Green Biomasses as a Source of Protein for Human Consumption

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Green Biomasses as a Source of Protein for Human Consumption

A PhD thesis

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Preface

This doctorial thesis was prepared at the National Food Institute at the Technical University of Denmark in fulfillment of the requirements for acquiring a doctorial degree in philosophy. The thesis was compiled in Overleaf using Laursen's XeLaTeX thesis template. Images included in the thesis is generated by the author, unless otherwise stated. Figures and diagrams were prepared using the MS Office package, unless otherwise stated. The project "Green Biomasses as a Source of Protein for Human Consumption" was carried out at DTU Food with Prof. Peter Ruhdal Jensen as supervisor and Assoc. Prof. Timothy John Hobley as co-supervisor. The project has received funding from the GUDP fund from the Ministry of Food, Agriculture and Fisheries of Denmark.

Kgs. Lyngby, Denmark, April 19, 2023

Mikkel Hansen

Summary

This PhD thesis presents the work done during the project "Green Biomasses as a Source of Protein for Human Consumption". By now it is well established that climate change is caused by human activities and to ensure food security for the growing population of the future, new climate friendly protein sources are needed. It was found early in the project that alfalfa *Medicago sativa* was the best suited candidate as one of the sources of new proteins for human consumption. Alfalfa contains all essential amino acids, contains only low amounts of anti nutritional factors, and the plant is already considered safe for consumption by EFSA. One of alfalfa's main proteins, RuBisCO, is known for it ability to emulsify and foam and the plant alfalfa is known to have low requirements with respect to irrigation and fertilizers. Lastly alfalfa protein concentrate has been approved since 2009, as a food supplement with an daily intake of 10 g pr day, thereby lowering the potential requirements for using it as a food ingredient to meet the food requirements of the future.

In the project an investigation of using screw presses for green protein recovery from alfalfa at laboratory and pilot scale was conducted. It was found that 16% of the total protein was recovered in one pressing, and that after re-hydrating and repressing the alfalfa up to ten times, 48% of the total protein could be recovered with a single screw press in pilot scale. The composition of the generated protein concentrate was analysed with respect to total protein, amino acid profile, protein digestibility, color, ash, fiber and fat content. It was found that repetitive pressings lowered the digestibility of the protein and lowered the total protein concentration due to dilution. In this study it was concluded that pressing should only be repeated once to obtain a protein concentrate with 32% soluble protein and more than 82% digestibility.

Following the investigation of the production of a green protein concentrate at pilot scale, a study was conducted to refine and improve the quality parameters with respect to taste, appearance and digestibility. This resulted in the development of a simplified method for the production of a white alfalfa protein concentrate. The produced concentrate still contained undesired flavours that limited its usage in foods, which was sought solved by including a step with supercritical CO_2 extraction in the proposed setup. Two concentrates were produced at lab scale and pilot scale, with yields of 0.012 g (lab scale) and 0.08 g (pilot scale), of protein per g of total protein introduced from the alfalfa plant in the process. The solubility of the protein produced at lab scale and pilot scale was approximately 30% and 15%, respectively and the digestibility of the pilot scale white protein was found to be above 92%. By including supercritical CO_2 extraction at 220 bar, 45 °C for 75 min, off-flavours were lowered to an acceptable threshold for the con-

sumer. It was found that the supercritical CO_2 extraction did not affect the digestibility or the functionality of the protein, when the protein was included in formulations of meringues and chocolate muffins. However, it was concluded that further studies would be needed to optimize the extraction parameters with respect to the taste.
Two other studies was carried out in the project, with respect to the side-streams generated from white alfalfa protein concentrate production. In the first study the residual pulp was utilized as a bio-polymer in a bio-composite with PLA in concentrations up to 50%. It was found that the properties of the bio-composite was lowered when increasing the concentration of alfalfa pulp (tensile strength from 64.54 MPa (blank) to 38.48 MPa (50%), elongation (from 4.11 % (blank) to 1.63 % (50%)). It was concluded that future studies should consider other pre-treatment methods of the pulp to increase the potential of using alfalfa pulp as a bio-polymer.
The other side-stream, brown juice, was also investigated in this project. Due to high concentrations of lactic acid in the brown juice, derived from the proposed processing method for the production of white alfalfa protein concentrate, an investigation of using the brown juice as a fermentation medium with lactic acid metabolising organisms was conducted. *Corynebacterium glutamicum*-ATCC-13032 (wildtype) and the α -amylase secreting GMO strain *Corynebacterium glutamicum*-SB025, was grown in brown juice with or without supplementation of yeast extract and glucose. After 24 h of growth *Corynebacterium glutamicum*-ATCC-13032 had a final OD_{600} of 44.6 (μ_{max} 0.55) in brown juice without supplements and an OD_{600} of 45.45 (μ_{max} 0.70) in the brown juice with yeast extract and glucose. The GMO strain, *Corynebacterium glutamicum*-SB025, had an final OD_{600} of 29.35 (μ_{max} 0.47) in the unsupplemented brown juice and an final OD_{600} of 50.8 (μ_{max} 0.545) in the brown juice with yeast extract and glucose. It was concluded that brown juice is a promising fermentation medium for *Corynebacterium glutamicum*, but further studies are needed to optimize the process and investigate the metabolism occurring while fermenting.
In the end of the study an application to EFSA was generated applying for an extended usage approval of white alfalfa protein concentrate. The application lists 18 different Foodex2 lvl 7 product that would lead to an average daily intake of 10 g of white alfalfa protein concentrate, thus being within the limit set by EFSA. The application for an extended usage of white alfalfa protein concentrate as a food ingredient, concluded that white alfalfa protein concentrate produced in this project would be safe for humans to consume.

Resumé

Denne PhD afhandling præsenterer arbejdet udført under projektet "Green Biomasses as a Source of Protein for Human Consumption". Det betragtes nu som en kendsgerning, at klimaændringer er menneskeskabte. For at sikre fødevaresikkerhed for fremtidens voksende befolkning er der behov for nye klimavenlige proteinkilder. Tidligt i projektet blev det fundet, at lucerne *Medicago sativa* var den bedst egnede kandidat som en af proteinkilderne til human ernæring. Lucerne indeholder alle essentielle aminosyrer, kun lave mængder af anti-ernæringsfaktorer, planten anses allerede for sikker til indtagelse af EFSA. Et af lucernes hovedproteiner, RuBisCO er kendt for sin evne til at emulgere og skumme og planten lucerne er kendt at have et lavt behov for kunstvanding og gødning. Ydermere har lucerne proteinkoncentrat været godkendt siden 2009 som kosttilskud med et dagligt indtag på 10 g pr. dag, og derved sænkes de potentielle juridiske krav for at bruge det som en fødevareingrediens i fremtidens fødevarer.
I projektet blev udvinding af grønt protein fra lucerne i laboratorie- og pilotskala undersøgt med skruepresser. Undersøgelsen viste at 16% af det totale protein blev udvundet i én presning, og at efter rehydrering og genpresning af lucerne op til ti gange, kunne 48% af det totale protein udvindes med en enkelt skruepresse i pilotskala. Sammensætningen af det genererede proteinkoncentrat blev analyseret med hensyn til total protein, aminosyre-profil, fordøjelighed af protein, farve, aske, fiber og fedtindhold. Det blev fundet, at gentagne presninger sænkede proteinets fordøjelighed og sænkede den totale proteinkoncentration på grund af fortynding fra rehydreringen. I dette studie blev det konkluderet, at presning kun skulle gentages én gang for at opnå et proteinkoncentrat med 32% opløseligt protein og mere end 82% protein-fordøjelighed.
Efter undersøgelsen af produktionen af grønt proteinkoncentrat i pilotskala, blev der gennemført en undersøgelse for at oprense og forbedre kvalitetsparametrene med hensyn til smag, udseende og fordøjelighed. Dette resulterede i udviklingen af en forenklet metode til fremstilling af et hvidt lucerne proteinkoncentrat. Det producerede koncentrat indeholdt stadig uønskede smagsstoffer, der begrænsede dets brug i fødevarer, hvilket blev søgt løst ved at inkludere et trin med superkritisk CO_2-ekstraktion i den forenklede metode. To koncentrater blev fremstillet i laboratorieskala og pilotskala med udbytter på 0.012 g (labskala) og 0.08 g (pilotskala) protein pr. g totalt protein indført fra lucerneplanten i processen. Opløseligheden af proteinet produceret i laboratorieskala og pilotskala var henholdsvis ca. 30% og 15%, og fordøjeligheden af det hvide protein i pilotskala viste sig at være over 92%. Ved at inkludere superkritisk CO_2-ekstraktion ved 220 bar, 45 °C i 75 minutter, blev de uønskede smage sænket til en acceptabel tærskel for forbrugeren. Det blev fundet, at den superkritiske CO_2-ekstraktion ikke påvirkede proteinets fordøjelighed eller funktionalitet, når proteinet blev inkluderet ved fremstilling

af hhv. chokolademuffins og marengs. I studiet blev det dog konkluderet, at yderligere forskning ville være nødvendig for at optimere ekstraktionsparametrene med hensyn til smagen.
I projektet blev der udført to andre studier i forhold til sidestrømmene genereret fra produktion af hvidt lucerne proteinkoncentrat. I det ene studie blev sidestrømmen pulp, undersøgt som biopolymer i en biokomposit med PLA i koncentrationer op til 50%. Det viste sig, at biokompositens egenskaber blev sænket ved forøgelse af koncentrationen af lucernepulp (trækstyrke fra 64.54 MPa (blank) til 38.48 MPa (50%), elasticitet (fra 4.11 % (blank) til 1.63 % (50%)). Det blev konkluderet, at fremtidige undersøgelser bør overveje andre forbehandlingsmetoder af pulpen for at øge potentialet ved at bruge lucernepulp som biopolymer.
I det andet sidestrømsstudie, blev brun juicen undersøgt. På grund af høje koncentrationer af mælkesyre i brun juicen, afledt af den foreslåede forarbejdningsmetode til fremstilling af hvidt lucerne proteinkoncentrat, blev brugen af brun juice undersøgt som fermenteringsmedie med mælkesyremetaboliserende organismer. *Corynebacterium glutamicum*-ATCC-13032 (vildtype) og den α -amylaseudskillende GMO-stamme *Corynebacterium glutamicum*-SB025, blev dyrket i brun juicen med eller uden tilsætning af gærekstrakt og glucose. Efter 24 timers vækst havde *Corynebacterium glutamicum*-ATCC-13032 en OD_{600} på 44.6 (μ_{max} 0.55) i brun juicen uden additiver og en OD_{600} på 45.45 (μ_{max} 0,70) i brun juicen med gærekstrakt og glucose. GMO-stammen, *Corynebacterium glutamicum*-SB025, havde en slut OD_{600} på 29.35 (μ_{max} 0.47) i den rene brun juice og en OD_{600} på 50.8 (μ_{max} 0.545) i brun juicen tilsat gærekstrakt og glucose. Det blev konkluderet, at brun juice er et lovende fermenteringsmedie for *Corynebacterium glutamicum*, men yderligere undersøgelser er nødvendige for at optimere processen og undersøge den metabolisme, der forekommer under fermentering.
I slutningen af projektet blev der udviklet en ansøgning til EFSA med ønske om udvidet brugsgodkendelse af hvidt lucerne proteinkoncentrat. Ansøgningen inkluderede 18 forskellige Foodex2 lvl 7 fødevarer, der ville føre til et gennemsnitligt dagligt indtag på 10 g hvidt lucerne-proteinkoncentrat og dermed være inden for grænseværdierne. Ansøgningen om udvidet brug af hvidt lucerne proteinkoncentrat som fødevareingrediens for EFSA blev udarbejdet, og konkluderede, at hvidt lucerne-proteinkoncentrat produceret i dette studie ville være sikkert for mennesker at indtage.

Abbreviations

- AA - Amino Acid
- APC - Alfalfa Protein Concentrate
- BJ - Brown Juice
- CFU - Colony Forming Units
- DM - Dry Matter
- EFSA - European Food Safety Authority
- EUR - European Union Regulation
- FAO - The United Nations Food and Agricultural Organization
- FDA - The U.S. Food and Drug Administration
- GHG - Green House Gasses
- GLC - Glucose
- GMO - Genetically Modified Organism
- IPCC - Intergovernmental Panel on Climate Change
- HPLC - High Performance Liquid Chromatography
- LSD - Least Significant Difference
- NA - Not Analyzed/Not Applicable
- OD - Optical Density
- RBCL - Large RuBisCO Subunit
- RBCS - Small RuBisCO Subunit
- PLA - Poly Lactic Acid
- PPI - Pea Protein Isolate
- SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

- SFE - Super Critical Fluid Extraction
- SMF - Soy Meal Flour
- SPI - Soy Protein Isolate
- STD - Standard Deviation
- WAPC - White Alfalfa Protein Concentrate
- WS - WAPC from Single screw
- WSS - WAPC from Single screw treated with SFE
- WT - WAPC from Twin screw
- WTS - WAPC from Twin screw treated with SFE
- YE - Yeast Extract

List of work done during the PhD Project

During the PhD project several activities were carried out, both with respect to dissemination and installation of new procured equipment. In the following section, an overview of all these activities, will be presented. Work listed here, that is not included in the main part of the thesis, can be accessed either through the links provided or in the appendix.

Peer reviewed articles in the thesis

Hansen, M.; Andersen, C.A.; Jensen, P.R.; Hobley, T.J. Scale-Up of Alfalfa (Medicago sativa) Protein Recovery Using Screw Presses. Foods 2022, 11, 3229.

Hansen, M.; Andersen, C.A.; Jensen, P.R.; Hobley, T.J. Treatment with Supercritical CO_2 Reduces Off-Flavour of White Alfalfa Protein Concentrate. Foods 2023, 12, 845.

Contribution to peer reviewed articles not included in the thesis

Zhou, F.; Hansen, M.; Hobley, T.J.; Jensen, P.R. Valorization of Green Biomass: Alfalfa Pulp as a Substrate for Oyster Mushroom Cultivation. Foods 2022, 11, 2519.

Popular scientific articles

Hansen, M.; Hobley, T.J.; Jensen, P.R. "Kan vi springe koens fire maver over og spise græsset selv?"

Conferences

Landbrug og Fødevarer - Ingredients Network Meeting
Speaker - The Future of Alfalfa Protein
https://lf.dk/for-medlemmer/virksomhedsmedlem-hos-os/ingredients-biotech-bulletin/2020/september-2020/netvarksdag

Landbrug og Fødevarer - Innograss Conference
Keynote Speaker - "Foreløbige fra resultater innoGrass projektet på DTU", "Procesteknik der gør grønt protein egnet til fødevarer", "Fødevarer med grønt protein"
https://orbit.dtu.dk/en/projects/innograss-b%C3%A6redygtig-anvendelse-af-protein-fra-gr%C3%B8n-biomasse-til--2

DTU Food - Healthy, Safe and Sustainable Foods of the Future 2022
PhD pitch Speaker and Poster presenter
https://www.youtube.com/watch?v=sL1Ofz_zPKw&list=PLDxOjeUXS7wJqcoeqvaLVzynB0rGzqZ2M&index=12

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Finally I would like thank all my colleagues both at DTU and in the rest of academic world for making the world we live in a better place every day through science.

Contents

CHAPTER 1

Introduction

In this chapter the challenge sought being solved by the this PhD project will be presented, followed by an overview of the following chapters.

1.1 The challenge

In the latest climate report from IPCC it is now regarded as a fact that the rapid changes in our climate have been accelerated by increased greenhouse gas (GHG) emmisions from human activities (1). By the end of 2022 the global population surpassed 8 billion and is prospected to surpass 10 billion in 2058 (2). This leads to a growing demand for food and an estimated need to produce 70% more food on a global basis to feed this growing population. From 1960 to 2020 the arable landmass pr. capita was halved, mainly due the increased population and partly due to climate changes (3). The increased demand for food and lower amount of arable land increases the requirement for optimal utilization of each available acre of arable land. It is estimated that 20-30% of the world's GHG originates from food production and around 70% of those GHG is derived from animal production (4; 5). 77% of the arable land is used for animal production (6) and in Denmark 79.8% of the arable land was producing feed in 2017 and only 10.7 % was used for making food for humans (7). To mitigate future consequences of this, a drastic change in the agricultural sector is needed (8). Therefore finding new alternative protein sources is required to ensure food security in the future while lowering the emissions of GHG.

1.2 Aims of the project

As described above there is a need to find new protein sources for the growing human population. The goal in this PhD project was to discover one of these new sources. The protein in question should be able compete with existing products on the market both with respect to functionality, price and taste, while having a lower climate impact, compared to the existing proteins it could substitute.

1.3 The optimal protein source

In this study protein sources have been divided into four categories illustrated in 1.1. Both the none processed and highly processed animal protein categories has high GHG emissions compared to the two other categories (9). Even though it is well established that eating a purely none or low processed plant diet will have the lowest climate impact, dietary factors, such as vitamins and digestibility of essential amino acids needs to be considered for a new protein source (4). Ruminants can, opposed to humans, utilize protein bound fibers efficiently through their digestion system. Although humans are able to solely consume grass and legumes, mall nourishment would most likely occur due to our less efficient utilization of protein bound fiber (10; 4). Due these factors the focus in this study has been to find a processed plant based protein source to substitute existing animal based protein sources.

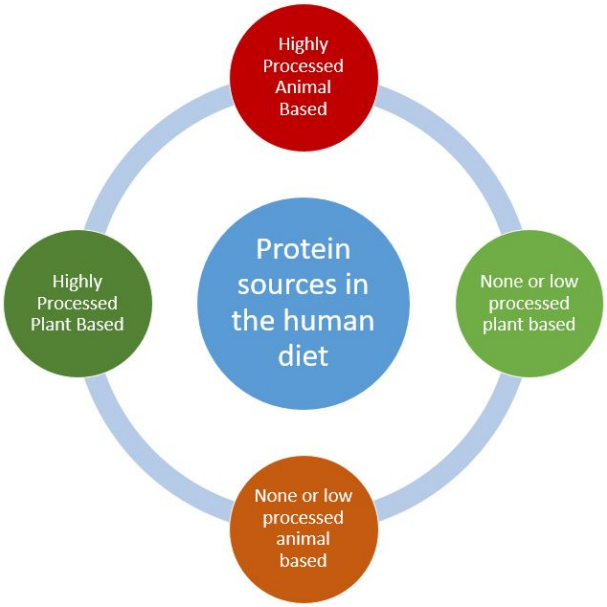

Figure 1.1: The four protein sources considered in this study in the human diet.

In this study 7 factors was chosen for defining and developing a new protein source which is attractive to the market:

1. Price on the protein
2. Availability of the protein
3. Safety of the protein
4. Functionality of the protein
5. Taste of the protein
6. Colour of the protein
7. Sustainability of the protein

1: Everything comes down to price. If the consumer is not willing to buy a product due to its price, the product cannot be sold. Therefore it is crucial that the cost price of a new protein source do not exceed what the market is willing to pay. 2: The protein should preferably be available in the agriculture sector already thereby making it easier to produce quantities big enough to substitute an existing protein. 3: The protein should be safe to consume for humans, in regulation with requirements set by EFSA or FDA 4: If the new protein is intended to substitute existing proteins it needs to be able to mimic the functionality the substituted protein. 5: The taste of the new protein should not differ from the protein it is substituting to increase the acceptance for the end consumer. 6: Preferably the colour of the end product should not be altered when using a new protein source. 7: If the processing is to extensive to meet the previous factors the new protein will have a lower sustainability both economically and environmentally.

All of the above listed factors have been considered in this project, when choosing to investigate a new protein source. Several green protein sources was investigated such as Rye grass, Clover, Spinach and Alfalfa. Grasses, such as rye grass, is known to contain large amounts of pollen, which is a known allergen (11, p. 104). This would increase the challenges with producing a protein that would be safe for all consumers. Even though clover is known for its large concentration of protein it is not regarded as a common food in the EFSA novel food catalogue (12; 13). Due to this it could be challenging to get protein from clover approved as a human food, compared to other plants that are regarded as a safe food by EFSA. Spinach is regarded as safe to eat, and has a high concentration of proteins (14). Early trials, in this project, with extracting protein concentrate from spinach, resulted in a protein powder with a strong metallic taste, that would be hard to accept by the consumers. Therefore it was considered to be of less relevance as a new protein source. However, it has been documented that leafs from alfalfa has been used as a substitute for spinach in South-Africa and that alfalfa have been commonly used in Asia in salads and soups(15; 16). Due to alfalfa's abundance across the world, high protein yield pr. acre, favorable amino acid profile, functionally

as a food ingredient and relatively low cost of production, alfalfa (*Medicago sativa*) was chosen as the candidate to investigate further (16; 17; 18; 19; 20). A full explanation for this can be read in chapter 2.

1.4 Thesis outline

This thesis is organized to give the reader insights into the development of a new protein source intended for human consumption. Going from idea, research and development, optimization and finally legal requirements. The following chapter (chapter 2) gives insights into the cultivation of alfalfa, its composition, anti nutritional factors and reasoning for choosing it as the focus point of this thesis. This is followed by a chapter with focus on optimizing the extraction of all proteins from alfalfa. To meet the criteria for an optimal new protein, listed in the section above, the sub-sequential chapter focuses on refining the protein extraction even further to produce a protein with a high enough quality to be accepted by the market. As a consequence of the processing methods, developed in the before mentioned chapters, new side streams are generated which needs to be addressed to minimize the carbon footprint of the whole production line. Chapter 5 give insights into possible solutions for utilizing these side streams developed during this PhD project. To ensure safety in foods all new foods have to be approved safe to consume by EFSA. Therefore the side stream chapter is followed by a chapter containing a manuscript for applying for extended use of the protein product presented in chapter 4. The final chapter presents a conclusion on the work done within producing a new protein source for human consumption from alfalfa.

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CHAPTER 2

Alfalfa *Medicago sativa* - a protein source for human consumption

2.1 Introduction

As mentioned in 1.3 the focus in this study was given to Alfalfa. In this chapter an explanation for this will be given. First an introduction to the agro-industrial history of alfalfa will be presented, this will be followed by an overview of current and future extraction methods used for bio refinement of alfalfa, leading to the reasoning for choosing alfalfa as a new protein source in the human diet.

2.2 Alfalfa - The Queen of forages

Alfalfa, called *"The Queen of Forages"*, is a perennial plant grown world wide (approx. 45 million ha annually), mainly as a forage crop to feed poultry and swine. It is regarded as the oldest plant being grown solely for forage, dating back more than 3300 years and is believed to originate from Iran from where it spread across the continents, mainly as fodder for horses (1; 2). Alfalfa is a perennial plant resistant towards drought due to its deep root system, and it has a low need for fertilizers due to its ability to fixate atmospheric nitrogen through symbiosis with *S. meliloti* (3, p. 395)(2). It is recommended to do crop rotation every fifth year and the plant can be harvested 3-8 times pr. year, depending on the climate and years since last crop rotation (4; 2). Alfalfa contains 18.3-27.7% protein (DM) and roughly half of the protein is stored in the leafs (4; 5). Reports have demonstrated yields of 3.6-6.6 tons kg protein pr ha, when cultivating alfalfa, which is up to a 10 times higher yield compared to soy (6; 7; 8). Alfalfa proteins contains all essential amino acids and the main soluble protein, RuBisCO, is known to have both emulsifying and foaming properties, making it able to replace egg and milk proteins in processed foods (5; 9). From a historical point of view alfalfa have been consumed in both raw and cooked form in various recipes. Consumption have been documented in Europe, Asia, Africa, and America. In Europe in newer times it has mainly been consumed as sprouts. The main reasoning for the limited usage of

alfalfa in foods is considered to be due to the high saponin content in alfalfa, which can be perceived very bitter by the consumer(1). Since the whole alfalfa plant have been consumed widely before 15-05-1997 it does not fall under the Novel food regulation and is therefor regarded as safe to consume(10). In 2009 alfalfa protein concentrate (APC) was approved as a novel food ingredient in food supplements with an daily intake of 10 g/day. But due to this limitation in only being approved as food supplement and not as a full food ingredient full commercial production of APC as a food ingredient within EU is not permitted.

2.3 Anti Nutritional Factors in Alfalfa

According to *Nelson et al 2006*, there are different allelopathic compounds in Alfalfa. When extracting green protein it is advisable to ensure that levels of these substances do not exceed maximum levels allowed in foods by EFSA. The main concerns discussed in Opinion on the safety of 'Alfalfa protein concentrate' as food 2009, was coumestrol, L-canavanine and β-carotene(11). Beside those concerns coumarin and albumin is of concern when digested in high concentrations, both present in alfalfa.

2.3.1 Coumarin

Coumarin is a natural flavouring, mainly derived from cinnamon and act in the plant as an anti-fungal metabolite (12; 13). According to EUR (EC) No 1334/2008 levels of coumarin content may not exceed 5 ppm in dessert and not exceed 50 ppm in traditional bakery containing coumarin (14). In alfalfa the concentration of coumarin is reported to be around 5 ppm for the leafs and 4 ppm for the stems(15). To ensure widespread usability for a alfafa protein as a food ingredient it should be checked that the coumarin content of the final product is below 5 ppm to ensure its usages in all foods.

2.3.2 L-Canavanine

Many legumes, among these alfalfa, contains L-canavanine in various amounts, which is used as nitrogen source and as a natural herbicide against pests. In alfalfa the concentration of canavanine is highest in seeds and sprouts, but are also found in the stems and leaves (16; 17). It has been reported that monkeys subjected to high intake of alfalfa seeds, developed systemic lupus erythematosus, which is thought to be due to the canavanine (18).

2.3.3 β -carotene

β -carotene plays a part in the synthesis of human retinal pigment, and 10 g of APC is believed to supply up 70% of the recommended daily intake(2). It is known to cause

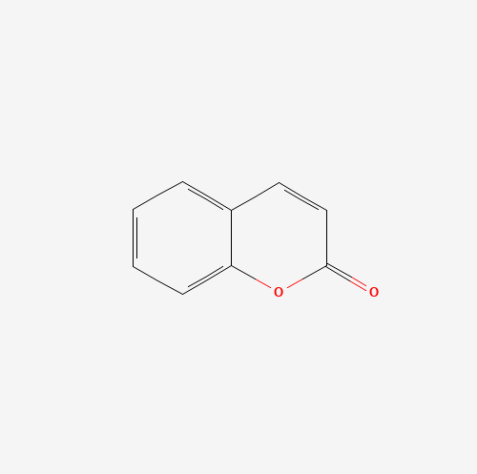

Figure 2.1: Structure of Coumarin. Source: PubChem CID: 323.

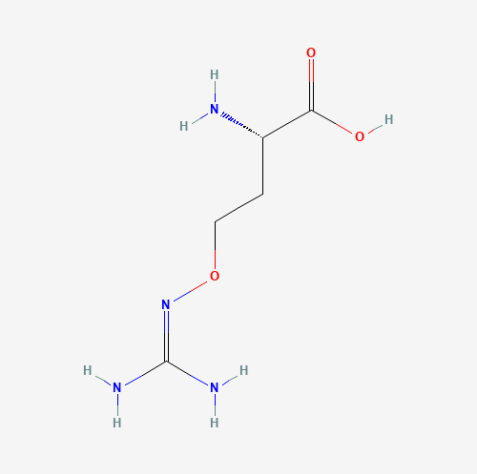

Figure 2.2: Structure of L-canavanine. Source: PubChem CID: 439202.

health benefits with intakes up to 10 mg pr day, and may cause an adverse effect in heavy smokers at intakes of 20 mg pr day (19).

2.3.4 Allergens

When looking at the list of allergens in food which requires labeling in Denmark (20) the only things that need to be considered as potential allergens in alfalfa would be sulphide and sulphur-dioxide if the content exceeds 10 mg/kg. In a study, done by *T. R. Pucek & J. B. Pyś*, in 1997, alfalfa cultivars grown in sulphur post-mining lands was studied. They found maximum levels of 0.5% sulphur (DM) in the plants, so the soil quality have to be evaluated before starting a production alfalfa protein for human consumption. (21). Beside the listed allergens, Malley et al 1974 found that albumin from the legume Pea,

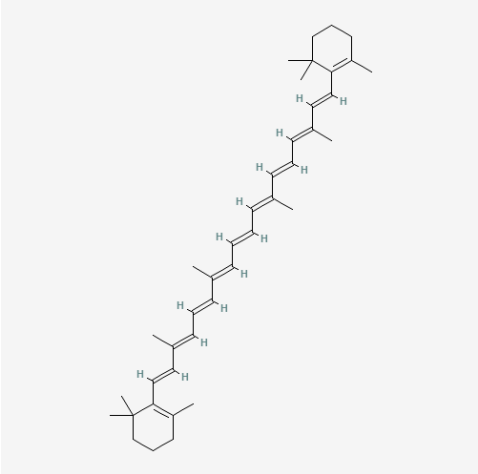

Figure 2.3: Structure of β -carotene. Source: PubChem CID: 5280489.

can cause allergic reactions in people sensitive to peas. This allergic reaction is however found limited when autoclaving the product (120 °C, >15 min)(22; 23).

2.4 Processing methods for Producing Alfalfa Protein Concentrate

Alfalfa protein concentrate (APC) is typically produced by a mechanical separation of the plant material, either only the leaves or the whole plant, followed by a separation of the liquid (green juice) and solid fraction (pulp). The green juice is then further treated in different manors to extract the proteins. Those methods can involve, centrifugation, heat precipitation, H_2O washing, acid precipitation, micro or ultra filtration, ultra sonication, and salt precipitation. This is followed by a step for removal of water, such as freeze drying, spray drying, tunnel drying or oven drying (24; 5; 25; 26; 27; 28). The before listed methods can be combined in various ways depending on the desired purity and quality of the end protein concentrate, which will end up with 3 or 4 fractions depending on whether extra refinement for the production of the purified white alfalfa protein concentrate (WAPC) is included (see Table 2.1).

2.5 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a processing method where a solvent (eg. CO_2) is put into a supercritical state, where it has penetration properties as gas and solvent properties as liquid. The main solvent can often be mixed with co-solvents (eg. ethanol) to increase the solubility and thereby extraction of certain compounds. The solvents

Fraction	Protein (% DM)	Source
APC	27.0-60.0	(19; 24)
WAPC	32.3-93.7	(27; 25)
BJ	18.2-24.6	(27; 26)
Pulp	3.1-7.6	(24)

Table 2.1: The four fractions derived from alfalfa protein production. APC=Alfalfa Protein Concentrate, WAPC=White Alfalfa Protein Concentrate, BJ=Brown Juice..

are then passed through a heated vessel where the product (eg. protein powder) is kept before meeting the automatic back pressure release valve (ABPR) which is used to adjust the pressure to the desired setting. After the ABPR a potential introduction of a make-up solvent might be introduced to ensure that potential volatile compounds or similar are kept in solution before being collected in a collecting vessel. The SFE system might, depending on the scale of the system, have systems such as CO_2 scrubbers to re-circulate the used CO_2 (29). Overview of the process can be seen in 2.4.

The SFE method was first described in 1879 by Hannay and Hogarth, and was first used in the asphalt industry in 1936 for de-oiling of asphalt and later in 1952 in the fish oil industry. These processes were mainly done with propane as the solvent. The usage of CO_2 as the main solvent was driven forth in the 1970's by the energy crisis which required less energy requiring extraction methods (30, Chapter 2,7 and 10). The main usage of SFE with CO_2 in the 1970's was in decaffeinating coffee and tea (31). SFE has in recent times been used for the extraction of lipophilic compounds from hops (*Humulus lupulus*). In the last decade the awareness of SFE has increased due to its advantages in extracting various compounds from *Cannabis sativa*. Due to the relatively low working temperature (above 31 °C) of SFE, temperature sensitive compounds can be extracted with low denaturation (32). This new awareness is most likely due to many states in USA legalizing the use of recreational cannabis, which in effect expands the market for

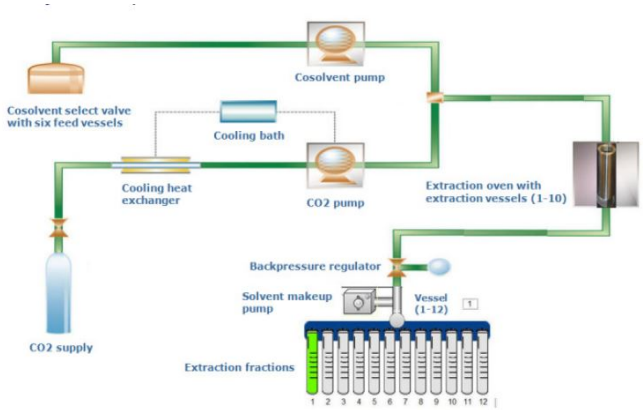

Figure 2.4: Diagram for the MV-10 SFE system from Waters A/S(29).

SFE and matures the SFE technology quicker due to market demands(33; 32).

In this study literature was found documenting the ability of SFE to extract, among others, lipids, phenolic compounds, iso-flavonoids, β-carotene and Xanthophylls, from plants (34; 35; 36; 37; 38; 31). Due to the SFE's ability to work at low temperature, thereby limiting potential denaturation of the protein and the ability to extract many compounds of concern with respect to the food safety for alfalfa protein concentrate, it was found as great candidate for refinement of the WAPC(19).

2.6 Alfalfa an optimal protein source?

With respect to finding optimal protein sources alfalfa protein concentrate, as it is, lives up to criteria 2, 3, 4 and 7 listed in section 1.3. 2: Alfalfa has been grown across the world for decades, so it is easily available. 3: Alfalfa protein concentrate is regarded as safe to consume, even though an extended approval is needed to give it status a food ingredient. 4: The RuBisCO fraction of the protein found in alfalfa have demonstrated abilities to emulsify and foam making it able to substitute other functional proteins with this functionality. 7: The low requirement for fertilizer and watering lowers the energy required to produce alfalfa protein. This included with the high yield of protein pr acre, compared to soy, makes alfalfa a promising candidate as a new protein source. The last three criteria listed section 1.3, 1: Price, 5: Taste of the protein and 6: Color of the protein is all criteria that needs to be addressed to make protein from alfalfa attractive for the market while maintaining the other four criteria within acceptable limits. In the following two chapters a potential solution to live up to those criteria will be presented.

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CHAPTER 3

Scale-Up of Alfalfa (*Medicago sativa*) Protein Recovery Using Screw Presses

foods

MDPI

Article

Scale-Up of Alfalfa (*Medicago sativa*) Protein Recovery Using Screw Presses

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Abstract: As a consequence of the increased demand for proteins for both feed and food, alternative protein sources from green plants such as alfalfa (*Medicago sativa*) have come into focus, together with methods to recover these proteins. In this study, we have investigated the use of screw presses for protein recovery from alfalfa at laboratory and pilot scale. We found that using a pilot scale screw press, with a working pressure of 6 bar, 16% of the total protein was recovered in one pressing, and that after rehydrating and repressing the alfalfa up to ten times, 48% of the total protein could be recovered. The green alfalfa protein concentrate was analyzed for total protein, amino acid profile, protein digestibility, color, ash, fiber and fat content. It was found that repetitive pressings lowered the digestibility of the protein pool and reduced the total protein concentration due to dilution. To achieve the best quality protein at the highest concentrations, it is recommended to press the alfalfa no more than twice, which results in an alfalfa protein concentrate with more than 32% soluble protein and greater than 82% digestibility.

Keywords: green proteins; sustainability; alternative proteins; green food

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1. Introduction

It is currently estimated that the production of protein needs to increase by 70% before 2050 to meet the demand for a growing and more wealthy population in the world [1]. As a consequence, new protein sources for animal and human diets must be developed, with low carbon footprints and high protein yields from the available arable land [1].

One of the potential new protein sources could be alfalfa, a perennial plant, grown world-wide as a feed for ruminants, pigs and hens. It is favorable as a protein crop with a protein yield per acre of land, which is up to 10 times higher than soybean and it has a high tolerance to lack of water due to its deep roots [2–4]. The deep root network also prevents washing out of nutrients from the soil to the aquatic bio-systems surrounding many fields [4]. Alfalfa also contains all essential amino acids. Furthermore, due to its ability to fix nitrogen from the air to the soil, it has been used for decades as a natural fertilizer. Recently alfalfa has also been suggested as a potential protein source in the human diet.

The leaves and sprouts of alfalfa have already been used in salads and soups and the plant as a whole is approved as safe to consume in the novel food catalogue [5,6]. It is therefore of interest to isolate an alfalfa protein concentrate, which could potentially be more sustainable than other plant protein alternatives such as soy. For animal feed, alfalfa is typically harvested 4–5 times per year and it is used to produce silage or it is transported directly to a biorefinery for protein extraction. The proteins are commonly extracted from either the whole plant or parts of it. A unit operation for mechanical separation of the plant material is used, followed by a press to produce a protein rich green juice. The green juice is then processed in various ways to isolate the proteins. They are then dewatered by centrifugation, followed by a drying step, yielding an alfalfa protein concentrate (APC) for animal feed supplementation [4,6–10]. The main protein found in alfalfa is RuBisCO which

is highly soluble and constitutes up to 70% of the soluble proteins found in alfalfa [11]. RuBisCO consists of a large subunit (55 kDa) and eight small subunits (15 kDa) [12].

With respect to human consumption, the green APC has been approved as a novel food supplement since 2009, with a daily intake of up to 10 g. Besides its favorable amino acid composition, it also contains several unsaturated fatty acids and vitamins, B, C, D, E and K [6]. Attempts to maximize yield can often negatively affect digestibility and functionality due to the processing conditions used. In 1972, Knuckles et al. investigated repetitive pressing for the production of alfalfa protein concentrate. Their study only investigated pressing up to three times and with a focus on using the protein obtained as animal feed [7]. Given the limitations and the age of the work of Knuckles et al., it is important to move the state of the art forward if protein from green biomass is to meet the demand for increased plant-based protein for food applications. In order for the technology to mature, it is important to understand how processing affects the quality of the protein produced and how to scale-up. The aim of the current study, therefore, was to examine how to maximize the yield of green protein from alfalfa using two different screw presses. Repetitive sequential pressing up to 10 times was conducted and it was determined if this had a negative impact on the properties of the protein concentrate.

2. Materials and Methods

2.1. Production of Alfalfa Protein Concentrate

Alfalfa was harvested in spring 2019, on the island of Fyn in Denmark, manually by scythe. It was transported directly to the laboratory where it was stored at −20 °C, until it was further processed. Before pressing, the alfalfa was thawed by submerging it in cold tap water. It was then drained until it had reached the weight it had before thawing and had equilibrated to room temperature (20–22 °C). The thawed alfalfa was processed at room temperature, either through a Vincent CP-4 single-screw press (Vincent Corporation, Tampa, FL, USA) [13] (Figure 1) or an Angel Juicer S8500 twin-screw press (Angel Juicer, Seoul, South Korea) used with its standard filter. For the Vincent CP-4, the length of the cylinder and screw were 800 and 540 mm, respectively, with a diameter of 50 mm and the pitch of the screw was 50 mm. It was operated at 10 rpm with a 0.5 mm filter. The press has a maximum capacity of 68–227 kg/h. The pressure on the discharge cone was 6 bar, which was the maximum possible. During setup of the machine, lower pressures were seen to give a lower yield of green juice, but they were not systematically investigated.

Figure 1. Pressing of alfalfa on Vincent CP-4 single-screw press. 1: Feeding with fresh alfalfa, 2: Screw and 0.5 mm filter matrix, 3: Discharge of pulp, 4: Discharge of the green juice, 5: Collection of the green juice. Photo: Flemming Leitorp.

Pressing of the alfalfa resulted in two fractions: A pulp and a green juice. The green juice was collected immediately and had the pH lowered to 3.5 with 3 M HCl. It was then centrifuged at 2500× g, for 15 min at 4 °C with a Thermo Scientific Multifuge X3R (Waltham, MA, USA), within 20 min from the pH adjustment. After centrifugation, two fractions were obtained: A green pellet and a supernatant, the weights of which are presented in Table A1, Appendix A. The green pellet was then freeze-dried using a Thermo Scientific Heto Drywinner DW8 (Waltham, MA, USA). The process started at −20 °C with a 1 °C/h increase until the temperature reached 20 °C. The temperature was then held constant, until no decrease in weight was observed. This resulted in a green protein rich powder called alfalfa protein concentrate (APC). Examples of the fractions obtained can be seen in Figure A1, Appendix A. After the first press, the alfalfa pulp was re-hydrated with an amount of tap water corresponding to two times the weight of the pulp produced, and it was then processed again as described above. This was repeated 10 times. The details of the amounts used are given in Table A1, Appendix A. Due to the mechanical forces in both of the screw presses, it can be expected that the temperature within would increase, however there was no provision for cooling in the presses used here. The temperature of the green juice was not observed to increase significantly above room temperature during the period of pressing and was therefore not accurately measured. After freeze-drying, a sample of APC from each press was checked for dry weight content, as described in the section below. The moisture content of the freeze-dried APC was found to be zero.

2.2. Dry Matter and Ash Content

Dry matter content was determined by first placing dry pre-weighed crucibles containing approximately 5 g of APC each in an oven at 104 °C overnight. The crucibles were then placed in a desiccator to cool before weighing. The dry matter content was then calculated from the loss in weight. The crucibles were then placed in a muffle furnace at 600 °C overnight and the ash content was determined from the loss in weight. With respect to the raw alfalfa, a known weight of approximately 200 g of frozen plant material was used to get a representative sample. It was dried to constant weight at 104 °C in an oven and the moisture content was determined by difference. The dried alfalfa was then homogenized by blending in a kitchen machine (KVL 6300, Kenwood, Yokohama, Japan). From the dried homogenized powder, three samples of around 3 g each were transferred to pre-dried crucibles. They were dried until constant weight at 104 °C, before being treated as described above, to determine the ash content.

2.3. Protein, Insoluble and Soluble Fiber and Available Carbohydrates

Protein content was determined using the DUMAS combustion method (rapid MAX N exceed, Elementar, Germany), using a nitrogen-protein conversion factor of 6.25 [14].

Fiber and carbohydrate composition were measured with the "Available carbohydrate/dietary fiber assay kit" (Megazyme, Ireland) [15]. In brief, the procedure consisted of 4 steps:

- Available carbohydrate determination through α-amylase, protease and amyloglucosidase incubation.
- Filtration with 96% ethanol to determinate the soluble fiber fraction.
- Protein and ash determination.
- Calculation of the insoluble fiber fraction from the equation below:

$$Insoluble\ fiber(\%) = \frac{Weight_{after\ step\ 2} - Protein - Ash - Blank}{Weight_{original}} \cdot 100 \quad (1)$$

Filtration was performed with 47 mm diameter microglass fiber filters (Thermo Fisher Scientific, Waltham, MA, USA). To ensure that the filter paper did not increase the fiber content of the sample, an extra blank sample was run in duplicate, which showed that the filters did not affect the results.

2.4. Preparation of Protein for SDS-PAGE and Bradford Soluble Protein Analyses

Solubilisation of the protein was done by taking 0.1 g of APC and mixing it with 0.9 mL of 0.1 M phosphate buffer (pH 8.00). It was vortexed for 20 s before being placed in a laboratory shaker where it was mixed for 10 min at 1000 rpm (TS-100C, Biosan, Riga, Latvia). After shaking, the samples were centrifuged for 5 min, at 10,000× *g* and room temperature (Microcentrifuge, Ole Dich, Hvidovre, Denmark) and the supernatant was used for SDS-PAGE or the Bradford assay.

2.5. SDS-PAGE

All reagents and equipment used in these analyses were from Biorad (Hercules, CA USA). 10 µL of sample prepared as described above, was mixed together with 5 µL of 4× Laemmli buffer, 4.75 µL of milliQ water and 0.25 µL β-mercaptoethanol. The solution was then incubated for 10 min at 95 °C in a TS-100C heating block (Biosan, Riga, Latvia). 10 µL of the incubated sample was then loaded into a well in a 4–20% Mini-PROTEAN® TGX gel, where the first well was loaded with 5 µL of Precision Plus Standard ladder, before running the gel (140 V, 400 mA, 50 min). The gels were then washed in milliQ water and stained for 1 h (Coomasie R-250). The gels were destained by first rinsing them in milliQ water and then they were left in milliQ water for 3 h; this was repeated three times. The gel was then scanned using a ChemiDoc XRS+ System.

2.6. Soluble Protein by Bradford

Soluble protein was measured using the Pierce Coomasie Plus Bradford kit following the instructions from the manufacturer for using microwell plates [16]. All analysis was conducted in triplicate on an Infinite M200 Pro plate reader (TECAN, Männedorf, Switzerland).

2.7. Protein Digestibility

Digestibility of the APC was determined following the procedures described by Saunders et al. 1973 [17]. In brief, 1 g of APC was suspended in a 50 mL centrifuge tube with 20 mL of 0.1 N HCl and it was mixed with 1 mL of 0.01 N HCl containing 50 mg of pepsin. The solution was incubated at 37 °C for 48 h with gentle shaking, followed by centrifugation at 2500× *g*, for 15 min, at 4 °C using a Thermo Scientific Multifuge X3R (Waltham, MA, USA). The supernatant was discarded, and the solids were resuspended in 10 mL of deionized water and 10 mL of phosphate buffer (pH 8) containing 5 mg of trypsin. The solution was incubated at 23 °C, with gentle shaking for 16 h, followed by centrifugation (2500× *g*, for 15 min at 4 °C). The supernatant was discarded, and the solids were washed with 30 mL of deionized water followed by centrifugation (2500× *g* for 15 min at 4 °C) and the supernatant was removed. The solids were filtered through a 1.2 µm pore size nitrogen free filter, air dried and analyzed for total nitrogen together with the filter paper. The digestibility was determined by comparing the total protein content before digestion with the total protein in the digested solid material using the DUMAS combustion method.

2.8. Amino Acid Analysis

Amino acid (AA) analysis was performed using the EZ:faast amino acid kit (Phenomenex, Torrance, CA, USA). Analysis was done on APC from press 1, 5 and 10 in duplicate. Acid hydrolysis of the APC was done first, by boiling 30 mg of sample in 0.5 mL of 12M HCl for 18 h in an oven at 104 °C. After hydrolysis, the samples were filtered through a 0.22 µm pore size sterile filter and processed according to the instructions for the assay [18]. The hydrolysate was analyzed by liquid chromatography using mass spectrometry (LC/MSD Trap, Agilent, Santa Clara, CA, USA) with an EZ:faast 4u AAA-MS Column (250 × 3.0 mm) (Phenomenex, Torrance, CA, USA).

2.9. Total Fat Analyses

Total fat was determined by using the Rapid NMR Fat Analyzer (CEM, Matthews, NC, USA) with the Powder method. All analyses were performed in duplicate.

2.10. L-a-b Color Measurement

L-a-b color was determined by placing around 1 g of APC from each respective press under a small glass plate. The sample was measured in triplicate through the glass plate using a LC 100 spectrocoloriometer (Lovibond, Amesbury, UK).

2.11. Statistical Analysis

Each analytical result is reported as the mean value of three replicate sample measurements, except where stated. Standard deviations and statistical differences were analyzed using MS Excel. Differences between the means of samples were analyzed by a single factor ANOVA test with least significant difference (LSD) test at a probability of 0.05.

3. Results and Discussion

The overall aim of this work was to examine how to maximize the yield of green protein from alfalfa using a screw press and to determine if this had a negative impact on the properties of the protein concentrate. As part of this, we examined whether scale-up from a laboratory based twin-screw press to a pilot scale single-screw press would affect the yield.

3.1. Comparison of Single- and Twin-Screw Presses for Extraction of APC

The small twin-screw press and large single-screw press were used to process 4.9 kg and 50.8 kg, respectively, of alfalfa in the first press, which amounted to a total input of 211 g and 2189 g of protein (derived from Table 1), respectively. The resulting pulp was then re-pressed 10 times. In Table 1, approximately 14.3% of the wet weight is unaccounted for, which is speculated to be soluble and insoluble fiber.

Table 1. Analyses of the raw alfalfa. Water content (%) n = 1, all others n = 3. Results are expressed in % wet weight.

Component	Protein (%)	Water (%)	Ash (%)	Fat (%)	Total (%)
Raw Alfalfa	4.3 ± 0.5	79.38	1.78 ± 0.21	0.25 ± 0.00	85.7

The results, presented in Figure 2, show that the protein content of the resulting APC was highest from the twin-screw press in the first two presses, and higher than the single-screw press. In contrast, the single-screw press produced the highest protein concentration in the third press. This difference in press behavior is thought to be due to the design of the respective extractors. The smaller twin-screw press has a section of its screw macerating the product before the extraction occurs, whereas the single screw does not. As a result, it is speculated that the alfalfa plant was not macerated sufficiently before the third press in the single-screw process. After 10 presses, 37% of the total protein was extracted by the twin-screw press (Figure 2). In contrast, 48% was extracted by the single-screw press (Figure 2). Both screw presses extracted approximately 25% of the total protein after the second press. The yield of the twin-screw press stagnated after 6 presses, whereas the single screw continued to extract APC up to the tenth press. Nevertheless, more than half of the protein is not released by either of the presses.

RuBisCo constitutes up to 70% of the soluble proteins in the alfalfa plant [10], and soluble proteins constitute ca. 33% of the total protein content in alfalfa (including stalks and leaves) [19]. In this work, we have used the entire harvested plant (i.e., including stalks and leaves). Therefore, it is not unexpected that the yield of protein is 50%. The composition and distribution of proteins in the plant is dependent on many factors, such as the harvest point and the alfalfa variety. Nevertheless, it is known that the stems have roughly only half

as much protein, compared to the leaves [20]. Therefore, when compared with experiments using only proteins obtained from the leaves, a lower yield is to be expected. Given that 33% of the proteins in alfalfa are soluble, and that this value was reached during the fourth press (Figure 2), it is to be expected that some insoluble proteins are also being extracted by the screw press, which might affect the digestibility and quality of the protein concentrate.

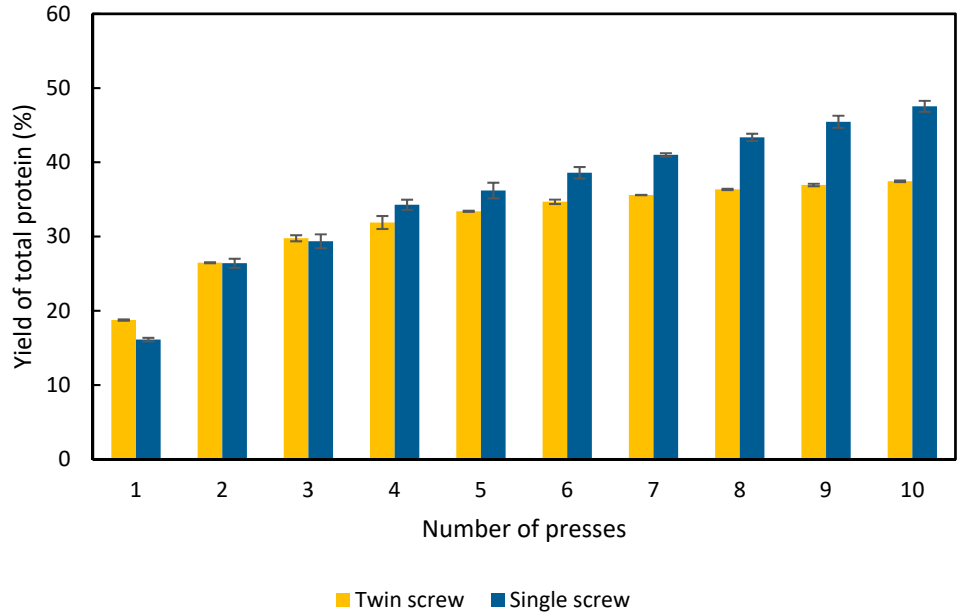


Figure 2. Cumulative yield of protein in the APC as a percentage of the total protein content in the raw alfalfa used in the experiment. Results are from the twin-screw and single-screw press; error bars show standard deviation, n = 3.

We obtained 6.6 g/L of total protein from the green juice in the first press and 6.7 g/L in the second press, with the single-screw. All the remaining presses from the single-screw, had significantly lower protein concentrations in their respective green juices, illustrated in Figure 3. In contrast, the twin-screw press produced a green juice with 10.4 g/L protein in the first press and 7.2 g/L in the second press. The concentration continued to drop in all of the further rounds of pressing. This corresponds well with the pattern observed in Figure 3, where it was observed that after the second press, less protein was extracted with the twin-screw press compared to the single-screw press.

3.2. Soluble Protein and Protein Profile by SDS-PAGE

The amount of soluble protein in the APC from each press was determined and it was found that 35% of the total possible soluble protein was extracted in the first press, 25% in the second press and in the third and fourth press only 8 and 13% of the total soluble protein was extracted, respectively (Figure 4A). As speculated above, the 50% yield of protein extracted from the alfalfa was likely due to recovery of soluble and insoluble protein, given that only ca. 33% of the total protein in alfalfa is soluble. Therefore, the proportions of soluble and insoluble proteins in APC from the single-screw press were determined. In the APC from the first press, the proportion of soluble protein was close to 33%, which was further enriched to 42.5% in the APC from the third press (Figure 4B). This is consistent with the results in Table 2 where the third press resulted in the highest percentage of protein in the APC. The APC produced from each of the first five presses had a proportion of soluble protein, which was almost twice as high as that in the second five presses (Figure 4B). This indicates that when the alfalfa is pressed more than 5 times, the APC produced contains greater and greater proportions of insoluble protein. A total of 1041 g of protein was extracted in the 10 presses (from a total possible protein amount of 2189 g), from this, 314 g was soluble, which corresponds to 30.1%. Therefore, in order

to generate an APC with the highest proportion of soluble protein, a maximum of five pressings should be used.

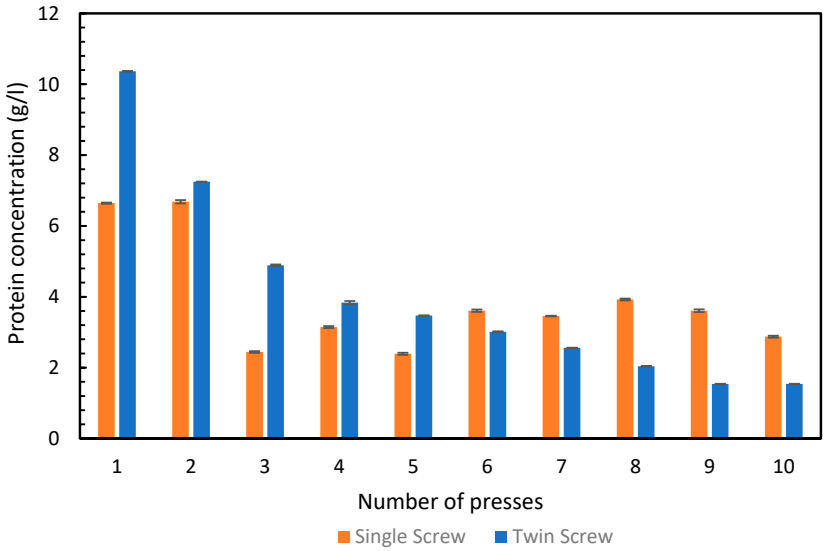
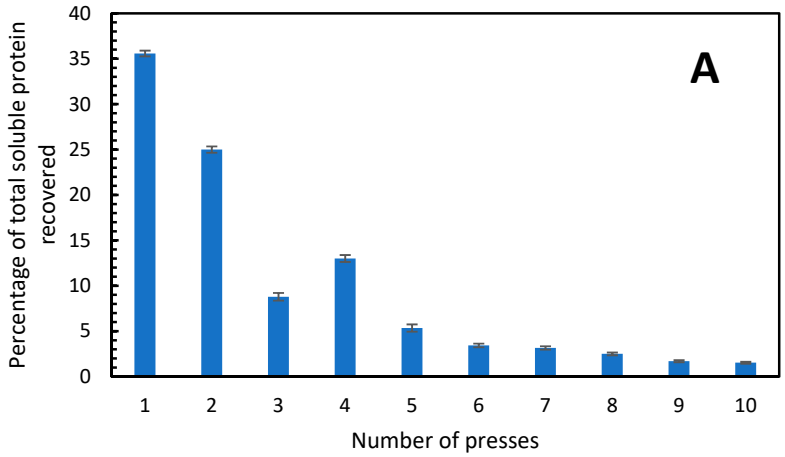


Figure 3. Concentration of total protein (g/L) in the green juice obtained from press 1–10 from the single- and twin-screw presses. Error bars show standard deviation, n = 3.



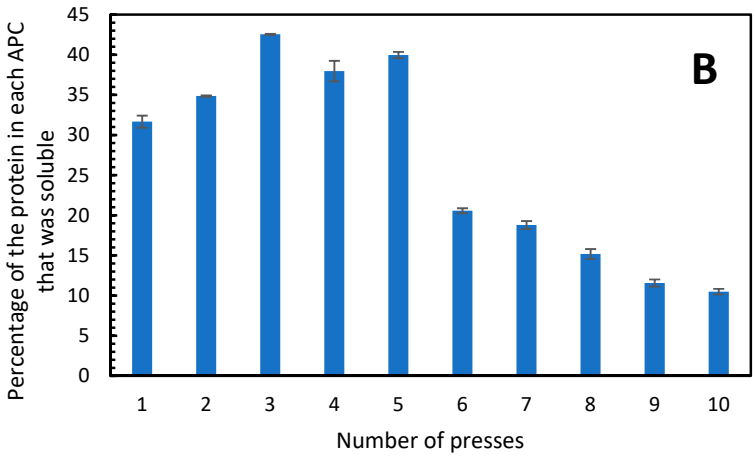


Figure 4. (**A**) Percentage of the total soluble protein in the fresh alfalfa that was present in the APC produced from each press. (**B**) Percentage of soluble protein in each APC fraction. The single-screw press was used. Error bars show standard deviation, n = 3.

Table 2. Composition of the extracted APC in press 1–10 (all results expressed % DM). Values are averages and standard deviations, n = 3 except for insoluble fiber and soluble fiber, where n = 1.

Component\Press	**1**	**2**	**3**	**4**	**5**
APC obtained (g)	818.4	511	136.9	268.5	99.7
Protein (%)	43.12 ± 0.26	44.08 ± 0.83	47.40 ± 0.93	40.07 ± 0.68	42.14 ± 1.06
Protein Digestibility (%)	87.36 ± 0.86	82.42 ± 1.30	76.20 ± 1.39	69.16 ± 0.49	70.43 ± 1.69
Insoluble fiber (%)	21.13	24.83	29.34	35.03	34.6
Soluble fiber (%)	1.67	1.21	2.17	2.22	2.41
Glucose (%)	3.15 ± 0.63	2.27 ± 0.48	1.79 ± 0.76	1.20 ± 0.65	2.14 ± 0.00
Fructose (%)	2.29 ± 0.47	0.30 ± 0.30	0.04 ± 0.06	0.38 ± 0.53	0.00 ± 0.00
Ash (%)	8.24 ± 0.28	3.15 ± 0.55	3.07 ± 0.37	2.72 ± 0.28	2.40 ± 0.18
Fat (%)	1.25 ± 0.15	3.77 ± 0.86	2.46 ± 0.25	2.41 ± 0.12	2.76 ± 0.09
Component\Press	**6**	**7**	**8**	**9**	**10**
APC obtained (g)	126.8	134.8	133.6	120.3	120.8
Protein (%)	41.29 ± 0.77	39.08 ± 0.13	38.69 ± 0.59	38.24 ± 0.82	37.84 ±0.80
Protein Digestibility (%)	62.28 ± 1.39	61.84 ± 0.82	64.07 ± 0.35	63.72 ± 0.43	65.02 ± 1.16
Insoluble fiber (%)	39.36	41.56	42.16	44.94	45.6
Soluble fiber (%)	1.51	1.71	1.82	1.77	1.86
Glucose (%)	1.61 ± 0.12	1.00 ± 0.12	1.06 ± 0.07	0.77 ± 0.12	0.55 ± 0.18
Fructose (%)	0.34 ± 0.36	0.38 ± 0.30	0.04 ± 0.06	0.17 ± 0.00	0.55 ± 0.78
Ash (%)	3.16 ± 0.61	2.95 ± 0.50	2.95 ± 0.57	2.52 ± 0.25	2.70 ± 0.29
Fat (%)	2.56 ± 0.04	2.71 ± 0.16	2.31 ± 0.04	2.13 ± 0.16	2.25 ± 0.00

When the protein composition of the APC was examined by reducing SDS-PAGE, it could be seen that the APC contained more than 20 proteins (Figure 5). The most distinct bands were at ca. 150, 75, 55, 37, 30 and 14 kDa. The small and large RuBisCO subunits are identified at 14 and 55 kDa, respectively [12]. The other bands are speculated to be enzymes which hydrolyze active and non-structural proteins, as suggested by Yakhlef et al., 2020 [21]. Interestingly, it could be observed that the pattern of the protein bands changed from press to press. The large RuBisCO subunit is visible in press 1–8 and the smaller subunit is only visible in press 2–7. Furthermore, the second, third and fourth pressings had three bands with molecular weights of 150 kDa and higher, which were not observed in the other pressings. These bands are most clearly visible for pressing 3.

3.3. Amino Acid Profile of APC

To determine whether the alfalfa protein concentrate had a suitable amino acid profile and whether that profile was changed by repetitive pressing, analysis was conducted on presses 1, 5 and 10 from the twin-screw press. The results in Figure 6 show that the amino acids present in the highest concentrations were valine, lysine, and glutamic acid. As expected from the results in Figure 2, the concentrations of each amino acid declined as more presses were conducted. However, the overall profile (percentage of each amino acid) did not change markedly (see Table A2, Appendix B).

The United Nations Food and Agricultural Organization (FAO) recommends that adult humans should consume 0.8 g of protein per kilogram of body weight per day [21]. Furthermore, the protein consumed should have a specific amino acid profile [21]. The APC produced here was compared to the FAO requirements and to two other popular plant based protein isolates, namely soy protein isolate (SPI) and pea protein isolate (PPI) in order to determine if APC had a similar quality. The data from SPI, PPI and the FAO reference values were obtained from Corgneau et al., 2019 [22]. For this comparison, the amount of each amino acid recommended by the FAO to be consumed per day was normalized to 100%. The data in Figure 7 shows that consuming 0.8 g of APC from press 1 per kg of body weight would supply markedly more of 7 out of the 10 essential amino acids when compared to the FAO recommendation. In particular, approximately three times the necessary amount of valine would be obtained. Consuming 0.8 g/kg bodyweight of

the APC from press 5 would supply more than enough of six of the essential amino acids, whereas the APC from press 10 had a low amount of protein (Table A3, Appendix B), which results in insufficient supply of all amino acids. When the APC from press 1 is compared to soy protein isolate and pea protein isolate, it can be seen that APC is enriched in valine and isoleucine, has similar proportions of threonine and lysine, and lower proportions of cysteine + methionine, histidine and phenylalanine + tyrosine. The results suggest that the proteins in APC obtained from the first pressings would complement the amino acid profile of soy and pea protein isolates and give a profile that is acceptable for human consumption.

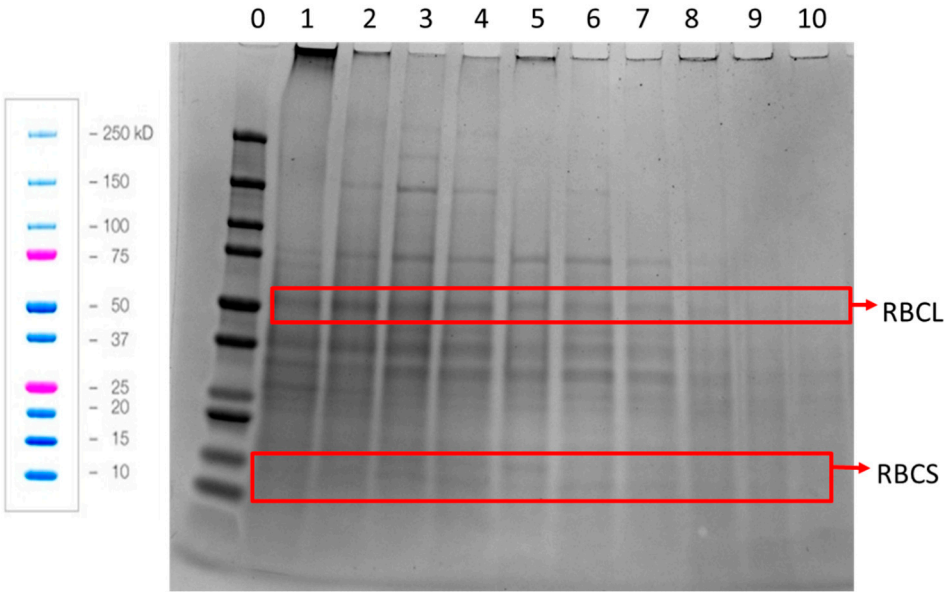


Figure 5. Reducing SDS-PAGE of the APC obtained from press 1–10 from the single-screw press. Lane 0, molecular weight standards. Lanes 1–10, APC from presses 1–10, respectively. RBCL = Large RuBisCO subunit, RBCS = Small RuBisCO subunit. Blue and pink colored bands are from Biorad's instructions showing how the standards are expected to run. The same mass of APC from each pressing was used to prepare the sample loaded onto each well of the gel.

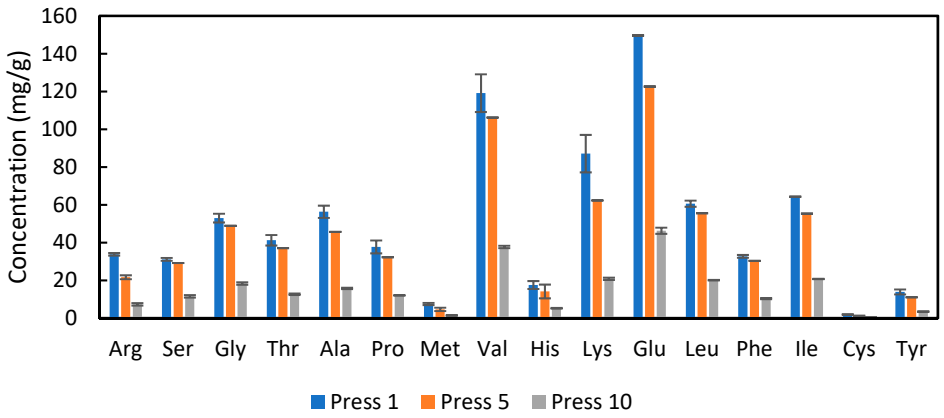


Figure 6. Concentration of amino acids on a dry weight basis for APC from press 1, 5 and 10, from the twin-screw extraction; error bars show standard deviations, n = 3.

3.4. Color of the APC

L-a-b is the analysis of the color spectrum of a sample in three dimensions. "L" is light from 0 (dark) to 100 (white), "a" is from −100 (green) to 100 (red) and "b" is from −100 (blue) to 100 (yellow) [23]. When the color of the APC was measured, it was seen that the amount of green color increased from press 1–3 (Figure 8). This suggests an increase in extraction of the green chlorophyll to the APC. The leaf of the alfalfa has the highest

concentration of protein and the highest concentration of chlorophyll in the plant [5,19]. This corresponds well with the amount of protein extracted presented in Table 2.

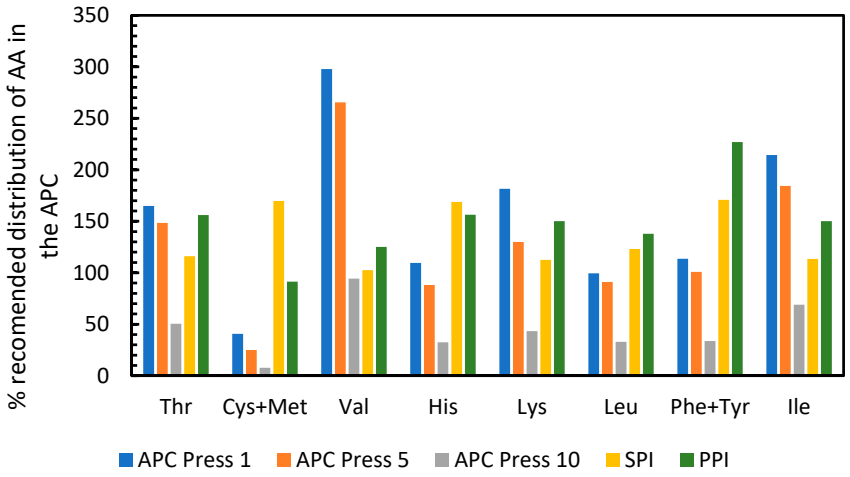


Figure 7. Percentage of each amino acid in APC and other protein sources normalized to the FAO recommended content of amino acids in protein sources. APC = Alfalfa Protein Concentrate, SPI = Soy Protein Isolate, PPI = Pea Protein Isolate. Data on PPI, SPI and reference values from Corgneau et al., 2019 [22].

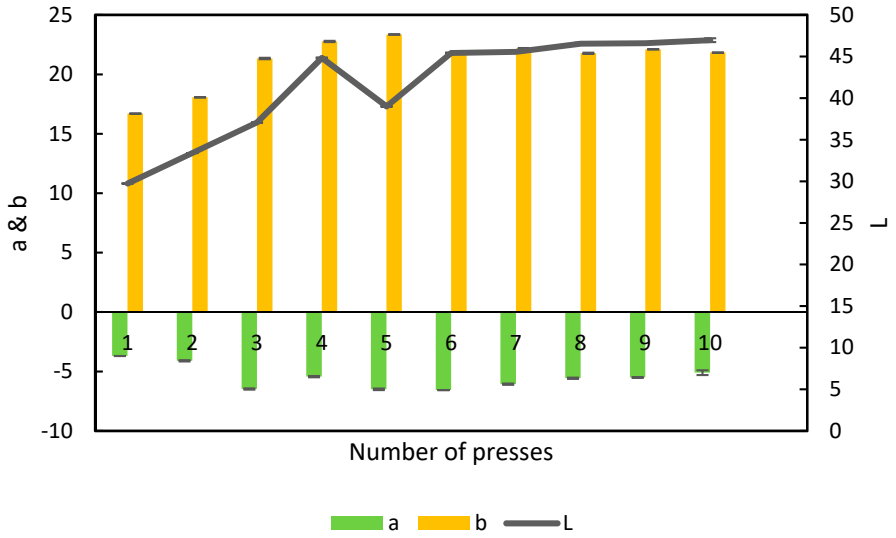


Figure 8. L-a-b color profile of alfalfa protein concentrate obtained in the 10 presses from the single-screw, n = 3, error bars show standard deviation.

The amount of yellow color increased from press 1–5 and was thereafter more or less stable. Yellow pigment is speculated to be xanthophyll, which is found in most parts of plants, including alfalfa [7,24].

3.5. Properties of APC Produced with Single-Screw Press

The results in Figure 2 showed that protein was recovered in all 10 rounds of pressing. Furthermore, it was seen that the concentration of protein present (on a dry weight basis) was over 37% even in the APC produced from the tenth press (as seen in Table 2). However, the most soluble proportion of protein was found in the first three presses. In order to further determine the quality of the APC, the protein digestibility was analyzed. The protein digestibility was highest in press 1 at over 87% and declined to ~70% in press 5, after which it fluctuated in the range of ~60–65% (Table 2). This confirms that in the first pressings, the most soluble and easily digestible proteins are released, and that repeated

pressings lead to release of poorly soluble, denatured or aggregated proteins as the alfalfa matrix is progressively broken down. It should be noted that the simple method used here most likely underestimates the digestibility, since it does not directly simulate the times and enzymes used in the mouth, stomach and gastric areas, as for example is the case with the Infogest assay.

Consistent with the pattern of digestibility, is that the highest concentrations of the highly soluble simple sugars, glucose and fructose, were in the first presses, which then decrease with further pressing. Breakdown of the alfalfa matrix is also supported by the observation that the amount of insoluble fibers in the protein concentrate increased when repressing the pulp (Table 2). Interestingly the amount of soluble fibers was similar in all ten presses.

The ash content was significantly higher in the first press (>8%) whereas it was around 3% in the following nine presses. The high ash content in the first press could be due to residual soil in the alfalfa, which was not washed away during the preparation of the raw material.

The total fat concentration was lowest in the first press and highest in the second. From the third to tenth press the total fat content was more or less similar. APC contains around 46% unsaturated fatty acids and 14% saturated fatty acids (i.e., values are as a percentage of the total fat). The main unsaturated fatty acid is alpha linoleic acid (34.1%) and the main saturated fatty acid is palmitic acid (12.3%) [6]. Since alpha linoleic acid is soluble in water and palmitic acid is not [25], it would be reasonable that many of the unsaturated acids are released in the second press after the plant material was partly opened up in the first press. A large reduction in the mass of pulp was observed from press 1, 2 and 3 (Table A1, Appendix A). From the fourth press until the 10th press, the decrease in the mass of pulp was low compared to first three presses. This might be a simple way to determine whether repetitive pressings should be stopped, or continued, when working with this in full scale.

In both of the cases used here, more than 10 cycles of pressing are required in order to extract all protein from the plant material. However, much of the protein is insoluble. Considering the single-screw press, 2189 g of protein was present in 50.8 kg of fresh alfalfa. Given that 33% of this protein is expected to be soluble, this equates to a maximum yield of soluble protein of ca. 722 g. In 10 presses a total of 1041 g of protein was recovered (Table 2) and 314 g of this was soluble protein (derived from Table 2 and Figure 4). This suggests that almost 30% of the soluble protein was recovered, and more than half of this was recovered in the first two presses.

4. Conclusions

Overall, the results indicate that small-scale laboratory trials with a twin-screw press can be scaled-up to a pilot scale single-screw press. However, the results also suggest that there is better maceration of the alfalfa with a twin-screw press. For scale-up it might therefore be beneficial to have a pre-maceration step, or to choose a screw design that macerates the plant material before pressing it, to maximize the protein yield.

Using 10 pressing cycles, is undesirable due to the amount of water consumed, the time taken, the risk of oxidation and energy used. However, since alfalfa is harvested several times during a harvest season, it is concluded that two rounds of pressing could be used to recover up to 25% of the total protein and ca. 25% of the total soluble protein. This would result in an APC product with the highest digestibility and lowest fiber content. The resulting pulp could then be used for animal feed or other applications, such as mushroom production [26,27].

The protein concentrate produced here was observed to be dark green and to smell of grass. It is thus concluded that it contains many impurities and although it might be suitable as a supplement, it is not a product ready to be used in large amounts as an ingredient, which would be acceptable for human food production. Therefore, a full techno-economic evaluation of alfalfa protein concentrate production is premature at this point. More work is required to identify the best process for protein ingredient production from alfalfa. It

is proposed that process development should proceed on two fronts: Optimizing protein recovery from alfalfa within two pressing steps and purification of the green protein.

Author Contributions: Conceptualization, M.H., T.J.H. and P.R.J.; methodology, M.H., C.A.A., T.J.H. and P.R.J.; software, M.H.; validation, T.J.H. and P.R.J.; formal analysis, M.H. and C.A.A.; investigation, M.H. and C.A.A.; resources, M.H., T.J.H. and P.R.J.; data curation, M.H., T.J.H. and P.R.J.; writing—original draft preparation, M.H.; writing—review and editing, M.H., T.J.H. and P.R.J.; visualization, M.H.; supervision, T.J.H. and P.R.J.; project administration, T.J.H. and P.R.J.; funding acquisition, P.R.J. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available in the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Masses (g) of the fractions produced during alfalfa processing with twin- and single-screw presses.

Twin-Screw Press				
Press Number	**Pulp**	**Green Juice**	**Brown Juice**	**Green Pellet**
1	932	3589	3474	321
2	681	2059	1897	182
3	555	1415	1303	118
4	485	1138	1023	108
5	469	963	855	101
6	432	942	836	98
7	413	861	797	62
8	391	815	754	56
9	378	775	718	54
10	358	746	693	48
Single-Screw Press				
Press Number	**Pulp**	**Green Juice**	**Brown Juice**	**Green Pellet**
1	16221	53131	47938	4834
2	12449	33703	42101	3959
3	9549	26566	25020	969
4	8769	34220	32297	1809.7
5	7494	17578	16814	693
6	7108	14514	13572	874
7	6587	15244	13696	1003
8	6183	13175	12091	1027
9	5849	12749	11789	974
10	5827	15900	14653	1113

(**A**) Frozen Alfalfa

(**B**) Pulp

(**C**) Green Juice

(**D**) Brown Juice

(**E**) Alfalfa Protein Concentrate

Figure A1. Examples of different fractions in the production of alfalfa protein concentrate.

Appendix B

Table A2. Percentage of each amino acid in the APC produced in press 1, 5 and 10 from the twin-screw press.

Press	Arg	Ser	Gly	Thr	Ala	Pro
1	4.18	3.85	6.56	5.10	6.98	4.67
5	3.19	4.30	7.21	5.47	6.74	4.76
10	2.99	4.72	7.53	5.18	6.45	4.94
Press	**Met**	**Val**	**His**	**Lys**	**Glu**	**Leu**
1	0.92	14.76	2.17	10.79	18.54	7.51
5	0.68	15.67	2.08	9.19	18.09	8.19
10	0.59	15.46	2.13	8.54	18.99	8.23
Press	**Phe**	**Ile**	**Cys**	**Tyr**		
1	4.05	7.97	0.23	1.72		
5	4.47	8.16	0.16	1.63		
10	4.23	8.49	0.14	1.41		

Table A3. Total protein concentration in the APC obtained from the twin-screw press in presses 1–10, n = 3.

Press Number	1	2	3	4	5
Protein (% DM)	51.35 ± 0.08	49.16 ± 0.07	39.45 ± 0.41	36.75 ± 087	33.80 ± 0.09

Table A3. *Cont.*

Press number	6	7	8	9	10
Protein (% DM)	31.21 ± 0.29	28.07 ± 0.03	28.43 ± 0.09	26.69 ± 0.17	24.53 ± 0.12

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CHAPTER 4

Treatment with Supercritical CO_2 Reduces Off-Flavour of White Alfalfa Protein Concentrate

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Treatment with Supercritical CO_2 Reduces Off-Flavour of White Alfalfa Protein Concentrate

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Abstract: White alfalfa protein concentrate from alfalfa (*Medicago sativa*) is a promising substitute for milk and egg protein due to its functionality. However, it contains many unwanted flavours that limits the amount that can be added to a food without affecting its taste negatively. In this paper, we have demonstrated a simple method for the extraction of white alfalfa protein concentrate followed by a treatment with supercritical CO_2. Two concentrates were produced at lab scale and pilot scale, with yields of 0.012 g (lab scale) and 0.08 g (pilot scale), of protein per g of total protein introduced into the process. The solubility of the protein produced at lab scale and pilot scale was approximately 30% and 15%, respectively. By treating the protein concentrate at 220 bar and 45 °C for 75 min with supercritical CO_2, off-flavours were lowered. The treatment did not decrease the digestibility or alter the functionality of white alfalfa protein concentrate when it was used to substitute egg in chocolate muffins and egg white in meringues.

Keywords: green protein; white protein; sustainable foods; supercritical CO_2; SFE

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1. Introduction

As the global population increases, so too does the demand for food. Currently, many new plant-based protein sources are being investigated as a means to meet the growing demand for food, while maintaining or lowering the total emission of greenhouse gases.

Since 2009, alfalfa protein concentrate (APC) has been regarded as safe for consumption as a food supplement in the European Union at concentrations up to 10 g/day [1]. However, the APC is not of high enough quality to substitute other alternative proteins, such as soy protein, which is the main plant-based protein on the market [2]. One of the problems with APC is the high fraction of fibre (Table 1), which consists mainly of hemicellulose and cellulose and a smaller fraction of lignin [3]. The high indigestible fibre fraction, which is known to bind proteins, is likely the reason why the digestibility of APC is lower than soy protein isolate (SPI), soy meal flour (SMF) and the white protein concentrate "Welpro" [4].

Table 1. Composition of APC, Welpro (WAPC), soy meal flour (SMF) and soy protein isolate (SPI). Data on SMF obtained from two sources ([a] = [5], [b] = [6]). DM = dry matter.

Component [Reference]	APC [1]	Welpro (WAPC) [7]	SMF [5,6]	SPI [8]
Protein (% DM)	45–60	88.7	40.7 [b]–49 [a]	90.5–92.2
Protein digestibility (%)	63.6 [9]	91.7 [10]	86 [a]	94.4–97.8
Fat (% DM)	9–11	<0.5	22.2 [b]	0.2–1.2
Fibre (% DM)	11–15	<0.5	10.4 [b]	-

Alfalfa is a perennial plant, grown worldwide as a feed for ruminants, pigs and hens. In the last decades, alfalfa has been of increasing interest as a new source of protein for

human consumption. This is due to its amino acid composition, which contains all essential amino acids and the high protein yield per acre of land [11–13]. Alfalfa is typically harvested 4–5 times per year and made into silage or transported directly to a biorefinery for protein extraction for animal feed. Extraction is typically carried out through a mechanical separation of green juice (protein rich wet fraction) from the pulp (fibre rich solid fraction). The green juice is subjected to further processing, during which the proteins can be precipitated and recovered by centrifugation. The green protein pellet is collected as the product. The supernatant, now called the brown juice, is typically used for biogas or fertilizer [3,4,11,14–16] even though it is rich in protein.

The green alfalfa protein concentrate looks, smells, tastes of grass and it is usually used as animal feed [17]. Nevertheless, alfalfa protein concentrates have been proposed to be incorporated into foods for several years. In all cases, the bitterness or "grass" taste has been a challenge for the consumer, and this has limited the amount of concentrate that could be included in the food formulation [7,18,19]. Studies have shown that the main contributor to this bitter taste is the saponin, zahnic acid, which is one the largest groups of saponins in alfalfa [20–22]. Other studies have shown that extraction of saponins from alfalfa is possible with supercritical CO_2 extraction (SFE), a method which is known to consume low amounts of energy and have the ability to preserve the functionality of the material extracted [23,24].

The main protein in alfalfa is RuBisCo, which constitutes 30–70% of the total protein content [25]. RuBisCo is one of the largest proteins found in nature (up 560 KDa), it contains all essential amino acids and it is known for its functionality with respect to emulsifying and foaming capabilities [11,26]. Due to its amino acid profile and functionality, RuBisCo is of high interest as a vegetable substitute for animal derived agents. RuBisCo is highly reactive and it is easily oxidized, which lowers its functionality. Thus, when working with this protein, limiting its oxidation is of high importance [27].

In 1975, a complex process, called the Pro-Xan II method, was proposed to produce edible white alfalfa protein concentrate (WAPC). The process consists of more than 19 processing steps and recovers roughly 0.08 g of purified white protein (Welpro in Table 1) per g of protein input in the process [7]. In a recent study, it was proposed to use ultrafiltration/diafiltration of the brown juice, instead of using acid precipitation, to obtain a WAPC with a protein concentration of 60.5% (DM). This was followed by an adsorptive removal of polyphenols, thus requiring a rehydration of the WAPC [28]. In 2015, Faris-Campomanes et al. demonstrated that SFE could be used for the extraction of polyphenols from lees [29]. Therefore, it seems possible that SFE could be used to extract saponins, and polyphenols from alfalfa protein concentrates.

In this study, we propose and investigate a new simple processing method to produce WAPC from brown juice. The entire process from raw alfalfa to WAPC consists of only eight steps including SFE, to produce a WAPC with acceptable taste, composition and functionality to substitute animal proteins in baked goods.

2. Materials and Methods

Two different batches of WAPC were produced during this study, one batch where a laboratory size twin screw press was used to press the alfalfa and one where a larger process scale single screw press was used. In order to differentiate between the different scales of production and whether the WAPC was treated with supercritical CO_2 or not, the nomenclature shown in Table 2 is used.

2.1. Production of White Protein Concentrate

Alfalfa was harvested in early spring, on Fyn (Denmark), manually with a scythe, packed in bags, then transported directly to the laboratory (within 3 h) and stored at −20 °C until it was further processed with a twin or single screw press. Before processing, roughly 200 g of raw alfalfa was withdrawn for composition analyses (protein, moisture, ash and fat) as described in Section 2.2. To recover white protein concentrate, the alfalfa was first

thawed by submerging it in cold tap water, followed by draining it. The plant was then processed through a twin screw press (Angel Juicer S8500 (Seoul, Republic of Korea)) or a single screw press (Vincent CP-1 (Vincent Corporation, Tampa, FL, USA)) resulting in two fractions; a protein rich green juice and a pulp fraction. For large-scale trials, the pulp was re-suspended with 2× its own weight of water before being pressed through the single screw-press again, resulting in a second fraction of green juice that was then pooled together with the first fraction. The green juice was continuously collected and stored at 4 °C to minimize potential oxidization of the proteins.

Table 2. Abbreviations of the four different types of white alfalfa protein concentrate produced and investigated in this study.

Treated with Supercritical CO_2	Type of Press	
	Single Screw	Twin Screw
Not treated	WS	WT
Treated	WSS	WTS

In order to determine the best temperature for green protein precipitation from the green juice, a simple test was first carried out. The pH was adjusted to 8 and the green juice was heated to 45, 50 or 55 °C at a rate of >2 °C/min. When it had reached the test temperature, it was centrifuged at 2500× *g* for 20 min at 4 °C (Thermo Scientific Multifuge X3R, Waltham, MA, USA). The green protein precipitate was removed and the soluble protein remaining in the supernatant (i.e., the protein depleted brown juice) was measured. The lowest amount of soluble protein left in the protein-depleted brown juice occurred at 50 °C. Based on those findings, it was decided to continue with 50 °C for removing the undesirable green protein fraction while maintaining the highest amount of soluble protein in the white protein fraction (see Appendix A for data).

The pH of the collected brown juice was lowered to 3.5 by addition of 3M lactic acid, while mixing it and then it was immediately centrifuged (2500× *g*, 20 min, 4 °C). The white protein pellet was then collected and freeze-dried (Thermo Scientific Heto Drywinner DW8, Waltham, MA, USA), starting at −20 °C with a 1 °C/h increase until the temperature reached 20 °C where it was kept, until no decrease in weight was observed. To ensure a uniform distribution during SFE treatment, manual crushing to a particle size below 1.25 mm (particle size distribution in Appendix C) was carried out with mortar and pestle. This resulted in a white alfalfa protein concentrate (WAPC). A simplified illustration of the process can be seen in Figure 1.

2.2. SFE Treatment of WAPC

A preliminary experiment was first conducted to determine the operating conditions for the SFE system. The WAPC was dried at room temperature in a vacuum oven over night (VT 6060, Thermo Fisher Scientific, Waltham, MA, USA). Then samples of 10 g were placed in 25 mL extraction vessels. Extraction was carried out for 75 min using either 180, 220 or 260 bar, with or without 1 or 2 mL/min of co-solvent, using the following conditions: 45 °C, 10 mL/min CO_2 flow, 60 min dynamic time, 15 min static time, make up flow 0.5 mL/min of 96% ethanol. The temperature of 45 °C was used to minimize solubility loss of the protein based on the test illustrated in Appendix A. A MV-10 (Waters, Milford, CT, USA) SFE system was used. *Chlorophyll* in the extract was estimated by spectrophotometry (Genesys 10S, Thermo Fisher Scientific, Waltham, MA, USA), using the equation described by Mouahid et al. [30]:

$$Chlorophyll_a = 12.74 \cdot A_{663} - 2.69 \cdot A_{645}$$
$$Chlorophyll_b = 22.9 \cdot A_{645} - 4.68 \cdot A_{663}$$
$$Chlorophyll_{total}(\mu g/mL) = Chlorophyll_a + Chlorophyll_b$$

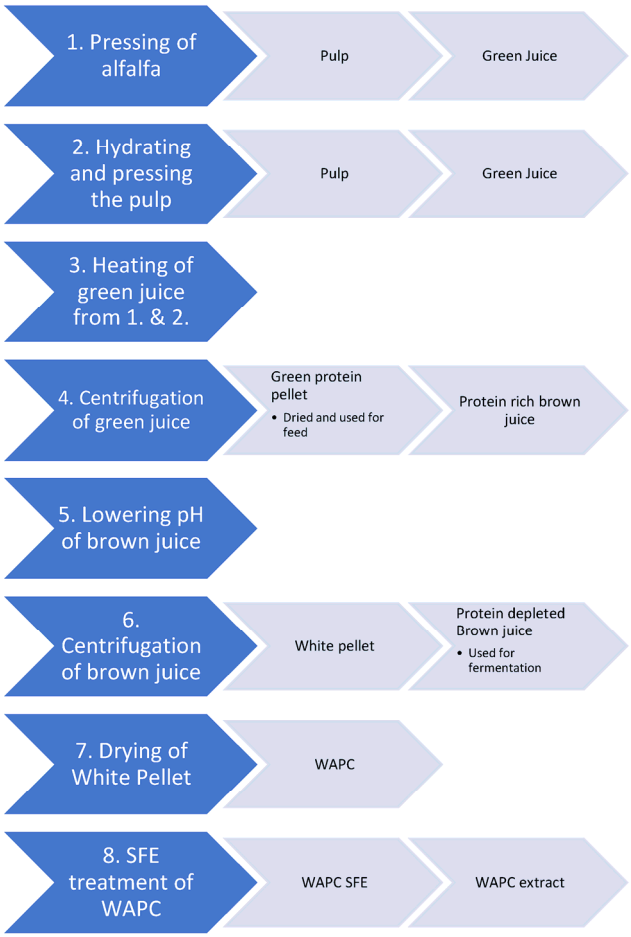

Figure 1. Process used for production of white alfalfa protein concentrate. Blue arrows = processing step, grey arrows = products from the processing step. WAPC = white protein concentrate, SFE = supercritical CO_2 extraction. When using the small twin screw press, step 2 was omitted.

The level of grass aroma was observed by sniffing the protein powder after extraction. The results showed (See Appendix B) that a combination of 220 bar without the use of co-solvents at 45 °C led to the best compromise between removal of *chlorophyll* and grass aroma. These conditions were used in all future work.

To extract enough WAPC for baking experiments, four samples of 10 g were placed in four 25 mL extraction vessels, and they were extracted using the optimal conditions described above. The four extracts were pooled and then the absorbance spectrum from 300–600 nm was measured (Genesys 10S, Thermofischer Scientific, Waltham, MA, USA) against a blank with 96% ethanol to inspect for the removal of colour compounds during extraction.

2.3. Moisture and Ash Content

Pre-dried crucibles were filled with roughly 3 g of sample and left in an oven at 104 °C overnight. The crucibles were then cooled in a desiccator before being weighed again. Moisture content was found from the loss of weight after drying. The raw alfalfa was treated in a similar way; however, due to the sample size of roughly 200 g, only one analysis was conducted for this. The dried alfalfa was then blended to a uniform powder, re-dried, and hereafter treated as the other samples for ash measurement.

After the moisture content was determined, the samples were placed in a muffle furnace at 600 °C overnight (D6450 M110, Heraeus, Hanau, Germany) and then placed in a desiccator for cooling. The weight of the cooled incinerated crucibles was then used to determinate the ash content.

2.4. L-a-b Colour Measurement

Around 1 g of sample was placed under a glass plate and L-a-b colour was measured using a LC 100 spectrocoloriometer (Lovibond, Amesbury, UK). Three measurements were carried out for each sample.

2.5. Total Fat Analysis

Total fat content was found by using the Rapid NMR Fat Analyzer (CEM, Matthews, NC, USA) with the Powder method. All analyses were carried out in biological duplicates.

2.6. Effect of pH Change on Protein Solubility

To investigate optimum solubility of the WT (Table 2), it was analyzed at different pH values. For this, 50 mg of WT was mixed with 2 mL of 0.1 M sodium phosphate buffer at different pH values of 7, 8, 9, 10 or 11 by vortexing for 20 s. They were then mixed in a laboratory shaker for 10 min at 1000 rpm (TS-100C, Biosan, Riga, Latvia), to ensure uniform distribution of the WAPC. After shaking, the samples were centrifuged for 5 min at 10,000× *g* and room temperature in a Microcentrifuge (Ole Dich, Hvidovre, Denmark). After centrifugation, the pH of the supernatant was measured using a LAB 845 pH meter (Xylem, Mainz, Germany). To measure the effect of pH changes on solubility of the WAPC, the concentration of soluble protein was determined using the Bradford analysis method (described in Section 2.7).

The effect on protein solubility of lowering the pH after it had been raised was also studied. In this case, the pH of WAPC was raised to values of 9, 10 and 11 as described above. The supernatant was recovered after centrifugation and then it was solubilized in a sodium phosphate buffer at pH 8, followed by centrifugation and analysis of the supernatant for soluble protein.

2.7. SDS-PAGE

All reagents and equipment used in these analyses were from Biorad (Hercules, NJ, USA). Samples were prepared by mixing 50 mg of WT with 2 mL of 0.1 M sodium phosphate buffer pH 8 (10 min, 1000 rpm (TS-100C, Biosan, Riga, Latvia)), then centrifuged for collection of the supernatant (5 min 10,000 rpm (Ole Dich, Hvidovre, Denmark)). An amount of 10 µL of the supernatant was then mixed together with 5 µL 4× Laemmli buffer, 4.75 µL of milliQ water and 0.25 µL of β-mercaptoethanol. The solution was then incubated for 10 min at 95 °C in a TS-100C heating block (Biosan, Latvia, Riga). An amount of 10 µL of the incubated sample was then loaded into a well in a 4–20% Mini-PROTEAN® TGX gel where the first well was loaded with 5 µL Precision Plus Standard ladder. The gel was run at 140 V, 400 mA for 50 min. The gel was then washed and stained for 1 h (Coomassie R-250) followed by a de-staining procedure. This consisted of replacing the staining reagent with water and leaving the gel with gentle shaking for 1 h, before replacing the water with fresh water. The last procedure was repeated 3 times before scanning the gel using a ChemiDoc XRS + System.

2.8. Protein Determination

Soluble protein was measured using the Pierce Coomassie Plus Bradford kit (Thermofischer Scientific, Waltham, MA, USA) following their protocol for using microwell plates [31]. All analysis was carried out in triplicate on an Infinite M200 Pro plate reader (TECAN, Männedorf, Switzerland).

Total protein was determined by using the DUMAS combustion method with a rapid MAX N exceed (Elementar, Langenselbold, Germany). Protein was calculated by using a protein factor of 6.25 [7].

2.9. Protein Digestibility

Protein digestibility was found by following the pepsin–trypsin method described by Saunders et al. [10]. First, 1 g of sample was suspended in 20 mL 0.1 M HCl containing

50 mg of trypsin (dissolved in 1 mL of 0.01 M HCl) and incubated for 48 h at 37 °C with gentle shaking. This was followed by centrifugation (2500× *g*, 15 min, 4 °C) using a Thermo Scientific Multifuge X3R (Waltham, MA, USA). The pellet was then suspended in 10 mL of distilled water and 10 mL of 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mg of trypsin was then added. This was incubated at 23 °C for 16 h before being centrifuged (2500× *g*, 15 min, 4 °C). The solids were then washed 3 times by suspending with 30 mL of distilled water each time and centrifuging (2500× *g*, 15 min, 4 °C), except for the last wash in which the solids were filtered through a 1.2 µm pore sized nitrogen-free filter. The filter was then dried in a VT 6060 vacuum oven (Thermofischer Scientific, Waltham, MA, USA) at room temperature, overnight. The digested dried pellet, including the filter, was then analyzed for total protein content as described above and the protein digestibility was found by comparing total protein content before and after digestion.

2.10. Foam Stability of WS and WSS Compared to Milk

To mimic the low fat content in WS and WSS, a semi-skimmed milk (Egelykke, Arla-foods, Viby, Denmark) with 3.5% protein and 1.5% fat (data from the ingredient list) was purchased in a retail shop. A 23.75 mL aliquot of the milk was transferred to a 50 mL plastic centrifuge tube. Samples of WS and WSS were dissolved in sodium phosphate buffer (pH 8.00) to a protein concentration of 3.5% and a final volume of 23.75 mL, in a 50 mL plastic centrifuge tube. The different solutions were then mixed vigorously for 10 s at 10,000 rpm with a T-25 Ultra Turrax disperser (IKA, Staufen, Germany) to create foam. Foam height and foam strength were measured with a TA.XT.plus texture analyzer (Staple Micro Systems, Godalming, UK), using a 25 mm cylinder probe. The test speed was 1 mm/s at a distance corresponding to the top of the liquid. The average strength of the foam was calculated based on the average force (N) required to lower the probe from the foam top until the liquid layer. Foam height was measured from the top of the foam to the liquid layer.

2.11. Production of Meringues

Three batches of meringues were produced to investigate the foaming capabilities of the WAPC when it was included in a food. The basic recipe was 1.6 g of powdered sugar per g of egg white, where the egg white was replaced by WT or WTS as appropriate. Pasteurized egg white (Dava Foods, Hadsund, Denmark) was purchased in a retail store, which contained 9.1% protein and 90% moisture (wet weight). WT and WTS were used to make an egg replacement by mixing an appropriate mass, to give the same protein content as egg white, with sufficient deionized water (pH 8.00 adjusted with $NaHCO_3$) to match that in egg white.

The pure egg white, WT or WTS egg replacements were foamed at 15,000 rpm with a T-25 Ultra Turrax disperser (IKA, Staufen, Germany), while powdered sugar was slowly added. After foaming, the three different batches were distributed in silicone moulds with cylindrical wells (35 mm diameter, 0.9 mm height), to a level just below the top of the form. They were then baked for 45 min at 85 °C, with low ventilation, in a Rational C11C95057013 Combi Oven (Rational, Landsberg am Lech, Germany) followed by 15 min at 120 °C. The meringues were then cooled for 20 min before further analyses.

2.12. Production of Chocolate Muffins

Four different batches of chocolate muffins were produced to demonstrate the WAPC functional properties: one batch without egg or WAPC as a control, one with egg, one with WS and one with WSS. In the two batches with WAPC, the amount of WAPC corresponded to the amount of protein added from eggs in the recipe and additional water was added to make up for the moisture content in the egg. The water content of egg was calculated based on data from the Danish National food database [32]. The muffin dough consisted of 60 g wheat flour, 50 g sugar, 1.5 mL baking soda, 1.5 mL vanilla sugar, 30 mL cocoa, 50 g egg and 30 g water. The ingredients were mixed together to give a uniform dough.

Subsequently, 15 mL of the dough was placed in a paper muffin mould (diameter 50 mm, height 35 mm) and baked at 180 °C for 20 min with low ventilation in a XVC 705 oven (UNOX, Cadoneghe, Italy) and left to cool for 20 min before further analyses.

2.13. Texture Analysis of the Chocolate Muffins and Meringues

The texture of the muffins and meringues was analyzed with a TA.XT.plus texture analyser (Staple Micro Systems, Godalming, UK). For both, springiness and chewiness were measured with a flat disc (100 mm diameter) with the following settings: test and post speed of 5 mm/s, strain at 75%, post waiting time of 5 s and trigger force of 0.049 N.

For the meringues, height and hardness were measured with the same disc and settings as for springiness and chewiness.

For the muffins, height and hardness were analyzed with a knife cutter in the same apparatus. Travel speed was set to 1 mm/sec and travel distance was set to 20 mm from the top of the muffin. Height was measured as the distance from the top of the muffin to the bottom plate.

2.14. Sensory Analysis of the Meringues

A focus group was used, consisting of 19 different persons. The participants consisted of 12 females and 7 males ranging from 18 to 62 years in age. They were asked to rank the two batches of meringues with respect to which one had the strongest grass taste. Before tasting the test meringues, the participants took a bite of the control meringue with egg white and drank a sip of water. The participants were all made aware that the meringues contained WAPC before the test, but not which batch contained the SFE-treated WAPC.

2.15. Sensory Analysis of the Chocolate Muffins

A focus group was used, which consisted of 11 employees from DTU Food. The participants ranged from 20 to 62 years of age and there were five males and six females. The focus group was asked to taste the four types of chocolate muffin and to rank them on a taste of grass scale (no grass taste, mild grass taste, strong taste). After the participants had ranked the muffin, a discussion about the muffins was carried out in plenum.

2.16. Statiscal Analyses

Each analytical result reported is the mean value of three replicate measurements or biological triplicates as stated in the captions to the data figures or tables, unless otherwise reported. Standard deviation and statistical differences were analyzed in MS Excel. Differences between the means were analyzed using the single factor ANOVA test with a least significant difference of 0.05.

3. Results

The overall aim of this work is to examine if white alfalfa protein concentrate is a suitable substitute for eggs in baked goods. This requires that the protein produced has a suitable amino acid profile, digestibility and functionality and that undesirable sensory properties can be satisfactorily ameliorated, in this case using supercritical CO_2 extraction.

3.1. Yield and Protein Concentration of the WAPC Obtained from the Single and Twin Screw Presses

From 4 kg of wet alfalfa, 17.5 g of WT was obtained, with a protein concentration of 57% using a twin screw press as shown in Table 3. This resulted in a yield of 0.012 g of protein per g of protein introduced into the twin screw press. The total protein content was found to be higher in the WTS, whereas no fat was present in the WTS. The colour in both WT and WTS was similar.

Table 3. Protein concentration, dry matter (DM), fat and L-a-b colour of the WAPC produced (from the twin screw press-based process), $n = 2$. Here, WT is WAPC without SFE treatment and WTS is WAPC treated with SFE.

Component	WT	WTS
Protein(% DM)	57.00 ± 0.44	64.56 ± 0.43
Dry matter (%)	93.83 ± 0.69	90.85 ± 1.26
Fat (% DM)	0.12 ± 0.16	0.00 ± 0.00
L-a-b colour	82.0, −6.9, 17.5	80.6, −6.9, 18.3

In order to generate enough WAPC for trials with muffins, a pilot scale production was made. Yield of the WAPC production was not part of the scope of this study, nevertheless, when 22.2 kg (wet) of alfalfa was used, 102.2 g of dry WS was recovered (6.17% moisture), which had a protein concentration of 57.73%. The protein concentration in the raw alfalfa was found to be 4.31% on a wet basis (derived from Table 4). This means that the yield was 0.06 g of protein per g of protein introduced into the screw press. However, this yield results from pooling the two fractions obtained from the first pressing of raw alfalfa, and the second pressing of the pulp. Since the main part of the plant material is lignocellulosic fibre, the rest of the WAPC is speculated to be mainly fibre and free carbohydrates; however, this was not investigated in the current study. The yield from the single screw press is 0.02 g/g lower than the method proposed by Edwards et al. in 1975 comprising 19 processing steps [7]. However, upon cleaning the screw press, it was noted that approximately 1.5 kg of pulp was left in the filter matrix due to its design.

Table 4. Composition of the raw alfalfa, the two types of WAPC (produced from the single screw press-based process), $n = 2$. Here, WS is not SFE treated; WAPC and WSS are SFE treated WAPC. NA = not analyzed or not applicable.

Component	Alfalfa	WS	WSS
Protein (% DM)	20.90 ± 2.42	57.73 ± 0.84	63.61 ± 0.07
Protein Digestibility (% DM)	NA	93.28 ± 0.89	92.65 ± 0.64
Soluble Protein (% of protein)	NA	14.98 ± 0.24	15.80 ± 0.42
Dry matter (%)	20.621	100 ± 0.00	99.8 ± 0.00
Ash (% DM)	8.61 ± 1.02	0.91 ± 0.14	Not determined
Fat (% DM)	1.22 ± 0.00	0.23 ± 0.25	0.00 ± 0.00
L-a-b colour	NA	63.6, 0.7, 26.5	61.3, 3.0, 22.0

Supercritical fluid extraction can be expected to remove small molecular weight polar compounds and fats from the WAPC in addition to unwanted flavour and aromas [33]. After SFE treatment, the protein content of the WSS was found to have increased slightly to 63.6%, which is expected to be due to the removal of fats (Table 4) and other non-protein compounds. A similar effect was seen for the twin screw press (Table 3). It has previously been shown that SFE can be used for the extraction of *chlorophyll* (green colour) and xanthophyll (yellow colour) from other plant materials [34,35]. As therefore expected, the SFE treatment reduced the green and yellow colour in the WSS, as shown by the LAB measurements in Table 4 and increased the colour in the extract recovered after the SFE treatment. The SFE extract had a visible yellow-green colour ($OD_{580(Yellow)}$ = 1.41, $OD_{550(green)}$ = 1.08), which is likely to be *chlorophyll* and xanthophyll pigments [15,33,36]. It should be noted that these measurements were made on the extract, which had been mixed with 35 mL of ethanol (96%) from the make-up flow during the extraction. The colour of the WAPC produced with the twin screw press was lighter, greener and less yellow (Table 3) compared to the WAPC produced with the single screw press.

No difference was observed with respect to *chlorophyll* extraction when the pressure was increased from 220 to 260 bar. Therefore, 220 bar was chosen to minimize the potential cost for a full-scale production. Future studies should include WAPC extracted at higher

pressures to investigate if this could lower the taste of grass for the consumer, while maintaining the functionality of the WAPC.

The digestibility of the protein was not affected by the SFE treatment as seen in Table 4. WSS had higher average protein concentration, better digestibility and lower fat compared to the APC in Table 1.

3.2. Solubility of the Proteins

The WT is at pH 3.5 after precipitation, and it was therefore of interest to see if solubilization could be enhanced by raising the pH. This was therefore tested at various pH values. From Figure 2, it can be seen that the protein was most soluble when raised to pH 8. Interestingly, when the pH was lowered to 7 from pH 8 or 8.5, the concentration of soluble protein was higher than if the protein had been raised to pH 7 in one step (Figure 2). The protein was therefore brought to a pH of 8 for the subsequent studies.

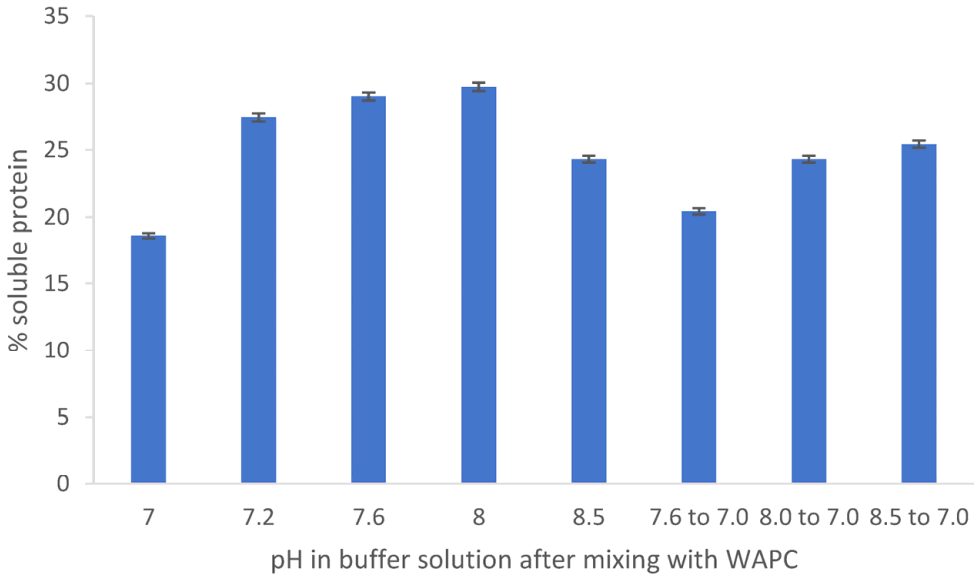

Figure 2. Percentage of soluble protein in the WT when suspended in sodium phosphate buffer solutions at various pH values. In the three bars to the right, the pH was decreased back to 7.0 after it had first been raised to a higher pH, $n = 3$.

The pattern of solubility seen in Figure 2 was also reflected in the results from analysis by SDS-PAGE (Figure 3), where intense bands were observed in lane 4 (i.e., pH 8), which also had the highest solubility in Figure 2. The least intense bands were observed for lanes 1 and 5, which had low solubility as well. Interestingly, lane 8 had the strongest bands in the gel, which suggest that more of the RuBisCo fractions were solubilized by increasing pH to 8.5 before lowering it to a neutral pH. Both the large and the small subunit of the RuBisCO protein were observed in all the lanes (55 kD and 14 kDa, respectively) [27]. The bands around 40 kDa are most likely the degraded species of the large subunit of RuBisCO [37].

The protein solubility of the WSS (15.80%) was observed to be 0.72 percentage points higher, which was not significant, compared to the untreated WS (14.98%). This is confirmed by the SDS-PAGE analysis in Figure 4, where there were only small differences observed between the two protein concentrates. The overall solubility of the WS was half of the WT (compare Table 4 and Figure 2). This is also illustrated in Figure 4 where the two RuBisCo fractions (around 55 Kd and 14 Kd) was less intense compared to the bands observed on the gel prepared with WAPC from the twin screw press (Figure 3). It could be speculated that the processing conditions in the single screw press increased oxidation of the proteins and thereby lowered the solubility of those proteins.

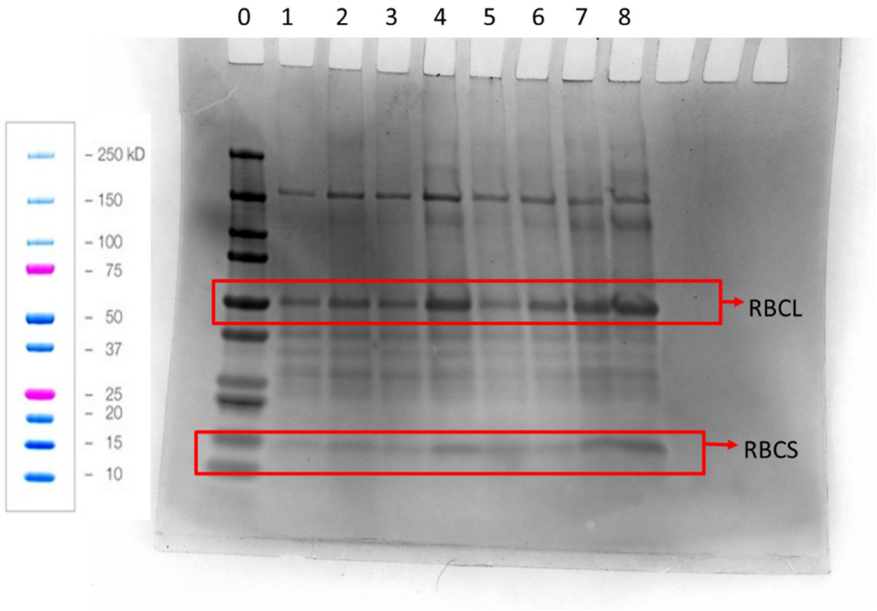

Figure 3. Reduced SDS-PAGE gel of WT, from the twin screw, solubilized in sodium phosphate buffer solution at various pH values (lanes 1–5) or raised to high pH and then lowered to neutral pH (lanes 6–8). Lane 0 = molecular weight standards, lane 1 = pH 7, lane 2 = pH 7.2, lane 3 = pH 7.6, lane 4 = pH 8, lane 5 = pH 8.5, lane 6 = pH 7.6 to 7, lane 7 = pH 8.0 to 7, lane 8 = pH 8.5 to 7. RBCL = large RuBisCO subunit, RBCS = small RuBisCO subunit. The figure on the left with blue and pink coloured bands show Biorad's instructions of how the standards are expected to run. The same mass of WAPC was used to prepare the sample loaded into each well.

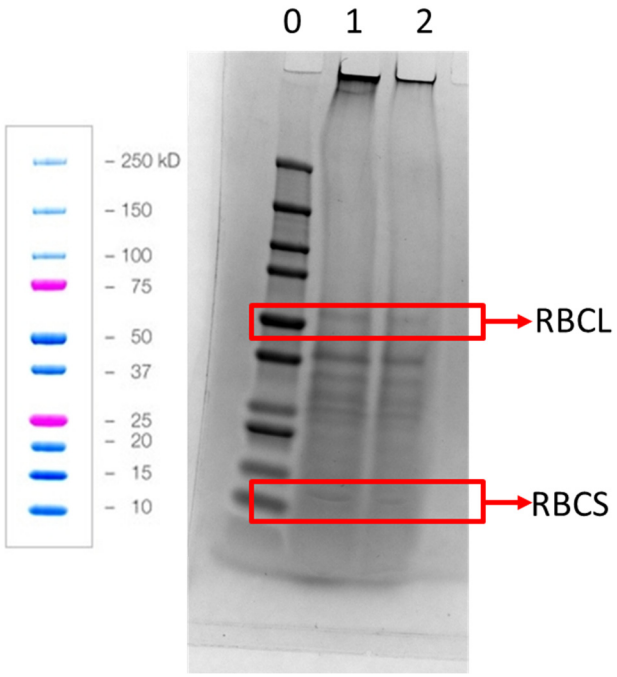

Figure 4. Reducing SDS-PAGE of the WS and WSS. Lane 0 = molecular weight standards, lane 1 = WS, lane 2 = WSS. RBCL = large RuBisCO subunit, RBCS = small RuBisCO subunit. Diagram on the left with blue and pink coloured bands is from Biorad's instructions showing how the standards are expected to run. The same mass of WAPC was used to prepare the sample loaded into the wells.

3.3. Foam Stability of the Proteins

The foaming properties of proteins are important functional attributes in food production. When a solution of WS or WSS was compared to semi-skimmed milk, it was seen that the WS and WSS (Table 5) produced a greater height and strength of foam. Furthermore, the SFE treatment had no negative effect on foaming properties. It was observed that 2 h after the test, more than 20 mm of foam was still present for both WS and WSS. In comparison, the semi-skimmed milk foam had dissipated within 2 min. The results here are consistent with previous work showing that soluble leaf protein from alfalfa has useful foaming capabilities [37–39]. Based on these results, it was decided to continue with developing batches of meringues to further study WAPC as a foaming agent.

Table 5. Comparison of foaming properties of WAPC (WS) and WAPC SFE (WSS) compared to semi-skimmed milk, $n = 1$.

Sample	Force (N)	Foam Height (mm)
Milk	0.061	20.49
WS	0.256	38.766
WSS	0.251	34.117

3.4. Texture Analysis of the Meringues

Three batches of meringues were produced as described in Materials and Methods. Even though the mass of the respective doughs was the same, the volume of the doughs made with WAPC was slightly lower compared to the control with egg white. This is illustrated by the number of produced meringues in the moulds in Figure 5A–C. This suggests a higher density of the doughs made with WAPC compared to the one with egg; however, this was not investigated further.

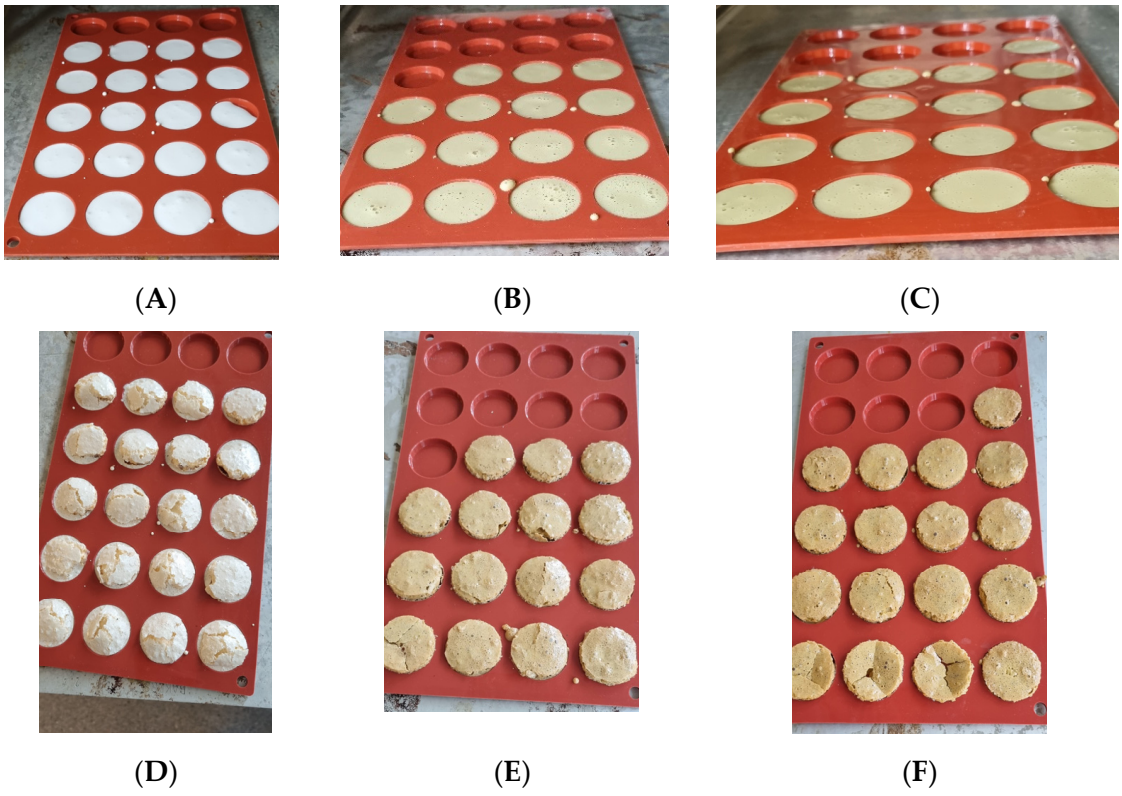

Figure 5. Pictures of the meringues before (**A**–**C**) and after cooking (**D**–**F**). Egg white (**A**,**D**), WT (**B**,**E**), WTS (**C**,**F**).

During cooking, the meringues with WAPC raised uniformly as can been seen in Figure 5E,F (the cracked WAPC meringues were broken manually after removal from the oven), whereas the standard meringues with egg white raised unevenly and cracked at the end of cooking (Figure 5D). The visual appearance of the dough made with WT and WTS (Figure 5B,C) was similar but was darker than the white appearance of the meringue made with egg white (Figure 5A).

The height of the meringues was measured from the point where the test probe registered resistance. The control with egg white had a cracked surface with a conical-like shape; therefore, the average height of the egg white meringues was in reality lower than the measured height presented in Table 6. The height of both meringue types produced with WAPC was almost identical, but the hardness of the crust was significantly higher for WTS compared to the WT ($p > 0.05$). The hardness of the WT meringue was lower than the other two batches, and this is speculated to be due the higher fat content, as described in Table 3. However, further investigation is needed to confirm this. The hardness of WTS meringues was not significantly different from that of the standard with egg white.

Table 6. Height and hardness of meringues produced with egg white, WT and WTS, $n = 3$; $p > 0.05$.

Protein Source	Height (mm)	Hardness (N)
Egg white	15.6 ± 0.6	27.3 ± 0.2
WT	13.0 ± 0.6	8.1 ± 0.3
WTS	12.8 ± 0.6	26.5 ± 3.56

3.5. Sensory Analysis of Merringues

All of the participants in the focus group could taste grass in the meringues produced with WAPC. However, 18 out of 19 participants found that the WTS meringue had the mildest taste of grass compared to the WT (presented in Table 7). The one participant who thought that the WT had the mildest taste explained that he thought that the taste of grass was milder in the WTS but lasted for a longer time, whereas the WT meringue had a strong taste of grass in the beginning of the taste experience and then quickly faded. Some of the participants liked the taste of the WTS meringue and others suggested masking the mild grass taste with ingredients such as ginger and liquorish.

Table 7. Number of focus group participants detecting the taste of grass in the meringues produced with different protein ingredients.

Type of Meringue	No Grass	Mild Grass	Strong Grass
Egg white	19	0	0
WT	0	0	19
WTS	0	18	1

3.6. Application of WAPC in Chocolate Muffins

The WS and WSS was examined to see if it could replace egg in baked goods. In light of the results from the meringue sensory testing, chocolate muffins were chosen as a test system, since the chocolate colour and flavour would be expected to mask any grassy notes or colour changes. In general, the results in Table 8 show that muffins with WS and WSS did not have a texture that was comparable to muffins with egg. The WS- and WSS-containing muffins were, in fact, not significantly different to the muffins without egg (Table 8). Nevertheless, muffins with egg and WS and WSS had all risen more than the control without egg (Table 8), although an ANOVA test shows that this is not significant ($p = 0.11$). From these data, it is clear that the standard muffin with egg had the best parameters with respect to its texture. As can be seen in Figure 6, the visual appearance of the four muffin types was quite similar. Saponins are known for their gel-forming capabilities, and it has been proven that most of the saponins are recovered in the green

pellet and not in the WS and WSS [40,41]. This could be an explanation of the lower springiness observed in the two batches of muffin with WS and WSS compared to those with egg. However, the saponin content was not measured and future studies should be carried out to verify this.

Table 8. Texture and height of chocolate muffins baked with WS, WSS or egg, $n = 3$. Control muffin has no egg.

Type of Muffin	Hardness (N)	Chewiness (N)	Springiness (%)	Height (mm)
Muffin control	229.18 ± 34.48	41.83 ± 9.35	36.09 ± 2.91	23.76 ± 0.92
Std. Muffin w. Egg	135.42 ± 32.16	38.04 ± 12.47	58.57 ± 3.46	29.43 ± 2.43
Muffin w. WS	217.56 ± 65.34	34.05 ± 15.54	36.45 ± 5.13	28.13 ± 3.16
Muffin w. WSS	239.89 ± 47.53	36.03 ± 10.55	35.80 ± 2.49	27.02 ± 3.14

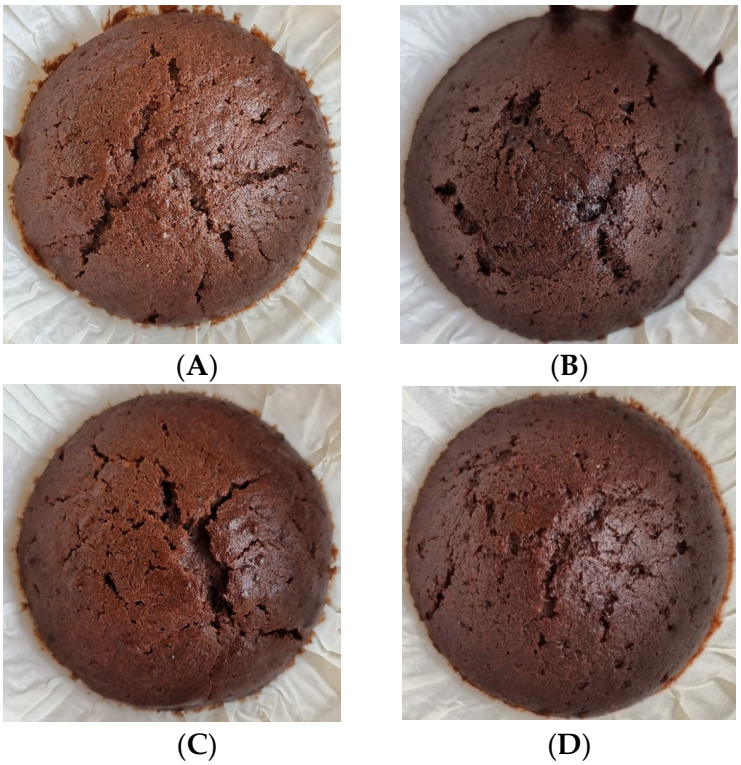

Figure 6. (**A**) Muffin control, (**B**) standard muffin with egg, (**C**) muffin with WAPC SFE treated, (**D**) muffin with WAPC not SFE treated.

3.7. Sensory Analysis of WAPC in Muffins

In order to determine whether the chocolate flavour was a way of masking the grass taste, the muffins were tested by a focus group. The main finding from the focus group analysis of the muffins was that the SFE treatment of the WSS lowered the taste of grass, compared to the muffins containing WS, which had not undergone SFE treatment as seen in Table 9.

Table 9. Intensity of grass taste in the different muffin types reported by the focus group.

Type of Muffin/Taste	No Grass	Mild Grass	Strong Grass
Muffin control	11	0	0
Std. Muffin w. Egg	11	0	0
Muffin w. WS	0	0	11
Muffin w. WSS	0	11	0

All participants in the focus group noted a mild grass taste in the muffin with WSS and a strong taste of grass in the muffin with WS. In the discussion in plenum, the muffin with WSS was mentioned as acceptable to chew, but they would have preferred if the taste of grass could be lowered. The muffin with WS was unpleasant overall and some found it disgusting. They mentioned that the control without egg was unpleasant to bite in and that the standard muffin with egg was pleasant overall. None of the participants in the test tasted any grass in the control muffin without egg and the standard with egg. No one observed any colour differences in the four muffin types.

4. Discussion

In this work, we have focused on two aspects for producing a protein ingredient from alfalfa: the design of a simple method of extraction for white protein concentrate and an off-flavour removal step that does not affect the functionality of the protein. Compared to the method proposed by Edwards et al. 1975 [7], our process is much simpler (9 compared to 19 steps), but gives a slightly lower yield of WAPC when using the large single screw press (0.06 g/g versus 0.08 g/g). Most of this is thought to be due to a loss of protein in the screw press due to the large dead volumes compared to the amount of alfalfa processed, since a yield of 0.12 g/g was possible with the smaller twin screw press. Some of the protein is recovered as green protein, which can be used for feed, but a large fraction is left unrecovered in the pulp. It is likely that much of the un-extracted protein is non-RuBisCo protein and thereby of a lower quality than the protein extracted. Future studies should investigate the composition of the remaining protein in the pulp to clarify whether or not developing a method for a higher yield of WAPC would be economically relevant.

The protein concentration in our process (57%) was around 30% lower compared to Edwards et al. (89%); however, the protein digestibility was similar (93% versus 92%). This was expected due to our simplified method. Even though it would be possible to refine and increase the protein concentration of the WAPC further by, for example, ultrafiltration and or acid washing, the potential cost of these procedures could make the resulting WAPC too expensive to compete with other vegetable protein concentrates on the market.

RuBisCo is known for its ability to emulsify and foam. However, the performance of WAPC as a substitute for egg, as an emulsifying agent was not found to be optimal. The low saponin content in WAPC is thought to be the reason for this. However, using WAPC as a substitute for foam-creating agents, such as egg white, was found to be favourable.

The WAPC we produced with a twin screw press had a higher solubility and our SDS-PAGE analysis had more intense bands with respect to the RuBisCo fractions in the WAPC produced with the single screw press. This suggests that a potential production of WAPC in full scale should be conducted with a twin screw press or in a setting where potential oxidation of the green juice during processing is minimized to maintain the highest levels of functionality in the RuBisCo protein.

5. Conclusions

We have demonstrated that our simplified nine-step extraction method, including a supercritical CO_2 step, can be used to produce white alfalfa protein concentrate with levels of grass taste and colour that are acceptable for substituting egg white as a foaming agent in baking.

Author Contributions: Conceptualization, M.H., T.J.H. and P.R.J.; methodology, M.H., T.J.H. and P.R.J.; software, M.H.; validation, T.J.H. and P.R.J.; formal analysis, M.H.; investigation, M.H.; resources, M.H., T.J.H. and P.R.J.; data curation, M.H., T.J.H. and P.R.J.; writing—original draft preparation, M.H.; writing—review and editing, M.H., T.J.H. and P.R.J.; visualization, M.H.; supervision, T.J.H. and P.R.J.; project administration, T.J.H. and P.R.J.; funding acquisition, P.R.J. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

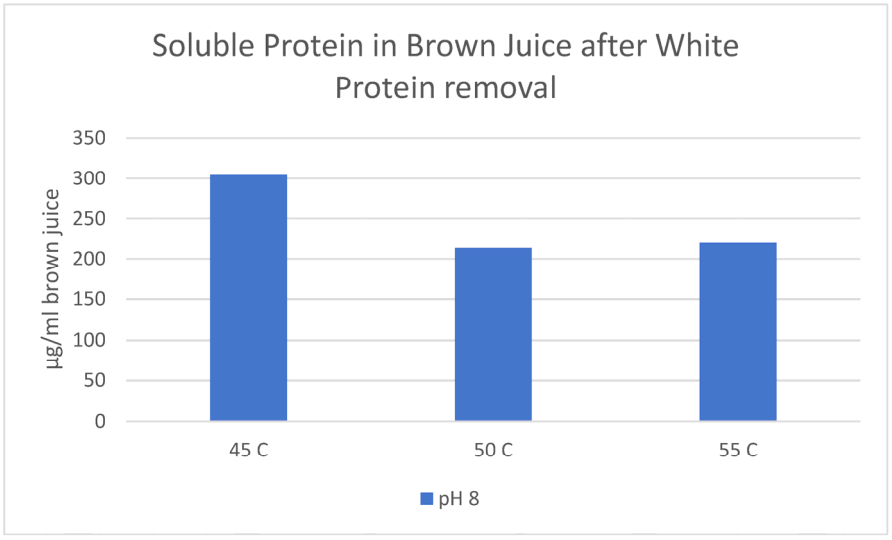

Figure A1. Soluble protein left in the brown juice after white protein removal at various temperatures.

Appendix B

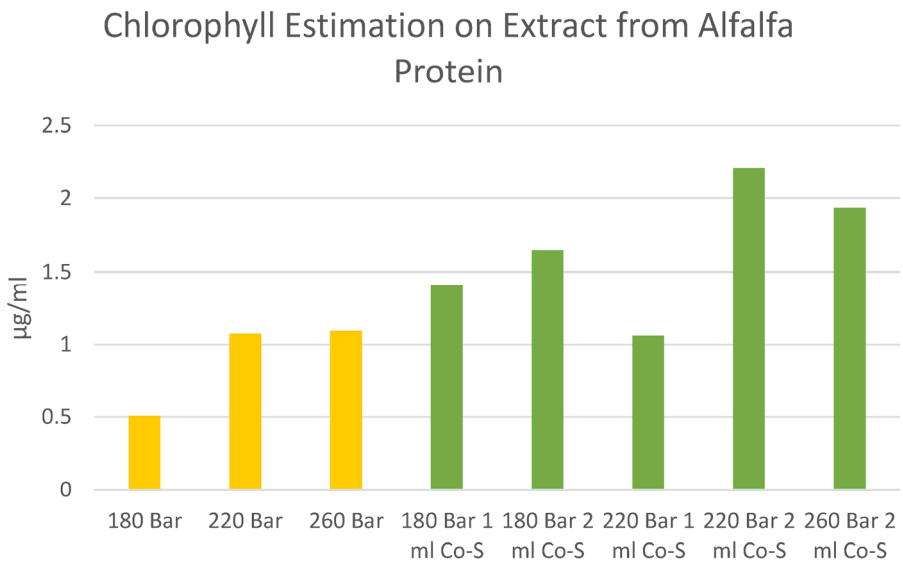

Figure A2. Chlorophyll concentration in the extract from supercritical CO_2 extraction of alfalfa protein. Yellow bars were run without co-solvent and had none to mild grass aromas, green bars were run with co-solvent and all had a strong grass aroma.

Appendix C

Table A1. Particle size distribution of WS after crushing in mortar and pestle, then sieving (Plansifter MLUA, Bühler, Beilngries, Germany, $n = 1$).

Size (mm)	>1.25	>1.00	>0.50	>0.25	>0.125	>0.00
Percentage (%)	0.00	6.62	12.79	46.04	21.25	13.30

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CHAPTER 5

Use of Side streams from the Production of White Protein Concentrate

5.1 Introduction

When producing foods all potential side streams should be taken into consideration as new raw material sources. By regarding all side streams as potential value streams, the potential CO_2 emissions from a food production might be lowered. With respect to production of white alfalfa protein concentrate there are two major side streams:

- Pulp
- Brown Juice

In this chapter the experimental work that have been done during the PhD project will be presented. It should be noted that much of the work presented in this chapter has not undergone peer review, however it still lives up proper scientific practices.

5.2 Pulp

5.2.1 Introduction

Depending on processing method, around 14-25% of pulp is being produced pr. kg of raw alfalfa, following a 2 press method as suggested by *Hansen et al 2022* (1). The pulp consist mainly of fibers and smaller amount of protein, fat and ash and it has recently been reported that alfalfa pulp can be used with advantage as a substrate for growing oyster mushrooms (2) and others have reported its potential as a feed-stock for ruminants (3). Other potential uses for the pulp could be hydrolysing the fiber fraction into free carbohydrates to be used as a second generation feed stock or using it as bio-composite in a bio-polymer. In the following section a manuscript will be presented where the author of this thesis and Efthymios Siamos have investigated incorporating alfalfa pulp from protein production into a PLA bio-composite.

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Mechanics of PLA reinforced with pulp from an alfalfa protein production

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Abstract: In this study the mechanics of PLA reinforced with pulp from an alfalfa protein production have been investigated. Biocomposites was produced containing alfalfa pulp powder in concentrations from 0-50% in 5% intervals. It was clearly demonstrated that increasing the concentration of alfalfa pulp in the biocomposites lowered its tensile strength (from 64.54 MPa (blank) to 38.48 MPa (50%), lowered its elongation properties (from 4.11 % (blank) to 1.63 % (50%)) and increased its initial modulus (from 2.38 GPa (blank) to 2.95 GPa (50%)). The results obtained limits alfalfa pulp usage as a biopolymer used in a biocomposite and suggest that future studies should focus on pretreatment of the pulp to optimize its usage.

Keywords: Alfalfa; Medicago sativa; Pulp; Biocomposites; PLA

1. Introduction

One of the greatest environmental challenges to resolve nowadays, is the pollution caused by plastic packaging. Most plastics, 60% of all plastic products, have a usage phase of 1 to 50 years, resulting in unmatched quantities of collected plastic waste and plastic production. The demand for packaging materials represents the largest end use application of plastics and one of the major contributors for plastic waste ending up in landfills and the oceans [1]. One way to tackle this problem is by developing new, alternative, biobased plastic solutions by utilizing new side streams, thus increasing the sustainability of the plastic production process and potentially the biodegradability of the produced biobased polymers.

There has been particular interest in the past few years for the development of these alternative solutions, usually produced by incorporating or reinforcing a biopolymer matrix with natural, renewable, and biodegradable filler materials. The production of such biocomposites can be achieved with the use of current plastic processing technologies, such as melt mixing, thermal extrusion, and injection molding. In this project, the fabrication of the biocomposites was achieved with the use of thermal extrusion, where the polymer matrix was melted in the appropriate conditions and a natural filler material was fed into the twin screw extruder, gradually getting homogeneously mixed and extruded into a biocomposite filament or pellets [2].

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To fabricate such biocomposites, a biodegradable, biopolymer matrix derived either-from renewable resources e.g., residual starch from the food industry to produce thermo-plastic starch biocomposites, or by a biopolymer matrix synthesized through biochemical processes such as Poly Lactic Acid (PLA)[3], [4]. PLA is a bio-based, biodegradable polymer that has various commercial applications, with similar processing properties to the fossil-based polymers, with great melting processability, high stiffness and transparency, making it an ideal candidate for compatibilizing with natural, fiber rich materials. Additionally, wide application of PLA in producing new biocomposites for packaging materials could significantly reduce the plastic waste impact on the environment, due to its increased biodegradability compared to the fossil-based alternatives. Furthermore, the incorporation of natural, fiber rich, filler materials could potentially increase its biodegradability, impart desirable mechanical and thermal properties, and significantly reduce the production cost of the packaging materials, creating revenue alongside a more sustainable and cleaner future[5]–[7].

The market for green proteins is in a significant rise currently, resulting in an increased side stream production of pulp. It has already been demonstrated that pulp can be used as feed or for biofuels. Meanwhile there is an increasing demand for biodegradable polymers in the market.

Alfalfa (*Medicago sativa*), also called the queen of forages, is a perineal plant grown across the world for forage feed[8]. It is known for its high protein yield and its high resilience towards pests and is one of the candidates in the emerging green protein market[9], [10]. In Denmark two factories are expecting to start production in 2021, where they process the alfalfa by pressing the raw material, resulting in two fractions, a protein rich green juice, which will not be discussed further in this article, and a fiber rich pulp[11], [12] . Alfalfa pulp from varies in its composition depending on both the processing method and seasonal variances since it is harvested more than 3 times pr. year [13]–[15].

Due to the future massive production of alfalfa pulp, other utilizations of this material should be investigated to maximize its usage and value.

In this study we investigate the effect of alfalfa pulp, as a bio-compositefiller material in concentrations from 0% to 60% in a PLA matrix, with the use of a twin-screw extruder, with respect to its thermomechanical properties.

2. Materials and Methods

2.1. Raw material

Polylactic Acid/PLA beads, Ingeo™ Biopolymer 2003D (NatureWorks LLC, USA) with a Melt Index of (210 °C/2.16 kg) = 6 g/10 min, was used.

Pulp was obtained by pressing whole alfalfa 3 times through a single screw press (Vincent CP-4, Vincent Corp. Florida, USA). After the 1st and 2nd press, the pulp was rehydrated in water before being pressed again. From the pulp samples were withdrawn for further analysis. The remaining pulp was transferred after pressing into an oven 70 °C for 24 H. The dried pulp was then blended in a home kitchen blender before separated in a universal laboratory sifter (Buhler, Switzerland) and divided into fractions of >1,25 mm,

>1,00 mm, >0,5 mm, >0,25 mm, >0,125 mm and <0,125 mm. Particles between 0,5mm and 0,25 mm were chosen and was manually mixed with the bag method, together with PLA beads, in ratios ranging from 0 to 45 % dried pulp content, with an increment of 5%. Subsequently, the mixed samples were placed in a vacuum oven (Thermofisher, Germany), at 70 °C for 8h, before the extrusion process.

2.2. Pulp Analysis

Dry matter was found by placing the pulp in dry crucibles in an oven at 104 °C for 24 H and comparing the weight before and after drying. Ash was determined by placing the dried samples in a muffle furnace at 600 °C for 24 H and comparing their weights before and after incineration.

Protein was determined through the DUMAS combustion method (rapid MAX N exceed, elementar, Germany) with a protein factor of 6.25.

Fat content and fiber composition was obtained according to ISO: 13906:2008. Detailed description will follow experiments has been done.

2.3. Extrusion

The fabrication of the biocomposites was done in a twin-screw extruder, Thermo Fisher Scientific, Rheomex PTW16, with a screw diameter of 16mm and L/D equal to 25 (Thermofisher, Germany). The screw alignment and the heating zone temperatures can be seen in Figure 1 below. The samples were compounded at 50rpm, due to the high load on the extruder motor caused by the high viscosity of the biocomposite melt, and then extruded through a 5 mm rod die and air cooled. The produced filament was cut and stored in polymer bags until used further, to produce dog-bone like, molded samples according to the method ASTM D638.

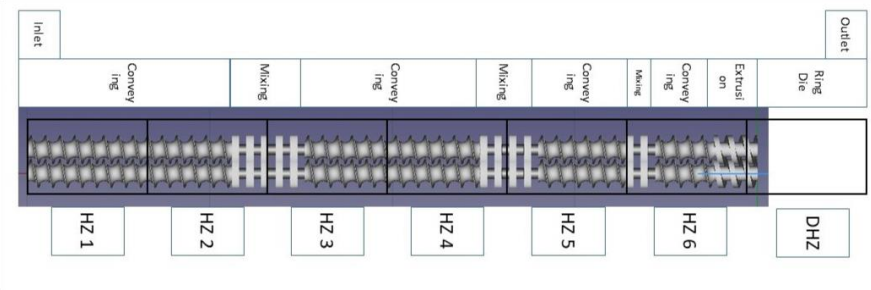

Figure 1 Extruder screw profile used in the experiment, HZ1-6=Heating zones, DHZ=Die Heating Zone (*Figure created in FreeCAD 0.19*).

2.5. Injection Molding

The cut filament was further chopped in a kitchen blender, for 60 seconds, in pulses of 3 seconds followed by 2 second stoppage to reduce the heat effect of the chopping process. Afterwards, the biocomposite beads were transferred in the handheld heating vessel of the injection molding equipment, HAAKE MiniJet Pro (Thermofisher, Germany), to melt for 60 sec, before injected to the mold. The injection process was according to ASTM D638 method, with a Type IV die with a temperature of 60 °C, at an Injection pressure of 400 bars and temperature of 210 °C, for 5 sec, followed by 150 bars of post injection pressure for 5 sec. The injected dog-bone samples were air blown and cooled and stored in PE plastic bags for further analysis.

3. Results

All results being presented with standard deviation (STD) in this section are done ass triplicates unless otherwise stated.

3.1 Raw Material Preparation

The biggest proportion of pulp particles was in the range of 0.5mm>0.25 mm>0.125mm with 665.4 grams as shown in Table 1. This was the particle size chosen for further treatment as a filler. The drymatter content was around 40% in the pulp and it had 20% protein content as seen in Table 2.

Table 1 Distribution of pulp particle size after sieving. All results expressed in g.

Total Dry Pulp	**>1.25 mm**	**>1.00 mm**	**>0.5 mm**	**>0.25**	**>0.125**	**<0.125**
1823.4	119.8	98.9	352.2	665.4	335.8	251.3

Table 2 Alfalfa pulp composition. Protein factor of 6.25 used as protein factor, n=3.

DM%(STD)	**Ash(STD)**	**Protein%(STD)**
39.71(0.86)	4.70(0.19)	19.14(0.58)

3.2 Biocomposite production

In the first pass of extruding, when the concentration of alfalfa pulp increased the torque on the motor increased as well. When passing the biocomposite through a second time the torque was consistently low.

As illustrated in Figure 2 PLA molds with 30% alfalfa pulp (top) and 10% alfalfa pulp (bottom)Figure 2 the color of the biocomposite went from dark green into towards deep brown as the concentration of alfalfa pulp increased in the blend.

Figure 2 PLA molds with 30% alfalfa pulp (top) and 10% alfalfa pulp (bottom)

3.3 Mechanical Properties

The tensile properties of the produced biocomposites were measured, evaluating the effect of the pre-treated alfalfa filler powder percentage in PLA-alfalfa biocomposites, in comparison to samples prepared with pure PLA and extruded PLA. The tensile properties: Initial Modulus at 0.1 % strain (GPa), Tensile Strength (MPa) and % Elongation of the produced samples are presented in Figure 3 (Full data can be seen in the Appendix).

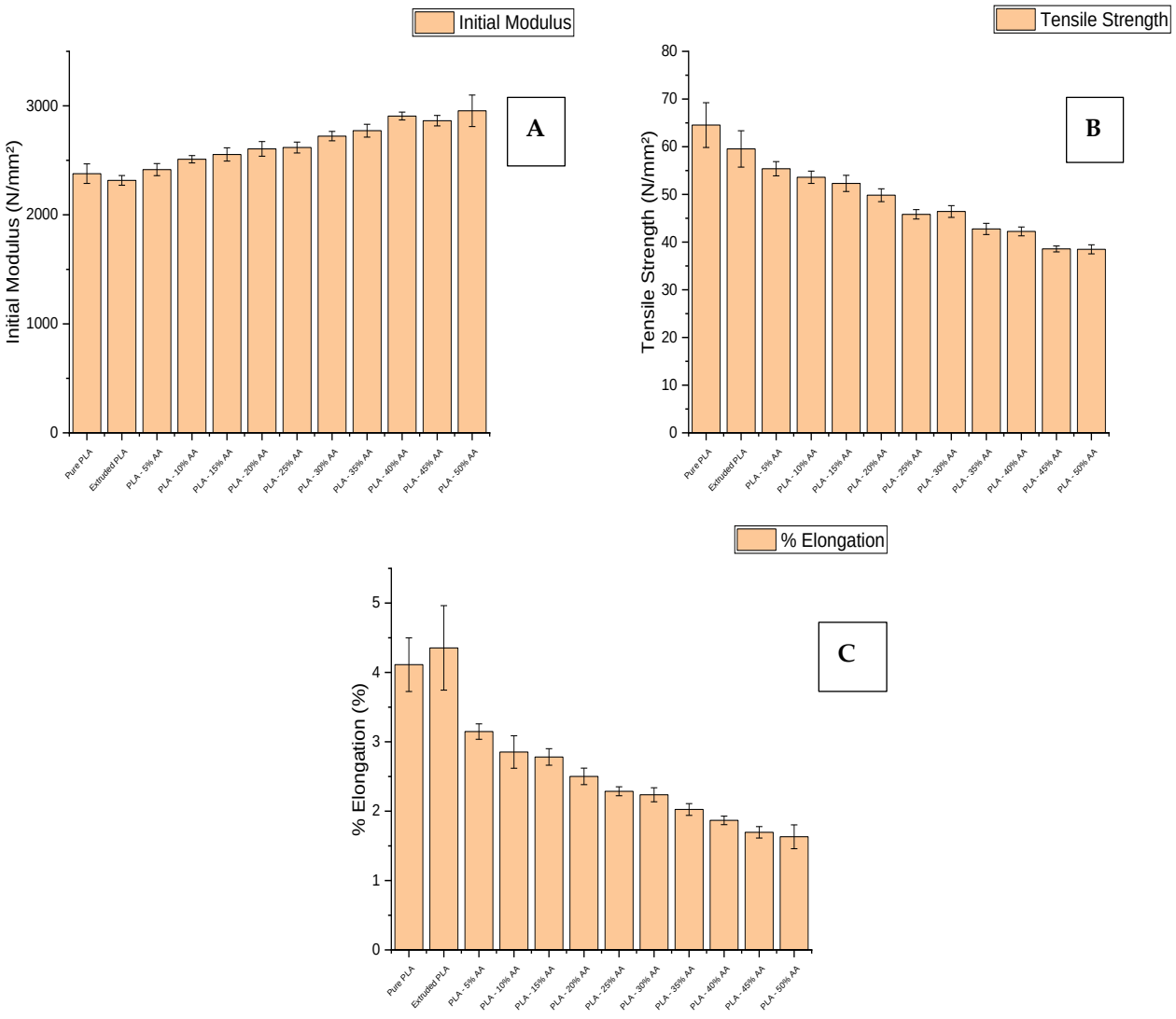

Figure 3 Initial Modulus (A) , Tensile Strength (B) and % Elongation (C) at break of the produced samples, AA=alfalfa pulp, n=3.

The samples that failed having a fracture in the narrow stress area of the produced dog-bones, as well as the samples that had obvious faults (e.g., bent, scratched, or filled with air bubbles), were not evaluated.

The pure PLA samples exhibits necking before fracturing, an effect that is strongly reduced in the extruded PLA samples and almost eliminated in the samples with 10% alfalfa powder or more. The addition of pre-treated alfalfa powder in a PLA matrix affected significantly all the tensile properties of the produced biocomposites, demonstrating a positive correlation of the percentage of alfalfa powder to the initial Modulus and negative for tensile strength and % elongation. All the composites, with 10% or more of alfalfa powder, showed higher moduli than PLA, increasing linearly with the increase in alfalfa powder concentration. In particular, samples with 10% alfalfa had a 5.5% increase in the tensile moduli, while samples with 20% had 9.3% higher and samples with 50% had 25% higher initial Moduli than pure PLA.

Despite the positive effect on the initial Moduli, the addition of alfalfa powder as a filler material, has a way higher magnitude decrease in the tensile strength as well as the % elongation at break, of the produced composites. Moreover, samples produced with 10% alfalfa as a filler, demonstrated 16% lower overall tensile strength and slightly more than 30% decrease in the % strain elongation before breakage. Samples produced with 50% alfalfa powder, demonstrated as low as 40% less tensile strength and up to 60% lower strain resistance before breakage, compared to the pure PLA samples. The magnitude of the effect seems to be reduced with the increase of the filler material, for concentrations higher than 30%. The reduced tensile strength and % elongation of the biocomposites, could be attributed to reduced adherence of the filler powder to the PLA matrix.

4. Discussion

4.1 Raw Material and its preparation

As demonstrated by *Morales et al.,* the composition of the filler has a huge impact on the final biocomposite [7]. It is well known that the composition of both the raw alfalfa and its pulp is affected by both seasonal variances and processing methods [13], [14].

However, in the context of this paper, we have focused on utilizing an existing side-stream from a production of green proteins for human consumption. Potential upstream processing of the pulp and the effects it may have on its properties as a biocomposite-filler are not discussed in this paper.

The major composition of the pulp used in this study was within the ranges previously described by *Knuckles et al.*.

In this study, only one particle size was investigated as a filler due to the amount available. Having a more efficient grinder that produced finer particles could have altered this decision.

4.2 Extrusion

Based on preliminary trials, a low rpm was chosen to ensure that the torque on the motor of the extruder remained within its limits. Throughout the first passing of PLA and alfalfa pulp, we observed an increase in the torque following the increase in the concentration of pulp. However, we observed a lower torque throughout the second passing of the biocomposite in the extruder. This suggests that having higher rpms when passing the biocomposite a second time should be possible in the future.

4.3 Injection Molding of PLA with alfalfa pulp as a filler

Incorporating cellulosic filler materials in PLA is well studied with respect to new packaging materials. Thorough investigations have been performed for various cellulosic materials such as Posidonia Oceanica(PO), wood particles or hemp [16]–[18]. Similar results have been presented by *Fiore et al*. when producing PLA composites reinforced with Arundo donax fillers [19]. They noted a strong increase in the tensile moduli, with detrimental effects on the tensile strength, % elongation and toughness of the produced composites. In their study, samples with filler concentrations af 10 and 20% demonstrated 24% and 35% lower tensile strength, respectively, than PLA, compared to 16 and 23% found in our study. Furthermore, alfalfa as a filler material seems to adhere better than acid detergent fiber, to the PLA matrix, as the decrease in the % elongation was significantly lower in our study [19]. Necking was also exhibited in the studies by Scaffaro et al. and Fiore et al. for the pure PLA samples and samples up to 10%, effect that the magnitude is reduced in all the studies for concentrations higher than 10% in filler material. Scaffaro et al. reported similar results with an increase in the tensile moduli and a decrease in the tensile strength and % elongation at break, even though the magnitude in their study was lower, as PO fibers seemed to be incorporated more strongly in the PLA matrix. They reported a decrease in tensile strength of 26% and 23% for composites produced with 10 and 20% PO fibers, respectively, and a decrease of 33 and 35% in % elongation compared to pure PLA samples. In both studies, the researchers increased the filler concentration up to 20%, without investigating the effect of higher concentrations. In our study, similar values of tensile strength (~39 MPa) and elongation at break were obtained, even though 60% more filler material was used in our case. This may have potential applications as the greater the amount of filler material used, the lower the production cost, although a more detailed investigation is required. In contrast, Smoca et al. investigated the effect of hemp fibers on reinforced PLA composites and noted that the tensile strength and elongation at break significantly increased with an increase in fiber content up to 40%, while there was a slight decrease for higher concentrations (up to 70%), even though samples with 70% hemp fibers were significantly stronger than pure PLA samples. In their study, both tensile strength and modulus increased in a nonlinear way, as the hemp fiber content increased up to 40% and decreased afterwards.

5. Conclusion

In the conditions described in this study it was demonstrated that incorporating alfalfa pulp power as a biofiller in PLA biocomposite, has a negative effect on it thermomechanical properties. This negative effect increased as the concentration of alfalfa pulp powder increased. Future research on alfalfa powder as filler material should investigate the effect of the particle size for composite production, addition of additives, or fiber pretreatment with novel technologies to increase the adherence of the filler material on the PLA matrix.

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Appendix

Table A Properties of the biocomposites produced in the study, n=3.

Sample	Initial Modulus *GPa*			Tensile Strength *MPa*			% Elongation		
Pure PLA	2.38	±	0.09	*64.54*	±	*4.70*	*4.11*	±	*0.39*
Extruded PLA	2.32	±	0.04	*59.54*	±	*3.81*	*4.35*	±	*0.61*
PLA - 5% AA	2.42	±	0.06	*55.39*	±	*1.49*	*3.15*	±	*0.11*
PLA - 10% AA	2.51	±	0.03	*53.61*	±	*1.29*	*2.85*	±	*0.23*
PLA - 15% AA	2.55	±	0.06	*52.31*	±	*1.70*	*2.78*	±	*0.12*
PLA - 20% AA	2.60	±	0.07	*49.83*	±	*1.35*	*2.50*	±	*0.12*
PLA - 25% AA	2.62	±	0.05	*45.82*	±	*0.98*	*2.29*	±	*0.06*
PLA - 30% AA	2.72	±	0.04	*46.42*	±	*1.23*	*2.24*	±	*0.10*
PLA - 35% AA	2.77	±	0.06	*42.75*	±	*1.18*	*2.02*	±	*0.09*
PLA - 40% AA	2.91	±	0.04	*42.24*	±	*0.93*	*1.87*	±	*0.06*
PLA - 45% AA	2.86	±	0.05	*38.56*	±	*0.62*	*1.70*	±	*0.08*
PLA - 50% AA	2.95	±	0.14	*38.48*	±	*0.95*	*1.63*	±	*0.17*
R^2	0.96475			0.96561			0.87712		

5.3 Brown Juice

5.3.1 Introduction

Brown juice obtained from APC and WAPC production is known for containing vitamins, soluble proteins and free carbohydrates, thus making it a potential candidate as a fermentation medium(4; 5; 6). Due to the usage of lactic acid for pH precipitation, in the production of WAPC, as described in chapter 4, finding new organisms with the ability to metabolise lactic acid is advisable when fermenting brown juice. In the following study the author of this thesis and Suvasini Balusubramanian have investigated different organisms for the valorization of brown juice from the production of WAPC.

MDPI

Article

Utilization of Brown Juice from Alfalfa (*medicago sativa*) protein production as a fermentation medium

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Abstract: In this study using brown juice, a side stream from alfalfa (*Medicago sativa*) protein production, as a fermentation medium, was investigated. First a screening trial was conducted where 4 different organisms was fermented in brown juice, with or without addition of 1% glucose. From the screening trial it was found that *Corynebacterium glutamicum*-ATCC-13032 (wildtype) was the best candidate. Secondly a growth-test with both the wildtype and a GMO strain *Corynebacterium glutamicum*-SB025, was done in brown juice with or without addition of 0.5% yeast extract and 1% glucose. After 24 h of growth *Corynebacterium glutamicum*-ATCC-13032 had a final OD_{600} of 44.6 (μ_{max} 0.55) in brown juice without supplements and an OD_{600} of 45.45 (μ_{max} 0.70) in the brown juice with 0.5% yeast extract and 1% glucose. The GMO strain, *Corynebacterium glutamicum*-SB025, grew to an final OD_{600} of 29.35 (μ_{max} 0.47) in the unsupplemented brown juice and a final OD_{600} of 50.8 (μ_{max} 0.545) in the brown juice with 0.5% yeast extract and 1% glucose. It was concluded that brown juice is a promising fermentation medium for *Corynebacterium glutamicum*, but further studies are needed to optimize the process and investigate the metabolism occurring while fermenting.

Keywords: alfalfa; medicago sativa; brown juice; side stream; corynebacterium glutamicum

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1. Introduction

As the the global population increases, so does the demand for food. Currently many new plant based protein sources are being investigated to meet the growing demand for food, while maintaining or lowering the total emission of green house gasses[1,2]. As a consequence of the many new food sources being developed, many new side streams are being generated as well, which needs to be utilized to maintain or lower the carbon footprint generated from the new food sources. In this study we focus on the side stream brown juice (BJ), generated during alfalfa protein production. BJ is a brown supernatant, generated after precipitation of the protein rich pellet, when producing either white alfalfa protein concentrate or green alfalfa protein concentrate [3–6]. Alfalfa is a perennial plant, grown world wide as a feed for ruminants, pigs and hens. In the last decades alfalfa has been of increasing interest as a new source of protein for human consumption. This is due to its amino acid composition, which contains all essential amino acids, and its high protein yield pr. acre of land. Alfalfa is typically harvested 4-5 times pr. year and is typically ensilaged or transported directly to a biorefinerey for protein extraction. Extraction is typically carried through a mechanically separation of green juice (protein rich wet fraction) and the pulp (fiber rich solid fraction). The green juice are further processed to extract its proteins and hereafter dehydrated by centrifuging[5,7–9].

BJ is composed of soluble nitrogen, vitamins, flavonoids and carbohydrates, where the distribution depends on variety, harvest time and processing parameters [10–12]. BJ

can be used as fertilizer or in biogas production and due to its compositione many studies have investigated using it for production of L-lactic acid through fermentation[3,5,12,13]. In this study a BJ obtained from a white alfalfa protein concentrate production has been used. In this production a combination of heat and lactic acid has been used to precipitate the protein, resulting in a BJ with relatively high concentration of lactic acid [6]. Thus investigating organisms known to metabolize lactic acid would be relevant to increase the value of BJ in the future.

2. Materials and Methods

2.1. Pasteurization trials

Prior to the experiments described below a pasteurization trial was conducted to investigate the optimal pasteurization unit (PU) for the BJ. It was found that a PU of 12 (70 °C, 54 s) was enough to ensure a CFU count of 0 at LB-agar plates (30 °C, 48h). However after pasteurization of the BJ both with a PU of 12 an higher no growth of organisms was observed when using the pasteurized BJ as fermentation medium. Interestingly, organisms grew well in non pasteurized sterile filtered BJ (0.45 µm). Which was why further trials with BJ was conducted with sterile filtered BJ.

2.2. Brown Juice Medium preparation

Brown Juice was obtained from a white alfalfa protein concentrate production, as described by Hansen et al 2023 [6]. The brown juice was collected and stored at -22 °C until needed. Before use, the brown juice was thawed in a 20 °C water-bath and was afterwards centrifuged (2500 g, 20 min, °C (Thermo Scientific Multifuge X3R, Waltham, USA)) to remove impurities. The pellet was then discarded and four different medium was formulated as listed below (All reagents was purchased from Sigma-Aldrich,Darmstadt, Germany):

- BJ control - Without additives
- BJ w. 1.0% Glucose
- BJ w. 0.5% Yeast Extract
- BJ w. 1.0% Glucose and 1% Yeast Extract

After addition of nutrients, the pH was altered to 7.0 with 1M NaOH, followed by a sterile filtration (0.45 µm).

2.3. Fermentation setup

The day before fermentation the given organism was grown overnight in an incubator in a shake flask (280 rpm, 32 °C) in the given medium the fermentation would occur in. 100 ml was transferred to 250 ml screw cap glass bottles containing a stirring magnet, under sterile conditions, before placing the glass bottles in a water bath (Julabo SW-20C, Germany) with a magnetic stirrer (150 rpm, 32 °C). The respective medium was left in the water bath for 10 minutes before inoculation to ensure uniform temperature in the mediums.

OD_{600} was then measured in the respective mediums against a blank containing demineralised water in a spectrophotometer (Genesys 10S, Thermo Fischer Scientific, Massachusetts, USA), OD_{600} was then measured in the inoculate against a blank containing demineralized water and subtracted the OD_{600} of the respective mediums. The inoculate was then transferred to the different glass bottles in a volume corresponding to a starting OD_{600} of 0.1 .

Just after inoculation samples was withdrawn for OD_{600} measurement as describe above and subsequent samples for OD_{600} measurement was withdrawn every half hour. When OD_{600} was measured above 0.9 the samples was diluted with their respective medium 10 fold.

In total 16 fermentation trials was carried out. Fermentation 1-8 was carried out in singles and 9-16 in duplicates. The first eight was a screening growing 4 different wildtype organisms in BJ with our without addition of glucose. This was followed by 8

more trials with a wildtype *C. glutamicum* and GMO *C. glutamicum* secreting α-amylase using various media formulations. Full overview of the various fermentations can be seen in table1

Table 1. Overview of the diffrent fermentations carried out in the study.

Fermentation number	Organism	Medium
1	*C. glutamicum*-ATCC-13032	BJ
2	*C. glutamicum*-ATCC-13032	BJ (1% Glc.)
3	*E. coli*-DH5α -AH236	BJ
4	*E. coli*-DH5α -AH236	BJ (1% Glc.)
5	*B. subtilis*-168-CS	BJ
6	*B. subtilis*-168-CS	BJ (1% Glc.)
7	*L. lactis*-Mg1363-CS3502	BJ
8	*L. lactis*-Mg1363-CS3502	BJ (1% Glc.)
9	*C. glutamicum*-ATCC-13032	BJ
10	*C. glutamicum*-ATCC-13032	BJ (1% Glc.)
11	*C. glutamicum*-ATCC-13032	BJ (0.5% YE)
12	*C. glutamicum*-ATCC-13032	BJ (1% Glc.+0.5% YE)
13	*C. glutamicum*-SB025	BJ
14	*C. glutamicum*-SB025	BJ (1% Glc.)
15	*C. glutamicum*-SB025	BJ (0.5% YE)
16	*C. glutamicum*-SB025	BJ (1% Glc.+0.5% YE)

2.4. Brown Juice Characterization

Soluble carbohydrates and organic acids in the BJ before and after fermentation trials (1-8) was measured using an Ultimate HPLC (Dionex, Hvidovre, Denmark) with an aminex HPX87H column (Biorad, Hercules, Canada) held at 60 °C and a Shodex RI-101 detector (Showa Denko K. K., Tokyo, Japan). A flow rate of 0.5 ml/min was used for the mobile phase (5mM sulfuric acid) and an injection volume of 20 µl was used. All samples was filtered through sterile filter (0.2 µm) before analyses. Results was quantified using the Chromeleon 2.0 software (Themofischer, Boston, USA)

3. Results

3.1. Screening of suited organisms

As illustrated in figure 1 A & B *C. glutamicum* grew above 15 OD_{600} within 8 h of growth, in both pure BJ and BJ with 1% Glucose. *B. subtilis* grew to 23.2 OD_{600} after 24 h in BJ medium supplemented with 1% glucose. As shown in table 2 those were the only two organisms that grew above 3 OD_{600}. From the HPLC analysis, shown in figure 2, it was observed that *L. lactis* increased the lactic acid concentration with 1% glucose supplemented, but had a decrease in lactic acid concentration without glucose. *B. subtilis* did not metabolise fructose when 1% glucose was supplemented to the BJ. *C. glutamicum* metabolised all fermentables measured without the addition of glucose, and did not metabolize all of the lactic acid (542 µg/mL) and acetic acid (26 µg/mL) after 24h of fermentation, when 1% glucose was added to the BJ. Since *C. glutamicum* was the only organism that grew well in both glucose supplemented and pure BJ, it was chosen as the best suited candidate for further testing.

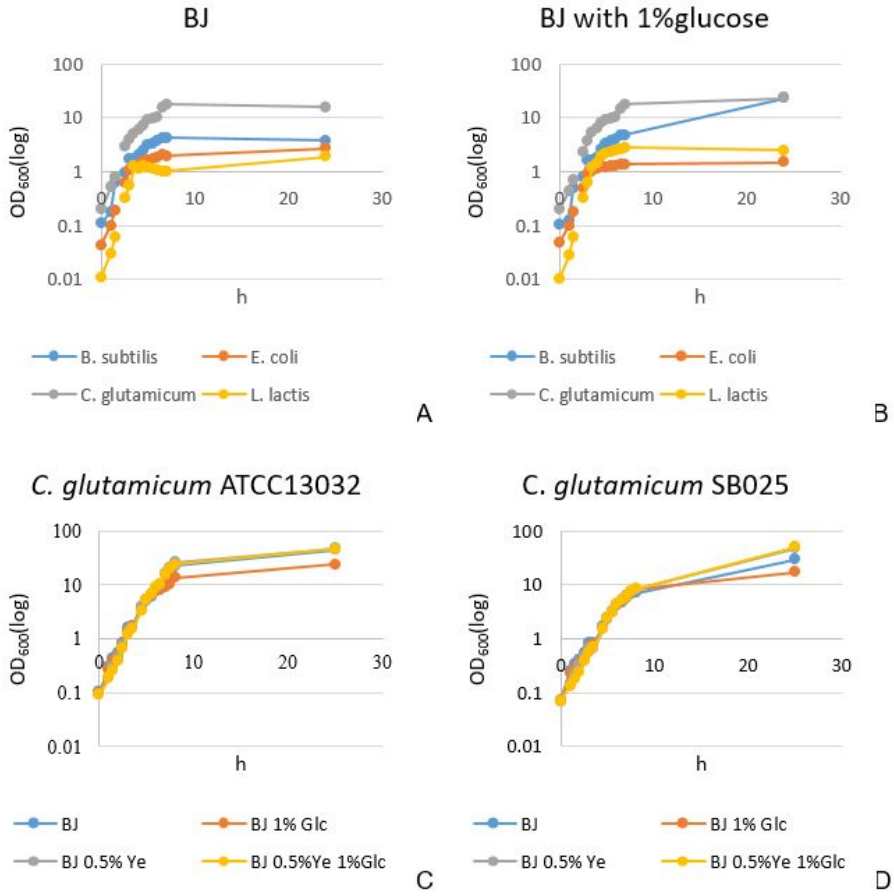

Figure 1. Growth curves for selected organisms in various brown juice based mediums. (**A**) Growth of *B. subtilis, E. coli, C. glutamicum* and *L. lactis* in pure brown juice. (**B**) Growth of *B. subtilis, E. coli, C. glutamicum* and *L. lactis* in brown juice with 1% glucose. (**C**) Growth of *C. glutamicum* ATCC 13032 (wildtype) in brown juice with various additives.(**D**) Growth of *C. glutamicum* ATCC SB025 (GMO) in brown juice with various additives. BJ=Brown Juice, YE=Yeast extract, Glc=Glucose.

Table 2. Fermentation of various organisms in BJ w/wo addition of 1% glucose, n=1.

No.	Organism	Medium	lag phase (h)	μ_{max}	Max OD_{600}
1	*C. glutamicum*-ATCC-13032	BJ	1	0.369	17.9
2	*C. glutamicum*-ATCC-13032	BJ (1% Glc.)	1	0.322	23.3
3	*E. coli*-DH5α -AH236	BJ	1	0.158	2.7
4	*E. coli*-DH5α -AH236	BJ (1% Glc.)	2	0.209	1.5
5	*B. subtilis*-168-CS	BJ	1.5	0.367	3.8
6	*B. subtilis*-168-CS	BJ (1% Glc.)	1.5	0.323	23.2
7	*L. lactis*-Mg1363-CS3502	BJ	2.5	0.667	2.8
8	*L. lactis*-Mg1363-CS3502	BJ (1% Glc.)	2.5	0.348	1.9

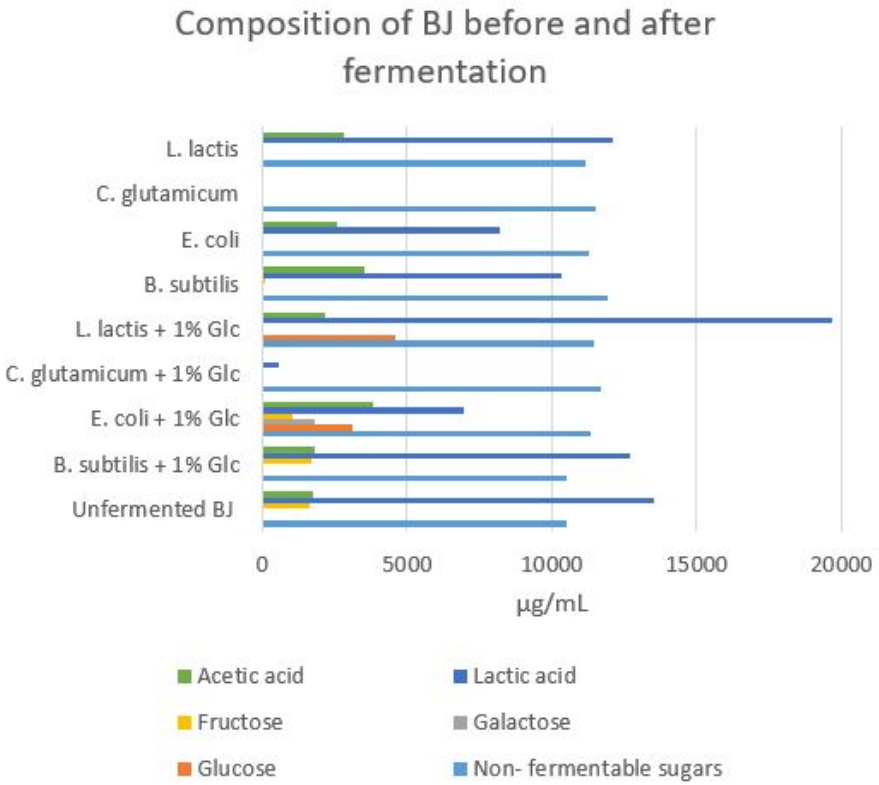

Figure 2. Composition of BJ before and after fermentation trial 1-8 with or without addition of 1% Glucose.

3.2. Optimal Brown Juice for growth of C. glutamicum

In this growth test it was investigated if supplementation of extra nutrients would benefit growth of *C. glutamicum*. In this test a GMO strain of *C. glutamicum* was included (SB025) as well as the wild type (ATCC-13032). As illustrated in figure 1 C & D both the wild type and GMO strain grew to an OD_{600} above 15 in all mediums. The highest OD was observed at 50.8 in fermentation number 16, where the BJ was supplemented with both yeast extract and glucose growing *C. glutamicum*-SB025. It was observed that both the wildtype and GMO strain had an lower OD after 24 h of growth with addition of only glucose and no yeast extract.

Table 3. Fermentation results from growing *C. glutamicum* SB025 and ATCC-13032 in BJ w/wo additives, n=3. .

No.	Organism	Medium	μ_{max}	Max OD_{600}
9	*C. glutamicum*-ATCC-13032	BJ	0.552	44.6 (± 1.4)
10	*C. glutamicum*-ATCC-13032	BJ (1% Glc.)	0.588	24.15 (± 2.05)
11	*C. glutamicum*-ATCC-13032	BJ (0.5% YE)	0.656	47.25 (± 2.85)
12	*C. glutamicum*-ATCC-13032	BJ (1% Glc.+0.5% YE)	0.702	45.45 (± 0.75)
13	*C. glutamicum*-SB025	BJ	0.469	29.35 (± 1.25)
14	*C. glutamicum*-SB025	BJ (1% Glc.)	0.504	17.1 (± 1.1)
15	*C. glutamicum*-SB025	BJ (0.5% YE)	0.545	47.65 (± 4.95)
16	*C. glutamicum*-SB025	BJ (1% Glc.+0.5% YE)	0.545	50.8 (± 0.8)

4. Discussion

Even though heat pasteurization trials were carried out only sterile filtrated BJ was fermentable by the organisms investigated in this study. Sterile filtration is a costly process compared to heat pasteurization and future studies should investigate other

pasteurization techniques to make fermentation with BJ economical attractive. In the screening trials, fermentation 1-8, done in this study, *C. glutamicum* performed better both with respect to growth and metabolism. However the screening was only done with single trials and future studies should include triplicates to confirm the results. For all trials (1-16), the fermentation was terminated after 24 h. Since residual fermentable content was observed in the HPLC analysis after 24 h, it would advisable that future studies continue fermentation until constant OD is observed.

In fermentation 9-16 no HPLC analysis was done on the fermented brown juice. Since no data was generated to illustrate the metabolism in trial 10 and 14, it would be wrong conclude that addition of glucose without surplus nitrogen limits the growth of *C. glutamicum* in BJ medium. Future studies should include HPLC analysis before, during and after fermentation to get insights into the mechanisms causing the lower OD after 24 h for those.

Comparing trial 9 and 12, where the wildtype of *C. glutamicum* was grown with pure BJ and supplemented with both glucose and yeast extract an OD difference of 1 and growth rate of 0.15 points higher in favor of the supplemented medium, was observed. However the same pattern was not observed with the GMO strain where only the nitrogen supplemented BJ fermentations, ended with a final OD above 40. This suggest that extra nitrogen when fermenting SB025 with BJ as medium is required for optimal growth. Future studies should include other nitrogen sources than yeast extract to investigate this further.

5. Conclusion

Due to high concentration of lactic acid present in brown juice from the production of alfalfa protein, growing lactic acid metabolising organisms is favorable to utilize the brown juice presented in this study when fermenting. The study demonstrates that *C. glutamicum* grow well in brown juice medium from alfalfa protein production, with a final OD of 44.6 without additional supplements. Thus we have demonstrated a simple method for producing a high concentration of *C. glutamicum*, that might be used as a single cell protein for humans, when using pure brown juice as a fermentation medium. However more studies are needed to make it scale-able into full scale production.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 6

Novel Food Application

6.1 Introduction

As mentioned in chapter 2 alfalfa protein concentrate was approved as a novel food in 2009, to be used as a food supplement with an intake of up to 10 g pr day. In this chapter an application to EFSA addressing this challenge is presented. Based on the data presented in chapter 4 and the fact that supercritical CO_2 extraction is known to extract and lower many of the listed concerns with respect to consumption of alfalfa protein concentrate by EFSA (see section 2.3 and 2.5), a new application for an extended use of WAPC have been generated. Here it is proposed to approve the usage of WAPC in 18 different Foodex2 lvl7 categories. Based on the average daily intake across Europe, of those 18 different food categories, the total intake would not exceed 10 g pr day and therefore it is believed that this extended approval will pose no threat with respect to food safety.

EFSA Novel food approval of white alfalfa (*Medicago Sativa*) protein concentrate as a substitute for egg in certain Foodex2 lv7 categories

Alfalfa (*Medicago Sativa*) has been grown across the world for centuries and considered the oldest cultivated feedstock in the world. Historical the whole alfalfa plant has been consumed in Asia, North and South America, whereas in the European market it is mainly the sprout that been consumed [1].

In the "Opinion on the safety of 'Alfalfa protein concentrate' as food" in the EFSA journal (2009) 997,1-19 is was concluded that using alfalfa protein concentrate (APC) as food supplement in concentration of 10 g pr. day was of no concern with respect to safety[2]. The main concerns, for the member states, with respect to consumption of APC was the content of coumestrol, L-canavanine and β-carotene and the potential allergenicity of the product. In this application we propose to include a refined APC (white protein concentrate (WAPC) as a food ingredient, substituting egg protein in certain processed foods where the egg protein concentration is lower than 2%.

Alfalfa

Cultivation of Alfalfa

Alfalfa (*Medicago Sativa*) is a perennial plant that is regarded to be oldest forage cultivated solely for feed purposes. The name is derived from the Iranian word "aspati" meaning horse fodder. It belongs to the genus of Medicago in the large group of leguminosae, where *Medicago sativa* is the purple flowered alfalfa. It has a high protein pr. acre concentration and can be harvested 3-5 times pr. season depending variety and climate conditions. Alfalfa has a deep root network, which minimizes its needs for irrigation, and it is naturally resistant towards most common pests in the agro industry. Due to its ability to fixate nitrogen, it is often sown to increase the nitrogen content of the soil[1], [3].

Consumption of Alfalfa

Alfalfa is has been consumed for decades in Russia, America, Asia, Africa and to some extent Europe. In Asia some farmers has treated alfalfa as a vegetable and in South Africa alfalfa leaves has been used to substitutes spinach in salads. In Europe the most common usage of alfalfa is its sprouts which are used in salads or sandwiches [1].

Consumption of Alfalfa Protein Concentrate in EU

The commission of the European communities decided the 13th of October in 2009 that alfalfa protein concentrate was safe as a food ingredient in serving portions of 10 g/day. To this day, no hazards has been observed within EU due to poisoning caused by APC[4].

Studies has been carried out where malnourished infants has been given APC as a substitute for milk powder. No adverse effects was observed in these studies and it was found that the acceptance of APC was good.

Production of alfalfa protein

To minimize potential carryovers from pesticides, all aspects of the production, from farm to table, meets the standards of organic food production as described in Council Regulation (EC) No 834/2007 [5].

White alfalfa protein concentrate (WAPC) is produced by harvesting organic alfalfa (*Medicago* sativa) and is processed it within hours from being cut. After transportation, the alfalfa is being pressed through a twin-screw press resulting in two fractions; a solid fraction (pulp) and a protein rich liquid fraction (green juice). The green juice is collected and stored at <5 °C. The pulp is then rehydrated with tap water and pressed again through the twin-screw press. The resulting pulp is used as feed and the green juice is pooled together with the green juice from the first press and further processed. The temperature of the green is then quickly raised to 50-52 °C by steam injection followed by a centrifugation. The centrifugation results in two fractions; a green slurry and a protein rich brown juice. The green slurry is then used as feed and the pH of the protein rich brown juice is lowered to 3.5-4.0 by addition of lactic acid. The protein rich brown juice is then centrifuged resulting in two fractions; a liquid fraction (brown juice) and a white protein rich slurry. The brown juice is used for biogas production and the white slurry is freeze-dried to a moisture content <6% and further processed in a supercritical CO2 system, to remove off flavors. The now finished WAPC is then packaged and stored in dry and dark conditions.

The whole process is done under aseptic conditions, living up to the standards of HACCP and GMP (ISO 22000) to minimize potential spoilage of the products.

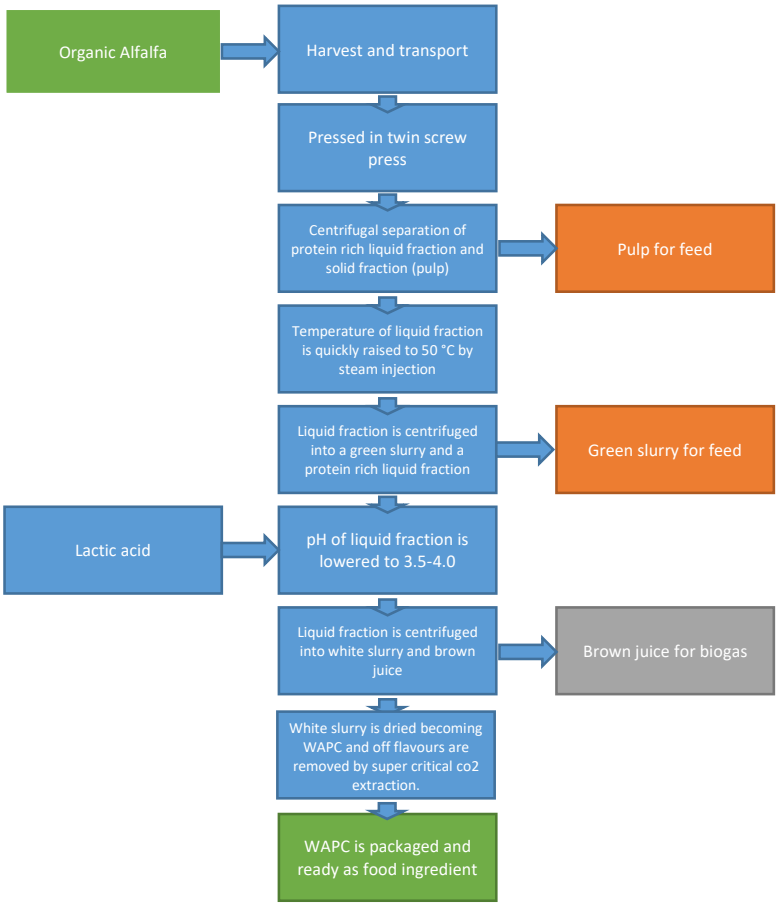

Figure 1 Production flow of white alfalfa protein concentrate for human consumption (Green=Food, Blue=Process, Orange=Feed, Grey=Biogas)

Safety considerations with respect to consuming White Alfalfa Protein Concentrate

According to the "Opinion on the safety of 'Alfalfa protein concentrate' as food" the main concern for consumption protein isolated from alfalfa was the presence of coumestrol, L-canavanine and β-carotene and the potential allergy of the product[2]. In the following chapter, considerations of these concerns will be presented.

Coumestrol

Coumestrol belongs to the group of phytoestrogens that show estrogenic activity. They was found in alfalfa and was the first phytoestrogen to be discovered in 1957. Coumestrol are found in high concentrations in sprouts from alfalfa and clover[6], [7].

Due to their stable structure and low molecular weight, they can pass the cell membrane and bind to estrogenic receptor. This may interfere with steroid metabolism and inhibit production of various enzymes[8].

It is speculated that coumestrol might prior to menopaus protect against breast cancer and after menopause induce the growth of breast cancer cells, due to the natural variance of estrogen in the cells. There are many conflicting studies on this matter and it has also been speculated that externals factors such life style, envoirement and diets affects the activity of coumestrol, when relating it breast cancer treatment[8].

According to *Knuckles et al. 1976* the coumestrol content is between 4-17 µg/g in WAPC depending on the processing method[9]. By consuming 10 g of WAPC, the consumer may have an additional intake of 0.017 mg of coumestrol pr. day. This is well within the threshold limits of 0.1 mg coumestrol pr. kg bodyweight. Furthermore previous studies have shown that supercritical CO_2 extraction of plant material lowers the potential coumestrol content[10].

Due to these reasons, using WAPC as a food ingredient with an average daily intake of 10 g, poses low risk with respect to increased coumestrol intake.

L-canavanine

L-canavanine is a non-protein amino acid and present in many leguminous plants as part of their natural defence against pests. Large consumption of L-canavanine may lead to development systemic lupus erythematosus syndrome[11]. In alfalfa, it is mainly found in the seeds (80-150 ppm) and in smaller concentration in the leaves (10 ppm). The content of L-canavanine in alfalfa is significant lower in APC (4.3 ppm) than that of other common foods such as onions (10 000 ppm)[12]. Since harvest of alfalfa for protein production has an optimum in early flowering stage, before seed production, the actual L-canavanine content is low. Therefore, it is considered that the L-canavanine is not of high concern when considering WAPC as an food ingrident with an average daily consumption of 10 g.

β-carotene

There is β-carotene natural present in alfalfa, and in the "opinion on the safety of 'Alfalfa protein concentrate' as food" they reported that a daily intake of 10 g of alfalfa protein concentrate would result in an intake of 2.2-7.2 mg β-carotene pr. day. Due to the last step of supercritical CO_2 extraction of WAPC the potential β-carotene content is lowered significant compared to APC. So having a daily intake of 10 g of WAPC as a food ingredient will not lead to increased intake of β-carotene.

Allergy

Studies has shown that mice sensitive to peanuts show slight allergic reactions, when consuming alfalfa protein concentrate. It was reported in 2008 by Jensen et al, that people with peanut allergy showed weak reactions in skin prick tests towards APC. So potential cross-link allergies between peanut allergy and allergy towards APC cannot be excluded [13].

When looking at the list of allergens in food which requires labeling in Denmark the only thing need to be considered as a potential allergen in alfalfa would be sulfite and sulphur-dioxide if the content exceeds 10 mg/kg [14]. In a study, done by T. R. Pucek & J. B. Pyin 1997, alfalfa cultivars grown in sulphur post-mining lands was studied. They found maximum levels of 0.5% sulphur (DM) in the alfalfa grown in the sulphur rich soil, so the soil quality have to be evaluated before starting a production alfalfa protein for human consumption[15].

Beside the listed allergens, Malley et al. 1974 found that albumin from the legume Pea, can cause allergic reactions in people sensitive to peas. This allergic reaction is however found limited when autoclaving the product (120 C, >15 min) [16], [17]. Since the products listed in Table 2 is subjected to heating greater than this, during processing, this should be of concern with respect to incorporating WAPC as a food ingredient in those listed foods.

Biological Stability of WAPC

Own data finds that the total CFU of the WAPC is <300 CFU/g, containing *Bacillus cereus, Bacillus subtilis, Bacillus pumilusa* and *Bacillus megaterium*, before treatment with supercritical CO_2 extraction, which in those concentrations is considered safe for human consumption. After treatment with supercritical CO_2 extraction, the CFU was 0 CFU/g of WAPC.

White Alfalfa protein concentrate composition

WAPC contains all essential amino acids and has a digestibility of roughly 75-90%, depending on the processing and its main protein RuBisCO is known for its foaming and emulsifying capabilities, thereby making it a great candidate to substitute egg protein[18], [19],[20]. Nutritional composition of the WAPC can be seen in Table 1.

Table 1 Composition of WAPC [20]

Compound	Concentration (%)	STD
Protein	63.61	0.07
Fat	0	0.0
Moisture	0.2	0.0
Ash	0	
Free carbohydrates	0.91	0.14

White alfalfa protein as a food ingredient

The current approval for APC is as a food supplement with an intake of up to 10 g/day. Due to the functionality of the RuBisCO protein, which is the main protein in WAPC, to substitute milk and egg protein we propose to include WAPC as a food ingredient in the 13 Foodex2 lv7 categories listed in Table 2 [20].

Table 2 Proposed foods where egg protein could be substituted with WAPC

Food code	Food name
A.01.05.003	Noodle, wheat flour, with eggs
A.01.07.001.006	Chocolate cake
A.01.07.001.017	Doughnuts
A.01.07.001.024	Gingerbread
A.01.07.001.032	Scone
A.01.07.001.042	Brioche
A.01.07.002.006	Butter biscuits
A.06.09.001.002	Bratwurst
A.06.09.001.004	Weisswurst
A.06.09.001.005	Bockwurst
A.10.04.014	Nougat
A.16.06.006	Mayonnaise, < 25% oil
I.01.07.001.068	Muffins, chocolate
I.16.08.003.008	Cream sauce, remoulade
I.19.01.003.018	Lasagna/Canelloni, vegetarian
I.20.02.001.009	Ice cream, milk-based, chocolate

By combining the average composition of the above listed foods with the food consumption data from the EFSA database, the estimated mean intake of WACP will stay below 10g/day for all population groups as can be seen in Table 3.

Table 3 Total WAPC intake (g/day) if the proposed foods egg protein were substituted with WAPC. Calculations based on composition and consumption data from the FoodEx2 database.

Population group	Mean (g/day)	5th percentile (g/day)	95th percentile (g/day)
Infants	2.65	0.58	6.37
Toddlers	4.11	1.20	9.06
Adolescents	9.12	2.65	20.46
Other Children	5.85	1.64	13.41
Adults	9.95	2.85	23.66
Pregnant Women	9.42	4.65	16.97
Lactating Women	5.82	0.00	10.42
Elderly	9.08	3.46	18.93
Very Elderly	8.47	3.58	15.78

Summary

Alfalfa protein concentrate (APC) has been accepted as a food ingredient since the 13th October 2009 by The Commission of the European communities with a daily intake of 10 g. We propose to approve white alfalfa protein concentrate (WAPC) as a food ingredient in 13 Foodex2 lv7 categories (listed in Table 2), produced under their conditions described by Hansen et al. 2023 [20]. The compounds of concern in the "Opinion on the safety of 'Alfalfa protein concentrate' as food" is all lower in WAPC compared to APC. Since the average daily consumption of all these food categories will lead to an intake of less than 10 g/day of WAPC, the total intake of l-canavanine, coumestrol and β-carotene will stay below the thresholds listed in the decision from the European council in 2009 with respect to the safety of APC as a food ingredient. Therefore, we conclude that including WAPC in the 13 Foodex2 lv7 categories (listed in Table 2) will pose no threat to human health.

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CHAPTER 7

Conclusion

In the beginning of this thesis 7 criteria was defined, that a new protein source should meet to be attractive for the market.

1. Price on the protein
2. Availability of the protein
3. Safety of the protein
4. Functionality of the protein
5. Taste of the protein
6. Colour of the protein
7. Sustainability of the protein

Through the development of a simplified method for the production of a white alfalfa protein concentrate with reduced off-flavour taste, through the usage of supercritical CO_2 extraction, it has been demonstrated that alfalfa can be used as new source of protein in the human diet, that lives up to 2-6 of the listed criteria. It was demonstrated that repetitive pressings of the pulp might be beneficial, depending on the pressing method.

Our simplified method for the production of white alfalfa protein concentrate, generate proteins with high digestibility (92.65%) and gave an yield of up to 0.012 g pr g of protein, which is double the amount that was generated through a much more complicated process proposed by Edwards et al. 1975. The white alfalfa protein concentrate produced in this study was found acceptable by the consumer, though improving the supercritical CO_2 extraction, to lower the taste, would be advisable in future projects. Methods for utilising the side streams pulp and brown juice was conducted and it was demonstrated that both side streams can be utilized in creating new products and thereby lowering the potential waste generated in a future white alfalfa protein production.

An application for an extended usage of white alfalfa protein concentrate as a food ingredient for EFSA was generated, concluding that white alfalfa protein concentrate would be safe to consume within the listed boundaries. By using the simplified method and utilizing the side streams to maximize the profit, the potential cost of a new white alfalfa protein concentrate might meet criteria 1 listed above, thus making the new protein attractive for the market. A full life cycle assessment is needed on the production of WAPC in full scale to document the sustainability of WAPC and there by meeting criteria 7. However the methods presented in this project are still premature and a full techno-economic evaluation, including a life cycle assessment, of the production is needed to justify a full scale production of white alfalfa protein concentrate as presented in this project.

CHAPTER 8

Appendices

As mentioned in the ”List of work done during the PhD project” in the beginning of this thesis, other work that was strongly related to the project, but not included in the main part of the thesis, was prepared or finished. These works are presented in the following sections.

APPENDIX A

Allergens in Rye Grass Protein

During this project digestibility of rye grass protein allergens was investigated. My contribution to this work was investigating different digestion techniques of the rye grass protein, followed by a separation of the digested protein by SDS-PAGE. The production of APC is fairly similar to the production of rye grass protein and thus many experiences was gathered in this project with respect to APC. The main finding was the digestion method applied to rye grass protein was not sufficient for APC and thus saved many hours of work in the lab with respect the digestion of APC. The work is not yet published, which is why a letter of documentation for my contribution to this work is attached here.

Title of article
In vivo and in vitro evaluation of the allergenicity of grass protein (in preparation)
Journal/conference
Molecular Nutrition
Author(s)
Ana I. Sancho, Mikkel Hansen, Kathrine Beck Sylvestersen, Jacob Ihlemann, Peter Ruhdal Jensen, Claus Heiner Bang-Berthelsen, Katrine Lindholm Bøgh
Name (capital letters) and signature of PhD student
MIKKEL HANSEN
PhD student's date of birth
03-08-1983
Declaration of the PhD student's contribution
For each category in the table below, please specify the PhD student's contribution to the article as appropriate (please do not fill in with names or x's)

Category	**Minor contribution to the work** *(please specify the nature of the PhD student's contribution)*	**Substantial contribution to the work** *(please specify the nature of the PhD student's contribution)*
Formulation of the conceptual framework and/or planning of the design of the study including scientific questions		
Carrying out of experiments/data collection and analysis/interpretation of results	Contributed by conducting the pepsin digestion assay followed by SDS-Page analysis of the protein fraction from rye grass.	
Writing of the article/revising the manuscript for intellectual content		

Signatures

_____

Technical University of Denmark

Title of article
In vivo and in vitro evaluation of the allergenicity of grass protein (in preparation)
Journal/conference
Molecular Nutrition
Author(s)
Ana I. Sancho, Mikkel Hansen, Kathrine Beck Sylvestersen, Jacob Ihlemann, Peter Ruhdal Jensen, Claus Heiner Bang-Berthelsen, Katrine Lindholm Bøgh
Name (capital letters) and signature of PhD student
MIKKEL HANSEN
PhD student's date of birth
03-08-1983

Date	Name	Title	Signature
1/11-2019	Claus Heiner Bang-Berthelsen	Senior scientist	
3/11-2019	Peter Ruhdal Jensen	Professor	

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APPENDIX B

Valorization of Green Biomass: Alfalfa Pulp as a Substrate for Oyster Mushroom Cultivation

During the PhD project I assisted my colleague Fa in one his studies where he investigated the utilization of the side-stream pulp from the WAPC production, as a substrate when growing oyster mushrooms. In this study Fa Zhou demonstrated one of the many potential usages of pulp from WAPC production, thus making it more economically attractive to produce WAPC in the future.

foods

MDPI

Article

Valorization of Green Biomass: Alfalfa Pulp as a Substrate for Oyster Mushroom Cultivation

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Abstract: In this study, the potential of alfalfa pulp as an alternative substrate to wheat straw for the cultivation of oyster mushroom (*Pleurotus ostreatus*) was investigated. The major components associated with different mushroom stages were evaluated, as well as changes in lignocellulolytic enzyme activities in substrates composed of alfalfa pulp, wheat straw or a combination of both. Based on the results, alfalfa pulp was demonstrated to be a better substrate than wheat straw for the production of oyster mushrooms, with a high biological efficiency of 166.3 ± 25.4%. Compared to the cultivation period on commercial straw (31 days), a shorter lifecycle for oyster mushroom was found on alfalfa pulp (24 days), which could help to reduce the risk of contamination during industrial production. Study of the spent substrate as well as the harvested mushrooms revealed that the biological efficiency was related to the higher protein content (17.42%) in the alfalfa pulp compared to wheat straw, as well as greater degradation of cellulose (57.58%) and hemicellulose (56.60%). This was, by and large, due to greater extracellular hydrolytic and oxidative enzyme activity from the mushroom growth in the alfalfa pulp. The quality and safety of the fruiting bodies produced on alfalfa pulp was evaluated, which showed that the protein content was 20.4%, of which 46.3% was essential amino acids, and levels of trace elements and heavy metals were below acceptable limits. Hence, oyster mushroom cultivation using alfalfa pulp provides an alternative method to produce a value-added product, while reducing the biomass wastes in the green protein bio-refinery, and may contribute to sustainable growth in the agricultural industry.

Keywords: green biomass; *Pleurotus ostreatus*; lignocellulose composition; lignocellulolytic enzyme; biodegradation

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1. Introduction

The supply of organic plant-based protein for feed and human consumption, with a suitable amino-acid profile and at a competitive price, is one of the major challenges for agriculture nowadays. Green bio-refineries can produce protein extracts, which contain the required specific amino acids and have the potential to alleviate those alternative protein needs [1]. Since 1969, leaf proteins have been utilized for human consumption as supplements in diets [2]. At the same time, as green biomass bio-refinery concepts become more attractive, it is important that these industries not only pay attention to the production of suitable protein sources, but also to processes that can deal with the huge amount of residues [3]. A recent example of such a green bio-refinery with a high technological readiness level is Biorefine in Denmark [4], and other examples have been recently reviewed by Xiu and Shahbazi [5]. For example, processing of alfalfa with a twin-screw press is an efficient way to extract proteins from the matrix, but, at the same time, it results in 50% dry matter of the raw material being produced as a fibrous residue [6]. Traditionally, most of these solid residues have only been used for animal feeding, and command a low price. However, given the global trend away from animal-based protein sources due to green-house gas emissions, and in order to render the green bio-refinery more sustainable and economically competitive, it is important to look for more sustainable,

higher value-added applications. The lignocellulosic residue from alfalfa has a physical structure allowing gas transfer and a suitable carbon/nitrogen ratio that could allow fungal growth. Hoa [7] examined C/N ratios of between 30–50 for growth of *Pleurotus* spp. on various substrates and found that the best performance was at C/N ratios close to 30. One such application could, therefore, be the cultivation of edible, protein-rich mushrooms for human consumption.

Mushrooms are a type of fungi with significant nutritional value and many beneficial properties and currently there are around 2000 edible species distributed around the world [8]. Among the edible mushrooms, oyster mushrooms (*Pleurotus* spp.) represent one of the most common species cultivated and account for more than 16% of the mushroom production industries [9]. In nature, they usually grow on waste materials and colonize dead organic materials, such as dead cottonwood, oak, or maple. During the cultivation period, the mushrooms are able to secret extracellular enzymes, which can degrade the large insoluble components of lignocellulosic materials and provide soluble, low-molecular-weight compounds for growth [10]. These enzymes include different types of peroxidases, such as manganese peroxidase, cellulase and xylanase. In addition to traditional bioconversion of organic wastes into edible protein products, there are other fields where the lignocellulolytic potential of oyster mushrooms may be economically relevant [11]. The residual enzymes in spent mushroom substrate could be a source of enzymes for lignocellulose conversion during second generation ethanol production, or can play a role in remediating soil and industrial wastewater in industry [12].

Generally, the common industrial substrates used for producing edible mushrooms are wheat straw [13], sawdust [14], by-products of cotton [15], and coffee pulp [16]. Important characteristics of these substrates are that they contain lignocellulose, which allows mushroom mycelia development. Though various culture conditions have been tested, the production of oyster mushrooms is usually divided into the following stages: Composting and filling, sterilization, inoculation, incubation, fruiting and harvest [17]. Compared to other species, the cultivation of *P. ostreatus* is generally easier, faster, and more cost effective. However, there is often the threat of contamination of the mushroom culture from foreign micro-organisms that affects the mycelia. Sub-optimum growth can result giving low yields of fruit through competition with the mycelia for space and nutrients [18]. Thus, there is an urgent need for an improved mushroom substrate with simple pretreatment, which supports fast mycelium generation and with high fruit productivity.

The present study examines the potential for using alfalfa pulp as a substrate for the production of oyster mushrooms and compares it to the commonly used substrate, wheat straw [13]. We demonstrated that even without the addition of nutrients, the mycelium production was superior to the reference substrate. A comprehensive analysis of the link between *P. ostreatus* cultivation and the secretion of enzymes was conducted to evaluate substrate degradation during the different phases of cultivation. Finally, the nutritional and chemical composition of the *P. ostreatus* fruiting bodies were examined, including dietary fiber, available sugar, protein, amino-acid profile and chemical composition.

2. Materials and Methods

2.1. Fungal Strain and Preparation of Alfalfa Pulp

Commercial dried straw and grain spawn of the oyster mushroom strain *Pleurotus ostreatus* were purchased from TagTomat ApS, Copenhagen, Denmark. An amount of 1 kg of fresh alfalfa was directly pressed using a twin screw juicer (Angel Juicer, Busan, Korea), the liquid was saved for green protein production and the resulting pulp was then collected and soaked with deionized water at a 1:1 mass ratio for 2 h before the second pressing. The second pressing was conducted in the same press as used for the first press. The liquid was used for green protein production. The pulp was collected, dried at 80 °C for 24 h to constant weight and then packaged in Ziploc bags and kept at room temperature until used.

2.2. Media Preparation and Inoculation

The alfalfa pulp or wheat straw was first ground or chopped with a Kenwood KVL4110W chef machine to a particle size of 0.5–1.0 mm as determined by sieving using a laboratory sifter (Buhler MLUA 230 V). The different dry media (i.e., alfalfa pulp, wheat straw or a mixture of both) were then rewetted with distilled water to give a final moisture content of 80% and sterilized at 121 °C for 15 min. When the substrate had cooled down, 70 g (corresponding to 14 g of dry matter) was placed into a 108 × 81 × 50 mm polypropylene box of 250 mL volume. The media was then inoculated with the spawn of *P. ostreatus* (10% of the dry weight of the substrate) by spreading the spawn over the substrate and covering with 0.5–1.0 cm of substrate; then, the prepared boxes were placed into a 300 × 200 mm plastic bag. Each bag was closed with 3 M micropore semi-permeable tape to prevent possible contamination by airborne organisms, while allowing air exchange. The cultures were then placed in an incubator (Aralab climatic chamber, FITOCLIMA 1200 PLH, Sintra, Portugal) where temperature, ventilation, relative humidity and light could be precisely controlled.

2.3. Culturing Conditions

Mushroom growth was divided into 4 stages: Fully grown mycelium, phase 1 (P1); primordium, phase 2 (P2); young fruiting bodies, phase 3 (P3), and; mature fruiting bodies, phase 4 (P4). In P1, the temperature and relative humidity were controlled at 24 °C and 85% respectively without lighting, until the substrate was completely colonized; it was assessed by visually observing, through the transparent plastic boxes, the mycelium spreading to the bottom of the substrate. For P2-P4, the culture bags were opened, then the temperature was reduced to 18 °C and the humidity was increased to 90%; additionally, 10% lighting intensity (60 μmoles m^{-2} s^{-1}) for 12 h per day was used until the end of P4.

2.4. Substrate Conversion

The first flush mushrooms at the end of P4 were harvested by twisting the mushroom at the base of the stem and the mass (both fresh and dry weight) produced by each substrate was determined. To determine the conversion of substrate at each phase of growth, three samples of each substrate were taken randomly then dried to constant weight. The dry weight was compared to that of three samples of uninoculated substrate, which were also dried to constant weight and the biological efficiency (BE) was determined according to Equation (1).

$$\mathrm{BE} = \frac{fresh\ mushroom\ substrate}{dry\ substrate} \times 100\% \quad (1)$$

2.5. Analysis of Lignocellulose Content in the Substrate

The collected substrate samples were dried at 50 °C for 24 h, then broken up with a Kenwood KVL4110W chef machine and sieved, as described above, into a size range of 0.5–1.0 mm prior to determining the composition and enzyme activities. The Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL) were used to measure the lignocellulose content [19]. In brief, 0.3 g of biomass was hydrolyzed with 3 mL 72% sulfuric acid for 1 h, then the hydrolyzed biomass was diluted with distilled water to 4% sulfuric acid and autoclaved at 121 °C for 1 h. The hydrolysate was filtered and oven dried to determine the acid insoluble lignin. The filtrate was collected for determining the acid-soluble lignin with the NREL method [17] using a UV-Vis spectrophotometer (Thermo Scientific, GENESYS 10S, Columbus, OH, USA) and monosaccharides by high-performance liquid chromatography. All the monosaccharides were quantified using a Dionex Ultimate 3000 HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) and a refractive index detector (Shodex RI-101; Showa Denko K.K., Tokyo, Japan) at 60 °C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL min^{-1}.

2.6. Analysis of Enzyme Activity in the Substrate

The substrate was first dried, milled and sieved as described above. Crude enzyme extracts were then obtained by adding 10 mL Na-citrate buffer (pH = 5.0) to 1 g of each sample. The samples were mixed at 4 °C for 24 h and then centrifuged for 20 min at 2500× *g*. The enzyme containing liquid solutions were filtered through 0.22 µm polyethersulfone membrane syringe-filters and used for the different enzyme activity determinations.

The total cellulase activity was determined by the filter paper activity assay according to the method from the standardized NREL Laboratory Analytical Procedure [20]. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper in 60 min has been designated for calculating filter paper cellulase units (FPU) of the enzyme solution. The released reducing sugars were assayed by adding 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent, boiling for 5 min, cooling and diluted with water, then measuring the absorbance at 540 nm.

The Xylanase activity was analyzed according to Bailey et al. [21] using birchwood xylan (1 g L^{-1}) as substrate. In brief, the released xylose in 5 min at 50 °C was determined by using dinitrosalicylic acid (DNS) reagent at 540 nm. One unit (U) was defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under the assay conditions.

The laccase (Lac) activity was measured by using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at a concentration of 0.5 mM in Na-acetate buffer (100 mM, pH = 5.0). The time-dependent oxidation of ABTS was determined by measuring the increase in A_{420} (ε = 36,000 M^{-1} cm^{-1}) [22]. One unit was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute.

The lignin peroxidase (LiP) activity was analyzed by measuring the oxidation of 40 mM veratryl alcohol in Na-citrate buffer (100 mM, pH = 4.9) with 0.1 mM H_2O_2, spectrophotometrically at 310 nm (ε = 9300 M^{-1} cm^{-1}) according to Haq et al. [23]. One unit was defined as the amount of enzyme that leads to the oxidation of 1 µmol veratryl alcohol per minute.

The manganese peroxidase (MnP) activity was assayed in a mixture of 0.9 mL Na-malonate buffer (100 mM, pH = 5.0) containing 1 mM of manganese ions (Mn^{2+}) and 0.1 mL of crude enzyme solution. The reaction was started by addition of 0.1 mM H_2O_2 and absorbance was measure at 270 nm. An extinction coefficient of ε = 11.59 mM^{-1} cm^{-1} was used to calculate the activity and one unit was defined as 1 µmol complex of Mn^{3+}-malonate formed per minute [24].

2.7. Properties of the Fruiting Bodies

The color of the fresh mushroom pileus was evaluated using a Hunter Lab Miniscan XE colorimeter (Reston, VA, USA). Prior to other analyses, samples of mushroom were frozen dried and crushed with a mortar to give a powder.

The total dietary fiber was analyzed by the Megazyme K-TDFC kit (Megazymes, Bray, Ireland). Briefly, 0.5 g of sample was incubated with α-amylase, protease and amyloglucosidase. Subsequently, the ash and protein content of the residue was determined, and the soluble carbohydrates were measured by the HPLC method described above. The total dietary fiber was then calculated as described in the kit.

The trace metals and heavy metals of the mushroom were analyzed by Hangzhou Yanqu Information Technology Co., Ltd. (Hangzhou, China). Briefly, the dried samples were wet-combusted in HNO_3 (65%) using a microwave technique (CEN Mars 5) and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) [25]. The related estimated daily intake (EDI) and target hazard quotient (THQ) were calculated in Equations (2) and (3) [26].

$$\mathrm{EDI} = \frac{C_{edible\ fungi} \times W_{edible\ fungi\ intake}}{B_{average\ weight}} \quad (2)$$

where $C_{edible\ fungi}$, $W_{edible\ fungi\ intake}$, and $B_{average\ weight}$ represent the concentrations of trace metals in the tested fungi (mg kg^{-1} DM), the weekly intake of edible fungi (30 g day^{-1}), and the average body weight (60 kg), respectively [27].

$$\mathrm{THQ} = \frac{EDI}{RfD} \quad (3)$$

where oral reference dose (RfD) for Cd, Cr, Cu, Mn, Ni, Fe, Zn, Hg (methyl mercury), and Pb are 0.001, 1.5, 0.04, 0.14, 0.02, 0.7, 0.3, 0.0001, and 0.0036 mg kg^{-1} d^{-1}, respectively [28].

The amino-acid composition was analysed by Hangzhou Yanqu Information Technology Co., Ltd. (Hangzhou, China). HPLC-MS was used, following 0.1 M HCl hydrolysis of the sample and derivatization. The amino acids were identified by comparing retention time and mass spectra of an external standard mixture. The accordingly recommended scoring pattern (RSP) is from the Food and Agriculture Organization (FAO) report [29].

2.8. Other Analyses

The total protein content of the samples was estimated by the Dumas (Rapid MAX N exceed cube N/protein analyzer, Elementar Analysensysteme GmbH, Hesse, Germany) using a conversion factor of 6.25 for substrate and 4.38 [23] for oyster mushroom fruit body. The ash content of the samples was determined gravimetrically after incineration at 600 ± 15 °C for 24 h. The extractive content was evaluated by subtracting the content of remaining substances [30].

2.9. Statistical Analysis

Each analytical result is reported as the mean value of three replicate sample measurements, except where stated. Standard deviations and statistical differences were analyzed using Origin 2021. Differences between the means of samples were analysed by Fisher's least significant difference (LSD) test at a probability of 0.05.

3. Results and Discussion

3.1. Mushroom Growth on Different Substrates

The objective of this study was to assess the potential of alfalfa pulp for the production of mushrooms. In order to do this, a comparison was made with growth on a conventional mushroom growth medium (straw) and a mixture of the two substrates, i.e., straw and alfalfa pulp. The composition of the three substrates was determined and the results showed that the alfalfa pulp had lower concentrations of cellulose, hemicellulose, soluble and insoluble lignin, total fiber and ash, but higher concentrations of protein, nitrogen and extractives than wheat straw (Table S1 in Supplementary Materials). In all cultivations, the same inoculum particle size (0.5–1.0 mm) and spawn inoculation level (10%) were used, without any nutritional supplements. In general, spawn running time on different substrates with different cultivation methods should allow for harvesting of mature fruiting bodies 5 days after emergence of the pins and the total cultivation period can normally be expected to be approximately 30–35 days [31].

The results in Table 1 show that the alfalfa pulp performed very well as a growth substrate and shortened the growth time at each phase when compared to straw and to a mixture of alfalfa pulp and straw substrate. Button-shaped mushroom bodies appeared in 22 days (P3), and two days after that the first mature fruit could be harvested (P4) when alfalfa pulp was used. The longest time for each phase was seen when straw only was used and the addition of alfalfa pulp to the straw was able to shorten these times (Table 1). The biological efficiency (BE) value (166.30 ± 25.44%) of *P. ostreatus* when grown on alfalfa pulp was much higher than the other two substrates and exceeded 100%. This could indicate that it is an ideal substrate. Furthermore, when compared with the literature for other substrates, the BE value of alfalfa pulp (166.30 ± 25.44%) is higher than wheat straw (50.2%), coffee pulp (86.5%) [16], and faba bean hulls (109%) [25]. For example, Vieira and de Andrade [32] investigated different substrates for commercial *P. ostreatus* growth

in a production-scale setting and found BE values of ca. 66% when using wheat straw without nutritional supplementation compared to ca. 80% using sugar cane straw without supplementation. The BE of the wheat straw substrate used in the current experiment with the same species was 22.72 ± 0.14%, which is lower than that reported by Vieira and de Andrade [32] and when compared with 54.2 ± 11.8% reported by Salmones et al. [16]. The difference could possibly be due to the small-scale experiment in this study, with initial dry substrate weight around 14 g per test, in contrast to the scale used by Salmones et al. [16], which was 200 g dry substrate. However, it is more likely that a difference in conditions is responsible for the difference in BE, since the yield can be improved by optimizing the culture conditions [17]. If so, then even higher BE values may be obtained in commercial production settings when using alfalfa pulp. Furthermore, the BE value of the straw could be raised dramatically from 22.72 ± 0.14% to 101.85 ± 16.88%, when it was mixed with alfalfa pulp. This suggests that the alfalfa pulp could also be used as an additive in a well-functioning mushroom production system.

Table 1. Cultivation parameters for *P. ostreatus* mushrooms produced on three substrates: Alfalfa pulp, mixture of straw and alfalfa pulp and straw only. Results are given as mean ± standard deviation, $n = 3$ biological replicates. P1–P4 designate the cultivation stage: P1, fully grown mycelium; P2, primordium; P3, young fruiting bodies; P4, mature fruiting bodies.

		Weight (g)	Cultivation Stages and Number of Days Needed				Substrate Dry Matter Loss (%)	Biological Efficiency (BE) (%)
			P1	P2	P3	P4		
Alfalfa pulp	Initial dry substrate	14.56 ± 0.09	6.67 ± 0.29	18.67 ± 1.53	22.33 ± 1.15	24.25 ± 0.56	41.51 ± 3.01	166.30 ± 25.44
	Spent dry substrate	8.51 ± 0.39						
	Fresh mushroom	14.10 ± 2.17						
Mixture (50:50)	Initial dry substrate	14.48 ± 0.35	7.33 ± 0.57	24.00 ± 1.73	26.50 ± 0.70	28.67 ± 2.08	30.29 ±5.20	101.85 ± 16.88
	Spent dry substrate	10.01 ± 0.68						
	Fresh mushroom	10.21 ± 1.39						
Straw	Initial dry substrate	14.70 ± 0.03	7.80 ± 0.80	26.67 ± 1.15	28.00 ± 1.00	31.33 ± 1.53	8.20 ± 2.42	22.72 ± 0.14
	Spent dry substrate	13.49 ± 0.33						
	Fresh mushroom	3.07 ± 0.05						

To understand why different substrates have different bioconversion efficiencies, a detailed analysis of the mushroom substrates composition at different stages was performed, see Figure 1. The results show that mushroom formation was related to the degradation of components in the substrate. Furthermore, the type of substrate has a major influence on the level of degradation, which is also reflected in the BE value (Table 1). The results in Figure 1 show that alfalfa pulp has more protein and less lignin, cellulose and hemicellulose than straw, or the mixture of alfalfa and straw. Furthermore, it can be seen that for almost all substrates, the amounts of protein, lignin, cellulose and hemicellulose decrease significantly during mushroom growth from the P1 to P4 phase. The only exception being for the straw substrate. The more than 3-fold higher protein content in the alfalfa pulp (Figure 1A) compared to the straw may indicate that straw lacks sufficient organic nitrogen content, thus leading to the best cultivation performance on the alfalfa pulp. It is known that nitrogen is also a major factor that affects enzyme secretion (cellulases, hemicellulases, and laccases), which is important in the degradation of cellulose, hemicelluloses and

lignin, respectively [33]. Although it is known that nitrogen excess can negatively affect the degradation of lignin [33], no evidence of that was seen when comparing the alfalfa pulp and straw degradation (Figure 1A). Supplementation of the straw with alfalfa pulp was seen to increase the productivity and biological efficacy of the oyster mushroom (Table 1), which is consistent with the increase in protein in the mixture, compared to the straw only (Figure 1A).

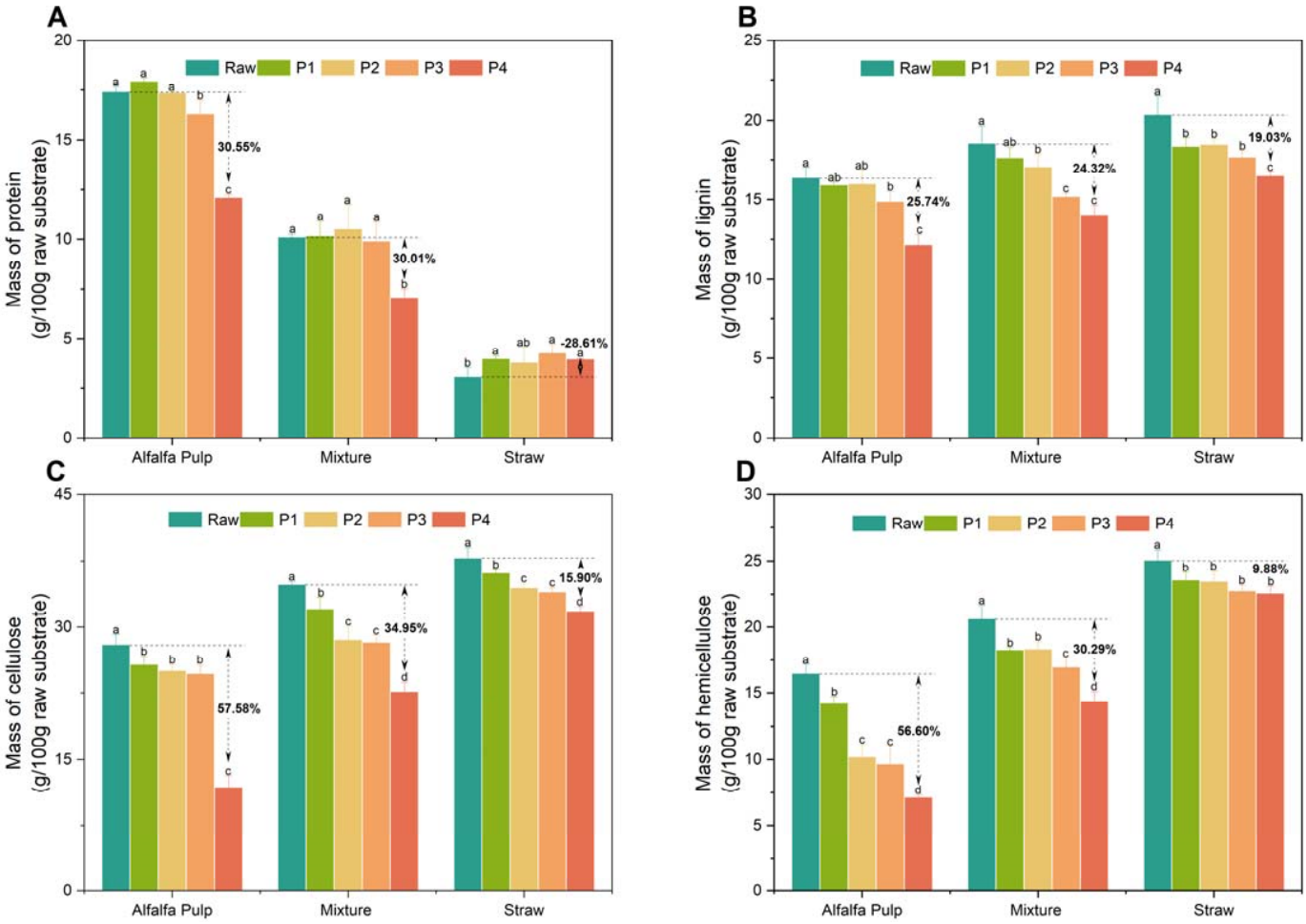

Figure 1. Major components of the mushroom substrates at different cultivation phases. Mass of: (**A**) protein; (**B**) lignin; (**C**) cellulose; (**D**) hemicellulose in g 100 g^{-1} raw substrate. The results are averages and standard deviations of 3 biological replicates. P1–P4 designate the cultivation stage. P1, fully grown mycelium; P2, primordium; P3, young fruiting bodies; P4, mature fruiting bodies. The letters a–d indicate if there is a significant difference ($p \leq 0.05$) between different stages in the substrate determined using Fisher's least significant difference.

A positive correlation was also observed between the BE value (Table 1) and cellulose and hemicellulose degradation in all spent substrates (Figure 1C,D). The degradation of cellulose was 57.58%, 34.95% and 15.90% in the alfalfa, mixture and straw, respectively. According to Owaid et al. [34], some mixed substrates were made to overcome the low bioconversion and render them more popular and acceptable in the mushroom cultivation industry than using straw alone. It was of interest in the current work that the greatest degradation of cellulose happened in the final stage (P4) when using alfalfa pulp and the mixture. Normally, the lignin, cellulose, and hemicellulose of the substrate will be utilized evenly during mushroom cultivation stages [35]. The differences in this study could possibly be due to changes in the levels of hydrolytic enzymes secreted during the different growth phases, which cause the simultaneous or selective degradation of cellulose and

hemicelluloses along with lignin [36]. Evidence of selective degradation of hemicellulose during different growth phases was observed in Figure 1D for alfalfa pulp, where there the majority of the decrease was seen from the P1-P2, in contrast to the mixture and straw only. Overall, more cellulose and hemicellulose was degraded compared to lignin for the alfalfa pulp compared to the straw. In contrast, Angel et al. [37] reported that *Pleurotus* spp. are efficient lignin degraders, and are able to remove more lignin selectively from non-woody lignocellulosic materials. It is speculated that the low lignin degradation of the non-woody alfalfa pulp can be attributed to the mechanical pressing pretreatment of the alfalfa. Here, the amorphous and crystalline cellulose matrix in the biomass residues might be disrupted, which may then positively affect the bioconversion [38].

P. ostreatus, as the traditional white rot fungi, uses extracellular enzymes to form a ligninolytic and a hydrolytic system to degrade lignocellulose. It can be expected that the selective degradation of components in the alfalfa pulp during the different stages of mushroom cultivation would be due to three main enzymes: cellulases, xylanase and the ligninolytic peroxidase. The activity of these enzymes during *P. ostreatus* growth on all test substrates was thereby examined, and the results are shown in Figure 2. The related morphology of four growth phases of *P. ostreatus* on three substrates are in Figure 2F.

Cellulase activity was highest on alfalfa (0.40 ± 0.05 U g^{-1}), next highest on the mixture and the least activity was observed on the straw. The activities increased most until the P1 stage and were then, more or less, constant until mushroom harvesting for all substrates. This is consistent with the degradation pattern seen in Figure 1C, where continuous cellulose degradation occurred during the cultivation process. It is also consistent with the highest BE values seen on the alfalfa pulp in Table 1. Cellulases have different specificities to hydrolyse the β-1,4-glycosidic linkages and convert the polysaccharides to oligosaccharides for fungi growth and metabolism [39]. The values found here for cellulase are similar to those found in a previous study [40], which recovered the same amount of enzyme in spent mushroom composts as a product.

Unlike cellulase activity, xylanase activity was low for the first three phases then increased dramatically at P4 (2.00 ± 0.42 U g^{-1}) in alfalfa pulp and to a lesser, though significant amount for the mixture (0.62 ± 0.14 U g^{-1}) (Figure 2B). The trend observed here is consistent with other studies, which have shown that xylanase activity on mushroom substrate increased over time and was associated with the fruiting body formation [24]. Because xylan is the major constituent in hemicellulose, this result is consistent with the hemicellulose loss (Figure 1D) seen for the alfalfa pulp and the mixture with straw. It is known that nitrogen sources have a dramatic effect on the production of xylanase [41]. Although straw has very high hemicellulose levels (25.06 ± 0.74%), the lack of nitrogen compared to the alfalfa pulp and the mixture appears to have limited xylanase production.

Lignin degradation is a complex process. Previous studies indicated that the lignin peroxidase is active in the primordium and the fruiting body formation stages. Here there is oxidation of the non-phenolic units of lignin, cleavage the C_α-C_β bond in lignin molecules, and opening of the ring of the aromatic skeleton [42]. Manganese peroxidase oxidizes a bound Mn^{2+} to Mn^{3+} in the presence of hydrogen peroxide generating an intermediate redox couple Mn^{2+}/Mn^{3+}. The Mn^{3+} complex can diffuse into the lignified cell wall, where it oxidizes phenolic or nonphenolic lignin components [43]. However, laccase is strongly inhibited by H_2O_2 [41]. It can, therefore, be expected that lignin peroxidase and manganese peroxidase activity in the substrate would increase during cultivation, but that laccase production would show a different pattern due to inhibition by H_2O_2.

When the results in Figure 2C, D are inspected, trends are observed that are consistent to what is expected from the literature above. There was a dramatic spike in laccase activity (Figure 2C) at the P1 phase for the alfalfa substrate and to a lesser extent for the mixed substrate, after which it declined to zero by phase P2. Ruiz-Rodríguez et al. [44] also found that laccase activity reached a maximum (1.2–2.1 U g^{-1}) after around 10–15 days of cultivation with six *Pleurotus* spp. strains on wheat straw, which was then followed by a significant decrease in activity [44].

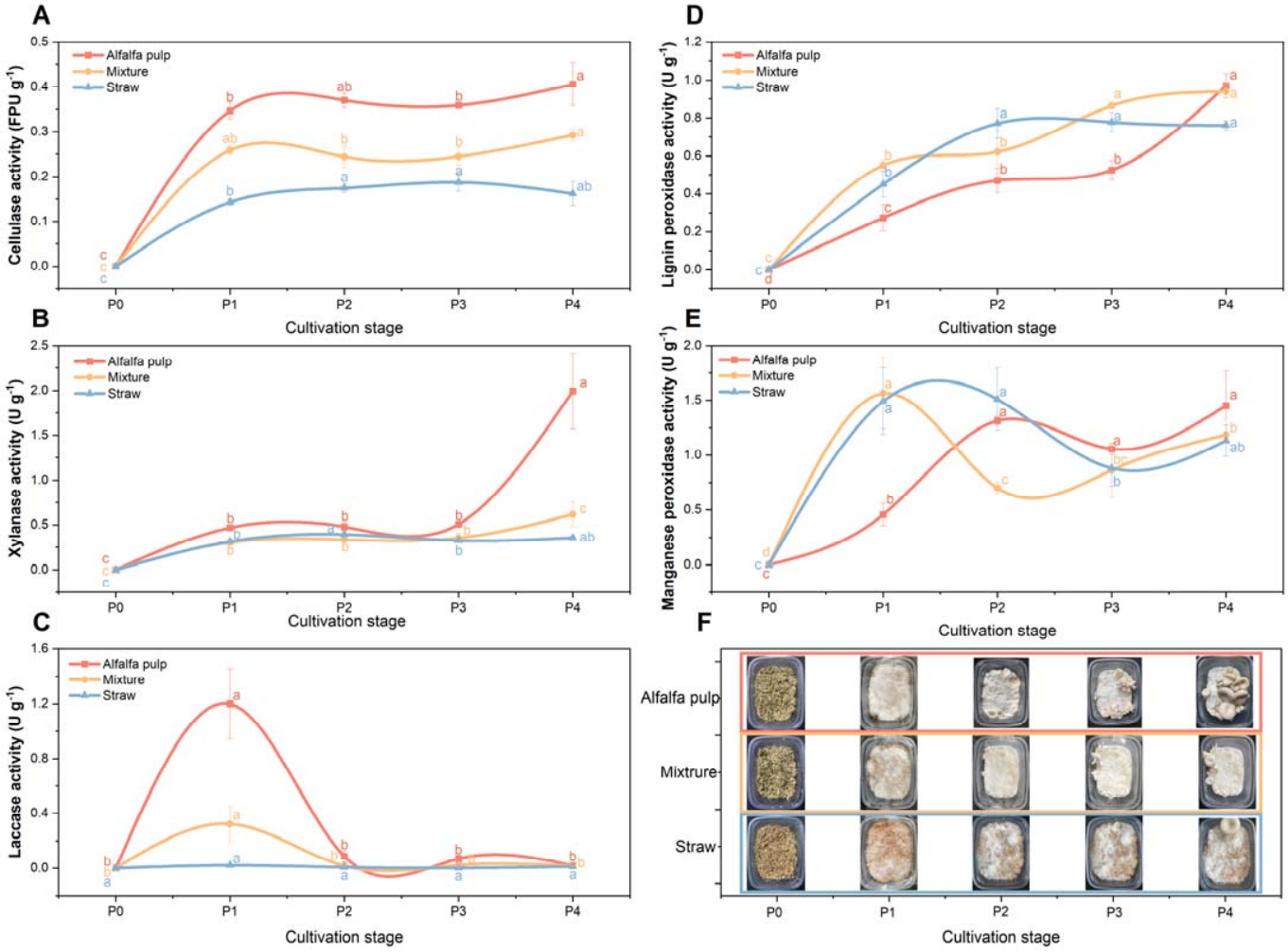

Figure 2. Enzyme activity during growth on substrates. (**A**) cellullases activity; (**B**) xylanases activity; (**C**) laccase activity; (**D**) lignin peroxidase activity; (**E**) manganese peroxidase activity; (**F**) morphology of the different cultivation stages of *P. ostreatus*. P1–P4 designate the cultivation stage. P1, fully grown mycelium; P2, primordium; P3, young fruiting bodies; P4, mature fruiting bodies. The results show averages and standard deviations of three biological replicates. The letters a–c indicate if there is a significant difference ($p \leq 0.05$) between different phases, determined using Fisher's least significant difference.

In contrast, the major ligninolytic enzymes lignin peroxidase (Figure 2D) and manganese peroxidase (Figure 2E) showed a trend of increasing during cultivation. The highest values for lignin peroxidase were seen in the alfalfa pulp (1.20 ± 0.26 U g^{-1}) and occurred when the fruiting bodies had formed, although the activities of this enzyme were generally lower than for the straw and the mixed substrate in the other growth phases (Figure 2). The lignin peroxidase activity gradually increased and reached a first peak at different stages for the different substrates. Maximum activity (0.77 ± 0.08 U g^{-1}) was observed at P2 for straw and with no significant increase during the rest of cultivation ($p \leq 0.05$). During growth on alfalfa pulp and the mixture, the point of this peak moved to P4 with 0.97 ± 0.06 U g^{-1} and P3 with 0.86 ± 0.02 U g^{-1}, respectively. The manganese peroxidase enzyme activity monitored in this study oscillated. A peak in activity was seen during P1 for the straw and mixture and at P2 for the alfalfa pulp. After these peaks, activity declined, then increased again until the fruiting body was harvested (Figure 2E) at P4. This is consistent with a report by Velázquez-Cedeño et al. [45], who observed manganese peroxidases appeared in coffee pulp until the end of the incubation period.

In lignocellulosic degrading systems, various enzymes act together to produce sugars that can be easily assimilated by the mushroom's mycelium. Although alfalfa pulp has a low lignocellulosic profile compared to other substrates utilized in this study, higher enzymatic activity observed in the early stage would partially explain the successful cultivation results obtained. Thus, in principle, a media which has a better capacity for *P. ostreatus* colonizing, synthesizing and secreting ligninolytic enzymes could produce higher fruiting bodies yields [44].

3.2. The Chemical Composition and Nutritional Value of Harvested Mushrooms

The above results indicated that 14.10 g of fresh mushrooms were produced from 14.56 g of dry alfalfa pulp and that this is an effective process to upcycle the pulp. However, it is also important that the mushrooms produced have the right quality, as described by their nutritional and chemical composition. It has been reported that the nutritional composition is highly variable when using different cultivation substrates [13]. These properties were therefore analysed and the results are shown in Table 2.

Table 2. The properties of *P. ostreatus* mushroom harvested from alfalfa pulp. Results are given as mean ± standard deviation, n = 3 biological replicates. DM = dry matter.

Parameters Examined		Values Measured
Moisture (g/100 fresh weight)		83.13 ± 0.01
Ash (g/100 DM)		1.84 ± 0.16
Protein (g/100 DM)		20.36 ± 2.90
Total dietary fiber (g/100 DM)		28.24 ± 0.01
Available carbohydrate (g/100 DM)	D-Trehalose	21.00 ± 1.15
	D-glucose	11.40 ± 0.11
	D-xylose	1.03 ± 0.04
Color	ΔL	73.00 ± 2.40
	Δa	−1.34 ± 0.52
	Δb	16.90 ± 0.82

The general trend of the results in Table 2 is that the mushrooms produced had similar, if not better, nutritional properties compared to what has been reported in the literature. For example, the total protein content on a dry weight basis was 20.36 ± 2.90% (Table 2). Koutrotsios et al., reported protein content of mushrooms grown on wheat straw and date-palm tree leaves of 14.64 ± 1.38% and 16.13 ± 1.22%, respectively [46]. The total dietary fiber, which is associated with anti-carcinogenic properties and immune regulatory functions [47], of the alfalfa pulp grown mushrooms was 28.24 ± 0.01% (Table 2), which is within the range reported for edible *P. ostreatus* (10.60–57.00%) [13]. Furthermore, the available carbohydrates consisted mainly of trehalose (21.00 ± 1.15%) and glucose (11.40 ± 0.11%), which were at similar levels to those previously found in other studies [48].

Lighting is an extrinsic factor affecting mushroom cultivation, which can change the color of the cap (pileus) of the oyster mushroom from bright white to dark, through releasing oxidized phenols by phenoloxidase and forming melanoidins. In this study, 12 h d^{-1} of lighting was used during the final cultivation stage (P3-P4) and the ΔL (lightness), Δa (redness), Δb (yellowness) of the resulting mushrooms were 73.00 ± 2.40, −1.34 ± 0.52, and 16.90 ± 0.82, respectively. These values indicated higher brightness and lower redness than other researchers measured [49], however, there is no relevant standard for this appearance quality.

The mineral composition of fruiting bodies of *P. ostreatus* produced from alfalfa pulp and the potential risk due to heavy metals was investigated and the results are shown in Table 3. The major minerals in the tested mushroom were found to be K (11,176 ± 1858 mg kg^{-1} DM), P (9843 ± 1391 mg kg^{-1} DM), and Ca (2785 ± 1633 mg kg^{-1} DM). This is consistent with other reports that pointed out that from a nutritional point of view, *P. ostreatus* has high levels of potassium and phosphorous, which are beneficial for control of blood pres-

sure [12]. The mushrooms provide higher Ca values than almonds (2640 mg kg^{-1}) and kale (1500 mg kg^{-1}) [35]. Oyster mushrooms are well-known for accumulating heavy metals, the concentration of which is affected by the growth substrates. Substrates high in a particular mineral produce mushrooms relatively high in the content of that mineral [50]. The results in Table 3 show that the estimated daily intake of Pb, Cd, Ni, Cr, and Hg from eating the tested mushrooms was lower than the provisional tolerable daily intake. Through further analysis of THQ, it could be seen that Cu, Mn, Zn, Fe, Pb, Cd, Ni, Cr, and Hg were all within the safe range (THQ $\leq$ 1) [36]. The value of total THQ (0.75) was lower than the edible mushrooms standard in Zambia (2.79), Serbia (2.30), Slovakia (1.65) Romania (1.59), China (1.39), Poland (0.98), Italy (0.89), and Greece (0.77), but higher than in Ukraine (0. 58), Bulgaria (0.45) and Korea (0.20) for adults [28].

Table 3. The concentration of elements (mg kg^{-1} DM) measured in the *P. ostreatus* mushrooms harvested from alfalfa pulp, the resultant estimated daily intake (EDI) and the target hazard quotient (THQ). Provisional tolerable daily intake (PTDI) for heavy metals appear in parentheses. Results are given as mean ± standard deviation, *n* = 3 biological replicates.

Trace Elements	Fruiting Body (mg kg^{-1} DM)	EDI (μg kg^{-1} Body $Weight^{-1}$ Day^{-1})	THQ
K	11,176.51 ± 1858.37	942.74 ± 156.75	-
P	9843.66 ± 1391.04	830.31 ± 117.33	-
Ca	2785.73 ± 1633.49	234.98 ± 137.78	-
Mg	1023.90 ± 141.71	86.37 ± 11.95	-
Na	256.67 ± 56.95	21.65 ± 4.80	-
Fe	135.35 ± 63.54	11.42 ± 5.36	0.03
Zn	71.52 ± 1.73	6.03 ± 0.15	0.14
Mn	25.82 ± 14.07	2.18 ± 1.19	0.17
Cu	12.02 ± 0.54	1.01 ± 0.05	0.03
Heavy Metals			
Pb	10.01 ± 0.62	0.84 ± 0.05 (3.57)	0.13
Cr	1.42 ± 0.77	0.12 ± 0.06 (100)	0.23
Cd	0.09 ± 0.01	$8.20 \times 10^{-3} \pm 5.89 \times 10^{-4}$ (1)	4×10^{-3}
Ni	0.85 ± 0.42	0.72 ± 0.04 (5)	6×10^{-3}
Hg	0.02 ± 0.02	$1.73 \times 10^{-3} \pm 1.27 \times 10^{-3}$ (0.71)	0.02

Nutritionally speaking, the protein quality of *P. ostreatus* is one of its major strengths because it has a high content of all the essential amino acids and excellent protein digestibility [51]. After the analysis of seventeen amino-acids in *P. ostreatus* produced on the alfalfa pulp, it was found that the total amino acid content (213.52 ± 5.35 mg g^{-1} DM; Table 4) was equivalent to the protein content measured (20.36 ± 2.90%; Table 2). The dominant amino acids in the mushrooms from alfalfa pulp were Asp and Glu (Table 4), which accounted for 19.52 ± 0.06 and 29.01 ± 0.62 mg g^{-1}, respectively, of the dry mushroom weight and are similar to the values reported previously in the literature [52]. In addition, the harvested mushroom meets the nutritional requirements of all essential amino acids for adults (Lys, Leu, Val, Ile, AAA: aromatic amino acids (Phe + Tyr)) and it was noteworthy that their composition meets the recommended scoring pattern (RSP) for adults. Threonine, SAA (sulphur amino acids: (Cys + Met)), His, and Lys are more than twice the recommended levels, and the aromatic amino acids are more than four times. Interestingly, the total essential amino acid content measured here is higher for *P. ostreatus* grown on perilla stalks (25.38%) and cotton-seed hull (27.69%) [15]. The oyster mushrooms grown on alfalfa pulp have an excellent nutritional profile and no negative characteristics as measured here. It can be speculated that the high quality proteins known to be present in alfalfa [53], may positively affect the amino-acid composition of *P. ostreatus* fruiting body.

Table 4. The amino acid concentration (mg g^{-1} DM) of harvested *P. ostreatus* mushrooms. Results are given as mean ± standard deviation, n = 3 biological replicates. RSP is the recommended scoring pattern.

Amino Acid	This Study (mg g^{-1} DM)	RSP (mg g^{-1} DM)
Aspartic acid (Asp)	19.52 ± 0.06	-
Serine (Ser)	9.81± 0.23	-
Glutamate (Gln)	29.05 ± 0.62	-
Glycine (Gly)	9.30 ± 0.28	-
Alanine (Ala)	12.18 ± 0.87	-
Arginine (Arg)	11.29 ± 0.05	-
Proline (Pro)	13.65 ± 0.50	-
Threonine (Thr)	10.17 ± 0.22	5.09
Valine (Val)	12.50 ± 0.37	8.14
Cystine (Cys)	0.77 ± 0.09	4.68
Methionine (Met)	7.36 ± 0.03	4.68
Isoleucine (Ile)	11.25 ± 0.27	6.10
Leucine (Leu)	14.96 ± 0.58	12.41
Tyrosine (Tyr)	9.19 ± 0.25	5.09
Phenylalanine (Phe)	16.61 ± 0.04	5.09
Histidine (His)	7.73 ± 0.28	3.23
Lysine (Lys)	18.16 ± 0.99	9.77
Total AAs content	213.52 ± 5.35	-
Total essential AAs content	98.75 ± 2.78	-
Essential AAs/FAAs (%)	46.25 ± 0.01	-

4. Conclusions

This study has demonstrated that alfalfa pulp had better performance than straw for producing oyster mushrooms, without the need for further nutritional additions. Compared to the cultivation period of 31 days on straw, alfalfa pulp produced the fruiting bodies within only 24 days, which is important for avoiding contamination. The reasons for higher biological efficiency achieved on alfalfa pulp are concluded to be a combination of the high protein content in the substrate compared to wheat straw, and higher production of enzymes needed for the breakdown of the lignocellulosic and hemicellulosic structure. Moreover, the *P. ostreatus* produced from alfalfa pulp have safe trace-metals concentration ranges, as well as excellent protein content and essential amino acids profile. Using alfalfa pulp for oyster mushroom cultivation is concluded to be a promising alternative application for this byproduct after protein extraction. Alfalfa pulp is recommended as a substitute for wheat straw given the faster production of fruiting bodies and superior biological efficiency.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11162519/s1, Table S1. Chemical composition of three test substrates. The values are averages and standard deviations of 3 biological replicates. DM = dry matter.

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APPENDIX C

Kan vi springe koens fire maver over og spise græsset selv?

Early in the PhD project our group was contacted by a popular scientific news website named ”videnskab.dk” (translates to science.dk). They asked if it was possible to generate a short article explaining our recent findings and coming research areas, within the project, in a manner that made it easy to read for all. This resulted in a article called ”Could we skip the four stomachs of the cow, and eat the grass instead?”.

Kan vi springe koens fire maver over og spise græsset selv?

Græsset kan i fremtiden havne på vores tallerkener i stedet for som køernes foder, hvis DTU-forskere har held med at udvikle spiselige proteiner fra 'det grønne guld'.

Med forskernes nye metode behøver vi ikke at efterligne koens drøvtyggemetode for at få næring ud af de grønne proteiner. (Foto: Shutterstock)

Mikkel Hansen - ph.d.-studerende ved DTU Fødevareinstituttet , Timothy John Hobley - Lektor ved DTU Fødevareinstituttet & Peter Ruhdal Jensen - Professor ved DTU Fødevareinstituttet

23 februar 2020 FORSKERZONEN MAD & ERNÆRING DYR KLIMA PLANTER DTU

FN forventer, at vi vil være næsten 10 milliarder mennesker på Jorden i 2050, hvilket medfører et øget behov for føde.

For at imødekomme dette behov anslås det, at fødevaresektoren skal producere op til 70 procent mere mad, hvis omfattende hungersnød skal undgås.

Kan en af løsningerne på dette være at bruge det græs, som landbrugets køer spiser i store mængder, til at brødføde mennesker i stedet?

Det er netop, hvad vi forsøger at finde ud af i forskningsgruppen for mikrobiel bioteknologi og bioraffinering under Fødevareinstituttet ved DTU.

Mindre landbrugsjord og flere mennesker skaber kritisk behov

Fakta
Forskerzonen

Blandt andet på grund af klimaforandringer er mængden af landbrugsegnet jord faldet støt siden 1960 samtidig med, at verdensbefolkningen er steget.

Denne tendens kan vi ikke forvente bliver ændret lige foreløbigt, og det er en de store udfordringer i at skulle brødføde fremtidens verdensborgere.

Mennesket har behov for forskellige elementer i vores føde: vitaminer, mineraler, fedtstoffer, kulhydrater og proteiner. Dyrekød er en af de fødevarer med det højeste proteinindhold, men er samtidig en af de proteinkilder, der forurener mest at fremstille.

Ifølge DTU Fødevareinstituttets nationale undersøgelse spiste danskere i 2011-2013 gennemsnitligt 14 kg okse/kalve kød om året.

Da animalske proteinkilder, såsom oksekød, optager langt mere landbrugsareal og har en højere klimabelastning, sammenlignet med planteprotein, er det nødvendigt at udvikle metoder til at erstatte dyreprotein med planteprotein, hvis vi skal øge fødevareproduktionen med 70 procent.

LÆS OGSÅ: Den klimavenlige ko er på trapperne

Hvad gør vi, hvis vi ikke vil tygge drøv?

Vi forsker i at udvinde og udnytte proteiner fra grønt plantemateriale såsom lucerne (*Medicago sativa)* og gøre disse proteiner spiselige for mennesker.

En af de største udfordringer ved at udvinde proteiner fra grønne planter er, at størstedelen af proteinet typisk er bundet i et komplekst fibernetværk. Dette netværk kan køer nedbryde ved hjælp et komplekst mavesystem kombineret med drøvtygning.

I princippet kunne vi stå og tygge på lucernen i flere timer, synke den, vente lidt og derefter gylpe den op igen, og tygge lidt mere på den, før vi spytter den ud igen.

Dette ville gøre proteinet lettere tilgængeligt for mennesker, men ville nok ikke være rentabelt i længden og heller ikke specielt appetitligt.

Så i stedet for at kopiere koens drøvtyggemetoder 1 til 1 har vi i stedet udviklet en metode til udvinding og forædling af det grønne protein.

Fremstilling af grønt protein: 1. Lucernen overføres til skruepresse, 2. Lucernen neddeles til pulp ('frugtkødet' fra presset) 3. Pulpen presses til grøn juice 4. Protein skilles fra den grønne juice 5. Den brune juice fjernes til senere forædling, 6. Det grønne protein tørres og vidrebehandles og er klar til at indgå i fødevarer, 7. Pulpen genvædes og presses derefter igen jævnført trin 2-7. (Figur: DTU Food)

Grøn juice og pulver kan være svaret

Ved at snitte og presse lucernen kan man udvinde en proteinrig grøn juice.

Proteinet kan herefter bundfældes ved hjælp syre, varme, salte og centrifugering i forskellige kombinationer afhængigt af ønsket produkt.

Ud fra disse trin kan vi skabe et proteinrigt grønt pulver med et proteinindhold på op til 50 procent protein. Til sammenligning har hvedemel omkring 11 procent protein og hakket oksekød omkring 40 procent protein (angivet på tørstofbasis).

LÆS OGSÅ: Studie antyder: Flere overvejer en plantebaseret kost til deres kæledyr

Lucern udleder mindre CO_2... men smager af hø

I fødevareindustrien har man i lang tid brugt sojaprotein som erstatning for animalske proteiner.

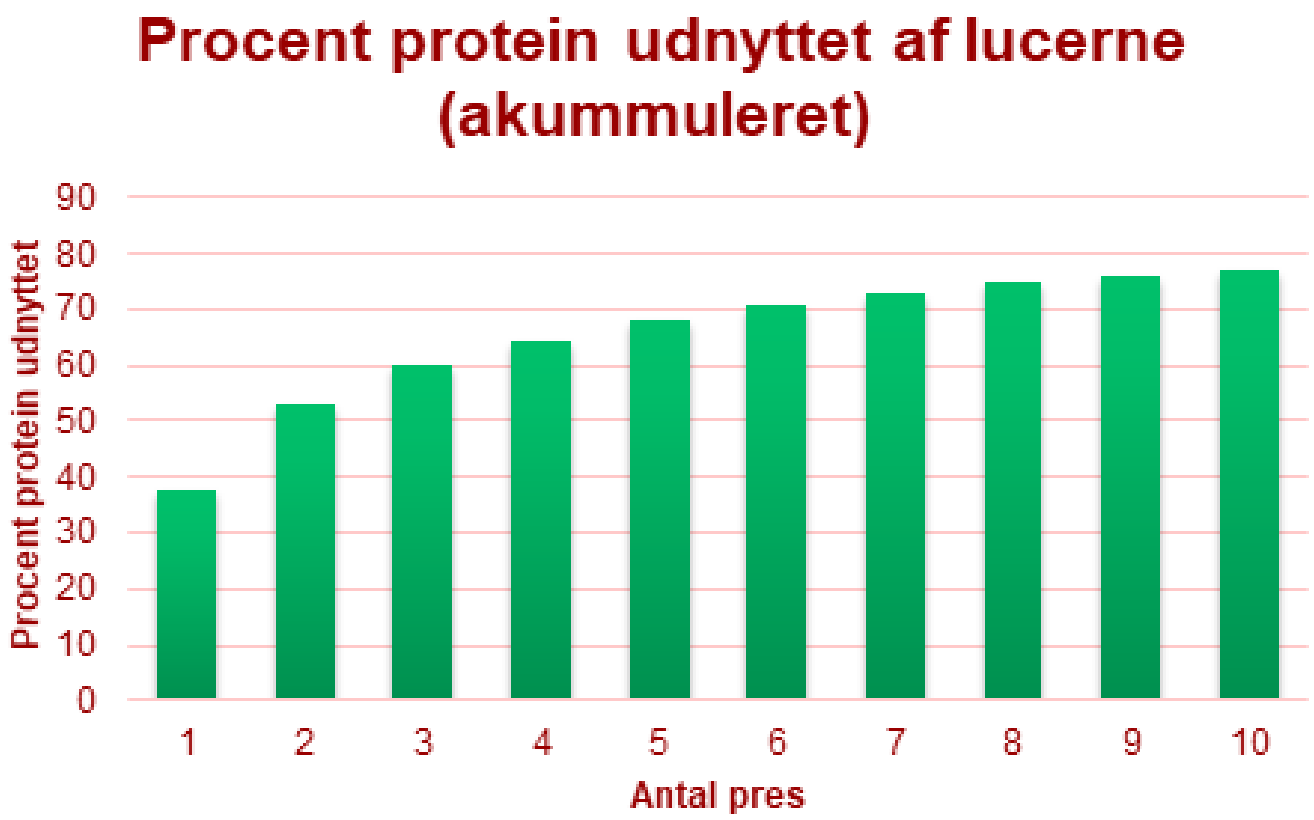
Der udledes dog op til fem gange så meget CO_2 pr gram protein fra fodersoja sammenlignet med foderlucernen, og derfor er lucernen potentielt bedre for miljøet som erstatning for animalske

proteiner.

Til gengælder er sojaprotein lettere at fordøje end lucerneprotein, og det er en af de udfordringer vi er i gang med at løse.

En anden udfordring ved det grønne protein fra lucerne er, at det smager og dufter af hø. I nogle interne smagstest er det faktisk blevet beskrevet som duften af hestestald.

Den lave fordøjelighed, den grønne farve og hestestalden gør, at proteinet ikke er klar til at blive spist, før vi har forædlet det yderligere.



Protein koncentration over flere pres. (Figur: DTU Food)

LÆS OGSÅ: Oksekød er otte gange værre for klimaet end kylling og laks

Vi arbejder på løsningerne

For nogen lyder disse udfordringer måske store – hvem gider at spise hø? – men vi er faktisk allerede godt på vej til at løse dem og regner med, at vi i samarbejde med vores partnere i InnoGrass-projektet, snart kan præsentere alternativer til både kød og soja, der er baseret på lucerne.

En af de simpleste metoder er at genpresse pulpen som illustreret i Figur 1 og Figur 2. Dette medvirker, udover at øge den samlede mængde af protein vi udvinder fra planten (vist i Figur 2), at uønskede smagsstoffer, såsom kumarin (kendt fra kanel), sænkes kraftigt.

Ved konventionel fødevarefremstilling produceres der ofte store mængder affald, men et af målene for forskningen på Fødevareinstituttet på DTU er at skabe værdi fra affaldsstrømmene i fødevareproduktionen.

Så ud over at kigge på selve proteinet, undersøger vi også nu, hvordan vi kan nyttiggøre de andre dele fra vores proteinproduktion. Den brune juice, som beskrevet i figur 1, indeholder store mængder af kulhydrater (sukkerstoffer). Disse kan man blandt andet fremstille alkohol af.

LÆS OGSÅ: MUHU! Sådan kan malkekøer blive mere klimavenlige

På vej mod at udnytte 100 procent af planten

Ud over protein indeholder vores grønne proteinpulver også kostfibre. De kan udnyttes som kosttilskud for at fremme en positiv bakteriekultur i vores tarmsystem.

Den tilbageværende pulp kan til sidst bruges som substrat til vækst af positive mikroorganismer i fødevareindustrien.

Så i stedet for at give lucernen til koen, der kun omsætter omkring 1/10 af den biomasse, den spiser, kan vi snart udnytte op mod 100 procent af planten uden at generere affald og på den måde være med til at føde verdens voksende befolkning.

Så tilbage til spørgsmålet i overskriften: Kan vi springe koens fire maver over og spise græsset selv?

I bogstavelig forstand er svaret nej. Men med den teknologi og viden, vi har udviklet, er vi tæt på at gøre os mindre afhængige af koens fire maver for at få protein på tallerkenen i fremtiden.

LÆS OGSÅ: Vegetarer lever længere, men ikke fordi de fravælger kød

LÆS OGSÅ: Kæmpestudie: Mad-revolution påkrævet, hvis vi skal redde verden

LÆS OGSÅ: Se på bøf, bil og bolig: Tre gode råd til en klimavenlig hverdag

- Artiklen må ikke redigeres og skal bringes i fuld længde (medmindre andet aftales med forskeren).
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Læs mere om Forskerzonen i Forskerzonens redaktionelle retningslinjer.

Kilder

- Mikkel Hansens profil (DTU)
- Timothy John Hobleys profil (DTU)
- Peter Ruhdal Jensens profil (DTU)
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APPENDIX D

DTU Food Conference Poster

At the DTU Food conference; Healthy, Safe and Sustainable Foods of the Future I gave short pitch about the project in the "PhD corner" (https://www.youtube.com/watch?v=sL1Ofz_zPKw&list=PLDxOjeUXS7wJqcoeqvaLVzynB0rGzqZ2M&index=12). At this conference I also presented a poster where all the work done during this PhD project was presented. The poster can be seen below.

DTU The National Food Institute
Healthy, Safe and Sustainable Food of the Future
13-10-2022

InnoGrass
Green proteins for humans

Mikkel Hansen, PhD Student, DTU Food, Microbial Biotechnology and Biorefining

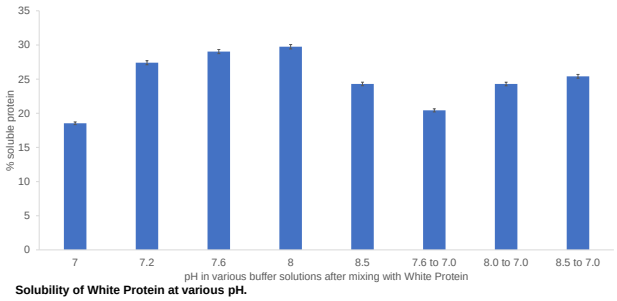
To produce 1 gram of beef protein we need to feed the cow 10 grams of plant based proteins. Why not eat the plant based protein instead of the cow?

Alfalfa is known for:

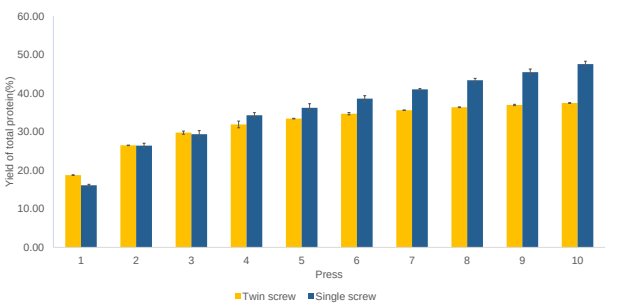
- Contains all essential amino acids
- 10 times higher protein yield per acre compared to soy
- No or low need for watering
- Resistant towards most pests
- Fixates nitrogen

The whole plant is considered safe to consume by EFSA, but not yet evaluated as a protein concentrate. The protein concentrate taste, according to some consumers, as “horse staple”, which limits its usages in formulation of foods.

Atribute	White Protein	White Protein treated with SFE
Protein(% DM)	57.00 ± 0.44	64.56 ± 0.43
Fat (% DM)	0.12 ± 0.16	0.00 ± 0.00
Digestibility	93.28 ± 0.89	92.65 ± 0.64



Solubility of White Protein at various pH.

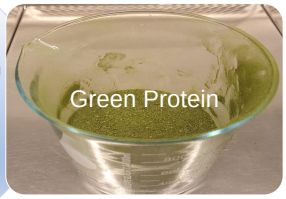


Two different extractors used to extract green protein from alfalfa. Here the resulting pulp has been repressed up to 10 times.

- Raw alfalfa is harvested and transported to production plant

- Alfalfa is macerated and pressed to produce green juice. Side-stream is pulp

- Juice is heated to precipitate fiber rich green protein for feed. Liquid fraction is further processed.



- pH of liquid fraction is lowered to precipitate white protein and afterwards dried. Side-stream is brown juice.

- White protein is processed through SFE to remove off flavours. Side-stream is natural yellow colouring (xanthophyll).

- White protein can substitute foaming and gelatinizing proteins, such as eggs, in various foods. Own studies has shown a significant decrease of the taste of “horse-staple” after SFE treatment on the white protein.

Chocolate muffins made to investigate texturizing properties of White Protein.

- Topleft=Control without any protein or egg
- Top-right=Standard with egg
- Bottom-left=Egg substituted with White protein
- Bottom-right=Egg substituted with white protein treated with SFE.

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