SCREENING FUNGI FOR THERMOSTABLE PHYTASES AND COMPARING THE THERMOSTABILITY OF A CURRENT PHYTASE PRODUCED UNDER LIQUID CULTURE AND SOLID SUBSTRATE CULTURE CONDITIONS

by

JOHN PATRICK DRIVER

BSc (Agric)

Submitted in partial fulfilment of the
requirements for the degree

MSc (Agric) (Animal Nutrition)

in the

Department of Animal and Wildlife Sciences
Faculty of Biological and Agricultural Sciences

University of Pretoria

Pretoria

2001

CONTENTS:

PHASE II THERMOSTABILITY OF TWO COMMERCIAL PHYTASES

Chapter 4 BROILER TRIAL.

List of Tables:

List of Figures:

CHAPTER 1. LITERATURE REVIEW

1.1. INTRODUCTION

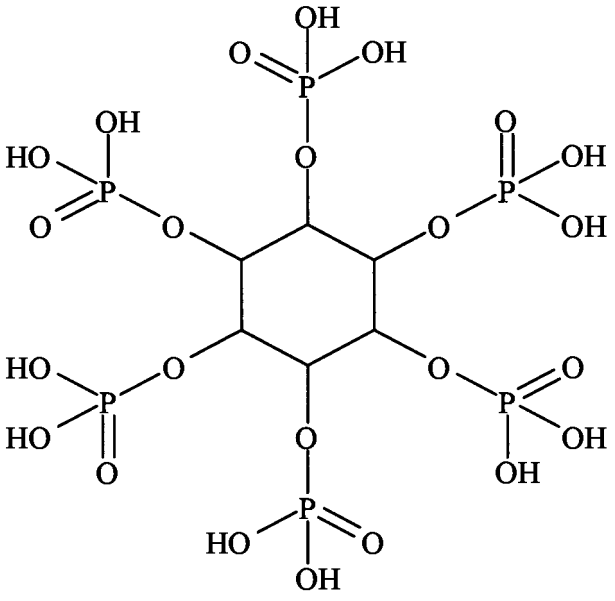
Phosphorus is the second most abundant mineral in the body accounting for over one-fourth of the total minerals present. It is essential in many organic compounds including nucleotides such as ATP, nucleic acids, phospholipids and a myriad of other compounds needed for metabolism. It also plays a part in almost every biochemical pathway in the body. However, only 20% of the body's phosphorus is found in the soft tissues where these reactions take place. The remaining 80% is found in the bone matrix (Pierce, J. L., 1999).

A phosphorus deficiency can lead to a number of disorders such as rickets, a reduction in bone mineralization, reduced egg production and decreased feed intake. It is for this reason that an adequate supply of dietary phosphorus must be supplied to the animal. This is of special importance for monogastric animals, as they do not possess the enzymes necessary to release inorganic phosphorus from phytic acid, the organic molecule that binds most phosphorus. Ruminants, however, are able to take advantage of this source of phosphorus through an abundance of microbial enzymes produced in the rumen (Pierce, J. L., 2000).

1.2. PHYTIC ACID

Phytic acid is the compound that accounts for the primary source of phosphorus and inositol in plant seeds that are used as feed ingredients ((Pierce, J. L., 1999). Phytic acid consists of an inositol nucleus with six radicals (Figure 1.1.). It exists as phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate), a complex salt, in plant tissue where it is deposited as globular inclusions in single-membrane storage microbodies in the seed (Raboy et al., 1990).

Figure 1.1. Phytate molecule



Interest in phytic acid first arose because of its strong binding action, especially for mono and divalent metal ions including Zn, Cu, Co, Mn, Fe, and Mg. Of these, Zn and Cu have the highest affinity for phytic acid (Maddaiah et al., 1964; Vohra, 1965). Furthermore, phytic acid is a strong acid, which enables it to bind certain proteins under the low and neutral pH conditions found in some portions of the gut (Sebastian et al., 1996). Maximum binding of Zn-Ca-Cu-phytate and Cu-Ca-phytate occurs at a pH of 6, the normal pH of the duodenum, where most of the divalent cations are absorbed (Oberleas, 1973). This accounts for the poor bioavailability of minerals in high phytic acid diets (Gibson and Ullah, 1990).

Phytic acid is the primary storage form of phosphorus in the plant and accounts for approximately 75% of the phosphorus in cereals and legumes ((Pierce, J. L., 1999). Although it is a compound that is found in many parts of the plant including the roots and tubers, vegetative tissue and even pollen, it occurs predominantly in the seed (Gibson and Ullah, 1990). Further, the phytic acid content of the seed varies according to the type of crop. For example, the phytic acid content of cereals ranges from 0.50% to 1.89% and from 0.40% to 5.2% in legumes and oilseeds (Reddy et al., 1982). Phytic acid accumulates in the seed during the ripening period. Production is localized to the aleurone grains in the aleuron layer of most cereals, while in legumes

and oil-seeds, the majority of phytic acid production takes place in the endosperm and cotyledons. The exception to the typical monocot localization patterns is maize where 88% of the phytic acid is found in the germ as opposed to the aleuron layer (Gibson and Ullah, 1990).

The role of phytic acid in the plant is, first and foremost, one of storage so that accumulated phosphorus can be liberated during germination and incorporated into ATP. However, recent studies have also implicated phytic acid as an intermediate in a number of cell transport systems ((Pierce, J. L., 1999). Further, inositol intermediates are thought to act as secondary messengers in plant and animal cells. Other possible physiological roles of phytic acid include its use as an energy source and an initiator of dormancy (Gibson and Ullah, 1990).

1.3. PHOSPHORUS POLLUTION

Plant material is the predominant ingredient in the diets of pigs and poultry. These monogastric animals are unable to utilize the phytic acid present, which makes up approximately two thirds of the available phosphorus in most plant feedstuffs. Endogenous phytase does exist within the gut, however it is not produced in sufficient quantities to be of any benefit to the animal. Microbes in the large intestine and caecum of the pig and chicken also produce phytase, but again this is of little significance to the animal as it is too late in the gut for most of the inorganic phosphorus released to be absorbed. It is for these reasons that phytic acid is, for all intense and purposes, unavailable to the animal. A number of publications place the phosphorous availability estimates for pigs and poultry in the range of between 10 and 30% for a maize soyabean diet (Nelson, 1967; Calvert et al., 1978; Jongbloed and Kemme, 1990).

It therefore becomes necessary for the producer to supplement the diet with inorganic phosphorus in order to avoid a deficiency. However, this leads to the problem of excessively high levels of phosphorus entering the environment, especially under intensive production. It is for this reason that both the swine and poultry industries have drawn sharp criticism for their role in phosphorus contamination of water systems. Cromwell (1999) surveyed a number of feed manufacturers and found that they commonly included between 20% and 30% more phosphorus in the feed for pigs

than that recommended by the NRC in order to maintain a safety margin. Table 1.1 gives an example of the possible effects of over supplementation of phosphorus in the diet.

Table 1.1. Effects of dietary phosphorous on the levels of phosphorous faecal excretion

	Phosphorous balance (g/d)		
Dietary P, %	Intake	Retained	Excreted
0.7	21	4.8	16.2
0.6	18	4.8	13.2
0.5	15	4.7	10.3
0.4	12	4.5	7.5
(NRC, 1998 Requirements)			(Cromwell, 1999)

Phosphorous contamination results from the propensity of phosphorus to bind tightly to soil particles. The phosphorus therefore does not leach into the soil but is rather carried out into rivers and streams through the process of erosion (Loer, 1984). A large quantity of phosphorus entering a water system causes large-scale deterioration of the water quality by promoting algal blooms and other forms of eutrophication. This rapid growth and increased metabolic activity in turn leads to increased plant decay. The result of this ongoing cycle of respiration and decomposition is a decline in the oxygen content of the water. When the depletion becomes excessive, the entire water system may die.

Current legislation in many Western European countries limits the amount of phosphorus that can be applied to the soil, e.g. 1998 Mineral Accounting System Legislation (Joengbloed and Lenis, 1998). This has been instituted in an attempt to reduce the amount of faecal phosphorus entering the ecosystem as a result of over-supplementation of inorganic phosphorus in livestock diets.

The most effective way to reduce this undesirable source of phosphorus is to either use feedstuffs with higher phosphorus availability such as low phytic acid maize, or to increase the digestibility of current ingredients (Pierce, 1999).

1.4. PHYTASE

Phytase (myo-inositol hexaphosphate phosphohydrolases) is the name given to a number of enzymes that catalyze the stepwise removal of orthophosphate from phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate). These enzymes are unique in that they hydrolyze phosphate from phytic acid as well as from a variety of natural and synthetic phosphorylated substrates (Gibson and Ullah, 1990).

The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (UIPAC-IUB) recognize two classes of phytase. The 3-phytase found in microorganisms and animals, and the 6-phytase found in higher plants. The 3-phytase-enzyme removes orthophosphate from 1, 2, 3, 4, 5, 6, hexakisphosphate and lower inositol phosphates in a stepwise fashion from the 3-position of phytic acid, while 6-phosphate starts removing orthophosphate from the 6-position (Pierce, 1999).

Characterization of fungal 3-phytase and plant 6-phytase has revealed that they have very little in common apart from similar pH and temperature optima. Fungal phytase has a much higher turnover number for phytic acid compared to the soyabean phytases tested (Gibson and Ullah, 1990).

The phytase most commonly used to supplement animal feed is a fungal 3-phytase. This phytase has two pH optima, the first at 2.5 where it reaches approximately 60% of its potential activity and the second at 5.5 where it functions at 100% of its potential activity (Kies, 1996). The temperature at which hydrolysis is optimal is between 55 and 58^{0}C (Gibson and Ullah, 1990).

The phytase enzyme is highly specific for the phytic acid molecule. It cleaves phosphorus from inositol far more readily than from other phosphorylated molecules by a process referred to as the "Ping-Pong" mechanism. This is the term used to describe how phosphate is first transferred from phytic acid to the enzyme and then from the enzyme to water. It is therefore a reaction that requires an aqueous environment (Kies, 1996).

Phytase supplementation of monogastric animals is a means by which the digestibility of the indigestible phosphorus fraction of a feed can be improved. The role of phytase

is three-fold. Firstly, to reduce feed costs by reducing the need to supplement with inorganic phosphorus. This includes that phosphorus supplied by animal protein sources. Secondly, to release metal-ions and proteins bound to phytic acid to be absorbed and thirdly, to reduce the burden of high levels of phosphorus on the environment (Pierce et al., 2000).

A number of studies have been conducted to determine the effect of phytase on the availability of phytic acid phosphorus as well as nitrogen retention in monogastric animals. There is a good deal of variation between these studies, however most come to the same general consensus: that phytase does substantially improve phosphorus absorption and utilization (Broz et al., 1994; Cantor., 1998; Davis., 1970; De Bryne., 2000; Gadien., 1998; Gerber and Wenk, 1998; Gibson and Ullah, 1990; Inborr and Bedford, 1994; Joengbloed and Kemme, 1990; Joengbloed et al., 1992; Kemme et al., 1997; Kiisskinen and Piironen, 1990; Leske and Coon, 1999; Mitchell and Edwards, 1996; Mitchell and Edwards, 1996; Morz et al., 1994; Nelson et al., 1971; Orban et al., 1999; Qian et al., 1997; Sebastian et al., 1996; Simons et al., 1990; Sohail and Roland, 1999; Verschoor, 1990; Yi et al., 1996 and Zanini and Sazzad, 1999).

The existence of naturally occurring plant phytases has been an established fact for many years. These were the only practical source of the enzyme until production of microbial phytase improved. Feeding cereals rich in phytase such as wheat, rye, triticale and their by-products is an effective means of improving the utilization of phytic acid phosphorous. Phosphorous availability in wheat is four or five times higher than that of maize due to the differences in endogenous phytase present (Coffey, 1994). The problem with plant phytase is its relatively low level of activity.

Fungal phytase production has only recently reached the levels of efficiency required to make large-scale commercial production a reality. Improvements in techniques have allowed for the widespread use of the enzyme in the pig and poultry industry.

1.5. PHYTASE SOURCES

Phytases have been detected in most varieties of organism including plants, microorganisms and a number of animal tissues.

Phytase occurs in a number of the higher order plants such as rice, wheat, maize, rye, soyabean, castor bean, and other leguminous oil seeds and in a number of vegetative tissues including orange juice, tobacco, spinach leaves and a number of roots and tubers (Nayini and Markakis, 1986). However, it is in the seed that most phytase is found. Levels of phytase are generally low in ungerminated seeds increasing rapidly as germination progresses. This increasing enzyme activity is associated with a concomitant decrease in phytic acid. Very large increases in phytase have been detected. Maize seeds demonstrate a 200% increase in phytase activity during germination (Chang, 1967) and some varieties of pea show an increase ranging from 800 to 3000% over the first five days of germination (Chen and Pan, 1977). There is still uncertainty as to whether phytase is produced in the growing seed or whether synthesis is triggered by phosphorus depletion during the process of germination (Gibson and Ullah, 1990).

Most of the 6-phytases that are found in higher plants have a pH optimum of between 5.0 and 5.6 and an optimum temperature in the range of 45 to 60^0C. The K_m (substrate concentration that gives a reaction velocity equal to one-half the maximum velocity) for these enzymes lies between 2.22×10^{-4} to 0.99×10^{-3} mM (Wodzinski and Ullah, 1996).

Most of the microbial phytase work has been concentrated on filamentous fungi as few bacteria are capable of synthesizing phytase in significant quantities extracellularly. This, combined with the fact that bacterial phytase has a pH optimum that is neutral to alkaline, limits the use of this source of phytase as a feed additive ((Pierce, J. L., 1999).

The prospect of using filamentous fungi as a source of phytase has been well researched and exploited. Extensive screening for phytase activity has revealed that phytase production is highest in *Aspergilli* (Shieh and Ware, 1968; Howson and Davis, 1983). The most prolific producer of extracellular phytase found to date is the strain *A. niger* 3135. This strain produces a phytase with two pH optima; one of 5.5 and the other of around 2.0 and a temperature optimum of between 50 and 55°C.

1.6. PHYTASE SUPPLEMENTATION

Phytase supplementation can substantially improve apparent phosphorus availability and therefore reduce the quantity of total phosphorus in the excreta. This improved utilization of dietary phosphorus is the primary reason for the superior growth and performance of animals fed phytase on low phosphorus diets. Orban et al. (1999) investigated the effect of feeding microbial phytase to White Pekin ducks between 3 and 6 weeks of age. They were able to demonstrate a linear increase in feed intake when low phosphorus diets were supplemented with dietary phytase. Furthermore, weight gain showed a quadratic response to the increased non-phytate phosphorus. It was concluded that supplementary phytase could reduce the requirement for inorganic phosphorus to the extent that it would become unnecessary to provide such phosphorus to meet the growth and performance requirements of ducks during the finisher phase (3-7 weeks).

Broz et al. (1993) also demonstrated a dose response pattern between chick performance and phytase supplementation on a low phosphorus diet. They were able to show significant gains in both growth rate and food intake with increasing levels of phytase (125, 250, or 500 PU/kg). This was attributed to improved phosphorus availability. Of significance is the fact that only moderate improvements in feed conversion were noted. These results are in agreement with research conducted by Kiiskinen and Piironen (1990) with leghorns and Sebastian et al. (1996) as well as Zanini and Sazzad (1999), again with broilers.

Simons et al. (1990) recorded similar results with their broiler experiments. However, they differed with regards to feed conversion response to phytase supplementation. They found that the addition of phytase to the diet improved feed conversions up until 2 weeks of age. This improvement decreased over the next two weeks so that, at 4 weeks of age, no significant response could be detected. Reasons for this phenomenon remain unclear, however it was postulated that phosphorus requirements at 4 weeks of age are much lower than phosphorus requirements at 2 weeks. The birds may also have become better adapted to utilizing phytic acid-phosphorus at the later stage of growth.

1.7. EVALUATING SUPPLEMENTARY PHYTASE

The phosphorous released into the blood stream as a result of phytase supplementation is partitioned between growth and bone mineralization. It is therefore necessary to evaluate both growth performance and bone development parameters. In poultry, bone ash, bone mineral content, bone density and bone breaking strength have all been successfully employed to measure phosphorus availability and bone mineralization response to phytase while body weight gain and feed intake are powerful physiological indicators of plasma phosphorus concentrations (Orban et al. 1999).

These growth and performance parameters have been used to establish equivalency values for phytase on a number of diets for feed formulation purposes. Yi et al. (1996) established inorganic phosphorus equivalency values for phytase by feeding graded levels of phytase to broilers on soyabean meal-based semi-purified diets and corn-soyabean meal-based diets. Body weight-gain and toe ash responses were used to generate equations, which were then used to calculate equivalency values. The results of these experiments gave average equivalency values of 1.146 U = 1g phosphorus and 785 U = 1g phosphorus for the soybean meal-based semi-purified diets and maize-soyabean meal-based diets respectively.

Leske and Coon (1999) used a bioassay to determine the effect of phytase on phytic acid hydrolysis and phosphorus retention for a number of different feed ingredients using broilers and layers. Each feed ingredient was included into the diet as the sole source of phosphorus and the amount of phytic acid phosphorus hydrolysed recorded, with and without the addition of supplementary phytase. This procedure is an alternative to the more traditional methods described above. It has the advantage of providing direct information about the effect of phytase on phytic acid phosphorous utilization and retention while avoiding the mandatory two-week growth period. The bioassay technique is also able to accurately quantify the retention of total phosphorus in individual feedstuffs. This makes it possible to identify all those factors that affect phytic acid retention enabling the accurate formulation of diets for phosphorus, thereby avoiding costly over-supplementation.

1.8. FACTORS AFFECTING SUPPLEMENTATION RESPONSE

A number of factors may lead to the variability in animal response often associated with phytase supplementation other than the variability in plant phytic acid phosphorus and the different phytase sources already discussed.

1.8.1. Calcium, phosphorous and cholecalciferol interactions

The effectiveness with which phytase is able to improve the utilization of phytic acid phosphorus and calcium is strongly influenced by the ratio of calcium to total phosphorus (Ca:tP) (Qian et al, 1996; Quamme, 1985; Wise, 1983; Pointillart et al., 1985; Qian et al., 1997) and the level of cholecalciferol (vitamin D_3) in the diet (Qian et al, 1996; Quamme, 1985; Wise, 1983).

The level of calcium in the diet is thought to be the most important factor to affect the phytase supplementation response. Excess calcium causes an increase in the Ca:tP ratio, which in turn results in increased binding of calcium to the available phytic acid present, forming an insoluble complex which is less accessible to phytase (Qian et al., 1996). Quamme (1983) hypothesised that the relationship between increased calcium and reduced phytase activity was as a consequence of an increased intestinal pH, brought about by higher levels of calcium in the gut. The higher pH values decrease the activity of supplementary phytases, which are most active at pH 2.5 and pH 5.5. The pH also has a drastic effect on the active transport of phosphorus due to the electrochemical nature of phosphorus uptake. Quamme (1985) reported that at pH 6.5 Na^+ and $H_2PO_4^-$ where transported across the intestinal wall at the same rate whereas at pH 7.5 two sodium ions were transported for each phosphate ion. Wise (1983) and Pointillart et al. (1985) proposed another theory: that calcium is able to repress phytase activity by directly competing for the enzyme's active site. However, this is improbable as calcium and phosphorus have different charges and therefore different binding affinities for the enzyme (Pierce, 1999). The level of non-phytate phosphorus (NPP) in the diet is also of critical importance as the depressing effect of calcium is far greater when NPP is low. Qian et al. (1997) established that the maximum response to phytase is achieved when the Ca:tP ratio was in the range of 1.1:1 to 1.4:1 for turkey poults.

Conversely, phytase may improve calcium absorption and retention by hydrolyzing phytic acid. This releases calcium bound to phytic acid as well as other insoluble salts. It appears that phytase supplementation improves the utilization of N, P, Ca and Zn on a percentage of intake basis by liberating these minerals from the phytic acid molecule (Zanini and Sazzad, 1999). Phytase supplementation also increases the concentration of calcium and zinc in the tibia (Zanini and Sazzad, 1999; Yi, 1995).

The vitamin D_3 (cholecalciferol) status of the animal also plays a crucial role in the utilization and retention of phosphorus in the body. Cholecalciferol enhances the enterocytes of the small intestine facilitating the uptake of phosphorus and its subsequent transport into the plasma compartment. This transport system appears to be independent of the intestinal calcium transport system (Deluca et al., 1989; Edwards, 1993). However, the Ca:tP ratio of a diet does affect the efficacy with which cholecalciferol enhances phytic acid phosphorus digestion (Qian et al., 1997). Mohammed et al. (1991) demonstrated that by lowering dietary levels of calcium while elevating cholecalciferol, greater utilization of phytic acid-phosphorus was achieved thus reducing requirements for inorganic phosphorus. Qian et al. (1997) added cholecalciferol at high levels to the diets of birds and were able to enhance calcium and phosphorus retention by 5 to 12 %.

Vitamin D_3 appears to have an independent yet synergistic relationship with supplementary phytase. This is emphasized by the superiority of the combination treatment of phytase and D_3 over either individual treatment on its own, suggesting that a different mechanism of action is at work for each. Once phytic acid is hydrolyzed by phytase, D_3 then increases the uptake of the freed phosphorus possibly by acting as a DNA replication ligand for the up regulation of phosphate transport receptors (Quamme, 1985). There is also evidence that suggests that cholecalciferol increases the intestinal phytase and alkaline phosphatase activity in chicks (Davies et al. 1996). Qian et al. (1997) achieved the maximum response with supplemental phytase when they fed it to broiler chicks at levels of 600-900 U of phytase/kg with dietary Ca:tP ratios of 1.1:1 to 1.4:1, and a D_3 level of 660µg/kg diet.

1.8.2. Influence of dietary energy

Zanini and Sazzad (1999) demonstrated that the AME content of the diet is positively correlated to the excretion of N, P, Ca, and Zn and negatively correlated to the concentration of phosphorus and calcium in the tibia. The lower absorption may be as a result of the formation of insoluble complexes or soaps between these minerals and free fatty acids. However, there are also strong interactions between phytase supplementation and AME. This is supported by the fact that birds fed on a high AME diet without supplemental phytase demonstrate a lower absorption of nutrients as a result of the formation of insoluble salts. However, if supplementary phytase is added to these high-energy diets, utilization of the above mentioned elements as a percentage of intake is significantly improved and results in a higher percentage of bone ash.

1.8.3. Influence of animal factors

Apart from the variation between different animal species in their ability to utilize phytic acid phosphorus, variation exists between breeds and strains as well as between animals of the same strain under similar or dissimilar physiological conditions.

The ability of poultry to utilize phytic acid-phosphorus has been well debated. The capacity of birds to utilize this source of phosphorus depends upon a number of factors which may include the bird's age, dietary fiber content, and the level and source of calcium and phosphorus. This has led to much controversy over the degree of utilization. Values vary from 0 % (Nelson, 1976; Matyka et al., 1990) to 60 % (Edwards, 1993; Mohammed et al., 1991) to as high as 82 % (Temperton and Cassidy, 1964). This is further complicated by the fact that phosphorus utilization and retention is enhanced in phosphorus deficient birds (Temperton and Cassidy, 1964; Edwards and Veltmann, 1983).

Edwards (1982) conducted a study to determine the effect of breed and strain of poultry on the utilization of phosphorus in diets that contain suboptimal levels of phosphorus. It was established that broiler cockerel chickens utilize phosphorus less efficiently than Single Comb White Leghorn cockerel chickens (SCWL) under low phosphorus conditions thus suggesting that breed differences do exist.

Punna and Roland (1999) investigated the variation in the ability of individual birds within the same broiler strain to utilize phytic acid-phosphorus by feeding male broilers a phosphorous deficient diet from day-old to four weeks of age. Livability, feed consumption, growth rate, and bone quality were used as test parameters. Faeces collection after Week 2 and Week 4 revealed that those birds which demonstrated normal growth, no leg problems and no visible signs of a phosphorus deficiency were able to utilize phosphorus far more efficiently than birds which did display problems. The identification of variation between individual broiler chicks of the same strain is important for future work on defining phosphorus requirements in poultry rations. It also serves as a tool for potential genetic selection. Work in these fields could help to reduce phosphorus requirements as well as phosphorous pollution.

Kemme et al. (1997) investigated the influence of physiological status on the ability of phytase to render phytic acid-phosphorus available in pigs. Five physiological classes of pig were used in the trial and all were fed diets identical in composition. It was found that phytase increased digestible phosphorus in all cases, however the improvements decreased in the order of lactating sows, growing-finishing pigs, sows at the end of pregnancy, piglets and finally sows in mid-pregnancy. The reason for this variation between animals of different physiological age was attributed to variation in gastric emptying, stomach capacity and pH of gastric contents. This is important, as the main site of activity for phytase is the stomach (Jongbloed et al., 1992). The importance of gastric conditions was emphasized by Morz et al. (1994) who used apparent total tract digestibilities (ATTD) and apparent ileal digestibilities (AID) to investigate the effect of feeding regimen (frequency and level) and microbial phytase on the apparent digestibility and retention of nutrients bound to phytic acid. They concluded that the feeding regimen employed determines the pattern of gastric emptying, digesta flow rates, and changes in the pH at different regions in the gastrointestinal tract (Low, 1989). These factors, in turn, influence the efficacy of the phytase present. It was established that phytase supplementation, increased total nutrient intake per unit time and increased feeding frequency all improved ATTD of dry matter although not as a percentage of the total intake.

These experiments point towards the existence of a significant degree of variation between breeds, strains, individual animals and different physiological conditions,

which could be exploited to select for improved phosphorus efficiency. These trials also demonstrate that diet formulation could be tailored to the requirement of the individual animal category.

1.8.4. Influence of pelleting on phytase activity

Pelleting is a fundamental part of modern feed processing and is used to improve feed efficiencies as well as feed handling characteristics. The principal reason for pelleting poultry diets is to kill pathogenic bacteria while at the same time reducing mould growth (Patterson, 1969; Mossel, 1971; Scott et al., 1975). However, the process also has a number of other beneficial effects that make it very useful.

Pelleting improves the performance of birds in terms of body weight and feed intake although the reasons for these improvements are not fully understood (Nir et al., 1994). Much of the increase in the body weight gains of birds fed pellets is attributed to raised intakes, however other factors play an important role. Improvements in performance are also achieved by increasing the availability of various nutrients. The most significant example of this is the gelatinization of starch under the high temperatures and pressures of pelleting. Gelatinization of starch increases its solubility (Nir, 1994; Theander and Westerlund, 1984). This in turn improves intestinal amylase efficiency so that only one fourth to one fifth of the amylase used in a ground maize diet is necessary to liberate the same amount of glucose from a pelleted maize diet. Pelleting also reduces the separation of ingredients into their different components thereby ensuring the animal receives a balanced diet (McEllhiney, 1985).

Feed manufacturers currently employ aggressive pelleting techniques to improve the quality of their feed. A commercially attractive phytase should therefore be able to withstand the high temperatures (60 to 90°C) reached. However, the capacity of enzymes such as phytase to withstand such temperatures is questionable (Inborr and Bedford, 1994).

A considerable number of trials have been undertaken in an effort to establish the thermostability of feed enzymes under a variety of pelleting conditions. Uncertainties exist as to the conditions under which enzyme stability is affected due to the fact that

no truly satisfactory method of accurately determining enzyme activity in the finished feed is available (Lyons and Walsh, 1993; Cowan and Rasmussen, 1993). Current procedures involve incubation of the finished feed over extended incubation times followed by a standard assay. Animal response trials provide an alternative to *in vitro* techniques and many have clearly demonstrated that enzyme supplemented feeds that have undergone pelleting still yield beneficial results for the animal. Lyons and Walsh (1993) and Inborr and Bedford (1994) attributed this to components of the feed in bulk, which they reasoned must exert a protective or stabilizing effect on enzymes.

Partial enzyme deactivation does occur during pelleting, however the degree of inactivation depends on the relationship between the pressure and temperature to which the feed is exposed and the length of time it is exposed for. This relationship was investigated by Inborr and Bedford (1994) who supplemented a broiler diet with a β-glucanase at 3 different levels, 0, 1 and 10 g kg^{-1} and pelleted at 75, 85 or 95°C. They concluded that pelleting temperature had a negative quadratic effect on enzyme stability. However, they also found that bird performance was only affected when feed was pelleted at temperatures over 85°C while conditioning time did not seem to influence chick performance or feed intake. Furthermore, Vranjes et al. (1994) were able to detect the effects of carbohydrases on broiler performance even after feed was extruded at 110°C.

Simons et al. (1990) reported that the effect of shear pressure on phytase activity appears to be small. Even after feed containing supplementary phytase was subjected to high-energy inputs through the pelleting process, most of the initial activity remained. It was concluded that it is mainly the high temperatures reached as a result of the high-energy inputs that are responsible for phytase deactivation (Table 1.2.).

Table 1.2. Phytase activity of pelleted feed produced under various pelleting conditions

Temperature of the meal before the press (°)	Temperature of the pellets after pelleting (°)	Energy input (Watt h/kg)	Phytase activity in the pellets (units/kg)	Remaining activity
50	78	20	240	96
50	81	25	234	94
65	84	17	208	83
65	87	23	115	46

The phytase activity in the meal before the pelleting process was 250 units/kg (Simons et al., 1990)

There is disagreement as to the effect of steam pelleting on the apparent absorption of phosphorus. This may be due to different concentrations of phosphorus in the diet or inconsistent conditions at pelleting between different trials. Jongbloed and Kemme (1990) performed three digestibility trials on pigs in which diets and pelleting conditions were precisely controlled so as to eliminate these confounding effects. They established that pelleting phytase-deficient diets and legume-seed diets had no effect on mineral digestibility while pelleting a phytase rich diet did significantly lower digestibility for DM and minerals. They found that when pelleting temperatures reach 80°C and above, there is a dramatic decrease in phosphorus digestibility due to a loss in phytase activity. Simoes Nunes (1993) reported that temperatures in excess of 60°C strongly reduce phytase activity and that loss of activity is particularly marked for temperatures higher than 75°C while only 50 % recovery of phytase activity was obtained when pelleting at 80°C.

Variation in the ability of different fungal phytases to withstand pelleting has also been investigated. Wys et al. (1998) investigated the thermostable properties of three histidine-acid phosphatases from *Aspergillus niger* and *Aspergillus fumigatus*. Both the phytases from *A. niger* and *A. fumigatus* were denatured when exposed to temperatures between 55 and 90°C. However, *A. fumigatus* phytase was able to regain most of its initial activity after being exposed to temperatures of up to 90°C due to its ability to refold completely into its native-like, fully active conformation after denaturation. In contrast, phytase from *A. niger* underwent irreversible conformational change and inactivation with losses in activity of between 70 to 80 %.

1.9. CONCLUSION

The importance of phosphorus for the monogastric animal necessitates the supplementation of low-phosphorus cereal-based diets with inorganic phosphorus. Unfortunately this has often led to negative consequences for the environment, which in turn has prompted increased legislation, particularly in Europe, restricting the amount of phosphorus that can enter the soil through livestock manure. Future legislation will continue to limit inorganic phosphorus supplementation all over the world forcing farmers to adopt different strategies to meet the phosphorus requirements of their animals.

Phytase is an enzyme that is able to catalyse the stepwise removal of orthophosphate from phytic acid thus increasing the phosphorus digestibility of current feed ingredients. Phytases are produced by a variety of plant and animal tissues, however commercial phytase is produced by filamentous fungi. The most prolific producer to date being the *Aspergillus niger* strain 3135. Adding phytase to the diet serves three functions. Firstly, it lowers feed costs by reducing the need to supplement with inorganic phosphorus. Secondly, it releases metal-ions and proteins to be absorbed and thirdly it reduces the amount of phosphorus entering the environment in the manure. Improved phosphorus utilization leads to superior growth and performance through increased feed intake and weight gain. However, whether improved phosphorus utilization increases the gain/feed ratio remains unclear.

The advantages of supplementing phytase are offset somewhat by the variability in animal response. One source of variation is the result of the calcium phosphorus and vitamin D_3 interaction. Calcium influences phosphorus digestion by forming insoluble complexes with phytic acid that are resistant to phytase attack. In addition to this, excess calcium may alter the gut pH thus reducing the activity of the enzyme. The gut pH also affects the active transport of phosphorus due to the electrochemical nature of the process. Vitamin D_3 appears to have an independent yet synergistic relationship with supplementary phytase. It is thought that D_3 enhances the ability of enterocytes to absorb phosphorus in the small intestine thus facilitating phosphorus uptake. Another factor that appears to affect phytase supplementation is the AME content of the diet. This may be due to the formation of insoluble soaps between

calcium and phosphorus and free fatty acids, decreasing the digestion and absorption of these elements and reducing their concentration in the bone.

The influence of animal factors cannot be ignored. Variation exists between breeds, strains, and individual animals within the same strain in similar or dissimilar physiological states. This variation is the raw material needed for future genetic selection of animals that will be better able to utilize phytic acid phosphorus.

Processing methods such as pelleting also influence the efficacy of feed enzymes such as phytase. The aggressive techniques employed by modern feed manufacturers results in the inactivation of much of the enzyme present. Differences in pelleting conditions, phosphorus concentrations, and amount and type of phytase present lead to yet more variability.

In spite of these sources of variation, all indications are that phytase has an important role to play in current monogastric nutrition practices and will probably play an even more prominent role in future when each feed ingredient will have to justify its place in the diet.

CHAPTER 2. MOTIVATION

2.1. INTRODUCTION

Environmental concerns and a well-informed public have given the search for biological alternatives to the traditional practice of supplying pigs and poultry with hefty safety margins for phosphorus, a new sense of urgency. The search has so far yielded a number of fungi able to produce the enzyme phytase. This enzyme is capable of liberating phosphorus from phytic acid, an otherwise inaccessible source of phosphorus for monogastric animals. Unfortunately phytase, while performing well when fed in mash feed, is unable to withstand the high temperatures of pelleting. This is extremely significant in view of the fact that poultry feeds must typically endure high pelleting temperatures that are used to control pathogens such as *Salmonella*. Simoes Nunes (1993) reported that steam-pelleting feed at temperatures in excess of 60^0C strongly reduces the activity of phytase produced by the fungus *Aspergillus niger*. It was further demonstrated that this reduction increases as temperatures increase with only 50% of the original activity being detected at 80^0C, a pelleting temperature frequently used. However, due to the nature of the *in vitro* trials available at present, which involve assaying samples over extended incubation periods, accurately quantifying levels of enzyme activity in complex mixes has proven inaccurate (Lyons and Walsh, 1993). It is for this reason that *in vivo* studies are still commonly employed to demonstrate phytase activity in the feed. Further, a number of publications have used animal response data to demonstrate the existence of appreciable levels of enzyme activity after pelleting (Inborr and Bedford, 1994; Spring et al., 1996; Vukic Vranjes et al., 1994 and Joengbloed and Kemme 1990).

2.2. PHYTASE RESEARCH PROJECT

This study was carried out to investigate the effectiveness with which existing phytase enzymes of fungal origin are able to withstand the high temperatures associated with steam pelleting and to establish whether other phytases of fungal origin could be produced that would better withstand high temperatures.

The project was carried out in two phases. Phase one involved testing a number of wild-type fungi for phytase production under solid substrate culture (SSC) conditions with a view to possible scaled-up production and animal trials. A number of

filamentous fungi capable of producing heat-stable phytases have been cited in the literature. However, these were only used as a source of foreign DNA. Alternative vectors were employed for the actual over-production of the enzyme under submerged liquid culture (LC) conditions. This phase of the study attempted to cultivate these original donor fungi under SSC conditions with a view to further stimulating over production of the phytase enzyme through manipulation of the solid substrate (SS) environment.

Liquid culture fermentation is the fermentation technique traditionally used for the production of microbially derived enzymes and involves the submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth. Two important criteria of LC fermentation include the ability of the system to operate aseptically for a number of days and provide adequate aeration and agitation to meet the metabolic requirements of the microorganism. Most common designs are based on a stirred upright cylinder with sparger aeration (Filer 2000).

SS fermentation is an ancient technology based on the growth of substrates on water-insoluble substrates in the presence of varying amounts of free water (Mitchell and Losane, 1992). The single most important feature of SS fermentation is the low water content of the medium, which favours the growth of filamentous fungi, which grow well at water activities of between 0.93 and 0.98 (Filer, 2000). Bacteria and yeasts grow at a water activity of above 0.99. The substrates used for SS fermentation are composite and heterogeneous products from agriculture or by-products of agro-industries (Filer, 2000).

Solid substrate culture conditions were chosen to cultivate these organisms for the following reasons:

- The production facilities and personnel at Alltech, Inc. laboratories are geared towards SS cultivation, as this is the method of commercial phytase production employed by the company.
- Time restraints and the nature of the project made this method the most suitable.

- There is increased public resistance to genetically modified organisms (GMO) entering the food system. Liquid culture production systems employ GMOs as a rule whereas all SSC that we examined utilised non-GMOs.
- The extract obtained from SSC contains a number of additional enzymes with their associated side actions, many of which can contribute to the overall effectiveness of the enzyme preparation (Mitchell and Lonsane, 1992).
- SSC employs media that are relatively simple. This means that there is less pre-treatment required compared to LC.
- Low water availability in SSC helps to select against undesirable contaminants. Low moisture content may also favour the production of specific compounds that may not be produced under LC conditions.
- Smaller reactors are needed for SSC due to the concentrated nature of the substrate.
- Forced aeration of SSC is easier as interparticle spaces allow for the diffusion of air to the thin films of water spread over the wide surface area of the solid substrate.
- Spores can be used to inoculate the SS media thereby avoiding the need for large seed tanks. Spores also facilitate the even dispersion of the culture throughout the media.

Phase two of the study was designed to investigate the extent to which currently available commercial phytases are able to withstand steam pelleting. Two phytases were tested, a Solid Substrate Enzyme Product (SSEP) phytase, which is produced under SSC conditions and a Liquid Enzyme Product (LEP) phytase, an enzyme produced under LC conditions. These phytases were added to commercial rations and steam pelleted. Enzyme activity was then tested using an *in vitro* enzyme incubation assay and an animal trial. The results obtained from the pelleted feed were then compared to those of the unpelleted mash.

PHASE I SEARCH FOR A THERMOSTABLE PHYTASE

CHAPTER 3. FUNGAL SCREENING STUDY

3.1. INTRODUCTION

The purpose of this trial was to identify organisms that could produce detectable levels of a heat stable phytase enzyme under SSC conditions using standard koji media (30% soyabean meal and 70% wheat bran) in flasks. Koji media is a common SS as it promotes good growth of filamentous fungi under SSC conditions and is relatively inexpensive (Raimbault, 1998). This study was embarked upon with a view to selection of cultures, and the manipulation of growth conditions for enhanced enzyme production, if significant levels of phytase had been discovered. The final goal of this phase of the project was to scale-up production of the enzyme using the SSC tray system for eventual use in animal trials, provided that preliminary laboratory scale trials indicated that such production was feasible.

Six types of filamentous fungi were selected from the literature for this trial, based upon the reported ability of each to produce a thermophilic phytase. They included *Aspergillus ficuum*, *Aspergillus terreus*, *Talaromyces thermophilus*, *Myceliophthora thermophila*, *Aspergillus fumigatus* and *Thermomyces languginosis*. All the phytases from these wild-type fungi have, in the past, been successfully cloned into and overexpressed in other fungi or yeasts.

It was hypothesised that alterations to the environment of the wild-type fungus on SSC might promote the over-production of phytase by stimulating the increased expression of the appropriate gene.

Proposed modifications to the standard culture conditions descried under the materials and methods were the:

1. growth of cultures on a variety of alternative broth substrates.
2. use of spores as a means of inoculating the SS instead of using a broth culture.
3. utilization of alternative solid substrate culture media.
4. use of a variety of different incubation temperatures.

5. use of a variety of different incubation periods for both the broth and the SSC flasks.

3.2. MATERIALS AND METHODS

3.2.1. Source of organisms

The *A. terreus, A. ficuum, T. thermophilus* and *M. thermophila* fungal strains were acquired from the American Type Culture Collection (ATCC). Their respective reference numbers are 1012, 66876, 20186 and 48102. All these organisms were delivered as freeze-dried double vial preparations. Procedures from the ATCC 2000 pamphlet guide: "Instructions for Rehydration of Freeze-Dried Cultures" were used to revive and transfer the organisms to test tubes containing liquid media. The fungi were grown up in these test tubes before finally being transferred to slants for storage until needed. The *A. fumigatus* and *T. languginosis* strains were obtained as slants from the Alltech, Inc. culture collection.

3.2.2. Preparation of the slant culture

The slants were prepared in 18 x 180mm test tubes. The slant media used were the same as those used for the liquid media (Table 3.1.) but for the addition of agar at a rate of 15g per litre. The media were mixed and boiled to dissolve the agar and then dispensed into test tubes (6ml per tube). The tubes were then sealed with cotton wool stoppers and sterilised at 121^0C for 20 min. The tubes were then allowed to cool on a slightly sloping surface so that the media solidified along the length of the tubes providing a large area for maximum growth. Inoculation of the slants was performed according to the ATCC 2000 pamphlet guide: "Instructions for Rehydration of Freeze-Dried Cultures".

3.2.3. Preparation of the broth culture

ATCC and Alltech, Inc. guidelines (Table 3.1.) were used to formulate a broth media preparation particular to each type of organism. Two hundred millilitres of this broth was decanted into each of a number of 500ml conical flasks. The flasks were then plugged using cotton wool stoppers and sterilised at 121^0C for 20 min. After sterilisation, the flasks were cooled to room temperature and inoculated with agar from working slants. They were then incubated in a shaking incubator at

temperatures recommended by ATCC and Alltech, Inc. guidelines (Table 3.1.) for approximately 72 hours. Those flasks devoid of contamination after the incubation period were selected to inoculate the SS flask cultures.

Table 3.1. Propagation guidelines for fungal cultures

Culture	Propagation medium	Incubation temperature
Aspergillus ficuum[1]	Potato dextrose broth	24^0C
Aspergillus terreus[1]	ME broth (Blakeslee's formula)	26^0C
Talaromyces thermophilus[1]	ME broth (Blakeslee's formula)	50^0C
Myceliophthora thermophila[1]	MRS broth	50^0C
Aspergillus fumigatus	ME broth (Blakeslee's formula)	30^0C
Thermomyces languginosis	YpSs broth	50^0C

[1]ATCC propagation guidelines obtained from www.ATCC.org

3.2.4. Preparation and inoculation of solid substrate flask cultures

Conical flasks (500ml capacity) were used to culture the organisms. Ten grams of dried SS product were placed into each flask. The standard solid substrate was a 30%/70% blend of soya and wheat bran respectively (koji media). Additional substrates were also used to culture the *A. fumigatus* and *T. languginosis* fungal strains as previous experimentation at Alltech, Inc. with these substrates suggested the possibility of enhanced phytase production with these organisms. These additional substrates included pure wheat bran, pure yucca fibre and a 30%/70% blend of yucca fibre and wheat bran. Once the SS was decanted into the flasks, the openings were sealed with a bio-shield and the flasks then sterilised at 121^0C for 20 minutes.

The inoculum was prepared by pippetting 2ml of broth culture into 80ml of sterile water in 125ml dilution bottles. The bottles were then shaken and 8ml of this solution was pippetted, under sterile conditions, into each of three conical flasks. The flasks were then shaken until the consistency of their contents became uniform. They were

then placed in an incubator for a period of 5 days at the same temperatures recommended for the incubation of the working broth (Table 3.1.).

3.2.5. Enzyme extraction

Tap water (200ml) at 40^0C was poured into each flask. The flasks were placed in an incubator at 37^0C for 2 hours after the contents of each were thoroughly mixed. Approximately 5ml of the extract from each flask was then filtered through a wire sieve into separate test tubes.

3.2.6. Enzyme assay

This *in vitro* phytase assay is based upon the measurement of the amount of ortho-phosphate released by the enzymatic hydrolysis of sodium phytate under controlled conditions. The assay was performed at 37^0C using phytate nonahydrate (Sigma Chemical Co. No. P-8810 from corn) as a substrate. Extract samples were dissolved in a 5mM acetate buffer solution (pH 5.5) according to the desired dilution rate. The samples were then readied by pippetting 0.5ml of each preparation into 10ml test tubes and allowing the solution to equilibrate to temperature. At time zero, 0.5ml of substrate solution was added to each sample and the mixtures then vortexed. The test tubes were then incubated for 10 min after which 2ml of a colour stop solution consisting of one part ammonium molibdate solution (10mM), one part 5N sulphuric acid and two parts acetone, was added followed by 0.1ml of 1M citric acid after a further 30 sec interval. Activity was measured spectrophotometrically at 380nm against a standard curve of KH_2PO_4 at concentrations of 0.1, 0.2, 0.3, and 0.4 µmol of phosphorus per ml. Blanks were prepared by pippetting 0.5ml of the sample solution into 10ml test tubes and adding 2ml of the colour stop solution before vortexing thoroughly. After this, 0.5ml of substrate solution was added to the test tubes and the tubes were then vortexed again. Finally, 0.1ml of the 1M citric acid solution was added after a 30 second interval and the solution vortexed. Phytase activity was expressed in phytase units per gram. One phytase unit (PU) is defined as the quantity of the enzyme required to release 1 micromole of phosphorus per minute from sodium phytate under assay conditions. The following formula was used to calculate the phytase activity per gram:

$$PU/g = (OD_T - OD_{TB}) \times F \times 2 \times 1/10 \times 1/W \times dilution$$

where OD_T equals the sample absorbance and OD_{TB} equals the absorbance of the blank solution. F represents the phosphate concentration (µmol/ml) corresponding to a change in absorbance of 1.0 obtained from the standard curve. W equals the sample weight in grams.

3.3. RESULTS

A threshold value of 400 PU/g was set for the flask cultures based upon previous experience at Alltech, Inc. It was decided that without this minimum level of activity, under the standard conditions listed in the materials and methods, any further experimentation with culture selection and alternative conditions would be unlikely to yield significantly improved results. Phytase production for the six fungi tested is shown in Table 3.2.

Table 3.2. Phytase yield for six varieties of fungi cultivated under solid substrate culture conditions

Culture	**Incubation temp**	**Replicates**	**Average yield (PU/g) per treatment[2]**			
			Soya/ Wheat bran[1]	**Wheat bran**	**Yucca fibre/ Wheat bran[1]**	**Yucca fibre**
Aspergillus ficuum	24^{0}C	10	ND	-	-	-
Aspergillus terreus	26^{0}C	4	ND	-	-	-
Talaromyces thermophilus	50^{0}C	8	ND	-	-	-
Myceliophthora thermophila	50^{0}C	4	ND	-	-	-
Aspergillus fumigatus	30^{0}C	2	8.7	8.5	9.7	9.6
Thermomyces languginosis	50^{0}C	1	ND	ND	ND	ND

[1] Respective 30%/70% blend of substrates.

[2] PU - phytase unit (1 PU = 1 micromole of phosphorus released/minute from sodium phytate under assay conditions).

ND - none detected.

None of the fungi tested demonstrated the ability to produce phytase enzyme to any appreciable extent and therefore all planned further testing was suspended.

3.4. DISCUSSION

Factors involved in the regulation of genes that control the synthesis of the phytase enzyme in filamentous fungi have been well researched. Ware and Shieh (1967) discovered that the available inorganic phosphorus content of the medium regulated the synthesis of the enzyme. It was established that *A. niger* 3135 produced the maximum levels of phytase when the inorganic phosphorus was maintained in the range of 0.001% to 0.005%. The reduction of activity at higher levels of phosphorus is a general phenomenon and is observed in all moulds and yeasts that produce phytase (Shieh and Ware 1968). Mandviwala and Khire (2000) demonstrated the same phenomenon when they used *A. niger* NCIM 563 for the production of extracellular phytase on SS media. They observed that enzyme production increased as the phosphate concentration in the medium increased from approximately 75U g^{-1} dry mouldy bran (DMB) at 0mg phosphate per 100g wheat bran up to 125.5U g^{-1} DMB at 10mg phosphate per 100g wheat bran. However, above this concentration the level of phytase detected decreased sharply and at 50mg phosphate per 100g wheat bran only 70U g^{-1} DBM phytase activity was detected. Mandviwala and Khire (2000) defined one unit of phytase activity (U) as the amount of enzyme that liberates 1µmol phosphorus per minute under the standard assay conditions employed. These conditions differ from the standard assay conditions described under materials and methods.

Although vigorous fungal growth on all substrates tested was obtained, it was not possible to significantly alter the regulation of phytase under the conditions tested. It was accepted that phytase production probably occurred under the culture conditions applied, but not at levels that could be detected with the assay procedure employed. Enzyme regulation and enzyme production were not uncoupled sufficiently so as to obtain the rate of phytase production necessary to perform animal trials. It was decided that further testing of these organisms was unlikely to produce significantly more phytase, even if the modified conditions proposed had been instituted.

3.5. CONCLUSION

Testing under the conditions of the assay revealed that none of the six fungi tested for phytase production produced sufficient quantities of the enzyme, under the test conditions employed, to warrant further steps. A more extensive screening process, using techniques such as UV mutagenesis and selective media, might successfully uncouple regulation and enzyme production for these six fungal types, to the extent that producing significant quantities of a heat stable phytase may become feasible.

PHASE II THERMOSTABILITY OF TWO COMMERCIAL PHYTASES

CHAPTER 4. BROILER TRIAL

4.1. INTRODUCTION

Recent investigations into the thermostability of feed enzymes have revealed that many are able to retain a high degree of activity after being subjected to the extreme temperatures and pressures associated with steam pelleting. Pelleting temperatures employed in industry can be as high as 90^0C and above, although they are more commonly in the region of 80^0C. Some reports from chick performance studies have suggested that feed enzymes improve nutrient utilization in unpelleted and pelleted diets to a similar extent, even though *in vitro* measurements have revealed a reduction in enzyme efficacy of 50% or more after pelleting (Vukic Vranjes et al, 1995). Inborr and Bedford (1994) demonstrated a significant improvement in broiler performance when enzymes were added to feed. This performance was only affected when feeds were pelleted at temperatures in excess of 85^0C. It is thought that additional interactions may occur between the enzymes and the starch and fibre components of the feed that have been modified due to pelleting which could explain this phenomenon (Vukic Vranjes et al, 1995).

The purpose of this trial was to determine the thermostability of two varieties of commercial phytase, the Solid Substrate Enzyme Product (SSEP) and the Liquid Enzyme Product (LEP) (i.e. two different commercial methods of producing the *A. niger* phytase described on pages 14 and 15), by means of a chick performance experiment. It was also the intention of this study to ascertain whether the SSC phytase product begets superior performance as a result of the detectable levels of side activities present, including cellulase, protease, and xylanase, which are not present in the LC product. Further, the trial set out to establish whether an *in vitro* in-feed assay properly reflects the responses obtained from chick performance experiments for feed supplemented with phytase.

4.2. MATERIALS AND METHODS

4.2.1. Broiler source

Four hundred male day-old Ross broilers were supplied from a commercial hatchery in the vicinity of the University of Maryland Eastern Shore (UMES) poultry facility in Princess Anne, Maryland, USA.

4.2.2. Vaccination

The chicks were vaccinated *in ovo* for Marek's disease at 18 days of incubation and spray vaccinated for Infectious Bronchitis and Newcastle Disease at one day of age.

4.2.3. Trial facilities

The chicks were housed in stainless steel batteries in an environmentally controlled room (Figure 4.1.). The temperature and ventilation of the poultry house was set to simulate that of a typical commercial production system. Fluorescent lighting was used to illuminate the facility 24 hours a day. The birds were fed from feed troughs placed in each battery. Jar drinkers were placed in each cage as the sole source of water at the onset of the trial and then simultaneously with external trough drinkers on day 10. The trough drinkers became the sole source of water from day 11 up until the end of the trial period.

Figure 4.1. Trial batteries with external trough drinkers and internal trough feeders

4.2.4. Bird allotment

Three hundred and seventy nine birds were wing banded and weighed on day 4. Weak and sickly birds were avoided. The chicks were ranked according to weight and a histogram was drawn up describing the weight distribution. From this, the most uniform group of 336 chicks was selected. Forty-eight groups of 7 birds each were then formed by grouping every seventh bird in the list together. Total and average weights were calculated for each replicate.

4.2.5. Experimental design

A randomised complete block design was used to conduct this trial. Ten treatments were decided upon, with 5 replicates per treatment with the exception of Treatments 3 and 6 where only 4 replicates were used due to the limitations of the facilities. Seven chicks were allocated to each replicate at 4 days of age. The study consisted of a 2 x 5 factorial arrangement with feed consistency and dietary non-phytate phosphorus (NPP) being the two main factors (Table 4.1.). Two feed consistencies were utilized (mash vs. pelleted) with five levels of dietary NPP (0.25%, 0.3%, 0.35%, 0.25% + 300 PU/kg SSEP, and 0.25% + 300 PU/kg LEP).

Table 4.1. Dietary treatments

Treatment	Feed consistency	Available P (%)	Enzyme supplement
1	Mash	0.25	-
2	Mash	0.30	-
3	Mash	0.35	-
4	Pellet	0.25	-
5	Pellet	0.30	-
6	Pellet	0.35	-
7	Mash	0.25	SSEP
8	Mash	0.25	LEP
9	Pellet	0.25	SSEP
10	Pellet	0.25	LEP

4.2.6. Experimental diets

All birds were placed on a standard maize-soyabean based starter ration containing 0.5% NPP from days 0-3. They were then fed the treatment diets from day 4 to day 18 after an initial overnight fast. The experimental treatment diets were maize-soyabean based with supplemental vitamins and minerals added (Table 4.2.). Dicalcium phosphate and limestone were added to the diets as sources of NPP and calcium. The dihydrate DCP used in this trial was assumed to be 100% available. All experimental diets were formulated to contain 1.00% calcium and graded levels of NPP (0.25%, 0.30%, and 0.35%). NRC (1994) recommendations for crude protein and ME were adhered to. Treatments 1-3 and 4-6 contained 0.25%, 0.30% and 0.35% NPP respectively. These diets were formulated so as to provide standard response curves (i.e. NPP levels below the requirements of the birds were used so as to obtain a linear relationship between NPP and growth and performance variables). Treatments 7 and 9 contained 0.25% NPP with the addition of 300 PU/kg SSEP. Treatments 8 and 10 also contained 0.25% NPP but with the addition of 300 PU/kg LEP. The inclusion rates for these enzymes were determined on an equivalency basis using the *in vitro* enzyme assay described under the materials and methods of the fungal screening study (3.2.6.). It was found that 0.03% of the SSEP or 0.01% of the LEP were equivalent to approximately 300 PU/kg of phytase activity.

Treatments 1, 2, 3, 7, and 8 remained unaltered as mash diets while Treatments 4, 5, 6, 9, and 10 were pelleted. The rations were steam-pelleted using a California Pelleting Mill Co. Master Model (30 hp) pelleter and conditioner. A 3.2mm pelleting die was employed with a width of 50.8mm and a relief of 12.7mm. A pelleting temperature of 80^0C was selected. Both pelleted and mash rations were passed through a hammer mill before feeding so that a consistent particle density was achieved for all treatments. Feeds were formulated to contain 0.25% Cr_2O_3 for phosphorus digestibility analysis. The chicks were allowed *ad libitum* access to their respective diets and water.

Table 4.2. Composition and calculated analysis of experimental diets

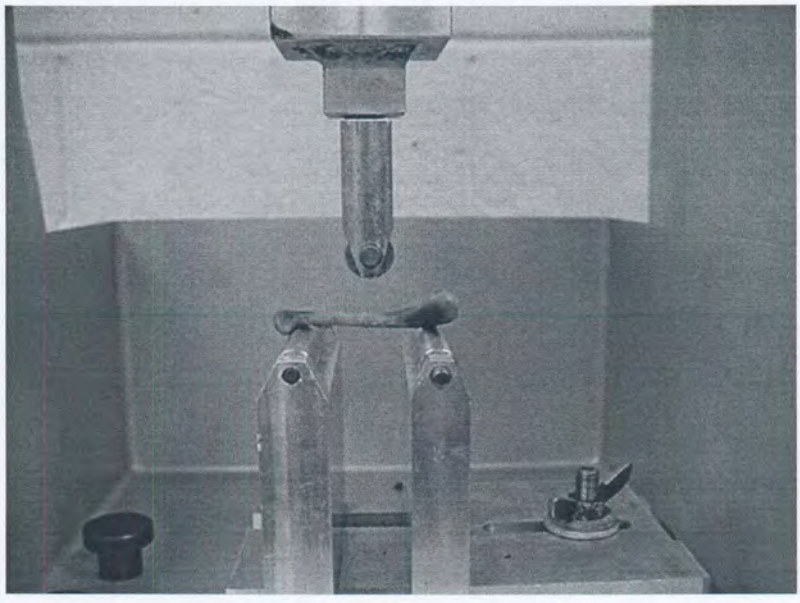
	Treatments					
	Starter	1 & 4	2 & 5	3 & 6	7 & 9	8 & 10
Ingredient	%	%	%	%	%	%
Maize	52.60	53.21	53.10	52.99	53.18	53.20
SBM, dehulled (48%)	38.50	38.50	38.50	38.50	38.50	38.50
Soyabean Oil	4.32	4.32	4.32	4.32	4.32	4.32
Dicalcium phosphate	2.00	0.68	0.93	1.18	0.68	0.68
Limestone	1.27	1.98	1.84	1.70	1.98	1.98
SSEP phytase	-	-	-	-	0.03	-
LEP phytase	-	-	-	-	-	0.01
Salt, iodised	0.50	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin/mineral mix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Coccidiostat[2]	0.05	0.05	0.05	0.05	0.05	0.05
Chromic oxide	0.25	0.25	0.25	0.25	0.25	0.25
Anti oxidant[3]	0.01	0.01	0.01	0.01	0.01	0.01
Calculated Analysis						
ME, kcal/kg	3191	3212	3208	3204	3211	3212
Crude Protein, %	23.10	23.15	23.14	23.13	23.15	23.15
Lysine, %	1.26	1.26	1.26	1.26	1.26	1.26
Methionine, %	0.60	0.60	0.60	0.60	0.60	0.60
Cystine, %	0.37	0.37	0.37	0.37	0.37	0.37
Met + Cys, %	0.97	0.97	0.97	0.97	0.97	0.97
Threonine, %	0.87	0.87	0.87	0.87	0.87	0.87
Calcium, %	1.00	1.00	1.00	1.00	1.00	1.00
Non Phytate P, %	0.50	0.25	0.30	0.35	0.25	0.25
Phytase activity, PU[1]/kg	0	0	0	0	300	300

[1]Supplies per kilogram of diet: vitamin A, 11,025 IU; vitamin D_3, 528 IU; vitamin E, 33 IU, B_{12} 0.03mg; vitamin K (as sodium menadione bisulfite complex) 2.8mg; riboflavin 7.7mg; d-pantothenic acid, 17.6mg; thiamine, 2.2mg; niacin 55mg; B_6 4.9mg; folic acid 1.1mg; choline, 479mg; d-biotin 0.22mg; Zn, 75mg; Fe, 40mg; Mn, 64mg; Cu, 10mg, I, 1.9mg; Se, 0.3mg.
[2]Amprolian 25%.
[3]Endox.

4.2.7. Variables monitored

Mortalities were monitored on a daily basis. Dead birds were weighed and their weights recorded. The feed intake of each pen was also recorded. Faecal samples were taken from each pen over a 24-hour period starting on day 16. A proximate and mineral analysis of all feeds as well as a faecal mineral analysis for each pen was performed at a commercial laboratory. The faeces were analysed for Sulphur, Potassium, Calcium, Phosphorus, Magnesium, Sodium, Iron, Manganese, Copper and Zinc. All birds were slaughtered and weighed on day 18. Feeding was suspended 12-hours prior to the cull. The right tibia of each bird was removed and frozen. The tibias were then thawed and the flesh and tibial caps removed. The bone breaking strength was subsequently determined by placing each individual bone across a set of rollers and applying a constant pressure to each using an Instron machine (Figure 4.2.). The force (kg) required to break through the centre of each bone was recorded. All pieces of bone were collected after breaking and pooled by pen for percentage bone ash determination, measured according to AOAC (1976) procedures. The third toe of each foot was severed between the third and fourth phalangeal joint, pooled according to pen and dried at 100^0C. The toes were then weighed, ashed and reweighed to determine percent toe ash.

Figure 4.2. Bone breaking strength determination using the Instron machine



4.2.8. Determination of phosphorus equivalency values for phytase

Phosphorus equivalency values for each variety of phytase were determined by employing inverse regressions as described by Draper and Smith (1981) (Figure 4.3.). Using this technique, the fitted straight line and the curves that give the endpoints of the 100(1 – α)% confidence intervals (α is 1 minus the probability that an estimated mean X-value lies in the calculated interval) for the true mean value of Y given X are drawn. A horizontal line is then sketched perpendicular to the X-axis at a height which corresponds to a particular Y value (Yo). Where this line cuts the confidence interval curves, perpendiculars are dropped onto the X-axis to give lower and upper 100(1 - α)% "fiducial limits" labelled X_L and X_U on Figure 4.3. The perpendicular from the point of intersection of the two straight lines onto the X-axis gives the inverse estimate of X, calculated by solving the straight line equation fitted to each regression ($Yo = b_o + b_1Xo$) for Xo.

$$Xo = \frac{(Yo - b_o)}{b_1}$$

The X_L and X_U values are obtained by setting the equation for the horizontal straight line

$$Yo = b_o + b_1 Xo$$

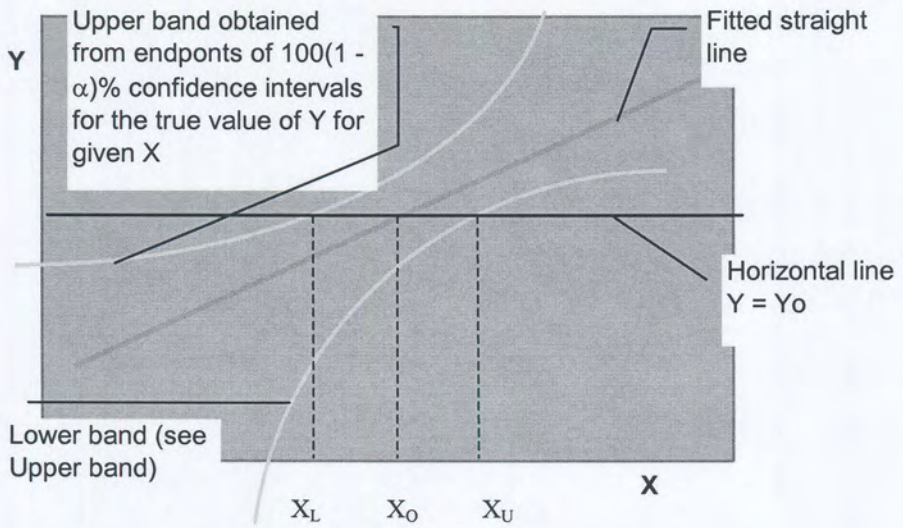
and the curve

$$Yo = Yx_{U/L} \pm ts\left\{\frac{1}{n} + \frac{(X_{U/L} - \overline{X})^2}{Sxx}\right\}$$

equal to each other to find the X-coordinate of the point of intersection. $Sxx = Yx_{L/U} = bo + b_1x_{L/U}$, $t = t(v, 1 - \alpha/2)$ is the usual t percentage point, and v is the number of degrees of freedom of s^2. Solving for X_L or X_U results in the following equation:

$$X_{U/L}\} = \overline{X} + \frac{b_1\left(Yo - \overline{Y}\right) \pm ts\left\{\left(Yo - \overline{Y}\right)^2 / Sxx\right] + (b_1^{\,2} + b_1^{\,2}n/n) - (t^2s^2/nSxx + Sxx)\right\}^{\frac{1}{2}}}{b_1^{\,2} - (t^2s^2/Sxx)}$$

Figure 4.3. Inverse regression description



(Draper and Smith, 1981)

The average quantities, and associated 95% fiducial limits, for NPP liberated by 300PU/kg of either variety of phytase, on both the mash and the pelleted diets was determined based upon the regression of Treatments 1-3 and 4-6 on percent bone ash, percent toe ash and the bone breaking strength data. For example the amount of NPP liberated by 300PU/kg SSEP on the mash diet, as determined by percent bone ash (Figure 4.4.), was calculated as follows:

$$Xo = (Yo - 25.63)/68.48$$

Yo = LS Mean of percent bone ash for birds on the 300PU/kg SSEP + 0.25% NPP = 48.18 %

$$= (48.18 - 25.63)/66.03$$

$$= 0.327\% \text{ NPP}$$

Therefore, Released NPP = 0.327% - 0.25% (0.25% NPP = basal NPP level inherent to the diet)

$$= 0.077\%$$

The 95% fiducial limits were calculated as follows:

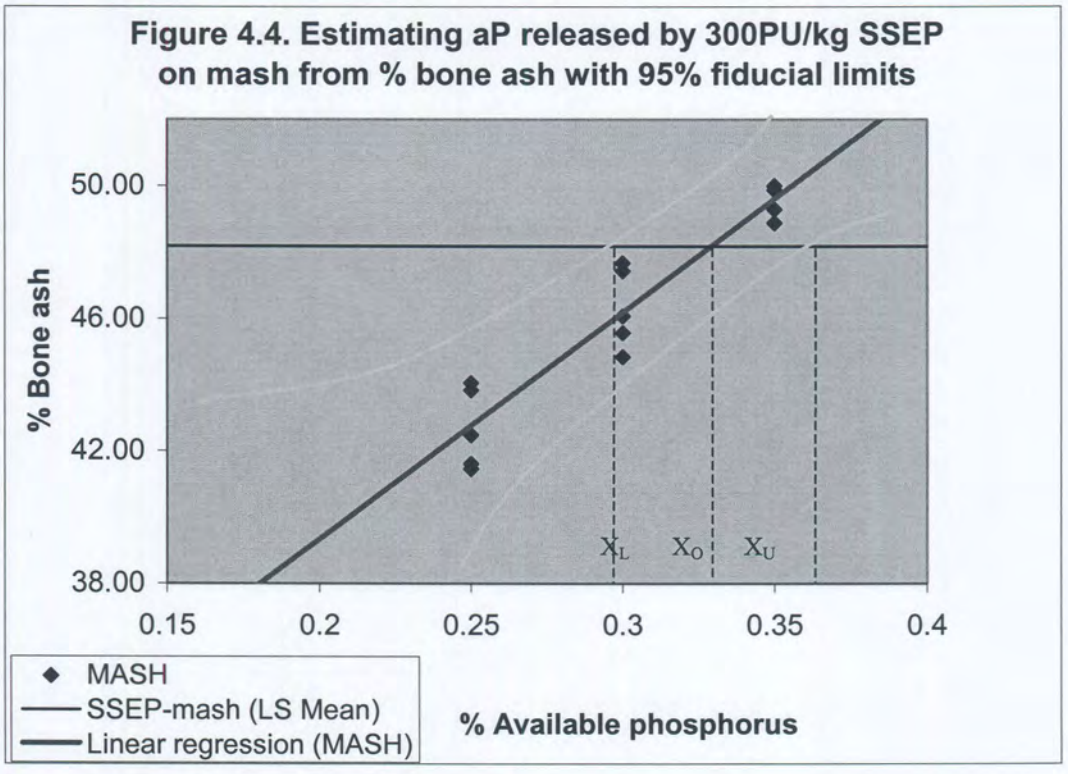
$$X_L = 0.296 + \frac{66.03(2.25) - ts\{(2.25^2/Sxx) + (66.03^2 + 66.03^2 n/n) - (t^2 s^2 n/nSxx + Sxx)\}^{1/2}}{66.03^2 - (t^2 s^2/Sxx)}$$

$$X_L = 0.290$$

$$X_U = 0.296 + \frac{66.03(2.25) + ts\{(2.25^2/Sxx) + (66.03^2 + 66.03^2n/n) - (t^2s^2n/nSxx + Sxx)\}^{1/2}}{66.03^2 - (t^2s^2/Sxx)}$$

$X_U = 0.376$

where $t = 2.18$, $s = 1.17$, $Sxx = 0.022$ and $n = 14$



4.2.9. Determination of phytase activity in feed

The in-feed assay follows the same principles as the standard assay procedure for determination of phytase activity described in the first phase of the study (3.2.6.). The phytase enzyme is incubated with a known concentration of the sodium salt of phytic acid under specified experimental conditions. During incubation, phytase liberates inorganic phosphate from phytic acid. The reaction is then terminated by the addition of a colour-stop (molybdate/vanadate) reagent. The liberated phosphate and the reagent form a yellow complex, which can be used to determine the phosphate concentration spectrophotometrically at a wavelength of 415nm.

The feed samples were prepared by grinding a small portion of each treatment into a fine meal using a coffee grinder and then weighing 5g of this meal into duplicate

125ml dilution bottles. The samples were then suspended in 50ml of pre-treatment buffer (acetate based- pH 5.45-5.55) and shaken for 60 minutes. The top layer from each bottle was then filtered off with Whatman No. 4 filter paper and 1ml of this filtered solution poured into each of three test tubes (duplicate reaction tubes and a single sample blank). At time zero, 4ml of phytic acid solution (2.10g phytic acid dodecasodium salt in 250ml acetate buffer- pH 5.45-5.55) was added to the first reaction test tube, the tube was then vortexed and placed in a water bath at 37^0C for exactly 60 minutes. This was repeated every ten seconds for all sample reaction tubes. After the incubation, 4ml of the colour-stop reagent was added to each sample and 15 minutes allowed for colour development to occur. All samples were then centrifuged for 5 minutes at 3, 000 x g before readings were taken on the spectrophotometer.

A separate phytase sample with a determined amount of enzyme activity was diluted so that a reaction curve consisting of six concentrations of the enzyme was obtained. The levels of activity for the six standards were 0.05, 0.10, 0.25, 0.50, 1.00 and 1.50 PU/ml respectively. Two millilitres of each standard dilution was poured into each of two test tubes. One of the tubes was used as a reaction tube and underwent the same treatment as the samples while the other tube was used as a blank.

Sample blanks were prepared by adding 4ml of colour-stop reagent to each tube containing the sample and then vortexing the mixture. Four millilitres of phytic acid was then added and the solution vortexed again. Once more, 15 minutes was allowed for colour development to take place before readings were taken.

Net absorbance was determined by subtracting the absorbance of the blank from that of the reaction tube. The absorbance and activity data were then graphed, activity on the X-axis and the absorbance on the Y-axis. The activity of the samples was then calculated by graphing absorbance against the standard curve using regression calculations and multiplying by the dilution factor according to the formula:

$$PU/g = (X)(\text{Dilution factor}) / \text{Sample Weight (g)}$$

4.2.10. Determination of Phosphorus digestibility

The digestibility of the total dietary phosphorus in each treatment was calculated from an indigestible indicator; chromium in the form of chromic oxide (Cr_2O_3). All treatments were supplemented with 0.25% Cr_2O_3. Samples of faecal matter excreted over a 24 hour period from day 16 of the trial were collected from each pen. The faecal samples along with samples from each treatment diet were then sent to a commercial laboratory for determination of the total phosphorus (tP) concentrations. The average tP concentration for the six diets containing 0.25% NPP was calculated from Treatments 1, 4, 7, 8, 9 and 10. Similarly, average tP concentrations were calculated for the 0.30 and 0.35% NPP diets by pooling tP concentrations for Treatments 2 and 5 and Treatments 3 and 6 respectively. Average tP concentrations for diets containing 0.25, 0.30 and 0.35% NPP were calculated to be 0.66, 0.75 and 0.78% respectively. Total phosphorus digestibility values were calculated according to the following formula:

$$tPdigestibility = 100 - \left[100 * \frac{\%\text{Cr Feed}}{\%\text{Cr Faeces}} * \frac{\%tPFaeces}{\%tPFeed} \right]$$

(Church, 1976)

4.2.11. Statistical analysis

The statistical analysis of the data was performed using the PC-SAS Version 8.01 commercial software. The LS Means test was used to determine the significance of differences between treatment means at a $P < 0.05$ level.

4.3. RESULTS

4.3.1. Growth results (days 4 – 18)

4.3.1.1. Average weight gain (g/bird)

There was no significant interaction between feed consistency and phosphorus level for the average weight gained by the birds over the course of the trial i.e. similar responses were obtained on both varieties of feed over the different levels of dietary NPP and visa versa, therefore only main effects were analysed.

Pelleting the feed had a significant effect on average weight gain (P = 0.0099) as demonstrated by Table 4.3.

Table 4.3. Least Square Means for average weight gains (g/bird) for the mash and pelleted treatments (P = 0.0099)

Feed	LS Means	SE
Mash	356	3.9
Pellet	341	3.9

The level of NPP in the diet also had a significant effect on the average weight gain of the birds. The Least Square (LS) Means for average weight gain over the different levels of NPP are listed in Table 4.4.

Table 4.4. Least Square Means for average weight gain (g/bird) over the different levels of NPP

NPP level	LS Means	SE
0.25%	330	6.02
0.25% + SSEP	345	6.02
0.25% + LEP	345	6.02
0.30%	355	6.02
0.35%	365	6.73

Increasing the NPP in the diet from 0.25% to 0.35% with inorganic phosphorus showed a corresponding increase in the LS Means for average weight gain although the difference in intake between the birds fed the 0.30% NPP and 0.35% NPP was not significant (P = 0.4507). There was no significant difference in weight gain between the 0.25% NPP + SSEP and 0.25% NPP + LEP treatments (P = 0.9997). Both enzyme-supplemented rations were compared to the treatments with graded levels of NPP. The average weight gained for either phytase-supplemented treatment was not significantly different from the 0.25 or 0.30% NPP diets, while quantitatively both were almost exactly in between (Table 4.4.).

4.3.1.2. Average feed intake (g/bird)

There was no significant interaction effect between feed consistency and phosphorus level on the average feed intake of the birds over the course of the trial, therefore only main effects were analysed.

Pelleting the feed significantly decreased average feed intake (P = 0.0475) as shown in Table 4.5.

Table 4.5. Least Square Means for average feed intake (g/bird) for the mash and pelleted treatments (P = 0.0475)

Feed consistency	LS Means	SE
Mash	521	6.22
Pellet	502	6.22

Furthermore, the level of NPP in the diet also had a significant effect on the average feed intake of the birds. The Least Square Means for feed intake are listed in Table 4.6.

Table 4.6. Least Square Means for average feed intake (g/bird) over the different levels of NPP

NPP level	LS Means	SE
0.25%	467	9.59
0.25% + SSEP	508	9.59
0.25% + LEP	518	9.59
0.30%	521	9.59
0.35%	543	10.73

Increasing the NPP in the diet from 0.25% to 0.35% with inorganic phosphorus showed a corresponding increase in the LS Means for average rates of feed intake, although the difference in intake between the birds fed the 0.30% NPP and 0.35% NPP was not significant (P = 0.1436). There was also no difference in intake found between the SSEP and LEP supplemented diets (P = 0.4547). These enzyme-

supplemented rations were compared with those treatments with graded levels of NPP. The average feed intake of the birds on the SSEP supplemented treatments was not significantly different from those on the 0.30% NPP diets, while the birds on the LEP supplemented diets had an average intake not significantly different from those on either the 0.30 or 0.35% NPP treatments. Numerically however, feed intakes of birds on the LEP supplemented diet were closer to those on the 0.30% NPP level.

4.3.1.3. Feed conversion ratio (gain/feed)

There was no significant interaction effect between feed consistency and phosphorus level on the feed efficiency of the birds over the course of the trial, therefore only main effects were analysed. Pelleting did not significantly influence the feed conversion ratio (P = 0.7391). The LS Means are presented in Table 4.7.

Table 4.7. Least Square Means for the gain/feed ratio for the mash and pelleted treatments (P = 0.7391)

Feed	LS Means	SE
Mash	0.685	0.005
Pellet	0.688	0.005

Numerically, feed efficiency was found to decrease as the level of NPP in the diet was increased from 0.25% to 0.35%, although these differences were not significant . The two phytase supplemented treatments differed significantly from each other (P = 0.0412). However, neither differed significantly from the other graded NPP treatments. The Least Square Means for feed conversion over the different levels of NPP are described in Table 4.8.

Table 4.8. Least Square Means for the feed/gain ratio over different levels of NPP

NPP level	LS Means	SE
0.25%	0.707	0.008
0.25% + SSEP	0.690	0.008
0.25% + LEP	0.667	0.008
0.30%	0.688	0.008
0.35%	0.679	0.009

4.3.2. Production results (days 4 – 18)

4.3.2.1. Percent bone ash

Significant interaction effects were found between feed consistency and NPP level for the percent bone ash parameter i.e. there was a significant difference in response between treatments for both varieties of feed over the different levels of dietary NPP and visa versa, therefore individual treatment means were compared. The data is presented in Table 4.9 and Figure 4.5.

Table 4.9. Percent bone ash of broilers fed two different sources of phytase with and without pelleting

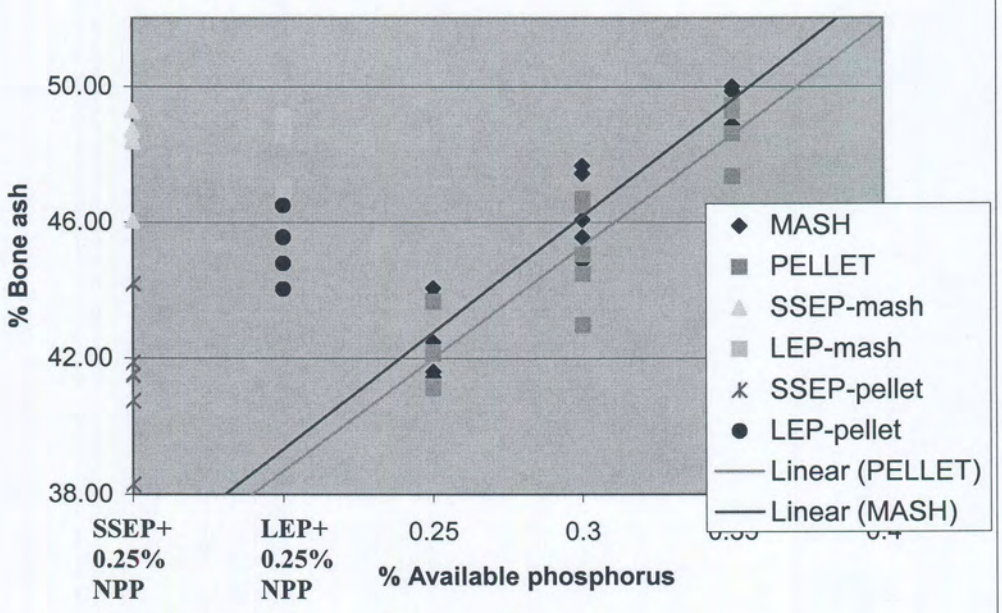
Treatment	Feed consistency	NPP (%)	Enzyme supplement	Bone ash (%)
1	Mash	0.25	-	42.68
2	Mash	0.30	-	42.36
3	Mash	0.35	-	49.51
4	Pellet	0.25	-	42.07
5	Pellet	0.30	-	45.14
6	Pellet	0.35	-	48.69
7	Mash	0.25	SSEP	48.18
8	Mash	0.25	LEP	48.02
9	Pellet	0.25	SSEP	41.32
10	Pellet	0.25	LEP	45.28

Increasing the percentage NPP in either the mash or the pelleted treatments produced a linear improvement in percent bone ash (Figure 4.5.). However, neither the pellet nor the mash treatments demonstrated a significantly superior ability to increase the percent bone ash accumulation over the other. No significant difference in percent bone ash was detected between the SSEP and LEP treatments when supplemented on the mash ($P = 0.8431$). Furthermore, birds on these two treatments produced approximately as much bone ash as those on the 0.35% NPP mash treatment.

When comparing the bone ash of the birds fed the pelleted rations it was found that those fed the 0.25% NPP + SSEP produced a similar amount of bone ash to those on the 0.25% NPP ($P = 0.3588$) indicating a loss in enzyme activity with pelleting.

Conversely, the percent bone ash of birds fed the 0.25% NPP + LEP on the pelleted ration did not differ significantly from those on the 0.30% NPP pelleted ration (P = 0.8632) which suggests that a degree of enzyme activity was retained after pelleting.

Figure 4.5. Effects of pelleting and phytase source on % bone ash



4.3.2.2. Percent toe ash

Significant interaction effects were found between feed consistency and phosphorus level for the percent toe ash parameter, therefore individual treatment means were compared. The data is presented in Table 4.10 and Figure 4.6.

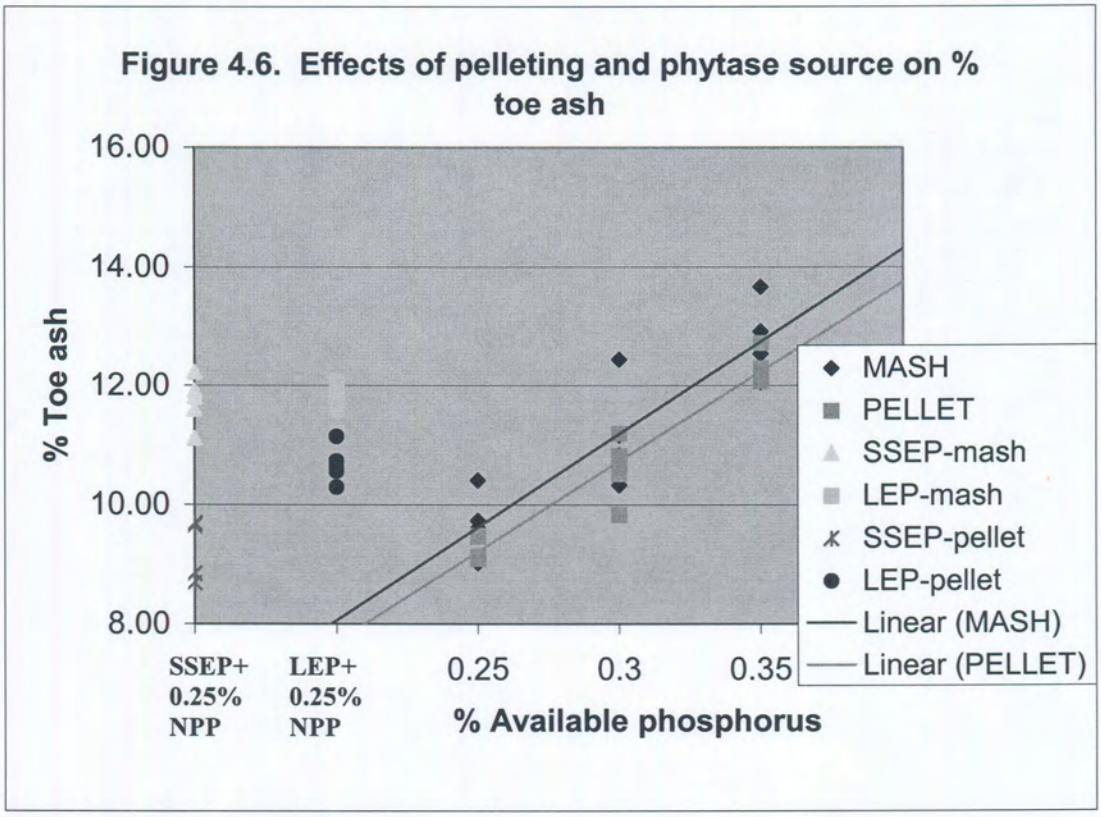
Table 4.10. Percent toe ash of broilers fed two different sources of phytase with and without pelleting

Treatment	Feed consistency	NPP (%)	Enzyme supplement	LS Means (%)
1	Mash	0.25	-	9.66
2	Mash	0.30	-	11.10
3	Mash	0.35	-	12.79
4	Pellet	0.25	-	9.26
5	Pellet	0.30	-	10.62
6	Pellet	0.35	-	12.29
7	Mash	0.25	SSEP	11.73
8	Mash	0.25	LEP	11.69
9	Pellet	0.25	SSEP	9.31
10	Pellet	0.25	LEP	10.66

As with percent bone ash, increasing the percentage NPP in either the mash or the pelleted treatments produced a linear improvement in percent toe ash (Figure 4.6.). Again, neither the pellet nor the mash treatments demonstrated a significantly superior ability to increase the percent ash accumulation over the other. No significant difference in percent toe ash was detected between the SSEP and LEP treatments when supplemented on the mash ($P = 0.8895$). Furthermore, birds on the LEP mash treatment produced a similar quantity of toe ash to those on the 0.30% NPP mash treatment ($P = 0.061$), while birds on the SSEP treatment produced significantly more toe ash than those on 0.30% NPP.

When comparing the toe ash of the birds fed the pelleted rations, it was found that those on 0.25% NPP + SSEP produced a similar amount of ash to those on the 0.25% NPP pelleted ration ($P = 0.8723$) indicating a loss in enzyme activity with pelleting occured. Again, the percent toe ash of birds fed the 0.25% NPP + LEP on the pelleted

ration did not differ significantly from those on the 0.30% NPP pelleted ration (P = 0.8942) which suggests that a degree of enzyme activity was retained after pelleting.



4.3.2.3. Bone breaking strength (kg)

Interaction effects for bone breaking strength were not significant according to the predetermined P value ($P < 0.05$), however the individual treatments did display a strong tendency towards interaction between feed composition and NPP level ($P = 0.0597$) therefore individual treatment means were compared. The data is presented in Table 4.11 and Figure 4.7.

Table 4.11. Bone breaking strength (kg) of broilers fed two different sources of phytase with and without pelleting

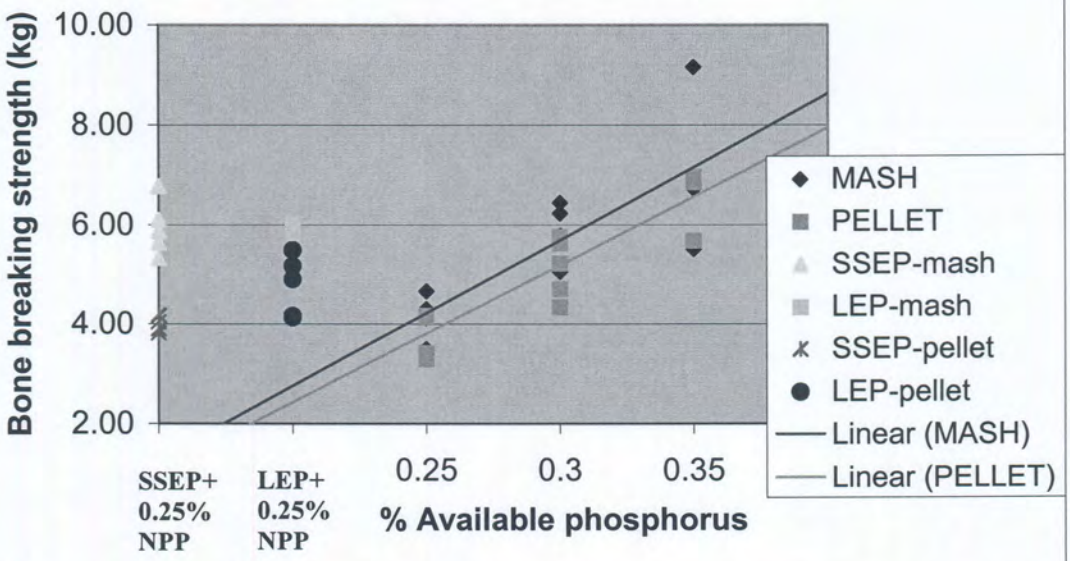
Treatment	Feed consistency	NPP (%)	Enzyme supplement	LS Means (kg)
1	Mash	0.25	-	4.14
2	Mash	0.30	-	5.85
3	Mash	0.35	-	7.05
4	Pellet	0.25	-	3.83
5	Pellet	0.30	-	5.13
6	Pellet	0.35	-	6.59
7	Mash	0.25	SSEP	5.94
8	Mash	0.25	LEP	5.70
9	Pellet	0.25	SSEP	4.02
10	Pellet	0.25	LEP	4.77

Bone breaking strength increased linearly as the percentage NPP in both the mash and pelleted treatments increased (Figure 4.7.). As with the bone and toe ash, neither the pelleted nor the mash treatments demonstrated a significantly superior ability to increase the resistance of the tibias to breaking over the other. No significant difference in bone breaking strength was detected between the SSEP and LEP treatments when supplemented on the mash ($P = 0.5486$). Furthermore, birds on both the LEP and SSEP mash treatments developed bones that were as robust as those on the 0.30% NPP mash treatment ($P = 0.8144$ and $P = 0.7143$ respectively for the SSEP and LEP mash treatments).

When comparing the bone breaking strength of the birds fed the pelleted rations it was found that the response of each of the enzyme supplemented treatments tended to be

different (P = 0.068). It was established that those birds fed 0.25% NPP + SSEP produced bones of similar strength to those on the 0.25% NPP (P = 0.6494) indicating a loss in enzyme activity with pelleting. Conversely, the bone breaking strength of birds fed the 0.25% NPP + LEP ration did not differ significantly from those on the 0.30% NPP ration (P = 0.373) which suggests that a degree of enzyme activity was retained after pelleting.

Figure 4.7. Effects of pelleting and phytase source on bone breaking strength



4.3.3. *In vitro* assay results

The phytase activity detected in the treatment diets as determined by the in-feed assay procedure described under the materials and methods (4.2.9.) was decreased by 88% for the LEP and by 100% for the SSEP, when the phytase supplemented unprocessed mash was compared with the pelleted feed.

4.3.4. Phosphorus equivalency values for phytase

The regression of Treatments 1-3 and 4-6 on percent bone ash, percent toe ash and bone-breaking strength produced the straight lines described in Table 4.12.

The average quantities, and associated 95% fiducial intervals, for the NPP liberated by 300PU/kg of either variety of phytase on the mash or pellets, based upon the calculated regressions (Table 4.12.), are presented in Tables 4.13. and 4.14. The net equivalency values and corresponding 95% confidence interval for the NPP released by each of the phytase enzymes were calculated by subtracting the basal amount of NPP (0.25%) added to each diet from the equivalent percentage NPP value and both the upper and lower 95% fiducial limits respectively, for each of the three parameters.

Table 4.12. Linear response equations for the percent toe and bone ash and bone breaking strength measurements over mash and pelleted maize soyabean diets

						Intercept			Slope		
Parameter	Feed Consistency	Formula	R square	SE	n	Lower 95%	Upper 95%	SE	Lower 95%	Upper 95%	SE
Bone ash (%)	Mash	Y = 66.027X + 25.486	0.855	1.17	14	20.369	30.604	2.35	48.917	83.137	7.85
Bone ash (%)	Pellet	Y = 68.484X + 25.63	0.891	1.03	14	21.129	30.132	2.07	53.435	83.534	6.91
Toe ash (%)	Mash	Y = 31.207X + 1.8173	0.813	0.65	14	-0.998	4.632	1.29	21.796	40.618	4.32
Toe ash (%)	Pellet	Y = 30.215X + 1.6553	0.931	0.36	14	0.712	0.103	3.21	25.025	35.41	2.38
Bone breaking strength (kg)	Mash	Y = 29.338X - 3.116	0.68	0.87	14	-6.899	0.667	1.74	16.691	41.985	5.80
Bone breaking strength (kg)	Pellet	Y = 27.566X - 3.0865	0.834	0.53	14	-5.4026	-0.770	1.06	19.822	35.309	3.55

Table 4.13. Calculated phosphorus equivalency values for mash and pelleted diets supplemented with 300PU/kg of SSEP phytase

Parameter	Mash							Pellet						
				Fiducial limits		(%) NPP Released[1]					Fiducial limits		(%) NPP Released[1]	
	LS Mean	Equivalent % NPP	Net Equivalency	Lower 95%	Upper 95%	Lower 95%	Upper 95%	LS Mean	Equivalent % NPP	Net Equivalency	Lower 95%	Upper 95%	Lower 95%	Upper 95%
Bone ash (%)	48.18	0.344	0.094	0.290	0.376	0.040	0.126	41.32	0.229	-0.021	0.202	0.276	-0.048	0.029
Toe ash (%)	11.73	0.318	0.061	0.270	0.370	0.020	0.120	9.31	0.253	0.003	0.224	0.280	-0.026	0.030
Bone Breaking Strength (kg)	5.94	0.309	0.059	0.236	0.387	-0.014	0.137	4.02	0.258	0.008	0.207	0.302	0.043	0.052

[1] % NPP released from 300PU/kg of the phytase enzyme

Table 4.14. Calculated phosphorus equivalency values for mash and pelleted diets supplemented with 300PU/kg of LEP phytase

Parameter	Mash							Pellet						
				Fiducial limits		(%) NPP Released[1]					Fiducial limits		(%) NPP Released[1]	
	LS Mean	Equivalent % NPP	Net Equivalency	Lower 95%	Upper 95%	Lower 95%	Upper 95%	LS Mean	Equivalent % NPP	Net Equivalency	Lower 95%	Upper 95%	Lower 95%	Upper 95%
Bone ash (%)	48.02	0.341	0.091	0.288	0.373	0.051	0.102	45.28	0.287	0.037	0.265	0.335	0.015	0.085
Toe ash (%)	11.69	0.316	0.066	0.269	0.368	0.026	0.11	10.66	0.298	0.048	0.271	0.325	0.021	0.075
Bone Breaking Strength (kg)	5.70	0.300	0.050	0.226	0.376	-0.003	0.106	4.77	0.285	0.035	0.238	0.330	-0.020	0.080

[1] % NPP released from 300PU/kg of the phytase enzyme

4.3.5. Phosphorus digestibility

Significant interaction effects were found between feed consistency and phosphorus level for phosphorus digestibility (P = 0.0024). The data are presented in Table 4.15.

Table 4.15. Percentage total phosphorus digestibility of broilers fed two different sources of phytase with and without pelleting

Treatment	Feed consistency	NPP (%)	Enzyme supplement	LS Means (%)
1	Mash	0.25	-	61.21
2	Mash	0.30	-	73.48
3	Mash	0.35	-	68.93
4	Pellet	0.25	-	66.25
5	Pellet	0.30	-	67.70
6	Pellet	0.35	-	68.10
7	Mash	0.25	SSEP	78.65
8	Mash	0.25	LEP	72.26
9	Pellet	0.25	SSEP	69.43
10	Pellet	0.25	LEP	68.29

Increasing the percentage NPP in the mash increased the phosphorus digestibility between the 0.25 and 0.30% NPP level significantly (61.21 vs. 73.48% tP digestibility respectively for the 0.25 vs. 0.30% NPP). However, increasing phosphorus availability between the 0.30 and 0.35% NPP levels led to a numerical, although insignificant, decrease in phosphorus digestibility (P = 0.0788). The pelleted treatments, by contrast, demonstrated a numerical improvement in percent tP digestibility with increasing NPP supplementation. However, the differences in digestibility between the three graded levels were not significant.

A significant difference in tP digestibility was detected between the SSEP and LEP supplemented mash treatments (P = 0.0198). The birds on the LEP diet demonstrated phosphorus digestibility values that were similar to those on the 0.30% NPP mash treatment (P = 0.6282), while birds on the SSEP treatment produced tP digestibility values that were significantly higher than birds on the 0.30 or 0.35% NPP levels.

No significant differences in tP digestibility were found when comparing birds fed the pelleted rations, although numerically, tP digestibility values for the chicks on both the SSEP and LEP diets were very similar (69.43 vs. 68.29% respectively) and greater than the tP digestibility values recorded for chicks on the 0.35% NPP diet.

A highly significant 9.22% decrease in tP digestibility from the mash to the pellet ($P = 0.0007$) was recorded for the SSEP, probably as a result of a loss of phytase activity. This differed markedly form the LEP where a far smaller 3.97% drop was recorded between the mash and the pelleted diets ($P = 0.1182$), suggesting a much lower level of enzyme deactivation.

4.4. DISCUSSION

Growth response

Positive body weight gain responses to phytase supplementation on unprocessed diets have been well documented (Yi et al., 1996; Broz et al., 1994; Sebastian et al., 1996 and Simons et al., 1990). Responses to phytase supplementation in this trial were obtained, however, these were not as substantial as those reported in the literature probably due to processing by way of pelleting. No significant interaction was found between the feed consistency and the level of NPP for average weight gain, therefore only main effects were analysed. Pelleting had a significant effect on weight gain ($P = 0.0099$) with birds on the mash diets gaining substantially more body weight than birds on the pelleted feed (356 vs. 341g respectively for mash vs. pellet). Significant differences for gain were also recorded between the 0.25 to 0.35% NPP levels due to the partial alleviation of the phosphorus deficiency. The average weight gain of birds on both the SSEP and LEP supplemented diets was very similar ($P = 0.9997$). Statistically, neither was significantly different from the 0.25 or 0.30% NPP, however, quantitatively both were amid these two NPP levels (330, 345, 345 and 358 respectively for the 0.25, 0.25 + SSEP, 0.25 + LEP and 0.30% NPP levels). The lower than expected body weight gains recorded for the 0.25% NPP + SSEP and 0.25% NPP + LEP treatments were probably due to the significant effect of pelleting on the survivability of the phytase supplements. Further testing is necessary to evaluate this.

The feed intake analysis followed a similar pattern to that of the average weight gain analysis. Again, no significant interaction was found between the feed consistency and the level of NPP in the diets, therefore only main effects were analysed. The average feed intake for birds on those diets that were pelleted, as described in the materials and methods (4.2.6), were markedly lower than birds fed the mash (521 vs. 502g respectively for the mash vs. pellet). This was probably due to a lower level of NPP in the pelleted diets as a result of phytase deactivation. Average feed intake increased numerically as the NPP in the diet increased from 0.25 to 0.35% NPP (467, 521 and 543g respectively for the 0.25, 0.30 and 0.35% NPP levels) although the difference between the 0.30 and 0.35% NPP levels was not significant ($P = 0.1436$). While neither of the two enzyme-supplemented NPP levels differed significantly from

the other and birds fed either type of phytase demonstrated average feed intakes similar to those on the 0.30% NPP rations, numerically, those birds on the LEP had higher feed intakes than birds on the SSEP (Table 4.6.). This again could be attributed to poorer survivability of the SSEP when subjected to pelleting.

The average feed intake response was in agreement with the results recorded by Broz et al. (1994). They reported that dietary phytase supplementation on low phosphorus diets significantly improved feed intake. Yet, not all publications concur as in the case of Zanini and Sazzad (1999) where no increase in intake was recorded. However, this may have been due to the higher levels of non-phytate phosphorus present in the experimental diets (0.4%).

The positive effect of microbial phytase on body weight gain and feed intake in this trial may in part have been as a consequence of improved protein and amino acid utilization. Phytic acid has been shown to bind with proteins at a low and neutral pH. Phytic acid might also form complexes with proteases such as trypsin, and pepsin (Camus and Laporte, 1976; Singh and Kirkorian, 1982). Yi et al. (1995) reported enhanced growth performance, ileal nitrogen and amino acid digestibility and apparent nitrogen and phosphorus retention in turkey poults fed maize soyabean meal diets supplemented with phytase. Furthermore, it appeared that by adding microbial phytase, the dietary CP level could be reasonably reduced without an adverse effect on the body weight gains of birds. Yi et al. (1995) attributed this phenomenon to the release of a more ideal pattern of available amino acids thus reducing the excretion of dietary waste products, a process that demands a significant amount of energy on its own.

There was no significant interaction between feed consistency and phosphorus level on feed efficiency. Feed efficiency (gain/feed) was not significantly affected by pelleting (0.6851 vs. 0.6875 respectively for the mash and pellet) and although differences in the gain/feed ratio were recorded over the graded levels of NPP, these were not significant (Table 4.8.). Only the enzyme supplemented diets differed significantly from each other (0.690 vs. 0.667 respectively for the SSEP and LEP).

The higher gain/feed ratio for the 0.25% NPP + SSEP diets may have been due to the measurable enzymatic side activities produced under SSC conditions. Further testing would be required to validate this. The lack of feed efficiency response is in keeping with the results obtained from a number of publications (Zanini and Sazzad, 1999; Broz et al., 1994; Sebastian et al., 1996 and Simons et al., 1990). Sebastian et al. (1996) attributed this phenomenon to the simultaneous increase in both body weight and feed intake.

4.4.2. Production response on mash diets

There was a significant interaction effect between feed consistency and phosphorus level for the percent bone and toe ash variables, therefore individual treatment means were compared. The percent bone and toe ash improved significantly with increased NPP levels regardless of whether birds were on the pelleted or mash diets. Linear response curves were produced for both (Table 4.12.). Birds on the mash demonstrated a superior ability to accumulate ash over birds on the pellets for all three levels of NPP and for both parameters, however the differences were not significant (Tables 4.9 and 4.11).

The percent bone ash accumulation was improved substantially on the mash supplemented with phytase, independent of the variety of the enzyme supplemented (48.18 vs. 48.02% respectively for the SSEP vs. LEP). Furthermore, the percent bone ash recorded for birds on the enzyme treated mash diets was not significantly different to those on the 0.35% NPP mash diets ($P = 0.1270$ and $P = 0.0885$ respectively for the SSEP vs. LEP), thus clearly illustrating the ability of the phytase enzyme to liberate phosphorus from phytic acid.

The pattern of toe ash accumulation with microbial phytase supplementation on the unprocessed mash was similar to the bone ash accumulation pattern on mash. Again, neither variety of enzyme product was able to significantly improve ash accumulation over the other. However, the percent toe ash of birds on the SSEP treatment was numerically superior to those on the LEP and significantly higher than birds on the 0.30% NPP mash treatment (11.73 vs. 11.69 vs. 11.10% respectively for the 0.25% NPP + SSEP vs. 0.25% NPP + LEP vs. 0.30% NPP treatments).

The bone and toe ash accumulation patterns obtained with phytase supplementation were in agreement with the literature. Zanini and Sazzad (1999); Broz et al. (1994) and Sebastian et al. (1996) all demonstrated that microbial supplementation of unprocessed, low phosphorus diets improves bone mineralization as a consequence of increased phosphorus retention and utilization. Furthermore, the percent toe ash data was consistent with the results obtained by Yi et al. (1996), where graded levels of NPP and phytase were added to unprocessed diets.

The consistency of the feed and the level of NPP demonstrated a strong tendency towards interaction for bone breaking strength, although this was not significant by predetermined limits (P = 0.0597). A comparison of individual treatment means revealed that the response pattern for bone breaking strength over the different levels of NPP, for both the mash and pellets, was similar to that described by the individual treatment means of both the bone and toe ash i.e. a linear response. The bone breaking strength was improved substantially on the unpelleted, phytase supplemented mash diets. No significant difference in breaking strength was detected between the SSEP and LEP mash treatments (5.94 vs. 5.70kg respectively for the SSEP vs. LEP) although, quantitatively, the SSEP again produced superior results. The bones from birds on the enzyme treated mash diets showed a similar resistance to fracturing when compared to those on the 0.30% NPP mash diets (P = 0.8144 and P = 0.7143 respectively for the SSEP and LEP). This reaffirms the suggestion that phytase supplementation on unprocessed diets leads to increased bone mineralization.

The results of the bone breaking strength analysis were found to be in agreement with the results of Sohail and Roland (1999). They investigated the influence of dietary phytase on phytic acid phosphorus availability using male broiler chicks and found that phytase on reduced phosphorus and calcium diets significantly improved bone strength.

4.4.3. Production response on pelleted diets

The ability of feed enzymes to withstand pelleting is controversial with many varying degrees of heat resistance having been recorded in the literature. Much of this disparity might be due to the conditions at pelleting in relation to the presence of native and supplementary enzymes (Jongbloed and Kemme, 1990).

Most publications to date have referred to the stability of those enzyme complexes involved in reducing the viscosity of cereal based monogastric feed. These include cellulase, xylanase, glycanase and pentosanase complexes. Partial β-glucanase enzyme recoveries, sufficient to significantly improve animal performance, have been recorded at pelleting temperatures in excess of 95^{0}C (Inborr and Bedford, 1994). Spring et al., 1996 reported that cellulase, fungal amylase and pentosanase can be pelleted at temperatures of up to 80^{0}C, at least. However, Vukic Vranjes et al. (1995) found that when pelleting the *Trichoderma viride* enzyme complex [cellulase, endo-β-(1:3)(1:4)-glucanase, xylanase], temperatures as low as 70^{0}C reduced enzyme efficacy by 52%.

The resistance of endogenous phytases to heat was shown to decreases dramatically once pelleting temperatures exceeded 80^{0}C (Jongbloed and Kemme, 1990). Simoes Nunes (1993) confirmed this, recording considerably reduced levels of activity for microbial phytase produced by *Aspergillus niger* at pelleting temperatures in excess of 60^{0}C. This was particularly marked for temperatures above of 75^{0}C, while only 50% of the original activity existed at 80^{0}C.

The thermostability of an enzyme is a product of its molecular structure. Wyss et al. (1998) demonstrated that the phytase from *A. niger* T213 was a monomeric protein that was not thermostable, nor did it have the capability to refold properly after heat denaturation. It was established that at temperatures between 50 and 55^{0}C, the enzyme undergoes an irreversible conformational rearrangement that is associated with losses in enzymatic activity of 70 to 80%.

The susceptibility of the SSEP and LEP phytases to the extreme temperatures and pressures of pelleting, as described under materials and methods (4.2.6.), differed when measured using percent bone ash ($P < 0.0001$), although both were manufactured from *A. niger* phytase genes. The 0.25% NPP + SSEP treatment produced similar quantities of bone ash to the 0.25% NPP treatments which intimates a high level of deactivation for the SSC enzyme during pelleting. The LEP showed more resilience towards pelleting, with bone ash accumulation results comparable to

those of the 0.30% NPP treatments. The level of enzyme activity retained after pelleting was calculated to be approximately 52%, which is similar to the levels found by Jongbloed and Kemme (1990) and Simoes Nunes (1993).

Percent toe ash, as with percent bone ash, was not significantly improved when diets supplemented with SSEP were pelleted before being fed, while birds on the pelleted 0.25% NPP + LEP treatment were again able to amass a similar amount of toe ash to birds on the pelleted 0.30% NPP diet. The degree of residual enzyme activity retained after pelleting for the LEP, calculated on a percent toe ash basis, was estimated to be approximately 54%.

The bone breaking strength for the pelleted SSEP and LEP enzyme treatments exhibited a strong tendency to differ from each other although this difference was not significant ($P = 0.0681$). The chicks fed the pelleted 0.25% NPP + SSEP treatment produced bones of similar strength to those on the pelleted 0.25% NPP treatment, 4.02 vs. 3.83kg respectively ($P = 0.6494$), while birds given the pelleted 0.25% NPP + LEP developed bones that were comparable in strength to those on the pelleted 0.30% NPP diet, 4.77 vs. 5.13kg respectively ($P = 0.373$). These results imply that little of the original enzyme activity in the SSEP mash was retained after pelleting, yet a portion of the LEP remained viable once it had undergone the same process. The degree of residual enzyme activity retained after pelleting for the LEP, calculated on a bone breaking strength basis, was also estimated to be approximately 54%.

Pelleting in itself may or may not affect the absorbability or availability of phosphorus. Summers et al. (1989); Bayley and Thomson (1969); Bayley et al. (1975) and Jongbloed (1967) all reported improved absorption of phosphorus with steam pelleting on mixed feeds including maize-soyabean meal diets. This phenomenon might have been as a result of the increase in crushed cells, which would enhance the hydrolysis of phytic acid by native phytases (Jongbloed and Kemme, 1990). Ross et al. (1983), on the other hand, found the NPP of maize unaffected by pelleting while Harrold et al. (1982) showed a reduction in NPP when pelleting barely, oats, maize or soyabean meal separately. The disparity in the literature shows that no definite conclusions can be reached regarding the effectiveness of steam pelleting on absorbability of phosphorus. This may be due to varying pelleting

conditions, native phytase concentrations and phosphorus concentrations in the diet (Jongbloed and Kemme, 1990).

In this trial no significant differences were found between the mash or pelleted diets for percent bone ash, percent toe ash or bone breaking strength when compared across increasing levels of NPP from 0.25 to 0.35% NPP. However, quantitatively the mash diets performed better than the pelleted diets for all three parameters and across all three levels of NPP. The apparently reduced ability of birds on the pelleted diets to absorb and assimilate calcium and phosphorus into the skeleton, may have been due to the destruction of native phytases through heat and pressure. Further research is needed to investigate this.

4.4.4. *In vitro* testing vs. animal response

Enzyme activity after pelleting was reduced by 100% and 88% for the SSEP and LEP respectively when measured using the *in vitro* in-feed assay technique (4.2.8.). Although sample numbers were limited, results reflected the disparity between *in vitro* and animal trials recorded in a number of different publications (Vukic Vranjes et al., 1995; Inborr and Bedford, 1994 and Spring et al., 1996). The reason for this disparity might be due to the greater inactivation of enzymes in buffered solutions as a result of different water contents and therefore different heat conductivities. The inaccuracies could be exacerbated by the protective effect of the feed components on enzyme activity (Spring et al., 1996). Further, the validity of current strategies that assay for enzyme activity in the final feed over extended incubation times is questionable, due to the low inclusion rates in the complex final feed product (Lyons and Walsh, 1993).

4.4.5. Phosphorus equivalency values

The percent bone ash variable provided the most precise estimation of the NPP liberated by SSEP on both the mash and the pelleted diets followed by the percent toe ash and then bone breaking strength (Table 4.13.). Based on percent bone ash, between 0.040 and 0.126% NPP is released by SSEP on the mash. This was subsequently reduced to between –0.042 and 0.026% NPP when the mash was pelleted at 80^0C in this experiment.

As with the SSEP, the efficacy of the LEP phytase was most precisely estimated from the percent bone ash data followed by the percent toe ash and bone breaking strength data respectively (Table 4.14.). Using bone ash, 95% fiducial limits of between 0.038 and 0.123% NPP were calculated for the LEP on the mash, which were reduced to between 0.015 to 0.085% NPP when the mash was pelleted.

This results from the enzyme on the mash analysis are in general agreement with the phosphorus equivalency values calculated by Yi et al. (1996) for maize-soyabean meal diets containing 0.24% phytic acid phosphorus. They found that 250 PU/kg released 0.02% NPP per 100 PU.

The calculated phosphorus equivalency values, based on the percent bone and toe ash and bone breaking strength data, reflected the apparent instability of the SSEP when pelleted. In contrast, the LEP appeared to retain enough residual activity to liberate a significant amount of otherwise unavailable phosphorus after being subjected to the same process.

4.4.6. Phosphorus digestibility

Although few of the differences between the treatment means for tP digestibility were significant, of importance is the fact that birds on the SSEP mash diet had significantly higher phosphorus digestibility values compared to the birds on the LEP mash diets ($P = 0.0198$). This is supported by the percent bone and toe ash and bone breaking strength parameters where similar, although insignificant, differences were found. The pattern suggests that the SSEP may be more effective than the LEP on the unprocessed mash. However, further trials are required to validate this.

Of greater consequence is the change in digestibility that took place as a result of pelleting. A substantial 9.22% difference in tP digestibility was recorded for the SSEP when pelleted, which strongly suggests that a high degree of phytase deactivation took place. Far less of the LEP appeared to be deactivated by the same process, as illustrated by the much smaller, insignificant reduction of 3.97% between the mash and pelleted diets.

4.5. CONCLUSION

Analysis of the bone ash data suggests that both the SSEP and LEP liberated between 0.04 and 0.12% phosphorus from phytic acid when supplemented on the unprocessed mash diet. This impacted positively on the birds that were on an otherwise phosphorus deficient diet, improving average weight gains, average feed intakes, percent bone and toe ash, and average bone breaking strengths. These improvements may have been enhanced by the increased availability of the Ca, Cu, Zn, amino acids, protein and digestive enzymes that are normally bound to phytic acid at the regular pH of the duodenum. The gain/feed ratio was not significantly altered by phytase supplementation, maybe due to the simultaneous increase in both body weight and feed intake (Sebastian et al., 1996).

It was found that chicks fed the SSEP exhibited significantly superior feed efficiencies and tP digestibilities and numerically, but not significantly, superior responses for average weight gain, average feed intake, feed efficiency, percent bone and toe ash and bone breaking strength to those on the LEP, when compared on the unprocessed mash. These differences may have been due to the measurable amounts of protease, xylanase, and cellulase produced under SSC conditions.

Pelleting depressed the activity of both varieties of phytase considerably, as demonstrated by the reduction in all growth and production parameters. However, the LEP proved more resistant to high temperature processing, retaining approximately 50% of its initial activity after pelleting at 80^0C. Phosphorus equivalency values for the LEP in the pelleted feed were in the range of 0.015-0.085% NPP while none of the SSEP appeared to survive. This disparity was also reflected by the tP digestibility values where a 3.97 and 9.22% drop in digestibility was recorded after pelleting for the LEP and SSEP respectively. Both enzyme products were derived from the same parent genetic material, therefore the dissimilarity in survivability was probably as a result of differences in downstream processing. Due to the proprietary nature of the information regarding the manufacturing process of each enzyme, the exact reason behind the dissimilarity was not established. However, processing by way of granulation, absorption of the enzyme to a carrier, coating of the enzyme, or a combination of these methods may have contributed to the discrepancy (Cowan and Rasmussen, 1993).

Pelleting, in itself, did not appear to increase phosphorus availability. Numerically, pelleting even decreased phosphorus availability when the mash vs. pelleted data was compared over the graded levels of NPP on a percent bone ash, percent toe ash and bone breaking strength basis. This may have been due to a reduction in the native phytases present in the feed.

The discrepancy between the *in vitro* and animal performance trial data was not unexpected due to the nature of current enzyme assay procedure, which involves incubating the final feed containing small amounts of enzyme, over extended periods of time. It follows that until alternative *in vitro* techniques are found, animal response trials will probably remain the most sensitive measure of in-feed enzyme activity (Lyons and Walsh, 1993).

The results of this phase of the study underline the importance of establishing the degree of enzyme deactivation that is likely to occur during feed processing before substituting a microbial phytase for inorganic phosphorus. The amount of deactivation that occurs is particular to the pelleting conditions as well as the enzyme.

4.6. CRITICAL EVALUATION

- The pelleting temperatures recorded were an overall value for the steamed meal. They did not take into account the localised higher temperatures of the feed that comes into direct contact with the injected steam (Stott et al., 1975).

- Due to the nature of the experiment, it was not possible to duplicate typical commercial broiler housing conditions exactly. This may have altered the growth response and feeding behaviour of the experimental birds in comparison to birds in a commercial operation.

- In order to ensure uniformity between treatments, all diets were ground to a powder before feeding. This may have altered the feeding behaviour and growth response of the experimental birds compared to those on commercial diets. Commercial diets are typically coarser and therefore less dense.

- The individual bird weights were not recorded at the end of the trial. Instead, whole pen weights were recorded. The average weight gain, average feed intake and feed efficiency variability could have been considerably reduced by weighing each bird at the end of the trial, as this would have led to the identification of outliers.

- Open feeders were used in this trial so that in the event of a feed jam the chicks would still have had ready access to their diets. A certain amount of wastage was caused by some of the chicks feeding from on top of the feeders rather than from the gravity-fed troughs along the sides. It was assumed that this wastage was relatively uniform for all the pens. Improved feeders may have provided more accurate results.

- Only two samples for each enzyme treatment, on either the mash or the pelleted diets, were tested using the *in vitro* techniques due to time constraints. Further sampling would have provided more reliable results.

4.7. FUTURE RESEARCH PROPOSALS

- In this experiment only one pelleting temperature and one rate of enzyme inclusion were applied to the test diets due to the limitations of the trial facilities. It would be of practical importance to compare the two enzymes across a range of different pelleting temperatures and enzyme inclusion rates to establish a pattern for commercial phytase supplementation.

- Valuable information might be obtained by comparing the two enzymes, again, across a range of different pelleting temperatures and enzyme inclusion rates, but on different poultry diets. It would be interesting to know how the enzymes behave on a number of the typical South African poultry diets.

- It would be beneficial to further define the effects of any possible side activities present in the extract produced under SSC conditions. Small improvements in efficiency may impact significantly on profitability in the context of large-scale commercial production. Further, if these additional enzymes do affect production, their ability to survive commercial pelleting conditions would be of interest.

- Further investigation into how downstream processing can be applied to improve the thermostability of the phytase enzyme, particularly the phytase produced under SSC conditions, could be undertaken.

CHAPTER 5. GENERAL CONCLUSIONS

The first phase of this study failed to produce a thermostable fungal phytase. A more extensive screening process of the six fungal types examined, using techniques such as UV mutagenesis and selective media, may successfully uncouple regulation and enzyme production to the extent that sufficient quantities of a heat stable phytase are produced.

Of the two commercial phytases tested in the second phase of the trial, the enzyme produced under liquid culture conditions (LEP) seemed to be better able to withstand the pelleting conditions described under the materials and methods (3.2.6). The enzyme produced under SSC conditions (SSEP) appeared to be completely destroyed at 80^0C. This contrasted with the performance of the two enzymes on the mash, where the SSEP appeared to generate slightly better results. The difference in thermostability was probably due to differences in downstream processing.

CHAPTER 6. REFERENCES

Allred, J. B., R. E. Fry, L. S. Jensen, and J. McGinnis. 1957. Studies with chicks on improvement in nutritrive value of feed ingredients by pelleting. *Poult. Sci.* **36**:1284-1289.

A.O.A.C. 1995. Official methods of analysis (16th ed.), AOAC International. Gaithersburg, Maryland, USA.

A.T.C.C. 2000 pamphlet guide: "Instructions for Rehydration of Freeze-Dried Cultures".

Bayley, H. S., et al. 1975. Influence of steam pelleting and dietary calcium level on the utilization of phosphorus by the pig. *J. Anim. Sci.*, **40**:857-863.

Bayley, H. S. and R. G. Thomson. 1969. Phosphorus requirements of growing pigs and effects of steam pelleting on phosphorus availability. *J. Anim. Sci.* **28**:484-491.

Broz, J., P. Oldale, A. H. Perrin-Voltz, G. Rychen, J. Schulze, and C. Simoes Nunes. 1994. Effect of supplemental phytase on performance and phosphorus utilization in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. *Br. Poult. Sci.* **35**: 273-280.

Calvert, C. C., R. J. Besecker, M. P. Plumlee, T. R. Cline, and D. M. Forsyth. 1978. Apparent digestibility of phosphorus in barley and corn for growing swine. *J. Anim. Sci.* **47**:420-426.

Camus, M. C., and J. C. Laporte. 1976. Inhibition de la proteolyse pepsique *en vitro* par de ble. Role de l'acide phytique des issues. *Ann. Biol. Biochem. Biophys.* **16**:719-729.

Cantor, A. H. 1998. Using enzymes to increase the phosphorus availability in poultry diets. Pages 349-353. In: Proceedings of the Fourteenth Annual Biotechnology in the Feed Industry Symposium. Lexington, KY, USA.

Chang, C. W. 1967. Study of phytase and fluoride effects in germinating corn seeds. *Plant Physiol.* **44**:129-142.

Chen, L. H., and S. H. Pan. 1977. Decrease in phytates during germination of pea seeds (*Pisum sativa*). *Nutr. Rep. Int.* **16**:125-131.

Choi, J. H., B. S. So, K. S. Ryu, and S. L. Kang. 1986. Effects of pelleted or crumbled diets on the performance and the development of the digestive organs of broilers. *Poult. Sci.* **65**:594-597.

Church, D. C. 1976. Excretion and Digestion. Pages 125-128. In: Digestive Physiology and Nutrition of Ruminants Volume 1 – Digestive Physiology. 2nd edition. Published by D. C. Church.

Coffey, R. D. 1994. Bioavailability of Phosphorus in Various Feedstuffs for Pigs and Chicks. Ph. D. Dissertation. University of Kentucky, Lexington.

Cowan, W. D., and P. B. Rasmussen. 1993. Thermostability of microbial expander and pelleting processes and application systems for post pelleting addition. Pages 263-267. In: Proceedings of the First Symposium of Enzymes in Animal Nutrition. Kartause Ittingen, Switzerland.

Cromwell, G. L. 1999. Modifying manure by modifying diets. Presented at the Nebraska Pork Exposition. February 3. Columbus, NE, USA

Davies, M. I., G. M. Ritcey, and I. Motzok. 1970. Intestinal phytase and alkaline phosphotase of chicks: influence of dietary calcium, inorganic and phytate phosphorus and vitamin D_3. *Poult. Sci.* **49**:1280-1286.

De Bruyne, K. 2000. New strategies in supplementation of phosphorus discussed. Pages 11-13, 22, 23. *Feedstuffs*. March 6, 2000.

Deluca, H. F. 1989. Vitamin D, calcium, and metabolic bone disease. Page 109. In: Nutrition and the Origins of Disease. H. F. Deluca, ed. Academic Press, Inc., New York, NY.

Draper, N. R. and H. Smith. 1981. Inverse Regression (Straight Line Case). Pages 47–51. In: Applied Regression Analysis, Second edition. John Wiley and Sons, Inc., New York, Chichester, Brisbane and Toronto.

Edwards, H. M. 1982. Effect of breed and strain on utilization of suboptimal levels of phosphorus in the ration. *Pout. Sci.* **62**:77-84.

Edwards, H. M., Jr. 1993. Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. *J. Nutr.* **123**:567-577.

Edwards, H. M., Jr., and J. R. Veltmann, Jr. 1983. The role of calcium and phosphorus in the etiology of tibial dyschondroplasia. *Poul. Sci.* **113**:1568-1575.

Filer, K. 2000. Production of enzymes for the feed industry using solid substrate fermentation. Pages 131-152. In: Biotechnology in the Feed Industry. Proceedings of Alltech's 16th Annual Symposium. Eds Lyons and Jacques. Nottingham University Press.

Gadient, M., L. Volker, and W. Schuep. 1993. Experiences with enzymes in feed manufacturing. Pages 255-262. In: Proceedings of the First Symposium of Enzymes in Animal Nutrition. Kartause Ittingen, Switzerland.

Gerbert, S., and C. Wenk. 1998. Allzyme phytase: The Swiss experiences. Pages 321-330. In: Proceedings of the Fourteenth Annual Biotechnology in the Feed Industry Symposium. Lexington, KY, USA.

Gibson. D. M., and A. B. J. Ullah. 1990. Phytases and their action on phytic acid. Pages 77-92. In: Inositol Metabolism in Plants. Wiley-Liss, Inc. New York.

Harrold, R. L., J. N. Johnson, W. D. Slanger, and C. N. Haugse. 1982. The bioavailable phosphorus content of some common North Dakota feedstuffs. *N. D. Farm. Res.* **40**:22-24.

Hayes, S. H., G. L. Cromwell, T. S. Stahly, and T. H. Johnson. 1979. Availability of phosphorus in corn, wheat and barley for the chick. *J. Anim. Sci.* **49**:992-999.

Howson, S. T., and R. P. Davis. 1983. Production of phytase hydrolyzing enzyme by some fungi. *Enz. Microbiol. Technol.* **5**:377-382.

Inborr, J., M. R. Bedford. 1994. Stability of feed enzymes to steam pelleting during feed processing. *Anim. Feed Sci. Technol.* **46**:179-196.

Jensen, L. S., L. H. Merill, C. V. Reddy, and J. McGinnis. 1962. Observations on eating patterns and rate of food passage of birds fed pelleted and unpelleted diets. *Poult. Sci.* **41**:1414-1419.

Jongbloed, A. W. 1987. Phosphorus in the feeding of pigs; effect of diet on the absorption and retention of phosphorus by growing pigs. I.V.V.O. Rep. No. 179, Leyland, The Netherlands.

Jongbloed, A. W., and P. A. Kemme. 1990. Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. *Animal Feed Science and Technology.* **28**:233-242.

Jongbloed, A. W., and N. P. Lenis. 1998. Environmental concerns about animal manure. *J. Anim. Sci.* **76**:2641-2648.

Jongbloed, A. W., Z. Morz, and P. A. Kemme. 1992. The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J. Anim. Sci.* **70**:1159-1168.

Johnston, S. L., and Southern, L. L. 2000. The effect of phytase addition on metabolisable energy in diets for chicks and pigs. Multi-State Poultry Meeting May 23-25.

Kemme, P. A., A. W. Jongbloed, Z. Morz, and A. C. Beynen. 1997. The efficacy of *Aspergillus niger* phytase in rendering phytate phosphorus available in pigs is influenced by pig physiological status. *J. Anim. Sci.* **75**:2129-2138.

Kies, K. 1996. Phytase-Mode of action. Pages 205-212. In: Phytase in Animal Nutrition and Waste Management: A BASF Reference Manual. BASF, Parsippany, NJ.

Kiiskinen, T., and J. Piironen. 1990. Effect of phytase supplementation on utilization of phosphorus in chicken diets. Pages 376-380. In: Proceedings of the 8th European Poultry Conference, Barcelona.

Leske, K. L., and C. N. Coon. 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. *Poult. Sci.* **78**:1151-1157.

Loer, R. C. 1984. Pollution control for agriculture. Academic press, Inc. Orlando Fl.

Low, A. G. 1989. Research into the digestive physiology of pigs. Pages 1-15. In: E. J. van Weerden and J. Huisman ed. Nutrition and Digestive Physiology in Monogastiric Farm Animals. Pudoc, Wageningen, The Netherlands.

Lyons, T. P., and A. Walsh. 1993. Application of enzymes in feed manufacturing. Pages 241-254. In: Proceedings of the First Symposium of Enzymes in Animal Nutrition. Kartause Ittingen, Switzerland.

Maddaiah, V. T., A. A. Kurnick, and B. L. Reid. 1964. Phytic acid studies. *Proc. Soc. Exp. Biol. Med.* **115**:391-393.

Mandviwala, T. N., and J. M. Khire. 2000. Production of high activity thermostable phytase from thermotolerant *Aspergillus niger* in solid state fermentation. *Journal of Industrial Microbiology & Biotechnology*. **24**:237-243.

Matyka, S., W. Korol, and G. Bogusz. 1990. The retention of phytin P from diets with fat supplements in broiler chickens. *Anim. Feed Sci. Tech.* **31**:223-230.

McEllhiney R. R. 1985. Feed Manufacturing Technology III. American Feed Industry Association. Arlington, VA, USA.

Mitchell, R. D., and H. M. Edwards. 1996. Effects of phytase and 1,25-dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chickens. *Poult. Sci.* **75**:95-110.

Mitchell, R. D., and H. M. Edwards. 1996. Additive effects of 1,25-dihydroxycholecalciferol and phytase on phytate phosphorus utilization and related parameters in broiler chickens. *Poult. Sci.* **75**:111-119.

Mitchell, D. A., and B. K. Lonsane. 1992. Chapter 1: Definition, Characteristics and Potential. Pages 1-13. In: H. Doelle, D. A. Mitchell, and C. E. Rolz ed. Solid Substrate Cultivation. Elsevier Applied Science, London and New York.

Mohammed, A., M. J. Gibney, and T. G. Taylor. 1991. The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. *Bri. J. Nutr.* **66**:251-259.

Morz, Z., A. W. Joengbloed, and P. A. Kemme. 1994. Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. *J. Anim. Sci.* **72**:126-133.

Mossel, D. A. A. 1971. Physiological and metabolic attributes of microbial groups associated with foods. *J. Appl. Bacteriol.* **34**:95-118.

Nayini, N. R., and P. Markakis. 1986. Phytases. Pages 101-118. In: Graf, E. ed. Phytic Acid Chemistry and Applications. Pilatus Press, Minneapolis, MN.

Nelson, T. S. 1967. The utilization of phytate phosphorus by poultry-A review. *Poult. Sci.* 46:862-871.

Nelson, T. S. 1976. The hydrolysis of phytin phosphorus by chicks and laying hens. *Poult. Sci.* **55**:2262-2264.

Nelson, T. S., T. R. Shieh, R. J. Wodzinski, and J. H. Ware. 1971. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. *J. Nutri.* **101**:1289-1294.

Nir, I., Y. Twina, E. Grossman and Z. Nitsan. 1994. Quantitative effects of pelleting on performance, gastrointestinal tract and behaviour of meat-type chckens. *Bri. Poult. Sci.* **35**:589-602.

NRC. 1994. Nutrient Requirements of Poultry (9th Ed.). National Academy Press, Washington, DC.

Oberleas, D. 1973. Phytates. Page 363. In: Toxicants Occuring Naturally in Foods. 2nd edition. National Academy Press, Washington, DC.

Orban, J. I., O. Adeola, and R. Stroshine. 1999. Microbial phytase in finisher diets of white pekin ducks: Effect on growth performance, plasma phosphorus concentration, and leg bone characteristics. *Poult. Sci.* **78**:366-377.

Pasamontes, L., M. Haiker, M. Wyss, M. Tessier, and A. P. G. M. van Loon. 1997. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environmental. Microbiol.* **63**:1696-1700.

Patterson, J. T. 1969. *Salmonellae* in meat and poultry, poultry plant cooling waters and effluent, and animal feeding stuffs. *Appl. Bacteriol.* **32**:329-337.

Pettersson, D., H. Graham, and P. Aman. 1991. The nutritive value for broiler chickens of pelleting and enzyme supplementation of a diet containing barley, wheat and rye. *Anim. Feed Sci. Technol.* **33**:1-14.

Pfirter, H. P., and M. V. Vranjes. 1993. Influence of extrusion of broiler feeds before pelleting on the effect of added enzymes. Pages 281-282. In: Proceedings of the First Symposium of Enzymes in Animal Nutrition. Kartause Ittingen, Switzerland.

Pierce, J. L. 1999. Nutritional assessment of conventional and low phytic acid corn for pigs and chickens. Ph. D. Dissertation. University of Kentucky, Lexington.

Pierce, J. 2000. Phytase, Production and Pollution. Pages 1-20. In: T. P. Lyons and D. J. A. Cole ed. Concepts in Pig Science. Nottingham University Press, Nottingham, UK.

Pointillart A., A. Pourdin, M. Thomasset, and M. E. Jay. 1985. Phosphorus utilization, intestinal phosphatase and hormone control of calcium metabolism in pigs fed phytic phosphorus: soybean or rapeseed diets. *Nutr. Rep. Int.* **32**:155-167.

Punna, S., and D. A. Roland. 1999. Variation in phytate phosphorus utilization within the same broiler strain. *J. Appl. Poultry Res.* **8**:10-15.

Qian, H., E. T. Kornegay, and D. M. Denbow. 1996. Phosphorus equivalence of microbial phytase in turkey diets as influenced by calcium to phosphorus ratios and phosphorus levels. *Poult. Sci.* **75**:69-81.

Qian, H., E. T. Kornegay, and D. M. Denbow. 1997. Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium:total phosphorus ratio in broiler diets. *Poult. Sci.* **76**:37-46.

Quamme, G. A. 1985. Phosphate transport in intestinal brush-border membrane vesicles: effect of pH and dietary phosphate. *Am J Physiol* 1985 **249**(2 Pt 1):G168-76.

Raboy, V., D. B. Dickinson, M. G. Neufer. 1990. A survey of maize kernel mutants for variation in phytic acid. *Maydica* **35**:383-390.

Ravindran, V. 2000. The effect of Natuphos phytase on the availability of protein and amino acids-a review. Proceedings of the Multi-State Poultry Meeting May 23-25. Indianapolis, Indiana.

Raimbault, M. 1998. General and microbiological aspects of solid substrate fermentation. Electronic Journal of Biotechnology. **1**:1-15. (www.ejb.orj)

Reddy, C. V., L. S. Jensen, L. H. Merill, and J. McGinnis. 1962. Influence of mechanical alteration of dietary density on energy available for chick growth. *J. Nutr.* **77**:428-432.

Reddy N. R., S. K. Sathe, D. K. Salunkhe. 1982. Phytates in legumes and cereals. *Adv. Food. Res.* **28**:1-92.

Ross, R. D., G. L. Cromwell, and T. S. Stahly. 1983. Biological availability of the phosphorus in high moisture and pelleted corn. *J. Anim. Sci.* **57**:96 (Abstract).

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P.C. Lague. 1996. The effects of supplemental microbial phytase on the performance and utilization of dietary calcium, phosphorus, copper, and zinc in broiler chickens fed corn-soybean meal diets. *Poult. Sci.* **75**:729-736.

Shieh, T. R., and J. H. Ware. 1968. Survey of microorganisms for the production of extracellular phytase. *Appl. Microbiol.* **16**:1348-1351.

Simoes Nunes, C. 1993. Evaluation of phytase resistance in swine diets to different pelleting temperatures. Pages 269-271 In: Proceedings of the First Symposium of Enzymes in Animal Nutrition. Kartause Ittingen, Switzerland.

Simons, P. C. M., H. A. J. Versteegh, A. W. Joengbloed, P. A. Kemme, P. Slump, K. D. Bos, M. G. E. Wolters, R. F. Beudeker, and G. J. Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Br. J. Nutr.* **64**:525-540.

Singh, M., and A. D. Krikorian. 1982. Inhibition of trypsin activity *in vitro* by phytase. *J. Agric. Food. Chem.* **30**:799-800.

Sohail, R. R., and D. A. Roland. 1999. Influence of supplemental phytase on performance of broilers four to six weeks of age. *Poult. Sci.* **78**:550-555.

Spring, P., K. E. Newman, C. Wenk, R. Messikommer, and M. Vukic Vranjes. 1996. Effect of pelleting temperature on the activity of different enzymes. *Poult. Sci.* **75**:357-361.

Stott, J. A., J. E. Hodgeson, and J. C. Chaney. 1975. Incidence of *Salmonellae* in animal feeds and the effect of pelleting on content of *Enteriobacteriaceae. J. Appl. Bacteriol.* **39**:41-46.

Summers, J. D., S. J. Slinger and G. Cisneros. 1967. Some factors affecting the biological availability of phosphorus in wheat by-products. *Cereal. Chem.* **44**:318-323.

Temperton, H. and J. Cassidy. 1964. Phosphorus requirements of poultry. I. The utilization of phytin phosphorus by the chick as indicated by balance experiments. *Br. Poult. Sci.* **5**:75-80.

Theander, O., and E. Westerlund. 1984. Chemical modification of starch by heat treatment and further reactions of the product formed. Pages 202-207. In: P. Zeuthen, J. C. Cheftel, C. Eriksson, M. Jul, H. Leniger, P. Linko, G. Varela, and G. Vos, ed. Thermal Processing and Quality of Foods. Elsevier, London.

Ullah, A. H. J., and K. Sethumadhaven. 1998. Differences in active site environment of *Aspergillus ficuum* phytases. *Biochemical and Biophysical Research Communications*. **243**:458-462.

Verschoor. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Bri. J. Nutr.* **64**:525-540.

Vohra, P., G. A. Grey, and F. H. Kratzer. 1965. Phytic acid-metal complexes. *Proc. Soc. Exp. Biol. Med.* **120**:447-449.

Vranjes, M., H. P. Pfirter, and C. Wenk. 1994. Influence of processing treatment and type of cereal on the effect of dietary enzymes in broiler diets. *Anim. Feed Sci. technol.* **46**:261-270.

Vranjes, M., H. P. Pfirter, and C. Wenk. 1995. Influence of processing on dietary enzyme effect and nutritive value of diets for laying hens. *Can. J. Anim. Sci.* **75**:453-460.

Ware, J. H., and T. R. Shieh. 1967. U.S. Patent No. 3,297,548.

Wise, A. 1983. Dietary factors determining the biological activities of phytate. *Nutr. Abstr. Rev.* **53**:791-806.

Wodzinski. R. J., and A. B. J. Ullah. 1996. Phytase. Pages 263-302. In: Advances in Applied Microbiology, Volume 42. Academic Press, Inc.

Wyss, M., R. Brugger, A. Kronenberger, R. Remy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. G. M. van Loon. 1999. Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties. *Appl. Environmental. Microbiol.* **65**:367-373.

Wyss, M., L. Pasamontes, A. Friedlein, R. Remy, M. Tessier, A. Kronenberger, A. Middendorf, M. Lehmann, L. Schnoebelen, U. Rothlisberger, E. Kusznir, G. Wahl, F. Muller, H. Lahm, K. Vogel, and A. P. G. M. van Loon. 1999. Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Appl. Environmental. Microbiol.* **65**:359-366.

Wyss, M., L. Pasamontes, R. Remy, J. Kohler, E. Kusznir, M. Gadient, F. Muller, and A. P. G. M. van Loon. 1998. Comparison of the thermostability properties of three acid phosphotases from moulds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl. Environmental. Microbiol.* **64**:4446-4451.

Yi, Z., E. T. Kornegay, V. Ravindran, and D. M. Denbow. 1996. Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values of phytase. *Poult. Sci.* **75**:240-249.

Zanini, S. F., and M. H. Sazzad. 1999. Effects of microbial phytase on growth and mineral utilization in broilers fed on maize soyabean-based diets. *Br. Poult. Sci.* **40**:348-352.