EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

OPINION OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON 3-PHYTASE EC 3.2.1.8 PRODUCED BY *ASPERGILLUS NIGER* CBS 491.94

(adopted on 22 January 2003)

1. BACKGROUND

SCAN adopted an opinion on the use of the 3-phytase EC 3.2.1.8 produced by *Aspergillus niger* CBS 114.94 [Natuphos®] as feed additive on 27 April 2000.

The applicant company has notified, through the rapporteur, that it intends to use a genetically modified micro-organism, *Aspergillus niger* CBS 491.94, to produce the above-mentioned 3-phytase.

The applicant, to address the additional safety requirements which might be requested due to the modified producing strain, has produced a complementary dossier which was presented by the Netherlands. The dossier concerning the modification of the producing strain was assessed in the course of 1999 by Member States and meets the current scientific requirements of the guidelines.

2. TERMS OF REFERENCE

The Scientific Committee on Animal Nutrition is requested to advise the Commission on the following questions:

(1) Is the product Natuphos® produced by *Aspergillus niger* CBS 491.94 safe to the consumer, the target animal categories (broilers, laying hens, piglets, pigs for fattening, sows) and the environment?

(2) Based on the efficacy data related to the 3-phytase EC 3.2.1.8 produced by *Aspergillus niger* CBS 114.94 and on the data of the present dossier, is the product Natuphos® produced by *Aspergillus niger* CBS 491.94 efficacious, when used as feed additive at the levels recommended by the Company in the above mentioned animal categories (see Table 1)?

Table 1

Proposal for publication on Annex 1 of 70/524/EEC for 3-phytase (Natuphos®) as produced with Aspergillus niger CBS 491.94 (FTU-11)

Additive n° E 1600	Chemical formula, description	Species or category of animal	Minimum content expressed in units of activity (FTU) per kg of complete feedingstuff	Other provisions
Category of additive				
3-phytase EC 3.1.3.8	Preparation of 3-phytase produced by *Aspergillus niger* (CBS 491.94) having a minimum activity of : Solid form : 5000 FTU ([1])/g Liquid form : 5000 FTU([1])/ml	Piglets with a maximum age of 2 months	500	Recommended dose per kilogram of complete feedingstuff: 500 FTU For use in compound containing more than 0,23% phytin bound phosphorous
		Pigs for fattening	280	Recommended dose per kilogram of complete feedingstuff: 400-500 FTU For use in compound containing more than 0,23% phytin bound phosphorous
		Sows	500	Recommended dose per kilogram of complete feedingstuff: 500 FTU For use in compound containing more than 0,36% phytin bound phosphorous
		Chickens for fattening	375	Recommended dose per kilogram of complete feedingstuff: 500 FTU For use in compound containing more than 0,23% phytin bound phosphorous
		Laying hens	250	Recommended dose per kilogram of complete feedingstuff: 500 FTU For use in compound containing more than 0,23% phytin bound phosphorous

(1) 1 FTU is the amount of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH 5,5 and 37°C

3. THE DATA PROVIDED BY THE COMPANY

The company has provided the working group with the following dossiers:

- Natuphos®, Supplementary data regarding the notification of a modification of the Natuphos® manufacturing process, October 2000.
- Evaluation of the ability of enzyme preparation from *Aspergillus niger* (FTU-11) to induce chromosome aberrations in cultured peripheral human lymphocytes.

4. CHARACTERISATION OF THE PRODUCT

Natuphos is a 3-phytase enzyme produced by *Aspergillus niger*. The minimum enzyme activity for the solid and liquid preparations is 5000 phytase units (FTU)/g. The particular production strain used in this application is FTU-11, a genetically

modified derivative of a parental strain GAM-53. At present, an other genetically modified production strain, FTU-8 is used for production purposes)

The phytase produced by FTU-11 has been compared to that of FTU-8 using SDS-page electrophoresis, isoelectric focusing, and assessing the pH and temperature dependence of the enzyme activity. According to the results there were minor differences in the isoelectric focusing patterns. However, no differences were observed in enzyme activity nor in the activity profiles at different pH and temperature conditions.

4.1. Details of the genetic modification

The parental strain, GAM-53, has been derived by UV-mutagenesis from the ancestral strain GAM-4 with a selection for improved glucoamylase production. Subsequently GAM-53 was transformed with pFYT3 expression cassette. No details of the structure of the construct or the selection system used was given, except that the cassette was the same that was used to construct a previous production strain FTU-8.

As a result of transformation individual transformant colonies were further purified and characterised for production parameters. A single colony isolate from the GAM-53 transformant # 575, subsequently designated as FTU-11, was selected for production purposes (FTU-8 was obtained by a similar procedure from transformant GAM-53 # 25).

FTU-11 has 36 phytase gene copies integrated at 10 different sites of the genome, while with FTU-8 the gene copy number is 15 and that of the integration sites 6. The methods used to analyse the copy number and the number of insertion sites were not given.

4.2. The safety aspects of the genetic modification

The company has done the several checks regarding the possible unintended effects of genetic modification. Screening of the production of antibiotic substances belongs to the routine control measures, and no antibiotic activity against *Staphylococcus aureus*, *Escherichia coli, Bacillus cereus, B. circulans, Streptococcus pyogenes,* and *Serratia marcescens* was detected using the paper disc method. The possibility of activation of mycotoxin production genes has also been monitored by an external expert laboratory by analysing “all important mycotoxins which may be significant in food”. No fungal metabolites were detected in FTU-11 phytase; in fact FTU-11 had ceased to produce malformin C and τ-naphtopyrone typical to the parent strain. In addition, the company routinely monitors the product for the presence of aflatoxin B1, T2 toxin, ochratoxin and zearalenone. According to the company, the phytase production cassette does not contain extra open reading frames and the 3’ sequence of the cassette does not indicate any possible read through and consequent production of fusion proteins of unknown activities in the host.

5. EFFICACY AND TOLERANCE

The 3-phytase enzyme itself is not changed by the change of production strain from FTU-8 to FTU-11. The same expression cassette, containing the same genetic information, was used to construct both the FTU-8 and FTU-11 production strain. Furthermore, the same technique (random integration) and the same host (*Apergillus niger GMA 53*) was used to construct as well FTU–8 as FTU-11. Therefore no differences in efficacy of 3-phytase in poultry and pigs should be expected.

Studies in piglets, pigs and sows were made to assess the efficacy of Natuphos® as produced by FTU-11. The tolerance was assessed for piglets, pigs, broilers and laying hens.

5.1. Pigs – Efficacy and tolerance

5.1.1. Piglets

280 piglets (initial body weight: 8 kg per animal) were divided into 10 groups (28 piglets per group; sub-divided into 4 pens per treatment and 7 animals per pen) and fed for 35 days a diet consisting of 35% maize, 30% barley, 11% soybean meal, 6% sunflower meal and some minor constituents. The negative control diet contained 1.3 g digestible P and 2.7 g phytic-P per kg feed. Positive control was supplemented with 1.5 g MCP-P.

Treatments 2 to 7 were carried out as a dose-response study with FTU-11 (Table 2). Furthermore FTU-8 and FTU-11 phytases were compared at similar levels (100 and 500 FTU/kg, compare treatments 2 and 8 as well as 4 and 9, Table 2).

Table 2 Efficacy trial with FTU-11 Natuphos phytase (CBS 491.94) in piglets (n= 28 per treatment, duration: 35 days)

Treatment		Enzyme added (FTU/kg)	Enzyme analysed (FTU/kg)	Average daily weight gain (g)	Feed conversion ratio (kg/kg)	Digestibility of P (%)
1	Negative control	0	0	369 ^{abc}	1.66	35.6^{a}
2	+FTU-11	100	93	369 ^{abc}	1.61	41.8 ^{b}
3	+FTU-11	250	259	377 ^{abcd}	1.61	49.3 ^{c}
4	+FTU-11	500	523	352 ab	1.64	55.0 ^{d}
5	+FTU-11	750	733	405 cd	1.62	60.2 ^{e}
6	+FTU-11	1500	1510	425 ^{d}	1.56	72.0 ^{f}
7	+FTU-11	15000	15400	426 ^{d}	1.56	82.1 ^{g}
8	+FTU-8	100	101	330 ^{a}	1.69	42.2 ^{b}
9	+FTU-8	500	509	382 ^{bcd}	1.65	53.5 ^{d}
10	Positive control (+ 1.5 g MCP-P)	0	0	376 ^{abcd}	1.64	49.6 ^{c}

^{abcdef} Means within columns with different superscript letters are significantly different ($p<0.05$)

Faecal digestibility was measured in weeks four and five of the experiment.

From this experiment it can be concluded that there are no important differences between microbial phytase by the production strain FTU-8 and FTU-11, in relation to animal performances or digestibility of phosphorus (Table 2). P-digestibility was significantly enhanced by the addition of microbial phytase. There was an experimental dose-response relationship between FTU-11 and digestibility of phosphorus. No signs of adverse effects of FTU-11 phytase on piglet health and performance were observed, even at a dose of 15.000 FTU/kg.

5.1.2. Fattening pigs

An efficacy and tolerance study on pigs for fattening was carried out (Table 3). The experiment involved 48 fattening pigs divided into six groups (8 pigs per treatment). The animals were housed in individual pens at an average weight of 30 kg and fattened to a weight of about 110 kg. The diets consist mainly of corn, barley and soybean extracts. The negative control diets contained 1.8; 1.3 and 1.0 g digestible P (dP) per kg and 2.5 g phytic P per kg in the three experimental periods. The diets of positive control were supplemented with MCP to increase the content of dP to 0.6g/kg. Natuphos® was added in increasing doses (Table 3). The findings on the apparent P digestibility show that the added phytase liberated phytate-bound phosphorus from the feed in a dose-dependent manner (Table 3). Phytase formulation proved to be effective on the digestibility of phosphorus in all three growing periods. No evidence of an adverse effect on metabolism or animal health was observed even at the extremely high does of 10.000 FTU/kg.

Table 3 Efficacy trial with phytase Natuphos® Prod.210518, Batok No 03-0151 in growing and fattening pigs (n=8 per treatment, trial with three periods)

Period (Body weight of pigs)	Treatment	Enzyme added (FTU/kg)	Average daily weight gain (g)	Feed conversion ratio (kg/kg)	Digestibility of P (%)
30-55 kg	1 Neg. control	0	624 [b]	2.36 [a]	33.0 [c]
	2 + phytase Natuphos®	100	676 [ab]	2.19 [b]	42.6 [b]
	3 + phytase Natuphos®	500	698 [ab]	2.14 [b]	45.0 [b]
	4 + phytase Natuphos®	1000	705 [ab]	2.12 [b]	54.3 [a]
	5 + phytase Natuphos®	10000	738 [a]	2.06 [b]	55.8 [a]
	6 Pos. control (+ 0.6 g dP /kg)	0	669 [ab]	2.13 [b]	40.1 [b]
55-90 kg	1 Neg. control	0	735	2.81	30.4[c]
	2 + phytase Natuphos®	100	736	2.83	37.9[d]
	3 + phytase Natuphos®	500	803	2.62	44.1[c]
	4 + phytase Natuphos®	1000	863	2.57	52.3[b]
	5 + phytase Natuphos®	10000	818	2.64	56.5[a]
	6 Pos. control	0	833	2.60	41.9[cd]
90-110 kg	1 Neg. control	0	857	2.99	27.5[e]
	2 + phytase Natuphos®	100	795	3.14	31.8[de]
	3 + phytase Natuphos®	500	812	2.98	42.2[c]
	4 + phytase Natuphos®	1000	822	3.06	49.3[b]
	5 + phytase Natuphos®	10000	807	3.07	55.8[a]
	6 Pos. control	0	766	3.23	36.9[d]
Total period 30-110 kg	1 Neg. control	0	728	2.68	
	2 + phytase Natuphos®	100	738	2.72	
	3 + phytase Natuphos®	500	771	2.57	
	4 + phytase Natuphos®	1000	798	2.56	
	5 + phytase Natuphos®	10000	792	2.56	
	6 Pos. control	0	762	2.61	

[abcd] Means within columns with different superscript letters are significantly different ($p<0.05$)

5.1.3. Pregnant and lactating sows

The efficacy of FTU-11 Natuphos® at a dietary level of 300 FTU/kg was studied in pregnant and lactating sows (Table 4). Phytase was added to diets consisting predominantly of barley, oat, wheat, rapeseed and soybean meal.

There was a significantly positive effect of Natuphos® on the apparent tract digestibility of nitrogen during pregnancy (+2.3%-units) and lactation (+1.9%-units, Table 4). Digestibility of Ca and P was also significantly increased by Natuphos® supplementation.

Table 4 Influence of FTU-11 Natuphos phytase (CBS 491.94) on apparent digestibility of some nutrients in pregnant and lactating sows (n= 8 per treatment)

Stage of pregnancy	Treatment	Enzyme added (FTU/kg)	Apparent digestibility (%)		
			Crude protein	Ca	Total P
Gestation	Negative control	0	79.1[a]	45.3[a]	42.0[a]
	+FTU-11	300	81.4[b]	49.7[b]	57.1[b]
Lactation	Negative control	0	80.5[a]	46.7[a]	44.6[a]
	+FTU-11	300	82.4[b]	49.9[b]	59.7[b]

[a, b] Means within columns with different superscript letters are significantly different ($p<0.05$)

5.1.4. Evaluation of efficacy in pigs

Three studies were carried out to assess the efficacy of FTU-11 phytase in piglets, growing and fattening pigs as well as pregnant and lactating sows.

In the experiment with piglets FTU-11 (from new GMO-strain) was compared with FTU-8 (from previous production strain) at levels of 100 and 500 FTU/kg. There were no significant differences for the most important parameters measured (see Table 2).

In the case of piglets, pigs and sows the company claims for 500, 280 and 500 FTU-11 per kg feed. Significant effects in piglets, pigs and sows could be measured for all claims.

No adverse effects were observed in the tolerance studies.

5.2. Poultry tolerance tests

5.2.1. Chicken for fattening

One experiment was carried out in order to assess the tolerance of phytase produced by the strain FTU-11 in broilers chickens. Three experimental groups were used. One negative control diet as a control group (deficient level of phosphorus without phytase supplementation) and two levels of phytase supplementation 1000 and 10 000 FTU Natuphos ® kg diet were used. From 360 birds, at day five, 270 were selected and allotted in 18 experimental units (pen or replicate), each pen housed 15 birds. The three experimental treatments were randomly distributed in 18 experimental units (3 treatments x 6 replicates).

The experiment was started when the chickens were five days old and continued until the age of 33 days. The experiment was divided in two period, first period 5-19 days and the second part from 19 to 33 days. The basal diet consisted in 50% maize , 30% soya , 5% tapioca, 3.8% fish meal, etc. The available phosphorus for the first and second period were 0.28 % and 0.22 % respectively.

Experimental procedure: Birds were weighed at 5, 19 and 33 days of age. Feed intake was determined (for both) the two experimental periods staring at 5 and 19 days of age, respectively, per experimental unit (15 birds in each). The feed conversion ratio was calculated by two week period. Water intake was measured at 21 and 25 days of age. The number of dead birds was registered in order to calculate mortality rate. Feed analysis and phytase activity were measured for experimental feed but the phytase recovery in the feed was not presented. Animal health and health status were monitored by visual observation by an animal technician by visual observation. In case of clinical signs of illness, a veterinary inspection was carried out.

Neither macroscopic examination of the organs of necropsied birds nor haematological studies, were conducted.

Results: During the experiment, the mortality rate was 3%. Mortality was not related to dietary treatment, since necropsy did not show any unusual cause of death. The performance parameters are presented in Table 5. In the first stage body weight and feed conversion were improved by the phytase supplementation. In the overall study, only the weight gain was significantly increased (+1000 FTU, Table 5)

Table 5. Body weight(BWG), daily feed intake(FI), feed conversion rate (FCR), daily water intake (WI) and water to feed ratio(WFR) of broiler chickens (Study number 809, annex IV, 8)

	Natuphos ® FTU/ kg				
	control	1 000	10 000	P	LSD
	First period 5-19 days				
BWG (g)	657[b]	734[a]	720[a]	0.001	32.2
FI(g)	64.0[b]	68.7[a]	67.4[a]	0.02	3.25
FCR(kg/kg)	1.362[a]	1.310[b]	1.312[b]	0.001	0.019
	Overall period 5- 33 days				
BWG(g)	1583[b]	1710[a]	1665[ab]	0.04	96.3
FI(g)	91.9[a]	98.0[a]	94.3[a]	0.10	5.57
FCR(kg/kg)	1.604[a]	1.604[a]	1,588[a]	0.54	0.035
WI (g/day)	251[a]	256[a]	251[a]	0.87	18.6
WFR	2.27[a]	2.16[a]	2.19[a]	0.16	0.099

[a, b] Means within rows with different superscript letters are significantly different ($p<0.05$)

5.2.2. Laying Hens

One experiment was carried out in order to assess the tolerance of phytase produced by the strain FTU-11 in laying hens. Three experimental groups were used. One control group with negative control diet (deficient level of phosphorus without phytase supplementation) and two levels of phytase supplementation (300 and 6 000 FTU Natuphos® kg per diet) were used. The experiment was carried out with a one week adaptation period and an experimental period of three weeks (22 to 25 weeks of age). At 21

weeks of age, 63 laying hens (7 hens per experimental unit x 3 experimental treatments x 3 replicates), were selected from a initial batch of 100 hens (LSL Lohmann brown) and were distributed to the experimental units based in body weight. The three experimental treatments were randomly distributed over the nine experimental units. The basal diet consisted in 60% maize, 18% soyabean, 8% tapioca, 3.8%, etc.

Experimental procedure: layers were weighed at 21 and 25 weeks of age . Feed intake was measured by week starting at 22 weeks of age per experimental unit (seven hens). The egg production was recorded daily by individual hen. Once a week the total egg production per experimental unit (seven hens) was weighed. Feed conversion rate was calculated as g of feed intake per g egg produced. Feed analysis and phytase activity were measured for experimental feed. Animal health and health status were monitored by visual observation by an animal technician. In case of clinical signs of illness, a veterinary inspection was carried out. Neither macroscopic inspection of the organs of necropsied birds nor haematological studies were conducted.

Results: The analytical results of the phytase recovery in the experimental feeds were 383 and 7030 FTU /kg of feed, as expected. The performance parameters of laying hens are given in Table 6. No mortality was recorded during the experiment. Only one hen from the group with the highest dose was euthanised in the third week due to an injury in the right leg. No significant effect was found on the performance of laying hens between the dietary treatments.

Table 6 Production performances of laying hens , measured during three weeks experimental period.(22 to 25 weeks of age, annex IV, 9).

	Nathupos ® FTU / kg				
	Control	300	6000	P	LSD
FI (g)	101.8	100.5	96.2	0.30	8.54
Laying rate%	98.6	99.5	98.8	0.51	1.89
Egg weight (g)	53.3	53.4	53.2	0.96	1.99
FCR	1.925	1.872	1.818	0.28	0.147
Body weight gain (g)*	192	160	123	0.28	70.9

*: including the pre-experimental period.
P: Significant of treatment effect
LSD: least significant difference at P<0.05

5.2.3. *Remarks on poultry tolerance tests*

Although, the experimental procedure used in tolerance test in poultry did not fit completely within the present guidelines; results demonstrated that a more than tenfold overdose of Natuphos® FTU-11 did not harm broiler chickens or laying hens.

6. SAFETY ASPECTS

6.1. Consumer safety

3-phytase EC 3.2.1.8 produced by Aspergillus niger CSB 491.94 (FTU-11) showed similar chemical structure, and similar chemical-physical properties as 3-phytase produced by the non-GMO-strain.

6.1.1. Mutagenicity

Two mutagenicity studies that did comply with GLP were undertaken.

In the first study, the enzyme preparation from *Aspergillus niger* (FTU-11) was tested in the Ames mutagenity test using *Salmonella typhimurium* and *Escherichia coli* tester strains in four histidine-requiring *Salmonella typhimurium* mutant strains: TA 98, TA 100, TA 1535 and TA 1537 and one tryptophan-requiring *Escherichia coli* mutant strain WP2uvrA, in two independent experiments. The assays were performed with 8 concentrations in triplicate, both with and without metabolic activation. Bacteria were exposed to 5 concentrations ranging from 100 to 5000 µg/plate. Sodium azide, 9-aminoacridine, daunomycine, methylmethanesulphonate, 4-nitroquinolone, and 2-aminoanthracene were used as positive controls. The enzyme preparation from *Aspergillus niger* (FTU- 11) did not induce a dose-related increase in the number of revertant colonies/plates ($hist^+$ in *S. typhimurium* or trp^+ in *E. coli*) in any of the strain used, with and without metabolic activation. Based on this study it is concluded than the test substance had an absence of mutagenicity at up to 5000 µg/plate.

In the second study, a chromosome analysis in cultured human peripheral lymphocytes treated *in vitro* with the enzyme preparation from *Aspergillus niger* (FTU- 11) was performed both in the absence and presence of a metabolic activation system in duplicate in two independent experiments. The enzyme preparation from *Aspergillus niger* (FTU- 11) was tested at doses of 100, 333, 1000, 3330 and 5000 µg/ml for several treatment and fixation times (Table 7). Cultures were treated with positive control mitomycin C (without metabolic activation) and cyclophosphamide (with metabolic activation). The mitotoxic index (dose range finding) of each culture was determined by counting the number of metaphase per 1000 cells; at least three analysable concentrations were used.

Table 7 Experimental conditions and results of the cytogenetic assay

		Treatment time (h)	**Fixation (h)**	**Results**
Experiment 1	+S9	3	24	Negative
	-S9	3	24	Negative
Experiment 2	+S9	3	48	Negative
	-S9	24	24	Negative

	-S9	48	48	positive

In the absence of metabolic activation, at 48h continuous treatment time, the enzyme preparation from *Aspergillus niger* (FTU-11) induced statistically significant increases in the number of cells with chromosome aberrations (including and excluding gaps) at the concentrations of 3330 µg/ml and 5000 µg/ml ($P<0.05$). The results of second experiment of this study indicate that enzyme preparation from *Aspergillus niger* (FTU-11) gave indications of clastogenicity at high doses.

This second *in vitro* cytogenetic study was repeated using similar experimental conditions to those used in the original study that gave a positive result. The same combination of exposure time and fixation time were used. The new study gave a negative result.

6.1.2. Comments on mutagenicity

Both cytogenic studies were well-conducted and it is not possible to discern which study gave the correct results. In order to safeguard consumer safety it is prudent to regard the enzyme preparation from *Aspergillus niger* (FTU-11) as a potential clastogen. The sponsor should investigate whether there is any *in vivo* mutagenic hazard from the enzyme preparation from *Aspergillus niger* (FTU-11). The Committee normally requires reassurance from negative results from in vivo mutagenicity studies using two different sites of somatic cells (e.g. bone marrow and liver) to demonstrate that *in vitro* mutagenicity is not expressed *in vivo*.

6.1.3. Short-term studies of toxicity

In a 14-day study of toxicity that comply with GLP, groups of 5 males and 5 females SPF-bred Wistar rats received enzyme preparation from *Aspergillus niger* (FTU-11) *by gavage* at a daily dose of 0, 500, 1500 or 4500 mg/kg bw. Clinical signs, body weights and food consumption were recorded weekly; gross pathology and organ weight were measured at termination. No histopathology of organs and tissues was performed. No deaths or signs of overt toxicity were noted in controls or in treated animals and there were no effects on body weights and body-weight gain during the study period. There were no substance-related effect on food consumption before or after allowance for body weight between treated groups and control animals. Treatment had no effects on haematology and blood chemistry end-points; prothrombin time and alanine aminotransferase (ALAT) values were decreased (slightly low for ALAT) in females at 4500 mg/kg bw per day. Gross pathology at necropsy did not reveal any alteration. No toxicologically relevant effects were seen on organ weights or organ:body weight ratios in animals dosed up to 4500 mg/kg bw per day. Kidney weight and kidney:body weight ratios

were increased in males treated with 4500 mg/kg bw per day. The NOAEL was 4500 mg/kg bw per day, the highest does tested.

In a 90-day study of toxicity (GLP-compliant), groups of 10 male and 10 female SPF- bred Wistar rats received enzyme preparation from *Aspergillus niger* (FTU-11) *by gavage* at a daily dose of 0, 500, 1500 or 4500 mg/kg bw per day. Clinical signs, body weights and food consumption were recorded weekly; functional observations (i.e. hearing ability, pupilary reflex, static righting reflex, grip strength) during week 12-13, ophthalmoscopic examination at pre-test (all animals) and at week 13 (control and at highest dose groups), gross pathology and organ weight were measured at termination. No histopathology of organs and tissues was performed. Treatment with enzyme preparation of *Aspergillus niger* (FTU-11) at doses up to 4500 mg/kg bw per day had no effects on general conditions, functional observations and the variation in motor activity, body weights, food consumption, ophthalmoscopic examinations, haemathology and blood chemistry end-points. The platelet count was decreased in males given the highest dose. Potassium content of serum was increased in males at 4500 mg/kg bw per day. Macroscopic examinations and organ weights revealed no susbstance-related effects in any of the dose groups. These finding were not accompanied by any indication of functional disturbance or morphological changes in the treated animals. The NOAEL identified for this study was 1500 mg/kg bw per day.

6.1.4. Conclusions on consumer safety

The bacterial mutation test showed no evidence of mutagenic effect. However, it must be noted that this enzyme preparation from *Aspergillus niger* (FTU-11) behaved as potential clastogen in the *in vitro* citogenetic assay and therefore *in vivo* mutagenicity studies using two different somatic cells are needed. SCAN recognises however exposure of consumers to the enzyme is not to be expected.

6.2. User Safety

6.2.1. Skin irritancy

In a GLP-compliant acute skin irritation/corrosion study of toxicity, 3 New Zealand rabbits were exposed to a single dose of 0.5 ml of the enzyme preparation from *Aspergillus niger* (FTU-11), applied into clipped skin for 4 h using a semi-occlusive dressing. The skin reactions were assessed at approximately 1, 24, 48 and 72 h after the removal of the dressing and the enzyme preparation from *Aspergillus niger* (FTU-11). Four hours exposure to 0.5 ml of enzyme preparation from *Aspergillus niger* (FTU-11) resulted in very slight erythema in the treated skin-areas of 2 animals, 1 h after exposure only. No oedema was noted and no irritant effects on the skin were observed in any of the animals. Moreover, no symptoms of systemic

toxicity were observed in the animals during the test period and no death occurred.

6.2.2. Eye irritancy

In a GLP-compliant acute eye irritation/corrosion study of toxicity, single samples of 0.1 ml of Natuphos® (FTU-11) were instilled in the conjunctival sac of one eyes of each of 3 New Zealand rabbits. Immediately, after the 24 h observation, a solution of 2% fluorescein in water was instilled into both eyes of each animal to quantitatively determine corneal epithelial damage. General conditions were recorded twice daily; toxicity at least once daily and day of treatment (prior to instalation). The eyes of each animal were examined approximately 1, 24, 48 and 72 h after instillation of the Natuphos®. Instillation of 0.1 ml of Natuphos® into 1 eye of 3 rabbits resulted in irritation of the conjunctivae, which was seen as redness of the sclera of 1 animal, 1 h after. Twenty-four hours after test substance instilation revealed no corneal epithelial damage in any of the animals and there was no evidence of ocular corrosion. No symptoms of systemic toxicity were observed in the animals during the test period and no death occurred.

6.2.3. Acute inhalation toxicity

In an acute (4-hour) inhalation toxicity study (GLP-compliant), one group of 5 male and 5 female SPF-bred Wistar rats each for a 4-hour period were exposed to a test atmosphere containing Natuphos® FTU-11 formulation at a limit concentration of 5.08 ± 0.31 g/m^3 (MMAD = 2.9 μm and the geometric standard deviation was 1.4.). After exposure, the animals were kept for a 14-day observation period.

Exposure to 5.08 g/m^3 produced laboured breathing (slight) in one female animal. However, no abnormalities were seen shortly after exposure and in the rest of the 14-day observation period and no death occurred. There were no effects on general conditions during the exposure and body weights recorded prior the exposure and on days 7 and 14. The macroscopic findings at necropsy were limited to a few petechiae on the lungs of one male animal. Based on this study, the 4-hour LC50 value of the Natuphos® FTU-11 formulation was greater than 5.08 g/m^3 for both sexes.

6.2.4. Conclusions on user safety

There was no evidence that the Natuphos® FTU-11 formulation may be irritant to skin and eyes.

No skin sensitisation assay was undertaken.

The results of the inhalation study based on the final product did not indicate any harmful effects.

6.3. Environmental safety

No studies on environmental safety are requested for enzymes.

7. CONCLUSIONS

The 3-phytase EC 3.2.1.8 produced by *Aspergillus niger* CSB 491.94 (FTU-11) showed no adverse effects in the target animal categories. However, the enzyme preparation gave indication for clastogenic activity in one of the *in vitro* cytogenicity test and therefore *in vivo* studies of genotoxicity should be conducted before the safety for the consumer and the user can be finally concluded.

The efficacy of the new formulation was demonstrated as equivalent to the existing FTU-8 product for pigs of all ages. Efficacy with poultry has not been demonstrated.