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**Surface Hydration
and Swelling rate of Pellets
in Water made of Microalgae with
Enzymes and Lignosulfonates**

Ana-Maria CATARGIU

Preface

The effort, time and performed work during my entire student work have brought me in this culminate point of writing the present master thesis. The scientific work presented henceforth was conducted at the Department of Mathematical Science and Technology of Norwegian University of Life Science (NMBU), Ås. The financial support and guidance of my supervisor Carlos Salas-Bringas is highly acknowledged.

This research could not have been conducted without the big support and encouragement of several people whose contributions are sincerely appreciated. I would like to thank my supervisor, Carlos Salas-Bringas, for his help, guidance and support. He allowed me to develop my research and laboratory skills, and helped me understand numerous scientific and technical concepts. I am truly grateful to him. I want, as well, to express my honest appreciation towards great teachers and co-supervisors, PhD student Nevena Mišljenović and Dejan Miladinovic.

An article based on the data from this thesis has been accepted for publishing in the Annual Transactions of the Nordic Rheology Society (Salas-Bringas et al. 2015).

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Ana-Maria Catargiu

Abstract

Water quality, pollution, shrimp growth and survival and decreasing shrimp disease have always been looked at as challenges regarding shrimp feed. These issues are related and could be resolved by improving the pellet physical quality parameters. The compounded diet for shrimp therefore, should be well bound to withstand feeding manipulation and the challenges that come with it. In order to optimize feed intake and reduce wastage, artificial diets for shrimps need to be formulated and manufactured by taking into account species behavior, physiological requirements and the physical processes of ingestion.

Lignosulfonate is another additive that can be used as a means of incorporating fat-soluble nutrients into water-based foods. This can help in improving pellet physical qualities. The usefulness of commercial lignosulfonate products comes from their dispersing, binding, complexing agent, and emulsifying properties.

Enzymes are defined as catalysts that increase the speed of the processes in which they are involved. The addition of enzymes to aquatic feed has the potential of improving the nutritional value of the feed by reducing their loss to the environment due to the inactivation of anti-nutritional factors, therefore reducing production costs.

Microalgae are able to raise the nutritional content and value of conventional food preparations and hence, to affect the health of humans and animals in a positive way. This is due to their original chemical composition that has a very good comparable level of protein some common foods used daily in human nutrition. Microalgae could substitute fish meal at a very high level and with these helps the environment by being sustainable in its production.

The last part of the present thesis contains an experiment that had its main goal in determining how addition (in a dry matter basis) of lignosulfonate and enzyme (NSP and protease) influences the physical quality of microalgae pellets. The pellets were produced using a single pellet press method. Four experimental diets were formulated: one control diet with 100% microalgae (C) and other three with addition of Lignosulfonate (LS), non-starch polysaccharides (NSP) and protease to C diet in a percentage of 0.5, 0.01 and 0.006 respectively. Characterization of their effect on tensile strength (durability of pellets), the water absorption rate and underwater swelling rate is presented. The results showed that the additives protease and non-starch

polysaccharide enzyme (NSP) decreased the tensile strength of pellets when added in their respective percentages. Lignosulfonate additive (LS) did not change the tensile strength when added in 0.5% (dry basis). Protease, NSP and LS increased the water activity, decreased the swelling of pellets under water, increased hydrophobicity and lowered absorption rate of a sessile water drop sitting on the pellet surface. Protease produced the highest water activity, the lowest swelling of the pellets and the highest initial contact angle (i.e. the most hydrophobic) followed by LS and NSP. Pellets made of pure microalgae had the lowest water activity, the lowest hydrophobicity and the highest swelling and absorption rate of the sessile drop.

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Chapter I - Introduction

The alarming increase in population and their respective demands for more and better food has drawn researchers' attention to find new sources for food and to improve the available ones. The struggle is with finding new protein sources as supplements (Becker 2007).

Aquaculture industry is facing a higher global demand for aquaculture product that is not feasible with the fishery resources available today; this has posed aquaculture development a necessity for the industry (Hardy 2000; Safarian et al. 2013). Since feed represents the major operational cost involving 50 - 60 % of the total cost in intensive farming, sustainable aquaculture depends on environmental friendly feeds, no limiting factor in their production and for them to be economically viable.

Fish meal represents the principal protein source for carnivorous fish with a 30 – 50 % addition (Hardy 1996). Due to its shortage and high cost, fish meal is generally being avoided in the feed and new protein sources and enzymes supplementation are some of the solutions looked upon in today's aquaculture industry. One of this new protein sources is microalgae. This days, microalgae are known for their increase in nutritional value (through improving the chemical composition) of food and animal feed. Their numerous applications on the market, made them important to be used in aquaculture (Spolaore et al. 2006). A large variety of animals are able to accept microalgae in their feed, like poultry, pig, pets and aquatic animals (Gouveia et al. 2008). Microalgae are a very good biological resource, representing one of the most promising sources for new products and applications (Pulz & Gross 2004). Microalgae are considered to be an unconventional protein sources (Becker 2007) and a sustainable and renewable source (Dufossé et al. 2005).

Plant protein sources have, generally, a lower digestibility than fish meal and addition of enzymes can help by improving their digestibility and reduce the content of anti-nutritional factors which will increase the protein concentration (Thiessen et al. 2003; Venou et al. 2003) and the utilization of dietary energy and amino acids (Hardy 2000), that will result in a better performance of crustaceans (Farhangi & Carter 2007; Shimei et al. 2007; Soltan 2009). One of the reasons for which enzymes have been introduced in aquafeed is that their addition doesn't

affect the fishes/shrimps health or the pond environment, due to the fact that they are natural products of fermentation (Behera 2013).

Lignin is the second largest component of wood. Calcium lignosulfonate (40-65) is an amorphous light-yellow-brown powder obtained from the sulfite pulping of softwood (Toledo & Kuznesof). It acts like an emulsifier and stabilizer in the addition of encapsulated fat-soluble active ingredients into water-based foods. It been used as a carrier for fat-soluble vitamins (A, D, E and K), carotenoids (e.g. β -carotene, carotenal, β -apo-8', lutein, lycopene, etc) and other functional ingredients in, e.g., fruit-based beverages, vitamin drinks, dairy products, and hard candies to assure the uniform dispersal and distribution of water (Toledo & Kuznesof)

Quality control of aquafeeds is another important parameter to reach sustainable aquaculture. High volume of diet consumption, high potent of environmental pollution and nutrient leaching, makes the physical quality control of commercial diets for aquatic animals a major factor in aquafeed industry (Safarian et al. 2013). Ighwela et al (2014) suggested that water stability, composition density and supplementation are the most important physical characteristics of aquatic animal feeds. Therefore, it exists an immediate need for formulating suitable pelleted feeds for the respective animal, by incorporating cheap locally available materials with the right addition of enzymes for the best physical and nutritional quality control.

Along the time and during many experiments, water stability has been analyzed and defined through different methods. Until today, there hasn't been found a standardized methodology to define water stability of a pellet. In the present paper, a new method for this purpose is introduced: pellet swelling rate measurement through imagine analysis.

In order to achieve economical efficiency through a sustainable aquaculture, appropriate nutritional characteristics and physical properties of an optimal diet for the considered animal is a critical step to follow (Safarian et al. 2013). One way of resolving some of today's issues concerning shrimp feed would be the development of all plant based proteins. In the present paper, the physical properties of microalgae pellets will be discussed.

The quality of shrimp diets is determined not only by their nutritional make-up but also by their physical properties, especially water stability. Shrimp pellets should be physically stable to

minimize disintegration and loss of water-soluble nutrients upon exposure to water, and during the ingestion process (Lim & Cuzon, 1994).

WS can be improved with the use of binders; also can be greatly improved by proper selection of feed ingredients, processing techniques and the use of proper processing equipment (Lim & Cuzon, 1994).

Chapter II – Literature review

2.1. Challenges within the Pelleted Shrimp Feed

In shrimp cultures, feed represents the most expensive production cost (Michael 1976). The quantity and quality of diets are primary factors influencing shrimp growth, nitrogen loading of the culture system and disease proliferation. For these reasons there is an increase interest in researching sustainable feed ingredients for optimal growth (Cho et al. 1994; Ochoa-Solano & Olmos-Soto 2006).

Challenges regarding shrimp feed have always been referred to pond water quality and pollution, enhancing shrimp growth and survival and decreasing shrimp disease. All of these issues are related to pellet quality parameters. For finfish (trout, salmon, channel catfish, tilapia) the pellets need to be stable in the water for only few minutes, since they are fast feeders (Storebakken et al. 1999). Shrimp, on the other hand, due to its benthic nature and being a selective and slow continuous feeder, requires a fast sinking feed with a much higher degree of water stability (Farmanfarmaian et al. 1982; Lim & Cuzon 1994). Shrimps take the feed with their chelate pereopods and masticate it outside the buccal cavity prior to ingestion. The compounded diet for shrimp therefore, should be well bound to withstand feeding manipulation and the challenges that come with it (Ahamed Ali 1988; Lim & Cuzon 1994).

While extensive shrimp farming operations may continue to depend upon live or fresh natural feeds, large-scale, semi-intensive commercial farms require formulated feed pellets (Farmanfarmaian et al. 1982). The physical instability of feed pellets and “leaching out” of specific hydrosoluble nutrients are serious problems in crustacean cultures since most species of interest are demersal continuous feeders, and grasp feed pellets with pincer like appendages and masticate externally (Farmanfarmaian et al. 1982). Binders and/or binding methods should not interfere with the animals’ ability to digest the feed nor allow the nutritive constituents to be altered or destroyed.

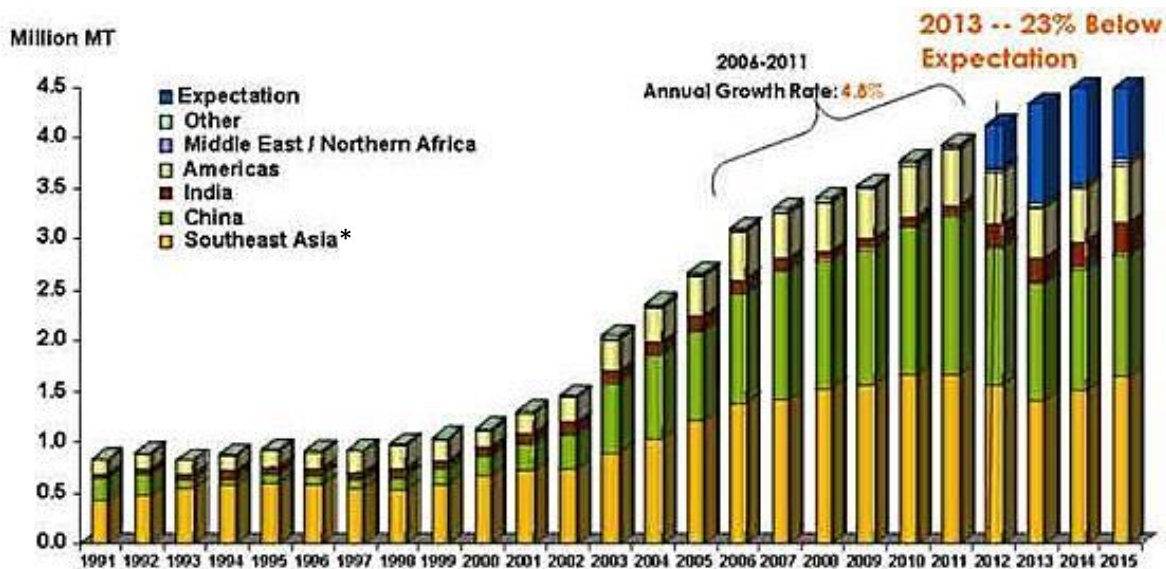


Figure 2.1. Global Production of Farmed Shrimp. EMS- Early Mortality Syndrome. FAO, 2013, for 1991 – 2011; GOAL (2013) for 2012 – 2015 (ShrimpNews 2013)

*Southeast Asia includes: Thailand, Vietnam, Indonesia, Bangladesh, Malaysia, Philippines, Myanmar and Taiwan.

After the severe loss in 2013 due to the Early Mortality Syndrome (EMS) outbreaks, the world shrimp production has started recover and is expected to reach the 2012’s level by 2016 (Anderson 2014). EMS is a recent disease of farmed Penaeid shrimp, also known by the name “acute hepatopancreatic necrosis disease” (AHPND) that was first reported in southern China in 2010 and subsequently in Vietnam, Thailand, and Malaysia (De Schryver et al. 2014). The EMS disease typically affects shrimp postlarvae within 20–30 days after stocking and frequently causes up to 100% mortality (De Schryver et al. 2014).

In 2013 (see fig. 2.1.), the global production of shrimp declined by about 15% from 2011 levels. Considering that the industry was expected to grow by about five percent a year, production was actually 23 percent below market expectations (ShrimpNews 2013).

2.1.1. Pond water quality and sustainability

When we talk about sustainable shrimp feed we have to take into account some aspects, like environmental concerns, over-fishing and ethical consideration regarding the use of fish products for aquatic feed when it can be used for direct human food (Davis et al. 2004).

Paez-Osuna (2001) has generated some factors that can generate environmental impacts of interest in shrimp farming:

1. Locations for shrimp pond construction;
2. Management and technology applied during the operation of shrimp ponds;
3. Size or scale of the production and the surface dedicated to it, and;
4. Capacity of the receiving waters.

Traditional intense shrimp culture uses open system with high water exchange that has a high negative effect on the pond water quality. This has brought an interest in developing an environmental friendly, closed or recirculating seawater systems with zero or nearly zero water discharges (Chuntapa et al. 2003). An explanation on how this systems can work has been described by Chuntapa et al. (2003): the organic nitrogen waste from uneaten feed and shrimp excretions decomposes into toxic inorganic nitrogen compounds, including ammonia (NH_3 or NH_4) and nitrite (NO_2); with aerobic conditions, ammonia and nitrite are converted into relatively nontoxic nitrate (NO_3), but high nitrate concentrations can stress shrimp.

A couple of studies have been published regarding pond water quality with the help of co-cultured algae (Chuntapa et al. 2003; Phang et al. 2000; Vilchez et al. 1997). Chuntapa et al. (2003) co-cultured a cyanobacterium (*Spirulina platensis*) with black tiger shrimp (*Penaeus monodon*) for water quality control with very good results. Biological nitrate removal using aerobic microalgae offers some advantage over anaerobic, microbial denitrification since both ammonia and nitrate nitrogen are immediately removed and the process is less complicated (Vilchez et al. 1997). Microalgae play a dominant role in stabilizing earthen pond water quality. However, there are disadvantages in using microalgae for this process. The algal cells are not that easy to remove from the culture system, which can allow nitrogen compounds to be released back into the water, and high concentration of microalgae can cause dissolved oxygen depletions during the night due to high respiration rates (Phang et al. 2000). *Spirulina* is one of the most widely studied microalgae, especially for wastewater treatment (Phang et al. 2000).

The impact of shrimp farming of most concern is the destruction of mangroves and salt marshes for pond construction. Compatibility with other users, the presence of buffer zones, maintaining an acceptable balance between mangroves and shrimp pond area, improved pond design, reduction of water exchange, and an improved residence time of water, size and capacity to

assimilate effluents of the water body, are examples of ways to mitigate the adverse effects. Healthy seed supply, good feed with the use of prophylactic agents (including probiotics), good water quality, and lower stocking densities are examples of actions suggested to control disease in shrimp farming (Paez-Osuna 2001).

Successful and sustainable aquaculture depends on economically viable and environmental friendly feeds.

2.1.2. Enhancing shrimp growth and survival / Decreasing shrimp disease

Diseases represent the biggest obstacle to the future of shrimp farming. Farms and hatcheries are susceptible to the invasion of protozoa, fungi, and bacteria, but viral diseases cause the greatest losses. As discussed in the beginning of this chapter, EMS has decreased the global production of shrimp in 2013 by about 15% from 2011 levels.

Healthy seed supply, good feed supplemented with the use of prophylactic agents, including probiotics (Primavera 1998), good water quality, and lower stocking densities are examples of actions suggested to attain disease control in shrimp farming (Paez-Osuna 2001).

Application of enzymes may be a solution to high larval mortality in aquatic animals. The intestinal tract of aquatic animal larvae is shorter and relatively undeveloped when compared to that of the adults. Behera (2013) affirms that larval feeding would benefit from enzyme application.

Growth and survival are mainly influenced by low oxygen tension, the water source or by the presence of phytoplankton, so by the quality of the pond water quality. Survival is rarely being associated with the quality of feed, poor feed will not harm the shrimp (Cuzon et al. 1994).

Ju et al. (2009) affirm that adding a whole diatom or Nanno biomass of microalgae to a control diet can significantly improve shrimp growth and survival. However, this might not be due to the microalgae contribution in macronutrients (protein, lipid, and energy), but may be a result of the presence of a growth factor in the algae.

However, increasing the use of vegetable ingredients may reduce the digestibility of the diet and therefore decrease the food efficiency. For these reasons, it is going to be necessary to use additives to improve the digestibility of the new ingredients and to convert these diets to an effective alternative in shrimp cultures (Ochoa-Solano & Olmos-Soto 2006).

2.1.3. Nutritional requirements

Most of the finfish and crustaceans species are either omnivorous or carnivorous species with high quality protein demand and special organoleptic properties of the feed (Davis et al. 2004). Fish meal fulfills most of the nutritional properties required for aquatic animals; this has made it one of the primary components of commercial feed formulation increasing its market value to such degree that nowadays new replacements are researched.

According to Davis et al. (2004) an all plant protein base diet will not fulfill all the nutritional need of the shrimp, as all the essential amino acids, lower level of minerals and highly unsaturated fatty acids. It can also provide anti-nutritional factors and a decrease in the palatability. In his experiment, Davis et al. (2004), has arrived at the conclusion that, both fish meal and marine oil sources could be removed from shrimp's diet with the condition that the substituent should be suitable enough to reach the amount of amino acids and fatty acids required by the shrimp. Nowadays, the microalgae biomass is broadly extracted, to obtain fatty acids, pigments, and other compounds (Guill-Guerrero et al. 2004). Even though microalgae, depending on the species, is rich in its chemical composition and has a comparative amino acid content with other animal based proteins, it is suggested to be used as a partial substitute for fish feed or other animal proteins, when fed to shrimps.

Microalgae are rich sources of vitamins, essential amino acids, minerals, essential fatty acids, and carotenoid pigments for aquatic animals (Takeuchi et al. 2002). Microalgae biomass was also tested as a protein or lipid source to partially replace fish meals or fish oil in fish feeds (Belay et al. 1996; Olvera-Novoa et al. 1998; Patnaik et al. 2006). The addition of whole algae also increased fatty acid contents in shrimp tails. The biomass of the algae samples was rich in carotenoids, and led to higher astaxanthin content and enhanced pigmentation in shrimp tail muscle samples (Ju et al. 2009).

In a study conducted by Patnaik et al. (2006) the complete replacement of fish meal and fish oil using plant protein and non-marine oil sources was researched. The experiment did not show apparent reduction in growth of Pacific white shrimp *L. vannamei* under the present diet conditions. In addition, the algal oil showed suitable characteristics as a fish oil substitute (HUFA source) in the practical diets for *L. vannamei*.

The protein requirement of Penaeid shrimps is an important nutritional consideration because protein is a major limiting nutrient for growth (Kureshy & Davis 2002) and expensive component of shrimp feeds (Shiau 1998). In general, fish feed contains 5–50% fishmeal, while shrimp feed contains a fishmeal level of between 30% and 50% (Dersjant-Li 2002). Fishmeal protein content can affect water quality and as a consequence the proliferation of diseases. Protein, assimilated for energy and not deposited for growth, contributes to the release of nitrogen metabolites into the culture medium (Cho et al. 1994).

2.1.4. Physical quality of the pellets

Pelleting process is used for improving feed nutrient utilization, for meeting customer expectations and for delivering pellets with improved physical quality when compared with mash meal (Behnke, 2001).

Pelleted feed has numerous advantages like, pellets have better flow properties, necessary for good transport in conveying equipment, and (gravitational) discharging behavior from silos, the bulk density of pellets is generally higher than that of meal, so that more tonnage can be carried by truck, the composition of the pellets as obtained from carefully blending and mixing ingredients remains fixed, no segregation of e.g. additives occurs.

Pellet quality can be measured through several techniques that can measure water activity (a_w), hardness and durability of pellets, water absorption rate and water stability by either disintegration rate or swelling rate in water.

More detailed information in the subject will be described in subchapter 4.1.

2.1.5. Economical issues

Due to an increase of the cost in the marine animal protein sources, such as fish meal, and the everyday concern about the environment issues that can be affected by this, the shrimp feed research has been focused on developing new protein sources (Amaya et al. 2007), that can substitute fish meal to a certain level without compromising the animal health or nutritional requirements and, alternatively providing a less expensive protein source.

According to FAO (2012), shrimps and prawns production has more than doubled from 2003 until 2012, followed by a similar increase in their respective cost. In 2012, marine shrimp were

the second most important world aquaculture species with a value of US\$ 19,4 billion (FAO, 2012).

In a study conducted by Amaya et al. (2007), an experimental treatment of four diets containing different percentages of fish meal (9, 6, 3 and 0%) in combination with poultry by-product (16%), a plant based feed (solvent extracted soybean meal, corn gluten and corn fermented soluble) containing 1% squid meal, and a commercial reference feed have been evaluated. The results have been evaluated on: mean final weight, percent weight gain, final net yield, feed conversion ratio and survival rate. There were no significant differences ($p \geq 0.05$) between the diets, which can drive us to the conclusion that plant base protein can be a good substitute for fish meal.

Use of plant based proteins as a good replacement (Becker 2007; Davis et al. 2004; Hardy 1996). They are produced in larger quantities, often less costly, they are not considered to be limited. Use of algae as a protein source supplement for aquatic feed (Appler & Jauncey 1983).

Crustacean penaeids adapt quite well to changes in diet composition by the induction of digestive enzymes synthesized and secreted in the hepatopancreas (Le Moullac et al. 1997). A better understanding of feeding preferences and the use of feed by shrimp is essential to optimize the use of nutrients and to reduce environmental pollution that originates from metabolite excretion and from uneaten artificial feed. A net waste of nutrients due to excessive feeding also represents an economic loss for the aquaculturist, as feed is the main variable cost and can represent up to 60% of the total costs in penaeid shrimp culture (Gamboa - Delgado et al. 2003). In addition, studies to examine the relationships between nutrition, diseases and the environmental factors should be given more attention.

In order to optimize feed intake and reduce wastage, artificial diets for shrimps need to be formulated and manufactured by taking into account species behavior, physiological requirements and the physical processes of ingestion.

2.2. Functional Properties of Lignosulfonates

Lignosulfonate is the main component in the liquid waste from chemical pulp mills (Ouyang et al. 2006). After cellulose, lignin is the most abundant organic polymer in the plant world whose content depends on plant species (roughly 15–30%) (El Mansouri & Salvadó 2006). Lignin in sulfonated form has been available as an industrial raw material since 1886, but purified lignosulfonates have been commercially available since the early 1930s (Browning 1975). It is estimated that there are more than 50 million tons of industrial lignin (lignosulfonate and alkali lignin) produced every year worldwide (Xiao et al. 2001), but only 10% of them are utilized, the rest being dumped as a waste and making it an expensive item (Browning 1975).

Lignin and lignosulfonates are recognized feedstocks for the manufacture of low molecular weight aromatic chemicals (Browning 1975). Their usefulness in the industry is mainly due to their colloidal properties.

2.2.1. Generalities – types, description, manufacturing process

Lignosulfonates are unique and multifunctional macromolecular polyelectrolytes. Commercial lignin is divided into two categories, the first category consists in conventional or sulfur containing lignin, which include Kraft lignin and lignosulfonates. These products have been available for many years. Lignosulfonate can be found in large quantities (around 1 million tons of solids per year) (Gosselink et al. 2004). Conventional lignin used industrially is mainly obtained from softwoods.

There are four types of lignin (table 2.1.):

- Lignosulfonate lignin
- Kraft lignin
- Organosolv lignin
- Soda lignin (Laurichesse & Avérous 2013)

Table 2.1. Properties of technical lignin (Laurichesse & Avérous 2013)

Lignin type	Sulfur-lignin			Sulfur-free lignin	
	<i>Kraft</i>	<i>Lignosulfonate</i>	<i>Soda</i>	<i>Organosolv</i>	
Raw materials	Softwood Hardwood	Softwood Hardwood	Annual plants	Softwood Hardwood	Annual plants
Solubility	Alkali	Water	Alkali	Wide range of organic solvents	

Lignosulfonates are commercially available as sodium and calcium salts and have been used by industry in a wide variety of applications. The usefulness of commercial lignosulfonate products comes from their dispersing, binding, complexing agent, and emulsifying properties (Toledo & Kuznesof).

Technological background of how calcium lignosulfonate (40-65) is functioning - These particles are produced by spray drying or powder catch technologies. Calcium lignosulfonates (40-65) role of emulsifier allows it to stabilize oil soluble /dispersible nutrients in water. In the moment

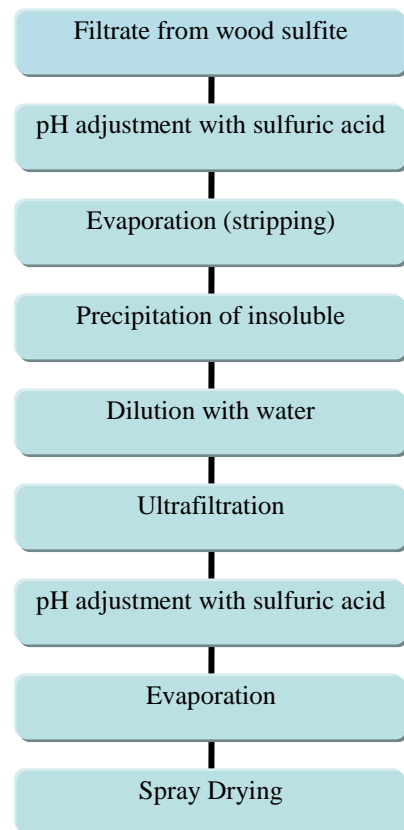


Fig.2.2. Process flow diagram of the Manufacturing process of LS (Toledo & Kuznesof)

when the fixed oil droplets are added to aqueous solutions they start dissolving while the oil droplets still have a coating of calcium lignosulfonate (40-65). The calcium lignosulfonate (40-65) forms an emulsion between the two immiscible phases (oil and water) (Statement 2011)

Manufacturing process (fig.2.2.)

Calcium lignosulfonate (40-65) is produced from softwood in the sulfite pulping method for manufacturing paper. The pH of the filtrate is adjusted by addition of concentrated sulfuric acid. After the first evaporation step, the filtrate may be diluted with water before it is send for further purification by ultrafiltration at moderately high temperatures. Ultrafiltration is a liquid/liquid separation method where the filtrate is separated by molecular size through a semi-permeable membrane.

The purified calcium lignosulfonate (40-65) from ultrafiltration of dilute sulfuric acid. This solution is then evaporated at a dry-matter content that is appropriate for spray-drying. The final

product is spray-dried to a moisture content in accordance with the specification for Loss on drying and filled into containers suitable for holding food (Toledo & Kuznesof)

2.2.2. Chemical characterization

Lignin is the second largest component of wood. The basic units of the polymeric structure consist of three aromatic propenyl alcohols: coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol (Toledo & Kuznesof). Impurities in calcium lignosulfonate (40-65) include monosaccharides from wood cellulose, sulfite residues, and potentially arsenic and various metallic elements. Filtration, evaporation and ultrafiltration have the role of reducing the content in the final product to not more than 5 % reducing sugars and not more than 0.5 % sulfite (Toledo & Kuznesof).

LS has a C9 formula (fig. 2.2.): $C_9H_{11.18}O_{4.94}N_{0.003}S_{0.40}(OCH_3)_{0.73}$ with a double bond equivalent (DBE) of 3.79, molecular weight 220.89 g/mol and a protein content of 0.12% (El Mansouri & Salvadó 2006).

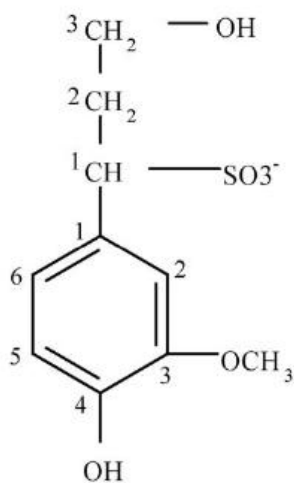


Fig. 2.3. Typical structural unit of lignosulfonate (El Mansouri & Salvadó 2006)

Data from a suitable animal model show that the use of calcium lignosulfonate (40-65) to incorporate fat-soluble nutrients is likely to be positively comparable in the gastrointestinal absorption of these nutrients with the use of another common substance like gelatin (Statement 2011). There is also indirect evidence suggesting that it also allows for normal digestion and absorption (Statement 2011). FSANZ (*Food Standards Australia New Zealand*) concludes that the use of calcium

lignosulfonate (40-65) as a means of incorporating fat-soluble nutrients into water-based foods is unlikely to result in any adverse nutritional outcomes (Statement 2011). For encapsulated nutrients, in case a barrier against oxygen is needed, gelatin is preferred. However, gelatin is of animal origin, and this imposes some impediments regarding kosher/halal, BSE and allergens. LS is of plant origin and can be used in replacing gelatin (Toledo & Kuznesof).

Lignosulfonate is an anionic surfactant, possessing a certain degree of surface activity with properties like promoting surface adsorption, foaming and further particle dispersion. It may be used in various industrial processes, like auxiliary substance for paper coating, binder, additive in oil drilling, and in concrete preparation. It is well known that surface activity and foaming capability of the water reducer will have a positive effect on solid particles dispersing into liquid (Ouyang et al. 2006).

Purified LS are a more effective form of LS that are substantially freed from wood carbohydrates. Such carbohydrates not only dilute the lignosulfonate, they also inhibit desired surface active properties (Browning 1975).

2.2.3. Colloidal properties

For a better and more useful application of LS in research and market development, is detrimental to understand how this properties act (Browning 1975). Purified lignosulfonates are predominately used in the domain of colloid science. Colloid science may be defined as “the science of large molecules, small particles, surfaces, and the forces that govern their interactions” (Browning 1975).

A few limited investigations have been published concerning the effect of lignosulfonates upon the stability and rheology of suspensions as related to adsorption and molecular weight (Ernsberger & France 1945).

Emulsification

As a conclusion in a colloid research at Rothschild (Browning 1975), it has been found that lignosulfonates were exceptional oil-in-water emulsion stabilizers. They were capable of stabilizing emulsions in saturated salt solutions, and were improved when subjected to shear and stable to centrifugation.

Other colloidal studies revealed that lignosulfonates could adsorb so strongly upon some surfaces that a three dimensional protective layer could be formed. These principles were developed into microencapsulation techniques in which free-flowing powders of liquid-in-solid or solid-in-solid dispersions could be prepared with lignosulfonates as the continuous phase. These encapsulation techniques were employed to prepare powders which when introduced into water formed "instant" emulsions of liquids, or dispersions of low melting solids (Browning 1975).

Commercial application of this microencapsulation technique can be found in the areas of pesticides, agriculture, mariculture, and construction, is an intriguing potential volume market (Browning 1975).

Deflocculation

The ability to disperse (deflocculate) or effect the colloidal stabilization of suspensions of solids is a property most commonly associated with commercial lignosulfonates. The commercial use of lignosulfonates in gypsum wallboard, kiln feeds, pesticides, industrial cleaners, ceramics, and oil well drilling fluids, all involve the ability of lignosulfonates to act as deflocculants in the presence of contaminating electrolytes (Browning 1975). Using lignosulfonates, stabilized suspensions of high solids concentration and low viscosity may be prepared, even in the presence of contaminating electrolytes (Browning 1975).

Chelation

The chelating properties of lignosulfonates have been used to facilitate the crosslinking of polysaccharides in nonclay type of drilling fluids. It appears that the chemistry of lignosulfonate coordination compounds is an important facet of lignosulfonate utilization and affords considerable development potential.

Adsorption

The industrial uses of lignosulfonates in many applications including emulsion stabilization, deflocculation, adhesives and water treating (boiler scale control) is fundamentally dependent upon adsorption (Browning 1975). The structures present in lignosulfonates are also capable of strong adsorption at some solid-liquid interfaces by forming surface complexes with the solid. Strong adsorption bonding by lignosulfonates and their ability to coordinate with some inorganic crystal lattices act to modify crystal habit during crystallization from solution. This property is used industrially in a number of applications including water treatment and boiler scale control. Experimental evidence indicates that lignosulfonate adsorption may be affected by one or more of the following: nonpolar Van der Waals' attraction, hydrogen bonding, covalent bonding or ion exchange.

Lignosulfonates behave in a manner common to polyelectrolytes. Higher molecular weight polymers are, in general, less soluble than low molecular weight polymers and thus tend to be more highly adsorbed.

FSANZ concludes that both the food additive technological functions of emulsifier and stabilizer are appropriate as the functions performed by calcium lignosulfonate (40-65) (Statement 2011). The additive calcium lignosulfonate has been used for a number of years in the food industry, serving, for example, as an emulsifier in animal feed, as raw material in the production of vanillin, and as a boiler water additive (Statement 2011). Lignin and lignosulfonates have demonstrated their commercial value as chemical feedstock and their value in industry as functional chemicals. Applied research has proposed commercial utilizations in thousands of patents and thousands of technical articles, most of them still in the research stage. It should be concluded that utilization efforts should be oriented toward applications in which the unique properties of lignosulfonates are emphasized.

The principal use of calcium LS is as a carrier in the production of encapsulated fat-soluble vitamins, carotenoids for their introduction into water-based foods. They can replace other additives, as gelatins, gum Arabic, soya protein hydrolysates and modified starches.

2.3. Functional Properties of Protease and NSP enzymes

Fish meal and other animal meals are progressively being replaced by plant meals for reasons of both cost and sustainability. The addition of protease results in a better amino acids digestibility, minimizing the anti-nutritional factors and can be utilized with a wide range of protein sources. The addition of carbohydrate degrading enzymes to the diet helps release energy to the fish and enhances its energy utilization.

Enzymes are defined as catalysts that increase the speed of the processes in which they are involved (e.g. the degradation of complex feed ingredients to digestible nutrients). The addition of enzymes to aquatic feed has the potential of improving the nutritional value of the feed by reducing their loss to the environment due to the inactivation of anti-nutritional factors, therefore reducing production costs (Behera 2013; Buchanan et al. 1997).

The importance of digestive enzyme analysis in the study of nutrition and composition of feed lies in different characteristics: the animal's dietary regimen, its ability to specifically hydrolyze certain nutrients from the diet, bacterial contribution to digestion, cyclic secretion and the changes that occur while the animal grows and matures (Lee et al. 1984).

According to Behera (2013) Enzymes are utilized due to a series of factors: increase need for quality food grain, animal products or by-products for fish/shrimp, researching new sources of food with better nutritive value, economic reasons (reduced cost : benefit cost) and the environmental awareness regarding sustainable aquaculture.

Feed enzymes, when added in a diet, have certain actions and benefits (Behera 2013): reduces in digesta viscosity, enhances digestion and absorption of nutrients especially fat & protein, improves Apparent Metabolizable Energy (AME) value of the diet, increases feed intake, weight gain and feed gain ratio, reduces ammonia production for a better environment, improves nutrient digestibility. The efficacy of the enzymes would also differ with the physiochemical conditions of the intestine and in particularly with the pH, which could differ from one fish species to another. In addition, the enzyme should also be resistant to intestinal proteases and should be active at the target site of action.

Types of Enzymes available for fishes / shrimps: *cellulose* , (*β -glucanases*), *xylanases* and associated enzymes like; *phytase*, *proteases*, *lipases* and *galactosidases*.

2.3.1. Proteases

A protease (also called *peptidase* or *proteinase*) is any enzyme that performs proteolysis, which refers to protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Different classes of protease can perform the same reaction by completely different catalytic mechanisms. Proteases can be found in animals, plants, bacteria, archaea and viruses (Barrett et al. 2012).

The use of proteases to improve protein digestibility has been extensively studied in feed industry. There are few studies on enzyme supplementation in diets for fish (Drew et al. 2005; Shimei et al. 2007).

Proteases are currently classified into six broad groups:

- Serine proteases - using a serine alcohol
- Threonine proteases - using a threonine secondary alcohol
- Cysteine proteases - using a cysteine thiol
- Aspartate proteases - using an aspartate carboxylic acid
- Glutamic acid proteases - using a glutamate carboxylic acid
- Metalloproteases - using a metal, usually zinc

Alternatively, proteases may be classified by the optimal pH in which they are active: acid proteases, neutral proteases (involved in type 1 hypersensitivity, includes the calpains) and basic proteases (or alkaline proteases).

Function and mechanism

Proteases are involved in digesting long protein chains into shorter fragments by splitting the peptide bonds that link amino acid residues. Depending on their function, they can be divided into two groups: exopeptidases (e.g. aminopeptidases, carboxypeptidase A) proteases that detach the terminal amino acids from the protein chain, and endopeptidases (e.g. trypsin, chymotrypsin, pepsin, papain, elastase) that can attack the internal peptide bonds of the protein.

Proteases can either break specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*).

There are 2 steps mechanisms that proteases are using: in the 1-step the hydrolysis is obtained by the enzyme using an acid to polarize water which then hydrolyses the substrate, the 2-step

hydrolysis is when a residue within the enzyme is activated to act as a nucleophile (Nu) and attacks the substrate. This forms an intermediate where the enzyme is covalently linked to the N-terminal half of the substrate, water is then activated to hydrolyze this intermediate and complete catalysis.

Catalysis is achieved by one of two mechanisms using different catalysts for the reactions:

1. Aspartic, glutamic and metallo proteases activate a water molecule which performs a nucleophilic attack on the peptide bond to hydrolyze it.
2. Serine, threonine and cysteine proteases use a nucleophilic residue in a (usually in a catalytic triad). That residue performs a nucleophilic attack to covalently link the protease to the substrate protein, releasing the first half of the product. This covalent acyl-enzyme intermediate is then hydrolyzed by activated water to complete catalysis by releasing the second half of the product and regenerating the free enzyme.

Autolysis refers to the reaction between proteases and other protease molecules, sometimes of the same variety, as a method of regulation of protease activity. Some proteases are less active after autolysis (e.g. TEV protease) whilst others are more active (e.g. trypsinogen). **Degradation** is the activity of the autolysis that can be a destructive change by eliminating a protein's function or digesting it to its principal components. It can be an activation of a function, or it can be a signal in a signaling pathway. By this interaction of proteases and proteins, the lifetime of other proteins with important physiological (hormones, antibodies) can be changed. This is one of the fastest "switching on" and "switching off" regulatory mechanisms in the physiology of an organism. Proteases are used in industry, medicine and as a basic biological research tool (Feijoo-Siota & Villa 2011; Thomas et al. 2006).

The activity of proteases is inhibited by **protease inhibitors** (Southan 2001). One example of protease inhibitors is the serpin superfamily, which includes alpha 1-antitrypsin, C1-inhibitor, antithrombin, alpha 1-antichymotrypsin, plasminogen activator inhibitor-1, and neuroserpin (Puente & López-Otín 2004). Natural protease inhibitors include the family of lipocalin proteins, which play a role in cell regulation and differentiation. Some natural protease inhibitors are used as defense mechanisms. The most common example of trypsin inhibitors are the ones found in the seeds of some plants like soybeans. Raw soybean is toxic to many animals, including humans. After the protease inhibitors have been denaturized, soybean can be consumed.

Although there are few reports on the effects of exogenous enzyme supplementation in diets for fish, a number of studies on the use of proteases to improve the digestibility of feed ingredients in poultry, pigs and cattle have been published (Shimei et al. 2007). In a study conducted by Shimei et al. (2007), the results on growth and feed utilization of tilapia, indicated that crude protein, energy and lipid and DM digestibility were improved by protease supplementation in plant-based diets.

2.3.2. Non Starch Polysaccharides (NSPs)

Non starch polysaccharides (NSPs) include all the plant polysaccharides other than starch which contains up to several hundred thousand monosaccharide units, joined through glycosidic linkages. They are the key components of the cell walls of various grains and cover a great variety of biological functions and chemical structures (Cummings & Stephen 2007; Kumar et al. 2012; Sinha et al. 2011). One of the main constraints in the utilization of plant ingredients in aquaculture is the presence of indigestible carbohydrates, which consist primarily of non-starch polysaccharides (NSPs), they can comprise up to 90% of the cell wall of plants (Kumar et al. 2012). These form a part of the cell wall structure of cereals and legumes. The presence of NSPs in the diet interferes with feed utilization and adversely affects performance of the animal. Supplementation of NSP-degrading enzymes in feed mitigates the adverse effects of NSPs. The effects of NSPs in pigs and poultry have been widely studied; however little information exists for fish (Sinha et al. 2011).

The antinutritive effect of NSP for monogastric animals is well defined in the literature (Bakker et al. 1998; Farhangi & Carter 2007) and mostly attributed to the soluble fraction (Choct 1997). The viscous properties may impair the diffusion and transport of lipase, oils and bile salt micelles (Farhangi & Carter 2007) and inhibit the diffusion of both digestive enzymes and nutrients (Farhangi & Carter 2007). High viscosity increases the weight of digestive tract, resulting in higher protein synthesis of gut tissue and increased energy expenditure for maintenance (Bakker et al. 1998).

The main limitation with plant-derived materials, such as legume seeds, soybean meal, different types of oilseed cake, canola (rapeseed) meal, sunflower oil cake, root tuber meal, is the presence of a wide range of anti-nutritional factors, such as protease inhibitors, non-digestible

carbohydrates, lectins, saponins, phytates and possibly allergenic storage proteins (Francis et al. 2001). In addition to these factors that hamper digestion in fish, non-starch polysaccharides (NSPs) play an important role.

However, in fish and other monogastric animals enzymes such as β -glucanases or β -xylanases that digest NSPs are scarce or nonexistent (Kuz'mina 1996). Consequently, the dietary NSPs remain indigestible and cannot be used as an energy source. NSPs are also thought to be responsible for a slower rate of gastro-intestinal passage of NSP-containing diets in fish (Storebakken et al. 1999). Many authors have demonstrated that marine shrimps have severe restrictions for the utilization of dietary CBH (Le Chevalier et al. 2000; Shiau & Peng 1992). Furthermore, the inability of the shrimp's α -1,4-glucosidase to cleave the α -1,6-bonds of amylopectin exhibits assimilation of starch, resulting in low growth rates (Arellano-Carbajal & Olmos-Soto 2002; Le Chevalier et al. 2000).

NSP vs Starch - NSPs differ from starch not only in the type of monomers present but also by the number and type of monomeric units linked together, the order in the chain and the types of linkages between the various monomers. Starch is composed entirely of glucose monomers, which are linked by α -glycosidic bonds while NSPs are composed of different kinds of monomers, which are linked predominantly by β -glycosidic bond.

Classification

The term NSP covers a large variety of polysaccharide molecules, excluding starch (Sinha et al. 2011). Bailey & Hunt (1973) proposed a classification of NSP into three main groups, namely cellulose, non-cellulosic polymers and pectic polysaccharides. Arabinoxylans, mixed-linked β -glucans, mannans, and xyloglucan come under the category of non-cellulosic polymers while polygalacturonic acids substituted with arabinan, galactan and arabinogalactan are included in the group of pectic polysaccharides (Asp et al. 1983).

NSPs can be *classified* into various groups based on their physicochemical properties, for example, viscosity, water-holding capacity, fermentation, and the capacity to bind organic and inorganic molecules. Moreover, based on the reaction with water, NSPs are classified as either soluble or insoluble.

- Soluble NSPs form dispersions when mixed with water and have the ability to increase the viscosity of digesta which slows down the diffusion of digestive enzymes and the absorption of nutrients.
- Insoluble NSPs do not enhance digesta viscosity, but can be characterized by their fecal-bulking capacity (Davidson & McDonald 1998).

Cellulose is insoluble whereas the other types of NSPs are soluble or partly soluble.

Table 2.2. Non-starch polysaccharide degrading enzymes in animal feed and their impact (Sinha et al. 2011)

Enzyme	Effect	Impact
Cellulases and hemicellulases	<ul style="list-style-type: none"> • Partial hydrolysis of lignocellulosic materials • Hydrolysis of β-glucans • Decrease in intestinal viscosity • Better emulsification and flexibility of feed materials 	<ul style="list-style-type: none"> ○ Improvement in nutritional quality of animal feed ○ Improvement in performance of ruminants and monogastrics
β-Glucanase and xylanase	<ul style="list-style-type: none"> • Hydrolysis of cereal β-glucans and arabinoxylans • Decrease in intestinal viscosity • Release of nutrients from grains 	<ul style="list-style-type: none"> ○ Improvement in feed digestion and absorption, and in weight gain of broiler chickens and hens
Hemicellulase with high xylanase activity Cellulases, hemicellulases and pectinases	<ul style="list-style-type: none"> • Increase in nutritive quality of pig feeds • Partial hydrolysis of plant cell wall during silage and fodder preservation 	<ul style="list-style-type: none"> ○ Reduction in the cost of pig feeds ○ Reduction in the cost of feeds for pigs ○ Contribution to production and preservation of high quality fodder for ruminants ○ Improvement in quality of grass silage

Function and mechanism / Properties of NSPs

The functional properties of NSPs include their ability to bind water, provide bulk to gut content, production of SCFAs by fermentation, be used in food products as fat replacers, dietary fiber, or prebiotics. Plant ingredients generally contain a mixture of both soluble and insoluble NSPs in a ratio that varies according to the type and stage of maturity of grains. NSPs have a high water binding capacity (Davidson & McDonald 1998) thereby playing a crucial role for the correct functioning of the digestive system.

Viscosity - Highly viscous NSPs have a low degree of branching and high ferulic acid content. However, viscosity is not specific to the sugar composition or linkage types present in the NSPs. Supplementation of NSPs in the diet of monogastric animals such as pigs and poultry have been shown to have an anti-nutritive effect. The adverse effect of NSPs in monogastric animals have

been attributed to the increased viscosity of gut contents. NSPs induced digesta viscosity is of prime concern in the animal feed industry (Kumar et al. 2012).

Water-Holding Capacity - The water-holding capacity of NSPs is the ability to incorporate water within their matrix which is influenced by the chemical structure, pH, and electrolyte concentration of the surrounding fluid, and by particle size (Knudsen 2001). Both soluble and insoluble NSPs have high water-holding capacities but insoluble NSPs are less well fermented, and therefore stimulate fecal bulking and shorten gut transit times (Davidson & McDonald 1998), whereas soluble NSPs may contribute to slow gastric emptying. Water binding capacity also permits NSPs to act as hydrocolloids influence the rheology in aqueous systems.

Fermentability - The colon of human beings is characterized by the presence of a large and diverse population of anaerobic bacteria that can ferment NSPs resulting in the formation of a variety of end-products including SCFAs (C2-C5 organic acids), gases (methane, hydrogen, carbon dioxide), as well as an increased bacterial mass. The water soluble NSPs could lead to complete fermentation while water insoluble such as cellulose results in partial fermentation (Kumar et al. 2012).

Application in edible films and coating - In recent years, NSPs are receiving much attention as coating materials for food protection and therefore, can prevent deterioration of products and maintain their sensory quality and safety. In general the NSPs which disperse or dissolve in water give a thickening or viscosity-building effect (Kumar et al. 2012) and form edible film which, unlike the coating, are freestanding structures, first formed and then applied to foods. Certain polysaccharide films may provide effective protection against surface browning, and oxidation of lipids and other food components (Nisperos-Carriedo et al. 1991).

Non-starch polysaccharides in fish feed. The NSPs in aquaculture feeds are present as an integrated part of the cell wall of plant ingredients and also in a purified soluble form, such as guar gum, to stabilize the pellet.

Fish in general have a limited capacity for carbohydrate utilization and processing methods, such as gelatinization, have been reported to improve the nutrient bioavailability to the fish. Gelatinization is a thermal modification of raw dietary carbohydrates. During this process carbohydrate granules are modified in such a way that their susceptibility to enzymatic action increases (Kumar et al. 2006), making digestion more complete. The plant tissues containing

starch are expected to contain NSPs, which could hinder the effect of processing on starch digestibility. Better understanding of the effects of different NSP types on gelatinization would aid in the efficient utilization of plant carbohydrates in fish nutrition.

In aquaculture the use of plant-based protein is increasing at a fast pace due to its relatively low cost and ample availability. Consequently, the use of non-starch polysaccharides (NSPs), a class of anti-nutrients present in plant-based diets, will increase in the future. Currently there is relatively little information on the effects of dietary NSP on fish nutrition and physiology. Non-starch polysaccharides, with their high water-holding capacity can affect digesta viscosity in fish; therefore the addition of NSP-degrading enzymes (NSPases) in diets containing plant sources could play a vital role in improving nutrient utilization in fish.

2.4. Functional Properties of Microalgae – Overview

The term SCP (Single Cell Protein) is being used for microalgae, but on the affirmation of Becker (2007) this is not entirely correct since microalgal material means much more than just protein, it's a rich material containing peptides, carbohydrates, lipids, vitamins, pigments, minerals and other valuable trace elements.

Single cell proteins, as a general definition, represents a group of microorganisms that includes unicellular algae, fungi, bacteria, cyanobacteria, and yeast. SCP are easy and cheap to produce for their use as natural fish food (El-Sayed 1999).

The most commonly mass cultured algae evaluated as fish diet protein sources are the unicellular microalgae *Chlorella*, *Scenedesmus* and *Spirulina* (Appler & Jauncey 1983).

2.4.1. Chemical composition – microalgal biomass profiles

Microalgae are able to raise the nutritional content and value of conventional food preparations and hence, to affect the health of humans and animals in a positive way. This is due to their original chemical composition (Gouveia et al. 2008).

Table 2.3. General composition of different human food sources and algae (% of dry matter) (Becker 2004)

Commodity	Protein	Carbohydrate	Lipid
Bakers' yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
<i>Anabaena cylindrica</i>	43-56	25-30	4-7
<i>Chlamydomonas reinhardtii</i>	48	17	21
<i>Chlorella vulgaris</i>	51-58	12-17	14-22
<i>Dunaliella salina</i>	57	32	6
<i>Porphyridium cruentum</i>	28-39	40-57	9-14
<i>Scenedesmus obliquus</i>	50-56	10-17	12-14
<i>Spirulina maxima</i>	60-71	13-16	6-7
<i>Synechococcus sp.</i>	63	15	11

The figures presented are estimates, since the composition of individual cells depends on environmental parameters (Becker 2007)

From the algae listed in Table 2.3., only a few of them have been selected for large-scale production: chlorophyceae *Chlorella sp.* and *Scenedesmus obliquus* and the cyanobacteria *Spirulina sp.* and *Athrospira sp. Chlorella* (Becker 2007). Table 1 shows a very good

comparable level in protein of the microalgae with some common foods used daily in human nutrition.

Proteins, Peptides and Amino Acids

Most of the figures published in the literature are based on crude protein. CP is obtained by hydrolysis of the algal biomass and estimation of the total nitrogen. The calculation results in an overestimation of the true protein content since nitrogen is found in other constituents of microalgae: nucleic acids, amines, glucosamides, cell wall materials (Becker 2007). Proteins are composed of different amino acids and hence the nutritional quality of a protein is determined by their availability and proportion (Becker 2007).

Table 2.4. Amino acid profile of different algae compared with conventional protein sources and the WHO/FAO (1973) reference pattern (g per 100 protein) (Becker 2007)

Source	Ile	Leu	Val	Lys	Phe	Tyr	Me	Cys	Try	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
WHO/FAO	4.0	7.0	5.0	5.5		6.0			1.0									
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0	-	6.2	11.0	12.6	4.2	2.4	4.2	6.9
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8
<i>Chlorella vulgaris</i>	3.8	8.8	5.5	8.4	5.0	3.4	2.2	1.4	2.1	4.8	7.9	6.4	9.0	11.6	5.8	2.0	4.8	4.1
<i>Dunaliella bardawil</i>	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
<i>Scenedesmus obliquus</i>	3.6	7.3	6.0	5.6	4.8	3.2	1.5	0.6	0.3	5.1	9.0	7.1	8.4	10.7	7.1	2.1	3.9	3.8
<i>Arthrospira maxima</i>	6.0	8.0	6.5	4.6	4.9	3.9	1.4	0.4	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2
<i>Spirulina platensis</i>	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
<i>Aphanzomenon sp.</i>	2.9	5.2	3.2	3.5	2.5	-	0.7	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

Due to the high content of protein in various microalgae species, they are considered these days as an unconventional source of protein (Spolaore et al. 2006). Additionally, they have a comparable positive amino acid pattern with other food protein sources (table 2.4.).

Carbohydrates

The carbohydrates in microalgae can be found in form of starch, glucose, sugars and other polysaccharides (mainly used as gelling and thickening agents). Their overall digestibility is high, which gives the possibility of using dried whole microalgae in foods or feeds (Becker 2004).

Lipids / Fatty Acids

The average lipid content of algal cells varies between 1% and 70% but can reach 90% of dry weight under certain parameters (Metting 1996). The lipids found in algae are composed of glycerol, sugars and saturated or unsaturated fatty acids (12 to 22 carbon atoms). Among all the

fatty acids in microalgae, some fatty acids of the omega-3 and omega-6 families are of particular interest.

Fish and fish oil are the common sources of long-chain PUFAs. As PUFAs are found in fish that consume microalgae in oceanic environments, it can be logical to believe that microalgae are a potential sources of PUFAs (Jiang et al. 1999). Table 3 presents the microalga content in PUFA; however, DHA is the only algal PUFA commercially available (Spolaore et al. 2006). The Martek (USA) and Nutrinova (Germany) companies are producing microalga DHA from *Cryptocodinium* and *Ulkenia* for application in infant formulas, nutritional supplements and functional foods (Pulz & Gross 2004; Spolaore et al. 2006).

Table 2.5. Particularly interesting microalga PUFAs (Spolaore et al. 2006)

PUFA	Structure	Potential application	Microorg. producer
γ -Linolenic acid (GLA)	18:3 ω 6, 9, 12	Infant formulas for full-term infants Nutritional supplements	<i>Arthrospira</i>
Arachidonic acid (AA)	20:4 ω 6, 9, 12, 15	Infant formulas for full-term/preterm infants Nutritional supplements	<i>Porphyridium</i>
Eicosapentaenoic acid (EPA)	20:5 ω 3, 6, 9, 12, 15	Nutritional supplements Aquaculture	<i>Nannochloropsis</i> , <i>Phaeodactylum</i> , <i>Nitzschia</i>
Docosahexaenoic acid (DHA)	22:6 ω 3, 6, 9, 12, 15, 18	Infant formulas for full-term/preterm infants Nutritional supplements Aquaculture	<i>Cryptocodinium</i> , <i>Schizochytrium</i>

Special attention has been given to the marine microalgae *Isochrysis galbana* and *Diacronema vlkianum* (Haptophyceae) for their ability to produce long chain polyunsaturated fatty acids that are accumulating in the form of oil droplets (Liu & Lin 2001). These species have been used as a feed for larval and juvenile molluscs, crustacean and fish species (Fidalgo et al. 1998).

Vitamins and minerals

Microalgae also represent a valuable source of nearly all essential vitamins (e.g., A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid) (Becker 2004; Brown et al. 1999). Its mineral content is represented by Na, K, Ca, Mg, Fe, Zn and trace minerals (Becker 2004). Vitamins content differs with environmental factors, the harvesting treatment and the biomass drying methods (Gouveia et al. 2008).

2.4.2. Nutritional qualities (bioactive molecules/compounds and functional products) and sustainability

Microalgae represent a very large market still not exploited at their full potential. Many of the microalgae species show their content in bioactive molecules which makes them unique and interesting functional products (Yamaguchi 1997). Microalgae can biosynthesize, metabolize, accumulate and secrete a large diversity of primary and secondary metabolites, this being a reason for their use in many applications in food, pharmaceutical and cosmetic industries (Yamaguchi 1997).

A main reason for their nutritional quality is due to the fact that microalgae can synthesize all amino acids (Guill-Guerrero et al. 2004), which can become a by-product of this algal process or even an amino-acid product under the appropriate conditions (Gouveia et al. 2008). This can make microalgae essential for human and animal consumption (Guill-Guerrero et al. 2004). There are different factors that can influence and modify their chemical composition: temperature, illumination, pH value, mineral content, CO₂, population density, growth phase and even algae physiology (Gouveia et al. 2008).

It what involves sustainability, microalga biotechnology has received a lot of attention in the last years due to their high productivity and the possibility of producing them in unsuitable climates like desert or seashore lands without having seasonality (they are producing all year long). Their production has a positive effect on the environment by reducing the excess of atmospheric CO₂, hence lowering the greenhouse effect, reducing the environmental heating and climate changes, can remove pollutants (nitrogen or phosphorus) from water (Gouveia et al. 2008).

Microalgae are known for their use as natural colorants and nutraceuticals and together with their sustainability of production and renewable nature, they can become a very important and essential market (Dufossé et al. 2005).

2.4.3. Microalgae in animal nutrition (poultry, pigs, ruminants and aquaculture, pets)

Microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals. 30% of the current world algal production is sold for animal feed applications (Becker 2004) and over 50% of the current world production of *Arthrospira* is used as feed supplement (Yamaguchi 1997). Algae positively affects the

physiology (by providing a large profile of natural vitamins, minerals, and essential fatty acids; improved immune response and fertility; and better weight control) and their external appearance (resulting in healthy skin and a lustrous coat) of animals (Spolaore et al. 2006).

A large variety of animals are able to accept microalgae in their feed. Gouveia et al. (2008) explains how the usage of microalgae affects every category of animals: for poultry feed, the use of microalgae has already been approved in several country; there are very good results for pigs with 25 to 33% incorporation; for ruminants little trials were done due to the high amount of algae necessary to conduct the experiments, even if it should be expected that they are most suitable for feeding because of their digestive system; in aquaculture it is mostly used for larvae, juvenile shell and finfish. There is an increase interest of using the microalgae potential for all aquaculture animals.

In poultry rations, algae up to a level of 5–10% can be used safely as partial replacement for conventional proteins. Prolonged feeding of algae at higher concentrations produces adverse effects (Spolaore et al. 2006), like influencing the color of broiler skin, shanks and egg yolk (Becker 2004).

Feasibility of microalgae as animal feed

The use of micro-algae as animal feed is more recent. A large number of nutritional and toxicological evaluations demonstrated the suitability of algae biomass as a valuable feed supplement or substitute for conventional protein sources (soybean meal, fish meal, rice bran, etc.) (Becker 2007). The target market for domestic animals is poultry. Another growing market is aquaculture.

To provide a better balanced nutrition and improve animal growth, several reports advice mixing the species, this gives better results than a diet composed of only one algal species (Yamaguchi 1997).

Radhakrishnan et al. (2014) – concluded that replacement of fish meal with *Spirulina platensis*, *Chlorella vulgaris* and *Azolla pinnata* enhanced the vitamin C and E and that these ingredients can be used as an alternative protein source for sustainable *Macrobrachium* shrimp culture.

Microalgae for value-added animal products - aquaculture

In 1999, the production of microalgae for aquaculture reached 1000 t (62% for mollusks, 21% for shrimps and 16% for fish) for a global world aquaculture production of 43×10⁶ t of plants and animals (Muller-Feuga 2000). Microalgae are valuable in aquaculture and have been used as live feeds for larval or juvenile crustaceans and finfish (Ju et al. 2009).

The most frequently used species in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Apt & Behrens 1999; Muller-Feuga 2000; Yamaguchi 1997).

Genus *Spirulina* is very much used as a feed additive in the Japanese fish farming industry, with inclusion levels of 0.5 - 2.5% (Hasan & Chakrabarti 2009). In an experimental paper it was shown that sturgeon fed with *Spirulina* based feed outperformed those receiving fish meal based diets (Kovač et al. 2013).

Table 2.6. Upper and lower estimates of microalgae biomass production required by the post-larvae of world aquaculture in 1997 (1998 for shrimps), and mid-term trends of this production (Muller-Feuga 2000)

	World aquaculture production (t/year)	Number of 10 ⁶ post-larvae per t of final product	Number of 10 ⁶ post-larvae for aquaculture production	Microalgae requirements per 10 ⁶ post-larvae (kg d. wt)	Microalgae biomass (t d. wt per year)		Trends
					Upper estimate	Lower estimate	
Mollusks	7,442,555	0.1	744,256	14.0	10,420	330	Sharp increase
Shrimp clear water	206,416	0.3	68,805	0.06	4	4	Decrease
Shrimp green water	530,784	0.4	224,786	0.65	146	146	Increase
Small larvae fish	169,167	0.005	845	60.0	51	51	Increase
Total	8,348,922				10,620	531	

The world microalgae requirements for hatcheries are represented in Table 4. It can be observed the total production of aquaculture in the world and the microalgae requirements. The major part of microalgae world requirements for aquaculture comes from mollusks (10,420 t d. wt per year) (Muller-Feuga 2000). The decrease in trends for the shrimp from clear water is due to the increase in formulated feed use.

Chapter III - Pellet Quality Analysis

Pelleting process is used for improving feed nutrient utilization and for meeting customer expectations (Behnke 2001). When compared with extruded process, steamed pellets don't give the same degree of quality as extruded ones (Hilton et al. 1981), but the process is more economically viable for the required quality in the market.

The feed industry is in a continuous development for finding new sustainable ingredients and new equipment and technology for evaluating their physical quality. For this to be done in a proper manner, a deep understanding of the factors that affect the pellet quality is a necessary step (Behnke 2001).

Aquatic feeds are often compressed by steam pelleting or extrusion method into particles that allow maximum utilization of the feed in the water, so of high durability to withstand handling and transportation and of good water stability to minimize disintegration and loss of nutrients upon exposure to water (Lim & Cuzon 1994).

The feeds are defined according to certain specifications with regard to nutritive composition based on specified descriptions for nutritional, hygienic and physical quality. Together, these specifications require knowledge of a vast number of different properties of ingredients to optimize processing while maintaining or controlling nutritional quality for a given feed form.

Different animal species require different physical properties for their respective feeds. This means that different quality standards are used. For fish-feeds, additional pellet characteristics such as flowability, sinking velocity, water absorption and water solubility are important. Furthermore, the hygienic quality of feeds is important. Hygienic quality involves the control of microbiological contamination of feeds based on levels of enterobacteriaceae and salmonella (McCapes et al. 1989).

Some advantages of pelleted feed versus mash meal:

- Pellets have better flow properties, necessary for good transport in conveying equipment, and (gravitational) discharging behavior from silos, than the meal they were prepared from.

- The bulk density of pellets is generally higher than that of meal, so that more tonnage can be carried by truck.
- The composition of the pellets as obtained from carefully blending and mixing ingredients remains fixed, no segregation of e.g. additives occurs.

However, the use of shaping equipment, like pellet presses, requires additional costs in terms of energy demand and necessary additional equipment; Pellets also need to have a basic form of physical quality in terms of e.g. hardness and durability to withstand the rigors of transportation. Such quality parameters can also be used to evaluate the effects of diet formulation, conditioning, expander treatment, pellet binders, die selection, etc.

For optimization of product quality in terms of physical characterization, knowledge of fundamentals for aggregating particles of a different size, hardness and shape is needed (Thomas & Van der Poel 1996).

Pellet quality can be measured through several characteristics, like water activity (a_w), hardness and durability of pellets, water absorption rate and water stability by either disintegration rate or swelling rate in water.

The durability of pellets it's not discussed in the present paper, more emphasis has been put on hardness of pellets.

3.1. Water activity (a_w)

Different procedures are available for measuring water activity (Rahman & Labuza 1999), but they usually fall into one of three general methods of approach. A_w can be measured through:

- Sample being brought into equilibrium with a closed atmosphere of known constant relative humidity (isopiestic technique or desiccators method)
- Equilibrium head space atmosphere surrounding sample is measured for its relative humidity with the sample (water activity meter)
- Dynamic method in which samples are exposed to atmospheres of various relative humidity and weighed simultaneously.

The first method of approach involves samples of known initial moisture content which go through the process of moisture gain (absorption) or loss (desorption) in a constant controlled relative humidity atmosphere. The sample weight will change over time as it gains or losses moisture, and is measured and recorded periodically until the sample has come into equilibrium with the relative

humidity of the surrounding atmosphere. At this point, when the sample will neither gain nor lose moisture with further time, the sample will have a water activity equal to the controlled constant relative humidity used throughout the experiment therefore this approach sometimes is called isopiestic technique.

The second approach is to place the food sample of known constant moisture content into a sealed enclosure with small head space. The atmosphere in the head space is then probed for measurement and monitoring of changes in relative humidity or partial pressure until equilibrium is reached. The relative humidity measured at equilibrium is taken as the water activity of the sample. Because time to reach equilibrium can be long e.g. several hours, some instruments are equipped with software for extrapolation to the water activity at which the sample will neither gain nor lose moisture.

In the third approach (dynamic method), samples of food can be exposed for a short period of time to flowing air at various relative humidity and weighed automatically. For this purpose, isothermal thermo gravimetric methods are useful techniques.

Regardless of the different approaches, it is important in all three methods to remember that sorption is strongly temperature depended, and the temperature must be kept constant and controlled while all the experiments are being carried out. It is also important to determine and note the distinction if the experiment is for the purpose of constructing desorption (drying) or adsorption (wetting) isotherm, because of hysteresis effects explained earlier (Figura & Teixeira 2007). In the case of desorption isotherm, the food sample will initially be very fresh with relatively high moisture content (fully hydrated), and will lose moisture and weight during the experiment. In the case of an adsorption isotherm, the food sample will initially be very dry (dehydrated), and will gain moisture and weight during the experiment.

3.2. Hardness of pellets

The difference between hardness and durability is: hardness is the force necessary to crush a pellet or a series of pellets at a time, while durability is the amount of fines returning from pellets after being subjected to mechanical or pneumatic agitation. Hardness is a quantity which is important for the nutrition of animals since it may play a role with preference of animals (Thomas & Van der Poel 1996). Also, availability of nitrogenous components for intestinal absorption has been reported to be affected by hardness (Thomas & Van der Poel 1996).

The hardness of the agglomerates is inversely related to the particle size distribution. They showed that with an increase in the filling of the pores with water, an optimum value for hardness exists. This optimum is dependent on the surface tension of the pelleting fluid (Thomas & Van der Poel 1996).

Hardness is determined by using equipment which measures the force needed to fragment a pellet. Nowadays, several devices are available for the evaluation of product hardness, each of which has different attrition or operating mechanisms. A first and common device used in industry to test pellet hardness is the ‘Kahl’ device (Fig.2.4.), analogous to the early developed, manually used Stokes tester. In the Kahl device, a pellet is inserted between two bars, and by increasing statical pressure applied by means of a spring, the force needed to crack the pellet is determined. The average of ten measurements is referred to as the ‘Kahl-hardness’ of the pellet.

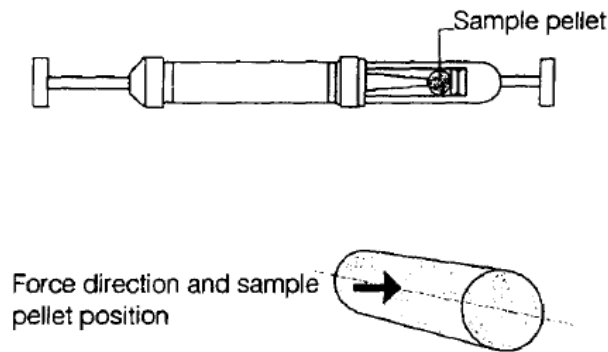


Fig. 2.4. Kahl pellet hardness tester (Thomas & Van der Poel 1996)

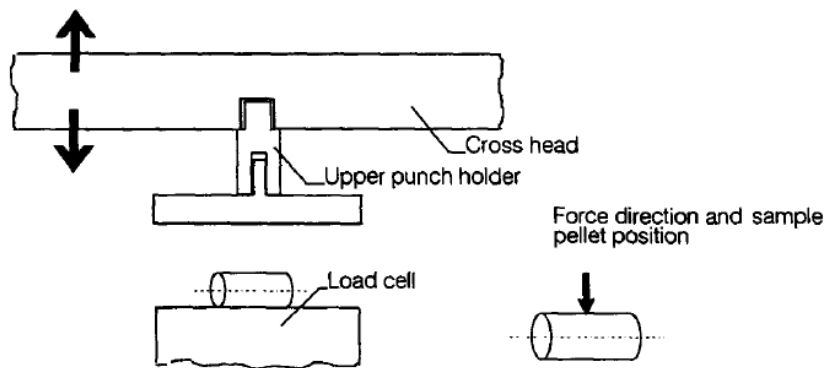


Fig. 2.5. Universal compression test device (Thomas & Van der Poel 1996)

Other devices use both compression and tension, and comprise so-called ‘universal tension and compression’ apparatus (Instron; overload dynamics). Their main advantage is the accuracy with which the different materials can be subjected to the different tests known in the field of feed and food engineering. These devices consist of a fixed plate containing a load cell and a moving bar with variable speed. On this instrument measuring bodies of a various geometry (either knife or plate for example) can be fitted and uniaxial compression, uniaxial tension, three-point bending and cutting experiments, respectively, can be performed. For the testing of pellets, uniaxial testing (plate) and cutting (knife) experiments are most appropriate. The force units needed to break the pellet (or a series of pellets) is recorded as a function of time. Both single and multiple sample pellet(s) can be tested.

3.3. Surface hydration / Contact Angle

The physical events involved in functional properties of flours (and more particularly in hydration properties) can be described by considering the surface wettability properties. The surface energy of a solid is a good way to characterize the wettability properties. The surface energy of solids determines the potential level of physical interactions that a solid is able to exchange. The knowledge of this parameter is important for the understanding and prediction of many surface and interface phenomena (e.g., adsorption, wettability, adhesion, and friction) (Yuan & Lee 2013).

The surface wettability properties of powders can be estimated by the measurement of contact angles. For the characterization of powders, values of contact angles have been determined by the technique of capillary rise (i.e. measurement of water penetration rates in a capillary that is filled with the powder). Contact angles are then calculated using the Washburn equation. The technique of capillary rise has also been used to estimate the water adsorption capacity of wheat flour (Roman-Gutierrez et al. 2003).

The wettability properties of material can also be evaluated by the measurement of contact angle of a liquid drop deposited on the surface of the solid. For powders, it is necessary to carry out a preliminary stage of compaction. A water drop or water drops can then be deposited onto the upper surface of the compacts. The liquid–solid contact angle can then be measured using a

protractor eyepiece on a goniometer or by photographing the drop and measuring the angle on the print. Secondary, a drop of maximum height can be produced and the measured height used to calculate a contact angle on the print. However, one of the major problems in measuring the contact angle on compacts is that the process of compaction may alter the outer surface. Plastic deformation may result in a measured contact angle, which is not representative of the original powder (Roman-Gutierrez et al. 2003; Yuan & Lee 2013).

3.4. Water Stability Measurements

Pellet water stability is an important quality parameter in the manufacture of aquaculture diets especially for shrimp. When we talk about duration of pellet water stability we think about the time required by the fish or shrimp to consume its ratio (Lim & Cuzon 1994). Water stability, as defined by Obaldo et al. (2002) represents the retention of pellet physical integrity with minimal disintegration and nutrient leaching while in the water until consumed by the animal.

The degree and duration of WS required can be lower if diets possess suitable texture, size or shape and contain attractants which enhance feed consumption. The pellet WS required can be reduced by proper feed distribution and daily feeding schedule and more frequent feeding (Lim & Cuzon 1994). Feed should be of high water stability to prevent increased cost of feeding and to provide the greatest proportion of available nutrients to the cultured animal. However, the degree and duration of water stability required could be lower if diets contain suitable attractants that enhance feed consumption (Lim & Cuzon 1994).

This subchapter reviews information on various factors which affect the water stability of pellets for shrimp, like composition of the diet, method of manufacturing and binders. Problems associated with the methods used for evaluation of water stability, and factors affecting the length of water stability required are also discussed.

Factors for improving pellet water stability:

- Method of diet preparation and processing
- Types of ingredients and diet composition
- Types of binding agents - Inclusion of different binders is a necessity in pellet milling (Ahamed Ali 1988; Cuzon et al. 1994; Fagbenro & Jauncey 1995). Binders affect pellet stability in three ways. They reduce void spaces resulting in a more compact and durable

pellet, they act as adhesives sticking particles together, and they exert a chemical action on the ingredients and alter the nature of the feed resulting in a more durable pellet (Ruscoe et al. 2005).

- Coating materials such as microcapsules

Pellet disintegration and nutrient leaching are more important in shrimp than in fish or other aquatic animals because of their benthic and slow feeding habits, which makes sinking and water stable feed a necessity for shrimp. A standard method must therefore be developed for routinely measuring and monitoring pellet water stability.

Several methods have already been used in evaluating pellet stability:

- Immersing pellets in static water and using mechanical agitation
- Water flow through a system to stimulate pellet and water movement in a particular culture system.

Despite this, there is no single method that has emerged to date that could serve as a standard for routine laboratory analysis (Obaldo et al. 2002). The object of this study was to develop a simple and accurate method for pellet water stability and nutrient leaching that can be adopted for routine laboratory analysis of dry pelleted and extruded shrimp feeds. Flexibility in the method to mimic actual shrimp culture conditions was an important consideration (Obaldo et al. 2002).

In table 2.7., some methods used to analyze water stability are described.

Table.2.7. Analysis of described methods for water stability

Nr. Crt.	Method	Description	Result measurements
1.	Brass wire mesh baskets immersed in water (Rout & Bandyopadhyay 1999)	Triplicate baskets containing ~20 g pellets spread in one layer were immersed in saline water flowing through a tank under mild agitation by air stones. Immersion times varied between 30 and 240 min.	Expansion ratio was measured as the ratio of average diameter of dry pellet to the die hole diameter. The results were reported as mean \pm S.D. of five measurements.
2.	Tanks simulation with submerged pellets / water stability of	Series 1 The physical appearance and integrity of the submerged pellets were recorded at different	1. Detailed notes were kept as to whether the pellets appeared entire and stable, partially intact,

<p>different feed pellets under tank conditions</p>	<p>time intervals: 1, 2, 4, 6, 8 and 22 h after immersion.</p>	<p>or totally disintegrated and dissolved. Each pellet type was tested 40 times using 10 pellets per trial.</p>
<p>(Fagbenro & Jauncey 1995; Farmanfarmaian et al. 1982)</p>	<p>Series 2 Ten pellets of each type were weighed and placed in small tarred sieves of 1.0 mm mesh</p> <p>Sieves remained submerged for 6-, 12-, 18- or 24-h periods</p>	<p>2. At the end of each interval, sieves were removed, oven dried at 75°C and reweighed. The difference between initial and final weight is a quantitative measure of the “leachout” rate of a particular feed</p>
<p>3. Horizontal shaking method</p> <p>(Obaldo et al. 2002)</p>	<p>It was used a Lindberg/BlueM refrigerated and heating circulating water bath with a shaker. The shaker tray held up to eight 250 mL flasks for each test run.</p>	<p>All solids were recovered using a Buchner filtration apparatus with Whatman filter paper (5µ). After, all samples were dried (105°C for 24h) and cooled. The WS was calculated as the ratio of dry matter recovered after leaching and dry matter of the original samples expressed as a percentage.</p>
<p>4. Static water method</p> <p>(Obaldo et al. 2002)</p>	<p>Technically the same as the horizontal shaking method with zero speed.</p>	
<p>5. Vertical shaking method</p> <p>(Obaldo et al. 2002)</p>	<p>A VanKel disintegration testing system was used. Sets of three baskets were placed into a basket rack assembly that was lifted and lowered in a 1000 mL beaker. Long cylindrical stainless-steel mesh basket with removable mesh cover</p>	<p>The same procedure was used as for the previous 2 methods.</p>

	was used to hold cca. 2 g of feed during leaching and during oven drying.	
6. Submerged pellets under water in cone shaped pouches made of nylon cloth. (Ahamed Ali 1988)	Pellets were cut into cca 5mm length. 50 pellets were kept in each pouch, initial weight was recorded. Nine sampler were weighted for each feed. The pouches with pellets were carefully lowered into the water and placed in petri dishes kept at the bottom of a plastic lined pool 90 cm and 40 cm depth of water salinity of 20%.	After one hour one pouch was carefully taken out of the water, the physical shape was noted and then dipped in a container of fresh water for five minutes. Then it was transferred to the oven and dried at 70°C for constant weighth. Same was done at every 2, 3, 4, 5, 6, 8, 12 and 24 h. The loss in weight was calculated by the difference in weight before and after the immersion.
7. Wet durability test (Ighwela et al. 2014)	Triplicate 5 grams samples of pellet of each diet were dropped into 15 glass beakers, which contained 800 ml tap water. The immersion times examined were 1h, 2h, 4h and 6h respectively. After immersion, the undissolved solids and water were filtered through filter paper and were dried in the oven (105°C for 30 min), followed by further drying at 65°C to a constant weight, then cooled in a desiccators.	The mean differences in weights of beakers containing the feed before immersion and after drying were used to calculate the percentage dry matter loss, which is a measure of the water stability of the pellets for the corresponding time intervals.

Table 2.7. describes different method of characterizing leaching rate of pellets immersed in water as a water stability parameter. In the last two decades the method has not changed much and it's still used with a various errors:

- Usage of filtered paper might not help in delivering the total wet material, some might get stuck on it;
- Trying to simulate tank condition by vibration might give to high movement in the water. Shrimps are slow movement crustaceans, their vibrations might not be as strong as the once used in the laboratory conditions.
- Testing leaching time only gives information about ponds environment and maybe some leak in nutrients, but does not give a direct information on how water stable, by a physical point of view, it can be.

Different methods for the evaluation of the attrition behavior of pellets supply different information. It is obvious that it is not possible to use each type of device with every type of feed pellets. The choice for a certain method therefore is partly determined by the objective of the measurement, related to handling or nutritional purposes or to study simulated production of fines caused by either fragmentation or abrasive stresses.

In the context of animal feed pellets, bending tests and tensile tests in general may give more information compared with compression tests (Thomas & Van der Poel 1996) but are of less value due to the type of attrition that feed pellets undergo between manufacturing in the feed mill and the animal feeding trough.

Since transport and handling involve both fragmentation and abrasion phenomena, it would therefore relate more closely to quantities measured with the Holmen pellet tester than with the tumbling can device, since the first device acts as an 'in between' simulator.

Chapter IV - Goals of the thesis

Human population is predicted to increase by 2050 to over 9 billion. This predicament has a high effect on the global demand for food. Due to the fact that some available food resources are already restricted, research for finding new sources or for improving the present ones is a must.

A few novel protein ingredients, recognized as ingredients with big potential are starting to be developed from single cell organisms. The quality control and their shelf life extension should be one of the major goals for the producers of the aquatic feeds and also the producers of these protein based ingredients. Single cell ingredients of interest for aquaculture are bio protein, microalgae and yeast protein concentrates.

The primary objective of the present master study was to evaluate the effect of lignosulfonate and enzyme addition on the physical characteristics of microalgae pellets, water absorption rate, swelling rate of the pellets as a water stability characteristic and hardness for a prolonged shelf life and an overall product quality for single cell microalgae protein concentrate. Previously to performing the experiments, a literature review has been done to understand the mechanisms and how the material chosen might act. A secondary objective was to introduce a new method for evaluating water stability in aquaculture industry, especially crustacean, by image analysis through a video microscope without the involvement of the animal.

Chapter V – Experiment

Characterization of the rheological properties for microalgae and the addition of LS and enzyme

5.1. Abstract

The main goal of this study is to determine how addition (in a dry matter basis) of lignosulfonate and enzyme (NSP and protease) influences the physical quality of microalgae pellets. The pellets were produced using a single pellet press method. Four experimental diets were formulated: one control diet with 100% microalgae (C) and other three with addition of Lignosulfonate (LS), non-starch polysaccharides (NSP) and protease to C diet in a percentage of 0.5, 0.01 and 0.006 respectively. Characterization of their effect on tensile strength (durability of pellets), the water absorption rate and underwater swelling rate is presented. The results showed that the additives protease and non-starch polysaccharide enzyme (NSP) decreased the tensile strength of pellets when added in their respective percentages. Lignosulfonate additive (LS) did not change the tensile strength when added in 0.5% (dry basis). Protease, NSP and LS increased the water activity, decreased the swelling of pellets under water, increased hydrophobicity and lowered absorption rate of a sessile water drop sitting on the pellet surface. Protease produced the highest water activity, the lowest swelling of the pellets and the highest initial contact angle (i.e. the most hydrophobic) followed by LS and NSP. Pellets made of pure microalgae had the lowest water activity, the lowest hydrophobicity and the highest swelling and absorption rate of the sessile drop.

5.2. Introduction

The alarming increase in population and their respective demands for more and better food has drawn researchers attention to find new sources for food and feed and to improve the available ones. The struggle is with finding new protein sources as supplements (Becker 2007). One of this new protein sources is microalgae. This days, microalgae are known for their increase in nutritional value (through improving the chemical composition) of food and animal feed. Their numerous applications on the market, made them important to be used in aquaculture (Spolaore

et al. 2006). On the other hand, novel additives like enzymes have demonstrated to improve the nutritional utilization of feed ingredients for various aquatic species. However, published data on how the enzymes affect the physical characteristics of aquatic feeds is scarce.

Lignin is the second largest component of wood. Calcium lignosulfonate (40-65) is an amorphous light-yellow-brown powder obtained from the sulfite pulping of softwood (Toledo & Kuznesof). It acts like an emulsifier and stabilizer in the addition of encapsulated fat-soluble active ingredients into water-based foods.

The rheological characteristics of feed pellets are important for trading, storage, transport, animal consumption and as a quality measure parameter. The strength of pellets is one of the most common and demanded quality parameter when buying. Another rheological characteristic, important for aquatic feed, is the deformation of the pellets under water, which is critical when feeding crustaceans because it can indicate how long a pellet can remain useful under water. Pellets that remain relatively cohesive are more likely to be eaten than disintegrated pellets. Pellet disintegration should also be avoided as they disperse nutrients into the aquatic environment, which means that, the ratio of deformation of a pellet under water (e.g. swelling), before disintegration takes place can be an important quality parameter to be brought to the aquaculture industry. This article presents a new method that estimates the rate of swelling of pellets under stagnant water, using a special testing arrangement coupled with image analysis. Another important parameter, still not used by the aquaculture industry, is to estimate the ability of a pellet to absorb water and to quantify how hydrophobic or hydrophilic a pellet surface is. For this purpose, contact angle measurements of a sessile drop over pellets are conducted for this article through image analysis. Water activity is also a parameter described in literature as having great importance for feed shelf life, but not often measured. The effects of enzymes and lignosulfonate over the mentioned parameters in microalgae pellets is studied in this article and presented with the new methods.

5.3. Material and methods

5.3.1. Raw material preparation and processing

The raw material was obtained from the Centre for Feed Technology pilot plant (FôrTek), located at the Norwegian University of Life Sciences, Ås, Norway. Commercial microalgae

(*Nanofrustulum sp.* and *Tetraselmis sp.*, produced by Cellana LLC), lignosulfonate (LignoBond DD Powder from Borregaard AS, Sarpsborg, Norway), non-starch polysaccharides (Econase XT 25 L from AB Enzymes GmbH, Darmstadt, Germany) and proteases (yeast based protease produced by AB Vista Feed Ingredients) were used to perform this experiment.

Microalgae biomass

Table 5.1. Technical information on microalgae biomass (Cellana LLC)

Technical information	
Species	<i>Nanofrustulum sp.</i> and <i>Tetraselmis sp.</i> (Percentage unknown)
Concentration	100%
Appearance and Odor	Green to brown. Powder. Fishy.
Total dust	15 mg/m ³
Specific gravity	< 1
Density	> 1 g/cm ³
Bulk density	608 kg/m ³
Water solubility	Negligible
Vapour density (air=1)	>= 1
Acute Toxicity	Non-hazardous

Table 5.2. Composition of microalgae (Cellana LLC)

CP	CF	Starch	DM	Ash	Carbohydrates
28,3%	5,1%	0%	96,2%	17%	49,6%

Lignosulfonate

LignoBond DD is a calcium lignosulfonate derived from spruce wood and produced as a brown powder with low hygroscopicity, medium sugar content and a bulk density of 550 kg/m³. Lignobond DD is specially prepared to reduce dust for ease of handling. Typical application is as an animal feed binder.

Table 5.3. Sales Specifications (Borregaard LignoTech)

		Test Method
Dry matter %	Min. 93.0	BLT-308
pH (10% solution)	4.3 ± 0.8	BLT-327

Table 5.4. Chemical data (Borregaard LignoTech)

Parameter	Unit
Calcium	6%
Reducing sugars	7%
Ash	16%

The NSP it's a enzymatic protein (by-product) containing xylanase, endo -1,4 -. Its physical state is liquid with a brown color, a pH value of 3.5 – 4.5 (10% solution), soluble in water and with a density of 1.1 – 1.2 g/cm³.

Four tests were performed, T1 consider the control contains pure microalgae as ingredient, in T2, T3 and T4 three different additives have been mixed, LS, NSPs and protease, respectively.

Table 5.5. Composition of the four tests

Test	C-control	C+LS	C+NSP	C+Protease
Microalgae	100%	99.500%	99.990%	99.994%
LS	0%	0.50%	0%	0%
NSPs	0%	0%	0.01%	0%
Protease	0%	0%	0%	0.006%

All the four tests, immediately after mixing, were stored in vacuumed bags and kept in a commercial fridge at approximately 1°C.

5.3.2. Moisture content

The moisture content of the microalgae was determined gravimetrically in a scale with Mettler Toledo LJ16 infrared Moisture Analyzer (Sigma Aldrich, U.S.) by addition of 2.5 g of raw material; approximately 15 min was the waiting time for the result.

Initially, the moisture content of the raw material under powder form has been determined, 3.3% microalgae and 6.3 % for lignosulfonate. To achieve 7% moisture as planned, an addition of approximately 4% water has been calculated for the mixture, in dry matter basis. The water addition for the last two tests (T3 - C+NSP and T4 - C+Protease) has been calculated (in dry matter) together with the percentage of enzyme addition, since they are commercially bought under liquid form. The enzymes have been added mixed with the respective percentage of water needed to achieve the desired moisture content.

5.3.3. Physical proprieties

Following analyses on raw materials were performed:

- particle size distribution
- bulk density

Particle size distribution. The particle size distribution of microalgae and lignosulfonate was measured by Mastersizer 3000 optical unit combined with Aero S dry dispersion unit (Malvern Instruments, U.K.) at room temperature. The particle size distribution is characterized by the mean diameter (D50) and by the dispersion (D90_D10)/D50 (Allen 1990). Ten measurements were carried out for each product.

The bulk density was determined by measuring the mass of a known volume of material that has been loosely poured into a graduated cylinder. The density of powder was determined by measuring the mass of know volume of powder in a graduate cylinder (± 0.2 ml) on analytical balance (± 0.0001 g)

5.3.4. Pelleting method

A laboratory single pellet press that was designed and fabricated at the workshop of the University was used to process microalgae pellets (fig.5.1.). All pellets used in this research were produced in the laboratory die pelleting rig presented by Salas-Bringas et al. (2010). The rig was assembled in a Lloyd LR 5K Plus texture analyzer. The die pelleting rig consists of a barrel made of brass having a compressing channel along the center. The compressing channel has a diameter of 5.5 mm and a 5.4 mm diameter rod was used to press the samples against a blank die. Using this configuration, the system can produce compacting stresses up to 218 MPa. To release the pellets from the compressing channel, the blank die was disassembled from the barrel.



Figure 5.1. Laboratory single pellet press

The barrel was heated by a jacket heater of 550 W which is controlled by a PID connected to a thermocouple in contact with the barrel surface (Salas-Bringas et al. 2010; Salas-Bringas et al. 2011). The temperature was set to 81 °C to manufacture all pellets. 81 °C is the minimum temperature that is required in Norway to disable *Salmonella* and to reduce the number of bacteria.

Pellets of pure microalgae were produced initially at four different compacting pressures (7, 11.6, 23.3 and 35 MPa) to observe the influence on density. For the production of all the pellets utilized for the desired analysis only one compacting pressure was used, 12 MPa.

The steel cylinder was first heated to a set temperature. After a steady temperature was reached, the channel was filled with material and the pressing rod was placed into the die. To obtain pellets with nearly equal length, the amount of material was weighted before pelleting. The average weight was $0.155 \pm 0.01\text{g}$. After 3 min of tempering, the biomass was compressed at a rate of 2 mm/min until the set pressure was reached. Afterward, the pressure was released, the bottom rod was removed, and the pellet was pressed out from the channel. The pressure required to initiate the pellet discharge, was not recorded. The total retention time of the material in the channel was 8 minutes for the first two tests (C and C+LS) and 10 minutes for the last two tests (C+NSP and C+Protease). The obtained pellets were stored in sealed plastic bags for about 48 h at room temperature and humidity (≈ 25 °C and $\approx 30\%$, respectively) until further testing.

5.3.5. Physical Characterization of Pellets – Hardness and Tensile Strength

The strength of the pellets was measured 48 h after production by a diametric compression test (fig.5.2.) using a 60 mm diameter probe connected to a Lloyd LR 5K texture analyzer (Lloyd Instruments, U.K.), as described by Salas-Bringas et al (2011). The compression speed was set to 1 mm/min. The maximum normal force at breakage was recorded. Prior to the strength analysis, the pellet density was calculated using the



Figure 5.2. Diametric compression test connected to a Lloyd LR 5K texture analyzer

weight and cylindrical shape of the pellet. Length and diameter of the pellet were measured with a digital caliper.

5.3.6. Water activity (A_w)

The water activity value (a_w) was measured by a Rotronic HygroLab C1 (Switzerland) instrument. Average temperature during a_w measurements was 21.2 ± 0.2 °C. The temperature is measured by the instrument temperature sensor. A_w value describes availability of free water in different products (typically food and feed). Free water affects products microbiological, enzymatic and chemical stability.

5.3.7. Measurements through Image Analysis – Pellet water stability method and swelling rate protocol

Recommendation for routine laboratory analysis of shrimp feed water stability using Image Analysis measurement

Describing the method

The method of measuring the swelling of a pellet under static water through image analysis employed a Krüss Tensiometer, a Micro Viper Portable Computer and Allen Compact Video Microscope Lenses for assembling (fig.5.3.). The Krüss Tensiometer provides the support for the video microscope (fig.5.7.), the glass container (fig.5.5.) and the light source. The Micro Viper Portable Computer contains the video microscope and the Micro Viper Portable software that allows us to capture the images at the desired time interval.

1. Purpose and scope

- Pellet water stability is an important analysis component for the complete characterization of the physical quality of aquafeeds.
- Pellet stability is referring to the ability of the pellet to maintain its form and nutrients while kept under water without being consumed by the animal.
- The pellet stability method employed for this analysis contains a video microscope, connected to a Micro Viper Portable Computer and attached to a Krüss Tensiometer. The same Krüss Tensiometer is used for the water absorption rate measurements. In

fig.5.4. different pieces of equipment used to place the camera and to assure a good stability of the pellet can be observed.

2. Equipment and test apparatus are presented in fig.5.3. and fig.5.8.
 - Video microscope
 - Micro Viper Portable Computer Krüss Tensiometer
 - Equipment pieces for complementing the Tensiometer
 - 100 mL graduated cylinder
 - Time chronometer
3. Test procedure (step by step)
 - a) Assemble the video microscope and mount it on the Tensiometer.
 - b) Mount the glass container on the DataPhysics Optical Tensiometer and introduce the pellet support bridge inside the container
 - c) Randomly select 3 pellets, obtain one pellet from three replicates
 - d) Add 100 mL distilled water in the glass container. Wait for ~5 minutes for the water to stabilize and make sure there are no water bubbles inside. With a laboratory pinzette sink the pellet in the water and slowly set it on the bridge.
 - e) Start the time chronometer from 0 time and continue by taking images at the time intervals that have been set.
4. Feed analysis and calculation
 - Analyze the images taken with the video microscope as described in Annex 1, in terms of area swelling during a certain time interval.



Figure 5.3. Equipment used for the evaluation of pellet water stability. Items are indicated by letters: (A) Krüss Tensiometer, (B) Allen Compact Video Microscope Lenses, (C) Micro Viper Portable Computer)

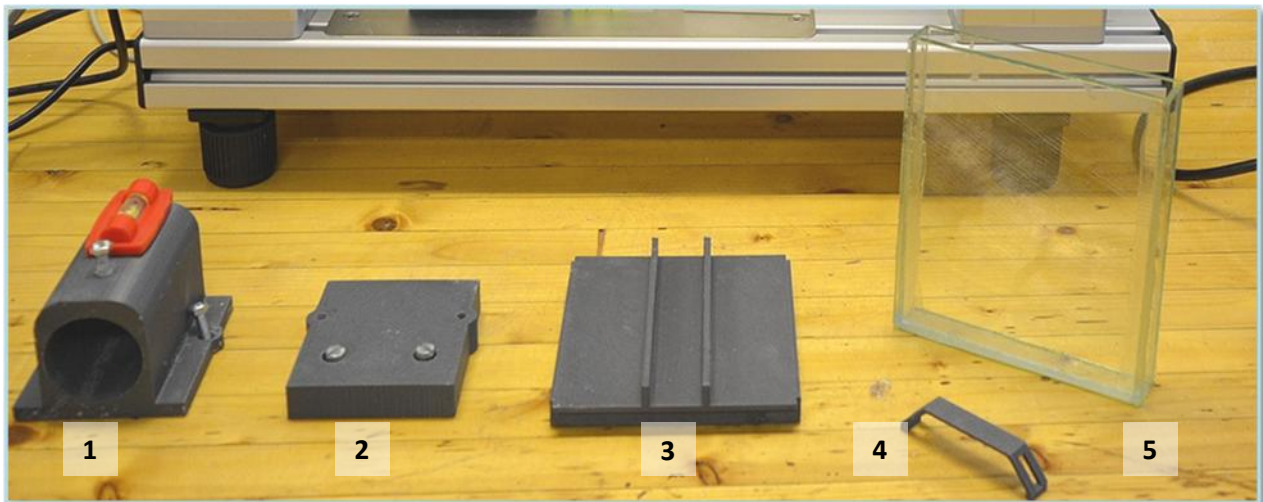


Figure 5.4. Materials made in the University's 3D printer for complementing the Tensiometer (Krüss G10). Items are indicated by numbers: (1) & (2) Support for stabilizing and mounting the video microscope on the Tensiometer, (3) Support for fitting the glass container to DataPhysics Optical Tensiometer, (4) Bridge for keeping and stabilizing the pellet while in the glass container, (5) Glass container

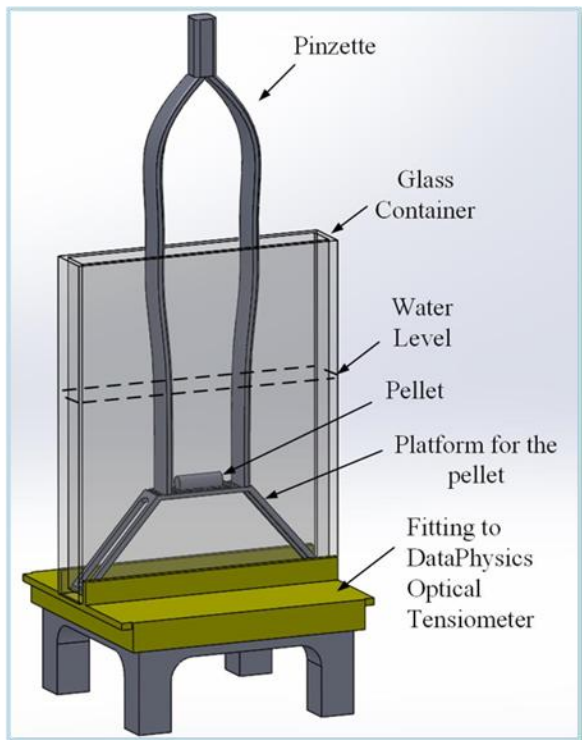


Figure 5.5. Assembled equipment for the image analysis of the swelling rate of the pellet (Salas-Bringas et al. 2015)



Figure 5.6. Assembling the video microscope. Items are indicated by numbers: (1) CVM Video probe head, (2) Zoom lens, (3) 20x – 120x basic lens, (4) Contact head adaptor, (5) 60x-420x contact head)

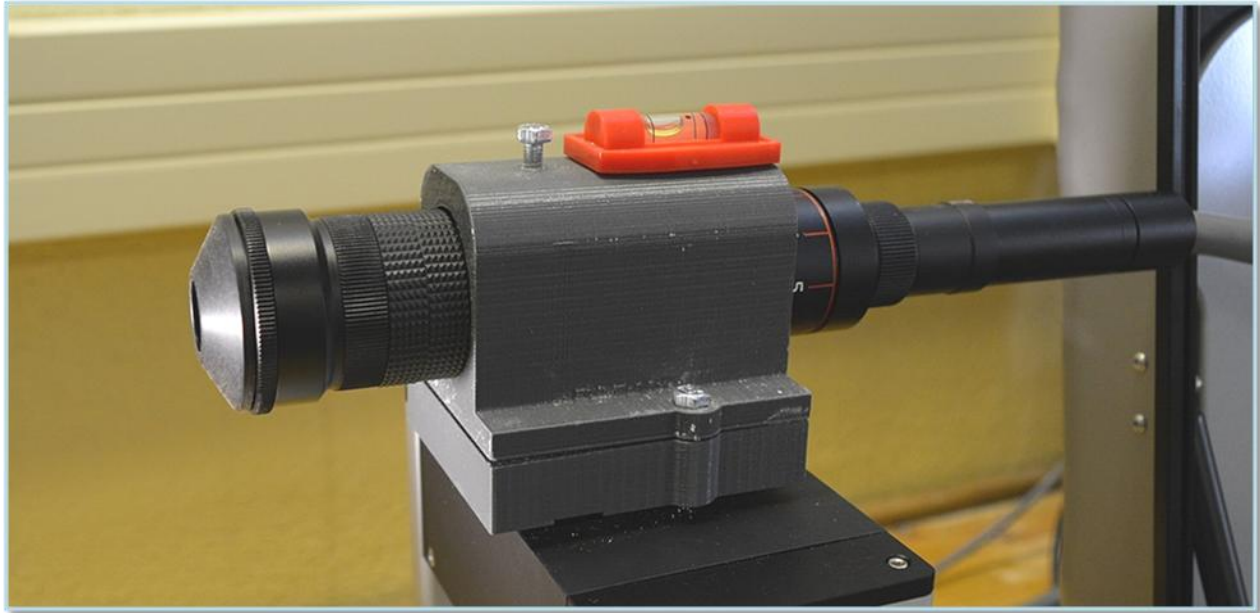


Figure.5.7. Final view of the assembled and fixed video microscope on the Tensiometer

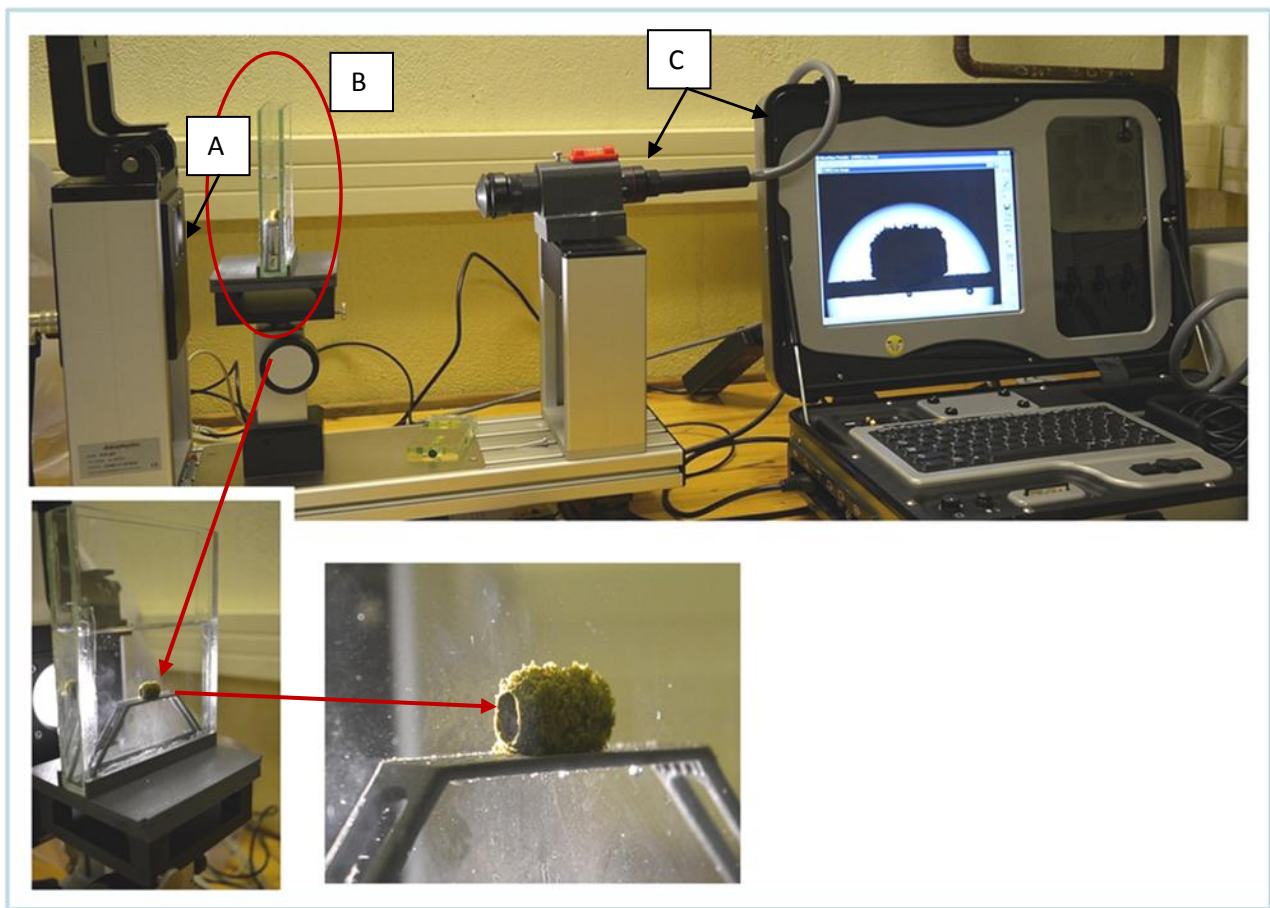


Figure 5.8. Experimental setup for the water stability measurement using Image Analysis. Items are indicated by letters: (A) light source; (B) Pellet in Glass Container; (C) Video Microscope

5.3.8. Surface hydration / Contact Angle

The surface hydration of the compacted material was performed by θ (angle) measurements on the pellet surface. For the respective measurements the material was compressed as described in the above Pelleting method. The θ measurements were conducted at room temperature based on optical θ measuring device OCA 15EC (DataPhysics Instruments GmbH, Germany) (fig.5.11.). A defined volume of distilled water (2 μl) was disposed from a dosing syringe on the upper plane surface of pellet and video of drop absorption was recorded. θ and its changes with time were calculated by SCA 20 software.

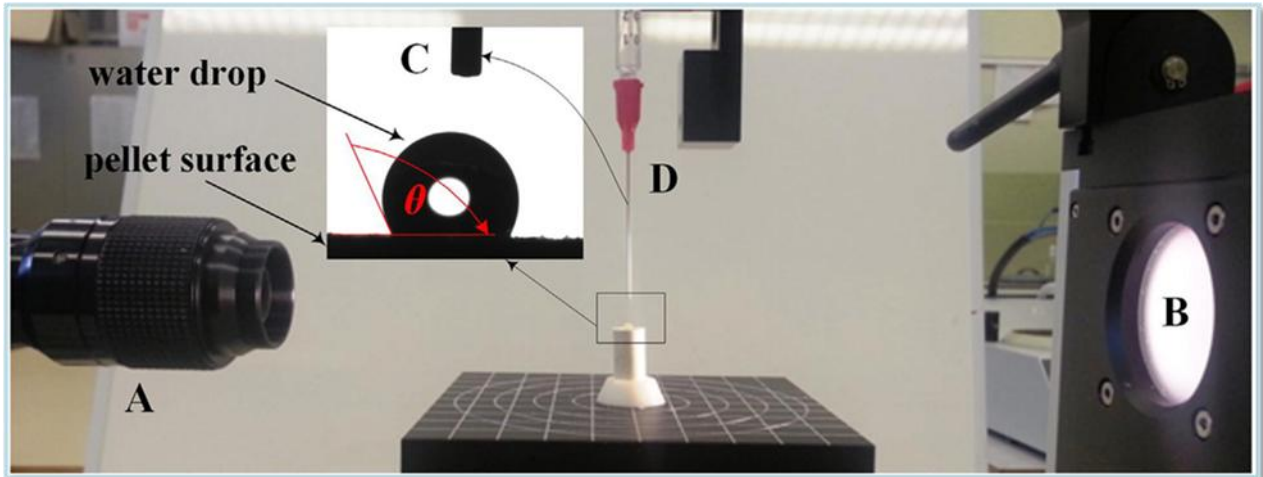


Figure 5.9. Experimental setup for θ measurements. Items are indicated by letters: (A) camera; (B) light source; (C) image of a drop on top of a pellet surface for θ tests; (D) dosing syringe with a needle (Mišljenović et al. 2015)

The initial contact angle and its change as a function of time were recorded. Apparent rate of water absorption was calculated as a slope of linear relationship between θ and time at linear period of water absorption. For each product, average values and standard deviations were calculated from 7-8 measurements.



Figure 5.10. Typical example for the measurement of contact angle by the method sessile drop of compact (based on hard wheat flour). The initial contact angle (A) and after 3 min (B) (Roman-Gutierrez et al. 2003)

5.3.9. Statistical analyses

The data was analyzed using ANOVA – one-way analysis of variance for determining significant ($P < 0.05$) differences among tests and Tukey method for determining the extend of these differences. Statistical analysis was conducted using Minitab software (Minitab Inc, USA). Linear or polynomial non-linear least squares regressions were applied to some curves.

5.4. Results

5.4.1. Particle size distribution

In figure 5.12 and 5.13 the average particle size distribution of 10 measurements can be observed, for microalgae biomass and lignosulfonate, respectively.

Table 5.6. Particle size distribution results for microalgae biomass and lignosulfonate powders

Result	Microalgae biomass	Lignosulfonate
Concentration	0.0055 %	0.0016 %
Uniformity	1.648	0.522
Specific Surface Area	337.8 m ² /kg	187.1 m ² /kg
D [3;2]	17.8 μm	32.1 μm
D [4;3]	167 μm	167 μm
Span	4.78	1.694
Result Units	Volume	Volume
Dx (10)	7.19 μm	17.0 μm
Dx (50)	81.7 μm	57.1 μm
Dx (90)	397 μm	114 μm

In addition to the tabular data which describes the raw materials, the volume based particle size distribution of microalgae and lignosulfonate are presented in Fig.5.14 & Fig.5.15. 90% of the particles had diameters lower than 397 μm and 10% had higher than 7.19 μm for microalgae and 90% of the particles had diameters lower than 114 μm and 10% had higher than 17 μm for lignosulfonate. Ground particles in commercial pellet production are typically coarser than the ones used here. Finer grinding was chosen because the single pellet press does not simulate the effect of the ‘second grinding step’ that normally occurs when material shears between die and rollers. The mean volume diameter of particles D [4;3] was 819 μm .

In the microalgae biomass there are 2 types of microalgae (*Nanofrustulum shiloi* and *Tetraselmis sp.*), it could be reasonable to expect that there should be 2 main particle size distributions (see figure 5.12.).

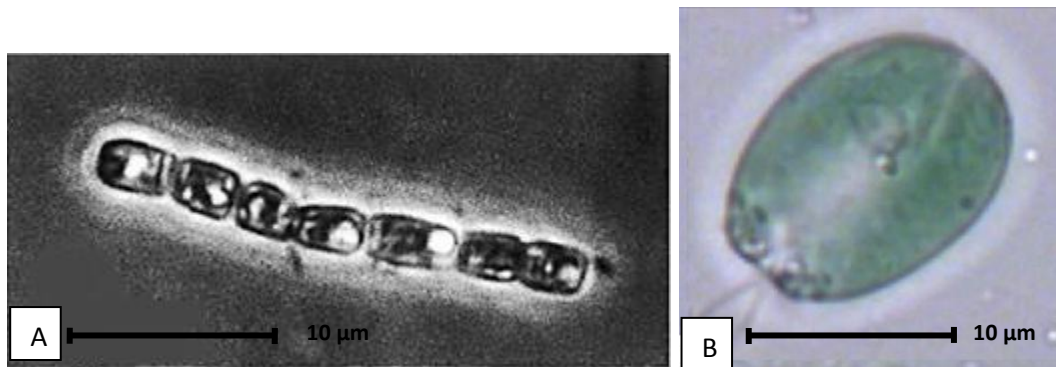


Figure 5.11. Microscope image of microalgae species. Letters indicate as follow: (A) *Nanofrustulum shiloi* in 10 μm scale bar (Sar & Sunesen 2003); (B) *Tetraselmis sp.* in a scale bar of 10 μm (Uduman et al. 2011)

In Figure 5.11. we can observe the two species of microalgae under the microscope at 10 μm scale bar with different shape and structure. As seen, *Nanofrustulum shiloi* has the cell size almost double than *Tetraselmis sp.* This might be a reason why we can observe in fig. 5.14. two main particle size distribution.

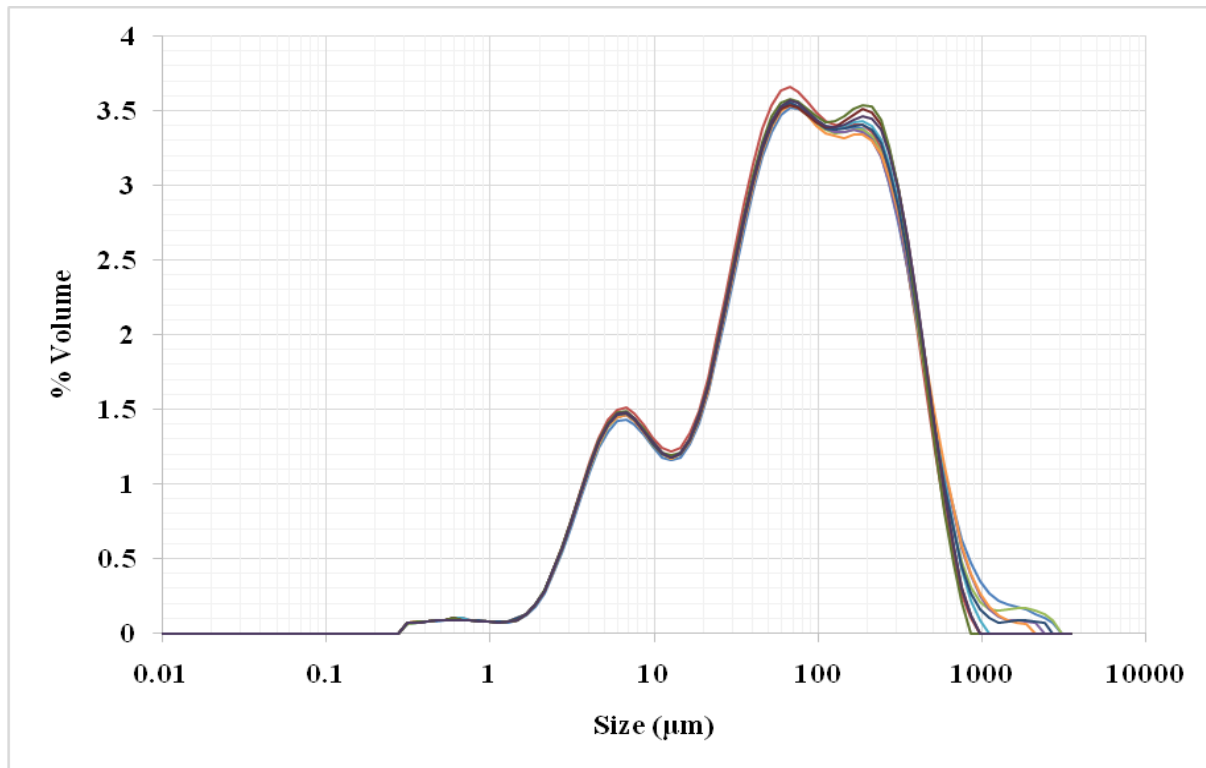


Figure 5.12. Microalgae particle size distribution for 10 measurements

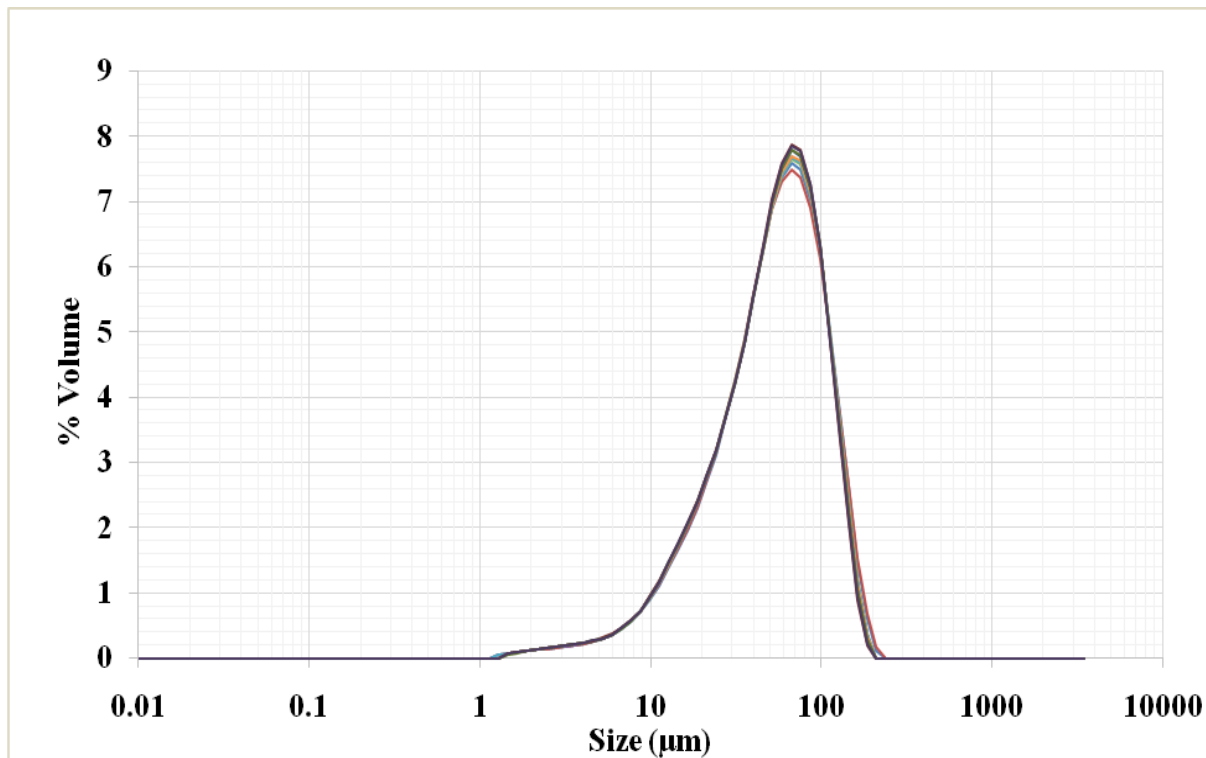


Figure 5.13. Lignosulfonate particle size distribution for 10 measurements

5.4.2. Moisture Content

Initial moisture content for microalgae biomass (control test) was ~3.3 % (96.7 % DM). To be in agreement with today's requirements of water addition in shrimp feed, a minimum of 7% moisture content was obtained after addition 4 % more of moisture. The moisture content average for all the mixes was 7.18 ± 0.5 %. The ANOVA – Tukey method in Minitab software showed no statistical differences ($p > 0.05$) between the moisture content of the four mixes (Table 5.7), this shows that a good mix of the four tests has been achieved. A confidence interval of 95% was used for the mean.

Table 5.7. Average moisture content of pellets.

	C	C+LS	C+NSP	C+Protease
Average moisture content	7.03 ^a	7.03 ^a	7.1 ^a	7.5 ^a

a– grouping number for ANOVA Tukey Pairwise Comparisons Method

5.4.3. Physical Characterization of Pellets – Hardness / Tensile Strength

Pellets produced in the single pellet press have a well defined cylindrical shape. In this experiment the control pellets and the mixed ones were well-formed, with smooth surface.

An experiment was performed for control (pure microalgae) with pellets made at four different pressures (7, 11.6, 23.3 and 35 MPa) to observe if it has any effect on the density, the hardness and tensile strength of the pellets. It was observed that the density did not change with increasing the compacting pressure (fig.5.16.); the mean value for density was 1.37 ± 0.02 g/cm³ ($p > 0.05$).

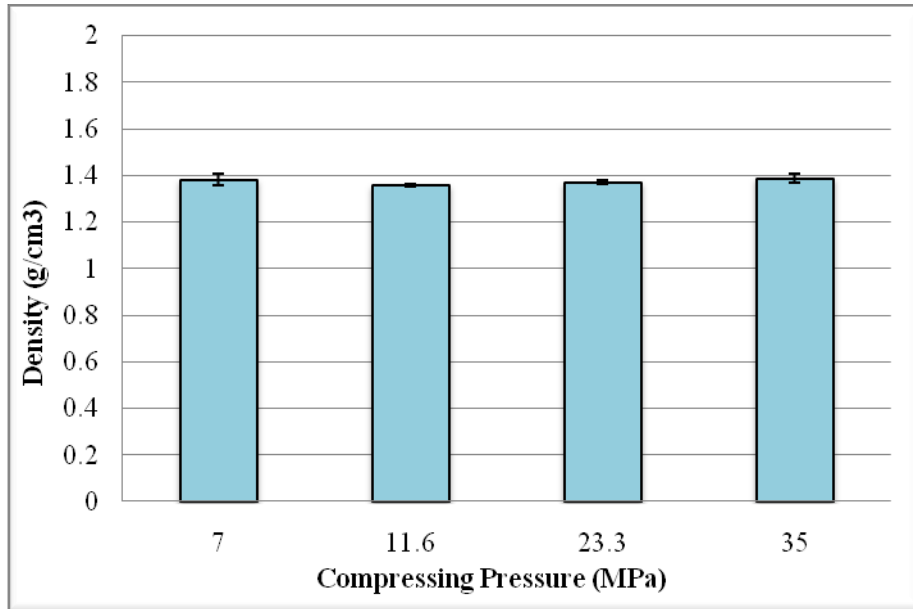


Figure 5.14. Density of control pellets at four different compressing pressures (7, 11.6, 23.3 and 35 MPa) with standard deviation error bars. Data represents the mean of three replicates.

The strength of the pellets was evaluated by a diametric compression test, where the peak force was recorded and the results are expressed as the maximum force per length of pellet (N/mm). The peak force represents the point where the pellet cannot keep its cohesive structure and breakage occurs. After observing the particle breakage of the microalgae pellets, tensile strength has become another important parameter to be taken under consideration (figure 5.16.), the pellets were brittle, which made them suitable for calculating the tensile strength through the Brazilian method. The Brazilian method is commonly used for indirect determination of tensile strength from the information obtained from a diametric compressive test (Sinka et al. 2007). Tensile strength can be defined as the force per unit of area required to simultaneously break all contacts in the fracture plane (Ghadiri et al. 2007). In practice it is important to determine the tensile strength as a function of compaction pressure, which is the principal process variable affecting strength. The formula used to calculate the tensile strength is: $\sigma_t = \frac{2P}{\pi Dt}$, where σ_t (MPa) is the maximum tensile strength, P (N) is the applied load at fracture, D (mm) the pellet diameter and t (mm) the pellet thickness/length (Sinka et al. 2007)

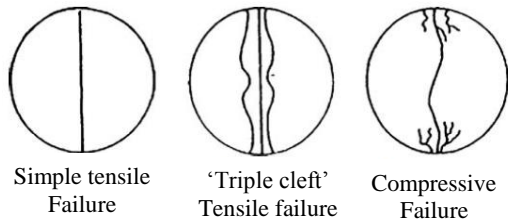


Figure 5.15. Failure modes in diametrical compression test of tensile strength (Sinka et al. 2007)

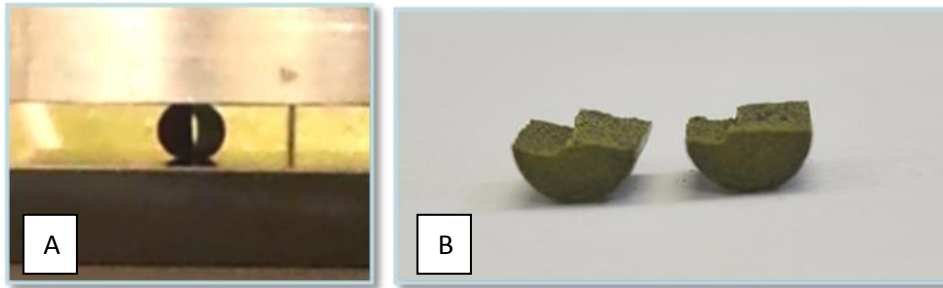


Figure 5.16. Simple tensile failure mode in diametrical compression of tensile strength for microalgae control pellets. Letters indicate as follows: (A) Initial breakage of pellet while compressed; (B) View after the pellet was broken by the compression test.

As seen in figure 5.15. there are three failure modes of tensile strength when a pellet is diametrically compressed: simple fracture mode involves the pellet being fractured into two almost equal pieces along the loaded diameter, in “triple cleft” the pellet is splitting into three or more pieces with fracture consisting of a central normal tensile and two nominally collinear fractures on either side of the central fracture (Ovri & Ndukwe 2014), for compressive failure the fracture is more of a collapse of the pellets particles.

In fig.5.17 it can be observed an increase in the tensile strength with the increase in compressing pressure. On the basis of post-hoc Tukey’s test ($p < 0.05$) there was no statistically significant difference in the tensile strength of pellets made at different compressing pressures, except between 11.6 MPa and 35 MPa.

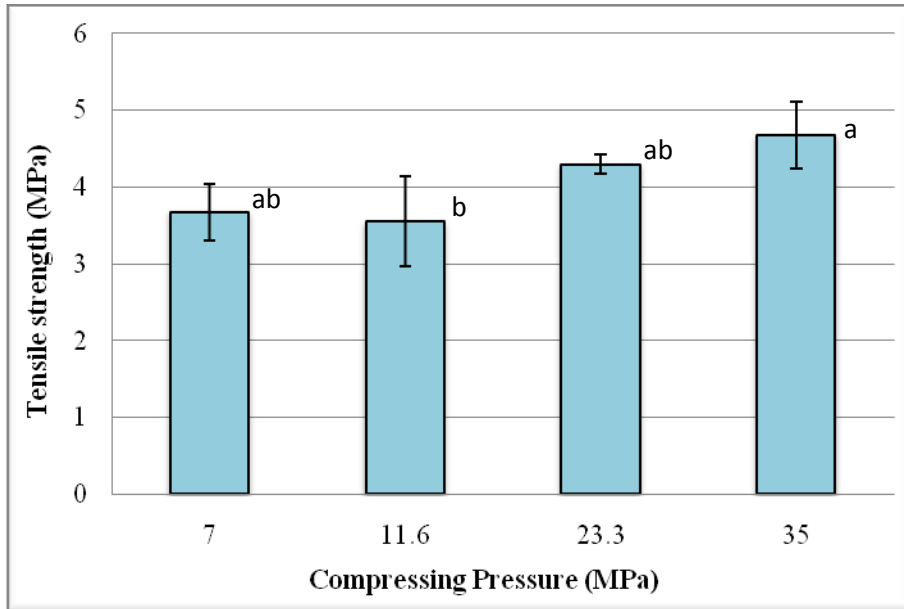


Figure 5.17. Tensile strength of control pellets at four different compressing pressures (7, 11.6, 23.3 and 35 MPa). Error bars represent the standard error of the mean ($n = 3$, where is the number of the replicates).

a.b - Letters for ANOVA Tukey Pairwise Comparisons test.

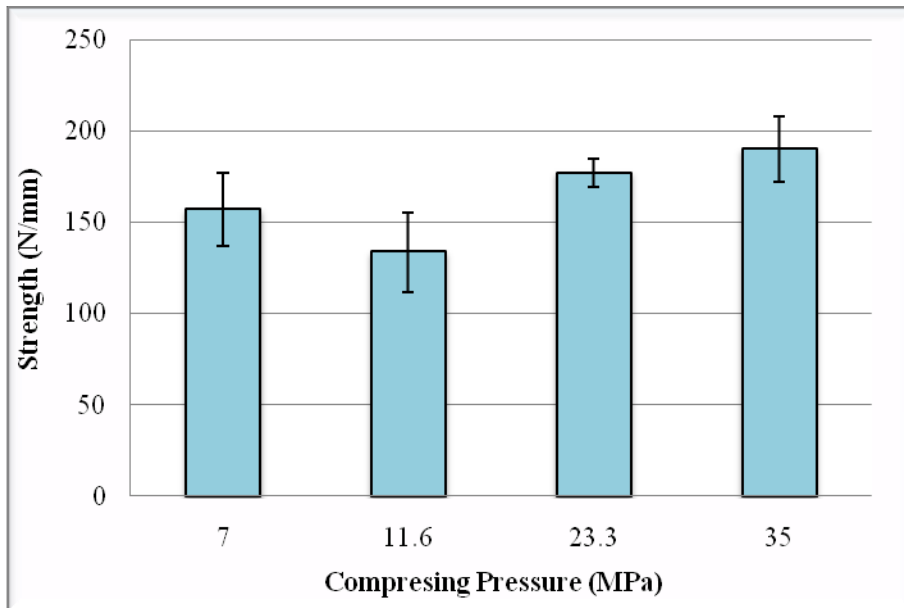


Figure 5.18. Hardness of control pellets at four different compressing pressures (7, 11.6, 23.3 and 35 MPa). Error bars represent the standard error of the mean ($n = 3$).

Due to the fact that the compressing pressure didn't influence the density of the pellets and the different pressures also didn't had a high effect on the tensile strength of the pellets, one

compacting pressure was chosen for the production of the four tests pellets, 12 MPa, representing the commercially used compacting pressure for shrimp feed.

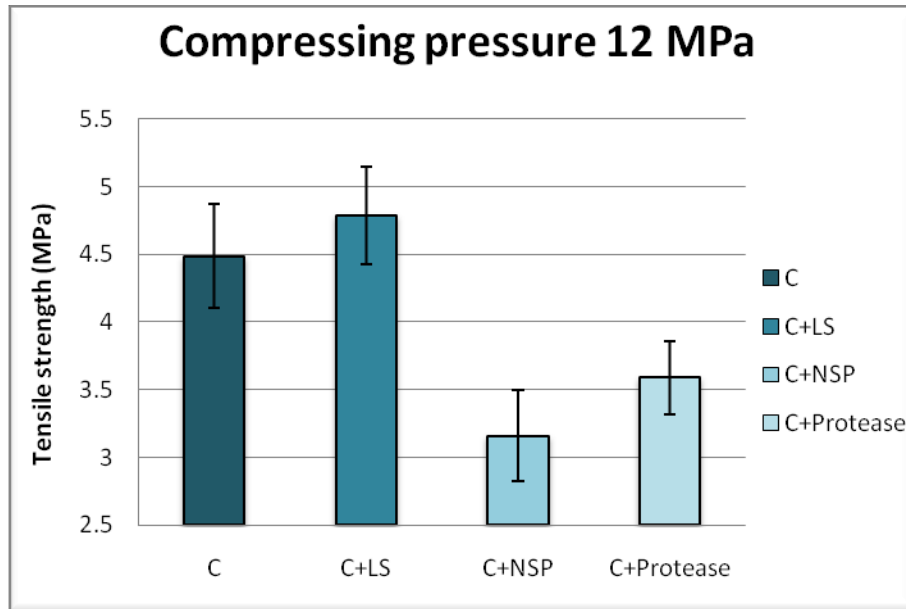


Figure 5.19. Tensile strength of the four tests (C, C+LS, C+NSP, C+Protease) produced at 12 MPa compressing pressures. Error bars represent the standard error of the mean ($n = 3$).

For hardness measurement the strength was taken under consideration as a parameter. On the basis of post-hoc Tukey's test ($p < 0.05$) there was no statistically significant difference in the strength. Hardness test indicates that pellets with addition of proteases are more durable than C+Protease, so more water stable, measured by the amount of strength that was necessary to break the pellet. C+LS gave the hardest pellets with the highest tensile strength (Table 5.8.) and highest a_w , while C+NSP was the weakest in all the parameters.

Table 5.8. Physical pellet quality characteristics with addition of LS and enzymes

Diets	Density g/cm ³	Tensile strength MPa	Hardness N/mm	Aw
C	1.38 ^a ± 0.01	4.48 ^{ab} ± 0.38	183.99 ^{ab} ± 11.80	0.378 ^a ± 0.005
C+LS	1.39 ^a ± 0.01	4.78 ^a ± 0.36	205.25 ^a ± 22.28	0.473 ^b ± 0.003
C+NSP	1.40 ^a ± 0.01	3.15 ^c ± 0.33	124.21 ^c ± 13.70	0.449 ^c ± 0.003
C+Protease	1.39 ^a ± 0.01	3.58 ^{bc} ± 0.26	150.20 ^{bc} ± 18.24	0.464 ^d ± 0.003

n=3

a, b, c, d – grouping letters for ANOVA Tukey Pairwise Comparisons test.

Values in verticals columns with the same letter superscript not significantly different ($p < 0.05$).

5.4.4. Water Activity (A_w)

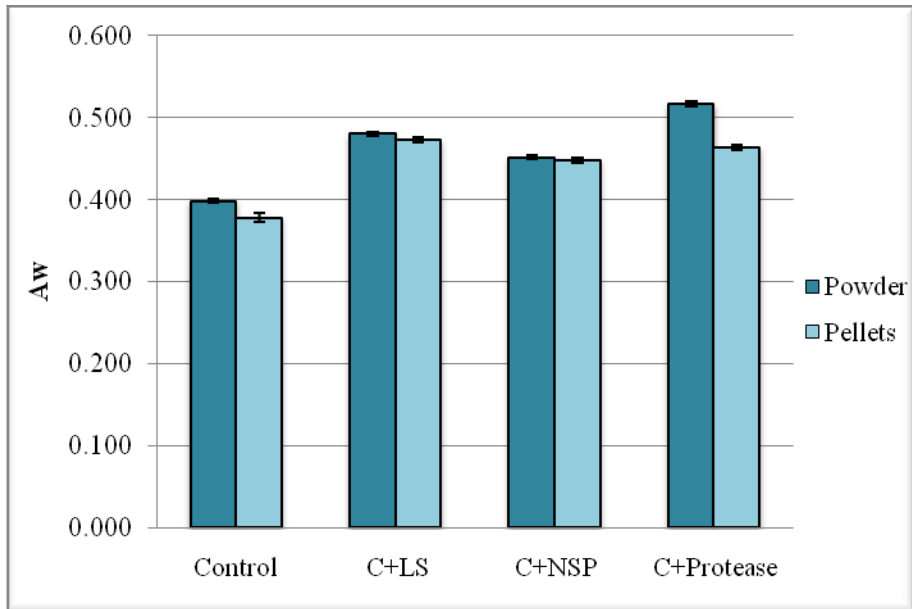


Figure 5.20. Water Activity of the four tests before and after pelleting, powder and pellets, respectively. Error bars represent the standard error of the mean ($n = 3$).

As observed in fig.5.20, a_w is increasing with addition of enzymes and LS in both powder and compressed form tests. According to Tukey's test ($p < 0.05$) there was no statistically significant difference in a_w , but as seen in table 5.9., the a_w for the analyzed tests is significantly different between all four tests. A decrease in a_w between powder form and pelleted form of the mixes was observed. This is in agreement with the theory, since compacted powders have less space to bind the water.

5.4.5. Measurements through Image Analysis

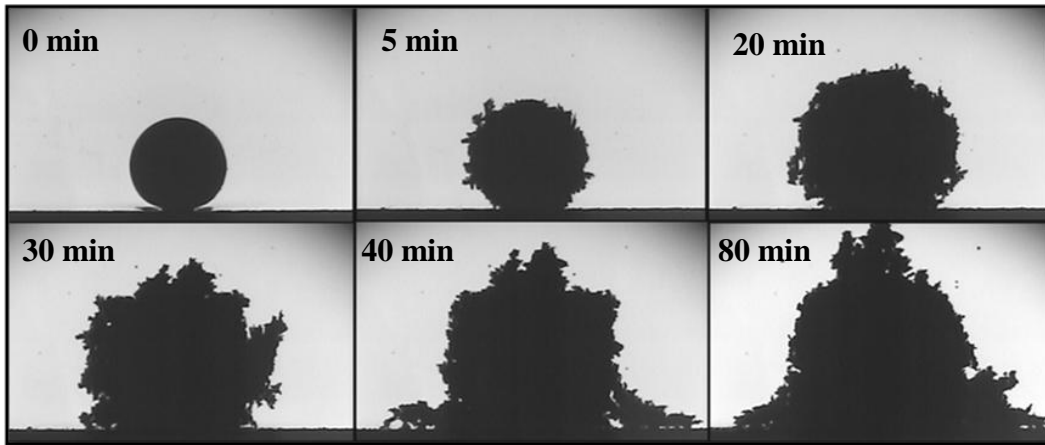


Figure 5.21. Pellet swelling rate at different time intervals measured through Fiji software image analysis as described in Annex 1.

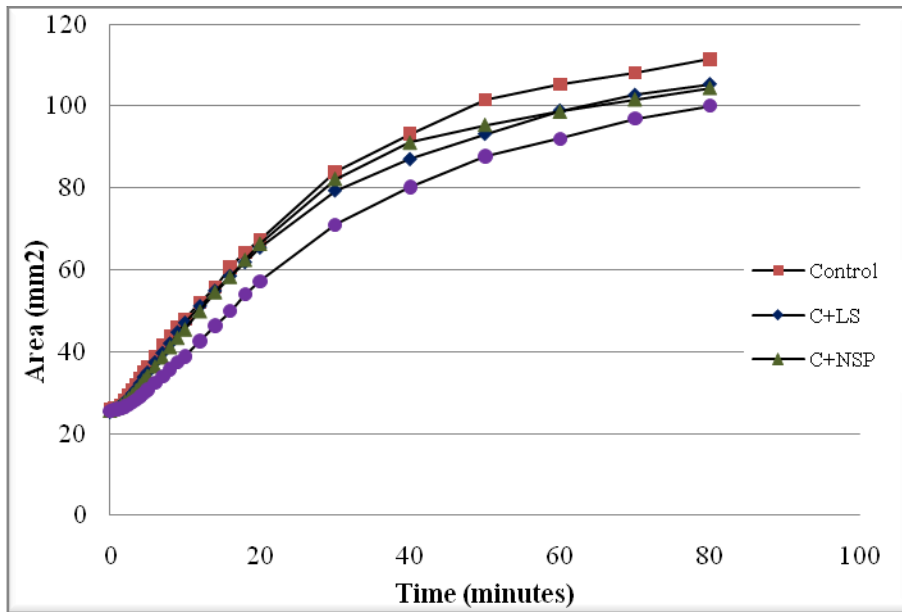


Figure 5.22. Pellet swelling rate

The pellet started deteriorating after only 5 minutes immersion with the outer shell becoming extremely soft and wet. However, even after 80 min of immersion, the inner core remained hard and easily to observe (Fig 5.23.).

Table 5.9. swelling rate of pellets after 80 minutes immersion in room temperature water (aprox 20°C).

Tests	Area increase (mm ²) after 80 min time - immersion
C	111.48 ^a ± 1.73
C+LS	105.33 ^{ab} ± 2.45
C+NSP	104.45 ^{ab} ± 1.25
C+Protease	100.06 ^b ± 7.00

n=3

Values in horizontal columns with the same letter superscript not significantly different ($p < 0.05$)

The results of the water stability test (swelling rate) are presented in Fig.5.22. At the initial 0 time of soaking in water, all diets looked similarly stable. From 5 min point a separation in the swelling rate is starting to be observed. Swelling rate was decreased with the addition of LS, NSP and protease. On the other hand water stability was increased with swelling rate decreasing. At the end of 80 min, the water stability of all diets was found to be not significantly different from a statistically point of view.

On the basis of post-hoc Tukey's test ($p < 0.05$) there was no statistically significant difference in the swelling rate of pellets after 80 min of stable water immersion.

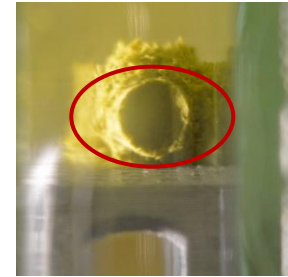


Figure 5.23. Swollen pellet in stable water after 80 min immersion time
Red circle represents the inner core of the pellet

5.4.6. Surface hydration / Contact Angle

The wettability properties of microalgae pellets were determined by direct measurements of θ of a sessile water drop deposited on the upper surface of the compacts. Water absorption was evaluated by changes in θ over time. The compaction of powders is a critical step for this measurement because it can alter the surface of pellet and porosity of pellet and in a way not represent the original powder properties. As a typical example, the changes in contact angle as a function of time for compacts based on microalgae powder are presented in Figure 5.24.

An initial contact angle of 68° was observed for compacted microalgae powder that served as control (Table 5.10). Immediately after the deposition of the water drop, a rapid absorption of the water inside the compact was observed. After few minutes (4-8 min, control and C+protease, respectively), the drop was absorbed by the compact. Apparent water absorption rates were

calculated from the polynomial equations with an order of fit of 10 that showed the decrease in θ as a function of time (Figure 5.24.).

On the basis of post-hoc Tukey’s test no statistically significant difference ($p < 0.05$) in initial θ and water absorption rate between control and enzyme addition tests was observed (Table 5.10.). According to the values of initial θ all surfaces can be considered as hydrophilic ($\theta < 90^\circ$).

Table.5.10. Averages of the initial contact angle

Tests	ICA
Control	68.43 ^b ± 2.79
C+LS	69.25 ^{ab} ± 3.03
C+NSP	70.88 ^{ab} ± 3.03
C+Protease	72.70 ^a ± 2.28

Values in horizontal columns with the same letter superscript not significantly different

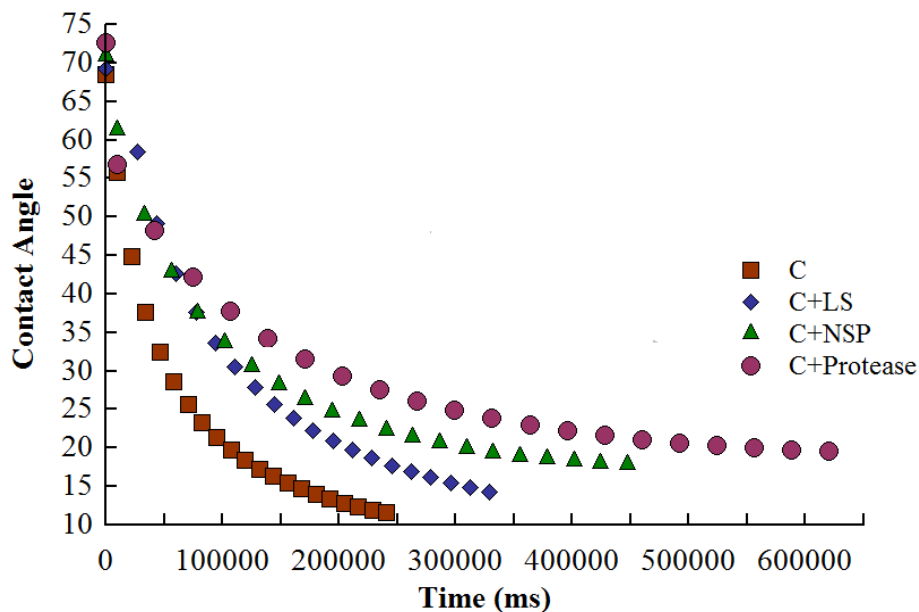


Figure 5.24. Water absorption rate

Pellets with added proteases absorbed water at a slower absorption rate than pellets with NSP and LS. They had the higher initial contact angle of the water drop which makes them higher in hydrophobicity.

The difference can also be observed from Figure 5.25 where the drops on the top pellet surfaces at different materials and different time intervals were presented. After 20 s water drop is much

more absorbed for C than for the other tests. When adding LS and enzymes the material becomes more hydrophobic and the water drop takes more time to be absorbed. This can be attributed to differences in binding between the particles once the additives are being introduced.

The water absorption through the pellet surface involves: 1) intra-particle diffusion, meaning the penetration of water inside the wood fibers; and 2) inter-particle diffusion, meaning the penetration of water through voids between wood particles. The first path can be limited due to collapsing of fibers exposed to high pressure during compaction. The second path depends on level of compaction and particle binding capability which can be correlated with the porosity of compacted surface.

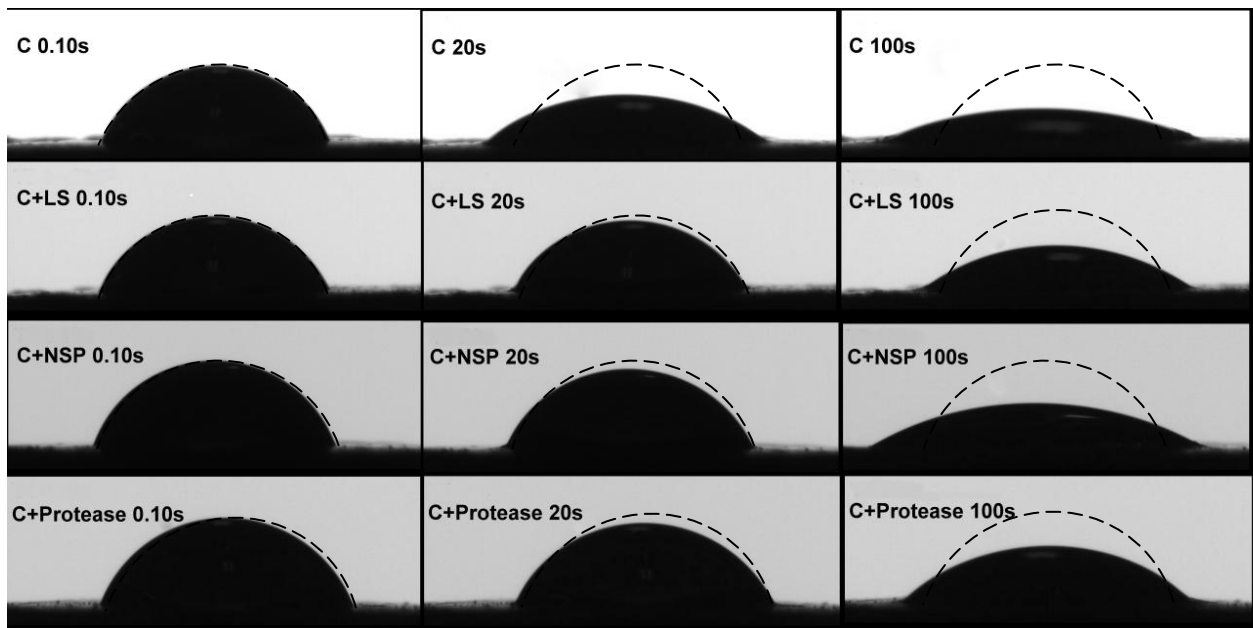


Fig.5.25. Sessile drop on the top pellet surface at different time intervals. Dash line represents initial drop profile.

5.5. Discussion

The results showed that addition of LS and enzymes clearly affects the physical characteristics of microalgae pellets. The small difference between the four experimental tests might be due to the microalgae compositions that already contain binding properties that give them good compacting properties with good water stability. The improvements that come with the addition regarding

strength, water stability and water absorption rate are in agreement with earlier studies (Buchanan et al. 1997; Lee & Lawrence 1985; Shimei et al. 2007).

Low a_w values of the tested mixture show that growth of most organisms is not possible, so there is no microbial proliferation (Figura & Teixeira 2007). Bacteria usually require at least 0.91, and fungi at least 0.7 (Rahman & Labuza 1999). A_w does not influence only the microbial growth, but also chemical and enzymatic reactivity. Water may influence chemical reactivity in different ways; it may act as a solvent, reactant, or change the mobility of the reactants by affecting the viscosity of the system. It influences nonenzymatic browning, lipid oxidation, degradation of vitamins and other nutrients, enzymatic reactions, protein denaturation, starch gelatinization and starch retrogradation. As the water activity is lowered, the rate of chemical degradative reactions decreases. Affects, also the texture of the feed, lower a_w gives more hard, dry and tough products. Generally, this information indicates that potential storage problems can rarely occur because of the nonexistent microbial proliferation. LS and enzyme can bind more water.

Fineness of grind can have a great deal of influence on pellet quality. As a rule, the finer the grind, either pre- or post- grind, the better the pellet quality. Particle size affects both the extent of conditioning and the way in which particle bonding occurs in the pellet itself (Behnke 2001), making it either more or less compact. Obaldo et al. (1999) indicated that pellet water stability, pellet durability, starch gelatinization, shrimp live weight, and weekly weight gain were significantly enhanced ($P < 0.05$) by reduction of ingredient particle size to 124 μ . The energy required to grind was lowest at 586 μ or coarser particle size and increased exponentially the ingredient particle size became smaller. Binding mechanisms during pelleting can be divided in five groups: solid bridges, adhesion and cohesion forces, surface tension and capillary pressure, attraction forces between solids and interlocking bonds.

The tensile strength increases as the compaction pressure is increased and this trend is observed for most pharmaceutical materials (Sinka et al. 2007). High pressures are, however, not always desirable. After a certain threshold defects such as cracks and lamination start appearing in most materials, thus the strength is reduced. Also, the porosity is decreased at high pressures, which can impede on fluid ingress (Sinka et al. 2007). Formulated pellet of control (pure microalgae)

with addition of LS is more compact needing a higher strength to break it. The potential for utilization of LS is of high interest and can give very good stable pellet for shrimp feed. In the context of animal feed pellets, bending tests and tensile tests in general may give more information compared with compression tests (Thomas & Van der Poel 1996) but are of less value due to the type of attrition that feed pellets undergo between manufacturing in the feed mill and the animal feeding trough.

Water stability is of considerable significance, both to prevent development of unfavorable pond conditions and to achieve a more reliable and economically feed conversion rate. Substituting fish meal with plant based proteins requires addition of certain enzymes to help the animal in digesting the feed (Shimei et al. 2007). In shrimp feed formulation, water stability and water absorption rate are the main issues. Findings showed that diet with addition of protease had the highest water stability by having the lowest swelling rate. The control feed had also good stability, probably due to the fact that microalgae are good binders by their composition in alginates. The addition of LS, NSP and protease helps in improving the water stability of the microalgae material. LS at the tested concentration had shown a small increase in water stability, Higher molecular weight polymers are, in general, less soluble than low molecular weight polymers and thus tend to be more highly adsorbed. This may be reversed if the lower molecular weight material has more hydrogen bonding groups or other bonding structures, or if the higher molecular weight material has more solubilizing groups. By measuring the θ and its changes with time, it was possible to estimate pellet hydrophobicity and how the materials will behave in direct contact with water, situation that can happen during wet storage conditions (rain and condensation). The method proved to be faster compared to conventionally used methods to test hydrophobicity. These characterizations can be used to compare and rank how stable a pellet can be under water or how hydrophobic it becomes with addition of different materials. C+Proteases has proven to give more hydrophobic character to the microalgae pellets by having a lower absorption rate of the water drop.

From the description and characterization of liginosulfonate it is easier to understand why lignin-based additives might be able to influence the shelf life of the novel aquatic feed ingredients in a positive manner. For a future experimental work on this subject it should be taken into consideration a lab-based digestibility study due to the energy concentration in the dilution that

lignin-based additives are producing. Lignosulfonates are a better alternative to the traditional stabilizers that are found now on the market, due to their renewable source and the negligible toxicity. Pre-addition of enzyme to the control microalgae pellets upgraded their stability and hidrophobicity and improved their physical quality.

In summary, proteases addition produced pellets of a superior durability and water stability.

Pellet physical qualities were improved with the addition of enzymes and LS.

There appear to be no previous reference to this type of study, characterizing physical properties of microalgae pellets with different additives. This is maybe due to a more intensified study on the chemical characterization of the use of microalgae biomass in aquatic feed and its effect on the animals' health than their rheological properties. From the practical point of view, future studies should incorporate pilot scale testing and finding optimal process parameters in order to compromise pellet quality and energy requirements for pelletizing.

Conclusion

Under the test condition for the present study experiment, a significant difference, even though minimal, has been observed between the addition of lignosulfonate and enzymes (NSP and protease). Of all the additives used in this study, protease showed the best improvement of the pellet physical qualities regarding water absorption rate, water stability and hardness. Based on the results, all the additives improved the microalgae pellet physical qualities.

The image analysis method introduced for water stability characterization has shown some promising results and a future prospect for further development of the method.

Enzymes can therefore play an important role in formulating eco-friendly aquafeeds. This may help to reduce the demand for fishmeal from the aquaculture sector in coming years. The use of enzymes as a feed additive has rapidly expanded and significant instrument for the use in aqua feed. Although the economic and social benefits of enzymes have been well established, the future of feed enzymes is a bright one in aquaculture industry. Although the efficacy of enzyme supplementation in improving protein digestibility is known, there are few published reports on the effect of adding enzyme to tilapia (shrimp) diets on feed utilization, growth performance or the secretion of endogenous enzymes.

The primary purpose of enzyme application in feeds is to improve digestion. The digestive processes will work better and result shown in improved feed efficiency by providing an extra dose of enzymes. Further, aquatic animals are lack certain digestive enzymes during early development or throughout their life. In the case of fishes / shrimps lacking certain enzymes even in adulthood, application of these enzymes results in better utilization of nutrient fractions that are digested by the enzymes.

After a nutritional and functional evaluation of these novel single cell aquatic feed ingredients – microalgae, it is shown that they are a pure sustainable source of high quality fatty acids, carbohydrates, oils, protein and vitamins. However, one future step for the industry will be to analyze how this novel ingredient influences on the stability of the aquatic feeds and how to improve its shelf life after processing.

References List

- Ahamed Ali, S. (1988). Water stability of prawn feed pellets prepared using different binding materials with special reference to tapioca. *Indian Journal of Fisheries*, 35 (1): 46-51.
- Amaya, E., Davis, D. A. & Rouse, D. B. (2007). Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture*, 262 (2): 419-425.
- Anderson, J. (2014). *Global shrimp production trends*. Asean Seafood. Available at: <http://aseanseafood.org/ca322-n28826-global-shrimp-production-trends.htm#>.
- Appler, H. & Jauncey, K. (1983). The utilization of a filamentous green alga (*Cladophora glomerata* (L) Kutzin) as a protein source in pelleted feeds for *Sarotherodon* (*Tilapia*) *niloticus* fingerlings. *Aquaculture*, 30 (1): 21-30.
- Apt, K. E. & Behrens, P. W. (1999). Commercial developments in microalgal biotechnology. *Journal of Phycology*, 35: 215–226.
- Arellano-Carbajal, F. & Olmos-Soto, J. (2002). Thermostable α -1, 4- and α -1, 6-glucosidase enzymes from *Bacillus* sp. isolated from a marine environment. *World Journal of Microbiology and Biotechnology*, 18 (8): 791-795.
- Asp, N. G., Johansson, C. G., Hallmer, H. & Siljestroem, M. (1983). Rapid enzymic assay of insoluble and soluble dietary fiber. *Journal of Agricultural and Food Chemistry*, 31 (3): 476-482.
- Bailey, R. & Hunt, W. (1973). Structural carbohydrate levels in kikuyu grass and ryegrass grown under identical conditions. *New Zealand journal of agricultural research*, 16 (2): 203-205.
- Bakker, G., Dekker, R., Jongbloed, R. & Jongbloed, A. (1998). Non-starch polysaccharides in pig feeding. *Veterinary quarterly*, 20: 59-64.
- Barrett, A. J., Woessner, J. F. & Rawlings, N. D. (2012). *Handbook of proteolytic enzymes*, vol. 1: Elsevier.
- Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances*, 25: 207–210.
- Becker, W. (2004). Microalgae in human and animal nutrition. In Richmond, A. (ed.) *Handbook of microalgal culture*, pp. p. 312–351. Blackwell, Oxford.
- Behera, P. C. (2013). *Importance of feed Enzymes for Shrimp and Fish–In Sustainable Aquaculture*. Engormix. Available at: <http://en.engormix.com/MAaquaculture/articles/importancefeedenzymesshrimpt2723/p0.htm> (accessed: 8/7).
- Behnke, K. C. (2001). Factors influencing pellet quality. *Feed Tech*, 5 (4): 19-22.
- Belay, A., Kato, T. & Ota, Y. (1996). Spirulina (Arthrospira): potential application as an animal feed supplement. *Journal of Applied Phycology*, 8 (4-5): 303-311.
- Brown, M. R., Mular, M., Miller, I., Farmer, C. & Trenerry, C. (1999). The vitamin content of microalgae used in aquaculture. *Journal of Applied Phycology*, 11: 247-255.
- Browning, W. C. (1975). THE LIGNOSULFONATE CHALLENGE. *Proceedings of the Eighth Cellulose Conference.I. Wood Chemicals - A Future Challenge*.
- Buchanan, J., Sarac, H., Poppi, D. & Cowan, R. (1997). Effects of enzyme addition to canola meal in prawn diets. *Aquaculture*, 151 (1): 29-35.
- Cho, C., Hynes, J., Wood, K. & Yoshida, H. (1994). Development of high-nutrient-dense, low-pollution diets and prediction of aquaculture wastes using biological approaches. *Aquaculture*, 124 (1): 293-305.
- Choct, M. (1997). Feed non-starch polysaccharides: Chemical structures and nutritional significance. *Feed milling international*, 191: 13-26.
- Chuntapa, B., Powtongsook, S. & Menasveta, P. (2003). Water quality control using *Spirulina platensis* in shrimp culture tanks. *Aquaculture*, 220 (1): 355-366.

- Cummings, J. & Stephen, A. (2007). Carbohydrate terminology and classification. *European Journal of Clinical Nutrition*, 61: S5-S18.
- Cuzon, G., Guillaume, J. & Cahu, C. (1994). Composition, preparation and utilization of feeds for Crustacea. *Aquaculture*, 124 (1): 253-267.
- Davidson, M. H. & McDonald, A. (1998). Fiber: forms and functions. *Nutrition Research*, 18 (4): 617-624.
- Davis, D. A., Samocha, T. M., Bullis, R. A., Patnaik, S., Browdy, C., Stokes, A. & Atwood, H. (2004). Practical diets for *Litopenaeus vannamei* (Boone, 1931): working towards organic and/or all plant production diets. *Avances en Nutricion Acuicola VII. Memorias del VII Simposium Internacional de Nutricion Acuicola*: 16-19.
- De Schryver, P., Defoirdt, T. & Sorgeloos, P. (2014). Early mortality syndrome outbreaks: a microbial management issue in shrimp farming? *PLoS pathogens*, 10 (4): e1003919.
- Dersjant-Li, Y. (2002). The use of soy protein in aquafeeds. *Avances en Nutricion Acuicola VI. Memorias del VI Simposium Internacional de Nutricion Acuicola*, 3: 541-558.
- Drew, M., Racz, V., Gauthier, R. & Thiessen, D. (2005). Effect of adding protease to coextruded flax: pea or canola: pea products on nutrient digestibility and growth performance of rainbow trout (*Oncorhynchus mykiss*). *Animal feed science and technology*, 119 (1): 117-128.
- Dufossé, L., Galaup, P., Yaron, A., Arad, S. M., Blanc, P., Murthy, K. N. C. & Ravishankar, G. A. (2005). Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science and Technology*, 16: 389-406.
- El-Sayed, A.-F. M. (1999). Alternative dietary protein sources for farmed tilapia, *Oreochromis* spp. *Aquaculture*, 179 (1): 149-168.
- El Mansouri, N.-E. & Salvadó, J. (2006). Structural characterization of technical lignins for the production of adhesives: Application to lignosulfonate, kraft, soda-anthraquinone, organosolv and ethanol process lignins. *Industrial Crops and Products*, 24 (1): 8-16.
- Ernsberger, F. M. & France, W. G. (1945). Portland Cement Dispersion by Adsorption of Calcium Lignosulfonate. *Industrial & Engineering Chemistry*, 37 (6): 598-600.
- Fagbenro, O. & Jauncey, K. (1995). Water stability, nutrient leaching and nutritional properties of moist fermented fish silage diets. *Aquacultural Engineering*, 14 (2): 143-153.
- Farhangi, M. & Carter, C. G. (2007). Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 38 (12): 1274-1282.
- Farmanfarmaian, A., Lauterio, T. & Ibe, M. (1982). Improvement of the stability of commercial feed pellets for the giant shrimp (*Macrobrachium rosenbergii*). *Aquaculture*, 27 (1): 29-41.
- Feijoo-Siota, L. & Villa, T. G. (2011). Native and biotechnologically engineered plant proteases with industrial applications. *Food and Bioprocess technology*, 4 (6): 1066-1088.
- Fidalgo, J. P., Cid, A. T. E., Sukenik, A. & Herrero, C. (1998). Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalgae *Isochrysis galbana*. *Aquaculture*, 166: 105-116.
- Figura, L. & Teixeira, A. A. (2007). *Food physics: physical properties-measurement and applications*: Springer Science & Business Media.
- Francis, G., Makkar, H. P. & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199 (3): 197-227.
- Gamboa-Delgado, J., Molina-Poveda, C. & Cahu, C. (2003). Digestive enzyme activity and food ingesta in juvenile shrimp *Litopenaeus vannamei* (Boone, 1931) as a function of body weight. *Aquaculture research*, 34 (15): 1403-1411.
- Ghadiri, M., Kwan, C. C. & Ding, Y. (2007). Analysis of milling and the role of feed properties. *Handbook of Powder Technology*, 12: 605-634.

- Gosselink, R., De Jong, E., Guran, B. & Abächerli, A. (2004). Co-ordination network for lignin—standardisation, production and applications adapted to market requirements (EUROLIGNIN). *Industrial Crops and Products*, 20 (2): 121-129.
- Gouveia, L., Batista, A. P., Sousa, I., Raymundo, A. & Bandarra, N. M. (2008). Microalgae in Novel Food Products. *Food Chemistry Research Developments*, Chapter 2.
- Guill-Guerrero, J. L., Navarro-Juárez, R., López-Martínez, J. C., Campra-Madrid, P. & Reboloso-Fuentes, M. M. (2004). Functional properties of the biomass of the three microalgal species. *Journal of Food Engineering*, 65: 511-517.
- Hardy, R. W. (1996). Alternate protein sources for salmon and trout diets. *Animal Feed Science and Technology*, 59 (1): 71-80.
- Hardy, R. W. (2000). New developments in aquatic feed ingredients, and potential of enzyme supplements. *Avances en Nutrición Acuicola V. Memorias del V Simposium Internacional de Nutrición Acuicola*: 19-22.
- Hasan, M. R. & Chakrabarti, R. (2009). Use of algae and aquatic macrophytes as feed in small scale aquaculture: a review. In *Bulletin: FAO Fisheries and Aquaculture Technical Paper*, No. 531: Rome: Food and Agriculture Organization of the United Nations (FAO).
- Hilton, J., Cho, C. & Slinger, S. (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.). *Aquaculture*, 25 (2): 185-194.
- Ighwela, K. A., Ahmad, A. B. & Abol-Munafi, A. (2014). Water Stability and Nutrient Leaching of Different Levels of Maltose Formulated Fish Pellets. *arXiv preprint arXiv:1409.5062*.
- Jiang, Y., Chen, F. & Liang, S.-Z. (1999). Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate *Cryptothecodinium cohnii*. *Process Biochemistry*, 34: 633–637.
- Ju, Z., Forster, I. & Dominy, W. (2009). Effects of supplementing two species of marine algae or their fractions to a formulated diet on growth, survival and composition of shrimp (*Litopenaeus vannamei*). *Aquaculture*, 292 (3): 237-243.
- Knudsen, K. B. (2001). The nutritional significance of “dietary fibre” analysis. *Animal feed science and technology*, 90 (1): 3-20.
- Kovač, D. J., Simeunović, J. B., Babić, O. B., Mišan, A. Č. & Milovanović, I. L. (2013). Algae in food and feed. *Food and Feed Research*, 40 (1): 21-31.
- Kumar, S., Sahu, N., Pal, A., Choudhury, D. & Mukherjee, S. (2006). Studies on digestibility and digestive enzyme activities in *Labeo rohita* (Hamilton) juveniles: effect of microbial α -amylase supplementation in non-gelatinized or gelatinized corn-based diet at two protein levels. *Fish physiology and Biochemistry*, 32 (3): 209-220.
- Kumar, V., Sinha, A. K., Makkar, H. P., de Boeck, G. & Becker, K. (2012). Dietary roles of non-starch polysachharides in human nutrition: A review. *Critical reviews in food science and nutrition*, 52 (10): 899-935.
- Kureshy, N. & Davis, D. A. (2002). Protein requirement for maintenance and maximum weight gain for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture*, 204 (1): 125-143.
- Kuz'mina, V. (1996). Influence of age on digestive enzyme activity in some freshwater teleosts. *Aquaculture*, 148 (1): 25-37.
- Laurichesse, S. & Avérous, L. (2013). Chemical modification of lignins: Towards biobased polymers. *Progress in Polymer Science*: Article in press.
- Le Chevalier, P., Sellos, D. & Van Wormhoudt, A. (2000). Molecular cloning of a cDNA encoding alpha-glucosidase in the digestive gland of the shrimp, *Litopenaeus vannamei*. *Cellular and Molecular Life Sciences CMLS*, 57 (7): 1135-1143.

- Le Moullac, G., Klein, B., Sellos, D. & Van Wormhoudt, A. (1997). Adaptation of trypsin, chymotrypsin and α -amylase to casein level and protein source in *Penaeus vannamei* (Crustacea Decapoda). *Journal of Experimental Marine Biology and Ecology*, 208 (1): 107-125.
- Lee, P. G., Smith, L. L. & Lawrence, A. L. (1984). Digestive proteases of *Penaeus vannamei* Boone: relationship between enzyme activity, size and diet. *Aquaculture*, 42 (3): 225-239.
- Lim, C. & Cuzon, G. (1994). Water Stability of Shrimp Pellet - A Review. *Asian Fisheries Science*, 7: 115-127.
- Liu, C. P. & Lin, L. P. (2001). Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP1324. *Botanical Bulletin Academia Sinica*, 42: 207-214.
- McCapes, R., Ekperigin, H., Cameron, W., Ritchie, W., Slagter, J., Stangeland, V. & Nagaraja, K. (1989). Effect of a new pelleting process on the level of contamination of poultry mash by *Escherichia coli* and *Salmonella*. *Avian diseases*: 103-111.
- Metting, F. B. (1996). Biodiversity and application of microalgae. *Journal of Industrial Microbiolog*, 17: 477-489.
- Michael, B. (1976). *A review of shrimp and prawn nutrition*. Proceedings of the annual meeting-World Mariculture Society: Wiley Online Library. 277-287 pp.
- Mišljenović, N., Mosbye, J., Schüller, R. B., Lekang, O.-I. & Salas-Bringas, C. (2015). Physical quality and surface hydration properties of wood based pellets blended with waste vegetable oil. *Fuel Processing Technology*.
- Muller-Feuga, A. (2000). The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology*, 12: 527-534.
- Nisperos-Carriedo, M. O., Baldwin, E. A. & Shaw, P. E. (1991). Development of an edible coating for extending postharvest life of selected fruits and vegetables. *J. Amer. Hort. Sci*, 107: 57-60.
- Obaldo, L. G., Dominy, W. G., Terpstra, J. H., Cody, J. J. & Behnke, K. C. (1999). The impact of ingredient particle size on shrimp feed. *Journal of Applied Aquaculture*, 8 (4): 55-67.
- Obaldo, L. G., Divakaran, S. & Tacon, A. G. (2002). Method for determining the physical stability of shrimp feeds in water. *Aquaculture research*, 33 (5): 369-377.
- Ochoa-Solano, J. L. & Olmos-Soto, J. (2006). The functional property of *Bacillus* for shrimp feeds. *Food microbiology*, 23 (6): 519-525.
- Olvera-Novoa, M., Dominguez-Cen, L., Olivera-Castillo, L. & Martínez-Palacios, C. A. (1998). Effect of the use of the microalga *Spirulina maxima* as fish meal replacement in diets for tilapia, *Oreochromis mossambicus* (Peters), fry. *Aquaculture research*, 29 (10): 709-715.
- Ouyang, X., Qiu, X. & Chen, P. (2006). Physicochemical characterization of calcium lignosulfonate—a potentially useful water reducer. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 282: 489-497.
- Ovri, J. & Ndukwe, A. (2014). The Densification and Diametral Compression Strength of Isi-Ogwuta Clay.
- Paez-Osuna, F. (2001). The environmental impact of shrimp aquaculture: causes, effects, and mitigating alternatives. *Environmental Management*, 28 (1): 131-140.
- Patnaik, S., Samocha, T., Davis, D., Bullis, R. & Browdy, C. (2006). The use of HUFA-rich algal meals in diets for *Litopenaeus vannamei*. *Aquaculture Nutrition*, 12 (5): 395-401.
- Phang, S., Miah, M., Yeoh, B. & Hashim, M. (2000). *Spirulina* cultivation in digested sago starch factory wastewater. *Journal of Applied Phycology*, 12 (3-5): 395-400.
- Primavera, J. H. (1998). Tropical shrimp farming and its sustainability. *Tropical Mariculture. Academic Press, London*: 257-289.
- Puente, X. S. & López-Otín, C. (2004). A genomic analysis of rat proteases and protease inhibitors. *Genome research*, 14 (4): 609-622.

- Pulz, O. & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*, 65: 635-648.
- Radhakrishnan, S., Bhavan, P. S., Seenivasan, C., Shanthi, R. & Muralisankar, T. (2014). Replacement of fishmeal with *Spirulina platensis*, *Chlorella vulgaris* and *Azolla pinnata* on non-enzymatic and enzymatic antioxidant activities of *Macrobrachium rosenbergii*. *The Journal of Basic & Applied Zoology*, 67 (2): 25-33.
- Rahman, M. S. & Labuza, T. P. (1999). Water activity and food preservation. *Handbook of food preservation*: 339-382.
- Roman-Gutierrez, A., Sabathier, J., Guilbert, S., Galet, L. & Cuq, B. (2003). Characterization of the surface hydration properties of wheat flours and flour components by the measurement of contact angle. *Powder Technology*, 129 (1): 37-45.
- Rout, R. K. & Bandyopadhyay, S. (1999). A comparative study of shrimp feed pellets processed through cooking extruder and meat mincer. *Aquacultural Engineering*, 19 (2): 71-79.
- Ruscoe, I., Jones, C., Jones, P. & Caley, P. (2005). The effects of various binders and moisture content on pellet stability of research diets for freshwater crayfish. *Aquaculture nutrition*, 11 (2): 87-93.
- Safarian, N. R., Safari, O. & Vakili, R. (2013). Study on the quality control of commercially cold-pellet and extrude diets of rainbow trout (*Oncorhynchus mykiss*)(a case study in the Khorasan Razavi Province farms). *International Research Journal of Applied and Basic Sciences*, 5.
- Salas-Bringas, C., Filbakk, T., Skjevrak, G., Lekang, O.-I., Høibø, O. & Schüller, R. B. (2010). Assessment of a new laboratory die pelleting rig attached to a texture analyzer to predict process-ability of wood pellets. Energy consumption and pellet strength. *Annual Transactions of the Nordic Rheology Society*, 18: 77-86.
- Salas-Bringas, C., Mišljenović, N., Wicklund, T., Lekang, O.-I. & Schüller, R. B. (2011). Influence of particle size on strength of pelleted feed. *Annual Transactions of the Nordic Rheology Society*, 19: 293-301.
- Salas-Bringas, C., Catargiu, A.-M., Miladinovic, D., Schüller, R. B. & Mišljenović, N. (2015). Effects of enzymes and lignosulfonate addition on tensile strength, surface hydration properties and underwater swelling rate of microalgae pellets. *Annual Transactions Of The Nordic Rheology Society*, 23.
- Sar, E. A. & Sunesen, I. (2003). *Nanofrustulum shiloi* (Bacillariophyceae) from the Gulf of San Matías (Argentina): Morphology, distribution and comments about nomenclature. *Nova Hedwigia*, 77 (3-4): 399-406.
- Shiau, S.-Y. & Peng, C.-Y. (1992). Utilization of different carbohydrates at different dietary protein levels in grass prawn, *Penaeus monodon*, reared in seawater. *Aquaculture*, 101 (3): 241-250.
- Shiau, S.-Y. (1998). Nutrient requirements of penaeid shrimps. *Aquaculture*, 164 (1): 77-93.
- Shimei, L., Kangsen, M. & Beiping, T. (2007). Effects of exogenous enzyme supplementation in diets on growth and feed utilization in tilapia, *Oreochromis niloticus* x *O. aureus*. *Aquaculture research*, 38 (15): 1645-1653.
- ShrimpNews. (2013). *Early Mortality Syndrome (EMS) - The Perfect Killer - A Webinar*. Available at: http://www.vietlinh.vn/library/aquaculture_shrimp/tom_benh_EMS_APA13.asp.
- Sinha, A. K., Kumar, V., Makkar, H. P., De Boeck, G. & Becker, K. (2011). Non-starch polysaccharides and their role in fish nutrition—A review. *Food Chemistry*, 127 (4): 1409-1426.
- Sinka, I. C., Pitt, K. G. & Cocks, A. C. F. (2007). The strength of pharmaceutical tablets. *Handbook of Powder Technology*, 12: 941-970.
- Soltan, M. (2009). Effect of Dietary Fish Meal Replacement by Poultry By-Product Meal with Different Grain Source and Enzyme Supplementation on Performance, Feces Recovery, Body Composition and Nutrient Balance of Nile Tilapia. *Pakistan Journal of Nutrition*, 8 (4): 395-407.

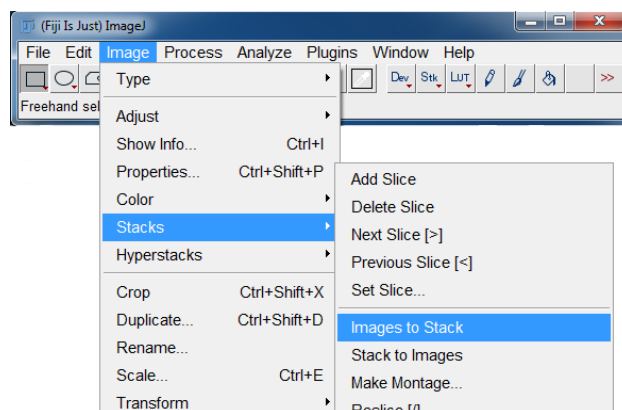
- Southan, C. (2001). A genomic perspective on human proteases as drug targets. *Drug Discovery Today*, 6 (13): 681-688.
- Spolaore, P., Cassan, C. J., Duran, E. & Isambert, A. (2006). Commercial Applications of Microalgae - Review. *Journal of Bioscience and Bioengineering*, 101: 87-96.
- Statement, E. (2011). Calcium lignosulphonate (40-65) as a food additive. *Food Standards*, Application A1030.
- Storebakken, T., Kvien, I., Shearer, K., Grisdale-Helland, B. & Helland, S. (1999). Estimation of gastrointestinal evacuation rate in Atlantic salmon (*Salmo salar*) using inert markers and collection of faeces by sieving: evacuation of diets with fish meal, soybean meal or bacterial meal. *Aquaculture*, 172 (3): 291-299.
- Takeuchi, T., Lu, J., Yoshizaki, G. & Satoh, S. (2002). Effect on the growth and body composition of juvenile tilapia *Oreochromis niloticus* fed raw *Spirulina*. *Fisheries Science*, 68 (1): 34-40.
- Thiessen, D., Campbell, G. & Adelizi, P. (2003). Digestibility and growth performance of juvenile rainbow trout (*Oncorhynchus mykiss*) fed with pea and canola products. *Aquaculture Nutrition*, 9 (2): 67-75.
- Thomas, D. A., Francis, P., Smith, C., Ratcliffe, S., Ede, N. J., Kay, C., Wayne, G., Martin, S. L., Moore, K. & Amour, A. (2006). A broad-spectrum fluorescence-based peptide library for the rapid identification of protease substrates. *Proteomics*, 6 (7): 2112-2120.
- Thomas, M. & Van der Poel, A. (1996). Physical quality of pelleted animal feed 1. Criteria for pellet quality. *Animal Feed Science and Technology*, 61 (1): 89-112.
- Toledo, M. C. F. & Kuznesof, P. M. CALCIUM LIGNOSULFONATE (40-65) Chemical and Technical Assessment. *JECFA*, 69.
- Uduman, N., Bourniquel, V., Danquah, M. K. & Hoadley, A. F. (2011). A parametric study of electrocoagulation as a recovery process of marine microalgae for biodiesel production. *Chemical Engineering Journal*, 174 (1): 249-257.
- Venou, B., Alexis, M., Fountoulaki, E., Nengas, I., Apostolopoulou, M. & Castritsi-Cathariou, I. (2003). Effect of extrusion of wheat and corn on gilthead sea bream (*Sparus aurata*) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. *Aquaculture*, 225 (1): 207-223.
- Vilchez, C., Garbayo, I., Lobato, M. V. & Vega, J. (1997). Microalgae-mediated chemicals production and wastes removal. *Enzyme and Microbial Technology*, 20 (8): 562-572.
- Xiao, B., Sun, X. & Sun, R. (2001). The chemical modification of lignins with succinic anhydride in aqueous systems. *Polymer degradation and stability*, 71 (2): 223-231.
- Yamaguchi, K. (1997). Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. *Journal of Applied Phycology*, 8: 487-502.
- Yuan, Y. & Lee, T. R. (2013). Contact angle and wetting properties. In *Surface science techniques*, pp. 3-34: Springer.

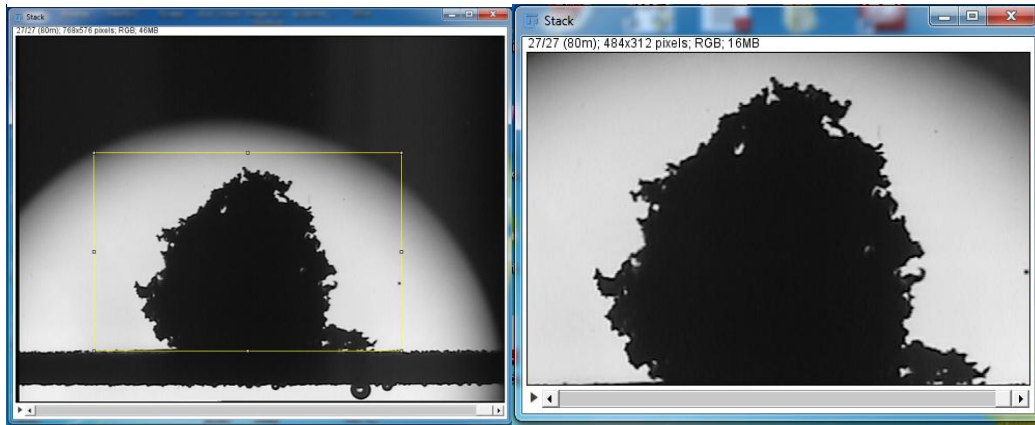
Annex 1

Measurement of pellet swelling rate using Fiji software for the analyze

Plug-in	Description
Drag and Drop	Pictures are selected in the respective folder; with left click on the selection, the pictures are dragged and dropped into the Fiji software.
Stacks	Combining the dropped pictures into a stack for a continuous analyze
Rectangular selection	Shaping the desired area for analyzing
Crop	Cutting/cropping the rectangular shape
Type – 8 bit	Converting the stack of images to 8 bit color (binary image) for Threshold.
Threshold	For applying the right color segmentation of the desired phase. The stack is being prepared for analyzing.
Ser scale	Command to specify what area statistics are recorded and add the unit of length used.
Analyze particles	Provides information about each particle in the image.
Results	Offers the calculated information for further use.
ROI Manager	A tool for working with multiple selections. The selections can be from different locations on an image or from different slices of a stack.

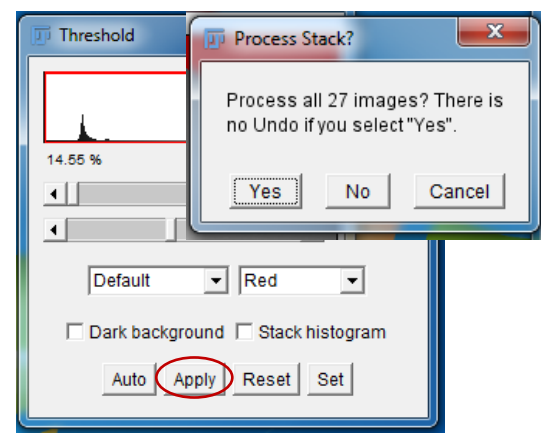
1. Open the *Fiji.app* directory on your desktop/computer and double-click on the executable (follow installation instructions from <http://fiji.sc/>).
2. In the folder containing the pictures taken with the video microscope, select all the pictures, maintain left click on the selection and drag it into the open software.
3. Go to Image command from menu and select Stacks → Images to Stack
4. Using a rectangular selection, select the area around the pellet (pay attention to select all the desired area) for a better view and calculation.
5. Go again to Image command from menu and select Crop.





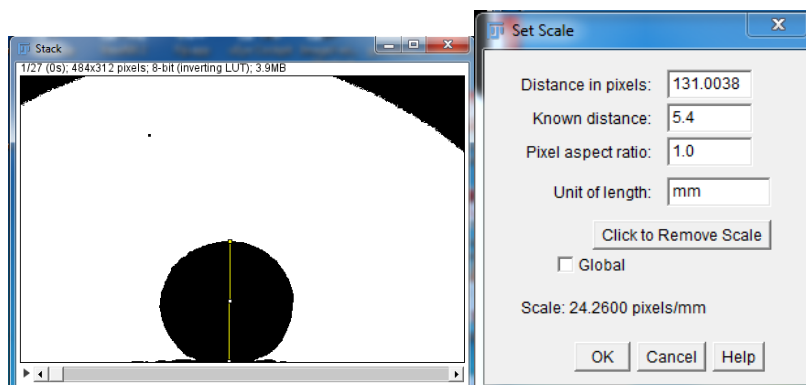
6. Go to Image command and select Type → 8 bit (color)

7. Go to Image command → Adjust → Threshold. A new window is going to open (Threshold) from where different options are available. Left click on Apply; from the new window, *Process Stack?*, you can select the option of processing all the images or to cancel the operation. In this case, left click on Yes. The picture stack will be converted into a 8-bit (inverting LUT) that can be analyzed now.

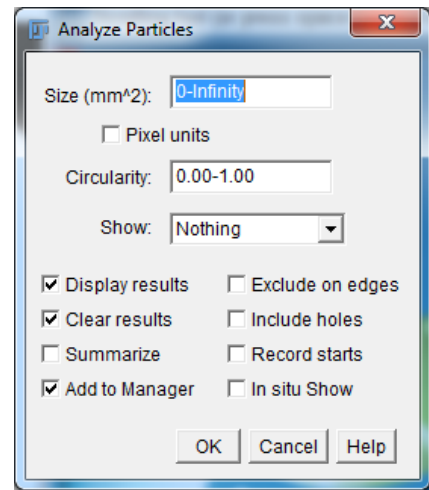


8. Close the Threshold window.

9. Select the Straight line from the Menu. Draw the line to form the pellet diameter. From Menu → Analyze → Set Scale we can set the known distance of the line drawn and the unit of length: 5.4 mm, in our case. Scale will be shown in pixels/mm.



10. Go to Menu → Analyze → Analyze particles. In the opened window different options are available for calculation and added in the Results. In our case the needed selected options are showed in the figure. Left click on OK. At the question opened now: *Process all images?*, left click on Yes. Two new Windows are going to open: Results and ROI Manager.

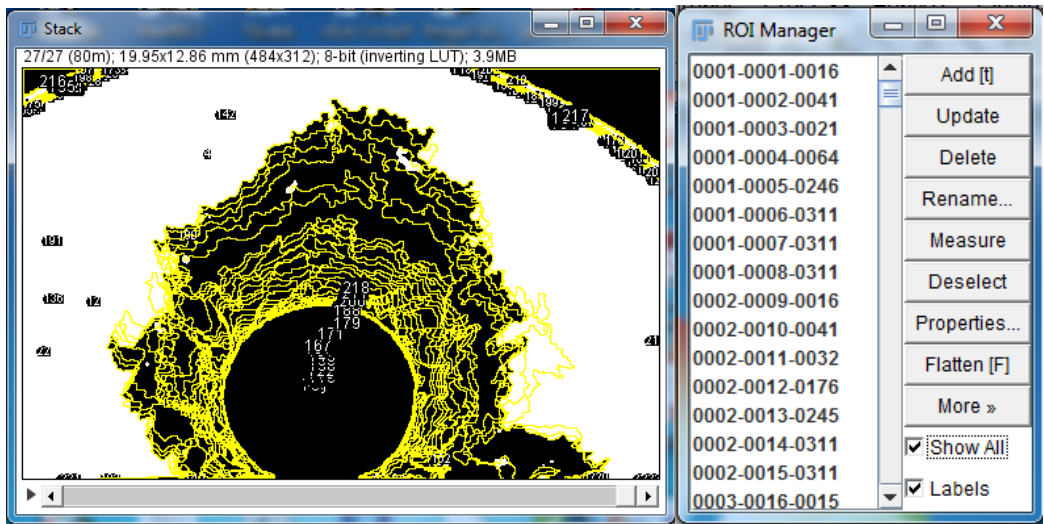


11. Result window gives all the information required from the program to calculate. The

	Area	Perim.	BX	BY	Width	Height
1	1.796	8.307	0	0	2.885	1.319
2	8.796	18.002	14.056	0	5.894	3.421
3	0.002	0.117	0.866	0.866	0.041	0.041
4	0.015	0.398	5.730	2.597	0.124	0.124
5	26.681	25.001	6.059	7.420	6.224	5.441
6	0.022	1.082	4.988	12.819	0.536	0.041
7	0.005	0.257	5.606	12.819	0.124	0.041

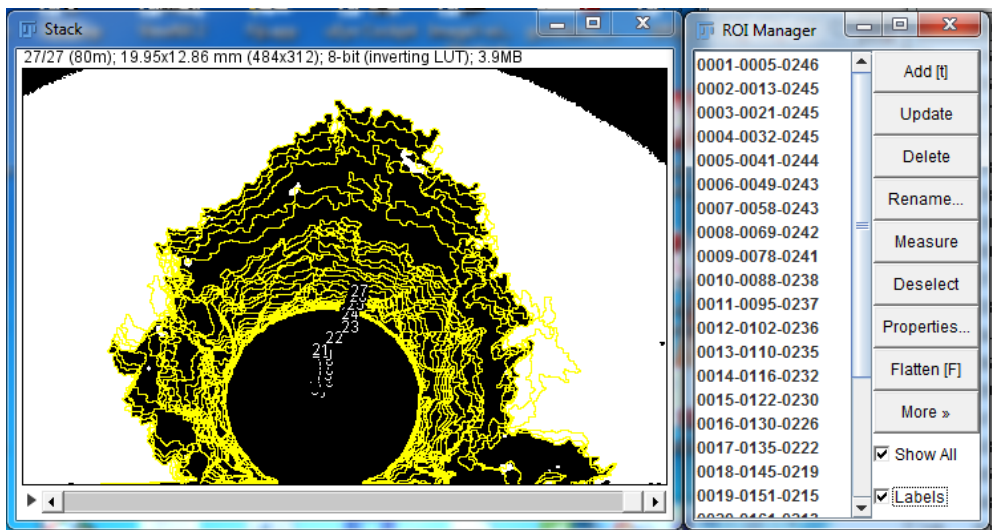
information (area, perimeter, width etc) are already calculated by the software.

12. In the ROI Management window there are the options of improving the results by removing some of the areas that are not necessary. Left click on Measure will send all the right measurements to the Results Window.



The program will give a calculation for all the areas and points that will be found in the stack pictures; this will require applying some improvement by removing some of the data. Removing data can be done by selecting the area in ROI Manager and clicking on Delete from the right side of the window.

The image above shows the picture stack before applying the improvement and removing the undesired areas.



After improvement, the final areas are ready to be sent in the result windows from where the data can be further analyzed in Microsoft Excel. The data analyzed in the present case was the area (first column in the result window)