Post-public consultation stakeholder event
21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Welcome and introduction

Andrew Chesson
Enzymes Working Group

Trusted science for safe food

efsa
European Food Safety Authority

Principles applied to developing the guidance

- **Primary audience**
  - Regulatory affairs personnel, consultants and those charged with the production of enzyme dossiers
  - Should assume a high level of technical expertise

Principles applied to developing the guidance

- **The guidance should be a stand-alone document**
  - When in force it will replace all other previous guidance documents and statements relating to the assessment of food enzymes

- *Where references are included in the guidance they are intended to:*
  - *Provide the origin and background of specific data requirements*
  - *Indicate compliance with risk assessment approaches developed by EFSA*

Principles applied to developing the guidance

- **The guidance should indicate why specific data is required** and how it relates to the overall risk assessment.
- **No guidance document can be exhaustive**, so should contain some flexibility
  - if data stipulated in the guidance are not considered by the applicant as relevant to a particular case, there should be an option to omit provided that the omission is fully justified.
  - The W/G should be able to request additional data or tests other than those indicated in this document if required for a specific evaluation

Principles applied to developing the guidance

- **The guidance should reflect the structure of the food enzyme opinions**

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

Risk management matters
State of play

Catherine EVREVIN
Policy Officer– Food Improvement Agents
Unit E2: Food Processing Technologies and Novel Foods
DG SANTE, European Commission

EFSA Stakeholder event on Enz 21-22 June 2021, virtual meeting

The views expressed are purely those of the speaker and may not in any circumstances be regarded as stating an official position of the European Commission

Food Enzymes Legislation

General food law, horizontal legislation – e.g. food hygiene, official controls

Specific legislation on FIA's (CAP, R. 1331/2008, R. 234/2011, R. 562/2012) and on enzymes (R. 1332/2008, R. 1056/2012) – scope, conditions of use, labelling, register, Union list

Risk Management matters discussed by the COM services and MS at the Working Party mtgs of Governmental Experts on Food Enzymes

In 2020 – 2 mtgs (April and December)

In 2021 – 2 mtgs (April and October)

Risk management matters

WGE 21 01:

1) Approval of the agenda
2) State of play of food enzyme dossiers submission
3) EFSA update on the evaluation process
4) Adopted opinions
5) Appraisal of the technological need for food enzymes
6) Working document describing the food processes in which food enzymes are intended to be uses
7) Draft Union list
8) AOB

Risk management matters

- **Register**
  fs_food-improvement-agents_enzymes_register.pdf (europa.eu)
- **New applications 44**

28 received in 2021 before the entry into application of the Transparency Regulation

- **New e-submission system in place since 27 March 2021**
- **Mandate to reopen the exposure part of certain EFSA opinion**

Risk management matters

Establishment of the Union list?

Register of all valid food enzymes to be considered for inclusion in the Union list published in April 2020

+

New applications fulfilling the criteria of Article 6 of Regulation 1332/2008

Name	Specifications	Foods	Conditions of use	Restrictions on the sale of the food enzyme to the final consumer	Specific requirement in respect of labelling of food

Risk management matters

Conditions of use

RA

*Do not pose **a safety concern** to the health of the consumer at the level of use proposed;*

RM

*Reasonable **technological need** for their use;*

*Its use does not **mislead the consumer***

Risk management matters

Appraisal of the technological need and misleading the consumer?

- **General approach for different food processes**

  Fruit&Vegetable processes

  Wine processes

- **Drafting of the EU list based on published opinion on a specific enzyme**

  Lipases

Risk management matters

Working document describing the food processes?

- **Updated version dated end of 2020**

- **New version under consultation by stakeholders**

Thank you for your attention!

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21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Production and Characterization of the Food Enzyme

Jose M Barat

Enzymes Working Group

Trusted science for safe food

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European Food Safety Authority

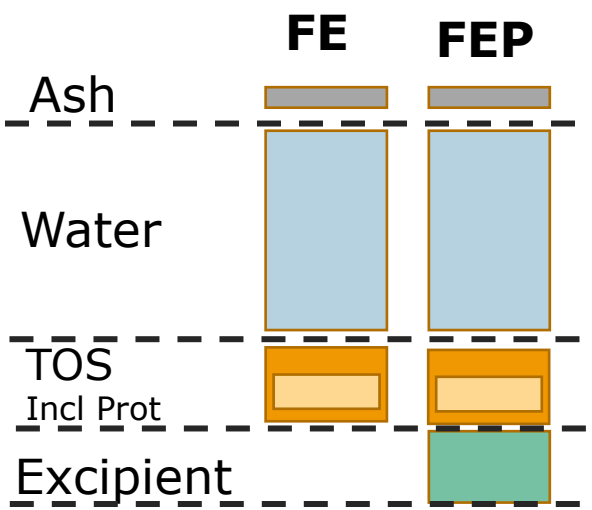
Production of the Food Enzyme

- Fermentation
- Downstream processing
  - Food enzyme preparations

picture: GettyImages-Artis777

Production of the Food Enzyme

- **Fermentation**
  - Safety of the commercial product can be affected by **all those elements that can end up in the food enzyme** as a consequence of this step, not only the very specific enzyme. They can also affect the potential allergenicity of the final product
    - The microorganisms or parts of them (e.g. AMR genes)
    - The metabolites produced during fermentation (e.g. mycotoxins)
    - The products used during fermentation (e.g. antibiotics)



The food enzyme will contain a lot of **unknown compounds** coming from the microorganisms. That is why the Total Organic Solids (**TOS**) concept is used.

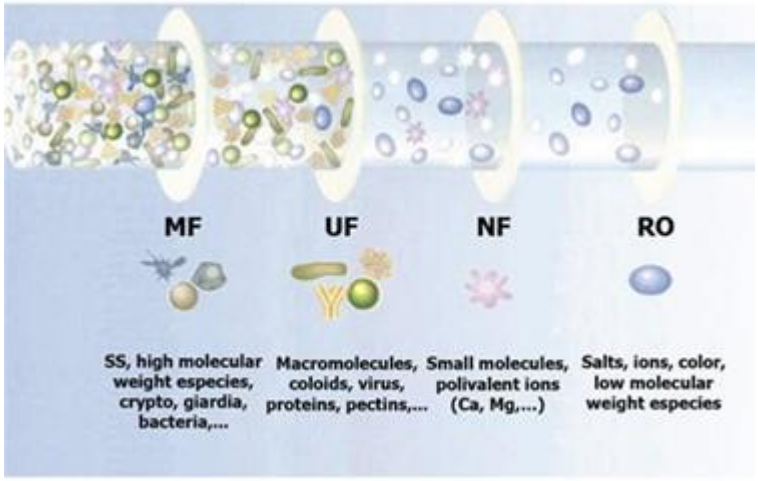
- For Food Enzymes (**FE**), TOS=100% - % water - % ash

- For Food Enzyme Preparations (**FEP**), TOS=100% - % water - % ash – % total added organic excipients

Production of the Food Enzyme

- **Downstream processing**

  The downstream processing is related to the safety of the food enzyme, not only because of being a potential source of substances of concern (e.g. microbial killing agents), but mostly because it modifies the properties of the fermentation products making them safer (e.g. killing of fermentation microorganisms, and purification steps).



Source: www.wateronline.com

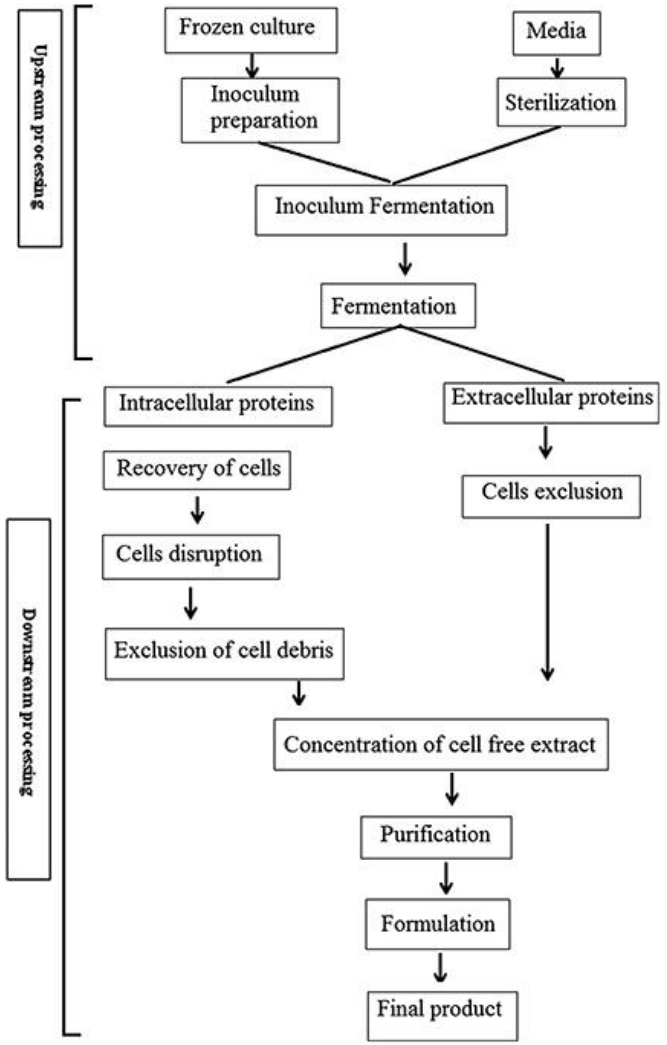
Source: bionet.com

Production of the Food Enzyme

- **Full details** of the production process for the food enzyme should be provided
  - **Flow chart** showing each step in the production and downstream process
  - Differences in the production process, depending on the production site, should be reported.

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

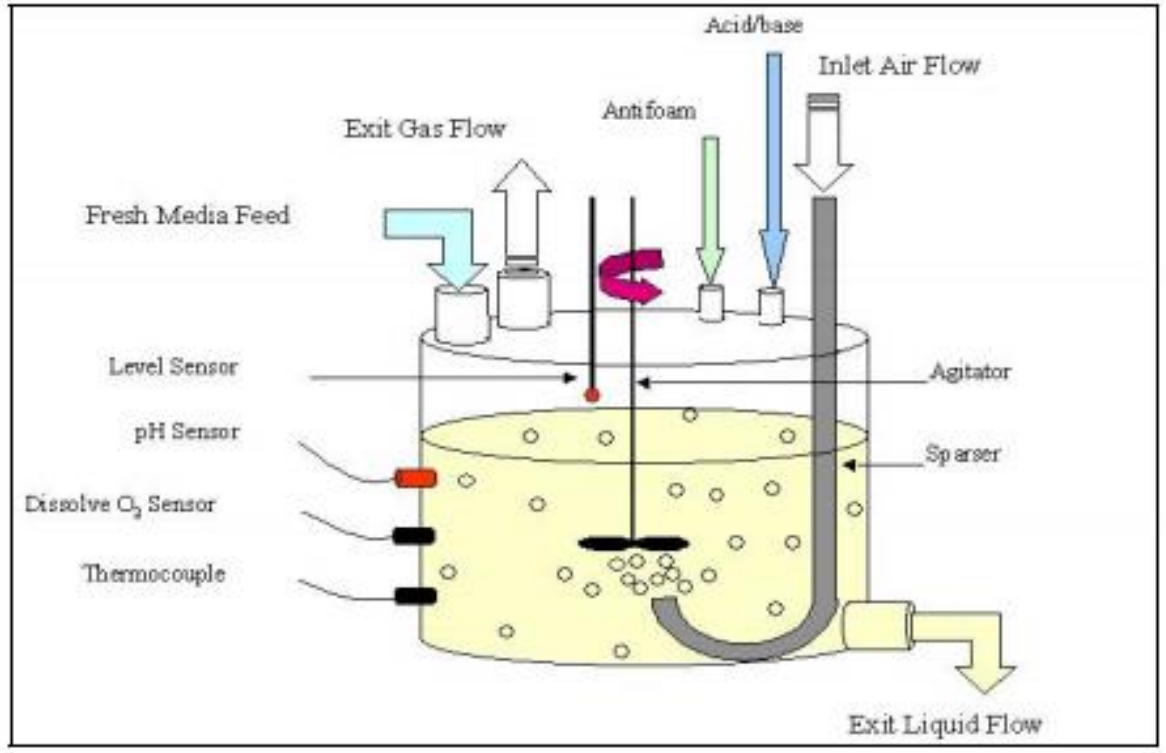
Statement: The production of the food enzyme will meet food safety management system principles (Commission Notice C/2016/4608) and accords with the Food Hygiene Regulation (Regulation (EC) No 852/2004). O**utside the EU:** similarity to the equivalent EU requirements confirmed.



Source: Introduction to Pharmaceutical Biotechnology. Vol 2. Enzymes, proteins and bioinformatics

Production of the Food Enzyme

- **Fermentation**
  - **Type of the fermentation** system used (e.g., continuous, fed-batch or solid state)
  - **List** of the raw materials, reagents and processing aids is required. These should be the actual materials used; **an indicative list will not be accepted**.



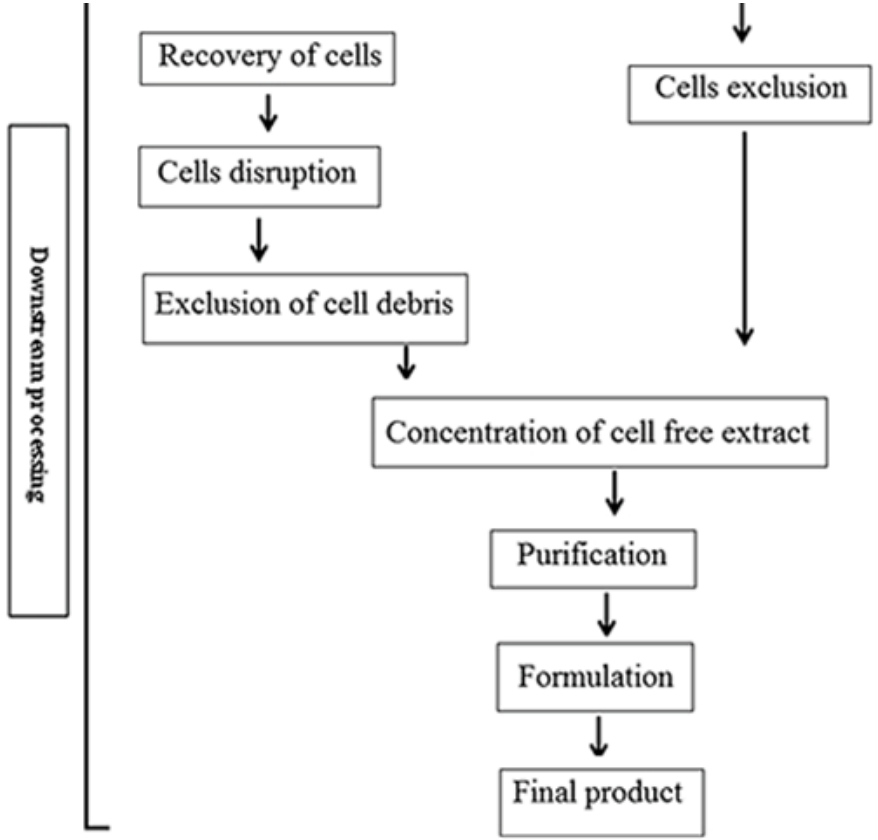
Source: Sci. Revs. Chem. Commun.: 2(4), 2012, ISSN ENZYME SYNTHESIS BY FERMENTATION METHOD : A REVIEW V. C. RENGE, S. V. KHEDKAR and NIKITA R. NANDURKAR

Production of the Food Enzyme

- **Downstream processing**
  - **Describe in detail:** Specific methods used to kill, disrupt and remove microbial biomass after completion of fermentation, to concentrate the enzyme liquor and to remove microorganisms from the food enzyme

  - Provide **a list** of all substances used, including the processing aids (Provide CAS if available and the function).

    An indicative list will not be accepted



Source: Introduction to Pharmaceutical Biotechnology. Vol 2. Enzymes, proteins and bioinformatics

Production of the Food Enzyme

- **Food enzyme preparations (1)**

- It is assumed that all food enzymes will be variously formulated to increase shelf-life and as a mean of standardisation. Information on the **method** and material used to produce a preparation is **generally not required**.

- The only **exceptions** would be:

  a) when the concentrated enzyme liquor which would normally constitute the **food enzyme is inherently unstable** and only data on a stabilised preparation can be generated

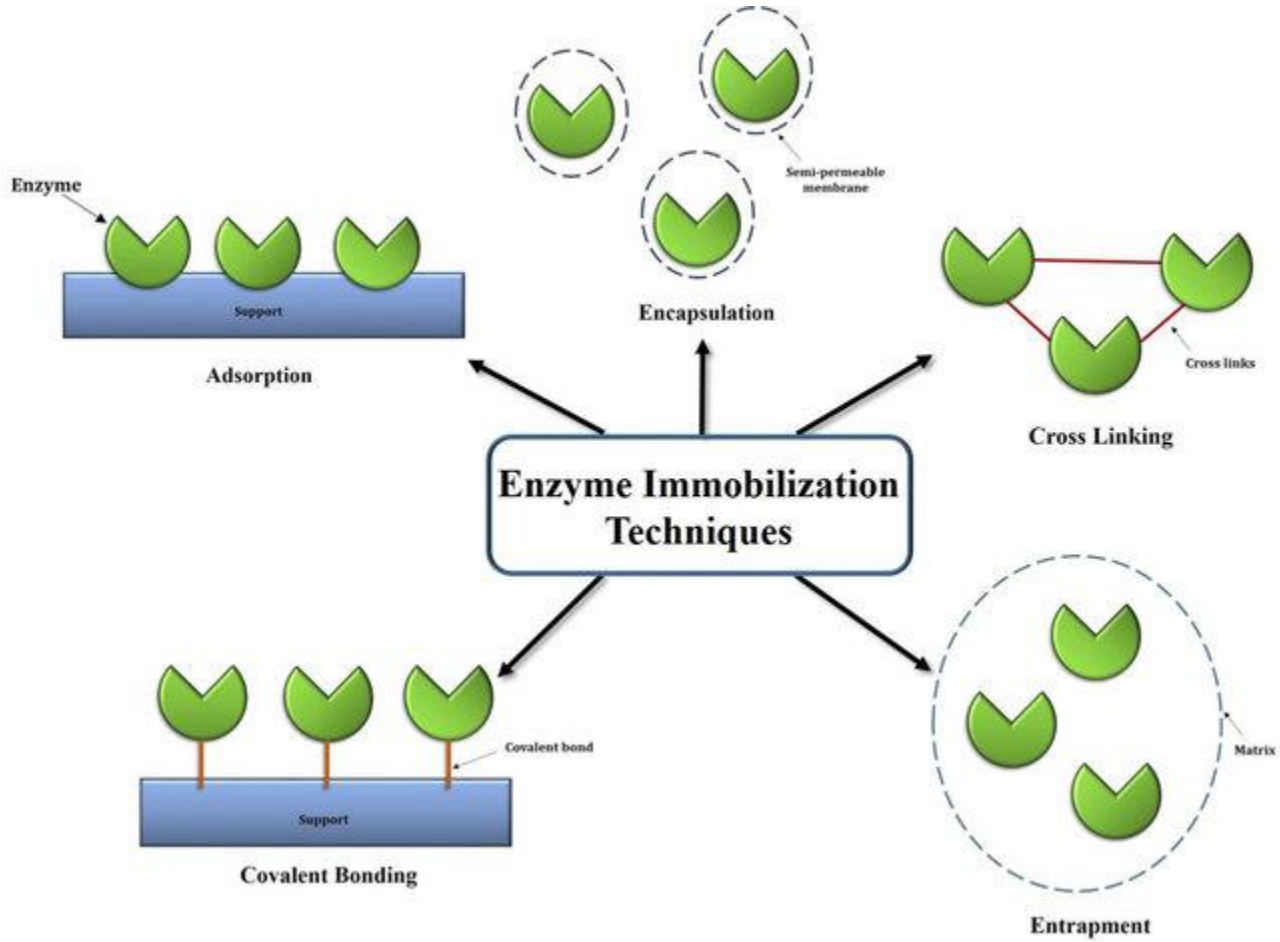
  b) when there is concern that the method used to produce the preparation may result in a **potential carry-over of hazardous material into a food**.

Production of the Food Enzyme

- **Food enzyme preparations (2)**

a) quantitative data on all added excipients is required to allow the calculation of the Total Organic Solids (TOS) arising from the fermentation.

b) Immobilisation or encapsulation of the food enzyme: 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.

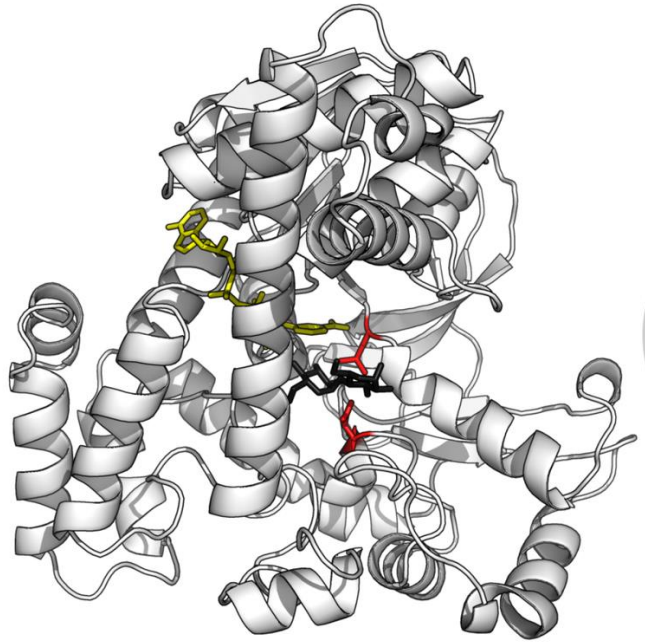


Source: Jun, LY; Yon, LS; Mubrak, NM; Bing, CH; Pan, S; Danquah, MK; Abdullah, EC; Khalid, M. (2019) An Overview of Immobilized Enzyme Technologies for Dye, Phenolic Removal from Wastewater. Journal of Environmental Chemical Engineering. 7, 102961

Characterisation of the food enzyme

SECTIONS

- Properties of the food enzyme
- Chemical parameters
- Purity
- Viable cells and DNA of the production strain
  - Viable cells of the production strain
  - DNA from the production strain



Characterisation of the food enzyme

- **Properties of the food enzyme (1)**

Amino acid sequence of the actual enzyme(s) under assessment; **reference to published sequences of enzymes with the same catalytic activity are not acceptable**.

Calculate **the molecular mass** of the enzyme (mature protein or with any signal sequence)

Available information on the subunit structure as well as degree of glycosylation should also be provided when relevant.

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

The **protein pattern** characteristic of **at least three batches** of the food enzyme and, additionally, any additional batch prepared for use in **toxicological** studies should be provided.

This may be done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or other analytical techniques such as size exclusion chromatography or mass spectrometry. Appropriate 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.

Characterisation of the food enzyme

- **Properties of the food enzyme (2)**

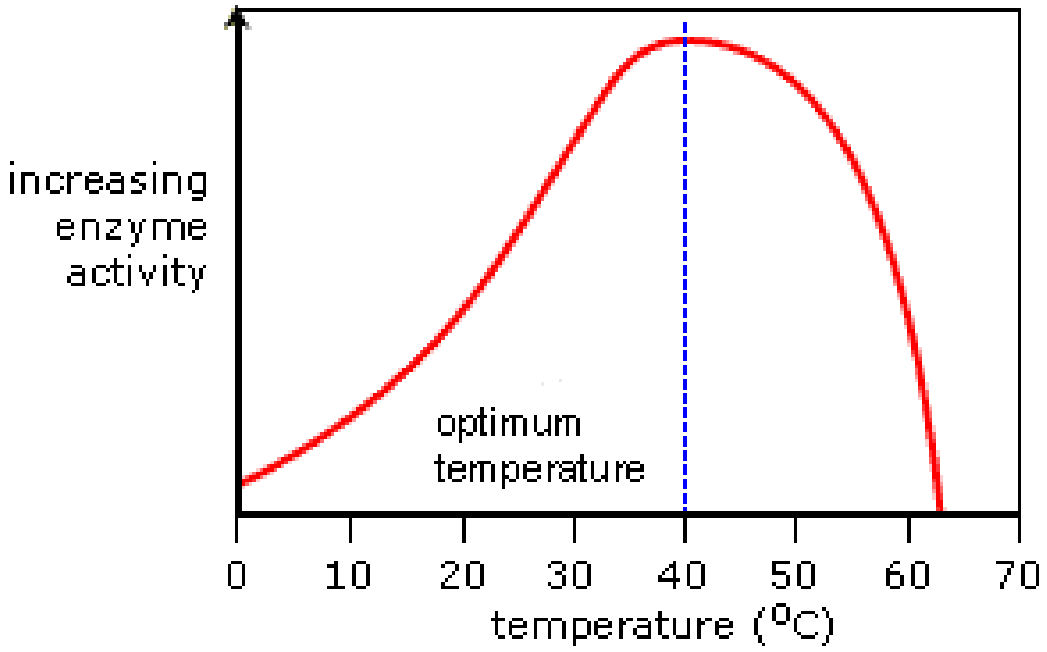
Enzyme activity

- **Description of the Standard Operating Procedure** (SOP) used for measuring the activity (enzyme activity units (U) per unit weight)

- **Temperature and pH range** with activity
- **Optimum values for pH and temperature**
- **Thermal stability**

  The chosen temperature range should reflect the technological role of the food enzyme

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.



Source: alevelnotes.com

Characterisation of the food enzyme

Chemical parameters

For **at least three batches** of the food enzyme representative of those intended for **commercialisation**. Preferably from a full-scale production run. Pilot plants may substitute for those food enzymes in a pre-production stage of development.

Parameters measured:

- **Enzyme activity or activities** under application (Units/g batch)
- Concentration (in % w/w) of:
  - **total protein**
  - **Ash**
  - **Water**
- Percentage of **TOS should be calculated** (as 100% - % water - % ash) and the **enzyme activity/mg TOS** determined.

Judgement: **food enzyme batches used for toxicological or other studies can be considered representative**?

Same data set is provided for all additional batches of the food enzyme used for the toxicological or other studies.

If food **enzyme preparation** is unavoidable: TOS= 100% - % water - % ash – % total added organic excipients

Provide: **Methods of analysis, certificates of analysis**

Characterisation of the food enzyme

Purity

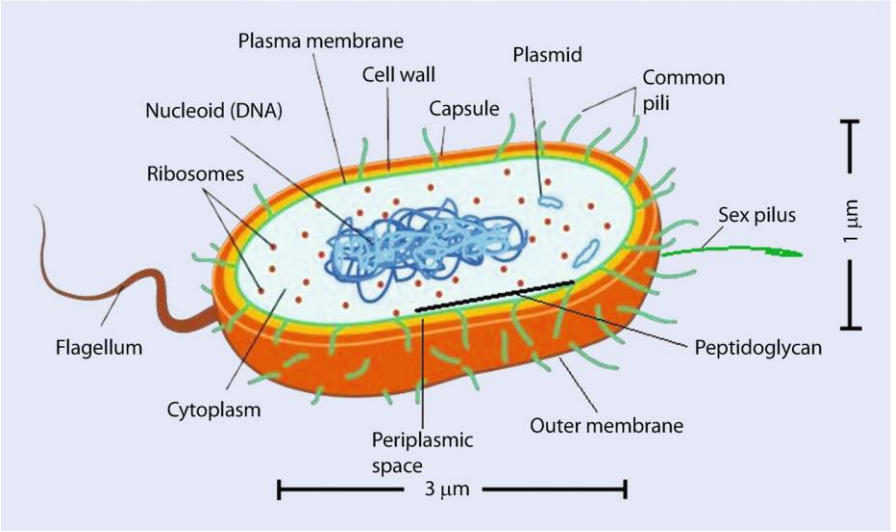
Analytical data to be given:

- **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.
- **Concentration of lead** in the food enzyme
- **Concentration** of potential **compounds of known toxicity** (e.g., mycotoxins) arising from the fermentation (trough literature searches or WGS analysis of the production strain). Should not give rise to concern.
- **Microbiological purity of the food enzyme:**
  - ***Escherichia coli*** not be detected in 25 g
  - **Enterobacteriaceae** should not exceed 10 CFU/g
- **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).

Provide: Methods of analysis together. Certificates of analysis, limit of detection (LoD) and the Limit of Quantification (LoQ)

Characterisation of the food enzyme

- Viable cells and DNA of the production strain
  - Viable cells of the production strain
  - DNA from the production strain



Source: Behera, SS; Ray, RC; Das, U; Panda, SK; Saranraj, P (2919) Microorganisms in Fermentation. In Essentials in Fermentation Technology (Springer Ed) pp 1-39.

Characterisation of the food enzyme

- **Viable cells of the production strain**

All food enzymes **except** for those obtained using non-GM QPS production strains.

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

a. culture-based method targeted to the detection of the viable cell
b. enable the recovery of stressed cells
c. consider specificity against contaminating microbiota possibly occurring in the sample
d. if the strain is able to form spores, their possible presence should be analysed
e. volume corresponding to at least 1 g or mL of food enzyme
f. at least nine samples obtained from a minimum of three independent batches
g. positive control with samples of the food enzyme

Characterisation of the food enzyme

- DNA from the production strain (1)

This section applies to:

a) food enzymes obtained using GM production strains;

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

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

a) in case the production strain contains AMR genes, whether GMM or not, primers should be designed to amplify a fragment not exceeding the size of the smallest antimicrobial resistance gene and in any case not exceeding 1 kb. If the production strain is a GMM not containing AMR genes, the targeted sequence should cover maximum 1 kb;

b) DNA from at least 1 g or 1 mL of product shall be extracted. Upstream intermediate products can be used as long as they are equally or more concentrated than the final product. For different production schemes, each of the product should be tested;

c) at least three independent batches of food enzyme should be sampled, each extracted in triplicate and analysed. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial-scale process. Samples from pilot-scale process are acceptable if it can be demonstrated that those from industrial process are not available. In this case it should be documented that the pilot-scale process (fermentation and downstream) is representative of the industrial-scale process;

d) to recover DNA from non-viable cells potentially remaining in the product, the DNA should be extracted using a methodology suitable for all cellular forms of the production strain (e.g., vegetative cells, spores).

Characterisation of the food enzyme

- **DNA from the production strain (2)**

The following controls and sensitivity tests should be included:

a) total DNA from the production strain, as a positive control for the PCR;

b) total DNA from the production strain, added to the product sample before the DNA extraction process, starting with a known quantity and in different dilutions until DNA extinction, to calculate the LoD;

c) a positive control with total DNA from the production strain, added to the DNA extracted from each of the three batches of the product tested, to check for any factors causing PCR failure;

d) a negative control without sample.

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

Thank you for your attention!!

Post-public consultation stakeholder event
21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Food enzymes of plant and animal origin

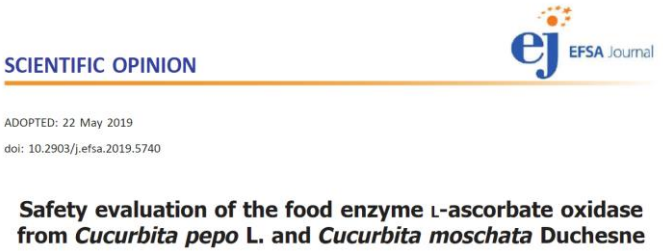
Holger Zorn
Enzymes Working Group

Trusted science for safe food

efsa
European Food Safety Authority

Food enzymes of plant and animal origin

EC 1.10.3.3	L-Ascorbate-Oxidase	*Cucurbita pepo*; *C. moschata*
EC 1.11.1.7	Peroxidase	*Glycine max* (L.)
EC 3.2.1.2	β-Amylase	*Hordeum vulgare*
		Glycine max (L.)
		Triticum
EC 3.2.1.17 Lysozyme		Albumen (*Gallus gallus domesticus*)
EC 3 .4.21.1	Chymotrypsin	Pancreas (*Sus scrofa domesticus*)
	Elastase	
	Carboxypeptidase	
EC 3.4.21.4	Trypsin	Pancreas (*Sus scrofa domesticus*)
EC 3.4.23.1	Pepsin	Abomasum (*Bos Taurus, Ovis aries, Capra aegagrus hircus*)
EC 3.4.23.4	Chymosin	

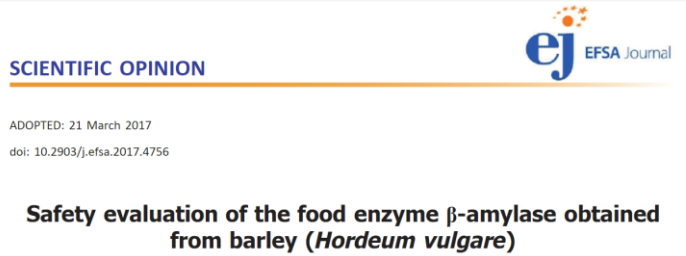

SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 22 May 2019

doi: 10.2903/j.efsa.2019.5740

Safety evaluation of the food enzyme L-ascorbate oxidase from *Cucurbita pepo* L. and *Cucurbita moschata* Duchesne

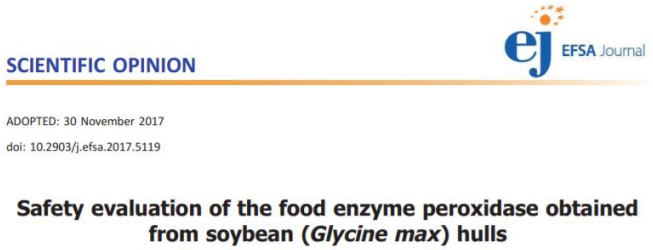

SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 21 March 2017

doi: 10.2903/j.efsa.2017.4756

Safety evaluation of the food enzyme β-amylase obtained from barley (*Hordeum vulgare*)


SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 30 November 2017

doi: 10.2903/j.efsa.2017.5119

Safety evaluation of the food enzyme peroxidase obtained from soybean (*Glycine max*) hulls

Food enzymes derived from GM plants and animals

PC comment: Page 21, line 793-795:
" … Accordingly, **food enzymes based on GM plant production organisms are treated differently from those based on GM microorganisms** as no such restriction applies here. A reason for this is not given and EFSA should critically question whether this unequal treatment is meaningful and scientifically justified. A scientifically sound justification should be provided as to why EFSA considers that if the source of the food enzyme is from a GM plant, the plant should have been assessed and received authorization under the Regulation (EU) No 1829/2003 for food and feed use in the EU in any case. In this regard, it should also be taken into account that the production and purification of food enzymes based on a GM plant production organism is also possible in a contained use system…"

PC comment: Page 23, line 911 et seq.:
"In the case of animals as source of the food enzyme, **GM animals are not even mentioned by name in Chapter 3**. In contrast, the Guidance Document designates specific requirements for both GMMs and GM plants, but these requirements are also not the same. In the case of GM animals, it is not clear whether and, if so, which specific requirements apply here. EFSA should provide detailed information and recommendations on this issue, also taking into account the comments previously made on GMMs and GM plants (if also useful for GM animals). As requested for microbial production strains on p. 10 (line 325-326) in the document, also for animal production organisms a clear statement on whether the production organism is genetically modified according to Directive 2001/18/EC should definitely be made. EFSA should also provide guidance here on how to describe the genetic modification."

Questions on legal aspects of food enzymes derived from GM plants and animals that have not been assessed and received authorization under the Regulation (EU) No 1829/2003 are not within the remit of EFSA.

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

Guidance structure

2	**Food enzymes of plant origin**
2.1	Source of the food enzyme
2.2	Production of the food enzyme
2.2.1	Extraction
2.2.2	Downstream processing
2.2.3	Food enzyme preparation
2.3	Characterisation of the food enzyme
2.3.1	Properties of the food enzyme
2.3.2	Chemical parameters
2.3.3.	Purity

3	**Food enzymes of animal origin**
3.1	Source of the food enzyme
3.2	Production of the food enzyme
3.2.1.	Extraction
3.2.2.	Downstream processing
3.2.3.	Food enzyme preparation
3.3	Characterisation of the food enzyme
3.3.1.	Properties of the food enzyme
3.3.2.	Chemical parameters
3.3.3.	Purity

2.3.1 Properties of the food enzyme

PC comment: Page 22: “Food enzyme presents the principal enzymatic activity i.e. the activity of the enzyme under application, but potentially other enzymatic activities too i.e. **subsidiary/side enzymatic activities**. Some enzymatic activities might cause adverse effects or generate metabolites from the food matrix. … Any subsidiary/side enzymatic activity should be characterised by applicant in the food enzyme.”

- Data on subsidiary /side enzymatic activities would not contribute to improve the safety evaluation of the food enzyme for the consumers.

PC comment: Page 22 line 861: “Data on the measurements of thermal stability of the enzyme should both include the **temperature of complete inactivation of the enzyme** (i.e. measurement of the specific activity of the enzyme after exposition to increasing temperatures) and the **temperature of denaturation** of the enzymatic protein (i.e. the lack of interaction with specific polyclonal antibodies in western blot experiments after exposition to increasing temperatures) because these two parameters are not necessarily correlated.” … “Moreover, **measurement of the enzymatic activity in final food or processed food products should be provided under industrial conditions**. Data in the literature demonstrate that food matrix can protect enzymes from both denaturation and inactivation by heat”.

- the panel generally considers the enzyme in its active state for its evaluation, so it does not make a difference to have data on denatured enzymes. No improvement of safety evaluation is obtained by these data.

- no data on the long-term stability of the enzyme are required

2.3.3. Purity

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

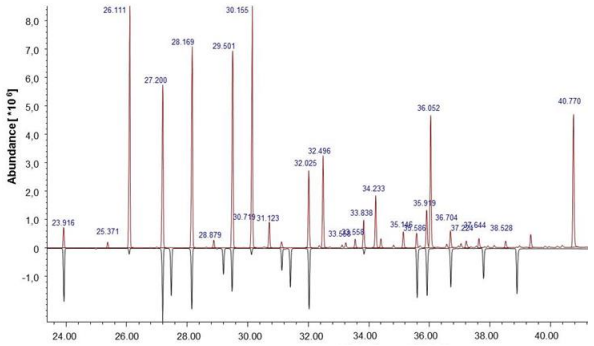
proposed modification of the guidance document:

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

2.3.3. Purity – Pesticide residues

PC comment: Page 23, line 894: “We would like to reword the sentence as follows adding “other than approved ones in EU”. The sentence would read, “it can be guaranteed that no pesticides other than approved ones in EU were used”

- waiving of analysis of potential pesticide residues is only possible if it can be guaranteed that no pesticides were used, independent from an approval of the pesticide(s) in the EU.
- data on (potential) residues are necessary to evaluate the safety of the food enzyme.



3 Food enzymes of animal origin
3.1 Source of the food enzyme

PC comment: Page 24 lines 923-924: “Proposal to modify the sentence in “The methods used to ensure the absence of any risk of infectivity (e.g. the agent of **transmissible spongiform encephalopathies** (TSEs), **parasites**, viruses or other zoonotic agents) should also be provided.”

- veterinary inspection & production process assure the absence of parasites
- no extraction from TSE risk material has been reported for food enzyme production or is expected

3.2.2. Downstream processing

PC comment: “L952: Suggestion to include "or inactivate" so the sentence reads: "...and to remove or inactivate microbes from the food...".

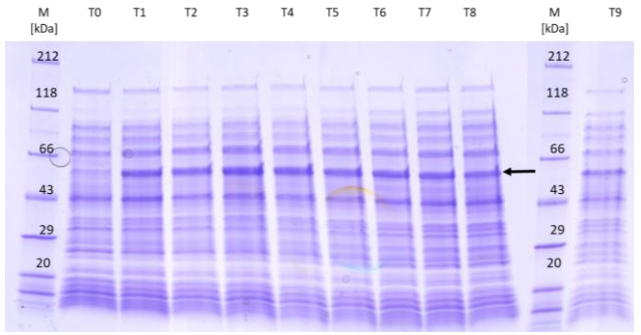
proposed modification of the guidance document:

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

3.3.1. Properties of the food enzyme

Page 25 before line 977: “The characterisation of the food enzyme is necessary too. The paragraph “The protein pattern characteristic of at least three batches of the food enzyme and, additionally, any batches prepared for use in toxicological studies should be provided. This may be done by SDS-PAGE or other electrophoretic techniques or size exclusion chromatography. Appropriate standards should be incorporated. The purpose of such studies is to provide evidence of the consistency of production and to confirm that food enzyme batches used in other studies are representative.” (lines 847-852) should be added.”

- **SDS-PAGE is not requested for animal enzymes because of the multiple proteins present**
- **no information on potential degree of glycosylation is required for enzymes from animals**



Post-public consultation stakeholder event
21-22 June 2021

Thank you very much for your attention!

Holger Zorn

Enzymes Working Group

Trusted science for safe food

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Post-public consultation stakeholder event
21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Food enzymes of microbial origin

Pier Sandro Cocconcelli

Enzymes Working Group

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Characterisazion and safety assessment of microorganisms for FE production: the timeline

Regulation (EC) No 1332/2008

2018: FEEDAP Guidance on the characterisation of microorganisms used as feed additives or as production organisms

2021: EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain

2021 CEP Scientific Guidance for the submission of dossiers on Food Enzymes

2009- Guidance on the Submission of a Dossier on Food Enzymes for Safety Evaluation

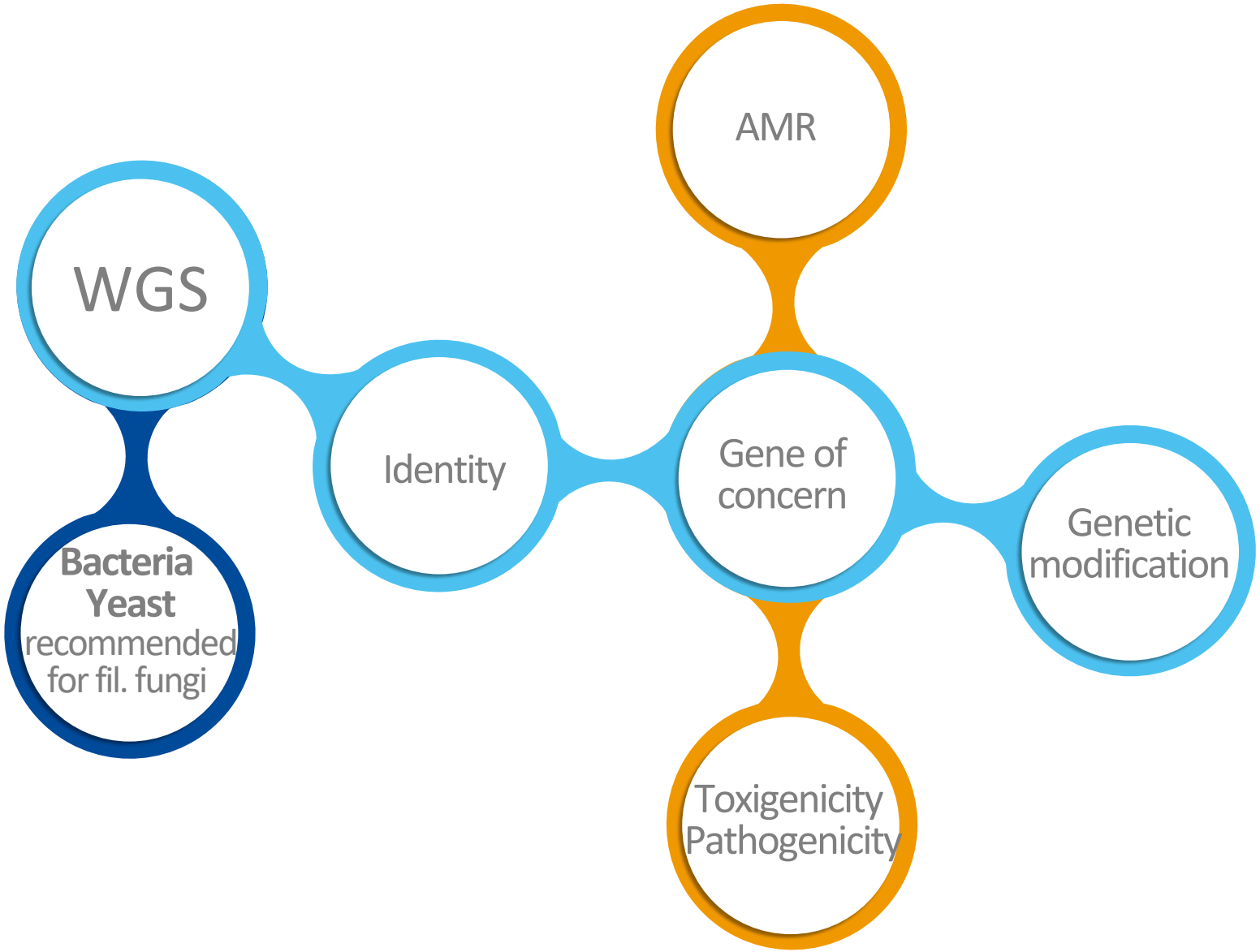
2019:CEP Statement characterisation of microorganisms used for the production of food enzymes

WGS- what's new?

- Application of the criteria defined by the **EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain**

- Improvement of the **CEP Statement characterisation of microorganisms used for the production of food enzymes (2019)** based on:
  - Experience of two years of application (e.g. no MIC determination for bacteria)
  - Rapidly evolving scientific methodologies (e.g. DNA sequencing and bioinformatics)
  - **Public consultations**

WGS-based Risk Assessment
THE SCHEME



The EFSA approach for WGS based Risk Assessment:

EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain

European Food Safety Authority (EFSA)

3.6 Provision of raw data and standard data formats

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 equivalent formats, compressed or not (e.g. gzip), pair or single end,
- Assembled sequences can be submitted in FASTA format (e.g.,*.fasta),
- Supported formats for annotation[14] 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.

Draft genomes acceptable if provide sufficient information for an adequate characterisation of the strain.

1.1.7 Identity
Unambiguous identification at the species level

BACTERIA

WGS based approach

no other methods accepted

- digital DNA-DNA hybridization >70%
- ANI>95 %
- For values below those thresholds, phylogenomic methods should be applied to identify the closest related species

YEAST

WGS based approach

no other methods accepted

- phylogenomic analysis (to produce a phylogeny against available related genomes)
- alignment to a complete reference genome from the same species

FILAMENTOUS FUNGI

WGS:

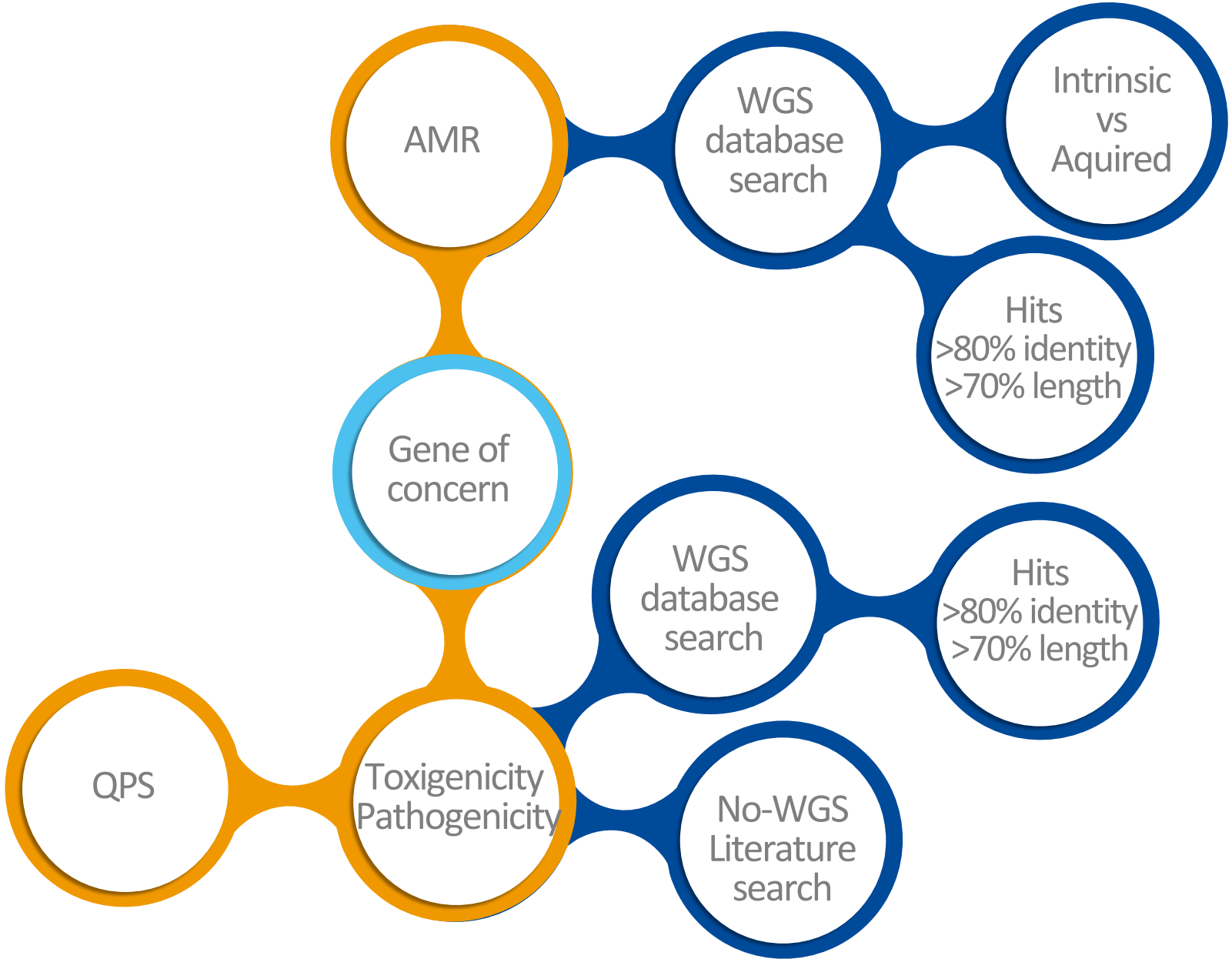
- phylogenomic analysis
- alignment to a complete reference genome from the same species.

WGS not determined:

sequence analysis of 18S rDNA/ITS

- When there is contradiction regarding taxonomical classification, this should be documented
- Mutagenesis steps should be reported

1.1.8 Identification of genes of potential concern



1.1.9 Antimicrobial Resistance

- The use of food enzymes should not add to the pool of AMR genes already present in human gut bacterial population or otherwise increase the spread of AMR.
- WGS interrogation from the presence of AMR genes
  - at least **two updated databases**
  - Report hits >**80% identity** (at protein or nucleotide level) and **70% length** of the subject sequence
  - When two or more fragments covering less than 70% length of the subject sequence with at least 80% identity to the same gene are detected, and it should be checked whether the full gene is present.
- Intrinsic *vs* acquired

Presence of acquired AMR genes:

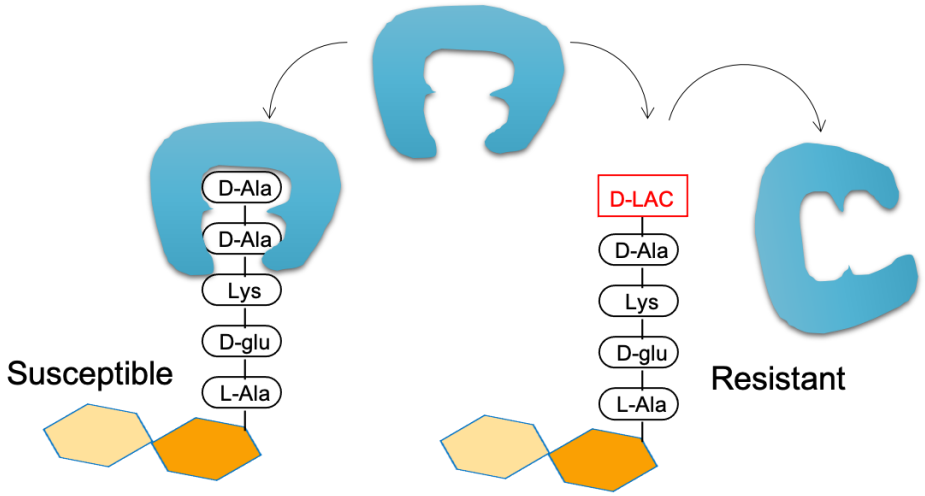
Demonstration of absence of viable cells and DNA of the production strain (Section 1.3.4.)

Intrinsic vs Acquired
The vancomycin resistance example

Acquired resistance: when a strain of a typically susceptible species is resistant to a given antimicrobial drug

Intrinsic resistance is inherent to a bacterial species and is typical of all the strains of that species.

Intrinsic antimicrobial resistance is generally not considered a safety concern.



Vancomycin Resistance - altered target site

heterofermentative *Lactobacillaceae*

Constitutive

D-ala D-lac Ligase

Acquired by HGT

Enterococcus

QPS strains: considered toxicologically safe

Unequivocal identification (species)

↓

QPS Strain level

↓

Bacteria: absence of AMR

→ Any other qualification

Bacillus:
cytotoxicity test on the production strain:
parental not acceptable

For GMMs: QPS parental = QPS concept applicable to the GM

1.1.10 TOXIGENICITY AND PATHOGENICITY
Non-QPS strains

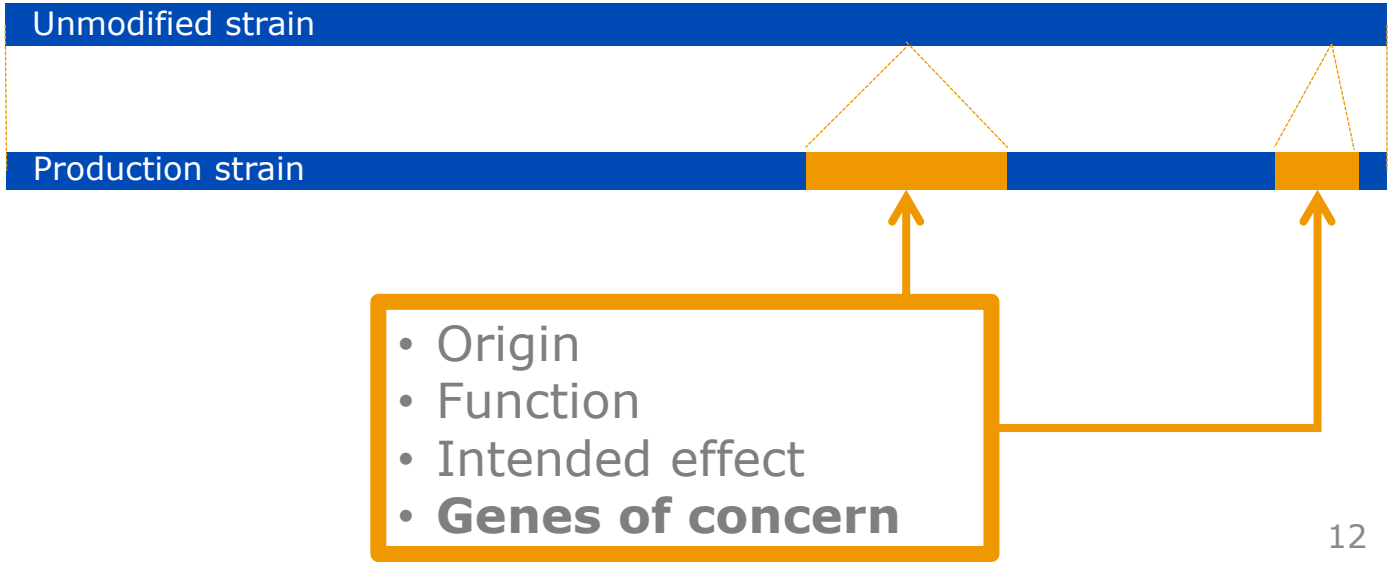
- **Bacteria**: WGS—› Search for known virulence factors.
  - Following the EFSA Statement on the requirement for WGS

- **Eukaryotes**:
  - If WGS available: targeted searches against known sequences encoding toxin production pathways (same parameters of bacteria)
  - No WGS: literature search to identify the capacity of the species or of closely related species to produce known toxic compounds. Database searched and the key words used to be reported

1.1.11 Genetic Modification - WGS

No changes with respect to the current CEP Statement on the Characterisation of microorganisms for FE (2019)

- **bacteria and yeasts** (optional for fungi) —› **WGS**
  - Map/graphic of all genomic regions harboring genetic modifications (ORFs and non-coding sequence/s)
  - sequences/databases and the methodology used for analyses and comparison



1.1.11 Genetic Modification – without WGS

No changes with respect to the current CEP Statement on the Characterisation of microorganisms for FE (2019)

- **all the steps** should be described.
- identification of **all genetic material** potentially introduced into the recipient/parental microorganism.
  - Characteristics of the **vector(s)**
  - Information relating to the **genetic modification process**
  - Structure of any **vector and/or donor** nucleic acid remaining in the GMM
  - **Genes of concern**

1.3.4 **Viable cells** and DNA of the production strain

No changes with respect to the current CEP Statement on the Characterisation of microorganisms for FE (2019)

- **Required for all cases except QPS**
- Culture-based method
  - Molecular methods less sensitive
- Production strain ≠ contaminating microbiota
- Recovery of possible stressed cells
- **≥ 1 g or ml of product**
- **9 samples from at least 3 batches**
- **Positive control**

1.3.4 Viable cells and **DNA** of the production strain

No changes with respect to the current CEP Statement on the Characterisation of microorganisms for FE (2019)

- **Requested for:**
  - GM production strains
  - non-GM production strains with acquired AMR genes
- **PCR-based methodology. Indications on:**
  - Target sequence (<1kb) or the smallest gene of concern (e.g AMR)
  - Amount of sample (**≥ 1 g or 1 ml**)
  - 3 batches – 3 replicates (3 x 3)
  - Controls
- **Threshold: 10 ng control DNA per g or mL of product**

Thanks for your attention

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Post-public consultation stakeholder event

21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Need for toxicological studies

Lieve Herman

Enzyme Working Group

Trusted science for safe food

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Toxicological studies are not always needed when derived from plant and animal sources

Toxicological studies might be waived for:

- Food enzymes derived from edible parts of plants and animals
  - Dietary exposure to the food enzyme TOS is within the same magnitude as the dietary intake of the fraction of the plant or animal material comparable to the food enzyme TOS

- Food enzymes derived from animal rennet (dietary exposure study not necessary because these enzymes have a long history of safe and wide consumption)

- When it can be demonstrated that there is no (or negligible) carry-over of the food enzyme TOS into the final food products e.g. in distillation processes, starch processing
  - No or negligible carry-over of the food enzyme TOS into the final products
    - See Annex E: > 99 % removal is acceptable
    - See Section 5.2 for guidance on the removal/absence of TOS during the manufacturing process

WAIVERS: Enzymes from microbial source

Toxicological studies might be waived :

- Food enzymes derived from microorganisms considered suitable for the qualified presumption of safety (**QPS**) approach for risk assessment (**also no search of the WGS for virulence/pathogenicity genes**)

QPS assessment

- QPS provides a generic **safety** status for **microorganisms** intentionally used in the food and feed chain (not for plants or products derived from plant origin)

- **The QPS approach can be extended to genetically modified production strains**
  - **is also including GM protists/microalgae;** *Euglena gracilis, Aurantiochytrium limacinum/ Schyzochytrium limacinum, Tetraselmis chuii)*

- QPS provides a safety status at the species level; **all strains belonging to a QPS species are following the QPS assessment (no danger to consumers due to intraspecies diversity)**

Requirements for QPS status strains

Even for food enzymes produced by QPS organisms, the application needs some information on the microorganism :

- **Taxonomic identification of the microorganism**
- Safety of the genetic modification
- Confirmation that the existing qualifications are met

Qualifications

- A qualification is a potential hazard that should be tested for strains belonging to the QPS species
- A generic qualification for all bacterial QPS species:
  - **AMR defined as 'absence of acquired genes conferring resistance to clinically-relevant antimicrobials'**
- Strains belonging to a QPS species with the qualification 'only for production purposes' needs confirmation in the dossier on the **absence of viable production organisms** in the enzyme product (also for non-GM)

  (e.g. some yeast species as *Candida* and *Komagaetella/Pichia*)

How microbes come on the QPS list?

- From **2008 till now**:
  - Further update of QPS list
    - based on notifications to **EFSA through application dossiers; actually no 2-step process available** to knew in advance if Tox studies would be needed

WAIVERS: Enzymes from microbial source

Toxicological studies might be waived for non-QPS microorganisms:

- Special situation: strains belonging to **species on the QPS list but which contains an AMR gene**
  - Tox studies can be waved when it is documented that **no DNA** of the AMR gene is present in the product

- For other non-QPS production microorganisms (e.g. filamentous fungi) tox studies from an other product produced by a very related microorganism may be used
  - **Evaluated on a case-by-case basis**

Case by case evaluation

- Substitutive dataset has to be produced from a microbial strain of the same strain lineage which means 'derived from a common progenitor'

- No conventional mutagenesis steps between the two different production strains have been carried out because conventional mutagenesis can have consequences for the phenotype

- The **genetic modification** should be compared carefully
  - All genetic modifications made in both strains should be documented in detail by WGS data (also WGS required for fungal strains in this case)
  - Modifications in the production strain may not alter the possible presence of products of concern in the end product
    - *Insertions in the production strain may not induce possible expression of genes of concern*
    - *Deletions: e.g. deletion of proteinase genes which could alter the break down of unwanted proteins in the end product*

Waiving toxicological studies

Application must also include:

- Experimental data demonstrating **absence of concern from the manufacturing process** and that the enzyme is sufficiently purified:
  - Residues
  - Impurities
  - Degradation products linked to the total production process (production, recovery and purification)

EFSA documents: QPS and REQUIREMENTS FOR WGS data

- The QPS list in KJ: https://zenodo.org/record/3336268#.Xjqg4GhKiUl
- The QPS topic: https://www.efsa.europa.eu/en/topics/topic/qualified-presumption-safety-qps
- EFSA journal virtual issue on QPS: https://efsa.onlinelibrary.wiley.com/doi/toc/10.1002/(ISSN)1831-4732.QPS

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Post-public consultation stakeholder event
21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Genotoxicity testing strategy for food enzyme

Claudia Bolognesi

Enzymes Working Group

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Genotoxicity of mixtures

Food enzymes are complex mixtures of unidentified components except the declared enzyme(s)

EFSA SC Statement (2019) Genotoxicity assessment of chemical mixtures

*The testing strategy for a **whole mixture** or its fraction(s) should follow the Scientific Committee testing **strategy guidance for individual chemical substances** (EFSA, 2011).*

*The demonstration of the identity and stability (batch-to-batch variability as well as stability over time) of a mixture is always required to ensure that the **mixture tested** is **representative of the mixture to be placed on the market.***

Genotoxicity testing strategy

EFSA SC Opinion (2011) Scientific opinion on genotoxicity testing strategies

A step-wise approach for the generation and evaluation of data on genotoxic potential

Tier 1: Basic battery of *in vitro* tests

- **Bacterial reverse mutation test** Ames test OECD TG 471

  End-point: **gene mutation**

- ***In vitro* mammalian cell micronucleus test (MN)** OECD TG 487

  End-points: **structural and numerical chromosome aberrations**

If the Ames test is not applicable a test for induction of gene mutations in mammalian cells could be performed and it needs to be justified

- ***In vitro* Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene** OECD TG 490
- ***In Vitro* Mammalian Cell Gene Mutation Test using the Hprt and xprt genes** OECD TG **476**

Genotoxicity testing strategy

Bacterial reverse mutation test Ames test OECD TG 471

- **Recommended combination of S.typhimurium strains**:
  TA100 and TA1535 base pair change
  TA98 and TA1537 or TA97 or TA97a frameshift mutation
  TA102 or E.coli WP2uvrA or E.coli WP2uvrA (pKM101)
  for oxidizing mutagens and cross-linking agents.
  Historical controls

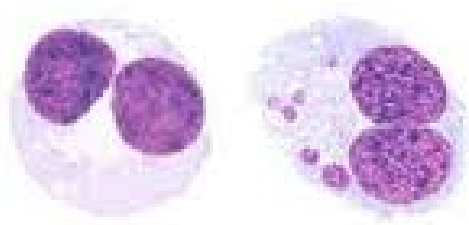
- **Methods:**
  Plate incorporation assay
  Preincubation assay
  Treat and plate assay recommended to avoid feeding effect

- **Range of concentrations** The recommended maximum test concentration for **soluble non-cytotoxic mixtures** is at least **5 mg TOS/plate**.
  to increase the concentration of each of the components of the mixture

- **Criteria for determining a positive result**: **reproducible increase** in the number of revertants
  concentration-related increase

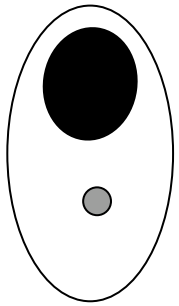
Genotoxicity testing strategy

In Vitro Mammalian Cell Micronucleus Test OECD TG 487

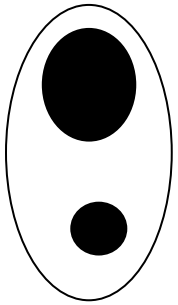


Cell lines: human peripheral blood lymphocytes
rodent cell lines (CHO, V79, CHL/IU, L5178Ycells)
human cell lines (TK6, HT29, Caco-2, HepaRG, HepG2 cells, A549)

Methods: use of CytoB as cytokinesis blocker
fixed and unstained slides stored for further analysis



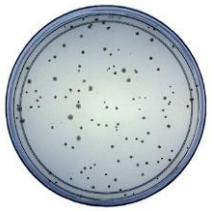
Range of concentrations: maximum concentration (MC) based on cytotoxicity
In the absence of precipitate or cytotoxicity the MC may be **higher than the limit of 10mM or 2mg/mL** (e.g., up to **5 mg TOS/mL)** to increase the concentration of each of the components of the mixture.



Criteria for determining a positive result:

- at least one of the test concentrations exhibits a statistically significant increase
- the increase is **dose-related** in at least **one experimental condition**
- any of the results are **outside the distribution of the historical negative control data**

Genotoxicity testing strategy



RESULTS

REVIEW OF THE RESULTS

FURTHER TESTING

Bacterial reverse mutation test

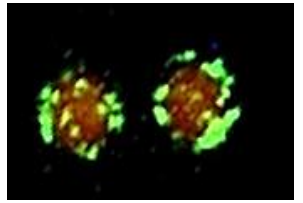
Positive results

- ✓ toxicity
- ✓ enzyme reactivity
- ✓ analytical data (e.g.aminogram)

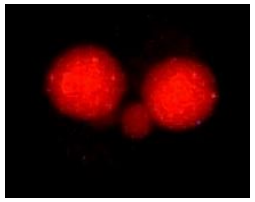
- ✓ Narrow conc. range
- ✓ Different method
- ✓ Inactivated enzyme

Negative results
No further testing
No concern for genotoxicity

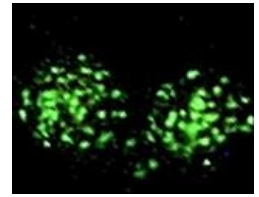
C-MN



Clastogenic



C+MN



Aneugenic

In vitro **mammalian cell micronucleus test**

Positive results

- ✓ toxicity
- ✓ slide scoring
- ✓ enzyme reactivity
- ✓ mechanism for MN induction

- ✓ FISH with DNA probes or CREST staining
- ✓ New experiments

Genotoxicity testing strategy

Tier 2: *in vivo* follow-up of *in vitro* positive results

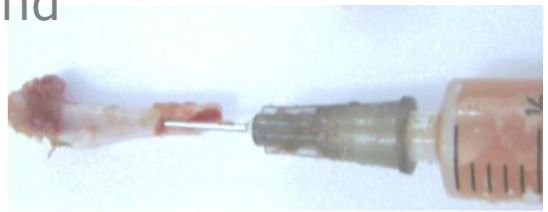
Gene mutations in bacteria:

- Transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488)
- *In vivo* Mammalian Alkaline Comet Assay (OECD TG 489)

Micronuclei frequency in mammalian cells

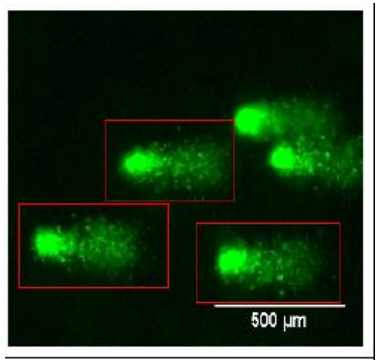
Clastogenic effects

- Combination of mammalian erythrocyte micronucleus test (OECD TG 474) and Mammalian Alkaline Comet Assay in stomach and or liver(OECD TG 489)



Aneugenic effects

- Mammalian erythrocyte micronucleus test (OECD TG 474)
  Possible combination with MN in liver (for *in vitro* positive with S9) or MN in gastrointestinal tract (for *in vitro* positive without S9)



Range of doses: **highest dose = maximum tolerated dose (MTD)**

Higher doses than the maximum limits in the OECD test guidelines (2000 mg/kg/bw/day) should be considered

The highest dose to be applied should be limited by the **maximum volume** that should be given to rodents: **1 mL/100** g body weight or **2 mL/100** g for aqueous solutions (OECD TG 474 and 489)

EFSA SC Statement (2019) Genotoxicity assessment of chemical mixtures

If the *in vivo* testing of an in vitro positive mixture provides negative results, the relevance of the findings obtained in the *in vivo* follow-up tests will depend:

- on the genetic effect assessed
- the test protocol applied (route of exposure, tissues, etc.)
- expert judgement on the reliability of the results obtained (including consideration of target tissue exposure)

- the assessment of systemic genotoxic effects (e.g. in the liver or bone marrow) may be limited by the fact that **target tissue exposure cannot be demonstrated**, as **any toxic effect elicited in the target tissue** by the mixture **cannot be unequivocally attributed to the *in vitro* genotoxic component**

Target tissue exposure

EFSA SC Opinion (2017) Clarification of some aspects related to genotoxicity assessment

Lines of evidence of bone marrow exposure

- Toxicity to the bone marrow in the in the mammalian erythrocyte MN test: decrease in the PCE/(NCE+PCE) ratio
- Toxicity to the bone marrow observed in toxicity studies (e.g. 90-day study)
- Systemic toxicity observed in the bone marrow micronucleus test
- Systemic toxicity observed in toxicity studies

Genotoxicity assessment of chemical mixtures

EFSA SC Opinion (2011) Scientific opinion on genotoxicity testing strategies

EFSA SC Opinion (2017) Clarification of some aspects related to genotoxicity assessment

EFSA SC Statement (2019) Genotoxicity assessment of chemical mixtures

EFSA SC Statement (ongoing) Guidance on aneugenicity assessment

Post-public consultation stakeholder event
21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Allergenicity

Henk Van Loveren
Enzymes Working Group

Trusted science for safe food

efsa
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Guidance

- The assessment of potential allergenicity considers a) the source of the food enzyme; b) the declared enzyme(s) and c) any proteinaceous material with known allergenic properties included in a fermentation medium.
- Quantifying the risk for allergenicity is currently not considered possible in view of the varying individual susceptibility to food allergens. Even trace amounts of protein transferred to food could potentially present a hazard for sensitized individuals. Lack of transfer will only be accepted if it can be demonstrated that food enzyme TOS is absent in the final food. For all other cases, the following information is required:
- Investigation of the amino acid sequence of the food enzyme. Any sequence identity greater than 35% to a known allergen using a sliding window of 80 amino should be reported. Hits found should be discussed in the context of available literature information.
- A search of the literature of potential sensitization or elicitation reactions by the specific enzyme(s) in the application. The route of exposure should be presented; for instance, the assessment of safety at the workplace (e.g., sensitization studies) should be submitted.
- A search for possible allergic reactions caused by enzymes of the same family of enzymes as the one under application should be performed. In the case allergic reactions have been observed, a rationale should be given on how these observations do or do not impact on the evaluation.

Questions

- The 35% cut off of sequence homology, using a rolling window of 80 amino acids, may lead to many false positives.

Questions

- Is it enough? Should follow up studies not be performed? IgE binding? Skin prick testing?

Questions

- Would a sliding window of 6-9 amino acids not be more appropriate?

Questions

- What about 3D Analysis?

Questions

- What about proteins inducting of celiac disease?

Questions

- Why referring to enzymes from the same family, even if the proteins may be different?

Questions

- Why indicating raw materials in the culture medium?

Questions

- What about adjuvanticity?

Post-public consultation stakeholder event

21-22 June 2021

New Guidance for Submission of Dossiers on Food Enzymes

Dietary Exposure

Yi Liu & Christina Tlustos

Enzyme Working Group

Trusted science for safe food

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Food Enzyme Dietary Exposure Summary

- Dietary exposure assessment considers the intake of EU consumers.
  - Actual consumption data from EU MS & Conservative scenarios
- The intake of the food enzyme TOS is not always calculated.
  - No residual transfer into foods-as-consumed, no calculation.
  - Extent of transfer is established by technical information and experimental data (so far) from food manufacturer associations.
- Guided by food processes in which enzymes may be used.
  - FoodEx categories and technical factors are consolidated via open call-for-data.
  - More call-for-data are being launched.
- Single process FEIM calculators are available for dossier preparation.
  - Calculators and Input data – both open access
- More than one food processes, EFSA will calculate in-house, until the web-form is developed.

Comments Collected from Public Consultation

1) What would be considered as a "comparable fraction of the source material"?
2) What if new applications are not all covered by the existent categories? Will EFSA update the categories as new information in dossiers are provided? E.g. use of food enzyme in cellular agriculture production.

Removal/absence of TOS transfer

3) Example of tests when referring to "strong experimental data"?
4) Does EFSA also require a method validation?

Q1: "*comparable fraction of the source material*"

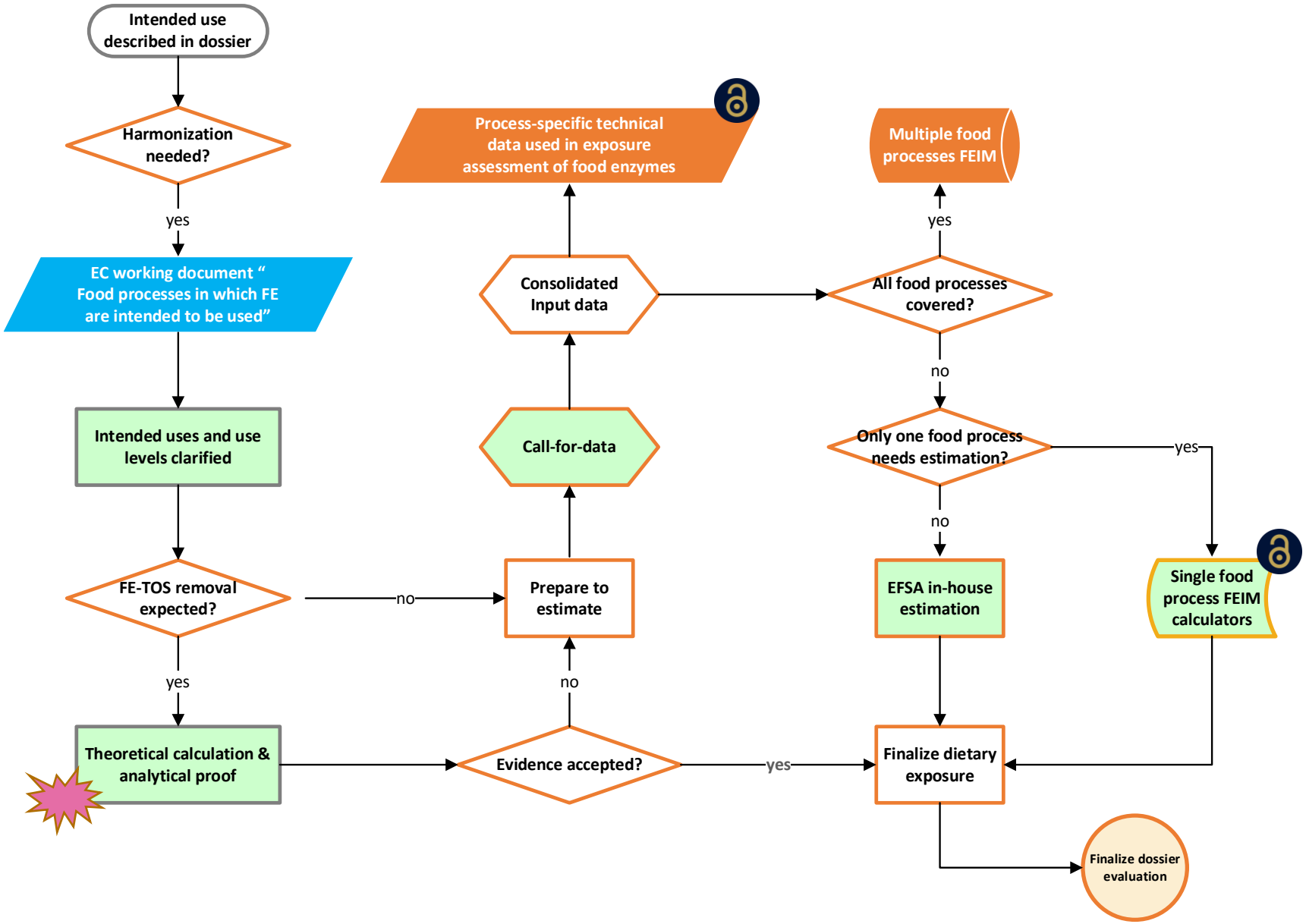
"consumed in a comparable way": Two sets of intake estimate: FE-TOS, SMT-equivalent

	Exposure to the FE-TOS from foods produced through a food process	**Intake of source material TOS equivalent**
Concentration data	TOS in raw material (e.g. vegetable peels)	Plant source: vegetable peels
	Provided in dossier	Edible from literature, recipe, etc
Consumption data	EFSA comprehensive Database (Individual food consumption data, six age groups)	
Food selection	Foods produced from intended processes	Foods containing or produced from the vegetable
Technical factors	**f1, f2**	**f1, f2, enzyme yield factor**
	Consolidated via open call-for-data	The **yield factor** is provided by an applicant. It refers to the amount (x kg) of food enzyme typically obtained from the amount (y kg) of the source material e.g. vegetable peels.

Q2: New food application, who does what?

European Food Safety Authority

Food Enzyme Dietary Exposure Scheme



Technical description needs to be detailed.

The same approach, but the individual applicant may have to bear more burden of the relevant technical info/data.

Q3-Q4: Removal/absence of transfer of the TOS

- "robust experimental data" examples
  - Both direct and indirect data are acceptable.
  - Data are generated from actual industrial scale: e.g. food manufacturing process, intermediate or final products, as appropriate.
  - Method validation is not required by EFSA, but the dossier needs to contain details sufficient to allow the judgement about the reliability of analytical values. This includes the description of the method, LoD, LoQ, *etc*.