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December 13, 2017

VIA FEDERAL EXPRESS

Susan Carlson, Ph.D
Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notification for Beta-Glucosidase From *Aspergillus niger*

Dear Dr. Carlson:

On behalf of DSM Food Specialties ("DSM"), we are submitting under cover of this letter, one paper copy, and one eCopy of a generally recognized as safe ("GRAS") notification for its beta-glucosidase enzyme preparation derived from *Aspergillus niger*. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. DSM has determined through scientific procedures that beta-glucosidase is GRAS for use in brewing fermented beverages such as beer.

The enzyme preparation is for use in brewing as a processing aid to hydrolyze the O-glycosyl bond between the terminal (non-reducing) glucose residue and the rest of the molecule in glycosides, with the simultaneous release of beta-D-glucose. Therefore, the beta-glucosidase enhances flavor in beer by hydrolysis of hop glycosides. The beta-glucosidase enzyme will be denatured during the beer pasteurization, so there are no active enzymes present in the final beer.

Pursuant to the regulatory and scientific procedures established by 21 C.F.R. § 170.36, the use of beta-glucosidase from *Aspergillus niger* is exempt from premarket approval requirements of the Federal, Food, Drug, and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require additional information to aid in the review of DSM's conclusion, please do not hesistate to contact me via email at gary.yingling@morganlewis.com or by telephone, (202) 739 -5610.

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Sincerely,

(b) (6)

Gary L. Yingling

cc: DSM Food Specialties

GRAS NOTICE

BETA-GLUCOSIDASE FROM *ASPERGILLUS NIGER*

November 2017

DSM Nutritional Products
PO Box 1
2600 MA Delft
The Netherlands
Tel: 31 611377088

RECEIVED
DEC 18 2017
OFFICE OF
FOOD ADDITIVE SAFETY

GRAS NOTICE: BETA-GLUCOSIDASE FROM *ASPERGILLUS NIGER*

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LIST OF ANNEXES

1.0 SIGNED STATEMENTS AND CERTIFICATIONS

1.1 Submission of GRAS Notice

DSM Nutritional Products (DSM) is hereby submitting a Generally Recognized as Safe (GRAS) notice in accordance with the provisions of 21 CFR part 170, subpart E.

1.2 Name and Address of Notifier

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands

1.3 Name of the Substance

The common or usual name of the notified substance is beta-glucosidase. It consists of beta-glucosidase produced by submerged fed-batch fermentation using a selected, pure culture of *Aspergillus niger*; the strain used to produce beta-glucosidase is not genetically modified. The beta-glucosidase preparation is produced and sold in liquid form, standardized with glycerol.

1.4 Intended Conditions of Use

The beta-glucosidase preparation is intended for use in brewing of fermented beverages such as beer. The enzyme preparation is applied during the mashing stage of the brewing process. The enzyme is heat denatured, and consequently inactivated, during the wort boiling step of beer manufacturing process. The use of beta-glucosidase can thus be regarded as a processing aid because it has no function in the finished food.

1.5 Statutory Basis for GRAS Conclusion

Pursuant to 21 C.F.R. 170.30, DSM has determined, through scientific procedures, that the beta-glucosidase enzyme preparation from a non-genetically modified strain of *Aspergillus niger* is GRAS for use as an enzyme for the hydrolysis of the O-glycosyl bond between the terminal (non-reducing) glucose residue and the rest of the molecule in glycosides, with the simultaneous release of beta-D-glucose, at levels not to exceed good manufacturing practices.

1.6 Exclusion from Premarket Approval Requirements

It is the view of DSM that the substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on the conclusion by DSM that beta-glucosidase is GRAS under the conditions of its intended use in brewing fermented beverages.

1.7 Availability of Information for FDA Review

The data and information that are the basis for DSM's GRAS determination are available for FDA's review, and copies will be provided to FDA upon request, in either electronic format or by paper copy. Requests for copies and arrangements for review of materials cited herein may be directed to:

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1.8 Exemptions from FOIA Disclosure

Parts 2 through 7 of this notification do not contain confidential or proprietary information, and therefore no FOIA exemptions are claimed.

1.9 Authorization to Share Trade Secrets with FSIS:

Should FDA find the need to share the information in this application with FSIS, DSM has no objections.

1.10 Certification

On behalf of DSM, I certify that, to the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and DSM, and pertinent to the evaluation of the safety and GRAS status of beta-glucosidase for use in brewing fermented beverages.

(b) (6)

Gary L. Yingling
Senior Counsel
Morgan, Lewis & Bockius LLP

2.0 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 Identity of Notified Substance

- Systematic name : beta-D-glucoside glucohydrolase

- Other names : gentiobiase; cellobiase; emulsin; elaterase; aryl-ß-glucosidase; ß-D-glucosidase; ß-glucoside glucohydrolase; arbutinase; amygdalinase; p-nitrophenyl ß-glucosidase; primeverosidase; amygdalase; linamarase; salicilinase; ß-1,6-glucosidase

- Accepted name : beta-glucosidase

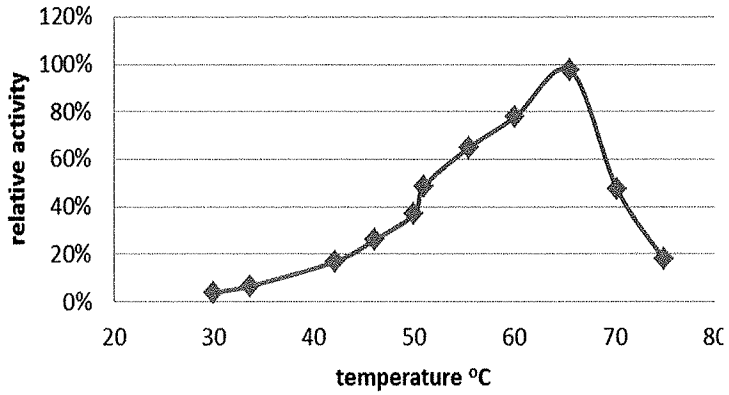
- IUPAC/IUB Number : EC 3.2.1.21

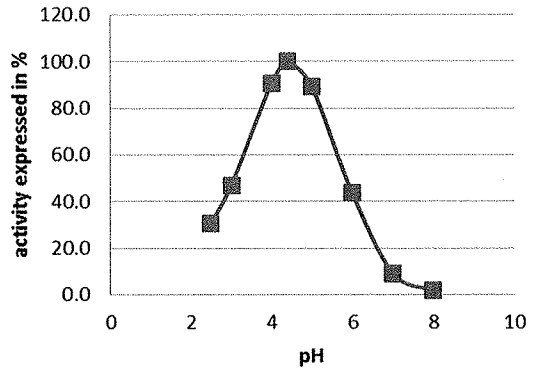
2.2 Principal Enzymatic Activity

Beta-glucosidase hydrolyses the O-glycosyl bond between the terminal (non-reducing) glucose residue and the rest of the molecule in glycosides, with the simultaneous release of beta-D-glucose.

The beta-glucosidase activity is expressed in WBDG units per gram or per ml. One WBDG is equivalent to the amount of enzyme that liberates 1 nmol PNP per second from the synthetic substrate paranitrophenyl beta-D-glucopyranoside at pH 4.4 and 37°C under the conditions of the assay. This method has been developed within DSM Food Specialties for determining the beta-glucosidase activity (see Annex 2).

The activity of beta-glucosidase from *Aspergillus niger* was measured under various pH and temperature conditions. The results are presented in the figures below.





As can be concluded from the figures above, the food enzyme beta-glucosidase from *Aspergillus niger* exhibits an optimal activity around pH 4 whereas the optimum temperature range lays between 60 and 70 °C. No enzyme activity is left at temperatures above 80°C.

2.3 Specifications for Food Grade Material

The common starting material for all formulations is the ultra-filtrate (UF) concentrate. Typically, its composition falls within the following ranges:

Enzyme activity	2500 - 6500 WBDG/g
Water (%)	82.5-92.5
Ash (%)	0-2
Proteins (Nx6.25, %)	5-10

Apart from the enzyme complex, the beta-glucosidase preparation will also contain some substances derived from the microorganism and the fermentation medium. These harmless constituents consist of polypeptides, proteins, carbohydrates, and salts.

In order to obtain a final formulation, the ultra-filtrated concentrate is diluted with glycerol to the desired activity.

The Total Organic Solids of the beta-glucosidase preparation were calculated for 3 commercial batches:

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (WBDG/g)	WBDG/mg TOS
414032901	83.2	0.64	16.2	6300	39
415062901	88.4	0.9	10.7	2710	25
415092902	89.2	0.57	10.2	3480	34
Mean					33

The TOS values of the final standardized enzyme preparations can be easily calculated on basis of values presented in the table above and taking the dilution factor into account.

Based on the above figures, the formulated commercial product with an activity of 4000 WBDG/g will have a TOS value of about 121 mg/g enzyme preparation.

The finished product is subjected to extensive controls and complies with JECFA and FCC specifications:

Parameter	Norm
Lead	< 5 mg/kg
Coliforms	< 15 CFU/g
Salmonella	0/25 g
Escherichia coli	0/25 g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

2.4 Description of the Production Organism

2.4.1 Name and designation

The strain used for the production of beta-galactosidase belongs to the species *Aspergillus niger*.

2.4.2 Source of the organism

The current *Aspergillus niger* strain used for production of beta-glucosidase, ARO, has been taxonomically identified by the expert on Aspergilli, Dr. R.A. Samson, of the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS), Annex 1. The strain ARO was determined as *Aspergillus niger* v. Tieghem.

2.4.3 Strain improvement

The parent strain of the *Aspergillus niger* ARO was obtained through an internal screening program focused on strains that have the capacity to release aroma compounds. *Aspergillus niger* ARO is derived from the parent *Aspergillus niger* strain by classical mutagenesis and selection on beta-glucosidase production level.

2.4.4 The classical taxonomy

The formal classification of *Aspergillus niger* is:

Kingdom: FUNGI

Division: EUMYCOTA

Genus: Aspergillus

Species: *Aspergillus niger*

2.4.5 Stability of parental or classical production organism in terms of relevant genetic traits

The *Aspergillus niger* ARO strain is regarded as a genetically stable strain. The ARO strain has been maintained for more than 20 years under laboratory conditions without any significant degeneration in yield or appearance of morphological variants

2.4.6 Nature of pathogenicity and virulence, infectivity, toxicity and vectors of disease transmission

Aspergillus niger does not appear on the list of pathogens in Annex III of Directive 2000/54/EC (EU, 2000) on the protection of workers from risks related to exposure to biological agents at work, as it is globally regarded as a safe microorganism:

- In the USA, *Aspergillus niger* is not listed as a Class 2 or higher Containment Agent under the National Institutes of Health (NIH) Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (USA, 2013);
- The US Environmental Protection Agency (EPA) has exempted *Aspergillus niger* from review by the Agency, due to its extensive history of safe use (USA, 1997);
- In Europe, *Aspergillus niger* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (Germany, 2002), the German Federal Office of Consumer Protection and Food Safety (BVL) (Germany, 2013), and the Dutch Commission on Genetic Modification (Netherlands, 2011). It is not mentioned on the list of pathogens on the Belgian Biosafety Server (Belgium, 2010).

As a result, *Aspergillus niger* can be used at the least stringent containment level at large scale, GILSP, as defined by OECD (1992).

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (*e.g.*, availability of nutrients, temperature and moisture) and biotic factors (e.g. competitors and predators). Their ever-changing environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In nature, this results in continuous adaptation of the microbes through induction of different biochemical systems; such as adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Klein and Paschke, 2004; Earl *et al.*, 2008). In contrast, culture conditions for microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of

the strain in question (see *e.g.* the review by Parekh *et al.*, 2000). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e. no external competitors or predators) with optimal abiotic conditions; and the fermentation process is terminated before or when the production strain enters the 'stationary growth phase'. Hence, there are no strong environmental signals that would induce stress (*e.g.*, lack of nutrient or low/high temperature) or defense mechanisms (*e.g.*, production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defense secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus superfluous, would result in wasting of energy, and is therefore highly unexpected (Sanchez and Demain, 2002). Furthermore, it is avoided from an economical perspective to optimize production efficiency and costs.

Aspergillus niger strains have been safely used for decades to produce citric acid as well as a wide variety of food enzymes (see below). In 1978, JECFA nevertheless expressed some reservations regarding food enzymes derived from non-pathogenic microorganisms commonly found as contaminants of foods, such as *Aspergillus niger*, and considered it necessary to establish purity specifications and to conduct short term toxicity experiments on such food enzymes (FAO/WHO, 1978). Because it was recognized that some strains of fungal origin, such as *Aspergillus niger*, might be able to produce certain secondary metabolites of potential safety concern, JECFA recommended to test food enzymes derived from fungal origin for the presence of the secondary metabolites aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone (FAO/WHO, 1984).

Based on the JECFA recommendations, a wide variety of food enzymes from *Aspergillus niger* have been tested already since decades for their safety as well as for the presence of potential unsafe secondary metabolites. More recently, JECFA (FAO/WHO, 2006) slightly changed its recommendation by stating that food enzymes from fungal origin should not contain toxicologically significant levels of secondary metabolites that theoretically could be produced by the species in question. Therefore, food enzymes from *Aspergillus niger* should be tested for the specific secondary metabolites that could theoretically be produced by the species, i.e. ochratoxin A and fumonisins (Pel *et al.*, 2007; Frisvad *et al.*, 2011).

The results of the high number of tests on secondary metabolites – which were evaluated by various competent authorities – confirm the general experience that *Aspergillus niger* strains do not produce toxicologically significant levels of secondary metabolites under industrial culture conditions, either because the industrial strains used have impaired ability to produce these secondary metabolites or because the culture conditions used for industrial production do not induce the formation of these secondary metabolites. The fact that culture conditions have an influence on the production of secondary metabolites has indeed been described in literature (Frisvad *et al.*, 2011).

2.4.7 Natural habitat, geographic distributions and climatic characteristics of the original habitats

Aspergillus niger is ubiquitous in soil and is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles and other decaying vegetation. Consequently, it is also commonly present as a contaminant in foods, such as rice, seeds, nuts, olives and dried fruits.

Aspergillus niger is a fungus that produces large black or brown conidia by phialids (a bottle-shaped structure within or from which conidia (conidiospores) are formed). The fungus is a saprophyte which is able to grow on a wide variety of complex substrates.

The optimal growth temperature for *Aspergillus niger* is 20-40 °C. It is capable of growing at a wide pH range, although acidic conditions are more favorable. In excess of sugars, *Aspergillus niger* will produce organic acids, like citric acid. Consequently, *Aspergillus niger* can acidify the culture medium; however, this occurs only under specific circumstances. *Aspergillus niger* prefers high oxygen concentrations and therefore grows best on the surface of organic substrates. It uses asexual reproduction via formation of conidiospores. Conidiospore formation is strongly inhibited during submerged fermentation.

Aspergillus niger has a long history of use in the food industry. It has been mostly used since the 1920s to produce citric acid and other organic acids by fermentation. Citric acid fermentation using *Aspergillus niger* is carried out commercially in both surface culture and in submerged processes (Berry *et al.*, 1977; Kubicek and Röhr, 1985), and it represents nowadays approximately 350,000 tons per year. The history of safe use of *Aspergillus niger* is primarily based on its use since the 1960s in the food industry for the production of a large number of food enzymes (Bennett, 1985a, 1985b; Schuster *et al.*, 2002). These food enzymes, including those derived from recombinant *Aspergillus niger* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Aspergillus niger* in the production of various foods, such as baking, brewing, juice production, wine production and the production of dairy products.

2.4.8 Good Industrial Large Scale Practice GILSP

DSM (as Gist-brocades) has produced pectinase with beta-glucosidase side activity for almost half a century, with various *Aspergillus niger* strain lineages and at various production scales, up to 200 m^3, at its enzyme production facility in Seclin, France. No adverse effects on the environment or health of the personnel employed in the fermentation facilities have been observed. At DSM, only submerged fermentations are used, during which no conidiospores are formed. Consequently, no health problems associated with exposure to Aspergillus spores are encountered.

The organism is non-pathogenic, does not produce adventitious agents and has an extended history of safe industrial use. Therefore, the beta-glucosidase production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD). In the facilities of DSM Food Specialties for the large-scale production of food enzyme

products, only fermentations not exceeding the GILSP level of physical containment are carried out.

2.4.9 Absence of Toxins

Although absence of mycotoxins was mentioned in the specification requirements for fungal enzymes as laid down by the Food Chemicals Codex ("FCC") and JECFA in the past, this requirement has recently been deleted.

Instead, the FCC (10th edition) mentions the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."

In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: "Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species."

The final enzyme preparations have been analyzed for mycotoxins, and no mycotoxins have been detected.

2.5 Method of Manufacture

2.5.1 Overview

Beta-glucosidase from DSM is produced by a controlled submerged fed-batch fermentation of a selected, pure culture of *Aspergillus niger* (see Section 2.4). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 3.

2.5.2 Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 6.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

The fermentation and fed medium used has been developed for optimum production of enzymes (in this case beta-glucosidase) by the *Aspergillus niger* host.

The antifoam used in the fermentation (Clérol EFC 3107) is used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998.

2.5.3 Fermentation Process

Beta-glucosidase from DSM is produced by a controlled submerged fed-batch fermentation of a pure culture of *Aspergillus niger*. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms.

The fermentation process consists of four steps: two successive pre-culture fermentation steps, followed by the seed fermentation and the main fermentation. The whole process is performed in accordance with Good Manufacturing Practices.

Biosynthesis of beta-glucosidase occurs during the main fermentation. To produce the enzymes of interest, a submerged, aerobic fed-batch fermentation process is employed, using a stirred tank fermentor. To control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points for pH or dissolved oxygen concentration.

Growth of the production organism and increase of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples.

2.5.4 Recovery Process

At the end of fermentation step, the cells of the production organism *Aspergillus niger* are killed by means of incubation of the biomass with sodium di-acetate. This treatment effectively kills the *Aspergillus niger* cells. The cell material is separated from the enzymes by means of a simple membrane filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ reduction filtration, and then concentrated by ultrafiltration (UF).

2.5.5 Formulation and Standardization Process

To obtain a liquid enzyme preparation, the UF concentrate is standardized with glycerol to the desired final enzyme activity.

2.5.6 Quality Control of Finished Product

The final beta-glucosidase preparation from *Aspergillus niger* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO in 2006 and the FCC (10^{th} edition). These specifications are described in Section 2.6.

2.6 General Production Controls (Good Manufacturing Practice)

Commercial demands require a strictly controlled fermentation process.

The enzyme manufacturing site at Seclin, France is operating since 1922 and has acquired ISO 9001-2000 certification.

2.6.1 Technical Measures

The batches of **primary seed material** are prepared, preserved, and stored in such a way that contamination and degeneration are avoided and genetic stability is secured. The vials are clearly labeled and strict aseptic techniques are applied during the recovery of the culture.

Only sterilized **raw materials** are used to prepare the nutrient medium for the fermentation.

The **fermentor** is a contained system. Only sterilized air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned, and replaced if necessary. Prior to inoculation, the fermentor is cleaned, rinsed, and sterilized. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermentor. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during **downstream processing** by several germ reduction filtrations. The filters are thoroughly cleaned for each production run.

2.6.2 Control Measures

After preparation of a new batch of **primary seed material**, samples are checked for identity, viability, and microbial purity. If these parameters are correct, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs. Each time a vial from such a certified batch of primary seed material is used for production, the viability, purity, and identity of the strain are checked.

The **raw materials** used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the **seed fermentation** samples are taken aseptically for analysis of pH, and microbiological quality in the laboratory.

During the **main fermentation** the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing** samples are being taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing will be discontinued.

3.0 DIETARY EXPOSURE

3.1 Estimated Dietary Intake

Based on the toxicological studies outlined in Part 6, the following estimation of the human consumption can be made:

Final food	Residual amount of (inactive) enzyme in final food (mg TOS/l beer)	95th percentile intake level (ml food/per person/day[1])	Estimated daily intake of enzyme (mg TOS/kg body weight/day)[2]
Beer	21-63[3]	360	0.126-0.378

The 28-day oral toxicity study resulted in a NOAEL of 5,000 mg enzyme preparation/kg body weight/day, corresponding to 440 mg TOS/ kg body weight/day. By applying an uncertainty factor of 6 for the duration of exposure (factor 3 from subacute to sub-chronic, and factor 2 from sub-chronic to chronic study[4]), the NOAEL extrapolated to sub-chronic exposure is estimated to be 73.3 mg TOS/ kg body weight/day.

The Margin of Safety is then calculated by dividing the extrapolated NOAEL by the Estimated Daily Intake (EDI). Therefore, the Margin of Safety lies between 194 and 582, which is high enough to ensure the consumer safety.

NOAEL (90-day study) = NOAEL (28-day tox study) / 6 — 440 / 6 = 73.3 TOS/ kg body weight/day

Margin of Safety = NOAEL (90-day study) / EDI — 73.3 / 0.378 = 194

73.3 / 0.126 = 582

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[1] Intake level of beer and ale based on Wilkinson *et al.* (1997). 90th percentile is approximately 2 times the intake level and 95th percentile approximately 4 times the intake level (US Food and Drug Administration, 2006). As alcohol intake is usually thought to be underreported in surveys US Food and Drug Administration, 2006), the 95th percentile is taken here as worst-case scenario.

[2] Calculated for a person of 60 kg.

[3] Considering a mean TOS value of 123.6 mg TOS/g enzyme preparation, determined in 3 different commercial batches.

[4] EFSA 2012 Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels, and Units in the absence of actual measured data.

3.1.1 Levels of Use

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. The levels of use expected to result in beneficial effect are described below.

The beta-glucosidase enzyme preparation is used in dose range of 1000 - 3000 ppm. The formulated commercial enzyme preparation Hoptimase is standardized to 4000 WBDG/g of beta-glucosidase activity.

Based on the information given above, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (denatured) enzyme in final food
Beer	1-3 g/kg wort	17%[5]	0.17-0.51 g/L beer

The beta-glucosidase enzyme preparation is added to the wort during the mashing stage of the brewing process to produce fermented beverages such as beer. The beta-glucosidase enzyme will be denatured during the wort boiling step of beer manufacturing process, so that no active enzymes will be present in the final beer.

3.1.2 Purposes

In principle, the enzymatic hydrolysis of the O-glycosyl bond between the non-reducing terminal glucose residue and the rest of the molecule in glycosides by beta-glucosidase can be used in the processing of all food raw materials which naturally contain these residues.

Beta-glucosidase is commonly used during manufacturing of beer, wine, and fruit juices.

The use of beta-glucosidase is widely described in literature. The flavor compounds in wine and fruit juices are covalently bound to sugar residues. These (glycosidic) flavor precursors are odorless. A sequential enzymatic hydrolysis is necessary to release the flavors. Beta-glucosidase releases a major proportion of the terpenols, benzyl alcohols and linalool oxides when applied in wine and passion fruit juice (Shoseyov *et al.*, 1990, Grassin, 1996). Thus, beta-glucosidase significantly accelerates the release of the aromatic compounds and so enhances the aroma of wine and fruit juices.

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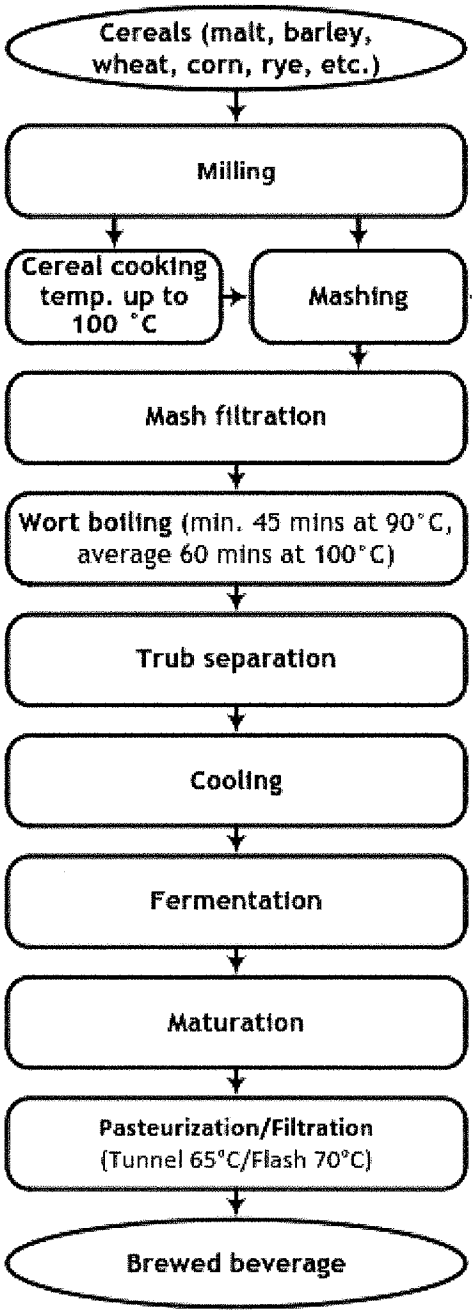
[5] The assumption used for calculation of dietary exposure is a yield of 100 L of beer per 17 kg of wort corresponding to a RM/FF ratio of 0.17 kg wort per L of beer.

DSM's intention is to use the beta-glucosidase from non-genetically modified strain of *A. niger* in the manufacturing of fermented beverages such as beer. The beta-glucosidase enhances flavor in beer by hydrolysis of hop glycosides (Daenen, 2007).

The different steps in the brewing process are shown below.

Brewing



3.2 Possible Effects on Nutrients

Beta-glucosidase acts, like any other enzyme, as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the enzyme protein itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The substrates for beta-glucosidase are certain glycosides, glycosidically bound flavor compounds, from hop. These glycosides are a sugar molecule, often a monosaccharide or disaccharides beta-linked to the hydroxyl group of flavor compounds such as linalool glucoside and geraniol glucoside.

The function of the beta-glucosidase is to hydrolyze the glycosides which are flavorless compounds. Once the glycosidic bond is broken the sugar molecule as well as the flavor active compound are released. For example, one study showed an increase in citrus, orange, grapefruit, and tropical pineapple flavors in a Cascade dry hopped beer that had beta-glucosidase enzymes added to it (Kirkpatrick, 2016).

Beta-glucosidases are wide spread in nature. Some fruits have been observed to have limited beta-glucosidase activity within themselves (Maicas 2005). Yeast and fungi are an important source of beta-glucosidases that can hydrolyze hop glycosides (Daemen 2007).

The reaction products of the enzymatic conversion of beta-glucosidase from hops in brewing are sugars and flavor compounds. These reaction products are naturally present in beer. Adverse effects on nutrients are not to be expected.

3.3 Consumer Population

Beta-glucosidase is a naturally occurring enzyme found in various microorganisms, plants and animals where the enzyme is involved in cellulose, starch and sucrose metabolism (Brenda http://www.brenda-enzymes.info/enzyme.php?ecno=3.2.1.21). In animals, including humans, an enzyme with beta-glucosidase activity was found in kidney and liver Berrin *et al.*, 2002; Dinur *et al.*, 1986; Gopalan *et al.*, 1992). Other edible sources are also mentioned in the scientific literature, such as papaya fruit (Hartmann-Schreier, J. and Schreier, P, 1986), orange (Barbagallo, R.N., 2007) and olive fruit (Kara, H.E. *et al.*, 2011).

In addition, the enzyme beta-glucosidase has a long history of use in food processing. Beta-glucosidase from different microorganisms are present in the list of enzymes used in food processing made by Pariza and Johnson in 2001 (Pariza, M.W. and Johnson, E.A., 2001). Beta-glucosidase is one of the enzyme activities in pectinase enzyme complex for which GRAS notification has been submitted and accepted by FDA with no questions for the use of pectinase enzyme preparation, GRN 89 (USA (2002))

Pectinase from *Aspergillus niger* containing beta-glucosidase as secondary activity has been evaluated by JECFA and attributed an ADI 'not specified' for their use in fruit and juice applications FAO/WHO (1989).

As is shown in Section 6.5 of this dossier, the amount of denatured enzyme in the final product is expected to be maximally 1289-3866 WBDG per L beer and the amount of enzyme TOS in the final product 21-63 mg/ L beer (0.0021-0.0063%).

Since the enzyme is present in food products at such low levels as an inactive protein, and because it is a naturally occurring substance in tissues commonly ingested by humans, the consumer population will be unaffected by the presence of the enzyme preparation in food.

4.0 SELF-LIMITING LEVELS OF USE

Enzyme preparations are generally used in quantum satis. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. Excessive amounts of enzyme would be expected to adversely affect the organoleptic and/or functional properties of the food.

5.0 COMMON USE IN FOODS PRIOR TO 1958

This section is not applicable to this application.

In addition to the positive evaluation of JECFA, countries which regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have approved the use of enzymes from *Aspergillus niger* in a number of food applications.

6.2 Safety of the Beta-Glucosidase Enzyme Preparation

As noted above, enzymes produced by *Aspergillus niger* have already been used for food production for several decades. In the USA, FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced by *Aspergillus niger* strains. FDA had no objections to each of these notifications.

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (Joint FAO/WHO Expert Committee on Food Additives, 1975a, 1975b, 1988). All these enzymes have received an ADI "not specified" by JECFA (see also Section 6.1).

Since it is generally known that commercial enzyme preparations of *Aspergillus niger* are not toxic and since beta-glucosidase has a history of safe use in food, it is not expected that beta-glucosidase will have any toxic properties.

Nevertheless, DSM conducted a limited package of toxicity studies, i.e. an acute (1 week) and a sub-acute (4-week) oral toxicity study in rats and two mutagenicity tests (Ames test and chromosome aberration test) to confirm the safety of the beta-glucosidase enzyme preparation (see Section 6.4). The toxicological studies were conducted for the pectinase enzyme preparation. However, since the DSM pectinase enzyme complex contains substantial amounts of beta-glucosidase as side activity, these studies are considered appropriate to assess the safety of beta-glucosidase.

The enzyme preparation showed no mutagenic or clastogenic activity, nor did it show toxic effects up to the highest dose level tested, which is considered as the no observed adverse effect level ("NOAEL").

The Margin of Safety is calculated by dividing the NOAEL derived from a sub-chronic toxicity study by the Estimated Daily Intake. In order to derive the NOAEL, an additional safety factor 6 was used to account for the duration of exposure in toxicological studies (factor 3 is taken for extrapolation from subacute to sub-chronic study and factor 2 from sub-chronic to chronic study, therefore a factor 6 is applied to extrapolate from subacute to chronic study[6]).

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[6] EFSA 2012 Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels, and Units in the absence of actual measured data.

Consequently, the Margin of Safety of beta-glucosidase lies between 194 and 582, which is high enough to ensure the consumer safety.

Given that the total intake is over-estimated to build additional safety into the calculations, considering that the enzyme will be inactivated during the wort boiling step and that the NOAEL is based on the highest dose level tested, DSM is convinced that the actual Margin of Safety will be much higher.

DSM has determined that its beta-glucosidase enzyme preparation can be used *quantum satis* in fermented beverages such as beer. The use of the enzyme for this intended use will not introduce any concerns of safety, supported by the toxicological and literature studies discussed above.

6.2.1 Allergenicity

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions.

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens.

The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food. The use levels are based on the activity of the enzyme, not the amount of the enzyme product.

Enzymes have a long history of safe use in food. Since new enzymes are generally (based on) existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure of the enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, 1994). To our knowledge, no reports exist on sensitization to enzyme products in the final commercial food after ingestion.

The absence of food allergenicity has been confirmed by an extensive literature search and survey of producers' files, in which no cases have been found of people who have been sensitized or persons who experienced an allergic reaction following the ingestion of food prepared with various enzymes (see Annex 6). Even among people who ingest high daily doses of enzymes as digestive aids for many consecutive years, there are no reports of gastrointestinal allergy to enzymes. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen *et al.*, 2006).For the purpose of this dossier the amino acid sequence of beta-glucosidase was compared with the amino acid sequences of known (food) allergens stored in the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated

February 12, 2013). AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm (Annex 6).

The exact amino-acid sequence of DSM beta-glucosidase from non-genetically modified strain *A.niger* was not determined. However the amino-acid sequence of beta-glucosidase from *A.niger* is available in public databases[7] and therefore this sequence was used in allergenicity screening. Based on these results, it is concluded that the beta-glucosidase protein has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

In addition, beta-glucosidase has been used worldwide for a number of years without any complaints from end consumers.

With regard to allergenicity of the fermentation media, DSM has concluded that the data that it has and the public data and information allow to conclude that there is no published or unpublished data that suggest there is a potential allergen from the fermentation media in the finished enzyme product. To reach that conclusion, DSM relies on the following data:

1. The Enzyme Technical Association in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA's website. The statement concludes that no potentially allergenic protein from the fermentation medium has been found in the finished enzyme, and states that regulatory bodies in both the EU and Japan have concluded that enzyme preparations do not pose an allergen risk that would require allergen labeling on the final product. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product's purity and stability, and would likely remove most proteins present in the fermentation media.

2. In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that because of the nature of enzymes as catalysts, they are used in very small amounts, and that the fermentation media are consumed during the enzymatic process. It is clear that any *de minimis* amount of protein present in the fermentation media that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscored the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicated that there is no reliable assay that could be used to detect the presence of most allergenic proteins in the final enzyme products, as the proteins would likely be degraded into fragments that would not reach levels of quantitation accessible with current commercial ELISA assays. The full August 2013 statement clearly concludes that any protein allergen present in the

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[7] *See* UniProtKB results for "beta glucosidase aspergillus niger" *available at* http://www.uniprot.org/uniprot/?query=beta+glucosidase+aspergillus+niger&sort=score.

final enzyme product would not be present at a level that requires labeling or raises a public health concern (see Annex 6).

It is therefore concluded that the beta-glucosidase protein is not likely to produce any allergenic or sensitization reactions by oral consumption.

6.2.2 Leading publications on the safety of beta-glucosidase enzymes or enzymes that are closely related

The safety of the production organism is the point of focus as to the safety of the enzyme which will be used in food processing. In this case, the production organism *Aspergillus niger* has been demonstrated to be nonpathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices ("cGMPs"). Pariza and Foster (1983) noted that a nonpathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, the authors include a list of the organisms being used in the industry of which *Aspergillus niger* is one.

The FDA has also accepted GRAS Notifications from the Enzyme Technical Association and DSM Food Specialties stating that carbohydrase, pectinase, protease, glucose oxidase and catalase (GRN 000089), lipase (GRN 000111; GRN 000158; GRN 000296), lactase (GRN 000132), phospholipase A2 (GRN 000183), asparaginase (GRN 000214), carboxypeptidase (GRN 000345) and peroxidase (GRN 000402) enzyme preparations from *Aspergillus niger* are generally recognized as safe. *Aspergillus niger* is listed as a production organism for enzymes (Pariza and Johnson, 2001) and has a long history of safe use (Schuster *et al.*, 2002).

Beta-glucosidase from *Aspergillus niger* is an enzyme present in nature (see Section 6.2). Beta-glucosidase is a member of the glycosidase enzyme family; glycosidases have a wide specificity for sugar moieties. Some members of the family also hydrolyze one or more of the following: β-D-galactosides, α-L-arabinosides, β-D-xylosides, β-D-fucosides. As part of the pectinase complex, beta-glycosidases have been affirmed as GRAS by FDA in 2002 (GRN 000089); they have been evaluated by JECFA and attributed an ADI 'not specified' for their use in several food applications (2000) and they have been included in the safety evaluation by Pariza and Johnson (2001). Beta-glucosidase itself is also included in the Pariza and Johnson paper, but it has not been affirmed as GRAS or evaluated by JECFA.

6.2.3 Substantial Equivalence

Several expert groups have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA appears to have accepted this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but

having differences in specific properties due to chemical modifications, or site-direct mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. Considering enzymes produced by micro-organisms the enzyme activity and intended use, the production organism and the process conditions should be taken into account. Beta-glucosidase (IUB 3.2.1.21) belongs to glycosidases (IUB 3.2.1). Other enzymes from this enzymes class have been notified as GRAS by the FDA: lactases, amylases, and xylanases, amongst others. Of all these enzymes, there are specific ones to which FDA responded with a letter stating they had no questions.

In addition to the safety of the beta-glucosidase enzyme itself, the current production strain is an *Aspergillus niger*. *Aspergillus niger* is a common food constituent of products like rice, seeds, nuts, olives, and dried fruits. In addition, *Aspergillus niger* has been used for several decades to produce organic acids and enzymes to be used in the food industry. The FDA summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer, Z.S. *et al.*, 2006).

Other food substances from *Aspergillus niger* were previously affirmed as GRAS. See 21 C.F.R. §§ 184.1033 (Citric acid); 184.1685 (Rennet and chymosin). Also, the FDA subsequently received GRAS notifications for additional enzyme preparations from *Aspergillus niger*, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132) and lipase (GRN 000111 and GRN 000158). FDA has no questions with these GRAS notifications.

The Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO has evaluated several enzymes from *Aspergillus niger* for their safety (Joint FAO/WHO Expert Committee on Food Additives, 1975a, Joint FAO/WHO Expert Committee on Food Additives, 1975b, Joint FAO/WHO Expert Committee on Food Additives, 1988). All these enzymes have received an ADI "not specified" by JECFA (see also Section 6.1).

Consequently, the *Aspergillus niger* strain used to produce beta-glucosidase is as safe as the production strains that have produced other GRAS enzymes subject to GRAS notifications. Accordingly, it can be concluded that the resulting enzyme product from the production strain is as safe as other enzymes produced by strains from the same safe strain lineage and processed the same way.

Since the production strain and production process are as safe as those used to produce other GRAS enzymes, and the beta-glucosidase itself is part of the pectinase complex as notified in GRN 000089, it can be concluded that the beta-glucosidase is GRAS.

6.3 Safety of the Manufacturing Process

The manufacture of the beta-glucosidase is performed under food GMP requirements and, in addition, the HACCP principles are followed. This is also described in Section 2.5. Moreover, it is indicated that ingredients are used that are acceptable for general use in foods, under

conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

The beta-glucosidase preparation meets the general and additional quality requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex.

6.4 Summary of Toxicology Studies

The safety of enzymes derived from *Aspergillus niger* has been evaluated by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (Joint FAO/WHO Expert Committee on Food Additives, 1990).

Various enzyme preparations derived from *Aspergillus niger* have been tested for their safety. The available toxicity data, which primarily consist of short-term feeding studies in rats and some studies in dogs, show that all the enzyme preparations tested were of a very low order of toxicity. The enzyme preparations tested were non-mutagenic in bacterial and mammalian cell systems. Studies on some strains of *Aspergillus niger* used to prepare carbohydrase showed no aflatoxin or related substance production. Based on these results JECFA established an estimate of Acceptable Daily Intake (ADI) expressed in terms of total organic acids (TOS) for enzyme preparations from *Aspergillus niger*, in general, of 0-1 mg TOS/kg bw for each of the enzyme preparations. For pectinases derived from *Aspergillus niger* when produced under Good Manufacturing Practice (GMP) the JECFA established a "not specified" Acceptable Daily Intake (ADI).

The above data strongly suggest that enzyme preparations from *Aspergillus niger* are safe for human consumption. Nevertheless, Gist-brocades (a company acquired by DSM) had performed a limited package of toxicity studies, *i.e.*, a sub-acute (4 weeks) oral toxicity study and two mutagenicity tests, to confirm the safety of the product.

The batch (batch no. RER 710) used for the toxicity studies, containing both beta-glucosidase activity (1750 BDG[8]/g) and pectinase activity (31,600 AVJP/g) and a Total Organic Solids content of 8.8%, was produced by the procedure used for the commercial preparation.

All studies were performed according to internationally accepted guidelines (OECD/EU/FDA) and comply with current internationally recognized Good Laboratory Practice Standards (OECD).

Summarizing the results obtained from the several toxicity studies performed as given below, the following conclusion can be drawn: The enzyme preparation from *Aspergillus niger* shows

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[8] The activity of beta-glucosidase in the tox-batch is expressed in BDG. Since 2010 the activity of the enzyme is expressed in WBDG, according to a new method. 1 BDG corresponds to 0.89 WBDG. Therefore, 1750 BDG correspond to 1562 WBDG. The Margin of Safety of the enzyme is calculated by using parameters - NOAEL and Estimated Daily Intake - which are expressed in mg TOS/kg bw/day. Since the TOS does not change, the Margin of Safety is not modified by the different method of analysis.

no mutagenic or clastogenic activity. Based on the results of the sub-acute (4 weeks) oral toxicity study the definitive No-Observed-Adverse-Effect level (NOAEL) was considered to be 5000 mg/kg/day, which is the highest dose level tested. This was equivalent to a daily intake of 8,750 BDG/kg body weight (corresponding to 7812 WBDG/kg body weight) or 440 mg TOS/kg body weight

6.4.1 Preliminary Toxicity Study by Oral Gavage Administration to CD Rats for 1 Week (Annex 7)

The objective of this study was to assess the systemic toxic potential of the tox-batch during its repeated daily oral administration to CD rats for 1 week and to select dosages for a four-week study in this species. Groups of five male and five female rats received the test substance, by oral gavage, at dosages of 500, 1500 and 5000 mg/kg body weight/day (volume-dosage of 10 ml/kg body weight/day) for seven days. A similarly constituted Control group received vehicle (water) alone.

The following parameters were evaluated: clinical signs, body weight, food consumption, food conversion efficiency, organ weights, and macroscopic pathology.

The results of this study can be summarized as follows:

- There were no deaths or signs related to treatment.
- Bodyweight gains were unaffected by treatment
- Females receiving 5000 mg/kg/day ate slightly less food than the Controls.
- Food conversion efficiency was unaffected by treatment.
- The absolute and bodyweight-relative lung and bronchi weights were high, in comparison with the Controls, in males which had received 5000 mg/kg/day and also in individual females in each of the treated groups.
- There were no macroscopic findings considered to be related to treatment.

In conclusion, under the experimental conditions adopted, treatment of CD rats with the tox-batch at dosages up to 5000 mg/kg/day did not result in any changes which were considered to be of toxicological significance.

Dosages of 500, 1500 and 5000 mg/kg/day were considered appropriate for use in the 4-week study in this species. Toxicity Study by Oral Gavage Administration to CD Rats for 4 Weeks (Annex 8)

The objective of the study was to aid the assessment of the toxicity of the tox-batch.

Based on the results of the preliminary toxicity study (see above) groups of ten male and ten female rats received the test substance, by oral gavage, at dosages of 500, 1500 and 5000 mg/kg body weight/day (volume-dosage of 10 ml/kg body weight/day) for 4 weeks. A similarly constituted Control group received vehicle (water) alone.

Examination during the study included clinical signs, body weight, food consumption, food conversion efficiency, ophthalmoscopy, haematology, blood chemistry, organ weights, macroscopic pathology, and histopathology.

Findings under the experimental conditions adopted were as follows:

- There were no deaths. There were no signs seen which were related to treatment with the test substance.
- Body weights, food consumption and food conversion efficiency were unaffected by treatment.
- There were no ophthalmic findings considered to be related to treatment.
- There were no haematological changes that were attributed to treatment.
- Slightly low plasma phosphorus concentrations, compared with the Controls, were observed in animals receiving 5000 mg/kg/day and in males receiving 1500 mg/kg/day.
- Organ weights were not affected by treatment and there were no macroscopic findings noted which were considered to be related to treatment.
- Microscopic examination revealed a higher incidence of inflammatory cell infiltrates in the lamina propria of the caecum in treated females when compared with the Controls. The number of animals affected in each group were three, two and seven for females which had received 500, 1500 or 5000 mg/kg/day, respectively.

Treatment of CD rats with the tox-batch at dosages up to 5000 mg/kg/day for four weeks was well tolerated, producing no significant effects.

The slightly low plasma phosphorus concentrations at 5000 mg/kg/day in both sexes and in males receiving 1500 mg/kg/day was not dosage-related in the males and there were no histopathological findings that would account for this finding. Slight changes in phosphate levels appear critical to health and as, an isolated blood chemistry finding, this change is not considered to be of toxicological significance.

Microscopic evaluation of the tissues revealed one finding associated with treatment. When compared with the Controls, a higher incidence of inflammatory cell infiltrates in the lamina propria of the caecum was seen in treated females. The presence of the test material in the alimentary tract may have caused minor irritation in the caecum. This finding is rodent-specific and is not considered to be a toxic effect of treatment.

In conclusion, treatment of CD rats with the tox-batch at dosages of 500, 1500 or 5000 mg/kg/day resulted in some minor changes. All changes were considered to be of no toxicological significance.

The No-Observed-Adverse-Effect Level (NOAEL) was considered to be 5000 mg/kg/day.

6.4.2 Bacterial Mutation Assay (Ames test) (Annex 9)

In this *in vitro* assessment of the mutagenic potential of the tox-batch, histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100,

and a tryptophan dependent mutant of *Escherichia coli*, strain CM891 (WP2uvrA/pKM101), were exposed to the test substance diluted in purified water, which was also used as a negative control.

Two independent mutation tests, using the treat-and-plate method, were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix), following preliminary tests to determine toxicity and to confirm that the test material did not inhibit the activity of the S9 mix at the concentrations tested.

Concentrations of up to 10 mg/ml were tested in the main mutation tests. This is higher than the standard limit concentration recommended in the regulatory guidelines this assay follows, and has been selected in order to compensate for the relatively short exposure time of the test method employed. Other concentrations used were a series of dilutions of the highest concentration (separated by *ca* half-$\log_{10}$ intervals). The concentrations are expressed in terms of the dry matter content of the enzyme preparation.

No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of the tox-batch in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the liver preparations.

6.4.3 *In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes (Annex 10)

The effects on chromosomal structure of exposure to the tox-batch were investigated in cultured human lymphocytes. Tests were conducted with and without the inclusion of a rat liver-derived metabolic activation system (S9-mix).

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohemagglutinin, and exposed to the test substance both in the presence and absence of S9-mix. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

To assess the toxicity of the test substance to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. Based on these data, the following concentrations were selected for metaphase analysis:

First test:

Without S9-mix - 3 hours treatment, 17 hours recovery: 1250, 2500 and 5000 µg/ml
With S9-mix - 3 hours treatment, 17 hours recovery: 1250, 2500, 5000 µg/ml

Second test:

Without S9-mix - 20 hours continuous treatment: 1000, 2000 and 3000 µg/ml
With S9-mix - 3 hours treatment, 17 hours recovery: 1250, 2500, 5000 µg/ml

In both the absence and the presence of S9-mix, the test substance caused no statistically significant increase in the proportion of chromosomal aberrations, at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No increases in the proportion of polyploid cells were seen.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9-mix.

It is concluded that the tox-batch has shown no evidence of clastogenic activity in this *in* vitro cytogenetic test system.

6.5 Basis for GRAS Conclusion

DSM has determined that its beta-glucosidase from a non-genetically modified strain of *Aspergillus niger* is a GRAS substance for use in the manufacturing of fermented beverages, including but not limited to beer. The enzyme preparation is added to the wort during the mashing stage of the brewing process to produce fermented beverages. Because the beta-glucosidase enzyme will be denatured during pasteurization of the final product, no active enzymes will be present in the final product.

The source organism, *Aspergillus niger,* is a well characterized, non-pathogenic organism that has a long history of safe use in the manufacture of food products, and particularly enzymes. The FDA recognized the safety of this enzyme in a publication (Olempska-Beer, Z.S. *et al.*, 2006), as well as through the review and issuance of "No Questions" letters for numerous GRAS notices which utilize *Aspergillus niger* (including GRN 89, GRN 111, GRN 158, and GRN 132).

Beta-glucosidase from *Aspergillus niger* is an enzyme present in nature. As part of the pectinase complex, beta-glycosidases have been affirmed as GRAS by FDA in 2002 (GRN 000089); they have been evaluated by JECFA and attributed an ADI 'not specified' for their use in several food applications (2000); and they have been included in the safety evaluation by Pariza and Johnson (2001). However, to further underscore the safety of the enzyme product, DSM conducted a number of safety studies, including a 28-day study in rats, the Ames assay, and an *in-vitro* chromosomal aberration assay. The safety studies, as discussed in Part 6.4 demonstrate the safety of DSM's beta-glucosidase preparation, showed the enzyme preparation is not mutagenic and not clastogenic. Further, a NOAEL was 5000 mg/kg/day was determined from the 28-day study, the highest dose tested. This is equivalent to a daily intake of 8,750 BDG/kg body weight (corresponding to 7812 WBDG/kg body weight) or 440 mg TOS/kg body weight

The data resulting from these studies, combined with the long history of safe use of similar enzymes from *Aspergillus niger*, support the conclusion that beta-glucosidase from a non-genetically modified strain of *Aspergillus niger* is a GRAS substance for use in the manufacturing of fermented beverages.

7.0 SUPPORTING DATA AND INFORMATION

Pursuant to 21 C.F.R. 170.255, the list of supporting data and information referenced in the GRAS notice is contained below.

7.1 List of References

Belgium (2010). Belgian classifications for microorganisms based on their biological risks (Last revision: March 8, 2010). Belgian Biosafety Server.
http://www.biosafety.be/RA/Class/ClassBEL.html, last visited on 11 November 2013

Bennett JW (1985a). Molds, manufacturing and molecular genetics. In: Molecular genetics of filamentous fungi. WE Timberlake, ed. Alan R. Liss, Inc., NY, 345-366

Bennett JW (1985b). Taxonomy of fungi and biology of the *Aspergilli*. In: Biology of industrial microorganisms. AL Demain and NA Solomon, eds. Benjamin Cummings Publishing Co., Menlo Park, CA, 359-406

Berrin JG., McLauchlan, W. R., Needs, P., Williamson, G., Puigserver, A., Kroon, P. A. and Juge, N. (2002), Functional expression of human liver cytosolic ß-glucosidase in *Pichia pastoris*. European Journal of Biochemistry, 269: 249-258

Berry DR, Chmiel A and Al Obaidi Z (1977). Citric acid production by *Aspergillus niger*. In: Genetics and physiology of Aspergillus. JE Smith and JA Pateman, eds. Academic Press, NY, 405-426

Bindslev-Jensen C, Stahl Skov P, Roggen EL, Hvass P and Sidelmann Brinch D (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology, 44, 1909-1915

Coulston and Kolbye (1990) REGULATORYT OXICOLOGYA ND PHARMACOLOGY1 2, S 114-S 128

Daemen L,Saison D., De Cooman L., Derdelinckx G. and Vanachtert H., (2007), Flavour enhancement in beer: hydrolysis of hop glycosides by yeast, Cerevisiae, 3224-36

Dinur, T., Osiecki, K. M., Legler, G., Gatt, S., Desnick, R. J., & Grabowski, G. A. (1986). Human acid beta-glucosidase: isolation and amino acid sequence of a peptide containing the catalytic site. Proceedings of the National Academy of Sciences, 83(6), 1660-1664.

Earl A M, Losick R and Kolter R (2008). Ecology and genomics of *Bacillus subtilis*. Trends in Microbiology, 16, 269-275

EU (2000). Directive 2000/54/EC of the European Parliament of the Council on the protection of workers from risks related to exposure to biological agents at work. Official Journal L262, p21, 17/10/2000.

http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:262:0021:0045:EN:PDF, last visited on 11 November 2013

FAO/WHO (1975a). Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain food additives. Microbial carbohydrase (*Aspergillus niger*). WHO Food Additives Series 6, JECFA 6, 124-128.
http://www.inchem.org/documents/jecfa/jecmono/v06je31.htm, last visited on 2 August 2013

FAO/WHO (1975b). Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain food additives. Microbial glucose oxidase (*Aspergillus niger*). WHO Food Additives Series 6, JECFA 6, 129-131.
http://www.inchem.org/documents/jecfa/jecmono/v06je32.htm, last visited on 2 August 2013

FAO/WHO (1978). Evaluation of certain food additives. Safety aspects of enzyme preparations used in food processing. WHO Technical Report Series, 617, JECFA 21, 8-9.
http://whqlibdoc.who.int/trs/WHO_TRS_617.pdf, last visited on 11 November 2013

FAO/WHO (1984). Specifications for identity and purity of food additives. FAO Food and Nutrition Paper 31/2, JECFA 28, 129-131

FAO/WHO (1988). Toxicological evaluation of certain food additives. Enzymes derived from *Aspergillus niger*. WHO Food Additives Series 22, JECFA 31, 11-20.
http://www.inchem.org/documents/jecfa/jecmono/v22je04.htm, last visited on 2 August 2013

FAO/WHO (1989). JECFA monograph of Pectinase from *Aspergillus niger*
http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-305.pdf

FAO/WHO (1990) Toxicological evaluation of certain food additives.
The 35th meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva

FAO/WHO (2006). General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of food additive specifications, sixty-seventh meeting. FAO JECFA Monographs 3, 2006 (ISBN 92-5-105559-9), 63-67.
ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, last visited on 30 May 2014

FAO/WHO (2009a). Safety evaluation of certain food additives. Asparaginase from *Aspergillus niger* expressed in *A. niger*. WHO Food Additives Series 60, JECFA 69, 3-13.
http://whqlibdoc.who.int/publications/2009/9789241660600_eng.pdf, last visited on 15 August 2013

FAO/WHO (2012). Safety evaluation of certain food additives. 3-Phytase from *Aspergillus niger* expressed in *Aspergillus niger*. WHO Food Additives Series 67, JECFA 67, 19-37.

http://apps.who.int/iris/bitstream/10665/77763/1/9789241660679_eng.pdf, last visited on 2 August 2013

FAO/WHO (2013). Inventory of Substances Used As Processing Aids (IPA), Updated List. ftp://ftp.fao.org/Codex/Meetings/ccfa/ccfa45/fa45_inf3x.pdf, last visited on 2 August 2013

Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA and Nielsen KF (2011). Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS ONE, 6 (8): e23496, 1-6

Germany (2002). Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA) (2002). http://www.baua.de/de/Themen-von-A-Z/Biologische-Arbeitsstoffe/TRBA/pdf/TRBA-460.pdf?__blob=publicationFile&v=3", last visited on 11 November 2013

Germany (2013). Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz. Organismen Liste. http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/register_datenbanken/organismenliste_2013.xls?__blob=publicationFile&v=4, last visited on 11 November 2013

Gopalan, V., Pastuszyn, A., Galey, W. R., & Glew, R. H. (1992). Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic beta-glucosidase. Journal of Biological Chemistry, 267(20), 14027-14032.

Grassin C. and Fauquembergue P. (1996). In Industrial Enzymology (second edition page 373) edited by Godfrey and West, ISBN 0-333-594649

Grimble (1994). The Significance of Peptides in Clinical Nutrition. Annual Review of Nutrition. 14:1, 419-447

Hartmann-Schreier, J.; Schreier, P. (1986), Purification and partial characterization of beta-glucosidase from Papaya fruit. Phytochemistry 25, 2271-2274

Kara, H.E.; Sinan, S.; Turan, Y.; J. (2011). Purification of beta-glucosidase from olive (Olea europaea L.) fruit tissue with specifically designed hydrophobic interaction chromatography and characterization of the purified enzyme. Chromatogr. B 879, 1507-1512.

K Kirkpatrick (2016), Optimizing hop aroma in beer dry hopped with Cascade utilizing glycosidic enzymes, Young Scientist Symposium, Chico, CA.

Klein DA and Paschke MW (2004). Filamentous fungi: The indeterminate lifestyle and microbial ecology. Microbial Ecology, 47, 224-235

Kubicek CP and Röhr M (1985). Aconitase and citric acid fermentation by *Aspergillus niger*. Appl. Environ. Microbiol., 50(5), 1336-1338

Kusters-van Someren MA, Samson RA and Visser J (1991). The use of RFLP analysis in classification of the black Aspergilli: reinterpretation of the *Aspergillus niger* aggregate. Curr. Genet., 19, 21-26

S Maicas and JJ Mateo (2005), Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review, Appl Microbiol Biotechnol. 67, 322-335

Netherlands (2011). Dutch Commission on Genetic Modification (COGEM): List of non-pathogenic fungi. http://www.cogem.net/index.cfm/nl/publicaties/publicatie/classificatie-apathogene-schimmels, last visited on 6 November 2013

OECD (1992). Safety Considerations for Biotechnology 1992. Organisation for Economic Cooperation and Development. Part One. Elaboration of Criteria and Principles for Good Industrial Large-Scale Practice (GILSP). http://www.oecd.org/dataoecd/8/3/2375496.pdf, last visited 11 November

Olempska-Beer ZS, Merker RI, Ditto MD and DiNovi MJ (2006). Food-processing enzymes from recombinant microorganisms – a review. Regul. Toxicol. Pharmacol., 45(2), 144-158

Parekh S, Vinci VA and Strobel RJ (2000). Improvement of microbial strains and fermentation processes. Applied Microbiology and Biotechnology, 54, 287-301

Pariza MW and Foster EM (1983). Determining the Safety of Enzymes Used in Food Processing. J of Food Protection 46, (5), 453-468

Pariza MW and Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Regul. Toxicol. Pharmacol., 33, 173-186

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JAE, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EGJ, Debets EGM, Dekker P, van Dijck PWM, van Dijk A, Dijkhuizen L, Driessen AJM, d'Enfert C, Geysens S, Goosen C, Groot GSP, de Groot PWJ, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JPTW, van den Hondel CAMJJ, van der Heijden RTJM, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJEC, Meulenberg R, Menke H, Mortimer AM, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJJ, Wedler H, Wösten HAB, Zeng A-P, van Ooyen AJJ, Visser J and Stam H (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* strain CBS 513.88. Nature Biotechnology, 25 (2), 221-231

Sanchez S and Demain A L (2002). Metabolic regulation of fermentation processes. Enzyme and Microbial Technology 31, 895-906

Schuster E, Dunn-Coleman N, Frisvad JC and van Dijck PWM (2002). On the safety of *Aspergillus niger* – a review. Appl. Microbiol. Biotechnol. 59, 426-435

Shoseyov O. *et al.* (1990). Immobilized endo-beta-glucosidase enriches flavor of wine and passion fruit juice. Journal of Agricultural and Food Chemistry 38.6, 1387-1390.

USA (1997). Environmental Protection Agency, Biotechnology Program under the Toxic Substances Control Act (TSCA). *Aspergillus niger* Final Risk Assessment. http://www.epa.gov/biotech_rule/pubs/fra/fra006.htm, last visited on 11 November 2013

USA (2002). FDA GRAS Notice Inventory. GRN No. 89. Five enzyme preparations from *Aspergillus niger*: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation. GRN 89, last visited on 1 August 2013

USA (2002b). FDA GRAS Notice Inventory. GRN No. 111. Lipase enzyme preparation from *Aspergillus niger*. GRN 111, last visited on 1 August 2013

USA (2003a). FDA GRAS Notice Inventory, GRN No.132. Lactase enzyme preparation from *Aspergillus niger*. GRN 132, last visited on 1 August 2013

USA (2005). FDA GRAS Notice Inventory. GRN No. 158. Lipase preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica*. GRN158, last visited on 1 August 2013

USA (2006a). FDA GRAS Notice Inventory. GRN No. 183. Phospholipase A2 enzyme preparation from *Aspergillus niger* expressing a gene encoding a porcine phospholipase A2. GRN 183, last visited on 2 August 2013

USA (2007). FDA GRAS Notice Inventory. GRN No. 214. Asparaginase enzyme preparation from *Aspergillus niger* expressing the asparaginase gene from *A. niger*. GRN 214, last visited on 2 August 2013

USA (2009). FDA GRAS Notice Inventory. GRN No. 296. Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger*. GRN 296, last visited on 2 August 2013

USA (2010). FDA GRAS Notice Inventory. GRN No. 345. Carboxypeptidase enzyme preparation from modified *Aspergillus niger*. GRN 345, last visited on 2 August 2013

USA (2012a). FDA GRAS Notice Inventory. GRN No. 428. Asparaginase enzyme preparation from genetically modified *Aspergillus niger*. GRN 428, last visited on 8 November 2013

USA (2012b). FDA GRAS Notice Inventory. GRN No. 402. Peroxidase enzyme preparation derived from a genetically modified strain of *Aspergillus niger*. GRN 402, last visited on 8 November 2013

USA (2013). National Institutes of Health (NIH) Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules. http://osp.od.nih.gov/sites/default/files/NIH_Guidelines_0.pdf, last visited on 2 September 2014

Wilkinson Enns, C., Goldman, J.D. and Cook, A. (1997) *Trends in food and nutrient intakes by adults: NFCS 1977-78, CSFII 1989-91, and CSFII 1994-95*, Family Economics and Nutrition Review 10 (4) 2-15

Annex 1

Taxonomic Identification of the *A. niger* strain ARO by the Dutch culture collection, the Centraalbureau voor Schimmelcutures (CBS)

[Logo] CENTRAALBUREAU VOOR SCHIMMELCULTURES
Institute of the Royal Netherlands Academy of Arts and Sciences

Mr. C. de Vogel
R&D Strain conservation
Gist-brocades
P.O. Box 1
2600 MA Delft

Baarn, 10-03-1997

Our Ref.: Det.: 20 RAS/as

Your ref.: order 6522653

Determination Service

Below are the results of the requested investigation.

ARO-1 (DS6047)= *Aspergillus niger* v. Tieghem

PEC-4 (DS2825) = *Aspergillus niger* v. Tieghem

FBG-1 (DS28601) = *Penicillium emersonii* Stolk (The teleomorph was not found in the culture)

DXL-1 (DS31362) = *Disporotrichum dimorphosporum* (von Arx) Stalpers

Annexed you will also find the invoices for these identifications.

Sincerely,

[signature]

Drs. R.A. Samson

CENTRAALBUREAU VOOR SCHIMMELCULTURES
Institute of the Royal Netherlands Academy of Arts and Sciences

De Heer C. de Vogel
R&D Stamconservering
Gist Brocades
Postbus 1
2600 MA DELFT

Baarn, 10-3-1997

Onze ref.: Det.: 20 RAS/as Uw ref.: order 6522653

DETERMINATIE DIENST

Hieronder vindt U de uitslag van het door U gevraagde onderzoek.

ARO-1 (DS6047) = *Aspergillus niger* v. Tieghem

PEC-4 (DS2825) = *Aspergillus niger* v. Tieghem

FBG-1 (DS28601) = *Penicillium emersonii* Stolk. (De teleomorph is in de cultuur niet waargenomen!)

DXL-1 (DS31362) = *Disporotrichum dimorphosporum* (von Arx) Stalpers

Tevens treft U de rekening aan voor deze identificaties.

Hoogachtend,

(b) (6)

Drs. R.A. Samson

Postal address: P.O.Box 273, 3740 AG BAARN, The Netherlands
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Annex 2

Method of analysis of beta-glucosidase activity

Analysis Service and solutions	METHOD OF ANALYSIS	No : 2510 Version : 02 Page : 1 of 15

Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

Product: - Fermentation samples - Downstream processing samples - Finished products	Validated method NO	Date of issue: 15 SEP. 2011

AUTHORIZATION

Compiled by	G. Hermans	(b) (6) Date: 11/8/2011
Approved by expert	J. Godefrooij	(b) (6) (b) (6) Date: 17/8/2011
Approved by QA/QC Analysis	M.M. Immerze (b) (6)	Date: 01-09-2011
Approved by Team Lead Service Lab Delft	M. Steenbeek	(b) (6) Date: 22/08/2011
Approved by external QA/QC (if applicable)	n.a.	Date:
Approved by external QA/QC (if applicable)	n.a.	Date:

Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

1 SAFETY AND ENVIRONMENT

Restrictions for working with chemicals and ML-I samples are mentioned in the work instructions concerning management, storage and use of chemicals, the handling of dangerous substances and standard rules for ML-I laboratories. These restrictions are also applicable for material that has been in contact with ML-I samples.

When working with strong acids, bases, carcinogenic matters and toxic matters take all necessary precautions.

When working with highly concentrated enzyme preparations take all necessary precautions. Avoid inhalation of dust and/or prolonged contact with unprotected skin.

2 PRINCIPLE

2.1 Application

This method is applicable to the determination of beta-D-glucosidase activity for White Biotech applications in fermentation broths, recovery samples and finished products. Many beta-D-glucosidases are susceptible to substrate inhibition to higher and lower extends. Comparing activity values of samples from different origin, measured at one single substrate concentration, will likely result in doubtful conclusions and should therefore be carefully considered.

2.2 Description of the method

Enzymatic ß-glucosidase activity is determined at 37°C and pH 4.40 using para-nitrophenyl-ß-D-glucopyranoside as substrate. Enzymatic hydrolysis of pNP-ß-D-glucopyranoside results in release of para-nitrophenol (pNP) and D-glucose. Quantitatively released para-nitrophenol, determined under alkaline conditions, is a measure for enzymatic activity. After 20 minutes incubation the reaction is stopped by adding sodium carbonate and the absorbance is determined at a wavelength of 405 nm. Beta-glucosidase activity is calculated making use of the molar extinction coefficient of para-nitrophenol.

2.3 Unit definition

ß-Glucosidase activity is expressed in **W**hite Biotech **B**eta-**D**-**G**lucosidase (WBDG) per gram or per milliliter. One WBDG is defined as the amount of enzyme that liberates one nanomol para-nitrophenol per second from para-nitrophenyl-beta-D-glucopyranoside under the defined assay conditions (4.7 mM pNPBDG, pH = 4.40 and T = 37°C).

2.4 Measuring range

The measuring range of this method is from 0.16 – 2.7 WBDG/mL.
Note that this range is **only** valid for ARO samples (like the control preparation). As parameters like Km values and possible substrate and/or product inhibition for beta-D-glucosidases from other

organisms are unknown, a linear dose/response range for such samples may deviate from the above mentioned measuring range.

2.5 Summary of the validation report

Not applicable.

3 APPARATUS AND CONDITIONS

3.1 Apparatus

Clinical analyzer	: Konelab Arena 30, Thermo
Konelab sample cups	: Thermo
Konelab sample racks	: Thermo
Konelab reagent vessels (20 mL)	: Thermo
Balance, accurately to 0.01 g	: Sartorius model2004 MP
Balance, accurately to 0.001 g	: Mettler AE200 or AJ100
Balance, accurately to 0.1 mg	: Mettler AT201
Diluter, provided with 0.5 and 5.0 mL cylinders	: Hamilton Microlab 500,
pH meter	: Radiometer PHM 82
Centrifuge, 1100 x g	: Megafuge 1.0 (Heraeus)
Disposable culture tubes (glass), 16 x 100 mm	: Corning

Or equivalent equipment.

3.2 Conditions

Not applicable.

4 MATERIALS

4.1 Chemicals

Sodium acetate . trihydrate, p.a.	: Merck 1.06267
Acetic acid 100%, p.a.	: Merck 1.00063
BSA, min 98% electrophoresis	: Sigma, A3803
p-nitrophenyl-beta-D-glucopyranoside	: Sigma N-7006
p-nitrophenol standard solution	: Sigma N-7660
Sodium carbonate (anhydrous), p.a.	: Merck 1.06392

Or equivalent quality

4.2 References, standards and controls

Standard:
para-Nitro-phenol standard solution, 10 mmol/L, with an officially assigned content. Store the standard solution in a tightly closed flask in the refrigerator.

Control:
ß-D-glucosidase (ARO) control preparation with an officially assigned activity.
The activity is expressed in WBDG/g. Store the stock of this control preparation and amounts for daily use in the freezer.

4.3 Reagents

- Water:
Ultra High Quality (UHQ) water, resistance >18.2 mega Ohm/cm and TOC<500 µg/L.

- Acetate buffer 100 mM pH 4.40:
Dissolve 27.2 g sodium acetate trihydrate in 1800 mL water in a 2 litre volumetric flask. Adjust the pH to 4.40 +/- 0.05 with acetic acid 100 %. Make up to volume with water and mix. This solution is stable for 3 months when stored in the refrigerator.

- Acetate buffer 100 mM pH 4.40, 0.1% BSA (dilution buffer):
Add 1.0 g BSA to 1 L of 100 mM acetate buffer pH 4.40 and mix.
This solution is stable for 1 month when stored in the refrigerator.

- pNP-BDG substrate solution, 5.0 mM (WBDGsub):
Dissolve 75.2 mg +/- 0.1 mg p-nitrophenyl-beta-D-glucopyranoside in 40 mL acetate buffer pH 4.40 in a 50 mL volumetric flask. Make up to volume with acetate buffer pH 4.40 and mix. Measure the pH, It should be within the range of pH 4.35 – 4.45. Transfer this substrate solution to a 20 mL Konelab reagent vessel. Always use a freshly prepared solution.

Note: Samples of different origin tend to suffer from substrate inhibition to higher or lower extends. (See annex 4) Multiple versions of the Konelab program are available for testing different substrate concentrations.

- Sodium carbonate solution 1 mol/L (WBDGstop):
Dissolve 26.5 g sodium carbonate (anhydrous), in 200 mL water in a 250 mL volumetric flask. Make up to volume with water and mix. Transfer this stop solution to a 20 mL Konelab reagent vessel. This solution is stable for 3 months when stored at ambient temperature.

5 PROCEDURE

5.1 Preparation

Not applicable

5.2 Pretreatment reference

Not applicable.

5.3 Pretreatment standard

Prepare a para-nitro-phenol calibration line by diluting the 10 mM pNP stock solution in acetate buffer 100 mM pH 4.40, 0.1% BSA according to the following table.

Table 1: Dilution table for preparation of the pNP calibration line

Code	pNP stock solution [mL]	Dilution buffer [mL]	Dilution factor
S0	0	3.000	-
S1	0.125	4.875	40
S2	0.200	4.800	25
S3	0.200	2.800	15
S4	0.250	1.750	8

Introduce approximately 1 mL in Konelab sample cups.

5.4 Pretreatment control

Before use, allow the control preparation to attain room temperature. Weigh accurately within 1 mg and in duplicate amounts of the control sample corresponding to approximately 4000 WBDG in 50 mL volumetric flasks. Add approximately 40 mL acetate buffer pH 4.40 and dissolve by stirring on a magnetic stirrer. Make up to volume with acetate buffer pH 4.40 and mix. Dilute 0.500 mL of this solution with 4.500 mL acetate buffer 100 mM pH 4.40, 0.1% BSA.
Subsequently dilute 0.200 mL of this diluted solution with 2.800 mL acetate buffer 100 mM pH 4.40, 0.1% BSA (Total dilution factor 7500; final activity approximately 0.53 WBDG/mL). Store these diluted control solutions on ice until starting the incubation.

5.5 Pretreatment samples

Fermentation samples and downstream processing samples:
Weigh approx. 1.0 g of sample, accurately to within 1 mg in a 50 mL volumetric flask. Add approximately 40 mL acetate buffer pH 4.40 and dissolve by stirring on a magnetic stirrer. Make up to volume with acetate buffer mix. Dilute with acetate buffer 100 mM pH 4.40, 0.1% BSA to a final activity of approximately 0.5 WBDG/mL. Centrifuge (if necessary) the diluted samples for 10 minutes at 1100 x g and measure the activity in the supernatant.
Finished products:
Weigh approximately 1.0 g of sample, accurately to within 1 mg in a 50 mL volumetric flask. Add approximately 40 mL acetate buffer pH 4.40 and dissolve by stirring on a magnetic stirrer. Make up to 50 mL with dilution buffer and mix. Dilute with acetate buffer 100 mM pH 4.40, 0.1% BSA to a final activity of approximately 0.5 WBDG/mL.

5.6 Preparation measurement

Introduce approximately 1 mL of final dilutions into Konelab sample cups.

Note 1: The Konelab will normally perform the incubations in multiple blocks of 12 cuvettes. As sample responses in individual incubations or in smaller blocks (e.g. total of 14 runs: block of 12 + block of 2 incubations) may result in an overestimate of the actual activity due to limitations to the Konelab incubator's heating capacity, such individual incubations (or blocks of less than 6 incubations) should always be prevented as much as possible. (So for instance when the sample response of just one sample is outside the measuring range, do not run a re-analysis of just this one sample, but include some other incubations as well, if needed incubations of plain dilution buffer)

Note 2: Extra care should be taken when analyzing WBDG samples after running an AGIs analysis (B2419). Excess levels of ß-glucosidase helper enzyme in the AGIs substrate solution may result in carry over into the WBDG analysis, thereby resulting in an overestimation of the actual activity in these samples.

5.7 Measurement

Using the Konelab Arena 30 analyzer:
- Log in the Konelab software as user "ara" and fill in the password.

- A yellow flagged message stating "startup needed" is visible in the main screen. Press F1-startup to perform startup operations and press OK.
- When startup is completed, check the water blanks by pressing F8, F2, F8 and F1, check water blank. Press F5 to show all tested wavelengths. If outliers are seen (above 2 mA), then repeat the water blank by pressing F1 twice.

Requesting samples:
- Press the Samples button on top of the main screen.
- Press F8-more
- Press F4-batch entry
- Type the sample name SLD using the analysis number (format: SLD00123, if necessary use an extra serial indication, e.g. BIO00123a) and press Enter.
- Select the used segments and used positions (see par. 5.6) from the pull-down menus.
- Select the tests to be run (**B25107S and B25107B**)

For standard runs only the blank test B25107B has to be run.
(Different versions of these tests are available for testing different substrate concentrations: B251072S and –B, B251073S and –B, etc)
- Press F2-Save changes
- Introduce the sample segments in the Konelab one by one by opening and closing the sample segment door. The Konelab automatically detects which segments are inserted.

Inserting reagents:

Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

- Press the Reagents button on the top of the main screen.
- Press on a free reagent position number
- Press F2-Insert reagent
- Select **WBDGsub** reagent from the list
- Press OK and follow on-screen instructions

(When multiple substrate concentrations are to be tested, repeat these last 4 steps for WBDGsub2, WBDGsub3,…)

- Repeat the last 4 steps for **WBDGstop**

Starting analysis:

- Go to the main screen and press the green start button, which is located on the keyboard at the right of the Home button. This is the only way the analyzer can be started.

Results:

- When analysis is complete, press the Results button on the top of the main screen
- Press F8-more, F4 to see all analysed samples
- Press F8-more twice, F1 to see details of a selected sample. Press F1 once more to turn details off.
- Press F5-Print results to print the results

Generating peak files:

- When the sample series is completely finished, go to the main screen and press F4-Reports and F8-more.
- Select items to report "samples" and "all" from the pull down menus
- Press F4-Results to file
- Select "one row per result" and press OK
- **Always** enter "results" as the filename

Subsequently use the available program to generate the actual peak file.

Cleaning up:

- Remove the sample segments from the Konelab when your series is finished by pressing Sample disk in the main screen
- Press one of the present segment numbers
- Press F3-remove segment and remove the segment from the Konelab.
- Press F6-remove all samples to clear the samples from the memory.
- Select all your other segments one by one from the pull down menu and repeat the two last steps until all segments are removed.

- Remove used reagents from the reagent tray by pressing Reagents on the main screen.
- Click on a reagent and then press F3-remove reagent and follow the on-screen instructions
- Repeat this until all reagents are removed.

Standby:

At the end of each series a Standby has to be done. The needles are then cleaned and the tension is
taken off the moving parts.
- Press F2-Standby in the main screen and follow the on-screen instructions

6 CALCULATION

Carry out the calculation with the aid of the computer program available for this analysis
If this program is not available carry out the calculation as follows:

Molar extinction coefficient (ε_{pNP}) of p-nitrophenol:
Prepare a calibration line by plotting the absorbance versus the exactly calculated para-nitro-phenol concentration of the standards S0 – S4. This calibration curve must be fitted according linear regression (y = ax + b, in which y = absorbance and x = concentration of pNP in the incubation solution (in mM). This curve is used to determine the molar extinction coefficient (MEC) of pNP according to the following formula:

$\varepsilon_{pNP} = 1000 * a$

Where:

ε_{pNP} = Molar extinction coefficient of p-nitrophenol at a wavelength of 405 nm [$M^{-1}.cm^{-1}$]
1000 = factor from $L.mmol^{-1}$ to $L.mol^{-1}$
a = slope of the pNP calibration line

Beta-D-Glucosidase activity calculation:
The molar extinction coefficient of para-nitro-phenol is used to calculate the activity in samples of interest.
Calculate the activity in the samples as follows:

$$WBDG / mL\ sample = 1\,000\,000 \times (Abs_{sample} - Abs_{blank}) / (\varepsilon_{pNP} \times 600) \times 125/10 \times Df$$

Or:

$$WBDG / g\ sample = [1\,000\,000 \times (Abs_{sample} - Abs_{blank}) / (\varepsilon_{pNP} \times 600) \times 125/10 \times Df)] / W$$

Where:

1 000 000	= factor from mol/L to nmol/mL
$A_{sample} - A_{blank}$	= Sample absorbance (test B2510S) corrected for blank absorbance (test B2510B)
ε_{pNP}	= Molar Extinction Coefficient of para-nitro-phenol [$L.mol^{-1}.cm^{-1}$]
600	= Incubation time [s]
125	= assay volume [µL]
10	= sample volume [µL]
Df	= Dilution factor
W	= sample weight [g]

7 ASSESSMENT

7.1 Requirements

- A (diluted) sample solution must have an activity fitted within the measuring-range.
- The level of each control value must fit in the range : $C_{assigned} \pm 3 \times SD_{overall}$
($C_{assigned}$ = Assigned control value; $SD_{overall}$ = overall standard deviation of the average control value calculated from past series).
- The *relative (absolute) difference* in level between (duplicate) *control* values within a daily series is not allowed to exceed a value of $2.8 \times RSD_{within\ day}$.
(Relative absolute difference in control values = $(|\text{control value 1} - \text{control value 2}| / \text{Average control value}) \times 100\%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past series using control values e.g. as determined in validation of the method).
- The *relative (absolute) difference* in level between (duplicate) *sample* values is not allowed to exceed a value of $2.8 \times RSD_{within\ day}$.
(Relative absolute difference in sample values = $(|\text{sample value 1} - \text{sample value 2}| / \text{Average sample value}) \times 100\%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past sample series with a comparable type of matrix e.g. as determined in validation of the method).
- A calibration curve point exceeding 5% deviation (after fitting) should be discarded and should be considered non-valid (= outlier). Refitting of the calibration curve is required.
- The fitted calibration curve should consist of at least 80% of the calibration curve points.
- The value of the molar extinction coefficient of para-nitro-phenol should be between 18100 and 20400 $L.mol^{-1}.cm^{-1}$.

The results of the control sample must be expressed as percentage of the assigned value.
The results of the control samples must be imported into the control charts available for this method of analysis. All results have to be evaluated.

7.2 Actions

Repeat the analysis of the sample with an adjusted dilution when the outcome is outside the measuring range.
Repeat the analysis whenever the calibration line or the resulting value for the molar absorbance coefficient does not comply with the requirements.
Repeat the analysis whenever the controls do not comply with the requirements.
Repeat the analysis of the sample whenever the difference between duplicate do not comply with the requirements.

7.3 Authorisation

After a training period by a for this method authorized laboratory technician, a technician will be authorized for this method when he/she succeeds on performing the test single-handed, whereby the standards and selected samples meet the criteria mentioned above.

8 REFERENCES

Memo: Transfer of beta-D-glucosidase assay to the Konelab analyzer: introduction of method B2510 (BIO00026). By J. Godefrooij, dated 20080819

9 REMARKS

Not applicable.

10 ANNEXES

Annex 1: Konelab sample program B25107S

Annex 2: Konelab blank program B25107B

Annex 3: Example pNP calibration line

Annex 4: Example of substrate inhibition for two different BDG samples

Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

Annex 1: Konelab sample program B25107S

Test	B25107S				
Full name	B25107S				
Online name		Test In Use			
Test type	Photometric		LOW	HIGH	
		Test limit	0.0000	*	abs
Result unit	abs	Initial absorbance	0.000	*	A
Number of Decim.	4	Dilution limit	*	*	abs
		Secondary dil 1+	0.0	0.0	
		Critical limit	*	*	abs
		Reflex test limit	*	*	abs
		Reflex test limit			
Acceptance	Automatic	Reference class	LOW	HIGH	In Use
Dilution 1+	0.0				
Sample type	Sample type 1	Correction factor	1.00		
		Correction bias	0.00	abs	
		Temperature	37.0	°C	
Calibration type	None				
Factor	1.00	Bias		0.00	
Bias correction in use	NO				
Manual QC in Use	NO	Routine QC in use		NO	
Blank	None				
		Normal cuvette			
Reagent	WBDGsub	Volume (ul)		83	
Disp. With	Extra	Add. Volume (ul)		30	
wash reagent	None				
Incubation		Time (sec)		300	
Sample		Volume (ul)		5	
Disp. With	Extra	Add. Volume (ul)		30	
Dilution with	Water	Wash reagent		None	
Incubation		Time (sec)		1200	
Reagent	WBDGstop	Volume (ul)		90	
Disp. with	Extra	Add. Volume (ul)		30	
Wash reagent	None				
Incubation		Time (sec)		900	
Measurement	End point				
Wavelength (nm)	405 nm	Side wavel. (nm)		None	
Meas. Type	Fixed timing				

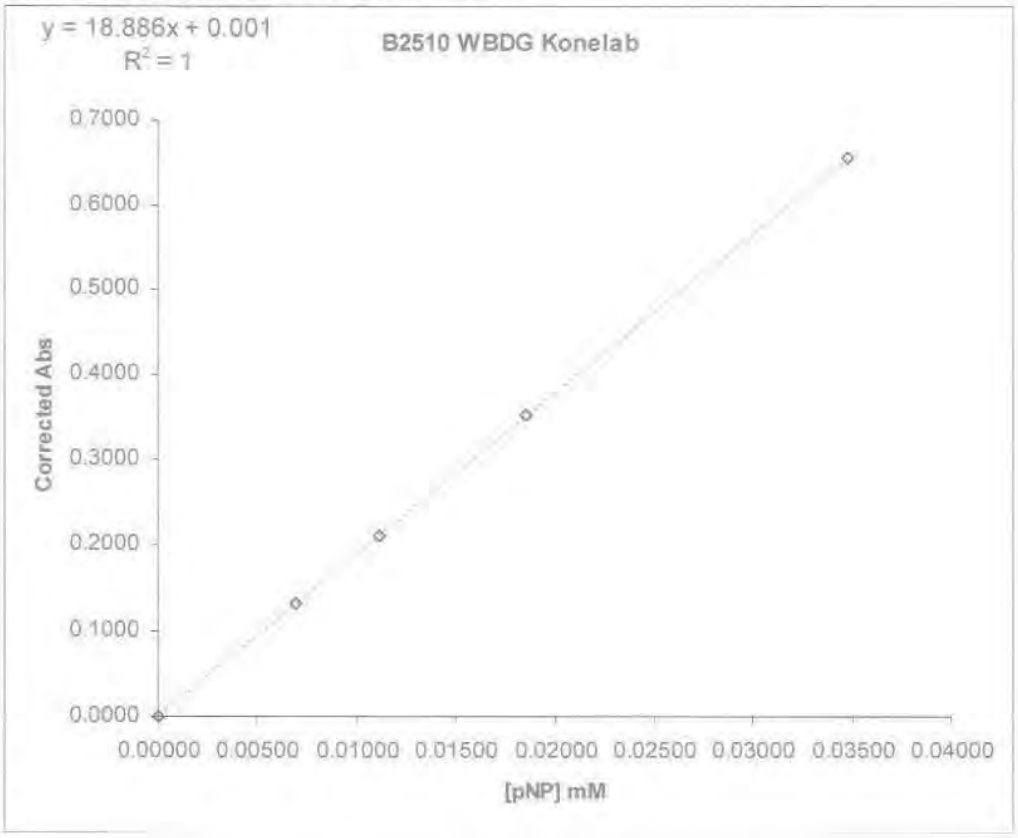
Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

Annex 2: Konelab blank program B25107B

Test	B25107B				
Full name	B25107B				
Online name		Test In Use			
Test type	Photometric		LOW	HIGH	
		Test limit	0.0000	*	abs
Result unit	abs	Initial absorbance	0.000	*	A
Number of Decim.	4	Dilution limit	*	*	abs
		Secondary dil 1+	0.0	0.0	
		Critical limit	*	*	abs
		Reflex test limit	*	*	abs
		Reflex test limit			
Acceptance	Automatic	Reference class	LOW	HIGH	In Use
Dilution 1+	0.0				
Sample type	Sample type 1	Correction factor	1.00		
		Correction bias	0.00	abs	
		Temperature	37.0	°C	
Calibration type	None				
Factor	1.00	Bias		0.00	
Bias correction in use	NO				
Manual QC in Use	NO	Routine QC in use		NO	
Blank	None				
		Normal cuvette			
Reagent	WBDGsub	Volume (ul)		83	
Disp. With	Extra	Add. Volume (ul)		30	
wash reagent	None				
Incubation		Time (sec)		1435	
Reagent	WBDGstop	Volume (ul)		90	
Disp. with	Extra	Add. Volume (ul)		30	
Wash reagent	None				
Incubation		Time (sec)		65	
Sample		Volume (ul)		5	
Disp. With	Extra	Add. Volume (ul)		30	
Dilution with	Water	Wash reagent		None	
Incubation		Time (sec)		900	
Measurement	End point				
Wavelength (nm)	405 nm	Side wavel. (nm)		None	
Meas. Type	Fixed timing				

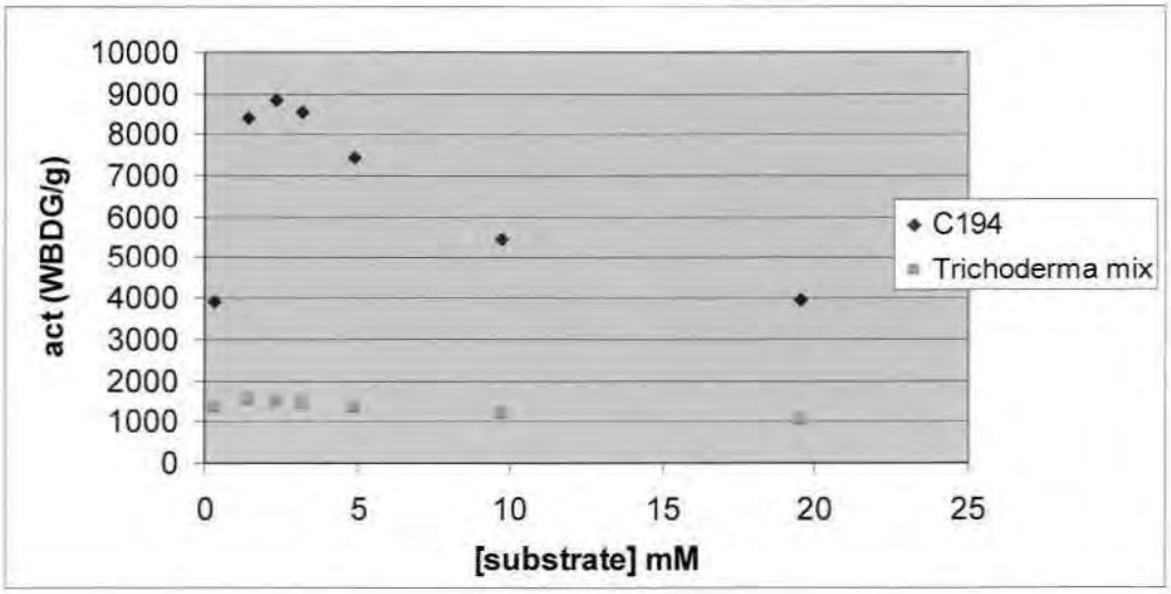
Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

Annex 3: Example pNP calibration line



Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

Annex 4: Example of substrate inhibition for two different BDG samples



Title:
Absolute determination of White Biotech beta-D-glucosidase activity using pNP-ß-D-glucopyranoside as substrate: absolute method (Konelab analyzer)

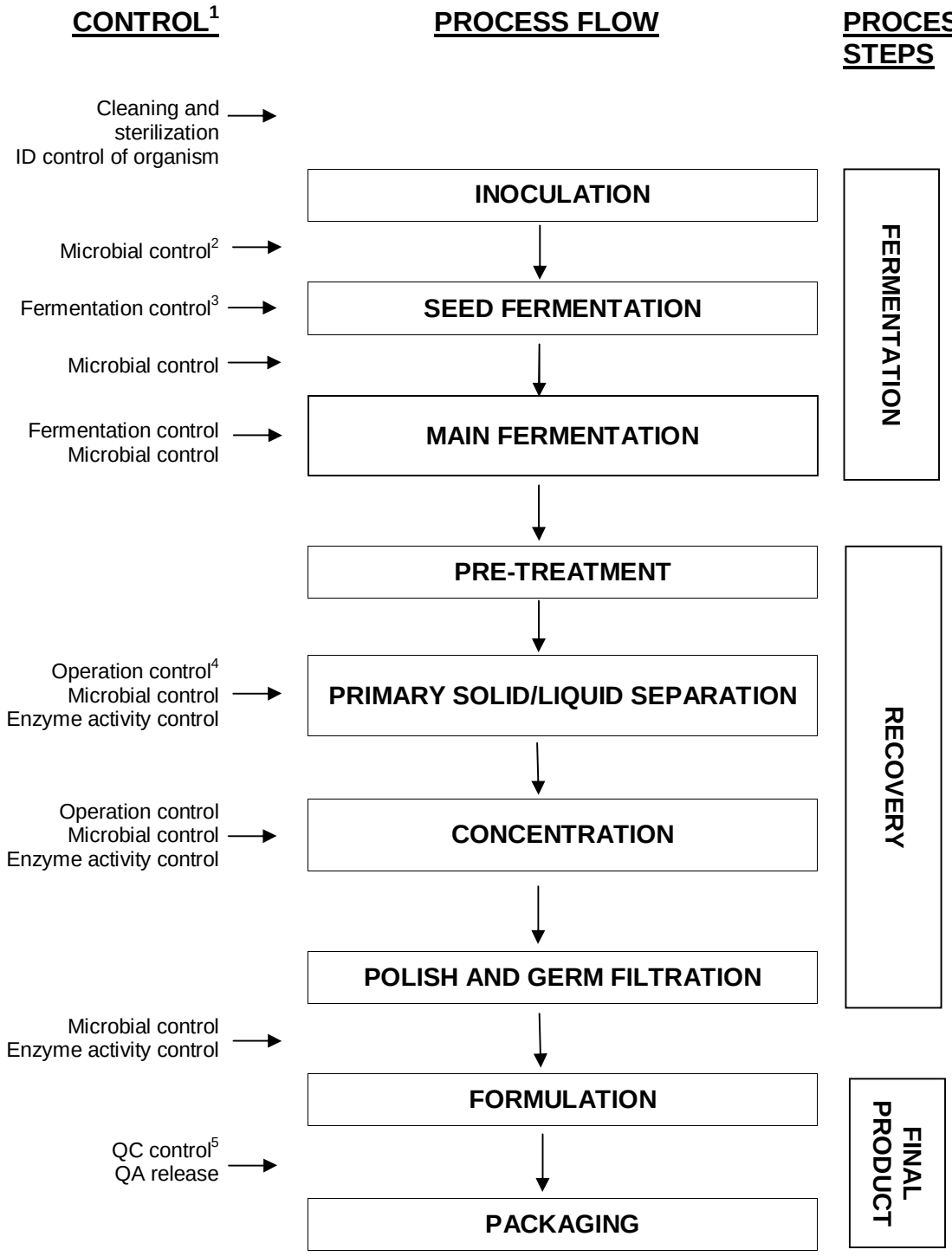
HISTORY

Version	Description of the modification
1	First version
2	August 2011 New DBC Lay out.

Annex 3

Flow diagram of manufacturing process

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

Annex 4

JECFA Safety Evaluation *A. niger* enzyme preparations, 1988

ENZYMES DERIVED FROM ASPERGILLIS NIGER

EXPLANATION

A. niger is a contaminant of food and was not considered in the same light as those organisms regarded as normal constituents of food. It is necessary to show that the strains used in enzyme preparations do not produce mycotoxins.

Microbial carbohydrases prepared from some varieties of A. niger were evaluated at the fifteenth meeting of the Committee, at which time a temporary ADI "not limited" was established (Annex 1, reference 26). A toxicological monograph was prepared (Annex 1, reference 27). An adequate 90-day study in rats was requested. Since the previous evaluation, additional data have become available on a number of carbohydrases, which are summarized and discussed in the following monograph. These enzymes were considered by the Committee to encompass the carbohydrases previously considered. The previously published monograph has been expanded and reproduced in its entirety below.

AMYLOGLUCOSIDASES (E.C. 3.2.1.3)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

Special studies on aflatoxin-related effects

Ducklings

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and mean liver weights were comparable, in all groups. No gross or histopathological lesions of the liver were seen (FDRL, 1963a).

Four groups of 5 ducklings received in their diet 0, 1, 5, or 10% enzyme preparation for 29 days. Growth, feed consumption, survival, behaviour, and development were comparable in all groups. No gross liver lesions were seen at autopsy and mean liver weights of treated animals were similar to those of controls. Histopathology of the livers was normal. No toxic elements were noted (FDRL, 1963b).

Acute toxicity[1]

Species	Route	LD_{50} (mg/kg b.w.)	Reference
Mouse	oral	> 3,200	Hunt & Garvin, 1963
		> 4,000	Hunt & Garvin, 1971
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
Rat	oral	10,000	Gray, 1960
		31,600	Kay & Calendra, 1962
		> 3,200	Willard & Garvin, 1968
		> 4,000	Garvin et al., 1966
		12,500 - 20,000	Kapiszka & Hartnage, 1978
Rabbit	oral	> 4,000	Garvin et al., 1966
Dog	oral	> 4,000	Garvin et al., 1966

[1] These data were obtained with several different commercial enzyme preparations.

Short-term studies

Rats

Three groups of 10 male rats received 0, 0.5, or 5% enzyme preparation in their diets for 30 days. No adverse effects related to treatment were observed regarding growth, appearance, behaviour, survival, food consumption, haematology, organ weights, or gross pathology (Garvin et al., 1966).

Two groups of 10 male and 10 female rats received either 0 or 5% enzyme preparation in their diets daily for 91 days. No differences from controls were observed regarding appearance, behaviour, survival, weight gain, haematology, organ weights, or gross pathology (Garvin & Merubia, 1959).

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g enzyme preparation/kg b.w./day) for 90 to 94 days. A control group of 20 male and 20 female rats were maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar between test and control groups. Differential blood counts were within the normal range at weeks 4 and 8 of the study in both test and control animals. At the end of the study serum clinical chemistry parameters, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin et al, 1972).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

Several short-term feeding studies in rats on amyloglucosidase preparations from A. niger have been performed. One study, in which the preparation was fed at up to 10% of the diet, was considered to be acceptable by current standards. No compound-related effects were observed in this study or in duckling tests that were performed to investigate potential aflatoxin-related effects.

The evaluations by the Committee of the carbohydrates and the protease from A. niger are summarized at the end of this section.

REFERENCES

FDRL (1963a). Unpublished report No. 84600e. Submitted to WHO by Laboratories, Inc., Elkhart, IN, USA.

FDRL (1963b). Unpublished report No. 84600f. Submitted to WHO by Laboratories, Inc., Elkhart, IN, USA.

Garvin, P.J. & Merubia, J. (1959). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.

Garvin, P.J., Willard, R., Merubia, J., Huszar, B., Chin, E., & Gilbert, C. (1966). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.

Garvin, P.J., Ganote, C.E., Merubia, J., Delahany, E., Bowers, S., Varnado, A., Jordan, L., Harley, G., DeSmet, C., & Porth, J. (1972). Unpublished report from Travenol Laboratories, Inc., Morton Grove, IL, USA. Submitted to WHO by Gist-brocades NV, Delft, Holland.

Gray, E.H. (1960). Unpublished report. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.

Hunt, R.F. & Garvin, P.J. (1963). Unpublished report. Submitted to WHO by Baxter Laboratories, Inc.

Hunt, R.F. & Garvin, P.J. (1971). Unpublished report. Submitted to WHO by Travenol Laboratories, Inc., Morton Grove, IL, USA.

Kapiszka, E.L. & Hartnage, R.E. (1978). The acute oral toxicity of Diazyme concentrate and Diazyme 325 in the rat. Unpublished report No. 16 from Miles Laboratories, Inc., Elkhart, IN, USA. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.

Kay, J.H. & Calendra, J.C. (1962). Unpublished report. Submitted to WHO by Miles Laboratories, Inc., Elkhart, IN, USA.

Willard, R. & Garvin, P.J. (1968). Unpublished report. Submitted to WHO by Travenol Laboratories, Inc., Morton Grove, IL, USA.

ß-GLUCANASE (E.C. 3.2.1.6)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

(The TOS of the enzyme preparation used for toxicity studies was 49%).

Special Studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using 5 strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537, and TA1538 both with and without metabolic activation (S-9 fraction). The preparation was not mutagenic or toxic at concentrations up to 40 mg/ml (McConville, 1980).

A cytogenic bone marrow study was performed using adult male Chinese hamsters. Groups of adult male hamsters received up to 5000 mg/kg b.w./day of the enzyme preparation for 5 consecutive days. Treatment did not result in an increased frequency of chromosomal aberrations in bone marrow (McGregor & Willins, 1981).

Acute toxicity

Species	Route	Sex	LD_{50} (ml/kg b.w.)	Reference
Mouse (NMRI)	oral	M & F	30	Novo, 1978a
Rat (Wistar)	oral	-	28.1	Novo, 1978b

Short-term studies

Rats

Three groups, each containing 5 male and 5 female Wistar/Mol SPF rats, were dosed orally by garage once a day for 14 days with enzyme preparation at dose levels equivalent to 2.5, 5.0, or 10 ml/kg b.w. No clinical changes were observed. Body-weight gains of test and control animals were similar. At termination of the study, measurements of organ weights showed no compound-related effects (Novo, 1978c).

In another study, 4 groups, each containing 15 male and 15 female Wistar/Mol SPF rats, were dosed by gavage once a day for 90 days with enzyme preparation at dose levels equivalent to 0, 2.5, 5.0, or 10 ml/kg b.w. Deaths, primarily in the high-dose group, appeared to be related to injury during dosing. No clinical signs were observed in the other test animals. Male rats in the high-dose group showed decreased weight gain and marked decrease in food intake. Haematology studies showed increased platelet counts and decreased clotting times

in the high-dose group at week 6, but this effect was not apparent at week 12. No other effects were reported. Clinical chemistry and urinalysis values at weeks 6 and 12 were within the normal range. At termination of the study, organ weight analysis showed a marked increase in relative weights of the spleen and testes of the males in the high-dose group. Gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Perry et al., 1979).

Dogs

Three groups, each containing one male and one female beagle dog, received single doses of 5, 10, or 15 ml/kg b.w. of the enzyme preparation over a 4-day period. Following a 7-day observation period the dogs were sacrificed and subjected to macroscopic post-mortem examination. No compound-related effects were observed, with the exception of vomiting during the first 4 days of the study. In another study, dogs were administered consecutive doses of 15 ml/kg b.w./day for 9 days, and 10 ml/kg b.w./day for 5 days. No deaths occurred during the course of the study. The only clinical sign noted was excessive salivation and emesis shortly after dosing. Body weights, electrocardiograms, haematological parameters, blood serum chemistry, organ weights, gross pathology, and histopathology showed no compound-related effects (Osborne et al., 1978).

In another study, three groups, each containing 3 male and 3 female beagle dogs, were dosed with the enzyme preparation by gavage once a day, seven days a week, for 13 weeks, at dose levels equivalent to 2, 5, or g ml/kg b.w./day. Two dogs in the high-dose group died during the course of the study, which the authors concluded was due to respiratory distress as a result of foreign material in the lungs. Vomiting was reported after dosing in the high-dose group. Haematological parameters at weeks 6 and 12 were within normal limits, with the exception of a significant increase in WBC count, specifically in the group mean neutrophil counts, in the high-dose group. Clinical chemistry values were within the normal range at weeks 8 and 12, with the exception of slight increases in blood glucose and cholesterol in the high-dose group. Urinalysis showed no compound-related effects. At termination of the study, organ-weight analyses and gross and histopathological examination of the principal organs and tissues showed no compound-related effects (Greenough et al., 1980).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. Short-term studies in rats and dogs resulted in no observed compound-related effects at levels up to 5 ml/kg b.w./day of enzyme preparation.

The evaluations by the Committee of the carbohydrases and the protease from A. niger are summarized at the end of this section.

REFERENCES

Greenough, R.J., Brown, J.C., Brown, M.G., Cowie, J.R., Maule, W.J., & Atken, R. (1980). ß-Glucanase 13 week oral toxicity study in dogs. Unpublished report No. 1630 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

McConville, M. (1980). Testing for mutagenic activity with S. typhimurium strain TA98, TA100, TA1535, TA1537, and TA1538 of fungal ß-glucanase. Unpublished report No. 1751 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

MGregor, D.B. & Willins, M.J. (1981). Cytogenic study in Chinese hamsters of fungal ß-glucanase. Unpublished report No. 2023 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

Novo (1978a). Acute oral toxicity of ß-glucanase given to mice. Unpublished report No. 1978-06-30 RKH/PNi from Novo Industri A/S, Bagsvaerd, Denmark. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

Novo (1978b). Acute oral toxicity of ß-glucanase given to rats. Unpublished report No. 1978-07-17 RICH/PNi from Novo Industri A/S, Bagsvaerd, Denmark. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

Novo (1978c). Oral toxicity of ß-glucanase given daily to rats for 14 days. Unpublished report No. 1978-08-21 RKH/PNi from Novo Industri A/S, Bagsvaerd, Denmark. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

Osborne, B.E., Cockrill, J.B., Cowie, J.R., Maule, W., & Whitney, J.C. (1978). Beta-glucanase, dog acute and maximum tolerated dose study. Unpublished report No. 1208 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

Perry, C.J., Everett, D.J., Cowie, J.R., Maule, W.J. & Spencer, A. (1979). ß-glucanase toxicity study in rats (oral administration by gavage for 90 days). Unpublished report No. 1310 from Inveresk Research International, Musselburgh, Scotland. Submitted to WHO by Novo Industri A/S, Bagsvaerd, Denmark.

HEMI-CELLULASE

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies

Special studies on mutagenicity

The enzyme preparation was tested for mutagenic activity using Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 both with and without metabolic activation (S-9 fraction). The test substance was not mutagenic or toxic at concentrations up to 5 mg/plate (Clausen & Kaufman, 1983).

In an in vitro cytogenetic test using CHO-K1 cells, both with and without metabolic activation (S-9 fraction), the enzyme preparation at test levels up to 2.5 mg (dry wt)/ml did not induce chromosomal aberrations (Skovbro, 1984).

Acute toxicity

No information available.

Short-term studies

Rats

Four groups, each containing 5 male and 5 female Wistar MOL/W rats, were dosed by garage once a day for 90 days with the enzyme preparation at doses equivalent to 0, 100, 333, or 1000 mg/kg b.w./day. No significant clinical changes were observed. Body-weight gain and food intake were similar among test and control animals. Haematologic and clinical chemistry measurements at termination of the study were within normal ranges. Post-mortem examinations, measurements of organ weights, and histopathology showed no compound-related effects. Slight increases in kidney and adrenal weights in the mid-dose group were not associated with histopathological effects, and did not show a dose response (Kallesen, 1982).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

This enzyme preparation was not genotoxic in microbial or in mammalian test systems. In a limited 90-day study in rats, no effects were observed at the highest dose administered (1 g/kg b.w./day). This enzyme preparation contained high levels of pectinase. The pectinase enzyme preparation summarized below may be identical to this hemi-cellulase preparation, which provides added assurance of the safety of this preparation.

The evaluations by the Committee of the carbohydrases and the protease from A. niger are summarized at the end of this section.

REFERENCES

Clausen, B. & Kaufman, U. (1983). Unpublished report from Obmutat Laboratiet. Submitted to WHO by Grinsted Products A/S, Brabrand, Denmark.

Kallesen, T. (1982). A 90-day toxicity study. Unpublished report No. 10023 from Scantox Biological Laboratory Ltd., Denmark. Submitted to WHO by Grinsted Products A/S, Brabrand, Denmark.

Skovbro, A. (1984). In vitro mammalian cytogenetic test (according to OECD Guideline No. 473). Unpublished report No. 10398 from Scantox Biological Laboratory Ltd., Denmark. Submitted to WHO by Grinsted Products A/S, Brabrand, Denmark.

PECTINASE (E.C. 3.1.1.11; 3.2.1.15; 4.2.2.10)

BIOLOGICAL DATA

Biochemical aspects

No information available.

Toxicological studies (The TOS of the commercial preparation is approximately 5%).

Acute toxicity

Species	Route	LD_{50} (ml/kg b.w.)	Reference
Rat	oral	18.8-22.1	Porter & Hartnagel, 1979

Short-term studies

Rats

Two groups of 10 male and 10 female ARS Sprague-Dawley rats were fed diets containing 5 or 10% of the test enzyme preparation (equivalent to 3.5 or 7 g of the enzyme preparation/kg b.w./day), for 90 to 94 days. A control group of 20 male and 20 female rats was maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar among test and control groups. Differential blood counts at weeks 4 and 8 of the study were within the normal range in test and control animals. At the end of the study serum clinical chemistry analyses, organ weight analyses, and gross and microscopic pathology showed no compound-related effects (Garvin et al., 1972).

Long-term studies

No information available.

Observations in man

No information available.

COMMENTS

In a short-term study in rats, no adverse effects were observed at dietary levels of the enzyme preparation up to the equivalent of 7 mg/kg b.w./day. This enzyme preparation may be identical to the hemi-cellulase preparation summarized above. The hemi-cellulase enzyme preparation summarized above also contained high levels of pectinase, which provides added assurance of the safety of this preparation.

REFERENCES

Garvin, P.J., Ganore, C.E., Merubia, J., Delahany, E., Bowers, S., Varnado, A., Jordan, L., Harley, G., DeSmet, C., & Porth, J. (1972). Carbohydrase from Aspergillus niger (pectinase, cellulase aud lactase). Unpublished report from Travenol Laboratories, Inc., Horton Grove, IL, USA. Submitted to WHO by Gist-brocades NV, Delft, Holland.

Porter, M.C. & Hartnagel R.E. (1979). The acute oral toxicity of a new pectinase product in the rat. Unpublished report No. 11 from Miles Laboratories, Inc., Elkhart, IN, USA. Submitted to WHO by Enzyme Technical Association, Washington, DC, USA.

PROTEASE

No information available.

GENERAL COMMENTS ON ENZYMES FROM A. NIGER

Aspergillus niger is a contaminant of food. Although there may be posible strain differences in A. niger, and different cultural conditions might be used to prepare the various enzymes, the available toxicity data, which consist primarily of short-term feeding studies in rats and some studies in dogs, show that all the enzyme preparations tested were of a very low order of toxicity. The enzyme preparations tested were non-mutagenic in bacterial and mammalian cell systems. Studies on some strains of A. niger used to prepare carbohydrases showed no aflatoxin or related substance production. These studies provide the basis for evaluating the safety of enzyme preparations derived from A. niger. It was also noted that the enzyme preparations tested exhibit a number of enzyme activities, in addition to the major enzyme activity. Thus, there may be considerable overlap of the enzyme activities of the different enzyme preparations so that safety data from each preparation provides additional assurance of safety for the whole group of enzymes.

Since the enzyme preparations tested were of different activities and forms, and most of the organic materials in the preparations are not the enzyme per se, the numerical ADI is expressed in terms of total organic solids (TOS) (see introduction to enzyme preparations section).

EVALUATION

Level causing no toxicological effect

All enzyme preparations tested showed no-observed-effect levels greater than 100 mg TOS/kg b.w./day in 90-day studies in rats.

Estimate of acceptable daily intake

0-1 mg TOS/kg b.w. for each of the enzyme preparations.

See Also:

Toxicological Abbreviations

Annex 5

Occurrence and significance of mycotoxins in *A. niger*

DATE: September 14, 1988

SUBJECT: Proposed Acceptable Daily Intake (ADI) Levels For Enzymes From Organisms Not Commonly Considered To Be Constituents of Food

FROM: J. W. Bennett, Ph.D.
Professor of Biology
Tulane University

I have read the report of the Joint FAO/WHO Expert Committee of Food Additives 31st meeting, Geneva, February 16-25, 1987 This report reiterates a conclusion reached at an earlier meeting of JECFA that an acceptable daily intake (ADI) should be established for certain enzyme preparations derived from microorganisms not normally used as food, or for enzyme preparations not removed from the food products to which they are added. This conclusion is based, in part, on the notion "that source organisms may produce toxins under certain conditions of growth" Neither the name of the putative toxins, nor the name of the organisms implicated as toxin producers, was given in the report. I would assume that the "offending" species are Aspergillus niger, Trichoderma harzianum, Trichoderma reesei, Penicillium funiculosum, and Aspergillus alliaceus since these are the producing organisms for enzymes for which the Joint FAO/WHO Expert Committee seeks to establish ADI's. Since none of these

species has been documented to produce mycotoxin in industrial applications, my comments below pertain more toward hypothetical situations, involving the introduction of new producing strains in the future, than to the species for which the ADI's are currently proposed. Based on the lack of documented evidence of toxin production in industrial settings, it is my opinion, that there is no reason to establish ADI's for the enzymes or species listed in the Table (ICS/87.13 Page 3 of the Summary and Conclusions of the Joint FAO/WHO Expert Committee on Food Additives entitled "Acceptable daily intakes, other toxicology information, and information on specifications" (Part A. Food additives, Enzyme preparations).

Before speaking to the questions raised by the report of the Joint FAO/WHO Expert Committee of Food Additives, it is important that certain terms be defined. Selected references, cited by author and date, are included in the text below. A bibliography is affixed at the end of the report.

Mycotoxins are fungal secondary metabolites that evoke a toxic response when introduced in low concentration to higher vertebrates, and other animals, by a natural route. Pharmacologically active fungal products such as antibiotics (which are toxic to bacteria and ethanol which is toxic to

animals but only in high concentration) are excluded from this definition (Bennett, 1987

Secondary metabolites are low molecular weight compounds of enormous chemical diversity and restricted taxonomic distribution that are normally synthesized after active growth has ceased Secondary metabolites are biosynthesized from small precursor molecules e.g., acetate, malonate, isoprene, amino acids) via a series of enzymatic conversions. Production of secondary metabolites is both species and strain specific Bennett & Ciegler, 1983).

Species are basic taxonomic units. Fungal species are named in accordance with the rules governed by the International Code of Botanical Nomenclature. The term "strain" derives from the International Code of Nomenclature of Bacteria. A strain constitutes the descendants of a single isolation in pure culture, sometimes showing marked differences in economic significance from other strains or isolations. Strain is analogous to "clone" in the International Code of Botanical Nomenclature (Jeffrey 1977; Bennett, 1985

The ability to produce a mycotoxin or other secondary metabolite is a characteristic of a species. Within the species different strains may vary in their biosynthetic potential: some strains may be high producers, some may be low producers, some

may be non-producers. The most common variant is the non-producer

Having defined the relevant terms, it is now possible to address certain issues raised by the report of the Joint FAO/WHO Expert Committee. The commentary below is organized as a series of questions and answers.

1. Do non-toxicogenic species of fungi develop strains that produce detectable levels of mycotoxins? Is the fact that mycotoxins are secondary metabolites relevant to this questions?

   No. Non-toxicogenic species of fungi do not become toxicogenic. However, the reverse is true. It is quite easy to isolate non-toxicogenic mutants and variants as clones "strains" from toxicogenic species

   The fact that mycotoxins are secondary metabolites is very relevant. Unlike enzymes, which are direct gene products synthesized directly from a structural gene via a series of RNA and amino acid intermediates, secondary metabolites are the result of numerous biosynthetic steps, each step enzymatically catalyzed. In most cases we do not know the exact number of steps in a biosynthetic pathway for a given secondary metabolite. Therefore, we do not know the number of genes required to encode for the enzymes of the pathway However, <u>all</u> secondary metabolites are biosynthesized by

multistep pathways with many genes and many enzymes involved.

2. Can conventional mutation (by mutagens or UV) or changes in medium or growth conditions cause a demonstrated non-toxin producer to begin producing toxins?

No. "You can't get something from nothing". Organisms which lack the structural genes for the enzymes of a mycotoxin pathway cannot be turned into toxin producers by simple mutation or changes in environmental parameters. In order for a non-toxicogenic species to become toxicogenic it would have to acquire the genes for an entire biosynthetic pathway.

A basic precept from genetics is analogous here: Deletions do not revert. Put another way, the absence of genetic material cannot mutate. Nor can it be expressed. Again note that the reverse is possible. Toxicogenic species may mutate to non-toxicogenic strains; and under certain growth conditions, toxicogenic strains may not express the genetic material for toxin production.

3. Since enzymes are primary metabolites which are ordinarily produced in the logarithmic phase of growth, what is the likelihood that mycotoxins, which are secondary metabolites, would be co-produced with the enzymes?

Usually there would be no co-production of secondary metabolites with the enzymes harvested during growth phase. Modern fermentation technology relies heavily on submerged cultures for growing production strains of fungi. Commercial enzymes are usually isolated from actively growing cultures. Because filamentous fungi grow in the form of thread-like hyphal cells, this early phase of growth, roughly analogous to logarithmic growth in single-celled organisms, has been given a special name: "trophophase" Similarly, in the jargon of fungal physiology, the period after active growth has ceased is called "idiophase". Idiophase is roughly analogous to lag phase or stationary phase for single-celled organisms. Most of the time, no secondary metabolites are produced during trophophase (Turner, 1971, pp. 18-20). Since this early growth phase is the phase during which most commercial enzymes are harvested, even in toxicogenic species it is possible to avoid accumulation of toxins by early harvesting of the fermentation cultures.

It is also relevant that the majority of mycotoxins are only sparingly soluble in water. Chemical separations of most mycotoxins use nonpolar solvents (Cole and Cox, 1981

Enzymes, on the other hand, are isolated with water and other polar solvents.

4. It is common practice for industry to test organisms for toxicogenicity and pathogenicity and products for non-specific toxicity before introducing them into commercial production and to test specifically for a toxin known to be associated with a given species. Is it appropriate for JECFA to impose testing for aflatoxin B1, ochratoxin A, sterig-matocystin, T-2 toxin and zearalenone in all fungal-derived enzyme preparations?

Once a producing species has been demonstrated as non-toxicogenic, it is a waste of time and money to test each lot of a commercial preparation for toxin production

If a species lacks the genetic material to biosynthesize a toxin, it will remain non-toxicogenic. Biosynthetic capacity is part of a species definition

A clumsy but colorful analogy could be drawn from the animal world. It would not make sense to test chickens and their eggs for milk production; nor would it be logical to assay cows and milk for the presence of feathers. Some vertebrates make milk; some make feathers. However, just because an organism is a vertebrate does not mean it will make either of these substances. Similarly, although some species of fungi make aflatoxin or T-2 toxin, it does not make sense to test <u>all</u> fungal preparations for aflatoxin and T-2 toxin

Specifically, there is no reason to test Aspergillus niger, Penicillium funiculosum, Trichoderma harzianum or T. reesei for aflatoxin B_1, sterigmatocystin, ochratoxin, T-2 toxin or zearalenone. Since some strains of Aspergillus alliaceus are known ochratoxin producers, enzyme preparations from this species might be tested for this one toxin. It would not be necessary to test A. alliaceus preparations for aflatoxin B_1, sterigmatocystin, T-2 toxin, or zearalenone.

BIBLIOGRAPHY

Bennett, J. W. 1985. Taxonomy of fungi and biology of the Aspergilli. In: Biology of Industrial Microorganisms (Eds: A. L. Demain and N. A. Solomon) pp. 359-406. Benjamin Cummings, Menlo Park, Ca.

Bennett, J. W. 1987. Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia. Mycopathologia 100: 3-5.

Bennett, J. W. and Ciegler, A. (Eds.). 1983. Secondary Metabolism and Differentiation in Fungi. Marcel Dekker, Inc., New York.

Cole, R. J. and Cox, R. H. 1981. Handbook of Toxic Fungal Metabolites. Academic Press, New York.

Turner, W. B. 1971. Fungal Metabolites. Academic Press, New York.

Sep. 1988

THE OCCURRENCE AND SIGNIFICANCE OF MYCOTOXINS

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THE OCCURENCE AND SIGNIFICANCE OF MYCOTOXINS

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1. Description of mycotoxins

A conservative estimate suggests that there are at least 100,000 species of fungi (Hawksworth, Sutton & Ainsworth, 1983) and many of these are able to produce one or more low molecular weight organic compounds known as secondary metabolites. These metabolites are a structurally diverse group of molecules (Turner & Aldridge, 1983) some of which have biological activity as antibiotics, phytotoxins and mycotoxins. The term mycotoxin is generally confined to those toxic metabolites produced by moulds growing on foods, animal feeds, or the raw materials and additives used in their manufacture.
The biological activity of mycotoxins is characterized by a toxic response when consumed by man or animals. Depending on the type of mycotoxin and animal species, even low concentrations of mycotoxins can create an acutely toxic, carcinogenic, oestrogenic or immuno-suppressive effect. A number of fungi producing macroscopic fruiting bodies (mushrooms and toadstools) also produce toxic metabolites and these are a hazard when such fruiting bodies are eaten. It is convenient to deal with these compounds separately and not include them as mycotixons.

2. Mycotoxins as natural contaminants in food

Of the several hundred known toxic mould metabolites (see Moreau, 1974; Wyllie & Morehouse, 1977; Watson, 1985) only about three dozen have been shown to occur as natural contaminants in food (Krogh, 1987). Table 1 lists the majority of these with the species of mould known to produce them.
A further selection of mycotoxins, such as the satratoxins, verrucarins, sporidesmins and slaframine, have been identified in animal feeds and fodders.

3. Ability of mycotoxin production depends on species as well as circumstances

Some mycotoxins are only produced by a limited number of strains of one or two species of fungi, whereas others may be produced by a large number of species. Thus the aflatoxins are only known to be produced by Aspergillus flavus and A. parasiticus, whereas ochratoxin is produced by several species of Aspergillus and Penicillium. It is not the case that species of mould traditionally used as constituents of food of producing mycotoxins.

Thus, Aspergillus oryzae, used extensively in the production of koji for the manufacture of a wide range of foods, is able to produce cyclopiazonic acid and β-nitro propionic acid, and Penicillium roquefortii, used in the manufacture of all the blue cheeses of the world, can produce PR-toxin, roquefortine and several other toxic metabolites. Because processes, and strain properties, are developed to optimise such qualities as biomass and industrial enzyme production (and are generally inversely related to those developed to optimize, or even permit, secondary metabolite formation), the production of koji and blue cheese is not associated with any known mycotoxin problem. In a sense, it is the process, rather than the organism, which is safe.

4. Species specific mycotoxins

Table 2 lists some of the secondary metabolites associated with species of mould used for the production of enzymes.
Only Aspergillus alliaceus is known to produce one of the mycotoxins (ochratoxin) included in those routinely tested for using the method of Patterson and Roberts (i.e. aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone).
The major justification for looking for these mycotoxins in products from species not associated with their production must presumably be concern for carry over from contaminated raw materials, or a failure to maintain a pure culture during the manufacturing process.

5. Effect of mutations on mycotoxin production

The biosynthetic pathways leading to the production of mycotoxins are frequently complex involving many steps (Steyn, 1980). The majority, if not all, of these steps will involve an enzyme which in turn will be coded for by a gene.
Thus many genes may be involved in the production of a particular mycotoxin. It is thus a common experience that the ability to produce a particular mycotoxin is readily lost during routine subculture of the producing strain. In fact, those who are trying to industrially produce secondary metabolites need to take special care to avoid this happening.
It is also relatively easy to lose the capability of producing a mycotoxin by a deliberate programme of mutation.
Since the chance to obtain a mutation defect in one of the many genes involved in mycotoxin synthesis is much higher than that of a mutation repair of one or more specific defects, the situation in which a non-toxigenic strain becomes toxigenic is far less common. Only one author (Benkhammar et al. (1985) has reported obtaining cyclopiazonic acid producing mutants of Aspergillus oryzae by treating a non-toxigenic strain with a mutagenic N-nitroso-guanidine derivative.

6. Mycotoxin and enzyme production: likelihood of co-production

The growth and morphological and biochemical differentiation of filamentous fungi involve the sequential induction, formation and repression of many hundreds of enzymes, some of which are involved in the biosynthesis of mycotoxins.
However, the relatively small number of enzymes of industrial interest are usually associated with the earlier stages of vigorous growth and their production is directly growth related. This is in contrast to the production of mycotoxins most of which occurs during the later stages of development and their optimum production is often associated with some form of stress on growth processes.
In a limited study of strains of Aspergillus flavus and closely related species at the University of Surrey, it was found that an inverse correlation occurs between the ability of strains to produce aflatoxin and the ability to produce and secrete high levels of growth related catabolic enzymes such as amylases. Such observations are entirely compatible with the suggestion that Aspergillus oryzae and A. sojae are "domesticated" forms of A. flavus and A. parasiticus respectively (Wicklow, 1984).

7. Mycotoxins and enzyme purification: likelihood of co-isolation

The enzymes of particular interest in the food industry are globular proteins which are high molecular weight water soluble compounds in contrast to the low molecular weight secondary metabolites many of which are more soluble in organic solvents than in water.
If secondary metabolites, including mycotoxins, were present in the production liquors from which enzyme are obtained, it is highly probable that some stages in down stream processing, such as ultra filtration, will effect a partial removal.

8. Bibliography

BENKHEMMAR, O. GAUDEMER, F. & BOUVIER-FOURCADE, I. (1985). Heterokaryosis between Aspergillus oryzae cyclopiazonic acid-defective strains: method for estimating the risk of inducing toxin production among cyclopiazonic acid-defective industrial strains. Applied and Environmental Microbiology. 50, 1087-1093.

HAWKSWORTH, D.L., SUTTON, B.C. & AINSWORTH, G.C. (1983). Ainsworth & Bisby's Dictionary of the fungi C.A.B. Kew

KROGH, P. (Ed.) (1987). Mycotoxins in Food. Academic Press, London.

MOREAU, C. (1974). Moulds, Toxins and Food J. Wiley, Chichester.

STEYN, P.S. (Ed.) (1980). The Biosynthesis of Mycotoxins, A Study in Secondary Metabolism. Academic Press, New York.

TURNER, W.B. & ALDRIGDE, D.C. (1983). Fungal metabolites II. Academic Press, London.

WATSON, D.H. (1985). Toxic Fungal Metabolites in food. Critical Reviews in food science and Nutrition. 22, 177-198

WICKLOW, D.T. (1984). Adaptation in wild and domesticated yellow-green aspergilli. In Toxigenic Fungi - their toxins and Health Hazard (Eds. M. Kurata & Y. Ueno). Elsevier, Amsterdam

WYLLIE, T.D. & MOREHOUSE, L.G. (Eds.) (1977). Mycotoxigenic fungi, Mycotoxins, Mycotoxicoses an Encyclopedic Handbook Vols 1, 2, 3. Marcel Dekker, New York.

TABLE 1: Mycotoxins identified as natural contaminants in food associated commodities.

MYCOTOXIN	MAJOR PRODUCING SPECIES
AFLATOXINS	Aspergillus flavus, A. parasiticus
OCHRATOXIN	Aspergillus ochraceus, Penicillium viridicatum
CITRININ	Penicillium citrinum
PENICILLIC ACID	Penicillium spp., Aspergillus spp.
PATULIN	Penicillium expansum, Aspergillus clavatus
STERIGMATOCYSTIN	Aspergillus versicolor
MYCOPHENOLIC ACID	Penicillium roquefortii
PENITREM A	Penicillium aurantiogriseum
P R TOXIN	Penicillium roquefortii
VIOMELLEIN	Aspergillus ochraceus Penicillium viridicatum
CYTOCHALASIN E	Aspergillus clavatus
CITREOVIRIDIN	Penicillium citreonigrum
CYCLOPIAZONIC ACID	Aspergillus flavus, Penicillium aurantiogriseum
ROQUEFORTINE	Penicillium roquefortii
ISOFUMIGACLAVINE	Penicillium roquefortii
ZERALENONE	Fusarium spp
ZEARALENOL	Fusarium spp
TRICHOTHECENES	Fusarium spp
MONILIFORMIN	Fusarium spp
TENUAZONIC ACID	Alternaria spp
ALTERNARIOL	Alternaria spp
ALTENUENE	Alternaria spp
ERGOT ALKALOIDS	Claviceps spp

TABLE 2: Examples of secondary metabolites reported to be produced by moulds used for the manufacture of enzymes.

MOULD SPECIES	METABOLITES
Aspergillus alliaceus	OCHRATOXINS A and B*
Aspergillus niger	RUBROFUSARIN B NIGERONE AURASPERONE NEOECHINULIN NIGRAGILLIN ASPERRUBROL
Aspergillus oryzae	B-NITROPROPIONIC ACID* MALTORYZINE* CYCLOPIAZONIC ACID* KOJIC ACID ORYZACIDIN ASPERGILLOMARASMINS
Penicillium funiculosum	11-DEACETOXY WORTMANNIN FUNICULOSIN SPICULISPORIC ACID
Trichoderma harzianum	IOSNITRINIC ACID*

* recognised as mycotoxins

APPENDIX 1

Search Strategy Used

Set	Items	Description
S1	5328	MYCOTOXIN
S2	16258	AFLATOXIN
S3	195	DIHYDROXYFLAV?
S4	736	DIACETOXYSCIRPENOL
S5	2352	OCHRATOXIN
S6	238	LUTEOSKYRIN
S7	0	EPOXY(W)TRICOTHECENE
S8	1226	STERIGMATOCYSTIN
S9	172721	TOXIN? ?
S10	2352	T(2W)2(2W)TOXIN
S11	2094	ZEARALENONE
S12	36	TRICOTHECENE
S13	394	RUBRATOXIN
S14	1506	PATULIN
S15	22846	S1 OR S2 OR S3 OR S4 OR S5
S16	173430	S6 OR S7 OR S8 OR S9 OR S10
S17	3802	S11 OR S12 OR S13 OR S14
S18	183474	S15 OR S16 OR S17
S19	1276181	ENZYME? ?
S20	261508	MANUFACTUR?
S21	2117	S19 AND S20
S22	80623	DEEP
S23	885707	CULTURE? ?
S24	2056	S22 AND S23
S25	4156	S21 OR S24
S26	77	S18 AND S25

The effect of the above strategy is that a reference is printed out if it contains one or more of the toxin keywords (S1 - S14) AND either Enzyme Manufacture OR Manufacture of Enzymes OR Manufacturing Enzymes etc., OR Deep Culture OR Deep Cultures. This gives a fairly wide coverage without overproducing results which swamp out relevant references and waste time, money and effort.

APPENDIX 2 - FILE SEARCHED

Files searched	Host	Major Journals Covered
Biotechnology	Orbit	Derwent Biotechnology Abst.
Current Awareness in Biotechnological Sciences	Orbit	Current Advances in Biotechnology Current Advances in Microbiol. Current Advances in Molecular Biol. Current Advances in Cell + Dev. Biol. Current Advances in Toxicology and many more
Biosis Previews	Dialog	Biological Abstracts
EMBASE	Dialog	Abstracts & Citations from 4000 worldwide Biomedical Journals
International Pharmaceutical Abstracts	Dialog	500 Pharmaceutical, medical + related Journals
Life Sciences Collection	Dialog	Industrial + Applied Microbiology, Microbiological abstracts
Chemcial Exposure	Dialog	Databank
Martindale on line	Dialog	Databank
Mèdline	Dialog	Index Medicus (3000 International Journals)
Occupational Safety & Health	Dialog	400 Journals 70,000 monographs
Chemical Regulations & Guideline system	Dialog	US Federal Databank on controlled substances
Drug information full text	Dialog	
Agrochemicals Handbook	Dialog	
CA Search	Dialog	Chemical abstracts
Merck index on line	Dialog	Merck index

Annex 6

Allergenicity assay beta-glucosidase from *A. niger*

Bioinformatics testing for putative allergenicity of A.niger beta-glucosidase

Applicant Paola Montaguti
Project
Project#
Scientist Hilly Menke
Date 7-07-2017

Used allergenicity database:
Database: AllergenOnLine
Last updated: 18-01-2017
Link: http://www.allergenonline.org/

Info of tested protein(s)
Enzyme name beta Glucosidase
Donor organism Na
Production organism *Aspergillus niger*

Protein sequence
>Beta-glucosidase
MRFTLIEAVALTAVSLASADELAYSPPYYPSPWANGQGDWAEAYQRAVDIVSQMTLAEKVNLTTGTGWELELCVGQ
TGGVPRLGIPGMCAQDSPLGVRDSDYNSAFPAGVNVAATWDKNLAYLRGQAMGQEFSDKGADIQLGPAAGPLGRS
PDGGRNWEGFSPDPALSGVLFAETIKGIQDAGVVATAKHYIAYEQEHFRQAPEAQGYGFNITESRSANLDDKTMHEL
YLWPFADAIRAGAGAVMCSYNQINNSYGCQNSYTLNKLLKAELGFQGFVMSDWAAHHAGVSGALAGLDMSMP
GDVDYDSGTSYWGTNLTISVLNGTAPQWRVDDMAVRIMAAYYKVGRDRLWTPPNFSSWTRDEYGFKYYYVSEGPY
EKVNQFVNVQRNHSELIRRIGADSTVLLKNDGALPLTGKERLVALIGEDAGSNPYGANGCSDRGCDNGTLAMGWGS
GTANFPYLVTPEQAISNEVLKNKNGVFTATDNWAIDQIEALAKTASVSLVFVNADSGEGYINVDGNLGDRRNLTLWR
NGDNVIKAAASNCNNTIVIIHSVGPVLVNEWYDNPNVTAILWGGLPGQESGNSLADVLYGRVNPGAKSPFTW
GKTREAYQDYLYTEPNNGNGAPQEDFVEGVFIDYRGFDKRNETPIYEFGYGLSYTTFNYSNLQVEVLSAPAYEPASG
ETEAAPTFGEVGNASDYLYPDGLQRITKFIYPWLNSTDLEASSGDASYGQDASDYLPEGATDGSAQPILPAGGAGG
NPRLYDELIRVSVTIKNTGKVAGDEVPQLYVSLGGPNEPKIVLRQFERITLQPSKETQWSTTLTRRDLANWNVETQD
WEITSYPKMVFAGSSSRKLPLRASLPTVH

Results

1. Blast against appropriate database
   As a control a blastP search is performed to verify the sequence and its origin.

Table 1: Blast results

Query sequence	Besthit Id	Donor-organism	database	%-age identity	description
Beta-glucosidase	UniRef100_A8WE01	*Aspergillus niger*	Uniref100	100 %	Beta-glucosidase

2. Check for signal sequence
   The signal sequence is removed upon secretion and is supposed to be degraded. Therefore it is assumed that the signal sequence is not present in the product. The signal prediction is performed with SignalPv2.0.

Table 2: Info signal sequence

Query sequence	signal	length	Signal sequence
Beta-glucosidase	yes	19	MRFTLIEAVALTAVSLASA

3. Full fasta alignment
   Full fasta alignment is used to identify allergens that are highly similar to the query sequence. Matches with E -values > 1 are not likely to be related in evolution or structure while matches with E -values > 10^{-7} are not likely to share immunologic or allergic cross-reactivity.

Table 3: Overview of full fasta alignment

Query sequence	Allergen	Source link	Sequence length	E-score
Beta-glucosidase	Asp n 14	gi:4235093	804	4.1e-06
Beta glucosidase	Asp n 14	gi:2181180	804	7.7e-06

4. FASTA alignments for an 80 amino acids sliding window
   Several hits were found between the query sequence and1 allergen (Asp o 21) in the Allergen Online database using a window of 80 amino acids and a suitable gap penalty.

Table 4: Hit info alignments for 80 aa sliding window

Query sequence	Allergen	Source link	%-age identity	Allergen host	Allergen type
Beta-glucosidase	Asp n 14	Asp n 14	36.3	*Aspergillus niger*	Non food
Beta-glucosidase	Asp n 14	Asp n 14	35.20	*Aspergillus niger*	Non food

5. Exact match for 8 Contiguous amino acids
   No exact matches for 8 contiguous amino acids have been found between the query sequence and all ALLERGEN ONLINE allergens.

Table 5: Hit info exact match for 8 aa

Query sequence	Allergen	Source link	Allergen host	Allergen type
Beta-glucosidase	No			

6. Available information of detected allergens (hits)
   (Link to the source, allergen host, type of allergen (food, nonfood)

Asp n 14	Biochemical name	Source link	Organism	Allergen type
Asp n 14	Beta-xylosidase	Asp n 14	*Aspergillus niger*	Non food

Conclusion

The potential allergenicity of the enzyme Beta-glucosidase from *Aspergillus niger* was evaluated by comparing the amino acid sequence of the enzyme with known (food) allergens. For the comparison, we made use of the database AllergenOnline.

Several stretches of 80 amino acids could be identified with an identity of more than 35% to 1 protein sequence in the AllergenOnline database.

1. Asp n 14 from Aspergillus niger is a non-food allergen

No identical stretches of 8 amino acids or more could be detected in the enzyme sequence as compared to the protein sequences in the AllergenOnline database.

Annex 7

Beta-glucosidase oral 7-day toxicity study in rats

DSM R&D-archives Delft

DSM

P.O. Box 1,
2600 MA Delft
The Netherlands

Report number: 15.805

Date: 24-11-1999

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1) PRELIMINARY TOXICITY STUDY BY ORAL GAVAGE ADMINISTRATION TO CD RATS FOR 1 WEEK

CRO-REPORT NO. GSB060/990021

Author(s): S. Cooper

Department: Huntigdon Life Sciences Ltd.

Experimental work:

Keywords: Toxicology
Oral
Subacute-tox
Beta-D-glucosidase
Aspergillus-niger
ARO-1
GLP-9708
0299-08-Tox-studies

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Signature author: Datum manuscript: 20-10-1999 Huntingdon Life Sciences Ltd Eye, Suffolk, UK	Signature department manager:

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

PRELIMINARY TOXICITY STUDY BY ORAL GAVAGE ADMINISTRATION TO CD RATS FOR 1 WEEK

Sponsor

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Draft: 23 February 1999
Final : 20 October 1999

CONTENTS

Huntingdon Life Sciences

COMPLIANCE WITH GOOD LABORATORY PRACTICE

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

PRELIMINARY TOXICITY STUDY BY ORAL GAVAGE

ADMINISTRATION TO CD RATS FOR 1 WEEK

This is a preliminary study and will be followed by a 4 week study with this test material.

The work performed in this study generally followed Good Laboratory Practice principles, however, no specific study-related Quality Assurance procedures or analysis of dose form were performed and the report may not contain all of the elements required by GLP. This is not considered to have compromised the scientific integrity of the study.

I consider the data generated by Huntingdon Life Sciences during the course of this study to be valid and that the final report fully and accurately reflects this raw data.

(b) (6)

20 October 1999

S. Cooper, B.Sc. C.Biol., M.I.Biol.
Study Director
Huntingdon Life Sciences Ltd.

Date

Huntingdon Life Sciences

RESPONSIBLE PERSONNEL

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

PRELIMINARY TOXICITY STUDY BY ORAL GAVAGE

ADMINISTRATION TO CD RATS FOR 1 WEEK

STUDY MANAGEMENT

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S. Cooper, B.Sc. C.Biol., M.I.Biol.
Study Director

PATHOLOGY

K.C.D. Liu, B.Sc., M.Sc., Ph.D.
Staff Pathologist

SUMMARY

Groups of five male and five female CD rats received Enzyme preparation from *Aspergillus niger* (ARO-1), by oral gavage, at dosages of 500, 1500 or 5000 mg/kg/day for seven days. A similarly constituted Control group received the vehicle (water obtained by reverse osmosis) alone.

There were no deaths or signs related to treatment.

Bodyweight gains were unaffected by treatment.

Females receiving 5000 mg/kg/day ate slightly less food than the Controls.

Food conversion efficiency was unaffected by treatment.

The absolute and bodyweight-relative lung and bronchi weights were high, in comparison with the Controls, in males which had received 5000 mg/kg/day and also in individual females in each of the treated groups.

There were no macroscopic findings considered to be related to treatment.

Conclusion

It was concluded that treatment of CD rats with Enzyme preparation from *Aspergillus niger* (ARO-1) at dosages up to 5000 mg/kg/day did not result in any changes which were considered to be of toxicological significance.

The dosages of 500, 1500 and 5000 mg/kg/day used in this study were, therefore, considered appropriate for use on the 4 week study in this species (GSB/061).

INTRODUCTION

Objective

The objective of this study was to assess the systemic toxic potential of Enzyme preparation from *Aspergillus niger* (ARO-1) during its repeated daily oral administration to CD rats for 1 week, and to aid in the selection of dosages for a 4 week study in this species (GSB/061).

Justification for the test system

The rat was chosen because of its acceptance as a predictor of toxic change in man and the requirement for a rodent species by regulatory agencies. The CD strain was used because of the historical control data available in this laboratory.

Justification for the treatment regimen

The oral route (gavage) was selected to simulate the conditions of human exposure during use of the test substance.

Dosages of 500, 1500 and 5000 mg/kg/day and the duration of treatment were selected by the Sponsor.

Study organisation

Testing facilities:

The principal laboratory was:

Huntingdon Life Sciences Ltd
Eye
Suffolk
IP23 7PX
England

Study timing:

Animals arrived (Experimental start)	:	06 January 1999
Treatment commenced	:	19 January 1999
Necropsy completed	:	26 January 1999
Experimental finish (Issue of macropathology report)	:	23 February 1999

Archives

Following completion of this study all raw data, specimens and samples, except those generated or used during any Sponsor's or supplier's analysis, were stored in the archives of Huntingdon Life Sciences. A copy of the final report was also retained.

MATERIALS AND METHODS

DESIGN CONDITIONS

Animals

A total of 25 male and 25 female rats of the CD strain, 26 to 30 days of age, were obtained from Charles River (UK) Limited, Margate, Kent, England. The males used on this study weighed 159 to 197g on the day treatment commenced, females weighed 141 to 172g.

Identification

After random allocation to groups each animal was assigned a number and identified uniquely within the study by a tail tattoo.

Acclimatisation and age at commencement

The animals were allowed to acclimatise to the conditions described below for 13 days before commencement of treatment. They were approximately 39 to 43 days of age when treatment started.

Environmental control

Animals were housed inside a barriered rodent facility.

Each animal room was kept at positive pressure with respect to the outside by its own supply of filtered fresh air which was passed to atmosphere and not re-circulated. Target values within the study room were 21°C for temperature (acceptable limits 19-25°C), 55% for relative humidity (acceptable limits 40-70%) and at least 15 air changes per hour. Lighting was controlled to provide a 12-hour light : 12-hour dark cycle.

The facility was designed and operated to minimise the entry of external biological and chemical agents and to minimise the transference of such agents between rooms. Before each study the room was cleaned and disinfected with a bactericide.

Access was limited to authorised personnel who were required to shower and change into clean protective clothing. Where practicable, materials and equipment entered the facility through an autoclave or a chamber in which their external surfaces were treated with a bactericide.

Alarms were activated if there was any failure of the ventilation system, or temperature limits were exceeded.

Periodic checks were made on the number of air changes in the animal rooms. Temperature and humidity were monitored daily. On one occasion before the commencement of treatment and on the last day of treatment the humidity was briefly below the target range of 40-70%. These minor deviations did not have any impact on the study. These data show that there were no other significant deviations from target values during the study and, therefore, they are not presented.

A stand-by electricity supply was available to be automatically brought into operation should the public supply fail.

Animal accommodation

The animals were housed five of one sex per cage, in RS Biotech cages from RS Biotech, Tower Works, Finedon, Northamptonshire, England, which were made of a stainless steel body, with a stainless steel mesh lid and floor. The cages were suspended above absorbent paper, which was changed at appropriate intervals. Cage trays and water bottles were changed once during the study.

Diet and water supply

The animals were allowed free access to an expanded rodent diet, Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England. This diet contained no added antibiotic or other chemotherapeutic or prophylactic agent. Weighed amounts of diet were provided at intervals during each week to each cage.

At the end of the treatment week the weight of uneaten food was recorded.

Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), was freely available, via polycarbonate bottles fitted with sipper tubes.

Quality control of diet and water

Each batch of diet was routinely analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates were scrutinised and approved before any batch of diet was released for use.

The quality of the water supply is governed by regulations published by the Department of the Environment. Certificates of analysis were routinely received from the supplier.

Since the results of these various analyses did not provide evidence of contamination that might have prejudiced the study they are not presented.

No other specific contaminants that were likely to have been present in the diet or water were analysed, as none that may have interfered with or prejudiced the outcome of the study was known.

Allocation to treatment groups

On arrival animals were non-selectively assigned to cages and treatment groups.

All animals were weighed during the acclimatisation period. Two males were discarded and replaced with spare animals from the same batch in order to obtain similar bodyweight means for each group.

As far as was practicable the distribution of animals in the room was designed to minimise the effect of any spatially variable component of the environment. The distribution is shown in Figure 1.

Composition and identity of treatment groups

Animals were assigned to the groups as follows:

Group	Treatment	Dosage (mg/kg/day) #	Cage numbers		Animal numbers	
			Male	Female	Male	Female
1	Control	0	1	5	1-5	21-25
2	*Aspergillus niger* (ARO-1)	500	2	6	6-10	26-30
3	*Aspergillus niger* (ARO-1)	1500	3	7	11-15	31-35
4	*Aspergillus niger* (ARO-1)	5000	4	8	16-20	36-40

Expressed in terms of the test substance as received.

Cage labels, identifying the occupants by experiment, animal number, sex and treatment group, were colour-coded.

TREATMENT

Test material

Nine aliquots of Enzyme preparation from *Aspergillus niger* (ARO-1) taken from batch no. RER710 were received from the Sponsor at Huntingdon Research Centre on 23 December 1998 and were subsequently transferred to Eye Research Centre on 6 January 1999. The test material was a brown liquid (frozen). Three aliquots weighed 250 g and the remaining six weighed 400 g (this information was supplied by Sponsor).

The test material was stored in a freezer (approximately -20°C) and protected from light.

Before use the identity, strength, purity and composition, or other characteristics which appropriately defined the batch from which the test material for this study was drawn, were determined by the Sponsor (Appendix 1). Stability of the test material and methods of synthesis, fabrication or derivation were documented by the Sponsor.

Before the consignment of the test material was used a 1 g representative sample was taken. This sample was placed in a well-closed glass container and stored in the archives under the conditions specified for the bulk supply of the test material.

All dosages and concentrations are expressed in terms of the material received.

Formulation

The Enzyme preparation from *Aspergillus niger* (ARO-1) was prepared for administration as a solution in water obtained by reverse osmosis to provide the required dosages at a constant volume-dosage of 10 ml/kg bodyweight. Control rats received the vehicle alone at the same volume-dosage.

For this preliminary study one 250 g container was allowed to thaw overnight at 4°C. This was used for the trial assessment and also to prepare the formulations for the study. Any residual test material was retained frozen at -20°C.

The test material was pre-weighed into a suitable container. The vehicle (water obtained by reverse osmosis) was then added until the required amount of bulk product was attained. The product was first hand stirred and then magnetically stirred for approximately two minutes to ensure that the test material was fully dispersed in the water. The bulk preparation was subdivided into seven aliquots which were retained at 4°C. Each day (in the afternoon) one aliquot was issued to the animal unit for use on the following day. Once received in the animal unit the aliquot was retained at 4°C until required.

Quality control of dosage form

Detailed records of compound usage were maintained. The amount of test material necessary to prepare the formulations and the quantity actually used were determined on each occasion. The difference between these amounts was checked before the formulations were dispensed.

Information received from the Sponsor indicated that the test material was stable in water for 48 hours where stored at 21°C and for 15 days when stored at 4°C. The formulations are stated by the Sponsor to form solutions and therefore homogeneity assessments were not included in this study.

A record of the weight of each formulation dispensed and the amount remaining after dosing was made. The balance was compared with the predicted usage as a check that the doses had been administered correctly. No significant discrepancy was found.

Before treatment commenced the suitability of the proposed mixing procedure was determined by a trial preparation. No samples were taken.

Administration

Animals received the test material or vehicle control formulations by oral gavage at a volume-dosage of 10 ml/kg bodyweight/day. All animals were dosed in sequence of cage-number, once each day, seven days per week. The volume administered to each animal was calculated from the most recently recorded scheduled bodyweight.

Duration of treatment

All animals were treated for seven consecutive days.

SERIAL OBSERVATIONS

All observations described below were performed in cage number sequence, except where otherwise indicated.

Signs

Animals were inspected at least twice daily for evidence of reaction to treatment or ill-health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

Daily detailed observations were recorded before and after dosing; these observations were recorded at the following times in relation to dosing:

Immediately before dosing.
Immediately after dosing on return of the animal to its cage.
On completion of dosing of each group.
Between one and two hours after completion of dosing of all groups.
As late as possible in the working day.

In addition a more detailed weekly examination, which included palpation, was performed on each animal.

Cages and cage-trays were inspected daily for evidence of animal ill-health, such as blood or loose faeces.

During the acclimatisation period, observations of the animals and their cages were recorded at least once per day.

Bodyweight

Each animal was weighed during the acclimatisation period, on the day that treatment commenced, twice during the treatment week and before necropsy.

Food consumption

The weight of food supplied to each cage of animals, that remaining and an estimate of any spilled was recorded for the week. From these records the mean weekly consumption per animal was calculated for each cage.

Food conversion efficiency

Group mean food conversion efficiencies were calculated for the week of treatment.

TERMINAL OBSERVATIONS

Euthanasia

All animals were killed at the end of the scheduled treatment period by carbon dioxide inhalation.

The sequence in which the animals were killed was selected to allow satisfactory inter-group comparison.

Macroscopic pathology

All animals were subjected to a detailed necropsy.

The necropsy procedure included a review of the history of each animal and a detailed examination of the external features and orifices, the neck and associated tissues and the cranial, thoracic, abdominal and pelvic cavities and their viscera. The requisite organs were weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Abnormalities and interactions were noted and the required tissue samples preserved in fixative.

Before disposal of the carcase the retained tissues were checked against the protocol and a senior prosector reviewed the necropsy report.

Organ weights

The following organs, taken from each animal, were dissected free of adjacent fat and other contiguous tissue and the weights recorded. The weight of each organ was expressed as a percentage of the bodyweight recorded immediately before necropsy.

Adrenals	Ovaries
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Thyroid with parathyroids, after partial fixation
Liver	
Lungs with mainstem bronchi	Uterus with cervix.

Tissues preserved for possible future histopathological examination

Samples of the following tissues were preserved in 10% neutral buffered formalin, except testes and epididymides which were placed in Bouin's fluid and subsequently retained in 70% industrial methylated spirit.

Adrenals	Oesophagus
Brain	Ovaries
Caecum	Prostate
Colon	Rectum
Duodenum	Sciatic nerve
Epididymides	Spinal cord
Head*	Spleen
Heart	Stomach
Ileum	Testes
Jejunum	Thymus
Kidneys	Thyroid with parathyroids
Liver	Trachea
Lungs with mainstem bronchi	Urinary bladder
Lymph nodes - mandibular	Uterus with cervix.
- mesenteric	

* Includes nasal cavity, paranasal sinuses and nasopharynx.

Samples of any abnormal tissues were also retained. In those cases where a lesion was not clearly delineated, contiguous tissue was fixed with the grossly affected region.

DEFINITION OF "DAY"

The first day of treatment started at midnight prior to treatment commencing and ended at midnight 24 hours following. Subsequent experimental days were of the same duration. Bodyweights taken on the first day of treatment, prior to the animals being dosed, are designated as Day 0.

TREATMENT OF DATA

This report contains serial observations pertaining to all days of treatment completed before commencement of the necropsies, together with signs data collected during the despatch of the animals to necropsy.

The name of the test material was abbreviated to ARO-1 for presentation in the data.

Group mean values were calculated from the individual values presented in the appendices, unless otherwise specified below.

The death code in Appendix 2 has the following meaning:

7 Terminal kill

Throughout the tables the following abbreviations are used:

N Number of animals examined.
SD Standard deviation.

Signs

A detailed history for all animals is presented in Appendix 2 and a summary of the total number of animals displaying any particular sign is presented in Table 1. Only animals with positive observations are presented.

The only sign evident at dosing was salivation on Day 7 for Animal 37. The sign was seen before dosing, immediately after dosing and on completion of dosing the group. As no other sign was evident, there are no data presented.

Bodyweight

Group mean weight changes for the appropriate period were calculated from the weight changes of individual animals.

Food consumption

Values are presented for the amount of food consumed in each cage during the experimental week.

Food conversion efficiency

The weekly cage values were calculated from the bodyweight gain of animals and the total weight of food consumed in the cage.

Organ weights

For bilateral organs, left and right organs were weighed together.

Pathology

Only tissues having macroscopic findings have been reported.

Statistical analysis

For organ weights and bodyweight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

Inter-group differences in macroscopic pathology were assessed using Fisher's Exact test.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the Controls ($p<0.05$).

RESULTS

Mortality and signs (Table 1; Appendix 2)

There were no deaths and the appearance and behaviour of the animals was unaffected by treatment.

Bodyweight (Figure 2; Table 2; Appendix 3)

There was no clear effect on the bodyweight gains of treated animals when compared with the Controls.

Food consumption (Table 3)

Slightly low food consumption was seen in females receiving 5000 mg/kg/day when compared with the Controls.

The food consumption of females receiving 500 or 1500 mg/kg/day and treated males was not considered to be affected by treatment.

Food conversion efficiency (Table 4)

Food conversion efficiencies were not affected by treatment.

Organ weights (Table 5; Appendix 4)

Slightly high absolute and bodyweight-relative lung and bronchi weights were evident for males receiving 5000 mg/kg/day. Similar findings were also noted in one female in each of the treated groups (2F 29, 3F 31 and 4F 38).

Macroscopic pathology (Table 6; Appendix 5)

There were no macroscopic findings which could be related to treatment with Enzyme preparation from *Aspergillus niger* (ARO-1). Findings seen in the animals were of the kind commonly seen in rats of this age and strain at these laboratories.

DISCUSSION AND CONCLUSION

Treatment of CD rats with Enzyme preparation from *Aspergillus niger* (ARO-1) at dosages of 500, 1500 or 5000 mg/kg/day resulted in slightly low food intake in females receiving 5000 mg/kg/day and high lung and bronchi weights in males which had received 5000 mg/kg/day and also in one female in each of the treated groups.

The slightly low food intake seen in females receiving 5000 mg/kg/day was not clearly associated with reduced bodyweight gains and, in the absence of a similar finding in the males, is not considered to be of any toxicological significance.

The high lung and bronchi weights seen in males which had received 5000 mg/kg/day and in several of the treated females (one in each dosage group) do not associate with any macropathological findings in these animals. In the absence of microscopic examination, the significance of this finding is unclear.

It was concluded that treatment of CD rats with Enzyme preparation from *Aspergillus niger* (ARO-1) at dosages up to 5000 mg/kg/day did not result in any changes which were considered to be of toxicological significance.

The dosages of 500, 1500 and 5000 mg/kg/day used in this study were, therefore, considered appropriate for use on the 4 week study in this species (GSB/061).

FIGURE 1

Cage arrangement in the battery

Group	:	1	2	3	4
Compound	:	Control		----- (ARO-1) -----	
Dosage (mg/kg/day)	:	0	500	1500	5000

Group/sex Cage number Animal numbers		
1M 1 1-5	4M 4 16-20	2M 2 6-10
3M 3 11-15		
1F 5 21-25	4F 8 36-40	2F 6 26-30
3F 7 31-35		

FIGURE 2A

Group mean bodyweight versus period of treatment - males

Group	:	1	2	3	4
Compound	:	Control		ARO-1	
Dosage (mg/kg/day)	:	0	500	1500	5000

—— Group 1M - - - Group 2M —— Group 3M ····· Group 4M

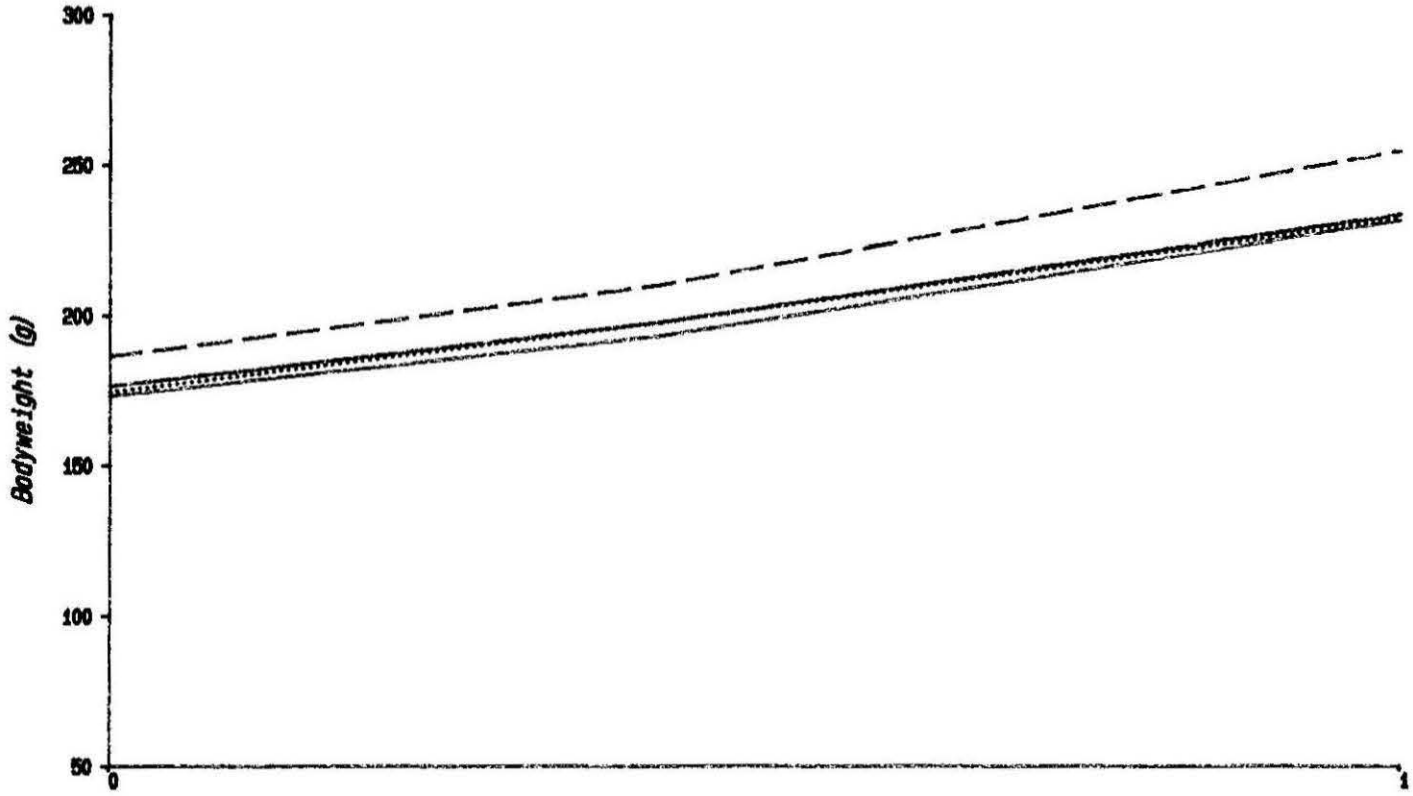

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FIGURE 2B

Group mean bodyweight versus period of treatment - females

Group	:	1	2	3	4
Compound	:	Control		ARO-1	
Dosage (mg/kg/day)	:	0	500	1500	5000

—— Group 1F — - Group 2F —— Group 3F ..... Group 4F

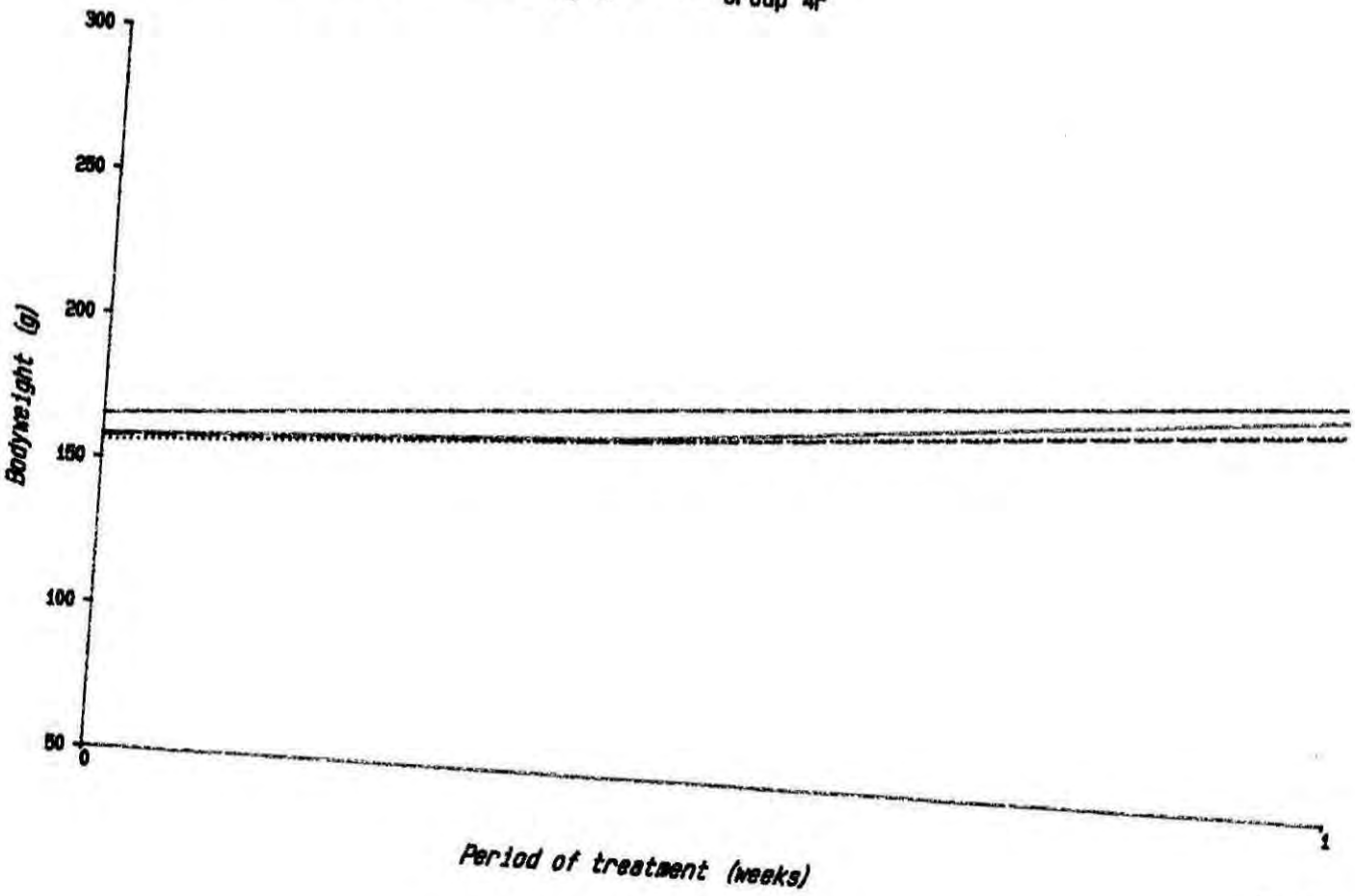

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TABLE 1

Signs - group distribution of observations

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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WEEKS 1-2

NUMBER OF ANIMALS AFFECTED

CATEGORY / KEYWORD / QUALIFIER	SEX:	MALE				FEMALE			
	GROUP:	1	2	3	4	1	2	3	4
	NUMBER:	5	5	5	5	5	5	5	5
*** TOP OF LIST ***									
BEHAVIOUR									
IRRITABLE		0	0	0	0	1	0	0	0
VOCALIZATION		0	0	0	0	1	0	1	1
COAT									
HAIRLOSS									
DORSAL BODY SURFACE		0	1	0	0	0	0	0	0
SKIN									
SCAB									
LEFT UPPER DORSAL THORAX		0	1	0	0	0	0	0	0
*** END OF LIST ***									

TABLE 2A

Bodyweights - group mean values (g)

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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	SEX:	MALE				FEMALE			
	GROUP:	1	2	3	4	1	2	3	4
DAY									
0	N	5	5	5	5	5	5	5	5
	MEAN	173	186	176	174	158	157	165	156
	S.D.	10.3	4.0	5.7	14.5	9.0	8.4	6.0	9.8
3	N	5	5	5	5	5	5	5	5
	MEAN	193	210	198	198	167	166	177	167
	S.D.	10.3	5.2	6.7	16.6	11.6	7.3	8.6	13.3
7	N	5	5	5	5	5	5	5	5
	MEAN	231	255	233	232	189	184	194	184
	S.D.	11.9	7.2	10.4	19.1	13.8	5.8	8.5	16.8

TABLE 28

Bodyweight gain - group mean values (g)

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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	SEX:	MALE				FEMALE			
DAY	GROUP:	1	2	3	4	1	2	3	4
0-3	N	5	5	5	5	5	5	5	5
	MEAN	20	24	21	23	9	9	12	11
	S.D.	3.3	1.5	1.3	3.4	3.1	2.4	3.6	4.1
3-7	N	5	5	5	5	5	5	5	5
	MEAN	38	44	35	34	22	17 a	17 a	18
	S.D.	3.6	2.4	6.0	2.8	2.7	1.7	1.4	4.1
0-7	MEAN	58	68 a	57	58	31	26	29	28
	S.D.	4.7	3.3	7.1	5.1	5.2	3.6	3.8	8.2

Significant when compared with Group 1: a - p<0.05; b - p<0.01

TABLE 3

Food consumption - group mean values (g/animal)

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Group	: 1	2	3	4
Compound	: Control	----	ARO-1	----
Dosage (mg/kg/day)	: 0	500	1500	5000

	SEX:	MALE				FEMALE			
	GROUP:	1	2	3	4	1	2	3	4
WEEK									
1	N	5	5	5	5	5	5	5	5
	MEAN	194	211	188	187	146	145	152	135
As % of Control			109	97	96		99	104	92

TABLE 4

Food conversion efficiency - group mean values (%)

Group	: 1	2	3	4
Compound	: Control	----	ARO-1	----
Dosage (mg/kg/day)	: 0	500	1500	5000

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SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
WEEK								
1	30.0	32.4	30.3	30.8	21.4	18.2	19.1	21.0

TABLE 5A

Absolute organ weights - group mean values (g) for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
TERMINAL BODY WEIGHT (g)								
		DUNNETT'S TEST				DUNNETT'S TEST		
N :	5	5	5	5	5	5	5	5
MEAN :	227.3	250.4 a	229.6	228.2	187.7	180.4	192.0	180.5
sd :	11.1	6.5	8.7	20.5	14.3	7.5	7.9	16.0
ADRENALS								
		DUNNETT'S TEST				DUNNETT'S TEST		
N :	5	5	5	5	5	5	5	5
MEAN :	0.035	0.035	0.037	0.035	0.043	0.046	0.047	0.044
sd :	0.004	0.003	0.004	0.005	0.012	0.007	0.006	0.009
BRAIN								
		DUNNETT'S TEST				DUNNETT'S TEST		
N :	5	5	5	5	5	5	5	5
MEAN :	1.83	1.84	1.84	1.85	1.78	1.77	1.78	1.73
sd :	0.09	0.10	0.07	0.04	0.06	0.04	0.03	0.05
KIDNEYS								
		DUNNETT'S TEST				DUNNETT'S TEST		
N :	5	5	5	5	5	5	5	5
MEAN :	2.06	2.17	2.01	1.93	1.69	1.60	1.78	1.63
sd :	0.13	0.08	0.13	0.13	0.09	0.05	0.08	0.21
EPIDIDYMIDES								
		DUNNETT'S TEST						
N :	5	5	5	5				
MEAN :	0.273	0.300	0.261	0.280				
sd :	0.055	0.047	0.045	0.018				

Significant when compared with Group 1: a - $p<0.05$

TABLE 5A - continued.

Absolute organ weights - group mean values (g) for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

Schedule number: GSB 060

SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
LIVER								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	12.5	14.0	11.8	11.9	9.2	9.0	9.4	9.1
sd :	1.6	1.8	0.9	0.7	0.7	0.5	0.5	1.5
OVARIES								
					DUNNETT'S TEST			
N :					5	5	5	5
MEAN :					0.065	0.063	0.079	0.056
sd :					0.010	0.010	0.011	0.019
SPLEEN								
	DUNNETT'S TEST				BEHREN'S - FISHER'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.625	0.699	0.660	0.619	0.607	0.532	0.528	0.500
sd :	0.099	0.068	0.098	0.044	0.156	0.050	0.048	0.052
TESTES								
	BEHREN'S - FISHER'S TEST							
N :	5	5	5	5				
MEAN :	2.61	2.36	2.11	2.36				
sd :	0.59	0.06	0.70	0.31				
THYMUS								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.537	0.660	0.587	0.592	0.575	0.557	0.595	0.542
sd :	0.099	0.098	0.084	0.028	0.125	0.137	0.083	0.119

TABLE 5A - continued.

Absolute organ weights - group mean values (g) for animals killed after 7 days of treatment.

Group	:	1	2	3	4
Compound	:	Control	----	ARO-1	----
Dosage (mg/kg/day)	:	0	500	1500	5000

Schedule number: GSB 060

SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
THYROIDS+PARAS								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.010	0.010	0.010	0.011	0.010	0.008	0.010	0.009
sd :	0.001	0.003	0.003	0.003	0.002	0.002	0.001	0.002
UTERUS + CERVIX								
					DUNNETT'S TEST			
N :					5	5	5	5
MEAN :					0.42	0.36	0.35	0.38
sd :					0.16	0.12	0.04	0.13
HEART								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.99	1.09	1.09	1.02	0.85	0.84	0.90	0.88
sd :	0.09	0.08	0.12	0.07	0.12	0.08	0.04	0.14
LUNGS & BRONCHI								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	1.22	1.34	1.27	1.51 b	1.09	1.22	1.25	1.19
sd :	0.05	0.07	0.14	0.06	0.16	0.17	0.19	0.28

Significant when compared with Group 1: a - $p<0.05$; b - $p<0.01$

TABLE 5B

Organ weights relative to bodyweight - group mean values (%) for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
TERMINAL BODY WEIGHT (g)								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	227.3	250.4 a	229.6	228.2	187.7	180.4	192.0	180.5
sd :	11.1	6.5	8.7	20.5	14.3	7.5	7.9	16.0
ADRENALS								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.0154	0.0141	0.0160	0.0154	0.0224	0.0256	0.0243	0.0246
sd :	0.0013	0.0011	0.0016	0.0025	0.0046	0.0035	0.0031	0.0060
BRAIN								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.808	0.734	0.803	0.817	0.950	0.984	0.927	0.965
sd :	0.062	0.053	0.028	0.057	0.052	0.049	0.026	0.078
KIDNEYS								
	DUNNETT'S TEST				BEHREN'S - FISHER'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.905	0.869	0.875	0.846	0.905	0.888	0.927	0.903
sd :	0.045	0.050	0.047	0.035	0.076	0.030	0.016	0.081
EPIDIDYMIDES								
	DUNNETT'S TEST							
N :	5	5	5	5				
MEAN :	0.1197	0.1203	0.1137	0.1240				
sd :	0.0214	0.0211	0.0203	0.0165				

Significant when compared with Group 1: a - $p<0.05$

TABLE 5B - continued.

Organ weights relative to bodyweight - group mean values (%) for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

Schedule number: GSB 060

SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
LIVER								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	5.51	5.56	5.14	5.21	4.92	4.96	4.90	5.02
sd :	0.50	0.56	0.22	0.26	0.15	0.14	0.28	0.49
OVARIES								
					DUNNETT'S TEST			
N :					5	5	5	5
MEAN :					0.0346	0.0348	0.0416	0.0312
sd :					0.0059	0.0050	0.0073	0.0103
SPLEEN								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.2745	0.2789	0.2868	0.2718	0.3202	0.2957	0.2748	0.2772
sd :	0.0374	0.0229	0.0359	0.0170	0.0572	0.0373	0.0152	0.0212
TESTES								
	BEHREN'S - FISHER'S TEST							
N :	5	5	5	5				
MEAN :	1.141	0.945	0.918	1.043				
sd :	0.212	0.044	0.300	0.181				
THYMUS								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.2361	0.2636	0.2569	0.2611	0.3042	0.3098	0.3094	0.2981
sd :	0.0409	0.0399	0.0435	0.0231	0.0473	0.0801	0.0374	0.0455

TABLE 5B - continued.

Organ weights relative to bodyweight - group mean values (%) for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

Schedule number: GSB 060

SEX:	MALE				FEMALE			
GROUP:	1	2	3	4	1	2	3	4
NUMBER:	5	5	5	5	5	5	5	5
THYROIDS+PARAS								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.0046	0.0040	0.0043	0.0047	0.0055	0.0046	0.0051	0.0049
sd :	0.0004	0.0011	0.0011	0.0014	0.0015	0.0013	0.0005	0.0012
UTERUS + CERVIX								
					BEHREN'S - FISHER'S TEST			
N :					5	5	5	5
MEAN :					0.224	0.203	0.183	0.215
sd :					0.097	0.077	0.015	0.095
HEART								
	DUNNETT'S TEST				BEHREN'S - FISHER'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.436	0.436	0.476	0.450	0.449	0.465	0.469	0.490
sd :	0.029	0.038	0.064	0.025	0.031	0.029	0.011	0.071
LUNGS & BRONCHI								
	DUNNETT'S TEST				DUNNETT'S TEST			
N :	5	5	5	5	5	5	5	5
MEAN :	0.537	0.537	0.554	0.666 b	0.581	0.677	0.649	0.656
sd :	0.031	0.038	0.062	0.048	0.057	0.095	0.082	0.111

Significant when compared with Group 1: a - $p<0.05$; b - $p<0.01$

TABLE 6

Macropathology - summary of individual findings for animals killed after 7 days of treatment.

Group : 1 2 3 4
Compound : Control ---- ARO-1 ----
Dosage (mg/kg/day) : 0 500 1500 5000

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Schedule number: GSB 060

ORGAN/TISSUE EXAMINED	MALE -1- (5)	MALE -2- (5)	MALE -3- (5)	MALE -4- (5)	FEMALE -1- (5)	FEMALE -2- (5)	FEMALE -3- (5)	FEMALE -4- (5)
** TOP OF LIST **								
EPIDIDYMIDES								
--APPEAR SMALL			0013					
KIDNEYS								
--HYDRONEPHROSIS			0012			0026 0029	0031	0036
L N THYMIC								
--CYSTIC		0010						
PITUITARY								
--CYSTIC				0017				
SKIN								
--ENCRUSTATION(S)		0010						
SUBMANDIB SL.GL.								
--AREA(S) OF CHANGE			0014					
TESTES								
--APPEAR SMALL			0013					
THYROIDS								
--APPEAR SMALL	0004							0037
TRACHEA								
--ABNORMAL CONTENTS			0015					
UTERUS								
--FLUID DISTENSION					0021	0030		0037
** END OF LIST **								