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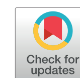
Research article

The effect of feed enzymes phytase, protease and xylanase on pelleting of microalgal biomass



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HIGHLIGHTS

- Enzymes help reducing consumption of the electrical energy during pelleting of microalgae.
- Reduction of flow resistance can be observed when using enzymes phytase and xylanase.
- Hydrolytic activities of the enzymes do not affect hardness of the microalgal pellets.
- Enzymes and their combination improved pellet stability under water.
- Enzymes decrease contact angle degree between pellet surface and oil droplet.

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ABSTRACT

Lower energy consumption for producing feed pellets is an important part of the economy in the feed mill. The same is if physical pellet quality is degraded. The interest in using of novel ingredients is increasing due to requirements for the sustainable development goals. Defatted microalgae as by-product from biodiesel production is one of many novel ingredients. The purpose of this experiment was to understand how the addition of small amount of enzymes can reduce the flow resistance in the die during pellet discharge, without affecting the physical quality of pellets. Thus, possibly reduce the total consumption of electrical energy during compaction. Three enzymes, phytase, protease, xylanase, and combinations of those were added to defatted *Desmodesmus subspicatus* microalgae at 3 inclusion levels. Feed enzymes xylanase and phytase helped lowering the flow resistance of the material in the die. Reduction of flow resistance was in average 17 times lover when all three levels of enzyme phytase were used. The same was observed when 0.01% xylanase was added. All feed enzymes and their combination have evidently lowered underwater pellet swelling due to their hydrolytic activity at the surface of the microalgal particles. The hydrolytic activities of the feed enzymes did not affect hardness of the microalgal pellets. Contact angle degree between pellet surface and oil droplet was lowered when xylanase and protease was used at all three dosage levels. However, contact angle degree between pellet surface and water droplets was unaffected by the hydrolytic activity of enzymes.

1. Introduction

Microalgae are an abundant source of protein, carbohydrates, lipids, and antioxidants (Pulz and Gross, 2004). Microalgae have high nutritional value when used for monogastric animal feed (Patil et al., 2005). Use of microalgae may, however, be hampered by components limiting their nutritional value (Bleakley and Hayes, 2017). Phosphate in the microalgal cells has low availability due to binding abilities of phytic acids to bind to cationic elements and protein (Konietzny and Greiner, 2003). Soluble non-starch polysaccharides increase the viscosity of digesta and limits digestion and absorption of lipids and lipid soluble materials (Sinha et al., 2011). These challenges may be addressed by using feed enzymes as digestibility enhancers. Feed enzymes are known to speed up hydrolytic processes during hydrothermal ingredient and feed processing, like steam conditioning of biological materials Such processing can thereby increase the nutritional value of the feed

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ingredients (Hardy, 2000; Svihus, 2010). The use of protease offers promising environmental benefits by enabling improved nitrogen utilization (Oxenboll et al., 2011). Protease showed to change physio-chemical properties of the feed product by affecting the plant-based proteins during and after feed manufacturing. Such change may possibly decrease the energy cost during feed manufacturing (Storebakken et al., 2015).

Reducing use of inorganic phosphorous in feeds, achieved by treating the feed ingredients with phytase (Robinson et al., 2002), can reduce the feed cost. Adding of phytase, combined with protease, has shown to cause improved protein solubility (Bae et al., 2013). Enzymes are used to reduce the energy dilution of the feed by carbohydrates or fibres, derived from vegetable protein concentrates (Hardy, 2000). Enzymes that hydrolyse non-starch polysaccharides (NSP) increase nutritional energy uptake from fibrous materials (Svihus, 2010). According to Miladinovic and Salas-Bringas (2014), the addition of xylanase in fibrous feed mixtures prior to pelleting resulted in a 28 % reduction in the consumption of electrical energy. Reducing die flow reduces compaction of the materials during the pelleting process. All this led to decreased physical quality of the pellets. Function of the enzyme xylanase is to release water from the hydrolysing medium and make it available for protein hydration (Hardt et al., 2014). Thus, when size of non-starch polysaccharides is reduced the general rheological properties of the medium may be changed.

1.1. Enzymatic treatment of microalgae

Microalgal cell walls have a low biodegradability rate (Schneider and Gerber, 2014). In biofuel production, cell wall degradation can be enzymatically increased (Gerken et al., 2013). Enzymatic degradation of the microalgal cell wall could reduce the energy inputs needed for feed pelleting. Influence of the enzymes on swelling of the defatted microalgal pellets, under stagnant water, was previously evidenced by Salas-Bringas et al. (2015).

1.2. Measuring quality characteristics

Animal feeds requires a variety of raw materials mixed in the form of mash prior to being pelleted. Fundamental insights of the processability and physical properties of the single ingredient are necessary to obtain targeted qualities of the pelleted matrix. It is important to evaluate physical properties for every ingredient included in such matrix. The powder-based material compacted into the pellet holds certain microstructures that influence on the texture, durability, hardness (Sun, 2017), hydrophilicity (Güttler et al., 2013), lipophilicity (Cao et al., 2007), and water activity of the final pelleted products (Roca et al., 2006).

Physical characteristics of the materials and their rheological properties can help in determining functionality of the ingredient during product development, shelf life estimation, evaluation of the texture, or overall product quality control (Stokes et al., 2013). Resistance of the mixed material to deformation during pelleting can influence consumption of electricity, final shape, texture, and physical quality of product. Quality parameters are dependent on the time scale of the deformation process, shear, and thermal history during manufacturing (Chen and Engelen, 2012). Controlled processing variables such as shear and temperature can influence the structure of the final products (Hermansson, 2000).

It is important to know how feed ingredients contribute to flow behaviour during compaction, final texture, and physical quality of compacted feed matrixes. Such knowledge sets manufacturers in a position to rationally design better nutritional and physical quality products prior to commercial manufacturing. For instance, the texture of feed products is physically and chemically dependent to various ingredients and additives that are influenced by temperature, dwell time and shear. This defines all mechanical, geometrical, and surface attributes of a final product.

Physical behaviour in the network of the food-polymer is influenced greatly by small particles, under 100 μm (Aguilera, 2005). Microalgae vary in their size. Such size variation may influence physical characteristics and overall texture and viscosity of the feed-products.

Physical quality properties of the dry and solid feed compacts, as hardness and durability, are important parameters for reliable quality measurements (Thomas and van der Poel, 1996). Under mechanical stress, the strength of feed pellets can be evaluated, and thus, the behaviour of the final product during storage or transportation can be assessed. The mechanical properties can be used to evaluate the physical contribution of a single feed material to the entire diet formulation under steam conditioning (Maier and Gardecki, 1993) or manufacturing parameters (Briggs et al., 1999).

Water activity (a_w) is a good indicator of the shelf life of feed products. Most degradation reactions are related to a_w. Proteins, carbohydrates and NSP quickly interact with water and increase the a_w. Such feed elements can lower their water pressure by polar-binding in small molecules or by surface interactions in large molecules (Maltini et al., 2003) when interacting with the enzymes. Therefore, it is crucial to understand how certain feed material react with water to predict their behaviour in the feed. The thermodynamic effect in a raw ingredient could control the texture of the final product at a given a_w during pelleting.

When developing a feed matrix, a major challenge can arise from the incompatibility of ingredients having different surface properties. Surface hydrophilicity and lipophilicity of feed pellets play an important role in post-manufacturing processes. The contact angle (θ) and surface energy of liquids can differ in each compacted medium, hence, the final product quality may also differ. The surface energy of a compacted medium can control the level of physical interaction between particles by adsorption, adhesion, friction, and wettability of the surface (Yuan and Lee, 2013). If the initial $\theta < 90^\circ$, the surface can be considered as hydrophilic, whereas $\theta > 90^\circ$ indicates a hydrophobic surface (Förch et al., 2009; Mišljenović et al., 2015).

Poor mechanical properties of the feed pellets may be expected if the feed ingredients have little or no interactions through chemical and mechanical bonding (Saheb and Jog, 1999). Chemically similar feed ingredients, originating from the same ingredient type, may have different initial water θ characteristics (Roman-Gutierrez et al., 2003; Güttler et al., 2013). The wettability of the ingredients can be evaluated by measuring the θ of any given liquid droplet placed at the surface of the compacted pellets. Nevertheless, the structural differences in compacted solids could alter θ. Different particle sizes in a feed matrix can influence porosity of the pellets (Vukmirovic et al., 2017). The θ measurements depend on liquid diffusion inside particles and liquid absorption in the voids between the particles (Roman-Gutirrez et al., 2003). Initial θ values and the droplet age of water and oil (i.e., at the time when the droplet is placed at the surface) were considered in this experiment to evaluate the contribution of enzymes to surface wettability of compacted microalgae.

The underwater swelling rate (UPS) is a quality parameter to indicate how long a pellet could remain consumable and nutritionally useful by farmed animals underwater. Slow UPS is an important requirement for shrimp feeds, due to the slow eating habits of the animals. Therefore, UPS is a quality indicator of how pellets remain underwater until they are consumed. Also, UPS measures particle detachment within the pellet structure, linked to the swelling rate. It has been a common practice to measure disintegration of pellets underwater. This is measured by several methods, commonly known as "pellet water stability". Obaldo et al. (2002) reviewed these methods as the horizontal shaking, static water method and vertical shaking. Measuring the swelling rate of the aquatic feed pellets submerged in stagnant water is vital for evaluating the possible leaching of micronutrients from the feed into the water. Leaching of nutrients from the feed, for example nitrogen, could lead to a decreased water quality and thus health risks for the farmed animals. Newly developed methods for measuring swelling rate of the feed pellets through image analysis may give more precise answer to disintegration of the pellets under water (Salas-Bringas et al., 2015).

The need for mapping the flow properties during pelleting, physical quality of pelleted products when including novel feed materials, and the effects of enzymatic additives, can be used to indicate the economic benefits of utilizing the enzymes. Such tool may help to decision makers where the emphasis is set towards sustainability, without threatening the final feed quality outputs. Usage of enzymes during feed manufacturing demonstrates a large potential for better use of feed constituents and their function (Bedford and Partridge, 2003). There is no published data on how the addition of enzymes correlates to the physical quality of compacted microalgal biomass. There is no reported evidence of how the enzymes during preparation of the microalgal biomass, as a feed material would influence production capacities and electricity consumption during manufacturing. The overall aim of this work was to understand the flow of the novel material when a single enzyme or a combination of enzymes, in various dosages and at low water content is added to microalgal biomass. If in low water-content environment the enzymes are altering chemical properties of compacted defatted microalgal materials, that could all lead to changed flowability of the material during pelleting. If the flowability changes the power consumption changes too by different pressure at incipient flow (p_{max}). This can all possibly lead to altered stability of pellets underwater, hydrophilicity or lipophilicity as well as physical strength of the compacted microalgal material.

Objectives of this research are based on the hypothesis that different enzymes and different dosages can lower the p_{max} of microalgal biomass during pelleting. Such change could allow the particles from microalgal biomass to pack better during compaction and that would further contribute to harder pellets. Harder pellets would maintain longer their structure if submerged underwater and will be longer available to intended usage, as for example feeding shrimps. In connection to better packing of the particles due the enzymatic hydrolyses this all may contribute to lower acceptability of oil or water to penetrate surface of the pellets.

2. Materials and methods

2.1. Characteristics of the experimental materials

Microalgal biomass of *Desmodesmus subspicatus* (Cellana, Kailua-Kona, HI, USA), as residual biomass from microalgae oil processing, was used.

The percentage of enzymatic dosage added to the biomass was based on the total batch size of 200 g dry matter. Each batch was weighed on a microscale (Mettler Toledo, model LJ16). Enzymes supplied by AB Vista (Marlborough, UK) in liquid form were beta 1–4, endo-xylanase (Econase XT), an *E. coli* derived phytase (Quantum Blue) and *Fusarium equiseti* protease (non-commercial product). The enzymes were applied in three different dosages. The recommended dosage (*R*) for all enzymes, was based on the optimal bioactivity of the enzymatic additives, as recommended by the supplier. All presented values are units of weight %. The R for xylanase was 0.01 %, half dosage (*H*) 0.005 %, and double dosage (*D*) 0.02 %. For phytase the dosages were R - 0.03 %, H - 0.015 %, and D - 0.06 %. For protease, R - 0.006 %, H - 0.003 %, and D - 0.012 %. Each dosage of enzymes was mixed with 2.5 % distilled water and thereafter sprayed over the biomass to ensure good mixing.

2.2. Preparation of the experimental mixtures and sampling

An intensive mixing of the biomass was performed to distribute well enzymes by using a high shear mixer, having three impellers and one tulip-form chopper (Diosna P1/6, Osnabrück, Germany). The mixing speed of the impellers was 250 rpm, and the chopper speed was 500 rpm. Spraying of the distilled water/enzyme solution was performed with a spraying lance (Model 970, Düsen-Schlick GmbH, Germany) assembled in the mixer. Samples for moisture analyses were taken from random areas in the mixer for each batch, three samples per batch. Thereafter, all three samples for each trial were mixed together to obtain a representative sample, assuming a homogeneous mixture. The moisture content presented in this work is an average of three measurements. Moisture was measured by the standard method (EU, No. 152/2009) and the average moisture measurement for all trials was 8 % w/w (+/- 0.3 %). Immediately after mixing that lasted for 60 s the water/enzyme solution was added and thereafter all the powder was collected, and vacuum packed to prevent moisture loss. The samples were stored at 4 °C for a maximum of 4 weeks until pelleting.

2.3. Pelleting

A single pellet press method (Salas-Bringas et al., 2010, 2011; Salas-Bringas, 2011) was used for compacting approximately 0.2 g of the microalgal biomass into cylindrical pellets (Figure 1).

The enzyme treated microalgal powder sample was poured into preheated die comprising channel. The temperature in the die was 81 °C as recommended to eliminate possible salmonella contamination (VKM, 2006). The material poured into the die was preheated for 3 min prior to compaction. Compaction was done with a compression rod with a diameter of 5.45 mm. The compression rod was set in the die immediately after the powder was poured, to prevent release of water from the sample during heating. When the sample reached a desired temperature, an initial pre-load pressure of 240 kPa was applied to the sample in the pelleting die before compression of the material started. This was done to rearrange particles of the microalgal biomass and avoid creating the air pockets in the die hole, without the risk of compaction. Pelleting was done by applying maximal force load of 285 N. Calculated compressibility of approximately 12 MPa was applied to microalgal biomass. The chosen compacting pressure of the trial mixture was used according to densities of products derived from the commercial animal feed ring-die pelleting process Salas-Bringas et al. (2011). The compaction speed was set to 10 mm/min through a rod inserted in a 5.5 mm compressing channel of the blank die. After compaction, the blank part of the die (i.e., closed end) was removed and thereafter, the pellets were discharged. Discharging of the pellet was done with a speed of 2 mm/min, which was low enough to avoid exceeding the compacting-pressure and hence avoid any further compaction. Total retention time of the materials in the compressing channel of the blank die was 9 min. The compacted pellets were 5.5 mm in diameter and a weight of 0.2 g. All compacted pellets were stored at 4 °C for a maximum of 30 days prior to analysing of physical properties.

2.4. Analytical techniques and measurements

2.4.1. Particle size distribution

A Malvern Mastersizer S instrument (Malvern Instruments Ltd, Worcestershire, UK) utilizing a laser diffraction method was used to determine the particle size distribution of the microalgal biomass. Combination of wet-sieving and laser diffraction method was used to determine final size of the particles. Untreated microalgal sample and its replica was placed in a particle dispersion unit one after another. The cell where the microalgal sample was located in the path of the beam of the scattered light. The sample was circulated through the cell that had depth of 2.4 mm. The setting of the focal length in the detector was 300 mm. The detector consisted of 44 photosensitive rings. Fraunhofer diffraction theory for spherical particles was used to calculate volumetric particle size distribution of the light energy on the detector (Allen, 1997).

The particle size distribution analyses of non-treated samples showed a particle size in the range of 1.45–549.5 μm. About 10 % of all particles had average size lower than 14.6 μm, whereas 50 % of particles were lower than 83.3 μm and 90 % of particles were under 275.5 μm. A De-Brouckere mean diameter volume of the particles for the representative sample had a diameter of 32.3 μm and specific surface area 0.19 m^2/g.

2.4.2. Compaction and pellet discharge

The observations during compaction and pellet discharge from the die were measured as maximal compaction pressure (Pa) as a product of

Figure 1. Single die pellet press. Adopted from Salas-Bringas et al. (2011).

maximal compacting force (N) divided by the area of a compact (mm^2). The results were recorded with NEXIGEN Plus software, attached to a Lloyd texture analyser (LR 5K Plus; Lloyd Instruments, U.K.).

2.4.3. Pressure at initial flow measurement

The pressure required to initiate pellet discharge from the die once the blank die was removed is referred to as the pressure at incipient flow (p_{max}). The p_{max} was recorded to quantify differences generated by friction on the die-pellet contact area. The die flow of the compressed pellets was analysed by recording the maximal pressure needed for the pellet to start flowing through the open die-diameter. Measurements were performed immediately after compaction and when the blank die was removed. The set discharge speed (2 mm/min) at the pelleting rig was set to ensure that the releasing pressure would not reach the compaction pressures. Such measurements indicate possible changes in electrical energy consumption due to changes in the resistance of the material to flow through the die. The analytical results for the tensile stress and maximal force prior to when the pellet started flowing through the die were further estimated with Eq. (1).

$$p_{max} = F/\pi r^2 \qquad (1)$$

where F is the load needed for the pellet to start flowing and r is the radius of the pellet and π the constant ratio of the circumference of the circle to its diameter.

2.4.4. Tensile strength

Tensile strength analyses were obtained through maximum force (F) applied on cylindrical specimens under diametral compression, on three randomly chosen pellets for each treatment. Strength for each pellet was measured by the first peak F occurring during a diametral compression at speed of 1 mm min^{-1}. For brittle cylindrical specimens, the stress (σ) was estimated by using Eq. (2) better known as the Brazilian test. The tensile fracture was produced in a pellet by compressive loading across the diameter. The σ measurements were done by using a probe with a flat surface of 60 mm in diameter, connected to a texture analyser (Lloyd LR 5K Plus, Lloyd Instruments, U.K.).

$$\sigma t = (2F/(PiDl)) \qquad (2)$$

where σt is the maximum tensile strength (MPa), F the load at fracture (N), Pi is constant ratio of the circumference of the circle to its diameter, D is pellet diameter (mm), and l is measured pellet length (mm).

2.4.5. Water activity (a_w) measurements

The change of a_w was determined by use of a HygroLab™ C-1 instrument (Rotronic AG, 8303 Bassersdorf). Three randomly chosen pellets for each treatment were used. The average temperature during a_w measurements was 23.5 °C (±0.4), and relative humidity (RH) of the air in the measuring cell was 37 % (±2) for all samples. The procedure for a_w measurement is explained in the user manual (IN-E-HyLab-V4_11) for Rotronic a_w devices (Rotronic AG, 8303 Bassersdorf).

2.4.6. Contact surface angle (θ) measurements

Characterization of the surface wetting of the pelletes with oil and water and was measured with an optical device OCA 15EC (Dataphysics Instruments GmbH, Germany). The θ measurements were made on three randomly chosen pellets, for each treatment, to compare how wettability of the pellets could be influenced by enzymes. The initial θ is defined as the moment when the drop is placed on the surface of the pellet (T0). The θ measurement was made with distilled water and rapeseed oil. The droplet volume for distilled water and rapeseed oil was 1 µl and 5 µl, respectively. To detach the oil droplet from the needle, a larger volume was needed, due to the larger surface tension of oil (Esteban et al., 2012). The droplets were disposed from a dosing system on the upper plain surface of a pellet (Figure 2). The recorded droplet absorption is presented in the results as the initial θ (T0) and final droplet-age, measured in seconds. Water and oil θ were recorded within 1.2 and 3 s, respectively. After those periods, the droplets fully penetrated the pellet. The measurements were carried out at a temperature of 20 °C (±2). The change of θ was observed by SCA20 software (Dataphysics Instruments GmbH, Filderstadt, Germany).

2.4.7. Underwater pellet swelling rate (UPS)

To monitor the swelling rate of a pellet under stagnant distilled water, a special arrangement was designed (Figure 3). The UPS measurements were made by assembling video microscope (microviper) with a

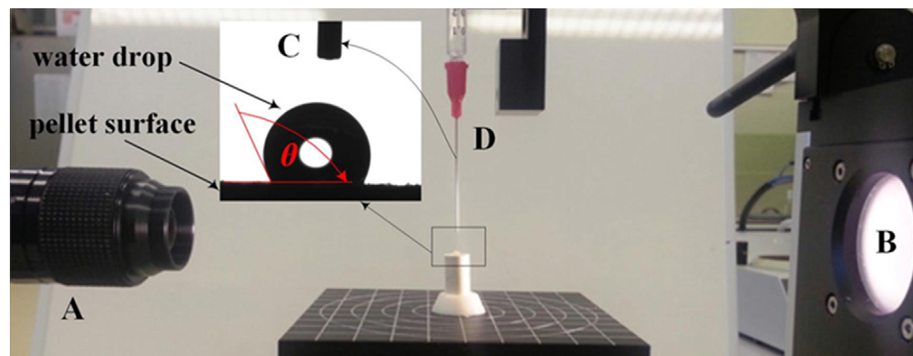

Figure 2. Contact surface angle measurement setup. Different letters indicate: A - video camera; B – light source; C – image of a drop on top of a pellet surface; D – dosing syringe. Adopted from Mišljenović et al. (2015).

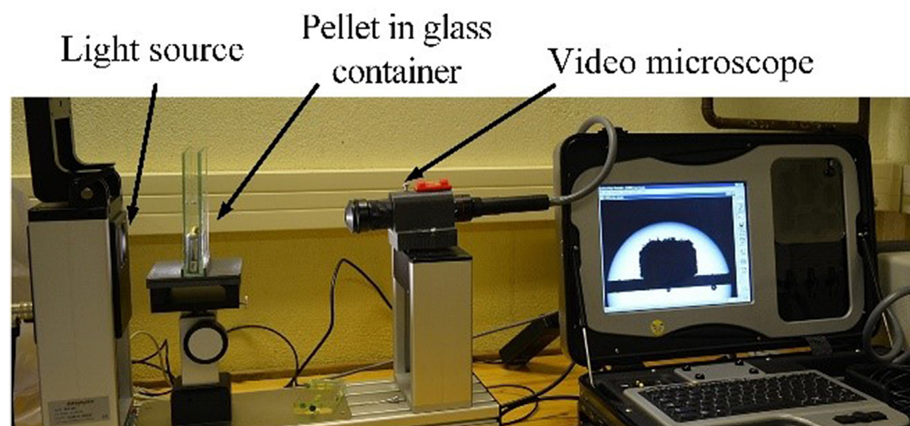

Figure 3. UPS experimental setup using image analysis (Salas-Bringas et al., 2015).

microscope lens (Allen ¼") into the optical tensiometer (OCA 15EC, Data Physics Instruments GmbH, Filderstadt, Germany) as shown in Figure 2. The image processing software Fiji (open source under the GNU, General Public License) was used for the optical monitoring of pellet swelling, according to Ferreira and Rasband (2012). Distilled water at a temperature of 19.5 °C (+/- 1) was added to the experimental glass container. Thereafter, a randomly chosen pellet was used for each treatment. The measurements were made in triplicate. A cross-sectional view of the non-swollen pellet with a diameter of 5.5 mm was used as a starting point. A picture was taken every minute for a total observation time of 14 min. The duration of the observation time was chosen as the time required for shrimp to find the pellet in open pond shrimp farming (Lovell, 1998). The total observation area occupied by the cross-section of a recorded pellet in the water during a total observation time of 14 min represents the UPS results.

2.4.8. Measuring of soluble protein and phosphorous

Phosphorous content was measured with a spectrophotometric method (FAO, 2011) with simplified sample preparation consisting of grinding the pellets with pestle and mortar. Soluble protein measurement was done on samples having lowest flow resistance of the pellets in the die (p_{max}). Soluble protein and phosphorous measurements were not performed on other samples because there was no observed change of the p_{max} in those samples. Compacted material treated with phytase was compared to control samples. Samples were held overnight on 550 °C temperature. Ash samples were cooked in the low acidic solution for total release of phosphorus and thereafter diluted in water. Samples are added to various reagents to obtain a colour reaction which was read spectrophotometrically. Measuring of soluble protein was done based on Licitera et al. (1996). During protein extraction, a stable pH and temperature was secured. A borate phosphate buffer with pH 6.75 was used to hold samples at 39 °C during incubations.

2.5. Data analyses

Variables were analysed as a 5 (enzyme treatments) x 3 (enzyme dosages) factorial arrangement in a randomized complete block design. Software used for plotting was Microsoft Excel and Minitab v.17 for statistical analyses. One-way analyses of variance (ANOVA) was used to examine possible effects of the enzymes and their dosages on the responses. Significant differences between treatments were determined by the Tukey–Kramer method, using a 95 % confidence interval. To analyse the existence of correlations between variables, a Pearson correlation test with a 95 % confidence interval was used.

3. Results

3.1. Flow resistance in the die during pellet discharge

Measurement of p_{max} showed that various enzymes and their combination influenced changing the resistance of the material to the flow in the die ($p < 0.05$). The lowest and significantly different p_{max} was observed for phytase and xylanase. ($p < 0.001$) (Figure 4).

All other enzymes, their combination and dosage level did not show to influence p_{max}.

3.2. Strength of pellets

The tensile strength of pellets was not influenced by adding the different enzymes independent of dosage either when added combined

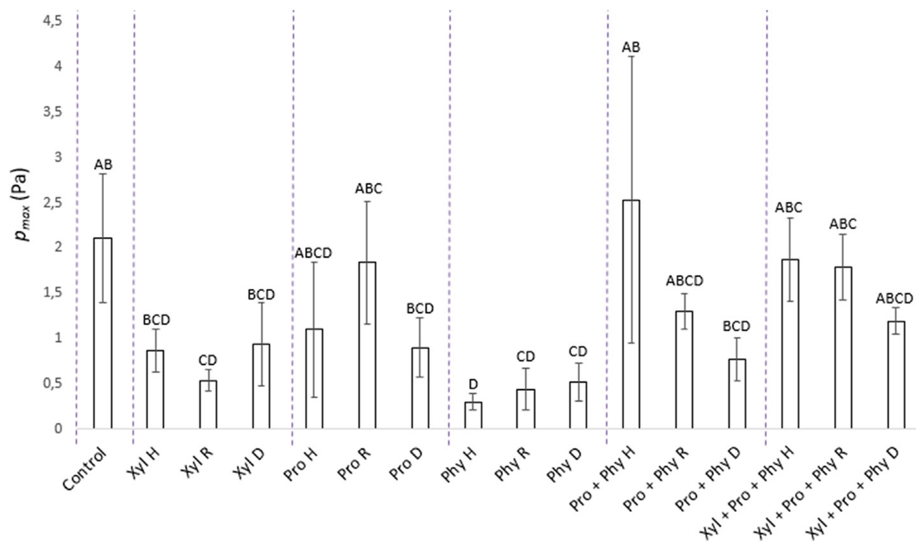

Figure 4. Mean values for p_{max}, derived from 3 replicates. H, R and D indicate half; recommended and double dosage, respectively. Xyl, Pro and Phy are enzymes xylanase, protease and phytase, respectively. Means that do not share a letter are significantly different, according to the Tukey pairwise comparisons test. Letters are organized in descending order and show significant differences ($p < 0.05$). The error bars represent 95 % confidence intervals.

enzymes or single enzymes (Table 1). No relationship between pellet strength and p_{max} was observed ($p = 0.82$; $r^2 = 0.011$).

3.3. Surface contact angle (θ) measurements for water and oil

Type and dosage of added enzymes did not have any influence on the initial θ (T0) either of water nor oil (Table 1). Enzyme type and dosage did not influence T0 oil θ. However, by having the oil droplet age for three seconds, xylanase and protease showed increased lipophilicity of the compacted microalgal biomass, when compared to the control treatment and treatments with different dosages of mixed protease and phytase (Table 1). Phytase alone or in combination with other enzymes, independent of the dosage, did not influence θ, when compared to the control treatment at after 1.2 s for the water or 3 s for the oil. The same was observed when mixture of protease and phytase was used or when all enzymes mixed together were added in various dosages. Increasing the dosage of all enzymes mixed together did not have any influence on oil θ at 3 s.

3.4. Underwater pellet swelling rate (UPS)

When comparing enzymatic treatments with the control treatment, it can be concluded that pellets treated with single enzymes or mixture of enzymes had decreased UPS (Figure 5). The total observation circular area of a non-swollen pellet was 23.7 mm^2. The slowest UPS 24.6 mm^2, for one minute was observed in treatment with phytase (Figure 5C). However, this was significantly different ($p < 0.001$) only by comparing the control treatment and the double dosage of mixed protease and phytase. Control treatment had UPS of 35.5 mm^2 and mixed protease and phytase 34.9 mm^2 (Figure 5D). Lowest UPS was observed at 14 min for a treatment with xylanase/protease/phytase added as a double dosage (Figure 5E). The highest UPS was observed in the treatment with

Table 1. Mean values and standard deviations for contact angle analyses (water and oil), pellet tensile strength and water activity (a_w).

Treatments	Dose level	CA oil; T_0	CA oil; 3 s	CA water; T_0	CA water; 1.2 s	Tensile strength (MPa)	a_w
Control	-	44.8 ± 13.3	34.7 ± 5.4 a	52.0 ± 16.2	29.6 ± 17.6	2.4 ± 0.7	0.3 ± 0.01 b,c
Xylanase	H	56.1 ± 4.3	21.4 ± 1.8 c	48.7 ± 5.5	20.6 ± 3.8	1.6 ± 0.2	0.2 ± 0.01 d
	R	51.6 ± 3.2	20.4 ± 3.1 c	58.1 ± 7.4	23.6 ± 4.4	1.3 ± 0.5	0.2 ± 0.01 d
	D	53.1 ± 14.2	21.8 ± 3.7 b,c	49.1 ± 8.8	17.5 ± 4.0	1.6 ± 0.3	0.3 ± 0.01 d
Protease	H	51.9 ± 6.0	19.1 ± 2.6 c	47.6 ± 2.6	26.0 ± 14.0	1.5 ± 0.3	0.2 ± 0.01 d
	R	53.9 ± 9.9	22.4 ± 1.2 b,c	43.6 ± 2.2	28.3 ± 6.1	2.0 ± 0.4	0.3 ± 0.01 d
	D	45.5 ± 2.4	22.1 ± 0.8 b,c	48.8 ± 0.4	29.2 ± 0.7	2.0 ± 0.4	0.2 ± 0.01 d
Phytase	H	46.6 ± 4.3	29 ± 4.7 a,b,c	44.8 ± 4.8	17.3 ± 2.4	2.7 ± 0.7	0.2 ± 0.01 d
	R	57.2 ± 6.7	27.7 ± 5.1 a,b,c	46.3 ± 2.2	15.6 ± 1.6	2.0 ± 0.3	0.2 ± 0.01 d
	D	52.2 ± 6.9	29.2 ± 4.9 a,b,c	48.4 ± 13.3	15.8 ± 2.5	2.4 ± 1.3	0.3 ± 0.02 c,d
Protease/Phytase	H	54.2 ± 3.8	39.4 ± 12.6 a	41.7 ± 3.4	16.1 ± 3.3	2.7 ± 0.3	0.4 ± 0.04 a
	R	51.1 ± 6.6	38.2 ± 3.9 a	40.0 ± 2.0	16.2 ± 5.0	2.0 ± 0.4	0.4 ± 0.06 a,b
	D	49.3 ± 6.7	34.7 ± 3.8 a	43.1 ± 3.7	17.2 ± 0.9	2.7 ± 0.7	0.4 ± 0.03 a,b
Xylanase/Protease/Phytase	H	47.0 ± 2.1	36.2 ± 0.8 a	51.2 ± 6.6	31.4 ± 6.5	2.2 ± 0.8	0.4 ± 0.04 a,b
	R	48.4 ± 5.2	32.7 ± 5.0 a,b	44.2 ± 6.8	21.5 ± 2.6	2.4 ± 0.9	0.4 ± 0.04 a,b
	D	41.9 ± 8.6	27.1 ± 2.9 a,b,c	57.5 ± 9.9	31.0 ± 3.3	1.9 ± 0.3	0.4 ± 0.06 a,b

Results without letters are not significant ($p \geq 0.05$).
H - half dosage; R – recommended dosage; D - double dosage. Means that do not share a letter are significantly different for the respective analyses, according to the Tukey-Kramer test (0.05). Letters are organized in descending order and show significant differences ($p < 0.05$) ± confidence intervals.

(a) Control VS Xylanase
Area (mm²)
Time (minutes)
Control, Xylanase H, Xylanase R, Xylanase D

(c) Control VS Phytase
Area (mm²)
Time (minutes)
Control, Phytase H, Phytase R, Phytase D

(b) Control VS Protease
Area (mm²)
Time (minutes)
Control, Protease H, Protease R, Protease D

(d) Control VS Protease+Phytase
Area (mm²)
Time (minutes)
Control, Protease/Phytase H, Protease/Phytase R, Protease/Phytase D

(e) Control VS Xylanase+Protease+Phytase
Area (mm²)
Time (minutes)
Control, Xylanase/Protease/Phytase H, Xylanase/Protease/Phytase R, Xylanase/Protease/Phytase D

Figure 5. Effect of enzymes and their dosage level on pellet swelling under stagnant water, observed within 14 min, and compared to control. (a) xylanase, (b) protease, (c) phytase, (d) protease and phytase, (e) xylanase, protease and phytase.

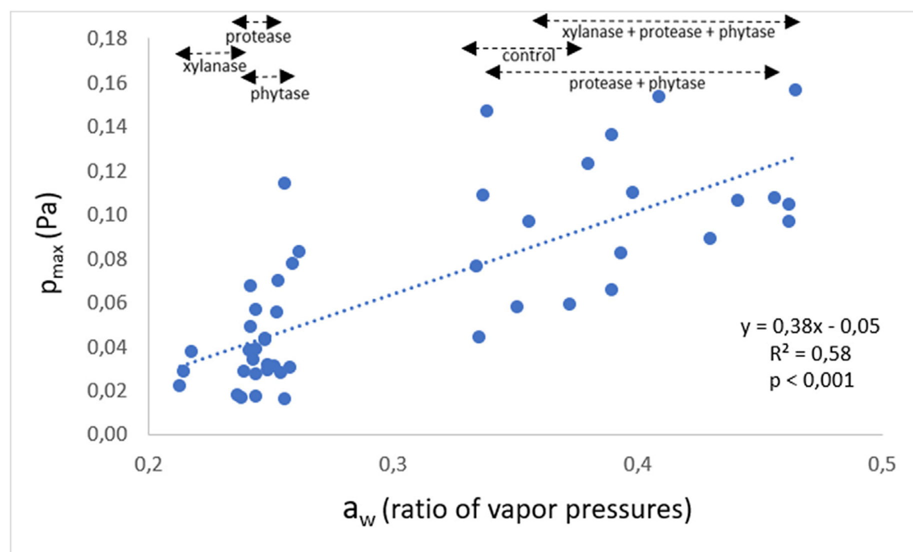

Figure 6. Water activity in direct correlation with compacted biomass flow (p_{max}) through cylindrical die.

protease/phytase (Figure 5D). At the first minute, the highest observed swelling was in the control treatment (Figure 5A) and the lowest was for the phytase H (Figure 5C). Five minutes of submerging a pellet under water, the largest swelling was observed in treatments with a protease/phytase double dosage and the control treatment (Figure 5D). However, by adding the protease/phytase mixture at the recommended dosage, after 6 min of UPS observation, a significant decrease ($p < 0.001$) of pellet swelling was observed (Figure 5D). When compared to the control, the treatments with xylanase were significantly lower ($p < 0.001$) in UPS, despite the dosage level (Figure 5A). The same was observed for treatments with the enzyme phytase (Figure 5C).

3.5. Water activity (a_w)

All enzymes, except the phytase D, added to microalgal biomass independent of dosage levels influenced a_w values when added separately. However, when added as mixes of enzymes no influence of a_w value was observed, except the mix of protease and phytase H (Table 1).

There was a correlation between a_w values and p_{max} ($R^2 = 0.46$) (Figure 6). By increasing a_w, the p_{max} values also increase. Additionally, by having higher a_w values, the tensile strength of the pellets increases ($R^2 = 0{,}12$) (Figure 7). However, the trend for this is unclear.

There was no correlation between a_w values either and to oil θ nor to water θ ($p > 0.05$)., The increased UPS rates were not correlated with changed a_w values ($p > 0.05$).

3.6. Effect of enzymatic hydrolyses on chemical change of pelleted microalgal material

Total phosphorous content did not influence flow resistance of the pellets (Table 2). When comparing to the control sample the soluble protein content was significantly higher in samples treated with phytase. Different dosages of phytase did not influence the changes of total soluble protein measurement in the pellets when compared to control (Table 2).

4. Discussion

The observed reduction of p_{max} for the recommended dosage of xylanase and all dosages of phytase may be explained by changes in the chemical structure of linearly layered polymers in the cell wall, well defined as xylans (Bajpai, 2014). Xylanase may hydrolase the bonds between xylans in the cell walls, decreasing the resistance to motion and friction.

Table 2. Chemical change influenced by the treatments: Total phosphorous and crude protein levels.

Treatments	Dose level	Total P (mg/g)	Crude protein (g/kg)
Control		1.83	55.1 a
Xylanase	H	1.56	-
	R	1.31	-
	D	1.47	-
Protease	H	1.32	-
	R	1.51	-
	D	1.42	-
Phytase	H	1.31	60.9 b
	R	1.41	58.8 b
	D	1.36	59.9 b
Protease/Phytase	H	1.08	-
	R	1.41	-
	D	1.39	-
Xylanase/Protease/Phytase	H	1.84	-
	R	1.43	-
	D	1.39	-

Results without letters are not significant ($p \geq 0.05$).

Also, the reduction of p_{max} is rationalized by hydrolysis caused by phytase. It was expected that phytase will influence structural changes of the phosphorous that is stored in the form of inorganic polyphosphate during "luxury phosphorous uptake" (Solovchenko et al., 2019). Polyphosphates are of pivotal importance in microalgae (Grobbelaar, 2004), and covalent binding to proteins that enable microalgae to acclimate to stress conditions (Sanz-Luque et al., 2020). However, according to the results presented in this research work this was not the case (Table 2). The experimental treatments did not provide any evidence that type of enzyme or its dosage is influencing any change in availability of phosphorus in the pellets (Table 2). Accumulated inorganic polyphosphate in *Desmodesmus sp.*, remain in the microalgal biomass, even after breaking the cell wall (Xin et al., 2010). By introducing phytase, the hydrolyses of the links between polyphosphate and protein occurs. Such hydrolyses reduced viscosity due to increased level of soluble protein, as showed in Table 2. Phytase, whether based on histidine acid or with alkaline base, can catalyse hydrolysis of a wide range of molecules containing phosphorous (Oh et al., 2004). Phytase, thus, enables the detachment of phosphate from a wide range of molecules. During such detachment the

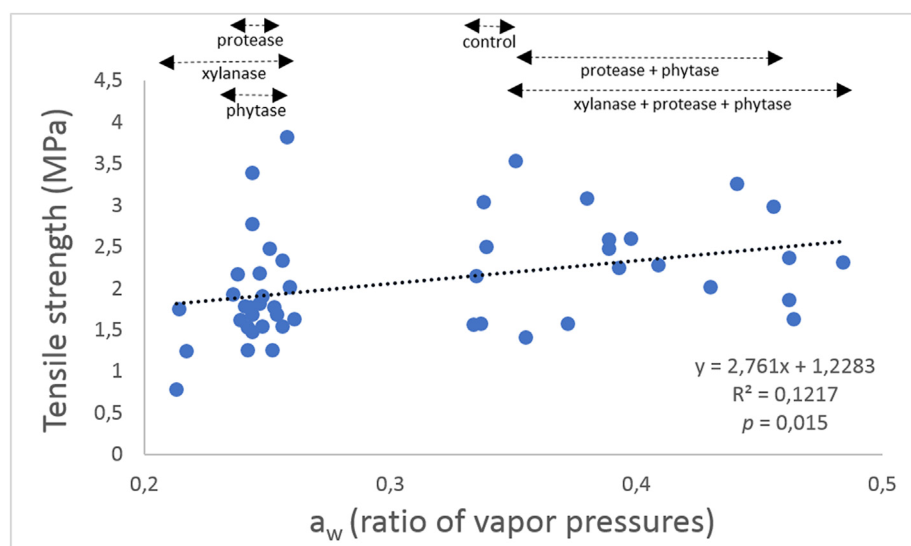

Figure 7. Pearson correlation between water activity and tensile strength.

chemical nature of protein surfaces may change (Hunter, 2012). When phosphate ester bonds detach from the protein, the protein may decrease the viscosity if being compressed by the forces, thereby, lowering p_{max} during pelleting.

Mixing phytase and protease did not significantly decrease p_{max}. This contrasts with Bae et al. (2013). The reduced p_{max} values, obtained when phytase was added to the microalgal biomass and thereafter pelleted, could be ascribed to hydrolyses, and changes of the chemical bonds in lipoprotein-complexes. Doubling the dosage of phytase, protease, combination of phytase and protease and all three enzymes combined did not affect p_{max}. Every enzyme in the combined enzymatic cocktails potentially had some influence on the rheological properties of microalgal biomass. Enzyme xylanase hydrolysed the xylans present in the cell walls, protease influenced protein solubility and phytase influenced phosphate ester bonds. Enzymes xylanase, double dosage and phytase all three dosages partially influence the change of complex molecules in microalgal biomass connected to the cell wall. This was evident as during compaction lowered p_{max} values. Such changes are directly influenced by catalysing the hydrolysis of the bonds between fibre and peptidoglycans from the cell walls (Bae et al., 2013). However, there was no indication that mixtures of xylanase, protease and phytase, in different dosages, influenced p_{max} when compared to control. One probable reason for the unchanged p_{max} is that proteolytic activity of protease may fully or partially deactivate other enzymes. The active sites of the enzymes may be affected. At those active sites protease may donated or accepted hydrogens, and destabilized the electrical charge build-up along the reaction mechanisms (Shafee, 2014).

Smaller particles of a powder material may result in lower resistance to flow. During densification and compaction processes, an interaction between enzymes and microalgal particles can influence lower resistance of a powder matrix in the die. At elevated and controlled temperature of the die this is even easier to control. Rheological properties of precompacting powders change due to enzymatic hydrolyses and chemical changes of the total protein (Table 2). The availability of protein bound to phytate, can be increased noticeably by hydrolysis of the phytate molecule by phytase. This agrees with Kies et al. (2001). Such changes may further influence the compacting properties of the system and lower p_{max}. This all may result in the decreased consumption of electricity during commercial pelleting. Lowering p_{max} during commercial pelleting and thus reducing retention time of the material in the pellet-die may increase the overall production capacity during pellet manufacturing. In a commercial microalgal biomass pelleting, the main concern is the ability of the powder material being pressed through the pelleting die. If the viscosity of material is high the increased temperature in the die will be generated by friction between the compacted powder and the die. Such temperature increase may cause burning of the compacted material and hence destruction of the nutrients. Mechanisms responsible for inter-particle reactions, through random distribution, are the rearrangement of particles in the clusters. This cluster-alike arrangement helps particles gaining the conformational entropy and the final stability of compacted powder materials (Pietsch, 1991; Walstra, 2003). Every system changes its volume when it is loaded. In other words, when particles of the microalgal biomass are loaded, they will have a strain response. Due to enzymatic reactions between phytase and protein linked to phosphorous, the enthalpies and the entropies could change their energy values and the differences in the p_{max} values. This agrees with Miladinovic and Salas-Bringas (2014). Using xylanase and phytase may possibly influence the reduction of electrical energy consumption during commercial pelleting, where non-starch polymers and microalgal biomass will be included. Further work related to the influence of enzymes must be done on the commercial scale and with direct measurement of electrical consumption, with clamp-on measurement devices.

With or without applied stress to all particles in compacted microalgal biomass, their macromolecules and atoms are subject to constant forces between them. In the present study, enzymes neither influenced the interparticle bonding (hardness) within the pelleted microalgal biomass, nor did the a_w changed in the pellets. Thus, it can be assumed that no chemical change involving enzymes influenced change in the entropy of the compacted particles at the level of pellet hardness. It seems that the molecules or the chemical groups in microalgal biomass compacts were not modified to produce changes in the strength of the pellets. The results did not reveal any changes regardless of dosage of enzymes. Reason for this may be nonsufficient water content in the microalgal biomass for fully functioning enzymes. The bulk powder, made of microalgal particles, may have partitioned the hydration water from the enzymatic solution. This is highly dependent on the polarity of organic solvents (Yang et al., 2004). In this way the active surface of the enzyme is not well hydrated to make any change at the surface of the particles. so that particles of the microalgal biomass could interlock and create harder pellets. This finding may be explained by some other factors influencing the observed correlation outputs, and thus, further investigation is necessary.

Particle detachment was observed when oil droplet was placed on the surface of the pellet. Statistical differences in θ could be explained by different interparticle distances in the compacts, which increased as the oil droplet was ageing. These results should not be interpreted as the enzymes did not affect the θ at a higher percentage of enzyme addition, which may be beyond the quantities used in this experimental work. These differences in θ may be explained by the activation entropy of the protein and the chemical components of the microalgal cell walls at processing temperatures where hydrophobic bonds may become weak (Liu et al., 2000). Therefore, the particles in compacts detached more quickly when oil was placed on them, thus increasing lipophilicity.

The amount of water added to the microalgal biomass was close to that in the commercial pelleting process. A low a_w indicates a low activity of enzymes. Lee and Kim (1995) explained that the enzyme reaction rate at a given water activity is different and that the reaction rate can be dependent on the property of the solvent. Enzymes need water to react and thus, a low a_w can indicate that the enzymes in the present experiment may not have reacted completely. The commercial pelleting process involves the moisture addition by steam conditioning. Such conditioning does not provide an adequate water content but provides the high enthalpy to the powder material. In such way the "nearly full" potential of the enzymes may be achieved prior to pelleting. The full potential of the enzymes may be accomplished with processes that include sufficient water content, such as extrusion technology. Decreased a_w of the microalgal biomass with addition of single enzymes at all three levels, can be explained by the enzyme trapping water between the particles. When enzymes were mixed and added to microalgal biomass, such change did not occur. This may be explained by proteolytic weakening of the function of other enzymes by protease. Additionally, it was expected that free water decreased friction between the compacted medium and the compaction die-wall, hence, decreased p_{max} values were expected. This did not occur.

The three enzymes, and their various dosages influenced the correlation between a_w and tensile strength of pelleted microalgal biomass. This could be explained by reduced adhesion of microalgal particles due to bound water presence. This was probably due to the free energy at the surface of the particles. This corresponds to the results obtained by Sun (2008). A significant positive relationship between a_w and tensile strength could be explained due to preferable water dynamics through hydrogen ionization, which occurs with the enzymatic reaction.

As hypothesized in this research, it has been shown that the UPS of the microalgal compacts can be positively influenced by adding any enzyme alone. This can be explained by the ability of enzymes to interact with a microalgal substrate, its disintegration and consequently systematic molecular packing and bonding of particles in the substrate. The overall tendency of the enzymes when added alone, independent of their dosage level, was to slow down the UPS process. However, the different effect of the protease/phytase on UPS at different dosages is still unclear. When all enzymes were mixed together in their double dosage, a lower UPS was observed. The complexity of such an enzymatic mixture may have allowed non-starch polymers, proteins and available phosphorous

to interact with each other, allowing the formation of strong molecular bonds. When all three enzymes were added together as the double dosage, most likely, xylanase helped hydrolysing the complex chain of polysaccharides and thus, increased hydrogen bonding between the molecules. When such a structure was formed, the double dosage of protease and phytase may have improved protein solubility, as explained by Bae et al. (2013). This study indicates that addition of enzymes is necessary to control the UPS, which could improve the environmental situation at the aquatic farms. The addition of the enzymes in such way may control the leakage of nutrients in the water. Which could help better situation in the aquatic farm.

5. Conclusions

Adding of the enzyme xylanase at recommended level or the enzyme phytase at all three levels reduced the resistance of microalgal pellets during discharge from a pelleting die. These enzymes could therefore possibly help to lower the electrical energy consumption during the pelleting process due to reduced friction between the pellets and the wall of the pelleting die (lower p_{max}). Adding of these enzymes was neither influencing the tensile strength of the pellets, nor the surface contact angle between the microalgal pellets and water or oil droplets.

Addition of enzymes xylanase, phytase or protease alone, used for this experiment, lowered the water activity. This may probably lower the microbiological activity in the microalgal pellets and lead to increased shelf life of the pelleted product. The reduced water activity did not influence underwater swelling or the surface contact angle, neither for oil nor for water droplets. Reduced water activity was directly correlated with reduced flow resistance of the compacted material in the pelleting die during pellet discharge. This happened most likely due to decrease of frictional forces between the pellet surface and the wall of the pelleting die.

The physical quality characterization of novel feed ingredients, if well used and understood, may have a positive economic impact for feed manufacturers. This is central when focus is the inclusion of novel ingredients into the feed matrix.

6. Further studies

Suggestions for further research includes water sampling for nutrient leakage by performing UPS analyses from the pellets treated with different enzymes. added alone or in combination. Also, future studies are needed to characterize different single cell organisms and their interaction with various enzymes and to assess their process-ability, nutritional and physical quality parameters during and after compaction. The use of higher amounts of water is suggested in the future for improved enzymatic hydrolyses. Preferably, water could be added with saturated steam after mixing and prior pelleting. Better understanding of enzymatic influence on the physical quality of the microalgal compacts may be achieved with an elaborate experimental plan. Such plan may include different compacting pressures, different temperatures, water addition, and enzyme concentrations beyond the levels used in this article, where focus must be given to power consumption during pelleting, underwater pellet swelling, hardness and durability of the pellets. Enzymatic activity is critical to consider in future scientific studies and for the commercial feed production where determination of pellet quality including microalgae would be essential. This could lead to better utilization of the raw materials and an overall better farm economy.

Declarations

Author contribution statement

Dejan Dragan Miladinovic: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Trond Storebakken: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Odd Ivar Lekang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Carlos Salas-Bringas: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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