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Department of Animal and Aqua-cultural Sciences
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Effect of steam-conditioning and enzymatic treatment on rheological properties of the shrimp feed, formulated with torula yeast (*Candida utilis*) as a novel feed raw ingredient.

Pashupati SUWAL

Master of Science in Feed Manufacturing Technology
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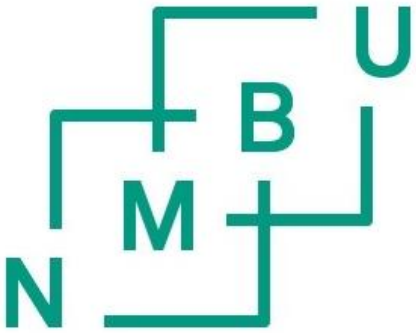

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Abstract

The use of yeast as an alternative source of protein has been in demand as a novel feed ingredient in shrimp feed nowadays. In order to optimize its use, more research and study is required. The objective of this study was to determine the possibility of using yeast as fishmeal replacer and observe the influence on the rheological properties of shrimp diet pellets due to steam conditioning and enzymatic treatment. Diets were formulated with the addition of different percentage of *Candida utilis* (CU) along with mixed enzymes (protease and endo/exo-1, 3 β- glucanase). The powder mash was compressed into pellets using a single pellet press method. Six experimental diets were formulated for the experiment. These includes: Diet 1 or positive control diet with 0% CU and 0% enzymes, diet 2 or negative control diet with 0% CU but with enzymes and remaining four diets with addition of CU to diet 2 in a percentage of 2.5% (diet 3), 5% (diet 4), 10% (diet 5) and 20% (diet 6) respectively. Rheological characters of these pellets on tensile strength (hardness), water activity (a_w), contact angle/wettability of pellets (θ_c), energy consumption (Pmax) and underwater pellet swelling rate (UPS) were studied. Tensile strength and Pmax were found to be dependent on the quantity of CU as diet 6 has the highest tensile strength and Pmax while diet 2 has the lowest. Although all the diets were found to have a_w below 0.5 indicating them as free from microbial growth, diet 5 and 6 contained slightly higher a_w enhancing their binding property. The UPS rate was significantly less for pellets with diet 6 from the starting time until the last (observation time was 40 minutes) while diet 2 having the high swelling tendency throughout the test.

The value of the initial water θ_c was not significant. However, the pellets from diet 6 and diet 1 had the high θ_c until the observation time of 2 seconds. In the oil phase, the pellets from diet 6 showed the least θ_c while diet 1 showed the highest θ_c until the observation time of 20 seconds. A significant positive correlation was observed between a_w vs. tensile strength, a_w vs. UPS rate and a_w vs. water/oil θ_c. Results showed that the presence or absence of CU in diets containing enzymes influenced rheological characters like tensile strength, Pmax, θ_c and UPS rate.

Thus, the addition of 20% CU with enzymes replacing fishmeal in shrimp diet pellets shows better results in the study of rheological characters. Further research needs to be performed before the commercial pilot feed production.

Key words

Candida Utilis (CU), protease, endo/exo-1, 3 β-glucanase, moisture content, tensile strength, water activity, Pmax, Contact angle, UPS rate

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Abbreviation

Anova	Analysis of Variance
a_w	Water activity
EFAs	Essential Fatty Acids
FM	Fishmeal
CU	*Candida utilis*
HUFA	Highly unsaturated fatty acids
L. vannamei	*Litopaneus vannamei*
Max.	Maximum
MCP	Mono Calcium phosphate
Mgo	Magnesium oxide
Mno	Manganese Oxide
MPa	Mega Pascal
mm	millisecond
ms	millisecond
MSP	Mono sodium phosphate
N	Newton
NY, NE	NO yeast, No enzyme
PUFA	Polyunsaturated fatty acids
p	probability of variance
P_{max}	Maximum pressure for pellet discharge
r	radius
SBM	Soyabean meal
SCPs	Single cell proteins
SPC	Soya Protein Concentrate
SE	Standard Error
s	second
UPS	Underwater pellet swelling rate
YY, YE	Yes Yeast, Yes Enzyme
θ_c	Contact angle
^oC	Degree centigrade
Wt.	Weight

1. Introduction

World has been changing day by day under the name of development. The present status of growth in population shows that the world's population will incline from 6.9 billion (2010) to 9.6 billion in 2050 with 38% increase rate. This leads to increase in death rate due to hunger and disease each year as per WHO estimation (Nalage et al., 2016). By that, the consumption demand of seafood by the year 2030 is predicted to reach between 150-160 million tonnes (Borgeson, Racz, Wilkie, White, & Drew, 2006). The market of world aquaculture had raised at an average annual rate of 16% for finfish and shell production in the years between 1984 & 1990 (Tacon, 1993). However, the expansion of aquaculture took much more rapidly than it was expected in 1989. In estimation, the total production of carnivorous fish and shrimp would be around 2.4 million tonnes by the year 2000. But in fact, the production of farmed marine shrimps and salmonids alone was exceeded 2.5 million tonnes by 1999(New & Wijkström, 2002). This makes the aquaculture, the fastest growing food production sector for both economy and nutritional diet.

The trend of growth rate in population indicates that an annual growth of 6.5% is necessary to fulfill the demand for seafood by 2025 (Chamberlain, 1993). Thus, there occurs the heavy reliance on fish meal and fish oil as the primary source for protein in aqua feed.

Fish meal is the major or even the sole ingredient as a protein source for the diets of shrimps and carnivorous fish. E.g. salmon, trout, marine fish (sea bream) etc. The reason behind the use of this ideal source is due to its high quality as animal protein and acts as a feeding stimulant for most finfish species (Tacon, 1997). Even, the carnivorous species need high dietary requirement for protein rich in essential amino acids (400-500g/kg diet) (Sargent & Tacon, 1999). It is a rich source of essential amino acids and fatty acids, macro and trace minerals, vitamins which are highly palatable (Oliva-Teles & Gonçalves, 2001) and highly acceptable due to its taste and texture (Sargent & Tacon, 1999). Hence, fish meal fulfils the requirement of farmed fish and provides reliable source of digestible energy (Tacon, 1993).

The increase in aquaculture production causes the need of high amount of fish meal and fish oil in aqua feeds. The main source for the fishmeal production includes pelagic species (small fish type). E.g. Chilean jack mackerel, the anchoveta, two sardines (the South American pilchard and the common sardine) etc. However, the extreme use causes the decrease in landings from these fisheries and also the decline in fishmeal production with respect to time (Bórquez & Hernández, 2009).

Though fishmeal and fish oil price had exceeded than that of soyabean oil in 1997-98, their use substantially increased over the last decade (11.6% compound growth/ year) (Sargent & Tacon, 1999). In 1996, the use of fishmeal for fish farming was huge. From the total global production, $2.0*10^6$ tonnes was used for making feeds for fish farming including shrimp farming that accounted for 20.3% of that tonnage. Likewise, in the same year, 576000 tonnes of fish oil was used in fish farming accounting 7.15% for marine shrimp farming out of total global production (Sargent & Tacon, 1999).

Thus, the extreme use of fishmeal in fish diets has led its resources limited causing serious concern on the future availability for its incorporation in such diets (Hardy, 1996) . Besides, the unstable prices, availability of cheaper alternative protein sources, threat of sustainability in environment have compelled the concerned industry to research for finding the alternative protein sources (Bórquez & Hernández, 2009). As per the study, fish meal prices doubled from USD 694 to 1379 per ton between July 2005 and July 2006 while fish oil prices almost doubled from USD 894 to 1700 per ton between march 2007 and march 2008 (Tacon & Metian, 2008).

Among the study of various alternatives, plant feedstuffs has been presented as the most promising one in recent years. However, this source has its own drawbacks. This includes low palatability, effects of anti-nutritional factors, essential amino acid unbalances, limited levels of highly unsaturated fatty acids (HUFA), reduced levels of minerals (Amaya et al., 2007). For example: soybean meal is a readily available plant protein source consisting of high protein quality and essential amino acids profile. However, it lacks methionine out of its amino acid profile and has wide variety of endogenous anti-nutrients (Tacon, 1997). According to viola et al., (1988), fish fed with soyabean meal based diet supplemented with lysine, methionine, lipid and di-calcium phosphate showed the same performance as that of fishmeal based diet (Viola et al., 1988). Study shows that the use of 30% SBM replacing fishmeal at optimum protein levels (32%) reduces the fish performance (Shiau et al., 1987). As a result, the use of plant feedstuffs as fishmeal replacement isn't accepted widely in the market.

Further research activities were carried out to find the sources that could replace fish meal. As result, single cell proteins (SCPs) came into existence. SCPs include microalgae, bacteria and yeast. They are rich in different nutrient elements such as proteins, B-vitamins, pigments, complex carbohydrates and glucan (Tacon, 1994). Among various sources of SCPs, yeasts have been used commonly in aqua feed production. Even, one of the types of yeast sources,

saccharomyces cerevisiae consists of immunostimulatory properties because of its complex carbohydrate components and nucleic acid content (Anderson et al., 1995).

But fishmeal and SCPs differ each other in comparison. The latter lacks one or more amino acids or say, there is an amino acid imbalance (Tacon & Jackson, 1985). Thus, it is necessary to supplement deficient amino acids in yeast based diets so as to achieve beneficial effects on fish growth (Bergstrom, 1978). Research works on fish diet using yeasts as sole dietary protein source showed poor performance on fish growth (Beck et al., 1978). However, yeast based diets added with methionine shows the equivalent growth rate as that of the control diet for rainbow trout (Tiews et al., 1979).

There are different types of microorganisms used as sources for yeast which are produced for commercial purposes. *Saccharomyces cerevisiae, candida utilis, kluyveromyces marxianus* are some of them (Øverland & Skrede, 2017). Among those, torula yeast (*candida utilis*) has been used commercially for producing yeast based diet for decades. In addition, there is no negative impact upon the feed acceptance and survival when yeasts are applied as replacement of fish meal partially (Gamboa-Delgado et al., 2016). The use of torula yeast replacing 40% of the protein from fishmeal shows a promising alternative protein source in diets for Atlantic salmon without negative impact on its growth performance and nutrient retention (Øverland et al., 2013).

These microbial products like yeast have been used as potential sustainable ingredients in aqua feeds as they have ability to convert low-value biomass obtained from forest and agricultural industry into high value feed materials. In addition, they use limited agricultural land, water and adapt in changing climatic conditions (Øverland et al., 2013). Depending upon the species, yeast can be produced either from fermentation of sugar based feed stocks i.e. sugarcane or from forest based lignocellulosic biomass. Development in advance fermentation technology has made the yeast production less costly and reliable to use as nutrient sources in aqua feeds (Kim et al., 1998).

In industries, the major cost of feed production occupies almost 55% of the total cost (Hardman et al., 1990). In addition, the higher cost for shrimp feed is due to its high dietary protein requirement. Thus, it is necessary to increase digestibility of the feed which in return lower the total cost production by reducing the nutrient expenditure per unit production. Besides using highly digestible feed ingredients, enzyme supplement is a good alternative to enhance the feed digestibility (Davis et al., 1998).

Thus, the study aim was to evaluate the effect of enzymes and conditioning process in shrimp feed with different percentage of yeast on rheological properties including hardness, Pmax, a_w, UPS rate and θ_c (in oil and water).

2. Literature Review

2.1 Pacific white shrimp: physiology and habitat

Pacific white shrimp, also called *Litopenaeus vannamei* is a marine crustacean with translucent body. Because of the pigmented chromophore, its body has bluish green hue and can grow up to 9 inches or 230 mm (in length). It's habitat varies from muddy shore bottoms down to depths of 72 meters (Galitzine, Morgan, & Harvey, 2009). The native habitat of this species ranges from the eastern pacific ocean in Sonora, Mexico to northern Peru (Sookying et al., 2013).

Litopenaeus vannamei is considered as an efficient species for farming due to its low and flexible protein requirement compared to other shrimp species like tiger prawn (*Penaeus monodon*) and the blue shrimp *Litopenaeus stylirostris* (Galitzine et al., 2009). Because of this feature, soy protein concentrate can be used to substitute approximately 75% of its diet excluding fishmeal and oil. However, DHA-omega 3-fatty acids need to be included in the feed as alternate source for shrimp growth. It is because crustaceans have a limited ability to synthesize them *de novo* (Galitzine et al., 2009).

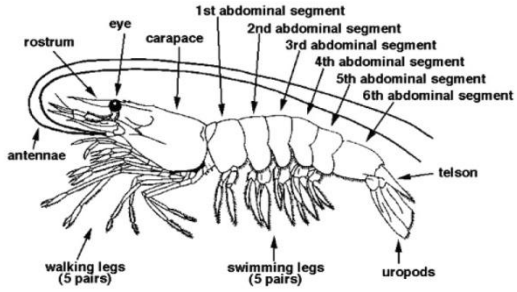

Figure 1: Basic anatomy of shrimp (Galitzine et al., 2009)

Shrimps belonging to *Penaidae* have potential to adapt changes in dietary composition inducing digestive enzymes synthesized and secreted by the hepatopancreas. They can tolerate different environment fluctuations related to salinity, pH and dissolved O_2 levels

(Rönnbäck, 2001). These species are produced in high quantity in the western hemisphere, which requires the average water temperature of 28^{o}C (Rönnbäck, 2001).

Salmonids (trout, salmon) are fast feeders as they take only few minutes to search for its feed in the water (Storebakken et al., 1999). But shrimps are a selective and slow continuous feeder taking long time to search and eat their food. Thus, it needs a fast sinking feed possessing high degree of water stability (Farmanfarmaian et al., 1982; Lim & Cuzon, 1994). Crustaceans have the ability to adapt changes in diet composition easily by the induction of digestive enzymes synthesized and secreted in the hepatopancreas (Le Moullac et al., 1997).

2.2 Nutrient requirement of shrimps

Feeding ingredients in shrimp diets need to possess certain nutritional properties such as low levels of fibre, starch (especially insoluble carbohydrates) and anti nutrients. Similarly, shrimp diets must also contain high protein content, favourable amino acid profile, high nutrient digestibility and reasonable palatability (Gatlin et al., 2007; Naylor et al., 2009).

The essential amino acids required for the optimum growth and maintenance of shrimp include arginine, histidine, and isoleucine. The list also includes leucine, methionine, lysine, phenyl alanine, threonine, valine and tryptophan (Kanazawa, 1989). The use of fish meal in shrimp diets is not only as protein source, but also as lipids, essential fatty acids, minerals and vitamins to the diet. Thus, it needs a variety of feed ingredients to be included for maintaining a well balanced nutrient profile without fishmeal (Sookying et al., 2013). Besides protein, lipids act as the major macronutrients that provide the energy and cellular binding blocks. They are necessary for the growth health welfare and reproduction in shrimp. Especially lipids rich in (n-6) and (n-3) HUFA i.e. marine fish oils are utilized better by shrimp than animal fats or vegetable oils (Lim et al., 1997).

Though fish meal is replaced, Essential Fatty Acids (EFAs) are important to be maintained in the shrimp diets. These include lipid content and the associated C18 polyunsaturated fatty acids (PUFA), linoleic (18:2n-6), linolenic (18:3n-3) acids as well as (n-3) and (n-6) HUFA. Similarly it includes eicosapentenoic acid (EPA, 20:5n-3), docosahexaeneic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) at levels between 5 and 10g/kg (Akiyama, 1991).

Cholesterol, a vital constituent of cell membrane and phospholipids are other necessary dietary ingredients responsible for the well growth of shrimps. Their interaction helps in the retention of total lipid and triglycerides in hepatopancreas as well as cholesterol is deposited in shrimps muscle (Sookying et al., 2013). Shrimps achieve their necessary minerals directly

from the aquatic habitat. They utilize soluble minerals like calcium, iron, magnesium, copper, phosphorus, sodium and zinc from the water source through their gills, epidermis or both (Sookying et al., 2013). Likewise the requirement of dietary phosphorus varies between 3.4 - 20 g/kg for juvenile while 20.9-22 g/kg for post larval L. vannamei (Davis et al., 1993; Niu et al., 2008).

Selenium is also a vital trace element which functions as the part of glutathione peroxidase enzyme in shrimp. It protects the cell from harmful effect of peroxides as it converts hydrogen peroxide and lipid hydro peroxide into water and lipid alcohols respectively (Davis & Gatlin III, 1996).

Table 1: List of nutrients required for L. vannamei on dry matter basis (Sookying et al., 2013; TNRC, 2011)

Item	Minimum requirement for *L. vannamei*
Typical energy and protein concentrations	
Digestible energy (kcal kg^{-1} diet)	3000
Digestible protein (g kg^{-1})	300
Amino acid (g kg^{-1})	
Lysine	16
Fatty acids (g kg^{-1})	
n-3 LC-PUFA	2.5–5.0 g kg^{-1}
Cholesterol (g kg^{-1})	1.3
Macrominerals (g kg^{-1})	
Magnesium	2.6–3.5
Phosphorus	3–7
Microminerals (mg kg^{-1})	
Copper	16–32
Selenium	0.2–0.4
Zinc	15
Fat-soluble vitamins	
A (mg kg^{-1})	1.4
E (mg kg^{-1})	100
Water-soluble vitamins (mg kg^{-1})	
Vitamin B6	80–100
Vitamin C	50–100

The requirements of shrimp nutrients are determined with highly purified ingredients defining their nutrient composition (Sookying et al., 2013).

2.3 Physical property of shrimp feed

These aquatic crustaceans are slow feed eaters and they manipulate their food extensively before ingesting it. Thus, feed diets provided for these animals should have the property of binding firmly so that particles remain intact in water for long time and avoid disintegration until feeding process is completed (Heinen, 1981). Hence, the feeding materials need to be selected tactfully so that the binding capabilities of particles would be higher and hence, the chance for better physical properties of the pellets would be maximum (Miladinovic & Salas-Bringas, 2014).

2.4 Problems with the pelleted shrimp feed

The production cost of shrimp feed is very high (Michael, 1976). Besides, the quality of diets also plays significant role in influencing shrimp growth, disease resistance and nitrogen loading. Furthermore, shrimp feed has challenges regarding water quality and pollution, physical instability of feed pellets and leaching out of specific hydro soluble nutrients too. To combat all of these issues, the quality concerned parameters of pellet need to be maintained including sustainable feed ingredients which help in optimum growth (Cho et al., 1994; Ochoa-Solano et al., 2006). In addition, binders need to be added in proper ratio which won't affect in the digestion ability of feed by animals.

2.5 Economical issues

Since feed cost occupies around 40-60% of the production cost in shrimp culture (Sookying et al., 2013), nutrient waste by excessive feeding is an economic loss to the business (Gamboa-delgado et al., 2003). Thus, the use of feed ingredients with high digestivity help in lowering the production cost, minimize nutrient loss and fecal waste. Replacement of expensive protein sources with cheaper and economical protein, lipid sources leads to the production of low economic feed (Sookying et al., 2013). For example: utilization of enzyme supplements is in a high priority nowadays (Davis et al., 1998).

2.6 Anti-nutritional factors in aqua feed

The availability of anti-nutritional factors in many plant materials adversely influence on the digestion of feed and its efficiency (Tacon, 1997). Effects of trypsin inhibitor, phytate and glucosinolates are visible in the digestivity of feed including plant materials. However, these factors can be inactivated by the use of heat and even soaking in water. Due to this drawback, plant derived feedstuffs aren't a favorite option though their source is large (Behera, 2013).

2.7 Need of single cell protein (SCP)

"The food supply increases in arithmetic ratio but the population of animals increases in geometric ratio" stated by Thomas Robert Malthus in the principles of population confirms the rapid growth of world population going far ahead of the food demand. Therefore, the limitation of protein rich food for the millions of lives have forced the researchers to search and study for any other protein sources which can substitute conventional and expensive sources like soy meal or fishmeal (Ravindra, 2000).

It is essential to explore the possibility of finding SCPs. It is so because the dietary needs with essential amino acids are difficult to get replaced. In addition, SCPs need less land to grow and can upgrade low protein organic material to high protein food (Nalage et al., 2016).

Single cell protein (SCP) has been a major concern at recent times for fortification of the protein-rich food supply. The major sources of SCP include algae, fungi and bacteria, which grow rapidly and contain high protein. Different fungal species rich in protein source include the yeast species like candida, hansenula, pitchian torulopsis and saccharomyces. They are even exploited for bioconversion of lignocellulose wastes (Ravindra, 2000).

Saccharomyces, candida and rodo torula are some of the species of yeasts commonly used for SCPs production. They grow in acidic condition (P^H 4.5-5.5) that helps in inhibiting bacterial contamination. Since proteins are made up of different amino acids, their nutritional quality also depends on types and content of amino acids. Thus in terms of amino acids, yeast is identical to the soya bean protein (Nalage et al., 2016). While, bacterial protein is similar to the fish protein.

Table 2:Composition of microbes (Nalage et al., 2016)

Constituents (% of dry wt. basis)					
Source	Crude protein	Nitrogen	Fat	Non amino acids	Ash
Bacteria	72-78	11.5-12.5	1.5-3	8.0-16	3.0-7
yeast	47-53	7.5-8.5	2.0-6	6.0-12	5.0-9.5
Filamentous fungi	31-50	5.0-8.0	2.0-8		9.0-14
Algae	47-63	7.5-10	7.0-20	3.0-8	8.0-10

The microbial use in the single cell proteins production is certainly innovative to solve the demand of food, which has ability to upgrade low protein organic material to high protein food. Candida species from yeast i.e. *arborea utilis* are the common options which solve the problem for replacement of essential amino acids (Nalage et al., 2016). SCPs are rich in nutrients elements like proteins , vitamin B pigments, complex carbohydrate and glucan (Tacon, 1996). Among them, yeasts are widely used for aqua feeds with *Candida* sp. and *Saccharomyces Cerevisiae* having immunostimulatory properties due to their carbohydrate components which are complex in nature and nucleic acid content (Oliva-Teles & Gonçalves, 2001). The feeding cost has been a major concern in aquaculture as it occupies almost 60% of the recurrent cost of aquaculture venture (Bob-Manuel & Erondu, 2011). This leads to the minimized profit margin of fish farmers and ultimately economic viability of the aquaculture industry. Fish meal has been a major ingredient in aquaculture diets since long time back, constituting almost 50-75% by weight. However, it exists as the most expensive ingredients due to its limited availability, human consumption and use in livestock industry (Misra et al., 2003). Hence , the partial or full substitution of fish meal protein is needed with alternative protein sources having good nutrient profile and moderate reduction in feed efficiency (Sogbesan et al, 2005). As a solution, plant feedstuffs also received a priority as a substituting ingredient of fish meal for protein source. However, the presence of anti-nutritional factors, low palatability, amino acid imbalances make these feedstuffs not well accepted (Oliva-Teles & Gonçalves, 2001).

Considering all these difficulties, researchers found a solution of using yeasts as a source for protein alternative. Furthermore, yeast species like Saccharomyces cerevisiae, Candida utilis have been considered as generally regarded as safe (GRAS) status assigned by the US food and drug administration (FDA) which means these substances aren’t hazardous to health and edible (Øverland & Skrede, 2017). Actually the use of torula yeast or Candida utilis has been

commercially started more than 70 years back during world war II where the Germans cultured torula yeast for protein source from pulp, paper and wood sugar by acid hydrolysis of wood (Øverland & Skrede, 2017).

Generally, yeast is a source of nutrients rich in vitamin B and lysine, the essential amino acid content. Since microbes have higher concentration of lysine and threonine than in wheat and cereals, there is possibility of using SCPs as a supplement in protein cereal diet.

Researchers believe that the nutritional value of yeast varies with origin and indicate that yeast grown in alkanes could be of better quality than that in carbohydrates (Olvera-Novoa et al., 2002). Experiments showed the acceptance of substituting the fish meal in salmonids diets from 25 to 50% which furthermore increases salmonids growth with the addition of methionine (Mahnken et al, 1980; Matty & Smith, 1978). However, the deficiency of Sulphur amino acids, high carbohydrate and nucleotide content restrict the furthermore use of yeast in salmon diet (Rumsey et al, 1992). In contrast, the common carp diet with yeasts (candida species) showed better results than that with soybean or meat (Olvera-Novoa et al., 2002). It can be used up to 62 to 88% in combination with fish meal or animal by-products (Alami-Durante et al., 1991; Atack et al., 1979). Torula yeast has been commercialized as a protein source in aqua diets since decades and even highest growth responses were observed when 30% of it used in tilapia fish diets (Gamboa-Delgado et al., 2016). Likewise, protein sources and nutrient contribution of both FM and CU complement each other due to their similar nitrogen contribution (Gamboa-Delgado et al., 2016).

2.7.1 *Candida utilis* (CU)

CU is a strain of yeast which is considered as a food supplement or for diet substitute. Torula yeast, torula utilis or torulopsis utilis are the alternative names for this yeast specie (Rentschler, 1971). Yeast is well known as source of vitamin, minerals and protein and even dried yeast consists about 50% of protein (Bressani, 1968; Bunker, 1968).

It has been used as food sources such as alcoholic beverage, cheeses, yogurt, soy sauces and even centuries before the Christ as mentioned by Hippocrates (Bunker, 1968). In historical time of world war I & II, CU was used for the first time as a protein source along with fat and vitamins replacing other food sources (Bressani, 1968; Bunker, 1968; Matelbs & Tannenbaum, 1968). Another reason of using CU was to convert industrial wastes or low-cost carbohydrate materials into animal feed including river pollution reduction (Pyke, 1958). The protein quality of CU (alkali extracted) gets improved by the methionine and arginine supplementation (Gitler et al., 1958). Cereal grains lack lysine, an essential amino acids in its

composition. Thus, yeast addition in cereal diet enhances the nutritional value with high protein content and amino acids. Similarly, the effect of feeding torula yeast with or without methionine supplement causes the improved nitrogen retention balance while the brewers' yeast does not (Goyco et al., 1959).

CU is the most popular yeast among yeast family which can ferment both hexoses and pentoses while *saccharomyces cerevisiae* (which are often used for baking purpose at home) can't ferment pentoses (Bunker, 1968; Dunn, 1958). It is robust nature, grows rapidly and can use all available carbohydrate as food (Silverman et al., 1966).

Since CU can use carbohydrate to synthesize protein and produces 29.48 kg of food yeast from 45.36 kg of sugar, it is beneficial to produce sufficient protein from cheap carbohydrate sources for solving the problem of protein malnutrition (Bressani, 1968).

The favorable parameters for the yeast growth rely on temperature, pH and oxygen supply. They grow well at optimum pH between 2.2 to 8.0, temperature of 20 to 30 ^{o}C and an abundant supply of oxygen (Morris, 1958; White, 1954).

Furthermore, the chemical composition of the yeasts depends upon different factors: substrate, salt concentration, degree of aeration, number of successive washes to remove impurities and drying technology (Rodríguez et al., 2011).

2.7.2 Chemical composition of yeast: CU

The comparison between amino acid content of CU and fish meal showed similarity on the basis of crude protein (Øverland & Skrede, 2017). However, the content of non protein nitrogen in the form of nucleic acid is higher in yeast while methionine & cysteine is low in yeast compared to fish meal. Thus, regardless of low methionine content , this yeast species contains a matchable amino acid composition compared with the aquatic requirements (Chanda & Chakrabarti, 1996; Øverland & Skrede, 2017). This yeast species is a good source of minerals like phosphorus, calcium, sodium, zinc, iron, copper, manganese, selenium and of course, vitamin B i.e. riboflavin, pantothenic acid and niacin.

The comparison among fish meal, soybean meal and CU on the basis of availability of amino acids is shown in the table 3.

Table 3: Average composition of amino acids (g 16 g N^{-1}) among fishmeal, soybean meal and candida utilis (Øverland & Skrede, 2017).

Indispensable amino acids	**Fish Meal**	**Soybean Meal**	***Candida utilis***
Arginine	5.74	7.38	5.2
Histidine	2.36	2.67	1.97
Isoleucine	4.53	4.94	4.29
Leucine	7.06	7.8	6.19
Lysine	8.18	5.53	7.71
Methionine	2.87	1.41	1.08
Phenylanine	3.84	5.26	3.64
Threonine	4	4.03	4.71
Tryptophan	1.05	1.41	1.17
Valine	4.87	5.51	5.08

Table 4: Protein quality of various kinds of yeasts ((Rentschler, 1971)

yeasts	**Biological value**	**digestibility**	**Net protein**	**Protein efficiency ratio**
Torula yeast	31.8	84.8	14.9	0.9
Torula yeast +0.5% methionine	88.3	90.2	44.1	2.0
Baker's yeast	58.9	80.7	22.7	1.4
Brewers' yeast	58.4	79.9	22.2	1.7

2.7.3 Digestibility of CU in aquatic animals

It is stated that the presence of thick and rigid cell walls present in yeast cause a problem inhibiting industrial production and utilization of dietary yeast protein (Nguyen et al., 1998; Øverland & Skrede, 2017). In addition, it lowers the chance of enzymatic access to cellular contents depending on yeast cell characteristics. Its cell wall contains approx. 26-32% of the cell dry weight including different proportion of mannan- oligosaccharides, β-glutan, chitin and nucleic acids as per species and strains.

Thus, it is essential to rupture the cell walls either chemically or enzymatic hydrolysis or mechanical rupture methods to enhance its digestibility (Nasseri et al., 2011; Pacheco et al., 1997).

One of the procedures that include enzymatic pretreatment followed by high pressure mechanical homogenization seems to be efficient for CU. Similarly, the increased addition of yeast extract as a fish meal substitution in shrimp diets (especially *litopenaeus vannamei*) showed the increased apparent digestibility of protein. This result is due to the combined effect of removing cell walls and increased proportion of water soluble low molecular weight proteins. Thus, the removal of immunostimulating and bioactive compounds like β glucan and mannan oligosaccharides by rupturing of cell walls, creates the most attractive feed ingredient which have high digestibility of protein and amino acid (Vidakovic et al., 2016).

2.8 Problems with yeast

Though the acceptance of using yeast as substitution of fish meal for the culture of fleshy shrimp is high, the problems including the decrement of apparent digestibility and/or palatability due to the wall of yeast cell (consisting 57% β glucan, 6.6% oligosaccharides and 22% glycoprotein) cause the difficulty in digestion (Rumsey et al, 1991). Therefore, the yeast extract is more suitable than yeast cells for replacing fish meal in aqua feed which is nucleotide rich and water soluble cell inclusion without cell wall (Zhao et al., 2017).

2.9 Yeast cell wall lysis

The yeast cell wall constitutes three major components including inner layer of glucans (β-1, 3 and β-1, 6-glucan), chitin (polymer if N- acetyl glucosamine) and outer layer of mannoproteins. This β-1,3 glucan is responsible for the rigidity of the cell wall (Rodriguez-Pena et al., 2013). β-1, 3-glucan laminaripentaohydrolase helps to degrade cell wall β-1, 3-glucan while protease, mannanase and endochitinase act on mannoproteins, mannan and chitin. Thus it is accepted that the combined effect of protease and β-1,3-glucanase enzymes helps in lysing yeast cell walls (Rodriguez-Pena et al., 2013; Zlotnik et al., 1984). Protease enzyme possesses a property which enhances the porosity of the cell wall enabling its lysis. The lysis of viable yeast cells is possible at optimum pH of 7.0-7.5 and temperature at 35^{o}. while the enzymes are stable at pH range 5-11 (Kitamura et al., 1971). Thus, glucanase alone is unable to lyse yeast cell walls without the synergistic enzyme activity from an alkaline protease (Scott & Schekman, 1980).

Glucan layer in yeast cell wall causes structural integrity and rigidity which is outer covered by mannoproteins layer. Thus, this layer needs to be modified before a lytic glucanase reaches its substrate for cell lysis. It means, the start of the lysis occurs by attack on the protein

portion of the manno protein, but not from the carbohydrate portion (Scott & Schekman, 1980). Protease helps to open up the outer protein structure releasing wall proteins and mannan. Then the rupture of the outer layer exposes the inner glucan surface which is attacked by glucanase and solubilizes the glucan (Salazar & Asenjo, 2007).

2.10 Effect of adding enzymes on feed

Enzymes are considered to be safe for fish/shrimp health as well as pond environment as they are the natural products of fermentation process. The inclusion of these ingredients in feed increases feed intake by enhancing fat and protein digestion. Reduction of ammonia production and digesta viscosity is possible due to its presence in feed diet (Behera, 2013).

The physiology of aquatic larvae shows that the intestinal tract is shorter and immature in comparison with adults. So, enzyme addition in feed helps in larval feeding which significantly reduces high larval mortality (Behera, 2013).

The enzyme addition in shrimp feed uplifts the nutritional value as well as helps in eliminating the effects of anti-nutritional factors, transforming complex feed components into simple absorbable nutrients. It also helps in utilizing dietary energy and amino acids. This ultimately improves the performance of fish/ shrimps (Farhangi & Carter, 2007; Liu & Lin, 2001).

Most of the enzymes added in the feed diet reduce power consumption and keeping high physical quality in the pellets. Likewise, for example: addition of lignosulfonate with 2% water enhances the pellet durability with reducing water activity on finely ground barley (Miladinovic & Salas-Bringas, 2014).

The optimum utilization of both enzymes protease and β-1,3-glucanase enzymes helps in lysing yeast cell walls (Rodriguez-Pena et al., 2013; Zlotnik et al., 1984). Protease enzyme has a potential for enhancing the porosity of the cell wall enabling cell wall lysis.

2.10.1 Proteases

A protease is any enzyme which causes proteolysis. It is also called peptidase or proteinase which leads to protein catabolism by hydrolysis of the peptide bonds linking amino acids together in a polypeptide chain. Though the classes of protease are different, the reaction caused is same with different catalytic mechanism. These enzymes are found in animals, plants, bacteria, achaea and virus (Barrett, Woessner, & Rawlings, 2012).

Studies are done extensively in feed industry regarding the use of proteases for improving protein digestibility through hydrolysis of structural proteins (DuPont, 2014).

Proteases work by digesting long protein chains into shorter fragments by splitting the peptide bonds that link amino acid residues.

These enzymes are divided into two groups depending upon the function: Exopeptidases, endopeptidases.

2.10.2 Exo/endo 1, 3-β-glucanase

β- glucanase is known as the fibre degrading enzyme that cleaves non-starch polysaccharides (e.g. arabinoxylans, β-glucans) present in barley, wheat, rye and other cereals. Presence of this enzyme in feed helps in improving nutrient digestion and feed utilization by assisting in digestion process, reducing digesta viscosity and improving nutrients uptake (DuPont, 2014).

Objectives

The main objective of this thesis was to study the effect on rheological properties of pelleted shrimp feed using Torula yeast/ CU in different percentage as a fishmeal replacer. The study was also focused on the role of conditioning and enzymes added to enhance the characters of CU on rheological quality parameters such as moisture content, tensile strength of pellets, UPS rate, a_w, water/oil surface contact angle (θ_c) and Pmax.

3. Rheological quality parameters of pellet

Rheology involves the study of determining physical qualities of the pellet and agglomeration of moist powders during pelleting (MacRitchie et al., 2002). Movement of water during pelleting includes two stages i.e. pushing of mash into the die and pushing of wet mass along the die. The induction stress inside the die causes migration of water molecule on the pellet surface and thus pellets agglomerate (MacRitchie et al., 2002). The variation of water content during these stages leads to the variation of moisture content after pelleting (Baert et al., 1992).

Increase in moisture level creates more number of moisture bridges that ultimately enhances total binding force (Thomas & Van der Poel, 1996). Pressure applied between particles reduces the distance between particles while increases the surface interaction (Thomas & Van der Poel, 1996).

There are different binding mechanism for food particles that includes solid-solid interactions, liquid necking (capillary forces in a three phase system: solid, water and air) and cohesive, adhesive forces between binders and particles (Thomas & Van der Poel, 1996). Bonds are

formed between particles during drying or cooling process in solid-solid interaction while liquid necking allows to held together particles in a porous agglomerate (pellets) distinguished in separate phases: solid, liquid and air (Thomas & Van der Poel, 1996). Among different methods like tensile test, simple compression and diametral compression, the latter one is commonly used in the study of compaction rheology i.e. animal feed pellets (Bringas, 2011).

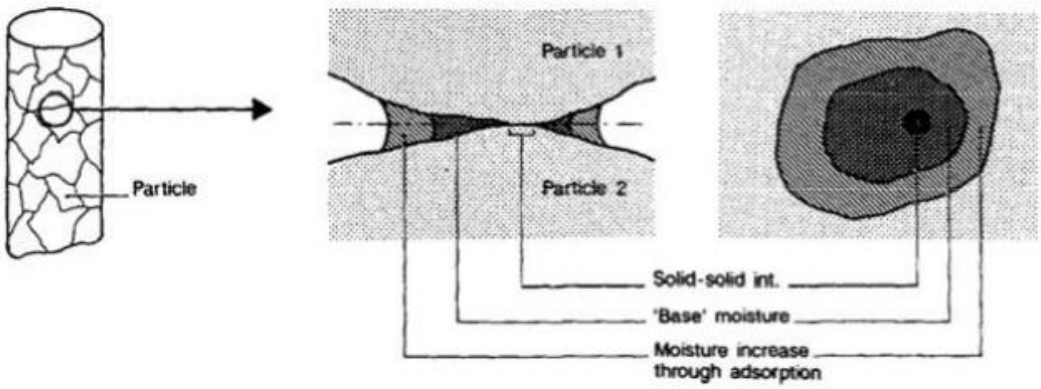

Figure 2: model figure showing the general binding forces between two particles (Thomas & Van der Poel, 1996)

3.1 Tensile strength

The measurement is fruitful when the material strength is determined without considering its shape and size. The ideal measurement is obtained by finding the area during the time when stresses are concentrated and specimen exhibits failure. In pelleted feed which is ductile in nature, it is difficult to know the correct point as it undergoes several cracks before the major failure occurs (Salas-Bringas et al., 2011). Thus, the ideal way to measure the strength is by considering the area of the applied stresses. It can be carried out by either uniaxial or diametral tests. However the second one shows a low variance indicating the repeatability of the testing method (Salas-Bringas et al., 2011). Once the particles of pellet are broken, the brittle nature of pellet makes it easy to calculate the tensile strength. This parameter is better calculated through the Brazilian method. The Brazilian or 'indirect tensile test' measures the tensile strength at the time when the tensile fracture occurs in a disc shaped material under diametric compressive test (Sinka et al., 2007). Tensile strength represents the force applied per unit area that can break the contacts in the fracture plane (Ghadiri et al., 2007).

The mathematical equation used to calculate the tensile strength for specimens which are cylindrical shape is:

$\sigma = F/\pi rl$, where r & l are the radius(mm) and length(mm) of pellets, σ(Mpa) is the maximum tensile strength, F (N) is the applied load at fracture (Sinka et al., 2007).

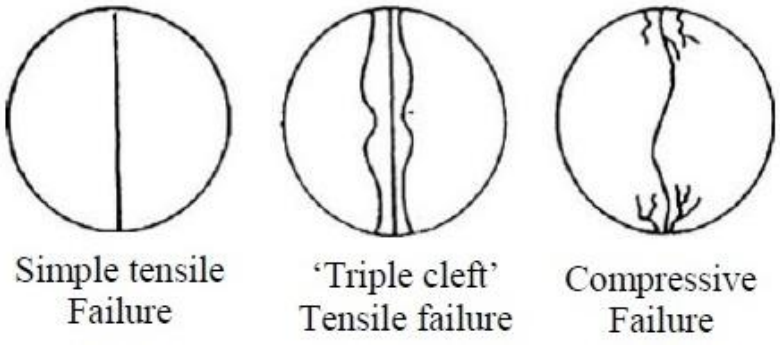

Figure 3: different failure modes of pellet under diametrical compression test (Sinka et al., 2007)

Figure 3 represents the three different failure modes of tensile strength when a pellet is diametrically compressed. One of the failure modes is simple tensile failure that involves the fracture of pellet into two different equal pieces along the loaded diameter. The triple cleft failure shows the splitting of pellet into three or more pieces consisting a central normal tensile and two other nominally collinear fractures on either side of central fracture (Ovri & Ndukwe, 2014). And the compressive failure shows a collapse of the pellet particles into pieces.

Pellet hardness is affected by particle size of ingredients, water addition and conditioning temperature (Salas-Bringas et al., 2012). High temperature and moisture help in forming the durable qualitative pellets as they activate natural binders like proteins and starch during conditioning (Hemmingsenet al., 2008). In addition, steam provides both moisture and heat at the same time using less amount of water. Thus, steam conditioning is preferred over water (Hemmingsenet al., 2008). Addition of moisture and temperature in high level leads to high tensile strength of pellets (Salas-Bringas et al., 2012). Fine particles controlled by grinding also results in improved pellet quality as they are well compressed in pellet die compare to larger particles (Hemmingsenet al., 2008).

3.2 Water activity (a_w)

The test of water activity measurement indicates the free water or bound water molecules present in pellets. It is of great interest to know a_w by both manufactures and customers as moisture content doesn't address all problems i.e. product stability, shelf life etc. It quantifies the active part of the total moisture content. The optimum range of aw varies from 0 to 1 that occupies absolutely dry to pure water (Jarvis et al., 2016). The growth of most of the pathogens are suppressed below a_w of 0.90, but in exception *staphylococcus aureus* can grow at 0.82 (Houtsma et al., 1996). Thus, higher the presence of a_w, higher is the chance for microbial growth, chemical and enzymatic reactivity. Water can act as different form as a solvent, reactant & viscosity modifier. Less water activity in pellets leads to less chemical degradation, more hardness and dryness in pellets which ultimately results in storage stability due to nonexistent microbial proliferation.

3.3 Contact angle/wettability

The study of wettability states the degree of wetting when a solid and liquid interact. It represents high wettability when contact angle is measured less than 90 degree while large contact angle represents low wettability at angle greater than 90 degree (Yuan & Lee, 2013). Likewise, the contact angle becomes zero when there is complete wetting at which the droplet turns into a flat puddle. There is a different case for super hydrophobic surfaces where the liquid and the surface have no contact. Contact angles are above 150 degrees at this state (Yuan & Lee, 2013). The study of contact angle with water and oil medium is very essential as it indicates the quality of feed. Pellets absorb water molecules during steam conditioning or extrusion and absorb oil molecules during vacuum coating of oil (Yuan & Lee, 2013).

Commonly used method to measure contact angle is sessile drop method where a sessile drop is illuminated from one side with a diffuse light source and the contour of the drop is observed from the other side. It is represented as θ_c.

Wettability of solid surface helps to understand the study of phenomena like adhesion, adsorption, friction and wettability (Yuan & Lee, 2013). The equilibrium relation between three interfacial tensions i.e. solid, liquid and gas interface shows the liquid contact angle on surface of solid. This relation is called Young's equation where θ_c is Young contact angle, Solid-water interface γ_{sl}, solid-vapor interface γ_{sv}, liquid-vapor γ_{lv} (Kwok & Neumann, 1999).

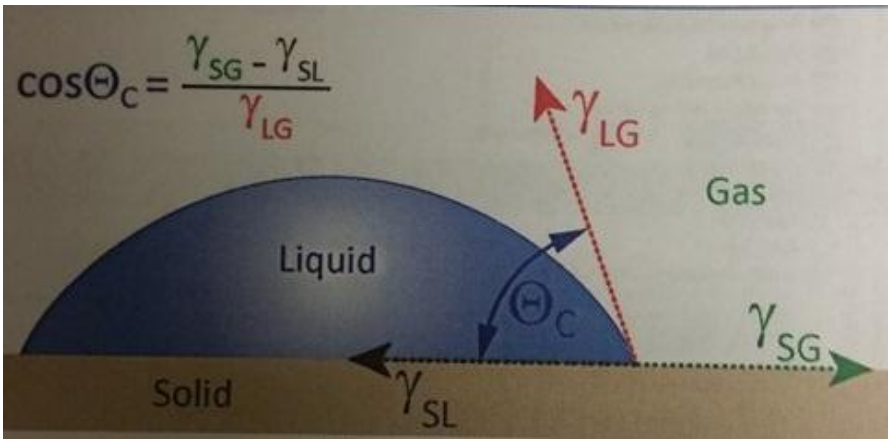

Figure 4: the sessile drop contact angle showing the quantities of Young's equation (Kwok & Neumann, 1999).

The behaviour of oil adsorption increases with time and remains consistent once it reaches the maximum equilibrium (Carmody et al., 2007). The other factors that influence the oil adsorption include air flow, oleophilic property of material and exposure time for adsorption (Carmody et al., 2007). The rate of oil adsorption is low when the surface is wet and in this stage, it depends upon the oleophilicity of adsorbent and morphology of the surface (Kumagai et al., 2007).

The cause for water adsorption in hydrophilic group of materials is H-bonding (Lagorsse et al., 2005). H-bonds are formed when water molecules contact with the solid surface. Interaction of water molecule with oxygen functional groups forms maximum H- bonds through unshared pairs of electrons (Lagorsse et al., 2005).

3.4 Underwater pellet swelling rate (UPS rate)

The study of pellet stability under water is an important process as it marks the quality parameter for the aquatic feed. It measures the resistance of pellet to swell under water. Aqua feeds are either compressed by extrusion or by steam pelleting for good water stability and high durability during transportation (Lim & Cuzon, 1994).

The water stability of pellets for longer duration is essential to avoid the increased cost of feeding and nutrient leaching until consumed by the animal (Obaldo et al., 2002). This problem could be sorted out using the diets with suitable texture, size and attractants that enhance feed consumption (Lim & Cuzon, 1994). Since shrimps are benthic in nature and slow in feeding, pellets need to have sinking property and stable. Thus, a standard method that could mimic actual shrimp culture conditions must therefore be conducted for monitoring pellet water stability

4. Materials and methods

4.1 Raw material

The raw materials that were required to conduct the experimental works include specific yeast *Candida Utilis* (CU) and cocktail of enzymes. They were supplied from Lallemand, Estonia and obtained from the Centre for Feed Technology pilot plant (Fôrtek), located at the Norwegian University of Life Sciences, Ås, Norway. Different percentage of CU was mixed with formulation diet starting from 0% to 20% addition. Enzymatic cocktail was consisted of protease (AB Vista, Marlborough, UK) and mix of endo/exo 1, 3-β-glucanase (Megazyme, Ireland).

Diet formulation was designed at the Norwegian university of Life Sciences, Ås (NMBU, Ås). Total of 6 diets were prepared for this experiment replacing fish meal with adding different percentage of torula yeast/CU starting from 0 to 20% in each total diet weight.

Table 5: Ingredients used for the formulation of shrimp diets in six treatments include SBM; Soya bean meal, SPC; Soya protein Concentrate, MCP; Mono Calcium Phosphate, MnO; Manganese oxide; Vit; Vitamins

Ingredients	**Weight (g) of specific ingredients in Torula yeast treatments**				
	0%	**2.50%**	**5%**	**10%**	**20%**
Wheat flour	90.00	90.00	90.00	90.00	90.00
FM	67.50	60.00	52.50	37.50	7.50
SBM	30.00	30.00	30.00	30.00	30.00
Poultry meal	25.50	25.50	25.50	25.50	25.50
Rice flour	18.00	18.00	18.00	18.00	18.00
SPC	18.00	18.00	18.00	18.00	18.00
Squad meal	15.00	15.00	15.00	15.00	15.00
Yeast	0.00	7.50	15.00	30.00	60.00
MCP	4.80	4.80	4.80	4.80	4.80
MgO	0.90	0.90	0.90	0.90	0.90
MnO	0.03	0.03	0.03	0.03	0.03
Vit/minerals	1.50	1.50	1.50	1.50	1.50
MSP	1.68	1.68	1.68	1.68	1.68
TOTAL	**273.81**	**273.81**	**273.81**	**273.81**	**273.81**

Two variable parameters were set up for the addition of cocktail enzymes among these diets shown in the Table 6.

Table 6: Formulation design of six different treatment diets.

Item no.	Diet1 /NY,NE (control+)	Diet2 /NY,YE (control-)	Diet 3/YY,YE,2.5%	Diet 4/YY,YE,5 %	Diet 5/YY,YE,10 %	Diet 6/YY,YE, 20%
Candida utilis (%)	0%	0%	2.5%	5%	10%	20%
Water %	10	10	10	10	10	10
Water spray (g)	16.5	15	15	15	15	15
Enzyme spray (g)	0	1.5	1.5	1.5	1.5	1.5

4.2 Methods

Different species of animals have different quality standards for their respective feeds. The quality of pellet can be measured through different tools like hardness, water activity, underwater pellet swelling rate and contact angle test. .

Chapter 1: Methods for preparing experimental diets

4.2.1 Mixing

To perform intense mixing and enzyme spray, the lab scale high shear mixer containing three impellers and a tulip form shopper (Diosna P1/6, Germany) was used. At the very first phase, raw materials for diets shown in Table 5 were already mixed thoroughly by another master candidate. Grounded starch ingredients were sieved at 2mm in the hammer mill and mixing was done for about 800 seconds (Mbuto, 2017). This lab scale mixing blender was used for mixing the provided diet powder (mixed formulation with varying proportion of CU i.e. 0%, 2.5%, 5%, 10%, 20%) with maintaining moisture percentage of 10%. The cocktail enzyme with protease and endo/exo-1, 3 β-glucanase was collected in freezing stage (kept at -80^{o}C) in order to avoid any activation of enzyme.

Before spraying the enzymatic solution in diet powder, they were kept in room temperature to get melted. During this phase, first the moisture of each provided diet powder was measured.

After that, 150g sample from each diet was weighed out and placed in mixing blender. This diet powder was then blended at 250 rpm for 3 minutes. Then the cocktail enzyme was sprayed at 0.06 bar air pressure at the blender speed of 100 rpm for 3 minutes. A spraying lance (Dusen Schlick GmbH, Germany, Model 970) assembled in the mixer was used for spraying the enzyme solution. Samples for each trial were taken randomly in the mixer and mixed together to have representative sample before measuring the moisture.

The moisture content was measured thrice for each trial as per the method defined by EU commission regulation for sampling and analysis of the official control of feed (No. 152/2009).

The average moisture measurement for all trials before and after enzyme addition was 9% w/w (±1%) and 18%w/w (±1%) simultaneously.

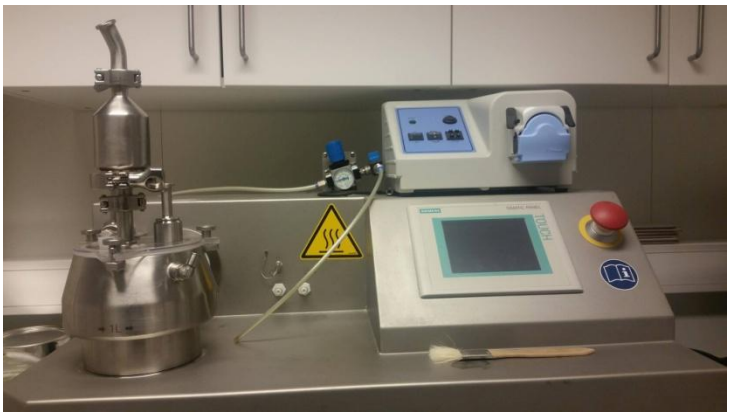

Figure 5: Lab scale mixing blender and fluid sprayer

4.2.2 Storage of samples

These powder samples ware then vacuum packed in plastic bag and sealed to avoid moisture loss. They were stored in freezer at -20^{o}C for 48 hours before proceeding for the conditioning process and pelleting. These samples need to be stored in fridge at -20^{o}C all the time. It is so because the cocktail of enzymes in the diets (protease and endo/exo-1, 3- β-glucanase) can be activated when kept at room temperature. Thus, the inhibition of enzyme activity is necessary as the main components of CU are complex protein and fibrous carbohydrates.

4.2.3 Steam Conditioning

First, the powder samples weighing 200±10mg from each diet were packed in eppendorf tubes. Then these tubes were tightly wrapped with thin parafilm at the opening end. It avoided

the caps being popped out due to steam during conditioning. Total 30 samples from each diet weighed in eppendorf tubes were submerged into the boiling water bath at 100 ^{o}C for 3 minutes and let them cool for 20 minutes before measuring the moisture. These samples were then kept in fridge at 4 ^{o}C before going for pelleting.

4.2.4 Pelleting

The single pellet press method described by publications (Misljenovic et al., 2015; Salas-Bringas et al., 2010; Salas-Bringas et al., 2011) was used for compressing the diet containing CU into the cylindrical pellets. This laboratory single pellet press was designed and fabricated at NMBU workshop, Ås. The laboratory die pelleting rig assembled in a Lloyd LR 5K plus texture analyzer consists of a barrel made of brass with compressing channel along the center. This channel has a diameter of 5.5 mm in which the rod with 5.4mm diameter was inserted to press the powder diet against a blank die. Then the blank die was disassembled from the barrel to release the compressed pellet from the channel. A jacket heater of 550 W was used to heat the barrel, controlled by a PID attached to a thermocouple along with the barrel surface (Salas-Bringas et al., 2010).

Once the steel cylinder reached the stable temperature of 81^{o} C, each sample from eppendorf tube was poured into the die hole (channel) of the preheated (81^{o}c) compressing die. The die heating temperature of 81^{o}C is recommended as the elimination of salmonella contamination in the animal feed is possible at this temperature (VKM, 2006).

It was heated up prior compaction for three mins at 81^{o} C. The compressing rod was inserted immediately after the sample was poured in to avoid release of moisture from sample during heating up. This temperature of 81^{o}C existed until during compaction and pellet extraction from the die.

Figure 6: Single die pellet press machine (Lloyd)

After the diet powder heated up for 3 min, the initial pre-load force of 5.6 Nm was applied with using maximal load force of 285 Nm and compressibility of about 12Mpa.

The compressing rate of pelleting was set to 10 mm/min with the help of a rod inserted in a 5.5 mm blank die. After compaction, the blank part of the die was unscrewed and removed so as to discharge pellets at a speed of 2 mm/min. This discharge speed is kept low to avoid any possible exceeding of the compacting pressure and hence avoid any further compaction.

The total retention time of the diet in the channel was approx. 9±1 minutes while the diameter of compacted pellets was 5.5±0.1mm and final pellet weight was 0.2±0.01 grams.

The compacted pellets were stored in sealed bags for further analysis in the fridge at temperature of 4^{o}C.

4.2.5 Moisture content

METTLER LJ16 and Axis moisture analyzer situated in IMT (NMBU, Ås) was used for the moisture content measurement. Moisture content was measured at different stages. It started with the mash provided, followed by mash after enzyme addition, conditioned and finally after pelleting process. The moisture analyzer was run at the pre-set standard temperature and time.

4.2.6 Tensile strength/Hardness

The diametric compression test was performed to evaluate the pellet strength. The peak force was recorded where the pellet can't hold its cohesive structure and thus, breakdown occurs. Hardness is expressed as the maximum force per pellet length (N/mm). It was analyzed by instrument used for pelleting process i.e. Lloyd LR 5k plus (Lloyd instrument, UK). The contracting cylindrical barrel was replaced with a flat probe (60 mm in diameter) connected to a Lloyd LR 5KN as shown in Figure 7.

Figure 7: Hardness testing machine (Lloyd LR 5K texture analyzer)

Three pellets from each treatment diets were randomly selected for hardness test. The pellet tensile strength analyses was set at the rate of 1 mm/min. Maximum force (N) used for pellet break was measured by the first peak when the pellet was crushed during a diametral compression at 1 mm/min. Hardness of cylindrical pellets are expected through maximum tensile stress (σ). Prior to the strength test, length and diameter of the pellet were measured using a digital caliper.

4.2.7 Water activity (a_w)

Five randomly chosen pellets from each diet were tested for water activity by using Rotronic Hygrolab C1 from Switzerland. The timescale to measure a_w was set up at 5 minutes and the temperature range was between 21.5 to 24.5 ^oC.

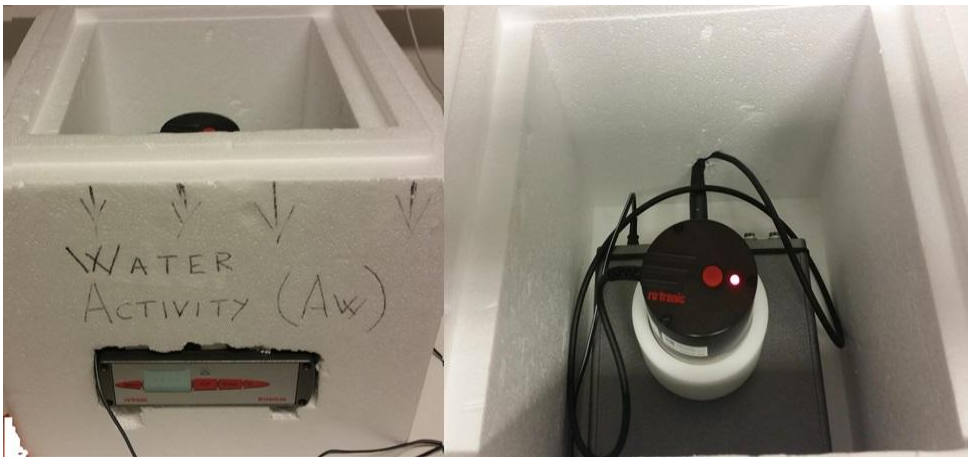

Figure 8: Water activity testing instrument (Rotronic Hydro Lab)

4.2.8 Underwater pellet swelling rate (UPS-rate)

The UPS rate was measured using a micro viper portable computer along with compact video microscope lenses. It recorded the images of the immersed pellet in 100 ml distilled water under static phase at room temperature starting from zero minute to 40 minutes. Forty images per each pellet were captured and analyzed by FIJI software (image analysis software) to determine the swelling rate of pellets under water. Three pellets from each diet were selected randomly for the test of UPS rate. The whole set of instrument used for the test includes:

1. Video lenses from Krüss Tensiometer and its light source
2. Glass container to fill distilled water and to immerse pellet (designed at Mathematical & Technical department, IMT, NMBU).
3. Allen Zoom compact video microscope lenses.
4. A Micro viper portable computer connected with Allen compact video microscope lenses.

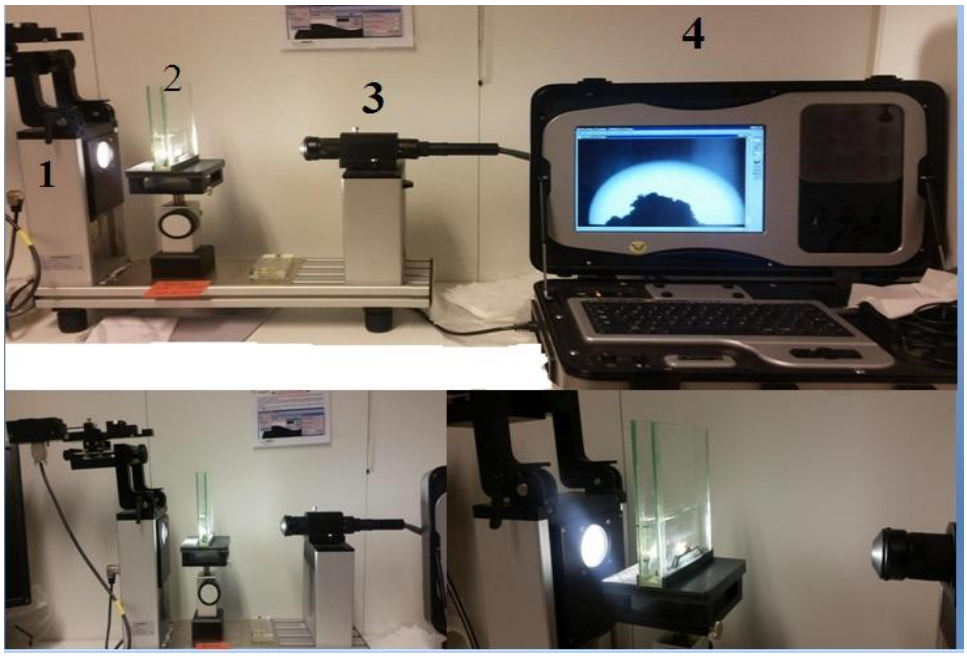

Figure 9: Full setup instrument for the test of UPS rate (left- Krüss Tensiometer, center- Allen zoom compact video microscope lenses, right- micro viper portable computer)

The images of individual parts of the UPS instrument are shown below.

Figure 10: Assembled and fixed video microscope lens on tensiometer.

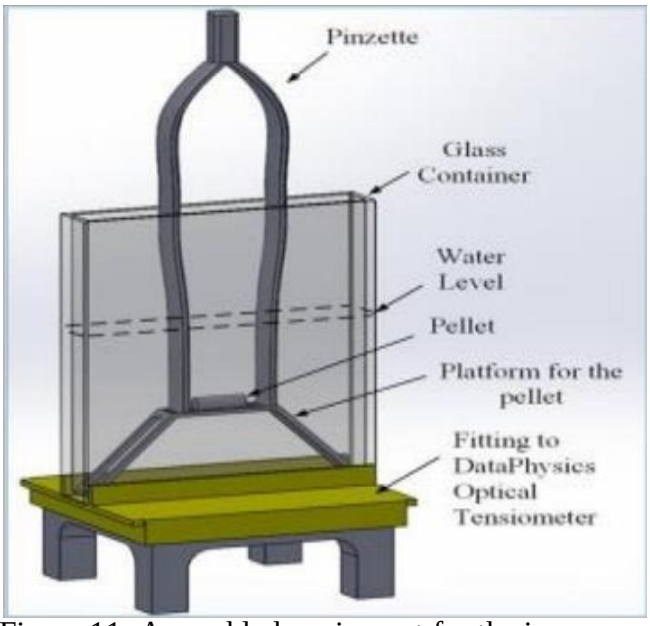

Figure 11: Assembled equipment for the image analysis of the UPS rate of the pellet (Misljenovic et al., 2015).


Figure 12: Setting of the video microscope: from right to left (in order of assembling): CVM video probe head from Micro Viper portable computer; Allen zoom lens, 20x – 120x basic lens; contact head adaptor; 60x-420x contact head (Misljenovic et al., 2015).

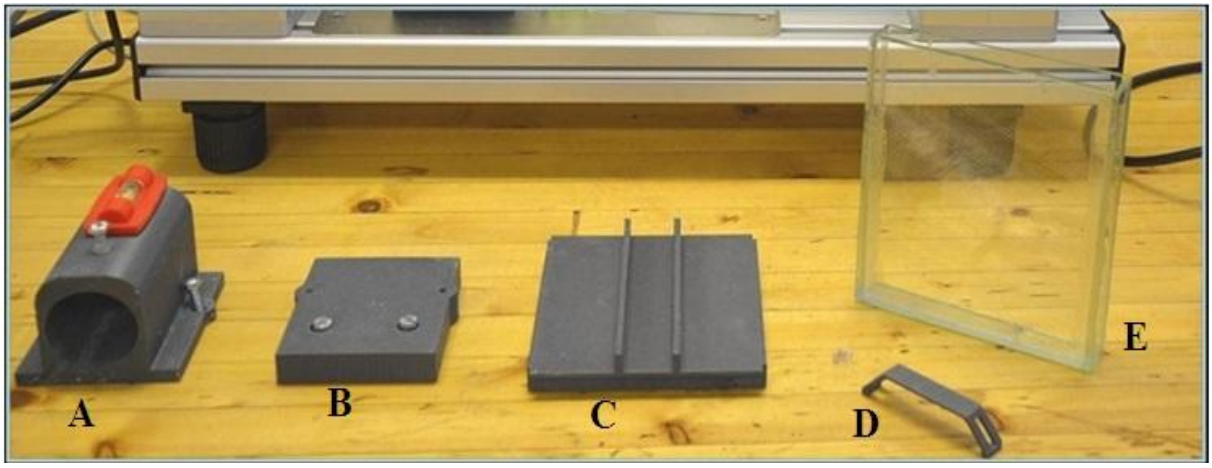

Figure 13: 3D printed parts for complementing the support for tensiometer lens (Krüss G10). A & B are for stabilising and mounting the video microscope on Tensiometer, C is for fitting the glass container, D is a bridge to stabilise the pellet while in the glass container & E is a glass container (Misljenovic et al., 2015).

4.2.8.1 Analytical procedure:

- One pellet at a time was selected randomly from the bag of chosen diet to observe the UPS rate. The task was performed thrice for the same diet in order to have the concurrent results or replicates.
- All the parts of the equipment were assembled as shown in the figures above.
- Then, 100 ml distilled water was poured in the glass container without having any air bubbles in water. The water surface should be stabilised or bubbles need to be removed with the long laboratory pinzette, if necessary.
- Dry laboratory pinzette was used to hold the pellet as wet pinzette could destroy its shape.
- Pellet was submerged quickly into the water with this long laboratory pinzette in the middle of the support bridge as possible. The Micro Viper portable computer screen was set up in such a way that only the circle of the compressed cylinder (pellet) was seen. If not, it should be adjusted by gently touching the pellet until the circle would be in frame. But this should be performed within 60 seconds. If not, another pellet should be taken.
- The stop watch was started immediately to avoid any delay, and clicked the first image as soon as the pellet was placed on the support bridge.
- Images were clicked once in a minute till it reached 40 minutes as per the experimental design.

- The complete analytical setup for the UPS ratio measurement is presented in figure 11.

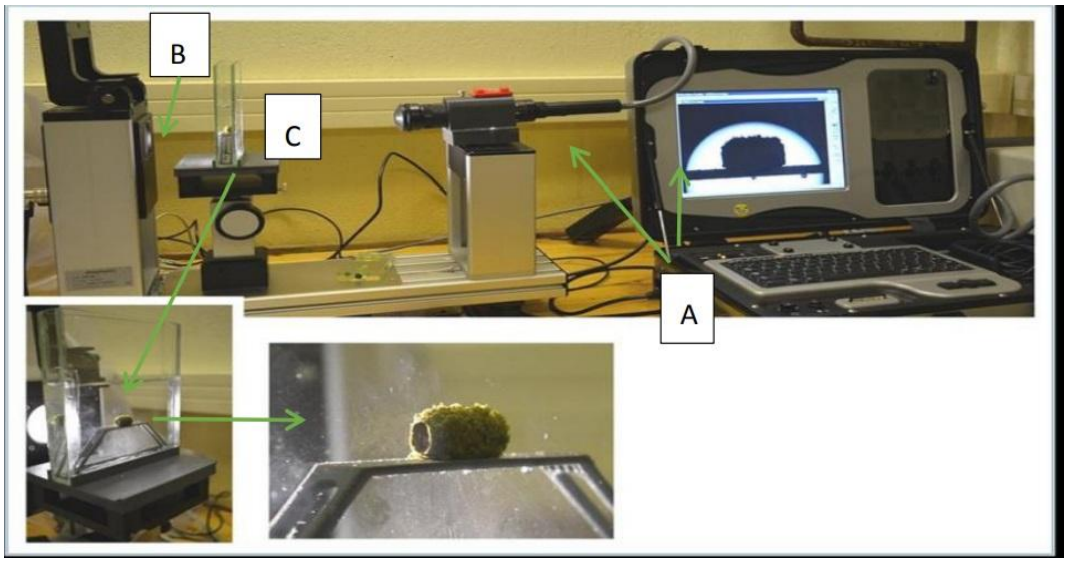

Figure 14: Complete analytical setup for the UPS ratio measurement using image analysis glass container; A – Video microscope; B– Light source; C – Pellet in glass container (Catargiu, 2015).

- FIJI software was used for the UPS rate analysis of the images taken by Micro Viper portable computer and lenses assembly. The change in the surface area of the pellet images indicates the underwater swelling rate during required time interval.
- Total 40 images from every pellet were stacked together and measured by Fiji software to determine the increased area of pellets for calculating swelling rate in distilled water. Increase in area was calculated using formula ($A=\pi r^2$) and followed by Minitab and Tukey posthoc test.

4.2.9 Contact angle, θ_c (with oil/water)

Contact angle measurement was performed to study the variation in contact angle which occurred due to the wettability effect of pellet influenced by enzyme cocktail and different CU percentage. The θ_c measurement was performed at room temperature based on optical θ_c measuring device OCA 15EC (Dataphysics Instruments Gmbh, Germany). During this test, one pellet at a time was taken randomly from the bag of chosen diet and repeated thrice to have a concurrent reading or replicates. While placing the pellet under water, lower side of all pellets used for analysis were showing darker colour when compared to upper side of a pellet.

Dosing volume for distilled water was 2 µl while for rapeseed oil was 5 µl. The dosing rate was 2 µl/s for both. Braun 1 ml disposable syringe was used to drop a liquid for contact angle measurement, so called sessile drop method. A defined volume of liquid was disposed on the upper plane surface of pellet and recorded the video of drop absorption. After determining the zero point of the drop, the initial angle and its changes in θ_c with time were calculated by SCA 20 software. The liquid-solid θ_c is measured using a protractor eyepiece on a goniometer[1] or by photographing the drop in order to measure its angle on the print.

The load of pressure is different for upper and lower side of pellet in the single pellet press method. The upper side of pellet receives higher pressure load than that of lower side. That's why; the upper side of pellet seems to be more compacted than the lower side. For the uniformity, all the calculation of θ_c was performed from the upper side of pellets as the absorptivity of oil would not be same if the measurement was considered randomly from upper and lower surface of pellets.

Figure 15: Experimental set up for CA measurement. Left: video being recorded with OCA software, Right: instruments with camera, light source and dosing syringe.

Once the set up of the instrument was complete, the chosen pellet was placed at the vertical state. When the command was given to eject the distilled water drop or oil drop, it dropped down from the needle by gravitational force and got absorbed due to surface tension when touched to the pellet. A video of water drop absorption was recorded for approximately 1-2

[1] An instrument for the precise angular measurement (allows an object to be rotated to a precise angle measurement).

minutes. Before the start of analyzing, the optimum moment of water drop was found just after the drop settled on the top of pellet. It means, the moment should be avoided till the water drop was still at the stage of following by its gravity. Then, the optimum moment was set as zero time and continued analysing the contact angle.
The analytical procedure is described in annex 2.

4.2.10 Statistical methods used for analyzing data

In order to organize large set of data into a meaningful order or groups, statistical tools are of practical importance. Data analyzing helps to identify the changes in rheological properties of the diets including enzymes and different ratios of CU. Minitab 17 software (Minitab Inc. USA) was used for the hypothesis testing including t-tests and ANOVA (mainly for analysis of variance). Since there were two parameters i.e. treatment and response in tests, one way Anova test was applied to assume an initial claim to be true, and then carry out the test to prove the claim using sample data from experiment.
Besides, Anova test was used to examine p-values to test whether the claim was significantly different or not ($p<0.05$). This was used for all the rheological parameters like Pmax, a_W, tensile strength, contact angle oil/water, UPS-rate and moisture content. Even, Tukey method was used to specify the significant differences between treatments among each other.
Pearson correlation (95% confidence) test was used to analyze the existence of correlations between a_W and hardness/tensile strength, underwater pellet swelling rate and CA for oil and water.

5. Results

5.1 Moisture content

Moisture data was collected three times at different stages including enzyme addition in powder, after conditioned and that of, pellets. The average moisture content of six different treatment diets with/without CU was 17.5±0.5% after the addition of the enzymes (Table 7). But one way Anova method showed there was statistical significant difference ($p<0.05$) among these diets. Similarly, treatment diets were conditioned and moisture content data were taken. The one way Anova method showed statistical difference ($p<0.05$) among the moisture of conditioned diets. A confidence interval of 95% was used for the mean value of moisture

content. Finally, the one way Anova method was applied for the moisture content of pellets which showed no statistical difference ($p>0.05$) among the pellets of six diets.

Table 7: Moisture content of treatment diets during enzyme addition in mash form conditioned and pelleted. Presented results are the mean values ± SE, based on three repetitions analyzed for each diet.

Treatment	Moisture after enzyme addition ($p<0.0001$)	After conditioned ($p< 0.0028$)	After Pelleting ($p>0.977$)
Diet1/NY, NE	19.067^{a} ±0.185	14.66^{a} ± 0.333	14.6^{a} ± o.333
Diet2/NY, YE	19.00^{a} ± 0.251	16^{ab} ± 0.577	14.3^{a} ± 0.329
Diet3/YY, YE, 2.5%	17.50^{c} ± 0.058	14.3^{ab} ± 0.333	14.3^{a} ± 0.333
Diet4/YY, YE, 5%	17.60^{bc} ± 0.196	12.6^{b} ± 0.333	14.0^{a} ± 0.00
Diet5/YY, YE, 10%	17.50^{c} ± 0.150	13.0^{b} ± 0.577	14.3^{a} ± 1.443
Diet6/YY, YE, 20%	18.367^{ab} ± 0.120	15.3^{a} ± 0.333	14.6^{a} ± 0.333

Different superscripts (a, b, c) indicated in vertical columns from Tukey method shows significantly difference ($p<0.05$).

5.2 Tensile strength

Six different diets were used to form pellets. The one way Anova analysis showed that the addition of enzymes and CU at different percentage on diets has significant difference ($p<0.05$) in terms of tensile strength. Pellet dimension and the maximum force (N) for breaking pellets were recorded to determine the tensile strength (N/mm^2).

Table 8: Results presented are the mean values ± SE of tensile strength, Pmax and water activity based on three repetitions analyzed for each diet.

Parameters	**Diet1**	**Diet2**	**Diet3**	**Diet4**	**Diet5**	**Diet6**
Tensile strength(N/mm^2) **p<0.001**	1.08^{bc} ±0.21	0.73^{c} ±0.12	0.96^{bc} ±0.05	0.91^{c} ±0.04	1.43^{b} ±0.02	2.22^{a} ±0.05
Pmax (MPa/mm^2) **p<0.001**	0.37^{bc}± 0.07	0.26^{c}± 0.04	0.34^{bc}± 0.02	0.34^{bc}± 0.01	0.51^{b}± 0.01	0.76^{a}± 0.02
Water activity; **p=0.0424**	0.46^{ab} ± 0.01	0.46^{ab}± 0.01	0.45^{ab} ± 0.01	0.44^{b} ± 0.01	0.48^{a}± 0.01	0.48^{ab}± 0.01

Superscripts a, b, c from Tukey method indicates significant difference among diets $p<0.05$.

Pellets containing 20% CU and enzymes (diet 6) had the increased tensile strength, while pellets without CU but with enzymes (diet2) possessed the least tensile strength.

5.3 Maximum force on extracting pellet (Pmax)

The maximum peak flow force while pelleting is called Pmax and expressed in MPa/mm^2. The one-way Anova- Tukey method showed statistical difference ($p<0.05$) on the use of different percentage of CU including cocktail of enzymes on different diets for Pmax.
Comparing with the control diets, it showed that the value of Pmax increases with increasing the percentage of CU in diet 6 i.e. 20% of CU showed higher influence on Pmax than diet 2 with 0% CU but having enzymes (Table 8).

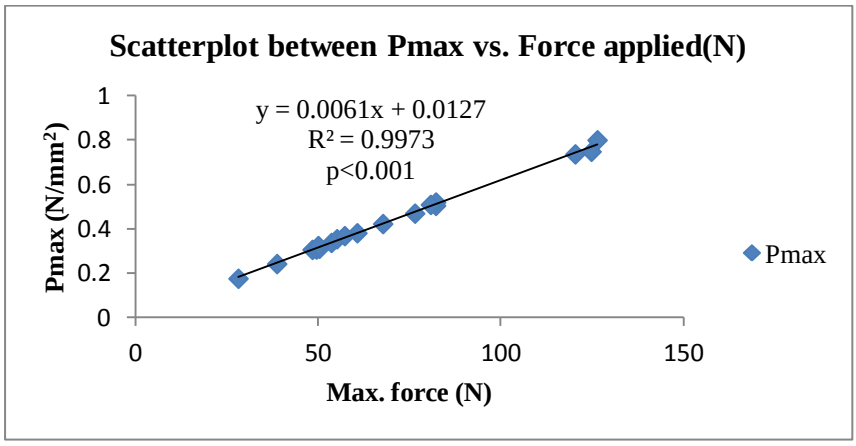

Figure 16: Correlation plot between Pmax and maximum force applied (N) on the pellet.

The scatter plot between Pmax and the maximum force applied till the breakage occurs, shows that there is a significant correlation between them and data are fitted to the regression line. Hence, with increasing the load or force applied on pellet, Pmax value also goes on increasing. Thus, a significant positive correlation occurs between Pmax and force applied.

5.4 Water Activity (a_w)

One way Anova and Tukey test showed that there is statistically significant difference ($p<0.05$) among the pellets containing varied percentage of CU on a_w.
However, the result showed diets 4 and 5 containing 5% and 10% CU are significantly different compared to other treatment diets (Table 8).

5.5 Underwater pellet swelling rate (UPS)

Using one way Anova-Tukey method, it shows that diets with different treatment (varies on CU percentage) have significant effect on underwater pellet swelling rate (UPS) in different time intervals i.e. 1, 20 and 40 minutes ($p<0.05$). Among these diets, the swelling rate of pellets is very less with increasing percentage of CU. It means, diet 6 with 20% yeast has the least swelling rate from minute one to forty.

Similarly, the table shows the mean value with different superscripts letter obtained from Tukey- comparison method and also standard error value.

Table 9: Mean values ± SE of UPS rate at (1, 20 & 40 minutes) for six different pellet diets are presented based on three repetitions analyzed for each diet.

Treatment	Total Area,mm^2 (1 min); p=0.003	Total Area,mm^2 (20 min); p=0.004	Total Area,mm^2 (40 min); p=0.003
Diet1	$48.33^{ab} \pm 6.78$	$80.51^{abc} \pm 7.82$	$106.51^{ab} \pm 4.96$
Diet2	$72.64^{a} \pm 2.49$	$109.63^{a} \pm 2.34$	$116.42^{a} \pm 2.09$
Diet3	$62.52^{ab} \pm 9.57$	$100.3^{ab} \pm 12.17$	$105.17^{ab} \pm 7.55$
Diet4	$53.46^{ab} \pm 5.82$	$73.97^{abc} \pm 5.88$	$96.96^{abc} \pm 5.85$
Diet5	$37.25^{b} \pm 1.13$	$73.97^{bc} \pm 0.18$	$92.06^{bc} \pm 3.02$
Diet6	$36.74^{b} \pm 0.53$	$66.39^{c} \pm 1.08$	$80.33^{c} \pm 2.65$

Different superscripts letter obtained from Tukey- comparison method indicate significant differences among treatments on UPS rate ($p<0.05$).

UPS rate of pellets for minute 1 to 40 showing the mean value with different superscripts letter obtained from Tukey-comparison method and also standard error value is placed in Appendix (annex A).

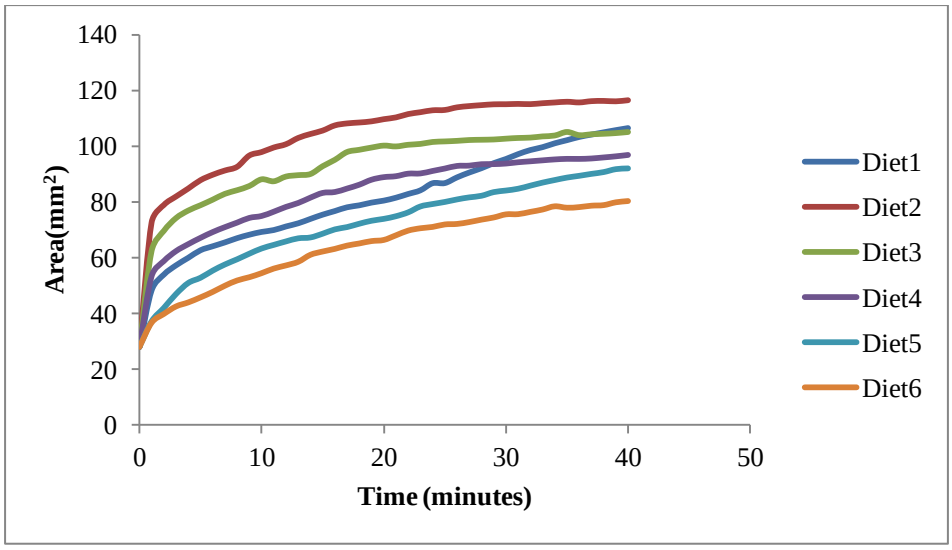

Figure 17: Underwater pellet swelling rate of different diets from minute 0 to 40 minutes.

Curve pattern from Figure 17 shows that the pellets started to deteriorate quickly once they were immersed into the water being soft and wet. From the initial zero time in water, deterioration of all diets started quickly. For the pellets from diet 2, 3 and 4, the deterioration rate continued in increasing order until 25 minutes. But after that, the rate slowed down and remained in a constant pattern until the test reached 40 minutes. However, the rate of deterioration was slow and consistent until the end of testing time period for the pellets of diet 1, 5 and 6. In overall, the pellet with 20% yeast showed the least deterioration rate and consistent pattern for the long testing time showing it as the best pellet type for the shrimps.

5.6 Contact angle (water)

The test for the wettability properties of pellets with cocktail of enzymes and different percentage of CU was carried out by the measurement of θ_c of a sessile water drop deposited on the upper surface of the pellet. The change in the θ_c with respect to time shows the rate of water absorption of pellet. The nature of compaction of powder can alter the surface of pellet and porosity affecting the water absorption rate. Thus, it ultimately changes in θ_c as function of time.

The initial θ_c for the pellets of controlled diets i.e.diet1 and 2 were $58.535^o/59.62^o$ simultaneously. Once the sessile water drop was deposited on the surface, a rapid absorption of the water was observed. In comparison between two diets for two seconds, diet containing enzyme had least contact angles showing high hydrophilicity (Table 10).

But the comparison of control diets with the testing diets i.e. diet 3, 4, 5 and 6 showed different results. Pellets of diet 3, 4 and 5 showed least contact angles making them higher in hydrophilicity than the control diets. But in reverse, diet 6 with 20% CU showed high contact angles making it high in hydrophobicity (Table 10).

Table 10: Comparison of water $\boldsymbol{\theta_c}$ among the different diets with change in time interval. Presented results were the mean values of θ_c ±SE, based on three repetitions analyzed for each diet.

Treatment	**0 Sec**	**0.5 Sec**	**1 Sec**	**1.5 Sec**	**2 Sec**
	p=0.6040	**p=0.0103**	**p=0.0070**	**p=0.0066**	**p=0.0034**
Diet1	$53.11^{a} \pm 2.49$	$46.49^{a} \pm 2.98$	$40.38^{a} \pm 2.28$	$36.63^{a} \pm 2.08$	$33.147^{a} \pm 2.24$
Diet2	$58.07^{a} \pm 1.14$	$45.42^{ab} \pm 1.20$	$37.89^{a} \pm 1.60$	$31.84^{ab} \pm 1.34$	$25.66^{ab} \pm 1.72$
Diet3	$59.00^{a} \pm 3.13$	$39.71^{ab} \pm 1.94$	$32.23^{ab} \pm 3.08$	$26.73^{ab} \pm 4.32$	$22.31^{ab} \pm 5.03$
Diet4	$56.21^{a} \pm 1.18$	$29.30^{b} \pm 2.19$	$19.17^{b} \pm 3.6$	$10.16^{b} \pm 5.88$	$4.62^{b} \pm 4.62$
Diet5	$57.11^{a} \pm 0.94$	$35.26^{ab} \pm 1.74$	$30.56^{ab} \pm 2.08$	$27.13^{ab} \pm 2.39$	$24.54^{ab} \pm 2.66$
Diet6	$58.87^{a} \pm 4.42$	$49.66^{a} \pm 6.97$	$44.73^{a} \pm 7.25$	$42.13^{a} \pm 7.90$	$40.06^{a} \pm 8.14$

Different alphabetical letters (a, b) from Anova-Tukey method indicate significant difference ($p<0.05$) among the diets except at zero second.

On the basis of one way Anova test, there was no statistically significant difference ($p>0.05$) in initial θ_c and water absorption rate between control diets and CU added diets. But with increase in time interval, statistically significant difference ($p<0.05$) in θ_c was observed (Table 10). The initial values of θ_c showed that all the surfaces were hydrophilic in nature ($\theta_c<90^{o}$).

The difference in the contact angle change can also be observed from Figure 18 where the sessile drop on the pellet surface of different diets were presented at different time interval. Water drop at 2 seconds seemed to be much more absorbed for diet 2 to diet 5. But diet 1 and diet 6 seemed to be more hydrophobic and the water drop took more time to get absorbed.

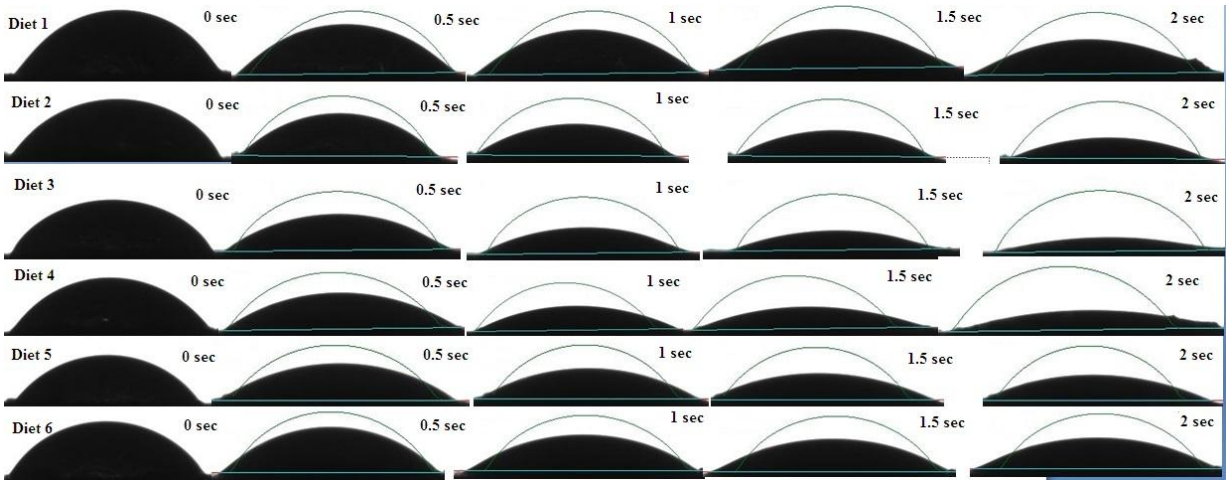

Figure 18: Change in the contact angle of sessile drop on pellet surface at different time intervals. Curve line represents initial drop profile.

5.7 Contact angle (Oil)

The initial θ_c for the pellets of control diets named as diet 1 and diet 2 were 53.087^o and 51.81^o simultaneously. The rate of oil absorption was slow compared to water drop test once the sessile oil drop was deposited on the surface.

In comparison between two control diets (with/without enzymes) for two seconds, diet containing enzyme had high θ_c showing high hydrophobicity (Figure 19).

Likewise, the comparison of control diets with other testing diets 3, 4, 5 and 6 i.e. diets with 2.5%, 5%, 10% and 20% CU showed the different results. All the pellets showed least contact angles making them higher in hydrophilicity than the control diets while observing from zero till twenty seconds.

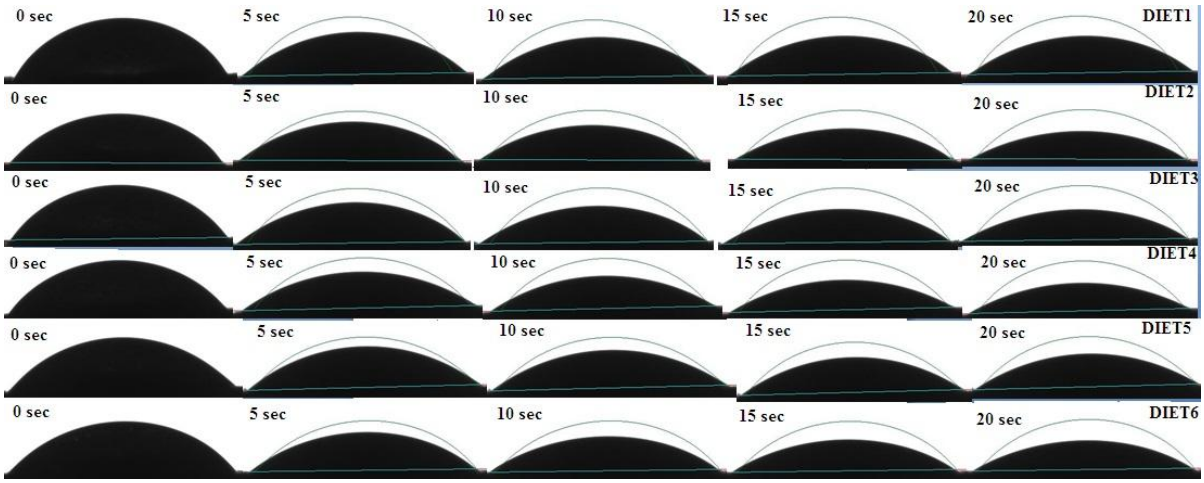

Figure 19: Change in the contact angle of sessile oil drop on pellet surface at different time intervals. Curve line represents initial drop profile

On the basis of one way Anova test, there was statistically significant difference from the start ($p<0.05$) in initial θ_c and oil absorption rate between control diets and CU added diets (Table 11). The initial values of θ_c showed that all the surfaces were hydrophilic in nature ($\theta_c<90^o$).

Table 11: Comparison of oil θ_c among the different diets with change in time interval. Presented results were the mean values of θ_c ±SE, based on three repetitions analyzed for each diet.

Treatment	**0 Sec**	**5 Sec**	**10 Sec**	**15 Sec**	**20 Sec**
	p<0.001	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**
Diet1	53.36^{a} ±0.52	42.24^{a}±0.577	41.28^{a}±0.57	38.49^{a}±0.31	37.99^{a}±0.31
Diet2	52.12ab±0.16	41.47^{a}±0.577	37.69^{b}±0.57	35.20^{b}±0.0.28	31.74^{b}±0.32
Diet3	50.98^{b}±0.48	41.407^{a}±0.39	36.71^{b}±0.57	33.916^{b}±0.16	31.59^{bc}±0.30
Diet4	52.12^{ab}±0.250	35.50^{b}±0.577	33.45^{c}±0.57	30.69^{c}±0.46	28.65^{d}±0.39
Diet5	45.91^{c}±0.07	36.070^{b}±0.57	32.72^{c}±0.83	31.106^{c}±0.16	29.68^{d}±0.39
Diet6	46.35^{c}±0.26	38.05^{b}±0.57	35.11^{bc}±0.57	31.947^{c}±0.38	29.92^{cd}±0.46

Different alphabetical letters (a,b,c,d) from Anova-Tukey method indicate significant difference ($p<0.05$) among the diets.

5.8 Pearson Correlation Comparisons

The comparison among the variables including water activity, tensile strength, underwater pellet swelling rate and water contact angle were conducted with Pearson correlation in order to determine the relationship among each other.

The p-values help in finding whether the correlation coefficients are statistically significant or not while R^2 represents a statistical measure of how close the data are fitted to regression line.

5.8.1 Water activity and tensile strength

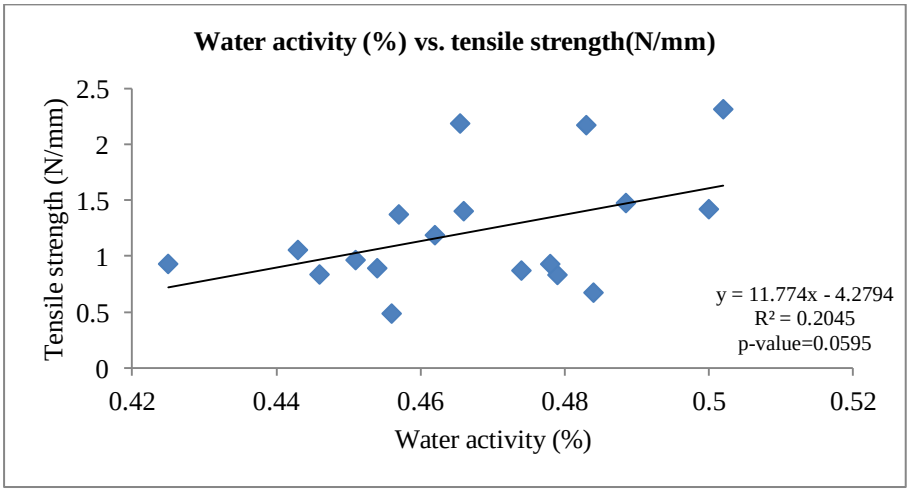

Figure 20: correlation between a_w and tensile strength (N/mm). p-value represents whether the correlation coefficients are statistically significant or not while R^2 shows the statistical measure of data being fitted to the regression line

A significant moderate positive correlation was measured between $\mathbf{a_w}$ and tensile strength (Figure 20).

5.8.2 Water activity and UPS rate

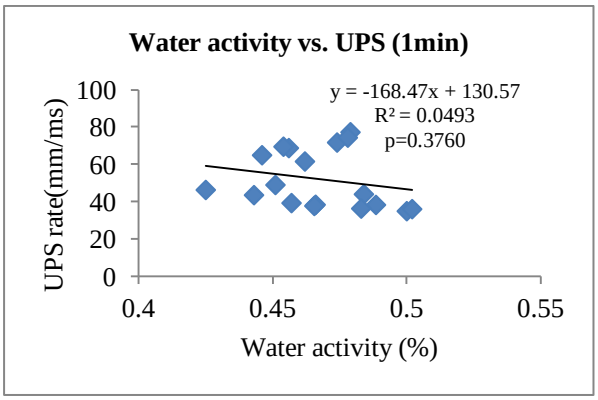

Figure 21: Correlation between UPS rate (mm/ms) for 1 minute and $\mathbf{a_w}$ (%)

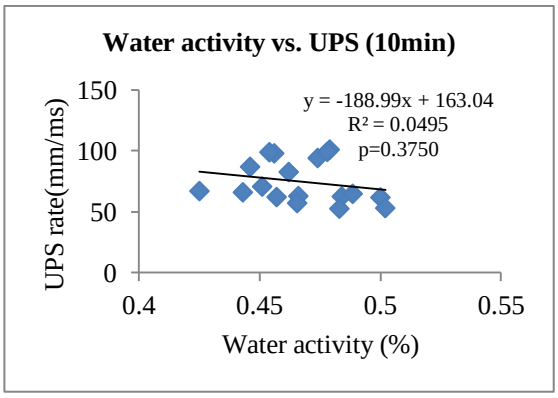

Figure 22: Correlation between UPS rate (mm/ms) for 10 min & $\mathbf{a_w}$ (%)

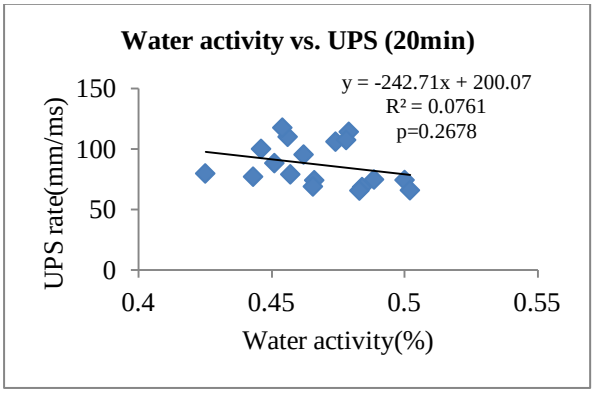

Figure 23: Correlation between UPS rate (mm/ms) for 30 minutes and a_w (%)

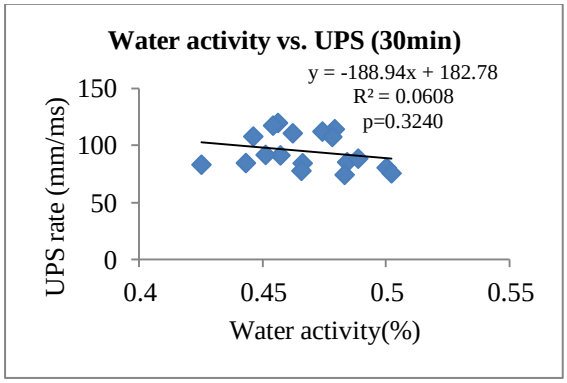

Figure 24: Correlation between UPS rate (mm/ms) for 30 minutes and a_w (%)

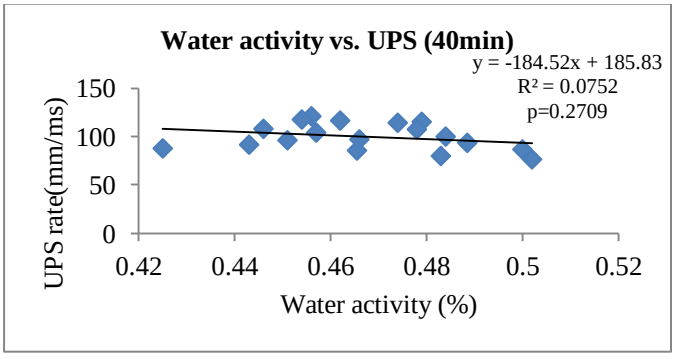

Figure 25: Correlation between UPS rate (mm/ms) for 30 minutes and a_w (%)

The above shown correlation graphs indicate that a significant weak positive correlation was measured between $\mathbf{a_w}$ and UPS rate (figure 21-25).

5.8.3 Water activity and water contact angle (θ_c)

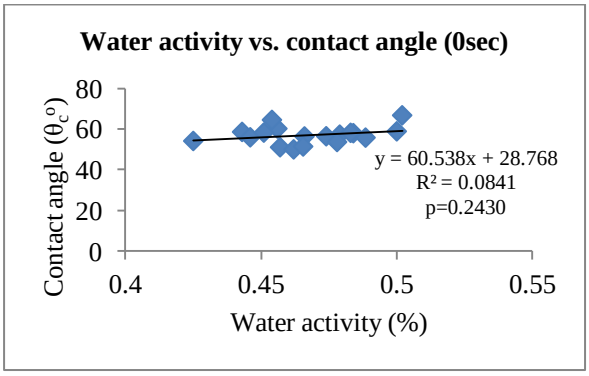

Figure 26: Correlation between (θ_c) for 0 second and a_w (%)

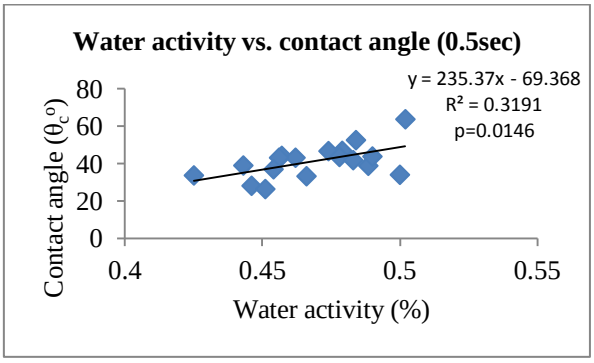

Figure 27: Correlation between (θ_c) for 0.5 second and a_w (%)

:

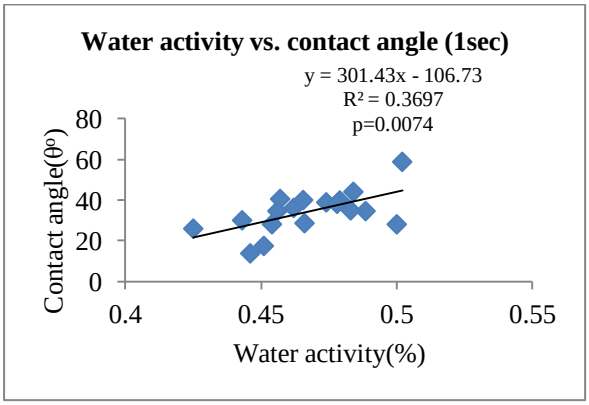

Figure 28: Correlation between (θ_c) for 1 second and a_w (%)

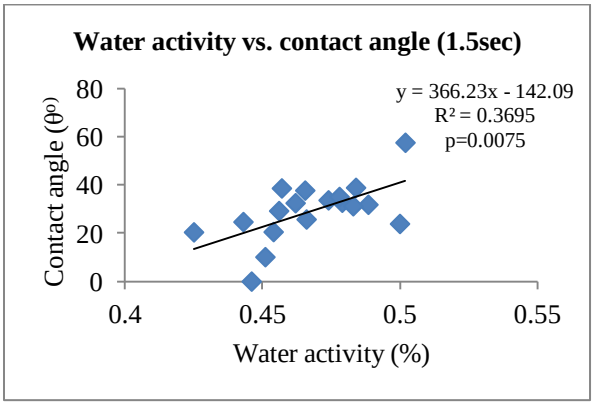

Figure 29: Correlation between (θ_c) for 1.5 seconds and a_w (%)

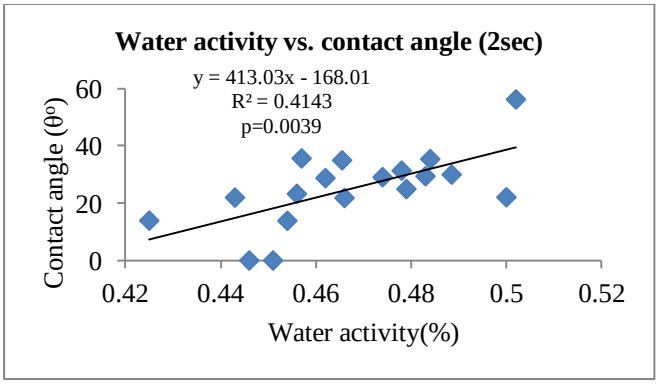

Figure 30: Correlation between θ_c for 2 seconds and a_w (%)

The above shown correlation graphs indicate that a significant moderate positive correlation was measured between a_w and water θ_c (figure 26-30).

6. DISCUSSION

6.1 Moisture content

The moisture content of powder diets during the addition of enzymes in mash was found high (Table 7) because of the interaction between water molecules and hydrophilic molecules in diets. The hydrophilic groups of protein in diets might have combined with the water molecules by H-bonds inside protein molecules. The significant difference in moisture content ($p<0.05$) among the powder diets after enzyme addition in mash might be due to variation in absorption behaviour. It is so because of the variation in ingredient quality. Diet 1 and diet 2 consists of 0% CU while rest diets include increasing percentage of CU i.e. diet 6 with 20% CU. Different ingredients with varied chemical composition and physical state have different capabilities to absorb moisture depending upon exposure time and temperature due to variation in physical and chemical surface conditions (Gilpin et al., 2002). Even the exposure of mash powder to steam during conditioning for 3 minutes at 100^{o}C enable the ingredients to enhance moisture absorption capacity since temperature has high influence on water absorption (Hemmingsenet al., 2008). The moisture content of powder mash after enzyme addition was found more than that after conditioning (table 7). It is so because ingredients exposed to water in the form of liquid state can influence more water absorption than when exposed into moist air (Hemmingsenet al., 2008; Svihus et al., 2004). Likewise, the moisture content in diets after being pelleted was found less which might be due to evaporation by high temperature in heating rod and die hole. In addition, compacting pressure in single die pellet press eliminates spaces for water molecules to stay in the pellet voids (Misljenovic et al., 2015).

6.2 Tensile strength

Among six experimental diet pellets, there is an increasing trend of tensile strength with increasing CU percentage (from 0% to 20%) in diets with enzymes (Table 8). It is because of the presence of CU quantity. Diet 1 and 2 consist of 0% while diet 6 consists of 20% CU. Since CU are small fine particles , they get hydrated and softened easily compared to larger particles aiding more gluing effect in the presence of water through liquid necking[2]. Hence it results in durable qualitative pellets (Hemmingsenet al., 2008). Even the tensile strength of pellets gets improved by conditioning process due to moisture and high temperature (Gilpin et

[2] Capillary forces existed between three phase system-solid, water and air.

al., 2002; Salas-Bringas et al., 2012). Natural binders that are present in the feed contents show binding effect due to conditioning enhancing the pellet tensile strength (Hemmingsenet al., 2008). More percentage of CU means more amount of released proteins and carbohydrates molecules which undergo protein denaturation and fibrous carbohydrate gelatinization under high temperature of 81^{o}. Thus, the strong adhesive effect and stickiness upon gelatinisation in the presence of water leads to excess hardness and tensile strength (Hemmingsenet al., 2008; Thomas & Van der Poel, 1996). Decreased tensile strength was observed among pellets with 0%, 2.5%, 5% CU as yeast was added in less amount. Pellets upon cooling can even influenced the hardness and durability of pellets as the new protein bonds are reassociated between feed particles (Thomas et al., 1997).

6.3 Pmax

As per the one way Anova & Tukey method, there is a significant difference ($p<0.05$) among diets in terms of Pmax. Table 8 shows that diet 6 with 20% CU has high Pmax value while diet 2 with 0% CU has the least Pmax. It is mentioned that the enzymes contain protease and exo/endo 1, 3 β glucanase. Protease acts upon breaking the protein molecules of yeast cell wall while endo/exo 1, 3 β glucanase focuses on releasing the fibrous molecules like β-glucan (Salazar & Asenjo, 2007). Since diet 6 has more amount of yeast, the release of protein and carbohydrates from cell membrane will also be high which ultimately causes high protein denaturation and fibrous carbohydrate gelatinization under high temperature. High temperature and moisture are necessary to activate natural binders such as protein and starch (Hemmingsenet al., 2008). This viscous nature contributes on causing more viscosity during single pellet press and hence increased Pmax at the same time. It means, there is high friction between the contact area of pellet surface and die surface. Therefore, the diet supplemented with 20% CU with enzymes cost more energy in pelleting and discharging process than the diets with less percentage or without CU.

6.4 Water Activity, a_w

Pellets from all diets under observation were found to have a_w below 0.5 which indicates that the growth of microbial organisms is not possible. Bacterial growth needs a_w at least 0.91 & fungi requires at least 0.7 (Rahman & Labuza, 1999).

There is no significant difference ($p>0.05$) in a_w among diets including diet 1, 2 without 0% CU and diet 6 with 20% CU (Table 8). Though diet 4 and 5 seem to be different, their

difference is not so big (average A_w=0.4, only difference in decimal). Thus, the high compacting pressure used during single pellet press contributes in lowering the A_w value eliminating void spaces for water molecules (Misljenovic et al., 2015).

6.5 Water Contact Angle

Results shown on table 9 indicate that the initial water θ_c of all the diets are under 65^o. Thus, they are hydrophilic in nature (Barkai et al., 2017) and have no significant changes ($p>0.05$) in terms of different dosages of CU percentage. But with interval of time period, significant changes ($p<0.05$) are seen among these diets. Since CU used in the diets was in fine powder form while other ingredients were in course form (sieved at 2mm) (Mbuto, 2017), the increase in CU% may have helped in making pellet surface smooth due to gluing of particles. As a result, water θ_c decreases with increase in CU% (2.5%, 5%, and 10%) due to better liquid penetration until the observation time of 2s. Also, the small particles are easily hydrated than big particles (Hemmingsenet al., 2008). Thus, it eases liquid penetration and decreases water contact angle (Nakae et al., 1998; Wenzel, 1949). However, protein solubility decreases at high temperature due to the progressive denaturation of the protein. Thus, the presence of H-bonds causes the increment in hydrophobicity of the molecules resulting less penetration of water (Voutsinas et al., 1983). That's why; the rate of water absorption is bit slower in the diet with more addition of yeast i.e. 20% CU compared to other diets.

Pellet diet 1 with 0% CU and 0% enzyme had less ability to penetrate liquid resulting high θ_c because of its rough surface on pellets. Smooth surface on pellets due to presence of yeast has less θ_c than that with rough surface without yeast (Kwok & Neumann, 1999).

6.6 Oil Contact Angle

The initial oil θ_c of all the diets are below 65^o. Thus, they are also hydrophilic in nature (table 10). However, diets with different percentage of CU have significant changes ($p<0.05$) on oil contact angle. Protein molecules possess emulsifying property and thus, have high oil binding ability (Voutsinas et al., 1983). Protease helps in binding with the hydroxyl group of oil. Thus, the absorption of the oil particles on the surface of pellet is much better in the diet 6 with 20% CU compared to other diets containing 0%, 2.5%, 5% and 10% CU though it is slow (table 10). Correlation study between interfacial tension and emulsifying activity of proteins describes the emulsification property of oil and proteins, explained by protein hydrophobicity (Voutsinas et al., 1983). Thus, absorption of oil is higher in diet 6.

Further, pressure and temperature used during single pellet press has a prominent role in the study of oil absorption of pellets. It is so because enzymes are also protein molecules whose molecular structures changes under high temperature and pressure (Hemmingsenet al., 2008). Thus, the use of high pressure makes the yeast particles more tighten resulting slow penetration of oil particles than usual

6.7 UPS rate

Among six different diets, diet 6 with 20% CU shows high stability under water till observed time of 40 minutes compared with diets containing CU (0%, 2.5%, 5% and 10%) (Table 9). Thus, it shows a significant difference in UPS rate ($p<0.05$) among these diets which might be due to the percentage of CU used, its nature and binding ability. High percentage of CU i.e. diet 6 with 20% CU releases more molecules of proteins and carbohydrates under heat exposure exhibiting hydrophobic character and under compaction reduces void spaces. The gluing effect of particles help in binding together either by reducing void spaces due to compression or by adhesion resulting more durable pellets (Ruscoe et al., 2005). The effect hydrophobicity is less in diet 1 and 2 as the use of CU % is 0. Since shrimps are the slow feeding creature, durability of pellets need to sustain around 40 minutes as they get sufficient time to detect and consume feed within that time period. Besides, diet formulation, particle size along with conditioning and die specification have pivotal role in maintaining the pellet quality (Behnke, 2001).

6.8 Correlation between parameters

The moderate positive correlation ($r=0.5$) between a_w and tensile strength shows that the presence of moisture influences the agglomeration of diet particles resulting high strength of pellets (Thomas & Van der Poel, 1996). Thus, the heat and moisture obtained from steam conditioning has important role in pellet strength (Salas-Bringas et al., 2012). There is a weak positive correlation ($r=0.2$) between a_w and UPS rate. However, it shows the increase in a_w enhances the binding capacity of water. It means, steam conditioning along with enzymatic treatment causes the release of protein and carbohydrate molecules from yeast cells present in diets which participate in water binding activity. These protein molecules under high temperature and pressure create more viscosity in presence of moisture which leads to bind molecules more tightly. Hence, the UPS rate of pellets decreases with the presence of high a_w.

Similarly, there is moderate positive correlation (r=0.6, except initial one) between a_w and water θ_c .Since the diet molecules are tightly bounded with high viscosity, the hydrophobic property of protein molecules repel water molecules being absorbed. Thus, increase in a_w increases θ_c on the surface of pellet.

7. Conclusion

The analysis of moisture content in powder mash from six different diets before pelleting showed significant difference ($p<0.05$). While, pellets showed no significant difference among the diets regardless of the presence or absence of enzymes and different percentages of added CU. Tensile strength showed the significant difference ($p<0.05$) among the diets where the pellet with 20% CU & enzymes showed the maximum value for tensile strength. The calculation for the Pmax of pellets from different diets showed that the addition of CU in diets enhances the peak flow force during discharge of pellets. For example, pellet with 20% CU had the highest Pmax value and 10% CU being the second. But the pellets with enzymes but no added CU had the least Pmax value. Significant difference ($p<0.05$) was observed among the pellets of different diets on a_w. Though a_w for 5% CU added pellets had the least a_w, the presence or absence of enzymes on diets showed no difference for a_w. Among the pellets of different diets, pellets with 20% CU and enzymes showed the least UPS rate from the starting minute until forty minutes. While the pellets without CU but with enzymes showed high swelling rate under water. The reason behind is, there were no released protein molecules and carbohydrates for gluing effect in presence of water and binding of molecules. It ultimately leads in shortening the time for swelling of pellets under water.

The water contact angle was significantly different ($P<0.05$) among the pellets of different diets with increasing time interval. Though all the contact angles were below 65^o and hydrophilic in nature, the pellet with 20% CU and enzymes had the highest contact angle compared to other diets. Likewise, the significant difference was observed among the pellets with different diets in oil contact angle. The high absorption rate of oil was observed in pellets with 20% CU and enzymes having lower contact angle at long time interval. While, the least oil absorption rate was seen in the pellets without CU but with enzymes having high contact angle at the same time interval.

CU has been studied widely as an alternative protein source for aquatic world in terms of nutrition and sustainability. However, the poor digestibility property of CU because of its rigid cell wall causes a hindrance for its wide application. As a solution, studies show that the

use of enzymes improves the digestion of feeds in aquatic creatures. Results from the thesis tell that the presence of enzymes in diets with high percentage of CU provides positive rheological properties. However, it has no positive effect when used alone.

Further, research can be done with varied proportion of enzymes for the same percentage of CU used in this thesis.

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9. Appendix

Annex A

The tables below show the mean values ± Std of UPS rate of pellets from three repetitions for each diet till 40 minutes. Different superscripts indicate significant differences on UPS rate obtained from Tukey- comparison method and also standard deviation value.

Treatment	Total Area;mm^2 (1 min); p=0.003	Total Area;mm^2 (20 min); p=0.004	Total Area;mm^2 (40 min); p=0.003
Diet 1	48.33ab ± 11.75	80.51abc ± 13.54	106.51 ab ± 8.59
Diet 2	72.64a ± 4.31	109.63a ± 4.06	116.42a ± 3.63
Diet 3	62.52ab ± 16.57	100.3ab ± 21.1	105.17 ab ± 13.08
Diet 4	53.46ab ± 10.07	73.97abc ± 10.19	96.96 abc ± 10.14
Diet 5	37.25b ± 1.96	73.97bc ± 0.314	92.06 bc ± 5.24
Diet 6	36.74b ± 0.914	66.39 c ± 1.88	80.33 c ± 4.59

Treatment-2min	Mean±Std; p=0.005
Diet 1	53.98a ±13.33
Diet 2	78.89ab ±5.6
Diet 3	69.7abc ±18.8
Diet 4	58.87c ±10.72
Diet 5	42.04abc ±2.82
Diet 6	39.88bc ±0.948

Treatment-3min	Mean±Std; p=0.006
Diet 1	57.31abc±12.97
Diet 2	81.94a ±7.28
Diet 3	74.3 ab±19.9
Diet 4	62.46abc±9.93
Diet 5	47.04bc±4
Diet 6	42.57c±1.466

Treatment-4min	Mean±Std; p=0.005
Diet 1	60.01abc±12.55
Diet 2	84.79a±7.29
Diet 3	77ab±19.3
Diet 4	64.95abc±10.48
Diet 5	50.99bc±3.88
Diet 6	44.02c±2.26

Treatment-5min	Mean±Std; p=0.004
Diet 1	62.71abc±12.11
Diet 2	87.78a±7.76
Diet 3	78.9ab±18.9
Diet 4	67.21abc±10.57
Diet 5	52.86bc±3.85
Diet 6	45.81c±2.72

Treatment-6min	Mean±Std; p=0.003
Diet 1	64.15abc±11.51
Diet 2	89.73a±6.81
Diet 3	80.9ab±18.4
Diet 4	69.29abc±9.59
Diet 5	55.42bc±3.6
Diet 6	47.78c±2.17

Treatment-7min	Mean±Std; p=0.003
Diet 1	65.53abc±11.57
Diet 2	91.25a±6.49
Diet 3	83ab±18.3
Diet 4	71.06abc±9.74
Diet 5	57.62bc±2.86
Diet 6	50c±2.95

Treatment-8min	Mean±Std; p=0.002
Diet 1	67.05abc±11.52
Diet 2	92.58a±5.04
Diet 3	84.3ab±17.9
Diet 4	72.68abc±9.19
Diet 5	59.49bc±3.23
Diet 6	51.87c±2.19

Treatment-9min	Mean±Std; p=0.002
Diet 1	68.29bc±11.63
Diet 2	96.62a±3.99
Diet 3	85.8ab±18.3
Diet 4	74.37abc±9.69
Diet 5	61.49bc±2.68
Diet 6	53.05c±2.24

Treatment-10min	Mean±Std; p=0.002
Diet 1	69.28bc±11.71
Diet 2	97.85a±3.46
Diet 3	88.2ab±19
Diet 4	75.06abc± 10.56
Diet 5	63.348bc±1.509
Diet 6	54.49c±2.52

Treatment-11min	Mean±Std; p=0.002
Diet 1	69.96bc± 11.98
Diet 2	99.49a± 3.69
Diet 3	87.5ab±17.8
Diet 4	76.6abc±10.77
Diet 5	64.65bc±1.077
Diet 6	56.14c±1.74

Treatment-12min	Mean±Std; p=0.002
Diet 1	71.24bc±11.81
Diet 2	100.67a±3.82
Diet 3	89.2ab±17.6
Diet 4	78.32abc±10.82
Diet 5	65.89bc±0.89
Diet 6	57.27c±1.65

Treatment-13min	Mean±Std; p=0.002
Diet 1	72.38bc±11.76
Diet 2	102.91a±4.79
Diet 3	89.7ab±18.1
Diet 4	79.73abc±10.52
Diet 5	67.002bc±0.789
Diet 6	58.56c±1.67

Treatment-14min	Mean±Std; p=0.002
Diet 1	73.95bc±11.68
Diet 2	104.33a±4.62
Diet 3	90.1ab±17.9
Diet 4	81.64abc±10.81
Diet 5	67.304bc±0.397
Diet 6	61.073c±1.557

Treatment-15min	Mean±Std; p=0.003
Diet 1	75.48bc±11.7
Diet 2	105.55a±4.9
Diet 3	92.9ab±19.6
Diet 4	83.34abc±11.96
Diet 5	68.65bc±0.201
Diet 6	62.25c±2.38

Treatment-16min	Mean±Std; p=0.003
Diet 1	76.77bc±12.14
Diet 2	107.4a±5.46
Diet 3	95.2ab±20.7
Diet 4	83.66abc±10.55
Diet 5	70.2bc±0.582
Diet 6	63.2c±2.16

Treatment-17min	Mean±Std; p=0.005
Diet 1	78.12abc±12.72
Diet 2	108.12a±5.1
Diet 3	98ab±22.9
Diet 4	84.91abc±10.45
Diet 5	71.03bc±0.58
Diet 6	64.37c±1.82

Treatment-18min	Mean±Std; p=0.005
Diet 1	78.82abc±12.72
Diet 2	108.44a±5
Diet 3	98.8ab±22.2
Diet 4	86.29abc±10.58
Diet 5	72.233bc±0.789
Diet 6	65.14c±2.17

Treatment-19min	Mean±Std; p=0.006
Diet 1	79.8abc±13.32
Diet 2	108.85a±4.6
Diet 3	99.6ab±21.7
Diet 4	88.08abc±11.29
Diet 5	73.295bc±0.534
Diet 6	65.99c±2.31

Treatment-20min	Mean±Std; p=0.004
Diet 1	80.51abc±13.54
Diet 2	109.63a±4.06
Diet 3	100.3ab±21.1
Diet 4	88.93abc±10.19
Diet 5	73.974bc±0.314
Diet 6	66.39c±1.88

Treatment-21min	Mean±Std; p=0.006
Diet 1	81.55abc±13.93
Diet 2	110.27a±3.53
Diet 3	100ab±20
Diet 4	89.34abc±11.92
Diet 5	74.921bc±0.607
Diet 6	68.05c±3.2

Treatment-22min	Mean±Std; p=0.006
Diet 1	82.89abc±14.37
Diet 2	111.46a±3.35
Diet 3	100.6ab±19.5
Diet 4	90.25abc±11.99
Diet 5	76.3bc±1.74
Diet 6	69.74c±3.01

Treatment-23min	Mean±Std; p=0.007
Diet 1	84.19ab±14.33
Diet 2	112.13a±3.19
Diet 3	100.9ab±19.12
Diet 4	90.33ab±11.79
Diet 5	78.42b±4.01
Diet 6	70.58b±2.41

Treatment-24min	Mean±Std; p=0.007
Diet 1	86.74abc±14.62
Diet 2	112.82a±3.05
Diet 3	101.6ab±18.4
Diet 4	91.22abc±12.05
Diet 5	79.29bc±4.33
Diet 6	71.06c±2.03

Treatment-25min	Mean±Std; p=0.008
Diet 1	86.79ab±14.42
Diet 2	112.93a±3.19
Diet 3	101.8ab±18.4
Diet 4	92.09ab±12.49
Diet 5	80.03b±4.12
Diet 6	71.95b±1.89

Treatment-26min	Mean±Std; p=0.008
Diet 1	88.84ab±14.45
Diet 2	113.87a±3.33
Diet 3	102ab±18.2
Diet 4	93.02ab±12.91
Diet 5	80.94b±3.99
Diet 6	72.13b±2.22

Treatment-27min	Mean±Std; p=0.008
Diet 1	90.57ab±14.12
Diet 2	114.33a±3.54
Diet 3	102.3ab±17.8
Diet 4	93.13ab±13.54
Diet 5	81.68b±3.62
Diet 6	72.8b±2.34

Treatment-28min	Mean±Std; p=0.009
Diet 1	92.13ab±14.08
Diet 2	114.66a±3.85
Diet 3	102.4ab±17.4
Diet 4	93.65ab±13.58
Diet 5	82.25b±3.54
Diet 6	73.67b±2.22

Treatment-29min	Mean±Std; p=0.008
Diet 1	93.93ab±13.33
Diet 2	114.96a±3.67
Diet 3	102.49ab±17.09
Diet 4	93.67ab±12.86
Diet 5	83.56b±4.08
Diet 6	74.439b±1.603

Treatment-30min	Mean±Std; p=0.008
Diet 1	95.42ab±13.21
Diet 2	114.99a±3.88
Diet 3	102.79ab±16.78
Diet 4	93.88ab±12.51
Diet 5	84.12b±3.98
Diet 6	75.56b±1.77

Treatment-31min	Mean±Std; p=0.007
Diet 1	97.17ab±13.29
Diet 2	115.1a±3.88
Diet 3	103.04ab±16.4
Diet 4	94.35ab±12.11
Diet 5	84.77b±3.9
Diet 6	75.64b±2.15

Treatment-32min	Mean±Std; p=0.008
Diet 1	98.63ab±13.26
Diet 2	115.02a±3.47
Diet 3	103.17ab±15.96
Diet 4	94.68ab±11.79
Diet 5	85.87b±3.97
Diet 6	76.45b±2.28

Treatment-33min	Mean±Std; p=0.007
Diet 1	99.66ab±12.78
Diet 2	115.35a±2.85
Diet 3	103.6ab±15.38
Diet 4	95.03ab±11.56
Diet 5	86.96b±4.3
Diet 6	77.29b±1.88

Treatment-34min	Mean±Std; p=0.007
Diet 1	101.05ab±12.27
Diet 2	115.62a±3.87
Diet 3	103.92ab±14.8
Diet 4	95.36ab±11.34
Diet 5	87.89b±4.67
Diet 6	78.447b±1.596

Treatment-35min	Mean±Std; p=0.003
Diet 1	102.17abc±11.67
Diet 2	115.88a±3.78
Diet 3	105.19ab±12.85
Diet 4	95.56abc±11.19
Diet 5	88.77bc±5.05
Diet 6	77.94c±3.31

Treatment-36min	Mean±Std; p=0.005
Diet 1	103.33abc±10.9
Diet 2	115.63a±3.14
Diet 3	104.04ab±14.8
Diet 4	95.54abc±11.25
Diet 5	89.4bc±5.04
Diet 6	78.16c±3.76

Treatment-37min	Mean±Std; p=0.004
Diet 1	104.18ab±10.27
Diet 2	116.09a±3.69
Diet 3	104.36ab±14.24
Diet 4	95.7abc±11.28
Diet 5	90.1bc±4.91
Diet 6	78.67c±3.95

Treatment-38min	Mean±Std; p=0.004
Diet 1	104.98ab±9.75
Diet 2	116.17a±3.87
Diet 3	104.54ab±13.9
Diet 4	96.07abc±10.87
Diet 5	90.74bc±5.06
Diet 6	78.87c±4.39

Treatment-39min	Mean±Std; p=0.004
Diet 1	105.77ab±8.97
Diet 2	116.03a±3.24
Diet 3	104.79ab±13.44
Diet 4	96.47abc±10.52
Diet 5	91.79bc±5.28
Diet 6	79.88c±4.59

Treatment-40min	Mean±Std; p=0.003
Diet 1	106.51ab±8.59
Diet 2	116.42a±3.63
Diet 3	105.17ab±13.08
Diet 4	96.96abc±10.14
Diet 5	92.06bc±5.24
Diet 6	80.33c±4.59

Annex B

Contact angle of shrimp pellets with water/oil is measured by video-based optical contact angle measuring device.

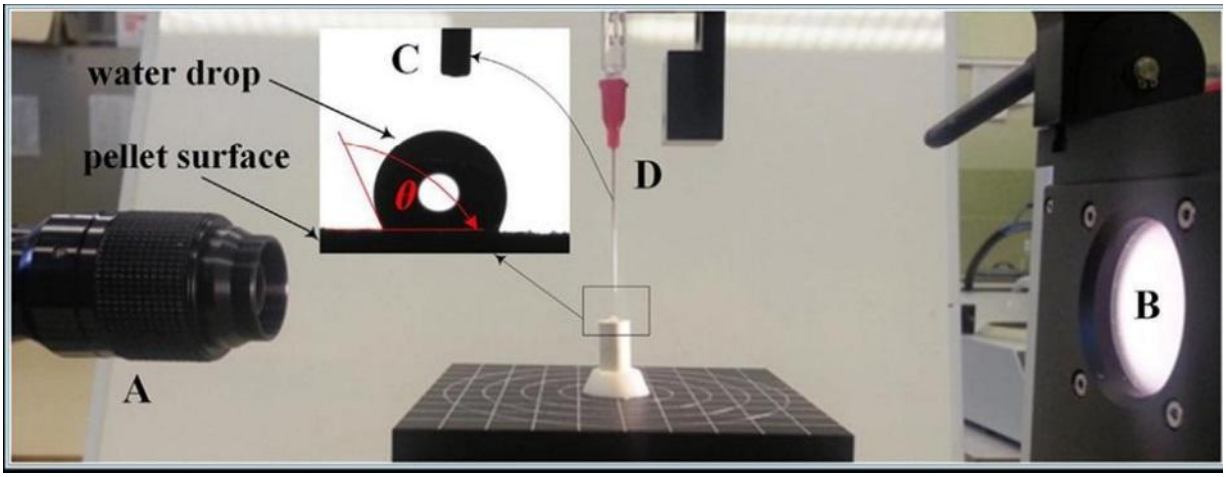

Figure 31: Experimental set up for θ_c measurements. Items are indicated by letters: (A) camera; (B) light source; (C) image of a drop on top of a pellet surface for θ tests; (D) dosing syringe with a needle (Misljenovic et al., 2015).

Information about using instrument (Operating manual OCA 15EC):

- Mounting the dosing of syringe: the syringe type used in this experiment is called Braun 1ml disposable syringe. Liquid used for the contact angle measurement includes water and glycerol. The density and viscosity of water are 0.998g/cm^3 and 1mPas respectively; the density and viscosity of glycerol (rapeseed oil=0.93g/cm^3)) are 1.26g/cm^3 and 1.41e^{+003}mPas, respectively.
- Positioning of the dosing needle: the dosing needle can be placed/adjusted vertically to the optical axis (X-axis) by means of the two adjustment screws.
- Positioning of the sample: pellet to be examined is placed on the the sample stage and can be freely movable in horizontal (X- and Y-axis) direction over the whole base plate by a magnetic slide system.

- Adjustment of the illumination: the brightness of the homogenous back lighting is adjusted with the control knob at the back of the illumination housing, or within the SCA software.
- Setting for the Frame grabber/ camera: Exposure time for most applications is 1ms and an illumination intensity of 10-15% is appropriate values. The windows Frame grabber Preferences contains 4 tabs, Images, Size, Timing, and Buffer. While, adjustment of the optics has parameters like Zoom, Fine focus, Tilting wheel, and Rotation.

Measurement operation

- The pellet was adjusted just under the needle keeping more at the centre.
- Caution was taken while dosing the drop to avoid air bubbles in the complete dosing system.
- When the SCA software was started, live video or drop image window opened automatically showing the live image from the camera.
- Video was recorded immediately once the command was given to dispense the water/oil drop.
- Approximately 1-2 minutes was given in order to have enough time for penetration.
- Then the recording was stopped and saved it for analyzing the contact angle.
- The zero time was determined for the drop on the surface and then contact angle was calculated.

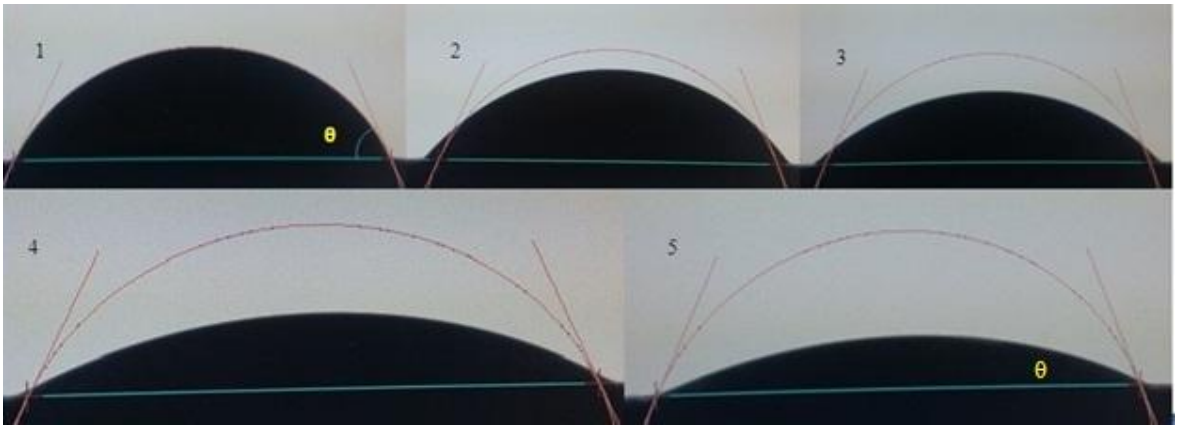

Figure 32: the explanation of contact angle of pellets by using sessile drop on pellet surface. θ_c is contact angle. 1 indicates initial contact angle and 5 indicates final contact angle.

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