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Rethink Tomorrow

August 25, 2017

GRN 728

GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
US Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Sir or Madam,

We are hereby submitting one paper copy and one eCopy, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' phosphoinositide phospholipase C enzyme preparation produced by a *Bacillus licheniformis* strain. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Novozymes has determined through scientific procedures that the Pi-phospholipase C is generally recognized as safe for use in the food industry as a processing aid in the degumming of vegetable oils.

Please contact me by direct telephone at 919 494-3187, direct fax at 919 494-3420 or email at jao@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

Janet Oesterling
Regulatory Affairs Specialist III

Enclosures

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road, P.O. Box 576
Franklinton, North Carolina 27525

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PART 1: Signed statement of the conclusion of GRAS (Generally Recognized as Safe) and certification of conformity to 21 CFR §170.205-170.260.

§170.225(c)(1) – Submission of GRAS notice:

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

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

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

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

The appropriately descriptive term for this notified substance is phosphoinositide phospholipase C from *Pseudomonas Sp. - 62186* produced in *Bacillus licheniformis.*

§170.225(b) – Trade secret or confidential:

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

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

The phosphoinositide phospholipase C enzyme (PI-PLC) will be used in degumming of vegetable oils intended for human consumption. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices. The "general" population is the target population for consumption.

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

This GRAS conclusion is based on scientific procedures.

§170.225(c)(6) – Premarket approval:

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

§170.225(c)(7) – Availability of information:

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

§170.225(c)(8) - FOIA (Freedom of Information Act):

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

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Novozymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

(b) (6)

2/16/17

Janet Oesterling Date
Regulatory Affairs Specialist III

A Phosphoinositide Phospholipase C from *Pseudomonas sp. - 62186* Produced in *Bacillus licheniformis*

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

February 2017

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

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a phosphoinositide phospholipase C enzyme preparation, hereby known as PI-PLC, produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism carrying the gene coding for phospholipase C from *Pseudomonas sp*.- *62186.*

Key enzyme and protein chemical characteristics of the PI-PLC are given below:

Classification	phosphodiesterases
IUBMB nomenclature:	phosphoinositide phospholipase C
EC No.:	3.1.4.11
CAS No.:	63551-76-8
Specificity:	catalyzes the hydrolysis of phospholipids
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Bacillus licheniformis* production strain, designated MaTa161, was derived via the recipient strain, AEB1953, from a natural isolate of *Bacillus licheniformis* strain DSM 9552.

Bacillus licheniformis complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (3) and several expert groups (4) (5) (6) (1) (7) (8) (9).

The expression plasmid, used in the strain construction, pMRT334, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced PI-PLC gene is based on the *lip* encoding sequence from *Pseudomonas sp*. 62186.

2.2(b) Recipient Strain

The recipient strain AEB1953 used in the construction of the *Bacillus licheniformis* production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of proteases. Also, the ability to sporulate was eliminated by deleting a gene essential for sporulation. Additionally, genes encoding

unwanted proteins that can be present in the culture supernatant were deleted. The absence of these represents improvements in the product purity, safety and stability.

2.2(c) PI-PLC Expression Plasmid

The expression plasmid, pMRT334, used to transform the *Bacillus licheniformis* recipient strain AEB1953 is based on the well-known *Bacillus* vectors pE194 (10) and pUB110 (11) from *Staphylococcus aureus*. No elements of these vectors are left in the production strain. The introduced DNA consist of a fragment of a hybrid *Bacillus* promotor with promotor elements from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus thurigiensis,* the PI-PLC coding sequence and finally a transcriptional terminator.

The PI-PLC gene is a synthetic gene encoding a variant of the wild-type PI-PLC from *Pseudomonas sp.* 62186 which has a single amino acid residue difference compared to the wild-type sequence. The gene has been codon optimized for expression in *Bacillus licheniformis*.

Following the terminator, a non-coding DNA sequence is inserted to enable targeted integration of the transforming DNA into the genome of the recipient strain. Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Bacillus licheniformis* MaTa161, was constructed from the recipient strain AEB1953 through the following steps:

1) Plasmid pMRT334 was integrated into three specific loci in strain AEB1953 by targeted homologous recombination to these loci using a two-step integration approach. Targeted integration of the expression cassettes at these loci allows the expression of the PI-PLC gene *lipPsp2.s* from the promoter.

2) The resulting PI-PLC strain containing one copy of the *lipPsp2.s* gene at each of the three target loci was named MaTa161.

Sequence confirmation of the inserted expression cassettes and the flanking regions at each of the integration loci was performed in the production strain.

2.2(e) Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern hybridization demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Bacillus licheniformis*

chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

2.2(f) Antibiotic Resistance Gene

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

2.2(g) Absence of Production Organism

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

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the PI-PLC enzyme which follows standard industry practices (12) (13) (14). The quality management system used in the manufacturing process for the enzyme preparation complies with the requirements of ISO 9001. It is manufactured in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (15). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (16).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the enzyme concentrate are standard ingredients used in the enzyme industry (12) (13) (14). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams

and flocculants dated April 10, 1998. The maximum use level of the antifoams and or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The PI-PLC enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Bacillus licheniformis* as described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Bacillus licheniformis.* Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

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

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

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

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

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

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

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation (if required)

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

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

5) Final concentration – evaporation and/or ultrafiltration.

6) Preservation and Stabilization of the liquid enzyme concentrate

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

2.4 COMPOSITION AND SPECIFICATIONS

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

2.4(a) Quantitative Composition

The PI-PLC enzyme preparation is sold in a liquid form. Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. Also, the enzyme preparation, that is the subject of this notification, does not contain any major food allergens from the fermentation media.

Table 1. Typical compositions of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	11%
Glycerol	>50%
Water	40 - 50%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The PI-PLC enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (15). In

addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (16).

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

Table 2. Analytical data for three food enzyme batches

Parameter	Specifications	PPW38943	PPW40876	PPW41767
PI-PLC activity	PLC(E)/g	6990	6160	4490
Total viable count	Upper limit 50,000	100	<100	100
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 4	< 4	< 4
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is phospholipase C (EC 3.1.4.11), commonly known as PI-PLC. PI-PLC will be used in degumming of vegetable oils intended for human consumption (17). Degumming is the first step in the refining of crude oil. The subsequent steps include bleaching and deodorization. In the standard degumming procedure, water and acid are used to remove phospholipids. In enzymatic degumming, phospholipase C will be added to the crude oil under conditions commonly used in the refining of edible oils. Under these conditions, PI-PLC will hydrolyse the major oil phospholipids, and phosphatidylcholine. The resulting phosphate esters, phosphorylinositol, will be solubilised in water and removed from the oil by centrifugation (18).

2.5(b) Use Levels

The PI-PLC enzyme preparation will be added to crude vegetable oils, such as soybean, corn, canola, rape, and sunflower at levels no higher than necessary to achieve an intended effect, and according to requirements for normal production following cGMP.

The dosage applied in practice by a food manufacturer depends on the particular process. It is based on an initial recommendation by the enzyme manufacturer and optimised to fit the process conditions.

The maximum recommended use level is 75 PLC(E) per kilo of oil.

2.5(c) Enzymes Residues in the Final Food

After the degumming reaction has been completed, the aqueous phase containing PI-PLC, is separated from the oil by centrifugation. The subsequent steps used in refining, i.e., repeated washing of the oil with hot water, bleaching, and deodorization, will remove the residual enzyme.

PART 3 - DIETARY EXPOSURE

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

The PI-PLC has an average activity of 5880 PLC(E)/g approximately 11% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 53.45 PLC(E)/mg TOS.

3(a) Assumptions in Dietary Exposure

The assumptions are highly exaggerated since the enzyme protein and the other substances are diluted or removed during certain processing steps. Furthermore, all processed foods and beverages produced with the enzyme are not always produced with the maximum recommended dosage. Therefore, the safety margin calculation derived from this method is highly conservative.

The exposure assessment represents a “maximum worst case” situation of human consumption. Overall, the human exposure to the PI-PLC will be negligible because the enzyme preparation is used as a processing aid and in very low dosages therefore the safety margin calculation derived from this method is highly conservative.

3(b) Food Consumption Data

The average daily consumption for added fats and oils is taken from the USDA-ERS Food Availability per capita consumption report for 2006-2011 (19). The estimate of 104.1g/person/day was calculated from the 2010 consumption of 83.8 pounds per annum of vegetable derived added oils and fats on a total fat content basis.

The average body weight of 88.8kg was used and was taken from the CDC Vital Health and Statistics anthropometric reference data. It is based on adult males over the age of 20 (20).

The PLC has an average activity of 5880mg PLC(E)/g and approximately 11% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 53.45 PLC(E)/mg TOS.

The maximum recommended dosage is: 75 PLC(E)/kg of oil

This will result in an exposure of:
1.40mg TOS/kg oil x 1.17g oil/kg bw/day / 1000 = 0.002mg TOS/kg bw/day

Therefore, the Total Maximum Daily Intake (TMDI) of the food enzyme by consumers is:

0.002 mg/TOS/kg bw/day.

The safety margin calculation derived from this method is highly exaggerated.

Theoretical Maximum Daily Intake (TMDI)

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13-week oral toxicity study in rats conducted on PI-PLC, PPW40064 was the highest dosage possible, 506mg TOS/kg bw/day. See Appendix 2 and Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	506
*TMDI (mg TOS/kg bw/day)	0.002
Safety margin	253000

*based on the worst case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following sections is the basis for our determination of general recognition of safety of the PI-PLC enzyme preparation. Our safety evaluation in Part 6 includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (4) (6) (1) (7) (8) (21). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Part 2.

6(a) Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (3) (2). The production organism for the Pi-PLC, *Bacillus licheniformis,* is discussed in Part 2 and also in this Part.

Bacillus licheniformis has a long history of safe industrial use for the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (22). *Bacillus licheniformis* is not a human pathogen and it is not toxigenic (23). If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (22). Pariza and Foster (2) 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”.

Bacillus licheniformis has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *Bacillus licheniformis* and are considered GRAS substances. See Table 4 below. In addition, *Bacillus licheniformis* is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *Bacillus licheniformis* recipient strain is derived from a safe strain lineage comprising production strains for more than ten enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay). The strains shown in Table 4 below were derived from the natural isolate DSM9552.

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Alpha-amylase (GRASP 0G0363)	3.2.1.1	*Bacillus licheniformis* Si3	*Bacillus stearothermophilus*	Yes
Alpha-amylase (GRN 22)	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Cyclodextrin glucanotransferase	2.4.1.19	*Bacillus licheniformis* SJ1707	*Thermoanaerobacter sp.*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1904	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus stearothermophilus*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus amyloliquefaciens*	Yes
Serine protease (GRN 564)	3.4.21.1	*Bacillus licheniformis* MDT223	*Nocardiopsis prasina*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Xylanase (GRN 472)	3.2.1.8	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Beta-amylase	3.2.1.1	*Bacillus licheniformis* PP3579	*Bacillus flexus*	Yes
Beta-galactosidase (GRN 572)	3.2.1.23	*Bacillus licheniformis* AEB1763	*Bifidobacterium bifidum*	Yes
Acetolactate decarboxylase (GRN 587)	4.1.1.5	*Bacillus licheniformis* AEB1763	*Bacillus brevis*	Yes
Pullulanase (GRN 645)	3.2.1.41	*Bacillus licheniformis* AEB1763	*Bacillus deramificans*	Yes

Table 3. Novozymes products derived from *B. licheniformis* strains. The predecessor strains show strains in the GM construction pathway that are in common with the Si3 strain lineage. At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13 week sub chronic oral toxicity study in rats.

Novozymes has used *B. licheniformis* production strains for over 20 years. As shown in Table 4, safety studies have been performed for the same enzyme in different strains in the lineage, supporting the fact that the genetic modifications performed in the *Bacillus licheniformis* strain lineage of the recipient do not result in safety concerns. Additionally, no safety issues are observed when different products that are produced in the same strain (e.g., amylases, xylanases, protease and pullulanase) are investigated, demonstrating that the safety of the strains in the lineage is not product-dependent.

Novozymes' has repeatedly used the procedures outlined by Pariza and Johnson (3) and has used the decision tree (Appendix 1) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed enzyme product is a phospholipase C. The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated

into the chromosome at five specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. Novozymes has repeatedly used the procedures outlined by Pariza and Johnson 2001 (3) to evaluate the enzymes derived from *Bacillus licheniformis* production strains. Therefore, following the evaluation outlined in this section this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

An evaluation of the genetically modified *Bacillus licheniformis* production organism embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (22), the EU SCF in 1991 (6), the OECD in 1992 (1), ILSI Europe Novel Food Task Force in 1996 (21), FAO/WHO in 1996 (8), JECFA in 1998 (16) and Pariza and Johnson in 2001 (3), demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Part 2.

Based on the information presented here it is concluded that the *Bacillus licheniformis* production strain is considered a safe strain for the production of PI-PLC enzyme.

6(b) Safety of the Donor Organism

The donor organism of the PI-PLC is *Pseudomonas sp-62186*. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the PI-PLC coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

6(c) Safety of the PI-PLC Enzyme

As indicated in Part 2, the subject of this GRAS notification is a phospholipase, EC 3.1.4.11. Enzymes, including phospholipase, have a long history of use in food (3) (2).

A wide variety of enzymes are used in food processing (2) (3). The active enzyme in the enzyme preparation is a phospholipase (EC 3.1.4.11). PI-PLC can be found in cells of plants, animals (including humans), bacteria and fungi (24) (25). Enzyme proteins do not generally raise safety concerns (3) (2). Phospholipase has been used safely in food production for decades and are widespread in nature being produced by a number of prokaryotic micro-organisms. Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the PI-PLC was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 1).

6(d) Allergenic/Toxigenic Potential of the PI-PLC Enzyme

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

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

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

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

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (26).

In order to further evaluate the possibility that the PI-PLC will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (27) and modified by Codex Alimentarius Commission, 2009 (28) the PI-PLC was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

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

Also, a search for homology of the phospholipase sequence from MaTa161 to known toxins was assessed on the basis of the information present in the UNIPROT

database (11-Feb-2016). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 20% indicating that the homology to any toxin sequence in this database is low and random.

Consequently, oral intake of the PI-PLC is not anticipated to pose any food allergenic or toxin concerns.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the PI-PLC which follows standard industry practices (14) (13) (12). The quality management system used in the manufacturing process for the PI-PLC complies with the requirements of ISO 9001. It is produced in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (15). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (16).

6(f) Safety Studies

This section describes the studies and analysis performed to evaluate the safety of the use of the PI-PLC.

The following studies were performed on test batch PPW40064 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Cytotoxicity Test: Neutral Red Uptake
- *In vitro* Micronucleus Test
- 13-week sub-chronic oral toxicity study

These tests are described in Appendix 2. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that PI-PLC represented by batch PPW40064, exhibits no significant toxicological changes under the experimental conditions described.

6(g) Results and Conclusion

Novozymes has reviewed the available data and information. We are not aware of any data and/or information that is, or appears to be, inconsistent with our conclusion of GRAS. Based on this critical review and evaluation, a history of safe use of *Bacillus licheniformis* and the limited and well defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; PI-PLC enzyme preparation, meets the appropriate food grade

specifications and is produced in accordance with current good manufacturing practices. Thus, it is generally recognized, among qualified experts, to be safe under the conditions of its intended use.

Part 7 – SUPPORTING DATA AND INFORMATION

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

APPENDICES

1. Pariza and Johnson Decision Tree Analysis

2. Summary of Toxicity Data. Phospholipase from *Bacillus licheniformis* PPW40064. 23, January 2017, File No. 2017-01294-01.

REFERENCES

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Appendix 3- This phosphoinositide phospholipase C preparation from *Pseudomonas sp. – 62186* produced in *Bacillus licheniformis* was evaluated according to the decision tree published in Pariza and Johnson, 2001 [(1)].

The result of the evaluation is presented below.

Decision Tree

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

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

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
      YES, *go to 3c.*

   c. Is the test article free of transferable antibiotic resistance gene DNA?
      YES, go to 3e.

   e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?
      YES*, go to 4**.*

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

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

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Toxicology & Product Safety

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SUMMARY OF TOXICITY DATA

Phospholipase, batch PPW40064, from *Bacillus licheniformis*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Phospholipase, batch PPW40064.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Envigo (UK) and Covance (UK) during the period March 2016 to January 2017.

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

- Phospholipase, batch PPW40064, was tested in a Neutral Red Uptake assay applying the BALB/c 3T3 cell line as test system and observed to reduce cell viability by 50% at and above 3 mg/mL.
- Phospholipase, batch PPW40064, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Phospholipase, batch PPW40064, did not induce biologically relevant increases in micronuclei *in vitro*, in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9).
- In a 13-week oral toxicity study in rats Phospholipase, PPW40064 was well tolerated and did not cause any toxicologically significant changes at any dose level tested.

2. TEST SUBSTANCE

The testsubstance is a phospholipase, (E.C. 3.1.4.11).

2.1 Characterization

The toxbatch Phospholipase, batch PPW40064, was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Phospholipase, batch PPW40064

Batch number	PPW40064
Activity	2610 PLC(E)DV/g
N-Total (% w/w)	0.66
Water (KF) (% w/w)	88.7
Dry matter (% w/w)	11.3
Ash (% w/w)	4.0
Total Organic Solids (TOS[1]) (% w/w)	7.3
Specific gravity (g/mL)	1.066

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

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Phospholipase, batch PPW40064 was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA pKM101) of *Escherichia coli*, both in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments. A 'treat and plate' procedure was used for all treatments in this study as Phospholipase, batch PPW40064 is a high molecular weight protein (which may cause artefacts through growth stimulation in a standard plate-incorporation test).

All Phospholipase, batch PPW40064 treatments in this study were performed using formulations prepared in water for irrigation (purified water).

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using final concentrations of Phospholipase, batch PPW40064 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL, plus vehicle and positive controls. Following these treatments, evidence of toxicity was observed at 5000 µg TOS/mL in all strains in the absence and presence of S-9 and also at 1600 µg TOS/mL in strain TA1537 in the absence of S-9.

Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S-9. The maximum test concentration of 5000 µg TOS/mL was retained for all strains. Narrowed concentration intervals were employed covering the range 51.2–5000 µg TOS/mL, in order to examine more closely those concentrations of Phospholipase, batch PPW40064 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, evidence of toxicity was again observed at 5000 µg TOS/mL in all strains in the absence and presence of S-9, and also at 800 and 2000 µg TOS/mL in strain TA1537 in the absence of S-9.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were all comparable with acceptable ranges for vehicle control treatments and were elevated by positive control treatments.

Following Phospholipase, batch PPW40064 treatments of all the test strains in the absence and presence of S-9, there were no clear and concentration-related increases in revertant numbers observed, and none that were ≥2-fold (in strains TA98, TA100 and WP2 uvrA pKM101) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of Phospholipase, batch PPW40064 mutagenic activity in this assay system.

It was concluded that Phospholipase, batch PPW40064 did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA pKM101) of *Escherichia coli* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg TOS/mL (a toxic concentration), in the absence and in the presence of a rat liver metabolic activation system (S-9)

using a modified ‘Treat and Plate’ methodology.

3.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

Error! Reference source not found. was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two **Error! Reference source not found.** donors in two independent experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test article was formulated in **Error! Reference source not found.**. The highest concentrations initially tested in Micronucleus Experiment 1 were determined following a preliminary cytotoxicity Range-Finder Experiment.

Treatments were conducted 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of **Error! Reference source not found.** on the replication index (RI). Micronuclei were analysed at three or four concentrations.

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in the vehicle cultures fell within current 95th percentile of the observed historical vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of S-9. Cells receiving these were sampled in the Micronucleus Experiments at 24 hours (CPA, MMC) or 48 hours (VIN) after the start of treatment. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei. The study was therefore accepted as valid.

Experiment 1 Treatments
Extended (24+24 hour) treatment of cells with **Error! Reference source not found.** in the absence of S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all treated cultures fell within the normal ranges.

Following pulse (3+21 hour) treatment in the absence of S-9, small but statistically significant increases in MNBN cells frequency were observed for the three higher concentrations analysed (500, 750 and 1000 µg TOS/mL). At each concentration one of the two replicate cultures exhibited a MNBN cell value that marginally exceeded the normal range though no concentration related effect was apparent. As no such increase was observed in the replicate cultures with the maximal response noted at each concentration similar to that observed within the concurrent vehicle control treatments, these marginal statistical increases were considered of highly questionable biological importance.

Following pulse (3+21 hour) treatment in the presence of S-9, a statistically significant increase in MNBN cell frequency was observed at the highest concentration analysed (750 µg TOS/mL), where post treatment precipitate was observed (and where precipitate was noted on the slide preparations). The MNBN cell values of both replicate cultures at this concentration marginally exceeded normal values. No such increases were observed for the two lower concentrations analysed. Although this increase did fulfil the protocol criteria for a positive response, because the increase was marginal and restricted to a single precipitating concentration, it was considered of questionable biological importance.

In order to better qualify these data, additional 3+21 hour treatments in the absence and presence of S-9 were conducted as part of Micronucleus Experiment 2.

Experiment 2 Treatments
Following pulse (3+21 hour) treatment in the absence and presence of S-9 with **Error! Reference source not found.**, frequencies of MNBN cells were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all treated cultures fell within the normal ranges. Maximum concentrations analysed were the lowest concentrations at which visible precipitate was present at the end of the treatment phase.

The weak increases in MNBN cells observed in Experiment 1 were not reproduced in Experiment 2 where similar concentrations (limited by the presence of post treatment precipitate) were analysed. Given the presence of precipitate (which can be a confounding factor in MN analysis), it was considered possible that this may have contributed to the weak effects observed in Experiment 1. Overall, the data from both Experiments indicate that there were no biologically relevant increases in MNBN cells as a result of treatment with Phospholipase, batch PPW40064.

It is concluded that **Error! Reference source not found.** did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations for micronucleus analysis were limited by the presence of post treatment precipitate.

4. GENERAL TOXICITY

4.1 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in BALB/c 3T3 Cell Culture

The aim of this study was to evaluate the cytotoxicity of Phospholipase, Batch PPW40064, using a Neutral Red Uptake (NRU) assay in 3T3 cells.

The growth of 3T3 cells treated with a range of concentrations of the test item was compared with vehicle control cultures after 48 hours exposure both visually and using neutral red uptake.

Phospholipase was toxic at approximately 50% viability when compared with the vehicle control at the highest three concentrations, 3 – 30 mg/mL, and less toxic at the lowest five concentrations, 0.01 – 1 mg/mL, according to the neutral red uptake results. A visual assessment of the cell monolayers showed that the test item produced around 10% confluency at the highest two concentrations, with slight precipitation of the test item at the highest concentration of 30 mg/mL. Confluency was approximately 20% at the third concentration and 40% confluency at the lower five concentrations. The vehicle control produced approximately 50% confluency.

The IC50 value of the positive control, sodium lauryl sulphate, was calculated to be 94.69 µg/mL which lay within the historical control range of this laboratory.

It was concluded that Phospholipase, batch PPW40064, demonstrated cytotoxicity at approximately 50% viability compared to the vehicle control in the concentration range 3 – 30 mg/mL, with less toxicity observed at the lower concentrations of 0.01 – 1 mg/mL.

4.2 Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks

The purpose of this study was to assess the systemic toxic potential of Phospholipase, batch PPW40064 (an enzyme used in the food industry) when administered orally, by gavage, to Han Wistar rats for 13 weeks. Three groups, each comprising ten males and ten females, received Phospholipase, batch PPW40064 at doses of 10%, 25% or 65% of the Phospholipase batch (equivalent to 77.8, 194.5 or 505.8 mg TOS/kg bwt/day, or 2782.3, 6955.7 or 18084.7 PLC(S)/kg bwt/day). A similarly constituted control group received the vehicle, reverse osmosis water, at the same volume-dose (10 mL/kg body weight).

During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, body weight, food consumption, water consumption, ophthalmoscopy, hematology (peripheral blood), blood chemistry, urinalysis, organ weight, macropathology and histopathology investigations were undertaken.

Results

The general appearance, sensory activity, grip strength and motor activity of the animals were unaffected by treatment and there was no death.

Bodyweight gain and food consumption was unaffected by treatment. There was a reduction of water intake in Week 1 and 4 by females receiving 25% or 65% Phospholipase, batch PPW40064 but this was considered to be incidental to treatment.

There was no treatment-related ophthalmic finding.

There was no treatment-related haematological finding in Week 13.

The biochemical examination of the blood plasma in Week 13 indicated a dose-related increase of glucose concentration in males receiving 25% or 65% Phospholipase, batch PPW40064, increased total plasma cholesterol concentration in animals, particularly females receiving 65% Phospholipase, batch PPW40064, and slightly low albumin concentrations in males receiving 65% Phospholipase, batch PPW40064. These findings were considered to be of no toxicological significance.

Urinalysis investigations revealed low urinary pH in males and females receiving 65% Phospholipase, batch PPW40064, a small reduction of urinary specific gravity in males receiving 25% or 65% Phospholipase, batch PPW40064 and a slightly high protein output in females receiving 65% Phospholipase, batch PPW40064. These findings were considered to be of no toxicological significance.

Kidney weights were increased slightly after 13 weeks in females given 25% or 65% Phospholipase, batch PPW40064; this was considered to be incidental to treatment.

There was no treatment-related macroscopic finding.

Histopathological changes that were due to treatment were confined to the stomach where there were eosinophilic globules in the glandular mucosa of the stomach, near the limiting ridge, at a higher incidence and at a slightly higher severity in males given 65% Phospholipase, batch PPW40064 and at a minimal severity in the majority of females given 65% Phospholipase, batch PPW40064, and minimal, focal, inflammatory cell infiltrate of the glandular mucosa/submucosa in males given 65% Phospholipase, batch PPW40064.

Conclusion

It is concluded that oral administration of Phospholipase, batch PPW40064 to Han Wistar rats for 13 weeks at doses up to 65% of Phospholipase, batch PPW40064 (equivalent to 505.8 mg TOS/kg bwt/day or 18084.7 PLC(S)/kg bwt/day) caused no overt evidence of toxicity but a non-adverse adaptive response to mild local irritation was evident in the stomach (eosinophilic globules and minimal, focal, infiltrate of inflammatory cells in the glandular mucosa). The no-observed-adverse-effect level (NOAEL) in this study was considered to be 65% Phospholipase, batch PPW40064 (equivalent to 505.8 mg TOS/kg bwt/day or 18084.7 PLC(S)/kg bwt/day).

5. REFERENCES

5.1 Study reports

Envigo: Study No.: PR80DV; Novozymes Reference No.: 20166014: Phospholipase, PPW40064: 3T3 Neutral Red Uptake test. (March 2016). LUNA file: 2016-04644.

Covance: Study No.: 8336166; Novozymes Reference No.: 20166001. Phospholipase, batch PPW40064: Bacterial Reverse Mutation Assay using a Treat and Plate Modification. (August 2016). LUNA file: 2016-12715.

Covance: Study No.: 8336168; Novozymes Reference No.: 20166002: Phospholipase, Batch PPW40064: *In vitro* Human Lymphocyte Micronucleus Assay. (July 2016). LUNA file: 2016-12346.

Envigo: Study No.: HQ56XG; Novozymes Reference No.: 20166020: Phospholipase, Batch PPW40064: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. (January 2017). LUNA file: 2017-01293.