iii. Monitoring of Production Strain

The following information shall be provided:

- Details of procedures for the control and monitoring of the microbial source selected for food enzyme production. This may include details on storage conditions of the strain, the industrial pre-culture and culture conditions and their effect on reproducibility between the different batches of food enzymes. Strain monitoring should be sufficient to demonstrate that the strain in use is the same as that described in the dossier.

- Details of procedures for control and monitoring to ensure pure culture and optimum enzyme productivity conditions during fermentation. This may include details of the culture and process conditions designed to ensure the absence of toxins or secondary metabolites harmful to human health.

- Details of procedures for the control of the hygienic conditions throughout recovery and treatments of the food enzyme.

- Details of strain identification methods and results, sufficient to distinguish the production strain from other strains of the same species.

iv. Production Strain Pathogenicity, Toxigenicity and Antimicrobial Resistance

- Information relating to pathogenicity and toxigenicity of the source organism, as well as other properties with potential impact on human health, *e.g.* the production of antibiotics as well as the presence of natural and/or acquired antibiotic/antimicrobial (TH) resistance genes.

- Details of data related to the presence of acquired antimicrobial resistance genes in accordance with the 'Opinion of the Panel on additives and products or substances used in animal feed (FEEDAP) on the updating of criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance' (EFSA, 2008).

3.2.2 Manufacturing Process

The production process for the food enzyme should be described as completely as possible. A flow chart diagram showing the most important steps in the process should accompany the description.

The following information is required:

i. Description of key steps involved in the production process

If the food enzyme is obtained from a microbial source, information on the fermentation process is required, *e.g.* on process parameters, fermentation media and chemical substances used throughout.

The purification procedure(s) used to obtain the food enzyme should be described including information on the techniques used to remove microbes from the food enzyme and information on extraction solvents, other chemicals, materials and equipment.

Analytical data on a statistically relevant number of manufactured batches representative of the commercial food enzyme demonstrating that the food enzyme complies with the specification set out in 3.1.2.2

ii. Description of operational limits including process controls and quality assurance procedures and how key parameters such as temperature are controlled during production.

iii. In the case of immobilised food enzymes, information on the immobilisation procedure is required, *e.g.* enzyme support materials[12] and immobilisation agents. Information on potential leakage of carriers, immobilisation agents and active enzymes into the food should be provided.

iv. Other relevant information, taking into account recent opinion of EFSA's Scientific Committee on "The potential risks arising from nanoscience and nanotechnologies on food and feed safety" (EFSA, 2009).

3.3 Reaction and Fate in Food

Information should be provided on the fate of the food enzyme during food processing (see **Section 3.1.2) and its behaviour in the food matrix. If relevant any data on intended and** unintended reaction products resulting either from enzymatic or chemical reactions of the food enzyme with food constituents or from the degradation of the food enzyme during storage and processing of the foodstuff. If for safety reasons certain food enzymes have to be inactivated experimental studies should be carried out and data from these studies presented to demonstrate the inactivation of both the principal and subsidiary/side enzymatic activities in the final food, if applicable.

In addition the following is required to allow safety assessment:

- Information on possible adverse effects on nutrients;
- Data related to any possible effects of food enzymes on existing micro-organisms in food (*e.g.* lysozyme can induce germination of microbial spores).

[12] Enzyme support materials should comply with rules for materials intended to come into contact with food under Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food.

3.4 Case of Need and proposed Conditions of Use

Information should be provided on:

i. The technological need/purpose and intended use of the food enzyme,

ii. The mode of action and reactions catalysed by the food enzyme,

iii. The type of foodstuffs in which the food enzyme is intended to be used,

iv. The amount of food enzymes to be added to specific foods (recommended use levels and maximum use levels),

v. The conditions of its use in food processing.

3.5 Dietary Exposure

Potential human exposure to the food enzyme and to any other constituent or by-product of concern should be assessed considering all proposed uses.

A conservative technique such as the "budget method" (Hansen, 1966; Hansen 1979; Douglass *et al.*, 1997; European Commission 1998; FAO/WHO 2008) should be used to assess potential dietary exposure in a standard adult of 60 kg body weight consuming large amounts of the categories of foods and beverages for which use levels have been proposed, assuming that they always contain the food enzyme at its proposed upper use level. If needed, the technique should be adapted to consider the potential higher consumption per kg body weight of these foods and beverages in children. All assumptions and data used for the dietary exposure assessment should be clearly described and justified.

In case the use of the food enzyme is proposed for products specifically designed for infants (0-12 months) or young children (12-36 months) as defined in the Commission Directive 2006/141/EC, *ad hoc* conservative exposure estimates must be produced taking specifically into account these population groups.

3.6 Information on Existing Authorisations and Evaluations

Information on any existing authorisations and evaluations and/or evaluations by other bodies should be provided. Evaluations performed by the national authorities of the EU Member States may be considered on a case-by-case basis.

4. Toxicological Data

4.1 Toxicological Testing

A decision on the need for toxicological testing on a food enzyme should be made on the basis of already available information, including the source of the enzyme, its composition and properties, any existing toxicological studies and any documented history of use of the enzyme in food as well as foreseen level of exposure.

The default assumption is that toxicological testing is necessary. Exceptions are detailed below (s. section 4.1.2).

4.1.1 The toxicological Data Set

The core set of toxicological data that is required is set out below.

i. Assessment of genotoxicity

This assessment should start with *in vitro* tests, covering both gene mutations and chromosomal effects (structural and numerical).

Two *in vitro* tests would normally be required:

- a test for induction of gene mutations in bacteria (Ames test; OECD guideline 471). If this assay is not applicable, alternatively a test for induction of gene mutations in mammalian cells, preferably the mouse lymphoma *tk* assay with colony sizing (OECD guideline 476), could be performed.
- an *in vitro* assay for the detection of chromosomal aberration (OECD guideline 473) or the *in vitro* micronucleus assay (Draft OECD guideline 487) or the mouse lymphoma *tk* assay with colony sizing (OECD guideline 476)

In any case at least two *in vitro* assays should be performed.

Positive results in any of the above *in vitro* tests may suggest that food enzyme and/or any residues, degradation products or substances originating from the production process that may be present in the food enzyme are mutagenic. A positive result in genotoxicity testing would then require further assessment to determine whether it is genotoxic *in vivo*. Deliberate addition of a genotoxic carcinogen to food is unacceptable (Barlow *et al*, 2006).

One or more positive *in vitro* tests normally require follow-up by *in vivo* testing, unless it can be adequately demonstrated that the positive *in vitro* findings are not relevant for the *in vivo*

situation. This is in line with the general strategy elaborated in the updated WHO/IPCS Harmonised Scheme on mutagenicity testing (Eastmond *et al.*, 2009).

The choice of the appropriate *in vivo* test is critical, due to different sensitivities, different endpoints and other variables. It requires expert judgement based on all available information, to be applied case-by-case. For this reason, a flexible approach is preferable to a fixed decision tree.

Guidance for the follow-up of positive results from *in vitro* assays could be taken from a guidance document issued recently by the European Chemicals Agency (ECHA 2008, ECB 2003) which recommends that any of the following tests may be conducted:

1. A rodent bone marrow or mouse peripheral blood micronucleus test (OECD guideline 474) or a rodent bone marrow clastogenicity study (OECD guideline475).
2. A Comet (single cell gel electrophoresis) assay
3. A test for gene mutations in a transgenic rodent model, *e.g.* using *lacI*, *lacZ* or *cII* as reporter gene present in every tissue.
4. A rat liver Unscheduled DNA synthesis (UDS) test

According to this ECHA guidance, "the nature of the original *in vitro* response(s) (*i.e.* gene mutation, structural or numerical chromosome aberration) should be considered when selecting the *in vivo* study. For example, if the test substance showed evidence of *in vitro* clastogenicity, then it would be most appropriate to follow this up with either a micronucleus test or chromosomal aberration test or a Comet assay. However, if a positive result were obtained in the *in vitro* micronucleus test, the rodent micronucleus test would be appropriate to best address clastogenic and aneugenic potential.

The rat liver UDS test may be appropriate for substances that appear preferentially to induce gene mutations, although the Comet and transgenic tests are also suitable (Speit, 2008). These latter test systems offer greater flexibility, most notably the possibility of selecting a range of tissues for study on the basis of what is known of the toxicokinetics and toxicodynamics of the substance. It should be realised that the UDS and Comet tests are indicator assays detecting putative DNA lesions. In contrast, the transgenic test measures permanent mutations." (ECHA 2008). A combination of the *in vivo* micronucleus assay and the Comet assay in a single study as suggested by Pfuhler *et al.* (2007) would also be acceptable.

Other studies (*e.g.* DNA adduct studies) could also be relevant in order to clarify the mechanism of genotoxicity.

It should also be taken into account that the sensitivity (ability to detect carcinogens as positive) and specificity (ability to give negative results with non-carcinogens) of such assays have recently been analysed by Kirkland and Speit (2008).

ii. Assessment of systemic toxicity

A subchronic oral toxicity study as described in OECD guideline 408 (OECD, 2000a) should be performed.

Toxicological studies should be conducted using internationally agreed protocols if available. Test methods described by OECD and other provisions adopted under European legislation are recommended. The most up-to-date edition of any test guideline should be followed. Studies should be carried out according to the principles of Good Laboratory Practice (GLP) described in Council Directives 2004/10/EC[13] and 2004/09/EC[14] and accompanied by a statement of GLP compliance of the laboratory conducting the studies.

The toxicological studies should be performed on a batch representative of the food enzyme before addition of other components of the food enzyme preparation.

There may be circumstances under which it may be appropriate to deviate from the above mentioned core set. Such deviations include exemption from certain tests, or use of alternative protocols or use of alternative assays or tests. In such cases a scientific justification should be provided and additional types of considerations or mechanistical studies may be needed.

In the event that the toxicological studies listed above are not sufficient for a safety assessment additional studies might be required on a case-by-case basis depending on the knowledge available with respect to the food enzyme's molecular and functional characteristics as well as its fate in food and the gastrointestinal tract and the extent of potential exposure.
For example, studies addressing possible health effects resulting from long-term exposure, including possible effects in the gastrointestinal tract, may be necessary, as may additional testing on the possible allergenicity of the food enzyme (see section 4.2). Decisions on whether additional studies are needed will be taken by EFSA on a case-by-case basis.

4.1.2 When toxicological Testing may not be needed

While administrative and technical data shall be provided for all notified food enzymes, the requirement for toxicological data may in some cases be reduced or completely waived; the justification for not supplying toxicological data may include:

- A documented history on the safety of the source of the food enzyme, the composition and the properties of the food enzymes as well as its use in food, demonstrating no adverse effects on human health when consumed in a comparable way, supported by any existing toxicological studies. In such cases, a detailed rationale must be provided to EFSA for evaluation, *e.g.* edible parts of animals and (non GM) plants.

- Food enzymes produced by micro-organisms that have been given a status of Qualified Presumption of Safety (QPS), if it can be demonstrated that there are no concerns related to any residues, degradation products or substances originating from the total production process (EFSA, 2005).

[13] OJ L 50, 20.2.2004, p. 44

[14] OJ L 50, 20.2.2004, p. 28

- If a food enzyme from a specific strain has been thoroughly tested and the manufacturing process does not differ significantly for other food enzymes from the same strain, the full testing battery may be waived for these food enzymes. This will be decided on a case-by-case basis.

The detailed justification shall be provided in the dossier. However, EFSA may request further clarification.

4.1.3 Data reporting

The data reported for standard toxicological tests should follow the recommendations for data reporting given in the relevant OECD guidelines. For each study performed it should be stated, and supported by analytical data for the specification as defined in section 3.1.2.2, that the test material is representative of the food enzyme as described in the dossier.

4.1.4 Review of the toxicological and exposure data and conclusions

For each toxicological study, the significant findings should be highlighted, together with the no-observed-effect level (NOEL) and/or the no-observed-adverse-effect level (NOAEL) if one has been determined, and any other relevant information. Where effects in animals are seen, the relationship between the dose giving rise to effects and likely dietary exposure from use of the food enzyme should be discussed to establish an appropriate margin of safety. The reasons for disregarding any findings should be carefully explained. Where relevant, the conclusions should include an interpretation of the significance of the findings.

4.2 Allergenicity

At present, validated testing methods to predict the allergenicity of the enzyme protein or its breakdown products after oral intake are not available. However, some information on the potential allergenicity of food enzymes can be obtained by applying the integrated, stepwise case-by-case approach used in the safety evaluation of the newly expressed proteins in genetically modified plants (EFSA, 2006a; FAO/WHO, 2001). The allergenicity of the source of the food enzyme should be considered and a search for amino acid sequence and/or structural similarities between the expressed protein and known allergens should be undertaken where possible. If there is cause for concern from this initial screening, further analysis may be undertaken, *e.g.* as described in Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006a).

If other studies are available, which may have been conducted for other purposes, such as the assessment of safety at the workplace (*e.g.* sensitisation studies), they should be submitted.

5. Conclusion

An overall assessment of the safety data and toxicological tests including rationales for the inclusion or exclusion of specific tests, discussion of their adequacy and any uncertainties, *e.g.* differences in specification between the tested and commercialised product or structural similarities to known allergens should be provided. The overall evaluation of potential human risk should be made in the context of known or anticipated human exposure.

6. Dossier Bibliography

In submitting a dossier, a full bibliography should be included and full copies of all references quoted should be provided. References should be quoted as follows:

i. Published Data
   - **Journals:** Author(s) (full list including all names and initials), date, title of article, journal, volume number, page numbers.
   - **Books:** Author(s), title of chapter/book, editor(s) (if relevant), publisher, location, date, page numbers (if relevant).
   - **Internet:** Organisation, title of report, website and access date

ii. Unpublished Data
   - Name of applicant, title of report, report reference, name of investigator(s) (if any), name of laboratory, address of laboratory, date.

iii. Appended Papers and Study Reports
   - Full copies from the references cited which are essential to the safety evaluation should be included in the dossier.

iv. Copies of all unpublished study reports should be submitted in full. Summaries or abstracts of unpublished studies are not sufficient.

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ABBREVIATIONS

AFSSA	Agence Française de Sécurité Sanitaire des Aliments
CAS	Chemical Abstract Service
COT	UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
DVFA	Danish Veterinary and Food Administration
EC	European Commission and Enzyme Commission
EC/IUBMB	Enzyme Commission of the International Union of Biochemistry and Molecular Biology
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Chemical Substances
ELINCS	European List of Notified Chemical Substances
EU	European Union
FAO	Food and Agricultural Organisation
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
GMM	Genetically Modified Micro-organisms
GMO	Genetically Modified Organisms
GMP	Good Manufacturing Practice
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
NOAEL	No-observed-adverse-effect level
NOEL	No-observed-effect level
OECD	Organisation for Economic Cooperation and Development
QPS	Qualified Presumption of Safety
SCF	Scientific Committee on Food
TSE	Transmissible Spongiform Encephalopathies
TOS	Total Organic Solids
WHO	World Health Organisation

ANNEX I Definitions

Enzyme activity unit (U) - The amount of enzyme which will catalyse the transformation of one micromole of the substrate per minute under standard conditions (IUPAC, 1974). **Enzyme activity unit (kat)** - Katal is the SI unit of activity consisting of the amount of enzyme which will catalyse the transformation of one mole of the substrate per second. Katal was proposed as a replacement for the enzyme activity unit (U) in 1978. One kat = 60 x 10^6 U.

Enzyme specific activity - Enzyme activity units (U) or SI units (kat) per unit weight.

Food enzyme[15] - A product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms, containing one or more enzymes capable of catalyzing a specific biochemical reaction and added to food for a technological purpose at any stage of manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

Food enzyme preparation[16] - A formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Food enzyme preparation - A formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution

Micro-organism - Word used to include prokaryotes, protozoa, microalgae, and all fungi (including moulds, yeasts and filamentous fungi). However, fungal basidiomycete fruiting bodies/ mycelia are considered together with plant sources.

Source materials - Animal, plant, basidiomycete fruiting bodies / mycelia or microbial sources that may be used for the production of the food enzyme.

Total Organic Solids (TOS) To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

$$\% \text{ TOS} = 100 - (A + W + D)$$

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

1) As defined in Regulation (EC) No 1332/2008 on food enzymes

[15] As defined in Regulation (EC) 1332/2008 on food enzymes
[16] As defined in Regulation (EC) 1332/2008 on food enzymes