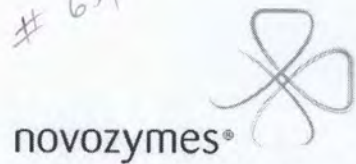


## ORIGINAL SUBMISSION

# 651



Rethink Tomorrow

GRN 000651

May 2, 2016

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 (a), for Novozymes' phospholipase A1 enzyme preparation produced by a genetically modified strain of *Aspergillus niger*.

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 phospholipase A1 is generally recognized as safe for use in the food industry as a processing aid for the de-gumming of oil.

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

Sincerely,

(b) (6)

Janet Oesterling  
Regulatory Affairs Specialist III

Enclosures



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Franklinton, North Carolina 27525

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[www.novozymes.com](http://www.novozymes.com)

000002

May 2, 2016

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) Novozymes North America Inc. hereby claims that phospholipase A1 preparations produced by submerged fermentation of a genetically modified *Aspergillus niger* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

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

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

A phospholipase A1 enzyme preparation produced by a genetically modified *Aspergillus niger*.

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The phospholipase A1 is used as a processing aid in the de-gumming of vegetable oil. 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.

Proposed § 170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at Novozymes North America, Inc. or will be sent to FDA upon request.

(b) (6)



Janet Oesterling  
Regulatory Affairs Specialist III

05-02-16  
Date



novozymes®

Rethink Tomorrow

**A Phospholipase A preparation produced by a genetically modified strain of *Aspergillus niger***

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

May 2016



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## 1. GENERAL INTRODUCTION

The subject of this notification is a phospholipase A1 enzyme preparation, hereby known as PLA, produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism carrying the gene coding for phospholipase from *Talaromyces leycettanus*.

This PLA enzyme preparation is intended for use as a processing aid to remove impurities that affect the taste, smell and visual appearance of vegetable oils. One important class of impurities is phosphatides, also called gums. Enzymatic degumming with PLA is an efficient way of removing these gum impurities. It also simplifies the degumming process by allowing less cooling and less pH adjustment during processing.

The active enzyme is phospholipase A (EC 3.1.1.32, CAS 9043-29-2).

The information provided in the following sections is the basis for our determination of general recognition of safety of the PLA enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

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 (1) (2) (Appendix 1). The production organism for the PLA, *Aspergillus niger*, is discussed in Sections 2 and 7. *Aspergillus niger* has a documented history of safe use in food and is considered to be a safe organism for the production of enzymes or ingredients used in food (3).

*Aspergillus niger* was exempted from EPA review under TSCA (4). *Aspergillus niger* has a long history of safe use. The FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* (GRAS notifications 000089, 000111, 000132) to name a few (5).

*Aspergillus niger* is generally considered to be non-pathogenic and non-toxicogenic and is often mentioned as an example of a well characterized and safe production strain with a long history of safe use. *Aspergillus niger* meets the US Environmental Protection Agency (EPA) criteria for non-toxicogenicity and non-pathogenicity, it is one of 10 recipient organisms eligible for Tier I exemption under the EPA regulations (6).

The production organism is derived from the *Aspergillus niger* recipient strain. *Aspergillus niger* has been used by industry since 1919 for the production of citric acid which can be an ingredient in foods such as soft drinks, fruit juices and jams. The US Food and Drug Administration (FDA) listed *Aspergillus niger* as a source of

citric acid (21 CFR 173.280). This species is well known to produce various enzymes for food use. The Association of Microbial Food Enzyme Producers (AMFEP) listed *Aspergillus niger* as a safe source of microbial food enzymes (7). In Section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

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 (8) (9) (10) (11) (12) (13). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, phospholipase A1 (PLA) enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices, thus making it GRAS for the intended conditions of use.

## 2. PRODUCTION MICROORGANISM

### 2.1 Production Strain

The *Aspergillus niger* production strain, designated 279-C2948-1 was derived from recipient strain C2948, a natural isolate of *Aspergillus niger* strain BO-1. This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (10). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (1) and several expert groups (8) (9) (10) (11) (12) (13).

The expression plasmid, used in the strain construction, *plhar279*, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *pla1* sequence encoding a phospholipase A from *Talaromyces leycettanus*.

### 2.2 Recipient Strain

The recipient strain C2948 (*Aspergillus niger*) used in the construction of the PLA production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of amylases and proteases. Furthermore the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted in C2948 together with the deletion of additional genes encoding unwanted proteins that can

be present in the culture supernatant. The lack of these represents improvements in the product purity, safety and stability.

### 2.3 PLA Expression Plasmid

The expression plasmid, *plhar279*, used to introduce the *pla1* gene in the recipient strain C2948 is based on the replication origin of *E. coli*. However, no fragments of the vector backbone are introduced into the production strain. The plasmid contains the expression cassette consisting of a fragment of the *Aspergillus niger* promoter, the *pla1* sequence encoding the PLA, a transcriptional terminator and finally a selective marker, *amdS*. The expression cassette and the *amdS* gene encoding an acetamidase are flanked by DNA regions used for targeted integration. Only this region is present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

### 2.4 Construction of the Recombinant Microorganism

The production strain, *Aspergillus niger* 279-C2948-1, was constructed from the recipient strain C2948 through the following steps:

1. The expression cassette from plasmid *plhar279* was integrated into four specific loci in strain C2948 by targeted homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the *pla1* gene from the promoter.
2. The selection of transformants was achieved by growing on a minimal medium and subsequent screening for expression of the PLA.

The resulting PLA production strain containing one copy of the *pla1* gene at each of the four target loci was named 279-C2948-1.

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

### 2.5 Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern blot hybridization. Analysis of samples from end of production using a *pla1* gene specific probe showed an identical band pattern compared to the reference production strain (279-C2948-1), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Aspergillus niger* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

## 2.6 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 Southern blot.

## 2.7 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (8) is satisfactorily addressed.

# 3. MANUFACTURING PROCESS

This section describes the manufacturing process for the PLA which follows standard industry practices (14) (15) (16). The quality management system used in the manufacturing process for the PLA complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (15) and 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 (17). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (18).

## 3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the PLA enzyme concentrate are standard ingredients used in the enzyme industry (14) (15) (16). 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 if used in the product is less than 1%.

### 3.2 Fermentation Process

The PLA enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Aspergillus niger* as described in Section 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.

#### 3.2.1 Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Aspergillus niger*, described in Section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

#### 3.2.2 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.

### 3.3 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.

### 3.3.1 Purification Process

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

- 1) Pretreatment - pH adjustment and flocculation
- 2) Primary Separation – vacuum drum filtration or centrifugation
- 3) Concentration - ultrafiltration and/or evaporation
- 4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
- 5) Final concentration – evaporation and/or ultrafiltration.
- 6) Preservation and Stabilization of the liquid enzyme concentrate

### 3.3.2 Formulation and Standardization Processes

The liquid enzyme preparation is standardized with sorbitol and glycerol and preserved with potassium sorbate and sodium benzoate. See Table 1 below.

### 3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in Section 5.

## 4. ENZYME IDENTITY

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

Classification	Phospholipase A1 (generic name)
IUBMB nomenclature:	Phosphatidylcholine 1-acylhydrolase
EC No.:	3.1.1.32
CAS No.:	9043-29-2
Specificity:	hydrolyses the fatty acyl ester bond at the sn-1 position of the glycerol moiety resulting in the formation of 2-acyl-1-lysophospholipids and free fatty acids.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

## 5. COMPOSITION AND SPECIFICATIONS

### 5.1 Quantitative Composition

The PLA 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.

**Table 1. Typical compositions of the enzyme preparations**

Substance	Approximate Percentage
Enzyme Solids (TOS*)	9.1%
Water	45 – 50%
Sorbitol	20 – 30%
Glycerol	20 – 30%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

### 5.2 Specifications

The PLA enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *Food Chemicals Codex* (17). 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 (18).

In the General Specifications for enzyme preparations laid down by JECFA in 2006 (18), the following is said: "Although nonpathogenic and non-toxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species."

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	Specification	PPW40302	PPW40087	PPW40360
Activity unit	PLA(L)/g	664	1170	1110
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Total Coliforms	Not more than 30/g	<4	8	16
Salmonella	Absent in 25g	ND	ND	ND
Escherichia coli	Absent in 25g	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Ochratoxin A	<LOD	<0.0003	<0.0003	<0.0003
Fumonisin B <sub>2</sub>	<LOD	<0.0003	<0.0003	<0.0003

## 6. APPLICATION

### 6.1 Mode of Action

The active enzyme is a phospholipase A1 (EC 3.1.1.32). PLA specifically acts on the fatty acid in position 1 in phospholipid substrates. It catalyzes the hydrolysis of sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and free fatty acid.

The PLA preparation is used as a processing aid during food manufacturing. The typical food process where this food enzyme is used is in the degumming of vegetable oil.

In the “degumming” of oil, phospholipids are removed from oil during the oil purification process in order to improve the taste and quality of the oil. Removal of phospholipids is required for good storage stability of the oil and facilitates downstream processing.

### 6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired 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 32mg PLA(L) per kg of oil.

### 6.3 Enzyme Residues in the Final Food

The PLA food enzyme is expected to be inactivated or removed during the production process for all degumming applications.

Phospholipases catalyze the hydrolysis of one or more ester and phosphodiester bonds of glycerophospholipids. The phospholipase object of the present dossier is a phospholipase A1 that specifically hydrolyses the fatty acyl ester bond at the sn-1 position of the glycerol moiety resulting in the formation of 2-acyl-1-lysophospholipids and free fatty acids.

In the de-gumming application, the PLA enzyme is applied in a water phase to the oil. The enzyme is soluble in water and insoluble in organic solvents, and will not migrate into the oil phase. After the reaction, the water phase that contains the PLA and lysophospholipids, is removed. Furthermore the oil will be subjected to oil refining methods, which would remove any potential residues.

## 7. SAFETY EVALUATION

### 7.1 Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (2) (1). If the organism is non-toxicogenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (19). Pariza and Foster (2) define a non-toxicogenic 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".

*Aspergillus niger* has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. *Aspergillus niger* is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in published scientific literature (1).

Carbohydrase, pectinase, protease, glucose oxidase, catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA, on request from the Enzyme Technical Association (ETA), converted into separate GRAS Notices (GRN 89, 111, 132) (5). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency did not question the conclusion that enzyme preparations from *Aspergillus niger* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 158, 183, 214, 296, 345, 402, 428 which all describe food enzymes produced by *Aspergillus niger* strains (5).

In 1997, *Aspergillus niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations (20). Also, *Aspergillus niger* was reviewed and was concluded to be a safe source organism by Olempska-Beer et al. (21) and Schuster et al. (3) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

An evaluation of the genetically modified production microorganism for the PLA, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (19), the EU SCF in 1991 (9), the OECD in 1992 (10), ILSI Europe Novel Food Task Force in 1996 (13), FAO/WHO in 1996 (12), JECFA in 1998 (18) and Pariza and Johnson in 2001 (1) 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 Section 2 and 3.

Novozymes' used the decision tree (Appendix 2) in Pariza and Johnson 2001 (1) as a basis for our safety assessment. The production strain is genetically modified as discussed in Section 2. The expressed enzyme product is phospholipase A1. The enzyme preparation is free of DNA encoding transferable antibiotic resistance DNA genes. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

The genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances.

Some *Aspergillus niger* strains can produce ochratoxin A (3), and the production of fumonisin B2 has also been shown in *Aspergillus niger* (22). Ochratoxin A and fumonisin B2 are the two mycotoxins of concern in terms of human and animal safety that can be produced by *Aspergillus niger* strains (23).

The BO-1 safe strain lineage were found to be unable to produce unwanted secondary metabolites (ochratoxin A and fumonisin B2) under conditions that are known to induce mycotoxin production in fungi. In addition analytical test results of three representative enzyme batches of this phospholipase (as discussed in section 5.2) confirm the absence of ochratoxin A and fumonisin B2.

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

### 7.1.1 Safe Strain Lineage

The *Aspergillus niger* production strains are derived from the *Aspergillus niger* C40 (parental strain) cell lineage. Strain BO-1, derived from the parental strain C40 solely by classical mutagenesis, was deposited as DSM 12665. The identification of strain BO-1 as *Aspergillus niger* has been confirmed. The *Aspergillus niger* production strains belong to a single strain lineage which has been used by Novozymes for production of a wide range of commercial enzymes.

The safety of the *Aspergillus niger* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (1) (8).

The recipient strain, *Aspergillus niger*, has been thoroughly characterized as shown in Sections 2.2. The introduced DNA is well-known and characterized in Section 2.3 and 2.4 and the introduced genetic material does not encode or express any known harmful or toxic substances.

The procedures used to modify the recipient organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (1) have been met.

Novozymes has extensive experience working with *Aspergillus niger* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry.

All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products but also indicate that no unexpected secondary effects of the genetic modifications have been observed. Furthermore the information included in this notification has been reviewed by Novozymes Regulatory Affairs staff for suitability.

Novozymes has used *Aspergillus niger* production strains derived from the C40 lineage for over 20 years and has performed a number of safety studies on different enzyme products manufactured using *Aspergillus niger*. Table 1 below outlines some of the Novozymes products produced by *Aspergillus niger* strains and the

safety studies conducted on those products. Section 7.5.1 outlines the testing completed and the appendix reference.

**Table 4. Safe strain lineage**

Enzyme	EC No.	Predecessor strain (a)	Donor strain	Safety studies (b)
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> BO-1	None	Yes
Pectin lyase	4.2.2.10	<i>Aspergillus niger</i> BO-1	<i>Aspergillus niger</i>	Yes
Lysophospholipase	3.1.1.5	<i>Aspergillus niger</i> BO-1	<i>Aspergillus niger</i>	Yes
Triacylglycerol lipase	3.1.1.3	<i>Aspergillus niger</i> BO-1	<i>Candida antarctica</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> JaL303	<i>Aspergillus niger</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> JaL303	<i>Talaromyces emersonii</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C878	<i>Trametes cingulata</i>	Yes
Alpha-amylase	3.2.1.1	<i>Aspergillus niger</i> C878	<i>Rhizomucor pusillus</i>	Yes
Alpha-amylase	3.2.1.1	<i>Aspergillus niger</i> C2218	<i>Rhizomucor pusillus</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C2218	<i>Gloeophyllum trabeum</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C2218	<i>Penicillium oxalicum</i>	Yes
Triacylglycerol lipase	3.1.1.3	<i>Aspergillus niger</i> C2218	<i>Candida antarctica</i>	Yes

**Table 4.** Novozymes products derived from *A. niger* strains where safety studies have been carried out.

<sup>a)</sup> The predecessor strain shows strains in the GM construction that are in common with the C40 strain lineage, see figure below.

<sup>b)</sup> 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 subchronic oral toxicity study in rats. The conclusions of these studies were in all cases favourable.

## 7.2 Safety of the Donor Organism

The donor organism of the PLA is *Talaromyces leycettanus*. As indicated in Section 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the PLA 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.

## 7.3 Safety of the PLA Enzyme

The principal enzyme activity is phospholipase A1. Phospholipases are classified as hydrolases which are a group of enzymes that hydrolyze various bonds. Phospholipases breakdown phospholipid substrates into smaller units that do not have toxic properties and that are readily metabolized by the human body

Phospholipase A1 is found in animal tissues (24) (25) (26) and plant tissues (27). It has been found in many cells and tissues of various organisms (28) including animal pancreas and small intestines (25) (26). Phospholipases are part of the digestive enzymes present in pancreatic juice of mammals including humans (29). FDA

Novozymes / Phospholipase A preparation produced by a genetically modified strain of *Aspergillus niger*

concluded that phospholipase A2 should be digested like any other protein in food (29). Because the PLA1 activity is also found in most cells and tissues including animal tissues that are consumed by man, we would also conclude that PLA1 should be digested like any other protein in food.

Animal derived lipases have been affirmed as GRAS substances (21 CFR§184.1415). These lipases are derived from porcine or bovine pancreas. Also animal derived phospholipase A2 has been safely used to manufacture enzyme modified lecithin (29). It is very likely that these preparations contain PLA1 but it was not detected as some studies indicate that PLA1 activity can be difficult to distinguish from other lipolytic activities (26).

A wide variety of enzymes are used in food processing (1) (2). Enzyme proteins do not generally raise safety concerns (1) (2). Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the PLA was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 2).

Based on the information above, Novozymes concludes that phospholipase A1 enzymes have a history of safe use in food.

### 7.3.1 Allergenic Potential of the PLA 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) (30).

In order to further evaluate the possibility that the PLA will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (31) and modified by Codex Alimentarius Commission, 2009 (32) the PLA 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>).

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 PLA 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 PLA 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.

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

#### 7.4 Safety of the Manufacturing Process

The PLA enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex*. As described in Section 3, the enzyme preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable 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 (14) (15) (16).

#### 7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the PLA.

##### 7.5.1 Description of Test Material

The following studies were performed on test batch PPW40307 with favourable results:

- Reverse Mutation Assay (Ames test)
- In vitro Cytotoxicity Test: Neutral Red Uptake

These tests are described in Appendix 3. Based on the present toxicity data and the history of safe use for the strain, it can be concluded that Phospholipase A1 exhibits no significant toxicological effects and does not induce gene mutations in bacteria under the experimental conditions described.

## 7.6 Estimates of Human Consumption and Safety Margin

As stated in Section 6.3 the enzyme activity is largely removed during de-gumming process of vegetable oil.

However, 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, if added to the crude oil at the highest recommended use rate, is retained in the final vegetable oil.

The assumptions are highly exaggerated since the enzyme protein and the other substances are diluted or removed in 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 average daily consumption for added fats and oils is taken from the USDA-ERS Food Availability per capita consumption report for 2006-2011 (33). 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 weight of 114kg 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, in the 90<sup>th</sup> percentile (34).

The PLA has an average activity of 981mg PLA(L)/g and approximately 9.1% TOS (Total Organic Solids) content. This corresponds to an activity/TOS ratio of 10.78 PLA(L)/mg TOS. The maximum recommended dosage is: 32mg PLA(L)/kg of oil

This will result in an exposure of:  
 $2.79\text{mg TOS/kg oil} \times 0.91\text{g oil/kg bw/day} / 1000 = 0.003\text{mg TOS/kg bw/day}$

### TMDI calculation

#### 7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL concluded in 13 week oral rat feeding studies conducted on Novozymes enzyme preparations is normally the

highest dosage that can be practically applied, i.e. limited by the concentration (TOS%) of the test batch and typically in the range of 500 to 1500mg TOS/ kg bw/ day. For the safety evaluation of this enzyme, a NOAEL level of (at least) 500mg TOS/kg bw/day is assumed and this value is used for the Safety Margin calculation. See Table 5 below.

**Table 5. NOAEL Calculation**

NOAEL (mg TOS/kg bw/day)	500
*TMDI (mg TOS/kg bw/day)	0.003
Safety margin	166,666

\*based on the worst case scenario

## 8. Results and Conclusions

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of safe use of *Aspergillus niger* and the limited and well defined nature of the genetic modifications, the phospholipase A1 enzyme preparation is safe for its intended use.

**9. List of Appendices**

1. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century Regulatory, Toxicology and Pharm 33: 173-186, 2001.
2. Pariza and Johnson Decision Tree Analysis
3. Summary of Toxicity Data. May 2016, LUNA No. 2016-07315-01.

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Pages 000027-000040 have been removed in accordance with copyright laws. The removed reference is:

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

## **Appendix 2-** Pariza & Johnson Decision Tree analysis of a phospholipase A1 produced by a genetically modified strain of *Aspergillus niger*.

This phospholipase A1 produced by a genetically modified strain of *Aspergillus niger* was evaluated according to the decision tree published in Pariza and Johnson, 2001.

The result of the evaluation is presented below.

### Decision Tree

1. Is the production strain genetically modified?

**YES**

***If yes, go to 2.***

The production strain *Aspergillus niger* designated 279-C2948-1, was derived via the recipient strain C2948 from a natural isolate of *Aspergillus niger* strain C40.

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**

As discussed in Section 7.3 of the notification.

***If yes go to 3c. If no, go to 3b***

- b. Is the NOAEL for the test article in the appropriate short-term oral studies sufficiently high to ensure safety? (Not required since 3a is YES however the answer here is also **YES**)

- c. Is the test article free of transferable antibiotic resistance gene DNA?

**YES**

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

***If yes go to 3e. If no go to 3d.***

- 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**

The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.

***If yes, go to 4.***

4. Is the introduced DNA randomly integrated into the chromosome?

**NO**

Site specific integration of the DNA was achieved at several selected loci of the *Aspergillus niger* chromosome. Sequence confirmation was performed in the production strain.

***If yes go to 5. If no, go to 6.***

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**

***If yes go to 6. If no go to 7.***

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

**YES**

The *Aspergillus niger* strain, 279-C2948-1, used in the construction of the phospholipase A1 production strain has previously been used as a host strain in several production strains for Novozymes enzyme products. These production strains were constructed by standard transformation procedures using well-known plasmid vectors and well characterized DNA sequences that were integrated into the *Aspergillus niger* strain chromosome by homologous recombination.

The safety of this production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients. The host strain has been thoroughly characterized as shown in Sections 2.2. The introduced DNA is well-known and characterized in Section 2.3 and 2.4 and the introduced genetic material does not encode and express any known harmful or toxic substances.

***If yes the test article is ACCEPTED.***

**Toxicology & Product Safety**

Date: May 03, 2016  
File: 2016-07315-01  
Ref.: LiNi

**SUMMARY OF TOXICITY DATA**

**Phospholipase A1, batch PPW40307 from *Aspergillus niger***

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## 1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Phospholipase A1, batch PPW40307.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Novozymes A/S (Denmark), Envigo (UK) and Covance (UK) during the period November 2015 to May 2016.

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

- Phospholipase A1, batch PPW40307 was tested in a Neutral Red Uptake (NRU) assay applying the BALB/c 3T3 cell line as test system. Only slightly reduced cell viability was observed, which is not considered to be of any toxicological concern because there was no dose-response relationship and hence no IC<sub>50</sub> value could be determined.
- Phospholipase A1, batch PPW40307 did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Phospholipase A1, batch PPW40307 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).

Based on the present toxicity data and the history of safe use for the strain it can be concluded that Phospholipase A1, represented by batch PPW40307, exhibits no significant toxicological effects under the experimental conditions described.

## 2. TEST SUBSTANCE

Phospholipase A1 (E. C. 3.1.1.32) catalyses the hydrolysis of fatty acids from phospholipids and will be used in the food and bioenergy sector for the degumming of vegetable oils.

### 2.1 Characterization

The batch Phospholipase A1, batch PPW40307 was used for the conduct of all the toxicological studies. The characterization data of the batch is presented in Table 1.

Table 1. Characterization data of Phospholipase A1, batch PPW40307

Batch number	PPW40307
Activity (PLA(L)/g)	679
N-Total (% w/w)	0.86
Water (KF) (% w/w)	86.4
Dry matter (% w/w)	13.6
Ash (% w/w)	0.7
Total Organic Solids (TOS <sup>1</sup> ) (% w/w)	12.9
Specific gravity (g/mL)	1.051

<sup>1</sup> % TOS is calculated as 100% - % water - % ash - % diluents.

### 3. GENERAL TOXICITY

#### 3.1 *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 A1, batch PPW40307 using a Neutral Red Uptake (NRU) assay in 3T3 cells.

The basis of the NRU assay is that 3T3 cells will normally grow in culture, continuously dividing and multiplying with time. A cytotoxic chemical will interfere with this process resulting in a reduction of the growth rate reflected by a decreased cell number. The growth of 3T3 cells treated with a range of concentrations of the test item is compared with vehicle control cultures after 48 hours exposure. The cell numbers are then estimated using the neutral red uptake technique, which depends on the ability of the viable cells to take up neutral red into their lysosomes. The amount of neutral red taken up by each treated culture is measured and is proportional to the number of viable cells present. The data are used to estimate the concentration producing 50% inhibition ( $IC_{50}$ ) relative to the vehicle control.

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

The relative toxicity assessed by neutral red uptake did not fall below 70% of the vehicle control at any concentration; therefore an  $IC_{50}$  value could not be estimated. Although toxicity as assessed by neutral red uptake was not substantial, the visual assessment of the cell monolayers at the end of the 48 hour treatment indicated that the cells were approximately 20% confluent at the highest concentration of Phospholipase A1 (30 mg/mL), 40% confluency at the second, third, fourth, fifth and sixth concentration (10, 3, 1, 0.3 and 0.1 mg/mL, respectively) and 50% confluency at the lower two concentrations (0.03 and 0.01 mg/mL, respectively). The vehicle control produced approximately 50% confluency.

The  $IC_{50}$  value of the positive control, sodium lauryl sulphate, was calculated to be 94.69  $\mu$ g/mL which lay within the historical control range of this laboratory.

Only slightly reduced cell viability was observed, which is not considered to be of any toxicological concern because there was no dose-response relationship and because the observed effect was just below level of significance.

### 4. MUTAGENICITY

#### 4.1 Bacterial Reverse Mutation assay (Ames test)

Phospholipase A1, batch PPW40307 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 A1, batch PPW40307 is a high molecular weight protein (which may cause artefacts through growth stimulation in a standard plate-incorporation test).

All Phospholipase A1, batch PPW40307 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 A1, batch PPW40307 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL, plus vehicle and positive controls. Following these treatments, there was some evidence of toxicity observed, but only at the highest treatment level of 5000 µg TOS/mL, and in strain TA98 in the presence of S-9 only.

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 160–5000 µg TOS/mL, in order to examine more closely those concentrations of Phospholipase A1, batch PPW40307 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, there was no clear evidence of toxicity observed, but a reduction in revertant numbers with strain TA1535 treatments at the higher concentrations in the absence of S-9 may have been due to test article related toxicity.

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 all fell within acceptable ranges for vehicle control treatments, and were elevated by positive control treatments.

Following Phospholipase A1, batch PPW40307 treatments of all the test strains in the absence and presence of S-9, the only notable increase in revertant numbers observed was a small increase in revertant numbers in strain TA1537 in the absence of S-9 in Experiment 1. This increase provided at least some evidence of a concentration-relationship, and approached (but did not achieve) the 3 fold increase over vehicle control level threshold. However, this increase was very small in magnitude (the increase in mean revertant numbers per plate above the vehicle control count was less than 10), and the increase was not reproduced in Experiment 2. Accordingly, this increase was not considered to have been a true compound related effect, and was not sufficient to be considered as evidence of Phospholipase A1, batch PPW40307 mutagenic activity.

As none of the other strain treatments provided any increases in revertant numbers that were  $\geq 2$ -fold (TA98, TA100 and WP2 *uvrA* pKM101) or  $\geq 3$ -fold (TA1535 and TA1537) the concurrent control, this study was considered therefore to have provided no evidence of any Phospholipase A1, batch PPW40307 mutagenic activity in this assay system.

It was concluded that Phospholipase A1, batch PPW40307 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 (the maximum recommended concentration according to current regulatory guidelines) in the absence and in the presence of a rat liver metabolic activation system (S-9) using a modified Treat and Plate methodology.

#### **4.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes**

Phospholipase A1, batch PPW40307 was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in a single experiment. 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 water for irrigation

(purified water) and the highest concentrations tested in the Micronucleus Experiment, 5000 µg TOS/mL (an acceptable maximum concentration for *in vitro* micronucleus studies according to current regulatory guidelines), was determined following a preliminary cytotoxicity Range-Finder Experiment.

Treatments were conducted for 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of Phospholipase A1, batch PPW40307 on the replication index (RI).

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 the 95th percentile of the current 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 rat liver S-9. Cells receiving these were sampled in the Micronucleus Experiment 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.

All acceptance criteria were considered met and the study was therefore accepted as valid.

Treatment of cells with Phospholipase A1, batch PPW40307 in the absence and presence 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 (all treatments). The MNBN cell frequency of all Phospholipase A1, batch PPW40307 treated cultures fell within normal ranges.

It is concluded that Phospholipase A1, batch PPW40307 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations were tested up to 5000 µg TOS/mL, a recommended regulatory maximum concentration for *in vitro* micronucleus assays.

## 5. REFERENCES

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Covance: Study No.: 8336737. Novozymes Reference No.: 20166007. Phospholipase A1, batch PPW40307: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli*. (May 2016). LUNA file: 2016-07297.

Covance: Study No.: 8336736. Novozymes Reference No.: 20166008: Phospholipase A1, batch PPW40307: *In Vitro* Human Lymphocyte Micronucleus Assay. (May 2016). LUNA file: 2016-07298.

SUBMISSION END