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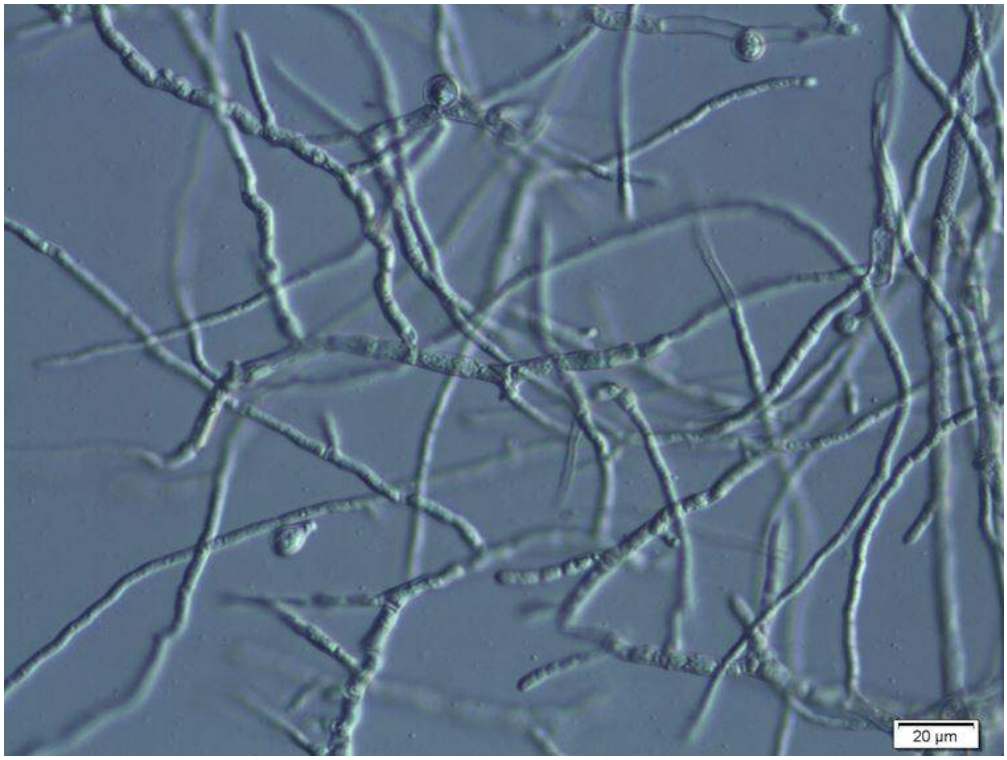

Investigating cell wall degradation treatments, pH-shifting and extrusion as methods to increase the quality of *Paecilomyces variotii* as a protein source in fish feed

BBTX03 Master thesis

FRIDA PERSSON

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Investigating cell wall degradation treatments, pH-shifting and extrusion as methods to increase the quality of *Paecilomyces variotii* as a protein source in fish feed
Frida Persson

Supervisors: Evelina Höglund and Sophia Wassén
Researchers at RISE Agrifood and Bioscience at the department Product Design and Perception

Examiner: Ingrid Undeland
Professor at Chalmers University of Technology in Food science

Master thesis 2019 in Biotechnology
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Abstract

The increased demand for seafood, due to both population growth and an increased health awareness, has resulted in an increased demand for fish feed in aquaculture production. This since wild fish capturing cannot fulfill the demand in a sustainable way. Traditionally, fishmeal has been the major protein ingredient in fish feed, providing the fish with the essential amino acids needed for optimal growth. However, the production of fishmeal has decreased rapidly the past decades. Instead, plant based protein sources has partly replaced fishmeal. The issues with this replacement is the different amino acid composition and the presence of antinutritional substances, resulting in decreased growth rates.

One alternative that is gaining interest is the use of single cell protein (SCP) as a potential protein ingredient in fish feed. SCP consists of microorganisms such as yeast, bacteria, algae and filamentous fungi. SCP has several advantages, such as similar amino acid composition to fishmeal, short generation time, no requirement to arable land and the ability to grow on various substrates. The filamentous fungus *Paecilomyces variotii* was selected in this project due to its high protein content and ability to grow on residual streams from the forest indutry.

Since the proteins in SCP are intracellular, cell wall degradation can result in higher digestibility. Further, if the polysaccharides in the cell wall are decreased, the protein content of the SCP would increase, resulting in a fish feed ingredient of higher quality. To achieve this, the following cell wall degradation methods were investigated: High Pressure Homogenization (HPH), enzymatic lysis with β-glucanase and a combination of the two methods. After the different treatments, pH-shifting was performed in order to precipitate the proteins. The pellets obtained were analyzed for protein content. Further, the carbohydrate concentration was determined in the supernatant to investigate the effect of the enzyme, and samples from the pellets were studied in a light microscope to study the cell wall degradation. Further, extrusion was investigated as a possible treatment method, and both the physicochemical and structural properties of the extruded samples were investigated. Growth and survival of *Artemia* was evaluated in a feeding trial.

The results showed that HPH resulted in the highest degree of cell wall degradation. The pH-shifting resulted in protein concentrate with a higher protein content (about 50 %) than the untreated biomass (35-40 %), with a yield of 53.81 %. When analyzing the physicochemical and structural properties of extruded samples, it was concluded that extrusion at 115 °C, with a screw speed of 120 rpm and with a moisture content of 40 % resulted in samples which held together well, both in the micro- and macrostructure, and had the lowest water solubility index (WSI) among the extruded samples. Finally, extrusion appeared to increase growth and survival in *Artemia* during the feeding trial, but no significant difference was found.

Keywords: single cell protein, fish feed, cell wall degradation, filamentous fungi.

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Frida Persson, Gothenburg, June 2019

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Introduction

The world population is constantly increasing, by 2050 it is estimated that it will reach 9.1 billion people (FAO 2009). Due to this increase, the Food and Agriculture Organization of the United Nations (FAO) predicts that an increase in food production with about 70 % will be necessary in order to feed the growing global population. Simultaneously, about 20-30 % of the environmental impact caused by human activity is related to food production (Hartmann and Siegrist 2017). Therefore, the need for sustainable protein sources is more relevant than ever due to global climate change, resulting in future challenges for the agriculture and food industries.

Seafood is an important source of nutritious food and protein. The demand for seafood is increasing due to both population growth and an increase in health awareness (Ayadi, Rosentrate, and Muthukumar 2012). This while wild fish capturing has remained at a relatively constant level of about 90 million tonnes the past two decades (see Figure 1.1). Additionally, 33.1 % of fish stocks were classified as overfished and 59.8 % classified as maximally sustainably fished in 2015 (FAO 2018). An illustration of the development of global trends in marine fish stocks between 1975-2015 is shown in Figure 1.2. Therefore, increasing the fishing is not a sustainable option since this could have serious consequences not only for the marine ecosystems, but also for the many people in coastal communities relying on fish as their main protein source (FAO 2018).

So, in order to meet the increasing demand for seafood, aquaculture has become an important source of seafood. The aquaculture production has an annual growth of around 7 %, making it one of the fastest growing animal food-producing sectors (Ayadi, Rosentrate, and Muthukumar 2012). Today, aquaculture almost contributes to half of the total world fish production, see Figure 1.2. Consequently, this leads to a high demand for fish feed.

Traditionally, fishmeal has been the main feed during aquaculture. Fishmeal is the crude flour obtained after milling and drying of fish or fish parts subjected to heating and pressing, and fish oil is a yellow/brown liquid obtained from the same process (FAO 2018). Fishmeal is still considered the most nutritious and digestible protein source option in fish feed, providing the fish with high-quality protein, essential amino acids and minerals (Ayadi, Rosentrate, and Muthukumar 2012).

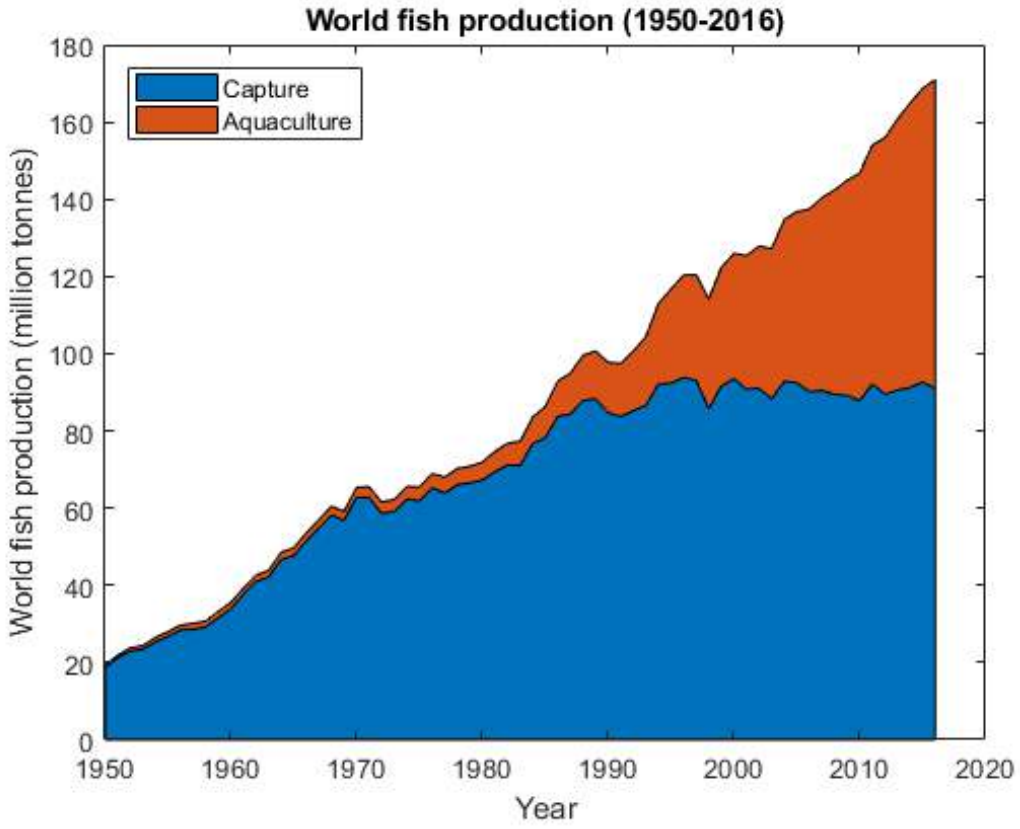


Figure 1.1: World fisheries and aquaculture production (million tonnes). Source: Data adapted from FAO 2018.

However, the production of fishmeal has decreased by about one third of the production since 1988 (FAO 2018).Today, fishmeal is more selectively used as an ingredient at lower levels for specific stages of production, even though it is still considered the main protein source for most species (FAO 2018). The reason behind the declined production is partly due to adoption of good management practices and certification schemes, resulting in lower volumes of wild caught species used for fish feed (FAO 2018). This fact, in combination with an increasing price, has resulted in scientists and companies searching for new alternative protein sources for fish feed.

One alternative source commonly used to partly replace fishmeal is plant based protein sources, such as soybean meal (Ayadi, Rosentrate, and Muthukumar 2012). One issue with replacing fishmeal completely with plant based sources is the different amino acid composition and the presence of antinutritional substances, for example protease inhibitors, phytates and alkaloids (Francis, Makkar, and Becker 2001). Further, soybean production has several sustainability issues. To begin with, soybean production is non-competitive in Europe compared to imports due to low yields (Tallentire, Mackenzie, and Kyriazakis 2018). This makes Europe dependent

on imported soybean protein for feed and food.

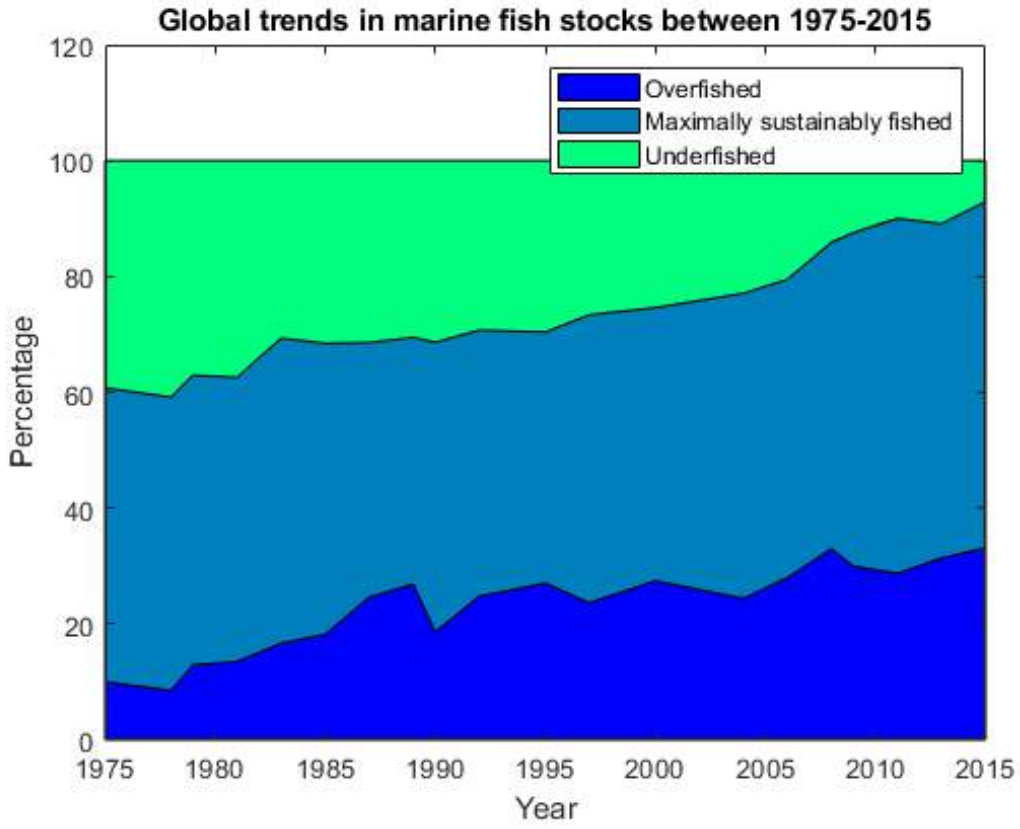


Figure 1.2: Global trends in the marine fish stocks between 1975-2015. Source: Data adapted from FAO 2018.

Secondly, soybean production worldwide has resulted in environmental issues, for example water pollution and loss of biodiversity in the producing countries (Sun et al. 2018). An example of the consequences from the increasing demand for soybean is the deforestation in the Brazilian Amazon (Sun et al. 2018). Due to the discussed sustainability issues for soybean, new sources of protein supply for feed and food are investigated. An alternative which is gaining interest is the use of single cell protein (SCP). SCP consists of microorganisms such as yeast, bacteria, algae and filamentous fungi. Unlike plant sources, many SCP have a similar amino acid composition to fishmeal, making them better suited as feed since deficiencies of essential amino acids results in decreased growth performance and feed efficiency ratios (Ayadi, Rosentrate, and Muthukumar 2012). Furthermore, microorganisms generally have a short generation time, requires no arable land and can be cultured on cheap and sustainable substrates. The filamentous fungus *Paecilomyces variotii* is known to grow on various substrates, one of them being residual streams from

the forest industry (Bajpai 2017). This opens up the possibility to turn forest raw-material into protein-rich fish feed (Alriksson et al. 2014). Further, *P. variotii* has a protein content of about 55 % (w/w) (Bajpai 2017), making it an interesting choice for production of fish feed. It is also interesting to investigate the functionality of the proteins to explore possible applications in food as well.

This master thesis was a part of the project SALMONAID, a cooperation between RISE, Processum, SLU and Domsjö fabriker. The project aims to produce SCP of *P. variotii* of high quality by cultivation on residual streams from the forest industry for replacement of soybean meal in fish feed.

This master thesis was a continuation of previous results from Marianthi Zioga, who examined different cell wall degradation methods on *P. variotii* and their effect on the protein concentration in the soluble phase (Zioga 2018). This master thesis will increase the knowledge of the functional properties of treated *P. variotii* such as protein solubility, water holding capacity (WHC) and water solubility index (WSI). This will increase the understanding of how different treatment methods can improve the functional properties of *P. variotii* when used as a protein source in fish feed. Furthermore, by investigating different cell wall degradation treatments along with the efficiency of a protein precipitation method this master thesis has the potential to find methods to increase the quality of *P. variotii* as a protein source by isolating the proteins from the fungal biomass.

1.1 Aim

One of the main aims with this master thesis was to investigate cell wall degradation treatments followed by pH-shifting as a method to isolate the proteins in the fungal biomass of *P. variotii*. The other main aim was to investigate the WHC, WSI and macro- and microstructure of the biomass after different extrusion conditions. Finally, a feeding trial in *Artemia* was performed to investigate growth and survival of the model organism when fed biomass from different treatments. Hopefully, this work will increase the knowledge of the functional properties of *P. variotii* and how different treatments can increase the quality of the fungus as a protein source in fish feed.

1.2 Clarification of the issue

This master thesis aimed to answer the following questions:

- Which cell wall degradation method is most efficient?
- What is the protein solubility after different cell wall degradation treatments, and at which pH value is the isoelectric point found for the proteins?
- Can pH-shifting be used to increase the protein content after cell wall degradation?

- How does different extrusion conditions affect the functional properties of the fungal biomass?
- Does cell wall degradation or extrusion of the biomass result in increased growth and survival of *Artemia* in a feeding trial?

1.3 Limitations

One limitation was that the original batch of fungal biomass was used up after the first six month of the project. This resulted in analysis of biomass from different batches during the project.

Another limitation was the nitrogen-to-protein (N:P) conversion factor. With less time restriction, a specific N:P conversion factor would be decided based on measurement of amino acid content of the fungal biomass (Sriperm, Pesti, and Tillman 2011). This since the traditionally used factor 6.25 results in an overestimation. Instead, the factor 5 was chosen based on a literature study (Grossmann et al. 2018; Safi et al. 2017; Phong et al. 2018; Wanzenb et al. 2017). Further, due to both financial, practical and time limitations, the feeding trial was performed on the model organism *Artemia* and with limited equipment and time. Optimally, a feeding trial on fish is recommended to be conducted since the aim of the SALMONAID project is to produce a protein source in fish feed. In addition, it would also be necessary to redo the feeding trial on *Artemia* in order to obtain significant results.

Another limitation was the extrusion conditions. With less time restriction, a factorial design and multiple regression analysis would have been performed to optimize the condition variables based on several physical and nutritional parameters.

1.4 Background

The following section will contain background information about the concept of SCP, including a brief history. Further, information regarding the use of SCP as fish feed will be provided, along with information about the fungus *P. variotii*. Then, a section about the different cell wall degradation methods is included, with advantages and disadvantages with the different methods, followed by a section about protein precipitation and pH-shifting. Finally, extrusion is explained, followed by sections about water holding capacity, water solubility index and *Artemia*.

1.4.1 Single cell protein

The term SCP refers to the dried cells of microorganisms such as bacteria, yeast, algae and filamentous fungi (Kuhad et al. 1997). The protein can either be consumed directly as part of the cell, or extracted and processed into food products.

The use of microorganisms in food production is not a new concept. Already 2500 BC *Saccharomyces cerevisiae* was used for production of bread (Kuhad et al. 1997). However, the use of microorganisms directly as food or feed is a relatively recent discovery. During World War I, Germany managed to replace half of their imported protein sources with the yeast *Candida utilis* (Nasseri et al. 2011; Bajpai 2017; Ugalde and Castrillo 2002). The interest in yeast as a source of protein declined when the war ended, but reappeared during World War II. The research about SCP continued between the 60's and 80's as a consequence of the Food and Agriculture Organization of the United Nations (FAO) enlightening the protein gap (Bajpai 2017). However, due to developments in the agricultural field, the process of SCP was outcompeted by the cheap agricultural crops (Bajpai 2017). Further, high nucleic acid content and low digestibility are the main factors limiting SCP for human consumption from a nutritional point of view (Nasseri et al. 2011).

Today, one successful example of SCP is Quorn™, a product containing mycoproteins from the filamentous fungus *Fusarium venenatum* (Ritala et al. 2017). The food product was launched in 1985, and is the only SCP at the moment used for human consumption (Ritala et al. 2017).

1.4.1.1 The use of Single cell protein as fish feed

Since the feed represents 40-70 % of the operating costs in an aquaculture process (Ayadi, Rosentrate, and Muthukumar 2012), it is crucial to find a cheap and sustainable protein source when searching for new feed alternatives. Many of the microorganisms used as SCP have the advantage of being able to utilize inexpensive feedstock and waste material for growth (Nasseri et al. 2011), making it possible to produce inexpensive and sustainable biomass for production of, among other things, fish feed. Another advantage with SCP is the high amount of protein in the cells, see Table 1.1.

Table 1.1: Average composition of the main SCP microorganisms (% dry weight) (Nasseri et al. 2011)

Composition	Fungi	Algae	Yeast	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1-3
Ash	9-14	8-10	5-10	3-7
Nucleic acid	7-10	3-8	6-12	8-12

To obtain a fish feed of high quality, many nutritional factors need to be considered. One important factor for optimal growth performance is protein content. The requirement varies between species, but one of the fish species with the highest protein requirement is salmon, which has a demand of 40-50 % or higher in their diet (Ayadi, Rosentrate, and Muthukumar 2012). Further, the amino acid composition is important when developing a fish feed. To obtain a high feed efficiency, it is desirable to have a similar amino acid composition as fishmeal. The general fish requirements of essential amino acids at different dietary protein levels are listed in Table 1.2.

Table 1.2: Amino acid requirements (% of dry matter) of fishmeal at different dietary protein levels (%) (Tacon 1987).

Amino acid	Dietary protein level (%)		
	45	50	55
Arg	1.94	2.15	2.37
Cys*	0.31	0.35	0.38
His	0.82	0.91	1.00
Ile	1.26	1.40	1.54
Leu	2.30	2.55	2.81
Lys	2.66	2.96	3.25
Met	0.87	0.96	1.06
Phe	1.31	1.45	1.60
Tyr*	1.04	1.15	1.27
Thr	1.45	1.61	1.77
Trp	0.27	0.30	0.33
Val	1.50	1.66	1.83

*Non-essential amino acids

The physical quality of a fish feed is another important factor, especially due to the increase of bulk transport, big packages and pneumatic delivery systems (Samuelsen, Mjøs, and Oterhals 2013). Exposure of the fish feed pellet to harsh environments can result in attrition and product loss. Therefore, the functional properties of the feed material after different treatments is highly relevant when developing new feed products. The feed quality is usually increased by addition of starch and other binding molecules, along with processing of the material using water, steam and mechanical energy to avoid fragmentation (Samuelsen, Mjøs, and Oterhals 2013).

1.4.1.2 Properties of *Paecilomyces variotii*

The filamentous fungus *P. variotii* has gained interest due to several beneficial properties. To begin with, *P. variotii* has a crude protein content of about 55 % (w/w) (Nasseri et al. 2011). This makes it very interesting as an alternative protein source in fish feed. Further, *P. variotii* has a favourable amino acid composition (see Table 1.3), has a relatively rapid growth, is easy to separate from the substrate and show no signs of toxic effects in test animals (Bajpai 2017). A study conduced by Alriksson et al. in 2014 analyzed the composition of *P. variotii*, and their results are shown in Table 1.3. Unfortunately, not all relevant amino acids were analyzed in the study.

Table 1.3: Biomass composition of *P. variotii* (g/100 g biomass) (Alriksson et al. 2014).

Biomass nutrient	***P. variotii*(g/100 g biomass)**
Protein	48
Fat	5
Carbohydrate	37
Water	5
Ash	5
Amino acid	**Composition (g/100 g biomass)**
Arg	2.7
Thr	1.8
Ile	2.0
Leu	3.1
Lys	2.9
Met	0.7
Val	2.4
Trp	0.6

In Finland, *P. variotii* was used between 1975-1983 in a process called the Pekilo process (Ugalde and Castrillo 2002; Silvennoinen and Koivo 1983). In the Pekilo process, *P. variotii* was cultured on spent sulfite liquor to produce SCP with high crude protein content (Bajpai 2017). The SCP was even approved as animal feed and food in Finland, though the process is not currently operating (Bajpai 2017; Ugalde and Castrillo 2002).

The cell wall composition of *P. variotii* varies between strains, but consists of the polysaccharides α-(1→ 3)-glucan and β-glucan-chitin complex (Domenech et al. 1994). For a general illustration of the fungal cell wall, see Figure 1.3. Chitin is the second most abundant polysaccharide after cellulose and has an important structural function in the cell wall of fungi, homologous with cellulose in plants (Pusztahelyi 2018). Similar with cellulose, chitin has a crystalline or semi-crystalline structure, making it a rigid and resistant material (Pusztahelyi 2018). Therefore, a lot of energy can be required in order to degrade the cell wall. The chemical structure of chitin is provided in Figure 1.4.

β-glucan is a polysaccharide consisting of D-glucose monomers linked by β-glycosidic linkages (Halpern 2013), see Figure 1.5.

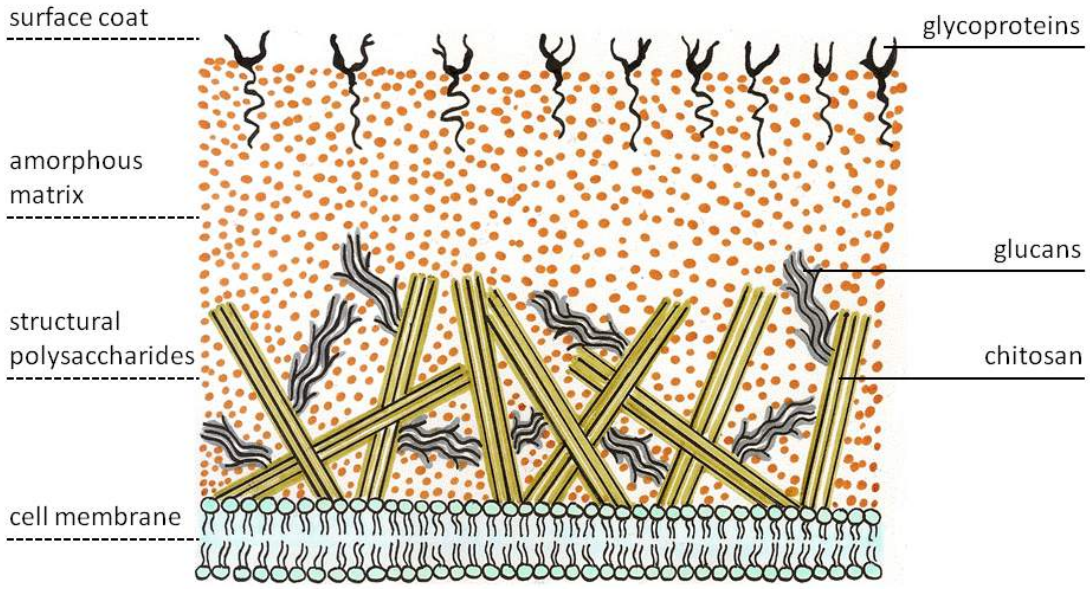


Figure 1.3: General cell wall structure of fungi. The picture originates from Wikimedia commons under the license CC-BY-3.0 (Maya and Rike 2013).

β-glucans in the cell wall of fungi can provide fibre when turning SCP from fungi into for example fish feed (Ritala et al. 2017). Further, β-glucans can even increase the value of the feed due to their immunostimulatory effect (Bajpai 2017; Halpern 2013). Steen (2014) studied the β-glucan content of *P. variotii*, and found a high value of 19.6 % (w/w dry biomass) (Steen 2014). However, it is uncertain if this type of β-glucan have the positive immunostimulatory effect.

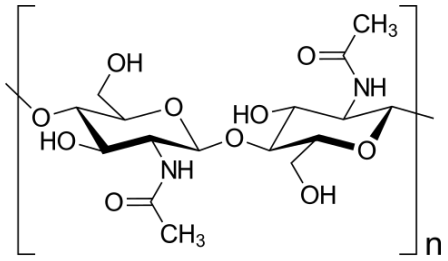


Figure 1.4: Chemical structure of chitin.

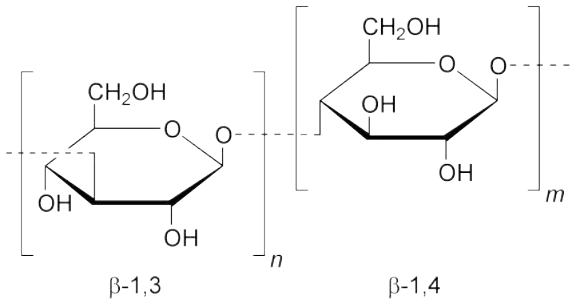


Figure 1.5: Chemical structure of β-glucan.

1.4.2 Cell wall degradation methods

Many of the valuable components produced by microorganisms are intracellular and are therefore not secreted, resulting in a need for cell disruption in order to obtain them and achieve high high nutrient digestibility (Uhlmann et al. 2013; Øverland and Skrede 2017). Since filamentous fungi is not traditional food for fish species, the fish generally lack the gastro-intestinal enzymes needed to degrade the cell walls (Øverland and Skrede 2017).

There are several different methods to achieve cell wall degradation, both chemical and mechanical. In this section, enzyme treatment, high pressure homogenization treatment and drying and milling will be explained, along with advantages and disadvantages with the methods. It should also be added that different methods can have different effects depending on the species and strain of the microorganism (Klimek-Ochab et al. 2011). Further, a combination of different treatments might be necessary in order to achieve an optimal cell disruption (Baldwin and Robinson 1994).

1.4.2.1 Enzyme treatment

Enzymes can be used either to completely disrupt cells or to achieve partial disruption (Nasseri et al. 2011). Cell degradation through enzyme treatment has the advantage of being conducted under gentle conditions, along with a high specificity (Klimek-Ochab et al. 2011), making it possible to for example target the cell wall. Further, less energy is required compared to mechanical treatments (Klimek-Ochab et al. 2011). Unfortunately, the method might be too expensive for industrial scale (Klimek-Ochab et al. 2011) and is very slow compared to mechanical methods (Nasseri et al. 2011).

Several studies have tested enzymatic treatment in combination with mechanical treatments (Nasseri et al. 2011). Baldwin and Robinson (2014) obtained an enhanced disruption of *Candida utilis* by first applying enzymatic treatment, followed by high pressure homogenization. Another study successfully increased the protein release from yeast cells by first disrupting the cells using high pressure homogenization (four cycles, 400 bars), then using lytic enzymes (Asenjo and Dunnill 1981).

1.4.2.2 High pressure homogenization

HPH is a mechanical disruption method suitable for large-scale applications (Baldwin and Robinson 1994). The method applies high pressure to the cells by forcing a liquid sample through a restricted homogenization valve (Klimek-Ochab et al. 2011; Balasubramaniam et al. 2016). When the pressurized sample is passing the valve, dissipation of kinetic energy results in the reduction of particle size and the compression results in heat generation (Balasubramaniam et al. 2016). There are several different types of valves and techniques available within high pressure homogenization.

The method requires relatively large energy inputs, therefore process optimization can be necessary to reduce the energy requirement (Baldwin and Robinson 1994). However, it can be very efficient in disintegrating cells of microorganisms with rigid cell walls (Safi et al. 2017).

1.4.2.3 Drying and milling

Primarily, the physical treatment drying is applied in order to remove large amounts of water in the biomass (Pohndorf et al. 2016). One disadvantage with the drying is the possible effect it can have on the product, for example on the protein quality and other cell components (Pohndorf et al. 2016). This, of course, depends on the drying conditions. A positive effect from the drying is an increased digestibility of for example yeast and microalgae (Nasseri et al. 2011). There are several different drying methods available, for example solar-drying, freeze-drying, oven-drying and spray-drying (Ansari et al. 2018).

Milling is a well-established mechanical disruption method used in several industries (Montalescot et al. 2015). Bead mills are commonly used when grinding paint

pigments, minerals and other products (Montalescot et al. 2015). It has also been shown to be a successful method for degradation of several microbial cells (Postma et al. 2017). The degradation can occur under mild conditions and requires low amounts of energy (Postma et al. 2017).

The combination of drying and milling requires relatively large amounts of energy, but is more beneficial financially compared to enzymatic or chemical methods (Montalescot et al. 2015). This due to the high costs of enzymes and the need to remove chemicals from the product in many processes (Montalescot et al. 2015). It is also applicable for large scale production.

1.4.3 Protein precipitation and pH-shifting

To separate proteins from other components in an aqueous solution, several different methods can be applied, such as salting out, isoelectric point (pI) precipitation and solvent precipitation (Grossmann et al. 2018). The pI of a protein is the pH value at which the net charge of the molecule is zero (Arancibia-Miranda et al. 2011), thus causing the protein to precipitate out of the aqueous solution. At pH values above pI, the surface of the protein is mainly negatively charged due to loss of protons. This results in repulsive forces against other negatively charged molecules (Novák and Havlíček 2016). On the other hand, if the pH value is below pI, the protein surface will predominantly be positively charged as a consequence of proton addition, and once again will repulsive forces occur between molecules with the same charge (Novák and Havlíček 2016). However, at pI, the repulsive forces will be reduced, and attracting forces will cause aggregation and precipitation (Novák and Havlíček 2016). The pI of most proteins is in the range of pH 4-7 (Novák and Havlíček 2016).

The effect on solubility of a protein by pH can be used to separate and concentrate proteins. This is called pH-shifting. To solubilize muscle proteins, the pH can either be lowered to about pH 3 or increased to around pH 11 (Hinchcliffe et al. 2018). Centrifugation can then be applied in order to sediment unwanted components from the solution. Then, the pH is adjusted to the pI of the proteins to precipitate them out of the solution.

1.4.4 Extrusion

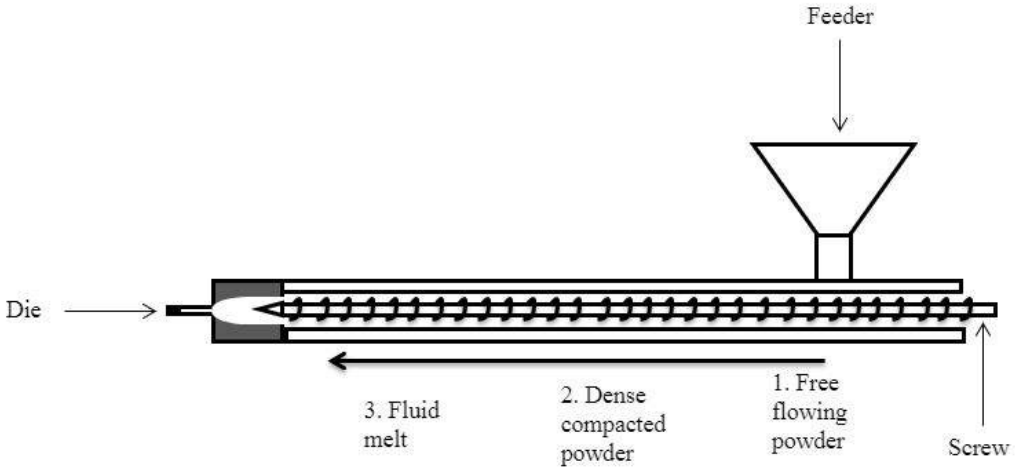


Figure 1.6: Illustration of an extruder.

Extrusion is a method using a combination of temperature, mechanical shear, pressure and moisture to process materials (Chen, Wei, and Zhang 2011). During extrusion, several chemical and structural transformations occur, such as starch gelatinization, protein denaturation and complex formations between compounds (Jafari, Koocheki, and Milani 2017). Several factors distinguish extrusion from other cooking methods. For example, the moisture content is usually between 10-40 % (Guy 2001; Camire 2001), which is low compared to conventional cooking methods. Another distinguishing factor is the temperature. Usually, temperatures between 100-180 °C are used, which is high compared to other methods (Guy 2001). This allows for short processing times and full transformation of raw material. In order to obtain a desired structure after the extrusion, different dies can be applied.

Extrusion is a common process method when producing fish feed pellets, due to several beneficial properties of the pellet compared to other processing methods. Extruded pellets have a higher durability, increased water stability and a higher water absorption ability than steamed pellets (Hilton, Cho, and Slinger 1981). Further, extrusion is also known to improve the bioavailability of carbohydrates, destroy antinutritional factors, increase the solubility of nitrogen and dietary fibre, denaturate deteriovative enzymes and thereby reduce lipid oxidation as well as destroying microbial pathogens (Irungu et al. 2019).

Different compounds have different functional roles during extrusion. Therefore,

each of the different functional roles will be explained in sections below.

1.4.4.1 Structure compounds

The structure of the raw material is dependent on the type of biopolymers, such as biopolymers of starch and protein (Guy 2001). These biopolymers create three-dimensional networks in which the other food components are located to form the texture of the material. The raw material thus have a strong impact on the structure and quality of the extruded product (Wild 2016). Wheat and maize flours are the most commonly used materials during extrusion (Guy 2001). The structure of an expanded extruded product is formed by creating a melted fluid from the biopolymers and through transportation of bubbles of water vapour into the fluid, creating a foam (Guy 2001). The biopolymers creates a cell wall around the bubbles, allowing them to expand until they burst. Afterwards, the temperature is rapidly decreased, resulting in a fast increase in viscosity as a result of moisture loss. The cellular structure becomes more rigid as a result of the increase in viscosity, followed by a glassy state formation (Guy 2001).

In recent years, high moisture extrusion (moisture content = 50-75 %) has been utilized to manufacture textured vegetable protein (TVP) products with a fibrous structure. To create TVP's the proteins are initially denaturated during the extrusion process with high moisture content and elevated temperatures. In a second step, the proteins form intermolecular covalent bonds in order to polymerize (Wild 2016). Finally, the protein alignments are stabilized during a cooling procedure, creating a protein network (Wild 2016).

1.4.4.2 Dispersed phase compounds

After extrusion with flour, a continuous phase of starch polymers can usually be observed in the microscopic structure. In this continuous phase, several different disruptive phases can be present. This could for example be proteins or fibres (Guy 2001). The size of the proteins will depend on their original size as well as their resistance to shear stress (Guy 2001). One effect of these dispersed-phase materials being present in the foamed structure is reduction of expansion (Guy 2001). Secondly, their presence can affect the elasticity of the extruded material when it leaves the die exit (Guy 2001).

1.4.4.3 Plasticizers and lubricants

In low moisture extrusion (moisture content$\leq$ 25%), the physical interactions causes frictional and mechanical energy in the extruder (Guy 2001). As a result, no external heating is usually necessary during the process. At higher moisture levels, the water acts as a plasticizer, causing a reduction in interactions (Guy 2001).

Other compounds which can affect the extrusion process is oil and fat. These compounds lubricate particles, resulting in a decrease in shear stress and thus in expansion (Guy 2001).

1.4.5 Water holding capacity and Water solubility index

The most important functional properties of proteins are related to their interaction with water. Water holding capacity (WHC) of a protein can be defined as the ability to prevent water from being released while influenced by pressure, centrifugation or heating (Zayas 1997). The water-protein interaction determines several important functional properties of proteins, such as solubility, swelling, viscosity and gelation (Zayas 1997). When adding proteins to a feed or food product, it is important to understand the mechanism of the protein-water interaction in order to find out if the proteins will function as a gel, a collodial dispersion or precipitate.

Water solubility index (WSI) is often used as an indicator for molecular degradation and starch conversion (Rashid et al. 2015). During extrusion, the mechanical forces results in shorter fragments of the large starch molecules being released and thereby increases the water solubility (Rashid et al. 2015).

1.5 *Artemia*

Artemia is a small zooplanktonic anthropod with a body shaped in a leaf-like structure (Abatzopoulos et al. 2002). The outer part of the body is covered with a thin exoskeleton made of chitin, with muscles attached to it. Adult *Artemia* are between 8-12 mm in length (FAO 2019). They can be found globally in hypersaline habitats, for example in salt lakes and coastal salt ponds (FAO 2019). Since their survival depends on their environment having a high enough salinity to eliminate nearly all potential predators, *Artemia* have adapted an exceptional osmoregulatory capacity (FAO 2019). This is achieved by transfer of ions and fluid from the gut into the haemolyph in approximately the same rate as fluid is lost to the environment from the body wall (Abatzopoulos et al. 2002).

Artemia can reproduce in two separate ways, either by nauplius or cyst production (FAO 2019). This is determined by the environmental conditions. If the conditions are favourable, *Artemia* will produce nauplius to replicate. On the other hand, during unfavourable conditions, for example when the *Artemia* are exposed to temperature stress, starvation or low oxygen levels, the females will produce an embryo which will become surrounded by a thick shell (FAO 2019). While at this stage, the embryo enters metabolic arrest and is called a cyst. When the conditions become favourable, the cyst will hatch and the embryonic development continues (FAO 2019). Under favourable conditions, *Artemia* nauplius can become adults in 8 days and live for several month (FAO 2019).

The natural feed of *Artemia* is purine-rich bacteria and algae (Abatzopoulos et al. 2002). However, *Artemia* are not able to feed during the early stages of nauplii (larval stage), since the digestive tract is under development (Abatzopoulos et al. 2002).

In research, *Artemia* is used as a model organism for different applications. One example is a study from 2018 which used *Artemia* as a model organism when studying

the effect of long term exposure to neurotoxic pesticides (Gambardella et al. 2018).

2
Method

In this section, the different methods will be explained in sections. Since there were several steps and methods performed during the master thesis, an overview of the methods used are presented in Figure 2.1.

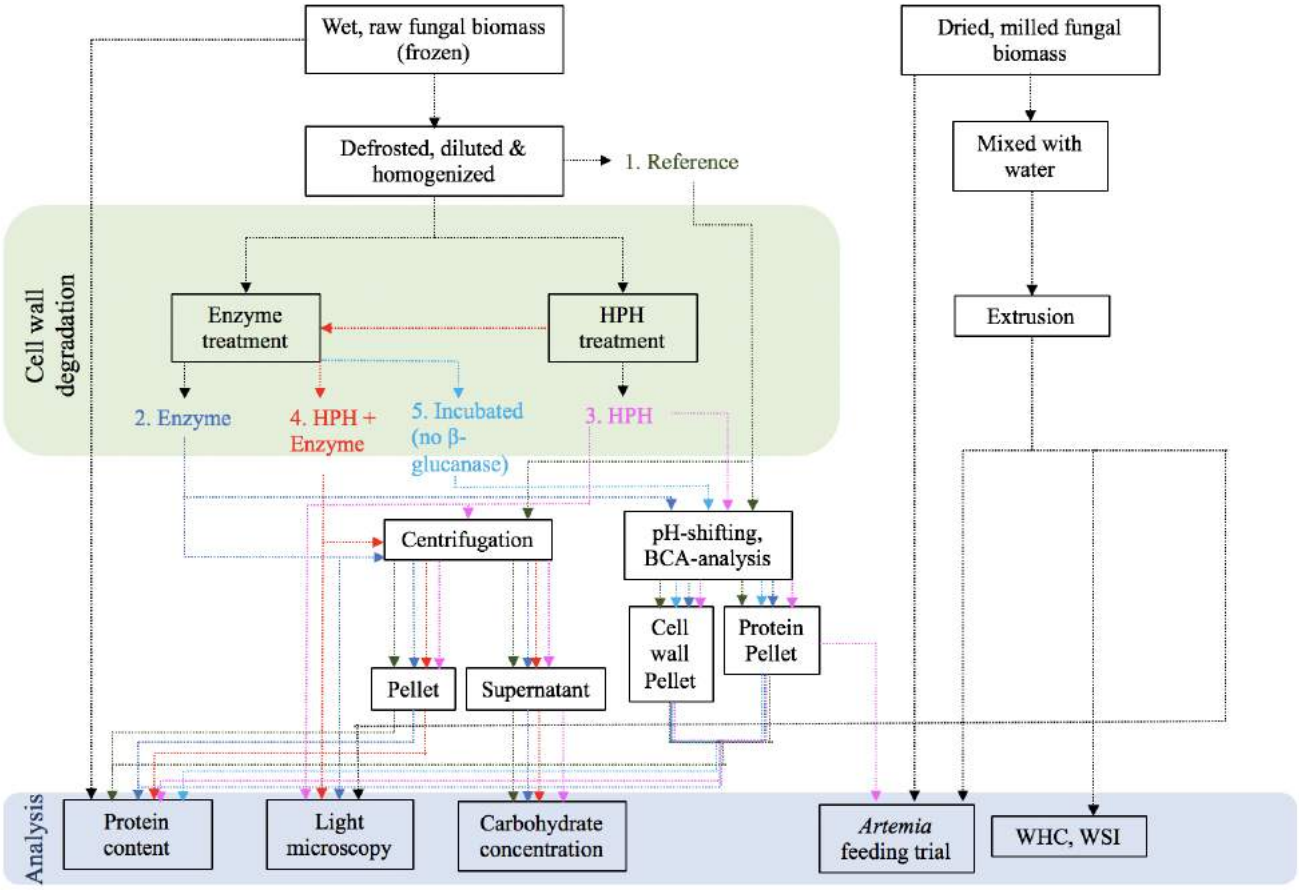


Figure 2.1: Flow-chart of all the different methods used. All samples have a distinctive colour and are numbered 1-5. The boxes either represent a treatment step, an analysis or a material (for example pellet obtained after a centrifugation step or dried, milled fungal biomass).

2.1 Biomass production and pretreatment

The filamentous fungus *Paecilomyces variotii* (CCUG 28186) was cultured on residual streams from the forest industry at RISE Processum AB in Örnsköldsvik, Sweden (Alriksson et al. 2014). The harvested biomass collected from the bioreactor was separated from the liquid fraction with the use of vacuum filtration. The obtained filter cakes were then washed with two volumes of distilled water. After the purification step, the filter cakes of raw, wet biomass were stored at -80 °C (Alriksson et al. 2014). Filter cakes from different batches were sent to the Department of Agrifood and Bioscience at RISE in Gothenburg and stored at -20 °C until further use.

Before the different treatment methods, pieces of the filter cakes were weighted and defrosted in a diluent at room temperature for 30 minutes, see Table 2.1 and Table 2.2 for details. Since the lowest volume necessary to run the HPH was 500 ml, 50 g of wet, raw biomass was defrosted in 500 ml of diluent. Since the enzyme used in the experiment had an optimal activity around pH 5, two different diluents were used depending on if the sample would undergo enzymatic lysis treatment or not. The preparation of sodium citrate buffer (pH 5, 8.14 mM) is presented in A.1. In Figure 2.2, the defrosting of three different samples of raw, wet biomass is shown (A).

Table 2.1: Pretreatment of the different samples. The frozen pieces of filter cake biomass were defrosted in either Milli-Q water or sodium citrate buffer (pH 5, 8.14 mM) at different ratios.

Treatment	**Diluent**	**Ratio Biomass/Diluent**
Reference	Milli-Q water	1:5
Enzyme	Sodium citrate buffer	1:5
HPH	Sodium citrate buffer/Milli-Q water	1:10

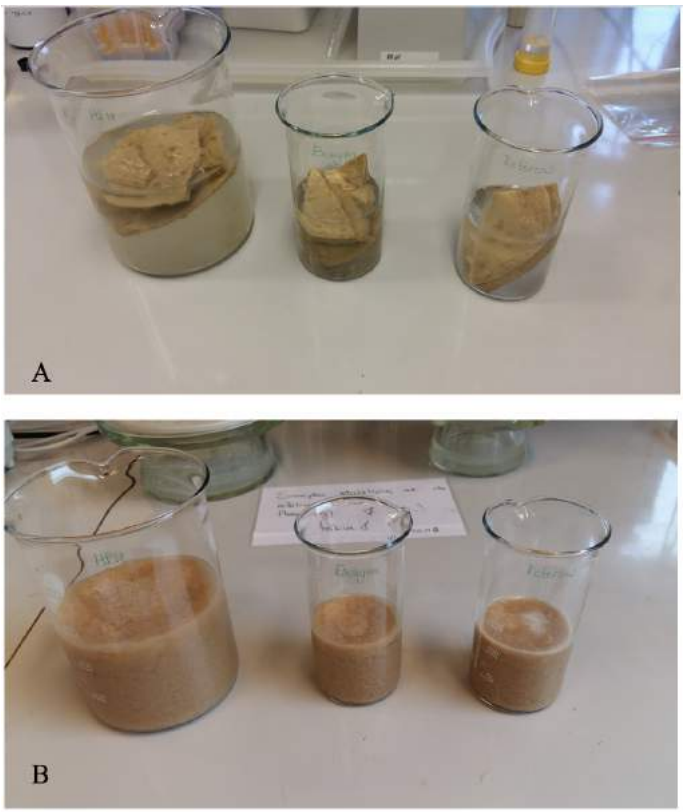


Figure 2.2: A. Defrosting of the filter cake biomass samples in diluent at room temperature. B. Samples after homogenization with Ultra Turrax.

When defrosted, the samples of diluted raw, wet biomass were homogenized with Ultra Turrax at 16 000 rpm for 4 min. Figure 2.2 B shows the samples after homogenization.

Table 2.2: Summary of the different treatments.

Treatment	**Raw, wet biomass (g)**	**Diluent (ml)**	**Enzyme (g)**
Reference	20	100	-
Enzyme	20	100	0.2
HPH (+Enzyme)	50	500	-(0.25)

2.2 Cell wall degradation treatments

In this section, the different cell wall degradation treatments will be explained. In Figure 2.3, a flow-chart of the different steps in the treatments are presented. In order to obtain both the insoluble and the soluble proteins after each cell wall degradation treatment, pH-shifting was introduced a few months into the project. Therefore, some protein content results are obtained from samples only undergoing sedimentation without a pH-shifting step (as shown in Figure 2.1). This will be clarified in the result.

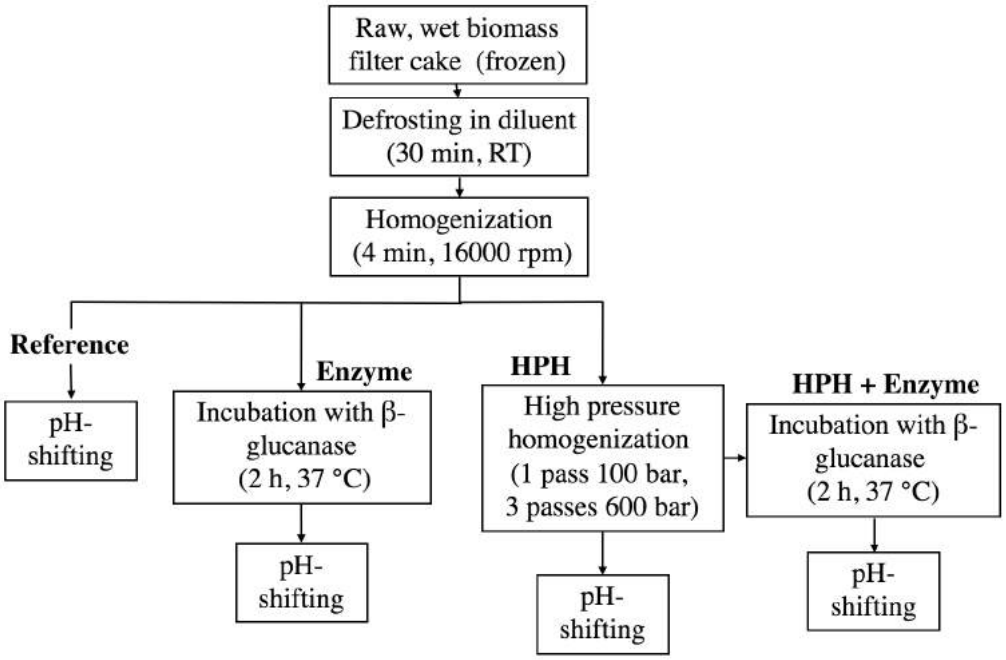


Figure 2.3: Flow-chart of the different steps during the cell wall degradation treatments.

2.2.1 Reference

The reference sample consisted of thawed fungal biomass which had been homogenized in MilliQ-water (see Figure 2.2). pH-shifting was also performed on the reference sample (more details can be found in section 2.3).

2.2.2 Enzyme treatment

After homogenization, the sample was incubated at 37 °C for 2 h after addition of the enzyme β-glucanase from *Trichoderma longibrachiatum*. The amount of enzyme

added was 1:100 of the amount of raw, wet biomass weighted initially (0.1 g enzyme per 10 g of raw, wet biomass).

2.2.3 High pressure homogenization

The high pressure homogenizer used was GEA Niro Soavi (NS100IL PANDA). The sample was added to the container of the HPH, and a beaker was placed to collect the sample. First, the sample was passed through the instrument at 100 bar since the biomass easily clogged the pipes on the first run. Then, the pipes were cleaned from lumps of biomass and the sample was passed 3 times at 600 bar. After the HPH treatment, the sample was divided in two (250 ml was used for HPH treatment, and 250 ml was used for HPH + Enzyme treatment).

2.3 pH-shifting

To isolate the proteins, pH-shifting was introduced as a solubilization and precipitation method after the cell wall degradation treatments (see Figure 2.1 and Figure 2.4).

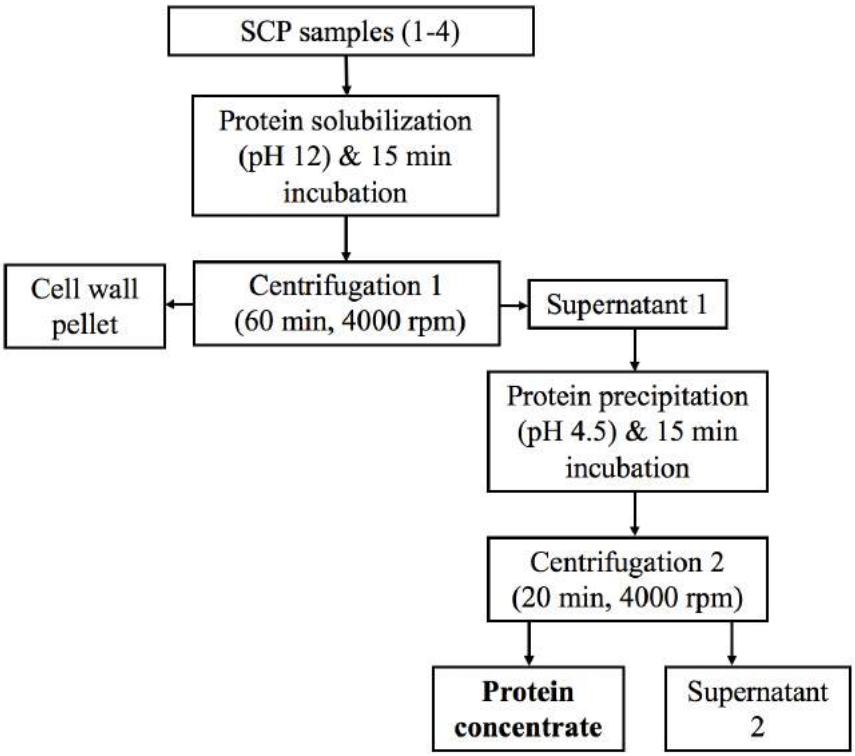


Figure 2.4: Flow-chart of the steps during pH-shifting. All steps were performed in room temperature.

To determine the protein solubility and isoelectric point of the proteins, Pierce™Rapid Gold BCA Protein Assay Kit (Thermo scientific) was used to measure protein concentration. The protein solubility and protein yields were calculated according to a study by Hinchcliffe et al. (2018), using the following equations (where the homogenate represents the treated samples 1-4 in Figure 2.3):

Protein solubility after centrifugation 1 (after pH 12):

$$\text{Protein solubility } (\%) = 100 * \frac{\text{Protein concentration}_{\text{Supernatant 1}}}{\text{Protein concentration}_{\text{Homogenate}}}$$

Protein solubility after centrifugation 2 (after pH 4.5):

$$\text{Protein solubility (\%)} = 100 * \frac{\text{Protein concentration}_{\text{Supernatant 2}}}{\text{Protein concentration}_{\text{Supernatant 1}}}$$

Protein yield centrifugation 1:

$$\text{Protein yield (\%)} = 100 * \frac{\text{Protein concentration}_{\text{Supernatant 1}} * \text{X ml Supernatant 1}}{\text{Protein concentration}_{\text{Homogenate}} * \text{X ml Homogenate}}$$

Protein yield centrifugation 2:

$$\text{Protein yield (\%)} = 100 * \left[1 - \frac{\text{Protein concentration}_{\text{Supernatant 2}} * \text{X ml Supernatant 2}}{\text{Protein concentration}_{\text{Supernatant 1}} * \text{X ml Supernatant 1}}\right]$$

First, a standard curve was prepared according to the manual from Thermo scientific, see Appendix A.2. Bovine serum albumin was used as a standard to create the curve. The absorbance was measured at 480 nm (Ultrospec 1000 UV/Visible Spectrophotometer Pharmacia Biotech). The equation obtained from the standard curve was used to convert absorbance into protein concentration in later measurements.

When the standard curve was accurate enough (the lower limit of R^2 was set to 0.98), the next step was to investigate the protein solubility at different pH values to be able to optimize the pH-shifting (performed both for the reference sample and the HPH sample). First, the aim was to find the pH at which the SCP had the highest solubility. Therefore, pH 8, 9, 10, 11 and 12 was first investigated. To begin with, a SCP reference sample was prepared (treatment method 1 in Figure 2.3). A sample from the reference was saved in order to measure the protein concentration in the homogenate (performed by diluting the homogenate sample x20 in MQ-water, followed my BCA analysis, see section below). The pH was adjusted during constant magnetic stirring using 1 M NaOH and 1 M HCl. The pH was measured using bench meter pH 1100 L. After pH adjustment, the samples were incubated at room temperature for 15 min, followed by centrifugation (4000 rpm, 20 min). The supernatant was collected for protein concentration analysis and the pellet was stored at -80 °C and was later analyzed for protein content. The procedure was repeated for an HPH sample.

The homogenate samples (both from the reference treatment and the HPH treatment) and the supernatant samples were diluted with Milli-Q water and vortexed. 80 μL of each sample was added to a 2.5 mL cuvette. Then, 2 mL of working reagent (prepared according to the manual obtained from Thermo scientific) was added to each cuvette. Since the reaction occur rapidly once the working reagent is added,

the samples were incubated for 5 min, then 500 μL of 1 M HCl was added to slow down the reaction. The absorbance was then measured at 480 nm. Duplicates were used to ensure accurate results, and the BCA-analysis was repeated two times to ensure reproducibility.
After investigating the SCP solubility at high pH-values, the pH in supernatant 1 (the supernatant after centrifugation 1) was adjusted to pH 3, 3.5, 4, 4.5, 5 and 5.5 to optimize the protein precipitation using 1 M HCl during constant magnetic stirring. The samples were then incubated (15 min, RT) followed by centrifugation (4000 rpm, 20 min). The supernatant (supernatant 2) was collected and the pellet (protein concentrate) at the bottom of the tubes were stored at -80 °C. The protein concentration in the soluble phase was measured in the supernatants using BCA-analysis. Duplicates were used to ensure accurate results. The procedure was repeated for a HPH sample.

After the optimization, the time during centrifugation 1 was changed to 60 min since larger sample volumes were centrifuged compared to the optimization phase.

2.4 Dry matter analysis using a vacuum oven

The dry matter (TS) of the pellets were determined using a vacuum oven. First, the containers were weighted. Then, the pellet samples were added to the containers and their combined weight were determined and written down. The samples were put in a vacuum oven (Fistreem vacuum oven) at 80 °C for 28 h. The containers were then weighted once again, and the dry matter (%) of the samples were calculated using the following formula:

$$TS(\%) = 100 * \frac{W_{container+dsample} - W_{container}}{W_{container+isample} - W_{container}}$$

Were $W_{container+dsample}$ is the combined weight of the container and dry sample (g), $W_{container}$ is the weight of the container (g) and $W_{container+isample}$ is the weight of the container and sample before the drying (g).

2.5 Protein content analysis and carbohydrate content analysis

The first pellet samples obtained (only protein precipitation, no pH-shifting step) were sent away for analysis of protein content at RISE Processum AB in Örnsköldsvik, Sweden. The pellet samples were first dried to a TS value of about 90 %. Then, the nitrogen content of the samples were determined using the Dumas method, and a factor of 5 was used to convert the result to protein content. The

values presented in the results are mean values of two measurements. Factor 5 was chosen after a literature study (Grossmann et al. 2018; Safi et al. 2017; Phong et al. 2018; Wanzenb et al. 2017).

To investigate the effectiveness of β-glucanase, supernatant from all the different treatments were sent to RISE in Borås for analysis of carbohydrate content. The method used was neutral sugar assay, which is based on rapid hydrolysis with high concentration of sulfuric acid, followed by derivatization of the hydrolysis products using phenol. The quantity was determined through absorbance measurement at 490 nm against an external standard curve of glucose.
Both the cell wall pellet and the protein pellet (also called protein concentrate) were analyzed for protein content at RISE in Borås using an elemental analyzer (Leco CHN628) to measure the nitrogen content. To convert the results to protein content, a factor of 5 was used. Before analysis, the samples were dried in an oven at 105 °C for 18 h and the dry matter of the samples were determined using the formula in section 2.4. The protein content was determined using a mean value from two to four measurements, depending on the amount of available sample volume.

2.6 Extrusion

Since extrusion is a common process method when producing fish feed pellet, the effect of extrusion on water holding capacity, water solubility index and structure of the fungal biomass was investigated. Before extruding the biomass, the texture of the fungal biomass at different water content values were investigated. An experimental design was performed taking into consideration the texture at different moisture contents, see Table 2.3.

Table 2.3: Experimental design for the extrusion. The table shows the weight of frozen, dried fungal biomass used (g) and the value within the brackets shows the amount of water mixed in (g). For exact values, see Appendix A.6.

Temperature	**Moisture content (%)**		
	30 %	40 %	50 %
115 °C	140 (60)	120 (80)	
125 °C		120 (80)	100 (100)

Dried biomass were mixed with water in a mixer until a homogeneous sample was obtained. The samples were transferred to labeled plastic bags. The single screw extruder (Teach Line E20T (Dr. Collin GmbH, Germany) was setup. The screw speed was set to 75 rpm and the die diameter utilized was 2 mm. The temperatures were set for each zone in the extruder before adding the samples one by one. For

details, see Appendix A.6. The extruded samples were then collected in plastic bags and stored at -40 °C until further use. An additional extrusion of fungal biomass was performed at SLU in Uppsala and evaluated in this project. Here, a twin screw extruder (Brabender KETSE 20/40, Germany) with a 3 mm die diameter was used. The biomass samples with a moisture content of 40 % were extruded at 115 °C and a screw speed of 120 rpm.

2.7 Sample preparation before microscopy

To investigate the effect on the cell wall from the different degradation treatments, the samples were placed on microscope slides and observed under a light microscope. To be able to distinguish the proteins, a staining procedure was performed (iodine and light green solution 1:2). About 4-10 drops of the staining solution was added to eppendorf tubes with sample depending on the sample volume, followed by an incubation of 10 min. A plastic pipette was used to transfer a small sample volume to a microscope slide, and the samples were covered with a cover glass. The samples were then observed under the light microscope (Nikon Microphot-FXA), and images were taken using the software NIS-Elements D 5.10.00.

For the extruded samples, freeze sectioning was performed prior to microscopy. Frozen extruded samples were placed on a circular plate, both in vertical and horizontal position. PELCO®Cryo-Embedding compound (TED PELLA INC.) was applied on the samples. The plates were then lowered into liquid nitrogen for rapid freezing. The samples were sectioned at -15 °C (LEICA CM1900) and the samples were placed on polysine glass slides (Thermo Scientific). The samples were fixated in a glass box containing glutardialdehyde solution (25 %) for 1.5 h. After fixation, the samples were stained using a diluted iodine and light green solution (3:5). One drop of staining solution was applied on the samples and incubated for 5 min before rinsing. A cover glass was then put over the samples before observation.

2.8 Water holding capacity and Water solubility index

A centrifugation tube was weighted. After documenting the value, approximately 0.1 g of sample was added to the tube and the weight of the sample was written down. Milli-Q water was added to obtain a total volume of 10 ml in the tube. The tube was vortexed for 20 s every 10 min during 1 h. The sample was then centrifuged (4000 g, 25 min). The supernatant was transferred to a preweighed glass plate and dried in an oven at 85 °C overnight. The final weight of the dried sample was then noted. The pellet obtained after centrifugation was dried in the tube at 50 °C for 25 min, and the final weight was equally written down.

To obtain accurate results, triplicates were used during the procedure. The WHC and WSI of the samples were calculated using the following equations:

A = Initial weight of sample
B = Final dried weight of remaining pellet
C = Final dried weight of supernatant

$$\text{WHC (g water bound/g solid)} = \frac{B - A}{A}$$

$$\text{WSI (\%)} = 100 * \frac{A - C}{A}$$

2.9 Feeding trial on *Artemia*

In the feeding trial, the following feeding samples were selected:

- Starvation sample
- Dried and milled *P. variotii* (reference)
- Milled extruded sample (115 °C, 40 % moisture content, 120 rpm)
- Protein concentrate from HPH

The trial was designed after a manual (Brine Schrimp Direct 2019). Approximately 5 L tap water was added to each of the four 5.2 L plastic boxes, and 150 g salt without iodine was added to each box during mixing. To de-chlorinate the water, the boxes were left open in room temperature for 48 h. 4 g of *Artemia* cyst were added to each box to hatch. Two light sources were placed above the boxes during the whole experiment, and water pumps were placed on the bottom of the boxes to ensure good circulation and oxygen levels in the water. The feed materials were prepared in different ways due to different dry matter contents. 0.5 g of dried and milled *P. variotii* was diluted in 6 ml tap water. Similarly, 0.5 g of milled, extruded sample was diluted in 5 ml tap water. This to obtain a dry matter content of about 10 % for all samples (the dry matter content of the HPH protein concentrate (the wet pellet obtained after pH-shifting of sample treatment 3, see Figure 2.3 and Figure 2.4)). Each day after hatching, 6 drops of each feed was added to the corresponding box.

To ensure a pH value between 7.5-8, the pH was measured daily. To buffer the pH, $MgSO_4$ was used since *Artemia* tolerate high concentrates of that substance (Hammer 1986). To analyze the growth, the length of 14-25 *Artemia* was measured each day in the light microscope for all samples (with some exceptions due to low

amounts of *Artemia*). The larval stage of the *Artemia* was also analyzed by comparing the larvae with the stages presented in Figure 2.5. To measure survival, the amount of *Artemia* in 1 ml was counted by transferring 1 ml of water sample from each box to a petri dish after mixing and counting the number of moving *Artemia*. This was performed in triplicates for all the samples.

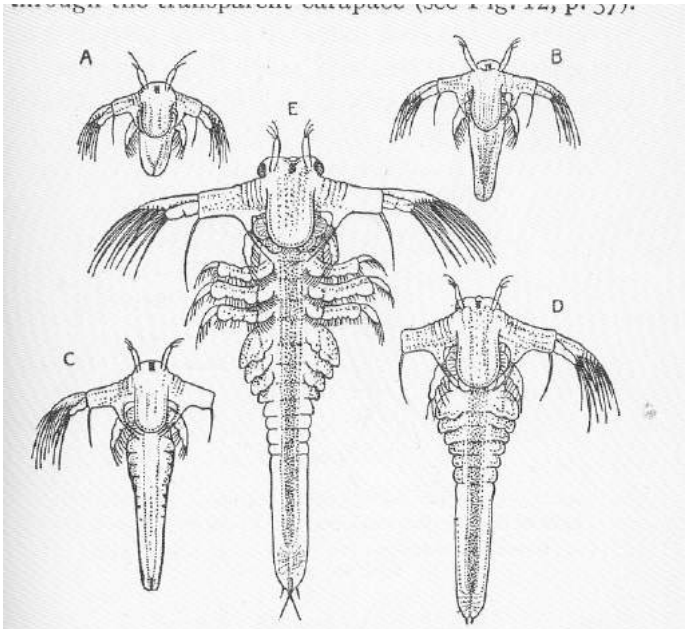


FIG. 33 — LARVAL STAGES OF THE BRINE SHRIMP (*Artemia salina*). (After Sars.)

A, Nauplius, just hatched; B—E, later stages, showing progressive increase in number of somites and appendages. The adult form of this species is shown in Fig. 55, p. 164

Figure 2.5: The larval stages of *Artemia* (Calman 1911).

2.10 Statistical analysis

During the different assays, 2-4 replicates were used depending on the amount of available material. To evaluate significance and variance, single factor ANOVA and t-test (when comparing only two groups) was used. A difference was considered statistically significant if $p<0.05$. Standard deviations are presented in graphs and tables. Calculations were performed in Microsoft Excel 2010, and graphs were created in MATLAB R2018a.

3
Result and Discussion

3.1 Cell wall degradation

To be able to obtain the intracellular proteins in the biomass of *P. variotii*, different cell wall degradation treatments were investigated. In this section, the result from the measurement of protein content in the untreated fungal biomass (Raw) and in pellets obtained after different cell wall degradation treatments followed by centrifugation is presented, as well as microscopy images to evaluate the efficiency of the degradation methods.

The result of the protein content analysis is presented in Figure 3.1. As illustrated, the treatment methods did not result in higher percentage of protein content in the pellet. The reason for the decrease in protein content could be a high degree of soluble proteins, resulting in a lower protein concentration in the pellet. It is also possible that harsh treatment methods such as HPH can result in disintegration of the proteins into peptides, making it more difficult to sediment them into the pellet phase. The result for the enzyme treatment indicates that proteins are degraded during the incubation at a higher rate than the enzymatic degradation of the carbohydrates. A reason for this could be activity of endogenous proteases during the incubation. This was later investigated by addition of a sample treatment undergoing the same incubation conditions as the Enzyme sample, except with no addition of β-glucanase. This sample treatment was named Incubation. To increase the protein content of pellets, pH-shifting was introduced.

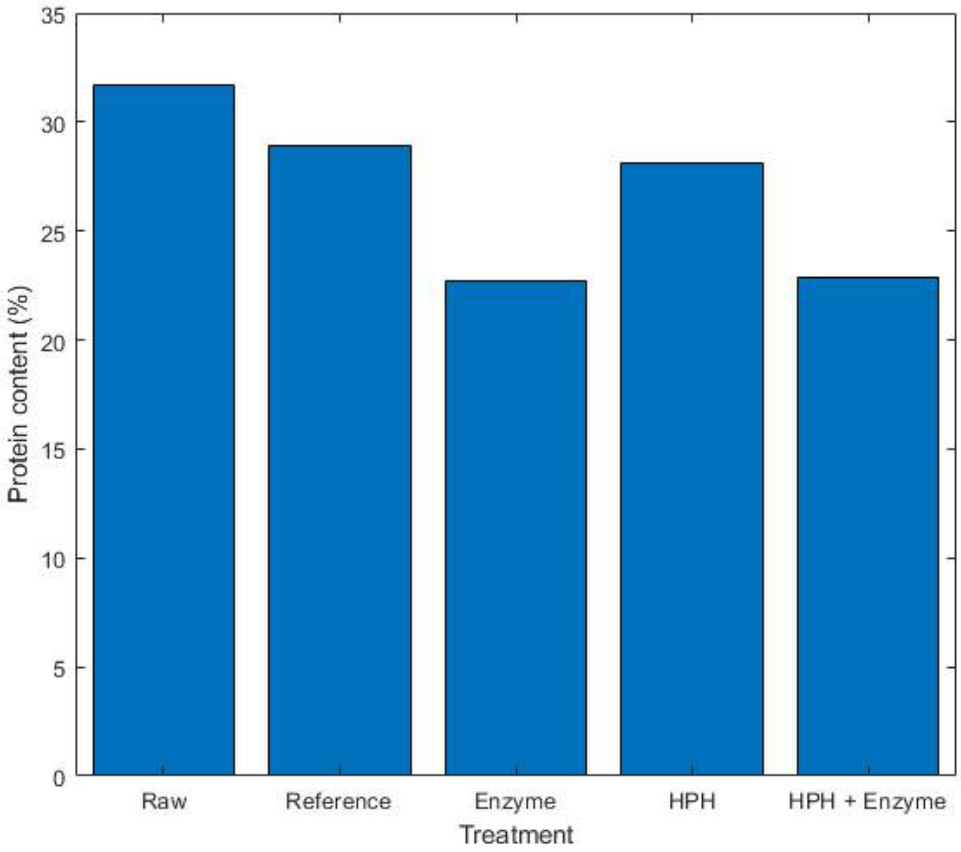


Figure 3.1: Protein content in dry matter basis (%) of pellets from the different cell wall degradation treatments after sedimentation (see Figure 2.3). The protein content in the raw, untreated biomass is also presented (Raw). The protein content was determined using the Dumas method, and the values in the figure are mean values from two measurements. Unfortunately only the mean values were obtained, therefore no error bars could be included in the graph.

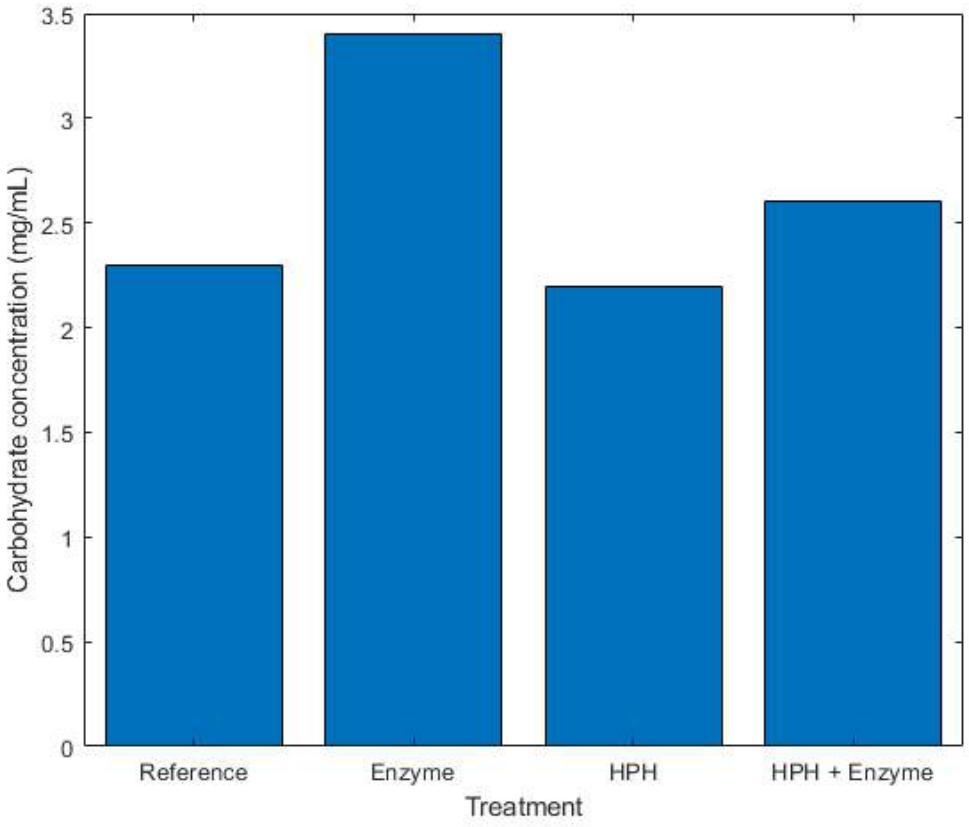


Figure 3.2: Carbohydrate concentration (mg/mL) in the supernatant of the differently treated samples.

In Figure 3.2, an increasing trend of carbohydrate concentration in the supernatant can be observed from the enzyme treatment. Since the concentration is higher in the Enzyme sample compared to the Reference (with the same increasing trend illustrated for the HPH and HPH + Enzyme samples), β-glucanase seems to be active and degrade the cell wall, thus releasing carbohydrates to the supernatant. Since only one measurement was performed for each sample, no significant difference could be determined.

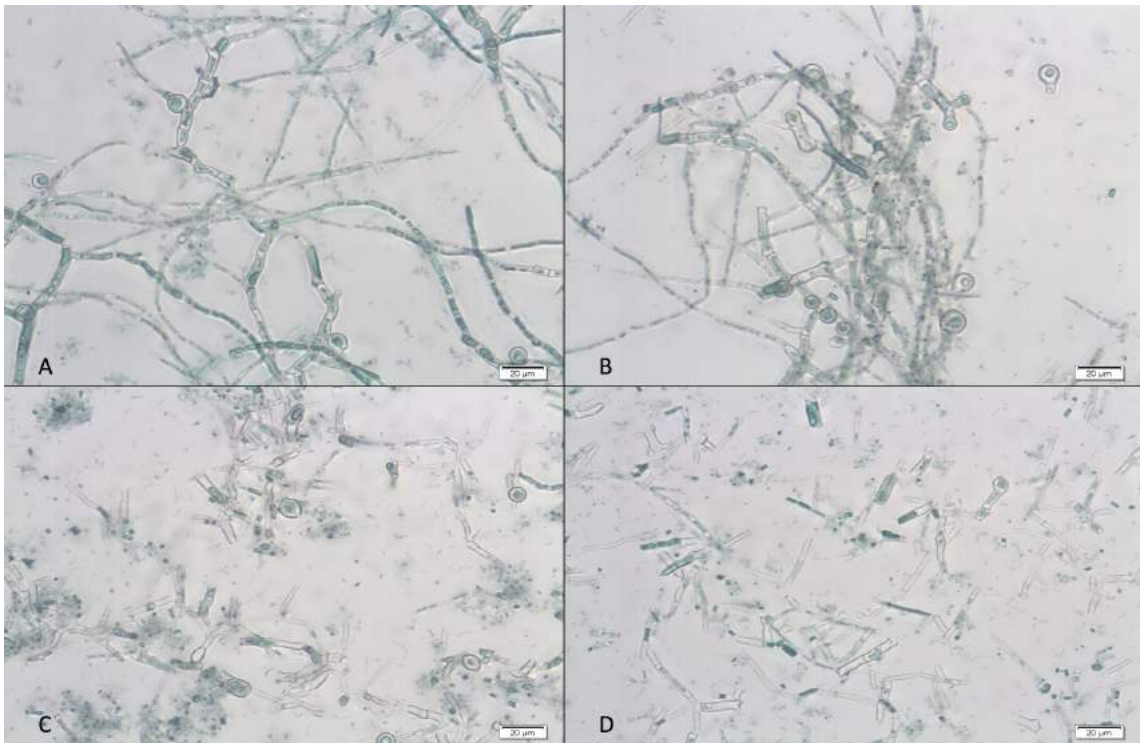


Figure 3.3: Observation of samples under light microscope. A. Reference sample. B. Enzyme sample. C. HPH sample. D. HPH + Enzyme sample. The samples are stained with iodine and light green.

In Figure 3.3 it can be seen that the long, branched mycelium structure is still intact in the reference (untreated, see Figure 2.3) and enzyme treated samples (A and B). The proteins (stained green) are located inside the cells in these samples, indicating that the enzyme treatment was inefficient in degrading the cell walls. The cell walls and fibres are unstained, and due to no visible impact from the iodine staining, low amounts of starch was present in all samples. Since enzyme treatment is considered a mild method, it might result in degradation while the cells keep their original shape (Safi et al. 2017).

In HPH and HPH + Enzyme samples (C and D), cell wall degradation is clearly visible since the long mycelium structure is interrupted. This indicates that the HPH method is effective in disrupting the cell walls. A study by Grossman et al. (2018) evaluated the cell wall degradation and protein precipitation in the microalgae *Chlorella protothecoides*. In their results, high pressure homogenization resulted in a high degree of cell wall disruption (99.9 % reduction after 6 passes at 150 MPa) (Grossmann et al. 2018). This supports the optical result on cell wall degradation in Figure 3.3.

The results in section 3.1 shows that HPH appears to be the most effective cell wall

degradation method when evaluating the protein content of the pellets and the disruption of the mycelium structure. Therefore, the other methods were deselected, and the results after this section will focus on HPH. However, since proteins are released into the soluble phase, a precipitation method is necessary to prevent protein losses during the cell wall degradation method.

3.2 pH-shifting

The pH-shifting method was implemented to precipitate proteins that had been released to the solution during the cell wall degradation treatments. The protein concentration in the supernatant was measured using BCA analysis and calculated using the equations presented in section 2.3.

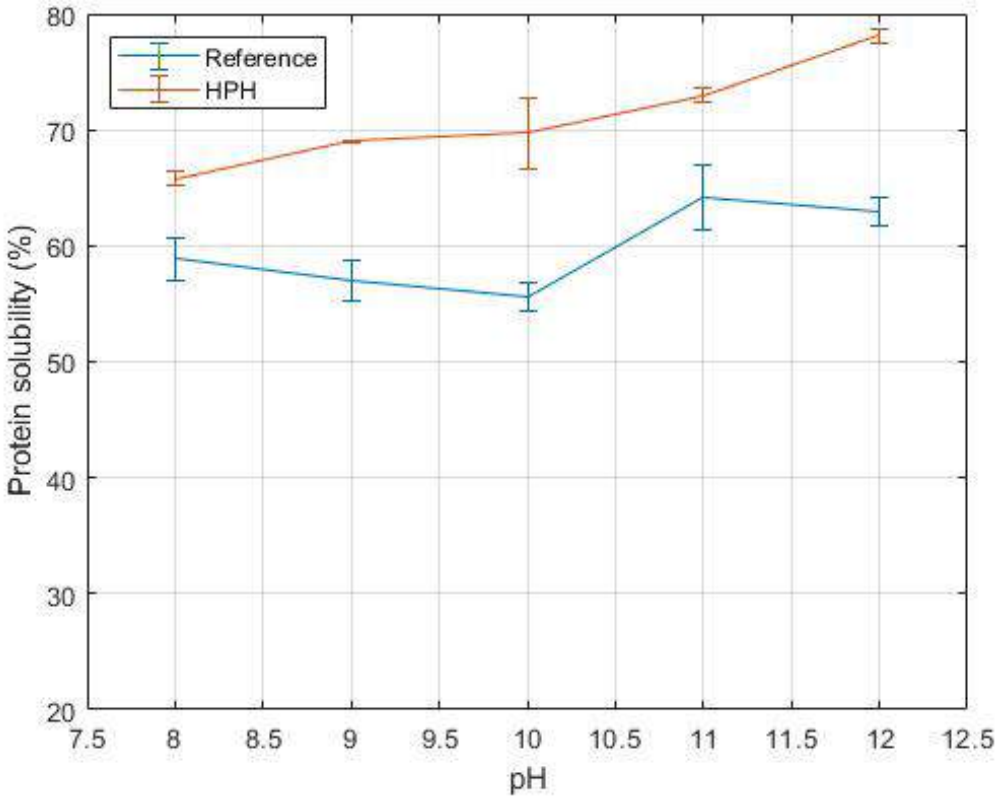


Figure 3.4: Protein solubility (%) at different pH values to optimize the solubilization step in Reference and HPH samples. The error bars show the standard deviation (n=3 with a few exceptions, see Appendix).

From the result in Figure 3.4, it was concluded that pH 12 should be used to solubilize the proteins, since the highest protein solubility was found at pH 12 for the HPH sample. Since the aim was to use pH-shifting on HPH treated samples, the pH-values used were based on the results for HPH. The Reference sample was measured in order to compare if the HPH resulted in a higher protein yield. In Figure 3.4, it is clearly illustrated that HPH treatment increases the amount of released proteins to the soluble phase. The differences in protein solubility at different pH were significant for both the Reference and the HPH samples ($p<0.05$). For details, see Appendix A.3.

The reason for the high values of protein solubility could be due to the chosen analysis method. The solubility is calculated by comparing the protein concentration in supernatant 1 with the protein concentration in the homogenate. Since BCA analysis measures protein concentration in the soluble phase, it is possible that a high amount of proteins are inside the cells in the homogenate samples, thus the BCA analysis results in an underestimation of the protein concentration in the homogenate. Consequently, this leads to an overestimation of the protein solubility.

To obtain fair measurements of protein concentration in the samples, addition of a base during homogenisation could of been added. This in order to keep the proteins in the liquid phase during the measurements.

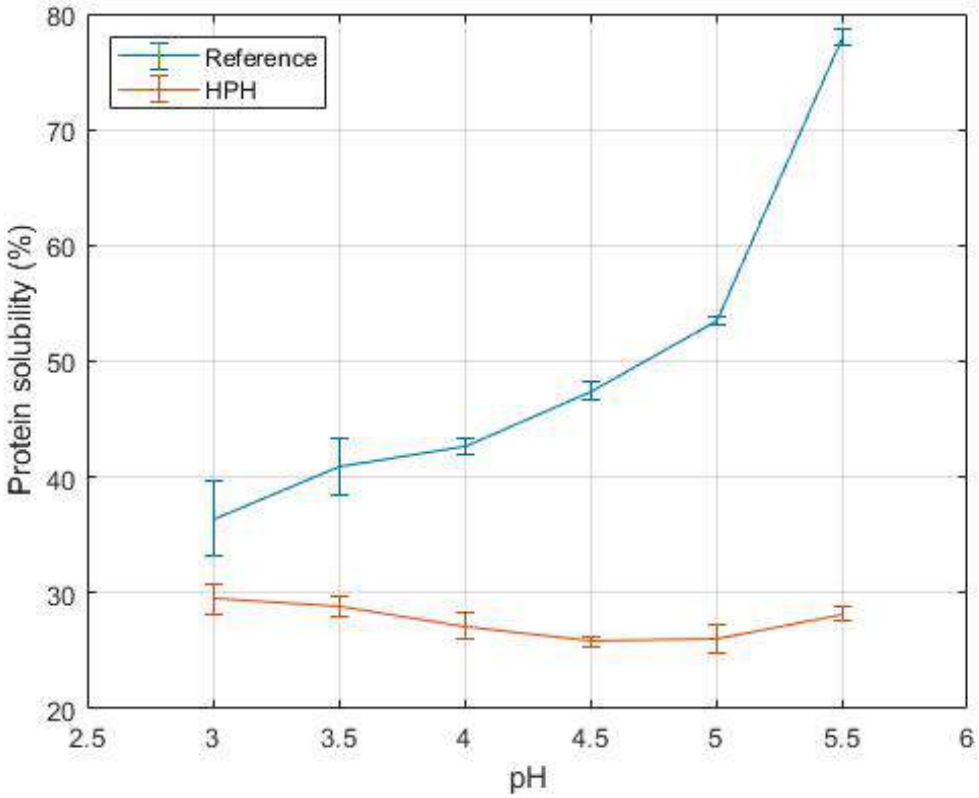


Figure 3.5: Protein solubility (%) at different pH values to optimize the protein precipitation. The error bars show the standard deviation (n=2 for all the samples except two, see Appendix A.3.)

In Figure 3.5, there is a distinct difference in protein solubility between the Reference and the HPH sample. The isoelectric point of the proteins were found at pH 4.5 for the HPH sample. However, a protein solubility of about 26 % is relatively high. To obtain a high yield (for values, see Table 3.1), it would be desirable to optimize the pH-shifting further, either by continuing to investigate different pH values and incubation times, or by investigation of other methods such as salting out and solvent precipitation. Another way would be to investigate different HPH conditions and the effect on protein solubility. This since harsh treatment conditions might result in peptide formation, making it difficult to precipitate the peptides. Grossman et al. (2018) investigated pH-shifting after cell wall degradation on *Chlorella protothecoides* using BCA analysis as well, and they obtained a very low degree of precipitation. They explained that a possible explanation could be a high hydrophilicity of proteins, preventing them from precipitating at their isoelectric point. Secondly, they mentioned that complex formation between proteins and polysaccharides could also increase the solubility.

Contrary, Hinchcliffe et al. (2018) obtained a total yield of about 60 % when using pH-shifting on herring by-products using a modified Lowry assay to determine protein concentration. Therefore, the efficiency of the method could be highly affected by the raw material, or the analysis method.

It is clear in Table 3.1 that HPH resulted in cell wall degradation and thus a release of proteins to the soluble phase. This due to the increased yield during the pH-shifting for the HPH treated sample compared to the Reference.

Table 3.1: Protein yields during pH-shifting on Reference and HPH samples. The total yield was calculated by multiplying the yields from centrifugation 1 and 2.

	Reference	HPH
Protein yield (%) Centrifugation 1	39.44 ±1.04	75.11 ±2.21
Protein yield (%) Centrifugation 2	56.17 ±1.16	70.31 ±1.68
Total yield (%)	22.16 ±0.61	53.81 ±1.73

In Figure 3.6, the protein content (%) of the protein pellet and cell wall pellet is shown. There are significant differences between the protein pellets and between the cell wall pellets (p-value < 0.05). Since the protein content has decreased in the cell wall fraction from HPH treatment compared to the other samples, it once again appears as if HPH is the most effective method for cell wall degradation. A protein content of about 50 % in the protein pellet is an increase compared to the raw, untreated biomass with a protein content of about 35-40 %. However, it would be desirable to obtain a protein content of 60-80 % to make the procedure financially viable.

When comparing the Incubation sample with the Enzyme sample, no significant difference was found in the cell wall fraction. However, there was a significant difference in the protein pellet fraction. Since the amount of enzyme added is not subtracted from the measurement, this could be a possible explanation.

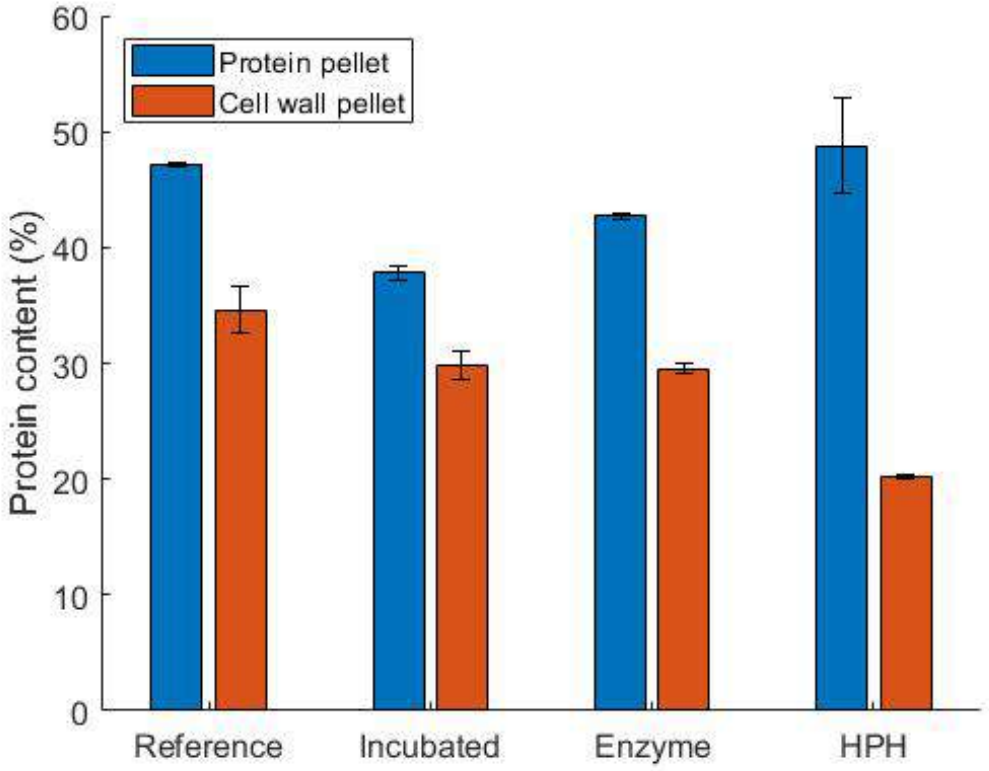


Figure 3.6: Protein content (%) (dry matter) in the precipitated cell wall pellet (sediment 1 during the pH-shifting) and protein pellet (also called protein concentrate, sediment 2 during the pH-shifting) from the different treatments. Only one measurement was performed for the Reference Protein pellet sample, therefore the error bar showing the standard deviation is not correct (see Appendix A.4 and A.5 for details).

3.3 Extrusion

Since extrusion is a common process method when producing fish feed pellets, the effect on structure and some physicochemical properties of the fungal biomass after different extrusion conditions was investigated.

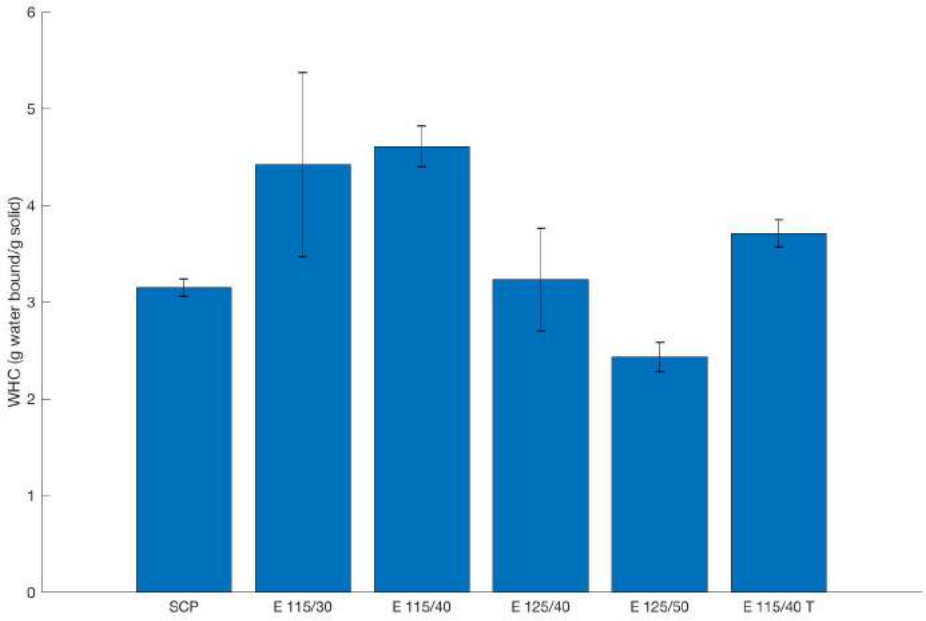


Figure 3.7: Water holding capacity (WHC) in g water bound/g solid before extrusion (SCP) and after different extrusion conditions. The first value after letter E shows the extrusion temperature, and the second value shows the moisture content. The T in the last sample in the figure stands for twin screw.

The result in Figure 3.7 shows that extruding the dried fungal biomass at 115 °C increases the WHC. From the ANOVA analysis, it can be concluded that there is a significant difference between the samples (see Appendix A.18). When performing t-test assuming equal variance between the samples, there are significant differences in WHC between the two temperatures, and between a moisture content of 50 % against the other two moisture contents (see Appendix for details). Therefore, a moisture content of 50 % seems to significantly decrease the WHC. If it is desired to obtain a high WHC of the extruded sample, the dried fungal biomass should be extruded at 115 °C and with a moisture content of 30 or 40 %. From these results, it can be concluded that the extrusion conditions have a significant effect on the fungal biomass, which needs to be considered when developing the production process.

Extrusion resulted in a significant increase in WSI for all the extrusion conditions, see A.20)and Figure 3.8. However, a significant difference was only found between the moisture contents 30 % and 50 %. There was a significant difference between the single and the twin screw, which shows that E 115/40 T had a significantly lower WSI than the other extruded samples. The increase in WSI compared to the SCP could be an issue when developing a fish feed, since a high water solubility

results in ingredient separation in water and thereby weakens the pellet structure and increases waste in the water (Irungu et al. 2019). Since E 115/40 T resulted in the lowest WSI among the extruded samples, it appears to be the preferred extrusion condition when considering WSI. It would, however, be necessary to continue to optimize the extrusion conditions to decrease the WSI.

Since extrusion result in functional changes of the biomass, it would be interesting to perform a factorial design and multiple regression analysis to optimize the extrusion process and improve the physicochemical properties of the dried fungal biomass. This was done in a study by Irungu et al. (2019), where the aim was to optimize the extrusion process for production of a fish feed from local food ingredients. In their study, they wanted to investigate how die diameter, temperature and pre-conditioning time affected expansion, bulk density, floatability, durability, water absorption, water solubility, water stability and in vitro protein digestibility. It would be very interesting to study these parameters as well in an extruded pellet containing both SCP of *P. variotii* as a protein source, along with other feed ingredients such as carbohydrates and lipids. One limitation with extrusion is the potential loss of heat sensitive vitamins and water soluble nutrients (Irungu et al. 2019). This should also be investigated when developing the fish feed.

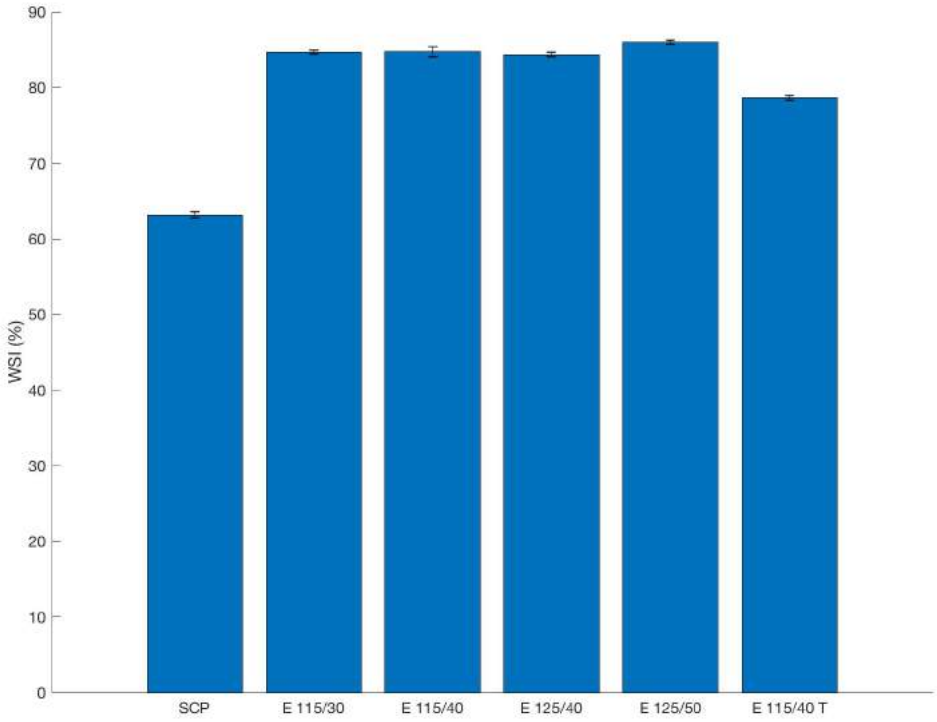
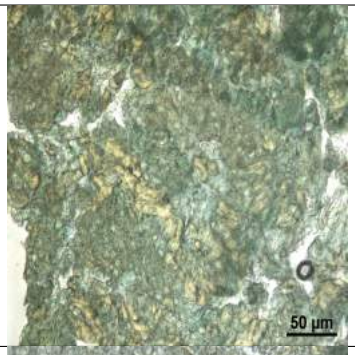
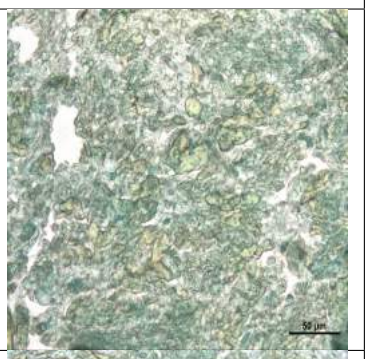
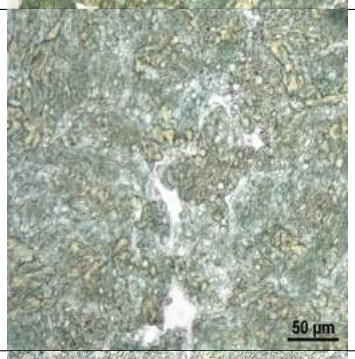
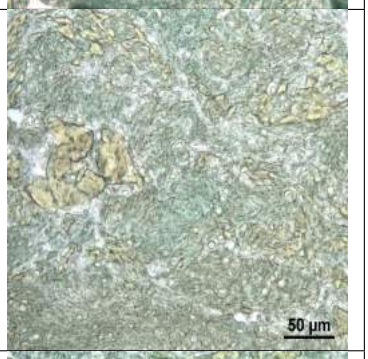
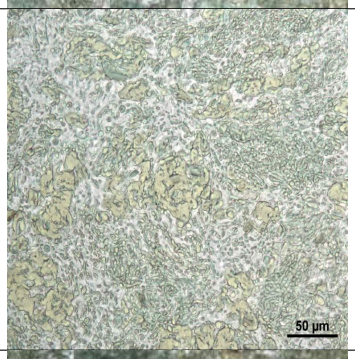
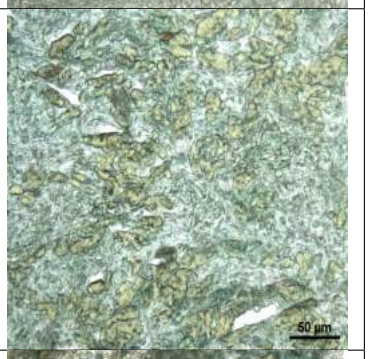
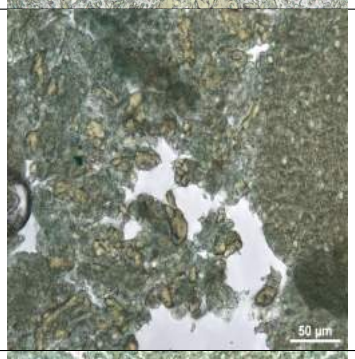
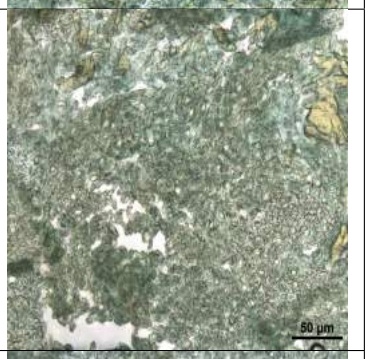
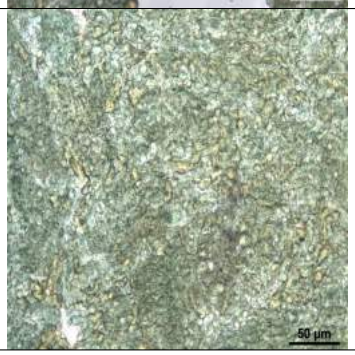
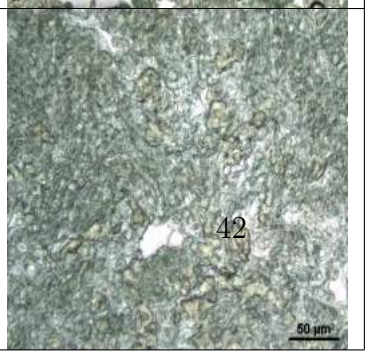


Figure 3.8: Water solubility index (WSI) in % before extrusion of dried fungal biomass and after different extrusion conditions. The first value after letter E shows the extrusion temperature, and the second value shows the moisture content. The T in the last sample in the figure stands for twin screw.

Table 3.2: The macro- and microstructure of the extruded samples. The extrusion conditions are presented in the following order: temperature, moisture content and screw speed. The samples are stained with light green and iodine, making the proteins stained green and the starch stained purple.

	Macrostructure	**Microstructure cross section**	**Microstructure longitudinal section**
115 °C 30 % 75 rpm			
115 °C 40 % 75 rpm			
115 °C 40 % 120 rpm			
125 °C 40 % 75 rpm			
125 °C 50 % 75 rpm			

In Table 3.2, an observation is the dryness of the samples extruded at 125 °C, which resulted in samples that did not hold together well lengthwise. Therefore, extruding at 120 rpm, 115 °C and with a moisture content of 40 % seems optimal when analyzing the macrostrucure.

A different microstructure was observed for all extruded samples compared to the unextruded, dried and milled fungal biomass, see Figure 3.9. In the dried fungal biomass, the mycelium structure had partly remained after the drying and milling. However, after extrusion, a phase stained partly in green is observed, with yellow, disruptive aggregates present. The unstained components are polysaccharides, and proteins are stained green.

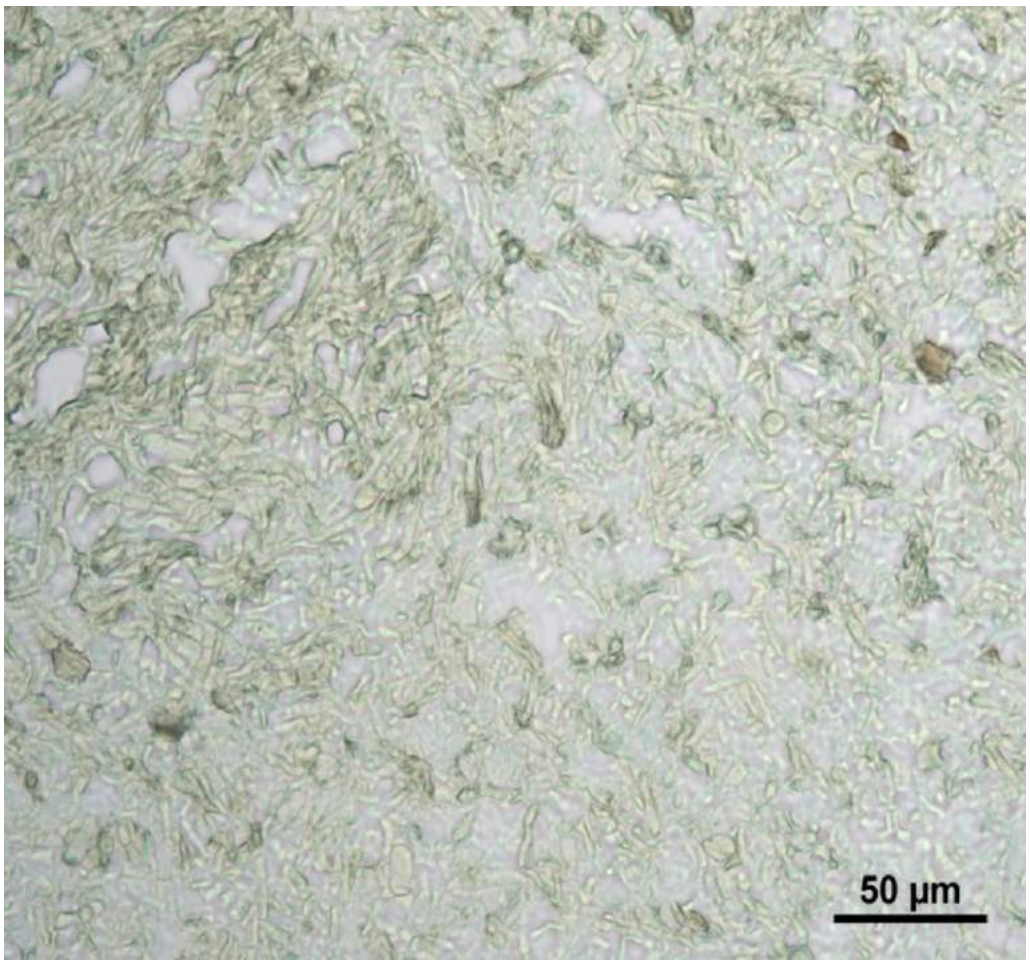


Figure 3.9: Light microscopy image of dried and milled fungal biomass. The sample was moistured with Milli-Q water, dipped in liquid nitrogen and sectioned at -15 °C (in the same way as the extruded samples) before staining and observation in the light microscope.

The reason for the variance in thinness between the fractions of the samples (as shown in Table 3.2) is the capability of the fractions to hold together. The sample extruded with a twin screw at 115 °C, 40 % and at 120 rpm had a microstructure which held together well in both cross- and longitudinal section. Therefore, an image of this sample in larger scale is shown in Figure 3.10.

In Figure 3.10, the different components are clearly distinguished. Proteins (stained green) appears to be included in the matrix with the polysaccharides (unstained), creating a structural network. The yellow aggregates could be proteins with different properties than the ones stained with light green or a combination of several components, but further studies are needed to confirm this. To continue, it would be interesting to use other techniques to find out for certain what the different components are in the structure. An example of this could be the usage of confocal microscopy and staining to for example locate the β-glucans, the cell walls or the proteins in the sample.

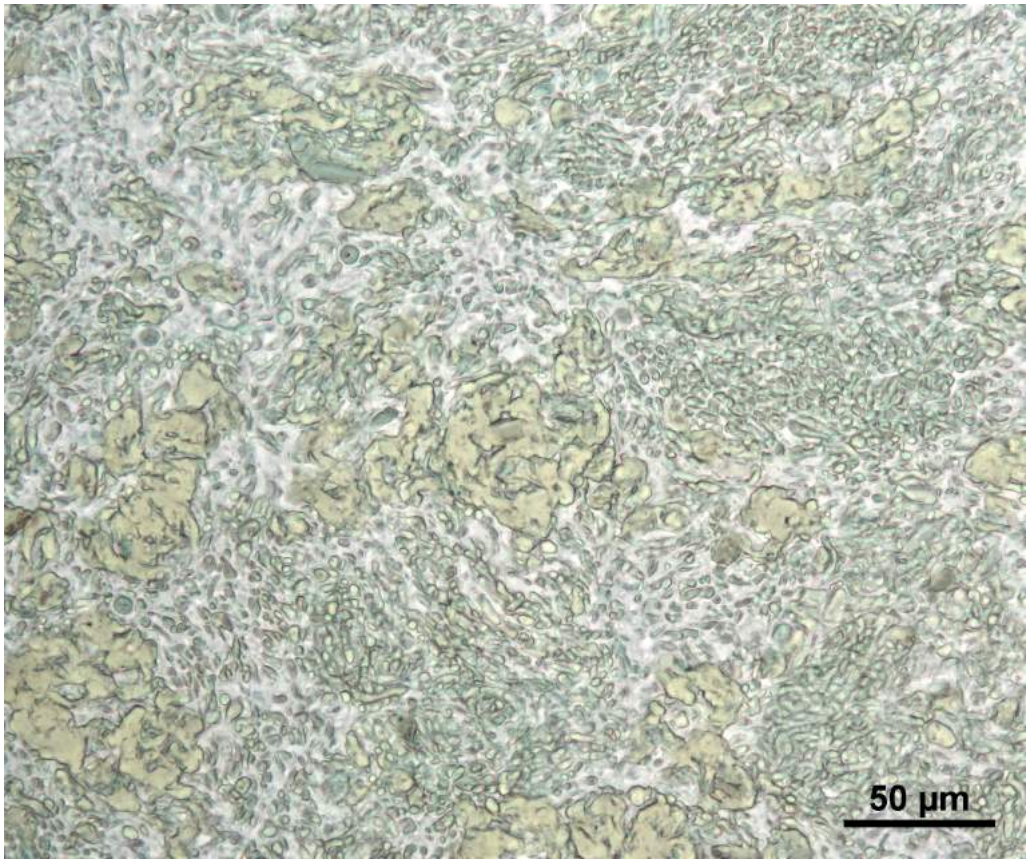


Figure 3.10: Light microscopy image of an extruded sample (115 °C, 40 %, 120 rpm).

3.4 Feeding trial on *Artemia*

In this project, Artemia was used as a model organism to investigate potential differences in growth and survival between samples from different treatments. Three different samples; dried and milled fungal biomass, wet protein pellet from HPH treatment and pH-shifting (see Figure 2.3 and 2.4) and milled extruded sample (twin screw, 115 °C, 40 % moisture content, 120 rpm), were evaluated in the Artemia feeding trial. It should be clarified that only a selection of individuals from each feeding sample was analysed during the feeding trial, and it was assumed that this selection group was representative for the whole population.

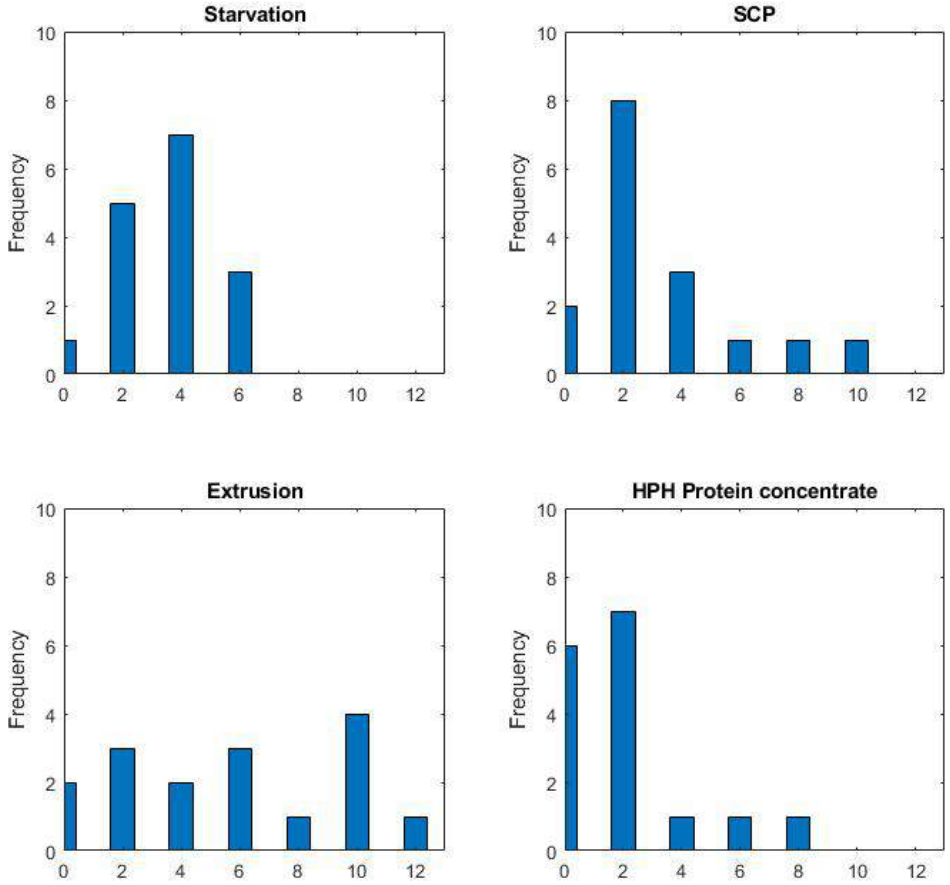


Figure 3.11: Histogram showing the frequency of number of counted *Artemia* in 1 ml water for each of the four feeding samples. The x-axis shows number of counted *Artemia*, the y-axis shows the frequency of which a certain amount of *Artemia* was counted in 1 ml sample.

In Figure 3.11, it is clearly illustrated that dried fungal biomass and HPH protein concentrate resulted in the lowest amount of live *Artemia* during the feeding trial. The Extrusion sample had the most evenly distributed frequencies over the time period and as shown in Figure 3.12 had a high number of living *Artemia* at the end of the feeding trial. This may indicate that extrusion as a treatment method results in a feed that can increase the survival when studying *Artemia*. To confirm this, it would be necessary to redo the feeding trial. Optimally, it would be desirable to perform the feeding trial in aquariums and for a longer time period. It can take 2-3 weeks for the nauplius to become adults, and afterwards they can survive for another 4-5 weeks (Ward's Science 2008). Therefore, it would be desirable to increase the feeding trial period to at least 4 weeks. Here, the feeding trial only lasted for 9 days due to time limitation.

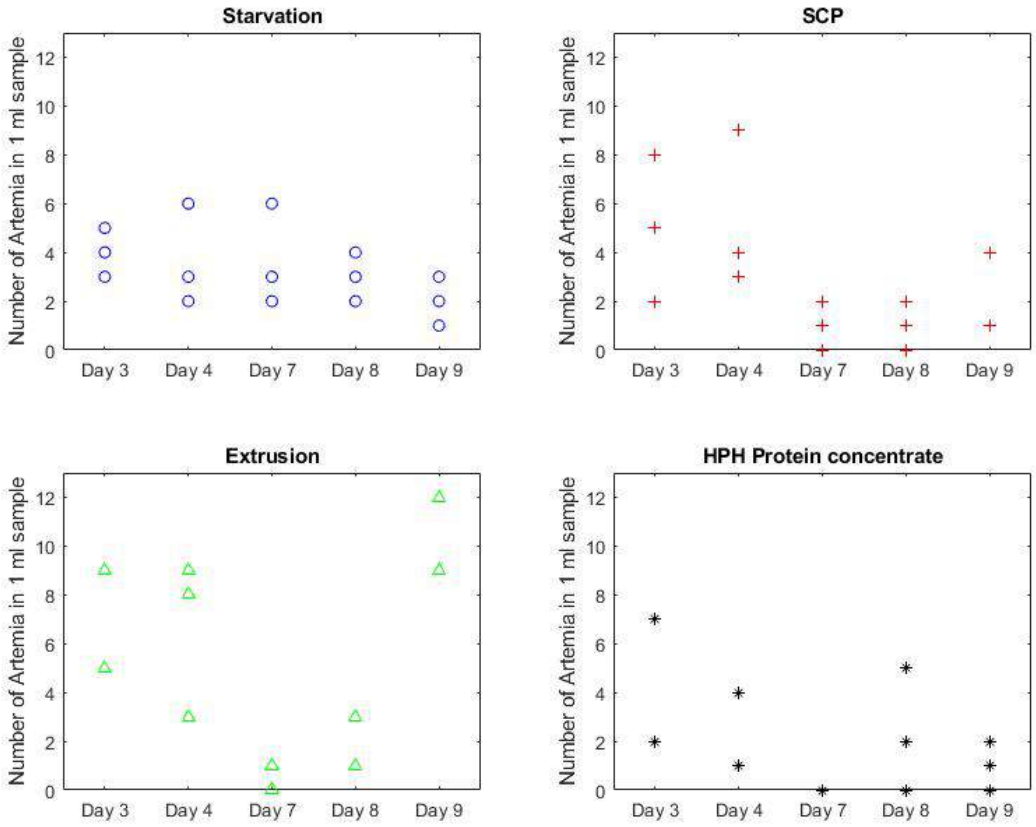


Figure 3.12: The number of *Artemia* in 1 ml sample (with three replicates) for each day and each feeding sample.

In Figure 3.12, there is a decreasing trend in the amount of *Artemia* in all samples except for the Extrusion sample. The *Artemia* were left unsupervised between day

5-6 (not able to count during the weekend), which seems to have resulted in a decrease. A reason for this could be the absence of feed, or a decrease in pH since no $MgSO_4$ were added these days.

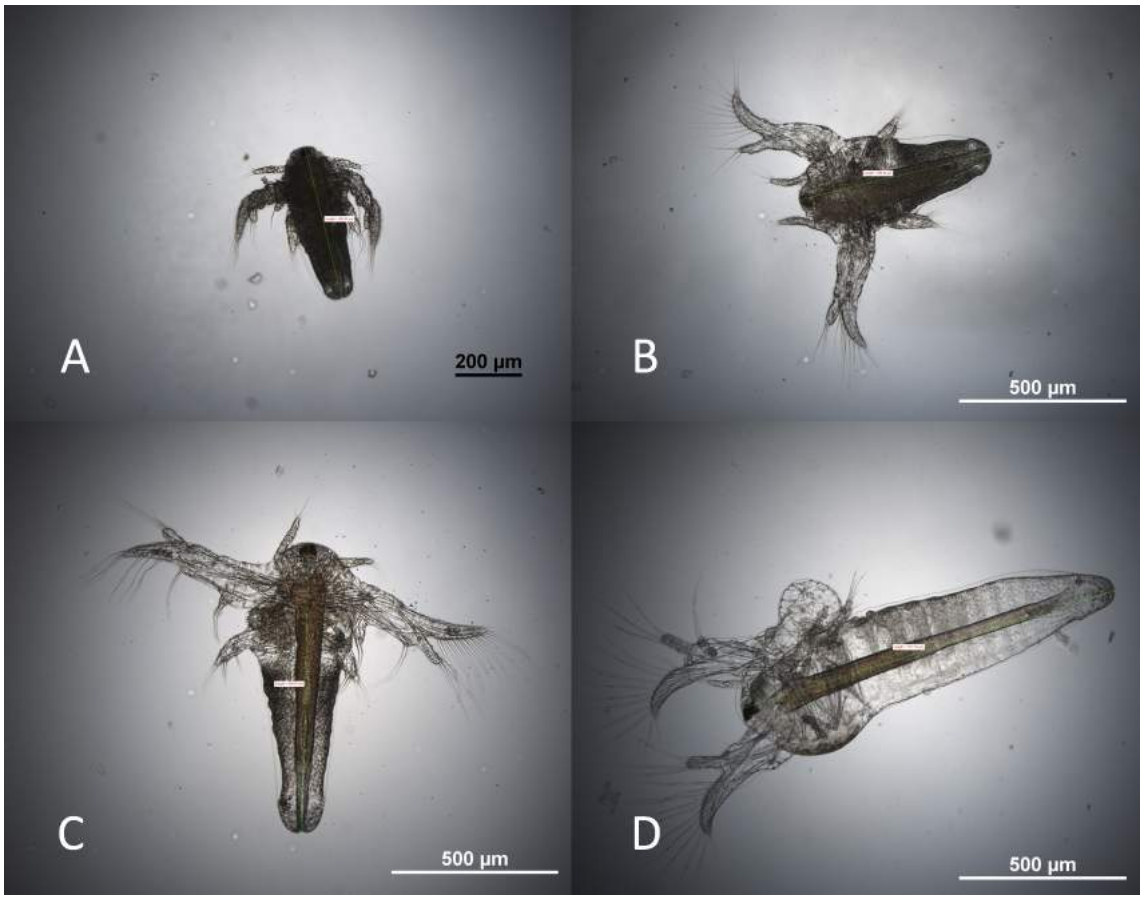


Figure 3.13: Images taken during the determination of the length of the *Artemia* in the light microscope. The images illustrates the different larval stages (A-D), see Figure 2.5.

In Figure 3.13, examples of the different larval stages are presented. It should be mentioned that the determination of the larval stage was subjective. Therefore, the results should only be used as an indication of the development of the *Artemia* during the trial period. No full grown *Artemia* (stage E) was found during the feeding trial. This was probably due to both the limited time, and since the conditions were not optimal for the *Artemia*. To obtain better conditions, it would be necessary to optimize oxygen levels, feed doses, salinity and temperature.

Figure 3.14: During the feeding trial, some *Artemia* in the Starvation sample appeared to starve. This is illustrated in the image above showing an *Artemia* from day 9.

In Figure 3.14, an example of an *Artemia* which appears to starve is shown (compare with image C in Figure 3.13). This phenomena was only found in the Starvation sample, which indicates that the *Artemia* needed addition of feed once they reached a certain larval stage.

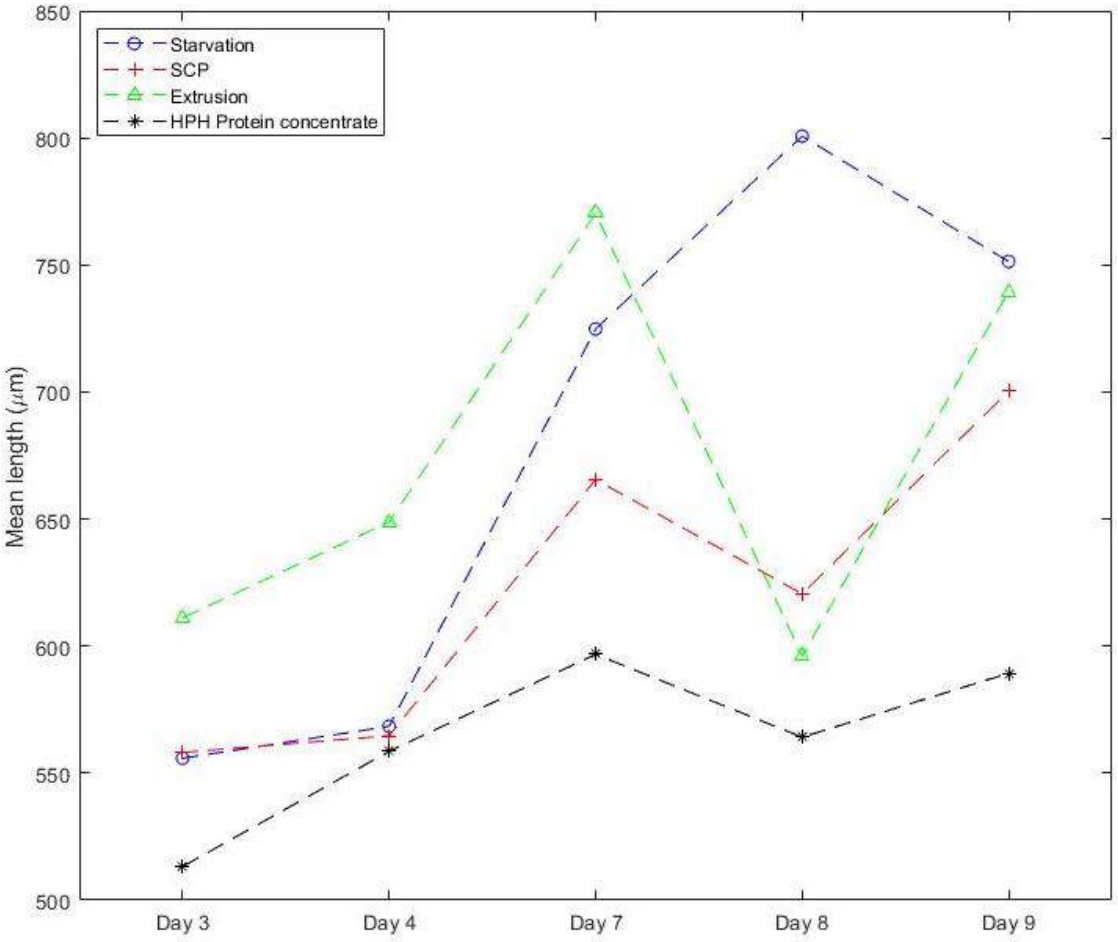


Figure 3.15: Mean length (μm) of the *Artemia* in the different feeding samples during the feeding trial.

In Figure 3.15, the mean length of the *Artemia* during the trial is shown (*Artemia* from all different larval stages was measured). It should be mentioned that some days, less than 14 *Artemia* were measured since there were so few in the sample (mainly HPH Protein concentrate), see Appendix A.25. There was a significant difference between the samples (see Table A.26), but no significant difference was found between the Starvation and the Extrusion samples. Therefore, it can be concluded that these two samples resulted in the fastest growth. Further, since new *Artemia* hatched during the whole feeding trial, the mean length does not necessarily illustrates the growth in a representative way. Therefore, the maximum length of the *Artemia* was also investigated, see Figure 3.16.

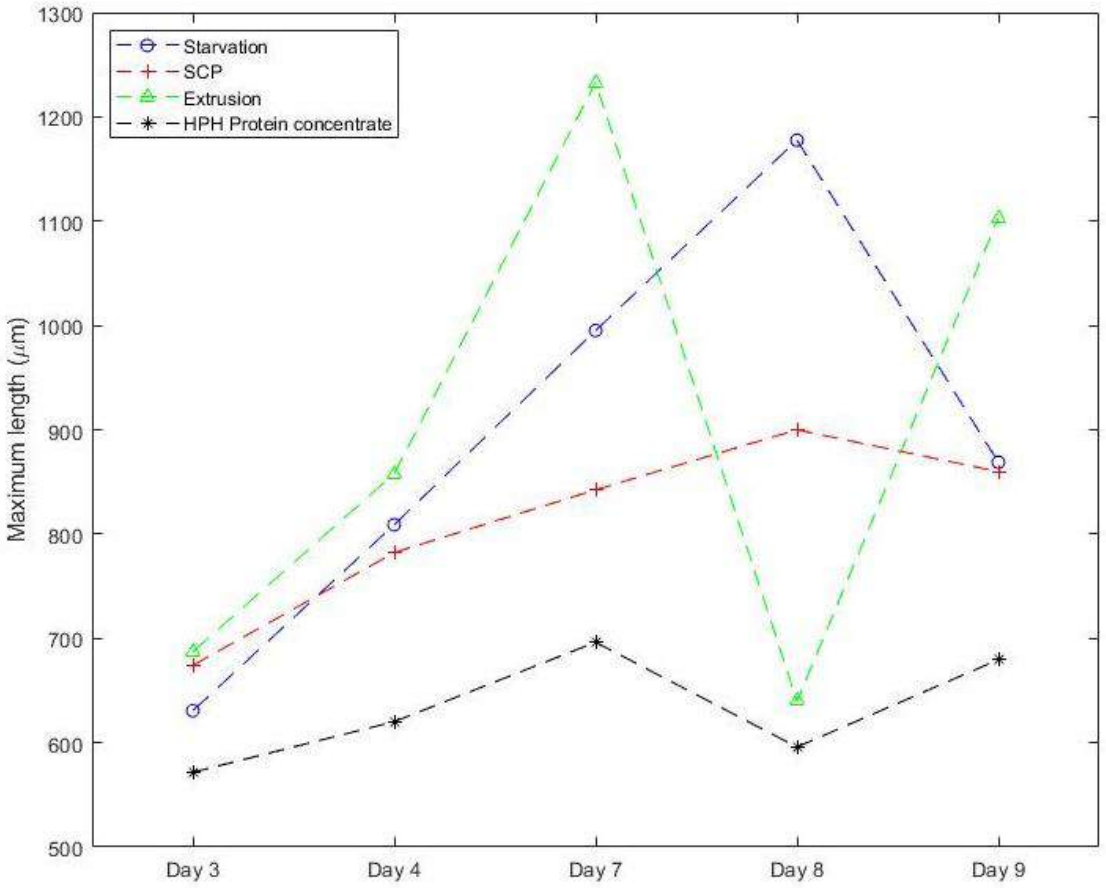


Figure 3.16: Maximum length (μm) of the *Artemia* in the different feeding samples during the feeding trial.

When analyzing the maximum length, it appeared as if Extrusion resulted in the maximum growth with one exception (day 8). However, the Starvation group also grew well. Once again, to draw conclusions it would be necessary to redo the experiment, but from these results it appears as if extrusion could increase the growth and survival. Since *Artemia* larvae do not eat during their early stages, it is important to also study the later stages in a feeding trial. Therefore, it would be desirable to perform the trial for at least 4 weeks. However, SCP and HPH Protein concentrate appears to decrease the survival and growth. This could be due to a decrease in water quality from the feed or faeces, or due to the fact that the *Artemia* are unable to digest the feed.

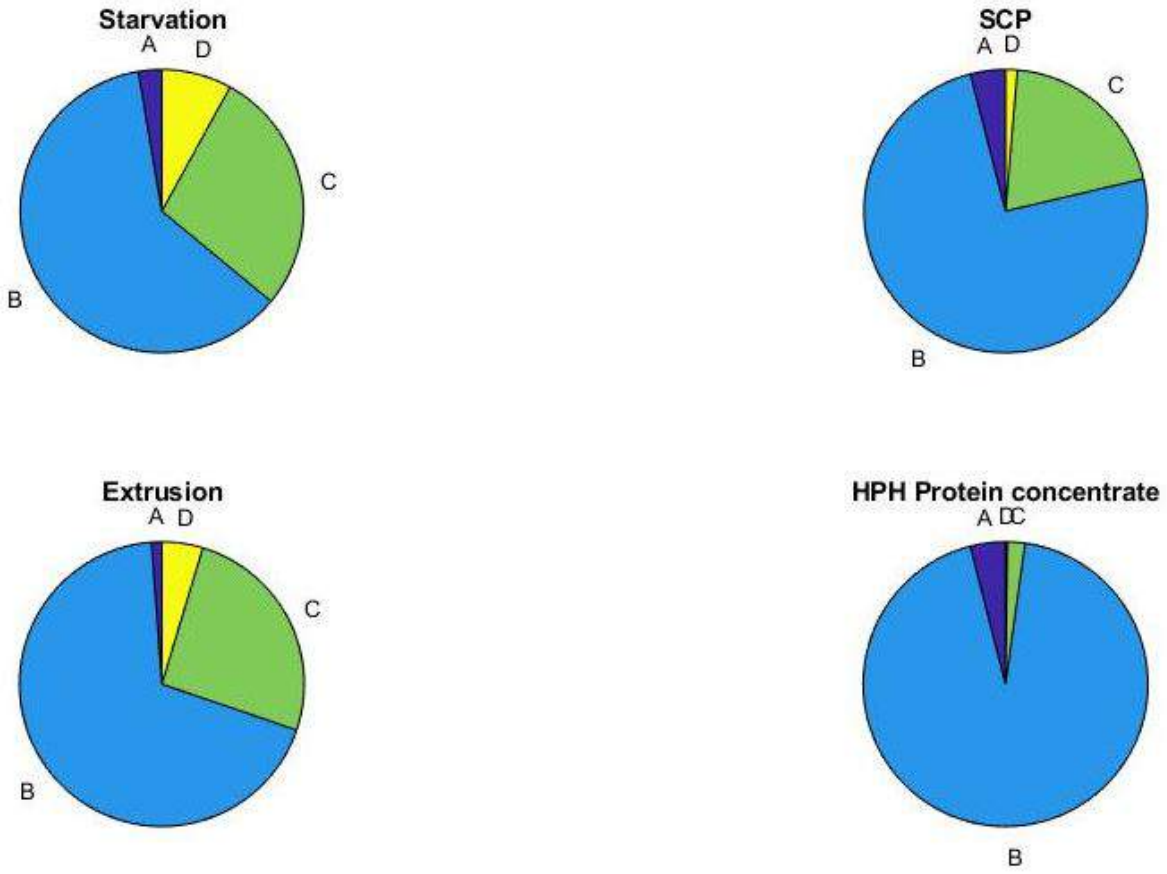


Figure 3.17: Pie charts showing the proportion of *Artemia* in each larval stage (A-D) during the feeding trial (see Figure 3.13).

The pie charts in Figure 3.17 shows once again that Starvation and Extrusion resulted in the fastest growth when comparing the proportion of *Artemia* in the larval stages.

To summarize, Extrusion appears to be a promising treatment method to stimulate survival and growth in *Artemia*. However, this sample did not significantly differ from the Starvation sample, therefore it would be necessary to redo the experiment for a longer period of time (e.g. 4 weeks). Due to time restrictions, no dose-response trial was performed for the different feeds prior to the trial. This would be necessary to make sure that the right amount of feed was added. A more advanced equipment with in- and outflow of water and with an ability to clean the equipment during the feeding trial would also improve the experiment.

In a study conducted by Alriksson et al. (2014), a feeding trial of the fish Tilapia

using 66 % fishmeal substitution with dried and milled *P. variotii* resulted in a 12 % increase in growth compared to the control. This indicates that the fungal biomass has high digestibility in Tilapia, and possibly in other species as well. Cell wall degradation could hopefully result in even higher degrees of growth during feeding trials, but studies are necessary to confirm this. In yeast biomass, disrupted cells provided a higher digestibility in rainbow trout than intact yeast cells (Øverland and Skrede 2017). Therefore, a next step would be to investigate the bioavailability of dried and milled *P. variotii* as well as biomass of the fungus undergoing different cell wall degradation treamtments such as extrusion in a fish feeding trial.

4

Conclusion

Cell wall degradation and pH-shifting can be used to obtain higher protein content in fungal biomass of *P. variotii*, but to make the procedure financially viable it would be desirable to obtain a protein concentration between 60-80 % (dry matter basis). HPH appears to be the most efficient cell wall degradation method, resulting in the highest yield of released protein isolate (about 50 % protein content on dry matter basis) during the pH-shifting (53.81 % total yield compared to 22.16 % for the Reference sample). It was also visible during the light microscopy analysis that HPH resulted in the highest degree of cell wall degradation (see Figure 3.3). However, since HPH followed by pH-shifting requires several different steps, some of them energy-consuming, it is possible that the procedure is too expensive for fish feed production.

The extrusion conditions significantly affected the physicochemical properties of the biomass such as WHC and WSI. Extruding at 115 °C, with a moisture content of 40 % and a twin screw (screw speed = 120 rpm) overall resulted in the best properties when considering WSI and stability (fractions which held together well during freeze sectioning when observed using a light microscope), but further optimization is necessary.

The results from the feeding trial indicated that extrusion could result in improved growth and survival in *Artemia* compared to the groups provided dried and milled fungal biomass or HPH protein concentrate, but the feeding sample did not significantly differ from the starvation sample during the trial. It would therefore be interesting to redo the experiment for a longer time period and analysing more features of the *Artemia*.

5
Future work

To continue, it would be interesting to investigate other cell wall degradation methods, for example High Power Ultrasound (HPU). This to investigate both the efficiency on cell wall degradation and the subsequent effect on protein solubility at different pH levels. As mentioned previously, it would also be interesting to optimize the HPH conditions using for example factorial design and regression analysis to study the effect on cell wall degradation and protein solubility from different condition variables.

To obtain high protein yields after cell wall degradation, it would be necessary to optimize the protein solubilization at high pH and the isoelectric precipitation. This could be achieved either by trying to optimize the pH-shifting, or by investigating other precipitation methods such as salting out and solvent precipitation. To get a better understanding of how to obtain a high yield of protein concentrate, it would also be valuable to study where the proteins are located in the fungal cells.

Extrusion resulted in good physicochemical properties such as WSI, but it would be necessary to continue to optimize the extrusion conditions and the physicochemical properties using for example factorial design and multiple regression analysis. It would be necessary to investigate the physiochemical properties of the dried and milled fungal biomass in combination with all feed ingredients. Some physicochemical properties that would be interesting to optimize with all feed ingredients are: WSI, durability, water absorption, bulk density, floatability and water stability. Further, it would be important to study how nutrients are affected by the extrusion, especially heat sensitive compounds.

To further understand how extrusion affects the dried and milled fungal biomass, it would be necessary to use other techniques such as confical microscopy and different staining procedures. For example, β-glucans could be stained using antibodies to distinguish them, making it possible to see how these polysaccharides are affected by extrusion. Another example would be to stain the cell walls or the proteins.

Finally, since no significant difference was found between the extrusion and the starvation sample during the feeding trial, it would be interesting to redo the experiment in *Artemia*. Since the larvae do not eat during their early stages, it would be interesting to increase the trial period to at least 4 weeks. It would also be desirable to use more advanced equipment with the possibility of in- and outflow of water or the ability to clean the equipment during the feeding trial. A dose-response experiment

should also be performed prior to the trial. To continue, it would also be important to study the bioavailability of the fungal biomass before and after different treatments in a fish species.

Bibliography

Abatzopoulos, Th. J. et al., eds. (2002). *Artemia: Basic and Applied Biology*. Dordrecht: Springer Netherlands. ISBN: 978-90-481-6073-0. DOI: 10.1007/978-94-017-0791-6. URL: http://link.springer.com/10.1007/978-94-017-0791-6.

Alriksson, Björn et al. (2014). "Fish feed from wood". In: *CELLULOSE CHEMISTRY AND TECHNOLOGY Cellulose Chem. Technol* 48.910, pp. 843–848. ISSN: 05769787. URL: http://www.cellulosechemtechnol.ro/pdf/CCT9-10(2014)/p.843-848.pdf.

Ansari, F.A et al. (2018). "Evaluation of various cell drying and disruption techniques for sustainable metabolite extractions from microalgae grown in wastewater: A multivariate approach". In: *Journal of Cleaner Production* 182, pp. 634–644. URL: https://ac-els-cdn-com.proxy.lib.chalmers.se/S0959652618304104/1-s2.0-S0959652618304104-main.pdf?_tid=48636483-4893-4927-9c58-c38da2d05b18&acdnat=1545314558_e667493572339e06acb9c8379e60825f.

Arancibia-Miranda, Nicolás et al. (2011). "Use of isoelectric point and pH to evaluate the synthesis of a nanotubular aluminosilicate". In: *Journal of Non-Crystalline Solids* 357, pp. 1750–1756. DOI: 10.1016/j.jnoncrysol.2011.01.012. URL: https://ac-els-cdn-com.proxy.lib.chalmers.se/S0022309311000615/1-s2.0-S0022309311000615-main.pdf?_tid=f372113f-ec16-4555-8f0d-38d20bc63182&acdnat=1547466174_67f20e9eda7f75a12fc4f7dddcd92b90.

Asenjo, J. A. and P. Dunnill (1981). "The isolation of lytic enzymes fromCytophaga and their application to the rupture of yeast cells". In: *Biotechnology and Bioengineering* 23.5, pp. 1045–1056. ISSN: 0006-3592. DOI: 10.1002/bit.260230512. URL: http://doi.wiley.com/10.1002/bit.260230512.

Ayadi, Ferouz Y., Kurt A. Rosentrate, and Kasiviswanathan Muthukumar (2012). "Alternative Protein Sources for Aquaculture Feeds". In: *Journal of Aquaculture Feed Science and Nutrition* 4.1, pp. 1–26. ISSN: 20701667. DOI: 10.3923/joafsnu.2012.1.26. URL: http://www.medwelljournals.com/abstract/?doi=joafsnu.2012.1.26.

Bajpai, Pratima (2017). *Single cell protein production from lignocellulosic biomass*. Ed. by Jaipur Sanjay K. Sharma. Kanpur, India: SpringerBriefs in Green Chemistry for Sustainability. ISBN: 9789811006869. DOI: 10.1007/978-981-10-5873-8{_}2.

Balasubramaniam, V M et al. (2016). *Food Engineering Series High Pressure Processing of Food Principles, Technology and Applications*. New York: Springer, pp. 10–11. DOI: https://doi-org.proxy.lib.chalmers.se/10.1007/978-1-4939-3234-4. URL: http://www.springer.com/series/5996.

Baldwin, Clark V. and Campbell W. Robinson (1994). "Enhanced disruption of-Candida utilis using enzymatic pretreatment and high-pressure homogenization". In: *Biotechnology and Bioengineering* 43.1, pp. 46–56. ISSN: 0006-3592. DOI: 10.1002/bit.260430107. URL: http://doi.wiley.com/10.1002/bit.260430107.

Brine Schrimp Direct (2019). *Hatching Brine Shrimp Cysts*. URL: https://www.brineshrimpdirect.com/about-us/articles/hatching-brine-shrimp-cysts/.

Calman, W. T. (1911). *Larval stages of the Brine Shrimp (Artemia salina)*. URL: https://commons.wikimedia.org/wiki/File:FMIB_46412_Larval_stages_of_the_Brine_Shrimp_(Artemia_salina).jpeg.

Camire, M.E. (2001). "Extrusion and nutritional quality". In: *Extrusion Cooking - Technologies and Applications*. Woodhead Publishing, pp. 108–129. ISBN: 9781855735590. DOI: 10.1533/9781855736313.1.108. URL: http://linkinghub.elsevier.com/retrieve/pii/B9781855735590500101.

Chen, Feng Liang, Yi Min Wei, and Bo Zhang (2011). "Chemical cross-linking and molecular aggregation of soybean protein during extrusion cooking at low and high moisture content". In: *LWT - Food Science and Technology* 44.4, pp. 957–962. ISSN: 00236438. DOI: 10.1016/j.lwt.2010.12.008. URL: http://dx.doi.org/10.1016/j.lwt.2010.12.008.

Domenech, Jezabel et al. (1994). "Cell wall polysaccharides of four strains of Paecilomyces variotii". In: *Current Microbiology* 28.3, pp. 169–173. ISSN: 03438651. DOI: 10.1007/BF01571060.

FAO (2009). *How to Feed the World in 2050*. Tech. rep. URL: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf.

– (2018). *The state of world fisheries and aquaculture - Meeting the sustainable development goals*. Tech. rep. Rome. URL: www.fao.org/publications.

– (2019). *Cultured aquatic species information programme - Artemia spp.* URL: http://www.fao.org/fishery/culturedspecies/Artemia_spp/en.

Francis, George, P S Makkar, and Klaus Becker (2001). *Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish*. Tech. rep., pp. 197–227. URL: www.elsevier.nlrlocateraqua-online.

Gambardella, Chiara et al. (2018). "Long term exposure to low dose neurotoxic pesticides affects hatching, viability and cholinesterase activity of Artemia sp." In: DOI: 10.1016/j.aquatox.2018.01.006. URL: https://doi.org/10.1016/j.aquatox.2018.01.006.

Grossmann, L et al. (2018). "Effect of precipitation, lyophilization, and organic solvent extraction on preparation of protein-rich powders from the microalgae Chlorella protothecoides". In: DOI: 10.1016/j.algal.2017.11.019. URL: https://doi.org/10.1016/j.algal.2017.11.019.

Guy, R. (2001). "Raw materials for extrusion cooking processes". In: *Extrusion Cooking - Technologies and Applications*. Woodhead Publishing, pp. 52–72. DOI: 10.1007/978-1-4615-2135-8{_}2. URL: https://app.knovel.com/hotlink/pdf/id:kt002QSSO3/extrusion-cooking-technologies/raw-materials-introduction.

Halpern, Georges M. (2013). "Fungal β-glucans". In: *Access Science*. DOI: 10.1036/1097-8542.YB133339. URL: https://www-accessscience-com.proxy.lib.chalmers.se/content/fungal-glucans/YB133339.

Hammer, U. T. (Ulrich Theodore) (1986). *Saline lake ecosystems of the world*. Dr. W. Junk Publishers, p. 287. ISBN: 9061935350.

Hartmann, Christina and Michael Siegrist (2017). "Consumer perception and behaviour regarding sustainable protein consumption: A systematic review". In: *Trends in Food Science & Technology* 61, pp. 11–25. ISSN: 09242244. DOI: 10.1016/j.tifs.2016.12.006. URL: https://linkinghub.elsevier.com/retrieve/pii/S0924224416302904.

Hilton, J.W., C.Y. Cho, and S.J. Slinger (1981). "Effect of extrusion processing and steam pelleting diets on pellet durability, pellet water absorption, and the physiological response of rainbow trout (Salmo gairdneri R.)" In: *Aquaculture* 25.2-3, pp. 185–194. ISSN: 0044-8486. DOI: 10.1016/0044-8486(81)90180-0. URL: https://www.sciencedirect.com/science/article/abs/pii/0044848681901800.

Hinchcliffe, James et al. (2018). "Aquafeed ingredient production from herring (Clupea harengus) by-products using pH-shift processing: Effect from by-product combinations, protein solubilization-pH and centrifugation force". In: DOI: 10.1016/j.anifeedsci.2018.07.014. URL: https://doi.org/10.1016/j.anifeedsci.2018.07.014.

Irungu, Francis Gichuho et al. (2019). "Optimization of extruder cooking conditions for the manufacture of fish feeds using response surface methodology". In: *Journal of Food Process Engineering* 42.2, e12980. ISSN: 0145-8876. DOI: 10.1111/jfpe.12980. URL: https://onlinelibrary.wiley.com/doi/abs/10.1111/jfpe.12980.

Jafari, Morteza, Arash Koocheki, and Elnaz Milani (2017). "Effect of extrusion cooking on chemical structure, morphology, crystallinity and thermal properties of sorghum flour extrudates". In: DOI: 10.1016/j.jcs.2017.05.005. URL: http://dx.doi.org/10.1016/j.jcs.2017.05.005.

Klimek-Ochab, Magdalena et al. (2011). "Comparative study of fungal cell disruption-scope and limitations of the methods". In: *Folia Microbiologica* 56.5, pp. 469–475. ISSN: 00155632. DOI: 10.1007/s12223-011-0069-2.

Kuhad, Ramesh Chander et al. (1997). "Microorganisms as an alternative source of protein." In: *Nutrition reviews* 55.3, pp. 65–75. ISSN: 0029-6643. DOI: 10.1111/j.1753-4887.1997.tb01599.x. URL: https://academic.oup.com/nutritionreviews/article-lookup/doi/10.1111/j.1753-4887.1997.tb01599.x%20http://www.ncbi.nlm.nih.gov/pubmed/9170892.

Maya and Rike (2013). *File:Cell wall structure of Fungi.png - Wikimedia Commons*. URL: https://commons.wikimedia.org/wiki/File:Cell_wall_structure_of_Fungi.png.

Montalescot, V et al. (2015). "Optimization of bead milling parameters for the cell disruption of microalgae: Process modeling and application to Porphyridium cruentum and Nannochloropsis oculata". In: *Biotesource Technology* 196, pp. 339–346. DOI: 10.1016/j.biortech.2015.07.075. URL: http://dx.doi.org/10.1016/j.biortech.2015.07.075.

Nasseri, A. T. et al. (2011). "Single cell protein: Production and process". In: *American Journal of Food Technology* 6.2, pp. 103–116. ISSN: 15574571. DOI: 10.3923/ajft.2011.103.116.

Novák, P. and V. Havlíček (2016). "Protein Extraction and Precipitation". In: *Proteomic Profiling and Analytical Chemistry*, pp. 51–62. DOI: 10.1016/B978-0-444-63688-1.00004-5. URL: https://www.sciencedirect.com/science/article/pii/B9780444636881000045.

Øverland, Margareth and Anders Skrede (2017). "Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture". In: *Journal of the Science of Food and Agriculture* 97.3, pp. 733–742. ISSN: 00225142. DOI: 10.1002/jsfa.8007. URL: http://doi.wiley.com/10.1002/jsfa.8007.

Phong, Win Nee et al. (2018). "Improving cell disruption efficiency to facilitate protein release from microalgae using chemical and mechanical integrated method". In: *Biochemical Engineering Journal* 135, pp. 83–90. DOI: 10.1016/j.bej.2018.04.002. URL: https://doi.org/10.1016/j.bej.2018.04.002.

Pohndorf, Ricardo S et al. (2016). "Production of lipids from microalgae Spirulina sp.: Influence of drying, cell disruption and extraction methods". In: *Biomass and Bioenergy* 93, pp. 25–32. DOI: 10.1016/j.biombioe.2016.06.020. URL: http://dx.doi.org/10.1016/j.biombioe.2016.06.020.

Postma, P R et al. (2017). "Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates". In: *Bioresource Technology* 224, pp. 670–679. DOI: 10.1016/j.biortech.2016.11.071. URL: http://dx.doi.org/10.1016/j.biortech.2016.11.071.

Pusztahelyi, Tünde (2018). "Mycology An International Journal on Fungal Biology Chitin and chitin-related compounds in plant-fungal interactions". In: ISSN: 2150-1211. DOI: 10.1080/21501203.2018.1473299. URL: http://www.tandfonline.com/action/journalInformation?journalCode=tmyc20.

Rashid, Summer et al. (2015). "Effects of extrusion cooking on the dietary fibre content and Water Solubility Index of wheat bran extrudates". In: *International Journal of Food Science & Technology* 50.7, pp. 1533–1537. ISSN: 09505423. DOI: 10.1111/ijfs.12798. URL: http://doi.wiley.com/10.1111/ijfs.12798.

Ritala, Anneli et al. (2017). "Single Cell Protein—State-of-the-Art, Industrial Landscape and Patents 2001–2016". In: *Frontiers in Microbiology* 8.OCT. ISSN: 1664-302X. DOI: 10.3389/fmicb.2017.02009. URL: http://journal.frontiersin.org/article/10.3389/fmicb.2017.02009/full.

Safi, C et al. (2017). "Energy consumption and water-soluble protein release by cell wall disruption of Nannochloropsis gaditana". In: *Bioresource Technology* 239, pp. 204–210. DOI: 10.1016/j.biortech.2017.05.012. URL: http://dx.doi.org/10.1016/j.biortech.2017.05.012.

Samuelsen, T A, S A Mjøs, and Å Oterhals (2013). "Impact of variability in fishmeal physicochemical properties on the extrusion process, starch gelatinization and pellet durability and hardness". In: *Animal Feed Science and Technology* 179, pp. 77–84. DOI: 10.1016/j.anifeedsci.2012.10.009. URL: http://dx.doi.org/10.1016/j.anifeedsci.2012.10.009.

Silvennoinen, E. and H.N. Koivo (1983). *Estimation of the Biomass Concentration in the Pekilo-Process From the Filtrate Flow Rate of the Concentrator*. IFAC,

pp. 219–224. DOI: 10.1016/B978-0-08-029978-5.50030-6. URL: https://linkinghub.elsevier.com/retrieve/pii/B9780080299785500306.

Sriperm, Nuntawadee, Gene M Pesti, and Paul B Tillman (2011). "Evaluation of the fixed nitrogen-to-protein (N:P) conversion factor (6.25) versus ingredient specific N:P conversion factors in feedstuffs". In: *Journal of the Science of Food and Agriculture* 91.7, pp. 1182–1186. ISSN: 00225142. DOI: 10.1002/jsfa.4292. URL: http://doi.wiley.com/10.1002/jsfa.4292.

Steen, Amanda (2014). "Production of Single Cell Protein from Residual Streams from 2 nd Generation Bioethanol". In: *The Royal\tInstitute of\tTechnology,KTH*, p. 84. URL: http://www.diva-portal.org/smash/get/diva2:801729/FULLTEXT01.pdf.

Sun, Jing et al. (2018). "Importing food damages domestic environment: Evidence from global soybean trade." In: *Proceedings of the National Academy of Sciences of the United States of America* 115.21, pp. 5415–5419. ISSN: 1091-6490. DOI: 10.1073/pnas.1718153115. URL: http://www.ncbi.nlm.nih.gov/pubmed/29735661%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6003527.

Tacon, A (1987). *The nutrition and feeding of farmed fish and shrimp -?- A training manual. 1. The essential nutrients.* Tech. rep. URL: http://www.fao.org/docrep/field/003/ab470e/AB470E02.htm#ch2.4.

Tallentire, C W, S G Mackenzie, and I Kyriazakis (2018). "Can novel ingredients replace soybeans and reduce the environmental burdens of European livestock systems in the future?" In: *Journal of Cleaner Production* 187, pp. 338–347. DOI: 10.1016/j.jclepro.2018.03.212. URL: https://doi.org/10.1016/j.jclepro.2018.03.212.

Ugalde, U. O. and J. I. Castrillo (2002). "Single cell proteins from fungi and yeasts". In: *Applied Mycology and Biotechnology* 2.C, pp. 123–149. ISSN: 18745334. DOI: 10.1016/S1874-5334(02)80008-9.

Uhlmann, E et al. (2013). "The First CIRP Conference on Biomanufacturing Development of a versatile and continuously operating cell disruption device". In: *Procedia - Social and Behavioral Sciences* 5, pp. 119–123. DOI: 10.1016/j.procir.2013.01.024. URL: www.sciencedirect.com.

Wanzenb, Elisa et al. (2017). "Wheat bran biodegradation by edible Pleurotus fungi e A sustainable perspective for food and feed". In: DOI: 10.1016/j.lwt.2017.07.051. URL: http://dx.doi.org/10.1016/j.lwt.2017.07.051.

Ward's Science (2008). *Conditions for Customer Ownership.* Tech. rep. URL: https://media.vwr.com/emdocs/docs/scied/Brine_Shrimp.pdf.

Wild, Florian (2016). "Manufacture of Meat Analogues Through High Moisture Extrusion". In: *Reference Module in Food Science*. DOI: 10.1016/B978-0-08-100596-5.03281-9. URL: https://www.sciencedirect.com/science/article/pii/B9780081005965032819.

Zayas, J F (1997). "Water Holding Capacity of Proteins 2.1 Introduction". In: *Functionality of Proteins in Food*. URL: https://link.springer.com/content/pdf/10.1007/978-3-642-59116-7_3.pdf.

Zioga, Marianthi (2018). "Final Report on Erasmus + Internship Protein extraction from fungal biomass for fish feed production". In: June, pp. 1–47.

A

Appendix

A.1 Sodium citrate buffer preparation

2.1 g of citric acid was added to approximately 900 ml of Milli-Q water. Then, the pH of the buffer was adjusted to pH 5 by addition of NaOH (1 M). When the desired pH was obtained, the volume was increased to 1 l by addition of Milli-Q water.

A.2 Standard curve BCA analysis

The procedure was performed according to the user guide obtained from Thermo scientific. The kit used was Pierce™Rapid Gold BCA Protein Assay Kit (Thermo scientific). Table A.1 contains the volumes used in the dilution scheme.

Table A.1: Dilution scheme for the standard curve procedure (working range = 20-2000 µg/mL).

Vial	Diluent (µL)	BCA (µL)	BSA concentration (mg/mL)
Stock	0	950	2
A	0	300 (Stock)	2
B	125	375 (Stock)	1.5
C	250	250 (Stock)	1
D	150	150 (Vial B)	0.75
E	250	250 (Vial C)	0.5
F	200	200 (Vial E)	0.25
G	150	150 (Vial F)	0.125
H	200	50 (Vial G)	0.025
I	250	0	0

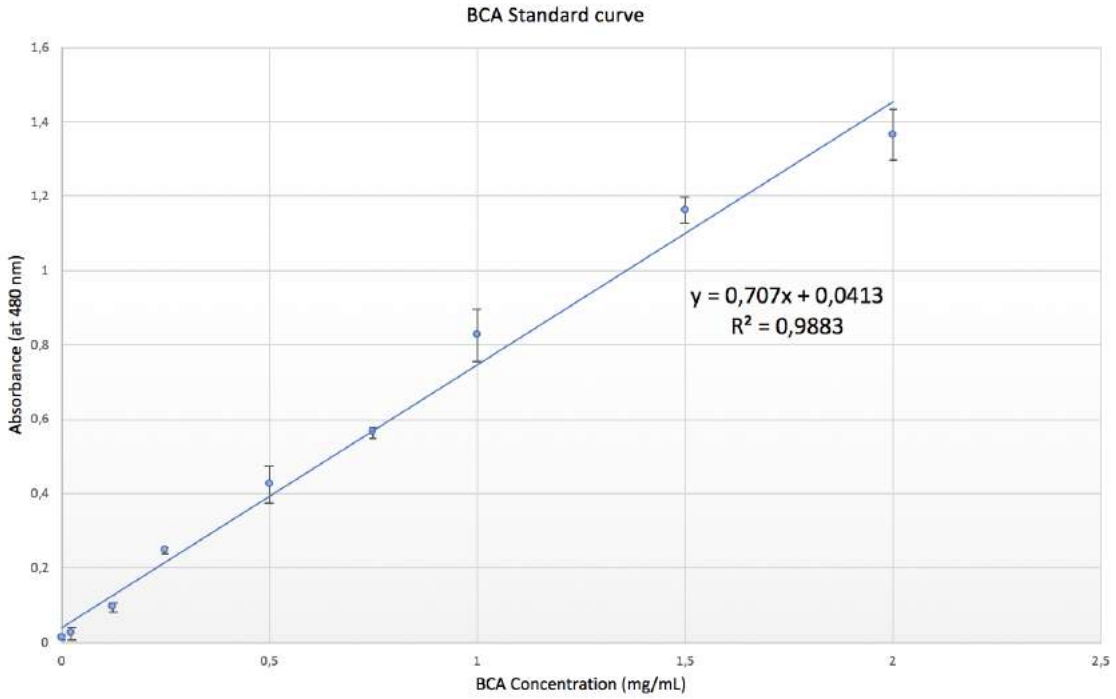


Figure A.1: Standard curve for the BCA analysis.

A.3 Protein concentration measurements from the pH-shifting

A.3.1 Reference

Table A.2: Data from the protein concentration measurement of the homogenized Reference sample.

Protein concentration (mg/mL)
11.277
14.040
13.389
13.795

Table A.3: Data from the protein solubilization of the Reference sample.

pH	Protein concentration (mg/mL)	Protein solubility (%)
8	7.861	49.438
	7.445	46.823
	7.878	49.545
9	7.615	47.890
	7.216	45.381
	7.607	47.837
10	7.233	45.488
	7.173	45.114
	7.488	47.089
11	8.056	50.665
	8.430	53.014
	8.786	55.255
12	8.141	51.200
	8.370	52.640

Table A.4: Data from the protein precipitation of the Reference sample.

pH	Protein concentration (mg/mL)	Protein solubility (%)
3	3.387	40.124
	2.882	34.143
	2.950	34.947
3.5	3.341	39.570
	3.629	42.989
	3.591	42.536
	3.260	38.616
4	3.642	43.139
	3.723	44.094
	3.434	40.676
	3.604	42.687
4.5	4.049	47.964
	3.964	46.959
5	4.490	53.192
	4.533	53.694
5.5	6.544	77.518
	6.625	78.473

Table A.5: ANOVA Table for the protein solubilization in the Reference sample.

Source	SS	df	MS	F	P-value
Between groups	107.388	4	26.8469	10.256	0.00104
Within groups	28.7944	11	2.61767		
Total	136.182	15			

Table A.6: ANOVA Table for the protein precipitation in the Reference sample.

Source	SS	df	MS	F	P-value
Between groups	2564.59	5	512.918	133.059	1.9E-09
Within groups	42.403	11	3.85482		
Total	2606.99	16			

A.3.2 HPH

Table A.7: Data from the protein concentration measurement of the homogenized HPH sample.

Protein concentration (mg/mL)
12.579
14.832
12.211
12.805
12.890

Table A.8: Data from the protein solubilization of the HPH sample.

pH	Protein concentration (mg/mL)	Protein solubility (%)
8	8.565	65.353
	8.684	66.260
9	9.040	68.979
	9.058	69.109
10	8.854	67.555
	9.423	71.893
11	9.516	72.605
	9.618	73.282
12	10.297	78.563
	10.186	77.721

Table A.9: Data from the protein precipitation of the HPH sample.

pH	Protein concentration (mg/mL)	Protein solubility (%)
3	2.925	28.559
	3.107	30.341
3.5	3.018	29.471
	2.891	28.228
4	2.696	26.322
	2.861	27.938
4.5	2.611	25.493
	2.679	26.156
5	2.573	25.120
	2.751	26.860
5.5	2.844	27.772
	2.929	28.600

Table A.10: ANOVA Table for the protein solubilization in the HPH sample.

Source	SS	df	MS	F	P-value
Between groups	159.686	4	39.921	20.814	0.00257
Within groups	9.590	5	1.918		
Total	169.276	9			

Table A.11: ANOVA Table for the protein precipitation in the HPH sample.

Source	SS	df	MS	F	P-value
Between groups	22.571	5	4.514	4.717	0.0428
Within groups	5.742	6	0.957		
Total	28.313	11			

A.4 Protein content in protein concentrates from the pH shifting

Since material was lost during the drying of a Reference sample, only one replicate was analyzed.

Table A.12: Protein content (%) in the protein concentrate obtained during the pH-shifting. The mean values are followed by the standard deviation.

Protein content (%)				
Reference	Incubated	Enzyme	HPH[1]	HPH[2]
47.164	38.300	42.492	54.895	45.960
	37.391	42.898	54.740	46.705
				46.031
				46.843
				46.427
Mean values				
47.164	37.843 ±0.640	42.695 ±0.287	54.818 ±0.109	46.393 ±0.393

Table A.13: ANOVA Table for the protein content in the protein concentrate. Since only one value for the Reference was obtained, the ANOVA was performed without including the Reference sample.

Source	**SS**	**df**	**MS**	**F**	**P-value**
Between groups	208.789	2	104.390	8.147	0.0118
Within groups	102.517	8	12.815		
Total	311.305	10			

A.5 Protein content in cell wall pellet obtained from the pH-shifting

Table A.14: Protein content (%) in the cell wall pellets obtained during the pH-shifting.

Protein content (%)			
Reference	Incubated	Enzyme	HPH
32.231	28.471	28.974	19.977
33.692	29.679	29.180	20.006
37.793	31.771	30.140	20.368
34.824	29.347	29.785	20.300
Mean values			
34.635 ±2.042	29.817 ±1.211	29.520 ±0.466	20.163 ±0.173

Table A.15: ANOVA Table for the protein content in the cell wall pellet.

Source	**SS**	**df**	**MS**	**F**	**P-value**
Between groups	439.663	3	146.550	74.716	5E-08
Within groups	23.538	12	1.962		
Total	463.200	15			

A.6 Extrusion

Table A.16: Amounts of SCP and water mixed before the extrusion.

Temperature (°C)	Moisture content (%)	SCP (g)	Water (g)
115	30	140.61	60.09
	40	119.59	79.46
125	40	119.96	79.86
	50	100.01	100.09

A.7 Water holding capacity and water solubility index

Table A.17: Water holding capacity (g water bound/g protein concentrate) for the extruded samples.

WHC (g water bound/g solid)					
SCP	E 115/30	E 115/40	E 125/40	E 125/50	E 115/ 40 T
3.047	4.150	4.819	2.618	2.269	3.874
3.186	5.477	4.395	3.468	2.558	3.656
3.210	3.640	4.618	3.590	2.476	3.611
Mean values					
3.148 ±0.088	4.422 ±0.948	4.611 ±0.212	3.225 ±0.529	2.434 ±0.149	3.714 ±141

Table A.18: ANOVA Table for the WHC of the extruded samples.

Source	SS	df	MS	F	P-value
Between groups	10.241	5	2.048	9.649	0.0007
Within groups	2.547	12	0.212		
Total	12.788	17			

Table A.19: Water solubility index (%) for the extruded samples.

WSI (%)					
SCP	E.115.30	E.115.40	E.125.40	E.125.50	E.115.40.T
63.103	84.364	83.935	84.211	86.076	78.858
63.577	84.747	84.872	84.091	85.741	78.690
62.797	84.765	85.337	84.637	85.741	78.204
Mean values					
63.159 ±0.39	84.625 ±0.23	84.715 ±0.71	84.313 ±0.29	85.853 ±0.25	78.584 ±0.34

Table A.20: ANOVA Table for the WSI of the extruded samples.

Source	SS	df	MS	F	P-value
Between groups	1145.50	5	229.11	1446	3E-16
Within groups	1.90	12	0.158		
Total	1147.4	17			

Table A.21: p-values from t-tests on WHC performed for the different extrusion variables (assuming equal variance, two-tailed)

Variables	t Stat	p-value
115 °C vs 125 °C	4.43	0.0007
30 % vs 40 %	1.16	0.272
30 % vs 50 %	3.59	0.023
40 % vs 50 %	3.49	0.0058
75 rpm vs 120 rpm	-0.065	0.949

Table A.22: p-values from t-tests on WSI performed for the different extrusion variables (assuming equal variance, two-tailed)

Variables	t Stat	p-value
115 °C vs 125 °C	-1.876	0.083
30 % vs 40 %	1.167	0.271
30 % vs 50 %	-7.140	0.002
40 % vs 50 %	-1.853	0.094
75 rpm vs 120 rpm	14.758	1.7E-09

A.8 Feeding trial

Table A.23: Number of moving *Artemia* in 1 ml water with 3 replicates during the feeding trial. Unfortunately, only one sample was calculated the first day, as shown in the table.

	Starvation			**SCP**			**Extruded**			**HPH Protein**		
Day 2	0	-	-	2	-	-	5	-	-	0	-	-
Day 3	4	3	5	8	5	2	9	5	5	7	2	2
Day 4	2	6	3	4	9	3	3	8	9	4	1	1
Day 7	6	3	2	2	1	0	1	0	0	0	0	0
Day 8	2	4	3	1	0	2	1	3	1	5	0	2
Day 9	2	3	1	1	4	1	9	9	12	0	2	1

Table A.24: pH of the samples during the feeding trial.

	Starvation	**SCP**	**Extruded**	**HPH Protein**
Day 1	7.71	7.54	7.58	7.81
Day 2	7.82	7.72	7.77	7.78
Day 3	7.56	7.58	7.61	7.57
Day 4	7.67	7.56	7.70	7.74
Day 7	7.43	7.44	7.52	7.63
Day 8	7.73	7.83	7.80	7.84

Table A.25: Measured length (μm) of the *Artemia* in the different feeding samples.

Day	Starvation	SCP	Extruded	HPH Protein
2		506.57		
		585.42		
		550.56		
		497.71		
		452.90		
		400.65		
		510.38		
		543.15		
		314.48		
		532.47		
		591.50		
		497.73		
		583.41		
		527.24		
3	567.28	674.07	687.01	553.68
	589.30	612.72	585.80	528.03
	518.29	580.50	597.18	511.37
	544.02	543.92	531.08	571.61
	528.86	575.81	602.15	498.98
	501.49	583.64	616.30	486.00
	580.54	527.50	591.86	499.44
	580.93	521.64	662.22	527.49
	524.50	555.91	615.16	424.23
	469.49	571.33	571.59	554.99
	582.80	528.80	631.66	533.57
	630.65	566.01	582.31	486.16
	557.40	449.20	568.41	474.13
	606.23	536.44	610.63	535.23
		563.46	627.69	
		536.37	665.16	
			642.68	
4	533.49	598.11	587.11	547.61
	469.23	782.22	798.97	620.12
	580.10	576.31	572.40	508.55
	569.3	555.62	554.67	568.95
	560.99	531.32	786.04	567.75
	557.51	550.03	651.32	585.65
	491.50	611.31	850.79	543.79
	586.45	477.71	578.08	513.63
	600.18	448.57	792.19	549.22
	688.55	559.15	562.78	520.71
	808.97	537.27	530.65	588.07
	587.97	581.80	522.25	552.95
	574.27	624.80	520.71	553.23
	542.28	529.03	857.88	599.65
	578.03	563.85	610.09	

	566.93	520.49	621.14	
	507.67	556.20	738.91	
	534.62	560.61	652.90	
	461.57		853.38	
			473.72	
			583.02	
			574.74	
7	623.46	543.29	588.54	596.10
	820.66	511.86	806.16	696.32
	539.59	842.38	487.72	627.53
	605.78	752.12	716.88	537.25
	781.38	772.42	847.86	526.58
	890.30	570.49	754.59	
	872.12	515.61	798.55	
	552.71	815.63	808.92	
	995.14	591.30	897.69	
	567.18	614.40	533.51	
		791.42	1233.04	
8	735.47	536.28	618.21	503.70
	971.04	619.36	620.36	569.47
	575.24	639.36	614.50	505.80
	606.35	595.76	626.96	578.72
	924.81	641.56	562.75	571.21
	815.65	626.03	513.54	591.24
	654.17	594.22	578.04	568.86
	651.92	899.96	547.59	586.66
	827.55	544.23	617.58	570.00
	949.66	585.50	627.71	595.85
	971.90	773.62	583.93	
	903.37	549.39	639.65	
	853.87	525.57	606.65	
	1177.70	557.11	586.96	
	680.90			
	650.83			
	661.21			
9	683.73	859.89	848.61	679.60
	590.86	599.32	718.41	590.90
	763.35	690.67	1027.70	598.10
	797.16	775.97	677.35	673.13
	868.87	789.93	1103.13	504.03
	791.47	535.28	714.80	561.36
	840.40	746.34	773.42	517.82
	784.54	614.07	625.47	
	840.04	628.05	840.84	
	638.16	743.27	636.94	
	668.05	623.56	830.48	
	848.71	837.36	744.21	
	653.35	601.59	672.04	
	671.73	781.98	770.76	
	829.66	640.03	711.88	

		737.57	763.56	
			577.10	
			855.57	
			585.36	
			610.36	
			589.69	
			738.87	
			590.97	

Table A.26: ANOVA Table for the measured length of the *Artemia* during the feeding trial.

Source	**SS**	**df**	**MS**	**F**	**P-value**
Between groups	673930	3	224643	15.05	3.8E-09
Within groups	4433332	297	14927		
Total	5107262	300			