GRAS NOTIFICATION FOR A NUCLEASE ENZYME PREPARATION PRODUCED BY *BACILLUS AMYLOLIQUEFACIENS*

c-LEcta GmbH
Perlickstr. 5,
04103 Leipzig
Germany

March 2023

TABLE OF CONTENTS

PART 1 - SIGNED STATEMENT OF THE CONCLUSION OF GRAS (GENERALLY RECOGNIZED AS SAFE) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260. 4

PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT 6

2.1 Identity of the notified substance 6

2.2 Identity of the source 6

2.2.1 Production strain, recipient strain and donor 6

2.2.2 Genetic modification 7

2.2.3 Stability of the introduced genetic sequences 8

2.2.4 Antibiotic resistance genes 8

2.2.5 Absence of production organism 8

2.3 Method of manufacture 8

2.3.1 Raw materials and processing aids 9

2.3.2 Fermentation process 9

2.3.3 Purification process and recovery 10

2.4 Product specifications and composition 10

2.4.1 Specifications 10

2.4.2 Quantitative composition 11

2.5 Applications 12

2.5.1 Mode of action 12

2.5.2 Intended use 12

2.5.3 Use levels 12

2.5.4 Enzyme residues in final food 12

PART 3 - DIETARY EXPOSURE 13

3.1 Assumptions for the dietary exposure assessment 13

3.2 Estimated daily intake 16

3.2.1 Estimated daily intake for the general population 16

PART 4 - SELF LIMITING LEVELS OF USE 16

PART 5 - COMMON USE IN FOOD BEFORE 1958 16

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS 16
6.1 Safety of the manufacturing process 17
6.2 Safety of the production strain 17
6.2.1 History of safe use 17
6.2.2 Safe strain lineage 19
6.2.3 Safety studies 20
6.2.4 Safety of the donor 21
6.3 Safety of the nuclease enzyme 21
6.3.1 Safety of use 21
6.3.2 Existing authorisations for nucleases 22
6.3.3 Publications on the safety of nuclease enzymes 23
6.3.4 Allergenicity 24
6.3.5 Identification of the NOAEL and Margin of Safety (MoS) 26
6.4 SUMMARY AND CONCLUSIONS 26
PART 7 - SUPPORTING DATA 27
7.1 Annexes 27
7.2 References 28

PART 1 - SIGNED STATEMENT OF THE CONCLUSION OF GRAS (GENERALLY RECOGNIZED AS SAFE) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260.

§170.225(C) (1) SUBMISSION OF GRAS NOTICE

In accordance with the 21 CFR § 170 subpart E, c-LEcta GmbH hereby is submitting a GRAS notification for its nuclease enzyme preparation produced by *Bacillus amyloliquefaciens*.

§170.225(C) (2) NAME AND ADDRESS OF THE NOTIFIER

Notifier:
c-LEcta GmbH
Perlickstr. 5,
04103 Leipzig
Germany

§170.225(C) (3) NAME OF THE NOTIFIED SUBSTANCE

Nuclease (EC 3.1.30.2, CAS 9025-65-4) from a *Bacillus amyloliquefaciens* strain expressing the gene encoding a nuclease from *Serratia marcescens*. The common or usual name of the substance is nuclease.

§170.225(C) (4) INTENDED CONDITIONS OF USE

The nuclease preparation is used as a processing aid for the hydrolysis of nucleic acids during manufacturing of microbial-derived ingredients. The enzyme may be used in different applications where nucleic acids from the microbial biomass can be cleaved by the nuclease. Some examples include the manufacturing of single cell protein, microbial cell extracts, or ingredients produced by precision fermentation, such as alternative protein or specialty functional ingredients. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. The target population for consumption is the general population.

§170.225(C) (5) STATUTORY BASIS FOR GRAS CONCLUSION

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §170.30 (a) and (b). It is of note that several enzyme preparations (protease, carbohydrase)

from the microbial host *Bacillus amyloliquefaciens* were affirmed as GRAS by US FDA [1] based on documented pre-1958 history of use [2].

§170.225(C) (6) PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of its intended use.

§170 225(C) (7) AVAILABILITY OF INFORMATION

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying during customary business hours at c-LEcta GmbH or will be sent upon request.

§170 225(C) (8) FOIA (FREEDOM OF INFORMATION ACT)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170 225(C) (9) INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favourable and unfavourable information, known to c-LEcta GmbH and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

DocuSigned by:

184013D60156494...

Paula Pescador
VP Regulatory Affairs
c-LEcta GmbH

Date: March 24, 2023

PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a nuclease enzyme produced by submerged fermentation of a genetically modified *Bacillus amyloliquefaciens* microorganism expressing the gene encoding for a nuclease from *Serratia marcescens*.

The chemical characteristics of the nuclease are given below:

Classification	Nuclease
Accepted Name	*Serratia marcescens* nuclease
Systematic Name	Nuclease
Synonyms	endonuclease (*Serratia marcescens*); barley nuclease; plant nuclease I; nucleate endonuclease, phosphodiesterase
(EC) Number	EC 3.1.30.2
(CAS) Number	9025-65-4
Molecular weight	26.70 kDa
Specificity	Catalyses the hydrolysis of the P-O-β' linkage to yield the 5'-phosphomononucleotide and 5' phosphooligonucleotide end-products. Nonspecific hydrolysis of both single and double stranded ribo- or deoxyribonucleic acids (RNA and DNA).
Amino Acid sequence	The total nucleotide and amino acid sequences have been determined

Nucleases are phosphodiesterase enzymes capable of cleaving the P-O bonds in nucleic acid polymers. According to the IUBMB nomenclature, the nuclease from *Serratia marcescens* belongs to the enzyme subclass of the hydrolases acting on ester bonds, and in particular to the endoribonucleases active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters.

2.2 IDENTITY OF THE SOURCE

2.2.1 PRODUCTION STRAIN, RECIPIENT STRAIN AND DONOR

Production organism:

The nuclease enzyme is derived from a genetically modified *Bacillus amyloliquefaciens* strain. The parental organism is the well-known *Bacillus amyloliquefaciens* strain P [3]. Strain GSB272 was derived from this strain by classical mutagenesis [4]. A strain isolated

without further mutagenesis steps (GSB272 L4) was obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany) and designated as LE2B100.

The *Bacillus amyloliquefaciens* species is generally regarded as non-pathogenic and is widely distributed in nature. It is classified as a Class 1 organism according to the NIH guidelines [5]. Risk Group 1 organisms are those not associated with disease in healthy adult humans. In addition, *Bacillus amyloliquefaciens* is included in the list of microorganisms with Qualified Presumption of Safety (QPS) status, introduced by the European Food Safety Agency (EFSA) [6]. The Qualified Presumption of Safety (QPS) is a safety assessment for microorganisms used in the food chain. The QPS concept uses existing knowledge about the safety of specific microorganisms to differentiate between those which are not of concern (and are therefore granted QPS status) and those which may represent a risk and should be subject to a full safety assessment [7].

The *Bacillus amyloliquefaciens* production strain has been deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The strain was unequivocally identified as *Bacillus amyloliquefaciens* by whole genome sequencing (WGS) and shows a degree of homology of over 99.9% with the type strain *Bacillus amyloliquefaciens* DSM 7.

This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large-Scale Practice) microorganisms [8]. It also meets the criteria for a safe production microorganism as described by Pariza et al. as well as by several expert groups [9]–[15].

Donor:

The DNA sequence for the introduced gene was *de novo* synthesized based upon the published sequence encoding a nuclease from *Serratia marcescens*. The gene sequence coding for the mature protein is fused to a leader sequence from *Bacillus spec*.

2.2.2 GENETIC MODIFICATION

For the development of the nuclease production strain, the *Bacillus amyloliquefaciens* LE2B100 host was modified at several chromosomal loci to improve the production process and the enzyme yield. A number of sequences encoding proteases as well as other unwanted proteins were deleted, leading to improved purity and stability of the enzyme preparation. In addition, one gene essential for sporulation was deleted. The resulting production strain LE2B125 is unable to sporulate and secretes high amounts of nuclease into the culture supernatant.

The expression plasmid used to transform the *Bacillus amyloliquefaciens* recipient strain is based on the well-known *Bacillus* vector pWV01 from *Lactococcus lactis* [16]. The expression cassette consists of a native *Bacillus amyloliquefaciens* promoter and terminator, a leader sequence from *Bacillus spec.*, and the sequence encoding the nuclease from *Serratia marcescens*. Only these elements are present in the final production strain, with no fragments of the vector backbone being introduced. For the construction of the production strain, the expression cassette containing the sequence coding for the nuclease was integrated into one chromosomal locus by targeted homologous recombination. Sequence confirmation of all introduced sequences and flanking regions was carried out by PCR analysis as well as whole genome sequencing.

2.2.3 STABILITY OF THE INTRODUCED GENETIC SEQUENCES

The introduced DNA is integrated into the *Bacillus amyloliquefaciens* chromosome, making it poorly mobilizable for genetic transfer to other organisms. Chromosomal integration is generally considered as a stable transformation strategy for industrial fermentation processes. The phenotypic and genetic stability of the modified *Bacillus amyloliquefaciens* production strain is demonstrated by its ability to produce stable levels of the nuclease enzymes, as shown by enzyme activity measurements in three independent batches of the food enzyme (see section 2.4.1). In addition, identical protein expression profiles were obtained for three enzyme batches by SDS-PAGE analysis, confirming the stability of the genetic modifications.

2.2.4 ANTIBIOTIC RESISTANCE GENES

No antibiotic resistance genes were introduced in the production strain as a result of the genetic modifications. The absence of antibiotic resistance markers or any other sequences of concern was verified by whole genome sequence analysis.

2.2.5 ABSENCE OF PRODUCTION ORGANISM

As established by the commercial product specification, the absence of the production organism is one of the parameters which are verified for each product batch. The nuclease food enzyme preparation is free of detectable, viable cells of the production organism. Absence of the production strain in the final product is confirmed by a validated in-house method.

2.3 METHOD OF MANUFACTURE

The nuclease enzyme is produced under a quality management system which complies with the requirements of ISO 9001. The manufacturing process for the enzyme is in accordance with current Good Manufacturing Practices [17], using ingredients that are

accepted for general use in foods, and under conditions that ensure a controlled fermentation [18]–[20]. An overview of the manufacturing process is presented in Annex A.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (FCC) [21]. It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [22].

2.3.1 RAW MATERIALS AND PROCESSING AIDS

All raw materials and processing aids used in the fermentation process are standard materials used in industrial enzyme production and comply with purity criteria and limits established in the FCC specifications or to internal specifications in line with FCC requirements, wherever applicable [21]. All raw materials are sourced from suppliers qualified and approved according to c-LEcta's supplier quality program.

Regarding potential major food allergens, soy peptone is used in the fermentation process and is consumed by the microorganism as a nutrient. Soy peptone is produced by hydrolysis of soy protein, whereby the hydrolysis step significantly reduces any potential allergenicity of the material. Several assessments performed by industry associations as well as by scientific and regulatory bodies have concluded that allergen proteins from fermentation media are not found in final enzyme preparations, and that enzyme preparations do not pose an allergen risk that would require allergen labelling on the final products (Annex B). In addition, c-LEcta has performed studies to evaluate the possible presence of soy residues. No soy allergens are detected in the final enzyme product (ELISA-based method, limit of detection: 2 ppm).

Any antifoams used in fermentation and recovery follow 21 CFR §173.340 and 21 CFR §172.820 and are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998 [23]–[25]. The maximum use level of the antifoams and/or flocculants, if used in the product, is not greater than 1%.

2.3.2 FERMENTATION PROCESS

The nuclease enzyme is produced by a controlled submerged fermentation of a selected, pure culture of the genetically modified *Bacillus amyloliquefaciens* strain described in Part 2.2. Nuclease is manufactured in compliance with cGMP, and a HACCP system is implemented. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination. All culture media are sterilized prior to use.

For each new batch of nuclease, a suspension of a pure culture (stock culture) of the production strain is aseptically transferred to an inoculum flask containing fermentation medium. Each new batch of the stock culture is thoroughly controlled for identity, microbial purity, and target enzyme expression before use. The culture is grown in the flask until a defined amount of biomass is obtained, which can subsequently be used as inoculum for the seed fermentation.

During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are carried out at regular intervals to ensure absence of foreign microorganisms and confirm strain identity.

2.3.3 PURIFICATION PROCESS AND RECOVERY

Fermentation is followed by a series of recovery and purification steps consisting of solid/liquid separation, concentration, and filtration. The purpose of the recovery process is to isolate the enzyme protein from the microbial biomass as well as to concentrate and stabilize the target enzyme protein.

During fermentation, the enzyme protein is secreted from the cells of the production strain into the medium. Consequently, the cells need to be removed from the fermentation broth containing the enzyme protein. During recovery, the enzyme product is separated from the cell biomass.

The recovery process steps are as follows:

1) Pre-treatment – flocculation (optional)
2) Primary separation – filtration or centrifugation
3) Prefiltration
4) Concentration – ultrafiltration
5) Final and polish filtration
6) Formulation: preservation and stabilization of the liquid enzyme concentrate.

The enzyme concentrate is stabilized with glycerol to obtain the final liquid product with the typical composition shown in the table below (see section 2.4.1).

2.4 PRODUCT SPECIFICATIONS AND COMPOSITION

2.4.1 SPECIFICATIONS

Food-grade specifications have been established for the nuclease from *Bacillus amyloliquefaciens*. These specifications comply with the current purity limits established for food enzyme preparations in the latest edition of the FCC and by JECFA [21], [22]. All

methods of analysis are nationally or internationally recognized or have been internally validated by c-LEcta.

Three representative batches of the nuclease preparation derived from *Bacillus amyloliquefaciens* were analysed to verify that the manufacturing process produces a consistent product that meets the specifications. The results of these batch analyses are as follows:

Parameter	Specification	Batch 1	Batch 2	Batch 3
Assay (Activity)	MU/L	81	77	112
Lead (mg/kg)	NMT 5 mg/kg	NMT 5	NMT 5	NMT 5
Cadmium (mg/kg)	NMT 0.5 mg/g	NMT 0.5	NMT 0.5	NMT 0.5
Mercury (mg/kg)	NMT 0.5 mg/g	NMT 0.5	NMT 0.5	NMT 0.5
Arsenic (mg/kg)	NMT 3 mg/g	NMT 3	NMT 3	NMT 3
Total viable plate count	NMT 50,000 CFU/g	NMT 10	NMT 10	NMT 10
Coliforms	NMT 30 CFU/g	NMT 30	NMT 30	NMT 30
Escherichia coli	Negative in 25 g	Negative	Negative	Negative
Salmonella spp.	Negative in 25 g	Negative	Negative	Negative
Antimicrobial activity	Negative	Negative	Negative	Negative

CFU = colony forming units; NMT = not more than.

2.4.2 QUANTITATIVE COMPOSITION

The nuclease enzyme preparation is commercialized in liquid form with the representative composition shown in the table below.

Substance	Approximate percentage (w/w)
Water	44-46%
Glycerol	52-55%
Ash	<1%
Total Organic Solids (TOS)	<1%

TOS = Total Organic Solids defined as 100% - (water + ash + diluents)

2.5 APPLICATIONS

2.5.1 MODE OF ACTION

The active enzyme is a nuclease (EC 3.1.30.2). Nucleases are phosphodiesterase enzymes capable of cleaving the P-O bonds in nucleic acid polymers. The enzyme hydrolyses single or double stranded DNA and RNA at the P-O3' bond, generating 5'-phosphate and 3'-OH products (mono- and oligonucleotides).

2.5.2 INTENDED USE

The nuclease enzyme is used as a processing aid to hydrolyze nucleic acids and reduce the polynucleotide content during the manufacturing of several microbial-derived food ingredients. The endonuclease activity of the enzyme leads to a rapid reduction of the molecular weight of nucleic acid polymers. The nuclease is used in microbial fermentation applications (such as the production of single cell protein, microbial cell extracts, microbial-derived alternative protein, or specialty functional ingredients produced by precision fermentation) to reduce the viscosity of the fermentation broth, increase the efficiency of the recovery process, to comply with regulatory requirements and/or to improve the nutritional and organoleptic profile of the products.

2.5.3 USE LEVELS

Enzyme preparations are used by food producers at the minimum levels required to achieve the desired effects and in accordance with Good Manufacturing Practices (GMP).

The final dosage applied by a food manufacturer depends on the specific process. The initial recommendation by the enzyme manufacturer represents the starting point for the subsequent optimization by the food producer. Typical use levels of the nuclease in food processing are 30.7-143.1 mg TOS per kilogram microbial biomass (dry cell weight).

2.5.4 ENZYME RESIDUES IN FINAL FOOD

The nuclease enzyme preparation is used during food processing, typically at the end of a microbial fermentation. During subsequent processing, and due to the process conditions used by food manufacturers, the enzyme is removed or rendered inactive in the final food (because of denaturation, substrate depletion, unfavourable pH conditions, etc.). Consequently, any food enzyme residues present in the final food will not exert any function.

PART 3 - DIETARY EXPOSURE

The nuclease is expected to be present only in negligible amounts and as non-functional residue in the final food. However, a worst-case scenario is provided below for the calculation of the theoretical maximal daily human exposure. To this end, it is assumed that the entire amount of enzyme preparation added during food processing will remain in the final food as TOS.

The overall general population is the target population for consumption.

3.1 ASSUMPTIONS FOR THE DIETARY EXPOSURE ASSESSMENT

Nuclease will be used as a processing aid for the manufacturing of microbial-derived ingredients such as single cell protein or microbial cell extracts as well as alternative protein and functional ingredients produced by precision fermentation. Starting from the same raw material (microbial biomass), the enzyme use levels on a mg TOS/kg basis may vary depending on the application, as shown in the table below. The yield of the target ingredient produced per kg raw material as well as the amount of the ingredient present in the final food (use levels of the ingredient) are also variable.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)
Production of single cell protein (fungal protein, etc.) microbial cell extracts, etc.	Microbial biomass	61.3-143.1
Production of microbial-derived alternative protein (dairy protein, egg protein, etc.)	Microbial biomass	14.3-30.7
Production of specialty functional ingredients (enzymes, flavorings, vitamins, etc.)	Microbial biomass	40.9-143.1

The nuclease food enzyme is used in the manufacture of a wide range of food ingredients. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method.

This method is widely accepted as a preliminary screening tool used to assess the intake of chemicals such as food additives [26]. The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys.

It must be noted that the estimates provided by this method are very conservative and should be considered as a highly exaggerated worst-case scenario. The total TMDI will be largely overestimated because of the following reasons:

1) It is assumed that all producers of the intended uses for both solid foodstuffs and beverages will use the food enzyme at the highest suggested dose.
2) For the calculation of the TMDI in food as well as in beverages, only those categories containing the maximum theoretical amounts of TOS are considered.
3) It is assumed that no amount of enzyme TOS will be removed in any of the food processing steps during manufacturing of the final food.
4) It is assumed that the final food containing the calculated theoretical amount of enzyme TOS is consumed daily over the course of a lifetime. The assumption is for processed food (50% of total solid food) and for soft drinks (25% of total beverages).

The following assumptions are made regarding the budget method for the consumption of solid food and beverages:

Budget method assumptions				
Average consumption over the course of a lifetime, per kg body weight and day	Total solid food (kg)	Total non-milk beverages (l)	Processed food = 50% of total solid food (kg)	Soft drinks = 25% of total beverages (l)
	0.025	0.1	0.0125	0.025

The ingredients manufactured using the nuclease enzyme can be added to a wide range of foods: as main ingredients (for example when replacing animal/vegetal protein), as specialty functional ingredients generally used at low levels, or as processing aids contained at even lower levels in the final food.

The recommended use level of the nuclease is given based on the raw material used in the food process and on the different applications. For manufacturing of alternative protein and of specialty functional ingredients, an average yield of 10% relative to the raw material (dry cell biomass) is assumed. The calculation also considers how much solid or liquid food is obtained per kg raw material in the different applications, and it is further assumed that all the TOS contained in the microbial-derived ingredient will end up in the final food product.

The assumed maximum amounts of TOS from the nuclease enzyme preparation in the different applications are summarized in the table below:

	Application	Raw material (RM)	Use level, mg TOS/kg RM	Final Food (FF)	Ratio RM/FF *	Maximum level in FF, mg TOS/kg
Solid foods	Production of single cell protein (fungal protein, etc.)	Microbial biomass	143.1	Mycelium- or plant-based meat and fish substitutes, dairy substitutes, grain products, baked goods, yeast extracts and spreads, etc.	0.25	35.77
	Production of microbial-derived alternative protein (dairy protein, egg protein, etc.)	Microbial biomass	30.7	Meat and dairy substitutes, egg substitutes, protein bars, confections, snack foods, etc.	3.5	107.4
	Production of functional ingredients (enzymes, functional proteins, specialty carbohydrates, etc.)	Microbial biomass	143.1	Multiple food categories	0.5	71.55
Liquid foods	Production of single cell protein (fungal protein, etc.)	Microbial biomass	143.1	Beverages and beverage bases, fruit and vegetable juices, fruit beverages, etc.	0.25	35.77
	Production of microbial-derived alternative protein	Microbial biomass	30.7	Nutritional beverages, sport drinks, high-protein performance drinks, etc.	2.5	76.5
	Production of functional ingredients (enzymes, functional proteins, specialty carbohydrates, etc.)	Microbial biomass	143.1	Beverages and beverage bases, nutritional beverages, sport drinks, high-protein performance drinks, etc.	0.5	71.55

*Calculated considering the ingredient yield (kg ingredient/kg RM) and the ingredient use levels in FF.

3.2 ESTIMATED DAILY INTAKE

3.2.1 ESTIMATED DAILY INTAKE FOR THE GENERAL POPULATION

Based on conservative assumptions for the Budget Method described in section 3.1 above, the TMDI of the nuclease enzyme from its intended uses as a processing aid in manufacturing of microbial-derived ingredients can be estimated. To represent a worst-case scenario, the TMDI for solid foods will be combined with the TMDI for beverages in the risk assessment. The TMDI from applications in the production of alternative protein represents the worse-case scenario and is used in the calculation as follows:

TMDI in solid foods (mg TOS/kg body weight/day)	TMDI in beverages (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
107.4 x 0.0125 = 1.34	76.5 x 0.025 = 1.91	1.34 + 1.91 = 3.25

PART 4 - SELF LIMITING LEVELS OF USE

This part does not apply.

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply.

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS

The information presented in the following paragraphs is used as a base for c-LEcta's conclusion of the general recognition of safety for the nuclease enzyme.

The safety of the nuclease enzyme preparation is assessed according to the guidelines developed by Pariza et al. and the International Food Biotechnology Council, which are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods [9], [11], [14]. The Pariza-Johnson decision tree outlining the safety evaluation of the nuclease enzyme is provided in Annex C.

The safety evaluation in Part 6 follows the approach described by the Enzyme Technical Association in a recent publication [27]. The safety assessment is based on an overview of the manufacturing process, of the safety of the production strain, of the introduced DNA and of the enzyme itself. Additional safety considerations (i.e., allergenicity of the enzyme)

are also addressed. All the information and data presented is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6.1 SAFETY OF THE MANUFACTURING PROCESS

As mentioned in section 2.3, the manufacturing process of nuclease follows the industry standard.

The quality management system used in the manufacturing process for the enzyme preparation complies with the requirements of ISO 9001. The enzyme is produced under a standard manufacturing process in accordance with current Good Manufacturing Practices using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation [18]–[20].

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the FCC [21]. It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA [22].

6.2 SAFETY OF THE PRODUCTION STRAIN

In assessing the safety of an enzyme food preparation, the main consideration should be the safety of the production strain [9], [14]. The definition of a non-toxigenic organism by Pariza and Foster is “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a non-pathogenic organism is “one that is very unlikely to produce disease under ordinary circumstances”[9]. Therefore, if an enzyme preparation is produced using a non-toxigenic and non-pathogenic strain and under current GMP, then it can be considered as safe [11].

6.2.1 HISTORY OF SAFE USE

Bacillus amyloliquefaciens is a soil-borne bacterium widely distributed in nature and generally regarded as non-pathogenic. It is classified as a Class 1 organism according to the NIH guidelines [5]. Risk Group 1 organisms are those not associated with disease in healthy adult humans. In addition, *Bacillus amyloliquefaciens* is included in the list of microorganisms with Qualified Presumption of Safety (QPS) status, introduced by EFSA [6], [7]. This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show acquired resistance to antibiotics of human and veterinary importance as well as the absence of toxigenic potential to be demonstrated.

Members of the *Bacillus* species are among the most important industrial sources of food grade enzymes. Because of their high grade of relatedness, *Bacillus subtilis* and *Bacillus amyloliquefaciens* were officially designated as separate species only in 1987 [28]. Food

enzymes derived from *Bacillus amyloliquefaciens* strains are mainly amylases and proteases. They have been evaluated by many countries which regulate the use of food enzymes, such as the USA [29], France [30], Australia/New-Zealand [31] and Canada [32], resulting in the approval of the use of food enzymes from *Bacillus amyloliquefaciens* or *Bacillus subtilis* in several food manufacturing applications, such as baking, brewing, juice production, wine production and the production of dairy products, as shown in the table below.

Authority	Food enzyme	References
Australia/ New Zealand	α-Acetolactate decarboxylase (EC 4.1.1.5) α-Amylase (EC 3.2.1.1) β-Amylase (EC 3.2.1.2) Amylomaltase (EC 2.4.1.25) Endo-1,4-beta-xylanase (EC 3.2.1.8) β-Glucanase (EC 3.2.1.6) Glutaminase (EC 3.5.1.2) Hemicellulase multicomponent enzyme (EC 3.2.1.78) Metalloproteinase Pullulanase (EC 3.2.1.41) Serine proteinase (EC 3.4.21.14)	Aus/NZ schedule 18 processing aids [31]
Canada	Amylase Glucanase Glutaminase Hemicellulase Pentosanase Protease	Canada List of permitted food enzymes [32]
France	α-Amylase β-Glucanase β-Glucanase Protease	France Arrêté 19 Oct 2006 [30]
USA	Amylomaltase Subtilisin and neutral proteinase Carbohydrase	Gras Notice Inventory [29] 21 CFR §184.1150 [1] 21 CFR §184.1148 [33]

In the US, the food enzyme industry has extensively used *Bacillus amyloliquefaciens* as a safe production organism for decades. Both carbohydrase (alpha-amylase and beta-glucanase) and protease enzymes from *Bacillus amyloliquefaciens* (subtilisin and neutral protease) are affirmed as GRAS by FDA [1], [33]. A petition from the Enzyme Technical Association, amended by several Federal Register notices, proposed affirmation that carbohydrase and protease enzyme preparations from *Bacillus amyloliquefaciens* are

GRAS for use in food. FDA relied on the history of safe use in food for its safety assessment and concluded that carbohydrase and protease enzyme preparations derived from *Bacillus amyloliquefaciens* and *Bacillus subtilis* were in common use in food prior to January 1, 1958 [34], [35]. FDA published its final GRAS affirmation Rule on April 23, 1999 (FR 64 (78)) as follows [36]: (1) carbohydrase enzyme preparation from *Bacillus subtilis*; (2) protease enzyme preparation from *Bacillus subtilis*; (3) carbohydrase enzyme preparation from *Bacillus amyloliquefaciens*; and (4) protease enzyme preparation from *Bacillus amyloliquefaciens*. The associated enzyme activities covered in the GRAS affirmation included: alpha-amylase (EC 3.2.1.1), glucanase (EC 3.2.1.6), subtilisin (EC 3.4.21.62), and neutral proteinase (EC 3.4.24.28).

6.2.2 SAFE STRAIN LINEAGE

Thoroughly characterized non-pathogenic and non-toxigenic microbial strains are logical candidates for developing a safe strain lineage, as described by Pariza and Johnson in 2001 and supported by several publications [9], [11], [14], [27].

In the assessment of the nuclease enzyme, c-LEcta considers that the safety of the *Bacillus amyloliquefaciens* production strain can be established based on the safe strain lineage approach. Specifically, the following conditions described by Pariza and Johnson are met: (1) thorough characterization of the host organism as non-pathogenic and non-toxigenic; (2) determination of the safety of all new DNA introduced into the host organism; and (3) use of modification procedure(s) appropriate for food use.

The long industrial use and wide distribution of *Bacillus amyloliquefaciens* in nature has never led to any pathogenic symptoms. Moreover, no cases demonstrating invasive properties of the species have been found in the literature [37]. *Bacillus amyloliquefaciens* is therefore generally accepted as a non-pathogenic organism. This conclusion has been repeatedly confirmed by EFSA with the inclusion of *Bacillus amyloliquefaciens* in the list of microorganisms with QPS status, including genetically modified microorganisms (GMMs) of this species for which the genetic modification does not give rise to safety concerns [6]. This list is regularly updated in a process that includes extended literature searches for any information that would affect the qualification of *Bacillus amyloliquefaciens* strains for the QPS status.

Over several decades, food enzymes from *Bacillus amyloliquefaciens* and *Bacillus subtilis* have been evaluated for safety using criteria comparable to those established by Pariza and Foster and IFBC, including toxicological testing [9], [11], [14]. These studies repeatedly showed that enzymes produced by use of *Bacillus amyloliquefaciens* were safe for their intended uses, independent of the specific enzyme.

Classical mutagenesis performed using standard microbiological methods is considered safe for food applications [11], [38], [39]. For strains improved using recombinant DNA techniques, the safety of the methods as well as of all new DNA introduced into the host organism must be determined.

The microbial host for the nuclease enzyme production strain was derived from a well-documented isolate of *Bacillus amyloliquefaciens* [3] and has a very high degree of homology (>99.9%) with the type strain DSM 7 (also known as ATCC 23350), as confirmed by c-LEcta using whole genome sequencing data. Thus, both the parental strain and the industrial production strain for the nuclease enzyme have been taxonomically identified and determined to belong to a non-pathogenic, non-toxigenic species. The intermediate strain LE2B100 was derived by chemical mutagenesis, and the strain was further modified by standard recombinant DNA techniques to develop the nuclease enzyme production strain. The specific methods and the modifications introduced have been described in Part 2.

Whole genome sequencing and analysis confirmed the correct integration and deletion of target genes in the chromosome as well as the absence of vector sequences not intended to remain in the production strain. The absence of antibiotic selection markers or any other acquired antimicrobial resistances was equally confirmed. In addition, the *Bacillus amyloliquefaciens* production strain was assessed via a cytotoxicity test to demonstrate the absence of toxigenic potential (see section 6.2.3). The results clearly showed the absence of cytotoxic effects, further confirming that none of the introduced changes (either by classical mutagenesis or by recombinant DNA methods) induced the production of cytotoxic substances.

Based on the above information, it is concluded that the *Bacillus amyloliquefaciens* production strain is part of a non-pathogenic, non-toxigenic safe strain lineage and is considered safe for use as the production organism for the nuclease enzyme.

6.2.3 SAFETY STUDIES

To demonstrate the absence of toxigenicity of the nuclease enzyme *Bacillus amyloliquefaciens* production strain, the cytotoxicity of culture supernatant towards Vero cells was tested based on a lactate dehydrogenase (LDH) release assay. The study was carried out according to the methodology recommended by EFSA [40]. Vero cells were exposed to the bacterial supernatants, after which the release of LDH was measured spectrophotometrically. The degree of cytotoxicity was quantified in relation to the amount of LDH released from cells treated with a detergent, which was considered as 100%. The threshold value for cytotoxicity was set at 20%. The relative value of LDH

release for the test supernatants was determined as 4.4%, well below the cytotoxicity threshold. Thus, the nuclease enzyme production strain was shown to be non-cytotoxic.

A large number of toxicological tests on enzymes derived from *Bacillus amyloliquefaciens* have been conducted over decades, including enzymes produced from recombinant strains. These toxicological studies provided the basis for several positive safety evaluations by JECFA. Mixed microbial carbohydrase and protease preparations from several different *Bacillus subtilis* strains (later classified as *Bacillus amyloliquefaciens*) were evaluated in 1971, and based on acute and repeated-dose oral toxicity tests, including a 90-day study in rats, it was concluded that enzyme preparations from *Bacillus subtilis* carry no toxicological hazard [41]. An alpha-amylase enzyme produced by *Bacillus amyloliquefaciens* ATCC 23350 DSM 7 (at that time still designated as *Bacillus subtilis*) was evaluated by JECFA in 1991 [42], and on the basis of acute oral toxicity studies in rat, an acceptable daily intake (ADI) "not specified" was assigned [42]. Recently, the safety of an amylomaltase produced by a recombinant strain of *Bacillus amyloliquefaciens* was assessed by a full toxicological package including an Ames test, a chromosomal aberration test, and a 90-day oral toxicity study in rats. The enzyme did not show any mutagenic or clastogenic activity. Moreover, no toxic effects were observed in the subchronic oral toxicity study for enzyme levels of up to 1000 mg TOS/kg body weight/day (GRN 507 [29]).

6.2.4 SAFETY OF THE DONOR

The DNA sequence for the introduced gene encoding the nuclease was *de novo* synthesized based upon the published sequence encoding a nuclease from *Serratia marcescens.* All the introduced DNA is well defined and characterized and does not code for any substances with known harmful or toxic properties. Therefore, it is the safety of the production organism that should represent the main element for assessing the safety of the food enzyme [9], [14].

6.3 SAFETY OF THE NUCLEASE ENZYME

As described in section 2.1, the nuclease is a well characterized enzyme that displays 5'-phosphodiesterase activity, hydrolyzing the phosphate- O-β' link to yield 5'-phosphomononucleotides and 5'-phosphooligonucleotides. This activity is well known as either phosphodiesterase or nuclease.

6.3.1 SAFETY OF USE

Nuclease activity is indispensable for life and, as such, ubiquitous in nature [43]. Nucleases cleaving DNA and RNA are involved in multiple biological processes ranging from DNA replication and recombination to RNA processing or the degradation of nucleic

acids as a source of nutrients. The production of nucleases by microorganisms for food processing uses has been described by Pariza and Johnson [14].

The extracellular nuclease from *Serratia marcescens* (*Serratia* nuclease) is the best characterized member of a family of closely related, non-specific nucleases with well-conserved amino acid sequences among prokaryotic and eukaryotic organisms, including humans [44]–[46]. Several naturally occurring sources of nucleases displaying the same activity as the *Serratia* nuclease, such as barley, baker's yeast (*Saccharomyces cerevisiae*), and cattle (*Bos taurus*), are part of the human diet [47]–[49]. All these enzymes catalyse the same reaction (EC 3.1.30.2). In particular, an enzyme with the same activity and derived from the sprout portion of malt barley has been traditionally used in the production of partially hydrolysed yeast extracts, which have been approved by the US FDA [50], [51]. In addition, nucleases obtained from other sources such as phosphodiesterase I from *Leptographium procerum* (recognized GRAS under GRN 505) or nuclease from *Penicillium citrinum* are considered safe for use as food enzymes in several food applications [29],[50], [52].

Consumption of these nucleases has not led to any adverse events or allergic reactions. Moreover, since the nuclease produced in *Bacillus amyloliquefaciens* is a normal protein composed of natural amino acids, it will be digested in the human gastrointestinal tract just as any other food protein.

A literature search was conducted on March 08, 2023, using the searching terms "nuclease", "endonuclease", "phosphodiesterase", "food", "safety", and combinations thereof. The search did not yield any reports that would raise any safety concerns or conflict with c-LEcta's conclusion of general recognition of safety for the nuclease enzyme.

Since it is generally accepted that nuclease/phosphodiesterase commercial enzyme preparations are not toxic, and since both the nuclease and the products of its reaction are natural constituents of several different microorganisms, plants and animals consumed as food, it is not expected that the nuclease from *Bacillus amyloliquefaciens* would have any toxic properties.

6.3.2 EXISTING AUTHORISATIONS FOR NUCLEASES

In the EU, a ribonuclease derived from *Penicillium citrinum* is currently listed as an authorized food enzyme at the national level in France for use in the hydrolysis of polyribonucleotide in the treatment of yeast extracts [30]. In addition, nuclease, listed as "phosphodiesterase", derived from *Penicillium citrinum* is currently marketed for use in food processing according to the Association of Manufacturers and Formulators of Food Enzymes (AMFEP) [53].

Nuclease, listed as "phosphodiesterase", from *Penicillium citrinum* is currently authorized for use in foods in Japan [54]. In China, phosphodiesterase I (nuclease) from *Penicillium citrinum* is an approved additive [55].

In Korea the phosphodiesterase (nuclease) from *Penicillium citrinum* is part of the list of authorised additives as an enzyme preparation [56].

In the US, the Food and Drug Administration (FDA) had no questions on the GRAS determination of phosphodiesterase I from *Leptographium procerum* based on scientific procedures (GRN 505 [29]).

The aforementioned authorizations of food enzymes are presented in the table below.

Authority	Micro-organism	References
Japan, MHLW	*Penicillium citrinum*	List of existing food additives [54]
China	*Penicillium citrinum*	List of approved additives [55]
France	Ribonuclease from *Penicillium citrinum*	France Arrêté 19 Oct 2006 [30]
USA	*Leptographium procerum*	GRN 505 [29]
Korea	*Penicillium citrinum*	List of authorized additives [56]

6.3.3 PUBLICATIONS ON THE SAFETY OF NUCLEASE ENZYMES

The nuclease/phosphodiesterase enzyme activity (produced by *Leptographium procerum* or *Penicillium citrinum*) was listed by Pariza and Johnson in 2001 as being commonly used by the food industry [14]. Moreover, several safety assessments have been performed on nuclease enzymes produced from these microorganisms.

The safety of the phosphodiesterase produced by *Leptographium procerum* was evaluated by Steensma et al. [57]. The production strain did not produce any toxic secondary metabolites. Likewise, the enzyme displayed no mutagenic or clastogenic activity, and no relevant effects were observed in a 28-day oral toxicity study in rats. Therefore, it was concluded that this enzyme is safe for food production [57].

The safety of a nuclease from *Penicillium citrinum* has also been previously evaluated in two different papers by both Burdock et al. and Kondo et al. The nuclease did not display mutagenic or clastogenic activity. Additionally, a no-observed-adverse-effect level (NOAEL) of 500 mg/kg body weight and of 317.4-345.9 mg/kg body weight was determined following 35-day and 13-week subchronic oral studies in rats [50], [52].

Finally, the safety of a nuclease from *Penicillium citrinum* was evaluated by Okado et al. A series of safety studies including an in vitro Ames test and chromosome aberration assay, an in vivo rat erythrocyte micronucleus assay and a 90-day oral toxicity study in rats were conducted. The results of the genotoxicity studies and the subchronic oral toxicity study support the safe use in food production of nuclease produced from *Penicillium citrinum*. The NOAEL was established at 1007 mg TOS/kg bw/day [58].

All these findings suggest that the use of nuclease in food applications is not likely to result in any adverse effects in humans.

6.3.4 ALLERGENICITY

As reported by Pariza and Foster, "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances.*"[9]. Because they are proteins, enzymes could theoretically have the potential to cause allergic responses. However, their oral ingestion is generally not considered to be of concern [27], [38], [59]. The allergenic potential of food enzymes was studied by Bindslev-Jensen et al. and reported in the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" [60]. The investigation included enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein-engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

To confirm that the nuclease does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted using the AllergenOnline database, version 21 (available at http://www.allergenonline.org; updated February 14, 2021) maintained by the Food Allergy Research and Resource Program of the University of Nebraska [61]. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. A full-length alignment search of AllergenOnline was conducted using default settings (E-value cut-off = 1 and maximum alignments of 20).

A total of 5 matches were identified. For all these matches, the percent identities of the amino acid sequence are at a maximum of 39%. The potentially cross-reactive structure of known allergens was exhaustively reviewed by Aalberse, who concluded that proteins with less than 50% identity throughout the length of the protein compared to an allergen are unlikely to be cross-reactive [62].

Regarding the E-value or expectation value (calculated value that reflects the degree of similarity of the target amino acid sequence to the identified matches), the values obtained were between 0.14 and 0.710. According to AllergenOnline, expectation value scores (E-scores or E-values) equal or higher than 1 are unlikely to be related in either evolution or structure. On the other hand, E-values of less than 0.02 might indicate that the sequences are related in evolutionary terms. However, when assessing the possibility of immunologic or allergic cross-reactivity, matches with E-values larger than 10-7 are not likely to be relevant [63].

A second homology search was conducted according to the approach outlined by JECFA and the Codex Alimentarius Commission [15] . In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1-80, 2-81, 3-82, etc.) derived from the full-length *Serratia marcescens* nuclease enzyme. The 80 amino acid alignment search was conducted using default settings (E-value cut-off = 1 and maximum alignments of 20). Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility [26]. Using this search strategy, no matches were identified.

A third homology search conducted using the exact 8-mer approach did not produce any matches.

The possible allergenicity of nuclease also was considered through a search of the available scientific literature; however, no relevant information was identified.

Based on the information provided above, no evidence exists that might indicate that *Serratia marcescens* nuclease enzyme would produce an allergenic response following consumption of foods to which the enzyme has been added. Additionally, there is no evidence from the available scientific literature indicating allergenicity to other nucleases in consumers of foods to which these enzymes have been added.

Regarding potential major food allergens used in the manufacturing process, soy peptone is used during fermentation and is consumed by the microorganism as a nutrient. Soy peptone is produced by hydrolysis of soy protein, which significantly reduces its potential allergenicity. Several published assessments have concluded that allergen proteins from fermentation media are not found in final enzyme preparations, and that enzyme preparations do not pose an allergen risk that would require allergen labelling on the final products (Annex B). In addition, for the nuclease enzyme subject of this assessment, no soy allergens have been detected in the final product.

Therefore, the use of nuclease is not anticipated to pose any allergenicity concerns in consumers.

6.3.5 IDENTIFICATION OF THE NOAEL AND MARGIN OF SAFETY (MOS)

Regarding the *Bacillus amyloliquefaciens* species, several toxicological studies have been conducted for food enzymes. These studies provided the basis for several positive safety evaluations by JECFA. In GRN 507, an amylomaltase produced by *Bacillus amyloliquefaciens* was assessed and a NOAEL of 1000 mg TOS/kg bw/day was established. In addition, the NOAEL for substantially equivalent nuclease enzymes was also considered: in the 90-day oral (gavage) study in rats for nuclease from *Penicillium citrinum* and of phosphodiesterase I from *Leptographium procerum,* recognised GRAS under GRN 505, NOAEL were established at 1007 mg TOS kg bw/day and 1000 mg TOS/kg bw/day, respectively.

Thus, the selection of a NOAEL of 1000 mg TOS/kg bw/day is appropriate for the safety assessment of the nuclease enzyme produced by *Bacillus amyloliquefaciens*.

The margin of safety is calculated by dividing the NOAEL by the TMDI. A margin of safety greater than 100 suggests that the available toxicology data support the proposed uses and application rates.

For the nuclease produced by *Bacillus amyloliquefaciens*, the margin of safety for the general population is calculated as follows:

$$\text{MoS} = \frac{(1000\ mg\ TOS/kgbw/day)}{3.25\ mg\ TOS/kgbw/day} = 307$$

6.4 SUMMARY AND CONCLUSIONS

As documented in this notification, the safety of the *Bacillus amyloliquefaciens* production strain has been thoroughly evaluated. *Bacillus amyloliquefaciens* has a very long history of safe use in the food industry. As noted in the previous sections, both the *Bacillus amyloliquefaciens* microbial host as well as several enzyme preparations derived from different strains have been recognized by qualified experts as being safe for their intended uses. Scientific reports, government laws and regulations, and reviews by expert panels such as JECFA further support this conclusion. The *Bacillus amyloliquefaciens* lineage has been demonstrated to be safe based on repeated testing and evaluation using the Pariza and Johnson decision tree [14]. Furthermore, the *Bacillus amyloliquefaciens* nuclease production strain and its well-characterized genetic modifications did not induce any concerns regarding toxigenicity, pathogenicity, or antibiotic production.

Nucleases, also known as phosphodiesterases, have a long history of safe use in food applications, as supported by several publications as well as existing authorizations. The nuclease enzyme from *Bacillus amyloliquefaciens* is produced according to the principles of cGMP for food, using food-grade ingredients or ingredients that are acceptable for general use in foods, and meets appropriate food-grade enzyme specifications.

Based on a critical review of the available data and information, c-LEcta concludes through scientific procedures that the nuclease enzyme produced by *Bacillus amyloliquefaciens* is safe for its intended uses.

PART 7 - SUPPORTING DATA

7.1 ANNEXES

Annex A: Manufacture process diagram

Annex B: Statements on allergen labelling requirements

Annex C: Pariza and Johnson decision tree analysis

7.2 REFERENCES

[1] Code of Federal Regulations, *21 CFR §184.1150 Bacterially-derived protease enzyme preparation*. 1999. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-184/subpart-B/section-184.1150

[2] US FDA, 21 CFR Part 184 Carbohydrase and Protease Enzyme Preparations Derived From *Bacillus Subtilis* or *Bacillus Amyloliquefaciens*; Affirmation of GRAS Status as Direct Food Ingredients, *Federal Register*, vol. Vol. 64, no. No. 78, pp. 19877–19895, Apr. 1999.

[3] N. E. Welker and L. L. Campbell, Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis*, *J Bacteriol*, vol. 94, no. 4, pp. 1124–1130, Oct. 1967, doi: 10.1128/jb.94.4.1124-1130.1967.

[4] G. Steinborn, M.R. Hajirezaei, and J. Hofemeister, *bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains, *Arch Microbiol*, vol. 183, no. 2, pp. 71–79, Feb. 2005, doi: 10.1007/s00203-004-0743-8.

[5] National Institute of Health, NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules, 2019.

[6] EFSA Panel on Biological Hazards (BIOHAZ), Update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA, *EFSA Journal*, vol. 21, no. 1, 2023, doi: 10.2903/j.efsa.2023.7747.

[7] EFSA Panel on Biological Hazards (BIOHAZ), Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update), *EFSA Journal*, vol. 10, no. 12, Dec. 2012, doi: 10.2903/j.efsa.2012.3020.

[8] OECD, *Safety Evaluation of Foods Derived by Modern Biotechnology, Concepts and Principles*. Paris, France: OECD Publications, 1993.

[9] M. W. Pariza and E. M. Foster, Determining the Safety of Enzymes Used in Food Processing, *Journal of Food Protection*, vol. 46, no. 5, pp. 453–468, May 1983.

[10] Berkowitz, *Report of the Eighteenth Session of the Joint FAO/WHO Codex Alimentarius Commission, Geneva, 3-12 July 1989*. Rome: Food and Agriculture Organization of the United Nations : World Health Organization, 1989.

[11] IFBC, Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms, *Regulatory Toxicology and Pharmacology*, vol. 12, no. 3, pp. S114–S128, Dec. 1990, doi: 10.1016/S0273-2300(05)80080-7.

[12] Commission of the European Communities, Reports of the Scientific Committee for Food (twenty-seventh series) - food science and techniques, 1991.

[13] D. A. Jonas et al., The safety assessment of novel foods, *Food and Chemical Toxicology*, vol. 34, no. 10, pp. 931–940, Oct. 1996, doi: 10.1016/S0278-6915(96)00061-0.

[14] M. W. Pariza and E. A. Johnson, Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, *Regulatory*

Toxicology and Pharmacology, vol. 33, no. 2, pp. 173–186, Apr. 2001, doi: 10.1006/rtph.2001.1466.

[15] Codex Alimentarius Commission, JECFA, Eds., *Foods derived from modern biotechnology*, 2. ed. Rome: Food and Agriculture Organization [u.a.], 2009.

[16] J. Kok, J. M. van der Vossen, and G. Venema, Construction of plasmid cloning vectors for lactic streptococci which also replicate in Bacillus subtilis and Escherichia coli, *Appl Environ Microbiol*, vol. 48, no. 4, pp. 726–731, Oct. 1984, doi: 10.1128/aem.48.4.726-731.1984.

[17] Code of Federal Regulations, *21 CFR §110 Current good manufacturing practice in manufacturing, packing or holding human food*. 1986. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-110

[18] K. Aunstrup, Production, Isolation, and Economics of Extracellular Enzymes, in *Applied Biochemistry and Bioengineering*, Elsevier, 1979, pp. 27–69. doi: 10.1016/B978-0-12-041102-3.50008-0.

[19] K. Aunstrup, O. Andresen, E. A. Falch, and T. K. Nielsen, Production of Microbial Enzymes, in *Microbial Technology*, Elsevier, 1979, pp. 281–309. doi: 10.1016/B978-0-12-551501-6.50014-0.

[20] O. Kirk et al., Enzyme Applications, industrial, in *Encyclopedia of Chemical Technology*, vol. Vol. 10, John Wiley & Sons, Inc., 2004.

[21] Food Chemicals Codex, 12th Edition, Enzyme Preparations. Dec. 2020.

[22] JECFA, *JECFA monographs. 1: Analytical methods, test procedures and laboratory solutions used by and referenced in the food additives specifications: all specifications monographds from the 1st to the 65th meeting (1956-2005)*. Rome: Food and Agriculture Organization of the United Nations, 2006.

[23] Code of Federal Regulations, *21 CFR §173.340 Defoaming agents*. 1977. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-173/subpart-D/section-173.340

[24] Code of Federal Regulations, *21 CFR §172.820 Polyethylene glycol*. 1977. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-172/subpart-I/section-172.820

[25] Enzyme Technical Association, Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food. Apr. 10, 1998.

[26] JECFA, Chapter 6 Dietary Exposure Assessment for Chemicals in Food, in *EHC 240: Principles for Risk Assessment of Chemicals in Food*, 2020. Accessed: Mar. 03, 2023. Available: https://cdn.who.int/media/docs/default-source/food-safety/publications/chapter6-dietary-exposure.pdf?sfvrsn=26d37b15_6

[27] V. Sewalt, D. Shanahan, L. Gregg, J. La Marta, and R. Carrillo, The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes, *Industrial Biotechnology*, vol. 12, no. 5, pp. 295–302, Oct. 2016, doi: 10.1089/ind.2016.0011.

[28] F. G. Priest, *Bacillus amyloliquefaciens sp.* nov. norn. rev., 1987.

[29] US FDA, GRAS Notice Inventory. https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices (accessed Feb. 22, 2023).

[30] JORF, Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires, updated 2021. https://www.legifrance.gouv.fr/loda/id/LEGITEXT000020667468 (accessed Feb. 22, 2023).

[31] Australian Government, Australia New Zealand Food Standards Code – Schedule 18 – Processing aids, 2022. https://www.legislation.gov.au/Details/F2022C00243 (accessed Feb. 22, 2023).

[32] Government of Canada, List of Permitted Food Enzymes (Lists of Permitted Food Additives), updated 2022. https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html (accessed Feb. 22, 2023).

[33] Code of Federal Regulations, *21 CFR §184.1148 Bacterially-derived carbohydrase enzyme preparation*. 1999. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-184/subpart-B/section-184.1148

[34] L. A. Underkofler, Cellulases and Their Applications, vol. 95. Washington, D.C.: American Chemical Society, 1969. doi: 10.1021/ba-1969-0095.

[35] L. A. Underkofler, R. R. Barton, and S. S. Rennert, Production of Microbial Enzymes and Their Applications, vol. 6, 1957.

[36] US FDA, *Federal Register Vol. 64, No.78, April 23, 1999*. 1999. Available: https://www.govinfo.gov/app/details/FR-1999-04-23/summary

[37] A. de Boer Sietske and B. Diderichsen, On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review, *Appl Microbiol Biotechnol*, vol. 36, no. 1, pp. 1–4, Oct. 1991, doi: 10.1007/BF00164689.

[38] Z. S. Olempska-Beer, R. I. Merker, M. D. Ditto, and M. J. DiNovi, Food-processing enzymes from recombinant microorganisms—a review, *Regulatory Toxicology and Pharmacology*, vol. 45, no. 2, pp. 144–158, Jul. 2006, doi: 10.1016/j.yrtph.2006.05.001.

[39] M. Galano, M. W. van den Dungen, T. van Rij, and H. E. Abbas, Safety evaluation of food enzymes produced by a safe strain lineage of *Bacillus subtilis*, *Regulatory Toxicology and Pharmacology*, vol. 126, p. 105030, Nov. 2021, doi: 10.1016/j.yrtph.2021.105030.

[40] EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guidance on the characterisation of microorganisms used as feed additives or as production organisms, *EFSA Journal*, vol. 16, no. 3, Mar. 2018, doi: 10.2903/j.efsa.2018.5206.

[41] JECFA, Toxicological Evaluation of some Enzymes, modified Starches and certain other Substances, 1972. https://www.inchem.org/documents/jecfa/jecmono/v001je02.htm (accessed Feb. 22, 2023).

[42] JECFA, WHO Food Additive Series 28, 1991. https://inchem.org/documents/jecfa/jecmono/v28je05.htm (accessed Mar. 03, 2023).

[43] W. Yang, Nucleases: diversity of structure, function and mechanism, *Quart. Rev. Biophys.*, vol. 44, no. 1, pp. 1–93, Feb. 2011, doi: 10.1017/S0033583510000181.

[44] M. J. Benedik and U. Strych, *Serratia marcescens* and its extracellular nuclease, *FEMS Microbiology Letters*, vol. 165, no. 1, pp. 1–13, Aug. 1998, doi: 10.1111/j.1574-6968.1998.tb13120.x.

[45] P. Friedhoff, B. Kolmes, O. Gimadutdinow, W. Wende, K. L. Krause, and A. Pingoud, Analysis of the Mechanism of the *Serratia* Nuclease Using Site-Directed Mutagenesis, *Nucleic Acids Research*, vol. 24, no. 14, pp. 2632–2639, Jul. 1996, doi: 10.1093/nar/24.14.2632.

[46] G. Meiss, F.U. Gast, and A. M. Pingoud, The DNA/RNA non-specific *Serratia* nuclease prefers double-stranded A-form nucleic acids as substrates, *Journal of Molecular Biology*, vol. 288, no. 3, pp. 377–390, May 1999, doi: 10.1006/jmbi.1999.2694.

[47] P. H. Brown and T.H. D. Ho, Barley Aleurone Layers Secrete a Nuclease in Response to Gibberellic Acid: Purification and Partial Characterization of the Associated Ribonuclease, Deoxyribonuclease, and 3'-Nucleotidase Activities, *Plant Physiology*, vol. 82, no. 3, pp. 801–806, Nov. 1986, doi: 10.1104/pp.82.3.801.

[48] P. Schäfer et al., Structural and Functional Characterization of Mitochondrial EndoG, a Sugar Non-specific Nuclease which Plays an Important Role During Apoptosis, *Journal of Molecular Biology*, vol. 338, no. 2, pp. 217–228, Apr. 2004, doi: 10.1016/j.jmb.2004.02.069.

[49] R. D. Vincent, T. J. Hofmann, and H. P. Zassenhaus, Sequence and expression of *NUC1* , the gene encoding the mitochondrial nuclease in *Saccharomyces cerevisia*', *Nucleic Acids Research*, vol. 16, no. 8, pp. 3297–3312, 1988, doi: 10.1093/nar/16.8.3297.

[50] G. A. Burdock, W. G. Flamm, and I. G. Carabin, Toxicity and mutagenicity studies of DN-50000® and RP-1® enzymes, *Food and Chemical Toxicology*, vol. 38, no. 5, pp. 429–442, May 2000, doi: 10.1016/S0278-6915(00)00018-1.

[51] Code of Federal Regulations, *21 CFR §172.590 Yeast-malt sprout extract.* 1977. Available: https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-172/subpart-F/section-172.590

[52] M. Kondo, S. Nishimura, N. Tanaka, and M. Flood, Safety Evaluation of Phosphodiesterase Produced from *Penicillium citrinum*: Summary of Toxicological Data, *Regulatory Toxicology and Pharmacology*, vol. 33, no. 1, pp. 2–11, Feb. 2001, doi: 10.1006/rtph.2000.1434.

[53] AMFEP, List of Commercial Enzymes April 2009, 2015. https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf (accessed Feb. 22, 2023).

[54] Ministry of Health, Labor and Welfare, Japan (MHLW), List of Existing Food Additives, updated 2022. https://www.ffcr.or.jp/en/tenka/list-of-existing-food-additives/list-of-existing-food-additives.html (accessed Feb. 22, 2023).

[55] Ministry of Health of the PRC, National Standard on Food Safety Standard for Use of Food Additives. Ministry of Health of the People's Republic of China (PRC), Beijing, People's Republic of China, 2014.

[56] Ministry of Food and Drug Safety Regulation, Korea, Food Additives Code, 2021.

[57] A. Steensma, P. W. M. van Dijck, and R. A. Hempenius, Safety evaluation of phosphodiesterase derived from *Leptographium procerum*, *Food and Chemical Toxicology*, vol. 42, no. 6, pp. 935–944, Jun. 2004, doi: 10.1016/j.fct.2004.01.017.

[58] N. Okado, K. Hasegawa, F. Mizuhashi, B. S. Lynch, T. D. Vo, and A. S. Roberts, Safety evaluation of nuclease P1 from *Penicillium citrinum*, *Food and Chemical Toxicology*, vol. 88, pp. 21–31, Feb. 2016, doi: 10.1016/j.fct.2015.12.001.

[59] G. S. Ladics, Safety assessment and regulation of food enzymes. In: *Enzymes: Novel biotechnological approaches for the food industry*. S. Kermasha, Ed. Academic Press, 2021.

[60] C. Bindslev-Jensen, P. S. Skov, E. L. Roggen, P. Hvass, and D. S. Brinch, Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry, *Food and Chemical Toxicology*, vol. 44, no. 11, pp. 1909–1915, Nov. 2006, doi: 10.1016/j.fct.2006.06.012.

[61] FARRP, Expert Opinion Statement, Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials. 2013.

[62] R. C. Aalberse, Structural biology of allergens, *Journal of Allergy and Clinical Immunology*, vol. 106, no. 2, pp. 228–238, Aug. 2000, doi: 10.1067/mai.2000.108434.

[63] R. E. Hileman et al., Bioinformatic Methods for Allergenicity Assessment Using a Comprehensive Allergen Database, *International Archives of Allergy and Immunology*, vol. 128, no. 4, pp. 280–291, 2002, doi: 10.1159/000063861.

Annex A

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186.

1. Is the production strain genetically modified?
   If yes, go to 2. **YES**

2. Is the production strain modified using rDNA techniques?
   If yes, go to 3. **YES**

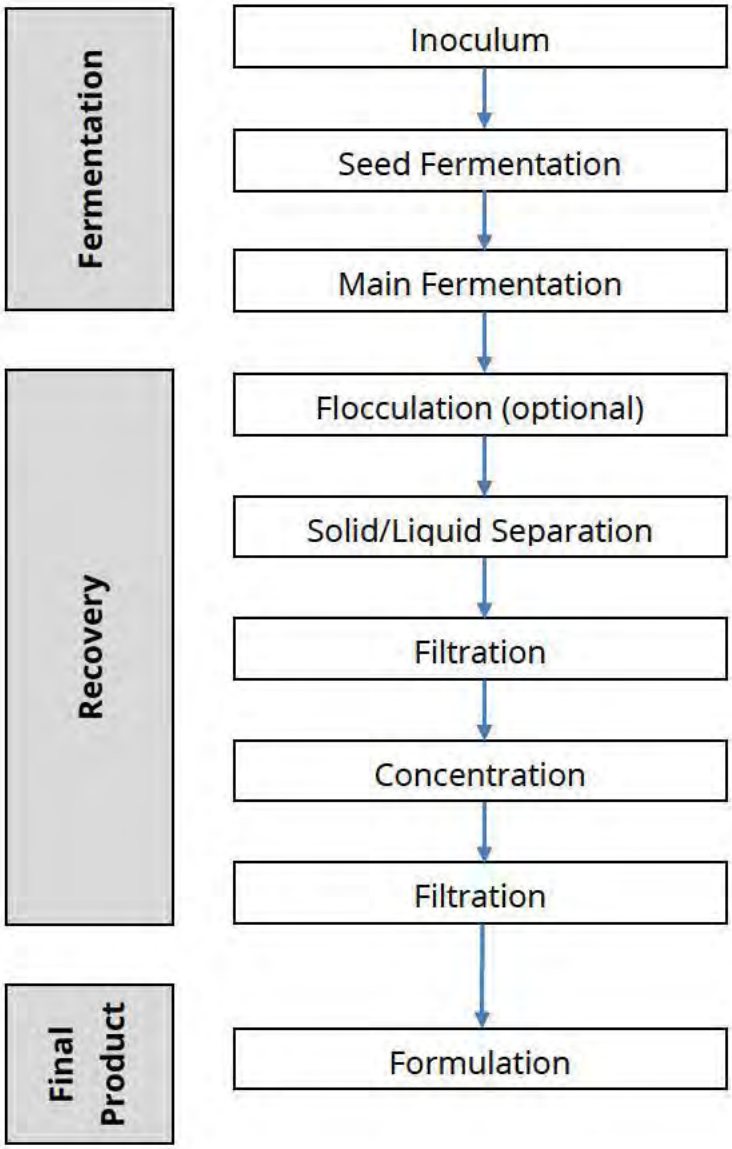
3. Issues relating to the introduced DNA are addressed in 3a to 3e.
   - 3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?
     If yes, go to 3c. **YES**
   - 3c. Is the test article free of transferable antibiotic resistance gene DNA?
     If yes, go to 3e. **YES**
   - 3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
     If yes, go to 4. **YES**

4. Is the introduced DNA randomly integrated into the chromosome?
   If no, go to 6. **NO**

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **YES**
   If yes, the test article is **ACCEPTED**

Annex B

Manufacturing Process Flowchart



Annex C

Statements on Allergen Labelling Requirements by the Food Allergy Research & Resouce Program (FARRP) at the University of Nebraska and the Enzyme Technical Association (ETA)

University of Nebraska Lincoln

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM

EXPERT OPINION STATEMENT
FOOD ALLERGY RESEARCH & RESOURCE PROGRAM
UNIVERSITY OF NEBRASKA

Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials

August 13, 2013

Prepared by: Steve L. Taylor, Ph.D., Co-Director
and
Joe L. Baumert, Ph.D., Co-Director

with assistance from Enzyme Technical Association

Microbially derived enzymes are used by food processors as additives and processing aids in a wide variety of foods. Enzymes obtained from microbial fermentation are directly derived from microorganisms fed on sterilized media[1] that may include protein sources obtained from one or more of the recognized commonly allergenic foods (e.g., milk, soybean) or from a cereal source of gluten (e.g., wheat, barley). This paper addresses the relevance of testing microbial enzymes for allergenic material from the fermentation growth media.[2]

It has been the long-standing position of the Food Allergy Research & Resource Program (FARRP) at the University of Nebraska that testing of the products of fermentation (with limited exceptions), including microbially derived enzymes is unreliable using enzyme-linked immunosorbent assays (ELISAs).

While various fermentation media may contain one or more of the major food allergens, the biochemical reactions that occur during fermentation result in the breakdown of the fermentation media proteins. The extent of proteolysis is dependent upon the fermentation culture and the resultant enzyme (e.g., some enzymes are proteases). As proteins are digested, the resulting amino acids, along with other nitrogenous material, are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein.

[1] Aunstrup, K., O. Andresen, E.A. Falch, and T.K. Nielsen (1979) *Microbial Technology*. (Perlman and Peppler, eds.) Academic Press, pp. 281-309.
[2] For this paper, FARRP's analysis is limited to microbially derived enzymes that are intended for additive and processing aid applications in food.

255 Food Industry Building / P.O. Box 830955 / Lincoln, NE 68583-0955
Phone (Taylor – Co-Director) (402) 472-2833 / (Baumert – Co-Director) (402) 472-3070
FAX (402) 472-5307 / Lab Phone (402) 472-4464

Upon completion of fermentation, remaining fermentation media that are not consumed by the microorganism are typically separated and/or purified from the enzyme in the recovery process. Enzymes are recovered from the fermentation broth by standard chemical engineering operations, such as filtration and centrifugation, broadly used in enzyme production.[3,4] (See Appendices for further information.) The recovery steps result in separation of microbial biomass and other fermentation solids from the enzyme, concentration of the enzyme, and removal of impurities prior to final formulation with food-grade ingredients.

Any potential residual fragments from the food allergen would be difficult to measure as there is no reliable assay. Commercial ELISAs are able to detect only intact proteins in most cases. Any peptides, even larger ones, would not likely be detected, although this possibility has not been well investigated. Results would typically be reported as below the limit of quantitation for the enzyme preparation. Further, if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven. Accordingly, testing of fermented product does not result in reliable or useful data.

In addition, due to the specific catalytic nature of enzymes, only very small amounts of enzymes are generally required and used by food processors to make the desired modifications to the property of a food, and therefore any *de minimis* amount of fermentation media protein that may survive the fermentation process will not pose a significant public health risk to the consumer.[5]

FARRP also notes that regulatory agencies in the European Union and Japan do not require allergen labeling of enzyme preparations for the raw materials used in the fermentation process.

---

[3] Atkinson, B. and F. Mavituna (1991) *Biochemical Engineering and Biotechnology Handbook*. (Atkinson, B. and Mavituna, F., eds.) Stockton Press, Hampshire, pp. 1146-1158.

[4] Kroschwitz, J.I. (1994) *Enzyme Applications in Encyclopedia of Chemical Technology*. 4th edition, Volume 9. (Kroschwitz, J.I., ed.), pp. 567-620.

[5] To the extent the enzyme producer uses an allergen as diluent to formulate the final product, labeling for such allergen is appropriate and required under Food Allergen Labeling and Consumer Protection Act.

ENZYME TECHNICAL ASSOCIATION

2050 M Street, N.W. | Washington, DC 20036 | Telephone: 202.719.4585 | Fax: 202.719.7049
www.enzymetechnicalassociation.org

December 17, 2020

ETA Position On Microbially Derived Enzymes Used as Food Processing Aids and Food Allergen Labeling Under FALCPA as it Applies to Fermentation Media Raw Materials[1]

Microbially derived enzymes themselves do not fit within the requirements of FALCPA because enzymes are not one of the eight major allergenic foods, often referred to as the Big 8. In addition, microbial enzymes are neither byproducts of nor are they derived from the major food allergens. Most commercial food enzymes are produced by fermentation using selected microorganisms. Most importantly, food enzymes are predominately used as processing aids in the production of food ingredients or final foods.

The enzymes are not derived from raw materials containing major food allergens but rather are produced by the microorganisms. While enzymes produced by microbial fermentation use media that may include protein from one or more of the major food allergens, these proteins and other nitrogenous material are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein. It is the intent of the enzyme manufacturer to supply enzymes; therefore, it is critical that the ratio of nutrient to enzyme yield is carefully controlled. It is also the intentof the manufacturer that these raw materials are added to the fermentation as food to be consumedby the microorganism. Further, down-stream processing typically includes filtration and purification steps enabling the further removal of any residual nutrients. Thus, the final food enzymes typically will not contain residual amounts of the media used during fermentation.

Even though microbially derived enzymes do not fit within the requirements of FALCPA, are neither byproducts of nor are they derived from the major food allergens, and are predominately used as a processing aids in the production of food ingredients or final foods, it is the responsibility of the food enzyme manufacturer to conduct a risk assessment regarding raw materials from food allergenic sources used in fermentation and to comply with labelling provisions for food enzyme preparations.

For consideration in the risk assessment process, ETA suggests the following:

The risk assessment should follow the weight-of-evidence approach and the following should be considered:

---

[1] This ETA position statement only pertains to IgE mediated food allergy responses subject to labeling requirements under FALCPA (e.g., milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans). The FDA final ruling on Gluten-Free Labeling of Fermented or Hydrolyzed Foods (21 CFR Part101.91) is out of the scope of this document as it concerns a non-IgE related food allergy response specificto gluten, which presents a different immune response.

ENZYME TECHNICAL ASSOCIATION™

2050 M Street, N.W. | Washington, DC 20036 | Telephone: 202.719.4585 | Fax: 202.719.7049
www.enzymetechnicalassociation.org

(1) Enzymes themselves are not one of the eight named proteins.

(2) Enzymes are not derived from any of these eight named proteins.

(3) Microbially derived enzyme preparations either do not contain or contain only negligible amounts of a major food allergenic protein.

(4) The separation and/or purification process substantially removes non-enzyme substances, including materials used in the fermentation process.

(5) The quantity of enzyme use in food processing is extremely low.

(6) ETA conducted a review of the published scientific literature and found no reports that suggest there has been an allergenic reaction to a component of the fermentation media used to feed the microorganism that produced the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001146	May 23, 2023
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Paula Pescador	VP Regulatory Affairs
Organization *(if applicable)*	
c-LEcta GmbH	
Mailing Address *(number and street)*	
Perlickstr. 5	

City	State or Province	Zip Code/Postal Code	Country
Leipzig	Saxony	04103	Germany

Telephone Number	Fax Number	E-Mail Address
(49)-341-3552140		paula.pescador@c-lecta.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Organization *(if applicable)*	
Mailing Address *(number and street)*	

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Nuclease

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8) and 170.250(d) and (e))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The nuclease enzyme is used as a processing aid to hydrolyze nucleic acids during the manufacturing of microbial-derived food ingredients to reduce the viscosity of the fermentation broth, increase the efficiency of the recovery process, to comply with regulatory requirements and/or to improve the nutritional and organoleptic profile of the products. The targeted population is the general population.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that c-LEcta GmbH

(name of notifier)

has concluded that the intended use*(s)* of Nuclease

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. c-LEcta GmbH *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Perlickstr. 5, 04103 Leipzig, Germany

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Paula Pescador Digital unterschrieben von Paula Pescador Datum: 2023.03.24 17:14:44 +01'00'	Paula Pescador, VP Regulatory Affairs	03/24/2023

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667_Nuclease Bacillus Amyloliquefaciens_2023-03-24.pdf	Administrative
	GRASNotice_Nuclease_Bacillus_Amyloliquefaciens_2023-03-24.pdf	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, **PRAStaff@fda.hhs.gov**. (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

GRN 1146 – FDA Questions

2/20/2024

1. For the administrative record, please provide the number of amino acids in the primary sequence and the method used to determine the sequence of the nuclease in this notification.

   The number of amino acids in the primary sequence of the nuclease enzyme is 245, as determined by PCR and by whole genome sequencing.

2. Please indicate the color of the liquid nuclease enzyme preparation.

   Brownish.

3. Section 2.2.5 (page 8) states that "the absence of the production organism is one of the parameters which are verified for each product batch." However, absence of production organism is not listed as a specification in the Table on page 11 and no batch analysis data are provided. Please confirm if absence of the production organism is a specification for the nuclease enzyme preparation and provide the results of three non-consecutive batch analyses. Additionally, please confirm that the in-house method used for this specification is validated and fit-for-purpose.

   We confirm that the absence of the production organism is part of the in-house specification for the nuclease enzyme preparation. Any enzyme batches not fulfilling this criterium would be rejected during batch release testing. The in-house method used for this parameter is a culture-based method appropriate for the detection of viable cells of *Bacillus amyloliquefaciens* and has been validated accordingly. The results of three non-consecutive batch analyses are provided below:

Parameter	**LOD / LOQ**	**Batch results**		
		1	**2**	**3**
Absence of viable cells of the production strain	1 cfu/g	Absent	Absent	Absent

4. Page 11 specifications:

   (1) Please provide the methods used to establish the specifications for the nuclease enzyme preparation and indicate if they have been validated for their intended purpose. Please confirm that the representative batch analyses are non-consecutive.

   Please find below details on the methods used to establish the specifications for the nuclease enzyme preparation. All methods are fit for purpose and have been validated. Analyses for heavy metals and microbes are performed by accredited external laboratories. Analysis results for three non-consecutive batches are provided under Question 4(2).

Parameter	Method
Activity	In-house method (validated)
Absence of viable cells of the production strain	In-house method (validated)
Antimicrobial activity	Combined Compendium of Food Additive Specifications. Volume 4" FAO JECFA Monographs
Lead	Standard DIN EN ISO 17294-2:2017-01
Cadmium	Standard DIN EN ISO 17294-2:2017-01
Mercury	Standard DIN EN 15763:2010-04
Arsenic	Standard DIN EN ISO 17294-2:2017-01
Total viable count	Standard DIN ISO 4833-2:2014-05
Coliforms	ISO 4832:2006-02
Escherichia coli	Standard DIN EN ISO 16649-3:2018-01
Salmonella species	Standard DIN EN ISO 6579-1:2017-07

(2) Please specify the limit of quantitation (LOQ) and limit of detection (LOD) for the heavy metals and microbial analyses and provide actual values for the analyses for heavy metals and microbes unless the results are below the LOQ.

The values of the LOD/LOQ for these analyses as well as the analysis results for three non-consecutive enzyme batches are provided below:

Parameter	LOD / LOQ	Batch results		
		1	2	3
Lead (mg/kg)	0.001 mg/kg	0.0095	< 0.001	0.0054
Cadmium (mg/kg)	0.0004 mg/kg	0.0011	< 0.0004	0.0004
Mercury (mg/kg)	0.002 mg/kg	< 0.002	< 0.002	< 0.002
Arsenic (mg/kg)	0.006 mg/kg	0.0069	0.0100	< 0.006
Total viable plate count	10 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Coliforms	10 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Escherichia coli	Negative in 25 g	Negative	Negative	Negative
Salmonella spp.	Negative in 25 g	Negative	Negative	Negative
Antimicrobial activity	Negative	Negative	Negative	Negative

(3) In line with FDA's "Closer to Zero" initiative, the specifications for heavy metals should reflect the amounts determined in the analyses of representative batches and be kept as low as possible.

Noted. c-LEcta will consider reducing the specifications for heavy metals to better reflect the results obtained in these analyses.

(4) You provided the units "mg/kg" and "mg/g" at the same time for arsenic, cadmium and mercury. Please clarify the correct units of these specifications.

The correct units for the analysis results for arsenic, cadmium and mercury are "mg/kg". The previous typographical error has been corrected in the table presented under Question 4(2).

5. Please provide a statement that the ingredient is not intended to be used in infant formula, products under the jurisdiction of the USDA, or in foods in which standards of identity preclude its use.

   We confirm that the ingredient is not intended to be used in infant formula, products under the jurisdiction of the USDA, or in foods in which standards of identity preclude its use.

6. Please provide a statement that that all food contact materials are safe and suitable for their intended use.

   We confirm that all food contact materials are safe and suitable for their intended use.

7. Please confirm that for the purposes of the dietary exposure estimate, the assumption is that the enzyme will remain active and present in the final food.

   The nuclease enzyme will be used as a processing aid in the manufacture of food ingredients. For some of the intended uses, the enzyme may be removed during processing and no longer be present in the final foodstuffs; for all other intended uses, once its intended technical effect has been achieved, the enzyme will be inactivated due to the conditions of temperature and/or pH applied during further food processing. For the purposes of the dietary exposure estimate, the assumption is that the enzyme will remain present in the final food. However, the enzyme will not have any technical or functional effect in that food.

8. On pages 23-24 you discuss the results of published toxicology studies for nuclease enzymes produced by *Penicillium citrinum*.[1,2,3] Please briefly discuss the relevance of these studies to the nuclease enzyme preparation that is the subject of this notice, including evidence of similarity between the enzymes and any expected differences between the enzymes reported in the studies and the enzyme that is the subject of this notice.

   According to the widely accepted Enzyme Evaluation Decision Tree proposed by Pariza and Johnson[4], a previous history of safe use in food is one of the relevant criteria for the safety evaluation of food enzymes. In that same article, the authors included the nuclease enzymes produced by *Penicillium citrinum* in the list of commercial enzymes used in food processing, confirming their long

history of safe use. The nuclease enzymes from *P. citrinum* and the one from *Serratia marcescens* that is the subject of this notice all belong to the functional class of the hydrolases acting on ester bonds and producing 5'-monophosphoesters. The nucleases from *P. citrinum* are zinc metalloproteins, whereas the enzyme subject of this notice requires Mg^{2+} as a cofactor. All three enzymes are endoribonucleases capable of hydrolyzing RNA. In addition, both Nuclease P1 and the nuclease from *S. marcescens* are able to cleave single- and double-stranded DNA. The products of these reactions are 5'-phosphorylated mono-, oligo-, and polynucleotides.

Thus, ribonuclease P, nuclease P1 and the nuclease from *S. marcescens* are very similar regarding their catalytic activity. Moreover, both the substrates and the reaction products are naturally present in a wide variety of food products, and nuclease enzymes are themselves ubiquitous and common constituents of the human diet. Based on these similarities, the results of the toxicological studies carried out for nuclease enzymes produced by *P. citrinum* may be considered to support the safety of the nuclease enzyme preparation that is the subject of this notice.

9. On page 26 you discuss the margin of safety calculation and select a NOAEL of 1000 mg TOS/kg bw/day. We note that there are two studies described in that paragraph with that same NOAEL. Please clarify which study you are using for your MOS calculation. Additionally, please provide a narrative for the relevance of that study to the nuclease enzyme preparation that is the subject of this notice, including evidence of similarity between the enzymes and any expected differences between the enzymes reported in the studies and the enzyme that is the subject of this notice.

   The study used to select an appropriate NOAEL for the MOS calculation is the one described in GRN 507: "Amylomaltase enzyme preparation from *Bacillus amyloliquefaciens* carrying an amylomaltase gene from *Thermus thermophilus*". The amylomaltase enzyme preparation from GRN 507 and the nuclease enzyme that is the subject of this notice are both produced using a *Bacillus amyloliquefaciens* microbial host. According to the scientific literature as well as relevant guidance documents from several international regulatory bodies, the safety of the microbial production strain is the key element for evaluating the safety of enzyme preparations. As recently reviewed by the US Environmental Protection Agency (EPA), *Bacillus amyloliquefaciens* strains have been safely used to produce commercial enzymes (including nucleases) for decades, and their non-toxicogenicity has been confirmed by a large number of toxicological tests on enzyme preparations, including several derived from recombinant strains.[5] Thus, for *Bacillus amyloliquefaciens* strains, several Safe Strain Lineages (SSL) as described by Pariza and Johnson,[4] and recently by Ladics *et al.*,[6] have been established. Following this concept, any food enzyme preparations from new strains in a safe lineage can also be considered safe based on pre-existing safety data, provided that safe recombinant DNA techniques have been used and

conclusive whole genome sequence data are available. The nuclease enzyme preparation that is the subject of this notice has been confirmed by whole genome analysis to have >99.9% homology with the *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* type strain DSM 7. Moreover, the correct integration and deletion of target genes as well as the absence of sequences not intended to remain in the production strain have been verified (s. page 20). Therefore, the available toxicological studies for other food enzyme preparations expressed in *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* microbial hosts, such as the amylomaltase enzyme preparation described in GRN 507, can be considered to support the safety of the nuclease enzyme subject of this notice.

1 G.A. Burdock et al., 2000. Toxicity and mutagenicity studies of DN-50000 and RP-1 enzymes. *Food and Chemical Toxicology*; 38(5): p. 429-442.

2 M. Kondo et al, 2001. Safety evaluation of phosphodiesterase produced from *Penicillium citrinum*: Summary of toxicological data. *Regulatory Toxicology and Pharmacology*; 33(1): p. 2-11.

3 N. Okado et al., 2016. Safety evaluation of nuclease P1 from *Penicillium citrinum*. Food and Chemical Toxicology; 88: p.21-31.

4 Pariza, M.W., Johnson, E.A., 2001. Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. *Regul. Toxicol. Pharmacol*. 33(2): p. 173-186.

5 EPA, OPTT. Risk Assessment of *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* for Consideration of Addition to the List of Eligible Recipient Microorganisms for the 5(h)(4) Exemptions from MCAN Reporting Requirements. July 2015.

6 G. Ladics et al., 2021. Safety assessment and regulation of food enzymes. In: *Enzymes: Novel biotechnological approaches for the food industry*. S. Kermasha, Ed. Academic Press.

PART 1 - SIGNED STATEMENT OF THE CONCLUSION OF GRAS (GENERALLY RECOGNIZED AS SAFE) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260.

§170.225(C) (1) SUBMISSION OF GRAS NOTICE

In accordance with the 21 CFR § 170 subpart E, c-LEcta GmbH hereby is submitting a GRAS notification for its nuclease enzyme preparation produced by *Bacillus amyloliquefaciens*.

§170.225(C) (2) NAME AND ADDRESS OF THE NOTIFIER

Notifier:
c-LEcta GmbH
Perlickstr. 5,
04103 Leipzig
Germany
Tel +49-341-3552140

§170.225(C) (3) NAME OF THE NOTIFIED SUBSTANCE

Nuclease (EC 3.1.30.2, CAS 9025-65-4) from a *Bacillus amyloliquefaciens* strain expressing the gene encoding a nuclease from *Serratia marcescens*. The common or usual name of the substance is nuclease.

§170.225(C) (4) INTENDED CONDITIONS OF USE

The nuclease preparation is used as a processing aid for the hydrolysis of nucleic acids during manufacturing of microbial-derived ingredients. The enzyme may be used in different applications where nucleic acids from the microbial biomass can be cleaved by the nuclease. Some examples include the manufacturing of single cell protein, microbial cell extracts, or ingredients produced by precision fermentation, such as alternative protein or specialty functional ingredients. The enzyme preparation is used at the minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices. The target population for consumption is the general population.

§170.225(C) (5) STATUTORY BASIS FOR GRAS CONCLUSION

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §170.30 (a) and (b). It is of note that several enzyme preparations (protease, carbohydrase) from the microbial host *Bacillus amyloliquefaciens* were affirmed as GRAS by US FDA [1] based on documented pre-1958 history of use [2].

§170.225(C) (6) PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of its intended use.

§170 225(C) (7) AVAILABILITY OF INFORMATION

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying during customary business hours at c-LEcta GmbH or will be sent upon request.

§170 225(C) (8) FOIA (FREEDOM OF INFORMATION ACT)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170 225(C) (9) INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favourable and unfavourable information, known to c-LEcta GmbH and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

DocuSigned by:
[redacted]
824A7CB9E6B44A9...

Dr. Marc Struhalla
CEO
c-LEcta GmbH

DocuSigned by:
[redacted]
D85BEED7A8DF444...

Nicole Albrecht
Regulatory Affairs
c-LEcta GmbH

Date: May 14, 2024