A Glucose Oxidase Enzyme Preparation

Produced with

Aspergillus niger

Expressing the Glucose Oxidase Gene

From

Aspergillus niger

Is Generally Recognized As Safe

For Use in Food Processing

Where science
& creativity meet

**Notification Submitted by Danisco US Inc.
(a Wholly Owned-Subsidiary of International Flavors & Fragrances)**

January 07, 2022

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §170. 225, Danisco US Inc. submits this GRAS Notice for glucose oxidase produced with submerged fermentation of *Aspergillus niger* carrying the gene encoding the glucose oxidase enzyme from *Aspergillus niger*.

The glucose oxidase enzyme is intended for use in baking applications, egg processing such as de-sugared eggs, mayonnaise, salad dressing, and in cheese applications such as shredded cheese, specifically to facilitate fast and complete removal of glucose or oxygen. The enzyme catalyzes the oxidation of D-glucose to D-glucono-1,5-lactone, while reducing molecular oxygen into hydrogen peroxide. In these applications, the glucose oxidase will be used as processing aid in baking and egg processing, which either not be present in the final food or will be present in insignificant quantities as inactive residue, having no function or technical effect in the final food. The glucose oxidase will be present in insignificant quantities as active residue with function or technical effect in the final food in cheese production.

The systematic name and IUBMB nomenclature of the principle enzyme activity is glucose oxidase. Other names used are glucose oxyhydrase; corylophyline; penatin; glucose aerodehydrogenease; *etc*., as described in Section 2.2.1 of this submission. For consistency, this enzyme will be presented by the name "GOX" throughout the dossier.

The EC number of the enzyme is 1.1.3.4, and the CAS number is 9001-37-0.

The enzyme catalyzes the oxidation of D-glucose to D-glucono-1,5-lactone, while reducing molecular oxygen into hydrogen peroxide.

The information provided in the following parts is the basis of our determination of GRAS status of this GOX enzyme preparation.

Our safety evaluation is consistent with the recent publication by the Enzyme Technical Association (Sewalt *et al*., 2016),[1] which includes an evaluation of the production strain, the enzyme, and the manufacturing process (Part 6 of this dossier), as well as a determination of dietary exposure (Part 3 of this dossier). This generally recognized methodology, based on the decision tree by Pariza and Johnson (2001) and inclusive of published safety information, provides the common knowledge element of the GRAS status of this GOX enzyme notified to the US Food and Drug Administration (FDA) (Sewalt *et al.*, 2017).[2]

The safety of the production organism is prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster, 1983).

[1] https://doi.org/10.1089/ind.2016.0011
[2] https://www.sciencedirect.com/science/article/abs/pii/S0278691517303605?via%3Dihub

The safety of the production organism (*A. niger*) is discussed in Part 2 and 6 of this submission. The other essential aspect of the safety evaluation of enzymes derived from genetically engineered microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; IFBC, 1990; SCF, 1991; OECD, 1993; Berkowitz & Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and characterized as described in Part 2 of this dossier. The safety evaluation described in Part 3 and 6 of this dossier shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 § 170.225 (c)(2) Name and Address of Notifier

Danisco US Inc.
(a Wholly Owned-Subsidiary of International Flavors & Fragrances)
925 Page Mill Road
Palo Alto, CA 94304

1.2 § 170.225 (c)(3) Common or Usual Name of Substance

The glucose oxidase enzyme preparation is produced with an *Aspergillus niger* strain expressing the gene encoding the glucose oxidase from *Aspergillus niger*.

1.3 § 170.225 (c)(4) Applicable Conditions of Use

The glucose oxidase is intended to be used in baking (bakery applications) at 3.40 mg TOS/kg RM (raw material), in egg processing at 7.72 mg TOS/kg RM, and in cheese processing at 1.54 mg TOS/kg RM.

1.4 §170.225 (c)(5) Basis for GRAS Determination

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §170.30 (a) and (b).

1.5 §170.225 (c)(6) Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in 21 C.F.R. §170.225, Danisco US Inc. has determined that its GOX enzyme preparation from a genetically engineered strain of *Aspergillus niger* expressing the glucose oxidase enzyme from *A. niger* is a Generally Recognized As Safe (“GRAS”) substance for the intended food applications and is, therefore, exempt from the requirement for premarket approval.

1.6 §170.225 (c)(7) Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 during normal business hours or can be sent to the Food and Drug Administration upon request.

1.7 §170.225 (c)(8) and (c)(9) Disclosure and Certification

This GRAS notice does not contain any data and/or information that is exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C §552).

We confirm that the data and information in this GRAS notice satisfactorily addresses Part 2-7 of a GRAS notice per 21 C.F.R. §170.230 to 170.255 as copied below.

§170.230	Part 2 of a GRAS Notice:	Identity, method of manufacture, specifications, and physical or technical effects
§170.235	Part 3 of a GRAS Notice:	Dietary exposure
§170.240	Part 4 of a GRAS Notice:	Self-limiting levels of use
§170.245	Part 5 of a GRAS Notice:	Experience based on common use in food before 1958
§170.250	Part 6 of a GRAS Notice:	Narrative
§170.255	Part 7 of a GRAS Notice	List of supporting data and information in your GRAS notice

Danisco US Inc. certifies that to the best of our knowledge this GRAS notice is a complete, representative, and balanced submission that includes unfavorable and favorable information known to us as well as relevant to the evaluation of the safety and GRAS status of the use of the notified substance.

Annie Han Digitally signed by Annie Han
Date: 2022.01.07 14:11:50
-08'00'

January 07, 2022

Annie Han Date
Global Regulatory Affairs
Danisco US Inc. (A Wholly Owned-Subsidiary of International Flavors & Fragrances)
925 Page Mill Road
Palo Alto, CA 94304
Work: 650-846-4040
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2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATION AND PHYSICAL OR TECHNICAL EFFECT

2.1 PRODUCTION ORGANISM

2.1.1 Production Strain

The production organism is a strain of *Aspergillus niger* that has been genetically engineered to express the glucose oxidase (GOX) gene from *A. niger*. *Aspergillus niger* is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U. S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees, and is also considered as suitable for Good Industrial Large-Scale Practice (GILSP) worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Foster (1983). GOX was overexpressed in *A. niger* strain by chromosomal integration of multiple copies of the GOX coding sequence. GOX was placed under the expression signals (promoter and terminator) of the *A. niger* glucoamylase gene, retaining the native GOX signal sequence. The transformation vector was constructed such that no antibiotic resistance markers of bacterial DNA sequences were introduced into the host strain.

2.1.2 Recipient Organism

The host organism *A. niger* strain AGME9 was obtained from Solvay (Elkhart, IN). The host strain AGME9 was obtained by classical UV mutagenesis of *A. niger* strain ATCC 14916 (Miles Laboratories), and selection for improved glucoamylase production. The host strain contains (a) native copy(ies) of GOX, which contains an internal stop codon and presumably yields a truncated protein. GOX activity is negligible in the host.

Since 1997 *A. niger* has been included as a Toxic Substance Control Act (TSCA) Tier 1 exempt recipient microorganism under 40 C.F.R. §725.420 for strain submissions to the U.S. Environmental Protection Agency (EPA) as EPA considers *A. niger* to be a well characterized and low hazard species. *Aspergillus niger* is a non-pathogenic fungus. It is not present on the list of pathogens used by the E.U. (Directive Council 90/679/EEC, as amended), and culture collections from Germany (Deutsche Sammlung von Microorganismen und Zellkulturen, DMSZ), The Netherlands (CentraalBureau Schimmelculturen, CBS), USA (American Type Culture Collection, ATCC), *etc*., and it is listed as being suitable for the construction of Genetically Modified Microorganisms (GMMs) of Risk Group 1 in Germany, The Netherlands, *etc*.

2.1.3 Glucose Oxidase Expression Plasmid

The expression of the *A. niger* glucose oxidase coding sequence in the final production strain is controlled using the promotor sequence of the *A. niger* glucoamylase gene and the terminator sequence of the *A. niger (tubingensis)* glucoamylase gene. The strain also expresses an acetamidase gene from *Aspergillus nidulans* as a selection marker.

All these modifications were performed in such a way that no bacterial vector DNA remains present in the strain. No antibiotic resistance markers were inserted into the new microorganism. The genetic constructions were evaluated at every step to assess the incorporation of the desired functional genetic information, and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *A. niger* strain had been made.

2.1.4 Stability of the Introduced Genetic Sequences

The introduced GOX gene in the production strain proved to be 100% stable after industrial scale fermentation as judged by glucose oxidase production.

2.1.5 Antibiotic Resistance Gene

No antibiotic resistance genes were used in the construction of the production microorganism, and therefore the final production strain does not contain any antibiotic resistance genes.

2.1.6 Absence of Production Microorganism in Product

The absence of the production microorganism in the final product is an established specification for the commercial product and utilizes an analytical method with a detection limit of 1 CFU/g.

2.2 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

2.2.1 Enzyme Identity

Classification:	Oxidoreductases
IUB Nomenclature:	Glucose Oxidase
IUB Number:	1.1.3.4
CAS Number:	9001-37-0
Reaction catalyzed:	Catalyzes the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone.
Molecular weight	64 kDa

2.2.2 Amino Acid Sequence

The amino acid sequence of the *A. niger* GOX is known and included in Appendix 1.

2.3 MANUFACTURING PROCESS

This section describes the manufacturing process for this GOX enzyme which follows standard industry practice (Kroschwitz, 1994; Aunstrup *et al*., 1979; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. §110.

2.3.1 Raw Materials

The raw materials used in the fermentation and recovery process for this GOX concentrate are standard ingredients used in the enzyme industry (Kroschwitz, 1994; Aunstrup, 1979 and Aunstrup *et al*., 1979). All the raw materials conform to the specifications of the Food Chemicals Codex, 12th edition, 2020 ("FCC"), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco US Inc. uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoams (also known as defoamers) used in the fermentation and recovery are used in accordance with cGMP per the Food and Drug Administration (FDA) correspondence to Enzyme Technical Association (ETA) acknowledging the listed antifoams and flocculants dated September 11, 2003.

Regarding potential major food allergens, glucose (which may be derived from wheat) will be used in the fermentation process and is consumed by the microorganism as a nutrient (or as nutrients). The final dry products for the bakery applications can be spray-dried on potato or wheat starch. Since bakery products are produced with similar allergen group (*e.g.*, wheat), no additional allergens are introduced into the final food. Therefore, the final enzyme preparation does not introduce any new major food allergens from the fermentation medium into the final food. No other major allergen substances are used in the fermentation, recovery processes, or formulation of this product.

2.3.2 Fermentation Process

The GOX enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *A. niger* described in Part 2. 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 in place and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

2.3.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process.

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

1. Primary separation –centrifugation or filtration;
2. Concentration – ultrafiltration;
3. Addition of stabilizers/preservatives; and
4. Polish filtration.

2.3.4 Formulation and Standardization Process

The final commercial formulation (microgranulate) contains 5-10% enzyme, 40-45% microcrystalline cellulose, 0.5% sodium benzoate, 25-30% wheat flour, 0.88% L-ascorbic acid, 1.31% potassium sorbate, and 17% inert ingredients (fermentation solids). The remaining portion of the formulation is water. The microgranulate is stabilized with the formulation ingredients listed above and tested to demonstrate that it meets the product specifications.

The final GOX formulation is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA") in 2006 and FCC, 12th edition (USP, 2020). These specifications are set forth in Section 2.4.

2.4 COMPOSITION AND SPECIFICATIONS

2.4.1 Quantitative Composition

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition for spray-dried commercialized product:

Glucose Oxidase	5-10%
Microcrystalline cellulose	40-45%
Sodium benzoate	0.5%
Wheat flour	25-30%
L-ascorbic acid	0.88%
Potassium sorbate	1.31%

The preparation includes TOS (total organic solids resulting from the fermentation), which is approximately 23.7% of the final commercial formulation.

2.4.2 Specifications

As mentioned, glucose oxidase preparation meets the purity specifications for enzyme preparations set forth in FCC, 12th edition (USP, 2020). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA (2006).

The results of analytical testing of the 3 lots of product is given in Appendix 3 verifying that it meets USP (2020) and JECFA (2006) specifications for enzyme preparations.

2.5 APPLICATION

2.5.1 Mode of Action

GOX catalyzes the hydrolysis of glucose to hydrogen peroxide and D-glucono-δ-lactone. In cheese manufacturing, glucose oxidase can aid in the removal of trace levels of O_2 before packaging. In bakery applications, glucose oxidase can facilitate the handling of the dough by improving the dough structure and behavior to ensure a uniform finished product. In the manufacture of mayonnaise and salad dressing, the fat may cause lipid oxidation with the presence of oxygen which may deteriorate the taste and reduce the shelf-life. Glucose oxidase is added to scavenge oxygen to prolong the shelf-life for mayo and salad dressing before sealing process. In the de-sugaring of eggs, Glucose oxidase is applied in egg whites to avoid the Maillard chemical reaction which results in brown color and loss of solubility.

2.5.2 Use Levels

The GOX preparation is intended for use in baking, egg processing, and in cheese applications.

The table below shows the recommended use levels for each application where the GOX may be used.

Application	Raw Material (RM)	Recommended Use Level (mg TOS/kg Raw Material)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	0.85-3.40	3.40
Egg processing	Eggs	2.32-7.72	7.72
Cheese	Milk	0.15-1.54	1.54

2.5.3 Enzyme Residues in the Final Foods

The GOX enzyme will be deactivated or removed during the subsequent production and refining processes for baking and egg processing. It will be active in cheese production. In the case that inactive or active GOX is present in the processed food and is ingested, it will not be absorbed intact. Instead, the enzyme is expected to be broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which is not expected to pose any human health risk.

3. DIETARY EXPOSURE

GOX will be used as a processing aid in baking, egg processing, and in cheese applications.

While we expect the GOX to be not present in the final food or present as inactive or active residue in negligible amounts, the following conservative calculations assume that 100% of the enzyme remains in the processed food, as total organic solids (TOS).

The exposure to GOX in baking, egg processing, and in cheese applications is outlined below via the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method has been used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001). The method enables calculation of a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake. The Budget Method is based on the following assumed consumption of targeted important foodstuffs and beverages (for less important foodstuffs, *e.g.*, snacks, lower consumption levels are assumed). The assumption is for processed food (50% of total solid food) and for soft drinks (25% of total beverages).

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	**0.0125**	**0.025**

The recommended use levels of the enzyme GOX are given, based on the raw materials used in the food process. The calculation considers how much solid or liquid food is obtained per kg raw material, and it is assumed that all the TOS will end up in the final product. Therefore, the concentration of TOS from GOX in the baking, egg processing, and cheese applications can be calculated/summarized as in the table below:

	Application	Raw Material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Rate RM/FF	Maximal level in FF (mg TOS/kg food)
Solid Food	Baking	Flour	3.40	Bread, Bun, Cakes, and *etc.*	0.71	2.41
	Egg Processing	Eggs	7.72	Egg and egg white products	0.30	2.32
	Cheese	Milk	1.54	Cheese	1	1.54

For selecting an overall maximum exposure via the consumption of solid food, the worst-case TOS concentration in baking (2.41 mg TOS/kg) is appropriate.

HUMAN EXPOSURE ASSESSMENT

In this assessment, the Budget Method is used. This method was previously used by JECFA (FAO/WHO, 2001) and contains the following assumptions:

1) Level of consumption of foods and beverages:

For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day.

2) Concentration of enzymes in foods:

The concentration of enzyme in foods is the maximum application rate.

3) Proportion of foods that contain the enzymes:
   a) A default of 50% of all solid foods is used to represent processed foods (*i.e.*, 12.5 g/kg bw/day).

4) Estimation of the theoretical maximum daily intake (TMDI).

To represent a worst-case scenario, TMDI for solid foods will be combined with the TMDI for beverages in the risk assessment.

Estimation of the TMDI for Solid Foods

The maximum dosage used in baking application is used for representation of worst-case scenario for solid food.

Solid food intake	25	g/kg bw/day
Processed food treated with enzyme (50%)	12.5	g/kg bw/day
Enzyme TOS in solid food as worse case	2.41	mg TOS/kg final food
TMDI solid food	0.03	mg TOS/kg bw/day

The Theoretical Maximum Daily Intake (TMDI)- Total

TMDI solid food	0.03	mg TOS/kg bw/day
TMDI total	0.03	mg TOS/kg bw/day

4. SELF-LIMITING LEVELS OF USE

As the enzyme will be used as processing aid in the food manufacturing process, there is no notable oral intake for humans. Therefore, self-limiting levels of use are not applicable.

In addition, as a processing aid the use levels are limited by economic reasons as customers are unlikely to use more enzyme than is needed to achieve the technical effects in order to minimize production costs.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Information regarding this enzyme's common use in food before 1958 is not provided as the statutory conclusion of our GRAS status, which is based on scientific procedures rather than common use before 1958.

6. SAFETY EVALUATION

6.1 SAFETY OF THE PRODUCTION STRAIN

The safety of the production organism is recognized as the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that common foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC 1990). Pariza and Foster (1983) 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." *A. niger* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

The ancestors of the production strain *A. niger* J39, ATCC 14916, and AGME9, are non-pathogenic and non-toxigenic and have been safely used for a long time. ATCC 14916, the direct ancestor of AGME 9, was the production strain of a commercial food-grade glucoamylase preparation, in the 1960s. During the late 1960s through the 1970s, AGME9 was the production strain of glucoamylases. The organism later, in the early 1990s, became the production strain of transglucosidase enzyme preparations. Enzyme preparations derived from AGME9 are routinely tested for mycotoxins using the standard JECFA method, in which for the past 10 years all the tested lots had negative results.

6.1.1 Safety of the Host

The safety of *A. niger* has been discussed in several review papers (Schuster, *et al*., 2002; Olempska-Beer *et al.*, 2006, Frisvad *et al.*, 2011, Sewalt *et al*., 2016, Frisvad *et al.*, 2018, Li *et al*., 2020). *Aspergillus niger* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production. It is concluded that the strain is non-pathogenic and non-toxic. *Aspergillus niger* is not listed in Annex III of EU Directive 2000/54/EC – which lists microorganisms for which safety concerns for workers exist-, as it is globally regarded as a safe microorganism:

- In the US, *A. niger* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (NIH, 2019). Data submitted in Generally Recognized as Safe (GRAS) notifications to the Food and Drug Administration (FDA) for numerous enzyme preparations from *A. niger* for human and animal consumption demonstrate that the enzymes are nontoxic.

- The Environmental Protection Agency (EPA) has exempted *A. niger* strains harboring new intergeneric trains from review by the Agency, due to its extensive history of safe use (Tier 1 exemption under 40 C. F. R. §725.420. EPA, 1997).

- In Europe, *A. niger* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (BAuA, 2010) and the Federal Office of Consumer Protection and Food Safety (BVL) (BVL, 2013), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010). As a result, *Aspergillus niger* can be used under the lowest containment level large scale, GILSP, as defined by OECD (1992).

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. It is one of the most important producers of

industrial enzymes (Uhlig, 1998, Aunstrup, 1979, and Li *et al.*, 2020). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. According to literature, relevant mycotoxins are Ochratoxin A and Fumonisin B2 (Schuster *et al.*, 2002; Nielsen, 2009; Blumenthal, 2004; Frisvad *et al*., 2011, and Frisvad *et al*., 2018). As required by the JECFA specifications for food enzymes preparations, Danisco US Inc. ensures that the GOX food enzyme preparations do not contain toxicologically significant levels of Ochratoxin A and Fumonisin B2 in addition to four other toxins (total aflatoxin, zaralenone, sterigmatocystin, and T-2 toxin) that could be produced by *A. niger*. *Aspergillus niger* is generally considered a safe production organism and is a common source organism for a range of enzyme products that are used as processing aids and direct additives in the international food and feed industries.

The GRAS affirmations and GRNs to support the use of *A. niger* as a safe production host include:

- Carbohydrase and cellulase enzyme preparation (21 CFR §173.120) (FDA, 1996[a]);
- Lipase enzyme preparation from *A. niger* (GRN 111);
- Lactase enzyme preparation from *A. niger* (GRN 132);
- Lipase enzyme preparation from *A. niger* (GRN 158);
- Phospholipase A2 enzyme preparation from *A. niger* expressing a gene encoding A2 from porcine phosphlipase A2 (GRN 183);
- Asparaginase enzyme preparation from *A. niger* expressing the asparaginase gene from *A. niger* (GRN 214);
- Lipase enzyme preparation from *A. niger* (GRN 296);
- Carboxypeptidase enzyme preparation from *A. niger* (GCN 345);
- Asparaginase enzyme preparation from *A. niger* (GCRN 428);
- Acid lactase from *A. niger* (GRN 510);
- Xylanase from A. niger (GRN 589);
- Phospholipase A1 from *Talaromyces leycettanus* produced in *A. niger* (GRN 651);
- Glucoamylase from *Penicillum oxalicum* produced in *A. niger* (GRN 657);
- Trehalase from *Myceliophthora sepedonium* produced by *A. niger* (GRN 699);
- Mannanase enzyme from *Talaromyces leycettanus* produced in *A. niger* (GRN 739);
- Beta-glucosidase from *A. niger* (GRN 750);
- *Triacylglycerol lipase* from *Rhizopus oryzae* produced in *A. niger* (GRN 783);
- Chymosin enzyme from *Camelus dromedarius* produced in *A. niger* (GRN 801);
- Acid prolyl endopeptidase produced by *A. niger* (GRN 832);
- Phospholipase A1 produced by *A. niger* (GRN 857); and
- Citric acid (21 CFR §173.280) (FDA, 1996[b]).

Food enzymes derived from *A. niger* strains (including recombinant strains) have been evaluated by many countries which regulate the use of food enzymes, such as the France, Australia, Brazil, China, Mexico and Canada, resulting in the approval of the use of food enzymes from *A. niger* in the production of various foods, such as baking, brewing, carbohydrate processing, egg processing, and dairy products.

A review of the literature search on the organism (1967 – 2021) uncovered no reports that implicate *A. niger* in any way with a disease situation, intoxication, or allergenicity among healthy adult humans and animals. The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Aspergillus niger has a long history of safe use in industrial scale enzyme production. Shuster *et al*. (2002) provided an overview of *A. niger* and its safety as an industrial production organism. The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries.

From the information reviewed, it is concluded that the organism *A. niger* strain provides no specific risks to human health and is safe to use as the production organism of GOX. The strain is non-pathogenic and non-toxigenic.

6.1.2 Safety of the donor source

The donor strain used as a source for the GOX gene was *A. niger* strain NRRL3, which is the same species as the host microorganism and a widely used strain for industrial scale production (Crueger & Crueger, 1990, Witt *et al.,* 1998). The safety of *A. niger* has already been discussed in Section 6.1.1.

Aspergillus nidulans acetamidase (*amdS*) gene was used as a selectable marker, to enable growth on acetamide medium. Only the *amdS* gene in isolated form was used. The gene was first described by Hynes *et al*. (1983). The strain was not described further than "a strain of genotype biA1" but it is certainly a derivative of the original *Aspergillus nidulans* isolate (Glasgow wild-type) deposited as strain A4 at the Fungal Genetics Stock Center, Kansas City, USA. Meanwhile, the description of the gene in GenBank (Accession number M16371) mentions the Glasgow wild-type *Aspergillus nidulans* strain as the source. Sequencing and

PCR experiments verified that the gene Danisco US Inc. used is the same as published by Corrick *et al*. (1987).

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce GOX is conducted in a manner like other food and feed enzyme production processes. It consists of a pure-culture fermentation process, cell separation, concentration, and formulation. The process is conducted in accordance with the current food good manufacturing practice (cGMP) as set forth in 21 C.F.R. §110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 12th Edition (US Pharmacopeia, 2020) and the general specifications for enzyme preparations used in food processing proposed by FAO/WHO (JECFA, 2006).

The fermentation process may use? glucose (which may be derived from wheat) that may contain trace amounts of wheat protein. This feedstock is expected to be consumed by *A. niger* as a source of nutrients. The final dry products for the bakery applications may be spray-dried on potato or wheat starch, but since bakery products are produced from ingredients from common allergen groups (*e.g.,* wheat), no additional allergens are introduced into the final food. Therefore, the final enzyme preparation is reasonably expected not to contain any major food allergens from the fermentation medium. No other major allergen substances are used in the fermentation, recovery processes, or formulation of this product.

6.3 SAFETY OF GLUCOSE OXIDASE

Glucose oxidase has a long history of safe use in food processing. Fungal glucose oxidases have been reported to be used in food since 1957 (Underkofler and Ferracone, 1957; Underkofler, *et al.,* 1958).

Glucose oxidase from *A. niger* is part of the GRAS petition GRP 3G0016 that was submitted to FDA by Enzyme Technical association (ETA) and filed by FDA on April 12, 1973. Glucose oxidase from *A. niger* is recognized as Generally Recognized As Safe (GRAS) according to GRN 89. In addition, the US Food and Drug Administration (FDA) has provided "no questions letters" to assert GRAS (Generally Recognized as Safe) status to various glucose oxidase enzyme preparations such as: glucose oxidase from *Penicillium* produced in *T. reesei* (GRN 707),[3] glucose oxidase enzyme preparation derived from *P. chrysogenum* (GRN 509),[1] glucose oxidase enzyme preparation from *Aspergillus oryzae* carrying a gene encoding glucose oxidase from *A. niger* (GRN 106),[1] and glucose oxidase enzyme preparation from *A. niger* (GRN 89)[1] for applications such as baking, egg processing, and cheese manufacture.

[3]https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=glucose%20oxidase

Various other countries also approved glucose oxidase preparations derived from *A. niger*, *e.g.*, Canada,[4] and Australia/New Zealand (Glucose oxidase, see Australian Standard 1.3.3). JECFA approved glucose oxidase produced by *A. niger* (JECFA 2006).

Glucose oxidase produced with production organisms other than *A. niger* have also been proven safe worldwide *e.g.*, in Australia/New Zealand glucose oxidase from *A. oryzae* has been approved (Australian Standard 1.3.3).

Canada has approved glucose oxidases as a food additive from *A. oryzae* and *Trichoderma reesei*. [5]

6.3.1 Allergenicity

According to Pariza and Foster (Pariza and Foster, 1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP, 1998) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that the enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy" (Bindslev-Jensen *et al*., 2006).

Despite this lack of general concern, the potential that GOX could be a food allergen was assessed by comparing the amino acid sequence with sequences of known allergens in a public database, which is described in more detail below. To conduct the bioinformatic analysis of subtilisin, three FASTA searches were performed: 1) a full-length amino acid sequence search, 2) a sliding 80-amino acid window search, and 3) an 8-amino acid search. Based on the sequence homology alone, it was concluded that the GOX is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics

[4] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes html
[5] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes html

et al. (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (*i.e.*, in the context of allergenicity there may be a potential cross reactivity). Both Codex and Ladics *et al*. suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The mature *Aspergillus niger* GOX (mature) sequence is given in Appendix 1. A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database,[1] February 14, 2021 V21, containing 2233 peer-reviewed allergen sequences listed in the database[2] (using E-value <0.1) yielded no matches > 35% identity.

There was also no match to allergens by identity across 80 amino acids exceeding 35%. FASTA alignment of the above sequence with known allergens also using the AllergenOnline database[6] revealed no match (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities.

Although cautioned in Codex Commission (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and AllergenOnline.org that there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and that could be missed by the conservative 80 amino acid match (35%), this database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous amino acids search on the GOX sequence also produced no sequence matches with known allergens.

Microbial enzymes acting as environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally

[6] http://www.allergenonline.org/index.shtml

challenged commercial enzymes in a double-blind placebo-controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a food allergy concern.

In conclusion, based on the sequence homology alone, *A. niger* GOX is unlikely to pose a risk of food allergenicity.

6.3.2 Safety of Use in Food

As noted in the Safety section 6.1, *A. niger* and enzyme preparations produced there with, including asparaginase, beta-glucosidase, carbohydrase, carboxypeptidase, chymosin, endopeptidase, glucoamylase, lactase, lipase, mannanase, phospholipase A1, phospholipase A2, trehalase, and xylanase enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as Danisco US Inc.'s own unpublished safety studies, support such a conclusion.

A. niger is widely used by enzyme manufacturers around the world to produce enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

In addition to the allergenicity assessment described above, the safety of this glucose oxidase has also been established using the Pariza and Johnson (2001) decision tree:

1. **Is the production strain[7] genetically modified[8,9]?** Yes, go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes, go to 3a.

[7] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this.
[8] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (*e.g.*, UV or chemically-induced mutagenesis) or rDNA technologies.
[9] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

3a. Does the expressed enzyme product which is encoded by the introduced DNA[10,11] have a history of safe use in food[12]? Yes, GOX has been used for years in food processing. It is homologous to the *A. niger* GOX affirmed as GRAS (GRN 89), and its protein sequence is not similar to known sequences of food allergens and toxins. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[13]? Yes. No antibiotic resistance genes were used in the construction of the production strain. Go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? Yes, inserted DNA is well characterized and free of unsafe attributes. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome? Yes. Go to 5.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? Yes. The inserted DNA is well characterized. The production strain does not produce toxic metabolites of concern as confirmed by mycotoxin analysis. Go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[14]? Yes. AGME9, a strain containing

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[10] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[11] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[12] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

[13] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (https://www.gpo.gov/fdsys/pkg/FR-1998-09-08/pdf/98-24072.pdf)

[14] In determining safe strain lineage, one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances, the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage

no foreign DNA, has a long history as a production strain for food-grade enzyme preparations with a safe lineage. Its safety as a production host and methods of modification are well documented and their safety have been confirmed through a battery of toxicology testing.

Conclusion: The test article is ACCEPTED, and it has been verified that the NOAEL derived from existing toxicological studies is sufficiently high to provide adequate margin of exposure (please refer to Section 6.4.2 of this notification).

6.3.3 Safety Studies

Aspergillus niger glucose oxidase is an enzyme preparation produced with *A. niger* that can be used in baking, egg processing, and cheese applications.

To assess the safety of glucose oxidase in baking, egg processing, and cheese applications; different endpoints of toxicity were investigated at Scantox/CiTox laboratories (Denmark) and the results are evaluated, interpreted, and assessed in this document. The test material, Ultra-Filtered Concentrate (UFC), used in all toxicology investigations had the following characteristics:

Lot No.	R-Gox-04004
Physical	Clear Brown Liquid
Enzyme	Glucose Oxidase
Enzyme Activity	2423 U/ml
pH	4.70
Specific gravity	1.023 g/ml
Total Protein (TP)	36.24 g/ml=65.10 mg TOS/ml
TOS	6.51%

Different endpoints of toxicity of this glucose oxidase were investigated as part of our safety program to satisfy international and external requirements globally. This battery of tests included:

1) Genotoxicity Studies (*in vitro* chromosomal aberration, mouse micronucleus, Ames)
2) 90-day oral toxicity study in rats
3) Primary eye irritation study
4) Dermal irritation study

A. Acute dermal irritation study in rabbits (sequential approach), 2005.

a. Procedure:

The objective of this study was to assess the local irritant effect of GOX. This study was conducted according to the method recommended in the OECD Guideline No. 404, April 2002 and evaluated according to Commission Directive 2001/59/EC of 6 August 2001. In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and readings were made at 1, 24, 48 and 72 hours post application. The skin was scored for erythema and edema formation and the mean score calculated.

b. Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. Very slight erythema was noted in one animal. No eschar or edema was observed at these test sites at any of the examinations throughout the study. The primary irritation score (PIS) for erythema was 0.1/8.0 and the PIS for edema was 0.0/8.0.

c. Evaluation

Based on the results obtained in this study, GOX is classified as non-irritant according to the Commission Directive 20001/59/EC of August 6, 2001 adapting to technical process for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances.

B. Acute Eye Irritation/Corrosion Study in the Rabbit, 2005.

a. Procedure

The objective of this study was to assess the ocular irritation potential of GOX. This study was conducted according to the method recommended in the OECD Guideline No. 405, 24 April 2002 and evaluated according to the Commission Directive 2001/59/EC of 6 August 2001. In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded at 1, 24, 48 and 72 hours later. The right eye served as control. At the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage. A confirmatory test was conducted with 2 rabbits. After termination of the study, 72 hours after treatment, the animals were sacrificed.

b. Results

In the confirmatory assay, slight conjunctival irritation was present (score 1) at the 1-hour period in one animal, but disappeared at subsequent observations (24, 48 and 72 hours after treatment). The primary irritation score was 0.0.

c. Evaluation

The primary eye irritation score was 0.0. According to the EEC Directive published in: EEC Directive published in: "Official Journal of the European Communities" No: L 383 A, volume 35, 29.12.1992, part B5: Acute toxicity (eye irritation) and No: L 110 A, volume 36, 04.05.1993, part 3.2.6.2 Ocular lesions (which is implemented in Commission Directive 2001/59/EC of 6 August 2001), GOX is classified as "non-irritant" to the eyes.

C. Bacterial Reverse Mutation Assay – Glucose Medium. 2006.

a. Procedure

The objective of this assay is to assess the potential of GOX to induce point mutation (frame-shift and base-pair) in five strains of *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The treat-and-plate method was selected since enzyme preparation can contain free histidine and tryptophan in amounts that are not compatible with a plate incorporation assay. In this method, bacterial suspension (in nutrient broth) was mixed with either S-9 mix (metabolic activation assay) or phosphate buffer (non-metabolic activation assay) and the test article (vehicle control, positive control or GOX). These mixtures were incubated at 37°C under shaking for 3 hours. At the end of the 3-hour period, the bacteria were sedimented by centrifugation, the supernatant was removed and the bacteria were resuspended in 2 ml of buffer. The cultures were then centrifuged, the supernatant was removed and the bacteria were re-suspended a second time in buffer and top agar was added. The contents of each tube were mixed and spread on selective agar plates. The plates were incubated for 72 hours at 37°C and then scored for revertants and viability.

The doses selected for the confirmatory phase were based on results from a preliminary toxicity test performed in strain TA 98. Triplicate plates were run for each dose level and the entire confirmatory assay was repeated twice. The positive controls used for assays without S-9 mix were 2-nitrofluorene, 9-aminoacridine, cumene hydroperoxide and sodium azide. The positive control used for assays with S-9 mix was 2-aminoanthracene. This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

In the preliminary phase, dose levels ranging from 50 to 5000 µg/plate were used. The highest dose level tested (5000 µg/plate) is the maximum required by OECD guideline. Severe cytotoxicity was noted at the three highest doses tested (500, 1600 and 5000 µg/plate) in the absence of S-9 mix and at the four highest doses tested (160, 500, 1600 and 5000 µg/plate) in the presence of S-9 mix. Due to cytotoxicity noted at higher dose levels, the dose levels selected for the main (confirmatory) study were as follows:

Strains	**Main Test 1**	**Main Test 2**
TA 98	0.5 to 5000 µg/plate	0.5 to 160 µg/plate
TA 100	0.5 to 5000 µg/plate	1.6 to 500 µg/plate
TA 1535	0.5 to 5000 µg/plate	1.6 to 500 µg/plate
TA 1537	0.5 to 5000 µg/plate	0.5 to 160 µg/plate
TA 102	0.5 to 5000 µg/plate	1.6 to 500 µg/plate

In the first main test, cytotoxicity was evident at > 500 µg/plate so the highest dose tested for the second main test was decreased to 160 µg/plate for strain TA98 and TA1537. The 500 µg/plate was the highest dose used for strains TA 100, TA 1535, and TA 102. Scattered incidences of statistically significant increases were noted at the highest dose level tested but none of these increases meet the criteria of a positive response (*i.e.*, 2-fold increase over vehicle control and reproducibility of findings between replicate plates). No biologically significant increases in the number of revertant colonies were observed in any tester strain after treatment with glucose oxidase at any dose level, either in the absence or presence of S-9 mix. Positive mutagenic response was observed with the positive control plates substantiating the validity of the assays.

c. Evaluation

Under the conditions of this assay, GOX is not a mutagen in *S. typhimurium* in both the presence and absence of metabolic activation.

D. Bacterial Reserve Mutation Assay – Fructose medium. 2006.

a. Procedure

The objective of this assay is to assess the potential of GOX to induce point mutation (frame-shift and base-pair) in five strains of *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537. The study was performed using selective agar plates containing fructose instead of glucose. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). A preliminary study was

performed first with the "plate incorporation" method, but increased growth of the background lawn was noted and this effect might be due to the presence of histidine and tryptophan in amounts that are not compatible with a plate incorporation assay. A second preliminary study was then conducted with the "treat-and-plate" method. In the latter, bacterial suspension (in nutrient broth) was mixed with either S-9 mix (metabolic activation assay) or phosphate buffer (non-metabolic activation assay) and the test article (vehicle control, positive control or GOX). These mixtures were incubated at 37°C under shaking for 3 hours. At the end of the 3-hour period, the bacteria were sedimented by centrifugation, the supernatant was removed and the bacteria were resuspended in 2ml of buffer. The cultures were then centrifuged, the supernatant removed and the bacteria were resuspended a second time in buffer and top agar was added. The contents of each tube were mixed and spread on selective agar plates. The plates were then incubated for 72 hours at 37°C and then scored for revertants and viability.

The doses selected for the confirmatory phase were based on results from a preliminary toxicity test performed in strain TA 98. Triplicate plates were run for each dose level and the entire confirmatory assay was repeated twice. The positive controls used for assays without S-9 mix were 2-nitrofluorene, 9-aminoacridine, cumene hydroperoxide and sodium azide. The positive control used for assays with S-9 mix was 2-aminoanthracene. This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

In the first preliminary phase (plate incorporation procedure), increased growth of the background lawn of non-revertant bacteria and small increases in the number of revertant colonies were observed at the two highest dose levels. These results suggested that histidine in GOX interfered with the test system. In the second preliminary phase (treat and plate procedure), GOX was toxic at 500, 1600 and 5000 µg/plate without S9 mix and at 5000 µg/plate with S9. The definitive phase consists of two main tests with doses ranging from 50 to 5000 µg/plate. The highest dose level tested (5000 µg/plate) is the maximum required by OECD guideline. GOX was toxic to some of the tester strains at higher dose levels (500, 1600 and 5000 µg/plate) causing reduced growth of the background lawn and reductions in the number of revertant colonies. A statistically significant increase was observed in strain TA 1535 in the first main test at 5000 µg/plate without S-9 mix but was not observed in the second main test. No biologically significant increases in the number of revertant colonies were observed in any tester strain at non-toxic doses (see table):

Strains	Without S-9 mix	With S-9 mix
TA 98	50 to 160 µg/plate	50 to 1600 µg/plate
TA 100	50 to 5000 µg/plate	50 to 5000 µg/plate
TA 1535	50 to 160 µg/plate	50 to 500 µg/plate
TA 1537	50 to 5000 µg/plate	50 to 160 µg/plate
TA 102	50 to 500 µg/plate	50 to 5000 µg/plate

Positive mutagenic response was observed with the positive control plates substantiating the validity of the assays.

c. Evaluation and Conclusion

The statistically significant increase noted in TA 1537 at 5000 µg/plate in the absence of S-9 mix does not meet the criteria for a positive response (*i.e., a* 2-fold increase over vehicle control and reproducibility of findings between replicate plates). No biologically significant increases in the number of revertant colonies were observed in any tester strain after treatment with glucose oxidase at any non-toxic dose level, either in the absence or presence of S-9 mix. Under the conditions of this assay, GOX is not a mutagen in *S. typhimurium* in both the presence and absence of metabolic activation.

E. Mouse Micronucleus Assay, 2006.

a. Procedure

The objective of this study was to investigate the potential of GOX as well as any other materials that could be present in the toxicology sample to induce chromosomal damage and aneuploidy *in vivo*. The genotoxic effect of the test material was obtained by comparing the frequency of micronucleated polychromatic (immature) erythrocytes (PCE) from the bone marrow of treated mice with the corresponding negative controls. A preliminary toxicity test was performed in both male and female SPF mice to select the highest dose level for the main study. Based upon the result of the preliminary study, the estimated maximum tolerated dose was 725 mg/kg. Because no relevant gender difference was observed in the preliminary study, only male mice were used in the definitive study. Three dose levels of GOX were given by gavage to groups of 5 male mice each on two occasions separated by 24 hours. All mice were sacrificed 24 hours after the last treatment. This study was conducted in accordance with OECD guideline No. 474 (July 1997) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

In the preliminary toxicity test, a dose level of 725 mg/kg/d was given by gavage to a group of 2 male and 2 female mice. None of the mice showed any adverse effects. The 725 mg/kg dose level was the maximum practical dose level based on a total protein content of 36.24 mg/ml and the maximum gavage volume (20 ml/kg bw). In the definitive study, groups of 5 male mice each were treated orally with 0, 181, 362 or 725 mg total protein/kg/day on 2 occasions separated by 24 hours. All animals were killed 24 hours after the last dosing. Cyclophosphamide at 20 mg/kg given by oral gavage served as positive control. Bone marrow smears from all groups were prepared on glass slides, stained and scored using a microscope. No adverse reactions to treatment were observed. No biologically or statistically significant increases in the frequency of PCE were seen in mice treated with the test material.

c. Evaluation and Conclusion

Under the conditions of this experiment, GOX and any other possible chemicals found in the test material did not demonstrate any genotoxic activity in the *in vivo* mouse micronucleus test.

F. A 13-week Oral (Gavage) Toxicity Study in Rats, 2006.

a. Procedure

The objective of this study was to investigate the potential of GOX to induce systemic toxicity after repeated daily oral administration to SPF Sprague-Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. The doses selected for this study were 0, 1.80, 3.60, or 10.87 mg total protein/kg bw/day corresponding to 3.23, 6.47, or 19.53 mg TOS/kg, respectively. GOX or vehicle control were given by gavage in a constant volume of 5 ml/kg and the volume administered to each animal was adjusted according to the most recent weekly body weight. All animals were observed daily for mortality and signs of morbidity. All groups were housed under controlled temperature, humidity and lightning conditions. Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and at study termination. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for the control and high dose rats. Hematology and clinical chemistry were measured at study termination prior to necropsy, which was performed on all groups. After a thorough macroscopic examination, selected organs were removed, weighed, and processed for future histopathologic evaluation. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

One high dose male (No. 61) was found dead on Day 29 before dosing and this death was attributed as intubation error as substantiated by the findings of fluid in the chest cavity and red lung. No clinical signs were seen in this study that could be related to treatment. There were no biological or statistical differences between the control and treated groups with respect to feed and water consumption, body weight, body weight gain, clinical chemistry and ophthalmologic examinations. In high dose males, there were statistically significant decreases in mean cell volume and mean cell hemoglobin and statistically significant increase in red blood cell count. No treatment related effects were observed on parameters from the Behavior and Functional Observation Battery tests conducted at study termination. No treatment-related changes in histopathology were found. All morphological changes were those commonly observed in laboratory rats of the age and strain employed.

c. Evaluation and Conclusion

Although statistical differences were noted in high dose males relative to some hematologic parameters, the toxicological significance of these findings is irrelevant considering the absence of associated morphological and pathological changes. Further, all values are still within the historical control range collected for this strain at the testing laboratory. There were no treatment related effects in other parameters investigated, from clinical observations to histopathologic examinations. Under the conditions of this assay, it can be concluded that oral feeding (gavage) of GOX in the diet for 90 continuous days did not result in systemic toxicity in rats. The NOAEL (no observed adverse effect level) is established at 10.87 mg total protein/kg bw (19.53 mg TOS/kg/day).

6.4 OVERALL SAFETY ASSESSMENT

6.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, a NOAEL was established at 10.87 mg total protein/kg bw/day corresponding to 19.53 mg TOS/kg bw/day. The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to *Aspergillus niger* GOX is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL: 19.53 mg TOS/kg bw/day = 10.87 mg TP/kg bw/day

6.4.2 Conclusion

Determination of the Margin of Safety

The margin of safety is calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst-case scenario) assessed in Part 3. If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{No Observed Adverse Effect Level (NOAEL)}}{\text{Maximum Daily Exposure}}$$

$$\text{Margin of Safety} = \frac{19.53 \text{ mg TOS/kg bw/day}}{0.03 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 651

6.5 BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the safety sections above, *Aspergillus niger* and its derived glucose oxidase (GOX) enzyme preparation is well recognized by qualified experts as being safe for its intended uses. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as IFF's own unpublished safety studies, support such a conclusion.

Aspergillus niger is used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

Toxicological studies for the subject enzyme are available. Genotoxicity assays were conducted with this glucose oxidase and, under the conditions of these assays, *A. niger* GOX is not classified as a mutagen, a clastogen, or an aneugen. The systemic toxicity of *A. niger* GOX was investigated in an oral study (90-day) and daily administration of *A. niger* GOX for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 19.53 mg TOS/kg bw/day.

Based on a worst-case scenario that a person is consuming GOX from the products of baking, egg processing, and cheese containing the glucose oxidase, the cumulative daily exposure of 0.03 mg TOS/kg bw/day.

Based on a margin of safety (651) greater than 100 (even in the worst-case), the proposed uses of glucose oxidase in baking, egg processing, and the manufacture of cheese are not a human health concern and are supported by existing toxicology data.

Based on the publicly available scientific data from the literature and additional supporting data generated by Danisco US Inc. (a wholly owned-subsidiary of International Flavor and Fragrances), and the decision tree analysis using generally recognized evaluation methodology (Pariza and Johnson, 2001; Sewalt *et al.*, 2016), the company has concluded that the glucose oxidase from *A. niger* strain is safe and suitable for use in baking, egg processing, and the manufacture of cheese. Collectively, the use of published information and evaluation methods provide a strong common knowledge element, based upon which this glucose oxidase can be considered Generally Recognized as Safe (GRAS) for its intended uses. In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by an external expert in the field, Dr. Michael Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 4).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES

Appendix 1: The Amino Acid Sequence of the Glucose Oxidase

Appendix 2: The Manufacturing Process

Appendix 3: Certificate of Analysis (3 lots)

Appendix 4: External Expert Opinion Letter from Dr. Michael Pariza

7.2 REFERENCES

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Appendix 1: The Amino Acid Sequence of the Glucose Oxidase

Aspergillus niger Glucose oxidase (GOX) (mature) sequence is given below in FASTA format.

SNGIEASLLTDPKDVSGRTVDYIIAGGGLTGLTTAARLTENPNISVLVIESGSYESDRGPIIEDLNAY
GDIFGSSVDHAYETVELATNNQTALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWDNV
AAYSLQAERARAPNAKQIAAGHYFNASCHGVNGTVHAGPRDTGDDYSPIVKALMSAVEDRGVPTKKDF
GCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNGTTPRAVGVEFGTH
KGNTHNVYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVDLPVGLNLQDQTTATVRSRITS
AGAGQGQAAWFATFNETFGDYSEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYRDWIVNHNV
AYSELFLDTAGVASFDVWDLLPFTRGYVHILDKDPYLHHFAYDPQYFLNELDLLGQAAATQLARNISN
SGAMQTYFAGETIPGDNLAYDADLSAWTEYIPYHFRPNYHGVGTCSMMPKEMGGVVDNAARVYGVQGL
RVIDGSIPPTQMSSHVMTVFYAMALKISDAILEDYASMQ

Appendix 2: Manufacturing Process Flow Diagram

PROCESS FLOW DIAGRAM

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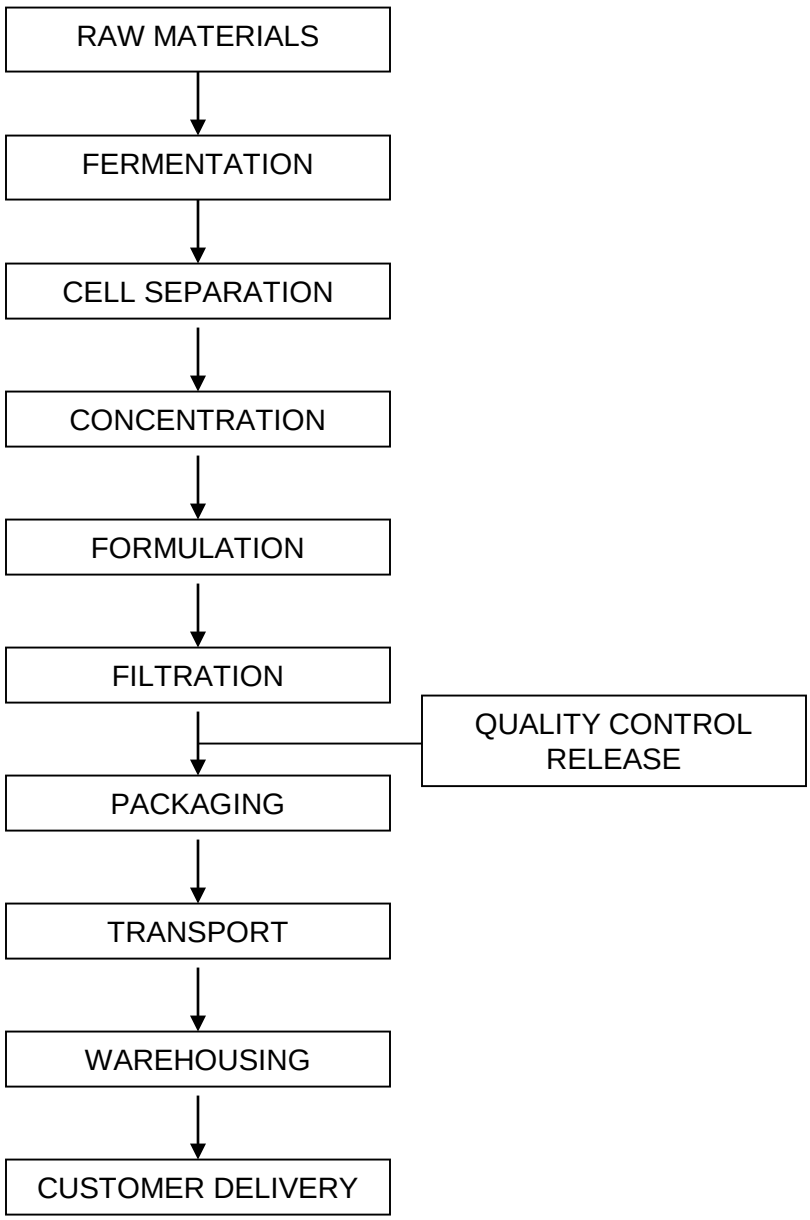

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Appendix 3: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **GC 199**
LOT NUMBER: **7203519227**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Glucose Oxidase	U/ml	1800-2000	1885
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1
Coliforms	CFU/ml	0-30	<1
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial activity	/ml	Negative by test	Negative
PHYSICAL PROPERTIES			
Specific gravity	%	1.15-1.20	1.17
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.05
Mercury	mg/kg	0-0.5	<0.05
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

12-Jul-2021
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

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CERTIFICATE OF ANALYSIS

PRODUCT: **GC 199**
LOT NUMBER: **7203520919**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Glucose Oxidase	U/ml	1800-2000	1861
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1
Coliforms	CFU/ml	0-30	<1
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial activity	/ml	Negative by test	Negative
PHYSICAL PROPERTIES			
Specific gravity	%	1.15-1.20	1.15
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.05
Mercury	mg/kg	0-0.5	<0.05
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

12-Jul-2021
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Where science
& creativity meet

CERTIFICATE OF ANALYSIS

PRODUCT: **GC 199**
LOT NUMBER: **7203879755**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Glucose Oxidase	U/ml	1800-2000	1869
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	15
Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial activity	/ml	Negative by test	Negative
PHYSICAL PROPERTIES			
Specific gravity	%	1.15-1.20	1.16
OTHER ASSAYS			
Lead	mg/kg	0-5	<5
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.05
Mercury	mg/kg	0-0.5	<0.05
Mycotoxins		Negative	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

29-Jul-2021
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 4: External Expert Opinion Letter from Dr. Michael Pariza

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

April 7, 2017

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the intended uses of DuPont's glucose oxidase enzyme expressed in *A. niger*

Dear Dr. Sewalt,

I have reviewed the information you provided on glucose oxidase (GOx) from *Aspergillus niger* AGME9 #J39, a genetically engineered strain that over-expresses a glucose oxidase gene derived from *A. niger* NRRL3. The intended uses of GOx are in cheese production such as shredded cheese, egg processing such as de-sugared eggs, mayonnaise, salad dressing, fruit and vegetable processing such as fruit juice and baking such as bread, the manufacture of gluconic acid derivatives which are used in a wide variety of foods as food supplements, sequestrants, stabilizers, thickeners and acidifiers, and as a fermentation aid in the manufacture of fuel ethanol with resulting distillers' grains destined for use in animal feed. In these applications the GOx enzyme will either be not present in the final food/feed or present at trace levels as inactive protein having no function or technical effect, except in the application of shredded cheese.

In evaluating GOx I considered the biology of *A. niger* and its history of safe use in food ingredient manufacture; the safe lineage of the GOx gene donor and recipient strains and their histories of safe use in food ingredient manufacture; safety evaluation studies on the GOx enzyme preparation; the history of safe use of glucose oxidases in food manufacturing applications; information that you provided regarding the safe lineage of the production organism, cloning methodology, manufacturing materials and procedures, and product specifications; and information that is publically available in the peer-reviewed scientific literature.

By way of background, *Aspergillus niger* is a mold that is commonly found in soil and on plants. It is an opportunistic pathogen that only rarely infects humans, typically those with compromised immune systems. The species does not possess the genetic elements needed to produce aflatoxin, but some strains produce ochratoxin and genome of one strain of *A. niger* contains a gene cluster that encodes for fumonisin (HJ Pel et al., Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnology 25 (2) 221-231, 2007).

Nontoxigenic strains of *A. niger* are widely utilized by food ingredient manufacturers for numerous applications including the production of enzyme preparations for use in human food and animal feed, including *A. niger* J39, ATCC 14916, and AGME9, the ancestral lineage strains for *A. niger* AGME9 #J39. The GOx gene donor was *A. niger* NRRL3, which also has a long history of safe use in food ingredient manufacture.

Glucose oxidase has a long history of safe use in food processing. For example, fungal glucose oxidases have been used in food since 1957, and glucose oxidase from *A. niger* is included in GRAS petition GRP 3G0016 that was submitted to FDA by the Enzyme Technical Association and filed by FDA in 1973. Glucose oxidases from *A. niger* and *Penicillum chrysogenum* are the subject of GRAS Notices to which FDA has responded with 'no questions' letters (GRNs 89 and 509, respectively).

The *A. niger* NRRL3 GOx protein was sequenced and studied for potential safety issues, specifically amino acid sequences that might elicit allergenicity or toxicity concerns. No such sequences were found.

The *A. niger* AGME9 #J39 GOx enzyme preparation was evaluated for acute dermal and eye irritation in rabbits, genotoxicity using bacterial and mammalian cell test systems, and subchronic toxicity (90 day oral gavage study) in SPF Sprague-Dawley rats. No dose-related adverse events were observed in any of these studies. The NOAEL for the *A. niger* AGME9 #J39 GOx enzyme preparation was established as the highest dose tested in to 90 day oral gavage study, 10.87 mg total protein/kg bw (19.53 mg TOS/kg/day). The cumulative exposure for human consumers to *A. niger* AGME9 #J39 GOx from all proposed sources was calculated as 0.038 mg TOS/kg bw/day, giving a Margin of Safety for GOx of 514. Similar calculations for animal feed use indicate Margins of Safety for cattle, pigs, and poultry, respectively, of 134, 107, and 124, respectively.

The cloning techniques and methodologies employed to construct *A. niger* AGME9 #J39 are appropriate for use in the genetic modification of production strains for food ingredient manufacture. In addition, the manufacturing process including the ingredients used for fermentation, extraction and concentration of GOx and the specifications for the GOx enzyme preparation, are appropriate for a food/feed ingredient.

Based on the foregoing, I concur with the evaluation made by DuPont that the *A. niger* AGME9 #J39 production strain is safe and appropriate to use for the manufacture of food-grade glucose oxidase (GOx). I further conclude that the GOx enzyme preparation, manufactured in a manner that is consistent with current Good Manufacturing Practice

(cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use in cheese production such as shredded cheese, egg processing such as de-sugared eggs, mayonnaise, salad dressing, fruit and vegetable processing such as fruit juice and baking such as bread, the manufacture of gluconic acid derivatives which are used in a wide variety of foods as food supplements, sequestrants, stabilizers, thickeners and acidifiers, and as a fermentation aid in the manufacture of fuel ethanol with resulting distillers' grains destined for use in animal feed. In these applications the GOx enzyme will either be not present in the final food/feed or present at trace levels as inactive protein having no function or technical effect, except in the application of shredded cheese.

It is my professional opinion that other qualified experts would also concur in this conclusion.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

Michael W. Pariza
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Annie Han	Senior Specialist, Global Regulatory Affairs

Organization *(if applicable)*
Danisco US Inc. (a Wholly Owned-Subsidiary of International Flavors & Fragrances)

Mailing Address *(number and street)*
925 Page Mill Road

City	State or Province	Zip Code/Postal Code	Country
Palo Alto	California	94304	United States of America

Telephone Number	Fax Number	E-Mail Address
650-846-4040	650-845-6502	annie.han@iff.com

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

Name of Contact Person	Position or Title
Annie Han	Senior Specialist, Global Regulatory Affairs

Organization *(if applicable)*
Danisco US Inc. (a Wholly Owned-Subsidiary of International Flavors & Fragrances)

Mailing Address *(number and street)*
925 Page Mill Road

City	State or Province	Zip Code/Postal Code	Country
Palo Alto	California	94304	United States of America

Telephone Number	Fax Number	E-Mail Address
650-846-4040	650-845-6502	annie.han@iff.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Glucose oxidase enzyme preparation produced with Aspergillus niger expressing glucose oxidase gene from Aspergillus niger

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

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

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

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

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

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

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – INTENDED USE

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

The enzyme is glucose oxidase (IUBMB 1.1.3.4) which catalyzes the oxidation of D-glucose to D-glucono-1,5-lactone, while reducing molecular oxygen into hydrogen peroxide. The enzyme is intended to be used as processing aid in baking at 0.85-3.4 mg TOS/kg RM (raw material), egg processing at 2.32-7.72 mg TOS/kg RM, and cheese production at 0.15-1.54 mg TOS/kg RM.

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

(Check one)

☒ Yes ☐ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☐ Yes ☒ No

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

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Danisco US Inc.
(name of notifier)

has concluded that the intended use*(s)* of Glucose oxidase enzyme preparation produced with Aspergillus niger expressing glucose oxid
(name of notified substance)

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

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

925 Page Mill Road, Palo Alto, California 94304, USA
(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Annie Han Digitally signed by Annie Han Date: 2022.01.07 14:19:41 -08'00'	Annie Han, Senior Specialist, Global Regulatory Affairs	01/07/2022

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667_GRASNotive_GlucoseoxidaseFromAspergillusniger_2022-01-07.pdf	Administrative
	GRASNotice_GlucoseOxidaseFromAspergillusniger_2022-01-7.pdf	Submission

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

Date: June 10, 2022

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To: Katie Overbey, Ph.D., M.S
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

From: Annie Han
Global Regulatory Affairs
Danisco US Inc.

RE: FDA Questions on GRN 1054 Glucose Oxidase Produced with *Aspergillus niger*

Dear Dr.Overbey,

Thank you for your review of our submission! We are providing this letter in response to FDA's questions that was sent via email on June 02, 2022 regarding our glucose oxidase submission. We have copied the request for information above our responses for your reference in this response letter:

1. Can you please clarify the intended uses of this ingredient? Your notice states intended uses in "baking, egg processing, and cheese processing" while your provided GRAS panel statement states "cheese production such as shredded cheese, egg processing such as de-sugared eggs, mayonnaise, salad dressing, fruit and vegetable processing such as fruit juice and baking such as bread, the manufacture of gluconic acid derivatives which are used in a wide variety of foods as food supplements, sequestrants, stabilizers, thickeners and acidifiers, and as a fermentation aid in the manufacture of fuel ethanol with resulting distillers' grains destined for use in animal feed."

   We confirm the intended uses in our GRAS notice are baking, egg processing, and cheese processing.

   The GRAS panel reviewed our self-GRAS determination on glucose oxidase (the subject enzyme of GRN 1054) and issued the GRAS opinion letter. The intended uses in the self-GRAS determination covered all the intended uses in US. In addition, we submitted the GRAS Notice on this glucose oxidase to

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support the submissions in other jurisdictions, therefore for this GRAS Notice we only included the intended uses of interest in these other countries.

2. Please clarify the specific egg processing uses for this ingredient.

   Egg processing includes de-sugared eggs, mayonnaise, salad dress, and *etc*. listed in the self-GRAS determination and concurred by GRAS panel.

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Date: March 08, 2023

To: Dr. Katie Overbey
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Tel: 240-402-7536
katie.overbey@fda.hhs.gov

From: Annie Han.
Global Regulatory Affairs
Danisco US Inc.

RE: GRAS Notice GRN1054 Glucose Oxidase Produced with Genetically Engineered *Aspergillus niger*

Dear Dr. Overbey,

Thank you for your review of our submission. We are providing this letter in response to FDA's questions that were sent via email on February 22, 2023 regarding our GRAS Notice submission on glucose oxidase produced with *Aspergillus niger* production strain. We have copied the request for information above our responses for your reference:

a. For the administrative record, please confirm the number of amino acids in the glucose oxidase primary amino acid sequence.

The number of amino acids in the glucose oxidase primary sequence is 583 amino acids.

b. Please state the strain designation of the *A. niger* production organism.

The *A. niger* production strain was designated as GICC03206.

c. Please state whether the *A. niger* production organism has been deposited in a recognized culture collection.

The *A. niger* production strain was deposited in Danisco's culture collection as GICC03206 and the private collection of the Westerdijk Fungal Biodiversity Institute (The Netherlands) as CBS 143966.

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d. Please indicate if the enzyme is secreted or lysed.

The glucose oxidase enzyme is secreted by the microbial production strain.

e. For the administrative record, please state the form (powder, liquid) and color of the final enzyme preparation.

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Various commercial formulations exist, with a range of enzyme activities for different applications. Please find the product description sheet for both liquid food and powder in Attachment 1.

a. Powder (spray dried): off-white
b. Liquid: brown

f. In section 2.4.1, you state that the enzyme preparation contains wheat as a formulation ingredient. Please clarify whether the final enzyme preparation composition for cheese and egg processing applications would also contain wheat.
   - Further, on page 18, you state that the fermentation process ***may*** use glucose and that final dry products ***may*** be spray-dried on potato or wheat starch. Please clarify the discrepancy between this and the information stated in the manufacturing section.

Various commercial formulations exist, with a range of enzyme activities for different applications.

The enzyme products used in egg and cheese applications are liquid form products, which will not contain wheat.

The spray dried powder products, which will contain wheat or potato starch, will be only used in baking applications.

Glucose (which may be derived from wheat) will be used in the fermentation process and is consumed by the microorganism as a nutrient (or as nutrients). The final dry products for the bakery applications can be spray-dried on potato or wheat starch. Since bakery products are produced with similar allergen group (*e.g.,* wheat), no additional allergens are introduced into the final food. Therefore, the final enzyme preparation does not introduce any new major food allergens from the fermentation medium into the final food. No other major allergen substances are used in the fermentation, recovery processes, or formulation of this product.

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g. Please confirm if the fermentation components and other raw ingredients are food grade and comply with United States rules and regulations.

We confirm that the fermentation components and other raw ingredients are food grade and comply with United States rules and regulations. The raw materials used in the fermentation and recovery process for this GOX concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemicals Codex, 13th edition, 2022 ("FCC"), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco US Inc. uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The product also meets or exceeds the Joint FAO/WHO Expert Committee on Food Additives (JECFA)/Food Chemical Codex (FCC) specifications for microbial and metal contaminants in food enzymes.

h. For the administrative record, please confirm that acetamide medium is not used to manufacture the final enzyme preparation and that acetamide medium used in the construction/ selection of the production strain is not expected to be present in the final enzyme preparation.

Acetamide is not used in the fermentation process or in the propagation medium used to select for the production strain. Therefore, acetamide should not be present in the final enzyme preparation.

i. In section 2.1.5, you state "no antibiotic resistance genes were used in the construction of the production microorganism." For the administrative record, please indicate if the parental strain contains any antibiotic resistance genes and describe the methods used to verify absence of functional antibiotic resistance genes.

The parental strain does not contain any antibiotic resistance genes. In order to confirm the absence of any antimicrobial resistance (AMR) genes in the *Aspergillus niger* production strain genome, we performed an antimicrobial resistance analysis using NGS data in combination with up-to-date antibiotic resistance gene and plasmid sequence databases. The outcome of the analysis confirmed the absence of any antibiotic resistance genes.

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j. Please address the following questions related to the specifications provided in Appendix 3:

a. For the administrative record, please state the specific methods used for establishing the specifications listed in Appendix 3 and confirm that they have been validated for their intended purpose.

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The specifications and methods are listed below.

Property	Reference Method	Specification
ENZYME ACTIVITIES		
Glucose Oxidase	Danisco US Inc. Method	Varies with product
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	ISO 4833 - "Microbiology of food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30℃ by the pour plate technique"" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/mL
E. coli	ISO 7251 – "Microbiology of food and animal feeding stuffs -Horizontal method for the detection and enumeration of presumptive Escherichia coli – Most probable number technique and FDA Bacterio-logical Analytical Manual; 8th Edition; AOAC International	Negative/25mL
Total Coliforms	ISO 4832 - "Microbiology of food and animal feeding stuffs -Horizontal method for the detection and enumeration of coliforms – Colony-count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/mL

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Property	Reference Method	Specification
Salmonella	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25mL
Production strain	Danisco US Inc. Method	Negative by test
Antibacterial Activity	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p217-218;	Negative by test
OTHER ASSAYS		
Arsenic	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.	Less than 3 mg/kg
Cadmium	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision	Less than 0.5 mg/kg
Mercury	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.	Less than 0.5 mg/kg
Lead	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.	Less than 5 mg/kg

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We confirm the methods have been validated for their intended purpose.

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b. You state that results found for lead and arsenic are below 5 mg/kg and 3 mg/kg respectively. Please provide the method for the analysis with detection limits as well as the actual result that was obtained.

	Lead		Arsenic	
	mg/kg	Method	mg/kg	Method
7203879755	ND	GB5009.12-2017 (1)	0.14	GB5009.11-2014 (1)
7203519227	ND		0.06	
7203520919	ND		0.31	

Lead results noted as not determined (ND) as lead levels were lower than the detection limits of 0.04 mg/kg.

The GB methods listed above refer to China National Methods below, which are consistent with the FCC 8th edition references.

- Lead is GB/T 5009.12 < National food safety standard: Determination of lead in foods>
- Arsenic is GB/T 5009.76 < Determination of Arsenic in food additives>

c. You list mycotoxins as a specification; please list the specific mycotoxins tested as part of the specifications.

Mycotoxin	Neogen Veratox ELISA kits (Patterson & Roberts, 1979)[1] AOAC TLC method	Less than 5 ppb total Aflatoxin Less than 2 ppb ochratoxin Less than 25 ppb zearalenone Less than 25 ppb T-2 Toxin Less than 100 ppb Sterigmatocystin Less than 100 ppb Fumonisin

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[1] Patterson, Deryck SP, and Basil A. Roberts. "Mycotoxins in animal feedstuffs: Sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin." *Journal of the Association of Official Analytical Chemists* 62.6 (1979): 1265-1267.

k. Please provide additional information about the composition of the 17% of fermentation solids that are included in the final enzyme preparation.

Inert Ingredients are solids carry over from the fermentation process from either the media or microbial metabolites. Fermentation media generically includes carbon sources, nitrogen sources and minerals needed to grow microorganism. The majority of the fermentation media ingredients are digested during the fermentation process and soluble solids which carry over are typically organic acids, salts, amino acids, etc. The Inert Ingredients are not themselves tested or further quantified.

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l. On page 16, the notifier lists "GRAS affirmations and GRNs to support the use of *A. niger* as a safe production host" but does not identify or summarize the relevant information from each GRAS notice. As each GRAS notice stands on its own, for the administrative record, please briefly summarize the information incorporated by reference from the GRAS notices listed on page 16.
Additionally, 21 CFR 173.120 ("Carbohydrase and cellulase enzyme preparation") and 21 CFR 173.280 ("Solvent extraction process for citric acid") are included in the list provided on page 16; please briefly discuss the relevance of these CFR references to the safety of your glucose oxidase enzyme preparation.

The GRAS Notices and 21CFR listed on Page 16 under Section 6.1.1 are to support the long history of safe use of the host microorganism in food not only for producing enzymes but also in the production of citric acid. The host strains of all the GRAS Notices are *Aspergillus niger* and after reviewing, FDA issued "no question" letters for them, which support our discussion below.

Beyond a history of safe use in GRAS Notices, in the public literature *"Aspergillus niger* has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. It is one of the most important producers of industrial enzymes (Uhlig, 1998, Aunstrup, 1979, and Li *et al.,* 2020). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production."

m. We note on page 16 that you reference GCN 345 and GCRN 428. Please clarify if these are typographical errors.

We apologize for these typos. It should be a reference to GRN 345 and GRN 428.

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n. Page 18 states “In addition, the US Food and Drug Administration (FDA) has provided ‘no questions letters’ to assert GRAS (Generally Recognized as Safe) status to various glucose oxidase enzyme preparations.” We note that the FDA’s no questions letters are not assertions or approvals and reflect FDA opinion on a manufacturer’s GRAS conclusion. Further, it is the ***intended conditions of use*** of a substance that must be concluded to be GRAS rather than the substance itself. It is not possible to establish a GRAS determination for an individual enzyme, rather, the specific use of the enzyme, including use level, manufacturing process, and intended product(s), may be evaluated as GRAS. The use of a substance is GRAS because of widespread knowledge among a community of qualified experts, not because of a listing or other administrative activity. Please remove this statement as it does not accurately reflect the nature of FDA’s response to GRAS notifications

We appreciate the clarification and confirm that we will remove the description in your question above. May we use the following statement instead in the future submissions?

“In addition, the US Food and Drug Administration (FDA) has provided “no questions letters” to various glucose oxidase enzyme preparations such as: glucose oxidase from *Penicillium* produced in *T. reesei* (GRN 707), glucose oxidase enzyme preparation derived from *P. chrysogenum* (GRN 509), glucose oxidase enzyme preparation from *Aspergillus oryzae* carrying a gene encoding glucose oxidase from *A. niger* (GRN 106), and glucose oxidase enzyme preparation from *A. niger* (GRN 89) for the intended conditions of use, such as baking, egg processing, and cheese manufacture.”[2]

o. You provide, in Section 6.3.3, a summary of toxicological studies done with *A. niger* glucose oxidase enzyme preparation. It is not clear the relationship between the test article and the article of commerce for GRN 1054. Please confirm whether or not the test article is the exact same preparation as the subject of GRN 1054 (*i.e*. GOX enzyme preparation produced with *A. niger* overexpressing GOX gene from *A. niger*).

We confirm the test article is exactly the same preparation as the subject enzyme of GRN1054.

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[2] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=glucose%20oxidase

p. You state on pg. 14 (Section 4):

"As the enzyme will be used as processing aid in the food manufacturing process, there is no notable oral intake for humans."
However, you also state on pg. 3 "the glucose oxidase will be present in insignificant quantities as active residue with function or technical effect in the final food in cheese production." Thus, your enzyme preparation will in fact be consumed by the consumer from this intended use (i.e., the enzyme preparation will not necessarily be inactivated or removed prior to consumption). Please provide a short narrative discussing why this use in which the enzyme preparation will remain in the final food is not a safety concern.

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The GOX (glucose oxidase) enzyme is expected to be active during cheese production but unlikely to have a technical effect or function in the final cheese products. In general, whether the enzyme has any enzymatic activity in the final food can be due to a combination of various factors and dependent on the application and the process conditions used by the individual food producer. For example, when GOX is used in the production of cheese that will be cooked (*e.g.,* cheese for pizzas and other baked goods), the GOX is expected to be heat inactivated given the high temperatures and residence times during cooking. GOX also becomes inactive when the substrates are depleted. GOX needs its two substrates to be active: glucose and oxygen. When it runs out of either one, the enzyme is no longer active.

In the case that inactive or active GOX is present in the processed food and is ingested, it will not be absorbed intact. Instead, the enzyme is expected to be broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which is not expected to pose any human health risk.

In addition, to assess the safety of glucose oxidase in baking, egg processing, and cheese applications; different endpoints of toxicity were investigated on the subject glucose oxidase of GRN 1054 and the results are evaluated, interpreted, and assessed in the original submission. In the 90-day oral (gavage) study in rats, a NOAEL was established at 10.87 mg total protein/kg bw/day corresponding to 19.53 mg TOS/kg bw/day.

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Based on a worst-case scenario that a person is consuming GOX from the products of baking, egg processing, and cheese containing the glucose oxidase, the cumulative daily exposure of 0.03 mg TOS/kg bw/day. Based on a margin of safety (651) greater than 100 (even in the worst-case), the proposed uses of glucose oxidase in baking, egg processing, and the manufacture of cheese is not a human health concern and are supported by existing toxicology data.

q. You state on pg. 7: “The host strain contains (a) native copy(ies) of GOX, which contains an internal stop codon and presumably yields a truncated protein.” Please provide a short narrative describing why the presumably truncated native GOX protein is not expected to impact the safety or activity of your GOX enzyme preparation.

The host strain produces a native, truncated, and inactive GOX protein sequence, which means that the GOX activity is negligible from the host. The native GOX amino acid sequence does not share homology with a toxin or venom sequence, nor does it pose a risk for food allergenicity. Taken together with data from a 90-day oral study, which included possible residues of the native and truncated GOX, this indicates that there is no safety concern from the truncated, native GOX protein.

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Attachment List

Attachment 1 Product Description

References

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes. In: Applied Biochemistry and Bioengineering, Volume 2, *Enzyme Technology*, Eds. Wingard, L.B., Katchalski-Katzir, E. and Golsdstein, L. pp. 28-68.

Li, C., Zhou, J., Du, G., Chen, J., Takahashi, S. and Liu, S., 2020. Developing Aspergillus niger as a cell factory for food enzyme production. *Biotechnology Advances*, p.107630.

Patterson, D.S. and Roberts, B.A., 1979. Mycotoxins in animal feedstuffs: Sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin. *Journal of the Association of Official Analytical Chemists*, *62*(6), pp.1265-1267.

Uhlig, H. 1998. Industrial enzymes and their applications. Translated by Linsmaier-Bednar, E. M. John Wiley & Sons, Inc.

U.S. Pharmacopeia, 2020. Food Chemicals Codex (FCC), 12th Edition. United States Pharmacopeial Convention (USP), Rockville, MD.

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ATTACHMENT 1 Product Description

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DuPont Industrial Biosciences
Bioactives, Food Enzymes
foodenzymes@dupont.com
www.food.dupont.com

Valid from: June 21, 2022

PRODUCT DESCRIPTION - PD 257840-4.0EN

FoodPro® GOL

Description

FoodPro® GOL is a liquid glucose oxidase derived from Aspergillus niger.
FoodPro® GOL catalyzes oxidation of glucose to gluconic acid by reaction with oxygen.

Application areas

Removal of glucose or oxygen from food
Production of gluconic acid derivatives

Potential benefits

- Desugarisation of eggs
- Oxygen scavenging for extending the shelflife of packaged food and beverages

Usage levels

Typical dose for egg desugarisation	80-250 ppm
Typical dose for deoxygenation of juices	5-15 ppm

However dose levels will highly depend on application and processes, and a test should be carried out to determine optimum dosage.

Composition

• Water	73-77 % (w/w)
• Sodium chloride	18 % (w/w)
• Glucose Oxidase	1-5 % (w/w)
• Sodium citrate dihydrate	2.56 % (w/w)
• Sodium phosphate monobasic	1.37 % (w/w)

Physical/chemical specifications

Physical form	liquid
Colour*	brown
Activity	1800-2000 U/ml
pH	5.2 - 5.5
Specific gravity	1.15 - 1.20

*Colour may vary from batch to batch.

Microbiological specifications

Total viable count	<50000 CFU/ml
Coliforms	<30 CFU/ml
E. coli	absent in 25 ml
Salmonella species	absent in 25 ml

Heavy metal specifications

Arsenic	less than 3 mg/kg
Cadmium	less than 0.5 mg/kg
Mercury	less than 0.5 mg/kg
Lead	less than 5 mg/kg

Nutritional data

Calculated values per 100 g

Energy	10/42 kcal/kJ
Fat	less than 0.5 g
Protein	less than 5 g
Carbohydrates	0 g
- Fiber	0 g
- Total sugar	0 g
- Added sugar	0 g
Moisture	73-77 g
Ash	16-26 g
Sodium	7804 mg
Potassium	0 mg
Calcium	0 mg

*other minor parameters not listed include Vit A, Vit C, Vit D, Iron, etc. are considered zero.

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Valid from: June 21, 2022

DANISCO
First you add knowledge...

PRODUCT DESCRIPTION - PD 257840-4.0EN

FoodPro® GOL

Storage

FoodPro® GOL should be stored dry and cool (max. 10°C/50°F) and sheltered against direct sunlight

Packaging

28 kg pail

Purity and legal status

This product complies with the current recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

Safety and handling

Enzymes are proteins. Enzyme exposure may cause respiratory allergy upon repeated exposure, use enzyme products under ventilation and/or closed processes. Respiratory protective equipment is recommended during open applications. Refer to the safety data sheet (SDS) or contact DuPont for more information on enzyme safety and handling practices.

Kosher status

FoodPro® GOL is certified kosher pareve by Union of Orthodox Jewish Congregations of America (OU).

Modern Biotechnology

The enzymes are manufactured by fermentation of microorganisms that are not present in the final product. The microorganisms have been optimized by means of modern biotechnology. This product does not contain genetically engineered material from the microorganisms.

Allergens

The table below indicates the presence (as added component) of the following allergens and products thereof.* Unless otherwise noted, the following listed allergens and products thereof have been used in the fermentation or recovery processes, or in the formulation of an enzyme product:

Yes	No	Allergens	Description of components
	X	Wheat	
	X	Other cereals containing gluten	
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	X	Soybeans	
	X	Milk (incl. lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sesame seeds	
	X	Sulphur dioxide and sulphites (>10mg/kg)	
	X	Lupin	
	X	Molluscs	
	X	Nuts includes: almond, Hazelnut, Cashew-nut, Brazilian-nut, Macadamia, Walnuts, Pecan, Pistachio, Pinoli and Chestnuts	
	X	Natural Latex	

*Local legislation has always to be consulted as allergen labeling requirements may vary from country to country. ** Based on risk assessments, DuPont Industrial Biosciences concludes that the amount of soybean or wheat proteins or protein fragments in the final food product to be de minimis and not likely to pose a risk to the final consumer.
https://amfep.org/_library/_files/amfep-statement-on-labelling-of-substances-allergies-or-intolerances-present-in-food-enzyme-preparations.pdf

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Bioactives, Food Enzymes
foodenzymes@dupont.com
www.food.dupont.com

Valid from: May 11, 2022

PRODUCT DESCRIPTION - PD 266578-5.1EN

GRINDAMYL® S 860

Bakery Enzyme

Description

GRINDAMYL® S 860 is a glucose oxidase which is produced by fermentation with a selected strain of fungus.

Application areas

Yeast-raised bread.

Potential benefits

- Increases tolerance towards variations in process parameters
- Improves dough handling
- Improves dough stability
- Reduces or supplements the use of chemical oxidants

Usage levels

Based on flour weight	5-20 ppm
corresponding to	0,5-2 g/100 kg

However, as different flours and procedures have different needs, tests should be carried out to find the optimum dosage.

Directions for use

GRINDAMYL® S 860 is mixed into flour, premixes or bread improvers together with other dry ingredients.

In formulations containing ingredients which are sensitive to oxidation i.e. fats and oils, care must be taken that off-flavour does not occur. The influence on taste should also be evaluated in procedures with very long fermentation times.

Composition

GRINDAMYL® S 860 is composed of:

- Glycine
- Wheat flour
- Protein (enzymes)
- Microcrystalline cellulose

Physical/chemical specifications

Physical form	powder
Colour*	off-white
Glucose oxidase activity	9350 - 12650 units/g

*Colour may vary from batch to batch.

Microbiological specifications

Total viable count	less than 10000 /gram
Coliforms	less than 30 /gram
E. coli	absent in 25 grams
Salmonella species	absent in 25 grams
Mycotoxins*	negative by test
Antibiotic activity	negative by test

* Aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenone

Heavy metal specifications

Arsenic	less than 3 mg/kg
Lead	less than 5 mg/kg
Heavy metals (as Pb)	less than 30 mg/kg

DuPont Industrial Biosciences
Bioactives, Food Enzymes
foodenzymes@dupont.com
www.food.dupont.com

Valid from: May 11, 2022

PRODUCT DESCRIPTION - PD 266578-5.1EN

GRINDAMYL® S 860

Bakery Enzyme

Nutritional data

Calculated values per 100 g of a typical batch composition.

Energy	376/1567 kcal/kJ
Fat	0 g
Protein	60-70 g
Carbohydrates	25-30 g
- Fiber	7 g
- Total sugar	0 g
- Added sugar	0 g
Moisture	2-8 g
Ash	1-10 g
Sodium	61 mg
Potassium	300 mg
Calcium	0 mg
Vitamin C	715 mg

*other minor parameters if not listed include Trans Fat, Saturated Fat, Cholesterol, Vit A, Vit D, Iron, etc. are considered zero.

Storage

GRINDAMYL® S 860 should be stored dry and cool (max. 10°C/50°F).

The shelf life of GRINDAMYL® S 860 is 12 months when stored as recommended in unbroken packaging.

Packaging

Polyethylene-lined paper bags of 20 kg net.

Purity and legal status

GRINDAMYL® S 860 meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

GRINDAMYL® S 860 is approved by most countries for use in food. However, as legislation regarding its use in food may vary from country to country, local food regulations should always be consulted concerning the status of this product. Advice regarding the legal status of this product may be obtained on request.

Safety and handling

Enzymes are proteins. Enzyme exposure may cause respiratory allergy upon repeated exposure, use enzyme products under ventilation and/or closed processes. Respiratory protective equipment is recommended during open applications. Refer to the safety data sheet (SDS) or contact DuPont for more information on enzyme safety and handling practices.

Country of origin

Brazil

Kosher status

This product is certified Kosher.

Modern Biotechnology

The enzymes are manufactured by fermentation of microorganisms that are not present in the final product. The microorganisms have been optimized by means of modern biotechnology. This product does not contain genetically engineered material from the microorganisms.

PRODUCT DESCRIPTION - PD 266578-5.1EN

GRINDAMYL® S 860

Bakery Enzyme

Allergens

The table below indicates the presence (as added component) of the following allergens and products thereof.* Unless otherwise noted, the following listed allergens and products thereof have been used in the fermentation or recovery processes, or in the formulation of an enzyme product:

Yes	No	Allergens	Description of components
X		Wheat	Wheat flour
	X	Other cereals containing gluten	
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	X	Soybeans	
	X	Milk (incl. lactose)	
	X	Nuts includes: almond, Hazelnut, Cashew-nut, Brazilian-nut, Macadamia, Walnuts, Pecan, Pistachio, Pinoli and Chestnuts	
	X	Celery	
	X	Mustard	
	X	Sesame seeds	
	X	Sulphur dioxide and sulphites (>10mg/kg)	
	X	Lupin	
	X	Molluscs	
	X	Natural Latex	

*Local legislation has always to be consulted as allergen labeling requirements may vary from country to country. ** Based on risk assessments, DuPont Industrial Biosciences concludes that the amount of soybean or wheat proteins or protein fragments in the final food product to be de minimis and not likely to pose a risk to the final consumer.
https://amfep.org/_library/_files/amfep-statement-on-labelling-of-substances-capable-of-causing-allergies-or-intolerances-present-in-food-enzyme-preparations.pdf

Date: April 11, 2023

To: Dr. Katie Overbey
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Tel: 240-402-7536
katie.overbey@fda.hhs.gov

From: Annie Han.
Global Regulatory Affairs
Danisco US Inc.

RE: GRAS Notice GRN1054 Glucose Oxidase Produced with Genetically Engineered *Aspergillus niger*

Dear Dr. Overbey,

Thank you for your review of our submission. We are providing this letter in response to FDA's question that was sent via email on March 30, 2023 regarding our GRAS Notice submission on glucose oxidase produced with *Aspergillus niger* production strain. We have copied the request for information above our responses for your reference:

1. In accordance with FDA's Closer to Zero action plan, we note that specifications for heavy metals should reflect the amounts determined in the analyses of representative batches and be kept as low as possible. Please consider reducing the specification for lead and arsenic to reflect the results from the batch analyses presented in the amendment dated March 8, 2023.

In our response to FDA's questions dated March 08, 2023, we provided the following specifications and methods for lead and arsenic.

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Property	Reference Method	Specification
OTHER ASSAYS		
Arsenic	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.	Less than 3 mg/kg
Lead	FCC 8th Edition, Elemental Impurities by ICP, General Tests and Assays, Appendix III.	Less than 5 mg/kg

In addition, we provided the analytical results on 3 batch glucose oxidase preparations for lead and arsenic.

	Lead		Arsenic	
	mg/kg	Method	mg/kg	Method
7203879755	ND	GB5009.12-2017 (1)	0.14	GB5009.11-2014 (1)
7203519227	ND		0.06	
7203520919	ND		0.31	

Lead results noted as not determined (ND) as lead levels were lower than the detection limits of 0.04 mg/kg.

The metals specifications on all our food grade enzyme products are consistent with the FCC and JECFA specifications for enzymes. The specifications are not set per individual product based on analytical data obtained or based on a small sample set.

The FCC enzyme specification for Lead is 0-5 mg/kg. There is no FCC enzyme specification for arsenic, however the FAO/WTO JECFA specification for Arsenic is 0-3 mg/kg.

As mentioned in our submission, glucose oxidase preparation, the subject enzyme preparation of GRN1054 meets the purity specifications for enzyme preparations set forth in FCC, 12th edition (USP, 2020). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA (2006).

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We appreciated FDA's comment to ask us to consider reducing the specifications for lead and arsenic for glucose oxidase, the subject enzyme in GRN1054. As the specifications were set for all food grade enzymes, we need to keep the enzyme specifications in this GRAS Notice consistent with the FCC and JECFA specifications for lead and arsenic to meet the global regulatory requirements. In addition, given the very low use rate of our enzyme products in food processing, these limits on lead and arsenic do not pose a safety issue.

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