydrate levels, fermentation temperature, and yeast extract and protein hydrolysate doses. Among the tested starter cultures, Culture A was found to impart desirable techno-functional properties and aroma to the pea protein medium, facilitating its processing through various equipment, with increased diacetyl levels, masking off-flavors of the pea protein, and enhanced its nutty aroma. The inoculation by the set of cultures resulted in fast acidification during lactic acid fermentation. In addition, the result of mitigating the off-flavors was repeatable in the experimental trials. Stirring during fermentation was found to be critical in maintaining the liquid form of the protein after fermentation, especially when pre-treated with homogenization. However, when the developed protein was compared to another fermented pea protein, people preferred the second one. As most people described the taste bitter and caustic, it is a safe assumption that the organoleptic properties affected the choice of the participants.

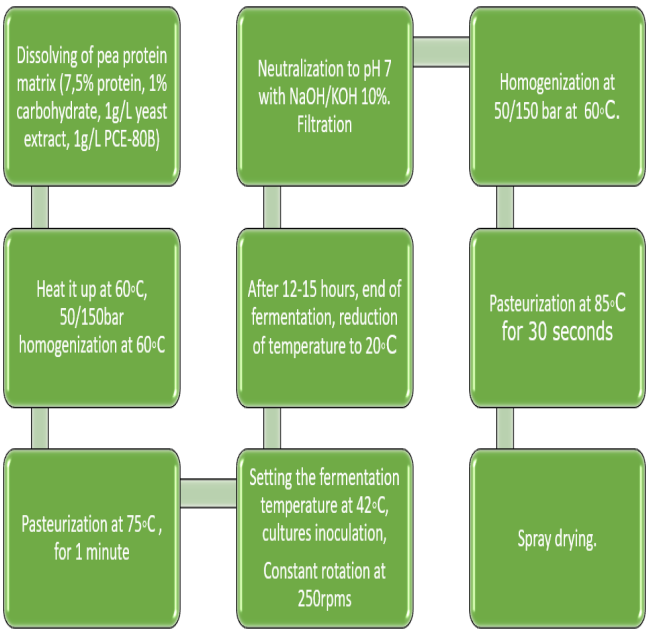

Figure 16 Proposed process diagram for the production of fermented pea protein powder.

7 Future recommendations

In order to advance our understanding of the production process of pea protein, additional research is necessary to determine how various process parameters affect the physicochemical, functional, and sensory properties of the end-product. Experimentation with alternative caustics should be explored to reduce the limey aftertaste left by the NaOH/KOH 10% mixture, which was crucial for people not to prefer the protein during the sensory tasting. In addition, to obtain more precise and reliable results, future sensory analyses should employ trained panelists and utilize more descriptive, qualitative and quantitative methods.Further exploration of the fermentation process could yield significant improvements in production time. Specifically, investigation into methods to reduce the fermentation time would be valuable. One potential strategy is to cease fermentation when the pH reaches 4.5 and determine if the same volatile compounds are produced. Additionally, adjusting the inoculum level could also lead to shortened fermentation times and thus, increased production efficiency. By continuing to explore these avenues, we can further optimize the production process of fermented pea protein powder.
Fermentation methods and microbial selection could be differed from that one mentioned here: it would be interesting to use LAF as a precipitation step during the production of pea protein isolates from pea concentrates. In this way, energy and production time is halved, as there is no need to spray dry the pea concentrate twice to produce a fermented pea protein. Lastly, nowadays there are various reports that state the fermentation by fungi could enhance the amino-acid quality of pea protein isolates and offer better nourishing products to the people (Massmann et al.,2022). In combination with LAF and mitigating the off-flavors, this would be something interesting to investigate. Lastly, as stated above, packaging requirements should be tested in a real-time experiment to determine the shelf life of the product.

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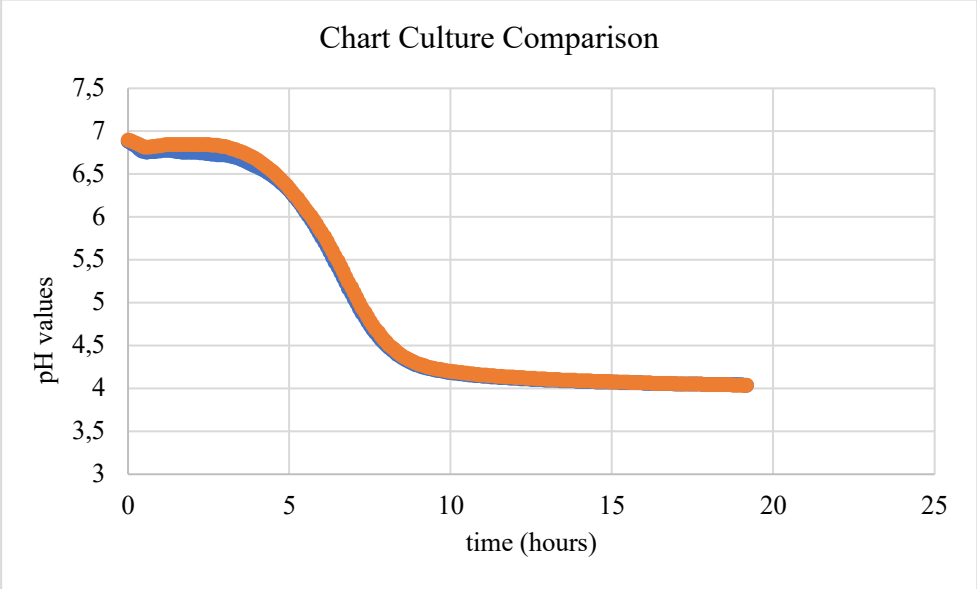
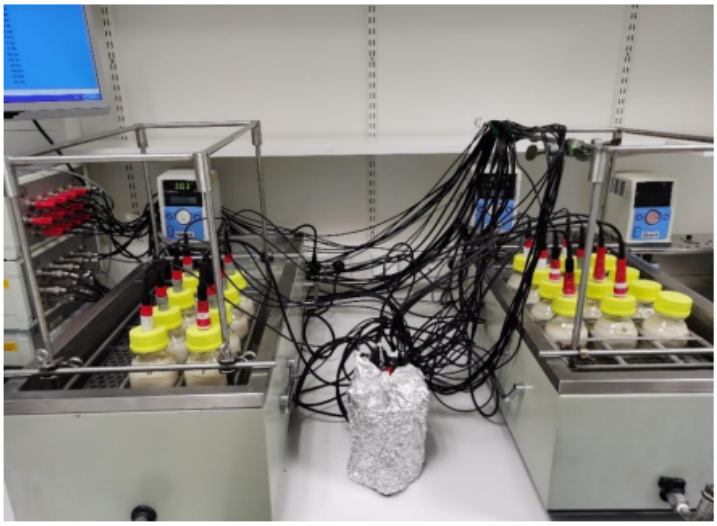
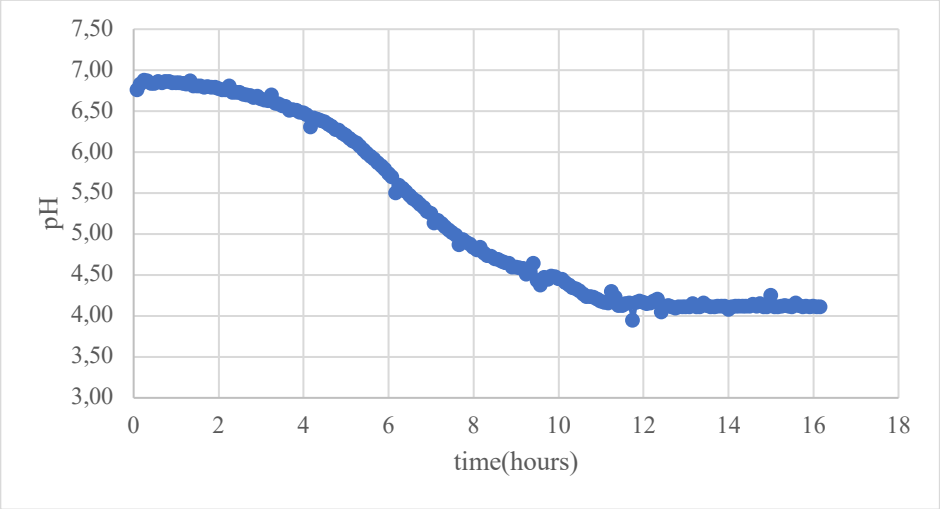
9. Appendix



APPENDIX A: pH kinetics measurement over time (hours), using the CINAC software.



APPENDIX B: Measurement of the pH using the CINAC system. Electrodes were put into Schott bottles to measure the pH over time.



APPENDIX C: pH kinetics measurement over time (hours), using the PASCO software, measuring the pH remotely.

APPENDIX D: Scaling up of the experiment and fermenting the product in buckets. Measuring the pH kinetics by using the PASCO system remotely.

APPENDIX E: Buschi mini spray dryer. Equipment used for the spray drying of the fermented pea protein