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Scientific Guidance for the submission of dossiers on Food Enzymes

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids

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 to 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 tissues. 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

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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) harmonized 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

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[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.

154 preparations generally will not be taken to substitute for data on food enzymes unless
155 demanded by stability issues or the use of whole cells.

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

161 Terms of Reference as provided by the requestor

162 In accordance with Article 29 of Regulation (EC) No 178/2002[3], the Commission requests EFSA
163 to update and consolidate the Guidance for the Submission of a Dossier on Food Enzymes
164 under Regulation (EC) No 1331/2008, taking into account the experience gained with the risk
165 assessment of enzymes and the numerous other relevant scientific and technical documents
166 that have been published by EFSA since the adoption of the current guidance related to food
167 enzymes.

168 Interpretation of the Terms of Reference

169 This revised Guidance relates only to the data needed to complete the safety assessment of
170 food enzymes.

171 All administrative information related to the preparation and submission of an application on
172 food enzymes are addressed in a separate EFSA document, i.e. 'Administrative guidance on
173 the preparation of applications on food additives, food enzymes and food flavourings' (EFSA,
174 2021a), which is applicable to applications submitted as of 27 March 2021.

175 Scope of the guidance

176 The purpose of this document is to assist applicants in the preparation and presentation of
177 dossiers for the safety evaluation of food enzymes in accordance with Regulation (EC) No
178 1332/2008, Regulation (EC) No 1331/2008 and its implementing Rules Regulation (EU) No
179 234/2011[4] and Regulation (EU) No 562/2012[5]. The application is initially made to the EC for
180 further transmission to EFSA, which is responsible for the safety assessment and providing an
181 opinion on the outcome of the evaluation. This document outlines the information necessary
182 to enable EFSA to make its safety assessment of a food enzyme and, where appropriate, the
183 rationale underlying the request for additional data.

184 No guidance document can be exhaustive. Information requirements may vary depending for
185 example on the food enzyme's function/activity, the properties of the source material, the
186 properties and amounts of any by-products and substances originating from the food enzyme
187 production processes and intended food manufacturing processes, as well as the intended use
188 and the resulting level of human dietary exposure. There may be circumstances where
189 additional data or tests to those indicated in this document are required for the evaluation.

[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.

[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, 11.03.2011, 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.

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 amalgamates and supersedes the original Guidance document developed and published at the request of the EC in 2009 (EFSA, 2009) and a number of 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 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 EC 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.

The data requirements in part A are organised to follow the broad structure of the opinions produced by EFSA which essentially consists 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. 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 tissues. Finally, the data requested under sections 4 and 5 are described independent of the source of the food enzyme.

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		

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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 common name (as described 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

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. 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

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266 applied. Total DNA (i.e. including chromosomal and extra-chromosomal elements), should be
267 extracted and subjected to WGS analysis.

268 1.1.3 Library construction

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

273 1.1.4 Sequencing strategy and quality control

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

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

282 The average read depth achieved should be at least 30-fold with a recommended target of
283 100-fold.

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

289 The sequencing reads can be *de novo* assembled (and annotated), mapped to a reference
290 genome/database or the two approaches can be used in combination (for GMM see section
291 1.1.14). For bacteria, complete genome sequence should be pursued but draft genome
292 sequence may be accepted.

293 1.1.5 *De novo* assembly and annotation

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

295 - *De novo* assembly including assembler software, version and parameters. If post-
296 assembly processing is carried out, approach, software, version and parameters should
297 also be reported.
298 - Contigs:
299 - for draft genomes, the total number of contigs produced by the assembler. For
300 bacteria, total contigs should be <500 and for yeasts and filamentous fungi
301 <1,000. If a higher number of contigs is produced, the applicant should provide
302 a justification;
303 - the total length of the contigs. Applicants should provide a justification if their
304 assembly size is not within +/- 20% of the expected genome size for the
305 species;
306 - N50 metric or similar quality parameters.
307 - For yeasts and filamentous fungi genomes, the number of highly conserved genes,
308 such as for example BUSCO genes, present in the assembly should be reported since
309 this parameter indicates the completeness and quality of the assembly

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310 (https://busco.ezlab.org/). Ideally, >90% complete matches to BUSCO gene set from
311 the most closely related group of yeasts/filamentous fungi should be present in the
312 assembly.

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

316 1.1.6 Reference-based read mapping

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

321 1.1.7 Identity

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

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

335 **Bacteria:** Taxonomical identification is expected to be made by computational approach using
336 WGS data. The identity of the organism under assessment should preferably be established
337 by digital DNA-DNA hybridization (dDDH; Auch et al., 2010a and 2010b), average nucleotide
338 identity (ANI; Goris et al., 2007) or phylogenomic methods. The sequencing data from the
339 microorganism under assessment should be compared with the reference genome of the type
340 strain of the expected species. In the case the reference genome of the type strain is not
341 available, another well-identified strain can be used. For identification at species level, dDDH
342 should reach >70% identity and ANI should reach >95 % (Chun et al., 2018).

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

348 **Filamentous fungi**: when WGS is available, identification should be made by phylogenomic
349 analysis (e.g., using a concatenation of several conserved sequences (e.g., AFToL genes

[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/

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350 including 18S rDNA/ITS) to produce a phylogeny against available related genomes) or by
351 alignment to a complete reference genome from the same species.

352 When the identification is done by WGS analysis using *de novo* assembly-based approach, a
353 summary of the method and sequence/s used for comparison, and results of the comparison
354 including sequence identity (percent of identity with the compared reference genome) should
355 be indicated. A phylogenetic tree is recommended, in particular for taxa where a high level of
356 identity between related species exists.

357 If read-mapping approach is used for identification, sequencing reads should be mapped
358 against a suitable reference genome(s) (e.g., type strain or well-known and well-identified
359 strain(s)). The choice of the reference genome(s) needs to be well considered, justified and
360 reported. The software used should be reported, including version number and all parameters
361 (if the default parameters are used this should be stated). The proportion of reads mapped,
362 the proportion of reference genome covered to at least 5x depth and the median depth of
363 mapping across entire genome should be reported.

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

367 The origin of the organism and history and purpose of modifications, including mutagenesis
368 steps performed during the development of the production strain, should be reported. Any
369 genetic modification should be characterised according to Section 1.1.11.

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

375 1.1.8 Identification of genes of potential concern

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

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

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

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

393 1.1.9 Antimicrobial resistance

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

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

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

409 In general, query sequence hits with at least 80% identity (at protein level or nucleotide level
410 as reported in the database) and 70% length of the subject sequence should be reported. In
411 the case two or more fragments covering less than 70% length of the subject sequence with
412 at least 80% identity to the same AMR gene are detected, these should be reported, and it
413 should be checked whether the full gene is present. Hits with at least 80% identity (at protein
414 level or nucleotide level as reported in the database) and 70% length of an acquired AMR
415 gene sequence are considered a hazard. The risk associated to this hazard is considered
416 absent if an applicant is able to demonstrate that DNA and viable cells of the production strain
417 cannot be detected in the food enzyme as defined in Section 1.3.4.2.

418 1.1.10 Toxigenicity and pathogenicity

419 *1.1.10.1 Qualified Presumption of Safety*

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

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

433 For production strains meeting the criteria for a QPS approach to safety assessment,
434 toxicological studies on the food enzyme will only be required in relation to possible safety
435 concerns identified elsewhere in the assessment process, e.g., manufacturing. In the specific
436 case of *Bacillus* and related species included in the QPS list, a cytotoxicity test should be made
437 with the production strain to determine whether it produces high levels of non-ribosomal
438 synthesised peptides. A recommended protocol for cytotoxicity testing is given in Annex C.

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

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439 *1.1.10.2 Organisms not included in the QPS list*

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

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

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

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

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

466 1.1.11 Genetic modifications

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

469 1.1.12 Purpose of the genetic modification

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

473 1.1.13 Characteristics of the modified sequences

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

477 For DNA from defined donor organisms:

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

481 a) the nucleotide sequence of all inserted elements including a functional annotation and
482 the physical map of all the functional elements;

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483 b) tabulated information on the size, origin and function of the inserted elements,
484 including coding and non-coding regions;
485 c) name, derived amino acid sequence(s) and function(s) of the encoded protein(s).
486
487 For designed sequences:

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

491 a) the rationale and strategy for the design;
492 b) DNA sequence and a physical map of the functional elements;
493 c) derived amino acid sequence(s) and function(s) of the encoded protein(s);
494 d) similarity with sequences in up-to-date databases (e.g., ENA, NCBI, UniProt). This
495 should identify the functional domains of the recombinant protein; the best hits should
496 be reported and described.
497
498 **Deletions:** A description of the intentionally deleted sequence(s) should be provided,
499 together with an explanation of the intended effect.

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

503 Structure of the genetic modification:

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

506 1.1.14 Structure of the genetic modification using WGS data

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

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

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

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

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526 ### 1.1.15 Structure of the genetic modification without WGS data

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

530 **Characteristics of the vector**

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

532 a) the source and type of the vector (plasmid, phage, virus, transposon). When helper
533 plasmids are used, they should also be described;
534 b) a map detailing the position of all functional elements and other vector components;
535 c) the map should accompany a table identifying each component, properly annotated,
536 such as coding and non-coding sequences, origin(s) of replication and transfer,
537 regulatory elements, AMR genes, their size, origin and role.
538
539 **Information relating to the genetic modification process**

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

541 a) the methods used to introduce, delete, replace or modify the DNA into the
542 recipient/parental and methods for selection of the GMM;
543 b) whether the introduced DNA is a replicative vector or is inserted into the
544 chromosome(s) and/or, for eukaryotic microorganisms, into DNA of organelles (e.g.,
545 mitochondria).
546
547 **Structure of any vector and/or donor nucleic acid remaining in the GMM**

548 This should include:

549 a) a map detailing the position of the sequences actually inserted, replaced or modified;
550 b) in case of deletion(s), the size and function of the deleted region(s) should be provided.
551
552 **Genes of concern**

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

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

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

561 This should be analysed by using appropriate methods, such as Southern analysis or PCR.

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

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

571 1.2 Production of the food enzyme

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

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

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

587 1.2.1 Fermentation

588 Information on the fermentation stage of the production of the food enzyme should specify
589 the type of the fermentation system used (e.g., continuous, (fed-) batch or solid state). A list
590 of the raw materials contributing to the medium and used as process controls is required.
591 These should be the actual materials used; an indicative list will not be accepted. For the
592 majority of raw materials which typically provide the nitrogen and carbon sources, which are
593 included to meet mineral and vitamin requirements or used in pH control, only qualitative data
594 is needed.

595 1.2.2 Downstream processing

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

602 1.2.3 Food enzyme preparations

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

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[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.

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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 chemicals 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 are 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 or includes any signal sequence. Available information on the subunit structure as well as degree and site 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 batches 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 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.

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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 large-scale pilot plants may substitute for those food enzymes in a pre-production stage of development.

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. 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. Analytical 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). Information on the level of other heavy metals (such as mercury or cadmium) or arsenic is required in case the raw materials used include potential sources of mineral contamination, or when extensive use of phyllosilicates is made as filter aids.

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 demonstrate by analysis that their presence in the food enzyme occurs at concentrations which do not give rise to concern. 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 16649-3:2014 or a validated alternative method. *Enterobacteriaceae* should not exceed 10 CFU/g measured according to ISO 21528-2:2017 or a validated alternative method.

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).

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

704 1.3.4 Viable cells and DNA of the production strain

705 *1.3.4.1 Viable cells of the production strain*

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

708 The techniques used to remove/inactivate microbial cells in the course of the downstream
709 processing should be described in detail. The absence of viable cells of the production strain
710 should be investigated using a well-described method for the detection:

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

735 *1.3.4.2 DNA from the production strain*

736 This section applies to:

737 a) food enzymes obtained using GM production strains;
738 b) food enzymes obtained using non-GM modified production strains carrying acquired
739 AMR genes.
740

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

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745 a) in case the production strain contains AMR genes, whether GMM or not, primers should
746 be designed to amplify a fragment not exceeding the size of the smallest antimicrobial
747 resistance gene and in any case not exceeding 1 kb. If the production strain is a GMM
748 not containing AMR genes, the targeted sequence should cover maximum 1 kb;
749 b) DNA from at least 1 g or 1 mL of product shall be extracted. Upstream intermediate
750 products can be used as long as they are equally or more concentrated than the final
751 product. For different production schemes, each of the product should be tested;
752 c) at least three independent batches of food enzyme should be sampled, each extracted
753 in triplicate and analysed. The exact phase of the manufacturing process from which
754 the samples are taken should be indicated. Samples should be taken from industrial-
755 scale process. Samples from pilot-scale process are acceptable if it can be
756 demonstrated that those from industrial process are not available. In this case it should
757 be documented that the pilot-scale process (fermentation and downstream) is
758 representative of the industrial-scale process;
759 d) to recover DNA from non-viable cells potentially remaining in the product, the DNA
760 should be extracted using a methodology suitable for all cellular forms of the
761 production strain (e.g., vegetative cells, spores).
762
763 The following controls and sensitivity tests should be included:

764 a) total DNA from the production strain, as a positive control for the PCR;
765 b) total DNA from the production strain, added to the product sample before the DNA
766 extraction process, starting with a known quantity and in different dilutions until DNA
767 extinction, to calculate the LoD;
768 c) a positive control with total DNA from the production strain, added to the DNA
769 extracted from each of the three batches of the product tested, to check for any factors
770 causing PCR failure;
771 d) a negative control without sample.
772

773 For the purpose of this assessment, the applicant should investigate whether the target
774 DNA is detected in analyses having a LoD of 10 ng of DNA per g or mL of product or lower.
775

776 2 Food enzymes of plant origin

777 2.1 Source of the food enzyme

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

786 The ability of the plant source to produce secondary metabolites that could be harmful to
787 humans should be assessed, particularly for non-edible plant tissue. A literature search should

[12] http://www.theplantlist.org/

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788 be made to identify the capacity of the species or a closely related species to produce known
789 toxic compounds.

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

793 It should be indicated whether the source is from a GM plant. If this is the case, the plant
794 should have been assessed and received authorisation under the Regulation (EU) No
795 1829/2003 for food and feed use in the EU.

796 2.2 Production of the food enzyme

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

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

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

812 2.2.1 Extraction

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

819 2.2.2 Downstream processing

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

825 2.2.3 Food enzyme preparation

826 Data on a food enzyme preparation is acceptable only when a) the concentrated enzyme liquor
827 which would normally constitute the food enzyme is inherently unstable or b) when there is

[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.

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828 concern that the method used to produce a preparation may result in a potential carry-over
829 of hazardous material into a food.

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

837 2.3 Characterisation of the food enzyme

838 2.3.1 Properties of the food enzyme

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

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

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

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

865 2.3.2 Chemical parameters

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

872 The parameters measured should be the enzyme activity or activities under application
873 expressed as Units/g batch, and the concentration (in % w/w) of total protein, ash and water.

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874 From these data, the percentage of TOS should be calculated (as 100% - % water - % ash)
875 and the enzyme activity/mg TOS determined. It is recognised that variation between batches
876 is to be expected in a food enzyme which represents an intermediate in the production process
877 before the introduction of excipients which allow a greater degree of standardisation. The
878 data will be used to judge the extent of variation encountered and, in particular, to judge in
879 conjunction with information on protein patterns, whether the food enzyme batches used for
880 toxicological or other studies can be considered representative. For this reason, it is essential
881 that the same data set is provided for all additional batches of the food enzyme used for the
882 toxicological or other studies.

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

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

890 2.3.3. Purity

891 Quantitative values for lead, cadmium, mercury and arsenic should be reported as a routine,
892 and other elements if they occur in concentrations which may give rise to concern. Batches
893 of the food enzyme should also be screened for pesticide residues unless cultivation is under
894 the direct control of the applicant and it can be guaranteed that no pesticides were used. A
895 screening for relevant mycotoxins should also be provided.

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

899 Microbiological purity should be established. *Escherichia coli* should not be detected in 25 g of
900 food enzyme (FAO/WHO 2006), measured according to ISO method 16649-3:2014 or a
901 validated alternative method. *Enterobacteriaceae* should not exceed 10 CFU/g measured
902 according to ISO 21528-2:2017 or a validated alternative method. Filamentous fungi and yeast
903 should not exceed 100 CFU/g in the food enzyme measured according to ISO 21527-1:2008.

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

909

910 3 Food enzymes of animal origin

911 3.1 Source of the food enzyme

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

915 Information should be provided on which animal tissue is used for the production of the food
916 enzyme, and how the tissue is sourced. Animal tissue should be suitable for human

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917 consumption according to Regulation (EU) No 2015/1162[14]. The history of human
918 consumption of the tissue(s) in question should be provided, in particular among European
919 populations.

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

925

926 3.2 Production of the food enzyme

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

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

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

942 3.2.1. Extraction

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

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

950 3.2.2. Downstream processing

951 The specific methods used to remove the animal tissue after extraction, to concentrate the
952 enzyme liquor and to remove microbes from the food enzyme should be fully described. All
953 processing aids used during concentration/purification should be specified. These should be
954 the actual materials used.

[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.

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955 ### 3.2.3. Food enzyme preparation

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

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

967

968 ## 3.3 Characterisation of the food enzyme

969 ### 3.3.1. Properties of the food enzyme

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

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

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

989 ### 3.3.2. Chemical parameters

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

996 The parameters measured should be the enzyme activity or activities under application
997 expressed as Units/g batch, and the concentration (in % w/w) of total protein, ash and water.
998 From these data, the percentage of TOS should be calculated (as 100% - % water - % ash)
999 and the enzyme activity/unit TOS determined. It is recognised that variation between batches

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1000 is to be expected in a food enzyme which represents an intermediate in the production process
1001 before the introduction of excipients which allow a greater degree of standardisation.

1002 The data will be used to judge the extent of variation encountered and whether the food
1003 enzyme batches used for toxicological or other studies can be considered representative. For
1004 this reason, it is essential that the same data set is provided for all additional batches of the
1005 food enzyme used for the toxicological or other studies.

1006 If the use of a food enzyme preparation is unavoidable, the TOS content equivalent to that of
1007 a food enzyme may be calculated as 100% - % water - % ash – % total added organic
1008 excipients as defined by JECFA (FAO/WHO, 2006).

1009 Methods of analysis together with certificates of analysis covering each measured parameter
1010 should be provided, whether made in-house or by a third party.

1011 3.3.3. Purity

1012 Data on chemical contamination is not required provided the food enzyme derives from
1013 animals considered fit for human consumption according to European standard.

1014 Microbiological purity should be established in three batches of the food enzyme. *Escherichia*
1015 *coli* should not be detected in 25 g of food enzyme (FAO/WHO 2006), measured according to
1016 ISO method 16649-3:2014 or a validated alternative method. *Enterobacteriaceae* should not
1017 exceed 10 CFU/g measured according to ISO method 21528-2:2017 or a validated alternative
1018 method. *Campylobacter jejuni* and *C. coli; E. coli* STEC (for ruminants); *Salmonella* spp. should
1019 not be individually detected in 25 g of food enzyme measured according to ISO methods 1027-
1020 1:2016, TS13136:2012 and 6579-1:2017, respectively or validated alternative methods. Data
1021 on filamentous fungi and yeast should not exceed 100 CFU/g in the food e enzyme measured
1022 according to ISO method 21527-1:2008 or validated alternative methods.

1023 For enzyme derived from pig tissues the absence of Hepatitis E virus should be demonstrated
1024 in three batches of the food enzyme.

1025 Methods of analysis together with certificates of analysis covering each measured parameter
1026 should be provided, whether made in-house or by a third party. The LoD/LoQ for each method
1027 should be given. The same batches as used for chemical characterisation should be analysed.
1028 The certificates of analysis should include sufficient identification of the test item to ascertain
1029 that it is in fact the food enzyme under application.

1030

1031 *The following sections apply to all food enzymes regardless of source.*

1032

1033 4 Toxicological studies

1034 Toxicological studies are required for all food enzymes unless specifically exempted. These
1035 will normally consist of *in vitro* tests for genotoxicity and *in vivo* studies for systemic toxicity.
1036 Only when the results of these tests indicate a potential issue, additional studies will be
1037 requested. Studies performed following guidelines which can be regarded as equivalent to the
1038 OECD Test Guidelines (TG) and OECD Good Laboratory Practice (GLP), respectively, can be
1039 accepted. In such cases comparability of the applied guidelines described in Council Directives
1040 2004/10/EC[13] and 2004/09/EC[14] should be provided along with a statement of GLP compliance
1041 of the laboratory conducting the study. The most recent version of any guideline should be
1042 applied.

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1043 ## 4.1. Exemptions from toxicity testing (other than allergenicity)

1044
1045 In the following circumstances, the need for toxicity testing is waived:
1046

1047 a) For food enzymes obtained from microbial sources (GM and non-GM) which meet the
1048 requirements of the QPS approach to safety assessment (namely, (i) the production
1049 strain is non-equivocally identified as belonging to a species included in the QPS list,
1050 (ii) it meets any QPS qualification, and (iii) no concerns are raised by the genetic
1051 modification), and in addition no safety issues are raised by the manufacturing process.
1052 For those cases where the QPS approach cannot be applied because of the presence
1053 of AMR genes, toxicity testing may still be waived if no viable cells and DNA are
1054 detected, as specified in section 1.3.4.2.
1055 b) For food enzymes obtained from microbial sources when appropriate substitute
1056 toxicological data are available. In general, substitute toxicological tests are
1057 acceptable, if they meet all the following:
1058 - the test material is a food enzyme from a microbial strain belonging to the
1059 same strain lineage as the production strain of the enzyme under assessment;
1060 - no additional conventional mutagenesis has been applied in the development
1061 of the production strain compared to the proposed substitute strain;
1062 - any difference in genetic modifications between the production strain
1063 compared to the proposed substitute strain is well characterised and of no
1064 concern (see Section 1.1.11). The strategy for the genetic modification should
1065 be based on targeted integration, deletion or editing at known genomic loci in
1066 the production strain. It should be determined whether any insertion (intended
1067 or unintended) in the production strain has interrupted any ORF involved in the
1068 regulation of the biosynthesis of mycotoxins of other metabolites of known
1069 toxicity. This should be studied by WGS analysis;
1070 - it should be demonstrated that the raw materials used and the manufacturing
1071 processes of both food enzymes are comparable. A full list of the actual raw
1072 materials used and a detailed description of the production process of the
1073 enzyme used as the substitute item should be provided.
1074 c) For food enzymes derived from plants and animals that are consumed by the European
1075 population, two criteria must be met: (i) no hazard is introduced through the
1076 manufacturing process, and (ii) when it can be demonstrated that the dietary exposure
1077 to the food enzyme TOS is within the same magnitude as the dietary intake of the
1078 fraction of the plant or animal material comparable to the food enzyme TOS.
1079 d) For animal derived rennet, provided that no safety issues are raised by the
1080 manufacturing process.
1081 e) When it can be demonstrated that there is no (or negligible) carry-over of the food
1082 enzyme TOS into the final food products.
1083

1084 ## 4.2 The test item and dose level

1085 The purpose of toxicity testing is to enable a conclusion about the safety of the food enzyme
1086 as a component of an enzyme preparation. The applicant should ensure that all other materials
1087 added to the food enzyme when formulating the preparation as placed on the market are
1088 compliant with EU food legislation and safe.

1089 The batch(es) used for the toxicological studies should be representative of the commercial
1090 batches, as judged by the unit:TOS ratio (with a similar (or higher) TOS content and a similar
1091 (or lower) enzyme:TOS ratio). It is recognised that practical constraints may require de-

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1092 watering or drying of the food enzyme before its use as a test item, however, this should not
1093 affect the enzyme:TOS ratio. The unit used to determine enzyme activity should be the same
1094 as that used in the commercial batches. If a different unit is used, a conversion factor should
1095 be provided.

1096 The batch(es) should be characterised for chemical composition and purity as described in
1097 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
1098 confirm that the test item is the food enzyme under application.

1099 Depending on the test, the units should be expressed as µg TOS/plate, µg TOS/mL or mg
1100 TOS/kg bw per day. The selection of the concentrations/doses should be justified.

1101

1102 4.3 Genotoxicity

1103 Food enzymes are complex mixtures of unidentified components except for the declared
1104 enzyme(s). The recommended approach for the genotoxicity assessment of such a type of
1105 mixture is to test the whole mixture (EFSA SC, 2019).

1106 The following two *in vitro* tests are recommended as the first step (EFSA SC, 2011):

1107 – bacterial reverse mutation assay (OECD TG 471), and
1108 – *in vitro* mammalian cell micronucleus test (OECD TG 487).

1109 This combination of tests fulfils the basic requirements to cover the three genetic endpoints
1110 with the minimum number of tests: the bacterial reverse mutation assay covers gene
1111 mutations and the *in vitro* micronucleus test covers both structural and numerical
1112 chromosomal aberrations.

1113 When the food enzyme activity may affect the performance of the *in vitro* tests (e.g.,
1114 inactivation of the post mitochondrial rat liver S9 fraction), inactivated food enzyme could be
1115 used as the test item. In such case, the use of inactivated enzyme should be justified.
1116

1117 **Ames test**

1118 In order to overcome potential problems with histidine or tryptophan in the food enzyme
1119 batch, it is recommended to expose the *Salmonella* and *E. coli* strains to the tested food
1120 enzyme in the liquid culture ('treat and plate assay'), instead of the traditionally 'plate
1121 incorporation assay'. A recommended protocol incorporating treat and plate is given in Annex
1122 D. The recommended maximum test concentration for soluble non-cytotoxic substances is at
1123 least 5 mg TOS/plate. This concentration is necessary to ensure sufficient level of exposure
1124 to detect the majority of known genotoxic compounds (Kenyon et al., 2007).

1125

1126 If the Ames test is not applicable, alternatively a test for induction of gene mutations in
1127 mammalian cells, preferably the mouse lymphoma *tk* assay (OECD guideline 476), could be
1128 performed, but it needs to be justified.
1129

1130 ***In vitro* micronucleus test**

1131 The highest test concentration should correspond to 2 mg TOS/mL, if no precipitate or limiting
1132 cytotoxicity is observed. However, the top concentration may need to be higher than
1133 recommended, e.g., up to 5 mg TOS/mL, to increase the concentration of each of the
1134 components in the absence of sufficient cytotoxicity (OECD TG 487).

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1135 ***In vivo* follow-up**

1136

1137 In case of one or more positive *in vitro* tests, further testing may be required to determine
1138 whether the hazard is expressed *in vivo*, unless it can be adequately demonstrated that the
1139 positive *in vitro* findings are not relevant for the *in vivo* situation.
1140 *In vivo* tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to
1141 appropriate target organs or tissues.

1142 In line with the recommendation of the EFSA Scientific Committee (EFSA SC, 2011 and 2017),
1143 the following *in vivo* tests are considered as suitable follow-up for substances positive in the
1144 *in vitro* basic battery:

1145 – *in vivo* mammalian erythrocyte micronucleus assay for *in vitro* clastogens and
1146 aneugens (OECD TG 474);
1147 – *in vivo* mammalian alkaline comet assay for substances which cause gene mutations
1148 and/or structural chromosomal aberrations (OECD TG 489);
1149 – transgenic rodent gene mutation assay to follow-up *in vitro* positive compounds for
1150 gene mutations (OECD TG 488);
1151 – a combination of an *in vivo* micronucleus assay and a Comet assay in the event of a
1152 positive *in vitro* micronucleus assay.

1153 For further guidance on the *in vivo* follow-up of substances positive in the *in vitro* basic battery,
1154 the Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA,
1155 2019) should be consulted.

1156

1157 4.4 Repeated dose 90-day oral toxicity study in rodents

1158 A sub-chronic oral toxicity study should be provided for assessment of systemic toxicity. The
1159 protocol according to the OECD TG 408 is recommended. A highest dose selected should be
1160 at least 1,000 mg TOS/kg bw per day, unless technological considerations will not allow doses
1161 of this magnitude.

1162

1163 Relevant historical control data should be provided to enable the judgment of the validity of
1164 the study as proposed in OECD TG 408 and in Commission Regulation (EU) No 283/2013[15].
1165 Decisions on whether additional studies are needed will be taken by EFSA on a case-by-case
1166 basis, in the event of the identification of an adverse effect.

1167

1168 4.5 Allergenicity studies

1169 The assessment of potential allergenicity considers a) the source of the food enzyme; b) the
1170 declared enzyme(s) and c) any proteinaceous material with known allergenic properties
1171 included in a fermentation medium.

1172

1173 Quantifying the risk for allergenicity is currently not considered possible in view of the varying
1174 individual susceptibility to food allergens. Even trace amounts of food enzyme protein
1175 transferred to food could present a hazard for sensitised individuals. Thus, an argument based
1176 on the lack of transfer will only be accepted if it can be demonstrated that food enzyme TOS
1177 is absent in the final food. For all other cases, the following information is required:

[15] 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.

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1178 1. An alignment-based study using the amino acid sequence of the food enzyme
1179 and reporting any sequence identity greater than 35% to a known allergen using
1180 a sliding window of 80 amino acids as proposed by FAO/WHO (2001). The database
1181 of known allergens used should be specified and the hits found discussed and
1182 supported by available literature information.

1183 2. A search of the literature for reports that the specific enzyme(s) under
1184 application may give rise to sensitisation or elicitation reactions. Any information
1185 available on the enzyme resulted from the search of the literature and taking into
1186 consideration the route of exposure should be presented. For instance, studies
1187 which may have been conducted for other purposes, such as the assessment of
1188 safety at the workplace (e.g., sensitisation studies) should be submitted.
1189

1190 3. A search for possible allergic reactions caused by enzymes of the same family
1191 of enzymes as the one under application should be performed. In the case allergic
1192 reactions have been observed, a rationale should be given on how these
1193 observations do or do not impact on the evaluation of the potential allergenicity of
1194 the enzyme under application.
1195

1196
5 Dietary exposure

1197 In the initial stages of the dietary exposure assessment the intended uses are reviewed with
1198 the purpose to decide if exposure estimates need to be derived. Where a full dietary exposure
1199 assessment is carried out, exposure estimates are compared to the reference point identified
1200 from the toxicological studies and the margin of exposure (MoE) is calculated. Alternatively,
1201 in the case of food enzymes that are derived from edible parts of plant or animals intended
1202 to be or reasonably expected to be ingested by humans, the food enzyme TOS is compared
1203 to a comparable fraction of the source material.

1204

1205
5.1 Intended use of the food enzyme

1206 All food manufacturing processes for which the food enzyme is intended to be used should be
1207 listed. The description of each food manufacturing process should, where possible, be aligned
1208 with the definition provided in Annex E of this guidance.

1209 Each intended use of the food enzyme should be supported by a description of the
1210 technological need and function of the food enzyme during manufacturing and describe the
1211 expected benefits of its use.

1212 Where the proposed use of the food enzyme cannot be (fully) aligned with one of the food
1213 manufacturing process listed in Annex E, full details of each food manufacturing process
1214 should be provided, illustrated by a flow chart. The stages where the food enzyme is added
1215 during the manufacturing process and in particular, any process (e.g. purification and filtration
1216 steps) that may result in the partial or complete removal of contaminating material (including
1217 food enzyme TOS) from the final food should be clearly indicated.

1218 It is recommended that information on intended use and use levels is provided using the
1219 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**

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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)

The amount of food enzyme to be added should be provided for each food manufacturing process whether or not included in Annex E. 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 Annex E 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 Annex E. 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 strong 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.

1261 In order to establish consumption in a comparable way, EFSA requires the following
1262 information:

1263 - evidence of consumption of the edible plant part or animal tissue, including quantity
1264 of consumption in the EU or elsewhere and reference to the source of information;
1265 - information on the enzyme yield factor, e.g., x amount (kg) of source material to obtain
1266 y amount (kg or g) of food enzyme (not food enzyme preparation).

1267 5.4 Calculation of exposure

1268 Dietary exposure is estimated where presence of TOS in the final food product cannot be
1269 excluded. Consequently, each food enzyme application will require assessment of each
1270 individual food processes for which the food enzyme is intended to be used.

1271 Given the complexity associated with assessing the exposure to food enzymes which are
1272 added to raw materials, which are then processed using different food manufacturing
1273 processes resulting in a carry-over of the food enzyme into food as consumed, EFSA developed
1274 a new methodology. This takes into account the nature of the use of food enzymes and their
1275 fate during food processing and the individual consumption data reported in the EFSA
1276 Comprehensive European Food Consumption Database. The Comprehensive Database
1277 represents the best available source of food consumption data across Europe at present
1278 covering infants, toddlers, children, adolescents, adults and the elderly.

1279 To aid applicants, process-specific exposure tools, namely the Food Enzyme Intake Models
1280 (FEIM), based on summary statistics, have been developed. Process-specific calculators (e.g.,
1281 FEIM-baking, FEIM-brewing) can be accessed on the EFSA webpage[16] or via Annex E of this
1282 guidance. Additional calculators are in the process of being developed. It is envisaged to
1283 create an overall assessment tool once all process-based models have been developed.

1284 Applicants should use all FEIM calculators available as appropriate and submit exposure
1285 estimates for each process separately, in the tabular format provided below. Exposure
1286 estimates to food enzymes should be reported for all six population groups (infants, toddlers,
1287 children, adolescents, adults and the elderly), as mg TOS/kg body weight, as appropriate. For
1288 both mean and 95th percentile intake, the range of minimum–maximum value observed
1289 across the selected surveys contained in the EFSA database should be reported.

1290

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**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)

1291 Applicants are required to report an exposure assessment only when an appropriate FEIM
1292 calculator is available. In case no suitable calculator is available, applicants should provide as
1293 much detail on the process, including all raw materials to which the food enzyme is added,
1294 the food groups in which the end product is going to be used, as well as information on use
1295 levels and yield factors, as applicable. This information will aid EFSA in developing additional
1296 food processes-based exposure models.

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

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1297 If the food enzyme is involved in more than one food manufacturing processes, EFSA will
1298 calculate combined exposure based on raw data contained in the Comprehensive Database.

1299

1300 5.5 Risk characterisation

1301 The purpose of deriving a quantitative estimate is to facilitate characterisation of any risk
1302 associated with such exposure to the European population. The risk is characterised through
1303 a comparison of the estimated human exposure with the reference point (e.g., no observed
1304 adverse effect level or benchmark dose level) determined in 90-day oral toxicity studies
1305 performed on animals. A ratio, referred to as the margin of exposure (MoE), between the
1306 reference point and the estimated exposure is used to conclude on the safety of the food
1307 enzyme.

1308 The MoE is calculated as follows: MoE = Reference point/highest P95 value

1309 The first estimate of the MoE is made using exposure data for all uses of a food enzyme. Only
1310 where the overall MoE is considered low, would the MoE for each food manufacturing process
1311 be separately calculated to establish whether the overall low value is a consequence of a
1312 single food manufacturing process.

1313 For applications concerning 'food enzymes that are obtained from edible parts of plant or
1314 animals intended to be or reasonably expected to be ingested by humans', a comparison is
1315 made between the exposure to the food enzyme resulting from its intended uses (following
1316 the described process above) and the exposure to a similar fraction of the source material
1317 resulting from the consumption of foods derived from this source. The outcome of this
1318 comparison is one of the criteria to justify that no toxicological data are required.

1319

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1320 # Part B. Data required for the safety assessment of modifications
1321 to an existing authorisation

1322 Article 14 of Regulation (EC) No 1332/2008 requires producers or users of food enzymes to
1323 inform the EC of any new scientific or technical information which might affect the assessment
1324 of the safety of a food enzyme. It is foreseen for those food enzymes already included in the
1325 Community list, that a significant change in the production methods or starting materials
1326 should be re-submitted for evaluation. In the view of EFSA, the use of a different strain of
1327 microorganism, the use of a different plant species or plant part or the use of a different
1328 animal species or tissues would constitute the production of a new food enzyme and thus
1329 require a full safety assessment as described in Part A of this guidance.

1330 Where a request to update the Community list of food enzymes involves a less significant
1331 change, defined in the Regulation (EC) No 1331/2008 as *'adding, removing or changing*
1332 *conditions, specifications or restrictions associated with the presence of a substance on the*
1333 *Community list'* the EC is only obliged to seek the opinion of EFSA if the proposed changes to
1334 the authorisation are thought liable to have an effect on human health.

1335 Changes which may impact human health could include any modification to a production
1336 system which leads to qualitative and quantitative changes in the TOS associated with the
1337 final food product or proposals for the use of the food enzyme in additional food manufacturing
1338 processes. For such cases, since the source of the food enzyme remains unchanged, a limited
1339 dataset is required.

1340 ## Data requirements

1341 Proposals to change an existing authorisation should reference the authorisation given in the
1342 Community list and indicate the proposed change(s). A statement should be made confirming
1343 that the source of the food enzyme remains the same. In case the manufacturing process also
1344 remains unchanged this should be confirmed.

1345 ## New manufacturing process of the food enzyme

1346 Applicants should provide a full description of the manufacturing process(es) as described in
1347 Sections 1.2, 2.2 and 3.2 of Part A of this guidance indicating the change(s) proposed.

1348 Modifications to a manufacturing process do not necessarily introduce concerns for human
1349 health and, thus, proposals for change would be evaluated on a case-by-case basis. It is the
1350 responsibility of the applicant to identify and characterise the hazard, usually starting by
1351 reference to the available literature.

1352 In case the intended change in the manufacturing process would lead to a change in the TOS
1353 composition the risk to consumers has to be assessed. The concentration of any substance of
1354 concern should be measured in the food enzyme TOS and the extent of carry-over into the
1355 final food established for each authorised food manufacturing process. If detected in the final
1356 food, the applicant should use the overall exposure assessment made in the course of the
1357 initial assessment to calculate exposure to the substance of concern in the various population
1358 groups based on the concentration present in the food enzyme TOS. In the absence of
1359 published data identifying and characterising the hazard, the applicant should provide a full
1360 set of toxicological data and the information needed to allow the characterisation of risks to
1361 human health. Toxicological tests should be performed as described in Part A of this guidance.

1362 The introduction of a specification for the use of an immobilised or encapsulated form of the
1363 food enzyme for one or more of the food manufacturing processes specified under the

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1364 conditions of authorisation is not expected *per se* to alter the conclusions on the safe use of
1365 the enzyme unless:

1366 a) the immobilisation support or encapsulating material is potentially hazardous and is
1367 leached into the final food, or if
1368 b) any cross-linking or other agents used to bind the food enzyme to the support material
1369 is potentially hazardous and is leached into the final food.

1370 In these cases, analytical data on the extent of leaching and the concentration into final
1371 food(s) should be established. In the absence of published data identifying and characterising
1372 the hazard, the applicant should provide a full set of toxicological data and the information
1373 needed to allow the characterisation of risks to human health. Toxicological tests should be
1374 performed as described in Part A of this guidance.

1375 Extension of the intended uses of the food enzyme

1376 Each proposal for an additional food manufacturing processes should be clearly indicated.
1377 Applicants should provide a full description of each modified use as described in Section 5.1
1378 of Part A of this guidance.

1379 To determine the need for a dietary exposure resulting from the extended uses, the applicant
1380 should follow Section 5.2 of Part A of this guidance. For calculation of dietary exposure, the
1381 applicant should follow Sections 5.3 and 5.4 of Part A of this guidance.

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1485 # Annexes

1486 Some necessary information has been included in this guidance in the form of Annexes rather
1487 than appearing in the body of the text. This is to allow modifications to be made without the
1488 need for a full revision of the guidance. In particular, this will enable the easier addition of
1489 food manufacturing processes and reference to additional food process-based exposure
1490 models (FEIM calculators) as they are developed.

1491

1492 ## Annex A - Background as provided by the requestor

1493 Regulation (EC) No 1332/2008 lays down the rules on food enzymes used in foods with a view
1494 to ensure the effective functioning of the internal market whilst ensuring a high level of
1495 protection of human health.

1496 Food enzymes shall be subject to safety evaluation by the European Food Safety Authority
1497 (EFSA) and approval via a Union list. The inclusion of a food enzyme in the Union list is
1498 considered by the Commission on the basis of the opinion from EFSA, taking into account also
1499 other general criteria such as technological need, consumer aspects and, where relevant,
1500 other legitimate factors. For every food enzyme included in the positive list, intended use(s)
1501 in food and specifications, including the criteria on purity and the origin of the food enzyme,
1502 shall be laid down.

1503 The establishment of the Union list will take place in a single step after the Authority has
1504 issued an opinion on each food enzyme for which an application complying with the validity
1505 criteria laid down in accordance with Article 9(1) of Regulation (EC) No 1331/2008 had been
1506 submitted in accordance with Article 17(2) of Regulation (EC) No 1332/2008.

1507 Pursuant to Article 9 of Regulation (EC) No 1331/2008 the Commission adopted implementing
1508 measures that are laid down in Regulation (EU) No 234/20113 as regards the content, drafting
1509 and presentation of applications submitted under each sectoral food law, arrangements for
1510 checking the validity of applications and the type of information that should be included in the
1511 opinion of EFSA.

1512 Article 5 of Regulation (EU) No 234/2011 requires applicants to take into account the latest
1513 guidance documents adopted or endorsed by the Authority available at the time of the
1514 submission of the application for the safety evaluation of a food enzyme.

1515 Since the beginning of the period established for which an enzyme application may be
1516 submitted for the inclusion in the Union list, the EFSA reference guidance document has been
1517 the Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and
1518 Processing Aids on the Submission of a Dossier on Food Enzymes. This document was later
1519 supplemented by the Explanatory Note for the Guidance of the Scientific Panel of Food Contact
1520 Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on
1521 Food Enzymes to assist applicants to compose a technical dossier.

1522 Based on experience gained in assessing submitted dossiers, EFSA, on its own initiative, has
1523 updated the assessment methodology on food enzymes resulting in the adoption of two
1524 statements by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and
1525 Processing Aids :

1526 • Statement on the exposure assessment of food enzymes (EFSA CEF Panel,
1527 2016)

1528 • Statement on the characterisation of microorganisms used for the production
1529 of food enzymes (EFSA CEP Panel, 2019)

1530 These documents are intended to assist in the preparation and presentation of applications in
1531 accordance with Regulation (EU) No 234/2011.

1532 The Commission considers that it is desirable to update the 'Guidance on the Submission of a
1533 Dossier on Food Enzymes' (2009) taking into account new approaches on certain aspects of
1534 the risk assessment, in particular the statements issued on the exposure assessment of food
1535 enzymes and the characterisation of microorganisms used in the production of food enzymes.
1536 In the updated guidance, other relevant documents, including guidance documents produced
1537 by the EFSA Scientific Committee, could be taken into account as appropriate.

1538 In the preparation of the updated guidance, EFSA should take into account the relevant
1539 provisions of Regulation (EU) No 2019/1381 of the European Parliament and of the Council
1540 on the transparency and sustainability of the EU risk assessment in the food chain and should
1541 ensure consistency with other sectors where similar updates are envisaged.

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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[17] are GFF format (*.gff), GenBank format (*.gbff, *.gbk and *.gb), Tabular format (*.csv) and the NCBI's Sequin ASN.1 (*.sqn).
- For the characterization 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.

A list of the relevant data and the information to be reported in the technical dossiers should be duly completed and signed by the applicants at the time of submission. Such list can be found in Annex A of the EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain (EFSA, 2021).

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

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Annex C - Recommended protocol for the detection of cytotoxicity in *Bacillus* and related species included in the QPS list

A cytotoxicity test should be made to determine whether the strain produces high levels of non-ribosomal synthesised peptides, as one of the qualifications of the QPS approach. They should be made preferably with Vero cells or other epithelial cell lines using culture supernatant following the protocol described by Lindbäck and Granum (2005). Detection based on 14C-leucine uptake is described, but other methods such as those based on lactate dehydrogenase release or propidium iodide (PI) uptake could be used alternatively.

Preparation of test substance

Bacterial cells should be grown in brain heart infusion (BHI) broth at 30°C and harvested after 6 h when it is anticipated that cells will have reached a density of at least 108 CFU/mL. Cells should be removed by centrifugation at room temperature. Toxicity is determined using 100 µL of supernatant in the Vero cells assay.

Cell assay

Vero cells should be grown in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum. Cells should be seeded into 24-well plates two or three days before testing. Before use, it should be verified that growth of the Vero cells is confluent and if so, the medium should be removed and the cells washed once with 1 mL preheated (37°C) MEM medium. Then, the subsequent steps should be followed: add 1 mL preheated (37°C) low-leucine medium to each well and then add the toxin to be tested (100 µL of non-concentrated supernatant), incubate the cells for 2 h at 37°C.

Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37°C) low-leucine medium. Mix 8 mL preheated low-leucine with 16 µL 14C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 h at 37°C.

Remove the radioactive medium and add 1 mL 5% trichloroacetic acid (TCA) to each well, incubate at room temperature for 10 min. Remove the TCA, and wash the wells twice with 1 mL 5% TCA.

After removing the TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 min. Transfer the content of each well to liquid scintillation tubes with 2 mL liquid scintillation cocktail. Vortex the tubes and count the radioactivity in a scintillation counter for 1 min.

Percentage inhibition of protein synthesis is calculated using the following formula: ((Negative control – sample)/Negative control) × 100; the negative control is Vero cells from wells without addition of sample. Above 20% inhibition is considered to indicate cytotoxicity. As a positive control, surfactin or supernatants from known cytotoxic *Bacillus cereus* strains may be used.

Propidium iodide uptake

An alternative method is to measure propidium iodine (PI) uptake in Vero cell suspensions using a spectrofluorimeter. Two-day-old confluent monolayers of Vero cells should be used as described above. Cell suspensions containing a final concentration of about 10^6 cells in 2 mL EC buffer containing PI (5 µg/mL) should be held in a thermostatically controlled (37°C) 1 cm quartz cuvette to which the toxin is then added. Cells should be continuously mixed by the use of a magnetic stirrer and 'flea'. Fluorescence should be monitored every 30 seconds using excitation/emission wavelengths of 575/615 nm and 5 nm slits for both. Results are used without subtraction of background fluorescence. For this alternative method with PI uptake or lactate dehydrogenase release, values above 20% of the fluorescence/absorbance obtained

1605 from the positive control (usually detergent-treated cells) are considered to indicate
1606 cytotoxicity.

1607 Annex D - Recommended protocol for the 'treat and plate' modification of 1608 the bacterial mutagenicity test

1609 Except for the treatment of bacteria, the methodology and the reagents (medium, metabolic
1610 activation system (S9 mix), preparation of test item, solvents, culture conditions, choice of
1611 doses, etc.) are the same as those used for the 'plate incorporation' or 'pre-incubation' Ames
1612 test (OECD guideline 471).

1613 ***Treatment of the bacteria***

1614 A 0.5 mL aliquot of S9-mix or phosphate buffer 0.2 M pH 7.4 is combined with 0.1 mL late log
1615 bacterial culture in a sterile container. A 0.1 mL aliquot of the test solution containing the food
1616 enzyme is added. Bacteria and treatment are incubated for 90 min with shaking at 37°C. After
1617 the 90 min pre-incubation, a large volume (10 to 15 mL) of a wash solution of Oxoid No 2
1618 nutrient broth in phosphate buffered saline is added and the washed bacteria are collected by
1619 centrifugation (e.g., at 2000 g for 30 min). All but about 0.7-1 mL of the supernatant is
1620 removed and discarded, and the bacteria are re-suspended in the residual supernatant prior
1621 to mixing with the overlay agar and pouring onto the surface of a minimal agar plate (1.5%
1622 agar, Vogel–Bonner medium E, 2% glucose). In some cases, it is possible to perform a second
1623 washing of the bacteria. The plates are inverted and incubated at 37°C for 48 to 72 h. After
1624 the incubation period, the number of revertant colonies per plate is counted.

1625 ***Controls***

1626 Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and
1627 without metabolic activation, should be included in each assay. Positive control concentrations
1628 that demonstrate the effective performance of each assay should be included. Sterility control
1629 is included in each experiment.

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Annex E - Food manufacturing processes and estimation of dietary exposure to the food enzyme TOS

1640 This annex provides description of food manufacturing processes to which food enzymes may
1641 be added, as well as, the way to estimate the dietary exposure. The food manufacturing
1642 processes listed below are aligned with those in the '*EC working document on food processes*
1643 *in which food enzymes are used*'[21].

1644
1. Baking processes

1645 Baking processes are those which rely on flour as the principal raw material and lead to the
1646 production of foodstuffs such as, but not limited to, bread, biscuits, cakes, pastries, tortillas
1647 and baking specialties. Food enzymes are usually added during the mixing of the dough
1648 ingredients.

1649 Dietary exposure can be estimated by using the calculator 'FEIM_baking', which can be
1650 downloaded from http://zenodo.org with the doi 10.5281/zenodo.4382037.

1651
2. Brewing processes

1652 Brewing processes are those which rely on cereals as the principal raw material, and following
1653 a fermentation, lead to the production of beer or other cereal-based beverages.

1654 Dietary exposure can be estimated by using the calculator 'FEIM_brewing', which can be
1655 downloaded from http://zenodo.org with the doi 10.5281/zenodo.4382046.

1656
3. Distilled alcohol production

1657 Distilled alcohol production processes are those which rely on starch or other carbohydrate-
1658 rich agricultural commodities as raw materials and lead to the production of products such as,
1659 but not limited to vodka, gin and whisky. The starch contained in raw materials is extracted
1660 and/or hydrolysed by enzymes, followed by a yeast fermentation. The ethanol produced
1661 during fermentation is subsequently recovered by distillation.

1662 Food enzyme TOS is not expected to be carried over with the distillate. Experimental evidence
1663 has been supplied by industry confirming this expectation (>99% removal). Consequently,
1664 the need for dietary exposure is waived for this process.

1665
4. Starch processing for glucose syrups production and other starch hydrolysates

1666 Starch processing relies on starch from various crops (e.g., cereals and potatoes) as the raw
1667 material. The starch fraction obtained from the raw material is transformed enzymatically into
1668 starch derivatives such as maltodextrins, maltose and glucose syrups and glucose, which are
1669 subsequently used in a variety of food products.

1670 Production of carbohydrate-rich syrups for food use, typically involves decolourisation with
1671 activated charcoal or similar and treatment with ion-exchange resins. These purification
1672 processes are expected to remove food enzyme TOS from the final food product. Experimental
1673 evidence has been supplied by industry confirming this expectation (>99% removal).
1674 Consequently, the need for dietary exposure is waived for this process.

1675
5. Cereal-based processes

1676 Cereal-based processes rely on flour as the principal raw material and lead to the production
1677 of products such as, but not limited to pasta, noodles, breakfast cereals and snacks (e.g.,
1678 muesli bars, popcorn, maize and rice crisps), extruded and/or puffed cereals.

1679 Dietary exposure can be estimated by using the calculator 'FEIM_cereal', which can be
1680 downloaded from http://zenodo.org with the doi 10.5281/zenodo.4382057.

1681

1682 ### *6. Grain treatment for the production of starch and gluten fractions*

1683 Grain treatment is the process whereby cereals (grains or grist) are milled and processed in
1684 order to be fractioned into starch, gluten and solubles. Solubles are typically used as feedstuff.

1685 Food enzyme TOS is water-soluble and is removed firstly in the soluble fraction, and further
1686 removed by repeated washing with water from the starch and gluten fractions. Experimental
1687 evidence has been supplied by industry confirming this expectation (>99% removal).
1688 Consequently, the need for dietary exposure is waived for this process.

1689 ### *7. Manufacture of speciality carbohydrates*

1690 Carbohydrate processing and conversion rely on disaccharides or oligosaccharides (e.g.,
1691 sucrose, lactose, inulin) or glucose syrups as the principal raw material and lead to the
1692 production of products such as, but not limited to, fructo-oligosaccharides, galacto-
1693 oligosaccharides and glucose-fructose syrups.

1694 When technical information (see section 5.1) and analytical data supporting the
1695 removal/absence of transfer of TOS (see section 5.2) are included in the submission, dietary
1696 exposure can be waived for these processes. Otherwise, the data needed to complete an
1697 exposure assessment are required.

1698 ### *8. Coffee bean demucilation*

1699 Coffee processing relies on raw coffee cherries as the principal raw material. Demucilation is
1700 the removal of the mucilage coat from the coffee cherries in the fermentation step which may
1701 be accelerated by the enzymatic treatment.

1702 Since the released beans are washed free of the degraded seed coat, the food enzyme TOS
1703 is not expected to remain and dietary exposure is waived for this process.

1704 ### *9. Coffee processing*

1705 Coffee processing relies on demucilated coffee beans as the principal raw material and covers
1706 any aspect of coffee processing after the fermentation step.

1707 EFSA is developing a FEIM calculator for this process.

1708 ### *10. Wine and wine vinegar production*

1709 Wine making processes rely on grapes as the main raw material in the production of wine and
1710 wine vinegar. The process involves fermentation of grapes or grape musts with yeast,
1711 optionally followed by a malolactic fermentation with bacteria.

1712 EFSA is developing a FEIM calculator for this process.

1713 ### *11. Egg processing*

1714 Egg processing relies on raw eggs (without shells) and/or fractions of eggs (yolk or egg white)
1715 as raw materials and leads to the production of products such as dried or pasteurised egg
1716 products.

1717 Dietary exposure can be estimated by using the calculator 'FEIM_egg', which can be
1718 downloaded from http://zenodo.org with the doi 10.5281/zenodo.4353056.

1719 ### *12. Modified lecithin production from egg*

1720 This food manufacturing process covers any modification to lecithin designed to improve
1721 functionality.

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1722 Dietary exposure should be estimated by multiplying the use level (mg of TOS/kg lecithin)
1723 with the summary statistics of consumption published by the EFSA ANS Panel (2017)[18].

1724 ***13. Refined and unrefined sugar production***

1725 Sugar production relies on sugar cane and sugar beet as the raw material and leads to the
1726 production of products such as, but not limited to, refined sugar, sugar syrups and molasses.

1727 Highly refined sugar (white and coloured) is a purified and crystalised product and is not
1728 expected to retain food enzyme TOS and so dietary exposure is waived for these products.
1729 Food enzyme TOS is, however, expected to remain with the less refined sugar products such
1730 as molasses.

1731 Dietary exposure can be estimated by using the calculator 'FEIM_molasses', which can be
1732 downloaded from http://zenodo.org with the doi 10.5281/zenodo.4354558.

1733 ***14. Hydrolysis of whey proteins for use in infant formula, follow-on formulae (FOF)***
1734 ***and food for special medical purposes***

1744 Because the intended use concerns vulnerable population groups, a separate process is
1745 established for enzymes that are used to produce whey protein hydrolysates for use in infant
1746 formula, follow-on formula and food for special medical purposes.

1747 The EFSA Scientific Committee derived a formula consumption value of 260 mL/kg bw per
1748 day, derived from 95th percentile consumption during the period of 14–27 days of life. This
1749 time reflects the highest relative consumption on a body weight basis and also covers the
1750 potential high consumption rates of preterm infants on enteral (formula) feeding.[19]

1751 The applicant can estimate the dietary exposure by using 260 mL formula/kg bw per day as
1752 the default consumption data. Dietary exposure should be estimated by multiplying the use
1753 level (mg TOS/kg protein) with a default factor of 1.96 g protein/100 mL formula and with the
1754 default consumption data of formula.

1755 ***15. Degumming of fats and oils***

1756 Crude oil extracted from different plant sources (e.g., oilseeds, fruit pulps) is the principal raw
1757 material. Water refining, usually called degumming, is the treatment of crude oils and fats
1758 with a small amount of water repeatedly to remove water-soluble impurities, followed by
1759 centrifugal separation to produce refined oils. The process is applied to many oils that contain
1760 phospholipids in significant amounts.

1761 Food enzyme TOS is water-soluble and is expected to follow the aqueous fraction and not the
1762 lipid fraction. Experimental evidence has been supplied by industry confirming this expectation
1763 (>99% removal). Consequently, the need for dietary exposure is waived for this process.

1764 ***16. Modification of fats and oils by interesterification***

1765 This food manufacturing process covers any modification of oils and fats designed to improve
1766 functionality through interesterification.

1767 When technical information (see section 5.1) and analytical data supporting the
1768 removal/absence of transfer of TOS (see section 5.2) and any material resulting from
1769 immobilisation are included in the submission, dietary exposure can be waived for this
1770 manufacturing process.

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[18] Available at https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2017.4742

[19] Available at https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2017.4849

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1771 Otherwise, dietary exposure can be estimated by using the calculator 'FEIM_modified fats',
1772 which can be downloaded from http://zenodo.org with the doi 10.5281/zenodo.4354782.

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Glossary and/or abbreviations and/or acronym

AMR	antimicrobial resistance
AMFEP	Association of Manufacturers and Formulators of Enzyme Products
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
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
ENA	European Nucleotide Archive
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
ITS	internal transcribed spacer
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kb	Kilo base
kDa	kiloDalton
LoD	limit of detection
LoQ	limit of quantification
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

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SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOP	Standard Operation Procedure
TOS	total organic solids
UniProt	Universal Protein
WHO	World Health Organization

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