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May 31, 2006

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GRAS Notice Review
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Center for Food Safety and Applied
Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
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REC'D JUN - 5 2005

Re: Notice of GRAS exemption for use of Diversa's BD16449 Phospholipase C, an enzyme preparation expressed in the yeast *Pichia pastoris* (strain DVSA-PLC-004), for degumming vegetable oils

Dear Dr. Mattia:

Pursuant to proposed 21 CFR § 170.36 and on behalf of our client, i.e., Diversa Corporation (4955 Directors Place, San Diego, CA 92121-1609), this submission – forwarded in triplicate (plus two extra copies for your convenience) – is intended to notify FDA of Diversa's and an expert panel's independent determinations – based on scientific procedures – that the above-referenced BD16449 Phospholipase C is generally recognized as safe (i.e., GRAS) when used as an enzymatic processing aid for degumming vegetable oils (intended for use in foods) at a recommended level of 100-1000 grams of formulated enzyme preparation per metric ton of vegatable oil (i.e., at 100-1000 ppm). Accordingly, and as with all GRAS substances, such BD16449 Phospholipase C enzyme preparation – when used as described above and in the attached information – is

exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and that section's implementing regulations.

In support of said notice, please find attached a summary discussion of the information relied upon by Diversa and others when determining that Diversa's BD16449 Phospholipase C enzyme preparation is GRAS. Such discussion is set forth in the format required by proposed 21 CFR § 170.36(c). (62 FR 18961 – 4/17/97). Please note that none of this discussion contains confidential business information.

After you and your colleagues have had an opportunity to review and consider the attached information, if you should have questions or need additional information, please let me know. All of the information that serves as the basis for this GRAS determination can be sent – upon request – to FDA and is available for review and copying at reasonable times. For your immediate convenience, a copy of the published evaluation of the safety of Diversa's BD16449 PLC enzyme preparation (i.e., reference "Ciofalo" and "Appendix A") is attached.

Thank you in advance for your and your colleagues' efforts on behalf of Diversa's notice.

Sincerely,

Charles L. Morin

Cc: Diane M. Shanahan
Associate Director, Regulatory Affairs
Diversa Corporation

DIVERSA

Diversa Corporation
GRAS Notification Concerning
BD16449
Phospholipase C Enzyme Preparation
From *Pichia pastoris*

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I. INTRODUCTION

The lipase enzyme preparation that is the subject of this Notification, referred to below as BD16449 phospholipase C, is intended for use as a processing aid to hydrolyze phospholipids in the refining of soybean oil and other vegetable oils intended for use in foods. This hydrolysis results in the following benefits:

- Reduction in the amount of gum phospholipids and overall gum mass
- Reduction in the total phosphorus contained in the oil
- Reduction in the total mass of neutral oil entrained in the phospholipid gum
- Increase in the quantity of diacylglycerol contained in the oil

Refining is a general term used by the industry to describe the process whereby crude vegetable oil is converted to a purified final product by removing non-triacylglycerol components that can negatively impact taste, color and stability, and make the oil unsuitable for particular applications. The first step in this refining process is known as **degumming**, and involves a procedure that is designed to remove contaminating phospholipids or phosphatides that otherwise interfere with the processing of high phosphorus oils such as soybean, canola, corn and sunflower oils. It is during this step that Diversa's BD16449 phospholipase C enzyme preparation will be used. After the degumming reaction has been completed, the water phase, containing BD16449 phospholipase C, is separated from the oil by centrifugation. Subsequent steps in refining, including repeated washing of the oil with water, bleaching and deodorization, remove any remaining BD16449 phospholipase C residues from the refined oil.

As discussed in greater detail in Sections V.F.4 and VI.C of this Notification, inactive and denatured residues of the BD16449 phospholipase C enzyme preparation in refined oil are not detectable at a sensitivity of 1 ppb when using current available analytical methodology. Therefore, the corresponding dietary concentration of BD16449 phospholipase C total organic solids (TOS) is (worst case) *de minimus*.

The BD16449 phospholipase C enzyme preparation is derived from a non-toxigenic, non-pathogenic, genetically modified strain of *Pichia pastoris*. The *P. pastoris* strain SMD1168 is a commercially available protease-derivative of the wild-type strain NRRL Y-11140 (ATCC, 2005; Gleeson, M.A. et al., 1998).

The safety of the BD16449 phospholipase C enzyme preparation, when used as described, is supported by a series of published safety studies (Ciofalo, V. et al., 2006), including a 90-day oral toxicity study in rats and *in vitro* and *in vivo* genotoxicity studies of the enzyme preparation. The NOAEL derived from the 90-day oral toxicity study in rats is approximately 133,000 higher than the "worst case" estimate of the total, daily, human consumption of BD16449 phospholipase C of 0.015 mg/kg/day (Ciofalo, V. et al., 2006). This safety margin assumes a "worst case" scenario for the calculation of possible, daily, human exposure by assuming that all of the enzyme, if added to the crude oil at the highest recommended use rate, is retained in the final refined vegetable oil.

II. ADMINISTRATIVE INFORMATION

A. Claim regarding GRAS status

Diversa hereby notifies the FDA of determinations by Diversa and an Expert panel that the BD16449 phospholipase C enzyme preparation, derived from *Pichia pastoris*, strain DVSA-PLC-004, expressing a gene encoding phospholipase C (PLC), is generally recognized as safe (GRAS), based on scientific procedures, when used as described below for the hydrolysis of phospholipids during the refining of soybean oil and other vegetable oils intended for use in foods.

Edward T. Shonsey
Chief Executive Officer
Diversa Corporation
4995 Directors Place
San Diego, CA 92121

Date

B. Name and address of notifier

Diane M. Shanahan
Assoc. Director, Regulatory Affairs
Diversa Corporation
4995 Directors Place
San Diego, CA 92121
dshanahan@diversa.com

C. Common or usual name of subject substance

Phospholipase C; (also referred to as BD16449 phospholipase C)

D. Conditions of use

BD16449 phospholipase C is intended for use as a processing aid to hydrolyze phospholipids in the refining of soybean oil and other vegetable oils, intended for use in foods. The enzyme preparation will be used at levels no higher than necessary to achieve the intended effect, generally in the range of 100 to 1000 grams of formulated enzyme preparation per metric ton of oil, depending on the oil to be treated and the reaction conditions. This is equivalent to a concentration range of 100 to 1,000 parts per million.

E. Basis for GRAS determination

The GRAS determination for the subject enzyme preparation is based upon scientific procedures, as described in detail below.

F. Statement of availability of data and information

The data and information that are the basis for the notifier's and expert panel's determination are available for review and copying at the office of Morin & Associates, 388 Market Street, Suite 1460, San Francisco, CA 94111 **or** will be sent to FDA upon request.

III. DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

A. Name: BD16449 phospholipase C

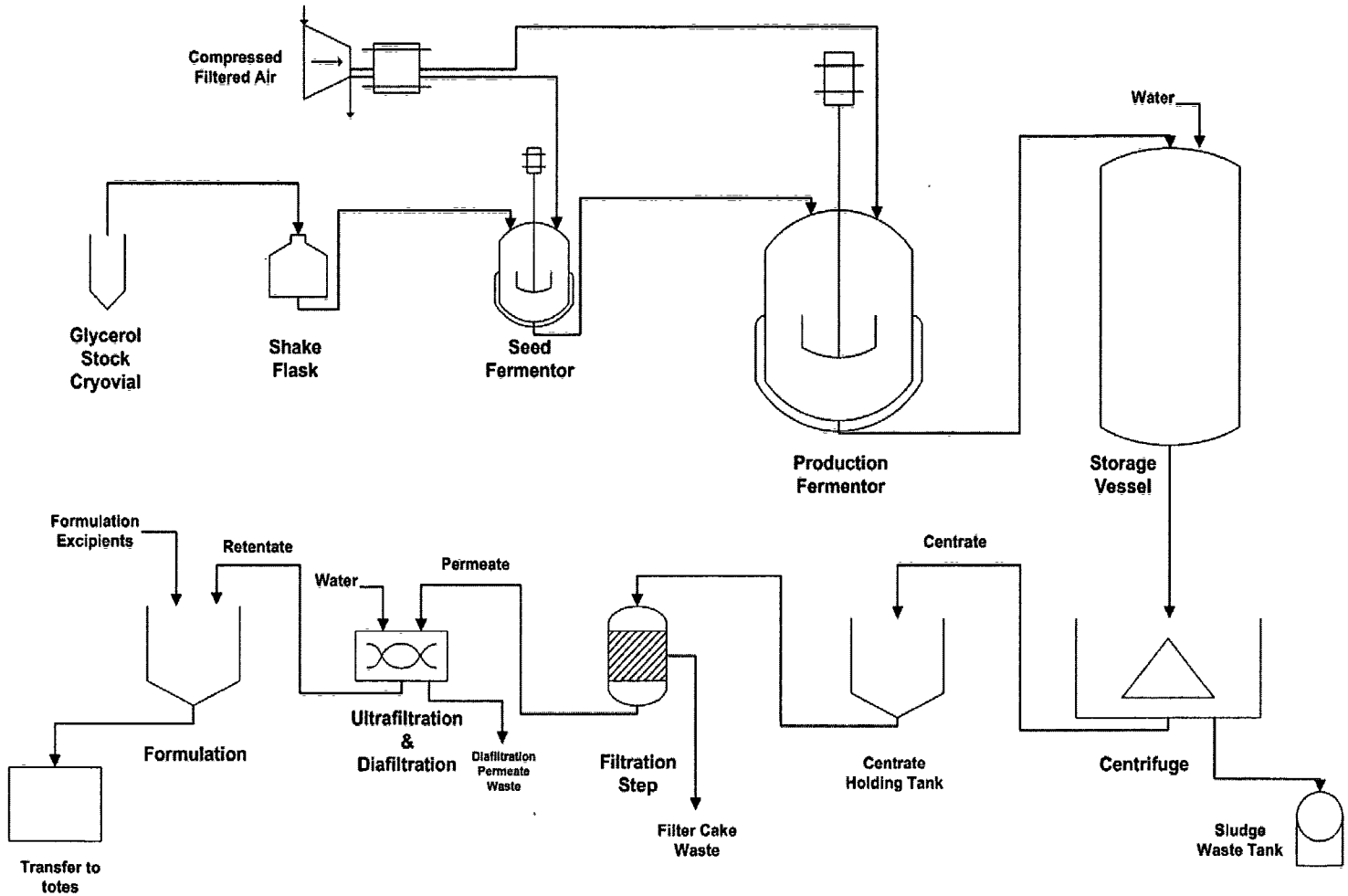
B. Physical description: Yellow to brown liquid

C. Method of manufacture

The BD16449 phospholipase C enzyme preparation is made using generally known and accepted methods for the production of microbial enzymes (Aunstrup, K. et al., 1979; Pariza, M.W. et al., 1983). In addition, the BD16449 phospholipase C enzyme preparation is manufactured in accordance with both current Good Manufacturing Practices for foods (cGMPs) and the Organization for Economic Co-operation and Development's criteria for Good Industrial Large Scale Practice (GILSP) (OECD, 1992). A diagram of the entire manufacturing process follows immediately on the next page. (See Figure 1).

Figure 1

Manufacturing Process for BD16449 Phospholipase C



1. Production microorganism

The *P. pastoris* parental strain SMD1168 is a commercially available protease-derivative of the wild-type strain NRRL Y-11140 (ATCC, 2005; Gleeson, M.A. et al., 1998). It has been genetically modified (strain DVSA-PLC-004) by procedures described in detail below (see Section V. A) for production of BD16449 phospholipase C.

Frozen cell bank cultures of the production organism, *Pichia pastoris* DVSA-PLC-004, are maintained as the source inoculum for enzyme production at -80°C in 15% v/v glycerol. The master cell bank is stored at multiple locations and serves as the parent to all working cell banks used for manufacturing. The manufacturing master cell bank (MCB1) was prepared and tested for cultural purity, acceptable productivity, and typical morphology and growth according to Diversa SOP MP0234. The manufacturing working cell bank (WCB1) was then prepared from MCB1 and qualified for the same criteria according to Diversa SOP MP0190.

Manufacturing master and working cell bank integrity under cold storage is verified by temperature records at each of the -80°C freezers. The master and working cell banks are also tested every 12 months to confirm cultural purity and the maintenance of good growth and productivity according to Diversa SOP MP0187.

2. Raw materials

Raw materials used in the fermentation and recovery process for BD16449 phospholipase C are standard ingredients used in the food/enzyme industry. The raw materials are of a purity and quality suitable for their intended use (Aunstrup, K. et al., 1979). They are food grade and GRAS, where available, or high-quality chemical or pharmaceutical grades (USP, NF, ACS, or reagent grades) from approved suppliers. All such raw materials have internal specifications (in line with *Food Chemical Codex* Fifth Edition requirements when available) and are quality inspected before use. These specifications include limits on lead and other pertinent heavy metals. If the raw material is from an approved supplier, the quality inspection consists of a review of the manufacturer's certificate of analysis to assure conformance with food grade specifications, along with a visual inspection or identification test. In other cases, raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Antifoaming agents used during manufacture and recovery of the BD16449 phospholipase C are of a suitable purity and appear on the Enzyme Technical Association's list of antifoaming agents evaluated and authorized by CFSAN (Enzyme Technical Association, 1998; FDA Center for Food Safety and Applied Nutrition, 2003; Pariza, M.W. et al., 1983).

3. Fermentation process

The BD16449 phospholipase C production begins with a submerged fed-batch pure culture of the genetically modified *Pichia pastoris* DVSA-PLC-004 (see Figure 1). Process parameters that are routinely monitored during the fermentation include pH, aeration, enzyme activity, agitation, temperature, and oxygen uptake rate. The fermentation broth is also routinely checked for purity. On the rare occasion when microbial contamination is detected that would affect either the product quality or safety, the broth is heat sterilized before being discarded.

After optimal BD16449 phospholipase C production is reached, the broth is transferred to the dilution tank where the enzyme is recovered from the fermentation broth by a chemical engineering operation broadly used in enzyme production (Atkinson, B. et al., 1991).

4. Recovery process

In the dilution tank, the broth is diluted with potable process water to allow passage and effective phase separation in the cell separation step. The cell mass is discharged as a sludge that is heat killed before going to waste. The centrate is passed to a filtration step, which removes any remaining cells and particulate solids. The retentate from this filter step is heat killed before going to waste. The clarified centrate is then concentrated by ultrafiltration, where the enzyme preparation is prepared at the appropriate concentration and then diafiltered to buffer exchange to the appropriate buffer salt concentration and pH (Cheryan, M., 1986). Food-approved stabilizers and preservatives are added to stabilize the finished product. The final enzyme preparation is then stored in containers held in a temperature controlled facility at 4-8°C.

D. Composition and specifications (of formulated product)

Diversa's BD16449 phospholipase C preparation complies with the General and Additional Requirements for Enzyme Preparations in the monograph on Enzyme Preparations in the Fifth Edition of the *Food Chemicals Codex* (FCC) (Committee on Food Chemicals Codex, 2004). In addition, the BD16449 phospholipase C preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing outlined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2001).

The BD16449 phospholipase C enzyme preparation is formulated as a liquid with the following typical composition:

Chemical Name	**Amount**
Total Organic Solids (TOS)[1]	approximately 5%
Sucrose	approximately 40%
Sodium chloride	approximately 10%
Zinc sulfate	approximately 0.15%
Methyl paraben	approximately 0.1%
Propyl paraben	approximately 0.05%
Water	Balance

The BD16449 PLC enzyme preparation specifications are as follows.

Specification	**Permissible Level**
Enzyme activity (units/mL)[2]	Not less than 30,000
Lead (mg/kg)	Not more than 5
Total viable count (cfu/g)	Not more than 50,000
Total Coliform (cfu/g)	Not more than 30
Antibiotic activity	Negative by test
E. coli (in 25g)	Absent
Salmonella (in 25g)	Absent
Absence of Production Organism	Absent

[1] The TOS level in the finished enzyme preparation is defined as 100% minus the sum of the moisture content, ash content, and any diluents or other substances added to the enzyme during formulation.

[2] One phospholipase C unit is defined as the hydrolysis of 1 μmole phospatidylcholine per minute at 37°C and pH 7.3.

The lead specification meets FCC and JECFA requirements. The *E. coli* and antibiotic activity specifications meet JECFA requirements, but are not included in FCC specifications. The total coliforms and *Samonella* specifications meet FCC and JECFA standards. The absence of production organism in the final product is a Diversa specification that is not included in FCC or JECFA specifications.

Three lots of BD16449 PLC enzyme preparation were analyzed for chemical and microbial composition to demonstrate that all three lots meet all of the specifications listed above for BD16449 PLC. The results can be found in Appendix B.

IV. ANALYTICAL METHODOLOGY

A. Introduction

In order to provide a consistent product, with uniform functionality in the end-use application, all finished lots of the BD16449 phospholipase C enzyme preparation will be analyzed for phospholipase C (PLC) activity prior to release for distribution and sale. As the BD16449 phospholipase C product is to be sold with a guaranteed minimum activity level of 30,000 units/mL, adequate methodology is needed to measure the amount of activity of the enzyme in its marketed form. The following discussion describes such methodology and includes the assays used for determining PLC activity levels of the formulated product.

B. Application and principle

The titrametric assay to determine PLC activity follows the conversion of phosphatidyl choline to 1,2-diacylglycerol and phosphorylcholine under controlled conditions. PLC is incubated in an emulsion containing phosphatidylcholine substrate, Triton X-100, and calcium chloride at 37°C and pH 7.3. As the enzyme PLC hydrolyzes phosphatidylcholine, acidic phosphorylcholine is released. Reaction rates are determined by monitoring the amount of base required to maintain the pH of the reaction mixture.

C. Source

A phospholipase A_2 (PLA2) activity assay using an auto-titration system was published in Journal of Lipid Research in 1973 (Dennis, E.A., 1973). The PLC assay method QC0225, as adopted at Diversa, optimizes the conditions of the PLA2 published assay system (including substrate concentration, buffer composition and pH, and incubation temperature) with respect to PLC.

D. PLC assay protocol

The detailed assay protocol is outlined in the Diversa standard operating procedure QC0225. The overview of the assay has been described above in Part B, Application and principle. One phospholipase C unit is defined as the hydrolysis of 1 μmole phosphatidylcholine per minute at 37°C and pH 7.3.

E. Assay validation

The phospholipase C assay procedure was validated under standard conditions at Diversa Corporation. The following analytical performance parameters were measured:

1. Linearity: The slope of the regression line and its variance provide a mathematical measure of linearity. The correlation coefficients for the regression lines for analyses of reaction rate *vs*. PLC concentration averaged 0.9948, based on data generated from two different lots.

2. Range: The range of the assay is the interval between the upper and lower levels of activity that have been demonstrated to be determined with precision and linearity, using the method as written. The range of the method is specified in QC0225 as 0.02 – 0.10 mL/min.

3. Limit of Detection and Limit of Quantitation: The limit of detection is the lowest PLC activity that can be detected and the limit of quantitation is the lowest PLC activity that can be measured, with acceptable precision. The standard deviation of the analytical response of a number of samples multiplied by the factors 3 and 10 provide estimates of the limit of detection and limit of quantitation, respectively.
   Limit of detection = 0.0039 mL/min
   Limit of quantitation = 0.0130 mL/min (equivalent to an activity of ~0.3 U/mg for a 100x dilution of a 5 mg/mL solution of a PLC sample with an activity of ~300 U/mg)

4. Repeatability: The repeatability of the assay was determined by measurement of the PLC activity of ten samples (two lots, five samples from each lot) on the same day, using the same analyst, substrate preparation and equipment, and calculation of the standard deviation and relative standard deviation (coefficient of variation). Two separate lots gave standard deviations of 30.2 U/mg and 11.4 U/mg and coefficients of variance of 7.8% and 3.8%, respectively.

5. Intermediate Precision: The intermediate precision of the assay was determined by assaying different sample preparations from two lots on six different days, preparing enzyme samples and substrate fresh daily and using the same reagents and equipment, and calculation of the standard deviation and relative standard deviation (coefficient of variation). Two separate lots gave

standard deviations of 28.5 U/mg and 15.8 U/mg and coefficients of variance of 8.1% and 5.0%, respectively.

6. Robustness: The robustness of the assay was determined by assaying a sample preparation under small variations of selected assay conditions (i.e., temperature, pH, and phosphatidylcholine (PC) substrate concentration) to be able to determine sensitivities of a control parameter. The results indicated that temperature is a critical control parameter, while the assay is relatively insensitive to small changes in pH setpoint and substrate concentration.

V. DETAILED SUMMARY OF THE BASIS FOR NOTIFIER'S GRAS DETERMINATION

A. Production microorganism

This section describes the historical activities associated with production of the *Pichia pastoris* (*P. pastoris*) production strain DVSA-PLC-004, the origin of the BD16449 PLC gene, and the methodology used to introduce the latter into the recipient strain *P. pastoris* SMD1168.

1. Production strain summary

DVSA-PLC-004 was constructed by electroporation of *P. pastoris* SMD1168, the recipient strain, with a purified DNA fragment containing the BD16449 PLC gene. The DNA fragment was obtained from a soil environmental library (see Section V.A.3 below). This genetically modified production organism complies with the OECD (Organization for Economic Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (OECD, 1992). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Berkowitz, D. et al., 1989; EU Scientific Committee for Food, 1992; FAO/WHO, 1996; International Food Biotechnology Council, 1990; Jonas, D.A. et al., 1996; OECD, 1993; Pariza, M.W. et al., 1983; Pariza, M.W. et al., 2001).

The specific DNA sequences used in strain construction include a PLC enzyme gene from an environmental library; the *Saccharomyces cerevisiae* α-mating factor secretion signal sequence; a fragment of the *P. pastoris* alcohol oxidase gene (3'*P.p. AOX1*); a *P. pastoris* selectable marker gene, *HIS4* (histidinol dehydrogenase) (Cregg, J.M. et al., 1985); and the well-characterized, non-coding *P. pastoris* regulatory sequences comprising the alcohol oxidase promoter (*AOX1*) (Tschopp, J.F. et al., 1987) and the transcriptional terminator from *AOX1*.

2. Recipient microorganism

The recipient microorganism is *P. pastoris* SMD1168. The general taxonomy of *P. pastoris* is as follows:

Name: *Pichia pastoris*

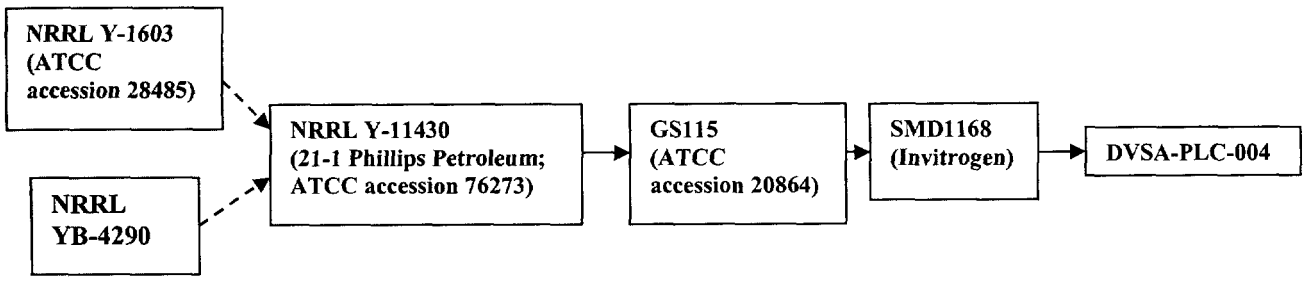
Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Hemiascomycetes
Order:	Saccharomycetales
Family:	Endomycetaceae
Genus:	Pichia
Species:	pastoris

According to the definitive source of yeast taxonomy (Kreger-van Rij, N.J.W., 1984) as well as a thorough literature search, there were no indications that *Pichia pastoris* has been associated with animal or human illness.

The following lineage for *P. pastoris* SMD1168 is based on literature sources and from discussions with experts in this area. The first *P. pastoris* strains (see Figure 2) were isolated from an oak tree and a chestnut tree and were deposited in the collection at the Northern Regional Research Laboratories (NRRL). The NRRL collection is now known as the Agriculture Research Service Culture Collection and is at the Microbial Genomics and Bioprocessing Research Unit (MGB) of the National Center for Agricultural Utilization Research (NCAUR) in Peoria, IL. Yeast strains screened by Phillips Petroleum for growth on methanol included two *P. pastoris* strains, designated NRRL Y-1603 (ATCC accession 28485) (ATCC, 2006b) and NRRL YB-4290 (NCAUR, 2006). Phillips Petroleum identified a *P. pastoris* strain with improved growth characteristics. The strain was designated 21-1 and deposited at NRRL as NRRL Y-11430 (Wegner, E.H., 1986). This strain is now available from ATCC as 76273 (ATCC, 2005). No records are available confirming that NRRL Y-1603 or NRRL YB-4290 is the progenitor of NRRL Y-11430, but it seems likely that one of them is the progenitor strain (Cregg, J.M., 2006; Kurtzman, C., 2006). NRRL Y-11430 was the progenitor strain for GS115, a histidine auxotrophic mutant (*his4-)* (ATCC, 2006a; Cregg, J.M. et al., 1985) and GS115 was the progenitor strain for SMD1168. SMD1168 is *his4-* and has a disrupted *PEP4* gene that normally encodes the Proteinase A protein (Gleeson, M.A. et al., 1998). SMD1168 was obtained from the Invitrogen Company of Carlsbad, California who received it from Phillips Petroleum. SMD1168 was selected because it allows for construction of a strain that lacks an antibiotic resistance marker. Also, the absence of the host proteases due to the *PEP4* disruption resulted in higher production of the PLC enzyme. See Figure 2 for a complete diagram of the strain lineage.

Figure 2

Strain Lineage



Additionally, the progenitor strain for SMD1168, *P. pastoris* GS115, that has an intact *PEP4* gene, has been approved by the FDA as a source of protein for use in broiler feed in concentration up to 10% of the total feed (FDA, 1993).

3. Phospholipase C gene source

DNA purified directly from a soil sample (Texas, USA) was used to construct an environmental library in *E. coli* using the plasmid vector pBK-CMV (Stratagene, San Diego, CA) (Robertson, D.E. et al., 1996; Short, J.M., 1999; Solbak, A.I. et al., 2005). High throughput screening of the library was used to identify clones expressing PLC activity. Secondary screens were performed to verify the absence of hemolytic activity. Discovery clones with PLC activity were sequenced, and the DNA sequence information was analyzed using in-house gene identification software. The predicted PLC open-reading frame was then manually curated to verify the software prediction. This analysis also confirmed that candidates of interest lacked the carboxy-terminal domain responsible for hemolytic activity. The PLC gene was isolated from all extraneous DNA using polymerase chain reaction (PCR) to specifically amplify the DNA, which encodes the PLC open reading frame, from the discovery clone. The native PLC gene ORF is 849 bp and encodes a secretion signal and a pro-sequence in addition to the mature enzyme. The DNA for the secretion signal and pro-sequence was deleted using PCR in order to improve expression of the enzyme in *P. pastoris*. This deletion resulted in a PLC coding sequence of 735 bp that encodes only the mature enzyme.

4. Construction of the expression vector

The final PLC expression plasmid pAO815-6xBD16449 PLC is a derivative of pAO815, obtained from Invitrogen Company of Carlsbad, CA. The construction of plasmid pAO815-6xBD16449 PLC involved several steps (see Figure 3). The coding sequence for the PLC enzyme was amplified by PCR from the pBK-CMV-PLC discovery clone in order to subclone it into pPICZαA (Invitrogen, Carlsbad, CA) using the primers shown below:

5'Primer

3'-Primer

The resultant PCR product was purified by gel electrophoresis and then the ends were digested with restriction enzymes *Xba* I and *Xho* I. The vector pPICZαA was digested with the same enzymes and purified by gel electrophoresis. The purified vector and the PLC gene were then ligated together and transformed into *E. coli* Top10 (Invitrogen, Carlsbad, CA) by electroporation using standard techniques to produce the expression vector pPICZαA-PLC. Plasmid DNA was purified from several *E. coli* transformants. Sequence analysis confirmed the presence of the *PLC* gene, that it was free of PCR-induced mutations and that it would be translated in frame with the *S. cerevisiae* α-factor secretion signal under the control of the *AOX1* promoter.

In order to improve PLC activity, three amino acids were substituted using a gene site directed mutagenesis method according to the manufacturer's protocol (Stratagene, 2006). These changes resulted in the elimination of unwanted post translational modifications of the enzyme. The changes have no impact on safety as there were no test article-related toxicity noted in the toxicological studies (see Section V.G). The modified PLC gene is designated as BD16449 PLC gene. The mature amino acid sequence of the BD16449 PLC gene is presented in Appendix C. The BD16449 PLC gene was moved into the pAO815 vector, resulting in pAO815-1xBD16449 PLC. This vector was modified further to create a multimerized form in which the expression cassette is multimerized in a head to tail fashion resulting in 6 copies of the cassette being present, generating the vector, pAO815-6xBD16449 PLC (see Table 1 and Figure 4). The predicted head-to-tail structure of the multimer (as shown in Figure 4) was verified using

restriction fragment length analysis. The expression cassette that is multimerized is defined as containing the genetic elements from position 1 to 2349 bp as outlined in Table 1. The multimerization method used was provided by the Invitrogen Company (Carlsbad, CA) and is a standard method for improving expression in *P. pastoris*. All the components for pAO815 are publicly available from the Invitrogen website (www.invitrogen.com).

Figure 3

Construction of pAO815-6xBD16449 PLC Expression Vector

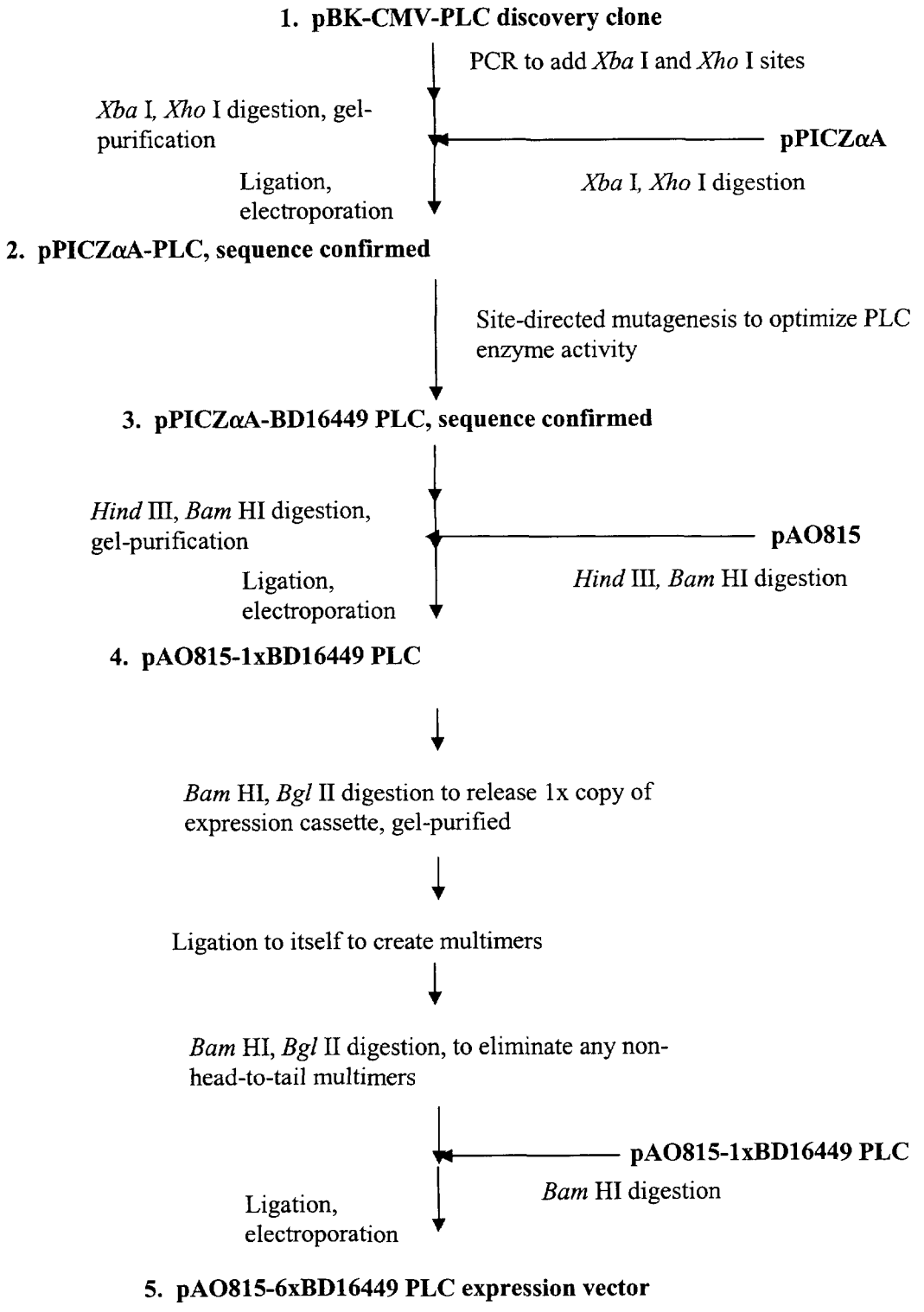


Table 1

Components of pAO815-6xBD16449 PLC Expression Vector

Position (bp)	Size (bp)	Element	Description
1-939	938	promoter	*P. pastoris AOX1* promoter for methanol-inducible expression; also fragment for homologous recombination
940-1200	261	secretion signal	*S. cerevisiae* α-factor for secretion
1201-1935	735	BD16449 PLC gene	Gene from an environmental library
1936-2008	72	vector	Vector sequence, no function
2009-2349	336	transcriptional terminator	*P. pastoris AOX1* transcriptional terminator
2350-4697	2348	Copy 2	Expression cassette*
4698-7044	2348	Copy 3	Expression cassette*
7045-9392	2348	Copy 4	Expression cassette*
9393-11740	2348	Copy 5	Expression cassette*
11741-14088	2348	Copy 6	Expression cassette*
14089-14471	383	vector	Vector sequence, no function
14472-17006	2535	*HIS4*	*P. pastoris HIS4* for selection of transformed cells
17007-17360	354	vector	Vector sequence, no function
17361-18117	757	*3' AOX1*	*P. pastoris AOX1 3'* fragment for homologous recombination

Everything listed above was integrated into the *P. pastoris* genome except the region from 18118-20517 bp; which is listed below.

18118-18546	429	vector	Vector sequence, no function
18547-19201	655	pBR322	pBR322 origin for replication in *E. coli*
19202-19345	144	vector	Vector sequence, no function
19346-20206	861	*bla* gene	Ampicillin-resistance for selection in *E. coli*
20207-20517	311	vector	Vector sequence, no function

* Contains the repeating genetic elements found in position 1 to 2349 bp.

5. Construction of the recombinant production organism DVSA-PLC-004

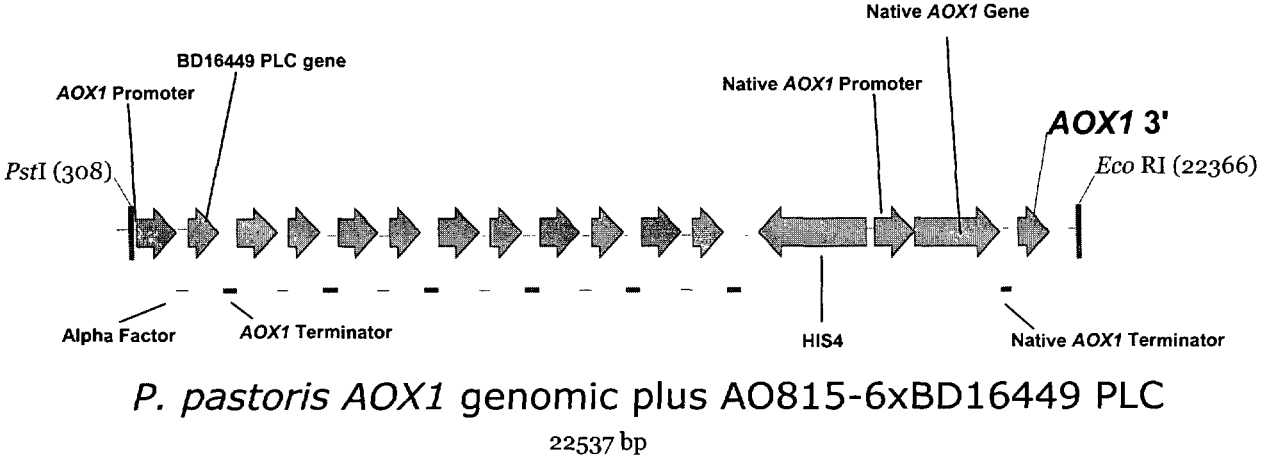
Prior to insertion into the host strain *P. pastoris* SMD1168, the expression vector pAO815-6xBD16449 PLC was linearized for greater transformation efficiency by digestion with *Bgl* II. This restriction digest also resulted in separation of the bacterial origin of replication (pBR322) and the ampicillin

resistance marker gene (*bla*) from the expression cassette. As the *bla* gene and bacterial origin portion of the vector sequence do not contain any *P. pastoris* DNA, such DNA will not integrate into the *P. pastoris* genome (see Figure 4).

P. pastoris SMD1168 cells were prepared for transformation following the manufacturer's suggested protocol (Invitrogen, Carlsbad, CA). An aliquot of the digested pAO815-6xBD16449 PLC DNA was electroporated into the recipient cells following an optimized protocol (Mo Bi Tec, 2003). The transformation mixture was then plated onto histidine deficient minimal dextrose solid medium and incubated at 30°C. Transformants growing after 3-5 days were selected and cultured. The culture supernatants were then tested for the presence of secreted BD16449 PLC enzyme by assaying for the ability to hydrolize phosphatidylcholine (see Section IV.B). Several transformants were screened for maximum expression of active BD16449 PLC protein. A single colony transformant was selected and designated DVSA-PLC-004. This clone served as the source for the production of the Master Cell Bank (MCB1) as described in Section III.C.1.

Figure 4

Diagram of predicted structure of BD16449 PLC DNA integrated into the *P. pastoris* genome



6. Stability of introduced sequences

An effort was made to insert multiple copies of the expression cassette into the *AOX1* locus of *P. pastoris*, with the assumption that the strain would produce higher levels of BD16449 phospholipase C with higher copy numbers of the

BD16449 phospholipase C gene. The final production strain, DVSA-PLC-004, was screened and produced the highest level of BD16449 phospholipase C. DVSA-PLC-004 contains 6 copies of the expression cassette. As demonstrated by the method of Southern blotting, the expression cassettes were successfully integrated into the *AOX1* locus and not anywhere else in the genome. All of the cells from master cell bank DVSA-PLC-004 and a majority of the biomass buildup runs retained the full six copy genotype. There is a fraction (which does not exceed 30%) of the cells from the biomass buildups runs that lost part of the 6x expression cassette. It is not surprising to have lost some of the copies of the expression cassette since multiple copies of the same sequence were arranged in tandem in the yeast. This is because it is a basic principle of yeast genetics and is generally recognized within the scientific community that DNA integrates into *P. pastoris* by homologous recombination (Sreekrishna, K. et al., 1997). It is most likely that the BD16449 phospholipase C gene lost from the genome was degraded or diluted during cell growth, since the expression cassettes (when looked for in the entire *P. pastoris* genome via Southern blot) were only found at the *AOX1* site. (Please note that the expression cassette lost from the genome cannot replicate itself and, therefore, the new daughter cells will not contain this free form of the expression cassette).

B. Enzyme identity

1. Identity

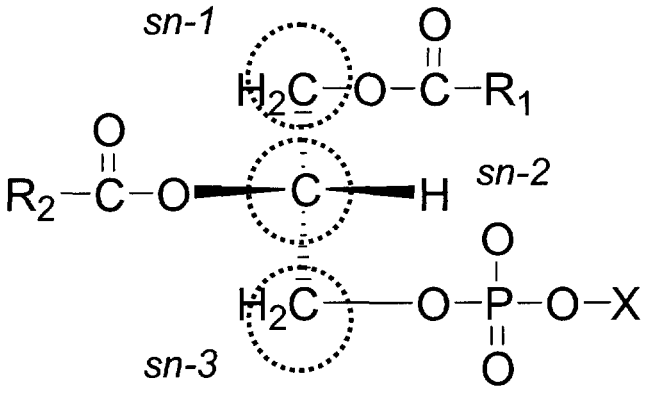
The lipase enzyme preparation that is the subject of this GRAS Notification is a phospholipase C as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

Enzyme Name:	Phospholipase C
Official Name:	Phospholipase C
Systematic Name:	Phosphatidylcholine cholinephosphohydrolase
Alternative Names:	PLC, Phosphatidylcholine-specific phospholipase, Lecithinase C, Lipophosphodiesterase I
EC Number:	3.1.4.3
CAS Registry No:	9001-86-9
Molecular Weight:	28,141 Daltons
Specificity:	Phospholipid + H_2O → 1,2-Diacylglycerol + Phosphate Ester

The PLC enzyme is responsible for the ester hydrolysis of the *sn*-3 phosphate head group from phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). BD16449 PLC shows the following substrate preference: PC > PE > PS >>>PA (phosphatidic acid). Figure 5 is a general phospholipid structure, where X represents the side chain of the phospholipid (for example, choline for the phospholipid phosphatidylcholine) and R_1 and R_2 represent fatty acid side chains attached to the glycerol backbone.

Figure 5

General Phospholipid Structure



2. Amino acid sequence

The phospholipase C protein, designated BD16449 PLC, is a 245 amino acid protein. As discussed below, this sequence contains the functional elements common to other food related phospholipases, but lacks (as do other food related phospholipases) the carboxy-terminal domain responsible for hemolytic activity. The identity of the protein as expressed in the *Pichia pastoris* (production strain DVSA-PLC-004) has been independently confirmed using mass spectroscopy, amino-terminal protein sequencing, and amino acid composition analysis. The complete amino acid sequence of BD16449 PLC is presented in Appendix C.

3. Sequence comparison to other lipases

Lipases, including phospholipases, are naturally found in foods and added to foods as processing aids. For example, porcine PLA2 is generally recognized as safe and it is routinely used for the production of enzyme-modified lecithin for

food processing (FDA, 1996). Although there is no significant primary sequence similarity between phospholipase C and phospholipase A_2 (PLA2), analysis of the crystal structure of the well-characterized *B. cereus* PLC has revealed structural similarities between PLC and porcine PLA2 (Hough, E. et al., 1989). The BD16449 PLC protein sequence is 82% identical to the PLC from *Bacillus cereus* (Hough, E. et al., 1989; Hough, E. et al., 1994). In addition to conservation of amino acid length, (*B. cereus* PLC also contains 245 amino acids) there is complete conservation of the amino acids involved in catalysis between BD16449 PLC and *B. cereus* PLC. The conservation of sequence is revealed both in the identity of the amino acids involved in catalysis and the position of the amino acids in the linear sequence of the two proteins (Hergenrother, P.J. et al., 2000; Hough, E. et al., 1989; Hough, E. et al., 1994).

Although phospholipase C is believed to be a natural component of foods, it is not as abundant in foods as other phospholipases. For this reason, *B. cereus* PLC is routinely used to mimic the action of the less abundant mammalian PLC enzymes for the study of PLC function in mammalian physiology. Antibodies raised to the *B. cereus* PLC cross-react with homologous proteins in mammalian cells (Hergenrother, P.J. et al., 2000; Hough, E. et al., 1989). As suggested by the relatively high level of sequence similarity between *B. cereus* PLC and BD16449 PLC, commercial polyclonal antibodies raised against *B. cereus* PLC also cross-react with BD16449 PLC.

In discussing the safety of porcine PLA2, the FDA stated "(a)ctive and inactive enzymes are constituents of many foods consumed by humans. Therefore, FDA concludes that inactive phospholipase A2 in enzyme-modified lecithin will be digested like any other protein present in food" (FDA, 1996). Given the sequence similarity between BD16449 PLC, *B. cereus* PLC, and mammalian PLC enzymes as well as the structural similarity between PLC and PLA2 enzymes, this argument can reasonably be extended to BD16449 PLC. The suggested safety of BD16449 PLC based on its similarity to phospholipases found in foods and used in food processing is further supported by the toxicological studies that have been performed with DV16449 test article in support of this GRAS submission (and which are identified and discussed below in section V.G).

4. Functional properties

Lipases have a long history of safe use in a variety of food processing applications (FDA, 1996; Pandey, A. et al., 1999; Vulfson, E.N., 1994). The use of a particular lipase in a food processing application depends on the composition

of the lipid substrate and the desired specificity of the enzymatic cleavage. When the substrate is a phospholipid, phospholipases are generally used (Guo, Z. et al., 2005; Ulbrich-Hofman, R., 2000). BD16449 PLC has been selected for use as a yield-enhancing processing aid in edible oil degumming because its functional properties are ideally suited for the application. When added to crude soybean oil under conditions commonly used by the edible oil refining industry, i.e., in low water (2-4%) at 60°C (Hodgson, A.S., 1996), BD16449 PLC efficiently hydrolyzes the major phospholipid substrates to create 1,2-diacylglycerol oil and the corresponding water soluble, phosphate ester. Similar results have been obtained with canola oil. It is envisioned that BD16449 PLC could also be used as an enzymatic degumming aid for other high phosphorus edible oils including corn oil, sunflower oil, and rapeseed oil because these edible oils are degummed using conditions similar to those used for soybean oil and canola oil and they contain the BD16449 PLC substrates, phosphatidylcholine and phosphatidylethanolamine.

C. Absence of production organism in enzyme preparation

Per requirements of Diversa's product specification for BD16449 PLC preparation, there are no detectable, viable production organisms present in the final product. This is ensured through both process design and testing. A number of process steps serve to prevent the presence of production organisms in the final product. In the cell separation step, most of the yeast cells are removed. In the clarification step, conventional (depth) filtration ensures that a cell-free process stream is provided for the ultrafiltration step. The polishing filtration step further ensures that no production organism is present in the final product.

The ability of these processing steps to remove the production organism from the enzyme preparation is verified by testing for the presence of *P. pastoris* in the final product. In the testing method (done pursuant to SOP QC0181 and QC0182), a 0.5 ml sample of the final product is streaked on an agar plate medium containing bacterial antibiotics ampicillin, gentamycin and streptomycin and cultivated for 48-72 hours to isolate any individual yeast colonies. If no yeast colonies are observed, then the product is free of any production organisms at a detection level of 2 cfu/ml. If any yeast colonies are observed, then they are examined microscopically for *P. pastoris* morphology and identified using the Biolog™ culture identification system. This sensitive system can type yeast colonies based on metabolic profiles on a number of different substrates. The test has been used to positively identify *P. pastoris* colonies (Praphailong, W. et al., 1997).

D. Absence of transformable DNA in enzyme preparation

Diversa's finished enzyme product does not contain transformable DNA. This has been established via Southern dot blot analysis. The experiment concludes that no encoding pieces of recombinant DNA are present in BD16449 PLC enzyme preparation. The level of detection of the Southern dot blot method is 0.01 picograms of recombinant DNA. Typical yeast transformations require microgram quantities of recombinant DNA for homologous integration; this is approximately 100,000,000 times more than the limit of detection of the Southern dot blot method.

E. Absence of antibiotic resistance gene in the production strain

A gene *bla*, coding for resistance to ampicillin was present in the original cloning vector pAO815-1xBD16449 PLC. However, it was removed by restriction digest with *Bgl* II. The absence of the *bla* gene in the *P. pastoris* production strain DVSA-PLC-004 was confirmed by Southern blot analysis. The detection sensitivity of the Southern blot method is <1 ng plasmid DNA, which is sensitive enough to detect a single copy of the *bla* gene in the genome.

F. Applications and residue measurements

1. Foods in which used and purpose of use

The lipase enzyme preparation that is the subject of this Notification, referred to as BD16449 phospholipase C, is intended for use as a processing aid to hydrolyze phospholipids in the refining of soybean oil and other vegetable oils, intended for use in foods.

Refining is a general term used by the industry to describe the process whereby crude vegetable oil is converted to a purified final product by removing non-triacylgylcerol components that can negatively impact taste, color and stability, and make the oil unsuitable for particular applications. (See Figure 6). Refining may be conducted as a series of batch processes, but most modern oil refineries utilize continuous processes.

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Figure 6

Oil Refining Process

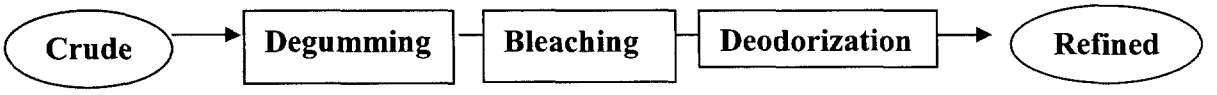
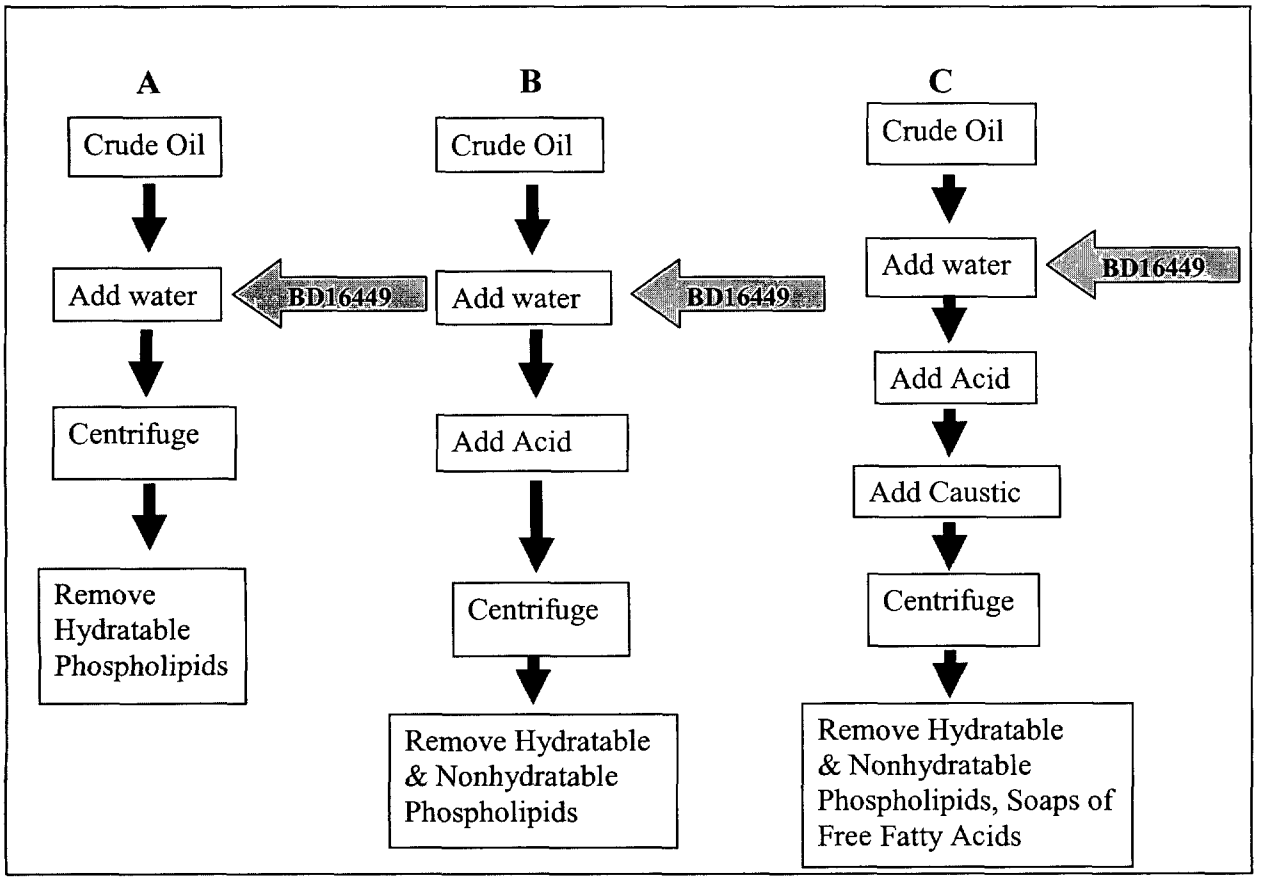


Figure 7

Degumming of Vegetable Oil[3]



[3] The flow diagram shown in **A** above depicts water degumming and, as indicated, this process will remove only the hydratable phospholipids from the crude vegetable oil. The hydratable phospholipids are removed from the oil using centrifugation to separate the aqueous phase from the oil phase. Flow diagram **B** depicts degumming associated with physical refining. Water and then acid are added to the crude oil to extract both hydratable and nonhydratable phospholipids from the oil. These phospholipids are removed from the oil using centrifugation to separate the aqueous phase from the oil phase. Flow diagram **C** depicts degumming associated with chemical refining. In this process, water and then acid are added to fully extract all phospholipids. Next, caustic is added to the oil to convert free fatty acids to soaps. Centrifugation is then used to separate the phospholipid/soap (soapstock) fraction from the oil. Also indicated in diagrams are the points in each degumming process where BD16449 PLC would be added in the process (shaded arrows).

The first step in refining is degumming and it is during this step that Diversa's BD16449 PLC enzyme preparation will be used (see Figure 7). Proper degumming is a procedure that is designed to remove, among other things, contaminating phospholipids or phosphatides that otherwise interfere with the subsequent processing of high phosphorus oils such as soybean, canola, corn, and sunflower oils (Hodgson, A.S., 1996; Lei, L. et al., 2003; Münch, E.W., 2005). These phospholipids originate in the oilseeds, are extracted with the triacylglycerol or neutral oil during solvent extraction (or mechanical extrusion) and typically represent up to 3% by weight of the oil. The major phospholipid species phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can together account for up to 70% of the the phospholipids, depending on the oil type; phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylserine (PS) make up the remainder. The phospholipids in crude vegetable oil are classified as hydratable or nonhydratable based on their tendency to be removed from the oil by the addition of small amounts of water, resulting in the formation of a gum. Addition of hot water to vegetable oil, followed by mechanical agitation, causes PC and PI to become hydrated, forming a gum that can be removed by centrifugation. PE and PA particularly, are considered nonhydratable, but will form a gum that can be removed by centrifugation if either citric or phosphoric acid is added to aid the hydration process (in the case of PA, the process works by dissociating the calcium and magnesium salts of PA that are present in the oil) (Dijkstra, A.J., 1998; Hvolby, A., 1971). Therefore, standard degumming protocols utilize water and then acid to remove all the phospholipids from the oil (Lei, L. et al., 2003). Recently, enzymatic degumming has become a well-established practice (Clausen, K., 2001; Dahlke, K., 1998; Münch, E.W., 2005). The benefits of enzymatic degumming include:

- Higher oil yield
- Less need for bleaching earth
- Significant cost saving (including lower usage of water and bleaching earth, and lower production of waste water)
- Simple to use with low environmental impact (and the enzyme is biodegradable and are inactivated during the process) (Münch, E.W., 2005).

When added with the water during degumming of vegetable oil, BD16449 phospholipase C hydrolyzes the major oil phospholipids, PC and PE, resulting in the following additional benefits:

- Reduction in the amount of gum phospholipids and overall gum mass
- Reduction in the total phosphorus contained in the oil
- Reduction in the total mass of neutral oil entrained in the phospholipid gum
- Increase in the quantity of diacylglycerol contained in the oil

Subsequently, acid is added to remove the remaining nonhydrated phospholipids. The contaminating free fatty acids naturally present in the oil are removed by either chemical or physical refining, according to the method then being used for removing the free fatty acids – either neutralization with caustic soda solution (a chemical reaction) or distillation (a physical process) (Allen, D.A., 1997; Anderson, D., 1996).

The most commonly practiced form of chemical refining is termed **alkali or caustic refining** (Allen, D.A., 1997). In chemical refining, a measured quantity of sodium hydroxide or other alkaline solution is added to the oil that is being refined, with the objective of forming a soap composed of the sodium or ammonium salts of the free fatty acids contained in the oil. The soapstock containing residual phospholipid gum and soap is removed by passing the oil/water mixture through a primary centrifugal separator. The oil that emerges from the primary separator is washed repeatedly with hot water to remove residual soap, recovered by passing the oil through a wash separator, and dried under vacuum to a low moisture content, for subsequent bleaching or intermediate storage. This is followed by a bleaching step in which undesirable impurities are removed from the oil by adsorption to bleaching clay that is added to the oil and subsequently removed by filtration (Hodgson, A.S., 1996). The final step in refining is deodorization or steam distillation, which is carried out at high temperature and reduced pressure, with the objective of removing trace levels of volatile compounds that might otherwise give the oil an unpleasant taste or odor (Carlson, K.F., 1996).

Physical refining is generally only practiced with low phospholipid vegetable oils that have been intensively degummed and contain preferably less than 10 ppm phosphorus (Allen, D.A., 1997; Hodgson, A.S., 1996). In this process, washed, degummed oil is first bleached as described for chemical refining, and the contaminating free fatty acids are then removed, together with other unwanted volatiles, during a deodorization or steam distillation step. When added to the 2-phase oil-water mixture during the degumming step, use of BD16449 phospholipase C enables subsequent physical refining by hydrolyzing the major oil phospholipids, PC and PE, resulting, after centrifugal separation of the residual phospholipids gum, in an oil containing less than 10 ppm phosphorus.

2. Mode of action

BD16449 phospholipase C catalyzes the hydrolysis of the phosphodiester bond linking the glycerol and phosphate moieties at the *sn*-3 position of glycerophospholids as shown below (Stryer L., 1988):

$$\text{Phosphatidylcholine} + H_2O \longrightarrow \text{1,2 diacylglycerol} + \text{phosphorylcholine}$$

$$\text{Phosphatidylethanolamine} + H_2O \longrightarrow \text{1,2 diacylglycerol} + \text{phosphorylethanolamine}$$

As can be seen in the foregoing chemical equations, the products of the hydrolysis of the major vegetable oil phospholipids, PC and PE, by BD16449 phospholipase C are diacylglycerol and water-soluble phosphate esters phosphorylcholine and phosphorylethanolamine respectively (Clausen, K., 2001; Dahlke, K., 1998).

3. Level of use

The enzyme preparation will be used at levels no higher than necessary to achieve the intended effect, generally in the range of 100 to 1000 grams of enzyme preparation per metric ton of oil, depending on the oil to be treated and the reaction conditions.

4. Enzyme residues in food ingredients

BD16449 phospholipase C is intended for use in the first step, i.e., the degumming step, in refining edible vegetable oils. This process step involves addition of the enzyme to a water-in-oil emulsion. BD16449 phospholipase C, like other proteins, is soluble in water and insoluble in organic solvents, and in a 2-phase water-in-oil emulsion, is known to partition in the aqueous phase. After the degumming reaction has been completed, the water phase, containing BD16449 phospholipase C, is separated from the oil by centrifugal separation. Subsequent steps in refining, including repeated washing of the oil with water, bleaching and deodorization, remove any remaining enzyme residues. For these reasons, inactivated and denatured enzyme may be present, if at all, in the fully refined vegetable oil at levels close to or below the limit of detection (LOD=1ppb) of available analytical methodology.

G. Pre-clinical safety testing

1. Test article production

The test article used to determine the safety of BD16449 phospholipase C was prepared following a process representative of the manufacturing process for the commercial enzyme, up to but not including, the final formulation step. The test article was produced in a Fermentation and Recovery Pilot Plant in accordance with current good manufacturing practices (cGMP) for foods. The following SOPs were used for the production of the test article: MP241 and MP242 for the fermentation protocol, MP243 for the recovery protocol, and MP204 for the lyophilization protocol. The test article was analyzed for chemical and microbial composition to ensure conformance to the specifications for enzyme preparations (see Appendix B), as outlined in the *Food Chemicals Codex*, Fifth Edition, 2004 (Committee on Food Chemicals Codex, 2004), and the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2001). The test article for BD16449 phospholipase C was designated as DV16449 (and used in all of the safety studies discussed below).

2. Genotoxicity studies

DV16449 (test article for BD16449 PLC) has been tested (by independent testing laboratories) to investigate its genotoxic potential in a range of *in vitro* and *in vivo* short-term genotoxicity screening assays. These include a chromosomal aberrations test in human lymphocytes, a mouse micronucleus assay, and a *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation assay (i.e., Ames assay). All studies were conducted pursuant to the OECD Principles of Good Laboratory Practice and in compliance with the FDA Good Laboratory Practice Regulations in Title 21 CFR Part 58. The results of these studies are summarized in the following table. A more complete description of these studies, as well as the studies themselves, may be found in Appendix D, E, and F.

Table 2

Results of Genotoxicity Studies

Test System	Test Object	Concentration of DV16449	Results
Chromosomal aberrations in human lymphocytes*	Cultured human peripheral blood lymphocytes	0-5000 μg/mL	Negative
Mouse micronucleus assay	Male CD-1® (ICR)BR mice	0-2000 mg/kg	Negative
Ames assay*	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A	0-5000 μg/plate	Negative

*With and without S-9 metabolic activation.

3. Animal studies

The safety of Diversa's DV16449 (test article for BD16449 PLC) was also evaluated via three different toxicology protocols – each of which demonstrates that BD16449 phospholipase C is safe for its intended use. Each is discussed below.

a. Acute oral toxicity study in rats (conducted by Calvert Laboratories, Inc.)

The purpose of this study was to determine the acute toxicity of DV16449 when administered to rats as a single dose by oral gavage (Gad, S.C., 1995; Speid, L.H. et al., 1990). The study was conducted pursuant to the OECD Principles of Good Laboratory Practice and in compliance with the FDA's Good Laboratory Practice Regulations in Title 21 CFR Part 58. Five female Sprague Dawley rats were dosed at 2000 mg/kg.

Mortality and clinical observations were evaluated daily. Body weights were recorded weekly.

Mortality was not observed in any of the animals at 2000 mg/kg. No clinical signs were observed in the animals during the study. No effect was seen on body

weight in the animals for Days 8 and 15. No visible lesions were observed in any of the animals at necropsy.

Since all five animals survived, testing was terminated and the oral LD_{50} for DV16449 was considered to be greater than 2000 mg/kg. (See Appendix G for additional details).

b. 14-Day dose range-finding oral toxicity study in rats (conducted by Calvert Laboratories, Inc.)

The purpose of this study was to evaluate the toxicity of DV16449 when administered via oral gavage, once daily for up to 14 consecutive days to Sprague Dawley rats (Gad, S.C., 1995; Speid, L.H. et al., 1990). The study was conducted pursuant to the OECD Principles of Good Laboratory Practice and in compliance with the FDA's Good Laboratory Practice Regulations in Title 21 CFR Part 58. The results of this study were used to determine the acceptability of doses for a 90-day study in Sprague Dawley rats. Groups of 5 male and 5 female Sprague Dawley rats were administered 0, 500, 1000, and 2000 mg/kg/day of DV16449. The dose volume for all groups was 10 mL/kg.

Mortality and clinical observations were evaluated daily. Body weights and food consumption were recorded on Days 1, 8, and 14. Blood for evaluation of hematology, coagulation, and clinical chemistry was collected on Day 15. Selected tissues were harvested at necropsy, and selected organs weighed.

No mortality was observed throughout the study in any dose group. Moreover, there were no treatment related clinical signs of toxicity, effects on body weight, body weight changes or food consumption values, effects in hematology or clinical chemistry parameters, gross findings, or effects on absolute organ weights, relative organ-to-body weight ratios or relative organ-to-brain weight ratios noted throughout the study for any of dose group.

Based on the results of this study, the No-Observed-Adverse-Effect-Level (NOAEL) following 14 consecutive days of oral administration of DV16449 to Sprague Dawley rats was deemed to be greater than 2000 mg/kg/day. (See Appendix H for further details).

c. 90-Day oral toxicity study in rats (conducted by Calvert Laboratories, Inc.)

The purpose of this study was to evaluate the toxicity of DV16449 when administered orally, via gavage, once daily for 90 consecutive days to Sprague Dawley rats (Gad, S.C., 1995; Speid, L.H. et al., 1990). The study was conducted pursuant to the OECD Principles of Good Laboratory Practice and in compliance with the FDA's Good Laboratory Practice Regulations in Title 21 CFR Part 58. Groups of 20 male and 20 female Sprague Dawley rats were administered 0, 500, 1000, and 2000 mg/kg/day of DV16449. The dose volume for all groups was 10 mL/kg.

Mortality and clinical observations were evaluated daily. Body weights and food consumption were recorded weekly. Ophthalmology examinations were performed prior to treatment initiation and during the last week of treatment. Blood for evaluation of hematology, coagulation, and clinical chemistry was collected on Days 91 or 92. Animals were sacrificed on Days 91 or 92. Selected tissues were harvested at necropsy, selected organs weighed, and selected tissues from the control and high dose groups evaluated microscopically.

There were three animals found dead during the study. One female (#3306) from the 500 mg/kg dose group was found dead on Day 10, one male (#3272) from the 2000 mg/kg dose group was found dead on Day 63 and one female (#3345) from the 2000 mg/kg dose group was found dead on Day 72. None of the animals had clinical signs of toxicity noted prior to being found dead. Necropsy findings included pale kidneys, dark spotted lung and black tipped spleen for #3306 and fluid filled thoracic cavity for animals #3272 and #3345. All three deaths were considered not to be treatment related, but rather to be related to improper gavage incidents. Such occurrence is fairly common and well-documented (Balcombe, J.P. et al., 2004; Brown, A.P. et al., 2000; Haschek, W.M. et al., 2002).

There were no test article-related clinical signs of toxicity noted.

All test article-treated males had decreased body weight gains during the first week of the study. This resulted in decreased group mean body weights compared to the concurrent controls on Day 8 for males at all dose levels. During the second week of the study, weight gains were increased for all test article-treated males compared to controls and, therefore, group mean body weights were comparable to controls in all groups by Day 15. Decreased group mean weights were also noted for males in the 1000 and 2000 mg/kg dose groups on Day 43 and the 2000 mg/kg

dose group on Day 50. The later changes did not result in differences in group mean body weights compared to the controls.

Group mean weight gains were increased for the 2000 mg/kg females on Day 15, the 1000 mg/kg females on Day 29, and decreased for the 1000 mg/kg females on Day 22. These changes had no effects on group mean body weight for the females.

Group mean food consumption was increased for the 1000 mg/kg males on Day 8. Group mean food consumption was decreased for the 1000 and 2000 mg/kg males on Day 15 and the 2000 mg/kg males on Days 36 and 57.

A statistically significant increased group mean food consumption was noted for the 500 mg/kg females on Day 15 and the 1000 mg/kg females on Day 22. However, as no dose-response was noted, these changes were considered incidental and unrelated to treatment.

There were no test article-related ophthalmological findings noted during the study. There were no test article-related changes in hematology, coagulation, or clinical chemistry parameters or erythrocyte morphology.

There were no test article-related gross necropsy findings. There were no test article-related changes in absolute or relative-to-body or relative-to-brain organ weights. There were no test article-related lesions noted in this study.

In conclusion, oral administration of DV16449 at doses of 500, 1000, or 2000 mg/kg/day, via oral gavage, once daily for 90 consecutive days resulted in only minimal, transient changes in group mean body weights, weight gains, and food consumption. Based on these findings, the No-Observed-Adverse-Effect-Level (NOAEL) was deemed to be greater than 2000 mg/kg/day. (See Appendix I for further details).

4. Worker Safety Studies

DV16449 (test article for BD16449 PLC) has also been tested (by independent testing laboratories) in order to evaluate the potential health hazards, if any, of its product with respect to inhalation exposure, dermal exposure, or eye exposure. These include an acute inhalation toxicity study, a primary eye irritation study, a primary dermal irritation study, and a delayed contact hypersensitivity study. All studies were conducted pursuant to the OECD Principles of Good

Laboratory Practice and in compliance with the FDA's Good Laboratory Practice Regulations in Title 21 CFR Part 58. The results of these studies are summarized in the following table (see Table 3). A more complete description of these studies, as well as the studies themselves, may be found in Appendix J, K, L, and M.

Table 3

Results of Worker Safety Studies

Test System	Test Object	Concentration of DV16449	Results
Acute, nose-only, inhalation toxicity	Sprague Dawley rats (5 males and 5 females)	2.34 mg/mL	LC_{50} >2.34 mg/mL
Primary eye irritation	New Zealand White rabbits (6 females)	2.0% solution	Minimal irritant (Class 3)
Primary dermal irritation	New Zealand White rabbits (3 females)	2.0% solution	Non-irritating
Delayed contact hypersensitivity (Buehler method)	Guinea pigs (10 males and 10 females)	2.0% solution	Negative

As the above described studies indicate, BD16449 PLC enzyme preparation does **not** pose any significant risk of harm to humans who might come into physical contact with it. Notwithstanding this conclusion, as an added precaution, the MSDS and the product information sheet associated with the product include instructions which indicate that one should wear appropriate coverings (i.e., rubber gloves, safety goggles, and other protective clothing) and have adequate ventilation when handling the enzyme preparation.

VI. SAFETY EVALUATION DISCUSSION

A. Lipase

1. Safety of food enzymes as processing aids

Pariza and Foster (Pariza, M.W. et al., 1983) and Pariza and Johnson (Pariza, M.W. et al., 2001) extensively addressed the issue of food processing enzyme safety and concluded that enzymes *per se*, that are used or likely to be used in food processing, are inherently nontoxic; thus, a safety evaluation should focus on possible contaminants which could be present in the final enzyme preparation. These authors suggest that the safety of the production strain should be the primary consideration when evaluating enzyme safety.

The fact that enzymes are a biological necessity for all living organisms and are ubiquitous in nature offers a high degree of safety assurance for these proteins. Enzymes have always been present as natural components of food; thus, the history of human exposure to enzymes is as old as human life itself. The application of enzymes to food processing has developed from traditional recipes such as those for fermented foods, whose preparation is older than recorded history.

2. History of use of lipases in food

Lipases are ubiquitous in nature and can be found in bacteria, plants, and animals. They have been studied for almost 100 years (Jaeger, K.E. et al., 2002) and used safely in food processing applications for over 50 years (Jaeger, K.E. et al., 2002; Pandey, A. et al., 1999; Pariza, M.W. et al., 1983; Vulfson, E.N., 1994). FDA has approved a number of lipases for use in food as described in the table below. Like other lipases, phospholipases (a subfamily of lipases) utilize a lipid substrate so it is not unexpected (Kessler, D.A. et al., 1992) to see that phospholipases have been used safely in food and are, therefore, a part of the list in Table 4 (see GRN000103, GRN000142, and GRN000145).

Table 4

Lipases Approved by FDA

Reference No.	Substance	Intended Use	Year of Authorization
21 CFR 173.140	esterase-lipase	Use as an enzyme to hydrolyze fatty acid glycerides	1983
21 CFR 184.1415	animal lipase	Use for the interesterification of fats and oils	1995
21 CFR 184.1420	lipase	Use as a flavor enhancer	1998
GRN 000043	lipase	Use in dough, baked goods, and the fats and oil industry as a processing aid for the interesterification or hydrolysis of glycerides	2000
GRN 000068	lipase	Use on fats and oils to produce fatty acids or glycerides at a level up to one percent of the weight of the fat or oil	2001
GRN 000075	lipase	Use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks as a processing aid	2001
GRN 000081	lipase	Use on fats and oils to produce fatty acids or glycerides at a level up to one percent of the weight of the fat or oil; use in the esterification of fatty acids to produce glycerides at a level up to one percent of the weight of the fatty acids	2001
GRN 000103	lipase (phospholipase A1)	Use in bakery products, egg yolks, whole eggs, and fats and oils as an enzyme	2002
GRN 000111	lipase	Use in dairy-based	2002

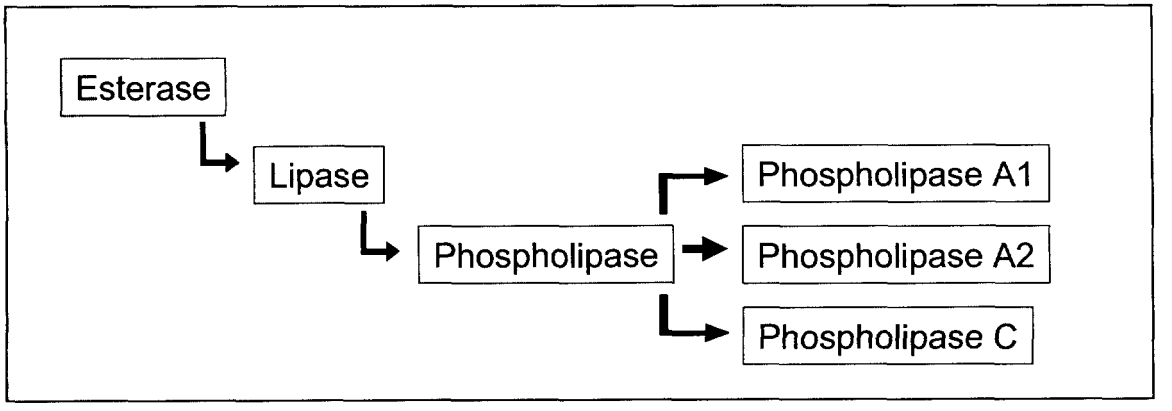
		flavoring preparations, cheeses, liquid and dried egg white, bread, flour, unstandardized bakery products, modified triglycerides, hydrolyzed lecithin, edible fats and oils, and modified egg yolk as an enzyme	
GRN 000113	lipase	Use in dairy-based flavoring preparations, cheeses, liquid and dried egg white, bread, flour, unstandardized bakery products, modified triglycerides, hydrolyzed lecithin, edible fats and oils, and modified egg yolk as an enzyme	2003
GRN 000142	phospholipase A1	Use as an enzyme in the production of cheese	2004
GRN 000145	phospholipase A2	Use as a enzyme to hydrolyze soy and egg yolk lechithins	2004
GRN 000158	lipase	Use as an enzyme in the production of triglyceride products	2005

3. Substantial equivalence

As discussed in section two above, members of the esterase, lipase, and phospholipase families have a long history of safe use in food. Lipases are a subfamily of esterases, and phospholipases are a subfamily of lipases. BD16449 is a member of the phospholipase C family. From a functional perspective, these enzymes can be classified as shown in Figure 8.

Figure 8

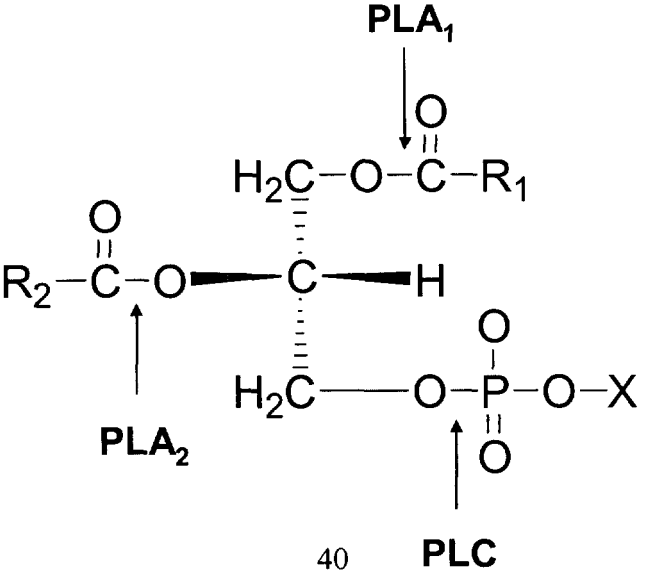
Functional Relationship of Esterases, Lipase, and Phospholipases



As shown in Figure 8, all of these enzymes are esterases. The lipases are distinguished from other esterases because they cleave the ester linkage of the long chain fatty acids from triacylglycerols (Fojan, P. et al., 2000; Silverman, R.B., 2002; Vulfson, E.N., 1994). Similar to lipases, phospholipases utilize lipid substrates possessing long chain fatty acids. The phospholipases are distinguished from other lipases because they perform ester hydrolysis on lipid substrates with a phosphate ester substitution on the *sn*-3 position of the glycerol backbone (Ulbrich-Hofman, R., 2000). The phospholipases are distinguished from each other based on the specificity of phospholipid ester cleavage (see Figure 9).

Figure 9

Specificity of Hydrolytic Cleavage Sites for Phospholipases



Members of both the phospholipase A1 (PLA1) and phospholipase A2 (PLA2) families have been approved for food processing applications (see Table 4 above) including use for the enzymatic degumming of vegetable oils (Clausen, K., 2001; Dahlke, K., 1998; Münch, E.W., 2005). Table 5 compares the properties of approved microbial phospholipases and BD16449 PLC.

Table 5

Substantial Equivalence of Microbial Phospholipases

Property	**BD16449 PLC**	**PLA1 (NOVOZYM 46016) GRN 000142**	**PLA2 (LysoMax) GRN 000145**	**PLA1 GRN 000103**
Enzyme class	Phospholipase	Phospholipase	Phospholipase	Phospholipase
Site of cleavage on glycerol backbone	*sn*-3	*sn*-1	*sn*-2	*sn*-1
Mode of action: alter emulsifying properties of phospholipids	YES	YES	YES	YES
Commercial enzyme	Focus of this submission	YES	YES	YES
Approved food use	Focus of this submission	Cheese manufacturing	Production of enzyme-modified lecithin	Referred to as Lipopan H for baking applications
Application includes oil degumming	YES	NO	NO	YES: referred to as Lecitase Ultra for oil degumming
pH optimum	7.5	NA	8.5	4.5
Temperature Optimum	60°C	NA	40°C	>40°C
Production organism	*Pichia pastoris*	*Aspergillus oryzae*	*Streptomyces violaceoruber*	*Aspergillus oryzae*
Produced as a secreted enzyme	YES	YES	YES	YES

As can be seen from the foregoing Table, there are a number of significant similarities between BD16449 PLC and the other microbial phospholipases that have been authorized by CFSAN for food use. In addition, as discussed in Section

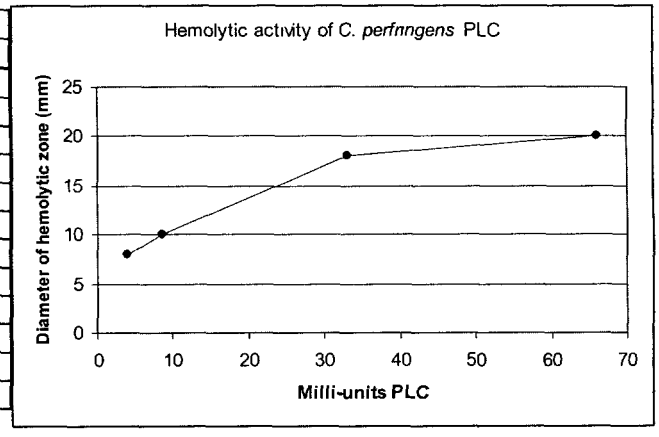
V.B.3. above, comparison of the crystal structures of the phospholipase C from *B. cereus* and porcine phospholipase A2, reveals structural similarities between the two enzymes (Hough, E. et al., 1989). This similarity is significant because BD16449 PLC is very similar to the *B. cereus* PLC. At the level of protein sequence, the BD16449 PLC amino acid sequence is 82% identical to the amino acid sequence of *Bacillus cereus* PLC (Hough, E. et al., 1989; Hough, E. et al., 1994). Importantly, similar to the *B. cereus* PLC sequence, the BD16449 PLC sequence does **not** encode the carboxy-terminal domain that has been demonstrated to be responsible for the hemolytic activity of some phospholipases (Titball, R.W., 1993). In order to confirm the absence of hemolytic activity, BD16449 PLC was evaluated in a blood agar hemolysis assay as shown in Figure 10. In addition, the similarity extends beyond amino acid sequence since the two PLC enzymes share similar physical and biochemical characteristics (Hergenrother, P.J. et al., 2000; Hough, E. et al., 1994). Although the crystal structure for BD16449 PLC has not been determined, it is likely BD16449 PLC and *B. cereus* PLC also share substantial structural similarities given the significant similarity between both their amino acid sequences and their biochemical properties.

Figure 10

BD16449 PLC: Absence of Hemolytic Activity[4]

	Diameter of hemolytic zone (mm)	
Milli-units activity	**BD16449 PLC**	***C. perfringens* PLC**
4	ND	8
8.5	ND	10
33	ND	18
66	ND	20
180	0	ND
390	0	ND
750	0	ND
1500	0	ND
3000	0	ND
6000	0	ND
12000	0	ND
30000	0	ND

ND = Not Determined



In summary, BD16449 PLC exhibits functional and biochemical properties that are substantially equivalent to other commercial microbial phospholipases. As noted by Kessler *et al.*, 1992 in discussing enzymes derived from microbial sources, "[generally], enzymes that are substantially similar to enzymes known to be safely consumed (including minor variations in structure or function) would not raise safety concerns" (Kessler, D.A. et al., 1992). Together with the toxicological data presented for BD16449 PLC, the substantial equivalence of BD16449 PLC to other microbial phospholipases with GRAS status as discussed here, strongly argues that BD16449 PLC should also be considered a safe and effective enzyme for use in edible oil degumming.

[4] **Hemolysis assay**. The hemolysis assay was performed by spotting active phospholipase samples on a sheep blood agar plate followed by incubation of the plate at 37°C overnight. Following this incubation, the plates were chilled to 4°C for 4 hours prior to visual inspection. Two phospholipase samples were evaluated in this assay: BD16449 PLC (lot PLC-16449-PD267B) and a positive control phospholipase from *Clostridium perfringens* (this enzyme possesses the C-terminal protein domain responsible for hemolytic activity). In this assay, hemolytic activity results in the production of a "clearing zone" on the agar plate as a result of hemolysis of the red blood cells. The table on the left shows the amount of phospholipase activity spotted onto sheep blood agar plates and the diameter of the resulting zone of hemolysis. BD16449 PLC did not produce a hemolytic zone when up to 30,000 milli-units (mU) activity were used in the assay. However, as little as 4 mU *C. perfringens* phospholipase produced a hemolytic zone in the assay. The graph on the right shows the positive correlation between *C. perfringens* phospholipase activity and the size of the hemolytic zone. Note: The *C. perfringens* PLC control was not tested beyond 66 mU of activity because the activity assay is not linear above that dose.

4. Assessment of allergenic potential

As discussed in Section V.G.4, the negative contact testing results confirm what is generally known about enzymes, i.e., that there is no scientific indication (after decades of testing) that such entities will cause any allergic contact dermatitis.

Rather, if an allergy is to develop, it would likely result only from inhalation of an enzyme in aerosol or other form. Given the above-referenced inhalation study results, developing such an allergy from exposure to the phospholipase C here in question is unlikely.

To understand the potential for allergic response from food processing enzymes, it is necessary to consider what enzymes are, how they act, how they are prepared, and how they are used. Since enzymes are proteins, they may have the potential to cause allergic responses. Although virtually all allergens are proteins, it is worth noting that only a small percentage of all dietary proteins are allergens. Enzymes are naturally present in the food we eat and for centuries numerous enzymes have been used safely in food processing. The unique role of enzymes in food processing is as a catalyst. Only a small amount of the enzyme is required to make the desired modification, and there is no intended functional effect in the finished food. Further processing of the food ingredient after the enzyme catalyst has performed the expected function often reduces or eliminates the enzyme from the product, resulting in an extremely low and insignificant potential exposure to the consumer of the final food. Even though there have been no known reports of consumer allergies caused by enzymes used in food processing (Pariza, M.W. et al., 1983) and Diversa is not aware of any allergic reactions caused by ingestion of phospholipase C, the allergenic potential of the protein should be assessed (FAO/WHO, 2001). A comparison of the amino acid sequence of the modified protein to known protein allergens is one step in a multilevel decision tree to assess allergenic potential (Metcalfe, D.D. et al., 1996).

A sequence evaluation scheme based on those formulated by Gendel (Gendel, S.M., 1998) and by the Joint FAO/ WHO Expert Consultation (FAO et al., 2001) was used to assess the similarity of BD16449 phospholipase C to known protein allergens. It has been suggested that an immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids or 35% identity over eighty amino acid residues. In order to assess the potential for BD16449 PLC to induce an allergy, a BLAST search was performed against the Food Allergy Research and Resource Program (FAARP) database

using the sequence of the BD16449 PLC protein. The FAARP database is accessible at http://www.allergenonline.com/asp/public/About.asp and contains a comprehensive list (1537 unique entries) of unique proteins of known and putative allergens (food, environmental, and contact). In addition to the full-length FASTA search at this website, there is also an option to automatically scan each possible 80 amino acid segment of the entered search protein against the Allergen database, looking for matches of at least 35% identity.

The results of the comparison of the amino acid sequence of BD16449 PLC protein to the 1537 protein allergens in the FAARP database demonstrate that BD16449 PLC protein does **not** have any significant homology to the allergens in the database. There were no cases where the homology exceeded 35% identity over an 80 amino acid span, and there were no regions of eight or more contiguous amino acids. The overall results demonstrate that BD16449 PLC shares no significant amino acid homology with known protein allergens that are present in the current releases of the FAARP database.

B. Production microorganism

This section addresses the safety of *Pichia pastoris* strain SDM1168, which was used as the host organism for production of the BD16449 phospholipase C. This strain was genetically modified to produce BD16449 phospholipase C and the resulting BD16449 phospholipase C production strain is *P. pastoris* strain, DVSA-PLC-004.

1. History of safe use

Pichia pastoris, a methylotrophic yeast, has been used for the production of over 300 recombinant proteins since the mid-1980's (Cereghino, J.L. et al., 2000). The majority of these proteins have been expressed as secreted proteins using the *Saccharomyces cerevisiae* α-mating factor pre-pro-peptide as a secretion signal. One of the advantages of using *P. pastoris* as a production host is that heterologous protein purification is enhanced due to the low levels of endogenous proteins secreted (Lin Cereghino, G.P. et al., 2001). Many human genes have been expressed in *P. pastoris* for pharmaceutical use. Two such recombinant proteins, Angiostatin and Endostatin, have completed Phase I clinical trials and are entering into Phase II clinical trials (Herbst, R.S. et al., 2002). Finally, *P. pastoris* is an FDA approved animal feed protein source and is approved for use in broiler feed up to 10% of the total feed (FDA, 1993).

2. Absence of pathogenicity and toxicity

As discussed in Pariza and Foster (Pariza, M.W. et al., 1983) and Pariza and Johnson (Pariza, M.W. et al., 2001), the two papers that set forth the gold standard used by the enzyme industry for assessing the safety of enzyme products, the primary consideration in the evaluation of microbial enzyme preparations to be used in food is the safety of the production organism. Yeasts are not known to produce toxins that are active by the oral route (Pariza, M.W. et al., 2001) and *Pichia pastoris* has been placed in the Biosafety Level 1 (BL-1) class by the ATCC organization, a category reserved for well-characterized agents not known to cause disease in healthy human adults and to be of minimal hazard to laboratory personnel and the environment (Center for Disease Control, 1999). In addition, *P. pastoris* itself has been approved by FDA as a source of animal feed protein for use in broiler feed up to 10% of the total feed (FDA, 1993). Toxicity studies done in support of the above-referenced *P. pastoris*-approved animal feed (including a pathogenicity study in mice, an acute oral toxicity study in rats, a subacute oral toxicity study in rats, and a two generation teratology study in rats) also demonstrated–per FDA's review in 1993–that *P. pastoris* is neither pathogenic nor toxigenic (FDA, 1993). Furthermore, results from toxicology and genotoxicity tests of BD16449 produced by *P. pastoris* demonstrate that this material does not contain toxic or genotoxic substances (Section V.G and Appendices D to M).

3. Inserted genetic material

Pariza's and Johnson's decision tree for evaluating microbial enzyme safety (Pariza, M.W. et al., 2001) asks several questions relating to the introduced DNA of the genetically modified production microorganism. The first question asks if the expressed enzyme product, which is encoded by the introduced DNA, has a safe history of use. While lipases themselves do have a long history of safe use in food (see section VI.A.2), the specific lipase of this GRAS Notification does not.

The decision tree then asks whether or not the No-Observed-Adverse-Effect-Level (NOAEL) for the test article in appropriate short-term studies is sufficiently high to ensure safety. The results of the safety studies pertinent to Diversa's product can be found in Section V.G, and the worst case dietary exposure calculations are set forth in Section VI.C.2. They verify that the NOAEL is sufficiently high to ensure safety.

The next question asks if the test article is free of transferable antibiotic resistance gene DNA. Southern blot analysis confirmed that *P. pastoris* production

strain DVSA-PLC-004 does not contain the *bla* gene, which codes for resistance to ampicillin (see Section V.E). BD16449 PLC enzyme preparation also does not contain transformable DNA (see Section V.D). Furthermore, no detectable antibiotic activity was found in the BD16449 PLC enzyme preparation (see Composition and Specifications, Section III.D). For these reasons, enzyme preparation made from *P. pastoris* production strain DVSA-PLC-004 is free of transferable antibiotic resistance gene DNA.

The decision tree then asks whether all other introduced DNA is well-characterized and free of attributes that would render it unsafe for constructing microorganisms to be used in producing food-grade products. The only other introduced DNA sequences in Diversa's construct are the *Saccharomyces cerevisiae* α-mating factor secretion signal sequence, a fragment of the *P. pastoris* alcohol oxidase gene (3'*P. pastoris AOX1*), a *P. pastoris* selectable marker gene, *HIS4* (histidinol dehydrogenase) (Cregg, J.M. et al., 1985), and the *P. pastoris* regulatory sequences comprising the alcohol oxidase promoter (*AOX1*) and transcriptional terminator from *AOX1*. Each of these sequences of DNA is known and has a defined function. The introduced DNA comes from well-characterized organisms, i.e., *P. pastoris* and *S. cerevisiae*. Both organisms are well-characterized and have a history of safe use.

The final question relevant to genetic modification that the decision tree asks is whether or not the introduced genetic material is randomly integrated into the chromosome. The multiple BD16449 PLC expression cassettes were targeted for integration into the *AOX1* locus. *P. pastoris* production strain DVSA-PLC-004 contains 6 copies of the expression cassette. As demonstrated by the method of Southern blotting, the BD16449 PLC expression cassettes were successfully integrated into the *AOX1* locus and not anywhere else in the genome. Therefore, there was no random integration into the chromosome.

The answers to the above questions are appropriate such that there are no safety concerns regarding the production strain and introduced DNA here in question; thus, all of the criteria used in the decision tree for evaluating the safety of a new enzyme (BD16449 PLC) have been met.

C. Enzyme product

1. Safety studies

The results of the safety studies for DV16449 - as outlined in Section V.G and published in Ciofalo et al. 2006 - show that the BD16449 PLC enzyme preparation is safe under its intended conditions of use. The NOAEL derived from the 90-day oral toxicity study in rats is approximately 133,000 higher than the conservative estimate of the total daily human consumption of BD16449 phospholipase C of 0.015 mg/kg/day. This safety margin assumes a "worst case" scenario for the calculation of possible, daily, human exposure by assuming that all the enzyme, if added to the crude oil at the highest recommended use rate, is retained in the final refined vegetable oil. The safety margin calculation can be found in the section immediately below.

2. Potential dietary exposure

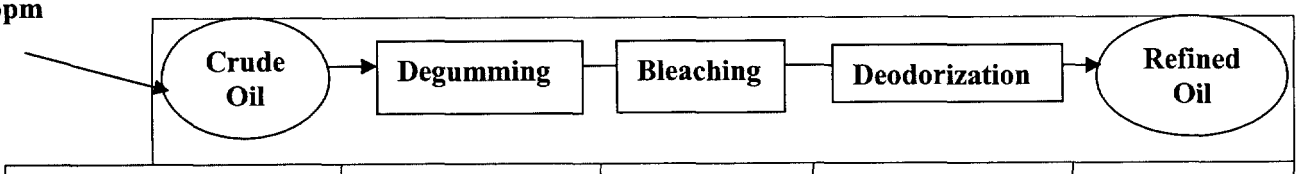
a. Vegetable oil

As mentioned in Section V.F.4, the process for refining vegetable oil includes several steps that inactivate and denature the enzyme. After the degumming reaction has been completed the water phase, containing BD16449 phospholipase C, is separated from the oil by centrifugal separation. Subsequent steps in refining, including repeated washing of the oil with water, bleaching and deodorization, remove any remaining enzyme residues. For these reasons, inactivated and denatured enzyme may be present, if at all, in the fully refined vegetable oil at levels close to or below the limit of detection (LOD=1ppb) of the available, highly-sensitive analytical method. (For the amount of the residue appearing at each step of the oil refining process see Figure 11). The method of quantitation used to determine these residue levels was an enzyme-linked immunosorbent assay (ELISA). Such ELISA sandwich assay protocol was developed and validated for detecting and quantifying BD16449 phospholipase C (PLC) residues in vegetable oil, phospholipid-containing gums and other samples.

Figure 11

Amount of PLC Residue (Determined by ELISA) During the Oil Refining Process

BD16449 PLC
10 ppm



Physical refining	2 ppb	<1 ppb	<1 ppb	<1 ppb
Chemical refining	1 ppb	<1 ppb	<1 ppb	<1 ppb

However, in order to provide a "worst case" scenario for the calculation of possible, daily, human exposure, an assumption was made that all of the enzyme, if added to the crude oil at the highest recommended use rate, is retained in the final vegetable oil. The highest recommended use rate of the active PLC enzyme (i.e., 10 ppm[5]; which is the same as 1000 ppm formulated product) would result in a maximum estimated daily intake (EDI) of 0.015 mg enzyme/kg/day or 0.092 mg TOS[6]/kg/day assuming the average per capita daily consumption of vegetable oils and fats for an average person (i.e., weighing 60 kg) is 90 g/person/day (Economic Research Service, 2005)[7].

[5] Please note 10 ppm = 10 mg/kg

[6] The Pariza and Foster (1983) paper recommends the use of the Total Organic Solids (TOS) concept when determining a reliable estimate of enzyme use and consumption. TOS is defined as the sum of the organic compounds, excluding diluents, contained in the final enzyme preparation. TOS (%) = 100 - % ash - % water - % diluent

[7] Please note the figure, 90g/person/day, was derived from the best available, current data supplied by USDA. The USDA-ERS consumption table for added fats and oils lists several sources of vegetable oils and fats, as well total vegetable oil and fat consumption per capita on a total fat content basis. Therefore, the "worst case" estimate of 90g/person/day was calculated from the 2003 per capita consumption of 73.1 pounds of vegetable-derived added oils and fats on a total fat content basis.

Maximum EDI Calculation:

$$0.015 \text{ mg PLC/kg/day} = 10 \text{ mg/kg PLC} \times \frac{90 \text{ g/person/ day}}{1000 \text{ mg/g}} \div 60 \text{ kg}$$

To provide the calculation in terms of total organic solids (TOS), the percent TOS for the DV16449 PLC test article (TA) is used. The TOS for the DV16449 PLC test article is 84%. Using a specific activity of approximately 2,300 U/mg for a highly purified PLC sample and an activity of 315 U/mg for the PLC test article, PLC represents 13.7% of the test article. Therefore, in order to achieve a 10 ppm dose (i.e., 10 mg/kg) of PLC, 73 ppm (i.e., 73 mg/kg) test article would be used (10 mg/kg ÷ 0.137) in the calculation below.

$$0.092 \text{ mg TOS/kg/day} = 73 \text{ mg/kg PLC TA} \times \frac{90 \text{ g/person/ day}}{1000 \text{ mg/g}} \div 60 \text{ kg} \times 0.84 \text{ TOS}$$

b. Safety margin calculation

The safety margin is calculated by dividing the NOAEL from the subchronic study by the maximum EDI.

The NOAEL or highest dose level fed to rats in the 90-day oral toxicity study without observable adverse effect was 2,000 mg/kg/day (10mL of a 200 mg/mL solution), which corresponds to 1,680 mg TOS/kg/day (2000mg x 0.84) since the percent TOS for DV16449 is 84%.

Thus, the safety margin calculated with or without TOS would be:

$$\frac{2{,}000 \text{ mg/kg/day}}{0.015 \text{ mg/kg/day}} = 1.33 \times 10^5 \quad \text{OR} \quad \frac{1680 \text{ mg TOS/kg/day}}{0.092 \text{ mg TOS/kg/day}} = 1.83 \times 10^4$$

A "worst case" safety margin of 133,000 (18,300 if expressed as TOS) is, Diversa believes, sufficient to insure safe intended use of Diversa's BD16449 PLC enzyme preparation.

A more realistic safety margin could also be calculated using the ELISA data for the refined oil from Figure 11. Assuming all the non-enzyme material from the enzyme preparation is removed during oil refining at the same rate at

which the PLC is removed, the maximum EDI and safety margin is calculated below using 1ppb PLC.

$\frac{2{,}000 \text{ mg/kg/day}}{0.0000015 \text{ mg/kg/day}} = 1.33 \times 10^9$ OR $\frac{1{,}680 \text{ mg TOS/kg/day}}{0.0000092 \text{ mg TOS/kg/day}} = 1.8 \times 10^8$

This more realistic safety margin of 180,000,000 is, Diversa believes, more than sufficient to insure safe intended use of Diversa's BD16449 PLC enzyme preparation.

D. Manufacturing process

As described in Section III.C, the BD16449 phospholipase C enzyme preparation is made using generally known and accepted methods for the production of microbial enzymes (Pariza, M.W. et al., 1983) (Aunstrup, K. et al., 1979). In addition, the BD16449 phospholipase C preparation is manufactured in accordance with both current Good Manufacturing Practices (cGMP) for food and the Organization for Economic Co-operation and Development's criteria for Good Industrial Large Scale Practice (GILSP) (OECD, 1992). The final preparation of BD16449 PLC meets the general and additional requirements for enzyme preparation of *Food Chemicals Codex* and JECFA. Accordingly, the manufacturing process for BD16449 PLC should be deemed safe.

VII. SCIENTIFIC EXPERT PANEL DETERMINATION

Diversa convened a panel of independent scientific experts – none of which currently serve or has served as a consultant to Diversa with regard to BD16449 phospholipase C, but all of whom were paid for their service on the panel – each of which had the appropriate education, training, and experience. The expert panel consisted of:

1. Eric A. Johnson, Sc.D., a food scientist and food microbiologist/ toxicologist (including expertise in bacterial physiology and genetics) whose principal research interests at the Food Research Institute, University of Wisconsin-Madison, include *Clostridium botulinum* toxins and food safety systems, and who is currently a Professor in the Department of Bacteriology and in the Department of Food Microbiology and Toxicology, University of Wisconsin-Madison;

2. Dharma R. Kodali, Ph.D., an organic chemist/ medicinal chemist and lipid technology expert who is currently an adjunct Professor at the University of Minnesota, Department of Biosystems and Agricultural Engineering and with Global Agritech Inc. (a consultant in lipid process and product development); and

3. Ian C. Munro, Ph.D., F.R.C.Path., M.Sc. (**Panel Chair**), a toxicologist/pharmacologist who is currently a Professor at the University of Toronto in the Department of Nutritional Science and with CANTOX Health Sciences International (consultants in health and environmental sciences) who has a long and distinguished career in virtually all food safety matters.

(for additional information, see attached curricula vitae, Appendices N-P). The panel had the aggregate expert capability to deal with all of the food safety issues inherent in this GRAS Notification. Such aggregate capability includes expertise in, among other areas, food safety, toxicology, allergenicity and immunology, use of recombinant technology, molecular biology, and oil matters.

Panel members were given a copy of this GRAS Notification **and** access to all information (including references and appendices) in support of such Notification – i.e., the same aggregate information relied on by Diversa in reaching its GRAS determination. The panel members reviewed such information, met to

discuss such information and had their questions answered, and then concluded – based on all such information – that Diversa' BD16449 phospholipase C enzyme preparation – is GRAS, based on scientific procedures, for its intended use. More specifically, the panel concluded:

> Based on our independent, collective, critical and in-depth evaluation of the available pertinent, scientific (both published and unpublished) and other information, we conclude that Diversa's BD16449 PLC enzyme preparation – which is derived from a genetically modified strain of *Pichia pastoris*, i.e., *Pichia pastoris* strain, DVSA-PLC-004, carrying and expressing a gene for BD16449 PLC – is manufactured in accordance with current good manufacturing practices (cGMP's) for food, meets the relevant food grade specifications and , based primarily on scientific procedures, is Generally Recognized As Safe (i.e., GRAS) for use in food as described within the GRAS Notification. (Expert Panel Report, page 3; see Appendix Q).

VIII. CONCLUSIONS

The lipase enzyme preparation that is the subject of this GRAS Notification, referred to as BD16449 phospholipase C, is derived from a genetically modified strain of *Pichia pastoris* SMD1168 that contains a recombinant gene for BD16449 phospholipase C.

Diversa has determined the BD16449 phospholipase C enzyme preparation to be GRAS, through scientific procedures, for use as a food processing aid in the edible oil refining industry. The enzyme preparation will be used at levels no higher than necessary to achieve the intended technical effects, generally in the range of 100 to 1000 grams of formulated enzyme preparation per metric ton of oil, depending on the oil to be treated and the reaction conditions.

The safety of the enzyme preparation has been evaluated using the safety scheme of Pariza and Johnson (2001) and others (FAO/WHO, 2001; International Food Biotechnology Council, 1990; OECD, 1992). Published and unpublished information is provided which assesses the safety of the following: recipient strain, introduced genetic material, production microorganism, lipases and their use in food, manufacturing process, final BD16449 phospholipase C enzyme preparation, and consumer exposure.

The safety of the production organism is a prime consideration when assessing the probable degree of safety of an enzyme preparation intended for use in food. If the enzyme production organism is nonpathogenic and nontoxigenic, and the enzyme is made according to current good manufacturing practices (cGMP) for food, then one can assume the food ingredient made from the production microorganism is safe to consume. *Pichia pastoris,* a methylotrophic yeast, is well-characterized, carries a Biosafety Level 1 (BL-1) designation, and complies with the OECD criteria for Good Industrial Large Scale Practice. *P. pastoris* has been used for the production of over 300 recombinant proteins since the mid-1980's (Cereghino, J.L. et al., 2000). Many human genes have been expressed in *P. pastoris* for pharmaceutical use (Herbst, R.S. et al., 2002). *P. pastoris* is also an FDA approved animal feed protein source and is approved for use in broiler feed up to 10% of the total feed (FDA, 1993).

The introduced DNA is well-characterized and shown to be safe. The ampicillin resistance gene *bla* which was present in the original cloning vector was

removed and was confirmed via Southern blot analysis to be absent from the final production strain DVSA-PLC-004. DNA from the PLC active discovery clones were sequenced, and the DNA sequence information was analyzed using in-house gene identification software. The predicted PLC open-reading frame was then manually curated to verify the software prediction. The PLC gene was isolated from all extraneous DNA using polymerase chain reaction (PCR) to specifically amplify the DNA, which encodes the PLC open reading frame, from the discovery clone. The toxicity studies performed using BD16449 PLC test article (DV16449) further show the introduced DNA is free of attributes that would render it unsafe for use in the production of a food enzyme.

The enzyme lipase has a long history of safe use in food processing. Lipases are ubiquitous in nature and published literature provides evidence that lipases have been studied for almost 100 years and have been used safely in food processing applications for over 50 years. Many GRAS lipases and phospholipases (a subfamily of lipases) are commercially available, several of which are protein engineered. The BD16449 phospholipase C from *Pichia pastoris* strain DVSA-PLC-004 exhibits functional and biochemical properties that are substantially equivalent to other known microbial phospholipases used in food.

In assessing the safety of the BD16449 phospholipase C preparation, Diversa conducted the following studies: acute oral toxicity study in rats; 14-day range finding gavage in rats; 90-day subchronic gavage in rats; chromosomal aberrations test in human lymphocytes, mouse micronucleus assay, and *Salmonella-Escherichia coli/* mammalian-microsome reverse mutation assay. The studies did not find any treatment related toxicity or induction of genetic mutation or chromosomal aberrations in tests using the alpha amylase test preparations derived from the production microorganism. Safety margin calculations indicate the worst case potential human exposure to the BD16449 phospholipase C enzyme preparation from the consumption of food made using the enzyme is negligible and well below the NOAEL seen in the oral toxicity studies.

The manufacturing process used to make BD16449 PLC enzyme preparation employs a pure culture submerged fermentation of the *P. pastoris* production strain, DVSA-PLC-004. Current good manufacturing practice for food is used throughout the process which utilizes generally accepted, published methods for enzyme manufacture and formulation. All raw materials used in the fermentation and recovery processes are of suitable purity and are standard materials used in the enzyme industry. The final BD16449 PLC enzyme preparation meets the general

and additional requirements for enzyme preparations as outlined in *Food Chemicals Codex* and by JECFA.

Based on the information provided in this GRAS Notification, Diversa concluded that the BD16449 PLC preparation derived from *P. pastoris*, carrying a BD16449 phospholipase gene, is GRAS under the intended conditions of use, as specified herein.

In addition, a panel of scientific experts was assembled to review the safety information compiled on the BD16449 PLC enzyme preparation from the genetically modified *P. pastoris* source. The panel also concluded that the enzyme preparation is GRAS for its intended condition of use.

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X. LIST OF APPENDICES

A. Ciofalo, V., Barton, N., Kreps, J., Coats, I., and Shanahan, D. *Safety evaluation of a lipase enzyme preparation, expressed in Pichia pastoris, intended for use in the degumming of edible vegetable oil.* Regulatory Toxicology and Pharmacology 45, 1-8 (2006)

B. Composition of formulated (final product) and unformulated (test article) BD16449 phospholipase C enzyme preparation

C. Amino acid sequence

D. Chromosomal aberrations in human lymphocytes

E. Mouse micronucleus assay

F. Ames assay

G. Acute oral toxicity study in rats

H. 14-day dose range-finding oral toxicity study in rats

I. 90-day oral toxicity study in rats

J. An acute nose-only inhalation study in rats

K. Primary eye irritation

L. Primary dermal irritation

M. Delayed contact hypersensitivity in guinea pigs (Buehler method)

N. *Curriculum Vitae* for Dr. Eric Johnson

O. *Curriculum Vitae* for Dr. Dharma Kodali

P. *Curriculum Vitae* for Dr. Ian Munro

Q. Expert panel's report

Appendix A

Ciofalo, V., Barton, N., Kreps, J., Coats, I., and Shanahan, D. ***Safety evaluation of a lipase enzyme preparation, expressed in Pichia pastoris, intended for use in the degumming of edible vegetable oil.*** **Regulatory Toxicology and Pharmacology 45, 1-8 (2006)**

Pages 000091 - 000098 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Reference List for Industry Submission, GRN 000204

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000091 - 000098	Ciofalo, Vince; Barton, Nelson; Kreps, Joel; Coats, Isabelle; Shanahan, Diane	Safety evaluation of a lipase enzyme preparation, expressed in Pichia pastoris, intended for use in the degumming of edible vegetable oil	2006	Regulatory Toxicology and Pharmacology	Volume 45, pgs 1-8

NA- Not applicable

AM

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June 29, 2006

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RECD JUL 3 2006

Re: Notice of GRAS exemption for use of Diversa's BD16449 Phospholipase C, an enzyme preparation expressed in the yeast *Pichia pastoris* (strain DVSA-PLC-004), for degumming vegetable oils

Dear Dr. Mattia:

When preparing the above-referenced GRAS Notification, we inadvertently included two citations i.e., "Clausen, K., 2001; Dahlke, K., 1998", at the end of the first full paragraph (under subsection 2, "Mode Of Action") on page 30 that should not have been cited at that location. Page 30 has been redone with the two cited references removed. Thus, please find a new page 30 (with no other changes) which should be inserted in Diversa's GRAS Notification instead of its current page 30.

Sorry for the inconvenience.

Sincerely,

(b)(6)

Charles L. Morin

2. Mode of action

BD16449 phospholipase C catalyzes the hydrolysis of the phosphodiester bond linking the glycerol and phosphate moieties at the *sn*-3 position of glycerophospholids as shown below (Stryer L., 1988):

$$\text{Phosphatidylcholine} + H_2O \longrightarrow \text{1,2 diacylglycerol} + \text{phosphorylcholine}$$

$$\text{Phosphatidylethanolamine} + H_2O \longrightarrow \text{1,2 diacylglycerol} + \text{phosphorylethanolamine}$$

As can be seen in the foregoing chemical equations, the products of the hydrolysis of the major vegetable oil phospholipids, PC and PE, by BD16449 phospholipase C are diacylglycerol and water-soluble phosphate esters phosphorylcholine and phosphorylethanolamine respectively.

3. Level of use

The enzyme preparation will be used at levels no higher than necessary to achieve the intended effect, generally in the range of 100 to 1000 grams of enzyme preparation per metric ton of oil, depending on the oil to be treated and the reaction conditions.

4. Enzyme residues in food ingredients

BD16449 phospholipase C is intended for use in the first step, i.e., the degumming step, in refining edible vegetable oils. This process step involves addition of the enzyme to a water-in-oil emulsion. BD16449 phospholipase C, like other proteins, is soluble in water and insoluble in organic solvents, and in a 2-phase water-in-oil emulsion, is known to partition in the aqueous phase. After the degumming reaction has been completed, the water phase, containing BD16449 phospholipase C, is separated from the oil by centrifugal separation. Subsequent steps in refining, including repeated washing of the oil with water, bleaching and deodorization, remove any remaining enzyme residues. For these reasons, inactivated and denatured enzyme may be present, if at all, in the fully refined vegetable oil at levels close to or below the limit of detection (LOD=1ppb) of available analytical methodology.