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Safety evaluation of a phytase, expressed in *Schizosaccharomyces pombe*, intended for use in animal feed

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Abstract

BD006 phytase is the product of the *Escherichia coli* B *appA* gene expressed in *Schizosaccharomyces pombe* strain ASP595-1. This enzyme preparation is intended for use in animal feed applications where it improves the bioavailability of phosphate for monogastric animals. BD006 phytase was tested as an unrefined (DV006U) and a refined (DV006R) preparation for its effects on genotoxicity and in acute, inhalation and subchronic toxicity studies. Dosages ranged from 5000 μg/plate for in vitro toxicity studies to a high of 2000*X* for oral in vivo toxicity studies. The highest oral dose tested (2000*X*) is 2000 times the highest expected consumption of product by poultry or swine ($X = 50$ units of phytase per kg bwt/day). There was no genotoxicity or any in vivo toxicity reported which could be directly related to systemic effects of the product. Other additional safety studies conducted were devoid of any relevant toxicity. The historic safety profile of the production organism *S. pombe*, and the results of the toxicity studies presented herein, attest to the safety of BD006 phytase for use in animal feed applications.

1. Introduction

Phytases are a class of phosphomonoesterases that catalyze the stepwise release of orthophosphate from myo-inositol hexakisphosphate (phytate) (Nys et al., 1996). Phytate-bound phosphorous is the major source of phosphorous present in plant derived feedstuffs; the primary components of the diets of monogastric animals (poultry and swine). However, the bioavailability of plant phosphorous in these feedstuffs is limited because monogastric animals lack intestinal phytase enzymes at the level needed to hydrolyze phytate and release the inorganic phosphorous (Nys et al., 1996).

Phosphorous is an essential nutrient for growing animals. It is important for bone formation and mineralization, cell metabolism, protein synthesis, and is a constituent of cell membranes and intracellular buffers for acid–alkaline balance. For these reasons, phytase is increasingly used as an ingredient in swine and poultry feeds to improve the bioavailability of phytate-bound phosphorous and reduce the need to supplement the feed with elemental phosphorous.

The use of phytase also decreases the phosphorous content of the manure. The presence of excess phosphorous in manure contributes to nutrient over-enrichment, which is recognized as the largest source of runoff-induced impairment of US rivers, streams, lakes, reservoirs, and estuaries (cleanwater.gov, 2002). Nutrient over-enrichment has been associated with various adverse environmental effects, such as harmful algal blooms, *Pfiesteria* proliferation, and fish kills that in turn can lead to, among other things, public health risks (cleanwater.gov, 2002; EPA, NOAA, USDA, USGS, US Department of Health and Human Services, and ASIWPCA, 1998).

Animal feeding operations are one of the most significant contributors to nutrient over-enrichment. Therefore, supplementation of animal feeds with phytase provides swine and poultry producers with a safe

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and effective management tool to reduce nutrient runoff by significantly reducing the amount of phosphorus excreted in the manure of the animals.

Although not naturally abundant in monogastric animals, phytases are widely distributed in plants, animal tissues, and microorganisms (Nys et al., 1996). Phytases from the microorganism *Escherichia coli*, including *E. coli* strain B, encode for phytase via the *appA* gene. This paper describes two preparations, DV006U and DV006R, of BD006 phytase derived from *E. coli* strain B (ATCC 11303), that is expressed as a secreted, glycosylated protein in the yeast, *Schizosaccharomyces pombe*. This phytase, like other *E. coli* appA phytases, is a 6-phytase (EC 3.1.3.26, CAS 9001-89-2) that initially catalyzes phosphate ester bond hydrolysis of phytate at position 6 of the inositol ring (Greiner et al., 1993).

$$\text{D-Myo-inositol } 1,2,3,4,5,6 \text{ hexakisphosphate} + H_2O \rightarrow \text{D-myo-inositol } 1,2,3,4,5 \text{ pentakisphosphate} + P_i$$

The initial hydrolysis step is extremely rapid and likely represents the major hydrolysis event monitored during initial rate measurements for phytase activity. Phytase continues to remove the remaining phosphates from the inositol ring, but at a slower rate (Wyss et al., 1999). The *E. coli* appA phytases exhibit specific activities that are among the highest of all reported phytases (Lim et al., 2000). BD006 phytase possesses a specific activity of 960 U/mg enzyme protein under the conditions of the assay. The enzyme has a pH optimum of 4.5 and a temperature optimum of 50 °C. In addition, *E. coli* appA phytases possess dramatically lower activity on other phosphate-containing substrates such as adenosine nucleotide phosphates, fructose 1,6-bisphosphate, and glucose 6-phosphate (Greiner et al., 1993). BD006 phytase exhibits properties that are substantially similar to other commercial microbial phytases (Lassen et al., 2001; Wyss et al., 1999).

The phytase *appA* gene from *E. coli* B was directly integrated into a non-toxigenic and non-pathogenic host, *S. pombe* (ATCC 38399), at the *leu 1* site using a yeast recombination method (Okazaki et al., 1990). The resulting production strain, ASP595-1, produces BD006 phytase via a submerged, fed-batch, pure culture fermentation. The enzyme is secreted as a glycosylated protein in three variants with molecular weights of 54.4, 51.9, and 49.0 kDa.

This enzyme and its production process have been evaluated following the guidelines and recommendations put forth by Pariza and Foster (1983), Pariza and Johnson (2001), International Food Biotechnology Council (IFBC) (1990), and the Organization for Economic Co-operation and Development (OECD, 1992).

As noted by Kessler et al. (1992), in discussing enzymes derived from microbial sources, "generally, enzymes that are substantially similar to enzymes known to be safely consumed (including minor variations in structure or function) would not raise safety concerns" (Kessler et al., 1992). Therefore, *a priori*, BD006 phytase should also be considered a safe and effective feed enzyme for use in poultry and swine diets.

The *S. pombe* production strain ASP595-1 was used to produce both an unrefined test article (DV006U) and a refined test article (DV006R), for the safety and toxicology testing discussed in this paper. The unrefined test article is a less refined preparation, and includes fermentation broth from which most, but not all, of the production organism cells have been removed, whereas the refined test article (representative of the final product) does not contain yeast cells.

2. Materials and methods

Unrefined and refined test articles were produced in a fermentation and recovery pilot plant following a process representative of the manufacturing process for the commercial enzyme, at scale. The test articles were prepared in accordance with current good manufacturing practices (cGMP) for foods.

S. pombe strain ASP595-1, originating from a frozen glycerol stock, was cultured in successive seed flask stages and ultimately in a 30 L fed-batch, pure culture fermentation. The batch and feed media, and process and quality control systems were representative of the manufacturing process.

For production of test articles, the recovery steps used in the manufacturing process were scaled-down to equivalent unit operations in the pilot plant.

For generation of the unrefined test article (DV006U), the fermentation broth was centrifuged in a continuous decanting centrifuge that removed approximately 80–90% of the initial cell mass.

For production of the refined test article (DV006R), the fermentation broth was centrifuged to remove most of the initial cell mass (>99%). The process stream was clarified using microfiltration to remove the remaining cells from the process stream and then concentrated by ultrafiltration. Finally, the enzyme concentrate was clarified by an additional filtration (polish filtration).

The majority of the unrefined and refined test article material was lyophilized, prior to storage and use for toxicology and safety studies. The product was lyophilized to increase shelf life and facilitate transfer to external sites for toxicological testing.

The unrefined and refined test articles were analyzed for chemical and microbial composition, prior to use in safety studies. The results demonstrated that the samples conformed to the microbial and chemical specifications established for enzyme preparations used in food processing, as published in the Food Chemical Codex (FCC), Fourth Edition, 1996 (Committee on Food

Chemicals Codex, 1996), and by the Joint FAO/ WHO Expert Committee on Food Additives (JECFA) (FAO/ WHO, 2001). Samples tested were also shown to be free of antimicrobial activity and mycotoxins.

2.1. Genotoxicity studies

2.1.1. Mutagenicity assay

The objective of this study was to evaluate the test article, DV006R, for the ability to induce reverse mutations at the histidine locus in four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and at the tryptophan locus in *E. coli* tester strain WP2*uvr*A(pKM101) in the presence and absence of an exogenous metabolic activation system (Arochlor induced rat liver, S9). Test cultures were exposed to the test article via the "treat and plate" modification (Ames et al., 1975).

A dose range-finding study was performed using tester strains TA100 and WP2*uvr*A(pKM101) and 10 doses of test article ranging from 6.7 to 5000 μg/plate in the presence and absence of S9. Normal growth was observed in both tester strains at all doses evaluated, with and without S9, as evidenced by a normal background lawn and no dose-related increase in revertant frequencies. In addition, the test article was found to be freely soluble at all doses evaluated with and without S9.

Based upon these results, the test article was evaluated in the initial mutagenicity assay in all tester strains at doses of 33.3, 100, 333, 1000, 3330, and 5000 μg/plate in the presence and absence of S9. Normal growth was again observed in both tester strains and the test article was again found to be freely soluble at all doses evaluated, with and without S9. Revertant frequencies for all doses of DV006R, in all tester strains, with and without S9, approximated or were less than those observed in the concurrent negative control cultures.

DV006R was re-evaluated in an independent confirmatory assay under identical conditions and similar results were observed. Normal growth was again observed with both tester strains and the test article was again found to be freely soluble at all doses evaluated, with and without S9. Revertant frequencies for all doses of DV006R, in all tester strains with and without S9, approximated or were less than control values. All positive and negative control values in both assays were within acceptable ranges, and all criteria for a valid study were met.

The results of the *Salmonella–E. coli*/Mammalian-Microsome Reverse Mutation Assay (Treat and Plate Method with a Confirmatory Assay) indicate that, under the conditions of this study, DV006R did not cause a positive increase in the mean number of revertants per plate with any of the tester strains in the presence or absence of an exogenous metabolic activation system.

2.1.2. Mouse lymphoma assay

The purpose of this in vitro assay was to evaluate the ability of DV006R to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test article formed a transparent, dark brown solution in the vehicle (water) at 50.1 mg/mL, which was the highest concentration prepared for use in the assay. The test article remained in solution at all concentrations tested.

A dose range-finding assay was performed with and without metabolic activation using a treatment period of approximately 4 h with concentrations from 9.9 to 5010 μg/mL.

In the absence of the rat liver S9 metabolic activation system, DV006R was noncytotoxic up to and including treatment at 1260 μg/mL, moderately cytotoxic at 2510 μg/mL, and weakly cytotoxic at 5010 μg/mL. In the presence of the S9 metabolic activation system, DV006R was noncytotoxic to weakly cytotoxic at all concentrations tested. The test article concentrations chosen for the mutation assays were based on these results.

In the initial nonactivation mutation assay, eight doses ranging from 39.3 to 5000 μg/mL were analyzed for mutant induction. No cytotoxicity, to weak cytotoxicity, was induced and none of the analyzed treatments induced a mutant frequency that exceeded the minimum criteria for a positive response.

A confirmatory assay was performed. In the confirmatory nonactivation mutation assay, eight treatments from 39.3 to 5000 μg/mL were analyzed. No cytotoxicity, to moderate cytotoxicity, was induced and none of the treatments induced a mutant frequency that exceeded the minimum criteria for a positive response. The test article was therefore determined to be negative without metabolic activation.

In the initial and confirmatory mutation assay in the presence of S9 metabolic activation, eight treatments from 39.3 to 5000 μg/mL were analyzed. No cytotoxicity was induced in the initial assay, and no cytotoxicity to weak cytotoxicity was induced in the confirmatory assay. None of the treatments in either trial induced a mutant frequency that exceeded the minimum criteria for a positive response and the test article was therefore determined to be negative with metabolic activation.

In conclusion, the test article, DV006R, was evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells, under activation and nonactivation conditions, at test article concentrations up to the testing limit for this assay.

2.1.3. Mouse micronucleus assay

This study was conducted to evaluate the test article, DV006R, for in vivo clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte (PCE) cells in Crl:CD-1 (ICR) BR mouse bone marrow (Heddle et al., 1983).

In the dose range-finding assay, the test article was dissolved in sterile water for injection and dosed by oral gavage to three males and three females per dose level. The animals were dosed at 500, 1000, or 2000 mg/kg and observed for up to 2 days after dosing for signs of toxicity and/or mortality.

Based on the results of the dose range-finding assay, the highest dose chosen was 2000 mg/kg; the maximum allowable dose based on regulatory guidelines. In the micronucleus assay, the test article was dissolved in sterile water for injection and dosed by oral gavage to six males for each dose level for each harvest time-point. The dose levels were 500, 1000, or 2000 mg/kg. Five animals dosed with the test article at 500 or 1000 mg/kg, and five animals dosed with the positive control article, were euthanized approximately 24 h after dosing for extraction of the bone marrow. Five animals dosed with the test article at 2000 mg/kg and five animals dosed with the positive control article were euthanized at approximately 24 or 48 h after dosing for extraction of the bone marrow. At least 2000 PCEs per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least 500 erythrocytes for each animal.

The test article, DV006R, induced no signs of clinical toxicity in any of the treated animals and was not cytotoxic to the bone marrow (i.e., as indicated by a statistically significant decrease in the PCE:NCE ratio). A statistically significant increase in micronucleated PCEs was not observed at any dose level or harvest time-point. The test article, DV006R, was evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

2.2. Acute toxicity study

2.2.1. Acute oral toxicity study in rats

The objective of this study was to determine the acute toxicity of DV006U when administered to rats as a single dose by oral gavage. Five rats/sex/group were administered DV006U at the $600X$ or $1000X$ level, where $X = 50$ units/kg body weight/day, which is the highest expected consumption of phytase by poultry or swine. An additional group of five rats/sex was administered sterile water for injection at a dose volume of 10 ml/kg body weight.

Evaluations were made of mortality, clinical observations and body weights. There were no deaths in this study and there were no clinical signs of toxicity observed following the administration of DV006U. Also there were no test article-related changes in body weight, weight gains or gross findings at necropsy on day 15.

The no-observed-effect level (NOEL) following the oral administration of DV006U to rats was considered to be greater than $1000X$.

2.3. Subchronic toxicity study

2.3.1. Ninety day oral toxicity study in rats

The objective of this study was to evaluate the toxicity of the unrefined (DV006U) and refined (DV006R) test articles when administered orally by gavage, once daily for 90 consecutive days to Sprague–Dawley rats. Based upon the results of a dose range-finding study, DV006U low-dose and mid-dose groups (20 rats/sex/group) were dosed at $100X$ and $600X$ levels, where X is equal to 50 units of enzyme activity/kg body weight/day, which is the highest expected consumption of phytase by poultry and swine. The DV006U high-dose and DV006R high-dose groups (25 rats/sex/group) were dosed at $1000X$ and $2000X$ dose levels, respectively. An additional group of rats (25 rats/sex/group) were dosed with sterile water for injection as the control group.

Evaluations were made of mortality, clinical observations, body weight, food consumption, clinical pathology parameters, ophthalmology, organ weights, and gross and microscopic pathology. Rats were sacrificed on day 91/92, or following a recovery period on day 105/103.

There were no test article-related deaths during this study. There were no test article-related clinical signs of toxicity observed and there were no test article-related changes in body weight, body weight gain or food consumption values. There were also no test article-related ophthalmologic changes during the course of this study.

After 90 days of treatment, there were no test article-related differences observed in the group mean white blood cell parameters, red blood cell parameters or red blood cell morphology. There were no changes in clinical chemistry parameters following 90 days of treatment, which were considered to be related to treatment with test article. There were no gross findings noted at necropsy, which were considered to be test article-related. There were no test article-related changes in absolute organ weights, relative organ to body weight ratios and relative organ to brain weight ratios. A few statistically significant values were noted but were not considered biologically relevant.

Inflammation occurred in the lungs, in some male and female rats (see incidence Table 1) given either DV006U or DV006R, at all dose levels administered. The incidence and severity was more profound in Group 4 (high dose DV006U) compared to Group 5 (high dose DV006R) rats. In addition, the incidence was dose-related in the rats given DV006U. Minimal to moderate inflammation in the lungs was noted in high-dose DV006U ($1000X$) and DV006R ($2000X$). Inflammation also occurred in recovery rats. The inflammation appeared as single to several relatively small foci involving the bronchioles and surrounding parenchyma. This chronic inflammatory response is of the bronchopneumonia

Table 1
Incidence and average severity of the lung inflammation in rats

Groups	Males					Females				
	1M	2M	3M	4M	5M	1F	2F	3F	4F	5F
Terminal sacrifice	0/20	8/20 (1.4)*	15/20 (2)	14/20 (2)	2/20 (1)	0/20	4/20 (1.8)	5/20 (2)	10/20 (1.8)	3/20 (1.3)
Recovery sacrifice	0/5			3/5 (1)	2/5 (1)	0/5			1/5 (2)	1/5 (1)

* Number in parentheses represents mean severity score of affected animals within treatment group. Groups 1 (sterile water), 2 (DV006U at 100*X*), 3 (DV006U at 600*X*), 4 (DV006U at 1000*X*), 5 (DV006U at 2000*X*).

type and is consistent with inhalation of a foreign substance.

The inflammation that occurred in the lungs is considered to be related to the physical characteristics of the test article and the residuum left behind by the dosing needle on the lining of the esophagus, in the vicinity of the larynx, leading to inhalation of the test article in proportion to concentration. Thus, this effect is considered to be a physical effect of the high concentrations administered, and not related to a systemic effect. Therefore, the no-observed-adverse-effect level (NOAEL) is considered to be greater than 1000*X* for DV006U and 2000*X* for DV006R.

2.4. Inhalation toxicity study

2.4.1. Acute, nose-only, inhalation toxicity study in rats

The 6-h nose-only inhalation toxicity of DV006U was evaluated in Sprague–Dawley rats. A limit test was performed in which a group of five male and five female rats received a 6-h nose-only inhalation exposure to a time-weighted average aerosol concentration (determined gravimetrically) of 2.20 mg/L. Following the exposure, the limit test rats were observed daily and weighed weekly. A gross necropsy examination was performed on all limit test animals at the time of scheduled euthanasia (day 14).

No mortality occurred during the study. The only notable clinical abnormalities observed included dark material around the facial area and decreased defecation. Body weight loss was noted in three females during the study days 0–7 and one female during the study days 7–14. Body weight gain was noted for all other animals during the test period. No significant gross internal findings were observed at necropsy on study day 14.

Under the conditions of this test, the acute inhalation LC_{50} of the unrefined test article (DV006U) was estimated to be greater than 2.20 mg/L in the rat.

2.5. Additional safety studies

2.5.1. Primary eye irritation in rabbits

This study was conducted to determine the potential irritant and/or corrosive effects of DV006U on the eyes of rabbits.

A dose of 0.1 ml of the test article (1691 units/ml) was instilled into the right eye of three male and three female New Zealand White rabbits, with the left eye (untreated) serving as a control. The eyes were examined at 1, 24, 48, and 72 h post-dose and scored for ocular irritation.

Non-positive ocular scores involving conjunctival redness were observed in four of the six animals at 1 h. No ocular scores were observed in any of the animals at 24, 48 or 72 h and the study was terminated after the 72 h scoring period.

Based upon the observations made in this study, DV006U was considered non-irritating (Class 1) according to the Modified Kay and Calandra Interpretation of Eye Irritation Test (Kay and Calandra, 1962).

2.5.2. Primary dermal irritation in rabbits

The objective of this study was to determine the irritant and/or corrosive effects of DV006U on the skin of rabbits.

Initially the test article, DV006U, was subjected to the in vitro technique of the Corrositex System with the intent of ruling out potentially corrosive test materials in the in vivo portion. The test article was classified as non-corrosive according to the Corrositex System (Gad, 1995).

The test article, DV006U (0.5 ml), was applied to a site on the clipped dorsal trunk of six New Zealand White rabbits (three males and three females). The exposure period for the site was 4 h. Observations for dermal irritation were recorded immediately after unwrap and at 24, 48, and 72 h (±1 h) after patch removal. Grading of irritation is according to the method of Draize (1959).

The following is a summary of the most severe dermal responses observed:

Exposure	Dermal responses	Comments
4 h	Very slight erythema and no edema	No other dermal signs noted

2.5.3. Delayed contact hypersensitivity in guinea pigs

The objective of this study was to determine the potential of the DV006U to elicit a delayed dermal contact

hypersensitivity response in guinea pigs by the measurement of skin reactivity.

For the induction phase of this study, 20 guinea pigs (10/sex) in the test article group were induced with three 6 h occluded dermal applications of DV006U with seven days between applications. A vehicle group of ten animals (5/sex) was induced in the same manner with sterile water for injection. A positive control group of six animals (3/sex) was induced with a known sensitizer; 1-chloro-2,4-dinitrobenzene (DNCB).

Fourteen days after the last induction, all animals were dermally challenged with occluded applications at naive test sites. Animals in the test article and vehicle control group were challenged with DV006U and/or sterile water for injection. Animals in the positive control group were challenged with DNCB (0.2% in acetone). Nineteen hours after the challenge, all animals were depilated and approximately 4 h later were scored for dermal irritation (24 h). Scoring was repeated at 48 h.

Under the conditions of this study, induction with DV006U did not elicit a delayed contact hypersensitivity response in guinea pigs when challenged with the test article as received.

Summary of challenge results

(Percentage of animals with scores after 24 and 48 h)

Group	Challenge	24-h scores					48-h scores				
		0	±	1	2	3	0	±	1	2	3
Vehicle	DV006U in sterile	100	0	0	0	0	100	0	0	0	0
	water for injection	100	0	0	0	0	100	0	0	0	0
Test article	DV006U in sterile	100	0	0	0	0	100	0	0	0	0
	water for injection	100	0	0	0	0	100	0	0	0	0
Positive control	DNCB (0.2% in acetone)	0	17	0	33	50	0	17	0	83	0

3. Discussion

The *S. pombe,* Linder teleomorph yeast strain (strain: ATCC38399), obtained from the American Type Culture Collection (ATCC, 2002), was the host strain used to produce the BD006 phytase enzyme product used in the studies presented in this paper. To thoroughly evaluate the safety of *S. pombe* for the heterologous expression of a food grade enzyme, two test articles, unrefined and refined, were used for the toxicology and safety studies. The unrefined test article contained some yeast cells from the host *S. pombe*, whereas the refined test article (representative of the final product) did not contain yeast cells.

Assessment of the safety of the production organism was conducted according to the safety guidelines of Pariza and Foster, which state that a nontoxigenic organism is "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" (Pariza and Foster, 1983). Upon evaluating various safety considerations (International Food Biotechnology Council (IFBC), 1990; OECD, 1992; Pariza and Foster, 1983; Pariza and Johnson, 2001) and the potential modes of toxicity such as allergenicity and the production of mycotoxins or antibiotics, *S. pombe* fulfills the criteria of a nontoxigenic and nonpathogenic microorganism.

Following this analysis, either the unrefined or refined enzyme preparations were evaluated in genotoxicity studies and in acute, inhalation, and subchronic toxicity studies. There was no genotoxicity reported in the mutagenicity assay, the mouse lymphoma assay, or the mouse micronucleus assay. There was no in vivo toxicity reported in the acute, subchronic, and inhalation toxicity studies which could be directly related to the systemic effects of the product. Additional dermal and eye irritation safety studies were also devoid of any relevant toxicity.

Extensive reviews of literature databases failed to generate documentation of toxigenic effects of *S. pombe*. Consistent with its history of safe use and consumption, *S. pombe* is classified by the National Institute of Health as an organism requiring the minimal safety precautions in handling and storage, i.e., a Biosafety Level I organism (US Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institute of Health, 1999).

S. pombe has a long history of safe use, as well as consumption (Barnett and Lichtenthaler, 2001; Lachance, 1995; Okafor, 1990; Pataro et al., 2000; Steinkraus, 1983), and fulfills the criteria of numerous safety evaluations. Also there is no documentation of it having any pathogenic or toxigenic effects, and on this basis *S. pombe* meets the criteria of a safe production organism. This assertion is substantiated by the toxicological and safety studies conducted on both unrefined and refined phytase preparations.

4. Conclusion

Information consistent with studies performed assures that the BD006 phytase product:

1. is derived from a production organism which has a long history of safe use,
2. is derived from a production organism which is non-toxigenic and non-pathogenic,
3. is derived from a controlled, pure fermentation process, and
4. does not induce any in vitro genotoxicity as well as any in vivo toxicity of the refined product in rats when inhaled, or consumed, up to 2000X; the intended use level in swine and poultry.

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