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Scientific Guidance for the submission of dossiers on Food Enzymes

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (EFSA CEP Panel), Claude Lambré, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn, Boet Glandorf, Lieve Herman, Jaime Aguilera, Magdalena Andryszkiewicz, Ana Gomes, Natalia Kovalkovicova, Yi Liu, Sandra Rainieri and Andrew Chesson

Abstract

Following a request from the European Commission, EFSA developed an updated scientific guidance to assist applicants in the preparation of applications for food enzymes. This guidance describes the scientific data to be included in applications for the authorisation of food enzymes, as well as for the extension of use for existing authorisations, in accordance with Regulation (EC) No 1331/2008 and its implementing rules. Information to be provided in applications relates to source, production and characteristics of the food enzyme, toxicological data, allergenicity and dietary exposure estimation. Source, production and characteristics of the food enzyme are first considered only for enzymes of microbial origin and subsequently for those enzymes derived from plants and for enzymes from animal sources. Finally, the data requested for toxicology, allergenicity and dietary exposure applies to all food enzymes independent of the source. On the basis of the submitted data, EFSA will assess the safety of food enzymes and conclude whether or not they present a risk to human health under the proposed conditions of use.

Keywords: food enzymes, guidance, applications

Requestor: European Commission

Question number: EFSA-Q-2020-00477

Correspondence: fip@efsa.europa.eu

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Panel members: José Manuel Barat Baviera, Claudia Bolognesi, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Vittorio Silano (until 21 December 2020 †), Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis and Holger Zorn.

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Introduction

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the regulatory frameworks of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008[1] on food enzymes entered into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. At the same time, Regulation (EC) No 1331/2008[2] established the European Union (EU) harmonised procedures for the safety assessment and the authorisation of food additives, food enzymes and food flavourings.

Regulation (EC) No 1332/2008 states that the use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market, as well as, all new food enzymes shall be subjected to a safety evaluation by EFSA and approval via an EU Community list.

Article 3 of Regulation (EC) No 1332/2008 provides a definition of a 'food enzyme' and makes a distinction from a 'food enzyme preparation'.

Food enzyme means a product obtained from plants, animals or microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

Food enzyme preparation means 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.

EFSA is mandated to establish the safety of a food enzyme which differs from the approach taken by other international bodies where the focus is on the enzyme preparation (e.g. the World Health Organization (WHO)). The distinction applied in Europe has the advantage that it gives flexibility to the manufacturer to produce different formulations for the same food enzyme without having to seek authorisation for each commercial product. It does, however, carry the disadvantage that a food enzyme normally represents an intermediate stage in the manufacture of an enzyme product and as such is open to a degree of interpretation. A food enzyme is normally considered by EFSA to be the enzyme-rich liquor obtained after removal of insoluble biomass and concentration. Any substance added should normally be limited to those essential to the maintenance of stability during these processes. Data on food enzyme preparations generally will not be taken to substitute for data on food enzymes unless demanded by stability issues or the use of whole cells.

In 2009, EFSA published the first Guidance on the Submission of a Dossier on Food Enzymes (EFSA, 2009). In 2020, the European Commission considered it desirable to update this guidance to take into account new approaches on certain aspects of the risk assessment and requested EFSA to revise the guidance. The full background provided by the European Commission is shown in Annex A.

Terms of Reference as provided by the requestor

In accordance with Article 29 of Regulation (EC) No 178/2002[3], the Commission requests EFSA to update and consolidate the Guidance for the Submission of a Dossier on Food Enzymes under Regulation (EC) No 1331/2008, taking into account the experience gained with the risk assessment of

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7 15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1 6.

[3] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

enzymes and the numerous other relevant scientific and technical documents that have been published by EFSA since the adoption of the current guidance related to food enzymes.

Interpretation of the Terms of Reference

This revised guidance relates only to the data needed to complete the safety assessment of food enzymes.

All administrative information related to the preparation and submission of an application on food enzymes are addressed in a separate EFSA document, i.e. 'Administrative guidance for the preparation of applications on food improvement agents (food enzymes, food additives and food flavourings)' (EFSA, 2021a), which is applicable to applications submitted as of 27 March 2021.

Scope of the guidance

The purpose of this document is to assist applicants in the preparation and presentation of dossiers for the safety evaluation of food enzymes in accordance with Regulation (EC) No 1332/2008, Regulation (EC) No 1331/2008 and its implementing Rules Regulation (EU) No 234/2011[4] and Regulation (EU) No 562/2012[5]. The application is initially made to the European Commission for further transmission to EFSA, which is responsible for the safety assessment and for providing an opinion on the outcome of the evaluation. This document outlines the information necessary to enable EFSA to make its safety assessment of a food enzyme and, where appropriate, the rationale underlying the request for additional data.

No guidance document can be exhaustive. Information requirements may vary depending for example on the food enzyme's function/activity, the properties of the source material, the properties and amounts of any by-products and substances originating from the food enzyme production processes and intended food manufacturing processes, as well as the intended use and the resulting level of human dietary exposure. There may be circumstances where additional data or tests to those indicated in this document are required for the evaluation. Conversely, if some of the data stipulated in the guidance are not considered by the applicant as relevant to a particular case, they may be omitted provided that the omission is fully justified.

A more restricted data set is described for applications concerning a modification of the conditions of use or specification for those food enzymes which have already undergone a full safety assessment and are included in the Community list.

The data requirements found in this guidance amalgamate and supersede the original guidance document developed and published at the request of the European Commission in 2009 (EFSA, 2009) and the Statements issued subsequently modifying or replacing elements of the original guidance. Foremost among these are the 'Panel statement on the exposure assessment of food enzymes' (EFSA CEF Panel, 2016) which replaced the use of the budget method in dietary exposure assessment with one based on the use of the EFSA Comprehensive Food Consumption database (EFSA, 2011) and the 'Statement on the characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019).

The data requested in this document are generally in line with and fulfil the requirements of the European Commission implementing rules (Regulation (EU) No 234/2011 as amended by the Regulation (EU) No 562/2012). However, some requirements imposed by the Implementing Regulations have proved to be of limited value for safety assessment purposes (e.g. documentation on previous applications/authorisations of similar food enzymes) or impractical (e.g. grouping of applications with the same microbial enzyme activity). Such information is not required by EFSA as part of the dataset.

Structure of the guidance

This document consists of two parts: Part A is dedicated to first-time submissions and Part B to submissions seeking modifications to an existing authorisation.

[4] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15 24.

[5] Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes.

The data requirements in Part A are organised to follow the broad structure of the opinions produced by EFSA which essentially consist of five sections and a conclusion.

1) Source of the food enzyme
2) Production of the food enzyme
3) Characteristics of the food enzyme
4) Toxicology
5) Dietary exposure estimation

Completion of Sections 1, 2 and 3 are required for all food enzymes regardless of source, while the requirements for data stipulated under Sections 4 and 5 may be waived in some circumstances (Table 1). In recognition that the majority of enzymes have a microbial origin, Sections 1, 2 and 3 are first considered only for enzymes produced by microorganisms and thereafter for those enzymes derived from plants and for enzymes from animal sources. Finally, the data requested under Sections 4 and 5 are described independent of the source of the food enzyme.

Table 1: Overview of the structure of the guidance

Part A. Data required for the evaluation of a food enzyme		
Microbial enzymes	**Plant enzymes**	**Animal enzymes**
1. Source	1. Source	1. Source
2. Manufacture	2. Manufacture	2. Manufacture
3. Characteristics	3. Characteristics	3. Characteristics
4. Toxicological data		
5. Dietary exposure estimation		
Part B. Data required for the safety assessment of modifications to an existing authorisation		

Applicants are encouraged to follow the structure of this guidance when providing technical data.

Part A. Data required for the evaluation of a food enzyme

A submission should start by indicating the identity of the enzyme activity or activities under application. This should be specified by the applicant and should be focused on the key activities necessary to fulfil the intended technological function of the food enzyme.

This should include for each declared activity:

a) the accepted name recognised by the International Union of Biochemistry and Molecular Biology (IUBMB);
b) the systematic name (when available);
c) any commonly used synonyms excluding trade names;
d) the Enzyme Classification Number of the IUBMB (when available);
e) the Chemical Abstract Service (CAS) Registry Number (when available);
f) the European Inventory of Existing Chemical Substances Number (EINECS) or European List of Notified Chemical Substances Number (ELINCS) (when available);
g) a brief description of the catalytic activity.

1. Food enzymes of microbial origin

1.1. Source of the food enzyme

The microbial sources encompass bacteria, archaea, yeasts, filamentous fungi and other eukaryotic microorganisms (e.g. microalgae) but excluding viruses.

1.1.1. Use of whole genome sequence for characterisation of microorganisms

Whole genome sequence (WGS) analysis (including chromosome(s) and extrachromosomal genetic elements, e.g. plasmids) is required for the identification and characterisation of bacterial and yeast strains intended for use as production strains. WGS analysis is also recommended for filamentous fungi and other eukaryotic microorganisms. WGS data provide information for the characterisation of the strains regarding their potential functional traits of concern (e.g. virulence factors, production of or

resistance to antimicrobials of clinical relevance, production of known toxic metabolites) (EFSA, 2021b). WGS data should be submitted in the formats specified in Annex B.

1.1.2. Microorganism and DNA Extraction

The microorganism(s) tested/under analysis should be the one(s) subject to the application for authorisation. The samples used for DNA extraction, sequencing, WGS data analysis and the results reported should correspond to the production organism, and this should be clearly stated.

Each microorganism should be cultivated before DNA extraction as a pure culture (for fungi, monosporic where possible). An adequate protocol/method for DNA extraction should be applied. Total DNA (i.e. including chromosomal and extra-chromosomal elements) should be extracted and subjected to WGS analysis.

1.1.3. Library construction

Library construction method, including DNA fragmentation method, and selection of fragments, when relevant, should be reported. Any selection of fragments by size should ensure that small plasmids are not lost. The manufacturer's instructions followed, including version number, and any deviations from that method should be provided.

1.1.4. Sequencing strategy and quality control

The report should describe the sequencing strategy, instrumentation used and any base-calling method applied, where applicable.

For short-read sequencing technologies, it is recommended to trim the sequencing reads to avoid assembly or read mapping artefacts, unless the assembler software discourages it. The trimming and adaptor removal criteria applied, including the software, version and parameters, should be reported. A Phred score threshold of at least 20 should be set for the quality trimming, and the number of reads and total base pairs of sequence data before and after trimming should be reported.

The average read depth achieved should be at least 30-fold with a recommended target of 100-fold.

Contamination in the sequencing reads should be investigated. Assigned reads to an unexpected organism should be less than 5%, if this is not the case then the applicants should provide an explanation. The tool used, the software version and any parameters used for detection of contamination should be provided along with the results. The database, its version (where available) and date of accession should be indicated.

The sequencing reads can be *de novo* assembled (and annotated), mapped to a reference genome/database or the two approaches can be used in combination (for genetically modified microorganism (GMM), see Section 1.1.11.3). For bacteria, complete genome sequence should be pursued but draft genome sequence may be accepted.

1.1.5. *De novo* assembly and annotation

If a *de novo* assembly-based approach is taken, then the following data are required:

- *De novo* assembly including assembler software, version and parameters. If post-assembly processing is carried out, approach, software, version and parameters should also be reported.
- Contigs:
  - for draft genomes, the total number of contigs produced by the assembler. For bacteria, total contigs should be < 500 and for yeasts and filamentous fungi < 1,000. If a higher number of contigs is produced, the applicant should provide a justification;
  - the total length of the contigs. Applicants should provide a justification if their assembly size is not within ± 20% of the expected genome size for the species;
  - N50 metric or similar quality parameters.
- For yeasts and filamentous fungi genomes, the number of highly conserved genes, such as BUSCO genes, present in the assembly should be reported since this parameter indicates the completeness and quality of the assembly (https://busco.ezlab.org/). Ideally, > 90% complete matches to BUSCO gene set from the most closely related group of yeasts/filamentous fungi should be present in the assembly.

If a genome annotation is carried out to provide any of the required information, the software name, version and parameters used should be reported. The public database(s), version (where available) and/or date of accession should be indicated.

1.1.6. Reference-based read mapping

There is the possibility to use reference-based read mapping as an alternative to *de novo* assembling approach or in combination with it, for the characterisation of the microorganism. In this case, the sequencing reads need to be mapped against reference genome(s)/database(s). The parameters to be reported are indicated in the next sections.

1.1.7. Identity

The following taxonomic information should be provided for the production organism: genus, species and strain name or code. If different names or codes are used for the production organism in-house or in third party data, a statement should be provided confirming they correspond to the production strain. A clear statement on whether the production organism is genetically modified according to Directive 2001/18/EC[6] should be made.

For bacteria, taxonomic identity is based on the internationally accepted classification, overseen by the International Committee on Systematics of Prokaryotes. The nomenclature of bacteria and the nomenclatural changes as cited in the Approved Lists of Bacterial Names or validly published in the International Journal of Systematic Bacteriology or in the International Journal of Systematic and Evolutionary Microbiology are reported in the web-site List of Prokaryotic Names with Standing in Nomenclature (LPSN).[7] The nomenclature and taxonomy of fungi, including yeasts, is covered by the International Code of Nomenclature for algae, fungi and plants (ICN). Applicants are referred to the website Mycobank.[8]

Bacteria: Taxonomical identification is expected to be made by computational approach using WGS data. The identity of the organism under assessment should preferably be established by digital DNA–DNA hybridisation (dDDH; (Auch et al., 2010a; Auch et al., 2010b), average nucleotide identity (ANI; (Goris et al., 2007)). The sequencing data from the microorganism under assessment should be compared with the reference genome of the type strain of the expected species. In the case the reference genome of the type strain is not available, another well-identified strain can be used. For identification at species level threshold values are: > 70% identity for dDDH, > 95% for ANI (Chun et al., 2018). In the case of values below the defined threshold, phylogenomic methods should be applied to identify the closest related species.

Yeasts: Taxonomical identification is expected to be made by computational approach using WGS data. Confirmation of identity should be done by phylogenomic analysis (e.g. using a concatenation of several conserved sequences (e.g. Assembling the Fungal Tree of Life (AFToL) genes including 18S rDNA/ITS) to produce a phylogeny against available related genomes) or by alignment to a complete reference genome from the same species.

Filamentous fungi: When WGS is available, identification should be made by phylogenomic analysis (e.g. using a concatenation of several conserved sequences (e.g. AFToL genes including 18S rDNA/ITS) to produce a phylogeny against available related genomes) or by alignment to a complete reference genome from the same species. When WGS is not available, identification may be made by comparing the 18S rRNA gene and/or internal transcribed spacer (ITS) regions and other characteristic genes (e.g. tubulin) with sequences deposited in databases.

When the identification is done by WGS analysis using *de novo* assembly-based approach, a summary of the method and sequence/s used for comparison, and results of the comparison including sequence identity (percent of identity with the compared reference/type strain genome) should be indicated.

If read-mapping approach is used for identification, sequencing reads should be mapped against a suitable reference genome(s) (e.g. type strain or well-known and well-identified strain(s)). The choice of the reference genome(s) needs to be well considered, justified and reported. The software used should be reported, including version number and all parameters (if the default parameters are used

[6] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.
[7] https://lpsn.dsmz.de/
[8] https://www.mycobank.org/

this should be stated). The proportion of reads mapped, the proportion of reference genome covered to at least 5× depth and the median depth of mapping across entire genome should be reported.

In the case that data do not allow the assignment of the production strain under assessment to a known microbial species, its phylogenetic position with respect to the closest relatives should be provided.

The origin of the organism and history and purpose of modifications, including mutagenesis steps performed during the development of the production strain, should be reported in all cases. In case of a GMM, all genetic modifications should be characterised according to Section 1.1.11.

The production strain under assessment should be deposited in an internationally recognised culture collection having acquired the status of International Depositary Authority under the Budapest Treaty (preferably in the EU) and maintained by the culture collection for the authorised period of the food enzyme. A valid certificate of deposition from the collection, which shall specify the accession number under which the strain is held, should be provided.

1.1.8. Identification of genes of potential concern

The WGS should be interrogated for the presence of genes of potential concern, which include those coding for or contributing to resistance to antimicrobials relevant to their use in humans and animals, virulence and toxigenicity.

A *de novo* assembled sequence can be analysed with a search/comparison-based approach against updated databases and the identified hits should be provided in a table. For each reported result, the subject sequence (i.e., the sequence in the database) name and accession number, function of the encoded protein, sequence identity and the percentage length of the subject sequence which is covered should be provided.

If a reference-based read mapping approach is used, the sequencing reads are compared to a reference database(s). The following statistics should be reported along with the subject sequence name, accession number and function of the encoded protein: sequence identity, the average depth of mapping and the percentage length of the subject sequence which is covered by reads. A minimum 5× median depth across the entire sequences should be used as a threshold.

The strategy, software and all relevant parameters (including algorithm if specified within the software) used to identify genes of interest should be reported. The database its version (where available) and the date when the database was accessed should be provided.

1.1.9. Antimicrobial resistance

This section is applicable to all bacteria used as production organisms.

The use of food enzymes should not add to the pool of antimicrobial resistance (AMR) genes already present in human gut bacterial population or otherwise increase the spread of AMR. When a strain of a typically susceptible species is resistant to a given antimicrobial drug, it is considered to have an 'acquired resistance' for that compound. In contrast, intrinsic resistance to an antimicrobial is understood as inherent to a bacterial species and is typical of all the strains of that species. Intrinsic antimicrobial resistance is generally not considered a safety concern.

WGS should be interrogated for the presence of genes coding for or contributing to resistance to antimicrobials relevant to their use in humans and animals (critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs), as defined by WHO, 2019). It is recommended to conduct the search against at least two updated databases. The search should be done applying the minimum available threshold in the database for the length of coverage. In addition, in case of microorganisms for which no or few AMR genes are present in databases, searches with hidden Markov model tools are recommended.

In general, query sequence hits with at least 80% identity (at the protein level or nucleotide level as reported in the database) and 70% length of the subject sequence should be reported. In the case two or more fragments covering less than 70% length of the subject sequence with at least 80% identity to the same AMR gene are detected, these should be reported, and it should be checked whether the full gene is present. Hits with at least 80% identity (at the protein level or nucleotide level as reported in the database) and 70% length of an acquired AMR gene sequence are considered a hazard. The risk associated to this hazard is considered absent if an applicant is able to demonstrate that DNA and viable cells of the production strain cannot be detected in the food enzyme as defined in Section 1.3.4.

1.1.10. Toxigenicity and pathogenicity

1.1.10.1. Qualified Presumption of Safety

A specific approach to safety assessment applies to those species of microorganisms included in the list of Qualified Presumption of Safety (QPS) status recommended biological agents.[9] QPS is a generic approach to the safety assessment of microorganisms intentionally introduced into the food and feed chain and also used as a source of fermentation products. To justify that a microorganism is suitable for evaluation according to the QPS approach, its taxonomic status should be unequivocally established, the species to which it belongs should be included in the QPS list and any qualification set in the list should be met.

In the case of food enzymes produced by GMMs for which the parental/recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment, and for which the molecular characterisation of the event does not give rise to concern, the QPS concept can be extended to the genetically modified (GM) production strain. Notwithstanding this, the absence of DNA from the production strain must be demonstrated in all products made with GMMs (as defined in Section 1.3.4.2).

For production strains meeting the criteria for a QPS approach to safety assessment, toxicological studies on the food enzyme will only be required in relation to possible safety concerns identified elsewhere in the assessment process, e.g. manufacturing. In the specific case of *Bacillus* and related species included in the QPS list, a cytotoxicity test of culture supernatant on Vero cells or other epithelial cell lines should be made with the production strain to determine whether it produces high levels of non-ribosomal synthesised peptides. Detection based on, for example, release of lactate dehydrogenase or uptake of propidium iodide (PI) may be used.

1.1.10.2. Organisms not included in the QPS list

Information relating to toxigenicity and virulence for humans should be provided for non-QPS production strains, including history of use of the strain or any close relative. This should be based on up-to-date literature searches.

Any strain development step (including mutagenesis and/or genetic modifications) aimed to reduce the toxigenicity and/or pathogenicity of the strain should be clearly documented.

Bacteria. WGS analysis should be compared against updated databases to identify genes coding for known virulence factors (e.g. toxins, invasion and adhesion factors) or to identify the presence/absence of genes known to be involved in toxigenicity. The search should be done by applying the minimum available threshold in the database for the length of coverage. In general, query sequence hits with at least 80% identity (at the protein level or nucleotide level as provided in the database) and 70% length of the subject sequence should be reported. In the case two or more fragments covering less than 70% length of the subject sequence with at least 80% identity to the same gene are detected, these should be reported, and it should be checked whether the full gene is present.

The presence of genes encoding virulence factors may trigger phenotypic testing (e.g. cytotoxicity tests).

Eukaryotic microorganisms. The potential pathogenicity or ability to produce metabolites that could be harmful to humans should be assessed for eukaryotic microorganisms. When WGS is available, targeted searches should be performed to identify the presence/absence of genes known to be involved in toxigenicity. In general, query sequence hits with at least 80% identity (at the protein level or nucleotide level as provided in the database) and 70% length of the subject sequence should be reported. Alternatively, a literature search should be carried out to identify the capacity of the species or of closely related species to produce known toxic compounds. Further information on known toxic secondary metabolites potentially produced by several microbial species can be found in scientific publications such as AINIA (2017) or Frisvad et al. (2018).

The presence of genes encoding toxic metabolites may trigger phenotypic testing.

1.1.11. Genetic modifications

If the strain is GM according to the definition in Directive 2001/18/EC the genetic modification should be described.

[9] https://zenodo.org/record/1146566

1.1.11.1. Purpose of the genetic modification

The purpose of the genetic modification should be described. A description of the traits and changes in the phenotype and metabolism of the microorganism resulting from the genetic modification is required.

1.1.11.2. Characteristics of the modified sequences

Inserted sequences: The sequences inserted in the GMM can be derived from defined organisms or may be designed. When the inserted DNA is a combination of sequences from different origins, the pertinent information for each of the sequences should be provided.

For DNA from defined donor organisms:

The taxonomic affiliation (genus and species) of the donor organism(s) should be provided. In case of sequences obtained from environmental samples, the closest orthologous gene(s) should be indicated. The description of the inserted sequence(s) should include:

a) the nucleotide sequence of all inserted elements including a functional annotation and the physical map of all the functional elements;
b) tabulated information on the size, origin and function of the inserted elements, including coding and non-coding regions;
c) name, derived amino acid sequence(s) and function(s) of the encoded protein(s).

For designed sequences:

Designed sequences are those not known to occur in nature (e.g. codon-optimised genes, rationally designed chimeric/synthetic genes, mutated alleles or genes harbouring chimeric sequences). In such cases, information should be provided on:

a) the rationale and strategy for the design;
b) DNA sequence and a physical map of the functional elements;
c) derived amino acid sequence(s) and function(s) of the encoded protein(s);
d) similarity with sequences in updated databases (e.g. ENA, NCBI, UniProt). This should identify the functional domains of the recombinant protein; the best hits should be reported and described.

Deletions: A description of the intentionally deleted sequence(s) should be provided, together with an explanation of the intended effect.

Base pair substitutions and frameshift mutations: Introduced base pair substitutions and/or frameshift mutations should be indicated, together with an explanation of their intended effect.

Structure of the genetic modification:

The characterisation of the structure of the genetic modification should be done using WGS data for bacteria and yeasts and is recommended for filamentous fungi.

1.1.11.3. Structure of the genetic modification using WGS data

The characterisation of the genetic modification can be done by comparing the WGS of the GMM with that of the non-genetically modified reference genome (preferably the parental strain). *De novo* assembly or read-mapping strategies can be used. For deletions and small modifications (e.g. regulatory elements) reference-based read mapping approach can be used, for other genetic modifications a *de novo* assembly approach or a combination of the two strategies may be needed.

Based on the alignment between the GMM and the reference genome, the actual genetic modification should be characterised. These alignments should be provided. A map or graphic presentation should be provided of all genomic regions (chromosome, contig or plasmid) harbouring genetic modifications, indicating:

a) the open reading frames (ORFs) actually inserted, modified or deleted. For each ORF, the gene products should be described in detail (at least amino acid sequence, function, metabolic role). Introduced genes of concern should be highlighted. Genes of concern are those known to contribute to the production of toxic metabolites and antimicrobials of clinical relevance, or to AMR;
b) the non-coding sequence(s) inserted/deleted/modified. The role and function of these sequences (e.g. promoters, terminators) should be indicated.

The sequences/databases and the methodology used for analyses and comparison should be described in detail.

1.1.11.4. Structure of the genetic modification without WGS data

For filamentous fungi for which WGS is not available, all the steps to obtain the genetic modification should be described. The information provided should allow for the identification of all genetic material potentially introduced into the recipient/parental microorganism.

Characteristics of the vector

The description of the vector(s) used for the development of the GMM should include:

a) the source and type of the vector (plasmid, phage, virus, transposon). When helper plasmids are used, they should also be described;
b) a map detailing the position of all functional elements and other vector components;
c) the map should accompany a table identifying each component, properly annotated, such as coding and non-coding sequences, origin(s) of replication and transfer, regulatory elements, AMR genes, their size, origin and role.

Information relating to the genetic modification process

The genetic modification process should be described in detail. This should include:

a) the methods used to introduce, delete, replace or modify the DNA into the recipient/parental microorganism and methods for selection of the GMM;
b) whether the introduced DNA is a replicative vector or is inserted into the chromosome(s) and/or, for eukaryotic microorganisms, into DNA of organelles (e.g. mitochondria).

Structure of any vector and/or donor nucleic acid remaining in the GMM

This should include:

a) a map detailing the position of the sequences actually inserted, replaced or modified;
b) in case of deletion(s), the size and function of the deleted region(s) should be provided.

Genes of concern

Any gene of concern as defined in Section 1.1.8 (such as genes encoding AMR, toxins and virulence factors) inserted in the GMM should be clearly indicated.

The absence of any sequence of concern (such as AMR genes) not intended to be present in the GMM should be confirmed experimentally. This includes:

a) the sequences used transiently during the genetic modification process including vectors and helper plasmids;
b) the sequences in plasmids/replicons from which a fragment was derived and used for transformation.

This should be analysed by using appropriate methods, such as Southern blot analysis or polymerase chain reaction (PCR).

Southern blots shall include appropriate positive and negative controls. The length and location of the probe(s) used should be indicated. The amount of DNA from the production strain loaded in the agarose gel should be provided, together with an image of the gel before blotting. Positive control shall be loaded in a concentration corresponding to approximately 10 copies of the target fragment. If several probes are used, they should be tested in separate experiments.

PCR experiments should include a positive control containing the same gene as that used during strain development, together with proper positive controls to exclude PCR inhibition and to ensure sufficient sensitivity. A negative control should also be included.

1.2. Production of the food enzyme

Full details of the production process for the food enzyme should be provided, including a flow chart showing each step in the production process. Where manufacture of the food enzyme occurs at more than one manufacturing site, differences in the production process, depending on the production site should be described in detail.

The chemical identity, the CAS or any other unique identification number (if available) and the function of agents used during the production process should be provided. Analytical data may be required if safety concerns exist and if there is a potential for carry-over into the food enzyme. Reference should be made to any available risk assessment data for the individual compounds.

A statement should be provided confirming that the production of the food enzyme meets or will meet food safety management system principles (Commission Notice C/2016/4608[10]) and accords with the Food Hygiene Regulation (Regulation (EC) No 852/2004[11]). For food enzymes manufactured outside the EU and subject to local regulations, these should be specified and their similarity to the equivalent EU requirements confirmed.

1.2.1. Fermentation

Information on the fermentation stage of the production of the food enzyme should specify the type of the fermentation system used (e.g. continuous, (fed-) batch or solid state). A list of the raw materials contributing to the medium and reagents used for process control is required. These should be the actual materials used; an indicative list will not be accepted. For the raw materials which typically provide the nitrogen and carbon sources, which are included to meet mineral and vitamin requirements or used in pH control, only qualitative data is needed. Quantitative data may be required for medium ingredients of potential concern.

1.2.2. Downstream processing

The specific methods used to kill, disrupt and remove microbial biomass after completion of fermentation, to concentrate the enzyme liquor and to remove microorganisms from the food enzyme should be described, when applicable. For all substances used during downstream processing, the chemical identity, the CAS or any other unique identification number (if available) and the function should be provided. These should be the actual materials used; an indicative list will not be accepted.

1.2.3. Food enzyme preparations

It is assumed that all food enzymes will be variously formulated to enable marketing as a solid or liquid preparation, to increase shelf-life and as a mean of standardisation. Information on the method and material used to produce a preparation is generally not required. The only exceptions would be:

a) when the concentrated enzyme liquor which would normally constitute the food enzyme is inherently unstable and only data on a stabilised preparation can be generated;
b) when there is concern that the method used to produce the preparation may result in a potential carry-over of hazardous material into a food.

In the case of a) quantitative data on all added excipients is required to allow the calculation of the total organic solids (TOS) arising from the fermentation. Situation b) is most likely to arise as a consequence of immobilisation or encapsulation of the food enzyme. In these cases, additional information is needed on the method of immobilisation/encapsulation, the support material and any chemical used in cross-linking. Where cross-linking agents are used, data will be required either showing the absence (below the limit of detection (LoD)) of cross-linking agents or quantifying their presence in the food to which the food enzyme preparation is applied.

1.3. Characterisation of the food enzyme

1.3.1. Properties of the food enzyme

Amino acid sequence data should be provided for each declared enzyme activity. The sequence should be that of the actual enzyme(s) under assessment; reference to published sequences of enzymes with the same catalytic activity is not acceptable. The amino acid sequence should be used to calculate the molecular mass of the enzyme, indicating whether the mass refers to the mature protein

[10] Commission Notice on the implementation of food safety management systems covering prerequisite programs (PRPs) and procedures based on the HACCP principles, including the facilitation/flexibility of the implementation in certain food businesses. C/2016/4608.
[11] Regulation (EC) No 852/2004 the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs.

or includes any signal sequence. Available information on the subunit structure as well as the degree of glycosylation should also be provided, when relevant.

If the food enzyme is modified by chemical treatment, the nature of the change and the rationale for the modification (e.g. modifying pH or thermal stability) should be provided.

The protein pattern characteristic of at least three batches of the food enzyme and, additionally, any batch prepared for use in toxicological studies should be provided. This may be done by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or other analytical techniques such as size exclusion chromatography or mass spectrometry. Appropriate molecular mass standards should be incorporated. The purpose of such analysis is to provide evidence of the consistency of production and to confirm that any food enzyme batch used in other studies is representative.

The Standard Operating Procedure (SOP) for the in-house method(s) used to measure and report the activity of the enzyme(s) under application should be described. Activity should be given in enzyme activity units (U) per unit weight. If an abbreviation is used to describe the unit, it should be given in full at first mention. Only one activity unit definition should be used throughout the dossier.

The temperature and pH range over which the food enzyme remains active, together with the optimum values for pH and temperature should be determined. This should preferably be done using one or more of the in-house methods for which a SOP is provided. Measurements of thermal stability in which the food enzyme is exposed to various temperatures for a fixed period before assay should also be provided. The chosen temperature range should reflect the technological role of the food enzyme, as the data will be used to judge the likelihood of the survival of activity. There is no requirement to provide data on long-term stability of the food enzyme as the shelf-life is out of the assessment's scope.

1.3.2. Chemical parameters

Chemical characteristics should be provided for at least three batches of the food enzyme representative of those intended for commercialisation. The selected batches should be those examined for their protein pattern and for purity (Section 1.3.3). These batches should preferably be taken from a full-scale production run. Enzymes from pilot plants are acceptable for those food enzymes in a pre-production stage of development, provided that the downstream processing is equivalent to production scale processes.

The parameters measured should be the enzyme activity or activities under application expressed as Units/g batch, and the concentration (in % w/w) of total protein, ash and water. From these data, the percentage of TOS should be calculated (as 100% – % water – % ash) and the enzyme activity/mg TOS determined. It is recognised that variation between batches is to be expected in a food enzyme which represents an intermediate in the production process before the introduction of excipients which allow a greater degree of standardisation. The data will be used to judge the extent of variation encountered and, in particular, to judge in conjunction with information on protein patterns, whether the food enzyme batches used for toxicological or other studies can be considered representative. For this reason, it is essential that the same data set is provided for all additional batches of the food enzyme used for the toxicological or other studies.

If the use of a food enzyme preparation is unavoidable, the TOS content equivalent to that of a food enzyme may be calculated as 100% – % water – % ash – % total added organic excipients as defined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2006).

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. When international standardised methods are used (e.g. ISO) a reference to this method will suffice. The certificates of analysis should include identification of the test item to ensure that the data derive from the food enzyme under application.

1.3.3. Purity

The need for data on chemical purity is determined by the nature of the fermentation process. Quantitative data should be provided on the concentration of medium ingredients added for purposes other than nutrition or pH control (e.g. expression inducing agents or antibiotics) which may be carried over into the food enzyme. The concentration of lead in the food enzyme should be determined according to JECFA Guidelines (FAO/WHO, 2006).

Where the possible presence of compounds of known toxicity (e.g. mycotoxins) arising from the fermentation is indicated by literature searches or WGS analysis of the production strain, the applicant

should determine the concentration of these compounds in the food enzyme. This applies generally, but not exclusively, to filamentous fungi used as the source of the food enzyme.

Microbiological purity should be established. *Escherichia coli* should not be detected in 25 g of food enzyme (FAO/WHO, 2006), measured according to ISO method 16649-3:2015 or a validated alternative method. Counts of Enterobacteriaceae or total coliforms should not exceed 30 CFU/g measured according to ISO 21528-2:2017 or ISO 4832:2006, respectively.

Antibacterial activity of the food enzyme should be determined according to JECFA recommendations, using the six indicator strains and the disc inhibition method prescribed (FAO/WHO, 2006, Volume 4).

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. When international standardised methods are used (e.g. ISO) by an accredited laboratory a reference to this method will suffice. The values of the LoD and the limit of quantification (LoQ) for each method should be given. The same batches as used for chemical characterisation should be analysed. The certificates of analysis should include sufficient identification of the test item to ascertain that it is in fact the food enzyme under application.

1.3.4. Viable cells and DNA of the production strain

1.3.4.1. Viable cells of the production strain

This section applies to all food enzymes except for those obtained using non-GM QPS production strains.

The absence of viable cells of the production strain should be investigated using a well-described method for the detection:

a) by means of a culture-based method targeted to the detection of the viable cell. Cultivation-independent methods are not acceptable;
b) the procedure should enable the recovery of stressed cells by cultivation in or onto media with a minimal selective pressure and/or by providing a longer (at least two times) incubation time compared to the normal culturing time;
c) the detection should also consider specificity against contaminating microbiota possibly occurring in the sample in case it interferes with the detection of the production strain;
d) if the strain is able to form spores, their possible presence should be analysed by using germination procedures (e.g. thermal treatment for bacteria) adapted to the organisms, and subsequent culturing;
e) viable cells should not be detected in a volume corresponding to at least 1 g or mL of food enzyme, obtained from a sample of at least 10 g or mL of product (e.g. from 10 g of product diluted in 90 mL, 10 mL should be analysed);
f) at least nine samples obtained from a minimum of three independent batches of food enzyme should be analysed. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial-scale process. Samples from pilot-scale process are acceptable if it can be justified that those from industrial process are not available. In this case, it should be documented that the pilot-scale process (fermentation and downstream) is representative of the industrial-scale process;
g) a positive control with samples of the food enzyme spiked with low counts (e.g. 10–300 cells/spores per plate) of viable cells of the production strain should be included to prove that the medium and cultivation conditions enable growth of any possible viable cells remaining in the product.

1.3.4.2. DNA from the production strain

This section applies to:

a) food enzymes obtained using GM production strains;
b) food enzymes obtained using non-GM production strains carrying acquired AMR genes.

The presence of DNA from the production strain should be tested in the food enzyme by PCR, targeting a fragment specific for this strain. A detailed protocol should be provided including cell lysis and DNA extraction methodologies, sample volumes, the specific target sequence, primers, polymerase used and amplification conditions:

a) in case the production strain contains AMR genes, whether GMM or not, primers should be designed to amplify a fragment not exceeding the size of the smallest antimicrobial resistance

gene and in any case not exceeding 1 kb. If the production strain is a GMM not containing AMR genes, the targeted sequence should cover maximum 1 kb;

b) DNA from at least 1 g or 1 mL of product shall be extracted. Upstream intermediate products can be used as long as they are equally or more concentrated than the final product. For different production schemes, each of the product should be tested;
c) at least three independent batches of food enzyme should be sampled, each extracted in triplicate and analysed. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial-scale process. Samples from pilot-scale process are acceptable if it can be demonstrated that those from industrial process are not available. In this case it should be documented that the pilot-scale process (fermentation and downstream) is representative of the industrial-scale process;
d) to recover DNA from non-viable cells potentially remaining in the product, the DNA should be extracted using a methodology suitable for all cellular forms of the production strain (e.g. vegetative cells, spores).

The following controls and sensitivity tests should be included in each of the three batches tested:

a) total DNA from the production strain, as a positive control for the PCR;
b) total DNA from the production strain, added to the product sample before the DNA extraction process, starting with a known quantity and in different dilutions until DNA extinction, to calculate the LoD;
c) a positive control with total DNA from the production strain, added to the DNA extracted, to check for any factors causing PCR failure;
d) a negative control without sample.

For the purpose of this assessment, the applicant should investigate whether the target DNA is detected in analyses having a LoD of 10 ng of DNA per g or mL of product or lower.

2. Food enzymes of plant origin

2.1. Source of the food enzyme

All plant sources of the food enzyme should be identified by genus and species using currently accepted nomenclature (see e.g. The Plant List).[12] Varietal names are not required unless a specific variety or cultivar is used. It should be specified which part of the plant (e.g. leaves, flowers, seeds, fruits, tubers, roots) is used to extract the enzyme, and any treatment or processing applied (e.g. peeling, seed removal), or whether the plant material used is a by-product of other processes. Any other characteristics (e.g. the degree of maturity) which determine the selection of source material should be given. Details of cultivation are not required.

The ability of the plant source to produce secondary metabolites that could be harmful to humans should be assessed, particularly for non-edible plant tissue. A literature search should be made to identify the capacity of the species or a closely related species to produce known toxic compounds.

The specifications set by the applicant to control the quality of the raw material should be described. This should include specific reference to agents applied to the raw material (e.g. pesticides[13]).

It should be indicated whether the source is from a GM plant.

2.2. Production of the food enzyme

Full details of the production process for the food enzyme should be provided, including a flow chart showing each step in the production process. Where manufacture of the food enzyme occurs at more than one manufacturing site, the production process for the main production site should be described in detail and any differences in the process occurring at other sites should be specified.

The chemical identity, the CAS or any other unique identification number (if available) and the function of agents used during the production process should be provided. Analytical data may be required if safety concerns exist and if there is a potential for carry-over into the food enzyme. Reference should be made to any available risk assessment data for the individual compounds.

[12] http://www.theplantlist.org/
[13] Regulation (EC) No 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/4141/EEC.

A statement should be provided confirming that the production of the food enzyme meets food safety management system principles (Commission Notice C/2016/4608) and accords with the Food Hygiene Regulation (Regulation (EC) No 852/2004). For food enzymes manufactured outside the EU and subject to local regulations, these should be specified and their similarity to the equivalent EU requirements confirmed.

2.2.1. Extraction

Information on the extraction stage of the food enzyme should specify the plant parts extracted. Details of any physical or enzymatic comminution and the extraction method should be provided. Processing conditions should be provided together with a full list of the actual raw materials used in this stage of manufacture. An indicative list will not be accepted. The chemical identity, the CAS or any other unique identification number (when available) and the function of each raw material used at this stage should be provided.

2.2.2. Downstream processing

The specific methods used to remove the plant biomass after extraction, to concentrate the enzyme liquor and to remove or inactivate microbial contaminants from the food enzyme should be fully described. All of the actual materials used during concentration/purification should be specified. The chemical identity, the CAS or any other unique identification number (when available) and the function of each material used at this stage should be provided.

2.2.3. Food enzyme preparation

Data on a food enzyme preparation is acceptable only when (a) the concentrated enzyme liquor which would normally constitute the food enzyme is inherently unstable or (b) when there is concern that the method used to produce a preparation may result in a potential carry-over of hazardous material into a food.

In the case of (a) quantitative data on all added excipients is required to allow the calculation of the TOS arising from the extraction. Situation (b) is most likely to arise as a consequence of immobilisation or encapsulation of the food enzyme. In these cases, additional information is needed on the method of immobilisation/encapsulation, the support material and any chemicals used in cross-linking. Data will be required either showing the absence of cross-linking agents or any other material resulting from the immobilisation process or quantifying their presence in the food to which the food enzyme preparation is applied.

2.3. Characterisation of the food enzyme

2.3.1. Properties of the food enzyme

The amino acid sequence should be provided for the enzyme activity(ies) under application. The sequence should be for the actual enzyme produced by the source plant but could be obtained by reference to any published sequence of an enzyme with the same catalytic properties and from the same species. These data will be used to assess the allergenic potential of the food enzyme. These data should be used to calculate the molecular mass of each declared activity, indicating whether the mass refers to the mature protein or includes any signal sequence. Available information on the subunit structure and the degree of glycosylation should also be provided, when relevant.

The protein pattern characteristic of at least three batches of the food enzyme and, additionally, any batches prepared for use in toxicological studies should be provided. This may be done by SDS–PAGE or other electrophoretic techniques or size exclusion chromatography. Appropriate molecular mass standards should be incorporated. The purpose of such studies is to provide evidence of the consistency of production and to confirm that food enzyme batches used in other studies are representative.

The SOP for the in-house method(s) used to measure and report the activity of the enzyme(s) under application should be described. Activity should be given in enzyme activity units (U) per unit weight. If an abbreviation is used to describe the unit, it should be given in full at first mention. Only one activity unit definition should be used throughout the dossier.

The temperature and pH range over which the food enzyme remains active, together with the optimum values for pH and temperature should be determined. This should preferably be done using

one or more of the in-house methods for which an SOP is provided. Data on the measurements of thermal stability, in which the food enzyme is exposed to various temperatures for a fixed period before assay, should also be provided. The chosen temperature range should enable a judgement to be made on the likelihood of the survival of activity under the intended conditions of use. There is no need to provide data on long-term stability of the food enzyme as the shelf-life is out of the assessment's scope.

2.3.2. Chemical parameters

Chemical characteristics should be provided for at least three batches of the food enzyme representative of those intended for commercialisation. The selected batches should be those examined for their protein pattern and for purity (Section 2.3.3). These batches should preferably be taken from a full-scale production run. Enzymes from pilot-scale production are acceptable for those food enzymes in a pre-production stage of development, provided that the downstream processing is equivalent to production scale processes.

The parameters measured should be the enzyme activity or activities under application expressed as Units/g batch, and the concentration (in % w/w) of total protein, ash and water. From these data, the percentage of TOS should be calculated (as 100% – % water – % ash) and the enzyme activity/mg TOS determined. It is recognised that variation between batches is to be expected in a food enzyme which represents an intermediate in the production process before the introduction of excipients which allow a greater degree of standardisation. The data will be used to judge the extent of variation encountered and, in particular, to judge in conjunction with information on protein patterns, whether the food enzyme batches used for toxicological or other studies can be considered representative. For this reason, it is essential that the same data set is provided for all additional batches of the food enzyme used for the toxicological or other studies.

If the use of a food enzyme preparation is unavoidable, the TOS content equivalent to that of a food enzyme may be calculated as 100% – % water – % ash – % total added organic excipients as defined by JECFA (FAO/WHO, 2006).

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. The certificates of analysis should include identification of the test item to ensure that the data derive from the food enzyme under application.

2.3.3. Purity

Quantitative values for lead, cadmium, mercury and arsenic should be reported as a routine (threshold values 5, 0.5, 0.5 and 3 mg/kg food enzyme, respectively), and other elements if they occur in concentrations which may give rise to concern. Batches of the food enzyme should also be screened for pesticide residues unless cultivation is under the direct control of the applicant and it can be guaranteed that no pesticides were used. A screening for relevant mycotoxins should also be provided.

Where the possible presence of compounds of known toxicity in the plant source is indicated by literature searches, the applicant should demonstrate by analysis that their presence in the food enzyme occurs at concentrations which do not give rise to concern.

Microbiological purity should be established. *Escherichia coli* should not be detected in 25 g of food enzyme (FAO/WHO 2006), measured according to ISO method 16649-3:2015 or a validated alternative method. Counts of Enterobacteriaceae or total coliforms should not exceed 30 CFU/g measured according to ISO 21528-2:2017 or ISO 4832:2006, respectively. Filamentous fungi and yeast should not exceed 100 CFU/g in the food enzyme measured according to ISO 21527-1:2008.

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. The LoD and LoQ for each method should be given. The same batches used for chemical characterisation should be analysed. The certificates of analysis should include sufficient identification of the test item to ascertain that it is in fact the food enzyme under application.

3. Food enzymes of animal origin

3.1. Source of the food enzyme

All animal sources of the food enzyme should be identified by genus and species (and subspecies if relevant) using currently accepted nomenclature. Animal breed names are not required unless a specific breed is used.

Information should be provided on which animal tissue and/or product is used for the production of the food enzyme, and how the tissue is sourced. Animal sources should be suitable for human consumption according to Regulation (EU) No 2015/1162[14]. The history of human consumption of the tissue(s) and products in question should be provided, in particular among European populations.

Any specification set by the applicant to ensure consistency and quality of the source material should be described. Information should be provided to attest that the animal tissues and products used for the preparation of the food enzymes comply with inspection requirements and are handled in accordance with good hygiene practice. The methods used to ensure the absence of any risk of infectivity from viruses or other zoonotic agents should also be provided. It should be indicated whether the source is from a GM animal.

3.2. Production of the food enzyme

Full details of the production process for the food enzyme should be provided, including a flow chart showing each step in the production process. Where manufacture of the food enzyme occurs at more than one manufacturing site, the production process for one site should be described in detail and any differences in the process occurring at other sites should be described.

The chemical identity, the CAS or any other unique identification number (if available) and the function of agents used during the production process should be provided. Analytical data may be required if safety concerns exist and if there is a potential for carry-over into the food enzyme. Reference should be made to any available risk assessment data for the individual compounds.

A statement should be provided confirming that the production of the food enzyme meets food safety management system principles (Commission Notice C/2016/4608) and accords with the Food Hygiene Regulation (Regulation (EC) No 852/2004). For food enzymes manufactured outside the EU and subject to local regulations, these should be specified and their similarity to the equivalent EU requirements confirmed.

3.2.1. Extraction

Information on the extraction stage of the production of the food enzyme should specify the tissue (s) and products extracted. Details of any physical comminution/maceration and the extraction method used should be provided. Processing conditions should be provided together with a full list of the actual raw materials used in this stage of manufacture. An indicative list will not be accepted.

The chemical identity, the CAS or any other unique identification number (when available) and the function of each raw material used at this stage should be provided.

3.2.2. Downstream processing

The specific methods used to remove the animal tissue and products after extraction, to concentrate the enzyme liquor and to remove or inactivate microbial contaminants from the food enzyme should be fully described. All processing aids used during concentration/purification should be specified. These should be the actual materials used.

3.2.3. Food enzyme preparation

Data on a food enzyme preparation is acceptable only when (a) the concentrated enzyme liquor which would normally constitute the food enzyme is inherently unstable or (b) when there is concern that the method used to produce a preparation may result in a potential carry-over of hazardous material into a food.

[14] Commission Regulation (EU) No 2015/1162 of 15 July 2015 amending Annex V to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies.

In the case of (a) quantitative data on all added excipients is required to allow the calculation of the TOS arising from the extraction. Situation (b) is most likely to arise as a consequence of immobilisation or encapsulation of the food enzyme; in that situation additional information is needed on the method of immobilisation/encapsulation, the support material and any chemicals used in cross-linking. Where cross-linking agents are used, data will be required either showing the absence of cross-linking agents or quantifying their presence in the food to which the food enzyme preparation is applied.

3.3. Characterisation of the food enzyme

3.3.1. Properties of the food enzyme

The amino acid sequence should be provided for the enzyme activity under application. The sequence should be for the actual enzyme produced by the animal species but could be obtained by reference to any published sequence of an enzyme with the same catalytic properties and from the same species. These data will be used to assess the allergenic potential of the food enzyme. These data should be used to calculate the molecular mass of each declared activity, indicating whether the mass refers to the mature protein or includes any signal sequence.

The SOP for the in-house method(s) used to measure and report the activity of the enzymes under application should be described. Activity should be given in enzyme activity units (U) per unit weight. If an abbreviation is used to describe the unit, it should be given in full at first mention. Only one activity unit definition should be used throughout the dossier.

The temperature and pH range over which the food enzyme remains active, together with the optimum values for pH and temperature should be determined. This should preferably be done using one or more of the in-house methods for which an SOP is provided. Data on the measurements of thermal stability, in which the food enzyme is exposed to various temperatures for a fixed period before assay, should also be provided. The chosen temperature range should enable a judgement to be made on the likelihood of the survival of activity under the intended conditions of use. There is no need to provide data on long-term stability of the food enzyme as the shelf-life is out of the assessment's scope.

3.3.2. Chemical parameters

Chemical characteristics should be provided for at least three batches of the food enzyme representative of those intended for commercialisation. The selected batches should be those examined for their protein pattern and for purity (Section 3.3.3). These batches should preferably be taken from a full-scale production run. Enzymes from pilot scale production are acceptable for those food enzymes in a pre-production stage of development, provided that the downstream processing is equivalent to production scale processes.

The parameters measured should be the enzyme activity or activities under application expressed as Units/g batch, and the concentration (in % w/w) of total protein, ash and water. From these data, the percentage of TOS should be calculated (as 100% – % water – % ash) and the enzyme activity/unit TOS determined. It is recognised that variation between batches is to be expected in a food enzyme which represents an intermediate in the production process before the introduction of excipients which allow a greater degree of standardisation.

The data will be used to judge the extent of variation encountered and whether the food enzyme batches used for toxicological or other studies can be considered representative. For this reason, it is essential that the same data set is provided for all additional batches of the food enzyme used for the toxicological or other studies.

If the use of a food enzyme preparation is unavoidable, the TOS content equivalent to that of a food enzyme may be calculated as 100% – % water – % ash – % total added organic excipients as defined by JECFA (FAO/WHO, 2006).

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. The certificates of analysis should include identification of the test item to ensure that the data are from the food enzyme under application.

3.3.3. Purity

Data on chemical contamination is not required provided the food enzyme derives from animals considered fit for human consumption according to European standard.

Microbiological purity should be established in three batches of the food enzyme. *Escherichia coli* should not be detected in 25 g of food enzyme (FAO/WHO, 2006), measured according to ISO method 16649-3:2014 or a validated alternative method. Counts of Enterobacteriaceae or total coliforms should not exceed 30 CFU/g measured according to ISO 21528-2:2017 or ISO 4832:2006, respectively. *Campylobacter jejuni* and *Campylobacter coli; E. coli* STEC (for ruminants); *Salmonella* spp. should not be individually detected in 25 g of food enzyme measured according to ISO methods 1027-1:2016, TS13136:2012 and 6579-1:2017, respectively or validated alternative methods. Data on filamentous fungi and yeast should not exceed 100 CFU/g in the food enzyme measured according to ISO method 21527-1:2008 or validated alternative methods.

For enzyme derived from pig sources, the absence of Hepatitis E virus should be demonstrated in three batches of the food enzyme.

Methods of analysis together with certificates of analysis covering each measured parameter should be provided, whether made in-house or by a third party. The LoD/LoQ for each method should be given. The same batches as used for chemical characterisation should be analysed. The certificates of analysis should include sufficient identification of the test item to ascertain that it is in fact the food enzyme under application.

The following sections apply to all food enzymes regardless of source.

4. Toxicological studies

Toxicological studies are required for all food enzymes unless specifically exempted. These will normally consist of *in vitro* tests for genotoxicity and *in vivo* studies for systemic toxicity. Only when the results of these tests indicate a potential issue, additional studies will be requested. Studies performed following guidelines which can be regarded as equivalent to the OECD Test Guidelines (TG) and OECD Good Laboratory Practice (GLP), respectively, can be accepted. In such cases comparability of the applied guidelines described in Council Directives 2004/10/EC[13] and 2004/09/EC[14] should be provided along with a statement of GLP compliance of the laboratory conducting the study. The most recent version of any guideline should be applied.

4.1. Exemptions from toxicity testing (other than allergenicity)

In the following circumstances, the need for toxicity testing is waived:

a) For food enzymes obtained from microbial sources (GM and non-GM) which meet the requirements of the QPS approach to safety assessment (namely, (i) the production strain is identified as belonging to a species included in the QPS list, (ii) it meets any QPS qualification, and (iii) no concerns are raised by the genetic modification), and in addition no safety issues are raised by the manufacturing process. For those cases where the QPS approach cannot be applied because of the presence of AMR genes, toxicity testing may still be waived if no viable cells and DNA are detected, as specified in section 1.3.4.

b) For food enzymes obtained from microbial sources, when appropriate substitute toxicological data are available. In general, substitute toxicological tests are acceptable, if they meet all the following:

- the test material is a food enzyme from a microbial strain belonging to the same strain lineage[15] as the production strain of the enzyme under assessment;
- no additional conventional mutagenesis has been applied in the development of the production strain compared to the proposed substitute strain;
- any difference in genetic modifications between the production strain compared to the proposed substitute strain is well characterised and of no concern (see Section 1.1.11). The strategy for the genetic modification should be based on targeted integration, deletion or editing at known genomic loci in the production strain. It should be determined whether any insertion (intended or unintended) in the production strain has interrupted

[15] Lineage is descent in a line from a common progenitor.

any ORF involved in the regulation of the biosynthesis of mycotoxins or other metabolites of known toxicity. This should be studied by WGS analysis;
- it should be demonstrated that the raw materials used and the manufacturing processes of both food enzymes are comparable. A full list of the actual raw materials used and a detailed description of the production process of the enzyme used as the substitute item should be provided.

c) For food enzymes derived from plants and animals that are consumed by the European population, two criteria must be met: (i) no hazard is introduced through the manufacturing process, and (ii) when it can be demonstrated that the dietary exposure to the food enzyme TOS is within the same magnitude as the dietary intake of the fraction of the plant or animal material comparable to the food enzyme TOS.
d) For animal derived rennet, provided that no safety issues are raised by the manufacturing process.
e) When it can be demonstrated that there is no (or negligible) carry-over of the food enzyme TOS into the final food products.

4.2. The test item and dose level

The purpose of toxicity testing is to enable a conclusion about the safety of the food enzyme as a component of an enzyme preparation. The applicant should ensure that all other materials added to the food enzyme when formulating the preparation as placed on the market are compliant with EU food legislation and safe.

The batch(es) used for the toxicological studies should be representative of the commercial batches, as judged by a similar (or lower) activity:TOS ratio. It is recognised that practical constraints may require de-watering or drying of the food enzyme before its use as a test item, however, this should not affect the activity:TOS ratio. The unit used to determine enzyme activity should be the same as that used in the commercial batches. If a different unit is used, a conversion factor should be provided.

The batch(es) should be characterised for chemical composition and purity as described in Sections 1.3.2 and 1.3.3, 2.3.2 and 2.3.3 or 3.3.2 and 3.3.3. Certificates of analysis should confirm that the test item is the food enzyme under application.

Depending on the test, the units should be expressed as μg TOS/plate, μg TOS/mL or mg TOS/kg body weight (bw) per day. The selection of the concentrations/doses should be justified.

4.3. Genotoxicity

Food enzymes are complex mixtures of unidentified components except for the declared enzyme(s). The recommended approach for the genotoxicity assessment of such a type of mixture is to test the whole mixture (EFSA Scientific Committee, 2019).

The following two *in vitro* tests are recommended as the first step (EFSA Scientific Committee, 2011):

- bacterial reverse mutation assay (OECD TG 471, 2020a), and
- *in vitro* mammalian cell micronucleus test (OECD TG 487, 2016a).

This combination of tests fulfils the basic requirements to cover the three genetic endpoints with the minimum number of tests: the bacterial reverse mutation assay covers gene mutations and the *in vitro* micronucleus test covers both structural and numerical chromosomal aberrations.

When the food enzyme activity may affect the performance of the *in vitro* tests (e.g. inactivation of the post mitochondrial rat liver S9 fraction), inactivated food enzyme could be used as the test item. In such case, the use of inactivated enzyme should be justified.

Ames test

In order to overcome potential problems with histidine or tryptophan in the food enzyme batch, it is recommended to expose the *Salmonella* and *E. coli* strains to the tested food enzyme in the liquid culture ('treat and plate assay'), instead of the traditionally 'plate incorporation assay'. A recommended protocol incorporating treat and plate is given in Annex C. The recommended maximum test concentration for soluble non-cytotoxic substances is at least 5 mg TOS/plate. This concentration is

necessary to ensure sufficient level of exposure to detect the majority of known genotoxic compounds (Kenyon et al., 2007).

If the Ames test is not applicable, alternatively a test for induction of gene mutations in mammalian cells, preferably the mouse lymphoma *tk* assay (OECD guideline 490, 2015), could be performed, but it needs to be justified.

***In vitro* mammalian micronucleus test**

The highest test concentration should correspond to 2 mg TOS/mL, if no precipitate or limiting cytotoxicity is observed. However, the top concentration may need to be higher than recommended, e.g. up to 5 mg TOS/mL, to increase the concentration of each of the components in the absence of sufficient cytotoxicity (OECD TG 487).

***In vivo* follow-up**

In case of one or more positive *in vitro* tests, further testing may be required to determine whether the hazard is expressed *in vivo*, unless it can be adequately demonstrated that the positive *in vitro* findings are not relevant for the *in vivo* situation.

In vivo tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to appropriate target organs or tissues.

In line with the recommendation of the EFSA Scientific Committee (EFSA Scientific Committee, 2011, 2017, 2021), the following *in vivo* tests are considered as suitable follow-up for substances positive in the *in vitro* basic battery:

- *in vivo* mammalian erythrocyte micronucleus test for *in vitro* clastogens and aneugens (OECD TG 474, 2014);
- *in vivo* mammalian alkaline comet assay for substances which cause gene mutations and/or structural chromosomal aberrations (OECD TG 489, 2016b);
- Transgenic rodent somatic and germ cell gene mutation assays to follow-up *in vitro* positive compounds for gene mutations (OECD TG 488, 2020b);
- a combination of an *in vivo* micronucleus assay and a Comet assay in the event of a positive *in vitro* micronucleus assay.

The range of doses to be applied in *in vivo* genotoxicity tests should reach the maximum tolerated dose (MTD) in line with the recommendations given in OECD test guidelines. If no toxicity is observed in an adequately designed range-finding study, it would be appropriate to test higher doses than the maximum limits mentioned in the OECD test guidelines, in order to increase the dose of each of the individual components of the mixture. The highest dose to be applied is limited by the maximum volume that should be given to rodents (1 mL/100 g body weight except in the case of aqueous solutions where a maximum of 2 mL/100 g may be used) (OECD TG 474 and 489).

For further information on the *in vivo* follow-up of substances positive in the *in vitro* basic battery, the Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019) should be consulted.

For evaluation and interpretation of results of genotoxicity studies, the test reports should include historical negative (solvent/vehicle) and positive control data, as recommended in OECD test guidelines.

4.4. Repeated dose 90-day oral toxicity study in rodents

A subchronic oral toxicity study should be provided for assessment of systemic toxicity. The protocol according to the OECD TG 408 (2018) is recommended. A highest dose selected should be at least 1,000 mg TOS/kg bw per day, unless technological considerations will not allow doses of this magnitude.

Relevant historical control data should be provided to enable the judgment of the validity of the study as proposed in OECD TG 408 and in Commission Regulation (EU) No 283/2013[16]. Decisions on whether additional studies are needed will be taken by EFSA on a case-by-case basis, in the event of the identification of an adverse effect.

[16] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market Text with EEA relevance. Part A, section 5.

4.5. Allergenicity studies

The assessment of potential allergenicity considers (a) the source of the food enzyme; (b) the declared enzyme(s) and (c) any proteinaceous material with known allergenic properties included in a fermentation medium.

Quantifying the risk for allergenicity is currently not considered possible in view of the varying individual susceptibility to food allergens. Even trace amounts of food allergens transferred to food could in principle present a hazard for sensitised individuals. Thus, an argument based on the lack of transfer will only be accepted if it can be demonstrated that food enzyme TOS is absent in the final food. For all other cases, the following information is required:

1) An alignment-based study using the amino acid sequence of the food enzyme and reporting any sequence identity greater than 35% to a known allergen using a sliding window of 80 amino acids as proposed by FAO/WHO, 2001. An updated database of known allergens should be used, its version and the date of accession specified, and the hits found discussed in the context of the available literature.
2) A search of the literature for reports that the specific enzyme(s) under application may give rise to sensitisation or elicitation reactions. Any information available on the enzyme resulted from the search of the literature and taking into consideration the route of exposure should be presented. For instance, studies which may have been conducted for other purposes, such as the assessment of safety at the workplace (e.g. sensitisation studies) should be submitted.
3) A search for possible allergic reactions caused by enzymes of the same family of enzymes as the one under application should be performed. In the case allergic reactions have been observed, a rationale should be given on how these observations do or do not impact on the evaluation of the potential allergenicity of the enzyme under application.

5. Dietary exposure

In the initial stages of the dietary exposure assessment, the intended uses are reviewed with the purpose to decide if exposure estimates need to be derived. Where a full dietary exposure assessment is carried out, exposure estimates are compared to the reference point identified from the toxicological studies and the margin of exposure (MoE) is calculated. Alternatively, in the case of food enzymes that are derived from edible parts of plant or animals intended to be or reasonably expected to be ingested by humans, the food enzyme TOS is compared to a comparable fraction of the source material.

5.1. Intended use of the food enzyme

All food manufacturing processes for which the food enzyme is intended to be used should be listed. The description of each food manufacturing process should, where possible, be aligned with the definition provided in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

Each intended use of the food enzyme should be supported by a description of the technological need and function of the food enzyme during manufacturing and of the expected benefits of its use.

Where the proposed use of the food enzyme cannot be (fully) aligned with one of the food manufacturing processes listed in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023) full details of each food manufacturing process should be provided, illustrated by a flow chart. The stages where the food enzyme is added during the manufacturing process and in particular, any process (e.g. purification and filtration steps) that may result in the partial or complete removal of contaminating material (including food enzyme TOS) from the final food should be clearly indicated.

It is recommended that information on intended use and use levels is provided using the tabular format shown below, when more than one food manufacturing process is proposed.

Food manufacturing process	**Raw material to which the food enzyme is added**	**Recommended use level of the food enzyme**
Food process	Raw material (e.g. flour, grain, cheese)	Recommended use level (in mg TOS/kg raw material) Maximum use level (in mg TOS/kg raw material)

TOS: total organic solid; bw: body weight.

The amount of food enzyme to be added should be provided for each food manufacturing process whether or not included in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023). Units of activity should be converted to the weight unit TOS/kg of physical raw material. The physical raw material may refer to a raw agricultural commodity (e.g. grain), to a food ingredient (e.g. flour) or to a food as consumed (e.g. cheese), but typically it is not the substrate for the enzyme *per se*. Where necessary, the conversion from Units to TOS should be based on the average activity:TOS ratio provided for the representative batches of the food enzyme used for commercialisation.

For a food process not listed in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023). where the produced food product is different from the physical raw material to which the food enzyme was added (e.g. milk treated with enzyme for the production of cheese) a yield factor should be specified, providing information on the amount of raw material (to which the food enzyme is added) required to obtain 1 kg of the final food product produced using the process (e.g. the amount of milk required to produce 1 kg of cheese).

5.2. Removal/absence of transfer of TOS during food manufacturing processes

By default, it is generally assumed that the entire food enzyme TOS is transferred into the food produced, unless evidence is available to prove the contrary.

Based on analytical data provided by the European Association of Manufacturers and Formulators of Enzyme Products (AMFEP) and other industrial bodies and the experience gained during the assessment of applications, EFSA has identified several food manufacturing processes where the probability of finding food enzyme TOS in the final food product is considered negligible. In these cases, the need for a dietary exposure assessment is waived. The food manufacturing processes to which this waiver applies is given in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023). To ensure that advances in technological processes are accounted for in the future, EFSA will periodically review the validity of the evidence supporting the waiver.

Additional food manufacturing processes may be added to the list of processes for which dietary exposure assessment may be waived, based on evidence on the removal of the food enzyme TOS or absence of transfer of food enzyme TOS into the final product. Any such claim should be supported by robust experimental data establishing the extent of the removal of both enzyme activity and markers of TOS (e.g. total nitrogen or any TOS-specific compound) in the food product(s). To generate analytical data, samples of the food enzyme itself and samples collected at different steps during food manufacturing, as well as the final products, should be analysed. Where sampling during manufacturing is not possible (e.g. closed system), laboratory samples, which should be representative of the manufacturing conditions, may be used. At least three independent samples should be analysed. Analytical methods and the LoD and LoQ should be provided.

5.3. Food enzymes that are derived from edible parts of plant or animals

Special consideration has been given to food enzymes that are derived from edible parts of plant or animals intended to be or reasonably expected to be ingested by humans. According to Regulation (EU) No 562/2012, the need for toxicological studies may be waived, in case there are no adverse effects on human health when consumed as food in a comparable way.

In order to establish consumption in a comparable way, EFSA requires the following information:

- evidence of consumption of the edible plant part or animal tissues and products, including quantity of consumption in the EU or elsewhere and reference to the source of information;
- information on the enzyme yield factor, e.g. x amount (kg) of source material to obtain y amount (kg or g) of food enzyme (not food enzyme preparation).

5.4. Calculation of exposure

Dietary exposure is estimated where presence of TOS in the final food product cannot be excluded. Consequently, each food enzyme application will require assessment of each individual food process for which the food enzyme is intended to be used.

Given the complexity associated with assessing the exposure to food enzymes which are added to raw materials, which are then processed using different food manufacturing processes resulting in a carry-over of the food enzyme into food as consumed, EFSA developed a new methodology. This takes

into account the nature of the use of food enzymes, their fate during food processing and the individual consumption data reported in the EFSA Comprehensive European Food Consumption Database. The Comprehensive Database represents the best available source of food consumption data across Europe at present covering infants, toddlers, children, adolescents, adults and the elderly.

To aid applicants, process-specific exposure tools, namely the Food Enzyme Intake Models (FEIMs), based on summary statistics, have been developed. Process-specific calculators (e.g. FEIM-baking, FEIM-brewing) can be accessed on the EFSA webpage[17] or via the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023). Additional calculators are in the process of being developed. It is envisaged to create an overall assessment tool once all process-based models have been developed.

Applicants should use all FEIM calculators available as appropriate and submit exposure estimates for each process separately, in the tabular format provided below. Exposure estimates to food enzymes should be reported for all six population groups (infants, toddlers, children, adolescents, adults and the elderly), as mg TOS/kg body weight, as appropriate. For both mean and 95th percentile intake, the range of minimum–maximum value observed across the selected surveys contained in the EFSA database should be reported.

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)
Min–max 95th percentile (number of surveys)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)	0.00–0.00 (n)

TOS: total organic solids.

Applicants are required to report an exposure assessment only when an appropriate FEIM calculator is available. In case no suitable calculator is available, applicants should provide as much detail on the process, including all raw materials to which the food enzyme is added, the food groups in which the end product is going to be used, as well as information on use levels and yield factors, as applicable. This information will aid EFSA in developing additional food processes-based exposure models.

If the food enzyme is involved in more than one food manufacturing processes, EFSA will calculate combined exposure based on raw data contained in the Comprehensive Database.

5.5. Risk characterisation

The purpose of deriving a quantitative estimate is to facilitate characterisation of any risk associated with such exposure to the European population. The risk is characterised through a comparison of the estimated human exposure with the reference point (e.g. no observed adverse effect level or benchmark dose level) determined in 90-day oral toxicity studies performed on animals. A ratio, referred to as the MoE, between the reference point and the estimated exposure is used to conclude on the safety of the food enzyme.

The MoE is calculated as follows: MoE = Reference point/highest P95 value

The first estimate of the MoE is made using exposure data for all uses of a food enzyme. Only where the overall MoE is considered low, would the MoE for each food manufacturing process be separately calculated to establish whether the overall low value is a consequence of a single food manufacturing process.

For applications concerning 'food enzymes that are obtained from edible parts of plant or animals intended to be or reasonably expected to be ingested by humans', a comparison is made between the exposure to the food enzyme resulting from its intended uses (following the described process above) and the exposure to a similar fraction of the source material resulting from the consumption of foods derived from this source. The outcome of this comparison is one of the criteria to justify that no toxicological data are required.

[17] https://www.efsa.europa.eu/en/applications/foodingredients/tools

Part B. Data required for the safety assessment of modifications to an existing authorisation

Article 14 of Regulation (EC) No 1332/2008 requires producers or users of food enzymes to inform the European Commission of any new scientific or technical information which might affect the assessment of the safety of a food enzyme. It is foreseen for those food enzymes already included in the Community list, that a significant change in the production methods or starting materials should be re-submitted for evaluation. In the view of EFSA, the use of a different strain of microorganism, the use of a different plant species or plant part or the use of a different animal species or tissues/ products would constitute the production of a new food enzyme and thus require a full safety assessment as described in Part A of this guidance.

Where a request to update the Community list of food enzymes involves a less significant change, defined in the Regulation (EC) No 1331/2008 as 'adding, removing or changing conditions, specifications or restrictions associated with the presence of a substance on the Community list' the European Commission is only obliged to seek the opinion of EFSA if the proposed changes to the authorisation are thought liable to have an effect on human health.

Changes which may impact human health could include any modification to a production system which leads to qualitative and quantitative changes in the TOS associated with the final food product or proposals for the use of the food enzyme in additional food manufacturing processes. For such cases, since the source of the food enzyme remains unchanged, a limited dataset is required.

Data requirements

Proposals to change an existing authorisation should reference the authorisation given in the Community list and indicate the proposed change(s). A statement should be made confirming that the source of the food enzyme remains the same. In case the manufacturing process also remains unchanged this should be confirmed.

New manufacturing process of the food enzyme

Applicants should provide a full description of the manufacturing process(es) as described in Sections 1.2, 2.2 and 3.2 of Part A of this guidance indicating the change(s) proposed.

Modifications to a manufacturing process do not necessarily introduce concerns for human health and, thus, proposals for change would be evaluated on a case-by-case basis. It is the responsibility of the applicant to identify and characterise the hazard, usually starting by reference to the available literature.

In case the intended change in the manufacturing process would lead to a change in the TOS composition the risk to consumers has to be assessed. The concentration of any substance of concern should be measured in the food enzyme TOS and the extent of carry-over into the final food established for each authorised food manufacturing process. If detected in the final food, the applicant should use the overall exposure assessment made in the course of the initial assessment to calculate exposure to the substance of concern in the various population groups based on the concentration present in the food enzyme TOS. In the absence of published data identifying and characterising the hazard, the applicant should provide a full set of toxicological data and the information needed to allow the characterisation of risks to human health. Toxicological tests should be performed as described in Part A of this guidance.

The introduction of a specification for the use of an immobilised or encapsulated form of the food enzyme for one or more of the food manufacturing processes specified under the conditions of authorisation is not expected *per se* to alter the conclusions on the safe use of the enzyme unless:

a) the immobilisation support or encapsulating material is potentially hazardous and is leached into the final food, or if
b) any cross-linking or other agents used to bind the food enzyme to the support material is potentially hazardous and is leached into the final food.

In these cases, analytical data on the extent of leaching and the concentration into final food(s) should be established. In the absence of published data identifying and characterising the hazard, the applicant should provide a full set of toxicological data and the information needed to allow the

characterisation of risks to human health. Toxicological tests should be performed as described in Part A of this guidance.

Extension of the intended uses of the food enzyme

Each proposal for an additional food manufacturing process should be clearly indicated. Applicants should provide a full description of each modified use as described in Section 5.1 of Part A of this guidance.

To determine the need for a dietary exposure resulting from the extended uses, the applicant should follow Section 5.2 of Part A of this guidance. For calculation of dietary exposure, the applicant should follow Sections 5.3 and 5.4 of Part A of this guidance.

References

AINIA Technology Centre, de Benito A, Ibanez C, Moncho W, Martınez D, Vettorazzi A and Lopez de 291 Cerain A, 2017. Database on the taxonomical characterisation and potential toxigenic capacities of 292 microorganisms used for the industrial production of food enzymes and feed additives, which do not 293 have a recommendation for Qualified Presumption of Safety. EFSA supporting publication 294 2017;14(7):EN-1274, 185 pp.

Auch AF, Klenk H-P and Göker M, 2010a. Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. Standards in Genomic Sciences, 2, 142–148. https://doi.org/10.4056/sigs.541628

Auch AF, von Jan M, Klenk H-P and Göker M, 2010b. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Standards in Genomic Sciences, 2, 117–134. https://doi.org/10.4056/sigs.531120

Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, Rooney AP, Yi H, Xu XW, De Meyer S and Trujillo MEE, 2018. Proposed minimal standards for the use of genome data for the taxonomy of 297 prokaryotes. International Journal of Systematic and Evolutionary Microbiology, 68, 461–466.

EFSA (European Food Safety Authority), 2009. Guidance of EFSA prepared by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. EFSA Journal 2009;7(5):1305, 26 pp. https://doi.org/10.2903/j.efsa.2009.1305

EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in exposure assessment. EFSA Journal 2011;9(3):2097, 34 pp. https://doi.org/10.2903/j.efsa.2011.2097

EFSA (European Food Safety Authority), 2021a. Administrative guidance for the preparation of applications on food improvement agents (food enzymes, food additives and food flavourings). EFSA supporting publication 2021;EN-6509, 37 pp. https://doi.org/10.2903/sp.efsa.2021.EN-6509

EFSA (European Food Safety Authority), 2021b. Statement on the requirements 3 for whole genome sequence analysis 4 of microorganisms intentionally used in the food chain. In press (due to be published in Feb 2021).

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food), 2017. Re-evaluation of lecithins (E 322) as a food additive. EFSA Journal 2017;15(3): 4742, 83 pp. https://doi.org/10.2903/j.efsa.2017.4742

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016. Panel statement on the exposure assessment of food enzymes. EFSA Journal 2016;14(11):4581, 9 pp. https://doi.org/10.2903/j.efsa.2016.4581

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), 2019. Statement on the characterisation of microorganisms used for the production of food enzymes. EFSA Journal 2019;17(6):5741, 13 pp. https://doi.org/10.2903/j.efsa.2019.5741

EFSA Scientific Committee, 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379, 69 pp. https://doi.org/10.2903/j.efsa.2019.2379

EFSA Scientific Committee, 2017. Clarification of some aspects related to genotoxicity assessments. EFSA Journal 2017;15(12):5113, 25 pp. https://doi.org/10.2903/j.efsa.2017.5113

EFSA Scientific Committee, 2019. Genotoxicity assessment of chemical mixtures. EFSA Journal 2019;17(1):5519, 11 pp. https://doi.org/10.2903/j.efsa.2017.5113

EFSA Scientific Committee, 2021. Guidance on aneugenicity assessment. EFSA Journal 2021 (In press).

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2001. Evaluation of Allergenicity of Genetically Modified Foods Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22 – 25 January 2001. Available online: http://www.fao.org/3/a-y0820e.pdf

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specificaions. 67th meeting. FAO JECFA Monographs, 3,3 63–67. Available online: http://www.fao.org/3/a-a0675e.pdf

Frisvad JC, Møller LLH, Larsen TO, Kumar R and Arnau J, 2018. Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Applied Microbiology and Biotechnology, 102, 9481–9515.

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P and Tiedje JM, 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. International Journal of Systematic and Evolutionary Microbiology, 57, 81–91. https://doi.org/10.1099/ijs.0.64483-0

Kenyon MO, Cheung JR, Dobo KL and Ku WW, 2007. An evaluation of the sensitivity of the Ames. Regulatory Toxicology and Pharmacology, 48, 75–86. https://doi.org/10.1016/j.yrtph.2007.01.006

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G and Durbin R, 1000 Genome Project Data Processing Subgroup, 2009. The sequence alignment/map format and SAMtools. Bioinformatics, 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

OECD, 2014. Test No. 474: Mammalian Erythrocyte Micronucleus Test, OECD Publishing, Paris, https://doi.org/10.1787/9789264224292-en

OECD, 2015. Test No. 490: *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, OECD Publishing, Paris, https://doi.org/10.1787/9789264242241-en

OECD, 2016a. Test No. 487: *In Vitro* Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264264861-en

OECD, 2016b. Test No. 489: *In Vivo* Mammalian Alkaline Comet Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264264885-en

OECD, 2018. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264070707-en

OECD, 2020a. Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264071247-en

OECD, 2020b. Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264203907-en

WHO (World Health Organization), 2019. Critically important antimicrobials for human medicine, 6th revision. ISBN: 978-92-4-151552-8

Abbreviations

AFToL	Assembling the Fungal Tree of Life
AMR	antimicrobial resistance
AMFEP	Association of Manufacturers and Formulators of Enzyme Products
ANI	average nucleotide identity
BUSCO	Benchmarking Universal Single-Copy Orthologs software
bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
CIA	critically important antimicrobial
dDDH	digital DNA–DNA hybridisation
EINECS	European Inventory of Existing Commercial Chemical Substances
ELINCS	European List of Notified Chemical Substances Number
ENA	European Nucleotide Archive
FAO	Food and Agricultural Organization of the United Nations
FEIM	Food Enzyme Intake Model
FoF	follow-on formulae
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
GMM	genetically modified microorganism
HIA	highly important antimicrobial
ICN	International Code of Nomenclature for algae, fungi and plants
ITS	internal transcribed spacer
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LoD	limit of detection
LoQ	limit of quantification
LPSN	List of Prokaryotic Names with Standing in Nomenclature

MoE	margin of exposure
N50	A metric used as a proxy for assembly quality that is defined as the length at which contigs of equal or longer length contain at least 50% of the assembled sequence
NCBI	National Center for Biotechnology Information
OECD	Organisation for Economic Cooperation and Development
ORF	open reading frame
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOP	Standard Operation Procedure
TOS	total organic solids
UniProt	Universal Protein
WGS	whole genome sequence
WHO	World Health Organization

Annexes

Some necessary information has been included in this guidance in the form of Annexes rather than appearing in the body of the text. This is to allow modifications to be made without the need for a full revision of the guidance. In particular, this will enable the easier addition of food manufacturing processes and reference to additional food process-based exposure models (FEIM calculators) as they are developed.

Annex A – Background as provided by the requestor

Regulation (EC) No 1332/2008 lays down the rules on food enzymes used in foods with a view to ensure the effective functioning of the internal market whilst ensuring a high level of protection of human health.

Food enzymes shall be subject to safety evaluation by the European Food Safety Authority (EFSA) and approval via a Union list. The inclusion of a food enzyme in the Union list is considered by the Commission on the basis of the opinion from EFSA, taking into account also other general criteria such as technological need, consumer aspects and, where relevant, other legitimate factors. For every food enzyme included in the positive list, intended use(s) in food and specifications, including the criteria on purity and the origin of the food enzyme, shall be laid down.

The establishment of the Union list will take place in a single step after the Authority has issued an opinion on each food enzyme for which an application complying with the validity criteria laid down in accordance with Article 9(1) of Regulation (EC) No 1331/2008 had been submitted in accordance with Article 17(2) of Regulation (EC) No 1332/2008.

Pursuant to Article 9 of Regulation (EC) No 1331/2008 the Commission adopted implementing measures that are laid down in Regulation (EU) No 234/20113 as regards the content, drafting and presentation of applications submitted under each sectoral food law, arrangements for checking the validity of applications and the type of information that should be included in the opinion of EFSA.

Article 5 of Regulation (EU) No 234/2011 requires applicants to take into account the latest guidance documents adopted or endorsed by the Authority available at the time of the submission of the application for the safety evaluation of a food enzyme.

Since the beginning of the period established for which an enzyme application may be submitted for the inclusion in the Union list, the EFSA reference guidance document has been the Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids on the Submission of a Dossier on Food Enzymes. This document was later supplemented by the Explanatory Note for the Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes to assist applicants to compose a technical dossier.

Based on experience gained in assessing submitted dossiers, EFSA, on its own initiative, has updated the assessment methodology on food enzymes resulting in the adoption of two statements by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids:

- Statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016)
- Statement on the characterisation of microorganisms used for the production of food enzymes (EFSA CEP Panel, 2019)

These documents are intended to assist in the preparation and presentation of applications in accordance with Regulation (EU) No 234/2011.

The Commission considers that it is desirable to update the 'Guidance on the Submission of a Dossier on Food Enzymes' (2009) taking into account new approaches on certain aspects of the risk assessment, in particular the statements issued on the exposure assessment of food enzymes and the characterisation of microorganisms used in the production of food enzymes. In the updated guidance, other relevant documents, including guidance documents produced by the EFSA Scientific Committee, could be taken into account as appropriate.

In the preparation of the updated guidance, EFSA should take into account the relevant provisions of Regulation (EU) No 2019/1381 of the European Parliament and of the Council on the transparency and sustainability of the EU risk assessment in the food chain and should ensure consistency with other sectors where similar updates are envisaged.

Annex B – Provision of raw data and standard data formats for whole genome sequence analysis

The WGS raw data should be submitted in the respective standard formats as indicated below.

- The sequencing reads, and trimmed reads where relevant, should be submitted in FASTQ or similar formats, pair or single end.
- Assembled sequences can be submitted in FASTA format (e.g.*.fasta).
- Supported formats for annotation[18] are GFF format (*.gff), GenBank format (*.gbff, *.gbk and *.gb), Tabular format (*.csv) and the NCBI's Sequin ASN.1 (*.sqn).
- For the characterisation of the genetic modification, the alignments should be provided in Sequence Alignment/Map format (SAM) or Binary Alignment/Map format (BAM) (Li et al., 2009) or similar file formats.

[18] In case the annotation format includes the nucleotide sequence, data in FASTA format is not required.

Annex C – Recommended protocol for the 'treat and plate' modification of the bacterial mutagenicity test

Except for the treatment of bacteria, the methodology and the reagents (medium, metabolic activation system (S9 mix), preparation of test item, solvents, culture conditions, choice of doses, etc.) are the same as those used for the 'plate incorporation' or 'pre-incubation' Ames test (OECD guideline 471).

Treatment of the bacteria

A 0.5-mL aliquot of S9-mix or phosphate buffer 0.2 M pH 7.4 is combined with 0.1 mL late logarithmic phase bacterial culture in a sterile container. A 0.1-mL aliquot of the test solution containing the food enzyme is added. Bacteria and treatment are incubated for 90 min with shaking at 37°C. After the 90-min pre-incubation, a large volume (10–15 mL) of a wash solution of nutrient broth in phosphate-buffered saline is added and the washed bacteria are collected by centrifugation (e.g. at 2,000 g for 30 min). All but about 0.7–1 mL of the supernatant is removed and discarded, and the bacteria are re-suspended in the residual supernatant prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate (1.5% agar, Vogel–Bonner medium E, 2% glucose). In some cases, it is possible to perform a second washing of the bacteria. The plates are inverted and incubated at 37°C for 48–72 h. After the incubation period, the number of revertant colonies per plate is counted.

Controls

Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be included. Sterility control should be included in each experiment.

Annex D – Food manufacturing processes and estimation of dietary exposure to the food enzyme TOS

Annex D is replaced by the publication of the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (https://doi.org/10.2903/j.efsa.2023.8094).