AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

Lysophospholipase from a genetically modified strain of *Trichoderma reesei*

AB ENZYMES GmbH

MAY 5, 2016

TABLE OF CONTENTS

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), AB Enzymes GmbH ("AB Enzymes") has determined that the lysophospholipase enzyme preparation from a genetically modified *Trichoderma reesei* strain is a GRAS substance for the intended applications based on scientific procedures and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, AB Enzymes' basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from harmful sequences.

Section 3 shows the enzymatic activity of the enzyme, along with comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 0. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for lysophospholipase. Section **Error! Reference source not found.** provides information on the mode of action, applications, and use levels of lysophospholipase and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that the lysophospholipase enzyme preparation from *T. reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1 Name and Address of Notifier

Notifier:

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Manufacturer:

Roal Oy[1]
Tykkimäentie 15
FIN-05200 Rajamäki
Finland

Person(s) Responsible for the Dossier:

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

[1] ROAL is a Joint Venture between Associated British Foods (UK) and Altia OY (Finland). Manufacturing and research and development activities are performed for AB Enzymes by ROAL Oy in Finland. ROAL coordinates its R&D activities independently while taking into account the market requirements reported by their sole distributor AB Enzymes GmbH.

1.2 Common or Usual Name of Substance

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. The enzyme is known as lysophospholipase (IUBM 3.1.1.5), also known as lecithinase B, lysolecithinase, phospholipase B.

1.3 Applicable Conditions of Use

For an enzyme to perform a technological function in the final food, certain conditions have to be met, such as the enzyme must be in its native, non-denatured form, and must be free to move, a substrate must be present and conditions such as pH, temperature and water content must be favourable for the particular enzyme.

1.4 Food Products Used in

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. This dossier is specifically submitted for use of lysophospholipase used in starch processing, ie. in the production of all kind of syrups (derived from wheat and corn/maize starches mainly).

1.5 Levels of Use

Application	Raw material (RM)	Maximal recommended use levels (mg TOS/kg RM)
Starch processing (production of syrups)	Starch (Wheat / corn)	Starch processing (production of syrups)

1.6 Purposes

In principle, the hydrolysis of lysophospholipids with the help of lysophospholipase can be of benefit in the processing of all vegetable based foods and food ingredients which naturally contain lysophospholipids.

This dossier is specifically submitted for the use of lysophospholipase in starch processing, i.e. in the production of all kind of syrups produced from starch, mainly wheat and maize/corn starches. Depending on the production process and the type of syrups to be produced, different enzymes are used (e.g. amylase, pullulanase) to degrade starch.

1.7 Technological Need

Lysophospholipds present in starch (mainly wheat starch) can form micelles which negatively affect the filtration rate of the starch hydrolysates (syrups). In addition, they are known to form a complex with amylase, leading to a formation of a cloud in the final syrup, thus affecting its characteritics.

Therefore, the benefits of the conversion of lysophospholipids with the help of lysophospholipase are listed below:

— Prevent the formation of lysophospholipid micelles

— Facilitate the separation of undesired components

— Improve filtration rate (better and faster filtration)

— Improve the characteristics (clearness) of the filtrate

— Improve the environmental impact and sustainability (energy saving due to the load mitigation and decreased production time)

The use of lysophospholipases in such process has been specifically approved for a number of years in a numerous EU countries and in the rest of the world – which together with the extensive use for decades, demonstrates the technological need of such food enzymes in this food process.

1.8 Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that the lysophospholipase enzyme preparation from a *Trichoderma reesei* strain object of this dossier, is GRAS for use in baking.

1.9 Availability of Information for FDA Review

A notification dossier providing a summary of the information that supports this GRAS determination is enclosed herein. The dossier includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration (FDA) for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request. Please direct all inquiries regarding this GRAS determination to:

Candice Cryne
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany
1-647-919-3964
Candice.cryne@abenzymes.com

2. PRODUCTION MICROORGANISM

2.1 Donor, Recipient Organism and Production Strain

The dossier concerns a lysophospholipase from genetically modified *Trichoderma reesei*. The *Trichoderma reesei* host strain is genetically modified to express an *Aspergillus nishimurae* lysophospholipase enzyme.

Name of the enzyme protein: **lysophospholipase**

Production strain: *Trichoderma reesei* RF7206

Donor:

The lysophospholipase gene, *lpl,* described in this application was isolated from a lambda EMBL3 genomic DNA library of the *Aspergillus nishimurae* (ex-*fumigatus*) strain RH3949, using a specific cDNA fragment as probe. The donor strain RH3949 was first identified as *Aspergillus fumigatus* and more recently as *Aspergillus nishimurae*. *Aspergillus fumigatus* RH3949 is an environmental isolate.

As the name *Aspergillus fumigatus* has been used in our publications, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used interchangeably in this dossier for the donor organism.

Aspergillus nishimurae belongs to the section Fumigati of *Aspergillus (Hong et al. 2008)*. The taxonomic lineage of *Aspergillus nishimurae* is shown below (according to http://www.uniprot.org/taxonomy/1220166):

Genus: *Aspergillus*

Species: *Aspergillus nishimurae*

Subspecies (if appropriate): not applicable

Generic name of the strain: RH3949

Previous or other name(s) (if applicable): *Aspergillus fumigatus*

A. fumigatus strains secrete multiple extracellular phospholipases (PL), including phospholipase A (PLA), B (PLB), C (PLC) and D (PLD). *Aspergillus fumigatus* is an ubiquitous filamentous fungus which plays an important role in natural environments in the aerobic decomposition of organic materials.

Several strains of *Aspergillus fumigatus* are known to be opportunistic pathogens afflicting primarily immunocompromised patients and the exoenzyme lysophospholipase has been considered as one of multiple possible pathogenicity factors. However, in the recent scientific literature it is discussed that lysophospholipase from environmental strains of *A. fumigatus* may be more important for growth in the environment than it is for clinical isolates growing in the body (Birch et al. 2004). According to Rementeria et al. (2005) "*there is no unique essential virulence factor for development of this fungus in the patient and its virulence appears to be under polygenetic control*". In another review article of Abad et al. (2010) the involvement of B-phospholipases (phospholipase, lysophospholipase and lysophospholipase transacylase) of *A. fumigatus* as virulence factor is almost excluded: "*Although these enzymes [phospholipases] have been considered virulence factors for other species such as Candida albicans or C. neoformans, in clinical isolates of A. fumigatus the production of B-phospholipases is lower than in environmental isolates, making unlikely, if not excluding, their involvement in the virulence of the fungus*."

Lysophospholipase from *A. fumigatus* is produced by the transformed strain *Trichoderma reesei* RF7206 under controlled conditions. After use of the enzyme as processing aid in the manufacture of glucose syrup the enzyme is inactivated by heat treatment. There will be no residual enzyme activity in foodstuffs. Accordingly, there will be no putative virulence activity from this enzyme in food.

Recipient Organism:

The recipient strain used for the genetic modifications in constructing RF7206 was *Trichoderma reesei* strain RF4847, a classical mutant deriving from the natural isolate *T. reesei* QM6a**.** This strain has been shown to be genetically stable.

The *T. reesei* recipient is a classical mutant strain originating from *T. reesei* QM6a. The identification of the strain as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands. It was identified based on the sequences of Internal Transcribed Spacer 1 and 2 and the 5.8S gene and Translation Elongation Factor 1α *(Kuhls et al. 1996)*.

T. reesei is an aerobic filamentous fungus (an ascomycete). It grows in mycelium form but starts to sporulate when cultivation conditions do not favor growth (e.g. due to lack of nutrients). *T. reesei* is a mesophilic organism which means that it prefers to grow at moderate temperatures. The cultures are typically fast growing at about 30° C (above 20°C and below 37°C). *T. reesei* prefers acidic to neutral pH (about 3.5 to 6) for growth. The colonies are at first transparent or white on agar media such as potato dextrose agar (PDA). The conidia are typically forming within one week of growth on agar in compact or loose tufts in shades of green. Sporulation is induced by daylight. Yellow pigment may be secreted into the agar by the growing fungal colonies, especially on PDA.

The taxonomic classification of the *T. reesei* is: *Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota*, Fungi, according to Index *Fungorum* database.

Roal Oy has been using *T. reesei* as an enzyme producer since the 1980´s without any safety problems. AB Enzymes GmbH filed a GRAS notice for pectin lyase enzyme preparation produced with *T. reesei* containing a gene from *Aspergillus niger* and FDA had no question and designated it as GRAS (Notice No. GRN 000032, *Appendix #1*). Further, AB Enzymes GmbH has submitted GRAS notices for enzymes produced with genetically engineered *T.reesei* strains, specifically GRN 000524 (phospholipase A2 enzyme preparation from *T. reesei* carrying a PLA2 gene from *Aspergillus fumigatus*), GRN 000566 (β-Mannanase enzyme preparation from a self-cloned *T. reesei*), GRN 000558 (pectin esterase enzyme preparation from *T. reesei* carrying a pectin esterase gene from *Aspergillus tubingensis)*, and GRN 000557 (polygalacturonase enzyme preparation from *T. reesei* strain expressing the gene encoding polygalacturonase from *Aspergillus tubingensis Mosseray* RH3544). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (e.g. cellulases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp

and paper industries). Currently, various *Trichoderma reesei* enzymes and enzymes produced in recombinant *T. reesei* strains are also used in the brewing process (β-glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases, hemicellulases), as a feed additive to livestock (xylanases, endoglucanases, phytases) and for pet food processing. *T. reesei* - wild type or genetically modified - is widely accepted as safe production organism for a broad range of food enzymes.

Production Strain:

The lysophospholipase enzyme preparation described in this dossier is obtained from *Trichoderma reesei* RF7206 carrying a recombinant gene encoding a lysophospholipase (*lpl*) from *Aspergillus nishimurae*. The expression cassette with the lysophospholipase gene was introduced into the genome of the recipient strain of *Trichoderma reesei* as several copies. *T.reesei* RF7206 is deposited in the "Centraalbureau voor Schimmelcultures" (CBS) in the Netherlands with the deposit number CBS125079.

The construction and the safety assessment of the production strain *Trichoderma reesei* RF7206 and the LPL product from this genetically modified microorganism have been described in notifications to the Finnish competent authority, Gene Technology Board, prepared according to Directive 90/219/EEC and the Finnish Gene Technology Law 377/1995.

The techniques used in transforming and handling *T. reesei* have been previously described *(Karhunen et al. 1993) (Penttilä et al. 1987)*. The production organism also meets the criteria for safe production microorganism (Pariza, Johnson 2001) (Decision Tree Analysis - *Appendix #2*). *T. reesei* strains are non-pathogenic and non-toxigenic and have been shown not to produce fungal toxins or antibiotics under conditions used for industrial enzyme production. Further they are considered as safe hosts for other harmless gene products *(Nevalainen et al. 1994; Olempska-Beer et al. 2006; Blumenthal 2004)*. The seed culture for the fermentation is inoculated with spores that have been stored at -80 ^{0}C. No additional growth cycles have been performed after the *T. reesei* RF7206 strain deposition to the culture collection.

2.2 Genetic Modification

The genetic modification, e. g. integration of the expression cassettes into the genome of the recipient strain *Trichoderma reesei* RF4847, results in the recombinant *Trichoderma reesei* strain RF7206. *T. reesei* RF7206 production strain differs from its original recipient strain (RF4847) in its high lysophospholipase production capability due to overexpression of the *lpl* gene driven by the cbh1 promoter. The transformation of RF4847 strain with the expression cassette was performed as described in *Penttilä et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The transformants were selected according to their ability to grow on acetamide plates. According to Southern blot analysis, at least two expression cassettes were integrated into the RF7206 genome.

Expression Cassette:

- The *lpl* gene is fused to a native *T. reesei* cellulase promoter. This promoter is strong and is used to overexpress *lpl* and to obtain high yields of lysophospholipase enzyme.
- *A. nishimurae lpl* gene.
- *Aspergillus nidulans amdS* gene: the gene has been isolated from *Aspergilus nidulans* VH1-TRSX6 *(Kelly, Hynes 1985)*. *Aspergillus nidulans* is closely related to *Aspergillus niger*, which is used in industrial production of food enzymes. The gene codes for an acetamidase that enables the strain to grow on acetamide as a sole nitrogen source. This characteristic has been used for selecting the transformants. The product of the *amdS* gene, acetamidase, can degrade acetamide and is not harmful or dangerous. The *amdS* marker gene has been widely used as a selection marker in fungal transformations without any disadvantage for more than 20 years.

Of the above genetic materials, the *Aspergillus nishimurae lpl* gene and *Aspergillus nidulans amdS* gene are not naturally present in the RF4847 genome.

The DNA fragments that have been transformed to *T. reesei* host strain RF4847 are well characterized, the sequences of the genes are known, and the fragments are free from any harmful sequences.

Southern blot analyses were performed to the genome of the production strain RF5427. According to Southern blot analysis, at least two expression cassettes were integrated into the RF7206 genome.

2.3 Stability of the Transformed Genetic Sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The transformed DNA does not contain any antibiotic resistance genes. The inserted DNA does not include any mobile genetic elements. Additionally, it should be highlighted that the *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome *(Kubicek et al. 2011; Martinez et al. 2008)*. This results in a low risk of transfer of genetic material.

The fermentation process starts always from the identical replica of the RF7206 seed ampoule. Production preserves at -80°C ("Working Cell Bank") are prepared from the "Master Cell Bank" (culture collection maintained at -150°C) in the following manner: A Petri dish is inoculated from the culture collection preserve (spore suspension) in such a way that single colonies deriving from one spore each, can be selected upon germination. Altogether at least 20 individual colonies are inoculated into three parallel slants in which strains are grown and let to germinate. Spores from one parallel slant, representing each of the individual colonies, are inoculated into shake flasks. The shake flasks constitute the culture stage.

A so-called productivity test is performed, i.e. shake flask cultivation being completed; the enzymatic activity is measured, which must correspond to a given value. If this value is not reached, the culture is discarded. This test serves to determine the characteristic metabolic efficiency of each strain (isolate), i.e. to establish its identity. The productivity test is redone in fermentor cultivations for the chosen isolates (out of at least 20) that showed the best productivity in the shake flask cultivations. The working cell bank ampoules with glycerol solution are then prepared from the slants whose productivity tests show the highest results. The suspensions thus obtained are frozen and stored divided into 0.5 ml aliquots at -80°C.

The annual production starts from these production preserves. Six of them are thawed for inoculation of six shake flasks and subsequent inoculation of the first process bioreactor is from these flasks. Mutation frequencies are low and in case mutations would occur, they only occur in the vegetative state during cell division. Owing to the above-described procedure, this vegetative state of the cultures is reduced to an inevitable minimum during production.

Potential changes in the genome of the production strain could theoretically occur during the propagation in the fermentation process. Therefore, Southern blot analysis was performed after fermentation process of the RF7206 strain. The results revealed that the recombinant lpl gene stays genetically stable in *T. reesei* genome over necessary time that is needed for industrial fermentation process of the RF7206 production strain.

Therefore, the production strain RF7206 is stable in terms of genetic traits. The genetic materials in the expression cassettes have been integrated as part of the genome and are as stable as any natural gene. The integrated genetic materials are not acting as mobilisable elements and they do not contain mobilisable elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek et al. 2011).

Additionally, the stability is also followed as equal production of the lysophospholipase activity in a number of fermentation batches performed for the strain RF7206. The activity measurements from parallel successful fermentations showed that the productivity of the RF7206 strain remains similar. The data of the analysis of enzyme activities from preparations from three different fermentation batches of the recombinant RF7206 strain is presented in Appendix 3.

These results confirm the genetic stability of the production strain RF7206.

2.4 Good Industrial Large Scale Practice (GILSP)

The *T. reesei* RF7206 enzyme production strain complies with all criteria for a genetically modified GILSP organism.

In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH, 1998) Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes produced by *T. reesei* are nontoxic. The Environmental Protection Agency (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[2], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD *(ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT 1992)*.

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). Indeed, the *T. reesei* RF4847 strain originates from the wild type strain QM6a from which it was developed by conventional mutagenesis programs. The wild type *T. reesei* strains have been isolated only at low altitudes and within a narrow belt around the equator *(Kubicek et al. 2008)*. The mycoparasitism-specific genes have been shown to be lost in *T. reesei (Kubicek et al. 2011)*.

Overall, industrial microorganisms modified to produce high levels of enzymes, in fermentation conditions (e.g. no competitive microorganisms, optimal nutrients and aeration that are not present in the natural environment) are not expected to have any competitive advantage against other

[2] Reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce

microorganisms in nature, which themselves are well-adapted in their natural environment. The fitness of the industrial strains to survive is very likely reduced by their high performance characteristic: most of the energy is needed for the production of proteins in high amounts.

The possible transfer of the recombinant DNA, if accidentally released into the environment, would not have any harmful or pathogenic effects on environmental processes. The DNA fragments used in the construction of the expression cassette are well characterized and do not contain any undefined or harmful fragments. It can be concluded that the DNA fragments in the expression cassette or their corresponding gene products are not biologically harmful and are common in surroundings. Also, the recombinant DNA used for transformation does not contain any antibiotic resistance markers.

Therefore, the *T. reesei* RF7206 production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the OECD. The production organism has been approved by the Finnish competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment.

2.5 Absence of the Production Organism in the Product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The *T. reesei* RF7206 is recovered from the fermentation broth by a widely used process (includes several filtering steps) that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch using an internal Roal method. This method has been validated in-house. The sensitivity of the method is 1 cfu/20 ml in liquid and 1 cfu/0,2 gram in dried semifinals. It is also important to notice that when the product is dried the drying step provides for an efficient way to kill *Trichoderma* strains, as the temperature is ca. 75°C of the air leaving the dryer, and fungi are not very tolerant to heat.

2.6 Structure and amount of vector remaining in the production strain

Trichoderma reesei RF7206 strain does not harbor any bacterial vector DNA. The expression cassette used for transformation was cleaved from the pUC19 vector backbone by restriction enzyme digestion followed by isolation of the expression cassette from an agarose gel.

A Southern blot hybridization experiment using the pUC vector as a labeled probe and genomic DNA of the production strain RF7206 was performed to confirm that no vector DNA is included in the genome of RF7206. It produced negative results (no hybridization), demonstrating that no part of the plasmid vector removed to generate the linear transforming DNA fragment was introduced into the *Trichoderma* production host.

2.7 Absence of Transferable rDNA Sequences in the Enzyme Preparation

The lysophospholipase is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, RF7206, are removed during the down-stream processing through a known acceptable filtration method.

After this the final product does not contain any detectable number of fungal colony forming units or recombinant DNA. Three separate enzyme samples (liquid semi-final concentrates) were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits. Please refer to *Appendix #3*.

2.8 Absence of Antibiotic Genes and Toxic Compounds[3]

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and*

[3] The Food Chemicals Codex ("FCC", 9th edition), states the following: "Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."

Agriculture Organization of the United Nations 2006) has been also tested for the fermentation products of the *T. reesei* strain RF7206. Adherence to specifications of microbial counts is routinely analysed. Several production batches produced by the production strain *T. reesei* RF7206 (3 liquid semi-final concentrates) were analyzed and no antibiotic or toxic compounds were detected (*Appendix #3*).

3. ENZYME IDENTITY

3.1 Common name of the enzyme

Systematic name	Lysophospholipase
Common names	lecithinase B; lysolecithinase; phospholipase B; lysophosphatidase; lecitholipase; phosphatidase B; lysophosphatidylcholine hydrolase; lysophospholipase A1; lysophopholipase L2; lysophospholipase-transacylase; neuropathy target esterase; NTE; NTE-LysoPLA; NTE-lysophospholipase; 2-lysophosphatidylcholine acylhydrolase
Production Strain	*Trichoderma reesei* RF7206

3.2 Classification of the enzyme

IUBMB #	3.1.1.5
CAS number	9001-85-8

The classification of the enzyme according to the IUBMB is as follows:

EC 3.	is for hydrolyases;
EC 3.1.	is for hydrolysases acting on ester bonds;
EC 3.1.1.	is for carboxylic ester hydrolases;
EC 3.1.1.5	is for lysophospholipase.

3.3 Characteristics of the enzyme preparation

Property	Requirement
Activity	Minimum 10,000 LPL/g.
Apperance	Light brown colour with characteristic odour.
Density	1.00-1.10 g/ml

4. CHEMICAL COMPOSITION AND PROPERTIES OF THE ENZYME AND THE ENZYME PREPARATION

4.1 Molecular mass and amino acid sequence of the enzyme

Depending on their physicochemical properties and on their origins, lysophospholipases often occur in different forms having molecular masses in a range of 30 - 100 kDa (Merkel et al. 1999; Masuda et al. 1991; Lee et al. 1994; Hsu et al. 2010; Shen et al. 2004; Patent US5965422).

4.2 Purity and identity specifications of the enzyme

Property	Requirement	Testing rate
Total viable counts	< 50000 g^{-1}	every lot
Yeasts and fungi	each < 1000 g^{-1}	every lot
E. coli	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Salmonella	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Coliform counts	< 30 g^{-1}	every lot
Arsenic	< 3 ppm	Spot samples from raw materials and semifinals
Lead	< 2 ppm	Spot samples from raw materials and semifinals
Heavy metals	< 30 ppm	Spot samples from raw materials and semifinals
Antibacterial Activity	not detectable	at least 1 lot/y

The proof that the food enzyme lysophospholipase complies with these specifications is shown by the analyses on 3 different stabilized liquid batches – see appendix #3

The results demonstrate reproducibility of the production process between batches and compliance with the required specifications.

4.3 Composition of the enzyme preparation

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Food enzymes, are concentrates containing a specific

enzyme protein, whose activity (also called main or principal activity) can be used for a specific, intended technological purpose in food processing. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing micro-organism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts. As has been established by JECFA (FAO/WHO, 2006), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS). The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.

The samples were collected at the end of the down-stream process after concentration, filtrations and stabilization. Food grade ingredients such as glyercol and sodium chloride can be used. Ash, Water, Protein contents (measured) and TOS value (calculated) in the 3 samples are not comparable within each other's as the volume of each liquid stabilised concentrate is different. Comparison is only possible on activity / TOS ratio parameter.

Batch Number	372062173	372062173	372062173
Size of the fermentation vessel	20 m^3	100m^3	Pilot 1m^3
Ash (%)	3.3	5.2	5.31
Water (%)	73.7	50.8	54.1
Protein (%)	3.0	9.0	7.0
TOS (%)	3.7	9.6	10.2
Activity (ALU/g concentrate)	35,500	64,800	60,400
Activity/mg TOS	959.9	674.6	590.1

The typical batch sizes range from 1 000 L to 150 000 L and are deeply depending on the market demand. Therefore, the frequency and the volume of production of the food enzyme vary. AB Enzymes is a small size company and the market demand for this specific enzyme is not so high to justify always full-scale fermentations on a frequent basis. This explains why the 3rd sample is a pilot scale fermentation.

TOS values were calculated using the following formula: % TOS = 100 % - (% Ash + % Moisture + % Diluents) as recommended by JECFA. Some diluents are already covered under the Ash fraction of the calculation.

4.4 Enzymatic Activity

The main activity of the enzyme preparation is lysophospholipase (IUB 3.1.1.5), which has been identified in many sources, including plants, microorganisms and animals (Wolf et al. 1979).

Lysophospholipase catalyzes the hydrolysis of an ester bond between a fatty acid and glycerol in lysophospholipids, resulting in the formation of free fatty acids and glycero-phosphatide. The reaction catalyzed can be described as follows:

2-lysophosphatidylcholine + H2O = glycerophosphocholine + a carboxylate

The substrates for lysophospholipase are lysophospholipids.

Phospholipids are major component of all cell membranes in animals, plants and micro-organisms (they naturally occur in most vegetable oils (e.g. soya, rapeseed, sunflower oils), marine oils, animal fats (e.g. bovine milk), chicken eggs, fish eggs, etc.-). In general, phospholipids are diacylglycerol molecules with the third carbon attached to a phosphate molecule.

Lysophospholipids (LPLs) are small (glycerol)phospholipids molecules, characterized by a single carbon chain and a polar head group, in which one of its two 0- acyl chains is lacking and then only one hydroxyl group of the glycerol backbone is acylated. They are formed during the phospholipid breakdown as a result of the action of phospholipases. Unlike phospholipids, LPLs are found only in small amounts in biological cell membranes (Birgbauer, Chun 2006) but LPLs and their receptors have been found in a wide range of tissues and cell types, indicating their importance in many physiological processes (Moolenaar, 2000; Torkhovskaya et al, 2007 as reviewed by D'Arrigo, Servi 2010).

Lysophospholipids are also known to be the predominant phospholipids found in wheat starch (Matser, Steeneken 1998).

Consequently, the substrate for lysophospholipase occurs naturally in nature, and in particular in vegetable (wheat) based foods and is therefore a natural part of the human diet.

Like the substrate, the enzyme also occurs in nature. Lysophospholipase activities were found in molds (Fairbairn, 1948), rice bran, several microorganisms, snake and bee venoms, insects, fish muscle and in various animal tissues. In mammalian tissue the enzyme was first described in beef pancreas. Relatively high levels were detected in intestine, lung, spleen, liver and pancreas, while lower levels were present in muscle, kidney, testes, brain and blood (as cited in Wolf et al. 1979). Lysophospholipase is a component of many animals and plant derived foods and thus has always been consumed by humans.

Reaction products: as a result of the catalytic activity of lysophospholipase low levels of free fatty acids and glycero-phosphatides are formed. These compounds are already present in the human diet. The method to analyse the activity of the enzyme is company specific and is capable of quantifying lysophospholipase activity as defined by its IUBMB classification. The enzyme activity is usually reported in LPL/g.

4.5 Secondary Enzymatic Activities

Food enzymes are biological concentrates containing, apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called 'main enzyme activity'), other organic substances.

These other substances may include various enzyme activities (defined as 'side activities' due to their lower relative amount compared to the amount of the main desired enzyme protein) derived from the producing microorganism. Like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, many enzymes are excreted to digest the material on which the microorganisms grow.

Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids (fats). These are the very same activities that play a role in the production of fermented food and in the digestion of food by – amongst others – the intestinal micro flora in the human body. In addition, if a food raw material contains a certain substrate (e.g. carbohydrate, protein or lipid), then, by nature, it also contains the very same enzymatic activities that break down such a substrate; e.g. to avoid its accumulation. Consequently, the presence in food of such enzyme activities and of the potential reaction products is not new and should not be of any safety concern. In addition, it is generally accepted that the enzyme proteins themselves do not pose any safety concern either.

During the production of food enzymes, the main enzyme activity is normally not separated from the other substances present. Consequently, the food enzyme contains a number of other enzymes excreted by the microbial cells or derived from the fermentation medium. Other strains of *Trichoderma reesei,* selected to produce other main enzyme activities, will produce and excrete the same set of enzymatic activities, albeit in various amounts. Consequently, the food enzymes from *Trichoderma reesei* which are approved and used in food processes already for many years, will also contain these activities. These activities are of no safety concern and their fate in the final food will be the same as that of the main enzyme activity. Thus, apart from lpl, the food enzyme also contains other enzymatic side activities in small amount, which are naturally and typically produced by the production organism *Trichoderma reesei*. Those include ß-glucanase and cellulase.

5. ENZYME PRODUCTION PROCESS

5.1 Overview

The food enzyme is produced by ROAL Oy by submerged fermentation of *Trichoderma reesei* RF7206 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).

T. reesei RF7206 described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. A manufacturing flow-chart is given in *Appendix #4*.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

5.2 Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

5.2.1 Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further confirmed by toxicology studies. The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The raw materials used for the formulation are of food grade quality.

The antifoam agents and flocculants used in the fermentation and recovery processes are used as described in the Enzyme Technical Association submission to FDA on antifoam and flocculants (April 24,

1998). The maximum use levels of antifoam and flocculants are used below ≤0.15% and ≤1.5% respectively.

5.2.2 Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals
- pH adjustment agents
- Foam control agents

5.2.3 Inoculum

A suspension of a pure culture of *T. reesei* RF7206 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

In order to have sufficient amount of biomass, the process is repeated several times. When a sufficient amount of biomass is obtained the shake flasks are combined to be used to inoculate the seed fermentor.

5.2.4 Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The seed fermentation is run at a constant temperature and a fixed pH. At the end of fermentation, the inoculum is aseptically transferred to the main fermentation.

5.2.5 Main fermentation

Biosynthesis of the lysophospholipase product by the production strain *T. reesei* RF7206 occurs during the main fermentation.

The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium. The fermentation in the main fermentor is run as normal submerged fermentation under well-defined process conditions (pH, temperature, mixing, etc.).

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

5.3 Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is secreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

5.3.1 Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above mentioned materials during recovery.

5.3.2 Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 2.5 %.

5.3.3 Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at defined pH and temperature ranges in order to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

5.3.4 Concentration

The liquid containing the enzyme protein needs to be concentrated in order to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation. Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

5.3.5 Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

5.4 Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in the USA according to ruling legal provisions.

The lipase enzyme preparations from *T. reesei* RF7206 are sold mainly as liquid preparations. For all kinds of food enzyme preparations, the food enzyme is adjusted to a declared activity, standardized and preserved with food ingredients or food additives (food grade quality).

The enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general testing requirements for Food Enzyme Preparations, and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations. Labels conform to relevant legislation.

5.5 Quality Control of Finished Product

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture*

Organization of the United Nations 2006) and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 10th edition (2015) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Property	Requirement
Total viable counts	< 50000 g^{-1}
Yeasts and fungi	each < 1000 g^{-1}
E. coli	not present in 25 g
Salmonella	not present in 25 g
Coliform counts	< 30 g^{-1}
Arsenic	< 3 ppm
Lead	< 2 ppm
Heavy metals	< 30 ppm
Antibacterial Activity	not detectable
Mycotoxins[4]	No significant levels

5.6 General Production Controls and Specifications

In order to comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore it is essential that the identity and purity of the microorganism is controlled.

[4] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules of the MCB. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination might result in decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:

- all the media are sterilized with steam injection in fermentors or media tanks (at 121°C for at least 20 min at pH 4.3 – 4.8.).

- Use of sterile air for aeration of the fermentors:
  - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
  - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
  - Maintaining a positive pressure in the fermentor
- Germ filtration

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
  - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (cGMPs). The whole process is controlled with a computer control system (Metso DNA) which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- CO2

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

Deviations from the pre-defined values at any of the preceding steps will lead to adjustment or actions ensuring an optimal enzyme products are achieved or to rejection of the product.

5.7 Stability of the enzyme during storage and prior to use

Food enzymes are formulated into various enzyme preparations in order to obtain standardized and stable products. The stability thus depends on the type of formulation, not on the food enzyme as such. The date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

6. PURPOSE

6.1 Technological purpose and mechanism of action of the enzyme in food

Like any other enzyme, lysophospholipase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product or products. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

In general, the technological need of the enzymatic conversion of lysophospholipids with the help of lysophospholipase can be described as: degradation of a component (the substrate lysophospholipids) which causes technical difficulties in processing of raw materials containing this component.

As described above, lysophospholipase is naturally present in many vegetable raw materials, including wheat, corn or barley. The natural enzymatic conversion of lysophospholipids in such materials would theoretically be of technological benefit in several industrial food manufacturing processes. However, the levels of endogenous lysophospholipase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial lysophospholipase is used during food processing. Typical use of lysophospholipase in food processing is starch processing (process of hydrolysing starch to produce caloric sweeteners, including syrups). In this process, the lysophospholipase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which

could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

This dossier is specifically submitted for the use of lysophospholipase in starch processing, ie. production of starch hydrolysates, more precisely production of all kind of syrups and sweeteners produced from starch (mainly wheat and maize/corn starches[5]).

Starch Processing

Lysophospholipids are the predominant phospholipids in wheat starch (0.5-0.8%) and are also found in corn starch (although in lower amount).

Lysophospholipids are water soluble and are efficient emulsifiers. This is because these compounds have both an ionic (hydrophilic, water soluble) and long chain non-ionic carbohydrate (hydrophobic, water insoluble, long chain fatty acid) group. Therefore, when concentrated (concentration > 0.025 g/kg) can form micelles which negatively affect the filtration rate of the hydrolysate (Matser, Steeneken 1998).

Use of lysophospholipase removes the emulsifying properties of the phospholipid by cleaving a fatty acid producing separate water insoluble (long chain fatty acid) and water soluble (glycerophosphatide) molecules. Therefore, lysophospholipase is used when the amount of lysophospholipids in a food processing raw material lies above the critical micelle concentration of 0.025 lysophospholipids per kg raw material.

In addition, lysophospholipds are known to form a complex with amylase leading to the formation of a cloud in the final syrup thus affecting its characteristics (clearness of the filtrate).

[5] Depending on the production process and the type of enzymes used, various syrups and sweeteners can be produced, such as maltodextrins, glucose, fructose, maltose, high fructose corn syrups, etc. The enzymatically produced syrups are used in various food industries, e.g. the beverage industry, dairy, baking, canning and confectionary industries.

Lysophospholipases hydrolyze lysophospholipids into free fatty acid (water insoluble) and glycerophosphate base (water soluble) molecules. Therefore, the benefits of the conversion of lysophospholipids with the help of lysophospholipase in starch processing are:

- Prevent the formation of lysophospholipid micelles
- Facilitate the separation of undesired components
- Improve filtration rate (better and faster filtration)
- Improve the characteristics (clearness) of the filtrate
- Improve the environmental impact and sustainability (energy saving due to the load mitigation and decreased production time)

Lysophospholipase is acting on one family of the components of the plant cell wall, and is often used together with other enzymes (enzyme systems) which modify other components of the plant cell walls. In particular, lysophospholipase is often applied together with (gluco-)amylases and/or pullulanase, that are alternatively applied during the production of syrups (Słomińska, Niedbacha 2009).

Therefore, lysophospholipase will improve the filtration rate of syrups produced from corn, potato, rice and sorghum starch.

Process flow-chart of starch processing:

**depending on type of syrup/sweetener to be produced*

6.2 Use Levels

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practices. The amount of enzyme activity added to the raw material

by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions.

Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more.

Consequently, from a technological point of view, there are no 'normal or maximal use levels' and lysophospholipase is used according to the QS principle. A food producer who would add much higher doses than needed would experience untenable costs as well as negative technological consequences.

Food enzymes also contain substances derived from the producing microorganism and the fermentation medium, and the presence of all organic material is expressed as Total Organic Solids[6] (TOS). This distinguishes the proportion of the enzyme preparation derived from the source material from that contributed by diluents, and other additives and ingredients.

Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS.

The Table below shows the range of recommended use levels for each application where the lysophospholipase from *Trichoderma reesei* RF7206 may be used:

Application and Raw Material	Raw Material	Maximal recommended use levels (mg TOS/kg RM)
Starch processing (production of syrups)	Starch (Wheat / corn starch)	1

[6] In the case of food enzymes, which are – per legal definition – not formulated, TOS is the same as Dry Matter minus ash. The amount of ash (e.g. mineral salts used in the fermentation) does generally not exceed a few percent.

6.3 Enzyme Residues or Activity in the Final Food

Like any other enzyme, lysophospholipase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

In general, enzymes perform their technological function during food processing. Like the endogenous enzymes present in food, they do not perform any technological function in the final food. The reasons why the enzymes do not typically exert enzymatic activity in the final food could be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer, such as:

- the enzyme protein must be in its 'native' (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favourable

In starch processing, the lysophospholipase exert its function during the production of starch hydrolysates, in particular syrups and sweeteners produced from wheat, corn (mainly) barley, potato, rice and sorghum starches, in order to contribute to an improved and consistent starch saccharification process (Słomińska, Niedbacha 2009). After saccharification, the syrup is heated to a temperature of 85°C at which all enzyme activity is inactivated. Further purification steps of the syrups such as activated carbon filtration and ion exchange refining will remove most of the inactivated enzyme which just represents a small fraction of protein in the final syrup.

Furthermore, under a lack of substrate and favourable pH conditions, the enzyme will be denatured (not active) in the final food application and would be similar to other endogenous enzymes in food.

As the enzymatically produced syrups are mainly used in the beverage industry, dairy, baking, canning and confectionary industries, it can be concluded that in the unlikely case of remaining functional enzyme, it will be denatured by the heat treatments applied during those dedicated processes anyway.

6.4 Possible Effects on Nutrients

The reaction products of the hydrolysis of lysophospholipids by lysophospholipase are free fatty acids and glycero-phosphatides. Like the substrate and the enzyme, these reaction products are also natural constituents in various organisms from bacteria to mammals. As a result, phosphatides and fatty acids are quite abundant in the human diet. Consequently, also the reaction products occur naturally in foods and adverse effects on nutrients are not to be expected.

Lysophospholipase activity is widely present in nature and in particular in food ingredients (as cited in Wolf et al. 1979). The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

T. reesei is an industrially important filamentous fungus and has been used as producer of different hydrolases such as xylanase and cellulase for food, animal feed, and pulp and paper industries. It is also used as host for production of heterologous proteins in the same areas. Like many other organisms with a long safe history of industrial use, *T. reesei* strains have been and are being used by many commercial companies in the construction of production strains by genetic engineering.

Species belonging to *Trichoderma reesei* are common in soil as well as on vegetable debris. However, *T. reesei* strains have been isolated from soil (compost material) only at low altitudes and within a narrow belt around the *equator (*+/- 20 degrees altitude *(Kubicek et al. 2008)*. According to *Kuhls et al. (1996)*,

T. reesei is a clonal, asexual derivative of the ascomycete *Hypocrea jecorina* and can be identified by PCR-fingerprinting assay and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene *(Kuhls et al. 1996)*. All the *T. reesei* strains used for industrial enzyme production derive from the same wild type isolate, QM6a.

T. reesei is regarded as non-pathogenic and non-toxigenic. The safety of this organism as an enzyme producer has been reviewed by *Pariza, Johnson; Olempska-Beer et al.; Nevalainen et al.; Blumenthal (2001; 2006; 1994; 2004)* and deemed to be safe. The review article by *Nevalainen et al. (1994)* reveals that some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics. However, strains of *T. reesei* used in industrial applications are proven to be absent of antibiotic activities *(Hjortkjaer et al. 1986; Coenen et al. 1995)*. Additionally, no genes have been introduced during the genetic construction that encodes antimicrobial resistance, and the absence of antibiotic activities was also confirmed from production batches (*Appendix #3*).

The transformed expression cassettes, fully characterized and free from potential hazards, are stably integrated into the fungal genome (see section 2.3) and are no more susceptible to any further natural mutations than any other genes in the fungal genome. Also, the transformation does not increase the natural mutation frequency. If there were any mutations in the genes affecting the relevant characteristics of the fungus, this would likely be noticed in the growth characteristics in the fermentation and/or in the product obtained, and no such changes have been observed. The possibility of mutations is further decreased by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. There is no indication that the genetic modification has a negative effect on the safety properties.

The lysophospholipase enzyme preparation from *T. reesei* RF7206, expressing the *lpl* from *Aspergillus nishimurae* was evaluated according to the Pariza and Johnson Decision Tree. The decision tree is based on the safety evaluation published by Pariza and Foster in 2001, adapted from their original evaluation in 1983. Based on the Pariza and Johnson decision tree analysis, AB Enzymes concludes that the lysophospholipase enzyme preparation is safe, see *Appendix #2*.

Therefore, it can be concluded that the *T. reesei* strain RF7206 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food processing.

7.2 Safety of lysophospholipase Enzyme

The gene for lysophospholipase protein overproduced by RF7206 originates from *Aspergillus nishimurae (ex A. fumigatus)*. Although *A. fumigatus* is known to be an opportunistic pathogen, recent literature (Birch et al. 2004; Rementeria et al. 2005; Abad et al. 2010) excludes lysophospholipase from *A. fumigatus* from being a virulence factor.

Enzyme proteins in food products are not regarded as harmful for humans/animals as shown in several safety evaluations, see section 7.4.

Lysophospholipase from *Aspergillus fumigatus (A. nishimurae)* showed high amino acid sequence identity (61-69%) to lysophospholipases isolated from *Aspergillus oryzae* and *Aspergillus niger*, which have been used commercially for improving the filtration of starch syrup (Słomińska, Niedbacha 2009). The Final Assessment Report of the Food Standards Australia New Zealand government agency concludes that the use of lysophospholipase from *A.niger* as a processing aid in food poses no public health and safety risk[7]. In addition, the lysophospholipase from *T. reesei* RF7206 has been previously concluded not to pose any risk for the consumer by the French Authorities and accordingly has recently been approved there (appendix #5).

Furthermore, lipases, including phospholipases and lysophospholipases, have been used in the food industry for many years. Commercial lysophospholipase enzyme preparations from various microorganisms (including genetically modified ones) are widely accepted and *Trichoderma reesei* whether or not genetically modifiedis has widely been accepted as a safe production organism for a broad range of enzymes that have been used e.g. as processing aids in the food industry for several decades (Pariza, Johnson 2001). AB Enzymes submitted a GRAS notification for phospholipase A2 from

[7] http://www.foodstandards.gov.au/code/applications/documents/A492_Lysophospholipase_FAR.pdf

Aspergillus fumigatus (A. nishimurae) expressed in *T. reesei* to the FDA and received a no objection letter in 2014[8].

It is generally accepted that known commercial enzyme preparations of *T. reesei* are non-toxic and since lysophospholipase is a natural constituent in the environment, it is concluded that the lysophospholipase enzyme from *T. reesei* RF7206 is safe as for use as a food processing aid in various applications.

To further confirm that the lysophospholipase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *T. reesei*, the following studies were conducted:

- Sub-chronic (90 day) oral toxicity study
- Ames test
- Chromosomal aberration test, *in vitro*

Based upon the results of these studies, it can be concluded that the lysophospholipase enzyme preparation does not produce adverse effects in rodents, nor was there any mutagenic or clastogenic activity detected, details are provided in section 7.4

7.2.1 Allergenicity

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, lysophospholipase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the lysophospholipase residues in food (mainly baked goods) seems remote. In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by *Bindslev-Jensen et al. (2006)* and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and

[8] http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm410988.pdf

comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products *(Daurvin et al. 1998)*. The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).
- In the case where proteins are denatured - which is the case for this lysophospholipase - due to the food process conditions (i.e baking process), the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins *(Valenta, Kraft 2002; Valenta 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006)*
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to

digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic

- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

In order to specifically evaluate the risk that lysophospholipase enzyme would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed.

Alignments of the *LPL* mature amino acid sequence to the sequences in the allergen databases were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme.

Similarity searches were performed to the sequences available in chosen public allergen databases, namely AllergenOnline (FARRP) and Allergen Database for Food Safety (ADFS).

According to the results obtained from the alignments and homology searches it can be concluded that the *LPL* enzyme does not show significant homology to any known allergen. Consequently the risk of *LPL* protein to cause an allergy is regarded as being low.

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is unlikely that the lysophospholipase produced by *Trichoderma reesei* RF7206 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

7.3 Safety of the Manufacturing Process

T. reesei RF7206 meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex (2014) as described in section 4.5.

As described in Section 4, the *T. reesei* RF7206 enzyme production strain is produced in accordance with cGMPs using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4 Safety Studies

This section describes the studies performed to evaluate the safety of the RF7206 lysophospholipase enzyme preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1 Summary of Safety Studies

The following studies were performed:

- Bacterial reverse mutation test
- *In vitro* mammalian chromosome aberration test
- 13 week oral toxicity study in rats

Lysophospholipase that has been tested is a dry ultra-filtrated concentrate, which is the most concentrated product before its formulation into a food enzyme preparation. The batch is a dried enzyme concentrate and does not contain any diluent or other formulation ingredient.

The composition and specifications of the toxicological batch is provided below:

Batch No	**LF 07102A3**
Ash (%)	1.5
Water (%)	5.8
TOS (%)	92.7
Activity (MNU/g)	666,877
Activity /mg TOS	719.4
Protein (%)	66.2
Lead (mg/kg)	<0.05
Salmonella sp. (per 25 g)	Not detected
Total coliforms (CFU per g)	<10
Escherichia coli (per 25 g)	Not detected
Antimicrobial activity	Not detected
Mycotoxins	No significant levels

Only the activity vs TOS can be compared between different batches, particularly when comparing the commercial batches (after concentration, filtrations and stabilization), and the liquid stabilized samples to the TOX batch. The 3 commercial batches were collected at the end of the down-stream process with added glycerol and NaCl, whereas the toxicologically tested batch is a dried enzyme concentrate, collected after concentration and filtration but not containing any diluent or other formulation ingredient.

In the three commercial batches, the average activity / TOS ratio is calculated to be 741.4 LPL/mg TOS. This figure is consistent with the activity/TOS ratio calculated for the toxicologically tested batch (719.4 LPL/mg TOS as shown in the table above).

It should be noted that in certain cases, enzymatic activity at the end of fermentation time could be higher in pilot scale fermentation than in full scale fermentations (because of energy input and aeration). Furthermore, in full scale production, we could also face activity losses because of the potential long lasting ultrafiltration procedure (which could cause physical stress to the enzyme structure leading to partly inactivation). This affects the activity vs TOS ratio accordingly.

Please note in addition that all figures above should be interperated cautiously due to diffent method accuracy between the TOX batch and the commericial batches. This can lead to some discrepancies in the measurements and in the accordant figures above.

Disregarding comparing a dry concentrate to a liquid semi-final concentrate with respect to certain values such as moisture, ash, etc., the activity (MNU/g) and activity/mg TOS are comparable, along with values for mycotoxins, which shows the TOX batch being respresentative of the commercial batches.

7.4.2 Results of the Safety Studies

Bacterial Reverse Mutation Test

The test, based on OECD Guidelines No. 471, was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany. The study was completed on February 14, 2008.

This study was performed to investigate the potential of lysophospholipase from *Trichoderma reesei* RF7206 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.
The test item was tested at the following concentrations :
— Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

— Experiment II: 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

The plates incubated with the test item showed normal background growth up to 5,000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with lysophospholipase at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, the lysophospholipase from *Trichoderma reesei* RF7206 was considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

Chromosomal Aberration Test

The test, based on OECD Guidelines No. 473, was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany. The study was completed on April 25, 2008.

The lysophospholipase from *Trichoderma reesei* RF7206 was assessed for its potential to induce structural and numerical chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in two independent experiments.

In each experimental group two parallel cultures were set up. Per culture 100 metaphases were scored for structural chromosome aberrations.

The highest applied concentration (5,441 µg/mL = 5,000 µg/mL adjusted to TOS) was chosen with respect to the current OECD Guideline 473. Dose selection for the cytogenetic experiments was performed considering the toxicity data.

No toxic effects indicated by reduced mitotic indices and/or reduced cell numbers of below 50 % of control were observed after treatment up to the highest required test item concentration.

In both independent experiments, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. However, in Experiment II in the presence of S9 mix a single significant increase (2.0 %) was observed but this value was clearly within the laboratory's historical control data range (0.0 – 4.0 % aberrant cells, excluding gaps) and is regarded as biologically irrelevant.

No relevant increase in the frequencies of polyploid metaphases was found after treatment with the test item as compared to the frequencies of the controls.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, no biologically relevant increases of chromosomal aberrations were observed.

Therefore the lysophospholipase from *Trichoderma reesei* RF7206 is considered to be non-clastogenic in this chromosome aberration test with and without S9 mix when tested up to the highest concentration required by the guideline and adjusted to TOS.

In vivo tests were not performed, as there was *no in vitro* mutagenicity detected.

90-Day Sub-Chronic Toxicity Study

The test was performed according to the following guidelines: OECD No. 408 at Harlan Laboratories Ltd (Itingen, Switzerland). The study was completed on May 29, 2009.

In this subacute toxicity study, lysophospholipase from *Trichoderma reesei* RF7206 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1,000 mg/kg body weight/day for a period of 13 weeks. A control group was treated similarly with the vehicle, bi-distilled water, only.

The groups comprised 10 animals per sex which were sacrificed after 13 weeks of treatment. Clinical signs, detailed behavioural observations, food consumption and body weights were recorded periodically during the acclimatization and treatment periods. Ophthalmoscopic examinations were performed during the acclimatization and at the end of the treatment period. Functional observational battery, locomotor activity and grip strength were performed during week 13.

At the end of the dosing, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were sacrificed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals, and all gross lesions from all animals.

Mortality / Viability: All animals survived until scheduled necropsy.

Clinical Signs (Daily and Weekly): No clinical signs of toxicological relevance were noted during daily observations in males and females at all dose levels.

Detailed Behavioural Observations: No clinical signs were recorded during the weekly detailed behavioral observations (weeks 1-12).

Functional Observational Battery: No clinical signs were recorded during the functional observational battery (week 13).

Grip Strength: No test item-related changes were noted in fore- and hind limb grip strength in male and female rats at any dose level

Locomotor Activity: The mean locomotor activity of males and females was not affected by the treatment with thetest item.

Food Consumption: A slight trend to reduced mean daily- and relative food comsumption was noted in test item treated animals of both sexes at all dose levels during the treatment period. Although these changes in mean daily- and relative food consumption were not accompanied by changes in body weight development of test item-treated animals, these findings were considered to be related to the treatment with the test item.

Body Weights: The mean body weight development in control and test item-treated animals of both sexes was comparable at any dose level during the treatment period.

Ophthalmoscopic Examinations: Typical background findings (corneal opacity, persistent hyaloid vessel in vitreous body, persistent pupillary membrane) were noted without relationship to dose or treatment

Clinical Laboratory Investigations:
Hematology: After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in hematology parameters in rats of both sexes at any dose level.

Clinical Biochemistry: After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in clinical biochemistry parameters in rats of both sexes at any dose level.

Urinalysis : After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in the urinalysis in males and females at any dose level.

Organ Weights: There were no differences indicating an effect of the test item. A few statistically significant deviations in average organ weights at the end of the treatment period were considered to be incidental, reflecting the usual individual variability.

Macroscopic / Microscopic Findings: At necropsy, performed at the end of the treatment period, no test item-related macroscopic findings were recorded. The test item, lysophospholipase produced no histological evidence of toxicological properties in the organs and tissues examined.

Conclusion: Oral administration of lysophospholipase to Wistar rats at doses of 100, 300 and 1000 mg/kg/day for at least 13 weeks resulted in no premature death, no clinical signs of adverse nature during daily observations, detailed behavioural observations and during the functional observational battery, no effects on fore- or hind limb grip strength, no effects on locomotor activity, no effects on body weight development, no test item-related changes observed during the ophthalmoscopic examinations, no effects on hematology, clinical biochemistry or urinalysis parameters, no effects on organ weight, no test item-related macroscopic findings of toxicological relevance. The test item, lysophospholipase produced no histological evidence of toxicological properties in the organs and tissues examined.

Insofar as the marginally reduced mean daily absolute and relative food consumption values noted in rats of both sexes were not accompanied by concomitant changes in mean body weight, and no other findings of toxicological relevance were noted, these differences were considered to be unrelated to the test item.

Therefore, the no-observed effect level (NOEL) and the no-observed-adverse-effect level (NOAEL) were considered to be above 1000 mg/kg/day (ie 955 mg TOS/kg/day1), the highest dose level used in this study.

7.5 Estimates of Human Consumption and Safety Margin

7.5.1 Estimate Dietary Exposure

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method, originally known as the Danish Budget Method *(Douglass et al. 1997; Hansen 1966)*. This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Consumption of food patterns:

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

In section 6.2, the recommended use levels of lysophospholipase are given based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products based on edible oils that are available to consumers.

Applications		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Starch processing (production of syrups)	Starch (Wheat / corn starch)	1	Liquid foods, in which syrups are used, mainly soft drinks	0.15	**0.15**
Solid foods	Starch processing (production of syrups)	Starch (Wheat / corn starch)	1	Solid foods in which syrups are used, e.g. baked products, confectionnary, etc.	0.25	**0.25**

* Assumptions behind ratios of raw material to final food:

Typically:

- starch hydrolysates (sweeteners, syrups) deriving from starch processing are used in a large range of food industries, mainly in soft drinks, dairy, bakery, confectionnary, etc. that fall in the categories of both solid and liquid foods;
- 1 kg of sweetener/syrup is produced per 1 kg starch, meaning that starch hydrolysates (syrups) are 100% starch.

Solid food:

- The most considerable final food applications are dairy and bakery with a maximum added starch content of 5%. Starch is also used in application area of confectionary, where it is used up to a content of 25%. Based upon the highest level of applications (confectionary), the corresponding RM/FF ratio is 0.25 kg starch per kg final food.

Liquid food:

- Syrups and sweeteners are mostly applied in soft drink beverages. Soft drinks typically contain 10-15% w/v HFCS. Therefore, the typical ratio of RM/FF is 0.15 kg starch per L final beverage.

The Total Theoretical Maximum Daily Iintake (TMDI) can be calculated on basis of the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be: TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
0.25 x 0.0125 = 0.003	0.15 x 0.025 = 0.003	0.006

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs (and beverages) use the specific enzyme lysophospholipase from *Trichoderma reesei*;
- It is assumed that ALL producers apply the HIGHEST use level per application; For the calculation of the TMDI's in food, only THOSE foodstuffs were selected containing the highest theoretical amount of TOS. Thus, foodstuffs containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels *(Douglass et al. 1997)*.

7.5.2 Safety Margin

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 955 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI), the Total TMDI of the food enzyme is 0.006mg TOS/kg body weight/day.

Consequently, the MoS is:

MoS = 955 / 0.006= **159,167**

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

8. CONCLUSION

Results of the toxicity and mutagenicity tests described in Section 7.4 demonstrate the safety of lysophospholipase preparation from *T. reesei* RF7206, which showed no toxicity or mutagenicity across a variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *T. reesei* derived enzymes and lipases in food processing, and in keeping with the conclusions found in a review of relevant literature presented. Based upon these factors, as well as upon the limited and well characterized genetic modifications allowing for safe production of the enzyme preparations, it is AB Enzymes' conclusion that lysophospholipase enzyme preparation from *T. reesei* RF7206 is GRAS for the intended conditions of use described herein.

10. LIST OF APPENDICES

1- GRN 32
2- Pariza and Johnson Decision Tree
3- Chemical Composition Report
4- Manufacturing Flow Chart
5- France Approval of Lysophospholipase RF7206

11. PUBLICATION BIBLIOGRAPHY

Abad, Ana; Fernández-Molina, Jimena Victoria; Bikandi, Joseba; Ramírez, Andoni; Margareto, Javier; Sendino, Javier et al. (2010): What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. In *Revista iberoamericana de micología* 27 (4), pp. 155–182. DOI: 10.1016/j.riam.2010.10.003.

Bindslev-Jensen, Carsten; Skov, Per Stahl; Roggen, Erwin L.; Hvass, Peter; Brinch, Ditte Sidelmann (2006): Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 44 (11), pp. 1909–1915. DOI: 10.1016/j.fct.2006.06.012.

Birch, Mike; Denning, David W.; Robson, Geoffrey D. (2004): Comparison of extracellular phospholipase activities in clinical and environmental Aspergillus fumigatus isolates. In *Medical mycology* 42 (1), pp. 81–86.

Birgbauer, E.; Chun, J. (2006): New developments in the biological functions of lysophospholipids. In *Cellular and molecular life sciences : CMLS* 63 (23), pp. 2695–2701. DOI: 10.1007/s00018-006-6155-y.

Blumenthal, Cynthia Z. (2004): Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. In *Regulatory toxicology and pharmacology : RTP* 39 (2), pp. 214–228. DOI: 10.1016/j.yrtph.2003.09.002.

Coenen, T. M.; Schoenmakers, A. C.; Verhagen, H. (1995): Safety evaluation of beta-glucanase derived from Trichoderma reesei: summary of toxicological data. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 33 (10), pp. 859–866.

D'Arrigo, Paola; Servi, Stefano (2010): Synthesis of lysophospholipids. In *Molecules (Basel, Switzerland)* 15 (3), pp. 1354–1377. DOI: 10.3390/molecules15031354.

Daurvin, T.; Groot, G.; Maurer, K. H.; Rijke, D. de; Ryssov-Nielsen, H.; Simonsen, M.; Sorensen T.B. (1998): Working Group on Consumer Allergy Risk from Enzyme Residues in Food. AMFEP. Copenhagen.

Douglass, J. S.; Barraj, L. M.; Tennant, D. R.; Long, W. R.; Chaisson, C. F. (1997): Evaluation of the budget method for screening food additive intakes. In *Food additives and contaminants* 14 (8), pp. 791–802. DOI: 10.1080/02652039709374590.

FAIRBAIRN, D. (1948): The preparation and properties of a lysophospholipase from Penicillium notatum. In *The Journal of biological chemistry* 173 (2), pp. 705–714.

Food and Agriculture Organization of the United Nations (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3).

Goodman, Richard E.; Vieths, Stefan; Sampson, Hugh A.; Hill, David; Ebisawa, Motohiro; Taylor, Steve L.; van Ree, Ronald (2008): Allergenicity assessment of genetically modified crops--what makes sense? In *Nature biotechnology* 26 (1), pp. 73–81. DOI: 10.1038/nbt1343.

Hansen, S. C. (1966): Acceptable daily intake of food additives and ceiling on levels of use. In *Food and cosmetics toxicology* 4 (4), pp. 427–432.

Hjortkjaer, R. K.; Bille-Hansen, V.; Hazelden, K. P.; McConville, M.; McGregor, D. B.; Cuthbert, J. A. et al. (1986): Safety evaluation of Celluclast, an acid cellulase derived from Trichoderma reesei. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 24 (1), pp. 55–63.

Hong, Seung-Beom; Shin, Hyeon-Dong; Hong, Joonbae; Frisvad, Jens C.; Nielsen, Per V.; Varga, János; Samson, Robert A. (2008): New taxa of Neosartorya and Aspergillus in Aspergillus section Fumigati. In *Antonie van Leeuwenhoek* 93 (1-2), pp. 87–98. DOI: 10.1007/s10482-007-9183-1.

Hsu, K-H; Wang, S-Y; Chu, F-H; Shaw, J-F (2010): Characterization and heterologous expression of a novel lysophospholipase gene from Antrodia cinnamomea. In *Journal of applied microbiology* 108 (5), pp. 1712–1722. DOI: 10.1111/j.1365-2672.2009.04569.x.

Karhunen, T.; Mäntylä, A.; Nevalainen, K. M.; Suominen, P. L. (1993): High frequency one-step gene replacement in Trichoderma reesei. I. Endoglucanase I overproduction. In *Molecular & general genetics : MGG* 241 (5-6), pp. 515–522.

Kelly, J. M.; Hynes, M. J. (1985): Transformation of Aspergillus niger by the amdS gene of Aspergillus nidulans. In *The EMBO journal* 4 (2), pp. 475–479.

Kikuchi, Yuko; Takai, Toshiro; Kuhara, Takatoshi; Ota, Mikiko; Kato, Takeshi; Hatanaka, Hideki et al. (2006): Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. In *Journal of immunology (Baltimore, Md. : 1950)* 177 (3), pp. 1609–1617.

Kubicek, Christian P.; Herrera-Estrella, Alfredo; Seidl-Seiboth, Verena; Martinez, Diego A.; Druzhinina, Irina S.; Thon, Michael et al. (2011): Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. In *Genome biology* 12 (4), pp. R40. DOI: 10.1186/gb-2011-12-4-r40.

Kubicek, Christian P.; Komon-Zelazowska, Monika; Druzhinina, Irina S. (2008): Fungal genus Hypocrea/Trichoderma: from barcodes to biodiversity. In *Journal of Zhejiang University. Science. B* 9 (10), pp. 753–763. DOI: 10.1631/jzus.B0860015.

Kuhls, K.; Lieckfeldt, E.; Samuels, G. J.; Kovacs, W.; Meyer, W.; Petrini, O. et al. (1996): Molecular evidence that the asexual industrial fungus Trichoderma reesei is a clonal derivative of the ascomycete Hypocrea jecorina. In *Proceedings of the National Academy of Sciences of the United States of America* 93 (15), pp. 7755–7760.

Lee, K. S.; Patton, J. L.; Fido, M.; Hines, L. K.; Kohlwein, S. D.; Paltauf, F. et al. (1994): The Saccharomyces cerevisiae PLB1 gene encodes a protein required for lysophospholipase and phospholipase B activity. In *The Journal of biological chemistry* 269 (31), pp. 19725–19730.

Martinez, Diego; Berka, Randy M.; Henrissat, Bernard; Saloheimo, Markku; Arvas, Mikko; Baker, Scott E. et al. (2008): Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). In *Nature biotechnology* 26 (5), pp. 553–560. DOI: 10.1038/nbt1403.

Masuda, N.; Kitamura, N.; Saito, K. (1991): Primary structure of protein moiety of Penicillium notatum phospholipase B deduced from the cDNA. In *European journal of biochemistry / FEBS* 202 (3), pp. 783–787.

Matser, Ariette M.; Steeneken, Peter A. M. (1998): Origins of the Poor Filtration Characteristics of Wheat Starch Hydrolysates. In *Cereal Chemistry* 75 (3), pp. 289–293. DOI: 10.1094/CCHEM.1998.75.3.289.

Merkel, O.; Fido, M.; Mayr, J. A.; Prüger, H.; Raab, F.; Zandonella, G. et al. (1999): Characterization and function in vivo of two novel phospholipases B/lysophospholipases from Saccharomyces cerevisiae. In *The Journal of biological chemistry* 274 (40), pp. 28121–28127.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

Nevalainen, H.; Suominen, P.; Taimisto, K. (1994): On the safety of Trichoderma reesei. In *Journal of biotechnology* 37 (3), pp. 193–200.

Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. (2006): Food-processing enzymes from recombinant microorganisms--a review. In *Regulatory toxicology and pharmacology : RTP* 45 (2), pp. 144–158. DOI: 10.1016/j.yrtph.2006.05.001.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (1992): Safety Considerations for Biotechnology. OECD, pp. 1–45.

Pariza, M. W.; Johnson, E. A. (2001): Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. In *Regulatory toxicology and pharmacology : RTP* 33 (2), pp. 173–186. DOI: 10.1006/rtph.2001.1466.

Penttilä, M.; Nevalainen, H.; Rättö, M.; Salminen, E.; Knowles, J. (1987): A versatile transformation system for the cellulolytic filamentous fungus Trichoderma reesei. In *Gene* 61 (2), pp. 155–164.

Rementeria, Aitor; López-Molina, Nuria; Ludwig, Alfred; Vivanco, Ana Belén; Bikandi, Joseba; Pontón, José; Garaizar, Javier (2005): Genes and molecules involved in Aspergillus fumigatus virulence. In *Revista iberoamericana de micología* 22 (1), pp. 1–23.

Shen, Da-Kang; Noodeh, Ali Dehghan; Kazemi, Abdolhassan; Grillot, Renée; Robson, Geoff; Brugère, Jean-François (2004): Characterisation and expression of phospholipases B from the opportunistic fungus Aspergillus fumigatus. In *FEMS microbiology letters* 239 (1), pp. 87–93. DOI: 10.1016/j.femsle.2004.08.019.

Słomińska, Lucyna; Niedbacha, Joanna (2009): Study on the influence of the so-called filtration enzyme action on the membrane filtration process of wheat starch hydrolysates. In *Desalination* 241 (1-3), pp. 296–301. DOI: 10.1016/j.desal.2007.10.101.

Takai, T.; Ichikawa, S.; Yokota, T.; Hatanaka, H.; Inagaki, F.; Okumura, Y. (2000): Unlocking the allergenic structure of the major house dust mite allergen der f 2 by elimination of key intramolecular interactions. In *FEBS letters* 484 (2), pp. 102–107.

Takai, T.; Yokota, T.; Yasue, M.; Nishiyama, C.; Yuuki, T.; Mori, A. et al. (1997): Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. In *Nature biotechnology* 15 (8), pp. 754–758. DOI: 10.1038/nbt0897-754.

Valenta, Rudolf (2002): The future of antigen-specific immunotherapy of allergy. In *Nature reviews. Immunology* 2 (6), pp. 446–453. DOI: 10.1038/nri824.

Valenta, Rudolf; Kraft, Dietrich (2002): From allergen structure to new forms of allergen-specific immunotherapy. In *Current opinion in immunology* 14 (6), pp. 718–727.

Wolf, M. de; Hilderson, H. J.; Lagrou, A.; Dierick, W. (1979): Lipolytic enzymes in bovine thyroid tissue. III. Lysophospholipase activity. In *Archives internationales de physiologie et de biochimie* 87 (1), pp. 1–18.