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Exogenous enzymes in aquaculture: Alginate and alginate-bentonite microcapsules for the intestinal delivery of shrimp proteases to Nile tilapia

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ABSTRACT

Shrimp processing waste holds digestive proteases with a great potential to be used as feed supplement for Nile tilapia *Oreochromis niloticus*. In the present work, we characterize both sodium alginate (A) and sodium alginate-bentonite (AB) microcapsules to entrap enzymes obtained from *Pleoticus muelleri* processing waste. Also, we evaluate these encapsulation methods as a strategy to improve intestinal delivery of exogenous enzymes in *O. niloticus* in order to enhance their digestion process. The effects of different storage methods, *in vitro* simulation of gastric pH conditions and exposition to 40 °C were studied. In order to evaluate if microencapsulated shrimp enzymes are active when they reach fish gut, animals were exposed to three treatments: (1) fast, (2) diet and, (3) AB capsules + diet. Alginate capsules were more affected by different storage methods than alginate-bentonite ones. SEM images showed a correlation between decreased enzyme activity and capsule microstructure changes. The best method to store the AB beads is at −20 °C. After incubating for 7 h at 40 °C, we observed a notorious reduction in the enzyme activities of both microcapsules. On the other hand, at pH 3 both microcapsules prevented enzyme irreversible denaturalization and kept 100% of their activity. The overall results indicate that AB capsules are better vehicles to deliver shrimp enzymes in Nile tilapia. In the bioassay, we observed that when fish were fed with treatment 3, the alkaline protease activity in their intestines was 27% higher than that of the diet fish group. Thus, encapsulated shrimp enzymes have a great potential to be used as a feed supplement in fish nutrition. Further trials involving grow bioassays are needed to verify if this shrimp enzyme contained in AB capsules improve fish digestion, feed conversion, body weight and survival rate.

1. Introduction

Fish aquaculture is an important source of protein for human diet. Traditional aquaculture production systems have changed towards modern intensive and super-intensive systems in order to cover the market demands of fish (Pauly and Zeller, 2017). This practice requires great amounts of fish diet that usually contains high levels of fishmeal, which increase the production costs > 40%. Therefore, this has a direct impact on cost-effectiveness of fish aquaculture (Little et al., 2016; Tveterås, 2002). Nile tilapia (*Oreochromis niloticus*) is one of the most farmed fresh-water species, and it is widely distributed around the world. Tilapia are being cultured in many tropical and subtropical regions due to several features of this species, such as resistance to diseases, low oxygen tolerance and their capacity to feed on a wide range of nutrient sources (Ogello et al., 2014; Zhao et al., 2010). Cultured tilapia species have increased, being more than 20 species farmed around the world; hence, an adequate feeding strategy is essential to achieve a reliable tilapia farming (El-Sayed, 2006). Since the past decade, many research efforts in aquaculture nutrition have focused on the replacement and supplementation of fishmeal based fed with other nutrients sources as plant-based meals and other co-products (Castillo and Gatlin, 2015; Mahmoud et al., 2014; Moesch et al., 2016). Tilapia obtains essential nutrients like amino acids, simple carbohydrates, *etc.* by metabolizing large polymeric compounds using digestive enzymes (*e.g.*, proteases, carbohydrases, and lipases); however, it is known that not all feed nutrient sources are efficiently digested. For instance, anti-nutritional factors (*e.g.*, phytin, protease inhibitors) could be present in feeds and affect fish growth (El-Sayed, 1999; Francis et al., 2001).

In the past decade, the supplementation of animal diets with exogenous enzymes has substantially increased. This new approach has

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favored complex polymeric compounds digestibility, hence improving the utilization of dietary nutrients, energy conversion, and reducing anti-nutritional factors during food-absorption processes (Farhangi and Carter, 2007). More recently, the use of exogenous enzymes in diets for several fish species – like rainbow trout (Rodehutscord and Pfeffer, 1995), catfish (Jackson et al., 1996), and salmon (ali Zamini et al., 2014; Refstie et al., 1999) – has been reported. Tilapia farming has not been an exception to advances indicating that enzyme supplementation is an excellent alternative to enhance nutritional properties of fish diets (Castillo and Gatlin, 2015; Mahmoud et al., 2014). Li et al. (2009) reported that supplementation with non-starch polysaccharides (NSPs) enzyme and phytase increase the enzymatic activity of amylase at both hepatopancreas and intestine of tilapia. Other authors have reported that added xylanase, β-glucanase, and cellulose can hydrolyze high molecular weight polysaccharides from NSPs fish diets and promote the reduction of gut viscosity, which increases NSPs digestion and modulates the intestinal microbiota (Adeoye et al., 2016; Yigit and Olmez, 2011). Furthermore, protease supplementation in aquaculture has been carried out in order to improve protein digestibility in fish species as Gibel carp (*Carassius auratus gibelio*) (Liu et al., 2017), Caspian salmon (*Salmo trutta caspius*) (ali Zamini et al., 2014) and tilapia species (Adeoye et al., 2016; Lin et al., 2007). These approaches have demonstrated that the addition of exogenous enzymes to aquafeeds enhance protein and carbohydrate digestibility, and thus, improve nutritional markers as feed conversion, body weight and the survival rate in fish aquaculture.

Nevertheless, some restraints on their utility can be found; for example, exogenous enzymes could be damaged by the action of endogenous proteases and other physicochemical factors (*e.g.*, pH) present in fish's digestive system. In addition, industrial processes during diet production, such as extrusion, pelletizing and heat drying, could affect exogenous enzymes structural integrity and therefore decrease its functionality. To deal these problems, many polymeric materials and lipids delivery systems have been developed to potentiate the efficiency of exogenous enzymes in both medical and food industries (Kumari et al., 2013; Yoon et al., 2001). Numerous techniques are effective to proper enzyme immobilization, such as microencapsulation in a gel matrix using polymeric compounds as alginate (Yoon et al., 2001), chitosan (DeGroot and Neufeld, 2001) and xylans (Chimphango et al., 2012). Briefly, microencapsulation comprises the entrapment of a substance (enzyme) into a particle, and as a result, the polymeric matrix protects the active molecule from external factors that affect its integrity (Chang and Prakash, 1999; Lovett and Felder, 1990). In fact, several microencapsulation methods have been developed. Chemical microencapsulation comprises changes in solvent properties or a chemical reaction that promotes the complexation of the material, generally polyelectrolytes as alginate and chitosan (Yoon et al., 2001). Alginate is extensively used to microencapsulate a diversity of substances; such as probiotic bacteria (Rosas-Ledesma et al., 2012), bioactive molecules (Ferrándiz et al., 2016; Yoo et al., 2006), and enzymes as glucose oxidase (Blandino et al., 2000), β-galactosidase (Taqieddin and Amiji, 2004), yeast invertase (Tanriseven and Doan, 2001), lipases and proteases (Mong Thu and Krasaekoopt, 2016).

Enzyme encapsulation has been used in food technology processes as dairy and meat fermentations and metabolite production (Gibbs et al., 1999; Nedovic et al., 2011). In fish aquaculture, alginate-calcium microcapsules have been proposed as an effective and low-cost method to deliver bioactive compounds as *Aeromonas hydrophila* (Rodrigues et al., 2006), bovine serum albumin (Polk et al., 1994), and nucleic acids vaccines (Nácher-Vázquez et al., 2015; Tian et al., 2008). However, reports of enzyme encapsulation in fish aquaculture are limited and, owing to their biochemical characteristics, enzyme retention in alginate microcapsules can be challenging. These biomolecules are water-soluble and unstable, losing their activity quickly during the encapsulation procedure. Bentonite – clay from volcanic ash with excellent ion exchanging properties (Adamis and Williams, 2005) – has been widely used in animal feeds production because of its binding and lubricating properties (Mumpton and Fishman, 1977); but also, this clay might improve enzyme encapsulation efficiency. Previous research observed that when 1% of bentonite was added to an alginate solution, the enzyme loss was significantly reduced and did not affect the enzyme activity (Dashevsky, 1998).

As mentioned above, exogenous enzyme supplementation has become the most usual strategy in aquaculture nutrition industry to improve fish feed. Most of these reports comprise the use of commercial enzymes that increase the food cost and therefore affects the fish marketing. As an alternative, the use of an enzyme-rich extract from marine fishery by-products has gained interest to improve fish feed digestibility in tilapia farming. Recently, Rodriguez et al. (2017) have reported the *in vitro* interaction of *O. niloticus* digestive proteases with enzyme extracts obtained from different fishery waste (*Pleoticus muelleri*, *Artemesia longinaris* and *Patagonotothen ramsayi*). The authors found that these enzyme extracts did not affect the function of tilapia enzymes, and indeed improve the digestibility of several fish-feed meal sources (fish, squid, shrimp and soybean meal). These findings suggest that enzymes recovered from fishery by-products could be supplemented in fish food (Rodriguez et al., 2017). In the present work, we characterize both alginate and alginate-bentonite microcapsules to entrap exogenous enzyme obtained from *P. muelleri* fishery by-products. Also, we evaluate these encapsulation methods as a strategy to improve intestinal delivery of exogenous enzymes in Nile tilapia in order to enhance its digestion process.

2. Material and methods

Animal care and experimental protocols were approved by the Institutional Animal Welfare & Ethical Review Committee at Mar del Plata National University (RD 200/15).

2.1. Microencapsulation of shrimp enzymes

2.1.1. Enzyme extract

Samples of shrimp *Pleoticus muelleri* processing waste were provided by manufacturing plants from Mar del Plata, Argentina (38° 02′ S, 57° 30′ W). All samples were immediately frozen and transported to the laboratory. After thawing, the cephalothoraxes of three shrimp were removed. Subsequently, using a glass-Teflon tissue homogenizer, each sample was separately crushed in ice-cold distilled water (1:2 w/v). The resulting material was then centrifuged at 10,000*g* for 30 min at 4 °C (Presvac EPF 12R). The supernatants obtained (shrimp enzyme extracts-SE) were frozen at −20 °C and stored until use.

2.1.2. Enzyme encapsulation

Microcapsules were elaborated according to an adaptation of Dashevsky (1998) methodology. Alginate (sodium salt of alginic acid for feed purposes; Química Industrial Kubo S.A., Mar del Plata, Argentina) at the concentration of 1.5% (w/v) and alginate-bentonite (Química Industrial Kubo S.A., Mar del Plata, Argentina) at 1.5:1.0% (w/v) were used to encapsulate the shrimp enzyme extracts (three replicates). The two polymers were prepared in distilled water. Shrimp enzymes (SE) were encapsulated in the mentioned hydro polymers by mixing 8 mL of enzyme extract in 12 mL of each polymer solution; these volumes were found to be the most appropriate since the microcapsules obtained had similar alkaline protease activity than the crude shrimp enzyme extract (alginate: $0.4 \pm 0.17\ U\,mL^{-1}$; alginate-bentonite: $0.4 \pm 0.21\ U\,mL^{-1}$; SE: $0.4 \pm 0.08\ U\,mL^{-1}$). Then, the solution obtained was stirred to suspend the bioactive material in the polymer. The cationic solution for gelling was 1 M $CaCl_2$. Next, using a pressure system, the polymer-enzyme solutions were extruded through a syringe with a needle, 0.8 mm diameter. When droplets were dropped into the cationic solution constantly magnetically stirred, beads were immediately formed. Consequently, two different shrimp enzymes