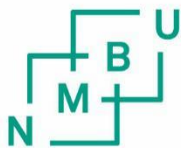

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Effect of different enzymes on compacting and physical pellet quality characteristics of torula yeast (*Candida utilis*)

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Table of contents

Abstract 3

Introduction 5

Methods and Materials 14

Part 1-----Description for experimental diets production line 14

1. Raw materials... 14

2. Diet formulation... 15

3. Mixing.. 16

4. Sampling 17

5. Leaving in fridge... 17

6. Conditioning 17

7. Pelleting 17

Part 2 ----Description for analyzing instruments and methods................ 19

1. Water activity(Aw) 19

2. Hardness... 19

3. Moisture content 20

4. Underwater pellet swelling rate (UPS-rate)................................... 20

Part 3 ----Description for statistical analyzing methods 26

Results 27

1.Maximum extraction force of pelleting (N).. 27

2. Surface contact angle of oil (θº) and rate of oil absorption (θº/ms) .. 27

3. Surface contact angle of water (θº) and rate of water absorption (θº/ms) 29

4. Hardness / tensile strength (Mpa) 30

5. Water activity (aW) 31

6. Moisture content (%) 32

7. Underwater pellet swelling (UPS) rate 33

8. Pearson comparisons 34

Discussion 39

1.Peak force (p-max) 39

2. Oil contact angle (θº) 39

3. Water contact angle (θº) 41

4. Hardness 42

5. Water activity or Aw 42

6. Moisture content(%) 43

7. Correlations between all parameters 43

Conclusion 44

Acknowledgment 46

References 47

Appendix 53

Abstract

The purpose of this study was to investigate the reaction between the yeast materials derived from *Candida utilis* (CU) and different enzymes, and to evaluate their interaction on physical pellet quality. In order to evaluate the effect of different enzymes on *Candida utilis*, three kinds of enzymatic products were used, to compare the influence of each enzyme on CU. Used enzymatic products were protease, endo/exo 1.3-beta-glucanase, and the mixture between those two products. A number of physical pellet quality parameters were analyzed to determine the enzymatic influence on hardness, underwater pellet swelling rate (UPS rate), moisture content, water activity (Aw), surface contact angle with water/oil. Ten diets were formulated with three different enzymatic dosages: the optimal dosage recommended by the producer, ten folds higher dosage and hundred folds higher dosage. Among three enzymatic products, the protease showed indication to influence changes on pellet quality when compared to endo/exo 1.3-beta-glucanase, mixture of protease and endo/exo 1.3-beta-glucanase. The results showed that among all enzymes, mixture of glucanase and protease had higher influences on P-max than single endo/exo 1.3-beta-glucanase and protease; The 1 dosage protease and 1 dosage mixture of protease and endo/exo 1.3-beta-glucanase increased the oil absorption rate; The water contact angle was significantly increased when optimal dosage (1 dosage) of protease and 100 dosages of endo/exo 1.3-beta-glucanase were added; The 100 dosages added protease increased predominantly the tensile strength, while endo/exo 1.3-beta-glucanase containing 10 dosages significantly decreased the tensile strength; Endo/exo 1.3-beta-glucanase decreased the aW, while

the increased dosage of protease up to 100 folds decreased the aW values; All enzymes increased the moisture content to different levels, but the effect of protease was more significant than other enzymes; the protease with 100 dosage dramatically decreased the UPS rate.

Key words

Candida Utilis (CU); Water activity (aW); P-max; Underwater pellet swelling (UPS) rate; Contact angle; Moisture content; Hardness; Tensile strength; Protease; endo/exo 1.3-beta-glucanase; Dosage;

Introduction

In recent years, the increase of global human population resulted an increase of the food demand. Finding sustainable food resources that will assure the food security is a challenging goal. Also, growing this sustainable foods should possess less influence on the environment, less usage of land and less burden on economic growth (Øverland and Skrede 2016). As the main source of protein and unsaturated fatty acids, seafood developed into one of the most popular foods around the world. However, this demand leads some challenges for aquaculture development: scarcity of resources, exploiting of agricultural land and environmental pollution (Martins *et al*. 2010). It is necessary to identify the importance of strategies and solutions to ensure the sustainable development of aquaculture. Finding a sustainable raw ingredients is one of conclusions for further expansion for aquatic production. At current situation, fish meal and soybean meal, as a protein source, are main dietary raw ingredients for aquatic animals. Aquatic animals, like fish and shrimp need higher content of protein in their diet because their energy production pathway depends, to some extent, on protein metabolism in the body (Avnimelech 1999). Traditionally the fish meal is the main protein source for fish and shrimp diets, as they can meet special need of fish, like amino acids composition as well as fishy smell (Jack *et al.* 1965). According to Food and Agricultural Organization of the United Nations (FAO), 7 million tonnes of fish meal is used as a protein source every year. Among plant materials, soybean is considered as an optimal protein source with nutritionally balanced composition of amino acids Protein content can vary from44 to 50 % (FAO). As we know, that protein is one of the most expensive nutrients

included in aquaculture feed. Most of the protein sources are considered as unsustainable. For example, need for the fish meal severely causes over-harvesting of a small fish because most of the protein source for fish feed originates from catching wild small pelagic fish (Julián *et al*. 2016). There are some negative reasons for using soybean meal (SBM) as a protein source. SBM is heavily demanding for arable land. Also, pretreatment of anti-nutrienal factors in plants itself. Therefore, it is necessary to find solutions to fill the gap for further maintenance of aquaculture development.

Application of novel ingredients There are several single-cell organisms presenting the biomass which was discovered to have important nutritional value, for both human food and animals feed. Those are bacteria, fungi and algae (Ravindra 2000). Single cell protein (SCP) referred to the protein which are extracted from those single cell microorganisms was coined by Carol L. Wilson in 1966 (Doelle 1994). More importantly, SCP possesses nutritional value for both human and animal health (Hezarjaribi *et al.* 2016). For feed production, SCP can be a supplementation or replacement of fish meal or soybean meal as a protein source for fish and shrimp. The application of SCP as nutritive source in animal diet mainly depends on its characteristics:

1) Chemical compositions of SCP: high content of crude protein, approximately 40-80% on dry weight bases, with the essential amino acids, like lysine and methionine (Hezarjaribi *et al.* 2016), but to notice that the content and compositions of single cell protein are depending on species (FAO). Besides that, those single cell microorganism also contains fat, carbohydrates, nucleic acids, vitamins and minerals (Ravindra 2000).

2) Unlimited harvesting time: The growing of single cell organisms is very fast and without depending upon climate (Hezarjaribi *et al.* 2016).

3) Environmental friendly: The single cell microorganisms are suitable for utilizing agricultural and industrial waste as a substrate in limited land (FAO, 2012). The nutritional value of all these microbial in food/feed are similar, but they come from different groups.

Bacteria, Algae and Yeast The nutritional value of any microbial protein is determined by its composition. Many bacterial species are utilized as SCP origins in feed, such as *Aeromonas hydrophilla*, Bacillus megaterium and Bacillus subtilis (Dhanasekaran, *et al.* 2011). Bacteria are capable of cultivating on many substrates, such as starch, sugars and some liquid hydrocarbons like methane. The chemical composition of bacteria are decided, to large extent, by substrates, but in general, they are rich in protein, about 80% (on a dry basis weight), high in nucleic acids (15%-16%) and essential AA like methionine (2.2%-3.0%)(Al□Harbi and Uddin 2005).

The time for utilization of algae were more earlier than bacteria in Mexico, and mostly used as foods after drying them. Algal SCP are mostly produced by some species, such as *Caulerpa rocemosa*, *Laminari*a *(micro)*, *Porphyra* and *Sargassum*. The basic requirement for fertilizing algae is carbon dioxide and sunlight. Micro algae rich in protein around 40-60 %, fats around 15% (but depends on the species and media for growing), vitamins (A, B, C, D and E), close to7% minerals, fiber, some essential AA

like methionine (~1.4%-2.6%)(Becker 2007). Algae contain lower content of nucleic acids comparing with bacteria, approximately 4.6% (Brock, 1989).

SCP from yeast have high nutrients value. Among these, some yeasts have been utilized widely in feed/food ingredients, like *Candida, Hansenula, Pitchia, Torulopsis* and *Saccharomyces cerevisiae* (Ravindra 2000). The chemical composition of yeast are crude protein ~55%, methionine ~1.8%-2.5%, B-group vitamins and nucleic acids ~9.7% (Singh *et al.* 1991). The growth media for yeast can be sulfite liquor waste and molasses (Nalage *et al.* 2016)The significant role of SCP extracted from yeast strains are determined not only by nutritional value, but also by its biological function. Torula yeast are used widely among all yeast strains. Torula yeast are utilized as a protein source for broiler since 1976 in Cuba and was used in white leghorn birds in 1981 (Valdivie, Compte et al. 1982) . Torula yeasts were used as a protein source for Pacific white shrimp *Litopenaeus vannamei* by Julián et al., in 2016. The result was pointed out that the proper amount of torula yeasts in diet has similar contribution on growth of shrimp as fish meal.

The greatest potential of these ingredients goes to their sustainability and environmentally friendliness (Øverland and Skrede 2016). Comparing with fish meal and plants originated materials, it is easier to get microbial proteins by avoiding use some resources that are directly available for human food production like water, energy and arable land (Maccaferri *et al.* 2012).

Benefits of Candida utilis utilization Generally speaking, several sort of yeast species are imported as nutritive ingredients in food/ feed production, like *Saccharomyces sp.* yeast as a byproduct of the alcohol industry; The promising benefits of utilization of the CU as protein source can be summarized in several fields. In economical aspects, growing yeasts are cheaper and yeast production is independent from climatic conditions. *Candida utilis* have shorter growth time and less dependence on arable land as long as they have proper substrate to growth like, like sugar waste from industries (Øverland *et al.* 2010). In terms of nutritive value, torula yeast contains high content of protein with high-value amino acids profile, which is similar to the amino acids ratio of the fishmeal, but lower in some essential sulfur containing amino acids like cytine and methionine (Øverland 2010). In addition, CU also contains nucleic acids, fatty acids, vitamins and minerals. Another advantage of the torula yeast in feed manufacturing is that it can help to improve the immune system (Sheikhzadeh *et al.* 2012) and to maintain the intestinal microbiology system in shrimp and fish etc (Campa-Córdova *et al.* 2002; Burgents *et al.* 2004)

Problems with yeasts as a protein source The component of yeast cell are mainly protein and carbohydrates, which amount to approximately 2/3 of cell components (Yamada and Sgarbieri 2005). The protective wall structure of US is a complex mixture of polysaccharides and protein: dietary fibrous composed the carbohydrates parts, like β-glucan and mannan (Fleet 1991); the inner layer composed by β-(1,3) glucan, β-(1,6) glucan, a small amount of chitin; the inner layer surrounds by mannoprotein, a mannan rich glycoprotein (Yoshida, Naito et al. 2009). Protease acts upon protein structures,

while endo/exo 1.3-beta-glucanase catalyse on fiber utilization (β-glucan) and furthermore, will increase the utilization of both protein and energy in the feed. Moreover, the structure of the yeasts' cell wall is composed by polysaccharides structured in carbohydrates and protein complex. Yeast cell wall consists at least two membranes and the main content of the first membrane is mannan with a small quantity of arabinose and galactose in which the main content of the second membrane is insoluble glycan (Yamada and Sgarbieri 2005). All these cell compositions are hard to digested by animals because most animals have poor secretion of enzymes that act on fibrous ingredients in their digestive tract. For fish, especially carnivores, and shrimp have few microbial activities in their digestive tracts. The digestibility of the yeast protein is little in nature when giving shrimp feed with fibrous materials in their diet, and consequently, most of the yeast protein in diet will be wasted intact in water (Rumsey *et al.* 1991). Another concern should be taken into consideration is the nucleic acids content in the diet when yeast is used as protein source. Even the shrimp belongs to cold environmental animal, the nuclease enzyme, especially deoxyribonuclease (DNase), has minor low temperature activity (Nilsen, Øverbø et al. 2010). Proper amount of nucleic acids can be the nitrogen source, but large level of nucleic acids will cause some disorders in animal body, like gut and kidney stone formation (Nasseri *et al.* 2011).

Solving in problems The CU contain 8%-11% nucleic acids in the basis of cell weight (Weatherholtz and Holsing 1976). The most economical method to reduce nucleic acids is done by the heat treatment in an acidic medium. The preferable

temperature for acidic treatment method is 90 C, which can protect amino acids and crude protein in to large extent (Zee and Simard 1975). The low digestibility of CU protein is a major question which needs to be solved. The explanation of low digestibility is that all nutrients of yeast are placed in cell cytoplasm and not accessible by human or animal. Nutrients are wrapped in the cell walls which are indigestible components like chitin and cellulose, therefore both human and animals are not able to break down the cell walls (Peng *et al.* 1984). Consequently, all protein and other nutritive components are excreted from host animals' body. In order to handle the indigestible characteristic of CU cell wall, enzymatic hydrolysis is invented. The mechanism of this method is that enzymes are applied to break down the yeast cell walls to liberate the nutrients inside. Enzymes are also implemented for the sake of increasing the digestibility of yeasts as the protein source for intensive shrimp farming and other fish. Enzymes are protein that are catalysts that act upon some complex protein structures into small pieces like amino acids so that the animals can building their body tissue. Some enzymes are not secreted by animal body but can be added in diets, while some enzymes are secreted by microorganisms in host animals' digestive tract. Application of enzymes have some significance improvements in torula yeasts' protein digestibility.

Aquatic feeds are more complicated than feeds for domestic animals. It should have specific density and under water swelling ability so that it can be ingested by underwater animals with the minimum level of wastage. For shrimp, the feed should have high physical quality as shrimp more prefer to take the certain time to eat all the

pelleted feed. The purposes of this study are 1) to see how is the reaction between yeasts and different content of enzymes and 2) how is this reaction influencing on pellet physical quality. In order to evaluate the effect of enzymes on the yeasts, three kinds of enzymes were used in first-feed production part to compare the specific influence of the each enzyme on torula yeasts: protease, endo/exo 1.3-beta-glucanase, and their mixture. The reason why these enzymes were chosen mostly determined by the composition of *candida utilis*. And several physical pellet quality parameters are analyzed in second-analyzing part to determine the physical pellet quality:hardness, underwater pellet swelling (UPS), moisture content, water activity(Aw), a contact angle with water and contact angle with oil(reason to chose these). Ten diets were formulated in with different enzymatic dosage: controlled diet - 200g torula with no enzyme; graded level of (0.012g, 0.12g, 1.2g) protease, (0.012g, 0.12g, 1.2g) gluconase, and (0.012g, 0.12g, 1.2g) protease/gluconase were incorporated into 200g torula, respectively.

Physical quality of feed The hardness of pellet is important indicator for feed quality, especially for feed for fish and shrimp, because fish feed need high stability under the water. The better the binding capacity of particles, the better the hardness. The hardness of pellet was always presented by tensile strength. Tensile strength means that the resistance of a pellet to break under certain tension, which is the main factor to evaluate the hardness. The Brazilian method is the common method to calculate the exact strength. In the formula of Brazilian test, tensile strength are: $\sigma_t = \frac{2P}{\pi DL} = \frac{P}{\pi RL}$, where P (N) in the applied maximum force for crack the pellet, radius R, L is length of pellet

(Claesson and Bohloli 2002). Aw content indicates the free water molecules in the pellet. The optimum water content is also a critical condition for producing high quality shrimp feed. Surface hydration/Contact angle with water/oil is an important indicator for feed quality because pellets needs to absorb water molecules during extrusion or absorb oil molecules during coating oil(Yuan & Lee 2013). UPS is also an important parameter for qualifying the aquatic feed. Unlike other animals' feed, feed for aquatic animals need to have certain water stability because feed might to be consumed by aquatic animals after certain time (Lim and Cuzon 1994). This certain time needs feed to stay contact. The higher the water stability, the lower UPS rate, therefore, the longer time the pellet would stay intact. That would help to decrease the economic cost by decreasing the wastage of feed (Lim and Cuzon 1994).

Methods and Materials

Part 1----- Description for experimental diets production line

1. Raw materials

The materials used for this experimental work were US (*Candida utilis*) and different enzymes. Spray dried US samples obtained from Lallemand BioIngredients Inc. (Canada), and were mixed with various enzymes in order to to produce different diets for this study. Enzymes were mixed into US in order to characterize the effect of enzymes on rheologial properties of US. The enzymes used for this study were commercial protease (AB, Vista, Marlborough, UK), cocktail product of endo/exo 1.3-beta-glucanase (Megazyme, Ireland) and mixture of this three enzymes. The chemical composition of torula yeast is shown in table 1.

Table 1

Average chemical composition (% by weight) of dried CU

H_2O	CP	CF	EE	Ash	NFE	Ca	P
7.0	48.0	2.1	2.7	8.0	32.2	0.49	1.52

Data presented by (Ling 1967). CP (crude protein), CF (crude fiber),EE(ether extract) NFE(nitrogen free extract), Ca(calcium), P (potassium)

Table 2.

Average chemical composition of essential amino acids of dried torula yeast (Candida Utilis)

Arg	Cys	Met	Thr	Iso	Leu	Lys	Val	Tyr	Tryp	Phen	Hist
2.62	0.60	0.79	2.62	2.88	3.51	3.77	2.93	2.00	0.51	2.93	1.36

All values were presented as % by weight on as fed-basis: Arginine-Arg, Cystine-Cyt, Methionine-Met, Threonine-Thr, Isoleucine-Iso, Leucine-Leu, Lysine-Lys, Valine-Val, Tyrosine-Tyr, Tryptophan-Tryp, Phenylalanine-Phen, Histidine-Hist. (Murray and Marchant 1986).

2. Diet formulation

Diet formulation was designed by and produced at, Norwegian University of Life Science, Ås, Norway. Ten diets were made for this study Each diet contained 200g of torula yeasts. Three variable parameters were designed in this diet formulation: enzymes (Protease, endo/exo 1.3-beta-glucanase and mixture of protease+endo/exo 1.3-beta-glucanase), and three different dosages of each enzyme: Optimal dosage suggested by enzyme producers, and marked as 1x (~0.012g), ten folds of optimal dosage, 10x (~0.12g), and hundred folds of optimal dosage, 100x (~1.2g).

Table 3

Diet Formulation

	Control	C+ Protease			C + Gluconase			C+Protease+gluconase		
	Diet1	Diet2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet10
Torula(%)	100	100	100	100	100	100	100	100	100	100
Water(%)	10	10	10	10	10	10	10	10	10	10
1Dosage(%)		0.006			0.006			0.006		

10Dosage(%)			0.06			0.06			0.06	
100Dosage(%)				0.6			0.6			0.6
Total (%)	110	110.006	110.06	110.6	110.006	110.06	110.6	110.006	110.06	110.6

Control/C means control diet. Glucanse were endo/exo 1.3-beta-glucanase

3. Mixing

To mix and spray the water-enzyme solution, an intensive mixing was performed to the MB using a high shear mixer having three impellers and a tulip-form chopper (Diosna P1/6, Germany). The mixing speed of the impellers was 250 rpm and a chopper speed 500 rpm. Spraying of distilled water and enzyme solution was performed with a spraying lance (Düsen-Schlick GmbH, Germany, Model 970) assembled in the mixer. Samples were taken from different areas in the mixer for every trial. Thereafter, all taken samples for each trial were mixed together to obtain the representative sample, assuming a homogeneous mixture. The moisture content presented in this work is a triplicate of each trial, done by using the method defined by EU Commission Regulation for sampling and analyses of the official control of feed (No. 152/2009). The average moisture measurement for all trials was 8 % w/w (+/- 0.3 %). Immediately after adding the water-enzyme solution, the powder was collected and packed into vacuum-sealed bags to prevent moisture loss. The samples were stored at 4 ºC for a maximum of 4 weeks until pelleting.

4. Sampling

Twenty six samples were weighted (around 0.2g) from each diet: 10 samples for testing moisture content after conditioning and 16 samples for further pelleting.

5. Leaving in fridge

The samples from mixing process were kept in fridge at 4 degree all the time because enzymes (endo/exo1.3-beta-glucanase, protease and mixture of endo/exo 1.3-beta-glucanase+protease) are activated when they are at room temperature. The activities of enzymes need to be inhibited because the main component of candida utilis are complex protein and some fibrous carbohydrates.

6. Conditioning

Every 26 samples for each diet were submerged into the boiling water and as being in eppendorf tube and conditioned for 3 minutes. after 24h .

7. Pelleting

The single pellet press method, presented in previous publications (Salas-Bringas *et al.*, 2010; Salas-Bringas *et al.*, 2011; Mišljenović *et al.*, 2015) was used for compacting the CU into the cylindrical pellets. Pellets were made to characterize the compressibility of CU. Approximately 0.2 grams of CU was used to produce each pellet. Each sample for every trial was poured into the channel of the preheated (81 ^{o}C) compressing die and it was heated-up prior compaction for 3 minutes on 81 ^{o}C. The same temperature of 81 ^{o}C was used during compaction and also pellet extraction from the die. The compressing rod with diameter of 5.4 mm was inserted immediately after the sample

was poured-in to prevent release of water from sample during heating-up. After the trail mixture was heated-up the initial pre-load force of 5.6 Nm was applied. Maximal force load of 285 Nm and compressibility of about 12 MPa was applied while continuous heat in the die of 81 °C was in use. The temperature of 81° C is recommended to eliminate possible salmonella contamination in the animal feed (VKM, 2006). Chosen compressibility (i.e. compacting pressure versus density) of the trial mixture was used according to densities of products derived from the commercial animal feed ring-die pelleting process, defined in detail by Salas-Bringas *et al.* (2011). The chosen pressure has been observed to be within the range of pressures used in ring die pellet press to produce other feed pellets (Salas-Bringas *et al.,* 2011; Salas-Bringas *et al.*, 2015). The rate of compression was set to 10 mm/min through a rod inserted in a 5.5 mm blank die. After compaction, the blank part of the die was removed and thereafter pellets were discharged. The pellet discharge was set to a speed of 2 mm/min which was low enough to avoid possible exceeding of the compacting-pressure and hence avoid any further compaction. The total retention time of the materials in the channel, tempered to 81° C, was about 9 (± 1) minutes. Compacted pellets were having diameter of 5.5 mm and final pellet weight was 0.2 (+/- 0.03) grams. All compacted pellets were stored on 4 °C for a maximum of 30 days prior analyzing.

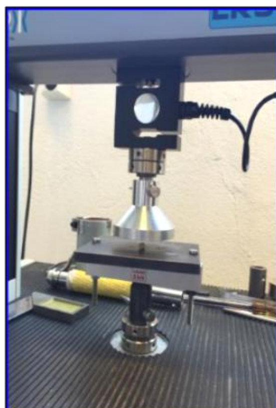
Part 2 ----Description for experimental diets analyzing instruments and methods

1. Water activity(Aw)

Aw indicates the free water in pellets, and have enough to inhibit the growth of some microorganisms such as molds. Four selected pellets from each diet were tested for Aw by the machine called Rotronic Hygrolab C1(Switzerland). The point for measuring time was scaled from 4.3 minutes to 5.5 minutes. The temperature was averagely 23.12 ±1.13℃.

2. Hardness

Diet hardness was analyzed by similar process descried in pelleting process. But the hardness were expressed through maximum tensile stress (σ) for cylindrical specimens under diametral compression on four pellets for each diet. The difference from pelleting process, the hardness test was held without applying of single compression die hole



channel, rod and blank. The contracting cylindrical barrel was replaced by a probe which was a flat side scaled 60mm in diameter, and the probe was connected to a Lloyd LR 15K (Lloyd instrument, UK) texture analyzer and a sensor which sends information to the computer. Four pellet from every diet were RANDOMLY chosen to do hardness for further comparison. Once a pellet was settled by horizontal state at the bottom flat plate, the probe pressing towards the pellet at the rate of 2mm/min. Strength for each pellet was measured by the first peak showing the force (F) in Newton (N) during a diametral compression at 1 mm/min^{-}. The first peak of force was recorded during crushing the pellet. The used force shows in the

computer as Newton/meters. The rate of probe press against the pellet was set up in computer manually at the beginning. The σ data for analyzing were obtained from the computer.

3. Moisture content

The moisture content was tested by METTLER LJ16 Moisture Analyzer in the lad at IMT, NMBU, Norway. The moisture content of samples was measured as replicate for 10 non-pelleted samples (mash), each diet measured right after conditioning and 4 pellets from every diet after pelleting to see how much water is lost at different stages.

4. Underwater pellet swelling rate (UPS-rate)

The water stability is one of the important pellet quality indicators for the aquatic feeds. The UPS rate was measured by recording images of the pellet silhouette every minute for 40 minutes. in 100 ml distilled water at room temperature. Forty images of per pellet were analyzed by FIJI software to determine the underwater pellet rate because water stability was expressed by UPS-rate. Every 4 pellets from 10 diets were tested by Krüss Tensiometer- a Micro Viper Portable Computer combining with Allen Compact Video Microscope Lenses. The rate of pellet UPS property indicates the quality of pellet resistant to swelling under water.

Pellets water stability were expressed by UPS rate. Forty images were taken after the pellets were settled under 100ml distilled water, and those images were measured by images analysis software. The image software include the video lenses from Krüss

Tensiometer and its light source; a Micro Viper portable computer with its software; and compact video microscope lenses (Allen)(figure1). Support for video microscope (figure 2) is the working body of the Krüss tensiometer. The glass container and all other equipment were designed, and 3D printed at Mathematical and Technical department, Norwegian University of Life Sciences (figure 3). The Micro Viper portable computer contains the video microscope and the Micro Viper software that captures the images of a solid submerged in water at the time interval desired by this experiment. Allen, compact video microscope lenses with different zooming capabilities are used-.Dependending on the required distance and size of the examined solid , by assembling them (figure 4) and connecting such setting to Micro Viper portable computer.

Figure 1. Equipment used for the evaluation of UPS swelling (left – Krüss tensiometer, center –Allen zoom compact video microscope lenses, right – Micro Viper portable computer.

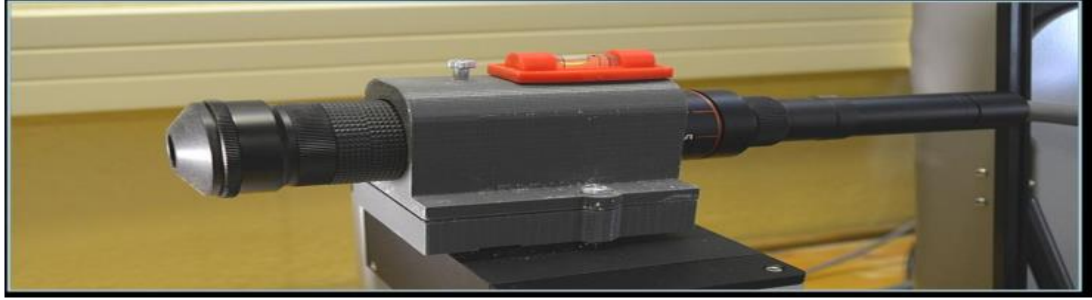


Figure 2. Assembled and fixed video microscope lens on tensiometer.

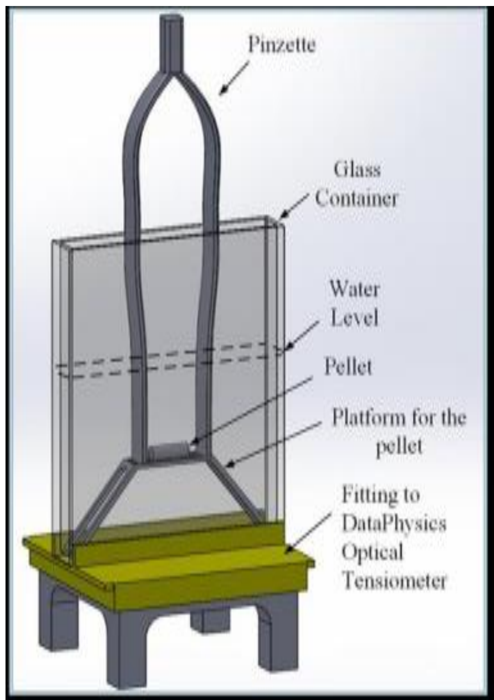


Figure 3. Assembled equipment for the image analysis of the UPS rate of the pellet (Salas-Bringas *et al*., 2015).

Figure 4. 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.

4.1. purpose:

--UPS rate is an important part of characterizing the physical quality of the aquatic feeds (fish and crustaceans).

--UPS has been describe historically as the "Pellet stability" which was referring to ability of feed pellet to maintain its form without leaking its nutrients while being submerged in the water, deprived of being consumed by the aquatic animal.

4.2. Scope and the equipment:

--The method for analyzing the UPS rate employs a video microscope lenses connected to a Micro Viper portable computer and attached to a Krüss tensiometer body. Figure 5 presents all parts for assuring the assembly of the equipment that is used to assure a good stability of the camera lens and overall good stability of the image or the observed object.

--video microscope

--micro Viper portable computer

--Krüss tensiometer

--assembly made in plastic by 3D printer

--100 mL graduated cylinder for measuring the 100 mL distilled water

--stop watch.

5

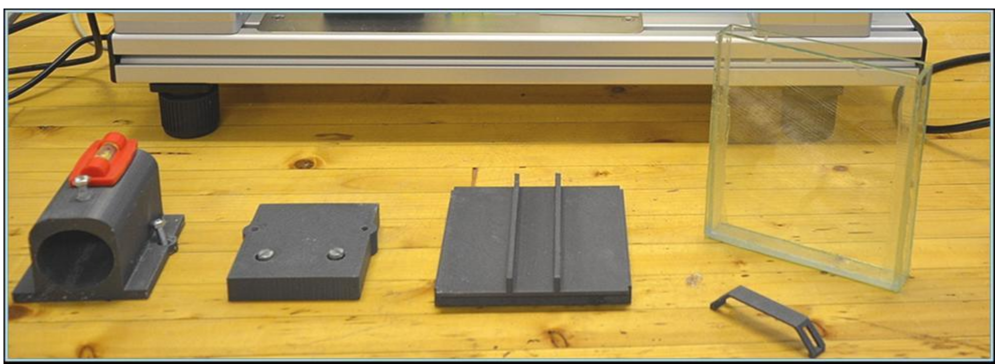


Figure 5. 3D printed parts for complementing the support for tensiometer lens (Krüss G10).

1 & 2: support for stabilizing and mounting the video microscope on the tensiometer (3, 4 and 5)

3 : support for fitting the glass container to Data Physics Optical Tensiometer

4 : bridge for keeping and stabilizing the pellet while in the glass container

5 :Glass container

4.3.Analytical procedure:

--take randomly one pellet from the pelleted diet/test/mixture you want to observe the UPS rate. The best results are obtained if minimum 3 pellets from the same diet/test/mixture are used (three randomly chosen pellets as replicates).

--assemble the video microscope lenses as suggested in figure 4 and mount it on the tensiometer (figure 1) with the 3D printed holder parts (figure 5).

--mount the glass container (figure 5, part 5) on the optical tensiometer, as suggested in figure 2 and introduce the pellet support bridge (figure 5, part 4) inside the container

--add 100 ml distilled water in the glass container. Ensure that there are no air bubbles in water. If necessary, wait five minutes for water to stabilize or destroy bubbles with the long laboratory pinzette.

--ensure that laboratory pinzette is not wet prior any pellet or solid to be subjected to further analyses will be touched with the pinzette.

--with a long laboratory pinzette submerge the pellet VERY FAST under distilled water and place it onto the sample support bridge (figure 5, part 2). Ensure that on the Micro Viper portable computer screen you see only the circle of the compressed cylinder (pellet). If you do not see only circle, adjust the submerged pellet by gently touching it as long as you can see only the circle, but under 60 seconds. In case it will take longer time, use another pellet.

--the complete analytical setup for the UPS ratio measurement is presented in figure 6

--start the stopwatch immediately and as fast/soon as possible to avoid any delay, and take the first image, as soon as you got to place the pellet on the support bridge. Continue taking images once in a minute, for minimum 40 minutes and at the requiredtime intervals for your experimental design.

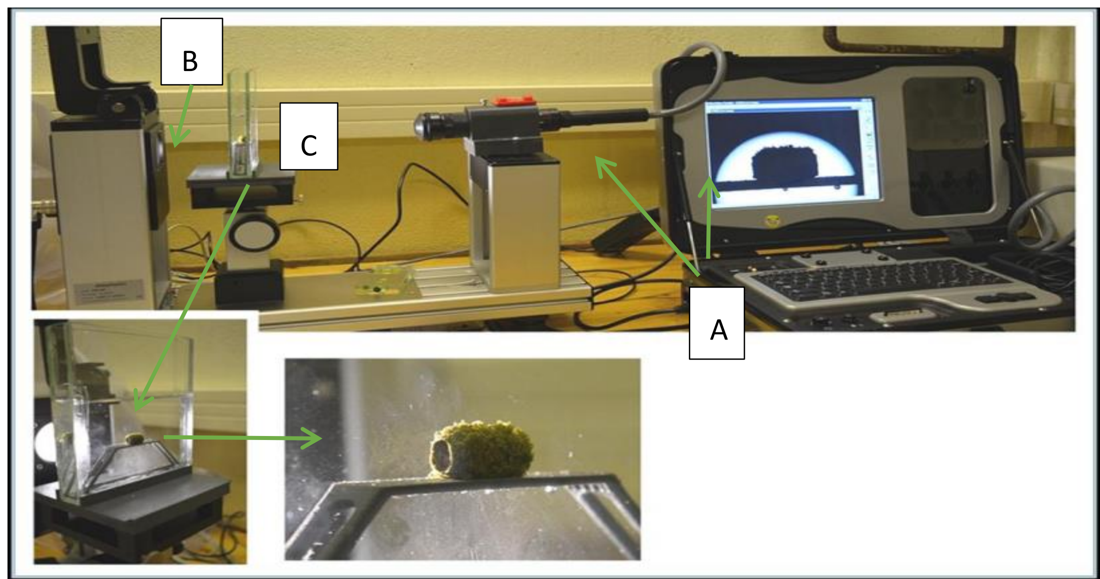


Figure 6. Complete analytical setup for the UPS ratio measurement using image analysis glass container; A – Video microscope; B– Light source; C – Pellet in glass container.

4.4 Analysis and calculations:

--FIJI software was used further steps for the UPS analyses of the images taken by Micro Viper portable computer and lenses assembly. The obtained results were defined as the underwater swelling rate during required time interval.

--all 40 images from every pellet were stacked together and measured by fiji to determine the increased swelling area for calculating swelling rate in distilled water.

5. Contact angle (θ) with water/oil and

In this study, all lower side of all pellets used for analyzed were showed darker color than upper sides in order to comparison. The analytical procedure is in appendix 1.

Part 3 ----Description for statistical analyzing methods

The main goal of data analyzing is to understand the change of rheological properties of the materials as CU. All the results from data analysis in this paper were performed by Minitab 17 software (Minitab Inc. USA). One of the most common methods used in statistical analysis is hypothesis testing. Minitab offers many hypothesis tests, including t-tests and ANOVA (mainly for analysis of variance). Usually, ANOVA is used when you perform a hypothesis test, you assume an initial claim to be true, and then test this claim using sample data.

--used ANOVA to examine p-values so that it more easy to figure out the possible effects of the enzymes and their dosages on these quality parameters: p-Max, aW, hardness, contact angle oil/water, UPS-rate and moisture content. Tukey–Kramer (95% confidence interval) was used to see how is the significant differences between treatments.

--used Pearson correlation (95% confidence) to analyze the existence of correlations between aW and other responses as CA for oil and water as well as UPS rate and the rest.

-- UPS-rate analysis method have to be mentioned here because the underwater pellet swelling parameter is important factor for aquatic feed. The raw data from pellet water stability got through images analyzing.in FIJI. Every 40 images of each pellet were stacked and the increased area were recorded at every minutes.

Results

1.Maximum extraction force of pelleting (N)

The maximum pelleting force was expressed by the peak flow force or P-max (N/mm^2). The peak flow force for pelleting extraction was analyzed by one-way ANOVA in order to evaluate the significant effect of different enzymes and dosages on P-max.

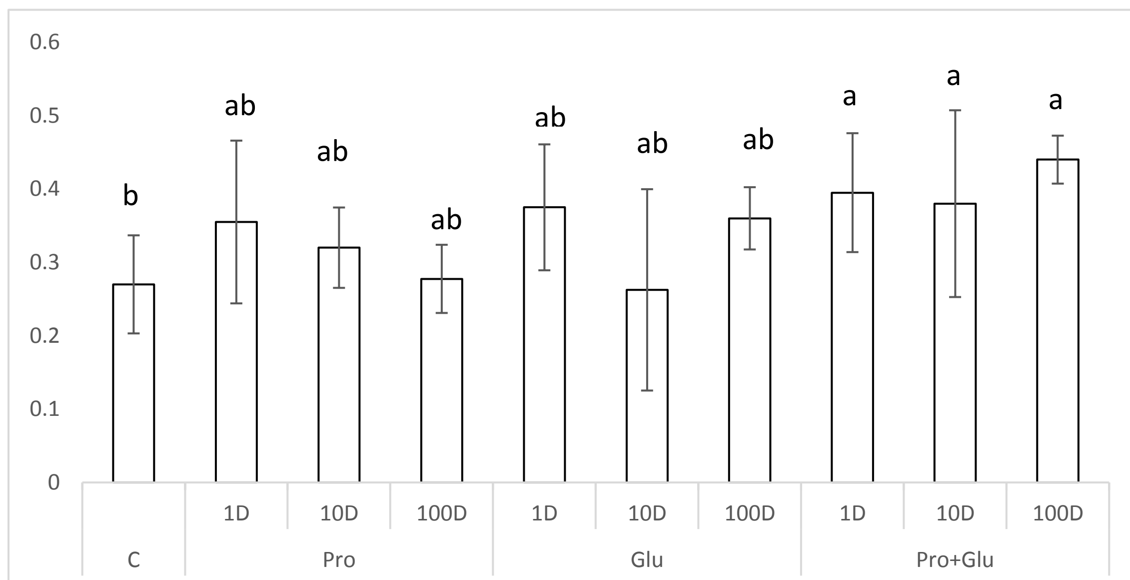


Fig.7. Relationship between P-max with enzymes and dosages of these enzymes were presented by the comparison of means and standard deviation. The standard deviation was represented by error bars; different letters from tukey method indicated the significant differences ($p < 0.05$); enzymes included protease (pro), endo/exo 1.3-beta-glucanase (glu), endo/exo 1.3-beta-glucanase +protease (glu+pro), C indicates control diet; 1D being 1 dosage(=0.012g enzyme), 10xD being 10dosage of enzyme(=0.12g), 100D being 100dosage of enzyme(=1.2g enzyme).

Comparing with control diet, the results from tested diets presented in figure 7 showed that P-max was increased by both, the enzymes and the dosages of the enzymes. However, the effect of dosages was not as significant ($p > 0.05$) as the changes caused by enzymes ($p < 0.05$) themselves. Among all enzymes, mixture of endo/exo 1.3-beta-glucanase and protease had higher influences on P-max than single endo/exo 1.3-beta-glucanase and protease.

2. Surface contact angle of oil (θº) and rate of oil absorption (θº/ms)

The rapeseed oil was chosen to test the surface contact angle on yeast-based pellets. The time for oil to penetrate into pellet was recorded just after the oil drop settled down on the pellet surface. The p-values derived from one-way ANOVA analysis considered dosages of enzymes and and oil contact angle. Those parameters were measured in order to evaluate the significant effect of the enzymatically treated CU material on the surface tension of the rapeseed oil, and oil absorption rate by the yeast-based pellets.

Table 4. Comparing the control diet with diets containing different enzymes (protease, endo/exo 1.3-beta-glucanase, protease+endo/exo 1.3-beta-glucanase, respectively) and different dosages (1 fold; 10 folds; 100 folds) on the sides of initial oil contact angles and the change of angles per millisecond. Means values ± SD, 4 pellets per diet were used. Different superscripts from Tukey method indicate significant differences for that particular column.

Enzyme	number of dosage	Initial θº	Rate of oil absorption (θº/ms)
Protease	1	$62.59^{C} \pm 2.05$	$0.50^{A} \pm 0.18$
	10	$57.23^{C} \pm 6.58$	$0.47^{A} \pm 0.08$
	100	$60.83^{C} \pm 1.41$	$0.38^{A} \pm 0.07$
glucanase	1	$59.49^{C} \pm 4.44$	$0.42^{AB} \pm 0.04$
	10	$62.42^{C} \pm 3.00$	$0.34^{AB} \pm 0.06$
	100	$64.89^{C} \pm 1.41$	$0.37^{AB} \pm 0.07$
glucanase+protease	1	$61.52^{C} \pm 3.02$	$0.60^{B} \pm 0.02$
	10	$56.37^{C} \pm 7.85$	$0.32^{B} \pm 0.06$
	100	$56.07^{C} \pm 8.84$	$0.21^{B} \pm 0.13$
control	0	$60.99^{C} \pm 1.54$	$0.41^{AB} \pm 0.12$

The results presented in table 4 show that both, enzymes ($p > 0.05$) and dosages ($p > 0.05$) changed the oil absorbency of pellets at different levels, however these differences were not statistically significant. On the other hand, the enzymes influenced the oil absorption rate ($p < 0.05$). The enzymatic dosages had no significant effect ($p > 0.05$) on oil absorption rate. Among enzymes, the optimal dosage of protease (1 fold) and optimal dosage of combined protease and endo/exo 1.3-beta-glucanaseglucanase (1 fold) increased the oil absorption rate. The combined protease endo/exo 1.3-beta-glucanase with 100 folds dosage decreased the oil absorption rate.

3. Surface contact angle of water (θº) and rate of water absorption (θº/ms)

The surface hydrophilicity and hydration properties of pellets were measured.

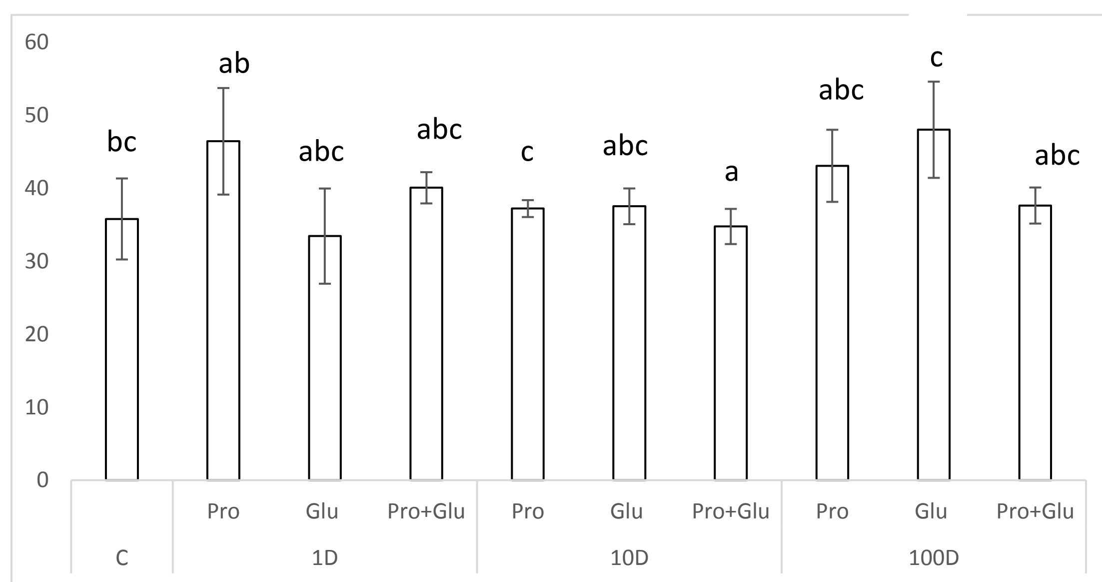


Figure 8. Comparison of average contact angle values of a distilled water drop settled on pellets from different diets (4 pellets per diet). Enzymes included protease (pro), endo/exo 1.3-beta-glucanase (glu), endo/exo 1.3-beta-glucanase +protease (glu+pro). Means values ± SD(error bars indicate the standard deviation). Different superscripts indicate significant differences on initial water contact angles (θº).

The results from one-way ANOVA analysis and tukey-kramer honestly significant difference method showed that the enzymes have statistically significant effect (p <

0.05) on water absorption-ability. Though, the dosages did not influence any changes ($p > 0.05$). Figure 8 showed that among all enzymes, the water contact angle was significantly increased when optimal dosage (1 dosage) of protease and 100 dosages of endo/exo 1.3-beta-glucanase were added. From the other side the water absorption-ability decreased when 1 dosage of endo/exo 1.3-beta-glucanase and 10 dosages of protease+endo/exo 1.3-beta-glucanase were used.

4. Hardness / tensile strength (Mpa)

The p-values of maximum load from one-way ANOVA analysis showed that enzymes cause similar changes on pellet hardness ($p=0.102 > 0.05$), but the effect were different with the changes of dosages ($p=0.018< 0.05$) of them. In this study, the maximum force (N) for breaking pellets and pellet length were recorded to determine the tensile strength of pellets (N/mm).

Table 5.

Compared three diets with different enzymes (endo/exo 1.3-beta-glucanase; protease; endo/exo 1.3-beta-glucanase+protease; respectively) to control diet (without any enzymes) and their effect on pellet tensile strength ($p > 0.05$). The tensile strength is represented as the force N/mm² when breaking the pellet. Presented results were the mean values ± SD, based on four repetitions analyzed for each diet. Different superscripts (from Tukey method) indicate significant differences for that particular column.

Enzymes	tensile strength(N/mm)
Protease 1x	$34.93^{b}\pm2.2$
Protease 10x	$36.22^{b}\pm1.89$
Protease 100x	$55.54^{a}\pm12.12$
Glucanase 1x	$41.38^{ab}\pm7.49$

Glucanase 10x	$25.74^{b} \pm 9.76$
Glucanase 100x	$37.30^{ab} \pm 9.77$
Glu + pro 1x	$35.76^{b} \pm 3.7$
Glu + pro 10x	$32.01^{b} \pm 10.66$
Glu + pro 100x	$34.83^{b} \pm 1.96$
Control	$29.97^{b} \pm 8.61$

Both, enzymes and their different dosages had significant effect on tensile strength ($p < 0.05$). The 100 dosages added protease increased predominantly the tensile strength, while endo/exo 1.3-beta-glucanase containing 10 dosages significantly decreased the tensile strength.

5. Water activity (aW)

One-way ANOVA analysis and tukey-kramer results showed that the effect of enzymes on water activity is statistically significant ($p<0.05$). Though, changes of water activity was not apparent with the supplementation of different dosages of enzymes ($p>0.05$).

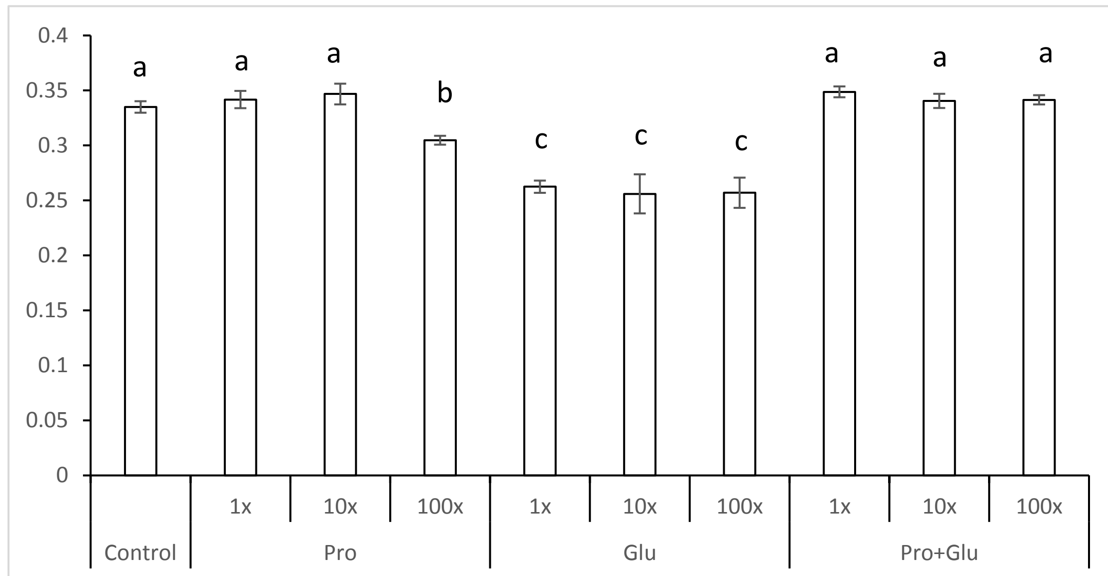


Figure 9. Comparison of average water activity values (aW) of pellets. Presented results are mean values from 10 diets based on,4 pellets for each diet. Enzymes included protease (pro), endo/exo 1.3-beta-glucanase (glu), endo/exo 1.3-beta-glucanase +protease (glu+pro) and different enzymatic dosages. Different superscripts indicate significant differences on water activity.

The results presented in fig.9 showed that endo/exo 1.3-beta-glucanase decreased the aW. Mixture of protease and endo/exo 1.3-beta-glucanase and alone protease partially did not influence significantly on aW. However, the increased dosage of protease up to 100 folds decreased the aW values.

6. Moisture content (%)

The moisture content of pellets were measured twice (before and after pelleting) to evaluate the effect of different enzymes with different dosage on moisture content; also to see the influence of pelleting process on moisture content.

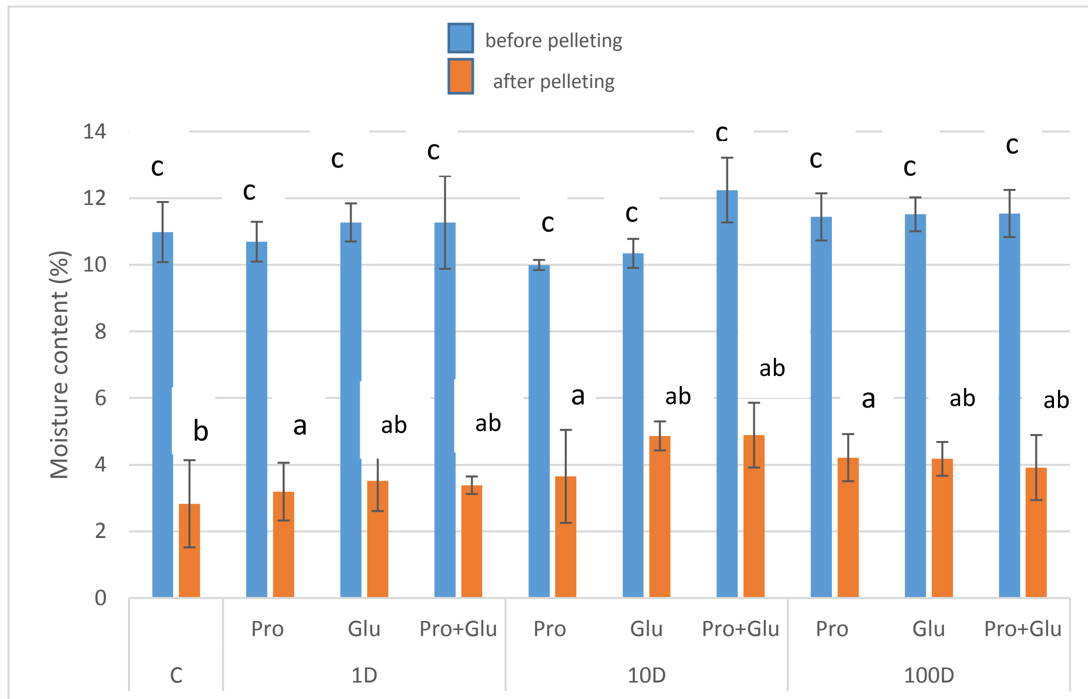


Fig 10. Comparison of the effect of enzymes and their dosages on moisture content (before pelleting and after pelleting): Mean values and±SD (error bars indicate the standard deviation) are based on ten samples for each diet before pelleting and four samples/each diet after pelleting. Different superscripts indicate significant differences on moisture content. C presents the control diet and 1D, 10D,100D presents 1 dosage, 10 dosage and 100 dosage, respectively.Enzymes included protease (pro), endo/exo 1.3-beta-glucanase (glu), endo/exo 1.3-beta-glucanase +protease (glu+pro).

The results presented in fig. 10 showed that both enzymes and their dosages have no significant effect on moisture content before pelleting ($p > 0.05$). However, the enzymes have significant differences on moisture content after pelleting process($P < 0.05$), but this changes were not related to their dosages ($p > 0.05$). All enzymes increased the moisture content to different levels, but the effect of protease was more significant than other enzymes.

7. Underwater pellet swelling (UPS) rate

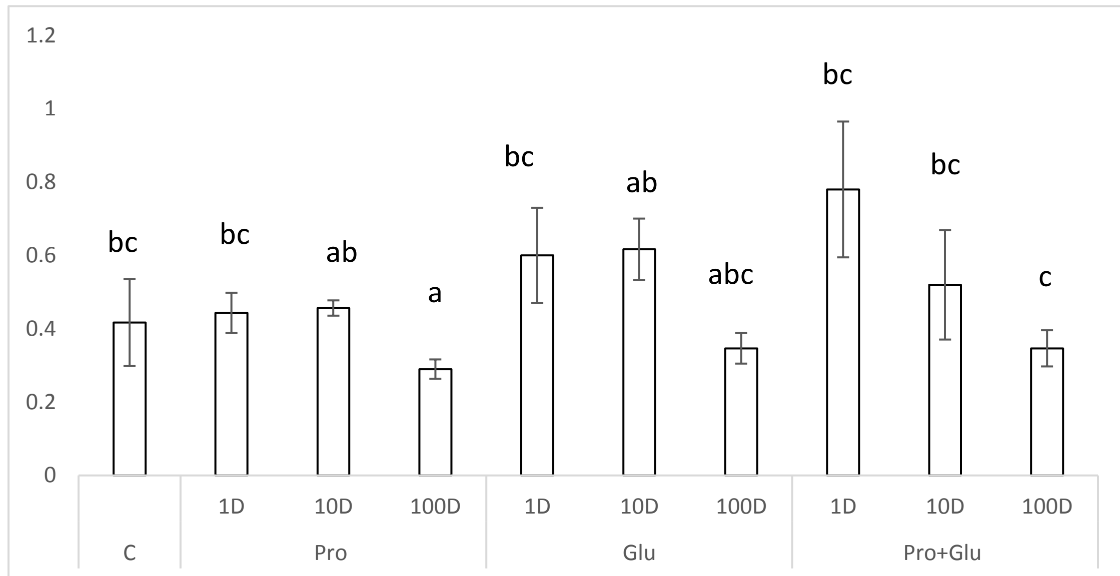


Figure 11. Comparison of average UPS rate of pellets. Mean values derived from 4 repetitions for each diet. Different superscripts indicate significant differences on water activity. Enzymes included protease (pro), endo/exo 1.3-beta-glucanase (glu), endo/exo 1.3-beta-glucanase +protease (glu+pro).

The outcome from one-way ANOVA with Tukey comparison method showed that both, enzymes and dosages have significant effect on UPS rate ($p < 0.05$).

The results presented in fig. 11 show that all enzymes have significant differences on UPS rate. Among these enzymes, the protease with 100 dosage dramatically decreased the UPS-rate, which means that 100 dosage protease helped to prolong the time of pellet swelling under water.

8. Pearson comparisons

The Pearson correlation between all variables were challenged by Minitab software in order to determine the relationship between p-max, UPS-rate, tensile strength, oil contact angle, water contact angle, moisture content and water activity. The p-values indicate whether the correlation coefficients are statistically significant or not and R^2 is a statistical measure which shows how close the data are fitted to regression line.

Among these, some of variables have positive relationship, while others have negative relationships to each other (fig.12; fig.13; fig.14; fig.15; fig.16)

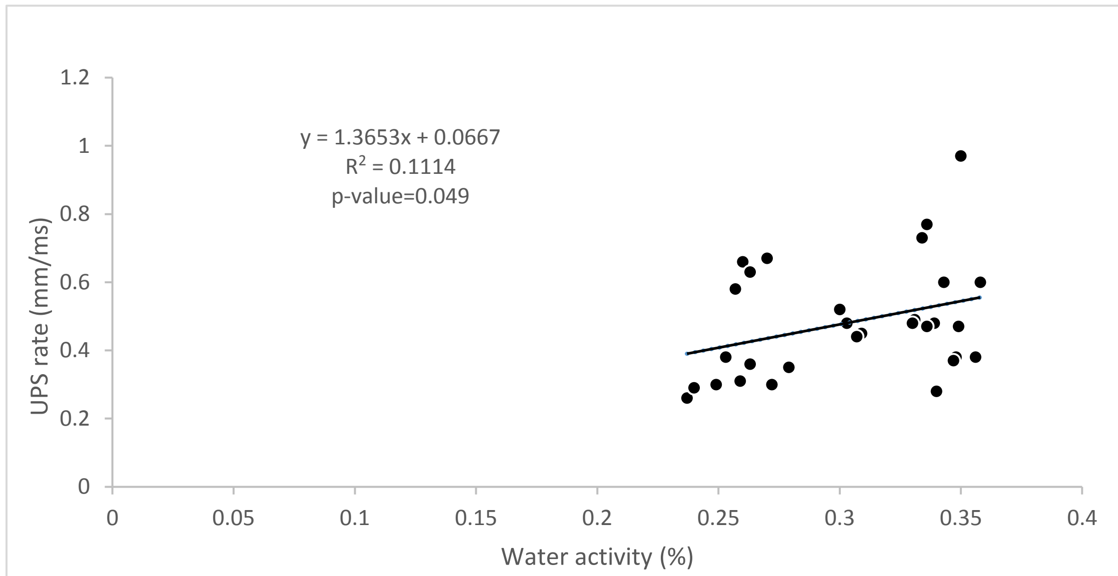


Fig 12. Correlation between UPS rate and water activity (%). P-values indicate whether the correlation coefficients are statistically significant or not. R^2 is a statistical measure showed the fitted regression line.

A significant positive correlation was measured between UPS-rate and water activity (fig. 12).

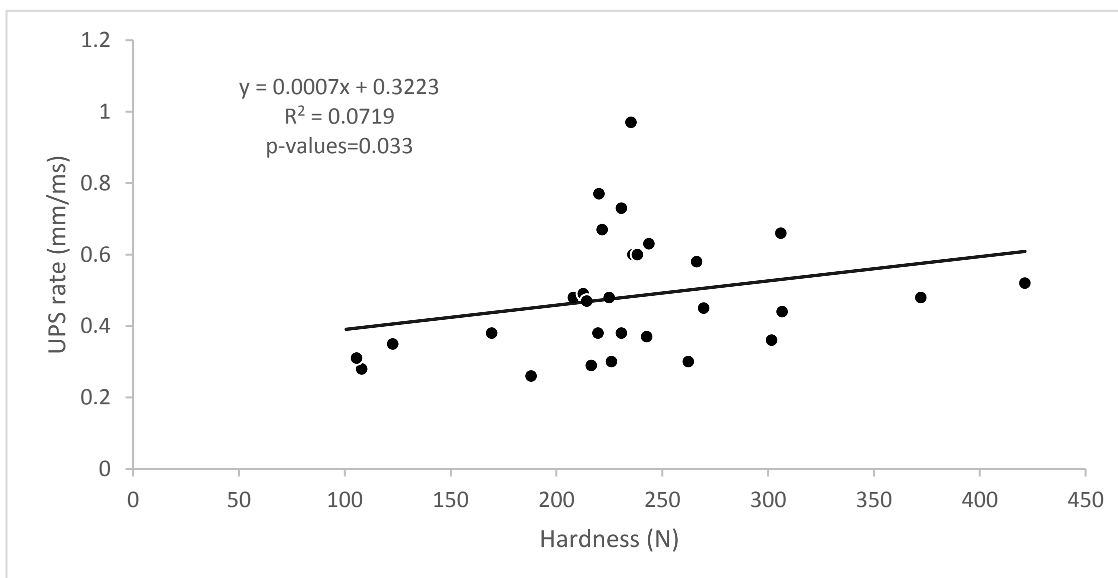


Fig 13 Correlation between UPS-rate and hardness. P-values indicate whether the correlation coefficients are statistically significant or not. R^2 is a statistical measure of how close the data are to the fitted regression line.

A significant positive correlation was measured between UPS-rate and hardness (fig. 13).

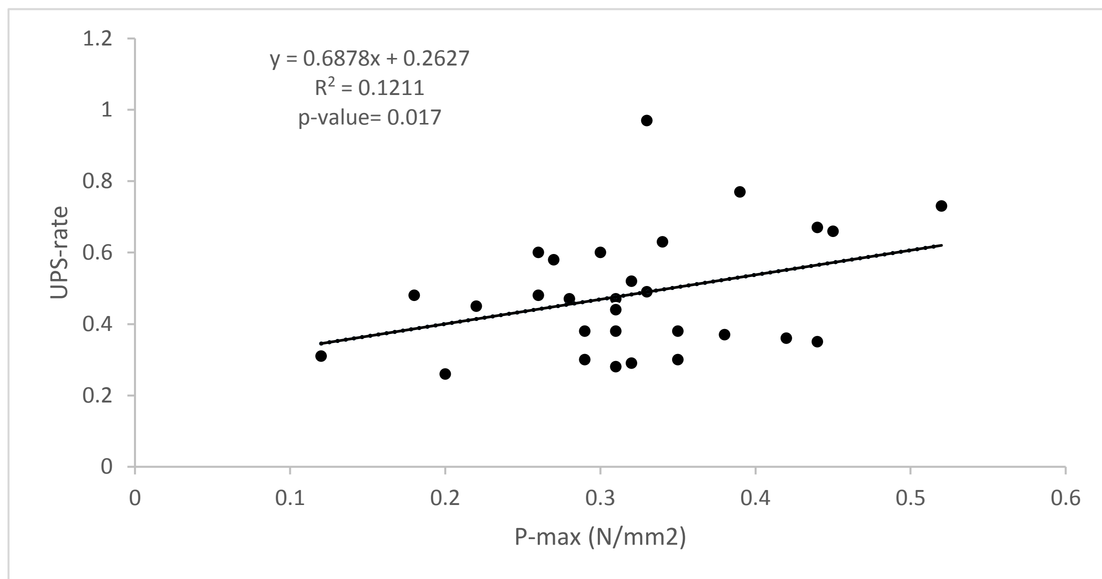


Fig 14. Correlation between UPS rate and P-max (N/mm^2). P-values indicate whether the correlation coefficients are statistically significant or not. R^2 is a statistical measure of how close the data are to the fitted regression line.

A significant positive correlation was measured between UPS-rate and P-max (Fig.14)

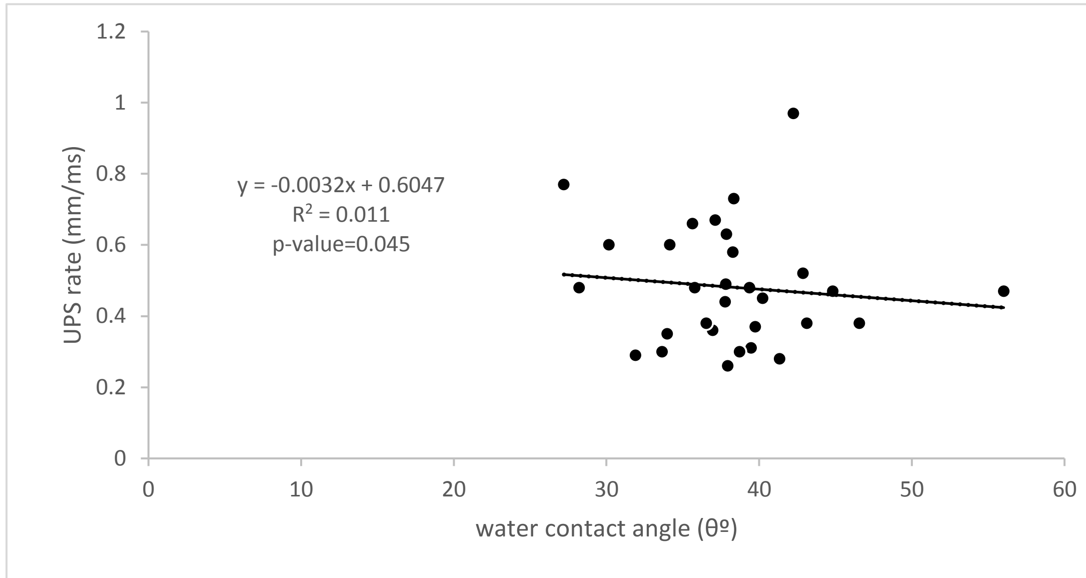


Fig 15. Correlation between UPS rate and water contact angle (θº). P-values indicate whether the correlation coefficients are statistically significant or not. R^2 is a statistical measure of how close the data are to the fitted regression line.

A significant negative correlation was measured between UPS-rate and water contact angle (Fig.15).

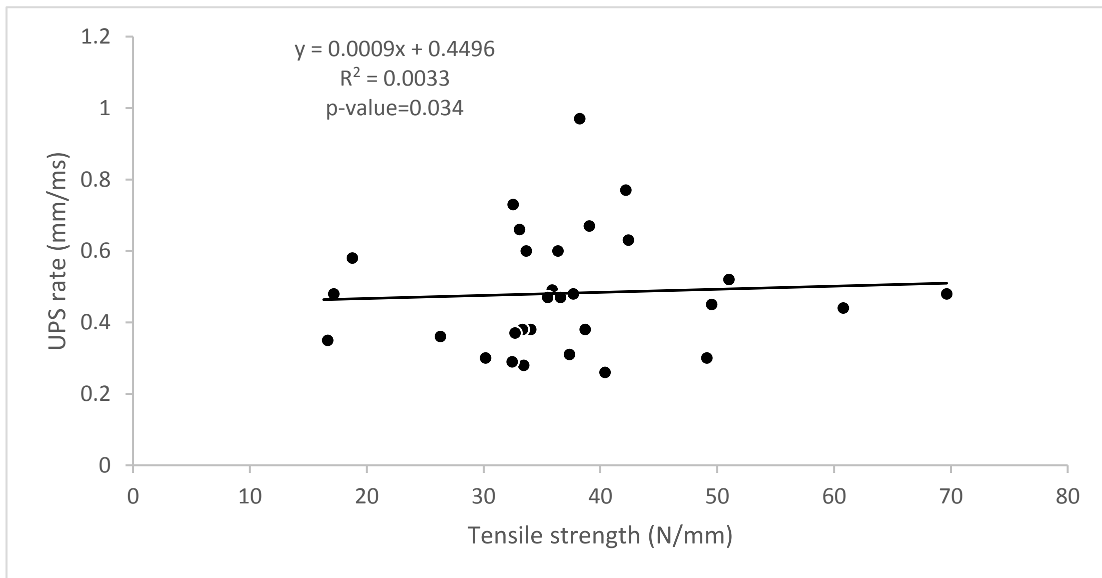


Fig 16. Correlation between UPS rate and tensile strength (N/mm). P-values indicate whether the correlation coefficients are statistically significant or not. R^2 is a statistical measure of how close the data are to the fitted regression line.

A significant positive correlation was measured between UPS-rate and tensile strength (Fig.16).

Discussion

1.Peak force (p-max)

Comparing with the effect of protease and exo/endo 1-3-beta glucanase, mixture of protease and exo/endo 1-3-beta glucanase increased P-max , which means that diets supplemented with mixture of two enzymes would cost more energy in pelleting process than diets contains protease or exo/endo beta glucanase (fig.1). The mechanism of why enzymes increased p-max can be explained at molecules levels: released molecules (proteins and carbohydrates) will contribute more on causing viscosity (by protein denaturation and fibrous carbohydrates gelatinization) under high temperature 81□. It was clear that protease will act only upon breaking more protein molecules, while exo/endo beta glucanase concentrate on releasing more fibrous molecules like β-glucan. Diets contain mixture of exo/endo beta glucanase and protease had higher P-max value than diets with protease or exo/endo beta glucanase. This is because of the cell membrane structure of torula yeast. The main main component of torula yeast cell membrane are protein and carbohydrates. More molecules protein molecules and β-glucans will be released in diets in total when adding enzymes in diets. Furthermore, all proteins and β-glucans molecules will cause high viscosity under high temperature, and this viscosity will cause high friction in die hole.

2. Oil contact angle (θº)

Results shown in table.1 indicate that all diets have a tendency of being lipophilic. In this study, both different enzymes and different dosages have no significant changes on

oil contact angle. Oil particles penetrate to between cell particles and inner cells.The main content of the cell walls of torula yeast are protein and carbohydrates such as β-glucan and small amount of chitin. Protein molecules have a high oil binding ability because of its emulsifying properties, which can explain why protease acted more active than glucanase. While fibrous carbohydrates like β-glucan (mainly β-1,3 glucan and β-1,6 glucan) and chitin are able to bind with the hydroxyl group of glycerol. But to notice that enzymatic effect will be influenced by heat treatment because enzymes are protein themselves, and their molecule structure will be changed under high temperature. The temperature used in this pelleting process was 81℃.Pressure and temperature used in single pellet press method should be considered when calculating oil absorption of pellets. High pressure will compact yeast particles more tightly, therefore obstruction of oil molecules penetration. The pressure contribution for compacting materials is different in this single pellet press methods. The upper side of pellet endures higher pressure than lower side of pellet, when compressing the pellet against the blank die. Therefore, the upper side of pellet appears to be more compacted than lower side (photo 1). That is the reason why some pellets used in this experiment showed cracks at the lower side. These pores will increase the oil absorption ability of pellets. All presented θ^{o} were calculated from upper side of pellets because amount of oil absorption for two sides would be different. The temperature effect for one pellet are also various. The temperature differences between blank and inside of die is usually 10±1℃. In this study, all lower side of all pellets used for analyzed were showed darker color than upper sides .This is because every sample mash were stayed extra 1.5min to warm up the

mash before compression in pelleting process. Raw materials located on the side of blank die were cooked better than materials were placed on the side of compacting rig. That' s why the lower side of pellets showed darker color than upper side.

Photo1. Lower side of the pellets presents the position of the compacts based on the blank side of the die, in single-pellet rig. These sides of all pellets showed darker color than upper sides (on the side of compressing rod in single pellet method).

3. Water contact angle (θº)

The diets were supplemented with different enzymes had different rate of water diffusion because enzymes could have impact on surface tension (Barkai *et al.* 2016). Protease will act upon protein molecules, and therefore more protein molecules were liberated to bind more water molecules; exo/endo beta glucanase also liberated fibrous carbohydrates, not because of water binding capacity, but for creating some spaces for water molecules to penetrate; mixture of two enzymes might contribute more to water diffusion ability.

4. Hardness

The pellets were compacted well under high temperature and high pressure. The effect of enzymes and dosage increased the tensile strength. As mentioned before, the majority compositions of CU are protein and carbohydrates. While for the component of yeast cell are mainly protein and carbohydrates, amount to approximately 2/3 of cell components (Yamada and Sgarbieri 2005). The structure of protein and carbohydrates were catalyzed by enzymes at different levels. These activities freed more fibrous carbohydrates and protein from compositions of both cell walls and inner of cells (Weihofen & Martoglio, 2003). Protein gelatinization and free β-glucan might cause high viscosity under high pressure and high temperature. This phenomenon can be also explained with high extraction force needed during extraction of pellet after pelleting. Pellet hardness was also affected by other factors, such as the level of oil content in torula yeast, particle size of ingredients, water addition and conditioning. The oil content of torula yeast is very low. Adding too high level of oil in the diet will give poor stability for feed, especially for aquatic feed (Tyapkova *et al.* 2016). Another considerable factor is the particle size of torula. The finer particles have better distribution in die whole, and compacted to well shaped pellet.

5. Water activity or Aw

In this part, exo/endo 1-3-beta glucanase with different dosages (1 dosage, 10 dosage and 100dosage) decreased the water activity. The exo/endo beta glucanase acts upon fibrous carbohydrates mainly β-glucan and chitin, and these composition are water

insoluble. The poor water binding ability might lose water content to some level. The high compacting pressure in single die pellet press method also contribute to eliminating spaces for water to stay in pellet (Mišljenović *et al.* 2015).

6. Moisture content(%)

The moisture content of tests diets were increased might be because of the interaction between water molecules and other molecules in diets. The special hydrophilic groups of protein might combine the waters molecules by hydrogen bonds insides protein molecules. The low moisture content in diets with glucanase might be due to the final water insoluble products of carbohydrates, therefore the moisture content might low in diet with exo/endo 1-3-beta glucanase.

7. Correlations between all parameters

The results regarding to USP rate decreased with increasing with extraction peak flow force can be explain in molecules levels. The high extraction force would give high compacted pellet, and which will help to increase water stability. High water stability will decrease the underwater pellet swelling rate.

High content of water activity indicates the molecules having high content of water binding capacity. The water activity in this experiment was increased by the adding enzymes in diets, which means more protein molecules were participated in water binding activity. High content of protein would give high content of viscosity under

high pressure and high temperature. High viscosity would bind molecules tightly, and therefore, USP rate would decrease with the water activity.

Conclusion

The p-max for pelleting was increased by adding enzymes in CU diet. Among all enzymes, the mixture of protease and endo/exo 1-3-beta glucanase significantly increased the p-max for pelleting than protease and endo/exo 1-3-beta glucanase. Both enzymes and dosages changed the oil absorbency of pellets at different levels, however these differences were not statistically significant. Among enzymes, the optimal dosage

of protease (1 fold) and optimal dosage of combined protease and endo/exo 1-3-beta glucanase (1 fold) increased the oil absorption rate. The combined protease +endo/exo 1-3-betaglucanase with 100 folds dosage decreased the oil absorption rate. The water contact angle was significantly increased when optimal dosage (1 dosage) of protease and 100 dosages of endo/exo 1-3-beta-glucanase were added. From the other side the water absorption-ability decreased when 1 dosage of endo/exo 1-3-beta-glucanase and 10 dosages of protease+endo/exo 1-3-beta-glucanase were used. Both enzymes and their different dosages had significant effect on tensile strength. The 100 dosages added protease increased predominantly the tensile strength, while endo/exo 1-3-beta-glucanase containing 10 dosages significantly decreased the tensile strength. Endo/exo 1.3-beta-glucanase decreased the aW. Mixture of protease and endo/exo 1-3-beta-glucanase and alone protease partially did not influence significantly on aW. However, the increased dosage of protease up to 100 folds decreased the aW values. Both enzymes and their dosages have no significant effect on moisture content before pelleting. However, the enzymes have significant differences on moisture content after pelleting process, but this changes were not related to their dosages. All enzymes increased the moisture content to different levels, but the effect of protease was more significant than other enzymes. Among these enzymes, the protease with 100 dosage dramatically decreased the UPS-rate, which means that 100 dosage protease helped to prolong the time of pellet swelling under water.

Enzymes has been mostly applied as good additive to help animals improving the digestion of feeds in digestive tract. However, there were not many research done to

evaluate the value of enzymes to improve the physical quality of feeds. In this study, enzymes were applied to determine whether it will help to improve the utilization of CU as feeds ingredient. CU is a good feed ingredients source for aquatic animals, both in nutrition and sustainability. But the existing problems for torula yeast as feed origins is its poor digestible property and cost for widely application. Enzymes can used to increase the utilization of CU both in digestion in digestive tract, also by improving the physical quality of feed. Future skilled technology would help to expand the production of CU ingredients. It would beneficial for production of sustainable feed ingredients by decreasing the economic cost.

Acknowledgment

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Appendices

Annex 1

Scanning electron microscopy

Pellet's contact angle with water/oil or is measured by Video-based optical contact angle measuring instrument (Operating manual OCA 15EC)(figure17). The measuring angle start with installing the equipment in the lab:

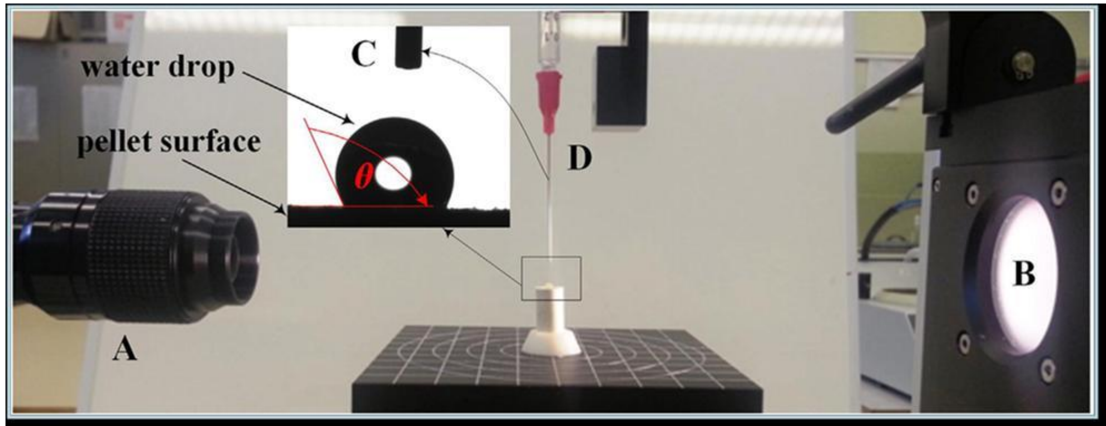

Figure 17. Experimental setup for θ measurements. Items are indicated by letters: (A) camera; (B) light source; (C) image of a drop on top of a pellet surface for θ tests; (D) dosing syringe with a needle (Mišljenović et al. 2015).

Steps for installation and setup:

--checkout the items for OCA15EC

--levelling the measuring device.

--mounting of SD-DM

--mounting of ES-D

--mounting of measuring table with markings

--montrols and connections at illumination housing

--PC-Setup

--driver installation

Operation:

--mounting the dosing of syringe: the syringe type used in this experiment is called (name).

--OCA15 -device preferences - liquid : in this paper, the contact angle with water and glycerol measured, the density and viscosity of water are 0.998g/m^2 and 1mPas

respectively; the density and viscosity of glycerol are 1.26g/cm^2 and 1.41e+003mPas, respectively.

--positioning of the dosing needle: the position of the dosing needle can be adjusted vertically and horizontally to the optical axis (X-axis) by means of the two adjustment screws.

--positioning of the sample: the sample stage is freely movable in horizontal (X- and Y-axis) direction over the whole base plate by a magnetic slide system. The vertical direction (Z-axis) the stage is precise adjustable via hand wheel.

--adjustment of the illumination: you can adjust the brightness of the homogenous back lighting with the control knob at the back of the illumination housing, or within the SCA software.

--setting for the Frame grabber/ camera : the default value for the exposure time in the SCA software will be read out from the video camera. For most common applications an exposure time of about 1ms and an illumination intensity of 10-15% are appropriate values. The windows Frame grabber Preferences contains 4 tabs, Images, Size, Timing, and Buffer.

--adjustment of the optics include Zoom, Fine focus, Tilting wheel, and Rotation.

Start the measurement:

--settle the pellet just under the needle, taking great care to avoid air bubbles in the complete dosing system (syringe- needle).

--live video/ drop image windows: the window live video will open automatically when the SCA software is started and shows the live image from the camera .

--result windows: record the video about 1 to 2 minutes and analyze the contact angles. The result window is used to set some important parameters for the measurement and for the tabular and graphical collection/ presentation of the measurement result.

Contact angle with water/ Hydration properties

The purpose of the measurement is to test the activities of different enzymes resulting in different contact angles with water. Once the pellet was settled at vertical state, the distilled water drop or oil drop drips from needle by gravitational reason and surface tension when touched to the pellet. The dosing volume and dosing rate for distilled water were 2μl and 0.2 μl/s, respectively. A video for water absorption was recorded. The recording time for every pellet was approximately 1 minute.The data collected from each pellet were analyzed. The data got through analyzing the videos which were recorded in the lab: First, need to find the optimum moment of water drop just after settled on top of pellet (avoid the moment that water drop still at the stage of following by its gravity), and set the optimum moment as zero time figure 18. The pellets have hydrophobic property if the contact angle is larger than 90℃, while the pellets show hydrophilic property if the contact angle is less than 90℃.

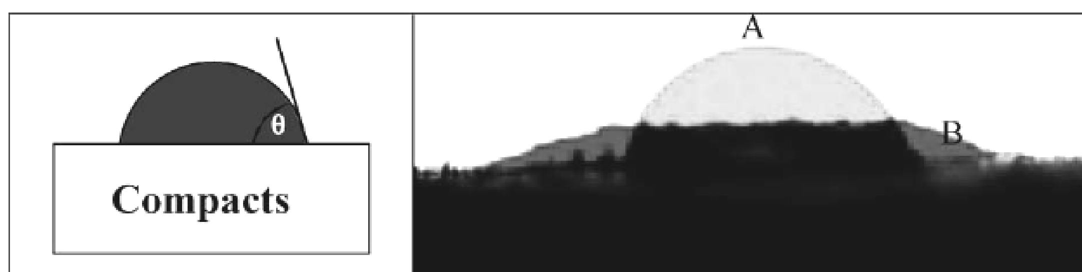

Figure 18. The explanation of contact angle of pellets by using sessile drop of compact. θ is contact angle. A indicates initial contact angle and B indicates final contact angle.

5.3 Contact angle with oil

The surface contact angle (θ) of the oil and the time (ms) for oil to penetrate into pellet was recorded just after the oil drop settled down on the pellet surface to measure the pellet rate of oil absorption. The process and measurement for contact angle with oil is same with the process held in water contact angle. The glycerol was used to determine

the oil absorption of pellets because we did not gave other oil in database. The dosing volume and dosing rate for glycerol were 5µl and 2µl/s, separately.