A Lipase Enzyme
from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DUPONT AUSTRALIA PTY LTD
Submitted by: AXIOME PTY LTD

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EXECUTIVE SUMMARY:

DuPont Industrial Biosciences (IB) is seeking approval for a "Lipase, triacylglycerol (EC 3.1.1.3)" enzyme for production of bakery products such as, but not limited to, bread, Chinese stem buns, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers, and waffles. Lipase 3 will also be used for the production of beer and other cereal-based beverages. The enzyme is designated as "Lipase 3" throughout the dossier.

The enzyme Lipase 3 is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress the Lipase 3 gene from *Aspergillus niger var. tubingensis (*hereafter referred to as *Aspergillus tubingensis)*.

The enzyme is intended for use in the baking and brewing processes. In baking, Lipase 3 performs its technological function during the dough or batter handling to improve the dough stability and dough handling properties. In brewing processes, Lipase 3 performs its technological function in the mashing and fermentation step for removal of the fatty lipids which otherwise affect the mash separation and the yeast fermentation.

In all of these applications, Lipase 3 will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

To assess the safety of the Lipase 3 for use in these applications, Dupont IB vigorously applied the criteria identified in the guidelines as laid down by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilizing enzyme toxicology/safety data, the safe history of use of enzyme preparations from *T. reesei* and of other lipase enzymes in food, the history of safe use of the *T. reesei* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

In addition, different endpoints of toxicity were investigated at MB Research Laboratories (Pennsylvania) and Harlan Laboratories (Switzerland) and the results are evaluated and assessed in this document. Lipase 3 is non-hazardous based on acute oral studies. In genotoxicity studies, Lipase 3 is not mutagenic, clastogenic or aneugenic. Daily oral administration of Lipase 3 up to and including a dose level of 160.6 mg total protein/kg bw/day or 123.15 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming Lipase 3 from the bakery products and brewing process, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.410 mg TOS/kg body weight/day. This still offers a 300× fold margin of safety.

Based on the results of safety studies and other evidence, Lipase 3 has been demonstrated as safe for its intended applications and at the proposed usage levels. Approval of this application would provide manufacturers and/or consumers with benefits of facilitating the baking or brewing process, lowering the manufacturing cost, and improving quality of final foods.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Axiome Pty Ltd on behalf of DuPont Australia Pty Ltd

(b) Company:

DuPont Australia Pty Ltd

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(e) Email Address:

See above

(f) Nature of Applicants Business:

DuPont Australia Pty Ltd – A subsidiary of E. I. du Pont de Nemours and Company, manufacturer/marketer of specialty food ingredients, food additives and food processing aids.

Axiome Pty Ltd – regulatory & scientific affairs consultants

(g) Details of Other Individuals etc.:

No other individuals, companies or organizations are associated with this application.

1.2 Purpose of the application

This application seeks to modify Schedule 18 section S18-4 Permitted Enzymes – Enzymes of Microbial Origin for Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application.

This application is made solely on behalf of DuPont Industrial Biosciences (IB), the manufacturer/marketer of the *Processing Aid*. When approved, the *Processing Aid* would be available for use by any food manufacturer in Australia and New Zealand.

Approval of this application would require amendment to Schedule 18 section S18—4(5) Permitted Enzymes – Enzymes of Microbial Origin:

Table 1: regulatory impact statement.

Lipase, triacylglycerol (EC 3.1.1.3)	*Aspergillus niger* *Aspergillus oryzae* *Aspergillus oryzae*, containing the gene for Lipase, triacylglycerol isolated from *Fusarium oxysporum* *Aspergillus oryzae*, containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginosa* *Aspergillus oryzae*, containing the gene for Lipase, triacylglycerol isolated from *Rhizomucor miehei* *Candida rugosa* *Hansenula polymorpha*, containing the gene for Lipase, triacylglycerol isolated from *Fusarium heterosporum* *Mucor javanicus* *Penicillium roquefortii* *Rhizopus arrhizus* *Rhizomucor miehei* *Rhizopus niveus* *Rhizopus oryzae* "*Trichoderma reesei,* containing the gene for Lipase, triacylglycerol isolated from *Aspergillus tubingensis"*

Currently no lipase from *T. reesei* is permitted as a Processing Aid, however other enzymes including Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* are listed in Schedule 18 section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Section 2.3 and Appendix A.

1.3 Justification for the application

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application

Lipase 3 is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the Lipase 3 from *A. tubingensis.* The enzyme is characterized as a Triacylglycerol lipase (EC 3.1.1.3). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

The enzyme is intended for use in the baking, and brewing processes. In baking, Lipase 3 performs its technological function during the dough or batter handling to improve the dough stability and dough handling properties. In brewing processes, Lipase 3 performs its technological function in the mashing and fermentation step for removal of the fatty lipids which otherwise affects the mash separation and the yeast fermentation.

More information on the benefit of this enzyme can be found in Section 2.2 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no lipase from *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade

The inclusion of triacylglycerol lipase from *A. tubingensis* expressed in *T. reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product, and reduce technical barriers to trade.

1.4. Support for the application

No marketing or promotional activities have been undertaken for Lipase 3 derived from *T. reesei* containing the gene for Lipase 3 from *A. tubingensis* in the Australia/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5. Assessment Procedure

This application seeks to modify Schedule 18 section S18-4(5) for Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, DuPont IB considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6. Confidential Commercial Information (CCI)

Certain (identified) technical and manufacturing information included in Appendix B1, B2, B3, B4, Appendix E and other information including amino acid sequences labelled with

Confidential Commercial information is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

1.7. Exclusive Commercial Capturable Benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8. International and other National Standards

Refer to Appendix D for further details

1.8.1 Codex Standards

Lipase 3 produced by *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Lipase 3 derived from *T. reesei* carrying the gene encoding the Lipase 3 gene from *A. tubingensis* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in production of bakery products and brewing process by a panel of scientific experts in the USA.

1.9. Statutory declaration

I,

of Australia, regulatory affairs consultant:

make the following declaration under the *Statutory Declarations Act 1959:*

1) The information provided in this application fully sets out the matters required

2) The information provided in this application is true to the best of my knowledge and belief

3) No information has been withheld which might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Signature: _____

Declared at _____ on _____ of _____

Before me,

Signature: _____

1.10. Checklist

CHECKLIST FOR STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD

This checklist will assist you in determining if you have met the information requirements as detailed in the Application Handbook. Section 3.1 – General Requirements is mandatory for all applications. Sections 3.3.1-3.3.3 are related to the specifics of your application and the information required is in addition to section 3.1.

General Requirements (3.1)

☒ Form of application	☒ Assessment procedure
☒ Applicant details	☒ Confidential Commercial Information
☒ Purpose of the application	☒ Exclusive Capturable Commercial Benefit
☒ Justification for the application	☒ International standards
☒ Information to support the application	☒ Statutory Declaration

Food Additives (3.3.1)

☐ Support for the application	☐ Analytical detection method
☐ Nature and technological function information	☐ Toxicokinetics and metabolism information
☐ Identification information	☐ Toxicity information
☐ Chemical and physical properties	☐ Safety assessments from international agencies
☐ Impurity profile	☐ List of foods likely to contain the food additive
☐ Manufacturing process	☐ Proposed levels in foods
☐ Specifications	☐ Percentage of food group to contain the food additive
☐ Food labelling	☐ Use in other countries (if applicable)

Processing Aids (3.3.2)

☒ Support for the application	☒ Information on enzyme use on other countries (enzyme only)
☒ Type of processing aid	☒ Toxicity information of enzyme (enzyme only)
☒ Identification information	☒ Information on source organism (enzyme from micro-organism only)
☒ Chemical and physical properties	☒ Pathogenicity and toxicity of source micro-organism (enzyme from micro-organism only)
☒ Manufacturing process	☒ Genetic stability of source organism (enzyme from micro-organism only)
☒ Specification information	☒ Nature of genetic modification (PA from GM micro-organism only)
☐ ~~Industrial use information (chemical only)~~	☒ List of foods likely to contain the processing aid

- ☐ ~~Information on use in other countries (chemical only)~~
- ☐ ~~Toxicokinetics and metabolism information (chemical only)~~
- ☐ ~~Toxicity information (chemical only)~~
- ☐ ~~Safety assessments from international agencies (chemical only)~~
- ☐ Anticipated residue levels in foods
- ☐ Percentage of food group to use processing aid
- ☐ Information on residues in foods in other countries (if available)

Nutritive Substances (3.3.3)

- ☐ Support for the application
- ☐ Identification information
- ☐ Information on chemical and physical properties
- ☐ Impurity profile information
- ☐ Manufacturing process information
- ☐ Specification information
- ☐ Analytical detection method
- ☐ Proposed food label
- ☐ Toxicokinetics and metabolism information
- ☐ Animal or human toxicity studies
- ☐ Safety assessments from international agencies
- ☐ List of food groups or foods likely to contain the nutritive substance
- ☐ Proposed maximum levels in food groups or foods
- ☐ Percentage of food group anticipated to contain nutritive substance
- ☐ Food consumption data for new foods
- ☐ Information on use in other countries
- ☐ Food consumption data for foods with changed consumption patterns
- ☐ Nutritional purpose
- ☐ Need for nutritive substance in food
- ☐ Demonstrated potential deficit or health benefit
- ☐ Consumer awareness and understanding
- ☐ Actual or potential behaviour of consumers
- ☐ Demonstration of no adverse affects to any population groups
- ☐ Impact on food industry
- ☐ Impact on trade

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The lipase (Lipase 3) enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the Lipase 3 gene from *A. tubingensis*.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code section 1.3.3-6 Enzymes.

2.2. Identity

2.2.1 Chemical/Common Name:

The systematic name of the principle enzyme activity is triacylglycerol acylhydrolase. Other names used are lipase (*ambiguous*); butyrinase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno *N*-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase, Lipase 3.

- EC number: 3.1.1.3
- CAS number: 9001-62-1

Biological source: The lipase (Lipase 3) enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei*, carrying the Lipase 3 gene from *Aspergillus tubingensis*.

2.2.2 Marketing Name of the Processing Aid:

An example marketing name of Lipase 3 could be Powerbake 6XXX. The exact marketing name of the enzyme preparation has not been confirmed at this stage.

2.2.3 Molecular and Structural Formula:

Lipase 3 is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

The function of Lipase 3 is to catalyse the hydrolysis of ester bonds primarily in 1 and 3 position of fatty acids in triglycerides with release of fatty acids and glycerol. Lipase 3 will be used as follows:

Baking:

Lipase 3 can be used in dough for bread making to improve the quality of the baked products. Wheat flour used for bread making typically contains ~2% lipids. Half of these lipids are non-polar lipids including triglycerides, diglycerides, sterols and sterol esters. The remainder of the lipids in wheat flour are polar lipids containing mainly galactolipids and phospholipids.

Lipase 3 can be used in dough to modify these endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids. The non-polar triglycerides in dough will also be modified during formation of mono- and diglycerides and free fatty acids. The properties of the endogenous lipids produced by addition of Lipase 3 contribute to improved dough stability and dough handling properties, and the polar lipids formed in the dough will produce bread with improved volume and a homogenous crumb structure with homogenous pores.

Noodles and pasta are normally produced by using the same raw material, wheat flour, which is used for the manufacture of bread. In pasta or noodle dough, Lipase 3 also modifies the endogenous wheat lipids and produces more polar lipids. This in turn improves the quality of the finished noodle or pasta product giving a product which has a lower cooking loss and improved quality upon consumption.

Brewing processes and cereal-based drinks:

Lipase 3 can be added to the brewing process to enhance the mash separation and fermentation process in production of primarily non-malted brewing products. In the mashing step Lipase 3 removes lipids to enhance the mash separation. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol (and sometimes organic acids). In the fermentation Lipase 3 helps remove the lipids from the wort which can otherwise incapsule the yeast and impeding the fermentation.

In all of these applications, the enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Appearance:

Depending on the application, the commercial enzyme preparation could be an amber to brown liquid, or off white powder.

Substrate specificity:

Lipase 3 catalyzes the hydrolysis of ester bonds primarily in 1 and 3 positions of fatty acids in triglycerides with release of fatty acids and glycerol.

Activity:

The activity of the Lipase 3 is defined in LIPU (Lipase Unit (Tributyrin)). 1 LIPU is defined as the amount of enzyme, which releases 1µmol H^+ per minute under the given condition.

Temperature optimum:

Approximately 30°C, with relatively high remaining activity up to 40°C.

Thermal stability:

The enzyme is relatively stable for 45 minutes at 70°C, while it is inactivated after 100 minutes of incubation at 70°C.

pH optimum:

Approximately pH 5.5-6.0.

pH stability:

Optimal stability is seen at the pH interval 5.0-7.0 and the enzyme is relatively stable in the pH range 4.0-9.0.

Interaction of the enzyme with different foods:

The Lipase 3 enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Nutritional implication:

Lipase 3 is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Lipase 3 are very low, and as with other enzymes that are currently approved and used as Processing Aids use of this preparation would not have any nutritional significance.

2.4. Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of Lipase 3 is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5. Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of Lipase 3 to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 10,000 CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25g
Salmonella	absent in 25g
Antibiotic activity	Absent in 1g of sample
Production strain	Negative by test
Physical properties:	
Appearance	amber to brown liquid, or off white powder, depending on the application

Standard for identity:

Lipase 3 meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

2.6. Allergenicity of the enzyme:

An allergen statement is given in Appendix A. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

3. Safety

Refer to Appendix B for further details

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e. to catalyze a specific chemical reaction. That reaction determines the IUBMB classification. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g. temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 1 below shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyze the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

Figure 1. Variation of enzymes in nature.

The expressed mature enzyme amino acid sequence of Lipase 3 shows a clear conserved 'Lipase (class 3)' sequence domain, characteristic for triacylglycerol lipases (IUBMB 3.1.1.3) of fungal origin. Our lipase 3 shows 94% identity to two lipase sequences annotated as obtained from *A. niger*, which is one of the approved lipase enzymes on Schedule 18 of the ANZ Food Standards Code. The identity among the FSANZ approved lipases range from 14% (*C. rugosa* to *F. heterosporum*) to 99% (*R. niveus* to *R. arrhizus*). Note that even available lipase sequences obtained from different strains of one species show variability. For instance, an alignment of just four of the available *A. niger* lipase amino acid sequences showed that these were 50-99% identical.

Lipase 3 enzyme derived from *T. reesei*, carrying the Lipase 3 gene from *A. tubingensis* has been determined to be GRAS in the United States, and been used for pizza, bread and other bakery applications in the U.S and for bakery in India since 2017. There have not been any adverse events reported since Lipase 3 has been in commercial use in these countries.

Please refer to section 1.8 and Appendix D for details on the different approval procedures in the countries listed above.

3.2. Toxicity of the enzyme

Toxin homology study

A BLAST search for homology of the mature *A. tubingensis* Lipase 3 protein sequence with known toxins and antinutrients was performed using the UniProtKB annotated Protein Knowledge database (Magrane et al., 2011; UniProt release 2016_07 of 06-Jul-16[1]). This database contains 551705 proteins[2], of which 5703 are manually annotated as toxins and 6143 as venom proteins[3].

From this search, the top 1,000 hits in the UniProt database were exported to MS Excel, with the appropriate annotation fields (protein name, key words, gene ontology, protein family), allowing for use of search terms "toxin" and "venom". The vast majority of hits were with Lipases, with none of the top 1,000 database hits being annotated as either toxin or venom.

Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilized by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the Total Organic Solid (TOS) originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Although *T. reesei* is scientifically determined by DuPont IB as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on *T. reesei* Morph Lip3 are thus one of the pillars supporting the DuPont IB *T. reesei* Safe Strain Lineage. The position of the food enzyme in the DuPont IB *Trichoderma reesei* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

[1] http://www.unitprot.org/
[2] http://web.expasy.org/docs/relnotes/relstat.html
[3] http://www.uniprot.org/program/Toxins

To assess the safety of Lipase 3, different endpoints of toxicity were investigated at MB Research Laboratories (Pennsylvania) and Harlan Laboratories (Switzerland) and are evaluated and assessed in this document:

Studies:

Acute oral Toxicity Study in Rats – up and down Procedure
Bacterial Reverse Mutation Assay – Ames assay
In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes
A 90-days Oral Toxicity (Gavage) Study in Wistar Rats

The safety of Lipase 3 from *T. reesei* strain Morph Lip3 as a food processing aid is assessed in a battery of toxicology studies investigating its acute oral, mutagenic and systemic toxicity potential. Lipase 3 is not acutely toxic. A battery of genotoxicity assays was conducted and under the conditions of these assays. Lipase 3 is not a mutagen, a clastogen, or an aneugen. Daily administration of Lipase 3 by gavage for 91/92 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 160.6 mg total protein/kg bw/day corresponding to 123.15 mg TOS/kg bw/day.

A summary of the results of the studies can be found in Appendix B.

In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3 Information on the source micro-organism

The production organism strain Morph Lip3 is a strain of *T. reesei* which has been genetically modified by DuPont IB to overexpress a lipase 3 gene from *A. tubingensis*.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally recognized as a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). The lipase 3 gene was placed under the expression signals of the endogenous *T. reesei cbh1* gene, and multiple copies of the expression cassette were integrated into the recipient chromosome using the *A. nidulans* acetamidase (*amdS*) gene as a selectable marker.

Full details of the gene and recombinant microorganism are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.4. Pathogenicity and toxicity of the source micro-organism

Trichoderma reesei was first isolated from nature in 1944. The original isolate, QM6a (Mandels and Reese, 1957), and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested that *Trichoderma reesei* be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1984). Subsequent evidence pointed out that the two species are not identical (Meyer *et al*. 1992) even though several regulatory jurisdictions still use both names interchangeably. The proposal by Khuls *et al*.

(1996) that *Trichoderma reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *Trichoderma reesei* as the anamorph of *Hypocrea jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, *Trichoderma reesei*, *Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different documents and national positive lists, but for historical reasons they refer to essentially the same microorganism species.

A literature search was conducted on August 28, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. A review of the literature search uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals. The species is not present on the list of pathogens used by the EU (Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Two authors reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo *et al.* 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing.

Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer *et al.* (2006), and various strains have been approved for the manufacture of commercial enzyme preparations by Food Standards Australia New Zealand, and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan.

3.5. Genetic stability of the source organism

The parental strain of the production strain *Trichoderma reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont IB and its parental companies, because of the stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using QM6a and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable after more than 60 generations of fermentation for Lipase 3 production. Refer also section 3.6.

3.6. Method used in the genetic modification of the source organism

The production organism of the Lipase 3 preparation, the subject of this submission, is *T. reesei* strain Mofph Lip3. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance lipase production levels. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. The donor organism is *A. tubingensis*. Lipase 3 expression cassette was integrated into the host genome. Full details of the genetic modifications are provided in Appendix E (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by genome sequencing. Broth samples were taken prior and after prolonged fermentation mimicking commercial fermentation conditions. Samples were then used for genomic DNA extraction and next generation sequencing. A complex integration site for lipase expression site was determined, and no change was observed between samples prior and after fermentation. The results demonstrate that the insertion cassette has been stably maintained through generations during the fermentation process.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4. Dietary exposure

Refer to Appendix C for further details

4.1. List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), Lipase 3 will be used in:

- bread, Chinese stem buns, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers, and waffles - 7 Bread and Bakery Products
- beer and other cereal based (alcoholic) beverages - 14.2.1 Beer and related products

4.2. Levels of residues in food

The proposed application rate of Lipase 3 in baking is 2.5-21.2 mg TOS/kg flour. The proposed application rate of Lipase 3 in brewing processes and other cereal based drinks is 5.2-52.2 mg TOS/kg cereals.

DuPont IB expects Lipase 3 to be inactivated or removed during the subsequent production and refining processes for all applications.

In baking, Lipase 3 performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. The Lipase 3 is denatured by heat during the baking or steaming step.

In brewing processes, Lipase 3 can be added in the mash separation step, before the final filtration and pasteurization step. Because of denaturation and aggregation of proteins under the mash and subsequent wort boiling steps, much if not all of the Lipase 3 enzyme is expected to be removed in the post boiling clarification processes.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). 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.

Based on the raw materials used in the various food processes, the recommended use levels of the enzyme Lipase 3, for the calculation of the TMDI, the maximum use levels are chosen. The TMDI is calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day. Consequently, the TMDI will be: 0.410 mg TOS/kg body weight/day. The NOAEL has been determined for Lipase 3 to be at 160.6 mg total protein/kg bw/day (equivalent to 123.15 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 300X fold margin of safety. It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value. Please refer to Appendix C for details.

4.3. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The enzyme would be used as a processing aid in about:

- 60% of the tonnage of bread and bakery products sold in Australia and New Zealand
- 30% of the tonnage of beer and related brewing products sold in Australia and New Zealand

4.4. Levels of residues in food in other countries

Applications and levels of use of the Lipase 3 preparation in other countries is the same as presented in section 4.2.

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January 11, 2018

APPENDIX B: Safety

Contents:

Appendices B

B1	Safe Strain Lineage (Confidential Commercial Information)
B2	Toxicology Test Summaries (Confidential Commercial information)
B3	Certificates of analyses of the test articles (Confidential Commercial Information)
B4	Risk assessment for potential food allergenicity
B5	Analysis of safety based on Pariza-Johnson Decision Tree

1 Toxicity of the enzyme

1.1 Toxin homology study

The *Aspergillus tubingensis* Lipase (Lipase 3) sequence is given below in FASTA format, without its secretion signal (**Confidential Commercial Information**).

Please refer to Appendix B Confidential Attachment

A BLAST search for homology of the mature *A. tubingensis* Lipase protein sequence with known toxins and antinutrients was performed using the UniProtKB annotated Protein Knowledge database (Magrane et al., 2011); http://www.unitprot.org/), UniProt release 2016_07 of 06-Jul-16. This database contains 551705 proteins (http://web.expasy.org/docs/relnotes/relstat.html), of which 5703 are manually annotated as toxins and 6143 as venom proteins (http://www.uniprot.org/program/Toxins).

From this search the top 1000 hits in the UnitProt database were exported to MS Excel, with the appropriate annotation fields (protein name, key words, gene ontology, protein family), allowing for use of search terms "toxin" and "venom". The vast majority of hits were with Lipases, with none of the top 1000 database hits being annotated as either toxin or venom.

1.2 Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilized by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the TOS originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Although *Trichoderma reesei* is scientifically determined by DuPont IB as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on *T. reesei* Morph Lip3 are thus one of the pillars supporting the DuPont IB *T. reesei* Safe Strain Lineage. The position of the food enzyme in the DuPont IB *T. reesei* Safe Strain Lineage is presented in Appendix B1.

1.3 Toxicological testing

To assess the safety of Lipase 3, different endpoints of toxicity were investigated at MB Research Laboratories (Pennsylvania) and Harlan Laboratories (Switzerland) and are evaluated and assessed in this document:

Studies

Acute oral Toxicity Study in Rats – up and down Procedure

Bacterial Reverse Mutation Assay – Ames assay
In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes
A 90-days Oral Toxicity (Gavage) Study in Wistar Rats

1.3.1. Test article description

The test material, Ultra-Filtered Concentrate (UFC), used in all toxicology investigations had the following characteristic (**Confidential Commercial Information**):

Please refer to Appendix B Confidential Attachment

1.3.2. Toxicological tests

A. Acute oral Toxicity Study in Rats – up and down Procedure

a. Procedure:

The objective of this study was to assess the acute toxicity of Lipase 3 when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a single female Wistar rat at 2000 mg/kg. Since this animal survived, the study was followed with four additional female rats dosed at 2000 mg/kg bw.

This study was conducted according to EPA Health Effects Test Guidelines, OPPTS 870.1100 (December 2002) and the OECD Guideline No. 425 (updated March 2006) and in compliance with Good Laboratory Practices regulations of the EPA 40 CFR §160 and 792, FDA 21 CFR §58, and as specified in Principles on Good Laboratory Practices published by OECD, 1997.

b. Results

No mortality was recorded in this study at 2000 mg/kg bw. There were no abnormal physical signs noted during the observation period. There were no abnormal findings at necropsy.

c. Evaluation

Under the conditions of this study, the oral LD_{50} was $\geq$2000 mg total protein/kg bw (corresponding to 1540 mg TOS/kg bw). Based on a LD_{50} > 2000 mg /kg, Lipase 3 is classified to category 5 [unclassified - practically non-toxic] according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), 2007.

B. Bacterial Reverse Mutation Assay – Ames assay

a. Procedure

The objective of this assay was to assess the potential of Lipase 3 to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherischia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). A pre-experiment test was performed with strains TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA using 8 concentrations ranging from 3 to 5,000 µg/plate. All dose levels were expressed in terms of total protein. The

highest dose level tested (5,000 µg/plate) is the maximum required by the OECD guideline. Subsequently, a main test was performed with all 5 strains in both the presence and absence of S-9 mix. Triplicate plates were used at each test point. Eight dose levels of Lipase 3 were used in the main test and ranged from 3 to 5,000 µg/plate. The positive controls used for assays without S-9 mix were sodium azide, 4-nitro-o-phenylene-diamine and methyl methane sulfonate. The positive control for assays with S-9 mix was 2-aminoanthracene. Negative control plates were treated by the addition of sterile deionized water.

This assay was conducted in accordance with OECD guideline No. 471 (July 21, 1997), EPA OPPTS 870.5100 (August 1998) and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

In the pre-experiment assay, Lipase 3 was not toxic to the test bacteria up to and including the highest dose level (5,000 µg/plate) in both the absence and presence of S-9 mix. Therefore, 5000 µg/plate was selected as the highest dose level for the main test.

In the main test, eight dose levels (3; 10; 33; 100; 333; 1,000; 2,500 and 5,000 µg/plate) were tested. The plates incubated with the test material showed normal background growth up to 5,000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

c. Evaluation

Under the conditions of this assay, Lipase 3 has not shown any evidence of mutagenic activity in the Ames assay. Lipase 3 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

C. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

a. Procedure

The objective of this assay was to investigate the potential of Lipase 3 to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen (e.g., phytohemagglutinin, PHA). Mitotic activity began at about 40 hours after PHA stimulation and reached a maximum at approximately 3 days.

Lipase 3 concentrate was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and one main test. Ten concentrations of Lipase 3 were used in the preliminary assay and at least 3 dose levels were then selected for analysis of chromosome aberration with the highest

dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity is characterized by the percentage of mitotic suppression in comparison to the controls by counting 1,000 cells per culture in duplicate. In the absence of cytotoxicity, the highest dose selected would be 5,000 µg/ml, as recommended by the OECD guideline.

In the preliminary test, all cultures (with or without S-9 mix) were treated for 4 hours. In the main test, cultures without S-9 mix were treated for 22 hours and those with S-9 mix for 4 hours. Three hours before harvesting, colcemid was added to all cultures at the concentration of 0.2 µg/ml to arrest all cells at the metaphase stage of mitosis. All cultures (with and without S-9 mix) were harvested by centrifugation 22 hours after the start of treatment. The supernatant was discarded and the cell pellets were re-suspended in a KCl hypotonic solution. The cell suspension was allowed to stand at 37ºC for 25 minutes and then centrifuged. The hypotonic solution was removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations:

i. Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1,000 cells examined). From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays.
ii. Metaphase analysis (i.e., evaluation of chromosomal aberration) was conducted on at least 100 metaphases per culture dose level.
iii. Ethylmethane sulfonate and cyclophosphamide were used as positive controls for cultures without S-9 mix and cultures with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test; February 1998) and complied with Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – In Vitro Mammalian Chromosome Aberration Test" dated May 30, 2008. The study was performed in compliance with the Chemicals Act of the Federal Republic of Germany (July 25, 1994; revised June 27, 2002) and the OECD Principles of Good Laboratory Practice (1997).

b. Results

Preliminary assay (Experiment I): Ten dose levels ranging from 32.5 to 5,000 µg/ml were used. Exposure period was 4 hours for both cultures with and without S-9 mix. No clear cytotoxicity was observed up to the highest concentration tested 5,000 µg/ml. No visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted in three highest dose levels selected for analysis (1,632.7; 2,857.1 and 5,000 µg/ml). Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary assay was designated as Experiment I and the results were analyzed for statistical significance.

Main assay (Experiment II: Exposure period was 4 hours for cultures with S-9 mix and 22 hours for cultures without S-9 mix. Ten dose levels ranging from 32.5 to 5,000 µg/ml were used. The chromosomes were prepared 22 hours after the start of treatment with the test material.

No visible precipitation of the test material in the culture medium was observed. In both the presence of S-9 mix (4-hour cultures) and absence of S-9 mix (22-hour cultures), no clear cytotoxicity was observed up to the highest concentration tested 5,000 µg/ml. No

biologically relevant increases in cells with chromosomal aberrations were noted in three highest dose levels selected for analysis (1,632.7; 2,857.1 and 5,000 µg/ml).

In both experiments I and II, no increase in polyploidy metaphases was noticed.

In both experiments, significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

c. Evaluation

Under the conditions of this test, Lipase 3 did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5,000 µg/ml) recommended by guidelines.

D. A 90-days Oral Toxicity (Gavage) Study in Wistar Rats.

a. Procedure

The objective of this study was to investigate the potential of Lipase 3 to induce systemic toxicity after repeated daily oral administration (gavage) to SPF-bred Wistar rats of both sexes. Dose levels were 0 (0.9% saline), 53.5, 80.3 and 160.6 mg total protein/kg bw/day (corresponding to, respectively, 0, 41.02, 61.57 and 123.15 mg TOS/kg bw/day or 0; 32,390; 48,610 and 97,225 LIPU/kg bw/day). Each group consisted of 10 animals/sex. Animals of the same sex were housed in groups of five in Makrolon-type 4 cages with wire mesh tops and softwood bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and at study termination. Urinalysis, clinical chemistry and hematology were conducted at week 13. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for all groups. All animals were sacrificed at the end of the treatment period. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice (May 18, 2005) and the OECD Principles of Good Laboratory Practice (1997).

b. Results

One control female (# 49) was sacrificed for humane reasons on day 57 of treatment.

There was no mortality in the low dose group (53.5 mg total protein/kg bw/day). In the mid dose group (80.3 mg total protein/kg bw/day), one male was found dead on day 46

due to gavage error (presence of test material in the lungs) and two females were found dead on days 31 and 45. The cause of death of the two mid-dose females was not determinable but was not considered treatment-related in the absence of mortality noted in mid-dose males and high-dose males and females. In the high dose group (160.6 mg total protein/kg bw/day), gavage error resulted in the death of one female (presence of test material in lungs; dark red discoloration of lungs).

There were no treatment-related statistical differences between the control and treated groups with respect to clinical observation, functional observation, body weight gains, feed consumption, hematology, clinical biochemistry, and urinalysis. Significantly higher mean hind-limb strength values were noted at all dose levels. However, these differences were considered to be incidental in the absence of similar findings in fore-limb grip strength. Increased locomotor activity was noted in mid and high dose males but the differences were not dose related. The mean absolute neutrophil count and plasma sodium were significantly elevated in high dose males when compared to concurrent control values. However, these differences were not considered as treatment-related since they were within the historical control data values for this species and strain collected at the testing laboratory. Higher plasma glucose was found in high dose females but the values were still within the historical control data range. At necropsy, a small number of statistically significant differences to the control values were noted in the mean absolute and/or relative organ weights. However, in the absence of accompanying histopathologic and/or functional changes and clear dose response relationship, these variations are considered as incidental. All microscopic lesions were within the normal background range of lesions found in laboratory animals of this strain and age.

c. Evaluation and Conclusion

Daily administration of Lipase 3 for 91/92 days by oral gavage to Wistar rats at doses of 0 (0.9% saline), 53.5, 80.3 and 160.6 mg total protein/kg bw/day (corresponding to, respectively, 0, 41.02, 61.57 and 123.15 mg TOS/kg bw/day or 0; 32,390; 48,610 and 97,225 LIPU/kg bw/day) resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment.

Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 160.6 mg total protein/kg bw/day corresponding to 123.15 mg TOS/kg bw/day or 97,225 LIPU/kg bw/day.

2 Information on the source micro-organism

The function of the genetic modification is to produce the Lipase 3 enzyme of the donor strain *Aspergillus tubingensis*, using a known safe *Trichoderma reesei* host strain.

2.1 The production strain

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under

conditions used for enzyme production. It is generally recognized as a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). The lipase 3 gene was placed under the expression signals of the endogenous *T. reesei cbh1* gene, and multiple copies of the expression cassette were integrated into the recipient chromosome using the *Aspergillus nidulans* acetamidase (*amdS*) gene as a selectable marker.

2.2 The host

The host organism *T. reesei* strain RL-P37 was obtained from Dr. Montenecourt. The derivation and characterization of strain RL-P37 has been published (Sheir-Neiss and Montenecourt, 1984). Strain RL-P37 is a cellulase over-producing strain that was obtained through several classical mutagenesis steps from the wild-type *T. reesei* strain (QM6a). Strain QM6a is present in several public culture collections, e.g. in the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina* (Khuls *et al*., 1996; Dugan, 1998).

2.3 The donor organism

The donor strain used as a source for the Lipase 3 gene was *A. tubingensis*. *A. tubingensis* is also called *Aspergillus niger var. tubingensis*. It is one of the species in the *Aspergillus* section Nigri (the black aspergilli). In the *A. niger* aggregate, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic.

A. nidulans acetamidase (*amdS*) gene was used as a selectable marker, to enable growth on acetamide medium. Only the *amdS* gene in isolated form was used. The gene was first described by Hynes *et al*. (1983). The strain was not described further than "a strain of genotype biA1" but it is certainly a derivative of the original *A. nidulans* isolate (Glasgow wild-type) deposited as strain A4 at the Fungal Genetics Stock Center, Kansas City, USA. Also the description of the gene in GenBank (Accession number M16371) mentions the Glasgow wild-type *A. nidulans* strain as the source. Sequencing and PCR experiments verified that the gene DuPont IB used is the same as published by Corrick *et al*. (1987). This gene has been used extensively over the last ten years as a marker both in academia and industry without any advert effects. Therefor this gene is regarded as part of a vector with an extended history of safe use and not described here as donor material.

2.4 The vector

The genetic modification of the *T. reesei* host involved recombinant DNA techniques to introduce multiple copies of the gene encoding the *A. tubingensis* Lipase 3 (*lip3*) gene into the *T. reesei* host. The expression cassette comprised the native *T. reesei* cellobiohydrolase (*cbh1*) promoter, which was used to drive expression of the *A. tubingensis* Lipase 3 (*lip3*) gene, the native *A. tubingensis* Lipase 3 gene, the native *T. reesei* cellobiohydrolase (*cbh1*) terminator, and the *Aspergillus nidulans* acetamidase gene (*amdS*) as a selectable marker.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *T. reesei* strain had been made.

3 Pathogenicity and toxicity of the modified micro-organism

3.1 The production strain

T. reesei was first isolated from nature in 1944. The original isolate, QM6a (Mandels and Reese, 1957), and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested that *T. reesei* be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1984). Subsequent evidence pointed out that the two species are not identical (Meyer *et al*. 1992) even though several regulatory jurisdictions still use both names interchangeably. The proposal by Khuls *et al*. (1996) that *Trichoderma reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *Trichoderma reesei* as the anamorph of *Hypocrea jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, *T. reesei*, *T. longibrachiatum,* and *Hypocrea jecorina* may appear in different documents and national positive lists, but for historical reasons they refer to essentially the same microorganism species.

A literature search was conducted on August 27, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. The full search output is on file in DuPont IB Product Stewardship & Regulatory. A review of the literature search uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals. The species is not present on the list of pathogens used by the EU (Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Two authors reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo *et al.* 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing.

Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*.

(1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer *et al.* (2006), and various strains have been approved for the manufacture of commercial enzyme products Food Standards Australia New Zealand and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan.

3.2 The donor

The donor used as a source for the Lipase 3 gene was *A. tubingensis*. *A. tubingensis* is also called *Aspergillus niger var. tubingensis*. It is one of the species in the Aspergillus section Nigri (the black aspergilli). In the *A.s niger* aggregate, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic.

The donor used in construction of the new microorganism as a source for the Lipase 3 gene is *A. tubingensis*. The Lipase 3 (triacylglycerol lipase) gene was not modified during construction of the subject microorganism. A literature search was conducted on August 17, 2016 in PubMED using the searching term "*Aspergillus tubingensis*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 53 records, which were reviewed and several of which were used in a brief literature review below.

The species *Aspergillus tubingensis* is a deuteromycetes with a full taxonomic lineage as:

> *cellular organisms; Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Pezizomycotina; leotiomyceta; Eurotiomycetes, Eurotiomycetidae, Eurotiales, Aspergillaceae, Aspergillus, Aspergillus niger; Aspergillus niger var. tubingensis*

A. tubingensis is a fungus of the genus *Aspergillus*. Black-spored Aspergillus section Nigri species has been identified for production of the mycotoxins ochratoxin A (OTA) and fumonisin B2 (FB2) which are toxic for human and animals. Ochratoxins and fumonisins are a small group of chemically related toxic fungal metabolites (mycotoxins).

A review of the abstracts revealed grapes (for wine and raisins) are the most commonly *Aspergillus* contaminated crop (Medina *et al.* 2005), and ochratoxin A is the most reported mycotoxin associated with *Aspergillus* species (*A. niger* being identified as the main source of Ochratoxin A). *Aspergillus* species has also been associated with myctoxin production in maize (Logrieco *et al*. 2014) and citrus fruits (Kanetis *et al*. 2015). *Aspergillus tubingensis* was identified in maize but not reported for mycotoxin production (Logrieco *et al*. (2014).

Storari *et al* (2012) has assessed six *A. tubingensis* strain from International culture collections for ochratoxin A (OTA) production. OTA was not detected in any of the tested samples. The non-toxigenic nature of *A. tubingensis* is further supported by several reports (Frisvad *et al.* 2011, Accensi *et al*. 2001, Nielsen *et al*. 2009 and others).

Our search found an article by Bathoorn *et al*. (2013), which reported *A. tubingensis* infections in immunocompromised patients.

Aspergillus nidulans acetamidase (*amdS*) gene was used as a selectable marker, to enable growth on acetamide medium. Only the *amdS* gene in isolated form was used. The gene was first described by Hynes *et al*. (1983). The strain was not described further than "a strain of genotype biA1" but it is certainly a derivative of the original *A. nidulans* isolate (Glasgow wild-type) deposited as strain A4 at the Fungal Genetics Stock Center, Kansas City, USA. Also the description of the gene in GenBank (Accession number M16371) mentions the Glasgow wild-type *A. nidulans* strain as the source. Sequencing and PCR experiments verified that the gene DuPont IB used is the same as published by Corrick *et al*. (1987).

As all the introduced genetic material was chromosomally integrated (*i.e*., not on a transferrable plasmid), this method of integration is generally recognized to be stable.

3.3 The host

T. reesei is not listed in Annex III of Directive 2000/54/EC – which lists microorganisms for which safety concerns for workers exist, as it is globally regarded as a safe microorganism:

- In the USA, *T. reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health (NIH) 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 *Trichoderma reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (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 requirements1, if this fungus was to be used in submerged standard industrial fermentation for enzyme production.
- In Europe, *T. reesei* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA) (BauA, 2010) and the Federal Office of Consumer Protection and Food Safety (BVL) (BVL, 2013), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010).

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992).

Cellulase, glucanase and glucoamylas from *T. reesei* have been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) “not specified” has been set (Monograph (FNP 52 (1992), JECFA, Monograph 14 (2013, JECFA 77th).

Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* have been approved as processing aid by FSANZ.

Cellulase from *T. reesei* was affirmed as GRAS by U.S. FDA (21CFR184.1250). Also the FDA has no questions to four GRAS notices on enzymes derived from *T. reesei*:

- Pectin lyase derived from *T. reesei* carrying a gene encoding pectin lyase from *Aspergillus niger* (GRN 32)
- Transglucosidase enzyme preparation from *T. reesei* expressing the gene encoding transglucosidase from *A. niger* (GRN 315)
- Acid fungal protease enzyme preparation (GRN 333)
- Chymosin enzyme preparation from *T. reesei* expressing the bovine prochymosin B gene (GRN 230)
- Glucoamylase enzyme preparation from *T. reesei* expressing the glucoamylase gene from *T. reesei* (glucoamylase enzyme preparation) (GRN 372)

T. reesei has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. During recent years, genetic engineering techniques have been used to improve the industrial production strains of *T. reesei* and considerable experience on the safe use of recombinant *T. reesei* strains at industrial scale has accumulated. From above, secondary metabolites are of no safety concern in fermentation products derived from *T. reesei. Th*us, *T. reesei* can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for other safe gene products.

3.4 Allergenicity of Lipase 3

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (Amfep) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that the enzyme residues in bread and other foods do not represent any unacceptable risk to consumers.

Despite this lack of general concern, the potential that Lipase 3 could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

A. tubingensis Lipase 3 (mature) sequence is given below in FASTA format (**Confidential Commercial Information**).

Please refer to Appendix B Confidential Attachment

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[1] containing 2035 peer-reviewed allergen sequences released on January 18, 2017[2] revealed no match to allergens by identity across 80 amino acids exceeding 35 %.

FASTA alignment of the above sequence with known allergens also using the AllergenOnline database[3] revealed no match (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities.

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al*. (2011) and on AllergenOnline.org, there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). This database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous amino acids search produced no additional sequence matches with known allergens.

According to Pariza and Foster (Pariza and Foster, 1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Microbial enzymes acting as environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a published study (Bindslev-Jensen *et al*., 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a concern with regard to food allergy.

In conclusion, based on the sequence homology alone, *A. tubingensis* Lipase 3 is unlikely to pose a risk of food allergenicity.

As for all enzyme products from DuPont IB, the Safety Data Sheet for the Lipase 3 product will include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

[1] http://www.allergenonline.org/index.shtml
[2] listed in http://www.allergenonline.org/databasebrowse.shtml

4 Genetic stability of the source organism

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by Lipase 3 production.

5 Pariza-Johnson Decision tree

Pariza and Johnson (2001) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations.

DuPont IB has evaluated Lipase 3 according to the safety scheme of Pariza and Johnson (2001) (Appendix B3) and determined that this enzyme preparation is safe for use in food as a processing aid. This determination employed an extensive review of published and unpublished safety data available on the enzyme, the production organism, the enzyme manufacturing process, and the enzyme product (Pariza and Johnson, 2001)

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Appendix B1: Safe Strain Lineage (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment

Appendix B2 Toxicology Test Summaries (Confidential Commercial information)

Please refer to Appendix B Confidential Attachment

Appendix B3: Certificate of analysis of test items (Confidential Commercial Information)

Please refer to Appendix B Confidential Attachment

Appendix B4: Risk assessment for potential food allergenicity

Sequence Analysis Based Risk Assessment for Potential Food Allergenicity of *Aspergillus tubingensis* lipase expressed in *Trichoderma reesei.*

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

Aspergillus tubingensis lipase (mature) sequence is given below in FASTA format **(Confidential Commercial Information)**.

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The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP)

AllergenOnline database (http://www.allergenonline.org/index.shtml) containing 2035 peer-reviewed allergen sequences released on January 18, 2017 (listed in http://www.allergenonline.org/databasebrowse.shtml) revealed no match to allergens by identity across 80 amino acids exceeding 35 % (Appendix A).

FASTA alignment of the above sequence with known allergens also using the AllergenOnline database (http://www.allergenonline.org/index.shtml) revealed no match (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities (Appendix A).

Although cautioned against in Codex (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). This database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous amino acids search produced no sequence matches with known allergens.

Microbial enzymes acting environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a concern with regard to food allergy.

In conclusion, based on the sequence homology alone, *Aspergillus tubingensis* lipase is unlikely to pose a risk of food allergenicity.

As for all enzyme products, an MSDS for the lipase product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

References

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Codex Alimentarius Commission. 2009. Foods Derived from Modern Biotechnology, Annex 1, Assessment of Possible Allergenicity, Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Rome, Italy, http://www.fao.org/docrep/011/a1554e/a1554e00.htm, pp. 21-23

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Appendix B5: Analysis of safety based on Pariza/Johnson Decision tree

Pariza and Johnson (2001), and Pariza and Cook (2010) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the Lipase 3 enzyme product is "ACCEPTED" as safe for its intended use.

1. Is the production strain[3] genetically modified[4,5]? Yes. *Go to 2.*

2. Is the production strain modified using rDNA techniques? Yes. *Go to 3a.*

3a. Does the expressed enzyme product which is encoded by the introduced DNA[6,7] have a history of safe use in food[8]? Yes, lipase has been used for years in food processing. The *Aspergillus tubingensis* lipase is relatively new as an isolate in food processing. However, it has high homology to other lipases used in food - e.g., 98% identity with *Aspergillus niger* lipase (GRN 111, GRN 296), 99% identity with *Aspergillus kawachii* lipase, and 56% with *Aspergillus oryzae* lipase (GRN 113), and its protein sequence is not similar to known sequences of food allergens and toxins. *Go to 3c.*

3c. Is the test article free of transferable antibiotic resistance gene DNA[9]? Yes.

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[3] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized;steps 6–11 are intended to ensure this

[4] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (e.g., UV or chemically-induced mutagenesis) or rDNA technologies.

[5] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[6] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[7] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[8] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

[9] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm)

No antibiotic resistance genes were used in the construction. *Go to 3e.*

3e. **Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes, inserted DNA is well characterized and free of unsafe attributes. *Go to 4.*

4. Is the introduced DNA randomly integrated into the chromosome? Yes. *Go to 5.*

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? Yes. The inserted DNA is well characterized. The production strain does not produce toxic metabolites of concern as confirmed by T-2 toxin analysis. *Go to 6.*

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[10]? Yes. The *Trichoderma reesei* RL-P37 safe lineage is established as discussed in Appendix B1 and B2. Its safety as a production host and methods of modification are well documented and their safety have been confirmed through repeated toxicology testing. A NOAEL from the 90-days oral rodent study has been established for *T. reesei* Morph Lip3.

Conclusion: Article is accepted.

[10] In determining safe strain lineage one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage

January 11, 2018

APPENDIX A: Technical information

Contents:

Appendices A

A1	EC number
A2	CAS number
A3	Activity of the enzyme complex
A4	Specifications of the commercial product
A5	Certificate of analyses
A6	Production process flow chart
A7	Food Chemical Codex, sixth edition
A8	General specifications for enzyme preparations used in food processing (JECFA)
A9	Allergen declaration

1 Identity

1.1 Lipase

The systematic name of the principle enzyme activity is triacylglycerol acylhydrolase. Other names used are lipase (*ambiguous*); butyrinase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno *N*-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase, Lipase 3.

The enzyme Lipase 3 is derived from *Trichoderma reesei* which is genetically modified to overexpress the Lipase 3 gene from *Aspergillus tubingensis*.

- EC number: 3.1.1.3 (Appendix A1)
- CAS number: 9001-62-1 (Appendix A2)

1.2 Other enzymes

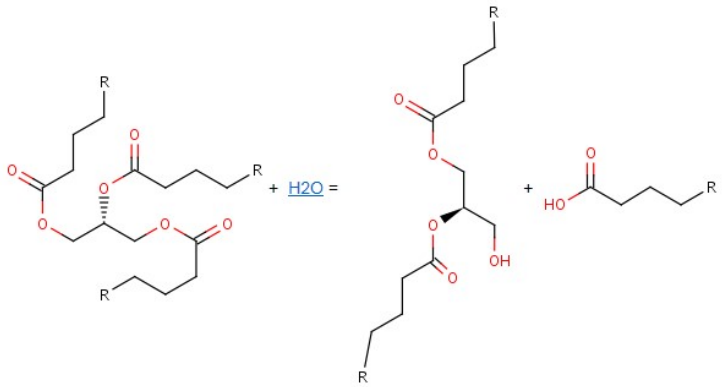
Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and traces of other enzyme activities (e.g. protease) might be found but their level will be very low.

2 Chemical and physical properties

2.1 Substrate specificity

Triacylglycerol lipase (IUBMB 3.1.1.3) hydrolyses ester bonds primarily 1 and 3 position of fatty acids in triglycerides. Lipases belong to the carbohydrate esterase family 10. Carbohydrate esterase family 10 consists of various esterases acting on non-carbohydrate substrates (http://www.cazy.org (Cantarel *et al.*, 2009)) and sharing a common (α,ß/α) sandwich domain. Substrates include lipids (triglycerides).

Reaction catalyzed by triacylglycerol lipase (3.1.1.3), lipoprotein lipase (3.1.1.34), hormone-sensitive lipase (3.1.1.79)



triacylglycerol + H2O = diacylglycerol + a carboxylate

2.2 Activity

The activity of the Lipase 3 is defined in LIPU (Lipase Unit (Tributyrin)). 1 LIPU (Lipase Unit (Tributyrin)) is defined as the amount of enzyme, which releases 1µmol H^+ per minute under the given condition. The assay is based on the Enzymatic hydrolysis of lipids releases

fatty acids. For each fatty acid released, an equivalent H^+ appears. By continuous titration of H^+, the lipase activity can be determined based on the consumption of base. This method is not applicable for samples containing calcium chloride. $CaCl_2$ might interfere with certain lipases.

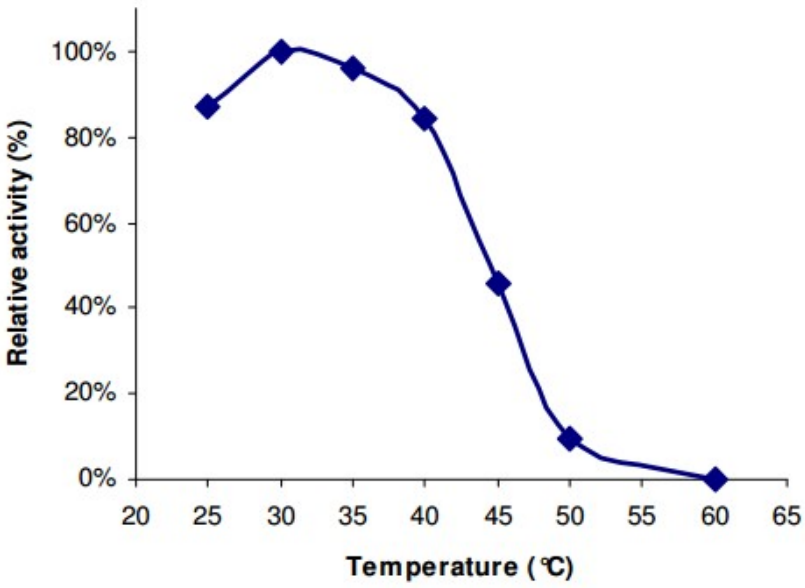
Lipase 3 preparations will have a minimum activity of 20 000 LIPU/g. A detailed assay method is present in Appendix A3.

2.3 Temperature optimum

Enzyme activity of purified Lipase 3 ferment was determined at various temperatures. The method is based on pH-stat titration using an automatic Titralab Tim856 titration manager. The substrate was a homogenised emulsion of 5% (v/v) Glycerol tributyrate, 0.10% (w/v) Gum Arabic, 7.5% (w/w) Glycerol, 51 mM NaCl, 0.50 mM KH2PO4, pH 5.5 and the titrant was 0.05 M NaOH. When not otherwise mentioned, the reaction pH was 5.5 and the reaction temperature was 30°C. Results are shown in **Error! Reference source not found.**.

Temperature optimum was determined to be 30°C with relatively high relative activity up to 40°C. At 45°C and above, the relative activity is reduced to <50 %.

Figure 1: Temperature profile of Lipase 3 in the LIPU titration assay. Activity is shown as



relative to the highest measured value.

2.4 Thermal stability

Thermal stability of Lipase 3 was determined by measuring residual lipase activity after incubation at 70°C for 0-100 minutes in 50 mM Sodium acetate buffer, pH 5.5. The enzyme is inactivated after 100 min. incubation at 70°C.

Lipase 3 samples were diluted in sample buffer containing 50 mM Sodium Acetate, pH 5.5, 0.1% BSA, 1.2% NaCl, and incubated at 70°C for 0, 5, 10, 20, 30, 40, 60, 80, 100 min. The samples were stored on ice and the residual activity was then determined by the following method: The substrate was diluted in substrate solution containing 120 mM Sodium Acetate, pH 5.5, 0.2% Glycerol Trioctanoate, 0.3% NaCl, 13% Triton X-100. The assay sequence was 3 min equilibration of 34 ml substrate at 37°C before addition of 20 ml enzyme sample

solution, and incubation for 30 min at 37°C before measurement of liberated free fatty acid. Blank measurements were performed by analysing samples without enzyme. Free fatty acid in the reaction mix was measured using the NEFA C kit (WAKO Chemicals, Neuss, Germany). 56 µl NEFA C reagent A was added to the enzyme reaction mix and incubated for 5 min at 37°C before addition of 113 µl NEFA C reagent B followed by incubation for 5 min at 37°C and, finally, OD520 was measured. The amount of liberated free fatty acid was calculated from an oleic acid standard curve with OD520 nm against mM FFA. 1 LIPU is defined as the quantity of enzyme that produces 1 microequivalent of free fatty acid per min under the conditions described. Results are shown in Figure 2.

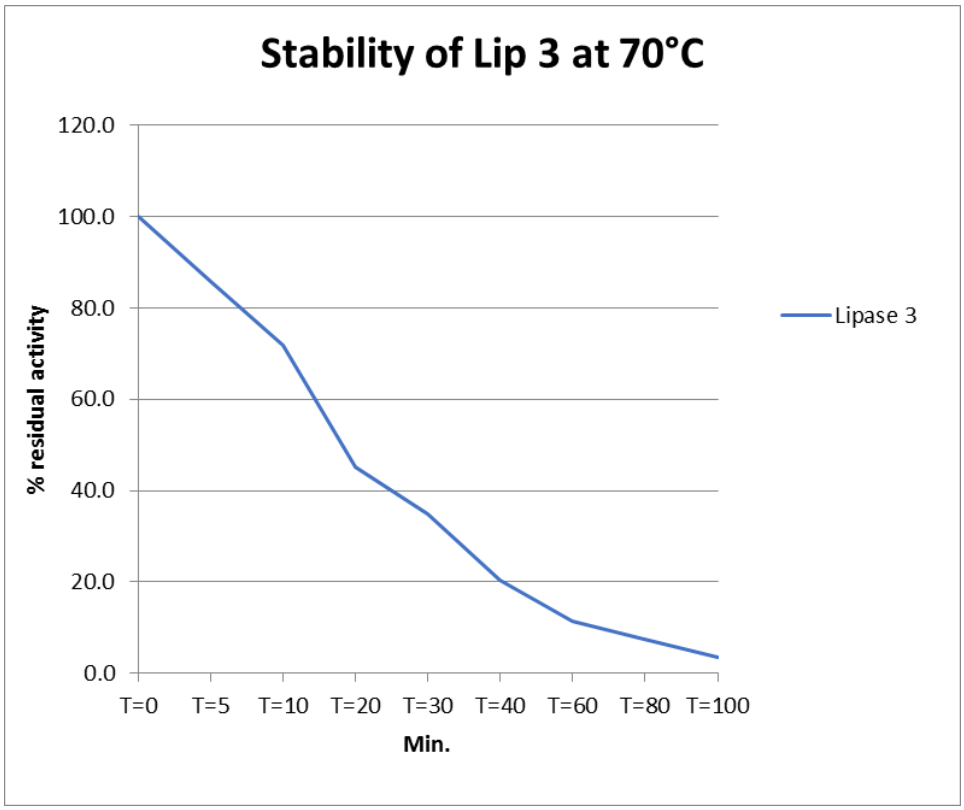


Figure 2: Activity was determined after 0-100 minutes of incubation in 50 mM Sodium acetate buffer, pH 5.5.

After 20 min incubation at 70°C, residual activity of Lipase 3 is 45 %. It is inactivated after 100 minutes of incubation at 70°C.

2.5 pH optimum

Enzyme activity of spray purified Lipase 3 ferment was determined at various pH-values. For titration for pH profile, the reaction pH's were as stated in Figure 3. 2 ml enzyme sample was added to 25 ml substrate, pre-equilibrated at stated pH and temperature, before the titration was started. Activity was calculated from the slope of the titration curve with consumption of titrant against reaction time. 1 LIPU is defined as the quantity of enzyme that produces 1 microequivalent of free fatty acid per min under the conditions described. Results are shown in Figure 3.

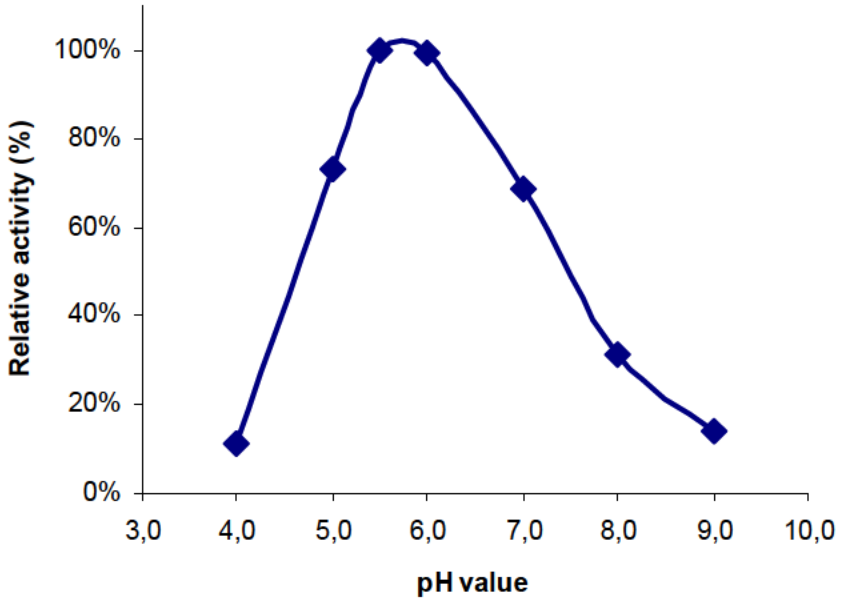


Figure 3: pH profile of Lipase 3 in the LIPU titration assay. Activity is shown as relative to the highest measured value

pH optimum is seen at pH 5.5-6 and the enzyme is active in the range from pH 4 to 9.

2.6 Storage stability

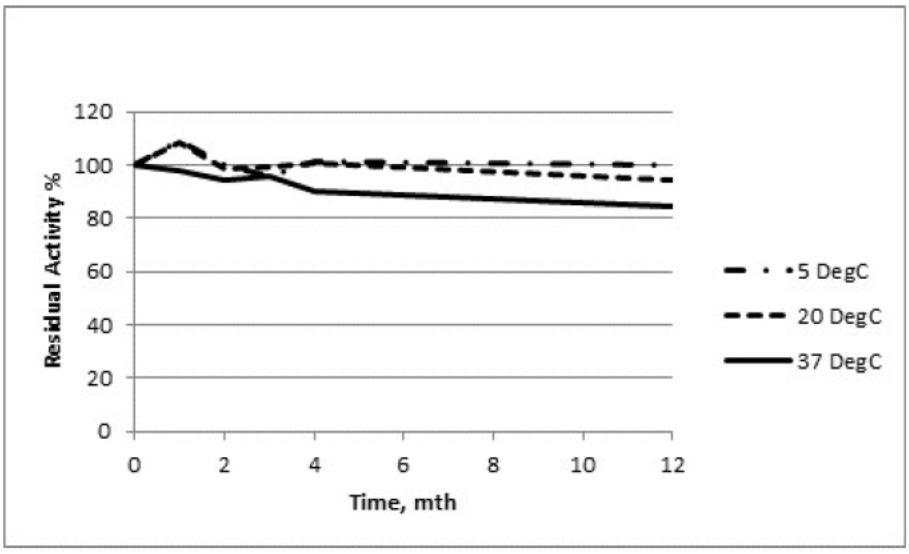


Figure 4. Storage stability of Lipase 3

As seen in the figure above, at 5°C and 20 °C, the enzyme is stable for more than 1 year without loss of activity.

3 Efficacy and benefits of the Lipase 3 enzyme preparation

3.1 Description

As noted above, the function of Lipase 3 is to catalyse the hydrolysis of ester bonds primarily in 1 and 3 position of fatty acids in triglycerides with release of fatty acids and glycerol.

Baking:

Lipase can be used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers and waffles.

Lipase 3 can be used in dough for bread making to improve the quality of the baked products. Wheat flour used for bread making typically contains ~2% lipids. Half of these lipids are non-polar lipids including triglycerides, diglycerides, sterols and sterol esters. The remainder of the lipids in wheat flour are polar lipids containing mainly galactolipids and phospholipids.

Lipase 3 can be used in dough to modify these endogenous lipids and produce more polar lipids like lyso-phospholipids and lyso-galactolipids. The non-polar triglycerides in dough will also be modified during formation of mono- and diglycerides and free fatty acids. The properties of the endogenous lipids produced by addition of Lipase 3 contribute to improved dough stability and dough handling properties, and the polar lipids formed in the dough will produce bread with improved volume and a homogenous crumb structure with homogenous pores.

Noodles and pasta are normally produced by using the same raw material, wheat flour, which is used for the manufacture of bread. In pasta or noodle dough, Lipase 3 also modifies the endogenous wheat lipids and produces more polar lipids. This in turn improves the quality of the finished noodle or pasta product giving a product which has a lower cooking loss and improved quality upon consumption.

Therefore the benefits of the conversion of lipids with the help of Lipase in baking and other cereal based processes, are:

- Improve handling of the dough (improved extensibility and stability).
- Improve the dough structure and behaviour during baking.
- Ensure a uniform and slightly increased volume and an improved crumb structure of the bakery product, which might otherwise be impaired by industrial processing of the dough.
- Add significantly softer textural characteristics to noodles and pasta (improved eating quality)
- Lower cooking loss (noodles and pasta)

Brewing processes:

Lipase 3 can be added to the brewing process to enhance the mash separation and fermentation process in production of primarily non-malted brewing products. In the mashing step Lipase 3 removes lipids to enhance the mash separation. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol (and sometimes organic acids). In the fermentation Lipase 3 helps remove the lipids from the wort which can otherwise incapsule the yeast and impeding the fermentation.

Therefore, the benefits of the conversion of lipids with the help of Lipase in Brewing processes, are:

- Increase flexibility in the choice of raw materials.
- Faster and more predictable mash separation.
- Increase filtration rate and reduce need for beer filtration aids.
- Higher extract yield due to the improved processing, and thereby less use of raw materials.
- Potential for higher alcohol yield
- Potential for use of less raw material
- Removal of beer haze

In all of these applications, the enzyme product will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

3.2 Efficacy examples

Bakery process

The function of Lipase 3 with triglyceride hydrolysing activity in bakery process is to modify the endogenous lipids from wheat flour and produce more polar lipids, which will contribute to improved dough stability and handling properties. The polar lipids formed in the dough will produce bread with improved volume and a homogenous crumb structure with homogenous pores.

As shown in Figure 5 below, addition of Lipase 3 improves volume and softness effect in white pan bread. Volume was increased from 4.12 to 4.36 when un-shocked, and from 4.05 to 4.58 when shocked process is used in the bakery process (Figure 5A). As shown in Figure 5B, addition of Lipase 3 improves softness and helps softness retention throughout the shelf life. On day 1, bread made with Lipase 3 has a softness at 466.5 compared to control at 584.7. On Day 8, control reaches 724.1 while that with lipase 3 remained at 579.7 (Figure 5B).

Figure 5A

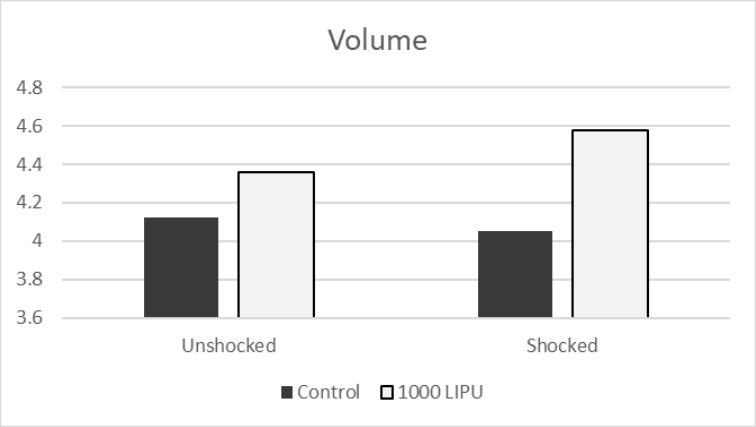


Figure 5B

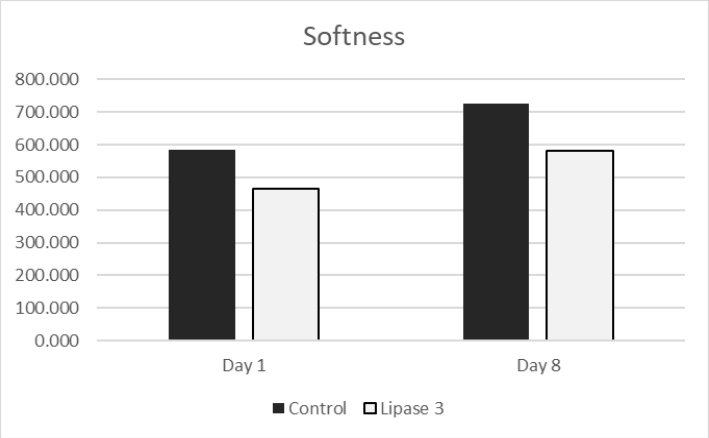


Figure 5. Adding lipase 3 increases white bread volume (Figure 5A) and improves softness (Figure 5B).

As shown in Figure 6 below, addition of Lipase 3 clearly shows an improvement of steam bun whiteness, shine, and smoothness from sensory evaluation. (Figure 6A), Specific volume increased from 2.34 ml/g as in no enzyme control to 2.46 ml/g with addition of Lipase 3.

Figure 6A

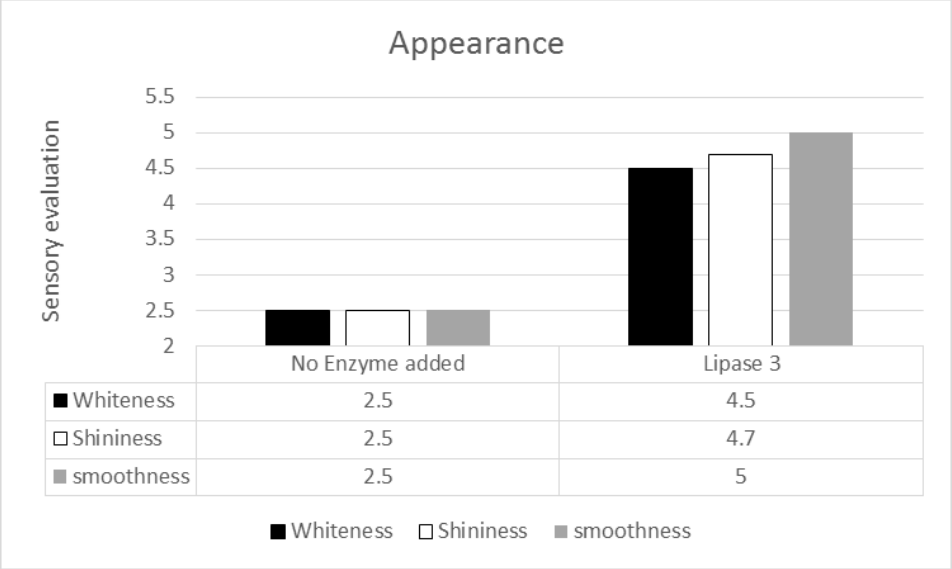


Figure 6B

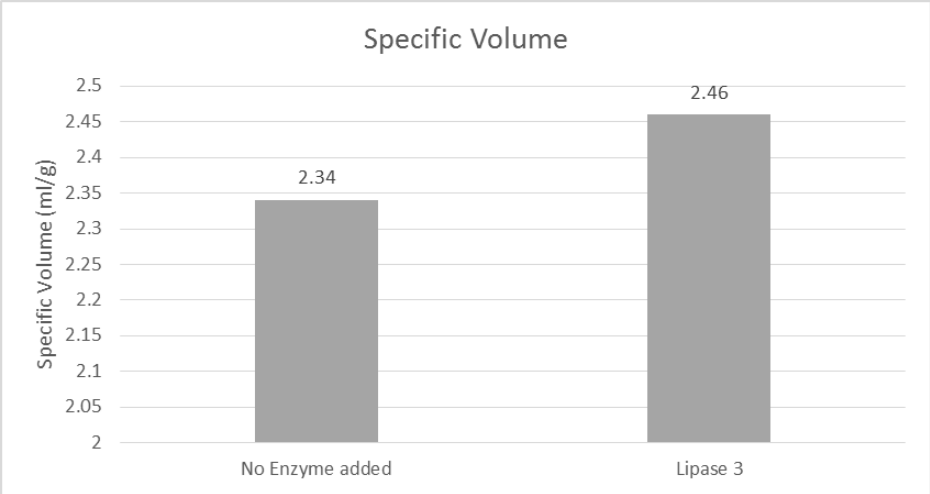


Figure 6. Adding lipase 3 improves steam bun whiteness, shine, smoothness (Figure 6A), and specific volume (Figure 6B).

Brewing process

The function of Lipase 3 is to catalyse the hydrolysis of ester bonds primarily in 1 and 3 position of fatty acids in triglycerides with release of fatty acids and glycerol. Lipase 3 can be added to the brewing process to enhance the mash separation and fermentation process in production of primarily non-malted brewing products. In the mashing step Lipase 3 removes lipids to enhance the mash separation. Current enzyme solution (amylase, xylanase glucanase, protease, and pullulanase) for barley brewing is functional up to 80% barley. Addition of Lipase 3 could help to achieve success with 100% brewing.

In Figure 7, it can be seen that addition of Lipase 3 to existing enzyme mixture (amylase, xylanase glucanase, protease, and pullulanase) could improve wort filtration and reduce the haze of the wort, providing more clear wort in 100% barley brewing. The increased filtration could be observed as early as 5 minutes. After thirty minutes of filtration, addition of Lipase 3 at 114 M LIPU/tn barley has approximately twice the volume of filtrate compared to the control.

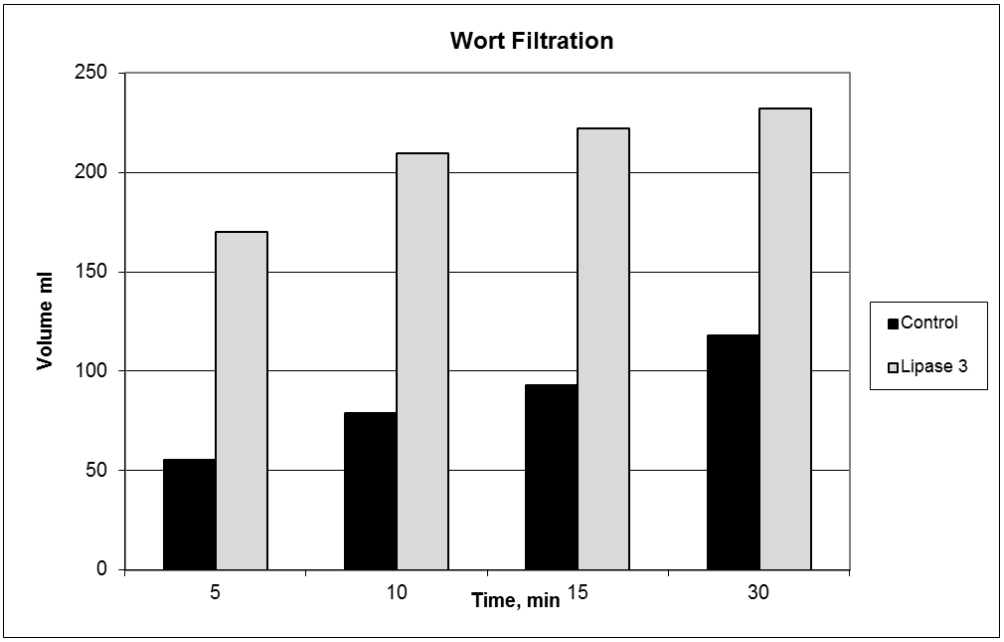


Figure 7. Adding lipase 3 improves wort filtration in 100% barley.

Control: Amylase, xylanase glucanase, protease, and pullulanase
Lipase 3: Lipase 3 + amylase, xylanase glucanase, protease, and pullulanase

In addition, Lipase 3 will increase extract yield of 100% barley brewing as presented by Original Extract (OE, Extract in the wort samples after mashing). Fermentable sugar as indicated by degree of polymerization (DP1) is also increased with addition of Lipase 3. Addition of Lipase 3 could increase plato percentage to 17.4% compared to control at 17.0% (Figure 8A), and fermentable sugar to 16.2% from 15.5% in control (Figure 8B).

Figure 8A

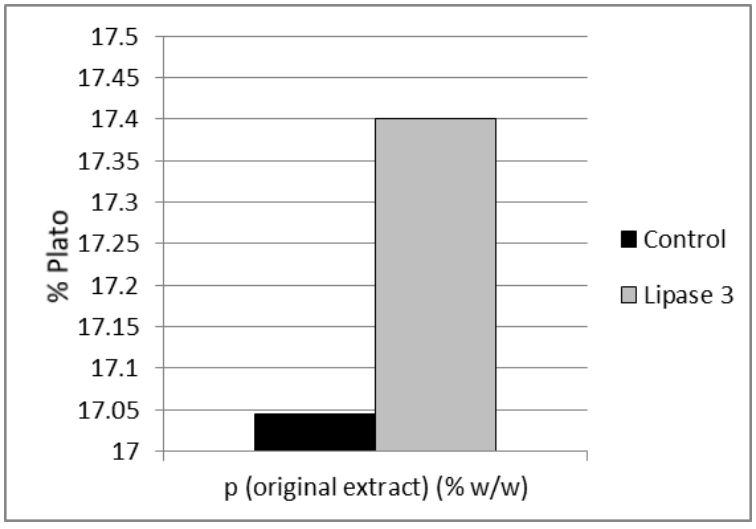


Figure 8B

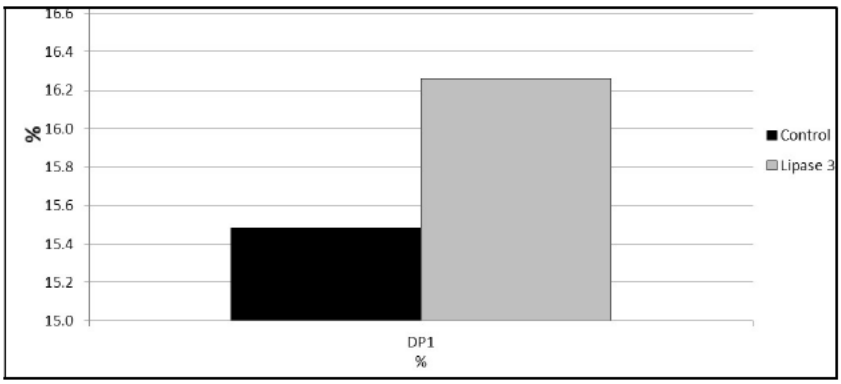


Figure 8. Adding lipase 3 improves extraction yield as indicated by OE (Figure 8A) and DP1 (Figure 8B).

Control: Amylase, xylanase glucanase, protease, and pullulanase
Lipase 3: Lipase 3 (114 M LIPU/tn Barley) + amylase, xylanase glucanase, protease, and pullulanase

4 Manufacturing process

The manufacturing process for the production of Lipase 3 will be conducted in a manner similar to other food and feed production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation/ drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (see, Enzyme Applications, 1994; Aunstrup et al, 1979; and Aunstrup, 1979).

4.1 Raw materials

The raw materials used in the fermentation and recovery process for the Lipase 3 enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008), except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. DuPont IB uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4.2 Fermentation

Lipase 3 is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* described in Appendix B. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures

are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new lyophilized stock culture vial of the *T. reesei* production organism is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

4.3 Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e. Lipase 3.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximize separation and is then fed into the filter or the centrifuge. The relatively solids free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g. glycerol to produce a liquid product.

A manufacturing flow chart is found in Appendix A6.

4.4 Formulation

The ultrafiltrated concentrate is then formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

5 Specification for identity and purity

5.1 Purity criteria

Appropriate GMP controls and processes are used in the manufacture of Lipase 3 to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits for the Lipase 3 product can be found in Appendix A4. Certificates of Analysis for three lots of product are given in Appendix A5.

The specifications for the Lipase 3 enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the Food Chemical Codex, 6th Edition (2008) (Appendix A7) and by the Joint FAO/WHO Expert Committee on Food additives (JECFA 2006) (Appendix A8).

5.2 Allergens

An allergen declaration of the enzyme concentrate can be found in Appendix A9.

6 References

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldsstein, L. pp. 28-68.

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. 1994. Kroschwitz, J.I., Volume 9, pp. 567-620.

Food Chemicals Codex (FCC) 6th Edition. 2008. US Pharmacopeia, Rockville, Maryland.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Appendix A1 : EC NUMBER

Source: IUBMB / http://www.chem.qmul.ac.uk/iubmb/

Accepted name: triacylglycerol lipase

Reaction: triacylglycerol + H_2O = diacylglycerol + a carboxylate

Other name(s): lipase; triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno N-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase

Systematic name: triacylglycerol acylhydrolase

Comments: The pancreatic enzyme acts only on an ester-water interface; the outer ester links are preferentially hydrolysed.

Links to other databases: BRENDA, EXPASY, GTD, KEGG, Metacyc, PDB, CAS registry number: 9001-62-1

References:

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3. Sarda, L. and Desnuelle, P. Action de la lipase pancréatique sur les esters en émulsion. *Biochim. Biophys. Acta* 30 (1958) 513-521.

4. Singer, T.P. and Hofstee, B.H.J. Studies on wheat germ lipase. I. Methods of estimation, purification and general properties of the enzyme. *Arch. Biochem.* 18 (1948) 229-243.

5. Singer, T.P. and Hofstee, B.H.J. Studies on wheat germ lipase. II. Kinetics. *Arch. Biochem.* 18 (1948) 245-259.

[EC 3.1.1.3 created 1961]

Appendix A2 : CAS NUMBER
Source: SciFinder Database
1. CAS Registry Number: 9001-62-1

CA Index Name:	Triacylglycerol lipase
Molecular Formula:	Unspecified
References in CAplus:	60059

Chemical Names

Other CA Index Names:	Lipase, triacylglycerol
Synonyms:	2212E; ABS Fungal Lipase L; AK Amano 20; AY Amano 30; AYS Amano; Acid lipase; Alfamalt LP 10066; Alkaline lipase; Allzyme Lipase; Altus 13; Altus 2; Amano AK; Amano AP; Amano AY 1; Amano B; Amano CE; Amano CES; Amano I; Amano II; Amano LPL 200S; Amano Lipase A; Amano Lipase AK; Amano Lipase IM; Amano Lipase PS; Amano Lipase PS-D-1; Amano M; Amano N-AP; Amano P; Amano PS 30; Amano PS-CI; Amano PS-IM; Arthrobacter lipase; Bakezyme L 8000; Bakezyme PH 800; Beamhouse BLE-N; Bile salt-activated lipase; Bioprase OP; Bioprase OP 10; Butyrinase; Buzyme 2103; Buzyme 2517; C-Lipase; C14 esterase; C8 esterase; Cacordase; Callera Trans L; Candida antarctica lipase A; Capalase; Capalase K; Capalase L; Cartazyme LP; Chirazyme 435; Chirazyme C 2; Chirazyme CHI L-2; Chirazyme L; Chirazyme L 1; Chirazyme L 10; Chirazyme L 2; Chirazyme L 2C2; Chirazyme L 3; Chirazyme L 5; Chirazyme L 6; Chirazyme L 7; Chirazyme L 8; Chirazyme L 9; Chirazyme L-2 C-1; Chirazyme c-f; ChiroCLEC-CAB; ChiroCLEC-CR; ChiroCLEC-PC; Cleanase NLA-P; CloneZyme ESL 001; DLIP 300; DeGreez; DeGreez (enzyme); Defat 50; E.C. 3.1.1.3; EnzOx A; EnzOx B; Enzylon PF; Esterase C14; Esterase C8; Esterase TL01; Esterzyme; Esterzyme B1; Esterzyme B2; Fetipase; Fluozim G 3Kh; GA 56; GA 56 (enzyme); GL 100; GS Clear; Glycerol ester hydrolase; Greasex; Greasex 100L; Greasex 50L; Hepatic lipase; Hepatic triacylglycerol lipase; ICR-107; ICR-113; ICR-116 lipase; IM 60; IMMAPF T2; IMMCALA-T2-150; Invigal DG; Italase; Italase C; KWI 56; L 8070; LE 100; LIP 300; LTK T 100; LVK F100; Leveking; Lilipase A 10; Lilipase A 10D; Lilipase A 10FG; Lilipase A 5; Lilipase B 2; Lillipase A-10FG; Lipase; Lipase 250; Lipase A; Lipase A 100L; Lipase A 10FG; Lipase A 15; Lipase A Amano 6; Lipase A CLEA; Lipase A12; Lipase AH; Lipase AK; Lipase AK Amano; Lipase AKG; Lipase AL; Lipase ALC; Lipase ALG; Lipase AP; Lipase AP 12; Lipase AP 6; Lipase AS; Lipase AS Amano; Lipase AY; Lipase AY 30; Lipase AY 30G; Lipase AY Amano; Lipase AY Amano 30 D-K; Lipase AY Amano 30 G; Lipase AY Amano 30 SD; Lipase AY Amano 50G; Lipase AY Amano 6; Lipase AYS; Lipase AYS Amano; Lipase Amano AY; Lipase BVP;

Lipase CCL; Lipase CE; Lipase CE Amano; Lipase CR; Lipase D; Lipase D Amano 2000; Lipase D Amano 350; Lipase DF 15; Lipase DF Amano 15; Lipase DF Amano 15K; Lipase EU-034; Lipase EU-093; Lipase F; Lipase F Amano; Lipase F-AP; Lipase F-AP 15; Lipase G LGD-N46-001; Lipase GC Amano 4; Lipase IM; Lipase L 5; Lipase L Amano 10; Lipase LAK; Lipase LP; Lipase LP ′S′; Lipase LPL; Lipase M; Lipase M 10; Lipase M Amano 10; Lipase M-AP 10; Lipase MER; Lipase MER Amano; Lipase MY; Lipase MY 30; Lipase N; Lipase OF; Lipase OFEX; Lipase P; Lipase PA; Lipase PF; Lipase PL; Lipase PL 266; Lipase PL 679; Lipase PLC; Lipase PLG; Lipase PN; Lipase PS; Lipase PS Amano IM; Lipase PS Amano SD; Lipase PS Amano SDH; Lipase PS LPSAN/2522; Lipase PS-30; Lipase PS-C; Lipase PS-C Amano 1; Lipase PS-C Amano II; Lipase PS-CI; Lipase PS-D; Lipase PS-D Amano I; Lipase PS-IM; Lipase PS-SDH; Lipase PSL-C; Lipase QL; Lipase QLC; Lipase QLG; Lipase QLM; Lipase QLMex; Lipase R; Lipase RH; Lipase RM; Lipase SAM-II; Lipase SL; Lipase SP 525; Lipase SP 539; Lipase SP-435; Lipase TL; Lipase TL IM; Lipase UH; Lipase UL; Lipase-esterase; Lipases; Lipazin; Lipex; Lipex 100L; Lipex 100T; Lipex 100TB; Lipex 50T; Lipidase 10000; LipoPrime; Lipolactine G 10x; Lipolase; Lipolase 100L; Lipolase 100L-EX; Lipolase 100T; Lipolase 30T; Lipolase EX; Lipolase L; Lipolase SP 400; Lipolase T100; Lipolase Ultra; Lipolase Ultra 50T; Lipolex; Lipolyve AN; Lipolyve CC; Lipolyve R; Lipomax; Lipomax 500G; Lipomax CXT; Lipomax CXT 1000; Lipomod; Lipomod 166P; Lipomod 187; Lipomod 187P; Lipomod 224P; Lipomod 29P; Lipomod 338P; Lipomod 34; Lipomod 34P; Lipomod 36; Lipoorizin; Lipoorizin G3X; Lipooryzin; Lipopan H; Lipopan S-BG; Lipopan S-FG; Lipopan S-OG; Lipoprime 50T; Liposam; Lipozyme; Lipozyme 10000L; Lipozyme 20000L; Lipozyme 3A; Lipozyme 62350; Lipozyme CALB L; Lipozyme IM; Lipozyme IM 20; Lipozyme IM 60; Lipozyme L 9; Lipozyme M; Lipozyme RM; Lipozyme RML; Lipozyme TLL; Llipozyme MML; Lumafast; Lumafast 2000G; Lypooryzin G3X; Lypozym lm45; M Amano 10; Meito 266; Meito MY 30; Meito Sangyo OF lipase; Monomax; NS 40013; NS 40042; NS 435; NS 44126; NS 51032; NS 51049; NS 51050; NS 51051; NS 51052; NZL-102-LYO; NZL-103-LYO; NZL-107-LYO; NovoSample 40013; Novocor ADL; Novozym 243; Novozym 345; Novozym 388; Novozym 51032; Novozym 677 MG; Novozym 677BG; Novozym 735; Novozym 868; Novozyme 388; Novozyme 398; Novozyme 40086; Novozyme 435 FG; Novozyme 51032; Novozyme 735; Novozyme 868; Novozyme 871; Olipase 4S; Orbazim HC 120Y; PEC High lipase; PLRP2 lipase; PPL; Palatase; Palatase 20000L; Palatase A; Palatase A 750L; Palatase M; Palatase M 1000L; Pancreatic lipase-related protein 2 lipase; Phytolipase; Piccantase A; Piccantase AN; Piccantase R8000; Postheparin lipase;

	Postheparin plasma hepatic lipase; Pregastric esterase; Randozyme SP 382; Remzyme PL 600; Resinase A 2X; Rizolipase; SG II; SP 398; SP 525 lipase; Saiken 100; Steapsin; Sumizyme CT-L; Sumizyme NLS; Takedo 1969-4-9; Talipase; TheraCLEC-Lipase; Toyozyme LIP; Transzyme; Triacetinase; Triacetinase I; Triacetinase II; Triacylglyceride lipase; Triacylglycerol ester hydrolase; Triacylglycerol hydrolase; Tributyrase; Tributyrin esterase; Tributyrinase; Triglyceridase; Triglyceride hydrolase; Triglyceride lipase; Triolein hydrolase; Trioleinase; Tween esterase; Tween hydrolase; Tween-hydrolyzing esterase; Tweenase; Ultra G; Validase fungal lipase 8000; Veron Hyperbake; YSSH
Other Identifiers	
Deleted Registry Numbers:	9004-01-7; 114558-45-1; 9014-49-7; 211049-97-7; 114558-43-9; 132823-04-2; 9001-70-1; 211255-77-5; 211049-96-6; 212955-16-3; 142615-72-3; 119663-46-6; 135105-44-1
Alternate Registry Numbers:	152923-53-0
Related Information	
Class Identifiers:	Manual Registration (MAN); Component (COM)
Document Types:	Patent (P); Journal (J); Dissertation (D); Conference (C); Book (B); Nonpatent (NPL); Report (R); Preprint (PP)
Dates	
Entered STN:	16 Nov 1984
Last update:	17 Jun 2017
Text updated:	17 Jun 2017

Appendix A3: Activity of the enzyme complex

Determination of lipase activity on Tributyrin

Substrate:

Substrate 5% (v/v) tributyrin:

Mix 15.00 ml tributyrin, 50.00 ml emulsifying agent and 235 ml dist. water for 20 sec on a homogenizer. Eventually adjust pH in the substrate to approx. 5.4 with 0.5 M NaOH. Check, when a new batch of tributyrine is used - see "Enzyminstruks nr. 5".

Assay procedure:

1. Prepare substrate and 0.05 N NaOH on the day of analysis.

2. Sample preparation: Make on enzyme solution of app. 1 LIPU/ml per samle.

The samples are dissolved in dist. water.

3. In a volumetric flask dissolve the weighed amount of enzyme in half of the final dilution and subject to magnetic stirring for 20 min.

4. After stirring, adjust to final dilution with dist. water. Any further dilution should be made with dist. water.

5. Thermostat 25.00 mL substrate at 30.0°C.

When using 718 STAT TITRINO (at DIGR), pH adjustment, titration, and calculation will take place automatically. Add sample when display shows "pause".

6. Adjust substrate pH to 5.50 with NaOH.

7. While stirring, add 2.00 mL sample, and initiate pH-stat titrator. The 2.00 mL sample is collected while stirring (eg. is mixed with the pipette before sampling).

8. Stop titration after 10 minutes and read slope of the titration curve.

Calculation:

Calculation of the activity LIPU/g enzyme:

$$\text{LIPU/g} = \frac{\text{ml/min.} \times N \times 1000 \times F \times \text{factor for tributyrin}}{A \times 2}$$

ml/min.: Slope of titration curve
N : Normality of NaOH
F : Dilution of sample
A : Gram sample weighed
2 : ml sample

Appendix A4: Specification of the commercial product

Property	Method number	Reference method	Specification
ENZYME ACTIVITIES			
Lipase	A0770		Varies by product
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	0238-83	Nordic Committee on Food Analysis; Aerobic microorganisms. Determination in foods at 37 °C, 30 °C, 25 °C, 20 °C, 17/7 °C or 6.5 °C by the colony count method. No. 86; 5. Ed., 2013) and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Not more than 10,000 CFU/g
Total Coliforms	0238-22	Nordic Committee on Food Analysis; Coliform bacteria. Detection in foods and feeds. No 44; 6.ed., 2004) and and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Not more than 30 per gram
Escherichia coli	0238-31	Ph. Eur. 2.6.13 equal to ISO 7521– "Microbiology – General guidance for the Enumeration Presumptive Escherichia coli – Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Absent in 25 g of sample
Salmonella sp:	0238-51	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No. 71; 5. Ed; 1999 and FDA Bacteriological Analytical Manual; 8th Edition AOAC International	Absent in 25 g of sample
Antimicrobial Activity	0238-63	Nordic Committee on Food Analysis; Coagulase positive staphylococci. Enumeration in foods. No. 66; 5.Ed; 2009)	Absent in 1 g of sample
OTHER ASSAYS			
Lead	DJA91	ISO 17294m:2005 –" Water quality -- Application of inductively coupled plasma mass spectrometry (ICP-MS) -- Part 2:	Less than 5 mg/kg

		Determination of 62 elements”	
Mycotoxin, T2		Neogen Corporation, Veratox T-2/HT-2 Toxins kit, V-T2HT2-0909	Negative by test
Production organism		SOP - Detection of production microorganism , R-SOP-SL-097-03	Negative by test

Appendix A5: Certificates of analyses

CERTIFICATE OF ANALYSIS

PRODUCT: **LIP3 Form UF Conc**

BATCH: **20160350**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Lipase	LIPU/g	Report value	120000
PHYSICAL PROPERTIES			
pH		Report value	4.4
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1
Total Coliforms	CFU/ml	0-30	<1
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

7-Jul-2017
Date

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **LIP3 Form UF Conc**

BATCH: **20160351**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Lipase	LIPU/g	Report value	124000
PHYSICAL PROPERTIES			
pH		Report value	4.2
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1
Total Coliforms	CFU/ml	0-30	<1
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

7-Jul-2017
Date

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **LIP3 Form UF Conc**

BATCH: **20168072**

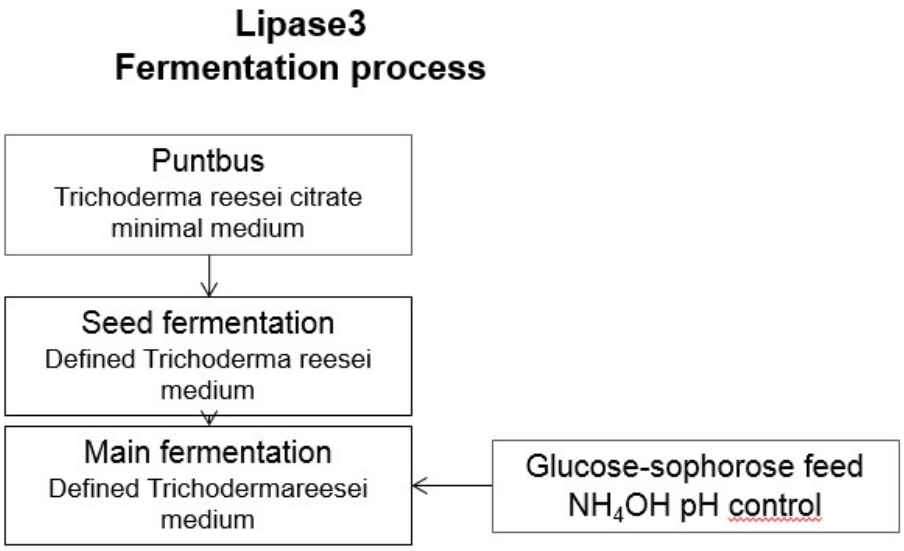
ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Lipase	LIPU/g	Report value	107901
PHYSICAL PROPERTIES			
pH		Report value	4.5
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	3
Total Coliforms	CFU/ml	0-30	<1
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

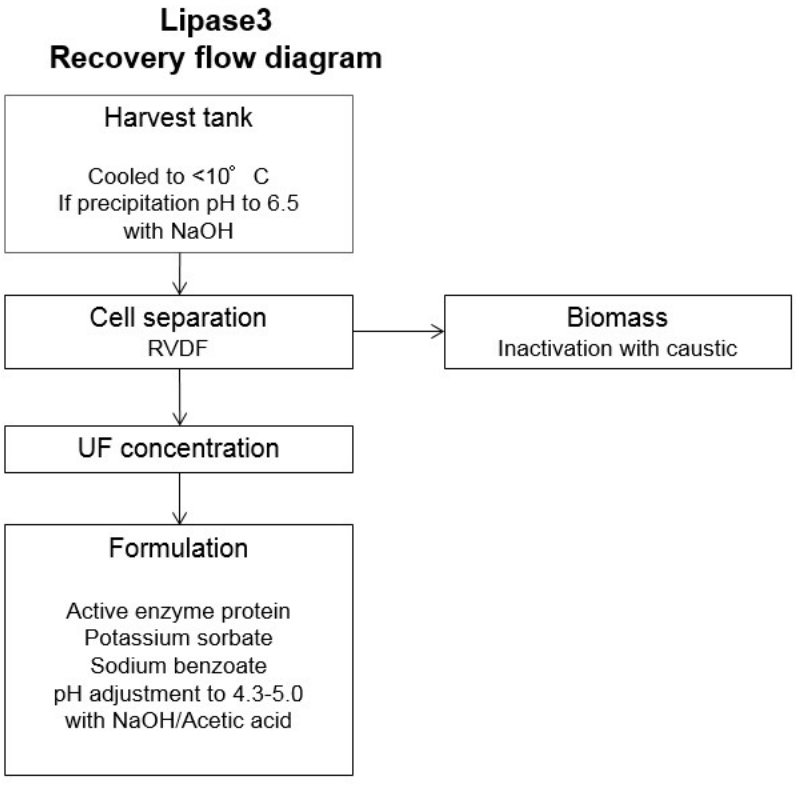
This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

7-Jul-2017
Date

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix A6: Production process flow chart





Appendix A7: Food Chemical Codex, 6th edition

Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt, Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Enzyme Assays,* Appendix V.

Function Enzyme (see discussion under *Classification*, below)

Packaging and Storage Store in well-closed containers in a cool, dry place.

IDENTIFICATION

Classification

- **ANIMAL-DERIVED PREPARATIONS**

Catalase, Bovine Liver: Produced as partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from purified extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3)

lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueous extracts made from the fourth stomach of calves. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (chymosin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of lambs or kids.

Trypsin: Obtained from purified extracts of porcine or bovine pancreas. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: used in baking, in the tenderizing of meat, and in the production of protein hydrolysates.

- **PLANT-DERIVED PREPARATIONS**

Amylase: Obtained from extraction of ungerminated barley. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble in water (the solution is usually colorless or light yellow and somewhat opalescent), but

practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

- **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase, pectin lyase*, and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosidase*, (6) *hemicellulase* (a mixture of enzymes, including *poly(galacturonate) hydrolase, arabinosidase, mannosidase, mannanase*, and *xylanase*), (7) *lactase*, (8) β-*glucanase*, (9) β-D-*glucosidase*, (10) *pentosanase*, and (11) α-*galactosidase*. Typical applications: used in the preparation of starch syrups and dextrose, alcohol, beer, ale, fruit juices, chocolate syrups, bakery products, liquid coffee, wine, dairy products, cereals, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in

water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortierella vinaceae* var. *raffinoseutilizer*) Produced as an off-white to tan powder or as pellets by controlled fermentation using *Mortierella vinaceae* var. *raffinoseutilizer*. Soluble in water (pellets may be insoluble in water), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*galactosidase*. Typical application: used in the production of sugar from sugar beets.

Carbohydrase: (*Rhizopus niveus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Rhizopus niveus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *glucoamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal, in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually

light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and cereal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase: (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*. The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is

soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat cereals, and other grain-based foods.

- **REACTIONS CATALYZED**

[NOTE: The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Acetolactatedecarboxylase: Decarboxylation of α-cetolactate to acetoin

Aminopeptidase, Leucine: Hydrolysis of *N*-terminal amino acid, which is preferably leucine, but may be other amino acids, from proteins and oligopeptides, yielding free amino acids and oligopeptides of lower molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-**Amylase:** Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrow O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + $O_2 \leftrightarrow$ D-glucono-δ-lactone + H_2O_2

β-D-**Glucosidase:** Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: Hydrolysis of α-1,4-glucan bonds

Lipase: Hydrolysis of triglycerides of simple fatty acids, yielding mono- and diglycerides, glycerol, and free fatty acids

Pancreatin:

α-Amylase: Hydrolysis of α-1,4-glucan bonds

Protease: Hydrolysis of proteins and polypepticles

Lipase: Hydrolysis of triglycerides of simple fatty acids

Pectinase:

Pectate lyase: Hydrolysis of pectate to oligosaccharides

Pectin depolymerase: Hydrolysis of 1,4 galacturonide bonds

Pectin lyase: Hydrolysis of oligosaccharides formed by pectate lyase

Pectinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase:

3-Phytase: *myo*-Inositol hexakisphosphate + H_2O ⇔ 1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + H_2O⇔an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α- and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

- **PROCEDURE**

Analysis: The following procedures, which are included under *Enzyme Assays,* Appendix V, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1]: Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

- **LEAD,** *Lead Limit Test,* Appendix IIIB

Control: 5 µg Pb (5 mL of *Diluted Standard Lead Solution*)

Acceptance criterion: NMT 5 mg/kg

SPECIFIC TESTS

- **MICROBIAL LIMITS**

[NOTE: Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.cfsan.fda.gov/.]

Acceptance criteria:

Coliforms: NMT 30 CFU/g

Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Change to read:

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source of derivation, they should cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used to produce enzymes must comply with the applicable U.S. meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used to produce enzymes or culture media used to grow microorganisms consist of components that leave no residues harmful to health in the finished food under normal conditions of use.

▲Preparations derived from microbial sources shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.▲ *FCC 6*

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

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[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
Monograph		(FI07) Food Ingredients Expert Committee

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Appendix A8 : General specifications for enzyme preparations used in food processing (JECFA)

General Specifications and Considerations for Enzyme Preparations Used in Food Processing

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (*1*) and published in FAO JECFA Monographs 1 (*2*). These specifications were originally prepared by the Committee at its twenty-fifth meeting (*3*) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (*4,5*). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (*6*). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 *(7*), and at the fifty-third meeting *(8*) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 *(9)*.

Classification and nomenclature of enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an

enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. 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 (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in processing and formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

% TOS = 100 - (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead:
Not more than 5 mg/kg.
Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:
Salmonella species: absent in 25 g of sample
Total coliforms: not more than 30 per gram
Escherichia coli: absent in 25 g of sample
Determine using procedures described in Volume 4.

Antimicrobial activity:
Absent in preparations from microbial sources.

Other considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.

2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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27. **Codex Alimentarius Commission**. *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms* (CAC/GL 46-2003; http://www.fao.org/ag/AGN/food/risk_biotech_taskforce_en.stm, accessed 20 July 2006).

28. **Health Canada.** *Guidelines for the Safety Assessment of Novel Foods Derived from Plants and Microorganisms (Draft),* 2003 (http://www.hc-sc.gc.ca/fn-an/consultation/init/consultation_guidelines-directives01_e.html, accessed 20 July 2006).

Appendix A9 : Allergen declaration on the enzyme liquid concentrate

Allergenic Ingredients

Product	**Lipase 3 Form UF Conc**
Article-no.	**C07002**

The table below indicates the presence (as added component) of the following allergens and products thereof *. **Unless otherwise noted, the following listed allergens and products thereof have been used in the recovery process or in the formulation of an enzyme product:**

YES	NO	Allergens	Description of components
X		Wheat	The final dry products for the bakery applications can be spray-dried on potato- or wheat starch but since bakery products are produced with similar allergen group (e.g. wheat) no additional allergens are introduced into the final food.
(X)		Other cereals containing gluten	Glucose, (used in fermentation)**
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
(X)		Soybeans	Soy bean grits (used in fermentation)**
	X	Milk (including lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sesame Seeds	
	X	Sulphur dioxide and sulfites >10mg/kg	
	X	Lupine and products thereof	
	X	Mollusk and products thereof	

*Local legislation has always to be consulted as allergen labeling requirements may vary from country to country.

**Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements

January 11, 2018

APPENDIX C: Dietary exposure

Contents:

1 Application areas

Lipase 3 will be used in baking for production of bakery products such as, but not limited to, bread, Chinese stem buns, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers, and waffles. In brewing processes, Lipase 3 will be used for the production of beer and other cereal-based beverages.

Consequently, according to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), Lipase 3 will be used in:

- bread, Chinese stem buns, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers, and waffles - 7 Bread and Bakery Products
- beer and other cereal based (alcoholic) beverages - 14.2.1 Beer and related products

2 Level of use

Baking:

Lipase 3 can be used in baking for production of bakery products such as, but not limited to, bread, Chinese stem buns, biscuits, steamed bread, cakes, noodles, pancakes, pasta, tortillas, wafers and waffles. Lipase 3 can be added to the dough. The proposed application rate of Lipase in baking is 2.5-21.2 mg total organic solid (TOS)/kg flour.

Brewing processes:

Lipase 3 can be used in brewing processes for production of beer and other cereal-based beverages, primarily for non-malted products. Lipase 3 can be added to the mash separation and fermentation step. The proposed application rate of Lipase 3 in brewing processes is 5.2-52.2 mg TOS/kg cereals.

3 Level of residues in food

3.1 Estimated Food Intake

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 Practice. 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 this 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 Lipase 3 is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

The dosage of a food enzyme depends on the activity of the enzyme protein (in this case Lipase 3) present in the final food enzyme preparation (i.e. the formulated food enzyme). However, the activity Units as such do not give an indication of the amount of food enzyme actually added. Microbial food enzymes contain – apart from the enzyme protein

in question – also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids[1] (TOS, FAO/WHO, 2006). 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 food enzyme may be used.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	2.5-21.2	21.2
Brewing processes	Cereals	5.2-52.2	52.2

Ratio between raw material (RM) and final food (FF)

Baking

Bakery products fall in the category of solid foods.

Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced. From 1 kg of flour one would produce 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of bread is higher than that of cracker, consequently bread is used as the assumption for the calculation of dietary exposure from bakery processes.

The yield of 1.4 kg of bread per 1 kg of flour correspond to a RM/FF ratio of 0.71 kg of flour per kg bakery product is used.

Brewing processes

Brewing processes adds to the class of liquid foods.

Raw materials used in brewing processes are various kinds of cereals (e.g. malt, barley, wheat, sorghum and maize). Yields will vary dependent on the type of cereal, process used and the type of drink produced.

Beer production has a range of RM/FF from 14-28 Kg of grist per 100L of beer, with 80-90 % of all beers produced at a RM/FF ratio of 14-20 kg of grist per 100 L of beer.

The assumption used for calculation of dietary exposure is a yield of 100L of drink per 17 kg of cereal corresponding to a RM/FF ratio of 0.17 kg grist per L of beer.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method

[1] 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.

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):

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 addition to the assumptions from the Budget Method, it is assumed that beer is consumed in the same amount as soft drinks (25% of total liquid intake).

3.2 Estimated intake of Lipase 3

The recommended use levels of the enzyme Lipase 3 are given (Section 2), based on the raw materials used in the various food processes. For the calculation of the Theoretical Maximium Daily Intake (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.

Application		**Raw material (RM)**	**Maximal recommended use level (mg TOS/kg RM)**	**Example Final food (FF)**	**Ratio RM/FF**	**Maximal level in FF (mg TOS/kg food)**
Beverages	Brewing processes	Cereal	52.2	Beer	0.17	**8.87**
Solid food	Baking	Flour	21.2	Bread	0.71	**15.05**

The Total TMDI can be calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day, which in this case is bread and beer. Consequently, the Total TMDI will 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)
15.05x0.0125=0.188	8.87x0.025=0.222	**0.410**

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 Lipase 3 from *Trichoderma reesei*.
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in foodstuffs as well as in beverages, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages 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).

4 Safety assessment

Lipase 3 is an enzyme produced from *T. reesei* which was genetically modified to express the lipase gene from *A. tubingensis*.

To assess the safety of Lipase 3 in foods, different endpoints of toxicity were investigated at MB Research Laboratories (Pennsylvania) and Harlan Laboratories (Switzerland) and are evaluated and assessed in this document. Lipase 3 is non-hazardous based on acute oral studies. In genotoxicity studies, Lipase 3 is not mutagenic, clastogenic or aneugenic. Daily oral administration of Lipase 3 up to and including a dose level of 160.6 mg total protein/kg bw/day or 123.15 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Identification of the NOAEL

In the 90-day oral (gavage) study in rats for Lipase 3 from *Aspergillus tubingensis*, a NOAEL was established at 160.6 mg total protein/kg bw/day equivalent to 123.15 mg TOS kg bw/day. The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice.

NOAEL: 160.6 mg TP/kg bw/day = 123.15 mg TOS/kg bw/day

Determination of the Margin of Safety

The margin of safety is calculated by dividing the NOAEL obtained from the 13-weeks oral (gavage) study in rats by the human exposure (worst case scenario). If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{NOAEL\ ((mg/kg\ bw)/day)}{Human\ Exposure\ ((mg/kg\ bw)/day)}$$

$$\text{Margin of Safety} = \frac{123.15\ mg\ TOS\ /kg\ bw/day}{0.410\ mgTOS\ /kg\ bw/day}$$

Margin of Safety = 300

5 Conclusion

The safety of Lipase 3 from *T. reesei* as a food processing aid in baking and brewing processes is assessed in a battery of toxicology studies investigating its acute oral, mutagenic and systemic toxicity potential. Lipase 3 is not acutely toxic. A battery of genotoxicity assays was conducted and under the conditions of these assays Lipase 3 is not a mutagen, a clastogen, or an aneugen.

Daily administration of Lipase 3 by gavage for 91/92 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 160.6 mg total protein/kg bw/day corresponding to 123.15 mg TOS/kg bw/day.

Based on a margin of safety of 300, the proposed uses of Lipase 3 in baking and brewing processes are not a human health concern and are supported by existing toxicology data.

6 References

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

January 11, 2018

APPENDIX D: International and other National Standards

Contents:

Appendices D

D1	GRAS panel letter
D2	EU legislation on enzymes

Food enzymes are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes will also contain some substances derived from the producing micro-organism and the fermentation medium. From a safety point of view, the similarity of the producing micro-organism is of higher importance than that of the enzyme protein in question. Therefore, sections below summarize not only authorized food enzymes with the same enzyme activity, but also authorized food enzymes from the same producing organism. As documented below, Triacylglycerol lipase from various micro-organisms (including genetically modified ones) are widely accepted and *Trichoderma reesei* - whether or not genetically modified - is widely accepted as a safe production organism for a broad range of enzymes.

1 Codex Standards

1.1 The enzyme

Lipase 3 has not been evaluated by the Joint Expert Committee on Food Additives (JECFA).

1.2 Supporting evaluations

Cellulase from *T. reesei* and Glucoamylase from *T. reesei* expressed in *T. reesei* have been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set (Technical Report Series 733, 1986).

2 International Legislation

2.1 United States

2.1.1 The enzyme

Lipase 3 has been determined to be GRAS as a food processing aid in baking and brewing processes by a panel of scientific experts in the USA (Appendix D1).

2.1.2 Supporting approvals

Cellulases from *Trichoderma reesei* were affirmed as GRAS by U.S. FDA (21CFR184.1250). Also the FDA has no questions to four GRAS Notices on enzymes derived from *Trichoderma reesei*:

- Pectin lyase derived from *T. reesei* carrying a gene encoding pectin lyase from *Aspergillus niger* (GRN 32)
- Transglucosidase enzyme preparation from *T. reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* (GRN 315)
- Acid fungal protease enzyme preparation (GRN 333)
- Chymosin enzyme preparation from *T. reesei* expressing the bovine prochymosin B gene (GRN 230)
- Glucoamylase enzyme preparation from *T. reesei* expressing the glucoamylase gene from *T. reesei* (glucoamylase enzyme preparation) (GRN 372)

In addition, lipases from following production organisms have been granted GRAS status by U.S. FDA.

- Lipase enzyme preparation from modified *Pseudomonas fluorescens* Biovar I (GRN 462)
- Lipase enzyme preparation derived from *Hansenula polymorpha* expressing a gene encoding a lipase from *Fusarium heterosporum* (GRN 238)
- Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger* (GRN 296)
- Lipase enzyme preparation from *Rhizopus oryzae* (GRN 216)
- Lipase prepration from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica* (GRN 158)
- Lipase enzyme preparation from *Aspergillus oryzae* (GRN 113)
- Lipase enzyme preparation from *Aspergillus niger* (GRN 111)
- Lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene (GRN 103)
- Lipase from *Candida rugosa* (GRN 81)
- Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Fusarium oxysporum* (GRN 75)
- Lipase from *Penicillium camembertii* (GRN 68)
- Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Thermomyces lanuginosus* (GRN 43)

2.2 Europe

2.2.1 The enzyme

Lipase 3 has neither been evaluated in France nor in Denmark yet.

In Europe, most of the enzyme preparations used in food processing are considered processing aids, meaning that they have their technological function in the food-processing stage and not in the final food. They are excluded from the Food Additives Framework Directive. On 16 December 2008 the European Parliament and the Council adopted Regulation 1332/2008 EC on food enzymes which aims to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU (Appendix D2). Several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable. Only France and Denmark have legislation covering all food-use enzymes. In Denmark and France, approval is needed prior to use. The information contained in the application dossier necessary for approval should follow the guidelines laid down by the SCF in 1992 or the EU Regulation 1332/2008. France has some additional national requirements specified in the Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la

fabrication de certaines denrées alimentaires as amended. In the other EU countries, enzyme preparation should be proved to be safe for use in food before being sold in EU according to the General EU Food Law. It is the producer's responsibility how to meet this requirement. DuPont IB uses the USA GRAS system as the backbone for this.

2.2.2 Supporting approvals

T. reesei, including genetically modified strains, has been approved for the production of amylase, cellulase, glucoamylase, xylanase enzymes in the food industry in Denmark and in France. In France, it is also approved for the production of Bêta glucanase and Lysophospholipase (Arrêté du 19 Octobre 2006 as amended).

Lipase from *Hansenula polymorpha* expressing the gene from *Fusarium heterosporum* has been approved in Denmark and Lipase from *Aspergillus oryzae (GM origin), Candida rugosa, Aspergillus niger (GM origin), Hansenula polymorpha (GM origin), Rhizopus oryzae, Rhizopus niveus* has been approved in France (Arrêté du 19 Octobre 2006)

2.3 Other countries

2.3.1 Supporting approvals

Trichoderma reseei, including genetically modified strains, has been approved for the production of amyloglucosidase, beta-glucanase, beta-glucosidase, celulase, esterase, hemicelulase and maltase enzymes in the food industry in Brazil (http://portal.anvisa.gov.br/documents/33916/391619/Resolu%25C3%25A7%25C3%25A3o%2BRDC%2Bn.%2B53_2014_Lista%2Bde%2Benzimas.pdf/680b654b-2bab-4571-a498-d77dd1cec8c4).

Strains of *T. reesei* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations" (https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html), as an authorized source for cellulase, glucanase, pentosanase and xylanase in several food applications.

Lipases from *Aspergillus niger, Aspergillus oryzae, Rhizopus oryzae, Rhizomocur miehei, Rhizopus niveus* and *Penicillium camembertii* have been approved for use in Canada ((https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html). Lipases from *Aspergillus niger, Aspergillus oryzae, Candida cylindracea, Rhizopus oryzae, Rhizomocur miehei,* and *Rhizopus niveus* have been approved in China (http://www.moh.gov.cn/zwgkzt/psp/201106/51947/files/b2413b87e525441ebb2882e61137242c.pdf).

Appendix D1 : GRAS panel letter

October 21, 2016

DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the intended use of DuPont's Lipase 3 enzyme preparation from *Aspergillus tubingensis* that is expressed in a non-pathogenic, non-toxigenic strain of *Trichoderma reesei*

Dear

I have reviewed the information you provided on DuPont's Lipase 3 enzyme preparation, which is produced by *Trichoderma reesei* Morph Lip3 (GICC03373), a production strain that has been genetically modified to over-express the native Lipase 3 from *Aspergillus tubingensis* (DuPont IB strain 1M341). The intended use of Lipase 3 is as a processing aid in brewing and baking, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect.

In evaluating Lipase 3, I considered the biology of *T. reesei* and *A. tubingensis* and their history of safe use in food-grade enzyme manufacture; safety evaluation studies on the Lipase 3 enzyme preparation; safety evaluation studies on other food grade enzymes expressed by DuPont's safe lineage of *T. reesei* production strains; history of safe use in foods of lipases from other microbial species; information that you provided regarding the safe lineage of the production organism, cloning methodology, manufacturing materials and procedures, and product specifications; and information that is publically available in the peer-reviewed scientific literature.

By way of background, *T. reesei* is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. DuPont's safe lineage of *T. reesei* production strains, including *T. reesei* Morph Lip3 (GICC03373), was derived through a series of modifications from *T. reesei* QM6a, the original non-pathogenic and non-toxigenic wild-type

parental strain used to produce this safe lineage of *T. reesei* enzyme production strains. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and DuPont's (legacy Genencor and Danisco) unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, transglucosidase, cellulases, glucoamylase, α-amylase, β-glucosidase/cellulase, acid fungal protease, α-glucosidase, lipase, phytase, trehalase, and xylanase. The enzyme products from 19 production strains within this safe lineage, and in two cases the production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (MW Pariza and EA Johnson. *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology 33: 173-186, 2001). Some of these enzymes are also the subject of GRAS notification documents that are listed on the FDA GRAS Notice Inventory, for example GRN 230, 315, 333, 372, and 567, all of which carry the decision statement, "FDA has no questions."

Aspergillus tubingensis is virtually indistinguishable from *Aspergillus niger* and is often referred to as *Aspergillus niger* var. *tubingensis*. The organism is classified within the '*Aspergillus* section Nigri', a species/subspecies group of closely related microorganisms that are widely used by enzyme manufacturers worldwide for the production of enzyme preparations that are used in human food, animal feed, and numerous other industrial enzyme applications.

Lipases from a number of microbial sources have long histories of safe use in food and feed manufacture. The Lipase 3 enzyme gene that was cloned into the *T. reesei* Morph Lip3 (GICC03373) production strain was obtained from *A. tubingensis* (DuPont IB strain 1M341). The Lipase 3 enzyme protein has been sequenced and studied for potential safety issues, specifically amino acid sequences that might elicit allergenicity or toxicity concerns. No such sequences were found.

The Lipase 3 enzyme preparation was evaluated for acute and inhalation toxicity in Wistar rats, dermal irritation in rabbits and mice, eye irritation in rabbits, genotoxicity in a number of test systems, and subchronic toxicity in a 90-day oral gavage study in Wistar rats. No dose-related adverse events were observed in any of these studies. The NOAEL for the Lipase 3 enzyme preparation was established as the highest dose tested in to 90-day oral gavage study, 160.6 mg total protein/kg bw/day equivalent to 123.15 mg TOS kg bw/day. The cumulative exposure for consumers to Lipase 3 from brewing processes and baking was calculated as 0.410 mg TOS/kg bw/day, giving a Margin of Safety for Lipase 3 from all uses of 300.

The cloning techniques and methodologies employed to construct *T. reesei* Morph Lip3 (GICC03373) are appropriate for use in the genetic modification of production strains for food ingredient manufacture. In addition, the manufacturing process including the ingredients used for fermentation, extraction and concentration of Lipase 3, and the specifications for the Lipase 3 enzyme preparation, are appropriate for a food ingredient.

Based on the foregoing, I concur with the evaluation made by DuPont that the *T. reesei* Morph Lip3 (GICC03373) production strain is safe and appropriate to use for the manufacture of food-grade Lipase 3. I further conclude that the Lipase 3 enzyme preparation, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in brewing and baking, where the enzyme is either not present in the final food or present at trace levels as inactive protein having no function or technical effect.

It is my professional opinion that other qualified experts would also concur in this conclusion.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

University of Wisconsin-Madison

Appendix D2 : EU Legislation on enzymes

Amfep/09/01 Association of Manufacturers and Formulators of Enzyme Products 18 January 2009

Amfep Statement on the EC Regulation 1332/2008 on Food Enzymes

On 16 December 2008 the European Parliament and the Council adopted Regulation 1332/2008 EC on food enzymes (OJ EU L 354/7, 31.12.2008).

The Association of Manufacturers and Formulators of Enzyme Products (Amfep) welcomes this EU legislation which is the first attempt ever to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU. The Regulation is set to improve the functioning of the internal market by removing disparities among member states and bringing more legal certainty to the market.

Only authorised food enzymes will be allowed to be commercialised and/or used in foods sold in the EU – irrespective whether they are used as processing aids or ingredients. This also applies to imported foods. The European Food Safety Authority (EFSA) will play a pivotal role in the authorisation process of food enzymes. On the basis of EFSA's scientific advice, the EU Commission will grant authorisations after consulting member states and the EU Parliament.

The publication of the Regulation in the Official Journal of the European Union only marks the beginning of an extensive implementation process. In fact, several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable.

The European Commission, supported by EFSA, has until 2011 to specify what information is required to be submitted for a risk assessment of food enzymes. After that, the industry will have another two years (until 2013) to submit dossiers for evaluation and authorization of food enzymes presently used in food on the EU market. Only after the EU Commission and EFSA have completed the evaluation of all these dossiers will the first EU (positive) list of approved food enzymes be established. The Regulation 1332/2008 EC does not set a deadline by which this evaluation is to be completed.

Apart from the authorization requirements, Regulation 1332/2008 also lays down specific provisions on labelling of food enzymes, food enzyme preparations and food prepared with enzymes. The provisions on labelling of food enzymes and food enzyme preparations will enter into force on 20 January 2010, whereas the provisions on labelling of food prepared with enzymes enter into force on 20 January 2009. The latter do not increase the scope of the previous food labelling provisions, although some changes are introduced to the way the small number of food enzymes that are not used as processing aids are declared.

Amfep is working closely with relevant European Stakeholders to ensure a seamless implementation of the new EU legislation for the benefit of food enzyme manufacturers, their clients, and consumers.

NB: The proposal for a regulation on food enzymes is a part of a so-called Food Improvement Agents package (FIAP). While harmonising EU legislation for food enzymes, FIAP is also aiming at upgrading existing EU legislation on food additives (EC Regulation 1333/2008) and food flavourings (EC Regulation 1334/2008) and establishing a transversal authorisation procedure (EC Regulation 1331/2008) The EU Regulation on food additives will include a positive list of additives and carriers that will be allowed in food enzymes and food enzyme preparations. This list will come into force on 1 January 2011.

For more information contact:
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