STATEMENT

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Characterisation of microorganisms used for the production of food enzymes

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Abstract

This document is intended to assist the applicant in the preparation and the presentation of an application, as foreseen in Article 17.3 of Regulation (EC) No 1332/2008, for the authorisation of food enzymes. It specifically covers the characterisation of microorganisms used as production organisms.

Keywords: microorganism, production strain, genetically modified microorganisms, food enzymes

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Background and terms of Reference as provided by EFSA

Regulation (EC) No 1331/2008[1] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. Regulation (EC) No 1332/2008[2] establishes the conditions for inclusion of a food enzyme in the Community list of approved food enzymes.

Therefore, the European Food Safety Authority (EFSA), asked the CEP Panel to:

1) Consider the existing FEEDAP Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018) and identify the relevant sections applicable to food enzymes
2) Based on the above, develop a statement indicating the data requirements and criteria for the characterisation of microbial strains used as production organisms of food enzymes.

Scope

This document is intended to assist in the preparation and presentation of applications to market food enzymes produced with microorganisms by fermentation in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes. For the purpose of this document, the term microorganism is taken to include archaea, bacteria, yeasts and filamentous fungi.

Only those aspects directly linked to the production organism, including the safety aspects of any genetic modifications, are considered. For other elements of the assessment of food enzymes, e.g. manufacturing process, toxicological studies, etc., applicants should refer to the other relevant CEF Panel documents, i.e. Guidance on the submission of a dossier for safety evaluation of a food enzyme (EFSA CEF Panel, 2009), and Statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

The characterisation of microorganisms used in the production of food enzymes should be made at the production strain level.

1. Characterisation of the microorganism

1.1. Identification

The following taxonomic information needs to be provided: genus, species and strain name or code. For bacteria, taxonomic identity is based on the internationally accepted classification of the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (Euzéby et al., 1997) and the modifications that appear in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (Oren and Garrity, 2016). The nomenclature and taxonomy of fungi, including yeasts, is covered by the International Code of Nomenclature for algae, fungi, and plants (ICN) (Turland et al. 2018). The organism under assessment should be identified unambiguously at species level based on up-to-date methodologies and current knowledge.

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

- Bacteria: Taxonomical identification is expected to be made by computational approach using whole genome sequence (WGS) data (e.g. phylogenomics or average nucleotide identity [ANI]). Identification made by comparing sequences commonly used for taxonomic identification (e.g. 16S rRNA gene), or other characteristic genes (e.g. housekeeping genes) may be acceptable and will be examined on a case by case basis.
- Yeasts: As for bacteria, WGS is the preferred option for the identification of yeasts. This should be done by phylogenomic analysis (e.g. using a concatenation of several conserved genes to produce a phylogeny against available related genomes).

[1] 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, p. 1–6

[2] 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, p. 7–15.

- Filamentous fungi: When WGS is available, identification should be made by a phylogenomic analysis comparing the genome against available related genomes. Alternatively, identification may be made by comparing the 18S rRNA gene and/or internal transcribed spacer (ITS) regions and other characteristic genes (e.g. tubulin) with sequences deposited in databases.

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

The origin of the organism and history of modifications, including mutagenesis steps performed during the development of the strain, shall be reported. Any genetic modification shall be characterised according to Section 1.5.

1.2. Use of whole genome sequence for characterisation of microorganisms

Whole genome sequence analysis (including chromosome(s) and extrachromosomal genetic elements, e.g. plasmids) is required for the 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).

The minimum set of information includes:

- the DNA extraction method
- the sequencing strategy and instrumentation used
- the assembly method applied (e.g. bioinformatic approach, *de novo* or re-seq strategy)
- the statistical measure of sequence quality (e.g. average Phred score, number of reads, coverage, N50 and K-mer)
- the FASTA file(s) of the WGS
- the total length of contigs relative to the expected genome size
- the annotation protocol used
- for fungi: information on the quality of the annotations obtained from relevant databases (e.g. BUSCO[3]).

1.3. Antimicrobial susceptibility

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

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

WGS should be interrogated for the presence of genes coding for or contributing to resistance to antimicrobials relevant to their use in humans and animals (critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs), as defined by WHO, 2016). For this purpose, a comparison against up-to-date databases should be performed (e.g. CARD,[4] ARG-ANNOT,[5] ResFinder[6]). The outcome of the analysis should be presented as a table focusing on complete genes coding for resistance to antimicrobials. The table should include at least gene identification, function of the encoded protein, percentage of identity and percentage length of reference sequence.

If the genetic analysis reveals complete acquired resistance genes coding for antimicrobials, the applicant should demonstrate that the food enzyme does not contain DNA of the production strain (Section 2.2).

In the case of uncertainty (e.g. for partial sequences, low percentage of identity, etc.), the determination of minimum inhibitory concentration (MIC) values for the concerned antimicrobials may be necessary in order to determine the functionality of the identified gene(s). Such determination

[3] http://busco.ezlab.org
[4] https://card.mcmaster.ca/
[5] http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot
[6] https://cge.cbs.dtu.dk/services/ResFinder/

should be made following Section 2.2.1 of the *Guidance on the characterisation of microorganisms used as feed additives or as production organisms* (EFSA FEEDAP Panel, 2018). Cut-off values for antimicrobials chosen to detect a wide range of resistance determinants are proposed for the most commonly used production organisms (Table 1).

Table 1: Microbiological cut-off values (mg/L)[7]

	Bacillus	***Corynebacterium* and other Gram-positive**	***Pseudomonas***	**Enterobacteriaceae**
Ampicillin	n.r.	1	n.r.	8
Piperacillin	n.r.	n.r.	16	n.r.
Vancomycin	4	4	n.r.	n.r.
Gentamicin	4	4	8	2
Kanamycin	8	16	n.r.	8
Streptomycin	8	8	n.r.	16
Erythromycin	4	1	n.r.	n.r.
Clindamycin	4	4	n.r.	n.r.
Tetracycline	8	2	n.r.	8
Chloramphenicol	8	4	n.r.	n.r.
Tylosine	n.r.	n.r.	n.r.	n.r.
Ciprofloxacin	n.r.	n.r.	0.5	0.06
Colistine	n.r.	n.r.	4	2
Fosfomycin	n.r.	n.r.	n.r.	8

n.r. not required.

1.4. Toxigenicity and pathogenicity

1.4.1. QPS

A specific approach to safety assessment applies to those species of microorganisms included in the list of Qualified Presumption of Safety (QPS) status recommended biological agents (EFSA, 2007).[8] QPS provides a generic approach to the safety assessment of microorganisms intentionally introduced into the food and feed chain and also used as a source of fermentation products. To justify that a microorganism is suitable for being evaluated according to the QPS approach, its taxonomic status should be unequivocally established, and the species to which it belongs included in the QPS list. In addition, any qualification set in the most recent QPS statement/opinion should be met.

- In the case of food enzymes produced by genetically modified microorganisms (GMMs) for which the parental/recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment, and for which the molecular characterisation of the event does not give rise to concern, the QPS concept can be extended to the genetically modified (GM) production strain (EFSA FEEDAP Panel, 2018). Notwithstanding this, the absence of DNA from the production strain must be demonstrated in all products made with GMMs (Section 2.2).
- For production strains meeting the criteria for a QPS approach to safety assessment, toxicological studies on the food enzyme will only be required in relation to possible safety concerns identified elsewhere in the assessment process, e.g. manufacturing. In the specific case of *Bacillus* species included in the QPS list, a cytotoxicity test should be made to determine whether the production strain produces high levels of non-ribosomal synthesised peptides, as one of the qualifications of the QPS approach (see Annex A).

[7] Values are taken from the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel et al., 2018), except for Pseudomonas, which are taken from EUCAST (http://www.eucast.org/mic_distributions_and_ecoffs/).

[8] The list of QPS status recommended biological agents for safety risk assessments carried out by EFSA is regularly updated and published at: http://efsa.onlinelibrary.wiley.com/hub/issue/10.1002/(ISSN)1831-4732.QPS/

1.4.2. Non-QPS

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

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

Bacteria

WGS analysis should be used to identify genes coding for known virulence factors. For this purpose, comparison against specific up-to-date databases (e.g. VFDB,[9] PAI DB,[10] MvirDB,[11] CGE[12]) should be performed. The outcome of the analysis should be presented as a table focusing on complete genes encoding recognised virulence factors (e.g. toxins) known to exist in the species or related species to which the strain belongs. The table should include at least the gene identification, function of the encoded protein, percentage of identity and percentage length of the reference sequence. The presence of genes encoding virulence factors may trigger further phenotypic testing (e.g. cytotoxicity tests).

Eukaryotic microorganisms

The potential pathogenicity or ability to produce metabolites that could be harmful to humans should be assessed for eukaryotic microorganisms. A literature search should be carried out to identify the capacity of the species or a closely related species to produce known toxic compounds. Further information on known toxic secondary metabolites potentially produced by several microbial species can be found in scientific publications such as AINIA (2017) or Frisvad et al. (2018). When WGS is available, targeted searches should be performed to identify the presence/absence of known metabolic pathways involved in toxigenicity.

Where the possible presence of compounds of known toxicity is suggested by literature searches or WGS analysis, the applicant should demonstrate by analysis and/or by relevant toxicological studies that their possible presence in the food enzyme is not of concern.

1.5. Genetic modifications

If the strain is GM according to the definition in Directive 2001/18/EC,[13] the genetic modification should be described.

1.5.1. Purpose of the genetic modification

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

1.5.2. Characteristics of the modified sequences

Inserted sequences

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

The following information should be provided:

[9] http://www.mgc.ac.cn/VFs/main.htm
[10] http://www.paidb.re.kr/about_paidb.php
[11] http://mvirdb.llnl.gov
[12] http://www.genomicepidemiology.org/
[13] Article 2(2) of Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms defines "genetically modified organism" as an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

DNA from defined donor organisms

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

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

Designed sequences

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

- rationale and strategy for the design
- DNA sequence and a physical map of the functional elements
- derived amino acid sequence(s) and function(s) of the encoded protein(s)
- similarity with sequences in up-to-date databases (e.g. ENA,[14] NCBI,[15] UniProt[16]). This should identify the functional domains of the recombinant protein; the best hits should be reported and described.

Deletions

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

Base pair substitutions and frameshift mutations

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

1.5.3. Structure of the genetic modification

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

Structure of the genetic modification using WGS data

Detailed information should be provided, including a map or graphic presentation of all genomic regions (chromosome, contig or plasmid) harbouring genetic modifications, indicating:

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

This can be done preferably by comparing the WGS of the GMM with that of the non-modified parental or recipient strain.

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

[14] http://www.ebi.ac.uk/ena
[15] https://www.ncbi.nlm.nih.gov/
[16] http://www.uniprot.org/

Structure of the genetic modification without WGS data

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

Characteristics of the vector

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

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

Information relating to the genetic modification process

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

- methods used to introduce, delete, replace or modify the DNA into the recipient/parental, and methods for selection of the GMM
- it should be indicated whether the introduced DNA is a replicative vector, or is inserted into the chromosome(s) and/or, for eukaryotic microorganisms, into DNA of organelles (e.g. mitochondria) if appropriate.

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

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

Genes of concern

Any genes of concern as defined in Section 1.5.3 (such as genes encoding AMR, toxins and virulence factors) inserted in the GMM shall be clearly indicated.

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

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

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

- Southern blots shall include appropriate positive and negative controls. The length and location of the probe(s) used should be indicated. The amount of DNA from the production strain loaded in the agarose gel should be provided, together with an image of the gel before blotting. Positive control shall be loaded in a concentration corresponding to approximately 10 copies of the target fragment. If several probes are used, they shall be tested in separate experiments.
- PCR experiments shall include a positive control containing the same gene as that used during strain development, together with proper positive controls to exclude PCR inhibition and to ensure sufficient sensitivity. A negative control should also be included.

2. Viable cells and DNA of the production strain

2.1. Viable cells of the production strain

This section applies to all food enzymes except 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:

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

2.2. DNA from the production strain

This section applies to:

- food enzymes obtained using GM production strains
- 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 product by PCR, targeting a fragment specific for this strain. Detailed information should be provided on the specific target sequence, primers and polymerase used and amplification conditions:

- 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
- 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
- at least three independent batches of product 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
- to recover DNA from non-viable cells potentially remaining in the product, the DNA should be extracted using a methodology suitable for all cellular forms of the production strain (e.g., vegetative cells, spores)
- the following controls and sensitivity tests should be included:

  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 limit of detection
  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

- if PCR failure is encountered, the causes should be investigated (e.g. PCR inhibition, presence of nucleases).

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

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Glossary

Acquired AMR gene	A gene acquired by a bacterium conferring antimicrobial resistance to an otherwise typically susceptible microorganism.
Antimicrobial	An active substance of synthetic or natural origin which destroys microorganisms, suppresses their growth or their ability to reproduce in animals or humans, excluding antivirals and antiparasitic agents. For the purposes of this document, antimicrobials are those relevant to their use in humans and animals defined by the WHO as critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs).

Gene of concern	gene known to contribute to the production of toxic metabolites and antimicrobials of clinical relevance, or to AMR.
History of use	Documented information on the microbial strain regarding its previous deliberate introduction or use in the food chain.
Microorganism	Any microbiological entity, cellular or non-cellular, capable of multiplication or of transferring genetic material, including viruses, viroids, animal and plant cells in culture. For the purpose of this guidance document, microorganisms cover archaea, bacteria, yeasts and filamentous fungi.
Parental strain	A non-genetically modified microorganism with direct genealogical link to the GMM.
Recipient strain	The strain that is subjected to genetic modifications which are subject of the application. The recipient strain can be the parental or its derivative, mutagenised or genetically modified. The recipient strain gives rise to the GMM.
Vector	is understood as the agent containing the introduced DNA sequence used as a vehicle to transfer such sequence into the transformed cell.

Abbreviations

AMR	antimicrobial resistance
ANI	average nucleotide identity
BHI	brain heart infusion
CIA	critically important antimicrobial
CFU	colony forming unit
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GM	genetically modified
GMM	genetically modified microorganism
HIA	highly important antimicrobial
ICN	International Code of Nomenclature
IJSEM	International Journal of Systematic and Evolutionary Microbiology
ITS	internal transcribed spacer
IUBMB	International Union of Biochemistry and Molecular Biology
LPSN	List of Prokaryotic Names with Standing in Nomenclature
MEM	Minimum Essential medium
MIC	minimum inhibitory concentration
ORF	open reading frame
PCR	polymerase chain reaction
PI	propidium iodide
QPS	Qualified Presumption of Safety
TCA	trichloroacetic acid
WGS	whole genome sequence

Annex A – Recommended procedure for the detection of cytotoxicity in *Bacillus* species included in the QPS list using epithelial cell lines

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. In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains, *in vitro* cell-based methods are the choice to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. In addition, 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 ^{14}C-leucine uptake is described, but other methods such as those based on lactate dehydrogenase release or propidium iodide (PI) uptake could be used alternatively (Fagerlund et al., 2008).

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 10^8 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-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 following 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 ^{14}C-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: ((Neg. ctrl – sample)/Neg. ctrl) × 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 *B. cereus* strains may be used.

An alternative method is to measure 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 contained 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, values above 20% of the fluorescence/absorbance obtained from the positive control (usually detergent-treated cells) are considered to indicate cytotoxicity.