The Efficacy of a Novel Microbial 6-Phytase Expressed in Aspergillus oryzae on the Performance and Phosphorus Utilization of Cold- and Warm-Water Fish: Rainbow Trout, Oncorhynchus mykiss, and Nile Tilapia, Oreochromis niloticus

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Abstract

The efficacy and tolerance of a novel microbial 6-phytase were investigated in rainbow trout, Oncorhynchus mykiss, and Nile tilapia, Oreochromis niloticus. Reference diets were sufficient in available phosphorus (P). The test diet limiting in available P was supplemented with phytase at 500, 1000, or 2000 phytase units/kg feed. The enzyme was effective in increasing total P apparent digestibility coefficient in relation to increasing the dose of phytase in rainbow trout and Nile tilapia. Zinc apparent digestibility improved in relation to phytase supplementation in rainbow trout. Prelease due to phytase supplementation ranged from 0.06 to 0.18% P/kg feed in rainbow trout and from 0.13 to 0.26% P/kg feed in Nile tilapia. A 58-d performance trial was conducted to evaluate tolerance of fish to phytase supplementation. Dietary treatments consisted of a basal diet without phytase or supplemented with 2000 and 200,000 phytase units/kg feed. Results indicate that this novel microbial 6-phytase is well tolerated by fish. Significant improvements for growth as well as feed conversion ratio were observed when the phytase was fed at 2000 phytase units/kg feed. This phytase is proven efficient in releasing P from phytate and could be added when plants are used for fish meal replacement in diets for salmonid and omnivorous fish.

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Approximately two thirds of the total phosphorus (P) in plants is in the form of phytate (Erdman 1979; Reddy et al. 1982) which is a salt of myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen-phosphate present in feedstuffs of plant origin where they serve as the principal storage form of P. Phytase is a naturally occurring enzyme that can degrade phytate to yield inositol and P (Liu et al. 1998). However, fish do not synthesize phytase. Thus, much of the phytate phosphorus present in the diet is eliminated in the excreta and represents a source of environmental pollution. Furthermore, phytic acid has strong chelating properties and can make up complexes with minerals, starch, and protein, thereby reducing their bioavailability. Singh and Krikorian (1982) also suggested that phytate may inhibit proteolysis by altering the protein configuration. Phytate is also known to inhibit a number of digestive enzymes such as pepsin, α-amylase (Deshpande and Cheryan 1984), and trypsin (Caldwell 1992). The inability of monogastric animals to efficiently utilize the phytate phosphorus necessitates the addition of inorganic phosphates (e.g., mono- or di-calcium phosphate) to the diets in order to meet their P requirement. Supplementation with microbial phytase allows almost complete, safe, and economic replacement of inorganic phosphate and reduces feed cost per kg feed.

The rationale for the use of phytase in aquafeeds is linked to the increasing use of plant-based feed ingredients as an alternative to fish meal protein source which is not only an expensive raw material but also a limited resource with an important ecological impact that will contribute to the development of a sustainable aquaculture production. Several studies, carried out to ascertain the efficacy of phytase supplementation on total P digestibility for various fish species, are reviewed in Kumar et al. (2012). In rainbow trout, Oncorhynchus mykiss, there is a positive response in growth due to increased total P availability when their diet is supplemented with phytase. Inclusion of phytase at 2000 phytase units/kg feed in comparison to non-supplemented diet improved P availability as indicated by higher P apparent digestibility, bone ash, plasma, and body P concentrations (Vielma et al. 1998). Indeed, the increase in bone ash and P in fish fed phytase-supplemented diets indicates an increase in mineral bioavailability by dietary manipulation, making it a sensitive indicator of the P status of the fish (Kumar et al. 2012).

In Atlantic salmon, Salmo salar L., fed diets based on canola meal, with and without phytase and inorganic P supplementation, P digestibility, and retention were significantly higher in fish fed supplemental phytase compared to fish fed supplemental inorganic P. Phytase increased P availability, therefore reducing the need to add inorganic P and reducing P waste for plant-meal-based diets (Sajjadi and Carter 2004). In another study, Denstadli et al. (2007) compared pre-treatment and in-feed application with phytase and found a positive effect of phytase with pre-treated raw material but not in the case of dietary phytase supplementation. A more recent study showed that juvenile Atlantic salmon fed a low fish meal diet with 60% soy protein concentrate had significantly improved growth when phytase was supplemented at 1000 phytase units/kg or more (Carter and Sajjadi 2011). P utilization was also significantly improved by dietary supplementation with phytase in sea bream, Sparus auratus, and sea bass, Dicentrarchus labrax (Oliva-Teles et al. 1998; Ai et al. 2007; Biswas et al. 2007).

In omnivorous fish, phytase supplementation has been proven efficacious in improving P digestibility in several species; among them are tilapia and catfish. However depending on the intestinal pH, phytase could be somewhat less efficacious in agastric fish like common carp, Cyprinus carpio (Cao et al. 2007). Phytase supplementation to a plant-based diet fed to Nile tilapia, Oreochromis niloticus, led to significant improvements in growth, feed conversion ratio (FCR), and specific growth rate (SGR). Nutrient utilization (energy, protein, and P) was also enhanced (Liebert and Portz 2005). Tudkaew et al. (2008) showed that dietary phytase supplementation led to growth improvement in sex-reversed red tilapia. Bone ash and bone P were higher in fish receiving inorganic P and/or phytase at 750 FYT phytase/kg feed (Tudkaew et al. 2008). Similarly, ash and...
P content in the Nile tilapia vertebra were significantly increased by supplementation of microbial phytase (Liebert and Portz 2005).

The preferred method for adding phytase into fish diets has been the addition of the liquid enzymatic form following extrusion and drying. This application procedure prevents activity loss during pellet formation. On-line spraying devices can be very effective and relatively simple to install on existing production lines. Moreover, post-extrusion liquid application allows great flexibility in formulation and the use of the enzyme. The vacuum coating process is routinely used in salmonid feed to allow coating of the oil onto the pellet. Through vacuum coating process, the enzyme and the oil are sucked into the pellet replacing the air in the space generated through gelatinization of starch through extrusion process. This process prevents any leakage of oil and enzyme.

Some microbial phytases have an optimum pH of 2.5–5.5. On the basis of the site of initial breakdown of the phytate phosphorus, microbial phytase are divided into two classes: the 3-phytases such as those from *Aspergillus niger*, initiate phytate degradation at the third carbon position and 6-phytases such as those from *Aspergillus oryzae* initiate phytate degradation from the sixth carbon position. Research efforts in recent years have focused on the isolation and development of new, heat stable microbial phytases from various microbial sources (Broz and Ward 2007; Selle and Ravindran 2007).

Toxicological and tolerance studies have been carried out to evaluate the safety of this microbial 6-phytase in living animals in line with the current European Union Regulation (EFSA 2003) on the additives use for animal nutrition. Toxicological assessment has been carried out in rats (Pariza and Cook 2009) and the product can be listed to be a Generally Recognized as Safe (GRAS) feed ingredient. The product was proven safe and efficacious in poultry (Aureli et al. 2011).

The objective of this work was to evaluate the efficacy of a microbial 6-phytase preparation expressed via the use of synthetic genes in *A. oryzae* known commercially as RONOZYME HiPhos, in cold- and warm-water fish.

**Materials and Methods**

A total of four studies were performed: a tolerance study and two digestibility studies in rainbow trout and a digestibility study in Nile tilapia. The determination of the optimum dosage of phytase was based on apparent digestibility coefficients (ADC) of total P measured by the indirect method with diets containing yttrium oxide (Y$_2$O$_3$) as inert tracer. The digestibility experiments were conducted using a basal diet supplemented with different dosages of phytase RONOZYME HiPhos (L) at 0, 500, 1000, and 2000 phytase units/kg feed and a control diet consisting of the basal diet supplemented with mono-calcium phosphate. The basal diets were formulated as a low-fish meal, practical type diet with several plant proteins as sources of phytic acid-bound P, so that, the phytate hydrolyzing role of supplementary phytase could compensate for the P marginal deficiency. In the tolerance study the effect of phytase on performance of rainbow trout was evaluated in fish fed a diet sufficient in P supplemented with 2000 and 200,000 phytase units/kg feed.

**Experimental Diets**

The product tested in the digestibility trials was a liquid product, RONOZYME HiPhos (L) tested at graded levels. In the tolerance study, RONOZYME HiPhos (L) was tested at 2000 phytase units/kg feed (PHY 2000) and a concentrate of the enzyme was used to evaluate the effect of the high dose of 200,000 phytase units/kg feed (PHY 200000).

In the rainbow trout efficacy study 1 (Trout 1), two basal diets (Table 1) were produced at the Fish Games and Fisheries Research Institute (FGFRI) in Finland: a control diet with supplemental inorganic P and an experimental diet without supplemental P but with graded levels of phytase added post-extrusion (0, 500, 1000, and 2000 phytase units/kg feed) and afterwards labeled as Control, PHY 500, PHY 1000, and PHY 2000. Y$_2$O$_3$ was used as an inert digestibility marker. Diets were extruded with a twin-screw extruder as 5 mm pellets. Phytase diluted in distilled water was sprayed onto pellets at 0.5% inclusion level. Thereafter fish
Table 1. Formulation of the experimental diets from the tolerance study (Tolerance) and the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Tolerance</th>
<th>Trout 1</th>
<th>Trout 2</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal diet</td>
<td>Ref</td>
<td>Test</td>
<td>Ref</td>
</tr>
<tr>
<td>Fish meal</td>
<td>330</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>–</td>
<td>80</td>
<td>80</td>
<td>–</td>
</tr>
<tr>
<td>Soybean protein concentrate</td>
<td>80</td>
<td>130</td>
<td>130</td>
<td>160</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>–</td>
<td>100</td>
<td>100</td>
<td>140</td>
</tr>
<tr>
<td>Pea protein concentrate</td>
<td>99</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>30</td>
<td>107</td>
<td>107</td>
<td>130</td>
</tr>
<tr>
<td>Wheat</td>
<td>150</td>
<td>105.6</td>
<td>119.6</td>
<td>85</td>
</tr>
<tr>
<td>Rice bran</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Corn</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Fish oil</td>
<td>210</td>
<td>230</td>
<td>230</td>
<td>205</td>
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<tr>
<td>Vitamin premix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Mineral premix&lt;sup&gt;b, c&lt;/sup&gt;</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>MCP/DCP</td>
<td>–/–</td>
<td>14/–</td>
<td>–/–</td>
<td>15/–</td>
</tr>
<tr>
<td>l-lysine/l-methionine</td>
<td>–/–</td>
<td>10/3</td>
<td>10/3</td>
<td>3/2</td>
</tr>
<tr>
<td>Guar gum</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Yttrium oxide</td>
<td>–</td>
<td>0.4</td>
<td>0.4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

MCP = mono-calcium phosphate; DCP = dicalcium phosphate; Ref = reference diet (positive control supplemented with MCP or DCP); Test = the basal diet used for control and phytase-supplemented treatments.

<sup>a</sup>Vitamins added to supply the following (per kg basal diet): retinol acetate, 8000 IU; cholecalciferol, 2000 IU; all-rac-a-tocopheryl acetate, 200 IU; menadione sodium bisulphite, 10 mg; thiamine.HCl, 10 mg; riboflavin, 20 mg; calcium D-pantothenate, 40 mg; biotin, 1 mg; folic acid, 6 mg; vitamin B<sub>12</sub>, 0.03 mg; niacin, 150 mg; pyridoxine.HCl, 15 mg; ascorbic acid (Stay C), 150 mg.

<sup>b</sup>Minerals added to supply the following (mg per kg basal diet) in digestibility trials: calcium, 1530; magnesium, 410; sodium, 1010; iron, 30; copper, 5; zinc, 50; manganese, 20; selenium, 0.2; iodine, 2; cobalt, 5.

<sup>c</sup>Minerals added to supply the following (mg per kg basal diet) in the tolerance study: calcium, 1460; phosphorus, 1080; magnesium, 410; sodium, 1010; iron, 30; copper, 5; zinc, 50; manganese, 20; selenium, 0.2; iodine, 2; cobalt, 5.

Oil was sprayed on top under vacuum in a Dinnissen (Sevenum, the Netherlands) vacuum coater.

For the other studies, diets were prepared on a Bühler twin-screw extruder at the experimental feed mill of the Research Centre for Animal Nutrition and Health of DSM Nutritional Products in Village-Neuf, France. The pellet density was adjusted in order that they sink slowly in the water column for trout studies while floating pellets were produced for the tilapia study. That guarantees that the fish have a good accessibility to the feed and that minimizes the loss of feed not eaten.

Protein and lipid levels of the diets followed those of typical commercial diets of similar size fish. The basal diet used in the tolerance study was formulated to be P sufficient (Table 1).

The products tested were added post-extrusion following the double coating method: the products were diluted in 2% demineralized water heated at 45 °C and sprayed onto the pellets kept at room temperature under a 300-mbar vacuum, using a Forberg vacuum coater F-60 RVC (Stoltz, Paris, France). In a second step, fish oil heated at 45 °C was added under vacuum at 450 mbars.

**Tolerance Study in Fish**

The experiment was conducted at the Research Centre for Animal Nutrition and Health of DSM Nutritional Products, Village-Neuf, France. All female rainbow trout of average weight 41.7 ± 1.1 g were randomly distributed into 15 250-L sub-square tanks in five replicates per
dietary treatment (35 fish per tank). The tanks are part of a recirculating unit supplied with ground water with partial renewal of water and temperature control. Mean water temperature was \(14.7 \pm 0.3\) °C and oxygen saturation was at 90% during the experimental feeding period. Ammonia, pH, nitrates, and nitrates levels were maintained within the recommended limits for the species. A 12:12 h light:dark cycle was applied during the trial.

Fish were fed manually twice a day (in the morning and in the afternoon) during the week and on belt-feeders during the weekends. Feed was placed on the belt-feeders to allow the distribution of the two meals at 4–6 h interval. All fish were fed according to commercial feeding ration tables of a comparable diet fed to fish maintained at 15°C and they received the same daily ration, which varied from 1.52% at the start of the experiment to 1.35% at the end of the experimental feeding period. The presence of waste feed was checked during the weekdays just after feeding. Fish were individually weighed at the beginning of the experiment and after 29 and 58 d of experimental feeding. At each time point, the following zootechnical parameters were measured and computed for each replicate tank: survival (%), growth, final body weight (g), weight gain (g), SGR (%/d), FCR. Macroscopic observation of the fish was performed at the end of the experimental feeding period to enable proper evaluation of the tolerance of the product by fish.

Macroscopic observation of the fish was performed by fish pathologists from the Centre for Fish and Wildlife Health (FIWI), Institute of Animal Pathology, University of Berne, Switzerland. Eight fish per tank were individually weighed and length was measured. Each fish was then examined externally for malformations and then dissected for observation of the following organs: global viscera, gut, liver, gallbladder, and spleen. Condition factor and hepato-somatic index were also determined and allowed, together with the external observation of the fish (skin, eye, fins), to determine the healthy condition as well as the good nutritional status of the fish. Proximate analyses of the experimental diets were performed at the Research Centre of Animal Nutrition and Health of DSM Nutritional Products, Village-Neuf (France) according to the methods described in the section “Chemical Analyses.”

**Efficacy Studies**

The rainbow trout efficacy study 1 was conducted at Laukaa Fish Farm, research facilities of FGFRI in Finland. Mixed-sex stock of rainbow trout initially weighing 670 g, were randomly allocated to 15 round fiber-glass tanks (1.5-m²) part of a recirculating system with three replicates per dietary treatment (20 fish per tank). Mean water temperature during the experimental feeding period was 14.9°C and oxygen saturation was at 85–90%. Ammonia, pH, nitrates, and nitrates levels were maintained within the recommended limits. Constant light was applied during the trial. Fish were fed according to commercial feeding table (1.0–1.2% body weight per day) for 6 h daily continuously with belt-feeders. Fecal samples were collected twice per diet by stripping. First sampling was carried out 21 d after the start of feeding the experimental diets. Fish were anesthetized in neutralized tricaine solution, and fecal matter from the last third between pelvic fins and anus was stripped into a container. Fecal matter stripped was freeze-dried. Dietary protein, lipid, ash, crude fiber, yttrium, and P analyses were carried out at Novalab Ltd. (Karkkila, Finland) and dietary energy contents were measured at FGFRI, according to methods described in the section “Chemical Analyses.”

The rainbow trout efficacy study 2 was conducted at the Research Centre for Animal Nutrition and Health of DSM Nutritional Products in Village-Neuf, France. All female rainbow trout of average weight 269 g were randomly distributed into 15 250-L sub-square tanks in three replicates per dietary treatment (19–20 fish per tank). The tanks are part of a recirculating unit supplied with ground water with partial renewal of water and temperature control. Water oxygen, temperature, and quality were regularly monitored. Mean water temperature was \(14.8 \pm 0.6\) °C during the experimental feeding period and oxygen saturation was at 90%. Ammonia, pH,
nitrites, and nitrates levels were maintained within the recommended limits for the species. A 12:12 h light : dark cycle was applied during the trial. Fish were acclimated to experimental conditions for 13 d, during which time all fish received the control-based diet without phytase supplementation. Fish were fed manually twice a day (in the morning and in the afternoon) during the week and on belt-feeders during the weekends. Feed was placed on the belt-feeders to allow the distribution of the two meals at 4–6-h interval. All fish were fed according to commercial feeding ration tables of a comparable diet fed to fish maintained at 15°C and they received the same daily ration, which varied from 1.5% body weight per day at the start of the experiment to 1.3% body weight per day at the end of the experimental feeding period. After 4 wk of feeding the experimental diets, feces were collected twice at 1-wk interval. Fish were lightly anesthetized (0.08 g/L tricaine methane sulfonate, MS222; PharmaQ Ltd., Overhalla, Norway) before handling. Feces from the two samplings were pooled by tank and stored at −18°C until freeze-drying and analysis. Proximate analysis of the feed samples was determined by DSM Nutritional Products. P and yttrium feed and fecal concentrations were also determined according to the methods described in the section “Chemical Analyses.”

The Nile tilapia efficacy study was conducted by Sparos at the experimental facilities of the University of Trás-os-Montes e Alto Douro (UTAD), Portugal. Nile tilapia of average body weight 133 g were randomly distributed into 60-L cylindro-conical tanks (13 fish per tank). Mean water temperature was 27 ± 1°C during the experimental feeding period and oxygen saturation was at 80–90%. Ammonia, pH, nitrites, and nitrates levels were maintained within the recommended limits for the species. A 12:12 h fluorescent light : dark cycle was adopted. Fish were adapted over 2 wk to rearing conditions and experimental diets. Fish were fed once a day, by hand in slight excess. Upon a thorough cleaning of the rearing tanks from any feed residues, feces were collected daily for the following 2 wk using the continuous outlet water filtration system (INRA system). After daily collection, feces were frozen at −20°C. Pooled feces from each group of fish were freeze-dried prior to analysis. Proximate analysis of the diets and feces were analyzed according to the procedures described in the section “Chemical Analyses,” as well as P and yttrium.

**Chemical Analyses**

Proximate analysis of the diets and feces was performed according to the following procedures: dry matter after drying at 105°C for 24 h, finely ground and reduced into ash by combustion at 550–600°C. Protein (N × 6.25) was calculated from total nitrogen determination using a LECO FP-428 nitrogen analyzer (LECO Instruments, Garges-les-Gonesse, France) or as Kjeldahl-nitrogen × 6.25. Gross energy was determined in an adiabatic bomb calorimeter (IKF, Staufen, Germany) and lipid content by gas chromatography (Büchi CPG, Büchi Sarl, Rungis, France) or by the Soxhlet method after petroleum ether extraction. For diet and fecal yttrium and P analyzes, samples were reduced to ash, digested in nitric acid according to AOAC (1995). All the samples derived from one study were analyzed using the same method. Samples from the tolerance study and the trout efficacy 2 were analyzed using the LECO nitrogen analyzer for the protein determination and the gas chromatography for lipid determination. The analyses performed in the rainbow trout efficacy 1 used Kjeldahl-nitrogen × 6.25 for protein and the Soxhlet method for lipid. The analyses performed in the Nile tilapia efficacy study used the LECO nitrogen analyzer for protein and the Soxhlet method for lipid determination.

Total P was determined by spectrophotometry according to the ISO/DIS 6491 method using the vanado-molybdate reagent, or quantified by ICP-AES. Y₂O₃ concentrations in feed and feces were determined using ICP-AES.

Phytates were separated by extraction and separation on an anion exchange column before ICP analysis of P (Harland and Oberleas 1986; Plaami and Kumpulainen 1991). Just after preparation of the diets, samples from each batch of feed from the various experiments were collected for determination of the phytase activity of the different dietary treatments.
Phytase activity of the experimental diets from the various studies presented in this article was measured at DSM Nutritional Products Analytical Research Centre (Kaiseraugst, Switzerland) according to the DSM Nutritional Products method PHY-101/05E, in accordance with the method ISO30024:2009 “Animal feeding stuffs – Determination of phytase activity.” One unit of phytase is defined as the amount of enzyme that releases 1 μmole inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and a temperature of 37°C.

**Determination of ADC and P Release**

ADC of dry matter and total P were calculated as a fractional net absorption of nutrients from diets based on Y$_2$O$_3$ as a non-absorbable indicator. ADC was calculated according to NRC (1993):

\[
\text{ADC of nutrient} = 100 - 100 \times \frac{\% \text{ Y in feed} \times \% \text{ nutrient in feces}}{\% \text{ Y in feces} \times \% \text{ nutrient in feed}}
\]

P release represents the % of P released due to phytase supplementation. It is calculated as follows: (% total P of the feed × P ADC at a phytase dose) – (% total P of the feed × P ADC of the test diet not supplemented with phytase).

P release is expressed in % P/kg feed for a phytase dose.

**Statistical Analyses**

Data of ADC are presented as mean of replicate tanks ± standard deviation. In the rainbow trout efficacy study 1, the effects of the dietary phytase concentration were studied by fitting linear models to the data. The full model included both linear and nonlinear effects. The second degree terms for the phytase effect were included to test nonlinearity of the response. The full model was as follows:

\[
\text{Yin} = p_0 + p_1 \text{P} + p_2 \text{P}^2 + \epsilon \text{in}
\]

where $p_0$, $p_1$, and $p_2$ are fitted model parameters, P is phytase concentration and $\epsilon$ is residual error. Models were compared at the significance level of 0.10 according to Akaike criteria by using the simplest possible model to adequately explain the relation between phytase and digestibility. In addition to regression analyses, pair wise differences were evaluated using ANOVA and Tukey’s test at the significance level of 0.05 (Systat 13). In the other studies, data were subjected to a one-way ANOVA followed by Newman–Keul’s multiple comparison tests (SPSS software from PASW Statistics 18 or StatBox Pro from Feedback & Co., Paris, France). Prior to ANOVA, ADC values were subjected to arcsine square root transformation. Statistical significance was tested at 0.05 probability level. Significant differences between dietary treatments are shown with different letters, for each parameter. In the case data were not distributed normally, a nonparametric Kruskal–Wallis test was performed followed by a t-test comparison of the data. In this case, statistical significance was tested at a probability level of 0.05/3 (3 = number of treatments).

**Results and Discussions**

**Experimental Diets**

Table 1 presents the formulation of the experimental diets manufactured for all trials and Table 2 presents the analyzed nutrient content of the experimental diets including dry matter, ash, protein, lipid, energy, total P, and phytate phosphorus contents as well as the analyzed Y$_2$O$_3$ content. Phytase activity of the different experimental diets is presented in Table 3. Results show a good recovery of the enzyme after post-extrusion application, irrespective of the dose.

Total P levels of the test diet averaged 0.66 and 0.68% in the non-supplemented diets and 1.06 and 1.1% in the reference diet supplemented with mono-calcium phosphate of the rainbow trout studies. The phytate phosphorus content of the test diet not supplemented with phytase and the reference diet supplemented with mono-calcium phosphate varies from 0.33 to 0.35%. In the Nile tilapia study, the total P averaged 0.99% in the test diet and 1.43% in the reference diet while phytate phosphorus in these diets reached 0.48%.
Table 2. Analyzed nutrient and yttrium content (% dry matter) of the experimental diets from the various studies from the tolerance study (Tolerance) and the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia).

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Tolerance</th>
<th>Trout 1</th>
<th>Trout 2</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal diet</td>
<td>Ref</td>
<td>Test</td>
<td>Ref</td>
</tr>
<tr>
<td>Dry matter</td>
<td>93.6</td>
<td>94.4</td>
<td>94.0</td>
<td>94.5</td>
</tr>
<tr>
<td>Ash</td>
<td>7.56</td>
<td>6.0</td>
<td>6.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Protein</td>
<td>46.5</td>
<td>38.1</td>
<td>38.3</td>
<td>40.4</td>
</tr>
<tr>
<td>Lipid</td>
<td>27.5</td>
<td>27.4</td>
<td>26.1</td>
<td>24.1</td>
</tr>
<tr>
<td>Gross energy, kJ/g dry matter</td>
<td>22.1</td>
<td>24.5</td>
<td>24.6</td>
<td>22.8</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>1.12</td>
<td>1.04</td>
<td>0.66</td>
<td>1.06</td>
</tr>
<tr>
<td>Yttrium oxide</td>
<td>ND</td>
<td>0.34</td>
<td>0.33</td>
<td>0.35</td>
</tr>
</tbody>
</table>

MCP = mono-calcium phosphate; DCP = dicalcium phosphate; Ref = reference diet (positive control supplemented with MCP or DCP); Test = the basal diet used for control and phytase-supplemented treatments.

Table 3. Phytase activity in phytase units per kg feed in the experimental diets from the tolerance study (Tolerance) and the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Tolerance</th>
<th>Trout 1</th>
<th>Trout 2</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
<tr>
<td>PHY 500</td>
<td>–</td>
<td>523 ± 22</td>
<td>517 ± 11</td>
<td>501 ± 1</td>
</tr>
<tr>
<td>PHY 1000</td>
<td>–</td>
<td>1,106 ± 26</td>
<td>1,038 ± 25</td>
<td>1,003 ± 39</td>
</tr>
<tr>
<td>PHY 2000</td>
<td>1,875 ± 8</td>
<td>2,393 ± 28</td>
<td>2,067 ± 33</td>
<td>1,914 ± 130</td>
</tr>
<tr>
<td>PHY 200000</td>
<td>189,934 ± 4902</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Reference diet</td>
<td>–</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
</tbody>
</table>

UDL = under detection limit.

Tolerance Study in Rainbow Trout

Table 4 and Figure 1 present data for survival, performance, and feed conversion ratio after 58 d of experimental feeding. Fish from the three experimental treatments tripled their initial body weight during the experimental feeding period of 58 d. Final body weight, weight gain, SGR, and feed conversion ratio were significantly improved when fish were fed the basal diet supplemented with RONOZYME HiPhos (L) at 2000 phytase units/kg feed in comparison to fish fed the basal diet not supplemented with phytase. Growth and feed efficiency parameters were also significantly improved when the recommended dose was increased by 100-folds, in comparison to rainbow trout fed the basal diet not supplemented with phytase. Responses were obtained after 29 d (data not shown) and at the end of the trial (58 d of experimental feeding).

Macrosopic observation of the fish was performed at the end of the feeding trial. Results show that in all groups fish were healthy and in good nutritional condition. The dietary treatments had no influence on condition factor (1.24 ± 0.02 for all treatments) and hepato-somatic index (1.05 ± 0.03 for all treatments). Any signs of disease or obvious lesions could be detected neither externally nor internally. Internally all fish presented with a high amount of perivisceral fat.

Efficacy Studies

Table 5 and Figure 2 present the ADC of total P from the three studies reported here and Table 6, the percent of P release per kg feed at each phytase dose.

In the rainbow trout efficacy study 1, the apparent digestibility of P was improved by phytase in a dose-dependent pattern. On the basis of Akaike information criteria, adequate fit was obtained by linear model for P. Using ANOVA approach, significant difference between 0 and 500 phytase units/kg was observed, whereas further improvements from 500 to 1000 phytase units/kg, and from 1000 to 2000 phytase units/kg were not different at the significance
Table 4. Survival, growth, and feed conversion ratio of rainbow trout after 58 d of experimental feeding in the tolerance study performed with rainbow trout.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>PHY 2000</th>
<th>PHY 200000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (%)</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>41.7 ± 0.1</td>
<td>41.7 ± 0.1</td>
<td>41.7 ± 0.1</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>136.1 ± 1.6\textsuperscript{c}</td>
<td>138.8 ± 0.5\textsuperscript{b}</td>
<td>143.7 ± 1.1\textsuperscript{a}</td>
</tr>
<tr>
<td>Specific growth rate (%BW/d)</td>
<td>2.04 ± 0.02\textsuperscript{e}</td>
<td>2.07 ± 0.01\textsuperscript{b}</td>
<td>2.13 ± 0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>0.71 ± 0.01\textsuperscript{a}</td>
<td>0.70 ± 0.00\textsuperscript{b}</td>
<td>0.67 ± 0.01\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values represent means ± SD of five replicates. Means in the same row with different superscript letters are significantly different (\(P < 0.05\)).

![Weight gain (g) of rainbow trout after 58 d of experimental feeding in the tolerance study performed with rainbow trout. Bars represent means ± SD of five replicates. Bars with different letters are significantly different (\(P < 0.05\)).](image_url)

level tested of \(P = 0.05\). Both 1000 and 2000 U phytase units/kg diet significantly improved digestibility in comparison to 0 phytase units/kg. The 2000 phytase units/kg dose significantly improved digestibility in comparison to 500 phytase units/kg. ADC of zinc also improved in a dose-dependent pattern with coefficients of 17.6% for the test diet not supplemented with phytase, and ranging from 24.3 to 40.5% with increasing phytase supplementation. The zinc ADC was of 25.3% in the reference diet. Pair wise post hoc comparison suggests that only the highest dose of 2000 phytase units/kg differs significantly from other phytase doses with a numerical improvement of 22.9% units.

The P content of feces decreased in relation with dietary phytase supplementation from 1.1% in the test diet not supplemented with phytase, to 0.7%, when dietary phytase supplementation reached 2000 phytase units/kg feed. The P level in the feces of fish fed the reference diet was 1.4%.

In the rainbow trout efficacy study 2, the ADC of total P (Table 5) in vegetable protein-based diet show that supplementing phytase significantly increased apparent digestibility of total P. RONOZYME HiPhos (L) at 500 phytase units/kg feed significantly enhanced the digestibility of P in comparison to the non-supplemented diet. Higher doses of phytase further increased the apparent digestibility of total P in rainbow trout. The P content of feces decreased in relation with dietary phytase supplementation from 1.7% in the test diet not supplemented with phytase to 1.1% when dietary phytase supplementation reached 2000 phytase units/kg. The P level in the feces of fish fed the reference diet was 2.0%.

Similar to our findings, several studies have demonstrated the potential of phytase to increase P digestibility in rainbow trout (Kumar et al. 2012). Other studies have shown a positive dose-dependent response to phytase, ranging from 400 to 4500 phytase units/kg feed on nutrient digestibility and retention (Sugiura et al. 2001; Vielma et al. 2004).

Our studies show that the recovery of the enzyme is good through post-extrusion application and that an effective dose of phytase in the range of 1000 phytase units/kg feed enables the reduction in inorganic phosphate supplementation. In a study with rainbow trout fed high plant protein based diets containing three graded levels of total P, phytase supplementation at 1400 phytase units/kg feed significantly improved the availability of total and phytate phosphorus (Dalsgaard et al. 2009). Both total- and phytate P waste output were...
Table 5. Apparent digestibility coefficients (ADC in %) of total phosphorus in the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Trout 1</th>
<th>Trout 2</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.3 ± 6.2c</td>
<td>26.1 ± 1.2c</td>
<td>36.6 ± 1.0d</td>
</tr>
<tr>
<td>PHY 500</td>
<td>60.2 ± 1.8b</td>
<td>40.9 ± 2.5b</td>
<td>50.4 ± 2.0f</td>
</tr>
<tr>
<td>PHY 1000</td>
<td>62.0 ± 4.3ab</td>
<td>44.5 ± 1.6b</td>
<td>56.0 ± 1.2b</td>
</tr>
<tr>
<td>PHY 2000</td>
<td>70.6 ± 2.5a</td>
<td>52.3 ± 2.1a</td>
<td>61.5 ± 2.3a</td>
</tr>
<tr>
<td>Reference diet</td>
<td>61.6 ± 1.6ab</td>
<td>43.6 ± 1.2b</td>
<td>57.8 ± 0.7b</td>
</tr>
</tbody>
</table>

Values represent means ± SD of three replicates. Means in the same column with different superscript letters are significantly different (P < 0.05).

Figure 2. Apparent digestibility coefficients (ADC in %) of total phosphorus in the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia). Bars represent means ± SD of three replicates. Bars in the same study with different letters are significantly different (P < 0.05).

Table 6. Phosphorus release from the experimental diets due to phytase supplementation expressed in % P per kg diet in the efficacy studies in rainbow trout (Trout 1 and 2) and Nile tilapia (Tilapia).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trout 1</th>
<th>Trout 2</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHY 500</td>
<td>0.07</td>
<td>0.09</td>
<td>0.13</td>
</tr>
<tr>
<td>PHY 1000</td>
<td>0.08</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>PHY 2000</td>
<td>0.13</td>
<td>0.18</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Regarding the effect of dietary phytase supplementation on performance of rainbow trout, results from a study performed by Vandenberg et al. (2012) the authors suggest that the microbial phytase tested at a dose of 3000 phytase units/kg feed can increase the apparent digestibility and bioavailability of a range of nutrients from plant protein-based diets for rainbow trout, leading to an enhanced performance of the fish as well as a better tissue mineralization. In this study data demonstrate the effect of the microbial 6-phytase on trout performance at a dietary supplementation of 3000 phytase units/kg feed in the context of a diet sufficient in P and containing both fish meal and vegetable protein sources. Our results confirm the potential of phytase to reduce the impact of negative effects of phytic acid. These results suggest that a dose of 1000 phytase units/kg feed should satisfy the P requirement of trout fed a plant-protein-based diet limiting in available P.

In the Nile tilapia efficacy study, P digestibility (Table 5) showed a wide variation, with the lowest values found in fish fed the control diet without DCP supplementation (36.6%) and the highest in fish fed the diet supplemented with 2000 phytase units/kg feed (61.5%). Significant enhancements of P digestibility were strongly related to increasing doses of phytase.
P digestibility in the test diet without phytase supplementation was 37% and was significantly increased to 50, 56, and 62%, with graded phytase supplementation levels of 500, 1000 and 2000 phytase units/kg feed, respectively. Diet with DCP supplementation showed a P ADC similar to that found in fish fed 1000 phytase units/kg diet. The P content of feces decreased in relation with dietary phytase supplementation from 2.2% in the test diet not supplemented with phytase to 1.4% when dietary phytase supplementation reached 2000 phytase units/kg. The P level in the feces of fish fed the reference diet was 2.1%. Similar to our findings with Nile tilapia, Furuya et al. (2001) observed that a microbial phytase supplementation between 500 and 1500 phytase units/kg improved calcium and P availability, performance, bone mineralization, and protein digestibility. Additionally, dietary phytase supplementation ranging 750–1000 phytase units/kg resulted in Nile tilapia growth rates and P utilization similar or superior to those obtained with a plant-based diet supplemented with inorganic P (Portz and Liebert 2004; Liebert and Portz 2005; Goda 2007). Moreover, these beneficial effects of phytase supplementation on plant-protein rich diets have also been reported for various other warm-water omnivorous fish species, such as common carp (Schafer et al. 1995; Nwanna and Schwarz 2007); channel catfish, Ictalurus punctatus (Jackson et al. 1996; Yan and Reigh 2002); African catfish, Clarias gariepinus (Van Weerd et al. 1999); and yellowtail catfish, Pangasius pangasius, fingerlings (Debnath et al. 2005).

When considering the efficacy of dietary phytase supplementation with either mono- or di-calcium phosphate, rainbow trout and Nile tilapia studies presented here all show that the digestibility of P was equivalent to that of the diet supplemented with 1000 phytase units/kg feed.

Total P release was increased from 0.18 to 0.36% with increasing dietary doses of phytase from 500 to 2000 phytase units/kg in Nile tilapia and in average from 0.08 to 0.16% in rainbow trout. This confirmed the results obtained by Dalsgaard et al. (2009) showing that phytase supplementation with 1400 phytase units/kg was proved very efficient, releasing on average 0.16% P/kg feed. The P release from the experimental diets only due to phytase supplementation is presented in Table 6. Values ranged from 0.07 to 0.18 and from 0.13 to 0.26 in rainbow trout and Nile tilapia, respectively.

In conclusion, the 8-wk tolerance trial performed with rainbow trout showed that this novel 6-phytase preparation expressed in A. oryzae is efficacious and tolerated by fish even when fed 100 times the maximum recommendation. This trial shows that dietary phytase supplementation at the maximum recommended dose could improve growth and feed conversion ratio of juvenile rainbow trout. The results of the efficacy studies performed in rainbow trout and Nile tilapia demonstrated that supplementation of a low fish meal diet with this novel microbial 6-phytase significantly enhanced P digestibility in a dose-dependent manner. The apparent utilization of P was significantly increased and consequently the amount of P excreted in the feces was reduced. Due to that, such phytase supplementation could reduce the necessity of inorganic P supplementation, thereby allowing further fish meal replacement by plant proteins and reducing water pollution.

Acknowledgments

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Literature Cited


