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Amelioration in growth and phosphorus assimilation of poultry birds using cell-bound phytase of *Pichia anomala*

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Summary

Cell-bound phytase of *Pichia anomala* was produced in glucose–beef extract medium in shake flasks and in a laboratory fermenter at 25 °C for 24 h at 250 rev/min. In the fermenter the biomass production increased and the fermentation time was reduced from 24 to 16 h. Two-week-old broiler chicks were fed with the biomass-supplemented feed [at 100 g/7.5 kg; 50-phytase units/bird/day]. The overall weight gain in the biomass-fed chicks was higher (90.2%) than that of the control group (77.7%). The biomass incorporation in the feed of broiler chicks also resulted in a better phosphorus retention (29% in the control, and 73.68% in the biomass-fed) in the body, consequently an improved growth. There was a decrease in the excretion of phosphorus in the faeces of the chicks fed with phytase-supplemented diet (188.9 mg/g dry matter) as compared to the chicks fed on unsupplemented broiler finisher ration (509.4 mg/g dry matter). This eliminated the need to supplement phosphorus in their diet and also reduced phosphorus pollution. The feed conversion ratio was also lowered for chicks, which were biomass-fed as compared to the control.

Introduction

Phytic acid is an abundant plant constituent comprising 1–5% by weight of edible legumes, cereals, oil seeds, pollens, nuts and others. It is an organic form of phosphorus, *myo*-inositol hexakis-dihydrogen phosphate (IP6). It is the primary source of inositol and storage form of phosphorus in plant seeds that are used as animal feed ingredients (oilseed meals, cereal grains and legumes) (Maga 1982). Most foods of plant origin contain 50–80% of their total phosphorus as phytates (Harland & Morris 1995).

The role of phytin–phosphorus in the plant was earlier speculated as a storage product. It is believed that a large amount of phosphorus is stored in the seed and it gets liberated on germination and incorporated into ATP. Recent studies have established the role of inositol phosphate intermediates in the transport of materials into the cell and as secondary messengers in signal transduction (Berridge & Irvine 1989).

Due to the interaction of phytic acid with other compounds, it acts as an antinutritional factor in several ways: (i) six reactive groups in the molecules of IP6 make it a strong chelating agent, which binds cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}. Under gastrointestinal pH conditions, insoluble metal phytate complexes are formed which make the metals unavailable for absorption in the intestinal tract of animals and humans (Maga 1982), (ii) phytates reduce digestibility of proteins, starch and lipids by forming insoluble complexes, and (iii) the action of certain enzymes such as amylase, trypsin, acid phosphatase and tyrosinase is inhibited by phytic acid and inositol pentaphosphate (Harland & Morris 1995). There are several methods for the reduction of phytic acid such as cooking, autoclaving, ion exchange, elution with buffer and germination of seeds. The use of these methods, in most cases, leads to loss of nutritional constituents. An alternate enzymatic treatment method using phytases is considered to be superior to the others (Bali & Satyanarayana 2001; Satyanarayana & Vohra 2003).

Phytases hydrolyse phytic acid to *myo*-inositol and phosphoric acid in a stepwise manner forming *myo*-inositol phosphate intermediates. The research on phytase spans 87 years from its discovery by Suzuki *et al.* (1907) until its commercialization in Europe in

1993–1994 by Gist-Brocades. The commercialization required not only a practical use and delivery of the enzyme, but also its production economically.

Phytases have been reported in a number of bacteria, yeasts and moulds. Among yeasts, extracellular phytases are produced by *Schwanniomyces castellii* (Segueilha *et al.* 1992), *Arxula adeninivorans* (Sano *et al.* 1999), *Pichia spartinae* and *P. rhodanensis* (Nakamura *et al.* 2000). An intracellular phytase occurs in *Saccharomyces cerevisiae* (Nakamura *et al.* 2000) and *Pichia anomala* (Vohra & Satyanarayana 2001, 2004).

Ruminant animals sustain the microflora that enzymatically release inorganic phosphorus from phytic acid. Monogastric animals such as humans, chickens and pigs however produce little or no phytase in the intestine. Hence, the phytic acid phosphorus is unavailable and the phytin-P is excreted (Mullaney *et al.* 2000; Satyanarayana *et al.* 2004). Phytic acid present in the manure of these animals is enzymatically cleaved by soil and water-borne microorganisms and the phosphorus thus released is transported into the water bodies causing eutrophication. This results in oxygen depletion due to excessive algal growth, which lowers the dissolved oxygen in water bodies resulting in fish kills (Vohra & Satyanarayana 2003). Supplementation of animal feeds with phytase provides swine and poultry producers with a safe and effective management tool to reduce nutrient run off by significantly reducing the amount of phosphorus excreted in the manure of the animals (Nelson, 1967; Bali & Satyanarayana 1999; Ciofalo *et al.* 2003; Chantasartrasamee *et al.* 2005). Furthermore, the reduction or elimination of inorganic phosphorus supplementation of animal feed reduces P in the manure by more than 33%, thus cutting the pollution burden by one-third (Bogar *et al.* 2003). *Pichia anomala* was shown to produce a cell-bound phytase (EC 3.1.3.8) (Vohra & Satyanarayana 2001), which was acid-stable as well as adequately thermostable, the characteristics that are desired for application in animal feeds (Vohra & Satyanarayana 2002b). This investigation was aimed at evaluating the beneficial effects of supplementation of poultry feed with the cell-bound phytase of *P. anomala*.

Materials and methods

Yeast strain and growth conditions

The yeast strain was isolated from dried flower buds of *Woodfordia fruticosa* and was identified as *Pichia anomala* (Hansen) Kurtzman (Barnett *et al.* 2003). The yeast strain was grown on malt–yeast–glucose–peptone (MYGP) [g/l: malt extract, 3; yeast extract, 3; glucose, 10; peptone, 5; pH 5.6] agar slants at 30 ± 1 °C for 2 days, and preserved at 4 ± 1 °C in a refrigerator. The culture is deposited at the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Phytase production

Pichia anomala was cultivated in glucose–beef extract broth (Vohra & Satyanarayana 2002a). Erlenmeyer flasks (250-ml) containing 50 ml liquid medium were inoculated with the yeast inoculum (1%, 3×10^6 c.f.u./ml) and incubated in an incubator shaker at 25 °C for 24 h at 250 rev/min. The cultures were harvested by centrifuging at $8000 \times g$ for 15 min at 4 °C. The pellet was collected and assayed for phytase. For the determination of yeast biomass, the pellet was washed twice with distilled water and dried in pre-weighed tubes at 80 °C to a constant weight (Vohra & Satyanarayana 2002b).

Pichia anomala was also grown in 22-l fermenter (Biostat C, B. Braun, Germany) containing 10 l of the production medium. The fermenter was operated at 25 °C, 250 rev/min with 1 v/v/m of aeration. The pH of the medium was adjusted to 6.0 after sterilization with 1 M NaOH, and the medium was inoculated with 5% (v/v) inoculum. The fermenter was run for 48 h, and samples were collected every 2 h. The absorbance of the culture fluid was monitored at 600 nm in a double-beam spectrophotometer (Shimadzu UV-1601). The samples were then centrifuged for determining the biomass gravimetrically and phytase assay. The pH and the dissolved oxygen content were constantly monitored. The yeast biomass obtained was then lyophilized, stored at 4 °C in a refrigerator and used as the source of phytase to supplement the chick feed.

Phytase assay

Phytase was assayed by measuring the amount of phosphate released using sodium phytate as the substrate (Fiske & Subbarow 1925). One unit of phytase is defined as the amount of enzyme that liberates 1 μmol inorganic phosphate/min at 60 °C under the assay conditions.

Poultry bird feeding trials

Ten chicks of 3 weeks age, were divided into two groups (control and biomass) of five birds each. The control group was provided with broiler finisher ration, while the biomass group received additional supplementation of crude yeast biomass at 100 g/7.5 kg (50-phytase units/bird/day), of the finisher ration. The broiler finisher ration was composed of maize (45%), deoiled rice bran (30%), soya deoiled cake (10%), marble powder (1%), bone meal (2%), molasses (35%), fish meal (4%), ground nut cake (5%), vitamin mixture (250 g/quintal) and mineral mixture (500 g/quintal). Feed consumption was recorded daily, and body weights were recorded at the beginning and then at weekly intervals to assess the growth and feed conversion ratio (FCR). The feed consumption was equal as the birds were maintained on a restricted feeding regimen as per routine commercial practice.

Faecal droppings were also collected and weighed every 48 h period on two occasions (3–4 and 13–14 days) of the trial for phosphorus utilization assessment. Estimation of acid-soluble phosphorus in feed and dropping samples was also carried out. A 5.0 g air dried feed/dropping sample was dried in an oven, followed by ashing at 600 °C for 4 h in a muffle furnace, and acid soluble minerals were extracted in dilute HCl. Phosphorus contents were estimated by colorimetric procedure (Fiske & Subbarow 1925). All the experiments were conducted in triplicate and their mean values are presented.

Results

In shake flasks, a phytase titre of 68 U/g dry biomass was attained after 24 h of incubation (Figure 1). There was no significant increase in the production levels in the fermenter, but the biomass increased from 5 to 8 g/l. Further, maximum phytase production was achieved in 16 h in the fermenter as compared to 24 h in the shake flasks (Figure 2).

The pH of the medium dropped from 6.0 to 3.7 after 16 h of incubation, which could be due to the production of acid, deamination of amino acids by the yeast strain. Phytase production increased linearly with increase in biomass accumulation till 16 h, and thereafter it declined. The sugar concentration decreased steadily with increase in phytase-containing biomass.

Table 1 shows the improved growth of chicks fed with yeast-biomass-supplemented feed for 2 weeks. The overall weight gain in the biomass-fed chicks (90.1%) was higher than that of the control group (77.7%). The biomass-fed chicks gained higher body weight as compared to the control, and therefore, their FCR was less than that of the control chicks. FCR is defined as the ratio of feed consumed per unit gain in body weight. Phytase being an enzyme that cleaves dietary phytic acid and liberates extra-dietary phosphorus for intestinal absorption, it was considered worthwhile to conduct a bioavailability trial. The biomass incorporation in the feed of broiler chicks also resulted in a better phosphorus retention (29% in the control, and 73.68% in the biomass-fed) in the body, consequently an improved growth (Table 2). There was a decrease in the excretion of phosphorus in the faeces of the chicks fed with phytase-supplemented diet (188.9 mg/g dry matter) as compared to the chicks fed on unsupplemented broiler finisher ration (509.4 mg/g dry matter).

Discussion

The production of metabolites in the fermenter provides advantages over that in shake flasks in better control of process parameters such as pH and aeration, and better mixing and uniform distribution of nutrients (Lambrechts *et al.* 1992; Babu 1994; Archana & Satyanarayana 1997). Thus, a peak in phytase production was achieved in 16 h as compared to 24 h in shake flasks.

On supplementing the broiler finisher ration with the biomass of *Pichia anomala*, the overall weight gain in the biomass-fed chicks was higher than that of the control group. This also resulted in better phosphorus retention in the body, consequently an improved growth. There was a decrease in the excretion of phosphorus in the faeces of the chicks fed with phytase-supplemented diet as compared to the chicks fed on unsupplemented broiler finisher ration.

Nelson *et al.* (1968) were the first to conduct feeding trials using corn–soya diet with culture filtrate containing

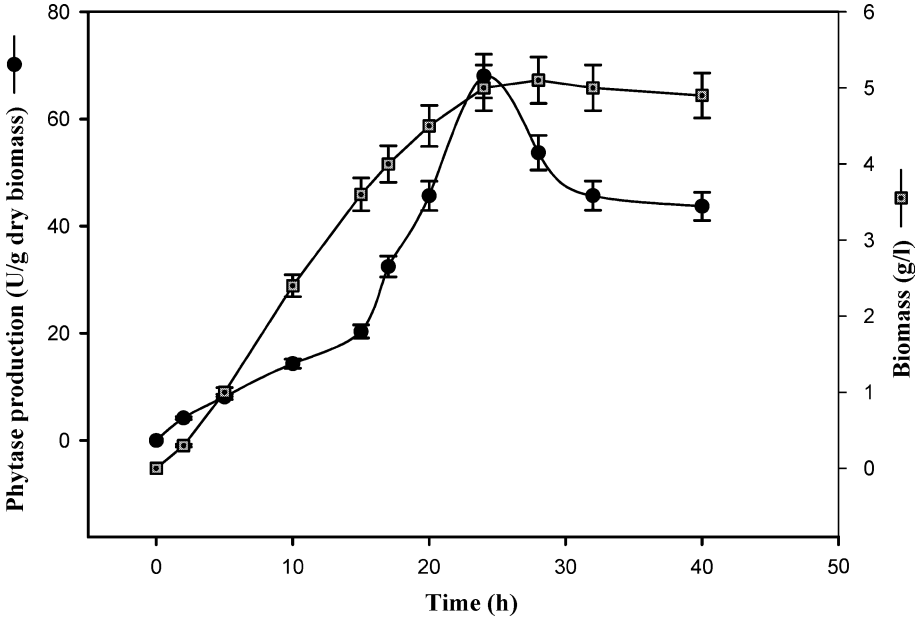

Figure 1. Phytase production by *Pichia anomala* during cultivation in shake flasks.

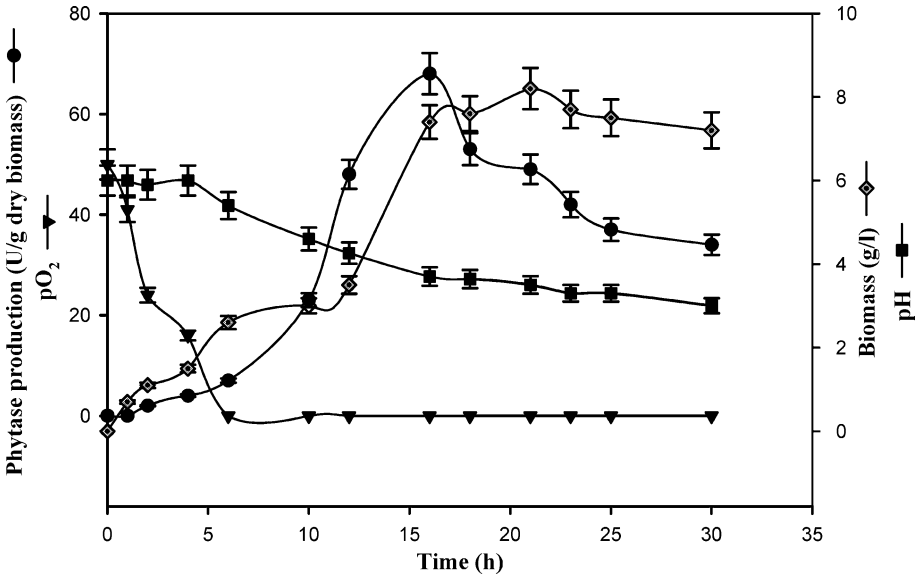

Figure 2. Fermentation profile of *P. anomala* in a laboratory fermenter.

Table 1. Comparison of weight gain and feed conversion ratio (FCR) in biomass-fed and control group of chicks (mean and standard error values) during 2 weeks feeding of *P. anomala* biomass-supplemented poultry feed.

Growth parameter	Control	Yeast-biomass-fed
Live weight (g) of a chick at start of experiment	792.0 ± 19.0	796.0 ± 13.2
Live weight (g) of a chick at first week	1040.0 ± 13.4	1076.0 ± 13.2
% Weight gain during first week	31.47 ± 1.95	35.13 ± 0.68[a]
Live weight (g) of a chick at second week	1408.0 ± 42.1	1514.0 ± 35.2
% Weight gain during second week	35.30 ± 2.74	40.67 ± 1.09[a]
Overall % weight gain for two weeks	77.70 ± 1.60	90.10 ± 2.06[b]
Feed conversion ratio (FCR)	2.272	1.949

[a]Significant at 5% level.
[b]Significant at 1% level.

phytases of *A. niger*. The chicks showed increase in bone ash due to the phytin-P released from the dietary substances by the action of phytases. A 3-week feeding trial with 180-day-old broiler chickens was conducted to study the efficacy of microbial phytase (Natuphos 1000) on growth performance, relative retention of P, Ca, Cu and Zn and mineral contents of plasma and bone (Sebastian *et al.* 1996). Phytase supplementation increased body weight in male and female chicks by 13.2 and 5.8%, respectively in 21 days. The supplementation of the low-P diet with phytase increased the relative retention of total P, Ca, Cu and Zn by 12.5, 12.2, 19.3 and 62.3% units, respectively. Research on enzymic cocktails comprising phytase A, phytase B, pectinase and citric acid in either soluble or intracellular form enhanced dephosphorylation and influenced phytate conversion rate. The enzymic cocktail strategy, when applied to poultry diets, also resulted in the high values

Table 2. Comparison of phosphorus retention and dropping phosphorus content in biomass-fed and control group of chicks.

Parameter	Control chicks		Yeast-biomass-fed chicks	
	Day 3–4	Day 13–14	Day 3–4	Day 13–14
(A) Droppings				
Dry weight of droppings (g)	212	348	203	390
Dry matter (DM) (%)	75.2	91.2	94.2	93.2
Ash content (% DM)	21.276	27.851	21.231	18.884
Acid soluble phosphorus (mg/g DM)	3.653	1.605	1.5122	0.5198
Phosphorus content in droppings (mg)	486.280	509.470	289.172	188.937
(B) Feed				
DM consumed (g)	961.86	1263.62	961.86	1263.62
Acid soluble phosphorus (mg/g DM)	0.5681	0.5681	0.5681	0.5681
Phosphorus intake (mg)	546.516	717.972	546.516	717.972
(C) Phosphorus retained (mg)	60.236	208.502	257.260	529.034
(D) Phosphorus retention (%)	11.021	29.041	47.08	73.68

of phosphorus retention (72–75% in broilers, 77–80% in turkeys) (Zyta 2001). The yeast *Schwanniomyces occidentalis* could be used to produce protein-rich feedstuff free of phytic acid, and phytates were removed from wheat bran and glandless cotton flour using the phytase of *S. castellii* at pH 4.4 and 70 °C (Simons *et al.* 1990). When microbial phytase was fed to broilers, the availability of phosphorus increased to 60% and the amount of phosphorus in the droppings declined by 50% (Nair & Duvnjak 1990). Canola meal, used as a feedstuff for livestock and fowl, was successfully dephytinized by *A. niger* NRRL 3135 in solid state fermentation (Segueilha *et al.* 1993; Ebune *et al.* 1995). Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish *Sebastes schlegeli* fed on diets containing soybean meal (Yoo *et al.* 2005).

At the close of the 20th century, annual sales of phytase as an animal feed additive were estimated to be $500 million. Evolution of the market for this feed additive can be attributed to a chain of events during the late 20th century that created the demand for the enzyme, and thus, provided a means for its commercial development (Abelson 1999).

The cell-bound phytase of *Pichia anomala* when added to chick feed improved the nutritional status of the feed by making phytate phosphorus available to the chicks. This resulted in improved weight gains, and also eliminated the need supplement phosphorus in the diet. There was an enhanced phosphorus retention and decreased phosphorus in the faeces, thereby reducing phosphorus pollution. Furthermore, the FCR was much lower with the yeast biomass supplemented feed than that with the unsupplemented.

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