

White paper

Innovation in enzyme development

Vibe Glitsoe (Novozymes), Jean-Paul Ruckebusch (DSM), Inge Knap (DSM)



Innovation in enzyme development

Vibe Glitsoe (Novozymes), Jean-Paul Ruckebusch (DSM), Inge Knap (DSM)

Discover our
RONOZYME®
portfolio >

Take home message

- **Feed enzymes are a widely accepted and adopted means to increase feed utilisation and ultimately save on feed costs**
- **Feed enzymes typically improve the environmental impact of animal production**
- **While the use of phytases is most developed and widespread, there is still more to be learned and more to be gained from the application of carbohydrases and proteases**
- **Enzyme activity assays and activity units are necessary for quality control and dosing purposes but cannot be used to compare or evaluate feed enzymes**
- **Enzyme development of innovative products is an interdisciplinary effort requiring a number of different competencies as well as dedication and investment**

Introduction

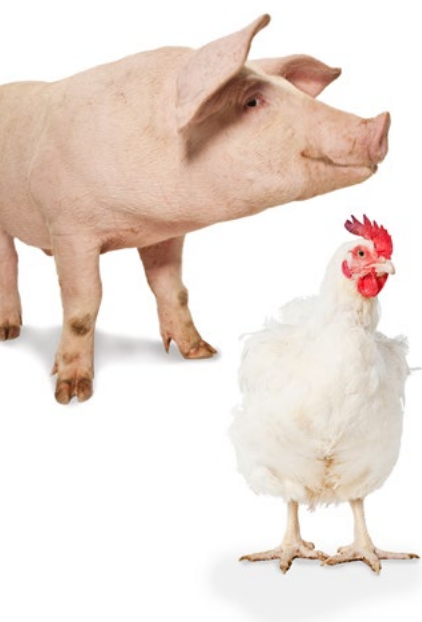
Enzymes are nature's catalysts. This means that they can increase the rate of a reaction without being consumed in the process – a characteristic which makes them a powerful tool in the optimization of a number of industrial applications including animal feed production. Enzymes are involved in the control of all reactions related to life and are therefore found in every living organism including animals, plants and microorganisms. These components have been used for thousands of years to naturally process and conserve agricultural products, but the rapid development in molecular biology has made it possible to significantly enhance the development and application of industrial enzymes.

Feed enzymes

While the first scientific publication on the potential use of enzymes to increase animal performance appeared approximately 100 years ago, it was only in the mid-1980s to mid-1990s that feed enzymes became commercially applicable to enhance the profitability of animal production.

Today, the use of microbial enzymes as feed additives is a well-established practice and the majority of feeds for poultry and swine in intensive farming contain feed enzymes. In particular the application of phytase has been widely adopted, but many other enzyme classes can significantly improve the utilization of feed such as xylanases, β -glucanases, pectinases, amylases and proteases. The feed enzymes market has been projected to reach about US\$1,280 million by 2019 from US\$900 million in 2014 (1).

The potential nutritive value of feed raw materials is often not realised at the animal level because of the limitations imposed by the presence of a range of anti-nutritional factors and the lack (or insufficiency) of digestive enzymes to break down specific chemical linkages that bind and prevent the release of nutrients. The need to efficiently utilise nutrients is the principal rationale for the use of feed enzymes in diets for monogastric animals. Feed enzymes are thus applied to improve feed utilization and the benefits of enzyme supplementation include reduced feed cost, improved animal performance and, last but not least, reduced environmental impact of agriculture. Still there are many other reasons for the increased implementation of feed enzymes.



Benefits of feed enzymes

1. Improved efficiency of utilisation of dietary components (protein, amino acids, starch, lipids and energy) in feedstuffs
2. Increased flexibility and increased range of feedstuffs in feed formulations. Feed enzymes may remove part of the constraint of inclusion limits of poorly digested ingredients. In effect this can reduce feed costs.
3. Reduced variability in the nutritive value of the ingredient. Enzyme supplementation reduces the variation between good and poor quality samples of a given ingredient and this, in turn, improves the degree of precision in feed formulation. It is generally recognised that the lower the ingredient quality, the greater the enzyme response will be.
4. Alteration of gut flora towards favourable bacterial species, which improves gut health and provides a protective effect on the overall health of the animal. This protective effect arises, in part, from the influence of flora on immune function.
5. As more nutrients are utilised and taken up by animal, less is secreted into the manure and ultimately into the environment

For all feed enzyme applications, two basic principles apply. First of all – from a biochemical point of view - an enzyme can be the solution to a problem, when the substrate that the enzyme can degrade is causing the problem. The most well described example is phytate, which renders the majority of plant phosphate unavailable to the animals and also acts as an anti-nutritional factor. Microbial phytase can degrade phytate and in this way significantly reduce the problems associated with phytate. Some enzyme products claim an array of enzyme activities, but it is not clear what problems the majority of these enzymes are addressing - hence the effects of these activities are unclear.

The second principle is that in many cases the use of feed enzymes must be accompanied by a concomitant change in the feed composition to get the advantage of the enzyme addition. In the above example with phytase, the content of inorganic phosphate in the diet must be reduced according to the effect delivered by the addition of phytase (which depends on the phytase product and dosage). If this is not done, the improved release of phosphorus from phytate will not add any benefit to the animals or, indeed, to the environment. A similar argument can be made for enzymes that increase the digestibility of feed nutrients, such as energy or protein. If the energy or protein content in a diet is already well above the animals' requirements, the optimal effect of the enzyme cannot be fully utilized. The re-formulation of the feeds has to be done with the use of matrix values.

While feed enzymes are in some cases added 'on-top' with the aim to improve animal performance, more often they are added by assigning a nutrient matrix value to a given enzyme product that will account for its value when feed millers optimise feed formulations. In the latter case, feed cost reductions are achieved by assigning a nutrient value to the enzyme that is caused by the enzymes effects on various dietary ingredients e.g. by releasing phosphate from phytate or by improving the digestibility of amino acids in a vegetable protein source.

As highlighted by Cowieson (2014) the manner in which enzymes are included in least cost formulation can profoundly influence their value, particularly when focus is given only to minimizing feed cost, while disregarding animal performance. Indiscriminate use of feed enzymes may result in inconsistent effects at an animal level, and it is suggested that the value of enzymes should be divided across feed cost saving and animal performance metrics as undue focus on feed cost alone can reduce the value proposition and may lead to erroneous formulation approaches (2). It is thus clear that enzyme application know-how is very important to optimise the value of feed enzyme solutions.

Feed enzyme applications

As mentioned above, the most well described feed application is that of **phytase** and its ability to hydrolyse phytate, or myo-inositol hexakisphosphate, which causes the majority of plant phosphate to be unavailable to the animals and also acts as an anti-nutritional factor by forming complexes with minerals (such as calcium, zinc and iron) and proteins. Mono-gastric animals are poorly equipped to utilise phosphorus present in phytate as they have little to no endogenous phytase activity. However, phytate and its degradation products (lower inositol phosphates) can be hydrolysed by exogenous phytase, and this significantly reduces the problems associated with phytate, which includes the release of excess phosphorus to the environment.

Although the use of phytase has been well established for some decades now, new insights are still generated and recent focus is on 'phytate-free nutrition' to fully alleviate the detrimental anti-nutritional effects of phytate, but also to benefit from the absorption of myo-inositol as such. These 'extra phosphoric effects' can be achieved at increased dosages of phytase and improve animal performance above what would be expected from the release of minerals (3,4). It is currently speculated that the extra-phosphoric effects of phytase is related to increased availability of myo-inositol, which may have an insulin-like effect stimulating the translocation of the GLUT₄ glucose transporter. Poultry have been shown to be insulin responsive, and there are some reports of improved performance in chickens by direct addition of myo-inositol to the diets (4).

Carbohydrases include NSP-degrading enzymes that can degrade fibre or the Non-Starch Polysaccharides present in plant well walls constitute another large feed enzyme segment. Included in this segment is xylanases, beta-glucanases, xyloglucanases, galactomannanases, pectinases, and debranching enzymes such as arabinofuranosidases and ferulic acid esterases. Probably the most important and most widely used enzyme class in this segment is the xylanases, as arabinoxylans constitute a major part of the NSP in the cereals used as feed ingredients. These enzymes reduce the anti-nutritional factors of NSP in plant material, by degradation of soluble fibre to reduce gut viscosity and improve nutrient absorption, and by solubilisation of the insoluble fibre and in this way liberate nutrients from plant cell wall 'cages'. In addition, the degradation of the polysaccharides yields oligosaccharides that can act as prebiotics benefiting the gut microflora in a positive way. A recent focus area for us has been the visualisation of enzyme effects by the use of microscopic techniques including that of specific antibodies to follow closely the degradation of specific cell wall polymers (5, 6).



Another carbohydrase class used in the feed industry is **amylases** that are used in fast-growing broilers to improve starch digestibility levels, which are typically 85-95% for corn and sorghum and 70-97% for wheat. One of the problems is that starch is harder to access in the gut when present in the form of pelleted feeds, as opposed to crushed feeds (7). Recent research has revealed other factors, beyond the biochemical challenges, which inhibit full utilization of the available starch content. Fast-growing modern broiler breeds have been shown to digest the starch in the diet less efficiently than slow-growing breeds. They therefore need more amylase in the diet. It is also known that the excretion of pancreatic amylase is limited for broilers during the grower and finisher phases, during which high levels of dietary starch are required.

The most recent feed enzyme segment is that of **proteases**. The use of proteases in feed has not been explored as extensively as other enzyme classes, and in many cases the proteases investigated have been present as part of an enzyme mixture (for example as Bacillus wild type fermentations) and in this situation it is not possible to evaluate precisely the effect and impact of a protease (8). Mono gastric animals, such as swine and poultry, produce digestive proteases, e.g. pepsin, trypsin, chymotrypsin, and carboxypeptidases, which digest feed proteins to a high degree. Yet a fraction of the ingested feed protein is excreted with the feces, and represents an opportunity for an exogenous protease to improve the utilization of protein in broilers (9,10). In vitro data suggests that there may be a synergistic effect between some microbial proteases and pancreatic protease, such as trypsin (Figure 1). In this experiment commercially toasted SBM was incubated with Pancreatic Trypsin Novo (PTN), RONOZYME® ProAct protease, or a combination of both for 3 hours at pH 7 and 40°C before the extent of the cleaved peptide bonds were analyzed using an OPA reagent. By addition of the microbial protease at a low level (that by itself does not give a response in this model system), the same level of protein hydrolysis could be obtained using only half the amount of the trypsin.

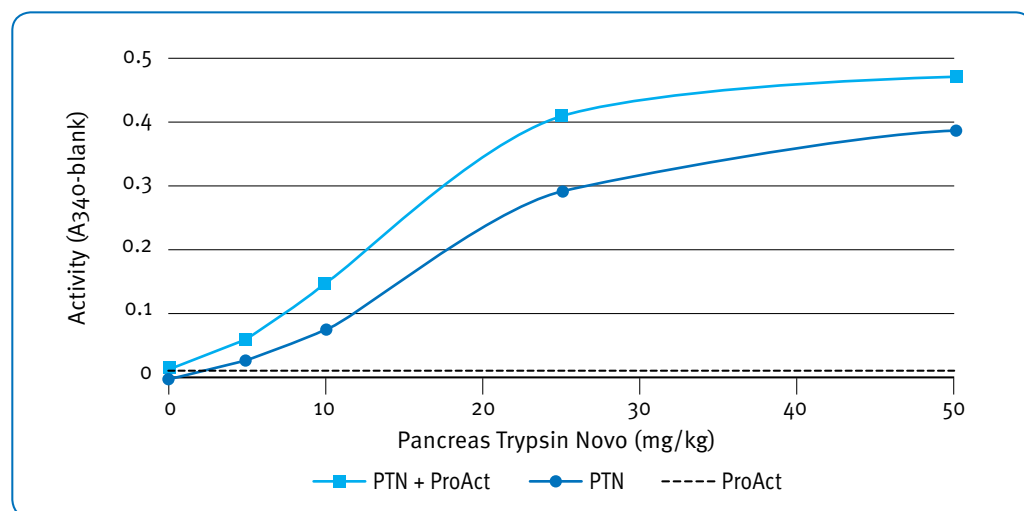


Figure 1: Synergistic effects of Pancreas Trypsin Novo and RONOZYME® ProAct (low dose)

Varying effects of proteases on broiler performance have been reported over the years and it is clear that the application knowledge on proteases is less than those of the other enzyme segments. However, in a recent meta-analysis of the effect of the RONOZYME® ProAct protease based on 804 data points from 25 independent digestibility studies (experiments run between 2006 and 2013 in Europe, the United States and Brazil), it was shown that the mean amino acid digestibility response was around 4% ranging from 5.6% for Thr to 2.7% for Glu. The apparent metabolizable energy was significantly increased by 49 Kcal/kg and fat digestibility was improved by 1%. It is worth noting that the inherent digestibility in the control diet explained around 47% of the variance in the response (11).

Enzyme assays and units

Enzyme activity is determined by enzyme activity assays and should not be confused for a quantification of the enzyme in a sample. It reflects the enzymes ability to perform a certain reaction under certain conditions (pH, temperature, substrate, buffer) and the activity of a given enzyme can thus be measured in many different ways using different substrates and different reaction conditions. Thus there is not one correct way to measure e.g. phytase activity. As a consequence, an activity number is always dependent on the exact assay conditions and therefore activity units and/or enzyme assays cannot be used to evaluate enzyme performance – this must be done under real application conditions. It should be noted that relative comparisons, e.g. the residual activity after a certain challenge such as in a pelleting trial, should give comparative results even when different methods are applied as the ultimate result is calculated relatively to the unchallenged control.

The use of enzyme activity assays is essential for quality control purposes and is also often used as a tool to calculate product inclusion rates. To this end, the enzyme producers design an assay that can most optimally be used for quality control purposes, for example one that can easily be automated and that has the lowest analytical variation e.g. by comparing to a standard enzyme sample instead of measuring an absolute number (e.g. release of phosphate in the phytase assay). It is often anticipated that an enzyme unit can be expressed along the lines of ‘the amount of enzyme required to release 1 micro-mole of inorganic phosphate from phytate per minute under certain pH, temperature, and buffer conditions’. However in some cases, e.g. where an artificial substrate is used that in no way resembles that of the substrate in the final application, this is not very meaningful. For quality control purposes, it is not an issue that the substrate in an assay is not the real one as the main purpose is to ensure reliable and consistent product quality.

Enzyme characterization

It is well known that enzymes are affected by the conditions of their surroundings (e.g. water concentration, temperature, pH) with respect to enzyme activity as well as stability, so a lot of attention has focused on biochemical characterisation of the enzymes. This is certainly true for pH activity curves and such data also gives important indications as to how and where an enzyme can act. However it should be noted that some caution is needed when interpreting data particularly when comparing across enzymes. Basically because the curves are almost always relative, meaning that for each enzyme the maximum activity is defined as 100%, the actual maximum activity in absolute numbers may vary significantly between the enzymes and the comparison of pH activity curves is distorted. Moreover, the conditions of the activity assays used to generate the data can have a considerable impact on the activity number. In our lab, changing the substrate from purified phytate as purchased from Sigma to a real feed substrate (SBM/maize feed mixture) shifted the pH curve of the RONOZYME® NP phytase one pH unit more acidic (Figure 2). In an example with proteases and different protein substrates, it was also clear that the relative performance of RONOZYME® ProAct and other commercial products claiming protease activity depended on which substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide; AZCL casein or skim-milk) was used to determine activity (Figure 3). It was also shown by Weaver et al (2009) that a change in buffer composition significantly changed the activity number when assaying an E.coli phytase (12).

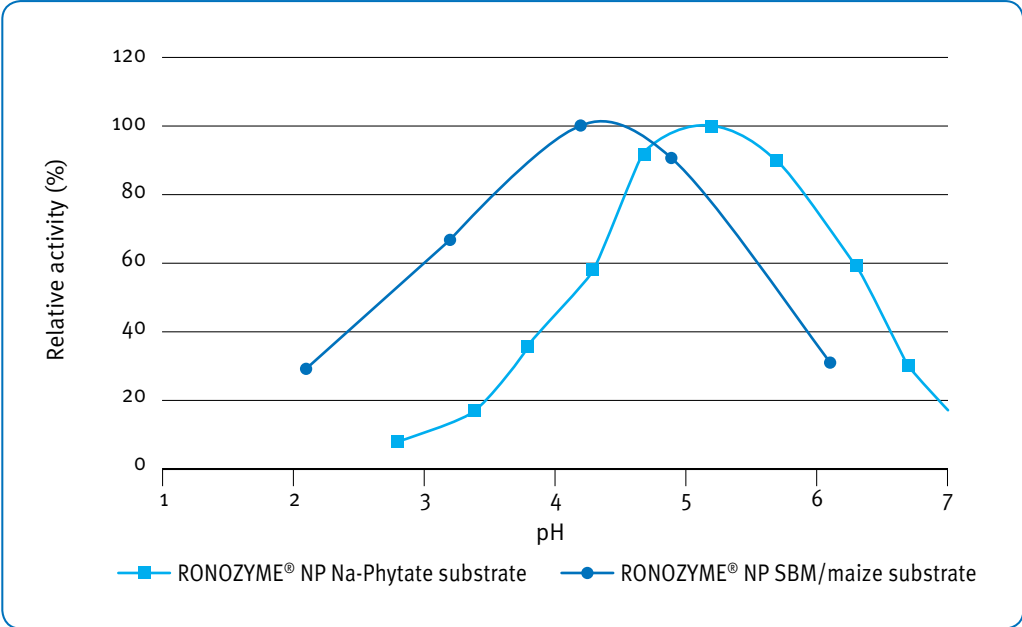


Figure 2: Relative activity as a function of pH for RONOZYME® NP measured with Na-phytate or SBM/maize feed mixture as substrate.

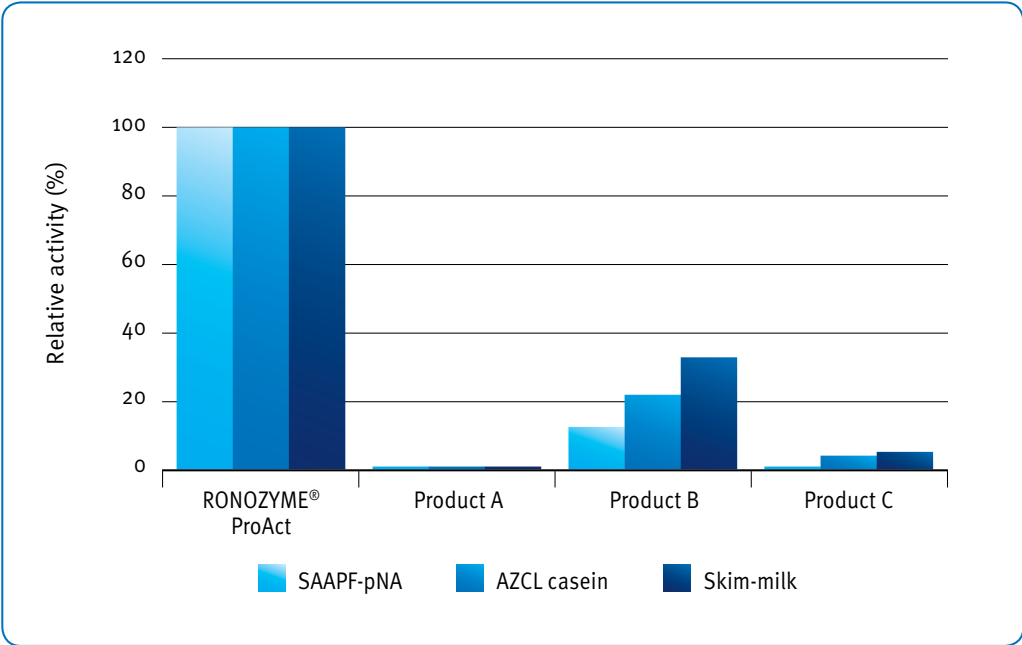


Figure 3: Comparative protease activity of five enzyme products claiming protease activity using three different protein substrates (all data for a given substrate shown relative to the activity of RONOZYME® ProAct set at 100%)

Development of feed enzymes

Numerous strategies can be employed to develop new enzyme solutions, but in general a high number of potential enzyme candidates to choose from enhance the probability of finding highly efficacious enzymes. Enzyme diversity can be a result of enzymes produced by naturally occurring wild type microorganisms or of (designed) protein engineered enzyme variants. In recent years, as the genomes of more and more microbes are sequenced and held as proprietary as well as public information, these sequences and their putative assignments has become another starting point for enzyme development. In the search for a new enzyme application it is of utmost importance at the initiation of the process to define as precisely as possible what the required action of the enzyme is and under what conditions it should be active. Another important prerequisite is that one must be able to design a series of assays that can select among the different candidates in a way that ensures that the final candidate will fulfil the desired criteria.

In the design of these assays, it is important to consider and balance the relevance of the assay conditions compared to those of the final application versus the complexity and the throughput of the analysis. A typical consideration in the development of feed enzymes is the ability to withstand and function in the conditions of the gastrointestinal tract. This includes resistance to low stomach pH and the digestive proteases secreted in the stomach and small intestine, while maintaining activity in the pH range of 3-7. In many cases the final selection is made after an application-relevant test such as *in vitro* testing, where the purpose is to mimic the passage of the gastro-intestinal tract and thus simulate a low pH stomach step in the presence of pepsin as well as a small intestinal step, where porcine pancreas enzymes are added to account for the digestive capacity normally present in the small intestine. Such a test can determine if a candidate has the potential to perform under the selected conditions of the *in vitro* assay and rank the candidates accordingly. Although *in vitro* tests are useful tools in the search for feed enzymes, the only way to determine if a candidate has the expected effect is to test it in the animal species of interest. This was also confirmed recently in an *in vitro* study on commercial phytases, where it was concluded that while the *in vitro* test could be a good screening tool, it was not able to mimic the *in vivo* conditions fully and could not be used to rank the enzymes with respect to their bio-efficacy in animals (13).

Having confirmed the beneficial effect of an enzyme candidate in animal feeding trials, the next step is to ensure that the enzyme will be stable in the entire distribution and feed production chain, i.e. during storage or processing of the feed, and in premixes and finished feeds. The ability to withstand the conditions of the pelleting process is particularly important for certain animal species. Finally, optimal production processes including fermentation, recovery and formulation must be established. Appropriate formulation technology such as granulation has proven to be the way to obtain the most pelleting stable products. In addition, granulation technologies can ensure that the products are safe and easy to handle (e.g. low dust and high flowability).

The dedicated and targeted development of a feed enzyme product is a challenging process, which requires the involvement and competence of many disciplines including microbial discovery, molecular biology, *in vitro* and *in vivo* testing, and enzyme production capabilities including formulation development. Before a product can be marketed, any new feed enzyme products must be submitted to thorough toxicology and safety examinations, and subsequently fulfil the requirements of the registration procedures in the different regions.

Environmental impacts of using feed enzymes

Public concern regarding the impact of agriculture, especially animal production, on the environment is increasing. For example when feed protein is not fully utilized in the animals, the undigested protein ends up in the manure, where proteins are converted to ammonia and nitrate. Ammonia emissions into the atmosphere cause water acidification, and nitrate eventually pollutes the water systems. The main purpose of feed enzymes is to improve nutrient utilisation by the animal and consequently less nutrients are released into the environment with the manure if the enzymes are applied correctly. Life cycle assessment (LCA) is a tool to quantify the environmental impacts of a certain process on the entire life cycle from cradle to grave. This includes raw material extraction, production, transport, and final disposal or recycling. One way to study the implications of an enzyme solution is to assess and compare the environmental impact of both a conventional and an enzyme-assisted solution delivering the same benefit.

A life-cycle analysis was carried out to determine the environmental impact of protease in poultry feed (14,15) using the principles for LCA described in ISO 1404017 and 1404418. The study showed significant benefits for all the environmental impacts that were considered. Most important was the potential to reduce water and air pollution with nitrous compounds, which can lead to eutrophication and acidification. The largest effects were observed when the protease was used in diets to compensate for reduced protein content (14). Similarly, LCAs have been carried out to document the benefits of phytase and xylanase (16,17). The use of phytase in intensive pig production led to much lower contributions to global warming, acidification and particularly nutrient enrichment. In another study, nutrient digestibility was increased by feeding xylanase to growing pigs and thus allowing for a change of feed ingredients, which potentially could result in a reduced contribution to global warming, acidification and photochemical ozone formation, as well as reduced use of energy.



Literature Cited

- 1: Feed Enzymes Market by Livestock, by Types & by Geography - Global Trends & Forecasts to 2019. Research and Markets, Dublin
- 2: Cowieson, A. J. (2014). Recent advances in exogenous enzyme application for monogastric nutrition. Abstract. 65th Annual Meeting of European Federation of Animal Science, 25 - 29 August 2014, Copenhagen
- 3: Zeng, Z.K., Wang, D., Piao, X.S., Li, P.F., Zhang, H.Y., Shi, C.X. & Yu, S.K. (2014). Effects of adding super dose phytase to the phosphorus-deficient diets of young pigs on growth performance, bone quality, minerals and amino acid digestibilities. *Asian-Australasian Journal of Animal Science*, 27: 237-246
- 4: Cowieson, A. J., Aureali, R., Guggenbuhl, P. & Fru-Nji, F. (2015). Possible involvement of myo-inositol in the physiological response of broilers to high doses of microbial phytase. *Animal Production Science*, 55: 710-719
- 5: Duy, M.L., Fojan, P., Azem, E., Pettersson, D. & Pedersen, N.R. (2013). Visualization of the anticaging effect of RONOZYME® WX xylanase on wheat substrates. *Cereal Chemistry*, 90(5): 439-444
- 6: Ravn, J.L, Martens, H.J., Pettersson, D. & Pedersen, N.R. (2015). Enzymatic solubilisation and degradation of soybean fibre demonstrated by viscosity, fibre analysis and microscopy. *Journal of Agricultural Science*, 7 (9): ISSN 1916-9752 E-ISSN 1916-9760
- 7: Svihus, B. & Hetland, H. (2001). Ileal starch digestibility in growing broiler chickens fed on a wheat-based diet is improved by mash feeding, dilution with cellulose or whole wheat inclusion. *British Poultry Science*, 42: 633-637
- 8: Angel C.R., Saylor W., Vieira S.L. & Ward N. (2011). Effects of a monocomponent protease on performance and protein utilization in 7- to 22-day-old broiler chickens. *Poultry Science*, 90: 2281-2286
- 9: Parsons C.M., Castanon F. & Han Y. (1997). Protein and amino acid digestibility of meat and bone meal. *Poultry Science*, 71: 133-140
- 10: Lemme A.V., Ravindran V. & Bryden W.L. (2001). Ileal digestibility of amino acids in feed ingredients for broilers. *World's Poultry Science Journal*, 60: 423-437
- 11: Cowieson, A.J. & Roos, F.F. (2014). Bioefficacy of a mono-component protease in the diets of pigs and poultry: a meta-analysis of effect on ileal amino acid digestibility. *Journal of Applied Animal Nutrition*, 2: 1-8
- 12: Weaver J.D., Ullah, A.H.J., Sethumadhavan, K., Mullaney, E.J. & Lei, X.G. (2009). Impact of Assay Conditions on Activity Estimate and Kinetics Comparison of *Aspergillus niger* PhyA and *Escherichia coli* AppA2 Phytases. *Journal of Agricultural and Food Chemistry*, 57(12):5315-20
- 13: Menezes-Blackburn, D., Gabler, S. & Greiner R. (2015). Performance of Seven Commercial Phytases in an in Vitro Simulation of Poultry Digestive Tract. *J Agric. Food Chem.*, 63: 6142-6149

- 14: Oxenboll, K.M., Pontoppidan, K. & Fru-Nij, F. (2011). Use of a protease in poultry feed offers promising environmental benefits. *International Journal Poultry Science*, 10(11): 842-848
- 15: Oxenboll, K.M., Pontoppidan, K., Fru-Nij, F. & Broz, J. (2011) Life cycle assessment of using RONOZYME® ProAct as a feed additive for poultry. <http://www.novozymes.com/en/sustainability/sustainable-solutions/life-cycle-assessments/published%20LCA%20studies/English.pdf>, accessed June 28th 2011
- 16: Nielsen, P.H. & Wenzel, H. (2006). Environmental assessment of RONOZYME® P5000 CT phytase as an alternative to inorganic phosphate supplementation to pig feed used in intensive pig production. *Int J LCA*, 7: 1-7
- 17: Nielsen, P.H., Dalgaard, R., Korsbak, A. & Pettersson, D. (2007). Environmental assessment of digestibility improvement factors applied in animal production: Use of RONOZYME® WX CT xylanase in Danish pig production. *Int J LCA*, 13(1): 1-8

This paper was first presented at the 36th Western Nutrition Conference, September 2015, Winnipeg, Manitoba, Canada