Int.J.Curr.Microbiol.App.Sci (2018) *7*(12): 2053-2074

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 12 (2018)**
Journal homepage: http://www.ijcmas.com

Review Article https://doi.org/10.20546/ijcmas.2018.712.236

An Evidential Review on Potential Benefits of Enzymes in Aqua Feed Industry

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Keywords

Enzyme, Fish meal, Phytase, Protease, Xylanase, Plant based ingredients

Article Info

Accepted:
15 November 2018
Available Online:
10 December 2018

A B S T R A C T

Enzymes are basically a type of protein in biological systems. They are generally used as catalysts in order to catalyse the rate of reaction. Feeding these enzymes in the aquaculture sector has some nutritional advances. Application of enzymes reducing the effects of anti-nutritional factors, improves the dietary energy resulting in better performance of fish/shrimps. Feed enzymes in the form of granules help enzymes to stay for longer time durations and are suitable for pelletisation process. Efficiency of feeds needs to be at maximum for economical operations. There are various kinds of enzymes which include phytase, xylanase, cellulase, lipase, protease, amylase and many more which can increase the nutrient availability, nutrient absorption during digestion, increase the rate of fish growth and assist survival of fish in early stages of life. In addition, it makes the feeds more economical. Enzyme application may give a solution of high larval mortality of aquatic animals. Feeding larvae with enzymes would be beneficial. Enzymes play a significant role in formulating cost effective, high quality and eco-friendly aqua feeds. At present, the use of enzymes in aqua feeds can reduce use of fishmeal which ultimately reduces the cost of fish production. This may help to reduce the demand for fishmeal from the aquaculture sector in coming years.

Introduction

The United Nations Food and Agriculture Organization (FAO) projected that world population will increase from the current 7.5 billion to 9.1 billion by 2050 (FAO, 2009). A significant increase in food production will be required to feed this population growth and the FAO in its report on "How to feed the world in 2050" estimated that food production in developing countries will need to double (FAO, 2009). Human diets are also shifting to more meat and dairy foods. However, the FAO data showed that world per capita meat consumption is increasing only for chicken and fish. As the conversion of feed to edible meat from fish is the most efficient for all animals farmed for meat, aquaculture is potentially the most viable source of future protein to meet global needs.

Economical fish and shrimp production requires maximum nutritional efficiency from feed. The main issue in aquaculture revolves

around fish meal reduction in feeds and fish oil substitution in high energy diets. By 2030, aquaculture production will contribute 62% or 93.6 million tonnes to global seafood production (The World Bank, 2013). The pattern of FM use has shifted nearly exclusively to aqua feed production from livestock (Hardy 2010). Aquaculture consumed 3.72 million tonnes or 60.8% of total FM produced (Tacon *et al.,* 2011) and 0.78 million tonnes (73.8%) of global fish oil (FO) in 2008 (FAO 2012), at the expense of the livestock sectors which have continued to reduce their usage of these marine commodities. By 2012, aquaculture's fish meal consumption rose to 68% while FO usage remained the same (74%) (Tacon and Metian, 2015). Despite efforts to improve fish meal availability and quality, global fish meal production has remained static (5 – 7 million tonnes) year over year due to fully/over-exploited fisheries while the production of cereal grains and oil seeds are trending upwards at 2.9 billion and 574.1 million tonnes respectively (USDA 2015). Further growth in the aquaculture production can therefore not depend on an increase in the catch volume of wild fish, but must rely on a further increase in the use of alternative feed resources. The main source of plant based protein aquatic feed includes soybean meal, corn gluten meal, sunflower meal, canola/rapeseed meal, peas and lupins. Soybean meal having highest proportion of plant protein in fish diets owing to high yield, relatively high crude protein content and easy and round the year availability. Nutritionists are investigating the ways of utilising proteins of plants origin, since they are cheaper, readily available, and easily accessible than animal protein sources. Plant ingredients have so far been the most cost efficient alternative, and cite an example, feeds for Norwegian farmed salmon have changed from a marine based diet (90 % marine ingredients) to a plant based diet (30 % marine ingredients) (Ytrestoyl *et al.,* 2014). The major high protein plant ingredients in Norwegian salmon diets are soy protein concentrate (24 %) and wheat/wheat gluten (17 %) (Ytrestoyl *et al.,* 2014), but increased use of other plant ingredients have to be considered for further growth in the aquaculture production.

Challenges with plant-based ingredients

The most important challenges with plant products as protein sources in feeds for fishes particularly for carnivorous fish are: low level of protein, low digestibility, high level of carbohydrates, adverse amino acid profile, other nutrients and the presence of anti nutritional factors (Gatlin *et al.,* 2007; Sorensen *et al.,* 2010). Poor amino acid composition and unfair nutrient composition can be balanced by combining ingredients of different origin and use of additives such as amino acids, vitamins and minerals. (Sorensen *et al.,* 2010).

Lower digestibility

Nutrient digestibility of plant-based ingredients is a critical component in determining the potential of raw feedstuffs for inclusion in fish feed. Digestibility refers the amount of the nutrients/energy in the ingested feed that is not excreted by the animal (NRC 2011). It is essential for optimising inclusion levels and minimising resource waste.

Compared to FM, plant-based ingredients have relatively lower digestibility. This is due to structural components (cellulose, hemicellulose *etc.*) and metabolites (ANFs) which interfere with the animal's digestive metabolism, lowering dietary nutrients absorption. Consequently, the nutritive value of a feedstuff also includes its nutrient and energy bioavailability (Altan and Korkut, 2011).

Anti-nutritional Factors (ANFs)

Plants commonly synthesize metabolites of low and high molecular weight called antinutritional factors as a defence mechanism against herbivores (Khokar and Apenten, 2003). ANFs are classified as endogenous compounds found in all plant-based ingredients which may negatively influence feed intake, nutrient digestibility and utilisation, growth, affect the function of internal organs and alter disease resistance (Krogdahl *et al.*, 2010). They include, but are not limited to, phytases, Protease Inhibitors (PIs), Non-Starch Polysaccharides (NSPs) (cellulose and hemicellulose), saponins, tannins, haemagglutinins or lectins, gossypols and cyanogenic glycosides (Soetan and Oyewole, 2009). The structure and chemical composition, specifically heat-sensitivity, of ANFs can determine which physical or chemical processes may be effective in reducing their biological effects in animals (Khokar and Apenten, 2003). ANFs can be removed or inactivated by selective breeding, genetic modification, heat treatment or extraction (extrusion, pelleting, alcohol extraction), or through supplementation (enzyme, mineral, *etc*.) (Krogdahl *et al.*, 2010) (Table 1).

Phytate binds naturally occurring plant P making it unavailable to monogastrics and impairs mineral absorption; NSP (soluble and insoluble) interferes with digestive processes limiting nutrient uptake while PIs depress the digestion of protein, hindering amino acid absorption (Krogdahl *et al.*, 2010).

Phytate-phosphorus

Phytate is the primary storage form of P in many plants accounting for 0.4 – 6.4% by weight and 60 – 90% of total P (Khokar and Apenten, 2003). Phytate consists of an inositol group, hexahydrocyclohexane in a chair configuration with six phosphate ester bonds (Haros *et al.*, 2005; Kumar *et al.*, 2012).

Phytate can strongly chelate with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts. This adversely affects the absorption and digestion of these minerals in fish (Papatryphon *et al.*, 1999). Around 50% to 80% of the total phosphorous content in plant seeds is stored in the form of phytate (Ravindran *et al.*, 1995). Phosphorus in this form is generally not bioavailable to monogastric animals (human, dogs, pigs, birds) and also to agastric animals because they lack the intestinal digestive enzyme, phytase, required to separate phosphorous from the phytate molecule (Jackson et al..1996). As a consequence of low digestibility of phytate by fish, most of the phytate-P ends up being excreted into the water and may cause algal bloom pollution (Baruah *et al.*, 2004). Moreover phytate can also integrate with cation groups on protein, amino acids, starch and lipids in feedstuff reducing the digestibility of these nutrients in fish, poultry and pig. Phytate depresses protein and amino acid digestibility and utilisation efficiency in fish and other higher animals. The concentration of phytate and phytase in the feedstuffs varies considerably. Phytate constitute between 0.7% and 2% of most cereal grains and oilseeds (Adeola and Sands, 2003). In general plant derived fish feed ingredients such as soybean meal, rapeseed meal, and sesame meal contain 1.0-1.5%, 5.0–7.5% and 2.4% phytate respectively (Francis *et al.*, 2001) (Fig. 1).

Non-starch polysaccharides

Dietary fibre is the portion of plant nutrient containing lignin and polysaccharides (cellulose and hemicellulose) (McDonald *et al.*, 2002; NRC, 2011). NSPs are, hemicellulose, a complex group of polysaccharides (with the exception of starch)

containing several hundred linked monomers of hexoses and pentoses (Sinha *et al.,* 2011). The main constituents are rhamnose, arabinose, xylose, glucose, galactose, mannose, glucuronic acid and galacturonic acid. Arabinoxylans (the arabinose and xylose fractions) make up 60 – 70% of the endosperm wall in most cereals with the exception of rice and barley where the percentages are 40% and 20% respectively.

Soybean meal the most highly utilised plant-based ingredient, contains significant amounts of NSPs (Ogunkoya *et al.,* 2006). Raw soya beans contain approximately 200 g kg^{-1} NSP (Refstie and Svihus, 1999) and cereals 100 – 200 g kg^{-1} of NSPs in soluble and insoluble forms (Castanon *et al.,* 1997). RB contains approximately 20 – 25% NSP which consist of equal portions of cellulose and arabinoxylans (Choct, 1997). Arabinoxylans are also the major NSP in maize (NRC, 2011) (Fig. 2).

Unlike the structure of starch, NSPs are composed of different monomers linked by β-glycosidic bonds. The digestion of starch is facilitated by α-amylase, α-glucosidase and oligo-1,6-glucosidase, specialized enzymes for hydrolysing α-glycosidic bonds (Sinha *et al.,* 2011).

In herbivores and some omnivores, the activities of these enzymes range from high to medium, negating the need for exogenous additives. Monogastrics, however, do not produce enzymes such as –xylanase or –glucanase that can hydrolyse the bonds found in NSPs (Sinha *et al.,* 2011).

Protease Inhibitors (PIs)

One of the main limitations of using high inclusions of plant-based feedstuff is their comparatively low quality protein content (López *et al.,* 1999). The presence of PIs reduce the activities of proteolytic digestive enzymes (*i.e.* protease). Proteases are enzymes that catalyse the hydrolytic cleavage of specific peptide bonds in their target proteins (Habib and Fazili, 2007). PIs are therefore proteins that form complexes with specific proteases (e.g. trypsin, chymotrypsin, etc) and suppress their activity along the GI trace (Krogdahl *et al.,* 2010). In essence Protease Inhibitors are natural anti-metabolic proteins which interfere with the digestive processes and protein utilization, similar to the effects seen with phytate (Alarcon *et al.,* 1999).

Protease Inhibitors are found in nearly all plants accounting for 1-10% of total protein and are abundant in storage organs such as seeds and tubers (Wait *et al.,* 2009). PIs represent 6% of the protein present in soybean and despite the efficiency of processing, residual levels may remain (Mikie *et al.,* 2009). Although some PIs are heat-labile and can be eliminated using thermal treatments (i.e. pelleting), some researches argue that technological treatments do not always guarantee elimination of trypsin inhibitor (a type of serine protease inhibitor) in feeds (Lopez *et al.,* 1999). However, other studies have confirmed that heat treatment typically used in the extrusion process (>120^{o} C) for fish feed may be sufficient to inactivate most of the trypsin inhibitor activity in untreated SBM (Romarheim *et al.,* 2005)

Enzymes

Enzymes are basically a type of protein in biological systems. They are generally used as catalysts in order to catalyze the rate of reaction. Enzymes catalyze the reaction to convert complex substances into absorbable substances. The catalysis reaction is very specific to substrates. Feeding these enzymes in the aquaculture sector has some nutritional advances. Since last few years and will also aid in reducing the effects of anti-nutritional factors, improve the dietary energy resulting in better performance of fish/shrimps.

Sources of enzymes

Enzymes are produced in all the living organisms right from simple unicellular organisms to complex higher forms of life. Various microorganisms are involved in enzyme production including bacteria from *Bacillus* group, fungus from *Aspergillus* groups and yeasts. There are few microbes in the digestive tract of animals which are potent in production of proteolytic enzymes and cellulose. Incorporation of live microbes in feed can produce enzymes. Microbial fermentation technique is widely used in large scale commercial applications.

Feed enzymes

Stability of enzymes is important in order to incorporate them in feed. Heat stability is an important parameter to be considered. Feed enzymes in the form of granules help enzymes to stay for longer time durations and are suitable for pelletization process. Efficiency of feeds needs to be at maximum for economical operations. There are various kinds of enzymes which include phytase, xylanase, cellulase, lipase, protease, amylase and many more which can increase the nutrient availability, nutrient absorption during digestion, increase the rate of fish growth and assist survival of fish in early stages of life. In addition, it makes the feeds more economical. Enzyme application may give a solution of high larval mortality of aquatic animals. Feeding larvae with enzymes would be beneficial.

Advantages of feed enzymes

Aid in improvement of digestion and absorption of nutrients such as fat and proteins

Improves metabolizable energy of diet

Lead to increased feed intake, gain in weight

Improves digestibility of nutrients

Reduces production of ammonia

Phytases

Phosphatases are a diverse group of enzymes that catalyse the hydrolysis of phosphomonoester bonds of various phosphate esters. Phytases are a sub-group of phosphatases with specificity for hydrolysing phytate into phosphoric acid and myo-inositol phosphate (Haros *et al.*, 2005), with complete hydrolysis yielding one molecule of inositol and six molecules of inorganic phosphate (Makhode, 2008). This action reduces the chelation capacity of phytate (Kumar *et al.*, 2012).

Phytase activity was first detected many decades ago in rice bran (Suzuki *et al.*, 1997). Warden and Schaible (1962) are the earlier to verify that exogenous phytase improve phytate-P use and bone mineralization in poultry. However, before 1990s, the application of phytase has mainly been confined to poultry and swine to improve utilization of plant P. Initial commercial phytase, Natuphos was created from *Aspergillus niger* and was released in market in 1991 (Selle and Ravindran, 2007).

Following the prologue of commercial phytase, more emphasis were given to evaluating the effects of supplemental phytase on nutrient utilization and growth of common aquaculture species such as rainbow trout. (Forster *et al.*, 1999), common carp (*Cyprinus carpio* L.) (Schaffer *et al.*, 1995), channel catfish *(Ictalurus punctatus*) Li and Robinson, 1997), African catfish (*Clarias gariepinus*) (Van Weerd *et al.*, 1999). Atlantic salmon (*Salmo salar*) (Storebakken *et al.*, 2000), stripped bass (*Morone saxatilis*) (Papatryphon *et al.*, 1999), and Nile tilapia (*Oreochromis niloticus*) (Liebert and Portz, 2005).

Phytase application in aquaculture

Enhancement in phosphorus bioavailability

Various scientists around the world reported a positive effect of phytase supplementation on total P availability in fish. The following table 3 shows that the bioavailability of P when phytase is added in the feed ingredients for different fishes.

It was seen that exogenous phytase was substantially efficient in enhancing the bioavailability of P and thus reducing the amount of faecal-P. Supplementation of phytase in fish feed reduces the phosphate load in water from fish and ultimately prevents phosphate induced algal bloom contamination. Any reduction in P excreted by fish and other animals is of benefit to both the environment and sustainable production.

Enhancement of bioavailability of other nutrients and minerals

The concentration of minerals in plasma, bone and whole body will be increased by the addition of phytase in fish feeds (Jackson *et al.,* 1996; Van Weerd *et al.,* 1999; Papatryphon and Soares, 2001; Debnath *et al.,* 2005; Liebert and Portz, 2005). Supplementation of phytase at a level of 1000 FTU/kg diet was sufficient to enhance Ca, Mg and Mn content of bone in channel catfish, and addition of phytase at a level of 8000 FTU/kg feed significantly increased the bioavailability of naturally occurring Zn from feed (Yan and Reigh, 2002). Phytase supplementation in rainbow trout increased the apparent absorption of Ca, Mg, Cu, Fe, Sr and Zn in low-ash soybean meal diet (Sugiura *et al.,* 2001). Baruah *et al.,* (2005) conducted an experiment on rohu fingerlings and found that Phytase-supplemented groups in general recorded significantly ($p < 0.05$) higher percentage of bone ash and also higher concentration of bone Ca and P compared with the non-supplemented group. These results were similar to those observed for rohu (Baruah *et al.,* 2005), common carp (Schafer *et al.,* 1995), and other fish species (Storebakken *et al.,* 1998; Papatryphon *et al.,* 1999; Yan and Reigh, 2002; Debnath *et al.,* 2005b; Liebert and Portz, 2005). From these studies it can be concluded that bone ash and bone P are sensitive indicators of the P status in fish. This is because the P requirement for maximum bone mineralization is greater than maximum body weight gain. Insufficient P intake leads to the mobilization of P from the bone and transfer to soft tissues and metabolic processes (Baeverfjord *et al.,* 1998). Phytase supplementation results increment in bone ash in fish feed that is an indication of the increased mineral bioavailability in fishes (Baruah *et al.,* 2005; Debnath *et al.,* 2005).

Phytase supplementation also enhances digestibility of minerals which are bound to phytate. Addition of phytase in a semi-purified diet containing 50% soybean meal in rainbow trout significantly improved the apparent digestibility of Zn (Cheng *et al.,* 2004). Moreover, dietary phytase have been shown to increase the apparent availability of protein, ash, Ca, Cu, Mg, Fe, Sr and Zn in low ash diets while little effect in high ash diets (Sugiura *et al.,* 2001).

Cheng and Hardy (2004) reported that graded level of phytase inclusion in the rainbow trout diet did not affect body composition; whereas, it was effective in releasing most minerals and trace mineral. This result showed that supplementation of trace minerals in rainbow trout diets can be reduced when phytase is added in the diet. Schafer *et al.,* (1995) observed that P excretion was lower by 30% on feeding a diet supplemented with phytase compared to a diet supplemented with mono calcium phosphate.

Enhancement of protein and amino acid digestibility

Nonselectively phytase binds with proteins and inhibits the activities of pepsin, trypsin and alpha-amylase (Liener, 1994) as well as to decrease protein digestibility. De-phytinization of dietary phytate by exogenous phytase accounts for increased protein utilisation in common carp (Schafer *et al.*, 1995), Atlantic salmon (Storebakken *et al.*, 1998; Sugiura *et al.*, 1998), Seabass (Oliva-Teles *et al.*, 1998), Tilapia (Heindl, 2002) and pangus (Debnath *et al.*, 2005b) by corrupting the pre-formed phytate–protein complex. Forster *et al.*, (1999) assessed the potential of using dietary phytase to improve the nutritive value of canola protein concentrate diets for rainbow trout. Similarly, chemical and enzymatic processing of canola meal efficiently lowered most of the anti-nutritional factors in rainbow trout. The digestibility and nutritional value of expeller and solvent-extracted Australian canola meals when included in the diets of juvenile red seabream (*Pagrus auratus*) was comparable to those of the fishmeal (Glencross *et al.*, 2004). 6.6% phytase supplementation of 500 FTU/kg diet improves digestibility of crude protein in Crucian carp (Lie *et al.*, 1999).

Phytase supplementation in expelled soybean diet of rainbow trout increased ADC of amino acid significantly compared to raw soybean but had no significant effect when added in extruded soyabean (Cheng and Hardy, 2003). Spraying soybean meal-based diets with phytase improves protein digestibilities in rainbow trout (Vielma *et al.*, 2004). Phytase supplemented diet in pangus increased apparent net protein utilisation (Debnath *et al.*, 2005) and apparent protein digestibility and were significantly ($p<0.01$) higher at a minimum supplement of 500 FTU/kg or higher in contrast to diet without phytase. There is discrepancy among authors for the positive impact of phytase on protein and amino acid bioavailability. Research conducted on rainbow trout by Predergast *et al.*, (1994) and Teskeredzic *et al.*, (1995) showed that pre-treatment of rapeseed protein concentrate with the enzyme phytase did not improve the protein utilisation by rainbow trout. Similarly, no positive effect of phytase on protein digestibility could be noted in rainbow trout (Lanari *et al.*, 1998), Atlantic salmon (Storebakken *et al.*, 1998) and striped bass (Papatryphon *et al.*, 1999)

Similarly Riche *et al.*, (2001) reported that Nile tilapia offered diet with and without phytase showed no difference in protein utilisation, and also concluded that the available methionine and lysine decreased with increasing incorporation of phytase pre-treated soybean meal. Phytase addition in poultry, pigs and swine diets also showed conflicting results as observed for fish. The probable reason for the neutral and/or negative interaction of phytase and amino acids is that removal of phytate may increase the efficiency of other anti-nutritional factors and protect amino acids from degradation, or decrease leaching of water soluble components (Cao *et al.*, 2007). More research is needed to obtain a better insight into the mechanisms for the phytase-protein interaction and availability of proteins and amino acids.

Enhancement of growth performance

Supplementation of phytase-containing diets neutralises the negative effects of phytate and increases growth in fish. Positive impact of phytase on growth of fish has been reported by a number of authors: Jackson *et al.*, (1996) in channel catfish, Vanweerd *et al.*, (1999) in African catfish, Papatryphon and Soares (2001) in striped seabass, Vielma *et al.*, (2000) in rainbow trout, Liebert and Portz (2005) in tilapia, Debnath *et al.*, (2005) in pangus. Nwanna *et al.*, (2005), in common carp and Baruah *et al.*, (2007a) in rohu. These authors have demonstrated phytase hydrolysis in

plant-based diets by phytase and improvement of fish growth and mineralization. Diet containing 250 FTU phytase per kg increases the feed intake and increases the weight than the control diet containing no phytase (Li and Robinson, 1997). Increase in weight gain from 243 to 459% in rainbow trout fed soybean meal-based diets with phytase and phosphorous supplementation (Vielma *et al.,* 2004). Similar results were reports in salmonids (Sugiura *et al.,* 2001). Nwanna and Schwarz (2007), Nwanna *et al.,* (2007) found better growth observed in common carp fed a diet (incubated plant feed ingredients) containing phytase than another diet (without incubated plant feed ingredients) with and without phytase. This is probable because incubation process reduce phytate content of feed improve phosphorous and mineral usage as compared to untreated diet. The optimal growth of Nile Tilapia is achieved by phytase supplementation at 750-1250 FTU/kg in plant-based diets (Liebert and Portz, 2005). Addition of phytase at 1500 FTU/kg diet in contrast to no inclusion of phytase enhanced the weight gain of rainbow trout (Vielma *et al.,* 2001). No considerable effect of phytase supplementation was noticed on performance of large sized rainbow trout fed diet supplemented with phytase at 1000 FTU/kg (Vielma *et al.,* 2000) (Fig. 3).

No effect on growth performance, protein digestibility, energy retention on phytase addition in the diet of sea bass (Olivia-Teles *et al.,* 1998). Forster *et al.,* (1999) and Sajjadi and Carter (2004) did not report any improvement in the growth of rainbow trout and Atlantic salmon when fed with canola protein concentrate incorporated with phytase. Similarly Masumoto *et al.,* (2001) and Yoo *et al.,* (2005) reported no effect of dietary phytase on weight gain of Japanese flounder and Korean rockfish (*Sebastrers schlegeli*). The discrepancy in above findings may be associated with differences in their diet composition and also with different rearing conditions, (Baruah *et al.,* 2007). Supplementing exogenous microbial phytase in feed ration exhort an enhancement in growth rate and performance which could be attributed to various factors, in individual and combine form namely better bio-availability of phosphorous (Rodehutscord and Pfeffer 1995; Vielma *et al.,* 2000; Baruah *et al.,* 2007) and minerals (Vielma *et al.,* 2004; Debnath *et al.,* 2005b), improved protein digestibility (Vielma *et al.,* 2004; Debnath *et al.,* 2005a; Liebert and Portz 2005; Baruah *et al.,* 2007a) and increased absorption of nutrients owing to well functioning of the pyloric caeca region of the intestine (NRC, 1993).

Reduction in pollution from aquaculture operation

Discharge of high levels of soluble P from fish culture systems into open water environment stimulate phytoplankton growth, resulting in wide fluctuations in dissolved oxygen concentrations (Li *et al.,* 2004). Many studies have reported a clear effect of phytase supplementation in reducing P excretion from fish. Total phosphorous effluent was significantly lowered when fish cultured with a diet enriched with phytase (200 FTU/kg) (Ai *et al.,* 2007). Similarly, soybean meal based diets supplemented with phytase decreased the excretion of phosphorous from red sea bream and maximum reduction was reported at 2000 FTU/kg feed (Biswas *et al.,* 2007b). Comparable results were observed in rainbow trout (Sugiura *et al.,* 2001). Faecal waste of P in rainbow trout was reduced by phytase supplementation in soybean protein concentrate diet (Vielma *et al.,* 1998) and a significant decrease was noticed when practical feed supplemented with phytase at a level of 2000 FTU/kg was fed (Vielma *et al.,* 2001). Phosphorus concentration in faecal matter was reduced when trout were fed a diet with phytase supplemented at 500 and 1000 FTU/kg compared to non-supplemented feed (Verlhac *et al.,* 2007). Soybean based phytase

supplemented diet considerably lower excretion of phosphorous compared to the fishmeal diet fed to Atlantic salmon (Storebakken *et al.,* 2000). Phosphorus content of faeces was also reduced in Atlantic salmon fed a phytase supplemented diet (Sajjadi and Carter, 2004). Microbial phytase supplementation in the diets of juvenile catfish reduced the excretion of faecal phosphorous about 60% (Li and Robinson, 1997). Many studies suggest potential environmental benefits to the extent of 30% to 40% reduction in P excretion (Omogbenigun *et al.,* 2003)

NSP-enzymes

A greater concern is the high content of indigestible carbohydrates such as non-starch polysaccharides (NSP) which dilute the dietary energy and protein concentration and reduce feed digestibility, content of anti-nutritional factors that affects fish health, nutrient utilization and growth, and reduced digestibility/bioavailability of nutrients due to extensive processing (Stone, 2003; Sørensen *et al.,* 2010). Therefore, processing are used to increase the protein content and reduce the level of NSP in plant ingredients used in feeds for carnivorous fish like Atlantic salmon (*Salmo salar*). Genetical selection and optimization of growing conditions can also be used to optimize nutrient content of plants.

Non-starch polysaccharides (NSPs) can be water soluble or insoluble. Soluble NSPs such as arabinoxylans swell and form viscous gels when hydrated in the intestine, thus preventing secreted enzymes from reaching digestible substrates, and impeding digested nutrients from migrating to the gut wall for absorption. Insoluble NSPs such as cellulose and lignin induce a "cage" effect, and nutrients are trapped within the folds of the NSP molecules. Ronozyme®WX (xylanase) works to reduce the viscosity of NSP gels, and breaks down insoluble NSPs as well as improving assimilation of digested peptides and fats.

Numerous studies have however recently shown beneficial effect from hydrolysed products from NSP, so called prebiotics, on fish growth and health (reviewed by Ringø *et al.,* (2010)). These could either be included in the feeds as prebiotics or indirectly given to the fish by adding exogenous enzymes in fish diets that hydrolyse NSP (Stone, 2003; Sinha *et al.,* 2011). In recent times, many researchers focussing on NSP enzymes in fish feeds. These have been studied and utilised in swine and poultry industry for several time (Khattak *et al.,* 2006). NSP-enzymes include glucanases, pentosanases, cellulosases and xylanases. These enzymes hydrolyze NSP to products available for bacteria as prebiotics or for the fish as digestible nutrients (Sinha *et al.,* 2011). Supplementations of these have also shown to improve protein utilization and growth in fish (Ai *et al.,* 2007; Jiang *et al.,* 2014) (Table 4).

Xylanase is a class of enzymes that degrades linear polysaccharides, and breaks drown hemicelluloses that are the major component of the cell wall from plant (Ganguly *et al.,* 2013). This enzyme have proven to be especially efficient in maize-soy-based diet to broilers where the enzyme disrupts the plant cell wall that allows water hydration and entering of endogenous enzyme to act for a better digestion of starch and proteins (Sinha *et al.,* 2011). Xylanases are naturally produced in numerous yeasts, fungi and bacteria (Goswami and Pathak, 2003). Ronozyme®WX (1000 U xylanase/g) from DSM Nutritional Products (Switzerland) has been used in several fish experiments. Ai *et al.,* (2007) showed that Japanese seabass (*Lateolabrax japonicas*) at 6 g fed a diet of plant protein as soybean meal (170 g/kg), rapeseed meal (100 g/kg) and peanut meal (100 g/kg), improved growth and protein utilization, by inclusion of 800 mg/kg diet of Ronozyme®WX. There are also available commercial enzyme complexes where xylanase is present in combination with other

enzymes like proteases and NSP enzymes. These were tested in several fish studies with variable results. Tilapia fed diets with soybean meal (170 g/kg), rapeseed meal (170 g/kg) and cottonseed meal showed improved growth, feed conversion and endogenous enzyme activities with increased inclusion (0, 1 and 1.5 g/kg) of a commercial enzyme complex (Yingheng Biotechnology, China) with xylanase (1600 U/g), protease and β-glucanase (Lin *et al.,* 2007). The ingredients was mixed and cold pelleted through an experimental feed mill. Shahsavai (2011) showed that common carp (30 – 50 g) fed diets with wheat bran (340 g/kg), soybean meal (150 g/kg) and cottonseed meal (140 g/kg) supplemented with 1, 2 and 3 g/kg diet of an enzyme complex (Endofeed W, GNC Bioferm, Canada), with xylanase (≥1200 IU/g), β-glucanase, cellulase and hemicellulase had no effect on feed conversion and growth. Farmazyme® (Famavet, Turkey) a multi enzyme complex containing fungal xylanase, glucanase and other enzymes have shown to improve growth and protein content in 46 g Africian Catfish (*Claris gariepinus)* (Yildirin and Turan 2010). The enzyme complex was mixed with water and a pulverized trout diet at 0, 0.25, 0.5 and 0.75 g/kg diet, and grinded with a 2 mm die plate. Growth and protein content was significantly improved at level of the enzyme complex above 0.5 mg/kg diet. As mention earlier, however, some of these herbivorous freshwater species have naturally occurring enzyme producing yeasts in their gut, which improve the carbohydrate digestibility. Therefore, supplementation of enzymes may perhaps have larger effects on carnivorous fish species.

Proteases

Digestibility of protein and amino acids in alternative ingredients of plant and animal origin can be improved by adding protease enzyme to feeds. ProAct protease (DSM, Switzerland) is at the moment the best solution for improving protein digestibility available to the feed industry. Experiments using an *in vitro* poultry gut model show significant improvements in ingredient digestibility when ProAct is provided on top of endogenous digestive enzymes, and results are not expected to be different with fish. The adoption of protease by the aquafeed industry is just beginning, so there is not much information available on the benefits of protease.

However, Dalsgaard *et al.*, (2012) were able to show a significant improvement in apparent digestibility of soy (34% inclusion level in the feed) and a significant decrease in solid N waste excretion when protease alone or protease combined with xylanase was added to rainbow trout feed. Plant ingredients such as soy, rapeseed and canola contain trypsin inhibitors that stop trypsin from cutting protein into peptides before further digestion by other proteases in the intestine.

ProAct has been shown to digest trypsin inhibitor proteins, thus improving digestive function; It is less specific in selecting active sites on proteins for digestion than trypsin, hence it actually accelerates the initial stages of protein breakdown. In an experiment with tilapia with three different protein levels and three different dosages of ProAct enzyme (Verlhac and Diaz, 2012), apparent protein digestibility was improved from 2–4% in a 31% crude protein (CP) diet, and from 3-8% for 28 and 26% CP diets (Table 2), suggesting that in feeds with lower quality protein the benefit of using protease may be greater Protease, then, has a lot of potential to improve digestibility of all types of protein ingredients, and will assist nutritionists in formulating feeds that are more digestible and less polluting, while at the same time offering the possibility of choosing less expensive ingredients to control formulation costs.

Table.1 Processing steps for removal/inactivation of ANFs (Nwanna 2007)

Anti-Nutrient	Heat Sensitivity	Extraction	Other Treatment
Phytic Acid	No	No	Phytase
Arabinoxylans (NSP)	No	No	Xylanase
Protease inhibitors	Yes	No	Protease
Hemagglutinin	Yes	No	No
Saponin	No	Yes	No
Phytoestrogen	No	Yes	No

Table.2 List of anti-nutrients in plant sources

Antinutrients	Chemical name	Plant source	Source
Phytic acid *or* Phytate-P	Myoinositol 1,2,3,4,5,6-hexakisdihydrogen phosphate	Cereal and legumes	(Khokar and Apenten, 2003)
Non-Starch Polysaccharides	*e.g.* Arabinoxylans (arabinose and xylose)	Cereals (wheat, rye, barley, rice, sorghum)	(Sinha *et al.*, 2011)
Protease Inhibitors	*e.g.* Trypsin inhibitor	Most plants particularly legumes and cereals	(Francis *et al.*, 2001; Krogdahl *et al.*, 2010)

Table.3 Total P and phytate-P in common plant-based ingredients. Source: Kumar *et al.*, (2012) and Ravindran *et al.*, (1994)

Ingredients	Total P **(g kg-1)**	Phytate-P **(g kg-1)**	Proportion of Phytate-P in Total P (%)
Maize	2.40	2.05	85.4
Corn	2.50	1.70	73.0
Rice	1.20	0.80	65.0
RB	17.51	15.83	90.2
Soya bean	5.55	3.08	55.5
SBM	6.66	4.53	68.3
Cassava	1.60	0.40	25.0

Table.4 NSP comparison of major plant-based ingredients (in g kg-1), NRC (2011)

Ingredients	Total NSP	Arabinoxylans[a]	Other fractions [b]
Rice Bran	218	85	133
Corn	81	52	29
Maize	97	52	45
Soya beans	192	47	145
SBM	196	42	154

Table.5 Commercially available microbial phytases (Sources: Hou, 2001: Srefan *et al.*, 2005; Cao *et al.*, 2007)

Company	Country	Phytase Source	Production Strain	Trademark
AB Enzymes	Germany	*Aspergillus awamari*	*Trichoderma reesei*	Finase
Aiko Biotechnology	Finland	*A.oryzoe*	*A.oryzoe*	SP, TP, SF
Alltech	USA	*A.niger*	*A.niger*	Allzyme Phytase
BASF	Germany	*A.niger*	*A.niger*	Natuphos
Biozyme	USA	*A.oryzoe*	*A.oryzoe*	AMAFERM
DSM	USA	*P.Lyci*	*A.oryzoe*	Bio-feed phytase
Fermic	Mexico	*A.oryzoe*	*A.oryzoe*	Phyzme
Finnfeeds international	Finland	*A.awamari*	*T.reesei*	Avizyme
Genencor International	USA	*Penicillium simplicissimum*	*Penicillium funiculosum*	Rovabio
Roal	Finland	*A. Awamari*	*T.reesei*	Finase
Novozymes	Denmark	*A.oryzoe*	*A.oryzoe*	Ronozyme

Table.6

References	Fish Feed ingredients (plant protein sources	P availability (%) without phytase	Phytase dose FTU Kg diet	P availability (%) with phytase
Rainbow trout (*Oncorhynchus mykiss*)				
Riche and Brown (1996)	Canola meal, solvent extracted soybean meal, full fat soybean, peanut meal, corn gluten meal, cotton seed meal, canola meal, Barley, wheat	4.8;(-13.4)8.4; 22.1; 30.7; NA	3.8 x 10^6	46.2; 46.6; 64.4; 75.6; 76.8; 56.3
Cherg and Hardy (2002)	Canola meal, Barley, Wheat	12.2; 79.4; 61.6	500	41.8; 82.7; 64.6
Cherg and Hardy (2003)	Raw soybean, expelled soybean; Extruded full fat soybean	21.2; NA; 12.5	750; 200; (200, 400, 600, 800, 1000)	NA; 31.7; (81.3. 92.2, 89.7, 95.2, 93.9,)
Vielma *et al.*, (2006)	Rapeseed meal, soybean meal; corn gluten meal, sunflower meal;	(-1.0); 48.3; 61; (-0.9); 45.0; 65.2	750	53.8; 85.2, 118; 45.7, 72; 846
Verlac *et al.*, (2007)	Soy protein concentrate; Pea meal, Faba bean meal	29.9; 74.1; 47.8	750	46.9; 80.3; 69.9
Nile Tillapia (*Oreochromis niloticus*)				
Verilac *et al.*, (2007)	Soybean meal. Palm kernel cake, rice bran, corn, cassava	47.9, 25.5; 35.,; 23.6; 72.4	750	76.9; 50.4; 59.5; 58.3; 92.6
Sea bass (*Dicentrarchus labrax*)				
Papatryphon and Soares (2001)	Isolated soy protein; soybean meal, corn gluten mean, wheat middings	48; 59; 52; (-10)	1000	74; 87; 70; 11

Fig.1 Chemical structure of phytate-phosphorus showing its chair-like conformation. Source: Adeola and Sands, (2003)

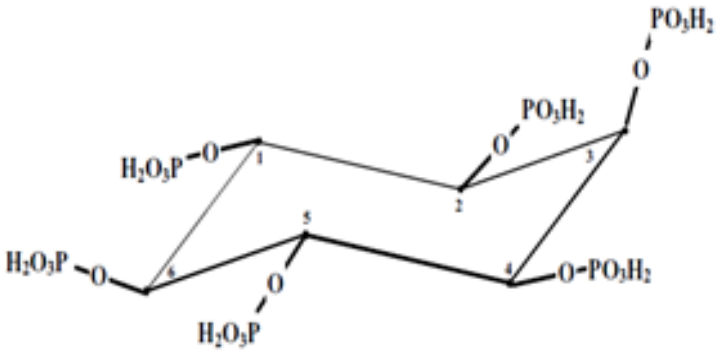


Fig.2 Chemical structure of arabinoxylan [Source: Sinha *et al.*, (2011)]

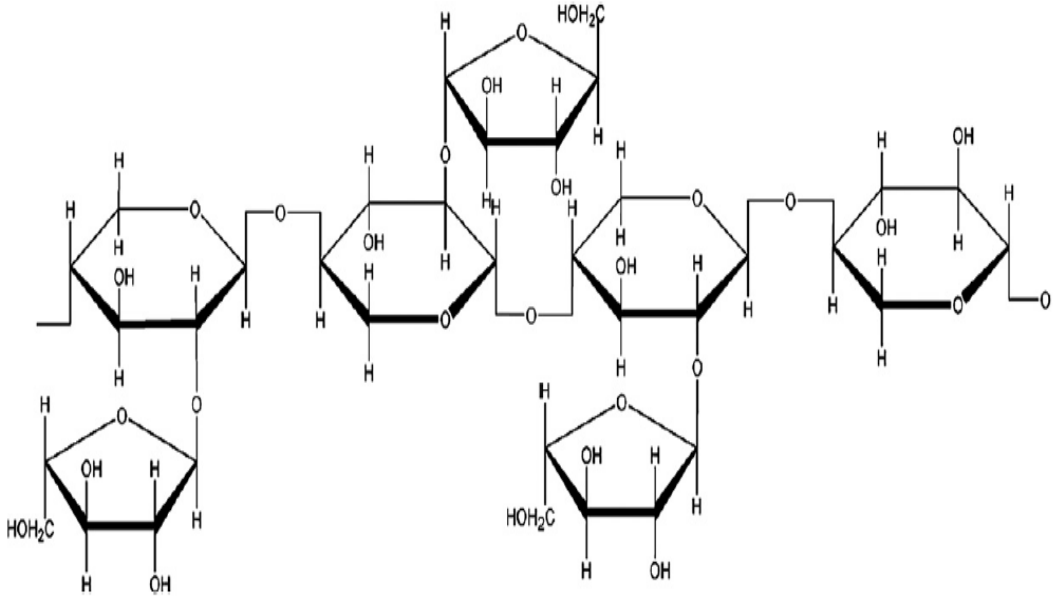
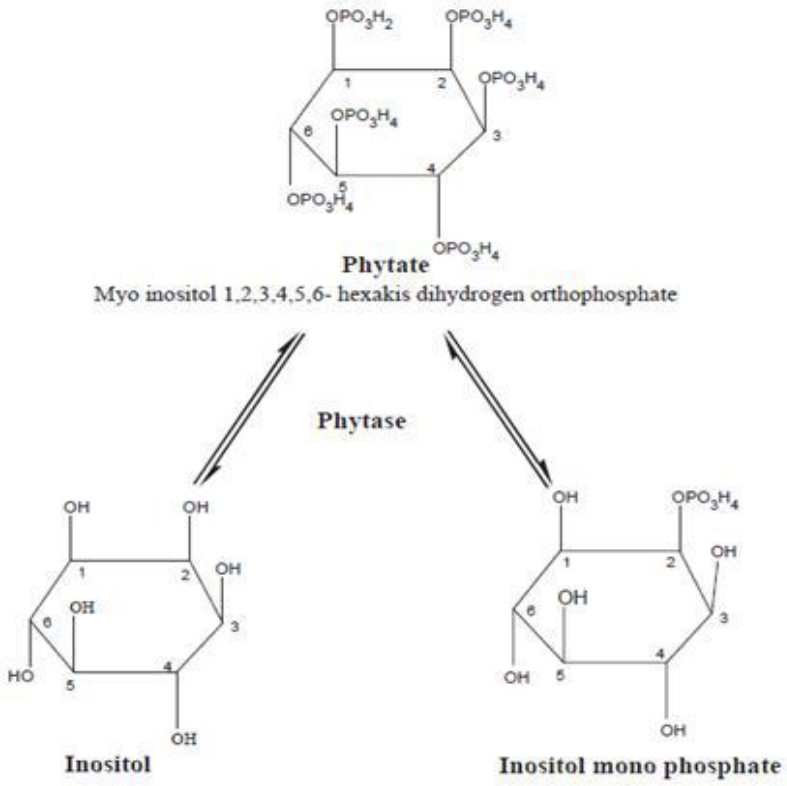


Fig.3 Hydrolysis of phytate by phytase. Source: Kumar *et al.*, (2012)



Benefits of combining enzymes

The cooperativity of enzymes to degrade feedstuff and their interactions require much research. The benefits of combining phytases and xylanases have been demonstrated to some extent in broilers (Bedford, 2000). Several enzyme companies (Novozyme/Royal DSM, Altech, Ameco-Bio & Cp., Canada Bio-Systems Inc etc.) are now producing enzyme cocktails to improve, even further, the efficiencies of feed utilisation, particularly those with high inclusions of plant-based ingredients, and the synergistic benefits for animal performances. Combining enzymes may provide additional benefits, in that, different enzymes act in different location along the GI tract and target different substrates (Walk, 2009).

Considerable effects of multi-enzyme supplementation on ADC of DM, CP, nitrogen free extract (NFE), P and GE in SBM-based diets fed to rainbow trout (Ogunkoya *et al.,* 2006). Using a similar commercial enzyme complex, higher FI was recorded with tilapia fed diets containing 0.15 g kg^{-1} but no difference were observed in protein, lipid and GE ADCs between treatments (0, 0.15 and 1.0 g kg^{-1}) (Lin *et al.,* 2007). Khalafalla *et al.,* (2010) also showed the addition of Amecozyme in diets at 0.5% and 1.0% enhanced the growth performance of *O. niloticus* fingerlings. Similarly, a cocktail containing protease, xylanase, glucanse, lipase, amylase and cellulase was used to supplement five grain diets fed to tilapia which improved fish performance, nutrient digestibility, carcass characteristics and faecal recovery (Soltan, 2009) (Table 5).

Economic benefits of supplementation

The use of enzymes must sufficiently demonstrate substantial improvements in feed conversion or product quality to cover any adjustments in formula cost resulting in higher profit margin (Chesson, 1993). In other words, they must somehow improve upon least-cost formulation by lowering input cost while maximizing outputs in terms of animal performance, health and cost to produce one unit of animal protein. Economic benefits of using phytase are by far more straight forward than those of xylanases and proteases. Phytase delivers direct cost benefit by replacing the need for inorganic phosphate (Bedford, 2000). The benefits of reducing P load and feed formulation cost are clear, and as a result phytase is now considered a standard feed additive. Though most enzyme studies acknowledge supplementation-related formula cost savings, rarely are these figures published for reference (Table 6).

Enzyme research for the future

With the increasing use of more plant ingredients such as rice bran, wheat bran, copra meal, and palm oil milling byproducts in aqua feeds, there is merit in improving digestion of plant cell walls to unlock valuable nutrients trapped inside cells. Cell walls of cereals (wheat, corn, barley, rice) are mainly made of arabinoxylans and β-glucans, whereas oilseed crops (soy, canola, rapeseed, sunflower) are mainly xyloglucans and pectins. Feed enzymes that digest cellulose, xylans, glucans, mannans and pectins are now widely used in livestock and poultry feeds, but have yet to be applied to aqua feeds. Adding phytase and xylanase together with protease improves protein utilization the most, with phytase reducing phytic acid-protein interactions, and xylanase improving protein, peptide and amino acid migration in the intestine in feeds containing large quantities of NSPs. Research is focussed to investigate combination of enzymes to further improve the feed efficiency of fishes.

In conclusion, flourishing and sustainable aquaculture depends on efficiently viable and environmentally responsible aqua feeds. Feed

is the major working cost involved in intensive farming of aquatic organisms. The major feed ingredient, fishmeal, is expensive and there is increasing competition with other livestock industries for the available supply. Hence, researchers are focussing to find alternatives to fishmeal. Substitution of fishmeal with plant proteins supplemented with feed enzymes is an effective alternative in aqua feeds. Enzymes play a significant role in formulating cost effective, high quality and eco-friendly aqua feeds. At present, the use of enzymes in aqua feeds can reduce use of fishmeal which ultimately reduces the cost fish production. This may help to reduce the demand for fishmeal from the aquaculture sector in coming years.

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How to cite this article:

Felix, N., E. Prabu, B. Kannan and Manikandan, K. 2018. Need of Enzymes in Aqua Feeds. *Int.J.Curr.Microbiol.App.Sci.* 7(12): 2053-2074. doi: https://doi.org/10.20546/ijcmas.2018.712.236