Production of exogenous enzymes for animal nutrition: future challenges and comparative study between poultry and pigs

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Enzyme Application to Feed
One of the most researched fields in poultry science

More than 2500 independent tests of feed enzymes in broilers

(Rosen, 2010)

Grown to be a >$550 million Industry that saves the global feed market

~ $3 to 5 billion per year (Adeola & Cowieson, 2011).

Phytase
A. niger
P. lycii
E. coli
Citrobacter spp
Buttiauxella spp

NSPase
Xylanase
β-Glucanase

Protease
Bacillus subtilis
Bacillus licheniformis

Other
Amylase
Mannanase
Galactosidase
Glucoamylase
Lipase
Key Decisions: Phytase

Which Phytase?  What dose?

How much AvP / Ca\(^{2+}\) contribution?

Energy and Amino Acids from Phytase?

### Table 1. Some examples of currently commercially available 3- and 6-phytases and their characteristics

<table>
<thead>
<tr>
<th>Type</th>
<th>Protein origin</th>
<th>Expression</th>
<th>pH optima</th>
<th>Temperature optima (°C)</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>A. niger*</td>
<td>A. niger</td>
<td>2.5–5.5</td>
<td>65</td>
<td>Natuphos®</td>
</tr>
<tr>
<td>3</td>
<td>A. niger*</td>
<td>A. niger, non-recombinant</td>
<td>6.0</td>
<td>–</td>
<td>Allzyme® SSF</td>
</tr>
<tr>
<td>3</td>
<td>A. niger*</td>
<td>Trichoderma reesei</td>
<td>2.5</td>
<td>–</td>
<td>Finase® P/L</td>
</tr>
<tr>
<td>6</td>
<td>Escherichia coli*</td>
<td>Schizosaccharomyces pombe (ATCC 5233)</td>
<td>4.5</td>
<td>55</td>
<td>Phyzyme® XP</td>
</tr>
<tr>
<td>6</td>
<td>Escherichia coli*</td>
<td>Pichia pastoris</td>
<td>4.5</td>
<td>–</td>
<td>Quantum®</td>
</tr>
<tr>
<td>6</td>
<td>Escherichia coli*</td>
<td>Trichoderma reesei</td>
<td>–</td>
<td>–</td>
<td>Quantum Blue®</td>
</tr>
<tr>
<td>6</td>
<td>Escherichia coli*</td>
<td>Pichia pastoris</td>
<td>3.4, 5.0</td>
<td>58</td>
<td>OptiPhos®</td>
</tr>
<tr>
<td>6</td>
<td>Peniophora lytica*</td>
<td>Aspergillus oryzae</td>
<td>4–4.5</td>
<td>50–55</td>
<td>Ronozyme®</td>
</tr>
<tr>
<td>6</td>
<td>Citrobacter braakii</td>
<td>Aspergillus oryzae</td>
<td>–</td>
<td>–</td>
<td>Ronozyme Hipo®</td>
</tr>
<tr>
<td>6</td>
<td>Buttiauxella spp.</td>
<td>Trichoderma reesei</td>
<td>3.5–4.5(^a)</td>
<td>60(^b)</td>
<td>Astra® PHY</td>
</tr>
</tbody>
</table>

\(^a\) Adapted from Le et al., with modifications;  
\(^b\) 3- or 6-phytase; —, no information available;  
\(^c\) personal communication (C Evans).

Dersjant-Li et al, 2015

Are decisions different in chickens & pigs?

Supplier Recommended Nutrient Contributions from STD Dose Phytase in Poultry

<table>
<thead>
<tr>
<th></th>
<th>E.Coli 1</th>
<th>E.Coli 2</th>
<th>E.Coli 3</th>
<th>Citrobacter</th>
<th>E.Coli 4</th>
<th>Buttiauxella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units/kg feed</td>
<td>500 FTU</td>
<td>500 OTU</td>
<td>500 FTU</td>
<td>1000 FYT</td>
<td>500 FTUQ</td>
<td>500 FTU</td>
</tr>
<tr>
<td>Digestible P%</td>
<td>0.11</td>
<td>0.11</td>
<td>-</td>
<td>0.117</td>
<td>-</td>
<td>0.134</td>
</tr>
<tr>
<td>“Available” P%</td>
<td>0.12</td>
<td>0.13</td>
<td>0.13</td>
<td>0.146</td>
<td>0.15</td>
<td>0.146</td>
</tr>
<tr>
<td>Calcium %</td>
<td>0.11</td>
<td>0.13</td>
<td>0.14</td>
<td>0.18</td>
<td>0.165</td>
<td>0.134</td>
</tr>
<tr>
<td>Phytase cost ($/Feed Ton)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Phytase Cost / 0.12% AvP</td>
<td>0.50</td>
<td>0.46</td>
<td>0.46</td>
<td>0.41</td>
<td>0.40</td>
<td>0.41</td>
</tr>
</tbody>
</table>

In practice, decisions of phytase source and dose are frequently determined on phytase cost /0.10% or 0.12% AvP release

Dose is usually < Max. profit from P replacement to risk

Commercial values, 2014

Barnard et al., 2014
Net feed Cost saving from Ca and P matrix above 500FTU/kg is small compared to perceived risk of removing more inorganic phosphate from feed.

Higher phytase doses in Pigs are less attractive economically and one quickly runs out of added inorganic phosphorus to replace.
Nutritionists are cautious about taking out more inorganic P too early… for good reason

**Bones must not only take the animal to market, but also hold up through processing**

Osteochondrosis  
Femur Fractures  
Culls due to Leg problems

Nutritionists are cautious about taking out more inorganic P too early… for good reason

However… using 500 FTU phytase to only replace 0.1% - 0.15% AvP is effectively only targeting 30 – 46% of undigested Phytate in feed by terminal ileum.

<table>
<thead>
<tr>
<th>Phytase dig. P replacement (%)</th>
<th>AvP Replacement</th>
<th>% Phytate P made available (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04 0.05</td>
<td>15.4%</td>
<td></td>
</tr>
<tr>
<td>0.06 0.08</td>
<td>23.1%</td>
<td></td>
</tr>
<tr>
<td>0.08 0.10</td>
<td>30.8%</td>
<td></td>
</tr>
<tr>
<td>0.1 0.13</td>
<td>38.5%</td>
<td></td>
</tr>
<tr>
<td>0.12 0.15</td>
<td>46.2%</td>
<td></td>
</tr>
<tr>
<td>0.14 0.18</td>
<td>53.8%</td>
<td></td>
</tr>
<tr>
<td>0.16 0.21</td>
<td>61.5%</td>
<td></td>
</tr>
<tr>
<td>0.18 0.23</td>
<td>69.2%</td>
<td></td>
</tr>
<tr>
<td>0.2 0.26</td>
<td>76.9%</td>
<td></td>
</tr>
</tbody>
</table>

*Based on diet with 0.26% Phytate P and 28.18% P / mol phytate.  
* Undigested phytate will depend on % phytate digested in absence of phytase (10-40%)
Ileal Phytate Hydrolysis in Broilers from Standard doses of phytase ~ 45-60%

Tamin et al., 2004

Shirley & Edwards, 2003

Plumstead et al., 2013

Similar results for some new “third generation” E.coli phytases

Beaulac et al. 2016
Phytate has been shown to have negative effects on live performance – independent of phosphorus

Phytate not only affect phosphorus digestibility, but also reduces amino acid digestibility in broilers & grower pigs

Average reduction in AA digestibility from increasing phytate

Broilers: - 2.01%

Pigs: - 3.23%
Linear correlation between phytate degradation & Amino acid digestibility in broilers and Pigs

Amerah et al., 2012

\[ y = 0.1292x + 72.203 \]
\[ R^2 = 0.9591 \]
\[ P < 0.05 \]

Dersjant Li et al., 2016

Our objective: 100% rapid degradation of phytate to reduce anti-nutritional effects on ME & AA

What dose? What Phytase?

What dose, what phytase??

Amerah et al., 2012

Dersjant Li et al., 2016
Phytase decisions on **Source** and **Dose** also need be based on phytate interactions with nutrients and understanding differences in biochemistry between phytase enzymes in the context of digestive physiology.

1. **Interactions of Phytate, Calcium, and Phytase Enzymes** – affects P contribution
2. **Interactions of Phytate with Protein, Starch, and Na** – Anti-nutrient effects on live performance & drives ME & AA digestibility improvement from phytase
3. **Differences in phytase enzyme pH optima and kinetics** - affect in-vivo results

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**What Biochemical criteria make a phytase great in terms of in-vivo efficacy?**

–For maximum Phytase performance benefits in the animal, IP6 (Phytate) needs to be hydrolysed as **as rapidly as possible, as completely as possible, in the proximal part of the digestive tract** (Acid stomach / Proventriculus / Gizzard) with IP5-IP2 in Duodenum / Early Jejunum.

**WHY?**
Interactions of phytic acid with dietary nutrients are pH dependent

Mineral cations also chelate at low pHs if soluble (Tamin et al., 2003)
Proteins and phytate acid also interact at higher pHs >6 in presence of Ca^{2+} Briggs (1959, Saio et al. (1967,1968)

**Gizzard / Proventriculus**

- Binds with basic AA of protein
- Binds with basic AA of protein
- Binds with basic AA of protein

**Duodenum / Ileum / Jejenum**

- Binds with divalent mineral cations
- Binds with divalent mineral cations
- Binds with divalent mineral cations

Nelson et al., 1968; Maga, 1982; Angel et al., 2002, Selle et al.,2009,2012; Walk et al.,2012

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**Interactions of IP6 with Ca and protein phytate**

( % of phytate P that precipitated)

- IP6 + Ca
- IP6 + ISP
- IP6 + ISP + Ca

ISP = protein soy isolate
IP6 = phytic acid

Kim et al., unpub
Slide from R. Angel
3 Objectives we Must achieve with Phytase

1. Prevent Interaction of Phytate with Calcium, Zn, and Protein in small intestine by hydrolysing 100% Phytate in Acid stomach

2. Prevent phytate from interacting with Protein and amino acids in acid to reduce antinutrient effects of phytate and maximize ME & AA dig.

3. Prevent phytate from interacting with Calcium in the Acid Stomach to maximize phytate hydrolysis here.

Why is objective Nr 1 important?

Mineral cations also chelate at low pHs if soluble (Tamin et al., 2003)

Gizzard / Proventriculus

1. Prevent Interaction of Phytate with Calcium, Zn, and Protein in small intestine by hydrolysing 100% Phytate in Acid stomach

Mineral cations also chelate at low pHs if soluble (Tamin et al., 2003)

Gizzard / Proventriculus

Nelson et al., 1968; Maga, 1982; Angel et al., 2002, Selle et al., 2009, 2012; Walk et al., 2012
Calcium negatively affects P digestibility

\[ \text{% P dig} = 72.112 - 51.75 \times \% \text{Diet Ca} \]

\[ R^2 = 0.81 \quad (P < 0.001) \]

Corn-SBM diet, no added inorganic phosphate
- tP = 0.44%, PP = 0.32%, nPP = 0.12%
- Limestone added to increase Ca
- Analyzed Ca 0.21 to 0.78

SEM 1.61
n = 9

Kim et al., 2013

Impact of diet Ca on P digestibility appears to be saturable

\[ \text{% P dig} \]

\[ \text{Diet Ca, %} \]

Slide presented by R. Angel, 2013
Kim et al., 2013
Impact of [Ca] on P digestibility

- Increasing diet [Ca] reduces P digestibility in broilers with/without phytase (Mohammed et al. 1991; Tamim et al., 2004; Adeola and Walk, 2013)

![Graph showing the impact of Ca on P digestibility](image)

**Speed of Hydrolysis of Phytate is important in Chickens**

![Diagram showing pH levels in different parts of the chicken's digestive system](image)

- **Crop**: pH 5.3 (4.3-6.5)
- **Proventriculus**: pH 1.9 (1.0-2.8)
- **Gizzard**: pH 2.5 (1.6-3.2)
- **Jejunum**: pH 6.5 (6.3-6.8)
- **Duodenum**: pH 5.5 (4.5-6.2)
- **Distal Duodenum**: pH 6.0 (5.7-6.4)
- **Ileum**: pH 6.8 (6.4-7.0)

<table>
<thead>
<tr>
<th>% soluble</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>97.9</td>
<td>35</td>
</tr>
<tr>
<td>84.7</td>
<td>40</td>
</tr>
<tr>
<td>62.1</td>
<td>45</td>
</tr>
<tr>
<td>51.7</td>
<td>50</td>
</tr>
<tr>
<td>26.4</td>
<td>60</td>
</tr>
<tr>
<td>11.1</td>
<td>65</td>
</tr>
<tr>
<td>7.9</td>
<td>75</td>
</tr>
<tr>
<td>1.5</td>
<td>84</td>
</tr>
</tbody>
</table>
New Research shows no/little effect of Ca on P digestibility in presence of phytase (subject to Ca solubilization rate)

Objective Nr 2 is critical for maximum ME and AA effects

Mineral cations also chelate at low pHs if soluble (Tamar et al., 2003)

Proteins and phytate acid also interact at higher pHs >6 in presence of Ca²⁺ (Briggs 1959, Saio et al. 1967, 1968)

Gizzard / Proventriculus

Duodenum / Ileum / Jejenum

2. Prevent phytate from interacting with Protein and amino acids in acid to reduce antinutrient effects of phytate and maximize ME & AA dig.

Binds with basic AA of protein

Binds w/ divalent mineral cations

SEM = 1.128

Overall P value <0.001

W/O Ca added 0,8 mm Limestone

Angel et al., Unpublished (1505)

All diets contained 0.221% PP from corn
N=8 (4 b/p), 24 d of age

Nelson et al., 1968; Maga, 1982; Angel et al., 2002, Selle et al., 2009, 2012; Walk et al., 2012
Phytic Acid Interactions with Protein

- Protein-Phytate complexes—form directly with phosphate group at low pH
- Tertiary bridges – via Ca and basic residues in the protein, at pHs>6
- Protein-phytate formation proportional to the ratio of Phytate:Protein

Protein-phytate complex formation is fundamental to phytate effects on protein/amino acid availability

Only IP6 and to a lesser extent IP5 has the ability to aggregate with soluble proteins at a pH of 2.5

Phytase must effectively degrade IP6-protein complexes rapidly at low pH
Phytic acid concentration (% w/v)

Relative activity of pepsin (%)

Phytate – Protein complexes are not broken down by Pepsin

Effect of phytic acid on the inhibition of porcine pepsin catalyzed azo-casein hydrolysis

Yu et al., 2012

Phytase reverses anti nutritional effects of phytate, allowing Pepsin to degrade protein – dose dependent benefits

Reaction conditions: pH 2.5, 40°C, 1 hr

Danisco, 2009 unpublished
Degradation of protein-phytate aggregates by phytase
Rate of breakdown is dose-dependent

Phytase dose effect on the clarification of soy protein aggregates induced by phytic acid

Turbidity of the soy protein-phytic acid complex

Reaction time (hr) of Phytase B at 37 °C

Different Phytases have different pH optima and different RELATIVE activity at low pH vs. pH 5.5.

All phytases are standardized at pH 5.5.
Rate of IP6 degradation of Buttiauxella vs. E.coli phytase

~ 3 x more rapid IP6 degradation in vitro confers substantial benefits in-vivo

Differences in enzyme kinetics and pH optima of phytases result in very different phytate dephosphorylation patterns and phosphate release during in-vitro simulation of digestion

Mendez et al., 2015, J.Agric Chem.

Enzymatic phytate dephosphorylation of wheat during in vitro simulation of poultry digestive tract in a high buffer system
Increasing Enzyme Substrate Ratio is a further tool to increase speed of phytate degradation.

Greater Relative Activity of Buttiauxella Phytase

Increasing dose of Buttiauxella Phytase

500 FTU 1500 FTU
0.26% Phytate 0.26% Phytate

Increased Enzyme:Substrate Ratio

More rapid IP6 degradation

Mechanism of Phytate Anti-nutrient effects

Binary protein-phytate complex refractory to pepsin digestion

Pepsin + Mucin contribute to endogenous amino acid flows

Increased endogenous amino acid flow results in reduced amino acid digestibility and lower efficiency

Myo-Inositol production and absorption ??
Meta analysis to model Model DE Contributions from Buttiauxella Phytase

- 12 Energy Digestibility Trials conducted from 2008 to 2011.
- 10 Weaner and 2 Grow-fin trials
- Range of Phytase dosing from 130 – 3200 FTU/kg feed (analyzed)
- Average Phytate P level = 0.24% (0.23% Wean, 0.24% Grow-fin)
- Increments GE digestibility determined at fecal (11 or Ileal level 1)
- DE contribution calculated by multiplying % increase energy digestibility x analyzed GE of the diets.
- Modelling used mixed models to assess interactions of stage (Weaner or Grow) on DE response.
- Significant for linear and quadratic terms (P<0.001) for DE response to added phytase.
- Significant interaction of stage*AFTU, suggesting the response in DE varied by stage.
- Each stage (Weaner and Grow-fin) modeled separately using non-linear regression models.

Overview of 12 trials for DE response to Axtra Phy
Treatments where Change in DE was negative (lower GE digestibility than NC) These were included in dataset for modeling
### Overview of diets – 5 WB, 7 CS diets

<table>
<thead>
<tr>
<th>Stage</th>
<th>Trial</th>
<th>FTU dose</th>
<th>FTU dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phytate</td>
<td>Wheat</td>
</tr>
<tr>
<td>GROW</td>
<td>1</td>
<td>2.39</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.50</td>
<td>36.1</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>2.45</td>
<td>36.1</td>
</tr>
<tr>
<td>WEEAN</td>
<td>1</td>
<td>2.04</td>
<td>57.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.05</td>
<td>59.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.36</td>
<td>45.0</td>
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<td>35.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.69</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.24</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
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<td>8</td>
<td>2.36</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.28</td>
<td>57.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.85</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>2.35</td>
<td>47.0</td>
</tr>
</tbody>
</table>

### Overview of GE digestibility from 12 Trial datasets included in Meta analysis to model GE digestibility from Buttiauxella Phytase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Trial</th>
<th>FTU dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GROW</td>
<td>1</td>
<td>84.99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84.10</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>84.55</td>
</tr>
<tr>
<td>WEEAN</td>
<td>1</td>
<td>89.92</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>91.25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>74.21</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>82.37</td>
</tr>
</tbody>
</table>

12 Trials with 487 data points included in analysis
Predicted digestible energy response to Buttiauxella using non-linear regression

Combined (weaner and grower-finisher) DE response to using non-linear regression

*SE = Standard error
Similar Results in Broilers: Average of 7 Trials in meta analysis

1) Mitcherlich model: \[ Y = A \times (1 - \exp(C \times (A_{FTU} - B))) \]

Our objective: 100% rapid degradation of phytate to reduce anti-nutritional effects on ME & AA

What dose? What Phytase? What dose, what phytase??

Amerah et al., 2012

Dersjant Li et al., 2016
Linear Relationship between AID phytate and mean AA digestibility in piglets

\[ y = 0.1292x + 72.203 \]

\[ R^2 = 0.9591 \]

\[ P < 0.05 \]

Lysine digestibility response to *Buttiauxella* Phytase, average of 7 trials in broilers

*Actual Lys dig. Coeff.*  *Predicted Lys Dig. Coeff.*

\[(0.26\% \text{ Phytate P})\]
Conclusions

Linear correlation between phytate degradation & Amino acid digestibility in broilers and Pigs

Amerah et al., 2012

\[ y = 0.1292x + 72.203 \]

\[ R^2 = 0.9591 \]

P < 0.05

Dersjant Li et al., 2016

\[ y = 0.207x + 69.5 \]

\[ R^2 = 0.678 \]

500 FTU dose not enough

\[ y = 0.1292x + 72.203 \]

\[ R^2 = 0.9591 \]

P < 0.05

500 FTU dose not enough
Speed of IP6 hydrolysis drives ME and Amino acid benefits of phytases

- Faster IP6 Hydrolysis
- Greater ME release
- Greater AA release

Greater Live-Performance

**Greater ME release**

62

43

**Greater AA release**

3.0

Significant Feed Cost Savings from ME and Amino acids IF you are confident on research underlying matrix values

<table>
<thead>
<tr>
<th>Phytase Dose</th>
<th>Feed Cost Saving/Tonne ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA+P Matrix only</td>
<td>$16.19 +2.02</td>
</tr>
<tr>
<td>Ca+P+75%ME/AA*</td>
<td>$18.22 +1.11</td>
</tr>
</tbody>
</table>

The value of energy/Amino acids from phytase is 3-4 x greater than the value of Calcium & Phosphorus

- $5.45 +0.56 |
- $5.84 +0.12 |
- $5.86 -0.33 |
- $5.63 -0.62 |
- $4.99

*Uses Full Ca and P contribution & 75% of ME and amino acid contribution of Axtra Phy phytase
Which came first, the chicken or the egg?