Solid-state fermentation of the microalgae *Scenedesmus* sp. for improved conservation and protein digestibility

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Master Thesis

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Abstract

As part of a research grant aimed at producing chicken feed from the novel microalgae *Scenedesmus* sp., called the ReMAPP project, this thesis investigates opportunities to increase the conservation potential and protein digestibility utilizing solid-state lactic acid fermentation and commercial enzyme additives. The lactic acid fermentation mimics the traditional agricultural ensiling process, where crops are anaerobically fermented at room temperature for upwards of a year. The ensiling process conserves the material by using lactic acid bacteria to convert water soluble carbohydrates in the biomass to mainly lactic acid, lowering the pH and preventing growth of pathogens. This approach to microalgae conservation has not been attempted previously. Enzyme additives are hypothesized to degrade cell wall polymers to monosaccharides and are used to increase the substrate for lactic acid production, further decreasing the pH and thus improving the silage quality of the microalgae. At the same time, the protein digestibility of the specific microalgae species is hypothesized to be limited by bioavailability as the incalcitrant cell wall of *Scenedesmus* could prevent access to intracellular proteins, rendering them indigestible. The same enzyme additives used to improve fermentation could also affect the protein digestibility, since degradation of cell wall polymers could release intracellular proteins.

To investigate the fermentation properties and protein digestibility, lab scale fermentations are carried out where *Lactoplantibacillus plantarum* and a variety of enzyme additives are mixed with *Scenedesmus* sp. biomass and left in vacuum-packed bags to ferment for 21 days. The results show bacterial inoculation by itself to be effective in decreasing pH to desirable levels but fermentation with β-glucanases, which seem to degrade polyglucans to glucose which are consumed by the lactic acid bacteria, proved the most effective. Only fermentations with β-glucanases achieved pH < 4.0, a benchmark for product safety.

The effect on protein digestibility did however seem to be largely negligible. Lactic acid fermentation by itself did increase the protein digestibility, measured in PDCAAS, from 0.79 to 0.83 which is a relevant increase but still below the gold standard set by soybean. Using additional enzymes had only a marginal effect on increasing protein digestibility, and as such the hypothesis of degrading the cell wall to increase protein digestibility cannot be confirmed by this study. Endoproteases increased protein digestibility the most, the do likely not increase protein digestibility by degrading the cell wall but rather by hydrolyzing peptides. The most successful non-protease in terms of protein digestibility was a pectinase which was unexpected since no galacturonic acid, the main monomer of pectin, could be detected in the sample. To determine the cause of increase in protein digestibility and the effect pectinases have on the biomass, in-depth characterization on the biochemical composition of *Scenedesmus* sp. would be required.

In conclusion, solid-state fermentation of *Scenedesmus* sp. biomass using lactic acid bacteria and enzyme additive was successful and could potentially be a useful conversation method for green microalgae in general. While some enzymes tested did yield an increase in protein digestibility where a pectinase and an endoprotease proved most effective, the hypothesis of releasing intracellular proteins to increase digestibility in combination with fermentation could not be confirmed.

Popular science summary

Microalgae are, as the name implies, microscopic individual algae cells which are present in almost every natural water source in the world. Thousands of different types of microalgae exist, where a few have been found to both be easy to grow in reactors and contain a lot of protein. One recently discovered kind, called *Scenedesmus* sp., is up to 55% protein and grows quickly which makes it an interesting candidate for creating cheap and sustainable chicken feed. The science of using microalgae in food and feed is very new, and a lot of research is required to make production cheaper and competitive with already existing feed alternatives like soybeans.

Two of many important questions to answer to create a successful product is how to store the algae without it rotting and how to draw out protein out of the algae cells. The first question is applicable to many foods. If the algae are left for too long dangerous bacteria will grow on it, making in unsuitable to eat. How can we prevent this? The second question is more specific to microalgae. The small cells have thick cell walls, which can be thought of as a shell and prevents the animals eating the algae from absorbing the protein since the protein is inside the shell. Can breaking the cell wall increase the amount of protein which the animals can absorb?

This project attempts to answer both questions using the same method: fermentation with both lactic acid bacteria and enzyme mixtures. Lactic acid fermentation is a very popular method in many foods, like when creating sauerkraut and kimchi. Essentially, safe bacteria (in this case one called *Lactoplantibacillus plantarum*) consume the sugars in the algae and create lactic acid, which makes the whole material very acidic. Other dangerous bacteria which cause rotting cannot grow in high acidity, and as a result the algae are preserved. To improve the fermentation, enzymes which break down the cell wall into smaller parts can be used. The cell wall is in large part composed of cellulose, which is a long molecule composed of many individual sugar molecules. The enzymes release the individual sugars from the cellulose, which the lactic acid bacteria can eat and create more acid to make the material even more inhospitable to dangerous bacteria. At the same time, it is hypothesized that the cell wall being broken down by the enzymes could also release proteins from the inside of the cell. The same enzymes which improve the fermentation quality could as a result also be found to increase the protein digestibility of the microalgae.

The study did find the fermentation part of the project to be successful. The acidity of the algae did increase significantly when only fermenting with the added safe bacteria. When also adding the cell wall degrading enzymes called β-glucanases, the algae became even more acidic, to the point where it is acidic enough deny any growth of dangerous bacteria. It was also found that a lot more of the sugar in the algae had been eaten when the enzymes were added, which indicates that the hypothesis was correct. However, no such clear confirmation could be found in increasing the protein digestibility. Even though the cell wall seemed to be damaged, almost no difference in protein digestibility was detected. Some enzymes did have an effect. Endoproteases increased protein digestibility, although they break down proteins into smaller parts which are more easily digested which means they work in a different way than what was hypothesized to increase the protein digestibility. Another enzyme, pectinase, was found to be somewhat successful in increasing the digestibility. However, pectinases only break down a substance called pectin, which could not be found in the algae. It is therefore unknown why it worked at all. To conclude, while fermentation seems to be a good way to conserve algae, it could not be said to have a meaningful effect on increasing the protein digestibility.

Table of Content

Abbreviations

Abbreviations appearing in the text, presented in alphabetical order.

Abbreviation		Meaning
AAS	-	Amino Acid Score
CAS	-	Chemical Abstracts Service
CWD	-	Cell Wall Degrading
DM	-	Dry Matter
HPAEC-PAD	-	High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
HPLC	-	High Performance Liquid Chromatography
LAB	-	Lactic Acid Bacteria
LOD	-	Limit Of Detection
LOQ	-	Limit Of Quantification
NaOAc	-	Sodium Acetate
NaOH	-	Sodium Hydroxide
NREL	-	National Renewable Energy Laboratory
PD	-	Protein Digestibility
PDCAAS	-	Protein Digestibility Corrected Amino Acid Score
SEM	-	Scanning Electron Microscopy
TRIS	-	Trisaminomethane
WC	-	Water Content
WSC	-	Water Soluble Carbohydrates
wt%	-	Weight percentage

1. Theory and Background

1.1 Introduction and gaps in science

Microalgae, which in day-to-day life is mostly seen as just a slight green tint in water, has huge potential in biotechnology applications. Chief among them is the application as a novel protein source, due to high protein content in some species. The variety among microalgae is as large as between land-based plants, of which one novel species of *Scenedesmus* with high protein content and growth rate was identified in a pond in Denmark. This sparked the ReMAPP-project which aims to create chicken feed from the microalgae, but some gaps in science remain to create an economically feasible product. The main aim of this thesis is to investigate whether the algal material can be fermented as a means of conservation, using bacterial inoculum and commercial enzyme additives. The thesis also aims to identify any effects the treatments have on protein digestibility, an important aspect of any food or feed application. Finally, the thesis aims to increase knowledge regarding the characteristics of the cell, since almost no previous documentation is available.

1.2 Microalgae - a diverse classification

Microalgae is a broad label and is applied to a large polyphyletic group of organisms. The term *alga* has no inherent taxonomic meaning and *micro* simple refers to the individuals being microscopic, and as such the different genus included are as varied as any land-based plants (de Carvalho et al., 2020). Primarily, most microalgae are photosynthetic organisms found in almost all aquatic environments and feature simple macrostructure, meaning they lack multicellular structures such as leaves or roots. However, all microalgae are not unicellular, and can often grow as colonies, as small groups of connected cells or even filament shapes. The group is extremely diverse and ill-defined. The common understanding of microalgae includes both procaryotic varieties such as cyanobacteria and blue-green algae, and eukaryotic varieties with all associated intracellular structures (Andersen, 2013). This makes research into and industrial applications onto microalgae a complex task, as the morphology and composition of each species will vary greatly, and universally applicable solutions are usually ineffective.

The ultrastructure of microalgal cells, referring to the intracellular structures and cell wall composition, is as diverse as the overall morphology. Microalgae are among the first organisms to develop in the history of life a lot of unique adaptions have evolved from their presence in a wide range of aquatic environments. Some cells have relatively simple ultrastructure, like cyanobacterial cells. These feature thylakoids, the reaction site for photosynthesis, but otherwise resemble many forms of bacteria. The eukaryotic varieties are vastly more complex and specialized. These varieties also feature thylakoids which make out the chloroplast, a defining organelle. The thylakoid plastids come in many different forms, for example as stacks like common plants within the charophycean class, or as forming lamellae in sheets within the haptophyte class. Due to the varying structures for energy production, different algal varieties will also feature different strategies for energy storage within the cell. For example, only green algae store the products of photosynthesis inside the plastids in the form of starch grains, while other species have carbohydrate storage outside the plastid (Andersen, 2013). Lipid bodies are also a common storage in many species, which can contribute to a high lipid content. The formation of lipid bodies is often dependent on growth conditions such as nutrient and light availability. The manipulation of growth conditions such as light intensity, nitrogen limitation and CO_2 availability to force a higher lipid or protein content is an important principle in industrial applications of microalgae (Alishah Aratboni et al., 2019). The protein content can

also be manipulated through growth conditions and generally varies depending on the growth phase. Some green algae can be composed of up to 60% protein per dry weight although the content is higher during the logarithmic growth phase. The exceptionally high protein content can make microalgae desirable as single-cell protein source for feed or food and can be an effective additive especially.

A very significant structure which comes in many different forms and biochemical compositions is the cell wall and membrane. Like all cell walls, these allow for control over the turgor pressure. For the microscopic organisms, the cell wall is also the main defense mechanism against external threats, and many species has as such developed incalcitrant cell walls, with structures such as scales, thecae and lorica which are various forms of protective armor. The organization of the cell wall is also connected with the reproduction of the species. For example, reproducing through a process called desmoschisis, where the cell partitions into two new daughter cells, allows the formation of a rigid wall encompassing the new biomass, whereas some cells partly digest the mother cell wall and use it as a scaffold for developing new daughter cells (occurs in some *Chlorella,* typica cell shown in figure 1f) and other completely discard the mother cell wall and forms new material in the daughter cells(Andersen, 2013; Trainor, 1996). Other genus, like *Scenedesmus,* reproduce through autospores (Hegewald, 1997)

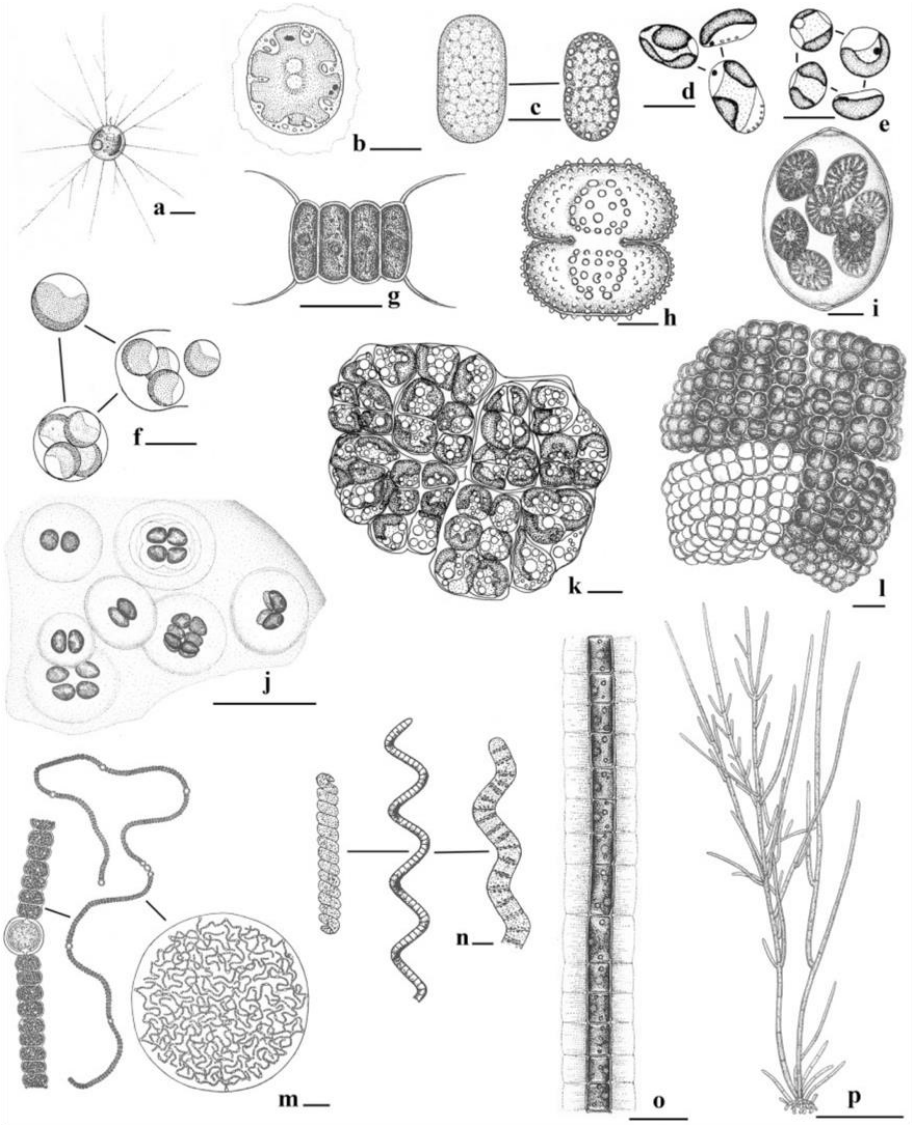

Figure 1. Examples of high microalgal morphological diversity. A) Chrysamoeba mikrokonta, amoeba with pseudopods. B) Porphyridium purpureum, unicellular red algae with chloroplasts. C) Synechococcus aeruginosus, freshwater cyanobacteria. D). Nannochloropsis salina, three coccoids.E) Nannochloropsis oculate, four coccoids. F) Chlorella Vulgaris, top as single cell, bottom with four autospores, right releasing autospores. G) Scenedesmus Maximus, connected cells with protruding structures. H) Cosmarium ornatum, a typical desmid. I) Oocystis gigas var. incrassata eight cells within original cell wall. Scale bar is 20 µm. J) Phacomyxa sphanicola, a palmelloid algae in gelatinous matrix. K) Botryococcus braunii, colonial species with high lipid quanitity (white spots). L) Chlorosarcina superba, cubic colony. M) Nostoc planctonicum, a trichome (enlarged on left, single in middle, colony on right) Scale bar is 5 µm, 25 µm, 50 µm left to right. N) Spirulina Arthrospira, morphological form of a trichome (Scale bar is 10 µm, 18 µm and 5 µm left to right). O) Ulothrix Moniliformis, unbranched filamentous algae. P) Cladophora sterrocladia, branched filaments. Scale bar is 250 µm. For C,D scale bar is 2 µm. For A, B, scale bar is 5 µm. For C, F, G, H, K, L, O scale bar is 10 µm (Andersen, 2013).

The differences are also evident in the biochemical composition of the cell wall. Cyanobacteria, like most procaryotes, have a layered structure with peptidoglycan. Others, like the diatoms commonly known as phytoplankton have outer layers of opaline silicon which give them unique biochemical properties. Some mineral based cell walls can potentially be utilized in the bioeconomy, for example the coccolithophorid algae which create layers of $CaCO_3$ from dissolved CO_2 increasing their use in carbon scrubbing. While these more exotic cell wall structures are unique among all plants, some species feature biochemical compositions more phylogenetically related to embryophytes (land plants). Green algae, like *Scenedesmus* and *Chlorella* are more related to land plants (Andersen, 2013).

The green algae, with discrete chloroplasts, are the genetic ancestor to all embryophytes and migrated onto land 470 million years ago (Domozych et al., 2012). Common compounds also found in higher embryophytes like cellulose, hemicellulose, pectin and extensin can be found in many green algae. However, large phylogenetic distance and genetic drift gives rise to differences in glycosylation patterns and prevalence of certain sugars and amino acids. Even among this more closely related group, many unusual structures and compounds can be found. There are varieties which do not possess any cellulose but rather construct a cell wall made compromise entirely of crystalline glycoprotein, and a long chain polymer known as algaenan are prevalent in some species as well (Domozych et al., 2012). Algaenan consist of highly aliphatic polymethylenic chains connected with ester and ether bonds (Blokker et al., 1998) and is highly relevant to industrial applications since it is resistant to many processing treatments (Scholz et al., 2014). *Scenedesmus* is in many cases described as a typical green algae, with the common biochemical features, as well as a large component of polymer of varying description in the cell wall (de Carvalho et al., 2020).

The overall biochemistry in green algae is also most like embryophytes, and starch is the most prevalent carbohydrate storage mechanism. In green algae, these starches contain the same β-1,4 glucan links with α-1,6 linked side chains as embryophytes whereas heterokonts like the diatoms utilize a polymer with β-1,3 linked glucan backbone. Starch is a prevalent storage mechanism, but some green algae, for example *Scenedesmus,* does produce large oil droplets under growth duress such as when nitrogen limited.

The Scenedesmaceae family are green algae belonging to the Chlorophycae taxon and includes genus like *Acutodesmus* and *Scenedesmus*. The species used in this project is identified as a *Scenedesmus* which grows in flat coenobiums, a type of arranged colony, with an even number of cells grown in two rows. Typically, each coenobium is composed of four cells but more and less are possible as well as individual free cells. The *Scenedesmus* genus reproduces by autospores grown within the mother cell, which form a new coenobium (Hegewald, 1997). The cells are elongated, which fits the genus, with acute angles at each cell pole. The outer cell surface is smooth, without warts or other structures which are sometimes present in *Scenedesmus.* Some taxa also feature elongated spikes at each corner of the coenobium, which the identified species seems to lack. Within the genus, six subgenera have been described and are differentiated by morphology. Due to some of the identified features; acute cell ends, cell arrangement, and lack of outer cell wall structures, the identified species seems to fit within the *Acutodesmus* subgenera. The *Acutodesmus* subgenera has some specific reproductive behavior, in that many species disrupt the coenobium into single cells prior to cell division, and that the formation of coenobium depends on the growth conditions (Hegewald, 1997). The subgenera also generally have three outer cell wall layers, beyond the inner fibrillar layer. These layers are sometimes described as pectinic, sporopolleninic or as being composed of algaenan; the specific literature description varies significantly and much remains unknown (de Carvalho et al., 2020; Hegewald, 1997; Spain & Funk, 2022). However, there seems to be consensus in that an outer layer containing some yet to be identified polysaccharide or aliphatic polymer is likely to be present. The inner cell wall layer is likely to be composed of some fibrillar polymers, commonly hemicellulose and cellulose, although the composition of carbohydrates and sugars present is still unknown.

Although faced with the challenge of high diversity, applications of microalgae can be considered an important frontier towards a sustainable bioeconomy and are very desirable due to the high protein and lipid content that is achievable. The heterogeneity is also one of the

benefits, as different varieties can be used to produce everything from biofuels and biopolymers to food products and vitamin supplements. The microalgae industry can still be considered to be in its infancy, with high potential for growth as development of cheap biomass production and processing techniques develop (de Carvalho et al., 2020).

1.3 Microalgae bioeconomy

The macro component compositions and unique biochemistry and unique biomass growth lend potential to microalgae as an industrially exploitable resource. Challenges remain in both cultivation and processing to make microalgae products economically feasible and a high impact industry.

Microalgae can be an essential part of creating a sustainable economy, yet the industry remains small and must grow to have a significant impact. Reports on state of the algae industry can best be retrieved from the Food and Agricultural Organization of the United Nations, which has reported statistics for fisheries and aquaculture production worldwide since 1950. Significant growth has occurred in the timeframe, with total algae production growing from 0.56 million tonnes annually to 32.7 million tonnes in 2016. With this increase, algae production has grown from 2.8% to 16.5% of the total fisheries and aquaculture sector. A significant trend has also been a shift in production methods and producing countries. In the 1950s European countries like France and Spain were among the top producers whereas the majority of the production today occurs in South Asian countries, like the Philippines and Indonesia (Vigani, 2020). The production shift can be explained by culturally accepted products being more established in Southern Asian countries, which mostly use macroalgae for food consumption. Of the total sales in the aquaculture sector, macroalgae are a significant majority (Vigani, 2020).

Algae can be grown in a variety of reactor setups, from photobioreactor with artificial light to large scale open-pond cultivations. Many algae can be grown both autotrophic conditions, using light as the energy source, in heterotrophic conditions using organic compounds for energy, or mixotrophic conditions where both are provided. Photobioreactors with either artificial or natural light are most common, since open-pond setups have very high risk of contamination unless specific species are used. Photobioreactors has greater control over important growing conditions such as pH, light, nutrients and water quality, allowing rapid algae growth without depending on arable land as with traditional crops (Fernández et al., 2020).

Autotrophic growth yields unique benefits, although higher costs and challenges come from it. Especially when using artificial light since the energy yield of light conversion is normally very low, below 10% (Tredici & Zittelli, 1998). Autotrophic growth does however allow for carbon dioxide sequestration since photosynthesis is utilized and can grow on non-organic carbon in very high concentrations. Highly concentrated carbon in gas streams is a resource available from most large-scale industries, from whose flue gas carbon dioxide concentrations reach up to 15%. These flue gas streams are normally difficult to utilize but can be dissolved in water at a rate limited by Henry's law. Microalgae utilize dissolved carbon as carbon source for growth when grown autotrophically and are thus uniquely positioned to generate biomass directly from industrial carbon capture. Algal growth can however be constrained by temperature and pressure requirements to increase aqueous carbon solubility, which limits how much carbon dioxide can be captured (Patricia Ruiz-Ruiz et al., 2020).

In addition, microalgae have high potential in wastewater treatment and growing on wastewater. Utilizing municipal wastewater seems to hold the highest potential, due to lower content of organic nitrogen and organic phosphorous. The organic N and P content is significantly higher in industrial and agricultural waste, which has been shown to inhibit growth. The microalgae can also be used to accumulate trace metals from various sources and generates valuable products in biomass generation as a byproduct from wastewater treatment. However, microalgae grown on wastewater has higher potential for toxicity due to accumulating compounds such as trace metals, and faces strict regulation to be used for pharmaceutical or food purposes (Martínez-Roldán & Canizares-Villanueva, 2020).

To further grow the industry, in particular the green microalgae, more products and production methods must be established and developed to be financially viable to exploit the unique growth characteristics. Microalgae products does have potential in both high-value, low volume products and low-value, high volume products. The main microalgae product categories being produced today are represented in figure 2, ordered from lowest value per weight unit in the bottom to highest at the top.

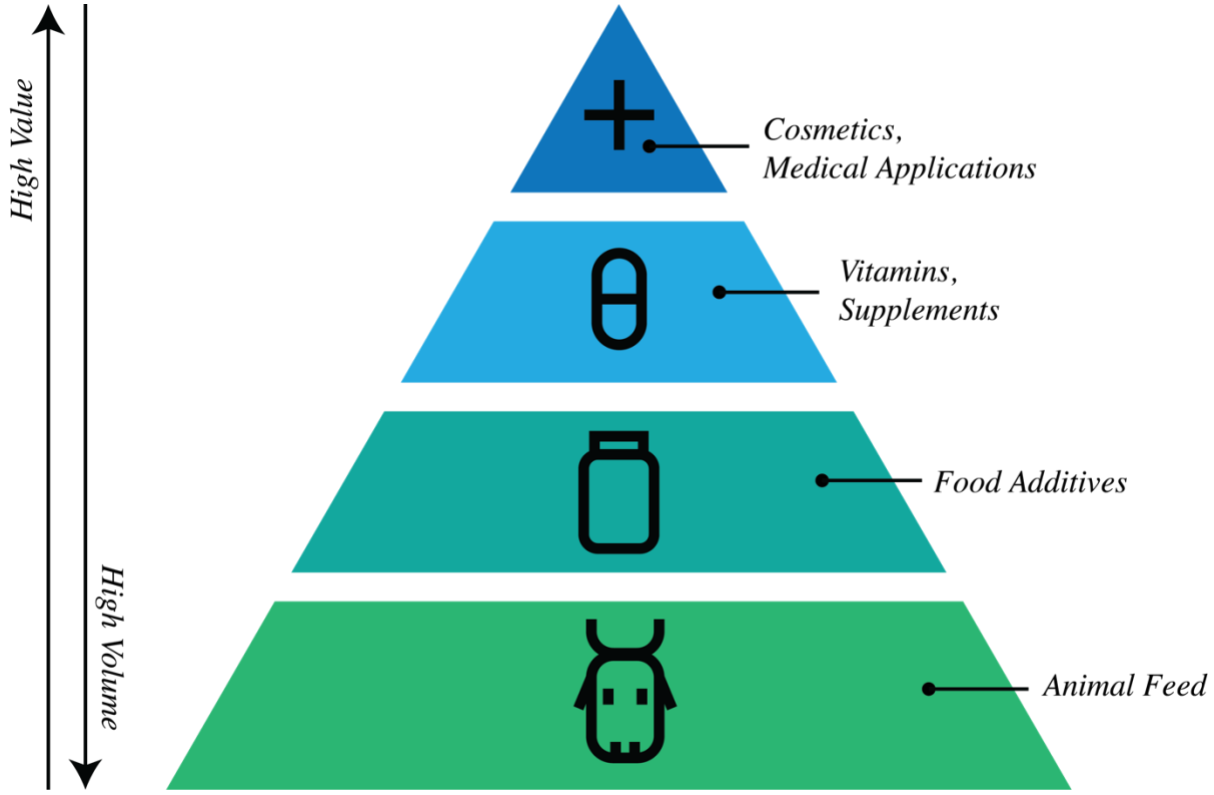

Figure 2. Broad categories of established microalgae products. Highest value, lowest volume products are the top segment.

Each of these product categories faces specific challenges that constrain their current growth. In terms of utilizing microalgae as single cell protein sources challenges both in processing, discovery and regulation (Acquaha et al., 2020).

First, discovery includes screening of relevant parameters regarding the choice of microalgae species. A lot of factors are pertinent to finding a suitable species for economically viable production, including overall protein digestibility, amino acid profile, macronutrient content, growth rate under varying conditions, identifying nutritional factors such as antioxidant content, processing requirements among others. These parameters can also change due to

growth conditions such as nitrogen deficiency, season, or temperature, making evaluation difficult and time consuming. High throughput screening methods and comprehensive testing could contribute to this end, with potentially high upside since many species show promise in terms of protein quality and growth rates. Microalgae are considered to have high potential in covering the protein gap as populations grow worldwide, and the discovery and evaluation of production candidates is an important step. Due to the high variability between species, the characteristics of each species must be assessed individually, making standardized and effective methods crucial. When creating animal feeds considerations to the specific interactions with the metabolic system of the consumer animals also have to be made (Acquaha et al., 2020).

Second, processing conditions and associated costs are significant challenges. The inclusion of microalgae in food products is hindered by poor knowledge of effective processing strategies and limited characterization, making development of algae products unattractive and difficult. For more developed species and production lines, scalability is a significant barrier(Acquaha et al., 2020). Microalgae protein production techniques have so far only been developed on small scales, and other techniques than the ones in current use are likely to be more economically viable (Bleakley & Hayes, 2017). Due to the incalcitrant nature of many species, production costs can be high, which is only exacerbated by the small scale of production. To create feasible production lines, large scale plants and tailormade techniques have to be developed (de Carvalho et al., 2020).

Third, as with all alternative food and feed sources, heavy regulation and product safety concerns are barriers to development. Some aspects of product safety are unique to algae and aquaculture, where depending on the growth environment, some toxic substances such as heavy metals can bioaccumulate to harmful levels. Due to being an effect of growth environment, regulation should be adapted to regional level or to production method (Schiavon et al., 2017). Since one large benefit of growing microalgae is its ability to be grown on industrial flue gas or municipal wastewater where toxic substances are prevalent, regulatory caution should be used (Acquaha et al., 2020). Applying for novel food certification within the EU, granting license to sell and consume the product, is an extensive process with high demands. Additional considerations have to be made to whether the product is to be sold as a whole cell or fragmented, which requires separate application (European Parliament, 2021). Since high expenses and time are required to certify a microalgae, only very few species such as *Chlorella vulgaris* are defined as food grade in the EU, severely limiting the potential products (Janssen-Bouwmeester et al., 2020).

A host of challenges balance out the potentially groundbreaking upsides from microalgae-based products, particularly in protein utilization. Upsides such as not using arable land, carbon fixation, growth on flue gas and wastewater, potentially high protein quality and high growth rate collectively make microalgae an attractive business. Challenges such as high rigidity in cell walls, insufficient screening of production species in terms of macronutrients or functional compounds, and inefficient processing hinder the growth of the industry. As a result of small scale production and undeveloped processing methods the price of microalgae protein is high compared to other plant protein sources, although the costs can be decreased by addressing the challenges (Janssen-Bouwmeester et al., 2020). Decreasing production costs and increase scale is especially significant for protein sources, due to the high volume required and relatively low value per kg.

1.4 The danish strain, *Scenedesmus sp*.

In a study from 2021, Olsen et al. isolate, cultivate and identify a microalgae strain from a freshwater pond in Lolland, Denmark. Through light microscopy and phylogenetic analysis, the strain was determined as belonging to the Scenedesmaceae family, a green algae belonging to the Chlorophyceae taxon (Olsen et al., 2021). A lot remains unknown about the species.

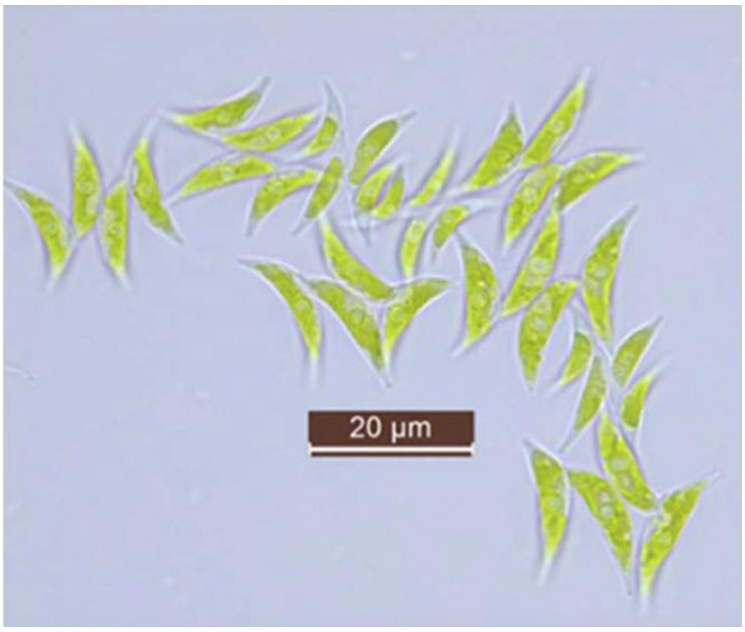

Figure 3. Light microscopy images of Scenedesmus *sp. isolate. Images produced by Olsen et al., 2021. The cells are consistent with previous descriptions of the* Scenedesmus *genus.*

The original paper produced light microscopy images which seem to fit the classification *Scenedesmus*. A light microscopy image showcasing the cell is presented in figure 3. Unpublished electron microscopy images confirm the absence of larger structures in the outer cell wall which is consistent with *Scenedesmus*, although there are some small granulations on the outside of the cell. While not fully explored, such granulations have in some cases of *Scenedesmus* been determined as mere precipitations rather than a cell wall structure (Hegewald, 1997). In other microalgae, like the industrially common *Chlorella vulgaris*, similar small hair-like fibers have been theorized to be a acetylglucosamine-based material (Gerken et al., 2013). From the electron microscopy images, large granules are visible in the cytosol. These are assumed to be starch storage granules, as is common in green algae. As previously mentioned, lipids can be stored in similar structures, but since the algae have not been grown in conditions that yield high lipid content it is more likely to be starch in this case.

From the descriptions of the *Scenedesmus* genus and the previously available images, a simplified diagram of the expected cell wall structure of the identified strain is presented below.

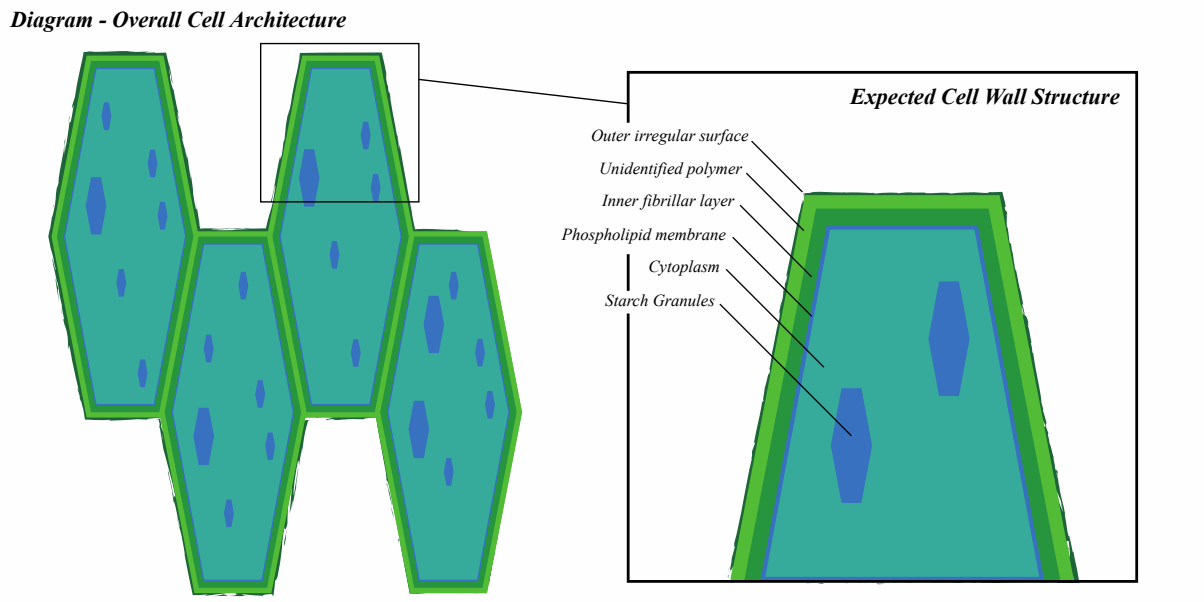

Figure 4. Hypothesized schematic outline of the identified Scenedesmus species with relevant cell characteristics noted.

The figure shows a crude overview of the main structures proposed to be relevant to the study. The previously published study on the strain used an acid Fuchsin staining and light microscopy to investigate the protein content and found high amounts of protein within the cytoplasm (Olsen et al., 2021).

1.4.1 Industrial qualities: chemical composition and growth rate

A key parameter of a successful microalgae product is high growth rate. When grown in pilot scale tube bioreactors, a volumetric growth rate of 0.083 g dry matter L^{-1} day^{-1} was achieved which is comparable and, in some cases, superior to other similar cultivations in a colder climate. In terms of growth rate per land area used, the *Scenedesmus* strain grew at a rate of 6.40 g dry matter m^{-2} day^{-1}. The growth measurements were taken from September from October, and will vary significantly depending on the time of year, mainly due to available light conditions (Olsen et al., 2021). When comparing to the growth of soybean per land area in Brazil, the biomass growth of the *Scenedesmus* species can be expected to be 8-10 times greater. The compound parameters of high growth rate and high protein content with the essential amino acids being present in sufficient levels makes the strain an attractive option for animal feed and food products.

The identified strain also has desirables composition for utilizing as a novel protein source. The previous study defined the overall biomass composition, as presented in figure 5. The crude protein content was determined by summarizing the sum of all amino acids, and the carbohydrate content was estimated as the rest when all other biomass components had been analyzed.

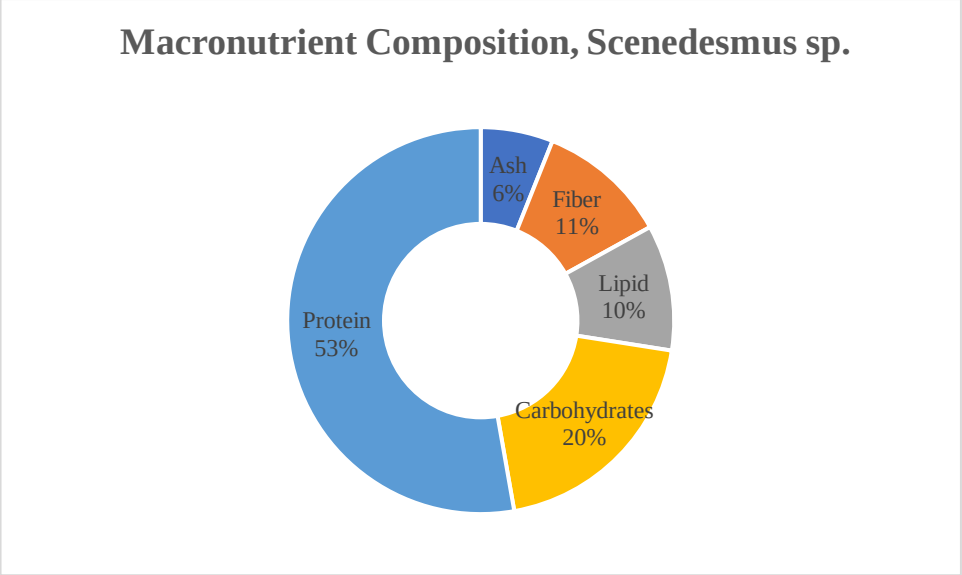

Figure 5. Macronutrient biomass composition of the identified Scenedesmus *sp., in wt% of dry mass (Olsen et al., 2021).*

With the exceptionally high protein content, especially in comparison with the mean protein content of soybean which only has around 31.76% protein content using the same method of analysis, the strain could potentially be valuable as a protein source. The value of a protein source also depends on the amino acid composition, especially the content of essential amino acids. Figure 6 shows the composition of all amino acids in the strain, as compared to the mean soybean content. Since the project is primarily focused on feed for poultry the essential amino acids for chicken feed, which differ slightly from human requirements, are presented with an asterisk in the figure (Chowdhury et al., 2021). Standardized protein quality measurements, which are utilized in the project, are however based on human amino acid requirements.

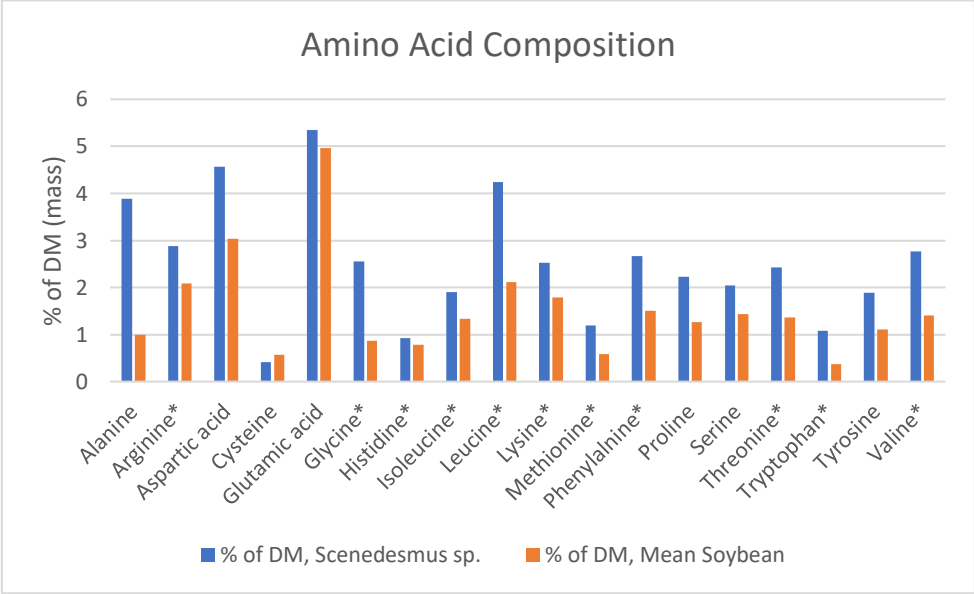

Figure 6. Amino acid composition of the Scenedesmus strain (blue) compared to the average Brazilian soybean amino acid composition (orange). Essential amino acids for humans are noted with a star (*) (Olsen et al., 2021).

Although the protein content is high in the cell, it is unknown whether animals or humans can utilize the high content and from where in the cell the high protein content stems. Limited research has been conducted on the cell wall compositions, although some assumptions can be

drawn from previous studies. The most ubiquitous components of green algae cell walls are neutral sugars, uronic acids, glucosamines and protein, where an early study found the *Scenedesmus obliquus* cell wall to be 39% neutral sugars, 1.2% uronic acids, 15% protein, completely lacking in glucosamines. 45% of the composition was not part of the ubiquitous categories, and could be other polymers like chitin or algaenan (Blumreisinger et al., 1983). Cell walls compositions of other *Scenedesmus* are reported to be a majority neutral sugars from dietary fibers material with an outer layer of algaenan, glycoproteins and glucosamine biopolymers. Although the literature is somewhat conflicting on exact components, the high protein content seems to stem in a minority of cell wall glycoproteins, with the vast majority of protein being intracellular (Machado et al., 2022). Studies on almost exclusively the green algae *Chlorella* , since it is one of the few microalgae accepted as food material, have connected higher protein digestibility to cell wall disruption techniques (Doucha & Lívanský, 2008). Reduced protein bioavailability is connected to recalcitrant cell walls, which must be disrupted (Safi et al., 2013).

The high protein content, presumed to be due a majority intracellular proteins, and the correlation between decreased protein digestibility and a complex, fibrous cell wall is key to the project. To create an economically feasible single cell protein product, both high protein content and high protein digestibility is required. It is assumed moving forward that degrading the cell wall should have an impact on the protein digestibility of the final product.

1.4.2 Proposed processing background of the ReMAPP project

The ReMAPP project, short for Resource Efficient Microalgae Protein Production, aims to utilize the identified *Scenedesmus* strain. The collaboration was established in 2018 with the goal of producing animal feed using the high quality protein from the local microalgae strain, using a circular economy approach (Pedersen, 2018). The project strives to outline a production method, from microalgae cultivation using residual gas from the biogas industry to final production formulation as animal feed targeting Danish chickens. An outline of proposed production steps is presented in figure 7, with cell concentration and solids weight percentage from typical, similar microalgae production lines (de Carvalho et al., 2020).

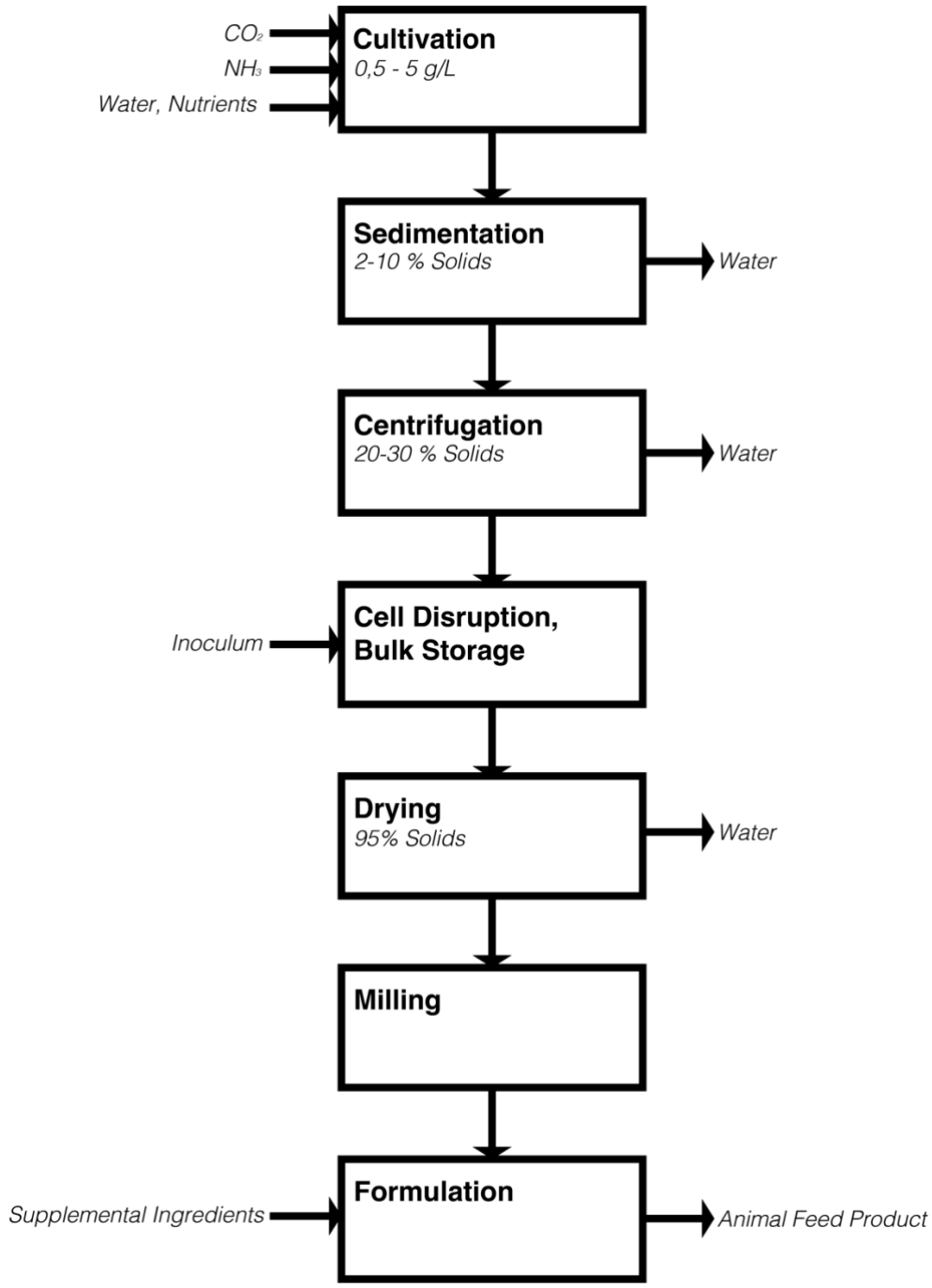

Figure 7. Standard flow chart of proposed production steps of the production of chicken feed from microalgae grown in photobioreactors.

In the final product, the algae will be utilized as a single cell protein (SCP) source for chicken feed. The highest cost step of most microalgae products, including utilizing the cell as SCP, is the drying steps, which can be up to 75% of operational costs (Rajshree Amrut Patil, 2020). Drying in this description includes all water content reducing steps, going from microalgae suspended in aqueous solution to a 95% DM powder. Drying is generally required chronologically early in the process, for product stability since high water activity can cause growth of contaminants. One *Scenedesmus* production plant in Almería identified freeze-drying and storage as accounting for 31.6% of equipment operation cost (Acién et al., 2012). This project focuses on energy efficient storage and protein extraction, an additional high-cost step for products targeting intracellular components.

Microalgae cell wall disruption

For processes which target intracellular products, additional cell disruption is required to access the product. These are generally categorized into mechanical and non-mechanical methods, which each come with pros and cons. Mechanical methods like bead-milling, sonication, high-pressure homogenization are often applied but are very energy intensive and lack scalability (Rajshree Amrut Patil, 2020). The energy requirements for mechanical disruption also scales with the thickness and structure or cell walls, where families with complex cell walls like *Chlorella* and *Scenedesmus* require significantly more energy input (Ometto et al., 2014). Non-mechanical disruption techniques have traditionally been chemical approaches, enzymatic hydrolysis, or thermal treatments. Chemical and thermal treatments are more scalable, but can to a larger extent cause damage to the products or be hazardous in the final product (de Carvalho et al., 2020). Enzymatic treatments are gentler to the material, less energy intensive and potentially scalable although the purchasing cost of enzymes can also be significant. Cell wall disruption through enzymatic hydrolysis can also be used in combination with other techniques as it can increase porosity and reduce structural integrity. However, using enzyme technology is not universally applicable to microalgae, as it requires high specificity to its substrate and the species vary in composition of targetable polysaccharides (de Carvalho et al., 2020).

There has been mixed success in previous studies targeting the cell walls of various microalgae. One paramount study was performed by Gerken et al. in *Chlorella vulgaris,* where lysozyme showed significant results in increasing the porosity of cell walls. The best results were achieved when using lysozyme in combination other enzymes like chitinases. The increase in effectiveness from using multiple enzymes was theorized to be due to the layered cell wall structure, where different enzyme activities are required for the separate layers or polymers, with lysozyme being particularly effective on the outermost layer (Gerken et al., 2013). Another study by Ometto et al. compared various disruption techniques on *Scenedesmus Obliquus, Chlorella vulgaris* and *Spirulina maxima,* where enzymatic disruption proved to increase yield and reduce energy requirements significantly. Different enzyme combinations where tested, where the combination of esterase + protease or cellulase + pectinase was most effective on all species. *Scenedesmus* showed susceptibility to single pectinase and single esterase as well. Another study by Zhang et al. found a combination of cellulase, xylanase and pectinase to be effective in increasing lipid extractability in *Scenedesmus,* for biogas production (Zhang et al., 2018).

A cell wall disruption method is presumed to be necessary to increasing the bioavailability of intracellular proteins. To increase the protein digestibility of the final single cell protein product, enzymatic hydrolysis will be investigated in the project. Enzymatic hydrolysis seems to be an energy efficient and scalable method, which is unlikely to damage the protein. However, since the cell wall composition of the microalgae is unknown, finding enzymes with relevant activity is a challenge.

Cell wall disruption could potentially also be correlated with drying costs. Drying is a complex process of combined mass and heat transfer, and the drying process is divided into two major phases. The initial drying is called the constant rate period and is limited by external heat and mass transfer. Water evaporates at a constant rate, limited by the transformation into gas phase. The falling rate period instead depends on internal heat and mass transfer. The falling rate period begins when the surface is no longer water saturated, and water must diffuse through the material before evaporating. The evaporation rate continually decreases as the water content

of the material decreases (Doran, 2013). The diffusion rate of water through the material depends on the porosity of the material, as seen in equation 1.

$$\frac{D_A \varepsilon}{\tau} = D_{eff} \quad (1)$$

D_A is the binary diffusion rate, τ is the tortuosity, and ε is the porosity of the material. As equation 1 shows, increasing porosity directly increases the effective diffusion rate, D_{eff}. (Joardder et al., 2016). As enzymatic hydrolysis has been shown to increase porosity and damage the diffusion restricting cell wall, there could potentially be a positive effect on drying from cell wall disruption. Degrading the cell wall could however also increase cell wall thickness, as the structures are loosened, which would have a negative effect (Gerken et al., 2013). Either way, evaluating drying parameters could be a relevant effect from enzymatic hydrolysis of microalgae which would have significant economic impact on operational costs.

Another challenge is the storage of microalgae, as it is susceptible to contamination and costly (Acién et al., 2012). In the ReMAPP project, algal growth depends on sunlight and will thus vary significantly seasonally (Olsen et al., 2021). Consistent supply from the photobioreactors would be an issue, and long-term storage upwards of a year would be required. A traditional approach to the issue, used extensively in agriculture, is ensiling.

1.5 Solid-state fermentation

Ensiling is a historically established method within agriculture and is used across the sector to store vegetable crops in a stable state, without large energy or material input over time. The principle of silage can be described as a form of anaerobic solid-state fermentation, using the biomass as substrate. In an agricultural setting, silage is commonly produced in silos with the process occurring in four stages. While ensiling is very established in traditional biomass like corn and wheat, the application to microalgae is novel. The same principles of ensiling agricultural biomass are assumed to be applicable to microalgae.

The initial aerobic phase occurs as the forage is packed into the silo, and the container is hermetically sealed. During this phase the biomass will continue to respire, reducing the oxygen content over time and creating an anaerobic growing environment without any extra input. The naturally occurring and varied population of microorganism on the harvest will begin growth during this stage, and the temperature and oxygen content allows for many potentially harmful or quality decreasing organisms to grow. To ensure a safe and nutritious silage, the initial phase should be minimized and is usually no longer than a few hours.

Once the oxygen content has been depleted, organisms utilizing anaerobic fermentation will outcompete the previously growing population if the harvest is a suitable feedstock and the seal is sufficient during what is known as the main fermentation phase. An important aspect of the feedstock is the inherent water-soluble carbohydrates (WSC) present and the dry matter content (Benjamim da Silva et al., 2022), where a higher dry matter content is generally desirable to achieve a stable fermentation. The water-soluble carbohydrates act as the available growth substrate for the organisms, and the strains that can grow the fastest on the substrate quickly become dominating. In most ensiling, high growth of lactic acid producing bacteria (LAB) is the desired outcome. The LAB ferment the WSC into organic acids, mainly lactic acid, which decreases the pH of the silage over time. As the conditions become more acidic, the LAB are generally favored because of the acidophilic nature of the flora and will outcompete all other organisms. The phase continues until the pH level stabilizes at a level where even the LAB can

no longer continue growth. The fermentation produces a high concentration of lactic acid, alongside some gas and other effluent, mainly other undesired organic acids. One determinant of a high-quality silage process during this stage is the dry matter loss. If the strains produce many byproducts which do not contribute to the drop in pH, more dry matter and carbohydrates will be converted into low nutrition products. High dry matter loss is usually a consequence of an undesired strain starting to dominate the fermenting population (Knický, 2005).

Once the pH has stabilized, the stable fermentation phase begins. At this point there is very low microbiological activity throughout the silage. Only certain LAB specific enzymes have continued activity under strongly acidic conditions, which can lead to some losses in protein quality and carbohydrates due to protein and polysaccharide hydrolysis. These activities contribute to a slow degradation in the quality of the silage, setting a soft limit on the storage time. Under the acidic conditions, pathogens are normally inactive although some endospores can remain dormant in the silage. If the main fermentation phase was not fast enough or the silo conditions are disturbed, some endospores or other dormant pathogens can resume some slow growth, potentially ruining the product if in storage for too long. The ability of the silage to remain acidic and not develop secondary fermentation is referred to as anaerobic stability, and can usually be detected through dry matter loss, high gas formation and the pH increasing (Knický, 2005).

After the main storage period, the final feed-out stage occurs when reopening the silo. The oxygen content resurges, allowing potential growth of aerobic (often pathogenic) organisms which can grow on the remaining WSC or the previously produced fermentation products. High quality silage can resist the growth for a longer period post returning to high oxygen levels, which is referred to as the silage having high aerobic stability. As previously, undesired growth can usually be detected by dry matter loss, gas formation and increasing pH (Knický, 2005).

While the general principles are assumed to be similar between agricultural and microalgae ensiling, some differences would be relevant. One major difference could be the bacterial flora present. The most common contaminations in traditional silage are *Escherichia coli* (O157:H7), *Listeria monocytogenes*, *Bacillus spp. Salmonella* and *Clostridium.* These bacteria most commonly contaminate the silage when the crops are irrigated with manure (Queiroz et al., 2018), which is not a concern in microalgae grown in photobioreactors. Since generally cleaner conditions are utilized in the closed photobioreactors, lower initial bacterial populations are to be expected, which is an important factor in creating high quality silage (He et al., 2018). The main cause for bacterial contamination in the photobioreactors is likely to be the water supply used, where *E. coli* remains a relevant concern (Harder-Lauridsen et al., 2013). The other contaminants, like *Listeria* and *Salmonella*, which are to be expected in lower initial populations, are still common and mostly correlated to poor fermentation conditions rather than excessive initial populations. Since they are relevant and relatively common food and feed pathogens in general, those contaminants are still considered a defining concern. Many studies have been conducted relating pH levels to the bacterial levels. To ensure safe levels of *E. coli*, the pH should stabilize below 4.0, likewise with restricting *Listeria* growth levels below 4.0 are desirable. Overall reducing the pH is directly related to decreased final populations, meaning lower pH is most desirable. Increasing the rate of acidification also decreased bacterial population (Queiroz et al., 2018).

Clostridium has slightly lower tolerance, and generally only grows at pH 4.6 and above. The proteolytic qualities of *Clostridium* are a potential issue in silage protein. In all silage, some amount of proteolysis is expected, from either inherent plant activity or bacterial growth, like

Clostridium. Such proteolysis products include for example glucosamines, histamine, or cadaverine and are categorized as non-protein nitrogen (NPN) (Huang et al., 2022). These are utilized to a lesser extent in animal digestion and could even be toxic in high levels. *Clostridia* also exhibit higher growth in biomass with lower dry matter content, DM < 30%. Both *Clostridium* growth and proteolysis is inhibited by decreasing the pH as low as possible and rapidly acidifying (Queiroz et al., 2018).

Beyond reducing pathogenic growth ensiling often improves the quality of the fermented biomass, although the particular effects depend on the biomass, inoculum and additives (Okoye et al., 2023). An increase in dry matter digestibility and ammonia, has been reported as a consequence of ensiling, which is generally desirable (Li et al., 2018). A decrease in methane produced by animals has also been reported, thought to be due to a higher degree of digestibility reducing the amounts of digestion products from the intestinal microbiome (Oskoueian et al., 2021). Solid state fermentation, like ensiling, also has significant effects on the sensory characteristics of the biomass. Due to the changes in pH and presence of lactic acid, chlorophyll is degraded, and acidic taste becomes dominating (Santra et al., 2021). A change in sensory profile would be positive for algae in general, as commercial, and cultural acceptance of it is low and often dominated by a bitter taste, grassy odor, and high umami in algal species with a high protein content. Algal products where the natural sensory characteristics have been disguised would are generally preferred, which the high acidity and degraded compounds would contribute to (Matos et al., 2022).

Ensiling is a traditional method for conserving agricultural biomass. So far, the implementation is largely novel in microalgae, where only some studies have investigated solid-state fermentation for producing hydrogen, extracting lipids or to prove the fermentative potential in some species of microalgae (Bhattacharya et al., 2023; Martelli et al., 2021; Phanduang et al., 2017). Due to the variety between microalgal species, results cannot be treated as universally applicable and the fermentative potential in the identified *Scenedesmus* species is unknown. Extended fermentations at room temperature (20 °C) with the intention of conservation is novel to the field of microalgae.

1.5.1 Ensiling optimization parameters

The quality of silage depends on a variety of parameters, and is as mentioned previously mostly measured by pH, dry matter content loss and presence of pathogens. Some main factors have been identified as having influence on the quality of silage, where some are inherent to the biomass utilized and some are connected to the fermentation process. The water-soluble carbohydrates are key to the process, in particular the content of glucose and to some extent fructose and sucrose since these are the main sugars metabolized by LAB. The WSC content is inherent to the crop used, although the concentrations can change dependent on the growth conditions and environment of the biomass and the time of harvest (Knický, 2005). The same remains true for microalgae, as the structure and composition varies a great deal depending on their current growth phase (Spain & Funk, 2022).

The dry matter content can be manipulated in most crops, by wilting or drying before fermentation and can be manipulated in microalgae by dewatering and drying. The dry matter content is mostly significant as it relates to the water activity and water availability to the microflora. Most LAB can thrive in conditions with lower water activity than the most prevalent pathogens, like *Clostridia*, since water activity is related to the dry matter content, lower dry matter content is generally desirable for safer ensiling (Knický, 2005).

Some other methods to manipulate the biomass can be utilized to control the silage quality. Mechanical treatments like laceration have been used to destroy macrostructures of the biomass, releasing water and WSC which accelerates the acidification process. Mechanical treatments also increase the packing density of biomass, which reduces air pockets and further increases the acidification process (Knický, 2005). Since microalgae lack macrostructures and can be packed with high density it seems a good candidate for ensiling, especially when combined with some method to release WSC.

A very common method to control silage quality is the use of various additives, most commonly various lactic acid bacteria. Some additives are used to stimulate the desired fermentation. As is industry convention, some lactic acid bacteria or combination of bacteria is added to control the fermentation products where rapid production of lactic acid is the most common desired outcome. The choice of LAB should generally fulfill the criteria of high and competitive growth in the biomass with tolerance for high dry matter content, being acid tolerant, feature high growth in the relevant temperature span, and high in lactic acid production. A commonly used candidate which fills these criteria is the facultative heterofermentative *Lactoplantibacillus plantarum,* although it can be supplemented with others to for example increase acetic acid production which is related to increased aerobic stability by inhibiting yeast growth (Muck et al., 2018). *Lactoplantibacillus plantarum* typically produces only lactic acid on hexoses but does produce both acetic acid and lactic acid when fed pentoses. It is desirable due to being able to metabolize many substrates common in plant biomass (Moraïs et al., 2013). Some chemical additives can also be employed, where these generally have the function of being fermentation inhibiting. Compounds like sodium nitrite can be added to degrade nitrite into nitric oxide and nitrous oxide, which inhibits growth of *Clostridia* and enterobacteria. Such additives are however limited by toxicity to animals and have to be carefully monitored (Knický, 2005).

More recently exogenous enzymes as silage additives have been explored. Enzyme additives serve the purpose of degrading cell material, usually the cell wall, in accordance with the principles of lacerating macrostructures. Cell wall material is mostly composed of non-digestible fibrous carbohydrates, like compositions of cellulose and hemicellulose. As such, most studies have focused on cellulases and hemicellulases as hydrolytic enzymes to release WSC from degraded fibrous material. The WSC acts as substrate for the LAB, which supports the acidification process and lowers pH in the final product. Some studies into proteases have been conducted in biomass with recalcitrant structures, like the corn endosperm which is a complex matrix of protein. Some success was found with the enzyme additives, regarding decreasing pH and increasing WSC which makes enzyme additives very relevant in biomass with limited WSC. However, mixed results were found in increasing digestibility because of enzyme additives and increased digestibility seems to depend on the biomass used. In the corn endosperm study, an increase in digestibility was found but the optimal levels was achieved after 10 months of fermentation, meaning that incalcitrant structures can require a lot of time (Muck et al., 2018).

Hydrolytic enzymes mostly have high substrate specificity and only act on specific bonds and compounds, and only have significant activity under specific temperature and pH. Using the correct enzyme therefore generally requires in-depth knowledge on the biomass to be used as substrate, in order to degrade relevant structures (Bhattacharya et al., 2023). Since microalgae are a relatively underutilized biomass with a lot of structural variety where in-depth

characterization is relatively lacking, and many enzymatic degradation trials are conducted with a trial-and-error approach (Gerken et al., 2013; Rojo et al., 2021).

1.6 Protein digestibility

Evaluating protein quality in food and feed sources is a complex process largely dependent on three parameters of the source; total protein content, amino acid composition and protein digestibility. Generally, the amino acid content and protein content remain mostly constant in a food source and are considered inherent properties. The final parameter, protein digestibility, is more susceptible to different treatments.

To properly assess the protein quality, all three parameters should be included. The Food and Agriculture Organization of the United Nations has since the 1980s worked to standardize the method for evaluating protein quality, which resulted in PDCAAS - Protein Digestibility Corrected Amino Acid Score (Leser, 2013). PDCAAS has been generally accepted and acted as a standard tool for several decades and combines a measurement of the amino acid score and the protein digestibility. Amino acid score, shortened AAS, and protein digestibility, shortened PD, must be measured independently for the particular biomass and treatments, and are then combined into the final PDCAAS.

1.6.1 Amino acid score

The amino acid score is a ratio derived from comparing the amino acid composition of the material to the dietary requirement as defined in a joint report by the FAO and the World Health Organization, WHO, in 1991. The score is calculated by comparing the content of each amino acid, as defined in equation 1, and choosing the lowest ratio as the amino acid score. The dietary requirements apply only to essential amino acids, as only they cannot be produced inside the body. The amino acid scores are therefore only calculated on essential amino acids (*Protein quality evaluation : report of the Joint FAO/WHO Expert Consultation, Bethesda, Md., USA 4-8 December 1989*, 1991).

$$\frac{Specific\ amino\ acid\ in\ food\ material,\ \frac{mg}{g\ protein}}{Dietary\ requirement, \frac{mg}{g\ protein}} \qquad (2)$$

The amino acid with the lowest ratio is defined as the limiting amino acid, which in the case of this untreated *Scenedesmus* species is lysine with a score of 0.958. A score of >1 means the protein source completely satisfies the human requirements, and a score <1 means more of the amino acid must be consumed from other sources. All essential amino acid ratios are presented in table 1. The amino acid content determination used a protein content determined by amino acid summary as described by Olsen et al.,2021 and a dry matter content of 20 g DM / 100 g.

Table 1. Amino acid content from the Scenedesmus species (Olsen et al., 2021) and dietary requirements from FAO/WHO report, 1991 (Protein quality evaluation : report of the Joint FAO/WHO Expert Consultation, Bethesda, Md., USA 4-8 December 1989, 1991)

Essential Amino Acid	**Amino acid content, *Scenedesmus* sp. (mg / g protein)**	**Dietary requirement from FAO/WHO report, 1991 (mg / g protein)**	**AAS (amino acid score)**
*L-Cysteine + L-Methionine**	35.58	25.00	1.423
*L-Tryptophan**	23.72	11.00	2.156
*L-Threonine**	53.37	34.00	1.570
*L-Valine**	60.62	35.00	1.732
*L-Isoleucine**	41.73	28.00	1.490
*L-Leucine**	93.13	66.00	1.411
*L-Tyrosine + L-Phenylalanine**	100.16	63.00	1.590
*L-Lysine**	55.57	58.00	0.958
*L-Histidine**	20.21	19.00	1.064

Most amino acids in organisms are mostly bound in protein, a small percentage exist in the cell as free amino acids (Christensen, 1964). The amino acid profile, and by extension the amino acid score, can largely be assumed to remain constant after milder unit operations, although the free amino acids can be more affected. Both unit operations and metabolic state of the protein source affect the free amino acids present (Christensen, 1964). For example, free amino acid content can be reduced or changed when the protein source is used as fermentation substrate as the amino acids could be used as a nitrogen source for bacterial growth (Han & Liu, 2010). High pressure and heat can also have effects on specific amino acids, like lysine becoming indigestible when reacting with carbohydrates in the heat-dependent Maillard reaction (Tomé, 2023). Due to the relatively small content of free amino acids and mild conditions used in this project, the amino acid score is assumed to be constant throughout.

1.6.2 Protein digestibility

Protein digestibility depends on several factors and is measured as a mass balance between nitrogen ingested and nitrogen excreted. The difference is the amount of protein that has been absorbed by the body. However, protein cannot be absorbed by the gut unless hydrolyzed into tri- and dipeptides or free amino acids, which occurs in several distinct steps. The first protein hydrolysis step occurs in the stomach facilitated by pepsins, a family of endoproteases, and highly acidic gastric acid (Tomé, 2023). The acidity is also vital for the action of the pepsin, as it is active at pH 1.0-2.0 (Blanco & Blanco, 2017). Pepsins feature broad protein hydrolysis activity, but has higher activity on phenylalanyl, leucyl and tyrosyl residues. The hydrolysis occurs on almost all proteins, with exceptions like keratins and mucoproteins (Blanco & Blanco, 2017).

A majority of the protein absorption occurs in the small intestine, where further protein hydrolysis occurs under more neutral conditions. These hydrolysis steps are facilitated by, among others, trypsin and chymotrypsin. Active transport systems are then used to transport free amino acids and specific di- and tripeptides into the blood stream, mainly in the duodenum and jejunum which are early parts of the small intestine. If undigested peptides pass through the small intestine they reach the distal intestine, where protein hydrolysis and absorption are instead dominated by the microbiome. The microbial flora utilize various extracellular enzyme

to degrade and absorb leftover proteins, and utilize them in anabolic or catabolic pathways (Tomé, 2023).

To calculate the protein digestibility, comparative measurements to the amount of ingested protein can be made either at the terminal ileum (the end of the small intestine) or in the feces of the organism. Fecal measurements are traditionally conducted and easier to access samples but are somewhat misleading. Since the microbial flora digest and absorb protein in the large intestine, the fecal amino acid profile and content also reflects microbial consumption. Terminal ileal measurements are considered more accurate, since amino acid absorption occurs almost exclusively in the small intestine. Measurements of ileal digesta are however more difficult to extract, and often include surgical procedures or euthanasia of animals (Adeola et al., 2016). Another distinction between true and apparent protein digestibility can be made, as not all absorbed amino acids can be utilized in metabolic processes. Such additional losses are both endogenous and always occur, and somewhat dependent on the diet. True protein digestibility measurements take such losses into account, while apparent measures only compare oral intake to fecal or terminal ileal digesta protein content into account. True terminal ileal protein digestibility is considered the best available measurement but is very work intensive to produce.

Because of the difficulties and animal rights concerns in producing high quality protein digestibility measurements, simplified analysis methods can be utilized. Assay kits which utilize approximative treatment steps, as incubating samples in pH 2.0 with pepsin and subsequent trypsin and chymotrypsin incubation, aim to fit the produced results to previous *in vivo* results. Such *in vivo* results are mostly produced from rat models. The assay utilized in this project, the Megazyme protein digestibility assay procedure - Animal-Safe Accurate Protein (ASAP) Quality Score method, uses previous literature and in-house generated *in vivo* rat model results to fit an *in vitro* procedure with a correlation score of 0.9205 for protein digestibility and 0.9784 for PDCAAS (Megazyme, 2018).

1.6.3 PDCAAS

The PDCAAS is the combined score of the amino acid score and the protein digestibility. To calculate the PDCAAS score of a given sample, the generated protein digestibility score and amino acid score are multiplied. Since the protein digestibility score is generated by assay and potential changes in the amino acid profile post fermentation and enzyme treatment are disregarded, some added uncertainty is added to the results generated in this study. While the method of evaluation introduces some uncertainty, the results can be utilized to investigate some potentially relevant factors to protein digestibility.

A lot of parameters influence PDCAAS. Principal among them is the structure of proteins in the cell, and their susceptibility to hydrolysis (Tomé, 2023). Long polypeptides with a fibrous structure such as collagen and keratin are generally less digestible, while globular proteins like enzymes and albumin are more soluble and digestible. In general, animal protein sources like meats, fish and eggs are the most digestible at 94-97%, followed by plant protein sources like beans and soy at 78-85%. Cereals such as wheat and oatmeal are in a similar region when refined (Tomé, 2023). Anti-nutritional factors (ANFs) are also an important consideration to PDCAAS and are most prevalent in plant protein sources. ANFs are a broad category of compounds which through different mechanism reduce nutritious values. Some prevalent such factors include tannins, phytates and various protease inhibitors. Protease inhibitors are a wide classification but mostly inhibit the activity of trypsin or chymotrypsin, or both. Since the group

is broad, both their inhibition effectiveness and the methods of inactivating them varies. Through pH and thermal treatment some increased PDCAAS is commonly observed (Francis et al., 2001).

Tannins and phytates are also common in several plant species and reduce the nutritional value in several ways. Tannins can bind to both digestion enzymes and feed components such as proteins and minerals, reducing the digestion capability. Phytates have a similar effect on proteins and can form phytate-protein complexes which are difficult to digest. Phytates affect not only proteins but can also bind to several kinds of minerals, further reducing the overall nutritional value. Further ANFs have a lot of different effect on digestibility and nutrition, and are important to consider food and feed products (Francis et al., 2001). A lot of studies on ANFs exist on common plant based feed and food products, like legumes and soy beans in particular, but relatively few studies have been performed in microalgae (Ahmad et al., 2022). One study on *Scenedesmus dimorphous* does however suggest the presence of tannins and trypsin inhibitors in relevant quantities, and that activity of the inhibitors can be reduced via oil extraction (Sun et al., 2022). To analyze ANFs, novel methods like antamers (Ma et al., 2023) or tandem liquid chromatography-mass spectrometry (Wen et al., 2021). Both *in vivo* and *in vitro* PDCAAS analysis methods are influenced by ANFs, making both valid alternatives for analysis. (Ketnawa & Ogawa, 2021; Rozan et al., 1997).

The PDCAAS does also depend on the physical availability of the proteins, and an increase in digestibility has been reported as a result of disrupting the incalcitrant cell wall of some microalgae species. Chemical hydrolysis to degrade the cell walls of *Chlorella vulgaris* and *Spirulina platensis* has previously been shown to increase *in vitro* protein digestibility by a factor of 2, which is significant since the two species feature very rigid cell walls (Kose et al., 2017). Previous literature has in large part focused on mainly *Chlorella vulgaris* and *Spirulina platensis* as feed in aquaculture, where treatments focusing on cell wall disruption has proven successful in terms of apparent digestion (Batista et al., 2020). Beyond microalgae, degradation of cell walls has been positively correlated to digestibility in many plant food sources (Grundy et al., 2022; Lu et al., 2023).

Protein quality is a complex measurement, which itself can depend on many factors beyond amino acid profile and protein quality. Even the measurement used comes at many different levels of quality - whether an assay is used, fecal matter analyzed or digesta extracted. Even then, assessing the protein digestibility requires further knowledge of the material analyzed, whether the digestibility deficiency depends on inhibiting compounds or incalcitrant cell structures. Protein digestibility in microalgae is a relatively novel subject, where these factors remain largely unknown. Some literature does however suggest that protein digestibility can be improved by degrading the cell wall.

1.7 Research questions

The projects aims to investigate the potential of small-scale ensiling on a specific microalgae, *Scenedesmus* sp. The protein quality of the fermented material will also be investigated using *in vitro* assay kits. The main parameters to be used are the addition of commercial enzyme additives and bacterial inoculums, and the overall effect will be evaluated. Some conclusions regarding the structure of the cell wall will also be evaluated based on the results.

The following questions are posed:

- What effect does lactic acid fermentation have on small-scale, time-limited ensiling of *Scenedesmus* sp. in terms of pH, dry matter loss, monosaccharide consumption and protein digestibility?
- What additional effect does using single commercial enzymes and enzyme cocktails have to the small-scale, time-limited ensiling?
- Can the observed effect be connected to saccharification of cell wall material, and can the effects be observed in microscopy?

2. Methods and materials

2.1 Overview

The data collection portion of the project was performed in three phases, chronologically. The first phase, designated "Batch 1" henceforth, focused on initial testing of the fermentation process. Batch 1 tested two types of bacterial inoculation strains to evaluate any potential difference, and a subset of enzyme additives which were deemed interesting from previous literature. The aim of batch 1 was to investigate whether the fermentation process would work, and to overview what issues would be relevant to improve in subsequent phases.

The second phase, titled "Screening", focuses on testing the suppliers' available enzymes in a accelerated process to gain enough results to draw relevant conclusions on which enzymes to proceed with. The overall aim of the screening phase was to choose a subset of enzymes or combination of enzymes which held the most potential in improving the fermentation process and increase protein digestibility. The screening phase acts as pilot studies for the full-scale fermentations performed subsequently.

The third phase, "Batch 2", aimed to use the results from the screening phase and test the enzyme additives in a longer fermentation setting, like the setup used in batch 1. In addition to testing the most successful additives, some other parameters like dosage and effect of mixing are evaluated.

Before fermentation batches and screening experiments, the material was homogenized, and the enzymes categorized. The method and materials section describes the initial preparations, then the fermentation and screening procedures and finally details the analysis methods used.

2.2 Biological sample preparation

The microalgae were supplied from an outdoor cultivation facility from the Danish Technological Institute in Taastrup, Denmark. The material was grown in three separate but identical tubular photobioreactors on level and black surfaces, where the algae growth occurred in 60 m long polyethylene tubes with a diameter of 10 cm. After harvesting the microalgae material was dewatered to reduce the dry matter content to 20-25%. Once dewatered, the material was transported to the University of Copenhagen and placed into frozen storage at -18 °C.

The material used for the project was gathered from different batches grown in separate photobioreactors. Three batches, which were received and frozen at the University of Copenhagen in September and November 2021, were mixed and refrozen at -18 °C to create a single, homogenous sample material to be used for all silage trials and as reference material. Since the composition and ultrastructure of many microalgae species can vary depending on the growth phase the algae is in when harvested (Spain & Funk, 2022), creating a homogenous sample is more representative of the average output of the cultivation facility. The material was mixed using a kitchen-grade Bosch ™ electronic mixer, running on low setting for 15 min.

All enzymes used in the silage trials were supplied by Tailorzyme, except for Alginate Lyase which was purchased from Sigma Aldrich (CAS 9024-15-1). The *Lactoplantibacillus plantarum* inoculum was also supplied from Tailorzyme. FeedTech Silage F10, purchased

from DeLaval, was also used as an inoculum and is a blend of freeze-dried *Lactoplantibacillus plantarum* (5.0 x 10^9 cfu/g), *Pediococcus acidilactici* (5.0 x 10^8 cfu/g) and *Enterococcus faecium* (3.0 x 10^9 cfu/g).

2.3 Enzyme additives

An assortment of enzyme solutions is provided from a research project partner, Tailorzyme, which repackages commercially sold enzyme solutions from other enzyme suppliers. The exact enzymes, enzyme concentrations and other components in the solutions is unknown. All enzyme solution used in the project and the by the supplier stated optimal pH and optimal temperature with respect to activity are presented in table 2.

Table 2. The product name, expected enzyme activity, optimal pH and temperature of each of the available enzyme extract. The optimal conditions were not disclosed for all products.

Tailorzyme ID	Enzyme activity	Optimal pH	Optimal Temperature
Tail56 (Filta-02L)	*β -glucanase, xylanase, cellulase*	3.0-6.5	30-70
Tail26 (Filta-01L)	*β-glucanase*	6.5	50
Tail175	*Xylanase, hemicellulose blend*	4.5-6.5	65-90
Tail200	*Trichoderma Cellulase*	4.5-5.5	40-60
Tail157	*Pectinase, Hemicellulase complex*	4.0-5.0	50-60
Tail73	*Pectinases blend*	-	-
Tail171	*Phospholipase A1*	4	40-70
Tail166	*Phospholipase PLC+PLA2*	5.5	60
Endocut-01L	*Endo-protease*	-	-
Tail204	*Aspergillopepsin I*	2.8	50
Tail31 (ViscoX-01L)	*Xylanase*	4.0-6.0	45-65
Extract-01L	*Endo-1,3(4)-β-Glucanase*	4.5	50
TZ266	*Cellulase/Hemicellulase*	5	50
Filta-04L	*endo-1,4- β xylanase*	-	-
Tail127	*Lipase 1,3*	6.5	30-40
Tail170	*Phospholipase A1*	4.5-6.5	40-55
Tail169	*Phospholipase A2*	-	-
Tail165	*Phospholipase C*	5.5-8.0	60
Tail55	*β -glucanase, pectinases, hemicellulases, xylanases*	3.5-5.5	45-55
CelluX-01L	*Cellulase β-Glucanase*	4.5-6.0	50-60
Sacchary-01L	*Glucoamylase*	-	-
Tail155	*Xylanase*	4.5-6.0	45-70
Alginate Lyase	*Poly α-I-Guluronate Lyase*	6.3	37
Lysozyme	*Lysozyme*	5.0-6.0	20

Detailed data regarding specific enzyme activity and the origin host for the enzymes was not disclosed by the supplier.

2.4 Fermentation and screening procedure

2.4.1 Batch 1 Fermentation procedure

2 kg of the *Scenedesmus* material was left to thaw at room temperature. While thawing, the enzyme solutions and inoculum solutions were prepared. Two different inoculums were screened in the first batch: pure *Lactoplantibacillus plantarum* and a commercially produced mixed strain product, FeedTech Silage F10. 200 mg of each inoculum, initially in freeze-dried powder form, were dissolved in 1 ml distilled water and vortexed. Of the enzyme additives lysozyme and alginate lyase were in powder form. 10.8 mg alginate lyase and 12.2 mg lysozyme was dissolved in 0.27 ml distilled water. The other enzymes were supplied premade liquid extract form, with undisclosed concentration.

Once the microalgae material was thawed, samples of 135 g were transferred into beakers. FeedTech Silage F10 was added to 6 beakers and pure *L. plantarum* was added to 6 other beakers in a 0.1% volume/weight ratio then mixed for 2 minutes with a kitchen-grade electronic mixer on low speed. Each enzyme was also added in a 0.1% volume/weight ratio to the corresponding beaker and mixed for the same duration. Once the samples were fully prepared, the pH was controlled using a Metrohm 827 pH lab with a Metrohm 6.0228.010 electrode. The contents of each beaker were divided into 3 SousVide™ polyethylene vacuum bags to create triplicate samples weighing 40 g each. The vacuum bags were sealed with a SousVideTools® IV3.0 Chamber-vacuum sealer on strong vacuum setting and normal sealing time. The triplicate bags were placed together in a larger vacuum bag, which was sealed on standard vacuum setting and normal sealing time. All bags were then stored at room temperature for 21 days, with frequent checks to document changes. A flow chart of the procedure is presented in figure 8.

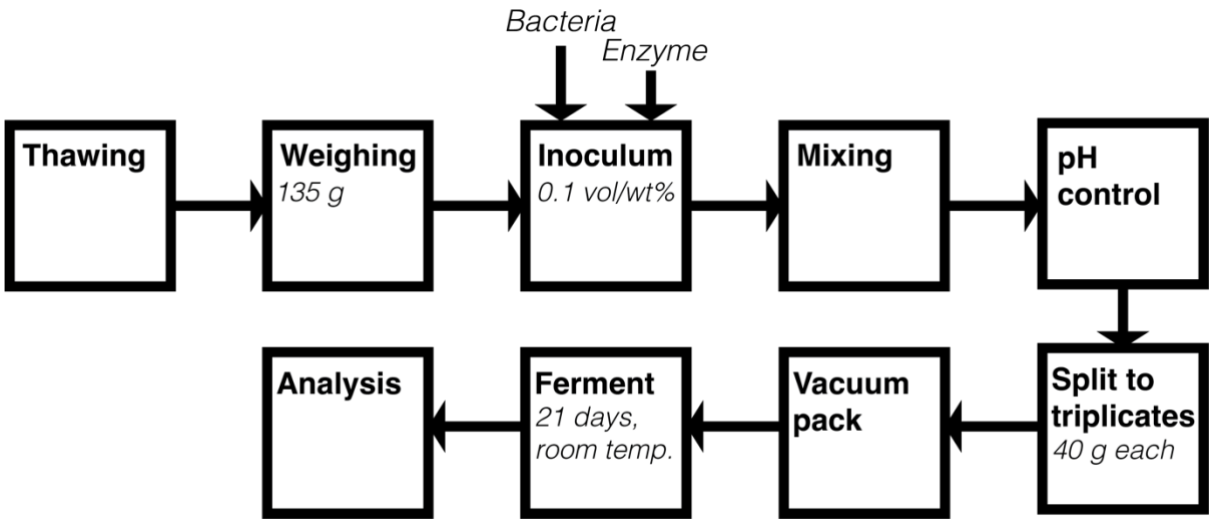

Figure 8. Batch 1 ensiling procedure. When analysis could not be conducted on the same day as unpacking, the samples were frozen at -18 °C.

When unpacking, each sample was weighed before opening, and post opening to control for gas formation. The pH was measured using the same electrode, and the samples then split into one sample which was taken to Lund University and freeze-dried for carbohydrate analysis, and one sample which was frozen at -18 °C at University of Copenhagen.

2.4.3 Enzyme screening procedure, initial trials

Since the project depended on utilizing the supplied enzymes at room temperature and pH which varies from around 6.0 to 4.0, the viability of some enzymes was compared between suboptimal and optimal conditions as designated by the supplier in a quick trial. The enzymes and respective optimal or close to optimal conditions, as disclosed by the supplier, are used in the conditions trial is compiled in table 3.

Table 3. Screened enzymes for impact on solubilized protein under varying temperature and pH, which are designated as optimal by the supplier.

Tailorzyme name	**Disclosed activity**	**pH**	**temperature**
Tail275	*Aspergillopepsin I*	3.8	55
Endocut-01L	*Subtillisin*	2.8	55
Tail204	*Aspergillopepsin I*	7	55
Extract-01L	*Endo-1,3(4)-β-glucanase*	4.5	55
TZ266	*Cellulase, xylanase*	5	55
Tail55	*β -glucanase, pectinases, hemicellulases, xylanases*	4.5	55
Tail54	*Cellulase betaglucanase*	5.5	55
Control	*none*	7	55

for each sample, 0.125 g of algae biomass were weighed into 2 ml Eppendorf tubes using a scale and a positive displacement pipette. In a separate beaker, the enzyme extracts are diluted in either 1.8 ml of Sodium Acetate buffer at the desired pH, or distilled water when working at pH 7. The sodium acetate buffers had been created from mixing anhydrous sodium acetate with glacial acetic acid in distilled water, then adding 2% NaOH until the desired pH is reached. The volume of enzyme used varies with the desired dosage, which is calculated as %vol/mass of wet algae sample. The algae sample is then dissolved in 1.6 ml of the prepared enzyme-buffer mixture and vortexed thoroughly. The sample is then incubated at 300 rpm for 20h at a fixed temperature for the desired enzyme activity. The trial aimed to compare optimal conditions to representative conditions during early stages of fermentation, where enzyme additives are assumed to be most important as they solubilize fibrous material for bacterial consumption. The conditions chosen were 22 °C (room temperature in the lab) and pH 5.5, which were also created through sodium acetate buffer.

After overnight incubation, the enzyme activity was deactivated with a 15 min heat shock at 95 °C. The sample is then vortexed again and 0.9 ml of the sample is diluted in 0.9 ml of distilled water in a separate Eppendorf tube, after which a water extraction at 40 °C for 40 min is performed to ensure the soluble protein is in the liquid phase. After extraction, the sample is centrifuged for 10 min, at 14100 rpm with an Eppendorf Mini spin Plus centrifuge. 0.5 mL of the supernatant is then diluted in 4.5 ml of 5.5 pH sodium acetate buffer. The results of the first trial screening, with the enzymes and conditions disclosed in table 2, are only used to determine whether enzyme activity remains relevant under poor conditions and inform the procedure moving forward. The results are presented only in the appendix. In all enzyme screenings, the

pH was controlled before further analysis using a Metrohm 632 equipped with a SI Analytic A157 electrode.

2.4.3 Enzyme screening procedure

To simulate the conditions of ensiling in a smaller and fast scale, a fermentation screening method was developed to use as a pilot study. The first screening of enzymes at optimal and suboptimal temperatures and pH showed that all enzymes screened did function at both high (50 °C) and low temperatures (23 °C). As a result, it was assumed that incubations at elevated temperatures would remain representative to incubations at low temperatures. When incubating *L. Plantarum* at 36 °C, the fermentation process is significantly faster when compared to room temperature (Zhou et al., 2015). By raising the temperature to 36 °C, much faster screenings under conditions more representative of the ensiling process can be achieved. All subsequent enzyme screening were conducted with a 36-hour fermentation period at 36 °C as a result.

Each screening was conducted with biological duplicates. Each sample was prepared by measuring 12.0 g of thawed algae sample into 20 ml sealable bottles using spoons and spatulas to transfer the material. Each sample was inoculated with 0.12 ml 200 mg/ml *Lb. plantarum* broth and 0.12 ml of the enzyme solution to be screened. All enzymes presented in table 3 were screened. The inoculation dosage of 1% volume-to-sample weight was chosen to overdose the sample to reduce the risk of insignificant effect from the enzyme. The inoculated sample was manually mixed with a glass rod for 1.5 minutes. To create the duplicates, the 12 g sample was divided into two Eppendorf tubes containing 2 g of sample each. The Eppendorf tubes were then incubated for 36 hours at 36 °C and 200 rpm. A lot of material was lost due to difficulty in transferring material between vessels, but the remaining material not incubated was frozen at -18 °C. A flow chart of the sample preparation is presented in figure 9.

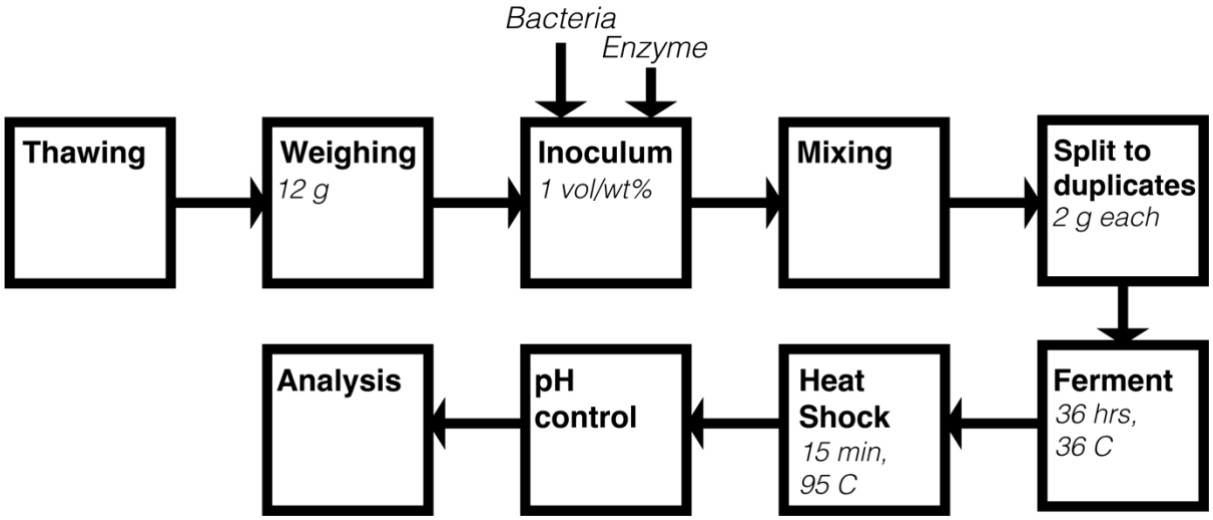

Figure 9. Sample preparation for individual enzyme screening

Once finished the first two fermentations, which screened individual enzymes, were heat shocked in a water bath at 95 °C for 15 minutes. The third and final screening trial was not heat shocked due to a suggestion by supervisor to reduce the variance of the setup. The pH of each sample was checked using an electrode, before following the protocol for solubilized protein screening as described above. The third fermentation screening was instead analyzed by protein digestibility assay.

The third screening focused on investigating potential positive interactions between already screened enzymes, since previous studies had found large positive effects when combining enzyme activities to degrade the cell wall (Gerken et al., 2013). The four best performing enzymes with regards to increased soluble amines were chosen by comparing the average L-glycine equivalent of each sample to the average of the control sample in the respective setup, since two screenings were performed in total. The comparison used is shown in equation 2.

$$\frac{Sample\ average\left[\frac{L-glycine\ eq.}{g\ DM}\right]}{Control\ average\ [\frac{L-glycine\ eq.}{g\ DM}]} * 100 - 100\% = Sol.Amine\ Increase\ [\%] \quad (2)$$

A full factorial experimental setup to test all 16 possible combinations was utilized to test interaction. 16 enzyme cocktails were designed, with the contents each described in table 4. The table described the amount added as volume-% to weight of the microalgae sample. To correct for volume effects each sample was diluted with distilled water so that the same volume had been added to all samples.

Table 4. Enzyme combinations prepared in the final screening experiment. Distilled water was added to each sample to correct for volume dependent errors.

Sample name	Tail73 [% vol/wg]	CelluX-01L [% vol/wg]	Extract-01L [% vol/wg]	Tail171 [% vol/wg]	Dist. Water [% vol/wg]
15	0	0	0	0	4
5	1	0	0	0	3
3	0	1	0	0	3
10	0	0	1	0	3
13	0	0	0	1	3
12	1	1	0	0	2
8	1	0	1	0	2
7	1	0	0	1	2
6	0	1	1	0	2
14	0	1	0	1	2
11	0	0	1	1	2
2	0	1	1	1	1
0	1	0	1	1	1
4	1	1	0	1	1
9	1	1	1	0	1
1	1	1	1	1	0

In addition to the 16 enzyme combinations tested, the best performing endoprotease Tail204 was also included in the screening with the intent to investigate effects of an endoprotease in the full protein digestibility assay.

2.4.2 Batch 2 Fermentation procedure

The material for fermentation batch 2 was prepared in an almost identical procedure as batch 1, with the only change being the enzyme an inoculum dosage which was increased to 1% volume to biomass mass, to remain consistent with the dosages used in the enzyme screening

trials. The samples created with their given sample name are presented in table 5. The different enzyme mixtures refer to the combinations created in the third screening experiment described in table 3. Since moisture content is relevant to being able to create a successful ensiling, investigating a lower moisture content could indicate whether the process is sensitive to water activity and should be more carefully considered as the research project moves forward. The diluted samples were diluted from 20% solids to 15% solids by adding distilled water. The mixed samples were stored in a rotating oven set at room temperature for the duration of the fermentation, with an interruption of a few hours due to booking errors and poor attachment to the rotating axle after 7 and 6 days respectively. Adding stirring to the fermentation could alleviate mass transfer issues, although the mixing achieved could be limited due to the highly viscous nature of the samples. The samples B, C and D compared different dosages of the most successful enzyme combination to investigate potential effects of increased enzyme concentration. The initial dosages used in batch 1 (0.1%) were advised by the supplier and should have been overdosages. Large effects from increasing dosages were not expected but worthwhile to investigate.

Table 5. Conditions tested in fermentation batch 2.

Sample Name	Condition
RAW	*Untreated Sample*
A	*Control*
B	*Enzyme Mixture 12, 1%*
C	*Enzyme Mixture 12, 0.1%*
D	*Enzyme Mixture 12, 5%*
E	*Mixture 12, 1%, Mixed*
F	*Mixture 12, 1%, Diluted*
G	*Mixture 12 1%, Diluted, Mixed*
H	*Mixed*
I	*Mixture 0, 1%,*
J	*Mixture 7, 1%*
K	*Mixture 9, 1%*
L	*Tail204, 1%*

The overall aim of the second ensiling batch was to validate the findings from the screenings, while investigating some concerns that had arisen over the course of the project.

2.5 Protein analysis and amine screening

A variety of analysis methods were using throughout the project, the two paramount variables to measure were protein digestibility, done by calorimetric assay, and sugar consumption, done by high-performance anion exchange chromatography.

2.5.1 Protein digestibility assay

The protein digestibility assay procedure was performed according to provided instructions by Megazyme, the supplier of the assay kit (Megazyme, 2018). The assay produces a reliable Kit Protein Digestibility Corrected Amino Score (K-PDCAAS), widely used for judging the qualitive of protein in a food or feed product, by *in vitro* simulating the physiological conditions of typical mammalian gastric and intestinal digestion. Two steps of enzymatic digestion were performed: the first with pepsin at 2.0 pH (HCl) for 60 min at 250 rpm and 37 °C, and the

second with trypsin and chymotrypsin at 7.4 pH (Tris-buffer) for 4 hours at 250 rpm and 37 °C. The undigested proteins were precipitated overnight with trichloroacetic acid, after which the supernatants are removed and treated with ninhydrin reagent. The treatment forms Ruhemann's purple in proportion to the amino acids present in the solution, detected in spectrophotometric analysis as an increase in absorbance at 570 nm.

To calculate the final digestibility scores and K-PDCAAS, the Mega-Calc calculation sheet also provided by Megazyme was used (Megazyme, 2019). It was assumed that the amino acid profile and total protein content remain constant after fermentation. These assumptions affect PDCAAS to a larger extent than the measured *in vitro* protein digestibility, and as such both results are presented in the results section, although they are correlated by a constant.

2.5.2 Soluble amine screening with ninhydrin colorimetric analysis

A key assumption of the project is that the cytoplasmic protein in the *Scenedesmus* cells is a good protein source, and that degrading the cell wall will release more protein into solution. It is assumed that due to the incalcitrant nature of the cell wall, the cytoplasmic protein inside the cells is indigestible. If the cell wall is broken, more protein becomes available for digestion. The assumption is therefore that an increased amount of protein in solution should correspond to an increase in protein digestibility.

Since the full protein digestibility assay is both extremely time consuming and expensive, a cheaper and quicker alternative is required to be able to screen all potentially relevant enzyme additives. Therefore, a ninhydrin screening measuring the soluble amines is used which, according to the key assumption of the project, should correlate to the protein digestibility.

Ninhydrin (2,2-dihydroxyindane-1,3-dion) is a widely used reagent for the detection and quantification of amines, like amino acids. Since many amino acids do not feature UV or visible light absorbing or high fluorescence functional units, derivatization processes are commonly used. Ninhydrin reacts with primary and secondary amines and forms the compound Ruhemann's purple as seen in figure 10 (Miyoshi et al., 2013).

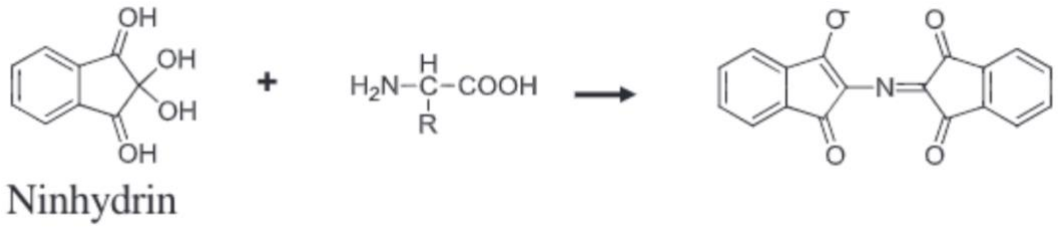

Figure 10. Ninhydrin reaction to form Ruhemann's purple with the N-terminal of an amino acid (Miyoshi et al., 2013).

By light absorption analysis at 570 nm, the response is proportional to the amine content in the sample and can be compared to a chosen standard. This detection method is used in the Megazyme PDCAAS assay, with a L-glycine standard for quantification. Ninhydrin can be used to quickly screen for amine content in a liquid sample, since the colorimetric reaction is relatively fast.

In practice, the ninhydrin screening is very similar to the methods employed in the digestibility assay described in section 2.5.1. By using an L-glycine standard, the screening method yields results quantifiable as L-glycine equivalents per g dry matter. While the unit is by itself not a

useful metric, it can be compared to a control sample to measure an increase or decrease in amines in the solution. As such, it is a useful measure to compare the effects of enzyme additives between each other and a control sample. The method is used to select for which enzymes hold the most potential in increasing the protein digestibility, under the assumptions of the project.

The step-by-step method for ninhydrin screening started with heat shocking the sample in a water bath at 90 °C for 15 minutes to inactivate any previously added enzymes. 125 ± 5 mg of the sample was then weighed in a 2 ml Eppendorf tube and suspended in 1.8 ml distilled water by vortexing. The diluted sample was incubated at 40 °C and 200 rpm for 40 min to extract soluble material. The sample was then diluted by a factor of 20 with 50 mM sodium acetate buffer to bring the sample to pH 5.5, the optimal for ninhydrin reaction (Perrett & Nayuni, 2014), as well as sufficient dilution to be within the range of the L-Glycine standard which ranged from 0 mM to 50 mM. In a 96-well plate, 0.1 ml duplicates of the prepared sample were transferred to the wells. Subsequently, 0.05 ml 2% Ninhydrin reagent was added to each well and the plate was inserted into a Thermomixer preheated to 70 °C at 300 rpm for 35 min. The reaction step is identical to the procedure described in the PDCAAS assay (Megazyme, 2018). Finally, 0.15 ml of 50 vol% ethanol was added to each well, where absorbance was then analyzed by SpectraMax i3 spectrophotometer from Molecular Devices at 570 nm after 15 seconds of pre-shake. The L-glycine standard was used to construct a standard curve, from which L-glycine equivalents per g dry matter was calculated after correcting for dilution and sample mass.

An important difference in the quick and full analysis is the TCA precipitation step only used in PDCAAS analysis. In order to only analyze digestible amines, which are only absorbed into the small intestine in the form of amino acids or very small peptides, remaining proteins are denatured before colorimetric analysis using TCA overnight (Koontz, 2014) in the PDCAAS analysis. This step is not included in the faster ninhydrin analysis. As a result, all proteins, peptides and measured rather than only peptides and amino acids as in the PDCAAS analysis.

While ninhydrin has been a standard for amino acid detection for many decades, the reagent is not problem free. Ninhydrin reacts with different amino acid at varying rates (Freitag, 2005), but since the same standard and the amino acid profile of the samples are assumed to be unchanged post fermentation the issue should be negligible. Ninhydrin does not exclusively react with amino acids, but proteins and peptides although at varying rates since the larger molecules are often sterically hindered (Bhagavan, 2002). Since ninhydrin reacts with amino acids more readily than proteins, and free amino acids have more available active sites, solutions where proteins have been hydrolyzed would have increased response although the initial protein content is unchanged. As such, comparing the effects of proteases to other cell wall degrading enzymes could yield false positive results in favor of the proteases. The reagent can also suffer from reduced sensitivity when not stored cold, dark storage removed from oxygen (Janssen-Bouwmeester et al., 2020).

2.6 Carbohydrate analysis

2.6.1 Neutral monosaccharide composition analysis

The monosaccharide composition was determined using a previously established method by (Allahgholi et al., 2020). The analysis is performed by acid extraction in two steps. First, by incubating 0.025 g of freeze-dried microalgae powder in 250 µL 72% H_2SO_4 for 60 min at 200 rpm and 25 °C. The sample is subsequently diluted with 7 ml MilliQ water and autoclaved for

60 min, 121 °C. The pellet is separated through centrifugation and the supernatant is neutralized by addition of 0.1 M Ba $(OH)_2 \cdot H2O$. For analysis, high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) from Thermo-Fischer was used, at a flow rate of 0.5 mL/min and using a mixture of 62.5% MilliQ-water and 37.5% 2 mM NaOH as eluent. A Dionex CarboPac PA-20 (Thermo-Fischer) column was used. As standards a dilution series containing known concentrations of fucose, arabinose, galactose, glucose, xylose, mannose, and mannitol was prepared.

An analysis of water-soluble carbohydrates was also performed in some samples. The same chromatographic method as described above was used, although the sample preparation was different. For each sample, 0.1 g was weighed and dissolved in 10 ml of MilliQ water**.** Once the desired dilution was prepared, the sample was vortexed thoroughly and left to extract the sugars at 40 °C, 300 rpm. The sample was then centrifuged at 3984 *g* for 2 minutes, and the supernatant pipetted into HPLC-vials.

2.6.2 Uronic acid analysis

Uronic acid analysis was performed on the same samples, only with different eluent mixture and different standards. As eluent a mixture of 55% MilliQ-water, 15% 1M NaOAc and 30% 200 mM NaOH was used. A dilution series containing known concentrations of galacturonic acid, mannuronic acid, guluronic acid and glucuronic acid was used as standard. The procedure was based on the same previous study by Allahghouli et al.

To investigate the validity of the uronic analysis, an amount of pure pectin was added to a sample before the hydrolysis steps, to evaluate whether the treatment had any unexpected hydrolytic effect on the galacturonic acid in the sample. The pectin used was isolated from lemon peel, with a galacturonic acid content of above 74% provided from Sigma Aldrich (CAS 9000-69-5). The pectin was dissolved in 2 ml MilliQ-water to create a solution of 10 mg pectin /ml, of which 0.1 ml was added to the sample to be hydrolyzed per standard procedure.

2.7 Drying time and total solids

2.7.1 Crucible drying

To calculate the total solids of the *Scenedesmus* reference material, it was initially freeze-dried to remove the majority of the moisture. The weight before and after freeze-drying was recorded to determine the water removed. To further calculate the total solids of the reference *Scenedesmus* material, triplicate samples of the freeze-dried powder weighed were weighed to 100 mg in porcelain crucibles and further dried for 22 h in a convection oven at 105 °C. The empty crucibles were pretreated at 575 °C for 3 h in a muffle oven, with 30 min ramping from room temperature. The crucibles were placed in a desiccator to cool, then weighed before adding the sample. After the convection oven drying, the samples and crucible weights were measured to determine the total solids.

To calculate the ash content, the same samples as for determining the total solids were used. After weighing, the samples were returned placed in a muffle oven for 3 h with 30 min ramping to incinerate the samples and leave only the ash content. After incineration, the crucibles were left to cool in a desiccator and weighed. The ash and solids determination protocol largely followed the National Renewable Energy Laboratory suggested procedure (Wychen & Laurens, 2013).

2.7.2 Drying rate estimations

To evaluate the impact of the enzyme treatment and silage process on the drying curve and drying time of the microalgae material, a HC103 Moisture Analyzer by Mettler-Toledo was used. 1500 mg ± 50 mg of raw *Scenedesmus* material is manually spread evenly on an aluminum plate and dried at 105 °C and atmospheric pressure while measuring the weight of the sample. It was assumed only water evaporates, and as such the weight difference of the sample per unit of time indicates the rate of water evaporation. Once the evaporation rate is below 1 mg/min for 1 minute, the drying terminates and total time elapsed recorded. The Moisture analyzer outputs the recorded weight in 5 second intervals, from the initial weight to the final weight.

The parameters of critical moisture content and constant rate period length are significant in industrial drying applications. Using the data from the HC103 moisture analyzer, the parameters are calculated by evaluating R-squared. Normally, drying occurs linearly during the constant rate period and becomes non-linear during the falling rate period, which is also the case for the *Scenedesmus* samples. Figure 11 shows a typical drying curve, plotting the water content per g DM. The interval B to C shows the constant rate drying period, and C to D the falling rate period.

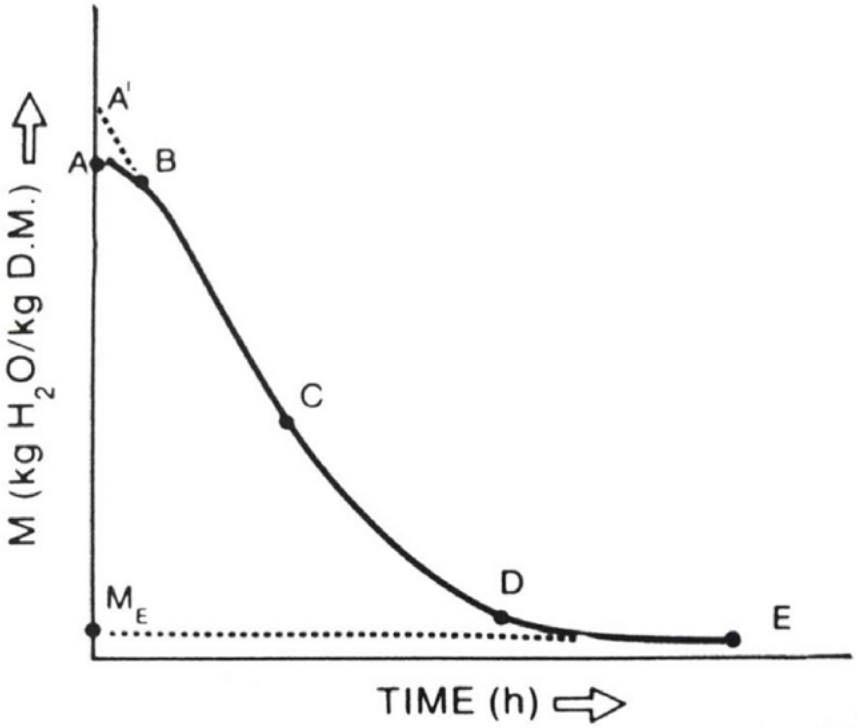

Figure 11. A typical drying curve of crops, where the interval B to C showcases the constant rate drying period. The point C is the critical moisture content (Chinweuba et al., 2016).

A linear approximation will have a good fit during the constant rate period consequently, but the fit will become worse as the falling rate period is reached. The critical moisture content is defined as the moisture content where the drying rate goes from constant to falling. This point coincides with where the R-squared value of the linear fit will start to decrease and is how the critical moisture content is calculated in this case. The constant rate period length is defined as the time measurement where the critical moisture content is reached.

2.8 Scanning electron microscopy

Scanning Electron Microscopy was used to evaluate the physical changes of the most successful enzyme cocktails from final room-temperature fermentation batch were used. One sample of around 1.5 ml raw material and three 1.5 ml samples of different fermented samples were analyzed. To chemically fixate each sample was mixed with 1 ml of Karnovskys's fixative for 2 hours. The samples were centrifuged, and fixative removed, and then washed with 1 ml distilled water for 10 min and centrifuged again. The washing procedure was repeated 3 times. To dehydrate the samples, they were washed with 1ml ethanol of increasing concentration, from 50% to 99%. The samples were washed for 10 minutes and centrifuged, removing the ethanol with pipette in each case. As a final drying step, 1 ml of hexamethyldisilazane was added and stirred for 15 min before being removed. The samples were then transferred onto paper and dried from all liquid overnight in a desiccator.

A small amount of the completely dried sample was then transferred onto black adhesive tape on aluminum knobs. The samples were then covered in gold via EM ACE200 sputter coater from Leica Microsystems. A layer of 6 nm gold was applied via diffusion coating, rather than directional. The samples were then left in a desiccator overnight, and then analyzed in a FEI Scanning Electron Microscope Quanta 200.

2.9 CIELAB color analysis

To analyze color changes from fermentation, the portable Spectrophotometer CM-700D was used. The spectrophotometer measures the color of a powder sample within the CIELAB color space, which is constructed of three axis of color each designated with their respective parameter. The L* parameter varies from 0 to 100, and is a measure of lightness, where 0 is complete black and 100 is complete white. The a* and b* parameters measure axis of color opponents instead, where a* measure the spectrum from green to magenta and b* measures blue to yellow. The parameters vary between -60 and +60 with the tool used, where negative values of the parameters are contain more green and blue, and positive more magenta and yellow. Figure 12 shows a representation of the color space and parameters.

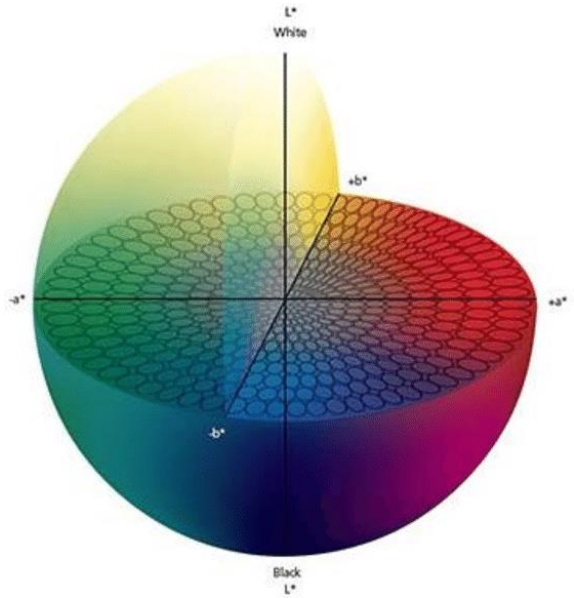

Figure 12. Visual representation of the CIELAB color space, and what colors are connected to which parameters (Vieira, 2021).

All samples from batch 1 and batch 2 were analyzed, and each sample was measure six times. The fermented samples were subsequently compared to the raw, untreated sample to see if a meaningful difference was visible. The CIELAB color scale was converted to RGB for digital displays.

2.10 Statistical analysis

The structure of experiments in this project relies largely on isolated comparisons to a control or reference sample. The goal of each analysis is to identify significant differences between either a raw untreated sample and some fermented sample, or a fermented sample without enzyme additives to a fermented sample with an enzyme additive. As a result, statistical tests to determine true difference, in this case reject the null hypothesis defined as "the tested value in the control sample is the same as the analyzed sample" are utilized in all analysis but are especially necessary in the ninhydrin screening and protein digestibility analysis to determine a significant difference. A typical and easy method is the Student's T-test, an effective measurement to compare two mean values (Mishra et al., 2019). However, when using the Student's T-test to compare many results, the issue of inflated α becomes relevant. Inflated α refers to the cumulative probability of incorrectly rejecting the null hypothesis at the designated level of significance as the number of hypothesis tests increases. Since many hypothesis tests are carried out in this project, the probability of incorrectly rejecting the null hypothesis is high. To reduce the risk, analysis of variance (ANOVA) tests which compare three or more samples, combined with *ad hoc* tests to compare specific samples among them are suitable. In this case, where many samples are compared against one control or reference Dunnet´s *ad hoc* test is most suitable (Lee & Lee, 2018).

Throughout the project all samples are created and measured in biological triplicates, except for the screening batches, where biological duplicates are used due to time restrictions. In tables and figures, all results presented are averages of the triplicates or duplicates, with standard error presented along the average. The measurements taken on all samples are assumed to be distributed by normal distribution for the tests to be applicable. Microsoft Excel with the add-on XLSTAT is used to perform all calculations and statistical tests.

3. Results

The results are structured by analysis method, after the screening results are initially presented to present the best performing enzyme cocktails used in batch 2 results. After screening results, data focusing on the effects of fermentation on the biomass are presented, and the results relating to PDCAAS are presented at the end of the section.

3.2 Enzyme screening

From initial testing of conditions and analysis methods in material from batch 1 and testing of various incubation conditions (results available in the appendix), it seems that the tested enzymes have relevant activity at lower temperatures and in the pH conditions of ensiling. To screen for relevant activity in as many of the supplied enzymes as possible, enzymes and inoculums were incubated for 36 hours at 36 °C in Eppendorf tubes, and then analyzed for pH and soluble amines.

Three enzyme screenings were performed, the first two to evaluating effectiveness in comparison to a control sample, and the final third fermentation to test combinations of the top performing enzyme extracts. The first two batches are analyzed with the simplified soluble amine analysis, and the third batch is analyzed with a full digestion analysis.

3.2.1 Fermentation screening batch 1

In the first batch duplicates of each sample were taken except for the control, of which four samples were taken. The control samples were inoculated with the *L. plantarum* strain like all other samples but did not contain any enzyme additives. Mainly enzymes with glucan activity were screened. Several enzymes of similar activity are screened since previous tests seemed to indicate that undisclosed differences between the enzymes, such as specific substrate or species of origin or extract age, are relevant to the response. The results are summarized in table 6 below with the average response and standard error.

Table 6. Amine solubility results from the first screening, where (*) signifies the samples not being significantly different to the control sample.

Sample	Activity	pH	L-Glycine Eq. (mM/ g DM)
Tail56	*β-glucanase, xylanase, cellulase*	4.1 ± 0	132.95 ± 21.69 *
Tail26	*β-glucanase*	4.3 ± 0	161.35 ± 21.6 *
Tail175	*Xylanase, hemicellulases*	4.1 ± 0.01	143.72 ± 14.59 *
Tail200	*Trichoderma cellulase*	4 ± 0.01	143.6 ± 7.81 *
Tail157	*Pectinase, hemicellulase complex*	4 ± 0	142.05 ± 12.61 *
Tail73	*Pectinases blend*	4.1 ± 0.01	344.22 ± 29.43
Tail171	*Phospholipase A1*	4.3 ± 0.01	182.64 ± 4.69 *
Tail166	*Phospholipase PLC+PLA2*	4.1 ± 0	125.67 ± 13.94
Endocut-01L	*Endoprotease*	3.96	359.6 ± 7.22
Tail204	*Aspergillopepsin I*	4.3 ± 0.01	737.8 ± 7.96
Tail31	*Xylanase*	4.3 ± 0.01	136.12 ± 2.59
Extract-01L	*Endo-1,3(4)-β-glucanase*	3.9 ± 0	188.27 ± 2.23
TZ266	*Cellulase, hemicellulase*	3.95	131.39 ± 13.95 *
Control Batch 1	-	4.2 ± 0	161.83 ± 20.46 *

Some increase in amine solubility was found from the first screening, although somewhat conflicting. As discussed previously, the proteases yield significantly positive effect which is likely due to a systematic error in the analysis method.

Beyond the proteases, only Extract-01L and Tail73 yielded significant increase in soluble amines. In the case of Tail73, a blend of unknown pectinases, the results are more than doubled in comparison to the control. It seems pectinases have high effect in increasing amine solubility which is unexpected since subsequent analysis detects no presence of galacturonic acid, the main monomer of pectin.

The final enzyme with increased L-glycine equivalents, although not significantly reliable, was Tail171, a phospholipase. The results seem conflicting when to the second phospholipase screened, Tail166. Tail166 has significantly lower soluble amines than the control, which could be explained by the enzyme having negative effect on the growth of *L. plantarum*, since some cell membrane degrading activity could be disrupting the membranes of the bacteria as well. Why the same effect is not present for Tail171 is unknown and could be due to some difference in substrate specificity. Another potential explanation could be more significant effect of phospholipase A1 on the algae. A previous study has theorized some potential increase in the permeability of algaenan being a possible effect of phospholipase A1, since the long aliphatic chains of algaenan hold some structural similarity to long tailed lipids (Gerken et al., 2013).

A trend in the results is a decrease in soluble amines in some samples, when compared to the control. The effect could be attributed to variance, but it could be due to some inhibitory action to the inoculum or some interaction with the solubilized amines.

3.2.2 Fermentation screening batch 2

The second fermentation screening batch aims to screen remaining enzymes, including some already tested. Since the first batch showed some potential promise with a lipase, several more are screened.

As previously, all enzymes are tested for solubilized protein in biological duplicates. The initial pH was 5.5. When analyzing in the spectrophotometer, each duplicate was analyzed twice, meaning. From the spectrophotometry results, outliers were identified and removed from the Filta-04L and CelluX-01L samples by q-test. The results are presented in table 7 below as averages with standard error.

*Table 7. Enzyme screening results of the second trial. Samples with * are not significant by Dunnett's test, $p < 0.05$.*

Sample	Activity	pH	L-Glycine Eq. (mM/ g DM)
FiltaX-04L	*Endo-1,4- β xylanase*	4.2 ± 0	155.17 ± 4.23 *
Tail127	*Lipase 1,3*	4.3 ± 0	171.57 ± 26.21 *
Tail170	*Phospholipase A1*	4.2 ± 0	150.07 ± 6.63
Tail169	*Phospholipase A2*	4.3 ± 0.01	166.96 ± 11.11 *
Tail165	*Phospholipase C*	4.4 ± 0	125.71 ± 21.86 *
Tail55	*β-glucanase, pectinases, hemicellulases, xylanases*	4 ± 0	187.27 ± 39.77 *
CelluX-01L	*Cellulase β-glucanase*	4.1 ± 0	229.84 ± 7.54
Sacchary-01L	*Glucoamylase*	4 ± 0	113.04 ± 17.53
Tail155	*Xylanase*	4.2 ± 0	136.43 ± 24.97 *
Alginate Lyase	*Poly alpha-I-Guluronate Lyase*	4.2 ± 0	177.97 ± 24.32 *
Control Batch 2	-	4.2 ± 0.045	168.66 ± 9.7

Many issues present in the first batch are prevalent in the second batch as well. Most of the added enzymes show the trend of decreasing the soluble amines below the control, while still to an insignificant degree. The Sachary-01L (glucoamylase) and Tail170 (phospholipase A1) perform significantly worse than the control. The response to Tail165, another phospholipase, was far below the control but the standard deviation was also very high. The reason for the trend of enzymes performing worse than the control cannot yet be established, but some hypothesis can be concluded.

High variance is an issue in both fermentation screenings. The high standard deviation was not present in the initial tests but seems to increase with time. Although the soluble protein analysis method was unchanged from the first screening, the average relative standard deviation had increased from 5.5% in the initial tests (in the appendix) to 10.7% in the first fermentation screening and finally 17.7% in the second fermentation screening. In addition, the r-squared fit of the L-glycine standard had been consistently decreasing from 0.99, to 0.97 and finally 0.92 in each consecutive screening. A new standard series was created to test, which still only achieved a fit of 0.93. Another potential issue could be the Ninhydrin reagent. Ninhydrin is reported to be sensitive to light, oxidation, and temperature. The reagent must be stored in refrigeration, darkness and under nitrogen. While the common procedure was followed and the headspace was flushed with nitrogen after each use, frequent use might have degraded the reagent and caused an inconsistent colorization. New Ninhydrin reagent was procured before the final fermentation screening.

High variance could also be due to insufficient mixing, both before and during fermentation. The inoculated container with enzymes added is mixed thoroughly with a glass rod before being pipetted into Eppendorf-tubes, which is the fermentation vessel. Although mixed for at least 1 minute the substrate is quite solid, and homogeneity might not be achieved. When pipetting into the fermentation vessel, the actual amount of enzyme and inoculum might vary significantly. The mixing might also be an issue during fermentation. The incubation occurs at 36 °C while shaking the rack at 200 rpm. However, as previously stated, the substrate is very solid and gelatinous and is likely not actually stirred to any meaningful degree in the incubator. The lack of stirring could create a very heterogeneous growth of lactobacillus, and high

variance in the effect of the enzymes. The enzymes are likely to be negatively affected by a lack of mixing since many enzymes are product-inhibited. If no mixing occurs, the enzymes will both be limited by a lack of substrate in the immediate vicinity and be further inhibited by high concentration of product, locally. The actual effect of all enzymes screened could be hindered by this effect, and a method for stirring during full-scale fermentation should be investigated.

From the second screening only one enzyme, CelluX-01L, has an unambiguous increase in soluble amines which can presumably be due to degrading the cellulose or some other glucan with β-linkage. Of all enzymes screened, 3 non-proteolytic enzymes have a significantly positive effect on the solubilization of protein; Tail73, CelluX-01L and Extract-01L. In addition, Tail171 seems to trend towards having positive interaction. These enzymes are deemed to be most successful among the non-proteolytic enzymes screened and were tested for increased effect when used in combination in a third screening setup.

To choose the most successful enzymes, the average response in L-glycine equivalents, which corresponds to the increase in soluble amines, from each was compared to the control average from the respective batch. The comparison is presented in figure 13. Tail73, CelluX-01L, Extract-01L and Tail171 are chosen as the best enzymes. Due to concerns from the animal feed producer, the proteases are not included among the best enzymes, but Tail204 is included in subsequent trials as validation.

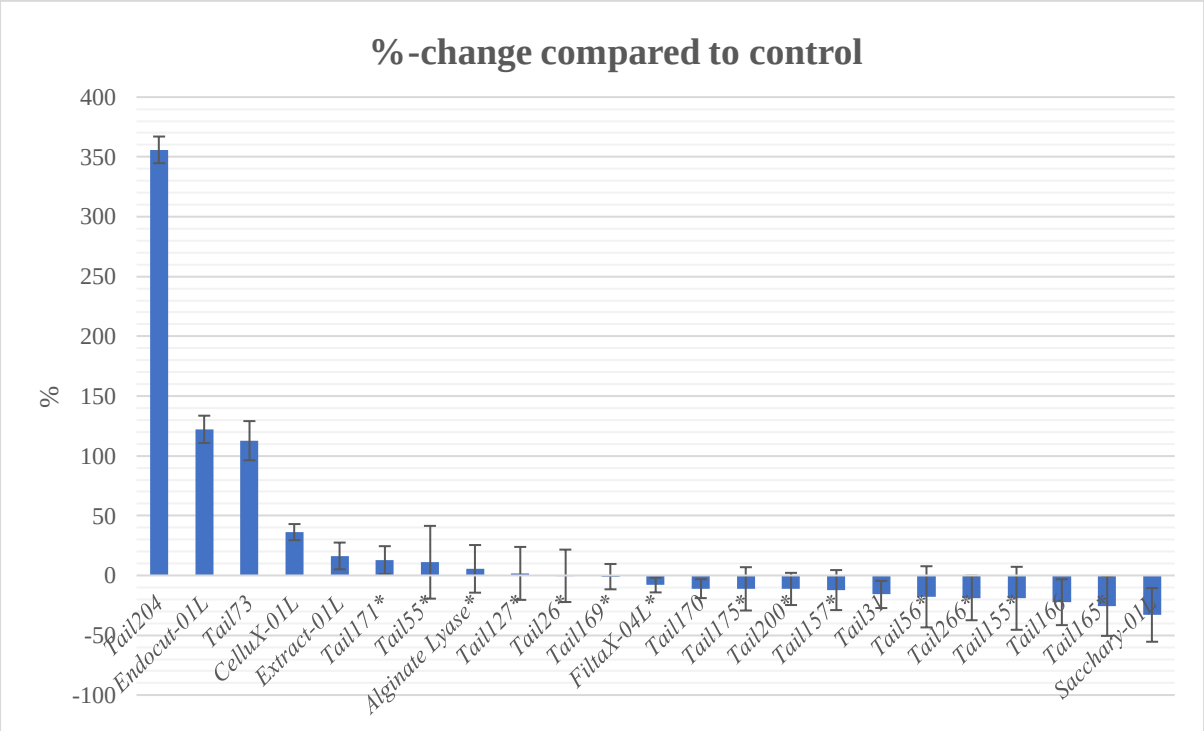

*Figure 13. Percentage increase and decrease of L-glycine equivalent / g DM compared to control sample. Samples marked with * were not statistically significant at $p<0.05$ by Dunnett's test. The error bars are standard deviation.*

3.3 Enzyme combination screening

To evaluate the effects on introducing several relevant enzyme activities at once, a full factorial experimental setup was used. Due to the issues identified in previous screenings, some additional changes to the method were introduced. The heat shock was disregarded and presumed to be a potential source of variance, while not being a vital step in the procedure. A

full protein digestibility assay was carried out as opposed to a soluble protein screening. The same volume of each enzyme solution as tested before was used, and the presence of an enzyme solution in the sample is indicated by a 1 in table. A 0 indicates the enzyme was not present in the sample.
To reduce volume dependent errors, a corresponding volume of distilled water was added in place an enzyme solution. Each 0 in the table thus corresponds to distilled water being added in the sample.

The order of the samples was randomized to reduce time or order dependent errors. The results sorted by enzymes added are presented in table 8 below. A heat map was used to evaluate positive effect, where high PDCAAS and low pH was desired. Dark green corresponds to highest desired effect, bright yellow to medium effect and dark red to lowest desired effect. The color coding is relative only to the results in the table.

*Table 8. Contents of each enzyme solution, and PDCAAS and pH results presented along standard error. Green corresponds to desired effect in both result columns. Samples with * did not pass Dunnett's significance test, $p < 0.05$.*

Sample No.	Tail73	CelluX-01L	Extract-01L	Tail171	PDCAAS	pH
15	0	0	0	0	0.805 ± 0.0002 *	4.20 ± 0.005
5	1	0	0	0	0.804 ± 0.0026 *	3.96 ± 0.005
3	0	1	0	0	0.799 ± 0.0001	3.98 ± 0.005
10	0	0	1	0	0.800 ± 0.0007 *	3.86 ± 0.015
13	0	0	0	1	0.804 ± 0.0003 *	4.16 ± 0
12	1	1	0	0	0.814 ± 0.0015 *	3.92 ± 0.005
8	1	0	1	0	0.809 ± 0	3.87 ± 0
7	1	0	0	1	0.810 ± 0.0012 *	3.96 ± 0.005
6	0	1	1	0	0.807 ± 0.0004 *	3.86 ± 0.005
14	0	1	0	1	0.798 ± 0.0006 *	4.03 ± 0
11	0	0	1	1	0.798 ± 0.0004	3.85 ± 0
2	0	1	1	1	0.805 ± 0.0008 *	3.85 ± 0.005
0	1	0	1	1	0.811 ± 0.0003	3.89 ± 0.005
4	1	1	0	1	0.802 ± 0.0007 *	3.92 ± 0
9	1	1	1	0	0.810 ± 0.0005	3.87 ± 0.015
1	1	1	1	1	0.810 ± 0.0018 *	3.88 ± 0.005
Tail204	-	-	-	-	0.878 ± 0.006	4.21 ± 0

The largest positive effects on the PDCAAS score seem to be connected to the presence of Tail73, although the same connection does not seem valid for decreasing pH. The decrease in pH seems to differ more significantly, while the PDCAAS increases only slightly. In a larger perspective, the increase in PDCAAS could be seen as marginal. Tail204 was also analyzed but not included in the factorial test, to validate the results seen in the previous screening. As before, there is significant increase in PDCAAS compared to the other results.

To compare the magnitude and importance of each effect, coefficients of a multiple linear regression were calculated, standardized, and plotted in figure 14. While the data has a poor fit to a linear model (R^2=0.53), the comparison of relative coefficient magnitude indicates the importance of each factor to the response. The calculations were performed in Excel, with the add-in XLSTATS.

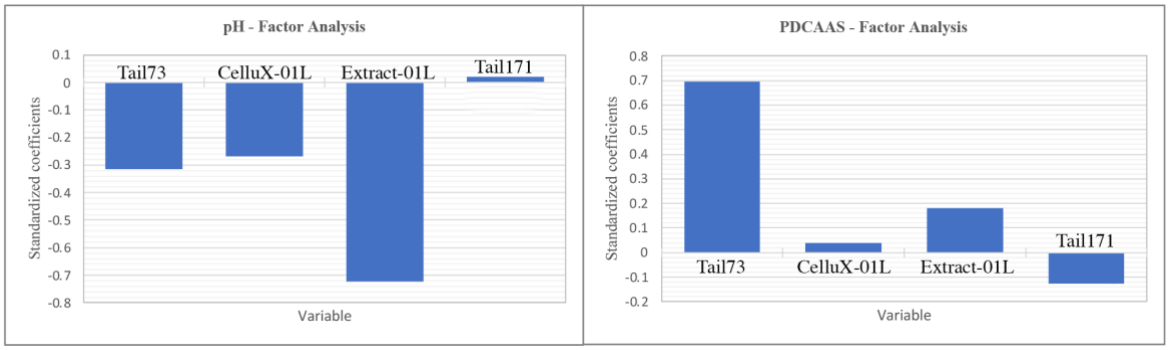

Figure 14. Factor analysis from the enzyme combination screening, where the magnitude of the number indicaties higher impact on the analyzed response.

Each model was dominated by one factor, Tail73 in the case of increasing PDCAAS and Extract-01L in the case of decreasing pH. Only the dominating term could be deemed significant at a significance level $p < 0.05$. While not significant, Tail171 tends to have the inverse effect of the desired outcome, perhaps suggesting that the positive trends visible in the screening attempts could be misleading. The low and insignificant impact of CelluX-01L in comparison to Extract-01L is also noteworthy, since the two enzymes are both degrade β-glucans. The difference could be due to more subtle differences between the enzyme mixtures, undisclosed by the supplier. The pH optimum of Extract-01L is 4.5, lower than CelluX-01L which is most active above 4.5. As a result, Extract-01L could perhaps be more active under the fermentation conditions.

The factorial experiment setup also allows comparison between PDCAAS and L-glycine equivalents, since the two results should be correlated for the hypothesis of the project to prove relevant.

3.3.1 Amine solubility to protein digestibility comparison

From the material in batch 2, both a glycine equivalent analysis and a PDCAAS score were calculated, using the ninhydrin procedure described above and the protein digestibility assay kit respectively. The results are presented in figure 1 for comparison, and table 9 below.

Table 9. Comparison of Glycine equivalents and PDCAAS, presented as an average of triplicates with standard error. Results with an asterisk (*) do not pass a statistical test and are not significantly different from.

Sample	Enzyme additive	L-Glycine Equivalents / g DM	PDCAAS
A	*None*	477.42 ± 14.99 *	0.805± 0.0002 *
B	*Tail73, CelluX-01L*	592.3 ± 2.32	0.814± 0.0018 *
I	*Tail73, Extract-01L, Tail171*	552.41 ± 15.831	0.811± 0.0004
J	*Tail73, Tail171*	588.69 ± 9.147	0.81± 0.0014 *
K	*Tail73, Extract-01L, CelluX-01L*	587.7 ± 7.236	0.81± 0.0006
L	*Tail204*	625.82 ± 2.585	0.844 ± 0.0014
RAW	*Not fermented*	233.1 ± 8.987	0.79 ± 0.0021

The results were compared to the RAW sample, which had not been fermented or enzyme treated. The results in percentage increase compared to the untreated sample is presented in figure 15.

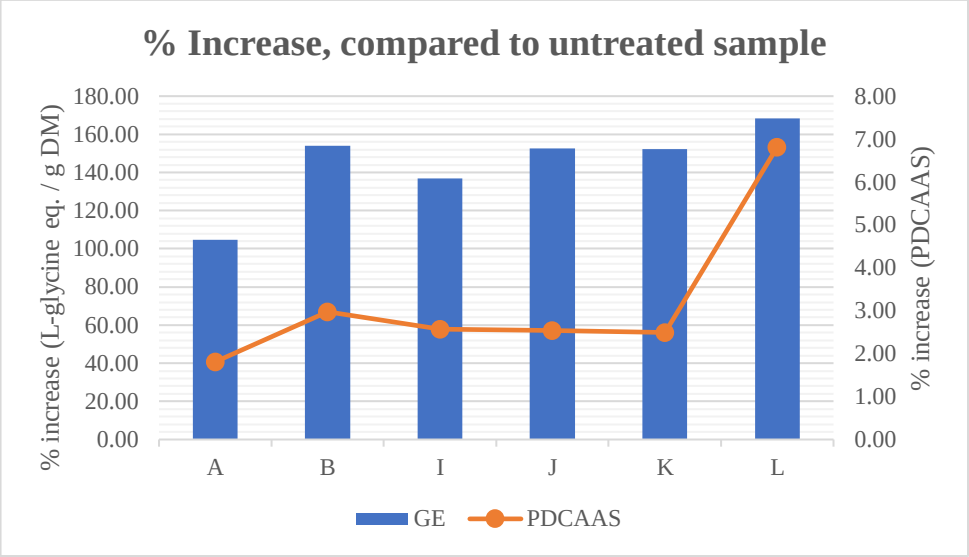

Figure 15. Percentage increase in both PDCAAS and L-glycine eq. / g DM from ensiling (A) and from ensiling with enzyme additives (B, I, J, K, L).

Although the nature of the relationship between the measurements was unknown, some correlation was expected. The changes in glycine equivalents are all statistically significant as determined by Dunnett's ANOVA *ad hoc* test compared to the control sample A, at $p < 0.05$, while similar changes are not achieved in PDCAAS. A trend of slight increases is apparent between fermented samples with and without enzyme additives. The large difference in glycine equivalents is between raw and all other samples, which had all been fermented, was not proportionally represented in an increase in PDCAAS.

The relationship between the two measurements of amine solubility and protein digestibility seems weak. This can either be interpreted as the choice of screening method being unsuitable, or as a failure of the initial hypothesis. Since the initial hypothesis stated that increasing the protein concentration in solution increases the digestibility, these results suggest that the proposed correlation is less impactful than expected. Instead, some other factor influencing digestibility could be more limiting.

Worth noting is the larger increase in PDCAAS in sample L, which was treated with the endoprotease Tail204. Endoprotease treatment is expected to hydrolyze peptide bonds. Since PDCAAS increase from increased protein hydrolysis, it suggests that the low PDCAAS score is partly due to some number of proteins not being hydrolyzed by pepsin, trypsin, and chymotrypsin even though the proteins must be available for reaction in solution. The endoprotease as such seems to increase the protein digestibility, which is also apparent in the substantial increase in amine solubility. The increase in amine solubility in the case of endoproteases is likely also due to protein hydrolysis making more primary amines available for reaction with the ninhydrin reagent.

3.4 Visual analysis

A variety of methods were used to control apparent physical changes from fermentation, along with analysis of microscopic changes with scanning electron microscope.

3.4.1 Sensory experience

The samples had changed noticeably in terms of smell, appearance and feel post fermentation. Except for the negative control, the samples had all changed in the same way which suggests the *L. plantarum* inoculation does affect the anaerobic stability, which is the ability to reduce secondary fermentation.

The inoculated samples all developed a viscous, sticky film with small pockets of gas distributed at the top of the unopened bag. In addition, the color changed from a very dark green to a dark brown, however still retaining some green coloration. The color change seemed homogenous, although more pronounced at the edges of the bag where the sample was thinner. A very slight change in feel could also be noticed, as the sample seemed slightly more liquid than before fermentation. When opening the vacuum bags, a greasy smell reminiscent of fatty foods emerged, seemingly from the clear viscous film. Force was required to separate the films and allow access to the fermented material, at which point the characteristic sea-like smell of the algae was more pronounced. The grassy smell of algae had been reduced. The changes were identical between both *L. plantarum* and F10 inoculated samples.

The negative control was dissimilar from the other samples. In all triplicate bags, large pockets of gas had formed although in different volumes. The negative control triplicates were also internally inconsistent, except for developing the same color shift towards brown as the inoculated samples. Negative control sample A had not developed the greasy layer as the other samples, and the smell was significantly different. Beyond the lack of greasy smell, negative control A had a more acidic smell, with a slight alcoholic note which overall gave a significantly less pleasant odor. Sample B had developed the characteristics, although not to the same degree, as the inoculated samples, indicating that similar strains had grown. Finally, sample C had the same sensory features as sample A, but more noticeably so. The different smell and internal inconsistency of the samples seems to indicate a low anaerobic stability with high formation of undesired byproducts and microorganisms.

In all samples, potential gas formation was measured by weighing each sample after vacuum packing and after opening post fermentation and allowing gas to escape. Significant gas formation was found only in the samples where no enzyme and no inoculum were added, titled negative control in sample batch 1. In the negative control sample, 0.087 ± 0.03 g of unidentified gas was formed. The gas formation variance was high in the negative control sample, varying between 0.153 g and 0.046 g of gas being formed.

Almost all enzyme additives had no apparent effect beyond those observed in inoculated samples. Only the endoprotease Tail204 had an observable effect, where small yellow spots had formed throughout the samples. The spots were firm but could be crushed by applying force, at which point the material was spreadable and grainy. Table 10 summarizes the apparent changes.

Table 10. Sensory experience as observed during and after fermentation.

Sample type	Observations during fermentation	Observations post opening
Inoculated samples	• Brown color shift • Less viscous • Very low gas formation, only visible the last week of fermentation	• Greasy smell upon opening • Clear film covering the inside of vacuum bag • Reduced grassy smell
Inoculated samples with non-protease enzyme additive	• Identical to inoculated samples • Rate of visual changes indistinguishable from inoculated samples	• Identical to inoculated samples
Inoculated samples with protease additive	• Identical to inoculated samples	• Yellow, hard, grainy spots dotted throughout samples • Otherwise identical to inoculated samples
Non-inoculated samples	• Large gas pockets forming within one week of fermentation	• More pungent, acidic, alcoholic smell • Reduced greasy smell and grassy smell • High variance in smell and gas formation between samples

The observed changes are also documented in figure 16 below. "Negative control" corresponds to non-inoculated samples in table 11, inoculated sample with protease additive corresponds to "Tail204+*L. plantarum* fermentation" and inoculated samples are "*L. plantarum* fermentation". All fermented samples appeared similar in visuals and smell, except for the negative controls and the samples fermented with protein.

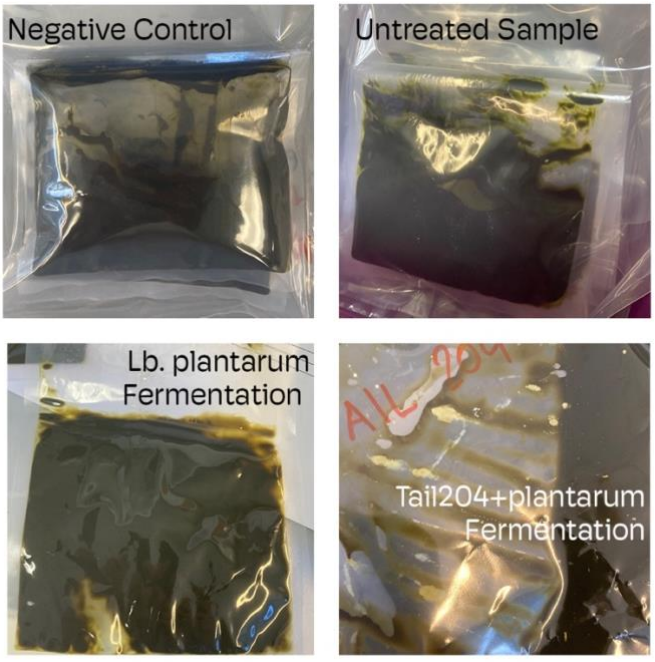

Figure 16. Images of notable visual changes from fermentation. Negative control picture, Lb. plantarum fermentation and untreated sample are from batch 1 and Tail204+plantarum fermentation is from batch 2. The untreated samples were highly viscous, and the negative control had a pungent smell and large gas pockets. Inoculated samples had negligible gas formation. Endoprotease caused firm yellow spots to form.

To validate and quantify the color changes, a portable spectrophotometer was used, measuring the color in the CIELAB color space. The results are presented in table 11. L* varies between 0 and 100, where 0 is fully black and 100 is white. The other parameters vary between -60 and 60, where to a* is green to magenta (negative is green) and b* is blue to yellow (negative is blue). The detected CIELAB color is converted to RGB and displayed in the right column.

Table 11. CIELAB colors and RGB color. All samples are significantly different from the Raw sample on all color axis.

	L*	a*	b*	RGB
Raw	26.6 ± 0.26	-1.1 ± 0.02	0.3 ± 0.11	
L. plantarum	40.8 ± 0.38	1.3 ± 0.08	13.3 ± 0.43	
F10	41.8 ± 0.32	1.3 ± 0.07	12.3 ± 0.36	
Negative	37.7 ± 0.44	0.4 ± 0.04	9.7 ± 0.22	
Tail55	39.5 ± 0.26	1.2 ± 0.08	12 ± 0.4	
TZ266	40.6 ± 0.3	0.9 ± 0.04	10.3 ± 0.25	
Tail54	41.3 ± 0.57	1.6 ± 0.07	13.3 ± 0.63	
Alginate Lyase	43.1 ± 0.37	1.6 ± 0.11	14.3 ± 0.51	
Lyzosyme	41.4 ± 0.36	1.6 ± 0.07	13.8 ± 0.36	
A	39.9 ± 0.74	0.9 ± 0.08	11.2 ± 0.43	
B	38.4 ± 0.49	0.6 ± 0.14	8.8 ± 0.84	
C	37.9 ± 0.36	1.3 ± 0.09	12.6 ± 0.48	
D	34.9 ± 0.61	0.4 ± 0.06	7.6 ± 0.2	
E	36.2 ± 0.31	0.1 ± 0.07	8 ± 0.22	
F	35.7 ± 0.36	1.2 ± 0.12	11.2 ± 0.45	
G	33.7 ± 0.51	1.1 ± 0.11	10.2 ± 0.56	
H	37.4 ± 0.25	1.2 ± 0.06	11.8 ± 0.27	
I	37.6 ± 0.34	0.7 ± 0.05	9.9 ± 0.24	
J	37.2 ± 0.44	0.5 ± 0.07	9.5 ± 0.27	
K	38.7 ± 0.88	0.5 ± 0.07	8.6 ± 0.53	
L	36.4 ± 0.64	-0.1 ± 0.07	6.3 ± 0.23	

The CIELAB analysis confirms a significant color difference from the raw sample, where all fermented samples had gained lightness, magenta and yellow. Since green was lost (a* increasing), there is potentially some effect on the chlorophyll in the samples.

3.4.2 Scanning electron microscopy

SEM was used to qualitatively infer the effects of fermentation and enzymatic treatment. Only four samples were analyzed due to time constraints. Untreated algae were used as a reference to investigate the effects fermentation without enzyme additive has on the substrate, along with samples that had been fermented with enzyme mixture 12 (containing CelluX-01L as a glucanase and Tail73 as a pectinase) and enzyme mixture 0 (Tail73, Extract-01L as glucanase and Tail171 as a lipase) respectively. Figure 17 shows a manually chosen representative image of each sample.

The image of sample 12 has been manually scaled from 3000x magnification to 5000x magnification to be more comparable to the other samples. Some manual adjustment of contrast and brightness was performed in Adobe Photoshop, also to make the images easier to compare.

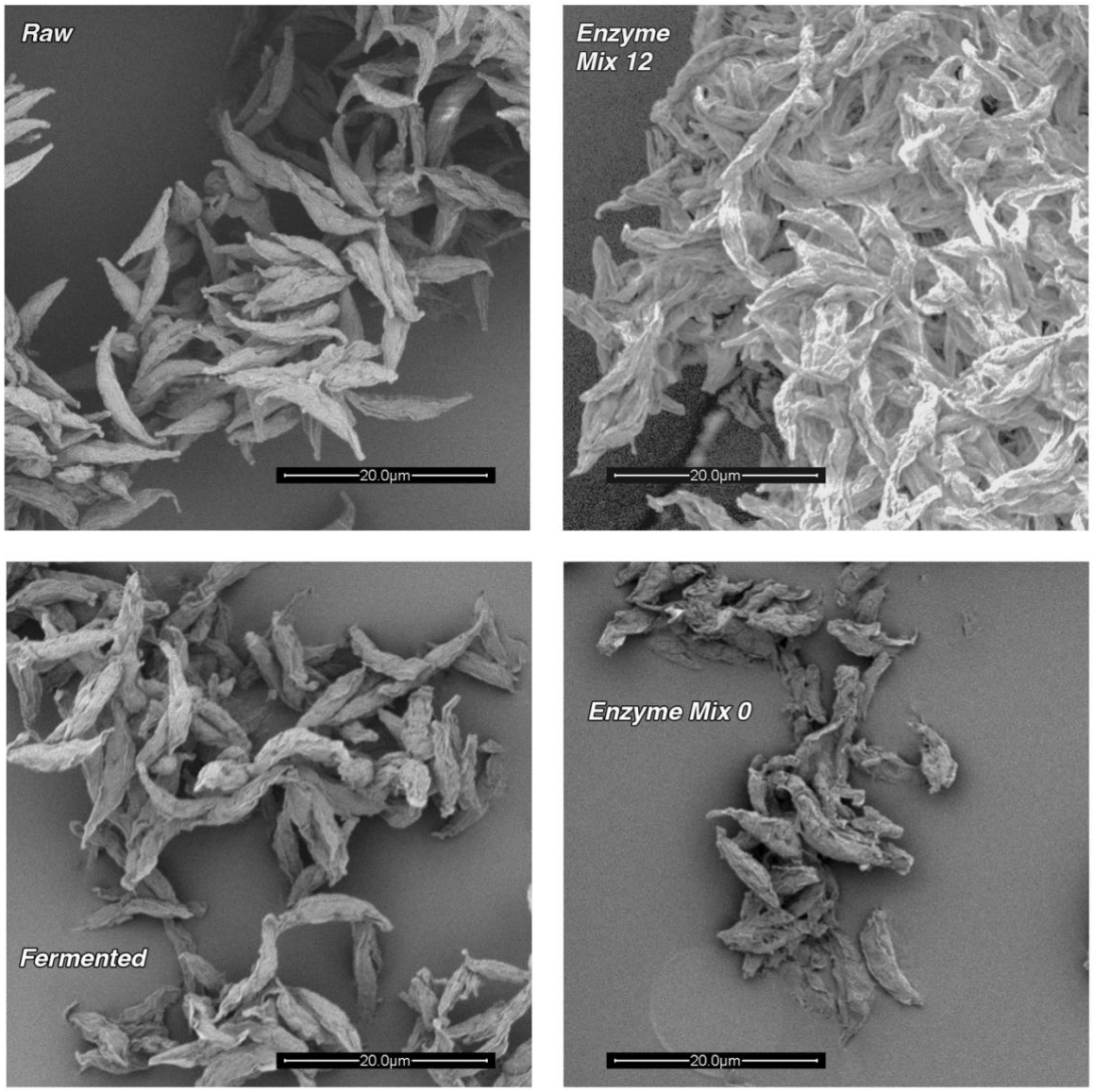

Figure 17. Scanning electron microscopy images of four different samples at 5000x magnification.

The samples were seemingly prepared with too high amounts of microalgae, which contributed to large clumps of algae. This caused difficulty in finding smaller, isolated groups which would have given a clearer image the effects of the treatments. The error was particularly pronounced in sample 12. Some issues during fixation and gold covering also resulted in images with lowered clarity.

Some defining characteristics could be observed in the raw sample. The raw samples had very defined and straight rigid structure, with a veiny structure connecting the distinctly acute ends of each cell. The cells seem to vary between approximately 8-12 µm long and 2-3 µm wide, with some smaller cells being present which could indicate many cells being at different stages of growth. While *Scenedesmus* typically grows in coenobium, mostly groups of four (Andersen, 2013), none could be identified in the raw or fermented samples. The lack of

coenobium can be attributed to the freezing and other pretreatment steps like dewatering. The algae samples should feature coenobiums, since previous study under light microscope shows the typical growth pattern (Olsen et al., 2021).

Some differences are identifiable between the raw and fermented samples. Most notably, some of the rigidity has been lost as many cells appear to be more curved and bent. The fermented cells also have a shriveled appearance, which is particularly noticeable when comparing the acute ends. While the ends of the raw samples are thin with a slight squared end, the fermented samples appear almost deflated and more paper-like in comparison. There could be a higher concentration of polysaccharides or other compound more susceptible to the added bacteria. Another noticeable feature is a darker indentation being present at the approximately same location in the fermented cells, around the midsection lengthwise on the cell surface. This could also be due to some more susceptible structure in the cell wall, or perhaps a membrane bound structure which factors into the structure of the cell.

When comparing the fermented control sample with the enzyme treated samples, the differences are less apparent, in part due to the quality of the images. The same deflated, bent, and shriveled features are present in the enzyme treated samples, to seemingly the same degree. From the images, there seems to be a higher presence of fully degraded cells in the form of debris particularly in enzyme mixture 0. This impression could be due to the choice of cells imaged, as enzyme mixture 12 has higher cell density than the other chosen images, and mixture 0 has a lot lower cell density. Overall, the differences between the fermented samples are negligible or undetectable with the imaged samples.

3.5 pH analysis

The pH drop from fermentation is a significant property in industrial application. Lower pH acts as an indication of more anaerobically stable silage, with lower risk of contamination and secondary fermentation.

3.5.1 Batch 1 pH

pH was used as an initial screening to evaluate which of the two inoculums used would be most appropriate to analyze further. The samples inoculated with *L. plantarum* were found to have generally lower pH than FeedTech F10 and were preferred in further analysis. pH measurements after fermentation in batch 1 are presented in table 12.

Table 12. pH from samples in fermentation batch 1, as average of triplicates with standard error and initial pH of 6.04. All samples were inoculated with L. plantarum *except for FeedTech F10 and negative control.*

sample	Enzyme Activity	pH
L. plantarum *control*	*None*	4.19 ± 0.02
Tail55	*β -glucanase, pectinases, hemicellulases, xylanases*	3.9 ± 0.02
TZ266	*Cellulase, Hemicellulase*	3.93 ± 0
Tail54	*Endo-1,3(4)- β -Glucanase*	4.02 ± 0.01
Alginate Lyase	*Poly alpha-I-Guluronate Lyase*	4.13 ± 0.02
Lysozyme	*Lysozyme*	4.01 ± 0.04
FeedTech F10	*None*	4.46 ± 0.01
Negative Control	*None*	5.47 ± 0.07

Using *L. plantarum* inoculation caused a significant drop in pH compared to both raw material and FeedTech F10 and the Negative control, which was not inoculated.

Using enzyme additives also has a significant impact on the pH, at a significance of ($p < 0.05$). The only exception is using Alginate Lyase, which only slightly decreased the average pH compared to only using *Lb. plantarum.* The lack of reduction is likely due to a lack of specific substrate being present in the microalgae sample. Alginate is a polymer mainly composed of mannuronic acid and guluronic acid and is common in many forms of red algae but seldom in green algae (Lee & Mooney, 2012). The lack of detectable results from the alginate lyase suggests alginate or similar compounds may not be a significant component of the cell wall structure. The lack of results could also be due to unsuitable conditions since the fermentation occurred at room temperature with pH varying from around 6 to 4, while the optimal conditions for the enzyme are 37 °C and pH 6.3 (Aldrich, 2023). When operating under such conditions, relevant enzyme activity might be negligible. Also, due to a lack of stirring during fermentation, available substrate and pH conditions might change more significantly locally and impact the overall results of the fermentation.

The lowest pH was achieved using enzyme additives which feature significant activity on fibrillar polysaccharides, like hemicellulose and cellulose. The group of enzymes is referred to as cell wall degrading enzymes, CWD-enzymes.

Overall, the fermentation process seems to create stable product with low pH when inoculating with *Lb. plantarum.* The process is also further improved by using enzyme additives, of which the commercial blends Tail55 and TZ266 which are CWD-enzymes seem to create the most desirable result.

3.5.2 Batch 2 pH

The pH was controlled in fermentation batch 2 as well. Results are displayed in table 13, where some notable trends are present.

Table 13. pH post fermentation in batch 2 of samples. The average initial pH was 6.04.

Sample	Conditions	pH
A	*Control*	4.29 ± 0
B	*Enzyme Mixture 12, 1%*	4.03 ± 0.01
C	*Enzyme Mixture 12, 0.1%*	4.09 ± 0
D	*Enzyme Mixture 12, 5%*	3.99 ± 0
E	*Mixture 12, 1%, Mixed*	4.06 ± 0
F	*Mixture 12, 1%, Diluted*	3.99 ± 0
G	*Mixture 12 1%, Diluted, Mixed*	4.00 ± 0
H	*Mixed*	4.28 ± 0.01
I	*Mixture 0, 1%,*	4.06 ± 0
J	*Mixture 7, 1%*	4.08 ± 0.01
K	*Mixture 9, 1%*	4.02 ± 0.01
L	*Tail204, 1%*	4.24 ± 0.01

Overall, the pH is consistently higher than batch 1 although both batches fermented for the same period of time, which could be due to miscalibration of the pH electrode. The same trend of CWD-enzymes reducing the pH 0.1-0.2 below the control is consistent between batch 1 and batch 2. Mixing seems to have no significant impact, as there is no notable difference between samples B and E or A and H respectively. The lack of difference could also be due to the material being too viscous for effective mixing. Dilution also has low impact when comparing samples F and B, which is positive and suggest the material is highly fermentable. Although samples B, I, J and K, which tested the different enzyme mixtures, caused pH drops of 0.1-0.2 as expected, the results do differ slightly from the screening due to sample I causing a slightly smaller pH drop than expected. Finally, dosage as compared between samples B, C and does seem to have an effect, since increasing the dosage from 0.1% to 5% causes a pH drop of 0.1.

3.6 Neutral monosaccharides content

The sugar content of a biomass is a portrait of the carbohydrate content in the mass and are important in this case to evaluate what type of carbohydrates and fibers are present in the *Scenedesmus* sp. An analysis of untreated algae sample and fermented samples with and without additives, was carried out to determine the difference in sugars. The difference observed corresponds to what types of sugars have been consumed and the amounts consumed.

The monosaccharide analysis also acts as a characterization tool of the untreated sample and is used to help determine what substrates are present and choose enzymes with corresponding activities to degrade the polymers. HPAEC-PAD was used to carry out the analysis of the first batch of fermented material along with an untreated sample. The content of each neutral monosaccharide, g per g dry matter, is presented as an average of analyzed biological triplicates along with standard errors of the measurements. The samples were frozen at -18 °C after fermentation before the analysis.

3.6.1 Batch 1 analysis

The first batch of fermented material was analyzed as described above. The results are presented in table 14. The average mass of each sugar was summarized as the total sugar content in the sample.

Table 14. Monosaccharide analysis of raw material and fermented material from batch 1. Triplicate averages are calculated and presented along with standard error. The

Sample	Fucose	Arabinose	Galactose	Glucose	Xylose	Mannose	Mannitol	Total Sugar
Raw	0.41 ± 0.03	0.49 ± 0.03	1.54 ± 0.08	4.46 ± 0.24	0.11 ± 0.02	4.05 ± 0.22	0.07 ± 0.02	11.14 ± 0.6
Negative Control	0.4 ± 0.02	0.49 ± 0.07	0.57 ± 0.1	0.68 ± 0.03	0.02 ± 0.01	2.3 ± 0.1	0.18 ± 0.01	4.65 ± 0.29
FeedTech F10	0.48 ± 0.02	0.6 ± 0.02	0.53 ± 0.02	2.69 ± 0.11	0.11 ± 0.03	3.32 ± 0.1	0.29 ± 0.08	8.02 ± 0.3
Lb. plantarum	0.42 ± 0.01	0.45 ± 0.01	0.44 ± 0.01	2.25 ± 0.04	0.1 ± 0.03	2.71 ± 0.09	0.04 ± 0.03	6.41 ± 0.08
Tail55 + Lb.	0.47 ± 0.01	0.53 ± 0.01	0.46 ± 0.01	0.36 ± 0.01	0.1 ± 0.01	2.56 ± 0.02	0.18 ± 0	4.66 ± 0.04
Alginate Lyase + Lb.	0.42 ± 0.03	0.46 ± 0.03	0.42 ± 0.02	2.3 ± 0.07	0.09 ± 0.01	2.59 ± 0.14	0.27 ± 0.03	6.54 ± 0.32
TZ266 + Lb.	0.45 ± 0.01	0.57 ± 0.01	0.5 ± 0	0.67 ± 0.02	0.11 ± 0.01	2.77 ± 0.06	0.32 ± 0.01	5.39 ± 0.05

Some differences are observed in comparison to previous examination of the *Scenedesmus* species. Unpublished analysis carried out at the University of Copenhagen indicated high amounts of glucose and xylose, in contrast to the results in this analysis where high amounts of glucose and mannose are instead detected. In the setups used, both at Lund University and the University of Copenhagen, the retention time of xylose and mannose are somewhat similar. In one of the trials, the analysis can have gotten the compounds confused.

The total amount of carbohydrates also differs significantly from previously published material by (Olsen et al., 2021). The total carbohydrate content in that publication is estimated at 19.9 g per 100 g dry matter, significantly higher than the estimated 11.1 g per 100 g dry matter from this analysis. In the previous publication, the carbohydrate content was estimated by analyzing the weight of each other major cell component and subtracting from the total weight. The remainder was assumed to be carbohydrates. The difference from this analysis can as such be some unidentified major component which were not quantified by previous analysis methods, or sugars which were included in the standard. The uronic acid content would also contribute to the total carbohydrates, although as the uronic acids are below the limit of quantification they are not included in the total.

Significant differences are detected when comparing the samples post fermentation to untreated microalgae biomass. Most notably, a significant amount of glucose has been consumed in all fermented samples but most of all in the negative control sample, the sample with Tail55, and the sample with TZ266. The decrease in glucose content supports that the glucan degrading enzymes have influenced the glucans in the cell and made the carbohydrates available as substrate to the bacteria. In the case of TZ266 and Tail54, this decrease in glucose content also correlates to a clear decrease in pH, which could indicate higher production of lactic acid. In negative control sample, the decrease in glucose does however not seem to be connected to a decrease in pH; instead, the sample has the highest pH of all tested samples. This could be due to uncontrolled and unknown bacteria growing in the sample and producing unknown byproducts with more neutral pH. No glucan degrading enzyme was added to the negative

control, which could indicate that some of the unknown strain growing in the sample feature more effective glucan polymer degrading activity than *L. plantarum* does naturally.

Although it seems the glucan polymer degrading enzymes have had an effect, it remains unknown whether it has led to significant penetration of the cell wall. Even if some cellulosic compounds of the cell wall could have been degrading the overall structure could remain unaffected, or a single layer of the cell wall could have been degrading without making the cytoplasmic compounds of the microalgae cell more available.

While the largest amount of glucose was consumed, significant amounts of galactose and mannose in all cases. The amount consumed does not seem to increase with any enzyme additive, but more mannose does remain when fermenting with the commercial mix Feedtech F10.

Xylose and fucose have not been significantly consumed, which could be due to a lack of relevant metabolic pathways in the strain used. In almost all cases, the level of mannitol has increased which could be interpreted as it being a product or metabolite of the fermenting strains. However, the mannitol content did not increase in the *L. plantarum* control but did increase in all samples containing both an enzyme additive and *L. plantarum*. Similarly, the arabinose content was unchanged in all samples except the F10 sample where the content increased. Arabinose could be a metabolite or product of one of the strains in the commercial mixture.

Overall, the total sugar content did decrease significantly which suggest the microalgae biomass did function as sufficient substrate for bacterial growth. The addition of glucanases (CWD enzymes) increased the consumption of sugars by degrading polymers and making their products available as substrate to the bacteria.

3.6.2 Batch 2 analysis

A selected subset of the samples fermented in batch 2 were analyzed with the same protocol as batch 1. Aselection of samples was made due to time constraints in sample preparation, and only the different enzyme cocktails as well as one mixed sample (E) were analyzed. Results are presented in table 15 and the averages summarized to total sugar content as previously.

Table 15. Monosaccharide content of a selection of samples fermented in batch 2. The total sugar is calculated by summing the identified sugars.

Sugar Content (g /100g DW)	**Fucose**	**Arabinose**	**Galactose**	**Glucose**	**Xylose**	**Mannose**	**Mannitol**	**Total Sugar**
A	0.72 ± 0.08	1.01 ± 0.12	0.52 ± 0.08	2.34 ± 0.27	0.08 ± 0.03	3.48 ± 0.45	0.44 ± 0.21	8.59 ± 1.16
B	0.65 ± 0.05	0.75 ± 0.08	0.47 ± 0.02	0.34 ± 0.01	0.02 ± 0.01	2.8 ± 0.15	0.19 ± 0.02	5.23 ± 0.29
E	0.67 ± 0.06	0.83 ± 0.15	0.4 ± 0.04	0.18 ± 0.02	0.03 ± 0.01	2.56 ± 0.16	0.15 ± 0.06	4.82 ± 0.46
I	0.6 ± 0.09	0.85 ± 0.14	0.38 ± 0.06	0.29 ± 0.03	0.02 ± 0.01	2.54 ± 0.21	0.14 ± 0.07	4.81 ± 0.59
J	0.55 ± 0.04	0.83 ± 0.07	0.4 ± 0.02	0.75 ± 0.03	0.02 ± 0.01	2.7 ± 0.13	0.25 ± 0.1	5.5 ± 0.34
K	0.52 ± 0.01	1.09 ± 0.07	0.21 ± 0.1	0.28 ± 0.01	0.02 ± 0.01	2.46 ± 0.14	0.52 ± 0.05	5.1 ± 0.24
L	0.36 ± 0.16	0.77 ± 0.38	0.21 ± 0.04	1.57 ± 0.17	0.02 ± 0.01	2.68 ± 0.26	0.26 ± 0.12	5.88 ± 0.8

The results are mostly in accordance with expectations. More glucose had been consumed in sample A then the *L. plantarum* control sample in batch 1, which should be comparable. Sample A only had a higher amount of inoculum added, which could explain the difference. Roughly the same sugar consumption trends as were apparent in batch 1 are present in batch 2 as well. Sample L, the endoprotease sample, did cause small increase in glucose and mannose consumption which not expected. Speculatively, hydrolysis of glycoprotein could cause saccharification, however the pH is not below the control.

3.6.3 Free sugar analysis

The soluble sugars in the algae were also analyzed in some samples from batch 2, along with a untreated sample. The analysis was made on the supernatant of a centrifuged sample which had previously been leeched in MilliQ water. The results are presented in the same structure as previous monosaccharide analysis in table 16.

Table 16. Soluble sugar analysis on material from batch 2, with the total sugar summarized of the sugars detected above the limit of quantification.

Free Sugar (g /100g DM)	**Fucose**	**Arabinose**	**Galactose**	**Glucose**	**Xylose**	**Mannose**	**Mannitol**	**Total Free Sugar**
RAW	0.03 ± 0.003	<LOQ	0.3 ± 0.017	0.62 ± 0.035	0.05 ± 0.009	0.51 ± 0.026	0.27 ± 0.005	1.79 ± 0.085
A	0.03 ± 0.002	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.03 ± 0.003
B	0.03 ± 0.004	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.03 ± 0.003

The dissolved free sugars show a different profile to the total sugar composition, which suggests some sugars are to a higher extent bound in larger polysaccharides. Almost no free sugars are detected after fermentation, with only fucose remaining above the limit of quantification. The results suggest the microbial activity has consumed all available sugars but is perhaps unable to metabolize fucose.

The total detected sugar composition and the changes to it from fermentation without enzyme additives, as well as the initial free sugar profile are presented in figure 18.

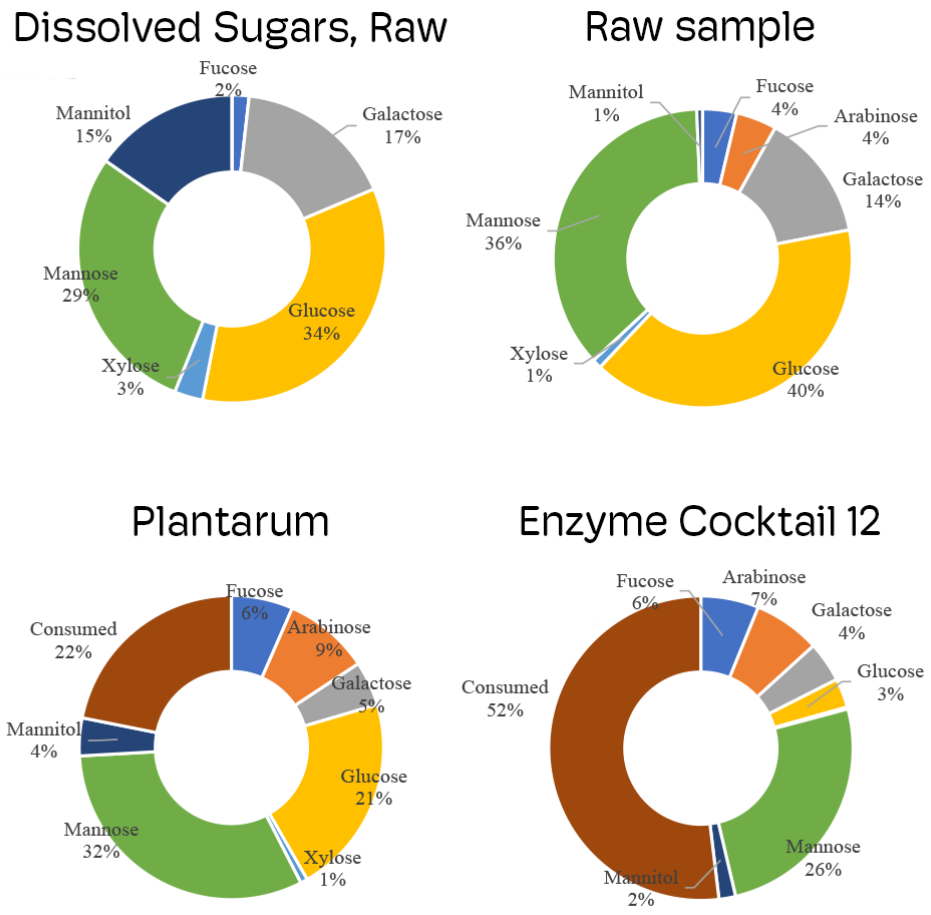

Figure 18. Overview of monosaccharide content. The percentages are of total initial sugar content of the Raw sample, to visualize how much of each sugar has been consumed. The exception is the dissolved sugars, where the percentage is calculated from total dissolved sugars.

The figure visualizes the total amount of monosaccharides consumed, and how the profile changes as a result. The two bottom figures show the monosaccharide contents in relation to the total sugar content of the untreated sample in order to visualize what total amount of sugar has been digested by the LAB. The dissolved sugar sample only shows what sugars were present in the supernatant before fermentation.

3.7 Uronic acid analysis

As evidenced by the difference in carbohydrate content between previous publication and this analysis, large amounts of unknown compounds are present in the microalgae biomass. One common component are polymers composed of uronic acids, like the pectin present in many plants and fruits. From literature of related *Scenedesmus* species, an outer layer of some pectinic or related polymer has been hypothesized. A characterization of both fermented and untreated material was performed to evaluate the content.

3.7.2 Batch 1

The first uronic acid analysis was performed on the fermented material from batch 1 and raw material. The average of biological triplicates along with standard error is presented in table 17, with the average dry matter content of each acid summarized as total uronic acids.

Table 17. HPAEC-PAD analysis of uronic acid content in fermented and untreated Scenedesmus sp. samples.

Uronic Acid Content (g / 100g DW)	Galacturonic Acid	Guluronic Acid	Glucoronic Acid	Mannuronic Acid	Total Uronic Acids
Raw	<LOD	<LOD	<LOQ	<LOQ	0
Negative Control	<LOD	<LOD	<LOQ	<LOQ	0
FeedTech F10	<LOD	<LOD	<LOQ	<LOQ	0
Lb. plantarum	<LOD	<LOD	<LOQ	<LOQ	0
Tail 55 + Lb.	<LOD	<LOD	<LOQ	<LOQ	0
Alginate Lyase + Lb.	<LOD	<LOD	<LOQ	<LOQ	0
TZ266 + Lb.	<LOD	<LOD	<LOQ	<LOQ	0

Contrary to the hypothesis, very limited to no uronic acids of the types screened for were detected. While some amount of glucuronic and mannuronic acid were detected, the amounts were below the limit of quantification and should be considered close to zero. Particularly the lack of galacturonic acid was notable since that is the main component of pectin, which was suspected to be a major component of the cell wall. The results is also contrary to previous analysis, where Tail73, a blend of pectinases, was found to be effective in increasing amine solubility. It is very unexpected to find no galacturonic acid content and by extension no pectin.

While the results seem to be reliable due to a good fit to retention times and standard curves seeming reliable, the sample preparation could produce faulty results. The hydrolysis could be too harsh and degrade the uronic acid, or the hydrolysis could be insufficient and the uronic acids remain in their polymer form (Garna et al., 2006). The procedure used is however well established and such concerns are unlikely to be valid. A validation experiment to confirm the hydrolysis procedure should be performed by spiking the sample with some known amount of pectin before hydrolysis or comparing the standard procedure with a longer period of hydrolysis.

Overall, the results were contrary to the expected, and the observed outer layer does not seem to be composed of uronic acids. The difference in carbohydrate content also remains unknown. If the results are correct, enzymes like pectinases or alginate lyase would be unlikely to exhibit significant effect on the biomass.

3.7.2 Batch 2 and method validation

Although batch 1 showed no evidence of significant amounts of uronic acids, a second analysis was performed on selected materials from batch 2 to validate the results. Included in the analysis is a sample spiked with pectin from orange peel containing >74% galacturonic acid when preparing the sample, to validate the hydrolysis method. The same subset of samples analyzed for monosaccharide content were analyzed for uronic acid content, and as previously all acids screened for were below the limit of quantification and are not presented. The sample spiked with pectin did however elicit a significant response, as presented in table 18.

Table 18. Uronic acid content of fermented material from batch 2, and one pure pectin spiked sample.

Pectin Validation		
Pectin Solution created	10.05	mg /ml
Expected Galacturonic acid in sample	22.7	µg / ml
Detected Galacturonic acid	21.88 ± 0.001	µg / ml

Prior to the hydrolysis, 0.1 ml of the 10.05 mg/ml pectin solution was added which after dilution should correspond to 22.7 µg / ml in the sample. The detected amount of galacturonic acid is consistent with the expected value. The sample preparation does not seem to affect the sample beyond the expected, which supports the reliability of uronic acid analysis method.

3.8 Solids and ash

Solid state fermentation can cause a reduction of dry matter content, due to consumption of solid substrates and formation of gaseous and liquid effluent. If high dry matter content losses are detected, secondary fermentation could be a concern. Table 19 summarizes the dry matter content and ash in each sample in batch 1.

Table 19. Total solids and ash from the NREL protocol.

Sample	**Solids (g/ g Wet Matter)**	**Ash (g /g Solids)**
Raw	0.20 ± 0.017	0.06 ± 0.001
Negative Control	0.16 ± 0.007	0.07 ± 0.002
FeedTech F10	0.15 ± 0.001	0.06 ± 0.002
Lb. Plantarum	0.15 ± 0.002	0.06 ± 0
Alginate Lyase + Lb.	0.16 ± 0.001	0.06 ± 0.001
Tail55 + Lb.	0.16 ± 0.004	0.06 ± 0.001
TZ266 + Lb.	0.14 ± 0.003	0.05 ± 0.009

The same drying procedure was repeated for the silage samples in batch 2, as presented in table 20.

Table 20. Total Solids and ash content from selected batch 2 samples, by NREL method.

Sample	**Enzyme additive**	**Solids (g/ g Wet Matter)**	**Ash (g /g Solids)**
A	Control	0.17 ± 0.004	0.02 ± 0.001
B	Enzyme Combination 12	0.16 ± 0.001	0.03 ± 0.011
E	Enzyme Combination 12, mixed	0.16 ± 0	0.06 ± 0.001
I	Enzyme Combination 0	0.16 ± 0.001	0.06 ± 0.003
J	Enzyme Combination 7	0.16 ± 0.001	0.06 ± 0.002
K	Enzyme Combination 9	0.13 ± 0.032	0.08 ± 0.011
L	Tail204	0.16 ± 0.003	0.06 ± 0

After fermentation the total solids did seem decrease for all samples, with the sample with added TZ266 decreasing the solids the most. The loss of dry matter is an important parameter, both as a product safety indicator but also for economic feasibility. The final animal feed product consists only of a dried powder, and as such large losses in dry material corresponds directly to loss of product. Fermenting to biomass seems to decrease the marketable material

by up to 25%, which will have significant impact on the viability of the process. Costs of drying the material will also increase.

3.8.1 Drying rate parameters

Dry content was also measured with a tabletop instrument using external ventilation to control constant airflow. The instrument was used to potentially get a more in-depth evaluation of how drying and dry matter was impacted by the fermentation. The results of batch are presented in table 21.

Table 21. Drying time and dry content parameters, generated with a tabletop Moisture Analyzer HC103. Averages of triplicates are presented alongside standard error.

Sample	Dry Matter (g/ 100g)	Critical Moisture Content (g Water /g g DM)	Slope of Constant Rate Period (g Water/ g DM s)	Constant Rate Period Length (s)
Raw	21.8 ± 0.5	2.2 ± 0.34	-0.016 ± 0.002	105 ± 40.1
Negative Control	21.7 ± 0.31	1.8 ± 0.3	-0.013 ± 0.0024	153.3 ± 41.77
F10 FeedTech	21.8 ± 0.75	2.2 ± 0.36	-0.015 ± 0.0023	108.3 ± 38.44
Lb. Plantarum	22.7 ± 0.45	1.9 ± 0.25	-0.013 ± 0.0018	130 ± 37.86
Lysozyme + Lb.	19.9 ± 3.43	2.2 ± 0.93	-0.016 ± 0.0058	170 ± 57.66
Alginate Lyase + Lb.	19.2 ± 2.34	3.3 ± 0.42	-0.023 ± 0.0028	51.7 ± 1.67
Tail54 + Lb.	22 ± 0.14	1.2 ± 0.08	-0.01 ± 0.0003	226.7 ± 11.67
Tail55 + Lb.	22.1 ± 0.65	2 ± 0.4	-0.014 ± 0.0027	136.7 ± 50.85
TZ266 + Lb.	17.3 ± 7.64	4.3 ± 2.36	-0.029 ± 0.0153	111.7 ± 54.34

The tool Moisture Analyzer HC103 generates real-time data of the water evaporation rate, which could potentially be used to create graphs and calculate key parameters useful in drying rate estimations. Figure 19 shows a characteristic graph of the untreated algae, and an estimation of the slope of the constant rate drying period. Although figures were created for all analyzed samples, the large variance makes the figures unreliable for analysis and as such they are not presented. Figure 19 is in included as an example that *Scenedesmus* does dry with the expected pattern of constant drying rate followed by falling drying rate.

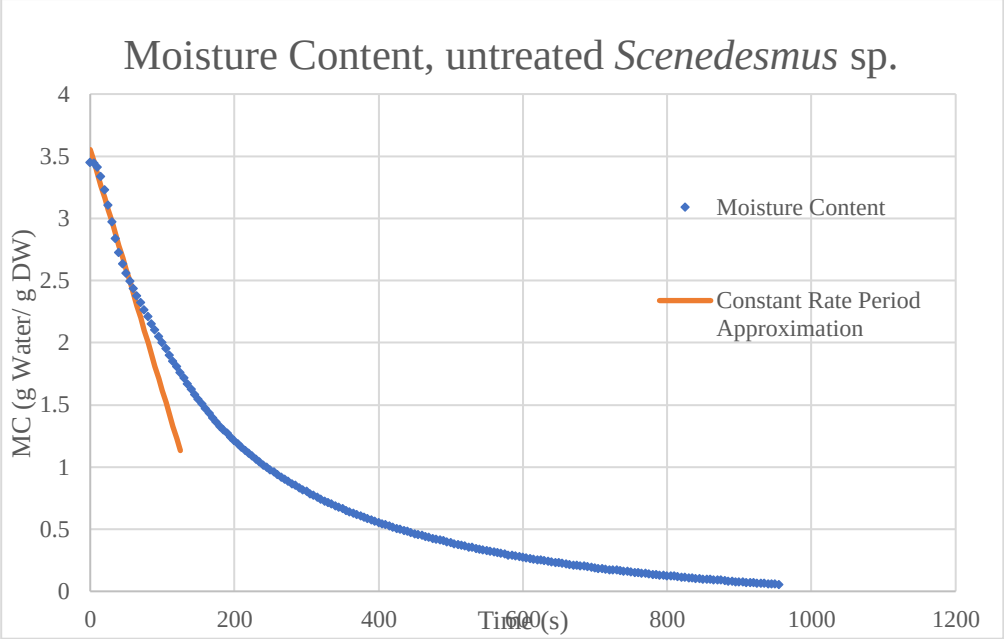

Figure 19. Moisture content (MC) per g dry matter in untreated Scenedesmus sample.

Because of the high standard error between observations, the results are not useful as evidence for change in drying time. When comparing to the standard crucible drying method, the results have significantly higher average and variance making it unreliable for calculating useful key parameters. The HC103 moisture analyzer was not used in further experiments, and any evaluation of economic impact to drying was not possible.

3.9 Protein digestibility

The protein digestibility results are presented for batch 1 and batch 2 separately. The calculated scores in PDCAAS are presented in tables with analysis of significant difference, while PDCAAS and *In vitro* protein digestibility are presented in comparative figures.

3.9.1 Batch 1

Using the Megazyme protein digestibility assay kit, the average in vitro digestibility and protein digestibility corrected amino acid score (PDCAAS) was calculated for each fermented sample. The results and statistical analysis are presented in the table 22, including an analysis of raw untreated *Scenedesmus* sp. as reference.

Table 22. Results and standard error (triplicate) from protein digestibility assay, presented as PDCAAS and protein digestibility score. Dunnett's test was used to validate a significant difference ($p < 0.05$) to untreated sample and sample fermented with L. plantarum. All samples which were not significantly different to the L. plantarum *control.*

Sample	Enzyme Activity	*In vitro* Protein Digestibility	PDCAAS
Raw	*None*	0.82 ± 0.003	0.79 ± 0.003
Negative Control	*None*	0.84 ± 0.003	0.82 ± 0.003*
FeedTech F10	*None*	0.84 ± 0.002	0.82 ± 0.002*
Lb. Plantarum	*None*	0.86 ± 0.002	0.83 ± 0.002
Lysozyme + Lb.	*Lysozyme*	0.86 ± 0.001	0.82 ± 0.001
Alginate Lyase + Lb.	*Poly alpha-I-Guluronate Lyase*	0.86 ± 0.002	0.83 ± 0.002*
Tail54 + Lb.	*Endo-1,3(4)-Glucanase*	0.86 ± 0.002	0.82 ± 0.002*
Tail55 + Lb.	*β -glucanase, pectinases, hemicellulases, xylanases*	0.86 ± 0.001	0.83 ± 0.001*
TZ266 + Lb.	*Cellulase, hemicellulase*	0.86 ± 0.002	0.82 ± 0.002*

Two separate Dunnett test qualifiers were calculated to verify the significance in the change in PDCAAS. The first test verified the difference to the untreated sample and indicates a significant increase in PDCAAS due to fermentation and enzyme additive where applicable. All samples score significantly higher, at a significance of $p < 0.05$, in this case.

The second test compares the results to the sample inoculated exclusively with *L. plantarum.* The aim of the second test is as such to verify a significant difference when co-digesting with enzyme additive to not using enzyme additive. The exception being for samples FeedTech F10 and Negative Control, where the test instead indicates a difference depending on the inoculum used. Except for when adding lysozyme, no significant difference is detected when using any enzyme additive or different inoculation. A visual representation of both protein scores for each analyzed sample is available in the appendix.

The results are contrary to the expected outcome. The reason for the insignificant change in protein digestibility could be due to the enzymes not having the desired effect on the microalgae cell structure. The enzymes used are most suitable for use at 40-60 °C and more acidic pH, while the solid-state fermentation is conducted at room temperature and uncontrolled pH. The enzyme additives could as a result be in a suboptimal state and exhibit negligible activity.

The results in reduced pH and increased sugar consumption does however indicate the enzymes being active, and in the case of CWD-enzymes having some effect on glucan-polysaccharides which are likely in the cell wall. The cell wall disruption could be negligible for increased protein digestibility, or the suboptimal protein digestibility could be due to other reasons than the cell wall lowering bioavailability.

3.9.2 Batch 2

Like previously, the Megazyme ™ K-PDCAAS assay was used alongside provided calculation aid in Microsoft Excel, using XLSTAT for statistical analysis. The average K-PDCAAS score and protein digestibility score from biological triplicate samples are presented in table 23, with the associated standard error.

Table 23. Protein digestibility assay results from the second fermentation batch. Results denoted with an asterisk were deemed insignificantly different from the control sample A by the Dunnet method and ANOVA analysis.

Sample Name	Condition	*In Vitro* Digestibility	PDCAAS
RAW	*Untreated Sample*	0.82 ± 0.003	0.79 ± 0.003
A	*Inoculated control*	0.85 ± 0.002	0.81 ± 0.002
B	*Enzyme Mixture 12, 1%*	0.86 ± 0.002*	0.82 ± 0.002*
C	*Enzyme Mixture 12, 0.1%*	0.85 ± 0.001*	0.81 ± 0.001*
D	*Enzyme Mixture 12, 5%*	0.86 ± 0.001	0.82 ± 0.001
E	*Mixture 12, 1%, Mixed*	0.87 ± 0.001	0.83 ± 0.001
F	*Mixture 12, 1%, Diluted*	0.84 ± 0.001*	0.81 ± 0.001*
G	*Mixture 12 1%, Diluted, Mixed*	0.85 ± 0.001*	0.81 ± 0.001*
H	*Mixed*	0.85 ± 0.002*	0.81 ± 0.002*
I	*Mixture 0, 1%,*	0.86 ± 0.001	0.82 ± 0.001
J	*Mixture 7, 1%*	0.86 ± 0.002	0.83 ± 0.002
K	*Mixture 9, 1%*	0.86 ± 0.001	0.83 ± 0.001
L	*Tail204, 1%*	0.88 ± 0.001	0.84 ± 0.001

The Dunnett *ad hoc* test was used to determine significant difference from control sample A. Similar results to previous fermentations were observed, characterized by minimal yet still significant difference from the control. The results are visualized in figure A2 in the appendix, which presents the averages with error bars from the standard deviation.

While the differences are small, samples E and L achieve the highest scores. Sample L is the only sample containing an endoprotease (Tail204), which hydrolyzes proteins to increase the reaction potential with ninhydrin. Sample E has the same additive concentration as sample B but was lightly mixed throughout the fermentation and achieved a higher score. The same

pattern is however not present between samples A and H, where H was stirred throughout, and A was not. The result could be influenced by the loss of one replicate of sample E during the mixing, as scratching had at some point torn a hole in the vacuum bag. Like the results in batch 1, the difference in PDCAAS achieved is minimal, and in most cases not statistically significant. Some samples do achieve a higher result compared to the control, which is positive compared to the results in batch 1 where the trend of sample with enzyme additives scoring slightly lower than the inoculated control. Overall, even after pilot studies to examine the best possible enzyme combinations the increase in PDCAAS is negligible.

4. Discussion

4.1 Summary

Overall, ensiling of *Scenedesmus* material seems to be successful when utilizing glucan degrading enzymes for saccharification, which allows higher production of lactic acid. Although some effect on the cell wall seems to be present from the glucose consumption, the effect on protein digestibility is largely insignificant. Some important discoveries regarding the composition of the cell were made, primarily the high levels of mannose and lack of uronic acid. The lack of uronic acid is in stark contrast to the seemingly high impact of pectinases, which should only have significant activity on galacturonic acid.

4.2 Effects of fermentation

A primary objective of the project was to investigate whether solid-state fermentation on *Scenedesmus* was possible, and to identify what concerns and solutions are relevant to the ReMAPP research project and similar future endeavors. Overall, fermentation on microalgae biomass seems successful and could bring many positives at low cost. Using additives of various enzymes and inoculating with lactic acid bacteria has significant and positive effects, mainly in decreasing the pH to safe levels.

The pH levels of samples is an effective and easily measured parameter of silage quality, from which a lot can be interpreted. In batch 1 a sample was fermented with no additives, referred to as negative control. The negative control sample had both on average significantly higher pH and higher pH variance than all other fermented samples. When looking at monosaccharide content, the negative control had among the lowest sugar content remaining after fermentation of all samples at 4.65 g / g DM, a 58.25 % loss of sugar. Most of the consumed sugar was glucose and mannose. The high sugar consumption suggests that there was high bacterial growth, although the high pH suggests that undesired acids like butyric acid, acetic acid and carbon dioxide from the high gas formation are more likely fermentation products. Rather than the desired homofermentative lactic acid production, high growth of contaminants like *Clostridium* has caused secondary fermentation. The high variance in pH also suggests several different bacteria or fungus could have grown between the produced triplicates. The growth of contaminants when fermenting without inoculum is according to expectations with such low dry matter content as the microalgae material since the increased water activity favors the undesired activity.

Adding *L. plantarum and* using the commercial additive F10 FeedTech, brings greater control to the fermentation. While the F10 additives brings down the pH to 4.46, using pure *L. plantarum* decreases the pH to even lower levels, likely due to more homofermentative production of lactic acid. The average pH of samples fermented only with *L. plantarum* was 4.2, with very low variance which suggests that the strain used successfully outcompeted other contaminating microorganisms at the dosage level.

The goal of ensiling to below pH 4.0, a benchmark for low populations of *E. coli* and *Listeria* growth, was however not achieved without enzyme additives. Two of the enzymes used in batch 1 did reach the set goal; Tail55, a mixture of β-glucanase, pectinases, hemicellulases, xylanases and TZ266, a cellulase and hemicellulase blend. Both mainly target fibrous material in the cell wall of many plants and green algae, which is seemingly also present in the novel *Scenedesmus* species.

An explanation for the reduced pH can be gleamed from the monosaccharide analysis. Both TZ266 and Tail55 addition resulted in increased sugar consumption compared to the raw sample and the *L. plantarum* control sample. The control sample resulted in 42.46% loss of total sugar, whereas TZ266 had 51.61% and Tail55 had 58.17% total sugar loss, where the increased sugar loss can be attributed almost entirely to increased loss of glucose, as the glucose is almost entirely depleted in the TZ266 and Tail55 samples and 2.25 g/ 100 g DM glucose remained in the control sample. This result seems to confirm the hypothesis of using hydrolytic enzymes targeting fibrous material, like cellulose and hemicellulose, to induce saccharification in the cell wall since more glucose was consumed and the pH dropped, indicating higher production of organic acids. From the soluble sugar analysis, almost no soluble sugars remain after fermentation, which would indicate that the LAB has consumed all available sugars since they absorb the sugars from the water interface.

Dry matter content of the material does drop significantly post fermentation, which could have economical and product safety implications. Although more carbohydrates are consumed in fermented samples with enzyme additives, no clear correlation is evident, and the total solids loss is roughly equal in all analyzed samples. The solids loss varies between 3-7%, whereas 2-4% solids is generally acceptable in agricultural lactic acid ensiling. Dry matter losses above 2-4% could be due to contamination. However, higher than desirable dry matter losses have previously been associated with higher initial wet matter which in this case is higher than normal and as such is not necessarily an indication of contamination (Knický, 2005). Negligible gas formation and low pH supports the fermentation being overall successful. Although the solids loss could be deemed safe, economic losses are still relevant. Since the final product will be sold as a dry powder, only the solids hold economic value. With the current dry matter losses, 22.3% of the vendible material has been lost. In addition, the solid material has been converted to liquid which requires additional separation inducing higher drying costs. Methods to reduce solids losses should be investigated to create an economically feasible process. Additives such as formic acid, propionic acid, sodium nitrate with organic acid salts and hexamine have been reported as successful in that regard although separate problems arise from using additives (Lingvall & Lättemäe, 1999; Pursiainen, 2001). Using microalgae material with higher initial dry matter could also reduce solids losses (Knický, 2005). The observed dry matter losses could be due to monosaccharide conversion into organic acids, although the mass balance of monosaccharide losses to the loss in mass does not fully explain the losses. Another potential explanation could be intracellular water being evaporated after the cell wall has lost integrity from fermentation.

Overall, using *L. plantarum* and a cell wall degrading enzyme for saccharification of cell wall components does produce silage with sufficiently low pH. To further evaluate and improve ensiling of *Scenedesmus*, several relevant investigations could be conducted.

- Extended time trials should be conducted to evaluate long-term stability.
- Microbial counts of the inoculum, *E. coli, Clostridium, Listeria,* and fungus.
- Aerobic stability of the silage should be evaluated by ventilating samples post opening, as well as monitoring pH and CO_2 production.
- Fermentation products analysis to validate hetero- or homofermentative metabolism
- Palatability and sensory trials. Unknown greasy films form from fermentation, which could be due changes in fatty acid profile or other byproducts (Ziarno et al., 2020).

- Acidification rate trials. Silage quality depends on the rate of pH drop, which has not been analyzed in this project. Acidification rate could vary significantly depending on the pH optimum of the hydrolytic enzyme used.
- Larger scale fermentation using tank/silo equivalents rather than vacuum bags, for scale-up investigation and sensitivity to oxygen.
- Evaluation of drying parameters post fermentation, since the trial failed in this project.

4.3 Effects on protein digestibility

Only very slight effects on protein digestibility could be confirmed, with most enzymes not yielding a significant difference. Lactic acid fermentation on its own did have a positive effect on protein digestibility. While even small increases in PDCAAS are notable and can increase the economic feasibility, the cost of enzymes have to be weighed against the gain.

Lactic acid fermentation by itself can have significant effect on a material in terms of nutrition. Although *Lactobacillus plantarum* can produce a host of glycosidic enzymes, it cannot inherently degrade cellulose or hemicellulose (Moraïs et al., 2013). The increase in protein digestibility from saccharification of fibrous cell wall material by endogenous *L. plantarum* activity is unlikely, although unknown polysaccharides could have been affected, since the total glucose content does decrease by 2.15 g/100 g DM, of which only 0.62 g/100g can be accounted for by free, soluble glucose. Lactic acid fermentation does however have some reported nutritional value increases as an effect on biochemical changes rather than increased bioavailability from cell wall disruption. A sharp reduction, 36%, in phytic acid content, a common ANF which affects protein digestibility in many plants, was found in solid-state fermentation on wheat bran (Spaggiari et al., 2020). Lactic acid fermentation has also been connected with a decrease in tannin and trypsin inhibitors, and which was directly correlated with an increase in *in vitro* protein digestibility (Ma et al., 2018). While ANFs are common among many plant species, it is unknown whether any are present in the identified *Scenedesmus* species and could be a cause for limited digestibility.

Introducing enzyme additives has very limited to no effect on protein digestibility. The limited effect could be due to the cell wall remaining largely unaffected, even though CWD do almost deplete the available glucose. This would likely signify the cell wall having other significant components which have not been degraded, like algaenan which has been identified in other *Scenedesmus* species. Since pectinase does have the most pronounced effect of any non-protease enzyme, some relevant substrate seems to be present. The presence of a pectinic outer cell wall would be in line with some previous literature (Spain & Funk, 2022), although other *Scenedesmus* reports very limited presence of any uronic acid (Blumreisinger et al., 1983). From the uronic acid analysis, no evidence of galacturonic acid, the majority component of pectin, was found nor any other of the analyzed uronic acids. Although the specific pectinase used is unknown, pectinase generally acts only on galactosiduronic linkages (Biz et al., 2014) which are not present in this *Scenedesmus*. To draw any conclusions, further characterization of the pectinase and potential targets in the cell wall would be required. One further, speculative, possibility could be that pectinases do not significantly extract more protein, but rather that some non-protein amine was made more available for reaction as a results of pectinase treatment. Other species of microalgae, and some other *Scenedesmus* do have amine containing compounds like chitinase in *Chlorella* or general glucosamines in the cell wall which could react with the ninhydrin in the assay. Such conclusions would however also require more thorough characterization.

The enzymes with glucan activity did consistently increase glucose consumption from non-digestible glucans. Two of the commercial enzymes had significantly increased response in the screenings, Extract-01L and CelluX-01L. Both are specified as β-glucanases by the supplier, which could speculatively indicate some interesting property of the glucan linkages in the cell wall. While the documented information about the other screened enzymes is somewhat lacking, Extract-01L specifically is capable of hydrolyzing both β-1,3 and β-1,4 glycosidic linkages. The target substrate for the enzymes has been assumed to be standard cellulose, which normally is unbranched and linked by only β-1,4 glycosidic linkages. Since Extract-01L is the only enzyme with specified activity on β-1,3 bonds and among the only enzyme targeting glucans with positive effect, it could be an indication of some cell wall polymer with β-1,3-glycosidic bonds being relevant. Some polysaccharides with those linkages are commonly occurring in nature, perhaps most significantly in β-glucans. β-glucans are primarily connected by isolated β-1,3-glycosidic bonds or with sets of β-1,4-glycosidic bonds, although macromolecular structure does vary significantly depending on the source. β-glucans commonly associated with high presence in cereals, varieties are also present in yeasts, fungal, bacterial and algal sources (Du et al., 2019).While not a highly researched subject, some amount of β-glucans has previously been detected in a few species of *Scenedesmus* (Schulze et al., 2016). The high presence of β-1,3 glycosidic linkages as a fibrillar structure in the cell wall of this *Scenedesmus* isolate seems like a possibility.

The identified high mannose content could also have some implication on cell wall structure. The high content implies high presence of mannans in the cell wall, since most carbohydrates in microalgae are situated there (Moreira et al., 2022). In addition, significant amounts of mannose remain after fermentation, where almost all of the consumed mannose can be attributed to the amount free soluble mannose. Mannose is frequently a component of various hemicellulosic polysaccharides and an important structural component which does not appear to have been enzymatically digested in any treatment. Including mannanases in future trials could have significant effect on disrupting the cell wall. Most of the hemicellulases used thus far have been xylanases since xylose content was presumed to be high.

Proteases, primarily Tail204, seems to have the most positive effect on protein digestibility but might come with some downsides. Introducing proteases in the fermentation induces formation of protein hydrolysate, a mixture of free polypeptides, oligopeptides and free amino acids. Previous literature on both plant and animal protein sources has shown protein hydrolysates to be an effective way of increasing protein digestibility and amino acid absorption rate. Rat studies similarly show increased muscle mass from feeding protein hydrolysates over free amino acids and standard whey protein (Liceaga & Hall, 2019). Although the nutritious benefits are notable and seem to be present in this study as well, some effect would only be noticeable in *in vivo* analysis. Feeding free amino acids and small peptides is more digestible, since less activity is required by the gastrointestinal digestion to produce units small enough for absorption in the lumen, but the faster absorption rate causes issues as well. A higher concentration of amino acids is transported into blood plasma at initial digestion, which causes a spike in insulin production and could be problematic. In addition, high concentration of amino acids causes some amount to be deaminated in the liver and used for catabolic metabolism, rather than the desired anabolic. As a result, more urea is produced which decreases the overall utilization of nitrogen in the animal. The effect is most pronounced in free amino acid digestion. Using protein hydrolysates and proteases in animal feeds for growth performance is not fully understood, and should be treated with some caution although there seems to be a distinctive benefit in digestibility (Eugenio et al., 2022).

However, since no direct analysis of cell wall degradation has yet been performed on this species of algae, connections between cell disruption and protein digestibility is mostly speculative. A direct analysis of cell wall disruption would also be beneficial to evaluating the screening technique used. A complementary analysis of total soluble protein, like CN-analysis, should also be performed to validate the findings in the screening experiments with ninhydrin. Since the effect of enzymes on the cell wall is generally difficult to measure and predict due to lacking characterization of the *Scenedesmus* species, other traditionally used methods to degrade the cell wall could be evaluated and compared. Mechanically treating the cells, for example through homogenizer has been shown to influence other microalgae and increase the PDCAAS. Such a comparison would more effectively connect the penetration of the cell wall to an increase in PDCAAS, without introducing other changes from fermentation and enzymatic treatment.

The other assumptions pertinent to the project should also be evaluated. It has been assumed that protein content and amino acid profile remain constant. Since the assumptions have a direct effect on the PDCAAS value generated, a complementary analysis would improve the quality of the data.

The viability of increasing protein digestibility through fermentation and enzymatic co-digestion seems limited. The protein quality of *Scenedesmus* is however inherently relatively high, and within the range of many plant protein sources which shows some promise as a feed protein source. To validate results and potentially increase the upside, some further investigations are proposed.

- Screening of inherent anti-nutritional factors such as trypsin inhibitors, phytate and tannins which are very relevant in other plant-based and algal protein sources. Analysis should include a comparison before and after fermentation.
- Investigation of mannanases, as mannose is the second most prevalent monosaccharide has not been significantly affected by the treatments.
- In-depth cell characterization, to identify the potential presence of algaenan or a similar non-standard polymer as well as an explanation for pectinase activity. All further insights into cell wall structure and components are significant for further treatments.
- Validation of the relation between cell wall degradation and increased protein digestibility and/or soluble amine concentration.
- Since it is unknown whether non-protein nitrogen sources, potentially formed from ensiling proteolysis, have significant impact on the nitrogen content of the microalgae or the ability of chickens to accommodate to protein, *in vivo* studies could complement and validate the results.
- Transmission electron microscopy would be beneficial to get a better understanding of the cell wall structure, specifically whether it is laminar in nature.

4.4 Future perspectives

The thesis project shows some potential in future algal treatments and has shown ensiling to be a candidate for combined storage and pretreatment of microalgae. The results produced should be validated for product safety and long-term viability to be certain, and protein digestibility results should be reproduced *in vivo* to ascertain unknown losses and effects.

The ReMAPP project overall has potential to produce microalgae products in entirely novel way, stemming from utilizing solid-state fermentation. Some factors hinder the economic viability of the product, chief among them the price of the competing animal feed protein sources. Imported soybean is among the most used and is competitively priced compared to all potential microalgae products. With the high dry matter losses and still requiring extensive drying to produce the final *Scenedesmus* product, it is likely to remain an expensive option. Using the microalgae powder as a supplement to already existing feed would be one application, due to its unique amino acid distribution which would complement other feed.

To introduce economic viability of any microalgae products relies on continually improving technology, procedures and scale. So far, the microalgae market is small and even established companies work with relatively small bioreactors, which due to higher operational and capital costs will be more expensive than the global supplies of soybean growing in open fields. In the end, the current and increasing production of soybean is not sustainable with long transport and high requirements on arable land leading to deforestation. Microalgae is a more sustainable alternative but is a subject still in its infancy. As prices of soybean and other feed sources like fishmeal could fluctuate in a world moving toward environmental crisis from deforestation and overfishing, microalgae could become an important and viable alternative protein source. Economic viability could also be increased by identifying high value products such astaxanthin, canthaxanthin, valuable fatty acids, or compounds for cosmetics which have been found in other green algae, in the identified *Scenedesmus.* Other products could act as a pivot for the project, or an expansion of value if non-destructive extraction is possible.

The successful application of ensiling shows potential in conservation, nutrition but also some indications on sensory changes. The high bitterness and fishy taste from the omega-3-fatty acids common in microalgae have historically been a barrier for food and feed applications. Fermentation has seemingly had some effect on the sensory profile, and could increase palatability. Although not very relevant for the ReMAPP project, since chickens are not very picky eaters, some potential lies in future applications for food products. The process of classifying any microalgae as an acceptable food product is expensive and time-consuming and severely limits marketable products, an any *Scenedesmus* food product is unlikely soon. The principles of the ensiling are instead more likely applicable to the related green algae *Chlorella vulgaris,* which also has a glucan backbone in the cell wall.

In summary, the ReMAPP project and this master thesis opens the door to the novel treatment of ensiling to microalgae, which could also be applicable to other green microalgae. A long road remains ahead for the economic viability of microalgae as a protein source. The unique upsides of microalgae do create a niche which is ripe for exploitation in the long term, specifically high growth rate on non-arable local land the ability to utilize industrial or municipal waste streams and grow viable protein sources locally. There is a future for microalgae products if research continues, and the ReMAPP project does contribute valuable knowledge to that potential future.

Figure 16. A very small cell with a strong smell and high potential to sell. Thank you for reading this thesis (colorized).

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Appendix

Condition Screening

The results of the first colorimetric screening is presented in table A1. Duplicates of each sample were produced, and averages of those duplicates are presented along with the standard error.

Table A1. Averages and standard deviations, soluble protein screening.

Sample	L-Glycine eq. (mM/ g DM), Optimal Conditions	L-Glycine eq. (mM/ g DM), suboptimal Conditions
Tail275	389.8 ± 12.37	425.56 ± 12.4
Endocut-01L	233.7 ± 15.85	556.87 ± 15.8
Tail204	578.1 ± 14.27	488.81 ± 14.3
Extract-01L	543 ± 8.89	360.17 ± 8.9
Tail266	410.7 ± 11.08	353.31 ± 11.1
Tail55	448.9 ± 9.69	328.39 ± 9.7
Tail54	371.9 ± 13.62	321.31 ± 13.6
Control	478 ± 25.83	299.24 ± 25.8

When comparing the optimal enzyme conditions with expected conditions during fermentation (considered as suboptimal conditions), some unexpected outcomes are present. Under optimal conditions, the control sample with no added enzymes (incubated at pH 7, 50 °C) had an inherently higher soluble protein fraction than all but two of the tested enzymes. This pattern is however not present for the same enzymes incubated under suboptimal conditions, where the soluble protein of the control sample is significantly lower. Extended heat treatment also seems to have a large effect on the extracted protein even without any added enzyme activity, since the control samples yielded very different outcomes (which were incubated at 50 °C and 22 °C).

Table 10. Comparative quotients and statistical tests for soluble proteins screening. Asterisk denotes not being statistically significant as determined by two-tailed T-test ($p < 0.05$).

Sample	Condition Factor	Enzyme Factor, Optimal Conditions	Enzyme Factor, suboptimal conditions
Tail275	0.92	0.82	1.42
Endocut-01L	0.42	0.49	1.86
Tail204	1.18	1.21	1.63
Extract-01L	1.51	1.14	1.2
Tail266	1.16*	0.86	1.18
Tail55	1.37	0.94*	1.1
Tail54	1.16	0.78	1.07
Control	1.6	1*	1*

Protein Digestibility

The protein digestibility scores vary only marginally, and to visualize the changes bar charts of each batch of results are created. Batch 1 is presented in figure A1 and batch in figure A2.

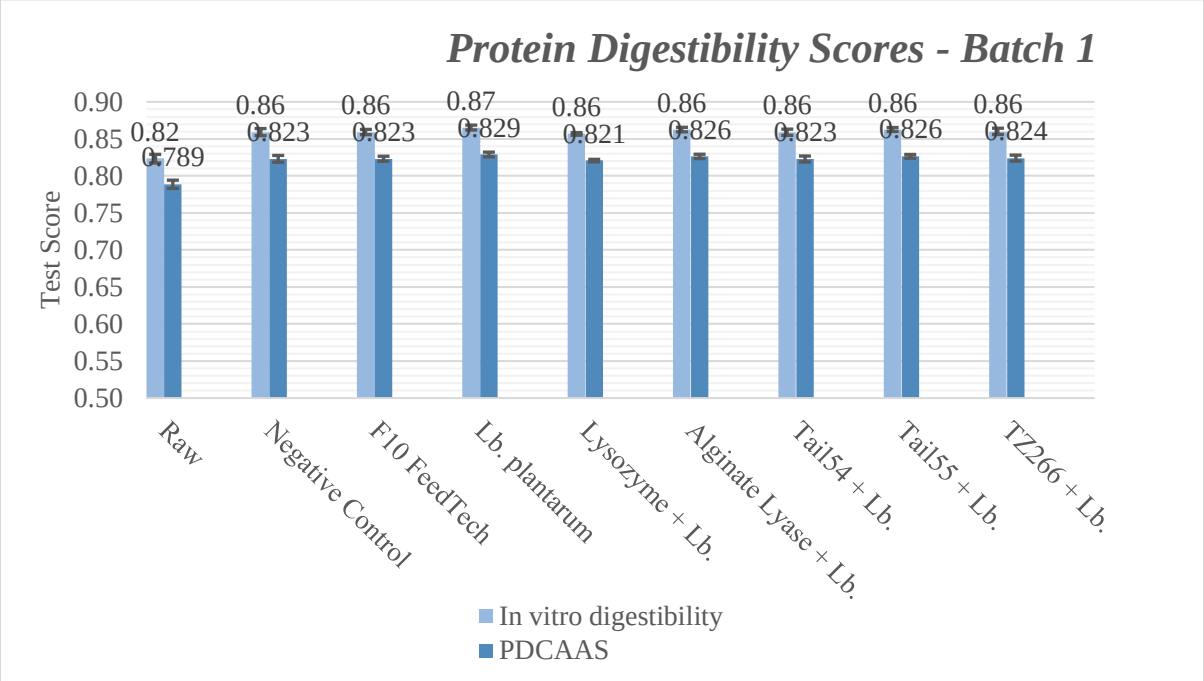

Figure A1. Protein scores of fermented samples. The samples which were inoculated with pure plantarum *are designated with Lb. Protein digestibility and PDCAAS can vary between 0-1, but the axis is limited to 0.5 for visibility.*

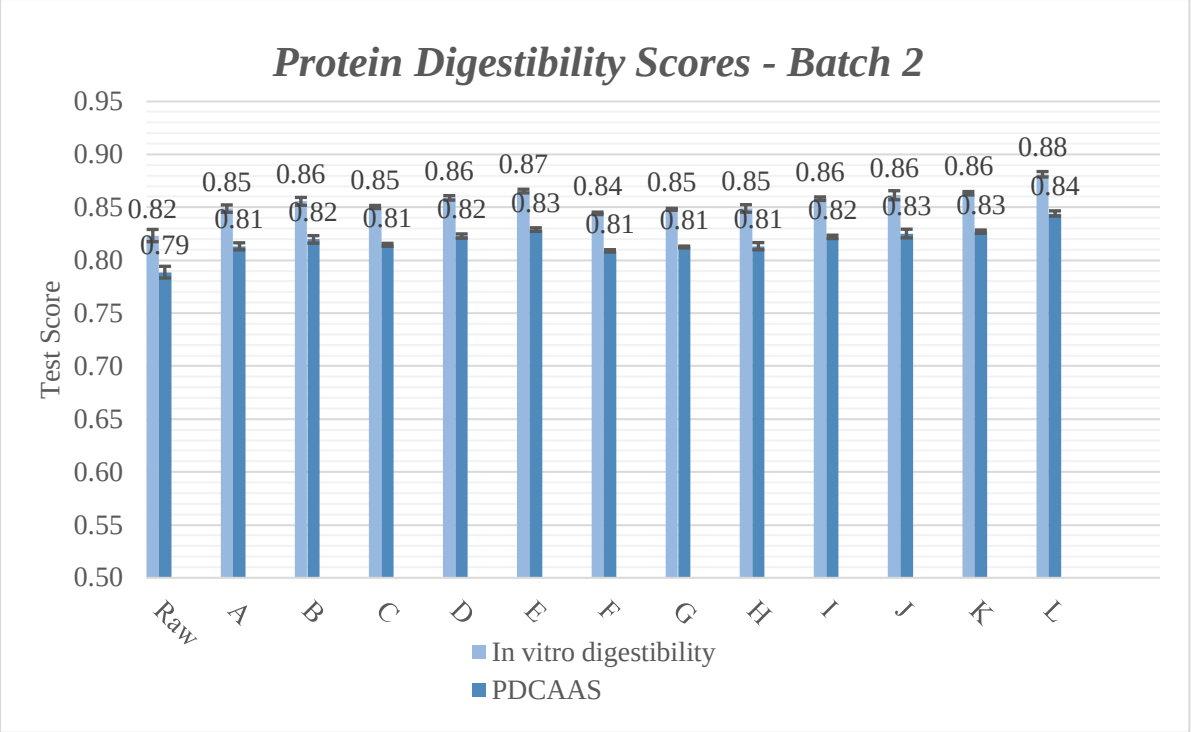

Figure A2. Visualization of the protein digestibility scores. In vitro digestibility and PDCAAS wary between 0 and 1. Protein digestibility and PDCAAS can vary between 0-1, but the axis is limited to 0.5 for visibility.