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3550 John Hopkins Ct., San Diego, CA 92121
800.523.2990

Use of BD29241 Palmitse as a Processing Aid for Reducing Palmitic Acid Levels in Triacylglycerol Oils Intended for Use in Food

Filed By

Verenium Corporation
3550 John Hopkins Court
San Diego, CA 92121

December 7, 2012

Law Offices Of
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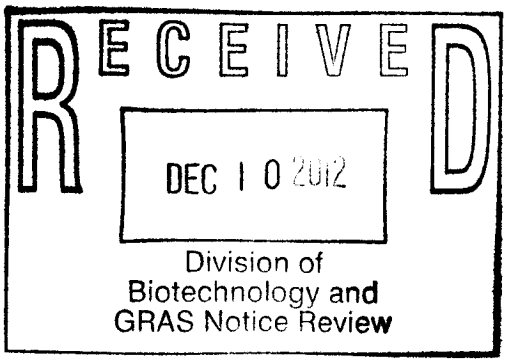
December 6, 2012



Antonia Mattia, PhD (HFS-255)
Director
Division of Biotechnology and
GRAS Notice Review

Office for Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Verenium Corporation
GRAS Notice
Notice of GRAS exemption for use of BD29241 Palmitase, an enzyme preparation expressed in the bacteria *Pseudomonas fluorescens* (strain BD29241), for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils

Dear Dr. Mattia:

Pursuant to proposed 21 CFR § 170.36 and on behalf of our client, i.e., Verenium Corporation (3550 John Hopkins Court, San Diego, 92121), this submission – forwarded in triplicate (plus two extra copies for your convenience) – is intended to notify FDA of Verenium's and an Expert Panel's independent determinations – based on scientific procedures – that the above-referenced BD29241 Palmitase enzyme preparation is generally recognized as safe when used as an enzymatic processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils (intended for use in food) at a recommended level of 50 to 100 kilograms of formulated enzyme preparation per metric ton of oil (i.e., at 50,000 to 100,000 ppm). Accordingly, and as with all GRAS substances, such BD29241 Palmitase enzyme preparation – when used as described above and in the attached GRAS Notice – 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 GRAS Notice, please find attached a summary discussion of the information relied upon by Verenium and others when determining that BD29241 Palmitase enzyme preparation is GRAS. Such discussion is set forth in the format required by proposed 21 CFR § 170.36(c). (62 FR 18961 – 04/17/97). 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 or Diane Shanahan, whose contact information is found below, know:

Diane Shanahan
Senior Director, Regulatory Affairs
Verenium Corporation
3550 John Hopkins Court
San Diego, CA 92121
Telephone: 858-481-8591
Email: diane.shanahan@verenium.com.

All of the information that serves as the basis for this GRAS notice 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 BD29241 Palmitase enzyme preparation (i.e., the document referenced in the GN either by "Halich" or "Appendix A") is attached.

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

(b) (6)

Sincerely,

(b) (6)

Charles L. Morin

Cc: Diane Shanahan
Senior Director, Regulatory Affairs
Verenium Corporation

COVER PAGE

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I. INTRODUCTION

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I. INTRODUCTION

The lipase enzyme preparation that is the subject of this GRAS Notification, referred to below as BD29241 Palmitase, is intended for use as an enzymatic processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils intended for use in food.

In order to generate a product with a specific fatty acid profile, a specific lipase may be used. BD29241 Palmitase may be used to selectively reduce the saturated fatty acid content (specifically palmitic acid, C 16:0) of a given oil. The profile of this modified oil would be that of a higher quality oil, conferring health benefits of reduced saturated fatty acids. To reduce palmitic acid levels in soybean oil and other triacylglycerol-based oils, BD29241 Palmitase is added to triacylglycerol-based oil and allowed to react with the oil before degumming begins in the physical refining of oil. Physical refining is described below.

Refining is a general term used by the industry to describe the process whereby crude vegetable oil is converted to a significantly more purified final product by removing non-triacylglycerol components that can negatively impact taste, color and stability, and make the oil unsuitable for particular applications. There are generally two types of refining – chemical and physical. BD29241 Palmitase enzyme product is used only in physical refining. 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, the oil is degummed using water and acid with the option to use commercially available enzymes, such as phospholipase C and phospholipase A, A1, and A2. The degummed oil is then bleached, whereby 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 contaminating free fatty acids are then removed, together with other unwanted volatiles, during a deodorization step. After the deodorization step, the oil is considered refined.

As discussed in greater detail in Sections V.F.4 and VI.C of this GRAS Notification, inactive and denatured residues of the BD29241 Palmitase enzyme preparation in refined oil are not detectable at a sensitivity of 60 ppb when using current available validated analytical methodology. Therefore, the corresponding dietary concentration of BD29241 Palmitase total organic solids (TOS) in finished product is (realistic case) *de minimus*.

The BD29241 Palmitase enzyme preparation is produced from a non-toxigenic, non-pathogenic, genetically modified strain of *Pseudomonas fluorescens*. The *P. fluorescens* strain DC454 is a derivative of the wild-type strain MB101.

The safety of the BD29241 Palmitase enzyme preparation, when used as described, is supported by a series of published safety studies (Halich, R. *et al.*, 2012) (see Appendix A), 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 2,442 higher than the "worst case" estimate of the total, daily, human consumption of BD29241 Palmitase of 0.819 mg/kg/day (Halich, R. *et al.*, 2012). 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

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II. ADMINISTRATIVE INFORMATION

A. Claim Regarding GRAS Status

Verenium hereby notifies the FDA of determinations by Verenium and an Expert Panel that the BD29241 Palmitase enzyme preparation, produced from *P. fluorescens*, strain BD29241, expressing a gene encoding palmitase, is generally recognized as safe (GRAS), based on scientific procedures, when used as described below for use as an enzymatic processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils intended for use in food – at a recommended level of 50 to 100 kilograms of formulated enzyme preparation per metric ton of oil.

(b) (6)

12/6/2012

James Levine
Chief Executive Officer
Verenium Corporation
3550 John Hopkins Court
San Diego, CA 92121

Date

B. Name and Address of Notifier

Diane Shanahan
Senior Director, Regulatory Affairs
Verenium Corporation
3550 John Hopkins Court
San Diego, CA 92121
diane.shanahan@verenium.com

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C. Common or Usual Name of Subject Substance

Palmitase, a palmitic acid specific lipase (also referred to as BD29241 Palmitase) (see Section V.B.1 for a detailed description).

D. Conditions of Use

BD29241 Palmitase is intended for use as a processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils intended for use in food. The enzyme preparation will be used at levels no higher than necessary to achieve the intended effect, generally in the range of 50 to 100 kilograms of formulated enzyme preparation per metric ton of oil, depending on the triacylglycerol-based oil being treated and the reaction conditions. This is equivalent to a concentration range of 50,000 to 100,000 parts per million (ppm) of formulated enzyme preparation.

E. Basis for GRAS Determination

The GRAS determinations for the subject enzyme preparation are 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 to the FDA for review and copying upon request.

III. Notified Substance

III. DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

A. Name

BD29241 Palmitase

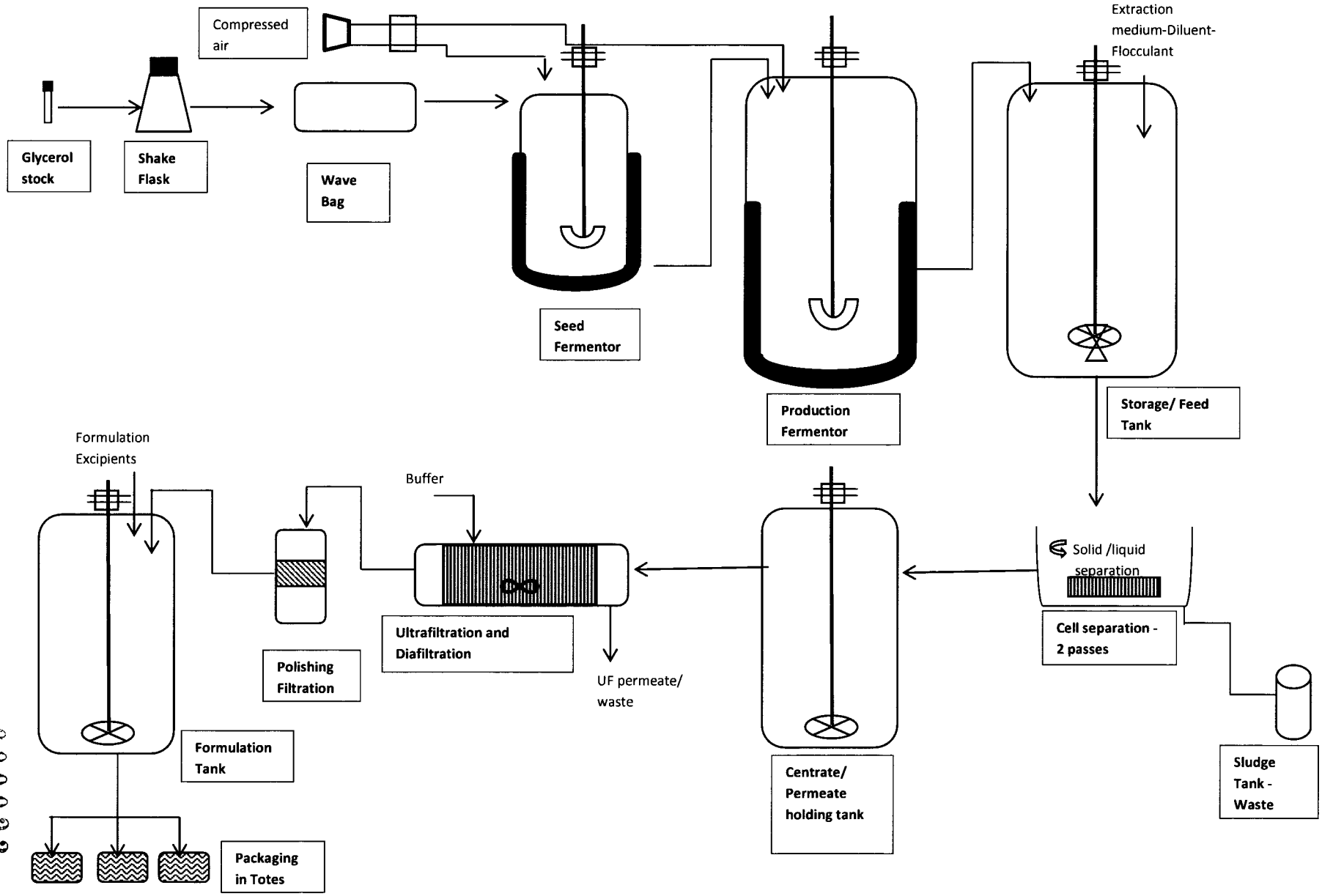
B. Physical Description

Yellow to brown liquid

C. Method of Manufacture

The BD29241 Palmitase 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 palmitase enzyme preparation is manufactured in accordance with both current Good Manufacturing Practices for foods (cGMPs) and the 1992 Organization for Economic Co-operation and Development's criteria for Good Industrial Large Scale Practice (GILSP) (OECD, Accessed 9/20/2012). A diagram of the entire manufacturing process follows immediately on the next page. (See Figure 1).

Figure 1. BD29241 Palmitase Manufacturing Process Diagram



1. Production microorganism

The parental strain of *P. fluorescens* DC454, a derivative of the wild-type strain MB101, was transformed with a plasmid containing an evolved, lipase gene coding for the BD29241 Palmitase enzyme. The resulting *P. fluorescens* strain was designated BD29241 and is intended for the production of a lipase with high selectivity for palmitate hydrolysis. The strain (BD29241) has been genetically modified by procedures described in detail below (see Section V.A.) for the production of BD29241 Palmitase enzyme.

Frozen cell bank cultures of the production organism, *P. fluorescens* BD29241, 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 Verenium SOP MP0314. The manufacturing working cell bank (WCB1) was then prepared from MCB1 and qualified for the same criteria according to Verenium SOP MP0315. Master Cell Bank integrity is verified by temperature logging at each of the -80°C freezers that store the cell banks.

2. Raw materials

Raw materials used in the fermentation and recovery process for BD29241 Palmitase 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* 8th Edition requirements when available) and are quality inspected before use. These specifications include limits on lead and other pertinent heavy metals. Raw materials used for the formulation are of food grade quality.

Antifoaming agents and flocculants used during manufacture and recovery of the BD29241 Palmitase are of a suitable purity and are used according to the Enzyme Technical

Association's list of flocculants and 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).

During fermentation, isopropyl β-D-thiogalactopyranoside (IPTG) is added as an inducer for the BD29241 Palmitase enzyme production. It is calculated that the "worst case" IPTG concentration in the final formulated product is 0.487 mg/L or 0.487 ppm. Based on the highest use rate of BD29241 Palmitase enzyme preparation (100 kilograms/metric ton of oil, which is equivalent to 100 g/L of oil), IPTG may be present in the final oil at the level of 48.7 ppb ("worst case" estimate). This is considered a "worst case" estimate because IPTG (with a molecular weight = 0.2383 kD) is expected to separate from the enzyme when the IPTG passes through the 10 kD membrane during the diafiltration step of the enzyme manufacturing process. Therefore, the actual residue level in the final oil is likely to be far below 48.7 ppb, if not zero.

3. Fermentation process

The BD29241 Palmitase production begins with a submerged fed-batch pure culture of the genetically modified *P. fluorescens* BD29241 (see Figure 1). Process parameters that are routinely monitored during the fermentation include pH, aeration, enzyme activity, agitation, temperature, dissolved oxygen, and oxygen uptake rate (OUR). During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

After optimal BD29241 Palmitase production is reached, the broth is ready for enzyme recovery. The BD29241 Palmitase enzyme is expressed intracellularly in the production strain BD29241. In order to begin recovery, the BD29241 Palmitase enzyme needs to be released into the broth. This is achieved by lysis of the cells using an alkaline pH chemical treatment. 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, along with the addition of FDA-approved flocculant processing aids to allow passage and effective phase separation in the solid/liquid separation step. A second separation step removes any remaining cells and particulate solids. The cell mass from both of these two steps is discharged as a sludge that is heat inactivated before going to waste. The clarified centrate is then concentrated by ultrafiltration, where the enzyme preparation is concentrated to the appropriate concentration target, and then diafiltered into 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)

BD29241 Palmitase preparation complies with the General and Additional Requirements for Enzyme Preparations in the monograph on Enzyme Preparations in the 8th Edition of the *Food Chemicals Codex* (FCC) (U.S.Pharmacopeial Convention, 2012). In addition, the BD29241 Palmitase preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing outlined by the 2006 Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, Accessed 9/24/2012).

The BD29241 Palmitase enzyme preparation is formulated as a liquid with the following typical composition:

Chemical Name	Amount
Total Organic Solids (TOS)[1]	Approximately 6% ± 3%
Glycerol	Approximately 40%
Methyl paraben	Approximately 0.1%
Sodium phosphate	Approximately 0.07%
Water	Balance

The BD29241 Palmitase enzyme preparation specifications are as follows.

Specification	Permissible Level
Enzyme activity (units/g)[2]	Not less than 1,000
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
Antimicrobial activity	Negative by test
E. coli (in 25g)	Absent
Salmonella (in 25g)	Absent
Absence of Production Organism	Absent

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

[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 unit of Palmitase activity is defined as the quantity of enzyme required to liberate 1 μmole of 4-methylumbelliferone from 400μM MU-palmitate during one minute at pH= 7.5 and 27°C.

Three lots of BD29241 Palmitase enzyme preparation were analyzed for chemical and microbial composition to demonstrate that all three lots meet all of the specifications listed above for BD29241 Palmitase. The results can be found in Appendix B.

IV. ANALYTICAL METHODOLOGY

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 BD29241 Palmitase enzyme preparation will be analyzed for palmitase activity prior to release for distribution and sale. As the BD29241 Palmitase product is to be sold with a guaranteed minimum activity level of 1,000 units/g, 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 palmitase activity levels of the formulated product.

B. Application and Principle

The fluorometric assay to determine palmitase activity follows the liberation of 4-methylumbelliferone from 4-methylumbelliferyl palmitate spectrophotometrically under controlled conditions. The palmitase enzyme sample is assayed in a 4-methylumbelliferyl palmitate substrate solution. The liberation of 4-methylumbelliferone is followed spectrophotometrically at an excitation of 360 nm and emission of 465 nm. The increase in fluorescence at 465 nm is converted to μmoles of 4-methylumbelliferone by using a standard curve at the defined conditions.

C. Source

There is no published activity method for palmitic acid selective lipases. The liberation of 4-methylumbelliferone from a variety of substrates is a common method for detecting enzymatic activity. Liberating this fluorophore provides a quick, quantitative assay method that amplifies the signal enabling use of minimal enzyme. This fluorophore may be purchased linked to a variety of fatty acids. These substrates may be used to detect lipase activity and/or selectivity. One such substrate is 4-methylumbelliferyl palmitate. This substrate was identified as the best surrogate substrate to detect palmitase activity. This surrogate substrate enables a quick, quantitative determination of enzyme activity by following the cleavage of palmitic acid.

Assay method QC0243 was developed to utilize the commercially available 4-methylumbelliferyl palmitate to quantify palmitase activity under appropriate conditions.

Validation of the assay was completed at Verenium using the standard conditions of use.

D. Assay Protocol

The detailed assay protocol is outlined in Verenium standard operating procedure (SOP) QC0243. In summary, the palmitase enzyme sample is diluted in HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and assayed at 27°C and pH 7.5 in 4-methylumbelliferyl palmitate substrate solution. The liberation of 4-methylumbelliferone is followed spectrophotometrically at an excitation of 360 nm and emission of 465 nm. The increase in fluorescence at 465 nm is converted to μmoles of 4-methulumbelliferone by using a standard curve at the defined conditions. One unit of activity is defined as the quantity of enzyme required to liberate 1 μmole of 4-methylumbelliferone from 800 μM 4-methylumbelliferyl palmitate substrate during one minute at 27°C and pH 7.5.

E. Assay Validation

The palmitase assay procedure was validated under standard conditions at Verenium 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 palmitase concentration *vs.* reaction rate averaged 0.9942, 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 QC0243 as a reaction rate of 6.5E+6 to 1.1E+7 RFU, with the minimum value corresponding to an activity of 0.21 units (μmole/min/mL).

3. Limit of Detection and Limit of Quantitation: The limit of detection is the lowest palmitase activity that can be detected, and the limit of quantitation is the lowest palmitase activity that can be measured. The standard deviation of the analytical response of a number of blanks multiplied by the factors 3 and 10 provide estimates of the limit of detection and limit of quantitation, respectively.

Limit of detection:	0.00041 units (μmole/min/mL)
Limit of quantitation:	0.00136 units (μmole/min/mL)

Note: assay limit of quantitation is set at 0.21 units based on the minimum reaction rate value set for the assay range.

4. Repeatability: The repeatability of the assay was determined by calculating the standard deviation and relative standard deviation (coefficient of variation) of five measurements of palmitase activity from each of two lots on the same day, using the same analyst, substrate preparation, and equipment. Two separate lots gave standard deviations of 1.09 U/mg and 0.61 U/mg and relative standard deviation of 2.61% and 1.62%, respectively.

5. Intermediate Precision: The intermediate precision of the assay was determined by calculating the standard deviation and relative standard deviation (coefficient of variation) of measurements of palmitase activity of two lots assayed over five different days, preparing enzyme samples and substrate fresh daily while using two instruments for determining sample activity. The two separate lots gave standard deviations of 1.98 U/mg and 1.67 U/mg and relative standard deviation of 5.36% and 4.93%, respectively.

6. Robustness: The robustness of the assay was determined by assaying a sample preparation under small variations of selected assay conditions (temperature, pH, and 4-methylumbelliferyl palmitate substrate stock solution concentration) to be able to determine sensitivities of a control parameter. In addition to the working substrate solution stability, temperature preparation of the working substrate solution and calculation of activity using relative and absolute RFU values were evaluated. The results indicated that temperature, pH, and substrate stock solution concentration are critical control parameters. Working substrate

solution was determined to be stable for up to two hours in the absence of light. Activity is best calculated by using the relative RFU values, as calculating with absolute RFU values potentially decreased the number reading used when calculating the kinetic reaction rates of samples. The assay was not sensitive to small temperature changes that may have occurred during preparation of the working substrate solution.

V. GRAS BASIS

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

A. Production Microorganism

This section describes the historical activities associated with construction of the *P. fluorescens* production strain BD29241, the origin of the BD29241 Palmitase gene, and the methodology used to introduce the latter into the recipient strain *P. fluorescens* BD29241. For a discussion on the safety of the production strain, please see Section VI.B.

1. Production strain summary

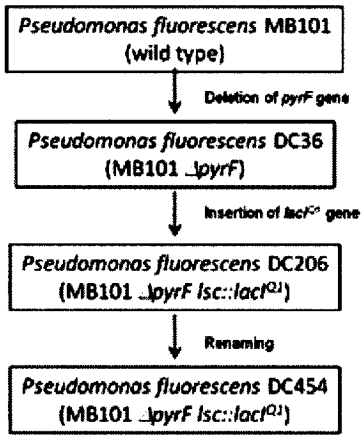
Production strain BD29241 was constructed by electroporation of *P. fluorescens* DC454, the recipient strain, with the expression vector pDOW1169_BD29241 that contains the BD29241 Palmitase gene. The native or wild type palmitase gene was obtained from a soil environmental library (see Section V.A.3 below). This genetically modified production organism complies with the 1992 OECD (Organization for Economic Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (OECD, Accessed 9/20/2012). 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; Jonas, D.A. *et al.*, 1996; OECD, Accessed 3/1/2012a; Pariza, M.W. *et al.*, 1983; Pariza, M.W. *et al.*, 2001).

2. Recipient microorganism

The recipient microorganism used for the construction of the BD29241 production strain is *P. fluorescens* DC454. Strain DC454 is a derivative of *P. fluorescens* Biovar I, MB101, which was originally isolated from nature by Mycogen Corporation. The identity of strain MB101 was independently verified by I. J. Misaghi, Ph.D., Prof. of Plant Pathology, University of Arizona, as *P. fluorescens* Biotype A, which is equivalent under current taxonomic standards to *P. fluorescens* Biovar I. With respect to its biotype designation, Prof. Misaghi found MB101 to be closely related to Biotype A of *P. fluorescens* ATCC 17397.

A derivative of *P. fluorescens* Biovar I, strain MB101 has been used as the recipient strain for another food-grade enzyme (GRAS Notice No. GRN000126, 2003). As mentioned above, strain DC454 is derived from strain MB101. The lineage of the DC454 recipient strain is illustrated below in Figure 2. As a first step to generate the strain DC454 (MB101 Δ*pyrF lsc::lacI*^{Q1}), the open reading frame of *pyrF* gene was deleted from the chromosome of MB101, resulting in *P. fluorescens* DC36 (MB101 Δ*pyrF*) (Schneider, J.C. *et al.*, 2005a; Schneider, J.C. *et al.*, 2005b). The *lacI* gene was then inserted into the chromosome of DC36 near the levansucrase (*lsc*) locus under the control of the *lacI*^{Q1} promoter using suicide plasmid technique (Schneider, J.C. *et al.*, 2005a). The new strain is called DC206. Later, strain DC206 was renamed as DC454 (Retallack, D. *et al.*, 2009).

Figure 2. Strain lineage



The taxonomic designation of strain DC454 is as follows:

Domain	Bacteria
Phylum BXII.	*Proteobacteria*
Class III.	"*Gammaproteobacteria*"
Order VII.	*Pseudomonadales*
Family I.	*Pseudomonadaceae*
Genus I.	*Pseudomonas*
Species	*fluorescens*
Biovar	I

3. BD29241 Palmitase gene source

DNA purified directly from a soil sample (Indonesia) 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 lipase activity. Discovery clones with lipase activity were sequenced, and the DNA sequence information was analyzed using in-house gene identification software. The predicted lipase open reading frame (ORF) was then manually curated to verify the software prediction. The lipase gene was isolated from all extraneous DNA using polymerase chain reaction (PCR) to specifically amplify the DNA, which encodes the lipase ORF, from the discovery clone. The native lipase gene ORF is 684 bp in size and was further modified (see page 15 for details) for palmitic acid selectivity. The modified lipase gene is referred to as BD29241 Palmitase gene.

4. Construction of the expression vector

The pDOW1169_BD29241 expression vector used to express the BD29241 Palmitase enzyme was constructed by subcloning the BD29241 Palmitase gene into the expression vector pDOW1169. The pDOW1169 vector was derived from pMYC5088, a self-replicating, non-conjugative, and non-mobilizable derivative of the well-known plasmid RSF1010 (Scholz, P. *et al.*, 1989) combined with fragments from well-known plasmids pRK2 (Pansegrau, W. *et al.*, 1994) and pKK223-3 (Brosius, J. *et al.*, 1984).

The expression vector pMYC5088 was used to express a food-grade alpha-amylase enzyme in a *P. fluorescens* system and was described previously in the GRAS Notification for BD5088 alpha-amylase enzyme preparation (GRAS Notice No. GRN000126, 2003). The pMYC5088 plasmid contains the *tetA* and *tetR* genes, which code for resistance to tetracycline and have been used as a selection marker to generate the production strain. However, during the construction of pDOW1169, the tetracycline resistance genes (*tetA* and *tetR*) were removed by a common molecular cloning technique (i.e. restriction digest) and replaced with an autotrophic selection marker, *pyrF* (orotidine-5'-phosphate decarboxylase). In addition, a second site of *lac*O

(lactose operator) was added into the promoter region during the construction of pDOW1169 to help with tighter regulation of the tac promoter.

Generation of the expression vector pDOW1169_BD29241 involved several steps. First, the native lipase gene was evolved to improve selectivity, expression, and fitness of the enzyme using Verenium's DirectEvolution® platform and codon optimization. The DirectEvolution® technologies that were employed were Gene Site Saturation Mutagenesis^{SM} (GSSM^{SM}) (Short, J.M., 2001), GeneReassembly^{SM} (Short, J.M., 2003), and Tailored Multi-Site Combinatorial Assembly^{SM} (TMSCA^{SM}) (Tan, X., 2009) technologies. Use of the DirectEvolution® technologies resulted in six amino acid changes and seven silent mutations. To improve expression of the evolved BD29241 Palmitase gene in *P. fluorescens* strain DC454, a codon optimized gene of the BD29241 Palmitase protein was designed. Second, the evolved, codon optimized gene, now referred to as the BD29241 Palmitase gene, was synthesized by DNA 2.0 Incorporated (Menlo Park, CA), and cloned into their cloning vector. Third, to facilitate cloning into pDOW1169, the restriction enzyme SpeI site and a ribosome binding site were added in front of the start codon ATG and the restriction enzyme XhoI site and an extra stop codon TGA were added after the stop codon TGA of the evolved, codon optimized gene. Fourth, both the plasmid containing the synthetic codon optimized BD29241 Palmitase gene and the pDOW1169 vector were digested with restriction enzymes SpeI and XhoI. Fifth, the digested BD29241 Palmitase gene and the vector were then purified by gel electrophoresis. Finally, the BD29241 Palmitase gene was ligated to the vector to generate the expression vector pDOW1169_BD29241.

The size of the pDOW1169_BD29241 plasmid is 9305 base pairs. The correct insertion of the BD29241 Palmitase gene into the plasmid was confirmed by sequencing. Once the correct insert was verified, the DNA sequence of the pDOW1169_BD29241 plasmid was also determined. An exhaustive open reading frame (ORF) analysis of the plasmid demonstrated that none of the predicted ORFs encode for a protein that is homologous to known toxins or other harmful proteins. Figure 3 provides a schematic diagram of the construction of pDOW1169_BD29241. Table 1 lists the genetic materials contained in pDOW1169_BD29241.

Figure 3. Construction of pDOW1169_BD29241

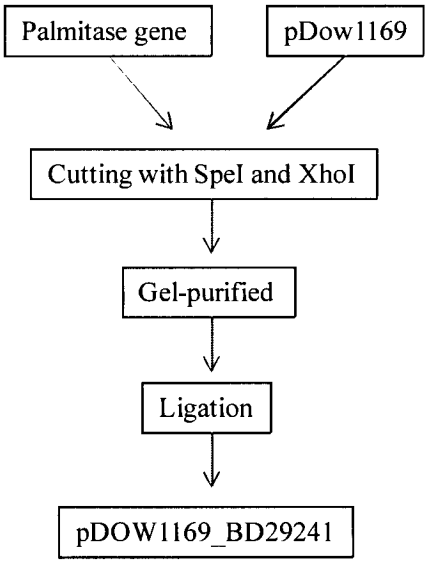


Table 1. Components of pDOW1169_BD29241 expression vector

Name	Start	End	Size (bp)	Source
Plasmid DNA	1	258	258	DNA from plasmid RK2
pyrF terminator	259	482	227	Downstream of *pyrF* gene from *P. fluorescens* MB214 host, proposed as terminator for *pyrF* gene
pyrF gene	483	1181	696	*P. fluorescens* gene, encodes orotidine-5'-phosphate decarboxylase, selectable marker
pyrF promoter	1182	1302	121	Upstream of *pyrF* gene from *P. fluorescens* MB214 host, proposed as promoter for *pyrF* gene

Name	Start	End	Size (bp)	Source
tac	1303	1421	119	Synthetic promoter based on *E. coli* trp and lac promoter with two *lac*O sites
RBS	1422	1433	12	Synthetic ribosome binding site and flanking sequence
BD29241Palmitase gene	1434	2117	684	Modified palmitase gene
Nonfunctional DNA	2118	2126	8	Extra stop codon and XhoI site
Terminator region	2127	2330	204	*E. coli rrnBT1T2* transcriptional terminator from pKK223-3
Plasmid DNA	2331	2351	21	DNA from plasmid pKK223-3
Plasmid DNA	2352	2367	16	Junction sequence with multiple restriction sites
Plasmid DNA	2368	2574	207	DNA from plasmid RSF1010
repC	2575	3525	951	RSF1010 replication machinery ORF
repA	3413	4252	840	RSF1010 replication machinery ORF
Plasmid DNA	4253	4762	510	DNA from RSF1010, encodes *orfE* and *orfF* which functions are unknown
mobA (partial)	4763	6154	1392	RSF1010 mobilization machinery mobA partial ORF, mutated to remove conjugational DNA transfer function

Name	Start	End	Size (bp)	Source
repB	4763	5734	972	RSF1010 replication machinery ORF
mobB	5731	6126	396	Mutated RSF1010 mobilization ORF B, mutated to remove conjugational DNA transfer function
mobA (partial)	6132	6908	777	RSF1010 mobilization machinery mobA partial ORF, mutated to remove conjugational DNA transfer function
Plasmid DNA	6909	6996	88	DNA from plasmid RSF1010
*ori*Tnic	6997	7153	157	Origin of replication from RSF1010
mobC	7107	7391	285	RSF1010 mobilization ORF C
*ori*V	7387	7811	425	Origin of replication from RSF1010, required for maintenance with the bacterium
Plasmid DNA	7812	8703	892	DNA from plasmid RSF1010
Plasmid DNA	8704	9305	602	DNA from plasmid RK2

5. Construction of the recombinant production organism BD29241

The recipient strain, *P. fluorescens* strain DC454, was used to generate the antibiotic resistance marker free production strain BD29241, which expresses the BD29421 Palmitase enzyme. DC454 is a derivative of the wild type *P. fluorescens* strain MB101. The MB101 strain was modified so that the *pyrF* gene (orotidine-5'-phosphate decarboxylase) on the chromosome was deleted (Schneider, J.C. *et al.*, 2005a; Schneider, J.C. *et al.*, 2005b) and the *lacI* gene was inserted into the chromosome near the levansucrase (*lsc*) locus under the control of the $lacI^{Q1}$ promoter (Schneider, J.C. *et al.*, 2005a). In order to improve selectivity, fitness, and expression

of the enzyme, the native lipase gene was modified, resulting in the BD29241 Palmitase gene. The modified gene was cloned into the pDOW1169 expression vector generating pDOW1169_BD29241. The pDOW1169_BD29241 plasmid contains the *pyrF* gene, which serves as a selective marker. Then the pDOW1169_BD29241 plasmid was transformed by electroporation into *P. fluorescens* strain DC454. A transformant with the appropriate sequence was designated as BD29241, the final production strain.

6. Stability of introduced sequences

One copy of the $lacI^{Q1}$ gene from *E. coli* K12 was introduced into the chromosome of the wild type *P. fluorescens* strain MB101 at the levansucrase (*lsc*) locus to generate the recipient strain *P. fluorescens* strain DC454. The *P. fluorescens* strain DC454 was transformed with the expression plasmid pDOW1169_BD29241, generating the palmitase production strain BD29241. The stability of the $lacI^{Q1}$ gene and the plasmid pDOW1169_BD29241 were evaluated during the fermentation step of the manufacturing process.

The stability of the $lacI^{Q1}$ gene in *P. fluorescens* strain BD29241 was determined by the presence of the $lacI^{Q1}$ gene at the targeted location at the end of fermentation process using colony PCR. The $lacI^{Q1}$ gene copy number was measured throughout the fermentation process using quantitative PCR. The $lacI^{Q1}$ gene was shown to be stable during the fermentation process and no loss of this gene was observed.

The stability of the expression plasmid pDOW1169_BD29241 in *P. fluorescens* strain BD29241 was measured by determining the plasmid copy number throughout the fermentation process using quantitative PCR. To determine if plasmid rearrangement occurred, plasmid restriction digest patterns and uncut plasmid DNA patterns were observed. Based on the results obtained, it was confirmed that the expression plasmid was stable throughout the fermentation process. No deletion or DNA rearrangement was observed. Furthermore, there was no significant variation in plasmid copy number within the BD29241 production organism throughout the fermentation process.

The results clearly demonstrate that the introduced expression plasmid pDOW1169_BD29241 and the integrated *lacI*^{Q1} gene are stable during the fermentation process in the production of BD29241 Palmitase enzyme preparation.

B. Enzyme Identity

1. Identity

The lipase enzyme preparation that is the subject of this GRAS Notification is a palmitic acid selective lipase that falls within the broader lipase sub-family as defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

Enzyme Name:	Lipase
Official Name:	Triacylglycerol lipase
Systematic Name:	Triacylglycerol acylhydrolase
IUBMB Number:	3.1.1.3
CAS Registry No:	9001-62-1
Molecular Weight:	24449.7 Daltons
Specificity:	Triacylglycerol + H_2O $\longrightarrow$ Diacylglycerol + Free fatty acid

2. Amino acid sequence

The lipase protein, designated BD29241 Palmitase, is a 227 amino acid protein. The identity of the protein as expressed in the *P. fluorescens* production strain BD29241 has been independently confirmed using mass spectroscopy, amino-terminal protein sequencing, and amino acid composition analysis. The complete amino acid sequence of BD29241 Palmitase is presented in Appendix C.

3. 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. In order to generate a product with a specific fatty acid profile, a specific lipase may be used. BD29241 Palmitase may be used to selectively reduce the saturated fatty acid content (specifically palmitic acid, C 16:0) of a given oil. This modified oil profile would be higher quality oil, conferring health benefits of reduced saturated fatty acids. When added to crude soybean oil, BD29241 Palmitase hydrolyzes fatty acids with sufficient selectivity to generate soybean oil with 1-2% palmitate. Standard soybean oil contains 11-12% palmitic acid. This enzyme also dramatically reduces palmitate in alternate oil sources (canola oil, high oleic sunflower oil). It is envisioned that the other oil types would generate similar fatty acid profiles. The hydrolyzed fatty acids may then be removed and used in alternate, non-food related applications.

C. Absence of Production Organism in Enzyme Preparation

Per requirements of the product specifications for BD29241 Palmitase enzyme 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. During enzyme release, most of the production organism is inactivated in the post fermentation, chemical treatment. Cell debris and any remaining cells are removed during the cell separation step. The polish 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. fluorescens* in the final product. In the testing method (done pursuant to Verenium SOP QC0214), a 0.1 mL sample of the final product is streaked on two agar plate medium and cultivated for 72 hours to isolate any individual bacterial colonies. If no bacterial colonies are observed, then the product is deemed to be free of any production organisms at a detection level of 10 cfu/mL. If bacterial colonies are observed,

then they are examined for the production of a fluorescent pigment under UV light. *P. fluorescens* is known to produce fluorescent, diffusible pigments (Holt, J.G. *et al.*, 1994).

D. Absence of Transformable DNA in the Enzyme Preparation

The plasmid pDOW1169_BD29241 is a derivative of pMYC5088. The plasmid pMYC5088 has been demonstrated to be incapable of self-transmission and is poorly mobilizable (GRAS Notice No. GRN000126, 2003). Like its parent pMYC5088, the plasmid pDOW1169_BD29241 was engineered to be defective in plasmid mobility by mutating the *mob* genes. DNA sequence analysis was done to characterize the mobilization potential of the plasmid. To further confirm that pDOW1169_BD29241 is poorly mobilizable, even in the presence of a helper plasmid, the conjugation frequencies were evaluated of pDOW1169_BD29241 from the BD29241 production strain to *P. fluorescens* and to *Escherichia coli*. Verenium has confirmed that plasmid pDOW1169_BD29241 is incapable of self-transmission and is poorly mobilizable. In addition, it has been demonstrated that the plasmid pDOW1169_BD29241 has poor transferability into other bacterial strains, like *E. coli*. Therefore, the BD29241 palmitase enzyme preparation is considered to be free of any transformable DNA.

E. Absence of Antibiotic Resistance Gene in the Production Strain

The expression vector pDOW1169_BD29241 was constructed by subcloning the BD29241 Palmitase gene into the expression vector pDOW1169. The pDOW1169 vector was derived from pMYC5088 (GRAS Notice No. GRN000126, 2003), which contains antibiotic resistance markers (ARMs) for tetracycline. However, those ARMs, the *tetA* and *tetR* genes, were removed by restriction digest during the construction of pDOW1169. The pDOW1169 expression vector is considered as an ARM-free vector. Consequently, the pDOW1169_BD29241 is also considered as an ARM-free vector. This was verified by bioinformatics analysis of the expression vector of pDOW1169_BD29241. During this sequence analysis, a fragment, which codes for a 57 amino acid long peptide, was identified. The N-terminal 46 amino acids of this peptide are identical to the N-terminus of the 267 amino acid long streptomycin phosphotransferase (StrA) protein (UniPort, Accessed 7/17/2012). It is only a

small portion of the *StrA* gene. Additionally, for a strain to be streptomycin resistance, it requires both StrA and StrB proteins to be expressed. Therefore, the expression vector pDOW1169_BD29241 is considered to be free of antibiotic resistance genes.

Antibiotic resistance markers were also used in the construction of the recipient microorganism, *P. fluorescens* strain DC454. Plasmids that contain either kanamycin resistance gene (for *pyrF* deletion) or tetracycline resistance genes (for $lacI^{Q1}$ insertion) were used. All of the antibiotic resistance genes mentioned above were removed during the counter selection process. Removal of the ARMs was demonstrated by genome sequencing. Therefore, the recipient microorganism, strain DC454, is considered to be free of antibiotic resistance genes.

Since expression vector, pDOW1169_BD29241, and the recipient microorganism, strain DC454, are both free of ARMs, the production strain, BD29241, is free of antibiotic resistance genes. Furthermore, the BD29241 Palmitase enzyme preparation is tested for absence of antimicrobial activity according to the recommendation from the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

F. Applications and Residue Measurements

1. Foods in which used and purpose of use

The lipase enzyme preparation that is the subject of this GRAS Notification, referred to as BD29241 Palmitase, is intended for use as a processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils for use in food. The fatty acid profile of this modified oil would be that of a higher quality oil, conferring health benefits of reduced saturated fatty acids.

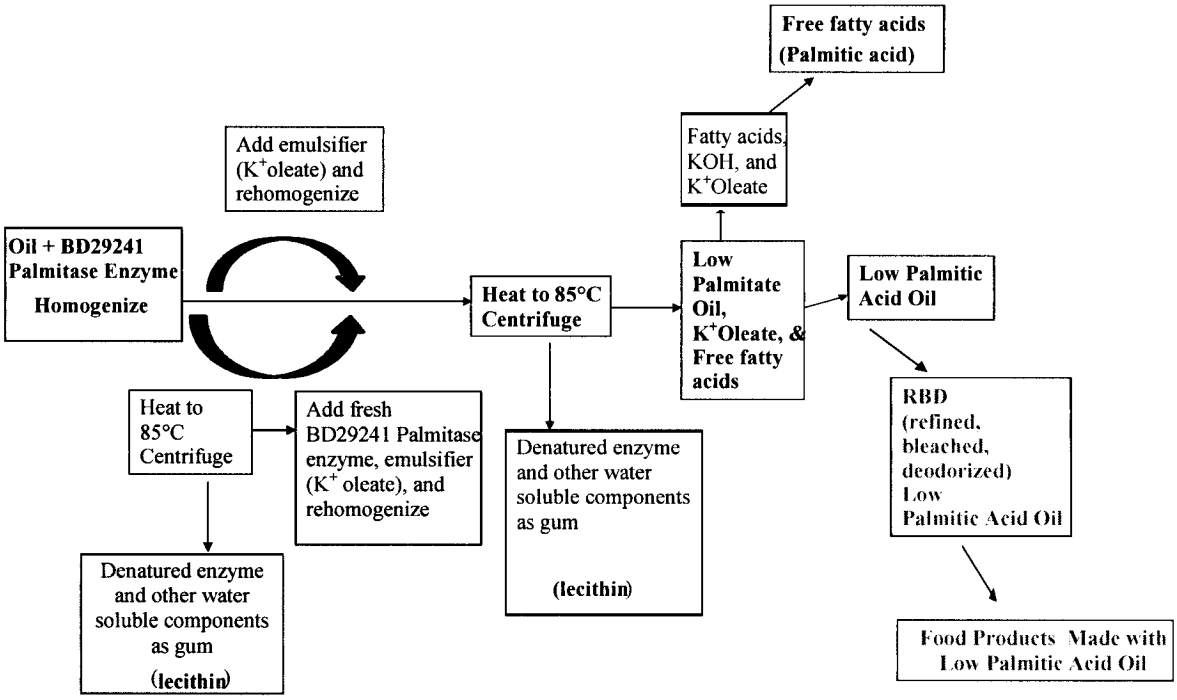
Refining is a general term used by the industry to describe the process whereby crude vegetable oil is converted to a purified final product by removing non-triacylgylcerol components that can negatively impact quality of the oil and make the oil unsuitable for particular applications. (See Figure 4). Refining may be conducted as a series of batch processes, but most modern oil refineries utilize continuous processes.

Figure 4. Oil refining process

There are generally two types of refining – chemical and physical. BD29241 Palmitase enzyme product is used only in physical refining. 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, the oil is degummed using water and acid with the option to use commercially available enzymes, such as phospholipase C and phospholipase A, A1, and A2. The degummed oil is then bleached, where 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 contaminating free fatty acids are then removed, together with other unwanted volatiles, during a deodorization step. After the deodorization step, the oil is considered refined.

To reduce palmitic acid levels in soybean oil and other triacylglycerol-based oils, BD29241 Palmitase is used during the physical refining process. BD29241 Palmitase is added to triacylglycerol-based oil. An emulsion is generated by high shear mixing. The enzyme reaction continues with mixing at the temperature appropriate for the triacylglycerol source, between 22°C and 40°C. The oil can be heated to 85°C, followed by centrifugation, cooling, and filtration. If the optional heating step is performed, potassium oleate and additional BD29241 Palmitase are added to replace the removed enzyme. The oil-enzyme-oleate mixture is emulsified by high shear mixing and incubated. The mixture is degummed; phospholipase enzymes can be added prior to heating to 85°C (breaking the emulsion and denaturing the enzyme) and centrifugation. The low palmitic acid oil is separated during this degumming process from the denatured protein, heavy phase, and water. Additional steps (e.g., bleaching, deodorization, etc.) separate the free fatty acids from the oil and further refine the low palmitic acid oil for commercial use. This RBD (refined, bleached and deodorized) low palmitic acid oil may be sold. (See Figure 5 below.)

Figure 5. Triacylglycerol-based oil refining process using BD29241 Palmitase

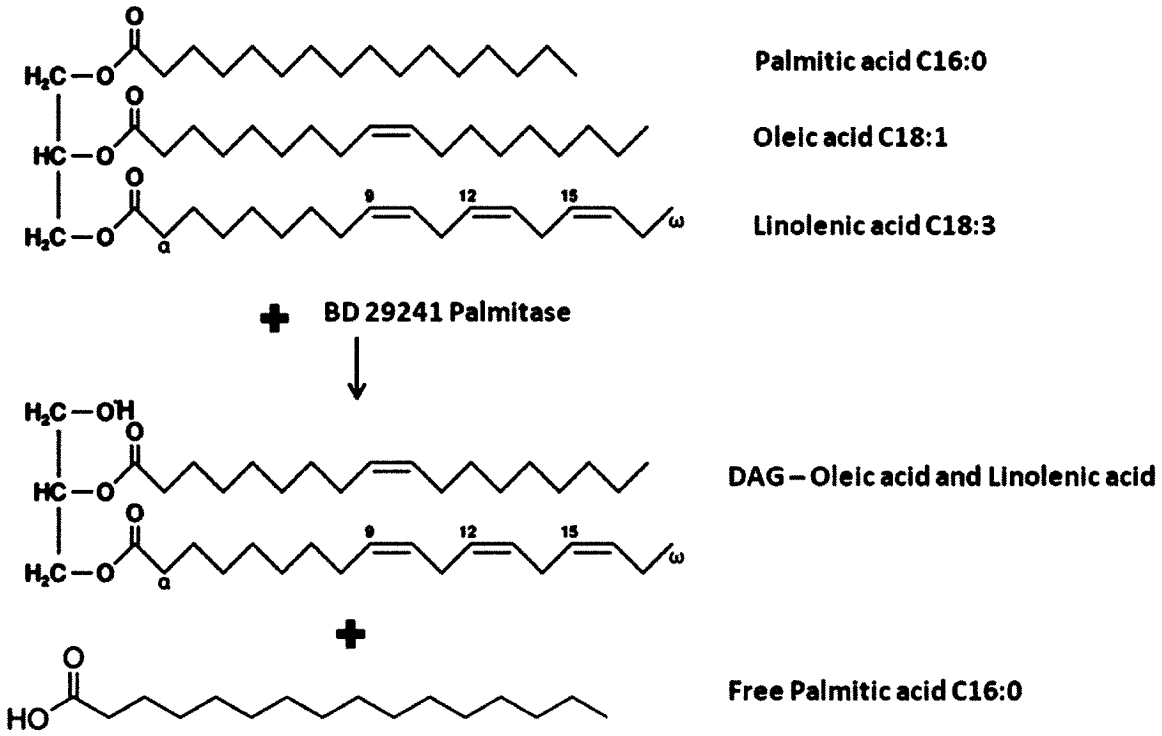


Black = main process, Blue = optional process, Green = low palmitic acid oil end products

2. Mode of action

BD29241 Palmitase enzyme is a lipase that is selective for palmitic acid, a 16-carbon saturated fatty acid. Due to the high selectivity of this lipase for palmitic acid, BD29241 Palmitase may most accurately be described as a palmitase enzyme. This palmitase enzyme cleaves the ester bond releasing fatty acids from the triacylglycerol yielding a diacylglycerol and a free fatty acid (palmitic acid also known as palmitic fatty acid). The selectivity of BD29241 Palmitase enzyme is illustrated below in Figure 6.

Figure 6. Hydrolysis of a triacylglycerol when using BD29241 Palmitase



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 50 to 100 kilograms of formulated enzyme product per metric ton of oil, depending on the oil to be treated and the treatment time.

4. Enzyme residues in food ingredients

BD29241 Palmitase is intended for use in the beginning of the triacylglycerol oil refining process. The BD29241 Palmitase is added to crude oil with water to create a water-in-oil emulsion. BD29241 Palmitase, 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 heavy phase, containing denatured BD29241 Palmitase enzyme residue and other water soluble components as gums, is separated from the oil by centrifugal separation. Subsequent steps in refining remove any remaining

enzyme residues from the refined oil. 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=60 ppb for soybean oil) of available analytical methodology.

G. Pre-clinical Safety Testing

Various safety tests – as described in detail below – were performed. All such testing and the results were reported in *Safety Evaluation of a Lipase Enzyme (BD29241 Palmitase) preparation, expressed in Pseudomonas fluorescens, Intended for Removing Palmitic Acid from Triacylglycerol* by Halich *et al.*, 2012[3] (see Appendix A).

1. Test article production

The test article used to determine the safety of BD29241 Palmitase 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 Verenium SOPs were used for the production of the test article: MP0320 for the fermentation protocol, MP0321 for the recovery protocol, MP0322 for the lyophilization protocol, and MP0323 for the milling and blending of BD29241 Palmitase powder. 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*, 8th Edition (U.S.Pharmacopeial Convention, 2012), and the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, Accessed 9/24/2012). The test article for BD29241 Palmitase was designated as BD29241 (Palmitase) (and used in all of the safety studies discussed below).

2. Genotoxicity studies

BD29241 (Palmitase) (i.e., test article name given to BD29241 Palmitase) has been tested (by independent testing laboratories) to investigate its genotoxic potential in a range of *in vitro*

[3] Please note the 14-day dose range-finding oral toxicity study in rats was not published in Halich *et al.*, 2012. The testing and results from this study are included in this submission and are described below in Section V.G.3.b.

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 Appendices D, E, and F.

Table 2. Results of genotoxicity studies

Test System	Test Object	Concentration of BD29241 (Palmitase)	Results
Chromosomal aberrations in human lymphocytes*	Cultured human peripheral blood lymphocytes	0-5000 μg/mL	Negative
Mouse micronucleus assay	CD®-1 mouse bone marrow	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 test article BD29241 (Palmitase) was also evaluated via three different toxicology protocols – each of which demonstrates that BD29241 Palmitase 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 assess the acute toxicity of the BD29241 (Palmitase) when administered to rats as a single dose by oral gavage (Chelius, M. *et al.*, 1994). This 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 was evaluated daily. Clinical observations were recorded prior to dosing, as well as at 30 minutes, 4 hours post-dose, and daily thereafter through Day 15. Body weights were recorded on the day of dosing (Day 1), and on Days 8 and 15.

For the dose of 2000 mg/kg, no mortality was observed. All animals appeared normal throughout the study at 2000 mg/kg. No biologically significant effect was seen on body weights on Days 8 and 15. Terminal necropsy revealed no visible lesions in any of the animals at 2000 mg/kg.

Based on the results of this study, the oral LD_{50} for the test article in rats was estimated 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 BD29241 (Palmitase) when administered orally, via gavage, once daily to Sprague Dawley rats for 14 consecutive days (Gad, S.C., 1995; Speid, L.H. *et al.*, 1990). This 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 five male and five female Sprague Dawley rats were administered 0, 500, 1000, and 2000 mg/kg of BD29241 Palmitase. The dose volume for all groups was 10 mL/kg.

Mortality/morbidity was performed twice daily. Clinical observations were evaluated prior to dose administration and approximately 1-2 hours following dosing. Animals were observed once prior to sacrifice on Day 15. Body weights were recorded at the time of randomization/selection, prior to administration on Days 1 and 8, and following the final dose on

Day 14. A fasted body weight was collected prior to sacrifice on Day 15. Food consumption was recorded weekly. Blood for evaluation of hematology, coagulation, and clinical chemistry was collected from animals prior to terminal sacrifice on Day 15. Selected tissues were harvested at necropsy and selected organs weighed.

There were no early deaths during the study. There were no test article-related clinical signs of toxicity noted during the 14 day treatment period. There were no test article-related effects on body weights, body weight gains, or food consumption noted during the study.

There were no test article-related effects on hematology, coagulation, or clinical chemistry parameters or erythrocyte morphology as determined on Day 15. There were no gross findings noted at necropsy. There were no test article-related changes in absolute or relative to body- or brain-weight ratios.

Based on the results of this study, the No Observed Effect Level (NOEL) following the administrations of 500, 1000, or 2000 mg/kg BD29241 (Palmitase) once daily by oral gavage for 14 days to Sprague Dawley rats is at least 2000 mg/kg. (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 BD29241 (Palmitase) when administered orally, via gavage, once daily to Sprague Dawley rats for 90 consecutive days (FDA, Accessed 9/18/2012; Gad, S.C., 1995; Speid, L.H. *et al.*, 1990). This 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 BD29241 (Palmitase). The dose volume for all groups was 10 mL/kg.

Animals were dosed once daily for 90 days. Mortality/morbidity was performed twice daily on Days 1 to Day 90 and prior to sacrifice on Day 91. Animals were observed daily prior to dose administration and at approximately 1–2 h following dosing. Animals were observed once prior to sacrifice on Day 91. Body weights were recorded at the time of randomization/selection, prior to dose administration on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64,

71, 78, 85, and following the final dose on Day 90. A fasted body weight was collected prior to sacrifice on Day 91. Food consumption was recorded weekly. Ophthalmology examinations were performed prior to treatment initiation and during the final week of treatment. Blood for evaluation of hematology, coagulation and clinical chemistry was collected on Day 91. Selected tissues were harvested at necropsy, selected organs weighed, and selected tissues for all rats were evaluated microscopically in Group 1 and Group 4 and for all animals that died.

There was no test article-related mortality or morbidity on this study. There were no test article-related clinical signs of toxicity noted during the 90-day treatment period.

There were no test article-related effects on body weight or food consumption. There were no test article-related ophthalmological findings noted during the study.

There were no test article-related effects on hematology, coagulation or clinical chemistry parameters or erythrocyte morphology.

There were no test article-related gross findings noted at necropsy. There were no definitive test article-related changes in absolute or relative to body- or brain-weight ratios. The test article, at 2000 mg/kg/day, had no adverse effects in any of the tissues examined microscopically.

Based on the findings in this study, the NOEL following administration of 500, 1000, or 2000 mg/kg/day of BD29241 (Palmitase) once daily by oral gavage for 90 days is ≥2000 mg/kg. (See Appendix I for further details).

4. Worker safety studies

Test article BD29241 (Palmitase) 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 BD29241 (Palmitase)	Results
Acute, nose-only, inhalation toxicity	Sprague Dawley rats (5 males and 5 females)	4.58 mg/L	LC_{50} >4.58 mg/L
Primary eye irritation	New Zealand White rabbits (3 females)	2%	Non-irritating
Primary dermal irritation	New Zealand White rabbits (3 males)	2%	Non-irritating
Delayed contact hypersensitivity (Buehler method)	Guinea pigs (10 males and 10 females)	2%	Does not elicit a delayed contact hypersensitivity

As the above-described studies indicate, BD29241 (Palmitase) 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

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.

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

Reference No.	Substance	Intended Use	Year of Authorization
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 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	2002

Reference No.	Substance	Intended Use	Year of Authorization
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 an enzyme to hydrolyze soy and egg yolk lechithins	2004
GRN 000158	lipase	Use as an enzyme in the production of triglyceride products	2005
GRN 000204	phospholipase C	Use as an enzyme in degumming vegetable oils for food use	2006
GRN 000212	phospholipase A2	Use as an enzyme to hydrolyze soy lecithin and egg yolk lecithin	2007
GRN 000216	lipase	Use as an enzyme to produce tailored triglycerides intended for use in infant formulas	2007

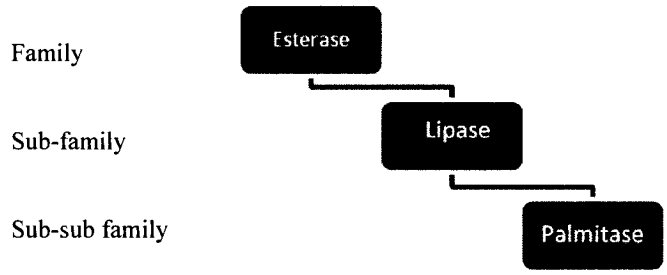
Reference No.	Substance	Intended Use	Year of Authorization
GRN 000238	lipase	Use as an enzyme to modify lipids in dough for bakery and noodle applications, in egg yolks for bakery applications and mayonnaise production, and in the refining process of soy bean oil	2008
GRN 000296	lipase	Use in baked goods to enhance the gas holding capacity of the dough, leading to an increased stability of the dough upon proofing	2009

3. Relationship to other lipases

As discussed above, members of the esterase and lipase families have a long history of safe use in food. Lipase enzymes, including our BD29241 Palmitase enzyme, use a catalytic triad to cleave the ester linkage of a fatty acid from triacylglyceride. Other commercial lipases share these catalytic triads and this enzyme function. Lipases are a subfamily of esterases, and palmitases are a subfamily of lipases. BD29241 enzyme is a palmitase and therefore also in the subfamily of lipases.

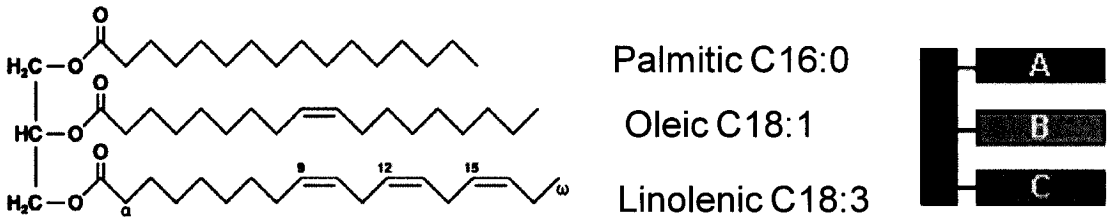
From a functional perspective, these enzymes can be classified as shown in Figure 7.

Figure 7. Functional relationship of esterases, lipases, and palmitase



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). In cleaving the ester bond, the fatty acid from the triacylglyceride is released, yielding a diacylglyceride and a fatty acid. The palmitase enzyme has a preference for the shorter 16 carbon chain palmitic acid than the 18 carbon fatty acids. Thus, BD29241 Palmitase, is a lipase that displays a preference for cleaving C16:0 over C18:0, C18:1, C18:2, or C18:3.

Figure 8. Specificity of cleavage sites for lipases and palmitases



Triglycerides may be visualized by the structure on the left or the simplified representation on the right. The structure on the left is comprised of a palmitic acid, oleic acid, and linolenic acid as indicated beside the structure. Palmitic acid is 16 carbons with no double bonds. Oleic acid is 18 carbons with one double bond. Linolenic acid is 18 carbons with three double bonds.

BD29241 Palmitase enzyme preparation is used as a processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils (where oil refers to triacylglycerols in a liquid or semi-liquid state, such as soybean oil at 22°C or lard at

30°C). Many lipases have been approved as processing aids in food applications, including for the treatment of vegetable oil.

Lipases are used as food processing aids to generate dietary ingredients, alter the physical or nutritional composition of fats and oils, and aid in other food and industrial applications. BD29241 Palmitase is a lipase that preferentially cleaves palmitic acid, thereby generating fats and oils with altered physical and nutritional compositions in a similar commercial space. As noted by Kessler *et al.* 1992 in discussing enzymes derived from microbial sources, "(g)enerally, 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). FDA has approved a number of lipases for use in food. A comparison of BD29241 Palmitase enzyme preparation and several commercially available lipases is provided in Table 5.

Table 5. Comparison of microbial lipases

Property	**BD29241 Palmitase**	**GRN 000068**	**GRN 000081**	**GRN 0000158**
Enzyme Class	Lipase	Lipase	Lipase	Lipase
Mode of Action: Hydrolyze fatty acid from triacylglcerol	Yes	Yes	Yes esterification	Yes carboxylesterase
Commercial Enzyme	Focus of this submission	Yes Lipase G Amano50 (Amano)	Yes Lipase AY (Amano)	Yes Lipozyme CALBL (Novozymes)
Approved Food Use	Focus of this submission	Dietary ingredients from fats and oils	Dietary ingredients from fats and oils	Triglyceride products
Application includes free fatty acid generation	Yes	Yes	Yes	Yes
Production Organism	*P. fluorescens*	*Penicillium camembertii*	*Canadia Rugosa*	*Aspergillus niger*

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 lipase 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 Verenium is not aware of any allergic reactions caused by ingestion of palmitase, the allergenic potential of the protein should be assessed (FAO/WHO, 2001; FAO/WHO Codex Alimentarius Commission, 2009; Ladics, G.S. *et al.*, 2011). 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).

As suggested by the Joint FAO/WHO Expert Consultation, amino acid sequence homology searches comparing the structure of the newly expressed proteins and the stepwise, contiguous, identical amino acid segments with all known allergens is an approach for the assessment of allergenic potential (FAO/WHO Codex Alimentarius Commission, 2009). Two

such searches were conducted using BD29241 Palmitase protein as the query sequence. A FASTA search to predict overall structural similarities and a search scanning each possible 80 amino acid segment (1-80, 2-81, 3-82, etc.) looking for matches of at least 35% identity were performed against the Food Allergy Research and Resource Program (FAARP) database. The FASTA search results demonstrated that the BD29241 Palmitase protein does not have any significant homology to the allergens in the database. The scan of each possible 80 amino acid segment showed that there were no cases where the homology exceeded 35% identity. This demonstrates that the BD29241 Palmitase protein shares no significant amino acid homology with known protein allergens that are present in the current release of the FAARP database. Based on this analysis, allergenicity (via the oral route) is not indicated to occur when using this enzyme.

B. Production Microorganism

This section addresses the safety of *P. fluorescens* strain DC454, which was used as the recipient organism resulting in the BD29241 Palmitase production strain P. fluorescens strain, BD29241.

1. History of safe use

P. fluorescens is a common saprophyte and potential plant pathogen that inhabits plant rhizosphere and phyllosphere environments (OECD, Accessed 3/1/2012b). The microorganism has been used in a variety of industrial applications (Warren, G.J., 1987; Wilson, M. *et al.*, 1993), to produce biological pesticides (Chew, L. *et al.*, 2005; Herrera, G. *et al.*, 1994), and in the control of diseases in the phyllosphere of plants (Wilson, M. *et al.*, 1993). The U.S. EPA established an exemption from the requirement of tolerance for residues of *P. fluorescens* in or on the raw agricultural commodity mushrooms (EPA, 1994). More recently, the U.S. EPA issued an exemption from the requirements of tolerance for residues of *P. fluorescens* strain CL145A, which is a Biovar I strain, in or on all food commodities when applied as a molluscicide (EPA, 2011).

Additionally, a derivative of *P. fluorescens* Biovar I, strain MB101 has been reviewed by a GRAS Expert Panel and by the U.S. FDA and was found to be a safe microorganism for the production of an alpha-amylase enzyme used in food production (GRAS Notice No. GRN000126, 2003). The French Food Safety Authority (AFSSA) also evaluated the above-mentioned microbial derivative and issued an opinion letter (AFSSA, Accessed 9/20/2012) concluding that the AFSSA "considers that the use of alpha-amylase produced by the genetically-modified strain of *Pseudomonas fluorescens* Biovar I presents no health risk for the consumer, under the conditions of use presented by the applicant."

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. A review of literature shows that *P. fluorescens* is generally considered a nonpathogenic and notoxigenic species to humans (OECD, Accessed 3/1/2012b). *P. fluorescens* has been shown to be an opportunistic pathogen in patients who are severely immunocompromised, but is of little concern to immunocompetent individuals (Chamberland, S. *et al.*, 1992; OECD, Accessed 3/1/2012b). Fluorescent pseudomonae have not been reported to be potent allergens; however, they do possess a lipopolysaccharide that may cause an allergic response in some individuals (OECD, Accessed 3/1/2012b). In addition, published studies have evaluated the pathogenicity and toxigenicity of *P. fluorescens* in mice; no evidence of pathogenicity or toxigenicity was seen under the conditions of the test (George, S.E. *et al.*, 1999; George, S.E. *et al.*, 2000). A pathogenicity evaluation of *P. fluorescens* Biovar I, strain MB101 demonstrated that the strain was avirulent after oral administration to mice and does not represent an infectious threat to humans (Landry, T.D. *et al.*, 2003). Furthermore, toxicology and genotoxicity tests we conducted using an alpha-amylase enzyme preparation produced by *P. fluorescens* determined that the material does not contain toxic or genotoxic substances (Landry, T.D. *et al.*, 2003). Lastly, results from toxicology and genotoxicity tests of BD29241 Palmitase produced by *P. fluorescens* 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 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 (Halich, R. *et al.*, 2012).

The next question asks if the test article is free of transferable antibiotic resistance gene DNA. *P. fluorescens* BD29241 does not contain any antibiotic resistance genes, which has been confirmed by bioinformatics analysis and genomic sequencing (see Section V.E.). Additionally, no detectable antimicrobial activity was found in the BD29241 Palmitase enzyme preparation (see Composition and Specifications, Section III.D). Furthermore, the expression plasmid pDOW1169_BD29241 is poorly mobilizable (see Section V.D.). For these reasons, enzyme preparation made from *P. fluorescens* production strain BD29241 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 sequences of the introduced DNA, expression vector pDOW1169_BD29241 and $lacI^{Q1}$, are known and their gene products are also known. Bioinformatics analysis was conducted on the expression vector. It was not found to contain genes that code for products that are homologous to known toxins or harmful factors. Bioinformatics analysis was also conducted on $lacI^{Q1}$ and its junction region and at its integration location on the chromosome. None of the putative ORFs generated fortuitously, and

none of the ORFs within the *lacI*^{Q1} expression cassette encoded any toxins or harmful factors. In addition, the allergenic potential assessment results for the BD29241 Palmitase protein and the toxicity study results using the test article for BD29241 Palmitase demonstrate that the introduced DNA is free of attributes that would render it unsafe for the proposed 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. *LacI*^{Q1} was integrated into the chromosome. The integration was targeted near the levansurcrase locus. The sequence bordering the integration site of was determined and showed that the *lacI*^{Q1} was integrated into the chromosome near the *lsc* locus in the recipient strain DC454. The expression vector introduced into strain BD29241 is a self-replicating, extrachromosomal plasmid and thus is not likely to be integrated into the chromosome. Therefore, there is limited possibility for random integration into the chromosome.

The answers to the above questions indicate 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 (BD29241 Palmitase) have been met. This is illustrated in Figure 9 below.

Figure 9. Pariza and Johnson Decision Tree

The following analysis is based on the 2001 Pariza and Johnson Decision Tree (Pariza, M.W. *et al.*, 2001). Decision points that do not pertain are included for completeness but crossed out.

1. Is the production strain genetically modified?
If yes, go to 2. If no, go to 6. **YES**

2. Is the production strain modified using rDNA techniques?
If yes, go to 3. If no, go to 5. **YES**

3. Issues relating to the introduced DNA are addressed in 3a–3e.

3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food? **No. Lipases are present in the food supply but there is no documented history of safe use for this specific palmitase, which is of unknown origin.**
If yes, go to 3c. If no, go to 3b

3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety? **YES**
If yes, go to 3c. If no, go to 12.

3c. Is the test article free of transferable antibiotic resistance gene DNA?
If yes, go to 3e. If no, go to 3d. **YES**

~~3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.~~

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3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?
If yes, go to 4. If no, go to 12. **YES**

4. Is the introduced DNA randomly integrated into the chromosome?
If yes, go to 5. If no, go to 6. **NO**

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
If yes, go to 6. If no, go to 7. **YES**

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **No, but another strain in this lineage (derived from the same parental strain) is used to produce food grade alpha-amylase; FDA had no questions regarding the conclusion that the uses of this enzyme are GRAS (GRN 000126).**
If yes, the test article is ACCEPTED. If no, go to 7.

7. Is the organism nonpathogenic?
If yes, go to 8. If no, go to 12. **YES**

8. Is the test article free of antibiotics?
If yes, go to 9. If no, go to 12. **YES**

9. Is the test article free of oral toxins known to be produced by other members of the same species?
If yes, go to 11. If no, go to 10. **YES**

~~10. Are the amounts of such toxins in the test article below levels of concern?~~
~~If yes, go to 11. If no, go to 12.~~

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety? **YES**
If yes, the test article is **ACCEPTED**.

~~12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.~~

C. Enzyme Product

1. Safety studies

The results of the safety studies for BD29241 (Palmitase) - as outlined in Section V.G and published in Halich *et al.* 2012 (see Appendix A) - show that the BD29241 Palmitase enzyme preparation is safe under its intended conditions of use. The NOEL derived from the 90-day oral toxicity study in rats is approximately 2442 higher than the conservative estimate of the total daily human consumption of BD29241 Palmitase of 0.819 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. Triacylglycerol-based oil

As mentioned in Section V.F.4, the process for refining triacylglycerol-based oil includes several steps that inactivate and denature the enzyme. After the reaction has been completed the heavy phase, containing denatured BD29241 Palmitase enzyme residue and other water soluble components as gums, is separated from the oil by centrifugal separation. Subsequent steps in

refining, including 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=60 ppb) of the available, highly-sensitive analytical method. (For the amount of the residue appearing at each step of the soybean oil refining process see Figure 10). 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 BD29241 Palmitase residues in vegetable oil, phospholipid-containing gums and other samples.

Figure 10. Amount of BD29241 Palmitase residues (determined by ELISA) during the soybean oil refining process

	Oil After 1st Enzyme Treatment	Oil After 2nd Enzyme Treatment	Degummed Oil	Final Oil (RBD)
AVE	1.23 ppm	21.7 ppm	1.05 ppm	<60 ppb

Soybean oil treated with the highest recommended use rate of the active BD29241 Palmitase enzyme (i.e., 506 ppm enzyme). Please note that 100,000 ppm of formulated enzyme product contains 506 ppm of active BD29241 Palmitase enzyme.

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 is 100,000 ppm formulated enzyme product, which contains 506 ppm[4] of the BD29241 Palmitase enzyme. Using the highest recommended use rate would result in a maximum estimated daily intake (EDI) of 0.819 mg enzyme/kg/day or 3.538 mg TOS[5]/kg/day assuming the

[4] Please note that 506 ppm BD29241 Palmitase enzyme is contained in 100,000 ppm formulated enzyme product. For its use in the maximum EDI calculation, 506 ppm = 506 mg/kg.

[5] 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

average per capita daily consumption of vegetable oils and fats for an average person (i.e., weighing 60 kg) is 97.1 g/person/day (USDA-Economic Research Service, Accessed 9/16/2011)[6].

Maximum EDI Calculation:

$$0.819 \text{ mg BD29241 Palmitase/kg/day} = 506 \text{ mg/kg BD29241 Palmitase} \times \frac{97.1 \text{ g/person/ day}}{1000 \text{ mg/g}} \div 60 \text{ kg}$$

To provide the calculation in terms of total organic solids (TOS), the percent TOS for the BD29241 Palmitase test article (TA) is used. The TOS for the BD29241 Palmitase test article is 83.4%. Using a specific activity of approximately 198 U/mg for a highly purified BD29241 Palmitase sample and an activity of 38.32 U/mg for the BD29241 (Palmitase) (test article), BD29241 Palmitase represents 19.4% of the test article. Therefore, in order to achieve a 506 ppm dose (i.e., 506 mg/kg) of BD29241 Palmitase, 2608 ppm (i.e., 2608 mg/kg) test article would be used (506 mg/kg ÷ 0.194) in the calculation below.

$$3.5 \text{ mg TOS/kg/day} = 2608 \text{ mg/kg BD29241 Palmitase TA} \times \frac{97.1 \text{ g/person/day}}{1000 \text{ mg/g}} \div 60 \text{ kg} \times 0.834 \text{ TOS}$$

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 (10 mL of a 200 mg/mL solution), which

[6] Please note that the figure 97.1 g/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. The most conservative estimate of 97.1 g/person/day was calculated from the 2009 per capita consumption of 78.1 pounds per annum of vegetable-derived added oils and fats on a total fat content basis.

corresponds to 1,668 mg TOS/kg/day (2000 mg x 0.834) since the percent TOS for BD29241 (Palmitase) is 83.4%.

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

$$\frac{2{,}000 \text{ mg/kg/day}}{0.819 \text{ mg/kg/day}} = 2.442 \times 10^3 \quad \text{OR} \quad \frac{1{,}668 \text{ mg TOS/kg/day}}{3.538 \text{ mg TOS/kg/day}} = 4.71 \times 10^2$$

A "worst case" safety margin of 2442 (471 if expressed as TOS) is, Verenium believes, sufficient to insure safe intended use of BD29241 Palmitase enzyme preparation.

A more realistic safety margin could also be calculated using the ELISA data for the refined oil from Figure 10. Assuming all the non-enzyme material from the enzyme preparation is removed during oil refining at the same rate at which the BD29241 Palmitase is removed, the maximum EDI and safety margin is calculated below using 60 ppb BD29241 Palmitase.

$$\frac{2{,}000 \text{ mg/kg/day}}{0.000097 \text{ mg/kg/day}} = 2.063 \times 10^7 \quad \text{OR} \quad \frac{1{,}668 \text{ mg TOS/kg/day}}{0.000417 \text{ mg TOS/kg/day}} = 4.048 \times 10^6$$

This more realistic safety margin of at least 4,048,000 is, Verenium believes, more than sufficient to insure safe intended use of BD29241 Palmitase enzyme preparation.

D. Manufacturing Process

As described in Section III.C, the BD29241 Palmitase 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 BD29241 enzyme preparation is manufactured in accordance with both current Good Manufacturing Practices (cGMP) for food and the 1992 Organization for Economic Co-operation and Development's criteria for Good Industrial Large Scale Practice (GILSP) (OECD, Accessed 9/20/2012). The final preparation of BD29241 Palmitase meets the general and additional requirements for enzyme preparation of

Food Chemicals Codex and JECFA. Accordingly, the manufacturing process for BD29241 Palmitase should be deemed safe.

VII. Expert Panel

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VII. SCIENTIFIC EXPERT PANEL DETERMINATION

Verenium convened a panel of independent scientific experts – none of which currently serve or has served as a consultant to Verenium with regard to BD29241 Palmitase, 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. Martina Newell McGloughlin, D.SC., an internationally recognized authority on biotechnology who is currently an Associate Adjunct Professor at the University of California Davis, Department of Plant Pathology and the Director of University of California Systemwide Biotechnology Research and Education Program. Her personal research experience has been in the areas of disease resistance in plants, scale-up systems for industrial and pharmaceutical production in microbes and microbiological mining;

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

3. Michael W. Pariza, Ph.D. (**Panel Chair**), a food scientist and food microbiologist who is currently an Emeritus Professor of Food Science and Emeritus Director, Food Research Institute, University of Wisconsin – Madison. He served as faculty and Chair of the Department of Food Microbiology and Toxicology, as Director of the Food Research Institute, and for over 15 years held a Wisconsin Distinguished Professorship. Dr. Pariza is widely recognized as the founder of the modern field of conjugated linoleic acid (CLA) research. Dr. Pariza has received numerous academic honors and awards and is internationally recognized as an expert in evaluating food enzyme safety. He developed the principal guidelines for evaluating the safety of microbially-derived food enzymes that are used by government regulators and food enzyme manufacturers throughout the world.

(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 Verenium 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 BD29241 Palmitase enzyme preparation – is GRAS, based on scientific procedures, for its intended use. More specifically, the panel concluded:

Following its independent and critical evaluation of the information provided by Verenium, the Expert Panel members convened in person and via conference call and unanimously concluded that the proposed use (noted above) of BD29241 Palmitase, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is Generally Recognized as Safe (GRAS), based on scientific procedures and corroborated by a history of safe use of another *P. fluorescens* production strain from this *safe lineage* for food enzyme manufacture (GRAS Notice No. GRN000126, 2003). (Expert Panel Report, page 2; see Appendix Q).

VII. Conclusions

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VIII. CONCLUSIONS

The lipase enzyme preparation that is the subject of this GRAS Notification, referred to as BD29241 Palmitase, is derived from a genetically modified strain of *P. fluorescens* DC454 that contains an expression vector, pDOW1169_BD29241, which includes the BD29241 Palmitase gene.

Verenium has determined the BD29241 Palmitase enzyme preparation to be GRAS, through scientific procedures, for use as a food processing aid in the edible triacylglycerol-based 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 50 to 100 kilograms 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, Accessed 9/24/2012; International Food Biotechnology Council, 1990; OECD, Accessed 9/20/2012). 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 BD29241 Palmitase 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. *P. fluorescens* is well-characterized and complies with the OECD criteria for Good Industrial Large Scale Practice. *P. fluorescens* has been used in a variety of industrial applications (Chew, L.C. *et al.*, 2005; Herrera, G. *et al.*, 1994; Warren, G.J., 1987; Wilson, M. *et al.*, 1993). The U.S. EPA established an exemption from the requirement of tolerance for residues of *P. fluorescens* in or on the raw agricultural commodity mushrooms (EPA, 1994). More recently, the U.S. EPA issued an

exemption from the requirements of tolerance for residues of *P. fluorescens* strain CL145A, which is a Biovar I strain, in or on all food commodities when applied as a molluscicide (EPA, 2011). Furthermore, a derivative of *P. fluorescens* Biovar I, strain MB101 has been reviewed by a GRAS Expert Panel and by the U.S. FDA and was found to be a safe microorganism for the production of an alpha-amylase enzyme used in food production (GRAS Notice No. GRN000126, 2003). The French Food Safety Authority (AFSSA) also evaluated the above-mentioned microbial derivative and issued an opinion letter (AFSSA, Accessed 9/20/2012) concluding that the AFSSA "considers that the use of alpha-amylase produced by the genetically-modified strain of *Pseudomonas fluorescens* Biovar I presents no health risk for the consumer, under the conditions of use presented by the applicant."

The introduced DNA is well-characterized and shown to be safe. The production organism BD29241 is free of antibiotic resistance markers as confirmed by bioinformatics analysis. DNA from the lipase active discovery clones was sequenced, and the DNA sequence information was analyzed using in-house gene identification software. The predicted lipase open-reading frame was then manually curated to verify the software prediction. The lipase gene was isolated from all extraneous DNA using polymerase chain reaction (PCR) to specifically amplify the DNA, which encodes the lipase open reading frame, from the discovery clone. The toxicity studies performed using BD29241 Palmitase test article 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 (Jaeger, K.E. *et al.*, 2002) and have been 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). Many GRAS lipases are commercially available, several of which are protein engineered. The BD29241 Palmitase from *P. fluorescens* strain BD29241 exhibits functional and biochemical properties that are substantially equivalent to other known microbial phospholipases used in food.

In assessing the safety of the BD29241 Palmitase preparation, Verenium 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 palmitase test preparations derived from the production microorganism. Safety margin calculations indicate the worst case potential human exposure to the BD29241 Palmitase enzyme preparation from the consumption of food made using the enzyme is negligible and well below the NOEL seen in the oral toxicity studies.

The manufacturing process used to make BD29241 Palmitase enzyme preparation employs a pure culture submerged fermentation of the *P. fluorescens* production strain, BD29241. 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 BD29241 Palmitase 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, Verenium and an Expert Panel conclude that the BD29241 Palmitase preparation derived from *P. fluorescens*, containing the pDOW1169_BD29241 expression vector that includes the BD29241 Palmitase gene, is GRAS under the intended conditions of use, as specified herein.

IX. References

IX. REFERENCES

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X. List of Appendices

X. LIST OF APPENDICES

A. Halich, R., Kline, K., Shanahan, D., and Ciofalo, V. *Safety evaluation of a lipase enzyme (BD29241 Palmitase) preparation, expressed in Pseudomonas fluorescens, intended for removing palmitic acid from triacylglycerol.* Regulatory Toxicology and Pharmacology 64, 87-94 (2012).

B. Composition of formulated (final product) and unformulated (test article) BD29241 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. Michael Pariza

O. *Curriculum Vitae* for Dr. Martina Newell-McGloughlin

P. *Curriculum Vitae* for Dr. Dharma Kodali

Q. Expert Panel's report

APPENDIX A
HALICH ET AL., 2012

Appendix A

Halich, R., Kline, K., Shanahan, D., and Ciofalo, V. *Safety evaluation of a lipase enzyme (BD29241 Palmitase) preparation, expressed in Pseudomonas fluorescens, intended for removing palmitic acid from triacylglycerol.* Regulatory Toxicology and Pharmacology 64, 87-94 (2012).

Pages 000096-000103 have been removed in accordance with copyright laws. The removed reference is:

Halich, R., Kline, K., Shanahan, D., and Ciofalo, V. *Safety evaluation of a lipase enzyme (BD29241 Palmitase) preparation, expressed in Pseudomonas fluorescens, intended for removing palmitic acid from triacylglycerol.* Regulatory Toxicology and Pharmacology 64, 87-94 (2012).

Shepherd, Lillian

From: Diane Shanahan <Diane.Shanahan@verenium.com>
Sent: Thursday, February 21, 2013 7:30 PM
To: Ramos-Valle, Moraima
Cc: Shepherd, Lillian
Subject: amended pages for Verenium GRN - BD29241 Palmitase
Attachments: Verenium GRN BD29241 palmitase.2.21.13pdf.pdf

Dear Moraima,
As we discussed this morning, please find attached a cover letter and the information you requested concerning our GRAS notice for BD29241 Palmitase.
Please confirm receipt and when everything is complete on your end so that I know that the submission has been filed.

I appreciate your help with this matter and I am available if anything else is needed.

Best regards,
Diane

Diane Shanahan
Senior Director, Regulatory Affairs
Verenium Corporation
3550 John Hopkins Court | San Diego | CA 92121

P: 858.431.8591 | F: 858.876.1857 | C: 858.774.7911
diane.shanahan@verenium.com

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800.523.2990

Feb. 21, 2013

Moraima Ramos-Valle
Office for Food Additive Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Verenium Corporation GRAS Notice – BD29241 Palmitase Enzyme

Dear Ms. Ramos,

Based on our conversation today, I have enclosed the modified version of pages 3 and 4. The modification on page 3, and at the bottom of this letter, includes my phone number.

The List of Appendices under tab X. does not need to be modified as it was meant to serve as a list of Appendices that could be provided to FDA upon request. We simply provided Appendix A from the list immediately to FDA because the published paper by "Halich et al." was referenced elsewhere in the GRAS notice and because the paper contains the published evaluation of safety of BD29241 Palmitase enzyme preparation.

After you have had an opportunity to review this information, if you should have any questions or need additional information, please contact me at 858-431-8591 or by email at diane.shanahan@verenium.com

Sincerely,

(b) (6)

Diane Shanahan

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FEB 22 2013
Division of
Biotechnology and
GRAS Notice Review

I. ADMINISTRATIVE INFORMATION

A. Claim Regarding GRAS Status

Verenium Corporation hereby notifies the FDA of the determination by Verenium that the BD29241 Palmitase enzyme preparation, produced from *P. fluorescens*, strain BD29241, expressing a gene encoding palmitase, is generally recognized as safe (GRAS), based on scientific procedures, when used as described below for use as an enzymatic processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils intended for use in food – at a recommended level of 50 to 100 kilograms of formulated enzyme preparation per metric ton of oil.

(b) (6)

2/21/2013

Date

/James Levine

Chief Executive Officer

Verenium Corporation

3550 John Hopkins Court

San Diego, CA 92121

B. Name and Address of Notifier

Diane Shanahan

Senior Director, Regulatory Affairs

Verenium Corporation

3550 John Hopkins Court

San Diego, CA 92121

diane.shanahan@verenium.com

Ph: 858-431-8591

C. Common or Usual Name of Subject Substance

Palmitase, a palmitic acid specific lipase (also referred to as BD29241 Palmitase) (see Section V.B.1 for a detailed description).

D. Conditions of Use

BD29241 Palmitase is intended for use as a processing aid for reducing palmitic acid levels in the production of refined soybean oil and other triacylglycerol-based oils intended for use in food. The enzyme preparation will be used at levels no higher than necessary to achieve the intended effect, generally in the range of 50 to 100 kilograms of formulated enzyme preparation per metric ton of oil, depending on the triacylglycerol-based oil being treated and the reaction conditions. This is equivalent to a concentration range of 50,000 to 100,000 parts per million (ppm) of formulated enzyme preparation.

E. Basis for GRAS Determination

The GRAS determinations for the subject enzyme preparation are 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 determination are available to the FDA for review and copying upon request at the office of Verenium Corporation, 3550 John Hopkins Ct., San Diego, CA or will be sent to FDA upon request.

SUBMISSION END