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Phytase production from fish viscera and used as probable aqua feed ingredient

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Abstract
Aquaculture pollution is a major concern among the entrepreneurs, farmers and researchers. Excess discharge of phosphorus and nitrogen into the water bodies is the principal pollutant responsible for this. Plant-based feed ingredients due to its high phytic acid content enhances both nitrogen and phosphorus discharge thereby increasing the pollution level. Dietary phytase treatment is probably the best answer to address this problem. Mozambique Tilapia (*Tilapia mossambica*) viscera were used for phytase extraction. Visceral extracts were prepared using water, KCl solution and EDTA homogenization. The extract with the highest phytase specific activities of 46.35 and 66.54 IU/mg protein, respectively. The enzyme was active at the optimum pH (7.0) and temperature (45°C) when sodium phytate was used as a substrate. The enzyme showed highest activity and purification when precipitated at 60-80% ammonium sulphate effectively improved specific activity of enzyme. Thus, the results revealed that active phytase enzymes could be prepared from tilapia viscera and could be attributed to better bio-availability of phosphorus and other minerals to fishes and improved protein digestibility in fishes. With the introduction of phytase in fish feed, fish nutritionists can formulate and produce a cheaper plant-based aqua feed. This will ultimately lead to profit maximization of aquaculture enterprises.

Keywords: phytase, aqua feed, fish nutrition, enzyme purification, tilapia

1. Introduction
The environmental impact of aquaculture activities is increasing and ecologists as well as governments are putting restrictions on this industry. Both marine and freshwater aquafarmers are facing increasing pressure concerning the discharges into the surrounding ecosystems. Aquaculture pollution occurs because of these discharges in general or because of loading of phosphorus into the environment in particular.

Phosphorus is a key mineral for marine organisms. Fish can absorb soluble phosphorus through the skin, fins, and gills; however, the concentration of phosphorus in fresh water and seawater is low (NRC, 1993) [11]. Therefore, the phosphorus requirement for fish is dependent on feed. It is also important to mention that phosphorus is a critical pollutant in the aquatic environment. Excessive phosphorus concentration is the most common cause of eutrophication of aquatic systems (Correll, 1999) [2].

Phytate is the primary storage form of phosphate in plants. Monogastric animals such as pigs, poultry and fishes utilize phytate phosphorus poorly because they are deficient in gastrointestinal tract- phytases and therefore an inorganic, non-renewable and expensive mineral supplement is used in diets for swine, poultry and fishes to meet their nutritional requirement of phosphorus. The unutilized phytate phosphorus from plant feeds is excreted as an environmental pollutant in areas of intensive live-stock production (LA Lawton *et al*, 1991) [7]. Excessive phosphorus in soil runs off to lakes and the sea, causing eutrophication and also stimulating growth of planktonic vegetation such as algal blooms and aquatic blooms (RA Vollenweider, 1971 and LC Bowling *et al*, 1996) [12, 8] that may produce neurotoxins, injurious to human beings (L Pizzolon, 1996 and LA Lawton *et al*, 1991) [6, 7]. Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated myo-inositol derivatives in the intestine of the monogastric animals is desirable. The different biotechnological aspects for lowering the phytic acid level in the common aqua feed ingredients, as an alternate approach to controlling the pollution level. Attempts to enzymatically hydrolyse phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals (JE Hill *et al*, 2007 and L Pizzolon, 1996) [5, 6].

Phytase may provide a remarkable platform for sustainable and eco-friendly farming. Phytase (myo-inositol hexaphosphate hydrolase) are phosphatase, enzymes that sequentially cleave orthophosphate groups from the inositol ring of phytic acid to yield available free inorganic phosphorus, a series of lower phosphoric esters (inositol pentaphosphate to inositol monophosphate) as intermediates, thereby decreasing phytates' affinity for different cations (Lie *et al*, 1993) [9]. The reaction ultimately leads to the production of free myoinositol (Harland and Morris, 1995) [3]. Phytases have a profound role in animal feed and various food industries due to the non-availability of phosphorus to the animals present in the feed, along with improving the digestion and absorption of the phosphorus and certain other poorly available nutrients. The environmental benefits of using phytase enzyme are: (1) less mineral supplementation is required and therefore less inorganic phosphate is required in the diet; (2) less organic phosphate (phytic acid) is excreted and thereby less phosphate loads into the environment in intensive aquaculture settings.

However, with the introduction of phytase, fish nutritionists can formulate and produce a cheaper plant-based aqua feed. This will ultimately lead to profit maximization of aquaculture enterprises.

Hence, the objectives of the study were to prepare the crude phytase extract from visceral organ wastes from tilapia and to evaluate properties of this enzyme. Positive effects of dietary phytase on the growth of fishes could be attributed to better bio-availability of phosphorus and other minerals, and improved protein digestibility. This research was carried out as a contribution to the utilization of fish by-products for producing enzymes and also to the reduction of waste disposal problems.

2. Material and Methods

Fish viscera

Experimental fish in the present study were Tilapia. They were taken from unpolluted private fish farm located in Ras Al Khaimah, UAE. The initial body length and weight of fish were (13-17.5 cm) and (69-83 g), respectively. All Tilapia (four) were transported in plastic containers with continuous aeration to the lab. Fishes were dissected. Viscera were removed and weighed, then stored in sealed plastic bags at -36°C until used for enzyme extraction (Saif Al Ghais *et al*, 2019) [13].

Preparation of Homogenate of Viscera

Digestive tracts (viscera) were partially thawed. 10% Sample Solution (viscera) was prepared by using tissue homogenizer (Tissue homogenizer, REMI RQ-127A, India) mixed with 0.1 M Tris HCL buffer with EDTA (pH 7.4). Pipetted 0.75 ml of viscera solution (10%) made final volume of sample up to 1.0 ml with water. Add 0.25 ml of 20% v/v Trichloroacetic acid and sample kept for incubation in refrigerator about 45 min, after incubation add 0.8 ml of water centrifuged at 2000 rpm for 20 min at room temperature. In the supernatant add 0.3 ml of 2M Tris base, and 0.1 ml of 0.01 M DTNB (5, 5 dithio bis 2-dinitrobenzoic acid, CAS No: 422592J, VWR UK). Absorbance measured at 412 nm after 10 min by spectrophotometer (UV spectrophotometer, GENWAY 7315, UK) (Saif Al Ghais *et al*, 2019) [13].

Phytase Assay

Quantitative phytase assay of the crude enzyme was done according to Yanke *et al*. (1999) using sodium phytate as the substrate. The colour that developed due to phytase activity was determined with a spectrophotometer (UV spectrophotometer, GENWAY 7315, UK) at 700 nm. One International unit (IU) of phytase activity is expressed as 1 µmol phosphate liberated per mL per min (µmol mL^{-1} min^{-1}).

Protein content

The protein content of the crude enzyme extract was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard (Saif Al Ghais *et al* 2018 and 2019) [14].

Partial Purification

The crude extract was fractionated by using ammonium sulphate at saturation level of 0-20%, 20-40%, 40-60%, 60-80% and 0-80%. Protein concentration of each fraction was determined by Lowry method using bovine serum albumin (BSA) solution as a standard (Vibha Bhardwaj *et al*, 2014) [16].

Enzyme kinetic method (Stability of crude enzyme extract)

pH optima

The pH optimum of the phytase enzyme was determined by preparing the substrate in various buffer solutions (0.2 M HCl–KCl buffer of pH 2.0, 0.2 M citrate phosphate buffer of pH 3–7 and 0.2 M Tris–HCl buffer of pH 7–12) and applying the enzyme extract to the substrate to assay the enzyme activity.

pH stability

The influence of pH on the stability of the phytase was determined by pre-incubating the enzyme in the above mentioned buffer solutions for 30 min at room temperature (25±1°C) then determined the remaining activity.

Temperature optima

The influence of temperature on the activity of phytase was determined at various temperature intervals (25-60°C).

Thermostability

The enzyme solution was incubated at various temperatures (25-60°C) for 3 hrs. Samples were removed at intervals of 30 min and residual activities of phytase was examined.

Statistical analysis

Experimental error was determined for triplicate assays and expressed as standard deviation (SD).

3. Results and Discussion

In the present investigation the viscera of Tilapia fish were used for phytase enzyme extraction and characterization. The weight of fish and viscera was taken (Table 1).

Table 1: Weight of fish and viscera

S. No.	Sample	Weight of fish(g)	Weight of viscera(g)
1	TF1	72	2.538
2	TF2	83	1.675
3	TF3	69	1.001
4	TF4	77	2.612

The purification steps, protein concentration, specific activity and yield of phytase are shown in Table. The specific activity and purification fold were 66.54 IU/mg protein and 1.44, respectively, when 60-80% ammonium sulphate used (Table 2).

Table 2: Purification steps of phytase from viscera of Tilapia

Purification step	Total phytase units (IU)	Protein(mg)	Specific activity(IU/mg)	Purification fold	% yield
Crude enzyme	10800	233	46.35	1	100
60-80% Ammonium sulphate fractionation	8650	130	66.54	1.44	69.66

Enzyme kinetics

pH optima

The partially purified phytase had the highest activity at pH 7.0 and it then decreased with increasing of pH (Fig 1.). Over pH 9.0, more than 50% of the relative activity was lost at pH 12. There was near complete loss of phytase activity at pH 12.0

This result was very close to those reported for phytase, the

optimum pH for the was found to be pH 8.0 at 35°C (Argha *et al*., 2013) [1].

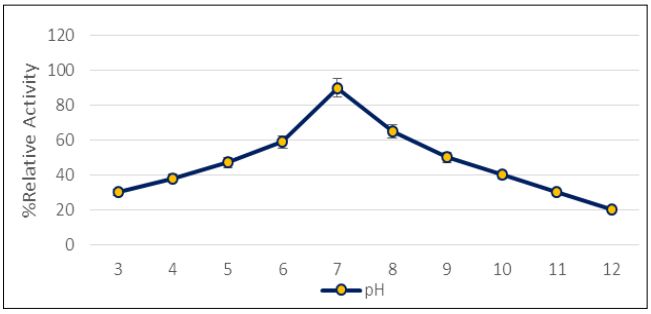

Fig 1: pH Optima of Phytase

pH stability

Fig 2. Illustrates pH stability of the phytase. The phytase retained more than 90% of its original activity in the pH range 5.0-7.0 and then decreased with increasing pH and reached its lowest relative activity at pH 12. These data clearly indicate that the phytase was most stable in the pH range 5.0–7.0 and least stable within the pH range 9.0–12.0.

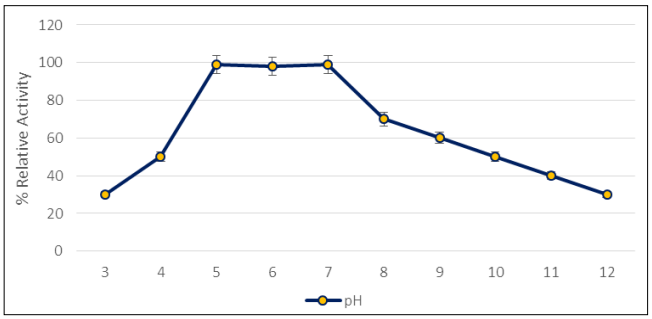

Fig 2: pH Stability of phytase

Temperature optima

The temperature stability profile of phytase activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 37°C to 50 °C (Fig 3) with highest activity at 45°C (Fig 3) incubation temperature for 1h. The relative activity increased with increasing the temperature from 25°C to 45°C and then decreased; however very less activity was detected at 60°C. Generally, these are similar with those reported by Iti Gontia-Mishra *et al*. (2013).

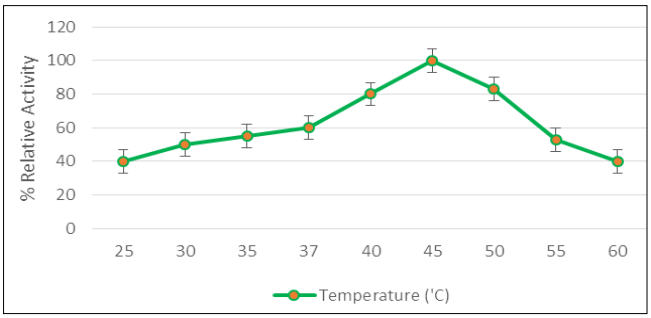

Fig 3: Temperature Optima of Phytase

Thermostability

Thermostability of the protease is shown in Fig 4. The phytase retained more than 50% of its activity after heating at 37°C and 45°C for 60 min, it lost 20% after heating at the same temperature for 120 min. A further increase in the reaction temperature caused significant drop in the protease activity. These results are in similar with those reported by Iti Gontia-Mishra *et al*. (2013).

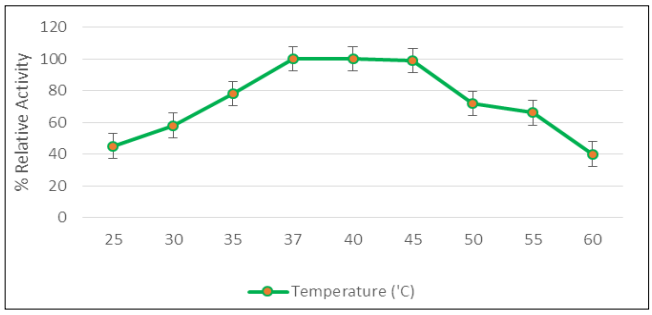

Fig 4: Temperature Stability of phytase

4. Conclusion

Expanded aquaculture production will require more fish feed, which will in turn require higher quantities of alternate protein sources to substitute fishmeal. Phytases are being recognized for their beneficial environmental role in reducing the phosphorus levels in manure and minimizing the need to supplement phosphorus in diets. Increasing the use of phytase in aquaculture offers a tremendous opportunity in order to allow the use of low-cost plant meals. From our experience, it seems that use of phytase in herbivorous fish diet is very encouraging. However, studies are needed to establish optimal dose of phytase for several other species of fish with varying feeding habits and different life stages.

In conclusion, the present study shows an efficient process of producing Phytase, which has wide applications prospects in animal feed, fish feed and agriculture. Our studies obtained high yields of Phytase from viscera of Tilapia fish. The Phytase from Viscera was found to be stable over wide range of temperature and pH and has thus shown the necessary potential for use as animal feed, fish feed supplement as well as on crops for field applications.

Conflicts of interest

The authors declare no conflict of interest pertaining to the research report in this manuscript.

Availability of data and materials

The relevant data and materials are available in the present study.

Competing interests

The authors declare that they have no competing interests. All procedures followed were in accordance with the ethical standards (institutional and national). All institutional and national guidelines for the care and use of laboratory animals were followed.

Authors' contributions

SAG supervised the entire project. VB performed all the experiments. The supervision of the laboratory work was performed by VB. OAS helped in fish dissection. VB analysed the data and wrote the manuscript. I would also like to thank Mr. Pramod in assistance.

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6. References

1. Argha Khan, Koushik Ghosh. Evaluation of Phytase Production by Fish Gut Bacterium, *Bacillus subtilis*, for Processing of *Ipomea aquatica* Leaves as Probable Aquafeed Ingredient Journal of Aquatic Food Product Technology. 2013; 22:508-519.
2. Correll DL. Phosphorus: a rate limiting nutrient in surface waters. Poult. Sci. 1999; 78:674-682.
3. Harland FB, Morris ER. Phytin: A good or a bad food component. Nutr. Res. 1995; 15:733-754.
4. Iti Gontia-M, Dhanshree D, Niraj T, Khushboo BB, Keerti T, Sharad T. Isolation, morphological and molecular characterization of phytate-hydrolysing fungi by 18S rDNA sequence analysis. Brazilian Journal of Microbiology. 2013; 44(1):317-323.
5. JE Hill, Kysela D, Elimelech M. Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVapeninsula. Environ. Microbiol. 2007; 9:3100-3107.
6. L Pizzolon. Importance of Cyanobacteria as potential factor of toxicity in continental waters. Interciencia. 1996; 21:239-245.
7. Lawton LA, Codd GA. Cyanobacterial (blue-green algal) toxins and their significance in UK and European waters. J Inst Water Environ Manag. 1991; 5:460-465.
8. Bowling LC, Baker PD. Major cyanbacterial bloom in the Barwon-Darling River, Australia, in 1991, and underlying limnological conditions. Mar Freshwat Res. 1996; 47:643-657.
9. Lei X, Pao KK, Miller ER, Ullrey DE, Yokoyama MT. Supplemental microbial phytase improves bioavailability of dietary zinc to weaning pigs. J. Nutr. 1993; 123:1117-1123.
10. Lowry OH, Rosenbrougj NJ, Farr AL, Randall RL. Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry. 1951; 193(1):265-275.
11. NRC (National Research Council). Nutrient Requirements of Fish. National Academy Press, Washington, DC, USA, 1993.
12. Vollenweider RA. Scientific fundamentals of eutrophication of lakes and flowing waters, with particular reference to nitrogen and phosphorus as factors in eutrophication. Organization for economic co-operation and development, Paris, 1971.
13. Saif Al Ghais, Vibha Bhardwaj. Utilization of fish viscera for protease production and used for digestion of waste in the pond. International Journal of Fisheries and Aquatic Studies. 2019; 7(3):112-115.
14. Saif Al Ghais, Vibha Bhardwaj, Pramod Kumbhar, Omar Al Shehhi. Effect of copper nanoparticles and organometallic compounds (dibutyltin) on tilapia fish, The Journal of Basic and Applied Zoology, Springer Nature. 2019; 80:32. https://doi.org/10.1186/s41936-019-0101-7.
15. Saif Al Ghais, Vibha Bhardwaj. Nannochloropsis Protein as Potential Fish Feed. International Journal of Science and Research (IJSR). 2018; 7(12):278-282.
16. Vibha Bhardwaj, Neelam Garg. Production, Purification of Pectinase from Bacillus sp. MBRL576 Isolate and its Application in Extraction of Juice. International Journal of Science and Research (IJSR). 2014; 3(6):648-652.
17. Yanke LJ, Selinger LB, Cheng KJ. Phytase activity of *Selenomonas ruminantium*: A preliminary characterization. Lett. Appl. Microbiol. 1999; 29:20-25.