



21 March 2019
[75-19]

Supporting document 1

Risk and technical assessment report – Application A1165

Lysophospholipase from *Trichoderma reesei* as a processing aid (enzyme)

Executive summary

The purpose of the application is to seek amendment of Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme lysophospholipase (EC 3.1.1.5) from a genetically modified strain of *Trichoderma reesei* for use in starch processing, including the production of syrups.

The evidence evaluated to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the form and prescribed amounts is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

Molecular evidence confirmed the taxonomy of the production strain of the lysophospholipase gene as *T. reesei*. This fungus is not toxigenic or pathogenic and has a long history of safe use in the production of a number of enzyme processing aids that are already permitted in the Code. No extraneous genetic material is carried across from the donor organism (*Aspergillus nishimurae*) as part of the genetic modification. The genetic modification has been shown to be phenotypically stable.

No public health and safety concerns associated with the use of lysophospholipase from genetically modified *T. reesei* were identified as a result of this hazard assessment.

The enzyme was not genotoxic in a bacterial reverse mutation assay (Ames test) or a chromosomal aberration test in Chinese hamster lung fibroblast V79 cells. No adverse effects were observed in a 90-day oral gavage study in rats at doses up to 1000 mg/kg bw/day, equivalent to 995 mg/kg bw/day when expressed as total organic solids (TOS). The Theoretical Maximal Daily Intake (TMDI) in humans under the proposed conditions of use is equal to 0.006 mg/kg bw/day TOS. Consequently, the Margin of Safety (MoS) between the human TMDI and the NOAEL in rats is 159,167.

Bioinformatic searches did not identify any significant homology of the amino acid sequence of the enzyme with those of known toxins or allergens. The enzyme preparation may contain

OFFICIAL

traces of wheat. As wheat is a major food allergen, risk management measures are indicated to protect wheat-allergic individuals.

Based on the reviewed toxicological data it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) of 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

Table of contents

EXECUTIVE SUMMARY	1
1 INTRODUCTION	2
1.1 OBJECTIVES OF THE ASSESSMENT	2
2 FOOD TECHNOLOGY ASSESSMENT	2
2.1 CHARACTERISATION OF THE ENZYME	2
2.1.1 <i>Identity of the enzyme</i>	2
2.1.2 <i>Technological purpose of the enzyme</i>	3
2.1.3 <i>Technological justification for the enzyme</i>	4
2.2 MANUFACTURING PROCESS.....	5
2.2.1 <i>Production of the enzyme</i>	5
2.2.2 <i>Specifications</i>	7
2.2.3 <i>Stability</i>	8
2.3 FOOD TECHNOLOGY CONCLUSION	8
3 SAFETY ASSESSMENT	8
3.1 HISTORY OF USE.....	8
3.1.1 <i>Host organism</i>	8
3.1.2 <i>Gene donor organisms</i>	9
3.2 CHARACTERISATION OF THE GENETIC MODIFICATION(S).....	9
3.2.1 <i>Description of DNA to be introduced and method of transformation</i>	9
3.2.2 <i>Characterisation of inserted DNA</i>	10
3.2.3 <i>Genetic stability of the inserted gene</i>	10
3.3 SAFETY OF LYSOPHOSPHOLIPASE	10
3.3.1 <i>History of safe use of the enzyme</i>	11
3.3.2 <i>Toxicology studies in animals</i>	11
3.3.3 <i>Genotoxicity assays</i>	12
3.3.4 <i>Potential for toxicity</i>	14
3.3.5 <i>Potential for allergenicity</i>	14
3.3.6 <i>Approvals by other regulatory agencies</i>	15
4 DISCUSSION	15
5 CONCLUSIONS	15
6 REFERENCES	15

1 Introduction

AB Enzymes GmbH has made an application to FSANZ seeking permission to use the enzyme lysophospholipase (EC 3.1.1.5) from a genetically modified strain of *Trichoderma reesei* as a processing aid for use in starch processing, including the production of syrups. The starch sources are mainly wheat and maize/corn.

AB Enzymes GmbH is an industrial biotechnology company that develops, manufactures and supplies enzyme preparations for industrial applications. The enzyme preparation that is the subject of this application has been authorised for use in France (2013) and the United States (US GRAS GRN 653, 2016). A dossier on the enzyme has also been submitted to the European Food Safety Authority (EFSA) (EFSA-Q-2015-00410) and is currently under review.

Currently, there is one permission for lysophospholipase (sourced from *Aspergillus niger*) in the Australia New Zealand Food Standards Code (the Code) (FSANZ 2018). There is no permission for use of this enzyme sourced from *T. reesei*. Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

1.1 Objectives of the assessment

The objectives of this technical and risk assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by a genetically modified organism, as a processing aid. Specifically by considering the:
 - history of use of the host and gene donor organisms
 - characterisation of the genetic modification(s), and
 - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

Accepted IUBMB¹/common name: lysophospholipase

Systematic name: 2-lysophosphatidylcholine acylhydrolase

¹ International Union of Biochemistry and Molecular Biology.

IUBMB enzyme nomenclature: EC 3.1.1.5

CAS² number: 9001-85-8

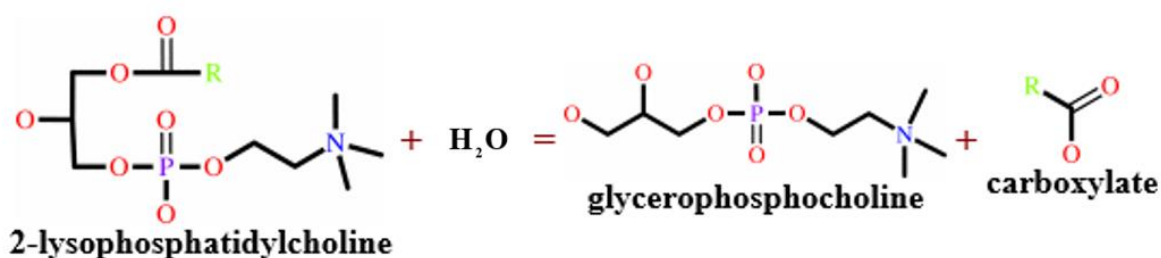
Other names: lecithinase B; lysolecithinase; phospholipase B; lysophosphatidase; lecitholipase; phosphatidase B; lysophosphatidylcholine hydrolase; lysophospholipase A1; lysophospholipase L2; lysophospholipase-transacylase; neuropathy target esterase; NTE; NTE-LysoPLA; NTE-lysophospholipase

Reaction: 2-lysophosphatidylcholine + H₂O = glycerophosphocholine + a carboxylate

2.1.2 Technological purpose of the enzyme

The enzyme is to be used as a processing aid in starch processing, including the production of syrups. The starch sources are mainly wheat and maize/corn.

Lysophospholipase catalyses the hydrolysis of an ester bond between a fatty acid and glycerol in lysophospholipids³. This results in the formation of glycerophosphatide and free fatty acids, as shown in Figure 1 below.



Source: https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/enzymes/GetPage.pl?ec_number=3.1.1.5

Figure 1 Representation of hydrolysis reaction catalysed by lysophospholipase

During starch processing, lysophospholipids present in starch can form micelles⁴. These can have a negative impact on the filtration rate of the starch hydrolysates (syrups), and can also form a complex with other constituents in the syrup (i.e. amylase), resulting in cloudiness. Lysophospholipase has a beneficial effect on starch processing by breaking down these lysophospholipids and, as such, preventing their negative effects.

The enzyme manufacturing process results in a concentrated lysophospholipase enzyme solution, which is formulated and sold mainly as a liquid enzyme preparation. The composition and physical properties of the lysophospholipase enzyme preparation sold under the commercial name of Rohalase® F, are presented below in Table 1 and Table 2,

² Chemical Abstracts Service.

³ Lysophospholipids are derivatives of phospholipids, a major component of the cell membranes of plants and animals. Lysophospholipids are also known to be the predominant phospholipids found in wheat starch (Morrison, 1988 as mentioned in Matser, Steeneken (1998)).

⁴ A micelle is a group of lysophospholipids that arrange themselves in a spherical form in aqueous solutions, with their polar heads forming the outside surface of the micelle, and their nonpolar tails facing into the micelle, away from the aqueous solution.

respectively.

Table 1 *Lysophospholipase enzyme preparation composition*

Composition of Rohalase® F (w/w)	
Water	31.35%
Glycerol	50%
Lysophospholipase	13.3%
Sodium chloride	5%
Sodium benzoate	0.35%

Table 2 *Lysophospholipase enzyme preparation physical properties*

Physical properties of Rohalase® F	
pH value	5-5.4
Density	1.00-1.10 g/ml
Appearance	Liquid product Light brown colour with characteristic odour Water soluble
Temperature range for function	45-70°C Optimum 60-65°C
pH range for function	4-7 Optimum 4-6
Min. enzyme activity	10,000 lysophospholipase Units/g.

Whilst the enzyme preparation contains predominantly lysophospholipase activity, there are also small amounts of other enzymatic side activities, which are known to be produced by *T. reesei*. These include xylanase, β -glucanase and cellulase. Supporting documentation showing the levels of these side activities, and the assays used to measure them was provided with the application. The documentation provided sufficient evidence that the side activities present in the lysophospholipase solution are not relevant to this application, mainly due to the small quantities involved, and when compared to enzyme preparations that have been standardized for those specific activities.

Use of commercial enzyme preparations are typically in accordance with Good Manufacturing Practice (GMP), whereby use is at a level that is not higher than the level necessary to achieve the desired enzymatic reaction. The recommended level of Rohalase® for initial trials on wheat starch hydrolysates is about 25–50 g/ton of dry solids. Food manufacturers will fine-tune the enzyme level based on this recommendation, and this will also be dependent on the application, the type and quality of the raw materials used, and the process conditions.

2.1.3 Technological justification for the enzyme

As mentioned above, lysophospholipids present during starch processing can have a

negative impact on the starch hydrolysates (syrup). The technological justification for using lysophospholipase is that it breaks down these lysophospholipids, resulting in the following beneficial effects:

- preventing the formation of lysophospholipid micelles
- facilitating the separation of undesired components
- contributing to a better and faster filtration rate
- improving the clarity of the filtrate.

Moreover, the lysophospholipase that is the subject of this application technologically justified because it has been found to have a higher enzyme activity compared to other enzyme products on the market. As a result, the level of use for a particular production situation is lower, and less excipients⁵ are added to the starch when the enzyme preparation is being applied during starch processing.

When used in wheat starch processing, this lysophospholipase improves the initial clarity of the syrup prior to further clarification steps. This results in further increases in the filtration rate, longer filtration runs, less use of diatomaceous earth (when using rotary vacuum filtration) and less membrane fouling and cleaning (when using cross-flow filtration).

The enzyme is inactivated after saccharification⁶, when the syrup is heated to 85°C and, as such, does not exert any residual enzymatic activity in the final syrups or the foods to which they are added.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by controlled submerged fermentation of the genetically modified production strain of *T. reesei*.

The production process comprises three main process steps: fermentation, recovery and preparation of the final product. Production begins with a pure culture of *T. reesei*, which is added to a shake flask containing fermentation medium. When sufficient biomass is obtained, the contents of the shake flasks are used to inoculate the seed fermenter. At the end of fermentation, the inoculum is then transferred to the main fermenter containing fermentation medium. Biosynthesis of lysophospholipase occurs in the main fermenter under well-defined process conditions governing the pH, temperature, time and mixing.

Once the fermentation is complete, the recovery process is initiated. The fermentation broth undergoes separation and concentration steps. The separation step involves removing the biomass from the fermentation medium containing the desired enzyme protein. This process is performed at defined pH and temperatures to minimise loss of enzyme activity. The concentration step ensures that the enzyme solution achieves the desired enzyme activity prior to formulation.

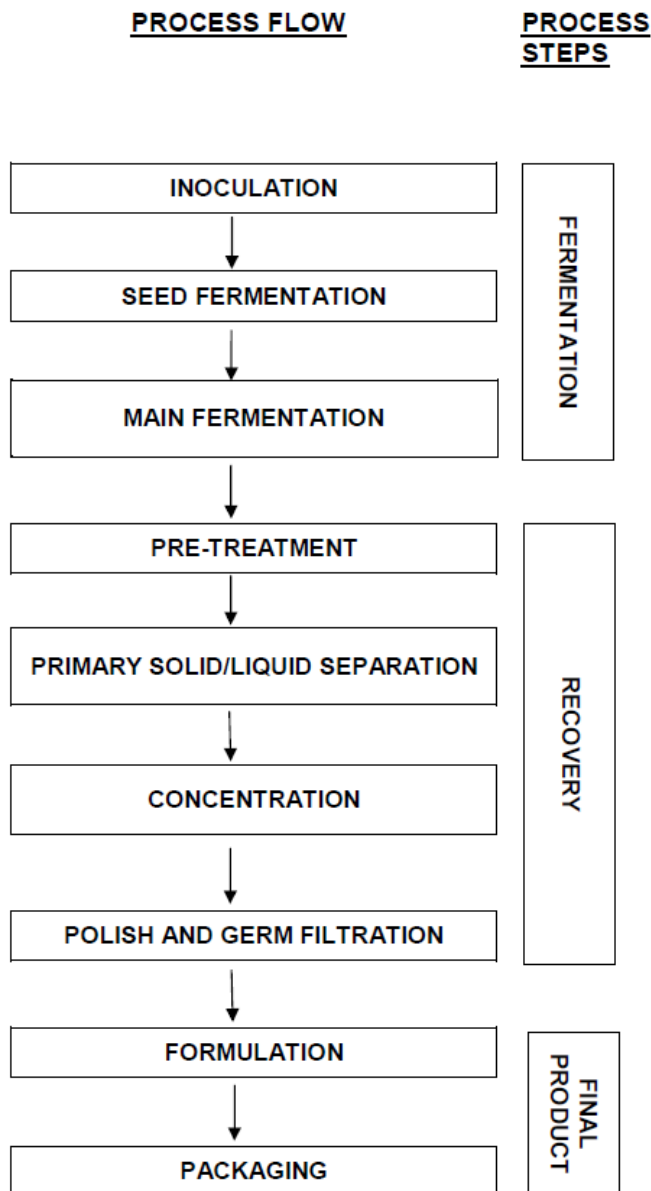
After concentration, residual cells of the production strain, microbial contamination and any other insoluble substances are removed via polish and germ filtration. The resultant concentrated enzyme solution is ready for formulating into a dry or liquid food enzyme preparation (see section 2.1.2) and then packaged and sold commercially.

⁵ Excipients are inactive substances in the enzyme preparation that serve as a vehicle for the enzyme.

⁶ Saccharification is the process of breaking down starch into sugars.

Materials used in the fermentation and recovery process are standard, food grade quality materials that meet quality standards and relevant specifications, including those outlined in Food Chemicals Codex (10th edition) (2016), that require that substances such as processing aids used to produce enzyme preparations be acceptable for general use in foods. Furthermore, production of the enzyme is in accordance with current GMP and Hazard Analysis and Critical Control Points (HACCP) principles, and in compliance with the food hygiene regulations that apply in the country of manufacture (e.g. [Regulation \(EC\) No. 852/2004](https://www.ecolex.org/details/legislation/regulation-ec-no-8522004-of-the-european-parliament-and-of-the-council-on-the-hygiene-of-foodstuffs-lex-faoc063426/) on the hygiene of foodstuffs⁷). The applicant has provided quality certificates demonstrating their adherence to these practices and principles.

The main steps of the manufacturing process are shown in Figure 2 below taken from the application.



⁷ <https://www.ecolex.org/details/legislation/regulation-ec-no-8522004-of-the-european-parliament-and-of-the-council-on-the-hygiene-of-foodstuffs-lex-faoc063426/>

Figure 2 Production process of lysophospholipase food enzyme from fermentation

2.2.2 Specifications

There are international specifications for enzymes used in the production of food. These have been detailed in the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Compendium of Food Additive Specifications (FAO/WHO 2016) and the United States Pharmacopeial Convention (2016) Food Chemicals Codex (10th edition). These primary sources of specifications are listed in Schedule S3—2 of the Code. Enzymes need to meet these enzyme specifications. Schedule 3 also includes specifications for heavy metals (section S3—4) if they are not specified within specifications in sections S3—2 or S3—3.

Table 3 provides a comparison of batch analysis of three liquid semi-final concentrates of lysophospholipase from the genetically modified *T. reesei* with the international specifications established by JECFA and Food Chemicals Codex, as well as those detailed in the Code (as applicable).

Table 3 Analysis of AB Enzymes lysophospholipase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Analysis	AB Enzymes batch analysis (3 different batches)	Specifications		
		JECFA	Food Chemicals Codex	Australia New Zealand Food Standards Code (section S3—4)
Lead (mg/kg)	<0.05 <0.05 <0.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.5 <0.5 -	-	-	≤1
Total heavy metals (As, Pb, Cd and Hg) (mg/kg)	<0.7 <0.7 -	-	-	≤1 (Cd) ≤1 (Hg)
Coliforms (cfu/g)	<1 <1 <1	≤30	≤30	-
<i>Salmonella</i> (in 25 g)	ND* ND ND	Absent	Negative	-
<i>E. coli</i> (in 25 g)	ND ND ND	Absent	-	-
Antimicrobial activity	ND ND ND	Absent	-	-

Mycotoxins	No significant amount of major ones in three samples	No toxicologically significant amounts	-	-
------------	--	--	---	---

*ND – not detected

Based on the above results of batch analysis, the enzyme meets international and Code specifications for enzymes used in the production of food.

2.2.3 Stability

The product technical data sheet for Rohalase® F indicates that the product should be stored in cool and dry conditions (<10°C). Under these storage conditions, Rohalase® F is best used within 24 months from the date of production.

2.3 Food technology conclusion

The use of this lysophospholipase enzyme to process starch, including the production of syrups, is clearly articulated in the application. This enzyme will be used to hydrolyse lysophospholipids in starch, which would otherwise have a negative impact on the filtration rate and clarity of the starch hydrolysates (syrups).

AB Enzymes' lysophospholipase is sourced from a genetically modified strain of *T. reesei*. Compared with other enzyme products on the market, this enzyme has been found to have a higher enzyme activity, which means the level of use is lower and less excipients are added during starch processing.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the form and prescribed amounts is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

3 Safety assessment

3.1 History of use

3.1.1 Host organism

T. reesei is a hypercellulolytic fungus commonly found in soil. The initial isolate came from deteriorating clothing and tent material found in the Solomon Islands after World War II. The initial isolate QM6a has been registered with the American Type Culture Collection (ATCC 13631) and has been classed as a Biosafety Level 1 organism, based on the [United States Public Health Service Guidelines](#)⁸, and is not considered pathogenic to humans. All strains of *T. reesei* used today in biotechnology have been derived from this particular isolate (Nevalainen et al 1994; Seidl et al 2009).

The production strain of *T. reesei* containing the lysophospholipase gene was characterized by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands as *Trichoderma reesei* E.G. Simmons. The characterisation was based on the DNA sequence of Internal Transcribed Spacer 1 and 2, and the 5.8S ribosomal RNA gene. The sequence had a 100% match with the *T. reesei* reference sequence for diagnosis of the International

⁸ <https://www.cdc.gov/biosafety/publications/bmbl5/index.htm>

Subcommission on *Trichoderma* and *Hypocrea* (<http://www.isth.info/>). Additional analyses with the Basic Local Alignment Search Tool (BLAST) also showed 100% similarity with the type species of *Trichoderma reesei*, confirming the taxonomy of the production strain.

Prior to generation of the final production strain (RF7206), the host organism was modified using a range of conventional mutagenesis and genetically engineered changes that are routinely used to optimise organisms for industrial use. During this development, a functional antibiotic resistance gene was spontaneously lost.

While lysophospholipase comprises the main enzyme in the product obtained from RF7206, other endogenous *Trichoderma* proteins (such as cellobiohydrolases, xylanases and endoglucanases) are also present in small amounts. Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al, 2016; [Schedule 18](#)⁹ of the Code). There is therefore a long history of safe use of *T. reesei* for the production of food grade enzymes.

3.1.2 Gene donor organisms

Aspergillus nishimurae

The gene sequence for the lysophospholipase enzyme was based on the gene from *A. nishimurae* (strain RH3949). This organism is a filamentous fungus, commonly found in soil (Takada et al. 2001; Hosoe et al. 2011). There is limited documentation on the toxicity or pathogenicity of this fungus. However, as the gene sequence for lysophospholipase has been manipulated through standard DNA cloning methods subsequent to the original isolation from the donor organism, extraneous material from *A. nishimurae* would not be carried across to the enzyme production organism.

Aspergillus nidulans

The gene sequence for acetamidase, used as the selection marker for positive transformants, was initially isolated from *Aspergillus nidulans* (Kelly and Hynes, 1985). The majority of *A. nidulans* strains have been classed at the Biosafety Level 1, however some strains have been associated with opportunistic infections in immunocompromised individuals (Gabrielli et al, 2014; Henriet et al. 2012). As the gene sequence has been manipulated through standard DNA cloning methods subsequent to the original isolation from the donor organism, extraneous material from *A. nidulans* would not be carried across to the enzyme production organism.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

T. reesei strain RF7206 was produced by a protoplast-mediated transformation procedure, using protoplasts prepared from mycelia (Penttilä et al, 1987; Karhunen et al, 1993). A purified linear DNA fragment (i.e. free from any vector-derived sequences) isolated from a plasmid and containing two coding regions was included in the transformation mix to create the production strain. The first coding region, driven by a single, strong native (*T. reesei*) promoter, contained the *lpl* gene from *A. nishimurae* with a native *T. reesei* terminator. The second coding region comprised the acetamidase gene (*amdS*) from *A. nidulans* with its own promoter (Kelly and Hynes, 1985), a 3' flanking region from *T. reesei* to facilitate targeting to

⁹ <https://www.legislation.gov.au/Details/F2018C00369/Download>

a specific locus in *T. reesei*, and a *T. reesei* terminator. This second gene allows for selection of transformants on acetamide-containing media and has been widely used as a selection marker in fungal transformations in the past.

The parental strain does not contain genes with antibiotic activities and no antibiotic resistance genes have been incorporated during transformation.

3.2.2 Characterisation of inserted DNA

Southern blotting was performed on genomic DNA extracted from the production and parental host strain. DNA samples were digested separately with two appropriate restriction enzymes, and hybridisation was performed with a probe that encompassed the complete expression cassette introduced into the production strain. The resulting hybridisation bands in the production strain DNA showed that at least one complete insert has been integrated into the host's genome. As expected, because of the presence of the native regulatory elements in the parental strain, some hybridisation bands were also observed in the parental strain DNA.

3.2.3 Genetic stability of the inserted gene

T. reesei strains are widely used in biotechnological processes because of their known stability.

Southern blotting was performed on genomic DNA extracted from spores of RF7206 grown without acetamide on potato dextrose agar over 10 generations. The DNA samples were digested with one of the restriction enzymes used for the characterisation described in section 3.2.2 and hybridisation was performed with the same probe. The results for the generations tested (1, 2, 3, 4, 5, 7, 10) were all similar as well as being similar to the equivalent Southern blot analysis discussed in section 3.2.2. This indicated that expression of the *lpl* gene was consistent across generations.

Phenotypic stability was confirmed by measurement of the same lysophospholipase activity across a number of fermentation batches.

3.3 Safety of lysophospholipase

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass this range of type and function, the safety assessment of the novel protein must consider if there is a history of safe use, and whether there are any potential toxic or allergenic effects.

The *lpl* gene sequence expressed in RF7206 is identical to that in the *A. nishimurae* donor.

The enzyme preparation as marketed is 13.3% w/w lysophospholipase. Other components are glycerol (50% w/w), water (31.4%), sodium chloride (5%) and sodium benzoate (0.35%). None of these components give cause for concern at the very low levels of exposure that would result from the use of the enzyme as a processing aid.

3.3.1 History of safe use of the enzyme

The specific enzyme that is the subject of this application has been approved for use in France since July 2013, and in the USA since October 2016 (see section 3.3.6).

3.3.2 Toxicology studies in animals

One study in rats was submitted as part of the application.

Ninety-day oral gavage study in Wistar rats (Harlan Laboratories Ltd., Study Number B99180, May 2009). Regulatory status: GLP. Conducted according to OECD Guideline No. 408.

The test article for this study was the enzyme that is the subject of this application; that is, lysophospholipase from *T. reesei* RF7206. The test article was provided by the Sponsor in dried form (96.3% dry matter) with a lysophospholipase content of 44.2 mg/g, equal to 6.67% of the total protein. The enzyme preparation comprised 66.3% protein, 28.8% carbohydrate, 0.8% ash and 0.4% lipid. The vehicle and control article was bidistilled water, in which the test article was administered as a suspension. Dose formulations were prepared weekly on the basis of stability data from a preliminary study. Concentration, homogeneity and stability of dose formulations were determined on Days 1 and 7, and concentration of dose formulations was also determined during Weeks 6 and 13. Dose concentrations were within 20% of nominal concentrations.

Wistar rats, 40/sex, were delivered at approximately 7 weeks of age and housed in groups of 5 in polycarbonate cages under standard laboratory environmental conditions. Water and standard pelleted rat diet were provided *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days prior to commencement of the study. From Day 1 of study, 10 rats/sex/group were gavaged once daily with 0, 100, 300 or 1000 mg/kg bw/day, at a dose volume of 10 mL/kg bw for 13 weeks.

Parameters measured in all rats during the in-life phase of the study were survival, clinical observations, food consumption, body weights and body weight changes, functional observational battery, grip strength and motor activity. Cageside mortality/moribundity checks were conducted twice daily, cageside clinical observations were made twice daily on Days 1-3 and daily thereafter, and detailed observations were made weekly. Detailed observations included assessment of appearance, motor function, behaviour, respiration, reflexes, muscle tone, foot colour and external examination of the eyes. Food consumption and body weights were recorded weekly. Functional observational battery (FOB), and assessments of grip strength and motor activity, were conducted during Week 13.

Ophthalmoscopic examinations were performed on all rats during the acclimatisation period, and in the control and 1000 mg/kg bw/day groups during Week 12 of the study. No differences were identified between control and 1000 mg/kg bw/day groups, and therefore no other groups were examined.

At the end of the in-life phase, all rats were fasted overnight in metabolism cages, with *ad libitum* access to water, and urine was collected. Rats were then lightly anesthetized with isoflurane, and blood was collected from the retro-orbital plexus. Comprehensive assessment of urinalysis, haematology, clinical chemistry and coagulation parameters was conducted, including measurement of methaemoglobin. Rats were then weighed, anaesthetized and killed. Weights of adrenal glands, brain, heart, kidneys, liver, spleen, thymus, and either testes and epididymides or ovaries and uterus, as sex-appropriate. A comprehensive list of organs was collected and fixed for histopathological examination.

Slides were prepared for all tissues of rats in the control and 1000 mg/kg bw/day groups, and for all tissues in which gross lesions were observed at necropsy.

All rats survived to scheduled termination and there were no treatment-related effects on clinical signs, performance on FOB, grip strength, locomotor activity, ophthalmological findings, haematology, gross necropsy findings, or histopathological findings. Group mean body weight of the 1000 mg/kg bw/day males was slightly lower than that of control males but the difference was not statistically significant.

A slight decrease in group mean food consumption, expressed as g/animal/day, was observed in all treatment groups when compared to control groups of the same sex, and this effect increased slightly with dose in males but not in females. The effect became apparent after 15-22 days in males, and 36-43 days in females. However the decreased food consumption did not result in decreased body weight or body weight gain. It was concluded that the effect was treatment-related but not adverse.

A small number of group mean values for clinical biochemistry parameters were significantly different to those of sex-matched controls, although they were not considered to be adverse because the values remained within the historical control range for the laboratory, there were no histopathological correlates, and a clear dose-response relationship was not evident. The differences were therefore not considered to be adverse or clearly related to the test article. Likewise, an increase in urinary bilirubin in 1000 mg/kg bw/day females remained within the historical control range.

The group mean heart weight of the 1000 mg/kg bw/day males was significantly less than that of male controls, but this effect was not evident when organ:body weight and organ:brain weight ratios were considered, which indicates that it is an effect of the slightly lower group mean bodyweight of the 1000 mg/kg bw/day males. Group mean kidney weights of females in all treatment groups were lower than those of control females, both in absolute terms and when compared to brain weight, but there were no histopathological correlates and the findings were therefore not considered to be adverse. Statistically significant differences to those of controls in absolute and relative ovary weight of 300 mg/kg bw/day females and in uterus:brain weight ratio in 100 mg/kg bw/day females did not show a dose-response relationship and had no histopathological correlates.

The NOAEL was the highest dose tested, 1000 mg/kg bw/day, equivalent to 995 mg/kg bw/day when expressed as total organic solids (TOS).

3.3.3 Genotoxicity assays

Two genotoxicity assays were submitted as part of the application.

Bacterial reverse mutation assay (RCC-Cytotest Cell Research GmbH, Study Number 1155101, April 2008). Regulatory status: GLP. Conducted in compliance with Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471 (July 21, 1997) and Commission Directive 2000/32/EC, L1362000, Annex 4D (May 19, 2000).

The test article for this study was the lysophospholipase from *T. reesei* RF7206. The purity of the batch was not provided to the testing laboratory by the sponsor. Test article was dissolved in deionised water (DI water) on the day of the experiment. No precipitation from the solution was observed up to the highest investigated dose.

The test systems were strains of *Salmonella typhimurium*. For frameshift mutations, *S. typhimurium* strains TA 1537 and TA 98 were used, and for assays for base-pair

substitutions, *S. typhimurium* strains TA 1535, TA 102 and TA 100 were used. For assays in the absence of metabolic activation (S9 mix), positive control substances were sodium azide in DI water for TA 1535 and TA 100; 4-nitro-o-phenylene-diamine in DMSO for TA 1537 and TA 98; and methyl methanesulfonate in DI water for TA 102. For assays in the presence of S9 mix for metabolic activation, 2-aminoanthracene in DMSO was used as the positive control for all test strains. As negative controls, concurrent untreated and solvent (DI water) control cultures were performed. All assays were performed in triplicate.

For Experiment 1, the test article concentrations were 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, whereas for Experiment II the test article concentrations were 33, 100, 333, 1000, 2500 and 5000 µg/plate. In a test-tube 100 µL test solution, negative control or positive control; 500 µL of S9 mix or S9 mix substitution buffer; 100 µL of bacterial suspension; and 2000 µL overlay agar were mixed. For Experiment 1 (plate incorporation method), the mixture was then poured on minimal agar plates, while for Experiment II (pre-incubation method) the mixture of test solution, S9 mix or substitution buffer and bacterial suspension was incubated at 37°C for 60 min prior to addition of the overlay agar and pouring onto minimal agar plates. After solidification all plates were inverted and incubated for at least 48 h at 37° C after which bacterial colonies were counted.

No toxic effects were observed in any test group, with or without metabolic activation, and no biologically significant increase in revertant colony numbers was observed in any of tester strains following incubation with any of the tested concentrations of the test article. The expected increases in induced revertant colonies were observed with all positive controls. It was concluded that under the conditions of the assays, there was no evidence that the test article induced either base pair changes or frame shift mutations in any of the test strains.

Chromosome aberration test in Chinese hamster V79 cells (RCC-Cytotest Cell Research GmbH, Study Number 1155102, April 2008) Regulatory status: GLP. Conducted in compliance with Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473 (July 21, 1997) and Commission Directive 2000/32/EC, L1362000, Annex 4A (May 19, 2000).

The test article for this study was the lysophospholipase from *T. reesei* RF7206. The purity of the batch was not provided to the testing laboratory by the sponsor. Test article was dissolved in DI water on the day of the experiment. The final concentration of water in the culture medium was 10% v/v. Positive control substances were ethylmethane sulfonate dissolved in nutrient medium for assays without metabolic activation, and cyclophosphamide dissolved in saline (9% w/v) for assays with metabolic activation (S9 mix). The test system comprised Chinese hamster lung fibroblast V79 cells.

A range-finding pre-test was performed to determine the cytotoxicity of the test article. V79 cells were incubated in the presence of the test article at concentrations of 0, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/mL. Incubations were conducted either with or without S9 mix for 4 hours, and without S9 mix for 24 hours. At the end of incubation, cells were stained and counted. All incubations were conducted in duplicate. No cytotoxic effects were observed. The highest dose tested was therefore selected as the high dose for the chromosomal aberration assays.

Two independent experiments were conducted. Concentrations of test article in both experiments were 156.3, 312.5, 625, 1250, 2500 and 5000 µg/mL, but only the three highest concentrations were scored. Both experiments were conducted in duplicate. In Experiment I, cells, with or without S9 mix, were exposed to the test article for 4 hours and then the culture medium was changed, and incubation continued for a further 14 hours, after which cells were stained and counted. In Experiment II, the same conditions as in Experiment I were applied

in the presence of S9 mix, but for the assay without S9 mix, the cells were exposed to the test article for the full 18-hour incubation. No structural chromosome aberrations were observed. The positive controls induced statistically significant increases in the incidence of structural chromosomal aberrations, confirming the validity of the assays.

It was concluded that lysophospholipase is not clastogenic under the experimental conditions used.

3.3.4 Potential for toxicity

A homology search was performed using the BLAST-P program, version 2.6.1+ (<http://blast.ncbi.nlm.nih.gov/>), using the amino acid sequence of the lysophospholipase that is the subject of this application as the query sequence. An additional search criterion was to limit the search to sequences related to toxins. To evaluate the significance of reported matches, control searches were conducted using five different shuffled versions of the amino acid sequence of the lysophospholipase that is the subject of this application. For all searches, the hit size value was increased from the default value of 100 to the maximum value of 20,000, while the E-value threshold was decreased from 10 to 100. Both of these alterations from the control values maximised the chance of even very weak matches being identified.

The search was conducted in October of 2017. No significant homology to any known protein toxin was found.

3.3.5 Potential for allergenicity

Sequence homology searches between the mature *A. nishimurae* LPL amino acid sequence and the sequences in two allergen databases were conducted. The allergen databases were AllergenOnline (FARRP) (<http://www.allergenonline.org/>) and Allergen Database for Food Safety (ADFS) (<http://allergen.nihs.go.jp/ADFS/>). The alignment methods were:

- a. Alignment of the entire amino acid sequence to sequences in the databases
- b. Alignment of sliding 80 amino acid windows of the enzyme amino acid sequence to known allergens
- c. For the ADFS search, the motif-based method.

These searches are consistent with recommendations of EFSA (2010) and FAO/WHO (2001). In addition, a search for sequences of 8 identical amino acids was conducted using the FARRP database. The alignment tools FASTA and BLAST were used, and the default alignment parameter settings, were used for each search. The database searches were performed in January 2015.

No alignments were identified between the PL mature amino acid sequence and sequences in the ADFS database. Alignments in the FARRP database were below the 35% identity limit identified by Codex (Codex Alimentarius, 2003). The allergen Tha p 2 from the pine processionary moth (*Thaumetopoea pityocampa*) showed 34.2% identity in a 38 amino acid overlap with the LPL sequence. There were also five hits of 33.8% identity in a 68 amino acid overlap with an allergen from soybean (*Glycine max*). E-values were in the range 0.31 to 0.82. E-values larger than 1×10^{-7} are considered unlikely to represent proteins that may share immunologic or allergic cross-reactivity (Hileman et al., 2002).

No matches with >35% identity were found using the 80 amino acid sliding window. No matches were found using the 8 amino acid sliding window in the FARRP database, and no hits were found in the ADFS database when the allergenicity was predicted using a motif-

based method.

The enzyme preparation may contain traces of wheat.

3.3.6 Approvals by other regulatory agencies

In 2013, the French National Agency for Food, Environmental and Occupational Health & Safety (ANSES) approved the enzyme for the intended use ([Application # 2012-SA-0259](#)¹⁰).

The United States Food & Drug Administration (FDA) responded to a Generally Recognised as Safe (GRAS) notification for the enzyme preparation under the intended conditions of use ([GRN000653](#)¹¹) in October 2016, stating that the agency had no questions.

4 Discussion

Molecular evidence confirmed the taxonomy of the production strain of *T. reesei* containing the lysophospholipase gene. This fungus is not toxigenic or pathogenic and has a long history of safe use in the production of a number of enzyme processing aids that are already permitted in the Code. No extraneous genetic material is carried across from the donor organism as part of the genetic modification. The genetic modification has been shown to be stably inherited.

No public health and safety concerns associated with the use of lysophospholipase from genetically modified *T. reesei* were identified as a result of this hazard assessment.

The enzyme was not genotoxic in a bacterial reverse mutation assay (Ames test) or a chromosomal aberration test in Chinese hamster lung fibroblast V79 cells. No adverse effects were observed in a 90-day oral gavage study in rats at doses up to 1000 mg/kg bw/day, equivalent to 995 mg/kg bw/day when expressed as TOS. The Theoretical Maximal Daily Intake (TMDI) in humans under the proposed conditions of use is TOS equal to 0.006 mg/kg bw/day. Consequently, the Margin of Safety (MoS) between the human TMDI and the NOAEL in rats is 159,167.

Bioinformatic searches did not identify any significant homology of the amino acid sequence of the enzyme with those of known toxins or allergens. However the enzyme may contain traces of wheat and risk management measures may be required for people with wheat allergy.

5 Conclusions

Based on the reviewed information concerning the source and donor organisms, the genetic modification and the toxicological data, it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) of 'not specified' is appropriate for the general population. A dietary exposure assessment was therefore not required.

6 References

Codex Alimentarius (2003) Codex Alimentarius (2003). Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms. (CAC/GL 46-2003).

¹⁰ <https://www.anses.fr/en/system/files/BIOT2012sa0259.pdf>

¹¹ <https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=653>

- Delaney B, Astwood JD, Cunny H, Conn RE, Herouet-Guichenev C, MacIntosh S, Meyer LS, Privalle L, Gao Y, Mattsson J, Levine M, ILSI Food Biotechnology Committee Task Force on Protein Safety (2008). Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46: S71-S97.
- FAO/WHO (2016) General specifications and considerations for enzyme preparations used in food processing. <http://www.fao.org/docrep/009/a0691e/A0691E03.htm>
- FSANZ (2018) Australia New Zealand Food Standards Code. <http://www.foodstandards.gov.au/code/Pages/default.aspx>
- Gabrielli E, Fothergill AW, Brescini L, Sutton DA, Marchionni E, Orsetti E, Staffolani S, Castelli P, Gesuita R, Barchiesi F (2014) Osteomyelitis caused by *Aspergillus* species: A review of 310 reported cases. *Clinical Microbiology and Infection* 20:559-565.
- Henriet SV, Verweij PE, Warris A (2012) *Aspergillus nidulans* and chronic granulomatous disease: a unique host-pathogen interaction. *The Journal of Infectious Diseases* 206: 1128–1137.
- Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, Astwood JD and Hefle SL (2002). Bioinformative methods for allergenicity assessment using a comprehensive allergen database. *International Archives of Allergy and Immunology* 128: 280-291.
- Hosoe T, Mori N, Kamano K, Itabashi T, Yaguchi T, Kawai K (2011) A new antifungal yellow pigment from *Aspergillus nishimurae*. *The Journal of Antibiotics* 64: 211-212.
- IUBMB (2017) EC 3.1.1.5. <http://www.sbcs.gmul.ac.uk/iubmb/enzyme/EC3/0101a.html#05>
- Karhunen T, Mäntylä A, Nevalainen KM, Suominen P. L. (1993) High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. *Molecular & General Genetics* 241 (5-6): 515–522.
- Kelly JM, Hynes MJ (1985) Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans* *The EMBO Journal* 4(2) 475 – 479.
- Matser A M, Steeneken P A M. (1998) Origins of the poor filtration characteristics of wheat starch hydrolysates. *Cereal Chemistry* 75(3) 289–293.
- Nevalainen H, Peterson R (2014) Making recombinant proteins in filamentous fungi - are we expecting too much? *Frontiers of Microbiology* 5:e75
- Nevalainen H, Suominen P, Taimisto K (1994) On the safety of *Trichoderma reesei*. *Journal of Biotechnology* 37 (3): 193–200.
- Paloheimo M, Haarmann T, Mäkinen S, Vehmaanperä J (2016) Production of industrial enzymes in *Trichoderma reesei*. In: Schmoll M, Dattenböck C (Eds) *Gene expression systems In Fungi: Advancements and Applications*. Springer International Publishing, Switzerland, p.23-57
- Penttilä M, Nevalainen H, Rättö M, Salminen E, Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61 (2): 155–164.
- Seidl V, Seibel C, Kubicek CP, Schmoll M (2009) Sexual development in the industrial workhorse *Trichoderma reesei*. *Proceedings of the National Academy of Sciences* 106 (33) 13909-13914; <https://doi.org/10.1073/pnas.0904936106>
- Takada M, Horie Y, Abliz P (2001) Two new heterothallic *Neosartorya* from African soil. *Mycoscience* 42: 361-367.
- The United States Pharmacopeia (2016) *Food Chemicals Codex 10th Edition*, United States Pharmacopeial Convention, Rockville, MD. <http://publications.usp.org/>