A Cellulase Enzyme from *Trichoderma reesei* Produced by *Aspergillus niger*

Janet Oesterling, Regulatory Affairs, Novozymes North America, Inc., USA

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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:

Novozymes North America Inc. is hereby submitting a GRAS (Generally Recognized as Safe) notice in accordance with subpart E of part 170.

§170.225(c)(2) - The name and address of the notifier:

Novozymes North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

§170.225(c)(3) – Appropriately descriptive term:

The appropriately descriptive term for this notified substance is: Cellulase enzyme from *Trichoderma reesei* produced by *Aspergillus niger.*

§170.225(b) – Trade secret or confidential:

This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:

The cellulase enzyme preparation is used as a processing aid during food manufacturing. The enzyme can be used in any food application where the starch that is present can be modified by the cellulase. Some examples of these applications include brewing and other cereal based beverages, fruit and vegetable processing, starch and grain processing, and baking. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices. The “general” population is the target population for consumption.

§170 225(c)(5) - Statutory basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures.

§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 the intended use.

§170 225(c)(7) – Availability of information:

This notification package provides a summary of the information which supports our GRAS conclusion of the notified substance. Complete data and information that are the basis for this GRAS conclusion is available to the Food and Drug Administration for review and copying during customary business hours at Novozymes North America, Inc. or will be sent to FDA 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 favorable and unfavorable information, known to Novozymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

[redacted]

03/08/2021

Janet Oesterling — Date

Regulatory Affairs Specialist III

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

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a cellulase enzyme produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism expressing the gene encoding for a cellulase from *Trichoderma reesei.*

Key enzyme and protein chemical characteristics of the cellulase are given below:

Classification:	Cellulase
Systemic Name:	4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase
Accepted Name:	cellulase
EC No.:	3.2.1.4
CAS No.:	9012-54-8
Molecular Wt:	35 kDa
Specificity:	catalyzes the hydrolysis of endohydrolysis of (1->4)-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans. Will also hydrolyse 1,4-linkages in β-D-glucans also containing 1,3-linkages.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Aspergillus niger* parental strain, C40, a natural isolate of *A. niger*, was modified by several rounds of mutagenesis to produce strain BO-1. The BO-1 (DSM 12665) strain was then genetically modified to produce the C3085 recipient strain. The C3085 recipient strain was further genetically modified to obtain the *Aspergillus niger* production strain, designated C3085-1870-2.

The expression plasmid used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The donor for the cellulase is the wild type *Trichoderma reesei* (ATCC 56765).

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 (1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (3) and several expert groups (4) (5) (1) (6) (7) (8).

2.2(b) Recipient Strain

The recipient strain, C3085, used in the construction of the *Aspergillus niger* production strain was modified at several chromosomal loci during strain development to inactivate genes encoding several amylases and proteases.

Furthermore, the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted in C3085 together with the deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity and stability.

2.2(c) Cellulase Expression Plasmid

The expression plasmid used to transform the *Aspergillus niger* recipient strain is based on the well-known vector pBluescript SK from E. coli. No elements of this vector are left in the production strain. The introduced DNA consists of the neutral amylase promotor from *A. niger*, the cellulase coding sequence and a transcriptional terminator and finally a selective marker; *amdS* encoding acetamidase.

The expression cassette and the *amdS* gene are flanked by DNA regions used for targeted integration. Only this region is present in the final production strain. This has been confirmed by whole genome sequencing.

2.2(d) Construction of the Recombinant Microorganism

The *Aspergillus niger* production strain, C3085-1870-2, was constructed from the recipient strain through the following steps:

1) The expression cassette from the plasmid was integrated into four specific loci in the recipient strain by homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the cellulase gene from the promoter.

2) The selection of transformants was achieved by growing on a minimal medium and subsequent screening for expression of the cellulase.

The insertion of the expression cassettes in the target locus of the production strain was confirmed by DNA sequencing.

2.2(e) Stability of the Introduced Genetic Sequences

The DNA is integrated into the *Aspergillus niger* chromosome. Thus, it is as such poorly mobilized for genetic transfer to other organisms and is considered mitotically stable. The phenotypic and genetic stability of the Aspergillus niger is proven by its capacity to produce a constant level of the cellulase enzyme. This was assessed by measuring the enzyme activity in three independent batches of the food enzyme, as outlined in Table 2 below. Furthermore, the protein spectrum for three batches showed identical expression profiles. Thus, stable enzyme production of the desired enzyme combined with the identical protein expression profile confirm the stability of the *Aspergillus niger* production strain

2.2(f) Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by genome sequence analysis.

2.2(g) Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC is satisfactorily addressed (4).

2.3 METHOD OF MANUFACTURE

The quality management system used in the manufacturing process for the enzyme preparation complies with the requirements of ISO 9001 and 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 (9) (10) (11).

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the cellulase enzyme concentrate are standard ingredients used in the enzyme industry (11) (10) (9). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC (12). For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams and/or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The cellulase is produced by pure culture, submerged, fed-batch fermentation of a genetically modified strain of *Aspergillus niger* as described in Part 2.

During fermentation, the cellulase enzyme that is produced by *Aspergillus niger* is secreted into the fermentation media.

All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Aspergillus niger*. Each new batch of the stock culture is thoroughly controlled for identity, absence

of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter before inoculation, at regular intervals during cultivation and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48-hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1) Contamination is observed in 2 or more samples by microscopy

2) Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

2.3(f) Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1) Pretreatment - pH adjustment and flocculation

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5) Final concentration – evaporation and/or ultrafiltration

6) Preservation and Stabilization of the liquid enzyme concentrate

The enzyme concentrate is stabilized with sodium chloride and sucrose. The liquid product is further formulated by the addition of water and preserved with potassium sorbate and sodium benzoate. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

The final products are analyzed according to the specifications given below.

2.4(a) Quantitative Composition

Table 1 below identifies the substances considered as diluents, stabilizers or preservative raw materials used in the enzyme preparation. The fermentation media used in the manufacturing of the cellulase enzyme preparation does not contain any major food allergens.

Table 1. Typical composition raw materials of the enzyme preparations

Substance	Approximate Percentage
Water	70 - 90%
Enzyme Solids (TOS*)	12.7%
Sodium Chloride	5 – 10%
Sucrose	5 – 10%
Sodium Benzoate	<1%
Potassium Sorbate	<1%

**Total Organic Solids: defined as: 100% - water – ash – diluents.

2.4(b) Specifications

The cellulase enzyme preparation complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex* (14). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (13).

This is demonstrated by analytical test results of three representative enzyme batches. See Table 2 below.

Table 2. Analytical data for three food enzyme batches.

Parameter	Specifications	PPC70666	PPC70825	PPC71072
Cellulase activity	FBG/g	2670	2540	2570
Total viable count	$\leq 10^4$/g	100	100	100
Lead	<5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	ND in 25g of sample	ND	ND	ND
Total coliforms	≤30/g	< 4	< 4	< 4
Escherichia coli	ND in 25 g of sample	ND	ND	ND
Antimicrobial activity	ND	ND	ND	ND
Production Organism	ND	ND	ND	ND
Ochratoxin A	<LOD = <0.0003 mg/kg	<LOD	<LOD	<LOD
Fumonisin B2	<LOD = <0.0003 mg/kg	<LOD	<LOD	<LOD

*ND: Not Detected
**LOD: Limit of Detection

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is a cellulase (EC 3.2.1.4). Cellulases catalyze the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin and cereal beta-D-glucans to break down the cellulose present in plants cell walls.

2.5(b) Intended Use

Cellulase enzymes are used as processing aids in a wide range of food products (15) (16) (17) (18). The typical food applications where this cellulase will be used are: Brewing and other cereal based beverages, fruit and vegetable processing, starch and grain processing, and baking. Stabilization of the manufacturing process, less batch to batch variability, higher yields and flexibility of raw material choices are just a few of the benefits of using this enzyme.

Brewing and Other Cereal Based Beverages:
Brewing processes rely on cereals (malted or not) as the primary raw material. And, are the primary raw material in the production of beer and other cereal based beverages.

Cellulase enzymes are typically added during the mashing step to reduce the viscosity of the wort and improve the separation of the wort from the spent grains. Also, the cellulase enzyme degrades the polymeric beta-glucans present in the endosperm cell wall of grain, into smaller less viscous molecules, thereby lessening the filtration time and reducing haze problems (19) (20). The enzyme is typically denatured during the lautering, mash filtration or during the pasteurization step after fermentation.

Fruit and Vegetable Processing:
In the juice industry, cellulases are applied in combination with other macerating enzymes. They are used to increase process performance and yield, improve extraction methods and clarify and stabilize juice. They can also reduce viscosity in nectars and purees (18). Here the enzymes are denatured during the pasteurisation steps included in the processing, rendering the enzyme inactive.

Starch/Grain processing:
Complex structures in cereals can cause processing issues when the grain is milled and when fractionated into starch, gluten and fiber. The use of cellulases during grain milling can provide a smooth and efficient processing of that grain, enable separation of the grain structures and ensure quality polysaccharide and gluten fractions.

Cellulases are typically added in grain processing during the milling, mixing and steeping processes. Enzymes are inactivated when used in further food processing, such as baking and starch liquefaction, and are considered non-functional.

Baking and other cereal based processes
During baking processes and other cereal based processes, flour is mixed with water and other raw materials into a dough. During this step, several components of the flour interact together, making the dough more or less firm and elastic. Cellulases typically perform their technological function during the dough or batter handling. Thus, improving the quality of bakery products (21).

2.5(c) Use Levels

Food enzyme preparations are used by food manufacturers according to the Quantum Satis (QS) principle i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction in accordance with Good Manufacturing Practices (GMP).

The dosage applied in practice by a food manufacturer depends on the process. The initial recommendation by the enzyme manufacturer is only the starting point for the food producer and is optimised by the manufacturer of the food to fit the process conditions.

From a technological position, there are no "normal or maximum use levels". But, a food producer who would add much higher doses than what is needed would experience untenable costs as well as negative technological consequences.

Table 3 below shows the maximum recommended use levels for each application where the food enzyme may be used.

Table 3. Recommended Use Levels

Application	Maximum Recommended Use levels (mg TOS/kg RM)
Brewing and other cereal based beverage processes	2.14 (per kg grist)
Fruit & vegetable processing	100 (per kg fruit/veg)
Starch/grain processing	70 (per kg starch/grain)
Baking	100 (per kg flour)

2.5(d) Enzyme Residues in the Final Food

The cellulase enzyme preparation is used during processing and does not exert any enzymatic activity in the final food. This is due to a combination of various factors and depends on the process conditions used by the individual food producer. These factors include; denaturation of the enzyme during heat processing, depletion of the substrate, physical removal of the enzyme, etc. In most cases, a heat treatment step is part of the manufacturing process for production of food ingredients and this process will be enough to inactivate or denature the enzyme protein.

Consequently, the presence of residues of food enzymes in the final food does not lead to any effect in or on the final food. The enzyme action has taken place during the food manufacturing process and is complete before the food product is available for delivery to consumers.

PART 3 - DIETARY EXPOSURE

To provide a "worst case" scenario for the calculation of the possible daily human exposure, an assumption was made that all the enzyme product is retained in the final food product. The general population is the target population for consumption. There is no specific subpopulation.

3(a) Assumptions in Dietary Exposure

Overall, the human exposure to the cellulase will be negligible because the enzyme preparation is used as a processing aid and generally at lower dosages.

The food enzyme is used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using both the Budget Method (22) (23) and specific human consumption.

An exaggerated human intake is estimated. The Budget method was used for the intake associated with starch/grain processing. Specific consumption data is used to estimate the intake associated with fruit/vegetable processing, brewing and other cereal based beverages and baking.

Data summarizing the intake of: juice from fruit/vegetable food products, brewing and other cereal based beverages and baked products consumption was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data from 17 countries and at the 95^{th} percentile (24) (25) (26).

The total TMDI represents a highly exaggerated value because of (among others) the following reasons:

- It is assumed that ALL producers of the intended uses mentioned above for both solid foodstuffs and beverages, use the food enzyme at the highest recommended level.
- For the calculation of the TMDI in food as well as in beverage, the TOS for each application was combined and the total sum was used as the factor for the TMDI in the MOS (margin of safety) calculation.
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.

Using these assumptions, the enzyme preparation will be consumed by humans at the maximum recommended dose for all applications. and will provide a highly conservative margin of safety.

Also, the consumption is further exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps. Therefore, the safety margin calculation derived from this method is highly exaggerated.

The cellulase enzyme preparation has an average activity of 2593 FBG per gram and approximately 12.7% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 20.4 FBG per mg TOS.

3(b) Food Consumption Data

Assumptions in the Budget Method

Solid food	The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight/day. 50 kcal corresponds to 25 g foods. Therefore, adults ingest 25 g foods per kg body weight per day. Assuming that 50% of the food is processed food, the daily consumption will be 12.5 g processed foods per kg body weight. It is further assumed that, in average, all processed food contains 25% starch (or starch-derived) dry matter = 3.12 g starch derived dry matter per kg body weight per day.
Liquids	The maximum intake of liquids (other than milk) is 100 ml/kg body weight day. Assuming that 25% of the non-milk beverages is processed, the daily consumption will be 25 ml processed beverages per kg body weight. It is further assumed that all processed beverages contain 10% starch hydrolysates = 2.50 g starch derived dry matter per kg body weight per day. It is assumed that the densities of the beverages are ~ 1.

TMDI calculation – Starch/Grain Processing

Solid Food:
The dosage given in Table 3 for starch/grain processing is 70 mg TOS per kg starch based raw material.

Based on this, 3.12-gram starch-derived dry matter in solid food will maximally contain:

70 mg TOS per kg ÷ 1000 g per kg x 3.12 g = 0.22 mg TOS per kg bw/day

Liquid Food:

The dosage given in Table 3 for starch/grain processing is 70 mg TOS per kg starch based raw material.

Based on this, 2.50-gram starch-derived dry matter in liquids will maximally contain:

70 mg TOS ÷ 1000 g per kg x 2.50 g = 0.18 mg TOS per kg bw/day

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:
0.22 + 0.18 = <u>0.4 mg TOS/kg body weight/day</u>

TMDI Calculation - Fruit and vegetable processing

The main and most consumed food application in fruit and vegetable processing is fruit juice. Raw materials used can be various fruits and vegetables and the yields will vary depending on the type of raw material used.

Typically, it takes 1.3 g fruit to make 1 g of fruit juice.

To demonstrate a worst-case calculation, an exaggerated human intake for fruit and vegetable juice was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for fruit and vegetable juices from 17 countries and at the 95th percentile (24). Based on this, the intake value of 33 g of juice is consumed per kg of body weight per day.

The dosage given in Table 3 for fruit and vegetable processing is 100 mg TOS per kg fruit/vegetable raw material.

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is:

100 mg TOS/ kg fruit x 1.3 g fruit/1 g juice x 33 g juice ÷ 1000 = 4.3 mg TOS per kg bw/day

TMDI calculation - Brewing and Cereal Based Beverage

Intake associated with beer and other cereal based beverage processes:

To demonstrate a worst-case calculation, an exaggerated human intake for beer and beer like beverages was used. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for beer and beer-like beverages from 17 countries and at the 95th percentile (25). Based on this, the intake value of 11.4 g of beer and beer-like beverage is consumed per kg of body weight per day.

The dosage given in Table 3 for beer and other cereal based beverages is 2.14 mg TOS/kg grist

As a rule of thumb, 1 kg of grist will be used to produce 6 kg of beer and an intake per kg bw per day of 11.4 g "Beer and beer-like beverage". This corresponds to a theoretical maximum daily intake (TMDI) of the food enzyme of:

11.4 g beer/kg bw/day x 2.14 mg TOS/kg grist ÷ 6 kg beer/g grits ÷ 1000 = 0.004 mg TOS/kg bw/day

TMDI calculation – Baking

The baking processes (mixing flour and water and other relevant ingredients followed by a heating step) on average results in 140 g of final baked product from 100 g of flour. The average intake of bread and similar products was used as bread is the most consumed bakery product. This intake value was taken from the EFSA Comprehensive European Food Consumption Database using the consumption data for grains and grain based products (bread and similar products) from 17 countries and at the 95th percentile (26). Based on this, the intake value of 12.10 g of grain and grain-based products is consumed per kg of body weight per day.

The dosage for the food enzyme in baking and other cereal based processes given in Table 3 is 100 mg TOS per kg flour.

100 mg TOS/kg flour x 100 g flour x 12.10 g grain/grain-based product/kg bw/day ÷ 1000 = 121 mg grain/grain-based product/kg bw/day ÷ 140 g baked product = 0.864 mg TOS/kg bw/day.

Total TMDI:

To represent a worst-case scenario for the maximum human exposure value, it is assumed that foods represented for each application are consumed daily. The final TMDI is calculated taking the TOS value for all applications.

Starch/Grain processing:	0.40 mg TOS/kg bw/day
Fruit/Vegetable processing:	4.3 mg TOS/ kg bw/day
Beer/other cereal based beverage processing:	0.004 mg TOS/kg bw/day
Baked Products:	0.864 mg TOS/kg bw/day
Total:	**5.56 mg TOS per kg bw/day**

Margin of Safety

The margin of safety is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption, TMDI. The safety margin calculation derived from this method is highly exaggerated.

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on alpha-amylase tox batch PPY35872 was the highest dosage possible, 1244 mg TOS/kg bw/day. See Table 4 below.

Table 4. Calculation of the Margin of Safety

NOAEL (mg TOS/kg bw/day)	1244
*TMDI (mg TOS/kg bw/day)	5.56
Margin of Safety	224

*based on the worst-case scenario

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 STATUS

The information provided in the following sections is the basis for our conclusion of the general recognition of safety for the cellulase enzyme preparation. The evaluation follows the generally recognized methodology and the decision tree by Pariza and Johnson 2001, (Appendix 1) (3). Our safety evaluation in Part 6 follows the approach described in the Enzyme Technical Association publication (Sewalt et al 2016, Appendix 2) which includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6(a) Safety of the Manufacturing Process

This Part describes the manufacturing process for the cellulase, which follows standard industry practices (11) (9) (10).

The quality management system used in the manufacturing process for the enzyme complies with the requirements of ISO 9001. It is manufactured 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.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

6(b) Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (2) (3).

If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (4). Pariza and Foster define a non-toxigenic organism as "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 as "one that is very unlikely to produce disease under ordinary circumstances" (2).

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. *Aspergillus niger* is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in published scientific literature (27).

Carbohydrase, pectinase, protease, glucose oxidase, catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA, on request from the Enzyme Technical Association (ETA), converted into separate GRAS Notices (GRN 89, 111, 132) (28). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the

agency did not question the conclusion that enzyme preparations from *Aspergillus niger* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 158, 183, 214, 296, 345, 402, 428 which all describe food enzymes produced by *Aspergillus niger* strains (28).

In 1997, *Aspergillus niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations (29). Also, *Aspergillus niger* was reviewed and was concluded to be a safe source organism by Olempska-Beer et al. (30) and Schuster et al. (31) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

An evaluation of this genetically modified production microorganism for the cellulase, embodying the concepts initially outlined by Pariza and Foster, 1983 (32) and further developed by IFBC in 1990 (4), the EU SCF in 1991 (5), the OECD in 1992 (1), ILSI Europe Novel Food Task Force in 1996 (8), FAO/WHO in 1996 (7), JECFA in 1998 (13) and Pariza and Johnson in 2001 (27) demonstrates the safety of this genetically modified production microorganism strain.

The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Some *Aspergillus niger* strains can produce ochratoxin A (31), and the production of fumonisin B2 has also been shown in *Aspergillus niger* (33). Ochratoxin A and fumonisin B2 are the two mycotoxins of concern in terms of human and animal safety that can be produced by *Aspergillus niger* strains (34).

The BO-1 safe strain lineage was found to be unable to produce unwanted secondary metabolites (ochratoxin A and fumonisin B2) under conditions that are known to induce mycotoxin production in fungi. In addition, analytical test results of three representative enzyme batches of this cellulase (see Table 2) confirm the absence of ochratoxin A and fumonisin B2.

Based on the information presented above it is concluded that the *Aspergillus niger* production strain is considered a safe strain for the production of the cellulase enzyme.

6(c) Safe Strain Lineage

The safety of this *Aspergillus niger* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4). The *Aspergillus niger* production strain is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

Novozymes has used *Aspergillus niger* as a production strain for a variety of enzymes for decades. Table 5 below outlines some of Novozymes enzyme preparations produced by

Aspergillus niger production strains within the safe strain lineage and the safety studies conducted on those enzyme concentrates.

Table 5: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain [1]	Donor strain	Safety studies [2]
Glucoamylase	3.2.1.3	*Aspergillus niger* BO-1	None	Yes
Pectin lyase	4.2.2.10	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Lysophospholipase	3.1.1.5	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Triacylglycerol lipase	3.1.1.3	*Aspergillus niger* BO-1	*Candida antarctica*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Aspergillus niger*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Talaromyces emersonii*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C878	*Trametes cingulata*	Yes
Alpha-amylase	3.2.1.1	*Aspergillus niger* C878	*Rhizomucor pusillus*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C2218	*Gloeophyllum trabeum*	Yes
Glucoamylase (GRN 657)	3.2.1.3	*Aspergillus niger* C2218	Penicillum oxalicum	Yes
Triacylglycerol lipase (GRN 158)	3.1.1.3	*Aspergillus niger* C2218	*Candida antarctica*	Yes
Mannanase (GRN 739)	3.2.1.78	*Aspergillus niger* C2218	*Talaromyces leycettanus*	Yes
Phospholipase A	3.1.1.32	*Aspergillus niger* C2948	*Talaromyces leycettanus*	Yes
Trehalase (GRN 699)	3.2.1.28	*Aspergillus niger* C2218	*Corynascus sepedonium (Myceliophthora sepedonium)*	Yes
Phospholipase	3.1.1.32	*Aspergillus niger* C2218	*Talaromyces bacillisporus*	Yes

Table 5. Novozymes products derived from *A. niger* strains where safety studies have been carried out.
[1] The predecessor strain shows strains in the GM construction pathway. [2] At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13-week sub chronic oral toxicity study in rats. The conclusions of these studies were in all cases favorable.

All toxicological studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view that strains derived from the *Aspergillus niger* strain lineage can be used safely for the production of food enzymes.

The fact that no issues are observed in safety studies on different enzymes (e.g. amylases, protease, xylanase) produced by strains derived from a common predecessor (*Aspergillus niger* MDT223), strongly supports the safety of the B. licheniformis strain lineage, independent of which enzyme is produced.

The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed cellulase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (3) have been met.

Based on the information presented in Parts 6 (a) and (b), it is concluded that the *Aspergillus niger* production strain is part of the safe strain lineage and is considered a safe strain for the production of the cellulase enzyme.

6(d) Safety of the Donor Organism

As noted above, it is the safety of the production strain that should be the primary concern when assessing the safety of an enzyme used for food.

The donor organism of the cellulase is *Trichoderma reesei*. As indicated in Part 2 the introduced DNA is well defined and characterized. The introduced DNA does not code for any known harmful or toxic substances.

6(e) Safety of the Cellulase Enzyme

A wide variety of enzymes are used in food processing (2) (27). Cellulases account for a significant share of the world enzyme market with major uses in the food and feed processing.

Cellulases catalyze the endo-hydrolysis of the 1,3- or 1,4- linkages in beta-D-glucans. Theses cellulases are widely distributed in nature and have been isolated from a variety of sources, such as fungi, yeasts, bacteria and plants (35) (36) (20). The FDA has reviewed beta-glucanase preparations in the past; GRN 149,195, 479 and 482, all of which received "No Questions" letters from the Agency. Several enzyme preparations of beta-glucanase have been evaluated by JECFA and assigned an ADI 'not specified' for their use in applications such as the preparation of fruit juices, beer and baking products (37).

GRAS notifications have been submitted and accepted by FDA with "No questions" for the use of cellulases (GRASP petition 9G0260, GRN 584, 479, 292 and 195) from a variety of production organisms (28). All the GRAS notifications mentioned above included sufficient toxicological testing data which showed no evidence of toxicological concern regarding the safety and consumption of cellulase enzymes.

All these notifications have provided sufficient toxicological testing data showing evidence that there is no toxicological concern regarding the safety and consumption of cellulase enzymes.

A literature search was performed in September 2019 for the periods 2000 to 2019 on cellulase, utilizing the database Web of Science and the keywords "cellulase enzyme", "food safety" and "toxicity". A total of 31 relevant hits were found. Novozymes reviewed the available abstracts and found no indication that that cellulase is associated with toxicity or other adverse effects in humans or animals and the findings did not contradict our determination of the general recognition of safety of the cellulase enzyme.

Novozymes also conducted a sequence homology to known allergens and toxins. The results showed no indication of allergenic or toxigenic potential of the cellulase.

From the information provided above, it is apparent that cellulase enzymes have a long history of use in food processing and are safe for human consumption.

6(f) Allergenic/Toxigenic Potential of the Cellulase Enzyme

The ingestion of a food enzyme protein is not considered a food allergy concern. This is based on the following considerations:

1) Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

2) The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal-based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

3) Enzymes in foods are added in concentrations in the low range of part per millions. The enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (38).

In order to further evaluate the possibility that the cellulase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (39) and modified by Codex Alimentarius Commission, 2009 (14) the cellulase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a gap penalty was done and showed no matches. Alignment of the cellulase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No homology was found between the cellulase and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

A sequence homology of cellulase to known toxins was assessed based on the information present in the UNIPROT database. This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 18% indicating that the homology to any toxin sequence in this database is random and very low.

On the basis of the available evidence it is concluded that oral intake of cellulase produced by *Aspergillus niger* is not anticipated to pose any food allergenic or toxigenic concerns.

6(g) Safety Studies Conducted

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate enzymes derived from *Aspergillus niger* production strains (3). As described in Part 6(c), Novozymes has concluded, that strains within the safe strain lineage of *Aspergillus niger* pose no safety concerns. Table 5 lists the strains within this lineage, with many having corresponding GRNs on file with the FDA, where toxicological safety studies have been performed.

The toxicological studies include genotoxicity, cytotoxicity and general toxicity activities. These toxicology studies have produced consistent findings indicating that the test article (enzyme concentrate) did not exhibit any toxic or mutagenic effects under the conditions of the test, thus supporting the safety of the enzymes produced by *Aspergillus niger* strains that are within this lineage.

It is reasonable to expect and conclude that enzymes produced by *Aspergillus niger* strains within this safe strain lineage will show similar toxicological profiles and further supports our conclusion that *Aspergillus niger* strains are safe hosts for the expression of enzymes. Therefore, we believe additional toxicological studies on the article of commerce (subject of this notification) are of little to no value, redundant and considered unnecessary (40) (41).

6(h) Description of the Test Article

The *Aspergillus niger* production strain (C3085-1870-2) for the cellulase enzyme (subject of this notification), was developed from the safe strain lineage listed in Table 4.

Novozymes has determined that the results of the toxicology studies on amyloglucosidase enzyme concentrate, batch PPY35872 from *Aspergillus niger*, can be bridged to support the toxicological outcome of the cellulase enzyme from *Aspergillus niger*, the subject of this notification.

This approach is in line with the Safe Strain Lineage concept as outlined by Pariza and Johnson, the EFSA (European Food Safety Authority) opinion (Question No. EFSA-Q-2013-00895) and the EFSA CEF Guidance on Food Enzymes regarding toxicological testing (42) (43) (27).

Based on the genetic modification performed and described in Part 2 of this dossier, the recipient strain (C3085), used to construct the *Aspergillus niger* production strain C3085-1870-2, is closely related to the predecessor strain C2218 mentioned in Table 5. There is no reason to assume that the C3085-1870-2 recombinant production strain should be less safe than the C2218 strain.

All toxicology studies performed on the amyloglucosidase enzyme concentrate, batch PPY35872, from the *Aspergillus niger* production strain, were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP).

The studies include a bacterial reverse mutation assay (AMES), *in vitro* micronucleus assay a 2-week oral (gavage) study in rats and a 13-week oral (gavage) study in rats. The studies were performed at Novozymes A/S (Denmark), Huntingdon Life Sciences (UK) and Covance Laboratories Ltd. (UK) during the period December 2013 to June 2014.

A summary of the toxicology studies for the amyloglucosidase enzyme concentrate batch PPY35872, produced by *Aspergillus niger*, is included in Appendix 3.

Based on the presented toxicity data, the history of safe use and the safe strain lineage of the *Aspergillus niger* production strain, it can be concluded that the test preparation; enzyme concentrate batch PPY35872, exhibits no toxicological effects under the experimental conditions described in the summary

6(i) Results and Conclusion

The enzyme industry has performed hundreds of toxicology studies using a variety of enzymes (e.g. cellulases, amylases, glucanases, lipases etc.) derived from multiple production organisms (e.g. *Aspergillus niger*, *Trichoderma reesei, Bacillus subtilis* etc.) with no adverse findings observed in the conducted studies (3) (44) (45).

Results of the toxicity and mutagenicity tests described in Appendix 3 showed no toxicity or mutagenicity of the test article; enzyme concentrate batch PPY35872 produced by *Aspergillus niger.*

A critical review and evaluation of the cellulase enzyme preparation (subject of this notification) and the amyloglucosidase enzyme preparation (subject of the test material PPY35872) was done following the concepts of the Pariza papers and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (46) (2) (3).

Based on the published, publicly available scientific information about *Aspergillus niger* production strains and cellulase enzymes used in food processing, along with the supporting data generated by Novozymes and using the decision tree evaluation method outlined by Pariza and Johnson (27), Novozymes considers the cellulase enzyme preparation (subject of this notification), produced by the *Aspergillus niger* production organism to be generally recognized as safe.

PART 7 – SUPPORTING DATA AND INFORMATION

All information indicated in the List of Appendices and References is generally available

APPENDICES

1. Pariza and Johnson Decision Tree Analysis

2. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology, Vol. 12, No. 5. October 2016.

3. Summary of Toxicity Data, *Amyloglucosidase* from *Aspergillus niger*, batch PPY35872. November 27, 2014, File No. 2014-04383-03.

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Decision Tree

Appendix 1- This cellulase enzyme preparation produced by *Aspergillus niger* was evaluated according to the decision tree published in Pariza and Johnson, 2001[(1)]. The result of the evaluation is presented below in the Decision Tree.

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.
2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3.
3. Issues relating to the introduced DNA are addressed in 3a-3e.

   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
   YES
   If yes, go to 3c.
   c. Is the test article free of transferable antibiotic resistance gene DNA?
   YES
   If yes, go to 3e.
   e. 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 products?
   YES
   If yes, go to 4.
4. Is the introduced DNA randomly integrated into the chromosome?
   NO
   If no, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   Yes
   Test article is accepted

LIST OF REFERENCES

1. Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.

Novozymes North America
Regulatory Affairs
77 Perry Chapel Church Rd
Franklinton, NC 27525
Tel. 919-494-3000
Fax 919-494-3420

Toxicology & Product safety

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SUMMARY OF TOXICITY DATA

Amyloglucosidase, batch PPY35872 from *Aspergillus niger*

Authors:
Birgitte Thue Ravn
Trine Østergaard Lund
Jens Lichtenberg

Issued by:
Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Amyloglucosidase, batch PPY35872.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Novozymes A/S, Denmark, Covance Laboratories, England, Jai Research Foundation, India and Huntingdon Life Sciences, England during the period Dec 2013 to June 2014.

The main conclusions of the safety studies can be summarized as follows:

- Amyloglucosidase, batch PPY35872 did not induce gene mutations in the Ames test, neither in the presence or absence of S-9 mix.
- Amyloglucosidase, batch PPY35872 did not show any clastogenic activity, neither in the presence or absence of S-9 mix, when tested in the *in vitro* micronucleus assay.
- In a 2 weeks oral toxicity study in rats Amyloglucosidase, batch PPY35872 was well tolerated and did not cause any toxicologically significant changes at any dose level.
- In a 13 weeks oral toxicity study in rats Amyloglucosidase, batch PPY35872 was well tolerated and did not cause any toxicologically significant changes at any dose level.

Based on the present toxicity data it can be concluded that Amyloglucosidase, represented by batch PPY35872, exhibits no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Amyloglucosidase is a liquid enzyme concentrate containing an Glucan 1,4 alpha-glucosidase (AMG) (E.C. number 3.2.1.3) which hydrolysis 1,4-alpha and 1,6-alpha linkages of dextrins from non-reducing end.

2.1Production organism

Amyloglucosidase is produced by a strain of *Aspergillus niger*, containing an Amyloglucosidase gene code which is originating from *Gloeophyllum trabeum.*

Aspergillus niger has a long history of safe use. This species has been used for decades in the production of enzymes, and for more than a decade as recombinant organism for production of a variety of bio-industrial products.

Strains of *Aspergillus niger* may have the potential to produce certain mycotoxins of medical importance. The production strain of Amyloglucosidase belongs to a strain lineage which has been thoroughly investigated for its potential to produce secondary metabolites. The results showed, that this strain lineage does not produce any known mycotoxins.

The test substance does not contain the production strain. Absence of the production strain is part of the complete specification of the product.

2.2 Characterization

The toxbatch PPY35872 was used for the conduct of all the toxicological studies. The characterization of the toxbatch is presented in Table 1.

Table 1. Characterization data of Amyloglucosidase, batch PPY35872

Batch number	PPY35872
Activity	454 AGU(D)/g
Water (KF) (% w/w)	86.9
Dry matter (% w/w)	13.1
Ash (% w/w)	1.2
Total Organic Solids (TOS[1]) % w/w	11.9
Specific gravity (g/mL)	1.045

[1] % TOS is calculated as 100% - % water - % ash - % diluents.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Amyloglucosidase, batch PPY35872 was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, and TA98 and *Escherichia coli* WP2uvrA. The study was carried out according to the OECD test guideline 471 (adopted in 1997) and in compliance with GLP.

Crude enzyme preparations, like the present batch of Amyloglucosidase, contain the free amino acids histidine and tryptophan, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.
To overcome this problem all strains were exposed to Amyloglucosidase in liquid culture ("treat and plate assay").

Two independent experiments were performed, with and without the inclusion of metabolic activation (S-9 mix). In each experiment cultures of bacteria were exposed to six doses of the test substance (5000, 2500, 1250, 625, 313, and 156 µg dry matter/mL) in a phosphate buffered nutrient broth for 3 hours. After incubation, the test substance was removed by centrifugation prior to plating.

The results obtained with the diagnostic mutagens and the solvent control demonstrated the sensitivity of the tests and the efficacy of the S-9 mix metabolic activation system.

No treatments of any of the bacterial strains with the test substance resulted in any increases in revertant numbers meeting the criteria for a positive or equivocal response, neither in the presence or absence of S-9 mix.

It was concluded that Amyloglucosidase, batch PPY35872 did not induce gene mutations in bacteria either in the presence or absence of metabolic activation when tested under the conditions employed in this study.

3.2*In vitro* Micronucleus assay

In order to assess the clastogenic and the aneugenic activity of Amyloglucosidase, batch PPY35872 its effects on the frequency of micronuclei was investigated in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted according to GLP, in compliance with the OECD guideline: Genetic Toxicology: OECD Guideline for the testing of chemicals. Guideline 487: *In vitro* micronucleus test (2010).

Heparinized whole blood cultures, pooled from two male donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Sets of duplicate cultures were treated with the solvent (purified water), test substance or appropriate positive controls. Treatments with the test substance covered a broad range of doses, separated by narrow intervals. The highest concentrations used was 5000 µg/mL (expressed in terms of the test substance as supplied), which is the highest dose level recommended in the guidelines for *in vitro* cytogenetic assays.

Cell cultures were exposed to the test substance for 3 hours in the presence and absence of metabolic activation (S-9 mix) and harvested 24 hours after the beginning of treatment (3+21 hour treatment). Additionally, a continuous 24-hour treatment without S-9 mix was included with harvesting 24 hours after removal og the test substance (24+24 hour treatment). After removal of the test substance the cultures were treated with cytochalasin-B. Three concentrations, covering an appropriate range of cytotoxicity, were selected for scoring of micronuclei by evaluating the effect of the test substance on the replication Index (RI). 2000 cells per concentration (1000 cells from each replicate culture) were scored.

The proportion of binucleate cells with micronuclei in all cultures of the vehicle controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with micronuclei, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

Treatment of the cells with the test substance, in the absence of S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all treated cultures fell within the normal ranges.

A small statistically significant ($p \leq 0.05$) increase in MNBN cells was observed following 3+21 hour treatments in the presence of S9 mix at the highest concentration tested (5000 µg/mL). However, this statistical increase was small and set against a low concurrent vehicle control response such that both treated cultures exhibited MNBN cell values that fell within the normal range and there was no evidence of a concentration related effect. All MNBN cell values for all test substance treated cultures (all concentrations analysed) fell within normal ranges. This isolated statistical increase was therefore not considered of biological importance.

It was concluded that Amyloglucosidase, batch PPY35872 did not induce micronuclei in cultured human peripheral blood lymphocytes either in the absence or presence of S-9 mix under the experimental conditions employed for this study.

4. GENERAL TOXICITY

4.1Toxicity Study by Oral Gavage Administration to Wistar Rats for 2 Weeks

The study was conducted to determine the adverse effects of Amyloglucosidase, PPY35872, in Wistar rats, when administered orally through gavage for a period of 14 consecutive days. The methods followed were based on study outline provided by the Study Sponsor and inspired from the guideline of the OECD N° 407 (October 3, 2008).

A total of 20 male and 20 female Wistar rats were randomly divided into four groups, each group comprising 5 male and 5 female rats. Rats were treated with Amyloglucosidase, PPY35872, at the dose levels of 474.4 Amyloglucosidase Unit AGU(D)/kg b. wt. corresponding to 124.4 g TOS/kg b. wt. (low dose), 1565.6 AGU(D)/kg b. wt. corresponding to 410.4 g TOS/kg b. wt. (mid dose) and 4744.3 AGU(D)/kg b. wt. corresponding to 1243.6 g TOS/kg b. wt. (high dose) for a period of 14 consecutive days through oral gavage. Concurrent vehicle control group rats received reverse osmosis (RO) water alone. The fixed dose volume of 10 mL/kg b. wt. was used. The dose formulation for mid and low dose groups were prepared by dissolving the test item in RO water and for the high dose group test item was used as supplied without any dilution.

All rats were observed twice daily for mortality, morbidity and clinical signs throughout the study period. The body weight of each rat was recorded on experimental days 1, 4, 8, 11, 14 (not fasted) and 15 (fasted). Food consumption was calculated as g/rat/day during experimental period 1-3, 4-7, 8-10 and 11-13. Hematological and clinical chemistry analysis was performed on blood and serum samples of all rats at the end of the treatment period.

All the rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination at the end of the treatment period. Absolute organ weights were recorded and relative organ weights were calculated for the organs viz., adrenals, brain, ovaries/testes, uterus/epididymides, heart, kidneys, liver, spleen and thymus.

No mortality or morbidity was observed during the study period and no clinical signs were observed throughout the study period. No significant changes were observed in mean body weight, mean body weight change and food consumption in treated rats at low dose, mid dose and high dose levels when compared with the vehicle control group throughout the treatment period. Haematology and clinical chemistry parameters did not reveal any treatment related significant alterations and no treatment related significant changes were observed in the terminal body weights, absolute organ weights and relative organ weights of treated groups when compared with the vehicle control group.

External and internal examination at the end of the treatment period did not reveal any findings in any of the animals.

In conclusion, Amyloglucosidase, batch PPY35872 did not cause any adverse effects up to the highest dose level applied 4744.3 AGU(D)/kg b. wt. (corresponding to 1243.6 mg TOS/kg b. wt.) when administered through oral gavage for 14 consecutive days to Wistar rats under the conditions and procedures followed in the present study. The NOAEL (No Observed Adverse Effect Level) for both male and female rats for Amyloglucosidase, batch PPY35872 was 4744.3 AGU(D)/kg b. wt., corresponding to 1243.6 mg TOS/kg b. wt.

4.2 Toxicity Study by Oral Gavage Administration to Sprague-Dawley Rats for 13 Weeks

The objective of this study was to assess the systemic toxic potential of Amyloglucosidase, batch PPY35872, when administered orally by gavage to Sprague-Dawley (Crl:CD(SD)) rats for 13 weeks. Three groups, each comprising 10 males and 10 females, received Amyloglucosidase, batch PPY35872 at doses of 10, 33 or 100% of the test enzyme (equivalent to 0.124, 0.410 or 1.244 g TOS/kg/day, or 474, 1566 or 4744 AGU(D)/kg/day). A similarly constituted control

group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg body weight).
During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, body weight, food and water consumption, ophthalmic examination, haematology (peripheral blood), blood chemistry, urinalysis, organ weight, macropathology and histopathology investigations were undertaken.
General appearance and behaviour, sensory activity, grip strength and motor activity were not affected by treatment and there were no deaths during the treatment period. There was no effect of treatment on bodyweight gain or food consumption. Water consumption was slightly high in females receiving 100% Amyloglucosidase, batch PPY35872.

There were no treatment-related ophthalmic findings. There were no treatment-related haematological findings or biochemical changes in the blood plasma.

urinalysis investigation revealed a slight reduction in pH for males receiving 33 or 100% of the Amyloglucosidase batch, the magnitude of which was dose-related.
Organ weights were unaffected and there were no treatment-related macroscopic or histopathological findings.

It is concluded that oral administration of Amyloglucosidase, batch PPY35872 to Sprague-Dawley rats at doses up to 100% of the Amyloglucosidase batch (equivalent to 1.244 g TOS/kg/day or 4744 AGU(D)/kg/day) was well-tolerated and did not result in any toxicologically significant change. A slight increase of water intake in females and slightly low urinary pH in males did not associate with any histopathological finding in the kidneys and were therefore of no toxicological significance. Consequently, the no-observed-adverse-effect level (NOAEL) was greater than 1.244 g TOS/kg/day or 4744 AGU(D)/kg/day.

5. REFERENCES

5.1 Study reports

Novozymes A/S: Study No.: 20138070. Amyloglucosidase, batch PPY35872: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. (March 2014). LUNA file: 2014-03645.

Covance Laboratories: Study No.: 8293662. Novozymes Reference No.: 20136083: Amyloglucosidase, batch PPY35872: Induction of micronuclei in cultured human peripheral blood lymphocytes. (March 2014). LUNA file: 2014-04062.

Jai Research Foundation: JRF Study No.: 410-1-02-7978. Novozymes Reference No.: 20136079: Repeated Dose 14-Day Toxicity Study of Amyloglucosidase, batch PPY35872, through Oral Gavage in Wistar rats. (March 2014). LUNA file: 2014-04378.

Huntingdon Life Sciences: Study No.: LKG0093. Novozymes Reference No.: 20136074: Amyloglucosidase, batch PPY35872, Toxicity Study by Oral gavage Administration to Sprague-Dawley Rats for 13 Weeks. (June 2014). LUNA file: 2014-09845.

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
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, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – 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)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

3b. 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):* _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person		Position	
	Company *(if applicable)*			
	Mailing Address *(number and street)*			
City		State or Province	Zip Code/Postal Code	Country
Telephone Number		Fax Number	E-Mail Address	

1b. Agent or Attorney *(if applicable)*	Name of Contact Person		Position	
	Company *(if applicable)*			
	Mailing Address *(number and street)*			
City		State or Province	Zip Code/Postal Code	Country
Telephone Number		Fax Number	E-Mail Address	

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

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

☐ Electronic Submission Gateway

☐ Electronic files on physical media with paper signature page

☐ 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 FDA's files by reference? *(Check one)*

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

5. The submission incorporates by reference 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 determination of GRAS status *(Check one)*

☐ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☐ No *(Proceed to Part IV)*

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, see attached Designation of Confidential Information

☐ 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

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☐ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1					
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s)),* and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☐ Any additional information about identity not covered in Part V of this form
- ☐ Method of Manufacture
- ☐ Specifications for food-grade material
- ☐ Information about dietary exposure
- ☐ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☐ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☐ Comprehensive discussion of the basis for the determination of GRAS status
- ☐ Bibliography

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

PART VII – SIGNATURE

1. The undersigned is informing FDA that _____
(name of notifier)

has concluded that the intended use*(s)* of _____
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☐ _____ agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.
(name of notifier)

_____ 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.
(name of notifier)

_____
(address of notifier or other location)

_____ agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. _____
(GRAS Affirmation Petition No)

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**

PART VIII – 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)

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 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, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (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.

novozymes
Rethink Tomorrow

Analytical methods

Detection of Escherichia coli (*E. coli*) in 25 g

Principle

E. coli is a Gram-negative, indole-positive, facultative anaerobic rod. It is considered to be a fecal indicator.

Detection of *E. coli* in 25 g is carried out as a qualitative analysis using nonselective enrichment in buffered peptone water (BPW) followed by isolation of β-D-glucuronidase-positive *E. coli* on a selective indicative agar medium (TBX agar). β-glucuronidase-negative E. coli strains (3–4%) form colorless colonies on TBX agar (e.g., *E. coli 0157*). The detection of *E. coli O157* is performed as ImmunoMagnetic Separation (IMS) using Dynabeads® anti-O157 and plating onto two selective indicative agar media (CT-SMAC agar and CHROMagar O157). Suspect *E. coli O157* colonies are verified using the *E. coli O157* latex test.
Suspect colonies from TBX agar and/or *E. coli O157* latex-positive isolates from CT-SMAC agar and/or CHROMagar O157 are reported as *E. coli* detected in 25 g.

Important: The media used have the following characteristics:

Media	Characteristics
BPW broth	Nonselective broth.
TBX agar	**Selective properties:** Growth of accompanying Gram-positive flora is largely inhibited by the use of bile salts. **Indicative properties:** The presence of the enzyme β-D-glucuronidase differentiates most *E. coli* spp. from other coliforms. *E. coli* absorbs the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-β-D-glucuronide). The enzyme β-glucuronidase splits the bond between the chromophore 5-bromo-4-chloro-3-indolyl and the β-D-glucuronide. *E. coli* colonies are colored blue-green. *NOTE:* For the recovery of sublethally injured *E. coli*, plates are incubated at 34–38 °C and not 44 °C as recommended by Merck (inhibits growth of accompanying Gram-positive flora).
CT-SMAC agar (Sorbitol MacConkey agar)	Polypeptone favors the growth of *E. coli O157:H7*. Sorbitol-negative bacterial (in particular *O157:H7*) colonies are colorless. Sorbitol-positive bacteria give rise to red colonies due to the change of the color of the pH indicator (neutral red). Contaminating bacteria are inhibited by the association of bile salts, crystal violet, cefixime, and potassium tellurite**.**
CHROMagar O157 and CT-CHROMagar O157	A typical *E. coli O157* will grow as a pink-mauve colony, whereas most other microorganisms are either inhibited or grow as blue or colorless colonies.

Definition of units

The result is stated as:

- DET (*E. coli* detected in 25 g) or
- ND (*E. coli* not detected in 25 g)

Standards

A positive reference strain can be used (e.g., *E. coli ATCC 11229*).
If a reference strain of *E. coli O157* is included, it must be *E. coli O157* without the genes coding for verotoxins (e.g., ATCC 43888).

Detection limit

Theoretical detection limit: 1 *E. coli* in 25 g

Equipment

Balance (± 0.1 g)
Magnetic stirrer
Incubator (34–38°C)
Sterile inoculation loops (1-µl)
Sterile swabs
Vortex mixer
Pipettes and sterile tips
For ImmunoMagnetic Separation (either mIMS or aIMS):

- For manual ImmunoMagnetic Separation (mIMS):

    - MPC-S rack and magnet (Invitrogen cat. no. 120.20) + Eppendorf tubes, 1.5-ml (Eppendorf cat. no. 0030 10.086) + MX-3 mixer (Dynal cat. no. 159.09) – mixer is optional.
- For automatic ImmunoMagnetic Separation (aIMS):
    - BeadRetriever™ (Invitrogen cat. no. 159-50) + tubes and tips (Invitrogen cat. no. 150-51)

Media and reagents

Buffered peptone water (BPW) (450 ml)
Chromocult® TBX agar plates (9-cm)
Cefixime-tellurite Sorbitol MacConkey agar (CT-SMAC agar plates, 9-cm)
CHROMagar O157 agar plates (9-cm) or CT-CHROMagar O157 (app. 5- or 9-cm)
Tryptone soya agar plates (TSA)
Dynabeads® anti-O157, Dynal cat. no. 710.04
Washing buffer (PBS-Tween 20 buffer), Sigma no. P-3563
E. coli O157 Latex Test Kit (for verification), Oxoid no. DR620

Safety

The *E. coli O157* Latex Test Kit (Oxoid DR0620) is labeled R22 – Harmful if swallowed due to 0.1% sodium azide.

Transfer of sample to BPW

25 g of sample is transferred to 450–900 ml of BPW, depending on the sample type

Enrichment

The nonselective enrichment is performed as follows:
Incubate BPW at 34–38°C for 16–20 hr (minimum 16 hr).

Detection of β-D-glucuroni-dase-positive *E. coli*

Detection of β-D-glucuronidase-positive *E. coli* is performed as follows:

- Streak the enriched sample onto the surface of a TBX agar plate using a sterile 10-µl inoculation loop. If using two BPW bottles, streak on one agar plate from each bottle
- Incubate the plate at 34–38°C for 18–24 hours
- Examine the plate for growth of typical *E. coli* colonies:

Organism	Growth on Chromocult® TBX agar
E. coli	Blue-green or dark-blue-to-violet colonies (Salmon-GAL and X-glucuronide reaction)
Coliforms (not *E. coli*)	Salmon-to-red colonies (Salmon-GAL reaction but no X-glucuronide reaction)
Other Gram-negatives	Colorless colonies, except for some organisms which possess β-D-glucuronidase activity. These colonies appear light-blue to turquoise

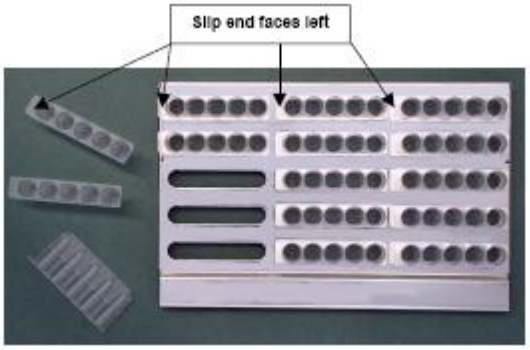
Detection of *E. Coli 0157*

ImmunoMagnetic Separation (IMS) is performed either as manual IMS (mIMS) or automated IMS (aIMS):

Manual IMS (mIMS):

Step	Action
1	Place one Eppendorf tube per sample in the rack without the magnet inserted. Gently vortex the Dynabeads® anti-O157 and add 20 µl of Dynabeads® anti-O157 to each tube. Use a lid opener to open the lids of the Eppendorf tubes
2	Gently add 1 ml of the pre-enriched sample to the Eppendorf tube. Use a new pipette/tip for each sample. Close the lid. *NOTE:* If the sample is divided between two BPW bottles, take 500 µl from each bottle
3	Incubate the tubes for approx. 10 min at room temperature. Gently rotate the rack without the magnet on an MX-3 mixer (Dynal) or by hand
4	Insert the magnet into the rack. Tilt the rack frequently for approx. 3 minutes to ensure complete collection of beads. With correct capture, a distinct circular-to-oval brownish pellet is formed at the tube site halfway between the top and bottom of the tube
5	Open the tubes gently using the lid opener. Place a Pasteur pipette at the water surface opposite the pellet. Gently pipette up the supernatant and liquid in the cap of the tube. Slow down pipetting when the surface of the liquid passes the pellet in order to make sure that no beads leave the tube through the pipette. If beads leave the sample, return the supernatant to the tube and repeat step 4. Use a new pipette/tip for each sample
6	Carefully add 1 ml of washing buffer to each sample. Do not touch the tube with the pipette/ tip as this can cross-contaminate the samples as well as the buffer. Close the lids and remove the magnet from the rack. Wash the bead complex by rotating the rack three times. Repeat steps 4 to 6 twice, but the last time only resuspending the pellet in 100 µl of washing buffer

Automatic ImmunoMagnetic Separation (aIMS):

Step	Action
1	Load one sample tube for each sample into a sample rack.  *NOTE:* Each sample tube consists of five tubes called tubes 1 to 5 (tube 1 is to the left (= slip end) and tube 5 is to the right)
2	Gently vortex the Dynabeads® anti-O157 until the pellet in the bottom of the tube disappears, then aseptically add 10 µl of properly mixed Dynabeads® anti-O157 into sample tubes 1 and 2
3	Aseptically add 500 µl of washing buffer to sample tubes 1 and 2. Aseptically add 1000 µl of washing buffer to sample tubes 3 and 4. Aseptically add 100 µl of washing buffer to sample tube 5
4	Add 500 µl of the enriched test sample to sample tubes 1 and 2; be careful not to contaminate other tubes.

	If the sample is divided between two BPW bottles, take 500 µl from each bottle
5	Repeat step 4 for the remaining samples
6	Aseptically insert the sterile protective sample tip combs into the instrument
7	Insert the rack of filled tubes into the instrument and lock it in place
8	Check that everything is properly aligned. Close the instrument door
9	Select the EPEC/VTEC program sequence by scrolling with the arrow key, then press the Start button

Streaking onto selective indicative agar plates:
Each IMS product (from mIMS or aIMS) is tested for the presence of *E. coli O157* using selective indicative agar plates:

Step	Action
1	Gently vortex the pellet (IMS product)
2	Streak 50 µl of IMS product onto the surface of a CT-SMAC agar plate, then streak another 50 µl of IMS product onto the surface of a CHROMagar O157 plate (or a CT-CHROMagar O157 plate) as follows: Spread the bead-bacteria complex over one half of the plate with a sterile cotton swab. This ensures the break-up of the bead-bacteria complexes. Dilute further by streaking with a loop
3	Incubate the plates at 34–38°C for 18–24 hours

Reading:

Agar	Description
CT-SMAC agar	On CT-SMAC agar, typical *E. coli O157* colonies are transparent and almost colorless with a pale yellowish-brown appearance and a diameter of approx. 1 mm. Sorbitol-positive organisms form bright-red (pink) colonies. In some cases, suspect colonies are so few that they can only be recognized in the bacterial lawn in the primary streaking zone. In this case, subculture suspect colony material onto a new CT-SMAC agar plate. If the growth is too weak after 18–24 hr, the plates can be reincubated for up to 24 hr. In this case, representative sorbitol-negative colonies (transparent) should be verified using the E. coli O157 Latex Test Kit from Oxoid (see below)
CHROMagar O157 and CT-CHROMagar O157	A typical *E. coli O157* will grow as a pink-mauve colony, whereas most other microorganisms are either inhibited or grow as blue or colorless colonies

Verification of *E. coli O157*:
Suspect colonies on CT-SMAC agar and CHROMagar O157 (or CT-CHROMagar O157) are verified as *E. coli O157* using the *E. coli O157* Latex Test Kit from Oxoid. The verification is performed according to the manufacturer's description.

Interpretation of results

E. coli detected (DET) in 25 g

- Presence of typical colonies on TBX agar
- Presence of O157 latex-positive colonies from CT-SMAC agar and CHROMagar O157 (or CT-CHROMagar O157), i.e., suspect *E. coli O157*

E. coli not detected (ND) in 25 g

- Absence of typical colonies on TBX agar

- Absence of O157 latex-positive colonies from CT-SMAC agar and CHROMagar O157 (or CT-CHROMagar O157), i.e., suspect *E. coli O157*

Sensitivity and specificity

Sensitivity: 100%
Specificity: 100%

References

ISO 16649-2, 1st ed. (2001) Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of presumptive *Escherichia coli* – Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.
ISO 16654, 1st ed. (2001): Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Escherichia coli O157.*
The detection of *E. coli O157* is in accordance with ISO 16654 with the following exception:

- Enrichment is performed using buffered peptone water at 34–38°C for 16–20 hours. ISO 16654 uses a modified TSB + novobiocin at 41.5°C ± 1°C for 18–24 hours
- ImmunoMagnetic Separation is only performed after 16–20 hours
  ISO 16654 states after 6 hr and again, if necessary, after 12–18 hours (i.e., a total elapsed time of 18–24 hours)
- Verification is performed using the *E. coli O157* latex test. ISO 16654 states the indole test and serological test

Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers.

Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation.
Avoid inhalation of dust aerosols and contact with skin and eyes.

Handling of chemicals and disposal of waste must be performed according to valid procedures.

Validity

Valid from November 2011.

Novozymes A/S
Krogshøjvej 36
2880 Bagsværd
Danmark

www.novozymes.com
info@novozymes.com

Novozymes A/S · Luna No. 2011-27232-02

DANISH TECHNOLOGICAL INSTITUTE

Method UA-255 Determination of elements in solid and liquid enzyme samples and samples of pasteurized yeast cell solutions by ICP-MS. Microwave-induced sample preparation.

The DTI method UA-255 is accredited* applicable for solid and liquid enzyme samples, for samples of polysaccharides and for samples of pasteurized yeast cell solutions for the ICP-MS determination of elements, incl.:

As, Cd, Hg, Pb.

The method determines the total content of the specified elements in the stated matrices. The results are used for control of production.

Sample preparation
0.5 g ± 0.1 g sample – accurately weighed – is by means of microwave induced heating prepared with 20 ml 7 M nitric acid. The resulting solution is diluted to 50 ml with Milli-Q water. Duplicate preparations are carried out. Blanks are made correspondingly.

Quantitative analysis by ICP-MS
Samples and blanks are analyzed for content of selected elements by ICP-MS with CCT in KED mode and with Helium as collision gas. Germanium, Rhodium and Rhenium is used as internal standards. Quantification by ICP-MS is carried out with traceable external standards of the elements. The calibrations are verified with independent traceable control samples.

Table 1. LOQ of elements

Element	**LOQ Mg/kg**
As	0.3
Cd	0.05
Hg	0.05
Pb	0.5

*DANAK accreditation number 90
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LAZI
2021-10-05

Determination of mycotoxins by LC-MS/MS

Principle

The sample is precipitated, and mycotoxins are extracted with formic acid and acetonitrile. The extract is centrifuged, and the supernatant is analyzed on LC-MS/MS.

Equipment

	Equipment
LC system	Ultra-performance liquid chromatography (UPLC) E.g. Waters Acquity UPLC
MS	E.g. Xevo TQ MS,
Vials	Glass vials
Software	MassLynx
Analytical column	Material: HSS T3, length: 50mm, size:1.8µm, ID: 2.1 mm
Needle type	Peak
Loop	20 µL

Chemicals and Gasses

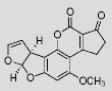
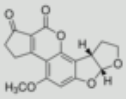
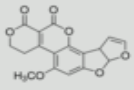
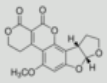
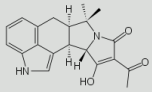
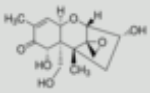
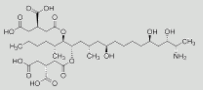
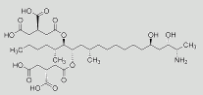
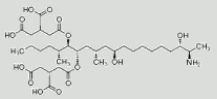
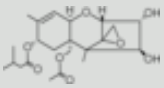
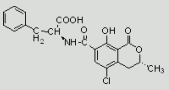
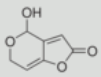
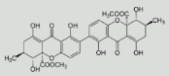
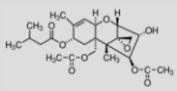
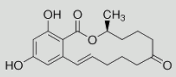
Chemicals used for eluents and mobile phases:

Chemical	Abbreviation	Brand /art. number	Purity
Acetonitrile	ACN	E.g Merck / 1.00029	-
Formic Acid	HCOOH	E.g Sigma / 33015	-
Methanol	MeOH	E.g Sigma / 34741	-
Argon	Ar	-	>99.999%
Nitrogen	N_2	-	>99%

NOTE: Other chemicals of same or superior quality can be used.

Chemicals and gasses

Chemicals used for stock or for preparing stock solution：

Chemical	Abbreviation	Structure	Vendor/ Part.no.	Conc. [mg/L]
Aflatoxin B1	AFB1		Sigma/33415	20
Aflatoxin B2	AFB2			
Aflatoxin G1	AFG1			
Aflatoxin G2	AFG2			
Cyclopiazonic acid	CPA		Sigma/C1530-10MG	Solid
Deoxynivalenol	DON		Sigma/34124	100
Fumonisin B1	FB1		Sigma/34139	50
Fumonisin B2	FB2		Sigma/34142	50
Fumonisin B3	FB3		Sigma/32606	50
HT-2	HT-2 toxin		Sigma/34136	100
Ochratoxin A	OTA		Sigma/34037	10
Patulin	PAT		Sigma/34127	100
Secalonic acid D	SAD		LGC Standards/B-MYC8110-1.2	52.5
Sterigmatocystin	STE		Sigma/32986	50
T-2 toxin	T2		Sigma/34071	100
Zearalenone	ZEA		Supelco/46916 U	50

IMPORTANT: Read the Safety Data Sheets (MSDS) for the chemicals

LC - Settings

<table>
<tr><th>Subject</th><th>Settings</th></tr>
<tr><td>Run time</td><td>16.9 min</td></tr>
<tr><td>Eluent A</td><td>0.15% formic acid</td></tr>
<tr><td>Eluent B</td><td>Acetonitrile with 0.15% formic acid</td></tr>
<tr><td>Gradient start</td><td>Relative to injection: 0 µL</td></tr>
<tr><td>Gradient table</td><td>
<table>
<tr><th>Time [min]</th><th>Flow</th><th>A-eluent [%]</th><th>B-eluent [%]</th><th>Curve</th></tr>
<tr><td>Initial</td><td>0.250</td><td>100</td><td>0</td><td>Initial</td></tr>
<tr><td>2.00</td><td>0.250</td><td>100</td><td>0</td><td>6</td></tr>
<tr><td>2.25</td><td>0.250</td><td>75</td><td>25</td><td>6</td></tr>
<tr><td>5.20</td><td>0.250</td><td>72</td><td>28</td><td>6</td></tr>
<tr><td>9.00</td><td>0.250</td><td>40</td><td>60</td><td>6</td></tr>
<tr><td>9.50</td><td>0.250</td><td>0</td><td>100</td><td>6</td></tr>
<tr><td>13.00</td><td>0.250</td><td>0</td><td>100</td><td>6</td></tr>
<tr><td>13.50</td><td>0.250</td><td>100</td><td>0</td><td>6</td></tr>
<tr><td>17.00</td><td>0.250</td><td>100</td><td>0</td><td>6</td></tr>
<tr><td>18.00</td><td>0.250</td><td>50</td><td>50</td><td>6</td></tr>
</table>
</td></tr>
<tr><td>Needle weak wash</td><td>10% acetonitrile with 0.15% formic acid</td></tr>
<tr><td>Needle Strong Wash</td><td>Acetonitrile with 0.15% formic acid</td></tr>
<tr><td>Needle Weak wash volume</td><td>600 µL</td></tr>
<tr><td>Needle strong wash volume</td><td>200 µL</td></tr>
<tr><td>Sample temperature</td><td>5°C</td></tr>
<tr><td>Injection mode</td><td>Full loop</td></tr>
<tr><td>Column temperature</td><td>35 °C</td></tr>
<tr><td>Injection volume</td><td>20 µL</td></tr>
</table>

MS settings

Detector Tune Parameters:

	Ion mode	
	ES-	**ES+**
Capillary (kV)	3.50	3.50
Extractor (V)	1.20	3.00
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	500	500
Cone Gas Flow (L/Hr)	30	30
Desolvation Gas Flow (L/Hr)	500	500
Collison Gas Flow (mL/Min)	0.13	0.13

MS method (MRM):

Component	#	Ion transition (m/z)		Dwell Time (secs)	Cone Voltage (V)	Collision Energy (eV)
		Parent	Daughter			
Aflatoxin B1	1	313.2	241.2	0.1	40	38
	2	313.2	285.1			22
Aflatoxin B2	1	315.10	259.10	0.1	42	29
	2	315.10	287.20			29
Aflatoxin G1	1	329.07	199.96	0.1	42	41
	2	329.07	243.08			25
Aflatoxin G2	1	331.00	245.00	0.15	45	30
	2	331.00	257.00			30
CPA	1	337.30	182.20	0.2	28	22
	2	337.30	196.20			22
DON	1	297.20	231.20	0.1	21	5
	2	297.20	249.20			13
Fumonisin B1	1	722.40	334.20	0.2	40	40
Fumonisin B2+B3	1	706.40	318.30	0.2	55	40
HT2 toxin	1	425.20	245.20	0.1	20	12
	2	425.20	263.20			12
Ochratoxin A	1	404.30	239.10	0.1	22	23
	2	404.30	358.20			13
Patulin	1	154.90	81.10	0.2	20	12
	2	154.90	99.10			10
Secalonic Acid D	1	639.30	183.20	0.2	35	35
	2	639.30	561.20			35
Sterigmatocystin	1	325.20	281.10	0.1	40	35
	2	325.20	310.10			25
T-2 toxin	1	489.30	245.20	0.1	38	24
	2	489.30	327.20			24
Zearalenon	1	317.20	130.90	0.1	40	32
	2	317.20	174.98			24

#: 1: primary ion transition used for quantification/calculation, 2: Secondary ion transition only used for identification

MS Instrument Configuration:

Parameter	Value
MS Inter-scan delay (secs)	0.005
Polarity/Mode switch Inter-scan delay (secs)	0.020
Enhanced switch Inter-scan delay (secs)	0.020
Inter-channel delay (secs)	0.005

Definition of unit

Reported in mg/kg.

Reagents

Reagent	Application
0.15% formic acid: H_2O: HCOOH (100:0.15)	Eluent A, Seal wash
Acetonitrile with 0.15% formic acid: ACN: HCOOH (100:0.15)	Eluent B, Strong Needle wash
10% acetonitrile with 0.15% formic acid: ACN: H2O: HCOOH (10:90:0.15)	Weak Needle wash

Eluent A, Seal wash: 0.15% formic acid
Example: Preparation of 1L

Step	Action
1	Add 1000 mL ultrapure water to a 1L Blue Cap bottle.
2	Add 1.5 mL formic acid. *IMPORTANT:* Always add acid to water and not vice versa.
3	Mix thoroughly.
4	Storability: 2 months at room temperature.

Eluent B, Strong Needle wash: Acetonitrile with 0.15% formic acid
Example: Preparation of 2.5 L

Step	Action
1	Open a bottle of 2.5 L acetonitrile.
2	Add 3.75 mL Formic acid.
3	Mix thoroughly.
5	Storability: 3 months at room temperature.

Weak Needle wash: 10% acetonitrile with 0.15% formic acid
Example: Preparation of 2 L

Step	Action
1	Transfer 200 ml of acetonitrile to a 2 L Blue Cap bottle.
2	Add 3.0 ml of formic acid.
3	Add 1800 ml ultrapure water
4	Mix thoroughly.
5	Storability: 2 months at room temperature

CPA Stock solution

Cyclopiazonic acid :50ppm

Step	Action
1	Weigh 5 ± 0.5 mg CPA and transfer quantitatively to a 100 mL volumetric flask. (if chemical is available in ampoule with certified amount, weighing is not required)
2	Add methanol to a total of 100 mL and mix.
3	Document the preparation of stock solution.
4	Storability: Freezer 6 months.

Standard stock solution

Prepare standard stock solution as described (the standard is used for calibration):

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Pipette, into a 25 mL volumetric flask, following volumes of stock solutions
<table>
<tr><th>Component</th><th>Vol. µL</th><th>Conc. µg/L</th></tr>
<tr><td>Aflatoxin B1, B2, G1, G2</td><td>50</td><td>40</td></tr>
<tr><td>CPA</td><td>375</td><td>750</td></tr>
<tr><td>DON</td><td>125</td><td>500</td></tr>
<tr><td>Fumonisin B1</td><td>20</td><td>40</td></tr>
<tr><td>Fumonisin B2</td><td>20</td><td>40</td></tr>
<tr><td>Fumonisin B3</td><td>20</td><td>40</td></tr>
<tr><td>HT-2 toxin</td><td>50</td><td>200</td></tr>
<tr><td>Ochratoxin A</td><td>50</td><td>20</td></tr>
<tr><td>Patulin</td><td>125</td><td>500</td></tr>
<tr><td>Secalonic Acid D</td><td>100</td><td>210</td></tr>
<tr><td>Sterigmatocystin</td><td>20</td><td>40</td></tr>
<tr><td>T-2 toxin</td><td>20</td><td>80</td></tr>
<tr><td>Zearalenon</td><td>20</td><td>40</td></tr>
</table>
Note: Limit the time of contact with plastic pipette for Secalonic acid D as chloroform may degrade the plastic.</td></tr>
<tr><td>2</td><td>Add 10 mL ACN by pipette.</td></tr>
<tr><td>3</td><td>Add 2 mL ultrapure water by pipette.</td></tr>
<tr><td>4</td><td>Add 1 mL formic acid by pipette.</td></tr>
<tr><td>5</td><td>Add methanol to a total of 25 mL and mix.</td></tr>
<tr><td>6</td><td>The solution is marked toxic and highly inflammable.</td></tr>
<tr><td>7</td><td>Document the preparation of stock solution.</td></tr>
<tr><td>8</td><td>Calculate concentrations</td></tr>
<tr><td>9</td><td>Storability: Freezer 3 months.</td></tr>
</table>

Spike solution

Prepare spike solution as described:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Pipette, into a 25 mL volumetric flask the following volumes of stock solutions
<table>
<tr><th>Component</th><th>Vol. µL</th><th>Conc. µg/L</th></tr>
<tr><td>Aflatoxin B1, B2, G1, G2</td><td>250</td><td>200</td></tr>
<tr><td>CPA</td><td>3750</td><td>7500</td></tr>
<tr><td>DON</td><td>1700</td><td>6800</td></tr>
<tr><td>Fumonisin B1</td><td>200</td><td>400</td></tr>
<tr><td>Fumonisin B2</td><td>200</td><td>400</td></tr>
<tr><td>Fumonisin B3</td><td>200</td><td>400</td></tr>
<tr><td>HT-2 toxin</td><td>200</td><td>800</td></tr>
<tr><td>Ochratoxin A</td><td>400</td><td>160</td></tr>
<tr><td>Patulin</td><td>1700</td><td>6800</td></tr>
<tr><td>Secalonic Acid D</td><td>800</td><td>1680</td></tr>
<tr><td>Sterigmatocystin</td><td>200</td><td>400</td></tr>
<tr><td>T-2 toxin</td><td>200</td><td>800</td></tr>
<tr><td>Zearalenon</td><td>200</td><td>400</td></tr>
</table>
NOTE:Limit the time of contact with plastic pipette for Secalonic acid D as chloroform may degrade the plastic.</td></tr>
<tr><td>2</td><td>Add methanol filling up the flask approximately half way.</td></tr>
<tr><td>3</td><td>Add 2 mL ultrapure water by pipette.</td></tr>
<tr><td>4</td><td>Add 1 mL formic acid by pipette.</td></tr>
<tr><td>5</td><td>Add methanol to a total of 25 mL and mix.</td></tr>
<tr><td>6</td><td>The solution is marked toxic and highly inflammable.</td></tr>
<tr><td>7</td><td>Document the preparation of stock solution.</td></tr>
<tr><td>8</td><td>Calculate concentrations of spike solution</td></tr>
<tr><td>9</td><td>Storability: Freezer 3 months.</td></tr>
</table>

Control sample stock solution

Prepare control sample stock solution as described:

Step	Action
1	Pipette 2.5 ml spike solution, into a 50 mL volumetric flask.
2	Add 4 mL ultrapure water by pipette
3	Add 2 mL formic acid by pipette
4	Add 20 mL ACN by pipette
	Add methanol to a total of 50 mL and mix.

Component	Conc. µg/L
Aflatoxin B1, B2, G1 G2	10
CPA	375
DON	360
Fumonisin B1	20
Fumonisin B2	20
Fumonisin B3	20
HT-2 toxin	40
Ochratoxin A	8
Patulin	360
Secalonic Acid D	84
Sterigmatocystin	20
T-2 toxin	40
Zearalenon	20

5	The solution is marked toxic and highly inflammable.
6	Document the preparation of stock solution.
7	Calculate concentrations of control sample stock and working solution
8	Storability: Freezer 3 months.

Standard working solution

Each sample sequence includes 2 injections of the standard working solution: One before the first sample and one after the last sample.

The standard working solution is prepared according to the following table:

Step	Action
1	Name one vial “STD”.
2	Transfer 750 µL standard stock solution to the vial.
3	Add 750 µL ultrapure water to the vial.

Control sample working solution

Each sample sequence includes several injections of control sample working solution. One injection before the first sample, between each sample and one injection after the last sample.

The control sample is prepared according to the following table:

Step	Action
1	Name one vial “Control”.
2	Transfer 750 µL control sample stock solution to the vial.
3	Add 750 µL ultrapure water to the vial.

Blank

Blank: 750 µL ultrapure water + 750 µL ACN

Procedure

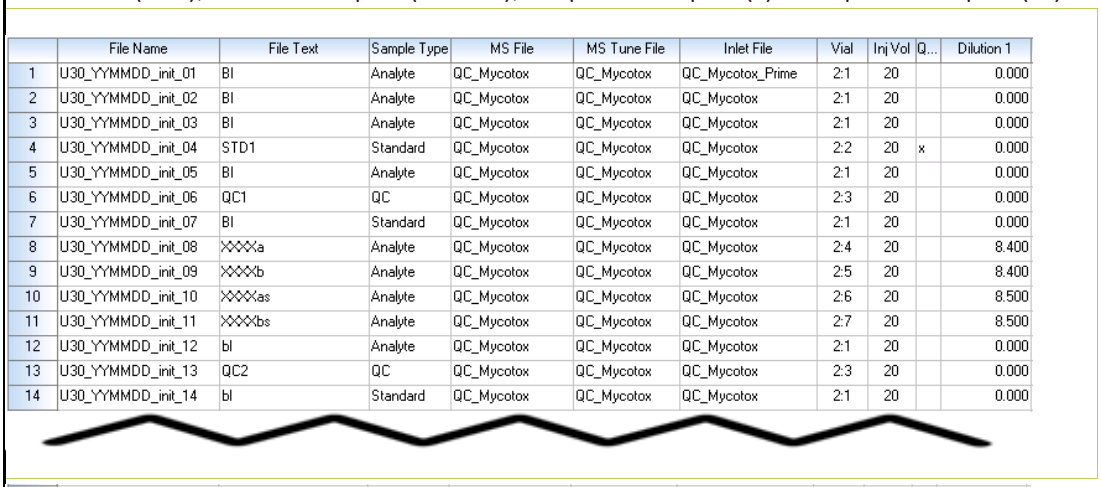
Procedure for preparation of all other samples types except wheat bran:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Document sample preparation</td></tr>
<tr><td>2</td><td>If dilution of sample needed before extraction (e.g. if interference issues, if recovery <25%, if area >STD (see Approval)):
Pipette (if possible) AND weigh sample into adequate measuring flask. Add ultrapure water to the mark
Example: 1:10 dilution liquid sample: Add 1 mL sample to 10 mL volumetric flask. Weigh. Fill to the mark with ultrapure water
Example: 1:10 dilution solid sample: weigh out 1 g sample in 10 mL volumetric flask. Fill to the mark with ultrapure water</td></tr>
<tr><td>3</td><td>Label 2 tubes (e.g. 15mL Nunc) with “a”, “b” “as”, “bs”</td></tr>
<tr><td>4</td><td>If diluted sample: prepare sample as Liquid according to table in step 6
NOTE: Sample amount in 4 tubes is not weighted. Sample weight from dilution (step 2) is used for calculations</td></tr>
<tr><td>5</td><td>If liquid undiluted sample:
Sample must be pipetted (if possible) AND weighed. If possible saturate pipette, if not, draw up once.
Sample weights within ±0.10 g of the amount specified in step 6 is acceptable.
If the sample is solid or too viscous to be pipetted, then weigh the sample within ±0.10 g of the amount specified in step 6
TIP: For ergonomic reasons, it is optional to take the tube out of the scale, while pipetting the sample.</td></tr>
<tr><td>6</td><td>Preparation of samples for extraction (in µL, solids in g as stated):

<table>
<tr><th></th><th colspan="2">Liquid/Viscous</th><th colspan="2">Secalonic Acid</th><th colspan="2">Solid</th></tr>
<tr><th></th><th>a</th><th>as+bs</th><th>a+b</th><th>as+bs</th><th>a+b</th><th>as+bs</th></tr>
<tr><td>Sample</td><td>1000</td><td>1000</td><td>500</td><td>500</td><td>0.25g</td><td>0.25g</td></tr>
<tr><td>Ultrapure water</td><td>-</td><td>-</td><td>500</td><td>500</td><td>1000</td><td>1000</td></tr>
<tr><td>ACN</td><td>3000</td><td>3000</td><td>3000</td><td>3000</td><td>3000</td><td>3000</td></tr>
<tr><td>Formic acid</td><td>200</td><td>200</td><td>200</td><td>200</td><td>200</td><td>200</td></tr>
<tr><td>Spike solution</td><td>-</td><td>50</td><td>-</td><td>50</td><td>-</td><td>50</td></tr>
</table></td></tr>
<tr><td>7</td><td>Vortex one tube for at least 5 sec. If sample is easily homogenized go to step 8. If sample dissolves with difficulty, vortex all 4 tubes until sample is as homogeneous as possible.
IMPORTANT: Ensure contact between solvents and samples</td></tr>
<tr><td>8</td><td>Shake samples for 30 minutes unless sample is not dissolved (Visual evaluation), then shake over night
NOTE: E.g. coated granulates may need to shake over night</td></tr>
<tr><td>9</td><td>Centrifuge (≥ 5 min, ≥ 3500 rpm).</td></tr>
<tr><td>10</td><td>Add 500 µL ultrapure water to each new vial and transfer 500 µL supernatant.</td></tr>
<tr><td>11</td><td>Turn vials up and down 8-10 times to mix supernatant and ultrapure water
TIP: Place all vials in rack and turn rack up and down</td></tr>
<tr><td>12</td><td>Analyze on LC-MS/MS
NOTE: It is very important to turn on the MS min. 30 min. before use. This is done by loading the MS method and let it run.</td></tr>
</table>

Sample list

To analyze on LC-MS/MS set up sample list in MassLynx:

Step	Action
1	Load sample list template
2	Example of sample list showing the order of analysis and setting of Sample Type for Blanks (Bl), Standards (STD), Control samples (Control), unspiked samples (a) and spiked samples (as):  *NOTE:* Blanks after Control samples are defined as Standard in Sample type

	File Name	File Text	Sample Type	MS File	MS Tune File	Inlet File	Vial	Inj Vol	Q...	Dilution 1
1	U30_YYMMDD_init_01	Bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox_Prime	2:1	20		0.000
2	U30_YYMMDD_init_02	Bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
3	U30_YYMMDD_init_03	Bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
4	U30_YYMMDD_init_04	STD1	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:2	20	x	0.000
5	U30_YYMMDD_init_05	Bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
6	U30_YYMMDD_init_06	QC1	QC	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:3	20		0.000
7	U30_YYMMDD_init_07	Bl	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
8	U30_YYMMDD_init_08	XXXXa	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:4	20		8.400
9	U30_YYMMDD_init_09	XXXXb	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:5	20		8.400
10	U30_YYMMDD_init_10	XXXXas	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:6	20		8.500
11	U30_YYMMDD_init_11	XXXXbs	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:7	20		8.500
12	U30_YYMMDD_init_12	bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
13	U30_YYMMDD_init_13	QC2	QC	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:3	20		0.000
14	U30_YYMMDD_init_14	bl	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
33	U30_YYMMDD_init_33	bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
34	U30_YYMMDD_init_34	QC5	QC	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:3	20		0.000
35	U30_YYMMDD_init_35	bl	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
36	U30_YYMMDD_init_36	XXXXa	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:20	20		8.400
37	U30_YYMMDD_init_37	XXXXb	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:21	20		8.400
38	U30_YYMMDD_init_38	XXXXas	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:22	20		8.500
39	U30_YYMMDD_init_39	XXXXbs	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:23	20		8.500
40	U30_YYMMDD_init_40	bl	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
41	U30_YYMMDD_init_41	QC6	QC	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:3	20		0.000
42	U30_YYMMDD_init_42	bl	Analyte	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:1	20		0.000
43	U30_YYMMDD_init_43	STD2	Standard	QC_Mycotox	QC_Mycotox	QC_Mycotox	2:2	20	x	0.000

Calculations MassLynx

The MassLynx software is used for calculation of mycotoxin concentration in the injected sample taking dilution during extraction into account.

Step	Action
1	Load sample list in MassLynx software
2	Enter/Check concentrations of Standards and control samples in sample list
3	Enter/Check values for User Dilution 1: **For...** / **Value...** Unspiked samples (a) / 8.40 Spiked samples (as) / 8.50 *NOTE:* Values in "Dilution 1" correspond to solvent/sample-ratio obtained during extraction multiplied by final extract dilution
4	Process relevant Standards, Blanks, control samples and samples with relevant process method *NOTE:* Due to carry over from the control samples calibrations for CPA and SAD include blanks after control samples. Regression is set to linear, exclude. Regression for all other components are set to linear, force (through 0.0)
5	In TargetLynx: Check identification of peaks and integrations
6	Check if quality criteria for Deviation on concentration of control samples are met
7	If Mycotoxins are detected check that peak area spiked samples < peak area STD
8	Transfer concentrations to spreadsheet for calculations

Calculations

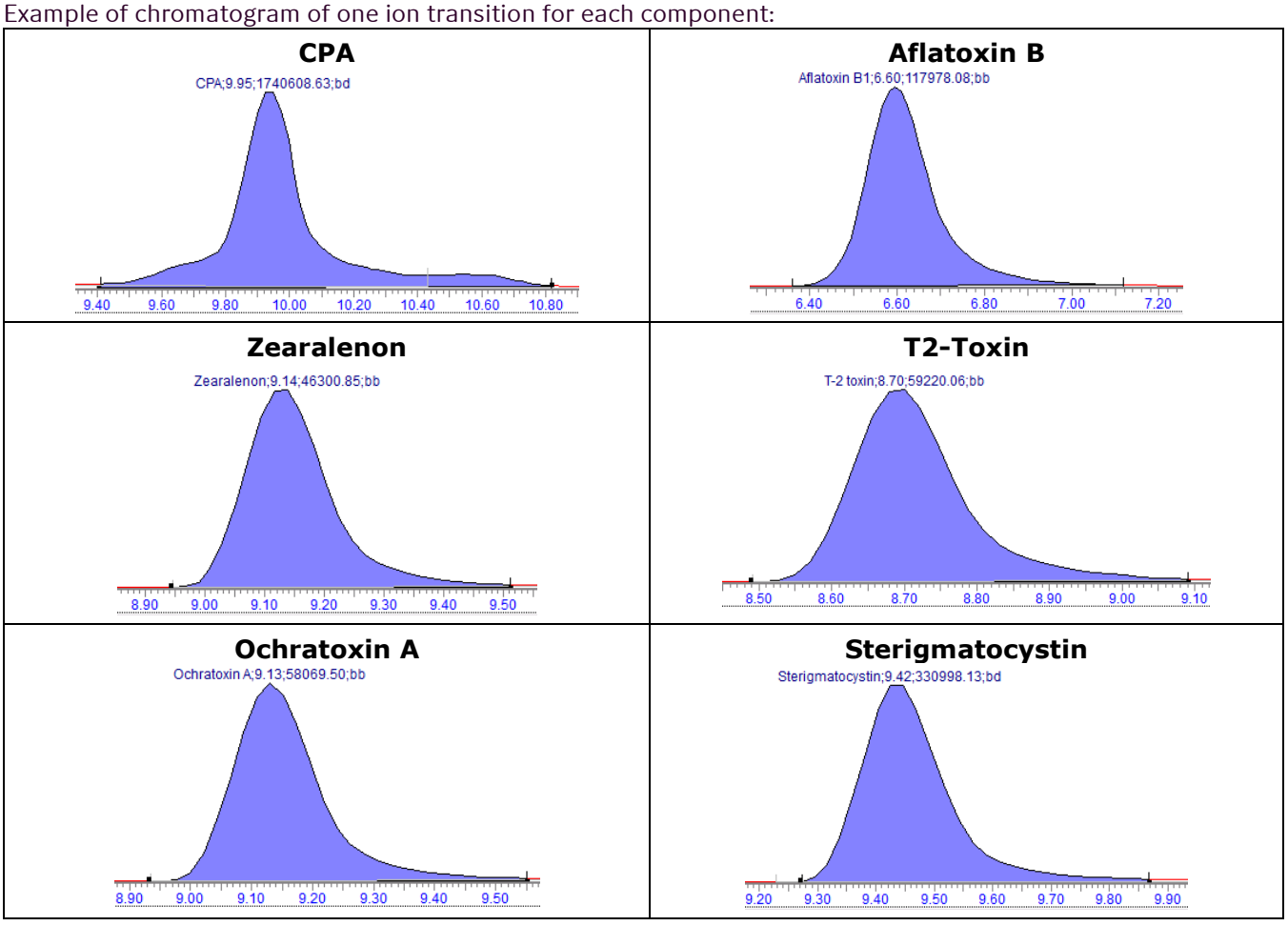
A= Concentrations for **a** transfer from MassLynx for each component in ppb
AS= Concentrations for **as** transfer from MassLynx for each component in ppb
W_a=Weighing of sample **a in g**
W_{as}=Weighing of sample **as in g**
C= The Calculated concentrations of spike solution in as in µg
V= Sample dilution total volume mL

$$Recovery\ of\ each\ compont\ \ \% = \frac{(\mathrm{AS} - \mathrm{A}) * W_{as}}{0.05\mathrm{C} * \ \mathrm{W_a}} * 100\%$$

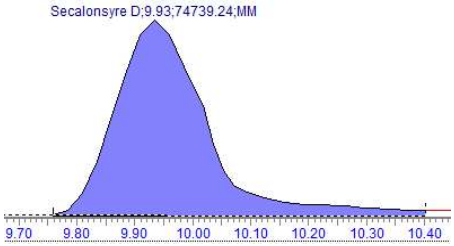
$$Concentration\ for\ \text{each Component mg/kg} = \frac{\mathrm{A}*\mathrm{V}}{W_a * \text{Recovery \%} * 1000}$$

Chromatograms

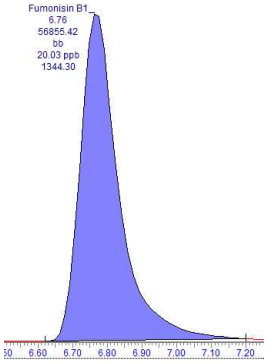
Example of chromatogram of one ion transition for each component:



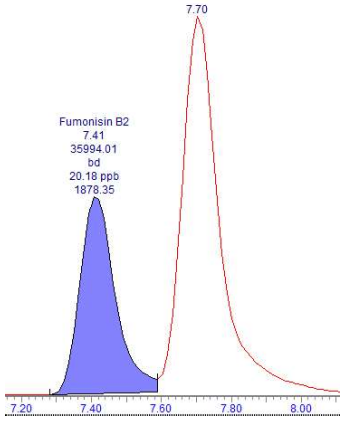
Secalonsyre D



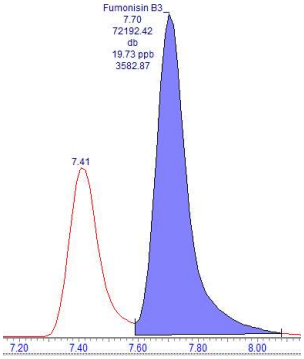
Fumonisin B1



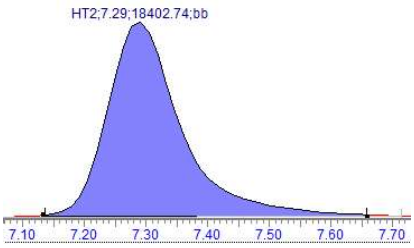
Fumonisin B2



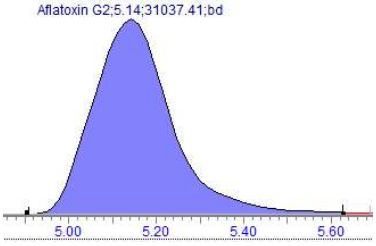
Fumonisin B3



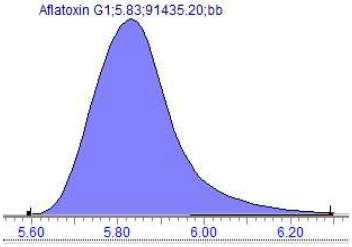
HT2



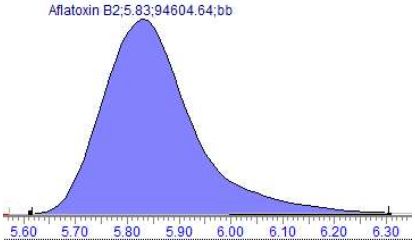
Aflatoxin G2



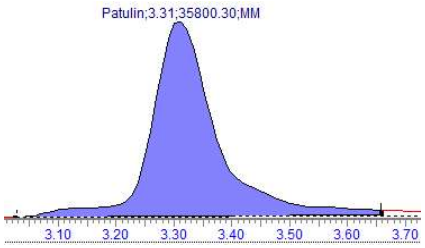
Aflatoxin G1



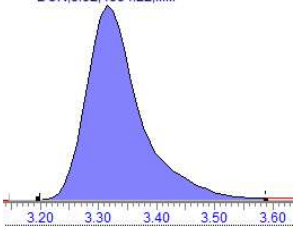
Aflatoxin B2



Patulin



Deoxynivalenol



Approval

Parameter	Accept criteria
Control sample	
Deviation on Concentration	± 25%
Spiked samples	
Peak area	< peak area of STD *NOTE:* If peak area of spiked sample > peak area of STD then dilute sample and re-analyze *NOTE:* E.g. calculate appropriate dilution as follows: $Dilution = \frac{Peak\ Area_{Spiked\ sample}}{Peak\ Area_{STD}} * 2$
Recovery	≥ 25% *NOTE:* If Recovery <25% Re-analyze using 1:10 dilution prior to extraction
RT	Within ± 0.03 min of Standards
CV%	≤ 25%
Samples	
RT	Within ± 0.03 min of Standards

Statement of analysis results

Results must be reported as follows:

Component	Reporting limit	> Reporting Limit	< Reporting Limit
Secalonic acid	1 mg/kg	Detected	Not detected
All other mycotoxins	LOD	2 significant digits	<LOD

Limit of determination

Mycotoxin	LOD (ppm)
Aflatoxin B1	0.0003
Aflatoxin B2	0.0008
Aflatoxin G1	0.003
Aflatoxin G2	0.0008
Cyclopiazonic acid	0.003
Deoxynivalenol	0.057
Fumonisin B1	0.0003
Fumonisin B2	0.0003
Fumonisin B3	0.0003
HT-2	0.006
Ochratoxin A	0.0003
Patulin	0.113
Secalonic acid D	0.003
Sterigmatocystin	0.0003
T-2 toxin	0.0006
Zearalenone	0.0006

Limit of Quantification

Mycotoxin	LOQ (ppm)
Aflatoxin B1	0.001
Aflatoxin B2	0.0025
Aflatoxin G1	0.0085
Aflatoxin G2	0.0025
Cyclopiazonic acid	0.01
Deoxynivalenol	0.190
Fumonisin B1	0.001
Fumonisin B2	0.001
Fumonisin B3	0.001
HT-2	0.02
Ochratoxin A	0.001
Patulin	0.375
Secalonic acid D	0.01
Sterigmatocystin	0.001
T-2 toxin	0.002
Zearalenone	0.002

Validity

Valid from June. 2019.

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
Denmark

Luna no. 2020-00647-01

novozymes®

October 7, 2022

FDA Questions for GRN 1030

1. On pg. 20, you state: "All these [GRAS] notifications have provided sufficient toxicological testing data showing evidence that there is no toxicological concern regarding the safety and consumption of cellulase enzymes." FDA notes that "cellulases" are classified according to their functional/enzymatic activities and not necessarily by their primary amino acid sequences. Thus, "cellulase" from one organism may have sufficiently differing primary amino acid sequences and/or secondary/tertiary structures compared to a cellulase from another organism. Furthermore, cellulases from different sources may have differences in thermal and/or proteolytic properties that could impact their safety.
    a. Please provide a short narrative comparing your cellulase to other cellulases that are already safely used in the food industry. Discuss any differences that would impact safety of your enzyme for its intended use.

Novozymes response:
The main concern when assessing the safety of a microbially derived enzyme used in food is the safety of the production organism. This concept has been widely accepted since at least 1983 with the publication of "Determining the Safety of Enzymes Used in Food Processing" by Pariza and Foster and later by Pariza and Johnson, (2001) and Olempska-Beer et al. (2006). Pariza and Foster (1983) says that "there has never been a health problem traced to the use of an enzyme per se in food processing" and "enzymes per se are inherently non-toxic". Olempska- Beer et al. (2006) noted that enzymes present in the human diet have not been associated with toxicity and are considered intrinsically safe.

These concepts still hold true today and Novozymes has no reason to believe that a cellulase enzyme is toxic. In section 6 (e) of our GRAS notice we provide reference to published information about the safety of enzymes in general and cellulases in general. We believe that that this public information that concludes the safety of other microbially derived cellulases supports the safety of this cellulase because there is no evidence that any cellulase used in food has ever been toxic. As you note, there is a range of natural variation among enzymes of a certain class. However, when a protein exhibits the function/enzymatic activity of a certain class of enzymes, we have never found reason to suggest that slight changes in primary amino acid sequences or secondary/tertiary structure would cause the enzyme to be toxic. Novozymes conducted a sequence homology assessment of this cellulase (subject of the GRN) to known toxins and allergens. This also confirms that this cellulase is not toxic.

In section 2.2 (a), we state, "The donor for the cellulase is the wild type *Trichoderma reesei* (ATCC 56765)." Cellulase from *T. longibrachiatum* is affirmed as GRAS by FDA in 21CFR184.1250. *T.longibrachiatum* was renamed *T. reesei*. The enzyme protein itself

Novozymes North America, Inc.
Regulatory Affairs
77 Perry's Chapel Church Road
PO Box 576
Franklinton, NC 27525
United States

Phone: 919-494-3000
Fax: 919-494-3420

www.novozymes.com

expressed from the wild type *T. reesei* gene would not be expected to "become toxic" simply because that gene is expressed by a safe *A.niger* strain. The cellulase that is the subject of this notice is essentially the same protein that would be found in a cellulase enzyme preparation produced by *T. reesei* which would be covered by the regulation 21 CFR 184.1250.

2. On pg. 22, you state: "Novozymes has determined that the results of the toxicology studies on amyloglucosidase enzyme concentrate, batch PPY35872 from *Aspergillus niger*, can be bridged to support the toxicological outcome of the cellulase enzyme from *Aspergillus niger*, the subject of this notification." FDA notes that while the two enzymes are produced by the same *A. niger* parental strain, the amyloglucosidase belongs to a different class of enzymes (3.2.1.3) compared to the cellulase (3.2.1.4). Therefore, the rationale for using the test article from the tox study of amyloglucosidase to support the safety of the article of commerce, i. e. cellulase, is not justified. Further, the use of the NOAEL obtained for the amyloglucosidase to report a 'margin of safety' for the cellulase enzyme preparation, the subject of GRAS Notice No. 0001030, is not an accurate measure of safety of the cellulase enzyme preparation.

   a. Please confirm that the provided toxicological data is to support the safety of the production strain and not the safety of the article of commerce (i.e. cellulase).

   Novozymes response:

   The results of the toxicology studies on amylogluosidase enzyme concentrate, batch PPY35872 support the safety of the *A. niger* production organism that is used to produce the enzyme concentrate that is the subject of this notice. The enzyme concentrate or enzyme solids is total organic solids (TOS) coming from the *A. niger* fermentation. The TOS is composed of enzyme protein and other organic material carried over from the fermentation. As noted above, it is the safety of the production strain that is the main consideration for assessing the safety of an enzyme preparation. Because the enzyme protein itself is known to be inherently safe (Pariza and Foster, 1983; Olempska Beer et al. 2006) it is essential to ensure that no substances of concern from the production strain (the TOS) could be expressed during fermentation. So the study on *A. niger* supports the part of the article of commerce that is carried over from the *A. niger* fermentation.

   b. Please further provide a short narrative on the publicly available data and information you consider pivotal to the safety of your cellulase enzyme.

   Novozymes response:

   Novozymes is supplying a list of publicly available data and information we consider pivotal to the safety of this cellulase enzyme and enzymes used in food processing in general:

   1) Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46:5:453-468, 1983. (Which lists cellulase from *T. reesei* as an enzyme used in food processing)
   2) Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001. (Which lists cellulase from T. reesei as an enzyme used in food processing)
   3) Olempska-Beer, Z.S. , Merker, R.I, Ditto, Mary D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganisms—a review. Reg. Tox and Pharm 45:144-158, 2006. (Which states that enzymes have not been associated with toxicity)

4) Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology, Vol. 12, No. 5. October 2016.
5) Hjortkjaer et al., Safety Evaluation of Cellulclast, an acid cellulase derived from *Trichoderma reesei*. Summaries of Toxicological Data. Fd Chem. Toxic. Vol. 24:55-63. 1986.

3. Please state whether *A. niger* strain C3085-1870-2 has been deposited in a recognized culture collection and provide the deposit number.

Novozymes response:
Novozymes confirms that the A.niger strain C3085-1870 has been deposited in a recognized culture collection.

4. For the administrative record, please confirm that the production strain is non-toxigenic and non-pathogenic.

Novozymes response:
Novozymes confirms that the production strain is non-toxigenic and non-pathogenic.

5. Please provide the number of amino acids in the primary sequence of the cellulase.

Novozymes response:
The primary sequence of the protein is 327 amino acids.

6. Please provide the methods used for establishing the specifications provided in Table 2. Additionally, please provide a statement that all methods were validated for their intended purposes.

Novozymes response:
Attached please find Novozymes methods for analysis of lead and microbiological specifications. The method used to determine enzyme activity is a method that has been developed and validated by Novozymes for the intended use.

7. Please consider reducing the specifications for lead to reflect the results from the batch analyses presented in the notice.

Novozymes response:
Novozymes enzyme preparations intended for use in food meet the requirements for food grade enzyme preparations as set by FCC and JECFA which includes a limit of 5 ppm for lead. The lead specification limit of 5 ppm considers the very low levels of enzyme preparations used in food processing. In most cases, enzymes are used at less than 0.5% in final foods and are often used in the manufacture of ingredients that are then used in final foods (meaning that any contribution of lead from an enzyme preparation to a final food would be further reduced). Novozymes is aware of FDA's Closer to Zero program with the current focus on contaminants such as lead. We acknowledge this request and will consider bringing this to the attention of the Enzyme Technical Association where the interests of the enzyme industry can be represented.

novozymes
Rethink Tomorrow

Analytical methods

Detection of *Salmonella* spp.

Principle
Detection of *Salmonella* spp. is carried out as a qualitative test.
The test is based on a non-selective enrichment of 25 g of sample in 450 ml of buffered peptone water for 18-24 hours followed by *Salmonella* specific PCR. Optionally, a secondary enrichment in the selective RVs broth may be added after enrichment in BPW.
The methods are in-house methods evaluated and validated at Novozymes.

Definition of unit
The result is stated as:
DET (*Salmonella* detected in 25 g)
ND (*Salmonella* not detected in 25 g)

Standards
A positive reference strain can be included in the test, e.g., *Salmonella adabraka*, *Salmonella havana*, or *Salmonella senftenberg*.

Detection limit
Theoretical detection limit: 1 *Salmonella* sp. in 25 g.

Equipment

General equipment	
Balance	-
Incubator for BPW and agar plates	(34–38°C)
Incubator or water bath for RVs	(40.0–42.0°C)
Vortex mixer	
Automatic pipettes and sterile tips	10-100 µl, 100-1000 µl, and 1 ml

PCR specific equipment and materials	
AB 7500 Fast Real-Time PCR System	-
Microcentrifuge	E.g. Ole Dich microcentrifuge
Heating block	E.g. Stuart Block Heater SBH200D
Automatic pipette dedicated to PCR	10-100 µl
Automatic pipette dedicated to PCR	100-1000 µl
Pipette tips dedicated to PCR, DNA and DNase free	100 µl and 1000 µl
Sterile pasteur pipettes	-
Powder free gloves (PCR)	-
FastReactionTubes 0,1ml 8/strip	-
Tube Cap Strips 8 Caps/Strip	-
Tubes RNase-Free 1.5mL	-

Mesia and reagents

Enrichment broths

Buffered Peptone Water (BPW) (450 ml)

Optional: Rappaport Vassiliadis soya peptone broth (RVs broth) (Oxoid CM0866)

PCR specific reagents

MicroSEQ® *Salmonella* spp. Detection Kit (Life Technologies Cat. No. 4403930).

PrepSEQ™ Rapid Spin Sample Preparation Kit (Life Technologies Cat. No. 4407760)

EP buffer, 90 ml buffered sodium chloride-peptone solution pH 7.0.

Nuclease-Free Water (e.g. Sigma Cat. No. 101210442)

Nonselective enrichment

The nonselective enrichment is performed in the following way:

Step	Action
1	Transfer 25 g or 25 ml of sample to 450-ml BPW preheated to 40-42°C
2	Incubate BPW at 34-38°C for 18-24 hours

Optional: Selective enrichment in RVs broth

The selective enrichment in RVs is performed in the following way:

- Transfer 500 µl or 0.5 ml from BPW to 10-ml RVs tubes equilibrated to minimum room temperature
- Incubate the RVs broth at 40.0–42.0°C for 22-26 hours

Note: If a water bath is used to incubate the RVs, there is no need to equilibrate the temperature of the broth.

DNA purification

Cautions: When performing DNA purification there is a cross contamination risk, therefore, the following precautions should be taken:

- Set up only the materials and reagents needed for the particular work process in the LAF bench
- Always use a new pipette tip for each sample at each step
- Always handle one sample at a time keeping the remaining samples physically separated
- When opening reagent bottles put lids/caps upside down behind the bottle

- Always keep open bottles/reagents separate from the waste bin and not in the path where the pipette is transferred
- Always close (eppendorf) tubes when they are not handled
- Avoid passing your hands and pipettes above open bottles/tubes

IMPORTANT: Work in LAF bench when performing DNA purification and use gloves when performing lysis procedure.
IMPORTANT: Mix any reagents before use.

1. Mix BPW or RVs broth
CAUTION: Be careful that the broth does not touch the lid when mixing.

2. Transfer 2 ml BPW broth (or optionally RVs broth) to a 2 ml eppendorf tube and microcentrifuge (hereafter named centrifuge) for 3 minutes at max speed, e.g. 15.000g
TIP: Store the BPW or RVs broth at a cool temperature (2-8°C) until PCR has been successfully completed. The RVs broth may be stored for max. 2 days at a cool temperature (Ref.**Error! Reference source not found.**).

3. Discard the supernatant without touching the pellet, e.g. using a sterile pasteur pipette

4. Add 650 µl EP buffer and re-suspend the pellet thoroughly

5. Insert a spin column into a labeled tube

6. Load 650 µl of sample onto the spin column and cap the column

7. Load the tube into the centrifuge. Make sure the lid points toward the center of the centrifuge. Then centrifuge for 3 minutes at max speed, e.g. 15.000g

8. Remove the tube from the centrifuge, and then discard the used spin column
CAUTION: Make sure that any liquid on the outside of the spin column is scraped off on the edge of the eppendorf tube.

9. Aspirate, and then discard the supernatant

10. Add 50 µl of Lysis Buffer to the pellet. Re-suspend by pipetting up and down, or vortex until the pellet is re-suspended

11. Cap the tube, and then incubate at 95±3°C for 10 minutes

12. Allow the sample to cool for 2 minutes at room temp, then centrifuge for 1 minute at max. speed, e.g. 15.000g

13. Add 250 µl of Nuclease-Free water, then centrifuge for 1 minute at max. speed, e.g. 15.000g

14. Proceed with PCR, or store the tube at ÷18°C. *Remark:* Avoid loading the black pellet when transferring to the lyophilized qPCR strip sample

TIP: Material may be stored at cool (2-8°C) for max. 2 hours after completion of step 3, 8 or 13.

PCR preparation
IMPORTANT: Use gloves or wash your hands thoroughly after the PCR preparation. The negative control contains 0 – 0.01 % Na-azide.
IMPORTANT: Use a Pathogen Detection Negative Control for each PCR run.

1. Open the storage pouch containing the assay beads (MicroSEQ® *Salmonella* spp. Detection Kit)
IMPORTANT: Do not remove the desiccant from the storage pouch.

2. Remove the appropriate number of individual tubes or 8-tube strips

3: NOTE: Frozen samples and/or controls only: thaw these completely, vortex, then briefly spin them down using a microcentrifuge

4. Examine the assay beads in the 8-tube strips. Gently tap the tubes as needed to settle all assay beads to the bottom of each tube

5. Gently remove, and then discard the concave caps. Avoid disturbing the beads from the bottom of the tubes

6. For each sample or control, transfer 30 µl into a tube containing the appropriate assay beads. Beads dissolve in 1 to 5 seconds.
IMPORTANT: Dispense all unknown samples first followed by the negative control

7. Add additional tubes as needed so that each strip contains a full set of 8 tubes

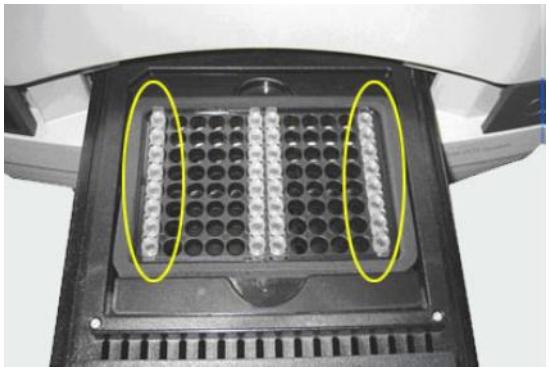
8. Cap the tubes, sealing each tube with the flat (transparent) optical strip caps provided in the kit. Cap the tubes firmly with the strip cap tool to avoid collapsing, bending, or misaligning the tubes.
IMPORTANT: Avoid scratching the optical strip caps as this will interfere with the reading of fluorescence

9. Confirm that the strips are straight and that each tube is in line with the adjacent tube

10. Make sure reagents are thoroughly mixed and at the bottom of the tubes

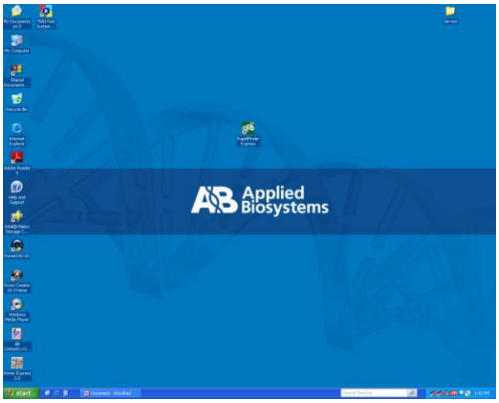
11. Carefully insert two or more 8-tube strips containing samples, starting from the center of the plate holder and moving out. This layout minimizes bending or misaligning the tube strips

12. If column 1 (leftmost) and column 12 (rightmost) of the Plate Holder are not used, insert two fully capped, empty, 8-tube strips into these columns (see below photo)

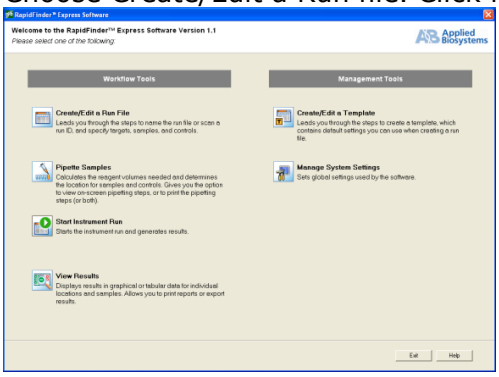


Run PCR reactions

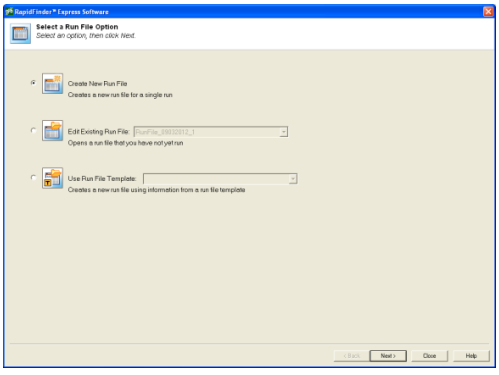
1. Turn on the PCR system first. Then turn on computer and open "RapidFinder Express"



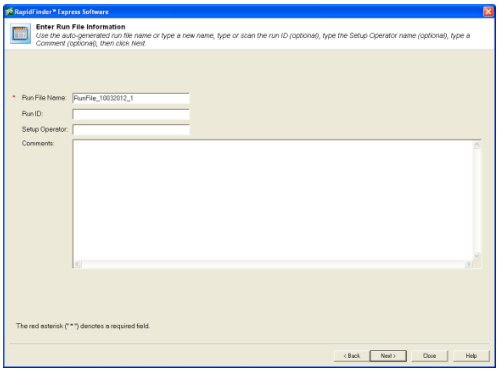
2. Choose Create/Edit a Run file. Click next



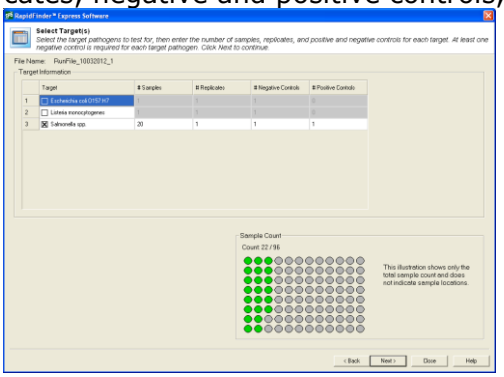
3. Choose Create New Run File. Click next



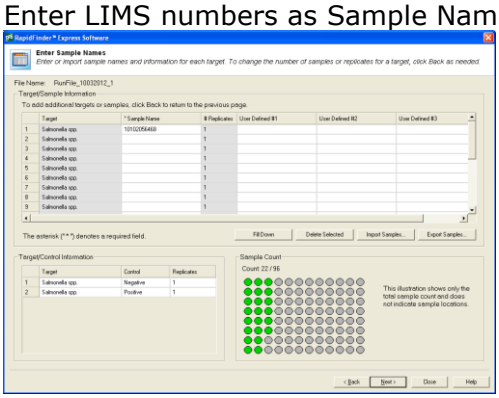
4. Enter Run File Information. Use default Run File Name and enter initials for Setup Operator. Click next



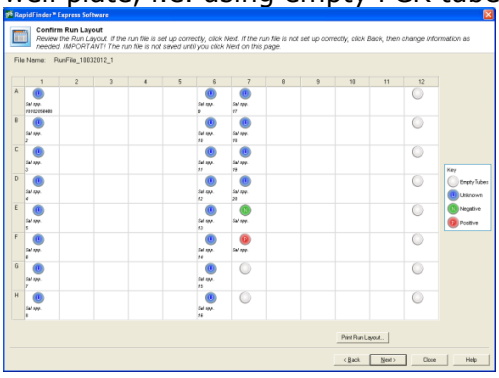
5. Select Targets: Choose *Salmonella* spp. Enter number of samples. Enter '1' for replicates, negative and positive controls, respectively. Click next



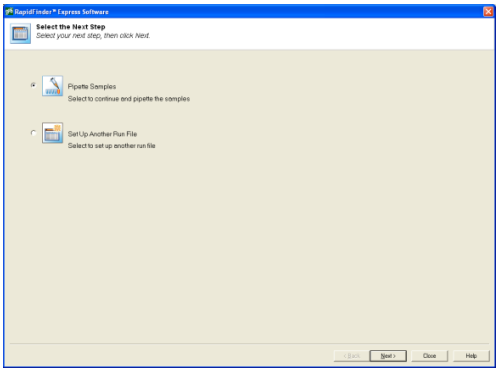
6. Enter LIMS numbers as Sample Names. Click next



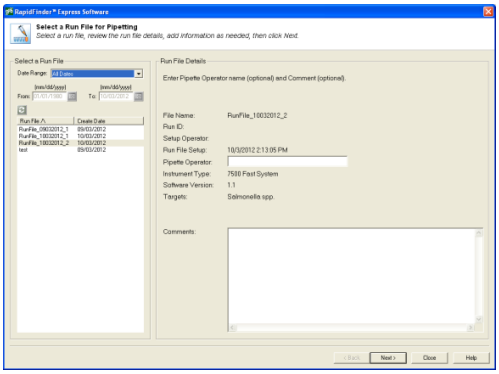
7. Confirm Run Layout: Make a layout of the program securing equilibrium on the 96-well plate, i.e. using empty PCR tubes. Click next



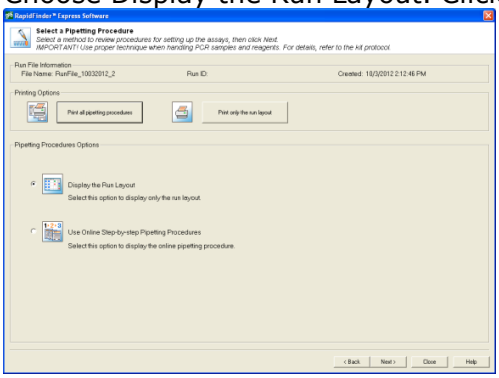
8. Choose Pipette Samples. Click next



9. Choose the above created Run File. Click next

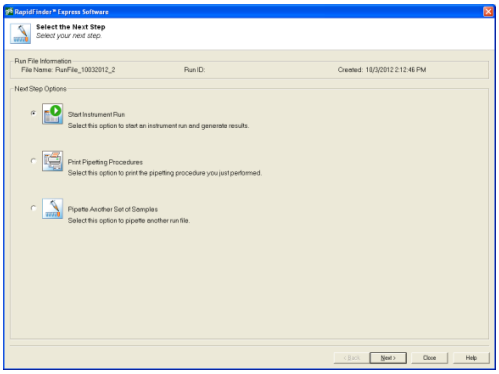


10. Choose Display the Run Layout. Click next



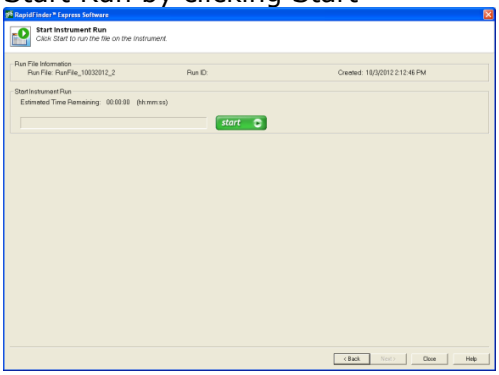
11. Confirm Run Layout. Click next

12. Choose Start Instrument Run. Click next

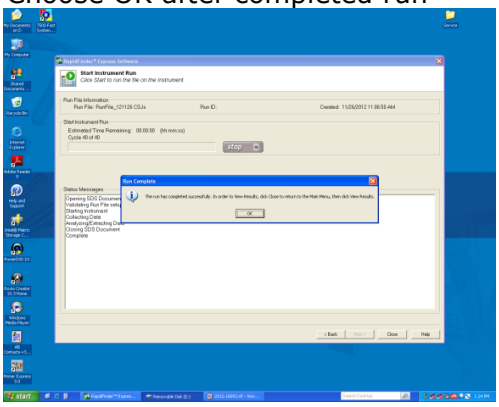


13. Load PCR tubes

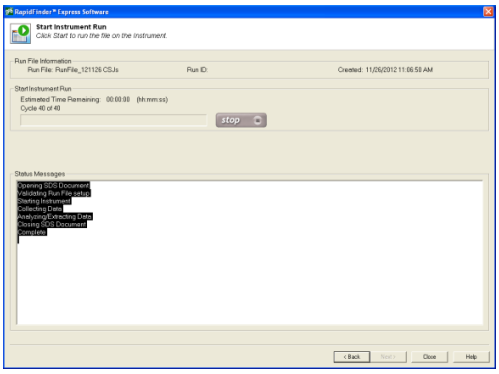
14. Start Run by clicking Start



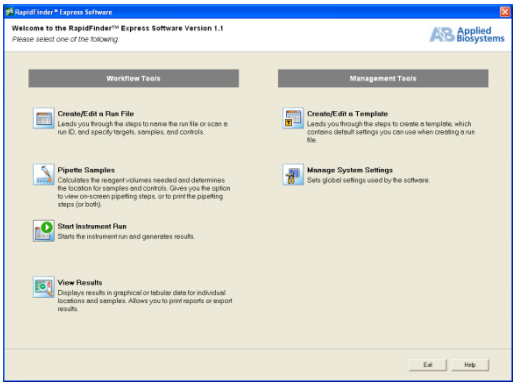
15. Choose OK after completed run



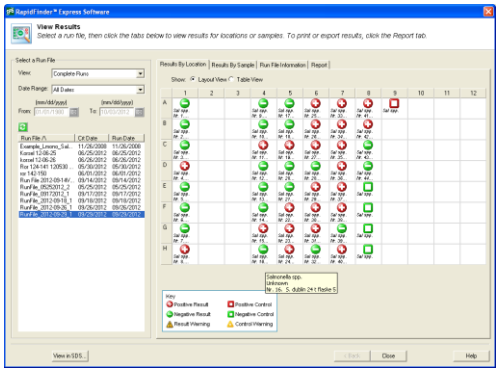
16. Choose Close in the Start Instrument Run window



17. Choose View Results. Click next



18. Choose relevant Run File and the sheet “Results By Location”



19. Interpretation of results

If…	**Then report result as…**
Negative control is marked as "+"	Re-perform PCR procedure with all "+" samples and negative controls
Sample is marked as "-"	*Salmonella* spp. not detected (ND)
Sample is marked as "+"	*Salmonella* spp. detected (DET)

TIP: The threshold value for a *Salmonella* positive sample is 35.69 cycles.
Note: If a positive PCR result is obtained, cultivation may be performed from the BPW or RVs broth if verification of the Salmonella type is desired. See flow chart or (Ref. **Error! eference source not found.**).

20. Click Close then Exit to close the RapidFinder Express software

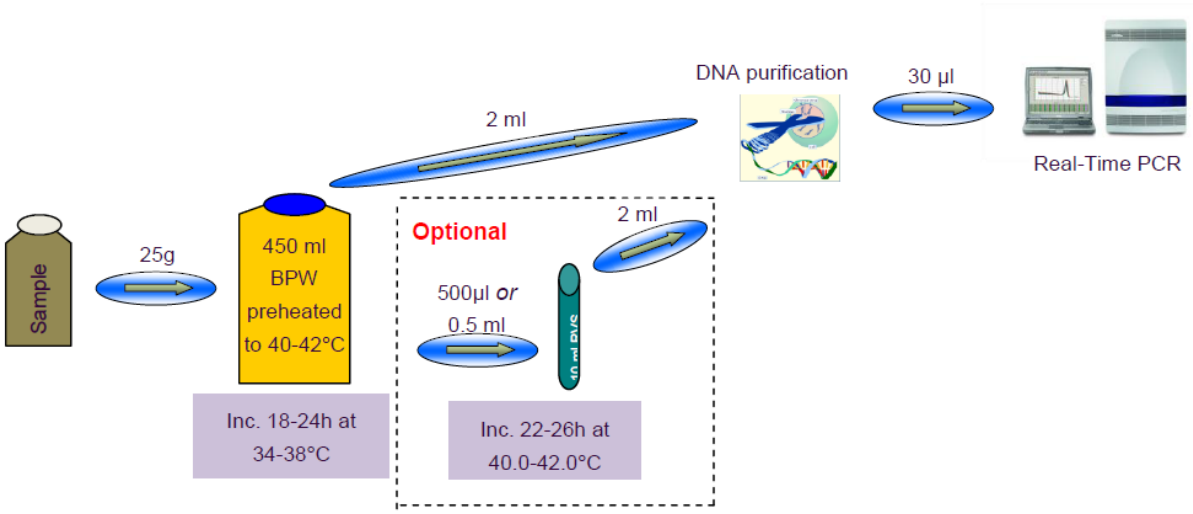
21. Choose Shutdown to turn computer off, then turn off PCR system

IMPORTANT: AFTER PCR run:

- **NEVER EVER** open tubes
- Throw tubes in the trash in the PCR room. Do not re-use tubes
- Before leaving the room:
  - Remove and throw gloves in the trash
  - Wash hands

Accuracy, sensitivity and specificity
Accuracy: 100% Sensitivity: 100% Specificity: 100%

Flow chart:


DNA purification
30 µl
Real-Time PCR
2 ml
Sample
25g
450 ml
BPW
preheated
to 40-42°C
Optional
2 ml
500µl or
0.5 ml
Inc. 18-24h at
34-38°C
Inc. 22-26h at
40.0-42.0°C

Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers. Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation.

Avoid inhalation of dust aerosols and contact with skin and eyes.

Handling of chemicals and disposal of waste must be performed according to valid procedures.

Validity

Valid from March 2013.

Novozymes A/S
Krogshøjvej 36
2880 Bagsværd
Danmark

www.novozymes.com
info@novozymes.com

Novozymes is the world leader in bioinnovation. Together with customers across a broad array of industries we create tomorrow's industrial biosolutions, improving our customers' business, and the use of our planet's resources. Read more at www.novozymes.com.

Novozymes A/S · Luna No. 2011-28477-04

Enumeration of coliform bacteria using violet red bile agar

This method is used for the analysis of glucose isomerase and liquid samples in general (except for liquid xylanase).

Principle

Coliform bacteria (coliforms) are broadly defined as Gram-negative, oxidase-negative, non-sporogonial rods, which grow under aerobic or facultative anaerobic conditions. More specifically, coliforms are capable of fermenting lactose (due to the production of β-galactosidase) in the presence of bile salts at 37°C.

Coliforms are not a taxonomically defined group of bacteria, and consequently there is no common agreement on which microorganisms truly belong to the coliforms. However, Novozymes defines coliforms as microorganisms belonging to the genera *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Hafnia*.

The presence of coliforms, especially *E. coli*, can be used as an indicator of the bacteriological hygiene of an enzyme product.

Violet Red Bile Agar (VRBA) is a selective and indicative agar:

Principles	Description
Selective principle	Crystal violet and bile salts inhibit growth of the Gram-positive accompanying microflora which then favors growth of the fast-growing Gram-negative *Enterobacteria*
Indicative principle	Degradation of lactose to acid is indicated by the pH indicator neutral red, which changes its color to red and precipitation of bile acids. Coliform bacteria are commonly defined by their ability to ferment lactose rapidly

Routine testing is performed in the following way:

Sample type	Technique	Volume spread	sample dilution	No. of plates	Plate size	Detection limit
Enzyme sample	Pour plate with cover layer	2.5 ml	10^{-1}	1	14 cm	4 CFU/g or ml

NOTE: Depending on the sample type, the level of contamination, and the detection limit needed for the specific sample, alternative procedures may be used

The method outlined below conforms to **ISO 4832:2006** with the following deviations:

- ISO 4832 describes the use of peptone salt solution or buffered peptone water as diluent. The Novozymes method uses Tween buffer 4%
- ISO 4832 describes the use of duplicates. The Novozymes method uses single test
- ISO 4832 describes that the agar plates are incubated at 30°C or 37°C for 24 ± 2 hours whereas the Novozymes method incubates agar plates at 34-38°C for 22-26 hours

Definition of unit

The result is stated as:
Coliform CFU/g or ml

Detection limit

The detection limit of this method is dependent on the sample volume and the dilution in use.

Equipment

Balance	± 0.1 g e.g. Sartorius, Mettler
Magnetic stirrer.	-
Sterile petri dishes	14 cm
Suitable sterile pipette for transfer.	2.5 ml or 10 ml
Incubator	34-38°C

Media and reagents

Tween buffer 4% (buffered peptone chloride solution pH 7.0, containing 4% tween 80)

Violet Red Bile Agar (VRBA), Merck 1.01406 – prepared in accordance with the recommendation of the supplier.

NOTE:

- Preparation of VRB agar should be executed in a flow bench to avid inhalation of the powder
- Ensure that media is thoroughly dissolved before melting procedure by regular shaking of the media. In addition, stir the agar immediately before cooling in water bath and again before pouring into petri dishes

IMPORTANT: Do not autoclave or overheat the VRB media

Always read the Safety Data Sheet (SDS) for all the chemicals

Procedure

Enzyme samples and other liquid samples are prepared using same procedure.

Sample preparation

Step	Action
1	Transfer 10 g of solid sample or 10 ml of liquid sample into 90 ml of Tween buffer 4%
2	Immediately homogenize the sample by stirring or shaking. NOTE: Solid samples are homogenized on a magnetic stirrer for approx. 20 minutes. TIP: Further 10-fold dilutions of any sample type can be prepared with Tween buffer 4%

IMPORTANT: All enzyme products must be analyzed from a 10^{-1} dilution due to possible growth inhibition of microorganisms in undiluted enzyme preparation

Plating

Plating is performed using pour plating technique and must be done within 15 minutes from end of homogenization. If this is not possible, the sample can be stored at 2-8°C for up to 4 hours.

Step	Action
1	Transfer 2.5 ml from the 10^{-1} dilution into an empty Petri dish (14 cm)
2	Pour approx. 40–45 ml of VRBL (47 ± 2°C) into the Petri dish (= bottom layer) and mix carefully. Leave this to solidify for max 10 minutes
3	Pour approx. 10 ml of VRBA (47 ± 2°C) onto the bottom layer (= covering layer). Leave this to solidify

Incubation

Incubate the plates at 34–38°C for 22-26 hours under aerobic conditions

Reading

Count the number of typical colonies:

Count colonies on plates with	Typical colonies
1–375 colonies per plate	Purplish red colonies with a diameter of ≥ 0.5 mm. Sometimes surrounded by a reddish zone of precipitated bile acid

NOTE: In case of doubt, the colonies should be examined in microscope as e.g. *Enterococci* might grow on VRB. Coliform bacteria will appear as small rods

Calculation

General principles:

The calculation is based on the number of colonies (C_x) on the plate and the sample volume analyzed (V_x).
The result is stated with two significant figures (e.g., 2.2 x 10^1).

When using results from	Then the result is	Where
1 dilution	$\frac{C_x}{V_x}$	C_x = no. of colonies V_x = volume analyzed
2 or more dilutions	$\frac{C_1 + C_2}{V_1 + V_2}$	C_1 = no. of colonies in lowest dilution C_2 = no. of colonies in next dilution V_1 = volume analyzed in lowest dilution V_2 = volume analyzed in next dilution

IMPORTANT: When using more than one dilution, the numbers from each dilution are compared (consider the likelihood of product inhibitions, contamination of the sample, analytical errors, etc.). In general, the highest dilution is used

When the sample volume is 2.5 ml, then V_x and C_x are:

Dilution	10^{-1}	10^{-2}
V_x	0.25 ml	0.025 ml
C_x	No. of colonies on the plate	No. of colonies on the plate

Examples of calculating with pour plate of 2.5 ml sample on a 14 cm agar plate:

C_x	V_x (g or ml)	Dilution	Result
0	0.25	10^{-1}	$\frac{0}{0.25}$ < 4 CFU/g or ml
1	0.25	10^{-1}	$\frac{1}{0.25}$ = 4 CFU/g or ml
3	0.25	10^{-1}	$\frac{3}{0.25}$ = 12 CFU/g or ml
412	0.25	10^{-1}	$\frac{>375}{0.25}$ = > 1.5 x 10^3 CFU/g or ml
53 8	0.25 0.025	10^{-1} 10^{-2}	$\frac{53+8}{0.25+0.025}$ = 2.2 x 10^2 CFU/g or ml

Accuracy and precision

CV% = 29%

References

ISO 4832:2006: *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coliforms – colony count technique.*

Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers.
Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation.
Avoid inhalation of dust aerosols and contact with skin and eyes.
Handling of chemicals and disposal of waste must be performed according to valid procedures.

Validity

Valid from December 2018.

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
Denmark

Jan 11, 2019 · Luna No. 2011-18492-05

Enumeration of total viable count

The method is used to determine total viable count in analysis of samples from Novozymes.

Principle

Total viable count (TVC) is defined as the number of microorganisms which form colonies on a rich non-selective agar medium (Tryptic Soy Agar, TSA) after aerobic incubation for 3 days at 30–35°C.

The method outlined below conforms to the principles of ISO 4833-2:2013, with the following exceptions:

- For NZ method, the solid media used for enumeration is TSA, whereas the ISO method uses the PCA media (Plate Count Agar)
- The diluent used in NZ is Tween buffer 4%, whereas the ISO recommend Buffered Peptone Water (BPW)
- Growth promotion test of TSA is performed according to in-house procedures and not as described in ISO 4833-2:2013

Samples are analyzed using either spiral plater (100 µl) or spread plate technique (100 µl or 1 ml) from suitable dilutions:

Technique	Dilution of sample	Volume spread	No. of agar plates	Plate size	Detection limit
Spiral plating or spread plating	10^{-1}	100 µl	1 plate	9 cm	100 CFU/g or ml
Spread plating	10^{-1}	1 ml	4 plates	14 cm	10 CFU/g or ml

Definition of unit

The result is stated as:

- Total Viable Count CFU/g or ml

Sample type
All sample types.

Detection limit
The detection limit of this method is dependent on the sample volume and the dilution in use (see “Principle”).

Equipment

Incubator	30 - 35°C
Balance	± 0.1 g e.g. Sartorius, Mettler
Magnetic stirrer	-
Spiral plater (for the spiral plate technique)	E.g. Eddy Jet 2
Stereomicroscope or microscope	E.g. Zeiss
Sterile petri dishes	9 cm or 14 cm
Sterile spatula (for the spread plate technique)	-
Sterile pipette for transfer	100µl or 1 ml (4X0.25 ml)

Media and reagents

Tween buffer 4% (Buffered peptone chloride solution pH 7.0, containing 4% tween 80)
Tryptone Soya Agar plates (TSA) - e.g. Oxoid CM0131 or Difco 236920, prepared in accordance with the supplier’s recommendation

Always read the Safety Data Sheet (SDS) for all the chemicals

Procedure
Enzyme samples and other solid samples are analyzed using the same procedure.

Preparation

Step	Action
1	Transfer 10 g of solid sample or 10 ml of liquid sample to 90 ml of Tween buffer 4%
2	Immediately homogenize the sample by stirring or shaking. NOTE: Solid sample are homogenized on a magnetic stirrer for approx. 20 minutes, however, glucose isomerase enzymes product is stirred for minimum 1 hour. TIP: Further 10-fold dilution can be prepared with tween buffer 4%

NOTE: All enzyme products must be analyzed from a 10^{-1} dilution due to possible growth inhibition of microorganisms in undiluted enzyme preparation.

Plating
Plating must be performed within 15 minutes from the end of homogenization. If this is not possible, the sample can be stored at 2-8°C for up to 4 hours.

Detection limit	Action
100 CFU/g or ml	Transfer 100 µl from the 10^{-1} dilution onto the surface of a TSA plate (9 cm). Repeat this for any of the necessary dilutions. Perform a spiral plating of 100 µl from the 10^{-1} dilution in accordance with the directions for the specific spiral plater.
10 CFU/g or ml	Transfer 1 ml from the 10-1 dilution to the surface of 4 TSA plates (14 cm) with approx. 0.25 ml onto each plate. Repeat this for any of the necessary dilutions.

Leave the plates on the table with their lids on until the sample has been fully absorbed by the agar.

Incubation

Incubate the plates at 30–35°C for 3 days.

Reading

Spread plate technique:
Count the number of colonies on the plates.

Size of agar plate	Count colonies on plates with
9 cm	1–300 colonies per plate
14 cm	1–750 colonies per plate

Spiral plate technique:
The number of colonies on each plate is counted and the result is calculated in accordance with the directions for the specific spiral plater.

IMPORTANT: Small colonies, e.g. *lactobacillus*, may erroneously be misread as product crystallizations. If in doubt, use stereomicroscope for macroscopic observation and/or prepare a slide culture of a colony for light microscopy.

Calculation

General principles:
The calculation is based on the number of colonies (C_x) on the plate and the sample volume analyzed (V_x).
The result is stated with two significant figures (e.g. 2.2×10^1).

When using results from	Then the result is	Where
1 dilution	$\frac{C_x}{V_x}$	C_x = no. of colonies V_x = volume analyzed
2 or more dilutions	$\frac{C_1 + C_2}{V_1 + V_2}$	C_1 = no. of colonies in lowest dilution C_2 = no. of colonies in next dilution V_1 = volume analyzed in lowest dilution V_2 = volume analyzed in next dilution

IMPORTANT: When using more than one dilution, the numbers of colonies from each dilution are compared (the likelihood of product inhibitions, contamination of the sample, analytical errors, etc. is considered). In general, the highest dilution is used. If the result is stated based on other dilutions, the reason must be given in the raw data

When the sample volume is 0.1 ml, then V_x and C_x are:

Dilution	**10^{-1}**	**10^{-2}**
V_x	0.01 ml	0.001 ml
C_x	No. of colonies on the plate	No. of colonies on the plate

Examples of calculating with spread plate of 0.1 ml sample:

C_x	**V_x (g or ml)**	**Dilution**	**Result**
0	0.01	10^{-1}	$\frac{<1}{0.01}$ = < 100 CFU/g or ml
334	0.01	10^{-1}	$\frac{>300}{0.01}$ = > 3.0 x 10^4 CFU/g or ml
253 24	0.01 0.001	10^{-1} 10^{-2}	$\frac{253+24}{0.01+0.001}$ = 2.5 x 10^4 CFU/g or ml

When the sample volume is 1 ml (4 x 14-cm agar plates with 0.25 ml on each plate), **then V_x and C_x are:**

Dilution	**10^{-1}**	**10^{-2}**
V_x	0.1 ml	0.01 ml
C_x	Sum of colonies on the 4 plates	Sum of colonies on the 4 plates

Examples of calculating with spread plate of 1 ml sample:

C_x	**V_x (g or ml)**	**Dilution**	**Result**
0	0.1	10^{-1}	$\frac{<1}{0.01}$ = < 100 CFU/g or ml
426	0.1	10^{-1}	$\frac{426}{0.1}$ = 4.3 x 10^3 CFU/g or ml
3134	0.1	10^{-1}	$\frac{>3000}{0.1}$ = > 3.0 x 10^4 CFU/g or ml
853 84	0.1 0.01	10^{-1} 10^{-2}	$\frac{853+84}{0.1+0.01}$= 8.5 x 10^3 CFU/g or ml

Accuracy and precision

CV% (surface plating) = 25%
CV% (spiral plating) = 29%

References

ISO 4833-2:2013: Microbiology of the food chain – Horizontal method for enumeration of microorganisms – Part 2: Colony count at 30°C by the surface plating technique.

Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers.
Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation.
Avoid inhalation of dust aerosols and contact with skin and eyes.
Handling of chemicals and disposal of waste must be performed according to valid procedures.

Validity

Valid from December 2018.

About Novozymes
Novozymes is the world leader in biological solutions. Together with customers, partners and the global community, we improve industrial performance while preserving the planet's resources and helping build better lives. As the world's largest provider of enzyme and microbial technologies, our bioinnovation enables higher agricultural yields, low-temperature washing, energy-efficient production, renewable fuel and many other benefits that we rely on today and in the future. We call it Rethink Tomorrow.

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
Denmark

Dec 21, 2018 · Luna No. 2011-18477-05

CODEX ALIMENTARIUS COMMISSION E

Food and Agriculture Organization of the United Nations

World Health Organization

Viale delle Terme di Caracalla, 00153 Rome, Italy - Tel: (+39) 06 57051 - Fax: (+39) 06 5705 4593 - E-mail: codex@fao.org - www.codexalimentarius.net

FA/44/ INF/03
February 2012

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON FOOD ADDITIVES

Forty Fourth Session

Hangzhou, China, 12 – 16 March 2012

INVENTORY OF SUBSTANCES USED AS PROCESSING AIDS (IPA), UPDATED LIST (INFORMATION DOCUMENT)

Prepared by New Zealand

BACKGROUND

1. The Codex Committee on Food Additives and Contaminants (CCFAC) at its 36th Session recognized that the development of a positive list of processing aids was not a realistic approach at the present time due to a lack of resources. However, the value of the Inventory of Processing Aids (IPA) itself, as a useful reference tool, has been recognized and the Committee agreed to maintain the IPA for the time being and decided that New Zealand would prepare updated versions of the IPA for consideration at subsequent sessions of the Committee[1].

2. The IPA includes:

- Substances in the original list in CAC/MISC 3
- Additions to the IPA agreed to by CCFA up to and including the 43rd session in March 2011.

The title of the Inventory has been changed to the *Inventory of Substances used as Processing Aids* as discussed at the 40th Session. This is to recognise that substances used as processing aids may also have other uses including as food additives and food[2]. For convenience and simplicity the acronym *IPA* continues to be used.

The 43rd Session agreed to develop a prototype of a new processing aid database with the aim of replacing IPA. The criteria for the entry of substances and the management of the database would be considered at a subsequent step[3].

CHANGES INTRODUCED IN THIS UPDATE

3. The enzyme Transglutaminase (*Streptomyces mobaraense*) has been updated to Transglutaminase (*Streptomyces mobaraensis*).

UPDATING ISSUES FOR FUTURE CONSIDERATION[4]

4. New Zealand is prepared to provide further annual updates to the IPA.

[1] ALINORM 07/30/12 paragraph 134.
[2] ALINORM 07/30/12 paragraph 133
[3] REP11/FA paragraph 172
[4] ALINORM 06/29/12 paragraph 95 and Appendix XV.

INVENTORY OF SUBSTANCES USED AS PROCESSING AIDS (IPA)

Prepared by New Zealand (February 2012)

BACKGROUND

1. The title of the Inventory has been changed to the *Inventory of Substances used as Processing Aids* to recognise that substances used as processing aids may also have other uses including as food additives and food[5]. For convenience and simplicity the acronym *IPA* continues to be used.

2. The IPA was originally a collection of information submitted by national authorities[6] to provide a list of those substances whose sole function is that of a processing aid.

3. At its 21st session in 1989, Codex Committee on Food Additives and Contaminants (CCFAC) agreed that the IPA be submitted to the CAC for adoption as a Codex advisory text. It was first published as a Codex advisory text in 1991 and included amendments agreed to at the CCFAC meetings in 1990 and 1991.

4. On initiation of the list, CCFAC's primary purposes for the IPA were to:

   a) develop information on substances used as processing aids; and

   b) determine priorities for the review of processing aids by JECFA.

5. CCFAC agreed that the IPA was not intended to be a positive list of permitted processing aids to be used, for example, by reference in Codex Commodity Standards. Further, CCFAC has not conducted its own risk assessment of the substances on the inventory.

6. CCFAC at its 36th Session recognized that the development of a positive list of processing aids was not a realistic approach at the present time due to a lack of resources. However, the value of the IPA itself, as a useful reference tool, has been recognized and agreed to maintain the IPA for the time being and decided that New Zealand would prepare updated versions of the IPA for consideration at sessions of the Committee.

INTRODUCTION

7. The Inventory is not intended to be complete or a "positive list" of permitted aids.

8. The Updated IPA includes:

   - Substances in the original list in CAC/MISC 3
   - Additions to the IPA agreed to by the Committee up to and including the 42nd session in March 2010.

9. Substances that may also function as food additives or foods are designated by an asterisk (*).

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[5] ALINORM 07/30/12 paragraph 133
[6] ALINORM 89/12A, Appendix VIII.

10. The Inventory is arranged in tabular format for presentation of information that will be necessary for the Committee to select substances for JECFA evaluation. The following information is provided:

- Category - the functional effect classification.
- Processing Aid - the chemical name or description of the substance used as a processing aid.
- Area of Use - the foods or food processing procedures in which the processing aid is utilised.
- Level of Residues - the level of processing aid remaining in food after processing. The levels should be designated with respect to those:

  (1) directly measured by analysis or

  (2) estimated by other means. Values are in mg/kg and values at the detection limit of available analytical procedures are reported as "less than" (<).
- Interaction with Food - describes the degree of chemical interaction with food components. Provides data on levels of interaction products in food.
- JECFA Evaluation – "Yes" indicates that the substance has been reviewed or considered by a JECFA. Note that JECFA consideration of a substance does not necessarily mean that JECFA has reviewed the processing aid use(s) of the substance, nor that JECFA assigned an ADI to the substance. Summary information is available on http://jecfa.ilsi.org/search.cfm
- JECFA specification – "Yes" indicates that there is a relevant monograph covering the identity and purity of the substance.
- ADI-the latest JECFA ADI in mg/kg body weight or other end point of their safety assessment. Abbreviations used in this column are :

  NS for ADI "not specified"

  NL for ADI "not limited"

  DP for decision postponed

  PTWI for provisional tolerable weekly intake

  MTDI for maximum tolerable daily intake
- JECFA comments includes any relevant comments in respect to the ADI or in some cases the specification.
- References - this includes the references from which the original 1989 list was developed (ALINORM 98/12A Appendix VIII) plus a notation when new substances have been added.

11. **Appendix A catalogues substances that are used as processing aids but not included in the main inventory as they have functions also as food additives or foods.**

(Note that substances already covered in the main IPA were formerly listed and annotated as (1.). These have been have been deleted to avoid repetition.)[7]

[7] CX/FAC 06/38/13.

12. The substances are annotated according to the following system:

    2. indicates those materials that are both food additives and processing aids (i.e. the substance functions as a processing aid in one food but may have a different function in another food).

    3. indicates those compounds that because of carry-over residues, would seem to usually be considered only as food additives.

    4. indicates those materials that might actually have simultaneous function as processing aids and functionality in the finished food.

(Appendix B of the earlier versions of the IPA has been deleted to avoid unnecessary duplication as it reproduces the Microbial Enzyme Preparation Section of the main Inventory.)[8]

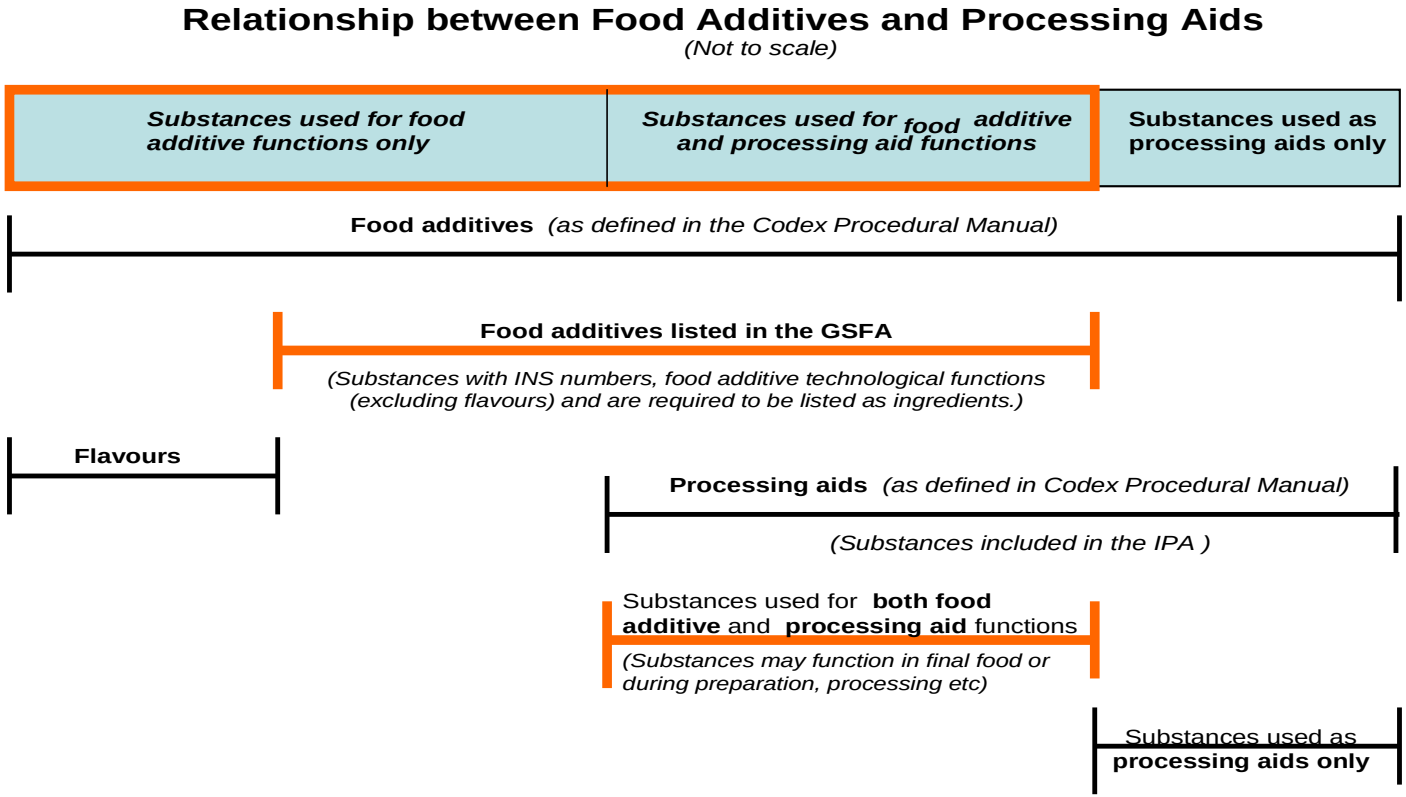
13. The Committee recognises that any food additive, even if not included in the inventory or the appendix, may be used as a processing aid and is eligible for addition to the appendix. In some cases, however, the processing aid use of the food additive may require a separate JECFA evaluation.

14. In general the list does not include substances used in the manufacture of food additives (but some substances used as solvents in the manufacture of flavourings and colourings are mentioned in the main list).

[8] CX/FAC 06/38/13.

THE RELATIONSHIP BETWEEN FOOD ADDITIVES AND PROCESSING AIDS IN THE CODEX SYSTEM

The diagram below shows the relationship between food additives and substances used as processing aids. The diagram takes into account the *Codex Procedural Manual* definitions and the scope of the *General Standard for Food Additives (GSFA)* and the IPA. It is important to note that the term *food additive* as defined in the *Codex Procedural Manual*, includes substances used as processing aids, and that the GSFA does not include flavours or substances used only as processing aids or any processing aids functions of listed food additives.



INVENTORY OF SUBSTANCES USED AS PROCESSING AIDS (IPA)

Main List

IPA CATEGORIES

Antifoam Agents
Boiler water additives
Catalysts
Clarifying agents/ filtration,aids
Contact freezing & cooling agents
Desiccating agent/anticaking agents
Detergents (wetting agents)
Enzyme immobilization agents & supports
Flocculating agents
Ion exchange resins, membranes, and molecular sieves
Lubricants, release and anti stick agents, moulding aids
Micro-organism control agents
Propellant and packaging gases
Solvents, extraction & processing
Washing and Peeling agents
Other processing aids
Enzyme preparations (including immobilized enzymes)*

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Antifoam Agents								
Fatty acid methyl ester	Vegetable protein							31
Fatty acid polyalkylene glycol ester (1-5 moles ethylene oxide or propylene oxide)	Vegetable protein							31
Fatty alcohols (C8-C30)	Vegetable protein							
Formaldehyde	Sugar beet processing	< 0.05	None					39
	processing	< 0.05	None					
*Hydrogenated coconut oil	confectionery Vegetable protein	May-15						36, 49
Mixtures of polyoxyethylene and polyoxypropylene esters of C8-C30 fatty acids	Vegetable protein							31
*Mono- and diglycerides of fatty acids from feed fat (E471)	Jams, jellies and marmalades			Yes	Yes	Mono and diglycerides differ little from food therefore use NL	NL	CCFA 41 ~~54~~, CCFA42 (IFU)
Oxoalcohols (C9-C30)								31
*Polydimethylpolysiloxane (INS 900a) Yeast	Beer Fats and oils Vegetable protein, Juice making	10 (Frying/deep frying purposes only) 10		Yes	Yes	Evaluated as antifoaming agent, anticaking agent Temporary ADI of 0-0.8.	0-1.5	57 Fats and Oils CCFAC 22 CCFA 41 JECFA 69 CCFA 42 (IFU)
Polyoxyethylene esters of C8-C30 fatty acids	Vegetable protein							31

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Polyoxypropylene esters of C8-C30 fatty acids	Vegetable protein							31
Polyoxyethylene esters of C9-C30 oxoalcohols	Vegetable protein							31
Polyoxypropylene esters of C9-C30 oxoalcohols	Vegetable protein							31
Boiler water additives								
Acrylamide-sodium acrylate resin	boiler water							
*Ammonium alginate	boiler water			Yes	Yes	Group ADI for alginic acid and its ammonium, calcium , potassium and sodium salts	NS	CCFAC 22
Cobalt sulphate	boiler water							CCFAC 22
1-Hydroethylidene-1,1-diphosphoric acid and its sodium and potassium salts	boiler water							CCFAC 22
Lignosulfonic acid	boiler water							CCFAC 22
Magnesium sulfate	boiler water			Yes	Yes	Evaulated as Nutrient	NS	CCFAC 22
Monobutyl ethers of polyethylene-polypropylene glycol produced by random condensation of a 1:1 mixture by wt. Of ethylene oxide and propylene oxide with butanol	boiler water							CCFAC 22
*Pentasodium triphosphate	boiler water			Yes	Yes	Expressed as P from all sources	MTDI 70	CCFAC 22
Poly (actylic acid co-hypophosphite), Na salt	boiler water							CCFAC 22
*Polyethylene glycols	boiler water			Yes	Yes	Evaluated as Carrier solvent and Excipient	0-10	CCFAC 22
Polymaleic acid and/or its sodium salt	boiler water							CCFAC 22
Polyoxypropylene glycol	boiler water							CCFAC 22
*Potassium alginate	boiler water			Yes	Yes	Group ADI for aliginic salts Evaluated as stabiliser, thickener,	NS	CCFAC 22

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
						gelling agent and emulsifier		
*Potassium carbonate	boiler water			Yes	Yes		NL	CCFAC 22
*Potassium tripolyphosphate	boiler water			Yes	Yes	Expressed as P from all sources specification as texturiser	MTDI 70	CCFAC 22
*Sodium acetate	boiler water			Yes	Yes		NS	CCFAC 22
*Sodium alginate	boiler water			Yes	Yes	Group ADI for alginates	NS	CCFAC 22
Sodium aluminate	boiler water							CCFAC 22
*Sodium carbonate	boiler water			Yes	Yes		NL	CCFAC 22
*Sodium carboxymethyl cellulose	boiler water			Yes	Yes	Group ADI for modified celluloses	NS	CCFAC 22
Sodium glucoheptonate	boiler water							CCFAC 22
*Sodium hexametaphosphate	boiler water			Yes	Yes	Expressed as P from all sources Evaluated as emulsifier, sequestrant, texurizer	MTDI 70	CCFAC 22
Sodium humate	boiler water							CCFAC 22
*Sodium hydroxide	boiler water			Yes	Yes		NL	CCFAC 22
Sodium lignosulfonate	boiler water							CCFAC 22
*Sodium metasilicate	boiler water							CCFAC 22
*Sodium nitrate	boiler water			Yes	Add.3/173 as anti-microbial and colour tentative	Expressed as nitrate ion; (or 0-5 mg/kg bw expressed as sodium nitrate) Evaluated as antimicrobial preservative, colour fixative	0-3.7	CCFAC 22
*Sodium phosphate (mono-, di-, tri-)	boiler water			Yes	Yes. Specification withdrawn for tri form	Expressed as P from all sources	MTDI 70	CCFAC 22
Sodium polyacrylate	boiler water							
*Sodium polyphosphates	boiler water				See sodium hexa-meta phosphate			CCFAC 22

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
*Sodium silicate	boiler water			Yes	Not prepared		NS	CCFAC 22
*Sodium sulfate	boiler water			Yes	Yes	Evaluated as colour adjuvant	NS	CCFAC 22
*Sodium sulfite	boiler water			Yes	Yes	Group ADI for sulfite ion	0-0.7	CCFAC 22
*Sodium tripolyphosphate	boiler water			Yes	Yes	Expressed as P from all sources	MTDI 70	CCFAC 22
*Starch, unmodified	boiler water							
*Tannin (including quebracho extract)	boiler water			Yes	Yes	Evaluated as a clarifying agent, flavouring agent, flavour adjunct. For use as a filtering aid where GMP ensures it is removed from food after use	NS	CCFAC 22
Tetrasodium diphosphate	boiler water				see Tetrasodium pyrophosphate below			CCFAC 22
Tetra sodium EDTA	boiler water							
*Tetrasodium pyrophosphate	boiler water			Yes	Yes	Expressed as P from all sources	MTDI 70	CCFAC 22
Catalysts								
Alloys of 2 or more listed metals	Hydrogenated food oils							5,22
Aluminum				Yes	Yes	Evaluated as a contaminant	PTWI 1 mg/kg bw	
Chromium	Hydrogenated food oils	< 0.1						1,22
Copper	Hydrogenated food oils	< 0.1		Yes		Evaluated as a contaminant. Provisional daily requirement/ maximum tolerable daily intake	PTDI 0.5	1, 22
Copper chromate								33
Copper chromite								45
Ferric chloride hexahydrate								CX/FAC 92/7

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Manganese	Hydrogenated food oils	<0.4						1, 22
Molybdenum	Hydrogenated food oils	< 0.I						1, 22
Nickel	Polyols	< 1						1, 36, 55
	Hardened oil manufacturing	< 0.8						6
	Hydrogenated food oils	0.2 to 1						22
Palladium	Hydrogenated food oils	< 0.1						1, 22
Platinum	Hydrogenated food oils	< 0.1						1, 22
Potassium metal	Interesterified food oils	< 1						1, 5, 22
Potassium methylate (methoxide)	Interesterified food oils	< I						22
Potassium ethylate (ethoxide)	Interesterified food oils	< 1						1,22
Silver	Hydrogenated. food oils	< 0.1		Yes		No info on use in/on foods insufficient data to evaluate	DP	5,22
Sodium amide	Interesterified food oils	< 1						1,22
Sodium ethylene (sodium ethylate)	Interesterified food oils	< 1						1, 22, 57
Sodium metal	Interesterified food oils	< 1						1,22
Sodium methylate (methoxide)		<1						

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Trifluomethane sulfonic acid	Cocoa butter substitute	< 0.01	None					38
Various metal oxides	Hydrogenated food oils	< 0.1						5,22
Zirconium								16
Clarifying agents/ filtration aids								
Absorbent clays (bleaching, natural or activated earths)	Starch hydrolysis Sugars Edible vegetable oil, Juice making	GMP						61 CCFA 42 (IFU)
Absorbent resins	Juice making	GMP						CCFA 42 (IFU)
Activated carbon	Sugars Oils Juice making	GMP				Evaluated as a adsorbent, decolouring agent	NL	32,55 CCFAC 25 CCFA 42 (IFU)
*Albumin								1
Asbestos				Yes	-	Evaluated as contaminant. Concerns of carcinogen characteristics	No tolerable intake est.	6, 17,25
Bentonite	Starch hydrolysis Juice making	GMP		Yes		No info on use or impurities for JECFA to evaluate	No ADI allocated	1, 6, 37,39,49 CCFA42 (IFU)
Calcium hydroxide	Juice making	GMP (Grape juice only)						CCFA 42 (IFU)
*Calcium oxide	Sugar			Yes	Yes	Evaluated as Alkali, dough conditioner and yeast food		6, 15
Cellulose	Juice making	GMP						CCFA 42 (IFU)
Chitin/ Chitosan	Juice making	GMP						CCFAC 22, CCFA 42 (IFU)

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Chloromethylated aminated styrene-divinylbenzene resin	Sugar processing	<1	None					58
Colloidal silica	Juice making	GMP						CCFA 42 (IFU)
Diatomaceous earth	Fruit juices Starch hydrolysis general use			Yes	Yes	Evaluated as filtering aid	DP	2,6,37,49
Divinylbenzene-ethylvinylbenzene copolymer	Aqueous foods (excluding carbonated beverages)	0.00002 (ex-tractives from copolymer)	None					58
Fuller's earth	Starch hydrolysis, Oils							CCFAC 25
Gelatin (from skin collagen))	Juice making			Yes			Yes	CCFA 42 (IFU)
Ion exchange resins (see ION EXCHANGE RESINS)	Juice making			Yes			Yes	CCFA 42 (IFU)
*Isinglass (Agar)	Juice making			Yes	Yes	Evaluated as thickener, emulsifier and stabliser	NL	1, CCFA 42 (IFU)
Kaolin	Juice making			Yes			Yes	b
Magnesium acetate				Yes	Not prepared	No info about manufacture or use	Not allocated	1, 32
Perlite	Starch hydrolysis Juice making			Yes			Yes	6, 37, 49 CCFA 42 (IFU)

CATEGORY * These substances may also function as a food additive or foods	**Use**	**Residues (mg/kg) (<= less than)**	**Inter-action with food**	**JECFA Eval.**	**Specifications**	**JECFA comments**	**ADI mg/kg bw**	**References**
Polymaleic acid and sodium polymaleate	Sugar processing	< 5	None					58
Polyvinylpolypyrrolidone	Juice making	GMP						CCFA 42 (IFU)
Potassium caseinate	Juice making	GMP						CCFA 42 (IFU)
Potassium tartrate	Juice making (grape juice)	GMP in grape juice only						CCFA 42 (IFU)
Precipitated calcium carbonate	Juice making (grape juice)	GMP in grape juice only						CCFA 42 (IFU)
Rice hulls	Juice making	GMP						CCFA 42 (IFU)
Silicasol	Juice making	GMP						CCFA 42 (IFU)
Sodium caseinate	Juice making	GMP						CCFA 42 (IFU)
Sulfur dixoide	Juice making (grape juice)	10 as $S0_2$ in grape juice only						CCFA 42 (IFU)
*Tannin (to be specified) Tannic Acid	Juice making	GMP		Yes	Yes	For use as filtering agent where GMP ensures it is removed from food after use.	NS	1, 6, CCFA 42 (IFU)
*Vegetable carbon (activated)	Starch hydrolysis			Yes	Yes	Evaluated as colour Also known as Carbon black	Not allocated	1, 6 23, 37 49,
Vegetable carbon (unactivated)								6
Contact freezing & cooling agents								
*Dichlorofluormethane	frozen food	100						1
Freon (to be specified)								1
*Nitrogen				Yes	Yes	Packaging gas, cryogenic freezant, propellant	Not neces-sary, inert	1
Desiccating agent/anticaking agents								
Aluminum stearate				Yes	Yes	Evaluated as anion and cation	PTWI for Al 1 mg/kg bw NS for stearates	61

CATEGORY * These substances may also function as a food additive or foods	**Use**	**Residues (mg/kg) (<= less than)**	**Inter-action with food**	**JECFA Eval.**	**Specifications**	**JECFA comments**	**ADI mg/kg bw**	**References**
Amorphhous hydrophobic silica								CCFA 42 (Brooke-Taylor &Co Pty Ltd)
Calcium phosphate (tricalcium phosphate)				Yes	Yes	Expressed as P from all sources	MTDI 70	28
Calcium Stearate				Yes	Yes		Not allocated	61
Magnesium oxide	anticaking agent and neutralising agent			Yes	Yes	Evaluated as anticaking agent	NL	14
Magnesium stearate				Yes	Yes		Not allocated	61
Octadecyl ammonium acetate (in ammonium chloride)								28
Potassium aluminum silicate								
Sodium alumino silicate				Yes	Yes	Anticaking agent Group ADI for silicon dioxide and certain silicates.	NS	28
Sodium calcium silicoaluminate				Yes	Yes	Anticaking agent	NS	61
Detergents (wetting agents)								
*Dioctyl sodium sulfosuccinate	Fruit drinks	<10		Yes	Yes	Evaluated as emulsifier or wetting agent	0-0.1	26
Magnesium Sulphate	Fats and oils							CCFAC 25
Methyl glucoside of coconut oil ester	Molasses	320						26
Quaternary ammonium compounds								
Sodium lauryl sulphate	Food fats and oils	< 1						221 39
Sodium xylene sulphonate	Food fats & oils	<1						

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Enzyme immobilization agents & supports								
Polyethylenimine (# ADI acceptable provided migration into food reduced to lowest technologically possible)				Yes	Yes	Evaluated as immobilizing agent. New method of analysis prepared at 29th to ensure < 0.1 mg/kg in enzyme preparations of ethylenimine.	Suitable #	42
Glutaraldehyde								33
Glass		Starch hydrolysis						33,49
Diatomaceous earth				Yes	Yes	Evaluated as filter aid	DP	33
Ceramics	Starch hydrolysis							37, 49
Diethylaminoethyl Cellulose								14, 33,
Ion exchange resins								55
Flocculating agents								
Acrylate-acrylamide resin	Sugar	(10 in sugar liquor)						3,24,56
Chitin/Chitosan								CCFAC 22
Complexes of soluble aluminum salt and phosphoric acid	Drinking water							57
								32
Dimethylmine -epichlorohydrin copolymer	Sugar processing	< 5	None					58
Fuller's earth (calcium analogue of sodium montmorillonite)								32
*Isinglass				Yes	Yes	Evaluated as thickener, stabilizer and emulsifier	NL	

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
*Dried and powdered blood plasma								
Modified acrylamide resin	Sugar, boiler water							3, 24
Polyacrylic acid	Sugar							1,15,17
Polyacrylamide	Sugar (beet)							
Sodium polyacrylate	Sugar (beet)							6, 17 6
*Trisodium diphosphate				Yes	Withdrawn (2004)	P from all sources Evaluated at stabiliser, leavening agent, emulsifier, nutrient	MTDI 70	28,16,57
*Trisodium orthophosphate				26	Comp /1559	P from all sources Evaluated as buffer, sequestrant, emulsion stabiliser	MTDI 70	28,16,57
Ion exchange resins, membranes, and molecular sieves.								
Resins:	Enzyme immob. Starch hydrolysis	<. 1 (calculated at Total Organic Carbon)						49
Completely hydrolyzed copolymers of methyl acrylate and divinylbenzene.								3
Completely hydrolyzed terpolymers of methyl acrylate, divi-nylbenzene and acrylonitrile.								3
Cross-linked phenol-formaldehyde activated with one or both -of the following: Triethylenetatramine Tetraethylenepentmine								3

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Cross-linked polystyrene, first chloremethylated then aminated with trimethylamine, dimethylamine, diethylenetriamine or dimethylethanolamine.								3
Diethylenetriamine, triethylenetetramine, tetraethylenapentamine cross-linked with epichlorohydrin								3
Epichlorohydrin cross-linked with ammonia.								3
Epichlorohydrin cross-linked with ammonia and then quaternized with methyl chloride to contain tot more than 18 percent strong base capacity by weight of total exchange capacity	Water used in food processing	None						58
Methacrylic acid-divinylbenzene copolymer.								3
Methacrylic acid-divinylbenzene copolymer with RCOO active groups.								6
Methyl acrylate-divinylbenzene copolymer containing not less than 2 percent by weight of divinylbenzene, aminolyzed with dimethylaminopropylamine.								3
Methyl acrylate-divinylbenzene copolymer containing not less than 3.5 percent by weight of divinyl benzene, aminolyzed with dimethylaminopropylamine								3

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Methyl acrylate-divinlybenzenediethylene glycol divinyl either terpolymer containing not less than 3.5 percent by weight of divinylbenzene and not more than 0.6. percent by weight of diethylene glycol divinyl ether, aminolyzed with dimethyl-aminopropylamine.								3
Methyl acrylate-divinylbenzene-diethylene glycol divinyl ether terpolymer containing not less than 7 percent by weight of divinylbenzene and not more than 2.3 percent by weight of diethylene glycol divinyl ether, aminolyzed with dimethylaminopropyl-amine and quaternized with methyl chloride.	Sugar processing	0.015 (extractives from resin)	None					58
Polystyrene- divinylbenzene reticulum with trimethylammonium groups.	Sugar, distilled liquors	Migrants from resin <1						17
Reaction resin of formaldehyde, acetone and tetraethylpentamine								3
Styrene-divinylbenzene cross-linked copolymer, first chlormethylated then animated with dimethylamine and oxidized with hydrogen peroxide whereby the resin contains not mor6 than 15 percent by weight of vinyl N,N-dimethyl-benzylamine-N-oxide,and not more than 6.5 percent by weight of nitrogen.								3

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Sulfite-modified cross-linked phenol-formaldehyde, with modification resulting in sulfonic acid groups on side chains								3
Sulfonated anthracite coal meeting the requirements of American society for Testing and Materials D388-38, Class 1, Group 2								
Sulfonated copolymer of styrene and divinylbenzene.								3
Sulfonated terpolymers of styrene, divinylbenzene and acrylonitrile. or methyl acrylate.								3
Sulfonated tetrapolymer of styrene, divinylbenzene, acrylonitrile and methyl acrylate derived from a mixture of monomers containing not more than a total of 2 percent by weight of acrylonitrile and methyl acrylate.								3
Counter ions for resins								3, 36
Aluminum								
Bicarbonate								
Calcium								
Carbonate								
Chloride								
Hydroniium								
Hydroxyl								
Magnesium								
Potassium								
Sodium								
Strontium								

CATEGORY * These substances may also function as a food additive or foods	**Use**	**Residues (mg/kg) (<= less than)**	**Inter-action with food**	**JECFA Eval.**	**Specifications**	**JECFA comments**	**ADI mg/kg bw**	**References**
Sulfate								
Membranes: Polyethylene - polystyrene base modified by reaction with chloramethyl ether and subsequent amination with trimethylamine, diethylenetriamine or dimethylethanolamine.								46

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Polymers and copolymers containing the following components: cellulosics (such as cellu-lose diacetate, cellulose triacetate, cellulose ethers, cellulose), Polysulfone - sulfonated polyethersulfone, Polyethersulfone - sulfonated polyethersulfone, Fluoropolymers (such as polyvinylidene fluoride, chlorotrifluoroethyl-ene-vinylidenefluoride copolymer, polytetra-fluoroethylene), Polysulfonamides, aliphatic/aromatic polyamide and copolyamides (such as polypiperazineamides, m-phenylene-diamine trimesamide polymer), Polyesters (such as polyethyleneterephalate), Polyolefins (such as polypropylene, polyethylene), Polya-mide - imide polymers, Polyimides, Polyacryl-onitriles, Polyvinylpyrrolidone, Polystyrene-sulonated polystyrene, chitin/chitosan and deri-vatives, polyureas - polyurethanes, Polyethers, and Polyamines.								

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Polymers and copolymers containing the following components: cellulosics (such as cellu-lose diacetate, cellulose triacetate, cellulose ethers, cellulose), Polysulfone - sulfonated polyethersulfone, Polyethersulfone - sulfonated polyethersulfone, Fluoropolymers (such as polyvinylidene fluoride, chlorotrifluoroethyl-ene-vinylidenefluoride copolymer, polytetra-fluoroethylene), Polysulfonamides, aliphatic/aromatic polyamide and copolyamides (such as polypiperazineamides, m-phenylene-diamine trimesamide polymer), Polyesters (such as polyethyleneterephalate), Polyolefins (such as polypropylene, polyethylene), Polya-mide - imide polymers, Polyimides, Polyacryl-onitriles, Polyvinylpyrrolidone, Polystyrene-sulonated polystyrene, chitin/chitosan and deri-vatives, polyureas - polyurethanes, Polyethers, and Polyamines.				29	Comp /265	Anticaking agent	NS	28

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Lubricants, release and anti stick agents, moulding aids								
Bentonite	Confectionery			Yes	Not prepared	Anticaking agent .No significant uses known, no data on impurities	No ADI allocated	2
*Dimethylpolysiloxane				Yes	Yes	ADI only applies to compounds with 200 – 300 subunits	0-1.5	16
Kaolin (Aluminum Silicate)	Confectionery			Yes	Yes	As anticaking agent	NS	2
Micro-organism control agents								
Acidified sodium chlorite (ASC)	Poultry Meats Vegetables Fruit Seafood	Chloride	None	Yes	Yes	The available toxicological data were sufficient to assess the safety of ASC by setting ADIs for chlorite and chlorate.	0.03 (chlorite) 0.01 (chlorate)	CCFA 40
*Chlorine dioxide #	Flour			Yes	Withdrawn (2000)	Flour treatment agent conditional, 30-75; acceptable level of treatment for flours to be consumed by man		
*Dimethyl dicarbonate	Wine Beverages	None		Yes	Yes	Acceptable for use as a cold sterilization agent in beverages when used according to good manufacturing practice up to a maximum concentration of 250mg/l	acceptable 57	58 CCFA 40

CATEGORY * These substances may also function as a food additive or foods	**Use**	**Residues (mg/kg) (<= less than)**	**Inter-action with food**	**JECFA Eval.**	**Specifications**	**JECFA comments**	**ADI mg/kg bw**	**References**
Formaldehyde	sugar							56
Hydrogen peroxide	Sugar, ~~fruit and vegetable juices~~			Yes	Yes	Small residues of hydrogen peroxide on food (which has been treated with antimicrobial washing solutions) at the time of consumption would not pose a safety concern~~.~~		14,24 CCFA 42 (IFU)
Hypcochlorite	Food oils							22
Iodophors	Food oils							22
Lactoperoxidase system (lactoperoxidase, glucose oxidase, thiocyanate salt)								47
Peracetic acid								
Peroxyacid antimicrobial solutions								CCFAC 38
Quaternary ammonium compounds	Food oils							22
Salts of sulfurous acid	Corn milling Starch hydrolysis	< 100						32,37,57
Sodium metasilicate (Sodium sulphate or sodium carbonate can be added to reduce silicate scaling on equipment)	Meat and poultry carcasses, half carcasses and cuts							CCFA 40
*Trisodium phosphate	Meat and poultry carcasses, half carcasses			Yes	Yes	Expressed as P from all sources	MTDI 70	CCFA 40

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
	and cuts							
Propellant and packaging gases								
*Air								45
Argon								45
*Carbon dioxide	Juice making	GMP						56, CCFA 42 (IFU)
Chloropentafluoroethane								1
Combustion product gas a variable mixture of gases produced by controlled combustion of butane, propane, or natural gas. The principle components are nitrogen and carbon dioxide,, with lesser amounts of hydrogen, oxygen, carbon monoxide (not to exceed 4.5%), any traces of other inert gases.								3,58
*Dichlorodifluoromethane (F 12)								56
*Helium								1
Hydrogen								
Isobutane	Propellent in vegetable oil pan spray (for professional use only)							CCFAC 37
*Nitrous oxide				Yes	Yes	At its twenty-ninth meeting (1985), the Committee concluded that use of nitrous oxide as a propellant for food was acceptable. At its fifty-fifth meeting (2000), the Committee	Use acceptable as a propellant	1, 6

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
						was requested by the CCFAC to evaluate the additional use of nitrous oxide as a packaging gas, but the Committee could not carry out this request because no information on intake of nitrous oxide for such use was available.		
*Nitrogen	Juice making	GMP		Yes	Yes	Packaging gas; cryogenic freezant, propellant	No ADI necessary	1.3,6 CCFA 42 (IFU))
Octafluorocyclobutane								1
Propane				Yes	Not prepared	Evaluated as propellant; extraction solvent	NS	1
Trichlorofluoromethane (F 11)								43.6
Solvents, extraction & processing.								
Acetone (Dimethyl ketone)	Flavourings, colours, food oils	< 30, 2, & 0.1		Yes	Yes	Extraction solvent, flavouring agent	Acceptable	1, 3, 4,17, 22, 14
Amyl acetate	Flavourings, colours			Yes	Yes	As carrier solvent, flavouring agent. Included in ADI for amyl butyrate expressed as isoamyl alcohol	0-3	2,59
Benzyl alcohol	Flavourings, colours, fatty acids			Yes	Yes	As carrier solvent, flavouring. ADI for total benzoate from all sources	0-5	2,59
*Butane	Flavourings, food oils	<1, 0.1		Yes	Not prepared	Propellant	Not allocated	1, 4, 17,22,19
Butane-1,3-diol	Flavorings	0-4		23	Comp/ 241	As carrier solvent	0-4	3
Butan-l-ol	Fatty acids flavourings, colours	<1000		Yes	Yes	Evaluated as extraction solvent, flavouring agent	Acceptable	2,4,19

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Butan-2-ol	Flavorings	1		Yes	Yes	Extraction solvent, flavour	Not allocated	56
*Butyl acetate				Yes	Yes	Evaluation as flavouring agent.	Acceptable	56
*Carbon dioxide				Yes	Yes	Carbonating agent, propellant, preservative, freezing agent, extraction solvent		
Cyclohexane	Flavourings, food oils	< 1		Yes	Yes	Extraction solvent	Not allocated	4.17.19
Dibutyl ether	Flavourings	<2					56	4,19
1,2 Dichloroethane	Decaf. Coffee	< 5		Yes	Not prepared	Evidence of genotoxicity and carcinogenicity; should not be used in food	Not allocated	1, 17
Dichlorodifluoromethane	Flavourings, colour	< 1		Yes	Not prepared	Propellant; Liquid Freezant	0-1.5	2,4,19,59,
Dichloromethane (methylene chloride)	Flavourings, decaf. Coffee, food oils	< 2,5,10		Yes	Yes	Should be limited to current uses (extraction solvent)		
Dichlorotetrafluoroethane	Flavourings	<1						4,19
Diethyl citrate	Flavourings, colours						2,4,17,22,19	2
Diethyl ether	Flavourings, colours	<2		Yes	Yes	Extraction solvent	Not allocated	2,4,19
Di- iso propoylketone								2
*Ethanol	Vegetable protein			Yes	Yes	Specification for extraction and carrier solvent	Limited by GMP	56
*Ethyl acetate				Yes	Yes	No safety concerns at current level of intakes when used as a flavouring agent	0-25	56

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Ethyl methyl ketone (butanone)	Fatty acids, fla-vourings, colour-ings. Decaffeina-tion of coffee, tea	< 2		Yes	Yes	Extraction solvent, flavouring agent	Acceptable	2, 4, 19
Glycerol tributyrate	Flavourings, colours							2
Glycerol tripropionate	Flavourings, colours							2,59
Heptane	Flavourings, food oils	< 1		Yes	Yes	Extraction solvent	Limited by GMP	1, 4, 6,22
Hexane	Flavourings. food oils,	< 0.1		Yes	Yes	Extraction solvent JECFA 65 recommended a re-evaluation of hexanes as there was insufficient information to change current specifications	Limited by GMP	1,3,4,
	Chocolate and chocolate products	1						CCFAC 37
*Isobutane	Flavourings	<1						4,19
Isoparaffinic petroleum hydrocarbons	Citric acid							3
Isopropyl myristate	Flavourings colours			Yes	Yes	Carrier solvent. No safety concerns at current level of intakes when used as a flavouring agent	Not allocated	2
Methylene chloride (dichloromethane)	Food oils	< 0.02		Yes	see above in dichlo-romethane			1,22
Methyl acetate	Coffee	20						56

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
	Decaffeination flavoring Sugar refining							
		1						
Methyl propanol-I	Flavorings	1						56
Nitrous oxide				Yes	Yes	evaluated as propellant Use acceptable as a propellant	acceptable	45
n-Octyl alcohol	Citric acid							3
Pentane	Flavourings, food oils	< 1						1,4, 22
Petroleum ether (light petroleum)	Flavourings, food oils	< 1		Yes	Yes	Extraction solvent	NS	1,4,6,22,19
*Propane	Flavourings, food oils	< 1, 0.1		Yes	Not prepared	Propellant; Extraction solvent Limited use and residue mean unnecessary to establish ADI	NS	4, 17,22,19
Propane-1,2-diol	Fatty acids flavourings, colours,							2,59
Propane-l-ol	Fatty acids, flavourings, colours			25	Comp/1205	Carrier/extraction solvent/ flavouring. Further tox studies required.	Not allocated	2,59
*Propylene Glycol				Yes	Yes	As solvent, humectant and glazing agent	0-25	CX/FAC 92/7
Tertiary butyl alcohol								38
1,1,2-Trichloroethylene	Flavourings, food oils	< 2		Yes	Withdrawn (2000)	Use as extraction solvent should be limited to ensure levels are as low as practicable	Not allocated	1,4,17,22, 19
Trichlorofluoromethane	Flavourings	<1						4,19,59
Tridodecylamine	Citric acid							3

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Toluene	Flavourings	<1		Yes	Yes	Residues of toluene occurring in food when this solvent is used in accordance with GMP would not pose any toxicological problems	NS	4, 19
Washing and peeling agents								
A mixture of alkene oxide adducts of alkyl alcohol and phosphate esters of alkylene oxide adducts of alkyl alcohols consisting of alpha-alkyl(C12-C18)-omega-hydroxy-poly(oxy-ethylene) (7.5-8.5moles) poly(oxypropylene) block copolymer having an average molecular weight of 810, alpha-alkyl- (C12-C18)-omega-hydroxy-poly(oxyethylene) (3.3-3.7 moles) polymer having an average molecular weight of 380, and subsequently esterified with 1.25 moles phosphoric anhydride; and alpha-alkyl (omega-hydroxy-poly(oxyethylene) (11.9-12.9 moles)/poly(oxypropylene) copolymer having an average molecular weight of 810 and sub-sequently esterified with 1.25 moles phosphoric anhydride	Fruits and vegetables	< 0.001 up to 0.01	None					3, 54
Alkylene oxide adducts of alkyl alcohols and fatty acids	Sugar beets	No Information Available						6,51,54
Aliphatic acid mixture consisting of valeric, caproic, enanthic, caprylic, and pelargonic acids	Fruits and vegetables	0.04-0-11	None					3,54

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Alpha-alkyl-omega-hydroxy-poly (oxyethylene)	Sugar beets	0.001in sugar beets, 0 in sugar						3,51.54
Ammonium chloride, quaternary	Sugar beets		None					53
Ammonium orthophosphate	Fruits and vegetables			Yes	Yes	Expressed as P from all sources	MTDI 70	
*Calcium chloride	Fruits and vegetables			Yes	Yes	Firming agent	NL	53
*Calcium hydroxide	Sugar beets			Yes	Yes	Specification for neutralizing agent; buffer; firming agent	NL	53
*Calcium oxide	Sugar beets			Yes	Yes	Specification for Alkali, dough conditioner, yeast food	NL	53
Carbamate	Sugar beets							53
Dialkanolamine	sugar beets	0.001 in sugar beets, 0 in sugar	None					3,54
Diammonium orthophosphate	Fruits and vegetables for canning			Yes	Yes	Expressed as P from all sources	MTDI 70	
Diammonium orthophosphate, (5% aqueous solution)	Fruits and vegetables for canning			Yes	Yes	Expressed as P from all sources	MTDI 70	
Dithiocarbamate	Sugar beets							53
Ethylene dichloride	Sugar beets	0.00001 in sugar beets, 0 in sugar	None	23				3,54
Ethylene glycol monobutyl ether	Sugar beets	0.00003 in sugar beets, 0 in sugar	None					3,54

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Hydrogen peroxide		No Information - Available		Yes	Yes	As antimicrobial agent	Acceptable	54
Linear undecylbenzenesulfonic acid	Sugar beets	0.001 in sugar beets 0 in sugar	None					3,54
Monoethanolamine	Fruits and vegetables, sugar beets	100						3,52
Monoethanolamine	Sugar beets	0.0001 in sugar beets, 0 in sugar	None					54
Monoethanolamine (8%)	Fruits and vegetables for canning							56
Organophosphates	Sugar beets							53
Peroxyacid antimicrobial solutions containing 1-hydroxyethylidene-1,1-				Yes		The peroxy compounds in these solutions (hydrogen peroxide, peroxyacetic acid and peroxy-octanoic acid) would break down into acetic acid and octanoic acid, and small residual quantities of these acids on foods at the time of consumption would not pose a safety concern. HEDP does not pose a safety concern at the levels of residue that are expected to remain on foods at the time consumption.		
Diphosphonic acid (HEDP) *Containing HEDP and three or more of the following components: peroxacetic acid, acetic acid, hydrogen peroxide, octanoic acid and peroxyoctanoic acid.*					Yes			
Acetic acid					Yes			
1-Hydroxyethylidene-1,1-diphosphonic acid (HEDP)					Yes			
Hydrogen peroxide					Yes			
Octanoic acid (as food additive)					Yes			

CATEGORY * These substances may also function as a food additive or foods	**Use**	**Residues (mg/kg) (<= less than)**	**Inter-action with food**	**JECFA Eval.**	**Specifications**	**JECFA comments**	**ADI mg/kg bw**	**References**
Polyacrylamide	Fruits and vegetables, sugar beets	< 1	None					3,51,54
Potassium bromide	Fruits and vegetables							3,54
Sodium dodecylbenzenesulfonate (alkyl group predominantly C12 and not less than 95 percent C1O-C16).	Fruits and vegetables, meat and poultry	< 2		None				3, 6, 54
Sodium 2-ethylhexyl sulphate	Fruits and vegetables	< 20		None				3,54
*Sodium carbonate				Yes	Yes	Alkali	NL	52
*Sodium hydroxide	Fruits and vegetables, sugar beets			Yes	Yes	Alkali	NL	53
Sodium hydroxide (10%, max.)	Fruits and vegetables for canning					See above		52
Sodium hydroxide (2%)	Mackerel for canning					See above		52
Sodium hypochlorite	Fruits and vegetables	No Information Available				No Information Available		3,52.54
Sodium mono- and di-methyl naphthalene-sulfonates (mol. wt. 245-260)	Fruits and vegetables	< 0.2	None					3, 54
Sodium n-alkylbenzenesulfonate (alkyl group predominantly C12 and C13 and not less than 95 percent C1O-C16).	Fruits and vegetables	Same as sodium dodecylbenzenesulfonate	None					3, 6, 54

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
*Sulfuric acid	Locust bean seeds			Yes	Yes	As acid		CCFAC 25
Tetrapotassium pyrophosphate	Sugar beets	0.00002 in sugar beets 0 in sugar	None	Yes	Yes	Specification as emulsifier, texturiser. MTDI for P from all sources	MTDI 70	3,54,57
Tetrasodium ethylenediaminetetraacetate	Sugar beets	0.000003 in sugar beets 0 in sugar	None					3,54
Triethanolamine	Sugar beets	0.00005 in sugar beets 0 in sugar	None					3, 54
Other processing aids								
Aluminum oxide								
Aluminum potassium sulphate				Yes	Yes	Acidity Regulator; firming agent, raising agent Group ADI for Al	PTWI 1 mg/kg bw expressed as Al	28
Ammonium nitrate								
Benzoyl peroxide	Bleaching whey			Yes	Yes	Treatment of whey with benzoyl peroxide at a maximum concentration of 100 mg/kg does not pose a safety concern.	Acceptable	
Beta – cyclodextrin	flavour adjunctor and cholesterol extraction in butter			Yes	Yes	As encapsulating agent for food additives, flavours and vitamins, thickening agent	0-5	CCFAC 25
*Erythorbic acid				Yes	Yes	Antioxidant	NS	58

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Calcium lignosulfonate (40-65)	Protective colloid/carrier for fat-soluble vitamins and carotenoids			yes	yes		0-20	CCFA 41 JECFA 69
Calcium tartrate				Yes	Not prepared	Acidity regulator	No ADI allocated	
*Citric Acid	Fats and Oils			Yes	Yes	As acidulant, antioxidant synergist, sequestrants,, flavouring agent	NL	CCFAC 25
Ethyl parahydroxybenzoate				Yes	Yes	Preservative As sum of ethyl, methyl and propyl esters of p-hydroxybenzoic acid	0-10	32
Gibberellic acid								
*Glucono -delta lactone	pre acidification of milk in cheese making			Yes	Yes	As acidifier , raising agent, sequestrant	NS	CCFAC 25
Glycerol ester of adipic acid								32
Hydrogen								
Magnesium tartrate				Yes	Not Prepared		No ADI allocated	
*Phosphoric Acid	Fats and Oils			Yes	Yes	As P from all sources Evaluated as acidulant sequestrant, antioxidant synergist	MTDI 70	CCFAC 25
Polyvinyl polypyrrolidone	Beverages			Yes	Yes	As colour stabiliser, colloidal stabiliser, clarifying agent	NS	13
Potassium gibberellate								

CATEGORY * These substances may also function as a food additive or foods	Use	Residues (mg/kg) (<= less than)	Inter-action with food	JECFA Eval.	Specifications	JECFA comments	ADI mg/kg bw	References
Propyl parahydroxybenzoate				Yes	Withdrawn (2006)	As preserevative In view of the adverse effects in male rats, propyl paraben (propyl p-hydroxybenzoate) should be excluded from the group ADI for the parabens used in food.	Withdrawn (2006)	32,58
Sodium								
*Sodium Hydroxide	Fats and Oils			Yes	Yes	As alkali	NL	CCFAC 25
Sodium hypochlorite								
*Sodium silicate				Yes	Not prepared		NS	

ENZYME PREPARATIONS (INCLUDING IMMOBILIZED ENZYMES)

Microbially-derived enzymes from genetically modified organisms are listed with the producing host organism name followed by a d-(name) to identify the source of the donor organism gene.

Note: Due to taxonomic changes of many micro-organisms used to produce enzymes, it would be necessary to mention all the synonyms in each case. This would make the table quite unreadable and require regular updating. Therefore please consult the following list of taxonomic changes for the current correct names of specific micro-organisms that produce enzymes.

- *Aspergillus niger* covers strains known under the names *Aspergillus aculeatus, A. awamori, A. ficuum, A. foetidus, A. japonicus, A. phoenicis, A. saitoi, A. usamii* and A. tubingensis.
- *Bacillus subtilis* formerly also covered the strain now known under the name *Bacillus amyloliquefaciens.*
- *Bacillus stearothermophilus is* also known as *Geobacillus stearothermophilus*)
- *Endothia parasitica* is the former name of *Cryphonectrica parasitica*
- *Humicola lanuginosa* is also known as *Thermomyces lanuginosus*
- *Klebsiella aerogenes* is the former name of *Klebsiella pneumoniae*
- *Micrococcus lysodeicticus* is the former name of *Microccocus luteus*
- *Mucor miehei* is the former name of *Rhizomucor miehei*
- *Penicillium emersonii* is the former name of *Talaromyces emersonii*. It is also known as *Geosmithia emersonii*
- *Rhizopus arrhizus* is the former name of *Rhizopus oryzae.*
- *Sporotrichum dimorphosporum* is the former name of *Disporotrichum dimorphosp orum*
- *Streptoverticillium mobaraensis* is the former name of *Streptomyces mobaraensis*
- *Trichoderma reesei* is also known as *Trichoderma longibrachiatum*
- *Verticicladiella procera* is the former name of *Leptographium procerum*

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Animal-Derived Enzyme Preparations:				
Alpha amylase (hog or bovine pancreas)				10,23
Catalase (bovine or horse liver)	yes	yes	Use limited by GMP	1
Chymosin (calf or kid or lamb abomasum)				
Chymotrypsin (bovine or porcine pancreas)				
Lipase (bovine stomach) (salivary glands or fore stomach of calf, kid, or lamb) (hog or bovine pancreas)	yes	yes	Use limited by GMP	1, 3, 10,13
Lysozyme (egg whites)		yes	Regard as food/preservative	44, 48, 57
Pancreatin (bovine or porcine pancreas)				
Pepsin				
(hog stomach)	yes	yes	Limited by GMP	1
(proventicum of poultry)	yes	yes		41
(porcine pancreas)				55
Phospholipase A				
(Bovine pancreas)				CCFA 43 (AMFEP)
(*Porcine pancreas)*				CCFA 40 (CRD14 AMFEP
Rennet				
(calf or kid, lamb stomach)	yes	yes	Limited by GMP	1
(bovine stomach)	yes	yes	Limited by GMP	
Trypsin (porcine or bovine pancreas)	yes	yes	Regard as food	1
Plant-Derived Enzyme Preparations:				
Alpha amylase (malted barley)				
Ascorbate oxidase *(Cucurbita pepo)*				CCFA 43 (AMFEP)
Beta amylase				
(malted or ungerrminated barley)				
(soya)				
Bromelain (*Ananas comosus*; *Ananas bracteatus*)	yes	yes	Limited by GMP	1
Chymopapain (*Carica papaya*)	yes	yes	Limited by GMP	

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Ficin (*Ficus glabrata*)	yes	yes	Nonedible plant derived enzyme preparation. No toxicological data	1, 3
Laccase				
(Trametes hirsuta)				CCFA 40 (CRD14 AMFEP)
(Trametes versicolour)				CCFA 40 (CRD14 AMFEP)
Lipases (origin?)	yes	yes		CCFAC 25/ (1993) Malaysia
Lipase *(Carica papaya)*				CCFA 43 (AMFEP)
Lipoxydase (soya)				55
Malt carbohydrases (alpha or beta amylase) (malted barley or barley)	yes	yes	Limited by GMP	1, 6, 40,49,55
Papain (*Carica papaya*)	yes	yes	Limited by GMP	
Peroxidase (soya)				
Protease (incl. milk clotting enzymes) *(Actinidia chinensis)*				CCFA 40 (CRD14 AMFEP)
Microbiologically derived Enzyme Preparations				
Acetolactate decarboxylase *(Bacillus subtilis d-Bacillus brevis)*	yes	yes		
Acetolactate decarboxylase (alpha) (*Sacccharomyces cerevisiae* d-*Enterobacter sp.*)				CCFA 40 (CRD14 AMFEP)
Acetylhexosaminidase, beta-L-N (*Streptomyces violaceoruber d-Streptomyces sp.*)				CCFA 43 (AMFEP)
Acid phosphatase (*Aspergillus niger*)				in CX/FAC 92/7
Alcohol dehydrogenase *(Saccharomyces cerevisiae*)				15
Alginate lyase (Sphingobacterium multivorum)				CCFA 43 (AMFEP)
Alpha amylase				
(*Aspergillus niger*)	yes	yes	Data required to show strains used do not produce mycotoxins	7
(Aspergillus niger d-Aspergillus niger)				
(*Aspergillus oryzae*)	yes	yes	Regard as normal constituent of food	7

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(*Bacillus amyloliquefaciens)*				CX/FAC 92/7
(Bacillus amyloliquefaciens d-Bacillus amyloliquefacien)				
(Bacillus amyloliquefaciens or subtilis d-*Thermoactinomyces sp.)*				CCFA 40 (CRD14 AMFEP)
(Bacillus licheniformis)				7
(Bacillus licheniformis containing a modified alpha amylase gene from *B. licheniformis)*	yes	yes		CCFAC 37
(*Bacillus licheniformis* with modified gene from *d-Geobacillus sp*.)				CCFA 43 (AMFEP)
(*Bacillus licheniformis d-Pseudomonas sp.)*				CCFA 43 (AMFEP)
(Bacillus licheniformis d-Bacillus stearothermophilus)				
(*Bacillus stearothermophilus*)	yes	yes		
(*Bacillus subtilis*)	yes	yes		7
(*Bacillus subtilis d-Bacillus megaterium*)	yes	yes		in CX/FAC 92/7
(*Bacillus subtilis d-Bacillus stearothermophilus*)	yes	yes		in CX/FAC 92/7
(Bacillus subtilis d-Bacillus subtilis)				
(Microbacterium imperiale)				
(Pseudomonas fluorescens with modified gene from *d-Thermococcus sp.)*				CCFA 43 (AMFEP)
(*Rhizopus delemar)*				7
(*Rhizopus oryzae)*				7
(Thermomonospora viridis)				
(Trichoderma reesei or longibrachiatum d-Aspergillus sp.)				CCFA 43 (AMFEP)
Alpha galactosidase or Melibiase				7
(*Aspergillus niger*)	yes	yes	Evaluated as carbohydrase	
(Aspergillus oryzae)				
(Aspergillus oryzae d-Aspergillus niger)				
(*Mortierella vinacea*)				7
(*Saccharomyces carlsbergensis*)	yes	yes	Evaluated as carbohydrase	7,31
(Saccharomyces cerevisiae d-Guar seed)				
Aminoacylase (*Aspergillus melleus)*				CCFA 40 (CRD14 AMFEP)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Aminopeptidase				
(Aspergillus niger)				
(Aspergillus oryzae)				
(Aspergillus oryzae d-Aspergillus sp.)				CCFA 43 (AMFEP)
(Lactococcus lactis)				
(Rhizopus oryzae)				
(Trichoderma reesei)				
AMP deaminase*)*				
(Aspergillus melleus				
(Aspergillus oryzae)				CCFA 43 (AMFEP)
Arabinanase *(Aspergillus niger)*				CCFA 40 (CRD14 AMFEP)
Arabinofuranosidase				CCFA 40 (CRD14 AMFEP)
(*Aspergillus niger*)	yes	yes	Evaluated as carbohydrase	31
(Aspergillus niger d-Aspergillus niger)				
Asparaginase				
(Aspergillus niger d-*Aspergillus niger.)*	yes	yes	ADI not specified when used under GMP	JECFA 69 CCFA 41
(Aspergillus oryzae d-Aspergillus oryzae)	yes	yes	ADI not specified when used under GMP in dough based and potato products prior to heating	AMFEP CRD14 JECFA 68 CCFA 40
(Aspergillus niger d-Aspergillus niger)	yes	yes	ADI not specified when used under GMP in bread and other cereal based products and baked and fried potato-based products prior to heating.	CCFA 41 JECFA 69
Beta amylase				
(*Bacillus cereus)*				7
(Bacillus lichenformis)				in CX/FAC 92/7
(*Bacillus megaterium)*				7, 8

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(*Bacillus subtilis)*	yes	yes	As mixed microbial carbohydrases and proteases	7
Beta glucanase				
(*Aspergillus niger)*	yes	yes	Temporary acceptance of microbial carbohydrase pending further short term tests	
(*Bacillus amyloquefaciens)*				in CX/FAC 92/7
(Bacillus amyloquefaciens d- Bacillus amyloquefaciens)				
(*Bacillus subtilis)*	yes	yes		
(Cellulosimicrobium cellucans)				CCFA 41 (CRD12 AMFEP)
(Disporotrichum dimorphosporum)				
(Humicola insolens)				
(Penicillium funiculosum)				
(Penicillium multicolor)				
(Pseudomonas paucimobilis)				
(Talaromyces emersonii)				
(*Trichoderma harzianum)*	yes	yes		20
(*Trichoderma reesei)*				in CX/FAC 92/7
(*Trichoderma reesei d-Trichoderma reesei*)				
Beta d-glucosidase or Cellobiase				
(Aspergillus niger)				7
(Penicillium decumbens)				
(*Penicillium multicolor)*				CCFA 40 (CRD14 AMFEP)
(Trichoderma harzianum)	yes	yes	As carbohydrases	
(Trichoderma reesei)				7, 20
(Trichoderma reesei d-Trichoderma reesei)				
Beta xylosidase (*Trichoderma reesei)*				55
Branching glycosyltransferase (*Bacillus subtilis d-Rhodothermus sp.*)				CCFA 43 (AMFEP) JECFA 71

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Carbohydrases, mixed (pectinase, cellulases,and hemicellulases) *(Aspergillus niger)*	yes	yes	Evaluated as carbohydrases	CX/FAC 92/7
Carboxypeptidase *(Aspergillus niger d-Aspergillus niger)*				
Catalase				
(Aspergillus niger)	yes	yes	Evaluated as carbohydrase	71.24,
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus oryzae)				
(*Micrococcus luteus*)				7
Cellobiose dehydrogenase (*Fusarum venenatum d-Microdochium sp.)*				CCFA 40 (CRD14 AMFEP)
Cellulase				
(*Aspergillus niger*)	yes	yes	Evaluated as carbohydrase	6, 7,55
(*Aspergillus oryzae)*	ADI not decided	Withdrawn 2000	Evaluated as carbohydrase	7
(Disporotrichum dimorphosporum)				7
(Humicola insolens)				
(Penicillium funiculosum)				
(*Rhizopus delemar)*				7
(*Rhizopus oryzae)*	yes	yes	Evaluated as carbohydrase	7
(Streptomyces lividans)				
(Talaromyces emersonii)				
(*Thielavia terrestris)*				7
(*Trichoderma reesei)*	yes	yes		
(Trichoderma reesei d-Trichoderma reesei)				
(Trichoderma viride)				
Chitinase (*Streptomyces violaceoruber d-Streptomyces sp.)*				CCFA 43 (AMFEP)
Chymosin A (*E coli K-12* d-calf stomach)	yes	yes		CCFAC 23 (1991)
Chymosin B				
(*Kluveromyces marxianus var. lactis* d-calf stomach)	yes	yes		CCFAC 23 (1991)
(*Aspergillus niger* var. *awamori* d-calf stomach)	yes	yes		CCFAC 23 (1991)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Cyclomaltodextrin glucanotransferase *(Bacillus licheniformis d-Thermoanaerobacter.)*				
Cyclomaltodextrin glucanotransferase *(Bacillus macerans)*				CCFA 40 (CRD14 AMFEP)
Dextranase				
(*Aspergillus ?)*				
(*Bacillus subtilis)*	yes	yes	Evaluated as mixed carbohydrases and proteases	
(Chaetomium erraticum)				
(Chaetomium gracile)				
(*Klebsiella pneumoniae*)				7
(Leuconostoc mesenteroides)				CCFA 41 (CRD12 AMFEP)
(*Penicillium funiculosum*)				7
(*Penicillium lilacinum)*				7
Dextransucrase *(Leuconostoc mesenteroides)*				CCFA 43 (AMFEP)
Endo beta glucanase				
(*Aspergillus niger*)	yes	yes	Evaluated as carbohydrases	7
(*Aspergillus oryzae)*	yes	yes	Evaluated as carbohydrases	7
(*Bacillus circulans)*				7
(*Bacillus subtilis)*	yes	yes	Evaluated as mixed carbohydrases and protease	7
(*Disporotrichum dimorphosporum*)				56
(Leuconostoc mesenteroides)				CCFA 43 (AMFEP)
(*Rhizopus delemar)*				7
(*Rhizopus oryzae)*	yes	yes	Evaluated as carbohydrase	7, 30
(Talaromyces emersonii)				7
(*Trichoderma reesei*)				
Esterase				from CX/FAC 92/7
(*Aspergillus niger)*				55
(*Rhizomucor miehei)*				7
(Trichoderma reesei)				55
Exo alpha glucosidase *(Aspergillus niger*)				

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Exo-alpha glucosidase (immobilized) (same source as above) no more than 10 mg/kg glutaraldehyde				
Ferulic acid esterase				
(Aspergillus niger)				CCFA 43 (AMFEP)
(Streptomyces werraensis)				CCFA 40 (CRD14 AMFEP)
Fructosyl transferase				
(Aspergillus niger)				
Glucanase (endo-1,3(4)-beta) *Cellulosimicrobium sp.)*				CCFA 40 (CRD14 AMFEP)
Glucanase (beta) *(Aspergillus oryzae* d-*Thermoascus sp.)*				CCFA 40 (CRD14 AMFEP)
Glucoamylase or amyloglucosidase				
(*Aspergillus niger)*	yes	yes		7, 9, 16, 49, 50
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus niger d-Talaromyces emersonii)				
(*Aspergillus oryzae*)	yes	yes	Microbial enzyme preparation	7
(Penicillium funiculosum)				
(*Rhizopus delemar)*				7
(*Rhizopus niveus)*				7
(*Rhizopus oryzae*)	yes	yes	Evaluated as carbohydrase	7
(*Trichoderma reesei*)				7, 30
(Trichoderma reesei or longibrachiatum d-Trichoderma sp.)				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum with modified gene from d-Trichoderma sp.)				CCFA 43 (AMFEP)
Glucanotransferase *(Bacillus amyloliquefaciens or subtilis d-Thermus sp.)*				CCFA 43 (AMFEP)
Glucose isomerase				
(*Actinoplanes missouriensis)*	yes	yes	Acceptable for use in food processing when immobilised.	7
(*Arthrobacter?)*	15		Evaluated as carbohydrase	7

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(*Bacillus coagulans*)	yes	yes	Non immobilised: No info on use No ADIallocated	7
			Immobilised: Use acceptable in food	
(Microbacterium arborescens)				
(*Streptomyces albus*)				7
(Streptomyces lividans)				
(Streptomyces murinus)				
(*Streptomyce olivaceus*)	yes	yes	acceptable when immobilised	7
(Streptomyces olivochromogenes)	yes	yes	acceptable when immobilised	12, 7
(Streptomyces rubiginosus)	yes	yes	acceptable when immobilised	9,20,21
(Streptomyces rubiginosus d-Streptomyces sp.)				CCFA 43 (AMFEP)
(Streptomyces ?)			See specific sp. above	17
(Streptomyces violaceoniger)	yes	yes		
Glucose isomerase (immobilized) .(same sources as above) not more than 10 mg/kg glutaraldehyde	yes	yes	See comments above	
(Microbacterium arborescens)				CX/FAC 92/7
(Streptococcus murinus)				CX/FAC 92/7
Glucose oxidase				
(Aspergillus niger)	yes	yes		1, 6, 7
(Aspergillus niger d- Aspergillus niger)				
(Aspergillus oryzae d- Aspergillus niger)				
(Penicillium chrysogenum)				
Glucosidase (exo-1.3-beta)				
(*Penicillium funiculosum)*				CCFA 40 (CRD14 AMFEP)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Trichoderma harzianum)				CCFA 43 (AMFEP)
Glutaminase				
(Aspergillus niger)				CCFA 43 (AMFEP)
(Bacillus subtilis)				
Glycerophospholipid cholesterol acyltransferase (*Bacillus licheniformis* d-*Aeromonas* sp.)				CCFA 43 (AMFEP)
Hemicellulase				
(Aspergillus niger)	yes	yes		
(Aspergillus oryzae)	yes	yes	Evaluated as carbohydrase	7
(Bacillus lentus)				
(Bacillus subtilis)	yes	yes	Evaluated as carbohydrase	7
(Bacillus subtilis d-Bacillus ?)				
(Disporotrichum dimorphosporum)				7
(*Rhizopus delemar)*				7
(Rhizopus oryzae)	yes	yes	Evaluated as carbohydrase	7
(Trichoderma reesei)				7,30
Hexose oxidase *(Hansenula polymorpha d-Chondrus crispus)*	yes	yes		CCFAC 38
Inulinase				
(*Aspergillus niger)*	yes	yes	Evaluated as carbohydrase	
(Aspergillus oryzae d-Aspergillus sp)				CCFA 41 (CRD12 AMFEP)
(Disporotrichum dimorphorsporum)				
(Kluyvercmyces fragilis)				7
(Streptomyces ?)	yes	yes		
Invertase				7
(*Aspergillus niger)*	yes	yes	Evaluated as carbohydrase	
(Bacillus subtilis)	yes	yes	Evaluated as carbohydrase	
(Kluyveromyces fragilis)				7
(Saccharomyces carlsbergensis)	yes	yes	Evaluated as carbohydrase	7
(Saccharomyces cerevisiae)	yes,	yes	Evaluated as carbohydrase	7, 17
(Saccharomyces ?)	yes	yes	Evaluated as carbohydrase	

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Isoamylase				7
(Bacillus cereus)				
(Pseudomonas amyloderamosa)	yes	yes	ADI not specified when used in applications as specified (starch processing)	CCFA 40
Laccase				
(*Aspergillus niger)*				CCFA 43 (AMFEP)
(*Aspergillus oryzae d-Myceliophthora thermophila)*	yes	yes		CCFAC 37
(Aspergillus oryzae d-*Polyporus sp.)*				CCFA 40 (CRD14 AMFEP)
(Trichoderma reesei or longibrachiatum d-*Thielavia sp.)*				CCFA 40 (CRD14 AMFEP)
Lactase or Beta galactosidase				
(*Aspergillus niger)*	yes	yes	Evaluated as carbohydrase	7
(*Aspergillus oryzae*)	yes	yes	Evaluated as carbohydrase	7,10
(Aspergillus oryzae d-*Aspergillus sp)*				CCFA 40 (CRD14 AMFEP)
(Bacillus circulans)				CCFA 40 (CRD14 AMFEP)
(*Candida pseudotropicalis)*				CX/FAC 92/7
(*Kluyveromyces fragilis)*				
(Kluyveromyces lactis)				
(Kluyveromyces lactis d-Kluyveromyces lactis)				
(Saccharomyces species)	yes	yes	Evaluated as carbohydrase	
Lactoperoxidase (Origin?)	yes	yes	under sodium percarbonate system for milk preservation	47,57
Lipase, monoacylglycerol (*Penicillium camembertii)*				CCFA 43 (AMFEP)
Lipase, triacylglycerol				
(Aspergillus niger)				7
(Aspergillus niger d-Candida antarctica)				
(Aspergillus niger d-Fusarium sp.)				CCFA 41 (CRD12 AMFEP)
(Aspergillus oryzae)	yes	Withdrawn 2000		1,7

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Aspergillus oryzae d-Rhizomucor miehei)				
(Aspergillus oryzae d-Humicola lanuginosa)				
(Aspergillus oryzae d-Fusarium oxysporum)				
(Aspergillus oryzae d-Thermomyces sp.)				CCFA 40 (CRD14 AMFEP)
(*Bacillus licheniformis d-Aeromonas sp*.)				CCFA 43 (AMFEP)
(Brevibacterium lineus)				46
(Candida lipolytica)				7
(Candida rugosa)				
(Mucor javanicus)				7
(Mucor pusillus)				
(Penicillium roqueforti)				
(Penicillium camembertii)				
(Pichia angusta or Hansenula polymorpha d-Fusarium sp.)				CCFA 43 (AMFEP)
(Rhizomucor miehei)				7
(Rhizopus nigrican)				7
(Rhizopus niveus)				
(Rhizopus oryzae)				
Lipoxygenase *(Escherichia coli* d-*Pea)*				CCFA 40 (CRD14 AMFEP)
Lysophospholipase				23
(Aspergillus niger)				
(Aspergillus niger d-Aspergillus niger)				
Malic acid decarboxylase (*Leuconostoc oenos*)				7
Maltase or alpha glucosidase				
(*Aspergillus niger)*	yes	yes		7
(*Aspergillus oryzae)*	yes	Yes		7
(Rhizopus oryzae)	yes	yes	Evaluated as carbohydrase	7
(Trichoderma reesei)				
Maltogenic amylase *(Bacillus subtilis d-Bacillus stearothermophilus)*	yes	yes		CX/FAC 92/7
Mannanase (endo-1.4-beta)				

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Aspergillus niger)				CCFA 40 (CRD14 AMFEP)
(Trichoderma reesei or longibrachiatum d-*Trichoderma sp.)*				CCFA 40 (CRD14 AMFEP)
Mixed xylanase, beta glucanase enzyme preparation *(Humicola insolens)*	yes			CCFAC 37
Nitrate reductase *(Micrococcus violagabriella)*				46
Pectate lyase (Bacillus subtilis)				CCFA 43 (AMFEP)
Pectinase or polygalacturonase				
(Aspergillus niger)	yes	yes		6, 7
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus oryzae)	yes	yes	Evaluated as carbohydrase	6, 7
(Aspergillus oryzae d-Aspergillus niger var. aculeatus)				
(Penicillium funiculosum)				
(Penicillium simplicissium)				7
(Rhizopus oryzae)	yes	yes	Evaluated as carbohydrase	7
(Trichoderma reesei)				7, 30
(Trichoderma reesei d-Aspergillus ?)				
Pectin esterase				
(Aspergillus niger)				
Pectin lyase				
(*Aspergillus niger)*	yes	yes	Evaluated as carbohydrase	20
(Aspergillus niger d-*Aspergillus sp.)*				CCFA 40 (CRD14 AMFEP)
(Aspergillus sojae)				CCFA 40 (CRD14 AMFEP)
(Penicillium funiculosum)				CCFA 40 (CRD14 AMFEP)
(Rhizopus oryzae or arrhizus)				CCFA 40 (CRD14 AMFEP)
(Trichoderma reesei or longibrachiatum d-*Aspergillus sp.)*				CCFA 40 (CRD14 AMFEP)
Pectin methylesterase or Pectinesterase				
(*Aspergillus niger)*	yes	yes	Evaluated as carbohydrase	20
(*Aspergillus oryzae d-Aspergillus sp.)*				CCFA 43 (AMFEP)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Aspergillus sojae sp.)				CCFA 40 (CRD14 AMFEP)
(Penicillium funiculosum)				CCFA 40 (CRD14 AMFEP)
(Rhizopus orzyae or arrhizus)				CCFA 40 (CRD14 AMFEP)
(Trichoderma reesei or longibrachiatum d-*Aspergillus sp.)*				CCFA 40 (CRD14 AMFEP)
Pentosanase				
(Aspergillus niger)				CCFA 43 (AMFEP)
(Bacillus amyloliquefaciens or subtilis)				CCFA 43 (AMFEP)
(Bacillus amyloliquefaciens or subtilis d-Bacillus sp.)				CCFA 43 (AMFEP)
(Humicola insolens)				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum)				CCFA 43 (AMFEP)
Peroxidase *Aspergillus niger d-Marasmius scorodonius)*				CCFA 43 (AMFEP)
Phosphodiesterase				
(Leptographium procerum)				
(Penicillium citrinum)				
Phospholipase A				
(Aspergillus niger)				CCFA 43 (AMFEP)
(Aspergillus niger d-*Aspergillus sp)*				CCFA 40 (CRD14 AMFEP)
(Streptomyces violaceoruber d-Streptomyces sp.)				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum d-*Aspergillus)*				CCFA 40 (CRD14 AMFEP)
(Trichoderma reesei or longibrachiatum d-*Thermomyces sp.)*				CCFA 40 (CRD14 AMFEP)
Phospholipase A1 (*Aspergillus oryzae d-Fusarium venenatum)*	yes	yes	ADI not specified when used in applications as specified in accordance with good manufacturing practice)	CCFA 40
Phospholipase A2				
(Aspergillus niger d-porcine pancreas)				
(Streptomyces chromofuscus)				
(Streptomyces violaceoruber)				
Phospholipase B				
(Aspergillus niger)				CCFA 43 (AMFEP)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(*Aspergillus niger d-Aspergillus sp.*				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum d-*Aspergillus sp.)***)**				CCFA 40 (CRD14 AMFEP)
Phospholipase C expressed in *Pichia pastoris*	yes	yes	ADI not specified when used in applications as specified in accordance with good manufacturing practice)	CCFA 41 JECFA 69
Phospholipase D *(Streptomyces cinnamoneus)*				CCFA 43 (AMFEP)
Phytase				CX/FAC 92/7
(Aspergillus niger)				
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus oryzae d-Peniophora lycii)				
(Trichoderma reesei d-Aspergillus ?)				
(Aspergillus niger d-Aspergillus niger)				
Polygalacturonase or Pectinase *(Aspergillus pulverulentus)*				CCFA 40 (CRD14 AMFEP)
Protease (including milk clotting enzymes)				
(Aspergillus melleus)				7
(Aspergillus niger)	yes	Not prepared		7
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus niger d-Camel stomach)				CCFA 43 (AMFEP)
(Aspergillus oryzae)	yes	yes		7
(Aspergillus oryzae d-Rhizomucor miehei)				
(Aspergillus sojae)				CCFA 40 (CRD14 AMFEP)
(Aspergillus sojae d-Aspergillus sp.)				CCFA 43 (AMFEP)
(Bacillus amyloliquefaciens)				
(Bacillus amyloliquefaciens d-Bacillus amyloliquefaciens)				
(Bacillus cereus)				7
(Bacillus clausii)				CCFA 43 (AMFEP)
(Bacillus licheniformis)				7
(Bacillus licheniformis d-Bacillus sp.)				CCFA 40 (CRD14 AMFEP)

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Bacillus licheniformis d-Nocardiopsis sp.)				CCFA 41 (CRD12 AMFEP)
(Bacillus stearothermophilus)				
(Bacillus subtilis)	yes	yes	Evaluated as mixed carbohydrases and proteases	1,7
(Bacillus subtilis d-Bacillus amyloliquefaciens)				
(Bacillus subtilis d-Thermus sp.)				CCFA 41 (CRD12 AMFEP)
(Bacillus thermoproteolyticus)				CCFA 40 (CRD14 AMFEP)
(Brevibacterium lineus)				46
(Endothia parasitica) -rennet from	yes	Withdrawn 2000		1,7
(Endothia parasitica d-Endothia parasitica				
(Fusarium venenatum d- Fusarium sp.)				CCFA 41 (CRD12 AMFEP)
(Geobacillus caldoproteolyticus)				CCFA 43 (AMFEP)
(Lactobacillus casei)				46
(Micrococcus caseolyticus)				56
(Mucor pusillus) -rennet from	yes	yes		1,7
(Penicillium citrinum)				CCFA 40 (CRD14 AMFEP)
(Rhizomucor miehei) -rennet from	yes	yes		1,7
(Rhizopus niveus)				
(Rhizopus oryzae)				
(Streptococcus cremoris)				46
(Streptococcus lactis)				
(Trichoderma reesei or longibrachiatum d-Trichoderma sp.)				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum d-Bos Taurus)				CCFA 43 (AMFEP)
Protein-glutaminase *(Chryseobacterium proteolyticum)*				CCFA 40 (CRD14 AMFEP)
Pullulanase				CX/FAC 92/7
(*Bacillus acidopullulyticus)*				30, 20
(Bacillus brevis)				CCFA 40 (CRD14 AMFEP)
(Bacillus circulans)				
(Bacillus licheniformis d-Bacillus deramificans)				

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
(Bacillus naganoensis)				
(Bacillus subtilis)				48, 49
(Bacillus subtilis d-*Bacillus acidopullulyticus)*				CCFA 40 (CRD14 AMFEP)
(Bacillus subtilis d-Bacillus naganoensis)				
(Bacillus subtilis d-Bacillus deramificans)				
(Klebsiella aerogenes)	yes	yes		7
(Klebsiella planticola)				CCFA 40 (CRD14 AMFEP)
(Klebsiella planticola d-Bacillus planticola)				
(Trichoderma reesei or longibrachiatum d-*Hormoconis sp.)*				CCFA 40 (CRD14 AMFEP)
Rhamnosidase				
(*Penicillium decumbens)*				
(*Penicillium multicolor)*				CCFA 40 (CRD14 AMFEP)
Serine proteinase				
(*Bacillus amyloliqu- efaciens)*				CX/FAC 92/7
(*Bacillus licheniformis)*				
(*Bacillus subtilis)*				CX/FAC 92/7
(*Streptomyces fradiae)*			Insufficient toxicological data available	23
Sulfhydryl oxidase *Bacillus subtillis* d-*Saccharomyces sp.)*				CCFA 40 (CRD14 AMFEP)
Tannase				
(*Aspergillus niger)*				7
(*Aspergillus oryzae)*				7
Transglucosidase				
(Aspergillus niger)				
(Trichoderma reesei or longibrachiatum d-Aspergillus sp.)				CCFA 43 (AMFEP)
(Trichoderma reesei or longibrachiatum d-Trichoderma sp.)				CCFA 43 (AMFEP)
Transglutaminase *(Streptomyces mobaraensis)*				
Urease *(Lactobacillus fermentum)*				

CATEGORY	JECFA Eval.	Specifications	JECFA comments	References
Xaa-Pro-dipeptidyl-aminopeptidase *(Lactococcus lactis)*				CCFA 40 (CRD14 AMFEP)
Xylanase				
(Aspergillus niger)				7
(Aspergillus niger d-Aspergillus niger)				
(Aspergillus oryzae d-Aspergillus niger var. aculeatus)				
(Aspergillus oryzae d-Humicola lanuginosa)				
(Aspergillus oryzae d-*Thermomyces sp.)*				CCFA 40 (CRD14 AMFEP)
(Bacillus amyloliquefaciens or subtilis)				CCFA 40 (CRD14 AMFEP)
(Bacillus licheniformis d-Bacillus licheniformis)				
(Bacillus subtilis d-Bacillus subtilis)	yes	yes		CCFAC 38
(Bacillus subtilis with modified gene from d-Bacillus subtilis)	yes	yes		CCFAC 38
(*Bacillus subtilis d-Pseudoalteromonas sp.)*				CCFA 41 (CRD12 AMFEP)
(Disporotrichum dimorphosporum)				7
(Fusarium venenatum d-Humicola lanuginosa)	61			CCFAC 37
(Humicola insolens)				
(Penicillium funiculosum)				CCFA 40 (CRD14 AMFEP)
(Streptomyces ?)				7
(Talaromyces emersonii)				CCFA 43 (AMFEP)
(Trichoderma reesei)				48
(Trichoderma reesei d-Trichoderma reesei)				
(Trichoderma viride)				CCFA 40 (CRD14 AMFEP)

REFERENCES (from main table of the IPA)

1. Appendix VI, ALINORM 79/12-A (Report of the 13th CCFA, List of processing Aids [prepared by Secretariat]).
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3. USA**,** letter (Witcher/Feberwee), 14.10.80
4. IOFI, letter (Grundschober/Ronk), 8.7-81.
5. Codex Comittee Fats and oils, letter (Burt/Rank), 16.7.81.
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7. AMFEP, letter (Noordervliet/Ronk), 20.8.81.
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11. European wax Federation, letter (Sayers/Ronk), 25.9.81.
12. UOP, Inc. letter (Moore/Ronk), 28.9.81.
13. (USA law firm), letter (Allera/Ronk), 29.9-81.
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17- France, letter (Gunzle/Ronk), 30.12.81.
I8. Crosfield Chemicals, letter (Burak/Ronk), 25.11.82.
19. IOFI, letter (Grundschober/Ronk), -7.12.82.
20. AMFEP, letter (Noordervliet/Ronk), 28.12.82.
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25. Sweden, letter (Agren/Ronk), 17.2.83.
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53. Italy, letter (Pricolo/Ronk), 2-2.88.

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APPENDIX A

CODEX INVENTORY OF COMPOUNDS USED AS PROCESSING AIDS WHICH ALSO SERVE OTHER FUNCTIONS

(excludes those substances already covered in the main IPA which were formerly annotated as (1.))

The substances are annotated according to the following system:

2. indicates those materials that are both food additives and processing aids (i.e. the substance functions as a processing aid in one food but may have a different function in another food).

3. indicates those compounds that because of carry-over residues, would seem to usually be considered only as food additives.

4. indicates those materials that might actually have simultaneous function as processing aids and functionality in the finished food.

Antifoam agents

(2) Aluminum stearate
(2) Butyl stearate
(3) Butylated hydroxyanisole (as antioxidant in defoamers)
(3) Butylated hydroxytoluene (as antioxidant in defoamers)
(2) Calcium stearate
(2) Dimethylpolysiloxane
(2) Fatty acids
(2) Hydroxylated lecithin
(2) Magnesium stearate
(3) Margarine
(2) Mineral oil
(2) Mono- and diglycerides of fatty acids
(2) n-Butoxypolyoxyethylene polyoxypropylene glycol
(2) Odourless light petroleum hydrocarbons
(2) Oleic acid from tall oil fatty acids
(2) Oxystearin
(2) Petroleum wax
(2) Petroleum wax (synthetic)
(2) Petrolatum
(2) Polyacrylic acid, sodium salt
(2) Polydimethylpolysiloxane (fruit juices at 10mg/kg CCFAC 37)
(2) Polyethylene glycol
(2) Polyethylene glycol (400) dioleate
(2) Polyethylene glycol (600) dioleate
(2) Polyglycerol esters of fatty acids
(2) Polyoxyethylene 40 monostearate
(2) Polypropylene glycol
(2) Polysorbate 60
(2) Polysorbate 65
(2) Polysorbate 80
(2) Potassium stearate
(2) Propylene glycol alginate
(2) Propylene glycol mono- and di-esters of fats and fatty acids

(2) Silicon dioxide
(2) Sorbitan monolaurate
(2) Sorbitan monostearate
(2) Soybean oil fatty acids
(2) Tallow
(2) Tallow, hydrogenated, oxidized or sulphated
(2) Tallow alcohol, hydrogenated
(3) Vegetable oil

Catalysts

(2) Ammonia
(2) Ammonium bisulfite
(2) Calcium chloride
(2) Ferrous sulfate
(2) Sodium chloride
(2) Sodium hydroxide
(2) Sodium metabisulfite
(2) Sulfur dioxide

Clarifying agents/filtration aids

(2) Acacia
(2) Agar
(2) Carbon dioxide
(2) Carrageenan/Furcelleran
(2) Casein
(2) Cellulose
(2) Cellulose powder
Chloromethylated aminated styrene-divinylbenzene resin
(2) Citric acid
(1) Diatomaceous earth
Divinylbenzene-ethylvinylbenzene copolymer
Fuller's earth
(2) Gelatin (edible)
(2) Phosphoric acid
Polyacrylamide/polysodium acrylate copolymer
Polymaleic acid and sodium polymaleate
(2) Polyvinylpyrrolidone
(2) Polyvinylpolypyrrolidone
(2) Potassium ferrocyanide
(2) Silicon dioxide amorphous - silica hydrogel
(2) Sodium alginate
(2) Stabilized aqueous silica sol
(2) Sulfur dioxide
(2) Tannic acid
(2) Wood flour/Sawdust

Colour stabilizers

(2) Dextrose
(2) Sodium acid pyrophosphate
(2) Sulphur dioxide

Contact freezing and cooling agents

(2) Brine (eg. salt brine)
(2) Carbon dioxide
Dichlorodifluoromethane
(2) Glycerol

Desiccating agent/anticaking agents

Aluminum stearate
(2) Calcium aluminum silicate
(2) Calcium silicate
Calcium stearate
(2) Magnesium carbonate, heavy
(2) Magnesium carbonate, light
(2) Magnesium oxide, heavy
(2) Magnesium oxide, light
(2) Magnesium silicate, synthetic
Magnesium stearate
(2) Magnesium trisilicate
(2) Silicon dioxide
(2) Silicon dioxide amorphous - silica gel
(2) Sodium aluminum silicate
Sodium calcium silicoaluminate
(2) Tricalcium diorthophosphate

Enzyme immobilization agents and supports

(2) Carrageenan (including Furcelleran)
(2) Gelatin
(2) Sodium alginate

Solvents (extraction and processing)

(2) Ammonia in methanol/ethanol
(2) Benzyl benzoate
(2) Butan-2-ol
(2) Butyl acetate
(2) Carbon dioxide
(2) Castor oil
(2) Diethyl tartrate
(2) Ethanol
(2) Ethyl acetate
(2) Ethyl lactate
(2) Glycerol
(2) Glycerol mono- di- and triacetate
Isobutanol (2-methylpropan-1-ol)
(2) Isopropyl alcohol
(2) Methanol
(2) Methyl acetate
Methyl propanol-1
(2) Nitric acid
(2) Propane-2-ol (isopropyl alcohol)

Trichlorofluoromethane
(2) Water

Fat crystal modifiers

(4) Lecithin
(4) Oxystearin
(4) Polyglycerol esters of fatty acids
(4) Polysorbate 60
(4) Sodium dodecylbenzene sulphonate
(4) Sodium lauryl sulphate
(4) Sorbitan monostearate
(4) Sorbitan tristearate

Flocculating agents

Acrylamide resins
(2) Aluminum ammonium sulfate
(2) Aluminum sulfate
(2) Citric acid
Dimethylamine-epichlorohydrin copolymer
(2) Gelatin
(2) Polyacrylic acid, sodium salt
(2) Silica
(2) Sodium alginate

Lubricants, release and anti-stick agents, moulding aids

Acetic acid esters of fatty acid mono- and diglycerides
(2) Acetylated monoglycerides
(2) Beeswax
(2) Butyl stearate
(2) Carnauba wax
(2) Calcium aluminum silicate
(2) Calcium carbonate
(2) Calcium phosphates
(2) Calcium silicate
(2) Calcium stearate
(2) Castor oil
(2) Edible bone phosphate
(2) Ethoxylated mono- and diglycerides
(2) Fats and waxes of vegetable and animal origin
(2) Fatty acids of tallow and vegetable oils
(2) Hydrogenated sperm oil
(2) Lecithin
(2) Magnesium carbonate
(2) Magnesium oxide, light and heavy
(2) Magnesium trisilicate
(2) Mineral oil based greases (lubricants for pumps)
(2) Mineral oil/Paraffin oil
(2) Mineral oils and waxes
Mono- and diglycerides of fatty acids
(2) Oxidatively polymerised soya bean oil

(2) Paraffin and paraffin oils
(2) Partially hydrogenated vegetable oil
(2) Polyglycerol esters of dimerised fatty acids of soya bean oil
(2) Polyglycerol polylinoleate
(2) Polyglycerol polyricinoleate
Shellac
Silicates (magnesium, potassium, sodium)
(2) Silicon dioxide
(2) Sodium aluminum silicate
(2) Starches
(2) Stearates (magnesium, calcium, and aluminum)
Stearates (potassium and sodium)
(2) Stearic acid
(2) Stearins
(2) Talc
(2) Tetrasodium diphosphate
(2) Tri-calcium phosphate
(2) Vegetable triglycerides
(2) Wax
(2) Wax coatings

Micro-organism control agents

(3) Disodium cyanodithioamidocarbonate
Disodium ethylene bis dithiocarbamate
Dimethyldicarbonate
(3) Ethylenediamine
(3) N-alkyl (C12-C16) dimethyl benzylchloride
(2) Natamycin
(2) Nitric acid
(3) Potassium N-methyldithiocarbamate
(3) Propylene oxide
(3) Sodium chlorite
Sodium dimethyldithiocarbamate
(2) Sulfur dioxide

Propellant and packaging gases

(2) Carbon dioxide
(2) Dichlorodifluoromethane
(2) Oxygen

Washing and peeling agents

(2) Ammonium chloride
Ammonium orthophosphate
(2) Calcium chloride
(2) Calcium hydroxide
(2) Calcium oxide
Diammonium orthophosphate
Dithiocarbamate
(2) Oleic acid
Organophosphates

(2) Sodium carbonate
(2) Sodium hydroxide
(2) Sodium hydroxide, 10%
(2) Sodium hydroxide, 2%
(2) Sodium tripolyphosphate
(2) Sulfuric acid

Yeast nutrients

(3) Ammonium chloride
(3) Ammonium sulphate
(3) Ammonium phosphates
(3) B-Complex vitamins
(3) Biotin
(3) Calcium carbonate
(3) Calcium phosphates
(3) Calcium sulphate
(3) Cupric sulphate
(3) Ferrous ammonium sulphate
(3) Ferrous sulphate
(3) Inositol
(3) Magnesium sulfate
(3) Niacin
(3) Pantothenic acid
(3) Potassium carbonate
(3) Potassium chloride
(3) Potassium hydrogen carbonate
(3) Yeast autolysates
(3) Zinc sulphate

Other processing aids

(2) Acetic acid
Acrylic resin with primarily tertiary amino groups
Alkylene oxide adduct
(2) Allyl isothiocyanate
(2) Ammonium bicarbonate
(2) Amyl acetate
(2) Benzyl alcohol
(2) BHA
(2) BHT
(2) Calcium carbonate
(2) Calcium chloride
(2) Calcium citrate
(2) Calcium hydroxide
Calcium oxide
(2) Calcium phosphates
(2) Calcium sulfate
(1) Calcium tartrate
(2) Caramel flavoring
Carbon dioxide

(2) Citric acid
(2) Coconut oil
(2) Disodium hydrogen phosphate
Ethylene oxide-propylene oxide copolymers
(2) Fatty acids of soybean oil
Fatty alcohol-glycol ether
(2) Fractionated soybean oil
(2) Fumaric acid
(2) Glycerol tripropionate
(2) Glycine
(2) Hydrochloric acid
(2) Hydrogenated soybean oil
Hydrophillic fatty acyl esters, linked to a neutral carrier
(2) Isopropyl alcohol
(2) Lactic acid
(2) Lactylated mono esters
(2) Magnesium chloride
(2) Magnesium citrate
Magnesium oxide
(2) Magnesium sulfate
(2) Magnesium hydroxide
(2) Magnesium phosphates
alpha-Methyl glycoside water
Methyl glycoside coconut oil ester
(2) Methyl paraben (Methyl parahydroxybenzoate)
(2) Mineral oil
Mixture of ethylene and propylene oxides, copolymers and esters, castor oil and polyethylene glycol ester
Mixture of naturally occurring and synthetic fatty acyl derivatives, with added emulgators
Modified higher alcohol
Mono- and diglycerides of fatty acids from feed fat (E471)
Mono- and diglycerides of fatty acids from feed fat, esterified with acetic acid, lactic acid and citric acid
Non-ionogenic alkylene oxide adduct with emulgator
(2) Oxalic acid
(2) Paraffin
(2) Phosphoric acid
Polyalkylene oxide, in combination with special fatty alcohols
Polyethoxylated alcohol, modified
Polyacrylate
Polyacrylate with carboxyl groups
Polyethylene glycol
Polyglycol copolymer
Polyphosphate
Polypropylene-polyethylene block polymer
(2) Polyvinylpyrrolidone
(2) Potassium carbonate
(2) Potassium chloride
(2) Potassium citrate

(2) Potassium nitrate
(2) Potassium phosphates
(2) Potassium sulfate
(2) Potassium tartrate
(2) Propyl gallate
(2) Propan-1-ol
(2) Propane-1,2-diol
(2) Shellac
(2) Sandarac gum
(2) Sodium chloride
(2) Sodium aluminosilicate
(2) Sodium bisulfite
(2) Sodium bicarbonate
(2) Sodium carbonate
(2) Sodium citrate
(2) Sodium hexametaphosphate
(2) Sodium hydroxide
(2) Sodium metabisulfite
(2) Sodium phosphate monobasic
(2) Sodium phosphate dibasic
(2) Sodium phosphate tribasic
Sodium polyacrylate
Sodium polyacrylate-acrylamide resin
(2) Sodium sulfate
Sodium sulfite
(2) Sodium tartrate
Solution of: anhyd. polyphosphate, polycarboxylic acid salt, polyalkylene glycol, sodium hydroxide
Sorbitan-fatty acyl esters and polyoxyethylene-20-sorbitan fatty acyl esters
(2) Soy lecithin
(2) Sulfuric acid
(2) Sulphur dioxide
Sulphonated copolymer of styrene and divinylbenzene
Surface-active esters with neutral carriers
Tannic acid with quebracho extract
(2) Tartaric acid
(2) TBHQ
Vegetable fatty acid esters
Vegetable fatty acyl (hydrophillic)
(2) Xylose

Fifteen pages have been removed in accordance with copyright laws. The removed reference citation is:

Z.S. Olempska-Beer, R. I. Merker, M. D. Ditto, M. J. DiNovi, “Food-processing enzymes from recombinant microorganisms—a review”, Regulatory Toxicology and Pharmacology, Vol. 45, pg 144-158, 2006.

Fourteen pages have been removed in accordance with copyright laws. The removed reference citation is:

M. W. Pariza, 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, pg 173-186, 2001.

February 2, 2023

RE: GRN 1030 – Additional FDA Question from Jan. 25, 2023

FDA Question for GRN 1030

a. The notifier states in the October 7, 2022 amendment that "Cellulase from *T. longibrachiatum* is affirmed as GRAS by FDA in 21 CFR 184.1250. *T. longibrachiatum* was renamed *T. reesei*" (p1). However, we note that 21 CFR 184.1250 states "Cellulase enzyme preparation is derived from a nonpathogenic, nontoxicogenic strain of *Trichoderma longibrachiatum* (formerly *T. reesei*)."

   For the record, please provide a reference and/or additional discussion to support the statement that "*T. longibrachiatum* was renamed *T. reesei*."

   Additionally, the October 7, 2022 amendment states that "the cellulase that is the subject of this notice [from the wild type *T. reesei*] is essentially the same protein that would be found in a cellulase enzyme preparation produced by *T. reesei* which would be covered by the regulation 21 CFR 184.1250." Please provide information to support this statement, noting that 21 CFR 184.1250 specifies *T. longibrachiatum*.

Novozymes response:

In our October 7, 2022 response where we said that "*T.longibrachiatum* was renamed *T. reesei*.", the choice of the word "renamed" was probably too strong. The names used to describe this organism have been inconsistent over the years and both *T. longibrachiatum* and *T.reesei* have been used. We are providing three articles that might be useful for supporting that both names have been used but that currently this organism is best described with *T. reesei*.

In Olempska-Beer et al 2006[1], the authors reviewed several articles on T. reesei and summarized the following:

T. reesei was first isolated from cotton canvas in the Solomon Islands in 1944 (Kuhls et al., 1996). The original isolate, QM6a, is the parent of practically all T. reesei industrial production strains (Nevalainen et al., 1994). During the 1980s, T. reesei was considered to be identical to Trichoderma longibrachiatum. More recent studies suggest that T. reesei is the asexual form of a tropical fungus, Hypocrea jecorina (Kuhls et al., 1996).

In 1999, FDA affirmed as GRAS the cellulase enzyme preparation derived from a nonpathogenic and nontoxicogenic strain of T. longibrachiatum (now known as T. reesei) (21 CFR 184.1250).

The Olempska-Beer et al 2006[1] article, seems to say that the subject of 21 CFR 184.1250 which was listed in 21 CFR as *T. longibrachiatum* (formerly *T. reesei*) in 1999, is "now known as *T.reesei*".

Novozymes North America, Inc.
Regulatory Affairs
77 Perry's Chapel Church Road
PO Box 576
Franklinton, NC 27525
United States

Phone: 919-494-3000
Fax: 919-494-3420

www.novozymes.com

By 2001, Pariza and Johnson[2] listed cellulase with the source as *Trichoderma reesei* (formerly *longibrachiatum*) in the Table 1, "Enzymes Used in Food Processing Today".

Finally, the Codex Alimentarius Commission[3] notes that *Trichoderma reesei* is also known as *Trichoderma longibrachiatum* on page 38 of the attached Inventory of Processing Aids.

The point that we were trying to make by mentioning the GRAS status of cellulase that is the subject of the FDA regulation in 21CFR 184.1250 is that this is a Novozymes product. This regulation is the result of a petition filed by AAC Consulting Group, Inc. on behalf of Novo Laboratories, Inc. which is now known as Novozymes A/S. Because this product is one of our commercial enzyme preparations, we are aware that the cellulase enzyme preparation (subject of 21 CFR 184.1250) is produced by a strain developed from *T. reesei* (ATCC56765) which is also the donor organism for the cellulase that is the subject of this GRN 1030 (as stated in Section 2.2(a) of the GRN). We believe this supports the GRAS status of the cellulase that is the subject of this GRN 1030.

Reference List

1. Olempska-Beer, Z.S. , Merker, R.I, Ditto, Mary D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganisms—a review. Reg. Tox and Pharm 45:144-158, 2006.
2. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Reg. Tox and Pharm 33: 173-186, 2001.
3. Codex Alimentarius Commission. Joint FAO/WHO Food Standards Programme. Codex Committee on Food Additives. FA/44/INF/03 China, March 2012.