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Development of Encapsulation and Coating for Protease on Shrimp Feed.

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Abstract: Aquaculture has the potential to revolutionize the global food supply chain by providing a source of nutrition for the world's growing population. This will require the ready availability of high-quality aquaculture feed, and especially its main ingredient, fishmeal. Yet legislation targeting IUU (Illegal, Unreported and Unregulated) fishing strictly controls the usage of fishmeal. Consequently, numerous fishmeal replacement products have been introduced to the market. Yet many such replacements, composed of plant materials, contain high levels of indigestible and antinutritional factors. Thus, supplementation with enzymes, such as protease, is a crucial way to increase products' digestibility by aquatic animals. A key limiting factor, however, rests upon the manufacturing techniques for aquaculture feed, which require the usage of high temperatures, as heat can diminish enzyme ability. This study is designed to find a solution to this problem, with the use of encapsulation and coating techniques. The ability of encapsulation of 0.25-1% alginate, and of coating materials – chitosan, Seal 4, pullulan and carboxymethyl cellulose (CMC) – to limit the leaching of protease from shrimp feed are observed. The retention capacity of alginate encapsulation is measured by determining the extent of protease leaching in calcium chloride solution. To test coating materials, feed is soaked in distilled water for 30 min, with the resulting solution from each treatment analyzed for protease activity. The results show that encapsulation with 1% alginate retains the most protease (87.63% and 80.56% from protease I-White and II-Brown respectively); and coating with pullulan and CMC results in the least protease leaching (0.200% and 0.210% respectively). To conclude, 1% calcium alginate gel is the most effective product for protease encapsulation, and pullulan is the most effective shrimp-feed coating in terms of its protease retention capacity.

Keywords: Protease, Encapsulation, Shrimp feed, Coating, Chitosan, Pullulan, CMC, Seal 4

1. INTRODUCTION

In recent decades, the aquaculture industry in Thailand has boomed. The shrimp industry in particular has experienced significant growth, and the commodity is now recognized as the country's most vital import and export product in terms of value (Jantarathin *et al.,* 2017). Aquaculture production has increased by more than 40 million ton/year over the past few years (FAO, 2020). However, the price of fishmeal, the raw material upon which production relies, has skyrocketed, rising to some 2,400 USD ($)/ton of fishmeal in 2014 (PFISHUSDM, 2021).

In addition, the supply of fishmeal has been substantially limited by regulation aimed to target Illegal, Unreported and Unregulated (IUU) fishing. As a consequence, most feed manufacturers have developed new formulations, replacing fishmeal with other protein sources, such as soybean meal, worm meal, and algae powder. However, plant-based protein is significantly harder for aquaculture animals to digest compared to fishmeal. Soybean meal, for instance, contains numerous antinutritional factors (ANFs), including tannins, trypsin inhibitors, phytate, cellulose and other fibers (Qiu & Davis, 2016). Some ANFs, namely tannins and trypsin inhibitors, are eliminated by the high temperatures that are a routine component of the feed manufacture process. However, heat does not destroy other common ANFs. Thus, manufacturers introduce a range of enzymes to counteract the negative impact of ANFs, with enzymes that break down nutrients from plant protein – cellulase, phytase, xylanase and protease – being the most common additions. Such enzyme supplementation targets a reduction in the effects of ANFs and an increase in the utilization of dietary energy and amino acids, leading to improved growth rates of aquaculture (Soltan, 2009; Bharathi *et al.,* 2019). In shrimp, digestive enzyme production is affected by animals' life stage including age, molting stage, circadian cycle, feed composition and their environmental conditions.

Amongst all enzymes, proteases are the most important for hydrolyzing dietary proteins, breaking them into smaller peptides to free amino acids (Liu *et al.,* 2014; Goda *et al.,* 2020). The protease activities in the digestive tract of an animal are induced by feeding, and are usually affected by genetics, age, life stage and environment. Protease supplementation is thus a crucial means to maximize the digestibility of aquaculture feed (Li *et al.*, 2015; Yao *et al*., 2019). Broadly speaking, proteases are divided into six groups: aspartate, cysteine, glutamate, metallo, serine, and threonine proteases. This classification is based on shared mechanistic features within individual groups (Li *et al.*, 2013). Nearly half of all proteases are known as proteolytic enzymes: serine proteases with an endo-proteolytic catalytic activity typically dependent on a triad of aspartate, histidine, and serine residues (Di Cera, 2009). In addition, the endogenous proteases, trypsin and chymotrypsin, belong to the largest family of serine protease. They cleave polypeptide chains at positively charged arginine and lysine residues, or large hydrophobic phenylalanine, tryptophan, and tyrosine residues, respectively (Walk *et al.,* 2018). Different proteases impact the environment and biological activity differently. As such, proteases are put to a variety of uses in industrial applications, selected according to their suitability for the task at hand.

Shrimp feed requires enzyme supplementation. However, the efficacy of supplementation is limited by the processes involved in shrimp feed production. Firstly, the temperatures required in the pelleting and post-pelleting stage of shrimp feed production are higher than 100 °C, directly affecting enzyme quality. As a result, the addition of enzymes to feed mixer is of limited utility. This problem firstly necessitates the introduction of encapsulation techniques, aimed at increasing the temperature resistance of enzymes before mixing with other feed ingredients. Secondly, pellets of shrimp feed are denser than those of fish feed: shrimp feed sinks, whilst fish feed floats. This means that shrimp feed less readily absorbs enzymes than fish feed, as enzymes are more easily leached. This problem can be solved by the use of materials to coat feed to reduce leaching (Mong Thu & Krasaekoopt, 2016).

This study investigates the optimum concentration of alginate for the encapsulation of powder protease, and compares the ability of four coating materials (chitosan, pullulan, carboxymethyl cellulose (CMC) and Seal 4) to reduce protease leaching from coated shrimp feed. Relative retention capacity is calculated based on the percentage of enzyme present in solution after samples have been soaked in water.

2. MATERIALS AND METHODS

Encapsulation of protease enzyme with alginate

- *Preparation of enzymes, encapsulation materials and experimental design*

Samples of two forms of protease enzyme, white (I-White) and brown (II-Brown) types (Jefo Nutrition Inc.), are tested. Both enzymes are in powder form, although brown protease has a larger particle size. Samples of both kinds of protease are thoroughly mixed with four different concentrations of alginate solution (0.25%, 0.5%, 0.75% and 1%) at a proportion of 20%. The resulting mixture is added to a calcium chloride (2%) solution. Thereafter, the protease droplets are filtrated and dried at 65 °C for 6 hours. The enzyme-retention capability of alginate encapsulation is measured by the amount of leached protease present in the calcium chloride solution.

$$\text{Retention capability (\%)} = 100 \times \frac{\text{Initial amount of protease-Leached protease in calcium chloride solution}}{\text{Initial amount of protease}}$$

The trial is designed in 2*4 factorials of 2 enzymes and 4 concentrations of alginate, as shown in Table 1.

Table 1: Experimental design for alginate encapsulation technique

Treatment	**Protease type**	**Alginate concentration (%)**
1	I-White	0.25
2	I-White	0.5
3	I-White	0.75
4	I-White	1
5	II-Brown	0.25
6	II-Brown	0.5
7	II-Brown	0.75
8	II-Brown	1

- *Statistical analysis*

All data were analyzed by two-way ANOVA (analysis of variance). Duncan's procedure was used for multiple comparisons on differences between treatment means. Alphabetical notation is used to mark differences at a significance level of alpha 0.05.

Coating materials for protease enzyme

- *Preparation of coating materials*

The coating materials for testing are: chitosan 5% (SSA190/3k8KF, VIV Interchem Co., LTD.); Seal 4 12.5% (Pathway Intermediates Thailand), pullulan 3% (MyskinRecipe); and carboxymethyl cellulose (CMC) 2% (FVH6-A) solute in acetic acid 3%. Shrimp feed and protease enzyme (II-Brown) are combined, with samples of the mixture then coated with each coating material. The trial is designed in 2*4 factorials of 2 enzyme conditions (feed without protease and feed with protease) and 4 coating materials. This results in 8 treatments, as shown in Table 2.

Table 2: Experimental design for coating materials technique

Treatment	Enzyme	Coating material
1	-	Chitosan 5%
2	-	Seal 4 12.5%
3	-	Pullulan 3%
4	-	CMC 2%
5	Protease II-Brown	Chitosan 5%
6	Protease II-Brown	Seal 4 12.5%
7	Protease II-Brown	Pullulan 3%
8	Protease II-Brown	CMC 2%

Brown protease (II-Brown) in powder form (Jefo Nutrition Inc., Canada) is used in the experiment at the recommended dose of 20g/ton of feed. After coating, samples of feed are dried for 30 min, before being soaked in distilled water at a proportion of 1:3 for a further 30 min. To determine the level of protease leaching, the resulting solutions are taken from each treatment and analyzed for protease activity by casein assay. The rate of enzyme leaching is calculated as follows:

$$\text{Leaching (\%)} = 100 \times \frac{\text{Enzyme in water after 30 min immersion}}{\text{Initial enzyme in feed}}$$

- *Analysis of enzyme: protease activity*

100 µl of solution is added to 170 µl of substrate casein (casein 0.2g, Tris-HCl 1 ml and buffer 9 ml). After incubation at room temperature (25℃) for 10 min, 1 ml of 1.2 M TCA is added. The sample is then centrifuged at 10,000 rpm for 5 min, followed by the addition of 200 µl of supernatant in 0.4 N NaOH 1 ml and incubation at 40 ^{o}C for 15 min. Thereafter 200 µl of Follin 1:1 is added, and the solution is incubated at room temperature (25^{o}C) for 10 min. The solution is then measured for protease absorbance at 660 nm by spectrophotometer (Modified Bisswanger, H., 2004; Kattakdad, Jintasataporn *et al.*, 2018).

- *Statistical analysis*

All data were analyzed by two-way ANOVA (analysis of variance). Duncan's procedure was used for multiple comparisons on differences between treatment means. Alphabetical notation is used to mark differences at a significance level of alpha 0.05.

3. RESULTS AND DISCUSSION

Despite the clear benefits it offers in terms of increased digestibility, enzyme supplementation of aquaculture feed is thus far limited by feed production techniques, especially the use of high temperatures, given the unstable structure and heat-sensitivity of enzymes. The encapsulation technique has been suggested as a means to overcome such problems (Ertan *et al.,* 2007). Encapsulation targets an increase in enzymes' storage stability and duration of time-release, hence relevant techniques, namely emulsion and extrusion, are routinely applied (Bhandari, 2009). The use of a syringe with a needle to inject organic solvents in feed leads to stable enzyme encapsulation (Anjani *et al*., 2007), and is thus a commonly used method. More specifically, encapsulation by alginate is in widespread usage, favored due to the advantages offered by calcium alginate gel: it is fast-acting, non-toxic, highly biocompatible, inexpensive,

with a stable acidic pH (Azarnia *et al*., 2008). Hence, calcium alginate gel was used in this study to determine its capability to limit protease leaching. The rates of leaching of both protease I-White and II-Brown after encapsulation with different concentrations of alginate are shown in Table 3.

Table 3: Protease leaching by encapsulation technique

Protease	**Alginate (%)**	**Leaching of protease (unit/kg sample)**
I-White	0.25	1.8261 ^{abc} ± 0.4369
	0.5	1.4858 ^{bc} ± 0.5904
	0.75	1.3810 ^{bc} ± 0.6788
	1	1.2370 ^{c} ± 0.8717
II-Brown	0.25	2.3759 ^{a} ± 1.1036
	0.5	2.1534 ^{ab} ± 0.6736
	0.75	2.1533 ^{ab} ± 0.6989
	1	1.9439 ^{abc} ± 0.6719
P-value protease		*0.000*
P-value alginate		*0.195*
*P-value protease*alginate*		*0.977*

Note: The presence of superscript letters a, b, c indicates a significant difference (P<0.05).

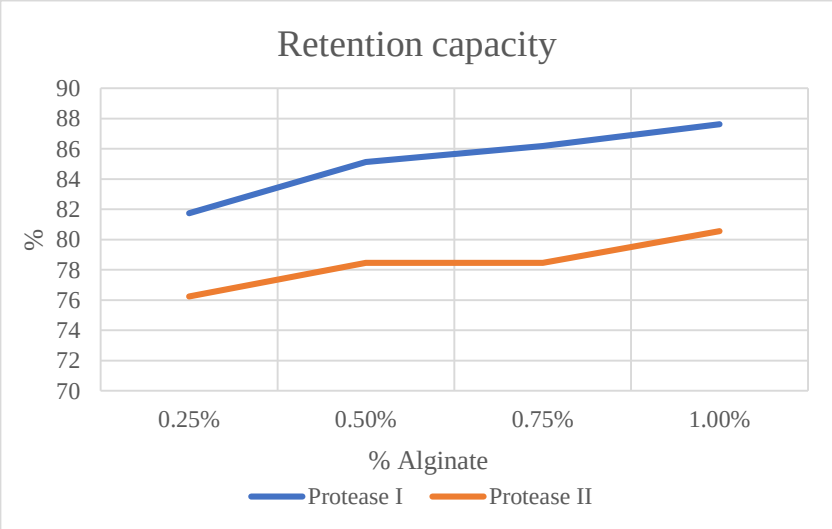

Figure 1: The percentage of protease retained by samples after encapsulation with different alginate concentrations and immersion in water

Table 3 and Figure 1 illustrate that encapsulation with alginate at 1% concentration has the greatest capacity to retain both protease I-White and II-Brown. Whilst both proteases follow the same overall trend, protease II-Brown shows higher (P<0.05) rates of leaching compared to protease I-White. After encapsulation with 1% alginate, the rates of leaching for protease I-White and protease II-Brown are 1.2370 ± 0.8717and 1.9439 ± 0.6719 unit/kg sample, respectively. Conversely, encapsulation with 0.25% alginate is least successful in terms of enzyme retention, leading to the highest rates of leaching with 1.8261 ± 0.4369 and 2.3759 ± 1.1036 unit/kg sample in protease I-White and protease II-Brown, respectively. However, the

data shows that there is no significant difference in terms of protease leaching between alginate encapsulation at concentrations in the range of 0.25-1% (P>0.05). Nevertheless, the results show that alginate is a suitable material for protease encapsulation due to its capacity to limit the loss of protease enzyme following immersion in water.

The range of alginate concentration investigated in this study was determined following research by Usama (2003) in which 0.5-10% alginate was used to experiment with protease encapsulation, alongside a later study that demonstrated the efficacy of encapsulation with 2% alginate (Mong Thu & Krasaekoopt, 2016). Protease I-White exhibits lower levels of leaching than protease II-Brown; hence, protease I-White is more suitable for use with this encapsulation technique. This could be because of evident differences in the two products' particle size and, consequently, their enzyme dispersion. The average particle size for protease I-White samples is 176.23 μm, compared to 97.07 μm for II-Brown samples (Figure 2). Protease I-White is a white powder with a large particle size, meaning that it attaches effectively to encapsulated materials. By contrast, protease II-Brown is brown powder with a small particle size, and thus particles are more readily released from the encapsulation materials. Ineffective encapsulation leads to high rates of protease leaching after immersion in water (Kurayama *et al*., 2012). In addition, Figure 1 illustrates the inverse variance between protease leaching and alginate concentration. Encapsulation with 1% alginate is the most effective in terms of protease retention, with 87.63% and 80.56% for protease I-White and II-Brown respectively. On the other hand, encapsulation with 0.25% alginate affords the lowest retention rates across all samples, with 81.74% and 76.24% for protease I-White and II-Brown respectively.

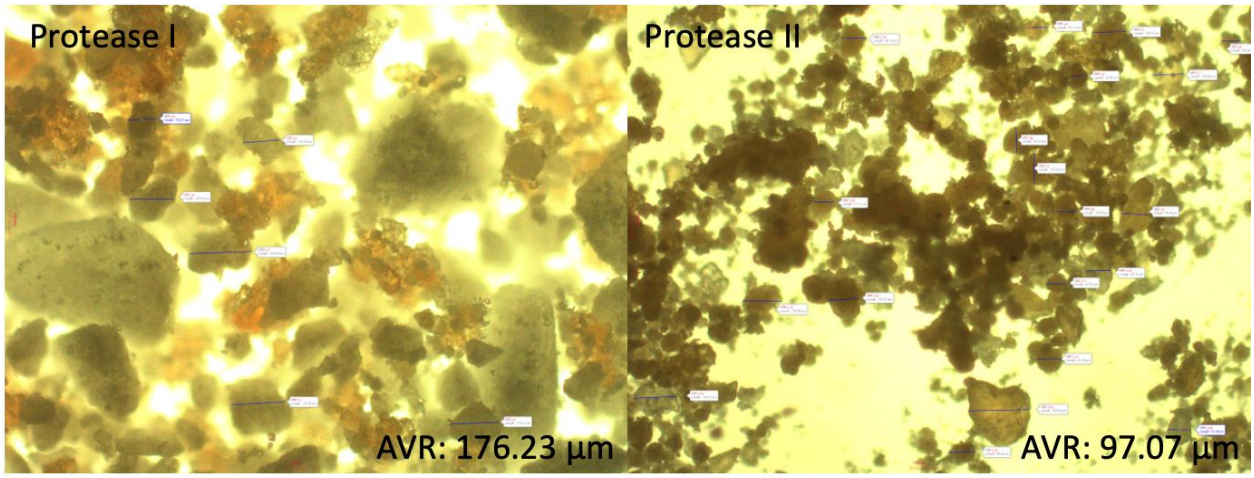

Figure 2: Particle size of protease I-White and II-Brown under light microscope (4x10)

The impact of coating materials on retention rates of supplemented protease in shrimp feed was studied, with a focus on the efficacy of four compounds: chitosan, Seal 4, pullulan and CMC. Figures 3 and 4 present the study's findings, showing rates of protease leaching for each coating material after water immersion, with enzyme-supplemented coated samples compared to coated samples without any protease supplementation. The data clearly demonstrates that the rate of protease leaching in samples coated by chitosan is significantly higher (P>0.05) than for all other coating materials under investigation. Actually, coating with chitosan is most effective not in overall protease retention, but in improving the retention compared to unsupplemented feed. Enzyme leaching from samples of coated feed without protease supplementation is caused by the release of naturally occurring enzymes in the feed's

raw materials, or the release of enzymes by microorganisms contaminating the pellet samples (Polonskaia *et al.*, 1977).

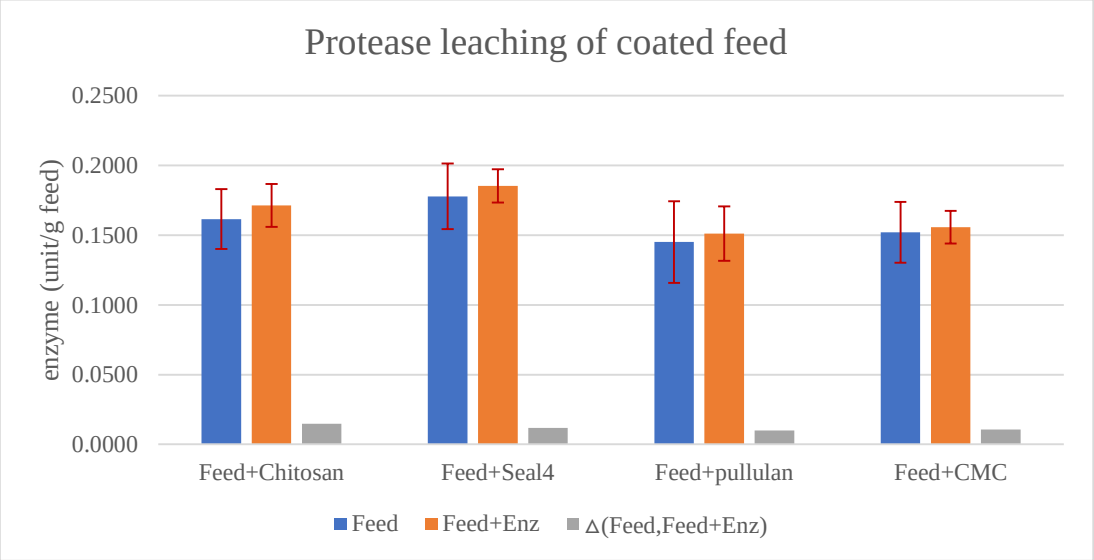

Figure 3: Protease leaching from samples of coated feed (with and without enzyme supplementation) after immersion in water for 30 min

Figure 3 demonstrates that coating feed with chitosan leads to the largest gap ($P<0.05$) in terms of protease leaching between coated feed without enzyme supplementation, and coated feed with enzyme supplementation. Chitosan is commonly used on aquaculture farms as a coating material applied post-pelletization, after the addition of additives to feed. As a biodegradable compound with good film- and shape-forming capacities, chitosan is particularly suitable for use as the external shell of feed capsules, by reaction of anionic polymers as alginate (Kailasapathy & Chin, 2000). It has been reported that chitosan, used as a feed coating, can enhance aquaculture animals' resistance to environmental stress, on the basis that chitosan is acquired from chitin by deacetylation in an alkaline media. In fact, chitosan is a copolymer comprised of (1–4)-2-acetamido-D-glucose and (1–4)-2- amino-D-glucose units (Abdou *et al.*, 2007). This explains its antimicrobial properties, in association with its cationicity and film-forming properties (Domard A & Domard M, 2001). Despite certain benefits, chitosan is a less effective coating in terms of protease retention than other materials tested in the study due to its stickiness, which limits the effective, even dispersion of enzymes throughout the coating. This can lead to wide variation in levels of protease leaching, as the enzyme may not be dispersed evenly over feed, but instead concentrated in certain areas.

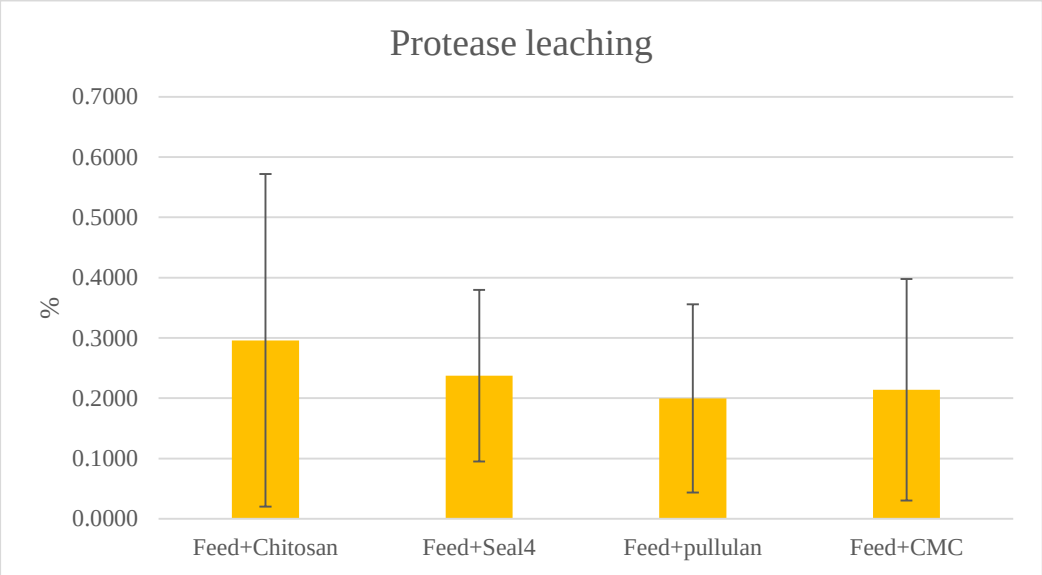

Figure 4: Percentage of protease leaching from shrimp feed coated by different materials after immersion in water for 30 min
Note: There is no significant difference (P>0.05).

Seal 4 offers the second highest difference, in terms of comparative levels of protease leaching between coated feed without enzyme supplementation, and coated feed with enzyme supplementation. This could be due to the fact that Seal 4 is composed of liquid gum and modified starch. Both raw materials are polysaccharide-based, and are used in many kinds of food products as thickening agents in various applications, including in hydrogels, microspheres, nanoparticles, and matrix tablets (Jain *et al*., 2008). The advantages of Seal 4 also include its biocompatibility and biodegradability (Shalviri *et al*., 2010). Whilst Seal 4's thickening properties are noteworthy, the product remains lower viscosity than chitosan. Hence, Seal 4 has a greater ability to disperse evenly over shrimp feed than chitosan. However, there is no significant difference (P>0.05) in protease leaching between samples of the two coating materials, due to the particle size of the enzyme.

Carboxymethyl cellulose (CMC) is used as a thickening agent in the food industry for its ability to retain enzymes, thanks to its structure. Figure 4 illustrates that feed coated with CMC releases a similar amount of enzyme to samples coated with pullulan (P>0.05). CMC is a polysaccharide commonly used for film blending, chosen for its benefits including high viscosity and non-toxicity (Duran and Kahve, 2016). As CMC consists of numerous hydroxyl and carboxylic groups, it demonstrates strong water-binding and moisture-absorption properties (Siracusa, 2012). Because of its polymeric structure and high molecular weight, CMC is also used in biocomposite film production to increase mechanical and barrier properties (Almasi *et al*., 2010). Despite such advantages, edible films made from CMC also exhibit weaknesses, such as lower tensile strength. As a result, CMC can be used to enhance the desirable characteristics of other materials, such as in a mixture film (Suderman *et al.*, 2016). Even though the stickiness of CMC affects its ability to coat samples evenly, its ability as a film agent supports the effective retention of enzymes in coated samples.

Pullulan exhibits low viscosity, and thus offers greater ability to coat pelleted feed evenly and thoroughly. This offers greater opportunities to collect enzyme from pullulan-

coated feed, when sampling feed to measure enzyme activity. Pullulan is obtained from the fermentation of the fungus-like yeast *Aureobasidium pullulans* (*Pullularia pullulans*). Its structure consists of maltotriose trimer by a-(1,6)-linked and (1,4)- a-D-triglucosides (Farris *et al.*, 2014). Nowadays, pullulan is used as a coating material due to its peculiar properties, such as its oxygen and carbon dioxide barrier properties. Previously, however, pullulan was utilized foremost as an edible coating, as a thin layer directly applied to the surface of a food product (Pavlath & Orts, 2009). This study shows that pullulan coating results in decreased rates of protease leaching from shrimp feed, suggesting that the biopolymer has many interesting properties that are yet to be fully explored in research, or applied in industry. Even though coating with pullulan demonstrates the best protease retention capacity, there is no significant difference evident across all treatments ($P>0.05$). Nevertheless, in a previous study investigating the capacity of various coating materials to retain phytase and xylanase in shrimp feed, pullulan was shown to be most effective (Uniyom *et al.*, 2021). The results of the present study could have been affected by the particle size of the protease enzyme used for supplementation. For this reason, it is recommended that the enzyme is used in liquid, rather than powder, form when coating materials are used.

4. CONCLUSION

Encapsulation with alginate at 1% capacity exhibits the greatest capacity to retain enzymes, for samples of both protease I-White and protease II-Brown. However, protease I-White is more suitable for alginate encapsulation due to its larger particle size. There is no significance difference in the performance of coating materials applied to protease-supplemented shrimp feed in terms of limiting enzyme leaching. Nevertheless, pullulan shows the greatest promise to retain protease, compared to other tested materials: its low viscosity means that it is easier to blend effectively with the enzyme in powdered form and to evenly disperse across shrimp feed pellets. The higher viscosity of chitosan and CMC limits their ability to be spread effectively on feed, leading to uneven dispersal of the enzyme compared to a liquid product. The results of this study are affected by the particle size of the powder-form of protease enzyme used. It is likely that if the enzyme were used in liquid form, a clearer picture would emerge of the differences between the coating materials under investigation.

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